

Commentaires de l'article « Turning lateral roots into nodules »

By Anthony Bishopp and Malcolm J. Bennett

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La fixation symbiotique de l'azote atmosphérique est réalisée par les plantes légumineuses au niveau de structures symbiotiques nommées nodules, le plus souvent racinaires, résultats d'une symbiose entre bactéries fixatrices d'azote et cellules de la plante hôte.

Cette symbiose offre en effet un environnement propice au fonctionnement de la nitrogénase bactérienne qui catalyse la transformation du diazote en ion ammonium assimilable par les plantes. Elle permet ainsi aux plantes légumineuses de pouvoir être cultivées sans utiliser d'engrais azotés chimiques.

Des études phylogénomiques récentes ont révélé la perte au cours de l'Evolution, pour ne subsister actuellement que chez les plantes légumineuses, de gènes clés de la formation des nodules. La connaissance de ces gènes peut permettre leur réintroduction/réactivation chez les plantes non-légumineuses, par exemple via les techniques d'édition des génomes.

La caractérisation de tels gènes est précisément l'objet des recherches rapportées dans le présent article publié par la revue Science (cf. ci-après). Selon les auteurs, de telles stratégies de réingénierie des plantes non-légumineuses amèneraient une véritable révolution en agriculture, réduisant potentiellement l'usage des centaines de millions de tonnes d'engrais azotés appliquées annuellement aux cultures, générant ainsi d'importantes économies d'énergie et le renforcement de la sécurité alimentaire dans les pays où l'accès aux engrais azotés est limité.

Il est clair que si ces résultats doivent être confortés par d'autres approches complémentaires, ils permettent un progrès considérable dans la connaissance de la biologie des plantes et de leur évolution. **Ils permettent de plus d'entrevoir des solutions radicalement nouvelles pour le développement d'une agriculture plus respectueuse de l'environnement tout en assurant la production des ressources alimentaires.**

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PERSPECTIVES | PLANT BIOLOGY

Turning lateral roots into nodules

The evolutionary origin of legume root nodules that help them grow is revealed

By Anthony Bishopp and Malcolm J. Bennett



Root nodules harbor nitrogen-fixing bacteria. Engineering plants to form root nodules could reduce fertilizer use. PHOTO: INGA SPENCE/SCIENCE SOURCE

Nitrogen is critical for plant growth but must be acquired from the soil in reduced forms such as nitrate. Often this occurs through biological fixation whereby nitrogen-fixing bacteria, such as rhizobia, live symbiotically in root nodules of legumes. Theories concerning the evolutionary origins of legume root nodules range from them being highly modified stems or lateral roots (1, 2). The latter origin is supported by rhizobia inducing lateral root-like nodules in nonleguminous *Parasponia* by triggering pericycle cell divisions, which normally produce lateral roots (3). However, in legume roots, rhizobia also induce cortical cell divisions, triggering many morphological differences to lateral root organogenesis (2). Two studies—by Soyano *et al.* (4) on page 1021 of this

issue and by Schiessl *et al.* (5)—report key regulatory components of lateral root organogenesis to have been hijacked in legumes to coordinate nodule formation. This could open avenues to engineer nitrogen fixation in nonlegume crops.

Root nodule organogenesis in legumes is initiated when rhizobia first colonize young root hairs (2). Nodulation factors released by rhizobia trigger root hair curling, cortical cell divisions that produce the nodule primordia, and infection thread formation (see the figure). The infection thread is a root-derived conduit that enables rhizobia to colonize dividing cortical cells, where they differentiate into nitrogen-fixing bacteroids and provide ammonium to the plant host. Root nodule organogenesis therefore represents a complex interplay between plant and bacterial symbiotic partners. Genetic studies have proved instrumental in dissecting the underlying regulatory mechanisms and signals controlling this important developmental program.

