NODULATION PHENOTYPES OF *Pisum sativum* MUTANTS

by

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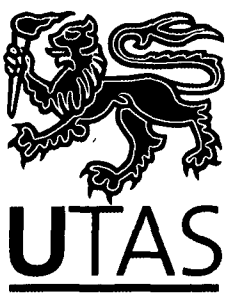

DECLARATION

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Brett J. Ferguson

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ABSTRACT

Nitrogen-fixing bacteria, collectively referred to as rhizobia, are able to trigger the organogenesis of novel organs on legumes, called nodules. The initiation and development of nodules requires a complex signal exchange involving both plant and bacterial compounds. Phytohormones have been implicated in this process, although in many cases direct evidence is lacking. In the work reported here, the root and nodulation phenotypes of various mutant lines of *Pisum sativum* L. are characterized, including those having alterations in their phytohormone levels and/or perception, and a homeotic mutant. Root systems having similar or elevated GA levels compared with that of their wild type developed wild type numbers of nodules, whereas those deficient in gibberellins or brassinosteroids exhibited reduced nodulation. Gibberellin application or grafting to a wild type root or shoot restored the nodule number of a gibberellin-deficient mutant to that of its wild type. In contrast, the shoot controlled the number of nodules that formed in graft combinations of a brassinosteroid-deficient mutant and its wild type. Interestingly, a strong correlation between nodule and lateral root numbers was observed in all lines assessed, consistent with a possible overlap in the early developmental pathways of the two organs.

Double mutants possessing the *na* mutation, which results in severe GA-deficiency, and the *sln* mutation, which results in elevated seedling GA levels, displayed abnormal nodules and a reduced capacity to autoregulate their nodule numbers. Constitutive GA signalling mutants also produced significantly fewer nodules than their wild type. However, these nodules were normal in appearance,

and significantly greater in number compared with that of *na* plants, regardless of whether or not they also possessed the *na* mutation. This indicates that intact GA signalling pathways are required for nodule development. Additional double mutants were created by crossing *na* with one of three independent mutations, *nod3*, *sym28*, and *sym29/nark/har-1*, that result in a plants inability to regulate its nodule number. Double mutant segregates from each of these crosses formed significantly more nodules than *na*, but these structures maintained the aberrant *na* nodule morphology. A significant increase in nodule numbers was also observed on *na* following treatment with an ethylene biosynthesis inhibitor, but these nodules were also aberrant. These findings suggest that GAs are required for late nodule development and that ethylene has a role in nodule initiation. The histology of *na* nodules further supported a role for gibberellins late in nodule development as the cells of the infected zone failed to enlarge.

The nodulation phenotype of the homeotic mutant, *cochleata*, which has stipules replaced by alternative leaf components, abnormal flowers and reduced fertility, was also investigated. Although the root system dry weight, root lengths and nodule numbers of *cochleata* were similar to those of its wild type, the nodulation phenotype of the mutant was unique. The nodules typically dichotomously branched and multiple callus and root structures emerged from their meristems. These nodule-roots incorporated a peripheral vascular bundle of the nodule into their own central vascular cylinder and both the nodules and roots of the hybrid structures appeared functional.

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GENERAL INTRODUCTION

Nodulation is a symbiotic process in which specific soil bacteria of the genus *Rhizobium* invade compatible leguminous host plants. The invasion ultimately leads to the formation of novel structures called nodules, in which the bacteria fix atmospheric nitrogen to be used by the host plant. As with any developmental process, nodulation is multifaceted, requiring specific signalling events regulated temporally and spatially (Ferguson and Mathesius, 2003). However, to date, little is known about the roles of many of the signals involved in legume nodule development.

Mutants have aided greatly in elucidating mechanisms required for nodule development (reviewed in Oldroyd and Downie 2004). Generally, mutants displaying abnormal nodulation phenotypes were created, following which various approaches were employed to identify their mutated genes and the role of the gene products in the nodulation process. A novel approach was adopted in the work reported here as the nodulation phenotypes of already well-characterized mutants were investigated. Many of the genes and gene products of the mutants investigated had formerly been identified, but the nodulation phenotypes had not been examined.

CHAPTER 1

Signaling Interactions During Nodule Development

The information contained in this chapter appears in part in the publication: Ferguson BJ, Mathesius U (2003) Signaling Interactions During Nodule Development. J Plant Growth Regul 22: 47-72.

Introduction

Nodule development involves the induction of cortical and pericycle cell divisions and their subsequent differentiation into a vascularized organ with a meristem. Concurrently, infection by the bacteria into root hairs and cortical cells in a so-called infection thread occurs until their eventual release into the developing nodule. Within the nodule, the invading bacteria differentiate into nitrogen-fixing bacteroids that provide reduced nitrogen to the plant in exchange for carbohydrates and shelter (for recent reviews see Crespi and Galves 2000; Stougaard 2001; Kistner and Parniske, 2002).

Precise interactions between phytohormones and various other signalling compounds are imperative for plant organogenesis, and in no case is this more apparent than in the process of nodulation. In this symbiosis, various signalling molecules are exchanged between the plant and the infecting bacteria to regulate nodule initiation, differentiation and functioning, as well as the number of nodules that develop. Nodule numbers are limited by at least two pathways. One pathway is a local regulation of infection in the root zone susceptible for infection (Vasse et al. 1993), while the second pathway is a negative feedback process termed autoregulation during which existing nodule meristems trigger a signal in the shoot that inhibits further nodule development on the root system (Delves et al. 1986). For this to occur, the timing and concentrations of hormones and other signalling compounds is crucial as alterations to either can result in the abortion of nodulation.

The following Chapter (Ferguson and Mathesius 2003) culminates much of what is known about the various signalling elements involved in nodulation and attempts to identify possible links existing between them. Due to the size of the topic, this chapter concentrates on the signals involved in nodule organogenesis and ignores many of the early signals, for example calcium, known to act in the root hair following Nod factor perception. However, a recent review by Lhuissier et al. (2001) covers this topic.

Signalling Interactions of the Classic Hormones

Earlier work on nodulation investigated hormones individually in an attempt to elucidate a role for each. For example, Thimann (1936) was one of the first to propose an involvement of hormones in nodule formation and implicated auxin in the process. Later, the finding that many soil bacteria, including rhizobia, synthesise plant hormones (reviewed by Costacurta and Vanderleyden 1995), initially seemed to suggest that rhizobia could provide the hormones that subsequently stimulate nodule formation (e.g. Phillips and Torrey 1972), although, this did not explain the specificity between legumes and their specific symbionts. Since then, nodule initiation has been shown to occur spontaneously in some legumes (Truchet et al. 1989) and can be triggered by altering the hormone balance, thus illustrating that the hormones act independently of the bacteria. In addition, the application of Nod factors can induce pseudonodule structures on certain hosts (Truchet et al. 1991), possibly by altering hormone levels within the host tissue. However, since Nod factor-induced nodule primordia typically fail to develop into differentiated nodules, it is possible that hormones or other signals produced by the bacteria during the infection process are also required.

During root nodule development, rhizobia stimulate differentiated cortex cells to re-enter the cell cycle, divide and differentiate. In 1973, Libbenga et al. recognised the need to assess hormone interactions during nodule development and suggested that gradients of both auxin and cytokinins are required for cortex proliferation and thus nodule initiation. Since the work of Libbenga et al. (1973), much has been

discovered about the complex signalling network required for nodule organogenesis. A central question in nodulation research is how changes in the hormone balance can affect the location (radially and longitudinally along the root), initiation, number and functioning of nodules on the root system. The following section discusses many of these findings and identifies the current knowledge of hormone signalling interactions in nodulation (summarised in Figure 1).

Abscisic Acid

The role of abscisic acid (ABA) in nodulation is poorly understood. Initially, ABA was thought to act as an inhibitor of nodule development, as application of the hormone reduced the number of nodules in *Pisum sativum* (pea) (Phillips 1971). ABA application to wild type soybean and its supernodulating mutant line NOD1-3 also caused a decrease in nodule numbers and dry weights in addition to isoflavonoid levels (Cho and Harper 1993). Moreover, Bano and Harper (2002) determined that nodule initiation, development and functioning were all inhibited by ABA in wild type and NOD1-3. Phillips (1971) speculated that ABA might act by reducing the cytokinin-stimulated cortical cell divisions associated with nodule formation, thus suggesting a putative ABA-cytokinin signalling interaction.

ABA and cytokinins have been shown to act in concert to affect numerous aspects of plant development, including root/shoot signalling (Davies and Zhang 1991) and symbiotic photosynthetic gas exchange (Goicoechea et al. 1997). Since the work of Phillips (1971), the ratio of the two hormones has been positively correlated with nodule suppression and autoregulation (Caba et al. 2000; Bano et al. 2002). The root ABA/zeatin riboside (ZR) ratio was found to be consistently higher in wild type *Glycine max* (soybean) relative to the supernodulating mutant *nts382* (Caba et al. 2000). Recently, Bano et al. (2002) proposed a model to explain possible influences of plant ABA/ZR ratios in nodule autoregulation. In this model, inoculation induces an initial decrease in the xylem ABA/ZR ratio. These authors speculated that the hormones of this ratio are then translocated to the leaves where they promote the synthesis of ABA. The increased ABA then moves via the phloem to the root where it inhibits further nodule formation, thus regulating the number of

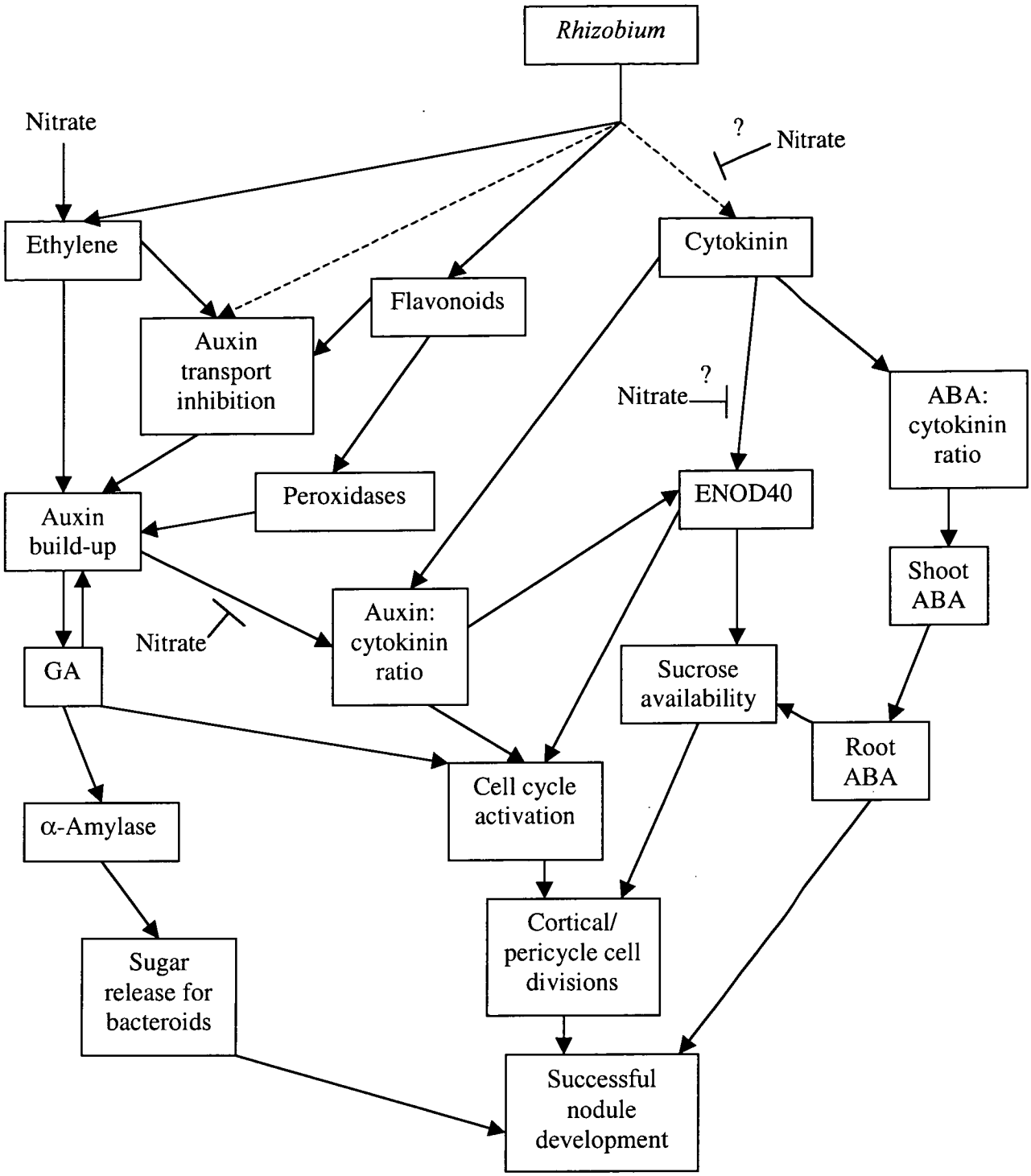

Figure 1. Proposed model for the interaction of hormones and other signals regulating the initiation of cell divisions and nodule development. See text for details. This figure summarises interactions that have been analysed separately and in different legume species. It should therefore not be seen as an accurate or complete overview for any particular legume. The flow diagram does not suggest a strict temporal but rather a functional overlap of interactions. Dashed arrows indicate that the interaction might be indirect and needs to be tested; see Conclusions and Outlook for details. The effect of nitrate on the signalling interactions is indicated in several places, but it will need to be tested whether some of the observed nitrate effects are indirect.

nodules that form. In supernodulating mutants, this pathway is effectively non-functional, as the initial decrease in the xylem ABA/ZR ratio does not occur and thus proper regulation of nodule number is not achieved (Bano et al. 2002). Caba et al. (2000) demonstrated that a final rise in root ABA concentration is absent in the mutant, consistent with the model.

In further support of this model, Gresshoff et al. (1988) illustrated via extrapolation that the concentration of ABA increased in the shoot at the onset of autoregulation in the wild type, but not in *nts382*. In addition, Bano and Harper (2002) demonstrated that the application of partially-purified phloem ABA-extracts, from either wild type or the supernodulating soybean mutant NOD1-3, inhibited nodule formation in the mutant. However, the phloem-ABA levels were similar in both lines and concluded that another signal may be present in the phloem that either inhibits nodule formation or counteracts the inhibitory effect of ABA in this autoregulatory process.

Further evidence supporting a negative role for ABA in nodule development was reported by Watts et al. (1983) who analysed the endogenous ABA content in nodules that form on the perennial *Alnus glutinosa* infected by the actinomycete *Frankia*. ABA levels were higher in nodules than in the surrounding root tissue, particularly in dormant, compared with actively growing, nodules. However, despite this finding, Watts et al. (1983) were unable to determine any obvious correlations between nodule ABA content and growth-rate.

The level of endogenous ABA is also reported to be higher in nodules of pea (Charbonneau and Newcomb 1985) and soybean (Williams and Sicardi De Mallorca 1982; Fedorova et al. 1992) compared with that of the roots. Moreover, increased amounts of ABA were detected in shoots, roots and nodules of soybean plants bearing VA mycorrhiza associations compared with nodulated non-mycorrhizal plants. This suggests that these fungal associations contribute to the ABA pool of the host, including that of the nodule (Murakami-Mizukami et al. 1991). Because ABA had previously been shown to activate a carbohydrate sink during the seed fill phase of soybean, Murakami-Mizukami et al. (1991) speculated that increased nodule ABA may act as a signal to induce a similar carbohydrate sink in the nodule. Thus, as

opposed to acting as an inhibitory factor, ABA could play a role in allocating photosynthates to the nodule to be used as an energy source for growth, development, rhizobial respiration and nitrogen fixation. Rhizobia synthesize ABA in culture when supplied with ABA-precursors (Dangar and Basu 1991) so perhaps this production is a mechanism used by the bacteria as a means of obtaining plant-derived carbohydrates. In the case of nitrogen fixation however, nitrogenase activity has been shown to decrease with increasing endogenous ABA levels in some species (Dangar and Basu 1984; 1987). As well, the daily application of ABA significantly reduced the level of nitrogen fixation in pea (González et al. 2001a), although this treatment may have exceeded an appropriate ABA concentration for optimum nodule functioning. This reduction in nitrogen fixation paralleled a decline in nodule leghemoglobin content, which the authors speculated resulted in a restriction of available oxygen required by the bacteroids for cellular respiration, thus inducing the decline in nitrogen fixation (González et al. 2001b).

In *Phaseolus vulgaris*, ABA application increased the accumulation of lipoxygenase (LOX, Figure 2) mRNA, which are enzymes associated with stress and development (Porta et al. 1999). These authors detected LOX in developing, but not mature, nodules suggesting a role for LOX in nodule growth. Moreover, *in situ* hybridisation revealed no exclusive LOX expression in the invasion zone of pea nodules; however, all LOX transcripts were expressed at the nodule apex (Wisniewski et al. 1999), thus further suggesting a role for the enzymes in nodule growth and development rather than a more direct role in the plant-microbe interaction or in host defence. Also in pea, Charbonneau and Newcomb (1985) noted an increased amount of ABA in the apical region of the nodule, possibly indicating a link between elevated levels of nodule ABA and LOX (Figure 3). If indeed LOX is required for nodule development and ABA is required to up-regulate the level of nodule LOX, it can therefore be argued that ABA is actually required for nodule growth. Furthermore, a role for LOX has been implicated in nitrogen storage and assimilate partitioning (Stephenson et al. 1998), which, if coupled with ABA, supports the hypothesis of Murakami-Mizukami et al. (1991) that ABA could have a role in inducing a carbohydrate sink in the nodule.

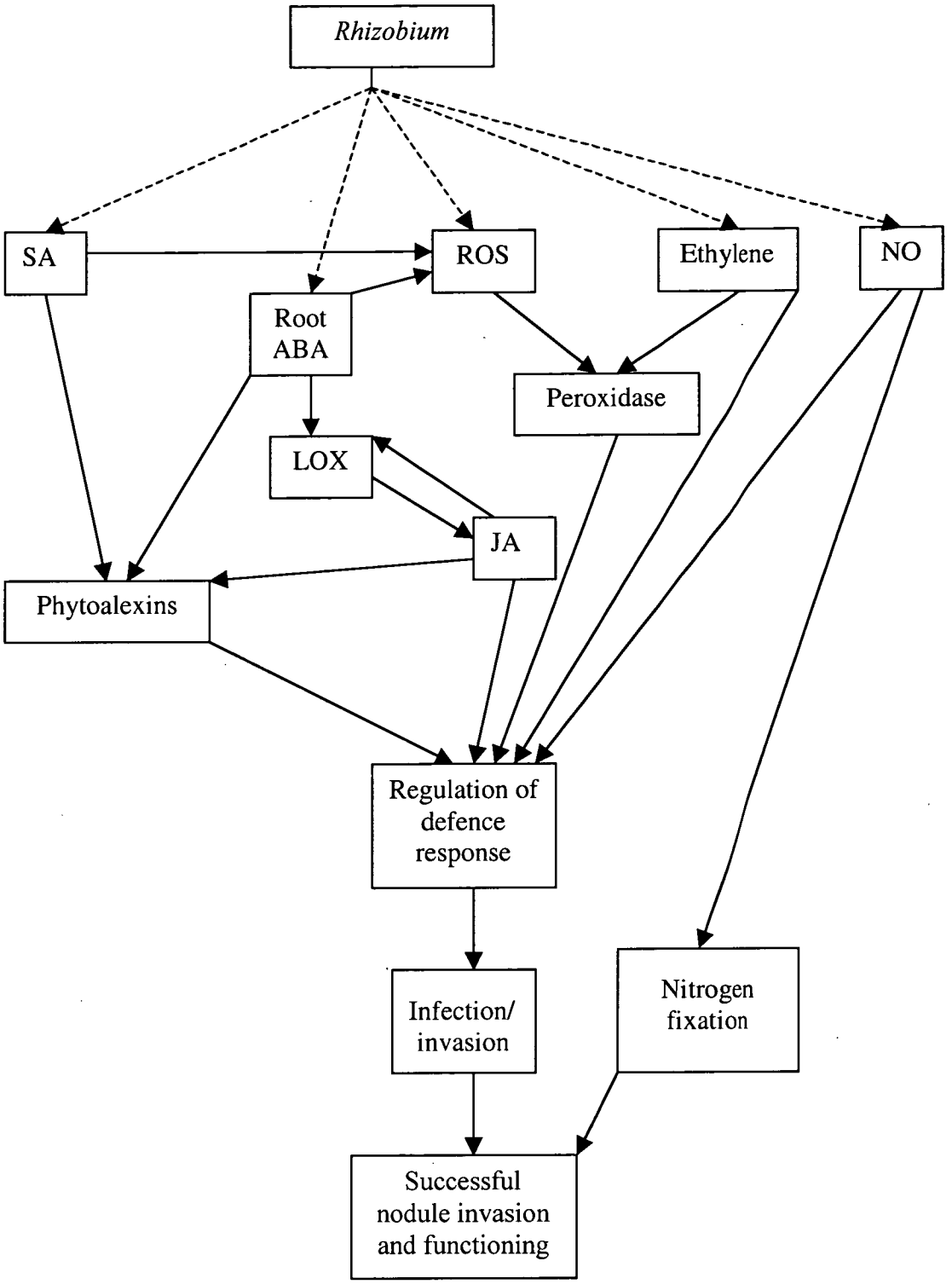

Figure 2. Proposed model for the interaction of signals regulating defence responses and nodule functioning. As in Figure 1, interactions that have been analysed separately and in different legume species are integrated in one diagram and should not be seen as an accurate or complete overview for any particular legume. The flow diagram does not suggest a strict temporal but rather a functional overlap of interactions. Dashed arrows indicate it is unknown whether *Rhizobium* independently activates these responses via different signals (e.g. Nod factors, exopolysaccharides, etc.) or whether *Rhizobium* induces one initial response that triggers further secondary events. This could be tested in mutants for ABA, SA, ethylene or NO.

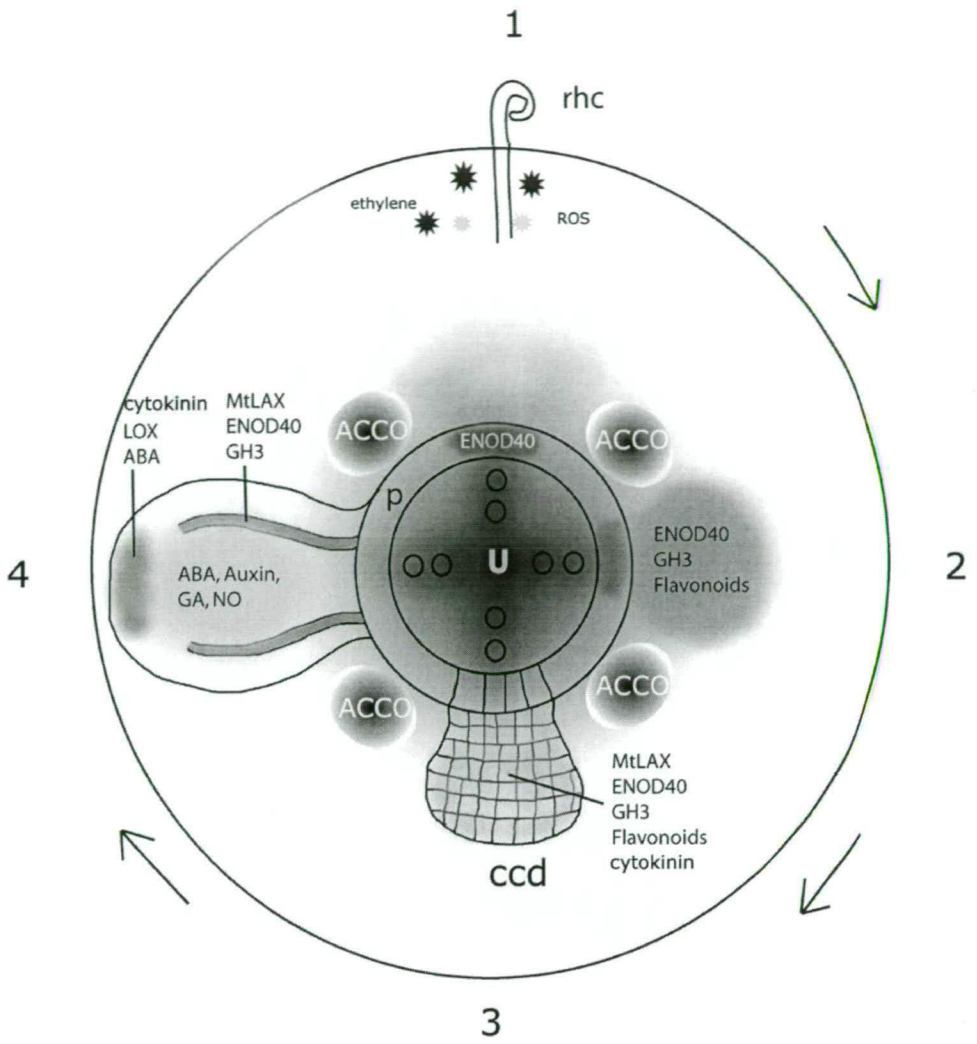

Figure 3. Spatial changes in hormone signals in relation to nodule development. The figure shows an idealised cross section through the root at the site of nodule formation, including the xylem poles (small circles) inside the stele, which is surrounded by the pericycle cell layer (p). A gradient of uridine (U) exists that emanates from the xylem. ACC oxidase (ACCO) is expressed opposite the phloem poles and might create local ethylene gradients that regulate possible sites for nodule initiation. Four developmental stages are shown in clockwise sequence: (1) initial infection of rhizobia at the site of root hair curling (rhc) accompanied by the induction of ethylene and reactive oxygen species (ROS) as well as *ENOD40* induction in pericycle cells within hours of inoculation. (2) Precursor cells of the cortex, which will divide to become a nodule, show increased expression of *GH3*, *ENOD40* and accumulation of specific flavonoids. (3) Early cortical cell divisions (ccd) show enhanced *AUX1*, *GH3* and *ENOD40* expression as well as flavonoid and cytokinin accumulation. (4) In a differentiating nodule, increased levels of ABA, auxin, GA and nitric oxide have been detected. *AUX1*, *GH3* and *ENOD40* expression are located in peripheral (probably vascular) tissue. Cytokinin, ABA and LOX levels are increased in the nodule meristem.

Reports suggesting that ABA is required for nodulation do not necessarily discredit the previously mentioned work indicating that ABA has an inhibitory role in nodulation. Instead, ABA may have a dual role in nodule development: one in negatively regulating nodule numbers and one in positively regulating the growth and development of individual nodules. As such, an increase in ABA (for example one brought about by exogenous application or stress) would directly inhibit nodule development, whereas a deficit of the hormone would fail to induce the signalling elements (such as LOX) required to meet the growth requirements of the nodule. This hypothesis may explain why some reports of ABA application (e.g. Bano and Hillman 1986) describe no effects of the hormone on nodule numbers as the authors may have applied a level of ABA below the threshold level required to achieve inhibition.

In support of this hypothesis, Charbonneau and Newcomb (1985) reported that pea nodule ABA levels were high in the first two weeks of nodule development followed by a two-week plateau and then a secondary period of elevated ABA. It is possible that the first rise in ABA is related to the regulation of nodule growth and number, the plateau corresponds to the period of nitrogen fixation and the second rise is associated with the onset of nodule senescence. These results suggest a putative third role for ABA in nodulation in which ABA increases in older nodules as part of a senescence-signalling pathway. In addition to pea, older nodules of *Lens* sp. (Dangar and Basu 1984), *Phaseolus aureus* (Dangar and Basu 1987), *Samanea saman* (Chattopadhyay and Basu 1989) and soybean (Federova et al. 1992) have elevated amounts of ABA when compared with younger nodules, which the authors of these studies also suggested was related to nodule senescence. The elevated level of ABA in soybean nodules led Federova et al. (1992) to speculate that ABA played a role in both the suppression of the formation of new nodule structures and in nodule senescence, which is consistent with the hypothesis reported here.

Auxin

Auxin is a plant hormone with multiple roles in cell division, differentiation and vascular bundle formation, three processes that also occur during nodule formation. Auxin is synthesised mainly in the shoot and is transported to the roots by an active transport process involving import into the cell by an auxin import protein (AUX1) and active auxin export by an export protein (PIN1 and PIN2/AGR/EIR1; reviewed by Muday and DeLong 2001). Additional control stems from negative regulators of auxin export by auxin transport inhibitors that bind to proteins interacting with the auxin exporter (Muday and DeLong 2001). Thus, the plant has several targets for regulating auxin homeostasis tightly to control organogenesis.

Compared with the roots, auxins levels have been reported to be elevated in the nodules of a variety of plant species (e.g. pea (Badenoch-Jones et al. 1984), *P. vulgaris* (Fedorova et al. 2000) and *A. glutinosa* (Wheeler et al. 1979)). Increased auxin levels in legume nodules, and in nodule-like structures of non-legumes, have also been observed after application of the synthetic auxin, 2,4-D (e.g. Ridge et al. 1992). Early experiments suggested that the ratio of auxins to cytokinins in the root was responsible for the initiation of cortical cell divisions and nodule formation (e.g Libbenga et al. 1973). In the soybean hypernodulating mutant *nts386*, the auxin:cytokinin balance was found to be lowered compared with the wild type, suggesting that the auxin:cytokinin ratio could be important for regulating nodule numbers (Caba et al. 1998). These experiments suggested that rhizobia might manipulate auxin levels in the plant. In addition, sensitivity to auxin in *Medicago sativa* (alfalfa) lines correlates with the rate of spontaneous nodule formation and nodulation efficiency can be increased by the introduction of *Agrobacterium rol* genes, which are known to affect auxin sensitivity and plant hormone levels (Kondorosi et al. 1993).

A number of experiments suggest that rhizobia manipulate auxin transport thus changing the auxin:cytokinin ratio in the root. For example, direct measurements of auxin transport using labelled auxin showed that rhizobia inhibit acropetal auxin transport (from the root base to the tip) capacity in *Vicia sativa* (vetch) roots (Boot et al. 1999). In addition, the expression of the auxin responsive

promoter *GH3* fused to the *GUS* reporter gene was reduced towards the root tip between 12 and 24 h following rhizobia inoculation or ballistic microtargeting of Nod factors in *Trifolium repens* (white clover; Mathesius et al. 1998a). High *GH3-GUS* expression levels were then seen 24 to 48 h following inoculation (Mathesius et al. 1998a) and in soybean, increased auxin levels were measured 48 h after inoculation (Caba et al. 2000). These results are consistent with the auxin burst hypothesis of nodulation which states that subsequent to the initial induction of nodule primordia, shoot derived auxin export into the root is stimulated, resulting in elevated auxin levels that inhibit further nodule primordia initiations, thus controlling nodule numbers (Gresshoff 1993). This auxin burst is assumed to be defective in supernodulation mutants, where increased auxin levels following inoculation could not be detected (Caba et al. 2000). Altogether, it is likely that auxin plays (at least) a dual role during nodulation: in the early stages, auxin transport inhibition might result in a reduced auxin:cytokinin ratio to allow cell division to start, and later divisions are inhibited by super optimal auxin levels (Figure 1).

The application of synthetic polar auxin transport inhibitors (PATIs), which interfere with the hormone balance, can induce pseudo-nodule structures on the root and are also sufficient to induce some of the nodulin genes inside pseudo-nodules, including *ENOD2* and *ENOD12* (Hirsch et al. 1989; Scheres et al. 1992; Wu et al. 1996). More recently, it has been shown that PATIs mimic the action on Nod factors on the repression of calmodulin expression in *P. vulgaris* (Camas et al. 2002).

In addition to PATIs, the inhibition of auxin transport could be achieved by regulating the number of auxin efflux carriers in the cells transporting auxin. Alternatively, Nod factors or chitin oligosaccharides could affect the affinity of endogenous auxin transport regulators to their binding site, similar to the effect of ethylene (Suttle 1988), and/or Nod factors could induce the synthesis or release of an endogenous auxin transport inhibitor. Other plant compounds, including ethylene, cytokinins and flavonoids (e.g. Brown et al. 2001; Jacobs and Rubery 1988; Murphy et al. 2000; Stenlid 1976), can also inhibit auxin transport and can regulate various peroxidases and IAA oxidases, the enzymes that break down auxin (Burgh and Burgh 1966; Lee 1971), thus leading to local shifts in the plant auxin:cytokinin ratio.

Peroxidase activity is elevated in *P. vulgaris* nodules, presumably to limit an auxin increase in maturing nodules (Fedorova et al. 2000). A temporal and spatial correlation was found between the accumulation of specific flavonoids that inhibit auxin breakdown by a peroxidase and the accumulation of *GH3:GUS* expression in nodule primordia (Mathesius 2001). Furthermore, the accumulation of other flavonoids that stimulate auxin breakdown was detected in cells that exhibit low *GH3:GUS* activity, further suggesting that a local accumulation of specific flavonoids could regulate auxin levels.

The expression of flavonoid genes (e.g. *PAL* (phenylalanine-ammonia lyase) and *CHS* (chalcone synthase)) is enhanced in nodules (e.g. Estabrook and Sengupta-Gopalan 1991; Djordjevic et al. 1997), and rhizobia and Nod factors can induce flavonoid gene expression and localised flavonoid accumulation (e.g. Djordjevic et al. 1997; Lawson et al. 1996; Mathesius et al. 1998b; Schmidt et al. 1994). Therefore, it has been suggested that Nod factors could have a role in inducing flavonoid accumulation at the infection site, followed by changes in the auxin balance (Hirsch 1992; Mathesius et al. 1998a). By micro-targeting flavonoids into roots of white clover carrying the *GH3:GUS* construct, it was shown that flavonoids had similar effects on auxin distribution as Nod factors and synthetic auxin transport inhibitors. While this suggests that flavonoids could mimic Nod factor action, it remains unclear if the exact flavonoids induced by rhizobia in the root would mediate this response in the concentration present in the tissue, and whether these flavonoids would be sufficiently mobile to reach their binding site.

There is also evidence that auxin distribution is regulated locally in nodule primordia and mature nodules, which would allow for spatial control of cell division in the root (Figure 3). Direct measurements of auxin (i.e. indole acetic acid, IAA) contents in *P. vulgaris* roots and nodules showed increased IAA levels in roots preceding nodule formation and during the early stages of nodule emergence, whereas auxin levels dropped in mature nodules (Fedorova et al. 2000). In white clover, expression patterns of *GH3:GUS* indicated that auxin levels and/or sensitivity are increased in early dividing cortical cells (Mathesius et al. 1998a). *GH3:GUS* expression then decrease in the differentiating nodule primordium and remain only in

developing vascular tissue, consistent with a role of auxin in triggering cell division and vascular bundle formation. Recent studies by de Billy et al. (2001) have expanded this idea by showing that in *Medicago truncatula AUX1*-related genes (termed *MtLAX*) are induced in early nodule primordia and developing vascular tissue. These expression sites mirrored those of *GH3:GUS* in white clover (Figure 3), which suggests that auxin might increase in early nodule primordia by regulation of auxin import into these cells.

The role of auxin in nodulation is tightly linked to the development of other root structures, including lateral roots and root galls, which require similar induction of new cell divisions and differentiation as nodules. Auxin transport is required for lateral root induction (Bhalerao et al. 2002) and auxin appears to accumulate not only in nodule but also lateral root primordia (Himanen et al. 2002) and root galls caused by nematodes (Goverse et al. 2000; Hutangura et al. 1999). Expression levels of *GH3:GUS* were very similar in developing nodule and lateral root primordia (Mathesius et al. 1998a). These similarities are likely due to auxin-induced activation of cell cycle genes that are required for the induction of new cell divisions during organogenesis (Doerner et al. 1996; John et al. 1993). A genetic link between regulation of root system architecture and nodulation has been found in the *Lotus japonicum* (lotus) *har1* (hypernodulation aberrant root formation) and the soybean *nts* mutants (Wopereis et al. 2000; Searle et al. 2002, respectively), which are both supernodulating mutants that show increases in the number of lateral roots in the uninoculated state and altered activities of the root apical meristem. Since auxin affects both lateral root, nodule and meristem formation, it is tempting to speculate, and pertinent to test, whether autoregulation exerts some of its effects via changes in auxin homeostasis, or whether additional, or different, signals are involved. The fact that lateral root frequency is not affected in the supernodulation mutant *astray* in lotus suggests the existence of nodule specific regulators in addition to regulation of all root meristems (Nishimura et al. 2002b).

Cytokinins

Cytokinins are a class of plant hormones having diverse roles in cell cycle regulation and differentiation. Re-activation of the cell cycle initiates nodule primordium formation (Foucher and Kondorosi 2000; Goormachtig et al. 1997; Yang et al. 1994) and cytokinins, together with auxin and ethylene, play a major role in cell cycle progression in plants (D'Agostino and Kieber 1999). Therefore, it is likely that cytokinins are also necessary for new cortical cell divisions initiated by *Rhizobium*. However, even though cytokinins have been reported to be synthesised by different bacteria, including rhizobia (Phillips and Torrey 1970; 1972), it is unlikely that cytokinins provided by rhizobia are the main factors necessary for nodule initiation, because purified Nod factors are sufficient to induce nodules in some legume species. Instead, it is more likely that Nod factors trigger changes in cytokinin synthesis, turnover or sensitivity in the roots during nodule initiation.

Either way, several pieces of evidence suggest that rhizobia do induce changes in the cytokinin balance of the root. Nodule cytokinin levels are reported to be elevated in numerous plant species when compared with the roots (e.g. pea (Badenoch-Jones et al. 1987), *Phaseolus mungo* (Jaiswal et al. 1981), *Myrica gale* (Rodriguez-Barrueco et al. 1979), and *Vicia faba* (Hensen and Wheeler 1976)). In pea, Newcomb et al. (1976) showed that nodule cytokinin levels were highest in young, developing nodules and decrease with maturity. Syono et al. (1976) demonstrated that the highest cytokinin levels in the pea nodule were located in the meristem (Figure 3). This agrees with the role of cytokinin in cell division and differentiation and also supports the results of Newcomb et al. (1976) as young nodules would be the most mitotically active and thus one would expect to contain elevated levels of the hormone.

The application of cytokinins induces the formation of pseudo-nodule structures on legumes and non-legumes, including *Nicotiana tabacum* (tobacco) (Arora et al. 1959), *A. glutinosa* (Rodriguez-Barrueco and Bermudez de Castro 1973), pea (Libbenga et al. 1973), *Macroptilium atropurpureum* (siratro) (Relic et al. 1994) and alfalfa (Cooper and Long 1994; Bauer et al. 1996). Cooper and Long (1994) transferred the *Agrobacterium* trans-zeatin secretion gene to either nodulation

deficient mutants of *R. meliloti* or to *E. coli*, and showed that synthesis of the cytokinin zeatin by these bacteria is sufficient to induce nodule-like structures in alfalfa. However, it is important to note that the concentration of externally applied cytokinin is important in determining whether cytokinins have stimulating or inhibiting effects on nodulation (Lorteau et al. 2001).

The roles of cytokinins during nodule development include, as expected, the activation of the cell cycle and genes associated with it (Jelenska et al. 2000). For example, cytokinins induce the expression of *Msgbl*, which is expressed in dividing cells of alfalfa, including those of the nodule primordia, and may be involved in hormone-mediated cell division including having a putative signal transduction role during nodule organogenesis (McKhann et al. 1997). Cytokinins may also be important for activating a number of early nodulin genes. For example, *ENOD2*, a gene expressed in nodules and nodule primordia can be induced by cytokinins in *Sesbania rostrata* (Dehio and deBrujin 1992) and in alfalfa (Cooper and Long 1994; Bauer et al. 1996). *ENOD12A*, coding for a hydroxyproline-rich glycoprotein that is expressed during nodule organogenesis, can also be induced by cytokinins in addition to Nod factor treatment (Bauer et al. 1996). Another early nodulin gene that may have an important role in organ formation is *ENOD40*, which is also induced by both *Rhizobium* and cytokinins in alfalfa (Fang and Hirsch 1998; Mathesius et al. 2000; Sinvany et al. 2002). Screening of molecular markers in alfalfa identified seven nodulin genes regulated by cytokinins, four of which were also inducible by auxin, suggesting partial overlaps between auxin and cytokinin regulated pathways during nodulation (Jimenez-Zurdo et al. 2000). Cytokinins have further been shown to affect ethylene levels in pea roots (Lorteau et al. 2001). However, Lorteau et al. (2001) were unable to demonstrate a direct correlation between cytokinin-induced ethylene and nodule inhibition, as inhibitors of ethylene synthesis did not restore nodulation in plants treated with high levels of cytokinin.

Cytokinins probably also play a role in setting up a carbohydrate sink for the developing nodule. Cytokinins can induce starch formation in the root cortex, similar to that of *Rhizobium* infection (Bauer et al. 1996). The use of a split root system in vetch has also shown that cytokinin treatment of a root can induce acidification of the

growth medium around a separate root of the same plant (van Brussel et al. 2002). These authors suggest that while cytokinins do not appear to be the autoregulation signal, they might create a sink in the inoculated root, which sends a signal to the shoot that regulates metabolism, including acid secretion, in the uninoculated roots. This cytokinin-induced root signal could play a role in autoregulation, in addition to the so far unidentified autoregulation signal from the shoot, which requires actively dividing cortex cells (van Brussel et al. 2002).

The analysis of legume mutants such as R50 (pea) and MN1008 (alfalfa) provide valuable tools for investigating the roles of cytokinins in nodulation. R50 develops abnormal infection threads that twist and bulge as opposed to properly progressing into the inner cortex (Lorteau et al. 2001). Lorteau et al. (2001) demonstrated that this characteristic could also be induced in wild type pea upon cytokinin application. Interestingly, nodulation is rescued in R50 by the application of inhibitors of ethylene biosynthesis or action. However, as stated above, the same ethylene inhibitors were unable to reverse the effects of cytokinin application on wild type pea. Recent work on R50 has shown that the shoot of the mutant is less sensitive to ethylene than its wild type and appears to overproduce the hormone in addition to having elevated levels of cytokinin (Ferguson et al. 2005b).

The application of cytokinins to the *Rhizobium* and Nod factor resistant MN1008 overcomes the nodulation block in this mutant (Hirsch et al. 1997), suggesting that this plant has low levels of the hormone or is unable to increase its cytokinin levels to meet the requirements for nodule initiation. PATIs were also reported to induce pseudo-nodules in this mutant (Hirsch and Fang 1994), suggesting again that the cytokinin:auxin ratio rather than cytokinins alone might be important for nodulation. The mutated gene in MN1008 was recently cloned and identified as a receptor kinase (Endre et al. 2002).

Further evidence that cytokinins play a role in cell division and autoregulation comes from the receptor kinase mutant *har1* of lotus (Krussel et al. 2002; Nishimura et al. 2002a). The *har1* mutant has a short root phenotype that can be mimicked in the wild type by application of cytokinin. However, in the presence of the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG), cytokinin caused root

elongation in the mutant in excess of untreated wild type levels, suggesting that *har1* has an altered response or sensitivity to cytokinin that is not mediated by ethylene (Wopereis et al. 2000).

Ethylene

Ethylene is a gas with multiple roles in plant development and defence. The role of ethylene in nodulation has recently been reviewed by Guinel and Geil (2002) and Wang et al. (2002). Ethylene might have a dual effect on nodulation, in that it causes a local inhibition of nodule formation in most legumes but might be required at certain levels for proper infection by the bacteria. The application of ethylene, or ethylene releasing compounds, is inhibitory to nodule organogenesis in numerous species including *P. vulgaris* (Grobbelaar et al. 1971), pea (Drennon and Norton 1972; Lee and LaRue 1992c), white clover (Goodlass and Smith 1971), *Melilotus alba* (sweet clover) (Lee and LaRue 1992c), *M. truncatula* (Penmetsa and Cook 1997), lotus, and siratro (Nukui et al. 2000). Grobbelaar et al. (1971) found that ethylene also reduced the level of nitrogen fixation in *P. vulgaris*. In pea, Lee and LaRue (1992c) determined that ethylene concentrations as low as 0.07 μL/L are able to inhibit nodule formation. It appears however, that soybean is less sensitive to the hormone as nodulation of this species is not affected by applied ethylene (Lee and LaRue 1992c; Schmit et al. 1999; Nukui et al. 2000). This finding suggests that different species display different requirements and regulatory mechanisms for hormones, a point that must be taken into consideration for any hormone when investigating its roles in processes such as nodulation.

Inoculation of roots with rhizobia has been reported to induce increases in the local ethylene concentration in alfalfa (Ligero et al. 1986), vetch (van Workum et al. 1995), and soybean (Suganuma et al. 1995), but this increase was not detected in pea (Lee and LaRue 1992b). These increases are likely due to an initial defence response elicited by the invading bacteria, which, interestingly, also synthesize the hormone (Billington et al. (1979).

The application of inhibitors of ethylene synthesis (e.g. AVG) or perception (e.g. silver ions) increased the number of nodules that formed on pea (e.g. Lee and

LaRue 1992a), alfalfa (Peters and Crist-Estes 1989; Caba et al. 1998), lotus and siratro (Nukui et al. 2000). These compounds also partially restored the nodulation phenotype of low nodulating mutants of pea including *sym5* (Fearn and LaRue 1991), *brz* (Guinel and LaRue 1992) and *sym21* (Markwei and LaRue 1997) and completely restored that of *sym16* (Guinel and Sloetjes 2000). Surprisingly, the nodulation phenotype of *sym17*, a pea mutant thought to overproduce the hormone, is not rescued with the application of ethylene inhibitors (Lee and LaRue 1992a). Interestingly, Yuhashi et al. (2000) illustrated that *Bradyrhizobium elkani*-produced rhizobitoxine, which acts as an inhibitor of ethylene synthesis, also enhances the nodulation of siratro and may help the bacteria overcome ethylene's inhibitory effects on nodulation. Additionally, Roddam et al. (2002) recently illustrated that the role of ethylene in nodulation can depend on the infecting *Rhizobium* cultivar as the application of AVG to *Trifolium subterraneum* (subterranean clover) enhanced the nodulation by some, but not all, strains of *R. leguminosarum*.

The mechanism of ethylene action as an inhibitor of nodulation is not known. One proposal is that ethylene induces plant chitinases, which subsequently destroy Nod factors and thereby limit the extent of nodule initiation (Mellor and Collinge 1995; Staehelin et al. 1994). Guinel and Geil (2002) proposed a model in which the rhizobia would not come into contact with ethylene in the root until after the epidermis, as this cell layer contains no ACC oxidase (the enzyme that catalyses the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene) and does not appear to perceive the hormone. Consistent with this model is evidence that in pea ethylene appears to block rhizobial entry into the root cortex, rather than the number of infection events (Lee and LaRue 1992c). This finding is supported by work with the *brz* mutant of pea, which has a third less infection events than its wild type. Although nodulation in *brz* is partially restored by ethylene inhibitors, the number of infection events is only slightly increased (Guinel and LaRue 1992).

Contrary to these findings with pea, ethylene does appear to negatively regulate rhizobial colonization of *M. truncatula* as the application of AVG increased the number of infection events, whereas ACC decreased them (Oldroyd et al. 2001). In addition, the ethylene insensitive *skl* mutant of *M. truncatula* has a significantly

increased number of infection events compared with that of its wild type (Penmetsa and Cook 1997; Oldroyd et al. 2001). The *skl* mutant is also unable to regulate the number of these events that develop into fully functional nodules and as such it hypernodulates (Penmetsa and Cook 1997). While ethylene is unlikely to be involved in systemic autoregulation (Nishimura et al. 2002c; Wopereis et al. 2000), it is likely that ethylene plays a role in regulating infection events locally in the susceptible root zone as demonstrated in the *skl* mutant.

Oldroyd et al. (2001) postulated that a block in nodulation induced by ethylene could occur very early during the signal transduction cascade. Evidence for this came from the finding that the sensitivity of root hair cells to Nod factors is significantly increased in the *skl* mutant, and that modulation of ethylene synthesis in the wild type had comparable effects on the sensitivity of Nod factor perception. Ethylene appears to influence a component at, or upstream of, calcium spiking in the Nod factor signal transduction pathway leading Oldroyd et al. (2001) to propose that, in addition to inhibiting the frequency of calcium spiking, the hormone determines the Nod factor concentration required for the root hair Ca^{2+} spiking response. These authors also illustrated that in *M. truncatula* ethylene regulates the expression of the early nodulin genes *ENOD11* and *RIP1* and thus might effect events downstream of the early influence on calcium spiking.

