Running Head: *CDC16* in root and nodule development

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Knock down of CELL DIVISION CYCLE 16 reveals an inverse relationship between lateral root and nodule numbers and a link to auxin in

Medicago truncatula

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Abstract

The post-embryonic development of lateral roots and nodules is a highly regulated process. Recent studies suggest the existence of cross talk and interdependency in the growth of these two organs. Although plant hormones including auxin and cytokinin appear to be key players in coordinating this cross talk, very few genes that cross-regulate root and nodule development have been uncovered so far. This study reports that a homolog of CELL DIVISION CYCLE 16 (CDC16), a core component of the Anaphase Promoting Complex, is one of the key mediators in controlling the overall number of lateral roots and nodules. A partial suppression of this gene in *Medicago truncatula* leads to a decrease in number of lateral roots and a 4 fold increase in number of nodules. The roots showing lowered expression of *MtCDC16* also show reduced sensitivity to phytohormone auxin, thus providing a potential function of CDC16 in auxin signaling.
Introduction

As in all eukaryotic organisms, cell division in plants is strictly controlled by a concerted action of several key regulators such as cyclin-dependent kinases (CDKs) and cyclins (De Veylder et al., 2007). The progression of the cell cycle from one phase to another requires the targeted degradation of selected cyclin molecules mediated by two ubiquitin-mediated proteolytic pathways. The (SKP)1-Cullin/F-Box-protein (SCF) pathway acts in the G1 to S phase transition by degrading the D-type cyclins and other substrate proteins (Yanagawa and Kimura, 2005). The second pathway, mediated by Anaphase Promoting Complex/Cyclosome (APC/C), regulates the sequential destruction of A and B-type cyclins in a D-box or a KEN-box-dependent manner resulting in chromosome segregation and exit from mitosis (Genschik et al., 1998; Pfleger and Kirschner, 2000). Evidence of the role of the APC/C in plant development comes from studies of the Arabidopsis hobbit (hbt) mutant that shows defects in root growth. The HOBBIT gene is required for both cell division and cell differentiation in root meristems and encodes CDC27, a core subunit of APC/C (Blilou et al., 2002; Perez-Perez et al., 2008). Cebolla et al. (1999) used the root nodule system of the model legume Medicago truncatula to study the function of an APC/C activator, CCS52, which is homologous to the yeast APC/C activator CDH1. A nodule-specific homolog of CCS52, CCS52A, was found to be required to initiate endoreduplication in the dividing cells, and its down regulation affected nodule development, resulting in lower ploidy, reduced cell size and inefficient rhizobial invasion and nodule maturation (Vinardell et al., 2003; Kondorosi et al., 2005). T-DNA insertions in the Arabidopsis CDC16 and APC2 genes result in gametophytic lethality, due to the failure to degrade mitotic cyclins (Capron et al., 2003b; Kwee and Sundaresan, 2003). Although the completed Arabidopsis genome has allowed the identification of homologs of almost all vertebrate
APC/C subunits in plants (Capron et al., 2003a), the functions of most of these subunits still remains largely unexplored.

Direct links between root growth and auxin signaling have been well documented. Several Arabidopsis mutants with decreased auxin sensitivity often exhibit an overall defect in both primary and lateral root development (Hellmann and Estelle, 2002; Casimiro et al., 2003; Hellmann et al., 2003; Vanneste et al., 2005). A number of these auxin-resistant mutants belong to the SCF proteolysis pathway, supporting a role for the SCF pathway in auxin signaling (Teale et al., 2006; Benjamins and Scheres, 2008). Auxin appears to control lateral root development by promoting G1 to S transition in selected xylem pericycle cells, perhaps by targeting KRP2, a CDK inhibitor and E2F, an S phase inhibitor to SCF-mediated proteolysis (del Pozo et al., 2002; Himanen et al., 2002). Unlike SCF, the role of APC/C in auxin-mediated plant development is not clear. The only report that has so far integrated APC/C with auxin signaling pertains to the hobbit (hbt) mutant, which shows an increased resistance to exogenous auxin due to accumulation of Aux/IAAs in the roots (Blilou et al., 2002).

As in lateral roots, auxin is an important player in the development of nodules on the roots of leguminous plants (Beveridge et al., 2007). Studies with auxin-responsive reporter gene constructs have shown auxin’s participation in cortical cell reactivation and initiation of nodule primordia (Mathesius et al., 1998). The exogenous application of Nod factor results in a transient inhibition of auxin transport capacity in roots of Vicia sativa (Boot et al., 1999) and Trifolium repens (Mathesius et al., 1998). Consistent with this, localized application of synthetic auxin transport inhibitors on alfalfa roots induces pseudonodules (Hirsch et al., 1989). Complementing these findings, a more recent study in Medicago truncatula has demonstrated that increased auxin transport, caused by silencing the flavonoid pathway, results in reduced nodule formation in response to rhizobia
(Wasson et al., 2006). Finally, hypernodulating mutants like *sunn* and *skl* show defective long distance transport of auxin, further suggesting the importance of polar auxin transport, not only in regulating nodule induction but also in controlling nodule numbers (Prayitno et al., 2006; van Noorden et al., 2006).

