The EMBO Journal vol.8 no.2 pp.343–350, 1989

Gene fusions to *lacZ* reveal new expression patterns of chimeric genes in transgenic plants

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Communicated by M.Van Montagu

The *lacZ* gene of *Escherichia coli*, coding for β-galactosidase, is a widely used reporter gene for gene expression studies in microbial and animal systems. To demonstrate that it is also a powerful reporter gene in plants, *lacZ* was fused to 5′ regulatory elements of several genes known to be functional in plant cells. By measuring LacZ activities in transgenic plants containing these gene constructs, we showed that the reporter is correctly monitoring the regulatory properties of the well-characterized promoters fused to *lacZ*. β-Galactosidase was assayed directly in plant extracts when they contained high levels of LacZ or, when LacZ was expressed at low level, by separating the endogenous and LacZ activities electrophoretically and detecting the enzymes with a fluorogenic substrate. The most outstanding property of the marker is its amenability to histochemical detection. Due to its stability, LacZ can be fixed in the tissue with glutaraldehyde without loss of activity and detected with high resolution by using XGal. We could reveal expression patterns unnoticed earlier for many of the regulatory elements studied. The chlorophyll *a/b* binding protein gene, expressed at very high levels in green tissues, is also expressed at a low level in the vascular cylinder of the root. The *Agrobacterium* T-DNA gene encoding octopine synthase is especially active in the epidermis of the root tip and the TR2′ gene was shown to be root specific in the intact plant and stimulated by wounding in the leaf tissue. The TR1′ gene, fused to *nptII*, shows similar characteristics suggesting co-regulation of this tightly linked dual promoter.

Key words: lacZ gene fusions/regulation of T-DNA genes/reporter gene/transgenic plants

Introduction

Gene fusions, where a marker gene coding for an easily detectable product has been fused to the 5′ regulatory regions of genes under study, have proven extremely useful in investigating gene expression and regulation in both prokaryotes and eukaryotes. In plants, genes for neomycin phosphotransferase II (*nptII*) and chloramphenicol acetylase (*cat*) can be expressed from chimeric constructions (Bevan *et al.*, 1983; Herrera-Estrella *et al.*, 1983) and they have been successfully employed as reporter genes. Sensitive assays based on radiolabelling techniques are available for both gene products. Fusions to *nptII* and *cat* have been used to demonstrate that the tissue specificity and light induction properties of nuclear genes involved in photosynthesis are located in the 5′ flanking sequences of these genes (Herrera-Estrella *et al.*, 1984; Simpson *et al.*, 1985). More recently, genes coding for the firefly luciferase (Ow *et al.*, 1986) and the *Escherichia coli* β-glucuronidase (GUS) (Jefferson *et al.*, 1987) have been introduced as reporter genes in the plant field. Both these new markers have been shown to have potential for sensitive quantification as well as for detection *in situ* in the plant tissues.

The most extensively used gene fusion marker in organisms other than plants is the *lacZ* gene of *E.coli* (Bassford *et al.*, 1978; Casadaban and Cohen, 1980; Hiromi *et al.*, 1985). The gene codes for β-galactosidase (LacZ), a cytoplasmic protein in *E.coli* for which a number of specific chromogenic substrates are available. The use of *lacZ* as a reporter in plants also, has been hampered by the background of endogenous activities. Plant cells have a wide range of β-galactosidases and some of them are active at neutral pH, near the optimum of the LacZ activity (Bhalla and Dalling, 1984). Therefore, the detection of the *lacZ* gene product in transgenic plants is complicated (Helmer *et al.*, 1984; Matsumoto *et al.*, 1988) and this has discouraged the use of *lacZ* as a reporter in plant cells. Animal cells seem to lack interfering amounts of neutral β-galactosidases (Nielsen *et al.*, 1983). This has led to the development of *lacZ* as a histochemically assayable marker gene (Lis *et al.*, 1983; Hiromi *et al.*, 1985; Goring *et al.*, 1987) which allows high-resolution mapping of gene expression in single cells of animal tissue sections.

Here we show that *lacZ* can also be used as a convenient reporter gene in plants. To separate the background activities from LacZ, a gel assay with *in situ* staining was developed. However, since the background activity is relatively low, *lacZ* fusions that are expressed at levels >50 units (or 60 ng LacZ) per mg of protein (e.g. 35S–*lacZ*) can be easily monitored with a direct enzyme assay and quantification of the gene product is accurate. What makes *lacZ* a very useful reporter gene is its amenability to histochemical detection of expression. The exceptional stability of LacZ allows efficient immobilization of the enzyme in the tissue by a cross-linking fixative, which simultaneously inactivates the endogenous enzymes. Subsequent to histochemical staining, the tissue can be either inspected as whole or thin-sectioned to reveal LacZ activity in each cell.

We analysed gene fusions between *lacZ* and two pea photosynthesis-related genes, three *Agrobacterium tume-*

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faciens T-DNA genes and the 35S transcript promoter of cauliflower mosaic virus. The gene fusions were transferred into the genome of tobacco cells by using an *Agrobacterium*-derived system. We could establish that the *lacZ* gene correctly monitors the level and tissue-specific pattern of gene expression of the well-characterized pea and CaMV regulatory elements in the transgenic plants. Applying the techniques to the less thoroughly characterized genes *ocs* and TR2′, we found unexpected patterns of expression. The *ocs* gene, considered to be a constitutively expressed gene (Ellis *et al.*, 1987), shows epidermis-specific activity in the root tips and the TR2′ promoter drives localized wound-stimulated expression of *lacZ* in the leaf. The TR1′ promoter, fused to the *nptII* gene, shows similar stimulation by wounding.

