The strigolactone biosynthesis gene **DWARF27** is co-opted in rhizobium symbiosis

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**Abstract**

**Background:** Strigolactones are a class of plant hormones whose biosynthesis is activated in response to phosphate starvation. This involves several enzymes, including the carotenoid cleavage dioxygenases 7 (CCD7) and CCD8 and the carotenoid isomerase **DWARF27** (D27). D27 expression is known to be responsive to phosphate starvation. In *Medicago truncatula* and rice (*Oryza sativa*) this transcriptional response requires the GRAS-type proteins NSP1 and NSP2; both proteins are essential for rhizobium induced root nodule formation in legumes. In line with this, we questioned whether MtNSP1-MtNSP2 dependent MtD27 regulation is co-opted in rhizobium symbiosis.

**Results:** We provide evidence that MtD27 is involved in strigolactone biosynthesis in *M. truncatula* roots upon phosphate stress. Spatiotemporal expression studies revealed that this gene is also highly expressed in nodule primordia and subsequently becomes restricted to the meristem and distal infection zone of a mature nodules. A similar expression pattern was found for MtCCD7 and MtCCD8. Rhizobium lipo-chitooligosaccharide (LCO) application experiments revealed that of these genes MtD27 is most responsive in an MtNSP1 and MtNSP2 dependent manner. Symbiotic expression of MtD27 requires components of the symbiosis signaling pathway; including MtDMI1, MtDMI2, MtDMI3/MtCCaMK and in part MtERN1. This in contrast to MtD27 expression upon phosphate starvation, which only requires MtNSP1 and MtNSP2.

**Conclusion:** Our data show that the phosphate-starvation responsive strigolactone biosynthesis gene MtD27 is also rapidly induced by rhizobium LCO signals in an MtNSP1 and MtNSP2-dependent manner. Additionally, we show that MtD27 is co-expressed with MtCCD7 and MtCCD8 in nodule primordia and in the infection zone of mature nodules.

**Keywords:** **DWARF27**, CCD7, CCD8, *Medicago truncatula*, Nodulation, Lipo-chitooligosaccharide, Nod factors, Rhizobium, Phosphate starvation, Strigolactones

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**Background**

Legumes evolved the capacity to live in an intimate endosymbiosis with nitrogen-fixing rhizobium bacteria. To host rhizobia intracellularly, nodules are formed on the root of the plant. These nodules provide optimal physiological conditions to the bacteria to fix atmospheric nitrogen gas into ammonia. Recent studies have suggested a role for strigolactones in rhizobium symbiosis [1–6]. Here we focus on expression of the strigolactone biosynthesis gene **DWARF27** during the legume-rhizobium interaction.

Nodule formation is initiated upon perception of lipo-chitooligosaccharide (LCO) signals excreted by compatible rhizobium bacteria [7]. These signals mitotically activate root cortical and pericycle cells, resulting in the formation of a nodule primordium [7, 8]. Rhizobium LCOs (also known as Nod factors) are also required to initiate an infection process to establish intracellular accommodation of the prokaryotic endosymbiont. This infection process starts in curled root hairs where a tube-like structure is formed intracellularly, which guides the rhizobia to the newly formed nodule primordium. There, the rhizobia are released as organelle-like structures (named symbiosomes), which remain surrounded by...
Medicago truncata), and a nuclear localized Ca\(^{2+}\)ortholog (Medtr1g471050) was expressed at 5 days. They are produced by phosphate starvation. However, MtD27 is expressed throughout plant root capacity to access nutrients, especially immobile phosphates. The plant receives these nutrients from the fungi at the expense of carbohydrates. Although strigolactones are not essential for establishment of an endomycorrhizal symbiosis, they contribute significantly to increasing root infection levels.

Several reports suggest also a role for strigolactones in legume nodule formation. In Medicago sativa and M. truncatula, application of 0.1 \(\mu\)M GR24 was shown to promote nodule formation, whereas slightly higher concentrations inhibited nodule formation in M. truncatula [1, 5]. In pea (Pisum sativum) and Lotus japonicus, strigolactone deficient mutants were shown to produce less nodules, which could be rescued by external application of GR24 [2–4]. Furthermore, in M. truncatula root hairs MtD27 and MtCCD8 expression is increased at 5 days post rhizobium inoculation [6]. This implies that MtD27 expression is regulated also in a symbiotic context. However, the precise regulatory network remains unknown.

Here, we show that in M. truncatula MtD27 expression is induced by rhizobium LCOs in an MtNSP1 and MtNSP2-dependent manner, similar as found for the induction of MtD27 by phosphate starvation. However, only induction of MtD27 by rhizobium LCOs requires the symbiosis signaling cascade. Using promoter-reporter constructs, we show that MtD27 is expressed throughout nodule formation. After early activation in the epidermis, its expression becomes restricted to the nodule primordium and subsequently to the nodule meristem and infection zone. Furthermore, we show that in nodule primordia and mature nodules MtD27 is co-expressed with MtCCD7 and MtCCD8.

**Results**

MtD27 is involved in strigolactone biosynthesis

The M. truncatula D27 ortholog (Medtr7g095920) was identified previously [17]. By searching the M. truncatula genome annotation Mt4.0, we noted the presence of 3 close homologs of the original MtD27 gene. To get insight in the relation of these genes to MtD27, we conducted a phylogenetic analysis based on an alignment of D27(-like) proteins of different plant species, including rice and Arabidopsis (Fig. 1). This showed that these sequences grouped in three separate phylogenetic clades (Fig. 1), consistent with a previous report [38]. Interestingly, the D27 clade contains 2 proteins from M. truncatula: MtD27 [17] as well as a close homolog Medtr7g095920. This shows that M. truncatula contains 2 putative D27 genes that could function redundantly. Expression of MtD27 and subsequent strigolactone production is known to be induced by phosphate starvation [17]. To get first insight whether Medtr7g095920 may also have a function in strigolactone production in M. truncatula roots, we examined its expression pattern. Analysis of publically available microarray...
data showed that Medtr7g095920 expression is relatively low, and has limited overlap with expression of MtD27 [39]. In roots, Medtr7g095920 expression is not induced by phosphate starvation. This in contrast to MtD27 (Additional file 1) [17]. This suggests that under phosphate limiting conditions only MtD27 might be involved in strigolactone biosynthesis in M. truncatula roots.

