

# Functional Analysis of the Metallothionein Gene *cgMT1* Isolated from the Actinorhizal Tree *Casuarina glauca*

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*cgMT1* is a metallothionein (MT)-like gene that was isolated from a cDNA library of young nitrogen-fixing nodules resulting from the symbiotic interaction between *Frankia* spp. and the actinorhizal tree *Casuarina glauca*. *cgMT1* is highly transcribed in the lateral roots and nitrogen-fixing cells of actinorhizal nodules; it encodes a class I type 1 MT. To obtain insight into the function of *cgMT1*, we studied factors regulating the expression of the MT promoter region (*PcgMT1*) using a  $\beta$ -glucuronidase (*gus*) fusion approach in transgenic plants of *Arabidopsis thaliana*. We found that copper, zinc, and cadmium ions had no significant effect on the regulation of *PcgMT1-gus* expression whereas wounding and H<sub>2</sub>O<sub>2</sub> treatments led to an increase in reporter gene activity in transgenic leaves. Strong *PcgMT1-gus* expression also was observed when transgenic plants were inoculated with a virulent strain of the bacterial pathogen *Xanthomonas campestris* pv. *campestris*. Transgenic *Arabidopsis* plants expressing *cgMT1* under the control of the constitutive 35S promoter were characterized by reduced accumulation of H<sub>2</sub>O<sub>2</sub> when leaves were wounded and by increased susceptibility to the bacterial pathogen *X. campestris*. These results suggest that *cgMT1* could play a role during the oxidative response linked to biotic and abiotic stresses.

Metallothioneins (MTs) belong to a superfamily of intracellular cysteine-rich metal-binding proteins that are widely distributed in a wide range of different organisms, including vertebrates, invertebrates, fungi, cyanobacteria, and plants. Typically, MTs have a low molecular weight (<7 kDa), high cysteine content (up to 30% of total amino acid residues), and the ability to bind metals such as zinc (Zn), copper (Cu), or cadmium (Cd) (Coyle et al. 2002; Hamer 1986; Vasak and Hasler 2000). In the past few years, the structure and function of mammalian MTs have been the object of intensive studies. A range of stimuli, including metals, hormones, cytokines, oxyradicals, inflammation, and stress, were shown to induce vertebrate MTs (Miles et al. 2000). However, their functional role in the cell has not yet been fully established. Studies conducted in animals first led to the assumption that MTs play a role in the detoxification of excess metal ions penetrating the cell, and act as a cellular reserve for Zn and Cu ions that are essential for metabolic processes (Kägi 1991). More recently, possible involvement of

MTs in the regulation of Zn finger proteins (Roesijadi et al. 1998) and in the cellular antioxidant defense system has been postulated (Sato and Bremner 1993; Viarengo et al. 2000). Thus, it is likely that MTs do not play a single role but that, as a result of evolution, some have acquired specific functions. Under normal conditions, constitutive MTs may play a background role in certain homeostatic mechanisms, whereas highly induced MT concentrations could help the plant to adapt to different environmental stresses (Miles et al. 2000).

The first plant MT gene was isolated from the flowering plant *Mimulus guttatus* (de Miranda et al. 1990). Since then, other plant MT genes have been isolated from a variety of plant species, including both monocots and dicots (Rausser 1999; Robinson et al. 1993). Unlike in animals where many MTs have been characterized, only Ec protein from wheat germ and MT1, MT2, and MT3 proteins from *Arabidopsis thaliana* have been purified in plants (Lane et al. 1987; Murphy et al. 1997). This is why the term “metallothionein-like protein” is preferred for plant MTs (Hsieh et al. 1995). Based on sequence structure, three classes of MTs have been described in plants (Cobbett and Goldsbrough 2002; Rausser 1999). Class I MTs (MT-I) are characterized by two cysteine-rich domains separated by a central cysteine-free spacer. MT-I recently were subdivided into two types according to the arrangement of cysteine residues in the amino- and carboxy-terminal domains (Yu et al. 1998). The typical representative of the MT-II class is the Ec protein from wheat embryos (Lane et al. 1987), in which the arrangement of Cys throughout the full-length sequence is not homologous with that of MT-I proteins. MT-III proteins, also called phytochelatin, consist of enzymatically-synthesized polypeptides based on repeating units of  $\gamma$ -glutamylcysteine.

Currently, over 100 sequences of plant MT-I and MT-II genes exist in databases. Those that have been characterized exhibit a diversity of gene expression patterns during plant development (Rausser 1999). MT-I type 1 transcripts accumulate preferentially in roots, whereas MT-I type 2 are expressed predominantly in aerial tissues, including leaves, stems, and flowers. Other MT-like transcripts accumulate at specific sites such as trichomes in *Vicia faba* (Foley and Singh 1994), leaflet abscission zones of elder (Coupe et al. 1995), or the tapetum in *Zea mays* (Charbonnel-Campaa et al. 2000). As reported for animals, gene expression of plant MT-like proteins is regulated by a range of endogenous and exogenous factors. MT-like genes can be induced by natural senescence, fruit ripening, ethylene, hormones, wounding, viral infection, heat shock,

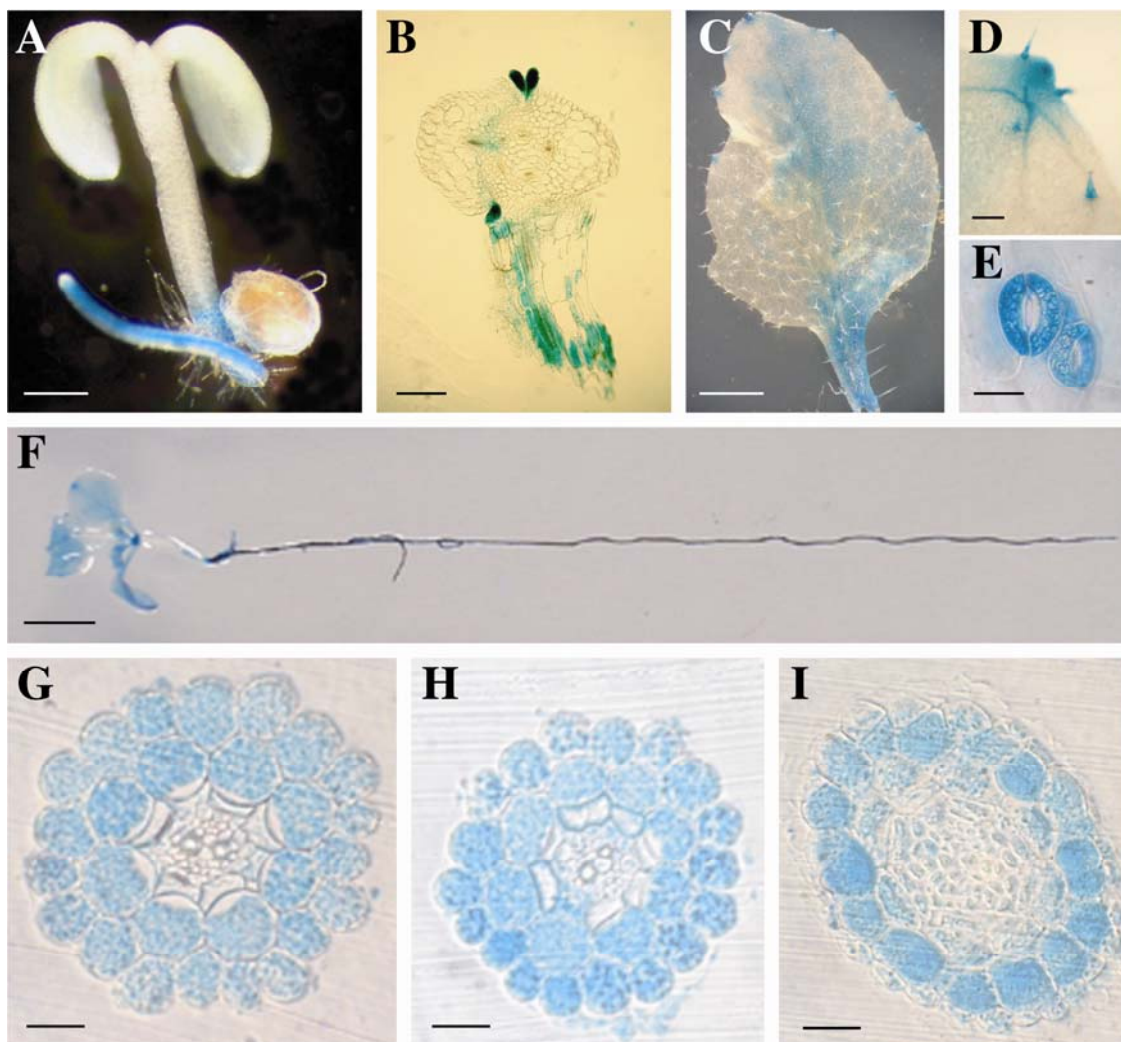
cold shock, salinity, and sucrose starvation (Rauser 1999). The effect of metals on the expression of plant MTs varies with the plant species, tissue, and MT type. For instance, metal-induced regulation was reported for MT genes in *Arabidopsis* (Zhou and Goldsbrough 1994), rice (Hsieh et al. 1995), and buckwheat (Brkljacic et al. 2004). However, because a large number of plant MTs do not respond to heavy metals, the possible role played by MTs in high-level metal tolerance remains elusive (Cobbett and Goldsbrough 2002).

