# Infection-Related Activation of the *cg12* Promoter Is Conserved between Actinorhizal and Legume-Rhizobia Root Nodule Symbiosis[1]

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Two nitrogen-fixing root nodule symbioses between soil bacteria and higher plants have been described: the symbiosis between legume and rhizobia and actinorhizal symbioses between plants belonging to eight angiosperm families and the actinomycete Frankia. We have recently shown that the subtilisin-like Ser protease gene *cg12* (isolated from the actinorhizal plant *Casuarina glauca*) is specifically expressed during plant cell infection by Frankia. Here we report on the study of *C. glauca cg12* promoter activity in the transgenic legume *Medicago truncatula*. We found that *cg12* promoter activation is associated with plant cell infection by *Sinorhizobium meliloti*. Furthermore, applications of purified Nod factors and mycorrhizal inoculation failed to trigger expression of the *cg12*-reporter gene construct. This indicates that at least part of the transcriptional environment in plant cells infected by endosymbiotic nitrogen-fixing bacteria is conserved between legume and actinorhizal plants. These results are discussed in view of recent data concerning molecular phylogeny that suggest a common evolutionary origin of all plants entering nitrogen-fixing root nodule symbioses.

Two groups of plants are able to form nitrogen-fixing root nodule symbioses with soil bacteria: legumes (plus Parasponia in the Ulmaceae family) associate with rhizobia, while the so-called actinorhizal plants belonging to eight angiosperm families interact with Frankia. Inside root nodules, bacteria protected and nourished by the plant find a favorable environment for nitrogen fixation and, in exchange, provide the plant with fixed nitrogen. Recent molecular phylogeny studies based on the chloroplast gene *rbcL* indicate that plants entering rhizobial or actinorhizal symbioses belong to the same clade (Rosid I; Soltis et al., 1995), suggesting that a predisposition to form nitrogen-fixing root nodule symbioses originated once in the history of flowering plants. However, the nature of this predisposition remains unknown.

Depending on the plant species, bacteria infect the root either by root hair infection or through cellular spaces between epidermal cells (crack entry). Root hair infection is characteristic of most temperate legumes and of several actinorhizal genera like Alnus and Casuarina. In this case, bacteria induce a localized degradation of the cell wall of the root hair; the plasma membrane then invaginates leading to the formation of a tubular structure called the infection thread (IT). ITs are filled with bacteria and surrounded by newly deposited cell wall material and spread bacteria by growing inside plant cells and from one cell to another. Whereas actinorhizal ITs never release the bacteria, in most legume species, the ITs that reach the nodule release bacterial cells that then differentiate into bacteroids and start fixing nitrogen (Pawlowski and Bisseling, 1996).

In legume-rhizobia symbioses, secreted bacterial Nod factors play an essential role by mediating specific recognition between the two partners and activating a series of responses involved in nodule formation (Lerouge et al., 1990; Denarié and Cullimore, 1993; Downie and Walker, 1999). Among these responses is the transcription of the so-called nodulin genes that are specifically transcribed in symbiotic tissues and may participate in the establishment of the symbiosis (Schultze and Kondorosi, 1998). Some of these nodulin genes are also activated in response to endomycorrhizal colonization of roots (Albrecht et al., 1998), and the analysis of plant mutants indicates that the signaling pathways involved in legume-rhizobia and mycorrhizal symbioses at least partially overlap (Duc et al., 1989; Wegel et al., 1998; Endre et al., 2002; Stracke et al., 2002). These results suggest that at least

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some of the molecular mechanisms of root nodule symbioses may have been recruited from the more ancient and widespread mycorrhizal symbiosis. As in legumes, several genes specifically induced during actinorhizal symbioses have been described, but little information concerning the signaling pathways involved in their regulation is available (Franche et al., 1998a).

