This article is from the
September 2003 issue of

Molecular Plant-Microbe Interactions®

published by
The American Phytopathological Society

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MPMI Vol. 16, No. 9, 2003, pp. 808–816. Publication no. M-2003-0714-02R. © 2003 The American Phytopathological Society

Comparison of Nodule Induction in Legume and Actinorhizal Symbioses: The Induction of Actinorhizal Nodules Does Not Involve *ENOD40*

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Submitted 5 May 2003. Accepted 2 June 2003.

Two types of root nodule symbioses are known for higher plants, legume and actinorhizal symbioses. In legume symbioses, bacterial signal factors induce the expression of *ENOD40* genes. We isolated an *ENOD40* promoter from an actinorhizal plant, *Casuarina glauca,* and compared its expression pattern in a legume (*Lotus japonicus*) and an actinorhizal plant (*Allocasuarina verticillata*) with that of an *ENOD40* promoter from the legume soybean (*GmENOD40-2*). In the actinorhizal *Allocasuarina* sp., *CgENOD40-GUS* and *GmENOD40-2-GUS* showed similar expression patterns in both vegetative and symbiotic development, and neither promoter was active during nodule induction. The nonsymbiotic expression pattern of *CgENOD40-GUS* in the legume genus *Lotus* resembled the nonsymbiotic expression patterns of legume *ENOD40* genes; however, in contrast to *GmENOD40-2-GUS, CgENOD40-GUS* was not active during nodule induction. The fact that only legume, not actinorhizal, *ENOD40* genes are induced during legume nodule induction can be linked to the phloem unloading mechanisms established in the zones of nodule induction in the roots of both types of host plants.

Additional keywords: promoter-GUS fusion.

Two root nodule symbioses are known between nitrogen-fixing soil bacteria and higher plants. In these interactions, the host plants form special organs, the root nodules, in response to bacterial signal factors. The microsymbionts fix nitrogen while being hosted inside nodule cells and supplied with carbon sources by the host plant. The symbiosis between rhizobia and legumes has been studied extensively (Cohn et al. 1998), while actinorhizal symbioses between actinomycetous bacteria of the genus *Frankia* and a diverse group of mostly woody plants from eight different families, collectively called actinorhizal plants, are less well understood (Pawlowski and Bisseling 1996). In legumes, several genes have been cloned whose expression is induced early in the process of nodule induction by bacterial signal factors, the Nod factors (Cohn et al. 1998). Due to the fact that the chemical nature of *Frankia* Nod factors is unknown, and the slower process of nodule induction on actinorhizal plants, such studies have not been performed in this system.

Legume nodules represent stem-like organs with a peripheral vascular system and infected cells in the central tissue, and their primordia appear in the root cortex. In contrast, actinorhizal nodules are coralloid organs consisting of multiple modified lateral roots with central vascular tissue and infected cells in the expanded cortex, and their primordia appear in the root pericycle like lateral root primordia. In spite of this apparent dissimilarity, cytological analysis has shown similarities in the early stages of nodule induction in both intracellularly infected legumes and actinorhizal plants. Rhizobial Nod factors induce root hair deformation (Heidstra et al. 1994) and lead to the induction of genes in the nodule epidermis (Ardourel et al. 1994; Horvath et al. 1993) and, within 12 h after application, to the induction of at least one gene, *ENOD40*, in the root pericycle (Compaan et al. 2001; Minami et al. 1996). At 24 h after Nod factor application, cortical cell divisions start and a nodule primordium is formed whose cells express *ENOD40*. Bacteria enter the root via an infection thread that is formed in a curled root hair, which grows toward the primordium cells and infects them. In intracellularly infected actinorhizal plants of the families Casuarinaceae, Myricaceae, or Betulaceae, the earliest response to the microsymbiont is root hair deformation (Pawlowski and Bisseling 1996); afterward, cortical cells begin to divide and form a so-called prenodule. Some of these dividing cells are infected by *Frankia* hyphae which enter the root via an infection thread-like structure formed in a curled root hair. At this point, however, the similarity with legumes ends. The nodule lobe primordium is formed in the pericycle. Infection thread-like structures grow from the prenodule to the nodule primordium and infect primordium cells.

Molecular phylogenetic studies have shown that legumes and actinorhizal plants go back to a common ancestor (Soltis et al. 1995); therefore, their nodule induction pathways can be expected to share some common features. In both systems, intracellular infection requires the induction of root hair deformation, polarization of cortical cells in order to support the tip growth of infection threads, and cortical cell division. Preliminary studies indicate that Frankia Nod factors do not represent lipochitooligosaccharides as rhizobial Nod factors do (Céremonie et al. 1999)

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but, nevertheless, some conservation of signal transduction pathways for elicitation of the abovementioned responses can be expected. It already has been shown that transcription factors of infected cells are conserved in legumes and actinorhizal plants, although the infected cells are located at different morphological positions in both systems: a legume leghemoglobin promoter-GUS fusion was expressed in infected cells of actinorhizal nodules (Franche et al. 1998), and an actinorhizal symbiotic hemoglobin promoter-GUS fusion was expressed in infected cells of legume nodules (Jacobsen-Lyon et al. 1995).

ENOD40 genes have been examined in several legume systems. They are present in symbiotic as well as nonsymbiotic plants (Kouchi et al. 1999), induced very early in nodule induction (i.e., prior to root cortical cell divisions), and expressed in the nodule vascular system throughout nodule development (Matvienko et al. 1994; Yang et al. 1993). In order to compare the induction patterns of legume and actinorhizal *ENOD40* gene promoters in legumes and actinorhizal plants, we isolated the *ENOD40* gene from one of the transformable actinorhizal plants, *Casuarina glauca. C. glauca* as well as a close relative, *Allocasuarina verticillata*, was transformed with a *CgENOD40* promoter-*GUS* fusion and an *ENOD40* promoter-*GUS* fusion construct from the legume soybean (*GmENOD40-2-GUS*) (Roussis et al. 1995) and their expression patterns were com-

