Auxin Influx Activity Is Associated with *Frankia* Infection during Actinorhizal Nodule Formation in *Casuarina glauca*[1][C][W][OA]

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Plants from the Casuarinaceae family enter symbiosis with the actinomycete *Frankia* leading to the formation of nitrogen-fixing root nodules. We observed that application of the auxin influx inhibitor 1-naphtoxyacetic acid perturbs actinorhizal nodule formation. This suggests a potential role for auxin influx carriers in the infection process. We therefore isolated and characterized homologs of the auxin influx carrier (*AUX1-LAX*) genes in *Casuarina glauca*. Two members of this family were found to share high levels of deduced protein sequence identity with Arabidopsis (*Arabidopsis thaliana*) AUX-LAX proteins. Complementation of the Arabidopsis *aux1* mutant revealed that one of them is functionally equivalent to *AUX1* and was named *CgAUX1*. The spatial and temporal expression pattern of *CgAUX1* promoter:β-glucuronidase reporter was analyzed in Casuarinaceae. We observed that *CgAUX1* was expressed in plant cells infected by *Frankia* throughout the course of actinorhizal nodule formation. Our data suggest that auxin plays an important role during plant cell infection in actinorhizal symbioses.

Actinorhizal plants, which belong to eight families of angiosperms, can form nitrogen-fixing nodules in symbiosis with the soil actinomycete *Frankia* (Benson and Silvester, 1993). The symbiotic interaction starts, under conditions of nitrogen deprivation, by an exchange of signals between the plant roots and bacteria. The chemical nature of *Frankia* nodulation factors is unknown, but data suggest that it has different biochemical properties from that of *Rhizobium* (Cérémonie et al., 1999). During intracellular infection, *Frankia* signals lead to root hair deformation, some of which become infected. At the same time, limited cell divisions are triggered in the cortex, creating a so-called prenodule. Prenodule function is not known, but it is an obligatory step of intracellular infection (Laplaze et al., 2000). Concomitantly, cell divisions occur in the pericycle in front of the xylem pole leading to the formation of a nodule lobe primordium. The growing nodule lobe is infected by *Frankia* hyphae coming from the prenodule. The structure of the new organ formed upon infection largely differs from legume nodules even if the infection mechanisms share common features (Pawlowski and Bisseling, 1996). Actinorhizal nodules are considered as modified lateral roots because (1) they originate from divisions in the pericycle in front of a xylem pole; (2) they have a lateral root-like structure with a central vasculature, infected cells in the cortex, and an apical meristem; and (3) in some species (e.g. *Casuarina* sp.) a so-called nodule root is produced at the apex (Obertello et al., 2003). Little is known about the mechanisms of actinorhizal nodule development.

The plant hormone auxin is involved in many developmental processes (Tanaka et al., 2006) and is the key signal controlling lateral root development (Casimiro et al., 2003). Auxin transport across the plant is polarized and perturbations of polar auxin transport using inhibitors such as naphthylphthalamic acid or mutants result in dramatic alteration of the plant

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developmental pattern (Reed et al., 1998). The existence of auxin transporters has been predicted for a long time to account for polar auxin transport (Goldsmith, 1977). Characterization of Arabidopsis (*Arabidopsis thaliana*) mutants perturbed in auxin transport or sensitivity led to the identification of auxin efflux and influx facilitators encoded by the *PIN* and *AUX-LAX* genes, respectively (Kramer and Bennett, 2006). The latter are encoded by a small gene family (four genes) in Arabidopsis (Parry et al., 2001b). Only one member of the *AUX-LAX* family has been characterized to date: *AUX1* is involved in gravitropism (Bennett et al., 1996) and lateral root initiation (Marchant et al., 2002). *AUX1* has recently been shown to encode a high-affinity auxin influx transporter by heterologous expression in *Xenopus* oocytes (Yang et al., 2006). The mechanism of transport remains to be elucidated, but is predicted to occur by proton symport (Kerr and Bennett, 2007).

Auxin transport is also thought to be involved in the establishment of legume symbiosis. Local auxin transport inhibition is triggered by spot inoculation of rhizobia, leading to subsequent accumulation of auxin at the site of infection as shown by the use of the *GH3:gusA* auxin response marker in white clover (*Trifolium repens*; Mathesius et al., 1998) and *Lotus japonicus* (Pacios-Bras et al., 2003). In legumes forming indeterminate nodules, flavonoids are produced as a response to bacterial lipochitin oligosaccharides (Mathesius et al., 2000) and act as inhibitors of auxin efflux transport (Brown et al., 2001), leading to local accumulation of auxin necessary for cell division and subsequent nodule primordium formation (Wasson et al., 2006). Moreover, the expression of auxin influx transporters in *Medicago* is associated with nodule primordium development and vasculature differentiation (de Billy et al., 2001).

A role of auxin during actinorhizal symbiont dialogue has also been suggested because some *Frankia* strains can produce different forms of auxin in culture (Gordons et al., 1988; Hammad et al., 2003). However, no link has been made between the production of hormones by *Frankia* and establishment of symbiosis. The symbiotic bacteria *Rhizobium* produce auxins that were proposed to be involved in establishing symbiosis with legume plants (Badenoch-Jones et al., 1983). Indeed, a *Bradyrhizobium japonicum* mutant producing 30-fold more indole-3-acetic acid (IAA) than the wild type has higher nodulation efficiency (Kaneshiro and Kwolek, 1985). Altogether, up to 80% of rhizobacteria are considered to produce auxins (Patten and Glick, 1996). However, nothing is known about the precise role of bacterial auxin during the processes of infection and symbiosis or how and when the plant cell perceives it.

In this study, we show that application of the auxin influx inhibitor 1-naphtoxyacetic acid (1-NOA) perturbs nodule formation. We therefore isolated a small family of *AUX-LAX* gene homologs in the actinorhizal plant *Casuarina glauca*. Among this family of genes, we identified *CgAUX1*, a homolog of *AtAUX1*, which carries an auxin carrier function as shown by functional complementation of the Arabidopsis *aux1* mutant. Expression of *CgAUX1* is found in all *Frankia*-infected cells from the root hair to nodule nitrogen-fixing cells. We also bring evidence of differences between the genetic programs of lateral root and actinorhizal nodule

