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cg12 Expression Is Specifically Linked to Infection of Root Hairs and Cortical Cells during *Casuarina glauca* and *Allocasuarina verticillata* Actinorhizal Nodule Development

Sergio Svistoonoff,[1] Laurent Laplaze,[1] Florence Auguy,[1] John Runions,[2] Robin Duponnois,[3] Jim Haseloff,[2] Claudine Franche,[1] and Didier Bogusz[1]

[1]Equipe Rhizogenèse, UMR 1098, Institut de Recherche pour le Développement, BP 64501, 34394 Montpellier cedex 5, France; [2]Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK; [3] Institut de Recherche pour le Développement, BP182, Ouagadougou, Burkina Faso

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***cg12* is an early actinorhizal nodulin gene from *Casuarina glauca* encoding a subtilisin-like serine protease. Using transgenic *Casuarinaceae* plants carrying *cg12-gus* and *cg12-gfp* fusions, we have studied the expression pattern conferred by the *cg12* promoter region after inoculation with *Frankia*. *cg12* was found to be expressed in root hairs and in root and nodule cortical cells containing *Frankia* infection threads. *cg12* expression was also monitored after inoculation with ineffective *Frankia* strains, during *mycorrhizae* formation, and after diverse hormonal treatments. None of these treatments was able to induce its expression, therefore suggesting that *cg12* expression is linked to plant cell infection by *Frankia* strains. Possible roles of *cg12* in actinorhizal symbiosis are discussed.**

Additional keywords: subtilase, Nod factors, genetic transformation.

Interaction between actinorhizal plants and the actinomycete *Frankia* results in the formation of nitrogen-fixing nodules on the roots of the host plants. Actinorhizal plants belong to eight families and 24 genera of angiosperms. All the host species are perennial woody shrubs or trees, with the exception of *Datisca glomerata*, which has herbaceous shoots. As in legume-rhizobium symbiosis, during the establishment of the actinorhizal symbiosis, both plant and bacteria undergo a complex series of developmental changes involving the expression of specific genes. By homology to legume nodulins, actinorhizal nodulin genes refer to plant genes expressed specifically or at high levels in actinorhizal nodules compared with roots. Since the isolation of *AgNOD-CP* (Goetting-Minesky et al. 1994), the first actinorhizal nodulin gene isolated in *Alnus glutinosa*, several putative symbiotic genes have been isolated from different actinorhizal species (Franche et al. 1998a).

We are working on the *Casuarina glauca–Frankia* symbiotic interaction, which represents a valuable system to study actinorhizal symbioses. *C. glauca* and its close relative *Allocasuarina verticillata* are the only actinorhizal plants that can be readily transformed and regenerated (Franche et al. 1997; Smouni et al. 2002). Such transgenic plants have proved useful, both for exploring the regulation of plant genes involved in the symbiotic process with the actinomycete *Frankia* (Laplaze et al. 2002) and for studying the evolution of nodulation and symbiotic genes (Franche et al. 1998b). *C. glauca* and *A. verticillata* are both infected by *Frankia* using the intracellular infection pathway (Franche et al. 1998a). Infection starts with the induction of root hair curling by *Frankia*. *Frankia* penetrate a curled root hair and hyphae are encapsulated by cell wall material that is believed to consist of xylans, cellulose, and pectin of host origin (Berg 1990). In response to the initial root hair infection, mitotic activity occurs in the root cortex and causes the formation of a swollen structure, the prenodule, whose cells are infected by *Frankia* hyphae. Recently, prenodule physiology and function were studied, using molecular markers, and it was shown that the prenodule is a primitive symbiotic organ (Laplaze et al. 2000a). As the prenodule develops, cell divisions are induced in the pericycle opposite the protoxylem pole, giving rise to an actinorhizal nodule lobe primordium. The pericycle-born nodule primordium develops into an indeterminate actinorhizal nodule lobe that resembles a modified lateral root without a root cap. New lobes arise continuously to form a coralloid nodule.

Subtilisin-like serine proteases or subtilases are a superfamily of proteases widely distributed in diverse organisms, including archaea, bacteria, fungi, yeasts, and higher eukaryotes (Siezen and Leunissen 1997). Based on their substrate specificity, two classes of subtilases have been described: i) degenerative subtilases mainly found in prokaryotes are involved in the degradation of a wide range of proteins and ii) processing subtilases mainly from eukaryotes cleave proteins or peptides at specific residues and activate or inactivate them. In yeasts and mammals, some subtilases have developed into highly specialized enzymes; examples are those belonging to the kexin and the proprotein convertase families that cleave inactive precursors to produce active peptides and proteins (Brake et al. 1984; Seidah and Chrétien 1999). Plant subtilases have recently been classified in the pyrolisin subfamily (Siezen and Leunissen 1997). Plant subtilase genes have been isolated from a variety of species including melon (Yamagata et al. 1994), tomato (Meichtry et al. 1999; Jordá et al. 2000), lily (Taylor et al. 1997), rice (Yamagata et al. 2000; Yoshida and Kuboyama, 2001), and Arabidopsis (Berger and Altman 2000; Neuteboom et al. 1999; Ribeiro et al. 1995; Tanaka et al. 2001). In Arabidopsis, 59 putative subtilase genes have been found in the whole genome sequence. The precise function of plant subtilase genes is only known for two

Corresponding author : Didier Bogusz; E-mail: bogusz@mpl.ird.fr.