In this report, we investigated the role of the APC/C component CDC16 in root and nodule development in *M. truncatula*. CDC16 was identified via microarray analysis as a gene that was significantly induced in roots of *M. truncatula* following inoculation by *Sinorhizobium meliloti* and in nodules relative to uninoculated roots (Kuppusamy, 2005), thus encouraging further functional analysis of this gene. To overcome the problem of the gametophytic lethality resulting from CDC16 knockout, as seen from analysis of an insertional mutation in *Arabidopsis* (Kwee and Sundaresan, 2003) we undertook an RNA interference (RNAi) approach to partially suppress the expression of CDC16 gene in *Agrobacterium rhizogenes*-transformed roots of *M. truncatula*. We report that roots transformed with the CDC16 RNAi construct (hereafter called Mtcdc16i roots) displayed a decreased sensitivity to auxin, defective primary root growth and fewer lateral roots. Interestingly, in response to *S. meliloti*, the Mtcdc16i roots showed almost 4-fold increase in number of nodules, suggesting that decreased sensitivity to auxin leads to a hypernodulation phenotype. Thus, this work highlights the importance of CDC16 in root and nodule development and indicates a possible role for this gene in auxin signaling.

**Results**

*MtCDC16* gene structure organization

The *MtCDC16* genomic and cDNA sequences were obtained from the *M. truncatula* BAC library (http://www.medicago.org-genome/blast.php) and the Medicago Gene Index, MtGI, version 8.0 (http://combio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=medicago) sequence databases respectively. Alignment
of the predicted \textit{MtCDC16} genomic region (~12 Kbp) with the full-length cDNA revealed that \textit{MtCDC16} is composed of 15 exons and 14 introns (Fig. 1A). As depicted in Fig. 1B, the \textit{MtCDC16} gene was predicted to encode a 540 amino acid sequence that exhibits approximately 70\% identity to the \textit{A. thaliana} CDC16 (Kwee and Sundaresan, 2003). Homologs of CDC16 in yeast and mammals contain 8-10 copies of a 34-amino acid tetra-tricopeptide repeat (TPR) motifs that form super helical structures that function in protein-protein interactions (D'Andrea and Regan, 2003; Passmore, 2004). The predicted \textit{MtCDC16} amino acid sequence contained a similar number and distribution of TPR motifs as seen in \textit{AtCDC16} (Fig. 1C). Southern blot analysis (Fig. 1D) and searches of the \textit{M. truncatula} Gene Index (MtGI, version 8.0) and the partially completed genomic sequence provided no evidence of additional \textit{MtCDC16} genes in \textit{M. truncatula} genome.

\textbf{Knockdown of \textit{MtCDC16} leads to reduction in lateral root number and root length elongation}

To silence \textit{MtCDC16} gene expression, the RNAi-inducing construct (pRNAi-CDC16) was designed to target the first two TPR units encoded in exon 1, along with part of the 5’-untranslated region (Fig. 1A) of the transcript. In each of three independently constructed populations of \textit{Mtcdc16i} roots (n=131 total roots examined), the root phenotype was found to vary in severity. To facilitate further analysis, we divided roots into two classes, using root length and lateral root numbers as the phenotypic criteria. The first class, designated as \textit{Mtcdc16i} (moderate) roots comprised on an average 30 \% of the transformed roots. This class of roots showed up to a 50\% reduction in the number of lateral roots, as compared to the control roots in which the GUS gene was silenced (henceforth called the \textit{gusi} or control roots). However, the root length of \textit{Mtcdc16i} (moderate) plants (Fig. 2B and D) was similar to that of the control plants (Fig. 2A and D). The second class of \textit{Mtcdc16i} roots comprised 60\% of the transformed roots (n
=131 roots) and showed almost 80% reduction in the number of lateral roots and an average of a 70% reduction in primary root length (Fig. 2C and D). Due to a more severe phenotype, this class was designated as *Mtcdc16i* (severe).

Quantitative RT-PCR analysis was performed on RNA isolated from three independent pools of control, *Mtcdc16i* (severe) and *Mtcdc16i* (moderate) roots (30 roots in each pool). This analysis revealed an average of 3-fold decrease in *CDC16* transcripts in *Mtcdc16i* (moderate) roots and an 8 to 9-fold decrease in the *Mtcdc16i* (severe) roots, consistent with the severity in the phenotype (Fig. 2E). To ensure that the decrease in transcript abundance was not limited to the region that was silenced, RT-PCR was carried out with a second pair of primers (Primer P2-P2') spanning a region downstream of the targeted region in addition to the primers targeting the silenced region (Primer P1-P1'). The two analyses showed a similar reduction in transcript accumulation of *MtCDC16* (Fig. 2E).

*MtCDC16*-suppressed roots display aberrant cell morphology

The *Mtcdc16i* plants with a severe phenotype often showed pronounced root tip defects. Microscopic analysis revealed that *Mtcdc16i* (severe) roots had cells that were irregular in shape and size at the distal end, with no clear meristem (Fig. 3B and D). This was in striking contrast to the regularity of the cell shape and tissue organization seen in the control (*gusi*) roots (Fig. 3A and C). This result indicates that a severe reduction in CDC16 expression leads to a dramatic disruption in the root meristem organization. Such root tip defects were not seen in *Mtcdc16i* (moderate) roots (Fig. S1) and hence detailed microscopic analysis was not carried out with these roots. Examination of the *Mtcdc16i* (severe) root elongation zone by light microscopy showed that the cortical cells in the elongation zone were comparable in length to that of the control roots (Fig. 3A). This suggested that the stunted root growth of the *Mtcdc16i* (severe) roots was likely due to the
defect in the root meristem caused by aberrant cell division rather than to a defect in cell elongation.

