Inhibition of Auxin Signaling in Frankia Species-Infected Cells in Casuarina glauca Nodules Leads to Increased Nodulation

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Actinorhizal symbioses are mutualistic interactions between plants and the soil bacteria Frankia spp. that lead to the formation of nitrogen-fixing root nodules. The plant hormone auxin has been suggested to play a role in the mechanisms that control the establishment of this symbiosis in the actinorhizal tree Casuarina glauca. Here, we analyzed the role of auxin signaling in Frankia spp.-infected cells. Using a dominant-negative version of an endogenous auxin-signaling regulator, INDOLE-3-ACETIC ACID7, we established that inhibition of auxin signaling in these cells led to increased nodulation and, as a consequence, to higher nitrogen fixation per plant even if nitrogen fixation per nodule mass was similar to that in the wild type. Our results suggest that auxin signaling in Frankia spp.-infected cells is involved in the long-distance regulation of nodulation in actinorhizal symbioses.

Nitrogen is one of the major elements that limit plant production worldwide. Most plants rely on inorganic nitrogen in the soil for their nutrition. However, during evolution, legumes and actinorhizal plants have acquired the unique ability to establish root nodule symbioses with nitrogen-fixing bacteria to enable them to acquire nitrogen through biological nitrogen fixation (Doyle, 2011). While legume and actinorhizal nodules differ in their ontogeny and structure (Pawlowski and Bisseling, 1996), phylogenetic studies have shown that all plants able to enter a root nodule symbiosis belong to the same clade, thus suggesting that they share a predisposition for symbiosis (Soltis et al., 1995; Doyle, 2011). The molecular bases of this predisposition are not yet known (Doyle, 2011).

Actinorhizal plants are important for N2-fixing root nodule symbiosis in large areas of the world (Santi et al., 2013). They occur in eight plant families and four orders with different distribution worldwide (Swensen, 1996; Pawlowski and Demchenko, 2012; Santi et al., 2013). Actinorhizal plants are mostly trees or shrubs and, because of their ability to fix nitrogen, are pioneer species widely used in land reclamation programs (Diagne et al., 2013).

The establishment of actinorhizal symbioses depends on a very tightly regulated signal exchange between Frankia spp. and its host plant (Perrine-Walker et al., 2011; Pawlowski and Demchenko, 2012; Svistoonoff et al., 2014). Plant root exudates and, in particular, flavonoids are important for early symbiotic signaling (Beauchemin et al., 2012; Abdel-Lateif et al., 2013). The nature of the nodulation signal(s) emitted by Frankia spp. is still unknown, but it has been shown that its perception depends on the common Nod signaling pathway (Gherbi et al., 2008; Svistoonoff et al., 2013). In the actinorhizal tree Casuarina glauca, the involvement of the phytohormone auxin in symbiotic signaling in plant cells infected by Frankia spp. has been suggested (Perrine-Walker et al., 2011). CgAUX1, a C. glauca gene encoding a functional auxin influx carrier, is expressed in Frankia spp.-infected cells during the formation of actinorhizal nodules (Péret et al., 2007). Moreover, the auxin influx inhibitor

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1-naphthoxyacetic acid inhibits nodulation, suggesting a role for auxin influx during symbiosis establishment (Péret et al., 2007). CgAUX1 is not expressed during intracellular colonization by the arbuscular mycorrhizal (AM) fungus Glomus intraradices (Péret et al., 2008). This shows that CgAUX1 expression during plant cell infection by the microsymbiont is not a general feature of endosymbioses but a specific response to Frankia spp. More recently, we showed that two auxins, indole acetic and phenylacetic acid, accumulate specifically in Frankia spp.-infected cells in C. glauca nodules (Perrine-Walker et al., 2010). This accumulation was shown to be the result of the specific expression pattern of plant genes encoding auxin influx (CgAUX1) and efflux (C. glauca PIN-FORMED1) carriers and of auxin production by Frankia spp. in nodules (Perrine-Walker et al., 2010). Altogether, these data raise the question of the function of auxin signaling in plant cells infected by Frankia spp.