Arabidopsis genes, Ale1 and Sdd1, which are involved in epidermal surface formation and stomatal density and distribution, respectively (Tanaka et al. 2001; Berger and Altman, 2000). Different functions have been proposed for other subtilases, including fruit ripening (Yamagata et al. 1994), response to pathogen attack (Jordá et al. 1999), cell wall loosening associated with lateral root development (Neuteboom et al. 1999), and microsporogenesis (Taylor et al. 1997). In tomato, a 50-kDa protein immunologically related to subtilases involved in the maturation of systemin has been identified (Schaller and Ryan, 1994).

In an earlier study, we identified *cg12*, a *C. glauca* nodule-subtilase gene homolog of *A. glutinosa ag12* (Laplaze et al. 2000b). Based on in situ hybridization, we found that *cg12* was expressed in young infected cells of the infection zone. In this work, the regulation of *cg12* and its possible role in the infection of nodules were studied, using transgenic *Casuarinaceae* plants carrying *cg12-gus* and *cg12-gfp* fusions. We found that *cg12* was induced in root hairs and in root and nodule cortical cells containing growing infection threads. We also studied *cg12* expression during mycorrhizae formation. In addition, we studied *cg12-gus* and *cg12-gfp* expression in transgenic *Casuarina* and *Arabidopsis* plants following different phytohormone treatments. In all cases, we found that *cg12* expression was not induced. These results indicate that expression of *cg12* is highly specific to actinorhizal symbioses and that the plant hormones tested are not directly involved in the activation of *cg12* expression.

RESULTS

Isolation and computer analysis of the *cg12* 5′ upstream region.

The *cg12* cDNA (Laplaze et al. 2000b) was used to amplify and clone a 1.5-kb fragment corresponding to the *cg12* 5′ upstream region, using the genome walking system. The sequence of the entire fragment was determined and is shown in Figure 1. This sequence overlaps with the first 221 nucleotides of *cg12* cDNA and with 11 coding codons corresponding to the N-terminal region of CG12 protein. Putative TATA and CAAT boxes situated, respectively, 211 bp and 275 bp upstream of the ATG initiation codon were observed. Analysis of the *cg12* promoter revealed the presence of several motifs. The consensus motif CTCTT found here has been shown to be involved in nodule-specific leghemoglobin expression and is conserved in legumes and nonlegume nodule-specific genes (Sandal et al. 1987), including in the promoters of the *C. glauca* hemoglobin (Andersson et al. 1997) and the *cgMT1* metallothionein genes (Laplaze et al. 2002). Moreover, a number of DNA motifs (P and SBF-1) that have been shown to contribute to the function of flavonoid biosynthetic gene promoters were found (Grotewold et al. 1994; Lawton et al. 1991).

