Factor affecting the endogenous β-glucuronidase activity in rapeseed haploid cells: How to avoid interference with the Gus transgene in transformation studies

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1. Introduction

The β-glucuronidase (GUS) gene originating from the enterobacterial Escherichia coli (Jefferson et al., 1986) is the most widely used reporter gene in plant transformation and gene expression studies (Gallagher, 1992). However, one of the problems associated with the use of the bacterial gus gene as a reporter is the presence of endogenous GUS’ activity in some species. Endogenous GUS activity has been reported in various organisms including: 1 — bacterial species, such as the enterobacterium E. coli and Shigella, nonenterobacterial such as Bacteroides and Clostridium (Hawkesworth et al., 1971), Streptococcus, Staphylococcus, Corynebacterium (Levy and Marsh, 1959), Alcaligenes (Jefferson, 1988) and many soil bacteria (Ritz et al., 1994); 2 — Some tissues of vertebrates such as kidney, liver and spleen (Kyle et al., 1992), human breast milk (Alonso et al., 1991; Ince et al., 1995); 3 — Invertebrates such as snails (Levy and Marsh, 1959), nematodes (Jefferson, 1985; Sebastiani et al., 1986) and insects (Langley et al., 1983; Levy and Marsh, 1959), housefly (Musca domestica) and various locusts (Levy and Marsh, 1959) and 4 — Plant species. In plants, endogenous GUS activity was first described in Arabidopsis by Jefferson et al. (1987). Hu et al. (1990) detected GUS activity in 52 plant species, including gymnosperms and all the key groups of angiosperms. This activity was predominantly found in fruit walls, seed coats and endosperms or embryos. Vegetative organs of tobacco (Nicotiana tabacum L.), potato (Solanum tuberosum L.), wheat (Triticum aestivum L.) and radish (Raphanus sativus L.) also exhibit GUS activity (Alwen et al., 1992). For various plant species, endogenous Gus or Gus-like activities have been reported in the male gametophyte, fruits and seeds (Alwen et al., 1992; Hänsch et al., 1995; Hodal et al., 1992; Hu et al., 1990; Wilkinson et al., 1994; Wozniak and Owens, 1994). Also, endogenous GUS activity has been found in pollen and in the tapetal and sporogenous cells of anthers from potato, tobacco, tomato (Lycopersicon esculentum L.) (Plegt and Bino, 1989) and haploid tissues (microspore and MDEs) of rapeseed (Brassica napus L.) (Abdollahi et al., 2011).
or absence of 28% methanol in the reaction buffer (pH = 5.8 ± 28% methanol, pH = 7 ± 28% methanol, pH = 7 ± 28% methanol, pH = 8 ± 28%). About 20 transformed MDEs (cv. Global and PF704), at globular stage were assayed in a 1.5 ml microfuge tube containing 500 μl of X-gluc solution. Each treatment replicated thrice. Explants were dark incubated at 38 °C for 24 h.

2.3.2. Experiment II

Different stages of rapeseed (cv. PF704) MDEs including heart, torpedo and cotyledonary embryos were incubated at various incubation temperatures (25 °C, 38 °C and 55 °C) in the dark. About 20 MDEs of heart, torpedo and cotyledonary stages were assayed in a 1.5 ml microfuge tube containing 500 μl of X-gluc solution as one replication. These 1.5 ml microfuge tubes were filled up to half with globular embryos (due to their small size). All explants were assayed in the reaction buffer with pH = 7 and without methanol.

2.4. Preparation of microspores and microspore transformation procedure

In microspore transformation experiments, microspores were isolated, centrifuged, and re-suspended in NLN-13 medium according to Abdollahi et al. (2009b). For bombardment, 100 μl culture droplets (with an optimal density of 10⁶ microspores) was deposited onto polyester filters (Millipore) over solid NLN (Lichter, 1982) medium (solidified with 0.6% agarose) containing 6.5% Sucrose and 0.2 M mannitol as an osmotic agent. Bombardment was performed with 1 μM DNA-coated gold particles, helium pressure of 1100 psi; stopping screen to target tissue distance (9 cm) and chamber vacuum pressure (28 inHg) using a Bio-Rad PDS-1000/He (Hercules, USA) biolistic particle delivery system. A pBGWFS7-64 binary vector (kindly provided by Dr. Kim Boutilier, Plant Research International, Wageningen, The Netherlands) carrying the gus reporter gene under the control of the pollen-specific p28640 promoter (AT2G28640, Exo70 gene family), was used for bombardment of microspores. For each treatment in GUS assay experiments, three Petri dishes containing the microspore samples were bombarded. Control samples were not bombarded. After bombardment, microspores were re-suspended and cultured in liquid NLN medium (with the same sugar and mannitol concentration used for bombardment) for 24 h at 32 °C and darkness. After incubation, microspores were collected again on polyester filters for GUS expression.

