Root developmental programs shape the *Medicago truncatula* nodule meristem

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SUMMARY

Nodules on the roots of legume plants host nitrogen-fixing rhizobium bacteria. Several lines of evidence indicate that nodules are evolutionary related to roots. We determined whether developmental control of the *Medicago truncatula* nodule meristem bears resemblance to that in root meristems through analyses of root meristem expressed *PLETHORA* genes. In nodules, *MtPLETHORA1* and *2* genes are preferentially expressed in cells positioned at the periphery of the meristem abutting nodule vascular bundles. Their expression overlaps with an auxin response maximum and *MtWOX5* that is a marker for the root quiescent centre. Strikingly, the cells in the central part of the nodule meristem have a high level of cytokinin and display *MtPLETHORA 3* and *4* gene expression. Nodule-specific knock-down of *MtPLETHORA* genes results in reduced number of nodules and/or in nodules in which meristem activity has ceased. Our nodule gene expression map indicates that the nodule meristem is composed of two distinct domains in which different *MtPLETHORA* gene subsets are expressed. Our mutant studies show that *MtPLETHORA* genes redundantly function in nodule meristem maintenance. This indicates that Rhizobium has recruited root developmental programs for nodule formation.

Keywords: *Medicago truncatula*, nodule meristem, *PLETHORA* genes, *DR5*
INTRODUCTION

The interaction between legumes and soil-borne bacteria collectively known as rhizobia leads to the formation of new organs, root nodules (Stougaard, 2001; Limpens and Bisseling, 2003). As nodules are formed on roots it has been hypothesized that the nodule developmental program is derived from the lateral root developmental program (Nutman, 1948; Hirsch et al., 1997; Mathesius et al., 2000; de Billy et al., 2001; Roudier et al., 2003; Bright et al., 2005; Desbrosses and Stougaard, 2011). Recently, the expression of several root meristem regulators has been observed in the nodule meristem (NM) (Osipova et al., 2011; Osipova et al., 2012; Roux et al., 2014), thereby creating molecular support for this hypothesis. However, whether the identified genes function in the formation of NM and root meristem (RM), a prerequisite for concluding that the nodule developmental program is derived from that of the root, has remained unclear so far.

Root tissues are continuously replenished by stem cells and in Arabidopsis these stem cells are surrounding the quiescent center (QC) cells (Dolan et al., 1993). The QC functions as a so-called organizer and is essential for maintenance of the surrounding stem cells (van den Berg et al., 1997) and together they form the stem cell niche. The daughter cells of these stem cells form files of transit-amplifying cells and together with the stem-cell niche they form the RM (Heidstra and Sabatini, 2014). Auxin accumulation is critical for the specification of the stem cell niche in the Arabidopsis RM that co-localizes with an auxin concentration and response maximum (Sabatini et al., 1999; Blilou et al., 2005; Petersson et al., 2009). Several Arabidopsis transcription factors have been identified that are required for proper formation and function of the root stem cell niche, among them WUSCHEL-RELATED-HOMEOBOX 5 (WOX5; Sarkar et al., 2007), SCARECROW (SCR; Di Laurenzio et al., 1996; Sabatini et al., 2003) and four PLETHORAs (PLTs; Aida et al., 2004; Galinha et al., 2007). WOX5 transcript accumulates specifically in the QC and mutant analyses revealed that it is required for columella stem cell maintenance (Sarkar et al., 2007). PLTs are part of the small AINTEGUMENTA-like (AIL) gene clade of transcriptional regulators within the large AP2/ERF family (Horstman et al., 2014). Among this clade, PLT1-4 are essential for root formation as their higher order mutants are root-less (Galinha et al., 2007). In double
mutants of *plt1,plt2* stem cells and transit-amplifying cells are lost, while ectopic *PLT1* and *PLT2* expression is sufficient to induce root niche formation (Aida et al., 2004; Galinha et al., 2007). This shows that a combination of *PLT1* and *PLT2* is most indicative for RM activity. A gradient of PLT activity controls root zonation and the highest PLT concentration localizes to the stem cell niche (Mähönen et al., 2014).

Legume nodule formation is initiated by dedifferentiation of cortical cells which divide and form the nodule primordium. Upon infection by the microsymbiont, the nodule meristem (NM) is formed at the apex of the primordium (Timmers et al., 1999; Stougaard, 2001; Limpens and Bisseling, 2003). In the model legume *Medicago*, that forms nodules with a persistent meristem at its apex, nodule development can be divided in 6 stages based on the sequential pattern of anti- and periclinal cell divisions in inner cortical cell layers C3-C5, endodermis and pericycle (Xiao et al., 2014). The cluster of cells formed up till stage V is called the nodule primordium. It consists of 6-8 cell layers derived from pericycle and endodermis, about 8 cell layers of infected cells derived from the inner cortical cell layers C5 and C4 and a few cell layers derived from cortical cell layer C3 that will develop into the nodule meristem (Xiao et al., 2014). From stage VI onward the *Medicago* nodule apical meristem becomes functional and adds cells to different nodule tissues; the central tissue, consisting of infected and non-infected cells, and the peripheral tissues including the nodule cortex, endodermis and parenchyma. The latter contains vascular bundles, that develop from nodule vascular meristems (NVM) (Roux et al., 2014). The part of the NM that adds cells to the central tissue forms a large domain at the apex and is composed of 4-6 cell layers. Transition of meristem cells to the central tissue cells is accompanied with a switch from mitosis to endo-reduplication in the cells that become infected by rhizobia (Cebolla et al., 1999).

