

## Distinct Hormone Regulation of Determinate and Indeterminate Nodule Development in Legumes

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### Abstract

Symbiotic legume nodules are classified as determinate or indeterminate depending on the persistence of nodule meristem. This review compares and contrasts the roles of different plant hormones during the development of these two types of nodules. Cytokinins promote the formation of both types of nodules while ethylene and abscisic acid inhibit them. Interestingly auxin, jasmonic acid, gibberellic acid and brassinolides appear to distinctly influence the two types of nodules.

**Keywords:** Legume; Nodule; Hormone; Auxin; Cytokinin; Jasmonic acid; Gibberellic acid

### Introduction

Symbiotic root nodules arise from a well-coordinated interaction between leguminous plants and diazotrophic soil bacterial collectively termed rhizobia. Legume roots release specific flavonoid compounds that are recognized by compatible rhizobia species. The rhizobia respond by producing lipochitooligosaccharide “nod factors” that are in turn recognized by LysM domain receptor-like kinases of compatible legume hosts. Recognition of root hair colonization by compatible rhizobia initiates a number of cellular and developmental responses in the plant host. These events can be broadly classified into (i) root hair and epidermal responses that occur early and (ii) cortical responses that follow in a coordinated manner. The earliest response is the initiation of calcium oscillations in the perinuclear region of epidermal and root hair cells. Decoding of these oscillations by a symbiotic calcium-calmodulin-dependent protein kinase subsequently results in the induction of several transcription factors in these cell types including those that potentially mediate hormone responses (e.g. ERN1). Constitutive activation of the symbiotic CcCaMK is sufficient to produce spontaneous nodules. Therefore, perception of nod factors at the root surface appears to generate and yet to be identified diffusible signal that initiates nodule organogenesis in the root cortex. A number of different transcription factors (e.g. NIN, NSP1, NSP2) and hormone signaling elements (see below) are activated in specific cortex cells that divide to form the nodule primordium. Simultaneously, infected root hairs form invaginations that transport rhizobia towards the inner root cell layers. The formation and elongation of these specialized “infection threads” are also strictly regulated. The infection threads ultimately reach the developing nodules in the cortex where the bacteria are released as membrane-enclosed structures. Cells surrounding the infection zone form the nodule parenchyma where distinct cell differentiation to form the nodule vasculature, cortex and endodermis occur. A number of genes potentially associated with all these different processes have been identified through genetic and functional genomic studies [1,2] (Figure 1).

There are two major types of nodules produced by legumes: (i) Determinate nodules that are characterized by a spherical shape and the lack of a persistent nodule meristem, and produced in general by tropical legumes viz. soybean and *Lotus japonicus*; and (ii) Indeterminate nodules that are characterized by a cylindrical shape and the presence of a persistent nodule meristem, and produced by temperate legumes viz. pea, white clover (*Trifolium repens*) and *Medicago truncatula* [3]. While the epidermal responses are very similar between these types

of nodules, cortical responses are somewhat distinct. For example, while determinate nodule initials arise from outer/mid cortical cells of the root, indeterminate nodule initials arise from inner cortical cells. However, almost all known signaling elements involved in nod factor signal transduction and nodule initiation, have a conserved function between the two types of nodules. A number of landmark studies have revealed the crucial roles of plant hormones in the development of both these types of nodules. Despite the similarities in nod factor signaling elements, some hormones appear to play distinct or even opposite role(s) during the development of these two types of nodules.

### Hormone Regulation of Root Hair and Epidermal Responses

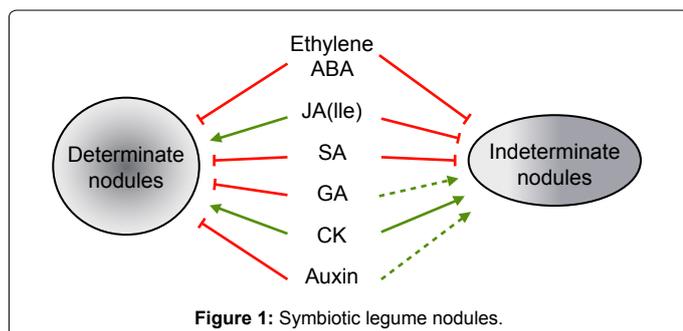
The application of exogenous hormones as well as genetic and reverse genetic approaches have demonstrated a clear role for ethylene, Jasmonic Acid (JA), Gibberellic Acid (GA) and Abscisic Acid (ABA) in regulating root hair and epidermal responses. Ethylene clearly inhibits root hair responses in both determinate and indeterminate nodule forming legumes [4,5] and acts to inhibit and delay the frequency of nod factor-induced Ca spikes [6]. Soybean is a notable exception where neither exogenous application of ethylene nor mutations conferring altered sensitivity to ethylene affected the number of nodules or rhizobial infection [7]. Interestingly, ethylene action is crucial for lateral root base nodulation, but inhibits nodulation occurring via root hair infection in *Sesbania rostrata* [8]. This appears to be an evolutionary mechanism for adaptation to submergence. Ethylene levels increase during submerged conditions promoting lateral root base nodules above the water while suppressing root hair infections below [9]. Indeed, ethylene signaling mutants that display altered response in typical assays such as triple response do not always have altered nodule numbers [10,11]. These observations suggest that ethylene signaling elements specific to nodule development might have

