ROLES OF PLANT HORMONES IN LEGUME NODULATION

P. K. Chan and P.M. Gresshoff

ARC Centre of Excellence for Integrative Legume Research, The University of Queensland, Australia.

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Summary

Legumes play a crucial role in agricultural and ecosystems based on their ability to convert atmospheric nitrogen gas into plant-available ammonium in symbiosis with soil bacteria commonly called ‘rhizobia’. This nitrogen-fixing ability makes legumes attractive as an agricultural, economical and environmentally friendly crop as their requirement for nitrogen fertilizer is reduced, leading to a reduction of fertilizer run-off into ground and surface waters. Such contamination may lead to human health and environmental quality effects. In recent years, the cost of industrial nitrogen fertilizers has increased dramatically due to the rising cost of fossil fuel. This makes legumes more attractive as they provide alternatives to nitrogen fertilizer in a sustainable production system for manufacture of both food and fuel.

1. Introduction

Legume plants are capable of developing an association with nitrogen-fixing bacteria generally known as rhizobia to form nodules. A nodule is a unique organ commonly found on the plant root where nitrogen fixing activities occur. Nodules provide a suitable environment for the nitrogenase enzyme complex of bacteria to convert nitrogen gas from atmosphere into ammonium which will be assimilated by the plant.

The symbiosis process begins with an interchange of molecular signals between both plant and rhizobia. The legume root secretes flavonoids to induce the synthesis of lipochitin-oligosaccharide signals, called Nod factors, by rhizobia. Nod factors activate nodule organogenesis on root plants through stimulation of cell divisions in the root
cortex and pericycle. Induced mutant or natural variants of some legumes are also available that nodulate in the absence of rhizobia. Many stages of nodule development resemble common plant development; for example, cells divide and differentiate, vascular tissue develops, nodules respond to external factors such as stress and nitrate, etc.). Accordingly, the analysis of multiple hormone effects on nodulation needs to be seen as a challenge as pleiotropic responses, resulting from the effect of phytohormones on plant growth per se, and need to be separated from direct functions in nodule ontogeny.

This review examines the roles of ‘classical’ plant hormones, abscisic acid (ABA), auxin, cytokinin, ethylene and gibberellins in nodule growth and development.

2. Abscisic Acid

Abscisic acid (ABA) is a naturally occurring compound in plants, derived from the mevalonic acid biosynthesis pathway that also leads to plant sterols (including brassinosteroids), and gibberellins. It plays crucial roles in various aspects of plant growth and development, including the mediation of responses to environmental stress such as cold, drought and high salinity. In nodulation, it has been reported to play multiple negative roles in nodulation at different stages of development.

The application of ABA through root irrigation was first shown to inhibit nodule numbers in *Pisum sativum*. ABA application to wild-type soybean and a supernodulating mutant NOD1-3 reduced both nodule numbers but also isoflavonoid levels. It has been shown that soybean flavonoids are responsible for the activation of nodulation and could have a role in nodule organogenesis. It was later revealed that ABA inhibited all phases of nodulation from nodule initiation, development and function, but seemingly independent of the nodulation control status.

The effect of ABA on white clover and the model legume *Lotus japonicus*, which form indeterminate and determinate nodules respectively, was analyzed. Both legumes showed a decrease in nodule number upon root application of ABA. Root hair deformation was observed suggesting that ABA blocked the steps between root hair swelling and curling. After application of abamine, an ABA biosynthesis inhibitor, nodule number increased which led to the hypothesis that the ABA concentration controls the number of root nodules.

Nodule development is also affected by ABA treatment resulting in arrested and small ineffective brownish colored nodules and early degeneration of bacteroid tissue was noted in developing nodules. The nitrogenase activity of nodules treated with ABA was lower compared to wild type in *Faba vulgaris*, pea and *Lotus japonicus*. This leads to the suggestion that the application ABA stimulated an abrupt stress situation mimicking severe drought which led to leghemoglobin reduction. In addition to that, ABA might create an oxygen barrier in nodules that resulted in the observed decline of nitrogen fixation. Histological sections of nodule inoculated with rhizobial expressing green fluorescence protein (GFP) revealed a fluorescent green “shell-like” structure on the outer layer of spherical *Lotus japonicus* nodules treated with ABA (Figure 1). Bacteroids in the nodule interior failed to express GFP suggesting that possible
degeneration of bacteroid tissues possibly caused by an ABA-induced oxygen barrier. This may relate to systemic stress responses in legume nitrogen fixation control, where stress treatment of one separated root portion results in a systemically transmitted drop of nitrogen fixation ability in a second root portion. Interestingly, nodulation is not suppressed in that situation suggesting that ABA mechanisms of nodulation control are local, while some act systemically. Of course general plant fitness effects would presumably be systemic.