Recent studies confirmed the expression of a number of known Arabidopsis root meristem regulators in the nodule, among them *MtWOX5, MtPLT2* and *MtBBM/PLT4* (Osipova et al., 2011; Osipova et al., 2012; Roux et al., 2014). These genes appeared to be expressed in the central meristem region and at the tip of the nodule vascular bundles, where also a maximum DR5 activity is observed (Couzigou et al., 2013), suggesting that a root–like developmental program is operational in the NM. To functionally address
whether the nodule developmental program is regulated by similar key factors controlling the Arabidopsis root developmental program, we studied the expression of MtPLT genes in the NM and the effect of their knock-down on nodule formation. Based on these results we propose that the NM consists of a distinct central and peripheral meristematic domain and four MtPLT (MtPLT1-4) genes redundantly control nodule formation and NM maintenance. This is reminiscent of the described function of AtPLT genes in root development and suggests that rhizobia recruited major regulators of root development.

RESULTS

Medicago truncatula orthologues of AtPLT genes

Recent studies showed that orthologues of the Arabidopsis PLETHORA (PLT) genes named MtPLT2(Medtr4g65370) and MtBBM/PLT4(Medtr7g080460) are expressed in the NM (Boutilier et al., 2002; Hofhuis et al., 2013; Limpens et al., 2013; Roux et al., 2014). We asked whether also the other Medicago PLT orthologs are expressed in the NM and performed reciprocal BLAST searches using the AtPLT protein sequences as a query to identify their homologs in Medicago (Mt4.0v1; http://blast.jcvi.org/erblast/index.cgi?project=mtbe; Table1, Fig. S1). Alignment of all Arabidopsis and Medicago PLT protein sequences using Vitis vinifera as an outgroup shows that there exist single Medicago orthologues of AtBBM/PLT4 and AtPLT5 which we named MtPLT4(Medtr7g080460) and MtPLT5(Medtr4g127930), respectively (Fig. S1). The phylogeny of the AtPLT1/2 and AtPLT3/7 subclades indicates that in Medicago ancestral gene duplications have occurred, independent from those observed in Arabidopsis, generating Medtr2g09180 and Medtr4g65370 that reside in the AtPLT1/2 clade and Medtr5g031880 and Medtr8g068510 that reside in the AtPLT3/7 clade. Because of the independent gene duplication events in Arabidopsis and Medicago a direct orthology link between genes in the PLT1/2 and PLT3/7 clades cannot be drawn. Nevertheless, comparison of the expression patterns indicates that AtPLT3 and Medtr5g031880 are expressed in the RM, while AtPLT7 and Medtr8g068510 are not (Galinha et al., 2007; Prasad et al., 2011; http://mtgea.noble.org/v3/). Based on these data and to keep in line with the previously designated MtPLT2 (Limpens et al., 2013), we utilize from now on
the following nomenclature: \textit{Medtr2g098180} (\textit{MtPLT1}), \textit{Medtr4g065370} (\textit{MtPLT2}), \textit{Medtr5g031880} (\textit{MtPLT3}) and \textit{Medtr7g080460} (\textit{MtPLT4}) (Table 1). The proposed gene annotations were subsequently used to design primers (Table S5) to enable gene expression studies by qPCR. Our data reveal that all four \textit{MtPLT} genes are expressed in nodules, albeit at lower levels than in roots (Fig. 1A).

A pre-existing and growing root that can be inoculated to induce nodulation, is crucial for the analysis of \textit{MtPLT} function in nodules. Therefore the maintenance of the RM, a process for which in Arabidopsis four redundantly acting \textit{PLT} genes are essential, (Aida et al., 2004; Galinha et al., 2007; Mähönen et al., 2014), should be ensured. To this end the function of \textit{MtPLT} genes must be tested in the Medicago RM. At present only for \textit{MtPLT1, 2} and \textit{MtPLT4} mutants are available (http://bioinfo4.noble.org/mutant/), hampering the generation of a quadruple mutant in Medicago as a tool to determine via genetics whether the four \textit{MtPLT} genes are the redundantly acting orthologs of Arabidopsis \textit{PLT1, 2, 3} and \textit{4}. Instead, we reduced the expression of \textit{MtPLT1} and \textit{MtPLT2} (\textit{MtPLT1i,2i}), \textit{MtPLT3} and \textit{MtPLT4} (\textit{MtPLT3i,4i}) and of all four \textit{MtPLT} genes (\textit{MtPLTi}) simultaneously by RNA interference (RNAi) under the control of the 35S promoter by \textit{A. rhizogenes}-mediated root transformation (Fig. S2) (Limpens et al., 2004).

Eight days after transferring the transformed plantlets to perlite, we counted the number of roots growing from transgenic callus. On 18 calli of empty vector transformed plantlets 58 transgenic roots of more than 3 cm in length were grown (Fig.S2A-C, arrow; Table S1). In contrast, no transgenic roots longer than 3 cm were grown from 16 calli of 35S\textit{MtPLTi} plants. On these calli only 4 transgenic roots of 1-2 cm in length were grown (Fig. S2 H, I, arrowhead) and numerous small outgrowths were detected (Fig.S2 E,F, red). Analyses of the transgenic short roots shows that the RM is absent here, indicating the rapid differentiation of meristematic cells (Fig. S2 G-I). On 20 calli of 35S\textit{MtPLTi,2i} transgenic plants 13 short and 9 long transgenic roots were grown, while on 27 calli of 35S\textit{MtPLT3i,4i} plants 12 short and 66 long transgenic roots were grown (Table S1). Thus down-regulation of \textit{MtPLT1} and \textit{MtPLT2} has a more profound effect on RM maintenance than down-regulation of \textit{MtPLT3} and \textit{MtPLT4}. This shows that in analogy to Arabidopsis (Aida et al., 2004; Galinha et al., 2007; Mähönen et al., 2014), \textit{MtPLT1-4} genes...
redundantly act on root formation and growth and that down-regulation of all four MtPLTs severely affects root formation.