*Casuarina glauca* Sieb. ex Spreng. is a tropical tree from the Casuarinaceae family that is native to Australia and Indonesia. Members of Casuarinaceae are actinorhizal woody plants that fix nitrogen when roots are nodulated by members of the soil actinomycete genus *Frankia*. Thus, these trees are able to grow in nitrogen-deficient soils and help to restore soil fertility in tropical and subtropical regions (National Research Council 1984). Therefore, this symbiotic interaction is of great agricultural importance. In order to better understand the molecular basis of actinorhizal nodule development and functioning, a nodule cDNA library from *C. glauca* was constructed. A cDNA clone of a transcript named *cgMT1* was more abundant in roots and nodules than in aerial parts. After sequencing, we established that *cgMT1* shares homology with class I type I MT-like genes. In situ hybridization studies performed on *C.*

*glauca* nodules showed that *cgMT1* transcripts were localized in the pericycle and the *Frankia* spp.-infected cells of the nitrogen-fixation zone, providing support for the hypothesis of *cgMT1* involvement in nodulation (Laplaze et al. 2002).

To further characterize *cgMT1*, we cloned a fragment of 1.15-kb corresponding to the promoter region (forming *PcgMT1*), fused to the  $\beta$ -glucuronidase (*gus*) gene and introduced via *Agrobacterium tumefaciens* into *Allocasuarina verticillata*, a close relative of *C. glauca* which can be more easily genetically manipulated. Strong reporter gene expression was observed in transgenic roots whereas, in the aerial parts of the genetically transformed plants, GUS staining was restricted to the oldest region of the shoot. Sections performed on transgenic nodules confirmed that *PcgMT1* drove expression in cortical nitrogen-fixing cells infected by *Frankia* spp. (Laplaze et al. 2002).

In the present study, we used the model plant *Arabidopsis thaliana* to gain more substantial insights into the possible role of *cgMT1*. Experimental data based on the study of transgenic plants expressing a fusion between *PcgMT1* and the reporter gene *gus* showed that wounding, H<sub>2</sub>O<sub>2</sub> treatment, and the bacterial pathogen *Xanthomonas campestris* pv. *campestris* induced the transcription of the promoter whereas metal ion treatments had no effect. We also showed that the constitutive expression of the coding sequence *cgMT1* in transgenic *Arabidopsis* con-



**Fig. 1.** Histochemical analysis of *Arabidopsis PcgMT1*- $\beta$ -glucuronidase (*gus*) expression patterns. **A**, Seedlings grown on Murashige and Skoog (MS) medium for 2 days. **B**, Stem cross-section of 7-day-old transgenic *Arabidopsis* showing expression in stipules. **C**, Activity in rosette leaf of 15-day-old transgenic *Arabidopsis* grown on MS medium with Gus activity restricted to apical and marginal hydathodes, **D**, trichomes, and **E**, in stomata. **F**, Seven-day-old *Arabidopsis* seedling. **G** through **I**, Cross-section of the main root of transgenic *Arabidopsis* grown in MS medium; **G**, mature, **H**, middle, and **I**, apex zone. Bars A and B = 200  $\mu$ m, bars C and F = 5 mm, bar D = 500  $\mu$ m, bar E = 50  $\mu$ m, bar F = 5 mm, and bars G to I = 20  $\mu$ m.

ferred increased susceptibility to the natural pathogen *X. campestris*. The possible significance of these data in relation to protection against oxidative stress is discussed.

## RESULTS

### *gus* expression directed by *PcgMT1* in *Arabidopsis*.

Twenty-one independent transgenic T1 plants were obtained by *Agrobacterium tumefaciens*-mediated genetic transformation of *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) with the binary vector pBI101.3 containing the *gus* gene driven by the 1.15-kb promoter *PcgMT1* (Laplaze et al. 2002). Among them, nine independent homozygous transgenic plant lines were established at the T2 generation and used for subsequent studies. Histochemical staining for *gus* expression during vegetative growth was carried out in plants grown axenically on solid Murashige and Skoog (MS) medium. Although variations in the levels of *gus* expression were observed among independent transformants, the qualitative pattern was always the same.

During the early stages of seedling development (within the first 48 h), *PcgMT1*-driven reporter gene activity was detected throughout radicles (Fig. 1A). GUS activity in the aerial parts of transgenic *Arabidopsis* plants carrying *PcgMT1-gus* revealed particularly strong expression at the base of petioles, stipules, trichomes, hydathodes, and stomata (Fig. 1B to E). Low *gus* expression also was found in mesophyll cells of old leaves from 15-day-old plants (Fig. 1C) and occasionally observed in the leaf vascular system (data not shown). In 7-day-old seedlings, strong blue staining was seen throughout the root system, including the main root, lateral roots, and root hairs; however, staining was absent from regions proximal to both primary and lateral root tips but present at the tips (Fig. 1F). Cross-sections of the main root indicated that the *gus* gene was expressed in the epidermis, cortex, and endodermis, whereas no or little expression was found in the stele layer (Fig. 1G to I). It should be noted that the intensity of blue staining decreased toward the root tips.

