



Choosing a reporter for gene expression studies in transgenic actinorhizal plants of the *Casuarinaceae* family

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Abstract

Transgenic *Casuarinaceae* and reporter genes provide valuable tools to study gene expression in transgenic actinorhizal nodules. In this paper, we discuss the use of β -glucuronidase for the histochemical localization and quantification of gene expression in transgenic plants of *Allocauarina verticillata* and *Casuarina glauca* nodulated by the actinomycete *Frankia*. We also report on the genetic transformation of *A. verticillata* by the *Agrobacterium tumefaciens* strain C58C1(pGV2260) containing the *35S-mgfp5-ER* construct encoding a modified green fluorescent protein of *Aequorea victoria* in a binary vector. The evolution of the GFP fluorescence was monitored through all stages of the regeneration process. The data indicate that GFP is not toxic in *Casuarinaceae* and that this reporter gene can be used for visual screening of transformed calli and transgenic plants. The fluorescence pattern of *gfp* provides a new tool for monitoring *in vivo* transgene expression in actinorhizal plants.

Abbreviations: BA – 6-benzylaminopurine; GFP – green fluorescent protein; GUS – β -glucuronidase; NAA – α -naphthalenacetic acid; PVPP – polyvinylpyrrolidone; X-gluc – 5-bromo-4-chloro-3-indolyl β -D-glucuronide

Introduction

A number of reporter genes have been used as convenient markers to visualize gene expression and protein localization in a wide spectrum of prokaryotes and eukaryotes. Each gene has specific, inherent characteristics that define both its limitations and the applications for which it will be useful. The *Escherichia coli gus* reporter gene (also referred to as *uidA*), which encodes β -glucuronidase (GUS), has been extensively used in plants (for review see Martin et al., 1992). The β -glucuronidase assay invented by Jefferson is very sensitive, and it is possible to obtain both qualitative (histochemical) and quantitative (fluorometric) data (Jefferson et al., 1987). This reporter gene is routinely used for promoter analysis and to study protein targeting; it is also an invaluable tool when used to follow

gene transfer and monitor the genetic transformation of plant species. However, the histochemical GUS assay is destructive for tissue and therefore not suitable for direct visual selection of transformed plants. Luciferase (LUC) is another extensively used reporter in plants. The formation of LUC can be monitored *in vivo* but requires an exogenous substrate (luciferin) and emits light at low intensity (Ow et al., 1986).

Recently, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was reported to function as a sensitive reporter of *in vivo* gene expression (Chalfie et al., 1994; Heim et al., 1994). This gene emits bright fluorescence upon excitation with ultraviolet (excitation max=395 nm) or blue light (excitation max=475 nm). The formation of the fluorescent chromophore requires no exogenous substrates or cofactors and is easily visible. GFP is stable and is only denatured under extreme conditions. So far, only molecular oxygen has been identified as a pos-

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sible cofactor for chromophore formation (Heim et al., 1994). These properties make GFP an ideal non-destructive marker for plant use. The wild-type GFP has been used for expression studies in plants in various transformation systems or virus-based delivery systems (Haseloff and Siemerling, 1998). However, to achieve efficient expression of GFP in plants, a cryptic intron sequence has to be removed from the coding sequence (Haseloff et al., 1997). Mutants of GFP with increased stability and enhanced fluorescence have been isolated (i.e. Davis and Vierstra, 1998; Haseloff and Siemerling, 1998; Stewart, 2001).

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

Our group is using *Casuarina glauca*, a tropical tree of the *Casuarinaceae* family, as a model system to study actinorhizal nodule development (Franche et al., 1998b). Simultaneously with the isolation and characterization of the plant genes involved in the symbiotic process, we have developed a genetic transformation procedure based on *Agrobacterium tumefaciens* which allow the regeneration of transgenic *C. glauca* and *Allocasuarina verticillata* trees (Franche et al., 1997). Transgenic *Casuarinaceae* plants containing chimeric actinorhizal nodulin promoters fused to reporter genes are valuable tools to study the regulation of symbiotic gene expression in nodules, roots and shoots (Franche et al. 1998a; Laplaze et al., 2001). Comparison of the regulatory mechanisms of legume and actinorhizal symbiotic genes, which is based on the analysis of transgenic nodules of *Casuarina*, also provides new insights into the evolution of symbiotic genes (Franche et al., 1998a).

