Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response

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SUMMARY

Nodulation is a form of de novo organogenesis that occurs mainly in legumes. During early nodule development, the host plant root is infected by rhizobia that induce dedifferentiation of some cortical cells, which then proliferate to form the symbiotic root nodule primordium. Two classic phytohormones, cytokinin and auxin, play essential roles in diverse aspects of cell proliferation and differentiation. Although recent genetic studies have established how activation of cytokinin signaling is crucial to the control of cortical cell differentiation, the physiological pathways through which auxin might act in nodule development are poorly characterized. Here, we report the detailed patterns of auxin accumulation during nodule development in *Lotus japonicus*. Our analyses showed that auxin predominantly accumulates in dividing cortical cells and that NODULE INCEPTION, a key transcription factor in nodule development, positively regulates this accumulation. Additionally, we found that auxin accumulation is inhibited by a systemic negative regulatory mechanism termed autoregulation of nodulation (AON). Analysis of the constitutive activation of *LjCLE-RS* genes, which encode putative root-derived signals that function in AON, in combination with the determination of auxin accumulation patterns in proliferating cortical cells, indicated that activation of *LjCLE-RS* genes blocks the progress of further cortical cell division, probably through controlling auxin accumulation. Our data provide evidence for the existence of a novel fine-tuning mechanism that controls nodule development in a cortical cell stage-dependent manner.

KEY WORDS: Autoregulation of nodulation, Auxin, CLE, Cytokinin signaling, *Lotus japonicus*, Nodule development

INTRODUCTION

Under appropriate environmental conditions, legumes can form nodular structures on their roots. Within the nodules, host plants can obtain a nitrogen source fixed by soil bacteria (rhizobia); in turn, the plants provide a carbon source for the rhizobia. This mutual interaction between plants and rhizobia is defined as root nodule symbiosis. During nodule development, plants respond to nodulation (Nod) factors produced by the rhizobia; perception of these factors by receptor kinases triggers a signaling cascade in the epidermis of the root. As a result, dedifferentiation of some of the cortical cells is induced; these cells subsequently divide to form the nodule primordia (Szczyglowski et al., 1998; Oldroyd and Downie, 2008; Oldroyd et al., 2011). During the course of nodule development, rhizobia invade the dividing cortical cells via a tubular structure called the infection thread (Murray, 2011).

A number of genes involved in the positive regulation of nodulation have been identified through analysis of nodulation-deficient mutants. Additionally, it has been postulated that another genetic mechanism, termed autoregulation of nodulation (AON), negatively regulates nodulation to moderate the number of nodules formed (Caetano-Anollés and Gresshoff, 1991; Oka-Kira and Kawaguchi, 2006; Ferguson et al., 2010; Kouchi et al., 2010; Reid et al., 2011b). The basis of AON is systemic long-distance signaling between root and shoot. In *Lotus japonicus*, HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1), which encodes a leucine-rich repeat receptor-like kinase, is hypothesized to function in shoots where it recognizes and responds to root-derived signals involved in the negative regulation of nodulation (Krusell et al., 2002; Nishimura et al., 2002). Among the candidates for such signaling molecules in *L. japonicus* are CLE-ROOT SIGNAL1 (*LjCLE-RS1*) and *LjCLE-RS2* (Okamoto et al., 2009). These two proteins belong to the CLE (CLAVATA 3/ESR) family of proteins that play significant roles as signaling molecules in cell-to-cell communication during a range of plant developmental processes including stem cell maintenance, vascular patterning and embryo development (Fletcher et al., 1999; Hirakawa et al., 2008; Stahl et al., 2009; Fiume and Fletcher, 2012). AON appears to have a conserved molecular mechanism among leguminous species, as functional counterparts of HAR1 and *LjCLE-RS1/2* have been identified in *Medicago truncatula* and *Glycine max* (Searle et al., 2003; Schnabel et al., 2005; Mortier et al., 2010; Reid et al., 2011a). However, little is known about the site of AON action in nodule development.