Next, we determined whether MtD27 represents a functional enzyme in the strigolactone biosynthesis pathway. To this end, we generated M. truncatula compound plants bearing transgenic roots in which MtD27 expression was reduced through RNAi-mediated knock-down. This reduced MtD27 expression by ~65 %, though with substantial variation (Fig. 2a). In contrast, expression of Medtr7g095920 was not reduced, but lower than MtD27 (Fig. 2a). Measurements of root extracts as well as root exudates collected from the MtD27 RNAi roots showed a 45-55 % reduction in strigolactone concentrations compared to that in the empty vector control (Fig. 2b and c). Taken together, this shows that MtD27 is involved in strigolactone biosynthesis and represents a functional ortholog of OsD27 from rice.

Expression of MtD27 is increased upon perception of rhizobium LCOs in an MtNSP1 and MtNSP2-dependent manner

To obtain insight in the symbiotic function of MtD27, we first determined whether its expression is responsive to rhizobium LCOs. Quantitative RT-PCR (qRT-PCR) reactions on RNA isolated from M. truncatula roots treated with Sinorhizobium meliloti LCOs (~10^{-9} M) for 3 h revealed that expression of MtD27 is strongly induced (Fig. 3). To determine whether Medtr7g095920 is also responsive to rhizobium LCOs, we quantified its expression as well. This showed that, unlike MtD27,
Medtr7g095920 is not responsive (Fig. 3). The low expression of Medtr7g095920 in combination with its non-responsiveness to phosphate starvation and rhizobium LCOs let us to decide to focus further studies on the symbiotic function of MtD27.

To acquire more insight in the symbiotic responsiveness of MtD27, we conducted a time series qRT-PCR experiment. To this end, M. truncatula seedlings were grown in Fåhraeus slides [40], a system optimized to study early responses induced by rhizobium LCOs in the root epidermis. M. truncatula roots of wild-type plants were treated with S. meliloti LCOs (~10⁻⁹ M) for 0, 1, 2 and 3 h. Subsequently, total RNA was isolated from the so-called susceptible zone, a region of about 1 cm just above the root meristem. Expression analysis by qRT-PCR showed a slight induction of MtD27 already at 1 h post LCO application, and a strong >20-fold induction after 2-3 h compared to mock-treated roots (Fig. 4a). This timing and induction level is comparable to that of MtENOD11; a gene frequently used as marker for rhizobium LCO-induced signaling in M. truncatula [16, 41] (Fig. 4a).

Induction of MtENOD11 expression by rhizobium LCOs requires a signaling module downstream of LCO perception consisting of MtDMI1, MtDMI2 and MtDMI3/ MtCCaMK (the common symbiosis signaling module) and the transcriptional regulators MtNSP1, MtNSP2 and MtERN1 [42–44]. To investigate whether induction of MtD27 expression by rhizobium LCOs is also dependent on this signaling module, we conducted qRT-PCR on LCO-susceptible root zones of these mutant plants, using MtENOD11 as control. This revealed that MtD27 expression is not induced in Mtdmi1, Mtdmi2 and Mtdmi3 mutant roots (Fig. 4b), indicating that symbiotic induction of MtD27 requires the common signaling module genes. Previously, we demonstrated that MtD27 expression in non-inoculated roots is dependent on MtNSP1 and MtNSP2 [17]. Here, we found that MtD27 expression is still induced by LCOs in an Mttnsp1, Mttnsp2 and Mttnsp1Mttnsp2 mutant background, albeit significantly lower than in wild-type roots (~2-3-fold vs ~15-fold, respectively) (Fig. 4c). Next, we monitored MtD27 expression in the Mtnsr1 mutant, in which LCO-induced MtENOD11 expression is blocked [45] (Fig. 4d). In this mutant, the induction of
MtD27 by rhizobium LCOs is about half of that in the wild type (Fig. 4e). Taken together, these results show that MtD27 is a rhizobium LCO-responsive gene whose expression in a symbiotic context is largely dependent on the common signaling module and the transcriptional regulators MtNSP1, MtNSP2 and in part MtERN1.
Previously it was shown in *Vicia sativa* that the LCO inducibility of early nodulin genes is indirect, as an inhibition of protein synthesis by cycloheximide (CHX) blocks early nodulin genes expression [46]. As *MtD27* displays a comparable expression pattern as *MtENOD11*, we tested whether this gene is a primary target of rhizobium LCO signaling. To this end, *M. truncatula* seedlings were grown in Fåhraeus slides for 3 days and pre-treated with 50 μM CHX for 30 min prior to LCO treatment (10⁻⁹ M, 3 h). The expression of *MtD27* and *MtENOD11* in the susceptible root zone was monitored by qRT-PCR. This showed that in control plants *MtD27* expression is elevated by CHX treatment (Fig. 4f), suggesting that active protein synthesis is required to keep *MtD27* expression at basal levels. Additionally it showed that, like *MtENOD11*, *MtD27* expression is induced by rhizobium LCOs in the absence of CHX, but not in the presence of CHX (Fig. 4f). This indicates that induction of *MtD27* expression by rhizobium LCOs requires new protein synthesis and therefore suggests that induction of *MtD27* by rhizobium LCOs is indirect.

**MtD27** is co-expressed with **MtCCD7** and **MtCCD8** during nodule formation

To obtain insight in the symbiotic function of *MtD27*, its spatiotemporal expression pattern is analyzed. For this, a ~1 kb fragment representing the 5’ region upstream of the translastional start site was cloned into a binary transformation vector in front of a β-glucuronidase (GUS) encoding sequence. This construct was used to create *M. truncatula* compound plants carrying transgenic roots. In non-inoculated plants, grown on buffered nodulation medium (BNM) containing no nitrate, but a relatively high phosphate concentration (0.5 mM PO₄³⁻), *MtD27* expression was observed in the vasculature and pericycle (Fig. 5a and b). LCO (~10⁻⁹ M) treatment for 3 h induced *MtD27* expression in the root epidermis (Fig. 5c).

Next, we determined the spatial expression of *MtD27* following inoculation with *S. meliloti* strain 2011. This showed that at 4 days post inoculation (dpi), the *pMtD27::GUS* transgenic roots showed a patched GUS staining, which was associated with rhizobium root hair infections (Fig. 5d). At 7 dpi, the expression in the root ceased, but GUS activity accumulated in nodule primordia (Fig. 5e). Sectioning nodule primordia revealed expression of *pMtD27::GUS* in dividing pericycle and cortical cells (Fig. 5f). This expression maintains in the developing nodule primordium (Fig. 5g), but becomes more restricted in the mature nodule, where it is visible only in the meristem and distal infection zone (Fig. 5h).