Our objective in this paper is to compare β -glucuronidase and green fluorescent protein reporter genes for expression studies in actinorhizal plants. We will first report on the limitations encountered with the

use of the β -glucuronidase gene in transgenic *Casuarinaceae*. We present assay conditions which eliminate artefacts observed in histochemical staining of GUS activity in transgenic actinorhizal nodules and minimize the effects of inhibitory compounds naturally present in *Casuarinaceae*. The regeneration of transgenic *A. verticilla* plants expressing the reporter gene *mgfp5-ER* (Haseloff and Siemerling, 1998) under the control of the *CaMV 35S* promoter is described and the potential of β -glucuronidase and green fluorescent protein genes for improving our knowledge of actinorhizal nodule development is discussed.

Materials and methods

Plant material and germination conditions

A. verticillata and *C. glauca* seeds were collected in Australia. Seeds were treated for 2 min in H_2SO_4 and washed for 30 min under running tap water. Then they were disinfected with 5% calcium hypochlorite for 30 min, and washed three times with sterile water. Seeds were germinated in sterile conditions on H medium (Le et al., 1996) solidified with 8 g L⁻¹ Difco bacto-agar. The plantlets were grown for 1–2 months at 28 °C with a 16 h photoperiod and a light intensity of 50 $\mu E m^{-2} s^{-1}$.

Bacterial strains, plasmids and culture media

Disarmed *A. tumefaciens* C58C1(pGV2260) containing the binary plasmid pBIN19 was used (Vancanneyt et al., 1990). Within the T-DNA borders, pBIN19 contains the coding region for kanamycin resistance under the regulatory control of the *nos* promoter. For the experiments with the β -glucuronidase gene, the intron-containing *gus* gene was driven either by the *CaMV 35S* promoter (Benfey and Chua, 1990) or by the *Parasponia andersonii* hemoglobin promoter (*P*) kindly provided by E. Dennis (Bogusz et al., 1990). The plasmid pBIN *mgfp5-ER* encoding an ER-targeted green fluorescent protein was obtained from J. Haseloff (University of Cambridge, UK); the *mgfp5-ER* gene was adapted for expression in plants and was driven by the constitutive *35S* promoter (Haseloff et al., 1998). Before cocultivation with the explants (see below), the *Agrobacterium* strain was grown overnight at 28 °C in Ag medium (Le et al., 1996) supplemented with appropriate antibiotics.

The *Frankia* Allo2 strain was used for inoculation of transgenic *A. verticillata* (Benoist et al., 1992) while transformed *C. glauca* plants were inoculated by the *Frankia* strain Thr (Girgis et al., 1990). *Frankia* strains were grown at 26 °C in a modified BAP medium (Benoist et al., 1992).

Agrobacterium tumefaciens transformation procedure

The transformation of *A. verticillata* and *C. glauca* was carried out as described by Franche et al. (1997) after making the following modifications to the protocol. Epicotyls were excised from plants 30–45 days old and cut into 1.5–2 cm fragments followed by three days of cocultivation on MSC medium with the *A. tumefaciens* strain C58C1(pGV2260) containing the appropriate binary vector. The agrobacteria were then removed by slowly swirling the explants in sterile water three times for 1 h. The transformed epicotyls were then placed in Petri dishes on the MSC medium (Le et al., 1996) supplemented with 0.5 μ M NAA, 2.5 μ M BA, 100 mg L⁻¹ kanamycin and 250 mg L⁻¹ cefotaxime. After two months, one to three calli had developed on at least 30% of the explants. When the calli were about 5 mm in diameter, they were placed in glass tubes (2.5 cm in diameter and 15 cm in length) closed by a cotton cap, and subcultured every three weeks. After four months, cefotaxime was omitted from the selection medium; in the absence of antibiotic, the adventitious regeneration process from the kanamycin resistant calli improved considerably. Shoots were excised when they were at least 3 cm in height and root formation was induced by a one-day-treatment with 25 μ M NAA.

Nodulation of A. verticillata plants

Transgenic and non-transformed control plants exhibiting a main root of 3 cm in length were transferred to tubes (Gibson, 1963) that were placed in a controlled-environment chamber as described above. This experimental system allows the shoot to grow outside while the root system is immersed in 1/4 strength sterile Hoagland liquid medium (Hoagland and Arnon, 1938) containing 17 mg L⁻¹ ammonium sulfate at pH 5.6. The nutrient solution was renewed every week. After one month, when the root system had reached the bottom of the tube, the liquid medium was discarded. Two ml of a dense suspension (25 μ g mL⁻¹ of protein) of *Frankia*, Thr or Allo2 were added to each tube. The tubes were kept lying horizontally for 1 h and were

then filled up to 5 mm from the top with nitrogen-free Hoagland solution at pH 6.8. Nodule initiation was monitored weekly using a stereomicroscope.

