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REVIEW PAPER

Auxin transport, metabolism, and signalling during nodule initiation: indeterminate and determinate nodules

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Abstract

Most legumes can form a unique type of lateral organ on their roots: root nodules. These structures host symbiotic nitrogen-fixing bacteria called rhizobia. Several different types of nodules can be found in nature, but the two best-studied types are called indeterminate and determinate nodules. These two types differ with respect to the presence or absence of a persistent nodule meristem, which consistently correlates with the cortical cell layers giving rise to the nodule primordia. Similar to other plant developmental processes, auxin signalling overlaps with the site of organ initiation and meristem activity. Here, we review how auxin contributes to early nodule development. We focus on changes in auxin transport, signalling, and metabolism during nodule initiation, describing both experimental evidence and computer modelling. We discuss how indeterminate and determinate nodules may differ in their mechanisms for generating localized auxin response maxima and highlight outstanding questions for future research.

Keywords: Auxin, crosstalk, legume, nodule, primordia initiation, rhizobia.

Introduction

Legumes are well known for their ability to form a symbiotic interaction with nitrogen-fixing bacteria collectively called rhizobia. These bacteria are housed intracellularly in specialized organs on the root called nodules. These organs are very different from lateral roots, making the legume root an interesting model from a developmental point of view. Although there are large overlaps in the signalling components and developmental processes involved in the formation of both lateral organs, there also exist striking differences (Hirsch *et al.*, 1997; de Billy *et al.*, 2001; Franssen *et al.*, 2015). Lateral root initiation is influenced by environmental signals, but ultimately the plant produces lateral roots in response to internal signals. Nodules, on the other hand, require the presence of a symbiont, and their initiation is triggered by specific rhizobially produced signalling molecules: lipochitooligosaccharides (LCOs), often referred to as Nod factors (Yang *et al.*, 1994). The required early signalling cascade for nodule initiation is largely co-opted from the much older (~450 Mya) and more widespread (~80% of all land plants) symbioses with arbuscular mycorrhiza (Catoira *et al.*, 2000; Maillet *et al.*, 2011).

Much of our current understanding on the role of auxin during nodule initiation is based on insights into auxin signalling during lateral root organogenesis (Mathesius, 2008). It seems that auxin signalling is crucial to the developmental programmes of both organs. Three main functions have been demonstrated for auxin during nodulation: cell cycle control,

vascular tissue differentiation, and rhizobial infection. During nodule development, auxin is a crucial signal controlling the cell cycle (Kondorosi *et al.*, 2005). Silencing of the cell cycle regulator *CDC16* in *Medicago truncatula* reduced auxin sensitivity and increased nodule numbers (Kuppusamy *et al.*, 2009), while the auxin-induced cyclin CycA2 is important for activation of the cell cycle in nodule meristems (Roudier *et al.*, 2003). Moreover, auxin plays a role in vascular differentiation in the nodule, with strong auxin responses occurring in the vascular tissue of nodules (e.g. Takanashi *et al.*, 2011) and aberrant auxin responses found in vascular tissues of nodules that formed central, rather than peripheral vascular bundles (Guan *et al.*, 2013). As an additional role in nodulation, auxin is also involved in the infection process in the root hair. For example, infection of rhizobia is significantly reduced in the auxin response mutant *arf16a* in *M. truncatula* (Breakspear *et al.*, 2014). The main focus of this review will be the role of auxin in the process of nodule initiation and development.

In both developmental programmes—lateral root and nodule—a tight correlation has been found between the position of auxin response and meristematic activity (Larkin *et al.*, 1996; Rolfe *et al.*, 1997; Mathesius *et al.*, 1998*b*; Pacios-Bras *et al.*, 2003; Takanashi *et al.*, 2011; Suzaki *et al.*, 2012; Herrbach *et al.*, 2014). In addition, meristematic markers including *PLT* (*PLETHORA*) and *WOX5* (*WUSCHEL-RELATED HOMEOBOX*) are expressed in both organs, with localization of four *PLT* and the *WOX5* genes in the nodule meristem as well as the root apical meristem, with, in both cases, expression overlapping with an auxin maximum in the meristem (Osipova *et al.*, 2012; Franssen *et al.*, 2015). Nevertheless, there are several indications that the processes leading to lateral root and nodule initiation are wired differently. For example, nodule-like structures can be induced by exogenous cytokinin application (e.g. Cooper and Long, 1994; Heckmann *et al.*, 2011), whereas this hormone has a strong inhibiting effect on lateral root initiation in both *Arabidopsis thaliana* and model legumes (Lohar *et al.*, 2004; Laplaze *et al.*, 2007; Marhavý *et al.*, 2011; Plet *et al.*, 2011). The number of lateral roots is increased by the application of auxin (Blakely and Evans, 1979; Woodward and Bartel, 2005), while external auxin application inhibits nodulation (van Noorden *et al.*, 2006; Li *et al.*, 2014). In addition, the initiation of lateral roots shows a strong preference for the convex side of root bends (Fortin *et al.*, 1989; Laskowski *et al.*, 2008; Deinum *et al.*, 2015), whereas nodules show no such bias (Deinum *et al.*, 2015). Last, but not least, the primordia are initiated from different cell layers. In Arabidopsis, lateral roots are exclusively founded from pericycle cells (Malamy and Benfey, 1997; Casimiro *et al.*, 2003). In model legumes, which all have multiple cortical cell layers, lateral root primordia are still predominantly pericycle derived in both indeterminate (e.g. Herrbach *et al.*, 2014) and determinate nodule-forming species (e.g. Held *et al.*, 2014). However, endodermal and some cortical divisions can also be observed, a feature shared with many non-legume plants (Mallory *et al.*, 1970; Lloret *et al.*, 1989; Casero *et al.*, 1993; Op den Camp *et al.*, 2011; Xiao *et al.*, 2014). Nodule primordia in the model legume *M. truncatula* are predominantly founded by the inner cortical cell layers, but pericycle and endodermis cells also contribute to the eventual nodule (Timmers *et al.*, 1999; Xiao *et al.*, 2014). The induction of these nodule primordia occurs in the so-called susceptible zone. The exact position of the susceptible zone along the root developmental axis differs among species, but it is transient and often begins where root hairs start to develop several millimetres behind the root tip (Bhuvaneswari *et al.*, 1981). This is similar to the zone where lateral roots are initiated, ~4 mm behind the root tip in *M. truncatula*, although lateral roots continue to emerge from dormant primordia in the mature root (Herrbach *et al.*, 2014).

In this review, we will focus on the role of auxin transport, metabolism, and signalling in controlling auxin accumulation during nodule initiation. How are auxin transport, metabolism, and signalling modified in response to Nod factor signalling? What are the commonalities and differences between different nodule types?

Different types of legume nodules

Several different types of nodules exist in nature. However, here we will mainly focus on the two most predominant and best-studied types: indeterminate and determinate nodules. A key difference between these two types of nodules is which cortical cell layers give rise to the nodule primordium (Hirsch, 1992; Sprent, 2007) (Fig. 1). While many legumes from all three subfamilies of Leguminosae form nodules with a persistent nodule meristem ('indeterminate nodules'), mature nodules of members of the Millettioid, Dalbergioid, and Loteae clades do not retain an active meristem ('determinate nodules') (Hirsch, 1992; Sprent, 2007). Correlated with meristem persistence is the position of the first cell divisions that give rise to the nodule primordium. In indeterminate nodules (such as those formed by species including *M. truncatula*, *Medicago sativa*, *Pisum sativum*, and *Vicia sativa*), cell divisions occur in the inner cortex and pericycle (Libbenga and Harkes, 1973; Timmers *et al.*, 1999; Xiao *et al.*, 2014), whereas in determinate nodules cell divisions are restricted to the middle (*Lotus japonicus*) or outer (*Glycine max*) cortex (Hirsch, 1992). The position of these primary divisions coincides with the position of auxin signalling in cortical cells, with additional expression in the pericycle and endodermis during nodule initiation (Fig. 1). This indicates that the initiation of cell division is correlated with the presence of an auxin maximum, as determined through *GH3::GUS* auxin reporter lines in species forming indeterminate (Mathesius *et al.*, 1998*b*; van Noorden *et al.*, 2007; Breakspear *et al.*, 2014; Ng *et al.*, 2015) and determinate nodules (Takanashi *et al.*, 2011). Further auxin maxima determined through *DR5::GFP-NLS* reporter lines in *L. japonicus* (Suzaki *et al.*, 2012), as well as *DR5::tDT* and *DR5::GUS* in soybean (Turner *et al.*, 2013) were found mainly in the proliferating outer cortical cells. Both nodule types contain peripheral vascular bundles and a central mass of mostly infected cells, where nitrogen fixation takes place, as well as some uninfected cells. However, the processes of infection, nitrogen fixation, and senescence of nitrogen-fixing tissue are spatially separated in indeterminate nodules, whereas such a separation does not exist in determinate

