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Fusions between green fluorescent protein and β-glucuronidase as sensitive and vital bifunctional reporters in plants

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Abstract

By fusing the genes encoding green fluorescent protein (GFP) and β-glucuronidase (GUS) we have created a set of bifunctional reporter constructs which are optimized for use in transient and stable expression studies in plants. This approach makes it possible to combine the advantage of GUS, its high sensitivity in histochemical staining, with the advantages of GFP as a vital marker. The fusion proteins were functional in transient expression studies in tobacco using either DNA bombardment or potato virus X as a vector, and in stably transformed *Arabidopsis thaliana* and *Lotus japonicus* plants. The results show that high level of expression does not interfere with efficient stable transformation in *A. thaliana* and *L. japonicus*. Using confocal laser scanning microscopy we show that the fusion constructs are very suitable for promoter expression studies in all organs of living plants, including root nodules. The use of these reporter constructs in the model legume *L. japonicus* offers exciting new possibilities for the study of the root nodulation process.

Introduction

Analysis of promoter activity in plants has been done almost exclusively using the *Escherichia coli* gene *gusA* encoding β-glucuronidase (GUS) as a reporter in gene fusions constructs. This is because there is almost no background GUS activity in most plant species and the levels of GUS can be easily quantified by using fluorescent substrates. Furthermore, GUS expression patterns can be analyzed histochemically [25]. However, GUS assays are destructive to the plant material which is inconvenient for following the expression of a given gene in time. For the latter strategy, the firefly reporter gene encoding luciferase has been used successfully, since its activity can be measured *in vivo* after addition of the substrate luciferine, using a sensitive video-imaging system [31, 32]. Disadvantages of this method are that these measurements have to be performed in darkness which interferes with the plants physiology, and the substrate for luciferase has to be introduced inside the plant. In contrast, the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* can be visualized directly without the addition of exogenous substrates or cofactors, and it is not toxic. GFP is a small protein of 238 amino acids which requires molecular oxygen for fluorophore formation [19]. Wild-type GFP absorbs blue light (major $\lambda_{ex} = 395$ nm and a minor peak at 470 nm) and emits green light (507 nm). The GFP complementary DNA was recently cloned and used as a vital marker gene in various heterologous organisms [4, 36, 53].

The wild-type GFP has been used for expression studies in plants in various transformation systems [22, 33] or virus-based delivery systems [1]. However, for efficient expression of GFP in plants a cryptic intron

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sequence had to be removed from the coding sequence [17, 37]. Mutants of GFP with altered spectral properties or enhanced fluorescence have been isolated [7, 18, 19]. A red-shifted variant of GFP (replacement of Ser65→Thr, S65T) has only one excitation peak (λ_{ex} = 485 nm), gives 6- to 7-fold increase in fluorescent intensity, and has reduced photobleaching [18]. Synthetic GFPs with optimized codon usage for humans and plants have been used successfully in maize, wheat, corn, tobacco and *A. thaliana* [6, 35, 39].

Modern new techniques which are suitable for the analysis of GFP include the use of single photon counting techniques, fluorescence correlation spectroscopy, fluorescence lifetime imaging microscopy and two-photon fluorescence spectroscopy [12, 38, 46]. However, for screening purposes in plants the usefulness of GFP is greatly limited due to the high levels of autofluorescence in various plant organs and calli. For this purpose, the reporter gene GUS remains unsurpassed in its sensitivity. A recently described fusion between GFP and β-galactosidase [44, 47] is not useful in plants because of the high endogenous β-galactosidase activity in plants. Therefore, we have combined the advantages of GFP and GUS by constructing bifunctional reporter genes making use of the fact that both GFP and GUS can tolerate N- or C-protein fusions [8, 13, 20].

Materials and methods

Plasmids and Agrobacterium strains

DNA manipulations were performed using standard procedures described by Sambrook *et al.* [40]. A detailed overview of the construction of the plasmids used in this work is depicted in Figure 1. The *nptII*-T*ocs* region of the *gusA::intr/nptII* fusion gene in pSDM5008 [9, 41], was exchanged with the s-*gfp*-*TYG*-T*nos* fragment of Blue-sGFP-TYG KS [6] via a series of cloning steps resulting in a promoterless *gusA::intr/s-gfp-TYG*-T*nos* gene in pMP2167a. The *egfp/gusA* fusion was made by cloning a *Bam*HI-*Eco*RI fragment of pBI101.2 [25] containing *gusA*-T*nos* in the *Bgl*II and *Eco*RI sites of pEGFP-C3 (Clontech) resulting in pMP3625. An intron was introduced into *egfp/gusA* by exchanging a *Sna*B1-*Bst*B1 fragment from pMP3625 with the analogous *gusA* fragment from GUS:intr/pBS, yielding pMP2845.The nucleotide sequence of the chimeric gene fusions was checked by the double strand dideoxy chain termination method [40]. For convenience, the fusion genes are indicated as *gusA::intr/gfp*, *gfp/gusA*, and *gfp/gusA::intr*, respectively. The double enhanced CaMV 35S promoter with optimized translation initiation sequences derived from pMOG18 [45] further on indicated as 35S was cloned 5′ of the *gusA::intr/gfp* and *gfp/gusA* fusion genes in pMP2167a and pMP3625 giving pMP2180 and pMP3628, respectively (Figure 1).

For the 35S-*gusA::intr/gfp* construct used for transformation of *A. thaliana*, the *Eco*RI fragment from pMP2180 harboring the fusion was excised and ligated into pBINPLUS [49], to make pMP2482. The same *Eco*RI fragment was also cloned in pMP2173, a pSLJ4644 [26] derivative with a modified right border of the T-DNA, resulting in pMP2182, which was used for *L. japonicus* transformation. The synthetic right border sequence of pMP2173 was made by the ligation of two compatible oligonucleotides, oMP215 (agcttatcgatacttggatcccacctggctacctaggaacctgcccgggcaggatatataccgttgtaatttcagct) and oMP216 (gaaattaccaacggtatatatcctgcccgggcaggttcctaggtagccaggtgggatccaagtatcgata) into *Sst*I and *Hin*dIII sites of pSLJ279 [26]; thereby eliminated the *Sst*I site. Subsequently, the *Hin*dIII-*Bgl*II fragment of this plasmid was cloned into pSLJ4644[26] yielding pMP2173 (Figure 1). The 35S-*gfp/gusA*-T*nos* fragment was transferred to the binary vector pZP212 [14] as an *Eco*RI fragment from pMP3628 giving pMP3629.