Histochemical GUS assays

Plant samples (shoots and roots) from *in vitro* propagated plants were taken 4–5 weeks after the rooting treatment, when the plants had reached a height of approximately 8 cm. One to two months after plant inoculation by *Frankia*, nodules displaying different stages of development were harvested. To assay GUS activity, whole explants or vibratome-sectioned tissues (see below) were flooded in a staining solution containing 1 mM X-gluc, and incubated for 16 h at 37 °C as recommended by Jefferson et al. (1987). To confine the localization of the blue staining, K₃Fe(CN)₆ and K₄Fe(CN)₆ were added as catalysts. Plant samples were fixed for several hours in a solution containing 5% formaldehyde, 5% acetic acid and 50% ethanol, washed several times in 70% ethanol and examined under a stereomicroscope (Le et al., 1996).

For higher resolution analysis, samples were embedded in 3% agarose and sliced into 45–50- μ m-thick longitudinal or transversal sections on a vibratome (Leica VT1000E) as described by Franche et al. (1998a). The explants were sectioned in the presence of phosphate buffer (50 mM, pH 7) containing 25 g L⁻¹ of insoluble polyvinylpyrrolidone (PVPP). The sections were mounted on glass slides with 50% glycerin and examined with a light microscope.

GUS quantification

GUS activity was quantified using the substrate 4-methylumbelliferyl β -D-glucuronide (MUG), as described by Jefferson et al. (1987) with or without the addition of insoluble PVPP (0.5 g g⁻¹ fresh weight tissue) to the extraction buffer (Serres et al., 1997). Fluorescence was then measured with excitation at 365 nm, and emission at 455 nm on a SFM25 spectrofluorimeter (Kontron) and was converted into μ moles 4-methylumbelliferone (4-MU) produced per hour per mg protein in transgenic plant extracts. Protein determination was carried out using Bradford reagent (Bio-Rad) and BSA as standard (Bradford, 1976).

Fluorescence microscopy

GFP expression *in planta* was analyzed using a (Leica MZ FLIII) stereomicroscope with a fluorescent light

source and equipped with filters giving an excitation spectrum between 475 and 495 nm and a barrier filter between 520–550 nm. Fresh sections were made of actinorhizal nodules using a (Leica VT1000E) vibratome. Sections were collected in a buffer containing 10 mM DTT, 1 mM MgCl₂, 100 mM NaCl and 10 mM Tris-HCl pH 8. Photomicrographs were made on iso (400) colour film.

PCR analysis

Plant DNA for PCR analysis was prepared as described by Bousquet et al. (1989). The primers used for analysis of the plants genetically transformed with *A. tumefaciens* were: 5'GAATGGTGATTACCGACGAAA3' and 5'CCAGTCGAGCATCTCTTCAGC3' for the *gus* gene (Jefferson et al., 1987), 5'ATTGCACGCAGGTTCTCCGG3' and 5'AGAAGTTCGTCGAAGAAAGGCA3' for the *nptII* gene (Mazodier et al., 1985), 5'CCTCGGCCGAATTCAGTAAAGG3' and 5'GGGGCAGATTGTGTGGACAGG3' for *mgfp5-ER* (Haselhoff and Siemering, 1998) and 5'ATGTCGCAAGGACGTAAGCCCA3' and 5'GGAGTCTTCAGCATGGAGCAA3' for the *virD1* gene of *A. tumefaciens* (Hamill et al., 1991). The predicted sizes of the amplified DNA fragments were 574 bp for the *gus* gene, 736 bp for the *nptII* gene, 818 bp for *mgfp5-ER*, and 450 bp for the *virD1* gene. PCR reactions were run on a Perkin Elmer thermocycler for 35 cycles at 94 °C (1 min), 55 °C (1 min) and 72 °C (2 min). PCR reactions with primers designed for the amplification of the *virD1* gene from *Agrobacterium* were carried out to check that transgenic plant tissues were not contaminated by persisting agrobacteria. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide using standard procedures (Sambrook et al., 1989). Control DNA from non-transformed plants were included in the experiments to ensure that reagents were not contaminated.