Among actinorhizal nodulin genes, *cg12* is one of the earliest induced after Frankia inoculation. This gene was isolated from the actinorhizal tree *Casuarina glauca* and encodes a subtilisin-like Ser protease (Laplaze et al., 2000). Using transgenic Casuarinaceae containing *cg12* promoter reporter gene fusions, we recently showed that *cg12* expression is specifically linked to the infection of root hairs and cortical cells by Frankia and that *cg12* could therefore be used as a marker gene of the early events that occur during cell infection by bacteria in actinorhizal plants (Svistoonoff et al., 2003). Moreover, *cg12* expression is not induced in endo- or ectomycorrhizae nor by Frankia root hair deforming factors (Svistoonoff et al., 2003). Here we show that the *cg12* promoter directs reporter gene expression specifically in rhizobia-infected cells in transgenic *Medicago truncatula* plants. This promoter is not activated either by infection by an endomycorrhizal fungus or by Nod factors. This indicates that a plant cell infection-related transcriptional environment is conserved between actinorhizal and rhizobial symbioses.

## RESULTS

### The *C. glauca cg12* Promoter Retains Its Cell-Specific Expression in Transgenic *M. truncatula*

To test whether or not regulatory sequences in the *cg12* promoter were recognized by legume transcription factors, transcriptional fusions between the *cg12* promoter and *gus*- or *gfp*-reporter genes were introduced in *M. truncatula*. Reporter gene expression was similar in four independent transgenic lines. No *gus* or *gfp* expression was detected in shoots and leaves. In noninoculated roots, no green fluorescent protein (GFP) fluorescence was detected; however, slight $\beta$-glucuronidase (GUS) activity was present in the pericycle around the vascular tissues (data not shown). This activity was very slight as the blue color could be seen only after >6 h of incubation and then only in small amounts (<0.5 mM) of FeCN. After *Sinorhizobium*