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evolved in legumes. Similar to ethylene, exogenous JA also inhibited root hair responses and nodule development in *M. truncatula* [11] and *L. japonicas* [12]. High concentrations of JA reduced both root hair- and lateral base nodulation in *Sesbania* [8]. However, examination of endogenous levels of JA-Ile, the active conjugate suggested that it might promote nodule development in determinate nodule-forming legumes (See below).

Exogenous application of ABA inhibited nodule formation in *L. japonicus* and white clover. In agreement, treatment with ABA biosynthesis inhibitors increased nodule formation. ABA appeared to inhibit root hair curling, but not initial swelling in response to rhizobium inoculation [13]. Subsequently, it was clearly shown that analogous to JA and ethylene, ABA also inhibits nod factor induced Ca spiking and modulated the interval between such spikes [14]. ABA insensitivity conferred by the dominant negative Arabidopsis *aba1-1* allele in *M. truncatula* increased nodule formation. Mutants specifically impaired in ABA-regulated nodule formation, but not other ABA responses have been isolated. On the contrary, wilty ABA insensitive mutants with no effect on nodule formation have also been identified [15]. It appears that legumes might have evolved specific components of ABA signaling that influence nodule formation. Indeed, legumes display increased LR formation in response to ABA unlike non-legume plants [16]. Salicylic acid (SA) also inhibits nodule development, but the site of action of SA is unclear. Exogenous SA clearly inhibited rhizobial association with root hairs and nodule primordium formation in indeterminate nodule forming legumes. Interestingly, exogenous SA did not affect determinate nodule formation [17]. However, reduction in endogenous SA levels by expressing *nahG* (a bacterial SA hydroxylase gene) increased rhizobial infection as well as nodule formation in both determinate and indeterminate nodule forming legumes. The possible inhibitory effect of SA on rhizobial growth raised questions on the direct role of SA in nod factor signaling. When plants were co-treated with nod factors and SA, root hair deformation responses were unaffected, but primordium initiation was significantly reduced [17,18]. This suggested that SA might not affect root hair deformation responses to nod factor, but inhibit rhizobial infection as well as downstream nod factor signaling.

Finally, GA also inhibits root hair deformation and nodule formation, but only in determinate nodule forming legumes. Exogenous application of active forms of GA or constitutive activation of GA signaling inhibited nodule formation in *L. japonicus*. GA also delayed the frequency of Ca spikes elicited in response to nod factor [19]. On the other hand, GA-deficient mutants of pea had significantly reduced nodule formation and this phenotype could be restored through exogenous application of bioactive GAs. These observations suggested that GA might play opposite roles on the formation of the two types of nodules. However, the concentration of GA appears to have a significant influence. While micromolar concentrations of GA have opposite

effects between the two types of nodules, higher concentrations of GA inhibit both determinate and indeterminate nodule formation [19,20]. In *Sesbania rostrata*, GA action is required for lateral root base nodulation, but inhibits nodulation via root hair infection via ethylene. Exogenous GA does not inhibit root hair deformation, but inhibits infection thread formation [21].

## Hormone Regulation of Cortex Responses and Primordium Formation

Cytokinin is a key plant hormone that influences cortex responses during nodule formation. Cytokinin-responsive gene expression was observed in nodule initial cells and therefore it might play a direct role in cortex cell division during nodule development. Cytokinin activity is crucial for the formation of both determinate and indeterminate nodules [22,23]. Indeed, constitutive activation of a cytokinin receptor or prolonged treatment with exogenous cytokinin is sufficient to initiate spontaneous nodules in *L. japonicus*. The expression of key transcription factors associated with nodule initiation (e.g. NIN, NSP1) act downstream of cytokinin. In addition, cytokinin specifically affects their expression in the cortex [24]. Additional components that play a role in cytokinin signaling (e.g. type-A RRs) also influence nodule development and cortex responses in both determinate and indeterminate nodule-forming legumes [25].

