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Goldacre paper:

Auxin: at the root of nodule development?

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Abstract. Root nodules are formed as a result of an orchestrated exchange of chemical signals between symbiotic nitrogen fixing bacteria and certain plants. In plants that form nodules in symbiosis with actinorhizal bacteria, nodules are derived from lateral roots. In most legumes, nodules are formed *de novo* from pericycle and cortical cells that are re-stimulated for division and differentiation by rhizobia. The ability of plants to nodulate has only evolved recently and it has, therefore, been suggested that nodule development is likely to have co-opted existing mechanisms for development and differentiation from lateral root formation. Auxin is an important regulator of cell division and differentiation, and changes in auxin accumulation and transport are essential for lateral root development. There is growing evidence that rhizobia alter the root auxin balance as a prerequisite for nodule formation, and that nodule numbers are regulated by shoot-to-root auxin transport. Whereas auxin requirements appear to be similar for lateral root and nodule primordium activation and organ differentiation, the major difference between the two developmental programs lies in the specification of founder cells. It is suggested that differing ratios of auxin and cytokinin are likely to specify the precursors of the different root organs.

Additional keywords: actinomycetes, auxin transport, cytokinin, flavonoids, lateral root, rhizobia, symbiosis.

General introduction

This review examines the questions of whether the phytohormone auxin is a regulator of both lateral root and symbiotic nodule development, and whether auxin has similar or divergent roles during the development of the two organs. Lateral roots and nodules are formed post-embryonically from endogenous cell types that are stimulated to divide, form an organ primordium, and later, differentiate and elongate (Fig. 1). In the case of lateral roots, cell divisions first occur in the pericycle. A lateral root primordium is formed after further divisions and the primordium differentiates into an organ with a central stele. An apical meristem becomes active and leads to lateral root elongation. Nodules usually arise from a combination of pericycle and cortical cell divisions, and after primordium formation a differentiated nodule forms, with an optional apical meristem and either central or peripheral localisation of vascular traces.

Lateral roots or branch roots have existed as parts of plant root systems for ~400 million years (Raven and Edwards 2001). In contrast, nodules only evolved relatively recently, ~60 million years ago, and their emergence could have been triggered by a lack of nitrogen in a CO_2-rich environment (Sprent 2007, 2008). Nodules develop only on the roots (and sometimes stems) of certain plants that form a symbiosis with nitrogen fixing bacteria. It has been argued that because nodule development emerged recently during evolution, it is likely that the mechanisms that regulate nodule development were co-opted from existing processes, most likely those that regulate lateral root formation (Hirsch and LaRue 1997). Therefore, this review will first give an overview of the diversity of nodules in different host plants, highlighting the existence of types of nodules that resemble lateral roots.

The developmental mechanisms of lateral root formation have been studied in great detail, and auxin has emerged as a central regulator of lateral root development (Fukaki *et al*. 2007). Thus, this review will examine whether auxin might play a similar role in nodule development as it does in lateral root development. There is evidence that auxin is synthesised by the nitrogen fixing symbionts of host plants, but more importantly, that the symbiont indirectly alters auxin transport and localisation inside the host root. To understand how the symbiont can interfere with the auxin balance in the host, we will examine the known mechanism of auxin signalling and transport in the plant, followed by how these mechanisms play a part in the regulation of lateral root formation. This will be compared with the involvement of auxin during different stages of nodule development and in the regulation of nodule numbers. This comparison leads to the hypothesis that auxin requirements differ between lateral root and nodule development at the earliest stage of organ formation, the

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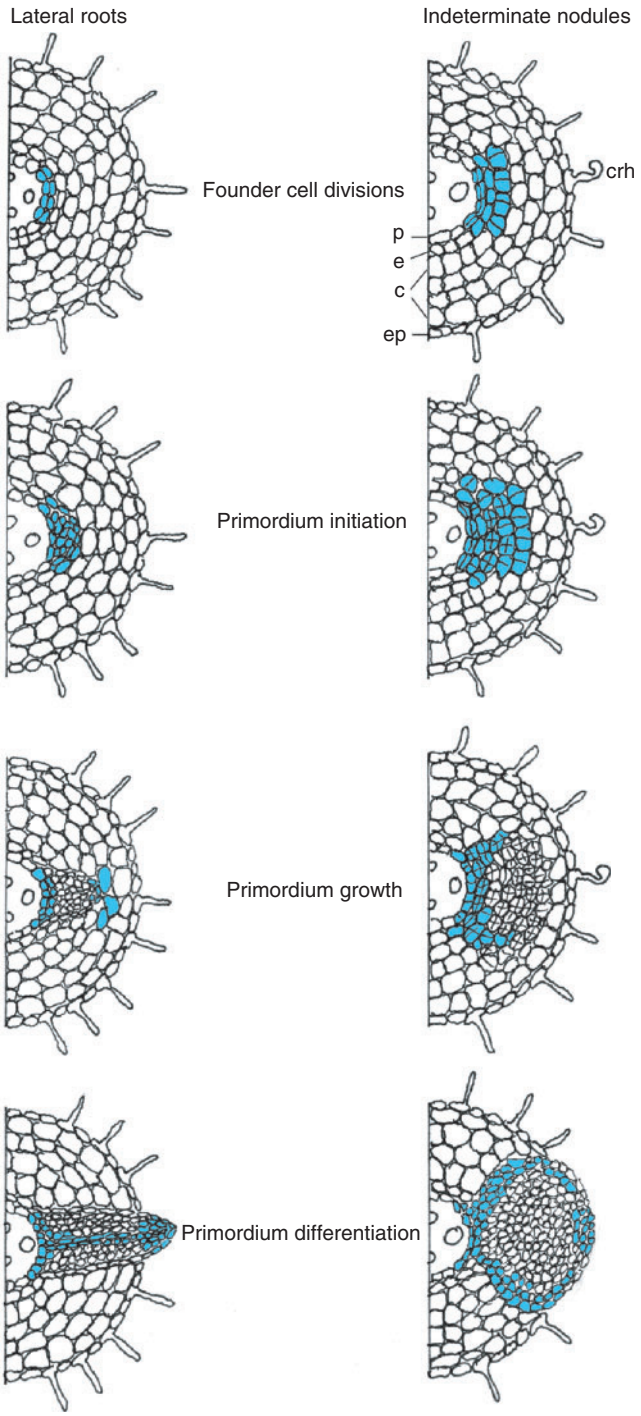

Fig. 1. Lateral root and indeterminate nodule organogenesis. Organogenesis of lateral roots (left column) and indeterminate nodules (right column) involves the first founder cell divisions, primordium initiation, primordium growth and primordium differentiation. The expression of the auxin responsive promoter *GH3* is shaded to highlight the overlaps in expression in both developmental programs. Expression patterns are modelled on data from *Medicago truncatula* and white clover (*Trifolium repens* L.). p, pericycle; e, endodermis; c, cortex; ep, epidermis; crh, curled roots hair, indicating the infection site of rhizobia.

specification of founder cell types, whereas auxin is likely to have similar roles in regulating cell division and differentiation once the organ has been specified. This hypothesis could be tested in the future by genetically manipulating auxin synthesis or responses in the specific founder cell types of both organs.

Diversity of nodule types in nitrogen fixing symbioses

Nitrogen fixing symbioses occur between a range of plants and bacteria, and only a subset of these form root structures classified as nodules (Sprent and Sprent 1990). An example of an ancient nitrogen fixing symbiosis is the association of cycads with cyanobacteria, in which the symbiont induces the formation of so-called collaroid roots (Sprent and Sprent 1990). The more recent symbioses of higher plants with nitrogen fixing bacteria that lead to the formation of root (or stem) nodules only arose in plant families belonging to the Eurosid 1 clade (Soltis *et al*. 1995). Actinorhizal plants of eight Angiosperm families associate with actinomycetes of the genus *Frankia*. The most common nodulated plants are species of the Leguminosae, many of which form a symbiosis with α-proteobacteria called rhizobia, as well as certain β-proteobacteria (Sprent 2008). The only known nodulating non-legume is the tropical tree *Parasponia* of the Ulmacae family.

Studies of the diversity of legume and actinorhizal nodulation suggest that both the invasion process as well as the development of the nodule can occur in several ways (Hirsch and LaRue 1997; Gualtieri and Bisseling 2000; Sprent 2007). Invasion can be via crack entry of bacteria between epidermal and cortical cells, often at sites of lateral or adventitious root emergence, or via infection threads. Nodule development can be based on modifications of existing lateral or adventitious roots or involve *de novo* induction of cell divisions in pericycle and cortical cells. Nodule formation in the non-legume species shows similarities to the development of lateral roots. In actinorhizal plants, *Frankia* first cause cortical cell divisions to form a pre-nodule that is colonised by hyphae, and then stimulate the division of pericycle cells to form a lateral root-like nodule. *Frankia* invade the cortical cells of this nodule, which retains a central stele, similar to lateral roots (Pawlowski and Bisseling 1996). Similarly, in *Parasponia*, rhizobia trigger the initiation of a lateral root which they later invade (Trinick 1979). Nodule structures in legumes are diverse, and typically characterised by the initiation of a nodule from pericycle or cortical cells *de novo*, resulting in a nodule with peripheral vascular strands (Hirsch 1992).

