The *Casuarina glauca metallothionein I* promoter in nodulated transgenic hairy roots of the actinorhizal plant *Datisca glomerata*

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Abstract. The activity of the promoter of a metallothionein gene expressed in actinorhizal nodules of *Casuarina glauca* Sieber ex Spreng., *CgMT1*, has previously been analysed in Casaurinaceae and in tobacco (*Nicotiana tabacum* L.), *Arabidopsis* and rice. In all these plants, the promoter showed high activity in the root cortex and epidermis, making it a useful tool for the expression of transgenes. Therefore, its activity was now analysed in transgenic root systems of *Datisca glomerata* (C. Presl) Baill, an actinorhizal plant from a different phylogenetic group than *C. glauca*, using the same *CgMT1*:: *GUS fusion* as in previous studies. However, in contrast with all other plant species examined previously, the *CgMT1*::*GUS* construct showed no activity at all in *D. glomerata* hairy roots: the expression pattern in nodules resembled that found in *C. glauca* nodules. This is probably due to the changed hormone balance in hairy roots since experiments on the *CgMT1*:: *GUS* construct in transgenic *Arabidopsis* showed that *CgMT1* promoter activity was repressed by auxin or cytokinin, respectively. Yet, in hairy roots of the model legume *Lotus japonicus* L. induced by the same *Agrobacterium rhizogenes* strain, the *CgMT1* promoter was active in roots and not in nodules. These results indicate that although the expression of pRi T-DNA genes leads to changes in root hormone balance, these changes do not abolish the differences in phytohormone levels or sensitivity between plant species. Therefore, gene expression data obtained using transgenic hairy root systems have to be viewed with care, not only due to the disturbed hormone balance, but also because the effects of the pRI-T-DNA genes can differ between species.

Additional keywords: *Agrobacterium rhizogenes*, *Lotus japonicus*, metallothionein, root nodules, roots.

Introduction

Two types of nitrogen-fixing root nodule symbioses are known: (i) legume–rhizobia symbioses between ~80% of all legumes and a group of Gram-negative soil bacteria, collectively called rhizobia (reviewed by Sprent 2006) and (ii) actinorhizal symbioses between Gram-positive actinomycetes of the genus *Frankia* and a group of plant species belonging to eight different families, collectively called actinorhizal plants (Benson and Silvester 1993). Three major phylogenetic subgroups of actinorhizal plants have been identified. The first subgroup, Fagales, includes the plant families Betulaceae, Myricaceae and Casuarinaceae. The second subgroup, Cucurbitales, includes the plant families Datiscaceae and Coriariaceae. The third subgroup, Rosales, includes the plant families Rhamnaceae, Rosaceae and Elaeagnaceae (Swensen and Mullin 1997).

Mature actinorhizal nodules are coralloid organs composed of multiple lobes, each of which represents a modified lateral root without root cap, with a superficial periderm and infected cells in the expanded cortex (Pawlowski and Bisseling 1996). The activity of the apical meristem leads to the formation of a developmental gradient of infected cortical cells, allowing the delineation of an infection zone, nitrogen-fixation zone and senescence zone in the cortex. In nodules formed on the roots of *Datisca* or *Coriaria* species, the pattern of infected cells is unusual; they form a continuous patch on one side of the acentric stele, not interspersed with uninfected cells (Newcomb and Pankhurst 1982; Hafeez *et al*. 1984; Fig. 1*a*).

