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REVIEW ARTICLE

Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue

J.E. Cooper

Department of Applied Plant Science, Queen's University Belfast, Newforge Lane, Belfast, United Kingdom

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Correspondence
James E. Cooper, Department of Applied Plant Science, Queen's University Belfast, Newforge Lane, Belfast BT9 5PX, United Kingdom. E-mail: j.e.cooper@qub.ac.uk

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Summary

The exchange of chemical signals between soil bacteria (rhizobia) and legumes has been termed a molecular dialogue. As initially conceived in the early 1990s, it involved two main groups of molecules: *nod* gene-inducing flavonoids from plants and the mitogenic lipochito-oligosaccharide Nod factors of rhizobia. This review considers how subsequent research revealed the existence of a more complex set of interactions, featuring expanded roles for the original participants and contributions from additional plant and bacterial metabolites. Rhizobia respond to chemoattractants and growth-enhancing compounds in root exudates, and several plant nonflavonoids possess *nod* gene-inducing properties. Expression of non-*nod* genes is induced by flavonoids; these include encoders of a type I secreted protein and the entire type III, and possibly also type IV, secretion systems. Many other genes and proteins in rhizobia are flavonoid-inducible but their functions are largely unknown. Rhizobia produce far more Nod factor variants than was previously envisaged and their structures can be influenced by the pH of the environment. Other symbiotically active compounds or systems of rhizobia, some of them universally present, are: the surface polysaccharides, quorum-sensing *N*-acyl homoserine lactones, plant growth-promoting lumichrome and two-component regulatory systems.

Introduction

With reference to plant-bacterium symbioses the term 'molecular dialogue' was first used by Dénarié *et al.* (1993) to describe the interchange of chemical signals that leads to infection of root hairs and the formation of root nodules on legumes by nitrogen-fixing bacteria collectively known as rhizobia. At that time, information on two of the foremost participants in the dialogue – flavonoids (released by the host plant) and lipochito-oligosaccharide Nod factors (synthesized by rhizobia) – was accumulating at a rapid pace. It had also been established that the regulatory and structural nodulation (*nod*) genes of rhizobia were involved in the reception of the former and synthesis of the latter, respectively. The first flavonoid *nod* gene inducers, luteolin from *Medicago sativa* and 7,4′ dihydroxyflavone from *Trifolium repens*, had been discovered in 1986 (Peters *et al.* 1986; Redmond *et al.* 1986) and the first structural formula of a Nod factor, from *Sinorhizobium meliloti*, was reported in 1990 (Lerouge *et al.* 1990). In the intervening years, more examples of *nod* gene-inducing flavonoids have been found in the tissues and epidermal exudates of legumes and the structures of many Nod factors from various rhizobia have been characterized. The roles originally ascribed to these molecules have been substantiated beyond question (at least in soil-free laboratory conditions) and both types have been found to operate in virtually every combination of legume and root nodule bacterium studied so far. However, the relatively simple two-signal model (supplemented by rhizobial surface polysaccharide involvement, already accepted in 1993) has undergone considerable modification. This has been necessary in order to accommodate a newly appreciated structural diversity among Nod factors as well as a number of other compounds of plant or bacterial origin that are now known to influence the course of root infection, even if precise functions remain to be determined for some of them. In addition

to flavonoids, plant signals to rhizobia include betaines, aldonic acids, xanthones, simple phenolics and jasmonates, all of which have been shown to act as *nod* gene inducers. Compounds synthesized by rhizobia encompass not only Nod factors and surface polysaccharides but also types I, III and IV secreted proteins, *N*-acyl homoserine lactones (AHL), bradyoxetin, hopanoids, lumichrome, indole-3-acetic acid (IAA), and a host of proteins (both characterized and putative) whose contributions to the dialogue, if any, have yet to be defined.

