WUSCHEL-RELATED HOMEOBOX5 Gene Expression and Interaction of CLE Peptides with Components of the Systemic Control Add Two Pieces to the Puzzle of Autoregulation of Nodulation[1][W]

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In legumes, the symbiotic nodules are formed as a result of dedifferentiation and reactivation of cortical root cells. A shoot-acting receptor complex, similar to the Arabidopsis (*Arabidopsis thaliana*) CLAVATA1 (CLV1)/CLV2 receptor, regulating development of the shoot apical meristem, is involved in autoregulation of nodulation (AON), a mechanism that systemically controls nodule number. The targets of CLV1/CLV2 in the shoot apical meristem, the WUSCHEL (WUS)-RELATED HOMEOBOX (WOX) family transcription factors, have been proposed to be important regulators of apical meristem maintenance and to be expressed in apical meristem "organizers." Here, we focus on the role of the WOX5 transcription factor upon nodulation in *Medicago truncatula* and pea (*Pisum sativum*) that form indeterminate nodules. Analysis of temporal *WOX5* expression during nodulation with quantitative reverse transcription-polymerase chain reaction and promoter-reporter fusion revealed that the *WOX5* gene was expressed during nodule organogenesis, suggesting that *WOX* genes are common regulators of cell proliferation in different systems. Furthermore, in nodules of supernodulating mutants, defective in AON, *WOX5* expression was higher than that in wild-type nodules. Hence, a conserved WUS/WOX-CLV regulatory system might control cell proliferation and differentiation not only in the root and shoot apical meristems but also in nodule meristems. In addition, the link between nodule-derived CLE peptides activating AON in different legumes and components of the AON system was investigated. We demonstrate that the identified AON component, NODULATION3 of pea, might act downstream from or beside the CLE peptides during AON.

Legume plants can thrive on nitrogen-poor soils because they interact symbiotically with soil-resident bacteria, the rhizobia. After a complex signal exchange between both partners, new root organs are formed: the nodules, in which the bacteria fix atmospheric nitrogen for the plant in return for a protective niche and carbon sources. Nodulation requires well-controlled bacterial invasion and initiation of cortical cell division after perception of the bacterially produced Nodulation (Nod) factors. Studies in several legumes have elucidated many elements of the signaling cascade (Catoira et al., 2001; Ben Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Lévy et al., 2004; Mitra et al., 2004; Kaló et al., 2005; Smit et al., 2005, 2007; Heckmann et al., 2006; Kanamori et al., 2006; Middleton et al., 2007). Mature nodules can be of the indeterminate or determinate type depending on whether an apical meristem is sustained through development or not. Which type grows relies on the host: typical models for indeterminate nodule development are *Medicago truncatula* and pea (*Pisum sativum*).

Nodule number and nitrogen fixation are strictly regulated by the legume plants to balance the nitrogen demand with nutrient supply and environmental

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conditions. As a result, the plant regulates the number of nodules and the size of the nodulation zone locally (Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007), but also by systemic mechanisms (Bauer, 1981; Pierce and Bauer, 1983; Kosslak and Bohlool, 1984; Caetano-Anollés and Gresshoff, 1991). The best studied systemic control mechanism is called autoregulation of nodulation (AON). During this AON process, the first nodules that are formed inhibit further nodule development on the entire root system via currently unknown long-distance signals (Kosslak and Bohlool, 1984). AON is activated early after the perception of the Nod factors, at the onset of cell division for primordium formation, and its strength increases as nodule development proceeds (Pierce and Bauer, 1983; Caetano-Anollés and Gresshoff, 1991; Li et al., 2009).

AON-defective mutants in *Lotus japonicus*, *Medicago truncatula*, soybean (*Glycine max*), and pea (Carroll et al., 1985; Sagan and Duc, 1996; Szczyglowski et al., 1998; Krusell et al., 2002; Nishimura et al., 2002; Penmetsa et al., 2003; Searle et al., 2003; Schnabel et al., 2005, 2010) developed many more nodules than the corresponding wild-type plants. Grafting studies and analysis of mutants have shown that AON involves a root-shoot signal communication (Delves et al., 1986; Olsson et al., 1989; Sheng and Harper, 1997; Wopereis et al., 2000; Penmetsa et al., 2003). Later, a shoot-acting receptor complex, similar to the receptors that control the balance between cell division and differentiation in the shoot apical meristem (SAM), were found to play a central role (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005). Indeed, individual mutants were affected in a gene encoding a CLAVATA1-like leucine-rich repeat receptor-like kinase (CLV1-like LRR-RLK; *har1/sunn/sym29/NARK*; Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005), whereas other mutants were impaired in CLV2-like LRR-RLK (*clv2/sym28*; Krusell et al., 2011). In addition, the supernodulating mutant *klavier* isolated from *L. japonicus* was altered in a gene encoding an LRR-RLK structurally unrelated to CLV1-like RLKs but closely homologous to the Arabidopsis (*Arabidopsis thaliana*) TOADSTOOL2/RECEPTOR-LIKE PROTEIN KINASE2 (TOAD2/RPK2; Oka-Kira et al., 2005; Miyazawa et al., 2010). All the Arabidopsis homologs transmitted the signal of the stem cell-specific peptide hormone CLV3 in the Arabidopsis SAM to control stem cell homeostasis by forming distinct homodimers or heterodimers (Oka-Kira and Kawaguchi, 2006; Bleckmann et al., 2010; Guo et al., 2010; Kinoshita et al., 2010; Miyazawa et al., 2010).

The elucidation of this shoot-acting receptor complex has led to the hypothesis that AON is initiated by a nodulation-dependent root-derived signal Q that is perceived in the shoot by the receptor, after which a shoot-determined inhibitor migrates from shoot to root to suppress further nodule formation (Gresshoff, 2003; Ferguson et al., 2010). Although both signals are currently unknown, the nature of the receptors (CLV1, CLV2, and TOAD2/RPK2-like components) might hint at a CLV3-like peptide as the Q signal. CLV3 belongs to the large group of CLAVATA3/EMBRYO-SURROUNDING REGION (CLE) peptides, which are small secreted peptides consisting of 12 to 13 conserved amino acids that are cleaved from the C-terminal end of CLE preproproteins (for review, see Wang and Fiers, 2010). Interestingly, in *L. japonicus*, *M. truncatula*, and soybean, when ectopically expressed, *CLE* genes that encode structurally similar peptides block or reduce nodulation systemically and depending on *HYPERNODULATION ABERRANT ROOT1* (*HAR1*)/*SUPERNUMEROUS NODULES* (*SUNN*)/*NODULE AUTOREGULATION RECEPTOR KINASE* (*NARK*; Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011; Saur et al., 2011). Albeit without ultimate proof, these nodulation-related CLE peptides have been hypothesized to act as the Q signal to activate the CLV-like receptor complex in the shoot for AON. In addition to the CLE peptides, several mutants have been identified that might be affected in genes involved in the control of AON in the root, such as *nod3*, *rdn1*, *rdh1*, *tml*, and *plenty* (Postma et al., 1988; Ishikawa et al., 2008; Magori et al., 2009; Novák, 2010; Yoshida et al., 2010; Novák et al., 2011; Schnabel et al., 2011). These mutants might be defective in the genes that control the root-derived signals or the root-to-shoot processing/transducing signals or vice versa (Li et al., 2009; Novák, 2010). Recently, *rdn1* and *nod3* have been found to encode unknown proteins that act in the vascular system, suggesting that these proteins might be involved in the vascular transport of a mobile signal acting between roots and shoots (Schnabel et al., 2011).

