

Evidence for non-proteinaceous inhibitor(s) of β -glucuronidase in wheat (*Triticum aestivum* L.) leaf and root tissues

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Abstract

The GUS gene of *E. coli*, encoding β -glucuronidase, has been widely used as a reporter gene in plant transformation. However, β -glucuronidase activity in transgenic wheat leaf or root tissue is rarely observed or reported. To address this question, we investigated three wheat lines transformed with the GUS reporter gene. We found all three lines expressed GUS mRNA as well as β -glucuronidase protein in their leaf and root tissues as detected by RNA gel blot, ELISA, and immunoblot analyses. However, β -glucuronidase enzyme activity was only detected in pollen grains from the transgenic plants. Fluorometric and histochemical assays performed in the presence of wheat tissue extracts indicated that wheat leaf and root tissues contain inhibitor(s) of β -glucuronidase activity, but pollen grains contain much lower concentrations. Further characterizations indicated that the inhibitor(s) is of low molecular weight (< 10 kDa) and is non-proteinaceous.

Abbreviation: GUS – β -glucuronidase

Introduction

Enzyme activity of the GUS (or *uidA*, encoding β -glucuronidase) reporter gene in transgenic plants is detectable by a convenient histochemical assay and can be simply quantified by fluorometric assay (Jefferson et al., 1987). These advantages make GUS a widely used reporter gene in plant transformation and plant biology studies (reviewed by Zvereva and Romanov, 2000). Leaf is the most often used tissue for such an assay. When GUS gene is used in genetic transformation of most cereal crops, the β -glucuronidase activity is usually detected in leaves of 48–75% of the transgenic plants when GUS gene is linked to a selectable marker gene in the

same vector (Somers et al., 1992; Wan and Lemaux, 1994) and in 18–36% transgenic plants when the two gene constructs are in separate vectors (Gordon-Kamm et al., 1990; Li et al., 1993).

However, wheat seems to be an exception. When GUS gene is used in wheat transformation, the β -glucuronidase activity is rarely detected in leaf or root tissues of transgenic wheat plants. For example, among 20 recovered wheat plants in our lab transformed with GUS driven by strong, near-constitutive promoters, such as from maize *ubil* (Christensen and Quail, 1996) or rice *Act1* gene (McElroy et al., 1991), none showed any detectable β -glucuronidase activity in their leaf tissues. Several other laboratories reported very

little β -glucuronidase activity in leaves or roots of transgenic wheat plants (Vasil et al., 1993; Weeks et al., 1993; He et al., 1994). Still others (Becker et al., 1994; Nehra et al., 1994) reported that β -glucuronidase activity varied significantly among individual leaves from transgenic plants, while their progeny showed consistent enzyme activity in their floral parts. Folling and Olesen (2001) reported that 50% of recovered albino transgenic wheat plants showed β -glucuronidase activity in leaf histochemical assays, while none of the green transgenic plants had detectable activity. Sorokin et al. (2000) did not obtain visually detectable histochemical β -glucuronidase activity in T₀ and T₁ wheat plants in spite of RT-PCR evidence of GUS gene expression. These reports and our own observation on GUS activity in transgenic wheat plants prompted us to investigate the possible cause of this phenomenon. In this correspondence, we show that wheat leaf and root tissues contain strong, non-proteinaceous inhibitor(s) of β -glucuronidase enzyme activity.

Materials and methods

Plant expression vectors

A 2.65 kb *EcoRI/PstI* fragment containing the gene construct CaMV 35S promoter::*bar::OCS* terminator derived from pTAB₁₀ (De Block et al., 1987) was ligated into pBluescript II SK to obtain pAB2. A 4.4 kb *XbaI/PstI* fragment containing the rice *Act1* promoter::GUS::*NOS* terminator cassette from pAct1D (McElroy et al., 1991) was inserted into pAB2 to obtain pAB5 (Figure 1), which was used for transformation experiments.

