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# Symbiotic and non-symbiotic expression of *cgMT1*, a metallothionein-like gene from the actinorhizal tree *Casuarina glauca*

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## Abstract

A clone for a type 1 metallothionein (*cgMT1*) was isolated from a *Casuarina glauca* nodule cDNA library. The corresponding gene belongs to a small family and is highly expressed in roots and nitrogen-fixing nodules, whereas low expression was observed in aerial parts of the plant. The promoter region of *cgMT1* was isolated and fused to the *β*-glucuronidase (*gus*) gene. Transgenic *Casuarinaceae* plants showed that the *cgMT1* promoter was most active in roots and in the oldest region of the shoot. *In situ* hybridization indicated that in nodules *cgMT1* transcript is present in mature *Frankia*-infected cells and in the pericycle. Possible roles for *cgMT1* in symbiotic and non-symbiotic tissues are discussed.

## Introduction

Metallothioneins (MTs) are defined as low-molecular-mass (<10 kDa) cysteine-rich proteins that bind heavy metals. They are widely distributed in diverse organisms including fungi, plants, cyanobacteria and mammals. In the past few years, the structure and the function of mammalian MTs have been the object of intensive study (Vasak and Hasler, 2000). Animal MT synthesis can be stimulated not only by various metals such as Cd, Zn and Cu, but also by mediators of physiological stresses, including hormones, reactive oxygen species and metal ions (Kägi, 1991). Studies in animals have suggested that MTs play a role in detoxification of heavy metals and in homeostasis of intracellular metal ions (Kägi, 1991). They may also participate in defence against intracellular oxidants (Sato and Bremner, 1993) and in the regulation of metal-containing enzymes and transcription factors (Andrews, 2000). Despite considerable progress in the knowledge regarding this class of proteins the primary function of mammalian MT remains enigmatic (Vasak and Hasler, 2000).

Plant MT genes have been isolated from a variety of plant species but their function is poorly understood (Robinson *et al.*, 1993; Rauser, 1999). Since in most cases the plant MTs have not been purified their designation as MT-like is preferred (Hsieh *et al.*, 1995). Based on the sequence pattern, three classes of MTs have been described in plants (Rauser, 1999). Class I MTs were characterized by two cysteine-rich domains separated by a central cysteine-free spacer and have recently been subdivided into four types according to the arrangement of cysteine residues in the amino- and carboxy-terminal domains (Yu *et al.*, 1998). For example, in type 1 there are exclusively Cys-Xaa-Cys motifs in the two terminal domains. Class II MT, represented by the wheat Ec protein, showed Cys residues scattered throughout the entire sequence. Finally, class III MT (phytochelatins) consists of enzymatically syn-

thesized peptides with a poly ($\gamma$-glutamylcysteinyl) glycine structure.

Plant MT gene expression is regulated by many factors including metal ions, different stresses such as plant pathogens, and developmental stages. For example, in wheat transcription of MT was enhanced by excess aluminium (Snowden and Gardner, 1993) whereas copper treatment decreased the mRNA level of MT-like genes in *Mimulus guttatus* (De Miranda *et al.*, 1990). A tobacco MT-like gene was induced by wounding and infection with tobacco mosaic virus (Choi *et al.*, 1996). Moreover, the promoter of a *Brassica napus* MT-like gene expressed during leaf senescence (Buchanan-Wollaston, 1994) was found to be induced by wounding and during the hypersensitive response in *Arabidopsis thaliana* (Butt *et al.*, 1998). Furthermore, a MT-like gene from potato (*STLB13*) was induced during an early stage of the hypersensitive response to *Phytophtora infestans* (Birch *et al.*, 1999). Although the exact function of plant MTs is not understood, the diversity in MT gene responses and in the sites of expression suggests that plant MTs might be involved in various biological processes such as the defence reaction to plant pathogens, apoptosis, development and heavy-metal metabolism.

Actinorhizal plants belong to eight families of angiosperms. They are capable of forming nitrogen-fixing root nodules in response to invasion by a soil actinomycete, *Frankia* (Benson and Silvester, 1993). Unlike legume nodules, actinorhizal nodules are structurally and developmentally similar to lateral roots (Pawlowski and Bisseling, 1996; Franche *et al.*, 1998b). We are presently working on the *Frankia-Casuarinaceae* symbiosis, a model system that is especially suited to the analysis of plant symbiotic gene expression since *Casuarina glauca* and its close relative *Allocasuarina verticillata* can be readily transformed and regenerated (Franche *et al.*, 1997, 1998b; Smouni *et al.*, 2000). In this study, we isolated and characterized *cgMT1*, a class I type 1 MT-like cDNA from the actinorhizal tree *C. glauca*. In addition, we describe the cloning of the promoter of the cgMT1 gene and its analysis by *gus* fusion. We show that *cgMT1* expression in nodules is associated with *Frankia*-infected cells. The possible functions of *cgMT1* in actinorhizal nitrogen-fixing symbiosis are discussed.

## Materials and methods

### *Plant materials*

*C. glauca* plants were grown in a glasshouse and inoculated with *Frankia* strain Thr (Girgis *et al.*, 1990) as previously described (Gherbi *et al.*, 1997). For stable transformation, *A. verticillata* was propagated in tissue culture under sterile conditions at 26 °C with a 16 h photoperiod (Franche *et al.*, 1997).

