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 (Szczypowski 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 Camp et al., 2011). The various reports described above suggest initiation. Downstream of cytokinin signaling, two putative

NIN

expression of 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 MiPIN genes is upregulated in the cre1 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-R5 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.

MATERIALS AND METHODS

Plant materials

The Miyakojima MG-20 ecotype of L. japonicus was used in this study. MG-20 plants were mutagenized with ethylmethane sulfonate (EMS) and cyclosp-6, nin-9 and har1-8 were isolated from M2 plants. Allelism tests indicated that all three genes were new mutants of cyclosp, nin and har1, respectively. The site of mutation in each mutant is shown in supplementary material Table S1. The snf2 mutant, which has an MG-20 background, has been reported previously (Miyazawa et al., 2010). The DRS::GFP-NLS construct was introduced into the mutants by crossing with transgenic MG-20 plants.

Constructs and transformation of L. japonicus

The primers used for PCR are listed in supplementary material Table S2. To generate the DRS::GFP-NLS construct, a 2.8 kb fragment of the DRS::gateway-cassette (GW) was excised from a vector kindly provided by J. Lohmann (Heidelberg University, Germany) and inserted between the SacI and KpnI sites of pCAMBIA1300. The translational fusion of GFP-NLS in pDONR221 (Invitrogen), which was kindly provided by D. Weigel (Max-Planck Institute for Developmental Biology, Tübingen, Germany), was inserted downstream of DRS using the LR recombination reaction. The resulting plasmid was introduced into Agrobacterium tumefaciens strain AGL1 and transformed into MG-20 as described previously (Nishimura et al., 2002).

To obtain the pCYCLOPS::mCherry-NLS construct, GFP was removed using Xhol from the binary vector pCYCLOPS::GW, p35S::GFP reported previously (Yano et al., 2008). The translational fusion of mCherry-NLS in pDONR221, which was provided by D. Weigel, was inserted downstream of the CYCLOPS promoter by the LR recombination reaction. For constitutive expression of NIN, the GFP in the pIGUBQ::GW, p35S::GFP binary vector (Maekawa et al., 2008) was removed using Xhol, and PCR-amplified mKO2 [Medical and Biological Laboratories (Sakae-Sawano et al., 2008)] was inserted into the Xhol site to create the new binary vector pIGUBQ::GW, p35S::mKO2. NIN cDNA was amplified by PCR and cloned into the pENTR/D-T0PO vector (Invitrogen). The NIN cDNA in pENTR/D-T0PO was inserted downstream of the LJUBQ promoter by the LR recombination reaction. To create pLJUBQ::LjLTI6b, p35S::GFP-LjLTI6b or pLjUBQ::LjLTI6b, first, a GFP from which the stop codon had been deleted was amplified by PCR and cloned into a pENTR1A (Invitrogen)-based vector (pJL-Blue), which has a multi-cloning site between the attL1 and attL2 sequences; the plasmid was kindly provided by D. Weigel. The resulting vector was named pENTR-GFP.

In order to identify a marker for the plasma membrane in L. japonicus (GFP-LjLTI6b), we performed a BLAST search (Altschul et al., 1990) of the L. japonicus genomic sequence database for the chs5.CM0048.40 gene using as the query the amino acid sequence of Arabidopsis LT16b, which encodes a plasma membrane-localized protein (Reddy et al., 2004). We designated the recovered gene sequence as LT16b. The LjLTI6b cDNA was amplified with a short alanine linker sequence by PCR and inserted downstream of the C-terminus of the GFP in pENTR-GFP to produce a translational fusion of GFP-LjLTI6b. The resulting GFP-LjLTI6b was amplified by PCR and used to replace the original GFP of the pIGUBQ::GW, p35S::GFP vector to form the new binary vector pIGUBQ::GW, p35S::GFP-LjLTI6b. The LjCLE-RS1, LjCLE-RS2 and GUS cDNAs in pDONR221 (Okamoto et al., 2009) were inserted downstream of the LJUBQ promoter using the LR recombination reaction. The recombinant plasmids were introduced into A. rhizogenes strain AR1193 and were transformed into roots of DRS::GFP-NLS plants by a hairy root transformation method described previously (Okamoto et al., 2009).

In situ hybridization

The primers used for PCR are listed in supplementary material Table S2. The in situ hybridization probes for GFP and HISTONE H4 were created by PCR amplification of 495 bp and 372 bp fragments from each, respectively. The fragments were inserted into a PGEM-T Easy vector by TA cloning (Promega). Probe synthesis, preparation of sections and in situ hybridization were performed as described previously (Suzuki et al., 2004). Signals were observed with a light microscope (BX-50, Olympus).
Expression analysis

The primers used are listed in supplementary material Table S2. LjTAA1 (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 RNeasy 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) (Benkóvá 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. 1N-P). 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/snf2 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. 2J; 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-
Auxin involvement in nodulation

**Fig. 2. Auxin accumulation patterns in cyclops and snf2 mutants.**

(A, B) Infection thread formation in wild-type (WT) (A) and cyclops-6 (B) *L. japonicus* plants at 7 dai. (C, D) Nodules in WT (C) and cyclops-6 (D) at 14 dai. (E) Spontaneous nodule in snf2 35 days after germination (dag) in the absence of rhizobia. (F, G) GFP expression patterns during nodule formation in DR5:GFP-NLS/cyclops-6 at 7 (F) and 14 (G) dai.