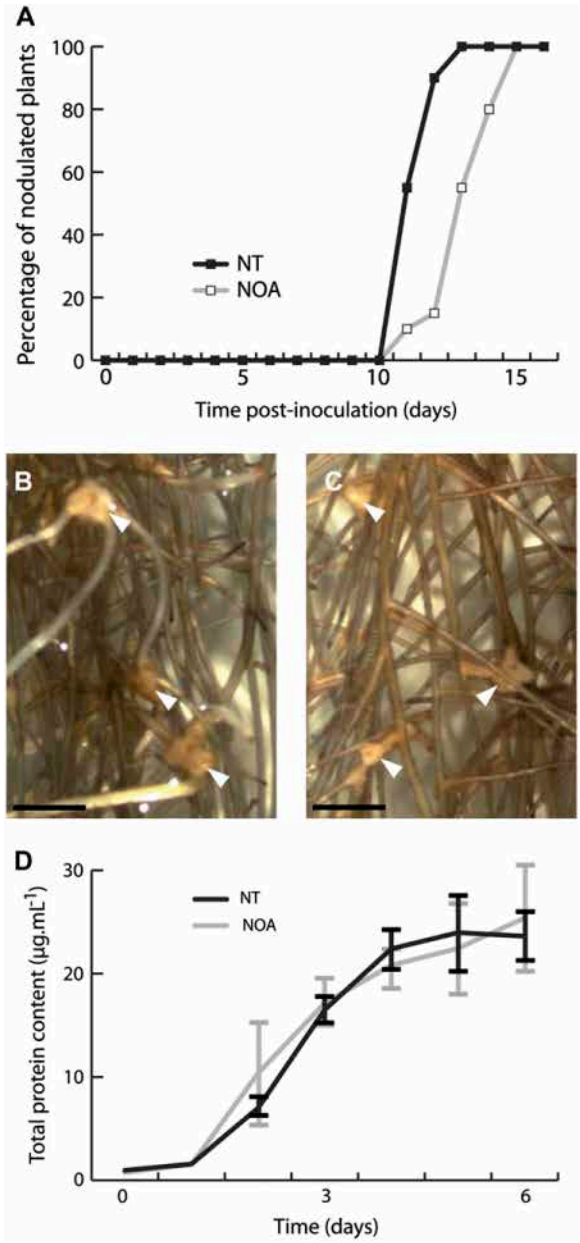

Figure 1. 1-NOA treatment perturbs the nodulation process by specifically inhibiting auxin influx transport. A, Percentage of plants bearing symbiotic structures (prenodules or nodules) after inoculation by *Frankia* in absence (NT, black squares) or presence of 25 μM 1-NOA (NOA, white squares). The data presented correspond to one representative of three experiments with similar results (20 plants/treatment). B, 24-d-old roots of plants bearing nodules (arrowheads) upon NT. C, 24-d-old roots of plants bearing big prenodules (arrowheads) upon NOA treatment. D, *Frankia* exponential growth indicated as total protein content upon NT or 25 μM 1-NOA treatment (NOA). Bars = 1 cm (B and C). [See online article for color version of this figure.]

Table I. *Degenerate primers designed in the most conserved regions of the AUX-LAX genes and used for C. glauca genomic DNA amplification*

Primer Name	Sequence (5′-3′)	Direction-Position
AD1	ATYCARCTHATWGCYTGYGC	Forward-exon 3
AD2	GACAARAGRACWTGGACWTA	Forward-exon 4
AD3	CACAT6GCRTGCATDATYTC	Reverse-exon 6
AD4	CCRAA6GCCCARTADAS6GC	Reverse-exon 6
AF2	CCACAT6GCRTGCATDATYTC	Forward-exon 3
AF3	TGGAC6TAYATHTTYGG6GC6TGY	Reverse-exon 5

primordium based on different patterns of *CgAUX1* expression. Altogether, our results shed light on the role of auxin influx transport during actinorhizal nodule formation.

RESULTS

Inhibition of Auxin Influx Transport Using 1-NOA Perturbs Nodule Formation

We analyzed the effect of 1-NOA, a competitive inhibitor of auxin influx, on the *C. glauca-Frankia* interaction. 1-NOA is known to specifically inhibit AtAUX1 (Yang et al., 2006) and to mimic the *aux1* mutant phenotype in Arabidopsis (Parry et al., 2001a). *C. glauca* plants were inoculated and grown in hydroponics in the presence of 25 μM 1-NOA. The number of nodulated plants (i.e. plants bearing prenodules or nodules) was checked every day after 10 d (Fig. 1A). We found in three independent experiments that 1-NOA treatments caused a 2-d delay in nodule appearance. The same effect was observed if the growth medium was changed every 3 d with fresh 1-NOA to prevent potential 1-NOA degradation (data not shown). Moreover, 24 d after inoculation, plants treated with 1-NOA mainly showed prenodules, whereas control plants showed nodules (Fig. 1, B and C). This 1-NOA effect on nodulation was not due to a more general effect on root growth because we found no significant differences in shoot or root weight in treated or nontreated plants (Student's *t* test; $P < 0.1$; no treatment [NT] roots, m = 0.059 g; NOA-treated roots, m = 0.062 g; NT shoots, m = 0.136 g; NOA-treated shoots, m = 0.137 g; dry weights; $n = 20$). Moreover, we also verified that addition of 25 μM 1-NOA had no deleterious effects on *Frankia* growth (Fig. 1D). We therefore conclude that inhibition of auxin influx transport using 1-NOA partially perturbs actinorhizal nodule formation in *C. glauca*.

Identification of a Small Family of Auxin Influx Carrier Genes in *C. glauca*

Our data suggest a role for auxin influx carriers encoded by *AUX1* homologs during actinorhizal nodule development. *AUX-LAX* gene homologs were therefore isolated from *C. glauca* by amplifying genomic DNA with different sets of degenerate primers (Table I) designed in conserved regions of AUX-LAX proteins of Arabidopsis, *Medicago truncatula*, and poplar (*Populus* spp.). Seven different PCR products were produced, sequenced, and found to correspond to two

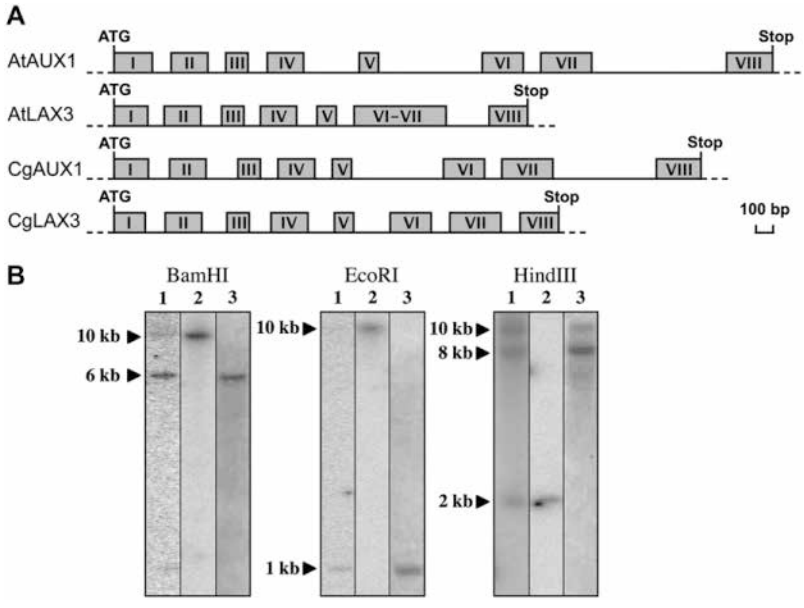

Figure 2. *C. glauca AUX-LAX* genes family. A, Exon-intron structure of *CgAUX1* and *CgLAX3* compared to *AtAUX1* and *AtLAX3*. Exons are shown as gray boxes and introns as black lines. B, Southern-blot experiments suggest that there are only two *AUX-LAX* genes in the *C. glauca* genome. Digested genomic DNA (with *Bam*HI, *Eco*RI, or *Hin*dIII) was hybridized with a probe designed in a conserved region (lane 1), a *CgAUX1*-specific probe (lane 2), or a *CgLAX3*-specific probe (lane 3).

different genes. The corresponding cDNAs (1,440 and 1,395 bp) were obtained by RACE-PCR. They were named *CgAUX1* and *CgLAX3* according to the sequence identity of the predicted proteins to Arabidopsis proteins, 85% for AUX1 and 87% for LAX3, respectively.