Auxin-responsive marker gene expression was also observed during nodule initiation. The application of polar auxin transport inhibitors resulted in nodule-like structures that expressed nodulation marker genes [26]. Indeed, flavonoid-mediated inhibition of polar auxin transport is crucial for the formation of indeterminate nodules [27]. However, the requirement of polar auxin transport inhibition appears to be not important for the initiation of determinate nodules [28]. Interestingly, auxin activity is suppressed in the nodule infection zone during post initiation stages of nodule development [29]. Such suppression appears to be crucial for proper nodule development. Recent results from my lab showed that enhanced sensitivity to auxin conferred by constitutive expression or nodule primordium/infection zone-specific expression of miR160 inhibited nodule formation in soybean. Notably, this did not inhibit nodule initial cell division (Turner et al., manuscript under review). There is at least one study that suggests that resistance to auxin results in enhanced nodule development in *M. truncatula* as well [30]. It appears that the requirements of auxin distribution as well as specific auxin concentrations during primordium development are distinct between determinate and indeterminate nodules. On the other hand, in both determinate and indeterminate nodules, auxin-responsive gene expression was observed in the nodule parenchyma and was associated with regions of nodule vascular development. Therefore, auxin appears to play multiple roles during nodule initiation requiring precise spatio-temporal regulation of its activity.

GA also inhibited the expression of key nodulation-associated transcription factors NIN and NSP1, and nodule formation in *L. japonicus*. In agreement, treatment with uniconozal P (GA biosynthesis inhibitor) resulted in increased nodule formation in *L. japonicus*. Similarly, enhanced/constitutive activation of GA signaling also inhibited formation of both determinate and indeterminate nodules [19,31]. However, as mentioned above GA deficient pea mutants had reduced and impaired nodule formation. Interestingly, suppression of ethylene biosynthesis in the GA-deficient pea mutant, *na* resulted in a partial increase in nodule formation, but these nodules were small and nearly devoid of typical nodule zones [31]. Therefore, repression of ethylene production can partially compensate GA's role in nodule

initiation, but normal GA levels are crucial for subsequent nodule development (at least in indeterminate nodule-forming legumes). Ethylene not only inhibits root hair responses, but also regulates local control of nodule numbers. Ethylene deficiency or insensitivity resulted in increased nodule formation due to the emergence of nodules in non-xylem pole positions of the root [32-34]. Enhanced ethylene production suppressed cytokinin-induced spontaneous nodules as well indicating that ethylene acts downstream of cytokinin in the cortex [35]. Current evidence suggests that ethylene plays an inhibitory role in the initiation of both determinate and indeterminate nodules.

## Hormones and Autoregulation of Nodulation

Legumes control the extent of nodulation through a shoot-regulated long distance signaling pathway ("autoregulation of nodulation"). A CLAVATA1-like receptor kinase in the leaves potentially perceives CLAVATA3-like peptide signals produced by developing nodules and inhibits subsequent nodule formation [36]. The shoot-derived inhibitory signal is thought to be perceived by an F-box protein (TML) in the roots resulting in the inhibition of cytokinin-mediated organogenesis of additional nodules. Interestingly, this signal does not appear to inhibit early nodulation responses in the epidermis [37]. Plant hormones have been implicated in autoregulation of nodule numbers as well. Increased auxin levels in response to rhizobium inoculation or high levels of nitrate were observed in soybean. Interestingly, such increase in auxin was not observed in supernodulating mutants [38]. This observation prompted the "auxin burst hypothesis" which postulates that enhanced auxin levels inhibit nodule development. Indeed, exogenous auxin inhibited nodule formation in soybean [39] and recent evidence indicates that auxin hypersensitivity also inhibits determinate nodule development. Interestingly, the opposite appears true in indeterminate nodule-forming legumes. There is increased transport of auxin from the shoot to the root in the *M. truncatula* supernodulating mutant, *sun* [40] suggesting that shoot-derived auxin promotes nodule formation in this species.

