Right time, right place: The dynamic role of hormones in rhizobial infection and nodulation of legumes

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ABSTRACT

Many legume plants form beneficial associations with rhizobial bacteria that are hosted in new plant root organs, nodules, in which atmospheric nitrogen is fixed. This association requires the precise coordination of two separate programs, infection in the epidermis and nodule organogenesis in the cortex. There is extensive literature indicating key roles for plant hormones during nodulation, but a detailed analysis of the spatial and temporal roles of plant hormones during the different stages of nodulation is required. This review analyses the current literature on hormone regulation of infection and organogenesis to reveal the differential roles and interactions of auxin, cytokinin, brassinosteroids, ethylene, and gibberellins during epidermal infection and cortical nodule initiation, development, and function. With the exception of auxin, all of these hormones suppress infection events. By contrast, there is evidence that all of these hormones promote nodule organogenesis, except ethylene, which suppresses nodule initiation. This differential role for many of the hormones between the epidermal and cortical programs is striking. Future work is required to fully examine hormone interactions and create a robust model that integrates this knowledge into our understanding of nodulation pathways.

Keywords: auxin, brassinosteroids, cytokinin, ethylene, gibberellin, nodulation

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INTRODUCTION

The endosymbiosis between plants and nitrogen-fixing bacteria is a unique model of an intimate interaction between a plant and bacteria that has major ecological impacts. Plant symbioses with nitrogen-fixing bacteria appeared about 100 million years ago in a clade of fabids, when it is likely that a common ancestor acquired a predisposition for this association (e.g., Doyle, 2011; Werner et al., 2014; van Velzen et al., 2019). The legume-rhizobial symbiosis occurs via two spatially and temporally separated root programs: infection in the epidermis and organogenesis of the nodule in the cortex (Roy et al., 2020) (Figure 1A). The development of a new nodule organ requires a complete reorganization of existing developmental programs to generate the ideal conditions for nitrogen fixation, including a low-oxygen microenvironment and specialized transporters to exchange nutrients between plant and symbiont.

Coordinating infection and nodule organogenesis requires tight spatial and temporal control as well as precise communication. Plant hormones, small, potent, and often mobile compounds, are unique regulators of nodulation events. In this review, we examine how the plant hormones cytokinin (CK), auxin, gibberellin (GA), brassinosteroid (BR), and ethylene regulate the tight temporal and spatial events during nodulation. As strigolactones appear to act as an ex planta signal on the rhizobium itself (López-Ráez et al., 2017), they will not be considered in this review. We do not seek to provide a comprehensive review of the evidence that these hormones play roles in nodulation, as this is established and reviewed in detail elsewhere (Miri et al., 2016; Kohlen et al., 2018; McGuiness et al., 2019; Lin et al., 2020; Mathesius, 2020). Rather, we will focus on highlighting our current understanding of how spatial and temporal regulation of hormone biosynthesis, catabolism, transport, and/or response regulate and coordinate infection and nodule organogenesis; how hormones may interact; and, importantly, how hormones integrate with key genes and signals of the nodulation pathway. This includes examination of the potential role of hormones as mobile signals that coordinate infection at the epidermis with
nodule formation in the cortex and as regulators of checkpoints during nodulation, as well as identifying actors upstream and downstream of hormones that enable plants to determine the right time and right place for nodulation to occur.

THE INFECTION AND NODULE ORGANOGENESIS PROGRAMS

Many legumes rely on root hair infection via infection threads (ITs), which involves inverted tip growth of the root hair cell wall to deliver the bacteria from the epidermis into the cortex (Oldroyd et al., 2011; Roy et al., 2020; Tsyganova et al., 2021; Figure 1A), and it is these species that are the focus of this review. Further description of the types of rhizobial infection has been provided elsewhere (Gage, 2004; Sprent, 2008; Ibáñez et al., 2017). Careful observations in the model legume *Medicago truncatula* after the addition of compatible rhizobia have revealed early events that facilitate the attachment of the bacteria, including swelling of the root hair tip at 1–3 h post inoculation (hpi), asymmetric tip growth at 3 to 6 hpi, root hair branching at 12 hpi, and hair curling after 16 hpi (Catoira et al., 2000; Larrainzar et al., 2015). About 24 hpi, microcolonies start to form in some of these root hairs in an infection pocket, and the IT starts to develop (Lohar et al., 2006; Larrainzar et al., 2015). In other curled root hairs, no infection occurs, indicating a checkpoint that limits the number of ITs formed. IT formation involves reorganization of actin microfilaments and endoplasmic microtubules, ultimately leading to inward polar growth of the cell wall surrounded by a plasma membrane and containing a matrix with enclosed bacteria, in a process that resembles pollen tube growth (Gage, 2004; Oldroyd et al., 2011). At 48 hpi, the IT reaches the base of the root hair (Larrainzar et al., 2015), and in some cases,
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Infection is halted and no concomitant nodule organogenesis occurs, marking another important check point to control the extent of the symbiosis (Gage, 2004, 2019). In infections that will go on to form nodules, the first cell division associated with nodule organogenesis begins at approximately 36 hpi in specific cells underlying the IT (Figure 1A).

Nodule organogenesis involves nodule initiation (activation of cell division and formation of the nodule primordium), uptake of the bacteria from the infection thread, and nodule development and maturation into a nitrogen-fixing organ (Figure 1A). Following nodule maturation, nodules can also undergo senescence. Legume species are categorized as either forming determinate nodules (e.g., Lotus japonicus and soybean; cell division occurs in the middle/outer cortex, and there is no meristem at maturity) or indeterminate nodules (e.g., M. truncatula, pea; cell division occurs in the inner cortex/pericycle, and there is a meristem at maturity). The IT traverses the cortex through the formation of a microtubule cytoplasmic bridge, termed a pre-infection thread (PIT), in cells ahead of IT entry (Gage, 2004). Concomitantly, nodule organogenesis proceeds as cells divide to form a nodule primordium (48 hpi) and the IT, often branched by this stage, enters the nodule primordium; the rhizobia are then released intracellularly and differentiate into nitrogen-fixing bacteria (Figure 1). Nodules then mature by a process that includes the formation of a meristem and vasculature (65–80 hpi) (Xiao et al., 2014). Once the nodule is mature, reduced carbon from the plant is exchanged for reduced nitrogen via integration and coordination of plant and bacterial metabolism. Nodules are de novo root secondary organs believed to have evolved in part by the recruitment of elements from the lateral root program (Hirsch & Schiel, 1997; Schiel et al., 2019; Soyanova et al., 2019, 2021; Shrestha et al., 2021). As nodules are energetically expensive, plants have evolved mechanisms to control the extent of infection and nodule formation through autoregulatory feedback loops (Ferguson et al., 2019; Chaulagain and Frugoli, 2021). These programs include shoot-root communication through mobile peptides and microRNAs.