**A controlled suppression of MtCDC16 results in an increase in nodule number**

In order to elucidate the role of *MtCDC16* in nodule development, we carried out nodulation assays using *Mtcdc16i* (moderate) roots. *Mtcdc16i* (severe) roots do not survive in the nitrate-free medium used for nodulation assays and hence were not analyzed. In the presence of *S. meliloti*, 54 out of 60 *Mtcdc16i* (moderate) roots developed ~4 times more nodules than the control roots (n = 60). While the control roots developed 8-10 nodules per seedling (Fig. 4A), *Mtcdc16i* (moderate) roots typically developed 35-40 nodules (Fig. 4B). Most of the nodules on *Mtcdc16i* (moderate) roots were restricted to the primary nodulation zone and were more densely packed (Fig. 4B) than nodules on control roots. The primary nodulation zone of *Mtcdc16i* (moderate) roots was also longer than that seen in the control roots (10 ± 0.8 cm in *Mtcdc16i* (moderate) roots and 6 ± 1.2 cm in control roots; n = 30 roots examined). To characterize the *Mtcdc16i* symbiotic phenotype at the cellular level, we examined nodule sections at 12 days post-inoculation from control and *Mtcdc16i* nodules (n = 22 *Mtcdc16i* nodules examined from 12 plants; n = 11 control nodules examined from 7 *gusI* plants). The organization of the tissue layers in *Mtcdc16i* nodules was very similar to the control nodules, and contained a clear meristem, an infection zone containing infection threads, and bacteroid-containing cells in the nitrogen-fixing zone (Fig. 5A, B). However, the length of the infection zone in the *Mtcdc16i* nodules (0.14 µM ± 0.006) was significantly longer than the control nodules (0.08 µM ± 0.009) (p<0.01) (Fig. 5A). Also, the infection zone of the *Mtcdc16i* nodules occupied an average of 21% of total nodule length in contrast to the infection zone of control nodules that occupied an average of 13% total nodule length. There was no significant difference in the size of the nitrogen-fixing zone between *Mtcdc16i* nodules.
and control nodules. To further verify the status of symbiotic development in the
*Mtcdc16i* nodules, nitrogenase activity was assessed in nodulating *Mtcdc16i* and
control roots using two independent pools of samples (n = 10 ± 2 roots examined
in each pool). On a per root basis, *Mtcdc16i* and control plants possessed similar
nitrogenase activity, as measured by acetylene reduction to ethylene (Fig. 4C),
suggesting that on a per nodule basis the amount of nitrogen that was fixed was
comparatively lower in *Mtcdc16i* roots than in control roots. Similar negative
correlation between nodule number and nitrogenase activity/nodule has been
described in hypernodulating mutants in *M. truncatula* (Penmetsa and Cook,
1997; Penmetsa *et al.*, 2003) and other legumes (Wopereis *et al.*, 2000). To ensure
that the hypernodulating phenotype seen in the *Mtcdc16i* (moderate) roots is due
to the suppression of *MtCDC16* expression, a comparison of *MtCDC16* transcript
abundance was carried out in nodulating and non-nodulating parts of *Mtcdc16i*
and control root systems in three independent experiments (n= 60 roots examined
per pool). A significant decrease in *MtCDC16* expression was noted in both
nodulating and non-nodulating parts of *Mtcdc16i* roots as compared to the
corresponding control root systems (Fig. 4D). Moreover, in both control and
*Mtcdc16i* plants, the nodulating parts of the roots showed a relatively higher
*CDC16* transcript level than the non-nodulating parts of the same pool of roots.

*MtCDC16* expression is activated in the zone of cell division of growing roots
and nodules

To examine the temporal and spatial expression pattern of *MtCDC16*, its putative
promoter region (Fig. 1A) was fused to a glucuronidase (GUS) reporter gene. The
transcriptional activation of the reporter gene was monitored with and without
rhizobial inoculation in the transgenic hairy roots of *M. truncatula* generated in
the wild type ecotype A17. In the uninoculated roots, strong GUS expression was
observed at the root tip (Fig. 6A, arrowhead). Blue staining was also visible at the
site of lateral root primordia and emerging lateral roots (Fig. 6B and C
respectively) and it subsequently became confined to the tip as the lateral roots fully emerged out of the primary root (Fig. 6 D, arrowhead). In response to rhizobia, GUS expression was noted in the nodule primordia and emerging nodules (Fig. 6E and F respectively), and then became restricted to the meristematic zone of fully developed nodules (Fig. 6G, arrowhead).