2.5. Histochemical GUS assay in rapeseed bombarded microspores

GUS expression analysis was used for rapeseed microspores with a modified procedure from Jefferson et al. (1987). The substrate buffer was contained 200 mM NaPO₄ buffer (pH 7.0), 1 mM K₃[Fe(CN)]₆, 1 mM K₄[Fe(CN)]₆, 20 mM EDTA, 13% sucrose and 0.1% (w/v) X-gluc. For each filter, the filters retaining the microspores were put on the 10×35 mm Petri dishes contained 3 ml of solid NLN-13 medium followed by addition of 0.5 ml substrate buffer. The samples were incubated at 38 °C in the dark for different periods including 4, 24 and 48 h. Finally, samples were placed at 4 °C until counting and analysis. In order to eliminate the endogenous GUS activity from rapeseed (cv. Global and PF704) microspores, two separate experiments were conducted. Both experiments were repeated thrice.

2.5.1. Experiment I

Microspore samples of rapeseed (cv. PF704) were dark incubated at 38 °C for 4, 24 and 48 h in the GUS assay solution with or without 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide.

2.5.2. Experiment II

Microspore samples of rapeseed (cvs. Global and PF704), were dark incubated at 38 °C and 55 °C for 4, 24 and 48 h in presence of 1 mM
potassium ferricyanide and 1 mM potassium ferrocyanide. In this experiment, pH of the reaction buffer was adjusted on pH = 7.

2.6. Induction of endogenous GUS activity by gibberellic acid

Rapeseed (cv. Global) MDEs at cotyledonal stage (35-day-old embryos) were treated with 2 μM GA3 (Sudan et al., 2006) for 12 h at 25±2 °C under continuous light. These embryos were analyzed both quantitatively and qualitatively for GUS activity. The pH of Assay buffer in this experiment adjusted on pH = 5.

2.7. Quantitative GUS assay

Enzyme extraction and determination of protein content in rapeseed MDEs were carried out by the method of Sudan et al. (2006). Rapeseed MDEs at different stages (globular shape, heart shape, torpedo shape and cotyledonal) were ground with extraction buffer 0.1 M citrate phosphate buffer, 1 mM EDTA, 5.6 mM β-mercaptoethanol, adjusted to pH 5.0 at the ratio 1:5 of fresh weight (g) to buffer volume (ml). The homogenate was centrifuged at 600 g for 5 min at 4 °C. The supernatant was re-centrifuged at 20,000 g for 20 min. All extraction procedures were performed on ice. An aliquot of this final supernatant containing a fixed amount of total protein in the range of 5–50 μg dissolved in 100 μl extraction buffer was added to 100 μl of 2 mM 4-methyl-umbelliferyl-beta-D-glucuronide (MUG) made in the extraction buffer. The mixture was vortexed briefly and incubated at 37 °C for 60 min. The reaction was stopped by the addition of 900 μl 0.2 M Na2CO3 and fluorescence was measured at 455 nm using a Shimadzu RF-540 spectrofluorometer set at an excitation wavelength of 365 nm. In this experiment, protein concentration was determined by using the Bradford’s dye binding assay (Bradford, 1976).

2.8. Statistical analysis

Each experiment was performed thrice with three replicates. Data shown are from a single representative experiment. Data presented were subjected to F test to detect differences between treatments and Duncan multiple comparison test was used to separate treatment means. SPSS statistical package (version 16, SPSS Inc., an IBM Company, USA) was used to analyze the data of this study.