In the case of flavonoids, research in the 20 years following the discovery of their *nod* gene-inducing attributes has served to accentuate the significance of their contribution to the dialogue. In addition to inducing *nod* gene expression and Nod factor synthesis they activate the production of all the proteins and some of the other compounds referred to before. The surface polysaccharides of rhizobia that are required for a successful symbiosis can also be modified by flavonoids either during or after their synthesis. Furthermore, endogenous root flavonoids act as developmental signals by inhibiting the transport of auxin to the sites of nodule organogenesis (Mathesius *et al.* 1998).

This article reviews the progressive disclosure of an increasing complexity in the cross-talk between legume hosts and their nitrogen-fixing microsymbionts. It focuses on signals received from the plant by rhizobia and the compounds that are in turn synthesized by these bacteria during the early stages of symbiotic development. The responses of the plant to rhizobial signals are mentioned briefly but a full consideration of, e.g. Nod factor reception and attendant signal cascades can be found elsewhere (see Miklashevichs *et al.* 2001; Geurts *et al.* 2005; Mulder *et al.* 2005).

Signals from Legumes to Free-living Rhizobia

Flavonoids

Flavonoids are plant secondary metabolites that are synthesized via the central phenylpropanoid pathway and the acetate-malonate pathway (Forkmann and Heller 1999; Aoki *et al.* 2000). When released from seed coats or roots they act as inducers of the structural *nod* genes of rhizobia that are required for Nod factor synthesis. In the case of soybean, endogenous isoflavonoids have been shown to induce *Bradyrhizobium japonicum nod* genes after the bacterium has entered the root (Subramanian *et al.* 2006). Approximately 30 *nod* gene-inducing flavonoids have been isolated from nine legume genera under axenic conditions (see Cooper 2004). They are either glycones or aglycones from a variety of flavonoid subclasses including chalcones, flavones, flavanones, isoflavones and coumestans, and their gene-inducing activity is frequently exhibited at low micromolar or even nanomolar concentrations. Mixtures of flavonoids can be more effective than single compounds (Bolanos-Vasquez and Werner 1997; Begum *et al.* 2001) and in some instances they can act as inducers for certain rhizobia and antiinducers (antagonists) for others. Such is the case with the isoflavones daidzein and genistein, which are inducers for *B. japonicum* and *Rhizobium* sp. NGR234 but antiinducers for *Rhizobium leguminosarum* bv. *trifolii* and bv. *viciae*. The release of more flavonoids from legume roots, with a consequent increase in *nod* gene-inducing activity of root exudates, can be prompted by the presence of compatible rhizobia in the rhizosphere (e.g. van Brussel *et al.* 1990). Rhizobial Nod factor can trigger this process (Schmidt *et al.* 1994) but the involvement of an even earlier, as yet unknown, bacterial signal should not be discounted.

The mode of action of flavonoids during *nod* gene induction is open to some speculation but certain aspects are well understood. Transcription of genes required for Nod factor synthesis is usually mediated by NodD proteins, regulatory products of the predominantly constitutively expressed *nodD* genes. NodDs are members of the LysR family of transcriptional regulators and rhizobia may contain between one and five homologues, depending on the species/biovar. They bind to conserved DNA sequences, known as *nod* boxes, which are found in the promoter regions of inducible *nod* genes, producing a bend in DNA at the binding site (Fisher and Long 1993). Both NodD protein and flavonoid coinducer are required to activate transcription of Nod factor biosynthesis genes and it has long been suggested that a NodD-flavonoid complex is formed at the *nod* box. The existence of such an entity remains unproven (Schlaman *et al.* 1998; Taylor and Grotewold 2005), but indirect evidence for an interaction between the two molecules is plentiful (see Peck *et al.* 2006). Recent research suggests that the presence of an appropriate flavonoid sharpens the bend in DNA at the location of NodD in the promoter site, thereby allowing RNA polymerase to initiate gene transcription (Chen *et al.* 2005). Interaction between flavonoid and NodD does not necessarily result in transcription: out of several flavonoids that could bind to NodD1 of *S. meliloti*, only luteolin was capable of activating *nod* gene expression (Peck *et al.* 2006). NodDs from different rhizobia respond to different sets of flavonoids but there appears to be no consistent correlation between the number of flavonoids capable of inducing *nod* genes in a rhizobium species or biovar and the host range of the bacterium. For instance, *Rhizobium* sp. NGR234 has a very broad host range and is responsive to a wide variety of flavonoid inducers whereas *R. leguminosarum* bv. *viciae*, which also responds to many inducers, has a narrow host range.