Results

Regeneration of transgenic *A. verticillata* plants

Epicotyls of *A. verticillata* and *C. glauca* were genetically transformed with the disarmed strain of *A. tumefaciens* C58C1(pGV2260) containing the binary vector pBIN19 which included the *35S-gus* (Vancanneyt et al., 1990), *P-gus* (Bogusz et al., 1990), or *35S-mgfp5-ER* (Haselhoff et al., 1998) constructs as

described in 'Materials and methods'. For each construct, two separate transformation experiments were carried out and transgenic plants were regenerated from at least 20 independent kanamycin resistant calli. As previously reported (Franche et al., 1997; Le et al., 1996), successful gene transfer in *C. glauca* plants was confirmed by the expression of the reporter genes and by polymerase chain reaction amplification using *nptII* and *gus* or *gfp* primers. The individual transformants showed quantitative, but never qualitative differences (data not shown) in the reporter gene expression.

Histochemical detection of β -glucuronidase activity in transgenic Casuarinaceae

The first genetic transformation experiments that were carried out in our laboratory were based on the use of the *gus* reporter gene to follow gene transfer into *A. verticillata* and *C. glauca* (Franche et al., 1997; Le et al., 1996). In *Casuarinaceae* shoots and roots from young transgenic plants cultivated in a growth chamber, the blue staining representing GUS activity was easy to localize and was used for heterologous promoter analyses (Franche et al., 1998a; Laplaze et al., 2000a). However, we encountered some difficulties with the detection of β -glucuronidase activity in transgenic actinorhizal nodules. After incubation with X-gluc as substrate, no blue colour was detected in whole nodules of *A. verticillata* and *C. glauca* expressing a *35S-gus* construct. Attempts to vacuum infiltrate the substrate X-gluc did not improve the data. Reporter gene activity could only be localized in 45 μ m thick nodule sections obtained on a vibratome (data not shown). This treatment was found necessary to compensate for the poor penetration of X-gluc in nodules of transgenic *Casuarinaceae*. No indigo-blue crystals were detected in nontransformed control nodule sections.

In order to confine the localization of blue staining in actinorhizal organs and tissues, 0.5 mM K₃Fe(CN)₆ and 0.5 mM K₄Fe(CN)₆ were routinely added as catalysts with the X-gluc substrate (Jefferson et al., 1987). Nevertheless, with promoters driving high β -glucuronidase activity, such as the hemoglobin promoter from *Parasponia*, diffusion still occurred during histochemical assays and resulted in localization artefacts (data not shown). To overcome this problem, we found it necessary to increase the concentration of K-ferricyanide/K-ferrocyanide from 0.5 mM to 3 mM. This modification improved the precision of the GUS test and permitted the study of strong promoters such

Table 1. β -glucuronidase activity in extracts of transgenic and untransformed nodulated plants of *C. glauca*

	Explant	Average β -glucuronidase activity ($\mu\text{mol MU h}^{-1} \text{mg}^{-1} \text{protein}$)	
		-PVPP	+PVPP
Shoots	35S- <i>gus</i>	374.6 \pm 61.1	724.7 \pm 70.6
	untransformed	1.3 \pm 1.2	2.9 \pm 0.4
Roots	35S- <i>gus</i>	57.0 \pm 7.5	574.7 \pm 41.4
	untransformed	0.6 \pm 0.8	1.2 \pm 0.3
Nodule	35S- <i>gus</i>	11.6 \pm 2.6	926.4 \pm 65.1
	untransformed	0.2 \pm 0.1	0.5 \pm 0.2

Transgenic *C. glauca* plants expressed the β -glucuronidase gene under the control of the 35S constitutive promoter. β -glucuronidase assays were conducted with and without the addition of 0.50 g PVPP g⁻¹ fresh weight. Values are the mean \pm standard error for ten samples.

as the hemoglobin promoter from *P. andersonii* (Franche et al., 1998a).

Fluorometric analysis of GUS activity in transgenic Casuarinaceae

The β -glucuronidase activity in transgenic plants of *C. glauca* expressing the *gus* gene under the control of the 35S constitutive promoter was quantified by fluorometry. Transgenic plants regenerated from 21 *C. glauca* transformed calli were analyzed. Shoots and roots were taken from transgenic plants one month after the rooting treatment, when the aerial part was about 6 cm in height. β -glucuronidase activity expressed as $\mu\text{moles 4-MU h}^{-1} \text{mg}^{-1} \text{protein}$ was found to be slightly higher in whole shoots than in roots (Table 1): In whole shoots it ranged from 98.9 to 944.6 with an average of 724.7 \pm 70.6, while in roots, it ranged from 81.6 to 816.1 with an average of 574.7 \pm 41.4. Without the addition of PVPP in the extraction buffer, the values measured in shoots decreased by a factor of 1.9 and in roots by a factor of 10.

