

***Light sensitive short hypocotyl (LSH) genes confer symbiotic nodule identity in the legume
Medicago truncatula***

Tak Lee^{1,2,8,10}, Martina Orvosova^{1,2,10}, Morgane Batzenschlager³, Marcelo Bueno Batista⁴, Paul C. Bailey⁵, Nadia A. Mohd-Radzman¹, Aram Gurzadyan¹, Naomi Stuer¹, Kirankumar S. Mysore^{6,7}, Jiangqi Wen^{6,7}, Thomas Ott^{3,9}, Giles E. D. Oldroyd^{1,2,12} and Katharina Schiessl^{1,11,12}

¹⁰Authors contributed equally to this work.

¹¹Lead contact

¹²Correspondence to: katharina.schiessl@slcu.cam.ac.uk, gedo2@cam.ac.uk

¹Sainsbury Laboratory, University of Cambridge, 47 Bateman Street, Cambridge CB2 1LR, UK

²Crop Science Centre, Department of Plant Sciences, University of Cambridge, 93 Lawrence Weaver Road, Cambridge CB3 0LE, UK

³University of Freiburg, Faculty of Biology, Schänzlestrasse, 79104 Freiburg, Germany

⁴Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

⁵Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond TW9 3DS, UK

⁶previous address: Noble Research Institute, LLC., 2510 Sam Noble Parkway, Ardmore, OK 73401, USA

⁷current address: Oklahoma State University, Stillwater, OK 74078, USA

⁸current address: Max Planck Institute for Plant Breeding Research, Carl-von-Linne-Weg, 1050829 Cologne, Germany

⁹CIBSS – Centre of Integrative Biological Signalling Studies, University of Freiburg, Schänzlestrasse, 79104 Freiburg, Germany

Summary

Legumes produce specialized root nodules that are distinct from lateral roots in morphology and function, with nodules intracellularly hosting nitrogen-fixing bacteria. We have previously shown that a lateral root program underpins nodule initiation, but there must be additional developmental regulators that confer nodule identity. Here, we show two members of the *LIGHT SENSITIVE SHORT HYPOCOTYL (LSH)* transcription factor family, predominantly known to define shoot meristem complexity and organ boundaries, function as regulators of nodule organ identity. In parallel to the root initiation program, *LSH1/LSH2* recruit a program into the root cortex that mediates the divergence into nodules, in particular with cell divisions in the mid cortex. This includes regulation of auxin and cytokinin, promotion of *NODULE ROOT1/2* and *Nuclear Factor Y-A1* and suppression of the lateral root program. A principal outcome of *LSH1/LSH2* function is the production of cells able to accommodate nitrogen-fixing bacteria, a key feature unique to nodules.

Keywords: nitrogen, endosymbiosis, rhizobia, nodule organogenesis, lateral organ development, legumes, *NODULE INCEPTION*, *LIGHT SENSITIVE SHORT HYPOCOTYL*, *Medicago truncatula*,

Introduction

To overcome nitrogen (N) deficiencies in the soil, legumes have evolved the ability to enter symbioses with beneficial N-fixing rhizobial bacteria. Legumes accommodate rhizobia inside cells of specialized root organs called nodules, that provide a favorable environment for N-fixation.

Initiation and progression of bacterial infection and nodule development are controlled by and dependent on the function of the nodulation-specific transcriptional regulator *NODULE INCEPTION (NIN)*, which is activated downstream of the symbiosis signaling pathway.¹⁻³ Epidermal infection and nodule organogenesis are genetically separable processes as evidenced by studies of different *nin* loss of function alleles and studies of the *cis* regulatory elements in the *NIN* promoter.²⁻⁷ To initiate the development of symbiotic root nodules in the inner tissue layers, *NIN* expression is activated via *CYTOKININ RESPONSE 1 (CRE1)*-mediated cytokinin signaling.^{7,8} Our previous work showed that cytokinin-induced *NIN* recruits a conserved program associated with lateral root development, to initiate the formation of a symbiotic root nodule, through the transcriptional regulator *LATERAL ORGAN BOUNDARIES 16 (LBD16)*, which promotes cell proliferation in the inner tissue layers via the upregulation of *STYLISH-like (STY-I)* transcriptional regulators and *YUCCA (YUC)* auxin biosynthesis genes.^{9,10} Consequently, nodules and lateral roots initiate from the same inner tissue layers of the primary root including the pericycle, the endodermis and the inner cortex in response to the local accumulation of auxin.^{9,11}

The commonalities between lateral root and nodule initiation imply that additional nodule-specific regulatory pathways must be recruited to confer nodule organ identity, in order to differentiate nodules from lateral roots in both morphology and function. While lateral root primordia predominantly develop from cells that are derived from the inner cell layers, the development of nodule primordia is in addition associated with the promotion of cell proliferation in the mid cortex.⁹⁻¹³ These cortical-derived primordial cells are key determinants in the

establishment of nodule organ identity as they give rise to the cells that are intracellularly colonized by rhizobial bacteria, released from infection threads.¹²

Several regulators have been implicated in the promotion of cortical cell divisions downstream of cytokinin-induced *NIN* expression. These include the transcriptional regulator *NUCLEAR FACTOR Y* (*NF-YAI*) and *NODULE ROOT1/2* (*NOOT1/NOOT2*), two BTB-ankyrin transcriptional co-activators orthologous to the *Arabidopsis* genes *BLADE-ON-PETIOLE 1/2* (*BOPI/2*).^{12,14-17} *NF-YAI* functions in cortical infection thread progression, nodule initiation, and the subsequent establishment and maintenance of the nodule meristem, which provides cells for continued rhizobial infection in the indeterminate nodule of *M. truncatula*.^{10,12,17,18} *NF-YAI* acts in part through the upregulation of *STY* transcription factors and *YUC* auxin biosynthesis genes.^{19,20} *NOOT1/2* appear to suppress the root-like initiation program induced by *NIN* via *LBD16* during nodule differentiation, with the *noot1/noot2* loss of function mutant developing root-like conversions from nodules.^{9,14,15} While nodule development is compromised in *nf-yai* and *noot1/noot2*, nodules still initiate in these mutants, that can at least in part support rhizobial colonization and N-fixation, suggesting that additional regulators of nodule organ identity must act either upstream, redundantly or in parallel with these known regulators. In this study, we demonstrate two members of the *LIGHT SENSITIVE SHORT HYPOCOTYL* (*LSH*) transcription factor family, that previously have been shown to predominantly control shoot meristem function, also control nodule organ identity, downstream of *NIN*. These genes are required to provide the unique organ identity of nodules, allowing intracellular bacterial colonization and N-fixation. Through the transcriptional control of and together with the known nodule organ identity regulators *NF-YAI* and *NOOT1/NOOT2*, *LSH1/LSH2* directly and indirectly recruit a growth-regulatory and differentiation program, highlighting the importance of the auxin-cytokinin

crosstalk and the pleiotropic function of shoot- and nodule-expressed meristem and growth regulators in the specification of this symbiotically-induced root organ.

RESULTS

***LSH1* and *LSH2* are upregulated during early nodule organogenesis downstream of *NIN*.**

To define potential regulators of nodule organ identity we screened a pre-existing comparative RNA-Seq time course of nodule and lateral root development, for transcription factors induced during the early stages of nodule development, with nodule-specific expression, in a manner dependent on *NIN*.^{9,21} This selection criteria identified *NF-YA1*, its interacting subunit *NF-YB16* and *NOOT1/NOOT2*^{15,22,23}, as well as two transcriptional regulators with yet uncharacterized functions in rhizobial symbiosis (Figure 1A). These novel regulators share an ALOG domain of identical amino acid sequence, with high sequence similarity to *LIGHT SENSITIVE SHORT HYPOCOTYL* (*LSH*) transcription factor 3 in *Arabidopsis* (*AtLSH3*) and with the recently characterized *SYMMETRIC PETALS 1* gene in *Pisum sativum* (*PsSYP1*) (Figure S1A).²⁴⁻²⁶ We named these two genes *LSH1* and *LSH2*. *LSH1* and *LSH2* are upregulated in roots from 16 hrs and 24 hrs post rhizobial spot inoculation (hpi), respectively. By contrast, neither *LSH1* nor *LSH2* were differentially expressed during the initiation and early development of lateral roots, suggesting that *LSH1* and *LSH2* may be part of a developmental program that distinguishes nodules from lateral roots (Figure 1A).⁹ The expression of *LSH1* and *LSH2* during rhizobial infection was dependent on *CRE1* and *NIN*; ectopic expression of *NIN* was sufficient to upregulate both genes (Figure 1A)^{9,21} and cytokinin treatment of *M. truncatula* roots induced *LSH1* in a *CRE1*- and *NIN*-dependent, but *NF-YA1*-independent manner (Figure 1B). Furthermore, a high confidence *NIN* DNA-binding site (found in three out of three biological replicates), 5,260 bp upstream of the

transcription start site (TSS) of *LSH1*, was identified by Chromatin-Immunoprecipitation (ChIP) in roots expressing *pLjUBI:GFP-NIN*, suggesting that *LSH1*, but not *LSH2*, is a direct target of NIN regulation (Figures S1B-C, Data S2).

To investigate their spatial expression patterns we performed mRNA *in situ* hybridization using anti-sense probes for *LSH1* and *LSH2* on wildtype root tissue 72 hpi, alongside *LSH* promoter-GUS analysis. Together, these approaches revealed that both genes are specifically expressed in dividing and newly divided cells of the nodule primordia, with no expression being observed in the overlaying outer cortical and epidermal cell layers that are traversed by infection threads (Figures 1C-D). In mature nodules, *LSH1* and *LSH2* are strongly expressed in the apical meristem region, in the infection and fixation zones and in the peripheral nodule vasculature (Figures 1C-D and S1D). These expression patterns for *LSH1* and *LSH2* are consistent with their cluster enrichment in recently obtained single cell RNA-sequencing of 14-day old nodules.²⁷ Consistent with their expression patterns in proliferating cells in nodule primordia and meristems, *LSH1* and *LSH2* expression was also observed in the proliferation zone of root meristems (Figure S1E). The observed gene expression patterns for *LSH1* and *LSH2* in early and mature nodules strongly overlapped with their protein localization patterns, assessed using GFP reporters translationally fused to the N-terminus of *LSH1* and *LSH2* genomic sequences and expressed from their endogenous promoter and terminator sequences (Figure 1E).

***LSH1/LSH2* have conserved floral development functions and negatively regulate lateral root emergence.**

To assess the roles of *LSH1* and *LSH2*, we identified loss of function mutants in *LSH1* (*lsh1-1*, *lsh1-2*) and *LSH2* (*lsh2-1*) and generated an *lsh1-1/lsh2-1* double mutant (Figure S2A). *lsh1*, but

not *lsh2*, showed significantly altered shoot organ morphologies including changes in petal shape and number, and a reduction in stipule complexity (Figures 2A-B and S2B-C, Table S1), revealing conserved floral functions across angiosperms.^{26,28-30}

No effect for *LSH* has been reported in root system architecture, and we found no function for *LSH1* or *LSH2* in the promotion of lateral root development. Rather, we observed a slight increase in the number of lateral roots in *lsh1* and *lsh1/lsh2* (Figure S2D), suggesting that in *M. truncatula* these genes may negatively regulate lateral root initiation. This was further corroborated by our observation that ectopic expression of either *LSH1* or *LSH2*, or both combined, resulted in a severe reduction in the number of emerged and elongated lateral roots (Figures 2C and S2E), with the majority of lateral root primordia in *LSH1*-overexpressing roots stalling/aborting at stage VI-VII¹¹ (Figures 2C and S2F-G and Table S2). Together, these results show that *LSH1/LSH2* are negative regulators of lateral root development and positive regulators of floral organ development.

***LSH1/LSH2* are required for nodule development and N-fixation**

For assessment of functions during nodulation, we inoculated plants with *Sinorhizobium meliloti* strain 2011 expressing *pNifH-GUS*, a bacterial promoter associated with the expression and activity of nitrogenase and used to approximate biological N-fixation in nodules.³¹ *lsh1* mutants showed a high frequency of white nodules, while the *lsh1/lsh2* double mutant developed almost exclusively white nodules, suggesting that N-fixation was severely attenuated (Figures 2D-F and S2H-I). The reduction and absence of N-fixation was confirmed in an acetylene reduction assay (Figure S2J). At 28 dpi *lsh1/lsh2* (Figure S2K) showed higher nodule frequency, a phenomenon frequently observed in *fix⁻* mutants.³¹ Nodule morphology was significantly altered in *lsh1* and

lsh1/lsh2, with frequent multilobed and fused nodules (Figures 2D-F and S2H-I). Nodule sections revealed a severe reduction in the number of cells with intracellular bacterial colonization in *lsh1/lsh2* nodules, but no obvious defects were observed in bacteroid differentiation in the few cells that possessed intracellular colonization (Figure S3A).

***LSH* genes are required for the development of nodule primordia that can support bacterial colonization**

To further investigate the cause of this severe reduction in rhizobial colonization, we assessed the progression of rhizobial infection and early nodule primordium development. In wildtype, the progression of rhizobial infection threads through the epidermis and cortex was temporally and spatially coordinated with the development of the nodule primordium (Figures 3A-B and S6A-B). We observed nodule primordium initiation and early establishment of epidermal infection threads in *lsh1* and *lsh1/lsh2*, however, the progression of infection threads through the mid cortex and subsequent internal colonization of primordium cells was severely impaired (Figures 3A-B and S6A-B). This resulted in a high proportion of *lsh1* and *lsh1/lsh2* nodules that were partially or completely uncolonized.

A recent study highlighted the importance of reduced mitotic potential and the modulation of the G2/M cell cycle transition as a key context for successful transcellular passage of cortical cells by rhizobial infection threads overlaying the developing nodule primordium.³² We used the dual-color H3.1/H3.3 histone reporter to assess the cellular state of cortical cells during rhizobial infection. We observed that the histone H3.1 eviction and enlargement of nuclei, which coincides with cortical infection thread progression, was comparable between *lsh1/lsh2* and wildtype (Figure 3C). This demonstrates that cortical cells associated with infection progression develop normally

in *lsh1/lsh2* and is consistent with infection-associated gene expression also appearing relatively unaffected in *lsh1/lsh2* (discussed later), this coupled with the fact that *LSH1/LSH2* expression is restricted to the newly divided cells in the nodule primordium (Figure 1D-E), strongly implies that *LSH1/LSH2* are not directly required for the genetic program associated with infection thread initiation and progression. Rather, we propose that the limitations to infection thread progression in *lsh1/lsh2* are the result, directly or indirectly, of *LSH1/LSH2* function in early nodule morphogenesis and subsequent host cell fate acquisition and maintenance.

In support of this hypothesis, while we found few differences between WT and *lsh1/lsh2* at 24 hours (Figure S3B), when early cell division is initiated by *LBD16*⁹, we observed a severe reduction in cell cycle activity in *lsh1/lsh2* primordia at 72 hpi compared to WT: cells in the mid cortex of WT primordia had undergone periclinal divisions generating cell layers that form the interface for the first intracellular bacterial colonization of the nodule primordium, whereas these cortical-derived cell layers were severely reduced or absent in *lsh1/lsh2* (Figures 3D and S3C). Concomitant to this reduction in cell cycle activity in the cortex, we observed continued cell cycle activity at the nodule initiation site in the inner tissue layers of the *lsh1/lsh2* mutant, suggesting that control of these cellular processes is at least in part independent and genetically separable from the function of *LSH1/LSH2*. Consequently, periclinal cell divisions originating from the inner tissue layers at the base of the nodule appeared to contribute substantially higher numbers of cells to the emerging *lsh1/lsh2* nodule in comparison to WT (Figures 3D and S3C-D), coincident with an increased number of cells of high nuclear H3.1 content (Figure S3D). The cellular origin of the structures that emerge in *lsh1/lsh2* are much more closely related to lateral roots than to nodules^{9,11,12}, and we propose it is this lack of appropriate mid-cortical cell division that leads to the severe reduction of bacterial infection.