Ethylene may also have a positive role in infection thread development as the number of infection threads aborted in *skl* is very low (Penmetsa and Cook 1997; Oldroyd et al. 2001). Guinel and Geil (2002) suggested that in pea ethylene could affect the cytoskeleton, pre-infection thread and infection thread formation. Using pea and vetch, Heidstra et al. (1997) demonstrated that ethylene is also likely to be involved in determining the positioning of nodule primordium development around the stele (Figure 3). These authors showed that the expression of ACC oxidase is elevated in the inner cortical cells located in front of the root phloem poles. These locations are between the positions at which nodules preferentially arise opposite the root xylem poles. In addition, inoculation of vetch with *R. leguminosarum* induces ethylene-related responses including a thick and short root phenotype and abnormal

nodule positioning on the root system, which is restored following AVG application (Zaat et al. 1989; van Spronsen et al. 1995).

Interestingly, ethylene also appears to change the phenotype of the nodules of *Sesbania rostrata*, a legume that grows in waterlogged soils and therefore is likely to be exposed to varying levels of the hormone (Fernández-López et al. 1998). The authors found that in the absence of ethylene (perception), nodules were of the indeterminate type, whereas in the presence of ethylene, determinate nodules with a terminal meristem were formed, suggesting a role for ethylene in meristem differentiation.

Gibberellin

Little is known about the signalling involvement of gibberellins (GAs) in nodulation. Early work focused on applying the hormone (generally GA_3) to the plant, which resulted in a decline in nodule formation (Thurber et al. 1958; Galston, 1959; Fletcher et al. 1959; Mes, 1959). In 1952, Nutman demonstrated that the removal of root tips and mature nodules from various red clover sp. promoted the formation of new nodules, presumably by removing the source of a compound inhibitory to nodulation. Based on the results of Nutman (1952), and evidence that nodules of pea and *P. vulgaris* contain elevated levels of GAs, Radley (1961) speculated that GAs regulate nodule formation. Since then, nodules of *Lupinus luteus* (Dullaart and Duba 1970), *A. glutinosa* (Henson and Wheeler 1977), *Phaseolus lunatus* (Evensen and Blevins 1981), soybean (Williams and Sicardi de Mallorca 1982), *Lens* sp. (Dangar and Basu 1984), *Phaseolus aureus* (Dangar and Basu 1987), *P. vulgaris* (Atzorn et al. 1988), *S. saman* (Chattopadhyay and Basu 1989) and *Vigna unguiculata* (cowpea) (Dobert et al. 1992b and c) have all been reported to contain higher levels of GAs than adjacent root tissue, yet to date no direct evidence implies a signalling role for GAs in the regulation of nodule formation.

In 1970, Dullaart and Duba reported in *L. luteus* that, in addition to having increased GA levels in nodule extracts compared with those of the surrounding root tissue, the application of GA_3 to nodule extracts stimulated IAA production from L-tryptophan. These authors speculated that a signalling interaction existed between the

two hormones in which GA_3 was able to either increase the bioproduction, or decrease the metabolism, of IAA (Figure 1), but the mechanism underlying this interaction has still not been demonstrated. However the reverse interaction has since been confirmed in stems, where the biosynthesis of GA_1 requires the presence of IAA (Ross et al. 2000). In addition, the application of PATIs to the stem reduces GA_1 levels below the site of PATI application, corresponding with the IAA level at these locations (Ross 1998). PATIs can induce the formation of pseudonodules on the root systems of various species, and as such it will be interesting to investigate what role(s) GAs, and possibly more importantly GA/IAA ratios, play in the formation of these outgrowths. Recently, IAA was shown to promote root growth in *Arabidopsis* by modulating cellular responses to GAs (Fu and Harberd 2003) and it seems possible that a similar interaction might exist between the two hormones in regulating nodule development.

Nodule GA levels appear to be influenced by the infecting *Rhizobium* strain in *P. lunatus* (Tripplett et al. 1981; Dobert et al. 1992a and c), contrary to a report on *P. vulgaris* nodules (Atzorn et al. 1988). Many reports have demonstrated that various *Rhizobium* strains are capable of synthesizing GAs in culture (e.g. Katznelson and Cole 1965; Rademacher 1994). Recently, putative GA biosynthetic enzymes were identified in *Bradyrhizobium japonicum* that function anaerobically, including under the symbiotic conditions bacteroids are subjected to in the symbiosome (Tully et al. 1998) suggesting that rhizobia might be capable of regulating GA levels both before and after bacteroid differentiation. However, whether or not the elevated GA levels of *P. lunatus* nodules stem directly from rhizobial synthesis, or if the bacteria induce the plant to increase GA production, is unknown (Dobert et al. 1992c). Dobert et al. (1992c) hypothesized that, in addition to the bacterial strain, nitrogen, ABA and even the host plant species may have a role in regulating nodule GA concentrations.

The application of GA_3, and to a lesser extent GA_4, induced the formation of nodule-like structures on the roots of lotus (Kawaguchi et al. 1996). These structures initiated from divisions of the pericycle and could be suppressed with the addition of nitrate. Thus, it appears that an interaction exists in lotus whereby GAs positively regulate the division of pericycle cells necessary for nodule organogenesis and that

nitrate modulates this process by acting as signalling elements that suppress these GA-induced divisions.

Nonetheless, it has been argued that an increased concentration of GAs might not be a requirement for nodule formation in some species, such as *P. vulgaris* (Atzorn et al. 1988). If elevated GA levels are not required for nodulation, then based on the previously mentioned work demonstrating that GAs are influenced by IAA, the increased GA levels detected in nodules may be no more than a consequence of the high IAA levels also present there.

As an alternative to having a role in nodule formation, GAs may act as signals for the hydrolysis of nodule starch to provide a substrate for rhizobial respiration requirements. GAs promote the production of α-amylase (e.g. Gubler et al. 1995), an enzyme involved in the metabolism of starch, and it may be worth investigating whether or not the activities of the hormone and the enzyme are interacting within the nodule. Evidence for a link between GAs and α-amylase in starch hydrolysis exists for various fungal species (reviewed in Rademacher 1994), but to the best of my knowledge, the idea that GAs might have a similar role in nodulation has not been proposed previously. If a correlation is established between GAs, α-amylase and starch in nodulation, it is possible that the bacteria are responsible for regulating nodule GA levels as a means of obtaining nutrients. As I hypothesized for ABA, this alludes to multiple roles for GAs in nodulation, including aiding in cell division and elongation and providing the energy requirements for the nitrogen-fixing bacteria. Elevated nodule GA levels have also been correlated with increased internode number and length and increased petiole length in *P. lunatus* (Tripplett et al. 1981; Dobert et al. 1992a and c) and cowpea (Dobert et al. 1992b and c). Thus, GAs may benefit both symbionts by increasing the plants size, thereby increasing the photosynthetic capability of the plant, resulting in more photosynthates for plant and nodule growth and functioning.

Signalling Peptides

Apart from the classical plant hormones, peptides have recently emerged as potential regulators of nodulation. Compared with animal peptide hormones, only a few plant signalling peptides have been discovered so far. However, this number is likely to rise because more and more receptor kinases are being identified as playing a role in plant development and nodulation, many of which could be activated by peptide ligands. For example, recent discoveries of receptor kinases responsible for early Nod factor perception/signal transduction ("NORK", Endre et al. 2002; Stracke et al. 2002) and for the autoregulation of nodulation ("NARK", Krusell et al. 2002; Nishimura et al. 2002a; Searle et al. 2002) indicate that peptides or proteins could be ligands for these nodulation related receptor kinases.

One putative peptide that plays an important role in nodulation is the early nodulin *ENOD40*. There has been some debate on whether or not *ENOD40* is actually translated. Several ORFs have been identified with stable predicted secondary structure, and it was initially suggested that *ENOD40* acts in the form of a stable RNA, a so-called "riboregulator" (Asad et al. 1994; Crespi et al. 1994). However, Sousa et al. (2001) found that translation of two small *ENOD40* ORFs is necessary for biological function (induction of cortical cell division) and Röhrig et al. (2002) reported detection of one of the ENOD40 peptides by immunoprecipitation and Western blotting. Mutational analysis suggests that the translated products might have a role in stabilising a biologically active *ENOD40* mRNA structure (Sousa et al. 2001). It is therefore possible that both the peptide and the mRNA are necessary for biological function as a ribonucleoprotein (Sousa et al. 2001), although no target or receptor has so far been found.

ENOD40 appears to play an important role in cell cycle control because over-expression (Charon et al. 1997) and microtargeting (Sousa et al. 2001) of *ENOD40* induces cortical cell divisions in alfalfa roots in the absence of rhizobia and causes teratomas in *Medicago* embryos. In the presence of rhizobia, overexpression of *ENOD40* was shown to accelerate nodulation (Charon et al. 1999). In contrast, silencing of *ENOD40* leads to arrest of callus growth in *Medicago* (Crespi et al.

1994). Recent evidence suggests that *ENOD40* might play a role in sucrose partitioning or unloading from the phloem in the nodule (and/or the whole plant), because synthetic ENOD40 peptides bind to nodulin 100, a sucrose synthase (Röhrig et al. 2002). A role in sucrose partitioning might be related to a role for ENOD40 in promotion of (cortical) cell division because incipient meristems are strong carbohydrate sinks. The expression of *ENOD40* in vascular tissue in roots and mature nodules (Kouchi and Hata 1993) supports a role in sucrose unloading.

ENOD40 has been identified in many legumes as well as the non-legume rice (Kouchi et al. 1999). In all legumes examined, *ENOD40* mRNA has been localised in dividing and meristematic cells (Figure 3; e.g. Asad et al. 1994; Corich et al. 1998; Crespi et al. 1994; Fang and Hirsch 1998; Mathesius et al. 2000; Yang et al. 1993), consistent with the hypothesis that *ENOD40* plays a role in cell division. *ENOD40* is thought to be involved in the earliest stages of nodule initiation because it is expressed within hours of inoculation with nodulating rhizobia (Corich et al. 1998; Fang and Hirsch, 1998) and its expression in the pericycle precedes nodule initiation (Figure 3; Compaan et al. 2001). In addition, *ENOD40* expression is induced by signal molecules that can initiate cortical cell divisions, including Nod factors (Fang and Hirsch 1998; Minami et al. 1996), cytokinins (Fang and Hirsch 1998; Mathesius et al. 2000), and auxin transport inhibitors (Fang and Hirsch 1998). *ENOD40* is also induced in the nodule primordium by *Rhizobium* strains that induce cell divisions but do not infect and invade the nodules (Yang et al. 1993), which is a further indication that *ENOD40* is involved in nodule morphogenesis, rather than the infection process. However, *ENOD40* is not specific to the nodulation process, and is also induced during the establishment of lateral root primordia (Mathesius et al. 2000) nematode-induced galls (Favery et al. 2002; Koltai et al. 2001) and mycrorrhizal interactions (Staehelin et al. 2001; Sinvany et al. 2002).

Defence-Related Signalling Compounds

In addition to its previously mentioned roles in nodulation, ethylene is involved in pathogenic defence as part of a signalling process termed systemic acquired resistance (SAR). Other components of SAR include salicylic acid (SA), nitric oxide (NO), reactive oxygen species (ROS), jasmonic acid (JA) and its methyl ester (MeJA) (reviewed in Ryals et al. 1996; Rojo et al. 2003). Although the mechanism is not fully understood, symbiotic organisms invade the host plant without fully inducing the SAR response. However, Vasse et al. (1993) demonstrated that some plant defence compounds do accumulate following the establishment of the first nodule primordia, resulting in increased abortion of infection threads and localized hypersensitivity response (HR) including necrosis. These authors suggested that this response is part of the autoregulatory mechanism used by plants to control the level of nodulation. Despite this and much work involving ethylene (described above), little is known about the signalling involvement of other SAR components in regards to nodulation; major-findings involving these compounds are addressed in the following section (see also Figure 2).

Salicylic Acid

Pre-soaking seeds with SA prior to sowing decreased the nodule number and protein content and root nitrogenase activity of *Vigna mungo* plants (Ramanujan et al. 1998). SA application prior to inoculation with rhizobia or purified Nod factor also decreased the number and dry weight, and delayed the emergence, of alfalfa nodules (Martínez-Abarca et al. 1998). van Spronsen et al. (2003) found that 0.1 mM SA application completely inhibited indeterminant nodule formation, including the mitogenic effect induced by Nod factors, in vetch, pea (including the hypernodulating mutant P88), alfalfa and white clover but did not affect determinant nodule formation in *P. vulgaris*, lotus and *Glycine soya*. In contrast to these findings, in soybean, 5 and 1 mM SA did decreased the nodule number and dry weight and suppressed photosynthesis and nitrogen uptake (Lian et al. 2000). Also in soybean, Sato et al. (2002) found that concentrations of SA as low as 0.1 mM applied 5 days prior to bacterial inoculation decreased the nodule number and dry weight in addition to the

level of nitrogen fixation. SA also reduced the nodule number and dry weight in supernodulating soybean mutants, but the decreases were less pronounced than in the wild type. Sato et al. (2002) proposed that SA, or SAR induced by SA, might be involved in an autoregulatory signalling pathway of nodulation.

Upon symbiont recognition, the root-SA level of alfalfa did not increase (as occurs upon plant-pathogen recognition), although it did increase in plants inoculated with either an incompatible or a compatible but Nod factor-deficient mutant of *Rhizobium* (Martínez-Abarca et al. 1998; Blilou et al. 1999). Thus, it was concluded that a function of Nod factors is to inhibit host SA-mediated defences. Interestingly, upon inoculation with a compatible rhizobial strain the root-SA level of the pea *sym30* mutant did increase, whereas upon inoculation with plant pathogens an increase was not detected (Blilou et al. 1999). Thus the *SYM30* gene product appears to function specifically with symbiotic microorganisms leading Blilou et al. (1999) to conclude that the *SYM30* gene product is likely required for symbiosis, as a suppressor of a SA-dependent defence response.

In *Rhizobium etli*, multi-drug resistance genes have been identified that act as bacterial efflux pumps that confer resistance to the accumulation of toxic compounds. Mutations to two of these genes, termed *rmrA* and *rmrB*, enhanced the sensitivity of the bacteria to plant toxins including phytoalexins, flavonoids and SA (González-Pasayo et al. 2000). These mutants displayed diminished growth on SA or naringenin, and the *rmrA* mutant formed 40 % fewer nodules on *P. vulgaris* than its wild type (González-Pasayo et al. 2000). It was concluded that by preventing the accumulation of toxic compounds, *R. etli* have established an advantage that improves their chances of nodulating the host. In addition, SA was found to promote isoflavonoid (e.g. genistein) synthesis and secretion from *L. luteus* roots (Kneer et al. 1999). Genistein can function as a phytoalexin due to its slight antimicrobial and fungistatic activity and thus rhizobia containing resistance genes to such a toxin should have an infectious advantage over bacteria lacking the efflux pump.

Nitric Oxide

In nitrogen-fixing rhizobia, heme-based sensors have been detected, such as the oxygen-regulated FixL protein kinase in *R. meliloti* (Gilles-González et al. 1994). When active, the deoxy-FixL protein induces a gene expression cascade required for nitrogen fixation. This process is inhibited by the presence of O_2, and possibly also by NO and CO, thus halting nitrogen fixation (Gilles-González et al. 1994). Therefore, NO may have a role in regulating gene expression required for nitrogen fixation within the nodule.

NO has been identified as an inhibitor of bacteroid nitrogenase (e.g. Trinchant and Rigaud 1982). Maskell et al. (1977) illustrated that NO tightly binds to leghemoglobin (Lb) in soybean and cowpea nodules forming nitrosyleghemoglobin complexes (NO-Lb) and suggested that Lb may actually have a higher affinity for NO than it does for O_2. Thus, the NO-Lb complex may act as a protective mechanism used by the nodule to prevent the inhibiting NO from reaching the NO-sensitive nitrogenase of the bacteroid. Alternatively, the accumulation of NO-Lb may result in the inhibition of nitrogenase activity (Kanayama and Yamamoto 1990) as the binding of NO to Lb may competitively inhibit the binding of oxygen, subsequently diminishing the oxygen supply available to bacteroids, thereby reducing nitrogen fixation (Mathieu et al. 1998).

Soybean nodules on roots exposed to high concentrations of nitrate mainly contained NO-Lb (Kanayama and Yamamoto 1990) and declines in nitrogen fixation rates paralleled the increase in NO-Lb in these nodules. Thus, the plant may induce NO synthase (NOS) in response to excess exogenous nitrate as a means of regulating nitrogen fixation activity. However, Mathieu et al. (1998) found that even in the absence of applied nitrate, some NO-Lb exists in soybean nodules. These authors found that the amount of NO-Lb was highest in young nodules, decreased with nodule age, and was nearly absent in senescent or H_2O_2-treated nodules. Moreover, in soybean plants grown in controlled-environmental conditions, NO-Lb was shown to comprise almost a third of the total nodule Lb content (Maskell et al. 1977), but to date no definitive evidence exists to explain this occurrence.

NOS activity has been detected in nodules of *Lupinus albus* (Cueto et al. 1996). Two putative NOS sites were detected: one in the vascular bundles and the other in the inner cells of the infected zone (Cueto et al. 1996). In contrast to root preparations, the synthesis of nodule NO was found to be Ca^{2+} independent and the authors speculated that nodule NOS could possibly be induced by compounds such as lipopolysaccharides of compatible *Rhizobia* sp.

Reactive Oxygen Species

To prevent pathogen invasion, reactive oxygen species (ROS) or active oxygen species (AOS), including hydrogen peroxide (H_2O_2), superoxide radicals (O_2^-) and the hydroxide radical (•OH), are upregulated in the plant upon pathogen recognition. Together, these compounds reinforce plant cell walls and trigger a localized hypersensitive response (HR) involving defence gene expression, the induction of SAR and programmed cell death (reviewed in Ryals et al. 1996). ROS are also induced in host plants upon inoculation with *Rhizobium* (e.g. Bueno et al. 2001; Santos et al. 2001) and thus it is imperative that the bacteria compensate for these defence molecules in order to achieve nodule organogenesis. Both plant and bacterial compounds exist that help protect against the harmful effects of ROS, including peroxidases, catalases and superoxide dismutase (SOD) among others, and *Sinorhizobium meliloti* genes induced upon host infection include those that protect against ROS (Oke and Long 1999). However, aside from having negative effects, ROS can also positively regulate the nodulation process.

Peroxidase activity increases shortly after inoculation at the site of root hair deformation (Salzwedel and Dazzo 1993). The activity appears to have a role in oxidative cross-linking of cell wall polymers at the site of rhizobial penetration resulting in a hardening of the cell wall structure. H_2O_2 can act as a substrate for peroxidase in this process, thus illustrating a potential role for low levels of certain ROS during nodulation. Salzwedel and Dazzo (1993) speculated that for successful infection to occur, the rhizobia must first suppress root hair peroxidase activity, therefore allowing the bacteria to penetrate the cell wall of the host. The authors suggested a rapid and transient decrease in peroxidase activity could be evoked by

rhizobial exopolysaccharides (EPS) which rapidly bind to root hairs, increase infection frequency and may aid the bacteria in avoiding the elucidation of SAR during invasion. Following penetration, highly localized peroxidase activity might be required to repair the eroded root hair cell wall at the site of rhizobial entry and infection thread initiation. Salzwedel and Dazzo (1993) also speculated that the plants might resist non-host bacteria and pathogens by rapidly increasing localized peroxidase levels to harden the root cell walls and prevent their invasion.

Prior to rhizobial infection of *M. truncatula*, Nod factors trigger a rapid and localized expression of the putative peroxidase-encoding *RIP1* early nodulin gene (Cook et al. 1995), as does ethylene (Olroyd et al. 2001). As a peroxidase, RIP1 could have a role in metabolising H_2O_2 and/or in peroxidase-mediated cross-linking of cell wall polymers. The *RIP1* transcript was localized to epidermal cells that subsequently were infected by the *Rhizobium* and were expressed for the duration of pre-infection (Cook et al. 1995) suggesting a possible involvement in cell wall repair at the site of infection. Recently, Ramu et al. (2002) demonstrated that RIP1 transcripts and ROS share a similar pattern of localization in *M. truncatula* and that Nod factor application elicits a rapid induction of each. Neither ROS nor *RIP1* expression was detected using a Nod factor-deficient mutant of *Sinorhizobium meliloti* or a mutant of *M. truncatula* impaired in Nod factor signal transduction. Moreover, Ramu et al. (2002) found that H_2O_2 specifically induced *RIP1* expression, leading the authors to speculate that Nod factor perception by the plant induces H_2O_2 production, which then mediates the Nod factor-induced expression of *RIP1*. This finding seems logical since H_2O_2 can act as a substrate for peroxidases, such as the putative RIP1.

In pea, Wisnewski et al. (2000) found that the insolublisation of matrix glycoproteins creates a barrier inhibiting the continued ingress of invading bacteria. These authors speculated that diamine oxidase activity could locally produce H_2O_2 that can be used by peroxidase to induce the insolublisation of the glycoproteins thereby modulating cell wall plasticity. Within the infection thread, the matrix glycoproteins are found to be insoluble at the tip and hardened elsewhere (Wisnewski et al. 2000). This allows invading rhizobia to progress towards the infection zone of

the nodule in the infection thread as long as the peroxidase level at the tip remains at a low enough level to avoid hardening of the infection thread tip walls.

In addition, actin monoubiquitylation is induced in developing nodules of *P. vulgaris* (Dantán-González et al. 2001). These actin modifications are likely part of a defence response against invading organisms and appear to provide microfilament stability against proteolytic degradation. This response can be mimicked in suspension cell culture by H_2O_2 application (Dantán-González et al. 2001), thus further suggesting that H_2O_2 has a role in modifying cell wall structures.

Salzar et al. (1999) demonstrated that H_2O_2 accumulates in *M. truncatula* cortical cells in the region occupied by arbuscular mycorrhiza. More specifically, H_2O_2 was concentrated around hyphal tips attempting to penetrate a host cell, similar to phenomenon described by Salzwedel and Dazzo (1993) following root hair penetration and infection thread formation by rhizobia. This was suggested to be indicative of an oxidative burst involved in the control of intracellular colonization of the host (Salzar et al. 1999).

In agreement with the above findings, Santos et al. (2001) detected an oxidative burst of H_2O_2 and O_2^- in the curled region of the root hair immediately following inoculation of *M. truncatula*. Interestingly, these elevated levels of ROS were also found in infected cells suggesting that this burst is prolonged and could have a role in regulating the infection process (Santos et al. 2001). van Spronsen et al. (2003) suggested that an oxidative burst could be prolonged by SA, which could bind to, and therefore inactivate, peroxidases such as RIP1.

In addition to modulating cell wall repair and plasticity, ROS can be detrimental to nodulation as they can damage and degenerate the proteins, DNA and lipids of both symbionts and their levels are often elevated in senescent nodule tissue. ROS such as O_2^- and •OH inhibit nitrogen fixation and it has been suggested that the inhibition by O_2^- may be due to its breakdown into the highly reactive and damaging •OH (Puppo and Halliwell 1988). To compensate for the stress of ROS, rhizobia are equipped with enzymes such as SOD, which detoxifies O_2^-. *M. truncatula* inoculated with *Sinorhizobia meliloti* defective in SOD nodulate poorly and display abnormal infection (Santos et al. 2000). In addition, most of the bacteria failed to differentiate

into nitrogen fixing bacteroids and senesced rapidly. This lead Santos et al. (2000) to speculate that oxidative stress interferes at numerous stages of the symbiosis and not simply at the level of nitrogen fixation. Thus rhizobial SOD is a requirement for nodule development as well as functioning.

As mentioned, in addition to rhizobial SOD, plants contain antioxidant defence enzymes that also can breakdown ROS. In leaves of *Zea mays*, treatment with 10 to 100 μM ABA induced the production of O_2^- and H_2O_2 followed by increases in the activities of antioxidant enzymes at levels sufficient enough to scavenge the elevated levels of O_2^- and H_2O_2 (Figure 1; Jiang and Zhang 2001). The authors of this report concluded that ROS have a dual role in plants depending on their quantity: acting as toxins inducing oxidative stress when abundant or as triggers eliciting the upregulation of antioxidant enzymes when elevated only slightly. It seems plausible that the invading *Rhizobium* could positively regulate the plants antioxidant enzymes, possibly via elevated ABA levels, to avoid the damaging ROS and thereby promoting nodulation.

Like ABA, Bueno et al. (2001) showed that inoculation of alfalfa plants with *Rhizobium* elevates both antioxidant enzyme activities and H_2O_2 generation. These elevated levels of scavenging antioxidant enzymes likely have a role in controlling the oxidative burst. Interestingly, among the enzymes elevated is LOX, which was earlier described as being influenced by ABA (Figure 2). Taken together with the previous paragraph, the complexity of signalling in nodulation becomes increasingly apparent.

Jasmonic Acid

JA both induces LOX mRNA accumulation (Figure 2; Porta et al. 1999) and is produced by the action of LOX upon polyunsaturated fatty acids (Gundlach et al. 1992). In addition, MeJA induces the transcription *PAL* (Gundlach et al. 1992), an enzyme that catalyses the first step in SA biosynthesis, and in *L. luteus* roots, its application promotes the synthesis and rhizosecretion of the isoflavonoid genistein (Kneer et al. 1999).

JA also appears to promote the colonization and development of mycorrhizal structures in *Allium sativum* (Regvar et al. 1996) and mycorrhizal colonization has been reported to elevate JA biosynthesis in *Hordeum vulgare* (barley) (Hause et al. 2002). It is possible that JA has similar roles in nodule formation and mutants impaired in JA synthesis or response would greatly aid in the understanding of this signalling molecule in nodulation.

Other Signalling Compounds

Brassinosteroids

Foliar application of epibrassinolide to *Arachis hypogaea* (groundnut) substantially increased the number and weight of nodules and promoted root nitrogenase activity (Vardhini and Rao 1999). In contrast, application of epibrassinolide to the roots of soybean (Hunter 2001) decreased the number of nodules and amount of nitrogen fixation. These differences between studies may be attributed to variation in methods or species used.

Endogenous BRs also appear to influence nodule formation. Recent evidence has shown that BR deficient mutants of pea form significantly fewer nodules than their wild type (Chapter 2; Ferguson et al. 2005a). Using grafting techniques, Ferguson et al. (2005a; Chapter 2) demonstrated that BRs appear to influence nodule formation via a mechanism of the shoot. However, precise roles of BRs in nodulation are unclear as no molecular evidence or signalling interactions pertaining to the roles of BRs in nodule organogenesis exist to date.

Flavonoids

Flavonoids have multiple roles in plant development, defence and nodulation (reviewed in Dakora 1995; Spaink 1999); they constitute a large class of compounds of the phenylpropanoid pathway, and their exact structure is important for their varied functions, including concomitantly inducing the chemotaxis of the *Rhizobium* to the root and elevating the production of Nod factors (e.g. Redmond et al. 1986; Stafford

1997). Flavonoid production is also induced by rhizobia in roots and nodules (e.g. Cooper and Rao 1992; Recourt et al. 1992) and different flavonoids are synthesised in response to rhizobia that up- and down-regulate Nod factor production, both before and during infection (e.g. Zuanazzi et al. 1998).

Flavonoids are distributed in a strictly tissue-specific pattern in many species. In particular, flavonoids are often located in dividing and meristematic tissues, including dividing cortical cells of nodules (Mathesius et al. 1998b). It is possible that flavonoids merely protect dividing cells from oxidative damage because of their activity as antioxidants (Rice-Evans 2001). However, as discussed above it could also be possible that flavonoids affect cell division either by regulating auxin transport or turnover (Figure 1), thereby regulating auxin accumulation (Figure 3), or by directly regulating cell cycle regulators. In animals, much evidence has been found that flavonoids regulate cell cycle activity, but in plants this evidence has so far been very tentative (e.g. Logemann et al. 1995; Jinsart et al. 1991). The existence of a flavonoid deficient mutant in Arabidopsis has shown that flavonoids are not essential for plant survival, although interestingly the mutant showed alterations in lateral root formation, root growth and plant height, which could be result of increased auxin transport due to the absence of flavonoids acting as PATI (Brown et al. 2001). At this stage, flavonoid-deficient mutants have not been isolated in legumes.

Uridine

The position of a nodule is not only determined by the initiation of cell divisions in either the inner or the outer cortex of indeterminate and determinate legumes, respectively, but also in respect to the protoxylem poles (Figure 3). In most legume species, the majority of nodule primordia are initiated in front of one of the protoxylem poles and it has been suggested that a signal (the "stele factor") diffuses out of the xylem and acts together with auxin and cytokinins to induce cell divisions comprising the nodule primordia (Libbenga and Harkes 1973).

The stele factor has been identified as uridine (Smit et al. 1995). In the presence of very low uridine concentrations, cell divisions can be induced in every

cortical cell by cytokinins in pea (Libbenga and Harkes 1973) and in inner cortical cells by chitin oligosaccharides following ballistic micro-targeting in vetch (Schlaman et al. 1997). Differences between the concentrations of uridine in front of xylem versus phloem poles could explain the preference for nodules to initiate opposite xylem poles. The fact that nodules are initiated in the outer cortex in determinate legumes and in the inner cortex in indeterminate ones could be explained by the fact that determinate and indeterminate species have different sensitivities for uridine, although definitive evidence is lacking so far.

Nitrate

Nitrate interacts with plant hormones to regulate nodule formation (Figure 1). The presence of nitrate in the soil at concentrations above 1-5 mM suppresses nodulation locally at several levels, including infection, nodule primordium initiation and nitrogen fixation (reviewed by Streeter 1988). How nitrate inhibits nodulation is not exactly known, although its purpose may be to limit the formation of nodules under conditions that provide sufficient nitrate.

The existence of mutants that hypernodulate even in the presence of nitrate shows that nitrate is not the inhibiting factor itself, but that it leads to secondary signals that suppress nodulation (Carroll et al. 1985). According to the auxin burst hypothesis (Gresshoff 1993), high auxin levels inhibit nodule formation, and it is hypothesised that nitrate increases the sensitivity of the root to auxin, thus reducing nodule formation. In the supernodulation *nts* mutants, the auxin burst control is altered and therefore these mutants can still nodulate in the presence of nitrate because not as much auxin is available in the root to suppress further nodule initiation. In support of that hypothesis, Caba et al. (2000) found that nitrate decreased auxin levels in inoculated and uninoculated roots of wild type and *nts* mutants, whereas root growth was not altered. The authors hypothesised that this represented an increased sensitivity to auxin in the presence of nitrate, which would be consistent with the auxin burst hypothesis; however, auxin sensitivity will need to be assessed by more direct means. An effect of nitrate on the auxin response pathway

has been found in Arabidopsis (Zhang et al. 1999) and it is possible that, in legumes, at least some of the effects of nitrate are also mediated by auxin.

The regulation of nodulation by nitrate could be imposed via an effect on flavonoid accumulation in the root, which can alter auxin transport or Nod gene activity (Coronado et al. 1995). There is also evidence for the involvement of ethylene in mediating the inhibitory effect of nitrate. The findings that inhibitors of ethylene synthesis or action (e.g. AVG and Ag^{+}, respectively) restore nodulation in the presence of nitrate suggest that nitrate induces the production of ethylene which then inhibits nodulation (Caba et al. 1998; Ligero et al. 1991). Since ethylene can regulate auxin transport (Burg and Burg 1966; Suttle 1988) and turnover (Ke and Saltveit 1988), the effect of nitrate via alterations in auxin levels could be mediated by nitrate-induced ethylene. Caba et al. (1999) found that the tolerance of the *nts* mutant to nitrate in respect to nodulation is paralleled by a tolerance for ethylene, which supports an involvement of ethylene in nitrate regulation. Unlike the *nts* mutants, in lotus, the nodulation phenotype of the recently characterized early- and hyper-nodulating mutant *astray* displayed normal sensitivity to ethylene and nitrate as its nodule number declined in the presence of both (Nishimura et al. 2002c). Interestingly, the mutated gene of *astray* was found to be the homologue of the Arabidopsis *HY5* gene (Nishimura et al. 2002b), which is involved in photomorphogenesis.

Nitrate also inhibits *ENOD40* induction by rhizobia, but not by cytokinins (Mathesius et al. 2000), suggesting two possibilities for the action of nitrate (see Figure 1): (1) if rhizobia induce *ENOD40* independently of cytokinins, nitrate would act between Nod factor perception and *ENOD40* induction, or (2) if rhizobia change cytokinin levels, which subsequently stimulate *ENOD40*, nitrate would inhibit the cytokinin changes induced by rhizobia.

Mutants are valuable to test the interactions between nitrate and hormone signalling. For example, the nitrate reductase deficient mutant *ANR1* and the auxin response mutant *axr4* were used in Arabidopsis to establish a role for the auxin response pathways during nitrate regulation of lateral root development (Zhang et al. 1999). Assuming that lateral root and nodule development share aspects of their

regulation by nitrate, it is possible that nitrate also acts via the auxin response pathway during nodulation. If this is the case, the effects of nitrate on cytokinin, *ENOD40* expression and ethylene could be indirectly caused by changes in auxin response.

Nod factors and other chitin derivatives

Nod factors are *Rhizobium*-produced lipochitin oligosaccharides and represent the major morphogenic molecule regulating nodule organogenesis. In addition to determining host specificity, Nod factors elicit root hair curling and deformation and cortical cell divisions in alfalfa (Truchet et al. 1991). There has been some debate about whether Nod factors are hormone-like signals *per se* or act indirectly, for example via changing the plant hormone balance as discussed above. While specific Nod factor action during nodulation has been extensively reviewed elsewhere (e.g. Cullimore et al. 2001; D'Haeze and Holsters 2002; Miklashevichs et al. 2001), the focus here is on the hormone-like roles of chitin oligosaccharides in general.

Whereas Nod factors are specific in their morphogenetic effect for certain host plants, Nod factor-related molecules have been suggested to play a more general role in plant development (Spaink et al. 1993; van der Holst et al. 2001). Chitin oligosaccharides play a role in animal development and have been detected in plants (Benhamou and Asselin 1989; Spaink et al. 1993), can be recognised by receptors for chitin oligosaccharides (Stacey and Shibuya 1997), and are substrates for chitinases, which have been shown to play a role in different aspects of plant development (Collinge et al. 1993). Expression of a chitinase was shown to rescue an embryonic mutant of carrot (de Jong et al. 1992) and modifying chitin structures by expression of the bacterial *nodA* and *nodB* genes, which modify Nod factors in rhizobia, led to changes in plant development (Schmidt et al. 1993).

Directed microtargeting of chitin oligosaccharides induced cortical cell divisions in vetch roots (Schlaman et al. 1997). Dyachok et al. (2000) found that Nod factors could stimulate embryogenesis in cell cultures of Norway spruce, a non-nodulating plant, and more recently isolated a lipochitin oligosaccharide like compound from these cultures which stimulated embryogenesis (Dyachok et al.

2002). Collectively, these experiments suggest that chitin perception could be widespread in both plants and animals and that chitin related molecules play a role in development. However, the mode of action of chitin derivatives remains elusive and identification of receptors and downstream response elements will be necessary to establish whether chitin oligosaccharides act via classical hormones or directly on target genes.

Conclusions and Outlook

This Chapter (Ferguson and Mathesius 2003) demonstrates the manifold effects of classical plant hormones and other compounds on nodule initiation, differentiation and numbers. Additional factors, such as soil nutrients, light, polyunsaturated fatty acids, CO_2, Ca^{2+}, PAL, CHS and *Rhizobium* EPS and LPS, etc. are all probably required for proper nodule development and functioning, but could not be fully discussed here.

Reports on classical plant hormones in nodulation are often ambiguous and contradictory because (1) nodulation is a fine balance between induction and repression of new nodule formation; (2) hormone requirements change with the varying stages of nodulation; (3) hormone levels and requirements change in different places in the shoot, root and nodule; (4) hormones interact leading to complex negative and positive feedback loops; (5) hormone requirements differ in different legume species, and (6) nodulation is regulated by both local and long distance signalling interactions involving varying actions of the same hormone in each regulatory pathway.

The search for homologues for many of the recently discovered Arabidopsis hormone response genes in legumes and their silencing or overexpression should help pinpoint the action of hormones during nodulation. For example, it should be tested whether *Rhizobium* directly affect cytokinin levels or whether cytokinin-related responses are the result of changing the auxin:cytokinin ratio due to changes in auxin transport or levels (see Figure 1). This could be tested in an inducible mutant for

cytokinin synthesis. Inducible or temperature sensitive mutants in polar auxin transport could be used to test whether auxin transport inhibition is necessary for nodule induction. Moreover, such mutants could help identify whether changes in auxin occur in the absence of PATI, for example via flavonoid-regulated changes in peroxidase activity as indicated in Figure 1. Accordingly, it could be tested whether auxin transport inhibition is a result of changes in ethylene induction in an ethylene synthesis deficient mutant. A mutant in ABA synthesis would also be useful for testing the functional relationships indicated in Figure 2. If elevated ABA levels are necessary for changes in phytoalexins, LOX, ROS and therefore indirectly for changes in peroxidase levels, JA and regulation of defence responses, these responses should be reduced in an ABA synthesis mutant.

There are challenging questions to address in future research. First, how does Nod factor perception lead to downstream events that could affect the plant hormone balance? Not much is known about how the early events in the root hair are linked to the events in the cortex, but the analysis of nodulation mutants is beginning to address that problem (Kistner and Parniske 2002). Secondly, there is a need for more large-scale experiments to discover the broad response pathways for plant hormones during nodulation, because each hormone usually has many targets and interacts with other hormones, which also have multiple effects. The use of mutants with hormone insensitivity, overproduction, or underproduction, the use of accurate reporters for different hormones, concentrating on model species for different types of analyses, as well as keeping an open mind about possible interactions should help to unravel the complex interactions of hormone-regulated signalling during nodulation. In addition, the recent identification of ESTs in *M. truncatula* has opened the door for expression analyses on the transcript (Federova et al. 2002) and proteome level (Mathesius et al. 2001). Thirdly, it is almost certain that new signalling compounds will be discovered apart from those presently known. Among them will be peptide hormones that might regulate receptor kinase activity. But other long-range signals are also likely to be discovered, including the autoregulatory signal from the shoot (Searle et al. 2002). The molecular and physiological characterization of these novel compounds should

help further the understanding of the intricate nodulation process that is just beginning to be understood.

CHAPTER 2

Nodulation Phenotypes of Gibberellin and Brassinosteroid Mutants of *Pisum sativum*

The information contained in this chapter appears in part in the publication: Ferguson BJ, Ross JJ, Reid JB (2005) Nodulation Phenotypes of Gibberellin and Brassinosteroid Mutants of *Pisum sativum*. Plant Physiol 138: 2396-2405.

INTRODUCTION

Beginning in the 1980s, mutagenesis experiments using *Pisum sativum* (pea) produced abnormal nodulation phenotypes including non-nodulating (nod-), poorly nodulating (nod+/-) and hypernodulating (nod++) mutants, as well as those that fix nitrogen poorly or not at all (fix-) (see references in Borisov et al. 2000). At present, over 200 nodulation mutants exist in pea (Borisov et al. 2000). Nodulation mutants have also been selected for in the model legume species *Medicago truncatula* and *Lotus japonicus*, which have smaller genomes than pea, making them more desirable tools for molecular studies. Mutants in these species have since been used to identify genes and gene products involved in nodule formation and functioning. This approach has been successful, and the orthologs of many nodulation genes discovered in *M. truncatula* or *L. japonicus* have subsequently been identified in important crop species such as pea (see references in Oldroyd and Downie 2004).

Here, a reverse approach to investigating nodulation is reported. In contrast to selecting for nodulation mutants and identifying their mutated genes, I identified the root and nodulation phenotypes of previously characterized mutants (Table I). The mutants examined here are all affected in their biosynthesis of, or responses to, the phytohormones gibberellin (GA) or brassinosteroid (BR). Moreover, the genes and gene products of these lines have all formerly been identified (reviewed in Reid et al. 2005; Table I).

Table I. *Overview of the various* P. sativum *lines investigated*

Genotype	Line Number	Gene Product	Hormone Level	Phenotype	References
Torsdag	107	wild type			
lk	212-	BR 5α-reductase	reduced total plant BRs	dwarf, thickened internodes	Reid, 1986; Ross and Reid 1986; Nomura et al. 2004
lka	5865	BR receptor	increased total plant BRs	dwarf, thickened internodes	Reid and Ross 1989; Nomura et al. 1997; Nomura et al. 1999; Nomura et al. 2003
lkb	5862	BR C-24 reductase	reduced total plant BRs	dwarf, thickened internodes	Reid and Ross 1989; Nomura et al. 1997; Nomura et al. 1999; Schultz et al. 2001
ls-1	181	copalyl diphosphate synthase	reduced total plant GAs	dwarf	Ait-Ali et al. 1997; Yaxley et al. 2001a
lh-2	5843	*ent*-kaurene oxidase	reduced total plant GAs	dwarf	Davidson et al. 2004; Yaxley et al. 2001a
le-3	5839	GA 3-oxidase	reduced shoot GAs, wild type root GAs	dwarf	Ingram et al. 1984; Yaxley et al. 2001a
NA	1766x1769	wild type			
na	1766x1769	*ent*-kaurenoic acid oxidase	reduced total plant GAs	extreme dwarf	Davidson et al. 2003; Yaxley et al. 2001a
SLN	250+	wild type			
sln	250-	GA 2-oxidase	elevated seed GAs leading to elevated total plant GAs	elongated internodes	Reid et al. 1992; Ross et al. 1993; Lester et al. 1999; Yaxley et al. 2001a

This study is, to the best of my knowledge, the first to investigate the nodulation phenotypes of previously characterized mutants. Unlike dwarf (*le*) cultivars used in many previous nodulation studies (e.g. Finale, Frisson, Rondo, Solara, Sparkle), the wild types studied here are all on a tall (*LE*) background. Interestingly, many pea lines used for agricultural purposes are on *le* backgrounds, and are therefore deficient in shoot GA_1 (Reid et al. 2005), as are many of the lines used for the selection of nodulation mutants. However, the effects of shoot dwarfism and reduced shoot GA_1 levels on nodulation have not been described previously. This thesis is also the first to investigate the role(s) of endogenous BRs in nodulation. As with GA deficiencies, reductions in BR levels cause shoot dwarfism, thus allowing for the use of two distinct hormone-mediated mechanisms to investigate the effects of shoot stature on nodulation and root development.

RESULTS AND DISCUSSION

Nodulation Phenotypes of Gibberellin Mutants

In the collection of GA-deficient mutants used here, *na-1* causes the greatest reduction in bioactive GA_1 levels in the root, followed by *ls-1* and *lh-2* (Yaxley et al. 2001a). In the current study, all three of these mutants developed significantly fewer nodules and significantly reduced root systems (fewer and shorter secondary and tertiary lateral roots; Fig. 1; Table II) than their wild types. The reductions in total nodule numbers were observed on a per plant (Fig. 2) and also on a per mg root DW basis (Table III). The severity of these reductions closely parallelled the reductions in the root GA_1 levels of the mutants (Yaxley et al. 2001a) and strongly indicates a requirement for GAs in root and nodule formation. Reduced root GA_1 levels may affect nodule formation directly by reducing successful *Rhizobium* infections and nodule development. Alternatively, reductions in root GA_1 levels may act indirectly by increasing the level of nodulation inhibitors, such as ethylene, and/or limiting root numbers and lengths, thereby reducing available *Rhizobium* infection sites.

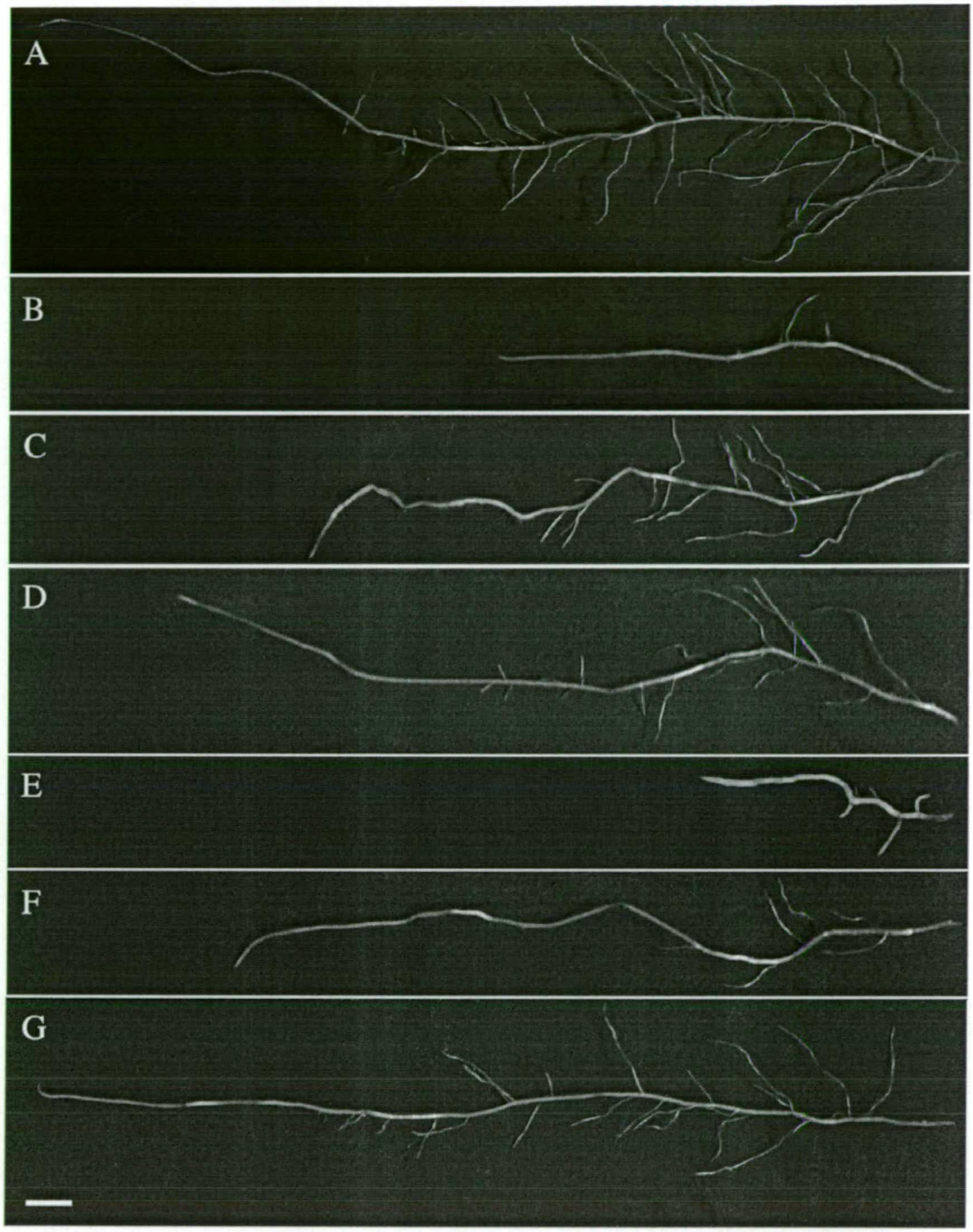

Figure 1. Detached secondary lateral roots of 17 day-old plants of (A) wild type (Torsdag) and (B) the BR-deficient *lk*, (C) the BR-receptor mutant *lka*, (D) the BR-deficient *lkb*, (E) the GA_1-deficient *na-1*, (F) the GA_1-deficient *ls-1*, (G) and the shoot GA_1-deficient *le-3*. The roots were collected from the most mature region of the plants, closest to the crown. The far right hand side of the secondary lateral root is the point at which it was detached form the primary root. Bar = 1 cm.

Table II. *Root numbers and lengths of 17 day-old GA and BR mutants and their wild types*
Indicated are the number of secondary lateral roots per plant in addition to the number of tertiary lateral roots per secondary lateral root, based on the average number located on the six uppermost secondary lateral roots. Also shown are the lengths of the shoot and the longest secondary and tertiary lateral roots per plant.

Genotype	Number		Length (cm)		
	Secondary Roots	Tertiary Roots	Shoot	Secondary Roots	Tertiary Roots
Torsdag	89 ± 3.6	20 ± 1.1	12.5 ± 0.3	19.7 ± 0.1	4.3 ± 0.2
lk	50 ± 3.6*	4 ± 0.4*	3.0 ± 0.2*	10.4 ± 0.3*	2.2 ± 0.1*
lka	82 ± 2.5	9 ± 0.8*	5.9 ± 0.4*	14.8 ± 0.8*	3.5 ± 0.2*
lkb	82 ± 4.1	13 ± 0.9*	5.9 ± 0.3*	16.9 ± 0.4*	3.7 ± 0.3
ls-1	63 ± 4.8*	7 ± 0.6*	2.9 ± 0.1*	16.7 ± 0.7*	2.2 ± 0.3*
lh-2	63 ± 4.7*	12 ± 1.0*	5.7 ± 0.2*	17.2 ± 0.7*	2.5 ± 0.2*
le-3	100 ± 1.7	20 ± 1.1	4.2 ± 0.2*	18.5 ± 1.2	3.8 ± 0.3
NA	107 ± 5.2	21 ± 1.1	21.4 ± 0.5	18.2 ± 0.7	5.9 ± 0.3
na	50 ± 2.4*	5 ± 0.4*	2.9 ± 0.2*	6.1 ± 0.3*	1.1 ± 0.1*
SLN	93 ± 5.4	13 ± 1.0	28.6 ± 0.7	19.0 ± 1.2	3.2 ± 0.4
sln	98 ± 1.4	13 ± 0.9	50.0 ± 3.8*	18.6 ± 1.2	2.9 ± 0.3

Results are means ± SE (n = 6). Values for each mutant trait followed by an * are significantly different from that of their respective wild type at the 0.01 level.