The flavonoid-NodD-*nod* box induction mechanism operates for most Nod factor biosynthesis genes. Taking *Rhizobium* sp. NGR234 as an example, 14 of its 16 genes required for Nod factor production (Freiberg *et al.* 1997) are distributed among five operons, each controlled by a different *nod* box (Kobayashi *et al.* 2004). However, *nod* boxes are not restricted to the promoters of these genes: *Rhizobium* sp. NGR234 possesses 13 other such elements in the promoter regions of a variety of genes that are unconnected with Nod factor synthesis (Kobayashi *et al.* 2004). Thus in this bacterium flavonoids, together with NodD1 (and in some cases also NodD2), are required for the expression of regulatory or structural genes involved in such diverse functions as secretion of type III proteins, synthesis or modification of extracellular polysaccharides and synthesis of IAA. Another important discovery by Kobayashi *et al.* (2004) was a regulatory mechanism which ensured that 36 flavonoid-inducible, *nod* box-controlled genes were not expressed simultaneously, but rather in a sequence starting with Nod factor biosynthesis, followed by type III protein secretion and somewhat later by production of rhamnose-rich lipopolysaccharide (LPS). In this way, the expression of a symbiotic gene is timed to coincide with the requirement for its contribution to a particular stage of root infection or nodule development.

Flavonoid-dependent induction extends to rhizobial genes that do not contain *nod* box motifs in their promoter regions. Such is the case with genes in the aforementioned type III secretion system (TTSS), which require a second transcriptional activator, TtsI (see next). Evidence that flavonoids induce the expression of many more rhizobial genes continues to accumulate; e.g. exposure of free-living cells of *Rhizobium* sp. NGR234 to daidzein enhanced the transcription of 147 open reading frames (ORFs) which were previously silent (Perret *et al.* 1999). To cite only the most recent of several other transcriptional studies: Zhang and Cheng (2006), using *in vivo* expression technology (IVET) to detect *S. meliloti* genes expressed during the early root infection stage of symbiosis, identified 23 alfalfa root exudate-inducible genes of which 17 were flavonoid (apigenin)-inducible. Most of the latter group of genes had not been previously characterized in this organism and none contained a *nod* box motif (H.-P. Cheng, personal communication). Proteome analyses of rhizobia have also detected new proteins whose expression requires the presence of flavonoids in the culture medium (Guerreiro *et al.* 1997, 1999; Chen *et al.* 2000; Süß *et al.* 2006). Although essential symbiotic functions have not yet been ascribed to most of these genes and proteins, it is nonetheless abundantly clear that the rhizobial response to host plant flavonoids comprises a wealth of new and varied gene expression, far in excess of that which is required for Nod factor synthesis.

Nonflavonoid *nod* gene inducers

Several nonflavonoids can induce rhizobial *nod* gene transcription. In most cases their inducing properties are displayed only at higher concentrations than flavonoids and their ability to substitute for them as coinducers with NodD1 has not been proven.