Two different nodule types have been studied in detail. Indeterminate nodules are formed on most temperate legumes, e.g. pea (*Pisum sativum* L.), clover (*Trifolium* sp.), alfalfa (*Medicago sativa* L.) and Barrel medic (*Medicago truncatula* Gaertn.), and are characterised by nodule initiation in the inner cortex and usually also the pericycle (Timmers *et al*. 1999). These nodules form a persistent nodule meristem, which allows continuous growth, and leads to the formation of elongated nodules. Determinate nodules are formed on many (sub) tropical plants, including soybean (*Glycine max* L.), bean (*Phaseolus* sp.) and Japanese trefoil (*Lotus japonicus* L.), and are initiated in the outer root cortex by cell enlargement and divisions. Cell divisions are later induced in the pericycle and inner cortex, and both cell division sites merge later on. These

nodules are typically spherical because the nodule meristem differentiates (Rolfe and Gresshoff 1988). In some legumes, nodules arise at sites of lateral or adventitious root initiation and this is usually associated with crack entry invasion. For example, in peanut (*Arachis hypogaea* L.), nodules only arise from cortical cells adjacent to an emerging lateral root (Allen and Allen 1940). A similar pattern of nodule initiation occurs in many species of the Dalbergiae and Aeschynomeneae (Sprent 1989). In white clover (*Trifolium repens* L.), which usually forms indeterminate nodules after inoculation at the young root hair zone, the most susceptible zone for nodulation, nodules can be induced at sites of lateral root initiation when roots are inoculated in the mature root zone (Mathesius *et al*. 2000*b*). In the aquatic legume *Sesbania rostrata* L., nodules can arise either *de novo* from the root cortex and pericycle or from adventitious or lateral root emergence sites. Under well-aerated conditions, root nodulation occurs via infection threads and is strictly dependent on nodulation (Nod) factor structure. Under water-logging conditions, adventitious root-based nodulation occurs, which is less stringent for Nod factor structure and invasion takes place via cracks through the epidermis (Goormachtig *et al*. 2004). *S. rostrata* can form both determinate and indeterminate nodules, depending on environmental conditions (Fernández-López *et al*. 1998). The switch between indeterminate and determinate nodules in *Sesbania* is likely to be regulated by ethylene (Fernández-López *et al*. 1998).

Nodulation – innovation by recruitment?

It is not clear what distinguishes plants forming nitrogen fixing symbioses from most other plants that do not form them, but it is likely that the presence of receptor kinases for bacterial Nod factors (lipochitin oligosaccarides) plays a key role in the ability to form symbioses (Spaink 2004; Zhang *et al*. 2007). The receptors that are necessary for the more ancient (~450 million year-old) symbiosis of mycorrhizal fungi with plants are thought to have been recruited for the more recent bacterial endosymbioses, as the same receptors are required for the interaction of legumes with rhizobia and of actinorhizal plants with *Frankia* (Gherbi *et al*. 2008; Markmann *et al*. 2008). A physiological characteristic of nodulating plants from a range of genera is an altered response to ABA: whereas ABA inhibits lateral root formation in non-nodulating plants, it stimulates their development in nodulating species (Liang and Harris 2005). Therefore, it is possible that changes in hormone response pathways are either a condition for or a consequence of the ability for nodulation.

Recently, it was found that the genomes of certain photosynthetic *Bradyrhizobium* species which infect the legume *Aeschynomene* via crack entry on the roots and stems do not contain any genes encoding the canonical Nod factor synthesis enzymes (Giraud *et al*. 2007). It is possible that nodulation may have started as a process involving infection of roots via crack entry and nodule formation based on a developmental program for lateral roots. Nodulation may have become more specific with the requirement of symbiosis for specific Nod factors, which allowed infection thread invasion (with the possible advantage of better selection of efficient rhizobial symbionts) and *de novo* formation of a nodule independent of lateral roots (Hirsch and LaRue 1997; Sprent 2007, 2008).

If nodule development has been recruited from lateral root formation, it could be expected that similar developmental signals regulate both processes. A major regulator of lateral root initiation, differentiation and meristem specification is auxin (Casimiro *et al*. 2003; Fukaki *et al*. 2007). In particular, auxin patterns in the plant determine subsequent developmental patterns (Heisler *et al*. 2005). Thus, auxin appears to be a pattern-determining global regulator, as well as a player in cell division, cell elongation and vascular tissue differentiation (Woodward and Bartel 2005; Teale *et al*. 2006). It has been suggested that auxin is also a regulator of nodule development (Thimann 1936; Hirsch 1992; Hirsch and Fang 1994). There are multiple ways by which symbiotic bacteria could alter root and nodule development through the involvement of auxin: via auxin synthesis by the microsymbiont, or through alteration of auxin synthesis, breakdown, signalling or transport in the host.

Importance of auxin synthesis by the microsymbiont

Auxin is known as a plant hormone and is synthesised by all higher plants (Ljung *et al*. 2002). The most abundant form of auxin in plants is indole-3-acetic acid (IAA). However, many plant-associated soil bacteria are also known to synthesise auxin, in particular IAA, and this could be part of a strategy to manipulate the growth of host plants (Spaepen *et al*. 2007). Auxin synthesis has been demonstrated in non-symbiotic plant growth-promoting bacteria (Dobbelaere *et al*. 1999), in symbiotic nitrogen-fixing cyanobacteria (Sergeeva *et al*. 2002), in the actinomycete *Frankia* (Wheeler *et al*. 1984) and in rhizobia (Kefford *et al*. 1960). The exudation of various compounds from plants has been shown to stimulate IAA synthesis in bacteria. Most importantly, bacteria are likely to use tryptophan exuded by plant roots as a precursor for auxin synthesis (Kefford *et al*. 1960). Flavonoids, which are exuded in particular from legume roots to stimulate Nod factor synthesis, have also been shown to stimulate IAA synthesis in *Rhizobium* sp. (Theunis *et al*. 2004).

There is evidence that auxin synthesis by bacteria alters root architecture in non-nodulating plants. For example, auxin synthesis by *Pantoea* (*Erwinia*) *agglomerans* pv. *gypsophila* stimulates the formation of tumours in its plant host *Gypsophila paniculata* L. (Clark *et al*. 1993). Auxin synthesis by plant growth-promoting rhizobacteria can partially explain some of the growth-promoting effects that these bacteria have on plants, including stimulation of root growth in wheat by *Azospirillum brasilense* (Dobbelaere *et al*. 1999), and stimulation of root elongation in canola by *Pseudomonas putida* (Xie *et al*. 1996).

Auxin synthesis in cyanobacteria that associate with cycads and certain Angiosperms was found to be more commonly the case in symbiotic than in free-living species (Sergeeva *et al*. 2002). It is possible that the auxin synthesised by these cyanobacteria is involved in the activation of mitotic divisions in the infection structures of cycads. Auxin synthesis by actinomycetes that form symbioses with actinorhizal plants could be involved in infection. In *Casuarina glauca* Sieber, the auxin import protein AUX1 is specifically induced in root cells colonised by *Frankia* (Peret *et al*. 2007). The authors of this

study suggested that *Frankia* synthesise IAA which is transported into colonised host cells via AUX1 and that this is a necessary step in plant cell infection. Similarly, the synthesis of IAA by rhizobia can contribute towards successful nodulation in legumes (Kefford *et al*. 1960). Although the early steps of nodule initiation can be induced by Nod factors alone, synthesis of IAA by rhizobia could be important at later stages of nodulation. Studies with *Rhizobium* mutants deficient in IAA synthesis have shown that nitrogen fixation can be impaired by a lack of rhizobial auxin, whereas increased nodulation efficiency can be reached with IAA overproducing strains, although this might differ between determinate and indeterminate legumes (Pii *et al*. 2007). It has been noted that non-legumes can also be stimulated to form nodule-like structures after application of auxin and the resulting structures can be colonised by diazotrophs, including *Azospirillum* and *Rhizobium* sp. which appear to infect via crack entry (Christiansen-Weniger 1998). Therefore, it could be hypothesised that auxin production by microsymbionts is a general and maybe ancient mechanism to alter root architecture and induce nodule-like structures in plants. However, in most studies it has not yet been demonstrated whether bacterial mutants deficient in auxin synthesis would also be deficient in symbiosis or other interactions. As discussed below, a more refined strategy of rhizobia to control nodule development is likely to be the indirect manipulation of auxin transport or turnover in the plant host.