In preliminary experiments, transgenic *C. glauca* nodules exhibiting an average of four to six lobes were homogenized in the standard extraction buffer without PVPP as described by Jefferson et al. (1987). Fluorogenic assays in the absence of PVPP showed that β -glucuronidase activity in transgenic nodules was surprisingly low (Table 1). The addition of PVPP during preparation of nodule extracts led to a spectac-

ular increase in β -glucuronidase activity in the assayed transgenic nodules. In extracts of transformed nodules obtained without PVPP, the average GUS activity was found to be 11.6 \pm 2.61 $\mu\text{moles 4-MU h}^{-1} \text{mg}^{-1} \text{protein}$; when nodules collected from the same plants were grounded with PVPP, GUS values increased 84 fold. These data suggest that some inhibitors of the β -glucuronidase activity are present in large quantities in *Casuarina* nodules.

Green fluorescent protein as a vital marker in transgenic actinorhizal plants

Using the *Agrobacterium* strain C58C1(pGV2260) carrying the binary vector pBIN35S-*mgfp5-ER*, we obtained 48 transgenic calli of *A. verticillata* after the genetic transformation of 174 epicotyls. *gfp* expression was followed throughout the regeneration process with particular emphasis on detection of fluorescence patterns in intact nodulated plantlets.

At the callus stage, a bright green fluorescence was observed in the kanamycin resistant calli (Figure 1A). Although weak greenish fluorescence occurred in control *A. verticillata* calli (Figure 1B), the identification of GFP-positive calli was unambiguous. Thirty nine transgenic *A. verticillata* plants were then regenerated and examined under blue light; plants obtained from non transformed calli were used as negative controls. It should be noted that we observed no difference in the regeneration process, in quantity, quality or duration, between epicotyls genetically transformed with *agrobacteria* containing a *gus* or a *gfp* binary plasmid.

In shoots, significant fluorescence was only detected in the young scales of the apex (Figure 1C). As development progressed, chlorophyll autofluorescence increasingly masked the GFP fluorescence which thus became more difficult to detect in tissues comprising more differentiated cells (data not shown). In the root system (Figures 1D), *gfp* expression was clearly visible in lateral roots especially in the vascular system. The high level of expression in the vascular bundle was to be expected since it has been previously reported that the *CaMV 35S* promoter is more strongly expressed in cells of the vascular bundle (Benfey and Chua, 1990). In the main root, fluorescence was strong in the apical region (Figure 2E) but decreased as roots became lignified (data not shown).

Some of the transgenic plants containing the *mgfp5-ER* construct were then nodulated by *Frankia*. Prenodules and nodules exhibited strong green GFP fluorescence making their identification easy on the