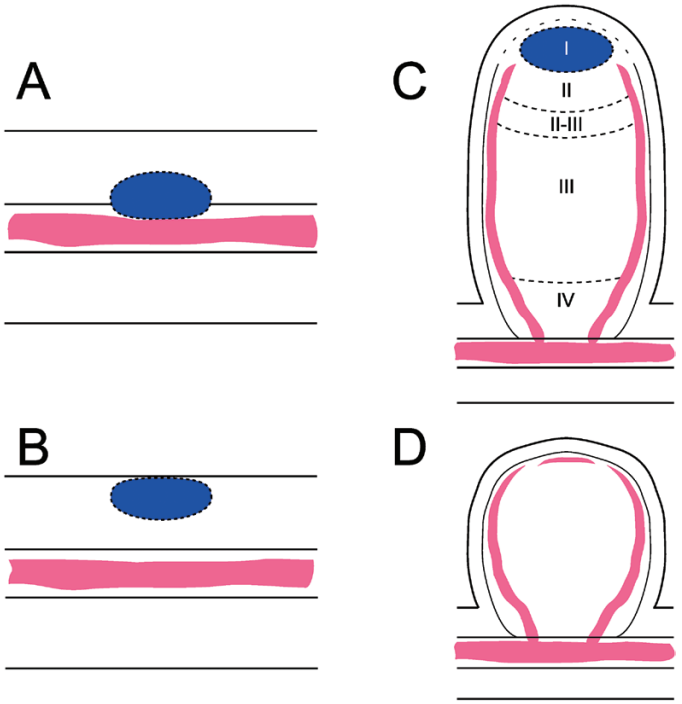

Fig. 1. Indeterminate (A, C) and determinate (B, D) nodules. Position of the first cell divisions (A, B), which coincides with a local auxin response. Mature nodule structure (C, D). Blue: cell divisions/meristematic tissue. Pink: vascular tissue, root stele and nodule vascular strands. Indeterminate nodules (C) maintain an active meristem (I) followed by an infection zone (II), transition zone (II–III), fixation zone (III), and, when the nodule gets older, a senescence zone (IV). Determinate nodules (D) lack this distinct zonation. When the nodule senesces, the process starts from the centre of the nodule. Zones after Hirsch (1992).

nodules (Fig. 1; Hirsch, 1992). Auxin responses are absent in the infected zone of both indeterminate and determinate nodules, but are retained in vascular tissue (Takanashi *et al.*, 2011; Suzaki *et al.*, 2012; Turner *et al.*, 2013; Breakspear *et al.*, 2014). Indeterminate nodules, which retain an apical meristem, also show auxin responses in the meristem (Guan *et al.*, 2013; Breakspear *et al.*, 2014; Franssen *et al.*, 2015)

An additional type of nodule can be found on the roots of the only non-legume genus known to form a root nodule symbiosis with rhizobia: *Parasponia*. Here, indeterminate nodules contain a central vascular bundle. In other words, these nodules are morphologically more similar to lateral roots than legume nodules (Price *et al.*, 1984). This different type of nodule shows that the peripheral vasculature is not essential for nodule function. Further morphological and developmental diversity can be found in other legumes such as lupin (*Lupinus albus*) and peanut (*Arachis hypogaea*) (Guinel, 2009). Unfortunately, these nodule types have hardly been studied using molecular approaches and no data are available on auxin responses in these nodule types.

The meaning of pseudonodules

A final 'type' of nodule that has had and still has great influence on the field is the pseudonodule. Pseudonodules are a collection of roughly nodule-shaped root outgrowths that can be induced in the absence of rhizobia in a number of ways. Few of these structures develop the typical peripheral vasculature, including pseudonodules induced by purified Nod factors on *G. max* and *M. sativa* (Truchet and Roche, 1991; Stokkermans and Peters, 1994), cytokinin application (Heckmann *et al.*, 2011), as well as the spontaneous (pseudo)nodules formed on roots with the constitutively active cytokinin receptor LHK1 (Tirichine *et al.*, 2007) or DMI3/CCaMK (Gleason *et al.*, 2006; Tirichine *et al.*, 2006). Other pseudonodules develop a central vasculature, which led to the suggestion that they are more like modified lateral roots (e.g. Allen *et al.*, 1953). Such pseudonodules include those formed by application of synthetic auxin transport inhibitors like TIBA (2,3,5-triiodo benzoic acid) or NPA (1-*N*-naphthylphthalamic acid) (e.g. Hirsch *et al.*, 1989), or the synthetic auxin 2,4-D (2,4-dichlorophenoxyacetic acid, e.g. Hiltenbrand *et al.*, 2016), although IAA (indoleacetic acid) itself does not induce pseudonodules (Mathesius *et al.*, 2000). A similar central vascular structure, however, is also observed in several uninfected rhizobia-induced nodules (Guan *et al.*, 2013). In addition, transport inhibitor-induced pseudonodules on *M. sativa*, *P. sativum*, and *M. truncatula* have been shown to express genetic markers typical for real nodules (Hirsch *et al.*, 1989; Scheres *et al.*, 1992; Rightmyer and Long, 2011).

Clearly, the occurrence of pseudonodules (particularly in response to 2,4-D) has to be interpreted with caution. Regardless, pseudonodules have been important in the hypotheses that auxin transport inhibition is part of the process that leads to nodule formation (Hirsch *et al.*, 1989), and that cytokinin signalling is sufficient to trigger nodule initiation (Tirichine *et al.*, 2007). A careful study of the timing and location of the earliest cell divisions in various pseudonodules would be informative. Nonetheless, as discussed below, differences exist among legumes in their potential to form pseudonodules, which could hint at underlying differences in the mechanisms of initiation and progression of nodule formation.

The ins and outs of auxin transport in legumes

It has been demonstrated that in response to Nod factor signalling, an auxin maximum—visualized by *GH3*::*GUS* and/or *DR5*::*GUS* expression—is established during the initiation of a nodule primordium (Fig. 1; Mathesius *et al.*, 1998*b*; van Noorden *et al.*, 2007; Takanashi *et al.*, 2011; Suzaki *et al.*, 2012). It has long been postulated that initiation of this maximum is regulated by changes in auxin transport capacity (Hirsch *et al.*, 1989; Mathesius *et al.*, 1998*b*). However, the molecular mechanisms by which this is achieved are still poorly understood. A contributing factor to this is that most legumes are far from ideal plant models. Cell biology has proven more difficult compared with the model species Arabidopsis (Barker *et al.*, 1990; Kouchi *et al.*, 2004). A chronic absence of stable transformation protocols, especially in *M. truncatula* where elevated levels of co-suppression hinder their usage, leads to a limited amount of available

genetic tools. In addition, the relative thickness of the root and a high abundance of secondary metabolites hinder state-of-the-art cell biology (Holmes *et al.*, 2008; Watson *et al.*, 2015). As a result, most, if not all, research on auxin homeostasis in model legumes such as *M. truncatula* and *L. japonicus* is based on fundamental research performed on the model Arabidopsis. However, Arabidopsis does not form root nodules and in many cases functionality is extrapolated from sequence homology only (e.g. Schnabel and Frugoli, 2004; Huo *et al.*, 2006; Plet *et al.*, 2011; Sańko-Sawczenko *et al.*, 2016). The genes involved in auxin transport, *PIN* (*PIN-FORMED*) and *AUX1*/*LAX* (*AUXIN RESISTANT 1*/*LIKE-AUX1*), are no exception. Please note that the numbering of the legume PINs and AUX1/LAXs is not always consistent with that of Arabidopsis. Although this is a recurring theme in plant biology, it is an important fact to keep in mind when dealing with functionality based on orthology.