Evidence for altered auxin content and distribution in host plants during nodulation

Auxin was first connected with nodulation with the discovery of increased auxin levels in legume nodules (Thimann 1936), and this has subsequently been confirmed in several legumes and actinorhizal plants. To examine spatial and temporal changes in auxin accumulation during nodulation, the auxin responsive promoters *GH3* and *DR5* have been monitored in legumes forming determinate and indeterminate nodules. In the legume white clover, which forms indeterminate nodules, rhizobia caused a reduction in *GH3* activation at and below the site of infection within 10 h (Mathesius *et al*. 1998*b*). This decrease was followed by an increase in expression at the site of nodule initiation ~24 h after inoculation. Similarly, *DR5* expression appeared to be interrupted below the site of nodule initiation in *M. truncatula*, but induced in the forming nodule (Huo *et al*. 2006). *GH3* expression could then be observed in the first dividing pericycle and cortical cells of a forming nodule in white clover (Mathesius *et al*. 1998*b*) and in *M. truncatula* (van Noorden *et al.* 2006) (Fig. 1). *GH3* expression was high in the early nodule primordium of white clover, but then disappeared from the centre of a differentiating nodule and remained only in the nodule meristem and the vascular bundles (Fig. 1; Mathesius *et al.* 1998*b*). Similarly, high *GH3* expression was found in the first dividing outer cortical cells in the determinate legume *L. japonicus* after *Mesorhizobium loti* infection (Pacios-Bras *et al*. 2003). During later stages of determinate nodule development, *GH3* expression was similarly present in peripheral vascular tissue and meristematic cells.

Studies of the localisation of *GH3* and *DR5* reporters during lateral root development in white clover and *M. truncatula*, respectively, found high expression in early dividing pericycle cells, whereas expression decreased in the forming primordium and was retained only in the apical meristem and central vascular tissue of a differentiated lateral root (Fig. 1) (Mathesius *et al*. 1998*b*; Huo *et al*. 2006). Studies in *Arabidopsis* using the *DR5* promoter also demonstrated high activity in the first dividing pericycle cells of a lateral root, with disappearing staining in the forming primordium (Benkovà *et al*. 2003). Therefore, changes in auxin distribution are likely to shape organ development during both nodule and lateral root development.

There are indications that the observed changes in auxin accumulation during nodulation are regulated by the plant upon Nod factor perception and are most likely due to changes in auxin transport. It was observed in several legumes that synthetic auxin transport inhibitors can induce nodules spontaneously in the absence of rhizobia (Allen *et al*. 1953; Wu *et al*. 1996), and this was accompanied by similar expression of nodulation genes as in normal nodules (Hirsch *et al*. 1989). In addition, the reduction of *GH3* expression observed during the early stages of indeterminate nodule formation can be mimicked by Nod factors and the synthetic auxin transport inhibitor 1-N-naphthylphalamic acid (NPA) (Mathesius *et al*. 1998*b*). The next section, therefore, examines possible mechanisms of auxin transport regulation in the plant.

How can auxin patterns be altered in the plant?

Auxin synthesis and translocation

Auxin is synthesised mainly in young shoot tissues and distributed from there to other tissues and organs via transport, although most other tissues can also synthesise auxin (Ljung *et al*. 2002). Auxin can occur as the free, active form, or be conjugated for storage. Tracking of radiolabelled auxin showed that there are a transport of auxin from the shoot to the root tip through the vascular tissue, and a transport in the root from its tip to its elongation zone through epidermal cells (Mitchell and Davies 1975; Tsurumi and Ohwaki 1978). In addition to long distance auxin transport, local transport of auxin along and across tissues is important for auxin localisation in small groups of cells, for example in an emerging lateral root or in the root cap during gravitropism (Jones 1998). Although auxin can be transported within the plant via the phloem from source to sink tissues, polar auxin transport can be regulated specifically by active polar auxin transport (PAT) through auxin transport proteins (Fig. 2).

Auxin import and export

Auxin is a weak acid; when present in the acidic cell wall environment, it takes a protonated form (IAAH) and can enter cells to a certain degree by diffusion. It can also enter into cells by auxin importers of the amino acid permease families AUX1 (Auxin resistant 1), LAX (like-AUX1) and PGP4, a member of the MDR/PGP (Multidrug resistance/P-glycoprotein) families (Terasaka *et al*. 2005; Yang *et al*. 2006). This involves proton symport for AUX1 and ATP-driven uptake for PGPs (Fig. 2). One expression site of AUX1 is in protophloem cells, and AUX1 has, therefore, been suggested to play a role in auxin unloading from the phloem and loading into the PAT system (Swarup *et al*. 2001).

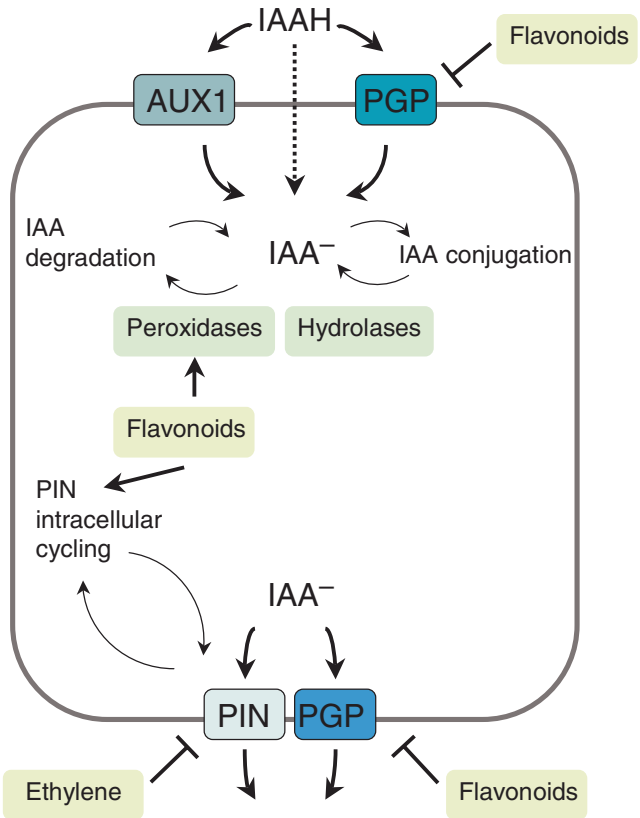

Fig. 2. Targets of auxin transport regulation by rhizobia. The figure highlights transporters, enzymes and metabolic regulators of auxin transport that could be targeted by rhizobia. Protonated auxin (IAAH) is imported into the cell by the importers AUX1 and PGP. Some auxin also diffuses into the cell (dashed arrow). Inside the cell most of the auxin dissociates and is present as IAA^-. Export from the cell requires the action of auxin exporters of the PIN and PGP families. Flavonoids that are activated by rhizobia could act on auxin transport by inhibiting PGP proteins or by interfering with intracellular cycling of PIN proteins. Active auxin concentrations within the cell are also determined by breakdown and conjugation of auxin. Breakdown by oxidation can be catalysed by peroxidases, some of which are regulated by flavonoids that accumulate during nodulation. Auxin can be hydrolysed from inactive auxin conjugates by auxin hydrolases that were shown to be induced by rhizobia. Ethylene can also inhibit auxin transport and could act on the expression of PIN proteins.

Because of the higher pH inside the cell, deprotonated auxin (IAA^-) cannot diffuse back out of a plant cell; it requires active export (Fig. 2). Auxin is exported by transporters of the PIN (Pin-formed) and PGP families, including PIN1 to PIN7, PGP1 and PGP19 from *Arabidopsis* (Geisler *et al*. 2005; Petrasek *et al*. 2006). In addition to their individual auxin transport activities, it is likely that PIN and PGP form complexes that enhance each other's activities (Blakeslee *et al*. 2007). The polarity of auxin transport is established by the polar localisation of PIN proteins on either the basal or apical side of the cell (Wisniewska *et al*. 2006). Different members of the PIN family are localised in a cell-and developmental-specific pattern, for example PIN1 is localised on the apical side of vascular cells in the root and mediates acropetal auxin flow, whereas PIN2 is localised at the basal side of epidermal cells in the root tip where it mediates basipetal auxin flow. Mutations or mis-expression of *PIN* genes causes changes in auxin accumulation and plant development (Friml 2003; Vieten *et al*. 2007).

Regulation of auxin transport proteins

Auxin transport can be altered by the regulation of the activity, localisation, and internalisation of auxin transport proteins. The expression and localisation of PIN proteins are regulated by PINOID, a serine-threonine receptor kinase that can direct PIN proteins to either side of the cell through changes in phosphorylation (Friml *et al*. 2004). Dynamic cycling of PIN and AUX1 proteins between the plasma membrane and internal vesicles leads to changes in transport protein availability. The cycling of PIN proteins involves transport via actin filaments and is regulated by GNOM, a GDP/GTP exchange factor for small G proteins (Geldner *et al*. 2003). The internalisation of AUX1 by vesicle cycling is regulated by ARX4 (Auxin resistant 4) but not by GNOM (Dharmasiri *et al*. 2006), suggesting two independent internalisation mechanisms. Auxin export can be inhibited by synthetic and natural auxin efflux inhibitors (AEIs), including NPA and TIBA (2,3,5-triiodobenzoic acid), which bind to the so-called NPA-binding proteins (NBPs). The NBPs have been suggested to interfere with PIN activity through a possible third protein (Muday and DeLong 2001). Although no NBPs have been identified with certainty, the *tir3* mutant of *Arabidopsis*, which shows reduced NPA binding, is defective in the large protein BIG, which mediates the effect of NPA on PIN trafficking within the cell (Gil *et al*. 2001). AEIs also affect auxin transport by inhibiting actin dynamics, which are required for PIN cycling (Dhonukshe *et al*. 2008). In addition, NPA inhibits auxin export by binding to MDR/PGPs (Noh *et al*. 2001; Murphy *et al*. 2002; Geisler *et al*. 2005).