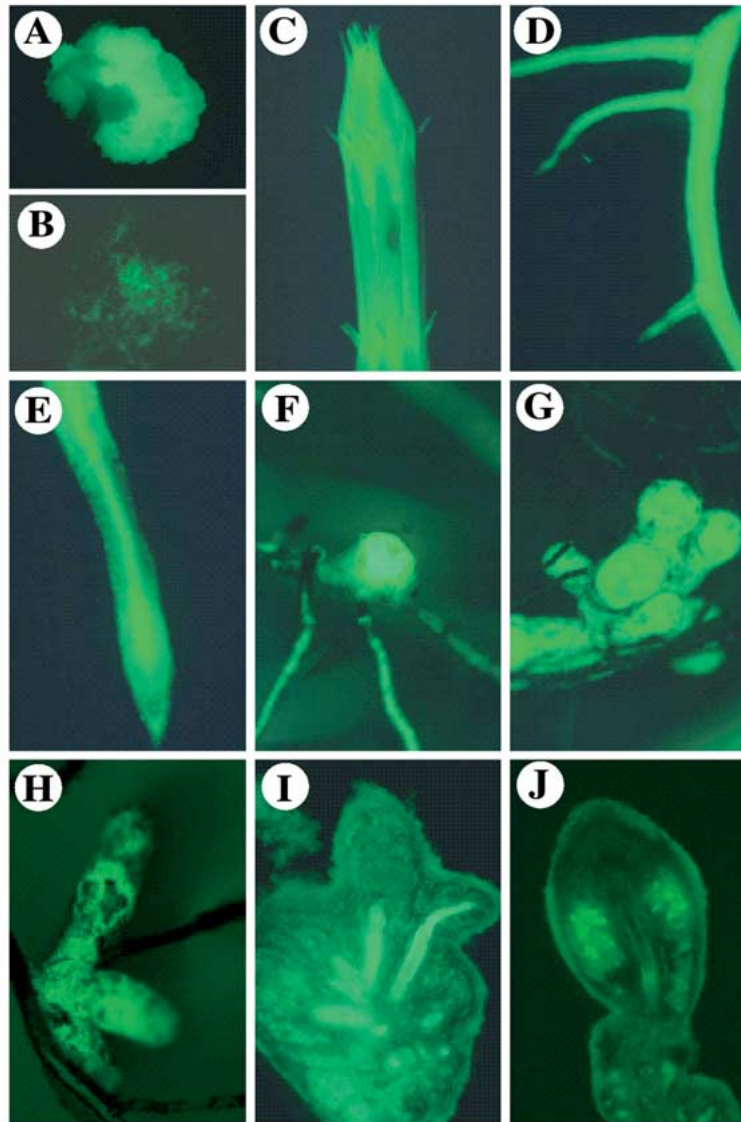


Figure 1. Expression of GFP in transgenic *A. verticillata* plants containing the *35S-mgfp5-ER* construct. (A) *gfp* expression in a transgenic callus developing from an epicotyl of *A. verticillata* two months after genetic transformation with the *A. tumefaciens* strain C58C1(pGV2260; pBINmGFP5-ER). (B) low autofluorescence in a non transformed callus. (C) strong *gfp* expression in the apex of a transgenic shoot. (D) *gfp* expression in transgenic lateral roots. (E) strong *gfp* expression in the vascular system of the apex of a transgenic primary root. (F) *gfp* expression in a prenodule developing three weeks following the inoculation of a transgenic plant by *Frankia*. (G) *gfp* expression in a two-month old transgenic nodule. (H) autofluorescence in a non transformed nodule. (I) *gfp* expression in a longitudinal section of a transgenic *A. verticillata* nodule; strong fluorescence was observed in the nodule vascular system. (J) control section of a nodule lobe from a non-transformed *A. verticillata* plant.

lignified root system (Figure 1F, G). In control nodules obtained after inoculation of non-transformed *A. verticillata* plants, a low level of green autofluorescence was observed (Figure 1H). Fluorescence was then examined on semi-thin vibratome sections of the nodules. The brightest fluorescence appeared associ-

ated with the vascular system and the phellogen of transgenic nodules (Figure 1I) whereas a strong autofluorescence was observed in the lignified cell walls of *Frankia*-infected cells from untransformed nodules (Figure 1J).

Discussion

The analysis of promoter-reporter gene fusions is one of the most widely used techniques for identifying sequences that control the temporal and spatial regulation of cloned genes. β -glucuronidase and green fluorescent protein genes have been incorporated with success into several plants and used as markers of gene expression (Martin et al., 1992; Stewart, 2001). According to the data from analysis of transgenic *Casuarinaceae* plants in our laboratory, the *E. coli* β -glucuronidase enzyme has proven to be a very useful reporter system. There is no endogenous GUS-like activity in *Casuarina* that interferes with the detection of the transgene encoded β -glucuronidase. This reporter gene has been used in a quick genetic transformation procedure for *C. glauca* based on *A. rhizogenes* (Diouf et al., 1995) and to obtain transgenic *A. verticillata* and *C. glauca* plants after gene transfer by a disarmed *A. tumefaciens* strain (Franche et al., 1997; Smouni et al., 2000). The *gus* gene is currently used in our laboratory to characterize the tissue specific expression conferred by promoter regions of *C. glauca* symbiotic genes (Laplaze et al., 2001). However, our results demonstrate that some care must be taken to obtain accurate information concerning the β -glucuronidase activity both at a qualitative and a quantitative level in transgenic *Casuarinaceae*.