Clearly, successful nodulation requires spatial and temporal coordination of the epidermal and cortical programs. The fact that nodules start to form before bacteria reach the cortical cell layers indicates that an as-yet unidentified mobile signal(s) is exchanged between the cell layers (reviewed by Guttair, 2018; Tsyanova et al., 2021). CK has been proposed as a possible mobile signal, but further studies are needed to clearly determine the involvement of hormones in the communication between cell layers (Gamas et al., 2017; Liu et al., 2019). In addition, although the programs are connected, they can be regulated independently, as illustrated by the fact that altering the extent of infection does not always translate into a concomitant change in nodule number (e.g., Murray et al., 2007; Madsen et al., 2010). For example, mutants have been identified that genetically uncouple these programs, such as rpg and symRK-14 in Medicago and Lotus, respectively (Arrighi et al., 2008; Kosuta et al., 2011), and nin mutants with altered cis-regulatory regions in Lotus and Medicago (Yoro et al., 2014; Liu et al., 2019).

The nodulation process is triggered in the plant by the recognition of Nod factors, specific lipo-chitooligosaccharide molecules produced by rhizobia and perceived by Nod factor receptors in the root epidermis (Wang et al., 2012). This perception triggers calcium (Ca2+) oscillations in the nucleus via specific nucleoporeins and ion channels (Harzil and Oldroyd, 2013). These Ca2+ oscillations are in turn perceived by the calcium and calmodulin-dependent kinase (CaMK) (Oldroyd et al., 2011), which interacts with and phosphorylates a protein complex containing the transcription factors CYCLOPS/IPD3. Downstream events include activation of the expression of key transcription factors such as Nodule Inception protein (NIN), ERN1, NSP1, and NSP2, which in turn regulate the expression of genes that promote infection and organogenesis (Roy et al., 2020). Nod factor signaling continues throughout IT growth and is essential for infection, as mutants in the above-mentioned genes fail to make infections. Interestingly, breakdown of Nod factors by plant hydrolases also appears to be important for these processes (Cai et al., 2018; Malolepsz et al., 2018). Although genes and pathways in addition to Nod factor signaling, such as exopolysaccharide and reactive oxygen species signaling, play important roles in nodulation (for review, see Roy et al., 2020), few studies have examined the connection of these elements to plant hormones, and this review will therefore focus on the integration of plant hormones with the Nod factor pathway.

It is interesting to note that the formation of nodule-like organs (often termed pseudonodules or spontaneous nodules) can be elicited in the absence of rhizobia by application of Nod factors, activation of the key downstream element CCaMK, and ectopic CK application or response (Niwa et al., 2001; Tirichine et al., 2007; Hayashi et al., 2010, 2014; Kosuta et al., 2011; Jin et al., 2016). Cell divisions leading to bumps in roots can also be induced by the application of auxin-modifying compounds and GA (Kohlen et al., 2018; Akamatsu et al., 2021). Nonetheless, it is important to note that the exact cells from which these structures are derived and their structure are not always reported and/or can in some cases differ in important ways from rhizobial-induced nodules (reviewed by Kohlen et al., 2018); therefore, care needs to be used in interpreting these results.

We have undertaken a critical review of the role of CK, auxin, GA, BR, and ethylene during the different stages of nodulation, with the aim of highlighting the distinct roles played by these hormones in epidermal infection and cortical nodule organogenesis. Clear gaps in our understanding are identified and future directions are outlined.

**EPIDERMAL INFECTION**

**Ethylene, GAs, and BRs negatively regulate epidermal infection**

Ethylene, GA, and BRs all suppress epidermal infection. This is best understood for ethylene; studies using the application of ethylene and ethylene synthesis inhibitors and mutants disrupted in ethylene signaling indicate that ethylene strongly inhibits root hair curling and IT formation (Penmetsa and Cook, 1997; Nukui et al., 2004; Lohar et al., 2009; Larrazanz et al., 2015; McAdam et al., 2018; Reid et al., 2018; McGuiness et al., 2020). Ethylene appears to inhibit Nod factor response via suppression of Ca2+ spiking (Oldroyd et al., 2001). Indeed, rhizobial-induced gene
expression is significantly upregulated in the skl ethylene-insensitive mutant of Medicago (Breakspear et al., 2014). Ethylene may also limit infection by activating defense responses during rhizobial invasion (Guinel, 2015) and influencing the expression of cytoskeleton genes important for the control of root hair deformation and IT progression (Larrainzar et al., 2015).

