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GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants

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We have used the *Escherichia coli* β-glucuronidase gene (GUS) as a gene fusion marker for analysis of gene expression in transformed plants. Higher plants tested lack intrinsic β-glucuronidase activity, thus enhancing the sensitivity with which measurements can be made. We have constructed gene fusions using the cauliflower mosaic virus (CaMV) 35S promoter or the promoter from a gene encoding the small subunit of ribulose bisphosphate carboxylase (rbcS) to direct the expression of β-glucuronidase in transformed plants. Expression of GUS can be measured accurately using fluorometric assays of very small amounts of transformed plant tissue. Plants expressing GUS are normal, healthy and fertile. GUS is very stable, and tissue extracts continue to show high levels of GUS activity after prolonged storage. Histochemical analysis has been used to demonstrate the localization of gene activity in cells and tissues of transformed plants.

Key words: chimaeric genes/plant transformation/reporter gene/ *Agrobacterium*

Introduction

Control of gene activity can be manifested at many levels, including the initiation of transcription or translation and the processing, transport or degradation of mRNA or protein. The use of precise gene fusions can simplify analysis of these complex processes and delineate the contribution of transcriptional control by eliminating the specific signals for post-transcriptional controls and replacing them with sequences from a readily assayed reporter gene. In addition, members of multi-gene families whose products are very similar can be regulated differentially during development. By using gene fusions to individual members of such families and introducing these fusions into the germline one can study the expression of individual genes separate from the background of the other members of the gene family. Analysis of mutationally altered genes in organisms accessible to transformation techniques is greatly facilitated by the use of sensitive reporter enzymes. By using a reporter gene that encodes an enzyme activity not found in the organism being studied, the sensitivity with which chimaeric gene activity can be measured is limited only by the properties of the reporter enzyme and the quality of the available assays for the enzyme.

To date, at least six reporter genes have been used in studies of gene expression in higher plants. Gene fusions using the *Escherichia coli* β-galactosidase (Helmer *et al.*, 1984) proved difficult to assay because of high endogenous β-galactosidase activity in plants. Use of the *Agrobacterium tumefaciens* Ti-plasmid-encoded genes nopaline synthase (Depicker *et al.*, 1982; Bevan *et al.*, 1983a) and octopine synthase (DeGreve *et al.*, 1982) promised to overcome problems associated with endogenous activity because the opines produced by these genes are not found in normal plant cells. However, these reporter genes are not widely used because the assays are cumbersome and difficult to quantitate, they cannot be used to demonstrate enzyme localization (Otten and Schilperoort, 1978), and octopine synthase cannot tolerate amino-terminal fusions (Jones *et al.*, 1985). The two most useful reporter genes to date have been the bacterial genes chloramphenicol acetyl transferase (CAT) and neomycin phosphotransferase (NPTII) which encode enzymes with specificities not normally found in plant tissues (Bevan *et al.*, 1983b; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983a,b). In addition, NPTII can tolerate amino-terminal fusions and remain enzymatically active, making it useful for studying organelle transport in plants (van den Broeck *et al.*, 1985). However, both CAT and NPTII are relatively difficult, tedious and expensive to assay (Gorman *et al.*, 1982; Reiss *et al.*, 1984). Competing reactions catalyzed by endogenous esterases, phosphatases, transferases and other enzymes also limit sensitivity and make quantitation of CAT or NPTII by enzyme kinetics very difficult. Recently, the firefly luciferase gene has been used as a marker in transgenic plants (Ow *et al.*, 1986), but the enzyme is labile and difficult to assay with accuracy (DeLuca and McElroy, 1978). The reaction is complex and there is little, if any, potential for routine histochemical analysis or fusion genetics.

We believe that future advances in the study of plant gene expression require the development of new gene fusion systems that are easy to quantitate and highly sensitive, thus allowing analysis of genes whose products are of moderate and low abundance. This is contingent on a complete absence of any intrinsic reporter enzyme activity in plants. Activity of the reporter enzyme should be maintained when fused to other proteins at its amino terminus to allow the study of translation and the processing events involved in protein transport. The reporter enzyme should be detectable with sensitive histochemical assays to localize gene activity in particular cell types. Finally, the reaction catalyzed by the reporter enzyme should be sufficiently specific to minimize interference with normal cellular metabolism and general enough to allow the use of a variety of novel substrates to maximize the potential for fusion genetics and *in vivo* analysis.