The intron of *gusA* in pMP2167a was removed by exchanging the *Sna*B1-*Bst*B1 fragment with the corresponding fragment of *gusA* in pMOG18 [45] in order to obtain a functional fusion gene *(gusA/gfp*) for PVX infection. The *Sma*I- *Hin*dIII *gusA/gfp* fragment was filled in with Klenow polymerase and ligated into the *Sma*I site of the PVX vector pPC2S [1] resulting in pMP2497 (Figure 1).

The binary vectors pMP2482, pMP3629 and pBINPLUS were introduced into *Agrobacterium tumefaciens* strain LBA1115 [21] using electroporation [10], whereas, pSLJ4644, pMP2173 and pMP2182 were mobilized to *A. tumefaciens* strain AGL1 [28] using the helper plasmid pRK2013 [11].

Transcription of viral RNA and plant inoculations

DNA of pMP2497 was linearized with *Spe*I and capped RNA transcripts were generated using the T7 RNA polymerase large scale *in vitro* transcription kit of Promega according to the manufacturer instructions (Promega Corporation). As controls, also RNA was pre-

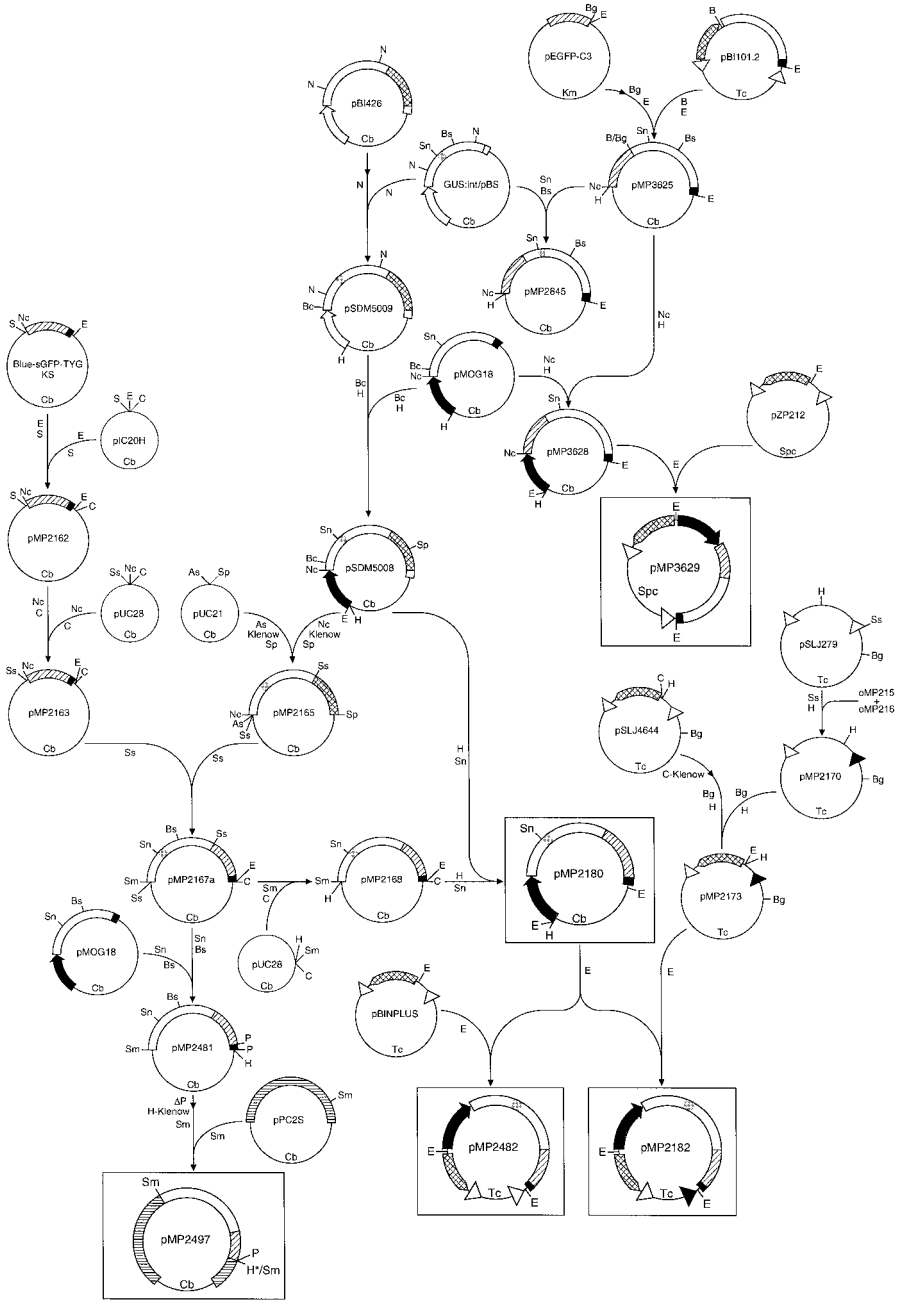

Figure 1. Cloning scheme of the *gusA::intr/gfp, gusA/gfp, gfp/gusA and gfp/gusA::intr* constructs. Several intermediate cloning steps in pUC21[52], pUC28 [2]or pIC20H [30] were required to obtain proper flanking restriction sites. Not all restriction sites are indicated, only those required for the understanding of the cloning steps. Plasmids are not drawn to scale. The most important constructs are boxed. The binary vectors, pMP2182 and pMP2482, contain the indicated *Eco*RI fragment from pMP2180 with *gusA::intr/gfp* under control of the enhanced CaMV 35S promoter and nopaline synthase terminator, and the plant selectable marker neomycin phosphotransferase between the right and left border. The PVX vector pMP2497 contains the intron-less *gusA/gfp*. The binary vector pMP3629 harbors an *Eco*RI fragment with *egfp/gusA* under control of the same promoter and terminator regions as used in pMP2182 and pMP2482. Restriction sites: As, *Asp*718I; B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; B/Bg, *Bam*HI/*Bgl*II junction; Bs, *Bst*BI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; H*/Sm, *Hin*dIII-Klenow/*Sma*I junction; N, *Nru*I; Nc, *Nco*I; P, *Pst*I; Sm, *Sma*I; Sn, *Sna*BI; Ss, *Sst*I; Sp, *Sph*I. Abbreviations: Cb, carbenicilin resistance gene; Spc, spectinomycin resistance gene; Tc, tetracyclin resistance gene; Km, kanamycin restistance gene; □, *gusA*; ▒, intron; ▨, *GFP*; ▤, PVX; ➡, enhanced CaMV 35S promoter; ⇨, CaMV 35S promoter; ▩, neomycin phosphotransferase; ◁, border sequence; ◀, synthetic border sequence; □, CaMV 35S terminator or octopine synthase terminator; ■, nopaline synthase terminator; Δ, deletion; →, additional cloning step.

