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No Home without Hormones: How Plant Hormones Control Legume Nodule Organogenesis

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ABSTRACT

The establishment of symbiotic nitrogen fixation requires the coordination of both nodule development and infection events. Despite the evolution of a variety of anatomical structures, nodule organs serve a common purpose in establishing a localized area that facilitates efficient nitrogen fixation. As in all plant developmental processes, the establishment of a new nodule organ is regulated by plant hormones. During nodule initiation, regulation of plant hormone signaling is one of the major targets of symbiotic signaling. We review the role of major developmental hormones in the initiation of the nodule organ and argue that the manipulation of plant hormones is a key requirement for engineering nitrogen fixation in non-legumes as the basis for improved food security and sustainability.

Key words: nitrogen fixation, symbiosis, hormones, nodule, legume

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INTRODUCTION

Nitrogen Fixing Symbioses

Nitrogen limits the production of food in all agroecosystems. Besides synthetic fertilizer, the second greatest source of nitrogen in agriculture is biological nitrogen fixation, such as that which occurs in the symbiotic relationship between legume crops and soil bacteria called rhizobia. Legumes are members of the FaFaCuRo clade (Fabales, Fagales, Cucurbitales, Rosales), which contains the only plant families that engage in this type of symbiosis (Soltis et al., 1995). A common feature of this symbiosis is the formation of a specialized organ (known as a nodule) that enables the reciprocal transfer of carbon from the plant and fixed nitrogen from the bacteria.

Importance of the Nodule Organ for Symbiotic Nitrogen Fixation

The nodule organ provides a number of favorable conditions for efficient nitrogen fixation. It is likely that no single function is sufficient to explain the requirement for a nodule, but together they provide an advantageous environment that necessitates the formation of such an organ. These functions include the facilitation of infection by host cell division (Breakspear et al., 2014), the development of a sink in which localized high gene expression levels facilitate nutrient and metabolite exchange (Clarke et al., 2014), the establishment of a low-oxygen region that facilitates nitrogenase activity (Layzell and Hunt, 1990), and the local relaxation of defense responses (Benezech et al., 2020).

There is some flexibility in the allowed structure of a nodule, as a number of related developmental programs are observed (Figure 1A). These differences are principally in the nodule initiation site (the cell layer varies from the pericycle to the outer cortex), the vascular arrangement, and the source and persistence of meristematic cells (Szczyglowski et al., 1998; Xiao et al., 2014, 2019; Shen et al., 2020). Although there are numerous determinants of nitrogen fixation efficiency, it is not clear whether any are directly related to the varied organ structures that have evolved (Terpolilli et al., 2012).

Plant Hormones Involved in Nodule Organogenesis

Plant hormones regulate all developmental processes, including nodule formation (Ferguson and Mathesius, 2014). The ubiquity of hormones means that they are often hubs that integrate plant environmental signals with developmental processes, including nodulation.

Here, we review the role of developmental hormones in the initiation of the nodule organ in legumes (Figure 1B). We pay particular attention to developmental hormones for which the greatest mechanistic insights have been uncovered: cytokinin (CK), auxin, ethylene, gibberellins (GA), and peptide hormones.

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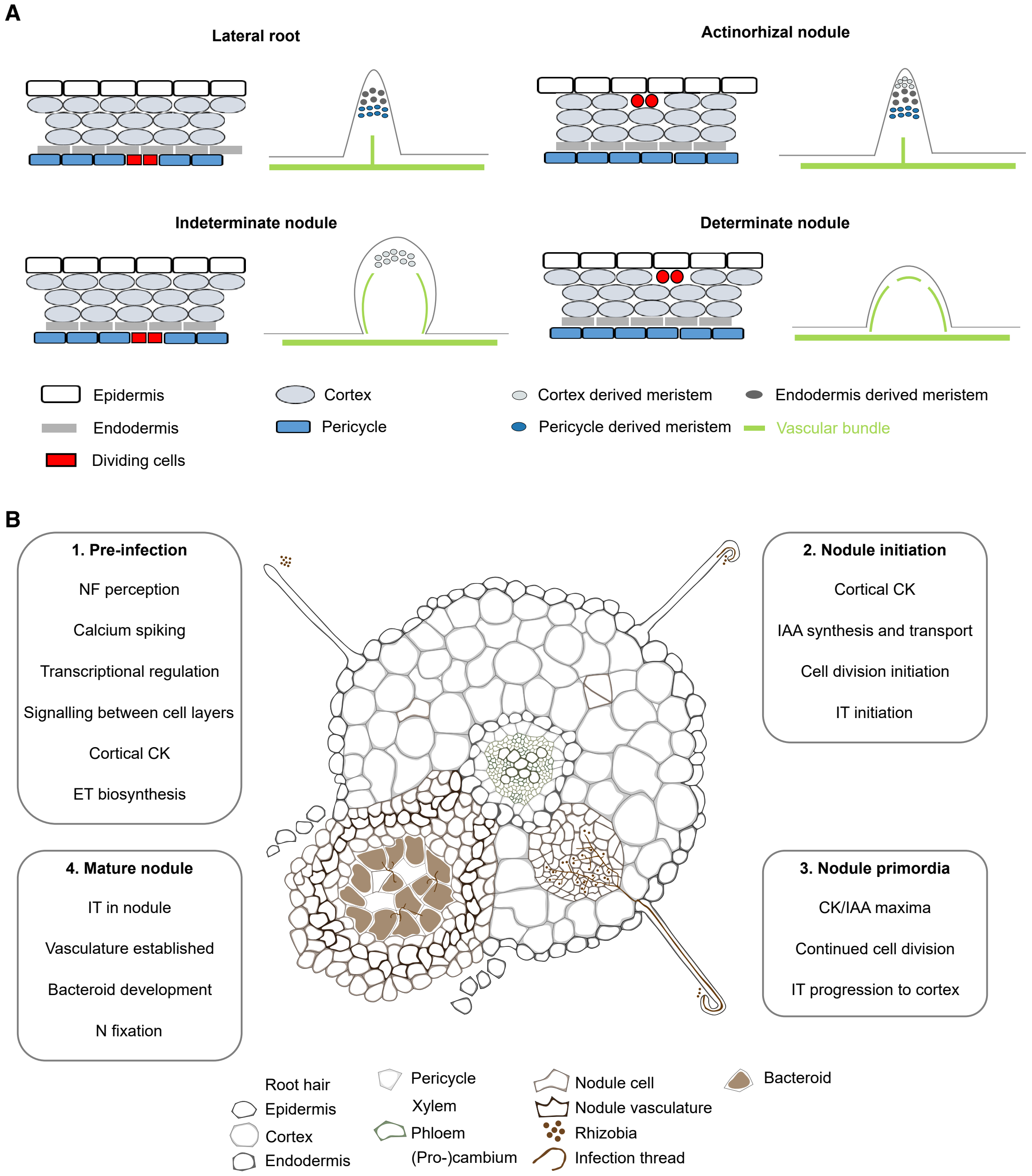

Figure 1. Nodule Development and Signaling Pathways.
(A) Comparison of the initiation sites and structures of lateral roots and several major nodule types. The first cell division (red) can vary within and between nodule types from the pericycle to the outer cortex. Vascular patterning and persistence of meristematic cells also differ.
(B) Major events during nodule organogenesis. Development of a determinate-type nodule as found in the model legume *Lotus japonicus* is exemplified. The establishment of cortical cytokinin (CK) and auxin signaling domains are major requirements for pre-infection signaling to stimulate nodule initiation and growth. NF, Nod factor; ET, ethylene; IT, Infection thread; IAA, indole-3-acetic acid (auxin).

Finally, the challenge of engineering nodule organogenesis in non-legumes is discussed in the context of plant hormone signaling as a key target.

INITIATION OF NODULATION AS A TARGET OF SYMBIOTIC SIGNALING

Nodule initiation in legumes occurs in response to the perception of Nod factors by the LysM receptors (LjNFR1/LjNFR5 and orthologs; Madsen et al., 2003; Radutoiu et al., 2003). Following receptor activation, perinuclear calcium spiking activates the common symbiosis pathway components, named for their common role in both nodulation and arbuscular mycorrhizal symbioses (2020) (for further details, see recent review by Roy et al., 2020). Transcriptional regulators are crucial for this early signaling and include CCAMK (MtDMI3) (Lévy et al., 2004), CYCLOPS (MtIPD3) (Messinese et al., 2007; Yano et al., 2008; Singh et al., 2014b), NSP1 (Smit et al., 2005), and NSP2 (Kaló et al., 2005). Additional transcriptional