CgMT1 represents a gene encoding a type I metallothionein (MT); the cDNA was isolated from a library prepared from mRNA from young nitrogen-fixing *Casuarina glauca* Sieber ex Spreng. nodules. MTs are defined as low molecular mass (<10 kDa) cysteine-rich proteins that bind heavy metals (Laplaze *et al*. 2002). They play a role in metal homeostasis and are
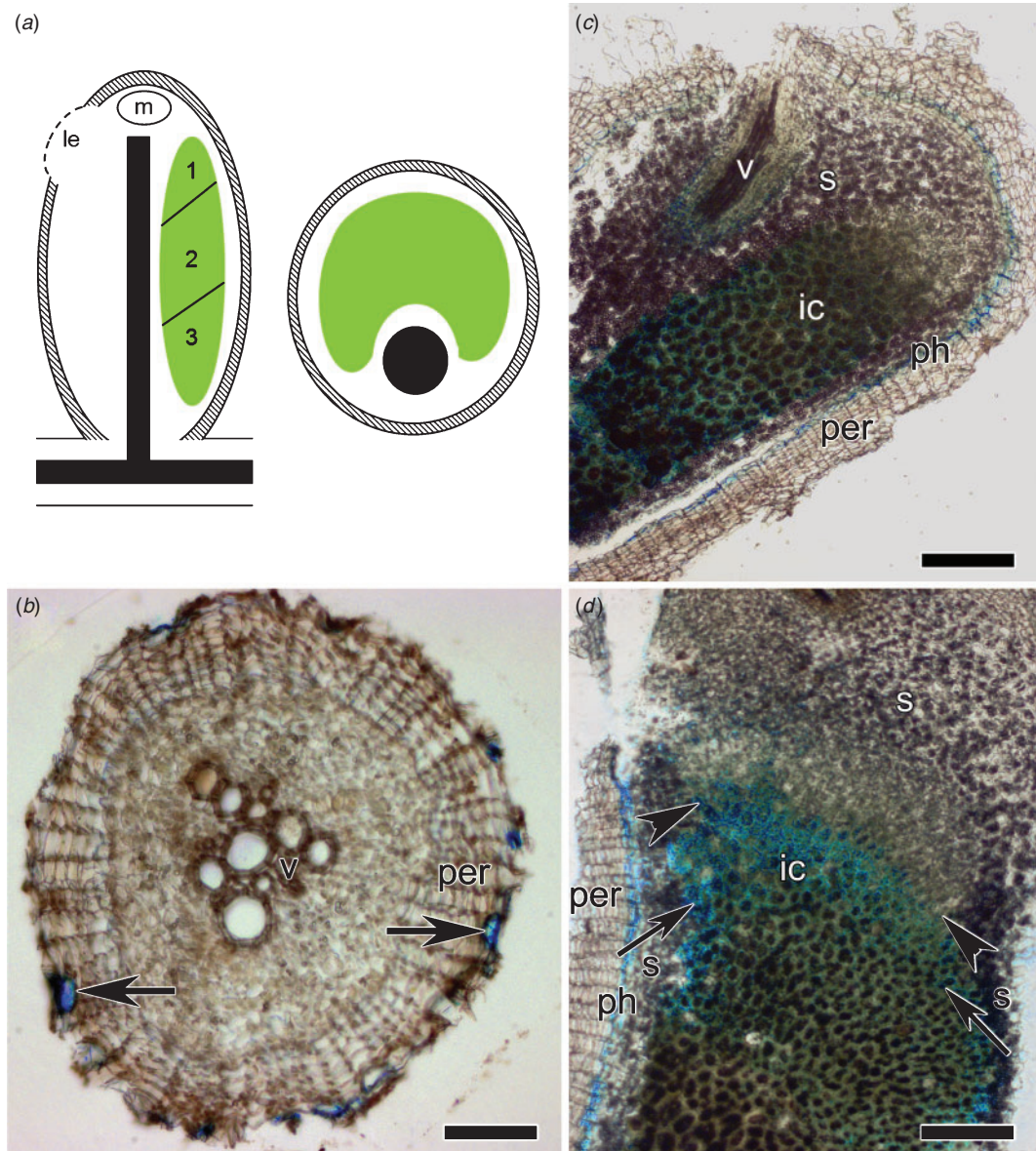
Fig. 1. Histochemical analysis of GUS activity in transgenic *Datisca glomerata* plants expressing the *CgMT1*::*GUS* construct. Roots and nodules were stained as described in Materials and methods. Blue staining indicates GUS activity. (*a*) Arrangement of infected cells in actinorhizal nodules of Datiscaceae and Coriariaceae as continuous patch (labelled in green) at one side of the acentric stele (labelled in black), shown in longitudinal section and in cross-section. The zonation of the infected cells is indicated (1, infection zone; 2, nitrogen fixation zone; 3, zone of senescence). m, meristem; le, lenticel. (*b*) Cross-section of a transgenic *D. glomerata* root. Arrows point to dead cells in the outer layer of the periderm that stain blue due to colonisation by soil bacteria. No blue staining is detected in living tissue. (*c*) Longitudinal section through a stained *D. glomerata* nodule lobe. GUS activity is seen in the phellogen (ph), the infected cells (ic) and in the pericycle of the nodule vascular bundle (v). No GUS activity is detectable in the starch-containing uninfected cells (s) of the nodule cortex or in the periderm (per). (*d*) Detail of the developmental gradient of infected cells. Uninfected cortical cells contain large amyloplasts (s, starch). Arrows point to the putative beginning of the nitrogen fixation zone, where *Frankia* has differentiated nitrogen-fixing vesicles in radial orientation that form a ring around the central vacuole of each infected cell. *CgMT1*::*GUS* expression starts before the onset of *Frankia* nitrogen fixation; arrowhead point to the onset of *GUS* expression. Bars denote (*b*, *d*) 50 μm, and (*c*) 100 μm.

involved in the detoxification of non essential metals like cadmium or arsenic (Miles *et al.* 2000; Cobbett and Goldsbrough 2002; Lee *et al.* 2004; Yang *et al.* 2009). Their expression is highly induced under different environmental stress conditions (Miles *et al.* 2000), which in some cases could be linked to a role in the removal of reactive oxygen species (ROS; Kim and Koh 2001; Xue *et al.* 2009; Yang *et al.* 2009; Zhu *et al.* 2009).

In *C. glauca* nodules, *CgMT1* transcripts were localised by *in situ* hybridisation. *CgMT1* mRNA was detected in the mature infected cells where bacterial nitrogen fixation takes place and in the pericycle of the nodule vascular system. No expression of *CgMT1* was found in young infected cells of the infection zone, the endodermis and the phloem (Laplaze *et al*. 2002). In transgenic *Allocasuarina verticillata* (Lam) L.A.S. Johnson nodules containing a *CgMT1* promoter::β-glucuronidase (GUS) fusion, GUS activity was consistently restricted to the infected cells of the nitrogen-fixation zone. No staining was seen in any part of the vascular bundle. The discrepancy between both sets of results – the absence of GUS activity in the nodule pericycle – might be explained by the assumption that the hybridising mRNA in the pericycle is due to cross-hybridisation with transcripts from another *MT* gene (Laplaze *et al*. 2002). It might also be due to the fact that the nodules used for *in situ* hybridisation experiments came from greenhouse-grown plants, whereas the nodules used for GUS staining came from tissue culture-grown plants (Laplaze *et al*. 2002).

The highest expression levels of the *CgMT1*::*GUS* construct in transgenic *A. verticillata* plants was observed in roots, specifically in the vascular pericycle and in the subepidermal cell layer. GUS activity was also detected in the root cortex and in the root epidermis including root hairs (Laplaze *et al*. 2002). The high expression levels suggested an essential role for this MT in root metabolism. In the aerial part of the *A. verticillata*, the apex of the young photosynthetic branchlets showed little or no GUS expression. Blue staining was found at the base of photosynthetic branchlets, especially in leaves with extensive expression in the mesophyll cells and in the vascular system (Laplaze *et al*. 2002).