Results

Construction of the lacZ gene fusions and their transfer into tobacco

To determine whether *lacZ* gene fusions can be used to study gene expression in plants, two light-inducible and tissue-specific regulatory elements from pea, coding for a small subunit of ribulose 1,5-bisphosphate carboxylase (*ss3.6* or *ss*) (Cashmore, 1983) and chlorophyll *a/b* binding protein (*AB80* or *cab*) (Cashmore, 1984), were attached to the coding sequence of *lacZ*. We also fused 5′ elements from two T-DNA-encoded opine synthase genes, the nopaline synthase (*nos*) (Depicker *et al.*, 1982) and the octopine synthase (*ocs*) (De Greve *et al.*, 1982) genes as well as the 35S transcript promoter of CaMV (35S) (Odell *et al.*, 1985) to *lacZ*. A construction, where *lacZ* is under the control of an octopine type Ti-plasmid right T-DNA gene 2′ promoter (TR2′) (Velten *et al.*, 1984), was also used. All of the constructs resulted in in-frame translational fusions to *lacZ*. The extent of the 5′ regulatory regions as well as N-terminal amino acid coding sequence coming from the plant genes in these fusions are indicated in Table I. The restriction enzyme maps of the constructions are shown in Figure 1.

Plasmids carrying the *lacZ* gene fusions were mobilized by conjugation from *E.coli* into *A.tumefaciens* and transferred to the tobacco genome in an *Agrobacterium* infection (see Materials and methods). To facilitate the transfer, each plasmid has a selectable *nptII* marker gene. In pJUH6 the *nptII* gene is located distally in the same transcription unit with *lacZ* and its translation relies on ribosomal reinitiation (Peabody and Berg, 1986). The effects and the efficacy of this arrangement are described elsewhere (G.Angenon, in preparation).

For each construction, three plants were propagated in the greenhouse for analysis, except for the *nos*−*lacZ* plants where only two plants were used. Apart from the two *nos*−*lacZ* plants, each set of three plants analysed showed qualitatively similar patterns of *lacZ* expression, although quantitative variation between the clones could be measured.

Tissue-specific expression of the fusions

To find out how the β-galactosidase produced by the gene fusions would reflect the tissue specificity of the regulatory regions directing the expression of the fusion genes, we assayed the enzyme in extracts of leaf, stem and root tissues in the transgenic plants by using a modification of the *o*-nitrophenyl-β-D-galactoside (ONPG) hydrolysis assay (Miller, 1972).

Table I. Structure of the *lacZ* gene fusions

Plasmid	*lacZ* fusion	Source and extent of the 5′ element		Extent of amino terminus foreign to LacZ
pHTT27	*ocs*−*lacZ*	*ocs*[c],	2965 bp	90[a] + 4[b] aa
pHTT31	*nos*−*lacZ*	*nos*[d],	270 bp	16 + 1 aa
pHTT32	*ss*−*lacZ*	*ss3.6*[e],	1274 bp	81 + 1 aa[i]
pHTT33	*cab*−*lacZ*	*AB80*[f],	6534 bp	247 + 1 aa
pJUH6	35S−*lacZ*	CaMV 35S[g],	972 bp	0 + 3 aa
pGSLac1	TR2′−*lacZ*	TR2′[h],	479 bp	0 + 3 aa

In each case, the eight N-terminal amino acids of LacZ were replaced by the indicated number of N-terminal amino acids (aa) of the plant gene product ([a]) and the ones coded for by the linking sequences ([b]). The 3′ element including the polyadenylation signal at the end of the fusion genes was from *ocs* except in pGSLac1 where it came from the TL-DNA gene 7 (Dhaese *et al.*, 1983).
[c]De Greve *et al.*, 1982; [d]Depicker *et al.*, 1982; [e]Cashmore, 1983; [f]Cashmore, 1984; [g]Odell *et al.*, 1985; [h]Velten *et al.*, 1984.
[i]Splicing assumed to take place.

The pattern of *lacZ* expression (Table II) in the *cab*−*lacZ* plants was in concordance with what Simpson *et al.* (1985) had shown for *cab*−*nptII* gene fusions. The expression was very high in green leaves, intermediate in stems (not shown) and negligible in roots. The CaMV 35S promoter drove *lacZ* in a more uniform manner. The expression was similar in leaf and stem but elevated in the roots. The TR2′ showed a root-specific pattern of expression.

The expression of the *nos*−, *ocs*− and *ss*−*lacZ* led to β-galactosidase levels, which were not detected easily above background activities (Table II). To monitor the expression pattern of these gene fusions, we had to separate the two activities from each other. This was accomplished by SDS−PAGE (Figure 2) where the endogenous and LacZ β-galactosidase bands were well separated and could be visualized by a fluorogenic stain. Although the level of LacZ produced by the *ss*−*lacZ* fusion was very low, it was specific to the leaf tissue. The *ocs*−*lacZ* showed constitutive expression in this assay and the two *nos*−*lacZ* plants showed different patterns (Figure 2). Since, at the moment, we do not have more of these plants, the leaf/root specificity of the *nos*−*lacZ* cannot be determined. Higher activity in roots than in leaves has been shown for the *nos*−*nptII* gene fusion (Simpson *et al.*, 1985; Teeri *et al.*, 1986) when it is measured relative to the amount of extracted protein.