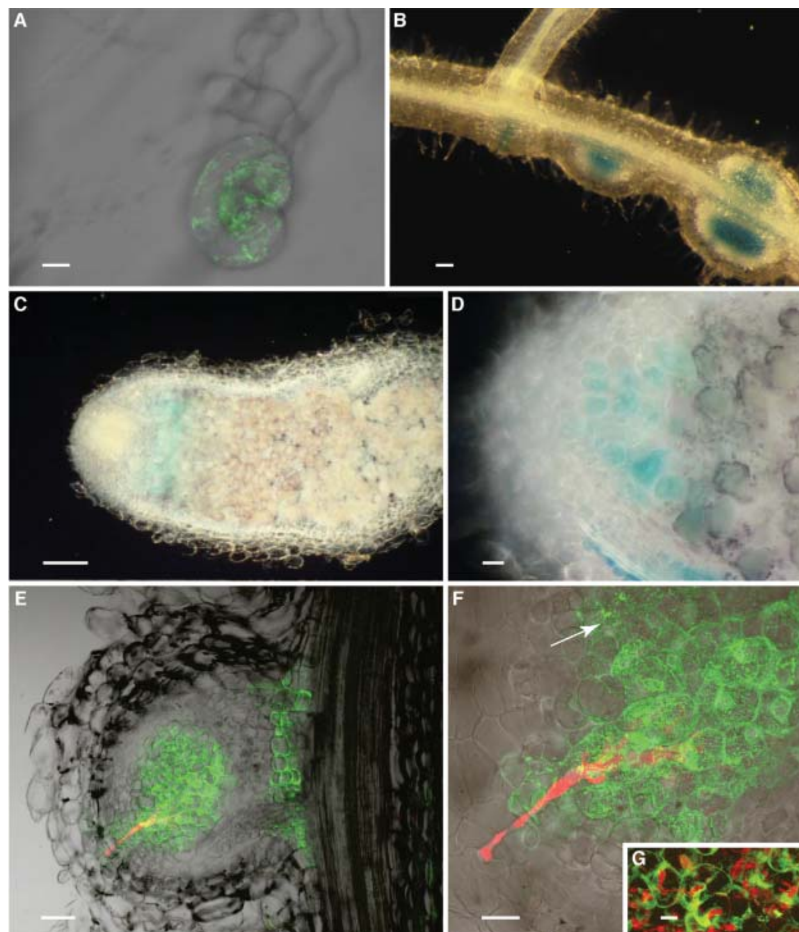


**Figure 1.** Analysis of reporter gene expression in transgenic *M. truncatula*. A to D, Plants inoculated with wild-type *S. meliloti*. A, Composite image of a root hair 10 dai showing GFP fluorescence. B, Lateral root showing three early developmental stages of nodule formation. C and D, Longitudinal sections of mature nodules. To determine different nodule zones, starch granules that accumulate in the interzone II-III were stained with Lugol's solution. E to G, Composite images of longitudinal sections of young nodules obtained 10 to 12 dai with transgenic *S. meliloti* constitutively expressing DsRed. GFP fluorescence is seen in green; DsRed fluorescence is seen in red. F, Detail of E showing the progression of the IT inside the nodule and the consecutive GFP fluorescence in the infected cells. Note that some cells not yet invaded by bacteria already show weak levels of GFP fluorescence (arrow). G, Cells infected by ITs strongly expressing GFP. A, Bar = 10 $\mu$m; B, D, and E, bar = 50 $\mu$m; C, bar = 500 $\mu$m; F and G, bar = 20 $\mu$m.

*meliloti* inoculation, root hair deformation occurred 24 h postinoculation, but the pattern of reporter gene expression did not change until 3 to 8 d postinoculation, when some deformed root hairs (1 out of 100–200) began to strongly express the reporter genes (Fig. 1A). Two to 3 d later, as a nodule primordium was formed beneath these root hairs, reporter gene expression was detected in the central tissues of the developing nodule (Fig. 1B). Strong reporter gene expression was then detected in bacteria-infected cells in the nodule primordia and in small nodules (Fig. 1B). In older nodules, reporter gene expression was detected in the infection zone (Fig. 1C) and decayed in the interzone II-III, as marked by starch accumulation, and in the fixation zone where cells begin to differentiate and fix nitrogen (Fig. 1, C and D). Infection with a *S. meliloti* strain that constitutively produces DsRed showed that all the cells containing ITs strongly express *gfp* (Fig. 1, E–G). GFP fluorescence was also detected in some uninfected cells close to the newly infected cells (Fig. 1, E and F). Consequently, the *cg12* promoter is activated in cells infected or just about to be infected by the endosymbiotic bacteria. This pattern of expression is similar to the one observed with the same constructs in transgenic actinorhizal plants (Svistoonoff et al., 2003).

To investigate whether the *cg12* promoter is also activated during arbuscular mycorrhizal (AM) symbiosis, transgenic *M. truncatula* were inoculated with the endomycorrhizal fungi *Glomus mossaeae*, *Glomus rosea*, and *Glomus deserticola*. GUS activity was not detected in mycorrhized roots (data not shown), suggesting that the *cg12* promoter is not activated in fungi-infected cells during AM formation.

### Nod Factors Are Necessary But Not Sufficient for *cg12* Promoter Activation in Transgenic *M. truncatula*

To determine whether Nod factor recognition is involved in *cg12* promoter activation, roots from transgenic *M. truncatula* plants were treated either with wild-type or a *nodA* mutant *S. meliloti* strain or purified *S. meliloti* Nod factors or water (control plants). The *nodA* mutant used in this study carries a polar mutation that abolishes synthesis of the three enzymes encoded by the *nodABC* operon that are responsible for elaborating the Nod factor core structure and is therefore unable to produce Nod factors. Inoculated roots were removed 2 or 7 d after inoculation (dai). GUS activity was analyzed on one-half of the samples. The rest of the inoculated roots were used to test the endogenous *MtENOD20* expression using a reverse transcription (RT)-PCR approach. *MtENOD20* is rapidly activated by infection and purified Nod factors (Vernoud et al., 1999) and was therefore used as a positive control. The results of both tests are summarized in Table I.

When plants were inoculated with wild-type *S. meliloti*, GUS activity was seen in some curled root hairs (Fig. 2A) and young nodule primordia 7 dai but not 2 dai. *ENOD20* expression was detected at both 2 and 7 dai (Fig. 2B). Control plants (treated with water) showed neither GUS activity (data not shown) nor *ENOD20* expression (Fig. 2B). No GUS activity nor *ENOD20* expression was found in root hairs or cortical cells following treatment with the *nodA* mutant *S. meliloti* strain. This shows that a *S. meliloti nodA* mutant unable to produce Nod factors is unable to activate *cg12* promoter. On the other hand, no GUS activity was found in transgenic plants treated with purified *S. meliloti* Nod factors (data not shown), whereas *ENOD20* expression was detected (Fig. 2B), therefore showing that the plants reacted properly to Nod factors. Taken together, these results indicate that Nod factors are necessary but not sufficient for *cg12* promoter activation in transgenic *M. truncatula* plants. Some GUS activity in the pericycle around the vascular tissues was seen for all treatments (data not shown).