The downstream processes activated via AON are still unknown, but typical cell proliferation regulators might be the targets of the shoot-derived signals to further restrict the nodule number. The phytohormones auxin and cytokinin are central in the control of cell division and differentiation, and both, but especially the cytokinins, are essential for nodule formation (Schnabel and Frugoli, 2004; Gonzalez-Rizzo et al., 2006; Tirichine et al., 2007; Crespi and Frugier, 2008; Frugier et al., 2008; Plet et al., 2011). Based on expression analysis, the nodulation-related MtCLE12 peptide has been proposed to control nodulation by negatively influencing cytokinin signaling (Saur et al., 2011). Additionally, the auxin marker *GH3*:*GUS* was up-regulated in roots ectopically expressing the same nodulation-related CLE peptide (Saur et al., 2011).

The similarities with the CLV3 signaling pathway might also hint at putative targets of the AON pathway. In the SAM, a cellular, nonautonomous feedback loop between CLV3 signaling and the homeodomain transcription factor WUSCHEL (WUS) regulates stem cell homeostasis (Schoof et al., 2000). WUS acts in the organizing center of the SAM and is essential for the specification and maintenance of stem cell proliferation in the central zone of the meristem (Mayer et al., 1998; Schoof et al., 2000). The CLV3 signaling pathway, including the CLV1/CLV2 receptor kinases and the CLV3 regulatory peptide, negatively controls *WUS*

expression, thereby restricting the size of the stem cell population (Brand et al., 2000; Schoof et al., 2000). In the root apical meristem (RAM), a similar signaling system involving the WUSCHEL-RELATED HOMEOBOX5 (WOX5) functions in the quiescent center (QC) to regulate the balance between cell division and differentiation (Kamiya et al., 2003; Haecker et al., 2004). Complementation experiments proved that WUS and WOX5 are functionally equivalent (Sarkar et al., 2007) and, therefore, could be involved in common regulatory pathways that control meristem maintenance and development in roots and shoots. In addition, a CLE peptide (CLE40) might control the *WOX5* expression domain in the RAM through the interaction with the RLK ARABIDOPSIS CRINKLY4 (Stahl and Simon, 2009).

In view of the similarities between the AON-controlling RLKs in legumes and the SAM and RAM stem cell homeostasis, we hypothesized that a WOX transcription factor might also be a target during AON. Previously, by means of gene expression studies, a few members of the *WOX* family in *M. truncatula* had been identified, including *WOX5*, which is expressed in root tissues upon somatic embryogenesis (Chen et al., 2009). We analyzed whether its expression is modified upon nodulation and is regulated by AON and CLE peptide signaling. Furthermore, we addressed the question of whether the *M. truncatula* CLE peptide, *MtCLE13*, which inhibits nodulation in a *SUNN*-dependent manner in gain-of-function analyses (Mortier et al., 2010), could also influence the nodulation of other known AON mutants. To this end, we tested whether the *MtCLE13* gain-of-function effect on nodulation also occurred in pea and studied its effect on the pea mutants *sym28*, *sym29*, and *nod3*. We present a comparative analysis of two legume plants that might be useful to understand the role of *WOX5* upon nodule development in indeterminate nodule-forming legumes.

RESULTS

Quantitative PCR Analysis of *WOX5* Expression upon Nodulation in *M. truncatula* and Pea

To identify a *WOX* gene with expression modulated upon nodulation, the Mt2.1 genomic sequence was searched with BLASTX followed by an analysis of the expression patterns of each hit via the *M. truncatula* Gene Expression Atlas (Benedito et al., 2008). *WOX5* (*CU326389*) was the only WOX transcription factor induced upon nodulation that already had been studied previously during somatic embryogenesis (Chen et al., 2009). The *WOX5* gene and mRNA were identified in pea (GenBank accession nos. JN603579 and JN603580) with degenerate primers designed based on the *M. truncatula WOX5* nucleotide sequences and subsequent RACE analyses (see "Materials and Methods"). The coding sequence of the *WOX* sequence of pea was 92.7% similar within and 85.5% similar outside the homeobox region of the *M. truncatula WOX5* (*CU326389*) and, therefore, was designated *WOX5* (Supplemental Fig. S1).

To study the temporal expression during nodule development, the relative transcript level of *WOX5* was analyzed at different stages after inoculation in both *M. truncatula* and pea. For *M. truncatula* (A17) and pea (cv Frisson), expression was tested from 3 to 28 d post inoculation (dpi; Fig. 1A) and from 5 to 22 dpi (Fig. 1B), respectively, and compared with the expression levels in uninoculated roots. The expression levels of *WOX5* transiently increased during nodule development to reach a maximum at approximately 11 to 12 dpi, whereafter they decreased again both in *M. truncatula* and pea (Fig. 1, A and B).

A correct auxin/cytokinin balance is a prerequisite for nodule formation (Oldroyd and Downie, 2008; Ding and Oldroyd, 2009). To determine whether

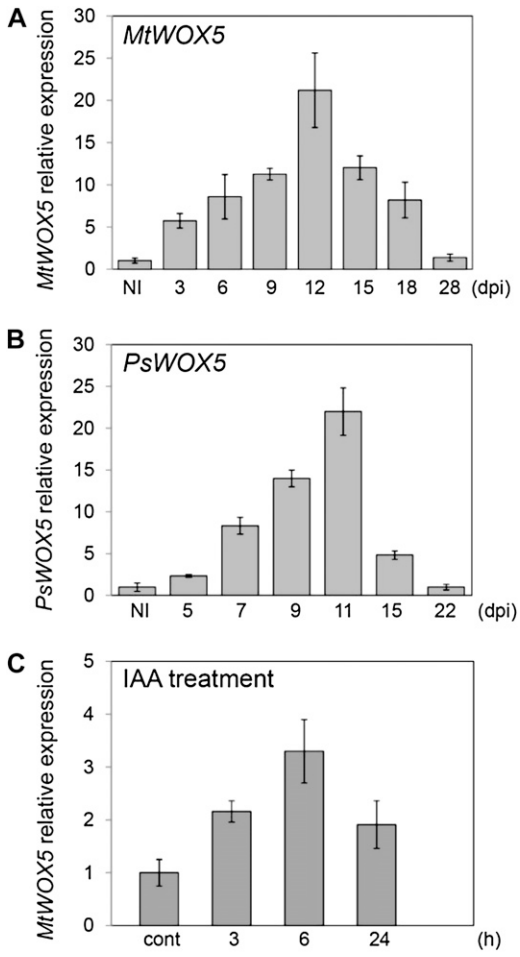

Figure 1. Temporal expression of *WOX5* during *M. truncatula* and pea nodulation and upon auxin treatment. A, qRT-PCR analysis of *WOX5* in noninoculated *M. truncatula* roots (NI) and at 3, 6, 9, 12, 15, 18, and 28 dpi. Expression is shown relative to the expression found in NI. B, qRT-PCR analysis of *WOX5* in noninoculated pea roots (NI) and at 5, 7, 9, 11, 15, and 22 dpi. Expression is shown relative to the expression found in NI. C, Effect of treatment with 10^{-6} M indole-3-acetic acid (IAA) on *WOX5* gene expression level at different days after treatment. Expression is shown relative to the expression found in untreated roots (cont). The error bars represent SD of three technical repeats. The graphs show the results of one biological repeat, representative for two additional independent experiments.