As strigolactone biosynthesis requires at least three enzymes, we tested whether besides *MtD27*, also *CCD7* and *CCD8* are responsive to rhizobium LCOs in *M. truncatula*. To this end, we first identified the putative orthologs of *CCD7* and *CCD8* from the *M. truncatula* genome based on homology to Arabidopsis genes. This revealed that one copy of *CCD7* (Medtr7g045370; *MtCCD7*) and two copies of *CCD8* (Medtr3g109610 and Medtr7g063800) are encoded in the *M. truncatula* genome (Additional file 2). Medtr3g109610 was described previously as *MtCCD8* [6]. Analysis of the Medicago gene atlas showed that *MtCCD8* and Medtr7g063800 show a similar expression pattern, though expression of *MtCCD8* is about 10- to 20-fold higher when compared to Medtr7g063800 [39]. This also applies to expression of both genes during phosphate starvation and rhizobium LCO application (Additional file 3), and therefore we decided to focus on *MtCCD8* for the remainder of this study. Expression analysis by qRT-PCR on samples taken from plants grown in Fåhraeus slides showed that *MtCCD7* and *MtCCD8* were not induced at 3 h post rhizobium LCO application (Fig. 6a). In contrast, in roots grown on agar-solidified Fåhraeus medium supplemented with aminooxyvinylglycine (AVG) expression of *MtCCD8* is induced ~5-fold 3 h after application of rhizobium LCOs, whereas *MtCCD7* expression was not substantially affected (Fig. 6b).

To determine the spatial-temporal expression pattern of *MtCCD7* and *MtCCD8* promoter-reporter GUS constructs were created and introduced in *M. truncatula* roots using *A. rhizogenes*-mediated transformation. We noted a basal expression pattern of both genes in the young root tip, including the susceptible zone and did not observe a discernible change in expression pattern of neither *MtCCD7* nor *MtCCD8* following LCO treatment (Additional file 4). This suggests that of the *MtD27-MtCCD7-MtCCD8* biosynthesis module *MtD27* expression is most strictly controlled in a spatial-temporal manner. However, upon inoculation with *S. meliloti* it showed that in young (two-day-old) nodule primordia *MtCCD7* and *MtCCD8* are co-expressed with *MtD27* (Fig. 5f, 7a and b). Moreover, in mature nodules, *MtD27*, *MtCCD7* and *MtCCD8* are co-expressed in the nodule meristem and distal infection zone (Fig. 5h, 7c and d).

Previous studies on strigolactone deficient mutants revealed that strigolactones at least in part contribute to root nodule formation and functioning [2–4]. To test whether *MtD27* function is required for rhizobium symbiosis, we determined the nodulation phenotype of *MtD27* RNAi roots. Under these conditions, the *MtD27* RNAi construct reduced *MtD27* expression by >90% (Additional file 5a). Examination of the *MtD27* RNAi roots showed that they can be effectively nodulated (Additional file 5b). Sectioning ~40 nodules did not reveal any discernible difference.
in nodule morphology between nodules formed on control or MtD27 RNAi roots (Additional file 5c and d). This suggests that either the reduction in MtD27 expression is not sufficient to cause a phenotype or that MtD27 is not essential for root nodule development and functioning.

The induction of MtD27 expression by phosphate deprivation is independent of the common symbiotic signaling cascade

Besides rhizobium LCOs, also phosphate starvation elevates MtD27 expression in an NSP1 and NSP2-dependent manner [17]. Induction of MtD27 by rhizobium LCOs requires MtDMI3/MtCCaMK and in part MtERN1. DMI3/CCaMK is positioned directly upstream of NSP1-NSP2 in the LCO signaling pathway [7, 47], whereas ERN1 functions in concert with NSP1-NSP2 to regulate expression of MtENOD11 [48]. In line with this, we questioned whether MtDMI3/MtCCaMK and MtERN1 are also required to induce MtD27 expression in response to a low phosphate status. First, we determined to what extent the spatial MtD27 expression in M. truncatula roots is affected by different phosphate regimes. To this end,
Fig. 6 Expression of MtD27, MtCCD7 and MtCCD8 upon treatment with rhizobium LCOs. a Relative transcript abundance as determined by qRT-PCR of MtD27, MtCCD7 and MtCCD8 in M. truncatula root susceptible zones 3 h after mock or rhizobium LCO (10^{-9} M) treatment. RNA was isolated from plants grown in Fåhraeus slides. b Relative transcript abundance as determined by qRT-PCR of MtD27, MtCCD7 and MtCCD8 in M. truncatula root susceptible zones 3 or 6 h after mock or rhizobium LCO (10^{-9} M) treatment. RNA was isolated from plants grown on agar-solidified Fåhraeus medium supplemented with 1 μM AVG. Data shown represent means of 2-3 biological replicates that each were analyzed in 3-fold (technical replicates) ± SEM. For each gene, transcript abundance was normalized against that of the mock-treated wild type. Different letters above bars indicate statistical difference (p < 0.05, students’ t-test).

Fig. 7 Spatial expression pattern of MtCCD7 and MtCCD8 in M. truncatula nodule primordia and mature nodules. Expression patterns were analyzed in M. truncatula transgenic roots expressing promoter-reporter GUS constructs. a Longitudinal section through a root expressing the pMtCCD7::GUS construct 2 days post inoculation (dpi) with S. meliloti strain 2011. b Longitudinal section through a root expressing the pMtCCD8::GUS construct at 2 dpi. Arrowhead points at an infection thread growing inside the root hair cell. c Longitudinal section through a mature nodule expressing the pMtCCD7::GUS construct. d Longitudinal section through a mature nodule expressing the pMtCCD8::GUS construct. Scale bars are equal to 50 μm. Sections were counterstained with Ruthenium Red.
transgenic *M. truncatula* plants carrying the *MtD27* promoter-GUS reporter construct were grown in perlite for 2 weeks at high phosphate (200 μM PO₄³⁻), and subsequently transferred to no phosphate (0 μM PO₄³⁻) medium. Plant roots were stained histochemically for GUS activity 5 days after the transfer and compared to control roots. In all plants, GUS staining could be observed in the stele of the roots, as well as in the root apical meristems (Fig. 8a and b). Phosphate-starved roots displayed a much more intense staining than control roots, which was most clear in the root apical meristem (Fig. 8c and d). However, unlike treatment with rhizobium LCOs, phosphate starvation did not change the spatial expression pattern of *MtD27*.

Next, we determined whether *MtDMI3/MtCCaMK* and *MtERN1* are also essential to mediate *MtD27* expression by phosphate starvation. To this end, *M. truncatula* *MtDMI3* and *MtERN1* knockout mutants were grown in an aeroponic system containing medium with high phosphate (200 μM PO₄³⁻) and subsequently transferred to the medium containing no phosphate for 2 days. To determine the expression of *MtD27*, qRT-PCR was conducted on RNA isolated from the bottom 2-3 cm of the root. This study reveals that in both mutants induction of *MtD27* in response to phosphate deprivation is similar to the induction found in roots of wild-type plants (Fig. 8e and f). This indicates that the phosphate response of *MtD27* expression is independent of the symbiotic signaling genes *MtDMI3/MtCCaMK* and *MtERN1*.