The expression pattern conferred by the *CgMT1* promoter suggested that this promoter could be a valuable tool for ectopically expressing transgenes in roots. To evaluate the usefulness of the MT promoter from *C. glauca* for biotechnological applications, the *CgMT1*::*GUS* fusion was introduced into tobacco and rice. In transgenic tobacco (*Nicotiana tabacum* L.) root systems containing the *CgMT1*::*GUS* construct, GUS activity was observed in the primary root cortex and the vascular system while all tissues of lateral roots, including the epidermis, showed strong GUS activity (Ahmadi *et al*. 2003). In rice (*Oryza sativa* L.) root systems, GUS staining was observed mainly in root tips, in the elongation zone of the primary and secondary roots and in lateral roots in the endodermis and pericycle cell layers as well as in the vascular parenchyma, whereas no GUS activity was detected in the root differentiation zone (Ahmadi *et al*. 2003).

Arabidopsis thaliana (L.) Heynh. plants carrying the *CgMT1*::*GUS* construct (Obertello *et al*. 2007) revealed strong blue staining all throughout the root system in 7-day-old seedlings, whereas staining was absent from regions close to root tips, but present at the tips. The *CgMT1* promoter was active in the root endodermis, in the cortex and in the epidermis. In summary, in dicotyledonous plants, CgMT1 promoter activity levels in roots were in the range of those of the CaMV35S promoter (Ahmadi *et al*. 2003), but in contrast with the CaMV35S promoter, the CgMT1 promoter showed particularly high activity in the root epidermis; that is, in the tissue where genes encoding components of the symbiotic signal transduction pathway are

expressed in legumes, and presumably also in actinorhizal plants (Ahmadi *et al*. 2003; Obertello *et al*. 2007).

Obertello *et al*. (2007) used the transgenic *A. thaliana* plants expressing *CgMT1*::*GUS* also to analyse the conditions that led to an increase of *CgMT1* mRNA levels on top of the developmentally regulated expression. They found that copper, zinc, and cadmium ions had no significant effect on *CgMT1*::*GUS* expression whereas wounding, infection by *Xanthomonas campestris* pv. *campestris* or H_2O_2 treatment led to an increase in reporter gene activity in transgenic leaves.

Under stress conditions plant cells increase the production of ROS (Torres *et al*. 2006). The stress conditions can be biotic (pathogens, herbivores) or abiotic (waterlogging, excess light, extreme temperature, salinity and drought). Thus, the increased need for CgMT1 caused by wounding, H_2O_2 application, and pathogen infection suggested a role for this protein in ROS detoxification.

To see whether the *CgMT1* promoter could be used to express genes in roots and nodules of actinorhizal plants from another subgroup, Cucurbitales, the *CgMT1*::*GUS* construct used previously (Laplaze *et al*. 2002; Ahmadi *et al*. 2003; Obertello *et al*. 2007), was introduced into *Datisca glomerata* (C. Presl) Baill. using hairy root transformation with *Agrobacterium rhizogenes*. The results showed that in contrast with all other systems examined earlier, the promoter was not active in *D. glomerata* roots. In order to find out whether this difference in promoter activity was based on the fact that this was the first attempt to express *CgMT1*::*GUS* in hairy roots, we examined the effect of phytohormones on the *CgMT1* promoter activity in *Arabidopsis*, and analysed the *CgMT1*::*GUS* construct in hairy roots of a legume, *Lotus japonicus* L.

Materials and methods

Plant and bacterial material

Datisca glomerata (Presl) Baill seeds were obtained from plants growing in Vaca Hills, California. Seeds were germinated on sand wetted with water and plantlets were transferred to pot soil (Weibull Trädgard AB, Hammenhög, Sweden) watered with 1/4 strength N-free Hoagland's medium (Hoagland and Arnon 1938). Light conditions in the greenhouse were 150–300 µmol photons $m^{-1} s^{-1}$. In the growth chamber the light conditions were 16 h light, 80–260 µmol photons $m^{-1} s^{-1}$, 8 h darkness with temperatures of 26 and 18°C, respectively.

D. glomerata plants were infected using crushed nodules from other *D. glomerata* plants containing a non-culturable *Frankia* strain originating from *Coriaria nepalensis* Wallich nodules from Pakistan (Mirza *et al*. 1992).

L. japonicus cv. Gifu plants with transgenic hairy root systems growing in a 1 : 1 mixture of perlite and vermiculite wetted with 1/4 strength N-free Hoagland's medium were infected *Mesorhizobium loti* strain TONO grown in YEB medium (Sambrook *et al*. 1989; Kawaguchi *et al*. 2002).

A. thaliana ecotype Columbia seeds containing the *CgMT1*::*GUS* construct (Obertello *et al*. 2007) were obtained from Claudine Franche (IRD Montpellier, France), surface-sterilised and germinated on MS medium (Murashige and Skoog 1962) containing 2% sucrose in vertical Petri dishes, starting with 2 days of vernalisation at 4°C in the dark, followed

by 3 days in a growth cabinet at 24°C and 60% RH and a light rhythm of 16 h light/8 h dark. Six days after germination, seedlings were transferred to vertical Petri dishes with test media.

Molecular biological methods

The *CgMT1*::*GUS* construct (Laplaze *et al*. 2002) was transformed into *Agrobacterium rhizogenes* strain LBA1334 by electroporation (Diaz *et al*. 2005). The electroporation conditions were 1.25 V; 200 Ω resistance; 25 µF capacity. Transformants were selected at 28°C on YEB medium containing 50 µg mL^{-1} kanamycin.