**Fig. 3. Relationship between NIN expression and auxin accumulation.** (A) GFP expression patterns in DR5::GFP-NLS/nin-9 L. japonicus plants at 7 dai. (B, C) Non-symbiotic root phenotype in DR5::GFP-NLS plants that have transgenic hairy roots, in which NIN and mKO2 are constitutively expressed. Transgenic roots were identified by means of mKO2 expression (C). Arrowheads indicate nodule/facial root-like organs. (D-F) GFP expression patterns during development of the nodule/facial root-like organs formed in DR5::GFP-NLS plants that have transgenic hairy roots constitutively expressing NIN in the absence of rhizobia. Arrow indicates locally accumulated GFP signal in cortical cells. Fluorescence was observed using side views of the root (A-D). For fluorescence analysis, at least 15 plants (A) or 15 plants having transgenic hairy roots (B-F) were analyzed at each developmental stage in three independent experiments. Scale bars: 100 μm in A,D-F; 1 mm in B,C.

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 transgenic 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 *LjLT16b* (the putative *L. japonicus* ortholog of *Arabidopsis* LT16b) because *LT16b* 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-*LjLT16b* 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-LjLT16b* 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
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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**Fig. 4.** Auxin accumulation patterns in the har1 mutant and the effects of constitutive activation of LjCLE-RS genes on nodulation.

(A) Nodulation phenotype of har1-8 L. japonicus plants at 14 dai. (B,C) GFP expression patterns during nodule development of DRS::GFP-NLS/har1-8 plants at 5 dai. Fluorescence and nodule formation were observed using front (B) or side (C) views of infected roots. For fluorescence analysis, at least 20 plants were analyzed at each developmental stage in three independent experiments. (D,E) Nodulation phenotype of DRS::GFP-NLS plants that have transgenic hairy roots constitutively expressing both GUS and GFP-LjLT6b (D), or both LjCLE-RS1 and GFP-LjLT6b (E). Transgenic roots were identified by their widespread distribution of GFP signals, derived from GFP-LjLT6b, compared with those of GFP-NLS that are localized in specific regions of the root. Infection threads appear as red spots in the roots. (F) Nodule numbers at 16 dai in DRS::GFP-NLS plants that have transgenic hairy roots constitutively expressing LjCLE-RS genes (n=13-17). Error bars indicate s.d. Scale bars: 1 mm in A,D,E; 100 μm in B,C.

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**Fig. 5.** The effects of constitutive activation of LjCLE-RS1 on cortical cell division and auxin accumulation.

(A-E) Infected DRS::GFP-NLS L. japonicus plants that have transgenic hairy roots containing pLjUBQ::GUS and p35S::GFP-LjLT6b. (F-I) Infected DRS::GFP-NLS plants that have transgenic hairy roots containing pLjUBQ::LjCLE-RS1 and p35S::GFP-LjLT6b. (J) Magnified image of I. Fluorescence and cortical cell division were observed using front (E,G,I,J) or side (A,D,F,H) views of the roots. Plant plasma membrane was visualized by the expression of GFP-LjLT6b. Green dots indicate auxin accumulation. Arrows indicate the regions where vestiges of cell division with diminished auxin accumulation were observed. Roots were at 6 (A,F) or 12 (B-E,G-J) dai. At least 20 plants having transgenic hairy roots were analyzed at each developmental stage in three independent experiments. Scale bars: 100 μm.
(TAR) because the TAA family produces indole-3-pyruvic acid (IPA), which is converted to indole-3-acetic acid (IAA) by the YUCCA family; this IPA pathway is thought to be the main route for IAA biosynthesis (Mashiguchi et al., 2011; Won et al., 2011). During nodulation, we found that expression of LjTAR1 was transiently increased at 3 dai, which is when local auxin accumulation started to occur, whereas LjTAA1 expression did not change following rhizobial infection (Fig. 1A; Fig. 6A,B).

**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 meristic activities are maintained within the nodule. Since the auxin distribution pattern during nodule development in *Lotus* resembles that of meristic 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 determinate 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 (Schauser 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. Soyano 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

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![Fig. 6. LjTAA1-like gene expression patterns during nodulation.](image-url)
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 Bielach, 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.

Acknowledgements
We thank Jan Lohmann for the DRS construct; Detlef Weigel for the GFP-NLS and mCherry-NLS constructs; Makoto Hayashi for the pCYCLOPS construct and for M. loti MAFF303099 expressing DsRED; Satoru Okamoto for LjCLE-RS1/2 constructs;Lens Stougaard for A. rhizogenes strain AR1193 and the nin-2 and har1-3 mutants; and Takashi Soyano and M. Hayashi for sharing unpublished data. Confocal images were acquired at the Spectrography and Bioimaging Facility, NIBB Core Research Facilities.

Funding
This research was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (22870035 and 23012038 to T.S., 22128006 to M.K.), grants from The NOVARTIS Foundation (Japan) for the Promotion of Science (to T.S.) and grants from The Sumitomo Foundation (to T.S.).

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.084079/-/DC1

References
Auxin involvement in nodulation