**Discussion**

Several lines of evidence indicate that strigolactones play a role in the legume rhizobium symbiosis [1–6]. Here, we showed that expression of *MtD27*, a gene that is acting in the strigolactone biosynthesis pathway, is strongly elevated by rhizobium LCO-induced signaling. Additionally, we found that this gene is co-expressed with *MtCCD7* and *MtCCD8* in nodule primordia as well as in the infection zone of mature nodules. This suggests a putative function for these strigolactone biosynthesis genes during several stages of the legume-rhizobium interaction.
Studies with the strigolactone analog GR24 revealed a nodule enhancing effect when applied exogenously [1, 5]. In line with this, a severe reduction in endogenous strigolactone levels due to mutations in ccd7 or ccd8 is linked to a moderate decrease in nodulation efficiency [2–4]. Foo and Davies [2] conclude that, although strigolactones influence nodule initiation they are not essential. We were unable to confirm these results in MtD27 RNAi knockdown roots of M. truncatula. We cannot rule out that in our MtD27 RNAi experiments MtD27 expression is not sufficiently reduced to cause such moderate nodule phenotype. Alternatively, phenotypes caused by an altered D27 function may be weaker than that of mutants of ccd7 or ccd8. This hypothesis finds support by studies in Arabidopsis and rice, where shoot branching phenotypes were much more severe in ccd7 and ccd8 mutants when compared to d27 [21, 38]. Based on this, Waters et al. [38] speculate on residual bioactive compounds present in Atd27 mutants. If such a residual bioactive compound also exists in M. truncatula, it is possible that its activity is sufficient for proper nodule initiation and development.

It remains currently unknown how strigolactones promote nodule initiation. One possible mechanism is through promoting the formation of a nodular auxin maximum. Mathematical modelling predicts that such maximum is most likely created through a local reduction in the auxin transport capacity in the root cortex [49]. Such reductions in root auxin transport capacity have been observed following rhizobial inoculation [50, 51]. Strigolactone-deficient mutants of Arabidopsis show elevated auxin transport in both shoots and roots [52]. It is proposed that strigolactones act by targeting the PIN auxin-efflux carriers at both the gene expression and protein level [53–57]. Therefore, it is possible that rhizobium-induced strigolactone biosynthesis will affect auxin transport and as such contributes to create and/or maintain an auxin maximum during nodule formation. However, it is unlikely that strigolactones alone are sufficient to reduce the auxin transport capacity upon perception of rhizobium LCOs, as strigolactone-deficient mutants still form nodules, although less numerous than wild-type plants [2, 3]. Possibly, they could function redundantly to another signal, like for example cytokinin [51, 58–60] or flavonoids [61].

MtD27 expression is elevated within 1-2 h post LCO application, by which it is among the earliest responsive genes. This transcriptional activation is under control of the rhizobium LCO signaling network, which includes MtDM12, MtDM11, MtDM13/MtCCaMK, MtNSP1, MtNSP2 and in part MtERN1. We found that also expression of MtCCD7 and MtCCD8 was induced following application of rhizobium LCOs. However, these responses were less pronounced when compared to MtD27. Expression of MtCCD7 was only slightly affected by application of rhizobium LCOs, whereas induction of MtCCD8 was dependent on the growth system. Spatial-temporal expression analysis revealed that in a symbiotic context especially MtD27 expression is strictly controlled in a spatial-temporal manner. Under non-symbiotic conditions MtD27 is mainly expressed in the stele of the root, whereas rhizobium LCOs activate expression in the root epidermis. Such clear spatial-temporal regulation was not observed for MtCCD7 nor MtCCD8 as both genes have a much broader expression pattern under non-symbiotic conditions. Why MtD27 is strictly controlled under symbiotic conditions remains unknown. Expression of MtCCD7 and MtCCD8 might not be rate limiting, or alternatively, induction of MtD27 by rhizobium LCOs might be part of a priming response that prepares epidermal cells for the infection process [6].

Our data hint at a putative role for strigolactones in mature nodules, as MtD27, MtCCD7 and MtCCD8 are co-expressed in the nodule meristem and distal infection zone. This may suggest that strigolactones promote meristem functioning and/or rhizobial infection. Recently, it was shown that MtD27 and MtCCD8 are transcriptionally induced in infected root hairs [6]. Our data also indicate expression of MtD27 and MtCCD8 in cells that contain growing infection threads in the root nodule, supporting a putative function for MtD27 and MtCCD8 in the infection process. Furthermore, induction of MtD27 expression by rhizobium LCOs is partly dependent on MtERN1, a transcription factor required for infection thread development [45].

MtD27 is also transcriptionally activated by phosphate starvation stress and this induction is dependent on MtNSP1 and MtNSP2 [17]. We studied the spatial regulation of MtD27 in response to phosphate starvation and found that the spatial expression pattern remains unchanged, but MtD27 expression is increased in the stele and apical root meristem. The transcriptional activation of MtD27 in response to the phosphate status in the environment coincides with an increased exudation of strigolactones [29, 30, 34]. Generally, it is anticipated that this response is contributing to the attraction of endomycorrhizal fungi, which enhance phosphate acquisition from the environment [33, 36]. We tested whether the induction of MtD27 by phosphate starvation is dependent on the common signaling pathway as well as whether this response is (partially) dependent on MtERN1. Neither signaling components were involved in the phosphate starvation induced MtD27 expression. This indicates that the signaling pathways regulating transcriptional activation of MtD27 by rhizobium LCOs and phosphate starvation only share NSP1 and NSP2 (Fig. 9). Interestingly, MtD27 transcript abundance is reduced in an Mtnsp1/Mtnsp2 mutant background when compared to wild type [17].
Furthermore, we found that induction of this gene upon phosphate stress or LCO signaling still occurs in these mutants, although at very moderate levels (Figs. 4e and 8f). Taken together, this supports the hypothesis that MtNSP1 and MtNSP2 function in a parallel pathway to facilitate induction of MtD27 by rhizobium LCOs and phosphate starvation stress, rather than being part of the primary signaling cascades [7, 15].

The D27-CCD7-CCD8 biosynthetic module is largely conserved in higher plants. As we showed that MtD27 is transcriptionally activated in a spatial-temporal manner in response to rhizobium LCOs in M. truncatula roots, this gene may represent an excellent marker gene to study rhizobium-induced signaling in a phylogenetic context.

