



## The promoter of a metallothionein-like gene from the tropical tree *Casuarina glauca* is active in both annual dicotyledonous and monocotyledonous plants

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### Abstract

A chimeric gene consisting of the  $\beta$ -glucuronidase (*gusA*) reporter gene under the control of the metallothionein-like promoter *cgMT1* from the tropical tree *Casuarina glauca* was introduced into *Nicotiana tabacum* via *Agrobacterium tumefaciens* and into *Oryza sativa* by particle bombardment. The strongest histochemical staining for GUS activity was observed in the root system of the transgenic plants, and especially in lateral roots. In contrast, a relatively low level of reporter gene expression was seen in the aerial tissues and GUS staining was located mainly in the plant vascular system. The average ratio of GUS activity between root and leaf was found to be 13:1 in tobacco and 1.5:1 in rice. The pattern of *cgMT1* promoter activity in floral organs was found to be different in tobacco and rice. High levels of *gusA* gene expression were detected in the ovules, pollen grains and tapetum, whereas in rice *PcgMT1* directs expression to the vascular system of the floral organs. These results suggest that *PcgMT1* is potentially useful in molecular breeding to express genes of interest whose products are preferentially needed in roots.

### Introduction

*Casuarina glauca* is a tall tropical tree of the *Casuarinaceae* family originating from Australia. In the field, *Casuarina* bears nitrogen-fixing nodules induced by the actinomycete *Frankia* (Baker & Mullin, 1992). Due to this symbiotic association, *C. glauca* has outstanding ability to grow on soils that otherwise would be too deficient in nitrogen to sustain growth. Current uses for *Casuarina* in tropical and sub-tropical areas with poor forest resources include sand stabilization, soil rehabilitation, fuel wood and timber production, shelterbelts, animal fodder and field crop

protection (National Research Council, 1984; Diem & Dommergues, 1990).

The symbiotic association between *Frankia* and actinorhizal plants is still poorly understood although it has striking differences with the *Rhizobium* legume symbiosis (for reviews see Pawlowski & Bisseling, 1996; Franche et al., 1998; Wall, 2000). However, in the past decade, some progress has been made in the knowledge of the plant genes that are expressed at different stages of actinorhizal nodule differentiation. Differential screening of cDNA libraries with root and nodule cDNA has resulted in the isolation of a number of nodule-specific or nodule-enhanced plant genes in several actinorhizal plants including *Alnus*, *Datisca*, *Elaeagnus* and *Casuarina* (reviewed in Mullin & Dobritsa, 1996; Pawlowski & Bisseling, 1996; Franche et al., 1998; Wall, 2000).

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From a cDNA library of young *C. glauca* nodules, our laboratory reported the isolation of a metallothionein (MT) cDNA named *cgMT1* (Laplaze et al., 2002). MTs are a group of low-molecular weight (10 kDa) cystein-rich proteins that are widespread throughout the animal and plant kingdoms (Hamer, 1986; Kagi, 1991; Robinson et al., 1993). In animals, two distinct classes of MTs exist based on their primary structure. Class I MTs display a typical arrangement of cystein residues resembling that of equine renal MT, the first MT isolated. Class II MT cannot be easily aligned with the equine renal MT. Extensive studies in animals show that MTs are induced by heavy metals such as zinc, cadmium and copper, and bind to *excess* metals for detoxification. Transcription of animal MT genes also increases in response to a range of stimuli including hormone signaling and stress conditions such as inflammation, UV and cytotoxic chemicals. In the past few years, MT-like genes have also been cloned from several higher plant species (Robinson et al., 1993). In some cases, plant MT-like genes are induced by an excess of heavy metals (Murphy & Taiz, 1995; Zhou & Goulsbrough, 1995). However, factors other than heavy metals have been reported to induce MT gene expression, that is, plant MT-like genes can be upregulated by wounding and virus infection, senescence, heat shock, fruit ripening and glucose starvation (for review see Rauser, 1999). These results imply that plant MTs may play an important role in other biological processes *in addition* to metal detoxification.

The MT-like gene from *C. glauca* encodes a 71-aminoacid long polypeptide with a calculated molecular mass of 7 kDa (Laplaze et al., 2002). According to the arrangement of cysteine residues, CgMT1 is a Class I type 1 MT and the corresponding gene belongs to a small gene family. The function of *cgMT1* in *C. glauca* remains unknown. Based on northern blot studies, *cgMT1* was found to be more highly transcribed in nodules and roots than in young leaves and stems. *In situ* hybridization studies carried out on nodules demonstrated that transcripts of *cgMT1* are located in mature *Frankia*-infected cells of the nitrogen-fixation zone, as well as in the pericycle cells. This expression pattern has led us to hypothesize that CgMT1 might provide metal ions that are necessary for the functioning of the nitrogenase complex.

To further characterize *cgMT1*, a fragment of 1.15 kb corresponding to the promoter region was isolated and fused to the  $\beta$ -glucuronidase reporter gene (*gusA*) (Laplaze et al., 2002). Transgenic

*Casuarinaceae* trees obtained after gene transfer by *A. tumefaciens* showed that *PcgMT1* directed strong GUS expression in roots. Intense blue staining was observed in secondary roots including root hairs. Thin longitudinal and transverse sections of primary roots revealed intense GUS activity in the pericycle zone and in the sub-epidermal cells and low activity in some cortical cells. In the aerial part of the transgenic trees, reporter gene activity was restricted to the oldest region of the shoots.