**MtCDC16 gene expression is auxin regulated**

Plant mutants that show defects in different subunits of the SCF ubiquitin ligase exhibit a decreased sensitivity to the plant hormone auxin (Hellmann *et al.*, 2003; Risseeuw *et al.*, 2003; Quint *et al.*, 2005). Since CDC16 functions as a component of APC/C (Passmore, 2004), another important member of the family of ubiquitin ligases, we hypothesized that *MtCDC16* is regulated by auxin. Previous reports have demonstrated that TGTCTC motifs in auxin response elements (AuxRE) confer auxin responsiveness to some auxin regulated genes (Ulmasov *et al.*, 1997; Ulmasov *et al.*, 1995). Sequence analysis of the promoter region (-1 to -1000 bp upstream of the putative transcription initiation site) of *MtCDC16* revealed two such motifs at -488 and -581 bp upstream of the putative transcription initiation site providing initial clues that *MtCDC16* could be auxin regulated. To investigate whether the expression of *MtCDC16* is indeed auxin mediated, the effect of exogenous auxin on its transcriptional activity was investigated. The roots of the wild type seedlings depleted of endogenous auxin were incubated in 5 or 50 µM concentrations of 2,4- dichlorophenoxy acetic acid (2,4 –D) for 30 min and 2h. Fig. 7A presents the transcript abundance of *MtCDC16* in response to exogenous auxin, relative to that seen in comparable samples without application of exogenous auxin. The transcript level increased about 2 fold by 30 min after exposure to auxin and remained at elevated levels at 2 h of auxin treatment. In general, the fold induction was similar for the two concentrations of auxin used. Consistent with this finding, transgenic hairy roots from three independent replicates (n=30) carrying the *CDC16:GUS* reporter construct displayed strong
GUS staining in the vascular bundle and the root tip when exposed to 0.5 μM of synthetic auxin, 2,4-dichlorophenoxy acetic acid (2,4-D) for 2 days (Fig 7C). In contrast, in the absence of applied auxin, the transgenic roots (n= 30) showed GUS expression only at the root tip but no expression was discernible in the vascular bundle (Fig. 7B).

To determine whether the Mtcdc16i roots show resistance to applied auxin in comparison to control roots, we performed a dose-response assay measuring auxin inhibition of root elongation. The root growth assay revealed that growth of control root was inhibited at 0.3 μM 2, 4-D and above (data not shown). At 0.5 μM 2, 4-D, the control roots from three independent experiments showed almost an 80% reduction in root length (Fig. 8A). The average length of the control roots with no auxin treatment was ~10.1cm. In contrast, the average root length in plants exposed to 0.5 μM 2, 4-D auxin was around 2.2 cm. This concentration of 2, 4-D was chosen to evaluate the root growth inhibition of Mtcdc16i (moderate) roots. We examined 15 Mtcdc16i (moderate) roots from three independent experiments with and without 2, 4-D (n = 15±2 roots examined per pool). This analysis revealed that Mtcdc16i (moderate) roots displayed less than 40% inhibition in root growth at this concentration of 2, 4-D, suggesting that they were much less sensitive to exogenous auxin than were the control roots (Fig. 8A). To further investigate the auxin responsiveness of Mtcdc16i roots, the expression of two transcripts corresponding to TC106907 and TC101963, that encode proteins similar to auxin responsive GH3 and an auxin induced protein, respectively, was evaluated in two independent experiments. As seen in Fig. 8B, their transcript levels were similar in the control roots and in Mtcdc16i roots in the absence of 2, 4-D treatment, when the constitutively expressed gene TC105607 was used to normalize values. By contrast, after exposure to 5.0 μM 2, 4-D for 30 minutes, the transcript abundance of TC106907 and TC101963 increased about 3-
5 fold in control roots, but showed only slight increase in Mtcd16i roots (n = 20 ± 2 roots examined in each pool).

Discussion
The continuous mitotic activity in the root apical meristem results in the indeterminate growth of primary roots. In primary roots, the activation of pericycle cells leads to de novo meristem and lateral root formation (Jiang and Feldman, 2005). Similarly, in leguminous plants, the presence of rhizobia elicits mitotic reactivation of root cortical cells and formation of meristems that develop into nitrogen fixing symbiotic nodules (Gage, 2004). Previous studies have identified several core cell cycle-regulating genes that function in both root and nodule formation (Foucher and Kondorosi, 2000; Himanen et al., 2002). The expression and functional analysis of a number of these genes provides a molecular basis that supports a possible integration in the development of these two organs, possibly via auxin signaling (Aloni et al., 2006).

In this study, we took a reverse genetic approach to demonstrate that MtCDC16, a homolog of a core component of the anaphase promoting complex (APC/C), plays a key role in the developmental pathway of both roots and nodules. The MtCDC16 silenced root system revealed impaired mersitem maintenance. This finding correlates well with the study by Vanstraelen et al. (2009) that showed that APC/C^CCS52A complexes control meristem maintenance in Arabidopsis roots. Interestingly, down regulation of MtCDC16 resulted in mature nodules that appeared similar to control nodules in terms of tissue organization except for an enlarged infection zone. Such discrepancies in the zonal length have been observed in nodules that exhibit defects in endoreduplication or in bacteroid differentiation (Vinardell et al., 2003; Vernie et al., 2008). Based on our results and the previous reports, we postulate that in Mtcd16i nodules, the timing of endoreduplication is impaired resulting in a delay in the conversion from
uninfected type cells with infection threads (an apoplastic compartment), to infected type cells containing bacteroids in intracellular compartments (symbiosomes). The delay of endoreduplication may impair the release of the rhizobia from the infection threads and/or impair conversion of type 2 to type 3 bacteroids thus slowing down the differentiation process and perhaps reducing the number of nitrogen fixing bacteroids in the nodules. Although further experiments will be needed to test these hypotheses, it is consistent with our data that on a per nodule basis the Mtcdc16i roots fixed relatively lower amount of nitrogen compared to the control roots.