Auxin signaling is mediated by two pathways that depend on the AUXIN BINDING PROTEIN1 (ABP1) and the TRANSPORT INHIBITOR RESPONSE1 (TIR1) AUXIN SIGNALING F-BOX1 (AFB1) to AFB5, respectively (Peer, 2013). ABP1 acts together with the plasma membrane receptor-like transmembrane kinase1 as a membrane receptor transmitting the auxin signal from the cell surface to the cytosol to regulate different cellular processes. ABP1 can mediate both transcriptional and nontranscriptional auxin responses, including polarized auxin transport, by regulating the endocytosis and exocytosis of PIN proteins (Grones and Friml, 2015). The auxin receptors TIR1/AFB1 to AFB5 are part of the Skp, Cullin, F-box containing ubiquitin ligase (E3) complex that directs the ubiquitination and proteasome degradation of auxin/indole-3-acetic acid (AUX/IAA) proteins in the presence of auxin. AUX/IAA proteins act as transcriptional repressors of the AUXIN RESPONSE FACTOR (ARF) transcription factors. Thus, at low auxin levels, AUX/IAAs repress ARF transcriptional activity, whereas at high auxin levels, AUX/IAA proteins are degraded and ARFs can regulate the transcription of their target genes.

The aim of this study was to characterize the role of auxin signaling in Frankia spp.-infected cells in C. glauca nodules. We identified genes encoding putative components of the auxin signaling pathway expressed in nodules and further characterized CgIAA7, a gene encoding a negative regulator of auxin signaling of the AUX/IAA family expressed in plants cells infected by Frankia spp. in C. glauca nodules. Taken together, our results suggest that auxin induces the production of a secondary signal in Frankia spp.-infected cells that is involved in the autoregulation of nodulation.

RESULTS

Identification of Genes Involved in Auxin Signaling in C. glauca

To study the role of auxin during the establishment of actinorhizal symbioses, we first performed an in silico search on a C. glauca EST database containing more than 35,000 ESTs (Hocher et al., 2011; http://esttk.cirad.fr/cgi-bin/public_quick_search.cgi) to identify genes involved in auxin signaling in C. glauca. We performed a TBLASTN search using Arabidopsis (Arabidopsis thaliana) TIR1 and IAA14 as queries. We found two homologs of the auxin receptors TIR1/AFB (renamed CgAFB1 and CgAFB2) and 13 consensus sequences for AUX/IAAs (named CgIAA1–CgIAA13). In addition, six consensus sequences for ARFs (named CgARF1–CgARF6; Supplemental Table S1) have been reported (Diédhiou et al., 2014). The expression of these genes in uninfected roots and nodules of C. glauca was analyzed using microarray data generated in our laboratory (Hocher et al., 2011). Both TIR1 homologs were expressed in roots and nodules, but CgAFB2 was expressed at significantly higher levels in nodules than in roots (Fig. 1). Among AUX/IAAs, only CgIAA7 was expressed at a significantly higher level in nodules compared with roots (Fig. 1), while transcriptomics data indicated that one ARF (CgARF6) out of six had significantly higher expression in nodules than in roots (Fig. 1).

We thus decided to focus our analysis on AUX/IAAs. Transcriptomics data were first validated using quantitative reverse transcription-PCR (RT-qPCR; Supplemental Fig. S1). Our data confirmed the gene expression data

Figure 1. Genes encoding potential components of auxin signaling expressed in C. glauca nodules. A schematic representation of the auxin signaling pathway is shown at left. Relative expression of genes encoding potential auxin receptors (CgAFBs) and regulators of gene expression (AUX/IAAs and ARFs) in C. glauca roots and nodules measured by microarray experiments (Hocher et al., 2011) is shown at right. Values are means of three biological replicates ± SD. Asterisks denote values significantly different from the control (Student’s t test, *P < 0.05 and **P < 0.01). NA, Nonapplicable (no signal was detected on the array).
obtained with the microarrays. In particular, we observed that out of 13 putative genes encoding AUX/IAAs, 12 were expressed at lower levels in nodules than in uninfected roots. Only CgIAA7 had higher expression in nodules than in uninfected roots. CgIAA7, therefore, was a candidate regulator of auxin signaling in C. glauca nodules and was selected for further studies.