pared from PVX.*gfp* and PVX.*gusA* plasmids [1, 5]. RNA was resuspended in water and manually inoculated on leaves of 5–6 week old *Nicotiana benthamiana* plants dusted with carborundum [5]. Plants were grown at 25 °C with a 16 h light period under Sylvania coolwhite tubes F18/133 T8. The spread of the virus was monitored 7–14 days post inoculation by analysis of both GFP and GUS expression as described below.

Plant transformation

Roots of 10-day-old *A. thaliana* plants (ecotype C24) seedlings were transformed with *A. tumefaciens* strain LBA1115 containing the binary vectors pBINPLUS, pMP2482 and pMP3629 according to the protocol by Valvekens *et al.* [48] which was optimized to our local conditions [51]. Transformants were selected by growth on kanamycin (50 mg/l). Hypocotyl explants of *L. japonicus* ('Gifu' accession number B-129) were transformed with the constructs pMP2182, pMP2173 and pSJL4644 using *Agrobacterium* strain AGL1 [28] as described by Handberg *et al.* [15] with minor modification as follows. Seedlings were grown for 5 days in the dark followed by one day in continuous light at 26 °C and hypocotyls were cut transversely in pieces of 6–8 mm before cocultivation. Transgenic calli were selected on callus induction medium containing 25 mg/l G418 (Sigma) until they reached a size of approximately 1 cm. During the shoot regeneration step the concentration of cefotaxime was reduced to 150 mg/l. Transgenic calli were cultured for 3 weeks on shoot induction medium. Subsequently, all calli, with or without emerging shoot structures, were transferred to shoot growth medium. Calli with emerging shoots of 0.5 cm were transferred to shoot elongation medium.

Immunoblot analysis

Transgenic *A. thaliana* seedlings were grounded in liquid nitrogen and powder was resuspended in 2 times their weight of extraction buffer containing 50 mM sodium phosphate buffer (pH 7.2), 5 mM dithiothreitol (DTT), 5% glycerol, 0.1% Triton X-100 and Complete^{TM} protease inhibitor cocktail (Boehringer Mannheim). Protein concentration were determined using Bradford reagent (Biorad) with bovine serum albumin as a standard. Equal volumes of 2× sodium dodecyl sulphate (SDS) sample buffer [40] was added and protein samples were loaded on 9% SDS-polyacrylamide gel according to Laemmli [27]. The separated proteins were blotted onto Immobilon-P membrane (Millipore) for 1 h, using a LKB NovaBlot Electrophoretic transfer unit at 0.8 mA/cm^2. Blots were blocked in 1% blocking reagent (Boehringer Mannheim) in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) for 1 h at room temperature. Rabbit polyclonal antibodies anti-GUS (Clontech) and anti-GFP (Molecular Probes) were used in 1:1500 dilutions in 0.5% blocking reagent in TBS. After washing in TBS, filters were incubated with peroxidase-conjugated goat-anti-rabbit IgG (1:3000 dilution; Sigma). Peroxidase activity was visualized using 3-3′ diaminobenzidine tetrahydrochloride as a substrate [43].

Detection of GFP and GUS expression

GFP expression *in planta* was analyzed using a stereo microscope (Leica MZ 12) with a fluorescent light source and a Leica GFP-*plus* filter set ($\lambda_{ex} = 480/40$ nm; $\lambda_{em} = 510$ nm LP barrier filter). Images of the plant tissues were taken using a color video camera (Sony CCD-iris with integration unit, Sony DKR700).

For GUS histochemistry, plant material was stained in 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) solution in 50 mM sodium phosphate buffer (pH 7.2), 0.1% Triton X-100, 10 mM EDTA, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ for 16 h at 37 °C in darkness. Tissue was cleared using 70% ethanol. GUS expression was examined with a stereo microscope (Wild Leitz M3Z) and images recorded with the CCD camera without integration. Pictures were corrected for brightness and printed using the ADOBE Photoshop 4 software.

Seeds of transgenic *A. thaliana* lines were sterilized and sowed on growth medium [51] containing 25 mg/l kanamycin and grown at 21 °C under a 16 h/8 h light/dark regime. 5-day-old seedlings were mounted in water and examined using a Leica TCS NT confocal laser scanning unit equipped with an inverted microscope (Leica DMIRB/E), a 16 A Argon/Krypton laser and FITC filter set.

Transgenic *L. japonicus* plants were nodulated with wild-type *Mesorhizobium loti* strain NZP2238 as described in López-Lara *et al.* [29]. Fresh sections were made of 2-week-old nodules using a vibratome (LeicaVT1000S). The sections were analyzed with a Biorad MRC1024ES confocal laser scanning module with a 16 A Argon/Krypton laser and FITC filter set connected to a Zeiss Axioplan microscope. GUS expression in the nodules was analyzed as mentioned above. The section were counter stained for 10 s in

0.05% safranin in 50% ethanol and briefly destained in 50% ethanol.