***LSH1* and *LSH2* function as key transcriptional regulators in nodulation**

To understand how *LSH* function is driving this nodule-specific cell division and associated nodule identity, we made use of our extensive transcriptomic profile during early and late nodule development (Figure 4A).⁹ We performed RNA-Seq on rhizobial spot inoculated root sections of *lsh1* and *lsh1/lsh2* compared to wildtype at 24 and 72 hpi (Figures 3D and S3B-C). Surprisingly, this revealed that >90% of rhizobial-responsive genes in wildtype were not differentially expressed in the *lsh1/lsh2* mutant (Figure 4A), with many of these gene expression differences already present at 24 hpi. This severe reduction in the overall transcriptional response to rhizobial inoculation emphasizes the major regulatory contribution of *LSH1/LSH2* to nodulation.

To identify the putative direct LSH targets, we undertook ChIP-Seq on roots ectopically expressing a *GFP-LSH1* translational fusion (*pLjUBI:GFP-LSH1*; using *pLjUBI-NLS-GFP* as control). In addition, we characterized the transcriptional effects of combined *LSH1/LSH2* overexpression (*LSH1+LSH2* OE) under non-symbiotic conditions (Figures 2C, S2E-F and S4A). These analyses revealed significant LSH1-DNA binding sites for 3,714 genes that were previously identified to be differentially expressed in response to rhizobial infection in wildtype⁹, suggesting that 18% (3,714/20,719) of the total number of nodulation-associated DEGs are under direct control of LSH1 (Figure 4A). We clustered the time-resolved nodule-associated DEGs (20,719) based on their log₂ fold changes into 17 clusters and assessed the representation of the 2,552 LSH1-ChIP-Seq and *LSH1/LSH2* OE-responsive targets (Figure 4A, Data S1). Putative direct *LSH*-responsive targets were equally represented in clusters of up- and downregulated genes, suggesting that *LSH* can act as both activators and repressors. High representation ($\geq$ the average of 18%) of LSH1-ChIP-Seq and *LSH1/LSH2* OE-responsive targets were found in the clusters of genes that

were induced during the 16-72 hpi-timepoints at the stages of cell proliferation and onset of differentiation , further corroborating a direct regulatory function during nodule organogenesis for *LSH1/LSH2*. By contrast, representation was lower ($\leq 12\%$) in clusters of genes that peaked in their differential expression very early or very late, indicating that *LSH1/LSH2* play a less significant role in early establishment of rhizobial infection and nodule initiation or in the maintenance of N-fixation (Figure 4A, Data S2). Consistently, marker genes for symbiosis signaling and early infection, such as *EARLY NODULIN 11 (ENOD11)* and *Rhizobium-directed Polar Growth (RPG)* were not directly targeted by *LSH1/LSH2* and still expressed in *lsh1/lsh2*. In contrast, genes associated with infection progression and N-fixation such as *VAPYRIN (VPY)* and *LEGHEMOGLOBINs (LB1/LB2)* were indirectly affected in the *lsh1/lsh2* mutant (Figure S4B).

The 3,714 nodulation-associated LSH1-ChIP-Seq targets were clustered by their log2-fold expression changes upon rhizobial spot-inoculation. The Gene ontology (GO) enrichment analysis on the clusters revealed an enrichment of GO terms related to cell division and shoot development, including auxin and cytokinin biosynthesis and signaling, in upregulated genes and response to biotic and abiotic stresses in downregulated genes (Figure S4C; Data S2). This included several key components of the auxin-cytokinin cross-talk such as *STYLISH-like (STY-I)* transcription factors promoting local auxin biosynthesis in nodulation, *PIN-FORMED 1 (PIN1)* and *PINOID (PID)* genes, as well as genes involved in the biosynthesis, catabolism, and transport of cytokinin including *ISOPENTENYL TRANSFERASE 1 (IPT1)*, members of the *LONELY GUY (LOG)*, *CYTOKININ OXIDASE (CKX)* gene families, the G subfamily of *ATP BINDING CASSETTE* transporters (*ABCG*), and cytokinin signaling genes such as *CRE1* and the response regulators *RR2* and *RR8* (Figures 4C-D).^{20,33-37} Cell cycle regulators were also directly controlled by LSH1, including D-type cyclin *CYCD3;1* and mitotic B-type cyclin *CYCB1;2*, while the

endoreduplication regulator *CSS52B* was indirectly dependent on *LSH1/LSH2*.^{38,39} Consistent with the suppression of lateral root development, we found the *PLETHORA* root meristem regulators (*PLT1* and *PLT2*)⁴⁰⁻⁴² to be under direct repression by *LSH1/LSH2* (Figure 4E).

Several LSH1-ChIP-Seq targets and *LSH1/LSH2*-responsive genes have previously been annotated to function as meristem and growth regulators in the shoot, highlighting the pleiotropic function of *LSH* transcription factors. This includes the *Medicago* orthologues of the transcriptional co-activators *BLADE-ON-PETIOLE*, *MtNOOT1/MtNOOT2*, two well characterized nodule organ identity genes.^{14,15,43}

***LSH1/LSH2* directly regulate *NOOT1/NOOT2* that function in the same pathway during nodule organogenesis**

NOOT1/NOOT2 are important regulators of nodule identity: in their absence nodules initiate, but revert to a lateral root identity, suggesting that *NOOTs* are key maintainers of the nodulation program.^{14,15} Our observations that *NOOTs* are direct targets of *LSH1/LSH2* suggest that the control of these transcription factors may be a key feature of *LSH* functionality. Promoter-GUS analysis of *pNOOT1:GUS-tNOOT1* and *pNOOT2:GUS-tNOOT2* in wildtype and *lsh1/lsh2*, revealed that *NOOT* gene expression in nodule primordia was *LSH*-dependent, although still present at the base of the developing *lsh1/lsh2* nodule (Figure 5A). Expression profiling of spot inoculated root sections of *noot1/noot2* revealed that loss of *NOOT1/NOOT2* affects a comparatively smaller subset of the rhizobial-induced genes than the loss of *LSH1/LSH2* ($\leq 60\%$ of rhizobial-responsive genes in the wildtype at 24 hpi and 72 hpi (Figures 4A and 5B). Interestingly, there was a complete overlap ($\geq 99.9\%$) between the genes that were affected in their response to rhizobial spot inoculation in the *noot1/noot2* and in the *lsh1/lsh2* mutants and a large

overlap between the expression profiles of the OE lines, suggesting that the effect on gene expression caused by gain and loss of *NOOT1/NOOT2* is embedded in the *LSH1/LSH2* function (Figures 5C and S5B). Gain or loss of *NOOT1/NOOT2* has no effect on *LSH1* or *LSH2* expression (Figures S5A), suggesting that *NOOT1/NOOT2* function downstream of *LSH1/LSH2*.

Comparing the early nodule developmental phenotypes of *lsh1/lsh2* and *noot1/noot2* revealed clear differences in the functions of these genes: whereas *lsh1/lsh2* show reductions in the periclinal cell divisions of the mid cortex, *noot1/noot2* primordia showed cell cycle activities comparable or greater than wildtype (Figures 3C and 6A-B) and wild-type rhizobial infection at these early time points, as previously reported.¹⁵ However, at later stages of nodule development, *noot1/noot2* showed similar defects to *lsh1/lsh2*, resulting in a large proportion of partially or completely uncolonized nodules (Figures 3A-B and S6A-B).

An *lsh1/noot1* double mutant recapitulated the phenotype of the *lsh1/lsh2* double mutant (Figures 6A-B and S6A-C), highlighting the genetic interactions between these two classes of genes. The phenotypic resemblance between the *lsh1/noot1* and *lsh1/lsh2* mutants was present also at early timepoints, with infection defects and reductions in periclinal cell divisions in the mid cortex of the root in both *lsh1/noot1* and *lsh1/lsh2* mutants (Figures 3D, 6C and S6A-B). A striking aspect of the *noot* mutant phenotype is the emergence of lateral roots from the tips of nodules.^{14,15} We observed this phenotype in *lsh1/lsh2*, but at a lower frequency to that observed in *noot1/noot2* (Figures 6A-B and S6C), consistent with the loss of repression of the root meristem genes *PLT1/PLT2* in both *lsh1/lsh2* and *noot1/noot2* (Figures 4E and S5A). Despite the genetic interactions between *NOOT* and *LSH*, the ectopic expression of *NOOT1/NOOT2* was not sufficient to rescue *lsh1/lsh2* ($n > 5$). Studies in other plant species have shown direct protein interactions between *LSH* and *BLADE-ON-PETIOLE (BOP)* genes, the orthologs of *NOOT1/NOOT2*^{26,44},

however, we have been unable to find any evidence for direct interactions between LSH1/LSH2 and NOOT1/NOOT2, using the BiFC assay in *M. truncatula* roots. We conclude that *LSH1/LSH2* directly control the expression of *NOOT1/NOOT2* in the nodule primordium, that in part explains the later developmental functions of *LSH*, but not the early specification of mid cortex cell divisions, with *NOOTs* maintaining the nodule identity preset by *LSH1/LSH2*.

LSH1/LSH2* partly functions through the cortical activation of *NF-YAI

In addition to *NOOTs*, *NF-YAI* has also been shown to function in nodule development^{10,12,18-20} and while we did not find *NF-YAI* to be a direct target of LSH1, we observed a partial dependence on *LSH1/LSH2* for appropriate *NF-YAI* expression during nodulation and activation of *NF-YAI* as a function of *LSH1/LSH2* OE (Figure 4B). Comparing *lsh1/lsh2* and *nf-ya1* mutant phenotypes, we found many similarities including an increased ratio of aborted cortical infection threads and a reduction in cell divisions and cell layers in the nodule primordium (Figures 2D-F, 3A-D, S7A-G). Consistently, comparison of rhizobial-responsive DEGs in *lsh1/lsh2* and *nf-ya1* and of *LSH1* and *NF-YAI* OE-responsive DEGs (Figures 7C and S7H-I, Data S1) revealed convergences in their gene regulation in genes associated with local biosynthesis, transport and conjugation of auxin and in cytokinin metabolism and signaling.^{19,20}

Promoter-GUS analysis of *pNF-YAI:GUS-tNF-YAI* showed expression in the inner tissue layers at the base of developing nodules and in nodule primordia (Figure 7A), with *NF-YAI* expression in the cortex-derived cells of the nodule primordium being *LSH1/LSH2*-dependent (Figures 7A). The reduced cortical expression of *NF-YAI* might at least in part explain the nodulation defects in *lsh1/lsh2*. To test this, we ectopically expressed *NF-YAI* under the constitutive *LjUBI* promoter or under both the *pLSH1* and *pLSH2* promoters in *lsh1/lsh2* roots.

Both modes of *NF-YAI* expression resulted in a partial rescue of *lsh1/lsh2*, leading to 25% of nodules with functional N-fixation, based on *pNifH-GUS* (Figure 7B, Table S4), revealing that *NF-YAI* regulation by *LSH* is a key aspect of *LSH* functionality, leading to N-fixing nodules.

NF-YAI is one of the earliest induced transcription factors in response to rhizobial recognition and plays a role in the establishment of bacterial infection^{9,10,45}. This is an important differentiator between *LSH* and *NF-YAI* functionality and consistently genes involved in early stages of rhizobial infection such as *RPG* were affected and responsive to loss and/or gain of *NF-YAI*, but not to *LSH1/LSH2* (Figure 7C, Data S1). While *NF-YAI* expression is in part controlled by *LSH1/LSH2*, we saw no evidence for the converse (Figures 1B and 7C), revealing that *NF-YAI* functions downstream of *LSH1/LSH2*.

DISCUSSION

The initiation of symbiotic root nodules and lateral roots converges on the upregulation of the transcription factor *LBD16*, that allows the initiation of cell divisions in the inner root tissues.^{9,10} Despite these similarities during the initiation stages, the development of nodules and lateral roots diverges, with nodules differentiating into organs that can intracellularly accommodate rhizobial bacteria and provide the environment for biological N-fixation. Here, we show that nodule-specific development is to a large extent directly orchestrated by *LSH1/LSH2*, two members of the *LSH* transcription factor family with previously reported pleiotropic functions in shoot development.^{26,29,44} Like *LBD16*, *LSH1/LSH2* are controlled by *NIN* downstream of cytokinin signaling, suggesting parallel activation of two conserved programs. *LBD16* and *LSH1/LSH2* appear to have spatially and temporally separable roles: *LBD16* allows the initial activation of cell divisions in the inner tissue layers, with the resultant divisions and developmental outcomes being

closely related to the initiation of lateral roots. In contrast, *LSH1/LSH2* control a very specific set of cell divisions with distinct identity and fate trajectory in the mid cortex of the root, that are unique to nodules and are directly associated with the creation of cells able to accommodate N-fixing bacteria.¹² *LSH1/LSH2* promote the cortex-specific expression of the previously identified nodule regulators *NOOT1/NOOT2* and *NF-YAI*, which converge and synergize in their regulatory functions on the modulation, amplification and maintenance of the auxin-cytokinin crosstalk, a key feature and differentiator between lateral root and nodule development.^{15,20}

Legumes appear unique in their ability to respond to cytokinin with the promotion of mitotic cell divisions in the root and this ability is the function of NIN promotion of *LBD16*, that upregulates local auxin biosynthesis.^{7,9,46} Cross talk between polar auxin transport and cytokinin signaling has previously been shown to determine cell division patterns and the onset of cell differentiation in the transition zone of the root and during lateral root development.⁴⁷⁻⁵¹ Equally, the maintenance of cytokinin homeostasis and cytokinin signaling have been shown to play vital roles in promoting nodule organogenesis and endosymbiotic host cell specification in legumes.^{8,33,38,52,53} Critically, the impacts of cytokinin are opposing in lateral root and nodule development.^{8,46,54} We demonstrate that the promotion of cytokinin signaling during nodulation is sustained by *LSH1/LSH2* via direct and indirect (via *NOOTs* and *NF-YAI*) mechanisms, providing a means for divergence between lateral roots and symbiotic nodules. The direct and indirect (via *NOOTs*) transcriptional repression of the lateral root program during nodulation, via repression of the root meristem regulators *PLT1/PLT2*, provides another means to modulate the auxin/cytokinin balance.^{41,42,51} Such differentiation in development is critical, since lateral roots and nodules initiate comparably⁹⁻¹², and only later diverge in their development.

The severe nodulation defects caused by loss of *LSH1/LSH2* can be partially explained by *LSH1/LSH2* regulation of *NF-YA1*. Previously, *NF-YA1* has been characterized as a direct downstream target of *NIN* and shown to be involved in nodule initiation and the progression of bacterial infection.^{10,23,45} Based on our results, we propose that *LSH1/LSH2* promotes *NF-YA1* expression specifically in the newly divided cells derived from the mid-cortical cell layers associated with the specification of the nodule primordium. Our data points towards an indirect regulation by *LSH1/LSH2*, perhaps via promotion of cytokinin and *NIN* in the cortex, subsequent and in parallel to its reported direct upregulation by *NIN* at the base of the developing nodule.^{10,55} Recent findings that implicate *NF-YA1* in infection competence via post-replicative cell cycle regulation at the G2/M phase transition and promotion of endoreduplication, provide an interesting mechanism by which our observed partial rescue of the *lsh1/lsh2* mutant by ectopic *NF-YA1* expression may be explained³², since endosymbiotic host cell colonization is facilitated by the switch from mitotic cell proliferation to endoreduplication.^{32,38,39,56}

A key feature of nodules is their ability to intracellularly accommodate N-fixing bacteria. However, the detailed regulatory mechanisms remain unclear. In the shoot, *LSH* genes have been shown to regulate the activity of meristems and the growth of lateral organs with a proposed function in promoting the appropriate amount of undifferentiated cells that can serve as a canvas for the differentiation of complex organs with intricate features and functions.^{26,29,30} This is exemplified by their role in the development of indeterminate compound inflorescences^{29,30} and the development of stipules with complex serrations, as demonstrated here. We propose that these previously described shoot functions of *LSH* and its co-recruited shoot-expressed regulatory subnetwork are of relevance during nodule development, where early cell patterning processes need to be spatially and temporally coordinated with the arrival of the colonizing bacteria. Perhaps

it is the maintenance of cells in an undifferentiated state, through the promotion of *LSH1/LSH2* expression, that allows their extensive colonization by bacteria, or alternatively perhaps it is the pausing of cellular differentiation to align with the timing of bacterial infection that is so critical. Further work is clearly required to understand the process of bacterial infection and accommodation in nodules: the transcriptional network controlled by *LSH1/LSH2* provides an excellent launchpad for these studies.