auxins and/or cytokinins affected *WOX5* expression, 10^{-6} M indole-3-acetic acid or 10^{-7} M 6-benzylaminopurine was supplemented to the growth medium of 5-d-old *M. truncatula* seedlings. The hormone-treated roots were harvested under each condition after 3, 6, and 24 h. Roots from plates without hormone addition were used as a negative control. *WOX5* expression was temporarily induced after auxin treatment (Fig. 1C), whereas 6-benzylaminopurine treatment had no clear effect (data not shown).

Tissue-Specific Expression Pattern of *WOX5* upon Nodulation in Pea and *M. truncatula* by Promoter-GUS Analysis

To localize *WOX5* expression during nodule development, a 2,310-bp *M. truncatula WOX5* promoter region was isolated and used to construct *pMtWOX5*:*GUS* (see "Materials and Methods"). This construct was introduced into both *M. truncatula* and pea roots. In noninoculated transgenic roots of both legumes, GUS staining was observed in the meristematic zones of the root tips (RAM), corresponding to the QC area (Fig. 2, A and B). *WOX5* was also expressed in primordia of lateral roots (Supplemental Fig. S2).

Next, expression was analyzed at various stages of nodule development both in *M. truncatula* and pea. At 3 to 4 dpi, GUS staining in *M. truncatula* was visible in some dividing cells of the pericycle, endodermis, and cortex located opposite the xylem poles, corresponding to the sites of nodule primordium initiation (Fig. 2, E and F). Also, a low expression level was seen in the outer cortical cells. At slightly more advanced stages (5–7 dpi), GUS staining was observed in the developing nodule primordium (Fig. 2G), and as nodule development progressed, *WOX5* expression gradually decreased and remained in the periphery of the developing nodules (12–14 dpi; Fig. 2H). Finally, in mature nodules (21–28 dpi), *WOX5* expression was limited to some cells at the tip of the vascular bundles (Fig. 2I).

The expression pattern was similar in transgenic roots of pea carrying the *pMtWOX5*:*GUS* construct. Before inoculation, GUS staining was visible in a local area in the RAM (Fig. 2B). *WOX5* expression was activated very early upon inoculation in the proliferation zone and afterward within developing primordia (Fig. 2C). The area of *WOX5* expression in mature nodules of pea was restricted to the tips of the vascular bundles, although it was slightly more expanded than that in *M. truncatula* (Fig. 2D). Thus, the tissue-specific expression pattern of *WOX5* upon nodulation was relatively similar in the two indeterminate nodule-forming legumes. The results of the *WOX5* expression analysis with the *pMtWOX5*:*GUS* fusion were consistent with the quantitative reverse transcription (qRT)-PCR data. The enhanced level of *WOX5* expression at early nodulation stages (7–12 dpi) corresponded to the stage when most of the nodule primordia developed. *WOX5* expression reached a maximum preceding the nodule meristem formation, whereas its decrease at later stages matched the restricted *WOX5* expression zone in developing nodules.

qRT-PCR Analysis of *WOX5* Expression in Mutants Affected in the CLV-Like Receptor Complex Involved in AON

To study the possible interaction of WOX5 with the CLV system during AON, we analyzed *WOX5* expression

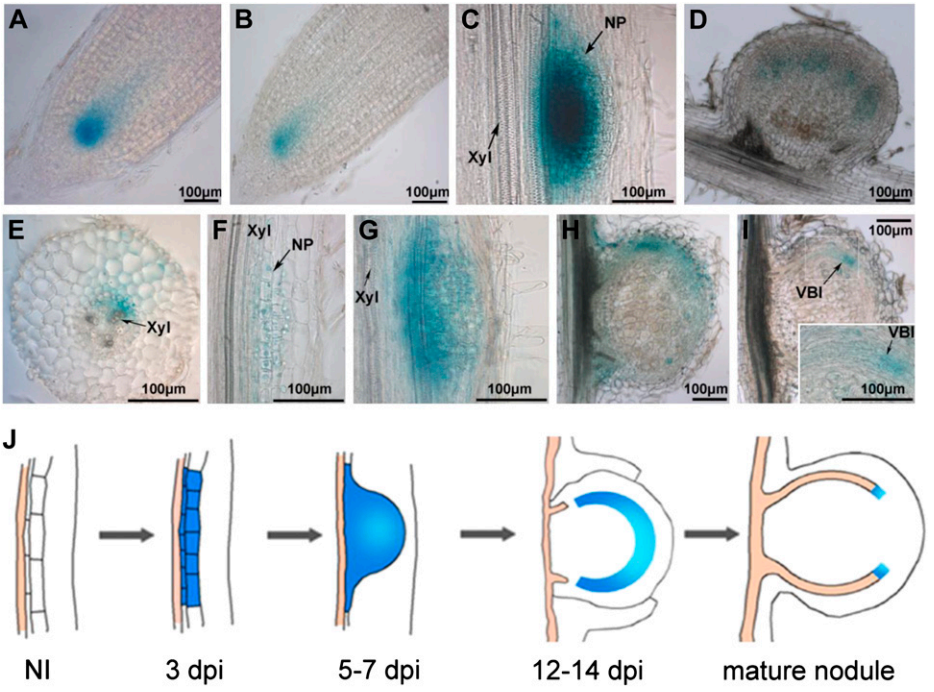

Figure 2. Analysis of the tissue-specific expression pattern of *WOX5* by promoter-reporter analysis in *M. truncatula* and pea. A, *WOX5* expression in root tips of pea. B, *WOX5* expression in root tips of *M. truncatula*. C and D, *WOX5* expression at different stages of nodule development in pea at 5 dpi (C) and 21 dpi (D). E to I, *WOX5* expression at different stages of nodule development in *M. truncatula* at 3 dpi (E and F), 5 dpi (G), 14 dpi (H), and 21 dpi (I). The inset in I shows an enlargement of the provascular strand. NP, Nodule primordium; VBI, vascular bundle initials; Xyl, xylem. Bars = 100 μm. J, Schematic representation of the *WOX5* expression domain at different stages of nodule development. On average, for each stage, approximately 30 to 50 transgenic roots were analyzed with similar results. NI, Noninoculated *M. truncatula* root.

in various supernodulating mutants. If any interaction between WOX5 and the CLV system existed, the defects in the components of the CLV system should be connected with changeable levels of *WOX5* expression. In *M. truncatula*, *WOX5* expression was tested upon inoculation of the *sunn-4* mutant (Fig. 3A), and in pea, it was tested upon inoculation of *sym29* (P88) and *sym28* (P64; Fig. 3B). For each mutant, the temporal expression pattern was compared with the respective wild-type controls (A17 for *sunn-4* and Frisson for *sym28* and *sym29)*.