The genomic sequences corresponding to *CgAUX1* and *CgLAX3* were amplified by PCR and found to be 2,942 and 2,224 bp long, respectively, from start to stop codon. Intron positions were conserved between Arabidopsis (Parry et al., 2001b) and *C. glauca AUX-LAX* genes. *CgAUX1* and *CgLAX3* have the same structure as *AtAUX1* (Fig. 2A), whereas *AtLAX3* (Fig. 2A) has one intron less in the C-terminal part of the gene, respectively. The same gene structure was found for all of the *M. truncatula AUX-LAX* genes (five genes; Schnabel and Frugoli, 2004), indicating common evolutionary origin. Interestingly, *AUX-LAX* gene structure is slightly different in rice (*Oryza sativa* sp. japonica 'Nipponbare,' five genes in the annotated genome; Tyagi et al., 2004), where three genes have seven exons like *AtAUX1* and the two others have five and two exons, respectively. However, the position of the conserved introns is similar (data not shown), suggesting that this gene structure preceded the divergence of monocots and dicots and that a loss of introns is responsible for the observed differences in intron-exon number.

To estimate the number of *AUX-LAX* genes in the *C. glauca* genome, we conducted Southern-blot experiments using three different probes: a nonspecific probe designed in one of the most conserved regions of *AUX-LAX* genes (exon VII) and two gene-specific probes designed in *CgAUX1* and *CgLAX3* 3′-untranslated regions. The conserved probe hybridized with a limited number of genomic DNA fragments in nonstringent conditions that could be assigned to either *CgAUX1* or *CgLAX3* using gene-specific hybridizations (Fig. 2B). This, together with the fact that we did not recover any other gene by PCR or in a *C. glauca* EST library (Hocher et al., 2006), suggests that auxin influx

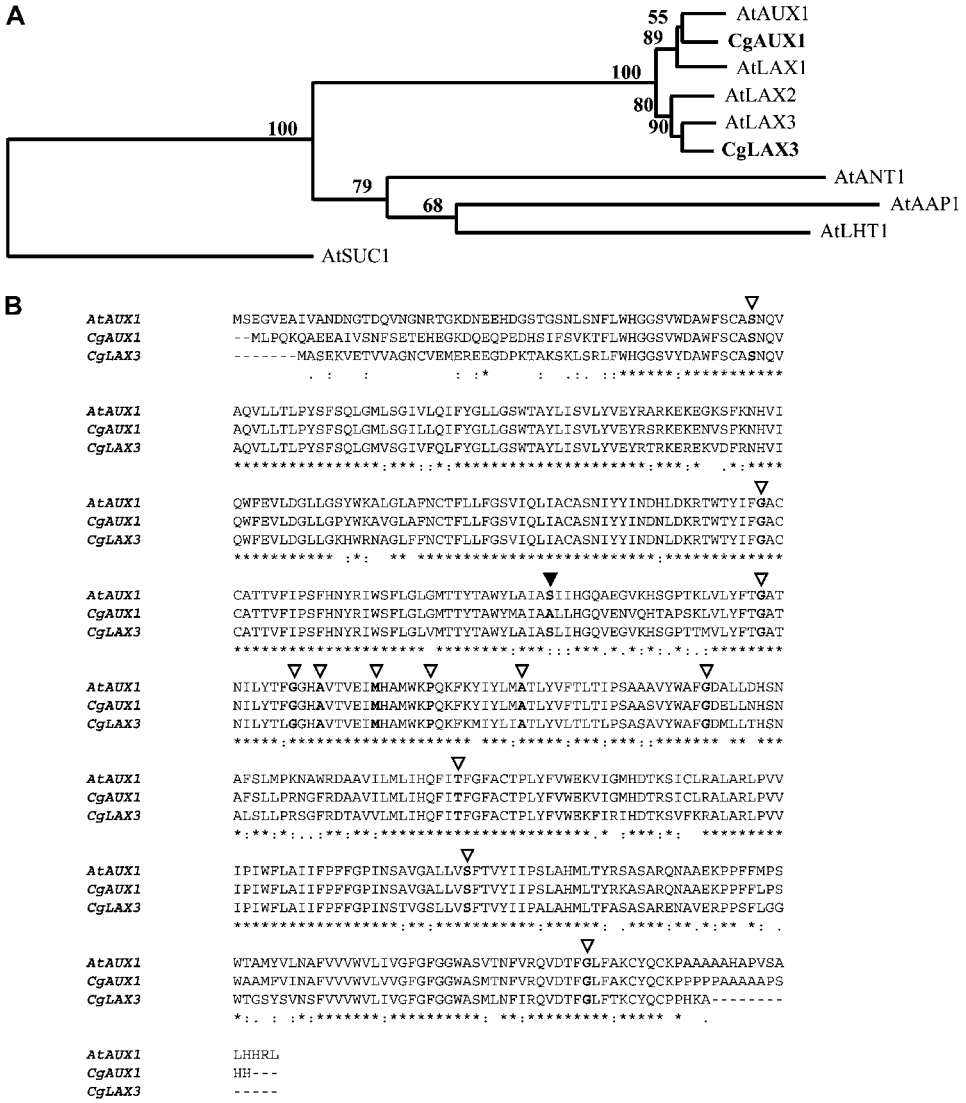

Figure 3. Sequence analysis. A, Arabidopsis and *Casuarina* AUX-LAX deduced protein sequences were aligned with a representative member of each subclass of the amino acid and auxin permease family: a Lys His transporter (AtLHT1, At5g40780), an amino acid permease (AtAAP1, At1g58360), and an aromatic and neutral amino acid transporter (AtANT1, At3g11900). The tree was elaborated using a neighbor-joining algorithm and rooted with the Suc transporter protein sequence (AtSUC1, At1g71880). Bootstrap analysis is shown for each branch (n = 100). B, Alignment of AtAUX1, CgAUX1, and CgLAX3 predicted protein sequences using ClustalW (Thompson et al., 1994). Amino acids known to be important for the activity of auxin influx carriers (Swarup et al., 2004) that are conserved are in bold and marked by a white arrowhead. The only amino acid that is not conserved in CgAUX1 is marked by a black arrowhead.

carriers are encoded by a small gene family (possibly only two genes) in *C. glauca*.