To meet these criteria, we have developed the *E.coli* β-glucuronidase gene as a reporter gene system for transformation of plants. β-Glucuronidase (GUS, EC 3.2.1.31), encoded by the *uidA* locus (Novel and Novel, 1973), is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides (Stoeber, 1961), many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. The β-glucuronidase gene has been cloned and sequenced, and encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson, 1985; Jefferson *et al.*, 1986; Jefferson *et al.*, 1987). In this paper we describe several useful features of GUS which make it a superior reporter gene system for plant studies. Many plants assayed to date lack

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detectable glucuronidase activity, providing a null background in which to assay chimaeric gene expression. We show that glucuronidase is easily, sensitively and cheaply assayed *in vitro* and can also be assayed histochemically to localize GUS activity in cells and tissues.

Results

Many higher plants contain no detectable β-glucuronidase activity

Roots, stems and leaves from wheat, tobacco, tomato, potato, *Brassica napus* and *Arabidopsis thaliana*, potato tubers, and seed from wheat and tobacco were homogenized with GUS extraction buffer containing a variety of protease inhibitors such as PMSF and leupeptin. The plant extracts were incubated in a standard assay at 37°C for 4 to 16 h, and the fluorescence of 4-methyl umbelliferone (MU) was measured. Endogenous activity was below the limits of detection. Extremely lengthy assays occasionally gave low levels of MU fluorescence, but the kinetics of MU accumulation were consistent with a slow conversion of the glucuronide into another form, possibly a glucoside, that was subsequently cleaved by intrinsic glycosidases. β-Galactosidase assays performed under similar conditions on tobacco and potato extracts were off-scale (at least 10 000 times higher than the minimal detectable signal) within 30 min. Reconstruction experiments were performed with purified GUS added to tobacco and potato extracts to demonstrate the ability of these extracts to support β-glucuronidase activity (data not shown).

Construction of plasmids for transformation of plants with GUS fusions

A general purpose vector for constructing gene fusions was made by ligating the coding region of GUS (Jefferson *et al.*, 1986) 5′ of the nopaline synthase polyadenylation site (Bevan *et al.*, 1983a) in the polylinker site of pBIN19 (Bevan, 1984). This vector, pBI101 (Figure 1), contains unique restriction sites for *Hin*dIII, *Sal*I, *Xba*I, *Bam*HI and *Sma*I upstream of the AUG initiator codon of GUS, to which promoter DNA fragments can be conveniently ligated. The cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al.*, 1985) as described in the expression vector pROK1 (Baulcombe *et al.*, 1986) was ligated into the *Hin*dIII and *Bam*HI sites to create pBI121. Similarly, the promoter from a tobacco gene encoding the small subunit of ribulose bisphosphate carboxylase (rbcS) Ntss23 (Mazur and Chui, 1985) deleted of rbcS coding sequences, was fused to pBI101 to make pBI131.

Chimaeric GUS genes are expressed in transformed plants

Nicotiana tabacum var. Samsun plants were transformed with *Agrobacterium* binary vectors (Bevan, 1984) containing transcriptional fusions of either the CaMV 35S promoter or the tobacco rbcS promoter with the coding region of GUS as shown in Figure 1. Several kanamycin resistant plants were regenerated from each transformation. Two rbcS−GUS transformants and two CaMV−GUS transformants were chosen for further study. We first assayed various organs of one plant from each transformation, axenically cultured in 3000 lux white light, 18 h day, 6 h night. The results of this analysis are shown in Figure 2, and tabulated in Table I using either of two normalization methods (see Discussion). The plant containing a rbcS−GUS fusion (rbcS−GUS 2) exhibited a pattern of gene expression consistent with earlier studies using heterologous rbcS gene fusions (e.g. Simpson *et al.*, 1986a). The highest sp. act., using either protein or DNA as a denominator, was found in older leaves (~8 cm long), with progressively less activity in very young leaves (<5 mm), stems and roots. The other rbcS−GUS fusion plant showed a similar pattern (data not shown).

The two plants transformed with the CaMV 35S−GUS fusion displayed a pattern of gene expression distinct from that of the rbcS−GUS fusion plants. The highest levels of activity were found in roots, with similar levels in stems. GUS activity was also high in leaves, consistent with previous observations that the CaMV 35S promoter is expressed in all plant organs (Odell *et al.*, 1985).

To verify that no significant rearangements of the transforming DNA had occurred, a Southern blot analysis was conducted as shown in Figure 3. Digestion of DNA extracted from all of the tranformants with *Hin*dIII and *Eco*RI released a single internal fragment of T-DNA consisting of the nopaline synthase polyadenylation site, the GUS coding region and the promoter