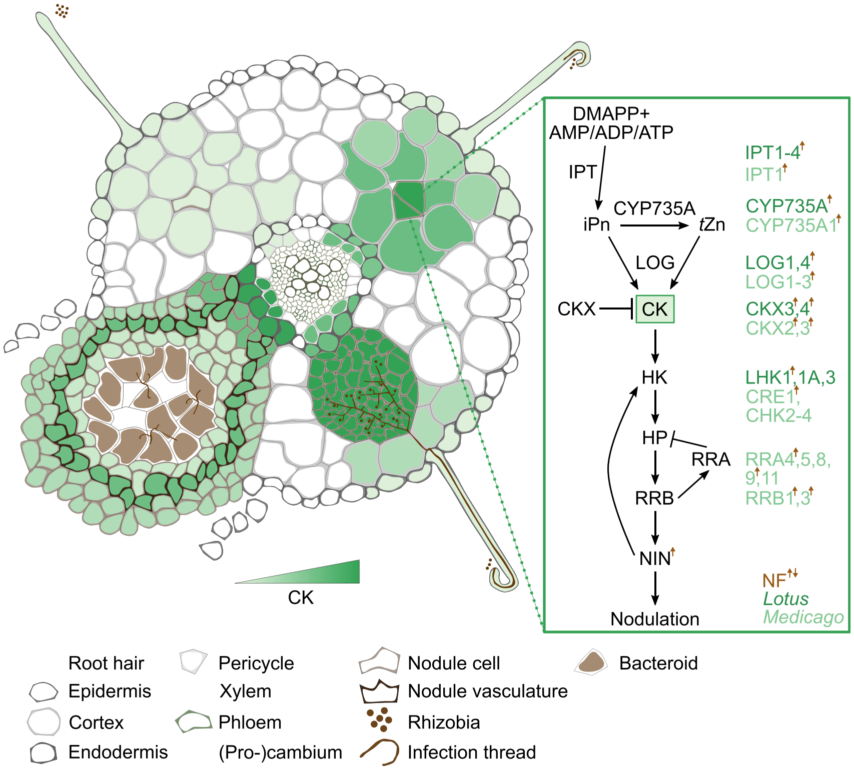

Figure 2. CK Biosynthesis, Metabolism, and Signaling.

Intensity of signaling output at different developmental stages is depicted based on reporter studies, transcriptome experiments, and signaling mutant phenotypes. Following the perception of rhizobia, CK level and signaling is rapidly induced in the cortex. Cortical CK remains increased during nodule initiation and growth, while surrounding tissue and epidermal responses are restricted. Mature nodules retain CK signaling in the vasculature, with reduced signaling in infected cells. Core biosynthesis, metabolism, and signaling components are depicted at the right, along with species-specific components, where known. NF-induced/repressed components are indicated with a brown arrow (up or down, respectively).

regulators that are specific to nodulation include ERN1 (Andriankaja et al., 2007), NIN (Schauser et al., 1999), and members of the NF-Y heterocomplex (Laloum et al., 2014; Laporte et al., 2014; Baudin et al., 2015). These transcriptional regulators are critical for the initiation of normal infection and organogenesis processes and are interlinked with many hormone signaling pathways (Buhian and Bensmihen, 2018). Transcriptome studies indicate that the expression of many, if not all, of these transcription factors is modulated by hormones. A key role of this early signaling is also to initiate hormone signaling, including that of cytokinin and auxin, which are the two major hormones that influence the cell-cycle initiation and progression required for nodule initiation. Further information is given on the mechanisms by which plant hormones interact with the nodulation pathway in each section below.

CYTOKININS

Cytokinin Synthesis, Metabolism, and Signaling

CKs were named after their ability to regulate cell division (Miller et al., 1955, 1956) and have been implicated in nearly all aspects of plant growth and development (reviewed in Werner and Schmülling, 2009; Kieber and Schaller, 2014; Cortleven et al., 2019).

Structurally, natural CKs are adenine derivatives that are either isoprenylated or carry an aromatic side chain at the N^6-residue (Sakakibara, 2006). Studies, mainly in *Arabidopsis*, suggest that N^6-isopentenyladenine (iP) and *trans*-zeatin (*t*Z) are the most biologically relevant CKs, with iP being primarily important for root development and *t*Z being crucial for most developmental processes in the shoot (Miyawaki et al., 2006; Kiba et al., 2013). They are initially synthesized by isopentenyltransferases (IPTs), forming iP nucleotides (Kakimoto, 2001; Takei et al., 2001), which may be converted further to *t*Z nucleotides by cytochrome P450 monooxygenases (CYP735As) (Takei et al., 2004; Kiba et al., 2013). Bioactive CKs are formed via dephosphoribosylation of CK riboside monophosphate precursors by LONELY GUYs (LOGs) (Kurakawa et al., 2007). They can be irreversibly degraded by either N-glucosylation or side-chain cleavage.

Bioactive CKs activate signaling through a histidine/aspartate phosphorylation cascade that bears similarities to the two-component signaling which is frequently seen in bacteria to couple membrane sensors with cellular responses (reviewed in Werner and Schmülling, 2009; Kieber and Schaller, 2014). Central components are the histidine kinase (HK) receptors (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001), which in turn phosphorylate the histidine residues of histidine phosphotransfer proteins (HPs) (Hutchison et al., 2006). The phosphorylation signal is transferred from HPs to type-B response regulator (RRB) transcription factors, activating them and ultimately leading to CK-dependent gene expression (Imamura et al., 1999). Acting antagonistically to these, RRAs act as negative regulators of signaling, establishing a feedback mechanism (Brandstatter and Kieber, 1998; Sakai et al., 2001).

As well as their roles in many aspects of plant development, CKs are central regulators of nodule organogenesis and root hair infection (Figure 2; reviewed in Frugier et al., 2008; Miri et al., 2016; Gamas et al., 2017).

Cytokinin in Nodule Organogenesis

Among the first indications that CK was of major importance for nodule organogenesis were the reports that CK application led to increased nodule formation in *Sesbania rostrata* (*t*Z) and alfalfa (BAP) (Dehio and de Bruijn, 1992; Cooper and Long, 1994; Bauer et al., 1996; Fang and Hirsch, 1998). A critical molecular link was established with the demonstration in *Lotus japonicus* and *Medicago truncatula* that the CK receptor *Lj*LHK1 was necessary for nodulation and, when activated, sufficient to initiate nodules spontaneously in the absence of bacteria (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007).

All isoprenoid class CKs, including the most relevant iP-type and *t*Z-type CKs, are induced upon infection with rhizobia or Nod factor (NF) treatment in *M. truncatula* and *L. japonicus* (van Zeijl et al., 2015; Reid et al., 2016). Multiple IPT-encoding genes have been identified as rapidly induced by rhizobia or NF treatment (Chen et al., 2014; van Zeijl et al., 2015; Reid et al., 2017; Mens et al., 2018). In *L. japonicus*, mutants in *Ipt4* or knockdown of *Ipt3* caused a reduced number of nodules, whereas *Ipt2*, which is the most significantly induced at the early stage, has not been characterized by reverse genetics (Chen et al., 2014; Reid et al., 2017). Intriguingly, nodulation of *ipt3ipt4* plants is indistinguishable from that of wild type (Reid et al., 2017), suggesting that the CK pool is sufficient for nodulation but that the balance with active forms may be altered. Active CKs are also rapidly formed, and several LOG family genes increase in abundance within 24 h of inoculation and throughout nodule primordia establishment (Mortier et al., 2014; van Zeijl et al., 2015; Reid et al., 2017). Although the functional relevance of these genes has not been demonstrated by analysis of loss-of-function mutants, overexpression of *LjLog4* leads to spontaneous nodule formation (Reid et al., 2017), and both knockdown and overexpression of *MtLog1* result in reduced nodule numbers compared with wild-type *M. truncatula* (Mortier et al., 2014). The relative activity of different *L. japonicus* LOG enzymes is unknown, although it would appear likely that overexpression of other LOG enzymes would achieve a similar accumulation of active cytokinin and thereby trigger organogenesis. Together, these studies suggest a key role for CK biosynthesis in the establishment of an early burst of CK to initiate cell division. Aside from the role of CKs in nodule organogenesis, biosynthesis of iP-type CKs by IPT3 in the shoot has also been suggested to play a role in the long-distance (systemic) negative regulation of nodule initiation (Sasaki et al., 2014). A similar shoot induction of CK biosynthesis has been found for soybean *GmIpt5* (Mens et al., 2018). The role of CK in the autoregulation of nodulation (AON) pathway is discussed further in later sections.

Whether iP- or *t*Z-type CKs play particular roles in nodulation remains unclear. An increased abundance of *LjCyp735a* transcripts and higher *t*ZR levels occur within 24 h of rhizobia infection in *L. japonicus* (Reid et al., 2016, 2017). In *M. truncatula*, *MtCyp735a1* is induced and *t*Z levels are increased 3 h after NF treatment (van Zeijl et al., 2015) or predicted by transcriptional networks in the epidermis 24 h after NF treatment (Jardinaud et al., 2016). However, only one predicted *Cyp735a* gene exists in *L. japonicus*, and *Ljcyp735a* mutants do not show a nodulation phenotype, indicating that *t*Z-type CK is not critical for nodulation in *L. japonicus* (Reid et al., 2017).

The regulation of CK biosynthesis appears complex: *MtIpt1* and *MtCyp735a1* are induced in a partially CRE1-dependent manner, whereas *LjIpt2* and *LjLog4* show increased expression in *Ljlhk1* mutants, indicating that regulation may differ between species (van Zeijl et al., 2015; Reid et al., 2017). Future studies to identify which combinations of *Ipt* and *Log* genes are required for nodule organogenesis and to characterize the regulation of these genes will be critical for defining how the initial burst of CK required for nodule induction is regulated by the symbiotic pathway.