CgAUX1 Encodes an Auxin Influx Carrier Functionally Equivalent to AtAUX1

Arabidopsis and *C. glauca AUX-LAX* deduced protein sequences were compared with a representative member of each class of the amino acid transporter family. A phylogenetic tree was generated using a neighbor-joining distance algorithm showing that AUX-LAX proteins belong to the amino acid and auxin permease (Young et al., 1999) family (Fig. 3A). Among the AUX-LAX proteins, two subclasses could be defined containing AtAUX1, CgAUX1, and AtLAX1 for the first subclass and AtLAX2, AtLAX3, and CgLAX3 for the second subclass (Fig. 3A). Comparison of protein sequences (Fig. 3B) shows that the N and C terminus sequences are the most divergent, whereas the central sequence is highly conserved. Out of 13 amino acids that have been shown to be important for AtAUX1 activity (Swarup et al., 2004), all are conserved in CgLAX3 and all but one in CgAUX1 (Fig. 3B).

We tested whether *CgAUX1* and *CgLAX3* encode functional auxin influx carrier proteins equivalent to Arabidopsis AUX1 by carrying out a complementation analysis. *CgAUX1* and *CgLAX3* open reading frames were inserted between *AtAUX1* promoter (Pro_{AtAUX1}) and terminator sequences in a binary vector and transformed into null *aux1-22* mutants. We then analyzed whether that was sufficient to restore gravitropic phenotype in T1 plants 8 d after germination. *aux1-22* plants transformed with an empty vector containing the *AtAUX1* promoter and terminator sequences are agravitropic (Fig. 4A). In contrast, transformation with a vector expressing the *AtAUX1* coding sequence under its own promoter and terminator rescued a wild-type gravitropic phenotype (Fig. 4A). In the same conditions, *CgAUX1* was able to rescue a gravitropic phenotype to *aux1* (Fig. 4A). However, expressing *CgLAX3* under the control of the *AtAUX1* promoter and terminator in *aux1-22* mutant background could not restore a wild-type phenotype (Fig. 4A) even if *CgLAX3* transcripts were detected in transgenic plants (Supplemental Fig. S1). We conclude that CgAUX1 is functionally equivalent to AtAUX1, whereas CgLAX3 is not. The inability of *CgLAX3* to complement the *aux1-22* mutant in the same conditions suggests that either *CgLAX3* is not a functional auxin influx carrier or it is regulated differently at the translational or posttranslational level. The phylogenetic tree shows that LAX3 and AUX1 proteins belong to different subgroups, thus suggesting that LAX3 and AUX1 proteins might have diverged and have different functions and/or modes of regulation. This is further confirmed by the fact that *AtLAX3* cannot complement the *aux1-22* mutant when expressed under the *AtAUX1* promoter and terminator (R. Swarup and M. Bennett, personal communication).

We also checked whether *CgAUX1* was sensitive to 1-NOA by attempting to disrupt the complementation of *aux1* root gravitropism by *CgAUX1*. Treatment with 25 μM 1-NOA leads to a reversion to the mutant agravitropic phenotype (Fig. 4B) as in wild-type plants. This result indicates that CgAUX1, like AtAUX1 (Parry et al., 2001a), is sensitive to the auxin influx inhibitor 1-NOA.

CgAUX1 Expression Is Associated with Plant Cell Infection by *Frankia*, But Is Excluded from Nodule Primordia

Expression of *CgAUX1* and *CgLAX3* was analyzed in different *C. glauca* organs. Reverse transcription (RT)-PCR experiments detected *CgAUX1* and *CgLAX3* transcripts in all the organs tested (Fig. 5), showing that both genes are expressed throughout the plant.

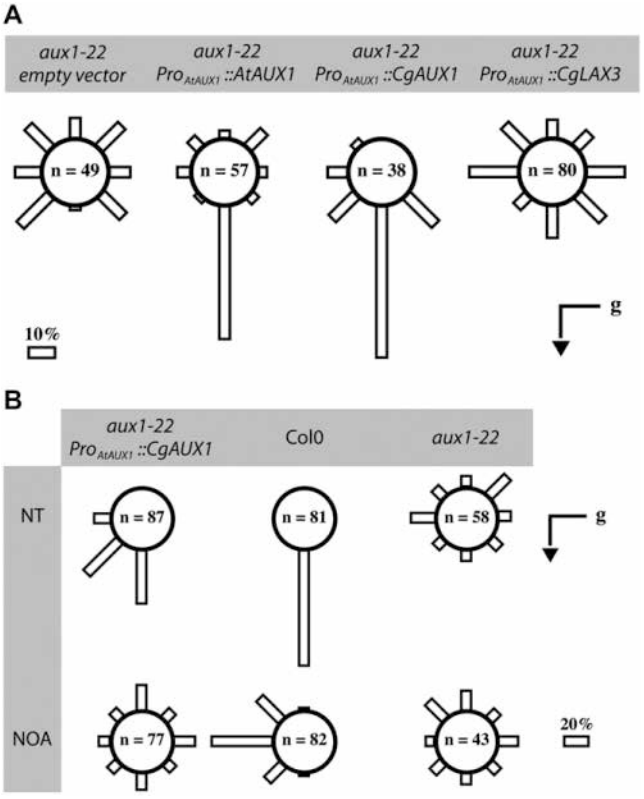

Figure 4. Gravitropic response of *aux1-22* Arabidopsis mutants complemented with *Casuarina* genes. *CgAUX1* and *CgLAX3* were expressed in *aux1-22* mutants under the control of the *AtAUX1* promoter and terminator sequences. A, Gravitropic response of T1 plants was assayed 24 h after a 90° gravistimulus, plants were grouped into eight classes, depending on the angle of the root apex. *aux1-22* plants transformed with an empty vector (*AtAUX1* promoter and terminator)-agravitropic phenotype. *aux1-22* plants expressing *AtAUX1* under the control of its own promoter-gravitropic phenotype. *aux1-22* plants complemented with *CgAUX1*-gravitropic phenotype. *aux1-22* plants complemented with *CgLAX3*-agravitropic phenotype. B, Gravitropic response of *aux1-22* mutants complemented with *CgAUX1* (homozygous T3 line), Col-0 plants, or *aux1-22* mutants upon NT or 25 μM 1-NOA treatment (NOA). The percentage of plants in each group is shown as oriented white bars and plant number (*n*) is indicated in the circle.

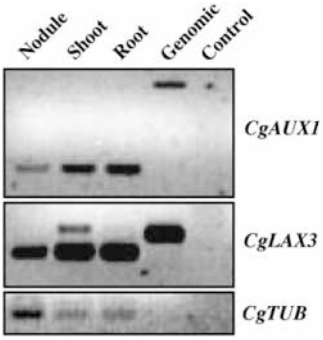

Figure 5. *CgAUX1* and *CgLAX3* are expressed in *Casuarina* root, shoot, and nodule. Nonquantitative RT-PCR analysis in mature nodule, shoot, and root using tubulin (*CgTUB*) as a control. A control without cDNA and a genomic DNA control were also included. The extra band in the *CgLAX3* shoot RT-PCR most probably indicates the presence of some genomic DNA in our RNA sample.

