The *Medicago truncatula* nodule identity gene *MtNOOT1* is required for coordinated apical-basal development of the root

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**Abstract**

**Background:** Legumes can utilize atmospheric nitrogen by hosting nitrogen-fixing bacteria in special lateral root organs, called nodules. Legume nodules have a unique ontology, despite similarities in the gene networks controlling nodule and lateral root development. It has been shown that *Medicago truncatula* NODULE ROOT1 (*MtNOOT1*) is required for the maintenance of nodule identity, preventing the conversion to lateral root development. *MtNOOT1* and its orthologs in other plant species—collectively called the NOOT-BOP-COCHLIKE (NBCL) family—specify boundary formation in various aerial organs. However, *MtNOOT1* is not only expressed in nodules and aerial organs, but also in developing roots, where its function remains elusive.

**Results:** We show that *Mtnoot1* mutant seedlings display accelerated root elongation due to an enlarged root apical meristem. Also, *Mtnoot1* mutant roots are thinner than wild-type and are delayed in xylem cell differentiation. We provide molecular evidence that the affected spatial development of *Mtnoot1* mutant roots correlates with delayed induction of genes involved in xylem cell differentiation. This coincides with a basipetal shift of the root zone that is susceptible to rhizobium-secreted symbiotic signal molecules.

**Conclusions:** Our data show that *MtNOOT1* regulates the size of the root apical meristem and vascular differentiation. Our data demonstrate that *MtNOOT1* not only functions as a homeotic gene in nodule development but also coordinates the spatial development of the root.

**Keywords:** *Medicago truncatula*, NOOT1, NOOT-BOP-COCHLEATA-LIKE, NBCL, Xylem cell differentiation, Rhizobium susceptible zone, NIN

**Background**

Legume plants (Fabaceae) can form unique lateral root organs to host nitrogen-fixing rhizobium bacteria, known as nodules. Legume nodules originate from root cells upon rhizobium-induced lipo-chitooligosaccharide (LCO) signalling. In the legume model *Medicago truncatula* (medicago) LCO signalling induces cell divisions in the pericycle and endodermis, followed by a coordinated mitotic activation of cortical cells. This will give rise to nodule primordia [1]. When fully developed, a medicago nodule possesses a large central zone with cells harbouring nitrogen-fixing rhizobia, surrounded by peripheral vascular bundles and a meristem at the apex allowing indeterminate growth [1].

Legume nodule formation is controlled by a network of transcriptional regulators, among which NODULE INCEPTION (NIN) is a master regulator [2, 3]. NIN expression is activated upon LCO signalling in a small zone of the root with elongating root hairs [4–7]. Expression of *NIN* in the root pericycle and dividing cortical cells is essential and sufficient to trigger nodule organogenesis [6, 8, 9]. Legumes recruited a BTB/POZ-ankyrin domain containing protein of the NOOT-BOP-COCHLEATA-LIKE (NBCL) family to maintain nodule identity in the newly formed primordium [10–12]. Knockout mutations in this gene—in *medicago* named NODULE ROOT1 (*MtNOOT1*)—cause a homeotic switch from nodule organogenesis towards lateral root formation [10]. This underlines the important functioning of
MtNOOT1 in nodule development. Besides nodules, MtNOOT1 is also expressed in young root tissue [13, 14]. However, its functioning during root development remains elusive.

MtNOOT1 is orthologous to the Arabidopsis thaliana (arabidopsis) BLADE-ON-PETIOLE1 (AtBOP1) and AtBOP2 genes. Studies in arabidopsis have revealed that BOP proteins function as co-transcriptional regulators involved in plant boundary formation (Reviewed in [15–19]). For example, AtBOP1 and AtBOP2 can promote expression of LATERAL ORGAN BOUNDARIES (LOB) genes to repress brassinosteroid signalling, which subsequently restricts cell growth and division in the boundary domain between the shoot apical meristem and lateral organs such as leaves [20, 21]. AtBOP1 and AtBOP2 also control proximal-distal leaf patterning by repressing the expression of genes that promote meristematic activity [22, 23]. Knockout mutations in AtBOP1/AtBOP2 lead to ectopic outgrowths of blade tissue along the petioles of cotyledons and leaves, due to misexpression of meristematic genes [22, 23]. Additionally, AtBOP1 and AtBOP2 are essential for abscission zone (AZ) formation at the junction between the leaving organ and the main plant body [24]. In line with this, a complete loss of floral organ abscission is observed in the arabidopsis Atbop1;Atbop2 double mutant [24].

Similar to arabidopsis, mutations in orthologous BOP genes in the legumes medicago MtNOOT1, pea (Pisum sativum) COCHLEATA1 (PsCOCH1), and lotus (Lotus japonicus) LjNBCL1 affect leaf patterning and AZ formation [25]. Arabidopsis Atbop1;Atbop2 double mutants do not form stipules [24, 26], which are also simplified or reduced in the medicago Mtnoot1 mutant and at early nodes of the pea Pscoch1 mutant [10]. In the lotus Ljnbcl1 mutant, nectary glands (proposed modified stipules) are completely absent [11]. Furthermore, in the medicago Mtnoot1, pea Pscoch1 and lotus Ljnbcl1 mutants, the abscission of petals is impaired [25], similar to what is observed in arabidopsis Atbop1;Atbop2 [24]. This indicates that the function of NBCL proteins in boundary specification in the proximal region of the leaf and in AZ formation is well-conserved.

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average size of cortical cells in the RAM is not affected by the Mtnoot1 mutation (Fig. 1d). This suggests that an increase in cell number in the RAM of the Mtnoot1 mutant underlies its accelerated growth. Taken together, these results suggest that MtNOOT1 either represses cell division in the RAM or/and promotes cell differentiation in the transition zone of the root.