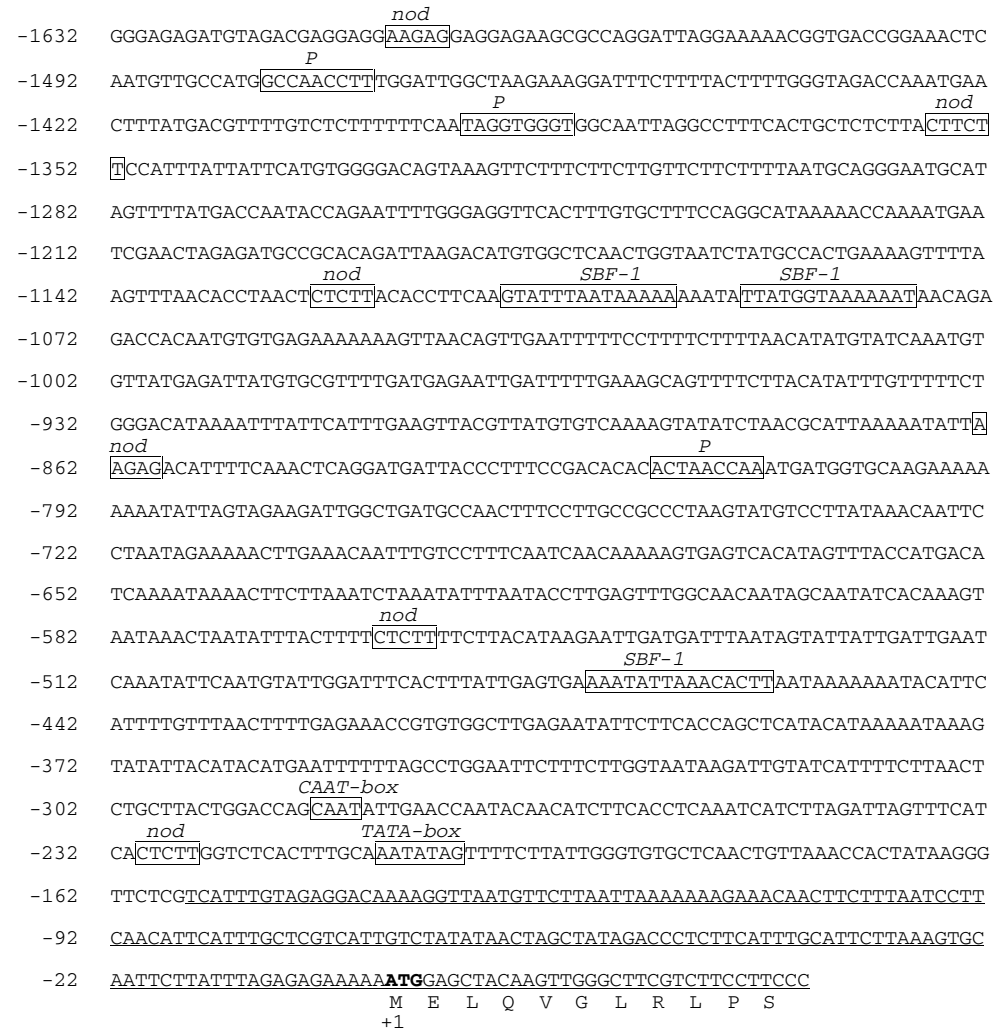

Fig. 1. Nucleotide sequence of the *cg12* 5′ upstream region. Numbering is with respect to the first base of the ATG codon (+1, in bold). The putative TATA box and CAAT box, the nodule specific motif (CTCTT), P, and SBF-1 boxes are framed. Sequence overlapping the 221 first bases of *cg12* cDNA is underlined.

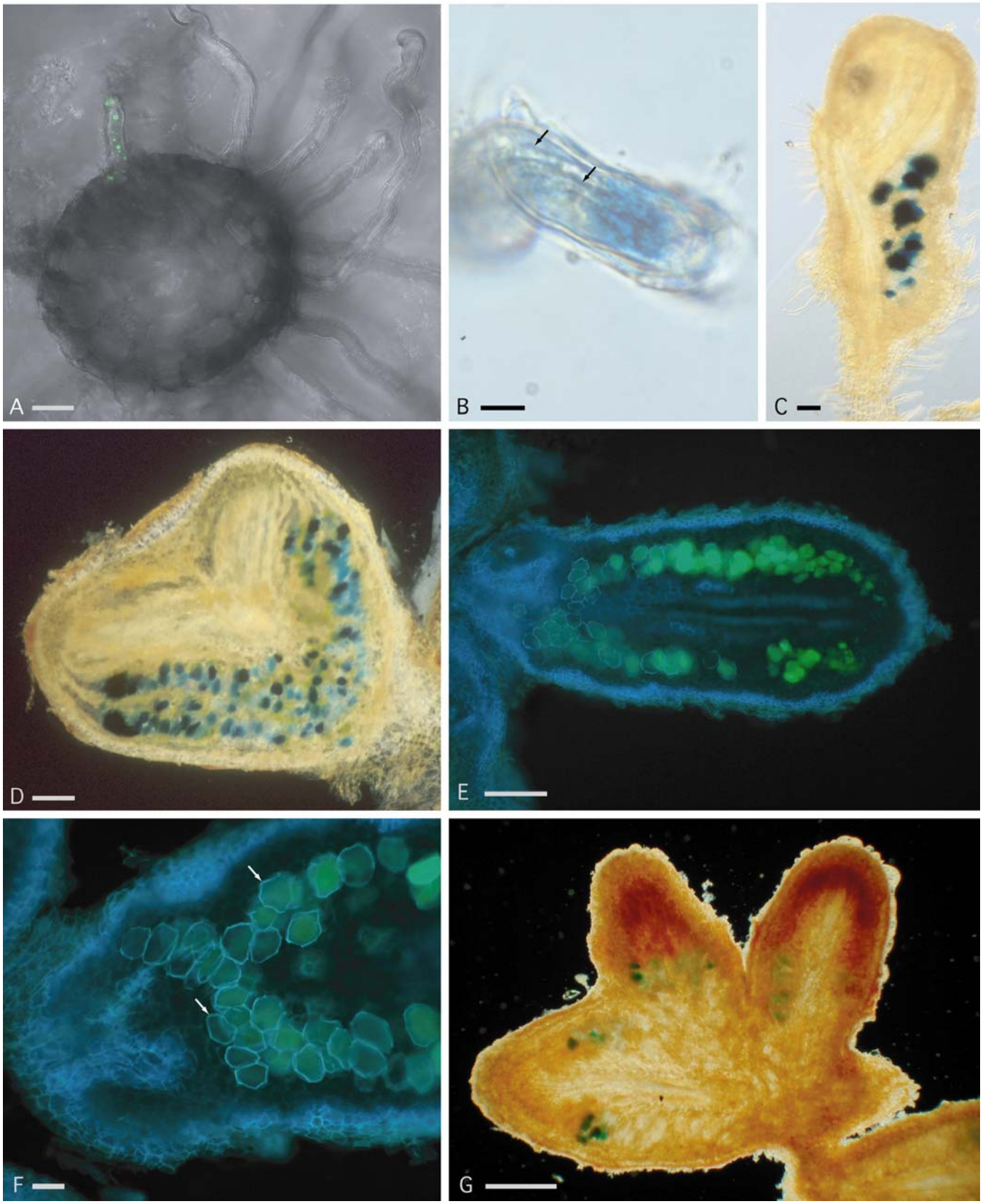

Fig. 2. Analysis of reporter gene expression in transgenic *Casuarinaceae*. **A,** Transverse section of an *Allocasuarina verticillata* lateral root 20 days after inoculation by *Frankia* showing green fluorescent protein fluorescence in a deformed root hair. Bar = 100 μm. **B,** Detail of a *Casuarina glauca* root hair showing GUS activity. Arrows indicate *Frankia* filaments. Bar = 5 μm. **C,** Longitudinal section of a *C. glauca* prenodule with a young nodular lobe. Bar = 100 μm. **D,** Longitudinal section of a young *C. glauca* nodule. Bar = 200 μm. **E,** Longitudinal section of a mature *A. verticillata* nodule observed under UV blue light. Bar = 200 μm. **F,** Detail of a longitudinal section of a mature *A. verticillata* nodular lobe. Arrows indicate lignified autofluorescent cell walls. Bar = 50 μm. **G,** Longitudinal section of a 6-month-old *A. verticillata* nodule. Bar = 250 μm.