Among the first examples found were the betaines stachydrine and trigonelline from seeds of *Medicago* species (Phillips *et al.* 1992); they are coinducers, with NodD2, of some *nod* genes in *S. meliloti*. Demethylation of stachydrine by *S. meliloti* may increase its gene-inducing activity (Goldmann *et al.* 1994). Lupin seeds also release compounds with inducing properties: the aldonic acids erythronic and tetronic acid (Gagnon and Ibrahim 1998). They induce Nod factor production in the cognate *Rhizobium lupini* and also in *Lotus*-nodulating *Mesorhizobium loti*, although their presence in *Lotus* seed coats or roots has not been reported. Indeed, the identity of *nod* gene inducers released by *Lotus* species remains a conundrum (Saeki and Kouchi 2000); a wide variety of flavonoids has been identified in this plant genus (Steele *et al.* 1999) but none of the many that have been tested is an inducer of *nod* genes in *M. loti* (López-Lara *et al.* 1995). Jasmonates are usually viewed as inducers of plant genes involved in response to wounding or defence against pathogens but they can also stimulate *nod* gene expression in *R. leguminosarum* and *B. japonicum*, either alone or synergistically in combination with a flavonoid inducer (Rosas *et al.* 1998; Mabood *et al.* 2006). *Bradyrhizobium japonicum nod* gene transcription can also be induced by xanthones (Yuen *et al.* 1995). Finally, the simple phenolics vanillin and isovanillin from a nonlegume, wheat, are capable of inducing *nod* genes in *Rhizobium* sp. NGR234 (Le Strange *et al.* 1990).

Chemoattractants, growth promoters and nodulation enhancers

In the legume rhizosphere rhizobia come under the influence of chemotactic and growth-promoting compounds, their combined effect being to increase root colonization. Rhizobia are positively chemotactic to unfractionated legume epidermal exudates and also to many individual flavonoids contained therein (reviewed by Cooper 2004). Other compounds in root exudates such as simple sugars, amino acids, dicarboxylic and hydroxyaromatic acids can also elicit a strong chemotactic response.

A role for flavonoids as growth promoters can be entertained on the grounds that rhizobia possess both the capacity to degrade them to monocyclic aromatics such as protocatechuic acid and 4-hydroxybenzoate (Rao *et al.* 1991; Rao and Cooper 1994) and the β-ketoadipate path-

way to channel these products into the citric acid cycle via succinyl CoA and acetyl CoA (Parke and Ornston 1986; Parke *et al.* 1991; Harwood and Parales 1996; Parke 1997). Another mechanism that confers enhanced growth in the host plant rhizosphere has been reported for *S. meliloti*: a regulatory locus in this bacterium, *bioS*, allows it to respond to biotin signals from *Medicago* by increasing its growth rate and its colonization of the root system (Streit *et al.* 1996; Heinz *et al.* 1999).

A functional *myo*-inositol catabolism pathway is found in some strains of *S. meliloti*, allowing them to degrade rhizopines synthesized by bacteroids in *Medicago* root nodules (Murphy *et al.* 1995; Galbraith *et al.* 1998); one such strain retained a competitive advantage for nodulation after 4 years in soil (Heinrich *et al.* 1999). Similar findings, over a shorter period of time in a non soil environment, were also reported for a *myo*-inositol degrading (but non rhizopine synthesizing/degrading) strain of *R. leguminosarum* bv. *viciae* (Fry *et al.* 2001) and a strain of *Sinorhizobium fredii* containing a functional *myo*-inositol dehydrogenase gene (Jiang *et al.* 2001). Several other researchers have proposed that catabolism of simple compounds in legume root exudates enhances the nodulating competitiveness of rhizobia (Jiminéz-Zurdo *et al.* 1995; Oresnik *et al.* 1998; Soedarjo and Borthakur 1998). The most recent report on this facet of rhizobial response to plant metabolites suggests that a mutation in erythritol catabolism impairs the nodulating competitiveness of a *R. leguminosarum* bv. *viciae* strain on pea plants (Yost *et al.* 2006). It should be noted, however, that preferential nodulation of a legume by a wild-type strain in admixture with a mutant that is defective in catabolism of a particular substrate does not constitute proof of a role for that catabolic function in competition under natural conditions.