We then focused our expression analysis on *CgAUX1* because it encodes a functional auxin influx transporter. We cloned a 1.7-kb promoter fragment and fused it to the *GUS* reporter gene sequence in a binary vector, thus creating the Pro_{CgAUX1}:*GUS* construct. This construct was introduced into *C. glauca* and its close relative *Allocasuarina verticillata* by *Agrobacterium tumefaciens*-mediated genetic transformation (Franche et al., 1997). Similar patterns of expression were obtained in these two species. *CgAUX1* is expressed in root tips (Fig. 6A) and in lateral root primordia (Fig. 6, B and C). Expression was also observed in the root (Fig. 6B) and shoot vasculature (data not shown). This expression pattern is very similar to the *AtAUX1* expression pattern in Arabidopsis (Marchant et al., 2002). This, together with the complementation results, suggest that *CgAUX1* is orthologous to *AtAUX1* and is involved in the same biological processes (gravitropism and lateral root development) as *AtAUX1*.

We then analyzed *CgAUX1* expression during symbiotic interaction with *Frankia*. Pro_{CgAUX1}:*GUS* expression was studied 2, 7, 10, 14, and 21 d after inoculation (eight transgenic *C. glauca* plants/time point). All of the plants showed the same expression pattern. *CgAUX1* expression was detected very early in very few root hairs from 10 d postinoculation (Fig. 7, A and C–F). Infecting *Frankia* hyphae were found in *CgAUX1*-expressing root hairs (Fig. 7F). At the same time, a higher expression level is clearly visible in the vasculature at the site of infection (Fig. 7, A–D). At later stages, *CgAUX1* expression is associated with the infection process. Nodule sections showing strong staining in cortical cells that are infected and no staining in noninfected cells further confirm this pattern of expression (Fig. 7, G–J). Surprisingly, *CgAUX1* is not expressed in the nodule primordium (Fig. 7B). This lack of expression in nodule primordia is confirmed by the analysis of nodule ramifications (Fig. 7K). We therefore found that *CgAUX1* expression was associated with *Frankia* infection from the first stage of infection, but was excluded from nodule primordia.

Frankia has been reported to synthesize different auxins (IAA and phenylacetic acid [PAA]). These bacterial auxins could be involved in the regulation of symbiotic genes in infected plant cells. *Cg12* encodes a subtilisin-like protease specifically expressed in *Frankia*-infected cells (Svistoonoff et al., 2003). We therefore tested the effect of these auxins on the expression of *CgAUX1* and *Cg12*. Plants were treated with different concentrations of IAA, naphthalene acetic acid, or PAA; roots were harvested and used to extract RNA. Quantification of gene expression by real-time RT-PCR experiments did not reveal any significant changes in *CgAUX1* or *Cg12* gene expression in response to any type of auxin (Supplemental Fig. S2). We also found no effect of plant nitrogen status on *CgAUX1* expression in response to auxin (Supplemental Fig. S2).

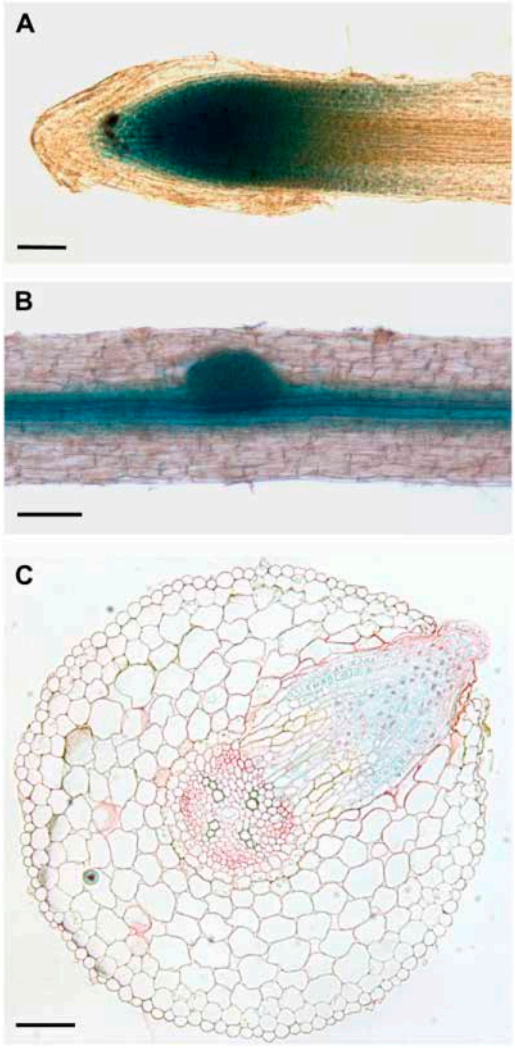

Figure 6. *CgAUX1* nonsymbiotic expression pattern in *A. verticillata*. *CgAUX1* expression is detected in root apex (A), mature root vasculature (B), and lateral root primordia (B and C). Bars = 50 μm (A and B) and 15 μm (C).

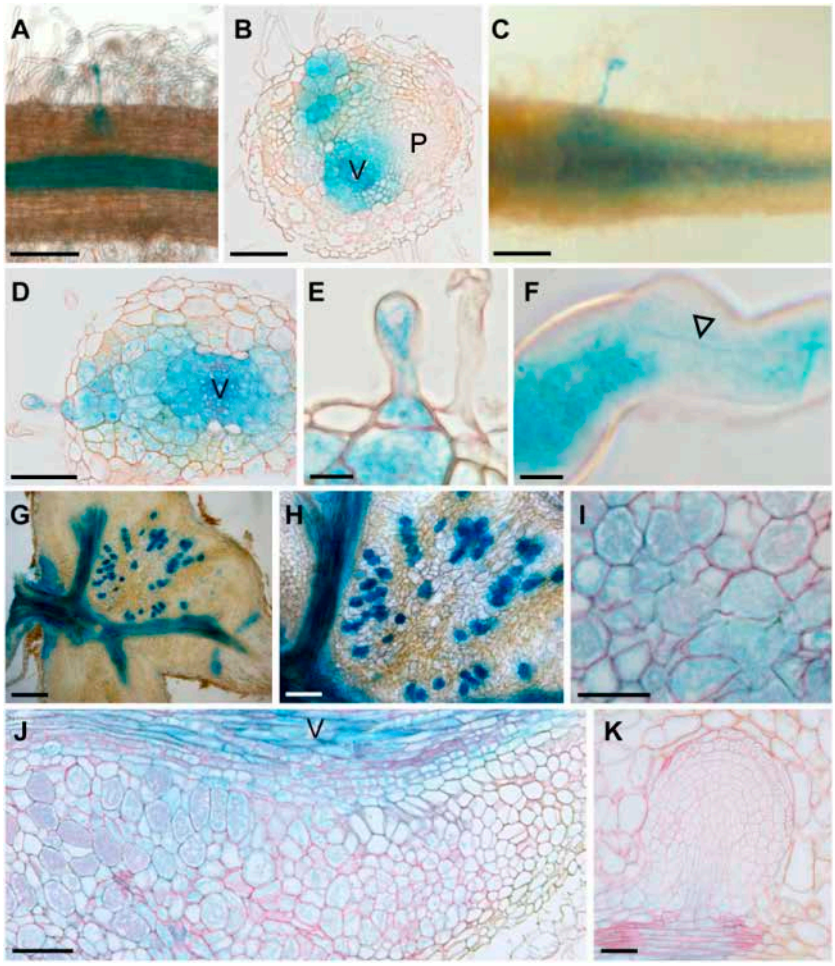

Figure 7. *CgAUX1* expression pattern upon *Frankia* infection in *C. glauca*. *CgAUX1* expression is observed in a few root hairs 10 d post-inoculation (A and C–F). The presence of *Frankia* hyphae in a root hair expressing *CgAUX1* is shown by an arrowhead (F). An increase in expression level is detected in the vasculature at sites of infection (A–D). *CgAUX1* expression is associated with infection in nodules (G–J). No expression of *CgAUX1* can be seen in primary nodule primordium (B) or nodule primordium ramification (K). V, Vasculature; P, nodule primordium. Bars = 5 μm (F), 10 μm (E), 25 μm (B and D), 50 μm (A, C, I–K), 125 μm (H), or 250 μm (G).