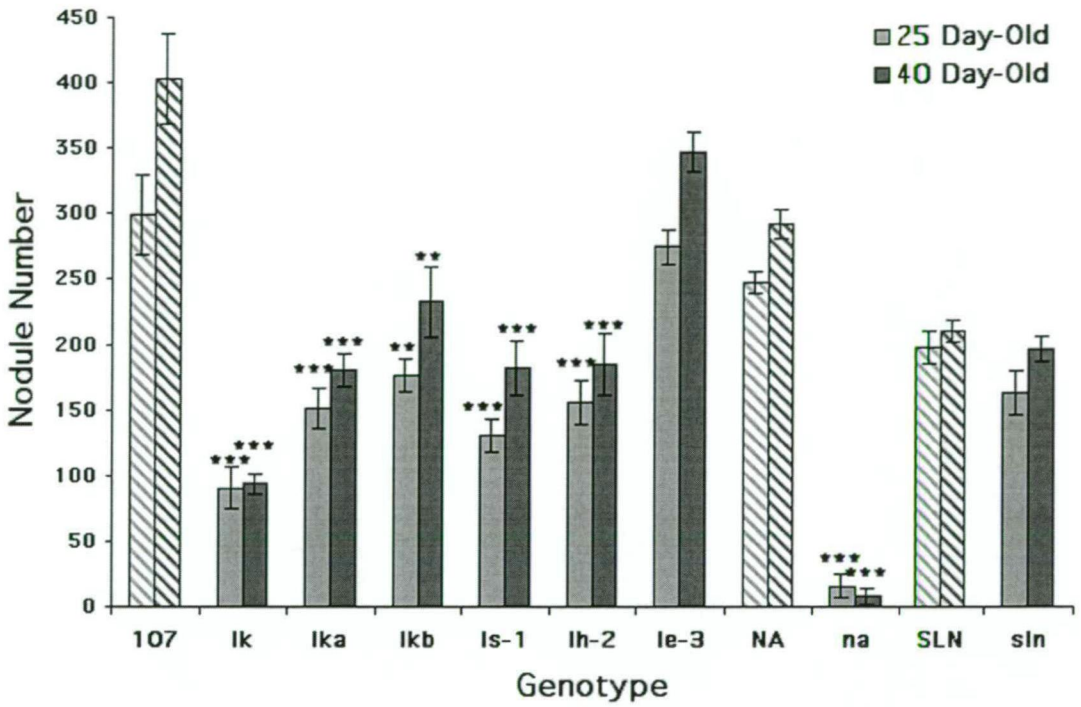

Figure 2. Nodule numbers of 25 and 40 day-old plants inoculated with *R. leguminosarum*. Results are means ± SE (n = 8). Dashed bars represent wild types of the mutants (solid bars) situated to their right. Mutant values denoted with an *, ** or *** are significantly different from that of their wild type at the 0.05, 0.01 and 0.001 level, respectively.

Table III. *Root, shoot and nodule dry weights and nodule numbers per root and shoot dry weight of 25 day-old GA and BR mutants and their wild types*

Genotype	Dry Weight				Number of Nodules	
	Shoot (mg)	Root (mg)	Nodule Total (mg)	Nodule Average (mg)	Per mg Shoot Dry Weight	Per mg Root Dry Weight
Torsdag	208 ± 12	158 ± 12	33.4 ± 3.8	0.11 ± 0.012	1.44 ± 0.12	1.95 ± 0.23
lk	142 ± 15*	115 ± 13	23.5 ± 3.5	0.29 ± 0.030*	0.62 ± 0.07*	0.77 ± 0.10*
lka	172 ± 13	173 ± 15	30.5 ± 2.3	0.21 ± 0.015*	0.87 ± 0.04*	0.87 ± 0.04*
lkb	236 ± 11	202 ± 16	42.6 ± 4.1	0.24 ± 0.012*	0.74 ± 0.03*	0.88 ± 0.04*
ls-1	111 ± 7*	132 ± 9	22.4 ± 2.1	0.18 ± 0.016*	1.17 ± 0.08	1.03 ± 0.13*
lh-2	162 ± 7*	159 ± 8	29.0 ± 3.7	0.19 ± 0.016*	0.94 ± 0.07*	0.99 ± 0.12*
le-3	203 ± 19	170 ± 15	39.7 ± 4.1	0.15 ± 0.013	1.41 ± 0.13	1.66 ± 0.14
NA	396 ± 24	197 ± 10	62.6 ± 4.1	0.25 ± 0.016	0.64 ± 0.04	1.28 ± 0.08
na	184 ± 12*	175 ± 14	1.3 ± 0.7*	0.06 ± 0.025*	0.08 ± 0.05*	0.09 ± 0.05*
SLN	357 ± 21	158 ± 9	63.6 ± 0.4	0.33 ± 0.017	0.55 ± 0.03	1.27 ± 0.11
sln	392 ± 28	142 ± 11	58.1 ± 5.1	0.37 ± 0.023	0.42 ± 0.04	1.20 ± 0.17

Plants were inoculated with *R. leguminosarum* five days following the time of sowing. Results are means ± SE (n = 8). Values for each mutant trait followed by an * are significantly different from that of their respective wild type at the 0.01 level.

Reductions in nodule numbers were observed in both 25 and 40 day-old plants (Fig. 2), indicating that the reduced root GA_1 levels are not simply delaying nodule development.

The *na-1* mutant exhibited the most dramatic nodulation phenotype as few to no nodules formed (Figs. 2 and 3). Those that did form were aberrant, being small and white and resembling emerged meristems that fail to develop further (Fig. 3). Unlike the nodules observed on the other lines investigated, the few aberrant nodules of *na-1* were often detected on the tertiary lateral roots of the mutant (Fig. 2b). As a consequence of their reduced size, the total DW, and average DW, of *na-1* nodules were significantly reduced compared with those of its wild type (Table III). Less dramatic reductions were detected in the total nodule DWs of *ls-1* and *lh-2* mutant plants (Table III) compared with that of their wild type. However, although the average nodule DW was reduced in *na-1*, it was actually significantly elevated in *ls-1* and *lh-2* (Table III). Thus, it appears that GAs may also influence nodule size with slight reductions being stimulatory (*ls-1* and *lh-2*) and large reductions inhibitory (*na-1*).

In an attempt to restore nodule numbers to that of the wild type, various concentrations of the bioactive GA_3 were applied to the roots of *na-1* mutants. Using this technique, concentrations of 10^{-6} M GA_3 were found to completely restore the *na-1* nodule appearance and numbers to that observed on the wild type control (Fig. 4). This finding lends further support to my evidence that GAs are required for nodule development. Low concentrations of the hormone also stimulated nodule formation in the wild type, but became inhibitory to both the wild type and the mutant as the applied concentration increased (Fig. 4). This finding is similar to that reported by Lorteau et al. (2001) for cytokinin, who found that the application of low concentrations of the phytohormone were stimulatory to pea nodule formation, but became inhibitory when increased beyond a threshold level.

Grafting studies were performed using various combinations of *lh-2* and its wild type (*LH*), Torsdag, in order to determine whether or not an *LH* shoot or root system could restore the reduced nodule number of the GA-deficient line (Table IV). This study revealed that either an *LH* root or shoot system was sufficient to restore

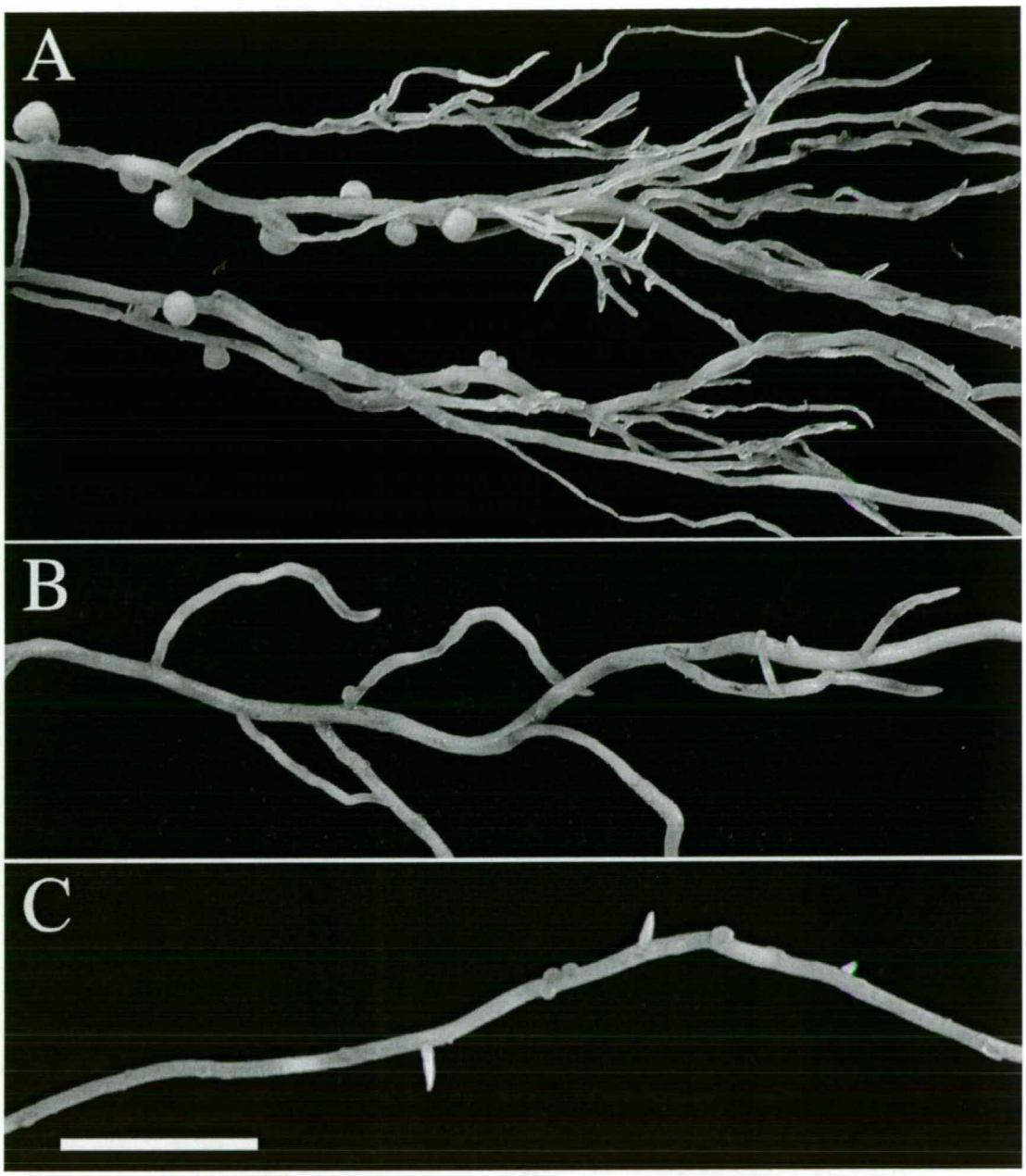

Figure 3. Nodulated lateral roots of 25 day-old (A) wild type and (B,C) *na-1* plants. Wild type nodules are large and display a white meristematic tip and a red center that represents the zone of nitrogen fixation. The few aberrant nodules that do develop on the *na-1* mutant are small and white and resemble an emergent nodule meristem that failed to develop further. Bar = 1 cm.

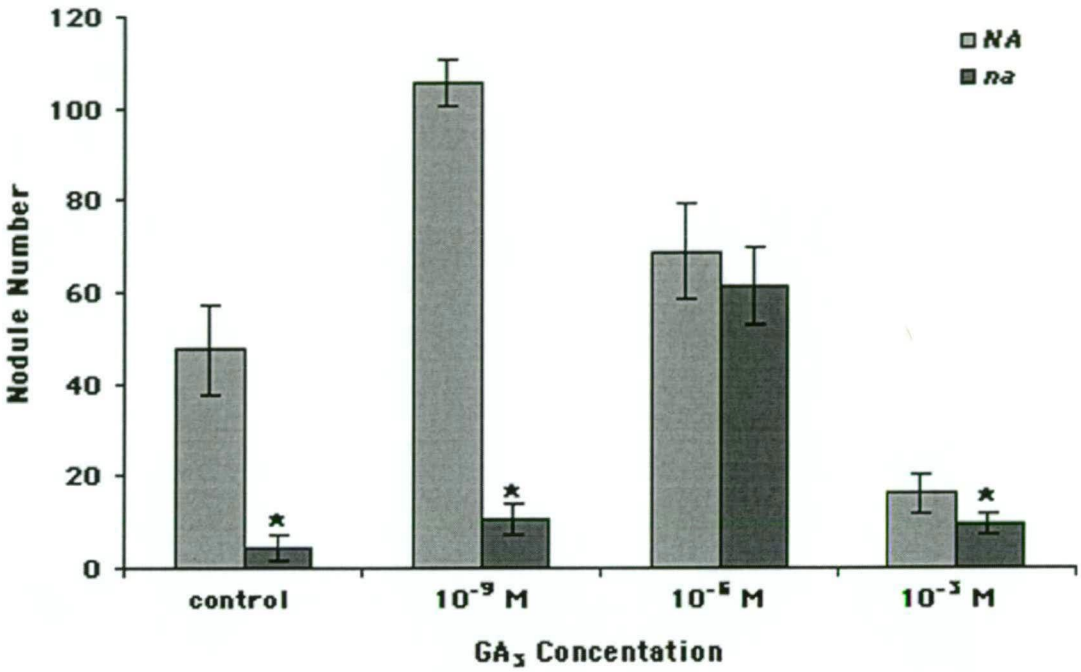

Figure 4. Nodule numbers of 20 day-old wild type and *na-1* plants inoculated with *R. leguminosarum* and treated with various concentrations of the bioactive GA_3. Results are means ± SE (n = 6). Mutant values denoted with an * are significantly different from that of the wild type control at the 0.01 level.

Table IV. *Root, shoot and nodule dry weights, and nodule numbers per plant and root and shoot dry weight of 30 day-old graft combinations of* LH *and* lh-2 *mutants*

Graft Type	Dry Weight				Number of Nodules		
	Shoot (mg)	Root (mg)	Nodule Total (mg)	Nodule Average (mg)	Per Plant	Per mg Shoot Dry Weight	Per mg Root Dry Weight
LH/LH	259 ± 12	81 ± 5	34.4 ± 2.8	0.42 ± 0.053	87 ± 9	0.34 ± 0.03	1.07 ± 0.07
LH/lh-2	249 ± 12	93 ± 6	31.3 ± 1.9	0.46 ± 0.066	78 ± 11	0.32 ± 0.05	0.87 ± 0.14
lh-2/LH	165 ± 17*	81 ± 8	23.7 ± 3.3*	0.32 ± 0.047	75 ± 6	0.49 ± 0.07	0.99 ± 0.12
lh-2/lh-2	151 ± 12*	62 ± 7	23.1 ± 2.6*	0.53 ± 0.062	44 ± 4*	0.30 ± 0.02	0.76 ± 0.07*

Plants were grafted six days after sowing and inoculated with *R. leguminosarum* at ten days. Results are means ± SE (n = 8). Values for each trait followed by an * are significantly different from the *LH/LH* graft combination at the 0.01 level.

the reduced nodule number of the mutant, both on a per plant, and a per mg root DW, basis. This finding implies that GAs are required for nodulation. Furthermore, the root system GA level appears to play a role in nodule development, as more nodules formed on *lh-2/LH* grafts than on those of *lh-2/lh-2* ($P < 0.001$), even though the shoots remained short, with a low DW (Table IV). *LH/lh-2* grafts also produced more nodules than *lh-2/lh-2* grafts, but it cannot be excluded that GAs were transported basipetally from the *LH* shoot into the mutant root system. Consistent with this suggestion is the significant promontory effect of *LH* shoots on the *lh-2* root DW, which increased compared with that of the *lh-2/lh-2* grafts ($P < 0.01$). Graft transmissibility of GA_1 precursors (but not of GA_1 itself) has been demonstrated previously (Reid et al. 1983). Interestingly, the total nodule DW was significantly reduced in grafted plants possessing an *lh-2* shoot, whereas the average nodule DW was slightly increased in grafts having *lh-2* roots (Table IV).

The *le-3* mutant, which has decreased shoot GA_1 levels, but wild type root GA_1 levels (Yaxley et al. 2001a), and the *sln* mutant, which has elevated root and shoot GA_1 levels early in development (Reid et al. 1992; Yaxley et al. 2001a), both had a similar number, and size, of lateral roots, and nodules, as their wild types (Figs. 1 and 2; Tables II and III). Importantly, the normal root and nodule phenotypes of the *le-3* mutant indicate that the effects of GA_1 deficiency on these characteristics, as observed in *na-1*, *ls-1* and *lh-2*, are not mediated by dwarfism of the shoot. Furthermore, the results with *le-3* are consistent with those of the grafting experiment with *lh-2* (Table IV), as neither dwarfism, nor a reduced shoot GA_1 level, impaired the root system DW, or the nodule number, of a root system having a normal level of GA_1. Moreover, the wild type level of GA_1 in the *le-3* root system is insufficient to rescue the shoot dwarfism of the mutant. This finding is consistent with that observed using the *lh-2* grafts (Table IV).

The elevated GA_1 levels of *sln* do not appear to influence the root system or the overall number of nodules that form per plant (Figs. 1 and 2; Tables II and III). Despite these findings, high GA_1 levels may actually be inhibitory to nodule organogenesis. The source of the elevated GAs of *sln* is the seed (Ross et al. 1993). As the *sln* seedling develops, this excess GA is mobilized throughout the plant until it

is eventually metabolized and maintained at near *SLN* levels (Ross et al. 1993). By this time, the primary roots of both *SLN* and *sln* are well established and appear similar. However, although numerous nodules formed on the primary roots of *SLN* plants, no nodules developed on the primary roots of *sln* mutants (Fig. 5). This may suggest that the elevated GA levels of the mutant prevented nodules from establishing, which is consistent with the finding that treatment with high concentrations of GA_3 reduced the number of nodule that formed on wild type plants (Fig. 4). This inhibition in *sln* is temporary, as nodulation was not prevented on lateral roots, of which many formed following the metabolizm of the majority of the excess GA_1. Elevated GA_1 levels might act directly to inhibit the infection process or nodule development, or indirectly, by affecting assimilate distribution.

Nodulation Phenotypes of Brassinosteroid Mutants

In the collection of BR mutants used here, *lk* has the most severe reduction in bioactive BRs in the shoot (Nomura et al. 2004), followed by *lkb* (Nomura et al. 1997). A reduction in BR levels in the roots has also been confirmed for *lkb* (Symons and Reid 2004). Here I demonstrate that, in addition to shoot dwarfism, the BR synthesis mutants *lk* and *lkb*, and the BR response mutant, *lka*, also have fewer and shorter lateral roots (Fig. 1; Table II). These findings support recent reports that BRs have a role in lateral root formation (Bao et al. 2004). Interestingly, despite all three BR mutants producing fewer and shorter lateral roots (Fig. 1; Table II), only the *lk* root system DW was significantly reduced compared with that of Torsdag (Table III).

Nodule numbers were reduced in all three BR mutants compared with that of Torsdag. These reductions occurred in both 25 and 40 day-old plants, indicating that nodule development was not delayed, but rather diminished, as was observed with the GA_1-deficient mutants (Fig. 2). The nodule numbers were also reduced on a per mg root DW basis (Table III), indicating that the reductions were not simply correlated with the size of the root systems. Instead, these diminished nodule numbers might be caused by reduced BR levels, or perception, directly or indirectly effecting nodule development, as is discussed above for mutants having reduced root GA_1 levels.

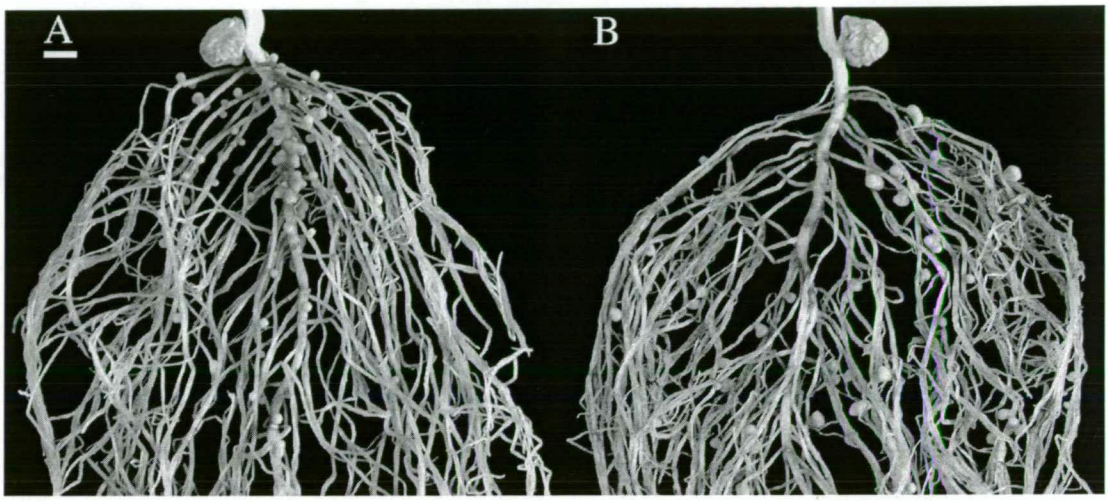

Figure 5. Nodulated root systems of the 25 day-old (A) wild type, *SLN*, and (B) the GA_1-overproducing, *sln*. *SLN*, like the other wild type lines investigated here, formed many nodules on both primary and secondary roots, whereas *sln* only developed nodules on secondary roots. Bar = 1 cm.

The average nodule DW was significantly increased for all of the BR mutants, compared with that of Torsdag (Table III). Thus, in the case of *lk*, although the root system DW decreased, the average nodule DW increased. This finding illustrates that nodule size is not simply a reflection of root system DW. Interestingly, with the exception of the severely reduced *na-1*, reductions in root GA_1 levels also resulted in increased nodule DWs. Producing large nodules may be a compensatory mechanism to increase nitrogen fixation in response to reduced nodule numbers.

Recently, BRs were shown to be relatively immobile within pea (Symons and Reid 2004). For this reason, BR application studies similar to that performed using GA_3 and *na-1* were not considered to be the best method to investigate nodulation here. In addition, a BR mutant similar to that of *le-3* having normal BR levels in the root, but decreased levels in the shoot, is not available. As a result, grafting studies involving *lkb* and its wild type (*LKB*), Torsdag, were the only method available to examine the effects of decreased root and shoot BR levels on nodulation. Results from these studies illustrate that the shoot controlled the number of nodules that formed in these graft combinations (Table V). This finding contrasted with that observed with the *lh-2* graft combinations (Table IV). Grafted plants having an *lkb* shoot developed fewer nodules than those having an *LKB* shoot on a per plant, as well as per mg root DW basis (Table V). In addition, the root and shoot DWs of grafted plants with an *lkb* shoot were not significantly reduced from those with an *LKB* shoot (Table V). This indicates that the reduced nodule numbers on grafted plants having an *lkb* shoot were not simply the result of a smaller root or shoot system. Instead, these findings suggest that BRs may be influencing a nodulation mechanism of the shoot that is involved in regulating the nodule numbers of the root. One such mechanism known to exist in the shoot involves the receptor kinase *HAR-1*/*SYM29*/*NARK* (e.g. Wopereis et al. 2000; reviewed in Oldroyd and Downie 2004). To date, it is unknown what effects, if any, BRs have on this receptor; however, the mutants examined in this report appear to be excellent candidates for investigating this potential relationship.

Recently, Symons and Reid (2004) demonstrated that BRs are not graft-transmissible. Thus, the level of BRs in an *lkb* root system would be reduced

Table V. *Root, shoot and nodule dry weights, nodule numbers per plant and root and shoot dry weight and root levels of IAA and GA_1 of 30 day-old graft combinations of* LKB *and* lkb *mutants.*

Graft Type	Dry Weight				Number of Nodules			Hormone Level	
	Shoot (mg)	Root (mg)	Nodule Total (mg)	Nodule Average (mg)	Per plant	Per mg Shoot Dry Weight	Per mg Root Dry Weight	IAA ($ng\ g^{-1}$ Fresh Weight)	GA_1 ($ng\ g^{-1}$ Fresh Weight)
LKB/LKB	410 ± 35	130 ± 15	65 ± 9.5	0.57 ± 0.085	136 ± 26	0.35 ± 0.03	1.09 ± 0.20	3.93 ± 0.69	0.022 ± 0.0005
LKB/lkb	420 ± 25	180 ± 23	75 ± 11.5	0.83 ± 0.252	129 ± 26	0.34 ± 0.09	0.94 ± 0.36	3.23 ± 0.32	0.020 ± 0.0020
lkb/LKB	310 ± 39	120 ± 12	48 ± 9.5	0.98 ± 0.239	56 ± 5*	0.20 ± 0.03	0.48 ± 0.06*	3.08 ± 0.06	0.020 ± 0.0025
lkb/lkb	430 ± 43	200 ± 15*	69 ± 10.6	1.58 ± 0.236*	46 ± 7*	0.11 ± 0.01*	0.23 ± 0.03*	3.16 ± 0.19	0.024 ± 0.0005

Plants were grafted six days after sowing and inoculated with *R. leguminosarum* at ten days. Results are means ± SE (n = 8) for physiological traits and means ± SE of two replicates, each consisting of six root systems, for hormone analysis. Values for each trait followed by an * are significantly different from the *LKB/LKB* graft combination at the 0.01 level.

compared with that of *LKB*, even if grafted to an *LKB* shoot. Therefore, the increased number of nodules observed on *lkb* roots grafted to an *LKB* shoot, cannot be explained by an increase in root BRs. In addition, despite having normal levels of BRs, *LKB* root systems grafted to an *lkb* shoot produced fewer nodules compared with those grafted to an *LKB* shoot. Together, these findings indicate that the root level of BRs does not have a direct effect on nodule numbers. Based on these results, I investigated whether or not shoot BRs regulate root and nodule development by altering the levels of other hormones in the roots. For example, the findings with the GA mutants indicate a role for GA in the development of roots and nodules. In addition, the phytohormone auxin is known to have a prominent role in both root and nodule development (Chapter 1; Ferguson and Mathesius 2003) and is produced at high levels in the shoot, followed by a reported acropetal transport to the root system. Thus, I measured the levels of GA_1 and the auxin, indole acetic acid (IAA), in the root systems of the various Torsdag and *lkb* graft combinations. This revealed that the levels of both GA_1 and IAA were similar amongst all of the graft combinations (Table V), demonstrating that the reduced BR levels of *lkb* do not alter the root GA_1 or IAA levels. Therefore, the reductions in root and nodule numbers of the BR mutants do not appear to be attributed to changes in the root levels of GA_1 or IAA.

Correlations Between Root and Nodule Formation

A correlation between the number of nodules and the number of lateral roots was detected across all of the mutant and wild type lines examined (Fig. 6). Correlations between nodule and lateral root numbers were first described by Nutman (1948) who noted that the more lateral roots a line of red clover developed, the more nodules it formed. These findings indicate that a strong correlation between nodule and root formation exists and may suggest that roots utilize an autoregulatory mechanism similar to that identified in nodulation (e.g. Caetano-Anollés and Greshoff 1991). Consistent with this suggestion is the observation that the hypernodulating mutant of *L. japonicus*, *har-1*, exhibits stimulated root initiation when grown in the absence of *Mesorhizobium loti* (Wopereis et al. 2000).

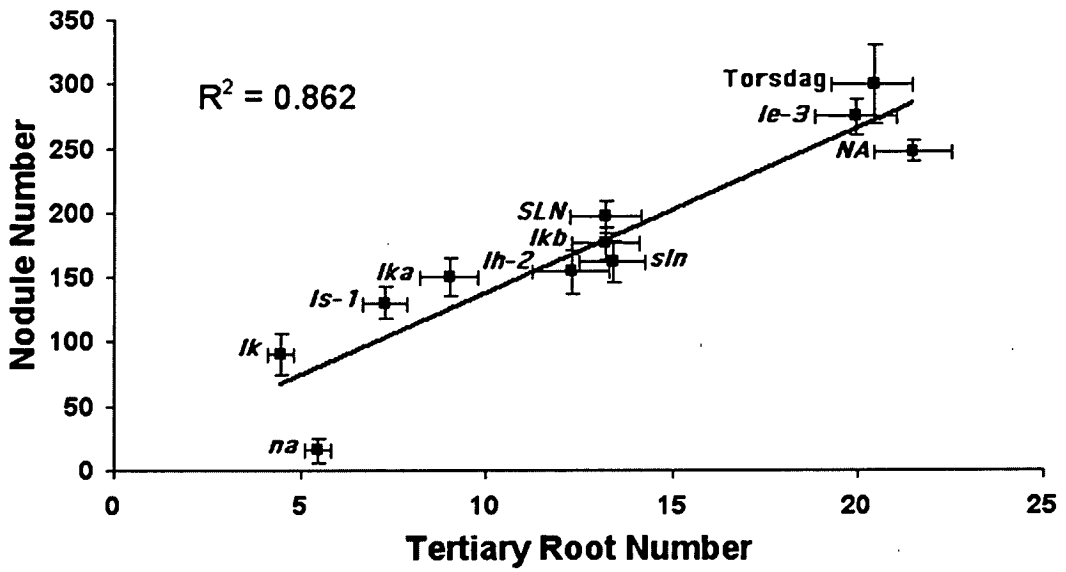

Figure 6. The correlation between the average number of tertiary lateral roots observed on the oldest six secondary lateral roots of 17 day-old plants and the number of nodules of 25 day-old plants inoculated with *R. leguminosarum*. Results are means ± SE for the nodule number (n = 6 to 8).

It has been postulated that nodulation evolved from pre-existing mechanisms of early lateral root development (Hirsch and LaRue 1997; Mathesius 2003). This theory is supported by root-nodule hybrids that have been observed on roots of *M. sativa* (Dudley et al. 1987) and *T. repens* (McIver et al. 1997) following inoculation with specific *Rhizobium* strains. Roots also emerge from apical meristems of actinorhizal nodules of *Casuarina cunninghamiana* (Torrey 1976) and *Myrica gale* (Torrey and Callaham 1978). The nodule apex can also be converted into a root apex by adjusting growing temperatures from low to high (see references in Dart 1977). Moreover, mycorrhizal nodules develop on Podocarpaceae species, even in sterile soil free of the fungus (Russell et al. 2002). These structures are not simply lateral roots modified by the endosymbiont, but rather novel outgrowths that have diverged from the root developmental pathway prior to their emergence.

Lateral roots and nodules share many aspects of their development. For example, they are both derived via post-embryonic mechanisms involving de-differentiating and dividing cells adjacent to xylem poles (Mathesius 2003). One proposed difference in their development is the site of initial cellular divisions; the pericycle for roots and the cortex for nodules. However, peanut nodules originate predominately form the pericycle (Allen and Allen 1940), and pericycle divisions do occur during nodule development of pea (Bond 1948) and *Trifolium repens* (McIver et al. 1997). In addition, non-leguminous Actinorhizal nodules, myconodules and *Parasponia* nodules are all derived from the pericycle (see references in Hirsch and LaRue 1997). Moreover, *ENOD40*, a signal thought to be involved in cell division, is expressed in the pericycle of *Medicago sativa* prior to nodule primordium initiation (Compaan et al. 2001). Furthermore, Kawaguchi et al. (1996) demonstrated that bioactive GAs induce pericycle divisions leading to nodule-like structures in *L. japonicus*. These structures were free of central vascular cells and were therefore not simply deformed lateral roots. Collectively, these findings point to a role for the pericycle in nodulation, possibly including cell divisions as are known to occur in lateral root development (e.g. Dubrovsky et al. 2000). The involvement of the pericycle may be mediated by hormones, which may explain why parallel declines in nodule and root numbers were observed in the mutants that have hormone-deficient

root systems. Transcript profiling of early lateral root initiation in *Arabidopsis* has detected numerous genes expressed in the pericycle (Himanen et al. 2004). Perhaps a similar investigation into the pericycle using a legume species, with and without *Rhizobium* inoculation, would help discriminate between gene products shared by, and unique to, root and nodule initiation.

Correlations between nodulation and the remaining characteristics measured were not observed. For example, there was no correlation between shoot stature and nodulation as *sln* was taller than its wild type, and *le-3* was shorter, but they both produced wild type numbers of nodules (Fig. 2). Also, there is no correlation between the rate of leaf expansion and nodulation because, when compared with their wild types, GA deficient mutants had fewer leaves, whereas BR mutants had more (data not shown), yet both formed fewer nodules (Fig. 2). Shoot and root DW also did not form a correlation with nodulation. The DW of *lh-2* shoots was similar to that of *le-3* (Table III), but *lh-2* formed significantly fewer nodules than *le-3* (Fig. 2). In addition, the BR mutants all formed significantly fewer nodules than Torsdag (Fig. 2), despite having differences in their root system DWs (Table III). Furthermore, the length of secondary lateral roots does not appear to be the limiting factor of the development of tertiary lateral roots and nodules. For example, *lkb* and *ls-1* secondary lateral roots are similar in length (Fig. 1; Table II), but *ls-1* developed fewer tertiary lateral roots (Fig. 1; Table II) and nodules (Fig. 2) than *lkb*.

CONCLUSIONS

The results presented here illustrate that reduced root levels of GAs significantly decrease the number of nodules in pea (Fig. 2). These decreases in nodule numbers were observed at both 25 and 40 days, indicating that they were not simply the result of a delay in nodule formation. The application of GA_3 restored the nodule number of *na-1*, suggesting a direct role for GAs in nodule development. In addition, grafting experiments illustrated that normal GA_1 levels in the root are sufficient to elicit the formation of a normal number of nodules. In contrast, BRs do

not have a direct effect on nodule numbers, but act to influence a shoot mechanism involved in regulating nodule numbers. Interestingly, with the exception of the severely inhibited *na-1*, significant increases in the average nodule DW were found on all GA and BR mutants having reduced nodule numbers (Table III). This might suggest the existence of a mechanism that compensates for changes in nodule numbers by regulating the size of individual nodules. Taken together, my findings support the theory proposed by Libbenga et al. (1973) that a delicate balance in hormone levels is required to achieve optimum nodule development. This theory is further supported by my finding that GAs, in addition to cytokinins (Lorteau et al. 2001), are stimulatory to pea nodule formation at low concentrations, but inhibitory when increased beyond a threshold level.

Reductions in root GA and BR levels also diminished lateral root numbers and lengths (Yaxley et al. 2001a; Table II). Interestingly, this appears to be opposite to the effects of cytokinins, which reportedly inhibit nodulation, but stimulate lateral root development (Lohar et al. 2004). It is likely that hormones have multiple roles in root and nodule development (Chapter 1; Ferguson and Mathesius 2003) and are required to different degrees at various stages of development. Overall, mutants have proven to be valuable tools for understanding the processes of root and nodule development, and for isolating genes relating to these processes. In pea, an extensive collection of nodulation mutants has been assembled (Borisov et al. 2000), but there remains a need for additional root mutants, which would aid in determining the developmental aspects that are shared in, and are unique to, the root-nodule relationship.

MATERIALS AND METHODS

Plant Growing Conditions

An overview of the various plant lines used in this report, including any mutated genes and their resulting effects on the plant, is provided in Table I. For

nodulation studies, plants were sown one per pot in 100 mm "Space Saver" pots (Reko, Australia) and for root analysis experiments, seeds were sown seven per pot in 200 mm "Plastamatic" pots (Melbourne, Australia). All pots contained a 1:1 mixture of grade 3 vermiculite (Australian Vermiculite and Perlite Co., Fairfield, Victoria, Australia) and 10 mm dolerite aggregate (HBMI, Kingston, Tasmania). This mixture was topped with approximately two cm of a pasteurized peat/sand potting mix composed of a 1:1 mixture of peat moss (Te - Em, New Brunswick, Canada) and coarse river sand (Island Resources, Scottsdale, Tasmania, Australia). Pasteurisation was achieved using a steam/air mix at 70 °C for 45 min. The pH was adjusted to 7.0 with dolomite lime and limestone.

Plants were grown in a controlled environment glasshouse with temperatures maintained at 20 °C day (18 h) and 15 °C night (6 h) +/- 1 °C. Relative humidity was maintained at a minimum of 40%. The photoperiod of 18 h consisted of natural daylight supplemented and extended morning and evening by 4 GE (Hungary) Lucagrow LU400/HO High Pressure Sodium 400 W globes and 2 incandescent globes (60 W Pearl, Thorn, Australia) delivering an additional approximately 150 μmol photons m^{-2} s^{-1} at the pot surface.

Plants were placed on capillary mats (Bottom Up Irrigation, Fertool Distributors, Hallam, Victoria, Australia) and watered using an automated overhead sprinkling system (70 lph @ 150 kPa) for 2 min each morning and evening. For nodule count studies, each pot was provided with 25 mL of *Rhizobium leguminosarum* bv. *viciae* 128C53K (Nitragin® Inoculants, Liphatech Inc., Milwaukee, WI) grown in yeast-mannitol broth and diluted with water to approximately OD_{600} 0.01, which represents 5 x 10^6 cells mL^{-1}. Based on a previous experiment, inoculation was delayed in these studies until 5 days after planting to maximize nodulation. For root characterization experiments, at the time of sowing 150 mL of the bacterial solution was applied. Plants grown in excess of 25 days were also provided with a modified Hoaglands solution containing only 1 mM NO_3^-, to prevent the inhibition of nodulation.

Nodule Count Studies

Investigation of mutant and wild type lines

Plants were harvested 25 days after planting. This timing allowed nodules to develop to a stage where they could be clearly distinguished and their appearance accurately assessed. For each plant, the number of nodes was recorded, counting the cotyledon as node zero. The roots and shoots were separated at the cotyledon, which was excised and discarded. The root system was gently rinsed clean of potting substrates and placed in a tray of water. Nodules were counted, removed with forceps and, together with the roots and shoots, placed in a 60°C oven for a minimum of three days to obtain their dry weights (DWs).

Additional plants were allowed to persist until 40 days after planting, coinciding with the flowering time of many of the lines, including wild types. The same traits examined using 25-day-old plants were then assessed. By 40 days, the formation of new nodule structures should be minimal due to the plants' autoregulation of nodulation (Caetano-Anollés and Gresshoff 1991). Thus, assessing the number of nodules at this age confirms that the numbers determined at 25 days have remained relatively stable and are not increasing indefinitely with age. This approach helps verify that autoregulation of nodulation is functional and provides confirmation of a reduction, as opposed to a delay, in nodule development.

Gibberellin treatments

The effect of gibberellin on nodule formation was examined using the gibberellin-deficient *na-1* and its wild type (Table I). Seeds of the two lines were sown according to the methods used for the root characterization experiments (described above). The roots of the seedlings were treated with 150 ml of either water (control) or various concentrations (10^{-9}, 10^{-6} or 10^{-3} M) of the bioactive GA_3. These treatments commenced three days after planting and continuing twice per week until harvest. The plants were harvested 20 days after planting, rinsed clean of soil substrates and their nodules counted.

Grafting experiments

For grafting experiments, seeds of Torsdag and *lkb*, or *lh-2* (Table I) were sown as detailed above for the nodule count investigation. At six days after planting, the seedlings were grafted using the methods of Reid et al. (1983). These mutants were chosen because of their common background (i.e. Torsdag; Table I) and their relative similarity in terms of both shoot stature and nodule numbers (Table III). At ten days after planting, the plants were inoculated with 25 ml of the bacteria, thus allowing the grafts to establish prior to inoculation. The graft combinations were then scored 30 days after planting.

Analysis of Root Characteristics

Plants were harvested 17 day after planting, allowing for the development of secondary and tertiary lateral roots. The plants were uprooted, gently cleaned in water, and placed in a tray of water. The length of the shoot and the longest secondary and tertiary lateral root was measured. The total number of nodes and secondary lateral roots were recorded. In addition, the number of tertiary lateral roots located on each of the upper (i.e. closest to the crown) six secondary lateral roots was counted.

Hormone Analysis

The roots of 30 day-old grafted plants were cleaned of soil, separated from their shoots and cotyledons and weighed. Indole acetic acid (IAA) and GA_1 were then extracted from these root systems, and their levels quantified, using the methods outlined in Ross (1998). Two replicates, consisting of 6 root systems per replicate, were analyzed.

Statistical Analysis

All statistics were determined using Student's *t*-tests.

CHAPTER 3

Further Characterizing the Nodulation Phenotypes of Gibberellin Mutants of *Pisum sativum*

INTRODUCTION

As shown in Chapter 2 (Ferguson et al. 2005a), GAs appear to have a role in nodule development. To investigate this role further, the nodulation phenotypes of additional GA mutants of *Pisum sativum* (see overview in Table I) were examined. This included investigating a number of both GA response mutants and GA biosynthesis mutants, including a variety of double mutants. In addition, the nodule histology of the severely GA-deficient *na-1* was investigated. Sections of the mutant and its wild type were examined using bright field microscopy to determine the effect of GA-deficiency on nodule development.

The roots and shoot of the GA-deficient *na-1* mutant are short and thick (Yaxley et al. 2001a; Davidson et al. 2003; Chapter 2; Ferguson et al. 2005a). These phenotypes are similar to those previously described for pea plants treated with ethylene (Ferguson et al. 2005b). Thus, the roles of ethylene in the *na-1* phenotype were also examined. To investigate these roles, precursors of ethylene, or inhibitors of ethylene biosynthesis, were applied to *na-1* plants, following which the phenotypes of the plants were assessed. Roles for both ethylene and GAs in nodule development are discussed.

Table I. *Overview of the various* P. sativum *lines investigated*

Genotype	Line Number or Cross	Mutated Gene Product	Effect of Mutation	Phenotype	References
NA	1766x1769	wild type			
na-1	1766x1769; 6074x (1766x1769)	*ent*-kaurenoic acid oxidase	reduced bioactive GAs	extreme dwarf with short, thick roots	Yaxley et al. 2001a; Davidson et al. 2003; Ferguson et al. 2005a; Chapter 2
sln	6074x (1766x1769)	GA 2-oxidase	elevated seed GAs leading to elevated bioactive seedling GAs	elongated internodes	Reid et al. 1992; Ross et al. 1993; Lester et al. 1999; Yaxley et al., 2001a; Ferguson et al. 2005a; Chapter 2
na-1 sln	6074x (1766x1769)	GA 2-oxidase; *ent*-kaurenoic acid oxidase	elevated, followed by reduced, bioactive GAs	elongated, followed by short internodes	Ross et al. 1995; Yaxley et al., 2001a
NA LA CRY^s	187	wild type			
NA la cry^s	197	probably DELLA protein	constitutive GA response	elongated internodes	Reid et al. 1983; Potts et al. 1985
na-1 la cry^s	188	*ent*-kaurenoic acid oxidase; probably DELLA protein	reduced bioactive GAs and constitutive GA response	elongated internodes	Reid et al. 1983; Potts et al. 1985
na-2 LA cry^s	81	*ent*-kaurenoic acid oxidase	reduced bioactive GAs	extreme dwarf	Davidson et al. 2003; Reid et al. 1983; Potts et al. 1985
LH LE	511x5839	wild type			
lh-1 LE	511x5839	*ent*-kaurene oxidase	reduced bioactive GAs	dwarf	Reid 1986; Davidson et al., 2004; Yaxley et al., 2001a; Ferguson et al. 2005a; Chapter 2
LH le-3	511x5839	GA 3-oxidase	bioactive GAs reduced in shoot, normal in root	dwarf	Ingram et al., 1984; Yaxley et al., 2001a; Ferguson et al. 2005a; Chapter 2
lh-1 le-3	511x5839	*ent*-kaurene oxidase; GA 3-oxidase	unknown	extreme dwarf	None available

Table I. *Overview of the various* P. sativum *lines investigated (Continued)*

Genotype	Line Number or Cross	Mutated Gene Product	Effect of Mutation	Phenotype	References
Frisson	wild type				
sym28	P64	unknown	unable to autoregulate nodulation	hyper-nodulation	Duc and Messager 1986; Sagan and Duc 1996
nod3	P79	unknown	unable to autoregulate nodulation	hyper-nodulation	Duc and Messager 1986; Sagan and Duc 1992
sym29	P88	receptor kinase	unable to autoregulate nodulation	hyper-nodulation	Sagan and Duc 1996; Krusell et al. 2002

RESULTS

Nodulation Phenotypes of GA Mutants

The na-1 *and* sln *mutations*

Plants possessing the *na-1* mutation produced few to no nodules (Table II). Those that did form were aberrant, being small, white, and probably not functional. In contrast, a healthy population of nodules was observed on mutants possessing the *sln* mutation (Table II), although few to no nodules were observed on the primary root of this mutant. These findings are consistent with those of Chapter 2 (Ferguson et al. 2005a) using different mutant lines of *na-1* and *sln*. Moreover, the nodule number observed on both *sln* and *na-1* mutants at 40 days was not significantly increased from that detected at 25 days (Table II), which is also consistent with the findings of Chapter 2 (Ferguson et al. 2005a). In addition, the total, and average, nodule DW of *na-1* was significantly reduced compared with that of *sln* at both 25 and 40 days.

Double mutants possessing both the *na-1* and *sln* mutations produced significantly fewer nodules at 25 days than mutants possessing the *sln* mutation only (Table II). However, the double mutant formed significantly more nodules than observed on plants possessing the *na-1* mutation only (Table II). The nodule number observed on *na-1 sln* double mutants at 40 days was significantly increased from that observed on the mutant at 25 days (Table II). This finding was in contrast to that observed on *na-1* and *sln* single mutants, whose nodule numbers were not significantly increased at 40 days from that observed at 25 days. In fact, the nodule number of *na-1 sln* mutants was actually significantly greater than that of *sln* at 40 days, despite having been significantly reduced compared to that of *sln* at 25 days (Table II).

The appearance of the *na-1 sln* nodules was abnormal, being pale and round, as opposed to the typical pink and cylindrical nodules of wild type and *sln* mutants (Fig. 1) or the small, white nodules of *na-1* (Chapter 2; Ferguson et al. 2005a). In addition, whereas the total nodule DW of *na-1 sln* mutants increased at 40 days

Table II. *Root, shoot and nodule dry weights and nodule numbers per root and shoot dry weight of 25 and 40 day-old* na-1, sln *and* na-1 sln *mutants*

Age	Genotype	Dry Weight				Number of Nodules		
		Shoot (mg)	Root (mg)	Nodule Total (mg)	Nodule Average (mg)	Per Plant	Per mg Shoot Dry Weight	Per mg Root Dry Weight
25	*na-1*	134 ± 9	121 ± 15	0.8 ± 0.3	0.15 ± 0.10	4 ± 3	0.02 ± 0.02	0.02 ± 0.02
	sln	429 ± 25	171 ± 21	41.9 ± 4.5	0.31 ± 0.02	138 ± 18	0.32 ± 0.04	0.93 ± 0.18
	na-1 sln	449 ± 37	264 ± 29	39.1 ± 7.3	0.43 ± 0.06	88 ± 5	0.21 ± 0.02	0.37 ± 0.06
40	*na-1*	221 ± 16	202 ± 13	0.5 ± 0.2	0.02 ± 0.01	17 ± 7	0.09 ± 0.04*	0.10 ± 0.04
	sln	1297 ± 84*	310 ± 28*	74.0 ± 6.5*	0.65 ± 0.08*	118 ± 7	0.09 ± 0.01*	0.41 ± 0.06*
	na-1 sln	996 ± 72*	420 ± 19*	115.4 ± 8.3*	0.47 ± 0.05	263 ± 31*	0.27 ± 0.04	0.64 ± 0.09*

Plants were inoculated with *R. leguminosarum* five days following the time of sowing. Results are means ± SE (n = 8). Values for each mutant trait followed by an * are significantly different from that of their respective 25 day-old value at the 0.05 level.