PIN proteins are a group of auxin efflux carriers extensively studied in Arabidopsis (Friml *et al.*, 2003; Furutani *et al.*, 2004; Blilou *et al.*, 2005; Paponov *et al.*, 2005; Huang *et al.*, 2010). However, their function in legumes has never been demonstrated. PIN proteins are specifically positioned on the cell membranes and therefore are responsible for the polarity of auxin transport. If the direction of auxin transport needs to change, PIN proteins can be re-localized accordingly, a process often required during organ initiation (Wiśniewska *et al.*, 2006; Benková *et al.*, 2003). In the Arabidopsis genome, eight PIN proteins have been identified, which can be divided into two distinct types referred to as long- and short-looped PINs based on their molecular structure. The long-looped PINs (AtPIN1, 2, 3, 4, and 7) co-facilitate auxin cell-to-cell transport (Vieten *et al.*, 2005; Ganguly *et al.*, 2010). The short-looped PINs (AtPIN5 and 8) are less well studied. These PINs are located to the endoplasmic reticulum (ER) and are believed to regulate cytosolic auxin homeostasis (Mravec *et al.*, 2009; Ding *et al.*, 2012). The only exception to this rule seems to be AtPIN6, which as a long-looped PIN was shown be located to the ER (Mravec *et al.*, 2009).

The model legumes *M. truncatula*, *L. japonicus*, and *G. max* genomes harbour 12, 11, and 23 PIN proteins, respectively (Wang *et al.*, 2015*a*; Sańko-Sawczenko *et al.*, 2016; Fig. 2A). The genome of *G. max* underwent a relatively recent whole-genome duplication, and—with the exception of PIN1a—all PINs can be found in duplicate (Schmutz *et al.*, 2010). In *L. japonicus*, several incomplete fragments resembling PIN proteins can be found. However, it is not clear whether these fragments represent genuine *PIN* genes, or are just artefacts since the *L. japonicus* genome is far from complete and almost no *L. japonicus* transcriptome data have been made publicly available. For Fig. 2A, the ORF of *LjPIN8* (Lj3g3v3735560) was extended by an additional 345 nucleotides before reaching a stop codon, and the two annotated *LjPIN6* fragments *LjPIN6a* (Lj0g3v0178829) and *LjPIN6b* (Lj1g3v0264160) were joined to form *LjPIN6a/b.* A similar correction was made in *GmPIN6a* (Glyma.13G038300-Glyma.13G038400). These changes provided sequences very similar to those of *M. truncatula* (Fig. 2A). However, whether these corrections are justified remains to be validated. In addition, two

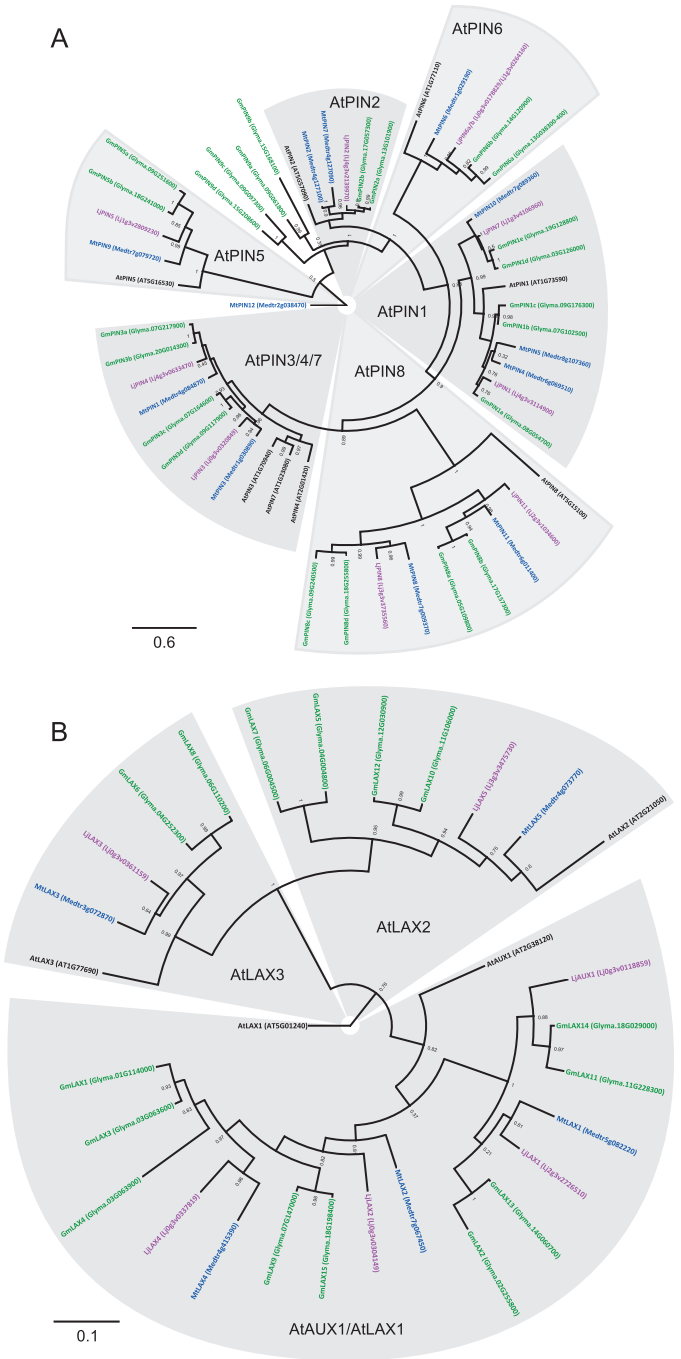

Fig. 2. Gene trees of *A. thaliana* (black), *M. truncatula* (blue), *L. japonicus* (pink), and *G. max* (green) PINs (A), and AUX1/LAXs (B). The trees are depicted as rooted for readability only. Numbers along branches represent bootstrap values of 100 resampling trees. Scale bars indicate substitutions per site. Trees were constructed based on MAFFT multiple sequence alignments (Katoh *et al.*, 2002) using the FastTree 2.1.5 algorithm, both using default settings of Geneious 9.0.4 (alignment: algorithm: default; scoring matrix, BLOSUM62; gap open penalty, 1.53; offset value, 0.123).

L. japonicus PIN1 genes (Lj4g3v3114900 and Lj2g3v0661480) with 100% identity at the nucleotide level were considered to be only one copy.

When analysing long PINs, three subgroups, so-called orthogroups, can be identified (Fig. 2A). The first group is comprised of AtPIN1, three *M. truncatula* PINs (MtPIN4, MtPIN5, and MtPIN10), two *L. japonicus* PINs (LjPIN1 and

LjPIN7), and five *G. max* PINs (GmPIN1a–e); together they form the PIN1 orthology group. Interestingly, MtPIN10, LjPIN7, and GmPIN1d-e represent an ancestral form, lost in Arabidopsis (Fig. 2A). Expression data are only available for *M. truncatula*, where it was shown that *MtPIN10* is highly expressed in both root and nodules (Roux *et al.*, 2014; Sańko-Sawczenko *et al.*, 2016). This makes MtPIN10 an excellent candidate for studying its involvement in nodulation. So far, no nodulation phenotypes have ever been described for these PINs. However, it is possible that this lack of phenotypes is due to redundancy with any of the additional PINs in this orthogroup. In line with this, *MtPIN4* is expressed in mature nodules (Roux *et al.*, 2014). RNAi knockdown of *MtPIN4* reduced nodule density (Huo *et al.*, 2006), but off-target effects of this construct on *MtPIN10* and/or *MtPIN5* were not excluded, leaving the question of possible gene redundancy unanswered. As little is known of the involvement of long PINs during nodulation, it would still be interesting to analyse double and/or triple mutants of this orthogroup in relation to nodule initiation. A second orthology group is comprised of three Arabidopsis proteins (AtPIN3, AtPIN4, and AtPIN7), two *M. truncatula* proteins (MtPIN1 and MtPIN3), two *L. japonicus* proteins (LjPIN3 and LjPIN4), and four *G. max* proteins (GmPIN3a–d). Closer inspection reveals that MtPIN1/LjPIN4/GmPIN3c/d are probably orthologues to AtPIN4, whereas MtPIN3/LjPIN3/GmPIN3a/b are more closely related to AtPIN3 and AtPIN7 (Sańko-Sawczenko *et al.*, 2016). Interestingly, *MtPIN1* is expressed in both *M. truncatula* roots and nodules. In Arabidopsis, *AtPIN4* expression is located around the quiescent centre (Friml *et al.*, 2002). Here it functions in transporting auxin towards the auxin maxima in the quiescent centre and columella (Blilou *et al.*, 2005). The expression of *MtPIN1* in both roots and nodules suggests that it has a function in both organs. Detailed analysis of gene expression, using laser microdissection of mature nodules combined with RNA sequencing, revealed that *MtPIN1* is most predominantly expressed at the nodule apex (Roux *et al.*, 2014). The *M. truncatula* root nodule has a functional meristem, and the expression domain of *MtPIN1* fits with a function during meristem maintenance. Mutants have not been reported so far, but could shed light on any putative MtPIN1 function during nodulation. As *L. japonicus* and *G. max* both have meristemless mature nodules, a differential spatial–temporal expression between *MtPIN1* and *LjPIN4*/*GmPIN3c/d* during nodule initiation and/or development could—at least in part—explain the absence of such a meristem. However, such expression data are currently not publicly available either for *L. japonicus* or for *G. max*. *MtPIN3* is highly expressed in the *M. truncatula* root, but absent from the nodule (Roux *et al.*, 2014; Sańko-Sawczenko *et al.*, 2016). Finally, MtPIN2, MtPIN7, LjPIN2, and GmPIN2a-b are orthologous to AtPIN2. Like *MtPIN3*, *MtPIN2* is expressed in the *M. truncatula* root but not in mature nodules. However, promoter activity was detected at the base of developing nodules (Huo *et al.*, 2006; Sańko-Sawczenko *et al.*, 2016).

