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Activity of β-glucuronidase in root tips of different types of transgenic sugar beet plants

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Abstract

Expression of the β-glucuronidase (GUS) reporter gene driven by the CaMV 35S, *rolC*, *nos* and *mas* promoters was assessed in the tips of 12 independent clones of transgenic sugar beet (*Beta vulgaris*) roots. Three questions were addressed: *1*) expression pattern specific for a given promoter, *2*) expression pattern variability, and *3*) relationship between gene expression and cell differentiation. Characteristic patterns of tissue-specific expression were distinguished for each promoter. Striking differences, however, were found between some clones, bearing the same construct. Statistical analysis of the pattern variability proved that the variability is significantly lower within the construct than between constructs. *rolC*-GUS clones exhibited the lowest and CaMV 35S clones the highest pattern variability. Comparisons between the four promoters showed consistent GUS activity in areas playing a key role in tissue determination (the elongation zone) where cells switch from frequent mitosis and mostly isodiametrical growth, typical for the promeristem, to rapid elongation and differentiation. All of the clones were highly GUS-positive in the elongation zone of stele. Activity was commonly localised in the stele of the maturation zone for CaMV 35S, *rolC* and *mas*-GUS clones. CaMV 35S-GUS clones were highly active in the promeristem.

Additional key words: expression variability, reporter gene, tissue-specific expression.

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Introduction

Understanding the regulation of gene expression is an essential requirement for the potential of plant biotechnology to be realised fully. One aspect of this understanding is the relationship between controlling gene expression and cellular differentiation. This is of particular importance in crop plants such as sugar beet, where productivity may be enhanced by switching on gene expression in the storage organ outer cambial rings to increase sucrose storage (Elliott and Weston 1993; Thomas *et al.* 1993). This relationship may be investigated using reporter genes, such as the β-glucuronidase (GUS) coding region, under the control of different regulatory sequences. During last years, attention has been paid to the study of tissue-specific and developmental regulation of many genes. Among them the T-DNA genes of *Agrobacterium* and the promoters of some plant viruses were of the most thoroughly investigated. In crown galls, genes encoded by the T-DNA direct the synthesis of opines that the *Agrobacterium* can utilize as carbon or nitrogen sources (Tempé and Petit 1982). The study of these genes is not only important for their potential in crop improvement, but also for the study of plant-microbe interactions and gene regulation in general. In this study, the CaMV 35S (Phillips *et al.* 1992), nopaline synthase (*nos*; An *et al.* 1990), *rolC* (Sugaya *et al.* 1989) and mannopine synthase (*mas*; Saito *et al.* 1991) promoters have been used to drive GUS expression in sugar beet root tips produced following Ri plasmid mediated transformation. These promoters have previously been characterised to varying degrees, but statistically valid comparative histochemical assessments of their regulatory effects have yet to be made in a single crop species at the same time. Sugar beet hairy roots have been chosen for this comparison since they have a relatively simple structure, from which gene expression patterns may be deduced (Phillips *et al.* 1992). The uniformity of GUS localisation within clones bearing the same GUS construct and between clones produced using different GUS constructs has been assessed. These localisation studies permit the regulatory effects of the four promoters to be characterised and related to tissue differentiation (Beneš *et al.* 1995) in a large number of root tips of a single cultivar at the same stage of development.

Materials and methods

Plasmids and vectors: *Agrobacterium tumefaciens* C58, harbouring pRi A4b (Phillips *et al.* 1992) and a plasmid encoding kanamycin resistance and β-glucuronidase was used for transformations. Four different constructs with variously promoted GUS gene were utilised: pBI 121 (CaMV 35S promoter - Jefferson *et al.* 1987), p8I101-012-pl (*rolC* promoter - Sugaya *et al.* 1989), pJPPl (*nos* promoter - An *et al.* 1990), and pJPP4 (*mas* promoter - Saito *et al.* 1991).