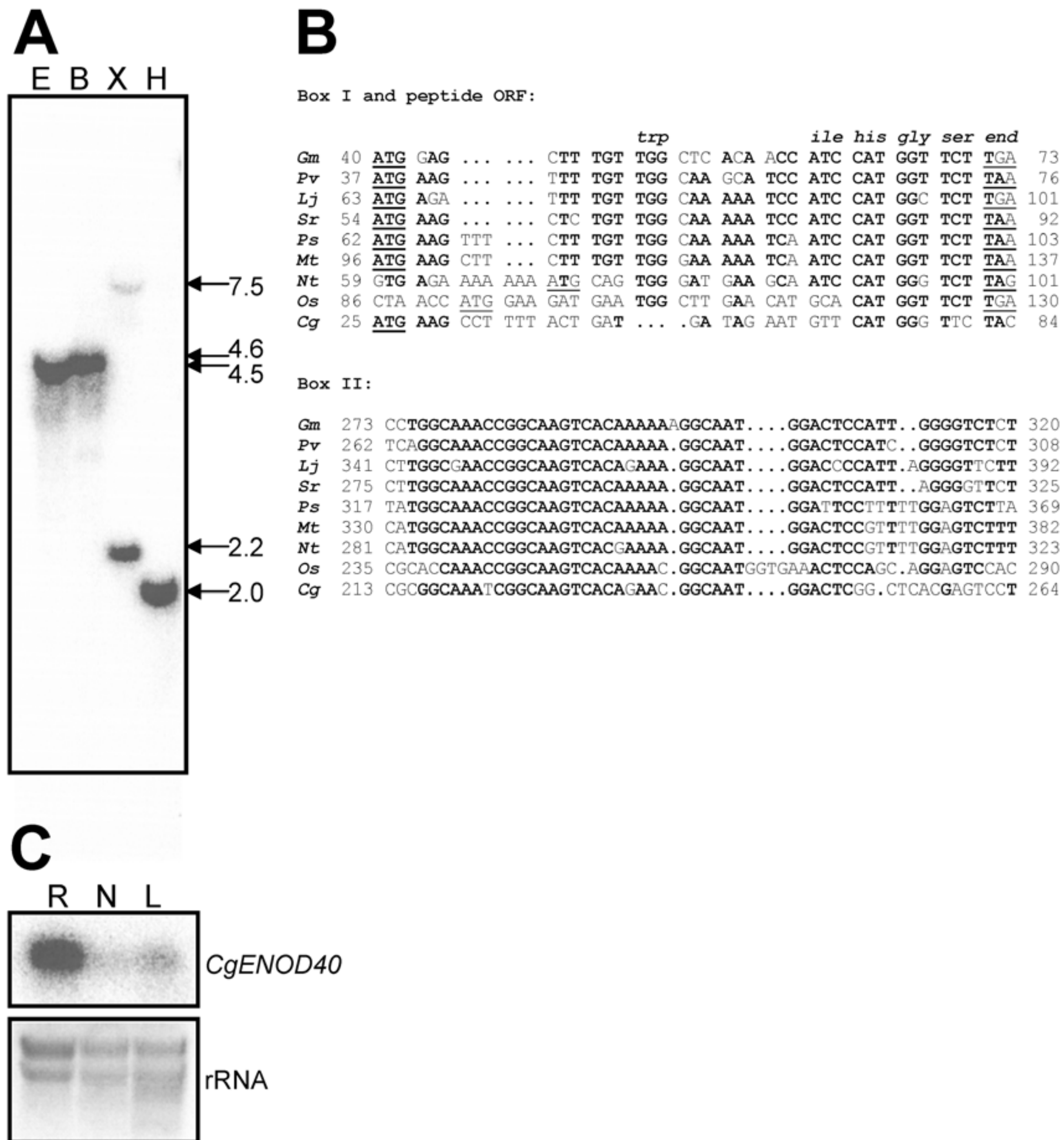


Fig. 1. A, *CgENOD40* seems to be encoded by a single gene. A DNA gel blot with total DNA of 10 µg/slot from *Casuarina glauca* plants digested with *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), and *Xba*I (X) was hybridized with the full size *CgENOD40* cDNA. The cDNA sequence contains one *Xba*I site, but no *Eco*RI, *Bam*HI, or *Hin*dIII site. **B,** Interspecific conservation of *ENOD40* cDNA sequences. The peptide ORF/box I and box II sequences of soybean (*Gm*; Yang et al. 1993), *Phaseolus vulgaris* (*Pv*; Papdopoulou et al. 1996), *Lotus japonicus* (*Lj*; Flemetakis et al. 2000), *Sesbania rostrata* (*Sr*; Corich et al. 1998), pea (*Ps*; Matvienko et al. 1994), *Medicago truncatula* (*Mt*; Crespi et al. 1994), *Nicotiana tabacum* (*Nt*; van de Sande et al. 1996), rice (*Os*; Kouchi et al. 1999), and *Casuarina glauca* are given. Nucleotides conserved in at least five sequences are labeled by bold print. In box I, the start and stop codons of the encoded peptides are underlined. The conserved part of the encoded peptide sequence (W-X_3-IHGS) is added on top of the DNA sequence comparison. *CgENOD40* contains most of the box I conservation on the DNA level as well as the start codon, but a 4-bp deletion between start codon and box I abolishes the conserved open reading frame. **C,** Expression of *CgENOD40* in different organs of *C. glauca.* Total RNA was isolated from roots (R), mature nitrogen-fixing nodules (N), and aerial parts (A) of *C. glauca* plants and approximately 10 µg per slot were separated on a 1.2% formaldehyde agarose gel. The amount of RNA per slot was quantified by photographing the 28S rRNA bands. The RNA gel blot was hybridized with the ^{32}P-labeled *CgENOD40* cDNA. Signal was determined by using a PhosphoImager (Molecular Dynamics, ImageQuant).

pared. In parallel, the legume *Lotus japonicus* was transformed with the *CgENOD40* promoter-*GUS* fusion construct, and its expression pattern was compared with that of *GmENOD40-2-GUS* (Martirani et al. 1999).

RESULTS AND DISCUSSION

Cloning of the *CgENOD40* promoter and construction of a promoter-*GUS* fusion.