When looking at the short-type PINs, three orthology groups can also be identified (Fig. 2A). AtPIN5 groups together with MtPIN9, LjPIN5, and GmPIN5a/b; AtPIN6 with MtPIN6, LjPIN6, and GmPIN6a-b; and AtPIN8 with MtPIN8, MtPIN11, LjPIN11, LjPIN8, and GmPIN8a–d. Overall, short-type PINs, apart from *MtPIN11*, are expressed at a low level in the *M. truncatula* root. On the other hand, expression of *MtPIN6*, *9*, and *11* is relatively high in the mature nodule. In particular, *MtPIN9* expression is strikingly high (Sańko-Sawczenko *et al.*, 2016). However, this is in contrast to previously published work that demonstrated expression of *MtPIN6* and *MtPIN9* to be low in mature nodules (Roux *et al.*, 2014). If the function of short PINs is evolutionarily conserved, even a low expression could indicate that MtPIN9 might be involved in nodule auxin homeostasis. In addition, although *MtPIN9* expression in the root is also low, it is strongly down-regulated in the early response to Nod factors (Plet *et al.*, 2011). This could suggest a function for MtPIN9 during the establishment of an auxin maximum prior to the development of a nodule primordium. However, it is too early to draw any conclusions. As for most legume PINs, limited data are currently available on the exact spatio-temporal expression patterns, localization, or function of MtPIN9. Overall, available results suggest a role for PIN-related auxin transport during nodulation.

In addition to efflux, auxin transport requires influx. This occurs in part by diffusion, but is also facilitated by a small multigene family of high-affinity auxin influx carriers (AUX1/LAX). In Arabidopsis, this family consists of four highly conserved genes, *AUX1*, *LAX1*, *LAX2*, and *LAX3* (Péret *et al.*, 2012; Swarup and Péret, 2012). Although this multigene family is larger in *M. truncatula*, *L. japonicus*, and *G. max* [5, 6, and 15, respectively (Chai *et al.*, 2016; Roy *et al.*, 2017)] (Fig. 2B), their sequences remain highly conserved even between these species. This suggests high evolutionary pressure on these genes, indicating the importance of active auxin influx in higher plants. As with *PIN* genes, nomenclature does not follow Arabidopsis. In *M. truncatula*, the genes are named *MtLAX1–5*, and similar names are used for the *L. japonicus* gene family, which has one additional member, *LjAUX1* (Sato *et al.*, 2008; Roy *et al.*, 2017). The *G. max* genes have been named by genomic position: with the first LAX on chromosome 1 called *GmLAX1*, and the last LAX on chromosome 18 *GmLAX15* (Fig. 2B; Chai *et al.*, 2016). Also here, the signature of the whole-genome duplication appears, as all—except *GmLAX4*—are found in pairs. Based on our phylogeny, the AUX1/LAX proteins can be divided into at least three orthogroups. The largest group, the AUX1/LAX1 orthogroup, consists of AtAUX1 and probably AtLAX1, combined with MtLAX1/2/4, LjAUX1, LjLAX1/2/4, and GmLAX1/2/3/4/9/11/13/14/15. This large group can most probably be divided into more subgroups. However, the conserved nature of these proteins makes it difficult to group them properly. The two additional orthogroups are more distinct. In the second group, AtLAX2 groups together with MtLAX5, LjLAX5, and GmLAX5/7/10/12, and in the last group AtLAX3 finds itself with MtLAX3, LjLAX3, and GmLAX6/8. A link between nodule development and auxin influx comes from *M. truncatula*, where it was demonstrated that *MtLAX2* is expressed during nodulation (Roy *et al.*, 2017).

MtLAX2 is not orthologous to AtLAX2, but belongs to a putative legume-specific subclade of the AUX1/LAX1 orthogroup (Fig. 2B). In *L. japonicus*, no data are available for the function of LjLAX during nodule initiation or development. However, in *G. max*, several *GmLAX* genes are highly expressed in roots (*GmLAX1*, *3*, *4*, *6*, *8*, *9*, *10*, *12*, and *15*), but only three are expressed in nodules, although at a relatively low level (*GmLAX6*, *13*, and *14*). Surprisingly, none of these can easily be considered orthologous to MtLAX2. Although this is just an observation, it could also indicate that auxin responses in the determinate nodulating species *G. max* are regulated differently or are less important.

So far key data are missing to draw any solid conclusions on how PIN and AUX1/LAX proteins contribute to nodule initiation and development in (in)determinate legume species. As additional legume genomes of sufficient quality become available, a more extensive phylogenetic analysis of the PIN and AUX1/LAX gene families becomes possible. Nevertheless, functional validation, combined with detailed spatio-temporal studies of PIN and AUX1/LAX during nodule initiation and development, remains crucial to uncover any differences between determinate and indeterminate nodule-forming species. It would be interesting to see where auxin transport-related research on nodulation will lead us in the near future and what new hypotheses this could yield in relation to the differences between both nodule types.

Auxin accumulation during nodule primordium induction: hypotheses from modelling work

With so many unknowns about auxin transport and metabolism, models were used in an attempt to understand the auxin accumulation patterns during the first steps of nodulation (Deinum *et al.* 2012; Xiao *et al.* 2014; Deinum *et al.* 2016). By necessity, these models used the broad PIN layout pattern from Arabidopsis (Laskowski *et al.*, 2008) placed over a *M. truncatula*-like legume root geometry representing the susceptible zone.

Several singular changes in auxin transport/metabolism were applied to a cluster of cells roughly the size of an early nodule primordium (Deinum *et al.*, 2012). Of these changes, a local reduction of auxin efflux (PIN function) produced a large and fairly homogeneous increase of the auxin concentration over the whole length of the cluster. In contrast, increased influx (LAX function) produced a large increase on the shootward or 'upstream' (single-cell wide) edge of the cluster, but much less in the remaining cells of the cluster; and locally produced auxin was mostly transported away. The difference between influx and efflux patterns depended on the polarity of the PIN proteins within the respective cell files, and disappeared if these cells had equal amounts of PIN protein located on their apical and basal ends (Deinum, 2013).

Interestingly, when local reduction of auxin efflux was triggered by a diffusive signal of epidermal origin—in response to a hypothetical rhizobial encounter—the strongest auxin accumulation occurred in the pericycle and inner cortex (Deinum *et al.*, 2016). These are the sites of the first cell divisions in indeterminate nodules forming on *M. truncatula* (Xiao *et al.*, 2014). These patterns appeared within the first hour of simulated time.

The conclusion that most probably a local reduction of auxin efflux underlies the earliest auxin accumulation during nodulation correlates closely with the range of observations on changes in auxin transport during the early stages of nodulation (Mathesius *et al.*, 1998*b*; Boot *et al.*, 1999; Wasson *et al.*, 2006). The strong single-edge pattern produced in a model of increased influx, on the other hand, contradicts the observations of auxin responses in a group of cells in experimental studies (Takanashi *et al.*, 2011; Ng *et al.*, 2015).

These modelling results, however, do not exclude a contribution of influx or production in combination with other changes in auxin transport; they only seem insufficient in isolation. Indeed, primordium-wide expression of *MtLAX2* has been observed at 16 h post-inoculation, and later in the meristem of *M. truncatula* nodules (Roy *et al.*, 2017). Additionally, increased expression of the auxin biosynthesis enzyme LjTAR1 (TRYPTOPHAN AMINOTRANSFERASE-RELATED 1) has been observed in developing *L. japonicus* primordia, peaking at 3 d post-inoculation (dpi) (Suzaki *et al.*, 2012), while no increased *PsTAR* expression was found in *P. sativum* nodule primordia (Dolgikh *et al.*, 2017; measured from 5 dpi). Future experiments with mutants defective in auxin synthesis would help to elucidate the extent to which local auxin synthesis is required for auxin localization and subsequent development of nodule primordia of either type.

