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Full Length Research Paper

Beta glucuronidase activity in early stages of rice seedlings and callus: A comparison with *Escherichia coli* beta glucuronidase expressed in the transgenic rice

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We have chosen rice as a model crop to ascertain endogenous β-glucuronidase (family 79) activity and to differentiate the same from *Escherichia coli* based β-glucuronidase (family-2). The investigation dwells on characterizing endogenous β-glucuronidase (GUS) activity during the early stages of seed germination and from rice callus. Also, similar studies were made from homozygous transgenic rice line expressing *E. coli* GUS under the control of *glyoxalase I* promoter. Endogenous GUS activity was detected in plumules, shoots and calli of rice, nevertheless, showing differential response to pH. Further, endogenous GUS in rice was specifically inhibited by saccharic acid 1,4-lactone (SL) but, the *E. coli* β-glucuronidase remain unaffected, indicating distinct biochemical properties of family-2 and family-79 β-glucuronidase.

Key words: Family 2, family 79, X-gluc, saccharic acid, 1,4-lactone.

INTRODUCTION

Glycosyl hydrolases hydrolyze the glycosidic bond in carbohydrates or between a carbohydrate and a non carbohydrate moiety. β-Glucuronidase (GUS) is classified into three glycosyl hydrolase families 1, 2 and 79. Found in the GH1 family, klotho is a type I membrane protein from mammals that hydrolyzes steroid beta-glucuronides (Tohyama et al., 2004). The family-2 β-glucuronidase is reported in a wide range of organisms. The bacterial gene uidA, encoding the family 2 β-glucuronidase, has been used extensively as a reporter gene in genetic transformation experiments (Jefferson et al., 1987; 1989). Another category of β-glucuronidase belongs to the family-79 of glycosyl hydrolases in the carbohydrate-active enzymes (CAZY) database, which also includes mammalian heparanases that degrade the carbohydrate moieties of cell surface proteoglycans, flavones specific beta glucuronidase (sGUS) from *Scutellaria baicaleinsis* (Sasaki et al., 2004) and an *Arabidopsis thaliana* beta-D-glucuronidase (AtGUS) was recently shown to hydrolyse glucuronic acids from carbohydrate chains of arabino-galactan protein (Eudes et al., 2008; Konishi et al., 2008).

Biochemical studies of family 79 GUS in plants are limited. Schulz and Weissenbock (1987) reported partial purification and characterization of a specific GUS from rye primary leaves and further, Hu et al. (1990) surveyed 52 plant species for intrinsic GUS like activity using either the quantitative flourimetric GUS assay where the tissues were assayed for 24 h or the histochemical assay which

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Table 1. Composition of GUS staining solution.

Ingredient	Volume (µl mL^{-1})
0.1 M EDTA (pH 8.0)	100
100 mM Phosphate buffer*	500
1% Triton X-100	100
50 mM potassium ferrocyanide	20
50 mM potassium ferricyanide	20
Methanol	200
100 mM X-Gluc	20
Sterile distilled water	40

*Citrate phosphate and sodium phosphate buffers were used to produce the pH gradient (3.0 - 7.0) in the GUS assay solution.

required an overnight incubation in the staining solution. Under these conditions, they reported that with few exceptions, the GUS activity was detected in certain part(s) of the fruit wall, seed coat, endosperm or the embryos of the tested plants. In addition, *S. baicalensis* has long been known to possess a GUS, called baicalinase (Levvy, 1954). The GUS enzyme from *S. baicalensis* has been purified and was found to provide protection to *Scutellaria* cells against oxidative stress (Matsuda et al., 1995; Morimoto et al., 1995, 1998). Muhitch (1998) reported endogenous GUS activity in the pedicel of developing maize kernels which had an estimated Mw of ca. 32 kDa, stimulated by assay at 60°C and showed inhibition of activity at high ionic strength or in the presence of EDTA. Sudan et al. (2006) used a pharmacological inhibitor of β-GUS, saccharic acid 1,4-lactone (SL) and demonstrated that its application to seedlings of model plant species, such as *Nicotiana tabacum*, resulted in the arrest of growth and the inhibition of root-hair development. Further, Schoenbeck et al. (2007) studied the beta-glucuronidase activity in seedlings of the parasitic angiosperm *Cusctua pentagona* and developmental impact of the beta-glucuronidase inhibitor saccharic acid 1,4-lactone. Also, *Coffea arabica* and *Coffea canephora* embryogenic calli and somatic embryos (Sreenath and Naveen, 2004), *Capsicum chinense* zygotic embryo (Solís-Ramos et al., 2010) and rapeseed microspores and microspore-derived embryos (Abdollahi et al., 2011) were found to have endogenous GUS activity which interferes with the expression of bacterial β-glucuronidase that was transiently expressed in these tissues.