The gel assay considerably increased the sensitivity of LacZ detection, e.g. low levels of LacZ activity could be detected in the root of the *cab*−*lacZ* plants, hidden by the background in the ONPG assay. This activity was not randomly localized as further shown by histochemical staining of LacZ (Figure 3).

Histochemical detection of LacZ

In animal cells LacZ activity can be visualized with the indigogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal) and the resulting pigment can be detected in single cells separately using thin-sectioned tissues (Goring *et al.*, 1987). We modified a *Drosophila* staining method (J.Szabad, personal communication) and could demonstrate that LacZ histochemistry works very well in plant tissues.

The LacZ enzyme was immobilized in root tips of transgenic and control plants by glutaraldehyde fixation and stained with XGal. The material was embedded in paraffin, thin sectioned and, after removal of paraffin, inspected under

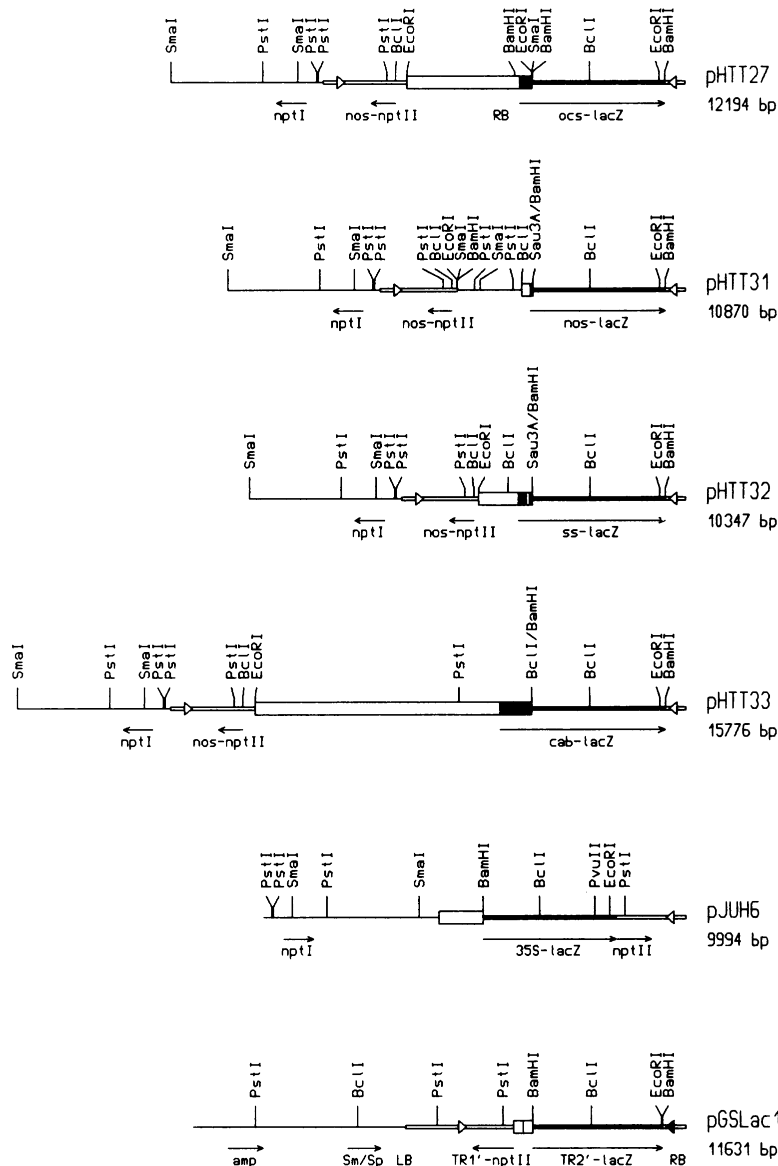

Fig. 1. Restriction maps of the *lacZ* gene fusions transferred to tobacco. Thick line, 5′ element linked to *lacZ*; filled line, sequences translated to the hybrid LacZ protein; triangle, polyadenylation site.

direct illumination in a microscope (Figure 3). The untransformed control material showed no detectable accumulation of indigo after fixation with glutaraldehyde. The 35S−*lacZ* showed prominent staining in all cells and clearly demonstrated that the substrate was penetrating to all parts of the tissue. The *ocs*−*lacZ* gene fusion showed very localized *lacZ* expression in the epidermis. The pattern was restricted to the root tip only. The activity of *cab*−*lacZ* in roots detected by the gel assay (Figure 2) was not unspecific either, but was localized to the vascular cylinder of the root (Figure 3). This pattern continued further up the root (Figure 4).