Results

Transient expression of gusA *and* gfp *fusion genes in plants*

In the various *gusA* and *gfp* fusion constructs two different red-shifted variants of *gfp* were used, a synthetic *gfp* (*s-gfp-TYG*) with optimized codon usage for humans which was shown to be highly expressed in plants [6] and an enhanced *gfp* (*egfp*) from Clontech. Expression in plants of *egfp* has not been shown before but since it differs only 4 nucleotides from *s-gfp-TYG* of which one is silent and the others result in only two amino acid substitutions (Leu64→Phe and His233→Leu), it was expected to be effective. An overview presenting the cloning steps of the fusion genes, *gusA::intr/gfp*, *gusA/gfp*, *gfp/gusA* and *gfp/gusA::intr*, is given in Figure 1. The functionality of various constructs was tested initially by DNA micro projectile bombardments. Based on the results obtained with these transient expression studies in calli of *L. japonicus* and in seedlings of *A. thaliana* and *Nicotiana sylvestris*, the fusion genes shown in Figure 2 were chosen for a more detailed analysis. With these constructs, transformed single cells were observed which were green fluorescent and GUS positive (data not shown).

An alternative method used to transiently express a heterologous gene is based on the use of plant viruses as a fast and convenient delivery tool of foreign genes (for a review see [42]). The fusion protein produced by one of the constructs was tested in detail by cloning the *gusA/gfp* gene in a potato virus X (PVX) vector. We removed the intron from the *gusA::intr/gfp* construct since in this system the genes are directly expressed from viral mRNA, which is produced in the cytoplasm and thus are avoiding the splicing machinery. The *gusA/gfp* fusion gene was cloned in the previously described PVX constructs using the subgenomic promoter duplication strategy [5] to express foreign genes (pMP2497; see Figures 1 and 2). Infectious RNA was obtained *in vitro* from run-off transcription of pMP2497 and RNA transcribed from PVX vectors containing either *gusA* or *gfp* alone was taken along as controls. All transcripts synthesized were infectious when inoculated on *N. benthaminiana* plants. For PVX.*gusA* and PVX.*gfp* mild mosaic symptoms were observed on systemic leaves 7 days post-inoculation, as was described previously (data not shown) [1, 5, 34]. However, PVX in which the *gusA/gfp* fusion gene was cloned did not spread systemically. The inoculated leaf, on which mild symptoms were visible, was analyzed with fluorescence microscopy, showing the presence of bright green fluorescent spots (Figure 3). Some very faint spots were also seen in the leaves growing out of the axilary bud of the inoculated leaf and in the vascular bundle of the internode above it. Histochemical analysis of GUS activity gave blue staining exactly at the same position were the green fluorescence was detected, as shown in Figure 3. Moreover, blue staining was observed in the shoot from the axilary bud, the vascular bundle of the internode above and the petiole of the next leaf. We conclude that the *gusA/gfp* fusion gene is functional but apparently interferes with systemic infection of the PVX virus. The results also show that the fluorescence emitted by the GUS/GFP fusion protein is sufficiently strong to be detected easily in strongly autofluorescent leaves using a stereomicroscope equipped with a fluorescent light source.

Analysis of expression of fusion genes in stably transformed plants

Root explants of *A. thaliana* were transformed with pMP2482 (35S-*gusA::intr/gfp*), pMP3629 (35S-*gfp/gusA*), and pBINPLUS (control). Similar transformation efficiencies with the reporter gene constructs and the control were observed. The kanamycin resistant calli obtained after transformation with pMP2482 and pMP3629 were brightly green fluorescent. The same calli were also positive for GUS when stained with X-Gluc (data not shown). Various organs of the primary transformants were monitored for GFP activity during the regeneration procedure (as an example is shown the expression obtained with the 35S-*gusA::intr/gfp* construct in Figure 4). Independent transgenic lines were regenerated and in all of them, bright green fluorescence was also observed in flowers, especially in the petals and the carpel (Figure 4a), and in siliques (Figure 4b). Due to the strong autofluorescence of the chlorophyll the expression of GFP is often detected as orange fluorescence. In the control plants only yellowish autofluorescence in the petals and siliques and the red fluorescence of chlorophyll in the leaves and carpels was detected, even with maximal integration time. In the leaves only highly expressing lines allowed GFP expression to be detected by standard fluorescent microscopic techniques. GFP was easy to detect in emerging roots since these tissues lack