The histochemical GUS reaction with X-gluc proceeds through an unstable colourless indoxyl intermediate that undergoes oxydative dimerization resulting in blue water-insoluble dichloro-dibromo indigo (Jefferson et al., 1987). It has been demonstrated by several authors that the precipitation of the indigo blue in cells other than those containing GUS activity occurs because the soluble indoxyl monomers can diffuse (Guivarc'h et al., 1996; Jefferson et al., 1987). To get rid of these artifacts, the dimerization process can be enhanced by a potassium ferricyanide/ferrocyanide mix, which is included during the enzymatic reaction. Our data on transgenic actinorhizal nodules demonstrate that the concentration of the potassium ferricyanide/ferrocyanide must be adapted to the level of β -glucuronidase expression conferred by the promoter introduced into *A. verticillata* or *C. glauca*. A concentration of 0.5 mM potassium ferricyanide/ferrocyanide is routinely used in preliminary experiments; if the blue colour conferred by the activity of the reporter gene is still diffuse, the concentration of oxydative catalysts is increased to 3 mM.

Another major problem that is sometimes encountered in fluorometric β -glucuronidase analyses performed on woody plants is linked to the presence of phenolic compounds, which have the capacity to interfere with the β -glucuronidase activity. The inhibitory effect of phenolics was first noted by Jefferson et al. (1987) and was also suggested by Vainstein et al. (1993) who studied extracts from transgenic carnation plants. It was recently demonstrated that the use of insoluble polyvinylpyrrolidone (PVPP) during preparation of transgenic extracts of cranberry could increase β -glucuronidase activity more than 200 fold (Serres et al., 1997). It has been suggested that PVPP could specifically absorb phenolic compounds that act as inhibitors of GUS enzymatic activity. Using the standard procedure to prepare the extracts of *C. glauca* transgenic nodules, we observed in fluorogenic assays a surprisingly low level of reporter gene activity. The addition of PVPP in the extracts led to a 84-fold increase in enzymatic activity in nodules and an increase to a lesser extent in roots. Our laboratory has previously reported histochemical and biochemical analyses on phenolic compounds extracted from *C. glauca* roots and nodules (Laplaze et al., 1999). Phenolics belonging to the flavan class of flavonoids were identified; they were about ten times more abundant in nodules than in roots. Our data suggest that at least some of these phenolic compounds may inhibit β -glucuronidase activity and that addition of PVPP minimizes the inhibition.

Because the GUS histochemical assay is toxic and destructive, it cannot be used to monitor gene expression *in vivo*. This is a major obstacle for kinetic analyses of symbiotic genes expression during actinorhizal nodule differentiation. Therefore *gfp* was tested as an alternative to the *gus* gene as a reporter in actinorhizal plants. The gene *mgfp5-ER* encoding a modified green fluorescent protein of *A. victoria* was introduced into *A. verticillata* using *A. tumefaciens*. The feasibility of generating stably transformed nodulated plants was established and no specific negative effects of this reporter gene were revealed by this study. GFP allowed successful screening for primary transformed calli and the specificity of expression conferred by the *35S* promoter in *A. verticillata* could be easily determined in young roots, in nodules and in the shoot apex. Autofluorescence in non-transformed control plants was less bright and yellow than fluorescence of GFP driven by the *35S* promoter. Although *mgfp5-ER* appears to be a good reporter gene for following gene transfer into *Casuarinaceae* trees, GFP

is less detectable than GUS when the expression is low. Analyses of actinorhizal plants genetically transformed with the *gfp* reporter gene driven by weak promoters will require the use of more sophisticated methods such as confocal microscopy (Haseloff and Siemerling, 1998).

In a recent work, the advantages of GFP as a vital marker and of GUS with its high sensitivity in histochemical staining were combined by constructing bifunctional reporter genes; the *35S-gus-gfp* construct was introduced into the model legume *Lotus japonicus* (Quaedvlieg et al., 1998). Analysis of the transgenic plants demonstrated a high level of expression of both reporter genes. This approach offers exciting new possibilities for the study of the root nodulation process in actinorhizal plants. The expression of *gfp* under the control of promoters from symbiotic actinorhizal genes could be followed *in vivo* in the root hairs and cortical cells during the early stages of *Casuarina* infection by *Frankia*, whereas *gus* might provide valuable informations in the late stages of nodule development. Our laboratory is currently focusing on the study of the *gfp* and *gus* expression patterns conferred by the promoter of *cg12*, an early symbiotic gene from *C. glauca* encoding a subtilisin-like protease (Laplaze et al., 2000b).

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