### *cg12* Promoter Is Induced after Inoculation with *S. meliloti exoH* Mutant Defective in Exopolysaccharide Synthesis

Since *cg12* promoter activation is linked to plant cell infection by ITs, we addressed the question of whether an *S. meliloti* mutant that forms aborted ITs still elicits activation of the *cg12* promoter. After inoculation of transgenic plants with the *exoH* mutant, root hair deformation occurred and was followed by cortical cell divisions as described (Yang et al., 1992). Six to 10 dai, small nodule-like structures devoid of bacteria

**Table I.** *GUS activity and MtENOD20 expression in Pcg12-GUS transgenic plants following treatment with wild-type or nodA mutant S. meliloti strains or purified Nod factors*

| Treatment | Days After Inoculation | | | |
|---|---|---|---|---|
| | 2 | | 7 | |
| | GUS[a] | MtENOD20[b] | GUS[a] | MtENOD20[b] |
| Noninoculated (water) | − | − | − | − |
| *S. meliloti* Rm2011 (wild type) | − | + | + | + |
| *S. meliloti* RM2011 nodA::Tn5 | − | − | − | − |
| *S. meliloti* Nod factors ($10^{-7}$M) | − | + | − | + |

[a]−, No GUS staining; +, GUS staining detected in some root hairs or cortical cells. [b]−, No MtENOD20 expression; +, MtENOD20 expression in the inoculated roots.

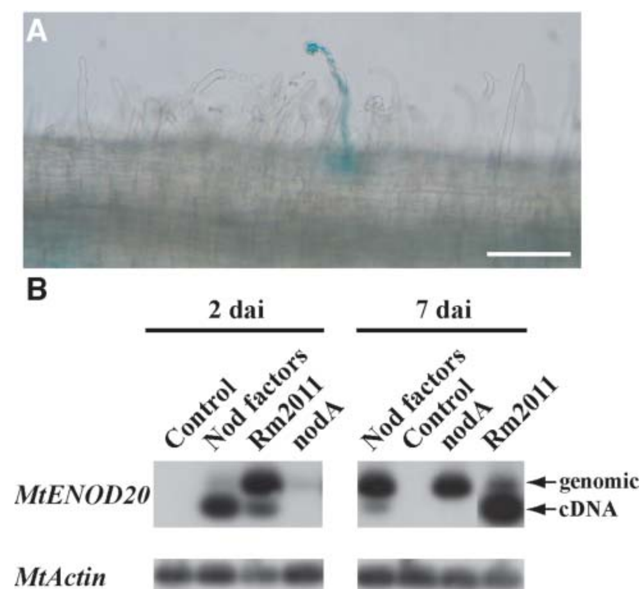


**Figure 2.** Analysis of *Pcg12::GUS* and endogenous *MtENOD20* expression in transgenic *M. truncatula* plants inoculated with wild-type (Rm2011), mutant (*nodA*) *S. meliloti*, purified Nod factors, or water (control) 2 and 7 dai. A, Detail of a root 7 dai with *S. meliloti* Rm2011 showing GUS activity in a deformed root hair and the adjacent cortical cells. B, Transcriptional activation of *MtENOD20* expression 2 and 7 dai. Inoculated roots were harvested, total RNA extracted, and *MtENOD20* expression analyzed by RT-PCR. *MtENOD20* primers are situated on both sides of the single *MtENOD20* intron (Vernoud et al., 1999) so that cDNA and genomic amplification products can be separated. Amplification of a constitutive actin cDNA (*MtActin*; Cohn et al., 2001) was used as a positive control. A, Bar = 100 $\mu$m.

appeared but did not evolve any further. Reporter gene activity was first seen in some deformed root hairs (Fig. 3A) then in cortical cells and in young nodule-like structures (Fig. 3, B and C). However, reporter gene expression was transient, and 2 weeks after inoculation reporter gene expression was no longer detected in the bacteria-free nodules.

