Use of ß-Glucuronidase (GUS) as a Marker for Transformation in Sugarbeet

C. A. Wozniak[1] and L. D. Owens

USDA-Agricultural Research Service, Northern Crop Science Laboratory[1], Fargo, North Dakota 58105 and Plant Molecular Biology Laboratory, Beltsville, Maryland 20705

ABSTRACT

Accurate detection of a genetic or biochemical marker introduced into sugarbeet *(Beta vulgaris* L.) is based on the absence of native sequences or activities in the plant that could confound the analysis of expression of the introduced marker. During the course of experiments designed to optimize DNA transfer from *Agrobacterium tumefaciens* to sugarbeet leaf disc cells, an endogenous enzyme activity was discovered which utilizes all the common substrates recognized by the marker enzyme ß-glucuronidase (GUS) from *E. coli.* This native sugarbeet enzyme (SB-GUS) was characterized immunologically and biochemically. GUS and SB-GUS were found to be distinct with regard to pH optima, thermal inactivation, reaction to denaturants and protein modifying reagents, inhibition by metals and saccharo-lactone, and molecular mass. The two activities are not immunologically related, as judged by Western blot and immunoprecipitation analyses. A protocol was developed to accurately quantitate introduced GUS in the presence of SB-GUS, by utilizing selective inhibition of GUS at pH 7.0 by saccharic acid 1,4-lactone. Under these conditions GUS activity is completely eliminated, while SB-GUS activity was unaffected.

Additional Key Words: *Agrobacterium, Beta vulgaris,* marker, sugarbeet glucuronidase, transgenic

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Selection of a marker gene that affords accurate quantitation of expression after introduction to the host, or provides for antibiotic selection of transformed cells, is dependent on the following criteria: 1) availability of a characterized DNA sequence, 2) development of assays that assess the effects of introduced DNA (e.g., visualization, quantitation of expression, integration), and 3) absence of endogenous (host) activity that is similar or identical to that encoded by the introduced foreign DNA. Numerous genetic markers are available for use in plant transformation experiments, but the most versatile and widely used is the ß-glucuronidase (GUS) enzyme from the *Escherichia coli gusA* (formerly *uidA)* locus (Jefferson et al., 1986).

GUS (EC 3.2.1.31) is a tetrameric protein with a M_r of 272 kD; it catalyzes the hydrolysis of ß-D-glucuronides from various ligands. The enzyme is stable at -80°C or 4°C in aqueous form, with a half-life of nearly 2 h at 55°C. Activity of the enzyme is measurable from pH 5 to 8 (Anonymous, 1972), inhibited by saccharo-lactones (Levvy, 1954), moderately inhibited by Zn^{2+} and Cu^{2+} (Jefferson et al., 1987), and moderately resistant to proteolysis (authors, unpublished results).

Several substrates are commercially available for quantitation of GUS activity *in vitro* by colorimetric or fluorometric assays (Martin et al., 1992). The colorimetric assay typically uses p-nitrophenyl-ß-D-glucuronide (PNPG) as the substrate and provides a sensitive, linear assay that is measurable with any standard spectrophotometer. A far more sensitive assay is based on fluorogenic substrates such as 4-methylumbelliferyl-ß-D-glucuronide (MUG), 3-carboxyumbelliferyl-ß-D-glucuronide (CUG), or resorufin-ß-D-glucuronide (REG). The most commonly employed fluorogenic substrate, MUG, is relatively inexpensive and can be measured in a substrate-dependent, linear fashion, over short or extended assay periods, with a spectrofluorometer. The product, 4-methylumbelliferone (4-MU), is excited at 365 nm and emits maximally at 455 nm, usually not within the spectrum of endogenous fluorescent plant compounds.

Histochemical analysis of GUS expression is a useful technique for localization of cellular and subcellular activity in intact tissues. The indigogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-gluc) forms an insoluble precipitate following cleavage from the aglycone by a glucuronidase, with subsequent oxidation and dimerization of the leaving group (i.e., X). This blue precipitate allows for visualization of GUS activity in transformed cells following co-cultivation with *Agrobacterium* or microprojectile bombardment. The

degree of precipitation can be influenced by internal pH and peroxidase activity (Stomp, 1992), hence the quantitation of expression is only an estimate at best.

The presence of an endogenous glucuronidase in higher plants has been controversial for some time (Jefferson et al., 1986; Stomp, 1992; Wilson et al., 1992) despite earlier reports (Levvy, 1954; Schulz and Wiessenbock, 1987) of its existence. Several recent studies examining endogenous glucuronidase activity in plants have demonstrated the presence of a plant encoded ß-glucuronidase (Sekimata et al., 1989; Plegt and Bino, 1989; Wenzler et al., 1989; Raineri et al., 1990; Alwen et al., 1992), including one in table beet (Hu et al., 1990) and sugarbeet (Hodal et al., 1992). In the majority of cases, however, the potential influence of contaminating microorganisms (e.g., residual *Agrobacterium;* endophytes; culture contaminants), which may express the enzyme, was not addressed.

