60 Ma of legume nodulation. What’s new? What’s changing?

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Received 5 June 2007; Revised 22 October 2007; Accepted 23 October 2007

Abstract
Current evidence suggests that legumes evolved about 60 million years ago. Genetic material for nodulation was recruited from existing DNA, often following gene duplication. The initial process of infection probably did not involve either root hairs or infection threads. From this initial event, two branched pathways of nodule developmental processes evolved, one involving and one not involving the development of infection threads to ‘escort’ bacteria to young nodule cells. Extant legumes have a wide range of nodule structures and at least 25% of them do not have infection threads. The latter have uniform infected tissue whereas those that have infection threads have infected cells interspersed with uninfected (interstitial) cells. Each type of nodule may develop indeterminately, with an apical meristem, or show determinate growth. These nodule structures are host determined and are largely congruent with taxonomic position. In addition to variation on the plant side, the last 10 years have seen the recognition of many new types of ‘rhizobia’, bacteria that can induce nodulation and fix nitrogen. It is not yet possible to fit these into the emerging pattern of nodule evolution.

Key words: Burkholderia, Cupriavidus, legume, nitrogen fixation, nodule, rhizobia.

Introduction
Current plant phylogeny places the family Leguminosae (Fabaceae) in the Eurosid I clade, which also includes all other higher plants known to form nitrogen-fixing root nodules (Soltis et al., 2000) as well as many that do not form these symbioses. Where this predisposition to nodulate comes from or why it is not always expressed is not clear nor is it clear why the actinorhizal plants (those nodulating with the filamentous bacterial genus Frankia) have nodules with a root-like structure of central vascular tissue and peripheral infected tissue and legume nodules have a central infected tissue and a peripheral vascular system. Pawlowski and Sprent (2007) have reviewed the differences between legume and actinorhizal nodules and this topic will not be covered here. Sprent (2007a, b) has discussed the evolution of nodulation in legumes from molecular and taxonomic aspects and this paper summarizes and extends these reviews. All generic and specific names of legumes are those accepted by the International Legume Database and Information Service (ILDIS).

Life before legumes
Current evidence suggests that legumes evolved about 60 million years ago (Lavin et al., 2005). What could the older plant groups provide that legumes could capitalize on to produce nodules? One of the first prerequisites was the ability of the two partners in the symbiosis to recognize each other. This is generally agreed to have developed from the ancient symbiosis between fungi and land plants, arbuscular mycorrhizas (AM), see, for example, Szczyglowski and Amyot (2003). In extant legumes, many of the signals are common between these symbioses. Another process that may have been ‘hijacked’ is that leading to pollen tube growth. This has much in common with the growth of infection threads down root hairs of both legumes and actinorhizal plants. Recent evidence suggests that gene duplication may have preceded this modification in function (Rodriguez-Llorente et al., 2004). What is not known is whether or not those legumes that do not have a hair infection and do not form infection threads have these duplicated genes. Another set of genes that has been replicated is that leading to the formation of haemoglobin. Active legume nodules are often characterized by their pink colour, due to the oxygen-carrying pigment haemoglobin. However, haemoglobin genes are very ancient and plant and animal
forms separated long before vascular plants evolved and extant plants may have one or both of two families of haemoglobin genes, often referred to as symbiotic and non-symbiotic forms. Legumes and actinorhizal plants may have one or more genes from each of these types, with variations among legumes from different taxonomic groups. Different forms of haemoglobin may be produced sequentially and be coupled to the induction of genes necessary for nitrogen fixation (Downie, 2005; Ott et al., 2005). There are other examples of gene duplication, for example, apyrases (Cannon et al., 2003) that will not be discussed here.

Regulation of differentiation in growing plants appears to have been highly conserved, possibly dating back over 400Ma (Floyd and Bowman, 2004). Modern molecular techniques, coupled with more sensitive analytical methods such as GC:MS have led to a resurgence in the study of the role of plant hormones in nodule processes and their similarities with, for example, root growth and nematode gall formation (Mathesius, 2003). For a general review of this area, see Manoury et al. (2007).