Wheat regeneration and transformation

Immature embryos of greenhouse-grown wheat (*Triticum aestivum* L.) cv. Hi-Line (Lanning et al., 1992) and cv. Giza 163 (an Egyptian cultivar) were used in the experiments. Conditions for tissue culture, particle bombardment, and selection were essentially the same as previously reported (Sivamani et al., 2000) except that some bombarded calli were not selected until they had been transferred to regeneration medium. Regenerated plantlets, after selection in rooting medium (half-strength MS salts medium containing 2 mg l⁻¹ bialaphos) for

2–3 weeks, were transferred to greenhouse potting mix (Sunshine Mix #1; Fison's Inc., Canada). Greenhouse day/night temperatures were 25 ± 2°C/19°C under a 16-h photoperiod with supplemental lights to provide 150 μmol m⁻² s⁻¹ PPFD. Leaf painting assays (Weeks et al., 1993) were performed using a 0.1% aqueous solution of Glufosinate 200™ (20% ammonium glufosinate, a gift from former AgrEvo USA, Montvale, NJ, USA) supplemented with 0.1% Tween 20 (Fisher, Fair Lawn, NJ, USA).

DNA, RNA gel blot analyses and PAT assays

DNA gel blot analysis was performed as previously described (Sivamani et al., 2000). For RNA gel blot analysis, total RNA was extracted from leaf and root tissues using the RNeasy kit (Qiagen, Valencia, CA, USA), 10 μg RNA per lane was subjected to gel electrophoresis, and blotted onto GeneScreen nylon membrane (PerkinElmer, Boston, MA, USA). A full-length GUS coding fragment was used to make [α -³²P]dCTP labeled DNA probes to a specific activity >1 × 10⁹ dpm μg⁻¹. RNA gel blots were hybridized and processed according to manufacturer's instructions (Perkin-Elmer, Boston, MA, USA). Phosphinothricin acetyltransferase (PAT) assays were carried out according to Spencer et al. (1990).

GUS protein assays

GUS polyclonal antiserum was raised against the purified β -glucuronidase enzyme (Roche Molecular Biochemicals, Mannheim, Germany) in rabbits according to Lister et al. (1983). Total soluble proteins from leaves and roots of transgenic plants and non-transgenic controls were extracted in extraction buffer containing 0.125 M Tris-HCl (pH 6.8), 2.5% SDS, and 10% glycerol. ELISA assays were performed (Edwards and Gooper, 1985) on 30 μg of plant protein (in 100 μl) using the anti-GUS polyclonal antiserum (diluted to 1/5000) and compared to positive controls containing purified β -glucuronidase (1 μg). Three replicate protein samples were extracted from each plant and absorbance (405 nm) was measured after 5 and 30 min, respectively. The data were subjected to ANOVA and means separated using Duncan's New Multiple Range Test (Duncan, 1955). Immunoblot analyses were performed using 30 μg soluble protein per dot

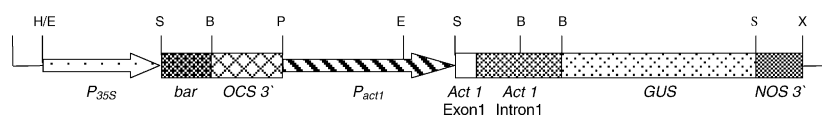


Figure 1. Plant expression cassettes of vector pAB5. DNA fragments used in the vector are labeled P (promoter) and 3' (transcription terminator). The abbreviations for the restriction sites are: B: *Bam*H1; E: *Eco*RI; H: *Hind*III; P: *Pst*I; S: *Sac*I; and X: *Xba*I.

with 1 ng purified β -glucuronidase as a positive control. Processing of dot blots was performed according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, nitrocellulose membranes were incubated at room temperature with diluted anti-GUS antiserum (diluted to 1/5000) for 30 min with gentle agitation, washed three times in TBST for 5 min each followed by incubation in goat anti-rabbit IgG alkaline phosphatase conjugate (diluted to 1/2500) for 30 min with gentle agitation and rewashing three times in TBST for 5 min each. β -glucuronidase protein was detected using 4-nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates.