It has been shown that the phytohormones cytokinin and auxin play fundamental roles in the control of cell proliferation and differentiation in many developmental regulatory processes. The putative cytokinin receptors LOTUS HISTIDINE KINASE 1 (LHK1) in *L. japonicus* and CYTOKININ RESPONSE 1 (CRE1) in *M. truncatula* are involved in nodulation (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). Nodule formation is impaired in plants carrying loss-of-function mutations of these genes, whereas nodule-like organs (termed spontaneous nodules)
are formed in the absence of rhizobia in the spontaneous nodule formation2 (snf2) mutant that has a gain-of-function mutation of LHK1. Exogenous application of cytokinin to L. japonicus roots has been shown to induce the formation of spontaneous nodules (Heckmann et al., 2011). Some response regulators, which are known to be components of the cytokinin signaling pathway, are reported to be involved in nodulation in both species (Op den Camp et al., 2011). The various reports described above suggest that activation of cytokinin signaling is a pivotal event in nodule initiation. Downstream of cytokinin signaling, two putative transcription factors, NODULE INCEPTION (NIN) and NODULATION SIGNALING PATHWAY2 (NSP2) (Schauer et al., 1999; Heckmann et al., 2006; Murakami et al., 2006), have been suggested to be involved in nodule organogenesis on the basis that spontaneous nodule formation in the snf2 mutant is suppressed if either of these genes has also been mutated (Tirichine et al., 2007).

Suggested to be involved in nodule organogenesis on the basis that either of these genes has also been mutated (Tirichine et al., 2007). NIN is thought to be required for nodule organogenesis because nodule formation is completely blocked in nin mutants (Schauer et al., 1999). Upon rhizobial infection, expression of NIN is strongly activated both in the epidermis and in the nascent nodule primordium. Recently, it has also been found that constitutive expression of NIN can induce ectopic cortical cell division in the absence of rhizobia (T. Soyano and M. Hayashi, personal communication). Thus, NIN appears to play a crucial role in the dedifferentiation and subsequent proliferation of cortical cells during nodule development.

In comparison to cytokinin, relatively little is known about the role of auxin in nodule development. There have been some physiological studies on auxin, mainly in the genus Medicago. More than twenty years ago, for example, it was shown that inhibition of polar auxin transport induces the formation of pseudonodules in the absence of rhizobia in M. sativa (Hirsch et al., 1989), a finding recently confirmed in M. truncatula (Rightmyer and Long, 2011). Furthermore, it was reported that silencing of the flavonoid pathway, which acts to inhibit auxin transport, causes a reduction in nodule number (Wasson et al., 2006). These observations suggest that localized accumulation of auxin at the sites of incipient nodule primordia might be a key step in nodule development. In addition, expression of some MtPIN genes is upregulated in the crel mutant of M. truncatula, suggesting that one role of cytokinin signaling in nodulation might be to establish local auxin accumulation through control of expression of such auxin transporters (Plet et al., 2011). Although some understanding of the behavior of auxin in nodulation has been achieved, there is still considerable uncertainty as to how and when auxin acts in the various genetic pathways that control nodule development.

Here we identify the site of auxin action during nodule development in L. japonicus. Our detailed analysis of the spatiotemporal induction pattern of auxin shows that cortical cell division occurs concurrently with strong induction of auxin accumulation. We show that auxin accumulation is under the positive regulation of cytokinin signaling and that NIN functions in the local accumulation of auxin at cortical cells. Our results further show that AON signaling, including HAR1 and LjCLE-RS1/2, acts to inhibit auxin accumulation. Moreover, a simultaneous analysis of the constitutive activation of LjCLE-RS genes in relation to auxin accumulation and cortical cell division patterns shows that signaling of these genes negatively regulates nodule development. We suggest that this effect might occur as a result of the blocking of further proliferation of cortical cells, probably by controlling auxin accumulation, although initiation of cell division is unlikely to be under the same control.
Expression analysis
The primers used are listed in supplementary material Table S2. LjTA2/1 (chr2.CM0008.610) and LjTAR1 (chr2.CM0008.590) were identified by a BLAST search of the L. japonicus genomic sequence database using the amino acid sequence of Arabidopsis TAA1 as the query. Total RNA was isolated from each plant tissue using the RNAeasy Plant Mini Kit (Qiagen). First-strand cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen). Real-time RT-PCR was performed using an ABI Prism 7000 (Applied Biosystems) with THUNDERBIRD SYBR qPCR Mix (Toyobo) according to the manufacturer's protocol. The expression of ubiquitin was used as the reference (Takeda et al., 2009). Data show the mean (± s.d.) of three biological replicates.