Spatial or temporal differences in ethylene production or response may permit the development of some infections and the abortion of others. In Lotus, an increase in ethylene production has been detected in whole roots as soon as 6 hpi, with a peak at 24 - 48 hpi, and this is dependent on elements of the Nod factor signaling pathway (Reid et al., 2016). This increase in ethylene production is consistent with elevated expression of the Medicago MtACC synthase (ACS) gene, which encodes a rate-limiting enzyme in ethylene biosynthesis, at 6 hpi and the expression of ASC::GUS in root epidermal cells exposed to bacteria (Larrainzar et al., 2015). Application of Nod factors also leads to rapid induction of MtACS expression (van Zeijl et al., 2015) and ethylene production (Reid et al., 2018). This elevation of ethylene biosynthesis coincides with uptake of the bacteria and initiation of nodule primordia (Figure 1). Interestingly, strong activity of the ACS::GUS reporter has been detected in arrested ITs, indicating that enhanced ethylene synthesis may inhibit the progression of some ITs (Larrainzar et al., 2015). Further insight into ethylene’s potential role in controlling infection checkpoints would be gained from understanding the spatial and temporal dynamics of an ethylene response reporter or biosensor during nodulation. For example, some transcriptional reporters generated for ethylene, such as 5xEBS::GUS, have not yet been evaluated in the nodulation context, and there is room for the development of additional fluorescence resonance energy transfer (FRET) or degradation-based biosensors for this hormone (Fernandez-Moreno and Stepanova, 2020). Potential interactions between ethylene and other plant hormones are outlined in detail below.

Bioactive GAs are also strong negative regulators of epidermal infection (Figure 1B). GA-deficient mutants, transgenic lines with reduced GA, and chemical suppression of GA biosynthesis result in increased infection (Ferguson et al., 2005; Kim et al., 2019; Maekawa et al., 2009; McAdam et al., 2018). Similarly, application of GA reduces IT numbers (Fonouni-Farde et al., 2016; Jin et al., 2016). Consistent with a positive role for DELLA in infection, in Medicago, a gain-of-function mutant version of the del1a protein resistant to GA degradation and fused to GFP is activated in root hairs within 6 hpi (Fonouni-Farde et al., 2016). In addition, loss-of-function del1a mutants show a reduction in IT numbers, and gain-of-function del1a mutants display elevated IT numbers (Fonouni-Farde et al., 2016; McAdam et al., 2018).

GA inhibition of epidermal infection may act independently of ethylene. Although GA-deficient na mutants in pea produce significantly more ethylene, these plants still form a large number of ITs (McAdam et al., 2018). This suggests that GA does not promote ethylene to inhibit IT formation. In addition, the introduction of an ethylene-insensitive mutation (ein2) into na mutant plants increases the number of ITs relative to that in ein2 single mutants, suggesting that the effects of both mutations on infection are somewhat additive (McAdam et al., 2018). It is interesting to note that GA biosynthesis and catabolism genes are strongly upregulated in an ethylene-insensitive mutant of Medicago within 48 hpi (Kim et al., 2019), although given the strong feedback regulation of the expression of genes in the GA pathway (e.g., Reid et al., 2002), it is not clear what influence this has on GA levels. To date, the crosstalk of GA with auxin in epidermal infection has not been examined directly, whereas the potential interaction between GA and CK during infection is outlined below.

In a similar manner to ethylene and GA, BRs also appear to suppress rhizobial infection. A small increase in IT number was observed in severely BR-deficient mutants of pea (McGuiness et al., 2020). This did not appear to be mediated through ethylene but was correlated with elevated auxin response as measured by DR5::GUS in the cortex in response to inoculation but, interestingly, not in infected root hairs (McGuiness et al., 2020). This suggests that BRs may negatively regulate early events in nodulation by influencing auxin accumulation in the cortex (McGuiness et al., 2020), and the role of auxin in this process is covered in more detail below. In Medicago, elevated expression of BR biosynthesis and signaling genes has been recorded in root hairs containing ITs (Breakspear et al., 2014), but it is not yet clear how this might translate into spatial or
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temporal changes in BR level or response. To date, there is no information on how the BR pathway may intersect with the Nod factor signaling pathway.

Cytokinin negatively regulates infection, possibly via feedback regulation

In Lotus, there is strong experimental evidence that CK is a negative regulator of epidermal infection. Mutation of the CK receptor (ihk1) leads to hyperinfection (Murray et al., 2007; Held et al., 2014; Miri et al., 2019), and conversely, elevated CK levels due to CK application or mutation of the CK catabolic enzyme CKX3 lead to suppression of infection (Reid et al., 2016). Elevated ITs in ihk1 mutants are arrested in the epidermis (Held et al., 2014), suggesting that CK signaling influences the checkpoint of epidermal to cortex progression of some ITs. CK action during epidermal infection is tightly regulated by catabolic enzymes, as cklx3 mutants develop fewer infections in Lotus (Reid et al., 2016). However, LjCKX3 expression is not detected in the epidermis during infection, indicating that CK levels in the epidermis may be regulated by CK produced in the cortex (Reid et al., 2016). This is supported by the fact that, in Lotus, the activation of the TCSn CK-responsive promoter occurs initially in the root cortex 24 hpi; TCSn activation only spreads to the root epidermis 48 to 120 hpi, and it is absent in epidermal or cortical cells with developed ITs (Reid et al., 2017). Thus, CK might be a negative-feedback signal that moves from the cortex to the epidermis to limit the extent of the infection (Miri et al., 2016). Whether CK acts directly as a mobile signal or via another signal such as ethylene is discussed below.

Studies in Medicago have revealed a somewhat different picture of the role of CK in infection. In Medicago, Nod factor signaling induces an increase in bioactive CK levels within hours (van Zeijl et al., 2015; Jarzyniak et al., 2021), and CK signaling, as analyzed by activity of the TCSn promoter, is activated in the root epidermis as early as 8 hpi, even before the cortical CK activity is triggered (Jardinaud et al., 2016). cre1 mutants or RNAi lines that target MtCRE1 in Medicago form a similar number of ITs as control plants, in contrast to the hyperinfection of similar Lotus mutants. However, as the majority of ITs in these plants were arrested in the epidermis, this does suggest a negative influence of CK on IT development (Gonzalez-Rizzo et al., 2006; Plet et al., 2011). Furthermore, a negative role for CK in epidermal infection of Medicago is supported by the fact that ectopic expression of the CK-degrading enzyme MtCKX3 in the epidermis enhanced nodule number (but not infection) and that Nod factor signaling is suppressed by exogenous CK (Jardinaud et al., 2016). Recently, the CK ATP-binding cassette (ABC) transporter MtABC56 was shown to act in the epidermis during interaction with rhizobium. Transgenic, mutant, and gene expression studies indicate that MtABC56 may enable export of CK from the epidermis, which may in turn positively influence nodule organogenesis in the cortex of Medicago (Jarzyniak et al., 2021). This suggests a role for epidermal-derived CK in communication between the epidermis and cortex. Furthermore, the small but significant decrease in ITs observed in abcg56 mutants compared with the wild type suggests that a buildup of CK in the epidermis might suppress infection thread formation (Jarzyniak et al., 2021).