Compounds Produced by Rhizobia

Nod factors

Nod factors are the products resulting from the concerted action of a suite of enzymes encoded by the mostly flavonoid-inducible structural *nod* genes of rhizobia. They are lipochito-oligosaccharides comprising β-1,4 linked *N*-acetyl-D-glucosamine residues with a fatty acyl chain attached at the nonreducing terminus. Variations in Nod factor structure arise from several sources: the length of the oligosaccharide backbone (between two and six *N*-acetylglucosamine residues), the type of fatty acid at the nonreducing terminus (common saturated/monounsaturated or specific highly unsaturated) and the number and types of substituent groups carried by the molecule (e.g. acetyl, arabinosyl, fucosyl, mannosyl, sulfate and so on). The organization and regulation of genes involved in Nod factor synthesis, as well as the structures and properties of Nod factors themselves, have been extensively reviewed (Schlaman *et al.* 1998; Perret *et al.* 2000; Spaink 2000; D'Haeze and Holsters 2002; Ovtsyna and Staehelin 2003).

Nod factors are essential signals in symbiotic development; without them rhizobia cannot enter legume roots (Relić *et al.* 1994). They are active at concentrations of *c.* 10^{-12} mol l^{-1} and they elicit a range of responses from the plant, including: deformation of root hairs; plasma membrane depolarization; rapid fluctuations in levels of intracellular free calcium in root hairs (known as calcium spiking); alterations in the root hair cytoskeleton; preinfection thread formation in deformed root hairs; cortical cell division at the sites of nodule primordia; inhibition of the reactive oxygen generating system; perturbation of auxin flow in roots (in conjunction with flavonoids); induction of plant genes (nodulins) at the preinfection, infection, nodule development and nodule function stages of the symbiosis (see Bartsev *et al.* 2004b). An interesting recent development in the study of Nod factor-induced changes in legume roots concerns the production of cytokinins via a calcium-dependent signalling pathway; through a cytokinin receptor these hormones appear to stimulate cortical cell division at incipient nodule sites. The pertinent research has been reviewed by Oldroyd (2007).

Much attention has been paid to the relationships between Nod factor structure and the host specificity of the producing bacterium. In some Nod factors certain features (e.g. sulfation in those of *S. meliloti*) are required for nodule formation on the cognate host (Lerouge *et al.* 1990) but for others (the large majority) this type of correlation has not been established (Ovtsyna and Staehelin 2003; Kannenberg and Carlson 2005). In fact, the number of different Nod factors synthesized by a single rhizobial species or biovar may be far higher than hitherto appreciated and it is difficult to see how such a wealth of structural diversity can be understood solely in terms of host specificity considerations. Of particular significance in this context is the discovery by Morón *et al.* (2005) that *Rhizobium tropici* strain CIAT899 produced 52 different Nod factors under acid conditions compared with 29 at neutrality, with only 15 structures being common to both treatments. As explained by Kannenberg and Carlson (2005) these findings are important for several reasons: they highlight a previously unacknowledged influence of rhizobial physiology and ecology on Nod factor production; they demonstrate that environmental parameters – in this case pH – can have a marked effect on the process; they suggest the existence of so far undiscovered genes and regulatory mechanisms involved in the biosynthesis of Nod factors as well as new functions for these compounds; finally, they indicate that the overall complexity

of Nod factor–plant interactions is much greater than it was previously thought to be.

Type I and type III secreted proteins

At least three mechanisms are responsible for the secretion by rhizobia of proteins that influence host range or suppress plant defence reactions. Two of them are considered here and the third in a later section.

NodO is a protein that has been found in only two rhizobial species (de Maagd *et al.* 1989a,b; van Rhijn *et al.* 1996) and that is released by a type I secretion system. It is a calcium-binding protein encoded by *nodO*, a flavonoid- and NodD-inducible gene that promotes infection thread development in root hairs (Walker and Downie 2000). Receipt of *nodO* by a rhizobium can extend the range of hosts nodulated (Vlassak *et al.* 1998) and the gene can also partially complement a nodulation defect in a *nodEFL* deletion mutant of *R. leguminosarum* bv. *viciae* (Downie and Surin 1990).