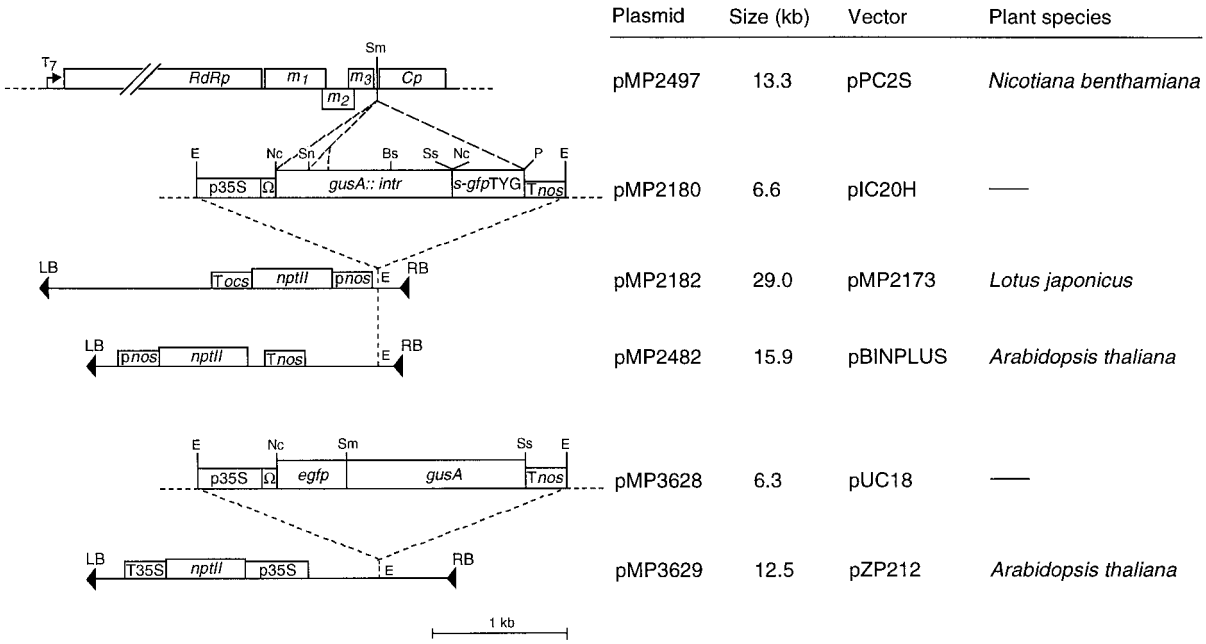

Figure 2. Fusion gene constructs and their use in plant assays. At the right, the size, the name of the parental vector and the plant species used to test the respective constructs are indicated. For abbreviations of the restriction sites see legend of Figure 1. Abbreviations: Ω, AlMV leader sequence; *nptII*, neomycin phosphotransferase; p35S, enhanced CaMV 35S promoter; p*nos* nopaline synthase promoter; T*nos*, nopaline synthase terminator; T*ocs*octopine synthase terminator; T35S, CaMV 35S terminator; LB, left border; RB, right border; T_7, promoter from bacteriophage T7; *RdRp* RNA-dependent RNA polymerase; *m1*, *m2* and *m3* movement protein genes; *Cp* coat protein gene.

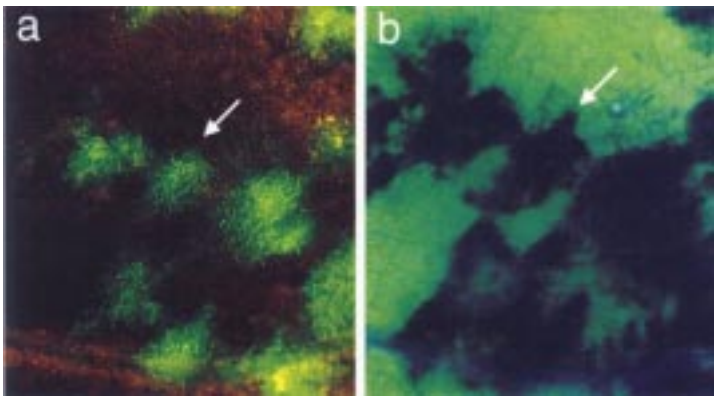

Figure 3. N. benthamiana infected with *gusA/gfp*-expressing PVX. (a) GFP expression in the inoculated leaf; (b) the same leaf stained with X-Gluc to detect GUS activity. The arrows point to the same position of the leaf.

chlorophyll (Figure 4c). The same plants were tested for GUS activity by X-Gluc staining. The results show that the detected green fluorescence is completely correlated with strong GUS activity in all parts of the plant tested. As an example, the results of a GUS assay of the same organs as shown with fluorescent microscopy detection are shown in Figure 4, panels a–c. The kanamycin resistant F1 progeny of transgenic lines carrying 35S-*gusA::intr/gfp*, 35S-*gfp*/*gusA* and the controls were also tested for GFP and GUS expression yielding similar results as shown in Figure 4 (data not shown) confirming stable integration and expression of the fusion gene.

Since vectors derived from plasmid pBINPLUS did not give good results in transformation of *L. japonicus*, a new vector was constructed derived from the binary