CK may negatively regulate infection in the epidermis by enhancing ethylene biosynthesis and/or response. In Medicago, the expression of the ethylene biosynthesis MtACS gene is activated during nodulation in a CRE1-dependent manner (van Zeijl et al., 2015), and an increase in ethylene production is seen in ihk CK receptor gain-of-function mutants in Lotus (Reid et al., 2018) and in pea after treatment with CK (Lorteau et al., 2001). The mechanism of CK-increased ethylene production may act through ACS stabilization (Hansen et al., 2009). Thus, negative-feedback regulation of infection by CK may occur via ethylene. This regulation might be triggered after a CK maximum is reached in the cortex 24 to 48 hpi, a time that coincides with the peak of ethylene production in L. japonicus (Reid et al., 2017, 2018). Cytokinin has also been proposed as a negative shoot-to-root signal in autoregulation of nodulation (Sasaki et al., 2014). Crosstalk between CK and auxin during infection is outlined below. Potential crosstalk between CK and GA has been observed in non-symbiotic roots of Medicago (Fonouni-Farde et al., 2017, 2018), although it is not yet clear if this interaction also occurs during nodulation. In pea, upregulation of the expression of CK biosynthesis genes was not seen in constitutive GA-response delta mutants, suggesting that GA may suppress CK. However, as this coincided with the timing of nodule development and not IT formation, this may reflect an interaction that occurs during organogenesis rather than infection (Dolgikh et al., 2019).

Auxin is a positive regulator of epidermal infection

Although auxin plays a key role in promoting nodule organogenesis (see below), there is mounting evidence that auxin may also positively regulate infection in the epidermis (Figure 1B). Blocking auxin biosynthesis through the chemical inhibition of the YUC auxin biosynthesis proteins reduces IT formation in Lotus (Nadzieja et al., 2018). Furthermore, increased auxin response as measured by the DR5 promoter or DII biosensor has been observed in root hairs exposed to rhizobium or following Nod factor treatment in Medicago (Breakspear et al., 2014) and Lotus (Nadzieja et al., 2018). In Lotus, DR5 was activated in root hairs with ITs (Nadzieja et al., 2018), although in pea, activation of DR5 in root hairs did not coincide with IT formation but rather appeared to precede infection (McGuiness et al., 2019). This elevated auxin response in Lotus is Nod-factor dependent and occurs upstream of CCaMK because ccamk mutants are still able to trigger early auxin accumulation in root hairs (Nadzieja et al., 2018). This indicates a possible role for auxin in regulating upstream nodulation responses or coordinating early events for bacterial entrance to the root. This early role for auxin in promoting infection is supported by transcriptome analysis of root hairs; expression of the MtARF16 auxin response factor was notably upregulated upon rhizobial inoculation, and mutation of this factor caused a reduction in the number of ITs in Medicago (Breakspear et al., 2014). However, a fine balance of auxin may be required, as overexpression of GmYUC2, which encodes an auxin biosynthesis enzyme in soybean, resulted in less root hair curling and fewer nodules, although it is not clear whether IT formation was affected (Wang et al., 2019).

Changes in auxin response during infection could be due to altered biosynthesis, transport, and/or response. Changes in
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auxin transport have been observed in the root following application of rhizobia. Nod factors, or flavonoids, leading to local auxin maxima (reviewed by Kohlen et al., 2018). However, to date, no mutant studies have identified a specific auxin biosynthesis enzyme or transporter that specifically influences IT formation. Although mutation of the auxin influx carrier MtLAX2 in *Medicago* resulted in fewer nodules, it is not clear whether this was due to altered IT formation (Roy et al., 2017). The role of PIN efflux carriers in nodulation has also been explored in *Medicago* using RNAi lines and the pin2 mutant. Although RNAi targeting of *MtPIN2*, -3, and -4 resulted in fewer nodules (Huo et al., 2006), mutation of the auxin transporter *MtPIN2* suppressed basipetal auxin transport in roots but did not influence nodulation (Ng et al., 2020). Similarly, in soybean, *GmPIN1a*, -b, and -c appeared to be required to promote nodule number, but infection was not assessed (Gao et al., 2021). An auxin biosynthesis enzyme encoded by *TAR1* was upregulated during infection of *Lotus*, but mutation of this gene did not change IT formation (Suzaki et al., 2012; Nadzieja et al., 2018). Overexpression of the soybean auxin receptor *TIR1* resulted in elevated root hair curling and nodule number, possibly via interaction with microRNA393, but it is not clear whether this rise in nodulation resulted from altered IT formation (Cai et al., 2017). Clearly, studies examining nodulation should assess both IT formation and nodule number to delineate the role of auxin in these processes, and future studies could test whether auxin moves between the epidermis and the cortex to coordinate these programs.

There is some evidence to suggest antagonism between auxin and CK during rhizobial infection. Auxin may influence CK levels in the epidermis of *Lotus* by reducing the expression of genes that encode the CK biosynthesis enzymes *LjLOG4* and *LjCYP735a* that accumulate during nodulation (Nadzieja et al., 2018). Downregulation of these genes may restrict CK accumulation in root hairs and enable infection progression. Interestingly, application of CK induced an epidermal auxin response, and the CK receptor mutant *lk1* displayed induction of an auxin response in both infected and neighboring uninfected *Lotus* root hairs (Nadzieja et al., 2018). This suggests feedback mechanisms that fine-tune hormone balance to enable infection to progress in a limited number of root hairs. Indeed, the low-nodulation phenotype of the CK receptor mutant *cre1* in *Medicago* can be restored by the local application of auxin transport inhibitors that allow the accumulation of auxin in nodule primordia (Ng et al., 2015), although it is not clear whether this is due to an influence on infection or only organogenesis. Interactions between auxin and ethylene have also been observed, as hyperinfected and hypernodulated ethylene-insensitive mutants of *Medicago* display altered auxin transport during nodulation (Prayitno et al., 2006a). However, it is not clear whether this is due to influences on infection, nodule organogenesis, or both. Conversely, given the interaction between CK and ethylene outlined above, it is possible that auxin interacts with ethylene, and ultimately infection, via CK.