Sugar beet cultivation and transformation: Sugar beet (*Beta vulgaris* L.) cv. Salohill seeds were surface sterilised and germinated on MSO medium at 22 °C (Phillips *et al.* 1992) and a 16-h photoperiod (irradiance of 70 μmol m^{-2} s^{-1}). Stem sections from 4-week-old plants were inoculated with *Agrobacterium tumefaciens* strains and incubated in the dark for a further four weeks. Individual tips of adventive roots emerging from the inoculation site were excised and transferred to agar solidified half strength Gamborg's B5 medium supplemented with 30 g dm^{-3} sucrose, 20 mg dm^{-3} kanamycin and 250 mg dm^{-3} cefotaxime (Gamborg and Miller 1968). After a further 3 weeks root clones, each derived from a single root tip, were tested for the presence of bacteria using nutrient broth. Only root clones shown to be free from bacteria were studied further. Twelve root clones (3 from each promoter type) were selected for further analysis. Each of these has been shown to be transformed with the GUS coding sequence by both DNA-DNA hybridisation and Polymerase Chain Reaction (PCR) analysis (Phillips *et al.* 1992, Dewar 1994). Hairy roots were cultivated in darkness, in shake flasks containing 50 cm^3 liquid Gamborg's B5 medium, 20 g dm^{-3} sucrose and 20 mg dm^{-3} kanamycin. The roots were subcultured at 6-week intervals. Material for histochemical analysis was passaged into 15 cm^3 of liquid B5 medium supplemented with 20 g dm^{-3} sucrose, 20 mg dm^{-3} kanamycin sulphate and cultivated in Petri dishes in darkness on a rotary shaker (80 rpm; 22 °C) for three weeks. For each clone, five Petri dishes were inoculated.

Histochemical GUS assay: Three-week-old cultures were fixed for 30 minutes in an ice cold solution of 4 % (v/v) formaldehyde in 50 mM sodium phosphate buffer (pH 7.0), and washed for 50 min in several changes of ice cold distilled water. Root tips 10 mm long were excised and incubated overnight at 37 °C in X-glucuronide solution [1 mg of 5-Br-4-Cl-3-indolyl-β-D-glucoronide, cyclohexylammonium salt, dissolved in 0.1 cm^3 of N,N-dimethylformamide and diluted to 10 cm^3 with 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM potassium ferricyanide and 0.5 mM potassium ferrocyanide]. After incubation, root tips were washed for several minutes in distilled water, mounted on slides and scored for the presence or absence (yes-no) of the blue final reaction product of GUS activity at 11 sites within the tip (Fig. 1). The relative intensity of staining was not taken into consideration. This way the

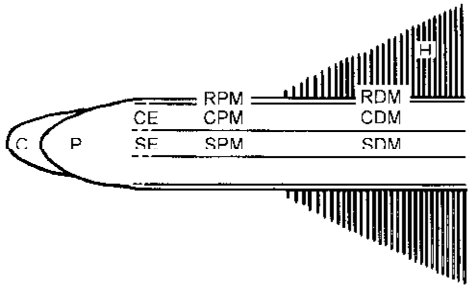

Fig. 1. Root tip sites assessed for GUS activity: C - root cap; P - promeristem; CE - cortex in elongation zone; SE - stele in elongation zone; RPM - rhizodermis in proximal part of maturation zone; CPM - cortex in proximal part of maturation zone; SPM - stele in proximal part of maturation zone; RDM - rhizodermis in distal part of maturation zone; CDM - cortex in distal part of maturation zone; SDM - stele in distal part of maturation zone; H - root hairs.

danger of misjudging the staining intensity - due to variable cell size and vacuolisation, as well as non-linear kinetics of the stain formation - was avoided. The experiment was repeated twice, each time using root tips from five Petri dishes per clone. In total, 347 to 977 root tips from each of three independent clones from each of the four different promoter constructs were assessed in this manner.