Here we propose a model for specific nodule development: cytokinin activation of *NIN* expression initiates the nodule primordium through induction of *LBD16*, which activates local auxin biosynthesis to drive cell divisions in a manner comparable to lateral root initiation. In parallel *NIN* activates the expression of *LSH1/LSH2*, to promote cell divisions specifically in the mid cortex and control and maintain nodule organ identity, in part through the transcriptional promotion of *NOOT1/NOOT2* and *NF-YA1*, as well as further promoting auxin-cytokinin levels and directly suppressing the lateral root developmental program. *LSH1/LSH2* drive cell divisions that provide cells able to accommodate N-fixing bacteria, possibly through a combination of promoting endoreduplication and maintaining the cells in an undifferentiated state, at least until the arrival of the infection threads.

While nodules are unique structures associated with symbiotic bacterial N-fixation, we have yet to see any evidence for de-novo gene evolution associated with the emergence of nodulation. Rather we find evidence for the re-networking of pre-existing developmental pathways, facilitating the emergence of this novel form of root development. The neo-functionalization of the nodule-specific transcription factor *NIN* and the associated evolution of cis-regulatory DNA binding sites in the promoter regions of its downstream targets, led to the recruitment of a lateral root organ initiation program into the symbiotic interaction with rhizobial

bacteria.^{9,10,13,57} Similarly, we hypothesize that further neofunctionalization of *NIN* provided the opportunity to recruit a growth regulatory network with pleiotropic functions in the shoot into the symbiotic root context, thereby promoting the expansion and diversification of the regulatory function of *LSH1/LSH2* and their associated downstream regulatory subnetworks into nodule development. This notion is in line with the common principle of morphological evolution as proposed by Carroll⁵⁸ in which changes in the spatial and temporal gene expression of pre-existing developmental regulators and their associated downstream networks, lead to trait divergence and the diversification of novel organ forms and functions. The parallel recruitment of a root initiation program and primordium identity program from the shoot that dictate nodule form and function are essential in non-legume species that are targets for engineering N-fixation.

ACKNOWLEDGEMENTS

We thank Raymond Wightman, Gareth Evans, Kim Findlay, and Elaine Barclay for support with microscopy, Anne Edwards for help with the acetylene reduction assay and Mandana Miri and Colleen Drapek for critical comments on the manuscript. We thank Pascal Ratet for providing *noot1/noot2* mutant seeds. K.S. thanks her orthopedic surgeon Dr. Erik Teumann, Eva Schiessl and the families of Ingrid and Franz Trimmel and of Bernadette Gerstl for their care and support. This work was supported by the Bill and Melinda Gates Foundation and the UK Foreign, Commonwealth and Development Office (OPP1172165) through Enabling Nutrient Symbioses in Agriculture (ENSA) project and the Gatsby Foundation (GAT3395/GLH).

AUTHOR CONTRIBUTIONS: K.S. designed and performed the experiments and analyzed the data with assistance from M.O., N.S, N.A.M.R and A.G.; T.L. analyzed RNA and ChIP sequencing

(RNA-Seq) data; M.B performed and T.O supervised dual histone reporter experiments; P.C.B. performed the phylogenetic analysis; M.B.B. provided resources and performed the acetylene reduction assay; K.S.M. and J.W. provided *Medicago* mutants; K.S. and G.E.D.O supervised the work and wrote the manuscript.

DECLARATION OF INTEREST: The authors declare no competing interests for this work.

Related to this work, we submitted a Patent Cooperation Treaty (PCT) Application (PCT/IB2023/055144) on May, 18th 2023 under the title “PROTEINS FOR REGULATION OF SYMBIOTIC NODULE ORGAN IDENTITY”.

FIGURES and FIGURE LEGENDS

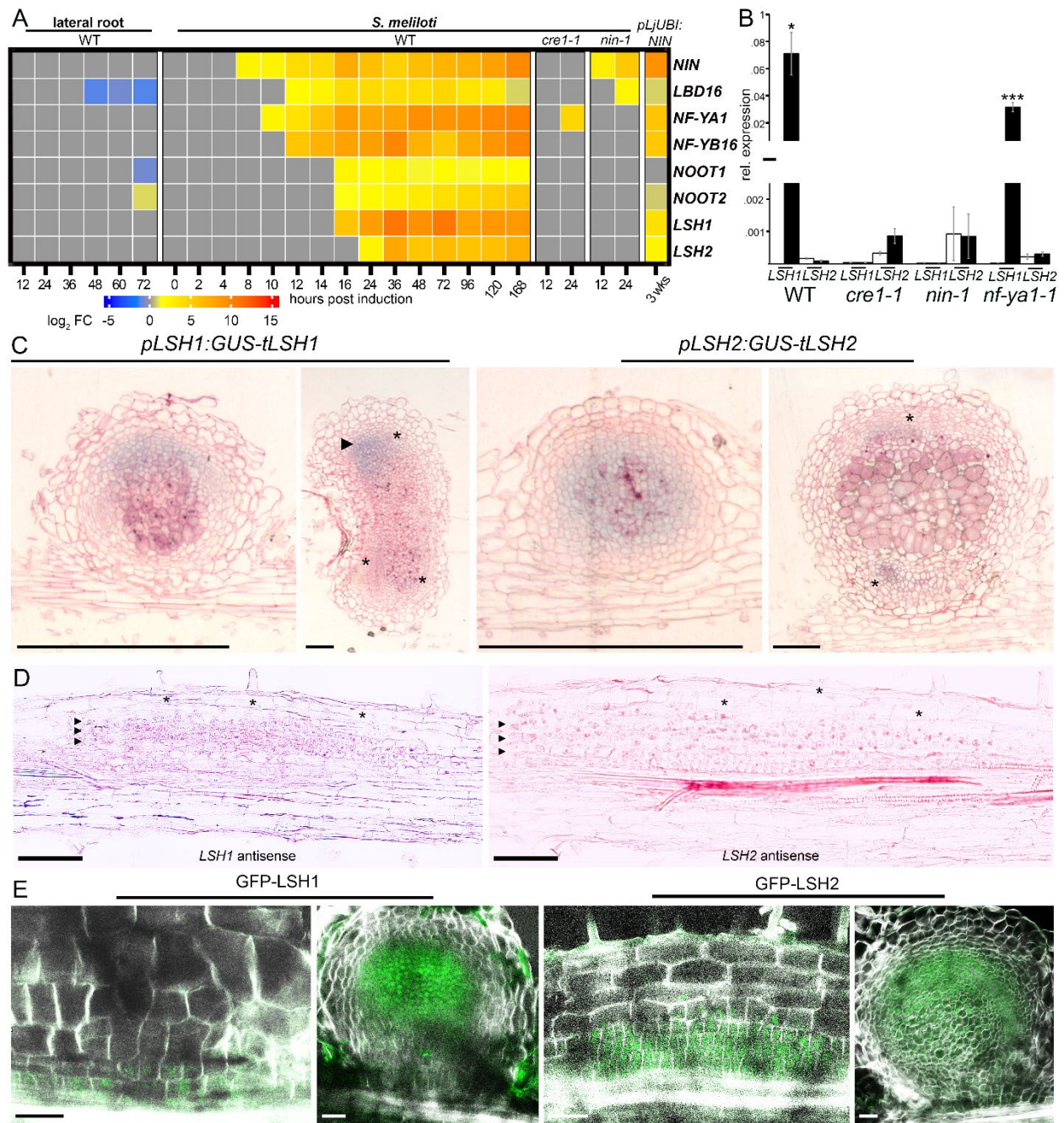


Figure 1: *LSH1* and *LSH2* are upregulated during early nodule organogenesis downstream of *NIN*. (A) Heatmap shows selected genes induced during lateral root and nodule development. Fold changes compared to controls are depicted in log₂ scale $\geq \pm 0.585$ (raw fold change of 1.5), p-value < 0.05. (B) Expression profiling on root segments treated with 100 nM 6-Benzylaminopurine (BAP) for 24 h by qRT-PCR normalized to *HH3*. Statistical comparisons between mock (white bars) and BAP (black bars). Values are the mean ΔC_t values of 3 biological replicates for *LSH1* and 2 for *LSH2* $\pm$ SEM (Student's t-test; asterisks indicate statistical significance *, P < 0.05; **, P < 0.01, ***, P < 0.001). (C) Promoter activity of *LSH1* and *LSH2* visualized by GUS (blue) (also see Figure S1D-E). Rhizobial expressed *LacZ* is stained magenta. Ruthenium Red demarks cell walls. Asterisks indicate expression in vascular bundles and arrows in the meristem. Scale bars: 500 μ m. (D) mRNA *in situ* hybridization on sections of WT roots 72 hours post rhizobial spot inoculation with *LSH1* or *LSH2* antisense probes; arrows indicate *LSH1/LSH2* expression in dividing and newly divided nodule primordium cells and asterisks indicate the lack of expression in the outer cortical and epidermal cell layers overlaying the nodule primordium. Scale bars: 100 μ m. (E) Optical sections of nodule primordia and mature nodules on hairy roots expressing *pLSH1:GFP-LSH1-t-LSH1* or *pLSH2:GFP-LSH2-t-LSH2* (in green) fluorescent brightener demarking cell walls (in white) and induced by spray inoculation with *Sm2011-mCherry* bacteria at 10 days post spray inoculation. Scale bars: 50 μ m.

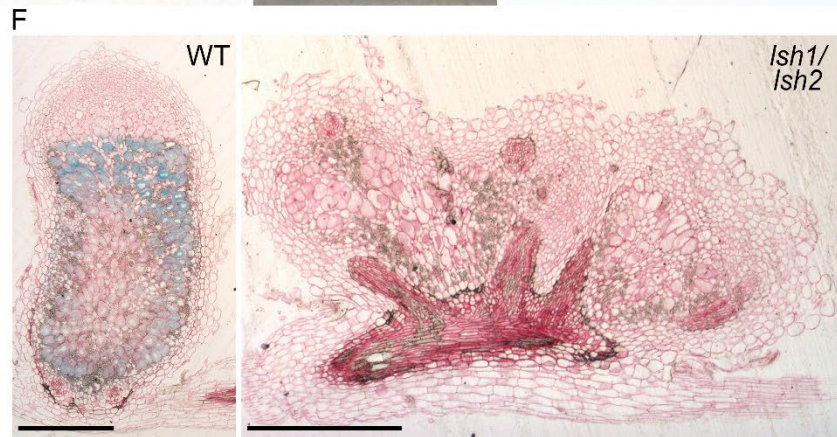
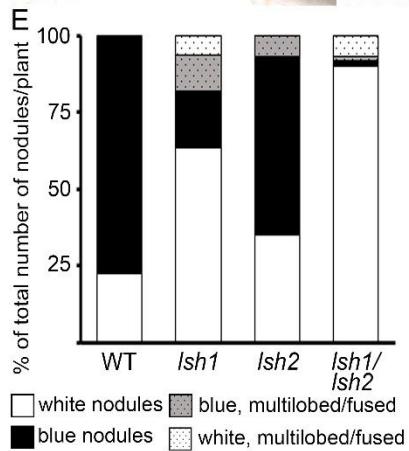
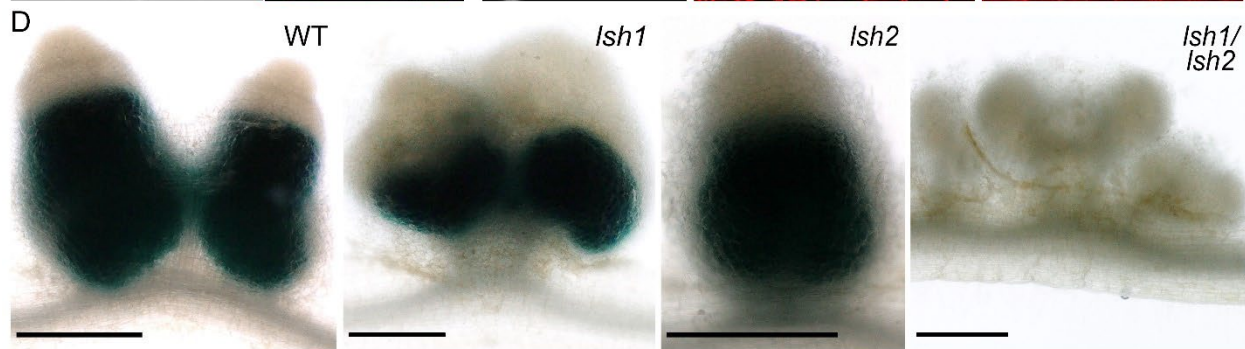
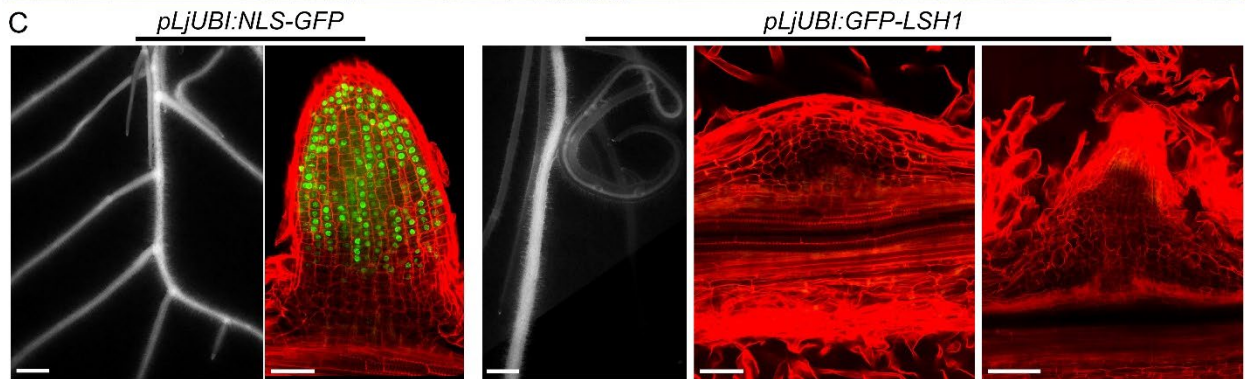
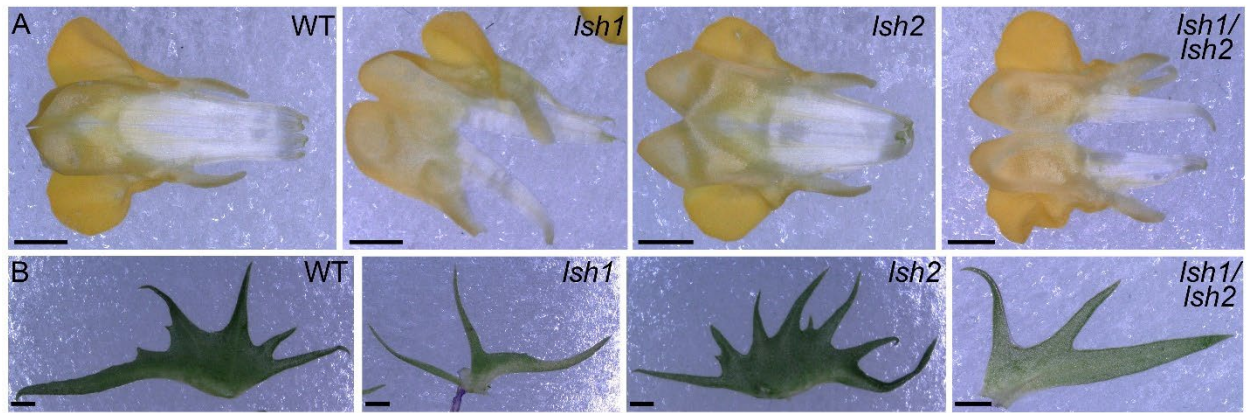


Figure 2: *LSH1/LSH2* are required for nodule development and N-fixation.