MtPLT genes are required for nodule development and NM maintenance

We next asked whether down-regulation of individual MtPLT expression influences nodule growth, as it was possible that individual members could have specific functions in nodules, despite redundant roles in root development. We reduced the expression of the individual MtPLT genes by RNA interference (RNAi) under the control of the 35S promoter by A. rhizogenes-mediated root transformation. We analysed nodules formed on at least 15 transgenic roots 15 days post inoculation in two experimental replicas. The level of MtPLT gene expression reduction was determined by qPCR on RNA isolated from roots and nodules (Fig. S3A-E). This showed that different degrees of RNA reduction were obtained for the different genes in roots as well as in nodules. Notably, RNAi was found to be specific for each of the targeted MtPLT genes (Fig. S3A-D). However, RNAi did not lead to a significant reduction in nodule number compared to the number of nodules formed on control roots in all replicas (Table S2). Next, we investigated in detail the effect of single MtPLT gene knock-down expression on nodule development by analysis of serial micro-sections of control and transgenic nodules and counting the cell layers in the meristem, infection and the fixation zone. Analyses of 20 control nodules, collected per replica, shows that the NM consists of 4-6 cell-layers and the central tissue of 16-19 cell layers distributed over 6-7 cell-layers in the infection zone and 10-12 cell layers in the fixation zone (Fig. 2A). We did not observe significant differences between the number of cell layers in single MtPLT knock-down and control nodules (Fig. S4, Table S3). Altogether, these results indicate that down-regulation of individual MtPLT genes had no significant effect on nodule development. Subtle effects, however, may remain uncovered due to the variation between transgenic roots obtained after a hairy root transformation (Limpens et al., 2004).

Down-regulation of MtPLT1 and MtPLT2 has a more profound effect on RM maintenance than down-regulation of MtPLT3 and MtPLT4. To demonstrate the effect of reducing gene expression of more than one MtPLT in nodules, we conducted RNA
interference using the *MtENOD12* promoter. During nodule ontogenesis this gene is activated in the nodule primordium, the NM and in the infection zone of mature nodules (Limpens et al., 2009; Limpens et al., 2013). We tested the effect of *ENOD12::MtPLTIi,2i, ENOD12::MtPLT3i,4i* and *ENOD12::MtPLTi* in three replicas on nodule growth and development. Importantly, *ENOD12::MtPLTi* did not affect transgenic root formation growing from callus upon *A. rhizogenes*-mediated transformation (Table S1).

The level of down-regulation of the *PLT* genes was determined by qPCR (Fig. 1B-D). We confirmed that *MtPLT1* and *MtPLT2* RNA levels were reduced in transgenic *ENOD12::MtPLTIi,2i* nodules, while *MtPLT3* and *MtPLT4* RNA levels were not (Fig. 1B). Similarly, *MtPLT3* and *MtPLT4* RNA levels were reduced in *ENOD12::MtPLT3i,4i* nodules, while *MtPLT1* and *MtPLT2* RNA levels were not (Fig. 1C). In transgenic *ENOD12::MtPLTi* nodules all four *MtPLT* genes were reduced in their expression, albeit to different levels (Fig. 1D). On transgenic *ENOD12::MtPLTi*, *ENOD12::MtPLTIi,2i* or *ENOD12::MtPLT3i,4i* roots the number of nodules was significantly reduced (Mann Whitney test, p<0.01 for *ENOD12::MtPLTi*; Mann Whitney test, p<0.05 for *ENOD12::MtPLTIi,2i* and *ENOD12::MtPLT3i,4i*; Table S4) compared to control roots.

All compound *ENOD12::MtPLT* RNAi transgenic nodules were smaller compared to nodules on control transgenic roots. To determine potential causes of the size reduction, we analysed longitudinal sections of transgenic nodules. We analysed the transgenic nodules collected from the three replicas, collected 15 days after inoculation and observed a high percentage of phenotypically aberrant nodules (Fig. 2, Table 2). We classified the observed phenotypes into two groups: class I nodules in which the number of cell layers in meristem and infection zone is reduced (Fig. 2B,D) and class II nodules that lack the NM and the infection zone (Fig. 2C,D). These class II nodules only consist of 6-10 layers of infected cells (Fig. 2C). Notably, a complete block of meristem formation still allows nodules with 6 layers of infected cells, which are derived from the C4 and C5 cortical cells (Xiao et al., 2014). These results indicate that *MtPLT* activity is needed for proper NM formation and maintenance, but not for the infection of
primordium cells. This does not exclude that for the infection of cell layers in the infection zone derived from the nodule meristem MtPLT gene activity may be required.

In nodules formed on ENOD12::MtPLT1i,2i and ENOD12::MtPLT3i,4i roots, the majority of the affected nodules grouped into class I (Table 2). In contrast, the majority of the ENOD12::MtPLTi nodules fall into class II (n= 11 out of 16, Table 2). These results show that the down-regulation of all four MtPLT genes simultaneously has a more dramatic effect on nodule meristem formation and maintenance than down-regulation of a combination of only two MtPLT genes. In conclusion, our results show that MtPLT genes redundantly affect NM formation.

MtPLT promoter activity marks the Medicago RM

The striking difference between PLT-directed root and nodule growth is that MtPLT3i/4i affects nodule growth, while Atplt3/Atplt4 knock-out and MtPLT3i/4i knock-down minimally affect root growth (Fig 2, Table 2, Table S1, Galinha et al., 2007). To seek for a putative explanation for this discrepancy, we compared expression patterns of the different MtPLT genes by using pMtPLT::GUS fusions in root and nodule and in situ hybridization (ISH) in nodule. MtPLT mRNA localization in nodules is in agreement with the GUS staining pattern observed from the respective promoter fusion indicating that the pMtPLT::GUS fusions reflect the true expression pattern of the corresponding genes (Roux et al., 2014; compare Fig. S5 and Fig. 6). In Arabidopsis, AtPLT3 and AtBBM/AtPLT4 are expressed in the RM in an overlapping but slightly different pattern compared to AtPLT1 and AtPLT2 (Galinha et al., 2007). Before testing the activity of MtPLT promoters in the NM, we first identified their activity pattern in the root and compared these to markers for auxin (DR5) and cytokinin (TCS) response and QC activity (WOX5).