The importance of CK degradation during nodule formation has been described in *L. japonicus*. *LjCkx3* is specifically expressed in xylem-pole pericycle cells and dividing cortical cells during early nodule development (Reid et al., 2016), and in *M. truncatula*, *MtCkx1* is a primary target of cytokinin signaling (Ariel et al., 2012). Mutants in *LjCkx3* have reduced infection and nodule numbers, which results at least in part from crosstalk with ethylene signaling (see below; Reid et al., 2016).

The active pool of CK triggers the CK signaling pathway, including the primary CK response by the RRBs. The CK-responsive TCS/TCSn promoters, which carry repetitive elements targeted by these response regulators, have been used to define the signaling domains of CK (Müller and Sheen, 2008; Zürcher et al., 2013). These promoters revealed that CK signaling in *L. japonicus* is activated in cortical cells within 24 h and is highly active throughout the development of young nodules, whereas the signaling is limited to vascular bundles of mature nodules (Held et al., 2014; Reid et al., 2017). A similar pattern in early nodule development is seen in *M. truncatula*, with the strongest CK signaling occurring in endodermal and pericycle cells (van Zeijl et al., 2015). There remains some uncertainty over the timing and relative signaling intensity of CK in the epidermis. In experiments with rhizobia inoculation in *L. japonicus*, early epidermal signaling activity was not detected, and later epidermal activity was mutually exclusive with infection (Reid et al., 2017). In *M. truncatula*, strong epidermal and outer cortical activity was detected in response to NF or *Sinorhizobium meliloti* inoculation through both tissue-specific transcriptomics and TCSn analyses (Jardinaud et al., 2016), and activity in the cortex, endodermis, and pericycle was detected in response to NF treatment using TCS (van Zeijl et al., 2015). The regulation of this process is highly dynamic and therefore difficult to compare between experimental systems. Whether CK accumulation and signaling differ between species with different nodule types and initiation locations will be key questions to resolve. Studies to directly identify CK accumulation rather than signaling at a cellular resolution may be key to resolving this question.

Most studies of CK signaling components in nodulation have focused on the role of CK receptors, and the current understanding is that three *L. japonicus* CK receptors act partially redundantly, with *Lj*LHK1 playing the predominant role in nodulation (Held et al., 2014). In hyperinfection thread1 (*hit1-1 = lhk1 har1)* roots, nodule primordia are aborted despite hyperinfection of root hairs, highlighting the different roles of CK in nodule organogenesis and root hair infection (Murray et al., 2007). Similarly, the *Lj*LHK1 homolog *Mt*CRE1 functions as the main CK receptor during nodulation in *M. truncatula*. *MtCre1* knockdown or loss-of-function mutants have a reduced nodule number but, in contrast to *lhk1-1*, no increase in infection events (Gonzalez-Rizzo et al., 2006; Plet et al., 2011), and the other three receptors (*Mt*CHK2–4) have partially redundant functions (Boivin et al., 2016). Gain-of-function alleles of *LjLhk1* named *spontaneous nodule formation2/5* (*snf2/snf5*) also demonstrated that increased CK signaling is sufficient to induce nodulation, independent of rhizobia infection (Tirichine et al., 2007; Liu et al., 2018b). Similarly, the application of CK or overexpression of its biosynthetic pathway achieves similar results (Heckmann et al., 2011; Reid et al., 2017). *LjLhk1* expression mostly overlaps with the expression pattern of TCS/TCSn during nodulation (Held et al., 2014), and its expression is induced upon CK treatment, suggesting a positive feedback loop during nodule organogenesis (Murray et al., 2007). In *M. truncatula*, the transcriptional regulator *Mt*NIN plays a key role in this positive feedback mechanism (Vernié et al., 2015). The cortical expression of *LjLhk1* is sufficient for both organogenesis initiation and infection regulation, highlighting the key role played by CK in linking these two processes (Miri et al., 2019). This cortex-dependent infection regulation may require ethylene as an intermediary and is discussed in more detail below.

Downstream of the well-studied CK receptors, the high degrees of redundancy in the core signaling pathway have hindered detailed study. Of the core components, several *Rr*s in *M. truncatula* are induced upon rhizobia infection and CK treatment (Gonzalez-Rizzo et al., 2006; van Zeijl et al., 2015). Among these, *MtRrb3* is most strongly expressed in nodules, and mutants exhibit fewer nodules, indicating that redundancy in signaling components is only partial (Tan et al., 2020). This work also identified a direct regulatory relationship with *Nsp2* and control of endoreduplication by CK in nodulation.

Interconnection between CK and Nodulation Signaling

Given the central role of CK in nodule organogenesis, the question arises: how do CK synthesis, metabolism, and signaling interact with other factors that are crucial for nodulation? Studies in *L. japonicus* suggest that CK acts downstream of the NF receptors *Lj*NFR1 and *Lj*NFR5 and the calcium signaling decoded by *Lj*CCaMK (Tirichine et al., 2007; Madsen et al., 2010). At the same time, CK biosynthesis and signaling act upstream of *Lj*NIN to positively regulate its expression (Murray et al., 2007; Tirichine et al., 2007; Madsen et al., 2010; Heckmann et al., 2011; Chen et al., 2014). This regulation of *LjNin* by CK occurs through a distal *cis*-regulatory region in the *LjNin* promoter that renders *Ljdaphne* (Yoro et al., 2014) and *Mtdaphne*-like mutants (Liu et al., 2019) unable to form nodules despite competence for infection events, as the distal promoter regions are not required for *Nin* expression in the epidermis (Liu et al., 2019). The exact factors that control these distant regulatory motifs remain to be identified. The contribution of CK to early symbiotic signaling in the epidermis remains a key point to resolve, as *Mt*CRE1 was reported to be required for the vast majority of symbiotic signaling (Schiessl et al., 2019), and null mutants or RNAi targeting of *MtCre1* results in reduced or aborted infection threads (Gonzalez-Rizzo et al., 2006; Plet et al., 2011). On the other hand, *Ljlhk1* mutants remain highly sensitive to NF and rhizobia inoculation, with hyperactive CK biosynthesis and hyperinfection (Murray et al., 2007; Reid et al., 2017). The precise genetic regulation of CK biosynthesis and signaling response by the symbiosis pathways is likely to be key to identifying how signals are propagated between the epidermis and cortex to initiate nodulation, and the analysis of signaling components such as RRs (as in RRB3; Tan et al., 2020) is likely to identify mechanisms that link CK with organ initiation and identity.

AUXIN

Auxin Biosynthesis, Metabolism, and Signaling

Auxins regulate essentially all plant developmental processes (Teale et al., 2006). The main natural auxin, indole-3-acetic acid (IAA), is predominantly synthesized through the tryptophan-dependent indole-3-pyruvic acid (IPA) pathway (reviewed in Zhao, 2012; Korasick et al., 2013). Initially, TRYPTOPHAN AMIDOTRANSFERASE OF ARABIDOPSIS1 (TAA1) and related (TAR) proteins convert tryptophan to IPA (Stepanova et al., 2008), which is subsequently converted to bioactive free IAA by YUCCA proteins (Zhao et al., 2001). IAA may be modified/inactivated in many ways, including oxidation or amino acid and carbohydrate conjugation. Intracellular IAA activates the auxin signaling pathway after perception by the TRANSPORT INHIBITOR RESISTANT1 (TIR1)/AUXIN signaling F-BOX (AFB) receptors (Dharmasiri et al., 2005a, 2005b; Parry et al., 2009). In the absence of IAA, TIR/AFBs are inactive, which allows AUX/IAA proteins to inhibit the function of AUXIN RESPONSE FACTOR (ARF) transcription factors through direct interaction (Ulmasov et al., 1997b, 1999b). Upon perception of IAA by TIR1/AFB receptors, a Skp1-Cullin-F-box (SCF)-TIR1/AFB complex is formed that inhibits Aux/IAA function by mediating their ubiquitination and thus their proteasome-dependent degradation. Consequently, ARFs are released from Aux/IAA repression and can form dimers to positively or negatively regulate the transcription of output genes through binding to auxin response elements (Ulmasov et al., 1997a, 1999a, 1999b).

The role of auxin in nodule organogenesis has been studied extensively, and greater mechanistic insights are now emerging (Figure 3; reviewed in Kohlen et al., 2018).

Auxin in Nodule Development

The importance of auxin for nodule development is perhaps best illustrated by the ability of auxin transport inhibitors to induce organogenesis and *Enod* gene expression (Hirsch et al., 1989; Scheres et al., 1992; Fang and Hirsch, 1998; Rightmyer and Long, 2011). In *M. truncatula*, auxin transport inhibitor application is also sufficient to partially rescue the organogenesis defect of the *cre1* mutant (Ng et al., 2015).