### *Isolation of* cgMT1 *cDNA and 5′- upstream region*

A cDNA library was constructed from mRNA isolated from *C. glauca* nodules as described previously (Gherbi *et al.*, 1997). The library was screened by differential hybridization of randomly picked clones with biotin-labelled root or nodule cDNA (Gherbi *et al.*, 1997). The insert of *cgMT1* was subcloned into pGEM-T vector (Promega, Madison, WI).

The *cgMT1* promoter region was cloned using the Universal Genome Walker kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Primers GSP1 (5′-CAGTTGCAGCCACTCCCGCCG CTGCAT-3′) and GSP2 (5'-CCGGAGCACAGCCAC AGGAAGACATT-3′) were designed to match the inverse complement of nucleotides 27 to 53 and nucleotides −1 to 26 of the *cgMT1* cDNA respectively. PCR was performed on *C. glauca* genomic DNA using Clontech's Advantage Tth polymerase mix. A 1150 bp product was amplified from the *Sca*I DNA library and cloned into pGEM-T generating pPcgMT1.

Both strands of the insert were sequenced using the Applied Biosystems 373A automatic sequencing system (Foster City, CA). Homology searches in databases were performed using the BLAST program through the NCBI server. Sequence alignments were carried out using multiple sequence alignment with a hierarchical clustering program (Corpet, 1988).

### *Construction of transcriptional* C. glauca MT1 *gene promoter* gus *fusion*

The *cgMT1* promoter fragment from pPcgMT1 was subcloned into the binary plant transformation vector pBI101.3 (Clontech) as follows. Two oligonucleotides were synthesized: a 5′ primer (5′-CCCAAGCTTGGGACTATAGGGCACGCGTGGTC G-3′) designed to match the first 21 nucleotides and to introduce a *Hin*dIII site, and a 3′ primer (5′-GCTCTAGAGCGATGAAGAGGTGGTTGGCCTG-3′) designed to match the inverse complement of 21

nucleotides starting 29 bp upstream of the initiation codon and to introduce a *Xba*I site. After PCR amplifications this *Hin*dIII/*Xba*I fragment was cloned into *Hin*dIII/*Xba*I-digested pBI 101.3. The sequence was confirmed by DNA sequence analysis.

*Genomic DNA blot analysis*

Genomic DNA was obtained from seedlings by a modification of the CTAB method and was restricted by *Bam*HI and *Bgl*II (Gherbi *et al.*, 1997). Hybridization was performed under high-stringency conditions (42 °C, in 50% formamide, 7% SDS, 0.25 M $Na_2HPO_4$, 2 mM EDTA, 100 $\mu$g/ml heparin, 100 $\mu$g/ml salmon sperm DNA). Filters were washed at 65 °C twice in 2× SSC, 0.1% SDS, twice in 1× SSC, 0.1% SDS and twice in 0.5× SSC, 0.1% SDS.

*RNA gel blot analysis*

Total RNA was isolated according to Bugos *et al.* (1995). Samples were subjected to electrophoresis on a 1.2% agarose gel containing formaldehyde together with a 0.24–9.5 kb RNA ladder (Gibco-BRL, Gaithersburg, MD) and blotted onto a nylon membrane. Pre-hybridization, hybridization and washing were done according to Laplaze *et al.* (2000b). Hybridization with a ribosomal probe (18S; Carnero Diaz *et al.*, 1997) was used to normalize the expression levels.

In situ *localization of* cgMT1 *mRNA*

*In situ* hybridization was performed as previously described (Gherbi *et al.*, 1997). Radioactive probes were used. The radioactive $^{35}$S-rUTP-labelled sense and antisense RNA were obtained according to Gherbi *et al.* (1997). For generation of an RNA probe, *CgMT1* was amplified with two primers homologous to the coding region, 5′-AATGTCTTCCTGTGGCTG-3′ and 5′-TCCAAACTCCTAAGTAGAG-3′. The 363 bp product was cloned into pBluescript KS+ (Stratagene, La Jolla, CA). The derivative plasmid was cut with *Eco*RI and transcribed with T7 RNA polymerase, or cut with *Bam*HI and transcribed with T3 RNA polymerase, to generate antisense or sense RNA probes, respectively.

*Transformation of* Allocasuarina verticillata *and histochemical localization of GUS activity*

Transgenic *Allocasuarina verticillata* plants were obtained through the transformation of mature zygotic embryos by engineered *Agrobacterium tumefaciens* as previously described (Franche *et al.*, 1997). Transgenic *A. verticillata* were recovered from nineteen transformed calluses representing different transformation events. As previously reported (Franche *et al.*, 1997), successful gene transfer was confirmed by the expression of the reporter gene and by polymerase chain amplification (PCR) with *nptII* and *uidA* primers. Additional PCR reactions with primers designed for the amplification of the *virD1* gene from *Agrobacterium* were carried out to check that tissues had not been contaminated by persisting *Agrobacterium.*

*Histochemical GUS assays and histochemistry*

Plant samples (shoots and roots) from *in vitro* propagated plants were taken 4 to 5 weeks after the rooting treatment, when the plants had reached a height of ca. 8 cm. Two months after plant inoculation by *Frankia*, nodules displaying different stages of development were harvested. To assay GUS activity whole explants or sectioned tissues were flooded in a staining solution containing 1 mM X-gluc, and incubated for 16 h at 37 °C. To confine the localization of the blue staining, 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ were added as catalysts. Plant samples were fixed and cleared, as previously reported, and examined under a stereomicroscope (Le *et al.*, 1996). For higher-resolution analysis, samples were embedded in 3% agarose and sliced into longitudinal or transversal sections 45–50 $\mu$m thick on a vibratome (Leica VT1000E) as described by Franche *et al.* (1998a).