**MtNOOT1 is expressed in the transition zone and root vasculature**

The accelerated primary root growth prompted us to determine the precise location of MtNOOT1 expression. Since we were unable to identify a functional promoter region of MtNOOT1, we decided to perform RNA in situ hybridization on longitudinal sections of root tips (1–2

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**Fig. 1** The primary root of the medicago Mtnoot1 mutant is longer than wild-type root. 

**a** Schematic representation of the zonation in a medicago primary root. Left panel: the size of the root apical meristem (RAM) is determined from the stem cell niche to the elongation/differentiation zone (EDZ). The apical border of the EDZ is defined by the first elongated cortex cell of second cortical layer, as indicated by arrowhead. Note that the exact position of the RAM-EDZ boundary can vary depending on the cell type, marking a region that named transition zone (TZ) [30, 31]. Cell types are indicated by color coding. Right panel: Nomaski image of the distal region of the RAM and apical region of the EDZ of a primary root at 6 Days After Germination (DAG). Arrowhead indicates the boundary between the RAM and the EDZ of the second layer of cortical cells. 

**b** Root length of the medicago Mtnoot1 tnk507 mutant is markedly longer at 4 DAG and 6 DAG when compared to primary roots of wild-type medicago seedlings (WT). 

**c** The RAM of medicago Mtnoot1 tnk507 seedlings is larger at 6 DAG when compared to the RAM of primary roots of wild-type medicago seedlings. The data represent means ± SD of three independent experiments, each experiment contains 16–20 roots. 

**d** The cortical cell size at the proximal part of the RAM is not affected in the root of the medicago Mtnoot1 tnk507 mutant seedlings. Given is the average cell size of cortical cells at the proximal part of the RAM of wild-type and Mtnoot1 mutant roots. The data represent means ± SD of two independent experiments, each experiment contains 16–20 roots. Student t-test was performed to assess significant differences (****: \(P < 0.0001\), ns: not significant). A representation of the RAM of wild-type (e) and Mtnoot1 mutant seedling (f) primary roots at 6 DAG. White dotted line marks the quiescent center/stem cell niche region, arrowhead marks the boundary between the RAM and the EDZ of the second layer of cortical cells. Scale bar: 50 \(\mu\)m (a), 100 \(\mu\)m (e, f).
mm) of wild-type seedlings at 6 DAG. This showed that MtNOOT1 is highly expressed in the region between the RAM and the elongation/differentiation zone and barely in the rest of the RAM (Fig. 2a). A magnification of the region with the most intense signals showed that MtNOOT1 is expressed in the transition zone and the distal part of the elongation/differentiation zone (Fig. 2b). This suggests that MtNOOT1 controls the size of the RAM by promoting cell differentiation in the transition zone.

Since it has been shown that AtBOP1 and AtBOP2 play a role in vasculature differentiation [27, 28, 32], we questioned whether MtNOOT1 may play a similar role in medicago roots. To investigate this, we first examined whether MtNOOT1 is expressed in the root vasculature. We divided the first 2 cm of young seedling roots (without visible lateral roots or primordia) into four zones of each ~5 mm long (basipetally, from zone 1 to zone 4, of which zone 1 includes the RAM till the mature root cells with elongated root hairs). These root segments correspond to different stages of vascular development and were used to conduct qRT-PCR studies (Fig. 2c). This showed that MtNOOT1 is expressed in all four zones, with relatively the lowest level of expression in zone 1 and the highest level in zone 4 (Fig. 2c). This is consistent with Gene Expression Atlas data (Additional file 1: Figure S1). RNA in situ hybridization on a cross section of wild-type zone 4 segment showed that MtNOOT1

![Fig. 2](image-url)
transcripts mainly occur in the procambium cells of the vasculature (Fig. 2d). This suggests that MtNOOT1 could also play a role in root vasculature development in medicago.

The primary root of the Mtnoot1 mutant is delayed in xylem cell differentiation
As MtNOOT1 is expressed in the root vasculature, we investigated whether the Mtnoot1 mutant is affected in root vasculature development. Preliminary observations suggested that Mtnoot1 mutant roots are thinner than wild-type roots. To quantify this, cross sections on Mtnoot1 mutant line tkn507 were made on the middle parts of zone 1 to zone 4 and the size of the cross-sectional area was determined. This showed that in zone 2 to zone 4 the cross-sectional area of the Mtnoot1 mutant roots is significantly smaller when compared to the counterparts of wild-type roots. The root cross-sectional area sizes of zone 3 and zone 4 of the Mtnoot1 mutants were more comparable to those of zone 2 and zone 3 in wild-type, respectively (Fig. 3a). A similar reduction in the root cross-sectional area size was also observed in the Mtnoot1 mutant line NF2717 (Additional file 3: Figure S3b). Further, the cross-sectional area sizes of the vasculature also showed that this is significantly smaller in Mtnoot1 (tkn507) root zone 2 to zone 4 when compared to wild-type roots. This suggests that the Mtnoot1 mutants have a delayed pattern of root radial growth, which correlates with the growth defects in its vasculature.

Vascular bundles are mainly built up of xylem and phloem cells, in medicago roots in a four-arch constitution (Fig. 3c, e). During the development of the root, vascular metaxylem cells differentiate and become lignified [33]. Upon toluidine blue staining, lignified metaxylem cells gain a lighter blue colouration than non-lignified cells [34]. Upon toluidine blue staining of wild-type and Mtnoot1 tkn507 roots, we found that 32% (19/60) of wild-type zone 3 showed lignified central metaxylem cells, in contrast to only 2% (1/60) of Mtnoot1 zone 3 showed lignification of central metaxylem cells. Furthermore, central metaxylem cells are lignified in 68% (41/60) of wild-type zone 4, but only in 27% (16/60) of Mtnoot1 zone 4, which is more similar to the lignification level of wild-type zone 3 (Fig. 4). A similar result was obtained by analysing the Mtnoot1 NF2717 mutant allele (Additional file 3: Figure S3c-g). These observations demonstrate that in Mtnoot1 mutants the lignification level of vascular metaxylem cells in zone 3 and zone 4 is lower when compared with wild-type, suggesting that the differentiation of Mtnoot1 root vasculature is delayed. This is consistent with the observation that the cross-sectional area sizes of Mtnoot1 root vasculature in zone 2 to zone 4 are significantly smaller when compared to wild-type (Fig. 3b).