Expression of *cg12* promoter gus and *gfp* fusions during *Frankia*-induced nodule development.

Transgenic *Casuarinaceae* have become a major tool to study symbiotic interaction with *Frankia* (Franche et al. 1998b; Laplaze et al. 2000a, 2002). In order to monitor the activity of the *cg12* promoter during the early stages of *Casuarina* infection by *Frankia*, transcriptional fusions of the 5′ upstream region with *gus* and *gfp* reporter genes were constructed. The chimeric genes were introduced in *A. verticillata* and *C. glauca* by *Agrobacterium*-mediated transformation (Franche et al. 1997; Smouni et al. 2002). Independent *A. verticillata* transgenic lines containing the *cg12-gus* and the *cg12-gfp* construction (45 and 35, respectively) were generated. For *C. glauca*, two independent transgenic lines containing the *cg12-gus* construct were obtained. The expression of the fusion genes was detected in nodules for two to five plants regenerated from each of the 82 independently transformed calli by histochemical GUS and green fluorescent protein (GFP) assays (Fig. 2). Reporter gene expression was further studied in at least five plants from 10 independent transgenic lines. The patterns of reporter gene expression were comparable in all the transgenic plants examined. Similar results were obtained with transgenic *A. verticillata* and *C glauca* when *gfp* or *gus* reporter genes were used.

No GUS staining or GFP fluorescence could be detected in control nontransformed plants or in nonsymbiotic tissues of transgenic *A. verticillata* and *C. glauca* (data not shown).

After inoculation by nodulating *Frankia*, a typical pattern of root hair deformation (Torrey 1976) was observed. Two weeks after inoculation with *Frankia,* rare deformed root hairs of *A. verticillata* (Fig. 2A) and *C. glauca* (Fig. 2B) showed reporter gene expression. These root hairs were always located on lateral roots, were long and deformed, and contained *Frankia* filaments (Fig. 2A and B).

Three weeks after inoculation, *gus* and *gfp* expression was found in prenodules. Figure 2C shows strong GUS activity in large infected prenodule cells. Reporter gene activity was further studied by examining sections of *C. glauca* and *A. verticillata* nodules. As shown in Figure 2D, the longitudinal section of a young *C. glauca* nodule lobe revealed that the *cg12-gus* gene was most active in the apical part of the nodule lobe, which corresponds to the infection zone (zone II) in which *Frankia* isolates had infected some of the new cells derived from the meristem. In mature nodule lobes, reporter gene activity was observed throughout the cortical cells that correspond to the *Frankia*-containing cells (Fig. 2E). However, toward the base of the nodule lobe, reporter gene expression progressively decreased; a micrograph taken under UV-blue light showed that the decrease in GFP fluorescence was accompanied by an increase in cell-wall lignification (Fig. 2E and F). Since the lignification of the cell walls is a cytological marker of differentiation of nodule cells for bacterial nitrogen fixation (Berg and McDowell 1987), we conclude that the activity of the *cg12* promoter is correlated with *Frankia* infection of the plant cell and slows down when nitrogen fixation starts. In old nodule lobes, reporter gene activity was weak and was occasionally detected in a few cortical cells near the apical pole (Fig. 2G). In nodules, no reporter gene activity was detected in the meristem, the uninfected cortical cells, or the vascular system.

These results are consistent with and extend previous analyses of *cg12* gene expression in the *C. glauca* prenodule and nodule (Laplaze et al. 2000a and b). Here, we show that *cg12* expression can be detected at a very early stage of the symbiotic interaction, namely in infected root hairs.

Effect of *Frankia* strain specificity and diffusible factors on *cg12* expression.

In order to investigate if *cg12* expression was specifically induced by infective strains, we inoculated transgenic *A. verticillata* roots with the Ac50 1 *Frankia* strain isolated from *Alnus cordata.* Ac50 1 did not induce root hair deformation and was not able to form nodules on *A. verticillata* (data not shown). Reporter gene expression could not be detected on the root system over a period of two months after inoculation, thus suggesting that only infective *Frankia* strains can induce *cg12* expression in the host plant.

In legumes, a number of plant genes have been identified whose transcription can be specifically elicited in root tissues in response to applications of Nod factors (Miklashevichs et al. 2001). We used Nod factors produced by the most promiscuous known *Rhizobia* strain NGR234 (provided by W. J. Broughton) to test whether we could induce *cg12* expression. Treatment of transgenic *A. verticillata* roots over a 1-month period with several concentrations (1 nM to 1 μM) of NGR234 Nod factors did not induce root hair deformation and expression of the *cg12-gfp* gene fusion.