One of the first nodule regulatory genes identified in legumes was *NODULE INCEPTION (NIN)* (6), which encodes a transcription factor that is required for rhizobia infection and nodule organogenesis (7). *NIN* promotes the expression of two *NUCLEAR FACTOR-Y (NF-Y)* genes which, when coexpressed with *NIN*, can activate cortical cell division during nodule development and also alter development of lateral roots. This provided a tantalizing link between lateral root and nodule organogenesis, but it remained unclear whether these root processes shared a common regulatory program. An observation that suggested that lateral roots and nodules develop through distinct regulatory programs was the contrasting roles of the phytohormones auxin and cytokinin. It was well documented that auxin accumulated at the sites of both lateral root and nodule primordia (8). By contrast, cytokinin promotes nodule formation in a *NIN*-dependent manner (7, 9) but antagonizes lateral root formation (10).

Soyano *et al.* and Schiessl *et al.* dissected the nodule gene regulatory networks operating in two model legume plants, *Lotus japonicus* and *Medicago truncatula*, using distinct approaches. Soyano *et al.* adopted a chromatin immunoprecipitation–based approach to pinpoint *NIN* target genes that are expressed following rhizobial infection of lotus roots. Each *NIN* target was overexpressed with *NF-Y* and only one gene, *LATERAL ORGAN BOUNDARIES DOMAIN 16 (LBD16)*, was able to promote cortical cell divisions. Schiessl *et al.* probed gene regulatory networks operating during lateral root initiation and nodule formation. This revealed that lateral root and nodule initiation programs overlap considerably. Common genes included many associated with auxin

and cytokinin action, genes known to be involved in nodule formation, such as the *ARF* family of auxin response factors and the *YUCCA* family of auxin biosynthesis genes, as well as genes previously associated with lateral root development. Soyano *et al.* and Schiessl *et al.* analyzed mutant alleles in these common genes and found that only *LBD16* is required for both lateral root and nodule formation.

LBD16 encodes a transcription factor, first identified in the commonly used model plant *Arabidopsis thaliana*, in which it is expressed in the pericycle and promotes asymmetric cell divisions in which daughter cells gain distinct identities that are required for lateral root morphogenesis (11). Soyano *et al.* revealed that *LBD16* expression in legume roots induced the expression of reporter genes in dividing cortical and pericycle cells. This observation raises a critical question: Does *LBD16* expression in dividing cortical cells provide the key step in creating populations of dividing pericycle and cortical root cells that form root nodules? Soyano *et al.* showed that the presence of a NIN-binding site in an *LBD16* intron led to its expression in nodule primordia. Moreover, these authors observed that the NIN-binding site was conserved in many species of legumes, but absent in nonlegume species. This raises the fascinating possibility that evolution of this NIN-binding site in an ancestral legume genome caused *LBD16* to be ectopically expressed in cortical cells. This event would have caused a gene regulatory network, normally restricted to pericycle cells and controlling lateral root development, to also be expressed in the cortex and induced by cytokinin (through NIN), leading to nodule formation (see the figure).