A possible mechanism of auxin promotion of infection could be related to an increase in host susceptibility to microorganism infection. Auxin is involved in increasing susceptibility of host plants to pathogen invasion, mainly by repressing the expression of pathogenesis response (PR) genes (Kazan and Manners, 2009). Rhizobial interaction with root hairs activates defense-related genes (Nakagawa et al., 2011; Gourion et al., 2015), and auxin could be implicated in reducing this response to allow infection. Auxin and CK play antagonistic roles in modulating the plant response to microbial infection in plant-pathogen interactions, as CK positively regulates plant defense-promoting PR gene expression (Naseem and Dandekar, 2012). Greater understanding of the role these components in plant-microbe interactions may shed further light on the evolution of nodulation.

**NODULE ORGANOGENESIS**

**Cytokinin is key to the activation of nodule organogenesis**

CK signaling appears to play a major role in nodule organogenesis (Figure 1B). Studies with the loss-of-function CK receptor mutants *lk1* and *cre1* indicate that cytokinin perception promotes nodule initiation in the cortex of *Lotus* and *Medicago* (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007; Held et al., 2014; Boivin et al., 2016). This is a direct effect on nodule organogenesis, as gain-of-function *lk1*/*cre1* mutants induce the formation of spontaneous nodules in the absence of rhizobia and do so downstream of *CCaMK* but upstream of *NSP1*, *NSP2*, and *NIN* (Murray et al., 2007; Tirichine et al., 2007; Hayashi et al., 2010; Madsen et al., 2010; Heckmann et al., 2011; Jin et al., 2016). Indeed, spontaneous nodules are only induced by exogenous CK application or ectopic expression of CK biosynthesis genes in nodulating legume species but not by CK application in non-nodulating species (Reid et al., 2017; Gauthier-Coles et al., 2019). CK also appears to influence nodule development and function, as disruption of CK signaling or biosynthesis resulted in delayed nodule development, reduced nitrogen fixation, and premature nodule senescence across species (Plet et al., 2011; Chen et al., 2014; Boivin et al., 2016; Reid et al., 2016). The activation of CK response in the cortex, as measured by the *TCSn* promoter and the expression of CK type-A and type-B response regulators, occurs within hours of inoculation or Nod factor application, and in some cases this has been localized to the cortex (Lohar et al., 2004, 2006; Plet et al., 2011; van Zeijl et al., 2015; Reid et al., 2017). Indeed, mutant studies indicate that these downstream CK type-A and type-B response regulators play important roles in promoting nodule number (Ariel et al., 2012; Tan et al., 2020). Recently, Nod factor-induced CK transported from the epidermis has been suggested to activate the CK response and hence nodule organogenesis in the cortex of *Medicago* (Jarzyniak et al., 2021). Interestingly, activation of the CK response in *Medicago*, including the development of spontaneous nodules in the cortex, requires *NIN* activation in the epidermis, suggesting an important link between *NIN* and CK during nodule initiation and development (Vernie et al., 2015). Indeed, CK appears to act both upstream and downstream of *NIN*, as *NIN* activation in the cortex may be promoted by CK (Heckmann et al., 2011; Liu et al., 2019). Tight spatial and temporal regulation of the expression of CK biosynthesis and catabolism genes in cells that go on to form nodules suggests that localized CK accumulation is important for nodule initiation and development in *Lotus* and *Medicago*. Elevated CK levels are seen in roots 24–72 hpi and also after...
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Nod factor application (van Zeijl et al., 2015; Reid et al., 2016). This appears to be due to a combination of changes in biosynthesis, catabolism, and transport. In response to inoculation, Nod factor signaling via CCA1, CYCLOPS, NSP1, and NSP2 activates the expression of CK biosynthesis genes (Chen et al., 2014; van Zeijl et al., 2015; Reid et al., 2016, 2017; Triozzi et al., 2022). CK biosynthesis genes show a range of spatiotemporal expression as evaluated by promoter::reporter fusions. Ipt3 is expressed mainly at the base of the nodule and in the vasculature of mature nodules in Medicago and Lotus (Chen et al., 2014; Reid et al., 2017; Triozzi et al., 2022). In Lotus, Lipt2 expression is found in the middle and outer cortical layers, whereas Lipt4 has a cortical expression 3 dpi and is present in the mature nodule vasculature, and Lipt4 is expressed in dividing cells and nodule primordia (Reid et al., 2017). When tight spatial and temporal regulation of multiple CK biosynthesis genes is deregulated, many fused nodules develop in response to inoculation, emphasizing the importance of discrete CK foci for normal nodule initiation and development (Reid et al., 2017). Elegant reporter studies indicate that CK produced via the activation of MtIPT3 in the pericycle at the base of the nodule primordium may move into the developing nodule (Triozi et al., 2022). Discrete CK catabolism may also be required to focus CK levels in cells that form nodules, as disruption of CK catabolism in Lotus chx3 mutants also impairs nodule initiation, development, and function (Reid et al., 2016). In addition, mutation of the CK transporter MtABC6 in Medicago suppresses nodule initiation, and as outlined above, this may be due to reduced capacity to transport CK from the epidermis (Jarzyniak et al., 2021).