While undertaking the molecular characterization of transgenic Pusa Basmati 1 carrying *Escherichia coli* uidA driven by rice *glyoxalase I* promoter, we carried out GUS assay, an intense blue color developed not only in the transformed explants but also in the untransformed control explants. Captivatingly, the present study was undertaken to characterize the endogenous beta glucuronidase in rice and to differentiate the same from *E. coli* beta glucuronidase.

MATERIALS AND METHODS

Plant material

Rice cultivars, Pusa Basmathi 1 and ADT 43 (obtained from Paddy Breeding Station, TNAU, Coimbatore) and transgenic Pusa Basmati 1 carrying *E. coli* uidA driven by rice glyoxalase I promoter (homo-zygous transgenic lines (T_4 generation) were developed by Dr. L. Arul in an independent study meant for isolating abiotic stress inducible promoters in rice, unpublished data) were used in this investigation. Seeds were surface sterilized with 70% ethanol and 0.1% $HgCl_2$, intervened by several washes with sterile double distilled water. Sterilized seeds were placed in jam bottles containing 0.6% agar for germination. All the cultures were maintained at a photoperiod of 16 h light and 8 h dark at 25±1°C for germination. Three days old plumules and five days old shoots were used for assaying GUS activity.

Callus induction

Calli were induced from ADT 43 and transgenic Pusa Basmati 1 (transgenic and non-transgenic) as per Sudhakar et al. (1998). Dehusked and surface sterilized seeds were placed in a Petri dish containing MS medium supplemented with 2.5 mg L^{-1} of 2,4-D and 3% sucrose. The seeds were incubated in dark at 25 ± 1°C. Fifteen days old calli were used for analysing GUS activity.

Histochemical assay

GUS assay was performed as suggested by Jefferson (1987) and, the assay was carried out at different pH conditions. The GUS assay solutions (composition shown in Table 1) buffered with different pH was prepared using citrate phosphate buffer (pH 3.0, 4.0 and 5.0) and sodium phosphate buffer (pH 6.0 and 7.0). Plant materials were kept in 1.5 mL Eppendorf tubes by completely immersing in GUS staining solution and, incubated overnight at 37°C. For removal of chlorophyll from the GUS stained tissues, the plant materials were immersed in 70% ethanol and incubated for 6 h at 37°C. The ethanol solution was changed twice at 2 to 3 h intervals.

Saccharic acid 1, 4-lactone (SL) treatment

Three days old plumules and five days old shoots of ADT 43 and transgenic Pusa Basmati 1 were soaked in 40 mM SL and incubated for 3 h at room temperature. SL solutions at pH 4.0 and 7.0 were prepared using citrate phosphate buffer and sodium phosphate buffer, respectively. Simultaneously, a set of plumules and shoots were maintained as controls at pH 4.0 and 7.0 without SL. The treated and control seeds were washed with sterile distilled water 3 to 4 times and GUS assay was performed at pH 4.0 and 7.0.

Microtome sectioning

GUS assayed tissues were preserved in 70% ethanol. Transverse section of plumules and shoots were taken without any additional staining and mounted on slides for long time preservation.