CgIAA7 Is Expressed in Frankia spp.-Infected Cells in C. glauca Nodules

AUX/IAAs are negative regulators of auxin signaling, and their expression is controlled by auxin, thus creating a negative feedback loop (Tiwari et al., 2001). We first checked the regulation of CgIAA7 expression by auxin. C. glauca roots were treated with 10 μM indole-3-acetic acid (IAA), RNA was extracted 0, 4, 8, and 24 h after treatment, and gene expression was tested by RT-qPCR. CgIAA7 expression was induced by auxin with a peak of expression after 8 h of auxin treatment (Fig. 2A). We then studied the expression of CgIAA7 during the establishment of the actinorhizal symbiosis with Frankia spp. C. glauca plants were inoculated with Frankia spp. strain CcI3, and roots were harvested at different times after inoculation and used to analyze gene expression by RT-qPCR.

![Figure 2](image-url)

Figure 2. CgIAA7 is an auxin-responsive gene expressed in roots and nodules. A, CgIAA7 expression is induced by auxin in C. glauca roots. Gene expression was analyzed by RT-qPCR and presented as relative expression in IAA-treated relative to control plants. Values presented are means of three biological replicates ± se. B, CgIAA7 expression is induced by Frankia spp. inoculation in C. glauca roots. Gene expression was analyzed by RT-qPCR and presented as relative expression in IAA-treated relative to control plants following inoculation with Frankia spp. Values presented are means of two biological replicates ± se. C, Expression of ProCgIAA7:GUS in C. glauca roots. Expression is found in the meristem, in vascular tissues, and in lateral root primordia. n = 19 independent hairy root plants. Bars = 250 μm. D, Expression of ProCgIAA7:GUS in C. glauca roots before (top left) and after 10 μM IAA treatment (right). Bar = 100 μm. Transverse-root cuts (insets 1 and 2; bars = 50 μm) reveal that the enhanced signal behind the root tip is due to additional expression in the endodermis and inner cortex. Expression was analyzed in three independent hairy root plants. E, Expression of ProCgIAA7:GUS in C. glauca nodules. Expression is found in the apical meristem (arrow), in the phellogen of the periderm (white arrowhead), in vascular tissues (yellow asterisk), and in Frankia spp.-infected cells (arrowheads). n = 15 independent hairy root plants. Bar = 250 μm.
CgIAA7 expression was induced 12 and 24 h after inoculation, suggesting that it might be involved in early steps of actinorhizal symbiosis formation (Fig. 2B).

In order to further characterize the CgIAA7 expression pattern, a 1,375-bp genomic DNA fragment upstream of the start codon was isolated and used to generate translational fusion with the GUS reporter gene (hereafter named ProCgIAA7:GUS). Transgenic ProCgIAA7:GUS C. glauca composite plants were obtained using Agrobacterium rhizogenes-mediated transformation (Diouf et al., 1995). ProCgIAA7:GUS expression was observed in the vasculature, root apical meristems, and lateral root primordia of uninoculated roots (Fig. 2C). Auxin treatment (10 μM IAA for 8 h) led to expression in the internal part of the cortex only in the root zone just behind the root apical meristem (Fig. 2D). Interestingly, this zone is the zone that is susceptible to Frankia spp. infection. We also observed an increase of the intensity of GUS staining in the vasculature (Fig. 2D). Finally, in nodules, ProCgIAA7:GUS was expressed in the meristems, including the secondary meristem responsible for periderm formation, in vascular tissues, and in Frankia spp.-infected cells (Fig. 2E).

We conclude from our data that CgIAA7 is auxin inducible and expressed in plant cells infected by Frankia spp. in C. glauca nodules.