Auxin correlates strongly with nodule organogenesis

There is strong spatial and temporal regulation of the auxin response in the cells that go on to form the nodule and during nodule development, probably achieved via spatial regulation of auxin transport and/or auxin biosynthesis. This suggests that auxin maxima are required to initiate and sustain cell division during nodule development. Similarly, there is a strong correlation between auxin response and the development of the nodule meristem and vasculature. Auxin response, as indicated by auxin responsive promoters or biosensors such as GH3, DR5, and LjDII, occurs in cells undergoing the first divisions leading to nodule primordia, and later in nodule development, it is restricted to cells surrounding the infected region and in the vascular bundle in species that form either determinate or indeterminate nodules (Mathiesius et al., 1998; Huo et al., 2006; van Noorden et al., 2007; Takanashi et al., 2011; Suzuki et al., 2012; Turner et al., 2013; Breakspear et al., 2014; Ng et al., 2015; Gao et al., 2021).

Although elevated auxin response occurs during noduleation, there are intriguing differences in the dynamics of auxin transport and biosynthesis between plant species that form determinate or indeterminate nodules. A clear but transient increase in auxin level and a decrease in acropetal polar auxin transport occur in Medicago 24 hpi below the site of nodule initiation (e.g., Ng and Mathiesius, 2018). This may be dependent on flavonoids, since the reduction of flavonoid biosynthesis in Medicago disrupts local inhibition of polar auxin transport, reducing auxin accumulation and nodule formation, and this appears to be a direct effect on organogenesis, as infection was not affected (Wasson et al., 2006). Flavonoids have also been shown to elevate the expression of the key auxin transport protein GmPIN1b in the cortex of soybean (Gao et al., 2021). Gene expression, mutants, and transgenic studies in Medicago indicate that this may involve both auxin efflux and influx carriers, as well as auxin biosynthesis (Huo et al., 2006; Roy et al., 2017; Schiessl et al., 2019). Indeed, suppression of auxin transport may be an important driver of nodule organogenesis across indeterminate species, as the addition of auxin transport inhibitors produces spontaneous nodules in a range of indeterminate, but not in determinate, species such as Lotus and soybean (reviewed by Kohlen et al., 2018). Interestingly, in Lotus and soybean, a decrease in auxin level is seen at the site below nodule initiation along with a sustained increase in acropetal polar auxin transport following inoculation with rhizobia (Ng and Mathiesius, 2018; Gao et al., 2021). This clear difference in auxin dynamics between determinate and indeterminate species may be due to the fact that different cell layers are activated early in nodule organogenesis (outer cortex versus inner cortex, pericycle, and endodermis, respectively) and that these cell layers have different auxin sensitivities. A recent study examining the role of PIN influx carriers in soybean demonstrated unique patterns of PIN protein distribution and polarity during various stages of nodule organogenesis, suggesting that fine-tuning of auxin distribution occurs during nodule development in this species. This study identified a key role for PIN1a, -b, and -c in the promotion of nodule number, although, as infection was not assessed, it is difficult to determine the role of these genes (Gao et al., 2021). There also appears to be a redundant role of GmPIN1a, -b, and -c with GmPIN9 in promoting nodule expansion by directing auxin supply into the nodule vasculature.

The correlation between auxin response and nodule organogenesis is striking. Legumes with disrupted auxin biosynthesis, transport, or response via mutation or transgenics provide a powerful tool to test this correlation, but in many cases, it is still not clear whether the influence of altered auxin in these lines is on infection, nodule initiation and development, or both, as often only nodule number is reported, as outlined above in relation to the influence of auxin on infection. Studies examining the interaction between auxin and CK have revealed that auxin may act downstream of CK, via the action of NIN, to control nodule organogenesis. Spatial regulation of auxin transport and response is disrupted in cre1 mutants in Medicago, and auxin transport inhibitors together with flavonoids partially rescue the reduced nodulation of cre1 (Plet et al., 2011; Rightmyer and Long, 2011; Ng et al., 2015). Indeed, CK-induced flavonoid production is required for auxin accumulation during nodulation (Ng et al., 2015). An upregulation of auxin by CK during nodule organogenesis is supported by the observation of elevated auxin response in spontaneous nodules induced by CK or gain-of-function Lkh1 CK receptor mutants (Mathiesius et al., 2000; Suzuki et al., 2012; Schiessl et al., 2019). In soybean, CK also directs GmPIN1b polarity during nodule development (Gao et al., 2021). The formation of auxin maxima is also required for lateral root primordia, but, distinct from its positive role in nodulation, CK suppresses lateral root initiation. Recent work in Medicago has identified LOB-DOMAIN PROTEIN 16 (LBD16), which activates local auxin biosynthesis to promote both lateral root and...
nodule formation. The expression of MtLBD16 is controlled by CK via the action of NIN, which in turn promotes the expression of the auxin biosynthesis genes STY and YUC, providing a nodulation-specific pathway (Schissel et al., 2019). A similar interaction occurs in Lotus, where LjLBD16a acts downstream of CK activation of NIN, which in turn activates the expression of auxin biosynthesis YUC genes (Soyano et al., 2019; Shrestha et al., 2021). Clearly, more studies are needed to fully understand how auxin and cytokinin regulate nodulation by acting through key developmental genes.