Rather more rhizobia possess a type III secretion system that is responsible for producing nodulation outer proteins (Nops), some of which may be delivered into host plant cells via pili on the bacterial surface (Krishnan *et al.* 2003). In contrast to the type I system, the genes required for the secretory apparatus, as well as those encoding secreted proteins, are dependent on a flavonoid and NodD for their induction. As noted before, most genes in a TTSS cluster do not contain a *nod* box sequence and induction is indirect, relying on other regulatory motifs in their promoter regions known as *tts* boxes; these respond to the transcriptional activator TtsI which is encoded by the *nod* box-containing, flavonoid- and NodD-inducible *ttsI* gene. A somewhat involved, but nonetheless effective, nomenclature for the various TTSS genes and proteins in rhizobia has been established by Viprey *et al.* (1998) and Marie *et al.* (2001, 2003). TTSS in general and Nops in particular can have a positive, negative or zero influence on nodulation, depending on the host plant involved; as an example of the second case, wild-type *Rhizobium* sp. NGR234 forms ineffective nodule-like structures on roots of *Crotalaria juncea* but a mutant strain with a disrupted TTSS forms effective nodules (Marie *et al.* 2001). In this instance, Nops from the wild-type strain were found to be impairing nodule development (Marie *et al.* 2003). Studies concentrating on NopL from NGR234 have shown that it suppresses plant defence reactions, probably by interfering with expression of genes activated by MAP kinase pathways, such as those encoding pathogenesis-related (PR) proteins (Bartsev *et al.* 2004a). Overviews of rhizobial TTSS components and their effects on host nodulation can be found in articles by Bartsev *et al.* (2004b) and Cooper (2004).

Surface polysaccharides

Rhizobia possess four main types of surface polysaccharides which contribute to various stages of symbiotic development including root colonization, host recognition, infection thread formation and nodule invasion. Specifically, they have been implicated in biofilm formation on root hair surfaces (Fujishige *et al.* 2006) and they are important for the evasion of plant immune responses and as protectants against reactive oxygen species (D'Haeze and Holsters 2004). They comprise extracellular polysaccharides (EPS), LPS, K polysaccharides (K-antigens, capsular polysaccharides or KPS) and cyclic glucans.

Extracellular complementation of EPS- (Niehaus and Becker 1998) and LPS- (Mathis *et al.* 2005) deficient mutants has confirmed the requirement (although not on the same host) for both types of polysaccharides in the development of a fully functioning nodule. With regard to EPS only, in *Rhizobium* sp. NGR234 Staehelin *et al.* (2006) proposed that the symbiotically active entity was in fact an exo-oligosaccharide (EOS) derived from EPS by enzymatic degradation with endo-β-1,4-glycanase encoded by the *exoK* gene. Comprehensive reviews of the synthesis, structures and functions of all classes of rhizobial surface polysaccharide are available (Breedfeld and Miller 1994; Price 1999; Becker *et al.* 2000, 2005; Fraysse *et al.* 2003).

In general, surface polysaccharide synthesis *per se* is not dependent on flavonoids for its induction; however, there is now ample evidence that flavonoids act to influence the final structures and symbiotic activity of these compounds either during or after their biosynthesis. Some apposite examples are as follows: EPS from *S. fredii* USDA193 had a lower average molecular mass and a reduced uronic acid content when the *nod* gene-inducing isoflavone genistein was present in the growth medium (Dunn *et al.* 1992). In *S. fredii* USDA205 the *O*-antigen polysaccharide (OPS) component of LPS is altered in carbohydrate composition and mass range in the presence of apigenin (Reuhs *et al.* 1994). Broughton *et al.* (2006) showed that flavonoid-inducible pathways were involved in the synthesis of a modified rhamnan *O*-antigen in a high molecular weight rhamnose-rich LPS from *Rhizobium* sp. NGR234; absence of this antigen adversely affected nodulation of several host legume species. LPS structural changes in some rhizobia, such as *Rhizobium etli*, appear to occur postsynthesis and involve methylation of particular OPS residues at low pH in the presence of anthocyanins (Duelli and Noel 1997; Noel and Duelli 2000).

