Running title: Role of CgAUX1 during Frankia Infection

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Research area:
Plants Interacting with Other Organisms
Auxin influx activity is associated with *Frankia* infection during actinorhizal nodule formation in *Casuarina glauca*

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ABSTRACT

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-naphtoxy acetic acid (1-NOA) perturbs actinorhizal nodule formation. This suggests a potential role for auxin influx carriers in the infection process. We therefore isolated and characterized homologues of the auxin influx carriers (AUX1-LAX) genes in Casuarina glauca. Two members of this family were found to share high levels of deduced protein sequence identity with Arabidopsis 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:GUS 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.
INTRODUCTION

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, in condition of nitrogen deprivation, by an exchange of signals between the plant roots and the bacteria. The chemical nature of Frankia nodulation factor(s) 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). Concomitently, cell divisions occur in the pericycle in front of 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 i) they originate from divisions in the pericycle in front of a xylem pole, ii) they have a lateral root like structure with a central vasculature, infected cells in the cortex and an apical meristem and iii) 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 (PAT) using inhibitors such as NPA (naphthylphthalamic acid) or mutants result in dramatic alteration of the plant developmental pattern (Reed et al., 1998). The existence of auxin transporters has been predicted for a long time to account for PAT (Goldsmith, 1977). Characterization of 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 (4 genes) in Arabidopsis (Parry et al., 2001a).
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. A local auxin transport inhibition is triggered by spot-inoculation of rhizobia, leading to a subsequent accumulation of auxin at the site of infection as shown by the use of the GH3:gusA auxin response marker in white clover (Mathesius et al., 1998) and in 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 the actinorhizal symbionts dialog 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 the establishment of the symbiosis. The symbiotic bacteria Rhizobium produce auxins that were proposed to be involved in establishing the symbiosis with Legume plants (Badenoch-Jones et al., 1983). Indeed, a Bradyrhizobium japonicum mutant producing 30-fold more IAA than the wild-type has a 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 genes homologues in the actinorhizal plant Casuarina glauca. Among this family of genes, we identified CgAUX1, a homologue of AtAUX1, that carries an auxin carrier
function as shown by functional complementation of the Arabidopsis aux1 mutant. The 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 primordium based on different patterns of CgAUX1 expression. Altogether, our results shed new 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-naphtoxyacetic acid (1-NOA), a competitive inhibitor of auxin influx, on 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 days (Fig. 1A). We found in 3 independent experiments that 1-NOA treatments caused a 2 days delay in nodule appearance. The same effect was observed if the growth medium was changed every three days with fresh 1-NOA to prevent a potential 1-NOA degradation (data not shown). Moreover, 24 days after inoculation plants treated with 1-NOA mainly showed prenodules while control plants showed nodules (Fig 1B and C). This 1-NOA effect on nodulation was not due to a more general effect on root growth as we found no significant differences in shoot or root weight in treated or non treated plants (Student test at P<0.1; NT roots m=0.059g ; NOA treated roots m=0.062g ; NT shoots m=0.136g ; NOA treated shoots m=0.137g ; 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 homologues during actinorhizal nodule development. AUX-LAX gene homologues 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. Seven different PCR products were produced, sequenced and found to correspond to two different genes. The corresponding cDNAs (1440 bp and 1395 bp) were obtained by rapid amplification of cDNA ends (RACE-PCR). They were named *CgAUX1* and *CgLAX3* according to 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 2942 bp and 2224 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 (5 genes; Schnabel and Frugoli, 2004) indicating a common evolutionary origin. Interestingly, AUX-LAX gene structure is slightly different in rice (*Oryza sativa* ssp japonica cv. Nipponbare - 5 genes in the annotated genome; Tyagi et al., 2004) where 3 genes have 7 exons like *AtAUX1*, and the two others have 5 and 2 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.

In order to estimate the number of AUX-LAX genes in *C. glauca* genome, we conducted Southern blot experiments using three different probes: a non-specific probe designed in one of the most conserved region 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 non-stringent conditions that could be assigned to either *CgAUX1* or *CgLAX3* using the 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 carriers are encoded by a small gene family (possibly only 2 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 transporters family (ATF). A phylogenetic tree was generated using neighbor-joining distance algorithm showing that AUX-LAX proteins belong to the amino-acid and auxin permease (AAAP; Young et al., 1999) family (Fig. 3A). Among the AUX-LAX proteins, two sub-classes could be defined containing AtAUX1, CgAUX1 and AtLAX1 for the first sub-class and AtLAX2, AtLAX3 and CgLAX3 for the second sub-class (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* ORF were inserted between *AtAUX1* promoter (*ProAtAUX1*) and terminator sequences in a binary vector and transformed into null aux1-22 mutants. We then analyzed if that was sufficient to restore a gravitropic phenotype in T1 plants 8 days after germination. aux1-22 plants transformed with an empty vector containing *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 *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 the transgenic plants (Sup Fig. 1). We conclude that *CgAUX1* is functionally equivalent to *AtAUX1* while *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 post-translational level. The phylogenetic tree shows that LAX3 and AUX1 proteins belong to different subgroups thus suggesting that the 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 \textit{AtAUX1} promoter and terminator (Ranjan Swarup and Malcolm Bennett, personal communication).