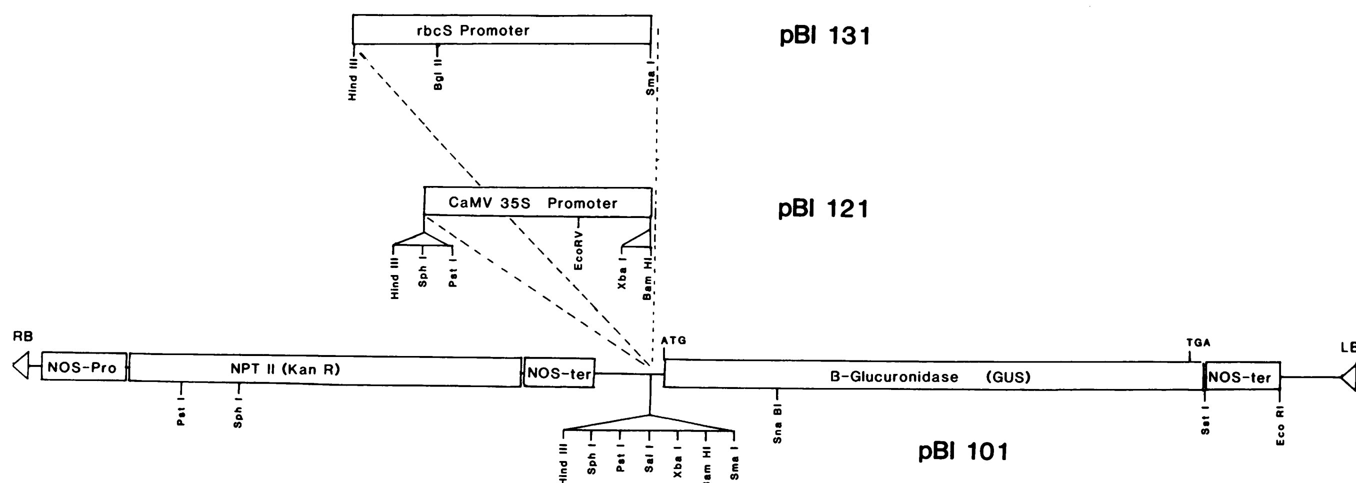

Fig. 1. Structure of expression vectors. **Bottom**: T-DNA region of pBI101, containing polylinker cloning sites upstream of the GUS, followed by the nopaline synthase polyadenylation site (NOS-ter). *Pst*I and *Sph*I are not unique to the polylinker. The expression cassette is within pBIN19, giving pBI101 a total length of ~12 kb. **Middle**: Chimaeric CaMV 35S−GUS gene in pBI121. An 800 bp *Hin*dIII−*Bam*HI CaMV 35S promoter fragment (Guilley *et al.*, 1982) was ligated into the corresponding sites of PBI101. The mRNA initiation site is approximately 20 bp 5′ of the GUS initiator codon. **Top**: Chimaeric rbcS−GUS gene in pBI131. A 1020 bp *Hin*dIII−*Sma*I fragment containing the promoter of a tobacco rbcS was ligated into the corresponding sites of pBI101. The mRNA initiation site is ~55 bp 5′ of the GUS initiator codon, and contains nearly the entire untranslated leader of the rbcS gene.

(CaMV 35S or rbcS). RbcS–GUS transformants contained three copies (rbcS–GUS 2, Figure 3, lane 6) and about seven copies (rbcS–GUS 5, lane 8) of the predicted 3.1 kb *Hin*dIII–*Eco*RI fragment. Digestion with *Eco*RI revealed multiple border fragments (Figure 3, lanes 5 and 7), confirming the copy number estimates deduced from the double digestions. Similarly, CaMV 35S–GUS plants had multiple insertions as shown in Figure 3, lanes 1–4. CaMV–GUS 21 had three copies of the predicted 2.9 kb fragment, while CaMV–GUS 29 had two copies. No hybridization of the labelled GUS coding region to untransformed plant tissue was observed (lanes 9 and 10).