Genome walking polymerase chain reaction (PCR) was performed on *Casuarina glauca* genomic DNA with a gene-specific primer derived from the conserved box II sequence of *Alnus glutinosa ENOD40* (5′-TCGTGACTTGCCGGTTTGC-CGTGGTTC-3′) and the adaptor primer AP1 from the Universal Genome Walker Kit (Clontech). From the 5′ end of the resulting 1.3-kb fragment, a new gene-specific primer was derived (5′-GTGTCTCGACCTCAGTGGCTAATCTG-3′) and used with the adaptor primer for the amplification of a 1.7-kb fragment which was cloned and sequenced. For the complete promoter, a primer derived from the 5′ end of the promoter (5′-TTCTCGGAGACCAAAATGGAAGTGCAATCA-3′) was used together with the first gene-specific primer to amplify a 2.6-kb fragment that contained 2.4 kb of the promoter and approximately 200 bp of the transcribed region based on comparisons with the *Alnus glutinosa ENOD40* cDNA sequence (data not shown). From this fragment, the 2.4-kb promoter fragment was amplified using the 5′ primer and a 3′ primer derived from the putative begin of the transcribed region (5′-GGGCTCTAACAAAAGCAGGTGGAGAAGC-3′). The resulting 2.4-kb promoter fragment was cloned and sequenced (EMBL accession no. AJ459787). For the construction of *CgENOD40-GUS*, this fragment was excised using *Not*I, treated with Klenow polymerase, and inserted in the *Sma*I site of pBI101.3 (Jefferson et al. 1987).

C. glauca ENOD40 is encoded by a single gene.

For cloning of the *C. glauca ENOD40* cDNA, PCR was performed on *C. glauca* nodule cDNA obtained using primer 5′-CTCGAGGATCCGCGGCCGCT$_{18}$-3′. Using a gene-specific 5′ primer derived from the sequence of the 1.3-kb genome walking PCR product (5′-CAGTTTCTCTTAGCTGATAGGC-3′) and the anchor primer, a 550-bp fragment was obtained, cloned, and sequenced (EMBL accession no. AJ487686). To find out whether the *CgENOD40* gene contains an intron or introns, PCR with 5′ and 3′ primers for the cDNA (5′-CAGTTTCTCTTAGCTGATAGGC-3′ and 5′-GATAGGAG-CACGAATATGCATGC-3′) was performed in parallel on total DNA from *C. glauca* leaves, and on nodule cDNA. Fragments of equal length were amplified (data not shown), indicating that, like other *ENOD40* genes (Crespi et al. 1994; Fang and Hirsch 1998), *CgENOD40* does not contain an intron. To analyze the gene family situation, total DNA from *C. glauca* was digested with *Eco*RI, *Bam*HI, *Hin*dIII, and *Xba*I and hybridized with the *CgENOD40* cDNA. The results corresponded to the predictions based on the cDNA sequence (Fig. 1A). Thus, we concluded that there is only one *ENOD40* gene in *C. glauca*.

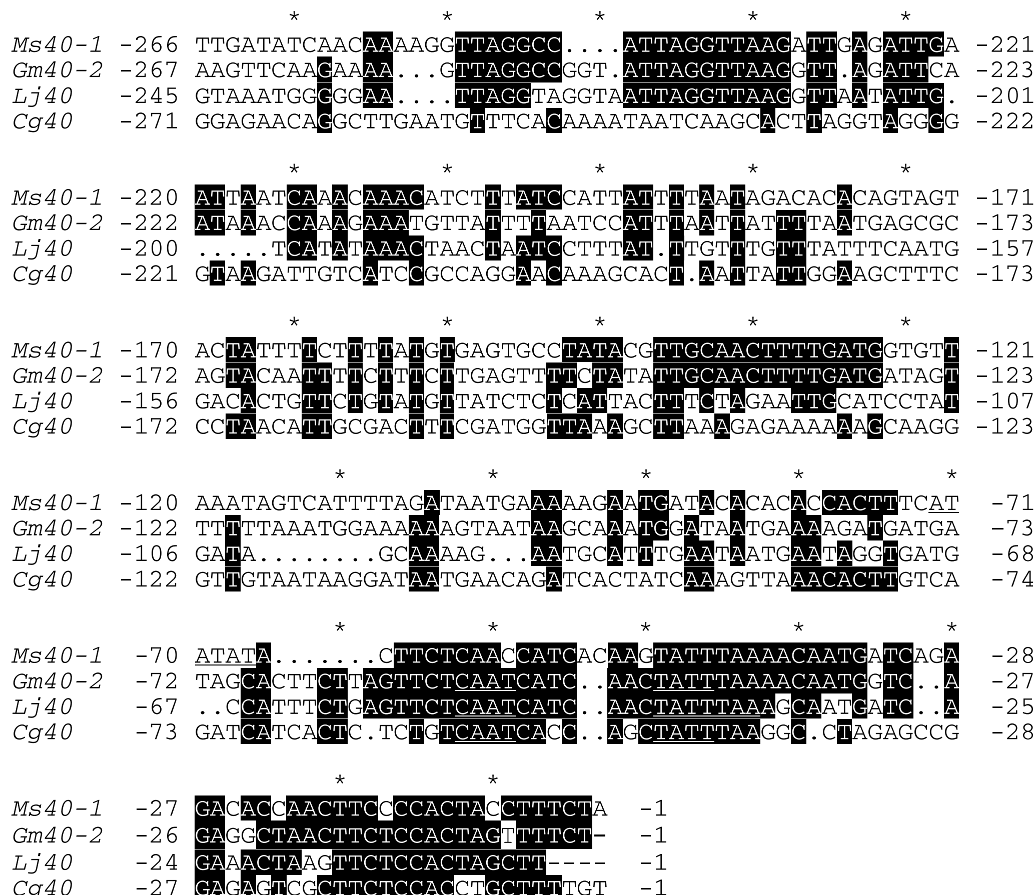


Fig. 2. Sequence comparison of the 3′ ends of *ENOD40* promoters from three legumes (*Medicago sativa* [*Ms*], Fang and Hirsch 1998; soybean [*Gm*], Yang et al. 1993; and *Lotus japonicus* [*Lj*], Chian and Gresshoff 1997) and *Casuarina glauca (Cg).* Only one of the two *M. sativa ENOD40* promoters is given because their sequences are identical in the 3′ region. Numbering is from the transcriptional start site backward (the transcriptional start site is putative in the case of *Lj* and *Cg*; in the case of *Ms40-1* it is determined based on the *MsENOD40* cDNA sequence [Asad et al. 1994]). Identical amino acids in conserved positions are labeled by inverse print. Putative CAAT and TATA boxes are underlined.

The 3′ part of the *Casuarina ENOD40* promoter shows homology with *ENOD40* promoters from legumes.