Microscopy
Bright-field and fluorescence microscopy were performed with an SZX12 stereomicroscope (Olympus) or with an A1 confocal laser-scanning microscope (Nikon). Images were acquired and analyzed using DP Controller (Olympus), NIS Elements (Nikon) or Photoshop (Adobe Systems).

RESULTS
Auxin accumulates predominantly in dividing cortical cells during nodule development
In order to determine the precise distribution of auxin during nodule development in L. japonicus, we created stable transgenic plants that express a GFP and nuclear localization signal (NLS) fusion protein (GFP-NLS) under the control of DR5, which is a highly active synthetic auxin-responsive element (Ulmasov et al., 1997). Strong GFP expression was observed in the root apex including the putative quiescent center, and during lateral root development, as has been reported in other plants (supplementary material Fig. S1A-C) (Benková et al., 2003). In addition, GFP expression was induced by exogenous application of auxin (supplementary material Fig. S1D). Our analysis showed that auxin distribution patterns during nodule development could be indirectly monitored in DR5::GFP-NLS transgenic plants.

The Mesorhizobium loti strain MAFF303099, which constitutively expresses DsRED, was used to visualize rhizobia in developing nodules following infection of plant roots. Three days after inoculation (dai) with rhizobia, infection threads had started to form in some root hairs, and GFP expression was observed in a small number of cortical cells beneath root hairs with infection threads (Fig. 1A,E). The nuclei in the cortical cells immediately before cell division appeared larger than those of surrounding cells. In roots at 5 dai, variable degrees of cortical cell proliferation had occurred and this cell division was coincident with strong GFP expression (Fig. 1B,C,F,G). Strong GFP expression continued until the actively dividing cortical cells produced the initial bulge of the nodule primordia, which was invaded by rhizobia via the growing infection threads (Fig. 1D,H). After rhizobial colonization of the developing nodule primordia, the strong GFP expression halted in the infected region of the nodule and was restricted to the surrounding tissues (Fig. 1I). This pattern was maintained after the nodules enlarged and the rhizobia expanded their region of infection (Fig. 1J). At this stage, GFP expression was also observed at the vascular bundle (lenticels).

Next, we carried out an in situ hybridization analysis to confirm the GFP expression patterns described above. This analysis showed that GFP transcripts were detectable in proliferating cortical cells (Fig. 1K); a similar expression pattern was found for the S-phase-specific marker HISTONE H4 (Fig. 1L). In developing nodules with rhizobial colonies, GFP transcripts were distributed around the region of infection (Fig. 1M). These distribution patterns were consistent with the results of the GFP fluorescence analysis described above.

It has been reported that the CYCLOPS gene is specifically expressed in dividing cortical cells during nodulation in L. japonicus (Yano et al., 2008). We performed hairy root transformation of DR5::GFP-NLS transgenic plants in order to obtain expression of mCherry-NLS under control of the CYCLOPS promoter. A co-localization analysis of DR5::GFP-NLS and pCYCLOPS::mCherry-NLS showed that GFP and mCherry were expressed in the same cells during nodule development (Fig. 1NP). This suggests that auxin accumulation occurs in cells in which the nodulation-related gene is expressed. We therefore conclude that auxin accumulation during nodule development occurs predominantly in proliferating cortical cells.