DISCUSSION

The results presented here suggest that auxin influx activity is important for symbiotic interaction between *C. glauca* roots and the soil actinomycete *Frankia*. We first show that competitive inhibition of auxin influx using 1-NOA delays nodulation and confirms the involvement of auxin carriers in the process. This led us to isolate two members of a small family of auxin influx carrier genes in *C. glauca*. We found that *CgAUX1* can complement the Arabidopsis *aux1* mutant, whereas *CgLAX3* could not. *AtAUX1* was demonstrated to encode an auxin influx carrier in the Xenopus oocyte (Yang et al., 2006). We therefore conclude that *CgAUX1* also encodes for an auxin influx carrier equivalent to AtAUX1.

The actinorhizal nodule is classically regarded as a modified lateral root (Pawlowski and Bisseling, 1996; Obertello et al., 2003). However, we observed that *CgAUX1* is expressed in lateral root primordia, but not in nodule lobe primordia. These results suggest that these two organs have, at least in part, divergent development programs. This is in agreement with previous observations showing that some heterologous promoters used as molecular markers, such as 35S and *AtUBQ1*, drive different expression patterns in lateral root and nodule primordia in Casuarinaceae plants (Obertello et al., 2005). Nevertheless, because our analysis is only based on a promoter-GUS fusion, we cannot completely rule out that *CgAUX1* is expressed in nodule primordia. We cannot exclude either that another *AUX-LAX* gene, such as *CgLAX3*, is involved in actinorhizal nodule primordium formation. Further studies will be needed to understand how much of the lateral root developmental program has been recycled during evolution to create the actinorhizal nodule developmental program. By comparison, in situ hybridization experiments suggest that *AUX-LAX* genes are expressed in vascular tissues and the nodule primordia during nodulation in the model legume *M. truncatula* (de Billy et al., 2001).

Interestingly, we found that *CgAUX1* expression is closely associated with *Frankia* infection of plant cells during nodulation (summarized in Fig. 8A). We observed *CgAUX1* expression already in *Frankia*-infected root hairs 10 d after infection. *CgAUX1* was later expressed in all *Frankia*-infected cells in the prenodule and in the nodule regardless of their development stage (infection, nitrogen fixation, etc.). *CgAUX1* expression