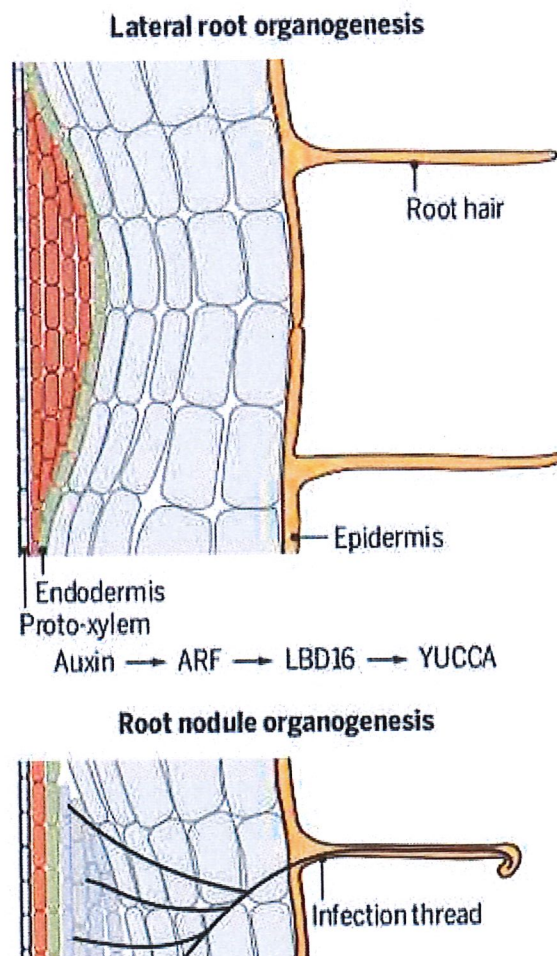
This “co-option” model helps answer a long-standing question relating to the morphological differences between lateral root-like nodules in nonlegumes versus root nodules in legumes. Lateral root-like nodules originate from dividing pericycle cells, whereas root nodules arise from dividing pericycle and cortical cells. This morphological difference may simply reflect the evolution of NIN-binding sites in the *LBD16* intron sequence of legumes that are missing in nonlegumes. It would be intriguing to test whether introducing NIN-binding sites in the *Parasponia LBD16* sequence is able to convert their lateral root-like nodules to true root nodules.

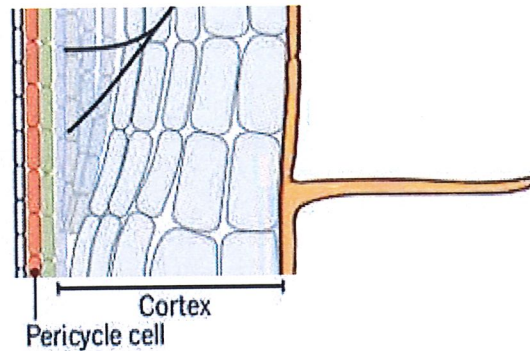
Stepping beyond nodule formation and even plant science, the question of how new structures emerge is an enduring conundrum that has intrigued evolutionary biologists. It is known from several model systems that the expression of key transcription factors has been fine-tuned through evolution to favor the development of one organ over

another. For example, in blind cavefish, sonic hedgehog genes *ShhA* and *ShhB* are amplified locally to negatively control eye development, but their overexpression increases jaw and taste bud development (12), providing an elegant example of how gene regulatory networks can be modified to promote the development of one organ rather than another. The discovery of enhanced LBD16 activity in cortical cells takes this phenomenon to a different level: Whether there is another example by which misexpression of a transcription factor in another tissue has hijacked an existing gene regulatory network to create an entirely new organ is unknown.

Lateral roots and nodules share common regulators

Lateral roots are derived primarily from dividing pericycle cells. Root nodule organogenesis initiates after rhizobia trigger root hair curling, cortical cell divisions, and infection thread formation. Despite being regulated by distinct hormone signals, lateral root and root nodule regulatory networks have common components.





Cytokinin → NIN → LBD16 → NF-Y → YUCCA

ARF, auxin response factor; LBD16, lateral organ boundaries domain 16; NF-Y, nuclear factor-Y; NIN, nodule inception.

GRAPHIC: N. DESAI/SCIENCE

The apparent co-option of the master regulator LBD16 raises the intriguing possibility that the number of genetic changes required for nodule formation in legumes may be relatively small. If so, it might be feasible to engineer legume-style nodules into other crops. Nodule organogenesis is also widely spread across four orders of flowering plants, and comparative genome sequencing studies recently revealed that inactivation of core nodulation genes, including *NIN* and *NF-Y*, have likely led to the loss of nodule-forming ability in different species (13, 14). In principle, this is good news because crops in these orders may require limited gene editing to reengineer their nodule-forming abilities. If either reengineering strategy could be achieved, it promises a revolution in agriculture, with symbiotic bacteria potentially lessening the hundreds of millions of tonnes of nitrogen fertilizer applied to crops annually (15), generating major energy savings (more than 3% of the global carbon budget is required for nonbiological nitrogen fixation) and aiding food security in countries with limited access to fertilizers.