During an evaluation of transformation methods in this laboratory, calli, petioles and laminae sampled from axenic clones of five sugarbeet lines were all observed to exhibit an endogenous activity that recognized the same substrates as GUS of microbial origin. In this paper we compare the native sugarbeet glucuronidase (SB-GUS) and GUS from *E. coli,* and report that substantial differences exist between the two enzymes. Further, a protocol was developed to allow accurate quantitation of introduced GUS activity in sugarbeet tissues expressing SB-GUS. Finally, the potential influence of microbial contaminants on detectable glucuronidase activity was evaluated and found to be inconsequential for the tissues utilized in this study.

MATERIALS AND METHODS

Plant Tissue Culture. Sugarbeet *(Beta vulgaris* L.) tissues and organs were derived from clonally propagated shoot cultures grown under axenic conditions. Lines 'REL-1', 'FC701', and 'EL48' were provided by Dr. J. Saunders (USDA-ARS, East Lansing, MI) and lines 'FC607' and 'FC901' by Dr. G. Smith (USDA-ARS, Fargo, ND). Shoot cultures were maintained on Murashige and Skoog (1962) medium (MS) supplemented with 0.25 mg/L BA, solidified with 0.3% Gelrite, at 24°C under constant fluorescent lighting.

Microbiological Evaluation of Tissue Cultures. Experiments assessing glucuronidase activity in transformed and non-transformed tissues were performed under sterile conditions in a biological safety cabinet. All solutions were autoclaved or filter sterilized, and all instruments autoclaved prior to use. Portions of tissue homogenate were

inoculated onto Luria-Bertani (LB), potato dextrose, Saboauraud agars and into tubes of fluid thioglycollate medium prior to addition of sodium azide (0.05% w/v) at assay initiation. Any data derived from tissue homogenates that yielded microbial growth were discarded.

Leaf Disc Transformation. Leaf discs of 7 mm diameter were cut from axenic shoot cultures with a cork borer. Overnight LB broth cultures of *Agrobacterium tumefaciens* A281 carrying plasmids pTiBo542 and pBI121 (provided by A. Binns, Philadelphia, PA) were pelleted at 3000 *g* for 10 min and resuspended in liquid MS medium at pH 5.3. Leaf discs were submersed in the culture of *A. tumefaciens* for 1 to 3 h prior to plating on solid MS medium in the dark at 24°C. After 3 days, leaf discs were washed in carbenicillin (100 mg/L) and tetracycline (5 mg/L) for 1 h and transferred to solid MS medium supplemented with 1 mg/L BA, and carbenicillin and Cefotaxime at 200 mg/L each. Discs were incubated at 25°C under fluorescent lights. Tissues and tumors were transferred to MS medium supplemented with Cefotaxime 200 mg/L, 150 mg/L kanamycin, and sampled for the presence of residual *Agrobacterium* cells.

Fluorometric Assays. Following the standard procedures for GUS analysis, as set forth by Jefferson (1987), callus, petiole and leaf explants were homogenized in GUS lysis buffer (50 mM $NaHPO_4$, 10 mM Na_2EDTA, 0.1% (w/v) Triton X-100, 10 mM 2-mercaptoethanol, pH 7.0; GUS-LB) and centrifuged for 4 min at 14000 *g*. The supernatant was cleared of particulates by the addition of cross-linked PVP and centrifuged again.

GUS used in comparison assays with SB-GUS was type IX (Sigma G7396; St. Louis, MO, USA), EC 3.2.1.31. GUS IX was solubilized in GUS-LB, pH 7.0, and stored at -80°C.

Portions of homogenate or GUS IX were added to GUS-LB, pH 7.0. Substrate (MUG, CUG, or REG) was solubilized at 5 mM in GUS-LB and all samples warmed to assay temperature for 10 to 15 min. When chemical treatments were included, these were added 15 min prior to addition of MUG. Substrate was added to the tubes at a final concentration of 1 mM at the start of the assay. At 15, 30, 60, 120, and 180 min after substrate addition, 100 mL aliquots were taken from reaction tubes and added to 1.9 Ml Na_2CO_3 (0.2 M). 2-Mercaptoethanol was omitted in REG assays.

Saccharic acid 1,4-lactone (S-L) was made fresh for each use in GUS-LB and added to the reaction mix at 5 or 10 mM final concentration 15 min prior to the addition of substrate. S-L inhibition assays were performed at pH 5.8 and 7.0.

Assays to determine the influence of pH on SB-GUS and GUS IX activity were performed in GUS-LB adjusted from pH 2.0 to 9.0, as indicated. Post-assay measurement indicated that pH varied <0.3 units in all cases. Sugarbeet callus was homogenized in 10 mM Na_2EDTA, 0.1% Triton X-100, pH 7.0, to avoid buffering capacity of $NaHPO_4$ in GUS-LB. 4-MUG was prepared in GUS-LB adjusted to the appropriate pH for each assay.

All fluorometric assays (and all other experiments) consisted of triplicate or quadruplicate assay tubes in each experimental treatment, with experiments repeated at least three times. All enzyme assays included substrate only controls to assess the affects of treatment on the substrate in the absence of enzyme.