### *PcgMT1-gus* expression in response to Cu, Zn, and Cd.

Cu, Zn, and Cd ions have been shown to upregulate MT-like gene expression in some plants (Rauser 1999). Therefore, the possibility to induce the full-length MT promoter from *C.*

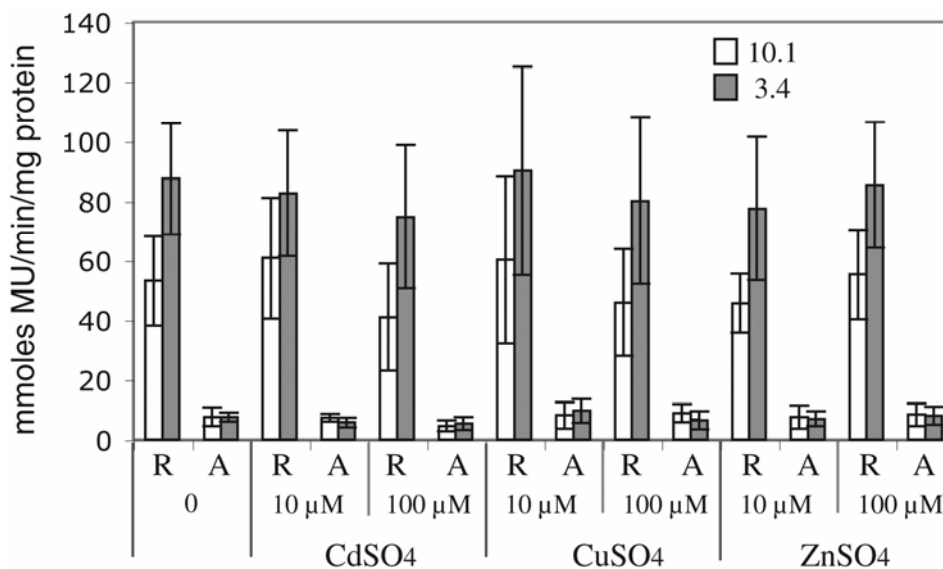
*glauca* with CuSO<sub>4</sub>, ZnSO<sub>4</sub>, or CdSO<sub>4</sub> was investigated. After incubation in 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc solution), no significant increase in blue staining resulting from *gus* expression was observed in roots and leaves of transgenic *PcgMT1-gus* plants incubated with metals compared with the one observed in transgenic plants treated with water (data not shown). Because the high basal activity in the root system could mask induction, fluorometric analyses were undertaken. Results based on the analysis of the root system and aerial part of transgenic plants from five different lines did not show any significant modification of *PcgMT1*-driven *gus* activity after metal treatments (Fig. 2).

### Wound induction of *PcgMT1-gus* expression.

Mechanical wounding was performed with a blade on mature leaves of soil-grown transgenic *Arabidopsis* and the induction of reporter gene activity was assayed by histochemical analysis. Although results shown in Figure 3B and C represent GUS activity at 24 and 72 h after treatment, respectively, induction was already visible 2 h after wounding. Strong GUS activity was observed within mesophyll cells at the cut surface and in the cells neighboring the wound site (Fig. 3B). After 72 h, the extent of blue staining area had increased around the cut edges of the leaf and *gus* activity also was observed in leaf veins (Fig. 3C). Both wounded and unwounded transgenic leaves exhibited blue staining in hydathodes.

### Induction of *PcgMT1-gus* by H<sub>2</sub>O<sub>2</sub>.

A role for MT genes in antioxidative response has been suggested recently in plants (Wong et al. 2004). Consequently, we studied induction of *PcgMT1* transgenic plants in response to different concentrations (1, 5, 10, 50, and 100 mM) of H<sub>2</sub>O<sub>2</sub>. After histochemical staining, *gus* expression was induced in leaves treated with a drop of H<sub>2</sub>O<sub>2</sub> (50 and 100 mM) (Fig. 3E and F), whereas no staining or very faint blue staining was observed in transgenic control leaves treated with a drop of water (Fig. 3D). At the lowest concentrations of H<sub>2</sub>O<sub>2</sub> (1 to 10 mM), the blue staining was observed mainly under the drop deposit (Fig. 3E); treatments with 50 or 100 mM H<sub>2</sub>O<sub>2</sub> resulted in reporter gene activity throughout the leaf. Fluorometric GUS quantitative assays were carried out to confirm the qualitative data obtained after histochemical analysis (Fig. 4). The



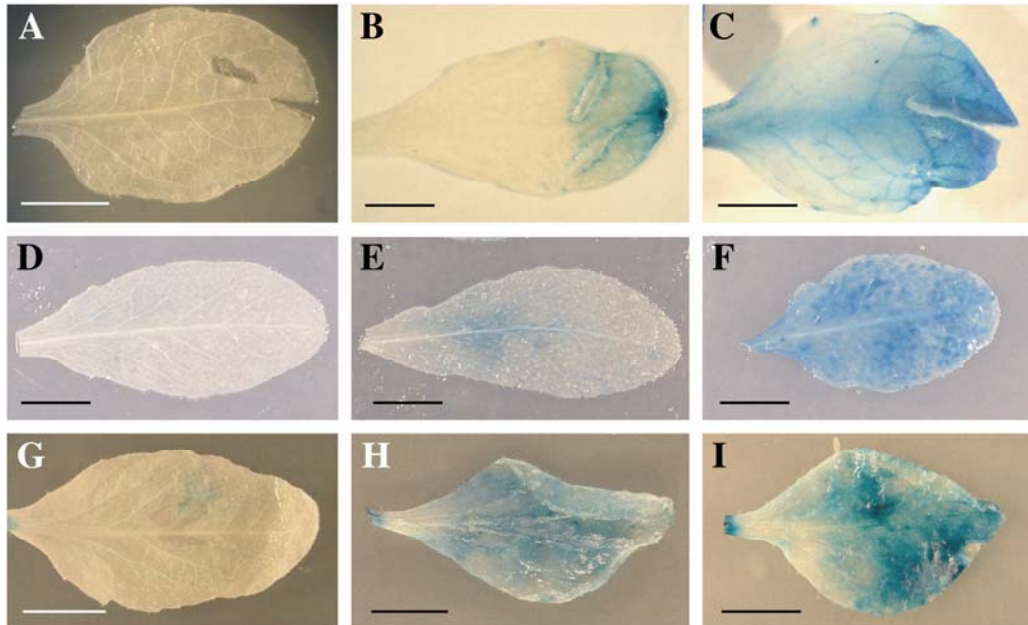
**Fig. 2.** Effect of metals on *PcgMT1*-β-glucuronidase (*gus*) expression in *Arabidopsis*. Ten-day-old transgenic plants were incubated for 24 h with CdSO<sub>4</sub>, CuSO<sub>4</sub>, or ZnSO<sub>4</sub> at a concentration of 10 or 100 μM. Results show the means resulting from the fluorometric analysis of the root system (R) or aerial system (A) from 10 plants of two independent lines (3.4 and 10.1) with the standard deviation.

results showed that the differences in *gus* activity between H<sub>2</sub>O<sub>2</sub>-treated leaves and control leaves (whether untreated or treated with sterile water) were significant.