In order to confirm (i) transformation and (ii) transfer of the binary vector, total DNA was isolated from ~50 mg of hairy roots of each plant according to Edwards *et al*. (1991). PCR was performed for the *CgMT1*::*GUS* construct (primers CgMT1pro5 5′-ACCC GGGACTATAGGGCACGCGTGG-3′ and EcGUS3 5′-CCGGC TTTCTTGTAACGC-3′; Laplaze *et al*. 2002) to confirm co-transfer of the binary vector, for the *rolB* gene from the T-DNA (primers 5′-GCTCTTGCAGTGCTAGATTT-3′ and 5′-GAAGG TGCAAGCTACCTCTC-3′; Alpizar *et al*. 2006) to confirm that the roots were transformed, for the *virD* gene of the pRi plasmid to check against contamination with agrobacterial DNA (primers 5′-ATGTCGCAAGGACGTAAGCCGA-3′ and 5′-GGAGTCTT TCAGCATGGAGCAA-3′; Alpizar *et al*. 2006) and for plant ubiquitin (Heidstra *et al*. 1997) as positive control.

Hairy root transformation

D. glomerata hairy root transformation was performed according to Markmann *et al*. (2008) with the following modifications: 6- to 7-week-old plants were infected with *A. rhizogenes* strain LBA1334 containing the *CgMT1*::*GUS* construct (Laplaze *et al*. 2002). Hairy roots appeared 4 weeks after infection with *A. rhizogenes*. After inoculation with crushed nodules containing non-culturable *Frankia* bacteria, the plants were watered with 1/4 strength Hoagland's medium containing 1 mM KNO$_3$, since previous experiments had shown that this concentration of KNO$_3$ did not inhibit nodulation, but prevented the plants to develop N deficiency symptoms when nodulation did not occur soon enough.

For control against artefactual GUS activity staining, wild-type nodulated *D. glomerata* root systems (seven plants with altogether 261 nodules) were stained with X-gluc. No blue staining could be detected. Furthermore, no GUS activity staining could be detected in nodulated hairy root systems of *D. glomerata* (eight plants with altogether 197 nodules) induced by infection with *A. rhizogenes* strain LBA1334 without a binary vector (data not shown). For one series of plants transformed with *A. rhizogenes* LBA1334 (pCgMT1::GUS) consisting of four plants, 100 mg of roots per root system were used for DNA isolation and the presence of the expression cassette in the binary vector and the T-DNA was confirmed by PCR. Control PCRs were performed for plant ubiquitin and *A. rhizogenes virD*, which is located on the pRi plasmid but not on the T-DNA. All plants were positive for ubiquitin and the T-DNA gene *rolB*, and only the three plants whose nodules showed GUS staining were positive for the presence of the *CgMT1*:: *GUS* cassette. All PCRs for *virD* were negative.

L. japonicus hairy root transformation was performed according to Markmann *et al*. (2008) with some modifications. Briefly, Petri dishes with sterile seeds were placed in a growth cabinet at 24°C and 60% of RH for 3 days in a slanted position in the dark. Then, plates were incubated under a photoperiod of 16 h light/8 h dark for 3 days. The hypocotyls were cut off above the root with a scalpel dipped in *A. rhizogenes* grown on YEB agar. The seedlings were transferred to plates containing B5 medium with 2% sucrose and 1% micro agar (Duchefa, Haarlem, The Netherlands). The plates were placed in a growth cabinet at 18°C and 60% of RH for 2 days in the dark, then kept under a photoperiod of 16 h light/8 h dark for 3 days. The seedlings were then transferred to new B5 medium plates with 300 µg mL^{-1} cefotaxim and kept at constant 24°C, photoperiod 16 h light/8 h dark and 60% of RH. When re-growth of *A. rhizogenes* was observed, the seedlings were transferred to fresh plates. When hairy roots had reached at least 1 cm in length (after 2–3 weeks), plantlets were transferred to pots containing a mixture of vermiculite/ perlite (1 : 1) and watered with 1/4 Hoagland's medium with 10 mM KNO$_3$. After 1 week, the plants were ready for infection by rhizobia. From that point on, they were watered with 1/4 Hoagland's medium without nitrogen.

Experiments on phytohormone effects

Four days after germination, *CgMT1*::*GUS Arabidopsis* seedlings were transferred to vertical Petri dishes containing MS medium with 2% sucrose and different concentrations of phytohormones (5 µM 6-benzylaminopurine (BAP), 10 µM BAP, 100 nM naphthylacetic acid (NAA), 1 µM NAA, 10 µM 1-aminocyclopropane-1-carboxylic acid (ACC), or 30 µM 1-naphtoxy acetic acid (NOA), respectively). After 2 days, seedlings were harvested for GUS activity staining.

GUS staining and documentation

For the detection of GUS activity, root systems of *D. glomerata* or *L. japonicus*, and *Arabidopsis* seedlings, were washed once in reaction buffer (50 mM sodium phosphate pH 7.2, 1 mM EDTA, 0.1% (v/v) Triton X-100), then vacuum-infiltrated with reaction buffer containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and incubated for 12–24 h at 37°C. Afterwards, samples were fixed for 12 h in a solution containing 5% formaldehyde, 5% acetic acid, and 50% ethanol and washed several times in 70% ethanol. Afterwards, *D. glomerata* and *L. japonicus* roots and nodules were embedded in 3% agarose and sliced into 40- to 60 µm thick sections on a vibratome (Leica VT1000E, Wetzlar, Germany). *D. glomerata* nodule sections and *L. japonicus* root and nodule sections were observed under an Axiovert 200M or an AxioImager.Z1 microscope (Zeiss, Jena, Germany), using bright field microscopy. *Arabidopsis* seedlings were observed under a Lumar V12 stereomicroscope (Zeiss). Pictures were taken using digital cameras AxioCam HRc or MRc5 (Zeiss).

The second *L. japonicus* hairy root series was used for detailed cytological analysis. For this purpose, 0.25 mM

potassium hexacyanoferrates were added to the reaction buffer to prevent diffusion of the GUS reaction products before oxidation and dimerization. After GUS staining and fixation, roots and nodules were embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to the instructions of the manufacturer. Sections of 5–6 μm were cut on a rotary microtome HM360 (Microm, Walldorf, Germany) and counterstained with 0.01% Ruthenium Red dissolved in 0.1 M borate buffer pH 9.2 for 5 min (Gutierrez-Gonzalvez *et al.* 1984).