## Results

*Isolation of* cgMT1 *cDNA*

As part of an ongoing programme to characterize plant genes expressed in actinorhizal nodules of *C. glauca*, a cDNA library was prepared with mRNA from 3-week old root nodules (Gherbi *et al.*, 1997). Among the cloned and sequenced cDNAs, the *cgTRI* clone was found to encode a MT-like protein and was renamed *cgMT1*. The DNA sequence of *cgMT1* cDNA is 600 bp long and contains an open reading frame of 216 bp encoding a 71 amino acid long polypeptide with a calculated molecular mass of 7 kDa (Figure 1a). The 5′- and 3′-untranslated regions are 56 and 328 bp long respectively. As shown in Figure 1a, the predicted amino acid sequence contains 12 cysteine residues

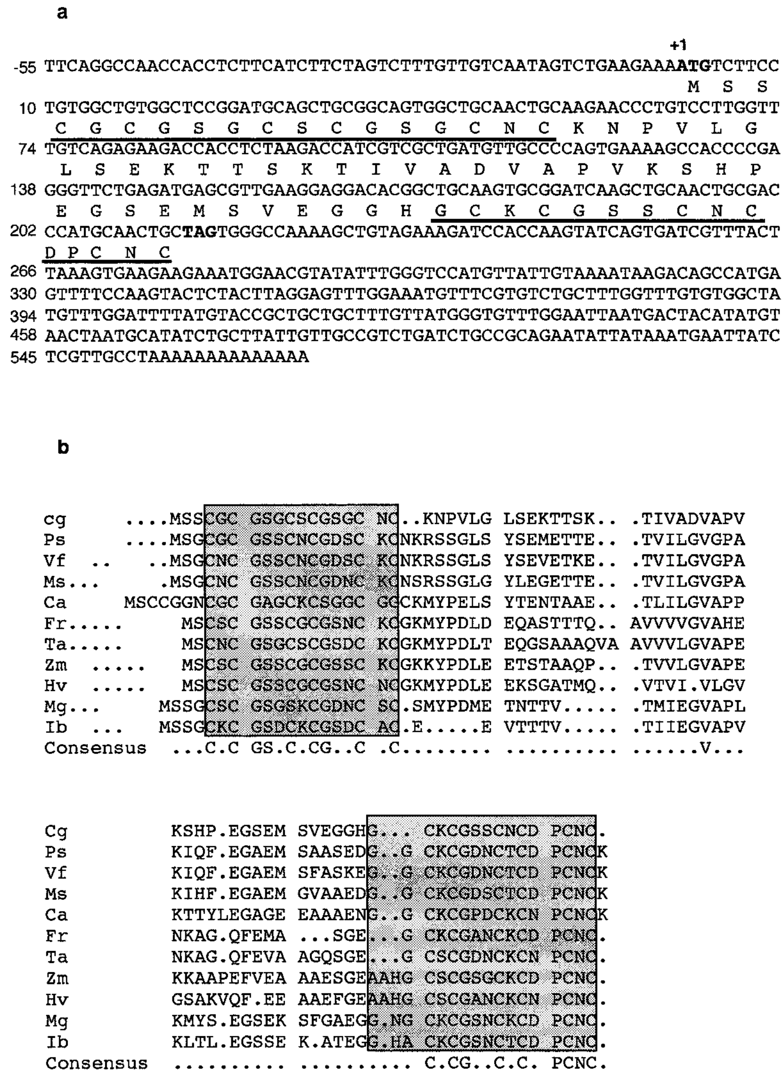

*Figure 1.* Nucleotide and deduced amino acid sequences and alignment to other type 1 MTs. a. Nucleotide and deduced amino acid sequences of *C. glauca* metallothionein-like cDNA (accession number Q39511). Numbering is relative to the first base of the ATG start codon (+1). Cys-rich-N and C-terminal domains are underlined. The start and the stop codon are in bold. b. alignment of some plant metallothionein–like proteins. Cg, *C. glauca* (accession number Q38511); Ps, *Pisum sativum* (P20830); Vf, *Vicia faba* (Q41670); Ms, *Medicago sativa* (AF 189766.1); Ca, *Coffea arabica* (P43396); Fr, *Festuca rubra* (O24528); Ta, *Triticum aestivum* (P43400); Zm, *Zea mays* (P30571); Hv, *Hordeum vulgare* (BAA23628); Mg, *Mimulus guttatus* (A34131); Ib, *Ipomae batatas* (AAD10220). The boxed and shaded areas represent the Cys-rich-N and C-terminal domains. The consensus identifies conserved amino acids in ten and eleven presented sequences.

distributed into N- and C-terminal domains (6 amino acids each), separated by a hydrophobic central region. The deduced protein has the typical structure of class I type 1 MTs where all Cys residues occur in Cys-X-Cys motifs (Yu *et al.*, 1998). cgMT1 displays a high degree of similarity to other plant class I MT proteins from both monocotyledonous and dicotyledonous plants. Figure 1b shows the alignment of cgMT1 to ten other plant class I type 1 MTs. The two cysteine-rich domains show the highest amino acid conservation.