Figure 3. CgIAA7 is a negative regulator of auxin signaling. A, CgIAA7 encodes a negative regulator of auxin signaling. B, Wild-type (WT) and mutated versions of CgIAA7 generated by site-directed mutagenesis. C, Effect of the mutated version of CgIAA7 on auxin signaling in Arabidopsis protoplasts. GUS activity was measured in ProDR5:GUS protoplasts transformed with the pMDC32 vector containing the wild-type or mutated version of CgIAA7 and treated with ethanol (mock) or 1 or 100 μM IAA. Transformation efficiency was normalized using a Pro35S:LUC construct.

To analyze the dynamics of protein localization, we fused CgIAA7WT, CgIAA7P96S, and CgIAA7P97L to the VENUS fluorescent protein and expressed these fusion proteins under the control of the constitutive 35S promoter in Arabidopsis protoplasts. The three protein fusions were localized in the nucleus (Supplemental Fig. S2). The fluorescence of CgIAA7WT-VENUS disappeared quickly upon auxin treatment, whereas CgIAA7P96S-VENUS and CgIAA7P97L-VENUS were still detected in the nuclei of transformed protoplasts (Supplemental Fig. S2). Hence, CgIAA7 is degraded upon auxin treatment, and the dominant-negative mutants CgIAA7P96S and CgIAA7P97L are stabilized versions of the protein.

We conclude from our data that CgIAA7 encodes a transcriptional repressor of the auxin response expressed in plant cells infected by Frankia spp. in C. glauca nodules.
Specific Inhibition of Auxin Signaling in *Frankia* spp.-Infected Cells Leads to Increased Nodulation and Nitrogen Fixation

The dominant-negative CgIAA7<sup>97P97L</sup> was then used as a tool to inhibit auxin-dependent gene expression specifically in *Frankia* spp.-infected cells in *C. glauca* nodules. The dominant-negative CgIAA7<sup>97P97L</sup> was cloned in a binary vector under the transcriptional control of the promoter of Cg12, a *C. glauca* gene encoding a subtilisin-like protease that is specifically expressed in *Frankia* spp.-infected cells throughout the symbiotic process (Laplaze et al., 2000; Svistoonoff et al., 2003). Two independent stable transgenic lines containing the Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup> construct were generated using *Agrobacterium tumefaciens* transformation (Smouni et al., 2002), and their nodulation phenotype was compared with a control line transformed with either a Pro<sub>Cg12</sub>GUS (Svistoonoff et al., 2003) or a Pro<sub>Cg12</sub>NOS-GUS construct (S. Svistoonoff, unpublished data).

In three independent experiments, plants from the two Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup> lines nodulated faster than control plants (Fig. 4A). Moreover, Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup> plants formed about three times more nodules than control plants (Fig. 4B), and these nodules were significantly bigger (Fig. 4C). Nitrogen fixation was measured by acetylene reduction assay (ARA) and found to be higher in Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup> plants than in control plants (Fig. 4D). However, the fixation rate per nodule mass was similar in controls and in Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup> plants (Supplemental Fig. S3), indicating that the increase in nitrogen fixation was due to an increase in the number and mass of the nodules rather than to an increase in nitrogen fixation efficiency. Sections of Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup>

DISCUSSION

Auxin plays an important role in many plant-microbe interactions (for review, see Spaepen and Vanderleyden, 2011; Denancé et al., 2013; Kazan, 2013). Auxin is important for arbuscule formation during the AM symbiosis (Etemadi et al., 2014). In the legume-rhizobia symbiosis, auxin is required for indeterminate nodule organogenesis (Mathiesius, 2008; Suzaki et al., 2013) but not for determinate nodule formation (Turner et al., 2013). Moreover, auxin signaling is required for the initiation of the rhizobial infection thread in *Medicago truncatula* (Breakspear et al., 2014). Local auxin accumulation in response to symbiotic signaling leads to the formation and development of nodule primordia, and auxin signaling is active in the indeterminate nodule meristem (Mathiesius et al., 1998). Similarly, we previously reported that treatment with the auxin influx inhibitor 1-naphtoxyacetic acid inhibits nodule formation and growth in *C. glauca* (Péret et al., 2007).