The expression of genes involved in xylem cell differentiation is delayed in Mtnoot1 mutant roots
In arabidopsis, secondary cell wall formation and subsequent programmed cell death (PCD) are two critical steps in the maturation of proto-/metaxylem and fibre cells [33]. Secondary cell wall biosynthesis is initiated by the master regulators VASCULAR-RELATED NAC-DOMAIN 7 (AtVND7) for protoxylem, AtVND6 for metaxylem and SECONDARY WALL–ASSOCIATED NAC DOMAIN PROTEIN 1 (AtSND1) for fibre differentiation [33]. These master regulators control secondary cell wall biosynthesis via a network of genes, which include the transcription factors AtMYB46, AtMYB58, AtMYB63, AtMYB83 and AtMYB85 that ultimately control lignin biosynthesis genes, and the peptidase encoding gene XYLEM CYSTEINE PEPTIDASE 1 (AtXCP1) involved in the PCD during xylem cell development [33, 35–37]. To find molecular support for the observation of the delayed vasculature development in the Mtnoot1 mutant, we aimed to analyse the transcript levels of the putative medicago orthologues of the above-mentioned genes. To identify such medicago orthologues we used phylogenetic reconstruction (Additional files 4-9: Figures S4-S9). Subsequent qRT-PCR expression studies on medicago root zone 3 and zone 4 revealed that both MtVND6 and MtVND7, but not MtSND1 are slightly lower expressed in zone 3 of Mtnoot1 mutant root (Additional file 10: Figure S10), while the putative downstream targets MtMYB46–1, MtMYB46–2, MtMYB83, MtMYB58/63, and MtMYB85 and MtXCP1 are markedly lower expressed in the Mtnoot1 root (Fig. 5a). In zone 4 of the Mtnoot1 mutant root, the expression of all these genes was restored to the wild-type level (Fig. 5a), indicating a delayed transcriptional regulation of genes controlling secondary cell wall biosynthesis in the apical-basal direction in the Mtnoot1 mutant roots. In contrast, the expression level of a phloem marker gene, ALTERED PHLOEM DEVELOPMENT (MtAPL) [38], is not affected in Mtnoot1 (Fig. 5b), suggesting that phloem cell development is not disturbed. Taken together, these results support our observation that xylem cell differentiation in the Mtnoot1 mutant is delayed.

The LCO susceptible zone of Mtnoot1 mutant roots is shifted basipetally
In medicago, the site where the interaction with rhizobia occurs is tightly linked to the developmental status of the root tissue. At the start of the differentiation zone, where young elongating root hairs can be found is called the susceptible zone, and it is here where rhizobium LCOs can trigger expression of symbiotic genes such as MtNIN [5–7]. The delayed root differentiation observed in the medicago Mtnoot1 mutant led us to question whether this may also affect the susceptibility of the root
To investigate this, we studied the expression of MtNIN in the four root zones upon application of ~10^{-9} M Sinorhizobium meliloti 2011 LCOs (dissolved in 1% DMSO) or mock (1% DMSO). qRT-PCR expression analysis showed that in wild-type roots the strongest induction of MtNIN occurred in zone 1 and a lower induction in zone 2. This indicates that the LCO susceptible zone of wild-type predominately locates in zone 1 (Fig. 6). In contrast, in the medicago Mtnoot1 mutant, the strongest MtNIN expression is observed in zone 2 (Fig. 6), implying that the Mtnoot1 mutation caused the LCO susceptible zone to shift to zone 2. This is consistent with the delayed differentiation of the Mtnoot1 root, which affects the LCO response by basipetally shifting the LCO susceptible zone.

Discussion
Here, we demonstrate that MtNOOT1 is required for coordinated development of the primary root of medicago along the apical-basal axis. A knockout mutation in the MtNOOT1 gene causes two root developmental affects; (1) the primary root growth is faster due to a larger...
RAM, meaning more dividing cells, and (2) the primary root is thinner with delayed root differentiation. This demonstrates that in roots MtNOOT1 not only functions as a homeotic gene in nodule development, but also coordinates root development.

In situ hybridization revealed that MtNOOT1 is expressed in the transition zone, which is located between the RAM and the elongation/differentiation zone. A larger RAM due to an increased number of meristematic cells indicates that the transition zone is positioned more distal from the stem cell niche. This suggests that MtNOOT1 controls the size of the RAM by defining the position of the boundary region (i.e. the transition zone) between the RAM and the elongation/differentiation zone. This is consistent with the aerial function of its orthologs in arabidopsis, where AtBOP1 and AtBOP2 promote the boundary specification between groups of cells with different fates [15–18]. In contrast to what we report for medicago Mtnoot1, a root growth and differentiation phenotype has not been reported in the arabidopsis Atbop1;Atbop2 double mutant [28]. The recruitment of NBCL family by legumes to maintain nodule symbiotic organ identity by defining nodule territories may have required adaptations in protein regulation and functioning [10]. Such adaptations could be causal for the difference in root functioning of MtNOOT1 in medicago and AtBOP1 and AtBOP2 in arabidopsis. Further, it has been proposed that arabidopsis has a closed RAM organization with distinct initial cells, while legumes have a RAM with a basic-open organization. In the latter case, the identity of cell files seems disorganized and cannot be predicted [39]. This deviation in structure of the RAM may explain the different functioning of BOP1/NOOT1 in arabidopsis and legume roots.