TR2′ and TR1′ are stimulated by wounding

It has been observed that a TR2′ promoter driving the expression of a *Bacillus thuringiensis* toxin (Vaeck *et al.*, 1987) leads to much better insect protection than a similar construction with the 35S promoter (J.Leemans, personal

Table II. Tissue-specific expression of *lacZ* gene fusions in tobacco—β-galactosidase activity (U/mg protein)

		Leaf	Root
SR1		8	18
ss−*lacZ*,	plant 1	11	19
	plant 2	6	18
nos−*lacZ*,	plant 1	8	18
	plant 2	47	18
ocs−*lacZ*,	plant 1	11	34
	plant 2	15	12
cab−*lacZ*,	plant 1	81	19
	plant 2	492	19
TR2′−*lacZ*,	plant 1	10	295
	plant 2	26	390
35S−*lacZ*,	plant 1	102	251
	plant 2	199	483

Individual plants analysed.

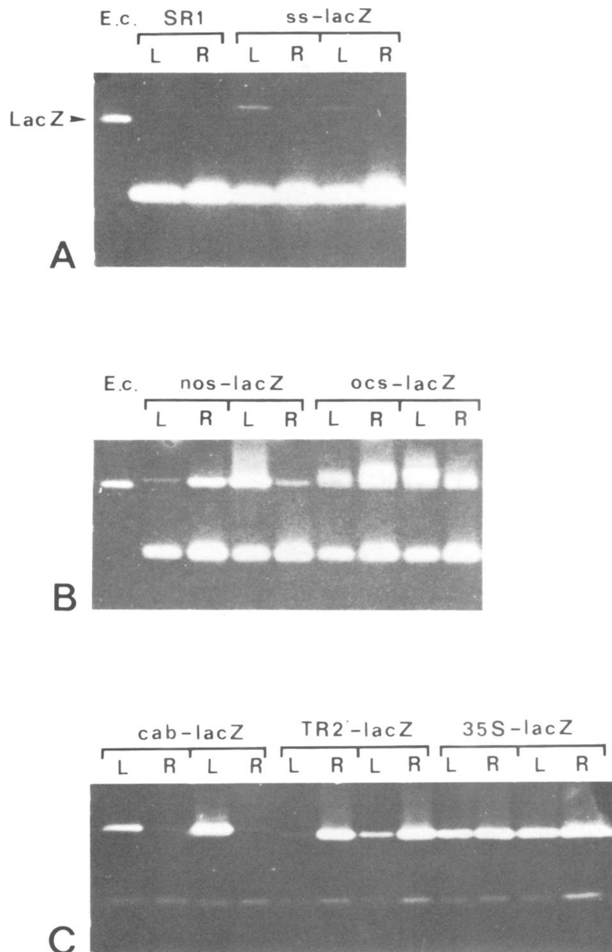

Fig. 2. The endogenous β-galactosidase migrates faster than LacZ in an SDS–polyacrylamide gel and the enzymes can thus be resolved and visualized with a fluorogenic stain. The enzymatically active form of LacZ is a tetramer and the enzyme forming the band probably migrates in this form. The fusion gene products have N-terminal extensions of different lengths (Table I). However, all bands in the gel migrate very close to the authentic LacZ extracted from *E.coli*, indicating proteolytic cleavage of the hybrid LacZ proteins. Ullman and Perrin (1970) have reported that while various proteases do cleave the LacZ polypeptide, the enzyme is still held together and active by noncovalent forces. The disproportionate resistance of the 'core' LacZ means that the processing observed in the gel is not necessarily an *in vivo* event but can be an *in vitro* artefact as well. The transgenic plants analysed are the same as those in Table II. The amount of protein loaded in each lane was 80 μg for gel A, 25 μg for gel B and 5 μg for gel C. L = leaf, R = root and EC = *E.coli* extract.

communication). This could suggest that the damage caused by the insects is actually stimulating TR2′.

To mimic insect chewing, we punched holes in leaves 24 h before assaying β-galactosidases from the 1 mm ring of tissue surrounding the hole. Whereas the expression of the 35S or *cab* fusions was not affected by this treatment, the expression of the TR2′–*lacZ* was stimulated 20-fold upon wounding (Table III). The stimulation of TR2′–*lacZ* was also demonstrated by histochemical analysis of wounded leaves. In addition, this analysis suggested that the wound induction of TR2′ was a relatively local event (Figure 5). The staining also indicated that the TR2′-regulated activity in the intact leaf tissue was localized to the veins. The *nos* promoter, fused either to *lacZ* or *nptII*, was also stimulated by wounding, but to a lesser extent (Tables III and IV).

Velten and Schell (1985) have suggested that the very closely linked promoters of the genes 1′ and 2′ of the octopine-type TR-DNA may be functioning coordinately although not following any known regulatory pattern. We determined, by measuring NPTII activity, the wound stimulation and tissue specificity of the TR1′–*nptII* gene fusion in the same plants, and found that it is similarly regulated to the TR2′–*lacZ* fusion (Table IV).

Discussion

We have constructed a collection of gene fusions between 5′ regulatory elements known to be functional in plants and the *lacZ* reporter gene of *E.coli*, coding for a soluble β-galactosidase with a neutral pH optimum (LacZ). By analysing the β-galactosidases in transgenic tobacco plants harbouring these gene fusions, we demonstrated that *lacZ* monitors correctly the activity of the 5′ element by using chromo- and fluorogenic β-galactosidase substrates and showed the power of histochemical staining by using an indigogenic substrate. New and more detailed information of the regulatory properties of the 5′ elements were obtained.