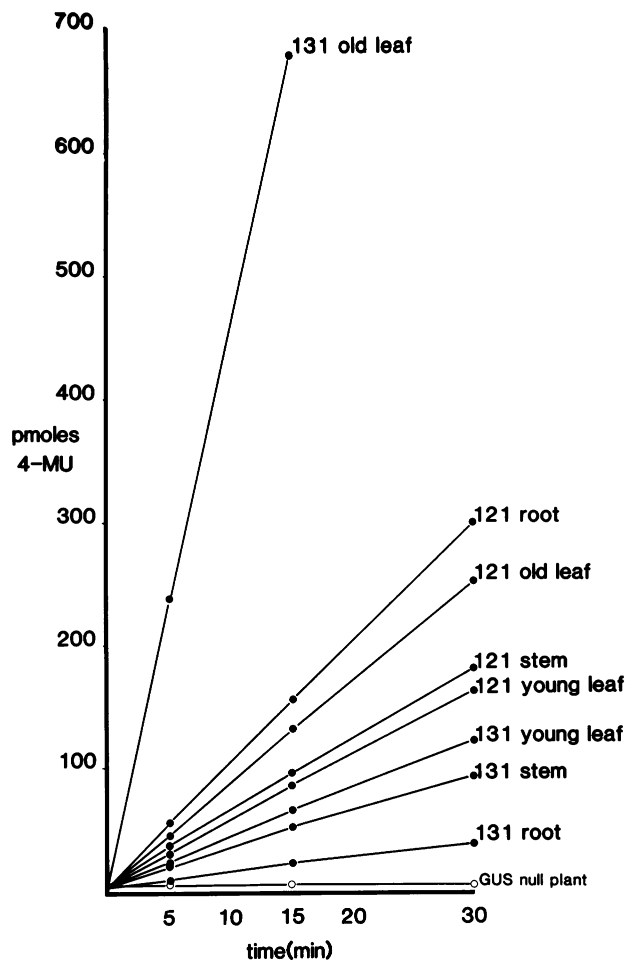

Fig. 2. β-Glucuronidase activity in extracts of different organs of transformed and non-transformed tobacco plants. Extracts were prepared from axenic tobacco plants using about 50 mg fresh weight of tissue ground in 500 μl extraction buffer. 5 μl of extract was assayed as described in Materials and methods. Mature leaves were lower, expanded leaves ~80 mm long, while young leaves were ~5 mm long, and were dissected from the shoot apex. All samples were taken from the same plant (either CaMV–GUS 21, rbcS–GUS 2 or non-transformed) at the same time. Leaf tissue was taken from a non-transformed plant for this assay, although all organs showed no GUS activity (data not shown).

GUS activity in plants can be visualized using histochemical methods

To determine whether we would be able to use histochemistry to investigate single-cell or tissue-specific expression of GUS gene fusions in plants, preliminary experiments were carried out on sections of stems of several independently transformed rbcS–GUS and CaMV–GUS plants. Typical results are shown in Figure 4. Stem sections were chosen both for their ease of manipulation and because most of the cell types of a mature plant are represented in stem. To illustrate the light-regulated nature of the rbsC–GUS fusion, the plants were illuminated from one side only for 1 week before sectioning. Sections from both plants stained intensely with the substrate while non-transformed tissue did not stain (Figure 4c). Stem sections of CaMV–GUS plants always show highest levels of activity in phloem tissues along the inside and outside of the vascular ring, most prominently in a punctate pattern that overlies the internal phloem and in the rays of the phloem parenchyma which join the internal and external phloem (Esau, 1977). There is also variable lighter staining throughout the parenchymal cells in the cortex and in the pith, and also in epidermal cells, including the trichomes (Figure 4a).

RbcS–GUS stem sections rarely if ever show intense staining in the trichomes, epidermis, vascular cells or pith, but tend to stain most intensely over the cortical parenchyma cells containing chloroplasts (chlorenchyma), with faint and variable staining in the pith. Although we most often see the strongest staining in a symmetrical ring around the vascular tissue just inside the epidermis, we sometimes observe an asymmetric distribution of staining in the cortical stem cells. Suspecting that this pattern was due to uneven lighting, we illuminated a plant from one side for 1 week before sectioning, and found that the staining was asymmetric, with intense staining in the chloroplast-containing cells proximal to the light source (Figure 4b). The staining patterns we observe for both the CaMV 35S–GUS and the rbcS–GUS transformants are consistent between several independent transformants. Untransformed plants never show staining with 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc), even after extended assays of several days (Figure 4c).

Discussion

We present here new methods for analysing gene expression in transformed plants that we feel will be of general utility. The β-glucuronidase gene from *E.coli* has been expressed at high levels in transformed tobacco plants with no obvious ill effects on plant growth or reproduction. The ability to quantitate gene expression through the routine use of enzyme kinetics greatly

Table I. GUS specific activity

Plant organ	pmole 4-MU/min/mg protein			pmol 4-MU/min/μg DNA	
	CaMV 35S–GUS	rbcS–GUS	Untransformed	CaMV 35S–GUS	rbcS–GUS
Leaf (5 mm)	283	205	<0.1	2530	4400
Leaf (70 mm)	321	1525	<0.1	5690	93 950
Stem	427	260	<0.1	13 510	2650
Root	577	62	<0.1	12 590	690

The rate data shown in Figure 2 were converted to sp. act. by measuring the protein concentration of the extracts using the Bradford reagent. The data are also presented as GUS activity/unit weight of DNA in the extract to account better for the differences in cell number between different tissues.