### cgMT1 *belongs to a small gene family and is expressed throughout the plant*

*cgMT1* cDNA was used in a genomic DNA blot analysis to study the genomic complexity of the corresponding gene. *cgMT1* probe hybridized to 2–3 bands in

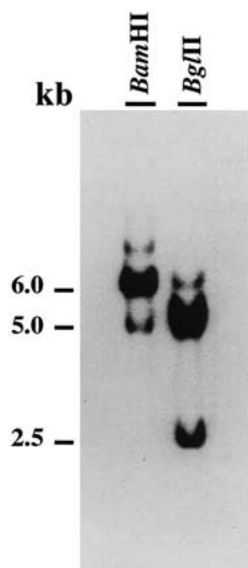


*Figure 2.* Southern blot of *C. glauca* genomic DNA. Genomic DNA (10 $\mu$g) was loaded in each track. Hybridization was performed with the [$^{32}$P]-dCTP-labelled *cgMT1* cDNA insert.

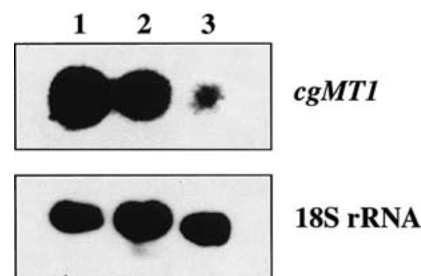


*Figure 3.* Northern blot analysis of *cgMT1* expression. Total RNA (10 $\mu$g per slot) from *C. glauca* organs (nodules, lane 1; uninfected roots, lane 2; young leaves and stems, lane 3) was hybridized with the [$^{32}$P]-dCTP-labelled *cgMT1* cDNA insert. The same blot was hybridized, as a loading control, with a ribosomal probe (Carneiro Diaz *et al.*, 1997).

each digest, suggesting that *cgMT1* gene belongs to a small gene family (Figure 2).

The accumulation of *cgMT1* transcripts in different organs was analysed by northern blot hybridization. Total RNA was extracted from nodules, uninfected roots and aerial parts of *C. glauca*. Since *C. glauca* is characterized by distinctive foliage consisting of deciduous, long, needle-like, articulate branchlets with reduced, scale-like leaves organized in whorls, the RNA from aerial part represent RNA from stem and leaves. The *cgMT1* transcript was detected in all organs tested (Figure 3). However, since we did not hybridize with a probe from the non-coding region we cannot exclude that the signal is the result of cross-hybridization with similar transcripts from different MT genes. Expression was high in both nodules and uninfected roots and weak in aerial parts. The size of the hybridizing mRNA was consistent with the size of the cDNA insert (0.6 kb) suggesting that the *cgMT1* clone contained a full-length cDNA.

### *Localization of* cgMT1 *transcripts in* C. glauca *nodules*

*cgMT1* transcripts were localized by *in situ* hybridization on longitudinal and transversal sections of *C. glauca* prenodules and nodules. Upon infection, *Frankia* induces cell divisions in the root cortex, leading to the formation of a small external protuberance called the prenodule (Angulo Carmona, 1974; Callaham and Torrey, 1977; Berry and Sunnel, 1990). Some of the prenodule cells become infected by *Frankia*. Concomitantly with prenodule formation, cell divisions occur in the pericycle opposite to a protoxyleme pole leading to the formation of a nodule primordium. Its cells become infected by bacterial hyphae coming from the prenodule. In a given nodule lobe, *Frankia* is restricted to the cortical cells (Figure 4). Due to the presence of a meristem at the apex of the nodule lobe, a developmental gradient exists and different zones have been defined using both morphological and gene expression studies (Angulo Carmona, 1974; Ribeiro *et al.*, 1995; Gherbi *et al.*, 1997). The apical meristem is designated as zone 1. The infection zone (zone 2) where *Frankia* infects some of the new cells derived from the meristem precedes the fixation zone (zone 3) where active nitrogen fixation takes place. Finally, in old nodule lobes, a senescence zone (zone 4) is observed, where both plant cytoplasm and bacteria undergo degradation.

*cgMT1* transcripts were detected in the mature infected cells (zone 3) where *Frankia nifH* is expressed (data not shown) and in the pericycle cells of the nodule (Figure 4). No expression of *cgMT1* was found in young infected cells of zone 2, the endodermis and the phloem. In prenodules, *in situ* hybridization showed that the level of expression of *cgMT1* in cells completely filled with *Frankia* is similar to that found in mature nodule lobes (data not shown). As mentioned before, hybridization might result of cross-hybridization with similar transcripts from different MT genes. No signal was detected using the *cgMT1* sense probe (data not shown).