(A-B) Images of WT, *lsh1-2*, *lsh2-1* and *lsh1-1/lsh2-1* dissected flower keels (A) and stipules (B), also see Figures S2B and C, Table S1). Scale bars: 500 μ m. (C) Whole mount images of hairy roots expressing *pLjUBI:NLS-GFP* (control) and *pLjUBI:GFP-LSH1*. Epifluorescence images: scalebars: 1 mm; Confocal images: EdU-labelled nuclei indicating DNA replication in green and propidium iodide-stained cell walls in red demarking cell geometry. Scale bars: 50 μ m. (also see Figures S2E-G and Table S2). (D) Whole mount images of WT, *lsh1-1*, *lsh2-1* and *lsh1-1/lsh2-1* nodules 28 days post *S. meliloti* inoculation. GUS staining (blue) indicates the expression of the bacterial *pNifH* promoter. Scale bars: 500 μ m. (E) Distribution of different nodule morphologies depicted as percentage of total nodule number per plant in WT and *lsh1-1* (n = 15), *lsh2-1* (n = 14), and *lsh1-1/lsh2-1* (n = 13) (also see Figures S2H-K). (F) Sections of 28-d old nodules in WT, *lsh1-1* and *lsh1-1/lsh2-1*, *pNifH:GUS* expression (blue), Ruthenium Red demarks cell walls. Scale bars: 500 μ m.

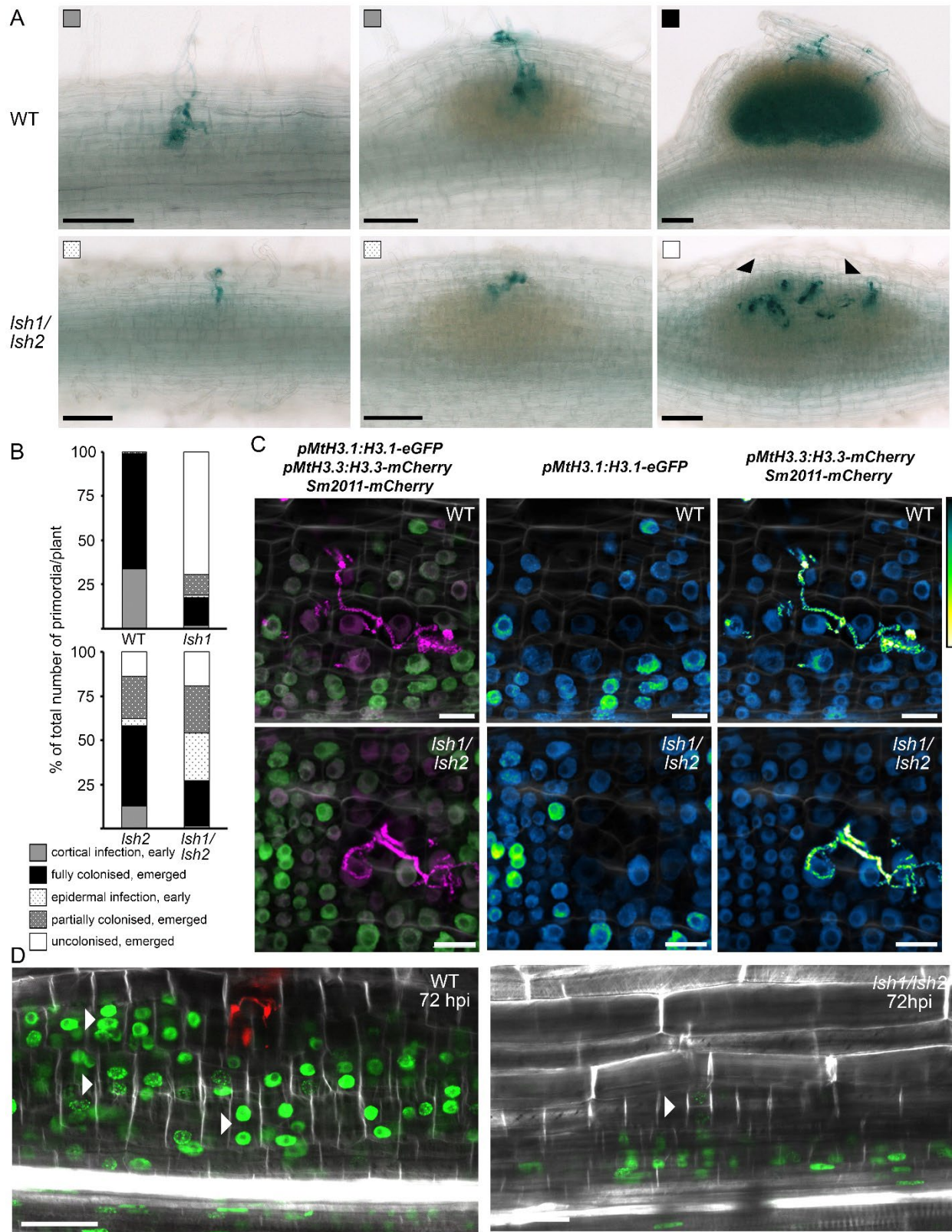


Figure 3. *LSH* genes are required for the development of nodule primordia that can support bacterial colonization. (A) Images of WT and *lsh1/lsh2* nodule primordia at different developmental stages, first initial divisions (left), multilayered (middle) and emerged primordia (right) observed 7 d post spray inoculation with rhizobial bacteria expressing *LacZ* (blue stain). Black arrowheads indicate infection threads that are restricted in their progression into the inner root tissue layers. Squares relate to the legend in Figure 3B. Scale bars: 500 μ m. (B) Distribution of bacterial colonization phenotypes observed in WT, *lsh1*, *lsh2* and *lsh1/lsh2* at 7 dpi depicted as percentage of the total primordia number per plant, WT (n = 30), *lsh1*, *lsh2* (n = 33), *lsh1/lsh2* (n = 32). (C) Confocal images of whole-mount transgenic roots co-expressing *H3.1-eGFP* (green) and *H3.3-mCherry* (magenta) in WT (n = 7 independent hairy root systems) and *lsh1/lsh2* (n = 10 independent hairy root systems) inoculated with *Sm2011-mCherry* (magenta) at 6 dpi (left). Images are maximum intensity projections (eGFP/mCherry signal either depicted in green/magenta (left panel) or in the Green Fire Blue colour range, blue to yellow indicating min to max fluorescence levels (middle and right panel) and calcofluor demarking cell walls (white). Scale bars: 20 μ m. (D) Optical sections of WT and *lsh1/lsh2* roots 24 and 72 h post spot-inoculation with *S. meliloti* (n > 30 per genotype and timepoint), also see Figures S3B-C and Table S3. At 72 hpi, *Sm2011-mCherry* bacteria in red, cell walls in white (fluorescent brightener) and EdU-labelled nuclei indicating DNA replication in green. White arrowheads indicate periclinal cell divisions. Scale bars: 50 μ m.

Figure 4.

***LSH1* and *LSH2* function as key transcriptional regulators in nodulation.**

(A) Heatmaps of all differentially expressed genes (DEGs) in response to *S. meliloti* spot inoculation in WT clustered according to their expression patterns into 17 clusters⁹, aligned with heatmaps of DEGs in WT (*R108*), *lsh1* and *lsh1/lsh2* at 24 and 72 hpi, and in response to combined ectopic expression of *LSH1/LSH2* (*pLjUBI:LSH1+LSH2*) compared to empty vector in 3-week old WT hairy roots. Expression levels are depicted as log₂ fold changes $\geq \pm 0.585$ (raw fold change of 1.5), p-value < 0.05. Absolute numbers indicate the number of DEGs in each genotype. Dark green, light green and grey colouring in the far right column depicts confidence levels of LSH1 DNA-binding sites associated with DEGs detected in $\geq 3/4$, $2/4$ and $\leq 1/4$ biological replicates comparing pull downs of GFP bound DNA fragments in *pLjUBI:GFP-LSH1* compared to *pLjUBI:NLS-GFP*, respectively. (B-E) Heatmaps showing expression levels of selected functional groups of DEGs in WT, *lsh1* and *lsh1/lsh2* root sections at 24 and 72 hpi and in response to combined ectopic expression of *LSH1/LSH2* (*pLjUBI:LSH1+LSH2*) compared to empty vector in 3-week old WT hairy roots, alongside LSH1 DNA-binding sites (far right column) according to their confidence levels. Fold changes compared to controls are depicted in log₂ scale $\geq \pm 0.585$ (raw fold change of 1.5), p-value < 0.05. Related to Figures S4A-C, 5B, S5A and 7C. Also see Data S1 and S2. (F) Representative ChIP-Seq peaks in 4 biological replicates comparing pull downs of GFP bound DNA fragments in *pLjUBI:GFP-LSH1* compared to *pLjUBI:NLS-GFP* visualized in the *MtrunAI7r5.0-ANR* Genome Browser.⁵⁹ Blue peaks denote regions where the immunoprecipitated samples show an increased number of mapped reads compared to the control samples and the red peaks denote the opposite. The purple rectangles indicate high confidence DNA-binding sites (found in $\geq 3/4$ biological replicates).

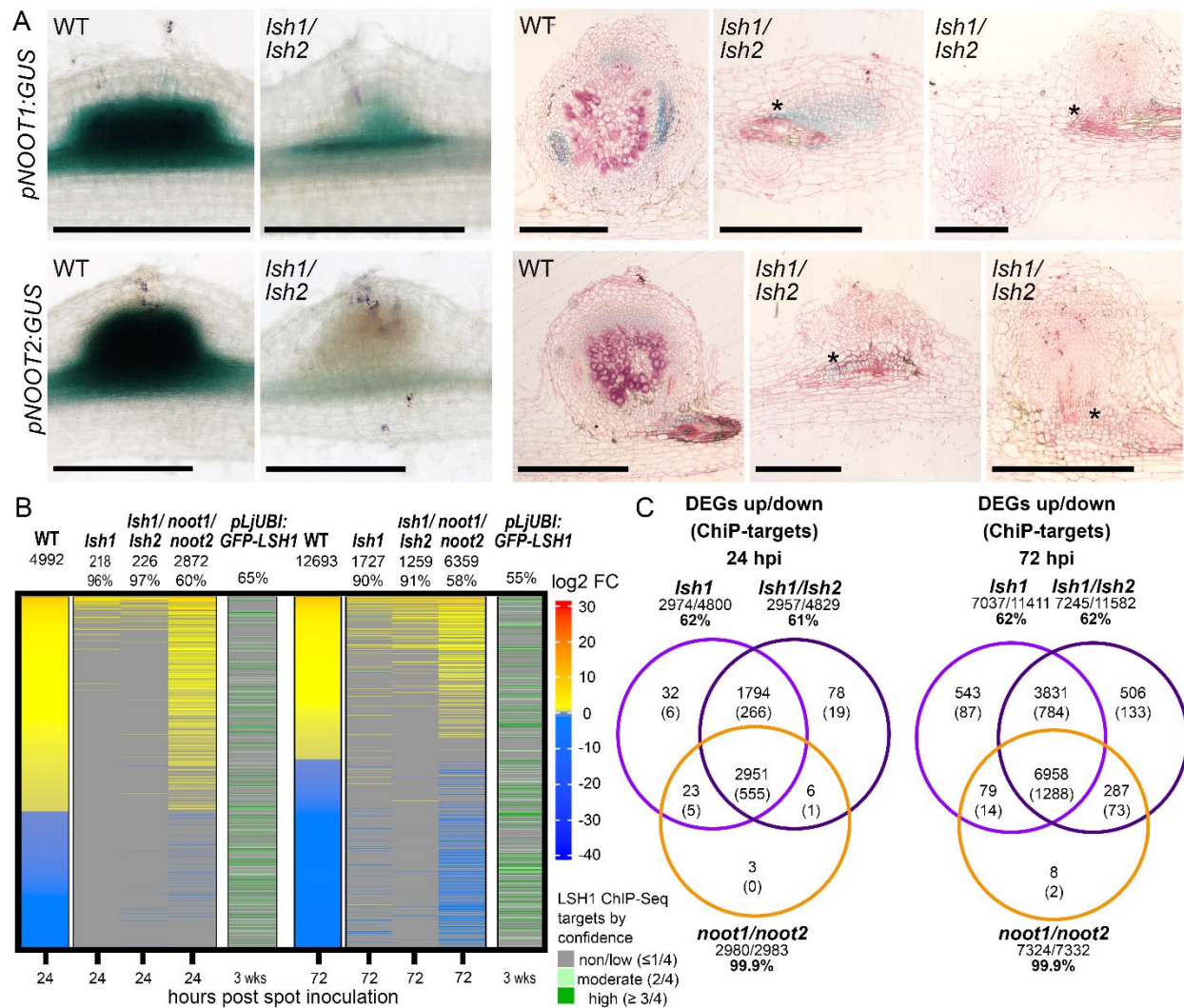


Figure 5. *LSH1/LSH2* promote the expression of and act together with *NOOT1/NOOT2*. (A) Expression patterns of *NOOT1* and *NOOT2* in WT and *lsh1/lsh2* nodules, visualized by GUS staining (blue) in whole mount images (left) and nodular sections (right). Rhizobial expressed *LacZ* is stained magenta. Ruthenium Red demarks cell walls in sections. Black asterisks indicate vascular expression at the nodule base. Scale bars: 500 μ m. (B) Related to Figures 4A and S5A-B, also see Data S1 and S2. Heatmaps of all DEGs in WT, *lsh1*, *lsh1/lsh2* and *noot1/noot2* at 24 and 72 hpi. Expression levels are depicted as log₂ fold changes $\geq \pm 0.585$ (raw fold change of

1.5), p-value < 0.05. LSH1 DNA-binding sites associated with rhizobial-responsive DEGs are depicted in dark green: high confidence; light green: moderate confidence; or grey: low/no confidence. To compare the overall transcriptional response to *S. meliloti* spot inoculation between WT and the mutants, we sorted all DEGs from the highest positive to the highest negative log₂ fold change values. Absolute numbers indicate responsive DEGs, percentages indicate the proportion of DEGs in WT that are not differentially expressed in the mutants and therefore affected by loss of *LSH1*, *LSH1/LSH2* and *NOOT1/NOOT2*. (C) Comparisons of all DEGs affected in *lsh1* (light purple), *lsh1/lsh2* (dark purple) and *noot1/noot2* (orange) up and down regulated at 24 hpi and 72 hpi. Numbers in brackets refer to high and moderate LSH1 ChIP-Seq targets. Genes with log₂ fold changes of $\geq \pm 0.585$ (raw fold change of 1.5), p-value < 0.05 were included in this analysis.