In primary Medicago roots, cell files converge to a group of cells that are suggestive to be QC cells (Fig. 3A, arrow). Distal to the QC cells are the columella cells that accumulate starch granules (Fig. 3A). Similar to the pattern observed in Arabidopsis (Sabatini et al., 1999), the highest level of DR5::GUS expression is detectable in the proposed stem cell niche in Medicago roots of plants into which a DR5::GUS construct was integrated (Fig.
Comparison of MtPLT1::GUS (Fig. 3C), MtPLT2::GUS (Fig. 3D), MtPLT3::GUS (Fig. 3E) and MtPLT4::GUS (Fig. 3F) expression patterns shows that these are mostly overlapping in the RM. The highest expression domains coincide with the root stem cell niche similar to AtPLT gene expression patterns (Galinha et al., 2007). However, the MtPLT3::GUS (Fig. 3E) and MtPLT4::GUS (Fig. 3F) expression patterns extend in the vascular tissue (Fig. S6). It is interesting that AtPLT3 and AtPLT4/BBM fusion protein accumulation extends in the vascular tissue of the Arabidopsis root as well (Galinha et al., 2007). It has been shown that the activity pattern of MtWOX5::GUS also marks the proposed stem cell niche (Osipova et al., 2012). Hence, MtPLT::GUS, DR5::GUS and MtWOX5::GUS expression patterns can be used to mark RM-like compartments in Medicago nodule organogenesis.

**MtPLT::GUS promoter activity in nodule primordia**

The dramatic reduction in nodule numbers on the MtPLTi root, indicates that MtPLT gene activity is crucial for nodule primordium formation. If so, MtPLT genes should be expressed in nodule primordia (NP). To test this, we analysed sections of pMtPLT::GUS containing transgenic hairy roots for promoter activation in stage II-V nodule primordia (Fig 4) (Xiao et al., 2014). Stage II-III NP (Fig 4 A, C, E, D) are characterised by the presence of cell divisions in pericycle and the most inner cortical cell layer, whereas endodermis cells do not yet divide (Xiao et al., 2014) and are distinct from Medicago lateral root primordia, where endodermis cell division precedes inner cortical cell division (Herrbach et al., 2014). The promoters of all four MtPLTs are active in cells of stage II-III NP (Fig 4 A, C, E, D) and remain active in the later stages of nodule primordium development (Fig 4, B, D, F, H). These analyses revealed that the promoters of MtPLT1, 2, 3 and 4 genes are indeed activated in nodule primordia (Fig. 4), corroborating their crucial role in nodule formation.

**Patterns of MtPLT activation and auxin-cytokinin response mark distinct domains in the NM**

Cells in the Medicago NM divide for a prolonged time, suggesting that stem cells may contribute to the maintenance of the NM. DR5::GFP (Couzigou et al., 2013) and
MtWOX5::GUS (Osipova et al., 2012) activity patterns have been allocated to distinct peripheral regions in the NM abutting vascular bundles (Fig. 5A, B arrows). Assuming that DR5::GUS and MtWOX5::GUS co-localize to areas of stem cell activity in nodules, in analogy to the situation in roots, this observation suggests that stem cells are present in the NM periphery. Recently, the expression of several auxin responsive genes in the central part of the NM (Limpens et al., 2013; Breakspear et al., 2014; Roux et al., 2014) has been reported, suggesting that auxin signaling occurs in this region of the NM.

Indeed, upon prolonged time of incubation (16 hours) DR5 activity becomes detectable throughout the vascular bundles and the nodule apex (Fig. 5C, arrowhead), including the central part of the NM. Such dynamics of GUS staining is only observed in DR5::GUS nodules and suggests that auxin signalling occurs in the entire NM, albeit at different levels in the central and peripheral part of the NM.

For both MtPLT1::GUS and MtPLT2::GUS, we observed GUS activity foci in discrete domains within the nodule apex (Fig. 5D and E, arrows). These domains of high MtPLT1 and 2 promoter activity appear embedded in a region with lower GUS activation encompassing the NM. In contrast, MtPLT3::GUS and MtPLT4::GUS are activated throughout the nodule apex (Fig. 5F, G arrowhead).

To determine whether the expression patterns of DR5::GUS, MtWOX5::GUS, MtPLT1::GUS and MtPLT2::GUS in the NM periphery overlap, we analysed serial sections from the nodule apex downwards. DR5 and MtWOX5 activity is present in a sub-population of cells within the apex adjacent to the vascular bundle (Fig. 6A, D). In subsequent sections, the radial tissue organization of a vascular bundle becomes apparent and all cells of this vascular bundle display DR5 and MtWOX5 activity (Fig. 6B, E). Finally, within this radially organized domain, xylem (white arrow) and phloem can be discriminated. At the developmental stage corresponding to this position, the activity of both DR5 and MtWOX5 decreases (Fig. 6C, F). Serial sections through MtPLT2::GUS nodules reveals that the highest GUS activity is restricted to cells that are contiguous to nodule vascular bundles (Fig.6G-I, arrow), resembling the DR5 (Fig. 6A-C) and the MtWOX5 (Fig. 6D-F) promoter activity pattern. MtPLT1::GUS also displays its highest activity in nodule vascular meristem cells (Fig. 6J, arrow). These analyses show that
MtPLT1, MtPLT2, MtWOX5 and DR5 are active in pro-vascular tissue and cells abutting the pro-vasculature, in analogy with their expression pattern in Medicago roots (Fig. 3B,D; Osipova et al., 2011; Osipova et al., 2012). A lower MtPLT1::GUS and MtPLT2::GUS activity is observed in cells in the central part of the NM (Fig. 6, G-J, arrowhead). In contrast, representative sections of MtPLT3::GUS and MtPLT4::GUS stained nodules show that both mark the entire NM and, in addition, are also activated in cells of the infection zone (Fig. 6K, L), albeit at lower levels.