Conclusions
Here we showed that in M. truncatula, MtD27 expression is rapidly increased upon perception of rhizobium LCOs. The gene remains expressed in the dividing cells of the nodule primordium and at subsequent stages its expression becomes confined to the nodule meristem and distal infection zone of the mature nodule. Analysis of the expression of MtCCD7 and MtCCD8 showed that they are co-expressed with MtD27 in nodule primordia and mature nodules. Additionally, we show that symbiotic expression of MtD27 as well as its expression during phosphate starvation is dependent on the GRAS-type regulators MtNSP1 and MtNSP2. This suggests that the NSP1-NSP2-D27 regulatory unit is co-opted to function in rhizobium symbiosis.

Methods
Plant materials and growth conditions
M. truncatula Jemalong A17, dmi1-1 (C71) [9, 42], dmi2-1 (TR25) [62], dmi3-1 (TRV25) [10], nsp1-1 (B85) [14, 42], nsp2-2 (0-4) [13, 43], nsp1 nsp2 [17] and ern1 (bit1-1) [45] were used in this study. Plants were grown in a growth chamber at 20 °C under 16/8 photoperiod at 50 μmoles of photons m⁻² s⁻¹. For gene expression studies, plants were grown as previously described [17]. This time, plants were grown for ~2.5 weeks before subjecting them to a two-day phosphate starvation regime. Subsequently, 2-3 cm root segments including the root tip were cut and snap-frozen in liquid nitrogen.

For analysis of transcriptional induction of MtD27 by phosphate starvation, plants were grown as previously described [17]. This time, plants were grown for ~2.5 weeks before subjecting them to a two-day phosphate starvation regime. Subsequently, 2-3 cm root segments including the root tip were cut and snap-frozen in liquid nitrogen.

Phylogenetic reconstruction
Predicted proteomes of Glycine max (Wm82.a2.v1) [63], Lotus japonicus (Lj2.5) [64], Medicago truncatula (Mt4.0v1) [65], Oryza sativa (v7.0) [66], Populus trichocarpa (v3.0) [67] and Vitis vinifera (Genoscope.12X) [68] were obtained through Phytozone 10 (http://phytozone.jgi.doe.gov/). These proteomes were searched by BLAST using A. thaliana proteins (TAIR10, www.arabidopsis.org) as query. For phylogenetic reconstruction, full length (predicted) protein sequences were aligned using MAFFT v7.017 [69].
implemented in Geneious R6 (Biomatters, Auckland, New Zealand), using default parameter settings. After manual inspection, alignments were used for tree building using MrBayes 3.2.2 [70] implemented in Geneious R6, using default parameter settings, with the exception of the rate matrix, for which was used. Midpoint rooting was performed for better tree visualization.

Vectors and constructs

For promoter-GUS reporter assays, a ~1 kb (MtD27) or ~2 kb (MtCCD7 and MtCCD8) fragment upstream of the translational start site was amplified from *M. truncatula* Jemalong A17 genomic DNA using the primers listed in Additional file 6. The pMtD27 fragment and a β-glucuronidase (GUS)-encoding sequence were recombined into a pDONR-L4L1 and pDONR-L1L2, thereby creating pENTR4-1_pMtD27 and pENTR1-2-GUS, respectively. These two constructs were combined with a pENTR2-3_t35S and subsequently recombined into the binary destination vector pKGW-RR-MGW by a multisite gateway reaction (Invitrogen, Carlsbad, USA) to obtain the binary construct pKGWS7-GUS, respectively. For RNAi-mediated knockdown of MtD27, a 268-bp fragment was amplified from *M. truncatula* Jemalong A17 root cDNA, using primer pairs MtD27i-F and MtD27i-R (see Additional file 6), and cloned into pENTR-D-TOPO (Invitrogen, Carlsbad, USA), creating pENTR4-1_pMtD27 and pENTR1-2-pMtCCD7 and pENTR1-2-pMtCCD8, respectively. Subsequently, both constructs were recombined into pKGWF57-RR, containing a GUS-GFP fusion reporter, by a single-site gateway reaction (Invitrogen, Carlsbad, USA), creating pKGWF57-RR_pMtCCD7-GUS and pKGWF57-RR_pMtCCD8-GUS, respectively.

For RNAi-mediated knockdown of MtD27, a 268-bp fragment was amplified from *M. truncatula* Jemalong A17 root cDNA, using primer pairs MtD27i-F and MtD27i-R (see Additional file 6), and cloned into pENTR-D-TOPO (Invitrogen, Carlsbad, USA). The MtD27 RNAi fragment was recombined into the DsRed-modified gateway vector pK7GW1WG2(II)-RR driven by the CaMV35S promoter [71] to obtain the binary construct pK7GW1WG2(II)-RR-p3SS-MtD27-RNAi. For the empty vector control, a pENTR containing a ~70 bp multiple cloning site was recombined into the DsRed-modified gateway vector pK7GW1WG2(II)-RR driven by the CaMV35S promoter (Invitrogen, Carlsbad, USA). The MtD27 RNAi fragment was recombined into the DsRed-modified gateway vector pK7GW1WG2(II)-RR driven by the CaMV35S promoter [71] to obtain the binary construct pK7GW1WG2(II)-RR-p35S-MtD27-RNAi. For the empty vector control, pENTR containing a ~70 bp multiple cloning site was recombined into pK7GW1WG2(II)-RR to obtain the binary plasmid pK7GW1WG2(II)-RR-p35S-RNAi-control.

All vectors used in this study contain pAtUBQ10: DsRED1 as selection marker [72]. All cloning vectors are available upon request from Plant Systems Biology (V.I.B.- Ghent University).

Plant transformation and treatments

*A. rhizogenes*-mediated root transformation of *M. truncatula* was performed as previously described [72]. For treatments with rhizobium LCOs, compound plants were transferred to agar-solidified buffered nodulation medium (BNM; 0.9 % Daishin agar (Duchefa, Haarlem, The Netherlands)) [73] containing 1 μM aminoethoxyvinylglycine (AVG) (Sigma, St. Louis, USA). After 3 days, ~100 μl of rhizobium LCOs (~10⁹ M) was pipetted on top of the root susceptible zone. After 3 h, roots were fixed in 90 % acetone and subsequently stained for GUS activity. For nodulation assays, compound plants were transferred to perlite and watered with Fähraeus [74] medium without nitrate. One week after transfer, plants were inoculated with *S. meliloti* strain 2011 (OD₆₀₀ = 0.05-0.1). For the phosphate starvation experiment, compound plants were transferred into perlite and watered with half-strength Hoagland medium [75]. After one week, plants were removed from perlite and washed three times with demineralized water to get rid of the nutrient salts. Plants were re-planted in fresh perlite and watered with half-strength Hoagland medium with (200 μM PO₄³⁻) or without (0 μM PO₄³⁻) phosphate, respectively. After 5 days, plants were removed from perlite and stained for GUS activity.