Flavonoids are natural auxin transport regulators

Flavonoids are a class of natural AEIs, some of which can regulate PIN activity and localisation (Peer and Murphy 2007). Flavonoids are synthesised by all plants. They have diverse structures and many functions, e.g. they can act as antioxidants, enzyme regulators, molecular signals for rhizobial *nod* gene expression, flower pigments, UV protectants and antimicrobials (Winkel-Shirley 2001). Flavonoids with specific structures, especially flavonols, were found to inhibit auxin transport by competing with synthetic AEIs for plasma membrane and microsomal binding sites (Stenlid 1976; Jacobs and Rubery 1988; Bernasconi 1996). Flavonoids are likely to have several targets in plant cells, as they have been shown to interact with PGP auxin transport proteins (Bernasconi 1996) as well as with an aminopeptidase (Murphy and Taiz 1999). The flavonol quercetin enhanced auxin uptake by PGP4 in a heterologous system (Terasaka *et al*. 2005) and reduced auxin export by PGP1 in a manner similar to that of NPA (Geisler *et al*. 2005). The action of flavonoids on MDR/PGPs in plants is similar to the modulation of many members of MDR/PGPs by flavonoids in animals (Morris and Zhang 2006). In addition to regulating PGPs, a lack of flavonoids in *Arabidopsis* altered the expression and localisation of certain PIN proteins, and it was suggested that

flavonoids could act by targeting PIN intracellular cycling, at least in the root tip (Peer *et al*. 2004). However, it is likely that PIN protein localisation is not directly regulated by flavonoids but by auxin localisation itself in a positive feedback loop (Peer *et al*. 2004). This could be regulated at the level of vesicle cycling as auxin was shown to inhibit internalisation of PIN proteins mediated by BIG, thus, auxin could increase its own transport (Paciorek *et al*. 2005), a phenomenon known as the 'canalisation hypothesis' (Sachs 1981). Auxin was also shown to increase *PIN* gene expression in a positive feedback loop (Vieten *et al*. 2005). Studies in flavonoid-deficient *Arabidopsis* mutants confirmed that these plants had higher rates of auxin transport whereas mutants over-accumulating flavonols show decreased auxin transport rates (Murphy *et al*. 2000; Brown *et al*. 2001; Peer *et al*. 2004). Flavonoids could be an ideal link between auxin transport and the environment because flavonoids are accumulated in response to a variety of environmental stimuli (Buer and Muday 2004; Taylor and Grotewold 2005). The co-localisation of flavonoids at sites of high auxin concentration supports their role in auxin transport control (Murphy *et al*. 2000; Peer *et al*. 2001; Buer and Muday 2004; Buer *et al*. 2006).

Auxin transport and response regulate lateral root development

The polar auxin transport system has been shown to be necessary for setting up plant developmental patterns (Friml 2003) and, not surprisingly, the correct auxin localisation and subsequent auxin response are crucial for lateral root development (Casimiro *et al*. 2003; De Smet *et al*. 2006; Fukaki *et al*. 2007).

Lateral root initiation

Lateral roots usually emerge from pericycle cells opposite xylem poles behind the root differentiation zone. Lateral root initiation is regulated developmentally, leading to an acropetal sequence of lateral root initiation, but environmental influences (e.g. drought, impedence, nutrient availability) can modify this pattern (Dubrovsky *et al*. 2000). During root development, lateral root initials ('founder cells') are probably specified in the root meristem. It is thought that pericycle founder cells of lateral roots remain in a meristematic state after emerging from the root apical meristem, i.e. they remain competent to divide in an otherwise differentiated part of the root. The founder cells, i.e. the pericycle cells opposite xylem poles, are mostly found in the G_2 phase of the cell cycle, whereas pericycle cells not forming founder cells are mainly found in the G_1 phase (Beeckman *et al*. 2001; Roudier *et al*. 2003). Following an asymmetric division, a small primordium forms from a specified number of cell divisions. The primordium later differentiates into different tissue types, after which it emerges from the root and continues to elongate (Fig. 1) (Malamy and Benfey 1997).

The role of auxin transport in founder cell specification

Studies in *Arabidopsis* have correlated the strict temporal and spatial pattern of lateral root initiation, with an oscillation of auxin activity occurring in two files of protoxylem cells in the root basal meristem, i.e. the zone between the root apical meristem and the elongation zone (De Smet *et al*. 2007). The source of this oscillation is not known, but could be due to auxin that is recycled by the basal meristem from the root tip through the root cap via AUX1-mediated auxin transport. Pericycle founder cells then require activation through auxin signalling to undergo cell cycling. This activation requires the action of auxin response proteins, especially members of the AUX/IAA (auxin/indole-3-acetic acid) family, which act as repressors of ARFs (auxin response factors), the transcriptional regulators of other auxin responsive genes (Badescu and Napier 2006; Parry and Estelle 2006). Degradation of AUX/IAA proteins occurs through the SCF^{TIR1} (SKP1, Cullin and F-box protein, in this case TIR1) complex after binding of auxin to its receptor TIR1 (transport inhibitor response 1), and leads to auxin-induced gene expression changes (Fig. 3). Auxin is directly involved in activating the cell cycle during lateral root initiation (Himanen *et al*. 2002) and the expression of downstream genes (Himanen *et al*. 2004; Vanneste *et al*. 2005). Mutants which overproduce auxin, like the *Arabidopsis superroot* mutant, have increased numbers of lateral roots (Boerjan *et al*. 1995) and similarly exogenous application of auxin increases lateral root numbers (Wightman *et al*. 1980; Laskowski *et al*. 1995). In contrast, mutants resistant to auxin show reduced numbers of lateral roots (De Smet *et al*. 2006).

Activation of the cell cycle in founder cells by auxin is not sufficient for lateral root initiation, which also requires cell fate re-specification by auxin through the auxin response protein SOLITARY ROOT/IAA14 (Vanneste *et al*. 2005). NPA application inhibits the induction of lateral roots, and at high (10 μM) concentrations it can block lateral root initiation at the earliest stage of founder pericycle cell division (Casimiro *et al*.

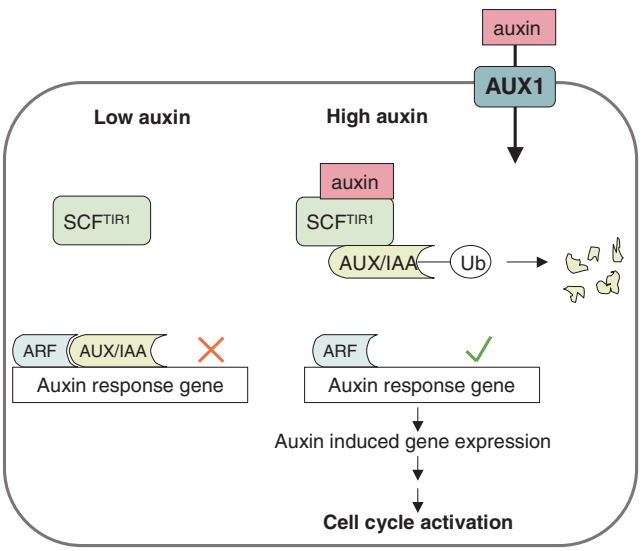

Fig. 3. Simplified model for the action of auxin on auxin response genes. At suboptimal levels of auxin in the cell, the expression of auxin response genes is repressed by a complex of AUX/IAA and ARF proteins. When auxin levels rise, for example through increased auxin import through AUX1, auxin binds to the receptor complex SCF^{TIR1}. This leads to the binding of AUX/IAA proteins to the SCF^{TIR1} complex, and the subsequent ubiquitination and degradation of AUX/IAA proteins. AUX/IAA proteins are not able to bind to ARF proteins any more, and expression of auxin response genes is released. This leads to the activation of further auxin-induced genes, including genes that regulate the activity of the cell cycle.

2001). NPA does not appear to be able to re-specify the identity of the pericycle founder cells as it does not alter the expression of a pericycle marker, nor does it prevent lateral roots from forming when it is applied at the same time as the auxin NAA (Casimiro *et al*. 2001). Thus, NPA appears to inhibit lateral root initiation by limiting auxin availability in the root. Measurements of IAA levels in roots treated with NPA show that increasing levels of NPA reduce IAA levels, except for the first 3 mm of the root tip where auxin accumulates (Casimiro *et al*. 2001). This is consistent with the expression pattern of the auxin responsive promoter *DR5* in *Arabidopsis* (Sabatini *et al*. 1999).