Localization pattern analysis: Pairwise nonreciprocal comparisons were made for pairs of roots both within each clone and between two clones. The number of sites which stained differently (*i.e.* only one root of the pair stained in the given site) was counted. This was called a 'difference' between the two roots. The 'mean differences' within the clone and between particular clones were calculated. The former characterizes variability of GUS staining pattern within the clone, *i.e.* intraclonal variability, the latter quantifies the pattern differences between the two clones, interclonal variability. The Welch test (Welch 1951) was performed to find if there are any significant differences among these values. A sequentially rejective multiple test procedure (Holm 1979) was used to reveal which variability values are mutually significantly different. The same approach was used to analyse pattern variability within and between the GUS constructs. "Between" specifies the 'mean differences' between roots carrying a given construct and all other roots bearing a different construct. All of the tests were performed at significance level $\alpha = 0.05$, whilst some also utilised the highly significant level $\alpha = 0.01$, where indicated.

Results

Reporter gene expression specificity: Characteristic patterns of GUS activity were distinguished for each construct. The 35S promoter drives expression predominantly in the promeristem, elongation zone, stele, and sometimes in the root cap. In *nos*-promoted material GUS activity was found less often and mostly in the elongation zone. *Mas*-GUS roots exhibited activity in the stele, cortex and less frequently in the elongation zone and root cap. In *rolC*-GUS roots activity was found mostly in the stele, elongation zone and often in the promeristem (Table 1).

The only site where all of the clones were GUS-positive was the stele of the elongation zone. With the exception of the *nos*-GUS construct, activity was also commonly localised in the stele of the maturation zone. In all the other sites considerable differences between clones and constructs were observed.

Pattern variability: During assessments of tissue specificity considerable variability of GUS localisation was found even between some clones bearing the same construct (Table 2). Welch Test analysis revealed significant differences among these values. Holm's procedure showed that in most cases, variability differed mutually at a significant level ($\alpha = 0.05$), with the exception of the intraclonal variability of the *nos*-promoted clone F70, which did not differ significantly from any other variability, probably due to the small number of roots which stained positively for this clone. Clone F 19 (*rolC* promoter) had the most uniform GUS localisation pattern, as indicated by the lowest intraclonal variability, while clone J10 (355 promoter)

Table 1. *In toto* histochemical GUS assessment in 11 sites of sugar beet hairy root tips (*n* - number of roots assessed, GUS+ - the percentage of roots GUS-positive in at least one site, GUS+ in a given site - the percentage of roots with GUS activity in a particular site relative to the total number of GUS-positive roots. For key to sites see Fig. 1.

Promoter	Clone	*n*	GUS+	GUS+ in a given site										
				C	P	CE	SE	RPM	CPM	SPM	RDM	CDM	SDM	H
35S	J3	577	56.2	0.3	68.5	16.4	47.8	1.2	0.0	47.8	0.0	0.0	66.7	0.0
	J8	641	92.0	37.5	60.5	53.7	35.6	10.0	13.7	49.7	0.0	0.0	99.0	0.0
	J10	849	37.1	18.1	79.7	42.2	85.1	7.3	0.0	27.3	0.0	0.0	69.8	0.0
mas	F28	660	59.4	23.0	7.7	2.8	41.6	7.1	34.7	50.5	0.0	0.3	14.3	0.3
	F35	977	32.9	0.9	0.0	0.3	3.1	0.0	2.2	76.0	0.0	3.4	45.8	0.0
	F85	811	50.8	7.8	6.2	2.9	36.4	0.2	39.1	53.4	0.0	0.2	24.0	0.2
nos	F70	851	0.5	0.0	0.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	F90-2	430	19.8	0.0	74.1	67.1	85.9	35.3	0.0	0.0	0.0	0.0	0.0	0.0
	F91-2	890	7.6	0.0	3.0	19.1	82.3	1.5	0.0	10.3	0.0	0.0	0.0	0.0
rolC	F1	691	99.7	0.2	0.0	0.0	17.3	0.0	0.0	98.6	0.0	0.0	100.0	0.0
	F19	605	100.0	4.1	28.9	12.1	98.8	7.9	2.8	99.8	0.0	0.0	97.5	0.2
	F22	347	99.7	0.0	48.0	37.3	96.5	29.8	0.3	100.0	0.0	0.0	99.7	10.1

Table 2. Intra- and interclonal variability of GUS localization pattern. The numbers along the diagonal represent staining pattern variability within each clone (intraclonal variability), while the other numbers characterize staining variability between clones (interclonal variability). To find the interclonal variability betwen particular two clones, locate the role of the first clone and look for the intersection with the column of the second one (*n* - number of roots exhibiting GUS activity in at least one site.