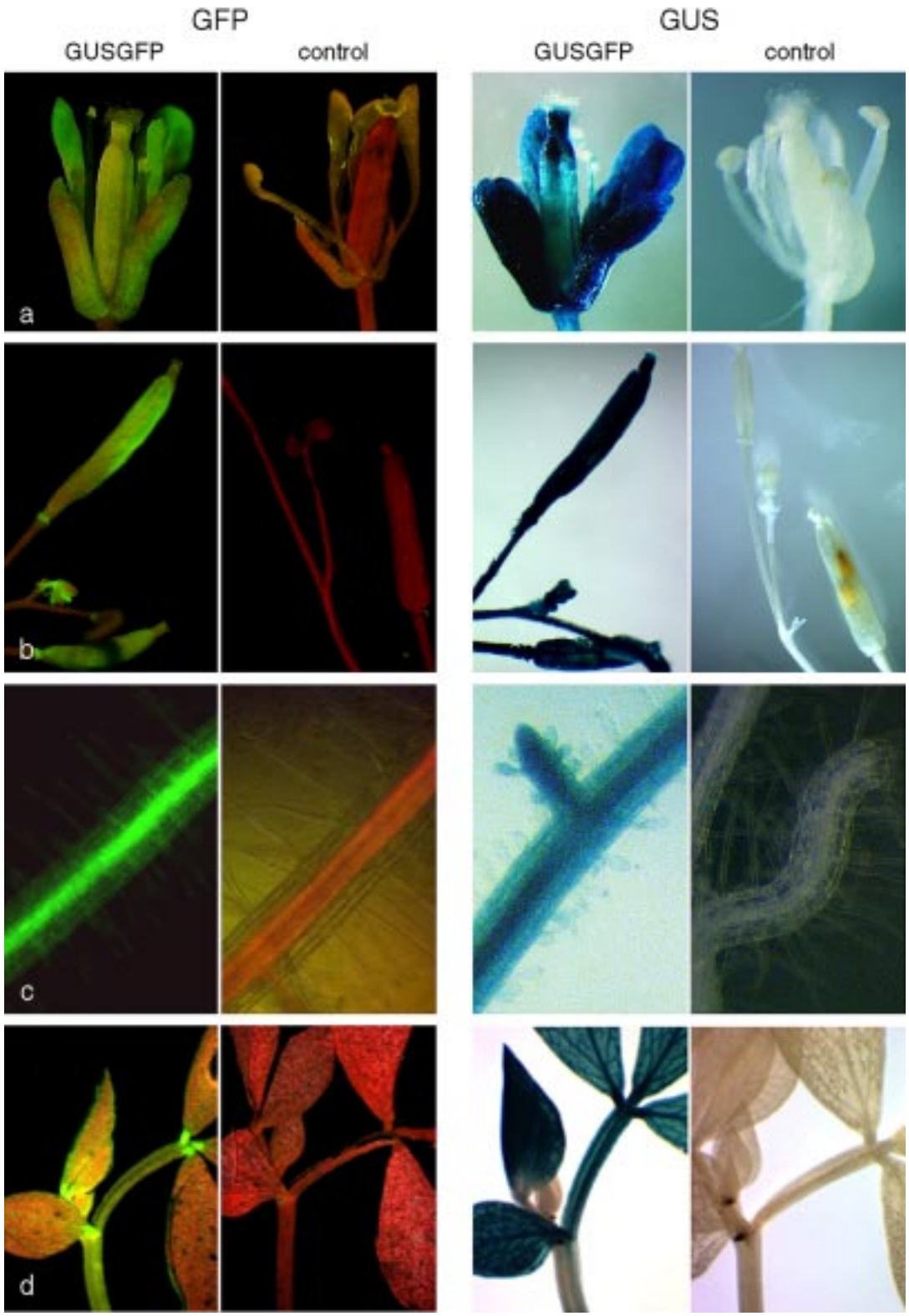

Figure 4. Expression of GFP and GUS in transgenic *A. thaliana* and *L. japonicus* plants. Plants containing 35S-*gusA:: intr/gfp* or control T-DNA are indicated as GUSGFP and control, respectively. Panel a, b, c: *A. thaliana* flowers, siliques and roots, respectively. To visualize the roots of the control plant under fluorescent light, the sample was illuminated indirectly with a white light source. Panel d: *L. japonicus* stem with leaf.

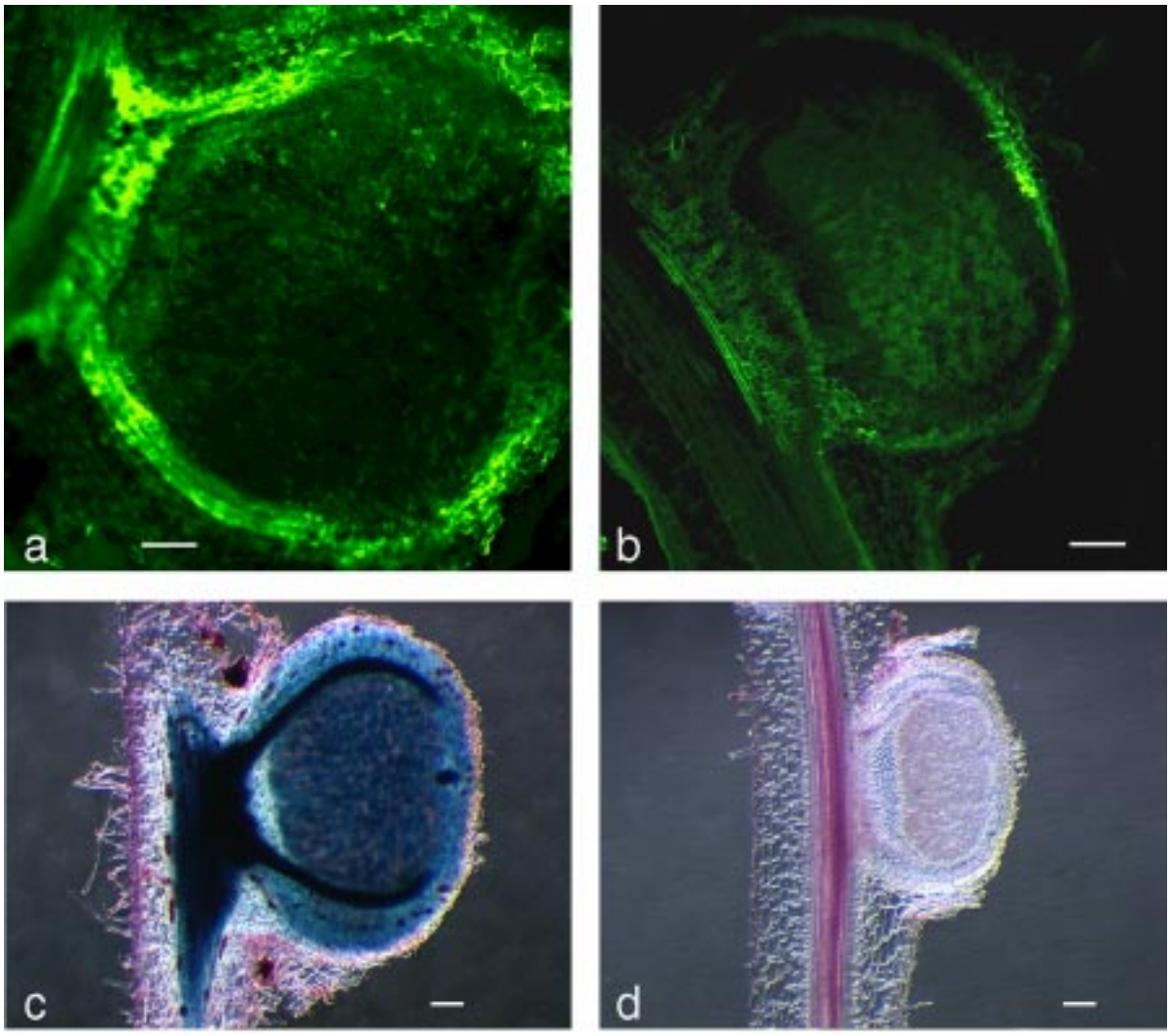

Figure 5. Expression of GFP and GUS in root nodules of transgenic *L. japonicus* plants. Nodules obtained after inoculation with *M.loti* on plants containing 35S-*gusA::intr/gfp* (a and c) or control T-DNA (b and d). Transverse sections of lotus nodules were analyzed for GFP with confocal microscopy (a and b) and stained with X-Gluc to detect GUS expression (c and d). Bar: 100 μm.

vector pSJL4644 [26]. In order to make the vector also suitable for future promoter-trapping strategies the construct was adapted to position the right border closer to the multiple cloning site within the T-DNA region, resulting in plasmid pMP2173 (Figure 1). Pilot experiment indicated that pMP2173 gave comparable transformation efficiencies as pSLJ4644 in *L. japonicus* (data not shown). The plasmid pMP2182, containing the *gusA::intr/gfp* fusion gene under control of the CaMV 35S promoter cloned into pMP2173, was used for the transformation of hypocotyl explants of *L. japonicus*. Transgenic calli were selected on G418 and 9 weeks after cocultivation green fluorescence was observed in calli transformed with pMP2182 and not in control calli obtained with pMP2173 or pSLJ4644 (data not shown). Regenerated plants resulting from these calli were tested for GFP and GUS activity. In shoots obtained with the 35S-*gusA::intr/gfp* construct GFP expression could be easily monitored in the stereo-microscope by the emission of orange fluorescence, whereas in the control shoots only the red autofluorescence of chlorophyll could be detected (Figure 4d). The petioles of the positive plants were brightly green fluorescent because these parts contain less chlorophyll. GUS activity was assayed showing that the 35S-*gusA::intr/gfp* leaves became indigo blue,