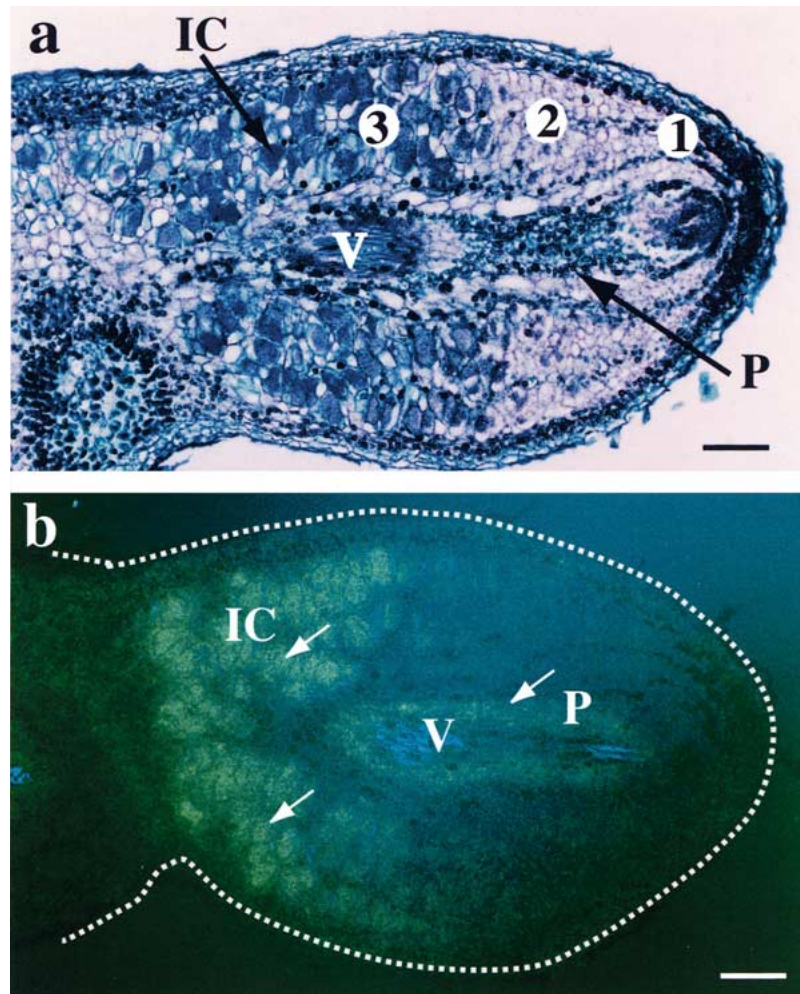


*Figure 4.* Localization of *MT* transcripts in *C. glauca* nodule lobes. a. Pseudo-longitudinal nodule lobe section of *C. glauca* stained with toluidine blue. Cortex of *Frankia*-infected cells appears in purple. Zone 1, apical meristem; zone 2, young infected and uninfected cells; zone 3, mature infected cells. b. Dark-field micrograph and epipolarized light; silver grains are visible as yellow dots. White arrows point at infected cortical cells of the fixation zone and the pericycle cells where *cgMT1* is expressed. No signal was found in control hybridization with sense RNA as a probe. P, pericycle; V, vascular bundle; IC, infected cells. Bar = 100 $\mu$m.

### *Isolation and computer analysis of the* cgMT1 *5′ upstream region*

In order to study the molecular mechanisms of *cgMT1* expression, the cDNA sequence was used to amplify and clone a 1.15 kb fragment corresponding to the *cgMT1* 5′-upstream region with the genome walking system. The sequence of the entire fragment was determined and is shown in Figure 5. This sequence overlaps with the 82 first nucleotides of *cgMT1* cDNA (not shown). A potential TATA box sequence (TATAAA) is situated 91 bp from the +1 site. Analysis of the *cgMT1* promoter revealed the presence of several sequence motifs. The two consensus motifs AAAGAT and CTCTTC have been shown to be involved in nodule-specific leghaemoglobin expression in legumes and are conserved in several legume and non-legume nodule-specific genes (Sandal *et al.*, 1987; Macknight *et al.*, 1995; Andersson *et al.*, 1997). A 17 bp long A/T-rich sequence starting at position −457 is identical to a sequence found in the promoter region of the *Phaseolus vulgaris* nodulin 30 gene (Npv30; Carsolio *et al.*, 1994). Interestingly, Npv30 bears two Cys-X7-Cys motifs resembling metal-binding domains. Sequences resembling H and G boxes that are involved in promoter activation in response to various developmental and

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                                                  Nod
1128 ACTATAGGGCACGCGTGGTCGACGGCCGGGGCTGGTACTAGAGCCTAAAGTAAATATGCT
1068 TCATTATGTGTAACTTTAAATTTAATGTCACTTGGCATGCAAAGATATATGTGTGAAAAA
      Nod
1008 AGAGGTCTCGTACCAAAAACATGATCCCTTTTGATGAGATTTAAATAAATAAAAAATACA
948 CTTTCAAATTTTAAGTTAACTTTTTAGTTAAGCCTTTAAATCTCTCAACATTTATGATAG
888 GATGATAGAATTTCTCTAAATAATTTGTTTCTTTATATCCATAGCTCATCTTCTCCTACA
828 ATATCTATCCCAAATATGTGCAGTACTGGTATAAAAACAAAAGGTCCGTCATCACGAAAA
768 TAATAAATTGAACAAAAACTGCCATACCTGTGATACGGACAATCACGAATCCCAAAGAAA
708 TTGATCGGTCAATACTAAGGCAGCTATAGGCTATCCATTTAACATTGCAAACGACAGATT
648 CCCGTATATCTTATTATTTTTCTGAGCCGTATCCACCTTAGAGCTTGTGTGAGATCGGTA
588 TCTTCCCGTTTGACAATGAATCATTCACCAGTTGGATTTTGATGGGTTCGTCTAAGCAAT
528 TGTTAAATCCGTCAACAGGACAAAATAGACTGACATTGTAGGGACATCTGGAATTAAAAT
     Npv30
468 AAAAATATTTTAAAGGGCTTATGTATAATTGATGTAGACCATCGAGGACTAACAATACTT
408 AAATCTTTTTTTCTATTAAAGAAAAATTAAATCTCCACAGAAACTGAAAATCAAACAAGT
348 CCTGACAAACCCAGATGATAATTTAGAAAATGATTCTGAAAACCCAACGCTGAAACGCCT
288 TGGAAGGTGATTAACCACCGGAATTAATAATCAATTAATCGACGACTTAGCTATACATGA
                                              ARE-like
228 ATGTGAATGGTTGGTGGTGCTCCACGTAGACGGGACCGTGACGTTCACTGCGAAAAAGTC
                                     G-box             H-box
168 TAAGACGCCATCAGTCTAAGATTTATACACGTGTACGGTGGATGCCTACCTCTCCCTGCC
     Nod
108 CTCTTCAAGTTTATAAATAGTAGCCAAGGCCCAACTCGAAAGTCATCCTACAGTTCAGGC
             Nod                                   +1
-48 CAACCACCTCTTCATCTTCTAGTCTTTGTTGTCAATAGTCTGAAGAAAATG
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*Figure 5.* Nucleotide sequence of the *cgMT1* 5′-upstream region. Numbering is with respect to the first base of the ATG start codon (+1). The putative TATA box and the ATG start codon are in bold. Underlined are the H-box (CCTACCT), the G-box (CACGTG), the nodule-specific motifs (AAAGAT and CTCTT) and the ARE-like element (GTGAC). The conserved sequence that is present in the *NPv30* promoter is double-underlined (AAATAAAAATATTTTA).