In conclusion, it is likely that multiple changes in auxin transport and metabolism occur during nodule development, the first of which may be a temporal reduction of auxin efflux, at least in indeterminate nodules. It remains unclear, however, whether auxin transport inhibition can also produce the observed auxin accumulation in the outer cortex for determinate nodules. In the models, the lateral position of the induced auxin maximum could be tuned by altering the amount of outward lateral PINs in the cortical layers, which strongly affected the auxin availability in the outer cortical layers and epidermis (Deinum *et al.*, 2012, 2016). Thus future experiments should be aimed at testing whether this lateral shift in PIN protein localization can explain the observed auxin responses in the outer cortex of determinate nodule-forming species.

Thus far, our understanding of the mechanism by which auxin transport is controlled in legumes is fragmented, partly due to our insufficient knowledge of auxin transporter biology in legumes. In the following section, we will discuss experimental evidence for the contribution of auxin export and import, auxin metabolism, and auxin signalling in defining the auxin maximum in nodule primordia.

Auxin transport, auxin metabolism, and auxin response contribute to auxin maxima formed in nodule primordia

Within 24 h of rhizobia infection, the auxin transport capacity below the initiation site of indeterminate nodules is reduced

(Mathesius *et al.*, 1998*b*; Wasson *et al.*, 2006). Moreover, it has been demonstrated that in *V. sativa*, application of specific Nod factors reduced auxin transport with 4 h, with a stronger reduction after 24–48 h (Boot *et al.*, 1999). These observations support the mathematical modelling that predicted auxin export inhibition to be the strongest driver of auxin accumulation. In contrast, auxin transport capacity in *L. japonicus* roots, forming determinate nodules, increases in response to inoculation with a compatible symbiont (i.e. *Mesorhizobium loti*) within 48 h (Pacios-Bras *et al.*, 2003). The formation of pseudonodules through auxin transport inhibitors NPA and TIBA has been reported for numerous species forming indeterminate nodules, such as Afghanistan pea (*P. sativum*; Scheres *et al.*, 1992), white sweetclover (*Melilotus albus*; Wu *et al.*, 1996), alfalfa (*M. sativa*; Hirsch *et al.*, 1989), and *M. truncatula* (Rightmyer and Long, 2011). However, induction of pseudonodules by application of auxin transport inhibitors has only been reported for one single species forming determinate nodules (i.e. *Macroptilium atropurpureum*; Relić *et al.*, 1993), unfortunately without a thorough description of the structures. Previous reports of pseudonodules formed in response to the auxin transport inhibitor 2-bromo-3,5-dichlorobenzoic acid in some determinate nodule-forming species described them as modified lateral roots of mainly pericycle origin and with central vasculature, and thus not true pseudonodules (Allen *et al.*, 1953). Despite the difference in the apparent requirement for auxin transport control, both legumes forming indeterminate nodules and those forming determinate nodules show an elevated auxin response in the cortical cells during the formation of a nodule primordium (van Noorden *et al.*, 2007; Takanashi *et al.*, 2011; Suzaki *et al.*, 2012; Turner *et al.*, 2013). This suggests that changes in acropetal auxin export are insufficient to explain the similarities in the auxin response maximum observed in indeterminate versus determinate nodule types.

It is likely that local auxin accumulation within the cortex is not just regulated by auxin efflux, but that auxin influx also plays a role. This is supported by *in situ* hybridization of *MtLAX2* (homologue of AtAUX1) auxin influx carriers during the early stages of nodule primordium formation (de Billy *et al.*, 2001). *MtLAX2* promoter activity has been demonstrated throughout early nodule primordia (at 16 h post-induction) as well as at specific locations in maturing and mature nodules (Roy *et al.*, 2017). Mutants defective in MtLAX2 exhibited reduced auxin responses and fewer nodules. In line with this, application of auxin influx inhibitors to wild-type roots similarly reduced nodule numbers (Roy *et al.*, 2017). This suggests that increased auxin influx capacity elevates the effectiveness of local auxin accumulation and thus improves nodulation success (Deinum, 2013). Whether this happens through a generic feedback of auxin concentration on AUX1/LAX production—similar to the auxin/AtAUX1 feedback in *A. thaliana* (Laskowski *et al.*, 2008)—or whether MtLAX2 is specifically induced as part of the nodulation programme remains to be investigated.

In addition to auxin transport, control of auxin metabolism and auxin responses also contributes to nodule initiation. Proteome and transcriptome studies suggest that responses to *Sinorhizobium meliloti* or their Nod factors, and auxin application to the roots of *M. truncatula* overlap extensively at the early stages (van Noorden *et al.*, 2007; Herrbach *et al.*, 2017), and increased auxin (IAA) content has been measured at the site of nodule initiation (Ng *et al.*, 2015). Support for local auxin biosynthesis can be found in the increased expression of auxin biosynthesis genes during nodulation in *L. japonicus* (Suzaki *et al.*, 2012). Campanella *et al.* (2008) showed an increased expression of several auxin conjugate hydrolase genes in response to *S. meliloti* infection, suggesting that the release of auxin from its conjugated form could be a mechanism contributing to increasing auxin concentration during nodulation. There is also indirect evidence that auxin breakdown in dividing cortical cells could be reduced by flavonoids accumulating in the same cells (Mathesius, 2001). However, under the (Arabidopsis-derived) assumption of continuous polar PIN activity in the whole cortex, local auxin biosynthesis or reduced breakdown would have to be accompanied by a reduction in auxin efflux at the same location to be effective. If not, the produced auxin is likely to be transported away (Deinum, 2013). This would make it unlikely that local auxin biosynthesis alone is sufficient to induce cell division and indicates that modification of the auxin transport machinery could be required for the establishment of such an auxin maximum. Rhizobia-synthesized auxins also positively affect nodulation, as an IAA-overproducing strain of *S. meliloti* increased nodule numbers in *M. truncatula* (Pii *et al.*, 2007). However, since rhizobia are not located in the inner cortex at the time that the first auxin maximum is observed, it is unlikely that this potential source of auxin contributes to generating the auxin maximum in the nodule primordium. Overall, there is little evidence to support host or symbiont auxin biosynthesis as a main strategy for increasing auxin concentrations early during nodulation.

An additional mechanism to increase auxin responses in the cortex is to increase the sensitivity of its perception. One way of regulating auxin responses in Arabidopsis is through several miRNAs that target auxin receptors and auxin response genes (e.g. Couzigou and Combier, 2016; Weijers and Wagner, 2016). Similar miRNAs are expressed in legume roots and at various stages of nodulation, and have effects on indeterminate and determinate nodule numbers (e.g. Subramanian *et al.*, 2008; Bustos-Sanmamed *et al.*, 2013; Turner *et al.*, 2013; Wang *et al.*, 2015*b*; Cai *et al.*, 2017; Table 1). It has been hypothesized in these studies that these miRNAs play a role in reducing auxin responses, and this may be relevant for controlling auxin responses in the growing nodule primordium (Turner *et al.*, 2013; Nizampatnam *et al.*, 2015). However, these data should currently be interpreted with some caution. First, the effects of these miRNAs on auxin signalling are mostly based on direct extrapolation of their effects on specific target genes in Arabidopsis, and this has not always been confirmed in legumes. Secondly, many studies, although not all (Nizampatnam *et al.*, 2015), have used ectopic overexpression of miRNAs, which may lead to expression of miRNAs and subsequent auxin responses in the cell types that do not usually divide, making interpretation difficult. Thirdly, a single miRNA may

Table 1. *Comparison of auxin transport, metabolism, and response phenotypes during the formation of indeterminate and determinate legume nodules*