The role of auxin transport in primordium initiation and lateral root emergence

The directional transport of auxin is crucial for lateral root initiation and emergence. Whereas the former requires auxin transport from the root tip into the basal part of the root, the latter is dependent on transport of auxin from the shoot to the root (Reed *et al*. 1998; Casimiro *et al*. 2001; Bhalerao *et al*. 2002). Mutants with reduced auxin transport, for example the *pinoid* (Benjamin *et al*. 2001) and *tir3* (Ruegger *et al*. 1997) mutants, are characterised by reduced lateral root numbers. Auxin exporters of the PIN and PGP family are also important for lateral root initiation. Individual *PIN* genes show overlapping but slightly distinct expression patterns in early lateral root primordia, and altered auxin localisation in lateral root primordia of *Arabidopsis pin* mutants is correlated with retarded lateral root initiation (Benkovà *et al*. 2003). GNOM, which is important for correct localisation of PIN, also affects lateral root initiation (Geldner *et al*. 2004). *PIN* gene expression is important for the creation of local auxin gradients in the lateral root primordium, and these gradients are likely to regulate cell specification (Vanneste *et al*. 2005; Vieten *et al*. 2005). The *Arabidopsis mdr1* mutant, which has reduced root acropetal auxin transport, was defective in lateral root elongation but not initiation (Wu *et al*. 2007). In contrast, decreased auxin uptake in the *pgp4* mutant correlated with elevated auxin levels and temporarily increased numbers of lateral roots in young *Arabidopsis* seedlings (Santelia *et al*. 2005). Flavonoid-deficient *Arabidopsis* mutants with increased auxin transport rates have a somewhat increased density of lateral roots (Brown *et al*. 2001). The auxin importer AUX1 might have a dual role during lateral root development, at least in *Arabidopsis*. During the initiation phase AUX1 appears to facilitate IAA unloading at the root tip, providing auxin to the initiating lateral roots. During the emergence phase, AUX1 facilitates export of IAA from the shoot and unloading of IAA at the site of a forming lateral root primordium (Marchant *et al*. 2002).

Auxin response changes during lateral root initiation

In addition to changes in auxin transport direction, the transition from lateral root initiation to lateral root emergence requires an altered auxin response. High auxin levels usually promote the initiation of lateral root primordia, whereas auxin levels need to drop afterwards in the primordium to allow its differentiation and elongation (Wightman *et al*. 1980; Laskowski *et al*. 1995). At a later differentiation phase, it gains 'autonomy' by synthesising its own auxin (Ljung *et al*. 2005), and in contrast with that of the primary root, elongation of the lateral root is stimulated by auxin (Muday and Haworth 1994). The changes in auxin response are reflected in the expression patterns of the auxin response gene *DR5*, which is localised in the earliest dividing pericycle cells and the early lateral root primordium, but disappears from an emerging lateral root, except for expression remaining in the lateral root tip (Benkovà *et al*. 2003). A microarray analysis confirmed these studies, showing that auxin response genes are activated during the very early cell divisions in a lateral root primordium, and later stages are characterised by downregulation of auxin biosynthesis genes and upregulation of auxin conjugation genes (Vanneste *et al*. 2005).

Negative regulation of lateral root initiation

Lateral root initiation and development are also under the control of negative regulators. Cytokinins inhibit lateral root initiation at the earliest stage of the asymmetric pericycle cell division, and it has been suggested that cytokinins could interfere with the cell fate re-specification mediated by *PIN* gene expression, because cytokinins inhibit PIN gene expression during lateral root initiation (Laplaze *et al*. 2007). In addition, early cell cycle activation in the pericycle is inhibited by cyclin dependent kinase (CDK) inhibitors, i.e. kip-related proteins (KRPs), which are repressed by auxin and are localised in cells not destined for lateral root initiation (Beeckman *et al*. 2001; Himanen *et al*. 2002).

How do rhizobia interfere with the root auxin balance?

Similar to lateral root development, the initiation of a nodule requires re-programming of pericycle cells. Unlike lateral root development though, cortical cells inside the root are also re-programmed during nodulation. Both cell types re-activate their cell cycle to form a new meristematic centre of actively dividing cells, although the cortical cells appear to be arrested in the G_0 phase of the cell cycle, rather than in G_2 or in an active state of cell cycling, as for xylem-pole pericycle cells (Foucher and Kondorosi 2000; Roudier *et al*. 2003). The group of early dividing cells is called a primordium, which is later invaded by rhizobia. Cells adjacent to the nodule primordium located in the central cortex divide to form the nodule meristem, which gives rise to the nodule parenchyma, vascular traces, vascular endodermis and nodule endodermis. A group of outer cortical cells divides and enlarges to give rise to the nodule cortex, and the nodule base is formed from cell divisions in the pericycle (Hirsch 1992). The centre of the emerging nodule is colonised by rhizobia which differentiate into bacteroids and fix nitrogen. The following section examines the roles of auxin at different stages of nodule development, in the early stage of nodule progenitor cell initiation (founder cell specification), in the stimulation of early cell divisions, in the differentiation of the nodule, and in the systemic regulation of nodule numbers.

Role of auxin in nodule founder cell specification

Nodules are initiated by Nod factors in pericycle and/or cortical cells, usually in front of xylem poles. There is a developmental 'window' of susceptibility of root cells to Nod factors that is located in the root elongation and differentiation zone (Bhuvaneswari *et al*. 1981). In addition, some legumes are susceptible to nodule formation at sites of lateral and

adventitious root emergence. It is not exactly known what specifies the nodule founder cells, or what determines the difference in founder cells between legumes that form indeterminate (inner cortex and pericycle) or determinate (initially outer cortex) nodules. Cell division is regulated by two crucial plant hormones that regulate cell cycle progression, auxin and cytokinin (Kondorosi *et al*. 2005). Both the concentration and the ratio of these two hormones can determine whether and where cells divide in the plant. Experiments with excised root sections have shown that altering the auxin to cytokinin balance specifies whether root cells divide in the pericycle or in the cortex of legumes (Libbenga *et al*. 1973).

The findings that NPA can induce spontaneous nodules (Hirsch *et al*. 1989), that rhizobia inhibit *GH3* expression within 10 h of inoculation in white clover and that this inhibition is mimicked by Nod factors, NPA and flavonoids (Mathesius *et al*. 1998*b*), suggest that rhizobia inhibit auxin transport in legumes forming indeterminate nodules before the onset of cell divisions (Fig. 4). This is supported by measurements of radio-labelled auxin transport in roots of garden vetch (*Vicia sativa* L.), which showed that rhizobia, and specifically functional Nod factors, inhibit polar auxin transport within 24 h of inoculation (Boot *et al*. 1999). Similar inhibition of auxin transport was found in *M. truncatula* (van Noorden *et al*. 2006; Wasson *et al*. 2006). However, no inhibition of auxin transport was detectable in *L. japonicus*, which forms determinate nodules, before nodule initiation (Pacios-Bras *et al*. 2003). Known regulators of auxin transport (Fig. 2) are flavonoids (Jacobs and Rubery 1988) and ethylene (Burg and Burg 1966). Both flavonoids (Mathesius *et al*. 1998*a*) and ethylene (Ligero *et al*. 1987) are induced early during nodulation. Ethylene is a negative regulator of nodulation (Guinel and Geil 2002), and is, thus, not a likely candidate for the early inhibition of auxin transport. Flavonoids are induced specifically in the precursor cells of a nodule after application of nodulating rhizobia or Nod factors (Mathesius *et al*. 1998*a*); they also accumulate after treatment of roots with cytokinin (Mathesius *et al*. 2000*a*). To test if flavonoids are required for auxin transport inhibition by rhizobia, Wasson *et al*. (2006) silenced the first enzyme of the flavonoid biosynthetic pathway, chalcone synthase, using RNA interference in *M. truncatula* hairy roots. These flavonoid-deficient roots did not nodulate, and auxin transport inhibition by rhizobia was abolished, confirming that flavonoids are