During nodule development, infection thread formation and cortical cell proliferation are closely related (Murray, 2011). To further investigate the relationship between infection thread formation and auxin accumulation, we examined auxin distribution patterns in a cyclops mutant (Yano et al., 2008). In contrast to wild type (WT), in which the infection threads grew through root hairs towards the cortical cells, they did not reach the cortical cells in the cyclops-6 mutant (Fig. 2A,B). This failure of infection thread growth is the likely cause of the premature arrest of nodule development in the mutant (Fig. 2C,D). DR5::GFP-NLS/cyclops-6 plants were produced by crossing DR5::GFP-NLS transgenic plants with cyclops-6 plants. During nodulation in DR5::GFP-NLS/cyclops-6 plants, however, induction of GFP expression was observed in cortical cells below the root hairs where the defective infection threads were located (Fig. 2F). GFP was still expressed in the developmentally arrested nodules formed in the mutant (Fig. 2G). These results suggest that it is not necessary for the infection threads to reach the cortical cells for the local accumulation of auxin to occur.

Cytokinin signaling regulates auxin accumulation during nodule development
In L. japonicus plants carrying the dominant snf2 mutation, the constitutive activation of the cytokinin signaling pathway causes spontaneous nodule formation in the absence of rhizobia (Fig. 2E) (Tirichine et al., 2007). To clarify the relationship between auxin accumulation and cytokinin signaling during nodule development, auxin distribution patterns were analyzed in snf2 mutant plants. DR5::GFP-NLS/snft2 plants were produced by crossing DR5::GFP-NLS transgenic plants with snf2 plants. During spontaneous nodule formation in DR5::GFP-NLS/snft2, induction of GFP expression was observed in the cortical cells that were proliferating to form the primordia of spontaneous nodules (Fig. 2H). GFP expression was maintained in the inner region of growing spontaneous nodules even when they were almost as large as normal nodules formed following rhizobial infection. In the latter, GFP expression is excluded from the inner regions colonized by the rhizobia (Fig. 1J; Fig. 2I). Thus, auxin accumulation patterns during spontaneous nodule development in the snf2 mutant suggest that cytokinin signaling positively regulates auxin accumulation and that rhizobia might have a role in the exclusion of auxin from infected cells.

NIN is involved in the local accumulation of auxin
NIN encodes an RWP-RK type transcription factor that acts in the downstream part of the cytokinin signaling pathway (Schauer et al., 1999; Tirichine et al., 2007; Madsen et al., 2010). To elucidate the relationship between NIN and auxin, we analyzed auxin
distribution patterns in a nin mutant and in the roots of transgenic plants that constitutively express NIN. In the nin-9 mutant, nodulation was completely inhibited, although excessive curling of root hairs was observed, as has been reported previously for other nin alleles (Schauser et al., 1999). DR5::GFP-NLS/nin-9 plants were produced by crossing DR5::GFP-NLS transgenic plants with nin-9 plants. No specific distribution of GFP was observed in the cortical cells beneath the curled root hairs in DR5::GFP-NLS/nin-9 (Fig. 3A). When NIN was expressed under the control of the L. japonicus ubiquitin promoter (pLjUBQ) in the hairy root of DR5::GFP-NLS transgenic plants, ectopic division of cortical cells was induced. The cortical cells formed nodule- and lateral root-like organs in the absence of rhizobia (Fig. 3B,C; T. Soyano and M. Hayashi, personal communication). During formation of these structures, localized expression of GFP was observed in the cortex of DR5::GFP-NLS roots that constitutively expressed NIN (Fig. 3D). This pattern of GFP expression continued in the bulge formed by the nodule/lateral root-like organs (Fig. 3E,F). Thus, our observations indicate that NIN plays a role in the local accumulation of auxin in the cortical cells of roots and that this probably leads to the activation of cortical cell division.