β -glucuronidase enzyme assays

For *in vitro* experiments, MUG assays (Jefferson et al., 1987) were performed by mixing pure β -glucuronidase enzyme with tissue extracts of non-transgenic cv. Hi-Line and cv. Giza 163 plants. In both cases, 1 g tissue samples from leaves, roots, or pollen were ground in 0.5 ml phosphate buffer (50 mM KH_2PO_4 , pH 7.0) at 0 °C using a mortar and pestle. The samples were centrifuged at 12,000g for 15 min at 4 °C, and protein concentrations of the supernatants were adjusted to 1 mg ml⁻¹ for each sample. MUG assay conditions included 10 μ g of purified enzyme (0.2 U), 250 μ l 2 \times GUS buffer, various volumes of tissue supernatants, and phosphate buffer to a final volume of 500 μ l. After incubation at 37 °C for 4 h, the reactions were stopped by addition of 2 ml of 0.2 M Na_2CO_3 . Samples were illuminated at 360 nm and fluorescence at 455 nm was measured. Tobacco (cv. Samsun) leaf extracts were prepared in the same way, and control treatments contained phosphate buffer only. Three replicates were performed for each sample and treatment means are presented in Table 1.

To examine the *in vivo* effect of potential inhibitors from wheat tissues on β -glucuronidase activity, immature embryos of cv. Giza 163 were bombarded with pAB5 and transient enzyme

activity was assayed 24 h after treatment. GUS histochemical assays were performed as described (Jefferson et al., 1987). Twenty-five bombarded embryos were incubated at 37 °C overnight in either 500 μ l GUS assay buffer (Jefferson et al., 1987), or in the same buffer supplemented with 70 μ l (1 μ g μ l⁻¹ total soluble protein) extract from wheat (cv. Giza 163) leaves or roots, or tobacco (cv. Samsun) leaves.

To estimate the molecular mass of putative inhibitor molecules, supernatant subsamples (2 ml) were fractionated using an ultrafiltration spin column (10 kDa cut-off) (Amicon Ultra-4 Centrifugal Filter Units, Millipore, Billerica, MA, USA). The flow-through (MW < 10 kDa) and supernatant (MW > 10 kDa; brought up to 2 ml total volume) fractions were collected and incubated with GUS enzyme as described above.

To determine whether the inhibitor is proteinaceous in nature, the supernatants of tissue extracts (500 μ l) were treated with 25 μ l proteinase K (10 mg ml⁻¹) for 15 min at 37 °C. The reactions were heated at 70 °C for 10 min, extracted with phenol, and the aqueous phase was incubated with GUS enzyme as described above.

Results

Three transgenic wheat plant lines transformed with pAB5, namely 290 and 306 (from cv. Hi-Line) and 314 (from cv. Giza 163) were used for experimentation. The transgenic nature of the lines was confirmed by leaf painting, PAT assays, and genomic DNA gel blot analysis (data not shown). RNA gel blot, ELISA, and immunoblot analyses were positive for GUS gene expression for all three lines (Figure 2), whereas no GUS enzyme activity was detected in leaf and root tissues in histochemical assays. However, when mature pollen grains were tested with histochemical assays, a ratio of 1 GUS⁺ : 1 GUS⁻ ($\chi^2 = 2.52$, Figure 3) was observed in each transgenic plant, indicating that the GUS transgene(s) was present at a

single locus in each plant genome. Further, the transgenes were expressed, as demonstrated by β -glucuronidase activity, in pollen grains.

These results indicated that the GUS reporter gene was expressed in transgenic wheat plants. However, there appeared to be no β -glucuronidase activity in leaf and root tissues, prompting us to hypothesize that there may be inhibitor(s) in these tissues. To test this hypothesis, we added various amounts of wheat leaf, root or pollen grain tissue extract supernatants to a MUG fluorometric assay of the purified GUS enzyme. The results were compared to reactions containing tobacco leaf extract and a negative control with no tissue extract added. The results clearly supported the hypothesis: 10 μ l of leaf or root

extract reduced β -glucuronidase activity by about 20%, while 100 μ l of extract inhibited β -glucuronidase activity by 90–100% (Table 1, Panel A). Pollen extract had a much less inhibitory effect, with only about a 30% decrease in enzyme activity from 100 μ l of extract. Tissue extracts from both wheat cultivars performed similarly. In contrast, tobacco leaf extract had virtually no inhibitory effect on enzyme activity (Table 1, Panel A and C). Together, these data support the idea that wheat leaf and root tissues contain inhibitor(s) of β -glucuronidase activity.