We also noted a basipetal shift of the root zone that is susceptible for rhizobium LCO signal molecules in the medicago Mtnoot1 mutant. Such shift of susceptibility to rhizobium can be explained by the delayed differentiation of root cells. In medicago, susceptibility to LCO signalling can only occur in defined window of development. By elongating the RAM, the differentiation zone gets shifted basipetally. As a result, the susceptible zone shifts basipetally as well.

In the arabidopsis root the transition zone is determined by the antagonistic interaction between auxin and

**Fig. 4** Vascular xylem differentiation is delayed in the primary root of Mtnoot1 at 6 DAG. The fraction of roots with lignified central metaxylem cells in zone 3 and zone 4 is decreased in the Mtnoot1 tk507 mutant when compared with wild-type seedlings. The lignification level of the central metaxylem cells in zone 4 of the Mtnoot1 mutant is similar to that of wild-type medicago zone 3, indicating that the differentiation of vascular xylem cells is delayed in the Mtnoot1 mutant. The presented data combines three independent experiments, each experiment contains 20 roots.
cytokinin. A well-defined auxin minimum is established in the boundary region, which is mediated by an active cytokinin signalling in the transition zone [40]. Activation of auxin signalling or inhibition of cytokinin signalling can lead to accelerated primary root elongation and a larger RAM with increased number of meristematic cells, which are similar to the phenotype of Mtnoot1 roots [30, 31]. Auxin and cytokinin are also involved in root vasculature development (Reviewed in [41, 42]). For example, mutations in cytokinin biosynthetic genes lead to abolished cambium formation and reduced thickening of the Arabidopsis root [43], and auxin signalling is

**Fig. 5** Genes putatively involved in xylem cell differentiation display reduced expression in Mtnoot1 roots. **a, b** Expression level of various genes putatively involved in xylem and phloem cell differentiation in zone 3 and zone 4 of wild-type and Mtnoot1 tnk507 roots determined by qRT-PCR. **a** The MYB-type transcription factors MtMYB46-1, MtMYB46-2, MtMYB83, and MtMYB85, and the XYLYME CYSTEINE PEPTIDASE 1 putative ortholog MtXCP1 are lower expressed in zone 3 of Mtnoot1 mutant roots when compared to wild-type. **b** The expression level of phloem marker gene MtAPL is not affected in Mtnoot1 mutant roots. The data represent means ± SEM of two independent experiments. Student t-test was performed to assess significant differences (*: P < 0.05, ****: P < 0.0001, ns: not significant)

**Fig. 6** Rhizobium LCO-induced MtNIN expression is spatially different in the medicago Mtnoot1 roots in comparison with wild-type roots. MtNIN expression is quantified using qRT-PCR in four root zones at three hours post application of 10^{-9} M S. meliloti LCOs. The data represent means ± SEM of two independent experiments using the Mtnoot1 tnk507 mutant
required for xylem differentiation and the organizer identity of vascular cambium cells in arabidopsis root [44]. In legumes, auxin and cytokinin participate in the LCO response and nodule organogenesis (Reviewed in [45–47]). For example, a mutation in cytokinin perception severely weakens the LCO response and perturbs nodule organogenesis [7, 48], whereas LCOs trigger auxin biosynthesis in the epidermis, leading to a rapid accumulation of auxin locally [49]. Recent findings suggest that in Mtnoot1 nodules the interplay between auxin and cytokinin is imbalanced [12]. In line with that, we speculate that the auxin/cytokinin signalling pathway is affected during the primary growth of Mtnoot1 roots, which will be targeted in future research.

We also showed that the lignification of xylem cells is delayed in Mtnoot1 primary roots, which correlates with a delayed induction of a cascade of transcription factors involved in xylem cell differentiation. This can be explained by the extended RAM, leading to delayed cell differentiation in the basal region of primary root. However, the expression of MtNOOT1 in theprocambium cells by in situ hybridization suggests that MtNOOT1 can positively regulates vascular cell differentiation in a cell-autonomous manner. This is in line with the function of arabidopsis AtBOP1 and AtBOP2 in stem vasculature, where they can induce lignin deposition in vascular cells by promoting the expression of lignin biosynthetic genes. Ectopic expression of AtBOP1/AtBOP2 in stem tissue leads to an expanded pattern of lignification [32]. Therefore, we hypothesize that MtNOOT1 promotes root vasculature differentiation by activating a cascade of genes involved in xylem cell differentiation.

Conclusions
Here, we showed that MtNOOT1 controls two aspects of root development; (1) the positioning of the transition zone, and (2) vasculature development by transcriptionally activating of a cascade of genes involved in xylem cell differentiation at a controlled distance from the root tip. Knockout of the MtNOOT1 gene not only leads to a delayed differentiation of the root, but also shift the root region susceptible to LCOs. Taken together, this demonstrates that MtNOOT1 not only maintains nodule identity, but also coordinates the primary root development along the apical-basal axis.

Methods
Plant materials and growth conditions
Medicago truncatula wild-type accession R108–1 plants and Mtnoot1 mutant lines tnk507 and NF2717 were used in this study [10, 12]. R108–1 was acquired from Toan Hanh Trinh [50], tnk507 and NF2717 were acquired from Pascal Ratet (IPS2, CNRS, Gif-sur-Yvette, France), they were identified by a forward genetics screen of Tnt1 insertion lines (Institut des Sciences du Végétal, France; Noble Foundation, Ardmore, USA). The surface-sterilization and germination of medicago seeds were performed as previously described by [51]. Note that medicago seeds germinated at room temperature for one day, before growing on Fahraeus agar medium [52] (including 0.75 mM Ca(NO₃)₂ in square petri dish (9 cm × 9 cm) and exposed to light for another five days. Plants were grown in an environmentally controlled growth chamber at 21 °C with a 16-h light/8-h dark.