The specificity of expression conferred by *PcgMT1* suggests that this promoter could be a valuable candidate for directing root specific gene expression in plants. To evaluate the usefulness of the MT promoter from *Casuarina* for biotechnological applications, the *PcgMT1-gusA* fusion was introduced into tobacco via *A. tumefaciens* and in rice by particle bombardment. GUS activity was examined in different tissues of these annual dicotyledonous and monocotyledonous transgenic plants. We found that the expression pattern conferred by *PcgMT1* in tobacco and rice was comparable to that in transgenic *Casuarinaceae* transformed with a similar construct.

## Materials and methods

### *Transformation vectors and bacterial strains*

The plasmid pBIN-*cgMT1-gusA* contained the 1150 bp MT promoter *PcgMT1* isolated from the tropical tree *C. glauca* (Laplaze et al., 2002) linked to the *gusA* reporter gene (Jefferson et al., 1987) in the binary vector pBIN19. The binary vector was maintained in *Escherichia coli* DH5 $\alpha$  and mobilized in *A. tumefaciens* C58C1(pGV2260) (Vancanneyt et al., 1990) by direct DNA transfer (An, 1987).

The purified pBIN-*cgMT1-gusA* plasmid was bombarded into rice together with the 5.1 kbp pILTAB227 vector consisting of the CaMV (cauliflower mosaic virus) 35S promoter with a duplicated enhancer sequence (*e35S*) (Kay et al., 1987) controlling the hygromycin phosphotransferase gene and followed by the 3' non-coding region of the nopaline synthase (*nos*) gene kindly supplied by Dr C. Fauquet (ILTAB, La Jolla, USA) (Chen et al., 1998).

To assess 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc) penetration in tobacco organs, transgenic *N. tabacum* plants expressing GUS under the control of the constitutive promoter *e35S* were used as positive controls. For the analysis of transgenic monocotyledonous plants, rice plants

genetically transformed with the *pAct1D* construct (Zhang et al., 1991) and the *p70* construct (T. Legavre, unpublished) were used as controls. *pAct1D* contains the promoter region and first intron of the rice actin 1 gene (McElroy et al., 1991) fused to the *gusA* gene and the *nos* 3' terminator inserted in pBluescriptII-KS (Stratagene). The *p70* construct contains the *e35S* promoter controlling the *gusA* gene and followed by the *nos* 3' end in the plasmid pUC18.

#### *Plant material*

Axenic cultures of *N. tabacum* were prepared by sterilizing seeds in 5% sodium hypochlorite and germination on Petri dishes containing Murashige and Skoog medium (1962) supplemented with 1% w/v sucrose and 0.8% w/v agar. Seedlings were grown in a chamber at 26°C with a 16-h photoperiod (50  $\mu\text{E m}^{-2} \text{s}^{-1}$ ). When transferred to a glasshouse, tobacco plants were grown under natural light conditions at 26°C on a 1:1:1 Perlite/vermiculite/sphagnum mixture irrigated with mineral nutrients. Disinfected T1 rice seeds were germinated on Whatman 1 filter papers imbibed with a 50 mg L<sup>-1</sup> hygromycin aqueous solution in Petri dishes in a growth chamber at 26°C with a 16-h photoperiod (50  $\text{E m}^{-2} \text{s}^{-1}$ ). Germinating seeds were transferred to peat trays in the greenhouse under natural light conditions at 29°C.

#### *Genetic transformation of N. tabacum by A. tumefaciens*

For tobacco transformation, 57 leaf discs of tobacco (*N. tabacum*) were infected with *Agrobacterium* strain C58C1(pGV2260) (Vancanneyt et al., 1990) using the co-cultivation method as described by Horsch et al. (1984). Twenty-three transgenic tobacco calli growing on MS medium containing 100 mg L<sup>-1</sup> of kanamycin were then selected and five putatively transformed T0 plants from each line were regenerated according to standard protocols (Rogers et al., 1986). These plants were transferred to soil, self-pollinated, and allowed to set seeds. Transformed kanamycin-resistant T1 plants were verified by GUS histochemical staining and by PCR analysis using *gusA* and *nptII* specific primers (Franche et al., 1997).

#### *Genetic transformation of Oryza sativa by particle bombardment*

The *pcgMT1* and *pAct1D* constructs were introduced into mature embryo scutellum-derived embryogenic

calli of the *japonica* rice cultivar (*O. sativa* L.) Taipei309 by microprojectile bombardment according to the procedure described in Chen et al. (1998), using the PDS1000/He particle gun device (BioRad laboratories, USA). Each construct was mixed in a 4:1 molar ratio with the pILTAB227 plasmid. Procedures for selecting transgenic rice plants based on resistance to hygromycin were those of Chen et al. (1998). Sixty and 32 shoot-forming, hygromycin-resistant cell lines were generated from the embryogenic callus pieces bombarded with the *pcgMT1* and *pAct1D* plasmids, respectively. The putative transformants were further confirmed by genomic Southern blot using a 1.8 kbp labeled probe corresponding to the *gusA* coding sequence. Upon analysis of the 60 plants regenerated from callus pieces bombarded with the *pcgMT1* and pILTAB227 plasmids, 40 proved to have both co-integrated the *pcgMT1* plasmid and to harbor at least one intact copy of the *gusA* expression cassette. T1 progeny seeds were obtained by selfing of primary transformants. Four independent T1 lines were selected for further analysis on the basis of the presence of intact *gusA* cassette and low (1–4) copy number of *pcgMT1*.