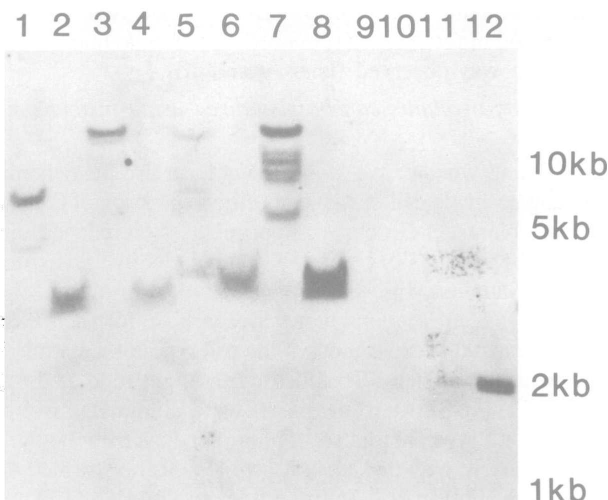

Fig. 3. Autoradiograph of a Southern blot of DNA extracted from transformed plants and digested with restriction endonucleases. The filter was hybridized with a ^{32}P labelled restriction fragment containing the coding region of the β-glucuronidase gene. Lane: (**1**) CaMV−GUS 21, *Eco*RI; (**2**) CaMV−GUS 21, *Eco*RI and *Hind*III; (**3**) CaMV−GUS 29, *Eco*RI; (**4**) CaMV−GUS 29, *Eco*RI and *Hind*III; (**5**) rbcS−GUS 2, *Eco*RI; (**6**) rbcS−GUS 2, *Eco*RI and *Hind*III; (**7**) rbcS−GUS 5, *Eco*RI; (**8**) rbcS−GUS 5, *Eco*RI and *Hind*III; (**9**) non-transformed, *Eco*RI; (**10**) non-transformed, *Eco*RI and *Hind*III; (**11**) single copy reconstruction of GUS coding region; (**12**) five copy reconstruction.

enhances the precision and resolution of the questions that we can ask. It should be emphasized that the determination of rates of enzyme activity eliminates the vagaries inherent in CAT, NPTII and luciferase assays, and allows accurate determination of a quantity of chimaeric gene product, even over an intrinsically fluorescent background. The fluorometric assay is very specific, extremely sensitive, inexpensive and rapid. Minute quantities of tissue can be assayed with confidence; recently we have measured GUS levels in isolated single cells of transformed plants (R.A. Jefferson *et al.*, in preparation).

β-Glucuronidase is very stable in extracts and in cells, with a half-life in living mesophyll protoplasts of ~50 h (unpublished data). Because of this, we feel it is reasonable to interpret GUS levels as indicative of the integral of transcription and translation, rather than the rate. In addition, GUS is not completely inactivated by SDS−PAGE, can tolerate large amino-terminal fusions without loss of enzyme activity (Jefferson *et al.*, 1986, 1987) and can be transported across chloroplast membranes with high efficiency (T.A.Kavanagh *et al.*, in preparation). We feel, therefore, that the system will also be very useful in studying the transport and targeting of proteins, not only in plants, but in other systems that lack intrinsic β-glucuronidase activity, such as *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Jefferson, 1985, 1986).

We have used a commercially available histochemical substrate to demonstrate GUS activity in transformed plant tissue. Other substrates are available and give excellent results (e.g. Jefferson *et al.*, 1987). We emphasize that meaningful interpretation of results of histological analysis in terms of extent of chimaeric gene activity, whether by *in situ* hybridization methods or by histochemistry, as presented here, is not a trivial or straightforward matter. There are numerous variables that must be dealt with (reviewed in Pearse, 1972). However, with these cautions, histochemical methods can be very powerful for resolving differences in gene expression between individual cells and cell-types within tissue.

We have observed a distinctly non-uniform distribution of GUS activity in stem sections of several CaMV−GUS transformed plants. Different cell-types within plants are expected to have differing metabolic activity with corresponding differences in rates of transcription and translation, and our results may reflect such a difference. Alternatively, since many of the cells of the phloem have very small cross-sectional areas, the intense dye deposition we see in these regions may simply reflect the greater cell number per unit area. The localization that we observe may also be due to a real difference in the level of expression of the CaMV 35S promoter between cell types. Recently, Nagata *et al.* (1987) have argued that the CaMV 35S promoter is preferentially active in cells during the S phase of the cell cycle. If this is true, then the pattern of GUS staining that we observe may reflect cell division activity in these cells. This observation is consistent with the proposed role of the 35S transcript of CaMV in viral replication (Pfeiffer and Hohn, 1983). It is also interesting that the other class of plant DNA viruses, the geminiviruses, replicates in the phloem parenchyma (Kim *et al.*, 1978). We conclude therefore that it is no longer adequate to describe the 35S promoter as 'constitutive' solely by the criteria of expression in all plant organs, when there may be a strong dependence of transcription on cell-type or cell cycle. This question is being investigated further.