### Putative *cg12* Homologs Are Expressed in *M. truncatula* Nodules

To find homologs of *cg12* in *M. truncatula*, we performed a BLAST search on *M. truncatula* cluster expressed sequence tag (EST) database (http://medicago.toulouse.inra.fr/Mt/EST). Many ESTs corresponding to subtilases and highly homologous to *cg12* were found. The expression pattern of the 25 genes most similar to *cg12* (Table II) was analyzed in silico using the iESTANT electronic northern facility (Journet et al., 2002). Four of those genes (MtC50058_GC, MtC10895_GC, MtD15912_GC, and MtC93070_GC) were predicted to be expressed in nodules or rhizobia-infected roots (data not shown) and might therefore represent orthologs of *cg12*.

## DISCUSSION

Infections of the legume *M. truncatula* by *S. meliloti* and the actinorhizal tree *C. glauca* by Frankia are morphologically very similar. The intracellular infection pathway shared by both plants begins with the penetration of bacteria through a deformed root hair. Invagination of the plasma membrane leads to the formation of an IT that progresses from one cell to another. In both symbiotic systems, the cells that are going to be infected by an IT undergo rearrangements leading to the movement of the nucleus toward the center of the cells and the formation of a phragmoplast-like structure, the preinfection thread (PIT), through which the IT can grow (Berg, 1999; Gage and Margolin, 2000). Our results suggest that activation of the *cg12* promoter in *M. truncatula* is strongly associated with plant cell infection: reporter genes are expressed in cells containing ITs and in cells located near infected cells that presumably contain PITs. A similar expression pattern has been described for the legume early nodulin gene *Enod5*. Pea (*Pisum sativum*) *Enod5* expression is detected in root 24 h after rhizobia inoculation (Albrecht et al., 1998) and in nodule cells recently invaded by rhizobia (Scheres et al., 1990). In contrast with other early-induced nodulin genes such as *Enod12* or *Enod40*, *Enod5* expression is mainly detected in cells that contain bacteria and is only slightly induced by Nod factor treatments (Heidstra et al., 1997). On the other hand, *Enod5* expression is induced in AM roots (Albrecht et al., 1998), while *cg12* is not. A factor produced in response to cell infection and acting over a short distance has been proposed to regulate *Enod5* expression (Scheres et al., 1990). Such a factor could also be involved in the regulation of *cg12* expression. In legumes, most early nodulin genes (such as *MtEnod11*, *Enod12*, *Enod12A*, and *Enod20*) respond to purified Nod factor treatments (Spaink, 1996; Niebel Fde et al., 1998). In this study, we showed that Nod factors are necessary but not sufficient to activate the *cg12* promoter. Interestingly, the same is true for PIT formation in alfalfa (*Medicago sativa*), a very close relative of *M. truncatula* (Timmers et al., 1999). On the other hand, Nod factors induce PIT structures in pea (Yang et al., 1994).

In a recent study, we showed that in *C. glauca*, the activation of *cg12* promoter is also strongly linked to plant cell infection by Frankia both in root hairs and in root and nodule cortical cells (Svistoonoff et al., 2003). This suggests considerable similarity between the transcriptional environments activated in response to plant cell infection by bacteria in both symbiotic systems. Similar results were obtained for hemoglobin genes whose expression is induced later in nodule development. The promoter of the soybean (*Glycine max*) *lbc3* gene retains its cell specificity in transgenic *Allocasuarina verticillata* (Franche et al., 1998b) and so does the *C. glauca* promoter in transgenic *Lotus japonicus* (Jacobsen-Lyon et al., 1995).