Colorimetric Assays. Experiments assessing the utilization of PNPG (5 mM) were performed at 37°C and stopped by addition of aliquots to 2-amino-2-methyl-propanediol.

Histochemical Assays. Assays were performed according to Jefferson (1987) or amended by the addition of 20% (v/v) methanol (Kosugi et al., 1990) or 10 mM S-L when evaluating inhibitors of SB-GUS. X-Gluc was used at 1 mg/mL in 100 or 150 mM $NaHPO_4$ (pH as indicated), 10 mM Na_2EDTA, 0.05% (w/v) NaN_3. Incubation at 37°C was for times as indicated, followed by washing in 100 mM $NaHPO_4$, pH 8.0, and clearing in 70% ethanol. Effects of S-L were evaluated following addition at assay initiation or after pre-incubation of tissue in GUS-LB without substrate for 1 h.

Immunoblotting and Immunoprecipitation. Total cellular protein of 'REL-1' was extracted from petioles, callus, and leaves as previously described (Wozniak and Partridge, 1988). Protein content was quantitated with the Bradford protein assay reagent (BioRad 500-0001, Richmond, CA, USA). Vertical polyacrylamide gel electrophoresis and transfer to nitrocellulose followed the method of Towbin et al. (1979). Western blots were probed with 1:1000 rabbit anti-GUS (RαG) antiserum (Clontech 1511-1, Palo Alto, CA, USA) and bound IgG was visualized with goat anti-rabbit IgG (GαR) coupled to horseradish peroxidase (1:2000).

Callus homogenate, prepared in the absence of 2-mercaptoethanol, was incubated 1 h with RαG-IgG, then coincubated with GαR-IgG linked to polyacrylamide beads (BioRad 170-5602) for 2 h at 24°C. Beads were collected (2 m, 1000 g), washed three times with TBS, and enzyme activity was quantitated fluorometrically. The supernatant fraction was saved and similarly quantified. GUS IX (5 ng) was incubated as above for comparison.

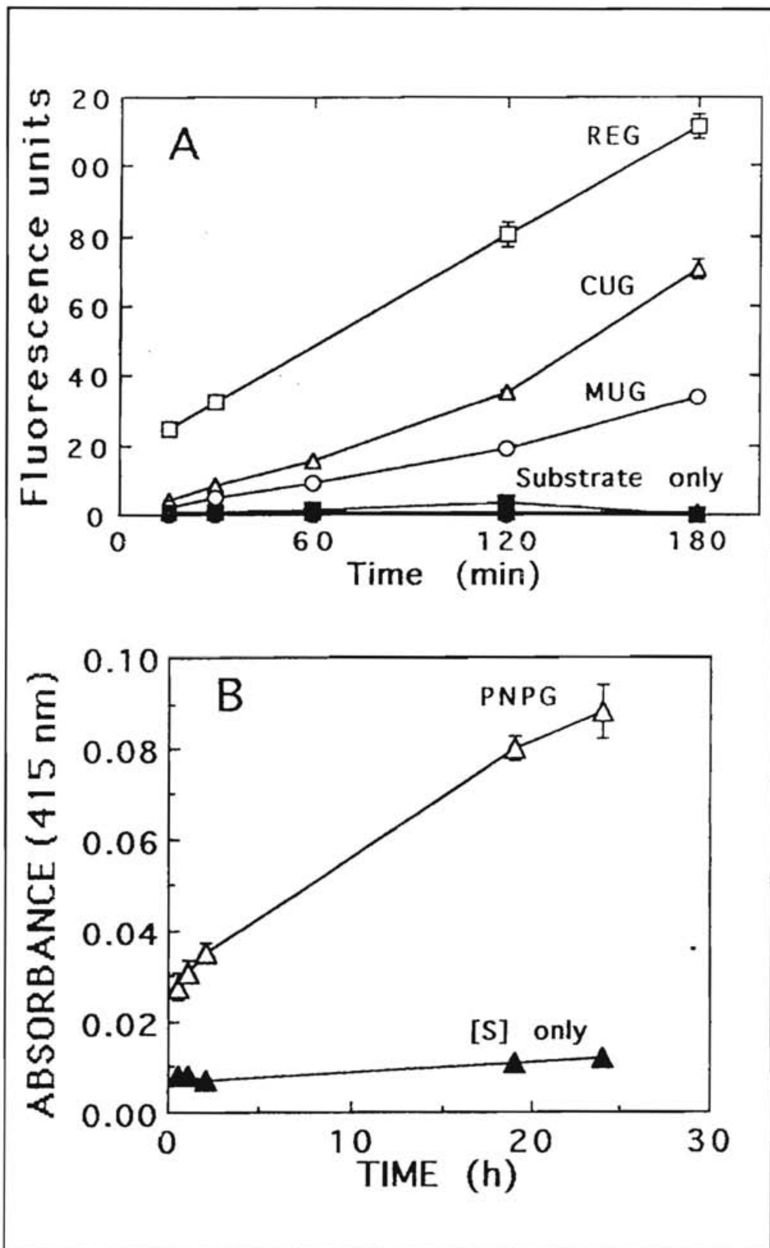

Figure 1. Utilization of (A) the fluorogenic substrates REG, CUG, and MUG; and (B) the colorigenic substrate PNPG, by callus extracts of 'REL-1' at pH 7.0, 37°C. Fluorometer was set to 100 units with 1 mM MU. Data are means ± SE of three experiments with triplicate assay tubes per treatment.