The distribution of GUS activity in the stem sections of plants transformed with rbcS−GUS genes is consistent with data that indicate a requirement for mature chloroplasts for maximal transcription of chimaeric rbcS genes (e.g. Simpson *et al.*, 1986b). Cortical parenchymal cells in the stem contain varying numbers of chloroplasts, while those in the pith and epidermis of the stem rarely contain chloroplasts.

Different cell-types present in each organ contribute differently to the patterns of gene expression and each organ consists of different proportions of these cell-types. We have undertaken to minimize this effect on quantitative analysis of extracts by suitable choice of a denominator. The parameter that needs to be studied with gene fusions is most often the expression of the gene fusion in each cell. When preparing homogenates from plant organs, the number of cells that contribute to the extract will vary, as will the protein content of each cell and cell-type. The DNA content of the extract will reflect the number of cells that were lysed (Labarca and Paigen, 1980) whereas the traditional denominator, protein concentration, will not. For example, a single leaf mesophyll cell contains much more protein than a single epidermal cell or root cortical cell (R.A.Jefferson *et al.*, in preparation). However, each will have the same nucleus with the same potential to express the integrated gene fusion.

Using this approach, we find that the differential expression of the rbcS−GUS fusion is much more pronounced between immature and mature leaf when we express GUS activity/μg of DNA (see Table I). When protein concentration is used as a denominator, the massive induction of GUS activity during leaf maturation is masked by the concomitant induction of proteins involved in photosynthesis.

The observation that the sp. act. of GUS produced by CaMV−GUS fusions is the same in immature and mature leaves when expressed using a protein denominator indicates that the rate of GUS accumulation closely follows the rate of net protein accumulation. The two-fold difference in GUS sp. act. using a DNA denominator illustrates the accumulation of GUS per cell over time. This quantitative analysis, together with our histo-