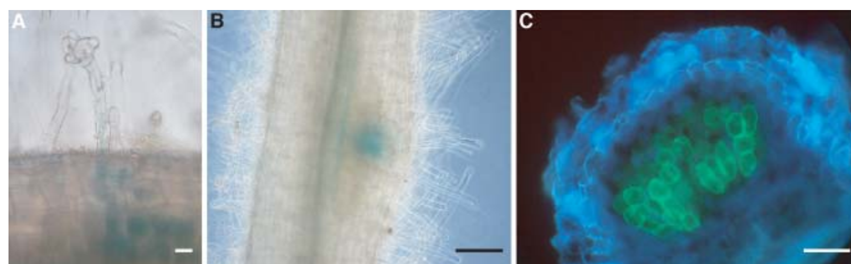


**Figure 3.** Analysis of reporter gene expression in transgenic *M. truncatula* plants inoculated with the Rm7225 *exoH* mutant. A, Detail of a lateral root showing GUS activity in a deformed root hair and in cortical cells beneath 10 dai. B, Nodule primordia 12 dai showing GUS activity in the pericycle and in primordium cells. C, Nodule-like structure obtained 2 weeks after inoculation showing GFP fluorescence in central parenchyma cells. A, Bar = 10 $\mu$m; B, bar = 60 $\mu$m; C, bar = 20 $\mu$m.

In *M. truncatula*, *cg12* promoter activity also occurred in pericycle cells located in front of a growing nodule primordium, whereas in *C. glauca* no expression in the pericycle cells was detected. The pericycle cells of the two systems behave differently in response to bacterial infection: in *C. glauca* the nodule primordia are formed in the pericycle, while in *M. truncatula* nodule primordia arise in the inner cortex. During the formation of legume nodule primordia, pericycle cells may be activated to enter the cell cycle (e.g. formation of nodule vasculature). *cg12* activation may be related to this cell activation in a way similar to that in cells that contain PITs, which were also shown to reenter the cell cycle (Yang et al., 1994). Alternatively, it is possible that the very high levels of reporter gene expression in *M. truncatula* in comparison to *C. glauca* allowed the detection of slight activation in *M. truncatula* that was too low to be detected in *C. glauca*.

The exopolysaccharide succinoglycan (EPS) has an important signaling function during the infection of legumes by their rhizobial endosymbiont. *exo* mutants deficient in EPS synthesis are inefficient at initiating and extending ITs (Gage and Margolin, 2000; Cheng and Walker, 1998). The involvement of bacterial EPS in *cg12* promoter activation was assessed using the *exoH* mutant that produces symbiotically nonfunctional high-molecular-weight EPS that lacks the succinyl modification. The *exoH* mutant is able to elicit root hair deformation, division of cortical cells, and even the formation of small nodule-like structures that are not colonized by bacteria (Cheng and Walker, 1998). ITs are initiated but fail to elongate and abort before reaching the middle of root hairs (Cheng and Walker, 1998). After inoculation with *exoH* mutant, the *cg12* promoter is transiently activated in root hairs and nodule primordia in transgenic *M. truncatula*. This

**Table II.** *BLAST search results*

CG12 protein sequence was compared to cluster *M. truncatula* consensus EST database.

| Rank | Name | BLAST Score | Expect Value | Identity | Positive | Gap |
|---|---|---|---|---|---|---|
| | | | | % | % | % |
| 1 | MtC50058_GC | 1,424 | 1.6e-146 | 41 | 60 | 3 |
| 2 | MtC10895_GC | 654 | 1.2e-124 | 39 | 56 | 4 |
| 3 | MtC10895_GC | 584 | 1.2e-124 | 43 | 62 | 1 |
| 4 | MtC40177_GC | 1,188 | 1.6e-121 | 41 | 57 | 3 |
| 5 | MtC40177_GC | 639 | 2.4e-63 | 39 | 58 | 5 |
| 6 | MtD05536_GC | 646 | 4.2e-119 | 65 | 78 | 0 |
| 7 | MtD05536_GC | 543 | 4.2e-119 | 51 | 72 | 3 |
| 8 | MtC45210_GC | 513 | 5.6e-85 | 39 | 57 | 3 |
| 9 | MtC45210_GC | 394 | 5.6e-85 | 39 | 59 | 2 |
| 10 | MtD04916_GC | 463 | 3.6e-83 | 50 | 70 | 2 |
| 11 | MtD04916_GC | 398 | 3.6e-83 | 55 | 71 | 1 |
| 12 | MtC60376_GC | 444 | 3.1e-80 | 38 | 56 | 4 |
| 13 | MtC60376_GC | 424 | 3.1e-80 | 41 | 57 | 6 |
| 14 | MtD01919_GC | 769 | 9.5e-77 | 43 | 60 | 3 |
| 15 | MtD15912_GC | 654 | 1.2e-64 | 39 | 56 | 4 |
| 16 | MtC00585_GC | 636 | 1.0e-62 | 40 | 60 | 2 |
| 17 | MtD02656_GC | 634 | 1.5e-62 | 38 | 55 | 2 |
| 18 | MtC93070_GC | 585 | 3.0e-57 | 44 | 61 | 1 |
| 19 | MtD06873_GC | 459 | 8.6e-57 | 45 | 66 | 1 |
| 20 | MtD06873_GC | 152 | 8.6e-57 | 37 | 55 | 3 |
| 21 | MtD07861_GC | 579 | 1.5e-56 | 40 | 55 | 2 |
| 22 | MtC10182_GC | 570 | 9.8e-56 | 41 | 58 | 0 |
| 23 | MtC61392_GC | 569 | 1.2e-55 | 44 | 60 | 1 |
| 24 | MtD24960_GC | 539 | 3.8e-52 | 49 | 69 | 1 |
| 25 | MtC91611_GC | 524 | 1.6e-50 | 46 | 63 | 5 |