An exchange of molecular signals must occur early during the infection process. The chemical nature of the signals exchanged is unknown to date, but a diffusible root hair deforming factor, which is different from Nod factors, is produced by *Frankia* cells (Cérémonie et al. 1999). In order to study the effect of *Frankia* diffusible factors on *cg12* expression, roots of transgenic *A. verticillata* plants were exposed to a filtrate of a culture of the *A. verticillata* infective strain Allo2. The root hair deformation induced by the culture filtrate was similar to that induced by the bacteria (data not shown). However, no GFP fluorescence or GUS staining was detected over a two-month period suggesting that *cg12* expression is not induced by a diffusible signal produced by *Frankia*.

Table 1. Treatments performed on transgenic *Allocasuarina verticillata*

Treatment	Source	Final concentration
1,4-dichlorophenoxyacetic acid (2,4D)	Sigma-Aldrich, Diesenhofen, Germany	1 μM (solution on roots)
		10 μM (solution on roots)
Methyl jasmonate (MeJa)	Interchim, Montluçon, France	10 μM (solution on roots)
		10 μl in 3-l tight box
Gibberellic acid (GA)	Sigma-Aldrich	10 μM (solution on roots)
Benzylaminopurine (BA)	Sigma-Aldrich	10 μM (solution on roots)
		100 μM (solution on roots)
Cis-trans-abcissic acid (ABA)	Sigma-Aldrich	10 μM (solution on roots)
Salicylic acid (SA)	Prolabo, Paris	1 mM (solution on roots)
		1 mM with 0.01% (vol/vol) Silwett L77 (spray)
N-1 Naphthylphthalamicacid (NPA)	Sigma-Aldrich	10 μM (solution on roots)
		100 μM (solution on roots)
Sinorhizobium fredii NGR234 Nod factors	W. J. Broughton	1 μM (solution on roots)
		1 μM (solution on roots)
Mas-7	BioMol Research Labs, Plymouth Meeting, PA, U.S.A.	0.2 μM (solution on roots)
		2 μM (solution on roots)

Since cross-talk between the bacteria and the plant must occur during the symbiotic interaction, we wondered whether *cg12* might be induced by a *Frankia* diffusible factor produced in response to a plant signal. To test this hypothesis, a dialysis membrane containing a culture of *Frankia* strain Allo2 was placed in contact with the root of *cg12-gus* and *cg12-gfp* transgenic *A. verticillata* plants. Once again, root hair deformation was observed but no *gfp* or *gus* expression could be detected in the deformed root hairs.

Therefore, *cg12* expression is specifically induced by infective *Frankia* strains. Diffusible molecules <6 to 8 kDa produced by *Frankia* are not able to induce *cg12* expression, even if they can induce root hair deformation. Taken together, these results suggest that *cg12* is a marker of plant cell infection and that its expression is linked to close contact with *Frankia*.

Effect of mycorrhization on *cg12* expression.

In order to analyze whether *cg12* expression was induced during other symbiotic interactions, we examined reporter gene expression in transgenic *A. verticillata* roots in response to ectomycorrhizal (ECM) and endomycorhizal (ENM) fungal inoculation. ECM roots started to appear one week after inoculation with the ECM fungus *Pisolithus alba* (data not shown). ENM colonization was detected in transgenic roots one month after inoculation (data not shown). Transgenic *A. verticilla* plants (10 and 14, respectively) were used for ecto- and endomycorrhization. No reporter gene expression was detected over a two-month period on roots where mycorrhizal structures were detected (data not shown). These results suggest that *cg12* is not induced during mycorrhizal symbioses.

Effect of hormonal treatments and signaling molecules on *cg12* expression.

In order to try to understand the signaling pathway leading to *cg12* expression, transgenic *A. verticillata* plants were treated with various signaling molecules, including plant hormones.

Salicylic and jasmonic acid are signaling molecules linked to plant-microbe interactions. They play an important role in the signaling network of plant defense pathways and are known to induce plant defense responses when applied exogenously. We investigated whether salicylic or jasmonic acid could induce *cg12* expression. Both the root system and the aerial parts of transgenic *cg12-gus* and *cg12-gfp* plants were treated with various concentrations of methyl jasmonate (MeJa) or salicylic acid (SA). Treatment with 1 mM of SA induced necrosis of the aerial tissues resembling an hypersensitive reaction, thus suggesting that the hormone was recognized by the plant (data not shown). Nevertheless, no GFP fluorescence or GUS activity could be detected.

We tested the effect of classical phytohormones (auxin, auxin transport inhibitors, cytokinin, abscissic acid, and giberellic acid) on *cg12-gfp* and *cg12-gus* expression, because several results indicate that phytohormones play a central role in legume and actinorhizal root nodule symbioses (Hirsch, 1992). Within one week after auxin treatment, a reduction in root growth and the induction of many lateral roots were observed. One month after treatments with cytokinin or auxin, pseudonodule formation occurred, whereas transport inhibitor application resulted in a continuous growth of the primary roots without branching (data not shown). The treatments listed in Table 1 failed to induce reporter gene expression in transgenic *A. verticillata* plants.

Table 2. Treatments performed on transgenic *Arabidopsis thaliana*

Treatment	Final concentration
1,4-dichlorophenoxyacetic acid (2,4D)	1 μM
	10 μM
Methyl jasmonate (MeJa)	10 μM
Gibberellic acid (GA)	10 μM
Benzylaminopurine (BA)	10 μM
Salicylic acid (SA)	10 μM
	100 μM

Recently, G proteins have been shown to participate in Nod factor signaling in the *Medicago-Rhizobium* symbiosis (Pingret et al. 1998). We tested the effect of a synthetic mastoparan analogue (MAS-7), a known G protein agonist (Pingret et al. 1998), on induction of *cg12* promoter. When transgenic *A. verticillata* plants were treated with various concentrations (0.2 to 2 μM) of MAS-7 for 2 h to 2 days, no reporter gene expression could be detected, suggesting that G proteins are not directly involved in the signal transduction pathway leading to *cg12* expression.