Results

CgMT1::GUS *activity in hairy root systems of* D. glomerata

To analyse whether the *CgMT1 promoter* from *C. glauca* was active in roots and nodules of *D. glomerata*, the *CgMT1::GUS* construct described by Laplaze *et al.* (2002) was transformed into *Agrobacterium rhizogenes* strain LBA1334 for hairy root transformation of *D. glomerata*.

The nodulated root systems of plants from three independent series, each consisting of 10 plants with hairy root systems, were stained for GUS activity as described in 'Materials and methods'. From each series, six plants showed GUS staining, i.e. co-transfer of the binary vector together with the Ri plasmid. All plants with hairy roots showed reduced nodulation compared with plants with wild-type root systems, as has also been observed for other constructs (B. Rashidi and K. Pawlowski, unpubl. obs.). The 18 plants that showed co-transfer had altogether 38 nodules, all of which stained blue, and were therefore available for analysis. The results are summarised in Fig. 1. No GUS staining was detected in the entire root systems of the *D. glomerata* plants whose nodules showed GUS staining (data not shown). The only GUS activity visible on roots was confined to individual dead cells of the outer layers of the periderm, presumably due to the presence of *GUS*-expressing rhizosphere bacteria (Fig. 1*b*). In all nodules examined, GUS activity was visible in the infected cells and in the phellogen (Fig. 1*c, d*). The onset of GUS expression preceded bacterial nitrogen fixation, as indicated by the differentiation of nitrogen-fixing vesicles, which, in nodules of actinorhizal Cucurbitales, form a 5–8 μm thick sphere around the central vacuole that is visible in light microscopy (Fig. 1*c, d*). Weak GUS activity was also found in the pericycle of the nodule vascular system (Fig. 1*c*).

Is the activity of CgMT1::GUS *affected by phytohormones?*

Despite its high expression levels in the root cortex in rice, *Arabidopsis* and tobacco, *CgMT1::GUS* did not display any activity in roots of *D. glomerata*. In the previous studies, rice plants were grown in peat, tobacco in perlite/vermiculite, *Arabidopsis* in soil and *A. verticillata* was grown in tissue culture (Laplaze *et al.* 2002; Ahmadi *et al.* 2003; Obertello *et al.* 2007), whereas in this study, *D. glomerata* was grown in soil; hence, the culture conditions could not explain the differences in promoter activity. However, all the three previous studies used stable transgenic plants whereas in the present study chimeric plants with transgenic hairy roots were used. The T-DNA of the root-inducing (Ri) plasmid of

Agrobacterium rhizogenes carries a set of genes encoding enzymes that control the biosynthesis of auxin and cytokinin (Schmülling *et al.* 1988) and change the cellular response to auxin (Maurel *et al.* 1991). Hence, hairy roots have a disturbed phytohormone balance that results in lack of geotropism and strong lateral branching. To see whether changes in phytohormone levels affected the activity of the *CgMT1* promoter, transgenic *Arabidopsis* plants with *CgMT1::GUS* (Obertello *et al.* 2007) were analysed for GUS activity on media with different phytohormone concentrations. The results are displayed in Fig. 2. Both auxin and cytokinin had a negative effect on the activity of the *CgMT1* promoter. GUS staining was significantly reduced after growth on medium with 5 μM benzyladenopurine (BAP) for 2 days and abolished when 10 μM BAP was used. *CgMT1::GUS* expression was also abolished with 100 nM or 1 μM NAA, whereas 10 μM ACC and 30 μM of the auxin transport inhibitor NOA had no significant effect except for some increase in GUS activity in the apical meristem and the cotyledons.

Activity of CgMT1::GUS *in hairy roots of the legume* L. japonicus *cv. Gifu*

In an independent attempt to determine whether the lack of activity of the *CgMT1* promoter in hairy roots of *D. glomerata* was due to the changed phytohormone balance in *A. rhizogenes* transformed as opposed to wild-type roots, the same *A. rhizogenes* strain and binary vector were used to induce hairy roots on the model legume *L. japonicus*. Two series consisting of 10 plants each were transformed, and eight and seven, respectively, of the plants showed co-transfer of the binary vector as proven by GUS staining. For the first series, PCR controls were performed. All plants that showed GUS staining were positive for the *CgMT1::GUS* construct. The control PCRs for *rolB* and ubiquitin were equally positive for all plants that showed GUS staining, while the control PCR for *virD* was negative for all 10 plants. All eight root systems containing the binary vector showed the same GUS activity staining pattern, namely, strong blue staining of roots (Fig. 3*a–c*) and no staining of mature nodules (Fig. 3*e*). In roots, GUS activity was detected in the epidermis including root hairs, in the cortex and in the vascular system (Figs 3*b, c*). A cross-section of a nodule shows the lack of GUS activity in the entire organ, except in the remains of the root cortex (Fig. 3*c*). This pattern was also found in all seven GUS-staining plants of the second series. Young nodules (10 days after infection), however, showed strong GUS activity in all cell types, particularly in the inner tissue (Fig. 3*d*).

Discussion

In this study, the cell-specific activity of the *Casuarina glauca MT1* gene promoter was examined in heterologous systems in order to assess its potential for the expression of transgenes in hairy root systems of actinorhizal plants.