Assays and histochemical detection of LacZ in plants

The assays for LacZ are complicated by the fact that tobacco contains endogenous β-galactosidase activity with a neutral pH optimum, as do other plant species tested (*Arabidopsis thaliana*, potato, barley and rice, data not shown). Thus a direct measurement of β-galactosidases in the transgenic plants can be used to quantify only LacZ activities well above the endogenous background. When applicable, a direct assay is invaluable in its simplicity and accuracy.

Many plant promoters, including those of the octopine and nopaline synthase genes of the *Agrobacterium* T-DNA, do not lead to very strong gene expression. To demonstrate the feasibility of *lacZ* as a reporter gene to analyse regulatory elements which express *lacZ* near or even much below the levels of endogenous β-galactosidases, we separated the activities from each other. SDS–PAGE resolved the enzymes efficiently and subsequent to electrophoresis they could be visualized with a fluorogenic substrate. Both activities were resistant to SDS at moderate temperatures.

We wanted also to extend our studies from tissue specificity to cell specificity of the plant regulatory elements attached to *lacZ*. The indigogenic β-galactosidase substrate XGal is ideal for these kinds of studies since the cleavage product precipitates as a coloured compound *in situ* provided that an oxidative agent is present. LacZ could be immobilized with glutaraldehyde fixation in the tissue without losing the enzymatic activity. The endogenous β-galactosidases are inactivated by glutaraldehyde and therefore LacZ could be visualized with XGal in conditions where no background was detectable. With thin-sectioned root tips stained with XGal we showed that while control material does not accumulate blue colour, the substrate is still available in every cell, since root tips from a 35S–*lacZ* plant stain uniformly (Figure 3).

Among the collection of existing reporter genes used in plant molecular biology, only the *uidA* gene coding for a β-glucuronidase (GUS) described by Jefferson *et al.* (1987) has as many applications as the *lacZ* gene to study gene expression. A very similar range of commercially available chromo-, fluoro- and indigogenic substrates is shared by

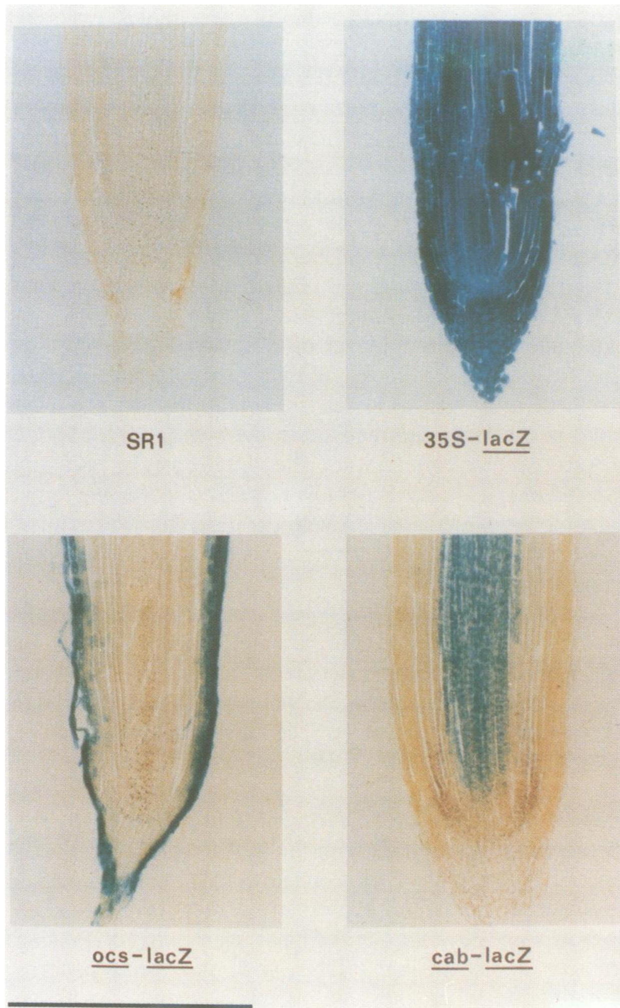

Fig. 3. Glutaraldehyde fixation immobilizes LacZ and differentially destroys the endogenous background in plant tissue. Root tips from transgenic and control (SR1) tobacco were fixed and stained with XGal, which visualizes β-galactosidase activity as a blue precipitate. The material was embedded in paraffin and thin sectioned. The nontransformed control shows no staining while the plant containing a 35S−*lacZ* gene fusion indicates that XGal is accessible throughout the tissue. The activity of the *ocs*−*lacZ* gene is localized to the epidermal tissue and the low *cab*−*lacZ* activity present in root, in the vascular cylinder. Root tips from the individual transformed plants 1, 3 and 1 containing the 35S−, *ocs*− and *cab*−*lacZ* constructions respectively are shown. Bar = 0.5 mm.