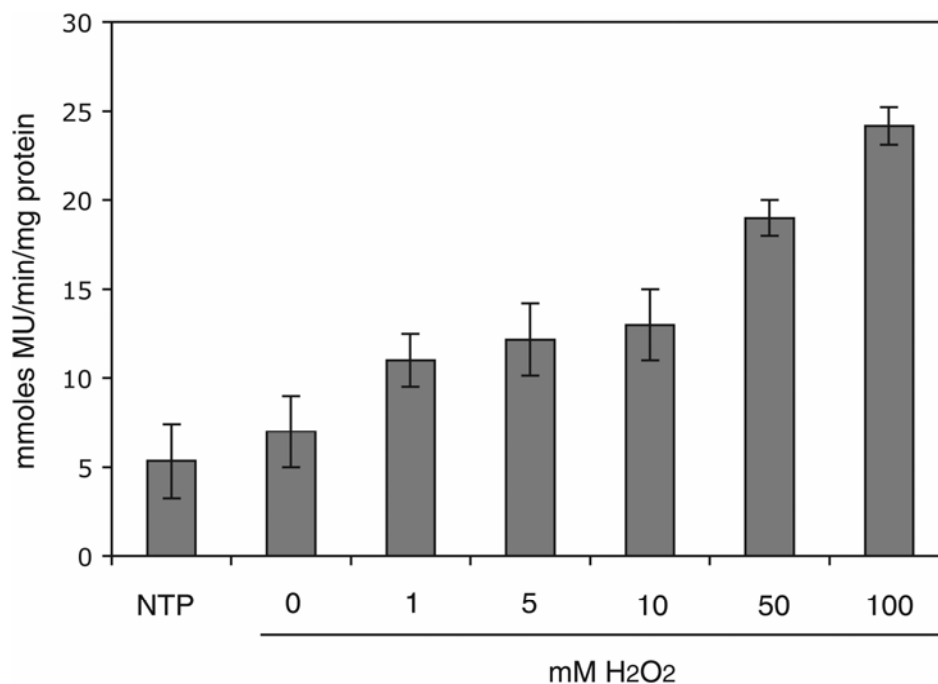
***PcgMT1-gus* expression in response to the pathogen *X. campestris* pv. *campestris*.**

*X. campestris* pv. *campestris*, the causal agent of black rot in the cruciferous family, has been identified as a potential pathogen that can induce a hypersensitive response (HR) in *A. thaliana* ecotype Col-0 (Lummerzheim et al. 1993). We tested

the effect on *PcgMT1-gus* expression of the hypervirulent isolate 147 at two concentrations (10<sup>5</sup> and 10<sup>8</sup> CFU ml<sup>-1</sup>). A spray inoculation procedure was preferred to infiltration in order to avoid the induction of the MT-like promoter by wounding or mechanical stress. The necrotic lesions characteristic of an HR were visible 2 days after treatment on 35% of the inoculated plants. Finally, studies were carried out 5 days after spray inoculation when signs of pathogen infection in leaves such as necrotic lesions were visible in the majority of inoculated plants. Disease was diagnosed by cell death at the infection



**Fig. 3.** Induction treatments in 15-day-old *Arabidopsis* transgenic plants carrying the *cgMT1* promoter driven by the  $\beta$ -glucuronidase (*gus*) reporter gene. **A to C**, Mechanical wound-induced treatment in *Arabidopsis* plants. **A**, Nontransgenic plant and **B** and **C**, *PcgMT1-gus* plants were collected 48 and 72 h, respectively, after treatment and incubated in the presence of 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide (X-gluc solution). **D to F**, *PcgMT1-gus* transgenic plants inoculated with **D**, water and **E** and **F**, 10 and 100 mM H<sub>2</sub>O<sub>2</sub>, respectively; leaves were collected 3 days after treatment. **G to I**, *PcgMT1* leaves were stained with X-gluc 5 days after spraying with **G**, sterile water or with *Xanthomonas campestris* pv. *campestris* at **H** and **I**, 10<sup>5</sup> or 10<sup>8</sup> CFU ml<sup>-1</sup>. Bars = 5 mm.



**Fig. 4.** Influence of H<sub>2</sub>O<sub>2</sub> treatment on the  $\beta$ -glucuronidase (*gus*) activity of transgenic *Arabidopsis* carrying the *PcgMT1-gus* construct. A single drop of H<sub>2</sub>O<sub>2</sub> was placed on *Arabidopsis* leaves. Nontreated plants (NTP) and plants treated with sterile water (0) were used as negative controls. GUS activity was measured by fluorometric assay 3 days after induction. Each result is the mean of at least 10 independent replicates.

site on leaves and by leaf deformation. Infection resulted in more intense GUS staining than in control plants inoculated with sterile water (Fig. 3G to I). Blue staining was particularly intense near the necrotic lesions (Fig. 3I). Leaves were collected and tested for GUS activity 7 days after infection. For each transgenic construct, two independent transformant lines were tested. Fluorometric analyses confirmed the significant increase in *gus* expression in the transgenic lines inoculated with *Xanthomonas* spp. compared with control plants (untreated transgenic plants and transgenic plants sprayed with water) (Fig. 5).

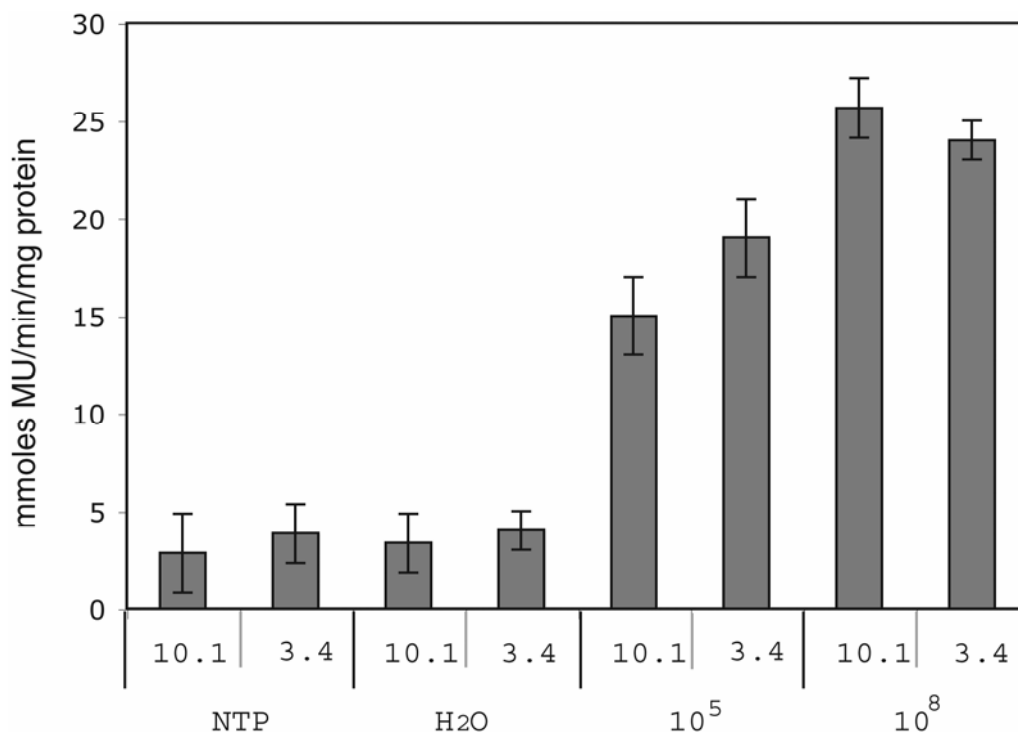
#### Genetic transformation of *Arabidopsis* with the 35S-*cgMT1* construct.

The 260-bp fragment corresponding to the coding sequence of the MT gene from *C. glauca* was placed under the control of the 35S promoter and cloned into the binary vector pKYLX71.