CgMT1::GUS *activity in roots of different plant species*

The activity of the *CgMT1* promoter had been stronger than that of the CaMV*35S* promoter in the root systems of the transgenic plant species tested so far, in particular, in root
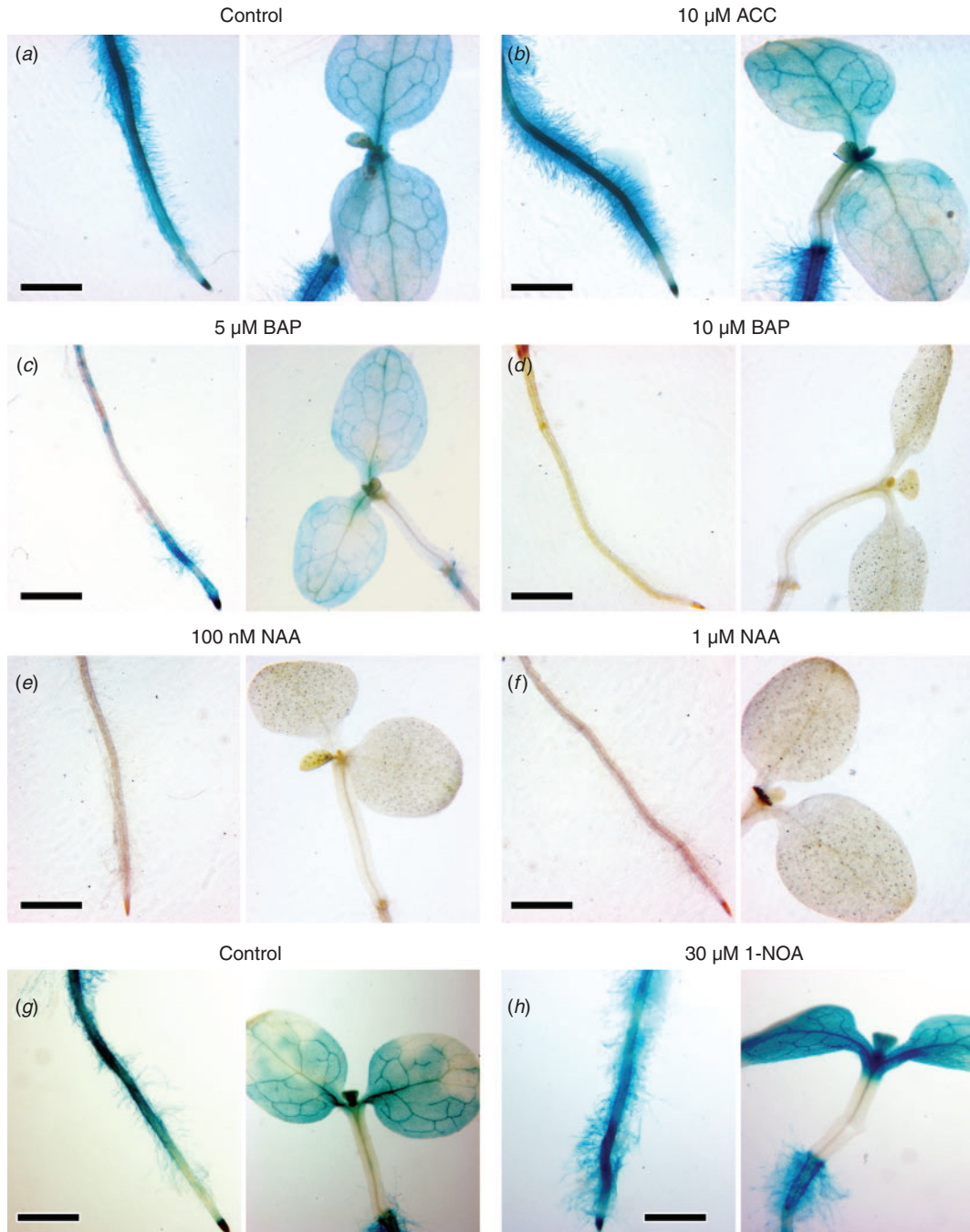
Fig. 2. Effect of phytohormones on GUS activity in transgenic *Arabidopsis thaliana* seedlings containing the *CgMT1*::*GUS* construct. (*a*–*f*) and (*g*, *h*) represent results of independent experiments. (*a*, *g*) Control seedlings without exogenously added phytohormones; (*b*) 10 µM ACC; (*c*) 5 µM BAP; (*d*) 10 µM BAP; (*e*) 100 nM NAA; (*f*) 1 µM NAA; (*h*) 30 µM 1-NOA. Bars denote 250 µm.