**Autoregulation of nodulation controls auxin accumulation**

Autoregulation of nodulation (AON) is a presumptive systemic regulatory mechanism that controls nodule number (Oka-Kira and Kawaguchi, 2006). We investigated the interaction between auxin and key components of AON, specifically, the HARI and LjCLE-
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RS genes. Compared with the WT, the number of nodules increased within a much larger nodulation zone in har1 mutants (Fig. 2C; Fig. 4A). DR5::GFP-NLS/har1-8 plants were produced by crossing DR3::GFP-NLS/har1-8 plants with har1-8 plants. In 5-dai roots of DR3::GFP-NLS/har1-8, GFP was not only strongly expressed but also expressed more widely than in DR5::GFP-NLS/WT plants (Fig. 1C,G; Fig. 4B,C). The GFP expression pattern in the har1 mutant suggests that excessive induction of cortical cell division leads to the increased number of nodules and that this is associated with a considerable increase in auxin accumulation in the mutant. Overall, our findings suggest that HAR1 might function to negatively regulate the accumulation of auxin.

LjCLE-RS1 and LjCLE-RS2, which encode small CLE peptides, are presumed to act as root-derived negative regulatory signals that function via receptor complexes, including HAR1, during nodulation (Okamoto et al., 2009; Kouchi et al., 2010). Although the expression of LjCLE-RS1 and LjCLE-RS2 is significantly increased in roots immediately after rhizobial infection, constitutive expression of either gene can suppress nodule formation (Okamoto et al., 2009). We therefore sought to identify the site of action of the negative regulation resulting from activation of the LjCLE-RS genes and to investigate its relationship to auxin accumulation. In order to monitor cell division patterns, we made use of LjlTI6b (the putative L. japonicus ortholog of Arabidopsis LTI6b) because LTI6b has been reported to be localized at the plasma membrane (see Materials and methods) (Reddy et al., 2004). The plasma membrane of host plants could be labeled by expressing a GFP-LjLTI6b fusion protein under control of the 35S promoter (Fig. 5A).

Nodulation phenotypes were examined in DR5::GFP-NLS plants that carried either pLjUBQ::LjCLE-RS1 or LjCLE-RS2; p35S::GFP-LjLTI6b in their hairy roots. In comparison to the hairy roots of control plants that expressed the GUS gene, constitutive expression of either of the LjCLE-RS genes reduced the number of nodules (Fig. 4D-F; supplementary material Fig. S2A) (Okamoto et al., 2009). Infection thread formation was observed in almost all hairy roots of control plants (19/20; Fig. 4D; Fig. 5A). Importantly, although nodule formation was suppressed by constitutive expression of LjCLE-RS genes, infection thread formation was observed in 85% of the plants (17/20; Fig. 4E; Fig. 5F), suggesting that the rhizobial infection process was unaffected by activation of the LjCLE-RS genes.

Comparison of cortical cell division patterns and auxin distribution in control roots showed that auxin accumulation was closely related to the progress of bulge formation in the nodule primordia (Fig. 5A-C). In hairy roots of plants constitutively expressing LjCLE-RS genes, auxin accumulation patterns immediately before the initiation of cortical cell proliferation were indistinguishable from those of control hairy roots (Fig. 5A,F). Furthermore, we noticed that some initial cortical cell divisions were accompanied by auxin accumulation in the roots (Fig. 5G; supplementary material Fig. S2B). Initial cortical cell division was observed in 21/25 pLjUBQ::LjCLE-RS1 plants and 17/20
plants. These results suggest that although the numbers of nodules decreased, initial cortical cell division still occurred in the presence of constitutive activation of LjCLE-RS genes. However, we also frequently observed vestiges of cell division with diminished auxin accumulation (Fig. 5H-J; supplementary material Fig. S2C,D). Such vestiges were present in 22/25 pLjUBQ::LjCLE-RS1 plants and 18/21 pLjUBQ::LjCLE-RS2 plants. This implied that premature arrest of cortical cell divisions occurred in hairy roots with constitutively activated LjCLE-RS genes. In control hairy roots, vestiges of cell division were also observed in 15/20 plants, although most cortical cell divisions proceeded to form nodule primordia (Fig. 5C-E). Thus, arrest of cortical cell divisions can also occur in WT nodule development in which the AON mechanism is anticipated to be fully functional. Overall, these findings indicate that activation of LjCLE-RS genes may act to inhibit further cortical cell divisions; the initial cortical cell divisions appear to avoid this negative regulation.