Promoter	Clone	J3	J8	J10	F28	F35	F85	F70	F90-2	F91-2	F1	F19	F22	*n*
35S	J3	1.548	2.490	2.354	2.860	2.564	2.819	3.292	3.351	2.532	2.032	2.300	2.958	324
	J8		2.193	3.196	3.875	3.498	3.599	4.722	3.940	3.463	2.427	2.778	3.611	590
	J10			2.305	3.417	3.515	3.435	3.042	2.594	2.754	3.177	2.666	2.687	315
mas	F28				2.192	2.105	2.183	2.443	3.607	2.196	2.659	3.188	3.811	392
	F35					1.054	1.787	2.309	3.724	2.353	1.067	2.484	2.683	321
	F85						1.903	2.347	3.615	2.021	2.312	2.794	4.037	412
nos	F70							1.333	1.267	1.091	3.004	3.727	3.115	4
	F90-2								1.839	2.291	4.587	3.925	3.290	85
	F91-2									0.789	2.771	2.618	3.948	68
rolC	F1										0.306	1.333	2.407	689
	F19											0.859	1.815	605
	F22												0.708	346

exhibited the highest pattern variability. Intraclonal variability was usually lower than interclonal one (Table 2).

The pattern variability within and between the constructs differed highly significantly ($\alpha = 0.01$). The variability within a particular construct was always lower than that between constructs. The lowest variability was obtained with the *rolC*-GUS construct, and the highest in 35S-GU5 clones (Table 3).

Table 3. GUS localization pattern variability within and between the four GUS constructs.

Contruct	Pattern variability within construct	between constructs
35S	2.467	2.991
mas	1.954	2.973
nos	1.711	3.357
rolC	1.307	2.746

In some clones bearing the 35S-GU5 or *mas*-GUS construct, considerable variability in GUS staining pattern was observed even among roots of the individual clone, regenerating from the same explant in the same Petri dish.

Discussion

Reporter gene expression: The patterns of GUS reporter gene expression obtained contrast with the findings of other workers. The CaMV 35S promoter has been described as constitutive (Benfey *et al.* 1989, Odell *et al.* 1985) and might therefore have been expected to express in all of the root sites assessed. The absence of demonstrable histochemical GUS activity from regions of the developing root tip, such as the rhizodermis and cortex from the distal maturation zone was in contrast to the findings of constitutivity and consistent with other workers reports of tissue specificity, including the cortex, phloem and vascular cambium (Nagata *et al.* 1987, Battraw and Hall 1990). In this study, the highest percentage 35S-GU5 activity was found in the promeristem, adjacent root cap and maturation zone stele regions. This suggests that the 35S promoter may be effective in areas of high metabolic activity. The 35S promoter has been shown to be influenced by transcriptional factors such as ASF 1 (Lam *et al.* 1989) present in root nuclear extracts, which can bind to TGACG (or as-1) sites found at two positions between -83 and -63 within the A domain (Benfey *et al.* 1989) known to direct strong expression in the root tip cortex, promeristem and vascular associated tissues. Both the *mas* and *nos* promoter sequences also contain as-1 sites (Velten *et al.* 1984, Lam *et al.* 1990) and GUS activity was also shown with these promoters in the elongation zone stele. CaMV 35S promoter also contains binding site for maize nuclear factor MNF1 in the region (-301 to -208) involved in the expression in the immature vascular tissue (Benfey and Chua 1990). Two other mutually structurally different DNA-binding proteins from maize interact with the MNF1 binding site: MNB1a and MNB1b and it was suggested that expression of MNB1b is developmentally regulated and correlates with cellular proliferation or differentiation (Yanagisawa and Izui 1993). Tabata *et al.* (1991) isolated a leucine-zipper type transcription factor of wheat, HBP-1b, that binds to the hexamer motif ACGTCA in CaMV355, *nos* and *mas* promoters. HBP-lb is believed to be a transactivator in cell-cycle dependent transcription. A conserved osc-element that binds transcription factor OCSTF, is a component of promoters of several T-DNA and plant viral genes, including CaMV 35S, *mas* and *nos* (Bouchez *et al.* 1989).