3. Results

3.1. Elimination of blue background from bombarded MDEs

The endogenous GUS activity in rapeseed MDEs was defined by visual observation of the blue color provided by the staining protocol. A screening for endogenous GUS activity in rapeseed bombarded MDEs was performed in the phosphate buffer adjusted to pH 5.8, 7 and 8. At pHs 5.8 and 7 with 28% methanol in the assay buffer, a less intense endogenous GUS activity (Figs. 1A and C) was observed in some of the rapeseed MDEs (about 40% and 20% of embryos, respectively). At pHs 7 and 8 in the absence of 28% methanol in the reaction buffer, rapeseed MDEs presented the endogenous GUS activity (Table 1; Figs. 1B and D and 2B and D). A substantial decrease or even a total absence of GUS activity was observed in phosphate buffer pH 8 and 28% methanol in almost all MDEs analyzed (Table 1; Fig. 1E). In the last treatment (pH = 8 + 28% methanol), the GUS activity originating from the introduced gus gene was well detectable (Figs. 2A and C).

Table 1

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>pH</th>
<th>pH=5.8 + 28% methanol</th>
<th>pH=7</th>
<th>pH=7 + 28% methanol</th>
<th>pH=8</th>
<th>pH=8 + 28% methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global</td>
<td>++</td>
<td>+++++++</td>
<td>+</td>
<td>++++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>PF704</td>
<td>++</td>
<td>+++++++</td>
<td>+</td>
<td>+++++</td>
<td>+++++</td>
<td>--</td>
</tr>
</tbody>
</table>

+++ +++: High activity; +: weak activity; --: no activity.

Different stages of rapeseed (cv. PF704) MDEs, revealed various levels of endogenous GUS activity when incubated at different temperatures. The heart shape, torpedo shape and cotyledonal embryos exhibited a
high level of endogenous GUS activity when incubated at 25 °C and 35 °C while the globular embryos showed a week activity of endogenous GUS at 25 °C and 35 °C (Table 2). Rapeseed MDEs at all stages exhibited no endogenous GUS activity at 55 °C (Table 2). In this experiment, the endogenous GUS activity was detected in phosphate buffer pH 7 without methanol in the reaction buffer.

3.2. Elimination of endogenous GUS from rapeseed bombarded microspores

3.2.1. Experiment I

In this experiment, use of 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide in the reaction buffer enhanced the expression rate of the gus transgene rather than endogenous GUS activity in rapeseed bombarded microspores. Bombarded microspores of rapeseed (cv. PF704), exhibited a high level of transgene expression in the presence of 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide, 4 h after histochemical GUS staining (Table 3). Furthermore, results showed a delay in the expression of endogenous GUS activity when 1 mM potassium ferricyanide and 1 mM potassium ferrocyanide were used in the reaction buffer. This result is supported by the medium level expression (++) of the endogenous GUS-like activity in microspores assayed in a reaction buffer with 1 mM potassium ferricyanide and potassium ferrocyanide and by the high level expression (++++) of this activity in the absence of potassium ferricyanide and potassium ferrocyanide, 24 h from GUS staining (Table 3).

3.2.2. Experiment II

Incubation of rapeseed (cvs Global and PF704) microspores for 24 h and 48 h under 38 °C did not decrease the endogenous GUS activity (Figs. 3A and C), while the dark incubation of microspores at 55 °C completely eliminated the endogenous GUS activity after 4, 24 and 48 h from GUS assay experiment (Table 4; Figs. 3B and D). Microspores with endogenous GUS activity show a light staining (Figs. 3A and C), while microspores with the gus transgene present a dark staining (Figs. 3C and D) in histochemical GUS analysis.

3.3. Induction of endogenous GUS activity by gibberellic acid

The hormones GA3 had a dramatic effect on GUS activity in rapeseed MDEs. Gibberellic acid (2 μM) enhanced the activity of GUS enzyme by 1.72 folds in 35-day-old embryos treated for 12 h (Fig. 4).

3.4. Developmental regulation of endogenous GUS expression in rapeseed MDEs

The developmental stage of the rapeseed MDEs was found to be critical for the detection of GUS activity. Globular embryos of B. napus, showed lighter stain for GUS activity (Fig. 5A), while the level of endogenous GUS activity was increased from heart stage toward torpedo and cotyledonary stages. The last developmental stages of rapeseed MDEs showed darker staining (Figs. 5B–E). Quantitative measurement of GUS activity in different stages of rapeseed MDEs revealed enhancement in endogenous GUS activity from heart shape embryo (Fig. 6). The level of endogenous GUS activity was increased 4.33 folds in heart embryos, 6.54 folds in torpedo embryos and 8.5 folds in cotyledonary embryos.