Previous studies indicated that auxins (indole acetic acid and phenylacetic acid) accumulating in nodules are produced by *Frankia* spp. and that the auxin and control nodules did not reveal any obvious changes in nodule structure and infection pattern (Fig. 5, A and B). Similarly, the size of infected cells was not modified in Pro<sub>Cg12</sub>CgIAA7<sup>97P97L</sup> nodules compared with controls (Fig. 5, C and D).

Hence, inhibition of auxin signaling in *Frankia* spp.-infected cells in *C. glauca* nodules leads to increased nodulation and nitrogen fixation per plant without changing nodule structure.

![Figure 4](http://www.plantphysiol.org/doi/10.1104/pp.15.02461/supplemental/)
transport machinery in C. glauca nodules concentrates auxins in infected cells (Perrine-Walker et al., 2010). In this study, we addressed the role of these auxins in plant cells infected by Frankia spp. in C. glauca actinorhizal nodules. We identified CgIAA7 as a plant gene that encodes a negative regulator of auxin signaling expressed in the vasculature, meristem, and infected cells of C. glauca nodules. Expression of a dominant-negative version of CgIAA7 that inhibits the transcriptional response to auxin specifically in Frankia spp.-infected cells led to increased nodulation and nitrogen fixation per plant. This indicates that the inhibition of auxin signaling in those cells promotes symbiosis at a distance. We also observed an impact on nodule mass. Again, this effect could be due to a long-distance signaling mechanism or to a local effect. However, the level of nitrogen fixation per nodule mass remained unchanged, suggesting that the difference in nitrogen fixation observed was due to increased nodulation rather than to more efficient nitrogen fixation in plant cells. This supernodulation phenotype suggests that changes in auxin perception in Frankia spp.-infected cells somehow perturb the regulation of nodulation at a distance in C. glauca. Similarly, it was recently reported that auxin-hypersensitive soybean (Glycine max) plants have reduced nodulation (Turner et al., 2013). Therefore, it was proposed that a negative feedback loop involving an inhibition of cytokinin signaling by auxin is involved in legume determinate development (Turner et al., 2013).

In actinorhizal plants, it has been suggested that an inhibitory signal produced in nodules negatively regulates symbiosis establishment (Wall, 2000). Our results indicate that in the context of plant cells infected by Frankia spp. in C. glauca nodules, auxin is a negative regulator of symbiosis establishment. It is tempting to speculate that auxin regulates the production of this as yet unknown inhibitory molecule in these cells. In such a model, bacterial proliferation would increase auxin production and perception in infected plant cells, leading to increased production of the inhibitory signal and, therefore, inhibition of nodulation (Fig. 6). Such negative feedback would be a simple way to regulate the infection of the host plant by Frankia spp. Interestingly, a similar long-distance regulation mechanism has been described in the AM symbiosis, and auxin was involved in this process (Meixner et al., 2005). As nitrogen-fixing root nodule symbioses are known to derive from the ancient AM symbiosis, this auxin-mediated autoregulation could have been conserved in actinorhizal symbioses.

Figure 5. ProCg12::CgIAA7P97L plants show normal nodule structure and infected cells size distribution. A and B, Control (A) and ProCg12::CgIAA7P97L (B) plant nodules colored with Toluidine Blue. Mature Frankia spp.-infected cells appear pink/violet in the fixation zone. Vascular tissues are marked by yellow asterisks. IZ, Infection zone; M, meristem. Bars = 100 μm. C, Distribution of infected cells and cell size in three control lines and two independent ProCg12::CgIAA7P97L lines (#10 and #42). n = 400 cells per line, sampled over fixation zones. D, Mean size of infected cells for the distributions presented in C. Error bars are so.
The process of autoregulation of nodulation finely regulates the number of nodules per plant to balance nitrogen gains with the carbon cost of symbiosis (Reid et al., 2011). This process has been documented in legumes (Reid et al., 2011) and actinorhizal plants (Wall, 2000) and relies on the exchange of signals between the root and the shoot. The effect of auxin that we report might be related to autoregulation or might be independent. In any case, it would ensure some feedback on nodulation to control the number of nodules and, therefore, ensure a right balance between the benefit (nitrogen source) and cost (carbon consumption) of the symbiosis. We hypothesize that this might also counterselect Frankia spp. strains that lost auxin production, as those strains would potentially lead to a reduced fitness of their host plant. Further work is now needed to understand the link between auxin and this long-distance regulation of nodulation in actinorhizal symbioses and to test the impact of this feedback loop on plant fitness.