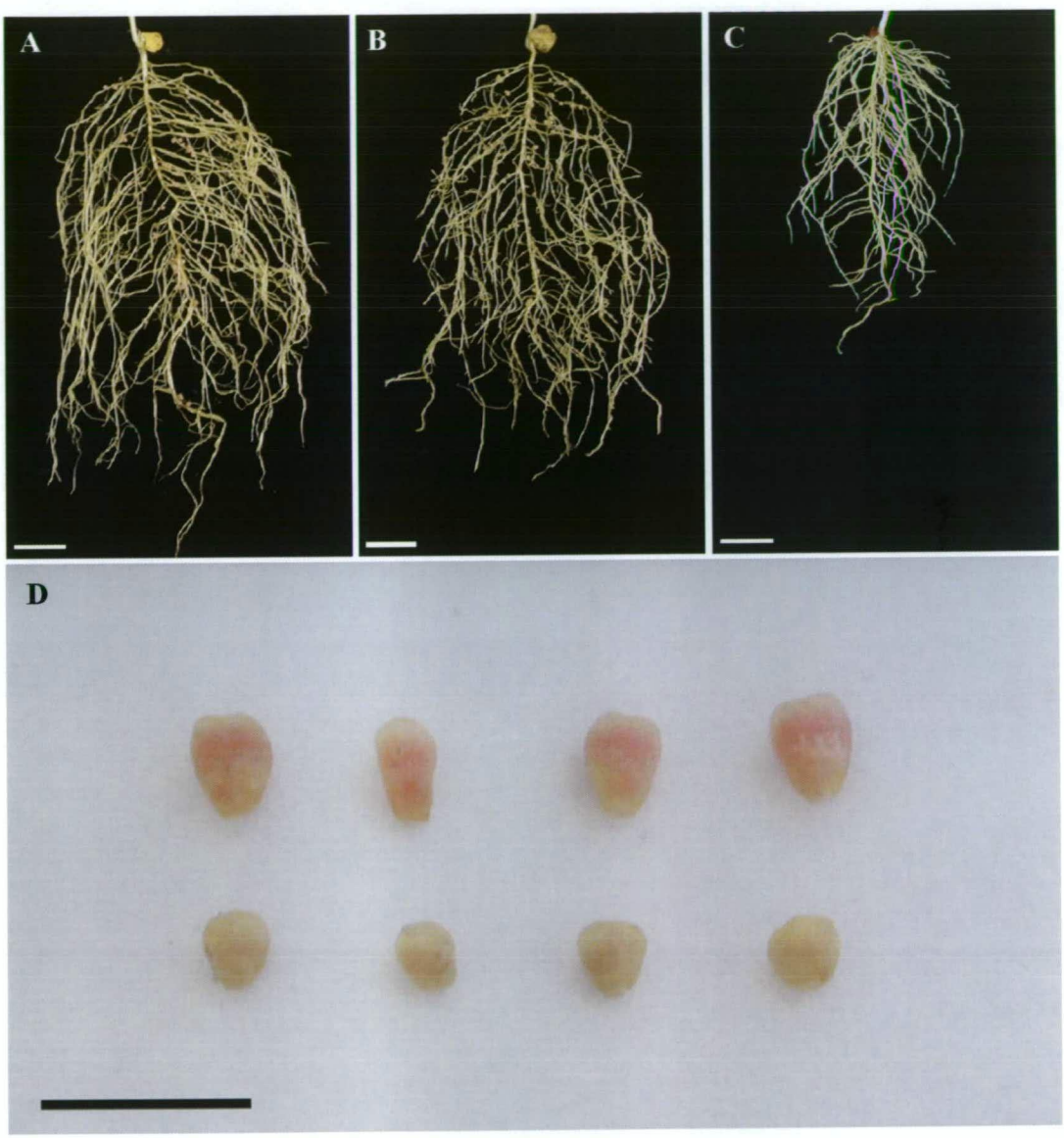

Figure 1. Root phenotypes of 25 day-old A) *sln*, B) *na-1 sln* and C) *na-1*. D) Red, cylindrical nodules of (top) *sln* compared with the more pale, round nodules of (bottom) *na-1 sln* at 25 days. A-C) Bar = 2 cm. D) Bar = 0.5 cm.

compared to that at 25 days, both the total, and the average, nodule DWs of *sln* plants increased. Similar to the trend in nodule numbers, the lateral roots of *na-1 sln* displayed an intermediate phenotype, being shorter and thicker than that of *sln*, but longer and thinner than that of *na-1* at 25 days (Fig. 1), consistent with the findings of Yaxley et al. (2001a).

The lh-1 *and* le-3 *Mutations*

Plants possessing the *lh-1* mutation formed fewer nodules, whereas those having the *le-3* mutation produced a similar number of nodules, compared with that of their respective wild types (Table III). These findings occurred on both a per plant, and a per mg root DW, basis, which is consistent with previous results reported in Chapter 2 (Ferguson et al. 2005a) obtained using a different allele of *lh* (*lh-2*) and a different mutation of *LE*. Also consistent with the findings reported in Chapter 2 (Ferguson et al. 2005a) was the similar appearance, location on the root system and DW (Table III) of the nodules of both *le-3* and *lh-1* mutants, compared with that of the wild type.

The *le-3* population examined segregated for the *lh-1* mutation, allowing for the selection of *lh-1 le-3* double mutants (Fig. 2). These double mutants formed significantly fewer nodules than any of the *LH LE*, *LH le-3* or *lh-1 LE* lines (Table III). Although *lh-1 le-3* is likely severely reduced in its shoot GA level, and its shoot resembled that of the extremely dwarf *na-1* mutant (Fig. 3), other phenotypes of this double mutant did not resemble those of *na-1*. For example, compared with that of their wild types, the reduction in nodule numbers of *lh-1 le-3* was not nearly as severe as that of *na-1* mutants (Tables II, III; Chapter 2; Ferguson et al. 2005a). However, differences in phenotypes may be attributed to differences in the genetic backgrounds of the mutants. In either event, the roots of *lh-1 le-3* were not as severely reduced in number, or increased in thickness (Fig. 3) and the nodules were not aberrant in appearance, compared with those of *na-1*. Furthermore, the nodule DW of *lh-1 le-3* was not significantly different from that of its wild type, or the *LH le-3* or *lh-1 LE* mutants (Table III), whereas that of *na-1* is significantly different from that of its wild type (Chapter 2; Ferguson et al. 2005a).

Table III. *Root, shoot and nodule dry weights and nodule numbers per root and shoot dry weight of 25 day-old wild type and* lh-1, le-3 *and* lh-1 le-3 *mutants*

Genotype	Dry Weight				Number of Nodules		
	Shoot (mg)	Root (mg)	Nodule Total (mg)	Nodule Average (mg)	Per Plant	Per mg Shoot Dry Weight	Per mg Root Dry Weight
LH LE	417 ± 34	184 ± 10	113 ± 7.5	0.24 ± 0.016	482 ± 23	1.19 ± 0.09	2.64 ± 0.13
LH le-3	397 ± 19	192 ± 12	106 ± 4.6	0.22 ± 0.013	492 ± 18	1.26 ± 0.08	2.62 ± 0.20
lh-1 LE	289 ± 26*	182 ± 11	83 ± 5.9*	0.24 ± 0.018	356 ± 17*	1.27 ± 0.08	1.97 ± 0.08*
lh-1 le-3	235 ± 38*	141 ± 7*	48 ± 8.6*	0.24 ± 0.014	196 ± 24*	0.84 ± 0.03*	1.38 ± 0.10*

Plants were inoculated with *R. leguminosarum* five days following the time of sowing. Results are means ± SE (n = 8; n = 2 for *le-3 lh-1*). Values for each mutant trait followed by an * are significantly different from that of their respective wild type at the 0.05 level.

Figure 2. Root and shoot phenotypes of 25 day-old seedlings of (left to right) *LH le-3*, *lh-1 LE*, *LH LE* and *lh-1 le-3*. Bar = 5 cm.

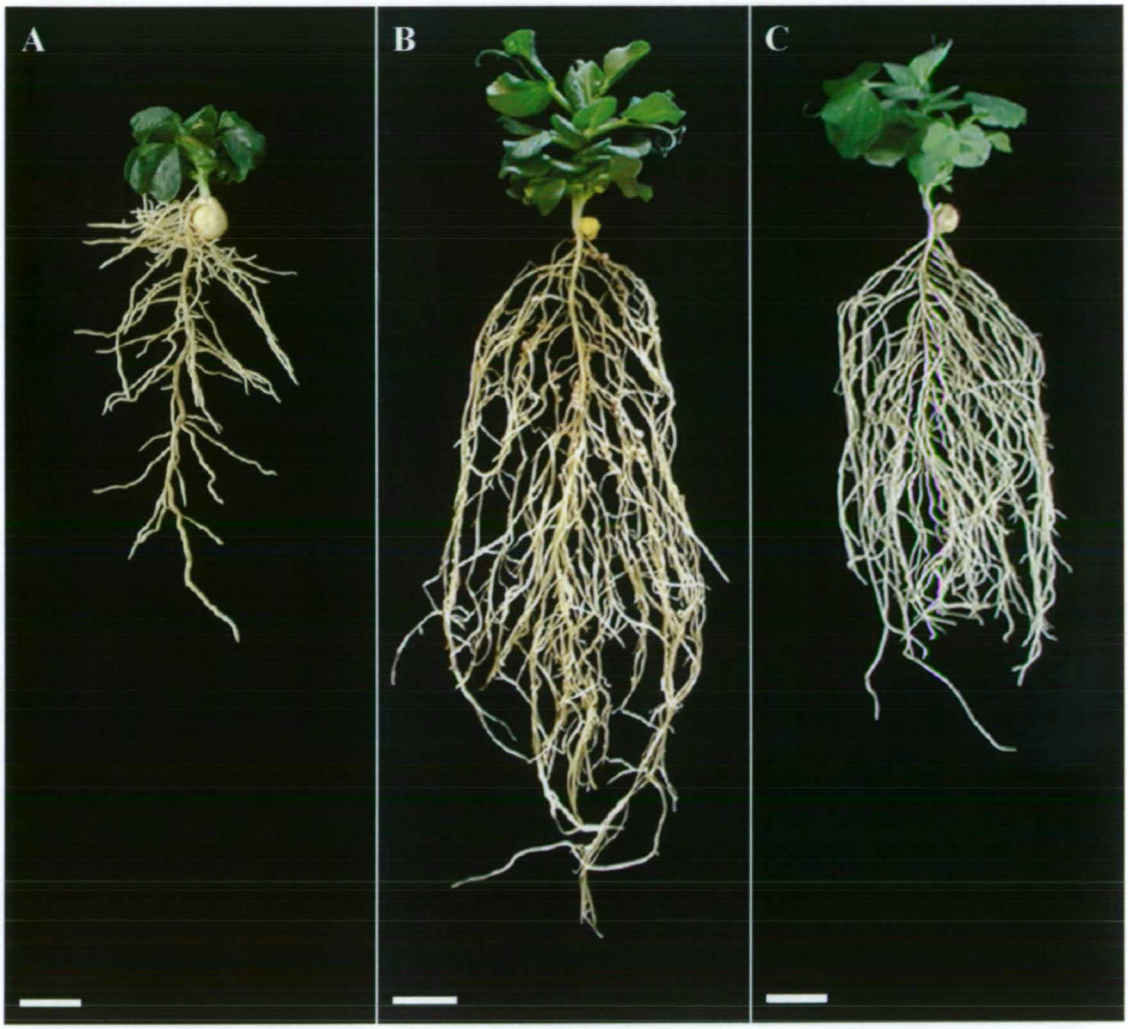

Figure 3. Root and shoot phenotypes of 25 day-old seedlings of A) *na-1 LA CRY LH LE*, B) *NA LA CRY lh-1 le-3* and C) *na-2 LA cry^{s}.LH LE*. Differences in root phenotypes are apparent despite similar, extreme-dwarf shoot phenotypes, although direct comparisons cannot be made between the three lines due to differences in their genetic backgrounds. Bar = 2 cm.

The la cry^s *mutations*

The *la cry^s* genotype, which results in constitutive GA signalling, formed significantly fewer nodules than the corresponding wild type at 25 days (Table IV). This reduction was on a per plant, and a per mg root or shoot DW, basis (Table IV). As a result of the fewer nodules formed, the total nodule DW per plant of the *la cry^s* mutant was also reduced compared with that of the wild type. However, the average nodule DWs of this mutant was similar to that of the wild type, indicating that its individual nodules were not reduced in size. In addition, the appearance of the *la cry^s* nodules, and their location on the root system, was similar to that of the wild type. The root system DWs of *la cry^s* was also similar to that of their wild type (Table IV). This mutant also exhibited an increased shoot length (Fig. 4) and a greater shoot DW (Table IV) than their wild type. Interestingly, the addition of the *na-1* mutation did not appear to affect any of the traits assessed, as there were no significant differences detected between *na-1 la cry^s* and *NA la cry^s* mutants (Table IV; Fig. 4), consistent with previous characterization of their shoot phenotypes (Reid et al. 1983; Potts et al. 1985).

In addition to the *la cry^s* double mutants, the *na-2 LA cry^s* mutant was investigated (Figs. 3,4; Table IV). Comparisons between this mutant and a wild type cannot be made because *na-2 LA cry^s* does not have an isogenic parent line. Moreover, *na-2 LA cry^s* is on a different genetic background from the other mutants reported here. These differences may contribute to the phenotypes of the mutants and need to be taken into consideration when making comparisons between the lines. Keeping this in mind, it was observed that the root system of *na-2 LA cry^s* was much more developed than that of *na-1* mutant lines (Table II; Chapter 2; Ferguson et al. 2005a), and it was phenotypically similar to those of *NA la cry^s* and *na-1 la cry^s* mutants (Figs. 3,4). Moreover, the *na-2 LA cry^s* mutant developed numerous nodules in comparison to any of the *na-1* mutant lines (Tables II, IV; Chapter 2; Ferguson et al. 2005a), despite exhibiting the extremely dwarf shoot system typical of these lines (Figs. 3). Thus, the combined shoot, root and nodule phenotypes of the *na-2 LA cry^s* mutant were more similar in appearance to that of the *le-3 lh-1* double mutant, than to

Table IV. *Root, shoot and nodule dry weights and nodule numbers per root and shoot dry weight of 25 day-old* na-1, la *and* cry^s *mutants and their wild type*

Genotype	Dry Weight				Number of Nodules		
	Shoot (mg)	Root (mg)	Nodule Total (mg)	Nodule Average (mg)	Per Plant	Per mg Shoot Dry Weight	Per mg Root Dry Weight
NA LA CRY	192 ± 10	76 ± 6	20.0 ± 0.8	0.16 ± 0.008	127 ± 9	0.66 ± 0.04	1.73 ± 0.13
NA la cry^s	276 ± 19*	93 ± 9	9.1 ± 2.4*	0.12 ± 0.020	68 ± 12*	0.25 ± 0.04*	0.76 ± 0.12*
na-1 la cry^s	284 ± 13*	106 ± 9*	8.3 ± 1.4*	0.13 ± 0.009*	61 ± 9*	0.22 ± 0.04*	0.63 ± 0.13*
na-2 LA cry^s	132 ± 8*	119 ± 7*	7.0 ± 2.4*	0.04 ± 0.008*	150 ± 30	1.10 ± 0.18*	1.28 ± 0.24

Plants were inoculated with *R. leguminosarum* five days following the time of sowing. Results are means ± SE (n = 8). Values for each mutant trait followed by an * are significantly different from that of their respective wild type at the 0.05 level. Note that the *na-2 LA cry^s* mutant has no respective wild type and one cannot exclude the fact that differences between this mutant and the other genotypes are background related.

Figure 4. Root and shoot phenotypes of 25 day-old seedlings of (left to right) *NA LA CRY*, *NA la cry*^{s}, *na-1 la cry*^{s}, and *na-2 LA cry*^{s}. The *na-2 LA cry*^{s} mutant is on a different genetic background from the other lines, which may contribute to its phenotype. Bar = 5 cm.

that of the *na-1* mutants (Fig. 3). However, the average nodule DW of the *na-2 LA cry*^{s} mutant was greatly reduced compared to that of the majority of other pea lines (Table IV), which is typical of the *na-1* mutants (Table II; Chapter 2; Ferguson et al. 2005a), but unlike that of the *lh-1 le-3* double mutant (Table III).

Nodulation Phenotypes of *na* Lines Unable to Autoregulate Their Nodule Numbers

Mutants exhibiting both severe GA-deficiency and an inability to autoregulate their nodule numbers were created by crossing a *na-1* mutant line with each of three hypernodulating mutant lines, *sym28*, *sym29* or *nod3*. As a result, double mutants exhibiting both severe GA-deficiency and hypernodulating phenotypes were isolated in the resulting F_2 populations of each of the three crosses (Table V; Fig. 5).

Typically, the root and shoot systems of the *sym28 na-1*, *sym29 na-1* or *nod3 na-1* double mutants appeared similar in size, thickness and stature to that of their *na-1* parent (Fig 5). However, the root systems of each of these double mutants exhibited numerous nodule structures, which is uncharacteristic of *na-1*, but typical of the hypernodulating lines, *sym28*, *sym29* and *nod3*. However, the phenotypes of the individual nodules formed on these double mutants were similar to those of the aberrant nodules of *na-1* (Fig. 5; Chapter 2; Ferguson et al. 2005a). Thus, phenotypes induced by both parent mutations were expressed in each of the three double mutant combinations identified. Probability tests revealed that the *SYM28*, *SYM29* and *NOD3* genes assorted independently from the *NA* gene (Table V), indicating that none of the autoregulation genes examined are linked to *NA*.

Since the genetic background of the three hypernodulating lines differed from that of the *na-1* line, additional phenotypes also segregated in the F_2 generation of all three crosses. For example, the hypernodulating mutant lines originated from the wild type, Frisson, which has a naturally occurring dwarf *le* background. In contrast, the *na-1* mutant line was derived from the tall *LE* background of its wild type, *NA*. As a result, in addition to extremely dwarf shoot phenotypes caused by the *na-1*

Table V. *Shoot and nodulation phenotypes of F_2 segregates from crosses between the hypernodulating mutants,* sym28, sym29 *and* nod3 *and the severely GA-deficient mutant,* na-1.
Shoot phenotypes include wild type (*NA*) and extreme dwarf (*na-1*) shoots. Nodulation phenotypes include the ability to regulate nodule numbers, nod+ (*SYM28*, *SYM29* and *NOD3*), and the inability to regulate nodule numbers, nod++ (*sym28*, *sym29* and *nod3*), resulting in an excessive number of nodules formed. Phenotypes were based on comparisons between the F_2 segregates, their single mutant parents and the wild types lines of these single mutant parents at 25 days. Contingency χ^2 (df = 1) testing for independence of the shoot elongation and nodulation phenotypes is shown.

Cross	Shoot Genotype	Nodulation Phenotype			χ^2	Probability
		nod+	nod++	total		
sym28 x *na-1*	*NA*	44	10	54	0.258	> 0.50
	na-1	12	4	16		
	total	56	14	70		
nod3 x *na-1*	*NA*	69	4	73	3.540	> 0.05
	na-1	12	3	15		
	total	81	7	88		
sym29 x *na-1*	*NA*	31	5	36	0.179	> 0.50
	na-1	10	1	11		
	total	41	6	47		

Plants were inoculated with *R. leguminosarum* at the time of sowing. Note that plants possessing the *na-1* mutation are considered as Nod+, despite the fact that they produced few to no nodules.

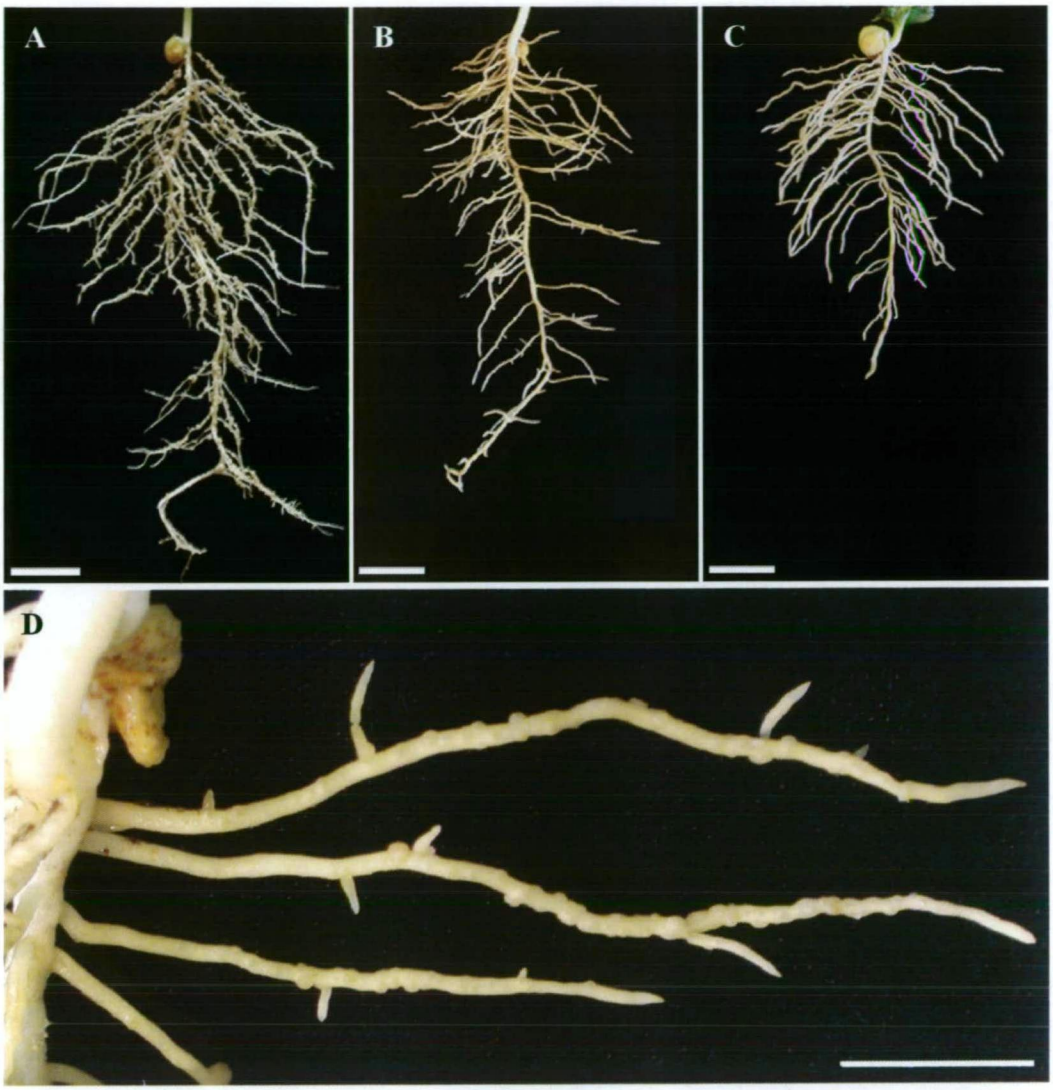

Figure 5. Root phenotypes of A) *sym29*, B) *sym29 na-1* and C) *na-1* at 25 days following inoculation with *Rhizobium leguminosarum*. D) Close up of lateral roots of *sym29 na-1* exhibiting numerous aberrant nodule structures. A-C) Bar = 2 cm. D) Bar = 1 cm.

Figure 6. Shoot systems of 60 day-old plants exhibiting (left to right) tall, dwarf and nana phenotypes.

mutation, dwarf (*le*) and tall (*LE*) shoot phenotypes segregated in the F_2 populations of the three crosses (Fig. 6). Interestingly, despite the fact that *sym28*, *sym29*, *nod3* and *na-1* all yield a reasonable number of seeds, the double mutants reported here produced very few seeds, often as little as one per plant. Due to this extremely poor yield, plants could not be sacrificed to obtain their root and nodule numbers and DWs, because they were required to perpetuate the line.

Effects of ACC and AVG Treatments on the *na* Mutant

Previously, it was demonstrated that GA application could restore the nodule, root and shoot phenotypes of the *na-1* mutant to that of its wild type, *NA* (Chapter 2; Ferguson et al. 2005a). These findings clearly indicated a requirement for GAs in the growth and development of this GA-deficient mutant. However, many of the phenotypes of untreated *na-1* plants, such as thick and short roots and a reduced capacity to form nodules, actually resemble those of pea plants exposed to high levels of ethylene (e.g. Ferguson et al 2005b). Thus, it was decided to continue the investigations into *na-1* by examining the role of ethylene in the phenotype of the mutant.

The application of the ethylene precursor, ACC, significantly reduced the nodule numbers of the wild type, *NA* (Table VI). In addition, moderate to significant reductions were also observed in the root numbers and lengths, shoot length and root and shoot DWs of ACC-treated *NA* plants (Fig. 7; Table VI). These findings are consistent with previous reports investigating the effects of ethylene in pea (Lee and LaRue, 1992c; Ferguson et al. 2005b). A significant reduction in nodule and root numbers was also observed on *na-1* following ACC treatments (Table VI). This indicates that the mutant is able to respond to elevated levels of ethylene. Thus, even if some of the phenotypes of *na-1* can be attributed to increased ethylene production, the ethylene receptors of the mutant are not fully saturated, allowing it to respond to increased levels of the hormone. Relatively unaffected by the elevated ethylene were the shoot length and shoot and root DWs of ACC-treated *na-1*, which were similar to

Table VI. *Nodule and root numbers, and root and shoot lengths and dry weights, of 20 day-old* NA *and* na-1 *lines treated with the ethylene biosynthesis precursor (ACC) or an inhibitor of ethylene biosynthesis (AVG).*
Indicated are the number of nodules and secondary lateral roots per plant, the average number of tertiary lateral roots located on the six uppermost secondary lateral roots, the lengths of the shoot and the longest secondary and tertiary lateral roots per plant, and the root and shoot system dry weights of *NA* and *na* plants treated with either water (control), ACC or AVG.

Line	Treatment	Number			Length (cm)			Dry Weight (mg)	
		Nodules	Secondary Roots	Tertiary Roots	Shoot	Secondary Root	Tertiary Root	Root	Shoot
NA	Control	116 ± 11.3	100 ± 4	20 ± 1.5	32.8 ± 0.9	24 ± 1.0	6 ± 0.6	246 ± 15	366 ± 28
	ACC	29 ± 4.1*	92 ± 5	11 ± 0.7*	22.5 ± 0.7*	21 ± 0.4*	4 ± 0.2*	223 ± 19	284 ± 13*
	AVG	124 ± 8.2	103 ± 1	15 ± 1.2*	33.9 ± 1.0	28 ± 0.5*	4 ± 0.4*	222 ± 6	371 ± 14
na-1	Control	1 ± 0.7	57 ± 1	6 ± 0.5	3.5 ± 0.2	7 ± 0.3	1 ± 0.1	140 ± 6	143 ± 10
	ACC	0 ± 0.0*	48 ± 2*	5 ± 0.5*	3.2 ± 0.1	9 ± 0.5*	2 ± 0.2*	147 ± 6	122 ± 6
	AVG	36 ± 4.7*	66 ± 3*	5 ± 0.4*	4.0 ± 0.1*	8 ± 0.4	1 ± 0.1	137 ± 4	194 ± 15*

Results are means ± SE (n = 6). Values for each trait followed by an * are significantly different from that of their respective control treatment at the 0.05 level.

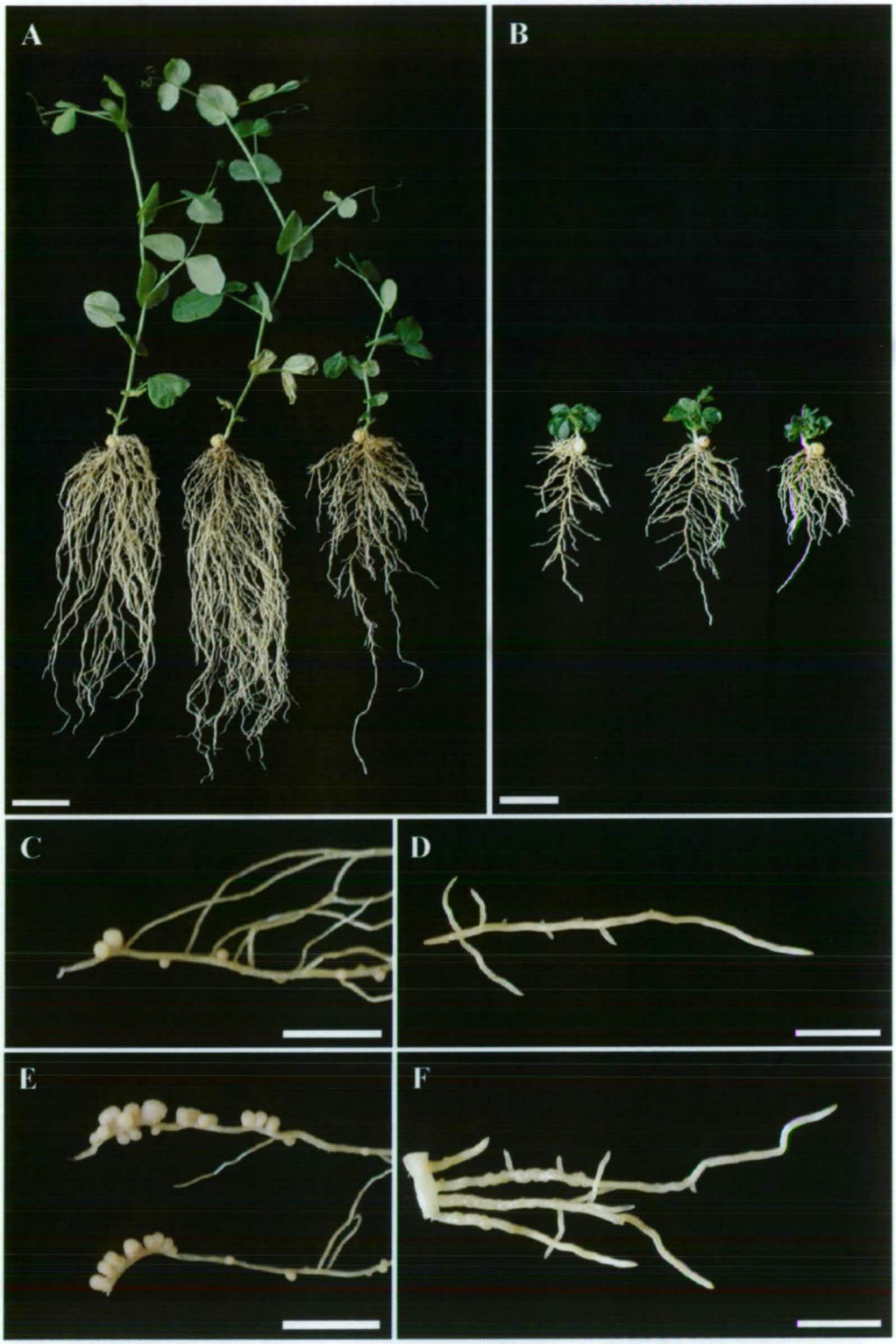

Figure 7. Seedlings of A) *NA* and B) *na-1* treated with (left to right) water (control), AVG or ACC. Excised secondary roots of C,E) *NA* and D,F) *na-1* treated with C,D) water or E,F) AVG. A,B) Bar = 5 cm. C-F) Bar = 1 cm.

those of untreated *na-1* plants, while significant increases were observed in the secondary and tertiary root lengths (Fig. 7; Table VI).

In contrast to ACC-treatments, the application of the ethylene biosynthesis inhibitor, AVG, resulted in a moderate increase in nodule formation on *NA*, and a significant, 36-fold increase on *na-1* (Table VI). This was likely a direct result of the AVG lowering the level of endogenous ethylene in the plant, thereby alleviating the inhibitory effect of the hormone on nodule formation, as detailed in Chapter 1, Ferguson and Mathesius (2003) and Ferguson et al. (2005b). This significant increase in nodule formation on *na-1* would therefore suggest that the mutant is indeed overproducing ethylene.

Interestingly, although AVG treatment increased the number of nodules on *na-1*, those that formed maintained the aberrant morphology characteristic of the few nodule structures observed on *na-1* control plants (Fig. 7; Chapter 2; Ferguson et al. 2005a). This indicates that reducing the ethylene level of the mutant is not sufficient to promote proper nodule growth and development, despite being able to increase the number of nodules that initiate. Thus, although ethylene appears to play a role in nodule initiation, GAs appear to be required for late stages of nodule development.

The application of AVG also significantly increased the number of *na-1* secondary roots, in addition to increasing the shoot length and DW compared with that of control-treated *na-1* plants (Fig. 7; Table VI). In contrast, none of these traits were found to be significantly different between control and AVG-treated *NA* plants (Fig. 7; Table VI). These findings suggest that ethylene may also has a partial role in the root and shoot phenotype of the *na-1* mutant.

Nodule Histology of the GA-Deficient *nana*

The nodule histology of the wild type, *NA* (Fig. 8), was similar to that reported for other wild type lines of pea (e.g. Bond 1948; Newcomb et al. 1979; Chapter 4; Ferguson and Reid 2005). The outer cortex of the nodule enclosed the three nodule histological zones: the meristematic, invasion and infected zones, in

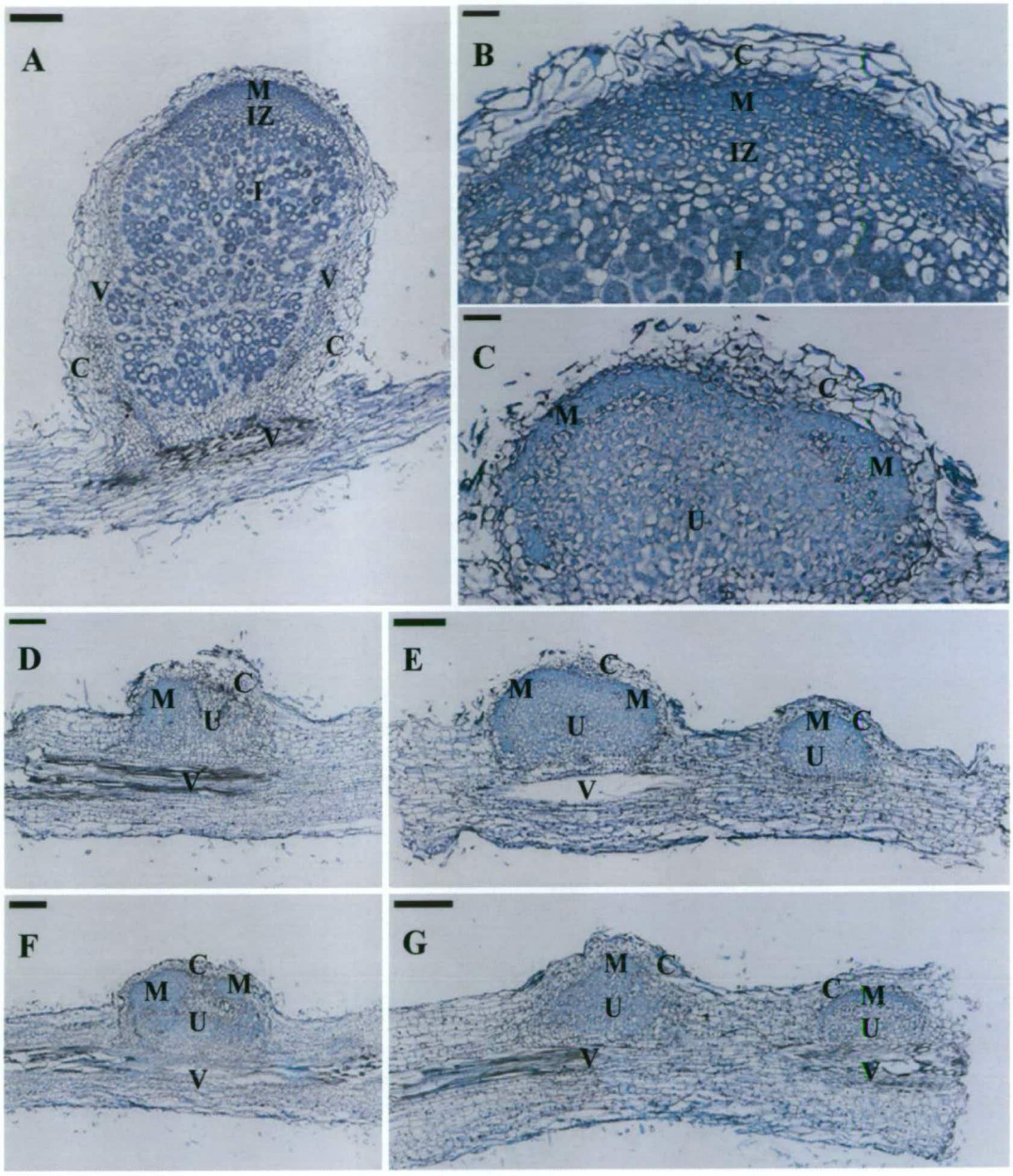

Figure 8. Nodule histology of A-B) wild type, *NA*, and C-G) *na* nodules. B-C) Magnified view of the nodule region exhibiting the transition between the various histological zones of B) *NA* and C) *na*. C=cortex; V=vasculature; M=meristem; IZ=invasion zone; I=infected zone; U=unenlarged cells of the invasion zone. A,D-G) Bar=200 μm. B,C) Bar=50 μm.

addition to the nodule peripheral vasculature (Fig. 8). The cells of the infected zone were enlarged and appear to have been invaded by the bacteria.

In contrast, the nodule histology of *na-1* was uncharacteristic (Fig. 8). The vasculature of the mutant was generally absent. The meristem was also reduced in size, often not extending across the entire width of the top of the nodule. In addition, the infected zone was greatly reduced in size. The cells of the infected zone were reduced in number, were not enlarged and did not appear to contain the bacteria, compared to those of *NA* (Fig. 8). As a result, the overall size of *na-1* nodules was significantly reduced in size compared to those of *NA*, despite the roots of the mutant appearing thicker than those of *NA*. These findings confirm previous reports regarding the size and thickness of *na-1* roots and nodules (Yaxley et al. 2001a; Chapter 2; Ferguson et al. 2005a).

DISCUSSION

Further Characterizing the Nodulation Phenotypes of GA mutants

The nodulation phenotypes reported here for *lh*, *le*, *na* and *sln* pea mutants were consistent with those reported in Chapter 2 (Ferguson et al. 2005a) using different lines, and in the case of *lh*, a different mutant allele. This supports the hypothesis that GA deficiency (*lh* and *na*) results in reduced nodule formation, whereas wild type root GA levels (*le*) or elevated total plant GA levels (*sln*) results in wild type numbers of nodules being formed.

The use of double mutants also provided considerable insight into the roles of GAs in nodule development. For example, although the lines are unrelated, the *lh-1 le-3* double mutant, like the *na-1* mutants, formed significantly fewer nodules than its wild type. Moreover, *lh-2 le-3* double mutants exhibited an extremely dwarf shoot phenotype, which is also similar to that of the *na-1* mutants and is consistent with recent findings by Davidson et al. (2005) using this double mutant. However, unlike the *na-1* mutants, which form few to no nodules, the *lh-1 le-3* double mutant

produced many nodules that were similar in size and appearance to those of the wild type. In fact, the *lh-1 le-3* double mutant formed approximately 50 fold more nodules per plant, and almost 70 fold more nodules per mg root DW, than observed on the *na-1* mutants reported here and in Chapter 2 (Ferguson et al. 2005a). This demonstrates that numerous nodules of normal appearance are able to develop on plants having an extreme-dwarf shoot. Instead, the differences between *na-1* and the *lh-1 le-3* double mutant may be directly related to the GA levels in the roots. Reductions in GAs caused by the *na-1* mutation are much more severe than those caused by either the *lh* or *le* mutations (Ingram et al. 1984; Reid 1986; Yaxley et al. 2001a; Davidson et al. 2003, 2004). In fact, the *le* mutations only affect the GA levels of the shoot (Ingram et al. 1984; Yaxley et al. 2001a). Thus, although the GA level of the *lh-1 le-3* double mutant shoot is likely severely reduced (perhaps even in the range of that detected in *na-1* as has been recently identified in the *ls-1 lh-2* double mutant shoot (Davidson et al. 2005)), the level of this hormone in the root may not be as severely reduced as that reported in *na-1* mutants. This could explain the longer, thinner roots and normal appearance of, and numerous nodules of, the *lh-1 le-3* double mutant compared with that of the *na-1* mutant. Further studies using a larger number of the *lh-1 le-3* double mutant are required to verify the nodulation phenotype reported here. In addition, quantifying the levels of GAs in *lh-1 le-3* double mutant roots and shoots, as has been done using *ls-1 lh-2* double mutant shoots (Davidson et al. 2005), will greatly aid in the understanding of the hormone in the shoot, root and nodule phenotypes of pea.

The *na-1 sln* double mutant also indicated a role for GAs in nodule and root development. The shoot, root and nodule phenotypes of this mutant appeared as intermediates of its *na-1* and *sln* mutant parents. For example, these structures appear to have grown and elongated, as occurs in wild type and *sln* mutants (Yaxley et al. 2001a; Chapter 2; Ferguson et al. 2005a), but as they mature, they become stunted, as has been described for *na-1* (Yaxley et al. 2001a; Chapter 2; Ferguson et al. 2005a). Similar to what was described above for the *le-3 lh-1* double mutant, the phenotypes of the *na-1 sln* double mutant are likely directly related to its GA level. For example, the elongated internodes of young seedlings are likely the result of elevated GA levels caused by the *sln* mutation (Reid et al. 1992), whereas the reduced lengths of

internodes developing on mature plants are likely due to reduced GA levels caused by the *na-1* mutation (Davidson et al. 2003). The same theory can be applied to the phenotypes of the nodules and roots. Further investigations into the histology of the nodules and roots, in addition to application studies using GAs and their inhibitors would help provide more insight into the phenotypes of the *na-1 sln* double mutant.

GAs Are Required Late in Nodule Development

The excessive number of nodules observed on *sym28 na-1*, *nod3 na-1* and *sym29 na-1* double mutants demonstrates that numerous nodules can indeed be initiated on the severely GA-deficient, and usually poorly-nodulating, *na-1* background. This indicates that GAs may not be required for bacterial recognition or infection or other early developmental aspects of nodule development. However, despite being numerous in number, the nodules observed on all three of these double mutants developed the aberrant phenotype that is characteristic of *na-1*. These findings could be a direct result of GA-deficiency on nodulation, or alternatively, they could be an indirect result of GA-deficiency causing severe shoot dwarfism, resulting in reduced resources available for nodule growth. The fact that numerous nodule structures of normal appearance were observed on *lh-1 le-3* double mutants, which have a similar extreme dwarf shoot stature to that of *na-1*, indicates that the former hypothesis is likely correct. The findings using *sym28 na-1*, *nod3 na-1* and *sym29 na-1* double mutants further suggest that GAs are required for proper nodule formation, specifically in the late growth and elongation stages of development. Interestingly, the hypernodulation phenotype was observed on all three double mutant combinations, indicating that each of SYM28, NOD3 and SYM29 affect the autorgulation pathway upstream of where GAs are required for nodule development. Based on this evidence, it appears that GAs do not influence any one of these gene products, nor the autoregulation pathway that they are apart of.

Further evidence that GAs have a role in late, but not early, nodule development emerged from the investigations into the effects of the ethylene biosynthesis inhibitor, AVG, on *na-1*. AVG treatment significantly increased the

number of nodules on *na-1* by 36 times that observed on untreated *na-1* control plants. This indicates that ethylene is inhibiting the initiation of nodules on the mutant. However, the nodules of these AVG-treated plants exhibited the aberrant phenotype characteristic of *na-1* control plants. This indicates that excess ethylene is not the cause of the aberrant nodule phenotype, but rather it is the reduction in bioactive GAs of the *na-1* mutant that is preventing the growth and elongation of the nodules. Thus, although ethylene is inhibitory to early stages of nodule development, as has been previously demonstrated using pea (Lee and LaRue, 1992c), GAs appear to be required late in nodule development for proper growth and elongation. This finding indicating a requirement for GAs in nodule development is consistent with that derived from the investigations into the nodulation phenotypes of *sym28 na-1*, *nod3 na-1* and *sym29 na-1* double mutants, in addition to that described for the GA mutants investigated in Chapter 2 (Ferguson et al. 2005a). Using microscopy techniques to examine the exact stage(s) in development where *na-1* nodules are blocked would help delineate between the roles for ethylene and GAs in *na-1* nodule development.

To the best of my knowledge, this is the first report suggesting a relationship between reduced GA levels and ethylene overproduction. Elevated ethylene levels may also have a partial role in the formation of other *na-1* phenotypes. For example, the shoot length and DW of AVG-treated *na-1* plants were both significantly increased compared to those of *na-1* control plants. Interestingly, like the number of nodules, AVG-treated *na-1* plants also developed significantly more secondary lateral roots compared to *na-1* control plants. This indicates that ethylene has a role in the inhibition of both nodules and roots of *na-1*, and is consistent with the theory that nodules and roots share aspects of their early developmental pathways (Nutman 1948, Hirsch and LaRue 1997; Mathesius 2003; Chapter 2; Ferguson et al. 2005a).

The histology of *na-1* nodules confirmed the morphological observations reported in Chapter 2 (Ferguson et al. 2005a) that these nodules are aberrant. The absence of a developed vascular system and the stunted cells of the infected zone, indicate that GAs are required for these tissues to develop properly. These findings lend further support to the theory that GAs are required late in nodule development,

as the majority of the affected cells are located in a region of the nodule that forms when the nodule emerges from the lateral root. It is possible that GAs are required for the persistence of the meristem, the extension of the vasculature, and the formation, and subsequent elongation, of the cells of the infected zone. In addition, GAs, or GA signalling, may be required for the release of the bacteria from the nodule infection threads into the cells of the nodule infected zone. Further investigations are required to confirm the exact roles of GAs in nodule development, including using SEM techniques to identify the existence of the bacteria in the infection threads and nodule infected zone.

Delineating The Roles of GA Signalling In Nodule Development

Mutants possessing *la cry*^{s} mutations, and thus exhibiting constitutive GA signalling, developed shoots that were slender and nodules that were normal in size and appearance. This was observed regardless of whether or not the *la cry*^{s} mutant also possessed the *na-1* mutation. This demonstrates that elongated shoots and normal nodules can form on the severely GA-deficient *na-1* background. More importantly, these findings indicate that the perceived GA_1 level is most important for internode elongation and nodule development, regardless of the actual GA_1 level of the plant. Thus these results further imply that GAs are required for nodule development, consistent with the findings reported previously in this thesis. Histological analysis of the nodules of the *la cry*^{s} mutants could also help to identify the importance of GAs in the nodulation process.

Despite the *na-2 LA cry*^{s} mutant lacking a comparable wild type at this stage, it still provided compelling insight into the roles of GAs in nodule development. Although on different genetic backgrounds, the *na-2 LA cry*^{s} mutant formed many more nodules than single mutant *na-1* lines (approximately 38 times more nodules per plant), similar to what was observed when comparing the nodule number of the *la cry*^{s} or the *lh-1 le-3* double mutants with that of *na-1*. However, unlike the *la cry*^{s} mutants or the *lh-1 le-3* double mutant, the *na-2 LA cry*^{s} mutant displayed the aberrant nodule phenotype that is characteristic of *na-1*.

The *na-2 LA cry*^{s} mutant also exhibited an extremely dwarf shoot phenotype, which is similar to the *lh-1 le-3* and *na-1* mutants, but unlike the *la cry*^{s} mutants. Together, these findings suggest that the *cry*^{s} mutation itself is not sufficient to result in slender shoot growth or normal-appearing nodule structures, although it may be sufficient to allow for the production of a large nodule population. This indicates that both of the *la* and *cry*^{s} mutations are required for a full impact on the aforementioned phenotypes, but not for nodule initiation. Alternatively, the *na-2* mutation might not reduce GA levels as severely as the *na-1* mutation, but genetic evidence does not support this view.

CONCLUSIONS

The work reported here lends further support to the findings of Chapter 2 (Ferguson et al. 2005a), that GAs are required for nodule formation. It appears that GAs are necessary for late nodule development, and that ethylene may have a role in limiting the initiation of nodules on GA-deficient plants. What remains to be unequivocally determined is the importance of GA signalling, as opposed to the GA level, on shoot, nodule and root development. Moreover, the signalling pathways in nodule development that GAs influence need to be identified at the molecular level.

MATERIALS AND METHODS

Plant Growing Conditions

An overview of the various plant lines used in this report, including their mutated genes, gene products and phenotypes, is provided in Table I. Plants were grown as outlined in Chapter 2 (Ferguson et al. 2005a). For nodulation studies, seeds were sown in 100 mm "Space Saver" pots (Reko, Australia) and for AVG and ACC treatment experiments, seeds were sown in 200 mm "Plastamatic" pots (Melbourne,

Australia). At the time of sowing, each pot was provided with either 25 mL (nodulation studies) or 150 mL (AVG and ACC treatment experiments) of *Rhizobium leguminosarum* bv. *viciae* 128C53K (Nitragin® Inoculants, Liphatech Inc., Milwaukee, WI) grown in yeast-mannitol broth and diluted with water to approximately OD_{600} 0.01, which represents 5 x 10^6 cells mL^{-1}.