The *cg12* promoter is not active in the nonsymbiotic plant *Arabidopsis thaliana.*

To investigate transduction pathways leading to *cg12* expression using genetic tools and mutants available in the model plant *Arabidopsis thaliana,* we introduced the *cg12-gus* and *cg12-gfp* constructs into *Arabidopsis thaliana* and analyzed the promoter activity of 16 independent transgenic lines during development. No reporter gene expression was detected in tissues at any stage of development. We also investigated whether the *cg12* promoter is inducible by different phytohormones (Table 2) in *Arabidopsis thaliana*. No expression was detected. The absence of reporter gene expression suggests that *Arabidopsis thaliana* transcription factors do not recognize *cis*-acting DNA regions of the *cg12* promoter.

DISCUSSION

cg12 is a *C. glauca* gene encoding a subtilisin-like protease induced during actinorhizal nodule formation. Previous studies have shown by in situ hybridization that *cg12* mRNA localize in infected cells of *C. glauca* prenodules and nodules (Laplaze et al. 2000a and b). Here, we report isolation of the *cg12* promoter and the use of transgenic *C. glauca* and *A. verticillata* plants expressing reporter genes under the control of this promoter to further analyze *cg12* expression. Patterns of reporter gene expression in transgenic prenodule and nodule are consistent with in situ hybridization analyses (Laplaze et al. 2000a and b).

The transgenic approach further revealed that the *cg12* reporter gene construct is activated very early during the infection process. We found reporter gene expression in a few deformed root hairs two weeks after infection. These root hairs were consistently infected by *Frankia* hyphae. Root hair infection in *Casuarina cunninghamiana*, a close relative of *C. glauca* and *A. verticillata,* is a rare event (Torrey 1976), with an average of only one infected root hair in 100. This suggests that the formation of each nodule does not involve multiple infection threads (Torrey 1976). This observation is in agreement with the frequency of appearance of *cg12* promoter activity. Due to the lack of genetic transformation tools or specific fluorescent staining of *Frankia,* we were unable to test whether there were any infected root hairs that do not express *cg12*.

In legumes, the expression pattern of the early nodulin gene *enod5* resembles the one described above for *cg12*. *enod5* is strongly expressed in nodule cells containing a growing infection thread (Scheres et al. 1990), but its expression shuts down as symbiotic cells become fully differentiated and start fixing N_2. *enod5* is strongly expressed prior to nodule formation in root cortical cells containing an infection thread and in root hairs after inoculation with rhizobia (Horvath et al. 1993;

Miklashevichs et al. 2001). However, in contrast with *cg12*, *enod5* expression is also induced in root hairs after a treatment with a diffusible signal, i.e., Nod factors (Horvath et al. 1993). *enod5* codes for an arabinogalactan-like protein and is presumed to be secreted to the extracellular compartment, where it could be involved in short distance cell signaling pathways in response to rhizobia infection (Scheres et al. 1990).

Although root hair deformation occurs about eight hours after inoculation with *Frankia* (data not shown), the first detectable reporter gene activities were detected two weeks later. This suggests that, during early interactions with the bacteria, it takes about two weeks between root hair deformation and root hair infection. *cg12* is the earliest actinorhizal nodulin gene, and the only one known to be expressed in root hairs to date. Moreover, we have shown that it is not induced by the noninfective *Frankia* strain Ac50 1, by infective *Frankia* culture filtrate, or by an infective *Frankia* culture separated from the plant root by a dialysis membrane, even if root hair deformation occurred in those cases. These results, together with the absence of activation of *cg12* promoter by mycorrhizal symbiosis or various signaling compounds, indicate that *cg12* expression is specifically associated with very close contact between the plant cell and *Frankia*. Moreover, the signal transduction pathways leading to *cg12* expression and root hair deformation are different.

The expression of defense-related genes in nodules has previously been reported in legume nodules. For example, it has been shown that, in *Medicago truncatula* nodules, the two pathogenic related genes *MtN1* and *MtN2* are expressed (Gamas et al. 1998). In actinorhizal symbiosis, expression of the cysteine proteinase *AgNOD-CP1* gene in *A. glutinosa*, the *Eleagnus umbellae* chitinase *EuNodCHT2*, and the chalcone synthase *CgCHS* gene in *C. glauca* nodules has also been reported to be part of a defense response to *Frankia* infection (Goetting-Mineski and Mullin 1994; Laplaze et al. 1999; Kim and An 2002). Some subtilases appear to have a role in pathogen attack responses; for example, two tomato subtilase genes have been induced in response to infection with *Pseudomonas syringae* or treatment with salicylic acid (Jordá at al. 1999). Salicylic and jasmonic acids are part of the signal transduction pathways leading to plant defense and disease resistance. Using transgenic *A. verticillata* and *Arabidopsis thaliana,* we were able to show that the *cg12* promoter was not induced by exogenous application of salicylic acid and methyl jasmonate.