The role of GAs in nodule organogenesis

Studies have reported both positive and negative effects of bioactive GA on nodule organogenesis. There is strong evidence for a positive role of GA in nodule initiation and development in species with determinate and indeterminate nodules (Figure 1B). Despite an increase in IT formation in some cases, transgenic or mutant lines with disruptions in biosynthesis leading to low GA levels in pea, soybean, and Medicago display low nodule numbers (Ferguson et al., 2005; McAdam et al., 2018; Kim et al., 2019; Chu et al., 2022), suggesting a direct role for GA in promoting nodule initiation. It is interesting to note that application of GA also promotes nodulation in Sesbania rostrata when the infection is via crack entry, which further indicates a positive and direct role for GA in the cortex program (Lievens et al., 2005). In addition, GA application can induce division of pericycle cells to form nodule-like structures in Lotus and Trifolium repens (Kawaguchi et al., 1996; Akamatsu et al., 2021). In Lotus, these GA-induced spontaneous nodules require NIN, and indeed the LjNIN promoter contains a GA-responsive element, suggesting that GA may interact with NIN to promote nodule initiation and development (Akamatsu et al., 2021). However, as these structures were not formed in the legume Medicago sativa following GA application, it is difficult to infer a general role for GA in the promotion of nodule-like structures in legumes (Kawaguchi et al., 1996; Akamatsu et al., 2021). In pea, normal GA levels are required for pea nodules to develop into large nitrogen-fixing organs (McAdam et al., 2018) with sufficient dicarboxylate metabolism to supply energy to the bacteria (McGuiness et al., 2021). Similarly, in soybean, CRISPR lines targeting GA biosynthesis GmGA2ox1 genes developed smaller nodules than control lines (Chu et al., 2022). GA also promotes nodule meristem bifurcation in pea (Serova et al., 2019). GA may promote nodule initiation by inhibiting ethylene production, as nodule number can be restored in GA-deficient pea lines with a mutation in the ethylene response element EIN2. However, as nodule size and function were not restored in double mutants that were ethylene insensitive and GA deficient, GA appears to act independently of ethylene to promote nodule development and function (Ferguson et al., 2011; McAdam et al., 2018). The fact that nodule function is not compromised in loss-of-function della mutants of pea is also consistent with a positive role for GA in nodule development (McAdam et al., 2018).

However, it is important to examine why elevated GA signaling or level can also result in reduction in nodule initiation and in some cases, nodule size, as observed in many species following GA application, della mutants, or transgenic lines with impaired GA catabolism (reviewed by McGuiness et al., 2019) (Chu et al., 2022). In some cases, this may be due to the negative role of GA in infection outlined above, which would be expected to have a knock-on negative effect on nodule number. In the case of soybean, overexpression of the GA catabolism gene GmGA2ox1 resulted in fewer nodules than control lines, although it was not clear whether this was due to elevated GA leading to a suppression of infection (Chu et al., 2022). However, the role of GA in nodule organogenesis may be complex, as there is also evidence of GA playing a direct negative role in nodule organogenesis, independent of the negative effect of GA on infection. GA application or loss-of-function della mutants inhibit spontaneous nodule formation in ccamk and CK receptor gain-of-function mutants and block the upregulation of expression of nodulation genes such as NSP2, NSP1, NIN, and ERN1 during the first 48 h after Nod factor stimulation, a time when nodule organogenesis is taking place and would require the activation of these genes in Lotus and Medicago (Maekawa et al., 2009; Fonouni-Farde et al., 2016; Jin et al., 2016). DELLA may have a direct role in organogenesis of Medicago, as the expression of della dominant active protein induces spontaneous nodules, although their number and detailed structure were not provided (Fonouni-Farde et al., 2017). However, although the expression of a DELLA dominant active gene in the cortex and epidermis recovered the low nodulation phenotype of della mutants, it did not induce the formation of more nodules than wild-type Medicago plants, indicating that DELLA alone does not increase nodule organogenesis (Fonouni-Farde et al., 2017).

Studies across several species have found elevated expression of GA biosynthesis and inactivation genes during infection and organogenesis, making it difficult to predict GA hormone levels during these processes. Indeed, as GA accumulation is tightly regulated by a feedback mechanism mediated by metabolic enzymes (Hedden and Sponsel, 2015), inferring GA levels from the expression of biosynthesis or catabolism genes is difficult. Both GA catabolic and biosynthetic enzymes are expressed in response to rhizobia inoculation within 6–48 hpi (Hayashi et al., 2012; Kim et al., 2019; Chu et al., 2022). As mentioned previously, the initial upregulation of these enzymes could be associated with infection, but their continued expression after 24 hpi indicates that these proteins may be involved in nodule initiation. Indeed, promoter fusion studies in soybean found that expression of the GmGA2ox1 GA biosynthesis gene was elevated in the outer cortex and pericycle at 3 dpi and later became concentrated in the nodule primordium and dividing and expanding cells of the developing nodule. Expression of the GA catabolism gene GmGA2ox1 was not apparent in the cortex until 5 dpi and was later associated with nodule primordia and dividing nodule cells (Chu et al., 2022). Similar overlapping patterns of GA biosynthesis and catabolism gene expression in nodule primordia have been shown in other species by promoter::GUS fusions (Lievens et al., 2005; Kim et al., 2019; Akamatsu et al., 2021), suggesting that tight spatial and temporal regulation of GA levels during these processes is important. Consistent with a role for GA in nodule initiation and development, GA response as measured by Gibberellin Perception Sensor 1 (GPS1) was observed in dividing and expanding cells of soybean nodules (Chu et al., 2022). Careful manipulation of GA levels and signaling, as has occurred for auxin and CK, is now needed to clarify the role of GA in nodule organogenesis.
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organogenesis. Given the central role of CKs in nodule organogenesis, further studies examining whether GA is acting upstream or downstream of CK during nodule initiation and development will also be informative. Furthermore, as previous studies have shown that auxin positively regulates bioactive GA accumulation by stimulating GA biosynthesis in elongating stems (Weston et al., 2009; O’Neill et al., 2010), closer examination of the interaction between auxin and GA during nodule organogenesis is needed.