The co-localization of MtPLTs and high DR5 activity in the periphery of the NM suggests that an auxin-driven root-derived developmental program is operational in the nodule. In addition, several genes in the cytokinin signaling cascade are reported to be activated in the NM (Frugier et al., 2008; Plet et al., 2011; Mortier et al., 2014). To determine the cytokinin response distribution in the NM we studied the expression of TCS::GUS, a synthetic cytokinin responsive promoter (Muller and Sheen, 2008), in transgenic Medicago root and nodules. In roots, TCS::GUS activity includes mainly the QC and root cap and fades in the vasculature (Fig. 7A) which is similar to the activity in Arabidopsis roots (Zürcher et al., 2013). In contrast to the DR5::GUS activity pattern (Fig. 5A,C), TCS::GUS activity is equally distributed over the apex of the nodules (Fig. 5H). Longitudinal sections of these nodules show that TCS::GUS activity is confined to cells in the central part of the NM (Fig. 7B).

DISCUSSION

Here we analysed the effect of down-regulation and the expression pattern of Medicago orthologs of Arabidopsis PLT1, PLT2, PLT3 and BBM/PLT4 genes during root growth and nodule formation and maintenance in Medicago.

By RNAi-mediated down regulation we show that MtPLT1-4 genes act redundantly in Medicago root formation demonstrating their orthology to the Arabidopsis PLT1-4 genes. Nodulation specific down-regulation of MtPLT genes hampers nodule formation and growth. This is reminiscent of the redundant AtPLT function in root formation and growth (Galinha et al., 2007). Therefore we conclude that root developmental programs are co-opted for nodulation. Interestingly, whereas root growth in Arabidopsis is
minimally affected in plt3,plt4 plants (Galinha et al., 2007), nodule growth is affected in MtPLT3i,4i nodules. To seek for an explanation we analysed MtPLT expression in Medicago RM and NM.

In Arabidopsis roots the highest expression levels of PLT1-4 genes co-localize in the stem cell niche (Galinha et al., 2007), which is also marked by AtWOX5 (Sarkar et al., 2007) and DR5 activity (Sabatini et al., 1999; Blilou et al., 2005; Petersson et al., 2009). The root expression patterns of the Medicago and Arabidopsis orthologs are similar, with the exception of the extension of MtPLT3 and MtPLT4 expression higher up in the meristem and elongation zone. Therefore, the pattern of pMtPLT3::GUS and pMtPLT4::GUS in the root may point to a difference in regulation of these genes in Medicago and Arabidopsis. In nodules MtPLT1 and MtPLT2 are highly expressed in regions located at the periphery of the NM corresponding to the NVM. The highest auxin response activity and the activation ofMtWOX5::GUS (Fig. 2B; Osipova et al., 2012; Roux et al., 2014) coincide with the NVM. These expression patterns indicate that the developmental program directing peripheral tissue formation bears similarities to root developmental programs involving PLT genes (Galinha et al., 2007). However, in the absence of a suitable promoter that marks the NVM specifically, the effect of knock-down of MtPLT genes could not be tested in the NM periphery.

In addition to the high peripheral NM expression, MtPLT1 and MtPLT2 are expressed at lower levels in the central part of the NM, while MtPLT3 and MtPLT4 expression levels are comparable in both central and peripheral zones of the NM. In conclusion, based on root meristem markers DR5, MtWOX5, MtPLT1, MtPLT2, MtPLT3, MtPLT4 and TCS, distinct gene expression signatures can be distinguished within the NM. One region at the periphery of the nodule that includes the NVM for which the gene activity patterns suggest that an auxin/PLT-directed root-like developmental program is active at each of the vascular bundle tips. A second domain is marked by high TCS, MtPLT3 and MtPLT4 activity. Cells within this second domain are centrally positioned within the NM and give rise to the central tissue. We will refer to this latter domain as the nodule central meristem (NCM). Based on our results we propose that the NM is built up of two adjacent
meristems, the NVM and NCM, respectively. We predict that the different levels of \textit{MtPLT} transcripts have specific effects in the NVM and NCM.

While the NVM is characterised by a high auxin response, the NCM is characterised by a higher cytokinin and a lower auxin response. The lower level of auxin signalling in the NCM is however sufficient to induce the expression of several auxin responsive genes (Limpens et al., 2013; Breakspear et al., 2014; Roux et al., 2014). The expression of cytokinin signaling and synthesis genes like \textit{MtCre1}, \textit{MtARR4} (Gonzalez-Rizzo et al., 2006; Plet et al., 2011) and \textit{MtLOG1} (Mortier et al., 2014) in the NM is in line with our observations on cytokinin response in the NM. To what extent differences in hormone regimes are instructive in shaping the NVM and the NCM remains to be elucidated. Likewise, whether the co-localization of \textit{TCS}, \textit{MtPLT3} and \textit{MtPLT4} activity in the infection zone is required for the formation of this zone remains to be determined.

Despite the differences in expression patterns of \textit{MtPLT1} and \textit{MtPLT2} versus \textit{MtPLT3} and \textit{MtPLT4} in the NM, the phenotypes of \textit{MtPLT1i,2i} and \textit{MtPLT3i,4i} nodules did not differ from each other. This might be due either to the fact that RNAi-mediated knock-down was directed using the \textit{ENOD12} promoter and not under a NVM specific promoter, or to redundant activity of \textit{MtPLT} genes. Therefore, it remains unclear whether the differences in \textit{MtPLT} activity in the NCM and NVM are instrumental for the formation of functionally distinct meristems. Comparing genes differentially regulated by either set of \textit{MtPLTs} and analyses of expression patterns of \textit{MtPLT} genes in nodules on the Medicago \textit{lin} (Guan et al., 2013; Xiao et al., 2014) and \textit{noot} (Couzigou et al., 2013) mutants, in which the development of nodule vascular bundles and the NCM are uncoupled, may be informative on this matter. Such knowledge may also uncover mechanisms underlying the communication between the NVM and NCM domains to enable proper nodule growth.