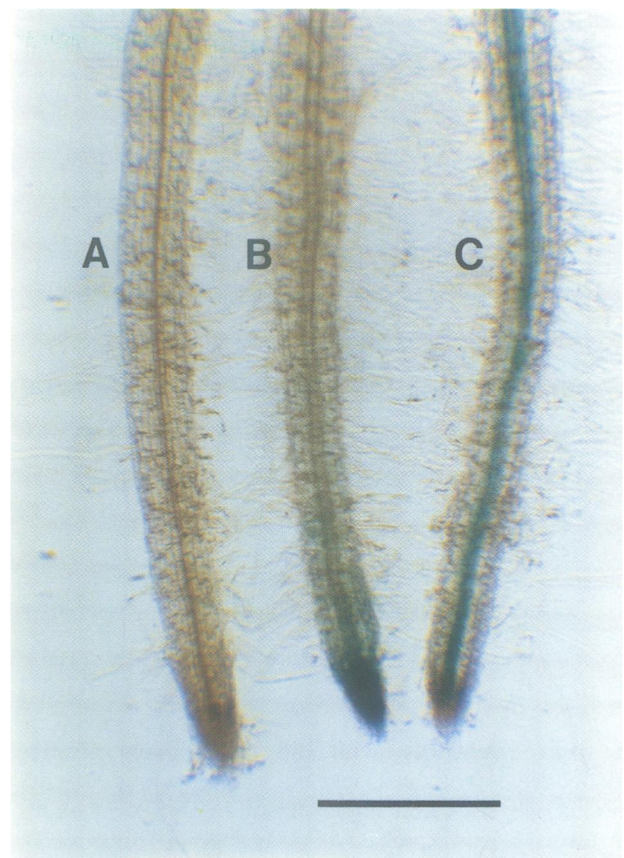

Fig. 4. Fixed and XGal-stained unsectioned roots of plantlets harbouring no *lacZ* sequences (**A**), an *ocs*−*lacZ* fusion (**B**) and a *cab*−*lacZ* fusion (**C**). The *ocs*−*lacZ* is most active in the tip of the root while the *cab*−*lacZ* is expressed in the vascular cylinder. Roots from the selfed progeny of the transformed plant 1, in both cases, are shown. Bar = 0.5 mm.

LacZ and GUS. Other tools, e.g. reporter gene cassettes, sequencing primers and immunoreagents recently made available for GUS, had already been at hand for LacZ earlier, due to its applications in microbial genetics. Perhaps the most praised benefit of *uidA* is that it provides an easily quantifiable reporter gene product, where the detection is sensitive due to the relative stability of the enzyme and lack of plant endogenous background (Jefferson *et al.*, 1987). Still, problems with background have been reported (Lee *et al.*, 1988).

The power of LacZ, in our opinion, is its excellent amenability to histochemical analysis. Histochemistry for GUS has been described (Jefferson *et al.*, 1987) and, although tissues stained for this marker enzyme can be extremely informative, a serious limitation of GUS is that it can tolerate only mild fixing. Tissues expressing *lacZ* can

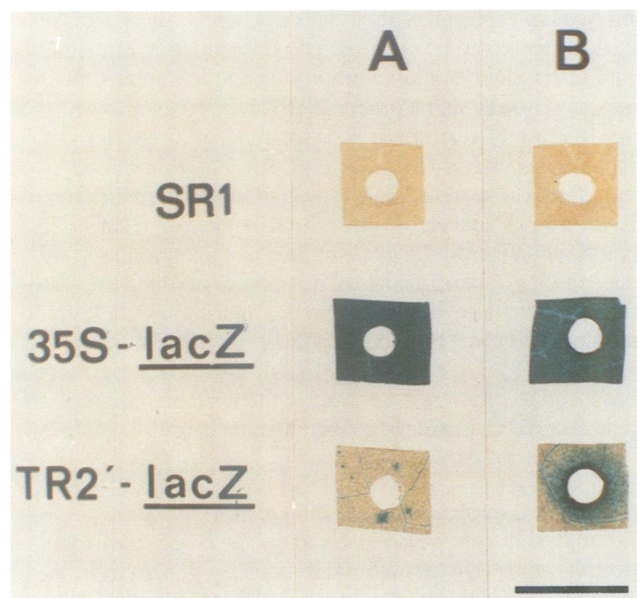

Fig. 5. The leaf pieces in column A were punched immediately before glutaraldehyde fixing and XGal staining, while the ones in column B were punched 24 h before staining. Chlorophyll was removed to better visualize the indigo colour. The control tissue shows no background β-galactosidase activity and the TR2′−*lacZ* shows strong local stimulation by wounding. Leaf pieces from the transgenic plants 3 (35S−*lacZ*) and 1 (TR2′−*lacZ*) are shown. Bar = 1 cm.

Table III. Wound stimulation of *lacZ* gene fusions in tobacco leaf tissue—β-galactosidase activity (U/mg protein)

	Intact leaf	Wounded leaf	Ratio
SR1	10	7	0.7
TR2′ – *lacZ*	14	278	20
35S – *lacZ*	102	90	0.9
cab – lacZ	225	201	0.9
nos – lacZ	27	68	2.5

Mean values from three transgenic plants (only two for the *nos – lacZ*).

Table IV. Wound stimulation of *nptII* gene fusions in leaf tissue and tissue specificity of the TR1′ – *nptII* fusion in tobacco—relative NPTII activity in counts per minute

	Intact leaf	Wounded leaf	Ratio
35S – *nptII*	3323	2996	0.9
TR1′ – *nptII*	176	2791	16
nos – nptII	330	1295	4
	Intact leaf	Intact root	Ratio
TR1′ – *nptII*	213	3292	15

Mean values from three transgenic plants.

be fixed extensively with the cross-linking fixative glutaraldehyde without losing the LacZ enzymic activity. This not only effectively immobilizes the enzyme in the cells but also excludes the problem of the endogenous background. Material stained for LacZ can be subsequently assigned to a whole variety of microscopic techniques.