Endophytic Bacteria. Microbial contaminants and endophytic organisms were isolated from some shoot cultures (not used in SB-GUS assays) and grown to turbidity in LB broth at 25oC, 250 rpm. Pelleted bacterial cells were treated with 2 mg/mL lysozyme for 5 min in 200 mL GUS-LB, pelleted again, and activity in the supernatant was quantitated fluorometrically. *Escherichia coli* MM294 carrying pMON9790 with *gusA* under the direction of the *mas* promoter (provided by Dr. C. Gasser, Monsanto Corp., Chesterfield, MO) was treated identically as a positive control for GUS.

RESULTS

Substrate Recognition by SB-GUS. Axenic calli of 'REL-1', 'EL48', 'FC701', and 'FC901' were homogenized in GUS-LB, pH 7.0, and evaluated for endogenous glucuronidase activity indicated by the fluorogenic substrate 4-MUG. All tissues were found to contain activity capable of hydrolyzing 4-MUG to 4-MU in a substrate dependent, linear fashion (Fig. 1A; data not shown for all genotypes). Background levels of fluorescence were determined for substrate incubated alone or homogenate incubated in the absence of substrate.

A comparison of available fluorogenic substrates indicated that this endogenous activity, called SB-GUS, was capable of recognizing each of the fluorogenic ß-D-glucuronides in a substrate dependent, linear manner (Fig. 1A). Similarly, PNPG, the colorimetric substrate, was also hydrolyzed by SB-GUS (Fig. 1B).

Differences in SB-GUS and GUS Properties. To further characterize SB-GUS activity, extracts were subjected to various treatments known to affect proteins and compared with similarly treated GUS IX. SB-GUS was more stable to treatment with SDS, heat, S-L, and N-ethyl maleimide (N-EM), suggesting substantial differences between the two proteins (Table 1). Additional differences in response to methanol addition and resistance to proteolysis were also noted. The most striking contrasts observed were the enhanced activity of SB-GUS at high temperatures, in the presence of Zn^{2+} and Cu^{2+}, and the lack of inhibition by S-L at pH 7.0. Additionally, the minimal inhibition of SB-GUS by N-EM, a sulfhydryl modifying reagent, suggested a protein with few available disulfide bonds (i.e., cysteine residues), in contrast to the tetrameric, N-EM sensitive nature of GUS IX.

Analysis of co-transformed tumor tissues, selected on kanamycin containing medium, yielded a MUG dependent, linear fluorogenic response at both pH 5.8 and 7.0 (Fig. 2A, B). Addition of 5 mM S-L to the reaction mix drastically decreased total ß-glucuronidase specific

Table 1. Comparison of SB-GUS and GUS IX activities following physical and chemical treatments. Unless otherwise noted, assays were performed at pH 7.0 for 3 h at 37°C. Extracts were incubated for 15 min prior to substrate addition (MUG) under treatment conditions, except 100°C, which was for 5 min. Formaldehyde (2% w/v) was determined to have no effect on fluorescence of MU. The specific activity of SB-GUS is expressed as pmoles MU/mg protein/min and GUS IX (10 ng) as mmoles MU/mg protein/min. Data are means ± SE from three experiments with triplicate assays in each.

Sample	Treatment	Specific activity	% Decrease or increase
SB-GUS	None	427 ± 96	——
	100°C, 5 min pretreatment	128 ± 26	70.0
	60°C during assay	1378 ± 137	(323)
	N-EM, 20 mM	348 ± 32	18.4
	Proteinase K, 40 μg/mL	207 ± 46	51.5
	SDS, 0.4% (w/v)	185 ± 50	56.7
	Formaldehyde, 2% (w/v)	3 ± 2	99.4
	Zn^{2+}, 10 mM	665 ± 37	(56.0)
	Cu^{2+}, 10 mM	524 ± 35	(22.7)
	S-L, 5 mM, pH 5.8	369 ± 25	13.5
	S-L, 10 mM, pH 7.0	436 ± 20	(1.0)
	Methanol, 20%	73 ± 1	82.8
	No substrate	< 0.1 ± 0	99.9
None	Substrate only[†]	0.2 ± 0.1	99.8
GUS IX	None	10.6 ± 0.6	——
	100°C, 5 min pretreatment	< 0.1 ± 0.1	99.7
	60°C during assay	0.6 ± 0.1	94.2
	N-EM, 20 mM	0.1 ± 0.1	98.7
	Proteinase K, 40 μg/mL	7.5 ± 0.8	29.4
	SDS, 0.4% (w/v)	0.1 ± 0.1	98.9
	Formaldehyde, 2% (w/v)	0.2 ± 0.1	98.2
	Zn^{2+}, 10 mM	8.2 ± 0.2	22.8
	Cu^{2+}, 10 mM	7.3 ± 0.3	31.1
	S-L, 5 mM, pH 5.8	0.2 ± 0.2	98.3
	S-L, 5 mM, pH 7.0	< 0.1 ± 0.0	100.0
	Methanol, 20%	5.9 ± 0.7	44.2
	No substrate	< 0.1 ± 0.0	100.0

[†]1 mM MUG in reaction buffer without extract.