#### *Histochemical staining for GUS activity*

To assay GUS activity, whole explants or sectioned tissues were incubated in a staining solution containing 1 mM X-gluc, for 16 h at 37°C, as recommended by Jefferson et al. (1987). To confine the localization of the blue staining, 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub> and 1 mM K<sub>4</sub>Fe(CN)<sub>6</sub> were added. Plant samples were fixed for several hours in a solution containing 5% formaldehyde, 5% acetic acid and 50% ethanol, washed several times in 70% ethanol and examined under a stereomicroscope.

For higher resolution analysis of transgenic *N. tabacum*, samples were embedded in 3% agarose and sliced into 45 to 50- $\mu\text{m}$ -thick longitudinal or transversal sections using a vibratome (Leica VT1000E). After overnight incubation with X-gluc, the sections were mounted on glass slides with 50% glycerin and examined with a light microscope.

After the histochemical assay was performed, specimens for histology were washed in 200 mM phosphate buffer (pH 7.2) then fixed in 2% paraformaldehyde, 1% glutaraldehyde, 200 mM phosphate buffer pH 7.2 for 24 h and cleared through a graded ethanol series. Sections (3  $\mu\text{m}$ ) of GUS-stained material were prepared from

2-hydroxyethylmethacrylate (Historesin Technovit 7100 Kulzer) embedded tissue with a LKB microtome. The cell wall was counterstained in purple by periodic acid schiff reaction (Gabe, 1968).

#### Fluorometric GUS assays

GUS activity was quantified using the substrate 4-methylumbelliferyl  $\beta$ -D-glucuronide (MUG), as described by Jefferson et al. (1987). Fluorescence was then measured with excitation at 365 nm and emission at 460 nm using a Fluoroscan (Labsystems) apparatus and was converted into nmoles 4-methylumbelliferone (4-MU) produced per minute per microgram of protein. Protein determination was carried out using Bradford reagent (Bio-Rad) and BSA as standard (Bradford, 1976).

GUS expression driven by *cgMT1* was determined in roots and leaves of three transgenic plants from nine T1 tobacco lines. Transgenic *N. tabacum* plants harboring the *e35S-gusA* construct and untransformed plants were used as positive and negative controls, respectively.

*gusA* gene expression under the control of the *cgMT1* promoter in rice was analyzed in leaf and root extracts from 18 T0 plants regenerated from callus pieces bombarded with the *pcgMT1*. Activities were also compared with that of 20 T0 plants transformed with the *Act1D-gusA* construct. Fifteen T0 plants derived from calli bombarded with the *e35S-gusA* construct in a parallel experiment following the same time-course were similarly analyzed. GUS activity was quantified in root and leaf tissues of five greenhouse-grown hygromycin-resistant T1 progeny plants at the 5–6-leaf stage derived from four *cgMT1-gusA* and one *Act1D-gusA* primary transformants. In each T1 plant, tissue samples were collected separately from blade and sheath tissues of the last fully expanded leaf and from the root system at the level of emission of lateral roots and individually analyzed for GUS activity. The values scored in the two leaf samples were averaged and a mean  $\pm$  SD leaf and root value calculated for each T1 line.

## Results

### *The PcgMT1 promoter directs strong GUS expression in N. tabacum roots*

The results presented here are based on the examination of a large number of tobacco plants from 23