Auxin production and response in root hairs occur rapidly in response to NF through both enhanced biosynthesis and transport regulation (Breakspear et al., 2014; Nadzieja et al., 2018). During nodule organogenesis, auxin response occurs in an *Lj*LHK1- and *Lj*NIN-dependent manner (Suzaki et al., 2012). In *M. truncatula*, this link is established by *Mt*NIN-dependent induction of *MtYuc2,8* expression via *Mt*LOB-DOMAIN PROTEIN 16 (*Mt*LBD16) (Schiessl et al., 2019). The *L. japonicus* ortholog of *Lbd16* (*Asl18a*) is regulated by *Lj*NIN through *cis* elements in the intron, as well as by auxin during lateral root formation (Soyano et al., 2019). *Shi/Sty* genes, which in *Arabidopsis* are known to regulate *Yuc* expression, are also regulated by NF-YA1 in *L. japonicus* (Hossain et al., 2016). One of the major roles of these transcriptional regulators is therefore

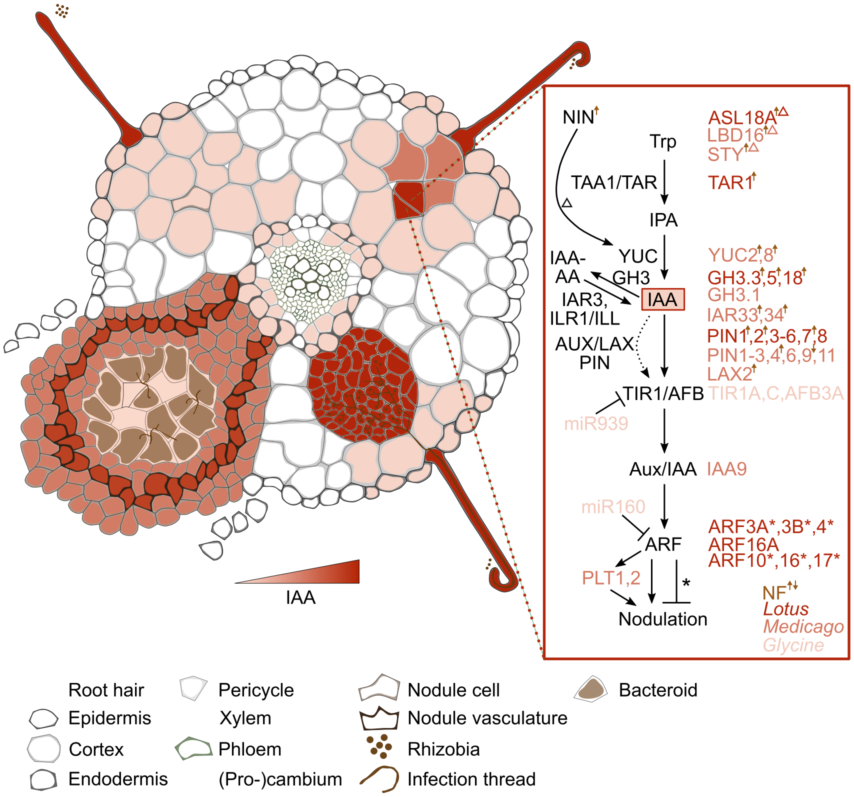

Figure 3. Auxin Biosynthesis, Metabolism, and Signaling.

Intensity of signaling output at different developmental stages is depicted based on reporter studies, transcriptome experiments, and signaling mutant phenotypes. Following rhizobia perception, auxin levels increase, and signaling output is initiated in the epidermis. Auxin biosynthesis and transport regulation contribute to increased signaling associated with nodule initiation and growth and are maintained until nodule maturity. Generally known biosynthesis, metabolism, and signaling components are depicted at the right with species-specific components, where known. A dotted line indicates IAA transport by AUX/LAX/PIN proteins. Factors through which NIN induces YUC are marked with a triangle. ARFs acting as negative regulators of nodulation are depicted with asterisks. NF-induced/repressed components are indicated with a brown arrow (up or down, respectively).

to establish connections between symbiotic signaling and the production and localization of an auxin maximum required for cell division and nodule initiation. This interconnection between auxin and organogenesis is also evident in other plant developmental processes, in particular lateral root initiation, where LBD16 and SHI/STYs are also central to the establishment of auxin signaling domains.

Induction of IAA conjugation by GH3s is a common feedback feature of auxin response in many developmental contexts, including nodulation, and is therefore commonly used as a marker for auxin signaling. Expression of *Gh3*s during both infection and nodule development highlights the importance of metabolic feedback on auxin signaling in these processes (Mathesius et al., 1998; van Noorden et al., 2007; Singh et al., 2014a; Breakspear et al., 2014). In addition, regulation of *Mt*IAR33 and *Mt*IAR34 during nodulation may indicate that IAA-Asp, IAA-Glc, and IBA-Ala are hydrolyzed during nodule organogenesis (Campanella et al., 2008).

Compared with auxin metabolism, more attention has been paid to the role of auxin transport during nodule organogenesis. Rhizobia inoculation of *M*. *truncatula* leads to a substantial reduction in auxin transport over time, correlating with a reduction in nodule formation (van Noorden et al., 2006). Gravitropic stimulation of roots, which results in asymmetric auxin distribution, is also sufficient to alter nodule positioning in *L. japonicus* (Nadzieja et al., 2019), and application of auxin import inhibitors before rhizobia infection reduced the number of nodules in *M*. *truncatula* (Roy et al., 2017). The requirement for auxin transport regulation is supported by the fact that several *MtPins* show enhanced expression during nodulation (Huo et al., 2006; Plet et al., 2011; Sańko-Sawczenko et al., 2016). Moreover, knockdown of *MtPin2*, *MtPin3*, or *MtPin4* results in a reduced number of nodules (Huo et al., 2006). In *L. japonicus*, all eight *LjPin* genes are expressed in young nodules and the vasculature of mature nodules (Sańko-Sawczenko et al., 2016). Auxin importers *Mt*LAX1 and *Mt*LAX2 are expressed during early stages of nodule formation and in the vasculature and the apex of mature nodules (de Billy et al., 2001; Roy et al., 2017), and *lax2* mutants form fewer nodules than wild type and have a reduced auxin response within nodules (Roy et al., 2017). The high degree of redundancy in the PIN family has hindered the establishment of additional mechanistic links between symbiotic signaling and auxin transport regulation (Ng et al., 2020).

Together, these transport studies indicate that auxin must accumulate during different stages of nodule development and subsequently activate auxin signaling. Studies with the auxin reporter constructs *pDR5*::*Gus*, *DII-Venus*, and *pGH3*::*Gus* in *L. japonicus* and *M. truncatula* demonstrated that auxin signaling starts to emerge in cell layers underneath infected root hairs and is strongest in the cortex and pericycle of young nodules (Pacios-Bras et al., 2003; Suzaki et al., 2012; Breakspear et al., 2014) and in the vasculature and apex of mature nodules (Takanashi et al., 2011; Suzaki et al., 2012; Guan et al., 2013; Breakspear et al., 2014; Franssen et al., 2015; Nadzieja et al., 2019). These observations support a requirement for auxin signaling in both infection and nodule organogenesis and may relate to the requirement for cell-cycle initiation and progression that occurs in both these processes.

Although the locations where auxin signaling occurs are relatively well established, few downstream signaling components have been demonstrated to have specific roles in nodulation. In soybean, six auxin receptor genes encoding either *Gm*TIR1 or

*Gm*AFB3 are important for proper nodule development, and their degradation depends on miRNA393 (Cai et al., 2017). *S. meliloti* infection of *M. truncatula* leads to increased transcript abundance of the auxin-responsive genes *MtIaa9* and *MtArf16a*, and *arf16a* mutants show a reduced number of infection events (Breakspear et al., 2014). In soybean, *Gm*ARF8a/8b, *Gm*ARF10, *Gm*ARF16, and *Gm*ARF17 are negative regulators of auxin signaling and are targets of miR167/miR160, which reduces their expression to increase the sensitivity to auxin (Turner et al., 2013; Nizampatnam et al., 2015; Wang et al., 2015). In *L. japonicus*, *Lj*ARF3A, *Lj*ARF3B, and *Lj*ARF4 can potentially act as negative regulators of nodulation. Their expression is increased in *rel3* plants, which are impaired in the biogenesis of *trans*-acting small interfering RNAs (siRNAs), have a reduced nodule number, and exhibit insensitivity to auxins but greater sensitivity to auxin transport inhibitors during nodulation (Li et al., 2014). Taken together, these results show that miRNAs and siRNAs contribute to the maintenance of robust auxin signaling during nodule formation. Additional tools, such as conditional knockouts of signaling components or transporters without pleiotropic phenotypes, may provide mechanistic insights into auxin signaling and help to clarify the relative contributions of biosynthesis, transport, and signaling components during nodule initiation.

Interaction of Auxin and Other Signaling Pathways during Nodule Organogenesis

During many developmental processes, auxin and CK interact with and mostly counteract each other by regulating each other's metabolism, signaling, and transport (Coenen and Lomax, 1997; El-Showk et al., 2013). In nodule development, auxin and CK have more synergistic activity, as the presence of both is required for nodule initiation.

Several studies have investigated how auxin and CK interact to regulate nodule development and suggest that LHK1/CRE1-dependent CK signaling acts upstream of auxin biosynthesis and transport. In *M. truncatula*, inhibition of polar auxin transport (PAT) upon rhizobia infection is *Mt*CRE1 dependent and accomplished through the regulation of *MtPin* expression and protein accumulation (Plet et al., 2011). The lack of PAT inhibition is causative for the reduced the nodulation of *cre1* plants and can be rescued by N-1-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) supplementation. Moreover, *Mt*CRE1-dependent flavonoid accumulation is required to inhibit PAT (Ng et al., 2015). As discussed earlier, *Mt*CRE1-dependent CK signaling is also required to induce *MtNin* expression. This CRE1-dependent induction is required to induce the expression of *MtSty*, *MtLbd16*, *MtYuc2*, and *MtYuc8*, resulting in auxin accumulation (Schiessl et al., 2019). Interestingly, miR160 regulates the sensitivity to auxin but also to CK during nodule organogenesis in soybean, and the nodulation phenotypes of miR160 mimicry and overexpressing lines can be rescued by application of one or both hormones (Turner et al., 2013; Nizampatnam et al., 2015). In *L. japonicus*, a connection was also established whereby, during root hair infection, *Lj*LHK1-dependent signaling restricts auxin accumulation after inoculation with rhizobia in the epidermal cell layer. It remains to be seen whether this requires LHK1 signaling in the cortex or epidermis. At the same time, auxin may counteract the inhibitory role of CK signaling in the epidermis by inhibiting the expression of the CK biosynthesis genes *LjCyp735a* and *LjLog4* (Nadzieja et al., 2018). These observations suggest a finely balanced antagonism between auxin promoting infection progression and CK inhibiting it in the epidermis, while both hormones positively influence the initiation and maintenance of cell division for nodule organogenesis.