Two additional experiments helped characterize the molecular mass and biochemical nature of the inhibitor(s). Fractionation experiments using the MUG assay indicated that the molecular

Table 1. Fluorometric assays of β -glucuronidase activity (pmol MU/min⁻¹/mg⁻¹ GUS protein) with addition of tissue extract. The control (no addition of tissue extract) reading is 368.5

Species/Variety	Extract volume (μ l) added ^a	Leaf	Root	Pollen
(A) Reactions with addition of tissue extracts				
Wheat/‘HiLine’	10	301.2a**	282.2a	325.3a
	50	157.4b	103.8b	309.0a
	100	20.6c	-15.1c	278.6b
Wheat/‘Giza 163’	10	273.6a	256.0a	301.4a
	50	99.5b	91.3b	270.8b
	100	7.4c	18.7c	261.1b
Tobacco	10	361.4a		
	100	354.6a		
(B) Reactions with addition of size fractions of wheat leaf extract				
Variety	Extract volume (μ l) added		< 10 kDa	> 10 kDa
‘HiLine’	10		216.5a	344.7a
	100		40.1b	341.8a
‘Giza 163’	10		205.3a	297.6a
	100		31.1b	303.2a
(C) Reactions with addition of proteinase K-treated tissue extract				
Wheat/‘HiLine’	10	312.9a	290.8a	347.6a
	50	161.5b	124.4b	322.8a
	100	31.7c	3.5c	298.0b
Wheat/‘Giza 163’	10	260.8a	270.1a	312.2a
	50	105.3b	119.9b	299.4a
	100	-12.2c	31.4c	260.0b
Tobacco	10	361.4a		
	100	354.6a		

^a The tissue extract supernatant was adjusted to 1 μ g μ l⁻¹ soluble protein.

** Values followed by the same letter in each column within a variety are not significantly different at $p = 0.05$ by Duncan’s Multiple Range test (Duncan, 1955).

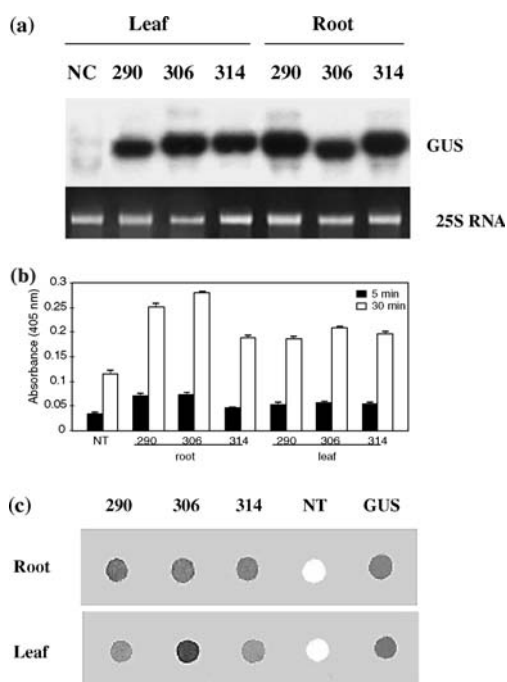


Figure 2. GUS transgene expression assays. (a) RNA gel blot analysis of GUS mRNA expression in three transgenic wheat plants. The 25S rRNA band of each sample, as stained by ethidium bromide, was also included as a reference in the figure. NC: negative control. (b) ELISA detection of GUS protein levels in roots and leaves of three transgenic plants (290, 306 and 314) and a non-transgenic control (NT) using polyclonal GUS antibodies. Bars indicate standard errors of the means ($n = 3$). (c) Immunoblot detection of GUS protein expression in roots and leaves (30 μ g protein per sample) of transgenic lines (290, 306 and 314, respectively), a non-transgenic control (NT) and a positive control (GUS, 1 ng of purified β -glucuronidase).

mass was < 10 kDa (Table 1, Panel B). Second, extracts treated with proteinase K remained inhibitory in the MUG assay, indicating that the inhibitor(s) are non-proteinaceous (Table 1, panel C).