Interactions between rhizobial EPS and carbohydrate-binding proteins, the lectins, on legume root hair surfaces have long been considered to be one determinant of host recognition. Remarkably, more than 30 years after the

pioneering studies of this phenomenon (Bohlool and Schmidt 1974; Dazzo and Hubbell 1975) the precise role of lectins in the recognition/infection process remains unclear (see Hirsch 1999; Hirsch *et al.* 2001 for the most recent reviews of this topic).

IAA, hopanoids, AHL, bradyoxetin and lumichrome

Several other compounds are produced by rhizobia, some of which may be required for the successful progression from root colonization to a functioning root nodule or, in the case of lumichrome, as an enhancer of plant growth prior to the onset of nitrogen fixation.

IAA is synthesized by several rhizobia (Prinsen *et al.* 1991; Theunis *et al.* 2004). In *Rhizobium* sp. NGR234 its production is controlled by *nod* box NB15 and is flavonoid-, NodD1-, NodD2- and SyrM2-dependent (Theunis *et al.* 2004). It might be expected that an IAA-deficient mutant would display at least some sign of nodulation impairment, but when such a strain was inoculated onto two host legumes normal nodule structures were formed, albeit with a reduced content of IAA and its conjugates compared with nodules containing the wild-type rhizobium (Theunis *et al.* 2004).

Hopanoids are pentacyclic triterpenoid lipids that are widely distributed in both gram-positive and gram-negative bacteria; they act as membrane reinforcers and are thought to confer resistance to various forms of environmental stress. In bradyrhizobia, hopanoids and their derivatives are present in amounts that are easily detectable by chromatographic and spectrometric analyses and they can account for more than 40% of the total cell lipid fraction (Kannenberg *et al.* 1995). Also, gene expression studies with *B. japonicum* have identified the enzymes squalene synthase and squalene-hopene cyclase, both of which are required for hopanoid synthesis (Perzl *et al.* 1997, 1998). In fast growing rhizobia however, no linkage between expression in the hopanoid biosynthesis gene cluster (*hpn*) and detection of hopanoids by chemical analysis has been established. Kobayashi *et al.* (2004) reported that hopanoid synthesis genes in *Rhizobium* sp. NGR234 are expressed in a flavonoid (daidzein)-, NodD1-dependent manner via *nod* box NB1, which could indicate a symbiotic function for these compounds. On the other hand, hopanoids themselves are undetectable in this bacterium after treatment with daidzein or a range of other flavonoids applied either individually or in combination (E.L. Kannenberg, personal communication). Other fast growing rhizobia have been found to be similarly devoid of detectable amounts of hopanoids (Kannenberg *et al.* 1995).

Regulation of gene expression in response to changes in bacterial population density can be effected by small, diffusible autoinducers know as quorum sensors. In rhizobia the most common signals of this type are AHL; they are detected within each cell by LuxR-type transcriptional activators and are responsible for inducing expression of genes whose products are required for host colonization and invasion. A non-AHL quorum sensor, bradyoxetin, operating through a two-component regulatory system in *B. japonicum*, represses *nod* gene expression as population density in the rhizosphere increases (Loh *et al.* 2002). In addition to influencing rhizobial gene expression AHL can also elicit changes in protein accumulation in at least one legume host: *Medicago truncatula* (Mathesius *et al.* 2003). In conjunction with density-dependent quorum sensing signals, an assortment of two-component regulatory systems (sensor protein kinase plus a response regulator) mediates the rhizobial reaction to environmental factors encountered during root infection. An example is VirA/VirG in *M. loti* which controls, via an unknown signal, expression of the *virB* operon encoding a type IV secretion system (Hubber *et al.* 2004). Incidentally, the presence of a *nod* box motif upstream of *virA* is indicative of roles for a flavonoid and NodD as primary coinducers of this particular two-component regulatory system. Reviews of both regulatory mechanisms have been prepared by González and Marketon (2003) and Soto *et al.* (2006).

Finally, a compound is produced by *S. meliloti* that enhances root respiration (thereby generating more exogenous carbon dioxide, upon which rhizobial growth is dependent) and improves the growth of *M. sativa* prior to the onset of nitrogen fixation by increasing its net carbon assimilation. This is lumichrome, a riboflavin degradation product (Phillips *et al.* 1999; Matiru and Dakora 2005).