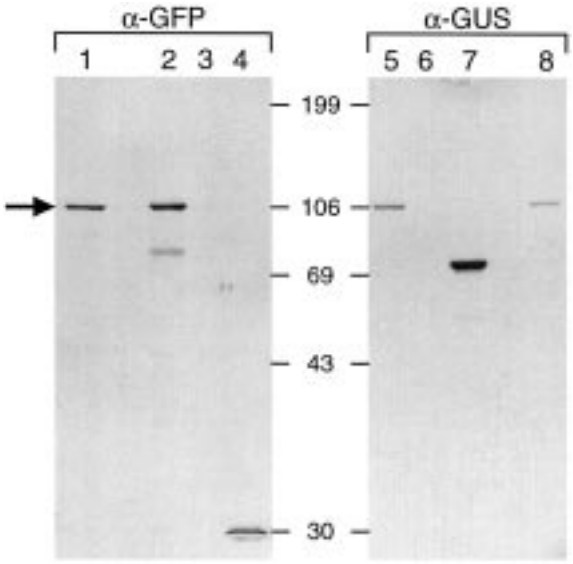

Figure 6. Immunoblot analysis of fusion proteins. Lanes contain protein extracts prepared from transgenic *A. thaliana* seedlings containing 35S-*gfp/gusA* (lane 1 and 8), 35S-*gusA::intr/gfp* (lane 2 and 5), pBINPLUS (lane 3 and 6), 35S-*gfp* (lane 4) or 35S-*gusA* (lane 7). Blots were incubated with polyclonal antibodies raised against GFP (α-GFP) or GUS (α-GUS). The position of the fusion proteins is marked with an arrow. The size of marker proteins is given in kDa indicated at the center.

whereas the control leaves were negative (Figure 4d). In the 35S-*gusA::intr/gfp* transgenic roots green fluorescence could be detected, especially on the position were lateral roots emerged. However, the green fluorescent in several parts of the roots was masked by autofluorescence. Staining these roots for GUS activity resulted in homogeneously blue roots (data not shown).

L. japonicus plants containing 35S-*gusA::intr/gfp* and which showed GFP and GUS activity in the leaves were inoculated with a wild-type *M. loti* strain. Transgenic lotus plants obtained with pMP2173 were taken along as control. Root nodules were analyzed both for GFP and GUS expression. Sections of 2-week-old nodules were analyzed for GFP expression by confocal laser scanning microscopy (Figures 5a and 5b). GFP was most clearly detectable in the vascular bundle of the 35S-*gusA::intr/gfp* nodule (Figure 5a). This could be expected since it has been described previously that the CaMV 35S promoter is more strongly expressed in cells of the vascular bundle [3, 25]. Control roots gave a background signal which was predominantly due to autofluorescence located in the epidermis (Figure 5b). Staining of the sections with X-Gluc to detect the GUS activity confirms the data obtained with confocal microscopy. The vascular bundle of the 35S-*gusA::intr/gfp* nodule stained most strongly blue (Figure 5c). The histochemical detection of GUS activity showed no background activity in the control plants (Figure 5d)

Immunochemical analysis of the fusion proteins

To analyze the different gene products on the molecular level, crude protein extracts of the kanamycin resistant F1 progeny of 35S-*gusA*::*intr*/*gfp* and 35S-*gfp/gusA A. thaliana* lines were subjected to immunoblot analysis. As controls, protein extracts prepared from seedlings expressing either GFP or GUS under control of the CaMV 35S promoter were used. Blots were incubated with polyclonal antibodies against GFP or GUS (Figure 6). The results showed that the estimated size of the fusion proteins of 100 kDa corresponds closely to the sum of the sizes of GUS (68 kDa; [24]) and GFP (28 kDa; [23]). Only in the lane loaded with 35S-*gusA*::*intr/gfp* plant material, an additional smaller GFP-antigenic band is detected (Figure 6, lane 2), suggesting some degradation of the fusion protein. In SDS-gels the GUS enzyme activity in protein extracts of independent transgenic lines was measured using X-Gluc or MUG as a substrate and activity was detected in a band with a lower mobility than GUS, confirming the immunoblot data. Furthermore, this result shows that both N- and C-terminal fusion proteins are produced at high levels (data not shown).

Localization of GUS/GFP *protein in transgenic* Arabidopsis *by confocal microscopy*

The CaMV 35S promoter is not evenly expressed in all plant tissues. Therefore, we tested the sensitivity of the GFP detection *in vivo* by confocal laser microscopy as compared to the method of GUS staining. A series of optical sections were made from the cotyledon and the lower part of the hypocotyl. A good example of the high sensitivity of the detection of GFP by fluorescence analysis is shown in Figure 7. The green fluorescence was found in the thin layer of cytoplasm between the cell wall and the vacuole. The cytoplasmic localization was most clear in the stomata of the cotelydon and in the hairs at the transition zone (Figure 7). Both these cell types have relatively small vacuoles. No GFP expression was detected in the nucleus and the vacuole. Furthermore, activities at the cellular level, such as cytoplasmic streaming, were clearly evident in the living cells. Further analysis of

some transgenic lines in which the fusion gene was relatively weakly expressed, using fluorescence correlation spectroscopy [38], showed that the constructs are very suitable for single molecule detection of the green fluorescent-GUS fusion protein (T. Visser and H.P.S., unpublished results).

Discussion

With the rapid advances in techniques for detection of fluorescence, the use of GFP from *A. victoria* offers exciting new possibilities for monitoring various processes, such as transcription, translation, translocation and interactions of proteins, in living cells. In order to optimize the study of transcription in plants, sensitive and vital bifunctional reporter genes were constructed. Two slightly different genes, both encoding red-shifted GFP variants and with altered codon usage, were cloned in frame at either the 5′ or 3′ terminus of the *gusA* gene (Figure 1). For both fusions we also tested the *gusA* gene containing an intron [50]. This has the advantage that it is possible to discriminate between the prokaryotic and eukaryotic expression of the reporter already at early steps in the *Agrobacterium*-mediated plant transformation procedure.