Microscopy and imaging
For microscopy studies, root segments were all collected at 6 DAG. For measuring the length of the RAM, ~ 5 mm root tips were cut and immersed in chloral hydrate solution at 4 °C overnight, analyzed under Axio Imager A1 microscope (Zeiss) with Nomarski optics. For measuring cross-sectional areas, root segments were fixed with 4% paraformaldehyde (w/v), 5% glutaraldehyde (v/v) in 0.05 M sodium phosphate buffer (pH 7.2) at 4 °C overnight. The fixed material was dehydrated in an ethanol series and subsequently embedded in Technovit 7100 ( Heraeus Kulzer) according to the manufacturer’s protocol. Sections (5 μm) were made with a R2035 microtome (Leica Microsystems) stained 1.5 min in 0.05% toluidine blue O. For phloroglucinol-HCl staining, root segments were fixed as abovementioned. The fixed material was washed with 1 x PBS (sodium phosphate buffer), and directly embedded in 6% low melting agarose dissolved in 1 x PBS. Sections (50 μm) were made with a VT1000 S vibratome (Leica Microsystems), stained with 2% phloroglucinol (in 95% ethanol) for 2 min and applied with a few drops of 37% HCl staining. Sections were all analysed by using a DM5500B microscope equipped with a DFC425C camera (Leica Microsystems).

In situ hybridization
Hybridization was performed twice on root segments at 6 DAG by using Invitrogen ViewRNAISH Tissue 1-Plex assay kit (Thermo Fisher Scientific), as previously described by [9, 53]. For user manual, visit https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0018633_viewRNAISH_UG.pdf. The probe sets for MtNOOT1 (catalogue number: VF1–16434, information is available on request) were designed and synthesized by Thermo Fisher Scientific. MtNOOT1 probe sets cover the region 2–913 nucleotide (nt) of the coding sequence (1449 nt, Medtr7g090020.1). A typical probe set contains ~ 20 oligonucleotide pairs of probes that hybridize to specific regions across the target mRNA. Each probe covers 20 nt, only a pair of two adjacent probes, which can target 40 nt, can form a site for signal amplification. By this principle, control probes are not needed [9, 53–56]. Sections were imaged as mentioned above.
Phylogenetic tree construction
The protein sequences of different orthogroups were obtained from [57]. For phylogenetic reconstruction, full length (predicted) protein sequences of at least two closely related orthogroups were aligned using MAFFT v7.017 [58], implemented in Geneious R6 (Biomatters, Auckland, New Zealand), using default parameter settings. After manual inspection, alignments were used for tree building by using W-IQ-TREE [59] with best-fit substitution model [60]. Branch support was assessed by using Ultrafast Bootstrap Approximation based on 1000 replicates [61].

RNA isolation and qRT-PCR analysis
RNA was isolated from root segments at 6 DAG using the EZNA Plant RNA mini kit (Omega), following the supplier’s manual. 1 μg total RNA was used to synthesize cDNA using iScript cDNA synthesis kit (Bio-Rad). Equal amounts of cDNA were used for qPCR using SYBR Green Super-mix (Bio-Rad) in a Bio-Rad CFX connect real-time system qPCR machine. Cycling conditions were: 95 °C for 3 min, [95 °C for 15 s, 60 °C for 30 s] (40 cycles), 95 °C for 10 s, followed by melt curve analysis (from 65 °C to 95 °C, at an increment of 0.5 °C, for 5 s). The gene expression was normalized using MtACT2 as reference gene. Three technical replicates per biological sample were performed. qRT-PCR primers used in this study are given in Additional file 11: Table S1.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-019-2194-z.

Additional file 1: Figure S1. Medicago MtNOOT1 is expressed in the root tip. Expression profiles are derived from the Medicago truncatula Gene Expression Atlas [29]. MtNOOT1 is targeted by the probe-sets Mtr.19586.1.S_at, Mtr.27707.1.S_at, and Mtr.39297.1.S_at. Root 3 mm tip: 3 mm root tip; [14]; adj tip: 1 cm root segment adjacent to 3 mm root tip [14]; Nod: nodules, all nodule samples are derived from [13]; dpi: days post inoculation. (DOCX)

Additional file 2: Figure S2. The primary root Mtnoot1 tnk507 mutant is longer than wild-type. Representative seedlings at 6 DAG are presented. (DOCX)

Additional file 3: Figure S3. The Mtnoot1 NF2717 mutant allele shows a similar phenotype as the Mtnoot1 tnk507 allele. a Root length of the medicago Mtnoot1 mutant (NF2717) is markedly longer at 4 DAG and 6 DAG when compared to primary roots of wild-type medicago seedlings (WT). b The cross-sectional area is significantly reduced in the medicago Mtnoot1 mutant (NF2717) at zone 3 and zone 4. The data represent mean ± SD of two independent experiments, each experiment contains 15–20 roots. Student t-test was performed to assess significant differences (***, P < 0.0001). Representative root cross sections of wild-type (c, e) and Mtnoot1 (NF2717) (d, f) vascular bundle at zone 3 stained with chlorogluconol-HCl to demonstrate lignin deposition at 6 DAG. Black arrow marks lignified metaxylem cells, which are not found in the Mtnoot1 mutant (NF2717). g Vascular xylem differentiation is delayed in the Mtnoot1 (NF2717) primary root at 6 DAG. The fraction of roots with lignified central metaxylem cells in zone 3 and zone 4 is decreased in the Mtnoot1 mutant when compared with wild-type seedlings. The presented data combines two independent experiments, each experiment contains 15–18 roots. Scale bar: 50 μm (d, f). (DOCX)

Additional file 4: Figure S4. Maximum likelihood tree of VND6, VND7 and related proteins. The protein sequences of OG0006787 (red) and OG0009959 (dark red), OG000118 (purple), OG0001465 (green and blue) are obtained from van Velzen et al. (2018). Species include arabidopsis (ATHALIANA), Eucalyptus grandis (Egrandis), Fragaria vesca (Fvesca), Glycine max (Gmax), medicago (Mtruncatula), Populus trichocarpa (Pttrichocarpa), Parasponia andersonii (Pan) and Trema orientalis (Tor). Numbers at the branches indicate support from 1000 ultrafast bootstrap replicates. OG0006787 including NAC1 was used as outgroup. (DOCX)