Since the development and structure of legume nodules resemble in some respects that of lateral roots, it has been proposed that their ontogeny may require the same molecular signals (Nutman, 1948; Ferguson and Mathesius, 2003). Supporting this proposition, nts mutants in soybean and har1 mutants in L. japonicus exhibit an excessive number of both nodules and lateral roots (Wopereis et al., 2000). The sequence of the HAR1/NARK protein is very similar to the Arabidopsis CLAVATA1-like receptor kinase that mediates cell-cell signaling during shoot meristem development (Nishimura et al., 2002). Interestingly, suppression of MtCDC16 in M. truncatula roots leads to a decrease in the number of lateral roots, but a simultaneous increase in the number of nodules. Such an inverse relationship was observed even at the stage of primordial development (data not shown) thus suggesting that the Mtcdc16i root and nodule phenotype is not due to an impaired outgrowth but most likely due to abnormal initiation of the two organs. Existence of such inverse relationship between nodule and lateral root development was also noticed in roots silenced for a homolog of CYTOKININ RESPONSE ELEMENT 1 (MtCRE1) in M. truncatula. MtCRE1-silenced roots showed significant increase in lateral root development with a simultaneous reduction in nodulation (Gonzalez-Rizzo et al., 2006).
Several studies have suggested that one of the common regulatory signals controlling the development of roots and nodules could be the plant hormone auxin. Using an auxin-responsive \textit{GH3} promoter, Mathesius and co-workers (Mathesius \textit{et al.}, 1998) showed that the two organs share similar auxin requirements during both initiation and emergence. Supporting this idea, de Billy et al. (de Billy \textit{et al.}, 2001) demonstrated that the \textit{MtLAX} genes, members of the AUX1 gene family in \textit{M. truncatula}, are expressed at two common stages during lateral root and nodule development: primordium formation and vasculature differentiation. Independent from this, it has also been found that rhizobia are able to “hijack” the cells that are activated during lateral root development to form nodules (Mathesius \textit{et al.}, 2000).

Detailed genetic and developmental studies have clearly implicated auxin as the major molecular signal in promoting lateral root development. A larger number of lateral roots occur in plants treated with auxin or those containing a higher level of this hormone (Boerjan \textit{et al.}, 1995; Fukaki \textit{et al.}, 2005), whereas auxin response mutants such as \textit{slr-1} (Fukaki \textit{et al.}, 2002, Vanneste \textit{et al.}, 2005), \textit{aux1}, \textit{axr1} and \textit{axr4} (Hobbie and Estelle, 1994) produce fewer laterals. The physiological studies with auxin transport inhibitors like naphthylphthalamic acid (NPA) suggest the importance of polar auxin transport in lateral root development. Reed and colleagues (1998) showed that application of NPA at the root-shoot junction causes a block in the acropetal transfer of auxin in the root, thus inhibiting the lateral root formation. The importance of auxin in root development has been further emphasized by its role in SCF mediated proteolysis. Mutations in the components of the SCF complex often confer auxin response defects (Dharmasiri and Estelle, 2004; Parry and Estelle, 2006). Such studies with respect to APC subunits have been fairly limited. Our study has provided strong evidence that \textit{MtCDC16} is an auxin responsive gene and that the suppression of \textit{MtCDC16} results in auxin insensitivity. This indicates a possibility that auxin
could be a key regulatory signal for APC-mediated proteolysis during cell division. Conversely, it is also possible that the defects seen in Mtcdc16i roots are not primarily caused by altered auxin response but in fact are a direct consequence of hampered cell division either due to partial knockdown of MtCDC16 or alternatively due to the disassembly of the APC complex as a result of the knock down of MtCDC16. Such a possibility was tested with the hbt mutant (encoding CDC27) by Blilou (2002), who found that although HBT influences the stability of some auxin response regulators, the defect seen in the mutant was not due to perturbed auxin perception (Serralbo et al., 2006). Future studies on MtCDC16 will be directed towards investigating these possibilities.

Numerous studies so far have suggested the involvement of polar auxin transport in nodulation. Application of auxin transport inhibitors like NPA to roots results in nodule-like structures on the roots of alfalfa (Hirsch et al., 1989). Also, application of the rhizobial Nod factor was found to cause a transient reduction in auxin transport in vetch roots (Boot et al., 1999). In parallel to this, Mathesius et al. (1998) demonstrated that in white clover roots, spot-inoculation with rhizobia leads to an acropetal decrease in transport of auxin followed by its basipetal accumulation resulting in nodule primordium formation. In general, it has been suggested that rhizobial Nod factors cause a localized induction of flavonoids in the roots that in turn may perturb the polar auxin transport by acting as endogenous auxin transport inhibitors (Hirsch, 1992). The disturbance in the mobilization of auxin results in its localized accumulation that further activates cortical cell division and nodule formation. Consistent with this idea, it has been recently shown that flavonoid deficient roots generated by silencing the flavonoid biosynthetic pathway are much less competent in forming nodules and also show altered auxin transport (Wasson et al., 2006).

After inoculation with rhizobia, the Mtcdc16i roots form 3–4 fold more nodules as compared to control roots. Based on this result, it is tempting to speculate that
auxin-insensitivity might lead to an increase in nodule number in Mtcdc16i roots. Alternatively, reduction in numbers of lateral root meristems as a result of defective cell cycle progression could trigger initiation of more nodule meristems. This alternative hypothesis opens up a new possibility of an as yet undefined mechanism wherein the plant imposes a regulatory control that balances out the reduction in lateral root meristems with a greater number of nodule meristems in the Mtcdc16i root. Further study is needed to distinguish between these possibilities.