Nodules are considered to be modified lateral roots. Like lateral root primordia, also nodule primordia are exclusively formed opposite the proto-xylem poles. In Arabidopsis, \textit{PLT}s are involved in lateral root formation (Hofhuis et al., 2013; Tian et al., 2014). Hence it is conceivable that also in Medicago \textit{PLT}s are involved in lateral root formation and have been co-opted by Rhizobium for nodule formation. Our phylogenetic analysis
indicates that \textit{PLT1/PLT2} and \textit{PLT3/PLT7} gene pairs in Arabidopsis and Medicago formed through independent gene duplication events. This suggests that despite the importance of the \textit{PLT1/PLT2} gene pair for root growth in both species (Aida et al., 2004, this study) a putative co-option mechanism for a function in nodulation was independent of the gene duplication event in Medicago. For the \textit{PLT3/PLT7} gene pair, in both species the \textit{PLT3} orthologs appear to be expressed in the primary root tip, whereas \textit{PLT7} orthologs are not (Hofhuis et al., 2013, this study, http://mtgea.noble.org/v3/). We show the importance of \textit{MtPLT3} for nodulation which suggests that also for this gene co-option was independent of the duplication event. \textit{PLT4} and \textit{PLT5} are present in both species as a single gene. It will be interesting to investigate whether Rhizobium has also co-opted existing pathways involving the additional \textit{MtPLT5} and \textit{MtPLT7} orthologs for the initiation and outgrowth of nodule primordia, in analogy to Arabidopsis lateral root formation (Hofhuis et al., 2013; Vilches-Barro and Maizel, 2015).

Finally, it may be revealing to identify Rhizobium-controlled genes involved in regulating the expression of \textit{MtPLT} genes to find out how root developmental programs are recruited to generate nodule primordia, form the NM and its subdomains and maintain nodule growth. This knowledge should uncover how Rhizobium has co-opted and subsequently modified existing developmental pathways.
Table 1. Accession numbers of *Arabidopsis thaliana* and *Medicago truncatula* PLETHORA genes.

The annotation for Medicago *PLT1* and *PLT2* genes is arbitrary (but following a previous annotation by Limpens et al., 2013) because both Medicago and Arabidopsis *PLT1* and *PLT2* genes were formed by independent gene duplication events (see Fig S1). *Medtr5g031880* is residing together with *Medtr8g068510* in the *PLT3/7* clade. Because the former one is, like *AtPLT3*, expressed in the RM and the latter not, we annotated *Medtr5g031880* as *PLT3*.

<table>
<thead>
<tr>
<th></th>
<th><em>Arabidopsis thaliana</em></th>
<th><em>Medicago truncatula</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PLT1</em></td>
<td>At3g20840</td>
<td>Medtr2g098180</td>
</tr>
<tr>
<td><em>PLT2</em></td>
<td>At1g51190</td>
<td>Medtr4g065370</td>
</tr>
<tr>
<td><em>PLT3</em></td>
<td>At5g10510</td>
<td>Medtr5g031880</td>
</tr>
<tr>
<td><em>PLT4 (BBM)</em></td>
<td>At5g17430</td>
<td>Medtr7g080460</td>
</tr>
</tbody>
</table>

Table 2. Distinct phenotypes were distinguished for *MtPLT* RNAi nodules.

N (#) is the total number of nodules collected over three independent biological replicas. Class I: reduced number of layers in C3 derived meristem cells and C4 and C5 derived infection zone. Class II: no meristem, no infection zone, only infected primordium cells derived from C4 and C5. I + II is the combined number of nodules with a phenotype. Significance of difference of phenotypes between *ENOD12::MtPLT1i,2i* or *ENOD12::MtPLT3i,4i* versus *ENOD12::MtPLTi* is P<0.05 (Fisher’s exact test). WT is wild type.

<table>
<thead>
<tr>
<th>RNAi</th>
<th>N (#)</th>
<th>WT (%)</th>
<th>Class I (%)</th>
<th>Class II (%)</th>
<th>I + II (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ENOD12::MtPLT1i,2i</em></td>
<td>54</td>
<td>19 (35)</td>
<td>25 (46)</td>
<td>10 (19)</td>
<td>35 (65)</td>
</tr>
<tr>
<td><em>ENOD12::MtPLT3i,4i</em></td>
<td>23</td>
<td>9 (39)</td>
<td>9 (39)</td>
<td>5 (22)</td>
<td>14 (61)</td>
</tr>
<tr>
<td><em>ENOD12::MtPLTi</em></td>
<td>21</td>
<td>5 (24)</td>
<td>5 (24)</td>
<td>11 (52)</td>
<td>16 (76)</td>
</tr>
<tr>
<td>control</td>
<td>50</td>
<td>47 (94)</td>
<td>3 (6)</td>
<td>0 (0)</td>
<td>3 (6)</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Constructs

DNA fragments of putative promoter regions of MtPLT genes (1.5 kb for MtPLT1; 1.3 kb for MtPLT2; 2.7 kb for MtPLT3 and 1.1 kb for MtPLT4) were generated via PCR on Medicago genomic DNA as a template using Phusion High-Fidelity DNA polymerase (Finnzymes) and specific primers (Table S5). Fragments were cloned into pENTR-D-TOPO (Invitrogen) verified by nucleotide sequence analysis and recombined into the modified Gateway vector pK7GWIWG2(II)-UBQ10::DsRED-GUS-GFP (Karimi et al., 2002).