The *gusA/gfp* fusion was active when PVX RNA carrying this fusion was inoculated on a tobacco leaf (Figure 3). However, the recombinant virus did not spread systemically throughout the plant. An effect of insert size on the systemic infection capacity of the PVX virus was already observed in *Nicotiana tabacum* cv. Samsun NN for the previously described PVX.*gusA* construct [5]. It was suggested that the relatively large insert size of 1.8 kb was partially responsible for this effect. This could also explain our results with the *gusA/gfp* fusion gene.

Two model plant species, *A. thaliana* and *L. japonicus*, were selected to test our bifunctional reporter genes in stably transformed plants. We have chosen the *gusA::intr/gfp* fusion gene for a detailed analysis of gene expression in various parts of the plants. We show that in transgenic *A. thaliana* and *L. japonicus* plants, the activity of both reporters, GFP and GUS, can be detected efficiently with a stereo microscope, using fluorescence, and histochemical staining, respectively (Figure 4). However, in root nodules of *L. japonicus* a significant level of green autofluorescence was detected (Figure 5). This implies that for detection of weak expression of *gfp* more sophisticated detection methods such as fluorescence lifetime imaging microscopy [12] should be used. Protein analysis using immunoblot assays shows that the GFP/GUS and GUS/GFP fusion proteins are relatively stable in transgenic plants since hardly any degradation products were detected (Figure 6). The high expression levels did not have a negative effect on the transformation efficiency since no differences in transformation frequencies with the control vectors were detected. Furthermore, the large majority of transformants was positive in the analysis of green fluorescence and GUS activity. These results are in contrast with the results of Haseloff *et al.* who reported a negative effect on the transformation of wild-type *gfp* into *A. thaliana* plants [17]. The difference between these experimental results can presently not be explained, but could be based on the use of a different variant of the *gfp* gene.

Confocal laser scanning microscopy gave the possibility to detect in much more detail the localization of the fusion protein as can be done by using the GUS assay. A disadvantage of histochemical detection of GUS activity is diffusion of the coloured or fluorescent product to surrounding cells. This feature, which fortunately does not count for GFP, makes precise cellular localization impossible. Recently, Haseloff and collaborators showed that GFP lacking a specific targeting signal was enriched in the nucleoplasm of *A. thaliana* cells [17]. In our analyse using confocal laser microscopy, the GUS/GFP protein could not be detected in the nucleus. The translocation of the fusion protein through the nuclear envelope or other membrane systems would also not be expected due to the large size of the fusion protein and the lack of a specific targeting signal. Therefore, an additional advantage of the use of the GUS/GFP fusion protein is that the detection of the reporter gene product is more strictly co-localized with gene expression.

The results with the transgenic *L. japonicus* plants carrying 35S-*gusA::intr/gfp* plants represent the first example of the use of the GFP protein for the detection of gene expression in a leguminous plant. Since *L. japonicus* is one of the most suitable model plants for genetic studies of the root nodulation process [16] our results are very useful for further studies on the molecular mechanism of this process. In future experiments we are planning to fuse the constructed fusion genes to promoters of various genes which are specifically expressed during nodule formation, the so-called nodulin genes, and introduce these constructs into *L. japonicus*. The resulting transgenic plants will be extremely useful to analyze the regulation of these

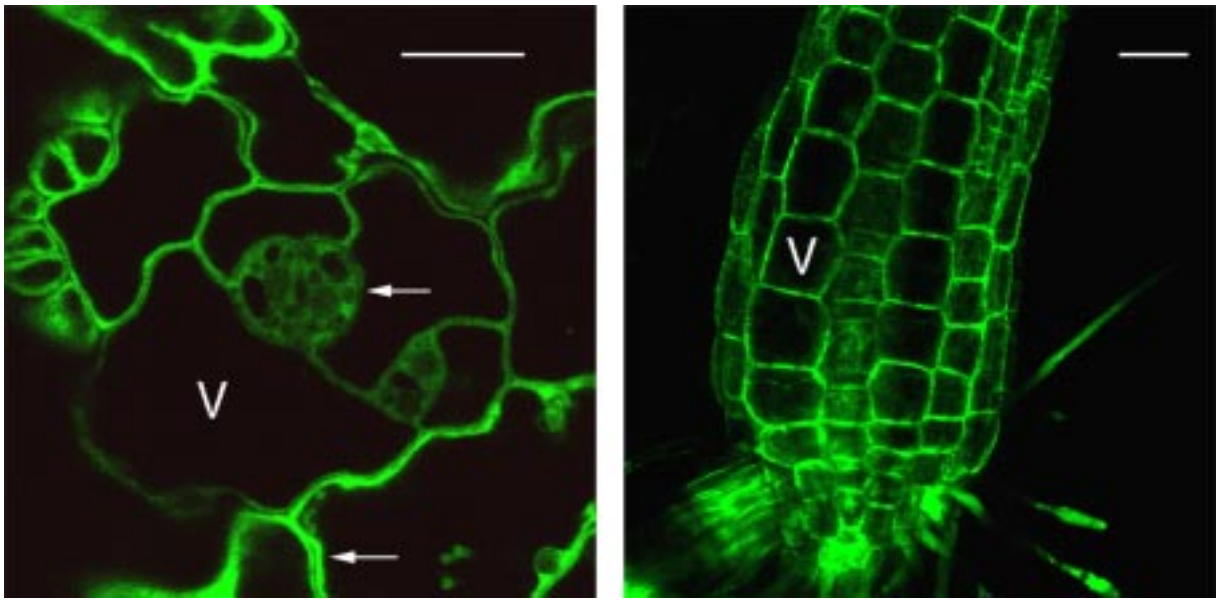

Figure 7. Confocal microscopy images of a transgenic *A. thaliana* seedling containing 35S-*gusA::intr/gfp*. Left panel: an optical section of the cotyledon. GFP in the cytoplasm of the stomata and the epidermal cells (arrow). Bar: 75 μm. Right panel: an optical section through the lower part of the hypocotyl and the transition zone. No GUS/GFP protein is present in the vacuoles (V). Bar: 25 μm.

promoters during the various stages of the root nodulation process.

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