Figure 1. Transverse sections of nodule of *L. japonicus* wild type (MG-20) and Beyma mutant. A, Wild type nodule 28 dpi. B, Wild type nodule of plant treated ABA. The nodule is has a fluorescent green “shell-like” structure on its outer spherical layer. C, Beyma mutant nodule 28 dpi. Beyma nodule is smaller than to wild type. D, Beyma mutant nodule of plant treated with ABA. The nodule is not affected by ABA treatment. Bar = 100 μM.

A possible role of ABA in autoregulation of nodulation (AON) was investigated in soybean supernodulating mutant *nts382* that shows a stronger supernodulation response than NOD1-3. The basal level of ABA in wild-type roots was higher than *nts382*, and further increased in wild-type root following inoculation with *Bradyrhizobium*. The concentration of ABA in the shoot increased at the onset of autoregulation in the wild type but not in *nts382*; similarly the root ABA-to-cytokinin ratio was found to be consistently higher in the wild type compared to *nts382*. This ratio had been suggested to be involved in the root-to-shoot signaling and symbiotic photosynthetic gas exchange in alfalfa. A model was proposed to explain the possible influence of ABA-to-cytokinin ratio in autoregulation of nodulation. In this model, inoculation-induced changes in the xylem resulted in a decreased ABA-to-cytokinin ratio that triggers the synthesis of ABA when moved up to the leaf. This ABA was speculated to be translocated to the root to autoregulate nodule development. However, it was later found that ABA may not be directly involved in autoregulation of nodulation as an ABA insensitive mutant of *Lotus japonicus* did not have altered AON, and the applied ABA effect on nodulation was local in a split root experiment with *Lotus japonicus*.

An ABA insensitive mutant in *Lotus japonicus*, *Beyma* was isolated based on the growth of root length. *Beyma* is an ABA response mutant that displays insensitivity to the inhibition of germination, stomata closing and nodulation. *Beyma* forms a similar nodule number compared to the wild type. This indicated that the AON in this mutant was not affected. However, nodules formed were smaller in size with reduced ability to fix nitrogen compared to wild type. The authors proposed that ABA is not directly involved in autoregulation but has a role in nodule growth rather than the control of initiation, consistent with data from pea.
3. Auxin

Auxins were the first class of major plant hormones discovered to be central to regulation of plant growth and development at all levels. The most important member of the auxin family is indole-3-acetic acid (IAA), a native auxin in plants, derived from the phenyl-propanoid biosynthetic pathway. The highest auxin concentration was found in cells undergoing cell division, elongation, differentiation and vascular bundle formation. Therefore, auxin has been speculated to play a significant role in nodule development.

Auxin is produced mainly in the shoot and moves to the root by an active transport process involving auxin efflux protein complexes. These proteins may regulate the auxin concentration in the plant. They are not evenly distributed along cell membrane and subjected to dynamic reallocation. Compounds like NPA (1-N-naphthylphthalamic acid) and TIBA (tri-indobenzoic acid) inhibit the acropetal auxin transport. Rhizobium-derived Nod factors and several classes of flavonoids have been reported to have a similar effect on auxin transport.

Early experiments suggested that the ratio of auxin-to-cytokinin in the root was responsible for the initiation of cortical cell divisions and nodule formation. This ratio was lowered in soybean hypernodulating mutant compared to wild type, indicating that hormones balance is important for regulating nodule number. Various plant compounds like ethylene, cytokinin and peroxidase could inhibit auxin transport which could lead to local shifts in the plant’s auxin-to-cytokinin ratio.