cortex and epidermis (Laplaze *et al*. 2002; Ahmadi *et al*. 2003; Obertello *et al*. 2007). In these previous studies, the organ specificity of the *CgMT1*::*GUS* construct in dicots (*A. verticillata*, tobacco, *Arabidopsis*) and monocots (rice) had been found to be similar in all plant species examined. Ahmadi *et al*. (2003) had suggested that the signals involved in the activation of *CgMT1* in *A. verticillata* roots were present in the other species examined, such as tobacco and rice, due to the fact that all plants contained *metallothionein* (*MT*) genes and that the mechanisms involved in their regulation could be expected to be conserved. However, our studies revealed that the *CgMT1* promoter showed no activity at all in hairy roots of the actinorhizal plant *D. glomerata*.
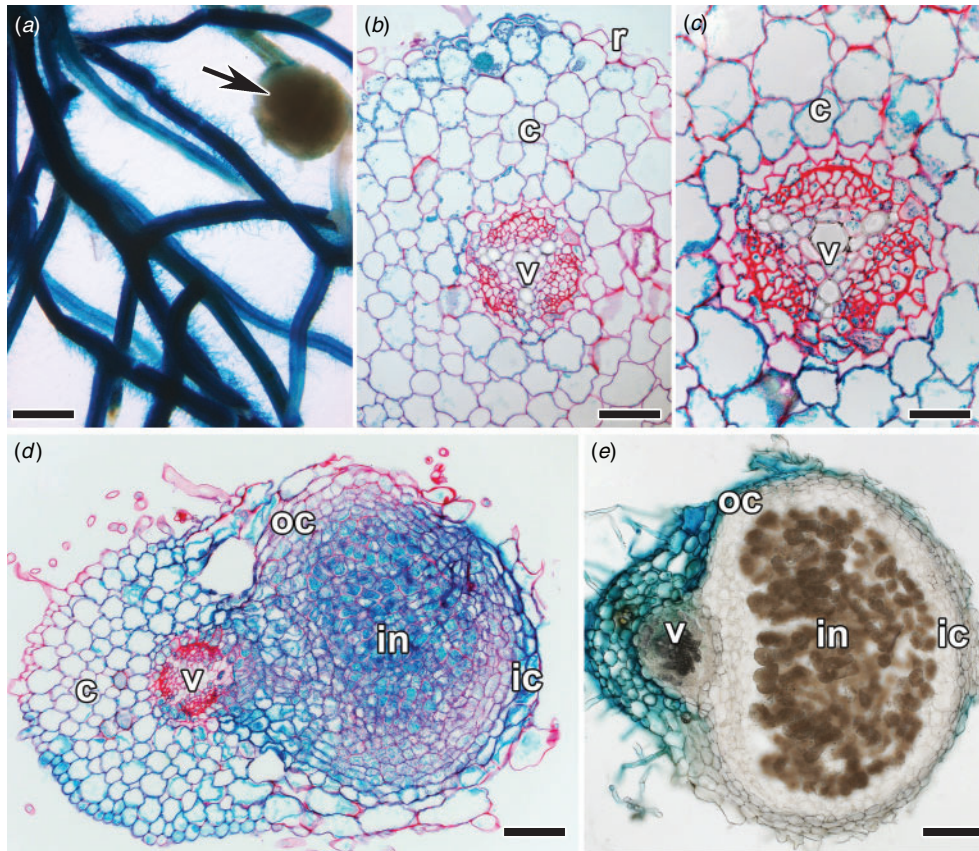
Fig. 3. Histochemical analysis of GUS activity in transgenic *Lotus japonicus* root systems containing the *CgMT1*::*GUS* construct. Blue staining indicates GUS activity. (*a*, *e*) Represent vibratome sections, (*b*, *d*) represent sections of nodules embedded in plastic, counterstained with Ruthenium Red. (*a*) Part of a stained root system shows GUS activity in roots and no activity in nodules. (*b*) Cross-section of a *L. japonicus* root. (*c*) Detail of (*b*) to show blue staining in all living cells of the vascular bundle. (*d*) Cross-section of a young *L. japonicus* nodule 10 days after infection. (*e*) Cross-section of a mature *L. japonicus* nodule 21 days after infection: v, vascular bundle in cross-section; r, rhizodermis; in, infected cells (appearing in brown); oc, outer nodule cortex; ic, inner nodule cortex. Bars denote (*a*) 250 μm, (*b*) 50 μm, (*c*) 25 μm, (*d*) 65 μm, and (*e*) 150 μm.

CgMT1::GUS *activity in nodules of different plant species*

The *CgMT1* promoter had been found to be active in infected cells of all types of actinorhizal nodules studied to date, namely, *C. glauca*, *A. verticillata* (Laplaze *et al.* 2002) and, as shown in this study, was also active in infected cells of *D. glomerata* nodules. However, no *CgMT1*::*GUS* activity was detected in mature infected cells of the legume *L. japonicus*, even though the promoter was active in all cell types of incipient nodules. These results indicate that *CgMT1* promoter activity in infected cells of actinorhizal nodules is likely to be due to cell-type specific signals, as induction by ROS (Obertello *et al.* 2007) would have been expected to work also in legume nodules (Günther *et al.* 2007), and the *CgMT1* promoter was inactive in mature nitrogen-fixing nodules of the model legume *L. japonicus*.

The previous studies had shown differences between the expression patterns of the *CgMT1*::*GUS* fusion in nodules of tissue culture-grown *A. verticillata* and the tissue-specific accumulation of *CgMT1* mRNA in nodules of soil-grown

C. glauca (Laplaze *et al.* 2002). *CgMT1* promoter activity was present in the vascular bundle of *C. glauca* nodules as detected by *in situ* hybridisation, but not of nodules formed on transgenic *CgMT1*::*GUS A. vertillata* plants grown in tissue culture. Before the results obtained in this study, it could have been assumed that the lack of promoter activity in the vascular pericycle of *A. vertillata* nodules was due to the fact that the *CgMT1*::*GUS* construct did not contain the full promoter sequence. However, the *D. glomerata* results obtained in this study show that the construct can direct expression in the pericycle of the nodule vascular system. Hence, *CgMT1* promoter activity in the pericycle of the vascular system of actinorhizal nodules seems to be due to physiological factors, the nature of which has not yet been deduced.

Apart from the nodule infected cells and the nodule vascular system, *CgMT1*::*GUS* was active in the phellogen of *D. glomerata* nodules. This follows since all *promoter*::*GUS* fusions tested to date in transgenic hairy root systems of *D. glomerata* were active in the nodule phellogen (B. Rashidi and K. Pawlowski, unpubl. data).

The CgMT1 *promoter is negatively regulated by auxin and cytokinin*

MTs have been implicated in metal uptake, heavy metal detoxification and in the detoxification of ROS (Cobbett and Goldsbrough 2002; Yang *et al*. 2009). All *MT* genes examined showed constitutive expression in some organs/tissues, and can be induced further by some biotic or abiotic stress factors. Usually, the function of an MT is inferred from the factors that induce the expression of the corresponding gene to higher levels (e.g. Omidvar *et al*. 2010; Samardžić *et al*. 2010).

Obertello *et al*. (2007) found that wounding, pathogen infection and H_2O_2 treatment led to an increase in *CgMT1* promoter activity, suggesting that the common factor in gene induction was H_2O_2. Production of ROS including H_2O_2 is also induced by abiotic stress (osmotic stress, drought and salt stress; reviewed by Dat *et al*. 2000). However, our study showed that the *CgMT1* promoter is also affected by exogenously added cytokinin or auxin, both of which inhibit promoter activity, although exogenously added ethylene had no effect. While there is limited knowledge available about the response of the promoters of other *MT* genes to changes in phytohormone levels, a recent study showed that the expression of a rice class II *MT* gene in roots is downregulated by cytokinins (Yuan *et al*. 2008).