ETHYLENE

Ethylene Biosynthesis and Signaling

Ethylene is a gaseous hormone involved in the regulation of numerous developmental processes, such as seedling growth, fruit ripening, leaf and root development, and senescence (Schaller and Kieber, 2002; Schaller, 2012).

Ethylene synthesis is a multistep process in which ACC synthase (ACS) (Liang et al., 1995; Yamagami et al., 2003) and ACC oxidase (ACO) (Linkies et al., 2009) are often considered the limiting steps, and their regulation is thus key for plant development.

In the absence of ethylene, ETHYLENE RESPONSE (ETR)/ETHYLENE RESPONSE SENSOR (ERS)/ETHYLENE INSENSITIVE4 (EIN4) receptors act as negative regulators of ethylene signaling (Ecker, 1995; Hua et al., 1995; Sakai et al., 1998), together with CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), a Ser/Thr kinase (Kieber et al., 1993). CTR1 phosphorylates EIN2 at its C terminus, causing its inactivation (Ju et al., 2012). In addition, it may be degraded in an F-box EIN2 TARGETING PROTEINs (ETPs)-dependent manner (Qiao et al., 2009). Without active EIN2, EIN3/EIN3-LIKE (EIL) transcription factors are degraded by EIN3-BINDING F-BOX PROTEINs (EBFs), resulting in an absence of ethylene response gene transcription (Guo and Ecker, 2003; Potuschak et al., 2003). Ethylene perception inactivates ETR/ERS/EIN4 and CTR1, enabling a phosphorylation-dependent interaction of EIN2 with ETR/ERS/EIN4 that protects EIN2 from degradation (Bisson and Groth, 2010). Cleavage and nuclear migration of the EIN2 C terminus stabilize EIN3/EIL proteins (An et al., 2010; Wen et al., 2012), resulting in the transcriptional regulation of ethylene response genes (Chao et al., 1997; Guo and Ecker, 2003).

Role of Ethylene in Nodule Organogenesis

Ethylene appears to function primarily as a negative regulator of nodulation in most legumes (Figure 4). Application of the ethylene precursor ACC reduces nodule number, whereas inhibition of ethylene biosynthesis, e.g., through the application of AVG or Ag^{+}, enhances nodulation in *Vicia sativa* (Heidstra et al., 1997), *M. truncatula* (Peters and Crist-Estes, 1989; Penmetsa and Cook, 1997), pea (Goodlass and Smith, 1979; Lorteau et al., 2001), and *L. japonicus* (Heckmann et al., 2011). This inhibitory effect acts very early in the signaling pathway, as ACC treatment not only inhibits nodule formation but also inhibits Ca^{2+} spiking in *M. truncatula* root hairs in response to NF (Oldroyd et al., 2001). Transcriptional studies in ethylene-insensitive mutants show a massive impact of ethylene on essentially all symbiosis-induced transcription (Larrainzar et al., 2015). The symbiotic induction of ethylene occurs rapidly, with rhizobia inoculation stimulating an

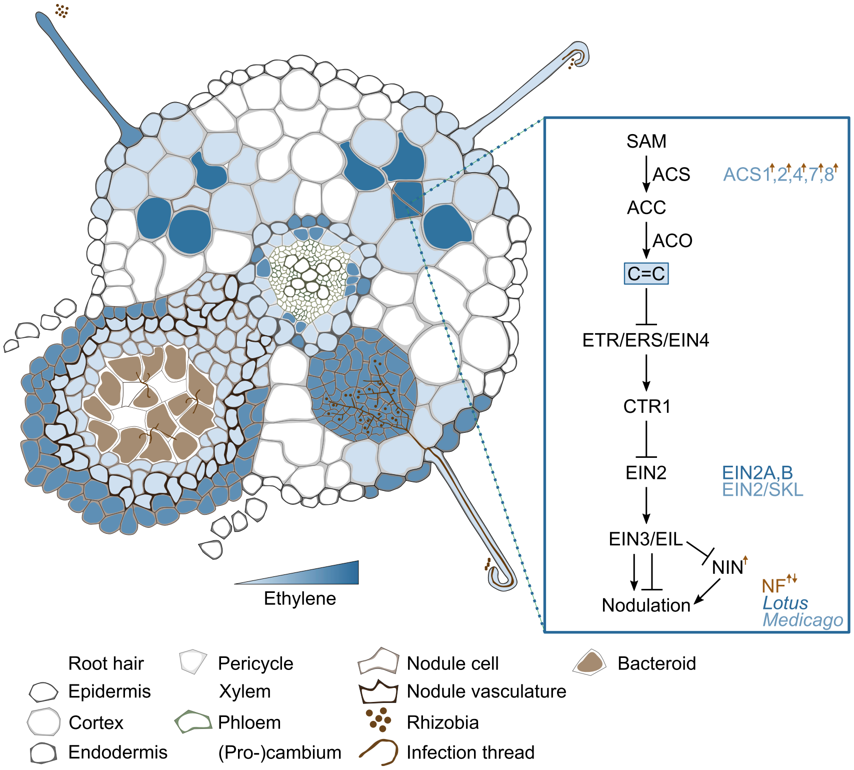

Figure 4. Ethylene Biosynthesis, Metabolism, and Signaling.

Following perception of rhizobia, ethylene levels and signaling are rapidly induced; however, precise localization has not been achieved, and signaling intensity at different developmental stages is depicted based on mutant phenotypes, indicating roles at all stages of nodule development and in nodule positioning. Core biosynthesis, metabolism, and signaling components are depicted in black at the right, together with species-specific components, where known. NF-induced/repressed components are indicated with a brown arrow (up or down, respectively).

increase in ethylene within six hpi (Reid et al., 2018), and *MtAcs1* and *MtAcs2* are induced upon NF application in *M. truncatula* (van Zeijl et al., 2015). *MtAcs4*, *MtAcs7*, and *MtAcs8* are expressed in the periphery of young nodules and in the meristem of mature nodules (Larrainzar et al., 2015). This rapid synthesis and inhibitory effect of ethylene thus create a rapid feedback mechanism to restrict symbiotic gene expression and nodulation.

In addition to its negative regulatory role, ethylene may act as a positional cue for nodule initiation, exerting stronger inhibitory effects at some positions within a tissue. For example, in *Vicia sativa*, *Aco* expression was found to accumulate in cells opposite phloem poles and in the first cortex layers adjacent to them (Heidstra et al., 1997). These observations were further supported by ethylene signaling mutants in *M. truncatula*: *skl* (*ein2)* mutants form nodules opposite xylem and phloem poles, in contrast to wild-type plants that only form nodules opposite xylem poles (Penmetsa et al., 2003; 2008). This suggests that ethylene signaling before rhizobia infection is required to suppress excessive nodule formation in general and specifically in cell layers over phloem-pole pericycle cells. *L. japonicus* possesses two EIN2 encoding genes, *LjEin2a* and *LjEin2b*, and the respective double mutant forms more nodules, akin to the *skl*/*ein2* mutant (Reid et al., 2018).

Although some ethylene responsive markers have been described, none so far developed offers the high-resolution and dynamic range available for CK and auxin (DR5/DII-VENUS reporter; Nadzieja et al., 2019), making it challenging to identify the precise location and signaling of ethylene responses during nodule organogenesis. Novel approaches such as single-cell sequencing might circumvent this restriction or lead to the development of such ethylene markers in the future.

Interaction between Ethylene and Symbiotic Signaling

Recent studies suggest that ethylene and CK balance each other's responses during nodule organogenesis in a feedback loop. In *M. truncatula*, induction of *MtAcs1* and *MtAcs2* expression upon NF application is *Mt*CRE1-dependent (as is regulation of CK biosynthesis/signaling genes), and *cre1* mutants are insensitive to the AVG-dependent enhancement of this NF effect (van Zeijl et al., 2015), suggesting that CK acts as a positive regulator of ethylene biosynthesis and signaling. The increased ethylene emission of uninoculated *L. japonicus* gain-of-function *lhk1* (*snf2*) plants provides additional support for this view (Reid et al., 2018) and is consistent with the CK-dependent induction of ethylene biosynthesis in *Arabidopsis* (Dugardeyn and Van Der Straeten, 2008). The question remains, where is ethylene active in this context? Given the inhibitory effect of ethylene on infection thread formation and the fact that reduced infection thread formation in *L. japonicus ckx3* mutants can be rescued by AVG supplementation (Reid et al., 2016), it is highly likely that CK-dependent ethylene synthesis inhibits infection thread formation, as proposed by Miri et al. (2016).