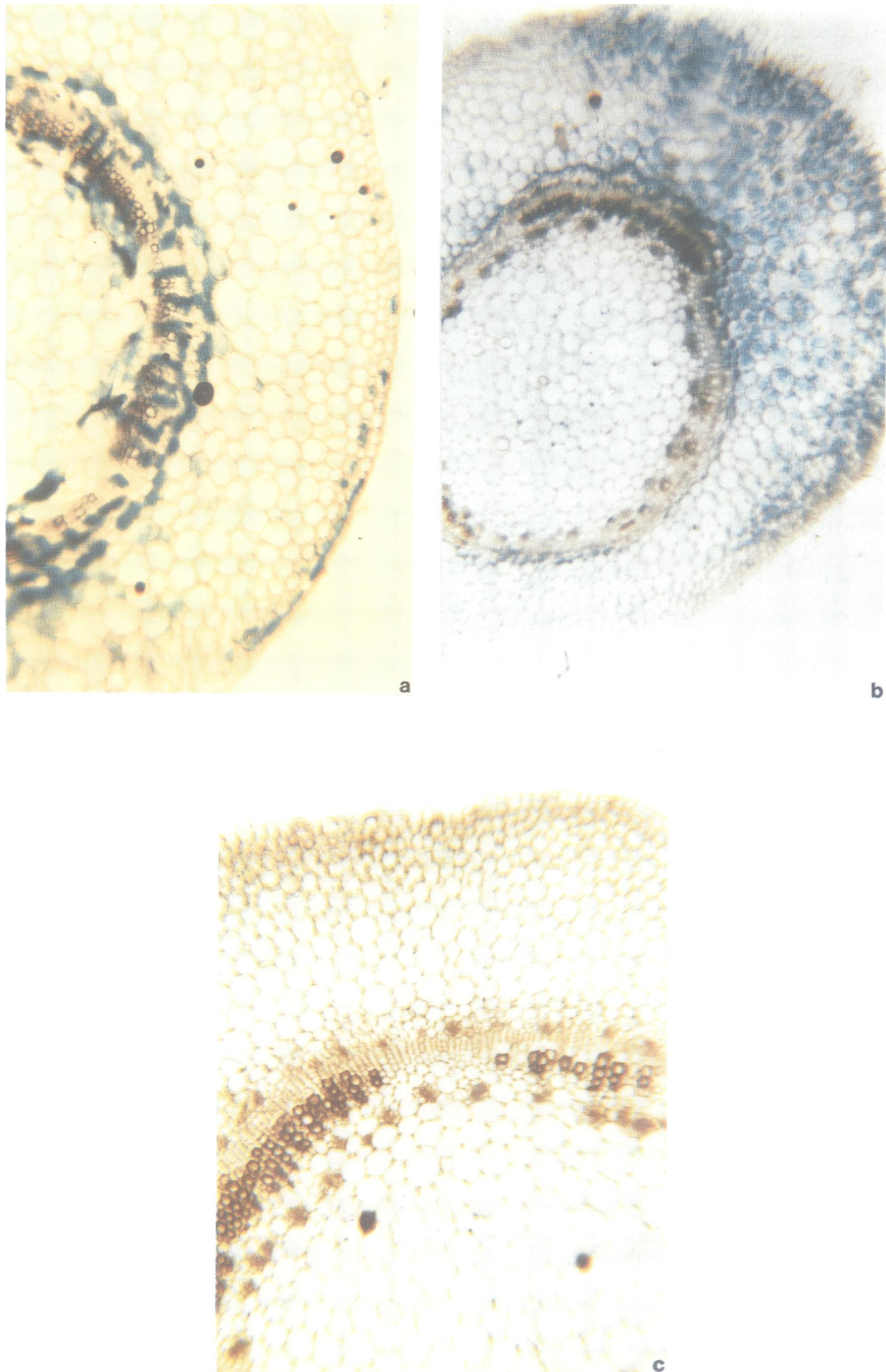

Fig. 4. Histochemical localization of GUS in transformed plant tissue. (**a**) Transverse stem sections from CaMV–GUS 21, stained with 2 mg/ml X-Gluc in NaH_2PO_4, pH 7.0, 1 h, 37°C. (**b**) Transverse stem section from rbcS–GUS 2, stained with X-Gluc, as above, for 3 h. (**c**) Transverse section of untransformed tobacco plant stained with X-Gluc for 16 h. All magnifications are ~ ×34.

chemical data, may indicate that the differences between GUS activity in the leaf, stem and root of CaMV–GUS fusion plants could reflect the larger proportion of phloem-associated cells in roots and stems compared to leaves. We feel that the choice of a DNA denominator best reflects the expression per cell and hence is a more accurate reflection of the true regulation of the gene.

Prospects of further development of the GUS system

There are many important questions arising from the use of currently available gene-transfer techniques in plants that can be addressed with this new technology. Both *Agrobacterium*-mediated transformation and direct DNA uptake methods result in cells and plants transformed with varying numbers of integrated copies of the foreign DNA and with different sites of integration, resulting in plants expressing different amounts of chimaeric gene product (e.g. Jorgensen *et al.*, 1987; Jones *et al.*, 1987). Previously, analysis of gene expression in transformed plants has been sufficiently laborious to preclude quantitative assays of the large numbers of plants necessary to finally delineate the contributions of local integration sites and copy number to the expression of transformed genes. Using the methods described here, it will be feasible to quantitate the variation that is often ascribed to differing sites and copy numbers of integrations, and obtain statistically significant answers to these questions.

The availability of routine histochemical analysis will greatly facilitate studies of the mechanism of transformation both by *Agrobacterium* and by direct DNA methods, as well as permitting a more detailed study of developmental regulation. These methods will also allow very rapid and sensitive screening of transformed cells and tissues. Using the indigogenic substrate X-Gluc, we can easily resolve GUS activity from single cells and small cell clusters from suspension cultures (data not shown).

GUS assay systems lend themselves very well to automation. The existing spectrophotometric and fluorogenic assays, and new assays using fluorogenic substrates that fluoresce maximally at neutral pH (Jefferson, 1985), will allow the use of automatic microtitre plate analysis of very large numbers of samples. The activity of GUS in lysed single cells can be measured with accuracy; using new fluorogenic substrates, we are conducting an analysis of GUS expression in single cells of transformed plants using the fluorescence activated cell sorter (R.A.Jefferson *et al.*, in preparation).

We have also used the GUS fusion system successfully to monitor the transient expression of chimaeric genes introduced into plant cells via electroporation and/or polyethylene glycol treatment (data not shown). We find the sensitivity to be very high, allowing expression to be reliably measured from a very small number of cells (R.A.Jefferson *et al.*, in preparation).

Because of the lack of intrinsic β-glucuronidase activity in all plants thus far assayed in our laboratory, and because the synthesis of β-glucuronides can be relatively straightforward, we are pursuing the use of the GUS system to begin 'fusion genetics'. Due to the complex genomes and long generation times of higher plants, fine scale genetic analysis of complex processes is unfeasible by conventional means. However, by using the GUS system and novel substrates, we may be able to generate positive and negative selections for GUS activity, thereby selecting mutations in the activity of gene fusions, both *in planta* and in tissue culture.

Finally, new methods and substrates are being developed to allow the GUS system to be used quantitatively and reliably *in vivo* and *in situ*.

Materials and methods

Nucleic acid manipulation

DNA manipulations were performed essentially as described (Maniatis *et al.*, 1982). Enzymes were obtained from New England Biolabs, Boehringer or BRL.