Additional file 5: Figure S5. Maximum likelihood tree of SND1 and related proteins. The protein sequences of OG0009898 (red) and OG0001875 (green) are obtained from van Velzen et al. (2018). Species include arabidopsis (ATHALIANA), Eucalyptus grandis (Egrandis), Fragaria vesca (Fvesca), Glycine max (Gmax), medicago (Mtruncatula), Populus trichocarpa (Pttrichocarpa), Parasponia andersonii (Pan) and Trema orientalis (Tor). Numbers at the branches indicate support from 1000 ultrafast bootstrap replicates. OG0009898 containing SMB was used as outgroup. (DOCX)

Additional file 6: Figure S6. Maximum likelihood tree of MYB46, MYB81 and related proteins. The protein sequences of OG000857 (red) and OG0001270 (green) are obtained from van Velzen et al. (2018). Species include arabidopsis (ATHALIANA), Eucalyptus grandis (Egrandis), Fragaria vesca (Fvesca), Glycine max (Gmax), medicago (Mtruncatula), Populus trichocarpa (Pttrichocarpa), Parasponia andersonii (Pan) and Trema orientalis (Tor). Numbers at the branches indicate support from 1000 ultrafast bootstrap replicates. OG000857 containing MYB50 was used as outgroup. (DOCX)

Additional file 7: Figure S7. Maximum likelihood tree of MYBS8, MYB63 and MYB81 proteins. The protein sequences of OG000538 (red) and OG0002420 (green) are obtained from van Velzen et al. (2018). Species include arabidopsis (ATHALIANA), Eucalyptus grandis (Egrandis), Fragaria vesca (Fvesca), Glycine max (Gmax), medicago (Mtruncatula), Populus trichocarpa (Pttrichocarpa), Parasponia andersonii (Pan) and Trema orientalis (Tor). Numbers at the branches indicate support from 1000 ultrafast bootstrap replicates. OG000538 containing MYB50 and MYB63 was used as outgroup. (DOCX)

Additional file 8: Figure S8. Maximum likelihood tree of XCP1 and related proteins. The protein sequences of OG0003401 (red) and OG0003952 (green) are obtained from van Velzen et al. (2018). Species include arabidopsis (ATHALIANA), Eucalyptus grandis (Egrandis), Fragaria vesca (Fvesca), Glycine max (Gmax), medicago (Mtruncatula), Populus trichocarpa (Pttrichocarpa), Parasponia andersonii (Pan) and Trema orientalis (Tor). Numbers at the branches indicate support from 1000 ultrafast bootstrap replicates. OG0003401 containing CEPI was used as outgroup. (DOCX)

Additional file 9: Figure S9. Maximum likelihood tree of APL and related proteins. The protein sequences of OG0009526 (red) and OG0006786 (green) are obtained from van Velzen et al. (2018). Species include arabidopsis (ATHALIANA), Eucalyptus grandis (Egrandis), Fragaria vesca (Fvesca), Glycine max (Gmax), medicago (Mtruncatula), Populus trichocarpa (Pttrichocarpa), Parasponia andersonii (Pan) and Trema orientalis (Tor). Numbers at the branches indicate support from 1000 ultrafast bootstrap replicates. OG0009526 containing sequences highly homologous to MAPL was used as outgroup. (DOCX)

Additional file 10: Figure S10. The NAC domain transcription factors MtNIN6 and MtNIN7, but not MtSN1, are lower expressed in zone 3 of Mtnoot1 tnk507 roots when compared to wild-type. The data represent mean ± SEM of three independent experiments. Student t-test was performed to assess significant differences (ns: not significant). (DOCX)

Additional file 11: Table S1. qRT-PCR primers used in this study, (DOCX)

Abbreviations
AZ: Abscession zone; DAG: Days after germination; DMSO: Dimethyl sulfoxide; ED2: Elongation/differentiation zone; LCO: Lipo-chitoioigosaccharide;
nt: Nucleotide; PBS: Sodium phosphate buffer; qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction; RAM: Root apical meristem; TZ: Transition zone

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Authors’ contributions
RG and TB supervised this research. DS designed and performed most of the experiments with help from KG and WK. OK and DS performed the in situ hybridization. DS drafted the manuscript with input from HF. RG contributed to writing and revising the manuscript, with input from TB. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this research and materials used in this research are available by contacting with the corresponding author (rene.geurts@wur.nl).

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Results

5. Yano K, Yoshida S, Muller J, Singh S, Banba M, Vickers K, et al. CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc Natl Acad Sci U S A. 2009;106:21146–21151.

6. Vernié T, Kim J, Frances L, Ding Y, Sun J, Guan D, et al. The NIN transcription factor coordinates diverse nodulation programs in different tissues of the Medicago truncatula root. Development. 2014;141:4311–9.

References
1. Xiao TT, Schilderink S, Moling S, Deinum EE, Kondorosi E, Franssen H, et al. A gene expression atlas of the model legume Medicago truncatula: root nodules. Development. 2014;141:3517–28.

2. Schauser L, Roussis A, Stiller J, Stougaard J. A plant regulator controlling development of symbiotic root nodules. Nature. 1999;402:191–5.

3. Marsh JT, Rakoczy A, Mita RM, Brocard L, Sun J, Eschtschuch A, et al. Medicago truncatula NIN is essential for Rhizobial-independent nodule organogenesis induced by autoactive calcium/Calmodulin-dependent protein kinase. Plant Physiol. 2007;144:324–35.

4. McKim SM, Stenvik G-E, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, et al. The BLADE-ON-PETIOLE1 genes are essential for abscission zone formation in Arabidopsis. Development. 2008;135:1537–46.