transient activation could be due to PIT formation in some cortical cells. This suggests that although the recognition of a proper EPS is not needed for *cg12* promoter activation, it requires the maintenance and extension of ITs in infected cells and in cells preparing for infection (containing PITs).

Several nodulin genes are also activated in response to endomycorrhizal colonization of roots (Miklashevichs et al., 2001), and the analysis of plant mutants indicates that the signaling pathways involved in legume-rhizobia and mycorrhizal symbioses at least partially overlap (Duc et al., 1989; Wegel et al., 1998; Endre et al., 2002; Stracke et al., 2002). In this study, we showed that *cg12* promoter is not activated by AM fungus infection in transgenic *M. truncatula*. Taken together, our results show that *cg12* promoter contains regulatory motifs that are specifically recognized during plant cell infection by the endosymbiotic bacteria in both rhizobial and actinorhizal symbioses. This suggests that at least part of the molecular mechanisms leading to the transcription of infection-related symbiotic genes is conserved between and specific to the two systems.

*cg12* codes for a subtilisin-like Ser protease secreted in the interface between the plant cell and Frankia (S. Svistoonoff, M. Nicole, and D. Bogusz, unpublished data). It may mature proteins and/or peptides involved in signal exchanges between the two partners or participate in cell wall loosening linked to IT growth. To our knowledge, no ortholog of *cg12* has been studied in legumes. We found genes highly similar to *cg12* among *M. truncatula* ESTs. Four of them are predicted to be expressed in nodules or rhizobia-infected roots and could therefore have a similar role in *M. truncatula* as *cg12* in *C. glauca*. It will be interesting to know if a true homolog of *cg12* exists in a model legume as this would allow the functional study of the role of this subtilase during the symbiotic infection process.

Actinorhizal plants are distributed among eight families that include symbiotic and nonsymbiotic plants. Phylogenetic studies have shown that together with legumes, all actinorhizal plants belong to the Rosid I clade (Soltis et al., 1995), thus suggesting that a predisposition to enter symbiosis appeared in the ancestor of this group. It is possible that key elements of this predisposition to enter symbioses with soil bacteria rely on their ability to recognize the symbiotic partner and to activate suitable responses to accommodate them. Our results suggest that actinorhizal plants and legumes share common mechanisms of transcriptional gene regulation activated specifically during bacterial infection. This conserved pathway may be part of the common heritage of legume and actinorhizal plants.

## MATERIALS AND METHODS

### Plant Material

*Medicago truncatula* cv Jemalong plants were grown in a growth chamber under a 16-h photoperiod and a day/night temperature of 24ºC/22ºC, respectively, with light intensity of about 100 $\mu$mol $E^{-2}$ $s^{-1}$ and relative humidity of 50%. Plants were cultivated on Murashige and Skoog medium with 3% Suc.

### Transformation of *M. truncatula*

Transcriptional fusions between the *cg12* promoter region and reporter genes *gus* and *gfp* generated as previously described (Svistoonoff et al., 2003) were transformed into *Agrobacterium tumefaciens* EHA105 strain (Trinh et al., 1998). Transgenic *M. truncatula* plants containing *Pcg12-gus* or *Pcg12-gfp* fusions were regenerated via somatic embryogenesis as described (Chabaud et al., 1996). Four transgenic lines coming from independent transformation events were selected for each construct. The transgenic nature of the plants was confirmed using kanamycin resistance, reporter gene expression, and PCR experiments performed with specific primers (data not shown).