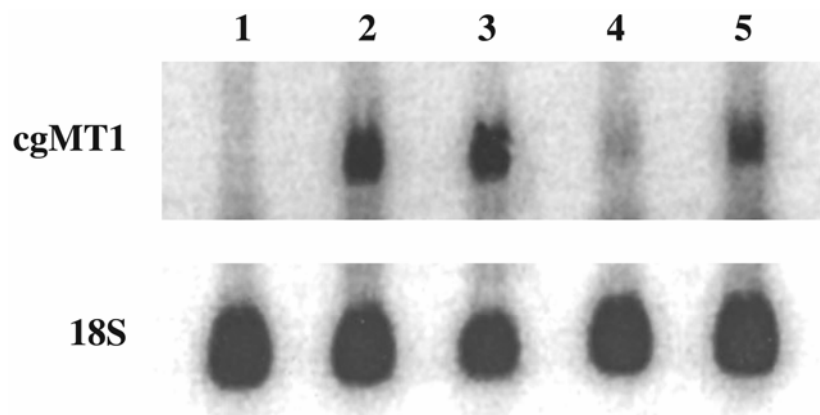
Transgenic T2 homozygous lines containing the 35S-*cgMT1* transgene were selected after genetic transformation of *A. thaliana* via *Agrobacterium tumefaciens*. Gene transfer was verified on four lines by Southern blot (data not shown) and *cgMT1* transcripts were detected by Northern blot analysis. No hybridization was observed in the wild-type Col-0 plants, whereas accumulation of *cgMT1* mRNA was found in three of the four transgenic lines tested (Fig. 6). The transgenic 35S-*cgMT1* plants did not exhibit any notable modification in their phenotype when grown in vitro or on soil (data not shown). The two transgenic lines T2-3 and T2-4 displaying a high *cgMT1* transcript accumulation were chosen for further studies.

#### Study of tolerance of 35S- *cgMT1* plants to metal ions.

In all, 10 *Arabidopsis* seeds of the two independent transgenic lines T2-3 and T2-4 carrying the 35S-*cgMT1* construct

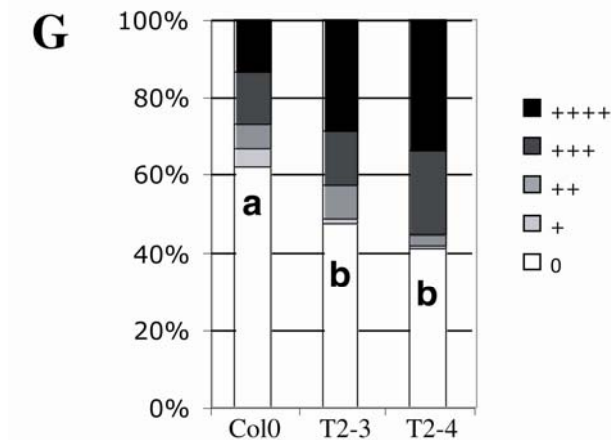
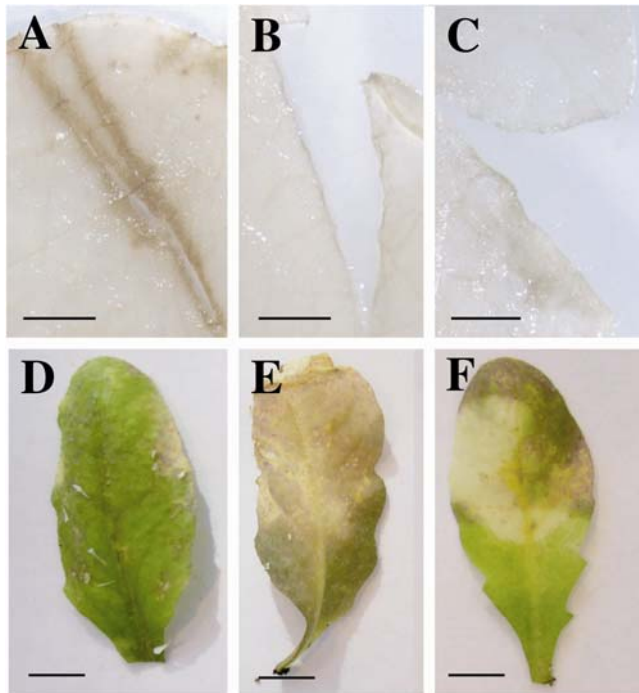


**Fig. 5.**  $\beta$ -Glucuronidase (*gus*) activity in transgenic *PcgMT1-gus Arabidopsis* plants following infection by the bacterial pathogen *Xanthomonas campestris* pv. *campestris* strain 147. Plants were inoculated with the strain 147 at two different concentrations: 10<sup>5</sup> CFU ml<sup>-1</sup> (10<sup>5</sup>) and 10<sup>8</sup> CFU ml<sup>-1</sup> (10<sup>8</sup>). Nontreated plants (NTP) and plants treated with sterile water (H<sub>2</sub>O) were used as controls. Infected leaves were collected 5 days after inoculation. Results show the means resulting from the fluorometric analysis of 10 plants from two independent lines (3.4 and 10.1) with the standard deviation.



**Fig. 6.** Northern blot analysis of transgenic *Arabidopsis* plants containing the 35S-*cgMT1* gene construct. RNA was isolated from four transgenic T2 lines of *Arabidopsis* and from one nontransgenic Col-0 plant. Lane 1, Col-0; lane 2, line T2-3; lane 3, line T2-4; lane 4, line T2-5; and lane 5, line T2-6. The same blot was hybridized with the [32-P]-dCTP-labeled *cgMT1* probe and with a 18S ribosomal probe as a loading control.

were sown together with 10 wild-type Col-0 seeds in petri dishes containing different concentrations of metal ions added in the MS nutrient medium. No germination was observed in seeds growing in the presence of 500  $\mu\text{M}$   $\text{CdSO}_4$  and  $\text{CuSO}_4$ . When exposed to 500  $\mu\text{M}$   $\text{ZnSO}_4$  and 100  $\mu\text{M}$   $\text{CdSO}_4$ , Col-0 and 35S-*cgMT1* seeds germinated but plants did not develop.



**Fig. 7.** Response of transgenic *Arabidopsis* 35S-*cgMT1* plants following wounding and infection by the pathogen *Xanthomonas campestris* pv. *campestris* 147. **A, B** and **D**, Assay for wound-inducible  $\text{H}_2\text{O}_2$  presence in leaves. Hydrogen peroxide was detected by the 3,3'-diaminobenzidine (DAB) uptake method. Leaves supplied with a solution of DAB for 4 h were wounded and further incubated for 6 h with DAB. **D, E**, and **F**, Disease lesions observed on leaves of *Arabidopsis* plants 5 days after inoculation by *X. campestris*. Plants were sprayed with a bacterial suspension at  $10^8$  CFU  $\text{ml}^{-1}$  and were photographed 5 days after inoculation. **A** and **D**, Wild-type (Col-0) *Arabidopsis*. **B** and **E**, Transgenic line T2-3. **C** and **F**, Transgenic line T2-4. **G**, Evaluation of symptoms on leaves in response to strain 147. The analysis was performed on 20 plants from each line. Chlorosis was scored after 5 days of infection: 0 = no symptoms; +, ++, and +++ = 25, 50, and 75%, respectively, of the leaf surface displaying symptoms; and ++++ = the whole leaf surface displaying symptoms. Statistical differences are shown as different letters ( $P < 0.05$ ). Bars **A**, **B**, and **D** = 1 mm; bars **E**, **F**, and **G** = 5 mm.

The root length of seedlings exposed to  $\text{CuSO}_4$  (10 to 100  $\mu\text{M}$ ),  $\text{ZnSO}_4$  (10 to 100  $\mu\text{M}$ ), and  $\text{CdSO}_4$  (10  $\mu\text{M}$ ) was scored after 12 days of growth. Statistical analyses revealed no differences between transgenic lines and nontransgenic lines (Col-0) (data not shown). Therefore, the expression of *cgMT1* in *Arabidopsis* cannot be linked to an increase of tolerance to Cu, Zn, or Cd.