Creating Double Mutant Lines

Pollen of *sym28*, *nod3* and *sym29* (lines P64, P79 and P88 respectively) was collected and transferred to individual flowers of *na-1* (line 1766x1769). The resulting F_1 seed of these three crosses were sown and the subsequent F_2 seed was then also collected and sown. Using this F_2 generation of these crosses, *sym28 na-1*, *nod3 na-1* and *sym29 na-1* double mutants were selected based on the combined identification of extremely dwarf shoots and hypernodulating phenotypes at 25 days. Following thier identification, the seed of the double mutant isolates was collected and sown to confirm the existence of the double mutant phenotype in the subsequent F_3 to F_5 generations.

Identifying Nodulation Phenotypes

Plants were harvested at 25 days, allowing the nodules to develop to a stage where they could be accurately assessed. For studies using *na-1 sln* double mutants, additional plants were harvested at 40 days. This allowed for the assessment of their nodules at the time of flowering, when the autoregulation of nodulation should be preventing the formation of new nodule structures from forming (Caetano-Anollés and Gresshoff, 1991). Double mutant lines consisting of *na-1* and a hypernodulating mutation yielded very few seeds. Thus, seedlings of these lines could not be sacrificed and instead their nodulation phenotypes were determined by gently pulling back the soil and examining their roots.

Harvested plants were severed at the cotyledon. The root system was rinsed in water and the nodules assessed, counted and removed. The cotyledon was

discarded and the roots, shoots and nodules of each plant were placed in an oven at 60°C for a minimum of three days in order to obtain their dry weights (DWs).

Assessing the Roles of Ethylene in *na*

The roles of ethylene in the phenotypes of the *na-1* mutant and its wild type were investigated by treating plants with 100 ml of either water (control), 0.1 mg•ml of the ethylene precursor, 1-amino-cyclopropane 1-carboxylic acid (ACC; Sigma-Aldrich®, St. Louis, MO), or 0.03 mg•ml of the ethylene biosynthesis inhibitor, aminoethoxyvinyl glycine (AVG; i.e. 0.2 mg•ml Retaine© containing 15 % AVG, Sigma-Aldrich®, St. Louis, MO). Treatments were initially administered three days after planting, following which they were repeated twice per week until harvest at 20 days. Upon harvest, the plants were rinsed of soil substrates and their nodules assessed as outlined above. The shoot length, in addition to the length of the longest secondary and tertiary lateral root was measured. The total number of secondary lateral roots was recorded, as was the number of tertiary lateral roots located on each of the upper (i.e. closest to the crown) six secondary lateral roots.

Histological Analysis

Nodules of *na-1* and its wild type, *NA*, were excised, serially sliced into 3 μm longitudinal sections, stained with Toluidine blue, viewed and photographed as outlined in Ferguson and Reid 2005 (Chapter 4).

Statistical Analysis

All statistics were determined using Student's *t*-tests.

CHAPTER 4

Cochleata: Getting to the Root of Legume Nodules

The information contained in this chapter appears in part in the publication: Ferguson BJ, Reid JB (2005) *cochleata*: Getting to the Root of Nodules. Plant and Cell Physiol 46: (in press).

INTRODUCTION

The *cochleata* (*coch*) mutant of *Pisum sativum* (pea) was first reported by Wellensiek (1959) as having altered flower and leaf phenotypes. The flowers of *coch* exhibit supernumerary and mosaic organs, in addition to abnormally fused parts and reduced fertility. The stipules of the mutant are replaced by alternative leaf parts. At some nodes, this involves the production of leaf-like structures consisting of petioles, leaflets and tendrils in place of the stipules. For this reason, *coch* can be described as a homeotic mutant. More recently, it was established that the stipule primordia of *coch* are reduced in size and retarded in their development (Gourlay et al. 2000; Yaxley et al. 2001b), which might explain the abnormal stipule phenotype. Gourlay et al. (2000) demonstrated that a gene involved in leaf complexity, *UNIFOLIATA*, is expressed in the stipule primordia of *coch* at a time when compound stipule architecture is predicted to form. This was not observed in wild type plants, suggesting that the *COCH* gene product might act to inhibit *UNIFOLIATA* expression, thereby preventing the formation of compound leaf structures. Thus, *COCH* may act as a signaling element for organ identity.

As a homeotic mutant with altered primordia, *coch* represents an excellent tool for investigating plant developmental processes, such as nodulation. Recently, a new nodulation mutant, SGE*amp*, was described as having a shoot phenotype similar to that of *coch* (Voroshilova et al. 2004). Based on this, I continued with the line of

investigation used in Chapter 2 (Ferguson et al. 2005a) and describe here the nodulation and root phenotypes of the homeotic mutant, *coch*.

RESULTS

Nodule and Root Morphology of coch Plants During Vegetative Growth

The *coch* mutant and its wild type, Torsdag, had formed a similar number of nodules by 25 days (Table I). However, the morphology of the nodules on *coch* plants was uncharacteristic. Typically, these nodules were dichotomously branched and displayed small emerging root structures, thus creating root-nodule hybrid structures (Fig. 1). The roots of these structures emerged from the meristems, generally protruding from the sides of the nodule lobes and displaying agravitropism (Fig 1). In addition, many of the nodule lobe meristems became swollen, resembling calli (Fig. 1D-F). The central zone of the nodule lobes appeared normal and characteristically exhibited a pink hue, representing the leghaemoglobin required for nitrogen fixation. The location of these hybrid structures on the root system was also normal, being dispersed throughout the mature portion of the root system, similar to those of Torsdag. This nodule phenotype was observed on three independently derived *coch* mutants, indicating that it is specific to the *coch* mutation.

The total, and average, nodule DW values of 25 day-old *coch* were similar to those of Torsdag (Table I), suggesting that the nodules of both lines were developing at similar rates. The lateral root lengths (Table II) and root system DW (Table I) of *coch* were also not significantly different from those of Torsdag, although there were moderate differences in the number of lateral roots formed (Table II).

Nodule and Root Morphology of coch Plants at Flowering

The hybrid roots of *coch* had markedly elongated by 40 days compared with those observed at 25 days (Fig. 2). They possessed root hairs and, in some instances,

Table I. Root and nodule dry weights, and nodule numbers of 25 day-old Torsdag and *cochleata*

Age (d)	Genotype	Dry Weight (mg)			Number of Nodules	
		Root	Nodule Total	Nodule Average	Per Plant	Per mg Root Dry Weight
25	Torsdag	177 ± 16	51.7 ± 8.2	0.41 ± 0.053	125 ± 8	0.74 ± 0.07
	coch	198 ± 18	62.1 ± 6.1	0.43 ± 0.058	148 ± 10	0.80 ± 0.11
40	Torsdag	236 ± 17	85.6 ± 17.3	0.60 ± 0.078	137 ± 12	0.59 ± 0.03
	coch	273 ± 28	149.4 ± 19.4*	1.09 ± 0.153*	141 ± 11	0.47 ± 0.09

Plants were inoculated with *R. leguminosarum* at the the time of sowing. Results are means ± SE (n = 7). Values for each *coch* trait followed by an * are significantly different from those of similarily aged Torsdag traits at the 0.05 level.

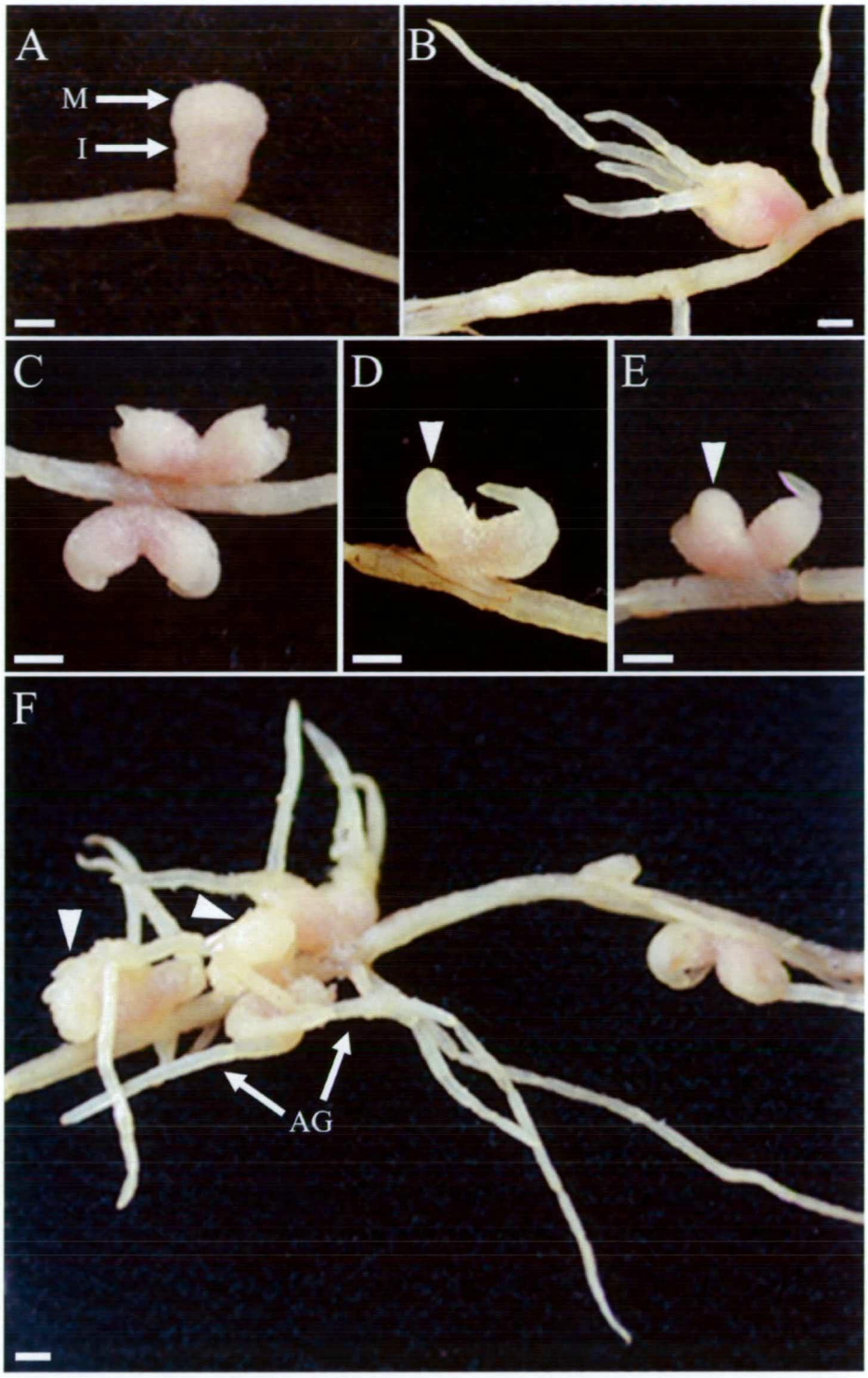

Figure 1. Lateral root nodules of 25 day-old A) wild type and B-F) *coch* plants inoculated with *R. leguminosarum*. White meristems and pink central infected zones were apparent in the nodules of both genotypes. The nodules of *coch* dichotomously branched and typically exhibited callus-like structures and agravitropic roots emerging from their meristems. M=meristem, I=infected zone, AG=agravitropic roots, arrowheads=callus. Bars = 1 mm.

Table II. Root numbers and lengths of 17 day-old Torsdag and *cochleata*
Indicated are the number of secondary lateral roots per plant in addition to the average number of tertiary lateral roots located on the six uppermost secondary lateral roots per plant. Also shown are the lengths of the shoot and the longest secondary and tertiary lateral roots per plant.

Genotype	Number		Length (cm)		
	Secondary Roots	Tertiary Roots	Shoot	Secondary Root	Tertiary Root
Torsdag	89 ± 3.6	20 ± 1.1	12.5 ± 0.3	19.7 ± 0.1	4.3 ± 0.2
coch	78 ± 2.7*	26 ± 0.8*	12.1 ± 0.8	20.2 ± 0.6	4.3 ± 0.1

Results are means ± SE (n = 6). Values for each mutant trait followed by an * are significantly different from that of Torsdag at the 0.05 level.

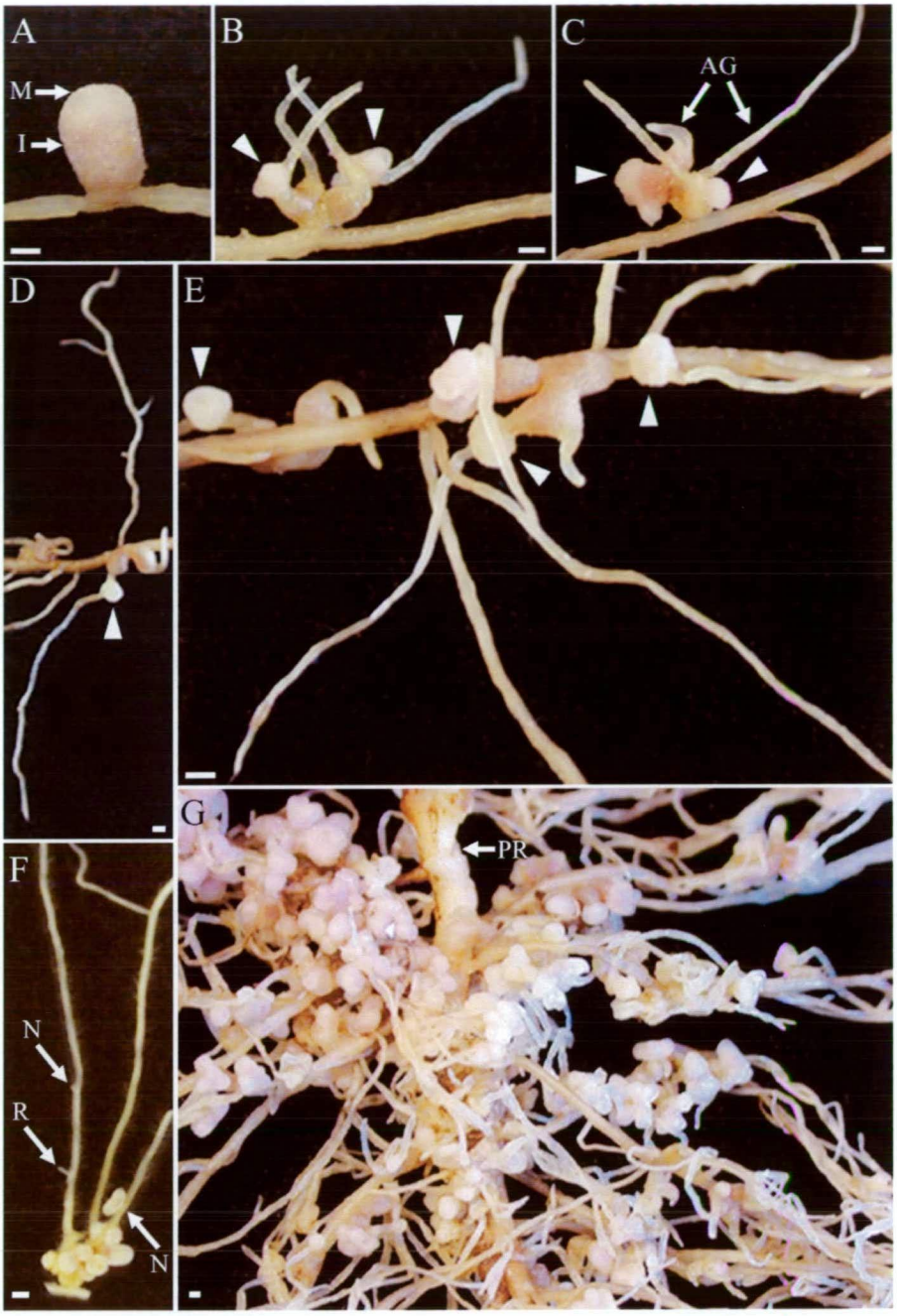

Figure 2. Nodules of 40 day-old A) wild type and B-G) *coch* plants inoculated with *R. leguminosarum*. As observed at 25 days (Fig. 1), the nodules of both lines possessed white meristems and pink infected zones, and those of *coch* typically branched and possessed root and callus-like structures. These structures were more numerous and markedly larger, compared with those observed at 25 days. D, E) The roots of these hybrids also exhibited callus-like structures, which tended to form in close proximity to the attachment site of the hybrid root on the nodule. F) Hybrid roots also occasionally gave rise to their own root and nodule structures. G) The complex root-nodule hybrids often engulfed the roots from which they arose. M=meristem, I=infected zone, AG=agravitropic roots, R=emerging root, N=emerging nodule, arrowheads=callus, PR=primary root. Bars = 1 mm.

even gave rise to new root and nodule structures (Fig. 2F). The nodule portion of the hybrids also continued to grow, often further branching and continuing to develop new root and callus-like structures. These findings illustrate that both the roots and nodules of the hybrids have their own, distinct meristems, allowing them to elongate at their own rates. Taken together, extremely complex hybrid structures consisting of a multitude of roots, nodules and calli, developed from a single initiation point (i.e. infection site) on the lateral roots of *coch*. In many cases, these hybrid structures grew until they engulfed the root from which they arose (Fig. 2G).

The nodule number of 40 day-old *coch* and Torsdag root systems remained similar to their respective values observed at 25 days (Table I), indicating that the autoregulation of nodulation was functioning properly in both genotypes. In addition, the mutant root system DW remained similar to that of Torsdag (Table I). However, due to the additional root and nodule structures, the total and average nodule DWs of *coch* were significantly greater than those of Torsdag at 40 days (Table I).

Effects of coch on Nodule Histology

Torsdag nodules were histologically similar to those previously described for wild type pea (e.g. Newcomb et al. 1979). Those of 25 day-old plants possessed an outer cortex, a peripheral vasculature connected to the central vasculature of the lateral root and three distinct histological zones: the meristematic, invasion, and infected zones (Fig. 3A). The majority of cells in the infected zone appeared to have been invaded by the bacteria and contained nitrogen-fixing bacteroids.

The nodules of 25 day-old *coch* plants also exhibited an outer cortex, and, although bifuricated, each lobe typically displayed the three histological zones observed in Torsdag (Fig. 3B-F). Meristematic tissue was not observed in the invaginated region between the nodule lobes of *coch*, although, the cells of the entire infected zone did appear to contain bacteroids. At least one vascular strand was observed in each nodule lobe of *coch*. Generally, the vasculature appeared thicker than that observed in Torsdag nodules, often branching and veering over the various

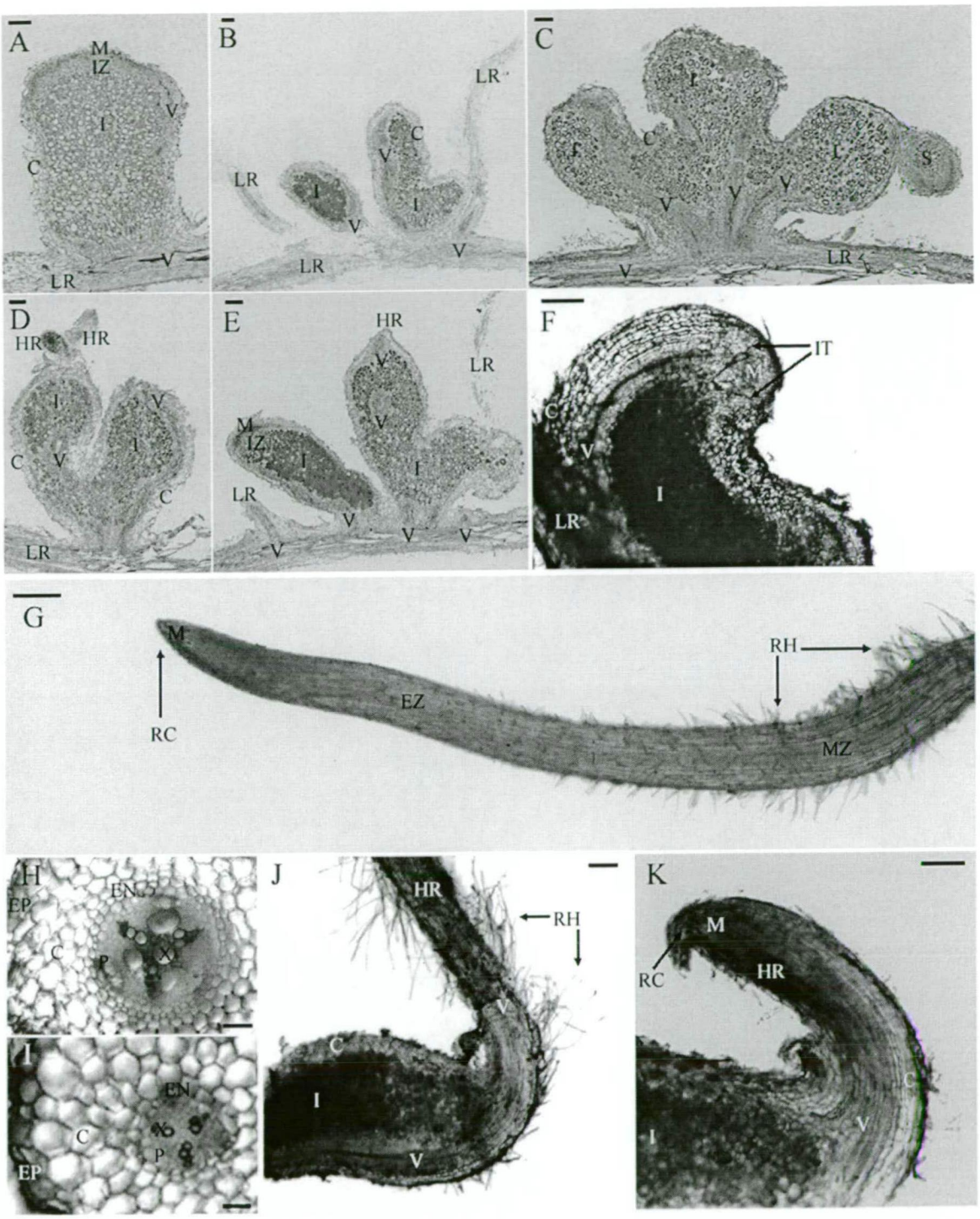
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Figure 3. Light micrographs of 25 day-old A) wild type and B-K) *coch* nodules. A-E) The meristems, invasion zones, and infected zones (which include cells harbouring the bacteria) are clearly distinguishable in the nodules of both lines. B-F) Meristematic tissue was not observed in the invaginated regions occurring between the mutant nodule lobes. The nodule vasculature of both lines is connected to that of the lateral root, however, the A) wild type vasculature is thin and peripheral, whereas that of B-D) *coch* is markedly thicker and frequently oriented away from the nodule periphery. B,E) Serial sections of the same nodule depicting thick vasculature extending from the lateral root through to a hybrid root emerging from the nodule meristem. C) A swelling developing from the meristem of a *coch* nodule lobe. D) Hybrid roots emerging from the meristems of nodule lobes. F-K) Hand sections of *coch* nodules and roots. F) Cross section of a *coch* nodule with vasculature extending towards the infection threads. G) Hybrid root exhibiting a root cap, meristem, root hairs and zones of elongation and maturation. Cross section of *coch* H) lateral and I) hybrid roots illustrating the endodermis, cortex, epidermis and similar triarch distribution patterns of xylem and phloem. The reduced size of the hybrid root is probably due to it being younger than the lateral root. J,K) Cross sections demonstrating the vascular connections existing between the nodule and hybrid roots of *coch*. M=meristem, IZ=invasion zone, I=infected Zone, V=vasculature, LR=lateral root, S=swelling, HR=hybrid root, IT=infection threads, RC=root cap, EZ=elongation zone, MZ=maturation zone, RH=root hairs, X=xylem, P=phloem, EN=endodermis, EP=epidermis, C=cortex. Bars = 200 μm (A-F,J,K), 25 μm (H,I).

histological zones. Collectively, the normal infected zone histology (Fig. 3) and the pink hue (Fig. 1,2) of the nodule, in addition to the healthy green shoots produced in the absence of applied nitrogen (data not shown), suggest that the process of nitrogen fixation is probably functional in *coch*.

The roots of *coch* hybrid structures also appeared functional. They consisted of a root cap, meristem, cortex, central vasculature and root hairs, similar to that of a lateral root (Fig. 3H-K). The vasculature was incorporated into the hybrid root from a vasculature strand of the nodule (Fig. 3J,K). The xylem and phloem of the hybrid roots was arranged in a triarch pattern of distribution, typical of a lateral root (Fig. 3H,I).

DISCUSSION

The homeotic pea mutant, *coch*, displays a highly unique nodulation phenotype. Typically, the nodules branch and develop roots, thus creating root-nodule hybrids. The roots of these hybrids emerge from the meristems of the nodule. They possess a central vasculature that is incorporated into the root from a peripheral vascular bundle of the nodule. Both components of the hybrid have their own meristems and are therefore able to elongate at distinct rates. Hence, the hybrid roots are slender and elongate well past the shorter, thicker, nodule. The nodule itself often becomes highly branched and forms a multitude of root, nodule and callus-like structures.

The fact that *coch* formed a similar number of nodules to that of its wild type, Torsdag, indicates that the *coch* mutation does not prevent bacterial recognition or infection, nor does it prevent the autoregulation of nodule formation. In addition, *coch* nodules appeared functional and its root system DW and lateral root lengths were similar to those of Torsdag, demonstrating that these parameters are also not affected by the mutation. Furthermore, *coch* does not affect the development of the shoot, other than the stipule and flower (Wellensiek 1959; Yaxley et al. 2001b), suggesting that the role of the *COCH* gene product in development is organ specific.

Alternatively, there may be redundancy of *COCH* in organs of the mutant that display a wild type phenotype. The fact that the *coch* mutation results in ectopic roots developing from the nodules and alternative leaf components replacing the stipules suggests that COCH may normally function to inhibit the development of these structures.

Root structures emerging from branching nodules have also been reported on *Sesbania grandifolia* Pior. (Harris et al. 1949). Moreover, root-nodule hybrids have been observed on *M. sativa* (Dudley et al. 1987), *T. repens* (McIver et al. 1997) and *Phaseolus vulgaris* (VandenBosch et al. 1985; Ferraioli et al. 2004) following inoculation with specific *Rhizobium* strains. However, these hybrids differed morphologically from those of *coch*, as the nodule zonation pattern, and multiple root, nodule and callus structures characteristic of *coch* hybrids, were not observed. It has also been reported that increasing the temperature can convert the nodule apex of *Medicago sativa* and various *Trifolium* sp. into roots and calli structures (Dart 1977; Day and Dart 1975), possibly suggesting that high temperatures interfere with the activity of the *COCH* gene product.

Nodules and roots share many aspects of their development, consistent with the theory that nodulation may have evolved from pre-existing mechanisms of early lateral root development (Hirsch and LaRue 1997; Mathesius et al. 2000; Mathesius 2003; Chapter 2; Ferguson et al. 2005a). The root-nodule hybrids of *coch* further support this theory, as do the nodule-like structures of many non-legumes, which are clearly derived from modified lateral roots (Hirsch and LaRue 1997). Although these structures are distinctly different from legume nodules, some aspects resemble those of *coch*, such as the agravitropic roots that emerge from the actinorhizal nodule meristems of *Casuarina cunninghamiana* (Torrey 1976) and *Myrica gale* (Torrey and Callaham 1978). In addition, mycorhizal nodules of Podocarpaceae species, which form even in the absence of the fungus, appear to be novel outgrowths that have diverged from the root developmental pathway (Russell et al. 2002).

Although the stipules of *coch* are replaced by alternative leaf structures, the nodules are not actually replaced by roots, but rather form concomitantly with them. This phenotype is analogous to that of *ufo/fim* homeotic mutants of *Arabidopsis* and

Antirrhinum, which mediate floral meristem and organ identity (Ingram et al. 1995). Yaxley et al. (2001b) hypothesized that there may be homology existing between certain meristems of pea because the primordia base of the petals and leaves is altered in *coch*. The meristems of *coch* nodules are also altered, giving rise to root and callus structures, possibly indicating common developmental abnormalities among these meristems. Yaxley et al. (2001b) proposed that *coch* stipule meristems might remain meristematic for a prolonged period of time, leading to greater meristematic flexibility and retarded stipule development. If this is true for *coch* nodule meristems, it may explain why they appear like swollen calli and why both nodule and root structures form.

The phytohormone auxin has been reported to induce the formation of meristems on the roots of rice (Ridge et al. 1993). These meristems consisted of nodule-like, modified root outgrowths that displayed a callus-like surface, but a differentiated internal anatomy, as observed in *coch* nodules. Auxin also regulates root gravitropism (Marchant et al. 1999), which is dysfunctional in *coch* and actinorhizal nodule-roots. Thus, altered levels or perception of auxin may have a role in the development of the root-nodule hybrids of *coch*. Temporally and spatially determining the auxin content of *coch* root-nodule hybrids would advance the understanding of this hormone in the development of these organs.

CONCLUSIONS

Unlike other meristematic processes, the initiation and location of nodule development can be tightly controlled in the laboratory, making nodulation an excellent process to study meristems. The *coch* mutant represents an exciting tool for such studies, particularly since its mutation appears to affect certain organs, but not others. In addition, *coch* could provide insight into nodule branching and could help delineate between which aspects of nodule development are shared with, and which are unique to, lateral root formation. The fact that *coch* nodules, stipules and flowers are abnormal, but the remainder of the root and shoot systems are not, suggests that

COCH may have been recruited into the process of nodule development from the flower and/or stipule developmental program(s). Of utmost importance will be to identify the initial cells from which the hybrid roots develop and to clone the *COCH* gene. Once the gene is available, the roles of COCH in organ identity, including its affects on organ initiation, hormone manipulation and development, can be ascertained.

MATERIALS AND METHODS

Plant Growing Conditions

The *coch* allele (Hobart line AF99) was produced by EMS mutagenesis by Dr. J Weller from its wild type, Torsdag (Hobart line 107) (Yaxley et al. 2001b). Additional, independently derived *coch* mutants, JI 2757 and JI 2165, were investigated to ensure that the nodule phenotype observed is due to the *coch* mutation. Plants were grown as outlined in Chapter 2 (Ferguson et al. 2005a). For nodulation studies, seeds were sown in 100 mm "Space Saver" pots (Reko, Australia) and for root analysis experiments, seeds were sown in 200 mm "Plastamatic" pots (Melbourne, Australia). At the time of sowing, each pot was provided with either 25 ml (nodulation studies) or 150 ml (root characterization experiments) of *Rhizobium leguminosarum* bv. *viciae* 128C53K (Nitragin® Inoculants, Liphatech Inc., Milwaukee, WI) grown in yeast-mannitol broth and diluted with water to approximately OD_{600} 0.01, which represents 5 x 10^6 cells ml^{-1}.

Nodule Count Studies

For nodule studies, plants were harvested 25 and 40 days after planting. Analysis at 25 days allowed for the development of the nodules to a stage where they could be clearly distinguished and accurately assessed. Delays in nodule development can be identified at 40 days, when the plants initiate flowers and the

formation of new nodule structures should be minimal due to the autoregulation of nodulation (e.g. Searle et al. 2002).

Upon harvesting, the roots and shoots were separated at the cotyledon, which was excised and discarded. The root system was rinsed and placed in water. The nodules were then characterized, counted and removed with forceps. The complex nodules of *coch* (which were comprised of multiple lobes, roots and calli) were counted as one nodule because they arose from a single initiation point (i.e. infection site) on the lateral root. The roots, shoots and nodules of each plant were then placed in a 60°C oven for a minimum of three days to obtain their dry weights (DWs).

Analysis of Root Characteristics

For root studies, plants were harvested 17 day after planting, allowing for the development of secondary and tertiary lateral roots. The plants were uprooted, rinsed, and placed in water. The length of the shoot and longest secondary and tertiary lateral root was measured. In addition, the total number of secondary lateral roots, and the number of tertiary lateral roots located on each of the upper (i.e. closest to the crown) six secondary lateral roots were recorded.

Histological Analysis

For histological examinations, portions of lateral roots bearing nodules were excised from 25 day-old plants. The specimens were fixed in 3.7 % (v/v) formaldehyde for 3 h, dehydrated in a graduated ethanol series for 5 h, followed by xylene treatment for 2 h, and embedded in Paraffin (Paraplast®, melting point 56°C; VWR Scientific, West Chester, PA, U.S.A.) using a vacuum infiltration processor (Tissue Tek® VIP™, Sakura Finetek, Japan). Longitudinal serial sections 3 µm thick were cut using a Leitz 1512 microtome (Ernst Leitz Westlar GmBH, Austria) and transferred to slides. Paraffin was removed by soaking the slides in xylene. The slides were then rinsed in ethanol, followed by water. All microtome and hand sections were stained with Toluidine blue, observed under an Axioskop 2 Plus

microscope (Carl Zeiss, Gottingen, Germany) using differential interference contrast illumination and photographed with an AxioCam HRc digital camera (Carl Zeiss, Gottingen, Germany).

Statistical Analysis

All statistics were determined using Student's *t*-tests.

OVERALL CONCLUSIONS

This thesis describes the nodulation phenotypes of previously characterized mutants of pea. Specifically, GA and BR mutants were assessed, in addition to the homeotic mutant, *cochleata*. Mutants have been useful in identifying genes involved in developmental processes such as nodulation. Previously, plants exhibiting impaired, or unusual, nodulation phenotypes were selected from mutagenesis experiments, following which an array of experiments were performed to identify the mutated gene, and the role of the gene product, in nodulation. This approach has been successful, yielding a number of genes involved in nodulation (e.g. see references in Oldroyd and Downie 2004).

However, the work reported here adopted a novel approach to using mutants to investigate nodulation. Well-characterized mutants were investigated, whose nodulation phenotypes had not been examined, but in many cases, whose genes and gene products were formerly identified. By investigating the nodulation phenotypes of well characterized mutants, it was anticipated that the effects, if any, of the mutation on nodulation would be revealed.

Investigating GA and BR mutants revealed that these hormones are required for nodule development. Mutants deficient in either of these hormones exhibited a reduced capacity to form nodules. The application of GAs, or the grafting of a wild type root or shoot, to a GA-deficient mutant was sufficient to fully restore the nodule number of the mutant. These findings indicated that there may be a direct requirement for GAs in nodule development. In contrast, grafting studies using a BR-deficient mutant indicated that BRs are indirectly affecting nodule numbers via a mechanism that operates in the shoot. A shoot mechanism involved in the autoregulation of nodule numbers has previously been identified, but the impact of BRs on this mechanism remains to be addressed. Further studies are required to identify precisely how BRs are influencing the nodulation process.

Additional studies using the severely GA-deficient mutant, *na*, indicated that ethylene might also have a role in some of the phenotypes of the mutant. Following treatment with an inhibitor of ethylene biosynthesis, the nodule number of the mutant

significantly increased. However, the nodules that formed were still aberrant, indicating that although ethylene may have a role in the initiation of nodules, GAs are likely required for their proper growth and development. Plants possessing mutations resulting in both GA-deficiency and hypernodulation also supported a role for GAs in late nodule development. Numerous nodules formed on these lines, indicating that GA-deficiency does not prevent bacterial recognition or infection, nor does it prevent early nodule development. However, these nodules were also aberrant in appearance, indicating that, in the absence of GAs, these nodules were unable to form properly in their late stages of development. The histology of nodules on *na* plants also supports a role of GAs in late nodule development. The meristem of these structures deteriorated and the vasculature and infected zone of the nodule failed to establish. These events should have occurred at the time when the nodule is emerging from the lateral root late in nodule development. The use of molecular markers and SEM will help identify where and when GAs are required in nodulation, and what impact this hormone has on the release and functioning of the bacteria in the infected zone of the nodule.

Mutants exhibiting constitutive GA signalling also provided exciting new insight into the roles of GAs in nodulation. These mutants formed nodules that were normal in appearance, even in lines that also possessed the *na* mutation. This suggests that the perceived GA level, rather than the actual level of GAs, may be most important for nodule formation. Further studies, using a variety of grafting techniques and the application of GAs and their inhibitors, are required to fully understand this process.

Upon examining the nodule and root phenotypes of the various mutants reported here, it became apparent that a correlation existed between the number of nodules and roots that developed per plant. This finding suggests that nodules and roots share aspects of their early developmental pathways, which is consistent with the theory that nodulation may have evolved from the root developmental process. Mutants will be useful for delineating which aspects of nodulation are shared with, and which are unique to, root development. In addition, identifying molecular mechanisms involved in nodule and root development will greatly advance the

understanding of the formation of these structures and allow for the optimization of these processes.

Further indicating that nodules and roots share aspects of their early developmental pathways was the nodule morphology of *coch*. The nodules of this mutant typically branched and exhibited callus and ectopic root structures emerging from their meristems. Histological examinations indicated that both the nodule and root portions of these hybrid structures appeared functional, with a vascular strand of the nodule being incorporated into the central region of the root. The *coch* mutation also affects the development of stipules and flowers, suggesting that *coch* may have a role in organ identity. Identifying the initial cells of the nodule that give rise to these roots, in addition to molecularly characterizing the *COCH* gene, and gene product, will also help advance nodule and meristem research.

In summary, using previously characterized mutants for the purpose of investigating the nodulation process proved to be successful. The work reported here has provided insight into the poorly understood roles of GAs and BRs in nodule development and has revealed putative overlaps in nodule and root development. However, a great deal more remains to be determined in order to establish the direct versus indirect effects of the mutations on nodulation, including identifying interactions that occur at the molecular level. Based on this, the present work can be regarded as the essential groundwork for a number of future studies in the field of signalling interactions in nodule development.

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Signaling Interactions During Nodule Development

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Abstract

Nitrogen fixing bacteria, collectively referred to as rhizobia, are able to trigger the organogenesis of a new organ on legumes, the nodule. The morphogenetic trigger is a *Rhizobium*-produced lipochitin-oligosaccharide called the Nod factor, which is necessary, and in some legumes sufficient, for triggering nodule development in the absence of the bacterium. Because plant development is substantially influenced by plant hormones, it has been hypothesized that plant hormones (mainly the classical hormones abscisic acid, auxin, cytokinins, ethylene and gibberellic acid) regulate nodule development. In recent years, evidence has shown that Nod factors might act in legumes by changing the internal plant hormone balance, thereby orchestrating the nodule developmental program. In addition, many nonclassical hormonal signals have been found to play a role in nodule development, some of them similar to signals involved in animal development. These compounds include peptide hormones, nitric oxide, reactive oxygen species, jasmonic acid, salicylic acid, uridine, flavonoids and Nod factors themselves. Environmental factors, in particular nitrate, also influence nodule development by affecting the plant hormone status. This review summarizes recent findings on the involvement of classical and nonclassical signals during nodule development with the aim of illustrating the multiple interactions existing between these compounds that have made this area so complicated to analyze.

Key words: Cell division; Defence response; Meristem; Nod factors; Nodulation; Organogenesis; Peptide signals; Plant hormones; Receptor kinase; Systemic acquired resistance

Introduction

Bacteria of the genus *Rhizobium* are capable of infecting the roots of host plants, resulting in the development of novel organs called nodules. Nodule development involves the induction of cortical and pericycle cell divisions and their subsequent differentiation into a vascularized organ with a meristem. Concurrently, infection by the bacteria into root hairs and cortical cells in a so-called infection thread occurs until their eventual release into the developing nodule. Within the nodule, the invading bacteria differentiate into nitrogen-fixing bacteroids that provide reduced nitrogen to the plant in exchange for carbohydrates and shelter (for recent reviews see Crespi and Galves 2000; Stougaard 2001; Kistner and Parniske 2002).

Precise interactions between phytohormones and various other signalling compounds are imperative for plant organogenesis, and in no case is this more

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apparent than in the process of nodulation. In this symbiosis, various signalling molecules are exchanged between the plant and the infecting bacteria to regulate nodule initiation, differentiation and functioning, as well as the number of nodules that develop. Nodule numbers are limited by at least two pathways. One pathway is a local regulation of infection in the root zone susceptible for infection (Vasse and others 1993), while the second pathway is a negative feedback process termed "autoregulation" during which existing nodule meristems trigger a signal in the shoot that inhibits further nodule development on the root system (Delves and others 1986). For this to occur, the timing and concentrations of hormones and other signalling compounds is crucial, as alterations to either can result in the abortion of nodulation. The following review culminates much of what is known about the various signalling elements involved in nodulation and attempts to identify possible links between them. Due to the size of the topic, we have concentrated on the signals involved in nodule organogenesis and have had to ignore many of the early signals, for example calcium, known to act in the root hair following Nod factor perception. However, a recent review by Lhuissier and others (2001) covers this topic.

Signalling Interactions of the Classic Hormones

Earlier work on nodulation investigated hormones individually in an attempt to elucidate a role for each. For example, Thimann (1936) was one of the first to propose involvement of hormones in nodule formation and implicated auxin in the process. Later, the finding that many soil bacteria, including rhizobia, synthesize plant hormones (reviewed by Costacurta and Vanderleyden 1995), initially seemed to suggest that rhizobia could provide the hormones that subsequently stimulate nodule formation (for example, Phillips and Torrey 1972), although, this did not explain the specificity between legumes and their specific symbionts. Since then, nodule initiation has been shown to occur spontaneously in some legumes (Truchet and others 1989) and can be triggered by altering the hormone balance, thus illustrating that the hormones act independently of the bacteria. In addition, the application of Nod factors can induce pseudonodule structures on certain hosts (Truchet and others 1991), possibly by altering hormone levels within the host tissue. However, because Nod factor-induced nodule primordia typically fail to develop into differentiated nodules, it is possible that hormones or other signals produced by the bacteria during the infection process are also required.

During root nodule development, rhizobia stimulate differentiated cortex cells to re-enter the cell cycle, divide and differentiate. In 1973, Libbenga and others recognized the need to assess hormone interactions during nodule development and suggested that gradients of both auxin and cytokinins are required for cortex proliferation and thus nodule initiation. Since the work of Libbenga and others (1973), much has been discovered about the complex signalling network required for nodule organogenesis. A central question in nodulation research is how changes in the hormone balance can affect the location (radially and longitudinally along the root), initiation, number and functioning of nodules on the root system. The following section discusses many of these findings and identifies the current knowledge of hormone signalling interactions in nodulation (summarized in Figure 1).

Abscisic Acid

The role of abscisic acid (ABA) in nodulation is poorly understood. Initially, ABA was thought to act as an inhibitor of nodule development, as application of the hormone reduced the number of nodules in *Pisum sativum* (pea) (Phillips 1971). ABA application to wild type *Glycine max* (soybean) and its supernodulating mutant line NOD1-3 also caused a decrease in nodule numbers and dry weights in addition to isoflavonoid levels (Cho and Harper, 1993). Moreover, Bano and Harper (2002) determined that nodule initiation, development and functioning were all inhibited by ABA in wild type and NOD1-3. Phillips (1971) speculated that ABA might act by reducing the cytokinin-stimulated cortical cell divisions associated with nodule formation, thus suggesting a putative ABA-cytokinin signalling interaction.

ABA and cytokinins have been shown to act in concert to affect numerous aspects of plant development, including root/shoot signalling (Davies and Zhang 1991) and symbiotic photosynthetic gas exchange (Goicoechea and others 1997). Since the work of Phillips (1971), the ratio of the two hormones has been positively correlated with nodule suppression and autoregulation (Caba and others 2000; Bano and others 2002). The root ABA/zeatin riboside (ZR) ratio was found to be consistently higher in wild type soybean relative to the supernodulating mutant *nts382* (Caba and others 2000). Recently, Bano and others (2002) proposed a model

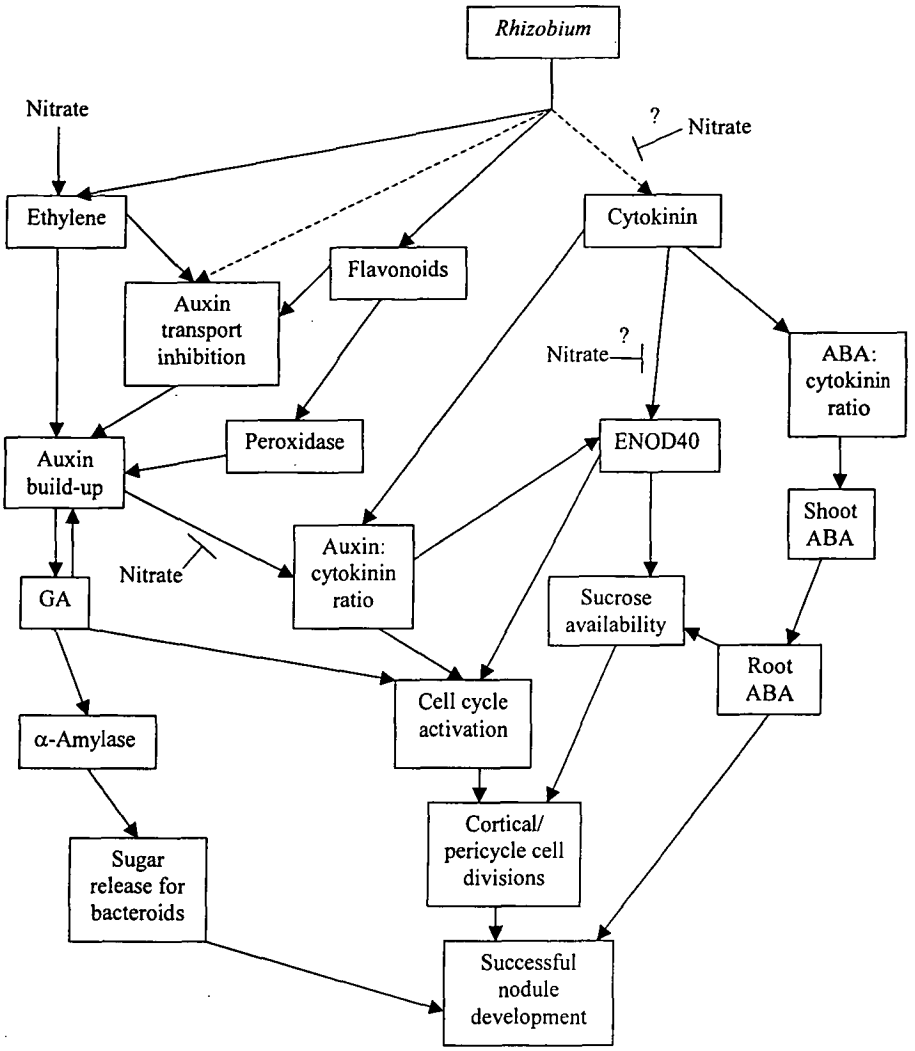

Figure 1. Proposed model for the interaction of hormones and other signals regulating the initiation of cell divisions and nodule development. See text for details. This figure summarizes interactions that have been analyzed separately and in different legume species. It should therefore not be seen as an accurate or complete overview for any particular legume. The flow diagram does not suggest a strict temporal but rather a functional overlap of interactions. Dashed arrows indicate that the interaction might be indirect and needs to be tested; see Conclusions and Outlook for details. The effect of nitrate on the signalling interactions is indicated in several places, but it will need to be tested as to whether some of the observed nitrate effects are indirect.

to explain possible influences of plant ABA/ZR ratios in nodule autoregulation. In this model, inoculation induces an initial decrease in the xylem ABA/ZR ratio. These authors speculated that the hormones of this ratio are then translocated to the leaves where they promote the synthesis of ABA. The increased ABA then moves via the phloem to the root where it inhibits further nodule formation, thus regulating the number of nodules that form. In supernodulating mutants, this pathway is effectively non-functional, as the initial decrease in the xylem ABA/ZR ratio does not occur and thus proper regulation of nodule number is not achieved (Bano and others 2002). Caba and others (2000) demonstrated that a final rise in root ABA concentration is absent in the mutant, consistent with the model.

In further support of this model, Gresshoff and others (1988) illustrated via extrapolation that the concentration of ABA increased in the shoot at the onset of autoregulation in the wild type, but not in *nts382*. In addition, Bano and Harper (2002) demonstrated that the application of partially-purified phloem ABA-extracts, from either wild type or the supernodulating soybean mutant NOD1-3, inhibited nodule formation in the mutant. However, they found that phloem-ABA levels were similar in both

lines and concluded that another signal may be present in the phloem that either inhibits nodule formation or counteracts the inhibitory effect of ABA in this autoregulatory process.

Further evidence supporting a negative role for ABA in nodule development was reported by Watts and others (1983) who analyzed the endogenous ABA content in nodules that form on the perennial *Alnus glutinosa* infected by the actinomycete *Frankia*. ABA levels were higher in nodules than in the surrounding root tissue, particularly in dormant compared with actively growing nodules. However, despite this finding, Watts and others (1983) were unable to determine any obvious correlations between nodule ABA content and growth rate.