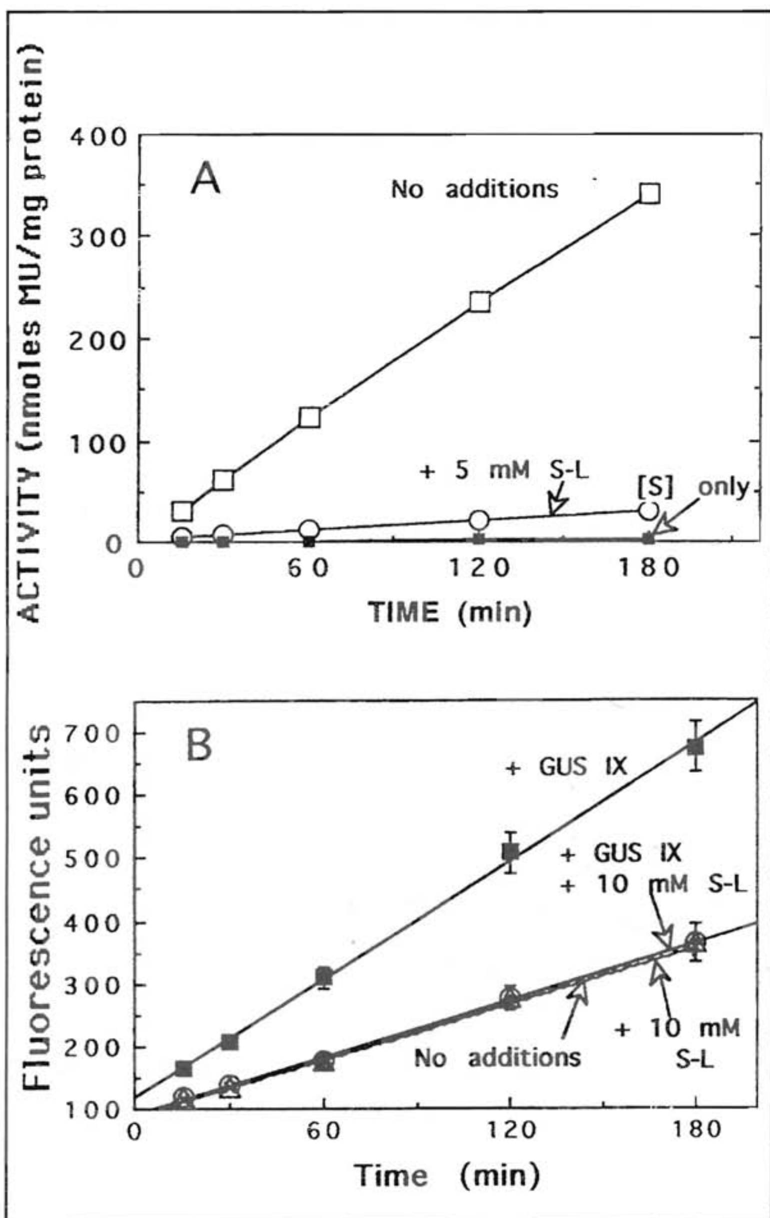

Figure 2. Inhibition of GUS activity by (A) 5 mM S-L at pH 5.8 in extracts from transformed tumor tissues and by (B) 10 mM S-L at pH 7.0 in extracts from non-transformed callus, in the presence or absence of added bacterial GUS (GUS IX) or S-L. Assays were performed at 37°C, with 1 mM MUG as the substrate. Data are means ± SE of three experiments with quadruplicate assay tubes per treatment.

activity in the extract. SB-GUS was shown to be minimally inhibited by 5 mM S-L (Fig. 2A, Table 1) at pH 5.8; hence, the remaining fluorogenic activity represents SB-GUS activity. Increasing the S-L concentration to 10 mM (to compensate for the decreased S-L stability at neutrality) allowed precise quantitation of both GUS IX and SB-GUS in a common extract. GUS IX was totally inhibited (Table 1) by S-L at pH 7.0, and SB-GUS was unaffected by S-L at pH 7.0, allowing for the subtractive quantitation of each enzyme's contribution to the total MUG hydrolysis (Fig. 2B).

Absence of Endophytes. Having noted these substantial differences in enzyme response, we further characterized SB-GUS to demonstrate conclusively its presence in sugarbeet tissues. The potential influence of microorganisms (e.g., endophytes, contaminants) on measurable glucuronidase activity is often overlooked with tissue cultured and field collected materials. Although the plant extracts used in experiments reported here tested negative for the presence of microbes, other cultures and lines of sugarbeet yielded bacteria from the test procedures employed. None of the six bacterial colony types evaluated yielded measurable glucuronidase in fluorometric assays (Fig. 3). Lysis of *E. coli* MM294/pMON9790 under identical conditions did allow quantifiable GUS activity as a positive control.