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

\textit{CgAUX1} expression is associated with plant cell infection by \textit{Frankia} but is excluded from nodule primordia

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

We then focused our expression analysis on \textit{CgAUX1} because it encodes a functional auxin influx transporter. We cloned a 1.7 kb promoter fragment and fused it to the β-glucuronidase (\textit{GUS}) reporter gene sequence in a binary vector thus creating the \textit{ProCgAUX1:GUS} construct. This construct was introduced into \textit{C. glauca} and its close relative \textit{Allocasuarina verticillata} by \textit{Agrobacterium tumefaciens} mediated genetic transformation (Franche et al., 1997). Similar patterns of expression were obtained in these two species. \textit{CgAUX1} is expressed in root tips (Fig. 6A) and in lateral root primordia (Fig. 6B and C). Expression was also observed in the root (Fig. 6B) and shoot vasculature (data not shown). This expression pattern is very similar to \textit{AtAUX1} expression pattern in Arabidopsis (Marchant et al., 2002). This together with the complementation results suggest that \textit{CgAUX1} is orthologous to \textit{AtAUX1} and is involved in the same biological processes (gravitropism and lateral root development) as \textit{AtAUX1}.

We then analyzed \textit{CgAUX1} expression during the symbiotic interaction with \textit{Frankia}. \textit{ProCgAUX1:GUS} expression was studied 2, 7, 10, 14 and 21 days after inoculation (8 transgenic \textit{C. glauca} plants/time point). All of the plants showed the same expression pattern. \textit{CgAUX1} expression was detected very early in very few root hairs from 10 days post-inoculation (Fig. 7A, C-F). Infecting \textit{Frankia} hyphae were found in \textit{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. 7A, B C and D). At later stages, $CgAux1$ expression is associated with the infection process. Nodule sections showing a strong staining in the cortical cells that are infected and no staining in the non-infected cells further confirm this pattern of expression (Fig. 7G, H, I and 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 (indole-3-acetic acid – 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, naphthylphthalamic acid (NAA) 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 (Sup Fig. 2). We also found no effect of the plant nitrogen status on $CgAux1$ expression in response to auxin (Sup Fig. 2).

DISCUSSION

The results presented here suggest that auxin influx activity is important for the 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 while $CgLax3$ 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, since 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 figure 8A). We observed CgAUX1 expression already in Frankia-infected root hairs 10 days after infection. CgAUX1 was later expressed in all Frankia infected cells in the prenodule and in the nodule irrespective of their development stage (infection, nitrogen fixation…). CgAUX1 expression was also detected in the vascular tissues in non-infected and infected roots and in 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 therefore 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, NAA 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 non-infected 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 cell (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 the 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 the infection by *Frankia*. Moreover, infected cells are hypertrophied (Berg, 1999b), a phenotype that has been classically associated to 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 (California, USA) were grown and inoculated by *Frankia* CcI3 strain as previously described (Franche et al., 1997). Arabidopsis 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 young shoot apex using a MATAB extraction method (Ky et al., 2000). Amplification of *AUX1* homologues 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 whole root system by ultracentrifugation (Chirgwin et al., 1979). Polyadenylated RNA was purified using 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 were amplified using primers 5’-ATAGCATTATTTTGTCTGTGGGTTG-3’ and 5’-
CAACCCACAGACAAAATAATGCTAT-3' for CgAUX1 5' and 3' RACE respectively and primers 5'-TCACTGGGGCTACCAACATTCTCT-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'-TGCTTTGGAAGCAAGGAGAAT-3'; LAX3F 5'-ACAATGGCTTCCAGAGGT-3'; LAX3R 5'-GGCTAAATTCAATCCCACCGTA-3', cloned into pGEM-T and sequenced.

**Genomic DNA blot analysis**

10 µg of DNA were digested with *Bam*HI, *Eco*RI and *Hind*III (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'-AGCTAACACCCCATAGTTTG-3' and 5'-AATAATAAGCCTATGCTTTGGAAG-3', a 234 bp CgLAX3 specific probe was synthesized using primers 5'GCGTGTAAAGAGATTGGCATTT-3' and 5'-TGAGCAAACACTACAACGCTAA-3' and a 174 bp AUX-LAX conserved probe was synthesized using primers 5'-CGTGTGGAAGAGATTGCCATTT-3' and 5'-TGAGCAAACACTACAACGCTAA-3'. Probes were labeled with \( \alpha \)-32P dCTP by random priming. Hybridization was carried out in high stringency conditions for CgAUX1 and CgLAX3 specific probes (65°C ; 10 min washes with 2X SSC, 0.1% SDS; 1X SSC, 0.1% SDS; 0.5X SSC, 0.1% SDS) and low stringency conditions for the AUX-LAX conserved probe (56°C ; 10 min washes with 2X SSC, 0.1% SDS; 1X 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.