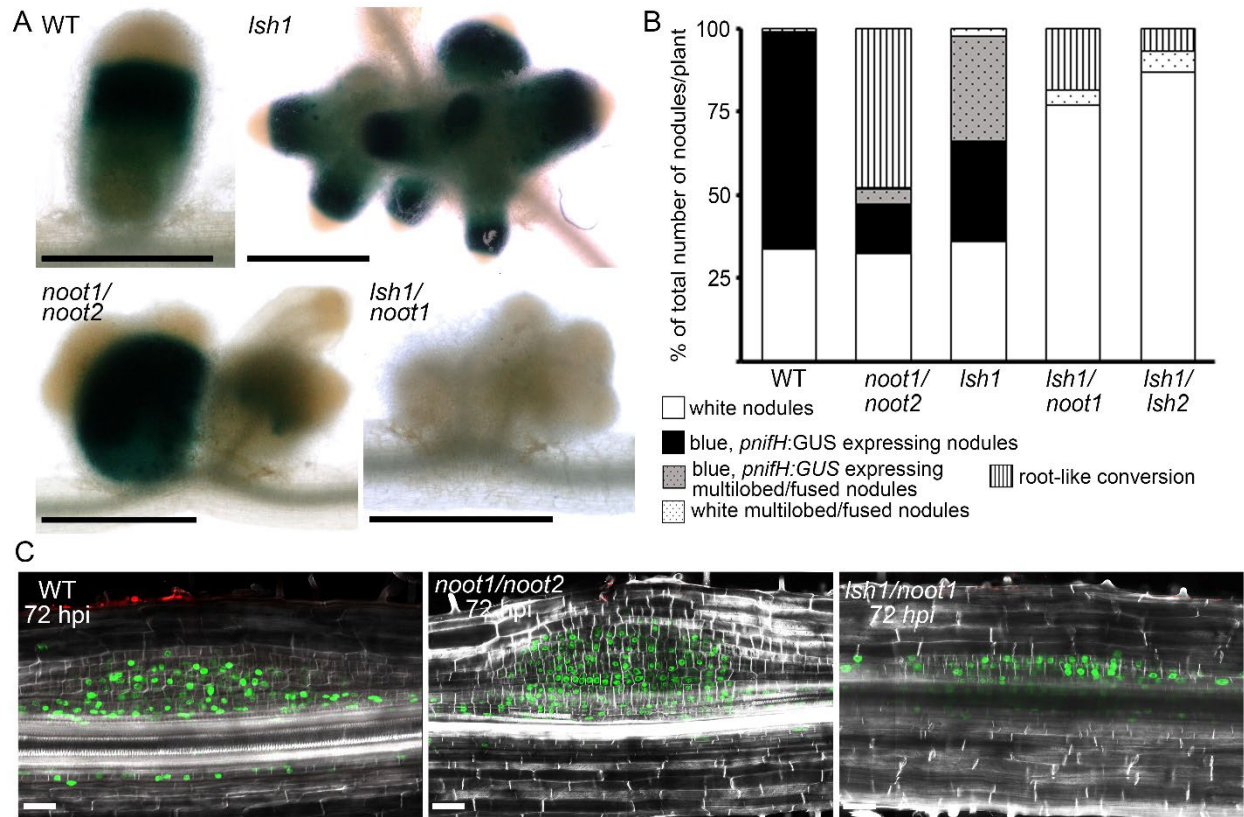


Figure 6. *LSH1/LSH2* and *NOOT1/NOOT2* function synergistically to confer nodule organ identity. (A) Whole mount images of WT, *lsh1*, *noot1/noot2* and *lsh1/noot1* nodules at 21 days post *S. meliloti* inoculation. GUS staining (blue) indicates the expression of the bacterial *pNifH*. Scale bars: 500 μ m. (B) Distribution of different nodule morphologies and N-fixation (*pnifH*:GUS staining) at 21 dpi depicted as percentage of the total nodule number per plant, WT (n = 37), *noot1/noot2* (n = 44), *lsh1* (n = 33), *lsh1/noot1* (n = 35), *lsh1/lsh2* (n = 40) (also see Figure S6C). (C) Optical sections of WT, *noot1/noot2* and *lsh1/noot1* root sections 72 h post rhizobial spot-inoculation (n > 15 per genotype). *Sm2011-mCherry* bacteria in red, cell walls in white (fluorescent brightener) and EdU-labelled nuclei indicating DNA replication in green. Scale bars: 50 μ m. For comparison to *lsh1/lsh2* also see Figures 3D and S3C and also see Table S3).

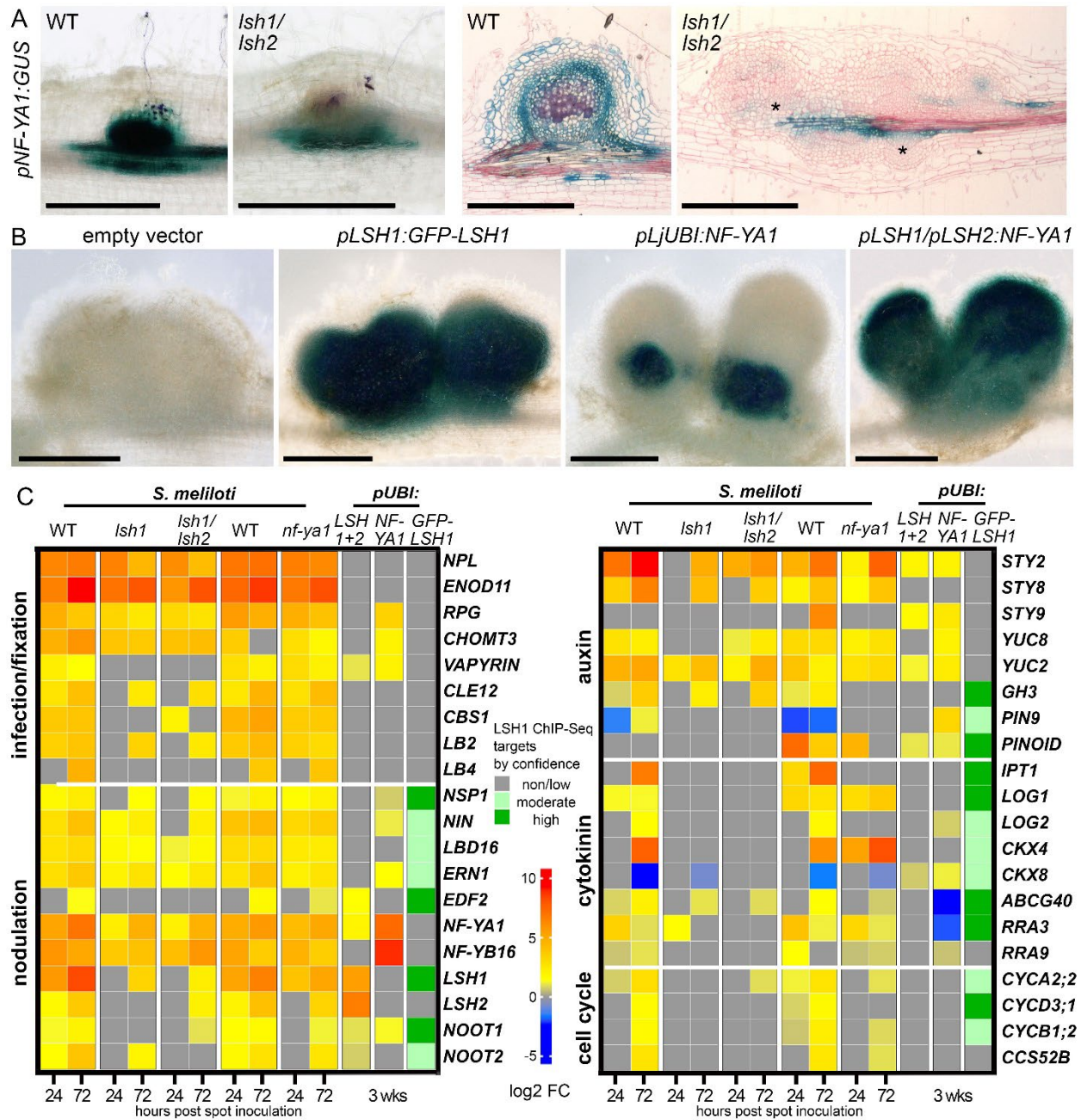


Figure 7. *LSH1/LSH2* partly function through the cortical activation of *NF-YA1*. (A) Expression pattern of *NF-YA1* in WT and *lsh1/lsh2* visualized by GUS staining (blue) in whole mount images (left) and nodule sections (right). Rhizobial expressed *LacZ* is stained magenta. Ruthenium Red demarks cell walls in sections. Black asterisks indicate vascular expression restricted at the nodule base. Scale bars: 500 μ m. (B) Whole mount images of nodules on hairy roots of *lsh1/lsh2* plants transformed with empty vector control, *pLSH1:LSH1*, *pLjUBI:NF-YA1*, and combined *pLSH1:NF-YA1/pLSH2:NF-YA1* at 28 dpi with *S. meliloti* expressing *pNifH:GUS*. GUS staining (blue) indicates the expression of the bacterial *pNifH* promoter (also see Table S4). Scale bars: 500 μ m. (C) Heatmap of selected functional groups of DEGs in WT, *lsh1*, *lsh1/lsh2* and *nf-ya1* at 24 and 72 hours post *S. meliloti* spot inoculation and in response to combined ectopic expression of *LSH1/LSH2* (*pLjUBI:LSH1/LSH2*) or *NF-YA1* (*pLjUBI:NF-YA1*) compared to empty vector control in 3-week old WT hairy roots under non-symbiotic conditions. Fold changes compared to controls are depicted in log₂ scale $\geq \pm 0.585$ (raw fold change of 1.5), p-value < 0.05. LSH1 DNA-binding sites associated with DEGs are depicted in dark green: high confidence; light green: moderate confidence or grey: low/no confidence. Also see Data S1 and S2.

STAR METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contacts, Giles Oldroyd (gedo2@cam.ac.uk) and Katharina Schiessl (ks846@cam.ac.uk), subject to material transfer agreements. This study did not generate unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material and growth conditions

M. truncatula ecotypes *jemalong*, cultivar Jester, and ecotype *R108* were used as wildtype in this study, dependent on the ecotype of the respective mutant. *Jemalong* was used to perform hairy root transformations and as wildtype for comparisons to *cre-1*, *nin-1*, and *nfy-a1-1*, previously described.^{2,60} All *Tnt1* retrotransposon insertion lines described in this manuscript (*NF17203 (lsh1-1)*, *NF1304 (lsh1-2)* and *NF14992 (lsh2-1)*) were derivatives of the *R108* ecotype and obtained from the *Tnt1* Retrotransposon Mutant Collection (Oklahoma State University, Stillwater, USA; previously Noble Research Institute, LLC., Ardmore USA).⁶¹ Previously described *NF2717 (noot1-1)*, *NF5464 (noot2-1)* were used.¹⁵ *R108* was used as the wildtype for analysis of these *Tnt1* mutants. Genotyping was performed using *Tnt1-F* and *Tnt1-R* oligos combined with the corresponding forward and reverse oligos encompassing the insertions (Table S5).

Seeds were scarified, surface sterilized with 10% (v/v) bleach solution, stratified for 3 days at 4 °C and germinated on water agar plates. Plants were grown in sterile conditions in controlled environment rooms at 22 °C (80% humidity, 16 h light/8 h dark, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity) on filter paper-lined agar media in sealed plates unless otherwise specified. For Chromatin-Immunoprecipitation and assessment of the root phenotype in the over-expression lines, hairy roots were grown on modified Fahraeus medium supplemented with 0.5 mM NH_4NO_3 ⁶² for 3 weeks.

For rhizobial spot inoculation seedlings were grown for 2 days on buffered nodulation medium (BNM)⁶³ supplemented with 1 μM aminoethoxyvinylglycine (AVG; Sigma-Aldrich Company Ltd, Darmstadt, Germany) at 22 °C (16 hours light/8 hours dark, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity). *Sinorhizobium meliloti* strain 2011⁶⁴ was grown in minimal medium supplemented with

3 μ M luteolin (Sigma-Aldrich Company Ltd, Darmstadt, Germany) and diluted to a final concentration of 0.02 OD 600 nm using Fahraeus medium⁶². The mock treatment consisted of Fahraeus medium with luteolin diluted to an equivalent concentration as the inoculum. Approximately 1 μ L of *S. meliloti* suspension or mock treatment was inoculated onto the susceptibility zone (where the root hairs first appear) and marked by puncturing the filter paper alongside the site of inoculation. After 24 and 72 hours, 2 mm sections of the root alongside the site of inoculation were harvested for RNA isolation or microscopy.

For spray inoculation of *S. meliloti* for plants grown on plates, seedlings were grown under similar conditions as described above. Roots of 1-day-old seedlings were covered with filter paper and sprayed with 2 ml *S. meliloti* of final concentration 0.02 OD 600 nm grown in minimal medium without luteolin. For inoculation of hairy roots and for phenotyping assays of mature nodules, plants were transferred to terragreen:sharp sand mix (1:1) (Oil-DriCompany, Wisbech, UK) in P40 trays and left to grow for 7 days before inoculation with *S. meliloti* 2011 (2 mL of overnight culture per plant diluted in liquid BNM to 0.02 OD 600 nm). Plants were grown for up to a further 4 weeks for nodule quantification and histochemical staining.

Bacterial strains

Sinorhizobium meliloti 2011 expressing *pXLGD4* (*hemA::lacZ*), *pNifH::GUS*, or *mCherry* were used in this study.^{31,64} *Agrobacterium rhizogenes* strain *AR1193* was used to introduce all binary vectors used in this study (*DsRed* as transformation marker) to *M. truncatula jemalong* seedlings following a previously published transient hairy root transformation protocol.⁶²

METHOD DETAILS

Construct production

The Golden Gate modular cloning system was used to prepare the plasmids.⁶⁵ All Level 0s used in this study are held for distribution in the ENSA project core collection (<https://www.ensa.ac.uk/>) and are listed along with the binary plasmid details in Table S1A. Sequences were domesticated, synthesized and cloned into pMS (GeneArt, Thermo Fisher Scientific, Waltham, USA). Sequence information for *LSH1* (*Medtr1g069825*), *LSH2* (*Medtr7g097030*), *NF-YA1* (*Medtr1g056530*), *NOOT1* (*Medtr7g090020*) and *NOOT2* (*Medtr1g051025*) were obtained from the *M. truncatula* Mt4.0v1 genome via Phytozome (<https://phytozome.jgi.doe.gov>).^{66,67}

Hormone and chemical treatments

6-Benzylaminopurine (BAP; Sigma-Aldrich Company Ltd, Darmstadt, Germany) were dissolved in water. Mock treatments were equal volumes of each solvent in the agar media. For BAP plate treatments (100 nM), 2-day old seedlings were grown on BNM plates for 24 hours with either BAP or mock and supplemented with 1 μ M AVG to replicate spot inoculation conditions.