Evidence for the influence of ethylene on CK biosynthesis or signaling was provided recently. In *M. truncatula skl*/*ein2* plants, iP concentrations are strongly increased due to both a compensatory effect and an inhibitory effect of ethylene signaling on symbiotic signaling and CK biosynthesis (van Zeijl et al., 2015). In support of the latter effect, the *skl*/*ein2* nodulation phenotype is almost completely suppressed by the introduction of the *cre1* allele (Plet et al., 2011), implying that *Mt*EIN2 may act as a negative regulator of *Mt*CRE1-dependent signaling. The identity of the molecular targets of ethylene-dependent inhibition of the symbiotic signaling pathway remains a key outstanding question. Studies in *Arabidopsis* suggest that ethylene and CK signaling may be interconnected through direct phosphorylation of AHPs by ethylene receptors (Zdarska et al., 2019). It will be interesting to determine whether and how the interconnection of these

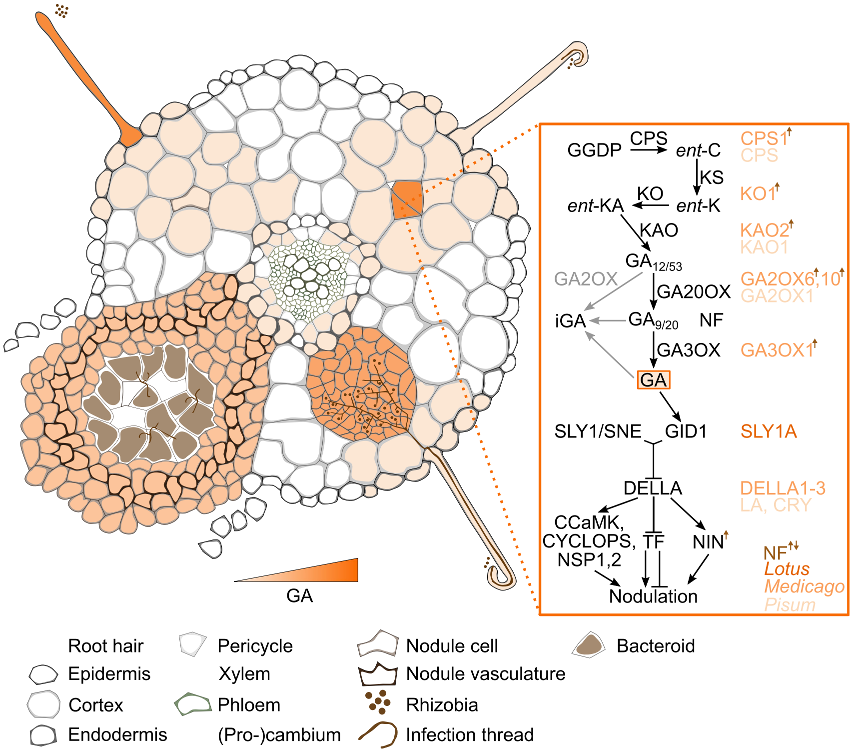

Figure 5. GA Biosynthesis, Metabolism, and Signaling.

Intensity of signaling output at different developmental stages is depicted based on transcriptome experiments and signaling mutant phenotypes; however, it has not been precisely localized using reporter studies. Following rhizobia perception, GA level/signaling accompanies the activation of symbiotic signaling. Core biosynthesis, metabolism, and signaling components are depicted in black/gray at the right with species-specific components shown where they have been investigated. NF-induced/repressed components are indicated with a brown arrow (up or down, respectively).

pathways has been recruited in nodule development or regulation.

GIBBERELLINS

Gibberellin Biosynthesis and Signaling

Gibberellins (GAs) are a large group of compounds formed through multiple consecutive reactions from geranylgeranyl diphosphate (GGDP); they are involved in many aspects of plant growth and development, including seed germination and the development of leaves and seeds (Sun, 2008; Yamaguchi, 2008). GGDP is converted further by a number of GA oxidases, ultimately leading to the formation of bioactive GAs (Mitchum et al., 2006; Hu et al., 2008; Sun, 2008).

Bioactive GAs activate a nucleus-localized signaling pathway that involves GIBBERELLIN INSENSITIVE DWARF1 (GID1) receptors, DELLA proteins GIBBERELLIC ACID INSENSITIVE (GAI)/REPRESSOR-OF-GA1-3 (RGA)/RGA-LIKE (RGL), F-box proteins SLEEPY1 (SLY1) and SNEEZY (SNE), and transcription factors, e.g., PHYTOCHROME-INTERACTING FACTORs (PIFs) (Sun, 2008; Schwechheimer, 2012; Davière and Achard, 2013). In the absence of bioactive GA, GID1 receptors are inactive (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006), allowing DELLA proteins to inhibit the GA signaling pathway by inhibiting the function of transcription factors such as PIFs (Sun, 2008; Schwechheimer, 2012; Davière and Achard, 2013). GA perception by GID1 receptors results in complex formation with F-box proteins SLY and SNE, mediating DELLA degradation (McGinnis et al., 2003; Dill et al., 2004; Ariizumi et al., 2011). As a result, formerly repressed transcription factors can mediate the transcription of output genes. A summary of GA-dependent nodulation in legumes has been given by Hayashi et al. (2014). We highlight recent advances in the mechanistic understanding of GA in nodule organogenesis (Figure 5).

Gibberellin Signaling in Nodulation

Our current understanding of the role of GA in nodulation is mainly based on research in pea and *M. truncatula* and suggests that levels of active GA must be precisely coordinated. Although there are contradictory reports that GA application either induces (Kawaguchi et al., 1996) or inhibits (Maekawa et al., 2009) the formation of nodules (and infection threads) in *L. japonicus*, nodulation promoting effect is seen in pea at low GA concentrations, whereas an inhibitory effect is seen at high concentrations (Ferguson et al., 2005). In *M. truncatula*, GA application inhibits nodule formation (Fonouni-Farde et al., 2016a; Jin et al., 2016). However, the GA antagonist paclobutrazol reduces nodule formation in pea (McAdam et al., 2018) but stimulates nodulation at low concentrations in *M. truncatula* (Fonouni-Farde et al., 2016a). These somewhat contradictory results may be explained by the sensitive GA balance during nodule development and the fact that GA is likely to cause artifacts when applied at high concentrations outside the normal range. Another possible explanation could be a bell-shaped dose–response curve similar to that described, e.g., for IAA/NAA-dependent protoplast swelling (Yamagami et al., 2004).

Extensive studies with pea mutants defective in GA biosynthesis and degradation have been conducted and show the importance of GA biosynthesis during nodule organogenesis. Generally, these mutants exhibit reduced nodule formation and also display pleiotropic effects on root and shoot development (Ferguson et al., 2005). Four of the mutants (*ls*, *na*, *lh*, and *sln*) with reduced nodule numbers (Ross et al., 1993; Yaxley et al., 2001) are loss-of-function mutants of *Ps*CPS, *Ps*KAO1 (Davidson et al., 2003), *Ps*KO1 (Davidson et al., 2004), and *Ps*GA2OX1 (Lester et al., 1999). Moreover, grafting experiments with *lh/ko1* and wild type revealed that normal GA synthesis in the shoot is sufficient to rescue the nodulation phenotype of *lh/ko1* (Ferguson et al., 2005). Crosses of *na-1* with the hypernodulation mutants *sym28*, *sym29*, or *nod3* show additivity of the nodulation phenotypes (Ferguson et al., 2011). Although biosynthesis mutants are not yet characterized in *L. japonicus*, *Medicago ga2ox10* plants display a reduced number of nodules (Kim et al., 2019). Moreover, rhizobia infection induces *MtGa3ox1*, *MtGa2ox6*, *MtGa2ox10*, *MtCps1*, *MtKo1*, and *MtKao2* expression between 1 and 12 dpi in *M. truncatula* (Breakspear et al., 2014; Kim et al., 2019) and *LjGa2ox* expression between 4 and 12 dpi in *L. japonicus* (Kouchi, 2004).

GA signaling components involved in the regulation of nodulation identified to date encode SLY1 and DELLA proteins (Maekawa et al., 2009; Fonouni-Farde et al., 2016b; Jin et al., 2016). In *L. japonicus*, overexpression of *LjSly1a* and a gain-of-function *LjSly1a* allele (SLY1A-d) reduce the number of nodules and also reduce *LjGa20ox* expression, indicating that constitutive GA signaling negatively regulates nodule organogenesis (Maekawa et al., 2009). This idea is further supported by the observation that GA application inhibits the expression of *LjNin* and *LjNsp1/2* (Maekawa et al., 2009). In pea, loss of the DELLA proteins LA and CRY leads to a reduction in nodule number, also indicating the repressive function of GA signaling in nodulation (Ferguson et al., 2011). Similar to pea, *Mt*DELLA1,2,3 are positive regulators of nodule organogenesis in *M. truncatula*. Expression of *MtDella1*,*2*,*3* is induced 1 d after inoculation with rhizobia in all root tissues, and these genes are also expressed in the nodule meristematic zone (Fonouni-Farde et al., 2016a, 2016b). Triple *della1*,*2*,*3* mutants display a strong reduction in nodule formation, and all three *Mt*DELLAs can physically interact with *Mt*NSP2, *Mt*NF-YA1, and *Mt*IPD3 to positively regulate the expression of nodulation genes such as *MtErn1*, *MtNin*, or *MtRip* (Fonouni-Farde et al., 2016a, 2016b; Jin et al., 2016).