**Non-quantitative and quantitative RT-PCR**

Total RNA was extracted on 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 Reverse Transcription System (Promega) and 3 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 sec at 94°C, annealing for 30 sec at 60°C and extension for 90 sec at 72°C. Target amplifications were performed with CgAUX1 or CgLAX3 specific primer pairs designed on each side of the last intron (CgAUX1 5’-GTTCTTCGGGCCATATAACT-3’ and 5’-TGCTTTGGAAGCAAAGGAAT-3’; CgLAX3 5’-ATTCTCTGCCCTAGCACAAT-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 recommendations of the manufacturer. PCR was carried out in 96-well optical reaction plates heated for 5 minutes to 95°C, followed by 40 cycles of denaturation for 10 seconds at 95°C and annealing-extension for 30 seconds 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’-CAGTTGCAGCAGCTGGATT-3’ and 5’-AAGAAGGGCGATCCCAAGACG-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 triplicates and the presented values represent means plus/minus standard deviation.

**Constructs and generation of transgenic plants**

For promoter studies, 1.7 kb genomic DNA fragments upstream of 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 (Col0 and aux1-22) was performed as previously described (Clough and Bent, 1998). Transformation of Casuarina 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 per 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 nodules 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

Supplementary Figure 1. RT-PCR detection of CgLAX3 transcripts in complemented aux1 mutants.

Supplementary Figure 2. Quantitative expression levels of CgAUX1 and CgI2 in response to auxin.

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FIGURE LEGENDS

**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 (twenty plants per treatment). B, 24 days-old roots of plants bearing nodules (arrowheads) upon no treatment. C, 24 days-old roots of plants bearing big prenodules (arrowheads) upon NOA treatment. D, *Frankia* exponential growth indicated as total protein content upon no treatment (NT) or 25µM 1-NOA treatment (NOA). Bars are 1 cm for B and C.

**Figure 2.** *Casuarina glauca* AUX-LAX genes family. A, Exon-intron structure of *CgAUX1* and *CgLAX3* compared to *AtAUX1* and *AtLAX3*. Exons are shown as grey boxes and introns as black lines. B, Southern blot experiments suggest that they are only two AUX-LAX genes in *C. glauca* genome. Digested genomic DNA (with BamHI, EcoRI or HindIII) 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).

**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 (AAAP) : a lysine histidine transporter (*AtLHT1* – At5g40780), an amino acid permease (*AtAAP1* – At1g58360), an aromatic and neutral amino acid transporter (*AtANT1* – At3g11900). The tree was elaborated using neighbor-joining algorithm and rooted with the sucrose transporter protein sequence (*AtSUC1* – At1g71880). Bootstrap analysis are shown for each branch (n = 100). B, Alignment of *AtAUX1*, *CgAUX1* and *CgLAX3* predicted protein sequences using Clustal-W (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 an empty arrowhead. The only amino acid that is not conserved in *CgAUX1* is marked by a full arrowhead.
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 AtAUX1 promoter and terminator sequences. A, Gravitropic response of T1 plants was assayed 24h after a 90° gravistimulus, plants were grouped into eight classes depending on the angle of the root apex. aux1-22 plants transformed with 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), Col0 plants or aux1-22 mutants upon no treatment (NT) or 25 µM 1-NOA treatment (NOA). The percentage of plants in each group is shown as orientated empty bars and plant number (n) is indicated in the circle.

Figure 5. CgAUX1 and CgLAX3 are expressed in *Casuarina* root, shoot and nodule. Non-quantitative 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.

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

Figure 7. CgAUX1 expression pattern upon *Frankia* infection in *Casuarina glauca*. CgAUX1 expression is observed in a few root hair 10 days post-inoculation (A, 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, B, C and 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 are 5 µm (F), 10 µm (E), 25 µm (B, D), 50 µm (A, C, I, J, K), 125 µm (H) or 250 µm (G).
**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. Panel 1, signal exchanges between the actinorhizal plant and *Frankia* lead to root hair infection. Panel 2, *Frankia* penetrates a deformed root hair showing *CgAUX1* expression and triggers cortical cell divisions. Panel 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. Panel 4, *Frankia* hyphae coming from the prenodule invade the cortex of the nodule primordium. Panel 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 non-specific signal, auxin, is also produced by the bacteria and act in synergy with the specific signal to trigger the infection related program by the plant.
Table I
Degenerate primers designed in the most conserved regions of the AUX-LAX genes and used for *Casuarina 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         | TGGAC6TAYATHTYGG6GC6TGY | Reverse - Exon 5    |
### A

| aux1-22 | aux1-22 | aux1-22 | aux1-22 |
|---------|---------|---------|---------|
| empty vector | Pro<sub>AtAux1</sub>:AtAux1 | Pro<sub>AtAux1</sub>:CgAux1 | Pro<sub>AtAux1</sub>:CgLAX3 |
| n = 49 | n = 57 | n = 38 | n = 80 |

10% g

### B

| aux1-22 | Col0 | aux1-22 |
|---------|------|---------|
| Pro<sub>AtAux1</sub>:CgAux1 | | |
| NT | n = 87 | n = 81 |
| NOA | n = 77 | n = 82 |
| | | n = 43 |