DNA of single MtPLT genes for RNA-interference constructs were generated via RT-PCR on cDNA made from Medicago nodule RNA using Phusion High-Fidelity DNA polymerase and gene specific primers (Table S5). These fragments were used as templates for obtaining DNA fragments for double and quadruple RNAi constructs.

The PCR strategy for obtaining these latter fragments is based on the In-Fusion HD cloning kit user manual (Clontech Laboratories, Inc.) which relies on the use of short overlaps to directionally clone multiple fragments by PCR. The strategy is outlined in Table S6 and the primers which map to exonic DNA are given in Table S5. To generate MtPLT1-MtPLT2 and MtPLT3-MtPLT4 DNA fragments for double RNA interference constructs, the DNA fragments of single genes were diluted 1:500 and used as a template in a first PCR to introduce short overlaps. Subsequently, PCR products were diluted 1:500 and used in a second PCR to create a single amplicon (Table S6). This final PCR fragment was cloned into pENTR-D-TOPO and recombined into the Gateway compatible binary vector pENOD12-pK7GWIWG2(II)-UBQ10::DsRED (Limpens et al., 2004; Ivanov et al., 2012) to create the final RNAi construct.

Similarly, for the quadruple RNAi of MtPLT genes, the above generated MtPLT1-MtPLT2 and MtPLT3-MtPLT4 PCR fragments were amplified using primer combinations shown in Table S5 to introduce short overlaps. The obtained fragments were diluted and combined in a second PCR to create a single amplicon which was cloned into pENTR-D-TOPO and subsequently recombined into the Gateway compatible binary vector.
pENOD12-pK7GWIWG2(II)-UBQ10::DsRED or in 35S-pK7GWIWG2(II)-UBQ10::DsRED (Limpens et al., 2004; Ivanov et al., 2012)

**Hairy root transformation**

All constructed binary vectors were introduced into *M. truncatula* A17 through *A. rhizogenes*-mediated transformation as described (Limpens et al., 2004). Plants carrying transgenic roots were grown in perlite for 8 days for root phenotype and for 15 days in the presence of *S. meliloti* 2011 to induce nodules. For each experiment, at least 15 individual roots and nodules were examined. Statistical analyses on nodule numbers were conducted using the Mann-Whitney test for non-normal distributions, under the assumption that nodule formation in two groups of analysed nodulated roots is independent and ordinal. (For critical U values for the Mann-Whitney U test see [http://users.sussex.ac.uk/~grahamh/RM1web/Mann-Whitney%20worked%20example.pdf](http://users.sussex.ac.uk/~grahamh/RM1web/Mann-Whitney%20worked%20example.pdf))

**Expression analysis and histochemical GUS staining**

Plant tissues containing promoter-GUS fusion were incubated at 37ºC in 0.1 M NaH$_2$PO$_4$-Na$_2$HPO$_4$ (pH 7) buffer including 3% sucrose, 0.05mM EDTA, 0.5mg/ml X-gluc, 2.5mM potassium ferrocyanide and potassium ferricyanide. Incubation time varied depending on tissues and different promoter-GUS fusions. GUS stained roots were cleared by using chloral hydrate (Mayer et al., 1991). Whole mount pictures of roots were taken by an Axio Imager A1 microscope (Zeiss) supplied with Nomarski optics.

**Histological analysis and Microscopy**

Root tips and nodules were fixed in 5% glutaraldehyde in 0.1M phosphate buffer (pH7.2) for 1-2 hours under vacuum, then washed with 0.1M phosphate buffer 4 times for 15min, once with H$_2$O for 15 min, and dehydrated for 10min in 10%, 30%, 50%, 70%, 90% and 100% EtOH, respectively, and sequentially embedded in Technovit 7100 (Heraeus Kulzer, Germany). Sections were made at 5-10 μm using a microtome (RJ2035, Leica Microsystems, Rijswijk, The Netherlands), stained either by 0.05% toluidine blue (Sigma, Germany) or 0.1% ruthenium red (Sigma, Germany), mounted in Euparal (Carl
Roth, GmbH, Germany), and analysed with a Leica AU5500B microscope equipped with a DFC425c camera (Leica, Microsystems, Wetzlar, Germany). At least 10 GUS-stained nodules from each transformation experiment were sectioned and analysed. Representative sections are depicted.

RNA *In situ* Hybridization

The 15 days-old nodules were fixed with 4 % paraformaldehyde mixed with 3% glutaraldehyde in 50 mM phosphate buffer (pH7.4) and embedded in paraffin (Paraplast X-tra, McCormick Scientific)). Nodule sections of 7 μm were made using a microtome (RJ2035, Leica Microsystems, Rijswijk, The Netherlands). RNA in *situ* hybridization was conducted according to the user manual “The QuantiGene ViewRNA ISH Tissue 2-Plex Assay” (http://www.panomics.com/UserDocs). RNA *in situ* probe sets were designed and produced by Affimetrix, USA. Each set contains 20 oligonucleotide probes, each consisting of a target-specific region and a unique sequence upon which signal amplification is built. Probe sets for *MtPLT1* covered the region between 122-1163bp of the full length mRNA (1569bp), for *MtPLT2* it covered the region between 317-1289bp of the full length mRNA (1632bp), for *MtPLT3* it covered the region between 123-1150bp of the full length mRNA (1545bp) and for *MtPLT4* it covered the region between 586-1529bp of full length mRNA (2070bp). Slides were analysed with a Leica AU5500B microscope equipped with a DFC425c camera (Leica, Microsystems, Wetzlar, Germany).

**ACKNOWLEDGEMENTS**

We thank Tom Guilfoyle for sharing DR5, Bruno Müller for TCS and Gabino Sanchez-Perez for help with the phylogenetic analysis. This work was supported by the Netherlands Organization for Scientific Research (WOTRO 86-160, X.W.)
AUTHOR CONTRIBUTIONS

HJF, OK and RH developed the approach; HJF, TTX, OK and XW performed experiments; HJF, OK, TTX, BS, TB and RH edited the manuscript prior to submission.
Fig. 1. Quantification of *MtPLT* expression levels in non-transgenic roots and nodules and RNAi nodules.