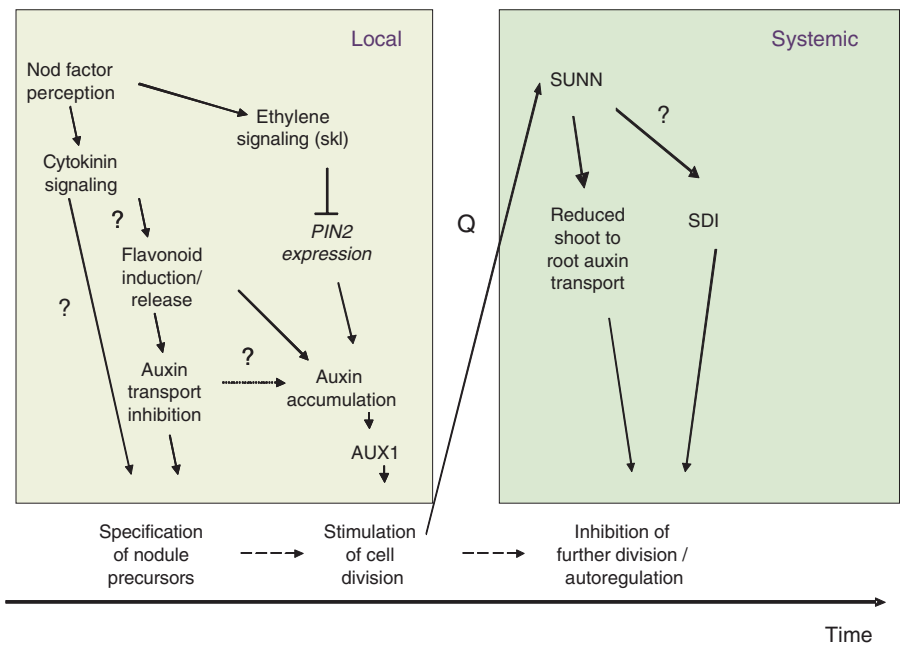

Fig. 4. Model for local and systemic regulation of auxin transport during nodulation in *Medicago truncatula*. Founder cell specification, primordium initiation and control of nodule numbers are thought to be regulated by auxin transport changes, at least in *M. truncatula*, on which this model is based. Nod factor perception causes local (i.e. at the inoculation site) auxin transport inhibition. This is dependent on the presence of flavonoids. It is possible that cytokinin signalling, which is activated by Nod factor perception, either provides an independent signal for founder cell specification, or is connected to flavonoid induction. Auxin transport inhibition could lead to the subsequent accumulation of auxin at the nodule initiation site. AUX1 is likely to be involved in transporting the accumulating auxin into the forming primordium. Ethylene signalling was found to downregulate this auxin accumulation via reducing *PIN* expression. An undefined step during the nodule initiation program stimulates a long distance signal (Q) to move to the shoot, where it activates the autoregulation receptor kinase, SUNN. SUNN mediates long distance inhibition of auxin translocation from the shoot to the root, which is associated with autoregulation. A separate inhibiting signal (SDI, shoot derived inhibitor) might be acting in parallel to auxin transport changes but it remains unidentified.

necessary for nodulation and for auxin transport inhibition in *M. truncatula* (Fig. 4). However, silencing of the isoflavonoid pathway in the legume soybean showed that isoflavonoids are crucial for nodulation as Nod gene inducers, but are unlikely to be required for auxin transport regulation in the development of a determinate nodule (Subramanian *et al*. 2006). Since no auxin transport inhibition was detectable preceding nodulation in soybean (Subramanian *et al*. 2006) or in *L. japonicus* (Pacios-Bras *et al*. 2003), it is possible that the early auxin transport inhibition is unique to legumes forming indeterminate nodules and required for nodule initiation from the pericycle and inner cortical cells (Wasson *et al*. 2006; Subramanian *et al*. 2007).

What is the effect of inhibiting auxin transport? Most likely, a reduction in auxin transport at the site of nodule initiation would initially reduce the auxin availability and therefore the auxin to cytokinin ratio in the inoculated root zone and root tip. In addition, there is strong evidence that rhizobia induce cytokinin signalling in the root before nodule initiation (Fig. 4) and that this is a required step for cortical cell divisions and for the induction of early nodulins like *ENOD40* (Fang and Hirsch 1998; Gonzalez-Rizzo *et al*. 2006; Murray *et al*. 2007). The localisation of the cytokinin-inducible *ENOD40* expression in pericycle and inner cortical cells several hours before the onset of cell division during nodulation supports a role for cytokinin in specifying the nodule founder cells (Charon *et al*. 1997; Mathesius *et al*. 2000*a*).

Both inhibition of auxin transport by NPA and the constitutive activity of the cytokinin receptor have been reported to be sufficient to initiate nodule-like structures in the absence of rhizobia (Hirsch *et al*. 1989; Tirichine *et al*. 2007). Either of those situations might be extreme when compared with what happens during nodule initiation by rhizobia, and a concomitant inhibition of auxin transport and increased cytokinin synthesis or response might be necessary for determining whether and where a nodule is initiated. So far it is unknown whether cytokinin signalling and auxin transport regulation are functionally linked, but there are indications that cytokinin can alter PIN gene expression (Laplaze *et al*. 2007) and can cause flavonoid as well as auxin accumulation in dividing cortical cells (Mathesius *et al*. 2000*a*). Future studies are needed to investigate whether the required ratios or sensitivities to auxin and cytokinin differ between legumes forming determinate and indeterminate nodules and specify the site of nodule initiation.

Role of auxin in nodule initiation and differentiation

Auxin transport inhibition in the hours preceding nodule initiation is followed by increased auxin transport and increased *GH3*::*GUS* expression in all cell layers at the site of nodule initiation in white clover (Mathesius *et al*. 1998*b*). Increased auxin levels were also found within 24 h of inoculation in bean (Fedorova *et al*. 2000), and strong induction of two auxin hydrolases, which release active auxin from conjugate forms, was found within 24 h of inoculation in *M. truncatula* (Campanella *et al*. 2008). Alternatively, the increase in auxin levels could be the result of the preceding inhibition of auxin export below the site of inoculation, which could cause acropetally-transported auxin to accumulate above that site (Fig. 4). Similar to the case of lateral root initiation, *GH3*::*GUS* expression experiments indicate that auxin is localised in the earliest dividing cells of a nodule primordium. In legumes forming indeterminate nodules, including white clover and *M. truncatula*, expression was localised in the pericycle and inner cortex (Mathesius *et al*. 1998*b*; van Noorden *et al*. 2007) and in the legume *L. japonicus*, which forms determinate nodules, expression was localised in the dividing outer cortex (Pacios-Bras *et al*. 2003). Therefore, in contrast to the requirement for auxin in the specification of founder cells, auxin appears to accumulate similarly in the early dividing cells of legumes forming either determinate or indeterminate nodules, and it is likely that auxin acts to stimulate cell cycle activity (Roudier *et al*. 2003).

Retention of auxin in dividing cells might be mediated by the spatially overlapping accumulation of flavonoids in the nodule precursor cells and early primordia. Certain flavonoids and other phenolics can inhibit the action of peroxidases and auxin oxidases (Furuya *et al*. 1962; Grambow and Langenbeck-Schwich 1983), and in white clover, those flavonoids accumulating in the inner cortical cells inhibit auxin breakdown by peroxidase (Mathesius 2001). Consistent with that, flavonoids have been shown to accumulate in outer cortical cells of the legume siratro (*Macroptilium purpureum* (DC.) Urb.), in which nodules are initiated in the outer cortex (Mathesius *et al*. 1998*a*), although their influence on auxin in legumes forming determinate nodules has not been investigated.

A study whereby the expression of members of the auxin import protein family MtLAX was localised showed that this transporter is strongly expressed in young nodule primordia in *M. truncatula* (de Billy *et al*. 2001). Likewise, the expression of the auxin export proteins PIN1 and PIN2 is localised in early nodule primordia in *M. truncatula* and their silencing by RNAi led to a reduction in nodule numbers (Huo *et al*. 2006). These studies strongly suggest that auxin transport into the initiating nodule could be responsible for the observed auxin accumulation in the primordium (Fig. 4). Whether or not changes in *PIN* and *LAX* gene expression or protein localisation are under the control of the flavonoid changes occurring during nodule initiation is so far unknown.

In a proteomic study comparing root responses to rhizobia with root responses to auxin (IAA) 24 h after each of their application, a high overlap (>80%) of protein changes was found in response to both treatments in *M. truncatula*, suggesting that increased auxin levels in the root could mediate some or many of the responses of the root to rhizobia (van Noorden *et al*. 2007). The necessity of auxin action during nodule initiation is supported by the fact that the auxin action inhibitor PCIB (*p*-chlorophenoxyisobutyric acid) reduces nodule numbers significantly (van Noorden *et al*. 2006). Auxin action is likely to be optimal only at a certain window of concentration, because increased exogenous auxin levels are known to inhibit nodulation, whereas very low exogenous auxin levels ($<10^{-8}$ M) stimulate nodulation (van Noorden *et al*. 2006). Unfortunately, no auxin response mutants have been available yet to test for the role of auxin response during nodulation.

The action of auxin during nodulation is linked with that of other plant hormones, for example cytokinin and gibberellic acid (GA). Cytokinins are likely to be required with auxin to sustain cell divisions in the nodule primordium. The cytokinin sensitive reporter *ARR5* (*Arabidopsis response regulator 5*) was localised to early nodule primordia in *L. japonicus* (Lohar *et al*. 2004), and

cytokinin-inducible *ENOD40* expression was also localised in nodule primordia (Crespi *et al*. 1994). In addition, cytokinin-insensitive plants are impaired in nodule initiation (Gonzalez-Rizzo *et al*. 2006; Murray *et al*. 2007).