Comparison of the 3′ parts of *ENOD40* promoters from *Medicago sativa,* soybean, *L. japonicus,* and *Casuarina* spp. shows that the sequences around the putative CAAT and TATA boxes are conserved among the four species (Fig. 2). Approximately 200 bp upstream of this region, conservation exists between the legume *ENOD40* promoters, but the corresponding sequences are not conserved in *Casuarina* spp. No sequence conservation was found between the 5′ parts of *ENOD40* promoters from legumes and *Casuarina* spp. (data not shown).

***CgENOD40* does not contain the conserved peptide open reading frame and its expression is not induced in nodules compared with roots.**

ENOD40 sequences are characterized by two conserved regions, box I and box II (van de Sande et al. 1996). Box I represents the 3′ part of an open reading frame (ORF) encoding a short peptide of 10 to 13 amino acids that is conserved in all *ENOD40* sequences analyzed thus far. A comparison of the *CgENOD40* cDNA sequence with *ENOD40* sequences from legumes and nonlegumes shows that both box I and box II sequences are conserved (Fig. 1B). However, the peptide ORF is missing due to a frame shift between the ATG and the conserved box II sequence. This lack of the peptide ORF also was found in the *ENOD40* gene of another actinorhizal plant, *Alnus glutinosa* (U. von Groll and K. Pawlowski, *unpublished data*).

RNA gel blot analysis with RNA from roots, nodules, and aerial parts of *C. glauca* plants revealed that *CgENOD40* was expressed strongly in roots and at very low levels in leaves and nodules (Fig. 1C). In contrast with all legumes examined, where *ENOD40* expression is strongly induced in nodules compared with roots, *CgENOD40* expression levels in nodules were lower than in roots. Furthermore, nodule expression levels were too low for in situ hybridization (data not shown). The absence of transcriptional induction in mature nodules indicates that, in contrast with legume nodules where *ENOD40* is expressed in the inner tissue, actinorhizal nodule function might not require *ENOD40.* However, this does not need to reflect on the function of *ENOD40* during nodule induction, where it is expressed in legume nodule primordia. Preliminary in situ hybridization experiments did not show *CgENOD40* expression in *Casuarina* prenodules (data not shown), but this could be due to the low overall expression levels of *CgENOD40* and the high detection limit of in situ hybridization experiments with woody plants. Therefore, transgenic actinorhizal plants containing the *CgENOD40-GUS* construct were required to examine *CgENOD40* expression during nodule induction.

The *Casuarina ENOD40* promoter and the soybean *ENOD40-2* promoter direct similar expression patterns during nonsymbiotic development of *A. verticillata*.

There are two transformable actinorhizal plants, *C. glauca* (Le et al. 1996) and *A. verticillata* (Franche et al. 1997). However, transformation and regeneration of *A. verticillata* is far more efficient than that of *C. glauca.* The efficiency of gene transfer is much lower in *C. glauca* than in *A. verticillata,* regeneration of transgenic plants takes 3 months longer, fewer transgenic shoots are obtained per callus, and the rooting efficiency of transgenic shoots is lower than in *A. verticillata.* Both plant species were transformed with the *CgENOD40-GUS* construct however, because only 10 transgenic *Casuarina* lines could be obtained in contrast with 28 transgenic *Allocasuarina* lines, only GUS staining results obtained with *Allocasuarina* lines will be presented here. GUS staining results of transgenic *Casuarina* lines were similar to those of *Allocasuarina* lines. The expression patterns of several promoter-*GUS* fusions have been compared in *C. glauca* and *A. verticillata* in the past. (Leg-)hemoglobin promoters from soybean, *Casuarina* spp., and *Parasponia* spp. (Franche et al. 1998); a metallothionein gene promoter from *Casuarina* spp. (Ahmadi et al., in press; Laplaze et al. 2002; C. Franche, *unpublished data*); the *cg12* promoter from *C. glauca* (Svistoonoff et al. 2003); as well as the promoter from *ENOD12B* from pea (C. Franche, *unpublished data*) directed the same expression patterns in both plant species. Slight differences were found between the expression directed by the CaMV *35S* promoter in aerial parts, but not in roots, of *C. glauca* versus *A. verticillata* (Franche et al. 1997; Smouni et al. 2002).