5. Couziou JM, Magne K, Mondy S, Cosson V, Clements J, Ratet P. The legume NOD-ORG-COCH-LIKE (NBL1) genes are conserved regulators of abscission, a major agronomical trait in cultivated crops. New Phytol. 2016;206:228–40.

6. Ichishita Y, Kawade K, Usami T, Horiguchi T, Takahashi T, Tsukaya H. Key proliferative activity in the junction between the leaf blade and leaf petiole of Arabidopsis. Plant Physiol. 2011;157:1151–62.

7. Liebsch D, Sunaryo W, Holmlund M, Norberg M, Zhang J, Hall HC, et al. Class I KNOX transcription factors promote differentiation of cambial derivatives into xylem fibers in the Arabidopsis hypocotyl. Development. 2014;141:4311–9.

8. Woerlem N, Allaham G, Popescu A, Corrigan L, Pautot V, Hepworth SR. Repression of BLADE-ON-PETIOLE genes by KNOX homeodomain protein BREVIPEDICELLUS is essential for differentiation of secondary xylem in Arabidopsis root. Planta. 2017;245:1079–90.

9. He J, Benedeto VA, Wang M, Murray JD, Zhao PX, Tang Y, et al. The Medicago truncatula gene expression atlas web server. BMC Bioinformatics. 2009;10:441.

10. Couziou JM, Magne K, Mondy S, Cosson V, Clements J, Ratet P. The legume NOD-ORG-COCH-LIKE (NBL1) genes are conserved regulators of abscission, a major agronomical trait in cultivated crops. New Phytol. 2016;206:228–40.

11. Couziou JM, Magne K, Mondy S, Cosson V, Clements J, Ratet P. The legume NOD-ORG-COCH-LIKE (NBL1) genes are conserved regulators of abscission, a major agronomical trait in cultivated crops. New Phytol. 2016;206:228–40.

12. Couziou JM, Magne K, Mondy S, Cosson V, Clements J, Ratet P. The legume NOD-ORG-COCH-LIKE (NBL1) genes are conserved regulators of abscission, a major agronomical trait in cultivated crops. New Phytol. 2016;206:228–40.

13. Benedito VA, Torres-Jerez I, Murray JD, Andriankaja A, Allen S, Kakar K, et al. A gene expression atlas of the model legume Medicago truncatula. Plant J. 2008;55:504–13.

14. Holmes P, Goeffard N, Weiller GF, Rolfe BG, Inman N. Transcriptional profiling of Medicago truncatula meristematic root cells. BMC Plant Biol. 2008;8:21.

15. Aida M, Tasaka M. Genetic control of shoot organ boundaries. Curr Opin Plant Biol. 2006;9:72–7.

16. Khan M, Xu H, Hepworth SR. BLADE-ON-PETIOLE genes: setting boundaries in development and defense. Plant Sci. 2014;215:216:157–71.

17. Žádníková P, Simon R. How boundaries control plant development. Curr Opin Plant Biol. 2014;17:116–25.

18. Hepworth SR, Pautot VA. Beyond the divide: boundaries for patterning and stem cell regulation in plants. Front Plant Sci. 2015;6:1052.

19. Wang Q, Hasson A, Rossmann S, Therres K. Divide et impera: boundaries shape the plant body and initiate new meristems. New Phytol. 2016;209:485–98.

20. Jun JH, Ha CM, Fletcher JC. BLADE-ON-PETIOLE1 coordinates organ and apical polarity in Arabidopsis by directly activating ASYMMETRIC LEAVES2. Plant Cell. 2010;22:62–76.

21. Bell EM, Lin W-C, Husbands AY, Yu L, Jaganatha V, Jablonska B, et al. Arabidopsis LATERAL ORGANS BOUNDARIES negatively regulates brassinosteroid accumulation to limit growth in organ boundaries. Proc Natl Acad Sci 2012;109:21146–21151.

22. Ha CM, Kim G-T, Kim BC, Jun JH, Soh MS, Ueno Y, et al. The BLADE-ON-PETIOLE1 gene controls leaf pattern formation through the modulation of meristematic activity in Arabidopsis. Development. 2003;130:161–72.

23. Ha CM, Jun JH, Nam HK, Fletcher JC. BLADE-ON-PETIOLE1 and 2 control Arabidopsis lateral organ fate through regulation of LOM domain and Adaxial-Abaxial polarity genes. Plant Cell. 2007;19:1809–25.

24. McKim SM, Stenvik G-E, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, et al. The BLADE-ON-PETIOLE1 genes are essential for abscission zone formation in Arabidopsis. Development. 2008;135:1537–46.

25. Couziou JM, Magne K, Mondy S, Cosson V, Clements J, Ratet P. The legume NOD-ORG-COCH-LIKE (NBL1) genes are conserved regulators of abscission, a major agronomical trait in cultivated crops. New Phytol. 2016;206:228–40.

26. Ichishita Y, Kawade K, Usami T, Horiguchi T, Takahashi T, Tsukaya H. Key proliferative activity in the junction between the leaf blade and leaf petiole of Arabidopsis. Plant Physiol. 2011;157:1151–62.

27. Liebsch D, Sunaryo W, Holmlund M, Norberg M, Zhang J, Hall HC, et al. Class I KNOX transcription factors promote differentiation of cambial derivatives into xylem fibers in the Arabidopsis hypocotyl. Development. 2014;141:4311–9.

28. Woerlein N, Allaham G, Popescu A, Corrigan L, Pautot V, Hepworth SR. Repression of BLADE-ON-PETIOLE genes by KNOX homeodomain protein BREVIPEDICELLUS is essential for differentiation of secondary xylem in Arabidopsis root. Planta. 2017;245:1079–90.

29. He J, Benedeto VA, Wang M, Murray JD, Zhao PX, Tang Y, et al. The Medicago truncatula gene expression atlas web server. BMC Bioinformatics. 2009;10:441.