The level of endogenous ABA is also reported to be higher in nodules of pea (Charbonneau and Newcomb 1985) and soybean (Williams and Sicardi De Mallorca 1982; Fedorova and others 1992) compared with that of the roots. Moreover, increased amounts of ABA are detected in shoots, roots and nodules of soybean plants bearing VA mycorrhiza associations when compared with nodulated nonmycorrhizal plants, suggesting that these fungal associations contribute to the ABA pool of the host, including the nodule (Murakami-Mizukami and others 1991). Because ABA had previously been shown to activate a carbohydrate sink during the seed fill phase of soybean, Murakami-Mizukami and others (1991) speculated that increased nodule ABA may act as a signal to induce a similar carbohydrate sink in the nodule. Thus, as opposed to acting as an inhibitory factor, ABA could play a role in allocating photosynthates to the nodule to be used as a source of energy for growth and development, rhizobial respiration and nitrogen fixation. Rhizobia synthesize ABA in culture when supplied with ABA-precursors (Dangar and Basu 1991) so perhaps this production is a mechanism used by the bacteria as a means of obtaining plant-derived carbohydrates. In the case of nitrogen fixation, however, nitrogenase activity has been shown to decrease with increasing endogenous ABA levels in some species (Dangar and Basu 1984, 1987). As well, the daily application of ABA significantly reduced the level of nitrogen fixation in pea (González and others 2001a), although this treatment may have exceeded an appropriate ABA concentration for optimum nodule functioning. This reduction in nitrogen fixation paralleled a decline in nodule leghemoglobin content, which the authors speculated resulted in a restriction of available oxygen required by the bacteroids for cellular respiration, thus inducing the decline in nitrogen fixation (González and others 2001b).

In *Phaseolus vulgaris*, ABA application increased the accumulation of lipoxygenase (LOX, Figure 2) mRNAS, which are enzymes associated with stress and development (Porta and others 1999). These authors detected LOX in developing, but not mature, nodules suggesting a role for LOX in nodule growth. Moreover, *in situ* hybridization revealed no exclusive LOX expression in the invasion zone of pea nodules; however, all LOX transcripts were expressed at the nodule apex (Wisniewski and others 1999), thus further suggesting a role for the enzymes in nodule growth and development rather than a more direct role in the plant-microbe interaction or in host defense. Also in pea, Charbonneau and Newcomb (1985) noted an increased amount of ABA in the apical region of the nodule, possibly indicating a link between elevated levels of nodule ABA and LOX (Figure 3). If indeed LOX is required for nodule development and ABA is required to upregulate the level of nodule LOX, it can therefore be argued that ABA is actually required for nodule growth. Furthermore, a role for LOX has been implicated in nitrogen storage and assimilate partitioning (Stephenson and others 1998), which, if coupled with ABA, supports the hypothesis of Murakami-Mizukami and others (1991) that ABA could have a role in inducing a carbohydrate sink in the nodule.

Additional evidence supporting a requirement of ABA in nodule development is the significantly reduced number of nodules that form on the ABA-deficient *wilty* mutant of pea (BJ Ferguson, JB Reid and JJ Ross unpublished). If the role of ABA in nodulation were of a strictly inhibitory nature, it would be expected that *wilty* would develop more nodules than its wild type. These findings, however, do not necessarily discredit the previously mentioned work regarding an inhibitory role for ABA in nodulation and it is possible that ABA has a dual role in nodule development: one in negatively regulating nodule numbers and one in positively regulating the growth and development of individual nodules. As such, an increase in ABA (for example, one brought about by exogenous application or stress) would directly inhibit nodule development, whereas a deficit of the hormone (as in *wilty*) would fail to induce signalling elements (such as LOX) required to meet the growth requirements of the nodule, thereby also inhibiting nodule formation. This hypothesis may explain why some reports of ABA application (for example, Bano and Hillman 1986) illustrate no effects of the hormone on nodule numbers.

In support of this hypothesis, Charbonneau and Newcomb (1985) reported that pea nodule ABA

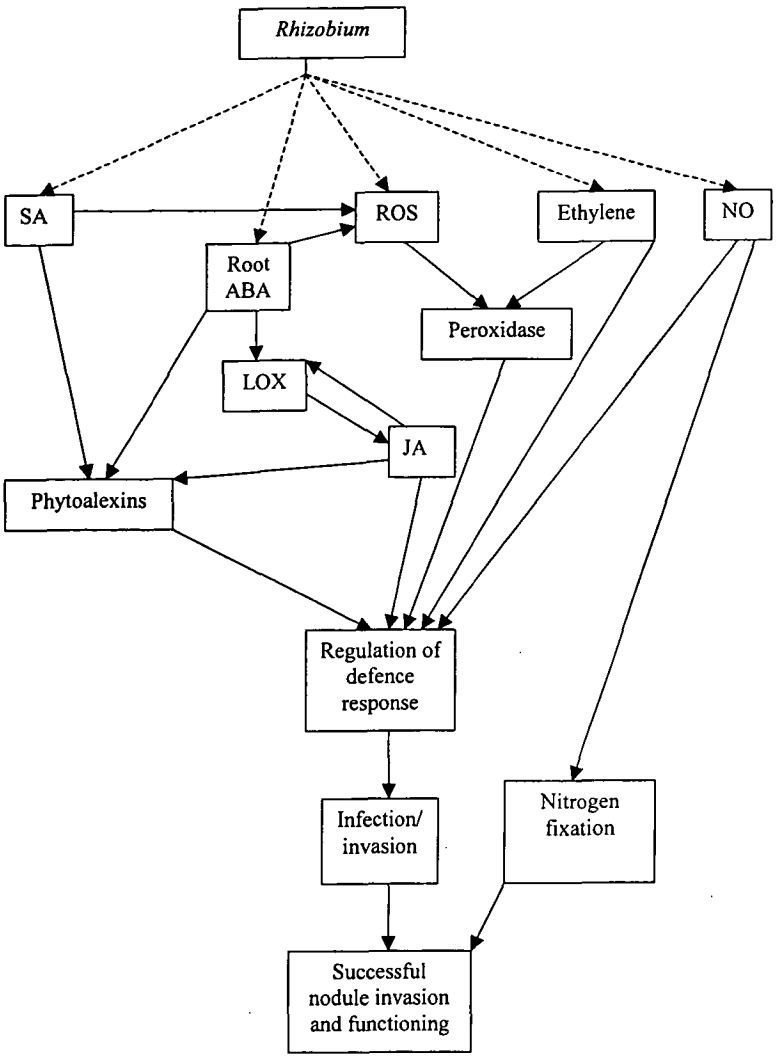

Figure 2. Proposed model for the interaction of signals regulating defense responses and nodule functioning. As in Figure 1, interactions that have been analyzed separately and in different legume species are integrated in one diagram and should not be seen as an accurate or complete overview for any particular legume. The flow diagram does not suggest a strict temporal but rather a functional overlap of interactions. Dashed arrows indicate that it is unknown whether *Rhizobium* independently activates these responses via different signals (for example, Nod factors, exopolysaccharides, and so on) or whether *Rhizobium* induces one initial response that triggers further secondary events. This could be tested in mutants for ABA, SA, ethylene or NO.

levels were high in the first 2 weeks of nodule development followed by a 2-week plateau and then a secondary period of elevated ABA. It is possible that the first rise in ABA is related to the regulation of nodule growth and number, the plateau corresponds to the period of nitrogen fixation and the second rise is associated with the onset of nodule senescence. These results suggest a putative third role for ABA in nodulation in which ABA increases in older nodules as part of a senescence-signalling pathway. In addition to pea, older nodules of *Lens* sp. (Dangar and Basu 1984), *Phaseolus aureus* (Dangar and Basu 1987), *Samanea saman* (Chattopadhyay and Basu 1989) and soybean (Fedorova and others 1992) have elevated amounts of ABA when compared with younger nodules, which the authors of these studies also suggested was related to nodule senescence. The elevated level of ABA in soybean nodules led Fedorova and others (1992) to speculate that ABA played a role in both the suppression of the formation of new nodule structures and in nodule senescence, which is consistent with our hypothesis.

Auxin

Auxin is a plant hormone with multiple roles in cell division, differentiation and vascular bundle formation, three processes that also occur during nodule formation. Auxin is synthesized mainly in the shoot and is transported to the roots by an active transport process involving import into the cell by an auxin import protein (AUX1) and active auxin export by an export protein (PIN1 and PIN2/AGR/EIR1; reviewed by Muday and DeLong 2001). Additional control stems from negative regulators of auxin export by auxin transport inhibitors that bind to proteins interacting with the auxin exporter (Muday and DeLong 2001). Thus, the plant has

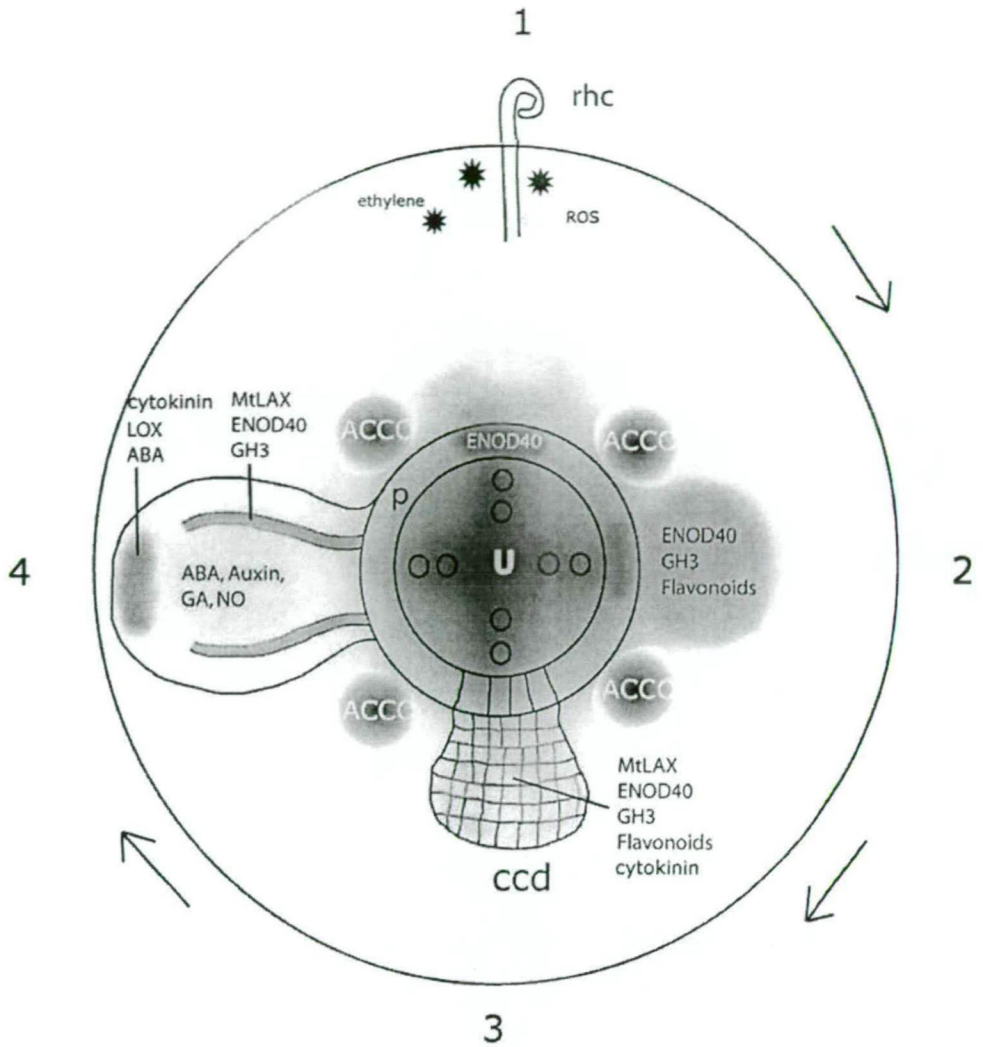

Figure 3. Spatial changes in hormone signals in relation to nodule development. The figure shows an idealized cross-section through the root at the site of nodule formation, including the xylem poles (small circles) inside the stele, which is surrounded by the pericycle cell layer (p). A gradient of uridine (U) exists that emanates from the xylem. ACC oxidase (ACCO) is expressed opposite the phloem poles and might create local ethylene gradients that regulate possible sites for nodule initiation. Four developmental stages are shown in clockwise sequence: (1) initial infection of rhizobia at the site of root hair curling (rhc) accompanied by the induction of ethylene and reactive oxygen species (ROS) as well as *ENOD40* induction in pericycle cells within hours of inoculation. (2) Precursor cells of the cortex, which will divide to become a nodule, show increased expression of *GH3*, *ENOD40* and accumulation of specific flavonoids. (3) Early cortical cell divisions (ccd) show enhanced *AUX1*, *GH3* and *ENOD40* expression as well as flavonoid and cytokinin accumulation. (4) In a differentiating nodule, increased levels of ABA, auxin, GA and nitric oxide have been detected. *AUX1*, *GH3* and *ENOD40* expression are located in peripheral (probably vascular) tissue. Cytokinin, ABA and LOX levels are increased in the nodule meristem.

several targets for regulating auxin homeostasis tightly to control organogenesis.

Compared with the roots, auxins levels have been reported to be elevated in the nodules of a variety of plant species (for example, pea (Badenoch-Jones and others 1984), *P. vulgaris* (Fedorova and others 2000) and *A. glutinosa* (Wheeler and others 1979)). Increased auxin levels in legume nodules, and in nodule-like structures of non-legumes, have also been observed after application of the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) (for example, Ridge and others 1992). Early experiments suggested that the ratio of auxins to cytokinins in the root was responsible for the initiation of cortical cell divisions and nodule formation (for example, Libbenga and others 1973). In the soybean hypernodulating mutant *nts386*, the auxin:cytokinin balance was found to be lowered

compared with the wild type, suggesting that the auxin:cytokinin ratio could be important for regulating nodule numbers (Caba and others 1998). These experiments suggested that rhizobia might manipulate auxin levels in the plant. In addition, sensitivity to auxin in *Medicago sativa* (alfalfa) lines correlates with the rate of spontaneous nodule formation, and nodulation efficiency can be increased by the introduction of *Agrobacterium rol* genes, which are known to affect auxin sensitivity and plant hormone levels (Kondorosi and others 1993).

A number of experiments suggest that rhizobia manipulate auxin transport thus changing the auxin:cytokinin ratio in the root. For example, direct measurements of auxin transport using labelled auxin showed that rhizobia inhibit acropetal auxin transport (from the root base to the tip) capacity in *Vicia sativa* (vetch) roots (Boot and others 1999). In addition, the expression of the auxin responsive promoter *GH3* fused to the *GUS* reporter gene was reduced towards the root tip between 12 and 24 h following rhizobia inoculation or ballistic microtargeting of Nod factors in *Trifolium repens* (white clover; Mathesius and others 1998a).

High *GH3-GUS* expression levels were then seen 24–48 h following inoculation (Mathesius and others 1998a) and in soybean, increased auxin levels were measured 48 h after inoculation (Caba and others 2000). These results are consistent with the auxin burst hypothesis of nodulation which states that subsequent to the initial induction of nodule primordia, shoot-derived auxin export into the root is stimulated, resulting in elevated auxin levels that inhibit further nodule primordia initiations, thus controlling nodule numbers (Gresshoff 1993). This auxin burst is assumed to be defective in supernodulation mutants, where increased auxin levels following inoculation could not be detected (Caba and others 2000). Altogether, it is likely that auxin plays (at least) a dual role during nodulation: in the early stages, auxin transport inhibition might result in a reduced auxin:cytokinin ratio to allow cell division to start, and later divisions are inhibited by super optimal auxin levels (Figure 1).

The application of synthetic polar auxin transport inhibitors (PATIs), which interfere with the hormone balance, can induce pseudo-nodule structures on the root and are also sufficient to induce some of the nodulin genes inside pseudo-nodules, including *ENOD2* and *ENOD12* (Hirsch and others 1989; Scheres and others 1992; Wu and others 1996). More recently, it has been shown that PATIs mimic the action on Nod factors on the repression of calmodulin expression in *P. vulgaris* (Camas and others 2002).

In addition to PATIs, the inhibition of auxin transport could be achieved by regulating the number of auxin efflux carriers in the cells transporting auxin. Alternatively, Nod factors or chitin oligosaccharides could affect the affinity of endogenous auxin transport regulators to their binding site, similar to the effect of ethylene (Suttle 1988), and/or Nod factors could induce the synthesis or release of an endogenous auxin transport inhibitor. Other plant compounds, including ethylene, cytokinins and flavonoids (for example, Brown and others 2001; Jacobs and Rubery 1988; Murphy and others 2000; Stenlid 1976), can also inhibit auxin transport and can regulate various peroxidases and IAA oxidases, the enzymes that break down auxin (Burgh and Burgh 1966; Lee 1971), thus leading to local shifts in the plant auxin:cytokinin ratio.

Peroxidase activity is elevated in *P. vulgaris* nodules, presumably to limit an auxin increase in maturing nodules (Fedorova and others 2000). A temporal and spatial correlation was found between the accumulation of specific flavonoids that inhibit auxin breakdown by a peroxidase and the accumulation of *GH3:GUS* expression in nodule primordia (Mathesius 2001). Furthermore, the accumulation of other flavonoids that stimulate auxin breakdown was detected in cells that exhibit low *GH3:GUS* activity, further suggesting that a local accumulation of specific flavonoids could regulate auxin levels.

The expression of flavonoid genes (for example, *PAL* (phenylalanine-ammonia lyase) and *CHS* (chalcone synthase)) is enhanced in nodules (for example, Estabrook and Sengupta-Gopalan 1991; Djordjevic and others, 1997), and rhizobia and Nod factors can induce flavonoid gene expression and localized flavonoid accumulation (for example, Djordjevic and others 1997; Lawson and others 1996; Mathesius and others 1998b; Schmidt and others 1994). Therefore, it has been suggested that Nod factors could have a role in inducing flavonoid accumulation at the infection site, followed by changes in the auxin balance (Hirsch 1992; Mathesius and others 1998a). By micro-targeting flavonoids into roots of white clover carrying the *GH3:GUS* construct, it was shown that flavonoids had similar effects on auxin distribution as Nod factors and synthetic auxin transport inhibitors. Although this suggests that flavonoids could mimic Nod factor action, it remains unclear if the exact flavonoids induced by rhizobia in the root would mediate this response in the concentration present in the tissue, and whether these flavonoids would be sufficiently mobile to reach their binding site.

There is also evidence that auxin distribution is regulated locally in nodule primordia and mature nodules, which would allow for spatial control of cell division in the root (Figure 3). Direct measurements of auxin (that is, indole acetic acid, IAA) contents in *P. vulgaris* roots and nodules showed increased IAA levels in roots preceding nodule formation and during the early stages of nodule emergence, whereas auxin levels dropped in mature nodules (Fedorova and others 2000). In white clover, expression patterns of *GH3:GUS* indicated that auxin levels and/or sensitivity are increased in early dividing cortical cells (Mathesius and others 1998a). *GH3:GUS* expression then decrease in the differentiating nodule primordium and remain only in developing vascular tissue, consistent with a role of auxin in triggering cell division and vascular bundle formation. Recent studies by de Billy and others (2001) have expanded this idea by showing that in *Medicago truncatula AUX1*-related genes (termed *MtLAX*) are induced in early nodule primordia and developing vascular tissue. These expression sites mirrored those of *GH3:GUS* in white clover (Figure 3), which suggests that auxin might increase in early nodule primordia by regulation of auxin import into these cells.

The role of auxin in nodulation is tightly linked to the development of other root structures, including lateral roots and root galls, which require similar induction of new cell divisions and differentiation as nodules. Auxin transport is required for lateral root induction (Bhalerao and others 2002) and auxin appears to accumulate not only in nodule but also lateral root primordia (Himanen and others 2002) and root galls caused by nematodes (Goverse and others 2000; Hutangura and others 1999). Expression levels of *GH3:GUS* were very similar in developing nodule and lateral root primordia (Mathesius and others 1998a). These similarities are likely due to auxin-induced activation of cell cycle genes that are required for the induction of new cell divisions during organogenesis (Doerner and others 1996; John and others 1993). A genetic link between regulation of root system architecture and nodulation has been found in the *Lotus japonicus* (lotus) *har1* (hypernodulation aberrant root formation) and the soybean *nts* mutants (Wopereis and others 2000; Searle and others 2003, respectively), which are both supernodulating mutants that show increases in the number of lateral roots in the uninoculated state and altered activities of the root apical meristem. Because auxin affects both lateral root, nodule and meristem formation, it is tempting to speculate, and pertinent to test, whether autoregulation exerts some of its effects via changes in auxin homeostasis, or whether additional, or different, signals are involved. The fact that lateral root frequency is not affected in the supernodulation mutant *astray* in *L. japonicus* suggests the existence of nodule specific regulators in addition to regulation of all root meristems (Nishimura and others 2002b).

Cytokinins

Cytokinins are a class of plant hormones having diverse roles in cell cycle regulation and differentiation. Re-activation of the cell cycle initiates nodule primordium formation (Foucher and Kondorosi 2000; Goormachtig and others 1997; Yang and others 1994) and cytokinins, together with auxin and ethylene, play a major role in cell cycle progression in plants (D'Agostino and Kieber 1999). Therefore, it is likely that cytokinins are also necessary for new cortical cell divisions initiated by *Rhizobium*. However, even though cytokinins have been reported to be synthesized by different bacteria, including rhizobia (Phillips and Torrey 1970, 1972), it is unlikely that cytokinins provided by rhizobia are the main factors necessary for nodule initiation, because purified Nod factors are sufficient to induce nodules in some legume species. Instead, it is more likely that Nod factors trigger changes in cytokinin synthesis, turnover or sensitivity in the roots during nodule initiation.

Either way, several pieces of evidence suggest that rhizobia do induce changes in the cytokinin balance of the root. Nodule cytokinin levels are reported to be elevated in numerous plant species when compared with the roots (for example, pea (Badenoch-Jones and others 1987), *Phaseolus mungo* (Jaiswal and others 1981), *Myrica gale* (Rodriguez-Barrueco and others 1979), and *Vicia faba* (Hensen and Wheeler 1976)). In pea, Newcomb and others (1976) showed that nodule cytokinin levels were highest in young, developing nodules and decrease with maturity. Syono and others (1976) demonstrated that the highest cytokinin levels in the pea nodule were located in the meristem (Figure 3). This agrees with the role of cytokinin in cell division and differentiation and supports the results of Newcomb and others (1976) as young nodules would be the most mitotically active and thus one would expect them to contain elevated levels of the hormone.

The application of cytokinins induces the formation of pseudo-nodule structures on legumes and non-legumes, including *Nicotiana tabacum* (tobacco) (Arora and others 1959), *A. glutinosa* (Rodriguez-Barrueco and Bermudez de Castro 1973), pea

(Libbenga and others 1973), *Macroptilium atropurpureum* (siratro) (Relic and others 1994) and alfalfa (Cooper and Long 1994; Bauer and others 1996). Cooper and Long (1994) transferred the *Agrobacterium* trans-zeatin secretion gene into *E. coli* and nodulation deficient mutants of *R. meliloti*, and showed that synthesis of the cytokinin zeatin of these bacteria is sufficient to induce nodule-like structures in alfalfa. However, it is important to note that the concentration of cytokinins is important in determining whether a stimulating or inhibiting effect on nodulation occurs (Lorteau and others 2001).

The roles of cytokinins during nodule development include, as expected, the activation of the cell cycle and genes associated with it (Jelenska and others 2000). For example, cytokinins induce the expression of *Msgbl*, which is expressed in dividing cells of alfalfa, including those of the nodule primordia, and may be involved in hormone-mediated cell division including having a putative signal transduction role during nodule organogenesis (McKhann and others 1997). Cytokinins may also be important for activating a number of early nodulin genes. For example, *ENOD2*, a gene expressed in nodules and nodule primordia, can be induced by cytokinins in *Sesbania rostrata* (Dehio and deBrujin 1992) and in alfalfa (Cooper and Long 1994; Bauer and others 1996). *ENOD12A*, coding for a hydroxyproline-rich glycoprotein that is expressed during nodule organogenesis, can also be induced by cytokinins in addition to Nod factor treatment (Bauer and others 1996). Another early nodulin gene that may have an important role in organ formation is *ENOD40* (see Signalling Peptides section below), which is also induced by both *Rhizobium* and cytokinins in alfalfa (Fang and Hirsch 1998; Mathesius and others 2000; Sinvany and others 2002). Screening of molecular markers in alfalfa identified seven nodulin genes regulated by cytokinins, four of which were also inducible by auxin, suggesting partial overlaps between auxin and cytokinin regulated pathways during nodulation (Jimenez-Zurdo and others 2000). Cytokinins have further been shown to affect ethylene levels in pea roots (Lorteau and others 2001). However, Lorteau and others (2001) were unable to demonstrate a direct correlation between cytokinin-induced ethylene and nodule inhibition, as inhibitors of ethylene synthesis did not restore nodulation in plants treated with high levels of cytokinin.

Cytokinins probably also play a role in setting up a carbohydrate sink for the developing nodule as they can induce starch formation in the root cortex, similar to that of *Rhizobium* infection (Bauer and others 1996). The use of a split root system in vetch has shown that cytokinin treatment of a root can also induce acidification of the growth medium around a separate root of the same plant (van Brussel and others 2002). These authors suggest that while cytokinins do not appear to be the autoregulation signal, they might create a sink in the inoculated root, which sends a signal to the shoot that regulates metabolism, including acid secretion, in the uninoculated roots. This cytokinin-induced root signal could play a role in autoregulation, in addition to the so far unidentified autoregulation signal from the shoot, which requires actively dividing cortex cells (van Brussel and others 2002).

Legume mutants such as R50 (pea) and MN1008 (alfalfa) also provide valuable tools for investigating the roles of cytokinins in nodulation. R50 develops abnormal infection threads that twist and bulge as opposed to properly progressing into the inner cortex (Lorteau and others 2001). Lorteau and others (2001) demonstrated that this characteristic could also be induced in wild type pea upon cytokinin application. Interestingly, nodulation is rescued in R50 by the application of inhibitors of ethylene biosynthesis or action. However, as stated above, the same ethylene inhibitors were unable to reverse the effects of cytokinin application on wild type pea.

The application of cytokinins to the *Rhizobium* and Nod factor resistant MN1008 overcomes the nodulation block in this mutant (Hirsch and others 1997), suggesting that this plant has low levels of the hormone or is unable to increase its cytokinin levels to meet the requirements for nodule initiation. PATIs were also reported to induce pseudonodules in this mutant (Hirsch and Fang 1994), suggesting again that the cytokinin:auxin ratio rather than cytokinins alone might be important for nodulation. The mutated gene in MN1008 was recently cloned and identified as a receptor kinase (Endre and others 2002).

Further evidence that cytokinins play a role in cell division and autoregulation comes from the receptor kinase mutant *har1* of *L. japonicus* (Krussel and others 2002; Nishimura and others 2002a). The *har1* mutant has a short root phenotype that can be mimicked in the wild type by application of cytokinin. However, in the presence of the ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG), cytokinin caused root elongation in the mutant in excess of untreated wild type levels, suggesting that *har1* has an altered response or sensitivity to cytokinin that is not mediated by ethylene (Wopereis and others 2000).

Ethylene

Ethylene is a gas with multiple roles in plant development and defense. Its role in nodulation has recently been reviewed by Guinel and Geil (2002) and Wang and others (2002). Ethylene might have a dual effect on nodulation in that it causes a local inhibition of nodule formation in most legumes but might be required at certain levels for proper infection by the bacteria. The application of ethylene, or ethylene-releasing compounds, is inhibitory to nodule organogenesis in numerous species including *P. vulgaris* (Grobbelaar and others 1971), pea (Drennon and Norton 1972; Lee and LaRue 1992c), white clover (Goodlass and Smith 1971), *Melilotus alba* (sweet clover) (Lee and LaRue 1992c), *M. truncatula* (Penmetsa and Cook 1997), *L. japonicus,* and siratro (Nukui and others 2000). Grobbelaar and others (1971) found that ethylene also reduced the level of nitrogen fixation in *P. vulgaris*. In pea, Lee and LaRue (1992c) determined that ethylene concentrations as low as 0.07 μL/L are able to inhibit nodule formation. It appears, however, that soybean is less sensitive to the hormone as nodulation of this species is not affected by applied ethylene (Lee and LaRue 1992c; Schmit and others 1999; Nukui and others 2000). This finding suggests that different species display different requirements and regulatory mechanisms for hormones, a point that must be considered for any hormone when investigating its roles in processes such as nodulation.

Inoculation of roots with rhizobia has been reported to induce increases in the local ethylene concentration in alfalfa (Ligero and others 1986), vetch (van Workum and others 1995), and soybean (Suganuma and others 1995), but this increase was not detected in pea (Lee and LaRue 1992b). These increases are likely due to an initial defense response elicited by the invading bacteria, which, interestingly, also synthesize the hormone (Billington and others 1979).

The application of inhibitors of ethylene synthesis (for example, AVG) or perception (for example, silver ions) increased the number of nodules that formed on pea (for example, Lee and LaRue 1992a), alfalfa (Peters and Crist-Estes 1989; Caba and others 1998), *L. japonicus* and siratro (Nukui and others 2000). These compounds also partially restored the nodulation phenotype of low nodulating mutants of pea including *sym5* (Fearn and LaRue 1991), *brz* (Guinel and LaRue 1992) and *sym21* (Markwei and LaRue 1997) and completely restored that of *sym16* (Guinel and Sloetjes 2000). Surprisingly, the nodulation phenotype of *sym17*, a pea mutant thought to overproduce the hormone, is not rescued with the application of ethylene inhibitors (Lee and LaRue 1992a). Interestingly, Yuhashi and others (2000) illustrated that *Bradyrhizobium elkani*-produced rhizobitoxine, which acts as an inhibitor of ethylene synthesis, also enhances the nodulation of siratro, possibly by helping the bacteria overcome ethylene's inhibitory effects on nodulation. Additionally, Roddam and others (2002) recently illustrated that the role of ethylene in nodulation can depend on the infecting *Rhizobium* cultivar as the application of AVG to *Trifolium subterraneum* (subterranean clover) enhanced the nodulation by some, but not all, strains of *R. leguminosarum*.

The mechanism of ethylene action as an inhibitor of nodulation is not known. One proposal is that ethylene induces plant chitinases, which subsequently destroy Nod factors and thereby limit the extent of nodule initiation (Mellor and Collinge 1995; Staehelin and others 1994).

Oldroyd and others (2001) postulated that a block in nodulation induced by ethylene could occur very early during the signal transduction cascade. Evidence for this came from the finding that the sensitivity of root hair cells to Nod factors is significantly increased in the *skl* mutant, and that modulation of ethylene synthesis in the wild type had comparable effects on the sensitivity of Nod factor perception. Ethylene appears to influence a component at, or upstream of, calcium spiking in the Nod factor signal transduction pathway leading Oldroyd and others (2001) to propose that, in addition to inhibiting the frequency of calcium spiking, the hormone determines the Nod factor concentration required for the root hair Ca^{2+} spiking response. These authors also illustrated that in *M. truncatula,* ethylene regulates the expression of the early nodulin genes *ENOD11* and *RIP1* and thus might effect events downstream of the early influence on calcium spiking.

Guinel and Geil (2002) proposed a model in which the rhizobia would not come into contact with ethylene in the root until after the epidermis, as this cell layer contains no ACC oxidase (the enzyme that catalyzes the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene) and does not appear to perceive the hormone. Consistent with this model is evidence that in pea, ethylene appears to block rhizobial entry into the root cortex, rather than the number of infection events (Lee and LaRue 1992c). This finding is supported by work with the *brz* mutant of pea, which has a third less infection events than its wild type. Although nodulation in *brz* is partially restored

by ethylene inhibitors, the number of infection events is only slightly increased (Guinel and LaRue 1992).

Contrary to these findings with pea, ethylene does appear to negatively regulate rhizobial colonization of *M. truncatula* as the application of AVG increased the number of infection events, whereas ACC decreased them (Oldroyd and others 2001). In addition, the ethylene insensitive *skl* mutant of *M. truncatula* has a significantly increased number of infection events compared with that of its wild type (Penmetsa and Cook 1997; Oldroyd and others 2001). The *skl* mutant is also unable to regulate the number of these events that develop into fully functional nodules and as such it hypernodulates (Penmetsa and Cook 1997). Although ethylene is unlikely to be involved in systemic autoregulation (Nishimura and others 2002c; Wopereis and others 2000), it is likely that ethylene plays a role in regulating infection events locally in the susceptible root zone, as demonstrated in the *skl* mutant.

Ethylene may positively influence infection thread development as the number of infection threads aborted in *skl* is very low (Penmetsa and Cook 1997; Oldroyd and others 2001). Guinel and Geil (2002) suggested that in pea, ethylene could affect the cytoskeleton, preinfection thread and infection thread formation. Using pea and vetch, Heidstra and others (1997) demonstrated that ethylene is also likely to be involved in determining the positioning of nodule primordium development around the stele (Figure 3). These authors showed that the expression of ACC oxidase is elevated in the inner cortical cells located in front of the root phloem poles. These locations are between the positions at which nodules preferentially arise opposite the root xylem poles. In addition, inoculation of vetch with *R. leguminosarum* induces ethylene-related responses, including a thick and short root phenotype and abnormal nodule positioning on the root system, which is restored following AVG application (Zaat and others 1989; van Spronsen and others 1995).

Interestingly, ethylene was also discovered to change the phenotype of nodules of *Sesbania rostrata*, a legume that grows in waterlogged soils and therefore likely to be exposed to varying levels of ethylene (Fernáandez-López and others 1998). The authors found that in the absence of ethylene (perception), nodules were of the indeterminate type, whereas in the presence of ethylene, determinate nodules with a terminal meristem were formed, suggesting a role for ethylene in meristem differentiation.

Gibberellins

Little is known about the signalling involvement of gibberellins (GAs) in nodulation. Early work focused on applying the hormone (generally GA_3) to the plant, which resulted in a decline in nodule formation (Thurber and others 1958; Galston 1959; Fletcher and others 1959; Mes 1959). In 1952, Nutman demonstrated that the removal of root tips and mature nodules from various red clover sp. promoted the formation of new nodules, presumably by removing the source of a compound inhibitory to nodulation. Based on the results of Nutman (1952), and evidence that nodules of pea and *P. vulgaris* contain elevated levels of GAs, Radley (1961) speculated that GAs regulate nodule formation. Since then, nodules of *Lupinus luteus* (Dullaart and Duba 1970), *A. glutinosa* (Henson and Wheeler 1977), *Phaseolus lunatus* (Evensen and Blevins 1981), soybean (Williams and Sicardi de Mallorca 1982), *Lens* sp. (Dangar and Basu 1984), *Phaseolus aureus* (Dangar and Basu 1987), *P. vulgaris* (Atzorn and others 1988), *S. saman* (Chattopadhyay and Basu 1989) and *Vigna unguiculata* (cowpea) (Dobert and others 1992b and c) have all been reported to contain higher levels of GAs than adjacent root tissue, yet to date no direct evidence implies a signalling role for GAs in the regulation of nodule formation.

In 1970, Dullaart and Duba reported in *L. luteus* that, in addition to having increased GA levels in nodule extracts compared with those of the surrounding root tissue, the application of GA_3 to nodule extracts stimulated IAA production from L-tryptophan. These authors speculated that a signalling interaction existed between the two hormones in which GA_3 was able to either increase the bioproduction, or decrease the metabolism, of IAA (Figure 1), but the mechanism underlying this interaction has still not been demonstrated. However, the reverse interaction has since been confirmed in stems, where the biosynthesis of GA_1 requires the presence of IAA (Ross and others 2000). In addition, the application of PATIs to the stem reduces GA_1 levels below the site of PATI application, corresponding with the IAA level at these locations (Ross 1998). PATIs can induce the formation of pseudonodules on the root systems of various species, and as such it will be interesting to investigate what role(s) GAs, and possibly more importantly GA/IAA ratios, play in the formation of these outgrowths. Recently, IAA was shown to promote root growth in *Arabidopsis* by modulating cellular responses to GAs (Fu and Harberd 2003) and it seems possible that a similar interaction might exist between the two hormones in regulating nodule development.

Nodule GA levels appear to be influenced by the infecting *Rhizobium* strain in *P. lunatus* (Tripplett and others 1981; Dobert and others 1992a, c), contrary to a report on *P. vulgaris* nodules (Atzorn and others 1988). Many reports have demonstrated that various *Rhizobium* strains are capable of synthesizing GAs in culture (for example, Katznelson and Cole 1965; Rademacher 1994). Recently, putative GA biosynthetic enzymes were identified in *Bradyrhizobium japonicum* that function anaerobically, including under the symbiotic conditions that bacteroids are subjected to in the symbiosome (Tully and others 1998), suggesting that rhizobia might be capable of regulating GA levels both before and after bacteroid differentiation. However, whether or not the elevated GA levels of *P. lunatus* nodules stem directly from rhizobial synthesis, or if the bacteria induce the plant to increase GA production, is unknown (Dobert and others 1992c). Dobert and others (1992c) hypothesized that, in addition to the bacterial strain, nitrogen, ABA and even the host plant species may have a role in regulating nodule GA concentrations.

The application of GA_3, and to a lesser extent GA_4, induced the formation of nodule-like structures on the roots of *L. japonicus* (Kawaguchi and others 1996). These structures initiated from divisions of the pericycle and could be suppressed with the addition of nitrate. Thus, it appears that an interaction exists in *L. japonicus* whereby GAs positively regulate the division of pericycle cells necessary for nodule organogenesis and that nitrates modulate this process by acting as signalling elements that suppress these GA-induced divisions.

Nonetheless, it has been argued that an increased concentration of GAs might not be a requirement for nodule formation in some species, such as *P. vulgaris* (Atzorn and others 1988). If elevated GA levels are not required for nodulation, then based on the previously mentioned work demonstrating that GAs are influenced by IAA, the increased GA levels detected in nodules may be no more than a consequence of the high IAA levels also present there.

As an alternative to having a role in nodule formation, GAs may act as signals for the hydrolysis of nodule starch to provide a substrate for rhizobial respiration requirements. GAs promote the production of α-amylase (for example, Gubler and others 1995), an enzyme involved in the metabolism of starch, and it may be worth investigating whether or not the activities of the hormone and the enzyme are interacting within the nodule. Evidence for a link between GAs and α-amylase in starch hydrolysis exists for various fungal species (reviewed in Rademacher 1994), but to the best of our knowledge, the idea that GAs might have a similar role in nodulation has not been proposed previously. If a correlation is established among GAs, α-amylase and starch in nodulation, it is possible that the bacteria are responsible for regulating nodule GA levels as a means of obtaining nutrients. As we hypothesized for ABA and ethylene, this alludes to multiple roles for GAs in nodulation, including aiding in cell division and elongation and providing the energy requirements for the nitrogen-fixing bacteria. Elevated nodule GA levels have also been correlated with increased internode number and length and increased petiole length in *P. lunatus* (Tripplett and others 1981; Dobert and others 1992a, c) and cowpea (Dobert and others 1992b, c). Thus, GAs may benefit both symbionts by increasing the plants size, thereby increasing the photosynthetic capability of the plant, resulting in more photosynthates for plant and nodule growth and functioning.

Signalling Peptides

Apart from the classical plant hormones, peptides have recently emerged as potential regulators of nodulation. Compared with animal peptide hormones, only a few plant signalling peptides have been discovered so far. However, this number is likely to rise because more and more receptor kinases are being identified as playing a role in plant development and nodulation, many of which could be activated by peptide ligands. For example, recent discoveries of receptor kinases responsible for early Nod factor perception/signal transduction ("NORK"), (Endre and others 2002; Stracke and others 2002) and for autoregulation of nodulation ("NARK") (Krusell and others 2002; Nishimura and others 2002a; Searle and others 2003) indicate that peptides or proteins could be ligands for these nodulation-related receptor kinases.

One putative peptide that plays an important role in nodulation is the early nodulin *ENOD40*. There has been some debate on whether or not *ENOD40* is actually translated. Several ORFs have been identified with stable predicted secondary structures, and it was initially suggested that *ENOD40* acts in the form of a stable RNA, a so-called "riboregulator" (Asad and others 1994; Crespi and others 1994). However, Sousa and others (2001) found that translation of two small *ENOD40* ORFs is necessary for biological function (induction of cortical cell division) and Röhrig and others (2002) reported detection of one of the ENOD40 peptides by

immunoprecipitation and Western blotting. Mutational analysis suggests that the translated products might have a role in stabilizing a biologically active *ENOD40* mRNA structure (Sousa and others 2001). It is therefore possible that both the peptide and the mRNA are necessary for biological function as a ribonucleoprotein (Sousa and others 2001), although no target or receptor has so far been found.

ENOD40 appears to play an important role in cell cycle control because over-expression (Charon and others 1997) and microtargeting (Sousa and others 2001) of *ENOD40* induces cortical cell divisions in alfalfa roots in the absence of rhizobia and causes teratomas in *Medicago* embryos. In the presence of rhizobia, overexpression of *ENOD40* was shown to accelerate nodulation (Charon and others 1999). In contrast, silencing of *ENOD40* leads to arrest of callus growth in *Medicago* (Crespi and others 1994). Recent evidence suggests that *ENOD40* might play a role in sucrose partitioning or unloading from the phloem in the nodule (and/or the whole plant), because synthetic ENOD40 peptides bind to nodulin 100, a sucrose synthase (Röhrig and others 2002). A role in sucrose partitioning might be related to ENOD40's role in promotion of (cortical) cell division because incipient meristems are strong carbohydrate sinks. The expression of *ENOD40* in vascular tissue in roots and mature nodules (Kouchi and Hata 1993) supports a role in sucrose unloading.

ENOD40 has been identified in many legumes as well as the non-legume rice (Kouchi and others 1999). In all legumes examined, *ENOD40* mRNA has been localized in dividing and meristematic cells (Figure 3; for example, Asad and others 1994; Corich and others 1998; Crespi and others 1994; Fang and Hirsch 1998; Mathesius and others 2000; Yang and others 1993), consistent with the hypothesis that *ENOD40* plays a role in cell division. *ENOD40* is thought to be involved in the earliest stages of nodule initiation because it is expressed within hours of inoculation with nodulating rhizobia (Corich and others 1998; Fang and Hirsch 1998) and its expression in the pericycle precedes nodule initiation (Figure 3) (Compaan and others 2001). In addition, *ENOD40* expression is induced by signal molecules that can initiate cortical cell divisions, including Nod factors (Fang and Hirsch 1998; Minami and others 1996), cytokinins (Fang and Hirsch 1998; Mathesius and others 2000), and auxin transport inhibitors (Fang and Hirsch 1998). *ENOD40* is also induced in the nodule primordium by *Rhizobium* strains that induce cell divisions but do not infect and invade the nodules (Yang and others 1993), which is a further indication that *ENOD40* is involved in nodule morphogenesis, rather than the infection process. However, *ENOD40* is not specific to the nodulation process, and is also induced during the establishment of lateral root primordia (Mathesius and others 2000) nematode-induced galls (Favery and others 2002; Koltai and others 2001) and mycrorrhizal interactions (Staehelin and others 2001; Sinvany and others 2002).

Defence-Related Signalling Compounds

In addition to its previously mentioned roles in nodulation, ethylene is involved in pathogenic defense as part of a signalling process termed "systemic acquired resistance" (SAR). Other components of SAR include salicylic acid (SA), nitric oxide (NO), reactive oxygen species (ROS), jasmonic acid (JA) and its methyl ester (MeJA) (reviewed in Ryals and others 1996; Rojo and others 2003). Although the mechanism is not fully understood, symbiotic organisms invade the host plant without fully inducing the SAR response. However, Vasse and others (1993) demonstrated that some plant defense compounds do accumulate following the establishment of the first nodule primordia, resulting in increased abortion of infection threads and localized hypersensitivity response (HR), including necrosis. These authors suggested that this response is part of the autoregulatory mechanism used by plants to control the level of nodulation. Despite this and much work involving ethylene (described above), little is known about the signalling involvement of other SAR components regarding nodulation; major findings involving these compounds are addressed in the following section (see also Figure 2).

Salicylic Acid

Pre-soaking seeds with salicylic acid (SA) prior to sowing decreased the nodule number and protein content and root nitrogenase activity of *Vigna mungo* plants (Ramanujan and others 1998). SA application prior to inoculation with rhizobia or purified Nod factor also decreased the number and dry weight, and delayed the emergence, of alfalfa nodules (Martínez-Abarca and others 1998). van Spronsen and others (2003) found that 0.1 mM SA application completely inhibited indeterminant nodule formation, including the mitogenic effect induced by Nod factors, in vetch, pea (including the hypernodulating mutant P88), alfalfa and white clover but did not affect determinant nodule for-

mation in *P. vulgaris, L japonicus* and *Glycine soya*. In contrast to these findings, in soybean, 5 and 1 mM SA did decrease the nodule number and dry weight and suppressed photosynthesis and nitrogen uptake (Lian and others 2000). Also in soybean, Sato and others (2002) found that concentrations of SA as low as 0.1 mM applied 5 days prior to bacterial inoculation decreased the nodule number and dry weight in addition to the level of nitrogen fixation. SA also reduced the nodule number and dry weight in supernodulating soybean mutants, but the decreases were less pronounced than in the wild type. Sato and others (2002) proposed that SA, or SAR induced by SA, might be involved in an autoregulatory signalling pathway of nodulation.

Upon symbiont recognition, the root-SA level of alfalfa did not increase (as occurs upon plant-pathogen recognition), although it did increase in plants inoculated with either an incompatible or a compatible but Nod factor-deficient mutant of *Rhizobium* (Martínez-Abarca and others 1998; Blilou and others 1999). Thus, it was concluded that a function of Nod factors is to inhibit host SA-mediated defenses. Interestingly, upon inoculation with a compatible rhizobial strain, the root-SA level of the pea *sym30* mutant did increase, whereas upon inoculation with plant pathogens, an increase was not detected (Blilou and others 1999). Thus, the gene product appears to function specifically with symbiotic microorganisms leading Blilou and others (1999) to conclude that the product is likely required for symbiosis, as a suppressor of a SA-dependent defense response.

In *Rhizobium etli*, multi-drug resistance genes have been identified that act as bacterial efflux pumps that confer resistance to the accumulation of toxic compounds. Mutations to two of these genes, termed *rmrA* and *rmrB*, enhanced the sensitivity of the bacteria to plant toxins including phytoalexins, flavonoids and SA (González-Pasayo and others 2000). These mutants displayed diminished growth on SA or naringenin, and the *rmrA* mutant formed 40% fewer nodules on *P. vulgaris* than its wild type (González-Pasayo and others 2000). It was concluded that by preventing the accumulation of toxic compounds, *R. etli* have established an advantage that improves their chances of nodulating the host. In addition, SA was found to promote isoflavonoid (for example, genistein) synthesis and secretion from *L. luteus* roots (Kneer and others 1999). Genistein can function as a phytoalexin due to its slight antimicrobial and fungistatic activity and thus rhizobia containing resistance genes to such a toxin should have an infectious advantage over bacteria lacking the efflux pump.

Nitric Oxide

In nitrogen-fixing rhizobia, heme-based sensors have been detected, such as the oxygen-regulated FixL protein kinase in *R. meliloti* (Gilles-González and others 1994). When active, the deoxy-FixL protein induces a gene expression cascade required for nitrogen fixation. This process is inhibited by the presence of O_2, and possibly also by NO and CO, thus halting nitrogen fixation (Gilles-González and others 1994). Therefore, NO may have a role in regulating gene expression required for nitrogen fixation within the nodule.

NO has been identified as an inhibitor of bacteroid nitrogenase (for example, Trinchant and Rigaud 1982). Maskell and others (1977) illustrated that NO tightly binds to leghemoglobin (Lb) in soybean and cowpea nodules forming nitrosyleghemoglobin complexes (NO-Lb) and suggested that Lb may actually have a higher affinity for NO than it does for O_2. Thus, the NO-Lb complex may act as a protective mechanism used by the nodule to prevent the inhibiting NO from reaching the NO-sensitive nitrogenase of the bacteroid. Alternatively, the accumulation of NO-Lb may result in the inhibition of nitrogenase activity (Kanayama and Yamamoto 1990) as the binding of NO to Lb may competitively inhibit the binding of oxygen, subsequently diminishing the oxygen supply available to bacteroids, thereby reducing nitrogen fixation (Mathieu and others 1998).