Process	Indeterminate nodules	Determinate nodules
Auxin transport inhibition in response to rhizobia preceding nodule initiation	Observed within 24 h of inoculation in *Medicago truncatula* and *Vicia sativa* (Boot *et al.*, 1999; Wasson *et al.*, 2006).	No evidence from *Lotus japonicus* (Pacios-Bras *et al.*, 2003) but untested in other species.
Auxin transport inhibitors induce pseudonodules	Observed in *M. sativa* (Hirsch *et al.*, 1989), *M. truncatula* (Rightmyer and Long 2011), *Pisum sativum* (Scheres *et al.*, 1992), and *Melilotus albus* (Wu *et al.*, 1996).	Reported in *Macroptilium atropurpureum* but structure not analysed in detail (Relić *et al.*, 1993).
Flavonoids required for nodulation and auxin transport control	Observed in *M. truncatula* roots lacking the whole flavonoid pathway (Wasson *et al.*, 2006).	No evidence that isoflavonoids are involved in soybean nodulation beyond their role as nod gene inducers, but other flavonoids remain untested (Subramanian *et al.*, 2006, 2007).
Auxin response in proliferating cortical cells	Observed in inner cortex in *M. truncatula* (van Noorden *et al.*, 2007) and *Trifolium repens* (Mathesius *et al.*, 1998*b*) using *GH3::GUS* reporter.	Observed in middle/outer cortex of *L. japonicus* and *Glycine max* (Turner *et al.*, 2013) using *GH3::GUS* (Takanashi *et al.*, 2011), *DR5::GUS* (Turner *et al.*, 2013), *DR5::GFP-NLS* (Suzaki *et al.*, 2012), and *DR5::tDT* (Turner *et al.*, 2013) reporters.
Increased auxin content, release, or synthesis during nodulation	Increased auxin (indole-3-acetic acid) content at 24 h post-inoculation in *M. truncatula* (Ng *et al.*, 2015). Increased expression of auxin conjugate hydrolases in *M. truncatula* (Campanella *et al.*, 2008).	Increased auxin synthesis gene expression at 3 d post-inoculation in *L. japonicus* (Suzaki *et al.*, 2012).
Altered auxin signalling in roots through miRNAs targeting the auxin receptor family TIR1/AFB	Overexpression of miR393 reduced nodule numbers in *M. truncatula* (Mao *et al.*, 2013).	Overexpression of miR393, did not alter nodule numbers in *G. max* (Mao *et al.*, 2013). Silencing of miR393, or overexpression of *GmTIR1* in *G. max* increased nodule numbers (Cai *et al.*, 2017).
Altered auxin signalling in roots through miRNAs targeting the auxin response factor ARF8a/b	Not tested.	miR167 inhibits ARF8a/b during nodulation, which enhances nodule numbers in *G. max* (Wang *et al.*, 2015*b*).
Altered auxin signalling in roots through miRNAs targeting the auxin response family ARF10/16/17	Overexpression of miR160 reduces nodule numbers in *M. truncatula* (Bustos-Sanmamed *et al.*, 2013).	Overexpression of miR160 enhances auxin responsiveness and reduces nodule numbers in *G. max* (Turner *et al*, 2013; Nizampatnam *et al.*, 2015).
Cytokinin signalling activates auxin response in cortex	The *M. truncatula cre1* mutant fails to show an auxin response in the cortex after infection with rhizobia (Ng *et al.*, 2015).	The *L. japonicus snf2* mutant, exhibiting constitutive cytokinin signalling and spontaneous nodule formation, activates an auxin response in the cortex (Suzaki *et al.*, 2012).
High auxin response/content in vascular tissue of a developing and mature nodule, while auxin response/content in the infected nodule zone is low	Observed in *M. truncatula* using the *DR5::GUS* reporter (Guan *et al.*, 2013; Franssen *et al.*, 2015), *GH3::GUS* in *T. repens* (Mathesius *et al.*, 1998*b*) and *M. truncatula* (Breakspear *et al.*, 2014), *SAUR1::GUS* (Breakspear *et al.*, 2014), and anti-IAA antibody (Fedorova *et al.*, 2005).	Observed in *L. japonicus* using the *GH3::GUS* reporter (Takanashi *et al.*, 2011) and the *DR5::GFP-NLS* reporter (Suzaki *et al.*, 2012), and in soybean using the *DR5::dTD* reporter (Turner *et al.*, 2013).
High auxin response/content in meristem of a mature nodule	Observed in *M. truncatula* using the *DR5::GUS* reporter (Guan *et al.*, 2013; Franssen *et al.*, 2015), *GH3::GUS* and *SAUR1::GUS* in *M. truncatula* (Breakspear *et al.*, 2014), and anti-IAA antibody (Fedorova *et al.*, 2005).	Not observed, no nodule meristem retained in mature nodules.
Increased auxin response or content in roots of supernodulating mutants	Increased auxin content in rhizobia-inoculated roots of the *M. truncatula sunn1* mutant (van Noorden *et al.*, 2006).	Increased *DR5::GFP-NLS* response observed in *L. japonicus har1* mutant (Suzaki *et al.*, 2012).
Increased shoot to root auxin transport in supernodulating mutants	Observed in *M. truncatula* (van Noorden *et al.*, 2006).	Not tested.

affect sensitivities to multiple plant hormones. For example, overexpression of miRNA160 in soybean reportedly resulted in auxin hypersensitivity as well as cytokinin hyposensitivity (Turner *et al.*, 2013), and nodule numbers in these plants could be rescued by cytokinin addition (Nizampatnam *et al.*, 2015). Currently, evidence of the involvement of miRNAs playing a role at the very earliest stages of nodule initiation that could explain an effect on the creation of an auxin maximum in the cortex is lacking.

Which signals modulate auxin transport during nodulation?

Nod factor signalling modifies auxin transport during the initiation of indeterminate and determinate nodules (e.g. Pacios-Bras *et al.*, 2003; Wasson *et al.*, 2006). However, it is unlikely that it controls the auxin transport machinery directly. Nod factors are produced at the epidermis by infecting bacteria, and have been demonstrated to be highly immobile

(Goedhart *et al.*, 2003). Thus, a secondary signal is required that is induced by epidermal Nod factor signalling, but acts in the inner cortex. One possible candidate for endogenous auxin transport modulation are the flavonoids (Peer and Murphy, 2007). Flavonoids are a large group of secondary metabolites derived from the phenylpropanoid pathway. Flavonoids accumulate in dividing cortical cells of legumes forming both nodule types (Mathesius *et al.*, 1998*a*), and flavonoid synthesis genes are induced at sites of nodule initiation (Chen *et al.*, 2015). In *M. truncatula*, forming indeterminate nodules, silencing of the first dedicated enzyme towards flavonoid biosynthesis (i.e. *CHALCONE SYNTHASE*) increased auxin transport rates, prevented inhibition of auxin transport in response to *S. meliloti*, and prevented nodule formation (Wasson *et al.*, 2006). External application of specific flavonoids to *M. truncatula* roots could inhibit auxin transport similarly to rhizobia (Ng *et al.*, 2015). How flavonoids function to reduce auxin transport in this process is unknown. Analysis of *MtPIN* gene expression in flavonoid-deficient *M. truncatula* roots showed no changes compared with control roots (Wasson *et al.*, 2006), suggesting that any effects involving PIN-mediated auxin transport should occur post-transcriptionally. The fact that nodule induction by application of auxin transport inhibitors was never observed in most determinate nodule type plants suggests that flavonoids might have a different function here. In soybean, which forms determinate nodules, silencing of isoflavone synthase reduced nodule numbers (Subramanian *et al.*, 2006). It has been demonstrated that (iso)flavonoids induce rhizobial Nod genes and subsequent Nod factor biosynthesis (e.g. Kosslak *et al.*, 1987), and in the soybean–*Bradyrhizobium* symbiosis this seems to be the case (Subramanian *et al.*, 2006, 2007). Interestingly though, increased auxin responsiveness and transport were observed in these knock-down lines as well (Subramanian *et al.*, 2006), indicating that flavonoids could have a function in controlling auxin transport in soybean. However, how this is related to nodule initiation is unknown. Detailed genetic analysis of the flavonoid pathway in different legume species could shed light on this matter.