Another advantage of *lacZ* as a reporter gene is the wide array of knowledge which has accumulated for this gene, its product and the particular use of *lacZ* as a reporter. Similar knowledge for GUS will with no doubt develop in time. The 27 first N-terminal amino acids of LacZ can be removed or replaced without loss of activity allowing translational fusions to be constructed (Casadaban *et al.*, 1983). LacZ provides a unique intramolecular complementation system (Ullman and Perrin, 1970) which has allowed the construction of very small α-donor-type *lacZ* reporter genes in bacterial systems (Messing *et al.*, 1977). It is well known that efforts to secrete LacZ through the inner membrane of *E.coli* do not succeed (Silhavy and Beckwith, 1985). This export incompatibilty may be limited to prokaryotic systems, since the LacZ can apparently be translocated into the endoplasmic reticulum of yeast (Ferenci and Silhavy, 1987). In addition, transport of LacZ into the yeast mitochondrion has been reported (Douglas *et al.*, 1984), and we have evidence that it can be efficiently targeted to the inside of chloroplasts (T.H.Teeri, G.Engler, M.Van Montagu and L.Herrera-Estrella, in preparation).

Regulated expression of lacZ in plants

We analysed gene fusions between *lacZ* and two tissue-specific and well-characterized nuclear genes from pea coding for chloroplast proteins, three T-DNA genes and the CaMV 35S transcript promoter. The pea genes *cab* and *ss*, coding for a chlorophyll *a/b* binding protein and a ribulose 1,5-bisphosphate carboxylase small subunit respectively, have been shown to be expressed preferentially in green tissues (Simpson *et al.*, 1986). Fusions of their 5′ regulatory regions and the beginning of their genes to *lacZ* were regulated in a very similar fashion. The *ss – lacZ* fusion is expressed at relatively low levels in the transgenic tobacco. With the gel assay we could detect LacZ in leaf extracts of these plants but not in the roots. The low expression level is not surprising since, together with another gene in the five-member family, the *ss3.6* gene contributes only 7% of the transcripts in pea coding for the small subunit (Fluhr *et al.*, 1986). The *cab – lacZ*, on the other hand, is very strongly expressed. Assuming that LacZ in the plant extract has similar specific activity (800 000 U/mg) to the enzyme purified from *E.coli* (Ullman *et al.*, 1968), nearly 0.1% of total soluble protein in leaves of the plants harbouring this gene fusion can be LacZ. With a direct β-galactosidase enzymatic assay we could determine that *cab – lacZ* is most active in the leaf, intermediate in the stem and not above background in the root tissue. The more sensitive gel assay revealed that the fusion is not totally inactive in the root tissue and a more detailed analysis with the histochemical method showed that the activity in roots is localized in the vascular cylinder.

The product of the 35S – *lacZ* fusion is also readily measurable directly. Approximately equal levels of β-galactosidase in leaf and stem tissue could be detected and two to three times more in the root tissue, a similar expression pattern of the 35S to that measured by other researchers (Odell *et al.*, 1985; Jefferson *et al.*, 1987).

Specific regulation of T-DNA genes in tobacco

The study of plant genes with the high resolution reporter gene *lacZ* immediately led to unexpected findings, such as the localized *cab – lacZ* expression in roots. What is even more striking is that the 'second hand' plant genes originating from the *Agrobacterium* T-DNA show interesting patterns of regulation when they are carefully analysed with an appropriate reporter gene system. The octopine synthase promoter shows very restricted expression in the root tip epidermis as is shown by the histochemical analysis. The *ocs* expression has earlier been considered not to be under any environmental or developmental control (Ellis *et al.*, 1987).

The TR2′ promoter is mostly root specific in the intact plant and the low activity in the leaf is localized in the veins. However, it is stimulated to a relatively high level of expression in the leaf tissue by wounding. We have not yet determined the time course of the induction or whether it could be reproduced by other kinds of environmental stress or plant hormones. The stimulation is very local and thus resembles more the induction of phytoalexins during pathogen attack than the systemic induction of proteinase inhibitors caused by insect wounding (Ryan, 1984). Lesser stimulation of the *nos* gene fusions by wounding is also evident.

The TR2′ is the other member of a tightly linked pair of promoters. Velten and Schell (1985) suggest that the two promoters are coordinately expressed in the terms of the general transcriptional state of the host genome at the site of T-DNA insertion. We discovered that TR1′, the other member of the pair, is also expressed at higher levels in roots than in leaves in intact plants and stimulated by wounding in leaf tissue, very similarly to TR2′. The co-regulation suggests that the two promoters, contained within only 479 bp of DNA, could be sharing transcriptional factors regulating their expression. It is becoming clear that the

properties of the T-DNA-encoded plant genes in *Agrobacterium* have been underestimated and that they have evolved to a much higher degree of sophistication than previously expected.

Materials and methods

Plasmid constructions

The recombinant plasmids (Figure 1) were constructed using standard DNA techniques as described in Maniatis *et al.* (1982). A full description of their structures can be received from the authors upon request. The plasmids pGSLac1 and pGSJ290 were kind gifts from J.Botterman. The latter contains a 35S–*nptII* construction.