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PLANT SCIENCE

A shared gene drives lateral root development and root nodule symbiosis pathways in *Lotus*Takashi Soyano^{1,2,3*}, Yoshikazu Shimoda⁴, Masayoshi Kawaguchi^{1,2}, Makoto Hayashi^{3,4*}

Legumes develop root nodules in symbiosis with nitrogen-fixing rhizobial bacteria. Rhizobia evoke cell division of differentiated cortical cells into root nodule primordia for accommodating bacterial symbionts. In this study, we show that NODULE INCEPTION (NIN), a transcription factor in *Lotus japonicus* that is essential for initiating cortical cell divisions during nodulation, regulates the gene *ASYMMETRIC LEAVES 2-LIKE 18/LATERAL ORGAN BOUNDARIES DOMAIN 16a* (*ASL18/LBD16a*). Orthologs of *ASL18/LBD16a* in nonlegume plants are required for lateral root development. Coexpression of *ASL18a* and the CCAAT box-binding protein Nuclear Factor-Y (NF-Y) subunits, which are also directly targeted by NIN, partially suppressed the nodulation-defective phenotype of *L. japonicus daphne* mutants, in which cortical expression of NIN was attenuated. Our results demonstrate that *ASL18a* and NF-Y together regulate nodule organogenesis. Thus, a lateral root developmental pathway is incorporated downstream of NIN to drive nodule symbiosis.

Root nodule symbiosis in legumes allows host survival in nitrogen-limiting conditions and partakes in the nitrogen cycle in terrestrial ecosystems. This symbiosis has evolved through the co-option and rearrangement of signaling pathways, following predisposition in a single ancestor of the nitrogen-fixing angiosperm clade (1–3). It is presumed that nodulation-specific factors, such as *Lotus japonicus* NODULE INCEPTION (NIN), function downstream of early signaling modules (common symbiosis pathway) recruited from arbuscular mycorrhizal symbiosis, which is widely distributed in plants (Fig. 1A) (4–6). NIN is related to proteins involved in responses to nitrate (7). Ectopic expression of NIN and its target Nuclear Factor-Y (NF-Y) subunit genes *NF-YA1* and *NF-YB1* alters development of lateral root primordia and activates cortical cell division for nodule organogenesis (8), implying that NIN and its target factors link nodule development programs with lateral root developmental programs.

To identify transcription factors that influence cell division with NF-Y, we searched for genes whose transcription is induced in response to rhizobia among NIN target candidates found by a chromatin immunoprecipitation sequencing (ChIP-seq) analysis (9), and we further overexpressed them in *L. japonicus* roots. Only one gene, *ASYMMETRIC LEAVES 2-LIKE 18/LATERAL ORGAN BOUNDARIES DOMAIN 16a* (*ASL18/LBD16a*), stimulated cell division when co-overexpressed with *NF-YA1*.

ASL18 genes have been duplicated at least once in an ancestral legume lineage (fig. S1). One or two NIN-binding nucleotide sequences (NBS-S1, or both NBS-S1 and NBS-S2) were found in *ASL18b* and *ASL18a* introns, respectively (Fig. 1B and fig. S2) (10). NBS-S1 and its flanking nucleotide sequences were conserved in leguminous *ASL18* introns—particularly in Papilionoideae, with the exception of a few species—but were not observed in nonleguminous orthologs (fig. S1). Hence, the evolution of NBS in *ASL18* intron sequences could have played a key role in recruitment of this lateral root regulator into the nodule signaling pathway in legumes. NIN was required for *ASL18a* expression in response to inoculation with *Mesorhizobium loti* (Fig. 1C). Furthermore, dexamethasone treatment of roots expressing NIN fused with a glucocorticoid receptor (NIN-GR) (8) increased *ASL18a* expression within 4 hours (Fig. 1D and fig. S3).