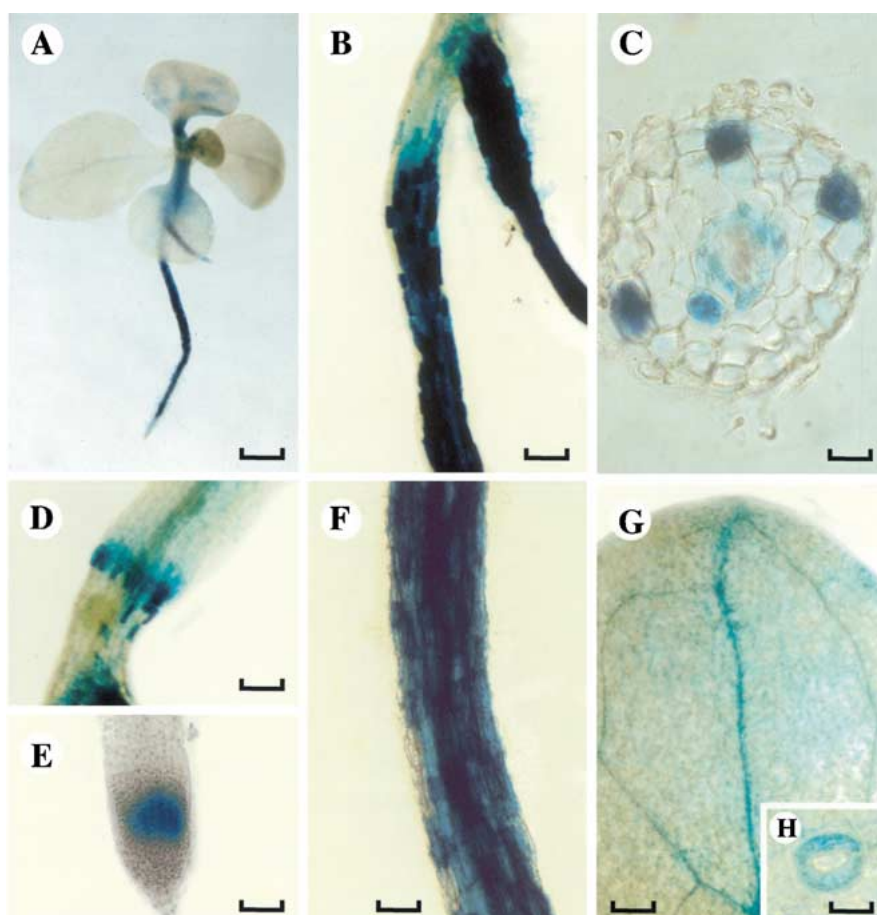
independent transgenic lines. Histochemical analysis was performed with T1 plants genetically transformed with the *cgMT1-gusA* construct. Plantlets germinated from wild-type seeds were used as negative controls and transgenic tobacco plants expressing GUS under the control of the CaMV promoter *e35S* were used as positive controls (data not shown).

Three-week-old *cgMT1-gusA N. tabacum* plants were incubated overnight in X-gluc. The strongest staining for reporter gene activity was observed in the root system (Figure 1(A)–(F)). In the primary root, blue staining was observed in the cortex and the vascular system. However, the cortical cells did not stain with equal intensity; some cells of the outer cortex were found to be very deeply stained (Figure 1(B) and (C)). As the primary root developed, the staining intensity decreased near the region located next to the base of the stem (Figure 1(B)) except in cells located around the crown that remained intensely stained. In two-month-old transgenic tobacco plants, the staining pattern of the primary root was found to be similar to that observed in three-week-old plantlets (Figure 1(D)). In secondary roots, deep blue staining was observed whatever the age of the plant (Figure 1(B)); root hairs were also found to express the *gusA* gene. In both primary and secondary roots, GUS activity was weak in the elongation zone (data not shown), whereas in the root apical region, strong *gusA* expression was detected in the meristematic zone (Figure 1(E)). When the transgenic plants were transferred in soil, reporter gene activity was found to decrease in primary roots, but remained high in lateral roots (data not shown).

In the aerial system, some diffuse reporter gene activity was observed in the cotyledons from three-week-old transgenic plants germinated *in vitro*, but staining was weaker than in the roots (Figure 1(A)). In young (Figure 1(A)) and fully expanded leaves (Figure 1(G)) GUS activity was restricted to the vascular system. Occasionally, blue crystals were observed in a few stomata generally located around the edges of the leaves (Figure 1(H)). The staining pattern in leaves remained restricted to the vascular system in two-month-old plants and in plants grown in soil in the glasshouse (data not shown).

### *The PcgMT1 promoter is active in a monocotyledonous plant*

Histochemical assays were repeatedly conducted on root and leaf tissues of 36 *in vitro*-grown and adult



**Figure 1.** Histochemical analysis of GUS activity in transgenic *N. tabacum* T1 plants expressing the *cgMT1-gusA* construct: (A) three-week-old plantlet (bar = 5 mm); (B) primary and lateral roots (bar = 1 mm); (C) transversal section of a primary root (bar = 100  $\mu$ m); (D) stem basis (bar = 500  $\mu$ m); (E) apex of the main root (bar = 500  $\mu$ m). Main root (F) (bar = 1 mm), leaf (G) (bar = 1 mm) and stomata (H) (bar = 200  $\mu$ m) from an eight-week-old plant.

T0 events and later in individual progeny plants from 48 T1 lines, selected on the basis of stable expression and simple integration pattern of the *pCgMT1-gusA* expression cassette.

All analyses revealed consistent GUS histochemical staining in root tissues of plants harboring the *pcgMT1* construct. Staining was mainly observed in root tips, in the elongation zone of the primary and secondary roots and in lateral roots, whereas no GUS activity was detected in the root differentiation zone (Figure 2(A)). Histological investigation of longitudinal and transversal primary, secondary and lateral root sections permitted detection of the presence of indigo-blue crystals in the endodermis and pericycle cell layers as well as in the vascular system (phloem and xylem cells) (Figure 2(B) and (C)). As previously ob-

served in transgenic tobacco plants, the root meristems and the lateral roots exhibited the most intense staining (Figure 2(A)). GUS products were also detected – albeit more faintly – in cells belonging to the subero-exodermic and external cortical parenchyma layers.