Response to pH. The effect of pH on SB-GUS activity was measured from pH 2.0 to 9.0 at 1.0 unit increments. The pH optimum was near 4.0 (Fig. 4).

Immunological Investigations. Analysis of Western immunoblots indicated no positive reaction near the M_r of GUS from *E. coli* when non-transformed callus extracts were probed with RαG IgG (Fig. 5). IgG was bound to several sugarbeet proteins, but these likely represent peptides with epitopes recognized by other fractions of antibodies in this polyclonal antiserum. Attempts to immunoprecipitate SB-GUS from solution with RαG antiserum at 1:20 dilution, with a secondary GαR IgG linked to polyacrylamide beads, indicated no significant recognition of SB-GUS epitopes by the antiserum raised against *E. coli* GUS. In contrast, GUS IX was effectively removed from solution by this method (Table 2).

Activation by Heat. The extraordinary heat stability of SB-GUS relative to GUS IX prompted an analysis of thermal inactivation of this enzyme. Reaction tubes were incubated in water baths or overlaid with mineral oil in a thermal cycler (Perkin-Elmer) at 37 to 99°C. SB-GUS activity was enhanced at 60, 70, 80, 90, 95 and 99°C relative

to 37°C (Fig. 6). The substantial variability in specific activity observed at 99°C may reflect the beginnings of thermal instability of SB-GUS. An increase to 100°C indicated some inactivation of SB-GUS (Table 1). In contrast, GUS IX was denatured at 60°C (Table 1).

Histochemical Assay Properties. When X-gluc was used as a histochemical indicator of SB-GUS activity under standard assay conditions (pH 7.0) with incubation times of less than 24 h, no significant indigo precipitate was formed in non-transformed tissues and organs (i.e., calli, petioles, laminae). In contrast, co-transformed, kanamycin-selected calli/tumors typically yielded dark blue precipitates in less than 4 h. When the assay was adjusted to pH 3.0, 4.0, or 5.0, however, significant hydrolysis of X-gluc occurred within 1 to 4 h in non-transformed tissues. All of these explants were dark blue by 24 h, while at pH 6.0, 7.0, 8.0, or 9.0 only faint or no visible reactions occurred

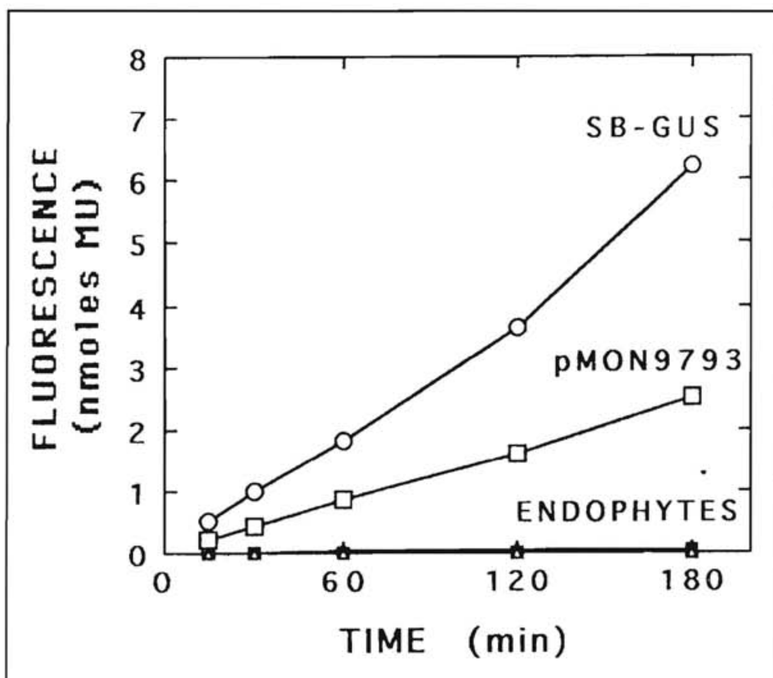

Figure 3. Comparison of glucuronidase activity in callus extracts (SB-GUS) and endophytic bacteria obtained from screening of sugarbeet cultivars. Lysis of *E. coli* MM294 carrying pMON9790 *(mas-gusA)* is shown as a positive bacterial control. Means of three experiments are presented. Assays were performed at 37°C, with 1mM MUG as the substrate.

Table 2. Immunoprecipitation of GUS IX or SB-GUS from solution (24°C, 3 h) with polyclonal antiserum (1:20) raised against GUS from *E. coli.* Activity bound to IgG was pelleted and measured fluorometrically with 1 mM MUG at 37°C for 3 h. Activities presented are means of three experiments with triplicate assay tubes per treatment.