Spatial expression patterns of *ASL18* genes were investigated using translational fusion

with β -glucuronidase (*GUS*) reporter (fig. S4A; *ProASL18a:ASL18a-GUS* and *ProASL18b:ASL18b-GUS*). Both translational fusions were expressed in early lateral root primordia derived from the pericycle (Fig. 2, A and B). Lateral root densities exhibited by *asl18a* knockout plants were lower than those of wild-type plants (Fig. 3A and fig. S5), which was consistent with the general function of *ASL18/LBD16* (11–13). The *ASL18a* promoter was responsible for expression in lateral root primordia and for response to auxin (Fig. 2, C and D, and fig. S6). In the presence of rhizobia, *ProASL18a:ASL18a-GUS* was expressed at infection foci in the root epidermis and nodule primordia formed in the cortex, similar to expression patterns of NIN and NF-Y subunit genes (Fig. 2, E and F, and fig. S4) (8). *ProASL18b:ASL18b-GUS* showed less expression at the basal region of the nodule primordia (Fig. 2G and fig. S4). The *ASL18a* intron was sufficient for conferring expression in nodule primordia and its transcription induced by NIN, whereas the *ASL18a* promoter was also active in primordia (Fig. 2, H to J, and figs. S4 and S7). Thus, multiple pathways are connected with *ASL18a* transcription downstream of NIN. The number and size of *asl18a* mutant nodules were reduced compared with those of the wild type, when KNO_3 was supplemented for partial inhibition of nodulation (Fig. 3B). This suggested that *ASL18a* was involved in nodule growth. The weakness of the *asl18a* phenotype is probably due to redundancy as observed in *Arabidopsis* (14). Indeed, nodule and lateral root development were inhibited when *ASL18a* was expressed as a fusion protein with an artificial repressor domain, SRDX, in hairy roots (fig. S8). *ASL18a* fused with a 35S minimal promoter partially suppressed the *asl18a* nodule phenotype (fig. S9).

The *asl18a* mutations enhanced nodulation phenotypes of *nf-y* subunit mutants. Nodule

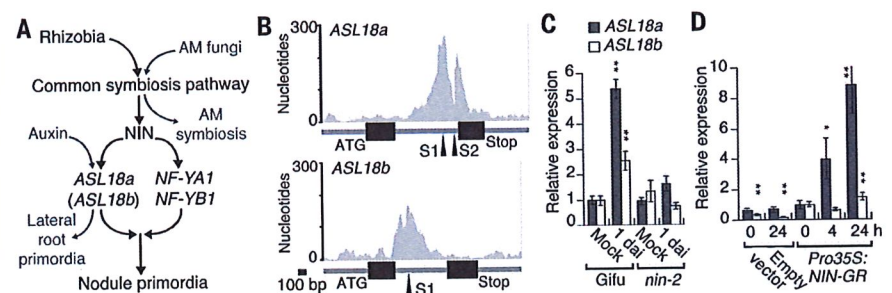


Fig. 1. Rhizobial infection activated *ASL18a* depending on NIN. (A) Schematic of a working hypothesis. AM, arbuscular mycorrhizal. (B) Read coverage in *ASL18* genes obtained by ChIP-seq analysis with NIN precipitation. Boxes indicate exons and arrowheads indicate putative NBSs (S1 and S2). bp, base pairs. (C and D) Quantitative reverse transcription polymerase chain reaction analyses of *ASL18* expression. (C) Wild-type (Gifu B-129) and *nin-2* roots inoculated with *M. loti* ($n > 10$ plants for each biological replicate). dai, days after inoculation. (D) Gifu B-129 roots transformed with either an empty vector or *Pro35S:NIN-GR* were treated with 10 μM dexamethasone ($n > 10$). * $P < 0.05$, ** $P < 0.01$ [one-way analysis of variance (ANOVA) with Tukey's post hoc test] versus wild-type mock (C) and *Pro35S:NIN-GR* 0 hours (D). Data are mean \pm SD of three biological repeats.