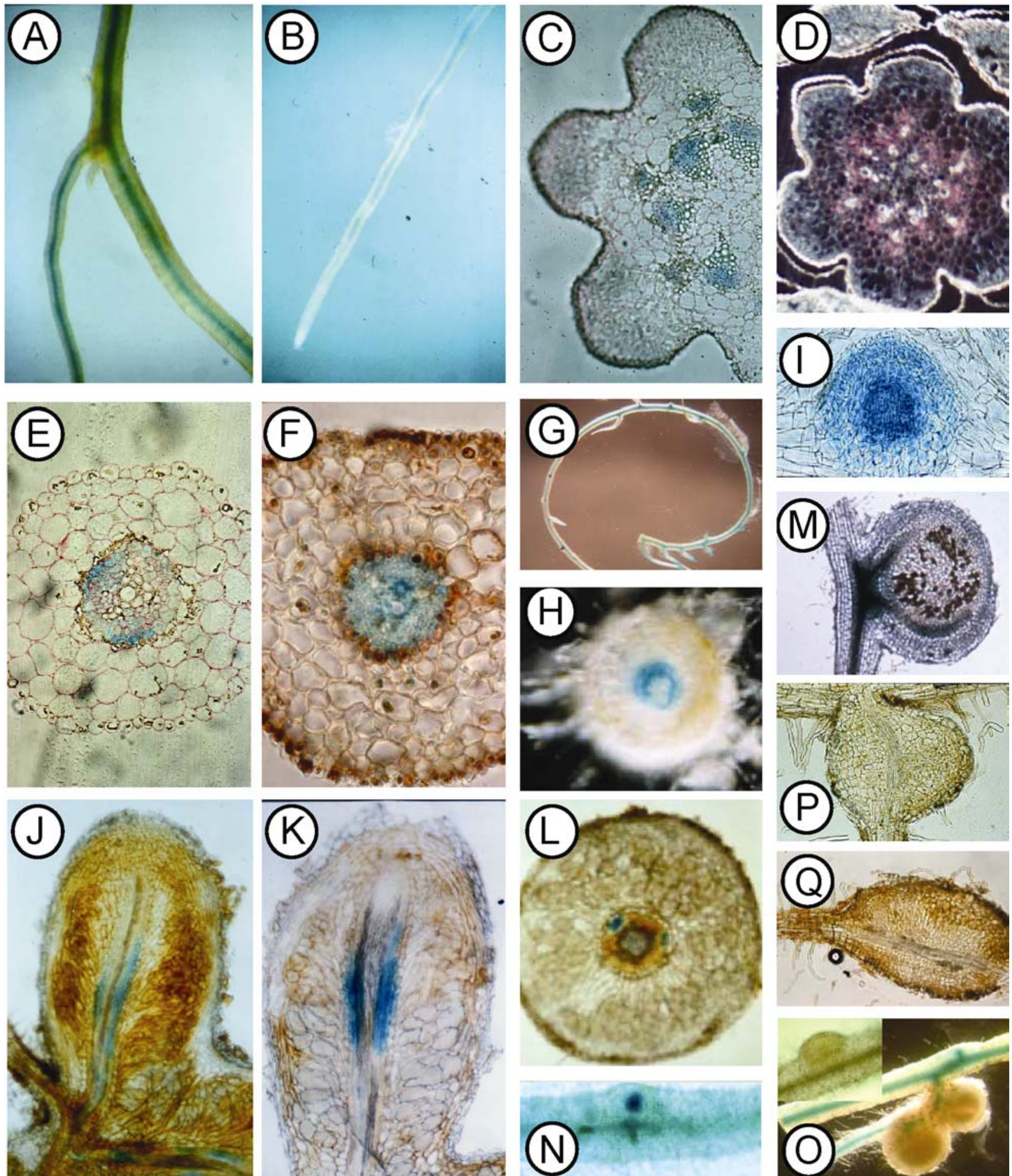
Roots and aerial parts from 28 transgenic *A. verticillate* lines containing the *CgENOD40-GUS* construct and 16 lines containing the *GmENOD40-2-GUS* construct (Martirani et al. 1999) were stained for GUS activity. In all cases, GUS activity was associated with the vascular system (Fig. 3A and B for roots, C and D for aerial parts). In both roots and aerial parts, GUS activity was found throughout the vascular system, but not in the apical zones. Cross sections showed that, in roots, *CgENOD40-GUS* was expressed strongly in the stele and in the pericycle at the phloem poles, and weakly in the phloem; whereas, in stems, it showed strong expression in the procambium/phloem of the vascular bundles (Fig. 3C and E). *GmENOD40-2-GUS* was expressed in the procambium and phloem of stems (Figure 3D); however, in roots, its expression was found not only in the phloem, but also in the xylem parenchyma (Fig. 3F).

The *Casuarina ENOD40* promoter directs expression in the vascular system of *L. japonicus* roots.

Transgenic roots containing the *CgENOD40-GUS* construct from 30 *L. japonicus* composite plants were stained for GUS activity. Blue precipitate denoting promoter activity was found in the root pericycle of 65% of these plants (Fig. 3G and H). In contrast with the situation in *A. verticillata* plants, *CgENOD40-GUS* was active in the tip of the primary root of *L. japonicus* plants (Fig. 3G) and also in lateral root primordia (Fig. 3I). The staining occurred in approximately 60% of the roots arisen from the *Agrobacterium rhizogenes*-mediated infection site, which is consistent with the efficiency of cotransformation of *GUS* and pRi-born T-DNAs previously reported (Le et al. 1996). The expression pattern of *GmENOD40-2-GUS* in *L. japonicus* had been examined already (Le et al. 1996) with the result that, in contrast to *CgENOD40-GUS, GmENOD40-2-GUS* did not show expression in uninfected roots. However, in soybean where *ENOD40* is encoded by a gene family of at least two members, in situ hybridization showed that *ENOD40* also is expressed during nonsymbiotic plant development, which may be accounted for by *GmENOD40-1* (Kouchi and Hata 1993; Yang et al. 1993). In *M. sativa, ENOD40* is encoded by a gene family with two members, one of which is expressed nodule-specifically (*MsENOD40-1*) while the other one is expressed in nonsymbiotic development as well as during nodulation (*MsENOD40-2*) (Fang and Hirsch 1998). *MsENOD40-2* is expressed in the root stele and in the stem procambium or phloem region of *M. sativa* (Fang and Hirsch 1998), resembling the expression pattern of *CgENOD40* in *Lotus* spp. Also, in *Sesbania rostrata, ENOD40* expression is found in the root stele in nonsymbiotic plant development (Corich et al. 1998). Altogether, the nonsymbiotic expression pattern of the only *ENOD40* promoter from the *C. glauca*, *CgENOD40,* in the legume genus *L. japonicus* resembles the nonsymbiotic expression patterns of legume *ENOD40* genes.

In nodules of *Allocasuarina* and *Lotus* plants, both the *Casuarina ENOD40* and the soybean *ENOD40-2* promoter direct expression in the vascular pericycle.

Longitudinal and cross sections of mature actinorhizal nodules from transgenic *A. verticillata* and *C. glauca* plants showed that, with both *ENOD40-GUS* constructs, blue staining is visible in parts of the multilayered pericycle of the central vascular bundle of a nodule lobe, namely at the phloem poles (20 plants positive out of 20 *A. verticillata* plants and five plants positive out of five *C. glauca* plants tested for *CgENOD40-GUS*, 20 plants positive out of 20 *A. verticillata* plants tested for *GmENOD40-2-GUS;* Fig. 3J, K, and 3L). In transgenic *L. japonicus* nodules, strong expression of *CgENOD40-GUS* was found in the vascular system (12 plants positive out of 20 plants tested; Fig. 3M). For *GmENOD40-2-GUS,* it already had been shown in transgenic *L. japonicus* as well as in transgenic *Vicia hirsuta* that expression in mature nodules is confined to the nodule vascular system (Martirani et al. 1999; Roussis et al. 1995), although this expression pattern of *GmENOD40-2* contradicts the expression patterns of the endogenous *ENOD40* genes as shown by in situ hybridization. In nodules of soybean and *L. japonicus*, *ENOD40* mRNA was found not only in the vascular system but also in the uninfected cells of the inner tissue of mature nodules (Flemetakis et al.