Enzyme activity	Rabbit $\alpha-$ GUS IgG (μl)	Goat $\alpha-$ Rabbit IgG (μl)	Activity bound to IgG (% of total)
GUS IX	0	0	0
GUS IX	0	20	0
GUS IX	5	20	98
SB-GUS	0	0	0
SB-GUS	0	50	0
SB-GUS	5	50	0.2

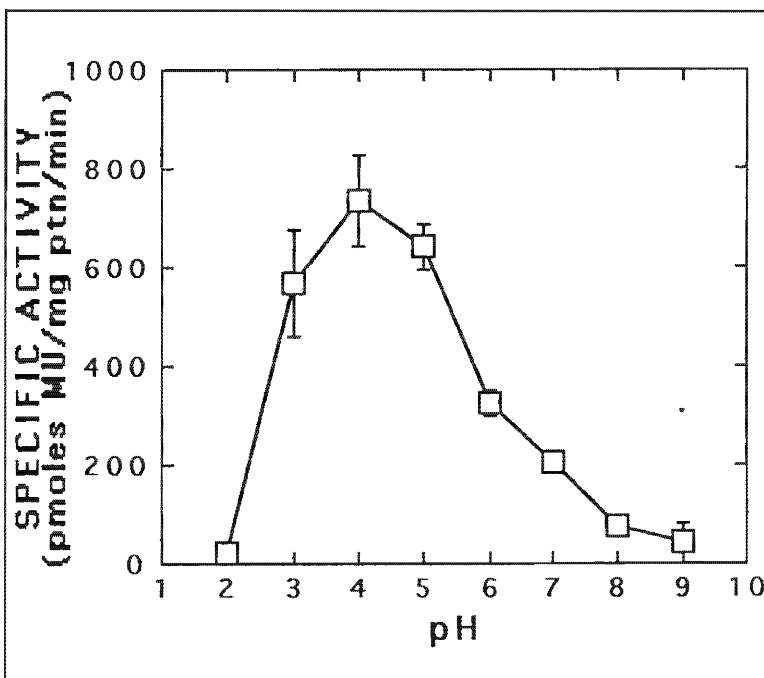

Figure 4. Effect of pH on SB-GUS activity in extracts from 'REL-1' callus as determined by MUG utilization. Data are means ± SE of three experiments with four replicates of each treatment. Assays were performed at 37°C, with 1mM MUG as the substrate.

(Table 3). Addition of 20% methanol reduced indigo precipitation (i.e., SB-GUS activity) at pH 3.0, 4.0, or 5.0, but blue cells were still visible following overnight incubations at 37°C (data not presented for all pH values). The addition of 10 mM S-L decreased the indigo precipitation to levels below that observed with 20% methanol.

DISCUSSION

The presence of a confounding glucuronidase activity in *B. vulgaris* tissues potentially complicates the use of *gusA* as a transgenic marker for this species. The biochemical characterization of SB-GUS reported here allowed development of a protocol for accurate estimation of introduced ß-glucuronidase activity in sugarbeet. Addition of 10 mM

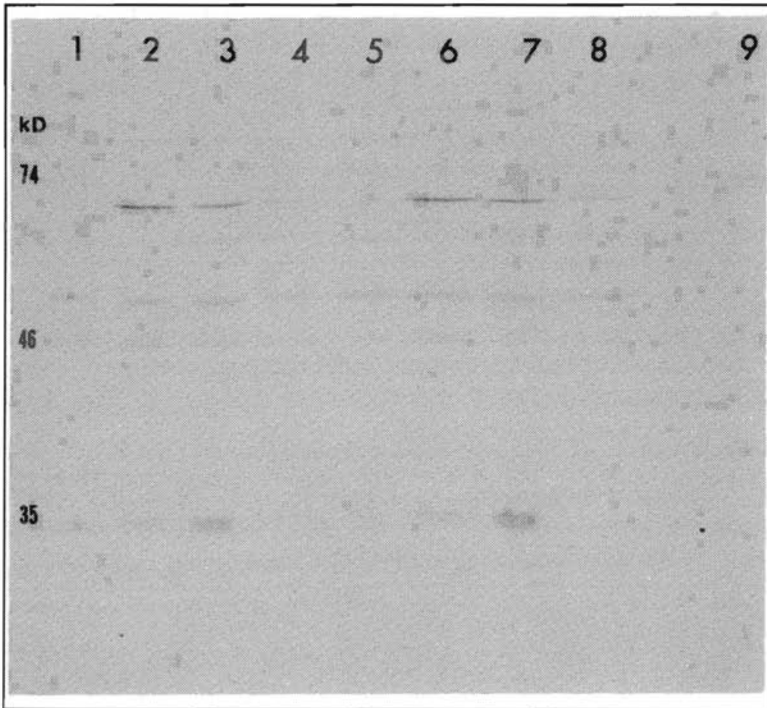

Figure 5. Immunoblot of total cellular protein of sugarbeet calli, petioles and leaves from axenic cultures of (lanes 1-3) 'REL-1', (lanes 4-6) 'REL-1-Hfr', and (lanes 7-8; leaf, petiole only) 'EL48' probed with rabbit anti-GUS antiserum (1:1000) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000). Color development was with 4-chloro-1-naphthol for 10 min. Lane 9 contains purified GUS IX. M_r markers are presented in kD.