30. Dello Ioio R, Lhaires FS, Scacchi E, Casamajana-Martinez E, Heidstra R, Costantino P, et al. Cytokinin determination Arabidopsis root-meristem size by controlling cell differentiation. Curr Biol. 2007;17:678–82.

31. Ioio RD, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Monta MT, et al. A genetic framework for the control of cell division and differentiation in the root meristem. Science. 2008;322:1380–4.

32. Khan M, Xu H, Murmu J, Tabb P, Liu Y, Storey K, et al. Antagonistic interaction of BLADE-ON-PETIOLE1 and 2 with BREVIPEDICELLUS and PENNYWISE regulates Arabidopsis inflorescence architecture. Plant Physiol. 2012;159:640–60.

33. Schuetz M, Smith R, Ellis B. Xylem tissue specification, patterning, and differentiation mechanisms. J Exp Bot. 2013;64:11–31.
34. Lars Hennig, Köhler C. Plant Developmental Biology Methods and Protocols 2010.
35. Zhong R, Lee C, Zhou J, McCarthy RL, Ye Z-H. A battery of transcription factors involved in the regulation of secondary Cell Wall biosynthesis in Arabidopsis. Plant Cell Online. 2008;20:2761–82.
36. Okash-Haz K, Oda Y, Fukuda H. Arabidopsis VASCULAR-RELATED NAC-DOMAINS directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. Plant Cell. 2010;22:461–73.
37. Zhong R, Lee C, Ye Z-H. Global analysis of direct targets of secondary wall NAC master switches in Arabidopsis. Mol Plant. 2010;3:1087–103.
38. Bonke M, Thiramadee S, Mähönen AP, Hauser MT, Helauny A. APL regulates vascular tissue identity in Arabidopsis. Nature. 2003;426:181–6.
39. Rost TL. The organization of roots of dicotyledonous plants and the positions of control points. Ann Bot. 2011;107:1213–22.
40. Kong X, Liu G, Liu J, Ding Z. The root transition zone: a hot spot for signal crosstalk. Trends Plant Sci. 2018;23:403–9.
41. Campbell L, Turner S. Regulation of vascular cell division. J Exp Bot. 2017;68:27–43.
42. Ruonala R, Ko D, Helauny A. Genetic networks in plant vascular development. Annu Rev Genet. 2017;51:335–59.
43. Matsumoto-Kitano M, Kusumoto T, Takikawa P, Kinoshita-Tsujimura K, Vaclavikova K, Miyawaki K, et al. Cytokinins are central regulators of cambial activity. Proc Natl Acad Sci. 2008;105:20027–31.
44. Smetana O, Mäkilä R, Lyu M, Amiryousefi A, Sánchez Rodríguez F, Wu M-F, et al. High levels of auxin signalling define the stem-cell organizer of the vascular cambium. Nature. 2019;565:485–9.
45. Blondin S, Fonouni-Farde C, Frugier F. How Auxin and Cytokinin Phytohormones Modulate Root Microbe Interactions. Front Plant Sci. 2016;7:1–12.
46. Gamas P, Braun M, Jardinaud MF, Frugier F. Cytokinins in symbiotic nodulation: where, when, what for? Trends Plant Sci. 2017;22:792–802.
47. Kohlen W, Ng JLP, Deenim EE, Mathiesius U. Auxin transport, metabolism, and signalling during nodule initiation: indeterminate and determinate nodules. J Exp Bot. 2016;69:229–44.
48. Gonzalez-Rizzo S, Crespi M, Frugier F. The Medicago truncatula CRE1 cytokinin receptor regulates lateral root development and early symbiotic interaction with Sinorhizobium meliloti. Plant Cell. 2006;18:2680–93.
49. Nadzieja M, Kelly S, Stougard A, Reid D. Epidermal auxin biosynthesis facilitates rhizobial infection in Lotus japonicus. Plant J. 2018;95:101–11.
50. Hoffmann B, Trieth TH, Leung J, Kondorosi A, Kondorosi E. A new Medicago truncatula line with superior in vitro regeneration, transformation, and symbiotic properties isolated through cell culture selection. Mol Plant-Microbe Interact. 1997;10:305–17.
51. Limpons E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, et al. RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula. J Exp Bot. 2004;55:983–92.
52. Fahraeus G. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J Gen Microbiol. 1957;16:374–81.
53. Kulkova O, Franken C, Bisseling T. Methods in Molecular Biology. In: In situ hybridization method for localization of mRNA molecules in medicago tissue sections. New York, NY: Humana Press; 2018. p. 145–59.
54. Katsushima K, Natsume A, Ohka F, Shinjo K, Hatanaka A, Ichimura N, et al. Targeting the Notch-regulated non-coding RNA TUG1 for glioma treatment. Nat Comm. 2016;7:1–14.
55. Osteen JD, Herzig V, Gilchrist J, Ernck JJ, Zhang C, Wang X, et al. Selective spider toxins reveal a role for the Nav1.1 channel in mechanical pain. Nature. 2016;534:494–9.
56. Roux B, Rodde N, Jardinaud MF, Timmers T, Sauvaci L, Cottret L, et al. An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. Plant J. 2014;77:817–37.
57. van Velzen B, Holmer R, Bu F, Fluten R, van Zeijl A, Liu W, et al. Comparative genomics of the nonlegume Parasponia reveals insights into evolution of nitrogen-fixing rhizobium symbioses. Proc Natl Acad Sci U S A. 2018;115: E4700–9.
58. Katoch K. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30:3059–66.
59. Trifinopoulos J, Nguyen LT, von Haeseler A, Minh BQ, W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. Nucleic Acids Res. 2016;44W232–5.
60. Kalyaanamoorthy S, Minh BQ, Wong TKF, Von Haeseler A, Jermin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods. 2017;14:887–9.
61. Minh BQ, Nguyen MAT, Von Haeseler A. Ultrafast approximation for phylogenetic bootstrap. Mol Biol Evol. 2013;30:188–95.

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