2000; Kouchi and Hata 1993; Yang et al. 1993). *ENOD40* expression has not been examined in *V. hirsuta* however, in nodules of *V. faba, ENOD40* was expressed in the vascular system and in uninfected cells of interzone II-III (Vijn et al. 1995). As discussed by Roussis and associates (1995), it is possible that the sequences necessary for expression in the inner tissue are located outside the 1.7-kb *GmENOD40-2* promoter fragment used in this study and by Martirani and associates (1999). Alternatively, the other *ENOD40* gene from soybean (*GmENOD40-1*) would have to be responsible for the expression in the uninfected cells of the inner tissue of mature determinate nodules. The homology between *GmENOD40-1* and *GmENOD40-2* is too high to allow the construction of gene-specific probes for in situ hybridization experiments.

Cell-specific expression of *ENOD40* in the inner tissue of mature nodules is not conserved among different legume species. Although, in determinate nodules, expression is confined to the uninfected cells (Flemetakis et al. 2000; Kouchi and Hata 1993; Papadopoulou 1996; Yang et al. 1993), the picture is more diverse for indeterminate nodules or intermediate nodule forms (Corich et al. 1998; Crespi et al. 1994; Fang and Hirsch 1998; Matvienko et al. 1994; Vijn et al. 1995). Hence, the lack of expression of an actinorhizal *ENOD40* gene promoter in the inner tissue of legume nodules does not necessarily imply a striking difference in regulation. The common feature of expression patterns of *ENOD40* genes in legume nodules is the expression in the vascular tissue. Altogether, *ENOD40* promoters from *C. glauca* and soybean show the same expression pattern in *A. verticillata* nodules and share a high activity in the vascular system of legume nodules.

C. glauca ENOD40-GUS is not induced in the pericycle of *L. japonicus* roots during nodule formation, nor is it expressed in nodule primordia.

In the course of nodule induction in *L. japonicus* plants, *GmENOD40-2-GUS* shows the same expression pattern as was determined by in situ hybridization in soybean: induction in the root pericycle at the infection site, then induction in the cells of the nodule primordium that is forming in the root cortex (Fig. 3N) (Le et al. 1996). When early infection stages of transgenic *L. japonicus* roots containing *CgENOD40-GUS* were stained for GUS activity, none of them showed either the induction of GUS staining in the root pericycle or staining in the nodule primordia of young nodules (Fig. 3O). GUS activity was confined to the root stele as in uninfected roots (of 15 plants examined, 60% showed staining in 62% of the roots; data not shown). Thus, the signal transduction pathway that leads to the induction of legume *ENOD40* early in nodule development (Compaan et al. 2001) does not seem to activate the actinorhizal *ENOD40* promoter. There are two possible explanations for this fact. (i) The lack of *CgENOD40-GUS* induction during legume nodule initiation is due to the absence of *CgENOD40* promoter elements that are activated by a Nod factor-dependent signal transduction pathway present in both legumes and actinorhizal plants. (ii) The lack of *CgENOD40-GUS* induction is due to the fact that the Nod factor-dependent signal transduction pathway responsible for legume *ENOD40* induction is not active in actinorhizal plants. The expression of *GmENOD40-2-GUS* during nodule initiation in *A. verticillata* plants was examined to decide between i and ii.

Neither *C. glauca ENOD40-GUS* nor soybean *ENOD40-2-GUS* are expressed in prenodules of *A. verticillata* plants.

When root systems of transgenic *A. verticillata* and *C. glauca* plants containing either *CgENOD40-GUS* or *GmENOD40-2-GUS* were tested for GUS activity 4 weeks after infection with *Frankia,* no blue stain was found in prenod-

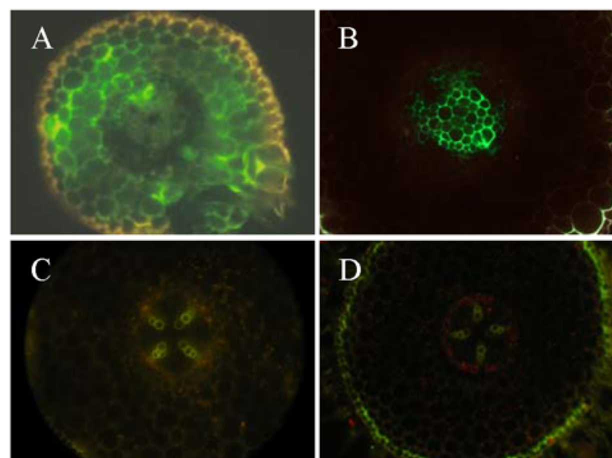


Fig. 4. Unloading of fluorescent tracers from the phloem in the root hair extension zone. **A** and **C,** Stems from *Casuarina glauca* plants and **B** and **D,** petioles from *Medicago truncatula* seedlings grown in pot soil were loaded with a 2.5 mg/ml solution of 8-hydroxypyrene-1,3,6-trisulfonic acid in H_2O. **A** and **C,** After 4 h (*M. truncatula*) and after 10 h (*C. glauca*), root hair extension zones were embedded in 3% agarose and vibratome cross sections were viewed under fluorescent light (450–490 nm) using a photonic microscope. **B** and **D,** Roots of untreated plants were used as negative controls. Green fluorescence in the root cortex indicating symplastic phloem unloading is found in **A,** *C. glauca* but not in **C,** *M.truncatula* roots.