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Fig. 2. Spatial expression patterns of ASL18 genes. GUS expression in lateral root primordia (A to D), infected root hairs (E), and nodule primordia (F to J). [(E) to (J)] Images merged with fluorescence from DsRed-labeled *M. loti*. Roots were transformed with *ProASL18a:ASL18a-GUS* [(A), (E), and (F)]; *ProASL18b:ASL18b-GUS* [(B) and (G)]; *ProASL18a:ASL18a (cDNA)-GUS* [(C) and (H)]; *Pro35Sminimal:ASL18a-GUS* [(D) and (I)]; and *Pro35Sminimal:ASL18a(cDNA)-GUS* (J) (see fig. S4A). Scale bars: 0.2 mm.

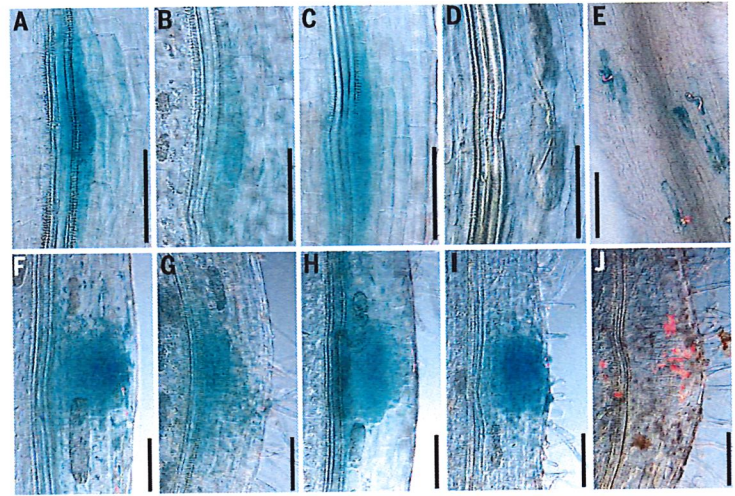
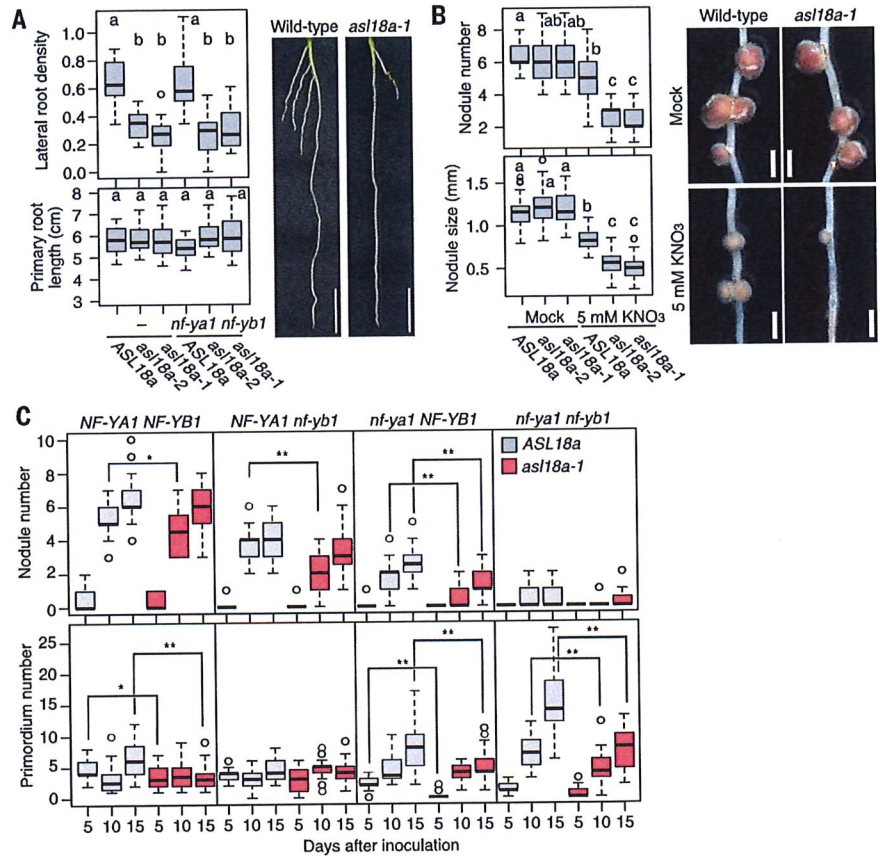