#### $\text{H}_2\text{O}_2$ detection in wounded transgenic leaves.

Leaves of the two independent transgenic lines carrying the 35S-*cgMT1* construct were wounded and examined for the presence of  $\text{H}_2\text{O}_2$  using 3,3'-diaminobenzidine (DAB). This compound captures  $\text{H}_2\text{O}_2$  and forms a reddish brown polymer at sites of peroxidase activity (Thordal-Christensen et al. 1997). DAB staining was observed around the wound site of the wild-type Col-0 plants and compared with transgenic plants constitutively expressing *cgMT1*. As can be seen in Figure 7A to C, the brown staining was hardly visible in 35S-*cgMT1*-expressing leaves. This result suggests that *cgMT1* may contribute to scavenging  $\text{H}_2\text{O}_2$  resulting from wounding.

#### Infection of 35S-*cgMT1* plants by *X. campestris*.

To examine the contribution of *cgMT1* during pathogen attack, 20 plants of the two lines T2-3 and T2-4 were tested and wild-type Col-0 plants were used as controls. *X. campestris* pv. *campestris* isolate 147 was tested at a concentration of  $10^8$  CFU  $\text{ml}^{-1}$  using a spray inoculation as described previously. Infection analyses were carried out 5 days after spray inoculation. Disease symptoms were characterized by cell death at the infection site, yellowish spots, and leaf deformation. Five days after inoculation, the transgenic leaves that overexpressed *cgMT1* exhibited spreading chlorosis (Fig. 7D to F). In order to compare the response of the transgenic lines with that of wild-type Col-0 plants, infectious symptoms were visually quantified as follows: symptom-free leaves were scored 0, leaves with infectious symptoms covering less than 25% of their surface were scored +, 50 and 75% of damaged surface were respectively scored ++ and +++, respectively, and, finally, whole leaves covered with necrotic lesions were scored ++++. Analysis of the 20 plants from each line showed that 35S-*cgMT1* plants were significantly more sensitive to pathogen infection than the nontransformed plants (Fig. 7G and H).

## DISCUSSION

The *gus* activity driven by the promoter *PcgMT1* in *Arabidopsis* was globally consistent with the GUS patterns previously reported by our team in transgenic *Allocausarina verticillata* (Laplaze et al. 2002), *Nicotiana tabacum*, and *Oryza sativa* plants (Ahmadi et al. 2003). The *PcgMT1* promoter was most active in the root system and expression in the leaves was restricted mainly to hydathodes and some trichomes and stomata in *Arabidopsis*. Although we have not reported previously on the expression of *cgMT1* in stomata of *A. verticillata*, careful examination of shoot transversal sections confirmed this cell-specific expression pattern in transgenic *PcgMT1-gus Casuarinaceae* (data not shown). In transgenic *N. tabacum*, reporter gene expression was noted in neither hydathodes nor trichomes and, in transgenic rice, the strongest histochemical staining was seen in the root system and in the vascular system of mature leaves (Ahmadi et al. 2003). The specificity of expression conferred by *PcgMT1* in *Arabidopsis* is also very close to that reported for the MT-like gene *PsMTA* in pea (Fordham-Skelton et al. 1997).

Based on the ability of MTs to bind metal ions, several authors have proposed that, in plants, MTs might be involved in metal transport, sequestration, and ion homeostasis (Robinson

et al. 1993). However, only a few plant MT-like genes have been found to be metal responsive, principally to copper toxicity (Brkljacic et al. 2004; Chattai et al. 1997; Hsieh et al. 1995; Liu et al. 2002; Zhou and Goldsbrough 1994). Our data established that treatments with metals did not result in significant alterations in *PcgMTI-gus* expression in transgenic *Arabidopsis* plants. Whereas metal-responsive elements (MREs) have been identified in some plant MT-like sequences such as pea and tomato (Evans et al. 1990; Whitelaw et al. 1997), the consensus MRE core sequence (Thiele 1992) was shown to be absent in *PcgMTI* (Laplaze et al. 2002). Moreover, the constitutive expression of the MT gene from *C. glauca* did not confer any tolerance to Cu, Zn, or Cd in transgenic *Arabidopsis* plants. Thus, at this stage of the functional analysis, we cannot propose a function related to metal binding for *cgMTI*.

The expression of MT-like genes in plants has been shown to be induced by a variety of stresses (Rauser 1999); therefore, we studied the response of the *PcgMTI* promoter to biotic and abiotic factors. Our data clearly established that *PcgMTI* was induced in leaves by wounding, H<sub>2</sub>O<sub>2</sub> treatments, and the bacterial pathogen *X. campestris* pv. *campestris*. In silico analysis of the 1.15-kb promoter region of *cgMTI* revealed the presence of several potential *cis*-acting elements which may be important for the transcriptional regulation of this gene (Laplaze et al. 2002). A recent search (*unpublished data*) indicated sequence homology with a WUN-responsive element (TCATTA CGAA) (Pastuglia et al. 1997), a W-motif TTGACC that is induced by wounding, pathogens (Rushton et al. 1996), and three TGACG-motives that have been reported to be *cis*-regulatory elements involved in the methyl jasmonate response (Penninckx et al. 1998). Deletion analysis of the promoter currently is underway to understand the real contribution of the potential *cis*-acting sequences to biotic and abiotic stresses.

An oxidative burst is a common response to wounding and pathogens. Thus, the increased need for MT caused by wounding, H<sub>2</sub>O<sub>2</sub> application, and pathogen infection suggests a role for *cgMTI* in reactive oxygen species (ROS) detoxification. The production of H<sub>2</sub>O<sub>2</sub> resulting from wounding was visualized in leaves infiltrated with DAB. Whereas H<sub>2</sub>O<sub>2</sub> was clearly detected in the wounded areas of nontransformed control leaves, no or very little brown staining was observed in transgenic *35S-cgMTI* leaves. In mammals, it has been shown in vitro that MTs display oxiradical scavenging capacity, suggesting that they can neutralize ROS (Viarengo et al. 2000). The highly toxic hydroxyl radical was quenched by class I MT with an affinity higher than that of reduced glutathione (Miura et al. 1997; Thornalley and Vasak 1985). The GTGAC sequence homologous to the core of the antioxidant element (5'-puGTG ACNNNGC-3') was found in the *cgMTI* promoter; however, the flanking regions were seen to be different (Rushmore et al. 1991). The contribution of this sequence to induction by ROS remains to be established.

Plants respond to pathogens with rapid and transitory production of ROS, which is referred to as an oxidative burst (Torres et al. 2006). During this response, ROS are produced by plant cells via the enhanced enzymatic activity of plasma-membrane-bound NADPH-oxidases, cell-wall-bound peroxylases, and amine oxydases in the apoplast. H<sub>2</sub>O<sub>2</sub> generated during this response is thought to diffuse into cells and, together with salicylic acid and NO that suppress the activity of ROS detoxifying enzymes, to activate many plant defenses, including plant cell death. During incompatible reactions, a biphasic ROS production is observed, with a first phase of rapid and transient H<sub>2</sub>O<sub>2</sub> accumulation of low amplitude, followed by a second phase of higher magnitude that correlates with disease resistance. During compatible interactions, only the first peak of H<sub>2</sub>O<sub>2</sub> accumulation is observed (Lamb and

Dixon 1997). In the wild-type genotype Col-0, *X. campestris* pv. *campestris* isolate 147 induces an HR that is characterized by the appearance at the site of attack of a restricted necrotic lesion delimited by healthy tissue (Godard et al. 2000). When *cgMTI* was constitutively expressed, generalized spreading of chlorosis was observed in the inoculated leaves, followed by cell death, indicating that *cgMTI* modulates susceptibility to this bacterial pathogen. Similar data have been reported for the MT-like gene *OsMT2b* from *O. sativa* (Wong et al. 2004). When transgenic rice plants overexpressed *OsMT2b*, an increase in susceptibility to bacterial blight and blast infection was observed. The function of this rice MT-like gene as a ROS scavenger was established further by studying H<sub>2</sub>O<sub>2</sub> levels in control and transgenic cell cultures induced by the sphingolipid elicitor.