Gene expression analysis

For rhizobial spot inoculation time-course experiments, roots were dissected as 2- to 3-mm segments around the spot of inoculation or mock treatment. For BAP response experiments, segments were dissected around the susceptibility zone marked at the time of treatment. About 50 to 60 segments were pooled to obtain 1 biological replicate, with 3-6 biological replicates per treatment/genotype were analyzed. RNA was extracted using the RNeasy Micro Kit (Qiagen, Germantown, USA) and the RNase free DNase kit (Qiagen, Germantown, USA) was used to remove genomic DNA. For reverse transcription of 1 μ g total RNA, Transcriptor First Strand

cDNA Synthesis Kit was used according to the manufacturer's instructions (Roche Diagnostics GmbH). Quantitative real-time polymerase chain reactions (qRT-PCR) were performed in technical triplicates in the LightCycler 480 System using LightCycler 480 SYBR green I master (04707516001, Roche Diagnostics GmbH, Mannheim, Germany) in a total reaction volume of 10 μ l. The primer pairs used for gene expression analysis are listed in Table S1A.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed using a combination of two protocols.^{68,69} Transiently transformed 3-week old *M. truncatula* hairy roots in the ecotype *jemalong* expressing *pLjUBI:GFP-LSH1*, *pLjUBI:GFP-NIN* or control *pLjUBI:NLS-GFP* grown under non-symbiotic conditions were used. Root tissue [2000–3000 mg (fresh weight) per sample] were fixed in 35 mL of fixation buffer [NaHPO₄ (100 mM) buffer, 1% formaldehyde, 100 μ M PMSF] under vacuum for 20 min on ice. Cross-linking was stopped with 100 μ M glycine for 10 min on ice. Tissue was washed twice with water, frozen in liquid nitrogen and stored at -80°C. Ground tissue was resuspended in 25 ml extraction buffer 1 (10 mM Tris-HCl pH 8, 10 mM MgCl₂, 0.4 M sucrose, 100 μ M PMSF, 10 mM β -mercaptoethanol, 1 tablet (per 50 ml of buffer) of protease inhibitor cocktail complete Mini, EDTA-free (11836170001, Roche)), filtered through miracloth and centrifuged at 1800 x g for 20 min at 4°C. The pellet was resuspended in 10 ml extraction buffer 2 (10 mM Tris-HCl pH 8, 10 mM MgCl₂, 0.24 M sucrose, 100 μ M PMSF, 10 mM β -mercaptoethanol, 1% Triton, 1 tablet (per 50 ml of buffer) of protease inhibitor cocktail complete Mini, EDTA-free (11836170001, Roche)) and centrifuged at 1800 x g for 10 min at 4°C. This washing step was repeated twice, followed by three washes with extraction buffer 3 (10 mM Tris-HCl pH 8, 10 mM MgCl₂, 1.70 M sucrose, 100 μ M PMSF, 10 mM β -mercaptoethanol, 0.15% Triton, 1 tablet (per 50 ml of buffer) of protease

inhibitor cocktail complete Mini, EDTA-free (11836170001, Roche)) as described in Cortijo et al. 2018. Purified nuclei pellets were resuspended in 1 mL of sonication buffer containing 500 mM Hepes, 150 mM NaCl, 5 mM MgCl₂, 10% (vol/vol) TRITON X-100, and one-half of a tablet of protease inhibitor mixture complete Mini, EDTA-free (Roche). Sonication was performed in a Bioruptor water bath sonicator at 4 °C (3 × 6 min high-power level with 30 s on/30 s off cycles), resulting in an average fragment size of 300-500 bp. After centrifugation, the supernatant was mixed with 500 µL of immunoprecipitation buffer containing 0.5 M Hepes, 150 mM NaCl, 5 mM MgCl₂, 10% (vol/vol) TRITON X-100, 1 mg/mL BSA, and 25 µL of anti-GFP µMACS Microbeads (Miltenyi Biotec); incubated on ice for 30 min; and loaded on a µ Column (Miltenyi Biotec) that had been equilibrated with 200 µL of immunoprecipitation buffer and placed into a magnetic µMACS separator (Miltenyi Biotec). After washing four times with 200 - 400 µL of immunoprecipitation buffer and twice with 200 µL of TE buffer [100 mM Tris (pH 8), 10 mM EDTA (pH 8)], DNA was eluted once with 20 µL and twice with 50 µL of preheated (96 °C) elution buffer containing 50 mM Tris (pH 8), 10 mM EDTA, 50 mM DTT, and 1% SDS. One hundred microliters of TE buffer and 9 µL of 25 mg/mL Proteinase K (Sigma) were added to the eluted samples and to the input control samples. Cross-linking was reverted in eluted and input samples at 37 °C overnight, followed by addition of 9 µL of 25 mg/mL Proteinase K and 8 h of incubation at 65 °C, phenol-chloroform extraction, and precipitation with ethanol overnight at -20 °C. After washing in 70% (vol/vol) EtOH, the air-dried DNA was resuspended in 100 µL of PCR-grade water (Roche), purified using a PCR purification Kit (catalog no. 18104; Qiagen), and stored at -80 °C.

Next generation sequencing for RNA and Chromatin-Immunoprecipitation

RNA sequencing (RNA-Seq) and sequencing following Chromatin-Immunoprecipitation sequencing (ChIP-Seq) were performed by Novogene Europe (Cambridge, UK). RNA-Seq and ChIP-Seq libraries were prepared with the Illumina TruSeq® Stranded mRNA HT kit and ChIP-Seq library kit, respectively. The sequencing of the libraries was performed on the Illumina NovaSeq 6000 next generation sequencing system with the read length of 150 bp paired-end reads resulting in 20 million reads and 15-30 million reads per sample for RNA-Seq and ChIP-Seq, respectively.

Assessment of shoot-related phenotypes

Flowers and stipules were dissected from the main shoot, arranged on double-sided tape and directly imaged using the Keyence VHX-5000 microscope equipped with a range of zoom lenses from 20x to 1000x (Keyence Ltd, Milton Keynes, UK).

Assessment of lateral root phenotypes

Seedlings were grown on modified Fahraeus medium⁶² plates for 14 days before lateral root number and length was scored.

Acetylene reduction assay

Nitrogenase activity was measured *in vivo* in mature nodules of terragreen:sand grown plants at 21 days post inoculation with *S. meliloti* 2011 by the acetylene reduction assay.^{70,71} Freshly harvested nodules were immediately transferred to BD Vacutainer tubes (3ml, 13x75mm, Thermo Fisher Scientific, Waltham, USA) prior to the injection of 10% (v/v) acetylene (C₂H₂). After 1 hour incubation, ethylene (C₂H₄) was quantified using a Perkin Elmer Clarus 480 gas

chromatograph equipped with a HayeSep® N (80-100 MESH) column. The injector and oven temperatures were kept at 100 °C, while the FID detector was set at 150 °C. The carrier gas (nitrogen) flow was set at 8 - 10 mL/min. An ethylene calibration curve was prepared from chemical decomposition of ethephon (Sigma C0143) in 10 mM Na₂HPO₄ pH 10.7 as described previously ⁷². Nitrogenase activity is reported as nmol of C₂H₄ per nodule per min.

Histochemical assays and cellular stains

For GUS staining roots were washed in water and immediately fixed in 90% acetone on ice for 1 hr. Subsequently, the acetone was replaced by a wash solution containing 50 mM phosphate buffer pH 7.2. The wash buffer was replaced by GUS staining buffer containing 50 mM phosphate buffer pH 7.2, 0.5 mM K₃Fe(CN)₆ (potassium ferricyanide), 0.5 mM K₄Fe(CN)₆ (potassium ferrocyanide) and 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (X-Gluc, Melford Laboratories Ltd., Ipswich, UK), vacuum infiltrated for 15 min and incubated at 37°C for 6 - 12 hrs. For X-Gal staining, the tissue was washed in 50 mM phosphate buffer pH 7.2 and fixed in 2.5 % glutaraldehyde by vacuum infiltration for 15 min and incubation at room temperature for 1 hr. Tissue was washed 3X in Z-buffer containing 100 mM phosphate buffer pH 7, 10 mM KCl, 1 mM MgCl₂ and incubated in X-Gal staining buffer (Z-buffer supplemented with 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ and 0.08% Magenta-5-Bromo-6-chloro-3-indolyl-B-D-galactopyranoside; (X-Gal, Melford Laboratories Ltd., Ipswich, UK) or 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (blue X-Gal, Sigma-Aldrich Company Ltd, Darmstadt Germany) at 28°C for 6-12 hrs and washed with water 3X. Subsequently, nodules and root tissue were dehydrated in an ethanol series and stored in 70% EtOH at 4°C. For imaging of whole mount tissue, stained samples

were mounted on glass slides in 70% EtOH and imaged using the Keyence VHX-5000 microscope equipped with a range of zoom lenses from 20x to 1000x (Keyence Ltd, Milton Keynes, UK).

Tissue Sections

Root segments and nodules were fixed with 4% formaldehyde (Sigma-Aldrich Company Ltd, Darmstadt Germany) in 1X PBS (10X PBS contains 1.37 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, and 18 mM KH₂PO₄) with vacuum infiltration for 15 min and at 4°C overnight. The fixed material was dehydrated in an ethanol series and subsequently embedded in Technovit 7100 (Kulzer Technik, Wehrheim, Germany) according to the manufacturer's protocol. Embedded tissue was sectioned (8-10 µm) using a Leica microtome (Leica, Milton Keynes, UK), mounted on glass slides, stained in 0.1% Ruthenium Red (Sigma-Aldrich Company Ltd, Darmstadt Germany) dissolved in distilled water for 15 min and rinsed. Sections were imaged using a Zeiss Axioimager.M2 light microscope (Carl Zeiss AG, Oberkochen, Germany).

Combined 5-ethynyl-2-deoxyuridine (EdU; Invitrogen, Thermo Fisher Scientific, Waltham, USA) and modified pseudo-Schiff-propidium iodide (PI; Sigma-Aldrich Company Ltd, Darmstadt, Germany) staining, clearing and imaging was performed as previously published.⁹ In addition, staining with 5-ethynyl-2-deoxyuridine (EdU; Invitrogen, Thermo Fisher Scientific, Waltham, USA) was combined with the cell wall stain Calcofluor white - Fluorescent Brightener 28 disodium salt solution (Sigma-Aldrich Company Ltd, Darmstadt, Germany). For this, roots were transferred to growth medium supplemented with 10 µM EdU and grown for 4 additional hours as previously described.⁹ Subsequently, 1 cm root sections centred around the susceptibility zone were dissected and fixed in 4% formaldehyde in 1X PBS with vacuum infiltration for 15 min and incubation for 1 hr at room temperature. After 3 washes in 1X PBS, the root sections were

incubated in solution containing 10 mM Alexa 488-azide (Invitrogen, Thermo Fisher Scientific, Waltham, USA) and 100 mM Tris pH 8.5 for 1 hr, followed by 30 min in solution containing 10 mM Alexa 488-azide, 100 mM Tris, 1 mM CuSO₄, 100 mM ascorbic acid, pH 8.5. The roots were subsequently washed in water 3X and transferred to ClearSee solution⁷³ for 24 – 72 hrs. For cell wall staining, root sections were transferred to ClearSee solution supplemented with 0.1% Fluorescent Brightener 28 disodium salt solution and incubated for 30 min.⁷³ For imaging root sections were mounted on glass slides in ClearSee solution and imaging was performed with a Zeiss 700 confocal scanning microscope using a 20X air lens objective with excitation and emission filters set to 405 nm/410–485 nm for Fluorescent Brightener 28 staining, at 488 nm/505–600 nm for EdU, and 488 nm/600–680 nm for rhizobial expressed *mCherry*, 488 nm/572–625 nm for propidium iodide (Carl Zeiss AG, Oberkochen, Germany). Images were processed using Zeiss software and FIJI⁷⁴. GFP-tagged LSH1/LSH2 proteins expressed in hairy roots were imaged using the ClearSee protocol and confocal microscope settings as described above. Imaging data of the dual histone reporter were obtained as previously described.³²

mRNA in situ hybridization

In situ hybridization was carried out as described previously.⁷⁵ For LSH1 and LSH2, near full length DNA fragments (400–600 bp) were amplified from GG plasmids containing the coding sequences synthesized using gene-specific forward and T7 tagged reverse primers (Supplemental Table S5). The anti-sense RNA probes were synthesized by in vitro transcription using the PCR products as templates with the DIG RNA Labeling Kit (Roche). The rhizobial spot inoculated root sections were fixed in FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) and embedded into paraffin wax in Leica ASP300 Tissue Processor. The samples were sectioned into 8µm slices.

After dewaxing, rehydration and dehydration, the sections were hybridized with gene-specific probes at 55 °C overnight. After 2h washes in SSC, the slices were incubated with an anti-digoxigenin-AP antibody (Roche) for 2h at room temperature. The hybridisation signals were detected by NBT/BCIP (Roche) colour reaction. Images were taken after 24h on a Leica SP8 microscope equipped with a HC PL APO CS2 20x/0.75 NA objective and Leica DFC7000T colour camera.

Transmission electron microscopy analysis

Mature nodules of plate grown plants at 14 days post inoculation with *S. meliloti* 2011 were cut from roots and immediately placed in a solution of 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate, pH 7.3 for fixation, and left overnight at room temperature. Samples were then placed in baskets and loaded into the Leica EM TP embedding machine (Leica, Milton Keynes, UK) using the following protocol. The fixative was washed out by three successive 15-minute washes in 0.05 M sodium cacodylate and post-fixed in 1% (w/v) OsO₄ in 0.05 M sodium cacodylate for two hours at room temperature. The osmium fixation was followed by three, 15-minute washes in distilled water before beginning the ethanol dehydration series (30%, 50%, 70%, 95% and two changes of 100% ethanol, each for an hour). Once dehydrated, the samples were gradually infiltrated with LR White resin (London Resin Company, Reading, Berkshire, UK) by successive changes of resin:ethanol mixes at room temperature (1:1 for 1hr, 2:1 for 1hr, 3:1 for 1hr, 100% resin for 1 hr then 100% resin for 16 hrs and a fresh change again for a further 8 hrs) then the samples were transferred into gelatin capsules full of fresh LR White and placed at 60°C for 16 hrs to polymerize. The material was sectioned with a diamond knife using a Leica UC6 ultramicrotome (Leica, Milton Keynes, UK) and ultrathin sections of approximately 90 nm were picked up onto 200 mesh copper grids which are formvar and carbon coated (EM Resolutions, Sheffield, England). The sections

were stained with 2% (w/v) uranyl acetate for 1hr and 1% (w/v) lead citrate for 1 minute, washed in distilled water and air dried. The grids were viewed in a FEI Talos 200C transmission electron microscope (FEI UK Ltd, Cambridge, UK) at 200kV and imaged using a Gatan OneView 4K x 4K digital camera (Gatan, Cambridge, UK) to record DM4 files. For overview images, sections were stained in 0.05% Toluidine Blue O for 5 min and imaged using a Leica DM6000 compound microscope 20X air objective with bright field settings (Leica Microsystems, Wetzlar, Germany).

Phylogenetic analysis

LIGHT SENSITIVE SHORT HYPOCOTYL (LSH) proteins were identified in the *Arabidopsis thaliana*, *Medicago truncatula*, *Lotus japonicus*, *Solanum lycopersicum* and *Pisum sativum* proteomes using HMMER3.1b2 HMMSEARCH (hmmer.org). The inputs to this program were the Pfam hidden Markov model (HMM), PF04852 (Pfam release 30), and the protein data sets from the genome annotations of *A. thaliana* (Araport11), *M. truncatula* (Phytozome, V10), *L. japonicus* (<http://www.kazusa.or.jp/lotus/>, build 3.0), *S. lycopersicum* (Phytozome, ITAG3.2) and *P. sativum* (<https://urgi.versailles.inra.fr/Species/Pisum>, v1a). The protein sequences detected were aligned back to the HMM using HMMER3.1b2 HMMALIGN. Gap columns in the alignment that were not part of the HMM were removed, sequences with less than 70% coverage across the alignment were removed and the longest sequence for each gene from the set of splice versions was used for phylogenetic analysis. Phylogenetic analysis was carried out using the MPI version of RAxML v8.2.9⁷⁶ with the following method parameters set: -f a, -x 12345, -p 12345, -# 100, -m PROTCATJTT. The tree was mid-point rooted and visualized using the Interactive Tree of Life (iTOL) tool.⁷⁷

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA-seq

The raw FASTQ files from the RNA-sequencing experiments were first quality checked with fastQC software.⁷⁸ Then the reads were mapped to the *M. truncatula* reference genome version v5.1.9⁵⁹ using STAR (v2.7.10b).⁷⁹ The raw counts of aligned reads were calculated with featureCounts in R package Rsubread (v2.12.0).⁸⁰ To account for potential batch effects, the R sva (v3.46.0)⁸¹ package was used and the number of surrogate variables were determined by the function sva(). Differentially expressed genes (DEGs) were identified by pairwise comparisons of raw counts of mock treatment versus experimental treatment, using the R package DESeq2 (v1.38.0)⁸² while addressing the surrogate variables. The threshold for the DEGs were absolute fold change of over 1.5 and a false discovery rate (FDR) corrected p-value significance of 0.05. To account for the false positive DEGs that arise from low counts, only the genes that had DESeq2 normalized counts more or equal to 10 in at least 3 samples were analyzed. The raw RNAseq data for time-course nodulation (wildtype Jester and *nin-1*, *cre1-1* mutants) and time-course lateral root development were from previously published data⁹, deposited in National Center for Biotechnology Information Gene Expression Omnibus, with the accession number GEO: GSE13361.