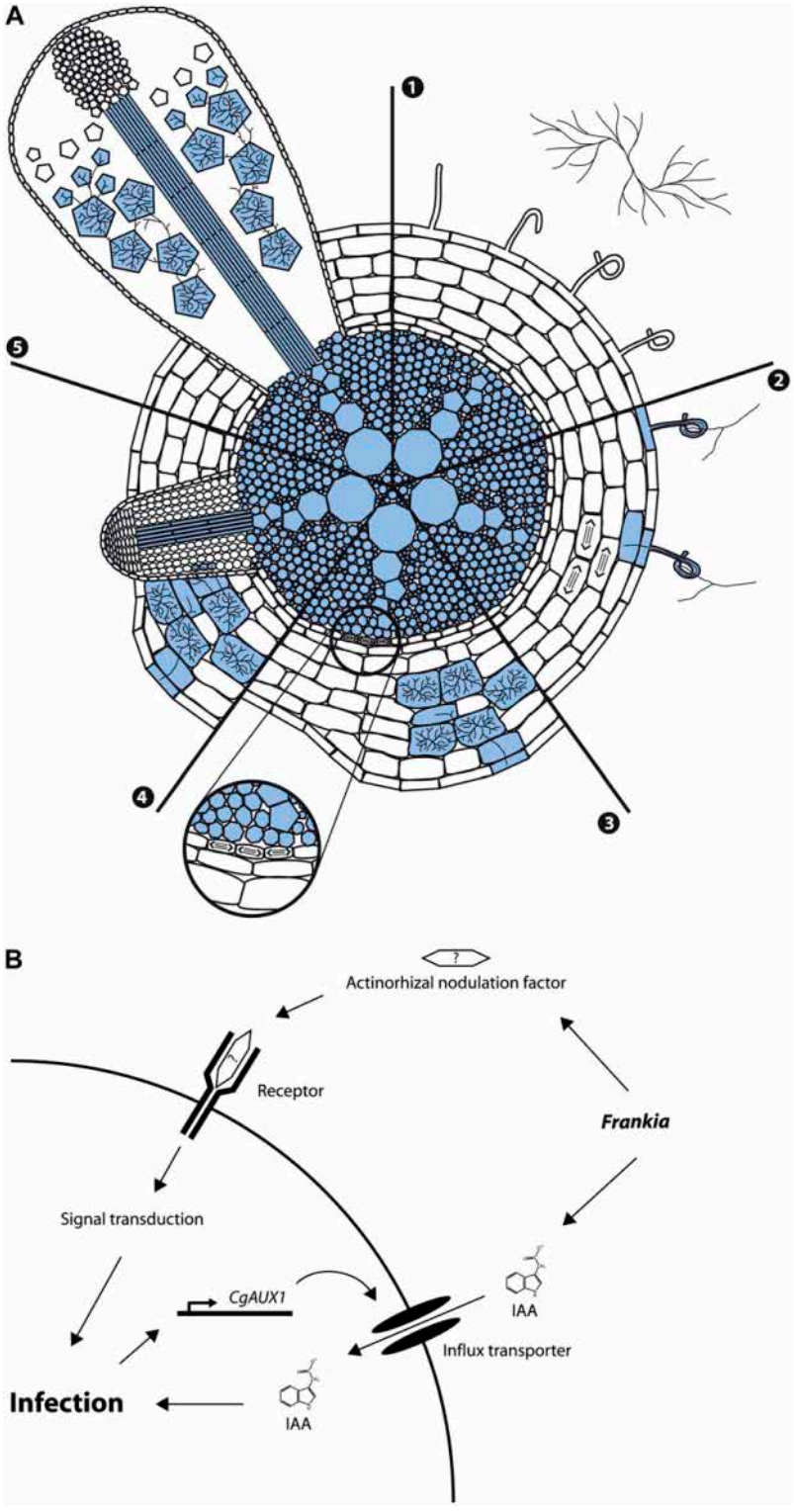

Figure 8. Putative *CgAUX1* function during actinorhizal nodule formation. A, Summary of *CgAUX1* expression pattern (blue color) at different steps of *C. glauca-Frankia* interaction. 1, Signal exchanges between the actinorhizal plant and *Frankia* lead to root hair infection. 2, *Frankia* penetrates a deformed root hair showing *CgAUX1* expression and triggers cortical cell divisions. 3, Dividing cortical cells are infected by *Frankia* hyphae and hypertrophy, thus leading to the formation of a prenodule. At the same time, pericycle cell divisions occur in front of a xylem pole to form a nodule primordium. 4, *Frankia* hyphae coming from the prenodule invade the cortex of the nodule primordium. 5, In mature nodules, *CgAUX1* expression is observed in infected cells and the vascular tissues. B, Proposed model of *CgAUX1* role during the infection process. We propose that two signals occur in synergy during actinorhizal symbiosis. A specific signal that remains unknown is produced by the bacteria and triggers the production of the auxin influx carrier CgAUX1. A nonspecific signal, auxin, is also produced by the bacteria and acts in synergy with the specific signal to trigger the infection-related program by the plant.

was also detected in the vascular tissues in noninfected and infected roots and nodules. As a comparison, no expression of *AUX-LAX* genes was detected by in situ hybridization in *Rhizobium*-infected cells in the model legume *M. truncatula* (de Billy et al., 2001). To our knowledge, this is the first report of an auxin influx activity linked to plant cell infection by a soil microorganism. The signal responsible for the infection-specific expression of *CgAUX1* is not known. We showed that auxin alone (IAA, naphthalene acetic acid, or PAA) cannot play this role. *CgAUX1* expression may be induced by a symbiotic signal produced

by *Frankia*. The expression of an auxin influx carrier in *Frankia*-infected cells would make them more permeable to auxin. Interestingly, some *Frankia* strains have been shown to produce different forms of auxin in culture, including IAA and PAA (Wheeler et al., 1984; Hammad et al., 2003). This could explain why actinorhizal nodules have been reported to contain more auxin than noninfected roots (Wheeler et al., 1979). We therefore speculate that *CgAUX1* expression allows the entry and perception of *Frankia*-produced auxin and restricts it to infected plant cells (Fig. 8B). Auxin alone or in synergy with a symbiotic signal could induce changes in gene expression, cell metabolism, etc., in infected cells to allow the establishment of intracellular symbiosis (Fig. 8B). For example, the infection process is associated with remodeling of the cell wall to create an infection thread (Berg, 1999a). Many cell wall remodeling genes have been found to be auxin inducible in Arabidopsis (Neuteboom et al., 1999; Overvoorde et al., 2005; Esmon et al., 2006; Osato et al., 2006). Auxin could therefore induce genes encoding cell wall remodeling enzymes necessary for infection by *Frankia*. Moreover, infected cells are hypertrophied (Berg, 1999b), a phenotype that has been classically associated with auxin response (Teale et al., 2006). Further experiments will be needed to understand the interaction between cell wall remodeling and auxin transport during nodule formation.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Casuarina glauca seeds (purchased from Carter Seeds) were grown and inoculated by *Frankia* CcI3 strain as previously described (Franche et al., 1997). Arabidopsis (*Arabidopsis thaliana*) Columbia-0 (Col-0) and *aux1-22* (Col-0 background) mutant seeds were obtained from the Nottingham Arabidopsis Stock Center. Plants were grown as previously described (Laplaze et al., 2005). Gravitropism assays were performed as previously described (Swarup et al., 2005).

Identification of *CgAUX1* and *CgLAX3* cDNA and Genomic Sequences

C. glauca genomic DNA was isolated from a young shoot apex using a MATAB extraction method (Ky et al., 2000). Amplification of *AUX1* homologs was performed on genomic DNA using different sets of degenerate primers (Table I). Amplified fragments were cloned into pGEM-T easy (Promega) and sequenced. Total RNA was extracted on the whole root system by ultracentrifugation (Chirgwin et al., 1979). Polyadenylated RNA was purified using the Amersham mRNA purification kit. Full-length cDNA sequences were obtained by performing RACE-PCR on a root cDNA library (Marathon cDNA amplification kit; CLONTECH). cDNA was amplified using primers 5′-ATAGCATTATTTTGTCTGTGGGTTG-3′ and 5′-CAACCCACAGACAAAATAATGCTAT-3′ for *CgAUX1* 5′ and 3′ RACE, respectively, and primers 5′-TCACTGGGGCTACCAACATTCTCTA-3′ and 5′-TAGAGAATGTTGGTAGCCCCAGTGA-3′ for *CgLAX3* 5′ and 3′ RACE, respectively.

Full-length cDNA and genomic DNA were amplified using AUX1F, 5′-GCAGATCAGCCGGAATTTAG-3′; AUX1R, 5′-TGCTTTGGAAGCAAAGGAAT-3′; LAX3F, 5′-ACAATGGCTTCCGAGAAGGT-3′; LAX3R, 5′-GGCTAAATTCAATCCCACCGTA-3′, cloned into pGEM-T and sequenced.

Genomic DNA-Blot Analysis

Ten micrograms of DNA were digested with *Bam*HI, *Eco*RI, and *Hin*dIII (New England Biolabs). DNA fragments were separated on a 1% agarose gel and capillary blotted onto a Hybond N^+ membrane (Amersham). A 175-bp *CgAUX1*-specific probe was synthesized using primers 5′-AGCTAACACACCCCATAGTTTG-3′ and 5′-AATAATAAGCCTATGCTTTGGAAG-3′, a 234-bp *CgLAX3*-specific probe was synthesized using primers 5′-GCGTGTAAAGAGATTGGCATTT-3′ and 5′-TGAGCAAACACTACAACGGCTAA-3′ and a 174-bp *AUX-LAX* conserved probe was synthesized using primers 5′-CGTTTGGATTCGCCTGCACTCC-3′ and 5′-GAGATCCAACAGTTGAGTTGA-3′. Probes were labeled with α-^{32}P dCTP by random priming. Hybridization was carried out in high-stringency conditions for *CgAUX1*- and *CgLAX3*-specific probes (65°C; 10-min washes with 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS; 0.5× SSC, 0.1% SDS) and low-stringency conditions for the *AUX-LAX* conserved probe (56°C; 10-min washes with 2× SSC, 0.1% SDS; 1× SSC, 0.1% SDS). Hybridization patterns were visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics). DNA extractions and hybridization were repeated at least twice.