(A) Relative *MtPLT* expression in 15 d old nodules (white bar) compared to their expression in roots (grey bar) shows that expression in nodules is lower than in roots (expression is normalized to 1 in roots for each *MtPLT* gene); (B-D) Relative *MtPLT* expression (white bars) in 15 d old transgenic nodules of *ENOD12::MtPLT1i,2i* (B); *ENOD12::MtPLT3i,4i* (C) and *ENOD12::MtPLTi* (D) with respect to expression in control nodules (grey bars, normalized to 1 for each *MtPLT* gene). Quantification was normalized using *MtACTIN-2* as reference gene. Shown are the means ±s.e.m. of two (A) or three (B-D) biological replicates. The value of each biological replicate is based on technical triplicates. Significance of expression reduction of tested *MtPLT* gene in RNAi versus control samples is indicated by * as P<0.05 in Student t test.
Fig. 2. RNA-interference of *MtPLT* genes affects *Medicago* nodule development.

(A) Control wild type nodule. Apart from wild-type looking nodules, two classes of nodules are formed when more than one *MtPLT* gene is knocked-down. (B) Representative class I nodule. The number of cell layers in meristem (M) and infection zone (IZ) is reduced. (C) Typical class II nodules lacking a meristem and infection zone. All infected cells in the fixation zone (FZ) originate from primordium cells derived from C4 and C5 cortex layers. (D) Comparison of the average number of cell layers in meristem (M), infection zone (IZ) and fixation zone (FZ) in 20 control (C) and 16 *ENOD12::MtPLTi* (5 class I and 11 class II phenotype) show that the meristem and infection zone are not present in class II nodules, whereas in class I nodules the number of meristem and infection zone cell layers is reduced (Student’s t-test, P< 0.05). Bars in A-C 75µm. Bars in D are s.e.m.
Fig. 3. *MtPLT* and *DR5* promoter activity in the Medicago root meristem.

(A) *Medicago truncatula* root tip stained with lugol to visualize starch granules. Cell files converge to a central point showing the presence of the presumptive QC cells (arrow). Distally are the columella cells that accumulate starch. (B) A *DR5::GUS* transgenic root shows *DR5* promoter activity in a cluster of cells encompassing the QC. (C) *MtPLT1::GUS*, (D) *MtPLT2::GUS*, (E) *MtPLT3::GUS* and (F) *MtPLT4::GUS* expression patterns are overlapping with the highest activity in and around the QC. Arrow indicates the location of the presumptive QC. Bars 75µm.
Fig. 4. *MtPLT* genes are activated in the nodule primordium.

(A, C, E, G) Nodule primordia at stage II (according to Xiao et al., 2014) showing *MtPLT1::GUS* (A), *MtPLT2::GUS* (C), *MtPLT3::GUS* (E) and *MtPLT4::GUS* (G) activity. Endodermis (arrow) cells did not divide yet, while cortex cells did. (B, D, F, H) Nodule primordia of stage III-V showing expression of *MtPLT1::GUS* (B, stage IV), *MtPLT2::GUS* (D, stage III), *MtPLT3::GUS* (F, stage III) and *MtPLT4::GUS* (H, stage V) activity. Bars 75µm.
Fig. 5. *DR5*, *MtWOX5*, *MtPLT* and *TCS* promoter activities in nodules.

(A, B) Top view of a *DR5::GUS* nodule (A) and *MtWOX5::GUS* (B) nodule shows GUS activity in distinct regions at the periphery of the NM (arrows). (C) Upon prolonged incubation time GUS activity becomes apparent throughout the NM in *DR5::GUS* nodules (arrowhead). (D, E) Top view on *MtPLT1::GUS* (D) and *MtPLT2::GUS* (E) nodules show highest GUS activity in discrete regions in the periphery in the NM (arrows), with a lower GUS activity throughout the NM (arrowhead). (F, G) *MtPLT3::GUS* (F) and *MtPLT4::GUS* (G) activity throughout the NM (arrowhead). (I) Top view of a *TCS::GUS* nodule marking the whole NM. All nodules were sampled 15 days after inoculation. Bars 75µm.
Fig. 6. DR5::GUS, MtWOX5::GUS and MtPLT::GUS expression patterns in nodules.

(A-C) Serial tangential sections of 2h-stained nodules to specifically localize the DR5::GUS activation region. DR5 activity first appeared in a group of cells (A) that morphologically appear different to surrounding cells in the NM. In subsequent sections, DR5 activity reached a maximum (B) and DR5 activity remains in cells that are part of the nodule vascular bundle (C). (D-F) Serial tangential sections of MtWOX5::GUS nodules shows a comparable pattern as DR5::GUS. White arrow indicates differentiation of xylem in the nodule vascular bundle (compare panel A and D, panel B and E, and panel C and F). (G-I) Serial sections of MtPLT2::GUS nodules show that the highest MtPLT2::GUS activity is in the NVM (arrows). A lower MtPLT2::GUS activity is in the central region of the NM (A-C, arrowhead). (J-L) Representative and illustrative serial sections showing that also MtPLT1::GUS expression is highest in the NVM (J, arrow), whereas MtPLT3::GUS (K) and MtPLT4::GUS (L) expression patterns show equal activity in NVM (arrow) and the central part of the NM (arrowhead). White arrow indicates differentiation of xylem in the nodule vascular bundle. Bars 75µm.
Fig. 7. *TCS::GUS* pattern in *Medicago* root and nodule.

(A) *TCS::GUS* stained root shows activity in columella and lateral root cap cells. (B) In nodules *TCS::GUS* activity is confined to the central region of the NM. Bars 75µm.
REFERENCES