The *WOX5* temporal expression pattern upon nodule development differed between wild-type plants and the supernodulating mutants (Fig. 3). In both *M. truncatula* and pea, the highest *WOX5* expression occurred earlier in the supernodulating mutant than in the wild-type plants. Upon inoculation of the *sunn-4* mutant, the maximal *WOX5* expression level was reached at 6 dpi, but only at 12 dpi in wild-type plants. Similarly, in pea *sym28* and *sym29*, the *WOX5* expression was maximal at 9 dpi, but only at 11 dpi in wild-type pea. At later nodulation stages in pea, *WOX5* expression was slightly higher in the supernodulating mutants than in the wild type. These results hint at an interaction between the AON shoot receptor complex and *WOX5* expression.

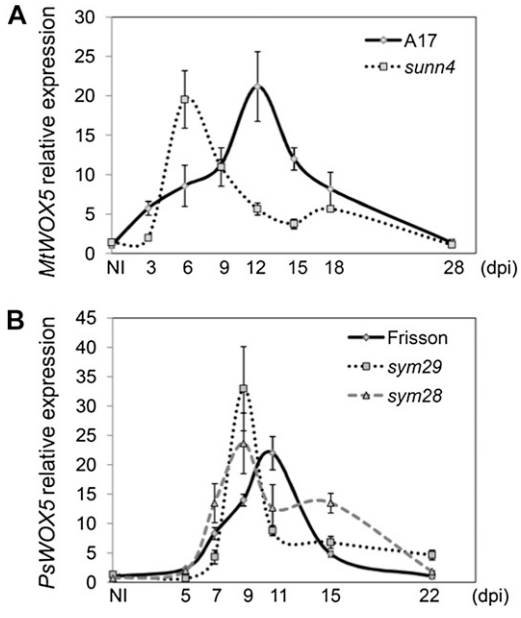

Figure 3. qRT-PCR analysis of *WOX5* gene expression upon nodule development in supernodulating mutants of *M. truncatula* and pea. A, Relative *WOX5* expression in noninoculated *M. truncatula* roots (NI) and at different dpi. Expression is shown relative to that in NI. B, Relative *WOX5* expression in noninoculated pea roots (NI) and at different dpi. Expression is shown relative to the expression in NI. The graphs show the results of one biological repeat, representative for one and two additional independent experiments in *M. truncatula* and pea, respectively. The error bars represent SD of three technical repeats.

Tissue-Specific Expression Pattern of *WOX5* in Supernodulating Mutants of *M. truncatula* and Pea

To control whether *WOX5* expression was altered locally within developing nodules of supernodulating mutants, we studied *WOX5* expression in the *M. truncatula sunn-4* and *sunn-3* mutants as well as in pea mutants *sym28* and *sym29*. For this purpose, the *pMtWOX5:GUS* construct was introduced into the mutant roots, and the expression patterns were compared with those of wild-type plants. For the comparison of wild-type and mutant alleles, GUS staining was done in an identical manner for all samples (see "Materials and Methods").

The GUS activity was significantly higher in nodules of the supernodulating pea mutants *sym29* and *sym28* than in those of the wild-type pea cv Frisson (Fig. 4, A–C). The expression in wild-type nodules was hardly visible in pea cv Frisson in the apical region, whereas in the AON mutants, GUS staining was seen all over the meristem and infection zone (Fig. 4, A–C). Consequently, *WOX5* expression was altered locally, at the level of a particular nodule, in supernodulating mutants of pea.

Next, *WOX5* expression was analyzed in two *M. truncatula sunn* mutants (*sunn-3* and *sunn-4* alleles), both carrying a nonsense mutation (Schnabel et al., 2005). At 5 to 7 dpi, GUS staining was more intense in *sunn-3* and *sunn-4* than in wild-type plants, although the expression pattern did not change (Fig. 4, D, H, and L). At later stages in nodule development, at 14 dpi (Fig. 4, E, I, and M [stereoscopic images] and Fig. 4, F, J, and N [agarose sections]) and at 21 dpi (Fig. 4, G, K, and O), the expression was still higher in *sunn-3* (Fig. 4, N and O) and *sunn-4* (Fig. 4, J and K) mutants than in wild-type nodules (Fig. 4, F and G). Interestingly, GUS staining was always more intense in *sunn-3* than in *sunn-4* nodules. Sectioning revealed that GUS staining was restricted to the vasculature tips in wild-type nodules, whereas this expression pattern extended over the meristem and infection zone and, to a lesser extent, in the fixation zone in *sunn-3* nodules (Fig. 4, N, and O).

In developing nodules of *sunn-3*, an additional expression pattern was seen that was clearly associated with the infection process when compared with the wild-type and *sunn-4* nodules (Fig. 4, P–R). GUS staining was observed in the epidermal and cortical cells that contained infection threads and was strong in the apical region, outside the meristem, and in mature nodules, corresponding to the initially infected, old root cortex (Fig. 4, P–R) during nodule primordium development. As a consequence, two *WOX5* expression domains were clearly visible in the *sunn-3* mutants: one associated with proliferating cells of developing nodules and one with infection threads penetrating the outer cortical cells. At later stages of nodule development (21 dpi), *WOX5* expression was still higher in *sunn-3* than in wild-type plants. In other words, the *sunn*, *sym28* and *sym29* mutations affect *WOX5* expression.