In addition to auxin, brassinosteroids (BR) and JA also appear to have distinct roles during autoregulation between determinate and indeterminate nodule-forming legumes. Application of BR inhibits nodule formation in the soybean hypernodulating mutant, EN6500. In agreement, application of brassinazole, a biosynthetic inhibitor of BRs resulted in increased nodule formation in wild-type plants [41]. This suggested that BRs could act as or is involved in generating the shoot-derived inhibitory signal during autoregulation in determinate nodule-forming legumes. On the other hand, BRs appear to play a crucial role in the formation of indeterminate nodules and play a positive role in promoting nodule number. For example, the BR deficient pea mutant, *lkb* has fewer nodules. In addition, shoot BR levels regulate the extent of nodule formation. Low BR levels in the shoot result in reduced nodulation irrespective of root BR levels [20]. JA also appears to play distinct roles in shoot-regulated nodulation between determinate and indeterminate nodule forming legumes. For example, rhizobium inoculation resulted in an inhibition of JA biosynthesis and action in leaves of *L. japonicus* and soybean [42,43]. Application of JA to the leaves promoted nodule development in both species especially under inhibitory environments (e.g. low R/FR light conditions in *L. japonicus*). Crucially, soybean supernodulating mutants had increased expression of key JA biosynthesis enzymes (and increased JA levels). Foliar application of JA biosynthesis inhibitors restored normal nodule numbers specifically in the mutant. It should however be noted that contradicting results were reported in another study where foliar application of JA was used to examine nodule formation in *L. japonicus*

[12]. Multiple studies have shown that JA inhibits indeterminate nodule formation (See above) although its role in autoregulation has not been studied in these species.

## Hormone Interactions During Nodule Development

In addition to specific and independent roles of plant hormones, there is also evidence for interaction between them during nodule development. While, both JA and ethylene suppress Ca-spiking at higher concentrations, they differentially influence the frequency of spiking at lower concentrations in *M. truncatula*. Interestingly, JA prolongs the frequency while ethylene increases the frequency. In agreement, absence of ethylene activity (resulting from genetic or pharmacological manipulations) enhanced the inhibition of Ca spiking by JA. In contrast, JA appears to require ethylene action to inhibit nodule development. For example, JA inhibition of nodule development was significantly attenuated in the absence of ethylene action [11]. Therefore, JA and ethylene interact in both antagonistic and synergistic manner to establish key checkpoints during indeterminate nodule development.

There is evidence to suggest that auxin and cytokinin might interact during nodule development. Recently, it was shown that auxin activity during nodule initiation occurs downstream of cytokinin perception and NIN activity in *L. japonicas* [29]. Cytokinin also appears to regulate polar auxin transport in response to rhizobial colonization [44]. However, cytokinin does not appear to mediate the suppression of auxin activity during post initiation stages [29]. Recent results from my lab confirmed that there is very low auxin activity during nodule initiation in soybean as well. In addition, our results suggest that deregulated auxin activity (achieved by over-expressing miR160) leads to cytokinin hyposensitivity and inhibition of nodule development in soybean (also a determinate nodule-forming legume). Interestingly, enhanced auxin sensitivity in nodule primordia is sufficient to inhibit nodule development. The requirement of precise spatio-temporal regulation of auxin and cytokinin activities during nodule development is demonstrated by the presence of central vasculatures in *lin-4*, a *M. truncatula* mutant that does not maintain cytokinin signaling, but displays ectopic auxin responsive gene expression in the nodule. This resulted in delayed and impaired nodule development.

## Conclusions and Future Perspectives

In summary, ethylene and ABA appear to play conserved role inhibiting root hair responses associated with both determinate and indeterminate nodule formation. On the other hand, GA, SA and JA might play distinct or in cases opposite roles during the formation of these two types of nodules. Cytokinins play an essential conserved role in the initiation of both determinate and indeterminate nodules while the distribution and levels of auxin required might be distinct between these nodule types. Finally, auxin, BR and JA play opposite roles in governing nodule autoregulation between determinate and indeterminate nodule forming legumes. In addition, spatio-temporal regulation of hormone activities especially through synergistic and antagonistic interactions between specific hormones is crucial for proper nodule development.

Studies using exogenous application of hormones have significantly contributed to our understanding of hormone function in nodule development. However, genetic and reverse-genetic approaches have yielded more conclusive results. This is likely due to dose-dependent response of different nodule development processes to hormones as well as cross-talk/pleiotropic effects induced by exogenous application.

The availability of high quality genome sequences for multiple legumes has enabled the identification of hormone biosynthesis and signaling genes in legumes [45-48]. Examination of their expression will identify candidates with specific roles in nodule development. Insertional and other mutagenesis programs in legumes [49-52] have enabled reverse-genetic screens to identify mutants impaired in specific hormone signaling elements. Together, we are set for an exciting decade discovering the distinct and crucial roles as well as mechanism of action of plant hormones in determinate and indeterminate nodule formation.

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