Plant cell transformations

For mobilization of the plasmids pHTT27, 31, 32, 33 and pJUH6 into the rifampicin-resistant *A.tumefaciens* strain C58C1 (Van Larebeke *et al.*, 1974) harbouring the disarmed Ti plasmid pGV3850 (Zambryski *et al.*, 1983), a triparental mating was performed between the *E.coli* strain GJ23, the one harbouring the plasmid intended to be transferred and the agrobacterium (Van Haute *et al.*, 1983). The plasmids pGSLac1 and pGSJ290 were mobilized in a similar way to C58C1-harbouring pGV2260 (Deblaere *et al.*, 1985). The plasmids are stabilized in the agrobacterium by integration into the Ti-plasmid. The T-DNA structures of the resulting agrobacteria were verified by Southern blot analysis of total bacterial DNA.

Transformation of *Nicotiana tabacum* SR1 was done by co-cultivating regenerating tobacco protoplasts with the agrobacterium strains harbouring the *lacZ* gene fusions as described by Zambryski *et al.* (1984) or by infection of tobacco leaf discs with agrobacteria (Horsch *et al.*, 1985) as described by Simpson *et al.* (1985).

Enzyme assays

β-Galactosidase assay in solution. Plant tissue was homogenized in Z-buffer (Miller, 1972) supplemented with extra β-mercaptoethanol (100 mM final). The extract was cleared in a microcentrifuge and its protein content was measured by the dye-binding method (Bio-Rad product profile). β-Galactosidase was assayed from the extract according to Miller (1972). To control the coloured compounds released from the tissue, a blank reaction with no substrate was used for each sample. One unit of β-galactosidase is defined as the amount of enzymic activity hydrolysing 1 nmol of ONPG per minute at 28°C (Ullman *et al.*, 1965).

β-Galactosidase assay in gel. The *E.coli* extract used as a control was obtained from an induced culture of a strain wild-type for the *lac* operon. The cells were lysed with lysozyme in 8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris–Cl, pH 8, diluted with the plant extraction buffer of Van den Broeck *et al.* (1985), cleared in a microcentrifuge and stored at −20°C after adding glycerol to 50%. Plant extracts were prepared as described for the assay in solution. The samples were normalized by measuring the total protein content of the extracts.

The extracts, kept on ice, were mixed with cold loading buffer and applied directly in an 8% polyacrylamide gel containing SDS (Laemmli, 1970). After electrophoresis at 4°C, the separation gel was washed once for 15 min (with agitation) in cold Z-buffer (Miller, 1972) and twice for 15 min in Z-buffer at room temperature. The fluorogenic β-galactosidase substrate 4-methyl umbelliferyl-β-D-galactoside was dissolved at 20 mg/ml in dimethyl sulphoxide and diluted 1000-fold in Z-buffer. The gel was agitated for 10 min in the substrate solution at room temperature, rinsed with water and the fluorescent enzyme bands were recorded by photography under UV illumination through a pale yellow filter (Kodak Wratten 2E).

β-Galactosidase assay in tissue. The histochemical staining is a modification of a *Drosophila* method (J.Szabad, personal communication). The reaction buffer Z′ is similar to the Z-buffer (Miller, 1972) except that the pH is 7.4 and β-mercaptoethanol is omitted. Tissue was first fixed with 1% glutaraldehyde in Z′-buffer for 2 h at room temperature. To inactivate the endogenous β-galactosidases, it is important that the fixative penetrates the material well. To aid penetration, the root tips were flash frozen in liquid nitrogen after 1 h of fixing and from the leaf material, grown in the greenhouse, the lower epidermis was removed. Plants grown in a closed chamber can be used when peeling of the epidermis is not acceptable. The glutaraldehyde was removed by rinsing twice with the Z′-buffer and replaced by the staining solution (880 μl Z′-buffer, 50 μl 100 mM $K_3[Fe(CN)_6]$, 50 μl 100 mM $K_4[Fe(CN)_6]$, 20 μl 8% 5-bromo-4-chloro-3-indolyl-β-D-galactoside in *N,N*-dimethylformamide). The tissue was incubated at 28°C until blue staining was visible, usually over night. The material was rinsed with Z′-buffer and to remove chlorophylls, postfixed for 1 h at room temperature with acetomethanol (1:3). After rinsing with absolute ethanol, the tissue was embedded in paraffin (melting point 55°C) and cut into 15-μm sections as described in Darlington and La Cour (1969) or soaked in 50% glycerol if photographed directly.

Neomycin phosphotransferase II assay in gel. This was performed according to a modification (Van den Broeck *et al.*, 1985) of the method of Reiss *et al.* (1984). The radioactive spots were quantified by cutting them from the ion exchange paper and counting in a scintillation counter.

Acknowledgements

Thanks are due to June Simpson and Sirpa Kurkela for critically reading the manuscript, Johan Botterman for providing plasmid constructions, Jan Leemans for useful discussions, Pirjo Rahkola and Eija Holma for excellent technical assistance, Barbara von Schoultz for preparing the thin sections and Seija Repo for the photographs. This research was supported by the Finnish National Fund for Research and Development (SITRA), Project 17005-1 YP/B, and the Academy of Finland. T.T. had part of the time on an Emil Aaltonen Foundation fellowship. Grants from Harry Federley Foundation, Oskar Öflund Foundation and Finnish Ministry of Education are gratefully acknowledged.

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Received on August 18, 1988; revised on December 2, 1988