Since fluorometric assays were used, the presence of quenching molecules in wheat leaf and root extracts cannot be excluded. To address this question, we bombarded wheat immature embryos with pAB5 and performed GUS histochemical assays in the presence of extracts from wheat leaves and roots, tobacco leaves, or with no added extracts. β -glucuronidase activity was very strong in control assays without added extracts, suggesting that immature embryos do not contain inhibitor(s). Although β -glucuronidase activity was not affected by tobacco leaf extract as seen above, activity was strongly inhibited in the pres-

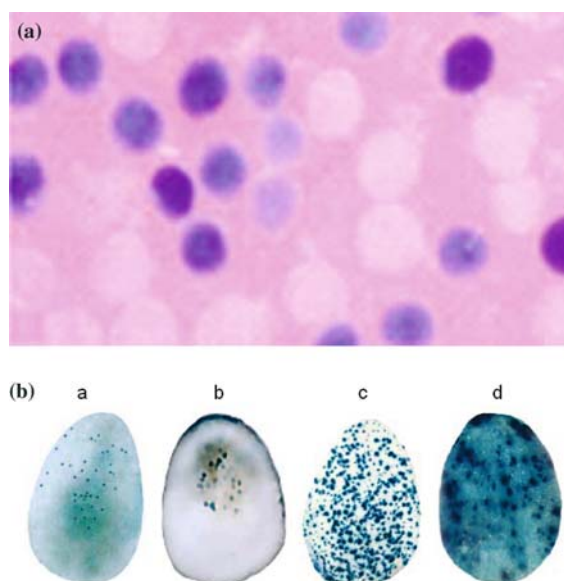


Figure 3. Histochemical assays of β -glucuronidase enzyme activity. (a) β -glucuronidase activity in pollen grains of transgenic wheat line 290. (b) Transient, β -glucuronidase activity in wheat immature embryos bombarded with pAB5 in the presence of (left to right) wheat leaf extract, wheat root extract, tobacco leaf extract, and blank control.

ence of wheat leaf or root extract (Figure 3B). These data do not support the quenching hypothesis for the MUG assay results, but instead further indicate the presence of β -glucuronidase activity inhibitor(s). Thus, we conclude that the presence of non-proteinaceous inhibitor(s) in vegetative tissues of transgenic wheat is a major reason for the frequent negative detection of β -glucuronidase activity.

Discussion

GUS is well known as a very useful and convenient reporter gene in plant biology studies. It has been successfully used in many species for developing and optimizing transformation protocols, and for both transient and stable transgene expression studies. However, in wheat transformation, the literature is replete with examples of failure to detect β -glucuronidase activity in transgenic plants, for unknown reasons. In this report, we observed β -glucuronidase activity in pollen grains but not in leaves or roots of the three transgenic wheat plants we examined. Since GUS mRNA and protein were detected by RNA gel blot, protein

immunoblot, and ELISA assays, GUS transgene silencing in leaf and root tissues can be excluded. We provide evidence from various assays using tissue extracts from two wheat cultivars to demonstrate that wheat leaf and root tissues contain low molecular weight, non-proteinaceous molecules that inhibit β -glucuronidase activity. The inhibitor(s) were also present in pollen grains, but at much lower levels (roughly 10%) than found in leaf and root tissues, which could explain why β -glucuronidase activity was detected there. No such inhibitor(s) were detected in tobacco leaves.

Occasional reports of β -glucuronidase activity in transgenic wheat leaves and roots could be explained by a combination of high GUS gene expression and low levels of inhibitor(s) in certain individual wheat plants and/or cultivars. Two inhibitors of *E. coli* β -glucuronidase enzyme have been reported in the literature: saccharic acid-1,4-lactone and glucuronic acid (Hodal et al., 1992; Muhitch, 1998). It remains to be elucidated whether the inhibitor(s) described here are one of these two or different compound(s).

Our results suggest the presence of endogenous inhibitor(s) of β -glucuronidase activity in wheat leaf and root tissues, with much lower levels in pollen grains. This scenario could explain the unusually low β -glucuronidase activities observed in a number of studies for vegetative tissues of transgenic wheat. We also suggest that the presence of such inhibitor(s) in other plant tissues or species should be considered as a possibly confounding factor in explaining β -glucuronidase enzyme activity data.

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