The action of auxin during nodule initiation could also be linked to the effects of GA. GA synthesis has been shown to require IAA (Ross *et al*. 2000), and GA can stimulate IAA synthesis in nodule extracts (Dullaart and Duba 1970). The observations that GA-deficient pea mutants are defective in nodulation (Ferguson *et al*. 2005) and that GA is required for nodule primordium formation during lateral root-based nodulation in *S. rostrata* (Lievens *et al*. 2005) suggest that GA and auxin could act synergistically during primordium formation.

As the nodule primordium differentiates, *GH3* expression is retained in peripheral cell layers of the primordium but disappears from the central tissue (Fig. 1). In mature nodules, high *GH3* expression is found in vascular tissues and the apical meristem and these expression patterns are similar to those in differentiating lateral roots (Mathesius *et al*. 1998*b*; Pacios-Bras *et al*. 2003). The expression pattern of *MtAUX1* is similar, with high expression in peripheral tissues of a nodule and central tissues of lateral roots, indicating that expression overlaps with regions of vascular tissue or endodermal differentiation in both organs (de Billy *et al*. 2001). These expression patterns support the role of auxin in vascular differentiation (Aloni *et al*. 2006) and in nodule meristem maintenance, for example through cell cycle activation (Roudier *et al*. 2003; Kondorosi *et al*. 2005). Like in lateral root formation, the expression patterns also suggest that auxin levels must drop at the differentiation stage relative to the primordium initiation phase (Laskowski *et al*. 1995). The loss of auxin in central parts of legume nodules could be regulated by peroxidases that destroy auxin accumulating inside the nodule (Fedorova *et al*. 2000; Mathesius 2001).

The *cochleata* mutant of pea forms hybrid structures of nodules and lateral roots, where the nodule meristem appears to be re-specified into a lateral root meristem (Ferguson and Reid 2005). As the *cochleata* phenotype also includes agravitropism and its nodules resemble auxin-induced nodule-like structures in non-legumes, the authors of this study suggested that an abnormal auxin response in this mutant could be responsible for the altered nodule meristem phenotype.

The localisation and role of auxin in the initiation of legume nodules appears to differ from that in actinorhizal nodules. Examination of the role of *AUX1* in Swamp Oak (*C. glauca*), which forms nodules with actinorhizal bacteria, showed that expression is localised in infected cells, first in the pre-nodule in cortical cells and later in the nodule (Peret *et al*. 2007). A possible role of the purported high auxin levels in infected cells could be to mediate cell hypertrophy or cell wall remodelling during infection (Peret *et al*. 2007). However, expression is absent from nodule primordia, even though the same gene is strongly expressed in lateral root primordia in *Casuarina*. These data suggest that despite the similarities of actinorhizal nodules to lateral roots, their initiation might require distinct auxin responses. In the actinorhizal plant *Eleagnus umbellate* Thundb., high levels of an auxin-responsive protein have been found in the nodule fixation zone, although it is not clear if this expression pattern reflects auxin levels (Kim *et al*. 2007). These two reports suggest that high auxin levels in actinorhizal nodules might be derived from auxin synthesis of the symbiont (Peret *et al.* 2007). Since no Nod factor-related signal molecules have been identified yet from *Frankia*, it remains unclear whether all the reported changes in *AUX1* expression are due to auxin from the symbiont, or to changes in auxin as a result of signal transduction events in the root.

Role of auxin in the regulation of nodule numbers

Nodule numbers are regulated by several mechanisms. If sufficient nitrogen is available in the growth medium, plants prefer nitrogen uptake from nitrate or ammonium over the costly establishment of a nitrogen-fixing symbiosis. Both nitrate and ammonium inhibit nodulation at different stages of infection, nodule development and nitrogen fixation, although the mechanisms are mostly unknown (Streeter 1988). Whether auxin is involved in this inhibition is unclear. Nitrate regulates lateral root initiation and elongation by both local and systemic mechanisms, and this regulation involves auxin signalling, suggesting that similar mechanisms might be involved in nodulation (Walch-Liu *et al*. 2006).

The plant also has an internal, systemic regulatory mechanism to limit the numbers of nodules on a root system. This mechanism has been termed autoregulation of nodulation (AON) and is dependent on the action of a leucine-rich repeat receptor-like kinase (LRR-RLK), also termed nodulation autoregulation receptor kinase (NARK) acting in the shoot (Kinkema *et al*. 2006). After the first few nodules are formed on a root system, autoregulation inhibits further formation of nodules, probably to limit the amount of carbon redirected towards nodules. Split-root experiments have shown that an early event during nodule formation sends a signal (termed Q) to the shoot, where it, or a derivative signal, is perceived by NARK and causes another signal (shoot-derived inhibitor, or SDI) to move back to the root system to limit further nodulation (Fig. 4) (Kinkema *et al*. 2006). These long-distance signals have so far not been identified. As auxin is known to be a long-distance signal from the shoot to the root, which is important for lateral root formation, the role of shoot-to-root transported auxin was investigated for its role in autoregulation. van Noorden *et al*. (2006) showed that the *M. truncatula* autoregulation mutant *sunn* (super numeric nodules) (Schnabel *et al*. 2005) transports approximately three times as much auxin from the shoot to the root as the wild type. Auxin concentrations in the zone of the root susceptible for nodule initiation were similarly increased in *sunn*. In addition, the auxin response gene *GH3* was expressed at much higher levels in inoculated *sunn* roots than in wild-type roots (Penmetsa *et al*. 2003). Within 24 h of inoculating the root tip with compatible rhizobia, long-distance auxin transport from the shoot to the root was reduced in wild-type seedlings, correlating with the onset of autoregulation in *M. truncatula* (van Noorden *et al*. 2006). However, no inhibition of long-distance auxin transport occurred in *sunn*, suggesting that SUNN regulates long-distance auxin transport changes in response to inoculation. Treatment of the shoot–root junction of *sunn* with NPA caused a reduction in nodule numbers to levels similar to the untreated wild type (van Noorden *et al*. 2006). In the model suggested by van Noorden *et al*. (2006), AON-regulated auxin

transport positively correlates with nodule numbers, in contrast with the finding that AON induces a shoot-derived inhibitor that negatively correlates with nodule numbers. So far it is not known whether, in addition to the changes in auxin transport, a separate SDI signal is under the control of SUNN (Fig. 4). It is also unknown whether long-distance auxin transport occurs as part of AON in other legumes, in particular in legumes forming determinate nodules.

In soybean, inoculation of wild-type roots led to increased root auxin content after 48 h, whereas this increase was not detected in the *nts382* (nitrate tolerant supernodulation) supernodulation mutant (Caba *et al*. 2000). It was, therefore, suggested that autoregulation is caused by a burst of auxin in soybean (Gresshoff 1993). Although no long distance auxin transport measurements have been made in legumes with determinate nodules, it is likely that legumes forming determinate and indeterminate nodules might differ in their perception or requirement for auxin in the regulation of nodule numbers.

It is important to note that the long-distance regulation of auxin transport during AON in legumes forming indeterminate nodules is regulated independently of local auxin transport inhibition that occurs at the root tip within hours of inoculation and is necessary for the initiation of the first nodules on the root (Fig. 4). The *sunn* mutant shows local auxin transport inhibition after inoculation with rhizobia similar to the wild type, despite the difference in long distance transport (van Noorden *et al*. 2006).

Nodule numbers are also regulated by ethylene, which is demonstrated in the hypernodulation phenotype of the ethylene insensitive *skl* (sickle) mutant (Penmetsa and Cook 1997). The gene mutated in the *skl* mutant has been shown to encode an orthologue of the *Arabidopsis* ethylene signalling protein EIN2 (Penmetsa *et al*. 2008). The effect of ethylene is local, i.e. ethylene acts in the root, as established from grafting experiments in *M. truncatula* (Prayitno *et al*. 2006*b*). Ethylene might have several roles, one in the regulation of defence responses that could restrict infection (Penmetsa and Cook 1997; Prayitno *et al*. 2006*a*; Penmetsa *et al*. 2008) and the other in the regulation of auxin transport. After inoculation, auxin transport inhibition at the root tip still occurred in *skl* (Prayitno *et al*. 2006*b*), consistent with the requirement of auxin transport inhibition for nodule initiation. Within 24 h, the increase in auxin transport observed in the wild type was exaggerated in *skl*, and this was accompanied by an increased expression of *PIN2* and increased numbers of nodules initiated at the site (Prayitno *et al*. 2006*b*). This suggests that ethylene synthesis or perception could downregulate the auxin accumulation at the site of nodule initiation (Fig. 4). This observation is in accordance with the ability of ethylene or its precursors to inhibit auxin transport (Burg and Burg 1966; Prayitno *et al*. 2006*b*). Ethylene is induced during nodule initiation (Ligero *et al*. 1986), and could be a signal to limit nodule numbers, as it also negatively influences Nod factor signalling (Sun *et al*. 2006). Ethylene also affects translocation of auxin from the shoot to the root. Although long-distance auxin transport was normal in uninoculated *skl* plants, the downregulation of auxin transport observed in wild type 24 h after inoculation with rhizobia did not occur in *skl* (Prayitno *et al*. 2006*b*). The relatively increased long-distance auxin transport in *skl* correlates with higher numbers of nodules formed in the root, in a manner similar to the higher long-distance auxin transport in the supernodulating mutant *sunn*.