Histochemical staining and microtome sectioning

For histochemical GUS staining of *M. truncatula* roots and nodules, samples were first rinsed three times with 100 mM phosphate buffer (PBS; pH = 7.2). Samples were transferred to GUS-staining buffer (contains 2 mM K₂Fe(CN)₆, 2 mM K₃Fe(CN)₆, 10 mM EDTA, 0.1 % Triton X-100 and 1 mg/ml X-Gluc salt (Duchefa, Haarlem, The Netherlands) in 100 mM PBS, pH = 7.2) and placed under vacuum for 30 min. Next, the samples were incubated in the dark at 37 °C for 3 h. Stained roots were rinsed with PBS (pH = 7.2) three times to stop the reaction.

For historesin embedding, roots and nodule samples were fixed with 5 % glutaraldehyde PBS (pH = 7.2) solution overnight. After fixation, the samples were rinsed with PBS (pH = 7.2) three times and dehydrated through ethanol gradients (20 %, 40 %, 60 %, 80 % and 100 %). Afterwards, the samples were embedded in Technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany), according to the manufacturer’s protocol. GUS-stained samples were sectioned to 7 μm using a microtome (Reichert-Jung, Leica Microsystems, Rijswijk, The Netherlands) and stained with 0.1 % Ruthenium Red for 15 min. Images were taken using a Leica DM5500B microscope equipped with a Leica DFC425C camera (Leica Microsystems, Wetzlar, Germany). Images were digitally processed using Photoshop CS6 (Adobe Systems, San Jose, USA).

qRT-PCR analysis

RNA was isolated from snap-frozen root material using the plant RNA kit (E.Z.N.A. Omega Biotek, Norcross, USA) following the supplier’s manual. cDNA was synthesized from 1 μg total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). qRT-PCR
reactions were set up in a 20 μl reaction system with 2× iQ SYBR Green Super-mix (Bio-Rad, Hercules, USA) and the iQ5 Real-time PCR detecting system according to the manufacturer’s manuals. All primers used in this study were designed using the qPCR settings of Primer3-Plus [76]. Relative expression values were calculated using the 2^ΔΔCt method, using M. truncatula ubiquitin (MtUBQ10) and poly pyrimidine tract-binding protein (MtPTB) as reference genes. Statistical significance was determined based on students’ t-test (unpaired, two tailed, equal variance). All primers used in this study are listed in Additional file 6.

Strigolactone analysis

M. truncatula root exudates and root extracts were purified and concentrated as previously described [17, 30] with minor modifications. Compound (MtD27 RNAi and Empty vector control) plants were grown on perlite and watered twice a week with 50 mL half-strength MS medium (Duchefa, Haarlem, The Netherlands). Seven days prior to strigolactone analysis pots were washed with 3 volumes half-strength MS medium without PO4.5 (Duchefa, Haarlem, The Netherlands) to initiate phosphate starvation. Strigolactone quantification was performed by comparing retention time and mass transitions with those of an available didehydro-orobanchol standard using ultra-performance LC coupled to MS/MS using [6,2H]2-epi-5-deoxystrigol as an internal standard, as previously described [77]. Didehydro-orobanchol MS/MS fragmentation spectra of M. truncatula were obtained as previously described [17]. Results were subjected to students’ t-test (unpaired, two tailed, equal variance).

Medicago gene atlas IDs

Probe IDs used for analysis of gene expression using the Medicago gene expression atlas [39] are listed in Additional file 7.

Availability of supporting data

All relevant supporting data can be found within the supplementary files accompanying to this article. The phylogenetic trees were deposited in TreeBase (www.treebase.org) and can be accessed using the following link: (http://purl.org/phylo/treebase/phylows/study/TB2:18414).

Additional files

Additional file 1: Relative transcript abundance of MtD27 and Medtr7g095920 upon phosphate starvation. Relative transcript abundance of MtD27 and Medtr7g095920 in roots of plants grown under low (20 μM) or high (2 mM) phosphate conditions. Data were obtained from the Medicago gene atlas [39]. Data represent means of three replicates ± SEM. Transcript abundance was normalized against MtD27 transcript abundance in roots grown under high (2 mM) phosphate conditions. Different letters above bars indicate statistical difference (p < 0.05, students’ t-test). (TIFF 737 kb)

Additional file 2: Bayesian phylogeny of CCD1, CCD7 and CCD8 proteins. Bayesian phylogeny of CCD1, CCD7 and CCD8 proteins from Arabidopsis (At), soybean (Glycine max) (Glyma), M. truncatula (Medtr), rice (Os), petunia (Petunia hybridia) (Ph), poplar (Populus trichocarpa) (Potri), pea (Pisum sativum) (Ps) and grapevine (Vitis vinifera) (VIT). CCD1 proteins were included as an outgroup. Medicago proteins are highlighted in red. Branch support is indicated by posterior probabilities. Terminals are labeled by their gene or genbank identifier. Mid-point rooting was applied for better tree visualization. (TIFF 3321 kb)

Additional file 3: Relative transcript abundance of MtD27, MtCCD7, MtCCD8 and Medtr7g063800 during phosphate starvation and upon rhizobium LCO treatment. (a) Transcript abundance of MtD27, MtCCD7, MtCCD8 and Medtr7g063800 in roots of plants grown low (20 μM) or high (2 mM) phosphate conditions. (b) Transcript abundance of MtD27, MtCCD7, MtCCD8 and Medtr7g063800 in mock-treated roots (+LCO) or roots treated with rhizobium LCOs (+LCO) for 6 or 24 h. Data were obtained from the Medicago gene atlas [39]. Data represent means of three replicates ± SEM. Transcript abundance was normalized against MtD27 transcript abundance in roots grown under high (2 mM) phosphate conditions (a) or in the 6 h mock-treated sample (b). Different letters above bars indicate statistical difference (p < 0.05, students’ t-test). (TIFF 2450 kb)

Additional file 4: Spatial expression pattern of MtCCD7 and MtCCD8 upon application of rhizobium LCOs. MtCCD7 and MtCCD8 spatial expression patterns were analyzed in M. truncatula transgenic roots expressing promoter-reporter GUS constructs. Roots were mock-treated or treated with S. meliloti LCOs (10^-3 M) for 3 h. Scale bars are equal to 0.5 mm. (TIFF 1705 kb)