Two hypernodulating mutants of M. truncatula, sunn (Schnabel et al., 2005) and skl (Penmetsa and Cook, 1997), show an increased transport of auxin from shoot to root as compared to the wild-type plants (Prayitno et al., 2006; van Noorden et al., 2006). The wild-type plants show an increased flow of auxin from shoot to the root immediately after inoculation with rhizobia but this is followed by a significant decrease 24 hours after inoculation. In contrast, both sunn and skl maintain increased auxin transport for a longer period, even a day after inoculation. SUNN, a homolog of HAR1, controls nodule numbers through the autoregulation of nodulation pathway whereas SKL is known to regulate nodule numbers through the ethylene pathway (Penmetsa et al., 2008; Oka-Kira and Kawaguchi, 2006). In spite of the two genes being regulated by two different pathways, it is very striking that the corresponding mutants show a similar auxin transport phenotype, suggesting that auxin is a key regulator of nodule numbers. It will be interesting to determine whether Mtcdc16i roots are also altered in auxin transport. Whether due to auxin insensitivity or altered auxin transport, our data provide the possibility of a third gene, MtCDC16 that controls nodule number in an auxin-dependent manner.

**Experimental procedures**

**Plasmid construction**
To create an RNAi construct of *MtCDC16*, a region corresponding to -143 to 354 nt (relative to the ATG start codon) was amplified from a corresponding cDNA clone (pKVKC-6A3; GenBank # BQ165117). The amplified fragment was introduced into the RNAi-inducing pHellsgate 8 vector (Wesley *et al.*, 2001) using the GATEWAY system (Invitrogen, Carlsbad, CA). The pHellsgate 8 vector carrying a fragment of the glucuronidase gene was used as a control. The resulting recombinant constructs were introduced into *A. rhizogenes* ARqua1 and used for plant transformation as described (Ivashuta *et al.*, 2005).

**Plant growth conditions**

*M. truncatula* L416 seeds (A17 containing a *PROENOD11-GUS* construct (Journet *et al.*, 2001) were used for transformation and generation of transgenic roots on Fahraeus-agar medium supplemented with 22.5-27 mg/l of Kanamycin. The transgenic roots thus generated were used for scoring visible root phenotypes using Nikon SMZ1500 zoom stereomicroscope (DIAPHOT 200, New York, USA) between 7-10 days after transformation. For nodulation experiments, plants showing a visible root phenotype were transferred to either aeroponic chambers or to Turface (Profile Products LLC) and inoculated with *S. meliloti* ABS7M as described previously (Kuppusamy *et al.*, 2004). Plants transformed with different constructs were grown independently in different aeroponic systems. Nodules were scored using Nikon SMZ1500 zoom stereomicroscope (DIAPHOT 200, New York, USA) between 10–15 days post inoculation (dpi) in plants grown in aeroponics and at 20 dpi in those grown in Turface.

**Gene structure and promoter-reporter fusion analysis of *MtCDC16***

The cDNA clone, pKVKC-6A3 (GenBank # BQ165117), the sequence of which is similar to *Arabidopsis CDC16*, was identified at the Medicago Gene Index-MtGI, version 8.0 ([http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=medicago](http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=medicago)). The clone was sequenced in its entirety from
the 3’ end. A search of the M. truncatula BAC library database (http://www.medicago.org/genome/blast.php) using the full-length cDNA sequence identified the MtCDC16 gene on a 11.9 Kb region of a BAC clone, mth2-4n3 (GenBank Accession AC121241). Using the GENSCAN program (http://genes.mit.edu/GENSCAN.html; Burge and Karlin, 1997) and the PileUP multiple sequence alignment algorithm from GCG (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA), alignment of the putative MtCDC16 gene sequence with the cDNA was carried out and the gene structure of MtCDC16 was predicted.

For generating a promoter-reporter construct, a PCR-amplified HindIII-HindIII fragment containing approximately 2.3-Kbp region upstream of the predicted translational start site of MtCDC16 was cloned into the HindIII site of binary plasmid vector pBI101.1 (Jefferson et al., 1987). The primers used were 5’-aagaagcttGAACGCCTGATGCATCCGC-3’ and 5’-aagaagcttCGAAGCTTCTATCTCTTG-3’ (the lower case letters in each primer represent the addition of HindIII site to the amplified fragment). This construct was sequenced to confirm the presence and the orientation of the cloned fragment with respect to the β-glucuronidase gene. The binary vector was transformed into Agrobacterium rhizogenes strain ARQua1 using standard methods. Transformed roots were created using M. truncatula A17 plants as described previously (Boisson-Dernier et al., 2001). Transgenic roots, before and after rhizobial inoculation, were stained with GUS assay buffer (Jefferson et al., 1987). Similarly, to test for auxin responsiveness of the promoter, the transgenic roots were incubated in Fahraeus-agar medium with or without 0.5 µM 2, 4-D and were stained, as described above. The stained roots were cleared by incubation in 0.24 N HCl and 20% ethanol at 57°C for 15 minutes, 7% NaOH in 60% ethanol for 15 minutes at room temperature, followed by 40, 20 and 10% ethanol for 5 minutes each. The cleared roots were mounted on slides with 50% glycerol and observed under a Nikon
microscope (DIAPHOT 200, New York, USA) using Nomarski optics. Pictures were taken with a Nikon E4500 digital camera. The expression pattern described was observed in a minimum of 15 independent transgenic roots.

**Nucleic acid isolation and analysis**

Genomic DNA was extracted from young leaves of A17 plants using the DNeasy plant maxi kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. Independent restriction digestion reactions were carried out overnight with HindIII, PvuII and XbaI each using approximately 15 µg of the DNA. Samples of the digested DNA were electrophoresed (2.5volts/cm) overnight and transferred to Hybond N+ nylon membrane (Amersham Biosciences, UK). The membrane was then hybridized with the labeled gene-specific probe, washed, and exposed to Hyperfilm ECL following the instructions from ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham Biosciences, UK).