Another option for controlling auxin transport during nodule initiation can be found in strigolactones. These plant hormones are known to affect PIN protein levels (Bennett *et al.*, 2006; Crawford *et al.*, 2010; Kohlen *et al.*, 2011; Ruyter-Spira *et al.*, 2011), but might also act independently of auxin transport, at least for shoot branching (Brewer *et al.*, 2015). Increased numbers of nodules have been reported after application of the synthetic strigolactone GR24 to *M. sativa* roots (Soto *et al.*, 2010). In *M. truncatula*, low concentrations of GR24 increased nodule number slightly, whereas higher concentrations had a reducing effect (De Cuyper *et al.*, 2015). Loss-of-function mutations or RNAi knockdown of strigolactone biosynthesis genes affect nodule numbers in legumes forming both indeterminate (*P. sativum*) and determinate (*L. japonicus*) nodules (Foo and Davies, 2011; Liu *et al.*, 2013). In *M. truncatula*, the strigolactone biosynthesis gene *MtD27* (*DWARF27*) is highly up-regulated upon Nod factor application within 3 h after inoculation (van Zeijl *et al.*, 2015*a*), and a clear link between *MtD27* expression and the Nod factor signalling pathway was demonstrated in the *nsp1* and *nsp2* (*nodulation-signaling pathway1* and *2*) mutants (Liu *et al.*, 2011). In addition, it was demonstrated that expression of the strigolactone biosynthesis gene *MtCCD8* (*CAROTENOID CLEAVING DEOXYGENASE8*) is up-regulated at the site of primordia formation (Breakspear *et al.*, 2014). However, no increase in strigolactone levels during early signalling was ever reported. Notably, however, the *Psrms1/ccd8* (*ramosus1*) mutant contains almost no strigolactones (Gomez-Roldan *et al.*, 2008), but produces only ~40% fewer nodules than the wild type (Foo and Davies, 2011). This suggests that if strigolactones are involved in regulating auxin transport upon Nod factor perception, they are not the only factor involved in this.

Other plant hormones such as cytokinins and ethylene play a role in nodule initiation, and there is strong evidence that they function in crosstalk with auxin. The gain-of-function mutation in the *L. japonicus LHK1* (*LOTUS HISTIDINE KINASE1*) cytokinin receptor produces dividing cortical cells and nodules in the absence of rhizobia. These nodules have a very similar developmental pattern to rhizobia-induced nodules (Tirichine *et al.*, 2007; Suzaki *et al.*, 2012). Similar spontaneous nodules are produced from the gain-of-function mutation in the orthologous *CRE1* (*CYTOKININ RESPONSE1*) receptor in *M. truncatula* (Ovchinnikova *et al.*, 2011). External application of cytokinin induces empty nodules in alfalfa (*M. sativa*; Cooper and Long, 1994), white clover (*Trifolium repens*; Mathesius *et al.*, 2000), siratro (*M. atropurpureum*; Relić *et al.*, 1993), *Aeschynomene* spp. (Podlevšáková *et al.*, 2013), *L. japonicus* (Heckmann *et al.*, 2011), and in the non-legume alder (*Alnus glutinosa*; Rodriguez Barrueco and Bermudez de Castro, 1973). These cytokinin responses have been linked to cortical auxin responses. For example, exogenous cytokinin treatment to white clover elicited auxin responses in associated divided cortical cells (Mathesius *et al.*, 2000). In *L. japonicus*, cortical auxin responses were observed in the *snf2* (*spontaneous nodule formation 2*) mutant, which harbours an autoactive LHK1 cytokinin receptor (Suzaki *et al.*, 2012). In *M. truncatula*, cytokinin signalling via the CRE1 receptor is required for the onset of auxin response in the inner cortical cells during nodule initiation (Ng *et al.*, 2015). This signal precedes the auxin maximum (Xiao *et al.*, 2014; van Zeijl *et al.*, 2015*b*), and could be mediated by the induction of flavonoids that inhibit auxin transport (Ng *et al.*, 2015). During the early cell divisions of the inner cortex in *M. truncatula*, auxin and cytokinin response maxima overlap (van Noorden *et al.*, 2007; Plet *et al.*, 2011). However, it is possible that cytokinins are initially produced in the epidermis and translocated inward towards the cortex as several genes belonging to the putative cytokinin transport facilitator family are up-regulated in the epidermis upon Nod factor application (Jardinaud *et al.*, 2016). At later stages of nodule development, the localization of auxin and cytokinin responses only partially overlaps. Auxin responses localize to vascular cells and the entire *M. truncatula* nodule meristem (Table 1), whereas cytokinin responses were observed in the nodule meristem for type-A cytokinin response regulators and throughout the nodule in type-B cytokinin response regulators (Plet *et al.*, 2011; Franssen *et al.*, 2015).

Ethylene is regarded as a negative regulator of nodulation. Evidence for this can be found in the fact that in wild-type plants, nodules are preferentially formed opposite protoxylem poles, a position where ethylene biosynthesis is assumed to be low (Heidstra *et al.*, 1997; Penmetsa and Cook, 1997). Moreover, ethylene-insensitive plants show massive numbers of nodules when inoculated with rhizobia (Penmetsa and Cook, 1997; Lohar *et al.*, 2009). In addition, ethylene inhibits the calcium spiking that otherwise follows LCO perception, and the ethylene-insensitive *skl*/*Mtein2* (*sickle*) mutant forms more infection threads compared with the wild type (Oldroyd *et al.*, 2001; Penmetsa *et al.*, 2008). Application of the ethylene precursor ACC (aminocyclopropane carboxylic acid) to the roots of *M. truncatula* reduced auxin transport (Prayitno *et al.*, 2006). The effects of both ACC and rhizobia on shoot to root auxin transport were abolished in the *skl* mutant (Prayitno *et al.*, 2006). The *skl* mutant also showed increased *MtPIN1* and *MtPIN2* expression at the site of nodule initiation. This suggests that ethylene signalling is required for the correct control of auxin transport during nodule initiation. This conclusion is supported by a significant reduction of pseudonodule formation induced by auxin transport inhibitors in the *skl* mutant (Rightmyer and Long, 2011). A similar control of auxin transport by ethylene has previously been described in Arabidopsis (e.g. Růžička *et al.*, 2007).

Ethylene also plays a role in controlling nodule numbers in species forming determinate nodules such as *L. japonicus* and *M. atropurpureum*, to the same extent as in *M. truncatula* and *M. sativa* (Nukui *et al.*, 2000; Lohar *et al.*, 2009). Some authors mention soybean as an exception (e.g. Schmidt *et al.*, 1999; Nukui *et al.*, 2000), but reports of strong hypernodulation in ethylene-insensitive soybean genotypes exist as well (Caba *et al.*, 1999). Further conflicting reports for species forming determinate nodules may be explained by multiple copies of the *EIN2* gene in *L. japonicus* (Miyata *et al.*, 2013) and/or large redundancies among ethylene receptors. The latter is well illustrated by Arabidopsis, where often quadruple or quintuple mutants of ethylene receptors are required to induce developmental phenotypes (Hua and Meyerowitz, 1998). There are no reports yet that ethylene reduces shoot to root auxin transport in species forming determinate nodules. Such measurements would be interesting in the light of the emerging picture that the importance of shoot to root auxin transport differs between indeterminate and determinate nodules.

In summary, several plant hormones and signals have been reported to interact with auxin transport during nodule initiation (Fig. 3) and others will have to be investigated in the future. While cytokinin signalling appears to be essential for auxin transport control in both indeterminate and

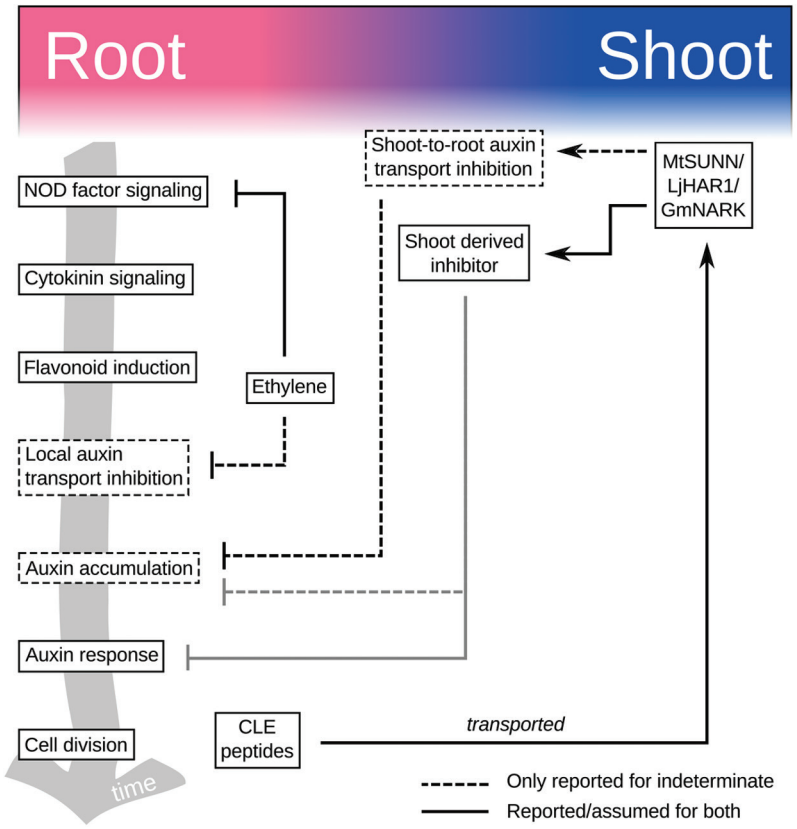

Fig. 3. Model for the involvement of auxin in local and systemic regulation of nodulation based on experimental evidence. Dashed lines/box outlines indicate that a feature has only been convincingly shown in legumes forming indeterminate nodules. Solid lines indicate features that play a role for both nodule types. The major root events are sorted in chronological order, insofar as known, on top of the large grey arrow. Grafting experiments have demonstrated that a shoot-derived inhibiting mechanism is present in both legume types. For determinate nodules, the nature of this inhibitor as well as its exact point of action remain elusive, and for indeterminate nodules it is unclear whether there is an additional signal apart from reduced auxin loading. Therefore, the respective arrows are drawn in grey. Ethylene probably can inhibit nodulation processes at multiple stages.