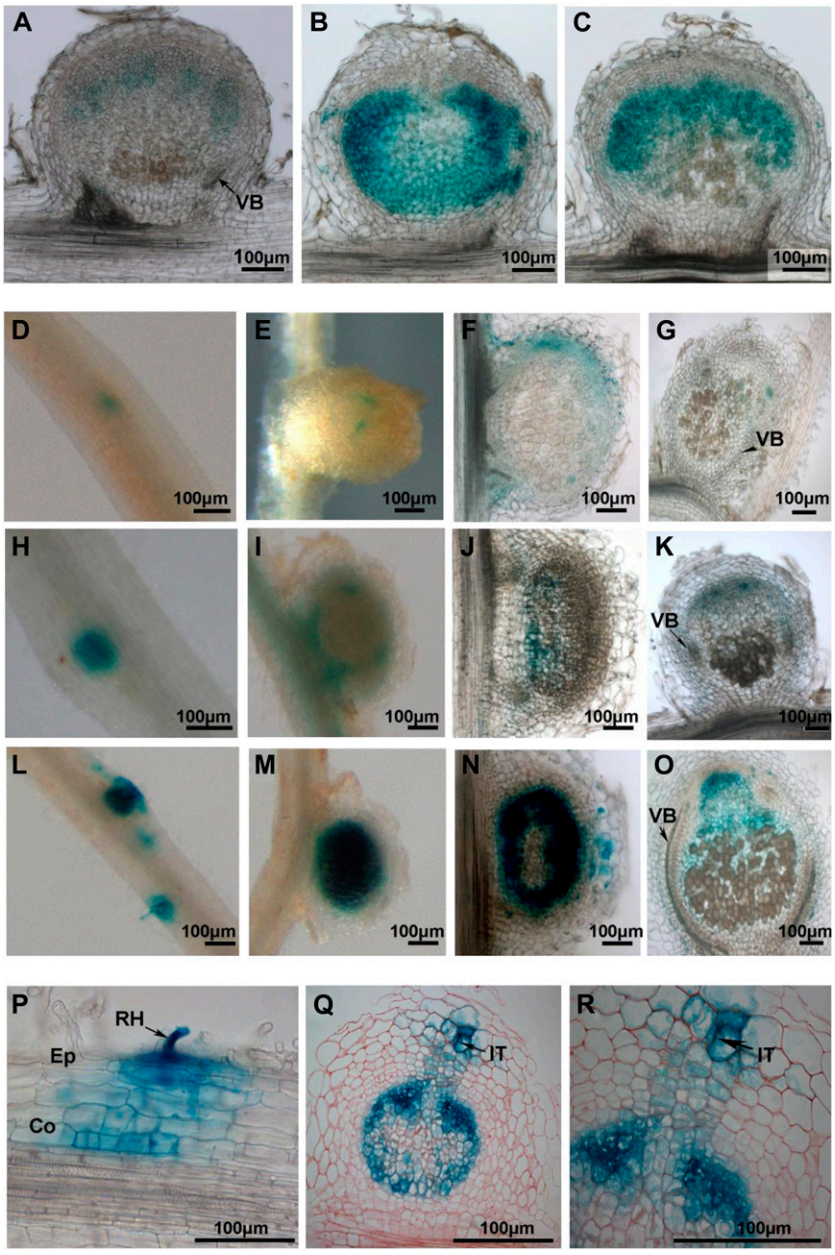

Figure 4. Analysis of the tissue-specific expression pattern of *WOX5* using promoter-reporter fusion in supernodulating mutants of *M. truncatula* and pea. A to C, *WOX5* expression in the wild type (A) and in *sym28* (B) and *sym29* (C) mutants of pea. GUS staining through a 21-dpi nodule is shown. D to R, *WOX5* expression at different stages of nodule development in the wild type (D–G) and in *sunn-4* (H–K) and *sunn-3* (L–R) mutants of *M. truncatula* A17. D, H, and L, *WOX5*:*GUS* staining during an incipient nodulation event. E, I, and M, *WOX5*:*GUS* staining in a young developing nodule at 14 dpi. F, J, and N, Young *WOX5*:*GUS*-stained nodule (14 dpi). G, K, and O, Mature elongated nodule (21 dpi). Stereoscopic images (D, E, H, I, L, and M) and light micrographs of agarose sections (50 μm; A–C, F, G, J, K, N, and O) are shown. P to R, Technovit sections (5 μm) stained with ruthenium red through *WOX5*:*GUS* developing nodules at 3 dpi (P) and 12 dpi (Q and R). Co, Cortex; Ep, epidermis; It, infection thread; RH, root hair; VB, vascular bundle. Bars = 100 μm.

Effect of the Ectopic Expression of *35S:MtWOX5* on Nodule and Root Development

To investigate the role of WOX5 in nodule organogenesis, transgenic roots overexpressing *35S*:*MtWOX5* were constructed and analyzed by microscopy. qPCR analysis confirmed the ectopic expression of *M. truncatula WOX5* in individual transgenic roots (Supplemental Fig. S3). Analysis of root length and nodule number revealed no significant changes in these parameters between *35S*:*MtWOX5* and *35S*:*GUS* control roots (Supplemental Fig. S3). However, the *35S*:*MtWOX5* roots often displayed slightly thickened root tips and roots with arrested growth (Supplemental Fig. S3). These roots contained more small dividing cells than those of the wild type, corresponding to the prolonged stimulation of cell proliferation. No visible changes in the organization of the *35S*:*MtWOX5* nodules were found (Supplemental Fig. S4).

Influence of Nodulation-Specific CLE Peptides on the Supernodulating Phenotype of Pea Mutants (*sym28*, *sym29*, and *nod3*)

Previously, ectopic expression of structurally related CLE peptide genes that were induced upon nodulation had been shown to inhibit or significantly reduce nodulation in *L. japonicus*, soybean, and *M. truncatula*. In addition, this gain-of-function effect depended on

HAR1, NARK, and SUNN, respectively (Okamoto et al., 2009; Mortier et al., 2010; Saur et al., 2011). To analyze this effect, we controlled whether an *M. truncatula* peptide, *MtCLE13*, could modulate nodulation in pea, and if so, we wanted to test the effect of *35S:MtCLE13* on the different AON mutants in pea, including the root-determined *nod3*.

Four-day-old pea seedlings were transformed with the *Agrobacterium rhizogenes* strain *Arqua*, containing either a *35S:MtCLE13* or a *35S:GUS* construct as a control. Plants were analyzed at 21 dpi, and only nodules on transgenic roots were counted (Fig. 5). In wild-type pea (Frisson and Rondo lines), ectopic expression of *MtCLE13* resulted in a severe reduction in nodulation. Whereas, on average, 35 and 50 nodules were obtained on Frisson and Rondo roots carrying the control construct, roots carrying the *35S:MtCLE13* construct only carried one to two and five nodules, respectively. Thus, *35S:MtCLE13* had a similar effect on *M. truncatula* and pea nodulation, indicating that *MtCLE13* is functional in pea (Fig. 5).

We also analyzed the effect of *MtCLE13* overexpression on the pea AON mutants *sym29*, *sym28*, and *nod3* (Fig. 6). In contrast to the wild type, in which a clear reduction in nodulation was seen by stereomicroscopy (Fig. 6, A–D), roots of mutants carrying a *35S:MtCLE13* construct developed as many nodules as control roots (Fig. 6, E–J). Hence, the effect of *35S:MtCLE13* expression on nodulation is abolished in these mutants.

DISCUSSION

We investigated the role of the WOX5 transcription factor upon nodule development in two legume plants, *M. truncatula* (a model legume) and pea. Analysis of the *WOX5* expression profile upon nodule development showed that *WOX5* expression increases transiently

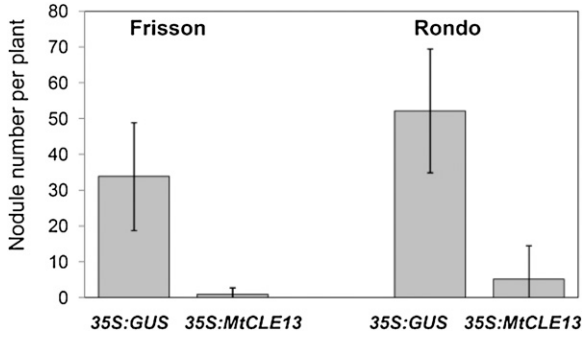

Figure 5. Influence of *35S:MtCLE13* overexpression on the nodule number in wild-type pea plants Frisson and Rondo lines). *35S:MtCLE13*, *MtCLE13*-overexpressing roots; *35S:GUS*, *GUS*-overexpressing roots (control). Error bars indicate SD on three technical repeats. The difference between *35S:GUS* control and *35S:MtCLE13* plants in nodule number is statistically significant (Mann-Whitney *U* test, $P < 0.001$).

early after rhizobium inoculation and is initially expressed in the pericycle, endodermis, and inner cortex of the infected root. At a later stage of nodule development, *WOX5* is expressed in cells of the developing nodule primordia and, during maturation of the primordia, gradually decreases and is restricted to the apical region of fully grown nodules, especially in cells of the provascular bundles.