Soybean nodules on roots exposed to high concentrations of nitrate mainly contained NO-Lb (Kanayama and Yamamoto 1990) and declined in nitrogen fixation rates paralleled by the increase in NO-Lb in these nodules. Thus, the plant may induce NO synthase (NOS) in response to excess exogenous nitrate as a means of regulating nitrogen fixation activity. However, Mathieu and others (1998) found that even in the absence of applied nitrate, some NO-Lb exists in soybean nodules. These authors found that the amount of NO-Lb was highest in young nodules, decreased with nodule age, and was nearly absent in senescent or H_2O_2-treated nodules. Moreover, in soybean plants grown in controlled environmental conditions, NO-Lb was shown to comprise almost a third of the total nodule Lb content (Maskell and others 1977), but to date, no definitive evidence exists to explain this occurrence.

NOS activity has been detected in nodules of *Lupinus albus* (Cueto and others 1996). Two putative NOS sites were detected: one in the vascular bundles and the other in the inner cells of the infected zone (Cueto and others 1996). In contrast to

root preparations, the synthesis of nodule NO was found to be Ca^{2+} independent and the authors speculated that nodule NOS could possibly be induced by compounds such as lipopolysaccharides of compatible *Rhizobia* sp.

Reactive Oxygen Species

To prevent pathogen invasion, reactive oxygen species (ROS) or active oxygen species (AOS), including hydrogen peroxide (H_2O_2), superoxide radicals $O_2$and the hydroxide radical (•OH) are upregulated in the plant upon pathogen recognition. Together, these compounds reinforce plant cell walls and trigger a localized hypersensitive response (HR) involving defense gene expression, the induction of SAR and programmed cell death (reviewed in Ryals and others 1996). ROS are also induced in host plants upon inoculation with *Rhizobium* (for example, Bueno and others 2001; Santos and others 2001) and thus it is imperative that the bacteria compensate for these defense molecules in order to achieve nodule organogenesis. Both plant and bacterial compounds exist that help protect against the harmful effects of ROS, including peroxidases, catalases and superoxide dismutase (SOD) among others, and *Sinorhizobium meliloti* genes induced upon host infection include those that protect against ROS (Oke and Long 1999). However, aside from having negative effects, ROS can also positively regulate the nodulation process.

Peroxidase activity increases shortly after inoculation at the site of root hair deformation (Salzwedel and Dazzo 1993). The activity appears to have a role in oxidative cross-linking of cell wall polymers at the site of rhizobial penetration, resulting in a hardening of the cell wall structure. H_2O_2 can act as a substrate for peroxidase in this process, thus illustrating a potential role for low levels of certain ROS during nodulation. Salzwedel and Dazzo (1993) speculated that for successful infection to occur, the rhizobia must first suppress root hair peroxidase activity, therefore allowing the bacteria to penetrate the cell wall of the host. The authors suggested that a rapid and transient decrease in peroxidase activity could be evoked by rhizobial exopolysaccharides (EPS) which rapidly bind to root hairs, increase infection frequency and may aid the bacteria in avoiding the elucidation of SAR during invasion. Following penetration, highly localized peroxidase activity might be required to repair the eroded root hair cell wall at the site of rhizobial entry and infection thread initiation. Salzwedel and Dazzo (1993) also speculated that the plants might resist non-host bacteria and pathogens by rapidly increasing localized peroxidase levels to harden the root cell walls and prevent their invasion.

Prior to rhizobial infection of *M. truncatula*, Nod factors trigger a rapid and localized expression of the putative peroxidase-encoding *RIP1* early nodulin gene (Cook and others 1995), as does ethylene (Olroyd and others 2001). As a peroxidase, RIP1 could have a role in metabolizing H_2O_2 and/or in peroxidase-mediated cross-linking of cell wall polymers. The *RIP1* transcript was localized to epidermal cells that subsequently were infected by *Rhizobium* and were expressed for the duration of pre-infection (Cook and others 1995), suggesting a possible involvement in cell wall repair at the site of infection. Recently, Ramu and others (2002) demonstrated that RIP1 transcripts and ROS share a similar pattern of localization in *M. truncatula* and that Nod factor application elicits a rapid induction of each. Neither ROS nor *RIP1* expression was detected using a Nod factor-deficient mutant of *Sinorhizobium meliloti* or a mutant of *M. truncatula* impaired in Nod factor signal transduction. Moreover, Ramu and others (2002) found that H_2O_2 specifically induced *RIP1* expression, leading the authors to speculate that Nod factor perception by the plant induces H_2O_2 production, which then mediates the Nod factor-induced expression of *RIP1*. This finding seems logical because H_2O_2 can act as a substrate for peroxidases, such as the putative RIP1.

In pea, Wisnewski and others (2000) found that the insolubilization of matrix glycoproteins creates a barrier inhibiting the continued ingress of invading bacteria. These authors speculated that diamine oxidase activity could locally produce H_2O_2 that can be used by peroxidase to induce the insolubilization of the glycoproteins thereby modulating cell wall plasticity. Within the infection thread, the matrix glycoproteins are found to be insoluble at the tip and hardened elsewhere (Wisnewski and others 2000). This allows invading rhizobia to progress towards the infection zone of the nodule in the infection thread as long as the peroxidase level at the tip remains at a low enough level to avoid hardening of the infection thread tip walls.

In addition, actin monoubiquitylation is induced in developing nodules of *P. vulgaris* (Dantán-González and others 2001). These actin modifications are likely part of a defense response against invading organisms and appear to provide microfilament stability against proteolytic degradation. This response can be mimicked in suspension cell culture by H_2O_2 application (Dantán-González and others 2001), thus further suggesting that H_2O_2 has a role in modifying cell wall structures.

Salzar and others (1999) demonstrated that H_2O_2 accumulates in *M. truncatula* cortical cells in the region occupied by arbuscular mycorrhiza. More specifically, H_2O_2 was concentrated around hyphal tips attempting to penetrate a host cell, similar to phenomenon described by Salzwedel and Dazzo (1993) following root hair penetration and infection thread formation by rhizobia. This was suggested to be indicative of an oxidative burst involved in the control of intracellular colonization of the host (Salzar and others 1999).

In agreement with the above findings, Santos and others (2001) detected an oxidative burst of H_2O_2 and O_2^- in the curled region of the root hair immediately following inoculation of *M. truncatula*. Interestingly, these elevated levels of ROS were also found in infected cells suggesting that this burst is prolonged and could have a role in regulating the infection process (Santos and others 2001). van Spronsen and others (2003) suggested that an oxidative burst could be prolonged by SA, which could bind to, and therefore inactivate, peroxidases such as RIP1.

In addition to modulating cell wall repair and plasticity, ROS can be detrimental to nodulation as they can damage and degenerate the proteins, DNA and lipids of both symbionts, and their levels are often elevated in senescent nodule tissue. ROS such as O_2^- and •OH inhibit nitrogen fixation and it has been suggested that the inhibition by O_2^- may be due to its breakdown into the highly reactive and damaging •OH (Puppo and Halliwell 1988). To compensate for the stress of ROS, rhizobia are equipped with enzymes such as SOD, which detoxifies O_2^-. *M. truncatula* inoculated with *Sinorhizobia meliloti*, defective in SOD, nodulate poorly and display abnormal infection (Santos and others 2000). In addition, most of the bacteria failed to differentiate into nitrogen fixing bacteroids and senesced rapidly. This led Santos and others (2000) to speculate that oxidative stress interferes at numerous stages of the symbiosis and not simply at the level of nitrogen fixation. Thus, rhizobial SOD is a requirement for nodule development as well as functioning.

As mentioned, in addition to rhizobial SOD, plants contain antioxidant defense enzymes that also can break down ROS. In leaves of *Zea mays*, treatment with 10–100 μM ABA induced the production of O_2^- and H_2O_2 followed by increases in the activities of antioxidant enzymes at levels sufficient enough to scavenge the elevated levels of O_2^- and H_2O_2 (Figure 1; Jiang and Zhang 2001). The authors of this report concluded that ROS have a dual role in plants depending on their quantity: acting as toxins inducing oxidative stress when abundant or as triggers eliciting the upregulation of antioxidant enzymes when elevated only slightly. It seems plausible that the invading *Rhizobium* could positively regulate the plants antioxidant enzymes, possibly via elevated ABA levels, to avoid the damaging ROS and thereby promoting nodulation.

Like ABA, Bueno and others (2001) showed that inoculation of alfalfa plants with *Rhizobium* elevates both antioxidant enzyme activities and H_2O_2 generation. These elevated levels of scavenging antioxidant enzymes likely have a role in controlling the oxidative burst. Interestingly, among the enzymes elevated is LOX, which was earlier described as being influenced by ABA (Figure 2). Taken together with the previous paragraph, the complexity of signalling in nodulation becomes increasingly apparent.

Jasmonic Acid

Jasmonic acid (JA) both induces LOX mRNA accumulation (Figure 2) (Porta and others 1999) and is produced by the action of LOX upon polyunsaturated fatty acids (Gundlach and others 1992). In addition, methyl jasmonate (MeJA) induces the transcription of *PAL* (Gundlach and others 1992), an enzyme that catalyzes the first step in SA biosynthesis, and in *L. luteus* roots, its application promotes the synthesis and rhizosecretion of the isoflavonoid genistein (Kneer and others 1999).

JA also appears to promote the colonization and development of mycorrhizal structures in *Allium sativum* (Regvar and others 1996) and mycorrhizal colonization has been reported to elevate JA biosynthesis in *Hordeum vulgare* (barley) (Hause and others 2002). It is possible that JA has similar roles in nodule formation and mutants impaired in JA synthesis or response would greatly aid in understanding this signalling molecule in nodulation.

Other Signalling Compounds

Brassinosteroids

Foliar application of epibrassinolide to *Arachis hypogaea* (groundnut) substantially increased the number and weight of nodules and promoted root nitrogenase activity (Vardhini and Rao 1999). In contrast, application of epibrassinolide to the roots of soybean (Hunter 2001) decreased the number of nodules and amount of nitrogen fixation. These differences between studies may be attributed to variation in methods or species used.

Endogenous brassinosteriods (BRs) also appear to influence nodule formation as preliminary evidence shows that BR-deficient mutants of pea form significantly fewer nodules than their wild type (B.J. Ferguson, J. Reid and J. Ross unpublished). However, precise roles of BRs in nodulation are unclear and no molecular evidence or signalling interactions pertaining to the roles of BRs in nodule organogenesis exist to date.

Flavonoids

Flavonoids have multiple roles in plant development, defense and nodulation (reviewed in Dakora 1995; Spaink 1999); they constitute a large class of compounds of the phenylpropanoid pathway, and their exact structure is important for their varied functions, including concomitantly inducing the chemotaxis of the *Rhizobium* to the root and elevating the production of Nod factors (for example, Redmond and others 1986; Stafford 1997). Flavonoid production is also induced by rhizobia in roots and nodules (for example, see Cooper and Rao 1992; Recourt and others 1992) and different flavonoids are synthesized in response to rhizobia that up- and down-regulate Nod factor production, both before and during infection (for example, see Zuanazzi and others 1998).

Flavonoids are distributed in a strictly tissue-specific pattern in many species. In particular, flavonoids are often located in dividing and meristematic tissues, including dividing cortical cells of nodules (Mathesius and others 1998b). It is possible that flavonoids merely protect dividing cells from oxidative damage because of their activity as antioxidants (Rice-Evans 2001). However, as discussed above, it could also be possible that flavonoids affect cell division either by regulating auxin transport or turnover (Figure 1), thereby regulating auxin accumulation (Figure 3), or by directly regulating cell cycle regulators. In animals, much evidence has been found that flavonoids regulate cell cycle activity, but in plants this evidence has so far been very tentative (for example, see Logemann and others 1995; Jinsart and others 1991). The existence of a flavonoid-deficient mutant in *Arabidopsis* has shown that flavonoids are not essential for plant survival, although interestingly the mutant showed alterations in lateral root formation, root growth and plant height, which could be the result of increased auxin transport due to the absence of flavonoids acting as PATI (Brown and others 2001). At this stage, flavonoid-deficient mutants have not been isolated in legumes.

Uridine

The position of a nodule is not only determined by the initiation of cell divisions in either the inner or the outer cortex of indeterminate and determinate legumes, respectively, but also in respect to the protoxylem poles (Figure 3). In most legume species, the majority of nodule primordia are initiated in front of one of the protoxylem poles and it has been suggested that a signal (the "stele factor") diffuses out of the xylem and acts together with auxin and cytokinins to induce cell divisions comprising the nodule primordia (Libbenga and Harkes 1973).

The stele factor has been identified as uridine (Smit and others 1995). In the presence of very low uridine concentrations, cell divisions can be induced in every cortical cell by cytokinins in pea (Libbenga and Harkes 1973) and in inner cortical cells by chitin oligosaccharides following ballistic microtargeting in vetch (Schlaman and others 1997). Differences between the concentrations of uridine in front of xylem versus phloem poles could explain the preference for nodules to initiate opposite xylem poles. The fact that nodules are initiated in the outer cortex in determinate legumes and in the inner cortex in indeterminate ones could be explained by the fact that determinate and indeterminate species have different sensitivities for uridine, although definitive evidence is lacking.

Nitrate

Nitrate interacts with plant hormones to regulate nodule formation (Figure 1). The presence of nitrate in the soil at concentrations above 1–5 mM suppresses nodulation locally at several levels, including infection, nodule primordium initiation and nitrogen fixation (reviewed by Streeter 1988). How nitrate inhibits nodulation is not exactly known, although its purpose may be to limit the formation of nodules under conditions that provide sufficient nitrate.

The existence of mutants that hypernodulate even in the presence of nitrate shows that nitrate is not the inhibiting factor itself, but that it leads to secondary signals that suppress nodulation (Carroll and others 1985). According to the auxin burst hypothesis (Gresshoff 1993), high auxin levels inhibit nodule formation, and it is hypothesized that nitrate increases the sensitivity of the root to auxin, thus reducing nodule formation. In the supernodulation *nts* mutants, the auxin burst control is altered and therefore these mutants can still nodulate in the presence of nitrate because not as much

auxin is available in the root to suppress further nodule initiation. In support of that hypothesis, Caba and others (2000) found that nitrate decreased auxin levels in inoculated and uninoculated roots of wild type and *nts* mutants, whereas root growth was not altered. The authors hypothesized that this represented an increased sensitivity to auxin in the presence of nitrate, which would be consistent with the auxin burst hypothesis; however, auxin sensitivity will need to be assessed by more direct means. An effect of nitrate on the auxin response pathway has been found in *Arabidopsis* (Zhang and others 1999) and it is possible that, in legumes, at least some of the effects of nitrate are also mediated by auxin.

Nitrate's regulation of nodulation could be imposed via an effect on flavonoid accumulation in the root, which can alter auxin transport or Nod gene activity (Coronado and other 1995). There is also evidence for the involvement of ethylene in mediating the inhibitory effect of nitrate. The findings that inhibitors of ethylene synthesis or action (for example, AVG and Ag^+, respectively) restore nodulation in the presence of nitrate suggest that nitrate induces the production of ethylene which then inhibits nodulation (Caba and others 1998; Ligero and others 1991). Because ethylene can regulate auxin transport (Burg and Burg 1966; Suttle 1988) and turnover (Ke and Saltveit 1988), nitrate's effect via alterations in auxin levels could be mediated by nitrate-induced ethylene. Caba and others (1999) found that the tolerance of the *nts* mutant to nitrate with respect to nodulation is paralleled by a tolerance for ethylene, which supports an involvement of ethylene in nitrate regulation. Unlike the *nts* mutants, in *L. japonicus*, the nodulation phenotype of the recently characterized early- and hyper-nodulating mutant *astray* displayed normal sensitivity to ethylene and nitrate as its nodule number declined in the presence of both (Nishimura and others 2002c). Interestingly, the mutated gene of *astray* was found to be the homologue of the *Arabidopsis HY5* gene (Nishimura and others 2002b), which is involved in photomorphogenesis.

Nitrate also inhibits *ENOD40* induction by rhizobia, but not by cytokinins (Mathesius and others 2000), suggesting two possibilities for the action of nitrate (see Figure 1): (1) if rhizobia induce *ENOD40* independently of cytokinins, nitrate would act between Nod factor perception and *ENOD40* induction, or (2) if rhizobia change cytokinin levels, which subsequently stimulate *ENOD40*, nitrate would inhibit the cytokinin changes induced by rhizobia.

Mutants are valuable to test the interactions between nitrate and hormone signalling. For example, the nitrate reductase-deficient mutant *ANR1* and the auxin response mutant *axr4* were used in *Arabidopsis* to establish a role for the auxin response pathways during nitrate regulation of lateral root development (Zhang and others 1999). Assuming that lateral root and nodule development share aspects of their regulation by nitrate, it is possible that nitrate also acts via the auxin response pathway during nodulation and that the effects of nitrate on cytokinin, *ENOD40* expression and ethylene could indirectly be caused by changes in auxin response.

Nod Factors and Other Chitin Derivatives

Nod factors are *Rhizobium*-produced lipochitin oligosaccharides and represent the major morphogenic molecule regulating nodule organogenesis, bringing us back to the start of the story. In addition to determining host specificity, Nod factors elicit root hair curling and deformation and cortical cell divisions in alfalfa (Truchet and others 1991). There has been some debate about whether Nod factors are hormone-like signals *per se* or act indirectly, for example, via changing the plant hormone balance as discussed above. Although specific Nod factor action during nodulation has been extensively reviewed elsewhere (for example, Cullimore and others 2001; D'Haeze and Holsters 2002; Miklashevichs and others 2001), we focus here on the hormone-like roles of chitin oligosaccharides in general.

Whereas Nod factors are specific in their morphogenetic effect for certain host plants, Nod factor-related molecules have been suggested to play a more general role in plant development (Spaink and others 1993; van der Holst and others 2001). Structurally related chitin oligosaccharides play a role in animal development and have been detected in plants (Benhamou and Asselin 1989; Spaink and other 1993). They can be recognized by receptors for chitin oligosaccharides (Stacey and Shibuya 1997), and are substrates for chitinases, which have been shown to play a role in different aspects of plant development (Collinge and others 1993). Expression of a chitinase was shown to rescue an embryonic mutant of carrot (de Jong and others 1992) and modifying chitin structures by expression of the bacterial *nodA* and *nodB* genes, which modify Nod factors in rhizobia, led to changes in plant development (Schmidt and others 1993).

Directed microtargeting of chitin oligosaccharides induced cortical cell divisions in vetch roots (Schlaman and others 1997). Dyachok and others

(2000) found that Nod factors could stimulate embryogenesis in cell cultures of Norway spruce, a non-nodulating plant, and more recently isolated a lipochitin oligosaccharide-like compound from these cultures which stimulated embryogenesis (Dyachok and others 2002). Collectively, these experiments suggest that chitin perception could be widespread in both plants and animals and that chitin-related molecules play a role in development. However, the mode of action of chitin derivatives remains elusive and identification of receptors and downstream response elements will be necessary to establish whether chitin oligosaccharides act via classical hormones or directly on target genes.

Conclusions and Outlook

This review demonstrates the manifold effects of classical plant hormones and other compounds on nodule initiation, differentiation and numbers. Additional factors, such as soil nutrients, light, polyunsaturated fatty acids, CO_2, Ca^{2+}, phenylalanine ammonia lyase, chalcone synthase, *Rhizobium* , exopolysaccharides, lipopolysaccharides, and so on are all probably required for proper nodule development and functioning, but could not be fully discussed here.

Reports on classical plant hormones in nodulation are often ambiguous and contradictory because (1) nodulation is a fine balance between induction and repression of new nodule formation; (2) hormone requirements change with the varying stages of nodulation; (3) hormone levels and requirements change in different places in the shoot, root and nodule; (4) hormones interact with each other, leading to complex negative and positive feedback loops; (5) hormone requirements differ in different legume species, and (6) nodulation is regulated by both local and long distance signalling interactions involving varying actions of the same hormone in each regulatory pathway.

The search for homologues for many of the recently discovered *Arabidopsis* hormone response genes in legumes and their silencing or overexpression should help pinpoint the action of hormones during nodulation. For example, it should be tested whether *Rhizobium* directly affect cytokinin levels or whether cytokinin-related responses are the result of changing the auxin:cytokinin ratio due to changes in auxin transport or levels (see Figure 1). This could be tested in an inducible mutant for cytokinin synthesis. Inducible or temperature-sensitive mutants in polar auxin transport could be used to test whether auxin transport inhibition is necessary for nodule induction and whether changes in auxin occur in the absence of PATI, for example, via flavonoid-regulated changes in peroxidase activity, as indicated in Figure 1. Accordingly, it could be tested whether auxin transport inhibition is a result of changes in ethylene induction in an ethylene synthesis-deficient mutant. A mutant in ABA synthesis would also be useful for testing the functional relationships indicated in Figure 2. If elevated ABA levels are necessary for changes in phytoalexins, LOX, ROS and therefore indirectly for changes in peroxidase levels, JA and regulation of defense responses, these responses should be reduced in the mutant.

There are challenging questions to address in future research. First, how does Nod factor perception lead to downstream events that could affect the plant hormone balance? Not much is known about how the early events in the root hair are linked to the events in the cortex, but the analysis of nodulation mutants is beginning to address that problem (Kistner and Parniske 2002). Secondly, there is a need for more large-scale experiments to discover the broad response pathways for plant hormones during nodulation, because each hormone usually has many targets and interacts with other hormones, which also have multiple effects. The use of mutants with hormone insensitivity, overproduction, or underproduction, the use of accurate reporters for different hormones, concentrating on model species for different types of analyses, as well as keeping an open mind about possible interactions should help to unravel the complex interactions of hormone-regulated signalling during nodulation. In addition, the recent identification of ESTs in *M. truncatula* has opened the door for expression analyses on the transcript (Fedorova and others 2002) and proteome level (Mathesius and others 2001). Thirdly, it is almost certain that new signalling compounds will be discovered apart from those presently known. Among them will be peptide hormones that might regulate receptor kinase activity. But other long-range signals are also likely to be discovered, including the autoregulatory signal from the shoot (Searle and others 2003). The molecular and physiological characterization of these novel compounds should help further the understanding of the intricate nodulation process that we are just beginning to understand.

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Nodulation Phenotypes of Gibberellin and Brassinosteroid Mutants of Pea[1]

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The initiation and development of legume nodules induced by compatible Rhizobium species requires a complex signal exchange involving both plant and bacterial compounds. Phytohormones have been implicated in this process, although in many cases direct evidence is lacking. Here, we characterize the root and nodulation phenotypes of various mutant lines of pea (*Pisum sativum*) that display alterations in their phytohormone levels and/or perception. Mutants possessing root systems deficient in gibberellins (GAs) or brassinosteroids (BRs) exhibited a reduction in nodule organogenesis. The question of whether these reductions represent direct or indirect effects of the hormone deficiency is addressed. For example, the application of GA to the roots of a GA-deficient mutant completely restored its number of nodules to that of the wild type. Grafting studies revealed that a wild-type shoot or root also restored the nodule number of a GA-deficient mutant. These findings suggest that GAs are required for nodulation. In contrast, the shoot controlled the number of nodules that formed in graft combinations of a BR-deficient mutant and its wild type. The root levels of auxin and GA were similar among these latter graft combinations. These results suggest that BRs influence a shoot mechanism that controls nodulation and that the root levels of auxin and GA are not part of this process. Interestingly, a strong correlation between nodule and lateral root numbers was observed in all lines assessed, consistent with a possible overlap in the early developmental pathways of the two organs.

Nodulation is a symbiotic process whereby bacteria of the genus Rhizobium invade compatible leguminous host plants (Mylona et al., 1995; Mathesius, 2003). The invasion ultimately leads to the formation of structures called nodules, in which the bacteria fix atmospheric nitrogen to be used by the plant. As with any developmental process, nodulation is multifaceted, requiring specific signaling events regulated temporally and spatially (Ferguson and Mathesius, 2003).

Beginning in the 1980s, mutagenesis experiments using pea (*Pisum sativum*) produced abnormal nodulation phenotypes including nonnodulating (nod−), poorly nodulating (nod±), and hypernodulating (nod++) mutants, as well as those that fix nitrogen poorly or not at all (fix-; see refs. in Borisov et al., 2000). At present, over 200 nodulation mutants exist in pea (Borisov et al., 2000). Nodulation mutants have also been selected for in the model legume species *Medicago truncatula* and *Lotus japonicus*, which have smaller genomes than pea, making them more desirable tools for molecular studies. Mutants in these species have since been used to identify genes and gene products involved in nodule formation and functioning. This approach has been successful, and the orthologs of many nodulation genes discovered in *M. truncatula* or *L. japonicus* have subsequently been identified in important crop species such as pea (see refs. in Oldroyd and Downie, 2004).

Here, we take the reverse approach to investigate nodulation. In contrast to selecting for nodulation mutants and identifying their mutated genes, we identified the root and nodulation phenotypes of previously characterized mutants (Table I). The mutants examined here are all affected in their biosynthesis of, or responses to, the phytohormones GA or brassinosteroid (BR). Moreover, the genes and gene products of these lines have all formerly been identified (for review, see Reid et al., 2004; Table I). Unlike dwarf (*le*) cultivars used in many previous nodulation studies (e.g. Finale, Frisson, Rondo, Solara, Sparkle), the wild types studied here are all on a tall (*LE*) background. Interestingly, many pea lines used for agricultural purposes are on *le* backgrounds and are therefore deficient in shoot GA_1 (Reid et al., 2004), as are many of the lines used for the selection of nodulation mutants. However, the effects of shoot dwarfism and reduced shoot GA_1 levels on nodulation have not been described previously. This report is also the first to investigate the role(s) of endogenous BRs in nodulation. As with GA deficiencies, reductions in BR levels cause shoot dwarfism, thus allowing us to use two distinct hormone-mediated mechanisms to investigate the effects of shoot stature on nodulation and root development.

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Table I. *Overview of the various pea lines investigated*

Genotype	Line Number	Gene Product	Hormone Level	Phenotype	References
Torsdag	107		Wild Type		
lk	212-	BR 5α-reductase	Reduced total plant BRs	Dwarf, thickened internodes	Reid (1986); Ross and Reid (1986); Nomura et al. (2004)
lka	5865	BR receptor	Increased total plant BRs	Dwarf, thickened internodes	Reid and Ross (1989); Nomura et al. (1997, 1999, 2003)
lkb	5862	BR C-24 reductase	Reduced total plant BRs	Dwarf, thickened internodes	Reid and Ross (1989); Nomura et al. (1997, 1999); Schultz et al. (2001)
ls-1	181	Copalyl diphosphate synthase	Reduced total plant GAs	Dwarf	Ait-Ali et al. (1997); Yaxley et al. (2001)
lh-2	5843	*ent*-Kaurene oxidase	Reduced total plant GAs	Dwarf	Yaxley et al. (2001); Davidson et al. (2004)
le-3	5839	GA 3-oxidase	Reduced shoot GAs, wild-type root GAs	Dwarf	Ingram et al. (1984); Yaxley et al. (2001)
NA	1766x1769		Wild Type		
na	1766x1769	*ent*-Kaurenoic acid oxidase	Reduced total plant GAs	Extreme dwarf	Yaxley et al. (2001); Davidson et al. (2003)
SLN	250+		Wild type		
sln	250-	GA 2-oxidase	Elevated seed GAs leading to elevated total plant GAs	Elongated internodes	Reid et al. (1992); Ross et al. (1993); Lester et al. (1999)

RESULTS AND DISCUSSION

Nodulation Phenotypes of GA Mutants

In our collection of GA-deficient mutants, *na-1* causes the greatest reduction in bioactive GA_1 levels in the root, followed by *ls-1* and finally *lh-2* (Yaxley et al., 2001). In this study, all three of these mutants developed significantly fewer nodules and significantly reduced root systems (fewer and shorter secondary and tertiary lateral roots; Fig. 1; Table II) than their wild types. The reductions in total nodule numbers were observed on a per-plant (Fig. 2) and also on a per-milligram root dry weight (DW) basis (Table III). The severity of these reductions closely paralleled the reductions in the root GA_1 levels of the mutants (Yaxley et al., 2001) and strongly indicates a requirement for GAs in root and nodule initiation. Reduced root GA_1 levels may affect nodule formation directly by reducing successful Rhizobium infections and nodule development. Alternatively, reductions in root GA_1 levels may act indirectly by increasing the level of nodulation inhibitors, such as ethylene, and/or limiting root numbers and lengths, thereby reducing available Rhizobium infection sites. Reductions in nodule numbers were observed in both 25- and 40-d-old plants (Fig. 2), indicating that the reduced root GA_1 levels are not simply delaying nodule development.

The *na-1* mutant exhibited the most dramatic nodulation phenotype as few to no nodules formed (Figs. 2 and 3). Those that did form were aberrant, being small and white and resembling emerged meristems that failed to develop further (Fig. 3). Unlike the nodules observed on the other lines investigated, the few aberrant nodules of *na-1* were often detected on the tertiary lateral roots of the mutant (Fig. 3B). As a consequence of their reduced size, the total DW, and average DW, of *na-1* nodules were significantly reduced compared with those of its wild type (Table III). Less dramatic reductions were detected in the total nodule DWs of *ls-1* and *lh-2* mutant plants (Table III) compared with that of their wild type. However, although the average nodule DW was reduced in *na-1*, it was actually significantly elevated in *ls-1* and *lh-2* (Table III). Thus, it appears that GAs may also influence nodule size with slight reductions being stimulatory (*ls-1* and *lh-2*) and large reductions inhibitory (*na-1*).

In an attempt to restore nodule numbers to that of the wild type, various concentrations of the bioactive GA_3 were applied to the roots of *na-1* mutants. Using this technique, concentrations of 10^{-6} M GA_3 were found to completely restore the *na-1* nodule appearance and numbers to that observed on the wild-type control (Fig. 4). This finding lends further support to our evidence that GAs are required for nodule development. Low concentrations of the hormone also stimulated nodule formation in the wild type but became inhibitory to both the wild type and the mutant as the applied concentration increased (Fig. 4). This finding is similar to that reported by Lorteau et al. (2001) for cytokinin; they found that the application of low concentrations of the phytohormone were stimulatory to pea nodule formation but became inhibitory when increased beyond a threshold level.

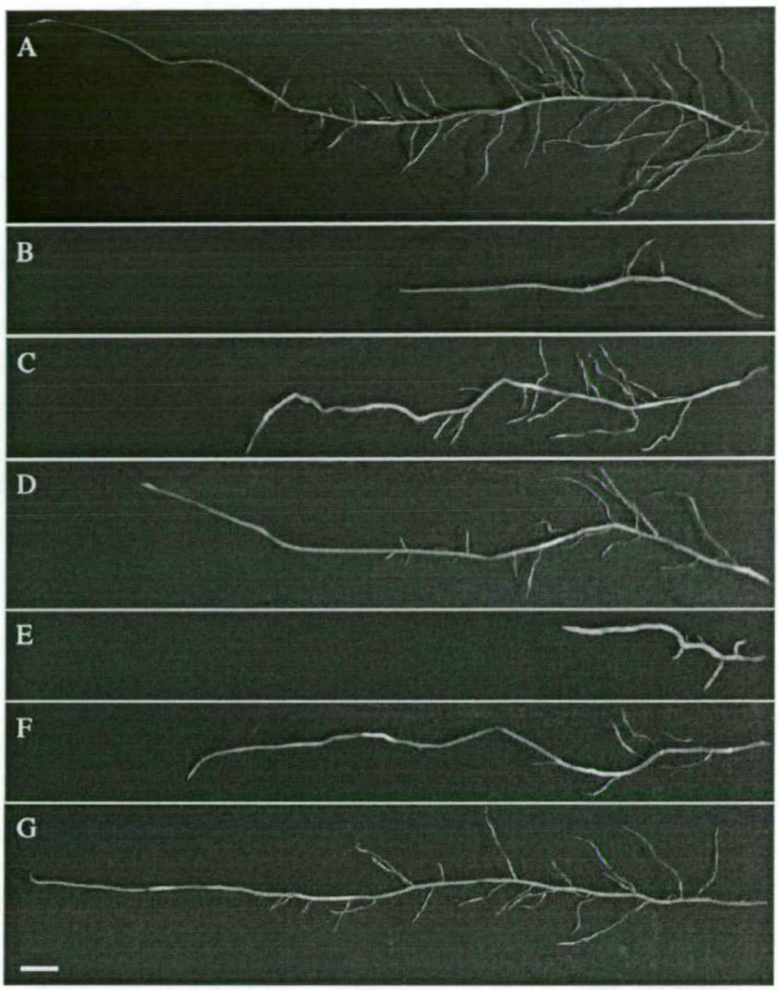

Figure 1. Detached secondary lateral roots of 17-d-old plants of (A) wild type (Torsdag) and (B) the BR-deficient *lk*, (C) the BR-receptor mutant *lka*, (D) the BR-deficient *lkb*, (E) the GA_1-deficient *na-1*, (F) the GA_1-deficient *ls-1*, (G) and the shoot GA_1-deficient *le-3*. The roots were collected from the most mature region of the plants, closest to the crown. The far right-hand side of the secondary lateral root is the point at which it was detached form the primary root. Bar = 1 cm.

Grafting studies were performed using various combinations of *lh-2* and its wild type (*LH*), Torsdag, in order to determine whether or not an *LH* shoot or root system could restore the reduced nodule number of the GA-deficient line (Table IV). This study revealed that either an *LH* root or shoot system was sufficient to restore the reduced nodule number of the mutant, both on a per-plant and a per-milligram root DW basis. This finding implies that GAs are required for nodulation. Furthermore, the root system GA level appears to play a role in nodule development, as more nodules formed on *lh-2/LH* grafts than on those of *lh-2/lh-2* ($P < 0.001$), even though the shoots remained short, with a low DW (Table IV). *LH/lh-2* grafts also produced more nodules than *lh-2/lh-2* grafts, but it cannot be excluded that GAs were transported basipetally from the *LH* shoot into the mutant root system. Consistent with this suggestion is the significant promotory effect of *LH* shoots on the *lh-2* root DW, which increased compared with that of the *lh-2/lh-2* grafts ($P < 0.01$). Graft transmissibility of GA_1 precursors (but not of GA_1 itself) has been demonstrated previously (Reid et al., 1983). Interestingly, the total nodule DW was significantly reduced in grafted plants possessing an *lh-2* shoot, whereas the average nodule DW was slightly increased in grafts having *lh-2* roots (Table IV).

The *le-3* mutant, which has decreased shoot GA_1 levels but wild-type root GA_1 levels (Yaxley et al., 2001), and the *sln* mutant, which has elevated root and shoot GA_1 levels early in development (Reid et al., 1992; Yaxley et al., 2001), both had a similar number and size of lateral roots and nodules as their wild types (Figs. 1 and 2; Tables II and III). Importantly, the normal root and nodule phenotypes of the *le-3* mutant indicate that the effects of GA_1 deficiency on these characteristics, as observed in *na-1*, *ls-1*, and *lh-2*, are not mediated by dwarfism of the shoot. Furthermore, the results with *le-3* are consistent with those of the grafting experiment with *lh-2* (Table IV), as neither dwarfism nor a reduced shoot GA_1 level impaired the root system DW nor the nodule number of a root system having a normal level of GA_1. Moreover, the wild-type level of GA_1 in the *le-3* root system is insufficient to rescue the shoot dwarfism of the

Table II. *Root numbers and lengths of 17-d-old GA and BR mutants and their wild types*

Indicated is the number of secondary lateral roots per plant in addition to the number of tertiary lateral roots per secondary lateral root, based on the average number located on the six uppermost secondary lateral roots. Also shown are the lengths of the shoot and the longest secondary and tertiary lateral roots per plant. Results are means ± SE (n = 6). Values for each mutant trait followed by an * are significantly different from that of their respective wild type at the 0.01 level.

Genotype	Number		Length		
	Secondary Roots	Tertiary Roots	Shoot	Secondary Roots	Tertiary Roots
			cm		
Torsdag	89 ± 3.6	20 ± 1.1	12.5 ± 0.3	19.7 ± 0.1	4.3 ± 0.2
lk	50 ± 3.6*	4 ± 0.4*	3.0 ± 0.2*	10.4 ± 0.3*	2.2 ± 0.1*
lka	82 ± 2.5	9 ± 0.8*	5.9 ± 0.4*	14.8 ± 0.8*	3.5 ± 0.2*
lkb	82 ± 4.1	13 ± 0.9*	5.9 ± 0.3*	16.9 ± 0.4*	3.7 ± 0.3
ls-1	63 ± 4.8*	7 ± 0.6*	2.9 ± 0.1*	16.7 ± 0.7*	2.2 ± 0.3*
lh-2	63 ± 4.7*	12 ± 1.0*	5.7 ± 0.2*	17.2 ± 0.7*	2.5 ± 0.2*
le-3	100 ± 1.7	20 ± 1.1	4.2 ± 0.2*	18.5 ± 1.2	3.8 ± 0.3
NA	107 ± 5.2	21 ± 1.1	21.4 ± 0.5	18.2 ± 0.7	5.9 ± 0.3
na	50 ± 2.4*	5 ± 0.4*	2.9 ± 0.2*	6.1 ± 0.3*	1.1 ± 0.1*
SLN	93 ± 5.4	13 ± 1.0	28.6 ± 0.7	19.0 ± 1.2	3.2 ± 0.4
sln	98 ± 1.4	13 ± 0.9	50.0 ± 3.8*	18.6 ± 1.2	2.9 ± 0.3

mutant. This finding is consistent with that observed using the *lh-2* grafts (Table IV).

The elevated GA_1 levels of *sln* do not appear to influence the root system or the overall number of nodules that form per plant (Figs. 1 and 2; Tables II and III). Despite these findings, high GA_1 levels may actually be inhibitory to nodule organogenesis. The source of the elevated GAs of *sln* is the seed (Ross et al., 1993). As the *sln* seedling develops, this excess GA is mobilized throughout the plant until it is eventually metabolized and maintained at near *SLN* levels (Ross et al., 1993). By this time, the primary roots of both *SLN* and *sln* are well established and appear similar. However, although numerous nodules formed on the primary roots of *SLN* plants, no nodules developed on the primary roots of *sln* mutants (Fig. 5). This may suggest that the elevated GA levels of the mutant prevented nodules from establishing, which is consistent with the finding that treatment with high concentrations of GA_3 reduced the number of nodules that formed on wild-type plants (Fig. 4). This inhibition in *sln* is temporary, as nodulation was not prevented on lateral roots, of which many formed following the metabolism of the majority of the excess GA_1. Elevated GA_1 levels might act directly to inhibit the infection process or nodule

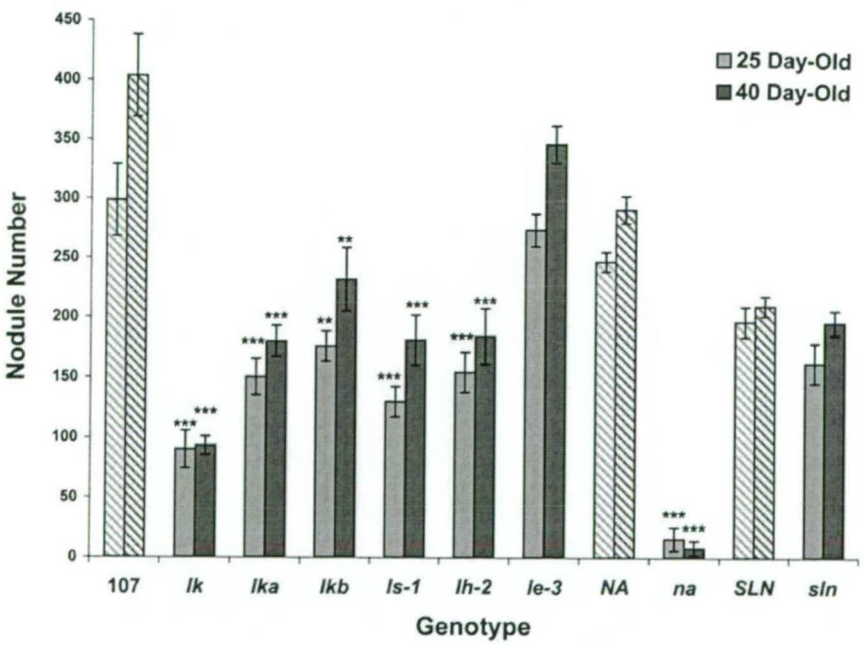

Figure 2. Nodule numbers of 25- and 40-d-old plants inoculated with *R. leguminosarum*. Results are means ± SE (n = 8). Dashed bars represent wild types of the mutants (black bars) situated to their right. Mutant values denoted with an *, **, or *** are significantly different from that of their wild type at the 0.05, 0.01, and 0.001 level, respectively.

Table III. *Root, shoot, and nodule DWs and nodule numbers per root and shoot DW of 25-d-old GA and BR mutants and their wild types*

Plants were inoculated with *R. leguminosarum* 5 d following the time of sowing. Results are means ± SE (n = 8). Values for each mutant trait followed by an * are significantly different from that of their respective wild type at the 0.01 level.

Genotype	DW				Number of Nodules	
	Shoot	Root	Nodule Total	Nodule Average	Per Milligram Shoot Dry Weight	Per Milligram Root Dry Weight
	mg	*mg*	*mg*	*mg*		
Torsdag	208 ± 12	158 ± 12	33.4 ± 3.8	0.11 ± 0.012	1.44 ± 0.12	1.95 ± 0.23
lk	142 ± 15*	115 ± 13	23.5 ± 3.5	0.29 ± 0.030*	0.62 ± 0.07*	0.77 ± 0.10*
lka	172 ± 13	173 ± 15	30.5 ± 2.3	0.21 ± 0.015*	0.87 ± 0.04*	0.87 ± 0.04*
lkb	236 ± 11	202 ± 16	42.6 ± 4.1	0.24 ± 0.012*	0.74 ± 0.03*	0.88 ± 0.04*
ls-1	111 ± 7*	132 ± 9	22.4 ± 2.1	0.18 ± 0.016*	1.17 ± 0.08	1.03 ± 0.13*
lh-2	162 ± 7*	159 ± 8	29.0 ± 3.7	0.19 ± 0.016*	0.94 ± 0.07*	0.99 ± 0.12*
le-3	203 ± 19	170 ± 15	39.7 ± 4.1	0.15 ± 0.013	1.41 ± 0.13	1.66 ± 0.14
NA	396 ± 24	197 ± 10	62.6 ± 4.1	0.25 ± 0.016	0.64 ± 0.04	1.28 ± 0.08
na	184 ± 12*	175 ± 14	1.3 ± 0.7*	0.06 ± 0.025*	0.08 ± 0.05*	0.09 ± 0.05*
SLN	357 ± 21	158 ± 9	63.6 ± 0.4	0.33 ± 0.017	0.55 ± 0.03	1.27 ± 0.11
sln	392 ± 28	142 ± 11	58.1 ± 5.1	0.37 ± 0.023	0.42 ± 0.04	1.20 ± 0.17

development, or indirectly, by affecting assimilate distribution.

Nodulation Phenotypes of BR Mutants

In our collection of BR mutants, *lk* has the most severe reduction in bioactive BRs in the shoot (Nomura et al., 2004), followed by *lkb* (Nomura et al., 1997). A reduction in BR levels in the roots has also been confirmed for *lkb* (Symons and Reid, 2004). Here, we demonstrate that, in addition to shoot dwarfism, the BR synthesis mutants *lk* and *lkb* and the BR response mutant *lka* also have fewer and shorter lateral roots (Fig. 1; Table II). These findings support recent reports that BRs have a role in lateral root development (Bao et al., 2004). Interestingly, despite all three BR mutants producing fewer and shorter lateral roots (Fig. 1; Table II), only the *lk* root system DW was significantly reduced compared with that of Torsdag (Table III).

Nodule numbers were reduced in all three BR mutants compared with that of Torsdag. These reductions occurred in both 25- and 40-d-old plants, indicating that nodule development was not delayed, but rather diminished, as was observed with the GA_1-deficient mutants (Fig. 2). The nodule numbers were also reduced on a per-milligram root DW basis (Table III), indicating that the reductions were not simply correlated with the size of the root systems. Instead, these diminished nodule numbers might be caused by reduced BR levels, or perception, directly or indirectly effecting nodule development, as is discussed above for mutants having reduced root GA_1 levels.

The average nodule DW was significantly increased for all of the BR mutants, compared with that of Torsdag (Table III). Thus, in the case of *lk*, although the root system DW decreased, the average nodule DW increased. This finding illustrates that nodule size is not simply a reflection of root system DW. Interestingly, with the exception of the severely reduced *na-1*, reductions in root GA_1 levels also resulted in increased nodule DWs. Producing large nodules may be a compensatory mechanism to increase nitrogen fixation in response to reduced nodule numbers.

Recently, BRs were shown to be relatively immobile within pea (Symons and Reid, 2004). For this reason, BR application studies similar to that performed using GA_3 and *na-1* were not considered to be the best method to investigate nodulation here. In addition,

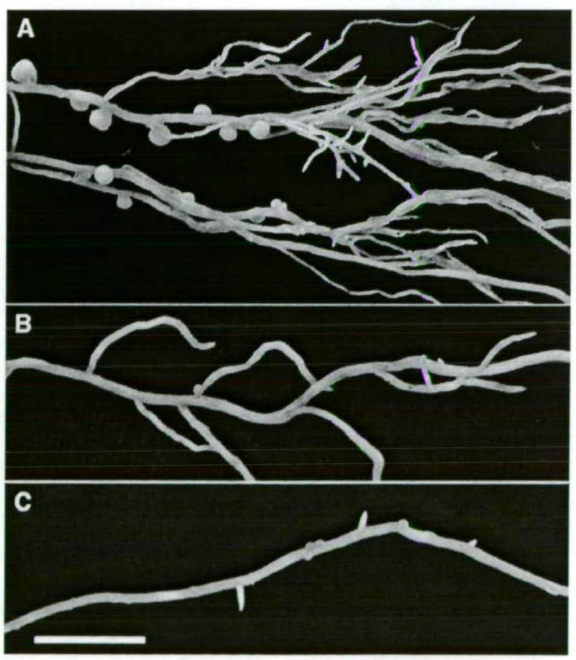

Figure 3. Nodulated lateral roots of 25-d-old (A) wild-type and (B and C) *na-1* plants. Wild-type nodules are large and display a white meristematic tip and a red center that represents the zone of nitrogen fixation. The few aberrant nodules that do develop on the *na-1* mutant are small and white and resemble an emergent nodule meristem that failed to develop further. Bar = 1 cm.

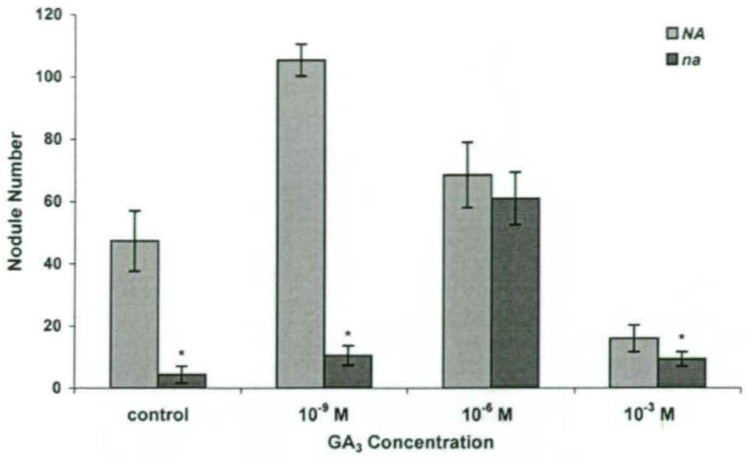

Figure 4. Nodule numbers of 20-d-old wild-type and *na-1* plants inoculated with *R. leguminosarum* and treated with various concentrations of the bioactive GA_3. Results are means ± SE (n = 6). Mutant values denoted with an * are significantly different from that of the wild-type control at the 0.01 level.

a BR mutant similar to that of *le-3* having normal BR levels in the root, but decreased levels in the shoot, is not available. As a result, grafting studies involving *lkb* and its wild type (*LKB*), Torsdag, were the only method available to examine the effects of decreased root and shoot BR levels on nodulation. Results from these studies illustrate that the shoot controlled the number of nodules that formed in these graft combinations (Table V). This finding contrasted with that observed with the *lh-2* graft combinations (Table IV). Grafted plants having an *lkb* shoot developed fewer nodules than those having an *LKB* shoot on a per-plant, as well as per-milligram root DW basis (Table V). In addition, the root and shoot DWs of grafted plants with an *lkb* shoot were not significantly reduced from those with an *LKB* shoot (Table V). This indicates that the reduced nodule numbers on grafted plants having an *lkb* shoot were not simply the result of a smaller root or shoot system. Instead, our findings suggest that BRs may be influencing a nodulation mechanism of the shoot that is involved in regulating the nodule numbers of the root. One such mechanism known to exist in the shoot involves the receptor kinase *HAR-1/SYM29/NARK* (e.g. Wopereis et al., 2000; for review, see Oldroyd and Downie, 2004). To date, it is unknown what effects, if any, BRs have on this receptor; however, the mutants examined in this report appear to be excellent candidates for investigating this potential relationship.