The expression of a TAA-like gene is induced upon rhizobial infection

The analyses described above identify the site of auxin accumulation and place this in a genetic context with respect to the regulation of nodule development. To determine the relationship between auxin production and nodulation, we examined the expression patterns of genes involved in auxin biosynthesis during nodulation. We focused on the L. japonicus homologs of TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and its paralog TRYPTOPHAN AMINOTRANSFERASE RELATED...
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DISCUSSION
In this study, we focused on the patterns of auxin accumulation during early nodule development, that is, the developmental stage from the initiation of cortical cell proliferation to the formation of nodule primordia. We show here that auxin predominantly accumulates in dividing cortical cells. The observed auxin distribution patterns are similar to those reported previously using the GH3 promoter (Pacios-Bras et al., 2003; Takanashi et al., 2011a); however, we have also been able to correlate auxin accumulation patterns with various genetic factors that are key to the formation of nodules.

Leguminous plants have two major and morphologically distinct types of nodule: indeterminate and determinate (Ferguson et al., 2010). In indeterminate nodules, such as those of Medicago and Pisum, the nodule meristem can be distinguished at the tip of nodules and this meristem stays active until the nodule becomes senescent. By contrast, in determinate nodules, such as those in Lotus, the activity of the nodule meristem appears to cease during early nodule development, although the identity and location of the nodule meristem is poorly characterized. Our observations apply particularly to the development of determinate nodules. After rhizobial colonization of developing nodules, the accumulation of auxin becomes restricted to the region surrounding the infected cells. This region appears to correspond to the nodule parenchyma, which has previously been reported to show expression of organ differentiation markers such as ENOD2 (van de Wiel et al., 1990; Niwa et al., 2001). As the size of a determinate nodule continues to increase by cell proliferation and elongation even after differentiation, it is likely that the meristematic activities are maintained within the nodule. Since the auxin distribution pattern during nodule development in Lotus resembles that of meristematic cells, the nodule parenchyma might be a candidate for the nodule meristem. Further analysis, for example defining the cell division patterns in the nodule, will be needed to clarify the identity and location of the nodule meristem in determine nodules. Auxin accumulation occurs not only in dividing cortical cells but also in the vascular bundle (lenticels) at later stages of nodule development. The formation of lenticels is inhibited by auxin transport inhibitors (Takanashi et al., 2011a; Takanashi et al., 2011b).

During spontaneous nodule formation in the snf2 mutant, auxin accumulation was observed at the inner nodular regions; by contrast, these inner regions are occupied by rhizobia in normal nodules. In comparison to normal nodules, some of the spontaneous nodules formed in the snf2 mutant were distorted in shape (data not shown). Thus, it is possible that persistent auxin accumulation in the inner region causes excess cell division and leads to the distorted shape of some spontaneous nodules.