Root samples (n = 20±2) were harvested from uninoculated plants growing on Fahraeus-agar medium and nodulating plants (20±2) growing in aeroponics. Samples from two independent biological replicates were pooled for RNA extraction using the RNeasy plant kits (Qiagen, Valencia, CA). DNase treatment, cDNA synthesis and qRT-PCR analysis were carried out as described previously (Kuppusamy *et al.*, 2004). The following sets of primers were used for RT-PCR of *MtCDC16* transcripts: P1 (sense): 5’-AACAAGAGATAGAGAAGCTTCG-3’; P1’ (antisense): 5’-GATCTTTATCCAAGTAGATA-3’; P2 (sense): 5’-AGAAGCACAAGTTTATCCAAG TAGATA-3’; P2’ (antisense): 5’-GAGAGAAATGAATGGACA-3’. P1 primers were designed from the sequence within the region used for the RNAi construct and primers P2 were from the region downstream of that used for the RNAi construct. To estimate the transcript abundance of TC106907 and TC101963, qRT-PCR analysis was carried out with two technical replications as described previously (Kuppusamy *et al.*, 2004). The
gene specific primers used were as follows: TC106907 (5’-TTATCACATATGAAGATCTGATT-3’; 5’-TTAGATTCCATCCAAGGATCTGATT-3’), TC101963 (5’-GTGGTTCCAATATCATACTTGTA-3’; 5’-TGCATTCATCAATCTTACCTCTC-3’) and TC105607 (5’-GGCAGGTCTGCCTATGGTTA-3’; 5’GGTCAGACGCACAGATTTGA-3’).

**Tissue processing, sectioning and staining**

For morphological studies, tissues were processed using a microwave-assisted processing method (Rangell and Keller, 2000). Briefly, root samples were fixed for 40 sec at approximately 240W under vacuum in 4% glutaraldehyde in phosphate buffer, pH 7.2 using a Pelco Laboratory Microwave oven, Model 3441 (Ted Pella Inc., Redding, CA). After 2 buffer rinses, the tissues were dehydrated in an incremental ethanol series for 40 sec each step at 240W in the microwave. Ethanol was replaced with methacrylate resin (Kulzer, Wehrheim, Germany) by immersion for 3 min each in 2:1 and 1:1 (v/v) mixtures of ethanol:resin in the microwave at 450 W under vacuum. This was replaced 2 or more times with pure resin to obtain a complete infiltration before polymerization at 60ºC overnight. A series of semi-thin (1 µm) sections were cut using RMC MT-7000 Ultramicrotome (Boeckeler Instruments Inc., Tucson, AZ) and placed on poly-L-Lys-coated slides (Corning, NY, U.S.A.). Sections were stained using 0.1% toluidine blue stain and observed using bright field optics.

Nodules were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer, pH7.2. Tissue was dehydrated through an ethanol and xylene series and embedded in Paraplast. Longitudinal sections (10µm) of *Mtcdc16i* and control nodules of comparable size were stained with toluidine blue. Nodule zone lengths were measured along the medial axis of the nodule.
sections. The proc ttest (SAS version 9.1) was used to compare zone lengths between the *Mtcdc16i* and control nodule sections.

**Acetylene reduction assay**

Nitrogenase activity of intact nodules on whole root systems was estimated by the acetylene reduction assay as previously described (Vance *et al.*, 1979). Samples consisting of a hairy root system harvested from an aeroponics chamber at 15 dpi were measured for acetylene reduction in 2 ml sealed vials in the presence of 10% acetylene. After 1 h of incubation in acetylene, a sample (1 ml) was injected into a Photovac 10S Plus Gas C (Vance *et al.*, 1979) chromatograph (Photovac Inc., Markham, ON, Canada). Samples were compared to a standard curve generated against a 5 ppm ethylene standard, and nitrogenase activity was expressed as pmoles of ethylene evolved (per minute/plant).

**Auxin response assay**

The wild type A17 seedlings were grown in aeroponics for 6 days. Approximately 60 mg of whole root tissue was cut into small pieces of 2-3 mm in length and incubated for a total of 4 hours at 30°C in KPSC buffer (10 mM K₂HPO₄- 2% sucrose- 50µg/ml of chloramphenicol) to deplete the endogenous auxin (Timpte *et al.*, 1994). The auxin-depleted root tissue was distributed into three aliquots of approximately 20 mg each and incubated in three different concentrations of synthetic auxin (2,4-D) – 0 µM, 5 µM and 50 µM in KPSC (Timpte *et al.*, 1994) for either 30 minutes or 2 hours and frozen separately. Samples were pooled from two independent biological replicates for RNA extraction and qRT-PCR analysis as described above. The P1 and P1’ primers described earlier were used for this analysis. Relative transcript abundance was determined by monitoring the transcript abundance of selected genes in the 2,4-D-treated samples relative to the buffer-treated samples.
Root growth assay for auxin resistance

Control plants (gusi) carrying the transgenic hairy roots were transferred to Fahraeus medium containing different concentrations of 2,4-D. Root growth was recorded after 6 days and expressed as a percentage of root growth on medium without 2,4-D. The concentration of 2, 4-D that showed root growth inhibition in the control plants (0.5 µM) was selected for the root growth assay of the *MtCDC16i* plants.