Clustering and Gene Ontology enrichment analysis

The union of DEGs from the time-course nodulation RNAseq data was subjected to clustering (20,719 genes). The DEGs were clustered by their log₂ fold change values across time points. The optimal number of clusters was determined by gap statistics⁸³, using the R package cluster (v2.1.4)⁸⁴. The final clusters were obtained by hierarchical k-means clustering using ward.D method and spearman correlation distance as distance measure.⁸⁵ The Gene Ontology⁸⁶ enrichment analysis

was performed with topGO (v2.50.0)⁸⁷ with the significance cutoff: $p\text{-value} \leq 0.01$. The GO term annotations and the functional annotations were obtained from *Medicago truncatula* A17 r5.0 genome portal (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>) and this was integrated with GO terms annotated to the previous genome assembly version that was used in Schiessl et al.⁹ The heatmaps were plotted with the R package ComplexHeatmap (2.14.0).⁸⁸

ChIP-Seq

At least 3 biological replicates for each construct (*pLjUBI:GFP-LSH1*, *pLjUBI:GFP-NIN* or control *pLjUBI:NLS-GFP*) were included in the full analysis. Reads from the ChIP-sequencing experiments were provided as raw fastq data. For analysis, the ENCODE-DCC ChIP-Seq pipeline2 version 2.1.2 (<https://github.com/ENCODE-DCC/chip-seq-pipeline2>)⁸⁹ was used with blacklisting the chlorophyll genome. Raw reads were quality-controlled and mapped to the *M. truncatula* reference genome version 5.0 (A17 r5.0, annotation version 1.9) (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>)⁵⁹ using Bowtie 2⁹⁰. Duplicate reads were removed from the aligned reads with Picard MarkDuplicates (<http://broadinstitute.github.io/picard>). Significant peaks ($q\text{-value} < 0.05$) were called from aligned and deduplicated reads and annotated using macs2 and chipseeker tools, respectively. The peaks were called by macs2 with the pooled control samples and the immunoprecipitated samples as input.⁹¹ The initial p-value threshold for peak calling was $p < 0.01$. Then they were subsequently filtered by the q-value threshold of $q < 0.05$ to correct for false discovery rate. The called peaks were annotated by ChIPseeker R package.⁹² Peaks that were called in $> 50\%$ of replicates (3 out of 4 replicates in *LjUBI:GFP-LSH1*, 2 out of 3 replicates in *LjUBI:GFP-NIN*) were merged using BEDToolsbedtools⁹³ and are considered as high confidence peaks. The bigwig files were generated

by deepTools2 bamCompare⁹⁴ and were visualized in Medicago genome browser (<https://medicago.toulouse.inra.fr/MtrunA17r5.0-ANR/>).

qRTPCR

Expression values of minimum three biological replicates in three technical replicates were analyzed using the Pfaffl method with *histone H3 (HH3)* as reference.⁹⁵ Statistical comparison was performed between WT and mutants or treatment and corresponding mock. Values depicted in bar charts are the mean of minimum 3 biological replicates $\pm$ SEM (Student's t-test; * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$).

Phenotyping

Data on the morphology of nodule primordia, rhizobial infection threads and mature nodules was depicted in bar charts and in boxplots representing the percentage of total nodule number. Total number of serrations per stipules, total number of nodules per gram root tissue and acetylene reduction rates were depicted as boxplots. Boxplots show the median (thick line), second to third quartiles (box), minimum and maximum ranges (lines), and outliers (single points). Normal distribution of data was tested using the Shapiro-Wilk normality test. For pairwise comparisons statistical analysis was performed using either unpaired Student's t-test, Wilcoxon test or Fisher's exact test. For multiple comparisons, one-way analysis of variance (one-way ANOVA) or one-way Kruskal-Wallis rank sum test, followed by Tukey multiple comparisons of means or Dunn test. The R statistical package was used for these analyses. Samples size n is provided in the figure legends and refers to the number of individual plants unless indicated otherwise. Statistical tests and significance levels are provided in the figure legends.

DATA AND CODE AVAILABILITY

The short-read sequencing data generated in this study have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus, with accession numbers GSE211680 and GSE243570. Lists of differentially expressed genes and ChIP-Seq targets and of downstream analysis are compiled in Data S1 and S2.

SUPPLEMENTAL INFORMATION

Document S1 includes Figure S1-S7 and Table S1-S6.

REFERENCES

1. Singh, S., Katzer, K., Lambert, J., Cerri, M., and Parniske, M. (2014). CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. *Cell Host Microbe* 15, 139-152. 10.1016/j.chom.2014.01.011.
2. Marsh, J.F., Rakocevic, A., Mitra, R.M., Brocard, L., Sun, J., Eschstruth, A., Long, S.R., Schultze, M., Ratet, P., and Oldroyd, G.E. (2007). *Medicago truncatula* NIN is essential for rhizobial-independent nodule organogenesis induced by autoactive calcium/calmodulin-dependent protein kinase. *Plant Physiol* 144, 324-335. 10.1104/pp.106.093021.
3. Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. *Nature* 402, 191. 10.1038/46058.
4. Yoro, E., Suzaki, T., Toyokura, K., Miyazawa, H., Fukaki, H., and Kawaguchi, M. (2014). A Positive Regulator of Nodule Organogenesis, NODULE INCEPTION, Acts as a Negative Regulator of Rhizobial Infection in *Lotus japonicus*. *Plant Physiol* 165, 747-758. 10.1104/pp.113.233379.
5. Vernie, T., Kim, J., Frances, L., Ding, Y., Sun, J., Guan, D., Niebel, A., Gifford, M.L., de Carvalho-Niebel, F., and Oldroyd, G.E. (2015). The NIN Transcription Factor Coordinates Diverse Nodulation Programs in Different Tissues of the *Medicago truncatula* Root. *Plant Cell* 27, 3410-3424. 10.1105/tpc.15.00461.
6. Liu, J., Rasing, M., Zeng, T., Klein, J., Kulikova, O., and Bisseling, T. (2021). NIN is essential for development of symbiosomes, suppression of defence and premature senescence in *Medicago truncatula* nodules. *New Phytologist* 230, 290-303. <https://doi.org/10.1111/nph.17215>.
7. Liu, J., Rutten, L., Limpens, E., Molen, T.v.d., Velzen, R.v., Chen, R., Chen, Y., Geurts, R., Kohlen, W., Kulikova, O., and Bisseling, T. (2019). A Remote cis-Regulatory Region Is Required for NIN

- Expression in the Pericycle to Initiate Nodule Primordium Formation in *Medicago truncatula*. *The Plant Cell*, tpc.00478.02018. 10.1105/tpc.18.00478.
8. Gonzalez-Rizzo, S., Crespi, M., and Frugier, F. (2006). The *Medicago truncatula* CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with *Sinorhizobium meliloti*. *Plant Cell* 18, 2680-2693. 10.1105/tpc.106.043778.
 9. Schiessl, K., Lilley, J.L.S., Lee, T., Tamvakis, I., Kohlen, W., Bailey, P.C., Thomas, A., Luptak, J., Ramakrishnan, K., Carpenter, M.D., et al. (2019). NODULE INCEPTION Recruits the Lateral Root Developmental Program for Symbiotic Nodule Organogenesis in *Medicago truncatula*. *Current Biology* 29, 3657-3668.e3655. <https://doi.org/10.1016/j.cub.2019.09.005>.
 10. Soyano, T., Shimoda, Y., Kawaguchi, M., and Hayashi, M. (2019). A shared gene drives lateral root development and root nodule symbiosis pathways in *Lotus*. *Science* 366, 1021-1023. 10.1126/science.aax2153.
 11. Herrbach, V., Rembliere, C., Gough, C., and Bensmihen, S. (2014). Lateral root formation and patterning in *Medicago truncatula*. *J Plant Physiol* 171, 301-310. 10.1016/j.jplph.2013.09.006.
 12. Xiao, T.T., Schilderink, S., Moling, S., Deinum, E.E., Kondorosi, E., Franssen, H., Kulikova, O., Niebel, A., and Bisseling, T. (2014). Fate map of *Medicago truncatula* root nodules. *Development* 141, 3517-3528. 10.1242/dev.110775.
 13. Dong, W., Zhu, Y., Chang, H., Wang, C., Yang, J., Shi, J., Gao, J., Yang, W., Lan, L., Wang, Y., et al. (2021). An SHR-SCR module specifies legume cortical cell fate to enable nodulation. *Nature* 589, 586-590. 10.1038/s41586-020-3016-z.
 14. Couzigou, J.M., Zhukov, V., Mondy, S., Abu el Heba, G., Cosson, V., Ellis, T.H., Ambrose, M., Wen, J., Tadege, M., Tikhonovich, I., et al. (2012). NODULE ROOT and COCHLEATA maintain nodule development and are legume orthologs of Arabidopsis BLADE-ON-PETIOLE genes. *Plant Cell* 24, 4498-4510. 10.1105/tpc.112.103747.
 15. Magne, K., Couzigou, J.-M., Schiessl, K., Liu, S., George, J., Zhukov, V., Sahl, L., Boyer, F., Iantcheva, A., Mysore, K.S., et al. (2018). MtNODULE ROOT1 and MtNODULE ROOT2 Are Essential for Indeterminate Nodule Identity. *Plant Physiology* 178, 295-316. 10.1104/pp.18.00610.
 16. Khan, M., Xu, H., and Hepworth, S.R. (2014). BLADE-ON-PETIOLE genes: Setting boundaries in development and defense. *Plant Science* 215-216, 157-171. <https://doi.org/10.1016/j.plantsci.2013.10.019>.
 17. Laloum, T., Baudin, M., Frances, L., Lepage, A., Billault-Penneteau, B., Cerri, M.R., Ariel, F., Jardinaud, M.-F., Gamas, P., de Carvalho-Niebel, F., and Niebel, A. (2014). Two CCAAT-box-binding transcription factors redundantly regulate early steps of the legume-rhizobia endosymbiosis. *The Plant Journal* 79, 757-768. 10.1111/tpj.12587.
 18. Combier, J.P., Frugier, F., de Billy, F., Boualem, A., El-Yahyaoui, F., Moreau, S., Vernié, T., Ott, T., Gamas, P., Crespi, M., and Niebel, A. (2006). MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes Dev* 20, 3084-3088. 10.1101/gad.402806.
 19. Hossain, M.S., Shrestha, A., Zhong, S., Miri, M., Austin, R.S., Sato, S., Ross, L., Huebert, T., Tromas, A., Torres-Jerez, I., et al. (2016). *Lotus japonicus* NF-YA1 Plays an Essential Role During

- Nodule Differentiation and Targets Members of the SHI/STY Gene Family. *Molecular Plant-Microbe Interactions* 29, 950-964. 10.1094/MPMI-10-16-0206-R.
20. Shrestha, A., Zhong, S., Therrien, J., Huebert, T., Sato, S., Mun, T., Andersen, S.U., Stougaard, J., Lepage, A., Niebel, A., et al. (2020). Lotus japonicus Nuclear Factor YA1, a nodule emergence stage-specific regulator of auxin signalling. *New Phytologist* n/a. 10.1111/nph.16950.
 21. Feng, J., Lee, T., Schiessl, K., and Oldroyd, G.E.D. (2021). Processing of NODULE INCEPTION controls the transition to nitrogen fixation in root nodules. *Science* 374, 629-632. 10.1126/science.abg2804.
 22. Baudin, M., Laloum, T., Lepage, A., Rípodas, C., Ariel, F., Frances, L., Crespi, M., Gamas, P., Blanco, F.A., Zanetti, M.E., et al. (2015). A Phylogenetically Conserved Group of Nuclear Factor-Y Transcription Factors Interact to Control Nodulation in Legumes *Plant Physiology* 169, 2761-2773. 10.1104/pp.15.01144.
 23. Soyano, T., Kouchi, H., Hirota, A., and Hayashi, M. (2013). Nodule inception directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus. *PLoS Genet* 9, e1003352. 10.1371/journal.pgen.1003352.
 24. Iyer, L.M., and Aravind, L. (2012). ALOG domains: provenance of plant homeotic and developmental regulators from the DNA-binding domain of a novel class of DIRS1-type retroposons. *Biology direct* 7, 39. 10.1186/1745-6150-7-39.
 25. Naramoto, S., Hata, Y., and Kyojuka, J. (2020). The origin and evolution of the ALOG proteins, members of a plant-specific transcription factor family, in land plants. *J Plant Res* 133, 323-329. 10.1007/s10265-020-01171-6.
 26. He, L., Lei, Y., Li, X., Peng, Q., Liu, W., Jiao, K., Su, S., Hu, Z., Shen, Z., and Luo, D. (2020). SYMMETRIC PETALS 1 Encodes an ALOG Domain Protein that Controls Floral Organ Internal Asymmetry in Pea (*Pisum sativum* L.). *Int J Mol Sci* 21. 10.3390/ijms21114060.
 27. Ye, Q., Zhu, F., Sun, F., Wang, T.-C., Wu, J., Liu, P., Shen, C., Dong, J., and Wang, T. (2022). Differentiation trajectories and biofunctions of symbiotic and un-symbiotic fate cells in root nodules of *Medicago truncatula*. *Molecular Plant* 15, 1852-1867. 10.1016/j.molp.2022.10.019.
 28. Cho, E., and Zambryski, P.C. (2011). Organ boundary1 defines a gene expressed at the junction between the shoot apical meristem and lateral organs. *Proceedings of the National Academy of Sciences of the United States of America* 108, 2154-2159. 10.1073/pnas.1018542108.
 29. MacAlister, C.A., Park, S.J., Jiang, K., Marcel, F., Bendahmane, A., Izkovich, Y., Eshed, Y., and Lippman, Z.B. (2012). Synchronization of the flowering transition by the tomato TERMINATING FLOWER gene. *Nature genetics* 44, 1393-1398. 10.1038/ng.2465.
 30. Yoshida, A., Sasao, M., Yasuno, N., Takagi, K., Daimon, Y., Chen, R., Yamazaki, R., Tokunaga, H., Kitaguchi, Y., Sato, Y., et al. (2013). TAWAWA1, a regulator of rice inflorescence architecture, functions through the suppression of meristem phase transition. *Proc Natl Acad Sci U S A* 110, 767-772. 10.1073/pnas.1216151110.
 31. Starker, C.G., Parra-Colmenares, A.L., Smith, L., Mitra, R.M., and Long, S.R. (2006). Nitrogen Fixation Mutants of *Medicago truncatula* Fail to Support Plant and Bacterial Symbiotic Gene Expression *Plant Physiology* 140, 671-680. 10.1104/pp.105.072132.