### Bacterial and Fungal Inoculation

The wild-type *Sinorhizobium meliloti* Rm2011 strain was used for nodulation experiments. A strain constitutively expressing DsRed was generated by introducing the pDG77 (ptrp-DsRed) plasmid (Gage, 2002) in the wild-type strain. The *S. meliloti nodA* mutant GMi5382 (Rm2011 *nodA*::Tn5 no.2208) that does not produce Nod factors was kindly provided by Dr J. Dénarié. The *S. meliloti* mutant defective in exopolysaccharide synthesis was the Rm7225 strain (exoH225; Leigh et al., 1987). Plants grown in petri dishes containing nitrogen-free Fahraeus medium (Fahraeus et al., 1957) were inoculated with *S. meliloti* strains resuspended in water at an $OD_{600}$ = 0.003.

Inoculum of the mycorrhizal fungus *Glomus mosseae*, *Glomus rosea*, and *Glomus deserticola* propagated on Trifolium roots was kindly provided by L.G. Wall (National University of Quilmes, Argentina). Transgenic plants from in vitro culture were transferred to pots containing a sterilized mixture of soil-vermiculite mixed with the fungal inoculum and allowed to grow for 1 month. The roots were harvested at different times during the infection process, and reporter gene expression was assayed. The presence of mycorrhizal structures was checked by staining fungal hyphae with trypan blue in the same samples used to assay reporter gene expression as described (Svistoonoff et al., 2003).

### Application of Nod Factors

*M. truncatula* plants were grown as described for *S. meliloti* inoculation. Twenty-two-week-old transgenic plants were used for Nod factor treatments. Solutions of purified Nod factors ($10^{-6}$ or $10^{-7}$ M) from *S. meliloti* (kindly provided by J. Dénarié, Institut National de la Recherche Agronomique Toulouse, France) were directly applied to the roots. Reporter gene expression was monitored 24, 48, 36, and 64 h, 1 week, and 3 weeks after inoculation.

### Histochemical GUS Assays and Microscopy

*M. truncatula* nodules were embedded in 3% agarose and sliced into 40- to 60-$\mu$m-thick sections on a vibratome (Leica VT1000E, Wetzlar, Germany). For the detection of GUS activity, explants from *Pcg12-gus* transformed plants were stained in a solution containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl $\beta$-D-glucuronide) and incubated for 0.5 to 12 h at 37°C. A total of 0.5 to 5 mM of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ were added to limit the diffusion of the blue staining. Samples were fixed for 12 h in a solution containing 5% formaldehyde, 5% acetic acid, and 50% ethanol and washed several times in 70% ethanol. Whole root segments or sections were observed under a Leica DMRB microscope. For starch staining, samples were incubated for 5 min in Lugol's solution then washed in water. Samples were observed using a Leica DMRB microscope with appropriate filter sets for GFP fluorescence observation. Confocal microscopy imaging was performed on agar-embedded sections of living material as described (Svistoonoff et al., 2003).

### RT-PCR Analysis of *MtENOD20* Expression

Roots inoculated with *S. meliloti* 2011 (wild type) or GMi5283 (2011 *nodA* mutant) strain or treated with purified Nod factors or water (control plants) were harvested and frozen in liquid nitrogen. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First strand synthesis, amplification, and detection of the *MtENOD20* cDNA fragment were done according to Vernoud et al. (1999).

Parallel amplification of a constitutively expressed *M. truncatula* actin gene (Cohn et al., 2001) was used as an internal control.

### BLAST Search and in Silico Analysis of Gene Expression

BLAST search was performed using the MtCD BLAST server (http://medicago.toulouse.inra.fr/Mt/EST). The predicted CG12 protein sequence was compared to MtCDJan2003 cluster DNA consensus EST database using TBLASTN. Gene expression was analyzed using the iESTANT electronic northern facility (http://medicago.toulouse.inra.fr/Mt/EST/; Journet et al., 2002).

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