Additional file 5: Nodule phenotype after knock-down of MtD27 through RNAi. (a) Relative transcript abundance as determined by qRT-PCR of MtD27 in M. truncatula transgenic roots expressing an empty vector control construct (EvI) or MtD27 RNAi construct (D27T). (b) Number of nodules formed on plants bearing transgen sic roots harboring an empty vector control construct (EvI) or MtD27 RNAi construct (D27T). (c) Section through a nodule formed on a root expressing the empty vector control construct. (d) Section through a nodule formed on a root expressing the MtD27 RNAi construct. Scale bars are equal to 100 μm. Data shown in (a,b) represent means of 6 (a) or 5 (b) biological replicates ± SEM. Different letters above bars indicate statistical difference (p < 0.05, students’ t-test). (TIFF 4222 kb)

Additional file 6: Primer sequences of all primers used in this study. The recombination sites in the sequences are shown in italic. (CSV 1 kb)

Additional file 7: Probe IDs used in this study as in the Medicago gene atlas. (CSV 180 bytes)

Abbreviations

BNM: Buffed nodulation medium; CHX: Cycloheximide; Dpi: Days post inoculation; GUS: β-glucuronidase; LCO: Lipo-chitooligosaccharide; PBS: Sodium phosphate buffer; qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

RG conceived of the study. RG, WCY and TB supervised the study. WK and AvZ performed functional characterization of MtD27. WL and TTX determined the spatiotemporal expression pattern of MtD27. WL and AvZ determined rhizobium LCO-induced expression of MtD27. AvZ determined the MtCCD7 and MtCCD8 spatiotemporal expression patterns. TTX and AvZ determined expression of MtD27 in response to phosphate starvation. AvZ and RG wrote the manuscript. WK, WL, TTX, WCY and TB assisted in editing the manuscript. All authors read and approved the final manuscript.

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References
1. Soto MJ, Fernández-Aparicio M, Castellanos-Morales V, García-Garrido JM, Ocampo JA, Delgado MJ, et al. First indications for the involvement of strigolactones on nodule formation in alfalfa (Medicago sativa). Soil Biol Biochem. 2010;42:383–5.
2. Foo E, Davies NW. Strigolactones promote nodulation in pea. Planta. 2011;234:1073–81.
3. Foo E, Yoneyama K, Huggill CJ, Quittenden LJ, Reid JB. Strigolactones and the regulation of pea nodule symbioses in response to nitrile and phosphate deficiency. Mol Plant. 2013;6:76–87.
4. Liu J, Novero M, Charnikhova T, Ferrandino A, Schubert A, Ruyter-Spira C, et al. Carotenoid cleavage dioxygenase 7 modulates plant growth, reproduction, senescence, and determinate nodulation in the model legume Lotus japonicus. J Exp Bot. 2013;64:1967–81.
5. De Cuypier C, Fromentin J, Ycsoo RE, De Keyser A, Guillotin B, Kunert K, et al. From lateral root density to nodule number, the strigolactone analogue GR24 shapes the root architecture of Medicago truncatula. J Exp Bot. 2014;66:137–46.
6. Breakspear A, Liu C, Roy S, Stacey N, Rogers C, Trick M, et al. The root hair "infestome" of Medicago truncatula uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. Plant Cell. 2014;26:6880–701.
7. Oldroyd GED. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol. 2013;11:252–63.
8. Xiao TT, Schilderink S, Moling S, Deinum EE, Kondorosi E, Franssen H, et al. Carotenoid cleavage dioxygenase 7 modulates plant growth, reproduction, senescence, and determinate nodulation in the model legume Lotus japonicus. J Exp Bot. 2013;64:1967–81.
9. Ané J-M, Kiss GB, Riely BK, Penmetsa RV, Oldroyd GED, Ayax C, et al. Nodulation and carotenoid cleavage dioxygenase 7 modulate plant growth, reproductive, senescence, and determinate nodulation in the model legume Lotus japonicus. J Exp Bot. 2013;64:1967–81.
10. Délaux P, Bécard G, Combier J. NSP1 is a component of the Myc signaling pathway. New Phytol. 2013;199:55–65.
11. Lin H, Wang R, Qian Q, Yan M, Meng X, Fu Z, et al. DWARF27, an iron-containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. Plant Cell. 2009;21:1512–25.
12. Alder A, Jamil M, Marzorati M, Bruno M, Vermathen M, Bigler P, et al. The path from beta-carotene to carotene, a strigolactone-like plant hormone. Science. 2013;339:1348–51.
13. Kalo P, Gleason C, Edwards A, Marsh J, Mitra RM, Hirsch S, et al. Nodulation of Medicago truncatula uncovers changes in cell cycle genes. Nature. 2011;469:58–63.
14. Maillet F, Poinssot V, André O, Puech-Pagès V, Haouy A, Gueinier M, et al. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature. 2011;469:58–63.
15. Supplemental data for "From lateral root density to nodule number, the strigolactone analogue GR24 shapes the root architecture of Medicago truncatula." J Exp Bot. 2014;66:137–46.
16. Strigolactones promote nodulation in pea. Planta. 2011;234:1073–81.
17. Oldroyd GED, Ayax C, et al. Carotenoid cleavage dioxygenase 7 modulates plant growth, reproductive, senescence, and determinate nodulation in the model legume Lotus japonicus. J Exp Bot. 2013;64:1967–81.
18. Breakspear A, Liu C, Roy S, Stacey N, Rogers C, Trick M, et al. The root hair "infestome" of Medicago truncatula uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. Plant Cell. 2014;26:6880–701.
19. Oldroyd GED. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol. 2013;11:252–63.
expressed during mycorrhization in arbuscle-containing cells. Mol Plant Microbe Interact. 2001;14:737–48.

42. Catoira R, Galera C, de Billy F, Pemnetta RV, Journet EP, Maillet F, et al. Four genes of Medicago truncatula controlling components of a nod factor transduction pathway. Plant Cell. 2000;12:1647–66.

43. Oldroyd GED, Long SR. Identification and characterization of nodulation-signaling pathway 2, a gene of Medicago truncatula involved in nod factor signaling. Plant Physiol. 2003;131:1027–32.

44. Andrianikaj A, Boisson-Demiér A, Frances L, Sauviciac L, Jaenouve A, Barker DG, et al. AP2-ERF transcription factors mediate nod factor-dependent MRED11 activation in root hairs via a novel cis-regulatory motif. Plant Cell. 2007;19:866–85.

45. Middleton PH, Jakab J, Pemnetta RV, Starker CG, Doll J, Kals P, et al. An ERF transcription factor in Medicago truncatula that is essential for nod factor signal transduction. Plant Cell. 2007;19:1221–34.