Many of the genes that have been shown to be involved in the positive regulation of nodulation function in the epidermis to signal the occurrence of infection (Kouchi et al., 2010). However, nodulation-related cytokinin receptors (LHK1 in L. japonicus and CRE1 in M. truncatula) are specifically involved in nodule development (Gonzalez-Rizzo et al., 2006; Murray et al., 2007; Tirichine et al., 2007). The RWP-RK type transcription factor NIN acts in the downstream part of the cytokinin signaling pathway (Schauer et al., 1999; Tirichine et al., 2007; Madsen et al., 2010). Recently, NIN has also been shown to be able to initiate cortical cell division: constitutive activation of NIN induces ectopic division of the cells in the absence of rhizobia (T. Suyano and M. Hayashi, personal communication). Here, we investigated the relationship between auxin accumulation patterns and the expression of genes that encode positive regulators of nodule development. Our results show that auxin accumulation was induced when cytokinin signaling was constitutively activated. Furthermore, in the roots of transgenic plants that constitutively expressed NIN, local auxin accumulations were observed. Based on these results, we propose a model for the molecular mechanism that regulates cortical cell division through control of auxin accumulation (Fig. 7). In the model, infection signals from the epidermis ultimately activate cytokinin signaling in the cortex, which causes a downstream transcription factor, NIN, to establish local auxin accumulation in some cortical cells, which in turn triggers division of these cortical cells. Since auxin accumulates in the dividing cortical cells until the formation of nodule primordia, then maintenance of auxin accumulation is required for nodule organogenesis. The establishment of local auxin accumulation appears to be related to the inhibition of polar auxin transport; previous studies have shown that inhibition of polar auxin transport in Medicago roots induces the formation of pseudonodules (Hirsch et al., 1989; Rightmyer and Long, 2011) and that expression of some auxin transporters is negatively regulated by cytokinin signaling (Plet et al., 2011). Here, however, we found that expression of a gene involved in auxin biosynthesis was contemporaneous with the beginning of local auxin accumulation. This observation suggests that inhibition of polar auxin transport in conjunction with de novo auxin production might contribute to the establishment of local auxin accumulation. Further investigation to identify the interactions of genes involved in the positive regulation of nodule development, polar auxin transport and auxin biosynthesis would clarify the molecular mechanism responsible for the local accumulation of auxin at the sites of incipient nodule primordia.

In legumes, AON is known as a genetic mechanism that controls the number of nodules via long-distance communication between...
Although constitutive expression of AON-related CLE genes in legumes causes inhibition of nodulation (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011a), the site of the negative regulation has remained elusive. Our simultaneous observation of cortical cell division and auxin accumulation patterns shows that early auxin accumulation and the initiation of cell division can occur even in the presence of constitutively activated LjCLE-RS genes. Thus, the developmental process does not seem to be affected by constitutive expression of LjCLE-RS genes. By contrast, we frequently observed vestiges of cortical cell division, implying its premature arrest, accompanied by reduced levels of auxin accumulation in transgenic roots. Importantly, these cell division vestiges were also observed in control roots, in which the LjCLE-RS genes were appropriately activated by rhizobial infection. Thus, the premature arrest of cortical cell division was not a secondary effect of constitutive activation of LjCLE-RS genes, as it is probable that such arrest occurs normally. Overall, we presume that activation of LjCLE-RS genes has a negative regulatory effect on cortical cell division after its initiation, possibly through inhibiting the maintenance of auxin accumulation (Fig. 7; supplementary material Fig. S3). Further investigation needs to be carried out to determine whether the link between activation of LjCLE-RS genes and auxin accumulation is direct or indirect.

Both nodules and lateral roots are formed as lateral organs of roots, and the regulatory mechanisms for these two organs seem to have some components in common and others that are organ specific (Desbrosses and Stougaard, 2011). On the basis of the results obtained here, we propose the existence of a novel molecular mechanism that controls cortical cell division in a developmental stage-specific manner during nodule development. This fine-tuning mechanism for regulation of cortical cell division is reminiscent of lateral root development, in which organogenesis initiation in the founder cells, the formation of primordia and lateral root emergence are regulated by distinct factors involved in auxin signaling (Benková and Biehl, 2010). In order to achieve a greater understanding of the characteristic features of nodule organogenesis, the identification of stage-dependent regulators of cortical cell division is required.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**Fig. 7. Model for the regulation of cortical cell division and the site of auxin action in nodule development**. The light-blue box indicates the developmental stage of cortical cell division that is negatively regulated by AON. The darker blue box beneath, which partially overlaps the light-blue box, indicates the developmental stage when maintenance of auxin accumulation occurs in all proliferating cortical cells. Green and red regions of cortical cells indicate the presumed sites of auxin accumulation and of rhizobia, respectively. CCD, cortical cell division; SDI, shoot-derived inhibitor. See text for explanation of the model.