All in all, DELLA accumulation, which promotes the transcriptional activity of multiple symbiotic signaling components and thus nodule initiation, is antagonized by increased GA levels. Therefore, the precise regulation of GA content and signaling is necessary for nodule initiation.

Interaction between GA and Other Hormones during Nodule Organogenesis

A number of interactions between GA signaling components and other nodulation factors have been demonstrated recently. A potential link between CK and GA was shown by GA supplementation experiments in *L. japonicus*. Both wild-type and *snf2* plants respond to GA supplementation with a decrease in nodule number caused by GA-dependent inhibition of *LjNsp1/2* and *LjNin* expression (Maekawa et al., 2009). In *M. truncatula*, the introduction of *della2 della3* into CRE1 gain-of-function plants suppresses their spontaneous nodule formation (Jin et al., 2016). As *Mt*DELLAs are positive regulators of *MtNin* and *MtErn1* expression (Jin et al., 2016), one possible explanation is that they act downstream of CK signaling to regulate *MtNin* and *MtErn1* expression during nodule organogenesis. Indeed, *Mt*DELLAs also antagonize cytokinin in the regulation of *Nsp2*, with dominant active DELLA forms triggering spontaneous nodule organogenesis (Fonouni-Farde et al., 2017).

There is also some evidence that GA and ethylene interact during nodulation. In pea, GA acts as a negative regulator of ethylene biosynthesis, as *na* plants show higher endogenous ethylene concentrations, which may result from increased *PsAcs1 and PsAco1* expression. Moreover, treating *na* plants with AVG can partially rescue the nodulation phenotype (Ferguson et al., 2011). This may indicate that a proper GA/ethylene balance is required for nodule organogenesis. However, crosses of *na-1* with *ein2* suggest that both hormones may act independently (McAdam et al., 2018).

GA signaling is therefore positioned as a key regulator of symbiotic signaling, with DELLA interacting with core members of the symbiosis pathway to positively regulate transcription of NF signaling genes (Liu et al., 2018a).

PEPTIDE HORMONES

It is becoming increasingly clear that peptide hormones perform myriad plant developmental functions alongside the classical small-molecule plant hormones (reviewed in Matsubayashi and Sakagami, 2006; Hirakawa and Sawa, 2019). In legume nodulation, several peptide hormones mediate systemic regulation of nodulation to ensure that organogenesis is balanced with internal and environmental signals (Figure 6).

Negative Regulators of Nodulation

The most studied plant peptide hormones in the CLAVATA (CLV)/EMBRYO SURROUNDING REGION (ESR)-RELATED PROTEIN (CLE) family play roles in the regulation of cell division and differentiation in various developmental contexts (Ito et al., 2006; Hirakawa and Sawa, 2019). After processing, CLE peptides typically consist of 12–13 amino acids (aa) and exert diverse effects on the shoot, inflorescence, vascular, and root meristem development in both monocots and dicots (Clark et al., 1996; Hirakawa et al., 2008; Xu et al., 2015; Je et al., 2018).

In legumes, several *Cle* genes are upregulated by rhizobia or NF to activate the AON pathway (see recent review by Ferguson et al., 2019). *LjCle-RS1/2/3*, *MtCle12/13*, and *GmRic1/2* are upregulated by rhizobia inoculation, and overexpression of these genes is sufficient to inhibit nodulation (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011; Nishida et al., 2016). *Lj*NIN can directly bind to the *LjCle-RS1/2* promoter to induce gene expression, and *Mt*NIN is also critical for *MtCle12/13* expression (Mortier et al., 2010; Soyano et al., 2014). In contrast to the early induction of *LjCle-RS1/2*, *LjCle-RS3* is upregulated at later stages of nodule organogenesis, implying that its regulation may differ from that of *LjCle-RS1/2* (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011; Nishida et al., 2016). CLE peptides also participate in nitrate regulation of nodulation, with nitrate inducing the expression of *Lj*Cle-RS2, *Lj*Cle-RS3, and *Gm*Nic1 (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011; Nishida et al., 2016).

After translation, CLEs are processed into 12–13-aa peptides and modified by tri-arabinosylation, which is essential for receptor binding and signal transduction (Okamoto et al., 2013). HYPERNODULATION RELATED GENE1 (*Mt*RDN1/*Lj*PLENTY) is a homolog of the *Arabidopsis* hydroxyproline O-arabinosyltransferases (HPAT1/2/3), which are required for this modification (Ogawa-Ohnishi et al., 2013; Kassaw et al.,

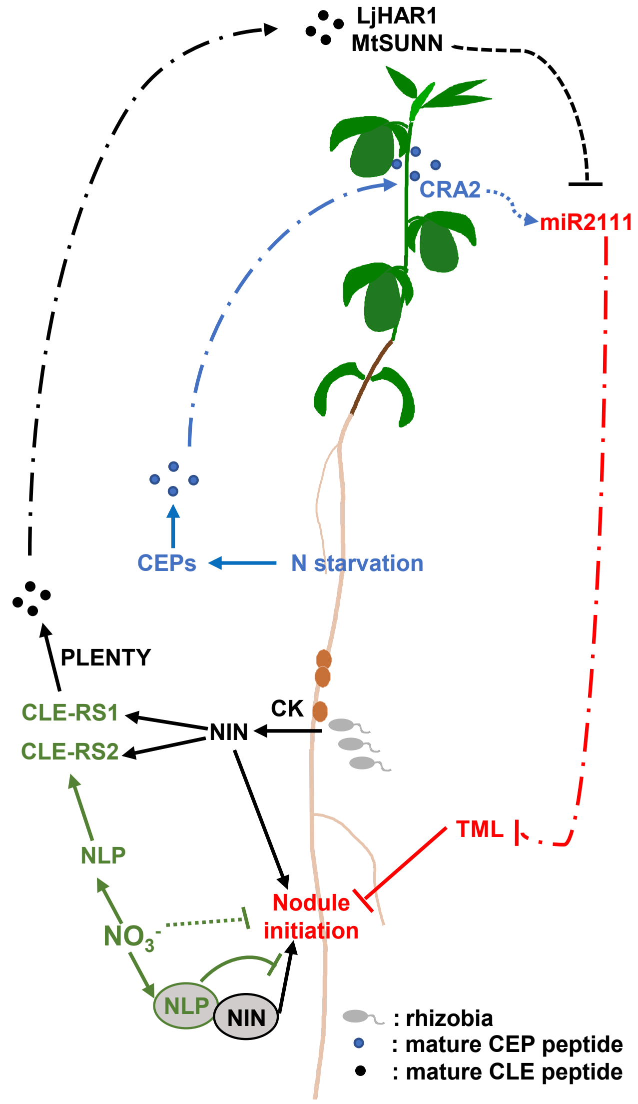

Figure 6. Peptide Hormones as Systemic Regulators of Nodulation.

CK associated with nodule initiation induces NIN and production of CLE-RS peptides. CLE-RS2 is also induced by nitrate via NLP4. Although CLE-RS2 participates in systemic regulation, NLP1 locally inhibits nodulation through competition with NIN for downstream regulatory elements. Processed CLE-RS peptides are transported to, and perceived in, the shoot by HAR1. HAR1 then inhibits miRNA2111 abundance to prevent negative regulation of TML, establishing AON. Nitrogen starvation responses are integrated into this pathway by CEP/CRA2 signaling, which acts antagonistically to HAR1 on miRNA2111 levels.

2017; Yoro et al., 2019). Tri-arabinosylation of *Mt*CLE13 (CLE13-TaP) is sufficient to inhibit nodulation and is dependent on *Mt*RDN1 (Kassaw et al., 2017). Intriguingly, *LjCle-RS3* overexpression reduces nodule number in an *Lj*PLENTY-dependent manner, whereas *LjCle-RS1/2* does not, indicating differences in substrate specificity, peptide stability, or functional redundancy, as in the case of *Mt*RND2 (Kassaw et al., 2017; Yoro et al., 2019).

Mature peptides are transported from root to shoot, where they are perceived by *Lj*HAR1 (Okamoto et al., 2013). Genetic evidence suggests that *Mt*SUNN, *Gm*NARK, *Ps*SYM29, and *Pv*NARK function as equivalent receptors in other legumes, exhibiting hypernodulation and nitrate tolerant nodulation phenotypes (Carroll et al., 1985; Wopereis et al., 2000; Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005; Ferguson et al., 2014). Mutants with similar phenotypes in other Leucine Rich Repeat (LRR) receptor-like kinases indicate that these likely function as part of an AON signaling complex (Miyazawa et al., 2010; Krusell et al., 2011). Activation of HAR1 leads to downregulation of the shoot-to-root mobile miR2111, which is expressed in the shoot but targets the *TML* mRNA for cleavage in the root. Subsequent increased TML levels negatively regulate nodule initiation and infection in the root (Tsikou et al., 2018). TML is a putative Kelch repeat-containing F-box protein and is predicted to target proteins for degradation by E3 ligase activity, but its target remains unknown (Takahara et al., 2013).