Recently, Symons and Reid (2004) demonstrated that BRs are not graft-transmissible. Thus, the level of BRs in an *lkb* root system would be reduced compared with that of *LKB*, even if grafted to an *LKB* shoot. Therefore, the increased number of nodules observed on *lkb* roots grafted to an *LKB* shoot cannot be explained by an increase in root BRs. In addition, despite having normal levels of BRs, *LKB* root systems grafted to an *lkb* shoot produced fewer nodules compared with those grafted to an *LKB* shoot. Together, these findings indicate that the root level of BRs does not have a direct effect on nodule numbers. Based on these results, we investigated whether or not shoot BRs regulate root and nodule development by altering the levels of other hormones in the roots. For example, our findings with the GA mutants indicate a role for GA in the development of roots and nodules. In addition, the phytohormone auxin is known to have a prominent role in both root and nodule development (Ferguson and Mathesius, 2003) and is produced at high levels in the shoot, followed by a reported acropetal transport to the root system. Thus, we measured the levels of GA_1 and the auxin, indole acetic acid (IAA), in the root

Table IV. *Root, shoot, and nodule DWs, and nodule numbers per plant and root and shoot DW of 30-d-old graft combinations of LH and lh-2 mutants*

Plants were grafted 6 d after sowing and inoculated with *R. leguminosarum* at 10 d. Results are means ± SE (n = 8). Values for each trait followed by an * are significantly different from the *LH/LH* graft combination at the 0.01 level.

Graft Type	DW				Number of Nodules		
	Shoot	Root	Nodule Total	Nodule Average	Per Plant	Per Milligram Shoot DW	Per Milligram Root DW
	mg	*mg*	*mg*	*mg*			
LH/LH	259 ± 12	81 ± 5	34.4 ± 2.8	0.42 ± 0.053	87 ± 9	0.34 ± 0.03	1.07 ± 0.07
LH/lh-2	249 ± 12	93 ± 6	31.3 ± 1.9	0.46 ± 0.066	78 ± 11	0.32 ± 0.05	0.87 ± 0.14
lh-2/LH	165 ± 17*	81 ± 8	23.7 ± 3.3*	0.32 ± 0.047	75 ± 6	0.49 ± 0.07	0.99 ± 0.12
lh-2/lh-2	151 ± 12*	62 ± 7	23.1 ± 2.6*	0.53 ± 0.062	44 ± 4*	0.30 ± 0.02	0.76 ± 0.07*

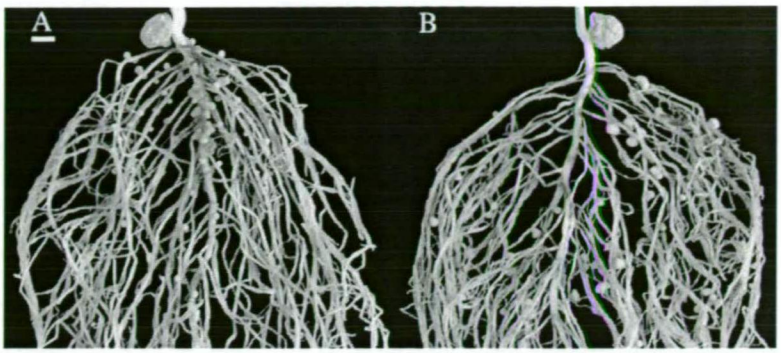

Figure 5. Nodulated root systems of the 25-d-old (A) wild type, *SLN*, and (B) the GA_1-overproducing, *sln*. *SLN*, like the other wild-type lines investigated here, formed many nodules on both primary and secondary roots, whereas *sln* only developed nodules on secondary roots. Bar = 1 cm.

systems of the various Torsdag and *lkb* graft combinations. This revealed that the levels of both GA_1 and IAA were similar among all of the graft combinations (Table V), demonstrating that the reduced BR levels of *lkb* do not alter the root GA_1 or IAA levels. Therefore, the reductions in root and nodule numbers of the BR mutants do not appear to be attributed to changes in the root levels of GA_1 or IAA.

Correlation between Root and Nodule Formation

A correlation between the number of nodules and the number of lateral roots was detected across all of the mutant and wild-type lines examined (Fig. 6). Correlations between nodule and lateral root numbers were first described by Nutman (1948) who noted that the more lateral roots a line of red clover developed the more nodules it formed. These findings indicate that a strong correlation between nodule and root formation exists and may suggest that roots utilize an autoregulatory mechanism similar to that identified in nodulation (e.g. Caetano-Anollés and Gresshoff, 1991). Consistent with this suggestion is the observation that the hypernodulating mutant of *L. japonicus*, *har-1*, exhibits stimulated root initiation when grown in the absence of *Mesorhizobium loti* (Wopereis et al., 2000).

It has been postulated that nodulation evolved from preexisting mechanisms of early lateral root development (Hirsch and LaRue, 1997; Mathesius, 2003). This theory is supported by root-nodule hybrids that have been observed on roots of *Medicago sativa* (Dudley et al., 1987) and *Trifolium repens* (McIver et al., 1997) following inoculation with specific Rhizobium strains. Roots also emerge from apical meristems of actinorhizal nodules of *Casuarina cunninghamiana* (Torrey, 1976) and *Myrica gale* (Torrey and Callaham, 1978). The nodule apex can also be converted into a root apex by adjusting growing temperatures from low to high (see refs. in Dart, 1977). Moreover, mycorrhizal nodules develop on Podocarpaceae species, even in sterile soil free of the fungus (Russell et al., 2002). These structures are not simply lateral roots modified by the endosymbiont, but rather novel outgrowths that have diverged from the root developmental pathway prior to their emergence.

Lateral roots and nodules share many aspects of their development. For example, they are both derived via postembryonic mechanisms involving dedifferentiating and dividing cells adjacent to xylem poles (Mathesius, 2003). One proposed difference in their development is the site of initial cellular divisions; the pericycle for roots and the cortex for nodules. However, peanut nodules originate predominately from the pericycle (Allen and Allen, 1940), and pericycle divisions do occur during nodule development of pea (Bond, 1948) and *T. repens* (McIver et al., 1997). In addition, nonleguminous Actinorhizal nodules, myconodules,

Table V. *Root, shoot, and nodule DWs, nodule numbers per plant, and root and shoot DW and root levels of IAA and GA_1 of 30-d-old graft combinations of* LKB *and* lkb *mutants.*

Plants were grafted 6 d after sowing and inoculated with *R. leguminosarum* at 10 d. Results are means ± SE (n = 8) for physiological traits and means ± SE of two replicates, each consisting of six root systems, for hormone analysis. Values for each trait followed by an * are significantly different from the *LKB/LKB* graft combination at the 0.01 level.

Graft Type	DW				Number of Nodules			Hormone Level	
	Shoot	Root	Nodule Total	Nodule Average	Per Plant	Per Milligram Shoot DW	Per Milligram Root DW	IAA	GA_1
	mg	*mg*	*mg*	*mg*				*ng g^{-1} fresh weight*	*ng g^{-1} fresh weight*
LKB/LKB	410 ± 35	130 ± 15	65 ± 9.5	0.57 ± 0.085	136 ± 26	0.35 ± 0.03	1.09 ± 0.20	3.93 ± 0.69	0.022 ± 0.0005
LKB/lkb	420 ± 25	180 ± 23	75 ± 11.5	0.83 ± 0.252	129 ± 26	0.34 ± 0.09	0.94 ± 0.36	3.23 ± 0.32	0.020 ± 0.0020
lkb/LKB	310 ± 39	120 ± 12	48 ± 9.5	0.98 ± 0.239	56 ± 5*	0.20 ± 0.03	0.48 ± 0.06*	3.08 ± 0.06	0.020 ± 0.0025
lkb/lkb	430 ± 43	200 ± 15*	69 ± 10.6	1.58 ± 0.236*	46 ± 7*	0.11 ± 0.01*	0.23 ± 0.03*	3.16 ± 0.19	0.024 ± 0.0005

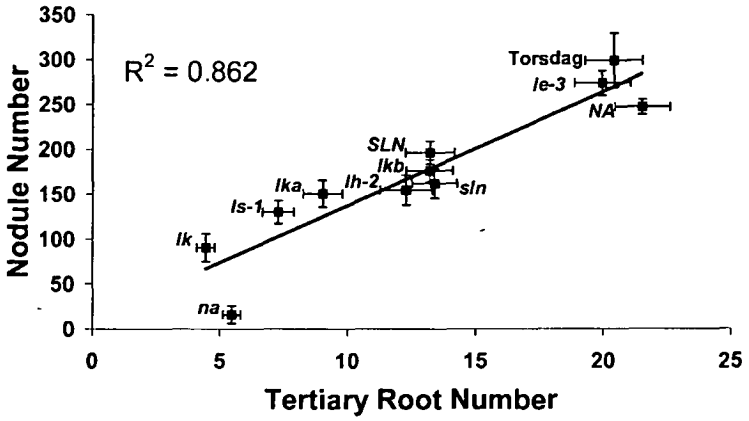

Figure 6. The correlation between the average number of tertiary lateral roots observed on the oldest six secondary lateral roots of 17-d-old plants and the number of nodules of 25-d-old plants inoculated with *R. leguminosarum*. Results are means ± SE for the nodule number (n = 6–8).

and *Parasponia* nodules are all derived from the pericycle (see refs. in Hirsch and LaRue, 1997). Moreover, ENOD40, a signal thought to be involved in cell division, is expressed in the pericycle of *M. sativa* prior to nodule primordium initiation (Compaan et al., 2001). Furthermore, Kawaguchi et al. (1996) demonstrated that bioactive GAs induce pericycle divisions leading to nodule-like structures in *L. japonicus*. These structures were free of central vascular cells and were therefore not simply deformed lateral roots. Collectively, these findings point to a role for the pericycle in nodulation, possibly including cell divisions as are known to occur in lateral root development (e.g. Dubrovsky et al., 2000). The involvement of the pericycle may be mediated by hormones, which may explain why parallel declines in nodule and root numbers were observed in our mutants that have hormone-deficient root systems. Transcript profiling of early lateral root initiation in Arabidopsis (*Arabidopsis thaliana*) has detected numerous genes expressed in the pericycle (Himanen et al., 2004). Perhaps a similar investigation into the pericycle using a legume species, with and without Rhizobium inoculation, would help discriminate between gene products shared by, and unique to, root and nodule initiation.

Correlations between nodulation and the remaining characteristics measured were not observed. For example, there was no correlation between shoot stature and nodulation, as *sln* was taller than its wild type and *le-3* was shorter, but they both produced wild-type numbers of nodules (Fig. 2). Also, there is no correlation between the rate of leaf expansion and nodulation because, when compared with their wild types, GA deficient mutants had fewer leaves, whereas BR mutants had more (data not shown), yet both formed fewer nodules (Fig. 2). Shoot and root DW also did not form a correlation with nodulation. The DW of *lh-2* shoots was similar to that of *le-3* (Table III), but *lh-2* formed significantly fewer nodules than *le-3* (Fig. 2). In addition, the BR mutants all formed significantly fewer nodules than Torsdag (Fig. 2), despite of no consistent differences in their root system DWs compared with Torsdag (Table III). Furthermore, the length of secondary lateral roots does not appear to be the limiting factor of the development of tertiary lateral roots and nodules. For example, *lkb* and *ls-1* secondary lateral roots are similar in length (Fig. 1; Table II), but *ls-1* developed fewer tertiary lateral roots (Fig. 1; Table II) and nodules (Fig. 2) than *lkb*.

CONCLUSIONS

The results presented here illustrate that reduced root levels of GAs significantly decrease the number of nodules in pea (Fig. 2). These decreases in nodule numbers were observed at both 25 and 40 d, indicating that they were not simply the result of a delay in nodule formation. The application of GA_3 restored the nodule number of *na-1*, suggesting a direct role for GAs in nodule development. In addition, grafting experiments illustrated that normal GA_1 levels in the root are sufficient to elicit the formation of a normal number of nodules. In contrast, BRs do not have a direct effect on nodule numbers, but act to influence a shoot mechanism involved in regulating nodule numbers. Interestingly, with the exception of the severely inhibited *na-1*, significant increases in the average nodule DW were found on all GA and BR mutants having reduced nodule numbers (Table III). This might suggest the existence of a mechanism that compensates for changes in nodule numbers by regulating the size of individual nodules. Taken together, our findings support the theory proposed by Libbenga et al. (1973) that a delicate balance in hormone levels is required to achieve optimum nodule development. This theory is further supported by our finding that GAs, in addition to cytokinins (Lorteau et al., 2001), are stimulatory to pea nodule formation at low concentrations but inhibitory when increased beyond a threshold level.

Reductions in root GA and BR levels also diminished lateral root numbers and lengths (Yaxley et al.,

2001; Table II). Interestingly, this appears to be opposite to the effects of cytokinins, which reportedly inhibit nodulation but stimulate lateral root development (Lohar et al., 2004). It is likely that hormones have multiple roles in root and nodule development (Ferguson and Mathesius, 2003) and are required to different degrees at various stages of development. Overall, mutants have proven to be valuable tools for understanding the processes of root and nodule development and for isolating genes relating to these processes. In pea, an extensive collection of nodulation mutants has been assembled (Borisov et al., 2000), but there remains a need for additional root mutants, which would aid in determining the developmental aspects that are shared in, and are unique to, the root-nodule relationship.

MATERIALS AND METHODS

Plant Growing Conditions

An overview of the various plant lines used in this report, including any mutated genes and their resulting effects on the plant, is provided in Table I. For nodulation studies, plants were sown one per pot in 100-mm Space Saver pots (Reko, Australia) and for root analysis experiments, seeds were sown seven per pot in 200-mm Plastamatic pots (Melbourne, Australia). All pots contained a 1:1 mixture of grade 3 vermiculite (Australian Vermiculite and Perlite, Fairfield, Australia) and 10 mm dolerite aggregate (HBMI, Kingston, Australia). This mixture was topped with approximately 2 cm of a pasteurized peat/sand potting mix composed of a 1:1 mixture of peat moss (Te - Em, New Brunswick, Canada) and coarse river sand (Island Resources, Scottsdale, Australia). Pasteurization was achieved using a steam/air mix at 70°C for 45 min. The pH was adjusted to 7.0 with dolomite lime and limestone.

Plants were grown in a controlled environment glasshouse with temperatures maintained at 20°C day (18 h) and 15°C night (6 h) ± 1°C. Relative humidity was maintained at a minimum of 40%. The photoperiod of 18 h consisted of natural daylight supplemented and extended morning and evening by 4 GE (Hungary) Lucagrow LU400/HO High Pressure Sodium 400 W globes and 2 incandescent globes (60 W Pearl, Thorn, Australia) delivering an additional approximately 150 μmol photons m^{-2} s^{-1} at the pot surface.

Plants were placed on capillary mats (Bottom Up Irrigation, Fertool Distributors, Hallam, Australia) and watered using an automated overhead sprinkling system (70 lines per hour at 150 kPa) for 2 min each morning and evening. For nodule count studies, each pot was provided with 25 mL of *Rhizobium leguminosarum* bv *viciae* 128C53K (Nitragin Inoculants, Liphatech, Milwaukee, WI) grown in yeast-mannitol broth and diluted with water to approximately OD_{600} 0.01, which represents 5×10^6 cells mL^{-1}. Based on a previous experiment, inoculation was delayed in these studies until 5 d after planting to maximize nodulation. For root characterization experiments, at the time of sowing, 150 mL of the bacterial solution was applied. Plants grown in excess of 25 d were also provided with a modified Hoagland solution containing only 1 mM NO_3^- to prevent the inhibition of nodulation.

Nodule Count Studies

Investigation of Mutant and Wild-Type Lines

Plants were harvested 25 d after planting. This timing allowed nodules to develop to a stage where they could be clearly distinguished and their appearance accurately assessed. For each plant, the number of nodes was recorded, counting the cotyledon as node zero. The roots and shoots were separated at the cotyledon, which was excised and discarded. The root system was gently rinsed clean of potting substrates and placed in a tray of water. Nodules were counted, removed with forceps and, together with the roots and shoots, placed in a 60°C oven for a minimum of 3 d to obtain their DWs.

Additional plants were allowed to persist until 40 d after planting, coinciding with the flowering time of many of the lines, including wild types. The same traits examined using 25-d-old plants were then assessed. By 40 d, the formation of new nodule structures should be minimal due to the plants' autoregulation of nodulation (Caetano-Anollés and Gresshoff, 1991). Thus, assessing the number of nodules at this age confirms that the numbers determined at 25 d have remained relatively stable and are not increasing indefinitely with age. This approach helps verify that autoregulation of nodulation is functional and provides confirmation of a reduction, as opposed to a delay, in nodule development.

GA Treatments

The effect of GA on nodule formation was examined using the GA-deficient *na-1* and its wild type (Table I). Seeds of the two lines were sown according to the methods used for the root characterization experiments (described above). The roots of the seedlings were treated with 150 mL of either water (control) or various concentrations (10^{-9}, 10^{-6}, or 10^{-3} M) of the bioactive GA_3. These treatments commenced 3 d after planting and continuing twice per week until harvest. The plants were harvested 20 d after planting, rinsed clean of soil substrates and their nodules counted.

Grafting Experiments

For grafting experiments, seeds of Torsdag and *lkb*, or *lh-2* (Table I) were sown as detailed above for the nodule count investigation. At 6 d after planting, the seedlings were grafted using the methods of Reid et al. (1983). These mutants were chosen because of their common background (i.e. Torsdag; Table I) and their relative similarity in terms of both shoot stature and nodule numbers (Table III). At 10 d after planting, the plants were inoculated with 25 mL of the bacteria, thus allowing the grafts to establish prior to inoculation. The graft combinations were then scored 30 d after planting.

Analysis of Root Characteristics

Plants were harvested 17 d after planting, allowing for the development of secondary and tertiary lateral roots. The plants were uprooted, gently cleaned in water, and placed in a tray of water. The length of the shoot and the longest secondary and tertiary lateral root was measured. The total number of nodes and secondary lateral roots were recorded. In addition, the number of tertiary lateral roots located on each of the upper (i.e. closest to the crown) six secondary lateral roots was counted.

Hormone Analysis

The roots of 30-d-old grafted plants were cleaned of soil, separated from their shoots and cotyledons, and weighed. IAA and GA_1 were then extracted from these root systems, and their levels quantified, using the methods outlined in Ross (1998). Two replicates, consisting of six root systems per replicate, were analyzed.

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Title:

***Cochleata*: Getting to the Root of Legume Nodules**

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Abbreviations:

coch, *cochleata*; DW, dry weight.

Footnotes:

none

Abstract:

The homeotic mutant of *Pisum sativum, cochleata*, has stipules replaced by alternative leaf components, abnormal flowers and reduced fertility. Although the root system dry weight, root lengths and nodule numbers of *cochleata* are similar to those of its wild type, the nodulation phenotype of the mutant is unique. The nodules typically dichotomously branch and multiple callus and root structures emerge from their meristems. These nodule-roots incorporate a peripheral vascular bundle of the nodule into their own central vascular cylinder. Both the nodules and roots of the hybrid structures appear functional. Roles for *COCHLEATA* in development are discussed.

Key words:

cochleata, homeotic, meristem, mutant, nodulation, *Pisum sativum*

Text:

The *cochleata* (*coch*) mutant of *Pisum sativum* (pea) was first reported by Wellensiek (1959) as having altered flower and leaf phenotypes. The flowers of *coch* exhibit supernumerary and mosaic organs, in addition to abnormally fused parts and reduced fertility. The stipules of the mutant are replaced by alternative leaf parts. At some nodes, this involves the production of leaf-like structures consisting of petioles, leaflets and tendrils in place of the stipules. For this reason, *coch* can be described as a homeotic mutant. More recently, it was established that the stipule primordia of *coch* are reduced in size and retarded in their development (Gourlay et al. 2000; Yaxley et al. 2001), which might explain the abnormal stipule phenotype. Gourlay et al. (2000) demonstrated that a gene involved in leaf complexity, *UNIFOLIATA*, is expressed in the stipule primordia of *coch* at a time when compound stipule architecture is predicted to form. This was not observed in wild type plants, suggesting that the *COCH* gene product might act to inhibit *UNIFOLIATA* expression, thereby preventing the formation of compound leaf structures. Thus, *COCH* may act as a signaling element for organ identity.

As a homeotic mutant with altered primordia, *coch* represents an excellent tool for investigating plant developmental processes. Nodulation is a symbiotic process in which specific soil bacteria of the genus *Rhizobium* invade compatible host plants. This process is complex, requiring multiple, tightly regulated, signalling interactions (Ferguson and Mathesius 2003) that lead to the formation of novel structures called nodules (e.g. Mathesius 2003). Within these nodules, the bacteria fix atmospheric nitrogen for the host plant in exchange for shelter and carbohydrates.

Legume mutants exhibiting abnormal nodulation characteristics have aided greatly in elucidating mechanisms required for nodule development (reviewed in Oldroyd and Downie 2004). Recently, a novel approach was undertaken by Ferguson et al. (2005), in which previously characterized mutants, whose nodulation phenotypes were unknown, were investigated. In addition, a new nodulation mutant, SGE*amp*, was described as having a shoot phenotype similar to that of *coch* (Voroshilova et al. 2004). Based on this, we continued with the line of investigation used by Ferguson et al. (2005) and describe here the nodulation and root phenotypes of the homeotic mutant, *coch.*

The *coch* mutant and its wild type, Torsdag, had formed a similar number of nodules by 25 days (Table I). However, the morphology of the nodules on *coch* plants was uncharacteristic. Typically, these nodules were dichotomously branched and possessed small, emerging root structures, thus creating root-nodule hybrid structures (Fig. 1). The roots of these structures emerged from the meristems, generally protruding from the sides of the nodule lobes and displaying agravitropism (Fig 1). In addition, many of the nodule lobe meristems became swollen, resembling calli (Fig. 1D-F). The central zone of the nodule lobes appeared normal and characteristically exhibited a pink hue, representing the leghaemoglobin required for nitrogen fixation. The location of these hybrid structures on the root system was also normal, being dispersed throughout the mature portion of the root system, similar to those of Torsdag. This nodule phenotype was observed on three independently derived *coch* mutants, indicating that it is specfic to the *coch* mutation.

See Table I

See Fig. 1

The total, and average, nodule DW values of 25 day-old *coch* were similar to those of Torsdag (Table I), suggesting that the nodules of both lines were developing at similar rates. The lateral root lengths (Table II) and root system DW (Table I) of *coch* were also not significantly different from those of Torsdag, although there were moderate differences in the number of lateral roots formed (Table II).

See Table II

The hybrid roots of *coch* had markedly elongated by 40 days compared with those observed at 25 days (Fig. 2). They possessed root hairs and, in some instances, even gave rise to new root and nodule structures (Fig. 2F). The nodule portion of the hybrids also continued to grow, often further branching and continuing to develop new root and callus-like structures. These findings illustrate that both the roots and nodules of the hybrids have their own, distinct meristems, allowing them to elongate at their own rates. Taken together, extremely complex hybrid structures consisting of a multitude of roots, nodules and calli, developed from a single initiation point (i.e. infection site) on the lateral roots of *coch*. In many cases, these hybrid structures grew until they engulfed the root from which they arose (Fig. 2G).

See Fig. 2

The nodule number of 40 day-old *coch* and Torsdag root systems remained similar to their respective values observed at 25 days (Table I), indicating that the autoregulation of nodulation was functioning properly in both genotypes. In addition, the mutant root system DW remained similar to that of Torsdag (Table I). However, due to the additional root and nodule structures, the total and average nodule DWs of *coch* were significantly greater than those of Torsdag at 40 days (Table I).

Torsdag nodules were histologically similar to those previously described for wild type pea (e.g. Newcomb et al. 1979). Those of 25 day-old plants possessed an outer cortex, a peripheral vasculature connected to the central vasculature of the lateral root and three distinct histological zones: the meristematic, invasion, and infected zones (Fig. 3A). The majority of cells in the infected zone appeared to have been invaded by the bacteria and contained nitrogen-fixing bacteroids.

See Fig. 3

The nodules of 25 day-old *coch* plants also exhibited an outer cortex, and, although bifuricated, each lobe typically displayed the three histological zones observed in Torsdag (Fig. 3B-F). Meristematic tissue was not observed in the invaginated region between the nodule lobes of *coch*, although, the cells of the entire infected zone did appear to contain bacteroids. At least one vascular strand was observed in each nodule lobe of *coch*. Generally, the vasculature appeared thicker than that observed in Torsdag nodules, often branching and veering over the various histological zones. Collectively, the normal infected zone histology (Fig. 3) and the pink hue (Fig. 1,2) of the nodule, in addition to the healthy green shoots produced in the absence of applied nitrogen (data not shown), suggest that the process of nitrogen fixation is probably functional in *coch*.

The roots of *coch* hybrid structures also appeared functional. They consisted of a root cap, meristem, cortex, central vasculature and root hairs, similar to that of a lateral root (Fig. 3H-K). The vasculature was incorporated into the hybrid root from a vasculature strand of the nodule (Fig. 3J,K). The xylem and phloem of the hybrid roots was arranged in a triarch pattern of distribution, typical of a lateral root (Fig. 3H,I).

The fact that *coch* formed a similar number of nodules to that of Torsdag indicates that the *coch* mutation does not prevent bacterial recognition or infection, nor does it prevent the autoregulation of nodule formation. In addition, *coch* nodules appeared functional and its root system DW and lateral root lengths were similar to those of Torsdag, demonstrating that these parameters are also not affected by the mutation. Furthermore, *coch* does not affect the development of the shoot, other than the stipule and flower (Wellensiek 1959; Yaxley et al. 2001), suggesting that the role of the *COCH* gene product in development is organ specific. Alternatively, there may be redundancy of *COCH* in organs of the mutant that display a wild type phenotype. The fact that the *coch* mutation results in ectopic roots developing from the nodules and alternative leaf components replacing the stipules suggests that COCH may normally function to inhibit the development of these structures.

Root structures emerging from branching nodules have also been reported on *Sesbania grandifolia* Pior. (Harris et al. 1949). Moreover, root-nodule hybrids have been observed on *M. sativa* (Dudley et al. 1987), *T. repens* (McIver et al. 1997) and *Phaseolus vulgaris* (VandenBosch et al. 1985; Ferraioli et al. 2004) following inoculation with specific *Rhizobium* strains. However, these hybrids differed morphologically from those of *coch*, as the nodule zonation pattern, and multiple root, nodule and callus structures characteristic of *coch* hybrids, were not observed. It has also been reported that increasing the temperature can convert the nodule apex of *Medicago sativa* and various *Trifolium* sp. into roots and calli structures (Dart 1977; Day and Dart 1975), possibly suggesting that high temperatures interfere with the activity of the *COCH* gene product.

Nodules and roots share many aspects of their development, consistent with the theory that nodulation may have evolved from pre-existing mechanisms of early lateral root development (Hirsch and LaRue 1997; Mathesius et al. 2000; Mathesius 2003; Ferguson et al. 2005). The root-nodule hybrids of *coch* further support this theory, as do the nodule-like structures of many non-legumes, which are clearly derived from modified lateral roots (Hirsch and LaRue 1997). Although these structures are distinctly different from legume nodules, some aspects resemble those of *coch*, such as the agravitropic roots that emerge from the actinorhizal nodule meristems of *Casuarina cunninghamiana* (Torrey 1976) and *Myrica gale* (Torrey and Callaham 1978). In addition, mycorhizal nodules of Podocarpaceae species, which form even in the absence of the fungus, appear to be novel outgrowths that have diverged from the root developmental pathway (Russell et al. 2002).

Although the stipules of *coch* are replaced by alternative leaf structures, the nodules are not actually replaced by roots, but rather form concomitantly with them. This phenotype is analogous to that of *ufo/fim* homeotic mutants of *Arabidopsis* and *Antirrhinum*, which mediate floral meristem and organ identity (Ingram et al. 1995). Yaxley et al. (2001) hypothesized that there may be homology existing between certain meristems of pea because the primordia base of the petals and leaves is altered in *coch*. The meristems of *coch* nodules are also altered, giving rise to root and callus structures, possibly indicating common developmental abnormalities among these meristems. Yaxley et al. (2001) proposed that *coch* stipule meristems might remain meristematic for a prolonged period of time, leading to greater meristematic flexibility and retarded stipule

development. If this is true for *coch* nodule meristems, it may explain why they appear like swollen calli and why both nodule and root structures form.

The phytohormone auxin has been reported to induce the formation of meristems on the roots of rice (Ridge et al. 1993). These meristems consisted of nodule-like, modified root outgrowths that displayed a callus-like surface, but a differentiated internal anatomy, as observed in *coch* nodules. Auxin also regulates root gravitropism (Marchant et al. 1999), which is dysfunctional in *coch* and actinorhizal nodule-roots. Thus, altered levels or perception of auxin may have a role in the development of the root-nodule hybrids of *coch*. Temporally and spatially determining the auxin content of *coch* root-nodule hybrids would advance the understanding of this hormone in the development of these organs.

Unlike other meristematic processes, the initiation and location of nodule development can be tightly controlled in the laboratory, making nodulation an excellent process to study meristems. The *coch* mutant represents an exciting tool for such studies, particularly since its mutation appears to affect certain organs, but not others. In addition, *coch* could provide insight into nodule branching and could help delineate between which aspects of nodule development are shared with, and which are unique to, lateral root formation. The fact that *coch* nodules, stipules and flowers are abnormal, but the remainder of the root and shoot systems are not, suggests that *COCH* may have been recruited into the process of nodule development from the flower and/or stipule developmental program(s). Of utmost importance will be to identify the initial cells from which the hybrid roots develop and to clone the *COCH* gene. Once the gene is available,

the roles of COCH in organ identity, including its affects on organ initiation, hormone manipulation and development, can be ascertained.

Materials and Methods

The *coch* allele (Hobart line AF99) was produced by EMS mutagenesis by Dr. J Weller from its wild type, Torsdag (Hobart line 107) (Yaxley et al. 2001). Additional, independently derived *coch* mutants, JI 2757 and JI 2165, were obtained form the John Innes Centre, UK, to ensure that the nodule phenotype observed is due to the *coch* mutation. Plants were grown as outlined in Ferguson et al. (2005). For nodulation studies, seeds were sown in 100 mm "Space Saver" pots (Reko, Australia) and for root analysis experiments, seeds were sown in 200 mm "Plastamatic" pots (Melbourne, Australia). At the time of sowing, each pot was provided with either 25 ml (nodulation studies) or 150 ml (root characterization experiments) of *Rhizobium leguminosarum* bv. *viciae* 128C53K (Nitragin® Inoculants, Liphatech Inc., Milwaukee, WI) grown in yeast-mannitol broth and diluted with water to approximately OD_{600} 0.01, which represents 5 x 10^6 cells ml^{-1}.

For nodule studies, plants were harvested 25 and 40 days after planting. Analysis at 25 days allowed for the development of the nodules to a stage where they could be clearly distinguished and accurately assessed. Delays in nodule development can be identified at 40 days, when the plants initiate flowers and the formation of new nodule

structures should be minimal due to the autoregulation of nodulation (e.g. Searle et al. 2002).

Upon harvesting, the roots and shoots were separated at the cotyledon, which was excised and discarded. The root system was rinsed and placed in water. The nodules were then characterized, counted and removed with forceps. The complex nodules of *coch* (which were comprised of multiple lobes, roots and calli) were counted as one nodule because they arose from a single initiation point (i.e. infection site) on the lateral root. The roots, shoots and nodules of each plant were then placed in a 60°C oven for a minimum of three days to obtain their dry weights (DWs).

For root studies, plants were harvested 17 day after planting, allowing for the development of secondary and tertiary lateral roots. The plants were uprooted, rinsed, and placed in water. The length of the shoot and longest secondary and tertiary lateral root was measured. In addition, the total number of secondary lateral roots, and the number of tertiary lateral roots located on each of the upper (i.e. closest to the crown) six secondary lateral roots were recorded.

For histological examinations, portions of lateral roots bearing nodules were excised from 25 day-old plants. The specimens were fixed in 3.7 % (v/v) formaldehyde for 3 h, dehydrated in a graduated ethanol series for 5 h, followed by xylene treatment for 2 h, and embedded in Paraffin (Paraplast®, melting point 56°C; VWR Scientific, West Chester, PA, U.S.A.) using a vacuum infiltration processor (Tissue Tek® VIP™, Sakura Finetek, Japan). Longitudinal serial sections 3 μm thick were cut using a Leitz 1512 microtome (Ernst Leitz Westlar GmBH, Austria) and transferred to slides. Paraffin was removed by soaking the slides in xylene. The slides were then rinsed in ethanol,

followed by water. All microtome and hand sections were stained with Toluidine blue, observed under an Axioskop 2 Plus microscope (Carl Zeiss, Gottingen, Germany) using differential interference contrast illumination and photographed with an AxioCam HRc digital camera (Carl Zeiss, Gottingen, Germany).

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Table I. Root and nodule dry weights, and nodule numbers of 25 day-old Torsdag and *cochleata*

Age (d)	Genotype	Dry Weight (mg)			Number of Nodules	
		Root	Nodule Total	Nodule Average	Per Plant	Per mg Root Dry Weight
25	Torsdag	177 ± 16	51.7 ± 8.2	0.41 ± 0.053	125 ± 8	0.74 ± 0.07
	coch	198 ± 18	62.1 ± 6.1	0.43 ± 0.058	148 ± 10	0.80 ± 0.11
40	Torsdag	236 ± 17	85.6 ± 17.3	0.60 ± 0.078	137 ± 12	0.59 ± 0.03
	coch	273 ± 28	149.4 ± 19.4*	1.09 ± 0.153*	141 ± 11	0.47 ± 0.09

Plants were inoculated with *R. leguminosarum* at the the time of sowing. Results are means ± SE (n = 7). Values for each *coch* trait followed by an * are significantly different from those of similarily aged Torsdag traits at the 0.05 level.

Table II. Root numbers and lengths of 17 day-old Torsdag and *cochleata*
Indicated are the number of secondary lateral roots per plant in addition to the average number of tertiary lateral roots located on the six uppermost secondary lateral roots per plant. Also shown are the lengths of the shoot and the longest secondary and tertiary lateral roots per plant.

Genotype	Number		Length (cm)		
	Secondary Roots	Tertiary Roots	Shoot	Secondary Root	Tertiary Root
Torsdag	89 ± 3.6	20 ± 1.1	12.5 ± 0.3	19.7 ± 0.1	4.3 ± 0.2
coch	78 ± 2.7*	26 ± 0.8*	12.1 ± 0.8	20.2 ± 0.6	4.3 ± 0.1

Results are means ± SE (n = 6). Values for each mutant trait followed by an * are significantly different from that of Torsdag at the 0.05 level.

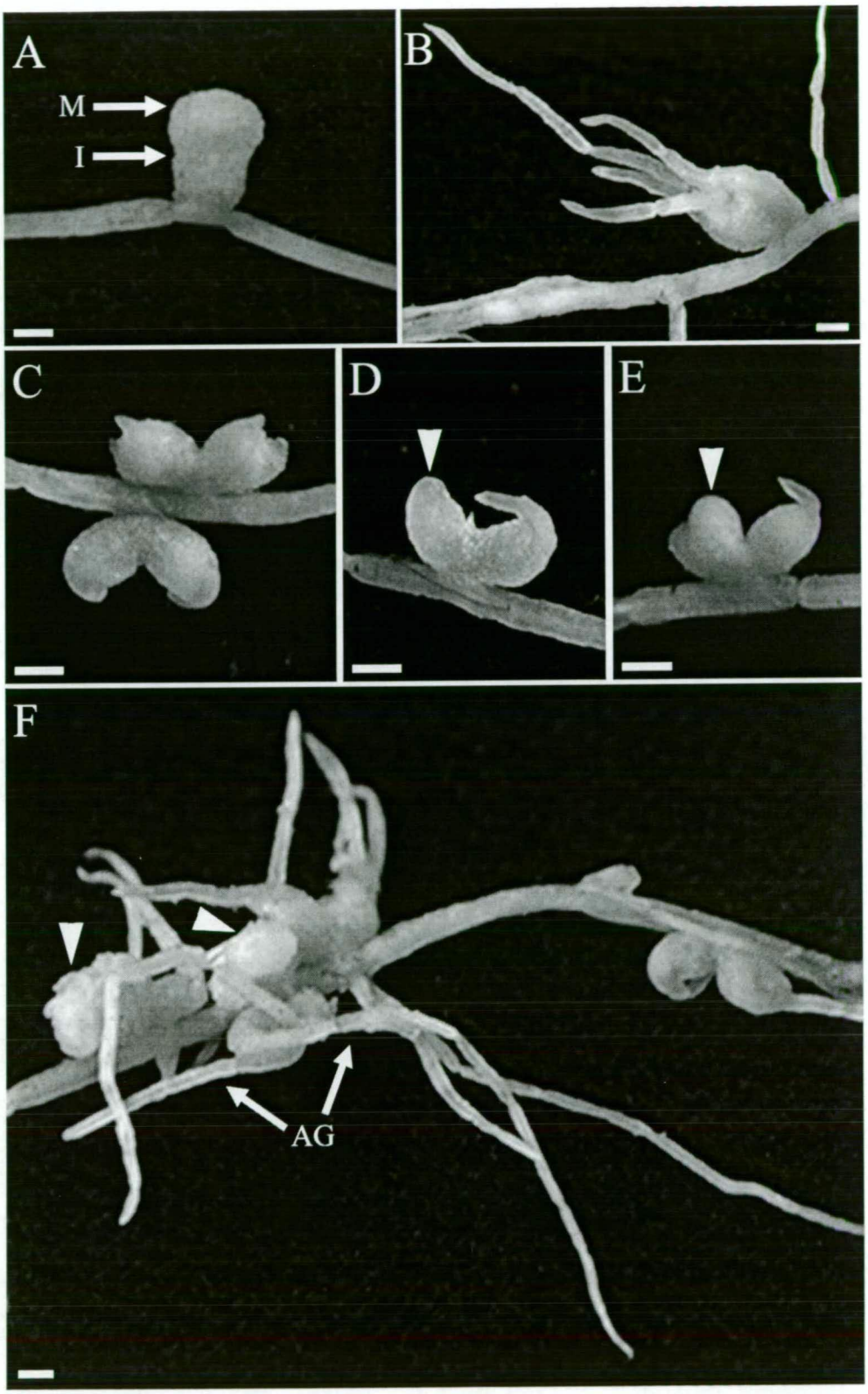
Figure Legends:

Figure 1. Lateral root nodules of 25 day-old A) wild type and B-F) *coch* plants inoculated with *R. leguminosarum*. White meristems and pink central infected zones were apparent in the nodules of both genotypes. The nodules of *coch* dichotomously branched and typically exhibited callus-like structures and agravitropic roots emerging from their meristems. M=meristem, I=infected zone, AG=agravitropic roots, arrowheads=callus. Bars = 1 mm.

Figure 2. Nodules of 40 day-old A) wild type and B-G) *coch* plants inoculated with *R. leguminosarum*. As observed at 25 days (Fig. 1), the nodules of both lines possessed white meristems and pink infected zones, and those of *coch* typically branched and possessed root and callus-like structures. These structures were more numerous and markedly larger, compared with those observed at 25 days. D, E) The roots of these hybrids also exhibited callus-like structures, which tended to form in close proximity to the attachment site of the hybrid root on the nodule. F) Hybrid roots also occasionally gave rise to their own root and nodule structures. G) The complex root-nodule hybrids often engulfed the roots from which they arose. M=meristem, I=infected zone, AG=agravitropic roots, R=emerging root, N=emerging nodule, arrowheads=callus, PR=primary root. Bars = 1 mm.

Figure 3. Light micrographs of 25 day-old A) wild type and B-K) *coch* nodules. A-E) The meristems, invasion zones, and infected zones (which include cells harbouring the

bacteria) are clearly distinguishable in the nodules of both lines. B-F) Meristematic tissue was not observed in the invaginated regions occurring between the mutant nodule lobes. The nodule vasculature of both lines is connected to that of the lateral root, however, the A) wild type vasculature is thin and peripheral, whereas that of B-D) *coch* is markedly thicker and frequently oriented away from the nodule periphery. B,E) Serial sections of the same nodule depicting thick vasculature extending from the lateral root through to a hybrid root beginning to emerge from the nodule meristem. C) A swelling developing from the meristem of a *coch* nodule lobe. D) Hybrid roots emerging from the meristems of nodule lobes. F-K) Hand sections of *coch* nodules and roots. F) Cross section of a *coch* nodule with vasculature extending towards the infection threads and meristem, which is swollen and appears to be initiating an ectopic root. G) Hybrid root exhibiting a root cap, meristem, root hairs and zones of elongation and maturation. Cross section of *coch* H) lateral and I) hybrid roots illustrating the endodermis, cortex, epidermis and similar triarch distribution patterns of xylem and phloem. The reduced size of the hybrid root is probably due to it being younger than the lateral root. J,K) Cross sections demonstrating the vascular connections existing between the nodule and hybrid roots of *coch.* M=meristem, IZ=invasion zone, I=infected Zone, V=vasculature, LR=lateral root, S=swelling, HR=hybrid root, IT=infection threads, RC=root cap, EZ=elongation zone, MZ=maturation zone, RH=root hairs, X=xylem, P=phloem, EN=endodermis, EP=epidermis, C=cortex. Bars = 200 μm (A-F,J,K), 25 μm (H,I).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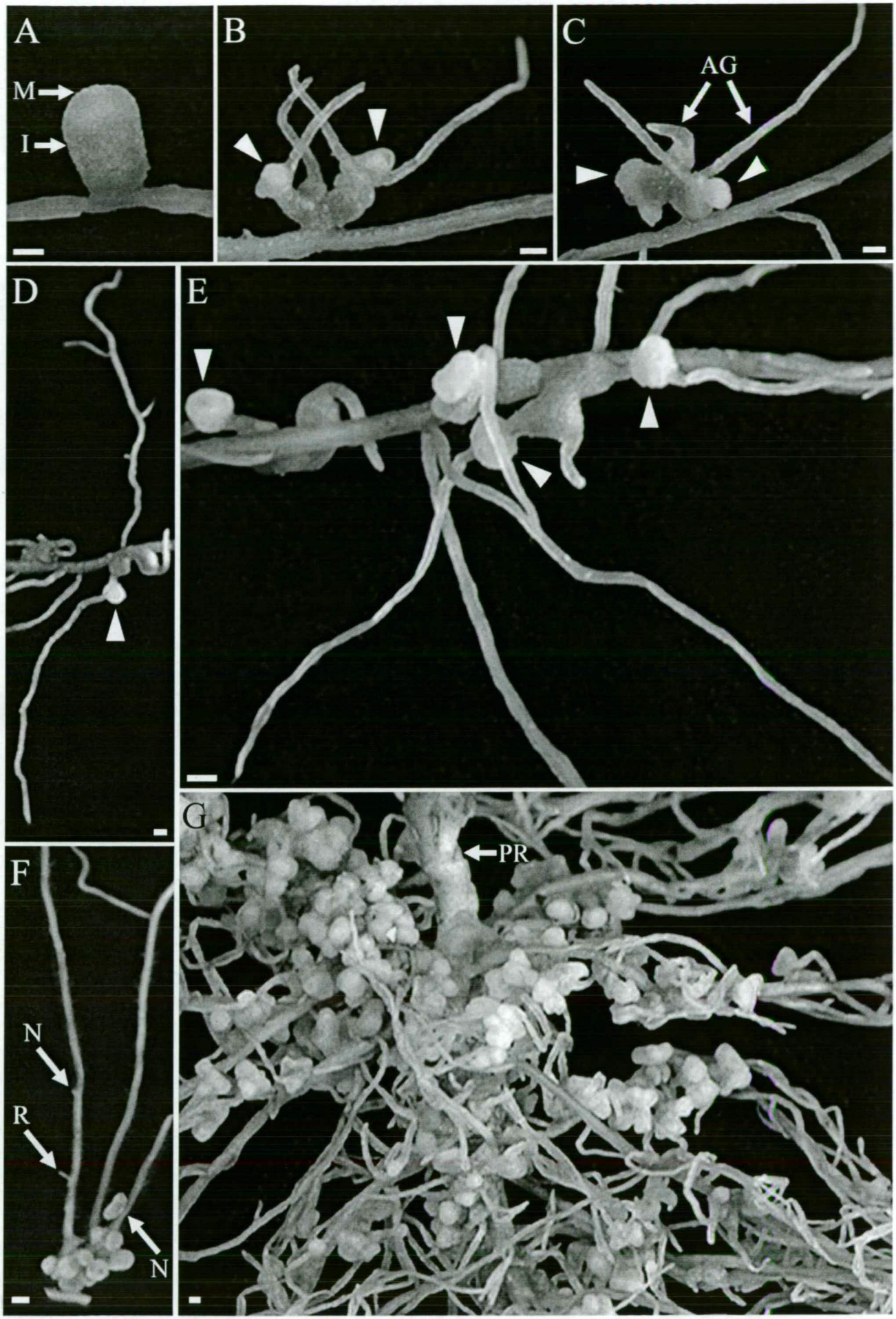
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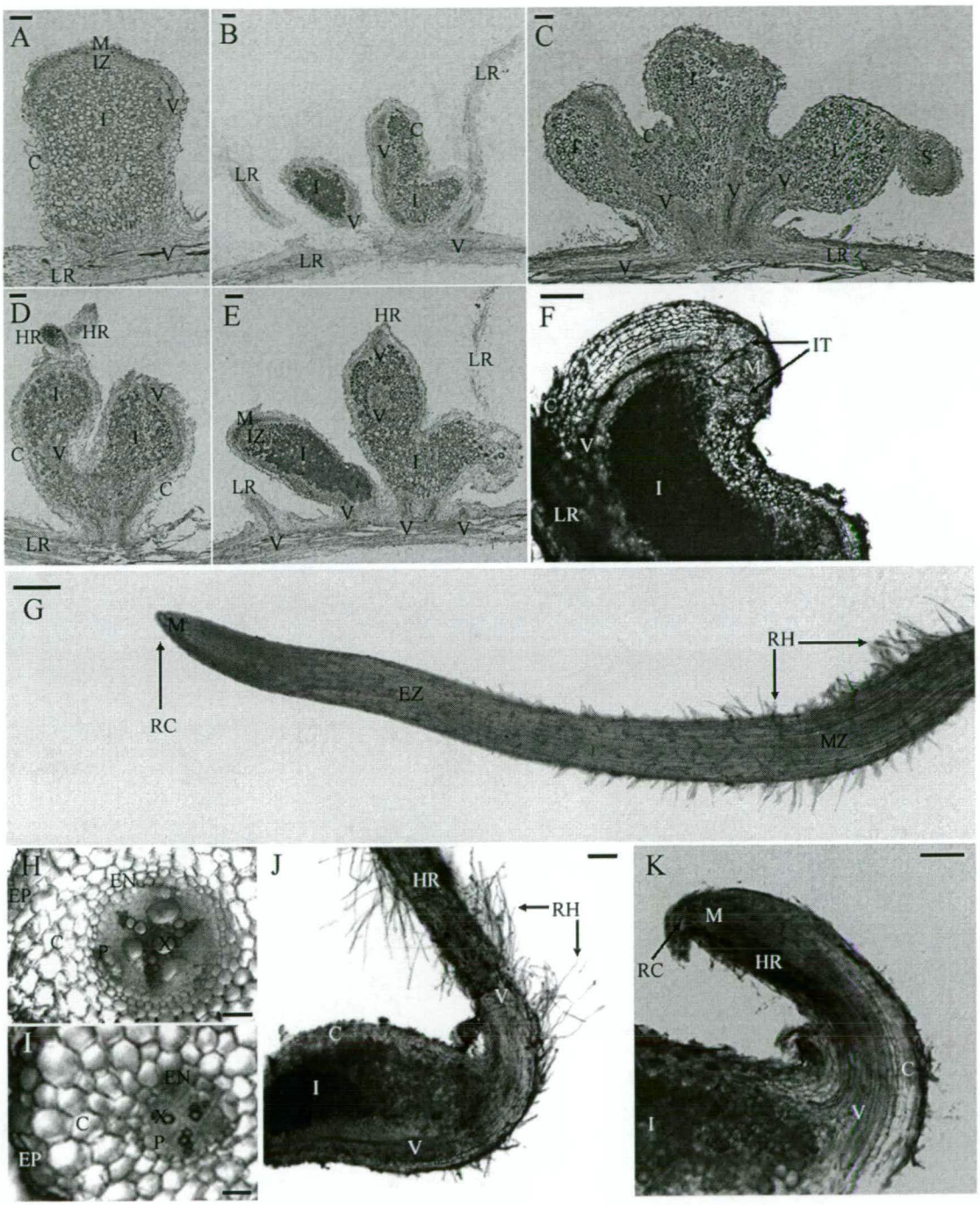
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