Nonquantitative and Quantitative RT-PCR

Total RNA was extracted on the whole-root system, shoot, or mature nodules by ultracentrifugation (Chirgwin et al., 1979). Poly(dT) cDNA was prepared out of 1 μg total RNA using the RT system (Promega) and three independent RT reactions were pooled for quantitative analysis. PCR reactions were carried out at 94°C for 5 min, followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 90 s at 72°C. Target amplifications were performed with *CgAUX1*- or *CgLAX3*-specific primer pairs designed on each side of the last intron (*CgAUX1*, 5′-GTTCTTCGGGCCCATAAACT-3′ and 5′-TGCTTTGGAAGCAAAGGAAT-3′; *CgLAX3*, 5′-ATTCCTGCCCTAGCACACAT-3′ and 5′-CCCACCGTAAAGAGATACCG-3′). Tubulin gene (*CgTUB*) expression was used as a control (*CgTUB*, 5′-CGCGGCCGCTGGAGAGGCGTC-3′ and 5′-GCAAGCTTTCGGATGCGATCC-3′). Quantitative PCR was performed on a Stratagene Mx3005P apparatus with the FullVelocity SYBR Green QPCR master mix (Stratagene) upon recommendation of the manufacturer. PCR was carried out in 96-well optical reaction plates heated for 5 min to 95°C, followed by 40 cycles of denaturation for 10 s at 95°C, and annealing extension for 30 s at 60°C. Target quantifications were performed with specific primer pairs designed using Beacon Designer 4.0 (Premier Biosoft International; *CgAUX1*, 5′-ACCAGGAGCAACCGGAAGAC-3′ and 5′-AGCACTTGCGCAACTTGATTG-3′; *CgLAX3*, 5′-CACTTGCGCGACCTGGTTAG-3′ and 5′-AAGAAGGCGATCCCAAGACG-3′; *Cg12*, see Hocher et al., 2006). Expression levels were normalized to ubiquitin (*CgUBI*; Hocher et al., 2006). All RT-PCR experiments were performed in triplicate and the presented values represent means ± SD.

Constructs and Generation of Transgenic Plants

For promoter studies, 1.7-kb genomic DNA fragments upstream of the *CgAUX1* and *CgLAX3* start codon (ATG) were amplified using the Universal GenomeWalker kit (CLONTECH) and cloned upstream of the *GUS* reporter gene in pBI101.3 binary vector (CLONTECH). For functional complementation, full-length *CgAUX1* and *CgLAX3* cDNA were fused with Arabidopsis *AtAUX1* promoter (1.7 kb) and terminator (0.3 kb) in a pMOG402 binary vector (MOGEN International). Vectors were introduced into *Agrobacterium tumefaciens* C58C1 pGV3101 by electroporation. Transformation of Arabidopsis (Col-0 and *aux1-22*) was performed as previously described (Clough and Bent, 1998). Transformation of *C. glauca* and *Allocasuarina verticillata* plants was performed as previously described (Franche et al., 1997).

Microscopy and Root Sections

GUS assays were performed as previously described (Svistoonoff et al., 2003). Tissues were cleared in 70% ethanol for 2 d and then immersed in 50% (v/v) ethanol/10% (v/v) glycerol for 2 h, 30% (v/v) ethanol/30% (v/v) glycerol for 2 h, and in 50% (v/v) glycerol for 2 h. Seedlings were then mounted in 50% (v/v) glycerol and visualized on a Leitz DMRB microscope. For thin root sections, samples were fixed (Svistoonoff et al., 2003) and cleared in 70% ethanol for 2 d. Ethanol dehydration was performed (90%, 100% twice) at room temperature (15 min/step). 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 2 min in aqueous 0.05% ruthenium red solution and mounted in Clearium Mountant (Surgipath). For thick nodule

sections, nodules were embedded in 3% agarose. Thick sections (55 μm) were cut with a Leica VT1000E vibratome.

Sequence data from this article have been deposited with the EMBL/GenBank libraries under accession numbers EF416279 and EF416280 for CgAUX1 gene and cDNA and EF416281 and EF416282 for CgLAX3 gene and cDNA.

Supplemental Data

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

Supplemental Figure S1. RT-PCR detection of *CgLAX3* transcripts in complemented *aux1* mutants.

Supplemental Figure S2. Quantitative expression levels of *CgAUX1* and *Cg12* in response to auxin.

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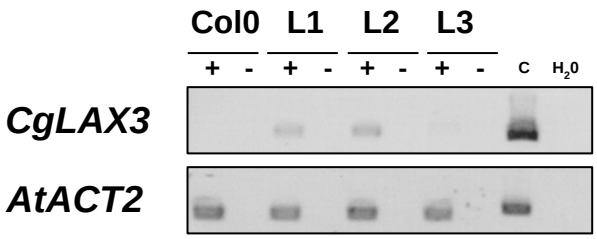
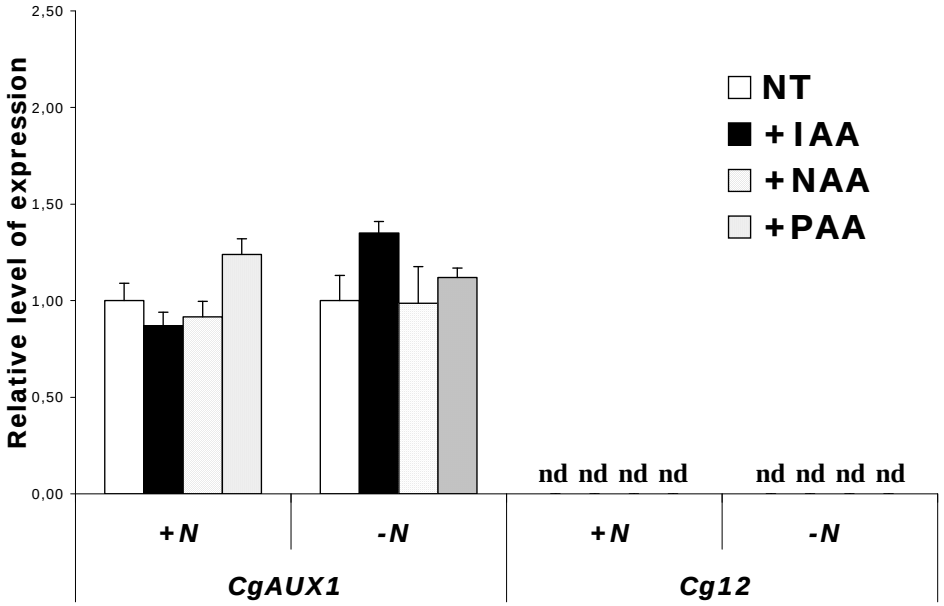


Supplementary Figure 1. RT-PCR detection of *CgLAX3* transcripts in complemented *aux1* mutants. *CgLAX3* mRNA presence was assayed by RT-PCR in Col0 plants and in three independent transgenic lines expressing *CgLAX3* under the control of *AtAUX1* promoter and terminator. Amplification of cDNA (+) or RNA control (-) are shown. A positive control (vector used for transformation – C) and negative control (water – H_20) are included.



Supplementary Figure 2. Quantitative expression levels of *CgAUX1* and *Cg12* in response to auxin. The expression level was determined by quantitative RT-PCR on RNA from plants after no treatment (NT) or 16 hours 10 µM IAA treatment (+IAA), 10 µM NAA treatment (+NAA), 10 µM PAA treatment (+PAA) in presence of 10mM nitrate (+N) or after one week nitrogen starvation (-N). Expression level were normalised to ubiquitin (*CgUBI*) expression level. nd : not detected.