Plant transformation and regeneration

Binary vectors containing CaMV–GUS fusions and rbcS–GUS fusions in *E.coli* MC1022 were mobilized into *A.tumefaciens* LBA4404 as described (Bevan, 1984). The integrity of the vectors in *Agrobacterium* was verified by preparing DNA from *Agrobacterium* immediately before plant transformation using the boiling method of Holmes and Quigley (1981). Leaf discs of *N.tabacum*, var. Samsun were transformed as described (Horsch *et al.*, 1984) and transformed plants were selected on MS medium (Murashige and Skoog, 1962) containing 100 μg/ml kanamycin. Plants were maintained in axenic culture on MS basal medium, 3% sucrose, 200 μg/ml carbenicillin and 100 μg/ml kanamycin, at ~2000 lux, 18 h day, 26°C.

Southern blot analysis

DNA was prepared from plants by phenol extraction and ethanol precipitation of plant homogenates, followed by RNase digestion, phenol extraction and isopropanol precipitation. DNA samples (10 μg) were digested with restriction endonucleases, electrophoresed in an 0.8% agarose gel and blotted onto nitrocellulose (Maniatis *et al.*, 1982). Filters were hybridized with oligomer-primed, ^{32}P-labelled GUS gene fragments (Feinberg and Vogelstein, 1984) and washed wth 0.2× SSC at 65°C.

Substrates

Substrates used included: 4-methyl umbelliferyl glucuronide (MUG; Sigma M-9130), X-Gluc (Research Organics Inc., Cleveland, OH, USA), resorufin glucuronide (ReG) (Jefferson, 1985; Molecular Probes Inc., Eugene, OR, USA).

Lysis conditions

Tissues were lysed for assays in 50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol (extraction buffer) by freezing with liquid nitrogen and grinding with mortar and pestle with sand or glass beads. Disposable pestles that fit into Eppendorf tubes (Kontes Glass) proved useful for homogenizing small bits of tissue (e.g. leaf). Extracts can be stored at −70°C with no loss of activity for at least 2 months. Storage of extracts in this buffer at −20°C should be avoided, as it seems to inactivate the enzyme.

Fluorometric assay

The fluorogenic reaction is carried out in 1 mM MUG extraction buffer with a reaction volume of 1 ml. The reaction is incubated at 37°C, and 200 μl aliquots are removed at zero time and at subsequent times and the reaction terminated with the addition of 0.8 ml 0.2 M Na_2CO_3. The addition of Na_2CO_3 serves the dual purposes of stopping the enzyme reaction and developing the fluorescence of MU, which is about seven times as intense at alkaline pH. Fluorescence is then measured with excitation at 365 nm, emission at 455 nm on a Kontron SFM 25 spectrofluorimeter, with slit widths set at 10 nm. The resulting slope of MU fluorescence versus time can therefore be measured independently of the intrinsic fluorescence of the extract. The fluorimeter should be calibrated with freshly prepared MU standards of 100 nM and 1 μM MU in the same buffers. Fluorescence is linear from nearly as low as the machine can measure (usually 1 nM or less) up to 5–10 μM MU.

A convenient and sensitive qualitative assay can be done by placing the tubes on a long-wave UV light box and observing the blue fluorescence. This assay can be scaled down easily to assay very small volumes (reaction volume 50 μl, terminated with 25 μl 1 M Na_2CO_3 in microtitre dishes or Eppendorf tubes).

If the intrinsic fluorescence of the extract limits sensitivity, it is possible to use other fluorogenic substrates. In particular, ReG has a very high extinction coefficient and quantum efficiency, and its excitation (560 nm) and emission (590 nm) are conveniently in a range where plant tissue does not absorb or fluoresce heavily. In addition, it fluoresces maximally at neutral pH, making it unnecessary to stop the reaction.

Protein concentrations of plant extracts were determined by the dye-binding method of Bradford (1976) with a kit supplied by Bio-Rad Laboratories.

DNA concentrations in extracts were determined by measuring the fluorescence enhancement of Hoechst 33258 dye as described by Labarca and Paigen (1980), with the calibrations performed by addition of lambda DNA standards to the extract to eliminate quenching artefacts.

Histochemical assay

Sections were cut by hand from unfixed stems of plants grown *in vitro*, essentially as described (O'Brien and McCully, 1981), and fixed in 0.3% formaldehyde in 10 mM MES, pH 5.6, 0.3 M mannitol for 45 min at room temperature, followed by several washes in 50 mM NaH_2PO_4, pH 7.0. All fixatives and substrate

solution were introduced into sections with a brief (~1 min) vacuum infiltration.

Histochemical reactions with the indigogenic substrate, X-Gluc were performed with 1 mM substrate in 50 mM NaH_2PO_4, pH 7.0 at 37°C for times from 20 min to several hours. After staining, sections were rinsed in 70% ethanol for 5 min, then mounted for microscopy.

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