The *WOX5* expression pattern observed at the onset of nodulation coincides with the site of nodule primordium formation (Mathesius et al., 1998a, 1998b; Timmers et al., 1999; Mathesius, 2008). In plant species forming indeterminate nodules, the first divisions are initiated in the pericycle, endodermis, and inner cortical cells, the sites where *WOX5* expression occurs. Accumulating data show that changes in cytokinin and auxin signaling underlie nodule development (Hirsch and Fang, 1994; Mathesius et al., 1998a, 1998b; Boot et al., 1999; Pacios-Bras et al., 2003; Huo et al., 2006; Wasson et al., 2006, 2009; Frugier et al., 2008; Mathesius, 2008; Grunewald et al., 2009; Plet et al., 2011). Upon inoculation, at the vasculature and inner cortical cells, cytokinin signaling has been proposed to perturb the expression and accumulation of PIN-FORMED auxin efflux carriers, temporarily inhibiting polar auxin transport to allow local auxin accumulation in the inner cortex for nodule primordium development (Plet et al., 2011). In agreement, a gene encoding an auxin influx carrier, AUXIN RESISTANT1-LIKE, is expressed in cells of the nodule primordia (de Billy et al., 2001; Schnabel and Frugoli, 2004). Moreover, in legumes forming indeterminate nodules, the auxin-responsive reporters *GH3:GUS* and *DR5:GUS* were expressed in developing primordia and later in nodule vasculature (Mathesius et al., 1998a; Huo et al., 2006). Because *WOX5* is activated by auxin (Chen et al., 2009; this study), the transcription factor might function downstream of cytokinin signaling and polar auxin transport inhibition to form nodule primordia. Expression in the developing nodule primordia is consistent with the function of other members of the WOX family that stimulate cells to proliferate while suppressing their differentiation (van der Graaff et al., 2009).

Interestingly, the *WOX5* expression pattern at the onset of the nodule development is similar to that of cytokinin-signaling and cytokinin-responsive genes (Plet et al., 2011). This colocalization raises the question of whether cytokinin signaling and *WOX5* expression might be linked similarly as in SAM. In Arabidopsis, *WUS* is known to be involved in the local regulation of the cytokinin response by repressing cytokinin A-type response regulators, which negatively control cytokinin action (Skylar and Wu, 2011), and, as a result, locally stimulates cytokinin action and cell proliferation. If a similar interaction existed for nodulation and because cytokinin is known to trigger nodule development, a higher nodule number would have been expected in the *WOX5*-overexpressing roots. However, no increase in nodule number was

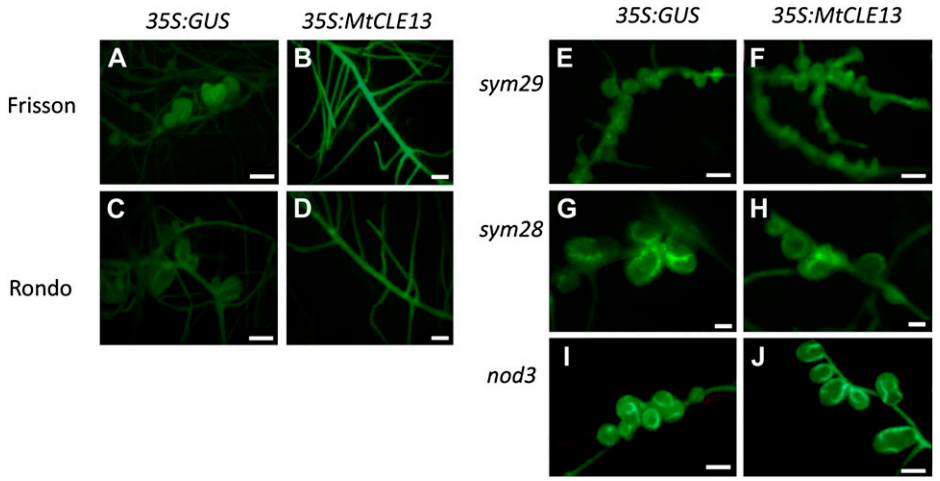

Figure 6. Effects of *35S:MtCLE13* on nodulation in the supernodulating pea mutants *sym29*, *sym28*, and *nod3*. A to D, Examples of the nodulation phenotype of GFP-positive transgenic roots carrying the *35S:GUS* (control) or *35S:MtCLE13* construct on Frisson (A and B) and Rondo (C and D) wild-type plants. E to J, Examples of the nodulation phenotype of GFP-positive transgenic roots carrying the *35S:GUS* (control) or *35S:MtCLE13* construct on the *sym29* (E and F), *sym28* (G and H), and *nod3* (I and J) mutants. Approximately 18 to 25 plants were analyzed with similar results. Bars = 1,000 μm.

observed on *35S:MtWOX5* roots, indicating that *WOX5* might not be involved in the amplification of cytokinin signaling. As the interaction might be more subtle, it would still be interesting to compare the expression of *WOX5* and the different cytokinin markers available for nodulation. In addition, analysis of *WOX5* in a *cre1* mutant background might give important information, as would analysis of the expression of cytokinin response regulators in *35S:WOX5* roots and nodules. In mature nodules of *M. truncatula* and pea, the *WOX5* gene is preferentially expressed near the tips of vascular bundles, which consist of small dividing cells that give rise to the vascular bundles. Also, in the tips of *M. truncatula* roots, *WOX5* was expressed at the site of vascular bundle initiation in cells that might correspond to the QC of the root meristem (Chen et al., 2009). Based on this expression pattern, the *WOX5*-expressing nodule cells might represent the organizing centers of the nodule meristems, in analogy to the RAM and SAM. In agreement, the transcription factor *NOOT* that suppresses root meristem identity in nodule meristems is also expressed at these sites (P. Ratet, personal communication). The origin of nodules has been debated in the literature, because they are unique structures with both shoot and root properties (Hirsch and LaRue, 1997; Markmann and Parniske, 2009). The central nodular tissue is surrounded by several vascular bundles that originate from independent provascular strands at the apical meristem. In an individual nodule of *M. truncatula* and pea, mostly two vascular bundles are found. Based on the similarity between the *WOX5* expression pattern in root tips and mature nodules, it is tempting to speculate that nodule development might involve the fusion of several (lateral) root-like structures. However, because nodules are unique structures, nodule meristem development will surely be more complex than a simple fusion between root meristems. Analysis of more QC markers together with mutant analysis and cell-specific transcript profiling will undoubtedly shed light on this interesting and undiscovered aspect of nodule ontogenesis.