When considered in detail, the influence of exogenously supplied phytohormones on *CgMT1* expression is quite complex. In this study, NAA in the growth medium abolished *CgMT1* expression in entire *Arabidopsis* seedlings; yet, studies on the effect of NAA in the growth medium on auxin distribution in the seedlings as determined by the activity of a *DR5*::*GUS* construct indicate that although auxin concentrations in the root system rise dramatically under these circumstances, auxin concentration in the cotyledons and hypocotyls remain unaffected (Desgagné-Penix and Sponsel 2008). Furthermore, the presence of the auxin transport inhibitor 1-NOA in the growth medium did not affect *CgMT1* expression in the *Arabidopsis* root system, which is consistent with the results of Desgagné-Penix and Sponsel (2008). In their study, 1-NOA in the growth medium only led to increased auxin concentrations in the cotyledons and the apical meristem, but did not affect auxin levels in roots. Hence, the effect of exogenously supplied phytohormones on *CgMT1* promoter activity may differ between roots and shoots and may differ between plant species.

Hairy roots induced by *Agrobacterium rhizogenes* have a disturbed phytohormone balance that leads to strong lateral branching and lack of geotropic growth and, as shown by the reduced actinorhizal nodule frequency on transgenic hairy root systems, also affects nodule induction by *Frankia* (B. Rashidi and K. Pawlowski, unpubl. data). The extent to which individual endogenous phytohormone levels are affected is unclear. For example, Prinsen *et al*. (1994) did not find enhanced cytokinin levels in transgenic tobacco harbouring the entire TL DNA from *A. rhizogenes* except in young shoot tips, but they analysed only aboveground organs, not roots. In general, the phenotype of hairy roots is ascribed to a hypersensitivity to auxin. However, the expression of the pRi T-DNA gene *rolC* in transgenic tobacco plants leads to effects similar to those of

exogenously applied cytokinin (Schmülling *et al*. 1988), and to increased cytokinin levels (Nilsson *et al*. 1996) although the mechanism of RolC action is not understood (Nilsson and Olsson 1997). Furthermore, the gene encoded by *orf13* of the *A. rhizogenes* T-DNA has cytokinin-like effects when expressed in tomato under control of the CaMV*35S* promoter (Stieger *et al*. 2004). The lack of *CgMT1*::*GUS* expression in transgenic hairy root systems of *D. glomerata* in combination with the results on phytohormone effects on *CgMT1*::*GUS* expression in *Arabidopsis* seedlings, could be explained by the fact that hairy roots of *D. glomerata* contain enhanced levels of cytokinins and/or auxin, or show enhanced sensitivity to cytokinins and/or auxin, which could lead to the suppression of promoter activity.

Cytokinin levels in nodules induced on hairy roots do not seem to be affected; at least, the expression pattern of *CgMT1*::*GUS* in *D. glomerata* nodules on hairy roots resembles that of the *CgMT1* promoter in *C. glauca* nodules on wild-type roots (Laplaze *et al*. 2002). This observation could be interpreted to mean that (i) in nodules, cell-specific or metabolic transcription factors override the auxin- and cytokinin-dependent repression of *CgMT1* promoter activity, or that (ii) the auxin- and cytokinin-dependent repression of *CgMT1* promoter activity is restricted to roots. The high *CgMT1* promoter activity in *L. japonicus* nodule primordia, which contain more auxin and cytokinin than mature nodules (Pacios-Bras *et al*. 2003; Lohar *et al*. 2004) in which the *CgMT1* promoter is not active, in combination with the high activity in infected cells of *C. glauca* nodules, which accumulate high amounts of auxin (Perrine-Walker *et al*. 2010), seem to favour the second hypothesis.

The organ specificity of the promoter in hairy root systems is reversed in L. japonicus *compared with* D. glomerata

Although the repression of the *CgMT1* promoter by auxin and cytokinin offers an explanation for its lack of activity in hairy roots of *D. glomerata*, the same promoter was active in hairy roots of the legume *L. japonicus* transformed with the same pRi T-DNA, but not in mature nodules formed on those hairy roots. In other words, the organ-specific expression was the opposite of that observed for *D. glomerata* hairy roots.

The activity of the *CgMT1* promoter in legume hairy roots seems to contradict the explanation for the results obtained in *D. glomerata* hairy roots. However, although *A. rhizogenes* transformation changes the root phytohormone balance, these changes occur on top of the endogenous phytohormone balance, which differs between plant species and families (Ross and Reid 2010). So whereas transformation of *D. glomerata* with *A. rhizogenes* LBA1334 seems to lead to auxin and/or cytokinin levels in hairy roots that preclude the activity of the *CgMT1* promoter, this does not seem to be the case for *L. japonicus*.

These results show that promoter activity data obtained using hairy root transformation can be misleading, not only due to the well known effects on phytohormone balance, but particularly when the comparison between different plant species is concerned.

Conclusions

It is concluded that the *CgMT1* promoter directs expression in infected cells of actinorhizal nodules from different phylogenetic subgroups, Fagales (Casuarinaceae) and Cucurbitales (Datiscaceae). The lack of *CgMT1*::*GUS* activity in *D. glomerata* hairy roots renders it useless for the introduction of transgenes in hairy roots in the course of research on signal transduction in actinorhizal nodulation, and might be explained by the changed phytohormone balance, although at this stage, alternative explanations cannot be excluded. Yet, the main results of this study are that (i) gene expression databased on experiments with hairy root systems have to be viewed with care, because they may not be consistent between different species, and (ii) that the effect of phytohormone levels on *MT* gene expression in different plant organs deserves further study.

Acknowledgements

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