Fig. 3. ASL18a is involved in both lateral root and nodule development. (A) Lateral root densities and primary root length ($n > 15$ plants) of wild type, *asl18a*, and *nf-ya1-5 nf-yb1-1* (14-day-old). (B) Numbers ($n = 15$ plants) and diameters ($n > 37$ nodules) of nodules formed under conditions supplemented with KNO_3 (15 dai). (C) Nodule and primordium numbers ($n > 20$ plants) of multiple mutants between *asl18a-1*, *nf-ya1-5*, and *nf-yb1-1*. One-way ANOVA with Tukey's post hoc test was used. Different letters represent classes with significant difference ($P < 0.05$). * $P < 0.05$, ** $P < 0.01$. Scale bars: 1 cm for (A), 1 mm for (B).



development was delayed and nodule number was reduced in *nf-ya1* (8, 15). Nodule development was affected more severely in *nf-ya1 nf-yb1* double mutants (figs. S10 and S11). We attribute enhancement of the nodulation phenotype to functional redundancies with other NF-Y subunits (16). Development of nodule primordia in *asl18a nf-ya1 nf-yb1* triple mutants was delayed further, and the numbers of primordia and visible cortical division sites

were approximately half of those observed in *nf-y* double mutants (Fig. 3C and fig. S11). This suggested the involvement of *ASL18a* in nodule development from early stages. Further, *ASL18a* seemed to genetically interact with NF-Y during nodule development. In contrast, *nf-y* mutations did not influence lateral root densities (Fig. 3A).

NF-Y requires other factors, including pioneer transcription factors, for an activation

of its targets (17). *Lotus* NF-Y subunits interacted with ASL18 proteins in vitro and in planta (Fig. 4A and fig. S12). NF-Y subunits were overexpressed with or without *ASL18a* in roots (fig. S13). Double expression of *NF-YA1* and *NF-YB1* increased lateral root densities to twice those of empty vector controls (Fig. 4C and fig. S14) (8). *ASL18a* alone exerted no effect. Coexpression of *ASL18a* with both NF-Y subunits increased lateral root densities sixfold