Histochemical assay of shoot sections of plants demonstrated that the immature blade of the innermost rolled leaf did not exhibit detectable staining whereas blade and sheath tissues of leaves of higher rank stained deep blue with a more intense GUS signal in the vascular system (Figure 2(D) and (E)). The specificity of staining in the vascular system in comparison with other hypodermal parenchyma and sclerified leaf tissues, appears to increase as the leaf matures (Figure 2(F)).

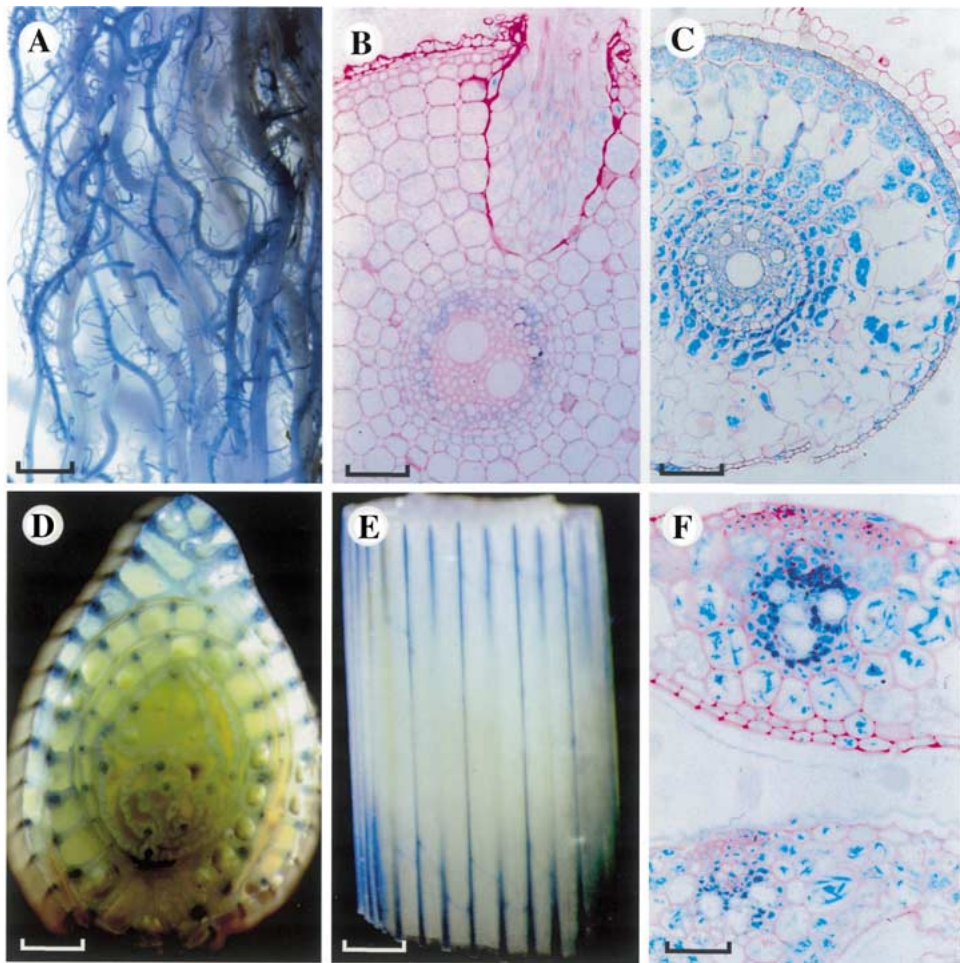


Figure 2. Histochemical localization of GUS specific activity in rice plants harboring the *pcgMT1-gusA* construct: (A) root system of a T1 progeny seedling at the 7–8 leaf stage (bar = 3 mm); (B) histological section of a main root at the site of emergence of a lateral root (bar = 45  $\mu$ m); (C) transversal section of a lateral root (bar = 90  $\mu$ m); (D) transversal section of a tiller of an adult plant (bar = 1 mm); (E) leaf sheath segment of a tiller from an adult plant (bar = 1 mm); (F) transversal section of a young leaf sheath (bar = 45  $\mu$ m).

*The pcgMT1 promoter directs a different pattern of expression in the floral organs of transgenic tobacco and rice plants*

GUS activity was monitored in the reproductive organs of *cgMT1-gusA* tobacco plants grown for three months in a glasshouse. In the initial developmental stages of flower buds, diffuse GUS activity was observed in the pistil, and in the filaments and the connective tissue of the anthers (Figure 3(A)). In the control buds developing on wild-type plants, no endogenous GUS activity was detected whatever the developmental stage (Figure 3(B)). At a more advanced stage, strong *gusA* expression was detected in ovules and pollen grains (Figures 3(C) and (D)). Transversal sections

of the anthers revealed intense blue staining in the tapetum and the vascular system of the anther (Figure 3(D)). Petals and sepals did not stain blue whatever the developmental stage of the flowers (Figure 3(A)).