**MATERIALS AND METHODS**

**Plant and Bacterial Material**

*Casuarina glauca* seeds were provided by the Australian Tree Seed Center (Commonwealth Scientific and Industrial Research Organization) and grown as described (Péret et al., 2007). Transgenic *C. glauca* plants were produced and analyzed as described (Péret et al., 2007; Gherbi et al., 2008). The Frankia spp. strain CcI3 (Normand et al., 2007) was grown and used to inoculate *C. glauca* as described (Péret et al., 2007). For auxin treatments, *C. glauca* plants were transferred to a hydroponic growth system 1 month after germination and were grown for 4 weeks in full Broughton and Dilworth medium before being transferred to new medium containing 10 μM IAA or without auxin (control). Whole root systems were harvested (four plants per sample) 4, 8, and 24 h after treatment and frozen in liquid nitrogen. For nodulation kinetics, *C. glauca* root systems were collected (32 plants per sample) at 0, 12, and 24 h after inoculation with Frankia spp. strain CcI3 and frozen in liquid nitrogen.
The Arabidopsis (Arabidopsis thaliana) Columbia-0 line containing the ProDR5:GUS construct was described previously (Benková et al., 2003).

RNA Ex extractions

Total RNA from C. glauca tissues was purified by ultracentrifugation (Hocher et al., 2006). Residual DNA was removed from RNA samples using the Turbo DNA free kit (Ambion), quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and qualitatively assessed using a Bioanalyzer 2100 according to the manufacturer’s instructions (Agilent).

RT-qPCR Experiments

C. glauca gene expression analyses were performed by RT-qPCR using specific primers (Supplemental Table S2) designed using Beacon Designer (Premier Biosoft International). Single-strand complementary DNA was synthesized from 500 ng of total RNA using the SuperScript III Reverse Transcriptase kit (Invitrogen Life Science). Three independent reverse transcription reactions were pooled to minimize potential heterogeneity in reverse transcription yield. RT-qPCR was performed on a Stratagene MX 3005 P apparatus (Agilent) with the Brilliant II SYBR Green QPCR Master Mix (Agilent) under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. Three RT-qPCRs were run for each biological replicate. Expression values were normalized using the expression level of the C. glauca Ulkakin gene (Hocher et al., 2006).

Constructs and the Generation of Transgenic Plants

To analyze the CgIAA7 expression pattern, a genomic DNA fragment upstream of its start codon (ATG) was amplified using the Universal Genome Walker kit (Clontech) and cloned upstream of the GUS reporter gene in the PMCI62-GFP vector as described previously (Iлина et al., 2012) to generate ProCgIAA7:GUS.

To analyze the function of CgIAA7, its coding sequence was amplified from EST CGLN02-P1-P04 by PCR using primers IAA-Rev (5’-GCATCTTG-3’) and IAA-mut (5’-CACCAAATGCCACCCGATAGCCACCTC-3’) and cloned in the pENTR D-TOPO vector (Invitrogen). Mutations were introduced by PCR using specific primers (Supplemental Table S2) in conjunction with primers IAA-Rev and IAA-mut and confirmed by sequencing. Wild-type and mutated versions of CgIAA7 were then cloned downstream of the 35S promoter in the pMDC32 vector (Curtis and Grossniklaus, 2003) by Gateway cloning. Versions without the stop codon of wild-type and mutated CgIAA7 were generated by pDONR 221 (Invitrogen). They were then combined with a 35S promoter in the pMDC32 vector (Curtis and Grossniklaus, 2003) by Gateway cloning. Versions without the stop codon of wild-type and mutated CgIAA7 were then cloned in the pENTR D-TOPO vector (Invitrogen). Mutations were encoded by sequencing. The two constructs in a p2rL7 plasmid and the effector constructs (Pro35S:GUS or the dominant-negative version or the empty vector pMDC32), with the two constructs in a p2rL7 plasmid and the effector constructs (Prom35S:GUS or the dominant-negative version or the empty vector pMDC32), were transformed into Agrobacterium rhizogenes (strain A4RS) for hairy root transformation.