The use of an auxin-regulated soybean promoter has given new insights into auxin distribution and expression in legumes. The GH3 promoter is active in tissue with high level of auxin and has quick and seemingly specific response to various auxin concentrations.

Its expression, monitored by GUS staining, was detected in dividing cells of the nodule and lateral roots. At early stages of nodulation, inferred auxin levels increased in early dividing cortical cells and decreased in differentiating nodule primordia and vascular tissue. Similar observations were made in *Medicago truncatula* AUX1-like genes (termed MtLAX). The MtLAX genes are expressed in nodule primordia at early stage of nodule development and vasculature emerged at the later stage.

The role of auxin in nodulation is closely linked to the development of other root structures like lateral root as these structures undergo a similar development program which involved cell division and differentiation. Both lateral roots and nodules are regulated by auxin-to-cytokinin ratio but in opposite way.

An increase in auxin stimulated lateral root formation while an increase of cytokinin concentration or inhibiting auxin transport induced the development of pseudo-nodules. The supernodulating mutant astray in *Lotus japonicus* has a normal frequency of lateral roots suggesting that the pathways regulating nodule and lateral root may share a common evolutionary origin but with existence of nodule-specific regulators. Analysis of ethylene responses on nodulation reflects a similar ‘inverse’ regulation of nodule and lateral root ontogeny (see below).
4. Cytokinin

Cytokinins are a class of plant hormones active in promoting cell division, and are also involved in regulation of many physiological processes during plant development, growth and adaptation to environment conditions. Cytokinins are implicated as the control of root architecture development including root nodulation.

One of the earliest indications was the observation from exogenous cytokinins application to roots induced formation of pseudo-nodule structures on legumes (garden pea and alfalfa) and even non-legumes (tobacco). These physiological studies revealed a role for this hormone for the control of root architecture and nodule development. Exogenous application of cytokinins on legume root induced responses similar to Nod factors. This includes cortical cell divisions, amyloplast deposition and induction of early nodulin gene expression. Transfer of trans-zeatin secretion gene (tzs; sharing sequence similarity to the ipt gene of plants) into Nod bacteria and non-symbiotic bacteria was sufficient to induce nodule-like structure formation at low frequency in alfalfa. Similar observation can be seen when cytokinin metabolism was altered by transgenic overexpression of cytokinin oxidase. On the other hand, suppression of a cytokinin receptor by RNA interference reduced nodulation in Medicago truncatula.

Further evidence that cytokinins are crucial for nodule growth comes from the work with Lotus japonicus. A gain-of-function mutant allele of a Lotus japonicus gene for histidine kinase (Lhk1) that leads to spontaneous nodule formation in absence of rhizobia and a loss-of-function allele, HYPERINFECTED1 (HIT1) of the same Lhk1 gene were identified. HIT1 mutant failed to established nodule and lead to a hyper-infected phenotype of roots. It will be interesting to dissect the downstream cytokinin-responsive network as it may lead to crucial information for transferring symbiotic relationship into other crop plant species.

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**Biographical Sketch**

**Pick Kuen Chan** studied genetic engineering and molecular biology in Malaysia and received a PhD from Universiti Putra Malaysia in 2003. She took up a postdoctoral position in the laboratory of Prof. Peter Gresshoff at the Australian Research Council Center of Excellence for Integrative Legume Research [CILR]. Her research focused in dissecting the role and involvement of hormone in nodulation. Her work on ethylene and ABA has made important findings in the area. She has accepted a research position at INRA, France to further her research in legume molecular genetics and physiology.

**Peter Gresshoff** studied genetics and plant biology in Germany, Canada and Australia, receiving a PhD from ANU in 1973 and a DSc in 1988. After initiating a research program investigating the regulation of soybean nodulation at ANU in 1980, he moved to the USA (University of Tennessee) to advance into the molecular genetic analysis of nodulation. Since 1999 he works at the University of Queensland, where he is Professor of Botany, and Director of the Australian Research Council Centre of Excellence for Integrative Legume Research (CILR). He has published over 300 papers, books and patents. He has organized several major international meetings, including the MPMI, Legume Genome and Genetics, and Nitrogen Fixation Congresses. He continues to research at the forefront of legume molecular genetics and physiology.