Preferential GUS activity found in the vascular system in rice leaves was also observed in floral organs (sterile glumes, lemma, palea, anther filaments) whereas pollen grains of both T0 and T1 plants showed no GUS staining even after being squeezed out from the anther loculi (Figure 3(E)). Absence of GUS-specific activity in pollen isolated from squeezed anthers of pCgMT1 rice plants was further confirmed by fluorometrical assay conducted in the four selected T1 lines using a pAct1D plant as positive control (data not shown).

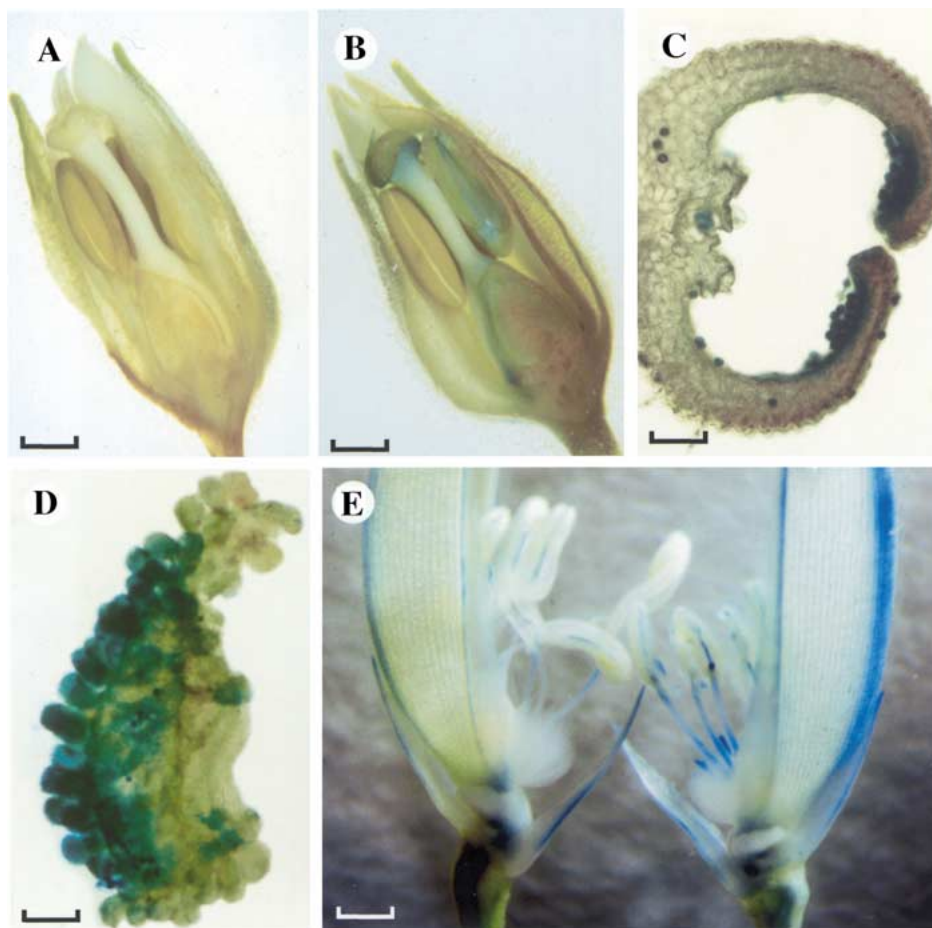


Figure 3. GUS activity in floral tissues of *cgMT1-gusA* transgenic *N. tabacum* and *O. sativa* T1 plants. (A) Longitudinal section of an untransformed *N. tabacum* flower bud (bar = 1 cm). (B–D) *cgMT1-gusA* flower buds from transgenic *N. tabacum*: longitudinal section of a flower bud (B) (bar = 1 cm); transversal section of an anther (C) (bar = 200  $\mu\text{m}$ ); ovules (D) (bar = 500  $\mu\text{m}$ ) and (E) flowers from transgenic *cgMT1-gusA* rice plants (bar = 2 mm).

#### Levels of expression of *cgMT1-gusA* in transgenic tobacco

Fluorometric analyses were undertaken on roots and leaves of one-month-old *N. tabacum* plants corresponding to nine transgenic T1 tobacco lines containing the *cgMT1-gusA* construct (see Figure 4(A)). GUS activity expressed as  $\text{pmoles 4-MU min}^{-1} \mu\text{g}^{-1}$  protein was found to be about 11-times higher in roots than in leaves: in roots it ranged from 7.5 to 100 with an average of 41.21 while in leaves it ranged from 2.2 to 5.8 with an average of 3.8. The ratio between root and leaf activity ranged from 1.2:1 to 40:1, with an average of 13:1. These results are in agreement with the histochemical expression profile previously observed. Compared to the activities measured in the transgenic lines containing the *e35S-gusA* construct, the reporter

gene activity driven by the MT promoter was found to be 13.6 times lower in leaves and 1.5 times lower in roots.