To analyze the function of ProCgIAA7P97L, its coding sequence was amplified using A. rhizogenes strain A4RS (Jouanin et al., 1986). Stable transformation of C. glauca using Agrobacterium tumefaciens C58C1 pGV3101 (Vancanneyt et al., 1990) was performed as described previously (Franche et al., 1997; Smouni et al., 2002).

Protoplast Transformation

Protoplasts were produced from Arabidopsis ProDR5:GUS plants and transformed following the protocol of Sheen et al. (2002). Protoplasts were cotransformed using polyethylene glycol as described (Sheen et al., 2002) with the pUA7 plasmid and the effector constructs (ProCgIAA7 or the dominant-negative version or the empty vector pMD32), with the two constructs in a 1:3 LUC vector:effector vector ratio. After 16 h of incubation at 25°C, protoplasts were treated with 1 or 100 μM IAA or ethanol (mock) and collected 8 h after treatment and frozen in liquid nitrogen. GUS and LUC activity tests were performed as described previously (Zarei et al., 2011). To analyze CgIAA7 intracellular localization, protoplasts were transformed with 10 μg of Pro35S: CgIAA7-VENUS plasmid.

ARA

Nitrogenase activity was determined using the ARA on ProCgIAA7::VENUS and ProCgIAA7:GUS (control) plants. For each construct, 10 plants were tested for ARA as described previously (Abdel-Lateif et al., 2013).

Microscopy and Nodule Sections

GUS assays were performed as described previously (Péret et al., 2007). Tissues were cleared in 70% (v/v) ethanol for 2 d and then immersed in 50% (v/v) ethanol/10% (v/v) glycerol for 2 h, in 30% (v/v) ethanol/30% (v/v) glycerol for 2 h, and in 50% (v/v) glycerol for 2 h. Roots were then mounted in 50% (v/v) glycerol and visualized on a Leitz DMRB microscope. For thin sections, samples were prepared as described (Péret et al., 2007). Samples were then embedded in Technovit 7100 resin (Heraeus Kulzer) according to the manufacturer’s instructions. Thin sections (5 μm) were cut with a Microm HM355S microtome. Sections were stained for 15 min in aqueous 0.01% (w/v) Toluidine Blue solution and mounted in Clearmount (Surgipath). Cell sizes were measured using the Fiji ImageJ package (http://fiji.sc/Fiji) along a sampling window defined over the fixation zone of imaged sections. At least 400 cells over 10 to 20 slices were measured for each line.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers FQ375455, FQ362957, FQ328316, FQ36922, FQ327942, FQ37867, FQ37867, FQ365113, FQ351497, FQ327875, FQ311723, FQ319075, FQ363257, FQ25843, FQ369042, FQ316474, FQ272274, FQ327320, FQ316457, FQ362350, FQ32692, FQ361670, FQ371608, FQ326137, FQ376114, FQ374600, FQ363551, FQ27614, and FQ365126.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression of CgIAAAs genes in C. glauca roots and nodules measured by RT-qPCR.

Supplemental Figure S2. CgIAA7 is degraded in presence of auxin.

Supplemental Figure S3. Measure of nitrogen fixation in ProCg12: CgIAA7:9PL and control plants.

Supplemental Table S1. C. glauca ESTs encoding potential auxin signaling regulators.

Supplemental Table S2. Primers used for qPCR.

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LITERATURE CITED


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