4. Discussion

A conspicuous endogenous β-glucuronidase activity was observed in histochemical assays of transformed rapeseed microspores and

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**Table 2**

<table>
<thead>
<tr>
<th>Explant</th>
<th>Endogenous GUS activity in different incubation temperatures</th>
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<tbody>
<tr>
<td></td>
<td>25 °C</td>
</tr>
<tr>
<td>Globular embryo</td>
<td>+</td>
</tr>
<tr>
<td>Heart embryo</td>
<td>++++</td>
</tr>
<tr>
<td>Torpedo embryo</td>
<td>++++</td>
</tr>
<tr>
<td>Cotyledonary embryo</td>
<td>++++</td>
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</tbody>
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+++ +: High activity; +: weak activity; −: no activity.

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**Table 3**

<table>
<thead>
<tr>
<th>Potassium ferricyanide and potassium ferrocyanide</th>
<th>Presence</th>
<th>Absence</th>
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<tbody>
<tr>
<td>Incubation periods</td>
<td>4 h  24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Endogenous GUS expressiona</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>Transgene GUS expressionb</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

| a | +++ +: High activity (100% of microspores); −: no activity; ++: medium activity (40% of microspores). |
| b | +: Expression; −: no expression. |
microspore-derived embryos, confounding the use of *Escherichia coli* gus as a reporter gene. The staining patterns of transformed and untransformed MDEs (cvs Global and PF704), assayed in the phosphate buffer with pH = 5.8, 7 and 8 with and without methanol in the reaction buffer were compared in the first part of this study. The endogenous GUS activity was greatly reduced when the pH of the buffer solution was maintained at 8 and 28% methanol (v/v) was used in the reaction buffer. These results are consistent with findings of previous researchers (Martin et al., 1992; Solís-Ramos et al., 2010; Sreenath and Naveen, 2004). It has also been reported previously that endogenous GUS activity in rye, potato slices, apple seeds and almonds is optimal between pH 4 and 5 and in rhubarb at pH 6 (Hodal et al., 1992). In contrast, *E. coli* GUS has an optimum activity at pH of 7.0 (Jefferson, 1987). The pH of the assay buffer is very critical for detection of the GUS activity in plants (Sudan et al., 2006). The researchers (Eudes et al., 2008) reported in situ localization of AtGUS2, an enzyme displaying GUS activity under acidic conditions in Arabidopsis. They characterized a knockout insertion line (*atgus2-1*) and also some transgenic lines over-expressing *AtGUS2* (*Pro35S: AtGUS2*). Endogenous GUS activity was absent in *atgus2-1* tissues and four times higher in *Pro35S: AtGUS2* lines. These studies also show the genetic control of endogenous GUS activity.

In this study, we also effectively used 28% methanol in the GUS reaction mixture to eliminate the endogenous GUS activity. Similar to previous studies (Kosugi et al., 1990; Sreenath and Naveen, 2004), methanol enhanced the activity originating from the introduced gus gene, lowering the endogenous GUS activity in all MDEs tested. This phenomenon may be explained by the effect of methanol on cell membranes and has been demonstrated that methanol does not specifically inhibit endogenous GUS activity (Wilkinson et al., 1994). It has been suggested that likely inhibitors of GUS activity are possibly belonging to the large class of phenolic compounds present in plant cells. If this holds true, phenolic compounds are most likely sequestered in vacuoles and cell walls, and disruption of cell membranes during the histochemical assay reaction is likely to cause

![Fig. 3](image)

**Fig. 3.** Elimination of endogenous GUS activity from bombarded and un-bombarded microspores of rapeseed. Endogenous GUS activity in rapeseed un-bombarded microspores, incubated at 38 °C and darkness for 48 h (A); Elimination of endogenous GUS activity from rapeseed un-bombarded microspores incubated at 55 °C and darkness for 48 h (B); Endogenous GUS activity has been interfered with the activity originating from the introduced gus gene in rapeseed bombarded microspores incubated at 38 °C and darkness for 48 h, white arrows show endogenous GUS activity while black arrows present the GUS activity from the introduced gus gene (C); A blue microspore expressing the gus transgene, after incubation at 55 °C and darkness for 48 h, (D); Bar 40 μm.

### Table 4

Histochemical assay of endogenous GUS activity in rapeseed microspores at different incubation temperatures and different incubation periods after GUS assay. Small crosses indicate relative intensity of staining.