Thus the stage seems to have been well set for the evolution of nodules, but important questions remain. Why is nodulation restricted to the Eurosid I clade? What was the driving force for the ability to form nitrogen-fixing symbioses? One suggestion is that it was associated with a sudden increase in atmospheric carbon dioxide around the time that legumes evolved (Sprent, 2007b). How did legumes evolve different nodulation processes? The next section will consider this question.

How did legumes evolve different nodulation processes?

Rhizobia and other bacteria can penetrate roots of a variety of plants. For example, Spencer et al. (1994) found that rhizobia could enter between cells of potato tissues. Bryan et al. (1996) suggested that this kind of entry, observed by them in the non-nodulating caesalpinoid legume *Gleditsia triacanthos* was the first stage in the evolution of nodulation. In their case, invading bacteria appeared later to be confined within infection threadlike structures, but these did not penetrate cells. It is important to distinguish such structures from the transcellular infection threads that are found in root hair infection and subsequent nodule development (Brewin, 2004). However, it is possible to have transcellular infection threads without having a root hair infection, as seen, for example, in the mimosoid legume *Neptunia plena* (James et al., 1992; Sprent and James, 2007), which is normally infected via wounds where adventitious roots emerge and in *Mimosa scabrella*, where infection may occur between epidermal cells (Faria et al., 1988). Thus the most likely origin of infection of legumes by rhizobia is directly through the epidermis or through breaks, for example, where lateral roots emerge (often referred to as crack infection). Subsequently, there were two distinct lines of evolution. In 75% of legumes, infection threads are found and these were a necessary prerequisite for root hair infection. All nodulating mimosoid and caesalpinoid legumes so far examined and over 50% of papilionoid legumes have transcellular infection threads in nodules. These infect some, but not all of the cells in the developing infected region; these infected cells enlarge greatly, giving the familiar nodule structure as found in crop species such as peas (*Pisum sativum*) and soybean (*Glycine max*). These two legumes have nodules with indeterminate and determinate growth, respectively (Sprent, 2001). Although legumes having crack infection have been known for many years, most notably in *Arachis hypogaea*, the groundnut or peanut, none has been subjected to the intense molecular study currently centred on the two model legumes, *Lotus japonicus* and *Medicago truncatula*. This is a pity as they typify a significant section of papilionoid legumes, the dalbergioid group (Lavin et al., 2001) and include important tropical forage genera such as *Stylosanthes* and prized timber trees such as some species of *Dalbergia* and *Pterocarpus*. Perhaps it is because they are largely tropical and have not been funded for large-scale agricultural production, that they are so poorly studied. However, it is now becoming clear that many temperate legumes also lack a hair infection pathway, bacteria entering between epidermal cells, sometimes after damage. The most widely studied of these is *Lupinus* which is an important grain legume in some areas and whose nodules frequently encircle the subtending root, forming a collar-like structure. Collar nodules are also found in in *Lotononis* a genus that has good potential as a forage legume in parts of Australia and South Africa (Yates et al., 2007). In both crack and epidermal infections, a few cells in the developing nodule are infected and these divide repeatedly giving infected tissue lacking uninfected cells. As with nodules formed after root infection there are two further branches of development, determinate (following crack infection, as in Dalbergioid legumes) and indeterminate (following epidermal infection). If one can infer infection process from nodule structure, then all those in which the central tissue is uniformly infected lack a hair infection process. This would include common temperate genera in tribe Genistaeae, such as *Genista, Cytisus*, and *Ulex* and possibly the related tribe Crotalarieae (Sprent, 2007b). Further, since most of the detailed studies on recognition processes between host and rhizobia have been carried out with hair infections, it is pertinent to ask whether these operate when the infection processes appear less complex. Evidence now emerging suggests that some nod genes may not be essential for crack entry (Giraud et al., 2007).

Endoreduplication in infected cells of nodules has been a subject of discussion for many years, but recent evidence suggests that it may be common, at least
in advanced papilionoid legumes. It has been demonstrated in determinate nodules of *Lotus japonicus*, indeterminate nodules of *Medicago truncatula* and *M. sativa*, and in nodules of *Lupinus albus* (reviewed in Maunoury et al., 2007).