Endogenous plant hormones are thought to play major roles in actinorhizal nodule organogenesis. Cytokinin treatment on *Alnus* roots leads to formation of bacteria-free, nodule-like structures called pseudonodules (Hirsch 1992). Inhibitors of polar auxin transport such as NPA (N-1 naphthylphthalamica-cid) or TIBA (triiodobenzoic acid) also induce pseudonodule formation in *Casuarina* spp. In legumes, expression of several early nodulin genes, such as *enod12* and *enod2*, can be elicited by hormone treatments (Hirsch 1992). It has also recently been shown in legumes that *enod12* expression could be induced using mastoparan, a G protein agonist that activates the G protein transduction pathway, thus suggesting a role for G proteins in cell signaling leading to nodule formation (Pingret et al. 1998). Treatment with several different phytohormones and a mastoparan analog, MAS-7, failed to induce *cg12* expression, suggesting that phytohormones and G proteins are not directly involved in the induction of *cg12* expression.

Nod factors play an important role in the process of establishing rhizobium-legume symbiosis (Miklashevichs et al. 2001). A very low concentration of Nod factors can trigger early morphological events leading to the formation of a nodule primordium and early nodulin gene expression—including e*nod5*—even in the absence of rhizobia isolates (Horvath et al. 1993; Miklashevichs et al. 2001). Recently, receptor-like kinases that are required for the transduction of both rhizobia and *mycorrhizae* symbiotic signals have been discovered in several legumes (Stracke et al. 2002). These authors suggest that this receptor is a component of the signaling pathway leading to perception of the rhizobia Nod factors. Application of purified Nod factors from the NGR232 strain also failed to induce *cg12* expression, suggesting that legume Nod factors are not recognized by *Casuarina* spp. as signal molecules leading to *cg12* expression. Previous experiments showed that application of the same Nod factor produces neither root hair deformation nor any morphological reaction in *C. glauca* or *A. glutinosa* roots (C. Santi, *personal communication*; Cérémonie et al. 1999). Structural and functional differences between *Frankia* root hair–deforming factors and NGR232 Nod factors have been found in *A. glutinosa* (Cérémonie et al. 1999), and so far, attempts to complement rhizobia mutants deficient in Nod factor synthesis with *Frankia* DNA have failed (Cérémonie et al. 1998), thus suggesting that actinorhizal and legume-rhizobia symbioses do not share similar signaling molecules.

cg12 expression appears to be associated with plant cell infection by *Frankia*. What could be the function of CG12 in this context? The answer depends on the specificity of CG12 cleavage. If CG12 has poor substrate specificity and belongs to the degenerative class of subtilases, it could participate in general cell wall–associated protein degradation, resulting in cell wall weakening during *Frankia* infection. This could enable infection threads to pass from one cell to another. Alternatively, if CG12 is active in the capsule, it could participate in the loosening of the capsule associated with infection thread growth. If CG12 is a processing subtilase, it could be involved in the maturation of a polypeptide as a part of a signaling cascade in response to *Frankia* infection. In order to better understand *cg12* function in actinorhizal symbiosis, biochemical characterization of the CG12 protein is needed. We are presently attempting to purify CG12 protein in order to determine its cleavage specificity.

MATERIALS AND METHODS

Plant material.

C. glauca seeds were provided by the Desert Development Center (Cairo). *A. verticillata* seeds collected in Australia were purchased from Versepuy Company (Le Puy en Velay, France). *A. verticillata* and *C. glauca* were grown and were inoculated as described (Franche et al. 1997). For stable transformation, *C. glauca* and *A. verticillata* were propagated as previously described (Franche et al. 1997). *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia (Col-0) seeds were obtained from the Nottingham Arabidopsis Stock Center. *Arabidopsis* plants were cultivated in growth chambers at 25°C under 50 $\mu E/m^{-2}/s^{-1}$ provided by cool fluorescent tubes (Gro-Lux; Sylvania OSRAM, Munich, Germany) with a 16-h photoperiod at an average of 45% humidity.

Nodulation and mycorrhization assays.

Frankia strains Allo2 (Franche et al. 1997) and Thr (Laplaze et al. 2002) were used to inoculate *A. verticillata* and *C. glauca*, respectively. *Frankia* strain Ac50 1 (provided by A. Moiroud) isolated from *Alnus cordata* nodules was assayed for *cg12* promoter induction. *C. glauca* and *A. verticillata* were inoculated with *Frankia* isolates as previously described (Franche et al. 1997). The ectomycorrhizal fungi *Pisolithus alba* IR100 was grown on petri dishes containing MNM medium (Marx 1969) at 28°C. For *Pisolithus* inoculations, sterile pieces of paper (2 × 4 cm) were laid on petri dishes containing the fungi. When the pieces of paper were covered with fungus,

they were removed under sterile conditions and put in contact with the root system of the transgenic plants grown as previously described for *Frankia* inoculation (Franche et al. 1997). Sterile spores of the endomycorrhizal fungi *Glomus intraradices* (strain accession number MUCL43194) were purchased from the MUCL Culture collection (Louvain-la Neuve, Belgium;). Ectomycorrhizae formation was assessed under a stereomicroscope. For *G. intraradices* inoculations, plants were grown as previously described for *Frankia* inoculation (Franche et al. 1997) with 17 mg of $(NH_4)_2SO_4$ per liter. Inoculum of *G. intraradices* consisted of about five spores embedded in agarose that were placed on the surface of young lateral roots of the transgenic plants.