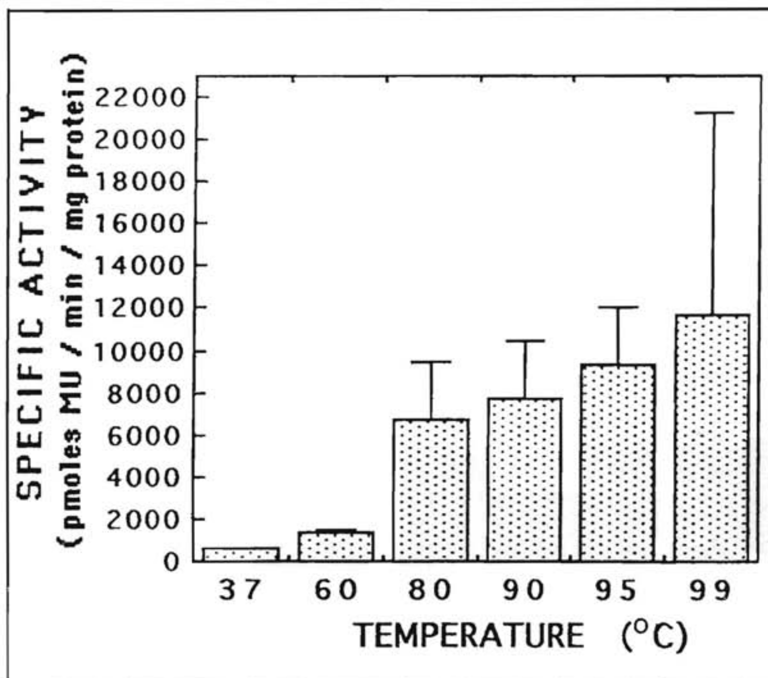

Figure 6. Incubation of callus extracts at 37, 60, 80, 90, 95, or 99°C in the presence of 1 mM MUG, pH 7.0. Means ± SE of three experiments with triplicate assay tubes per treatment are presented. All extracts were preheated 15 min at assay temperature before substrate addition.

Table 3. Effects of pH, methanol, and saccharic acid 1,4-lactone on histochemical staining† of sugarbeet leaf, petiole and callus tissues in the presence of X-gluc (1 mg/mL) at 37°C.

pH	% (v/v) methanol	mM S-L	4 h	24 h
7.0	0	0	-	-
6.0	0	0	-	+/-
5.0	0	0	+	+++
4.0	0	0	++	++++
3.0	0	0	+	+++
2.0	0	0	+/-	+
7.0	20	0	-	-
4.0	20	0	+	++
7.0	0	10	-	-
4.0	0	10	-	+/-

†Symbols: +/- indicates occasional faint blue; +, ++, +++, ++++ are increasing degrees of blue precipitate in tissue.

S-L to fluorometric assays eliminated ß-glucuronidase activity derived from *gusA* expression with no decrease in endogenous SB-GUS activity (at pH 7.0). Hence, the difference between the total ß-glucuronidase activity and that observed in the presence of S-L is the contribution of GUS from the introduced *gusA* gene.

With the use of X-gluc in histochemical localization of GUS activity, we have found the standard protocol (i.e., 37°C, pH 7) adequate, provided that the assays were less than 24 h in duration. Addition of 10 mM S-L or 20% methanol was not warranted under these conditions.

Part of the controversy associated with demonstrating the existence of glucuronidase in plants concerned the potential influence of microbial contaminants or endophytes in the organs sampled. We have demonstrated the absence of culturable heterotrophic, aerobic or microaerophilic microorganisms in materials used for measuring SB-GUS and have evaluated some commonly encountered endophytic organisms in sugarbeet tissue cultures. Although they were devoid of ß-glucuronidase activity, that condition cannot be assumed in untested situations.

Biochemical and immunological data from our study, and in some others involving higher plant species (Hu et al., 1990; Raineri et al., 1990; Alwen et al., 1992), have indicated a lack of similarity between the plant and microbial glucuronidases. The pH optimum, reaction to inhibitors of GUS, thermal stability, and failure to recognize any common epitopes, demonstrated a disparity between these two activities at the structural level. Our reported pH optimum and thermal inactivation point, however, should only be considered estimates for SB-GUS, as purified enzyme is required for accurate determination of these features.

The common recognition of substrates classifies both GUS and SB-GUS as glucuronohydrolases. We have recently found that two new histochemical substrates for GUS evaluation, Magenta X-gluc and Salmon X-gluc (Biosynth Inc., Skokie, IL, USA), are also recognized by SB-GUS in sugarbeet laminae, petioles and calli (data not shown). These substrates provide alternative colored precipitates to X-gluc that can be used where the coloration of tissues precludes adequate viewing of the indigo precipitate prior to clearing in alcohol.

The thermal stability of SB-GUS and the ability to interact positively with Cu^{2+} and Zn^{2+} make this enzyme worthy of further study. We are currently purifying this enzyme for use in antibody production, gene cloning, and characterization of the protein.

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