The ectopic expression of *WOX5* did not result in changes in nodule number or in root length. Microscopic analyses revealed no modification in nodule morphology and only a minor effect on root morphology. In Arabidopsis, expression of *35S:WOX5* did not produce any morphological changes (Gonzali et al., 2005), whereas the direct activation of WOX5 by a dexamethasone-inducible method resulted in altered differentiation of columella cells (Sarkar et al., 2007). The observed differences between the two methods used in Arabidopsis might be explained by a tight control on *WOX5* expression by posttranscriptional and posttranslational mechanisms. Hence, the lack or small effect of *35S:MtWOX5* expression on nodulation and root growth might also be due to posttranscriptional and posttranslational regulations that control WOX5 protein levels. Hence, the analysis of WOX5 levels would provide more information.

In legume plants, nodule number and SAM homeostasis are controlled by a similar shoot-localized CLV signaling complex consisting of different receptors that perceive CLE peptides (Krusell et al., 2002, 2011; Nishimura et al., 2002; Searle et al., 2003). In Arabidopsis, CLV3 suppresses *WUS* expression to provide the fine balance between cell proliferation and differentiation at the SAM. We addressed the question of whether the CLV signaling complex that controls AON in the shoot also exerts its effect on the expression of *WOX5* in the nodules by long-distance regulation. Indeed, qRT-PCR as well as *pMtWOX5:GUS* analyses demonstrated that *WOX5* expression in the nodules changed in *sunn* and in *sym29* and *sym28* mutants. Interestingly, in these mutants, the level and area of *WOX5* expression had significantly increased in individual nodules. Instead of a restricted expression in the provascular strands as seen in the wild-type nodules, *WOX5* expression occurred in the meristem, the infection zone, and the young fixation zone. Hence, signaling via CLV1-like and CLV2-like proteins

repressed *WOX5* expression in developing nodules. *sunn* mutants did not show the inhibition of shoot-to-root auxin transport that is usually seen at the onset of nodulation (van Noorden et al., 2006). As *WOX5* is auxin inducible, changes in auxin levels might mediate the influence of the CLV signaling complex on *WOX5* expression. Thus, a WUS/WOX-CLV regulatory pathway seems to act in the nodule meristem similarly to that of RAM and SAM in plants. A characteristic property of RAM and SAM homeostasis is that both the WUS/WOX proteins and the CLV signaling complex act at short distances in neighboring cells to influence each other's expression. Although the legume CLV1-like RLK has been shown to be produced in the vasculature of roots and shoots, grafting has indicated that its activity in the shoot controls AON (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003; Schnabel et al., 2005). Whether the shoot or root activity regulates *WOX5* expression during nodulation is currently unknown, but tests via grafting experiments would be interesting.

Microscopic analysis of *sunn*, *sym29*, and *sym28* mutants did not reveal differences in nodule organization, indicating that high *WOX5* expression does not disorganize the central nodule tissue. Accordingly, no essential structural changes in the *35S*:*WOX5* nodules were found. Interestingly, in *sunn-3*, but not in the other AON mutants, the *WOX5* promoter was active also in the outer cortical cells through which infection threads grow toward the nodule primordium. This expression pattern is still visible in remnants of this region at the apex of mature nodules. Outer cortical cells preparing for infection thread passage also dedifferentiate, enter the cell cycle, but, in contrast to the inner cortical cells, get arrested in G2 to make the preinfection threads through which the infection threads will pass (Yang et al., 1994). In agreement with the suppressive effect of the CLV signaling complex on *WOX5* expression, a low level of *WOX5* might exist in these wild-type cells, and it might be needed transiently to organize this dedifferentiation step. Why this expression pattern was not observed in the other AON mutants is currently unknown. As both *sunn-3* and *sunn-4* carry nonsense mutations, they might represent strong alleles (Schnabel et al., 2005, 2010; Mortier et al., 2012a), but additional functional analysis, including of *sunn-1* and *sunn-2* mutants, is undoubtedly needed to assess this issue.

Nodule-derived CLE peptides have been shown to activate AON in different legumes. As a result, ectopic expression of these CLE peptides systemically inhibited nodulation that depended on a functional CLV signaling complex (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011). However, an unsettled question is whether these CLE peptides are able to migrate to the shoot and interact with the CLV receptor complex during AON (Mortier et al., 2012b). To answer this question, the effect of ectopic expression of the *MtCLE13* on the wild type and AON mutants of pea was assessed. The *M. truncatula* CLE13 peptide was active in pea roots, because the ectopic expression of *35S*:*MtCLE13* strongly reduced pea nodulation, similar to that in *M. truncatula* (Mortier et al., 2010), indicating that the perception of the nodule-derived AON CLE peptide is conserved between legume species. This observation is also supported by the conserved amino acid sequence of the AON-related CLE peptides of various legumes (Mortier et al., 2011). The effect was absent in the *sym28* and *sym29* mutants, indicating that PsSYM29 (CLV1 kinase) and PsSYM28 (CLV2 receptor) act downstream of the CLE peptide, consistent with previously published data for *L. japonicus*, *M. truncatula*, and soybean (Okamoto et al., 2009; Mortier et al., 2010). Hence, these data confirm the existence of a conserved mechanism of AON in different species of legume plants.

Similar to *sym28* and *sym29* mutants, the ectopic expression of the CLE peptide did not affect nodulation in the pea *nod3* mutant that, defective in a gene orthologous to the *M. truncatula ROOT DETERMINED NODULATION1* (*RDN1*), is disturbed in a root-determined component of AON (Schnabel et al., 2011). Experiments have shown that it might control the production/transportation of a root-derived signal that is sent to the shoot at the onset of AON rather than be involved in the perception of the return signal from the shoot (Li et al., 2009; Novák, 2010; Schnabel et al., 2011). In view of this hypothesis, our results on the *nod3* mutant demonstrate that AON CLE peptides might act upstream or at the level of the RDN1/NOD3 protein. Although we cannot rule out that RDN1/NOD3, synthesized in the vasculature, might aid the CLE peptide transport toward the shoot, our data might also hint at an indirect interaction between nodule-related CLE peptides and the CLV receptor complex in the shoot. As a result, different CLV signaling systems in the root and shoot might act on a common component, such as auxin homeostasis, to control the nodule number.

The relation between *WOX5* and *MtCLE13* expression is currently unresolved. At the onset of nodulation, the expression of *WOX5* overlaps with that of *MtCLE13*. In mature nodules, *MtCLE13* is expressed predominantly in the apical region of the nodules (Mortier et al., 2010), whereas *WOX5* is more restricted to the provascular strands. Interestingly, infrequently, the *MtCLE13* expression level is enhanced in these provascular strands (Mortier et al., 2010). Hence, short-distance communication between the CLE peptides and *WOX5* is very plausible. The expression of *WOX5* and *MtCLE13* remained unchanged in *35S*:*CLE13* and *35S*:*MtWOX5* roots, respectively (data not shown). More sensitive investigations, involving stable marker lines and careful microscopy, will be needed to unravel these interactions.