46. Vijn I, Martinez-Abarca F, Yang W-C, Neves L, Brussel A, Kammern A, et al. Early nodulin gene expression during Nod factor-induced processes in Vicia sativa. Plant J. 1995;8:111–9.

47. Madsen LH, Trinchese L, Jukiewicz A, Sullivan JT, Heckmann AB, Belk AS, et al. The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nat Commun. 2010;1:10.

48. Cerri MR, Frances L, Laloum T, Auriac M-C, Niebel A, Oldroyd GED, et al. The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nat Commun. 2010;1:10.

49. Deinum EE, Geurts R, Bisseling T, Mulder BM. Modeling a cortical auxin maximum for nodulation: different signatures of potential strategies. Plant Cell. 2012;19:362–73.

50. Mathesius U, Schlaman HR, Spank HP, Of Sautter C, Rolfe BG, Djordjevic MA. Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. Plant J. 1998;10:134–33.

51. Piet J, Wasson A, Ariell F, Le Signor C, Baker D, Mathesius U, et al. MiCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in Medicago truncatula. Plant J. 2011;65:222–33.

52. Domagalska MA, Leyser O. Signal integration in the control of shoot branching. Nat Rev Mol Cell Biol. 2011;12:211–21.

53. Bennet T, Sieberer T, Willett B, Boker J, Luschning C, Leyser O. The Arabidopsis MAX pathway controls short branching by regulating auxin transport. Curr Biol. 2006;16:553–63.

54. Crawford S, Shinohara N, Sieberer T, Williamson L, George G, Hepworth J, et al. Strigolactones enhance competition between shoot branches by dampening auxin transport. Development. 2010;137:2905–13.

55. Shinohara N, Taylor C, Leyser O. Strigolactone can promote or inhibit shoot branching by triggering rapid depletion of the auxin efflux protein PIN1 from the plasma membrane. Plant Physiol. 2011;151:5174–7.

56. Sasse J, Simon S, Gubeli C, Liu G-W, Cheng X, Friml J, et al. Asymmetric localization of the ABC transporter PaPDR1 trace paths of directional branching by triggering rapid depletion of the auxin efflux protein PIN1 from the plasma membrane. Plant Physiol. 2011;151:5174–7.

57. Koltai H. Strigolactones are regulators of root development. New Phytol. 2011;190:545–9.

58. Dello Ioio R, Linhares FS, Sabatini S. Emerging role of cytokinin as a signal transduction pathway. Plant Cell. 2007;19:2866–85.

59. Middleton PH, Jakab J, Pemnetta RV, Starker CG, Doll J, Kals P, et al. An ERF transcription factor in Medicago truncatula that is essential for nod factor signal transduction. Plant Cell. 2007;19:1221–34.

60. Vijn I, Martinez-Abarca F, Yang W-C, Neves L, Brussel A, Kammern A, et al. Early nodulin gene expression during Nod factor-induced processes in Vicia sativa. Plant J. 1995;8:111–9.

61. Madsen LH, Trinchese L, Jukiewicz A, Sullivan JT, Heckmann AB, Belk AS, et al. The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nat Commun. 2010;1:10.

62. Cerri MR, Frances L, Laloum T, Auriac M-C, Niebel A, Oldroyd GED, et al. The molecular network governing nodule organogenesis and infection in the model legume Lotus japonicus. Nat Commun. 2010;1:10.

63. Deinum EE, Geurts R, Bisseling T, Mulder BM. Modeling a cortical auxin maximum for nodulation: different signatures of potential strategies. Plant Cell. 2012;19:362–73.

64. Mathesius U, Schlaman HR, Spank HP, Of Sautter C, Rolfe BG, Djordjevic MA. Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. Plant J. 1998;10:134–33.

65. Piet J, Wasson A, Ariell F, Le Signor C, Baker D, Mathesius U, et al. MiCRE1-dependent cytokinin signaling integrates bacterial and plant cues to coordinate symbiotic nodule organogenesis in Medicago truncatula. Plant J. 2011;65:222–33.

66. Domagalska MA, Leyser O. Signal integration in the control of shoot branching. Nat Rev Mol Cell Biol. 2011;12:211–21.

67. Bennet T, Sieberer T, Willett B, Boker J, Luschning C, Leyser O. The Arabidopsis MAX pathway controls short branching by regulating auxin transport. Curr Biol. 2006;16:553–63.

68. Crawford S, Shinohara N, Sieberer T, Williamson L, George G, Hepworth J, et al. Strigolactones enhance competition between shoot branches by dampening auxin transport. Development. 2010;137:2905–13.

69. Shinohara N, Taylor C, Leyser O. Strigolactone can promote or inhibit shoot branching by triggering rapid depletion of the auxin efflux protein PIN1 from the plasma membrane. Plant Physiol. 2011;151:5174–7.

70. Sasse J, Simon S, Gubeli C, Liu G-W, Cheng X, Friml J, et al. Asymmetric localization of the ABC transporter PaPDR1 trace paths of directional strigolactone transport. Curr Biol. 2015;25:647–55.

71. Koltai H. Strigolactones are regulators of root development. New Phytol. 2011;190:545–9.

72. Dello Ioio R, Linhares FS, Sabatini S. Emerging role of cytokinin as a regulator of cell differentiation. Curr Opin Plant Biol. 2008;11:23–7.

73. Marhavy P, Bielach A, Abas L, Abuzeineh A, Dutcherqa J, Tanaka H, et al. Cytokinin modulates endocytic trafficking of PIN1, auxin efflux carrier to control plant organogenesis. Dev Cell. 2012;21:796–804.

74. Van Zeijl A, Op den Camp RMH, Deinum EE, Chamikova T, Franssen H, Op den Camp HJM, et al. Rhizobium lipochitooligosaccharide signaling triggers accumulation of cytokinins in Medicago truncatula roots. Mol Plant. 2015;8:1213–26.

75. Wasson AP, Pellerone Fl, Mathesius U. Silencing the flavonoid pathway in Medicago truncatula inhibits root nodule formation and prevents auxin transport regulation by rhizobia. Plant Cell. 2006;18:1617–29.

76. Endre G, Kereszt A, Kevei Z, Milea S, Kalf P, Kiss GB. A receptor kinase gene regulating symbiotic nodule development. Nature. 2002;417:962–6.

77. Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, et al. Genome sequence of the palaeopolyploid soybean. Nature. 2010;463:178–83.

78. Sato S, Nakamura Y, Kaneko T, Asanuma E, Kato T, Nakao M, et al. Genome structure of the legume, Lotus japonicus. DNA Res. 2008;15:227–39.