Conclusions

This review has assessed the extent to which the concept of a molecular dialogue between rhizobia and legumes has changed after more than 13 years' research since the term was first introduced. Data collected over this period reveal two main trends: consolidation and elaboration. Both of them are applicable to flavonoids, Nod factors and surface polysaccharides while the latter covers the various additional elements in the communication network that have come to light; these include quorum sensing molecules, type I, III and IV protein secretion systems, lumichrome, nonflavonoid *nod* gene inducers and rhizobial growth enhancers.

The requirement for flavonoids as coinducers, with NodD1, of *nod* gene transcription (and therefore of Nod factor synthesis) has been confirmed many times over and this despite the discovery of several alternative inducing compounds, none of which has been shown to

replace flavonoids in this capacity. Transcription of numerous other rhizobial genes is either directly or indirectly dependent on flavonoid inducers and it has been clearly demonstrated that many of them are symbiotically active. The functions of others remain to be determined and new examples continue to be reported. Degradation of flavonoids by rhizobia and other soil micro-organisms with the attendant release of potential new signal molecules (see reviews by Cooper 2004; Shaw *et al.* 2006) are further phenomena whose consequences for plant–microbe interactions have not yet been explored. For all these reasons flavonoids have emerged as the dominant components in an array of plant and bacterial signals and, on account of their prime functions as *nod* gene inducers and auxin transport regulators during nodule organogenesis (Subramanian *et al.* 2006; Wasson *et al.* 2006), a *sine qua non* for root nodule formation on virtually all legumes studied to date (Cooper 2004). Moreover, they are now thought to act as interconnecting signals in tripartite symbioses between legumes, rhizobia and arbuscular mycorrhizal fungi (Antunes *et al.* 2006).

The apparently invariable need for bacterial Nod factors during root infection ['a key to the legume door' – Relić *et al.* (1994)] is another conclusion that can be drawn from a worldwide research effort on a diverse range (although, it should be borne in mind, still a small minority) of host–rhizobia associations. The structures of many of these compounds have been resolved and the relationships between structure and host specificity have received considerable attention. In recent years much effort has been expended, and significant progress made, on the search for Nod factor receptors and ensuing signal cascades in legume hosts. This work has identified commonalities as well as differences in plant responses to infection by rhizobia or mycorrhizal fungi (see Kistner *et al.* 2005; Oldroyd *et al.* 2005). Without detracting from the quality and importance of the results emanating from these lines of research (or, indeed, underestimating the difficulty of obtaining them!), they can be seen in terms of a logical and, to some extent, an anticipated verification and concretization of pre-existing concepts regarding Nod factor function. In contrast, new perspectives on Nod factor involvement in the molecular dialogue have been opened up following the discoveries that rhizobia produce far more variants of these compounds than was previously envisaged and that environmental parameters exert a strong influence on the range of structures synthesized (Morón *et al.* 2005).

Despite its sophistication, and its frequently displayed selectivity in favour of a single rhizobial species or biovar, the infection process cannot ensure the exclusion of ineffective (Fix^-) variants that will be of no benefit to their host. Such strains possess exactly the same capacity to nodulate roots as do effective (Fix^+) ones. Once established as bacteroids inside a nodule, however, weakly effective or ineffective rhizobia may be subjected to sanctions by the plant. Evidence for this is available from both cultivated and wild legumes and one mechanism appears to be restriction of oxygen supply to nonfixing nodules (Kiers *et al.* 2003; Denison and Kiers 2004; Simms *et al.* 2006). It has been argued (especially for rhizobia forming replicable bacteroids in determinate nodules) that sanctions of this kind can counter the evolution of parasitism and stabilize a mutualistic symbiosis by favouring the dissemination of effective populations; these would otherwise be disadvantaged by devoting a portion of their metabolism to supplying their host with nitrogen (Denison 2000; Kiers *et al.* 2003; Denison and Kiers 2004).

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