In summary, we have shown that *WOX5* expression in nodules is suppressed by the CLV signaling complex that controls AON. Thereby, we provide another component of the AON mechanism in addition to the CLV signaling mechanism in the shoot and the CLE peptides in the root. Hence, a conserved WUS/WOX-CLV regulatory system might regulate cell proliferation

and differentiation not only in RAM and SAM (regular plant apical meristems) but also in nodule meristems. The identified AON component, NOD3, might act downstream or alongside the CLE peptides during AON. The long-distance model in which root-derived CLE peptides would migrate to the shoot to be perceived and activate AON would be more complex than anticipated previously. Thus, although the exact interaction between the different AON components remains unsolved, we have found two more pieces of the AON puzzle that can be used in the future to gain an integrated insight into how AON works.

MATERIALS AND METHODS

Plant Material, Bacterial Strains, and Growth Conditions

Medicago truncatula Jemalong plants (wild-type A17 and supernodulating mutants *sunn-3* and *sunn-4*) and pea (*Pisum sativum*) cv Frisson (wild type and supernodulating mutants P64 [*sym28*] and P88 [*sym29*]) and cv Rondo (wild type and supernodulating mutant P79 [*nod3*]) were grown in growth chambers (16-h/8-h day/night regime, 21°C, and 75% relative humidity). *M. truncatula* plants were inoculated with *Sinorhizobium meliloti* strains Sm2011 pBHR-mRFP and Sm2011 pHC60-GFP and pea plants with *Rhizhobium leguminosarum* CIAM1026 from the All-Russia Research Institute for Agricultural Microbiology collection (http://www.arriam.spb.ru/eng/lab10/WDCM 966). Seeds were surface sterilized with concentrated sulfuric acid for 10 min and washed five to six times with sterile water. For temporal expression during nodulation, *M. truncatula* plants inoculated with Sm1021 pHC60-GFP were grown in aeroponic systems with nitrogen-poor SOLi medium (Blondon, 1964). Nodules were obtained at different stages after inoculation from the infected sites of roots by visualizing the green fluorescent bacteria with an MZFLII stereomicroscope (Leica Microsystems) equipped with a blue light source and a GFP Plus filter set (λ_{ex} = 480/40, λ_{em} = 510-nm long pass barrier filter; Leica Microsystems). Pea plants were grown in vermiculate-containing pots, and infected root tissue was harvested at different stages after inoculation with *R. leguminosarum* CIAM1026. Only segments between emerged lateral roots were collected to avoid harvesting of lateral root primordia.

Auxin Treatment

Five-day-old in vitro-grown plants were treated with 10^{-6} M indole-3-acetic acid supplemented to the medium. As a control, plants were grown without supplemented hormones. The growth conditions of the seedlings were the same as above. After 0, 3, 6, and 24 h of incubation, the roots of five to seven plants under each condition were harvested and analyzed by qRT-PCR. The experiment was repeated four times with comparable results.

Molecular Cloning

The *WOX5* gene fragment of pea was amplified with degenerate primers, cloned in the pAL-TA vector (Evrogen), and subsequently sequenced. The Mint RACE cDNA amplification kit (Evrogen) was used according to the manufacturer's protocol. As a result, an 872-bp sequence of the *WOX5* gene was identified. The *WOX5* promoter (1,965 bp) of *M. truncatula* was first cloned into the pENTR-D/TOPO vector (Invitrogen) and then in pBGFWS7.0 with the LR Clonase enzyme (Invitrogen). The primers used were 5′-GGGGACAACTTTGTATAGAAAAGTTGCAATTTTTGGCGACCAGATT-3′ and 5′-GGGGACTGCTTTTTTGTACAAACTTGCATGCTCTCTTCCATATTTCAATTC-3′. The coding sequence of *WOX5* was cloned into the pENTR-D/TOPO vector (Invitrogen) and then in pB7WG2D with the LR Clonase enzyme (Invitrogen).

qRT-PCR Analysis

Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. After a DNase treatment, the samples were purified through NH_4Ac precipitation, quality controlled, and quantified with a Nanodrop spectrophotometer (Isogen). RNA (1 μg) was used for cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad). The qRT-PCR experiments were done on a Lightcycler 480 (Roche Diagnostics), and SYBR Green was used for detection. All reactions were done in triplicate and averaged. Cycle threshold values were obtained with the accompanying software, and data were analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The relative expression was normalized against the constitutively expressed 40S ribosomal S8 protein (TC100533; *Medicago* Gene Index) for *M. truncatula* gene expression analysis and the ubiquitin gene in the experiments with pea. The following primers were used for qRT-PCR analysis: *MtWOX5*_forward, 5′-GCCTGATAGAGTGATTGAGAC-3′; *MtWOX5*_reverse, 5′-GGGTGTTCCATTTGTTCTCC-3′; *PsWOX5*_forward, 5′-GGTTTCAAAATCATAAGGCTAGGGA-3′; and *PsWOX5*_reverse, 5′-TCAACCGTAAGTCTAATGGTGGATG-3′. Each experiment was repeated at least three times with independent biological tissues.

Agrobacterium rhizogenes-Mediated Plant Transformation

A. rhizogenes-mediated *M. truncatula* plants were transformed as described (Mortier et al., 2010). For pea, the *M. truncatula* protocol was used and 3- to 4-d-old seedlings were infected by stabbing the hypocotyls with a fine needle containing an *A. rhizogenes* culture. After 7 to 10 d, calli appeared with emerged transgenic roots at the infection site. Plants were transferred into pots with vermiculite, watered with nitrogen-poor SOLi medium, grown for approximately 10 d at 21°C with a 16-h photoperiod and light at 70 μE m^{-2} s^{-1}, and subsequently inoculated with rhizobia.

Histochemical Localization of GUS Activity

GUS activity in cotransformed roots and nodules was analyzed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as substrate (Van den Eede et al., 1992). Roots and nodules were vacuum infiltrated during 20 min and subsequently incubated in GUS buffer at 37°C. After staining, root nodules were fixed, embedded with agarose (3%), and sectioned (50 μm) with a microtome with a vibrating blade (650V; Thermo Scientific). For thin sections (3–5 μm), plant tissues were embedded with the Technovit 7100 kit (Heraeus Kulzer) according to the manufacturer's instructions and sectioned with a microtome (Leica Microsystems). For tissue-specific staining, thin sections were submerged in a 0.05% (w/v) ruthenium red solution (Sigma-Aldrich), washed in distilled water, and dried. Finally, sections were mounted with Depex (BHD Chemicals). Photographs were taken with a Diaplan microscope equipped with bright-field and dark-field optics (Leitz) or a laser confocal microscope (LSM 510 META NLO; Carl Zeiss).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JN603579 and JN603580.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Alignment of *WOX5* coding sequences of pea and *M. truncatula*.

Supplemental Figure S2. *WOX5* expression in lateral root primordia analyzed by promoter-reporter fusion in *M. truncatula* and pea.

Supplemental Figure S3. Phenotype of *WOX5*-overexpressing roots of *M. truncatula*.

Supplemental Figure S4. Phenotypes of *WOX5*-overexpressing and control nodules of *M. truncatula*.

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