environmental signals are also present in the *cgMT1* 5′-upstream region (Loake *et al.*, 1992).

The sequence of the *cgMT1* promoter region was also compared to previously isolated plant and animal MT gene promoters. Searches did not reveal any metal regulatory element (MRE) as have been found in pea *PsMTA* (Fordham-Skelton *et al.*, 1997) and tomato *LeMTB* (Whitelaw *et al.*, 1997) MT genes. Nevertheless, the *cgMT1* promoter region contains a motif bearing a certain degree of similarity to the core antioxidant response element (ARE) (consensus ARE: GTGACnnnGC) as described by Rushmore *et al.* (1991).

*Analysis of the expression driven by the 5′-upstream region of* cgMT1 *in transgenic* A. verticillata *plants*

A translational fusion of the 5′-upstream region and the *gus* reporter gene was constructed. The chimaeric gene was introduced in *A. verticillata* by *Agrobacterium tumefaciens*-mediated transformation (Franche *et al.*, 1997). Fifteen independent transgenic *A. verticillata* plants were regenerated and expression of the fusion gene in shoots (stems/leaves), roots and nodules was analysed by histochemical GUS assay (Figure 6). The pattern of *gus* expression was comparable in all the 15 transgenic plants analysed.

The apex of the young photosynthetic branchlets showed little or no *gus* expression (Figure 6a). When visible, reporter gene activity was detected in the vascular bundle of the leaves (Figure 6b). Considerable blue staining was found at the base of photosynthetic branchlets, especially in leaves with extensive expression in the mesophyll cells and in the vascular bundle (Figure 6c). Intense blue staining was observed in roots. Strong GUS activity was observed particularly in the root tips of primary roots (Figure 6d), and appeared constitutively in secondary roots including hairy roots (Figure 6e). Thin longitudinal and transverse sections of primary roots revealed intense staining in the pericycle zone and in the sub-epidermal cells and faint staining in some cortical cells (Figure 6f–g). In transgenic nodules, GUS activity was