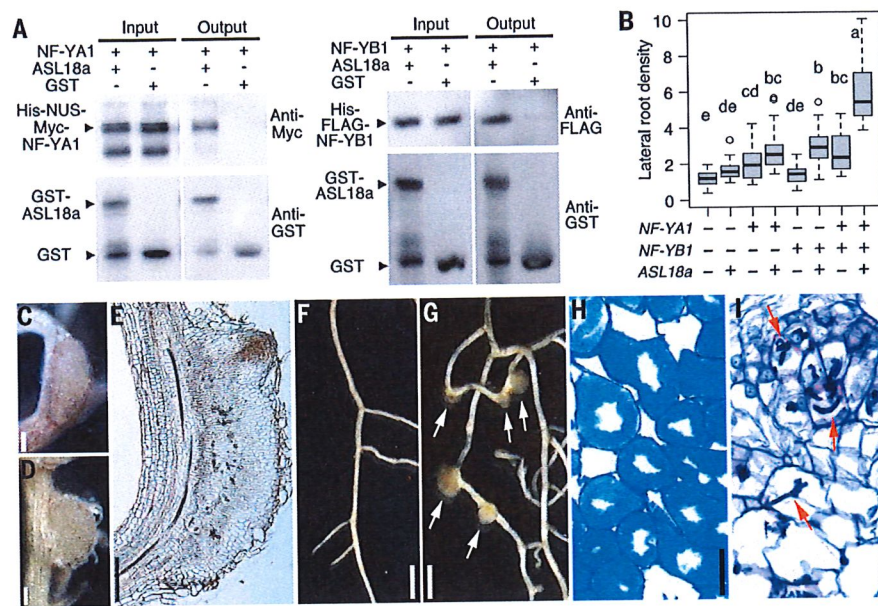


Fig. 4. Interaction of ASL18a with NF-Y stimulated lateral root formation and ectopic cell division.

(A) Pull-down assay in vitro. (B) Lateral root densities of wild-type (MG-20) roots constitutively expressing ASL18a, NF-YA1, and NF-YB1 ($n = 30$ roots). Different letters represent classes with significant difference ($P < 0.05$, one-way ANOVA with Tukey's post hoc test). (C to E) Bumps formed in MG-20 (C) and *nin-9* (D) roots coexpressing ASL18a with both NF-Y subunits. (E) A longitudinal section of (C). (F to I) Empty vector-transformed *daphne* roots (F) or a construct to constitutively express ASL18a with either NF-Y subunits [(G) and (I)] or NF-YA1 (H) were inoculated with *M. loti* (4 weeks). [(H) and (I)] Sections of infected nodules stained with toluidine blue. Arrows in (G) and (I) indicate infected nodules and infection threads, respectively. Scale bars: 0.2 mm for (C) to (E), 2 mm for (F) and (G), 0.1 mm for (H) and (I).

over controls, which was compatible with the protein interactions. Furthermore, roots ectopically expressing ASL18a and NF-YA1 generated bumps (fig. S14 and table S1). Likewise, triple overexpression showed bumps in both wild type and *nin-9* (Fig. 4, C to E). Thus, ASL18a stimulated cell division in collaboration with NF-Y subunits. This effect was not specific to legumes (fig. S15). However, it did not increase nodule numbers (fig. S14). To examine whether ectopic cell division is associated with symbiotic events, we expressed ASL18a and NF-Y subunits in *daphne* mutants, in which a chromosomal translocation upstream of NIN diminishes its expression in root cortex, thereby the mutant roots host infection threads in root epidermis but do not produce nodule primordia (18). Expression

of ASL18a with NF-Y subunits led to the appearance of infected nodules on *daphne* roots (Fig. 4, F and G, and table S1). Infection threads penetrated into nodules formed in *daphne* roots, and rhizobia were released into host cells when ASL18a was expressed with NF-YA1 (Fig. 4H). Although the efficiency of production of infected nodules was higher in roots coexpressing ASL18a and both NF-Y subunits, inhibition of rhizobial release suggested that a correct expression pattern is required for endosymbiosis (Fig. 4I).

The evolutionary origin of root nodules has been previously discussed (19, 20). Here, we show that a gene involved in lateral root development is co-opted for nodule organogenesis downstream of NIN. Replacement of NIN function by ASL18a in collaboration with

NF-Y suggested the recruitment of ASL18a in organogenesis. An organogenesis-regulating molecular network has evolved through the interplay between the nodulation-specific and lateral root developmental pathways. Our findings thus clarify how legumes acquired the ability to produce root nodules.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S15
Tables S1 to S3
References (21–30)

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A shared gene drives lateral root development and root nodule symbiosis pathways in *Lotus*

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Common gene yields different structures

Nodules form on legume roots to house symbiotic, nitrogen-fixing bacteria. Lateral roots, characteristic of a much broader range of plants, extend to take up nutrients and water from the soil. Soyano *et al.* found common ground in the developmental pathways that build nodules and lateral roots (see the Perspective by Bishopp and Bennett). Evidence from *Lotus japonicus*, a legume that can fix atmospheric nitrogen, shows that the nodule-forming pathway shares components with the lateral root pathway.

Science, this issue p. 1021; see also p. 953

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