Fig. 3. GUS activity staining of transgenic *Allocasuarina verticillata* or *Lotus japonicus* plants containing the *CgENOD40-GUS* or the *GmENOD40-2-GUS* construct. In **A, B, C, E, F, G, H, I, J, K, M, N, P**, and **Q,** GUS activity denoting promoter activity is visible as blue stain. **D** and **O,** Taken under fluorescent light; GUS activity is denoted by a purple precipitate. **A,** Part of a root system of an *A. verticillata* plant containing *CgENOD40-GUS*. Blue staining can be seen in the stele. **B,** Root of an *A. verticillata* plant containing *GmENOD40-2-GUS*. Blue staining can be seen in the stele. **C,** Cross section through the stem of an *A. verticillata* plant containing *CgENOD40-GUS.* GUS activity is present in the procambium/phloem of the vascular bundles. **D,** Stem cross section of a *GmENOD40-2-GUS A. verticillata* plant with GUS activity in the procambium or phloem of the vascular bundles. **E,** Root cross section of an *A. verticillata* plant containing *CgENOD40-GUS.* GUS activity is present in the pericycle at the phloem poles and in the phloem. **F,** Root cross section of a *GmENOD40-2-GUS A. verticillata* plant. GUS activity is present in the entire stele. **G,** Tip of a primary root of a composite *Lotus japonicus* plant containing *CgENOD40-GUS.* GUS activity can be seen in the stele and in the root tip. **H,** Cross section of a *L. japonicus* root containing *CgENOD40-GUS.* GUS activity is confined to the root pericycle. **I,** Section through a lateral root primordium of a *L. japonicus* plant with *CgENOD40-GUS.* GUS activity is found in the primordium. **J,** Longitudinal section of a nodule of an *A. verticillata* plant containing *CgENOD40-GUS.* GUS activity can be seen in parts of the phloem. **K,** Longitudinal section of a nodule of a *GmENOD40-2-GUS A. verticillata* plant with GUS activity in parts of the phloem. **L,** Cross section of a *CgENOD40-GUS A. verticillata* nodule. GUS activity is confined to the phloem poles. **M,** Longitudinal section through a young nodule of a *CgENOD40-GUS*-containing *L. japonicus* plant. GUS activity is confined to the vascular system. **N,** *Mesorhizobium*-treated root of a *L. japonicus* plant containing *GmENOD40-2-GUS.* GUS activity can be seen in the nodule primordium. **O,** *Mesorhizobium*-treated root of a *Lotus* plant containing *CgENOD40-GUS.* No GUS activity can be seen in the nodule primordium (small picture) or in young nodules (main picture). **P,** Prenodule on a root of an *A. verticillata* plant containing *CgENOD40-GUS*. No GUS activity is detectable. **Q,** Prenodule on a *GmENOD40-2-GUS A. verticillata* root. No GUS activity is detectable.

ules but GUS activity was present in the root stele as in uninfected roots (50 *A. verticillata* and 10 *C. glauca* transformants analyzed for *CgENOD40-GUS*, 40 *A. verticillata* transformants analyzed for *GmENOD40-2-GUS*) (Fig. 3P and 3Q). Thus, the Nod factor-dependent signal transduction pathway responsible for the induction of legume *ENOD40* induction is not active in intracellularly infected actinorhizal plants.

What is the function of *ENOD40* during the induction of legume nodules?

Although, in legumes, *ENOD40* transcription is induced by Nod factors (Minami et al. 1996), the induction is not specific to Nod factor structure as is the case for the induction of, for example, *ENOD12* expression (Ardourel et al. 1994). *ENOD40* expression in legume roots also is induced or enhanced by undecorated chitin oligomers (Minami et al. 1996) (i.e., possibly by pathogens, and by mycorrhization [van Rhijn et al. 1997]). Hence, while the induction of *ENOD40* is an integral part of legume nodulation and *ENOD40* expression levels are a limiting factor in legume nodule development (Charon et al. 1999), its increased expression plays a role in legume–microbe interactions other than root nodule symbiosis (Staehelin et al. 2001; van Rhijn et al. 1997). When the expression pattern of *ENOD40* in nonsymbiotic plant development is studied, it usually is found to be associated with the vascular system (Fang and Hirsch 1998; Kouchi and Hata 1993; Kouchi et al. 1999; Yang et al. 1993). Some *ENOD40* genes also are expressed in organ primordia (Fang and Hirsch 1998; Corich et al. 1998; Papdopoulou et al. 1996). In summary, *ENOD40* is expressed in the vascular system and in some sink tissues, and it is induced in root symbioses where the sink strength of roots has to be increased in order to supply microsymbionts with carbon sources. The question arises whether (i) *ENOD40* has a function in increasing phloem unloading, sink strength, or both and (ii) whether different mechanisms are employed for these processes in legumes versus actinorhizal plants. A function of *ENOD40* in phloem unloading or sink strength increase would be consistent with the effects of its over- and underexpression in *Medicago* plants (Charon et al. 1999; Staehelin et al. 2001). Recently, Röhrig and associates (2002) have shown that *ENOD40*-encoded peptides bind to sucrose synthase. An increase of sucrose synthase activity could be instrumental in increasing sink strength (Sturm and Tang 1999).

Phloem unloading in legumes versus actinorhizal plants.

Phloem unloading and post-phloem transport can occur symplastically via plasmodesmata or apoplastically via the release of sugars into the apoplast and their uptake into the next cells via sugar-proton symporters exploiting the proton motive force (Patrick 1997). Apoplastic transport mechanisms cannot take place between cells with lignified cell walls. The root systems of all actinorhizal plants are lignified; therefore, although most legumes whose root nodule symbiosis has been studied in detail are herbaceous plants, it is plausible that the regulation of carbon transport mechanisms in roots would be different in legumes than in actinorhizal plants.

For *Arabidopsis thaliana*, symplastic phloem unloading and symplastic post-phloem transport in the root tip has been shown using fluorescent symplastic tracers (Oparka et al. 1994; Wright and Oparka 1996) and additional apoplastic mechanisms have been implied based on the expression patterns of sugar transporters (Truernit and Sauer 1995). It should be noted that no significant symplastic phloem loading has been reported for the root hair elongation zone where nodule induction occurs, indicating that, in this area, apoplastic mechanisms dominate (Oparka et al. 1994; Wright and Oparka 1996). For the roots of woody plants with secondary cell wall modifications that would block apoplastic transport, it is likely that apoplastic post-phloem sugar transport plays only a minor role. An analysis of symplastic phloem unloading and post-phloem transport in the root hair extension zones of *C. glauca* and *M. truncatula* roots was performed using fluorescent tracers (carboxyfluorescein and 8-hydroxypyrene-1,3,6-trisulfonic acid). The results indicate that, in the root hair extension zone of *C. glauca* roots where nodule induction takes place, unloading is mostly symplastic, whereas no symplastic unloading was detected in the corresponding region of *M. truncatula* roots (Fig. 4). Thus, if *ENOD40* is involved in either inducing symplastic sugar transport or increasing apoplastic sugar transport, the activation of its expression leading to local enhancement of phloem unloading would represent a crucial part of nodule induction in nodulated herbaceous plants with mostly apoplastic transport mechanisms in the places of nodule induction; whereas, in nodulated woody plants with mostly symplastic phloem unloading mechanisms in the root hair extension zone, *ENOD40* would not play a role in nodule induction. This would explain why *ENOD40* is induced only during the induction of legume, not *C. glauca* nodules (Fig. 3N and Q).

MATERIALS AND METHODS

Plant and bacterial growth conditions.