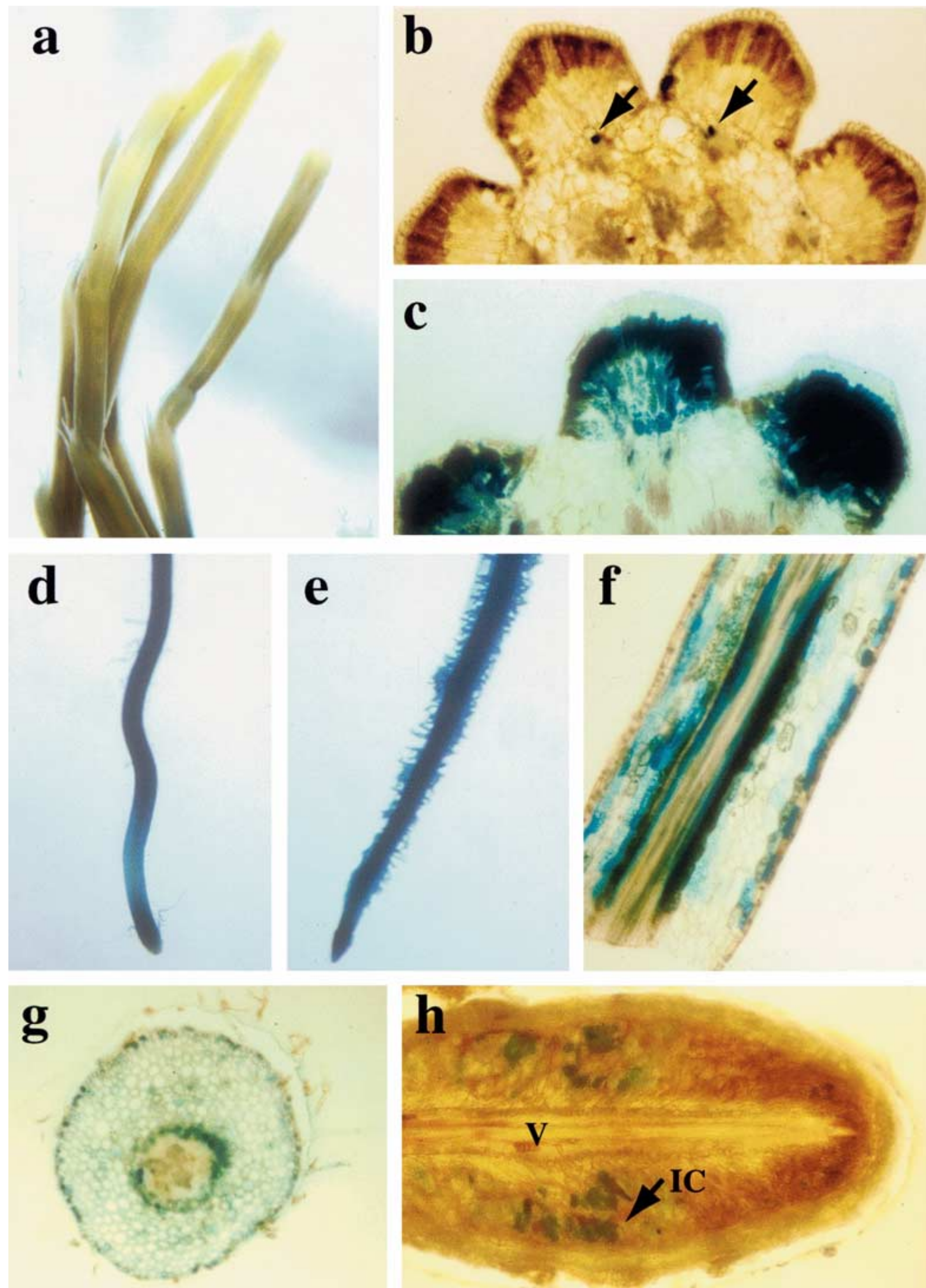


*Figure 6.* Histochemical analysis of GUS activity in transgenic *A. verticillata* plants expressing the *cgMT1-gus* construct. Different tissues were stained as described in Materials and methods. Blue precipitate indicates the location of GUS activity. a. Shoot. b. Transverse section of the shoot near the apex; dark arrows indicate GUS activity. c. Transversal section of the shoot near the base of the stem. d. Primary root. e. Secondary root. f. Longitudinal section of a primary root. g. Transversal section of a primary root. h. Longitudinal section of a mature nodule lobe. V, vascular bundle; IC, *Frankia*-infected cells.

consistently restricted to the large *Frankia*-infected cells of the nitrogen-fixing zone (Figure 6h). No staining was seen in uninfected cells of the cortex, the periderm, or the vascular bundle. The only *cgMT1-gus* expression obtained in transgenic nodules that was not consistent with *in situ* hybridization data was the absence of GUS activity in the pericycle.

## Discussion

In this study we have identified and characterized a *C. glauca* cDNA (*cgMT1)* encoding for a MT-like protein, and its putative promoter region that confers enhanced expression in uninfected roots and in root nodules. This is the first investigation of the occurrence of MTs in nitrogen-fixing root nodules. Based on the arrangement of the cysteine residues the deduced polypeptide can be classified as a class I type 1 MT-like protein. High-stringency Southern blot hybridization analysis of *C. glauca* genomic DNA with a *cgMT1* cDNA fragment suggests that *cgMT1*, like other type 1 plant MT-like proteins, belongs to a small gene family of few related genes (Evans *et al.*, 1990; de Framond *et al.*, 1991; Hudspeth *et al.*, 1996). The presence of different members of a type 1 MT-like family suggests that different homologues may perform related but distinct functions in different tissues.

In addition to northern blot and *in situ* hybridization experiment the expression pattern of the *C. glauca cgMT1* gene was studied at the cellular level with *A. verticillata* plants transformed with a promoter-*gus* gene fusion. *A. verticillata* is a close relative of *C. glauca* and both plants have a similar infection pathway.

The staining patterns of transgenic *A. verticillata* plants transformed with the *cgMT1-gus* fusion were consistent with the RNA blot analysis and showed that expression of *cgMT1* occurred in all plant organs tested with high expression in nodules and uninfected roots whereas expression levels in the aerial parts were low. Our data in non-symbiotic tissues are comparable to that of other genes of type 1 MT-like proteins that are expressed predominantly in roots, whereas genes encoding type 2 MT-like proteins are expressed primarily in leaves (Robinson *et al.*, 1993; Foley *et al.*, 1997).

In aerial parts, *gus* expression analysis showed that the *cgMT1* promoter is preferentially active in the leaf and stem vascular system (Figure 6b). In the oldest regions of the shoots, near the base of the stem, reporter gene activity was observed in nearly all the tissues. This expression pattern showing that GUS activity increases with distance from the shoot apex (Figure 6c) suggests that in aerial tissue *cgMT1* expression is correlated with ageing. A similar correlation with the cell maturation process has been reported for the expression of some other plant MTs (Buchanan-Wollaston *et al.*, 1994; Ledger and Gardner, 1994; Hsieh *et al.*, 1995; Butt *et al.*, 1998). One possible interpretation of this uneven expression in young versus ageing leaves could be that MT-like protein may function in binding metal ions released during cell senescence and could be involved in a protective mechanism against oxidative damage or in the transport of metal ions from the oldest tissues to developing areas (Buchanan-Wolaston *et al.*, 1994; Butt *et al.*, 1998).