The interaction of auxin and ethylene during nodulation is supported by the finding that root growth in *sunn* was less sensitive to ethylene than in the wild type (Penmetsa *et al*. 2003). Ethylene inhibits root growth via effects on auxin (Stepanova *et al*. 2007). If auxin is already at super-optimal levels for root growth in *sunn*, it is possible that ethylene has a relatively reduced effect on inhibition of root growth in *sunn*.

Differences and similarities between lateral root and nodule development

Despite the clear similarities between the development of lateral roots and nodules, in most legumes these organs are distinct. Lateral roots are pre-specified during plant development; they arise from pericycle cells and form a central stele. Nodules in many legumes are initiated *de novo* at unspecified times during plant development; they arise from both pericycle and cortical cells, and typically have peripheral vascular strands. The data discussed above suggest that the major difference lies in the specification of the founder cells of these organs, whereas their development might be regulated similarly (Fig. 5). The separate specification of the founder cells is supported by different requirements for auxin and cytokinin. Whereas high auxin concentrations increase the numbers of lateral roots formed, high auxin concentrations inhibit the formation of nodules (van Noorden *et al*. 2006). Cytokinin has the opposite effect, and inhibits lateral root formation but increases nodule numbers (Lohar *et al*. 2004), and can lead to spontaneous nodule formation (Gonzalez-Rizzo *et al*. 2006). Lateral roots and nodules also differ in their requirement for flavonoids. Whereas nodule initiation requires the presence of flavonoids, presumably because of the temporary action of flavonoids in inhibiting auxin transport, flavonoid-deficient *M. truncatula* plants still form lateral roots (Wasson *et al*. 2006) and lateral root numbers are slightly increased in *Arabidopsis*, possibly due to higher auxin transport in flavonoid-deficient mutants (Brown *et al*. 2001). In addition, distinct flavonoids mark the precursor cells of lateral roots and nodules (Mathesius *et al*. 1998*a*; Morris and Djordjevic 2006).

It might, therefore, seem surprising that despite the differences in organ initiation, several studies have shown a genetic link between the numbers of nodules and lateral roots formed on a legume root system. This was first studied by Nutman, who observed that different cultivars of red clover showed a positive correlation between the number of lateral roots and nodules (Nutman 1948). One of the determinants of lateral root and nodule numbers could be certain plant hormones. For example, in pea mutants deficient in GA and brassinosteroids, nodule numbers were reduced, in concert with reduced lateral root numbers (Ferguson *et al*. 2005). Another shared determinant of lateral root and nodule numbers appears to be the autoregulation gene. The autoregulation mutants *nts* of soybean (Searle *et al*. 2003), *har1* (hypernodulation aberrant root) of *L. japonicus* (Wopereis *et al*. 2000) and *sunn* of *M. truncatula* (van Noorden 2006) form more lateral roots than the wild type in the absence of rhizobia. However, in these studies inoculation led

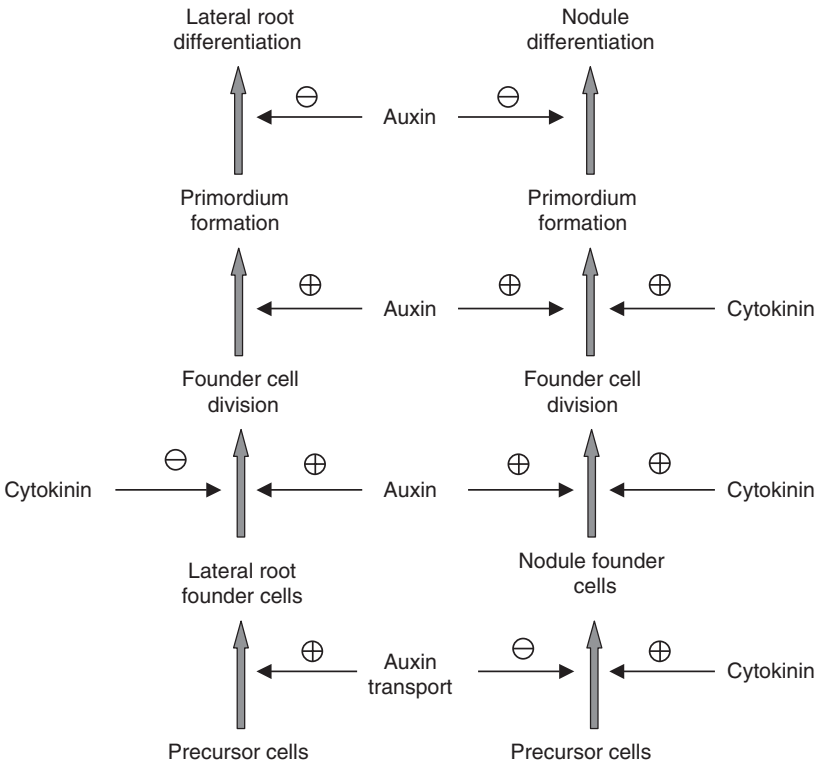

Fig. 5. The role of auxin and cytokinin in lateral root and nodule organogenesis. The organogenesis programs of lateral roots and nodules are similar in that founder cells divide, give rise to a primordium, the cell of which then divide and later differentiate. The difference is the specification of founder cells in either the pericycle (lateral roots) or the pericycle and inner cortex (indeterminate nodules). Lateral root founder cell specification is likely to be regulated by auxin oscillations in the basal root meristem. Nodule founder cells are likely to be specified by reduced auxin and increased cytokinin levels or signalling. Auxin is a positive signal for cell cycle activation of the founder cells and in the early primordium, whereas auxin levels presumably need to drop to allow organ differentiation. In contrast, cytokinin is a positive regulator for cell cycle activation in nodule primordia, whereas it is a negative regulator of lateral root founder cell division.

to a significant reduction of lateral root numbers of the root system, although this can be transient and total numbers of lateral roots can be increased in nodulated mature root systems. Therefore, it is possible that the plant regulates the total number of lateral root organs on a root system systemically, perhaps through an autoregulatory system related to the AON mechanisms. The systemic regulation might be acting independently of the local specification of organ founder cells. This could simply reflect a system to balance resource availability in the whole root system, and it would be interesting to test whether the long-distance transport of auxin could determine resource allocation from the shoot to the root.

A model for the role of auxin in nodulation

A hypothesis suggested by the sum of the data discussed above is that the requirement of lowering the auxin to cytokinin ratio is a specific step in the initiation of nodules. This is opposite to the initiation of lateral roots, which is promoted by high auxin to cytokinin ratios. Both the reduction of auxin transport by Nod factors and the induction of cytokinin signalling, which precede nodule initiation, could be crucial steps in the specification of nodule precursor cells in legumes forming indeterminate nodules (Fig. 5).

Once the organ is specified, it is likely that auxin plays similar roles in activating cell cycle activity in both types of primordia. A drop in auxin levels or response might be a shared requirement for the subsequent organ differentiation, although auxin remains a positive regulator for vascular differentiation and ongoing meristem activity (Fig. 5). This is supported by the similar patterns of auxin accumulation during lateral root and nodule formation after founder cell specification (Fig. 1). Independent of the local role of auxin in nodule initiation, long distance auxin transport is a mechanism that has been shown to control both lateral root and nodule numbers. Another shared aspect of lateral root and nodule differentiation is that the emergence of the organs, i.e. the activation of the meristem, requires the action of the same gene, *LATD*, which regulates ABA response in the root (Bright *et al*. 2005; Liang *et al*. 2007).

Future directions

Key questions about the role of auxin in nodulation remain; specifically, the differences in auxin requirements in founder cell

specification in determinate and indeterminate nodule types, the mechanism of auxin transport regulation by the autoregulation of nodulation process in indeterminate nodulation, as well as the interaction between auxin and cytokinin signalling during nodule initiation. The biggest impact in our knowledge of lateral root regulation by auxin has been through the analysis of various auxin mutants of *Arabidopsis*. Such mutants are currently lacking in legumes, but would be very useful for future research. For example, if autoregulation mutants could be rescued by mutations in the auxin transport machinery, this would support a link between auxin transport and nodule number regulation. Auxin response and auxin transport mutants of legumes forming determinate and indeterminate nodules could be used to test whether different auxin responsiveness is required for these two nodule types. Alternatively, cell-specific silencing of auxin response genes in inner and outer cortical cells could be expected to selectively inhibit the initiation of indeterminate or determinate nodule types. Advances in our understanding of auxin and cytokinin interactions could be made by studying auxin responses in cytokinin-insensitive mutants during nodulation. These and other genetic studies are likely to happen in some of the legume model species (Smit and Bisseling 2008). However, a wealth of information could be gained from the analysis of non-model legumes with a variety of nodule organogenesis programs that range from modified lateral roots to *de novo* formed nodules (Hirsch *et al*. 2001; Sprent and James 2008).

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