In *C. glauca*, MT genes account for 1.4% of nodules expressed sequence tags (ESTs) and, among these, *cgMTI* is the most abundantly expressed MT gene, representing 1.2% of all transcripts (Hochoer et al. 2006). This high level of expression suggests a key role for this MT in actinorhizal nodules. In situ hybridization experiments and the analysis of transgenic *PcgMTI-gus Casuarinaceae* plants have shown that *cgMTI* was highly expressed in nitrogen-fixing cells of actinorhizal nodules (Laplaze et al. 2002). Based on the functional analysis of *cgMTI* in *Arabidopsis*, it can be proposed that this MT gene from *C. glauca* helps prevent ROS accumulation in nitrogen-fixing cells of actinorhizal nodules. Indeed, symbiotic nitrogen fixation is a major source of nodule active oxygen species production and these molecules are involved in all stages of nodule development, from initiation to senescence (Becana et al. 2000; Hérouart et al. 2002; Matamoros et al. 2003). In legumes, an oxidative burst is observed when *Medicago sativa* is inoculated with rhizobia and hydrogen peroxide is found in the infection threads (Santos et al. 2001). Nod factors contribute to stimulate a spatially localized production of ROS in roots of *M. truncatula* (Ramu et al. 2002). H<sub>2</sub>O<sub>2</sub> produced during this response appears to induce the peroxidase *rip1* gene that is involved in the modulation of root hair growth and infection thread formation. Recently, the abolition of ROS production in roots has been shown to suppress root hair curling and infection thread formation, thus confirming the role of these molecules in regulating early stages of the symbiotic interaction (Peleg-Grossman et al. 2007). During nodule development, activities of several antioxidant enzymes such as catalase, superoxide dismutase, ascorbate peroxidase, and glutathione reductase have been shown to increase in the roots while there is a concomitant decline of ROS. Nodules have high rates of respiration due to the extensive energy demands of N<sub>2</sub> fixation, which results in a high flux of O<sub>2</sub> into the nodule and, hence, an elevated risk of ROS formation (Becana et al. 2000). Modulating activities of related antioxidant enzymes further confirmed the role of ROS in nodules. When a symbiotic thioredoxin gene, which confers tolerance to H<sub>2</sub>O<sub>2</sub>, was down-regulated in soybean, nodule infection and development were severely affected (Lee et al. 2005). Ascorbate, glutathione, and homoglutathione have been identified as antioxidants in nodules from legumes (Frendo et al. 2005; Matamoros et al. 2003). A recent study showed that nodules will not form on roots if glutathione synthesis is blocked (Frendo et al. 2005).

In actinorhizal plants, there are very few data related to the role of ROS in nodulation. As observed during the symbiotic process with *Rhizobium* spp., members of the actinomycete genus *Frankia* induce defense-like reactions (Berg 1983). Analysis of ESTs from *C. glauca* revealed that ESTs related to defense genes were found to be more abundant in nodules than ESTs in roots (Hochoer et al. 2006). Extracts of *Alnus rubra* root nodules have been shown to have high levels of ascorbate peroxidase activity (Dalton et al. 1987), suggesting that high levels

of hydrogen peroxide are present. The presence of superoxide dismutase, catalase, and hydroperoxidase also has been reported in *Frankia* spp. (Puppo et al. 1989; Tavares et al. 2003).

In conclusion, we suggest that *cgMT1* could be a part of the antioxidant system to prevent ROS accumulation in the nitrogen-fixing cells of the actinorhizal nodule. Efficient silencing of symbiotic genes recently has been reported using an RNAi approach in primary transformed roots and nodules of the legumes *M. truncatula* (Limpens et al. 2004) and *Lotus japonicus* genetically transformed by *Agrobacterium rhizogenes* (Kumagai and Kouchi 2003). We currently are developing this approach with the actinorhizal tree *C. glauca* because composite plants can be obtained after *A. rhizogenes* inoculation, and nodulated by *Frankia* spp. (Diouf et al. 1995). Silencing of *cgMT1* in actinorhizal roots and nodules should help to determine whether nodule development or functioning are impaired by the repression of *cgMT1*.

## MATERIALS AND METHODS

### Plant material.

*Arabidopsis thaliana* Col-0 seeds were obtained from the Nottingham Arabidopsis Stock Center. Axenic cultures were prepared by surface sterilizing seeds in 20% (vol/vol) Domestos bleach (Lever Industrial Ltd., Runcorn, U. K.) for 10 min and rinsing extensively with sterile distilled water three times. Seeds then were sown on vertical petri plates containing half-strength MS medium (Murashige and Skoog 1962) supplemented with 2% (wt/vol) sucrose and 1.2% (wt/vol) phytigel (Sigma, St. Louis). The plates were placed at 4°C for 48 h and transferred in a growth chamber at 24°C under a light intensity of 50  $\mu\text{E m}^{-2} \text{s}^{-1}$  provided by cool fluorescent tubes (Gro-Lux; Sylvania OSRAM, Munich, Germany) with a cycle of 16 h of light and 8 h of darkness and an average of 45% humidity. In some experiments, germinated seedlings were transplanted to individual pots containing soil/vermiculite (1:1) with a cycle of 12 h of light and 12 h of darkness.

### Plasmid constructs.

Construction of the plasmid *pBIN-cgMT1-gus* containing the 1,150-bp promoter *PcgMT1* linked to the *gus* reporter gene was described previously by Laplace and associates (2002).

The *Agrobacterium* spp.-based plasmid *pKYLX71-35S* (Scharidl et al. 1987) was used to generate the *pKYLX71-35S-cgMT1* construct containing the *cgMT1* coding sequence under the control of the 35S constitutive promoter from the *Cauliflower mosaic virus* (Olszewski et al. 1982). Using *cgMT1* cDNA (accession number no. Q39511) as template, a 260-bp fragment corresponding to the coding sequence was generated by polymerase chain reaction (PCR) with two oligonucleotides designed in the 5' and 3' coding regions (5'-CCAAGCTTcca ggccaaccacctcttc-3' and 3'-GCTCTAGAGcccactagcagtgcatgg-5'). The 5' and 3' primers introduced a *Hind*III restriction site and an *Xba*I site, respectively. The PCR product then was purified and cloned into the *Hind*III/*Xba*I site of *pKYLX71-35S2*. To check that base changes had not been introduced, the *cgMT1* region was sequenced. All DNA manipulations were carried out as described by Sambrook and associates (1989).

### Plant genetic transformation.

The *pBIN-cgMT1-gusA* and *pKYLX71-35S2 cgMT1* plasmids were introduced by electroporation into the disarmed *Agrobacterium tumefaciens* GV3101 strain (Koncz et al. 1994) according to Shen and Forde (1989). The presence and the integrity of the chimeric gene fusions were verified by Southern blot analysis of digested bacterial DNA (data not shown). Transgenic *A. thaliana* plants were obtained using the floral

dip method (Clough and Bent 1998). Seed from transformed plants were harvested, surface sterilized as previously described, and sown in petri dishes on solid MS medium (0.8% agar) supplemented with kanamycin at 50 mg liter<sup>-1</sup>. Kanamycin-resistant seedlings (T1) were transplanted into soil and were self-pollinated. Transgenic T2 homozygous plants were used for all further studies.