determinate nodulation, a role for flavonoids in controlling auxin transport has only been demonstrated for indeterminate nodulation. For strigolactones, influences on auxin transport and nodule number have been established in isolation, but how and if these hormones influence auxin transport, metabolism, or signalling during nodulation remains to be shown. Ethylene signalling is required for correct auxin transport control during indeterminate nodulation, but its role in controlling auxin during determinate nodulation will require further investigation.

A role for auxin transport in the autoregulation of nodulation

Whether nodules are initiated in response to compatible rhizobia largely depends on several environmental factors. A sophisticated system—called autoregulation of nodulation (AON)—systemically regulates nodule numbers on the root in response to signals derived from the shoot. AON is co-regulated by Nod factors as well as nitrate (Reid *et al.*, 2011*b*), and some evidence suggests a role for auxin in this process (van Noorden *et al.*, 2006; Suzaki *et al.*, 2012).

During AON, small regulatory peptides of the CLE (CLAVATA3/endosperm-surrounding region-related) family are induced. These CLE peptides bind to leucine-rich repeat receptor-like kinases (LRR-RLKs) and subsequently inhibit further nodules from forming. In soybean, nitrate induces the peptide GmNIC1, which is predicted to bind locally to the GmNARK (nodulation autoregulation receptor kinase; Searle *et al.*, 2003; Reid *et al.*, 2011*a*) receptor to inhibit nodule initiation. The same receptor is expressed in the shoot where it is hypothesized to bind GmRIC1, a second CLE peptide. This triggers the movement of a shoot-derived, nodule-inhibiting signal to the root (Reid *et al.*, 2011*a*; Okamoto *et al.*, 2013). In *M. truncatula*, Nod factors induce MtCLE12 and MtCLE13, which negatively regulate nodule numbers via the MtSUNN1 (SUPERNUMERARY NODULES 1) receptor in the shoot (Schnabel *et al.*, 2005; Mortier *et al.*, 2010). An equivalent signalling pathway has been identified in *L. japonicus* via the receptor LjHAR1, which binds CLE-RS peptides (Nishimura *et al.*, 2002; Okamoto *et al.*, 2009, 2013).

While the shoot-derived inhibitor has not been identified, both auxin and cytokinin movement from the shoot to the root have been implicated in AON. In *L. japonicus*, inoculation of roots with rhizobia led to increased translocation of cytokinin from the shoot to the root in an LjHAR1-dependent manner (Sasaki *et al.*, 2014). It is possible that this source of cytokinin interacts with auxin signalling in the root, as the increased numbers of nodules in the *Ljhar1* mutant were accompanied by an increased area of auxin response (Suzaki *et al.*, 2012). In *M. truncatula*, inoculation of roots with rhizobia led to a decrease of shoot to root auxin transport, and this was dependent on MtSUNN1 (van Noorden *et al.*, 2006). In addition, nodule numbers in the *Mtsunn* mutant are significantly reduced by application of an auxin transport inhibitor at the shoot/root junction. This suggests a positive correlation between the amount of shoot to root auxin transport and the number of nodules being formed (van Noorden *et al.*, 2006). Similar to the increased zone of auxin response in the *Ljhar1* mutant, *Mtsunn1* mutants exhibited increased auxin (IAA) concentration at the root zone responding to rhizobia (van Noorden *et al.*, 2006).

It has been demonstrated in *M. truncatula* that the presence of an external nitrogen source affects root auxin responses during nodulation. It led to an elevated and diffuse auxin response in the whole cortex following rhizobia infection, preventing a local accumulation of auxin typical for an incipient nodule primordium (van Noorden *et al.*, 2016). However, nitrate did not prevent the inhibition of auxin transport by Nod factors in vetch (Boot *et al.*, 1999). It is possible, though, that experiments with rhizobia in the presence of nitrate are affected by the reduction in Nod gene induction of flavonoids (Coronado *et al.*, 1995). At a whole-plant level, the presence of nitrate at levels inhibiting nodulation altered shoot to root auxin transport in *M. truncatula*, and this was dependent on the MtSUNN1 receptor (Jin *et al.*, 2012). Collectively these studies suggest that AON control of nodule numbers involves changes in the concentration, transport, and response to auxin in the root zone susceptible to rhizobia. However, the precise mechanisms underlying this involvement are still poorly understood.

Conclusion: indeterminate and determinate nodules—minor variations on the same developmental programme, or fundamentally different?

The Nod factor signalling pathways for the interaction between legumes and rhizobia are shared between indeterminate and determinate nodule formation, as well as most known plant signalling components required for the induction of nodule organogenesis. However, so far it remains unknown what determines the location of the first cortical cell divisions. In both nodule types, the location of the first cell divisions is accompanied by auxin responses (e.g. van Noorden *et al.*, 2007; Takanashi *et al.*, 2011; Fig. 1; Table 1). In addition, there is evidence of increased auxin synthesis, content, or release in both nodule types (Campanella *et al.*, 2008; Suzaki *et al.*, 2012; Ng *et al.*, 2015). Similarly, cytokinin responses are found in early dividing cells of nodule primordia in both nodule types (e.g. Plet *et al.*, 2011; Held *et al.*, 2014), and it has been shown that cytokinin responses occur upstream of auxin responses in those cells (Plet *et al.*, 2011; Suzaki *et al.*, 2012; Ng *et al.*, 2015; Fig. 3; Table 1). However, the mechanisms that induce these responses at their respective location may differ between indeterminate and determinate nodules, either by degree or fundamentally.

The modelling-derived hypothesis—that differences in the cortical PIN distribution could shift the radial position of an induced maximum through altered auxin availability (Deinum *et al.*, 2012, 2016)—remains to be verified experimentally.

Alternatively, it may be the case that the mechanism of inducing a local auxin maximum through auxin transport inhibition is effective for indeterminate nodules only, and

other mechanisms for locally increasing auxin availability and/or the sensitivity of auxin perception are more important in the formation of determinate nodules. Evidence for this alternative hypothesis falls into two categories: (i) local auxin transport inhibition can induce pseudonodules in a range of legumes forming indeterminate nodules, but has only been reported for one species forming determinate nodules, *M. atropurpureum*, with marginal description (Relić *et al.*, 1993); and (ii) auxin transport inhibition in response to rhizobia has been measured in legumes forming indeterminate (e.g. Boot *et al.*, 1999), but not determinate legumes (Pacios-Bras *et al.*, 2003), and a role for flavonoids in this auxin transport inhibition has also only been clearly demonstrated for indeterminate nodules. It appears, therefore, that auxin transport inhibition explains auxin accumulation and subsequent nodule primordium initiation for indeterminate, but not determinate nodules.

Thus, the main difference between indeterminate and determinate nodules appears to be the mechanism that different legumes use to achieve the initial buildup of an auxin maximum in different layers of the cortex. Future investigations will need to be directed at explaining how an auxin maximum in the outer cortex of legumes forming determinate nodules can be achieved, for example through lateral auxin transport or through altered auxin synthesis or sensitivity, which could be regulated by specific miRNAs. It will also be important to compare long-distance auxin transport in supernodulation mutants of indeterminate and determinate nodule-forming species.

Currently, our understanding of auxin signalling in legumes is limited, making experiments to answer how auxin maxima are formed in both nodule types difficult. For example, many legume auxin mutants remain uncharacterized, and a very limited number of reporter lines for auxin transporters have been described. In addition, few studies have directly compared different legumes. However, with increasing species-specific molecular and genetic tools at our disposal, this will improve. The great diversity in root nodule morphologies and development in different legume species has the potential to become an important resource for fundamental research questions about plant development.

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