By contrast, in roots, the *cgMT1-gus* construct was highly expressed in young tissues. A strong and uniform blue colour was seen at the tip of the main and lateral roots. The strongest expression of the *cgMT1-gus* construct was observed in the root stele and in the cortex (Figure 6f, g). Thus, under standard growth conditions *cgMT1* appears to be highly expressed in roots at high levels, suggesting an essential role for this MT. Similarly, Fordham-Skeleton *et al.* (1997) reported for transgenic *Arabidopsis* that high levels of expression of a *gus* fusion of the promoter of the pea type 1 MT-like gene *PsMTA* construct had been observed in roots. Furthermore, Hudspetht *et al.* (1996) have shown that a *gus* construct with the promoter of the cotton type 1 MT-like gene was highly expressed near the root tip in the transformed roots of cotton. The function of cgMT1 in roots remains unknown. Whether or not cgMT1 is able to bind metal ions remains to be established biochemically. The high expression of the *cgMT1-gus* construct in the vascular bundle suggests a role in metal transport throughout the plant. Biochemical characterization of the purified cgMT1 protein together with the study of *cgMT1* expression in response to metal deficiency or excess should help achieve a better understanding of the function of this gene.

*In situ* hybridization experiments showed that *cgMT1* is expressed in nodule cortical infected cells and in the pericycle of *C. glauca* mature nodule lobes. *cgMT1* transcripts were also detected in *Frankia*-infected cells of the prenodule. This finding is in agreement with previous data (Laplaze *et al.*, 2000a) suggesting that similar cell differentiation states oc-

cur in the corresponding cell types in the nodule and prenodule. Transgenic *A. verticillata* plants containing a *cgMT1-gus* construct showed GUS activity in infected cells, but no blue staining was detected in the pericycle. This difference may be due to the presence of a *cis* element for tissue-specific expression upstream of the cloned region or in the 3′ UTR as has been demonstrated for the early nodulin gene *SrEnod2* (Chen *et al.*, 1998). Another possibility is that the signal detected in the pericycle corresponds to cross-hybridization with transcript from another MT gene of the same family. Beta-glucuronidase expression was never detected in stems, roots or nodules of untransformed plants, thus indicating that *A. verticillata* and the symbiont have no detectable GUS activity (Franche *et al.*, 1997).

Computer analysis of the *cgMT1* promoter revealed the presence of several sequences showing similarity with regions and *cis*-acting elements previously described. The 17 bp sequence AAAATAAAAATATTTTA is similar to the sequence found in the promoter region of the *P. vulgaris* nodulin (*Npv30*) gene (Carsolio *et al.*, 1994). *Npv30* belongs to a family of several nodulins containing highly conserved cystein clusters that are not structurally related to MTs and for which a role in metal transport has been proposed (Sandal *et al.*, 1987). Since the regulatory role of this motif has not been shown, its significance for the activity of the *cgMT1* promoter has to be demonstrated by a site-specific analysis of this sequence. Computer searches for motifs involved in mediation late nodulin gene expression identified multiple copies of the common nodulin sequences AAAGAT and CTCTT (Jorgensen *et al.*, 1991). However, the typical arrangement of AAAGAT and CTCTT sub-elements found in the OSE/NICE (organ-specific element/nodule-infected cell expression) element of legume haemoglobin promoters (Stougaard *et al.*, 1987; Szczyglowski *et al.*, 1994) was not fulfilled (data not shown). Thus, the expression of *cgMT1* in infected cells might be due to metabolic regulation, or to an unknown signal that can determine the expression of genes in infected cells.

For nitrogen fixation to occur, the plant has to supply various metal ions to the endosymbiont in the infected cells. For instance, iron and molybdenum are required for nitrogenase function. Thus, *cgMT1* expression in nodule pericycle and infected cells might be related to metal ion transport and homeostasis. cgMT1 might also be part of the antioxidant defences against reactive oxygen species (ROS) or might prevent their formation, since it has been suggested that some other plant MT genes induced during plant-pathogen interactions are involved in the detoxification of activated oxygen (Choi *et al.*, 1996; Butt *et al.*, 1998). Moreover, in animals, experimental evidence supports a role for MT as an antioxidant (Sato and Bremner, 1993). Indeed, the nitrogen fixation process has the potential to generate ROS as by-products. The high capacity of nodules to produce ROS is due to the high rate of respiration and the presence of several proteins such as leghaemoglobin and nitrogenase that are capable of reacting with $O_2$ to generate ROS (Becana *et al.*, 2000). The presence of a putative consensus antioxidant response element (ARE) in the *cgMT1* promoter would support this hypothesis. Further studies of *cgMT1* expression and promoter sequence will help to clarify the role of this gene in symbiosis.

In conclusion, the pattern of expression of *cgMT1* in non-symbiotic tissue was comparable to the data available in the literature for type 1 MT. This study and other works cited herein have demonstrated that type 1 MT exhibit a specific expression pattern within the plant. Many plant functions are commonly attributed to MT, most being related to their putative metal binding capacity and antioxidant property. *cgMT1*, which is preferentially expressed in particular cells of nodules provides a good model to understand the function of these proteins in nitrogen-fixing symbiosis.

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