32. Batzenschlager, M., Lacey, B., Zhang, N., Su, C., Egli, S., Krohn, P., Salfeld, J., Ditengou, F.A., Laux, T., and Ott, T. (2023). Competence for transcellular infection in the root cortex involves a post-replicative, cell-cycle exit decision in *Medicago truncatula*. *bioRxiv*, 2023.2003.2028.534635. 10.1101/2023.03.28.534635.
33. Mortier, V., Wasson, A., Jaworek, P., De Keyser, A., Decroos, M., Holsters, M., Tarkowski, P., Mathesius, U., and Goormachtig, S. (2014). Role of LONELY GUY genes in indeterminate nodulation on *Medicago truncatula*. *New Phytologist* 202, 582-593. <https://doi.org/10.1111/nph.12681>.
34. Triozzi, P.M., Irving, T.B., Schmidt, H.W., Keyser, Z.P., Chakraborty, S., Balmant, K., Pereira, W.J., Dervinis, C., Mysore, K.S., Wen, J., et al. (2022). Spatiotemporal cytokinin response imaging and ISOPENTENYLTRANSFERASE 3 function in *Medicago* nodule development. *Plant Physiol* 188, 560-575. 10.1093/plphys/kiab447.
35. Jarzyniak, K., Banasiak, J., Jamruszka, T., Pawela, A., Di Donato, M., Novák, O., Geisler, M., and Jasiński, M. (2021). Early stages of legume-rhizobia symbiosis are controlled by ABCG-mediated transport of active cytokinins. *Nat Plants* 7, 428-436. 10.1038/s41477-021-00873-6.
36. Jamruszka, T., Banasiak, J., Pawela, A., Jarzyniak, K., Xia, J., Biała-Leonhard, W., Plačková, L., Iacobini, F.R., Novák, O., Geisler, M., and Jasiński, M. (2022). *Medicago truncatula* ABCG40 is a cytokinin importer negatively regulating lateral root density and nodule number. *bioRxiv*, 2022.2011.2010.516000. 10.1101/2022.11.10.516000.
37. Lohar, D.P., Schaff, J.E., Laskey, J.G., Kieber, J.J., Bilyeu, K.D., and Bird, D.M. (2004). Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. *The Plant Journal* 38, 203-214. 10.1111/j.1365-3113X.2004.02038.x.
38. Tan, S., Sanchez, M., Laffont, C., Boivin, S., Le Signor, C., Thompson, R., Frugier, F., and Brault, M. (2020). A Cytokinin Signaling Type-B Response Regulator Transcription Factor Acting in Early Nodulation. *Plant Physiology* 183, 1319-1330. 10.1104/pp.19.01383.
39. Vinardell, J.M., Fedorova, E., Cebolla, A., Kevei, Z., Horvath, G., Kelemen, Z., Tarayre, S., Roudier, F., Mergaert, P., Kondorosi, A., and Kondorosi, E. (2003). Endoreduplication Mediated by the Anaphase-Promoting Complex Activator CCS52A Is Required for Symbiotic Cell Differentiation in *Medicago truncatula* Nodules. *The Plant Cell* 15, 2093-2105. 10.1105/tpc.014373.
40. Franssen, H.J., Xiao, T.T., Kulikova, O., Wan, X., Bisseling, T., Scheres, B., and Heidstra, R. (2015). Root developmental programs shape the *Medicago truncatula* nodule meristem. *Development* 142, 2941-2950. 10.1242/dev.120774.
41. Santuari, L., Sanchez-Perez, G.F., Luijten, M., Rutjens, B., Terpstra, I., Berke, L., Gorte, M., Prasad, K., Bao, D., Timmermans-Hereijgers, J.L., et al. (2016). The PLETHORA Gene Regulatory Network Guides Growth and Cell Differentiation in *Arabidopsis* Roots. *Plant Cell* 28, 2937-2951. 10.1105/tpc.16.00656.
42. Galinha, C., Hofhuis, H., Luijten, M., Willemsen, V., Blilou, I., Heidstra, R., and Scheres, B. (2007). PLETHORA proteins as dose-dependent master regulators of *Arabidopsis* root development. *Nature* 449, 1053-1057. 10.1038/nature06206.

43. Carrere, S., Verdier, J., and Gamas, P. (2021). MtExpress, a Comprehensive and Curated RNAseq-based Gene Expression Atlas for the Model Legume *Medicago truncatula*. *Plant and Cell Physiology* 62, 1494-1500. 10.1093/pcp/pcab110.
44. Xu, C., Park, S.J., Van Eck, J., and Lippman, Z.B. (2016). Control of inflorescence architecture in tomato by BTB/POZ transcriptional regulators. *Genes & Development* 30, 2048-2061.
45. Laporte, P., Lepage, A., Fournier, J., Catrice, O., Moreau, S., Jardinaud, M.-F., Mun, J.-H., Larrainzar, E., Cook, D.R., Gamas, P., and Niebel, A. (2014). The CCAAT box-binding transcription factor NF-YA1 controls rhizobial infection. *Journal of experimental botany* 65, 481-494. 10.1093/jxb/ert392.
46. Gauthier-Coles, C., White, R.G., and Mathesius, U. (2019). Nodulating Legumes Are Distinguished by a Sensitivity to Cytokinin in the Root Cortex Leading to Pseudonodule Development. *Frontiers in plant science* 9, 1901-1901. 10.3389/fpls.2018.01901.
47. Ioio, R.D., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A Genetic Framework for the Control of Cell Division and Differentiation in the Root Meristem. *Science* 322, 1380-1384. 10.1126/science.1164147.
48. Laplace, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., et al. (2007). Cytokinins Act Directly on Lateral Root Founder Cells to Inhibit Root Initiation. *The Plant Cell* 19, 3889-3900. 10.1105/tpc.107.055863.
49. Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., and Benková, E. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. *Developmental Cell* 21, 796-804. <https://doi.org/10.1016/j.devcel.2011.08.014>.
50. Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J., Schwechheimer, C., Murphy, A., and Benková, E. (2014). Cytokinin Controls Polarity of PIN1-Dependent Auxin Transport during Lateral Root Organogenesis. *Current Biology* 24, 1031-1037. <https://doi.org/10.1016/j.cub.2014.04.002>.
51. Dello Ioio, R., Linhares, F.S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., and Sabatini, S. (2007). Cytokinins Determine Arabidopsis Root-Meristem Size by Controlling Cell Differentiation. *Current Biology* 17, 678-682. <https://doi.org/10.1016/j.cub.2007.02.047>.
52. Reid, D.E., Heckmann, A.B., Novák, O., Kelly, S., and Stougaard, J. (2015). CYTOKININ OXIDASE/DEHYDROGENASE3 Maintains Cytokinin Homeostasis during Root and Nodule Development in *Lotus japonicus*. *Plant Physiology* 170, 1060-1074. 10.1104/pp.15.00650.
53. Gamas, P., Brault, M., Jardinaud, M.F., and Frugier, F. (2017). Cytokinins in Symbiotic Nodulation: When, Where, What For? *Trends Plant Sci* 22, 792-802. 10.1016/j.tplants.2017.06.012.
54. Cooper, J.B., and Long, S.R. (1994). Morphogenetic Rescue of *Rhizobium meliloti* Nodulation Mutants by trans-Zeatin Secretion. *The Plant Cell* 6, 215-225. 10.1105/tpc.6.2.215.
55. Soyano, T., Hirakawa, H., Sato, S., Hayashi, M., and Kawaguchi, M. (2014). Nodule Inception creates a long-distance negative feedback loop involved in homeostatic regulation of nodule organ production. *Proc Natl Acad Sci U S A* 111, 14607-14612. 10.1073/pnas.1412716111.

56. Fan, W., Xia, C., Wang, S., Liu, J., Deng, L., Sun, S., and Wang, X. (2022). Rhizobial infection of 4C cells triggers their endoreduplication during symbiotic nodule development in soybean. *New Phytol* 234, 1018-1030. 10.1111/nph.18036.
57. Griesmann, M., Chang, Y., Liu, X., Song, Y., Haberer, G., Crook, M.B., Billault-Penneteau, B., Lauressergues, D., Keller, J., Imanishi, L., et al. (2018). Phylogenomics reveals multiple losses of nitrogen-fixing root nodule symbiosis. *Science*. 10.1126/science.aat1743.
58. Carroll, S.B. (2008). Evo-Devo and an Expanding Evolutionary Synthesis: A Genetic Theory of Morphological Evolution. *Cell* 134, 25-36. <https://doi.org/10.1016/j.cell.2008.06.030>.
59. Pecrix, Y., Staton, S.E., Sallet, E., Lelandais-Brière, C., Moreau, S., Carrère, S., Blein, T., Jardinaud, M.-F., Latrasse, D., Zouine, M., et al. (2018). Whole-genome landscape of *Medicago truncatula* symbiotic genes. *Nature Plants* 4, 1017-1025. 10.1038/s41477-018-0286-7.
60. Plet, J., Wasson, A., Ariel, F., Le Signor, C., Baker, D., Mathesius, U., Crespi, M., and Frugier, F. (2011). MtCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in *Medicago truncatula*. *Plant J* 65, 622-633. 10.1111/j.1365-313X.2010.04447.x.
61. Cheng, X., Wang, M., Lee, H.-K., Tadege, M., Ratet, P., Udvardi, M., Mysore, K.S., and Wen, J. (2014). An efficient reverse genetics platform in the model legume *Medicago truncatula*. *New Phytologist* 201, 1065-1076. doi:10.1111/nph.12575.
62. Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C., and Barker, D.G. (2001). *Agrobacterium rhizogenes*-Transformed Roots of *Medicago truncatula* for the Study of Nitrogen-Fixing and Endomycorrhizal Symbiotic Associations. *Molecular Plant-Microbe Interactions* 14, 695-700. 10.1094/MPMI.2001.14.6.695.
63. Ehrhardt, D., Atkinson, E., and Long (1992). Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* 256, 998-1000. 10.1126/science.10744524.
64. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 344, 781-784. 10.1038/344781a0.
65. Weber, E., Engler, C., Gruetzner, R., Werner, S., and Marillonnet, S. (2011). A Modular Cloning System for Standardized Assembly of Multigene Constructs. *PLOS ONE* 6, e16765. 10.1371/journal.pone.0016765.
66. Rokhsar, D.S., Fazo, J., Putnam, N., Hayes, R.D., Neupane, R., Howson, R., Shu, S., Mitros, T., Hellsten, U., Dirks, W., and Goodstein, D.M. (2011). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Research* 40, D1178-D1186. 10.1093/nar/gkr944.
67. Tang, H., Krishnakumar, V., Bidwell, S., Rosen, B., Chan, A., Zhou, S., Gentzbittel, L., Childs, K.L., Yandell, M., Gundlach, H., et al. (2014). An improved genome release (version Mt4.0) for the model legume *Medicago truncatula*. *BMC Genomics* 15, 312. 10.1186/1471-2164-15-312.
68. Cortijo, S., Charoensawan, V., Roudier, F., and Wigge, P.A. (2018). Chromatin Immunoprecipitation Sequencing (ChIP-Seq) for Transcription Factors and Chromatin Factors in *Arabidopsis thaliana* Roots: From Material Collection to Data Analysis. *Methods Mol Biol* 1761, 231-248. 10.1007/978-1-4939-7747-5_18.

69. Schiessl, K., Muiño, J.M., and Sablowski, R. (2014). Arabidopsis JAGGED links floral organ patterning to tissue growth by repressing Kip-related cell cycle inhibitors. *Proceedings of the National Academy of Sciences* 111, 2830-2835. 10.1073/pnas.1320457111.
70. Dilworth, M.J. (1966). Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. *Biochimica et biophysica acta* 127, 285-294. 10.1016/0304-4165(66)90383-7.
71. Schöllhorn, R., and Burris, R.H. (1967). Acetylene as a competitive inhibitor of N-2 fixation. *Proc Natl Acad Sci U S A* 58, 213-216. 10.1073/pnas.58.1.213.
72. Zhang, W., and Wen, C.-K. (2010). Preparation of ethylene gas and comparison of ethylene responses induced by ethylene, ACC, and ethephon. *Plant physiology and biochemistry : PPB* 48, 45-53. 10.1016/j.plaphy.2009.10.002.
73. Ursache, R., Andersen, T.G., Marhavý, P., and Geldner, N. (2018). A protocol for combining fluorescent proteins with histological stains for diverse cell wall components. *The Plant Journal* 93, 399-412. <https://doi.org/10.1111/tpj.13784>.
74. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods* 9, 676. 10.1038/nmeth.2019 <https://www.nature.com/articles/nmeth.2019#supplementary-information>.
75. Yang, W., Cortijo, S., Korsbo, N., Roszak, P., Schiessl, K., Gurzadyan, A., Wightman, R., Jönsson, H., and Meyerowitz, E. (2021). Molecular mechanism of cytokinin-activated cell division in Arabidopsis. *Science*, eabe2305. 10.1126/science.abe2305.
76. Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312-1313. 10.1093/bioinformatics/btu033.
77. Letunic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Research* 44, W242-W245. 10.1093/nar/gkw290.
78. Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. . Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.
79. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2012). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15-21. 10.1093/bioinformatics/bts635.
80. Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923-930. 10.1093/bioinformatics/btt656.
81. Leek, J.T., Johnson, W.E., Parker, H.S., Jaffe, A.E., and Storey, J.D. (2012). The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 28, 882-883. 10.1093/bioinformatics/bts034.
82. Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* 15, 550. 10.1186/s13059-014-0550-8.

83. Tibshirani, R., Walther, G., and Hastie, T. (2001). Estimating the Number of Clusters in a Data Set via the Gap Statistic. *Journal of the Royal Statistical Society. Series B (Statistical Methodology)* 63, 411-423.
84. Mächler, M., Rousseeuw, P., Struyf, A., Hubert, M., and Hornik, K. (2012). *Cluster: Cluster Analysis Basics and Extensions*.
85. Ward, J.H. (1963). Hierarchical Grouping to Optimize an Objective Function. *Journal of the American Statistical Association* 58, 236-244. 10.1080/01621459.1963.10500845.
86. Consortium, G.O. (2004). The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Research* 32, D258-D261. 10.1093/nar/gkh036.
87. Alexa, A., and Jörg Rahnenführer (2009). Gene set enrichment analysis with topGO. *Bioconductor Improv* 27.
88. Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32, 2847-2849. 10.1093/bioinformatics/btw313.
89. Landt, S.G., Marinov, G.K., Kundaje, A., Kheradpour, P., Pauli, F., Batzoglou, S., Bernstein, B.E., Bickel, P., Brown, J.B., Cayting, P., et al. (2012). ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. *Genome research* 22, 1813-1831. 10.1101/gr.136184.111.
90. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods* 9, 357-359. 10.1038/nmeth.1923.
91. Zhang, Y., Liu, T., Meyer, C.A., Eeckhoute, J., Johnson, D.S., Bernstein, B.E., Nusbaum, C., Myers, R.M., Brown, M., Li, W., and Liu, X.S. (2008). Model-based Analysis of ChIP-Seq (MACS). *Genome Biology* 9, R137. 10.1186/gb-2008-9-9-r137.
92. Yu, G., Wang, L.G., and He, Q.Y. (2015). ChIPseeker: an R/Bioconductor package for ChIP peak annotation, comparison and visualization. *Bioinformatics* 31, 2382-2383. 10.1093/bioinformatics/btv145.
93. Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841-842. 10.1093/bioinformatics/btq033.
94. Ramírez, F., Ryan, D.P., Grüning, B., Bhardwaj, V., Kilpert, F., Richter, A.S., Heyne, S., Dündar, F., and Manke, T. (2016). deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Research* 44, W160-W165. 10.1093/nar/gkw257.
95. Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research* 29, e45-e45.