C. glauca and *A. verticillata* seed were collected in Australia and obtained from Versepuy (Le Puy-en-Velay, France). Seed were surface sterilized and germinated as described previously (Le et al. 1996). The *Frankia* Allo2 strain used for the inoculation of *A. verticillata* was grown in a modified BAP medium (Benoist et al. 1992). *Agrobacterium tumefaciens* strain C58C1(pGV2260) (Vancanneyt et al. 1990) was used for the genetic transformation of *A. verticillata.* For nucleic acid isolation, *C. glauca* seed were germinated and grown in soil for 3 months, before being transferred into aerated liquid culture in one-quarter-strength nitrogen-free Hoagland's medium as described for *Alnus glutinosa* (Ribeiro et al. 1995). There, they were inoculated with *Frankia* strain Thr (Girgis and Schwencke 1993) grown in BAP medium (Fontaine et al. 1986). For RNA isolation, nodules were harvested 4 to 12 weeks after inoculation. Only nodules produced between April and October were used for experiments. Roots for RNA isolation were harvested from plants grown in one-quarter-strength Hoagland's medium containing KNO_3. Plants were grown in a greenhouse at day and night temperatures of 22 and 18°C, respectively, with photons at 200 to 400 µmol m^{-2} s^{-1}. *L. japonicus* ecotype GIFU F10 seed were sterilized (20 min in 25% commercial bleach [1% hypochlorite], 0.1 % Triton) and washed six times in sterile water. After overnight incubation at 4°C seed were spread on the surface of 1% agar plates and left overnight at 4°C. Then the plates were transferred to a culture cabinet (23°C, 16-h photoperiod). MSU440 (Sonti et al. 1995) and *Mesorhizobium loti* NZP2235 (DSIR culture collection) strains were grown at 28°C in Luria Bertani (LB) or TYR medium (Taté et al. 1998), respectively.

Molecular biological methods.

DNA was isolated from aerial organs of *C. glauca* as described for *Alnus glutinosa* (Ribeiro et al. 1995). RNA from roots, nodules, and aerial parts of *C. glauca* was isolated using the InvisorbSpin Plant RNA Mini Kit from Invitek (Berlin, Germany). Genome walking was performed using the Universal Genome Walker Kit from Clontech (Palo Alto, CA). PCR products were cloned in pGEM-T Easy (Promega Corp., Madison, WI). Sequencing reactions were performed using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany). Samples were separated using an ABO PRISM 310

Genetic Analyzer (Perkin-Elmer). The full size promoter was sequenced by SEQLAB (Göttingen, Germany).

RNA was separated on 1.2% formaldehyde-agarose gels and DNA on 0.8% agarose gels, then transferred to Amersham Hybond N membranes (Amersham Pharmacia Biotech, Freiburg, Germany) as described (Sambrook et al. 1989) using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridizations with ^{32}P-labeled DNA probes were performed according to the protocol provided by the manufacturer. Blots were washed at 65°C for 20 min in 2× SSC, 0.1% sodium dodecyl sulfate (SDS) and for 20 min in 0.5× SSC, 0.1% SDS.

Genetic transformation and nodulation of *A. verticillata*.

The plasmids pBI*GmENOD40-2-GUS* and pBI*CgENOD40-GUS* were transferred to the *Agrobacterium tumefaciens* strain C58C1(pGV2260) by electroporation. Transgenic *A. verticillata* were obtained through the transformation of epicotyls as described (Franche et al. 1997). Success of gene transfer was confirmed by PCR using *nptII* and *gus* primers. Untransformed plants were regenerated from nontransgenic calli and used as negative controls. Transgenic and nontransformed control plants exhibiting a main root of 3 cm in length were transferred to tubes as described (Gibson 1963) containing one-quarter-strength Hoagland's medium with ammonium sulfate at 17 mg/liter, pH 5.6. After 1 month, the inoculation with a *Frankia* was performed as described (Diouf et al. 1995).

Transformation of *L. japonicus*.

Agrobacterium rhizogenes strain MSU440 (Sonti et al. 1995), containing the *pGmENOD40-2-GUS* plasmid, has been described previously (Martirani et al. 1999). *pCgENOD40-GUS* was transferred into MSU440 by electroporation. Surface-sterilized seed were germinated and grown vertically on 1% agar plates. Six-day-old seedlings were transferred on NLN medium (Duchefa, Haarlem, The Netherlands) containing 1% sucrose. After 2 days, the primary roots of the seedlings were cut at different distances from the root tip. The freshly cut surfaces were inoculated with the *A. rhizogenes* strain grown overnight in LB medium. After 2 days of co-cultivation, the seedlings were washed and blotted on sterile paper before transfer on Murashige-Skoog (MS) medium (Duchefa) supplemented with 1% sucrose and cefotaxime at 200 mg/liter. Calli appeared at the wound site 5 days after infection. After 10 days, microcalli with emerging hairy roots appeared. The composite plants with hairy roots were maintained on MS medium for at least 2 weeks, until a massive root system had been obtained. During this period, roots arising above the wounded site were eliminated with scissors.

Inoculation of composite plants.

Composite plants (wild-type shoot over a transgenic root system) were transferred to petri dishes with slanted NLN medium containing 1% agar. In order to avoid growth of the roots into the agar, sterile filter paper was placed over the upper part of the slope where the root system would be located. Petri dishes were placed vertically in a growth cabinet at 22°C with a 16-h photoperiod. The roots were kept in the dark by covering with aluminum foil. Each primary meristem was inoculated with a drop of *Mesorhizobium loti* NZP2235 suspension containing approximately 10^6 bacteria.

Histological analysis and GUS activity.

Histochemical localization of GUS activity was done as described previously (Franche et al. 1998). Sections were prepared after staining and fixation of whole roots or nodules. Sections (40 µm) were cut with a vibratome (Leica VT1000E). Alternatively, plant material was fixed and embedded into Histo-Technovit 7100 resin as described (Laplaze et al. 2000) and 4-µm sections were cut with a microtome (Historange 2218 LKB). Sections then were mounted on glass slides with Clearium Mountant (Surgipath Medical Instruments, Richmond, IL, U.S.A.).

ACKNOWLEDGMENTS

K. P. was supported by the Deutsche Forschungsgemeinschaft (DFG grant Pa709/1-2). M. C. was supported by MIPAF (Ministero delle Politiche Agricole e Forestali, Progetto speciale: Risorse genetiche di organismi utili per il miglioramento di specie di interesse agrario o per una agricoltura sostenibile). We thank U. Wedemeyer for taking care of the plants.

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