### Plant treatments by metal ions.

To test the response of *PcgMT1-gus* to Cu, Zn, and Cd, five transgenic *Arabidopsis* lines were used. Metal ion treatments were performed following the procedure published by Zhou and Goldsbrough (1994). Ten-day-old transgenic plants were incubated for 24 h with CuSO<sub>4</sub>, ZnSO<sub>4</sub>, or CdSO<sub>4</sub> at a concentration of 10 or 100  $\mu\text{M}$ . Control plants consisted of transgenic *Arabidopsis* incubated with water. GUS activities were measured in leaves and roots by histochemical and fluorometric analyses.

For metal ion treatments of 35S-*cgMT1* plants, surface-sterilized seed were germinated on MS agar medium supplemented with ZnSO<sub>4</sub>, CdSO<sub>4</sub>, or CuSO<sub>4</sub> at a final concentration of 10, 100, or 500  $\mu\text{M}$ . Twenty-five seed from each line were germinated on square petri dishes in a vertical position to enable visualization of the roots. Root measurements were made at 9 and 15 days using the ImageJ 1.31 program.

### Wounding of transgenic Arabidopsis plants.

To examine the effect of wounding on the expression of *PcgMT1-gus*, *Arabidopsis* plants first were grown for 5 weeks on soil. Selected mature leaves (Medford et al. 1992) were wounded by cutting with a scalpel blade, then left undisturbed for 3 days on the plant. Wounded leaves then were harvested to assay GUS activity as described below. Unwounded leaves of the corresponding transgenic lines were harvested at the same time and used as negative controls.

### H<sub>2</sub>O<sub>2</sub> treatment.

Ten transgenic *Arabidopsis* plants from each line were grown for 5 weeks on soil and used for oxidative stress treatments with H<sub>2</sub>O<sub>2</sub> as described by Penninckx and associates (1998). A 5- $\mu\text{l}$  droplet of H<sub>2</sub>O<sub>2</sub> at a final concentration of either 1, 5, 10, 50, or 100 mM was applied to a leaf. Transgenic control plants were treated at the same time with a 5- $\mu\text{l}$  drop of water. Leaves were collected 3 days after treatment, stained in X-gluc solution to detect *gus* expression, or frozen in liquid nitrogen for quantification of GUS activity.

### In vivo detection of H<sub>2</sub>O<sub>2</sub> in Arabidopsis leaves.

H<sub>2</sub>O<sub>2</sub> was visually detected in mature leaves by using DAB as substrate (Thordal-Christensen et al. 1997). Leaves were excised at the base with a razor blade and supplied through the cut petioles with a 1 mg ml<sup>-1</sup> solution of DAB, pH 3.8, for 4 h at 25°C in the dark. DAB-treated leaves then were wounded with a razor blade and supplied with DAB solutions for another 6 h (Orozco-Cardenas and Ryan 1999). Deep brown polymerization product produced by the reaction of DAB with H<sub>2</sub>O<sub>2</sub> was observed after removal of chlorophyll from the leaf with 70% ethanol.

### Infection by the pathogen X. campestris pv. campestris.

*X. campestris* pv. *campestris* isolate 147 (kindly provided by Dr. D. Roby, INRA Toulouse, France) was cultivated at 30°C on YPG medium (0.5% wt/vol yeast extract, 0.5% wt/vol bacteriological peptone, and 0.5% wt/vol glucose, pH 7.2) solidified with 1.5% wt/vol bactoagar (Difco Laboratories, Detroit). An overnight liquid culture of the *Xanthomonas* sp. was washed once in sterile water and adjusted to the appropriate bacterial concentration using a spectrophotometer (Jalloul et al. 2002). At

5 weeks of age, leaves of transgenic and wild-type *Arabidopsis* plants grown on soil were sprayed with the bacterial solution. The low-dose infection corresponded to a bacterial concentration of  $10^5$  CFU  $\text{ml}^{-1}$  and the high-dose infection to  $10^8$  CFU  $\text{ml}^{-1}$ . After inoculation, plants were kept under a green cover to maintain high humidity and transferred to a growth chamber with 80% humidity as previously described. For the promoter studies, control plants included *PcgMT1-gus Arabidopsis* that were sprayed at the same time with sterile water. Leaves from each individual plant were collected for further *gus* analyses. For the functional analysis of the *cgMT1* coding sequence, the symptoms resulting from the infection of *35S-cgMT1* transgenic plants by *Xanthomonas* spp. were compared with those observed on nontransformed infected plants.

#### Histochemical staining for GUS activity and microscopy.

Excised plant tissues were immersed in 1 mM X-gluc solution at 37°C for 16 h as described by Jefferson and associates (1987). To confine the localization of the blue staining, 0.5 mM  $\text{K}_3\text{Fe}(\text{CN})_6$  and  $\text{K}_4\text{Fe}(\text{CN})_6$  were added as catalysts (Guivarch et al. 1996). After staining, tissues were fixed in FAA (10% formaldehyde, 5% glacial acetic acid, and 42.5% ethanol) and chlorophyll was removed by washing the samples in 70% ethanol. Plant samples then were mounted in 50% glycerol and examined under a stereomicroscope.

#### Fluorometric assay of GUS activity.

GUS activity was assayed in leaf and root extracts of transgenic plants using 4-methylumbelliferone  $\beta$ -D-glucuronide as a substrate according to the procedures recommended by Jefferson and associates (1987). Plant material was ground in extraction buffer (50 mM sodium phosphate, pH 7.0, 5 mM dithiothreitol, 1 mM EDTA, 0.1% [wt/vol] sarcosyl and 0.1% Triton X-100). Protein concentrations were estimated by the dye-binding method of Bradford (1976) using bovine serum albumin as standard. GUS activity was quantified with a Fluoroscan II fluorimeter (Labsystems) and expressed as nmol of MU  $\text{min}^{-1}$   $\text{mg}^{-1}$  of extracted proteins. After three independent experiments, mean values and their standard deviations were calculated.

#### RNA isolation and Northern blot analysis.

Total RNA was isolated from transgenic and untransformed control plants using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Northern blot analysis was performed using the Northern-Maxtm-Gly kit (Ambion, Austin, TX, U.S.A.). Total RNA (10  $\mu\text{g}$ ) was denatured in a glyoxal/dimethyl sulfoxide mix for 30 min at 50°C, separated by gel electrophoresis in 1% wt/vol agarose gel, and transferred to a positively charged nylon membrane. The membrane was prehybridized and then hybridized with a  $^{32}\text{P}$ -labeled *cgMT1* probe obtained by PCR using the primers 3'-gccactagcagttgcatg-5' and 5'-ccaggccaaccactctt-3'. The 260-bp amplified fragment was radiolabeled using  $\alpha^{32}\text{P}$ -dCTP (50  $\mu\text{Ci}$ ) random prime labeling Megaprime DNA labeling system (Amersham, Tokyo). Hybridization was carried out for 16 h (overnight). The nylon membrane then was washed twice for 10 min in  $2\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate), sodium dodecyl sulfate (SDS) 0.1%;  $1\times$  SSC, SDS 0.1%; and  $0.5\times$  SSC, SDS 0.1%. Blot hybridizations were quantified with a phosphorimager Typhoon 9400 and normalized with reference to a ribosomal RNA (18S) hybridization.

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