#### Levels of expression of *cgMT1-gusA* in transgenic T0 and T1 rice plants

GUS-specific activities were first quantified by fluorometry in root and leaf tissues of a population of 18 young T0 plants regenerated from callus pieces bombarded with the *pcgMT1* construct (Figure 4(B)). The data were compared with those scored on populations of plants regenerated from callus pieces transformed with the *pAct1D* and *p70* constructs. The range and average values observed in root tissues of the *pcgMT1* population were comparable to those of the *e35S* population but appeared to be lower than those of

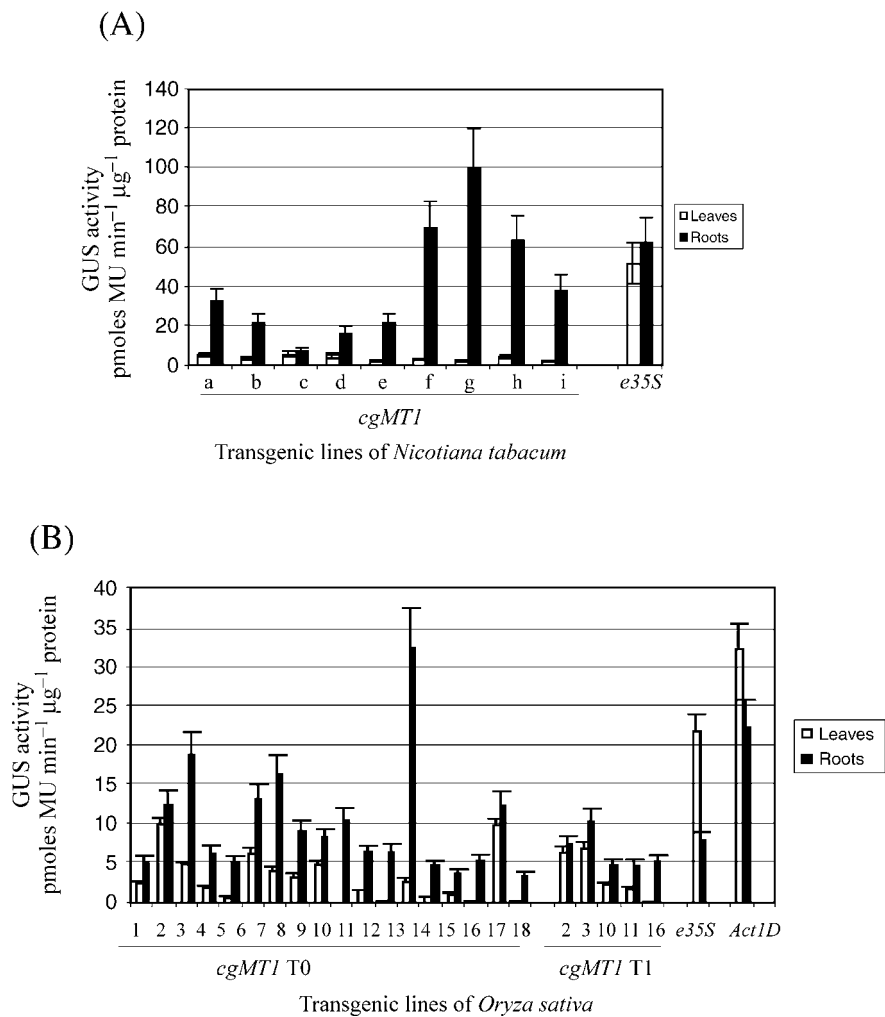


Figure 4. *cgMT1-gusA* activity in leaf and root extracts from transgenic plants of *N. tabacum* and *O. sativa*. Specific activities were determined by fluorometric analysis as described in Material and methods. The mean specific activity of nine T1 tobacco lines is shown in (A) with bars representing  $\pm$  standard error. GUS activities were determined in T0 and T1 transgenic lines of *O. sativa* (B). Transgenic plants containing the *gusA* gene under the control of the *e35S* and *Act1D* promoters were used as positive controls.

the *Act1* population. The average ratio between activities measured in roots and leaves was generally higher than 1 and proved to be higher than that scored in pAct1D plants (data not shown).

The GUS activity was then quantified in five hygromycin-resistant T1 plants for each of the five selected transgenic lines. The ratio of activities between root and leaf samples ranged from 1.2:1 to 4:1 and appeared higher than in the pAct1D control line 161 (0.8:1) (Figure 4(B)). Compared to the fluorometric analysis of T0 plants, the reporter gene activity was found slightly lower in T1 transgenic plantlets.

## Discussion

Since the analysis of the first MT-like gene in *Mimulus guttatus* in 1990 (de Miranda et al., 1990), the list of characterized plant MT-like genes has grown to at least 58 sequences isolated from a wide range of plants and tissues (for review see Rauser, 1999). The *cgMT1* gene isolated from *C. glauca* belongs to the type 1 MTs which are predominantly expressed in roots, whereas type 2 MTs are expressed primarily in leaves. The objective of our work with the *cgMT1* promoter was to determine the potential of this sequence that originated in a tropical actinorhizal tree for driving transgene

expression in roots of transgenic dicotyledonous and monocotyledonous plants. The activity conferred by the promoter was assessed by determining the pattern of GUS activity in transgenic *N. tabacum* and *O. sativa* containing the *cgMT1-gusA* construct.