In summary, AON functions to systemically control nodule number. Before inoculation, roots remain susceptible to rhizobia infection due to high miR2111 levels and relatively low levels of TML mRNA and protein. When nodule organogenesis events commence, the CLE-dependent induction of the negative regulatory loop is initiated (Figure 6).

Positive Regulators of Nodulation

C-TERMINALLY ENCODED PEPTIDEs (CEPs) are 14- or 15-aa peptides first found to regulate root development in response to nitrogen starvation in *Arabidopsis* (Ohyama et al., 2008). In *M. truncatula*, nitrogen deprivation can also induce the expression of CEPs (Imin et al., 2013). In contrast to *At*CEP, *Mt*CEP1 overexpression or peptide application does not affect root length but instead increases nodule formation, reduces lateral root number, and triggers periodic circumferential cell proliferation (Ohyama et al., 2008; Imin et al., 2013). Processing and modification are crucial for CEP peptides; for instance, hydroxylation or arabinosylation of prolines in *Mt*CEP1 increases or reduces its activity, respectively (Mohd-Radzman et al., 2015; Patel et al., 2018; Taleski et al., 2018).

In *Arabidopsis*, mature *At*CEP1 is transported from root to shoot to be perceived by the LRR receptor kinase *At*CEPR1/2 (Tabata et al., 2014). Similarly, *Mt*CRA2, the homolog of *At*CEPR1, plays a vital role in systemic promotion of nodule formation while acting locally to control root architecture (Huault et al., 2014). Perception of CEP peptides in *Arabidopsis* shoots leads to the production of the shoot-to-root mobile signals *At*CEPD1/2 and *At*CEPDL1/2, which regulate nitrate uptake and assimilation (Ohkubo et al., 2017; Ota et al., 2020). In *M. truncatula*, *Mt*CEPD1 and *Mt*CEPD2 are regulated in the shoot in an *Mt*CRA2-dependent manner, and unlike their *Arabidopsis* orthologs, they also show root expression (Gautrat et al., 2020).

Integration of Systemic Signaling Pathways

Integration of environmental signals into the nodulation program is critical for growth optimization under available conditions, balancing cheap soil nitrate or comparatively expensive fixed nitrogen with the carbon budget. The maintenance of numerous positively and negatively acting pathways is likely to allow finer control over this integration. Both negative feedback through

CLE/HAR1 and positive regulation through the CEP/CRA2 pathways can be induced by cytokinin and NIN (Laffont et al., 2020) and appear to converge on a common regulator in miR2111 (Figure 6; Laffont et al., 2019; Gautrat et al., 2020). HAR1-dependent CK synthesis in the shoot also appears to play a role in systemic nodule regulation (Sasaki et al., 2014), although it has not yet been clearly established whether this also acts through the regulation of miR2111 or through additional, parallel pathways. Regulation of auxin transport is also linked to both CLE and CEP signaling, as *sunn* and *cra2* mutants show increased auxin transport from the shoot (van Noorden et al., 2006; Mathesius et al., 2019; Chapman et al., 2020). Identification of the molecular players that act downstream of CRA2 and HAR1 in the shoot is likely to help clarify these relationships.

OTHER HORMONES

In addition to the four major hormones discussed here, all plant hormones appear to play roles in fine-tuning the nodulation program in response to environmental conditions or through crosstalk with the hormones discussed above. Although further mechanistic insight into their roles will be critical, physiological studies have shown that strigolactone, abscisic acid, brassinosteroids, salicylic acid, and jasmonic acid all play roles in nodule development and have been reviewed elsewhere (Ferguson and Mathesius, 2014).

MANIPULATION OF PLANT HORMONES TO ENGINEER NODULE ORGANOGENESIS IN NON-LEGUMES

The massive environmental impact of agriculture demands the development of more sustainable farming systems. Excessive nitrogen use has already exceeded the safe limits for humanity and requires urgent attention (Rockström et al., 2009; Springmann et al., 2018). While associative nitrogen fixation can provide substantial benefits and is already a part of agricultural practice, the efficiencies derived from symbiotic nitrogen fixation in a specialized organ make engineering an analogous symbiosis in cereals highly desirable.

Induction of lateral root organs (both Lateral roots - LRs, and nodules) by plant hormones, particularly auxin, is a conserved feature of vascular plants. Legume nodules share a transcriptional and developmental program with lateral roots (Xiao et al., 2014, 2019; Bensmihen, 2015; Schiessl et al., 2019; Soyano et al., 2019), and some actinorhizal plants accommodate actinorhizal bacteria in structures that are somewhat intermediate between lateral roots and legume nodules (Hirsch et al., 1997; Pawlowski and Bisseling, 1997). Auxin is critical to both nodule and lateral root processes, with NIN found to directly target *LjAsl18a/MtLbd16*, which coordinates auxin signaling in both nodule and LR initiation (Schiessl et al., 2019; Soyano et al., 2019). In addition, *Lj*NF-YA1 directly targets members of the SHI/STY family, which in *Arabidopsis* regulate auxin biosynthesis and lateral root development (Hossain et al., 2016). The existence of a common developmental program is further supported by the ability of cereals to develop nodule-like structures, which are presumably modified lateral root primordia, when treated with high levels of auxin (Hiltenbrand et al., 2016; Thomas et al., 2018, 2020). In legumes, similar responses can be triggered by auxin transport inhibitors (Rightmyer and Long, 2011; Li et al., 2014; Ng et al., 2015). The existing lateral root developmental program in cereals may therefore be considered a developmental blueprint for engineering a new lateral organ capable of supporting nitrogen fixation if manipulation of auxin signaling can be restricted to a small subset of cells in the root, thereby avoiding detrimental impacts on general root development. Further understanding of the similarities between lateral roots, actinorhizal nodules, and legume nodule development may help to clarify the requirements for a nodule developmental program and inform the engineering of cereals.

Cell division of the indeterminate nodule occurs first in the pericycle before extending to inner cortical cells, which develop into the meristem (Xiao et al., 2014; Bensmihen, 2015; Kohlen et al., 2018). On the other hand, cell division of the determinate nodule occurs first in the cortical cells, without the establishment of a persistent meristem (Xiao et al., 2019). This flexibility indicates that the site of induction is not critical per se, but rather the acquisition of nodule identity factors. The requirement for acquiring and maintaining nodule identity is perhaps best exemplified by the *Noot/Cochleata* regulators, loss of which causes meristem identity and vascular arrangement to alter periodically between lateral root and nodule characteristics (Ferguson and Reid, 2005; Couzigou et al., 2012; Magne et al., 2018; Shen et al., 2020). This indicates that, together with the initiation of a new lateral root organ, manipulation of legume regulators required for nodule identity (e.g., NIN, NOOT) (Griesmann et al., 2018; van Velzen et al., 2018) should be a priority in nodule engineering. A key adaptation of legumes appears to be the susceptibility of the cortical cells to cell division induction by CK (Gauthier-Coles et al., 2019). In addition to their role in nodule initiation, CKs appear to play a key role in the coordination of nodule identity. Gain-of-function mutations in symbiotic (*CCamK*) or cytokinin (*Lhk1*) signaling components give nodule-like structures with characteristics closer to those of true nodules (Gleason et al., 2006; Tirichine et al., 2006, 2007), whereas manipulation of other hormones, including auxin, gibberellin, and regulators such as *Nin* and *Nf-ya1*, induces organogenesis without conferring all nodule characteristics (Soyano et al., 2013; Fonouni-Farde et al., 2017; Schiessl et al., 2019).

In our view, successful engineering of a nodule organ in cereals will therefore require: initiation of lateral root organs through precise manipulation of auxin; establishment of nodule identity on the newly initiated organ, which in legumes is achieved with cytokinin signaling and specific nodule identity regulators; and organ-specific gene expression to support the unique requirements for hosting symbiotic bacteria.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plant hormones are universal regulators of development, yet they play a number of specific roles during the initiation of a nodule organ. Ongoing efforts to identify the symbiotic pathway components that regulate, and are regulated by, plant hormones together with the timing and location of each hormone's action are important next steps. Establishing the profiles of transcripts, proteins, and metabolites at cellular resolution is rapidly emerging as a feasible means to address these challenges. These efforts may help to unravel the nature of signaling between the root surface and initiation of a nodule in deeper cell layers, as well as the underlying characteristics that render a specific root zone susceptible to organ initiation. In our opinion, parallel studies are now needed in multiple species and within ecotypes of a species to identify the degree of flexibility that exists in these regulatory pathways. This understanding of the core nodule initiation program will be critical not only for our ability to improve legume cultivation but also for ongoing efforts to engineer an organ that is competent for nitrogen fixation in diverse non-legume crop species.

Although the engineering of cereal crops for efficient nitrogen fixation is a desirable goal, it may remain out of reach for some time. Ongoing research in nodule development must also focus on meeting demands for sustainable food and feed production. Adverse environmental conditions for crop production, such as insufficient or excess water, mineral toxicities and deficiencies, and varying nutrient conditions, all affect nodule development. To develop improved legume crops that maintain robust nodule development and nitrogen fixation in varied conditions, we must understand how these environmental signals are perceived and integrated with hormone signaling pathways and the nodule development program. This work will also necessitate a switch to greater focus on research in legume crops rather than model legumes for our understanding of nodule development. Improved genomic resources and targeted mutagenesis capabilities in many legumes are already facilitating such a transition.

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