**Where do the rhizobia fit into all of this?**

In the early years of research into legume–rhizobial interactions, two kinds of nodulating bacteria were known, slow and fast growing. The former were thought to be rather promiscuous with the latter showing various levels of host plant/rhizobial specificity (Nutman, 1987). These ideas were largely formulated in studies driven by the need for nitrogen-fixing legumes in countries such as Australia and the USA whose economies depended on them. Over the last 30 years, the situation has changed dramatically. First, the number of genera and species of ‘rhizobia’ increased greatly and then, in the last ten years, bacteria outside the family Rhizobiaceae have been found to nodulate legumes. Other bacteria have been isolated from legume nodules, but unless they have been shown to nodulate the host of isolation (or at least some legumes) their ability to nodulate must be regarded with suspicion. For example, Chou et al. (2007) isolated *Labrys neptuniae* from nodules of the aquatic mimosoid legume *Neptunia oleracea*, but were unable to re-infect the host plant with it.

Current bacterial taxonomy divides a group of gram negative bacteria, the Proteobacteria into a number of branches, α, β, γ, δ, and ε (Brenner et al., 2005). The α-branch has seven orders, one of which is the Rhizobiales, which in turn has 11 families. Four of these, Bradyrhizobiaceae, Methylobacteriaceae, Phyllobacteriaceae, and Rhizobiaceae contain genera that can nodulate and fix nitrogen in association with legumes. The first and last families correspond to the old categories of slow and fast growing rhizobia, respectively. In the Methylobacteriaceae, the genus *Methylobacterium* is of particular interest. The species *M. nodulans* was isolated from some species of *Crotalaria* (Sy et al., 2001), but before it was fully identified was found only to nodulate the species from which it was isolated, but not some other species of the genus (Samba et al. 1999). This clear infrageneric separation of host species has now been echoed by the report of another species of *Methylobacterium*, not yet fully described, which nodulates only some species of the closely related genus *Lotononis* (Yates et al., 2007). Such a clear-cut separation is rare in other nodulating α-proteobacteria. The β-proteobacterial branch also contains seven orders. One of these, the Burkholderiales is divided into five families and one of these, the Burkholderiaceae contains two genera, *Burkholderia* and *Cupriavidus* having species known to nodulate legumes. Both genera have been segregated from the old genus *Pseudomonas*, long known to be polyphyletic. *Cupriavidus* has been separated from *Ralstonia*, with a short time spent as *Wauterisia* and the only species known to nodulate legumes is *Cupriavidus taiwanensis* (Chen et al., 2003). Several species of *Burkholderia* have been shown to nodulate legumes, with varying levels of host specificity (Sprent and James, 2007). Some are capable of fixing nitrogen ex planta (Elliott et al., 2007a). Although all the early reports are from mimosoid legumes, there is now evidence that papilionoid legumes such as *Cyclopa* (Elliott et al., 2007b) and *Dalbergia* (Rasolomampianina et al., 2005) may also nodulate with *Burkholderia* spp. It remains to be seen how common these β-rhizobia are and whether they are especially important in certain environments.

**What’s next?**

Major advances in our understanding of the relations between legumes and rhizobia are being made using model legumes, but as Doyle and Luckow (2003) pointed out, these are only the tip of the iceberg. In the real world, the legume/rhizobium symbiosis varies greatly, being essentially parasitic to highly effective, imposing various levels of constraints on the partners (Sprent, 2003). These are poorly understood. For many years it has been the goal of some to have nitrogen-fixing cereals and other non-legume crops. Others believe that a better starting point may be to understand why some legumes cannot nodulate. With modern molecular techniques this is a realistic goal. During the course of legume evolution, some species have acquired all the necessary information to make nodule structures in the absence of rhizobia (summarized in Sprent and James, 2007). However, such structures have only been reported for a few papilionoid species with infection threads and interstitial cells in their infected tissue, arguably the more complex branch of nodule evolution.

**References**


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