The highest percentage activity with the *mas*-GUS construct was found in the maturation zone proximal cortex and stele, the elongation zone stele and the root cap. This is consistent with data from licorice and tobacco hairy roots (Saito *et al.* 1991), rice roots (Meijer *et al.* 1991) and in contrast to the constitutive regulation reported elsewhere (Deblaere *et al.* 1987, Stefanov *et al.* 1991, 1994). Little *mas*-GUS activity was detected in the rhizodermis, cortex or root hairs of the maturation zone. The *mas* promoter is considered to be wound inducible (Sanger *et al.* 1990) and contains a consensus auxin inducible promoter element GCAN-CATRCRY (N - any nucleotide; R - purine; Y - pyrimidine), which may explain high percentage activities in the auxin rich hairy roots studied (Gartland *et al.* 1991).

A very different typical GUS expression pattern was obtained with the *nos*-GUS construct. Activity was located almost exclusively in the promeristem and elongation zone. This was surprising given that the *nos* promoter sequence contains an as-1 site between -131 and -111 (Lam *et al.* 1990) and is thought to be both wound and auxin inducible (An *et al.* 1990). The reasons why no GUS activity was detected in the maturation zone with the *nos*-GUS construct require further investigation.

Percentage *rolC*-GUS activity was consistently several-fold higher in the vascular tissue of all three root developmental zones than in the peripheral tissues. This is consistent with data from rice roots (Matsuki *et al.* 1989) and of phloem specific expression from tobacco (Sugaya *et al.* 1989). The *rolC* promoter is activated by sucrose, which may regulate the phloem-specific expression (Yokoyama *et al.* 1994).

Pattern variability: Significant variations were observed in the histochemical staining patterns obtained with each of the four different promoters, as might have been expected. Variations were, however, also observed between clones produced using the same chimaeric gene, and within tips from the same clone. This variation was caused neither by minor differences in the composition of incubation medium, nor by variations in incubation conditions (Vitha *et al.* 1994). Thus, the variability seems to be an intrinsic attribute of the clones tested. Despite this variability, it was possible to find characteristic patterns of GUS expression for each promoter tested. Breyne *et al.* (1992) observed strong influence of flanking sequences on the quantitative level of expression of the GUS reporter gene downstream of *nos* promoter. They did not asses the tissue specificity of reporter gene expression. It is likely that in the presented work the differences in GUS staining pattern between clones bearing the same GUS construct are a result of the differences of the site of T-DNA insertion into the host DNA. We suggest that in these clones the expression of the reporter gene is under combinatorial control of the introduced promoter and nearby regulatory sequences of the host. This resembles the situation with enhancer-traps, where the reporter gene downstream the minimal promoter fragment is used for transformation and the tissue- and cell-specific expression is a result ofthe host's controlling sequences (Sundaresan *et al.* 1995). The interesting point in our case is that the position effect can modify or override the expression specificity of strong promoters, such as 35S, *mas*, *nos* or *rolC*.

Root tip differentiation and reporter gene expression: Expression pattern assessments for the 355, *nos*, *mas* and *rolC* promoters showed consistent GUS activity in areas of the root tip that play a key role in tissue determination, namely the stele of the elongation zone, and stele of the maturation zone. These are sites where switching in cell differentiation takes place: in the elongation zone, where the transition from meristematic parenchyma to the cylindrical cells of the stele occurs, and in the maturation zone as stele cells differentiate into vascular elements. This suggests that these promoters may be regulated in both spatial and temporal contexts. Understanding of the relationship between cellular differentiation and the control of gene expression will benefit from systematic comparisons of promoter and regulatory element effects, particularly in yield determining tissues of commercially important crop plants. Increased knowledge of these influences upon gene expression will enhance the ability of biotechnology to fulfil its economic potential.

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