<table>
<thead>
<tr>
<th>Incubation temperatures</th>
<th>Endogenous GUS activity in different incubation temperatures</th>
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<tbody>
<tr>
<td></td>
<td>38 °C</td>
</tr>
<tr>
<td>Incubation periods</td>
<td>4 h</td>
</tr>
<tr>
<td>Global</td>
<td>−</td>
</tr>
<tr>
<td>PF704</td>
<td>−</td>
</tr>
</tbody>
</table>

+++ +++: High activity (100% of microspores); +++: medium activity (60% of microspores); −: no activity.

![Fig. 4](image)

**Fig. 4.** Effect of GA3 on endogenous GUS activity in 35-day-old MDEs of rapeseed. Bars with the same letter are not statistically significantly different from each other (P<0.05).
mixing of the inhibitors with the cytoplasmic GUS and suppressing the endogenous GUS activity (Fior and Gerola, 2009). These findings support the likely effect of methanol on cell membrane disruption.

In another part of this study, incubation of rapeseed microspores and microspore-derived embryos at 55 °C rather than other incubation temperatures effectively eliminated the endogenous GUS activity. These findings are supported by other researchers (Hänsch et al., 1995). They reported that pretreatment of the barley bombarded scutella for 60 min followed by staining at the same temperature in a modified buffer can completely suppress endogenous GUS activity. In tobacco, the endogenous enzyme activity is quickly lost at 45 °C (Alwen et al., 1992), but in rye endogenous GUS showed an optimum activity at 55 °C (Fior and Gerola, 2009). High incubation temperature has been shown by others researchers to be useful in removing endogenous GUS activity (Hodal et al., 1992; Muhitch, 1998; Schulz and Weissenböck, 1987; Vitha et al., 1995).

In order to increase the expression rate of the gus transgene in rapeseed bombarded microspores and also delay in endogenous GUS activity, histochemical GUS assay was carried out in presence of potassium ferricyanide and potassium ferrocyanide. Use of 1 mM concentration of these products. Furthermore, potassium ferricyanide and potassium ferrocyanide in the incubation medium accelerates the formation of the blue reaction product (indigo). It also protects the formed indigo from further oxidation, which would convert it to colorless or yellowish products. Furthermore, potassium ferricyanide and potassium ferrocyanide provide more precise localization. Optimal concentration of the potassium ferricyanide and potassium ferrocyanide should be tested for a given sample to find the best compromise between the intensity of staining and the precision of localization. Generally, concentrations between 0.5 and 5 mM each are used for most samples, 1 mM being a good starting point (Vitha, 1995; Vitha et al., 1995).

The developmental stage of the tissue/organ is another critical factor for the detection of endogenous GUS in plants. In this study, quantitation of GUS activity in 35-day-old rapeseed MDEs showed more GUS activity when treated with GA3 (Fig. 4). The plant hormones BAP and GA3 also enhanced GUS activity in specific tissues of Z. mays, N. tabacum and O. sativa. (Sudan et al., 2006). In these studies, the intensity of GUS staining increased in the region of intercalary meristem and the elongating zone of rice inter-nodes when treated with GA3 (Sudan et al., 2006). This is the region where GA3 is known to bring about growth. Thus higher level of GUS activity seems to be associated with growing regions in rice (Cho and Kende, 1998). The present study also examined endogenous GUS expression levels in MDEs through the globular, heart, torpedo and cotyledonary stages. The level of endogenous GUS activity was low in globular embryos (Figs. 5A and 6) while this activity was increased markedly at heart embryos (Figs. 5B and 6) and enhanced up to 8.5 folds in cotyledonary embryos (Figs. 5E and 6). Mandel (1992) has examined changes in GA concentration in MDEs at several earlier stages, beginning at day 3 after culturing and continuing to globular, heart, torpedo and cotyledonary stages. For two growth-active GAs, GA1 and GA3, concentrations in MDEs remained low until the heart and heart/torpedo stages, when 3- to 8-fold increases occurred (Mandel, 1992). These results indicate that GA is essential to axis elongation and the increasing of endogenous GUS activity from heart embryo to cotyledonary stages seems to be associated with endogenous GAs concentrations and growth in rapeseed MDEs.

5. Conclusions

Finally, this study enabled us to eliminate the endogenous GUS activity from haploid tissues of rapeseed and determine the introduced gus gene expression more reliably and precisely. Furthermore, we suggest that the procedures here reported for elimination of endogenous GUS activity could be applied for other plant species in transformation studies, especially via particle bombardment method.

References