The distribution of GUS in roots and leaves of transgenic tobacco and rice lines was consistent with the accumulation of *cgMT1* transcripts previously determined by northern blot analysis in *C. glauca* (Laplaze et al., 2002). Blue staining was most intense in roots, and especially in lateral roots, whereas in the aerial part the indigo-blue crystals were mainly restricted to the vascular system. Fluorometric analyses carried on *Nicotiana* plants confirmed these data since the measured GUS activity was also greater in root extracts than in aerial tissues. In rice, though the events analyzed also exhibited preferential GUS activity in roots, we observed less dramatic differences between the specific activities detected in the aerial and root system than in *Nicotiana*. Such differences may simply reflect the difficulty in collecting samples of the same tissue, architecture and maturity in monocotyledonous and dicotyledonous plants genetically transformed with a tissue-specific and developmentally regulated promoter such as the *cgMT1*.

The pattern of expression conferred by *PcgMT1* in tobacco and rice was also found to be similar to that observed in transgenic *Allocasuarina verticillata* trees transformed by *A. tumefaciens* with the *cgMT1-gusA* construct (Laplaze et al., 2002). These results suggest that the signals involved in the activation of *PcgMT1* in *C. glauca* roots are also present in unrelated species such as tobacco and rice. The ubiquitous presence of MT genes in plants together with their conserved mechanisms of regulation in unrelated species suggest that these genes play fundamental roles in plant roots. It has been hypothesized that plant MTs could be involved in metal tolerance, in homeostasis of metals under normal growth conditions, in developmental processes and in responses to environmental stresses (Rauser, 1999).

*PcgMT1* was found to direct *gusA* gene expression at lower levels in rice than those obtained in tobacco. This result might be linked to the absence of an intron between the promoter and the reporter gene; efficient promoters for the expression of foreign genes in transgenic monocots usually require the addition of introns (McElroy et al., 1991). In transgenic *O. sativa*, the level of expression in T1 plants compared to T0 plants also appeared to be reduced in most plant organs. This apparent reduction in the progeny may be due to a si-

lencing phenomenon that could be more pronounced in transgenic plants obtained via particle bombardment (Matzke & Matzke, 1995). Alternatively, the apparently reduced expression in the progeny may be associated with variations between experiments, such as differences in the physiology of regenerated T0 and seed-derived T1 plants.

Because it takes about five years to obtain flowers in the actinorhizal tree *A. verticillata*, the GUS expression pattern driven by *PcgMT1* has not yet been established in floral tissues. From the analysis of transgenic *N. tabacum* and *O. sativa* plants, it appears that the expression pattern differs in monocot and dicot floral organs; intense staining was observed in the ovules, the tapetum and pollen grains of tobacco, whereas in rice blue staining was restricted to the vascular system and no GUS activity was detected in pollen whatever the developmental stage of the anthers. This implies that some of the *cis*-acting sequences of *PcgMT1* responsible for expression during the development of the floral organs are not recognized in a monocot. Analysis of transgenic *PcgMT1-gusA* inflorescences from *C. glauca* will be necessary to confirm the exact regulation conferred by the MT promoter in a homologous system. Previous reports discussed expression of MT-like genes in inflorescences (for review see Rauser, 1999). For instance, the *gusA* expression pattern conferred by the promoter region of the pea MT-like gene *PsMTA* was studied in detail in *A. thaliana* (Fordham-Skelton et al., 1997). The authors established that this promoter which was highly expressed in roots was also expressed during the late stages of flower development in petals and anthers; as flower development continued, there was increased staining in the stigma and filaments. Another report based on *in situ* hybridization studies on a MT gene from the monocot *Zea mays* established that a MT gene called *MZm3-4* was predominantly expressed in tapetal cells of the anthers (Charbonnel-Campaa et al., 2000). The authors postulated that the MT could provide heavy metals to the tapetum, which, in turns, provides nutrients, enzymes and structural components to the haploid cells. They also suggested that MT gene expression could be related to senescence since the tapetal cells undergo a cell death process during microsporogenesis.

Deletion analysis of *cgMT1* is currently underway to localize the regulatory elements responsible for the expression in roots, in the vascular system of leaves and in the inflorescences. Due to the conservation of the *cgMT1-gusA* expression pattern in heterologous plants, this analysis will be possible in model plants

such as *N. tabacum* or *A. thaliana*. A similar approach has already been undertaken for the pea MT-like gene *PsMTA* that was studied in *A. thaliana* (Fordham-Skelton et al., 1997). Truncation of this promoter has established that two independent regulatory element pathways were involved in expression in roots and in leaves. Additional characterization of *PcgMT1* will include the possibility to induce its expression by heavy metals, wounding of pathogen infection.

Many genes originating in trees have already been identified and many more will follow as a result of tree genomic projects (for review see Ahuja, 2000; Merkle & Dean, 2000). To date, most transformation experiments in trees have used promoters obtained from herbaceous plants or plant viruses (Jouanin & Pilate, 1997; Ahuja, 2000). Relatively few promoters from forest trees have been isolated and characterized, and the use of these sequences to drive expression of valuable genes in heterologous plants is still poorly documented. On the basis of our results it appears that *PcgMT1* might be a valuable sequence to drive transgenes whose gene products are preferentially needed in roots and that tree genes might be a useful reservoir to increase the repertoire of tissue-specific plant promoters.

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