Isolation of *cg12* 5′ upstream region and construction of *cg12* promoter-reporter gene fusions.

The *cg12* promoter region was cloned using the Universal Genome Walker kit (Clontech, Palo Alto, CA, U.S.A.), according to the manufacturer's instructions. Primers cg12-Gsp1, 5′-CTGATGCTGAACTACCAGGGAGAGTCAGCA-3′, and cg12-Gsp2, 5′-GGGAAGGAAGACG AAGCCCAACTTGT-AGC-3′, were designed to match the inverse complement of *cg12* cDNA. A 1,583-bp product was amplified from a *C. glauca* DNA library and cloned into pGEM-T (Promega, Madison, WI, U.S.A.), generating cg12-pGEM-T. The *cg12* promoter fragment from *cg12*-pGEM-T was subcloned into plant transformation vectors as follows: two oligonucleotides were synthesized, a 5′ primer (5′-CGGGATCCCGTTTTTC-TCTCTAAATAAGAATTGC-3′), designed to introduce a *Bam*HI site, and a 3' primer (5′- CCCAAGCTTGGGAGAGA-TGTAGACGAGGAGG-3′), designed to introduce a *Hin*dIII site. After PCR amplification, the *Bam*HI/*Hin*dIII fragment was cloned into *Bam*HI/*Hin*dIII-digested pBI101.3 (Clontech) and pBI101.gfp (discussed below) upstream of the reporter gene to yield *pcg12-gus* and *pcg12-gfp*, respectively. Cloning was confirmed by DNA sequence analysis using specific primers. pBI101.gfp was made by replacing the *uidA* gene in pBI101.3 (Clontech) by the 0.6-kb *Bam*HI/*Sac*I *gfp* fragment from pBIN35S-mgfp5ER (Haseloff et al. 1997).

Generation of transgenic plants.

Agrobacterium tumefaciens containing *cg12-gus* or *cg12-gfp* transgenes was used to transform *A. verticillata* and *C. glauca*, as previously described (Franche et al. 1997; Smouni et al. 2002). Transgenic *Arabidopsis thaliana* containing the chimeric reporter genes *cg12-gus* and *cg12-gfp* were generated by *Agrobacterium tumefaciens*–mediated transformation, as previously described (Clough and Bent 1998). Transformants were selected on Murashige and Skoog (MS) agar medium with 50 mg of kanamycin per liter, were transferred to soil, and were allowed to self-pollinate.

Histochemical GUS assays, histochemistry, and microscopy.

A. verticillata and *C. glauca* explants were embedded in 3% agarose and were sliced into 40- to 60-μm-thick longitudinal or transverse sections on a vibratome (Leica VT1000E, Wetzlar, Germany), as previously described (Franche et al. 1998b). For histochemical analysis, explants from *pcg12-gus* transformed *Arabidopsis thaliana, C. glauca*, or *A. verticillata* were stained in a solution containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and were incubated for 16 h at 37°C. $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (2 mM of each) were added as catalysts to limit the extent of the blue staining. Plant samples were fixed for 12 h in a solution containing 5% formaldehyde, 5% acetic acid, and 50% ethanol and were washed several times in 70% ethanol. Whole root segments or sections were observed under a Leica DMRB microscope. Epifluorescence was observed using an A filter (excitation 340 to 380 nm, stop 425 nm, Leica).

Endomycorrhizal fungi were stained as follows: roots were cleared for 10 min at 70°C in 50% KOH, were washed 10 min in acetic acid, were stained in 0.1% trypan blue for 30 min, and were washed several times in H_2O. Endomycorrhizae formation was assessed under a microscope.

Confocal microscopy imaging of GFP was performed on agar-embedded sections of living material, using a Leica DMRXA microscope and Leica TCS SP confocal software. A long pass 500 nm dichroic was used as the beam splitter. Emission maxima were 510 nm for GFP. Bandwidths of 20 to 40 nm were used. Objectives used were Leica 63× PlanApo NA 1.2, 20× PlanApo NA 0.7, and 10× PlanApo NA 0.4.

Application of hormones and signaling molecules.

A. verticillata: Three month-old transgenic plants placed in plastic petri dishes containing 20 ml of solid Hoagland medium (Franche et al. 1997), with the roots lying horizontally on the bottom and the stem passed through a hole pierced in the lid of the dish. The petri dishes were covered with aluminum foil to avoid illumination of the root system. Roots were immersed in 10 ml of liquid Hoagland medium containing the chemical factors listed in Table 1. The aerial parts of some plants were also sprayed with a solution containing 0.01% (vol/vol) Silwett L77 solution (OSI Specialties, Danbury, CT, U.S.A.) and 1 mM SA. MeJa treatments were also performed on rooted plants put in a 3-liter tight box containing a filter paper with 10 μl MeJa.

For mastoparan treatments, plants were removed from Gibson tubes and treated with 0.2 to 2 μM MAS-7, as described by Pingret and associates (1998) (Table 1). Plants were incubated for at least 6 h, and reporter gene expression was monitored over a period of three days.

Arabidopsis: Hormone treatments were performed in liquid culture. Two-week-old transgenic plants were transferred into liquid MS media on a shaker under constant light, containing 10 μM of either 2,4D, BA, GA, ABA, or MeJa or 1 mM SA. Reporter gene expression was monitored for one week after induction treatments.

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