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References


**de Billy F, Grosjean C, May S, Bennett M, Cullimore JV** (2001) Expression studies on *AUX1*-like genes in *Medicago truncatula* suggest that auxin is required at two steps in early nodule development. Mol Plant Microbe Interact, **14**: 267-277.


Figure Legends

Figure 1. Structural analysis of *M. truncatula* *CDC16* gene. (A) Gene structure of the *MtCDC16* gene. Translated regions of exons (E) are shown as gray boxes. The boldface line before the translational start site represents the 5’-untranslated and the putative promoter regions. The 500 bp fragment indicated below, which includes portions of exon 1 and the 5’-untranslated region, marks the area that was used for constructing the RNAi vector. The location of the two primer sets (P1-P1’ and P2-P2’) used for RT-PCR analysis of *MtCDC16* transcripts is indicated. (B) Alignment of the predicted *M. truncatula* CDC16 protein with the *A. thaliana* CDC16 protein. Blocks of amino acid sequences marked in red indicate the TPR motifs. (C) Graphical representation of the distribution of TPR motifs within *M. truncatula* CDC16 and *A. thaliana* CDC16 proteins. Gray boxes indicate the TPR motifs. (D) Southern blot analysis to estimate the copy number of *MtCDC16* in *M. truncatula* genome.

Figure 2. *MtCDC16*-suppressed (*Mtcdc16i*) roots show decreased lateral root number and root length. *A. rhizogenes* carrying the pRNAi-GUS or pRNAi-CDC16 construct was used to transform L416 seedlings (see Material and Methods). (A) Control root (*gusi*), showing normal primary and lateral root growth. (B) *MtCDC16*-suppressed root with a moderate phenotype [*Mtcdc16i* (moderate)], showing reduced lateral root development. (C) *MtCDC16*-suppressed root with a severe phenotype [*Mtcdc16i* (severe)], showing extreme reduction in root length and lateral roots. (D) Root length and lateral root numbers from three independent sets of control (*gusi*) and *Mtcdc16i* roots. Error bars represent standard deviation from the mean. (E) qRT-PCR analysis of *MtCDC16* expression in *Mtcdc16i* roots. P1 indicates the primers designed within region used for the RNAi construct and primers P2 were designed downstream of the region used for the RNAi construct.
Figure 3. Severe suppression of MtCDC16 results in aberrant cell morphology in the root meristem. Median longitudinal, toluidine blue-stained sections of the root tips of a control root (A) and an Mtcdc16i (severe) root (B). The double-sided bracket marks the root apical meristem (RAM) of the control root and the corresponding region of the Mtcdc16i root. (C-D) Higher magnification image of the bracketed regions in A and B. (C) shows regularity in cell morphology at the RAM in the control roots. (D) shows irregularity in cell morphology in comparable zone of the Mtcdc16i (severe) root. Bars = 50 μm.

Figure 4. Partial suppression of MtCDC16 results in hypernodulation phenotype. Control roots (A) and Mtcdc16i roots (B) inoculated with S. meliloti and grown in an aeroponic chamber for 4 weeks. (C) Acetylene reduction assay. Nitrogenase activity of intact nodules on control roots and Mtcdc16i roots was measured on a whole root basis. The error bars indicate standard deviation from the mean from three independent experiments. (D) qRT-PCR analysis shows significant suppression of MtCDC16 in Mtcdc16i roots and nodules. Each point represents the mean of three replicates with error bars representing the SD.

Figure 5. Longitudinal sections of root nodules from Mtcdc16i and control roots. Longitudinal section of a control nodule (A) and Mtcdc16i (moderate) nodule (B). The length of the infection zones is designated by brackets. Nodule zones are labeled according to Vasse et. al. (1990) meristem (I), infection zone (II), interzone (+), nitrogen-fixing zone (III). Bar = 0.5 mm.

Figure 6. Promoter-GUS fusion analysis of MtCDC16 promoter activity in transgenic hairy roots of M. truncatula. The expression of GUS is indicated by blue staining. A-D, GUS activity in the uninoculated plant roots. GUS expression observed at the root tip of primary root (PR) (A), lateral root primordium (LRP) (B), emerging lateral root (ELR) (C) and at the tip of fully emerged lateral root
(LR) (D). E-G, Induction of GUS during nodule development. Blue staining observed in the nodule primordium (NP) (E), emerging nodule (EN) (F) and at the meristematic zone of a mature nodule (N) (G). Bars = 50 µm.

**Figure 7. Auxin response phenotype of MtCDC16.** Histochemical GUS staining patterns of CDC16:GUS in transgenic hairy roots in the absence (A) and presence (B) of 0.5 µM 2, 4-D; Bars = 50 µm. (C) qRT-PCR analysis to estimate the transcript abundance of MtCDC16 in wild type roots in the presence and absence of 2, 4-D. Each point represents the mean of three replicates with error bars representing the SD.

**Figure 8. Auxin resistance phenotype of MtCDC16.** (A) Root growth assay for auxin resistance. Inhibition of root growth in the presence of 0.5 µM 2, 4-D was assayed in control (gusi) and Mtcdc16i roots. (B) qRT-PCR analysis to estimate the transcript abundance of TC101963 and TC106907 in gusi and Mtcdc16i (moderate) roots in the presence and absence of 5 µM 2, 4-D. In A and B, each point represents the mean of three replicates with error bars representing the SD.

**Figure S1.** Longitudinal section of Mtcdc16i moderate root meristem. Bar =50µm