Ethylene negatively regulates nodule organogenesis

Evidence that ethylene is a negative regulator of nodule initiation and development (Figure 1) is supported by studies on the application of ethylene and biosynthesis inhibitors and work with ethylene-insensitive mutants (Lee and LaRue, 1992; Pennmetsa and Cook, 1997; Schmidt et al., 1999; Oldroyd et al., 2001; Nukui et al., 2004; Lohar et al., 2009; Foo et al., 2016; Berrabah et al., 2018; Reid et al., 2018). The inhibitory effect of ethylene on nodule organogenesis is independent of rhizobial infection, as ethylene reduces spontaneous nodules formed by ccamk and lhk gain-of-function mutants and induced by the application of CK in Lotus (Tirchine et al., 2006; Heckmann et al., 2011). Ethylene may be involved in the positioning of the nodule and the regulation of nodule numbers. Studies have shown that wild-type nodule primordia do not develop at phloem poles, where ACC oxidase is expressed, whereas mutants impaired in ethylene perception form nodules at both phloem and xylem poles, indicating that ethylene accumulation may send a signal to position nodule primordia formation (Heidstra et al., 1997; Gresshoff et al., 2009; Lohar et al., 2009; Miyata et al., 2013; Foo et al., 2016). Ethylene appears to negatively regulate nodule size and nodule meristem formation (e.g., Xiao et al., 2014). Mature nodules of ethylene-insensitive ein2 pea mutants have a nodule metabolome and nitrogen-fixation capacity similar to those of wild-type nodules (McAdam et al., 2018; McGuinness et al., 2021), and a similar mutant in Lotus forms a large number of bacteroids per symbiosome (Lohar et al., 2009). The reduced nitrogenase activity in Lotus ethylene-insensitive lines compared with the wild type may be an indirect effect of smaller, underdeveloped nodules, as nitrogenase activity was not expressed per unit nodule weight but rather per plant (Reid et al., 2018). Therefore, overall, ethylene does not appear to promote nodule function. Indeed, application studies suggest that high levels of ethylene in nodules may trigger plant defense responses via EIN2, and suppression of this response may be an important factor in maintaining nodule function (Berrabah et al., 2018), suggesting that elevated ethylene can suppress nodule function.

The mechanism of ethylene inhibition of nodule initiation may involve the regulation of CK and auxin responses. Ethylene-insensitive mutants have an elevated Nod factor response and increased CK accumulation and signaling, whereas in the wild type, ethylene may reduce CK signaling activation and CK sensitivity (Pennmetsa et al., 2008; Plet et al., 2011; van Zeijl et al., 2015). The introduction of an ethylene-insensitive mutation into the cre1 loss-of-function mutant partially restores nodule defects of this mutant (Plet et al., 2011), suggesting that the ethylene pathway negatively influences organogenesis independently of the CK signaling pathway. CK may also influence ethylene biosynthesis during nodule organogenesis, as gain-of-function CK receptor mutants of Lotus that develop spontaneous nodules in the absence of rhizobia produce more ethylene than the wild type (Reid et al., 2018). In addition, a study with the ethylene-insensitive mutant sickle in Medicago has shown an altered expression of MtPIN auxin transporters and increased shoot-to-root auxin transport, indicating that ethylene may negatively regulate auxin accumulation (Prayitno et al., 2006b). Ethylene also interacts with GA in nodule initiation, as outlined above.

The exact time and place where ethylene accumulates have not yet been determined. As discussed above, ethylene production and ACS expression occur within 6 hpi and peak 24 to 48 hpi at a time when nodule primordia are initiating in Lotus and Medicago (Larrainzar et al., 2015; Reid et al., 2018). MtACS promoter-GUS fusions showed an increase in the expression of MtACS in roots 48 hpi, but details of the spatial and temporal patterns of expression have not been reported (Larrainzar et al., 2015). In mature nodules, the expression of these enzymes is maintained in the meristem and periphery of the nodule (Larrainzar et al., 2015). The expression of MtACS in the meristem of mature nodules correlates with a possible role of ethylene in meristem development. A more detailed observation of the expression of the ethylene biosynthesis enzymes ACS and ACO during nodulation through promoter::GUS fusions will provide useful information on the dynamics of ethylene biosynthesis in the wild type and different mutants and at different stages of nodulation (Figure 1). In addition, manipulation of ethylene production using transgenic tools will enable a better understanding of the role of ethylene in different tissues.

CONCLUDING REMARKS AND PERSPECTIVES

Exciting progress has been made in our understanding of the role of plant hormones in nodulation. In particular, the characterization of legume mutants and transgenic lines with altered hormone biosynthesis, catabolism, signaling, or transport has been key to integrating hormone action into the nodulation pathway. Key questions still remain. Given that many hormones act in specific ways during the different spatial and temporal phases of nodulation, including epidermal infection and cortical nodule organogenesis and development, going forward it is essential that analysis of such lines takes care to separate these distinct spatial and temporal events. Insights into the precise role of cytokinin and auxin have been greatly assisted by hormone-responsive promoters and biosensors, and similar tools for GAs, BRs, and ethylene will underpin new insights into the action of these hormones during specific stages of nodulation. Furthermore, direct measurement of hormone levels by mass spectroscopy imaging techniques (Shiono and Taira, 2020) during nodulation events combined with tissue-specific, cell-specific, and inducible promoters to manipulate hormone level or response in precise ways will enable us to pinpoint hormone action in these complex processes. This includes clarifying whether hormones act as mobile elements in the coordination between epidermal and cortical events. An integrated model of how hormones interact with each other and key nodulation pathways, such as Nod factor signaling and the autoregulation pathway, is now emerging.
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Another important point to note is that our current understanding of the action of some hormones differs between species. For example, information from the two model species Medicago and Lotus differs on some occasions. Whether this reflects real differences in the role of specific hormones in determinate and indeterminate nodulation, different techniques used, or the conflation of different components of the whole nodulation process requires attention. Work on other hormonally controlled processes in which such differences were initially reported has frequently been resolved with further detailed work. For example, despite earlier reports of species-specific effects, GA was later shown to play a conserved role in de-etiolation across species (Symons et al., 2008).

How will this information on the various components of nodulation inform the ambitious goals of deploying nodulation outside of legumes in our staple food crops (Huisman and Geurts, 2020; Kröner and Radutoiu, 2021)? Given the shared evolutionary history of the infection program of nodulation and the arbuscular mycorrhizal symbioses, an understanding of the shared and unique roles of plant hormones in these two processes will offer tools that may enable functional nodulation to be developed in non-legumes. Similarly, the exciting discovery of the key roles for hormones in the shared and similar regulatory networks of lateral root development and nodule organogenesis means that we are beginning to unravel how legumes retooled root development to take advantage of the boon of nitrogen fixation.

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E.F. conceived the basis of the manuscript. K.V. prepared the figure. K.V., J.B.R., and E.F. wrote the manuscript.

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