RESULTS

Histochemical assay shows GUS activity in rice Non-transgenic, and transgenic rice carrying GUS reporter

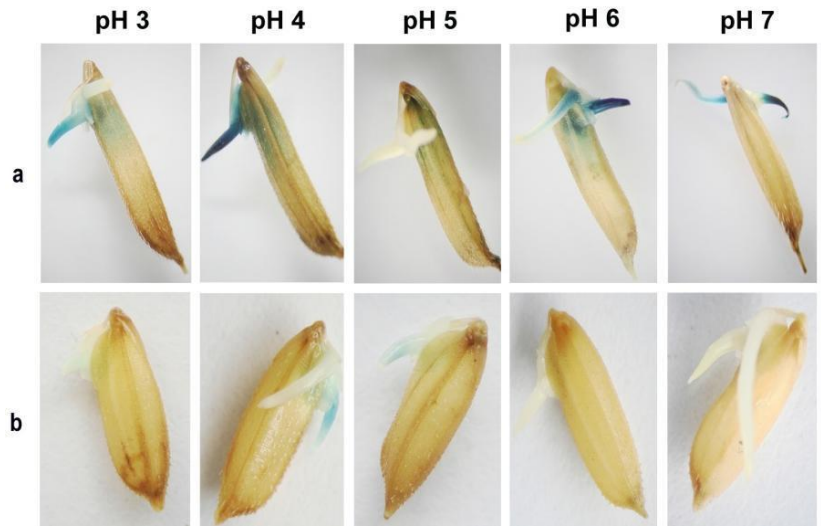

Figure 1. Plumules of transgenic rice (a) and non-transgenic rice (b) showing differential GUS activity between pH 3 and 7.

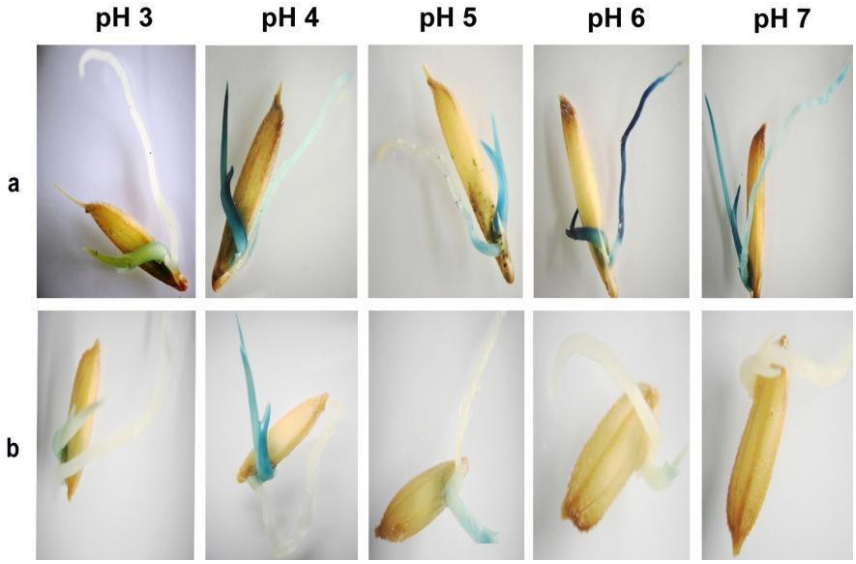

Figure 2. Shoots of transgenic rice (a) and non-transgenic rice (b) showing differential GUS activity between pH 3 and 7.

under the control of rice *glyoxalase I* promoter, showed differential GUS staining when assayed for β-glucuro-nidase activity with X-gluc at varying pH conditions. Three days old plumules, five days old shoots and fifteen days old scutellar derived calli were used for assaying GUS activity. Transgenic rice harbouring *E. coli* borne uidA showed continuous β-glucuronidase activity bet-ween pH 3.0 and 7.0, the maximal colour development was observed at pH 4.0 and 7.0, respectively (Figures 1, 2, 3 and 4). In the case of non-transgenic rice, plumules, shoots and calli showed blue colour but restricted to lower pH 3.0, 4.0 and 5.0, respectively (Figures 1, 2, 3 and 4). In the latter, maximum histochemical staining was observed at pH 4.0.

Saccharic acid 1,4-lactone (SL), an inhibitor of GUS activity

Non-transgenic and transgenic plumules and shoots were treated with 40 mM saccharolactone prior to GUS assay. The results of the study revealed the complete absence of histochemical staining at pH 4.0 and 7.0, in non-transgenic rice (Figures 5 and 6). In contrast, plumules and shoots of transgenic rice treated with SL gave rise to blue colour only at pH 7.0, but lacked histochemical staining at pH 4.0 (Figures 5 and 6).

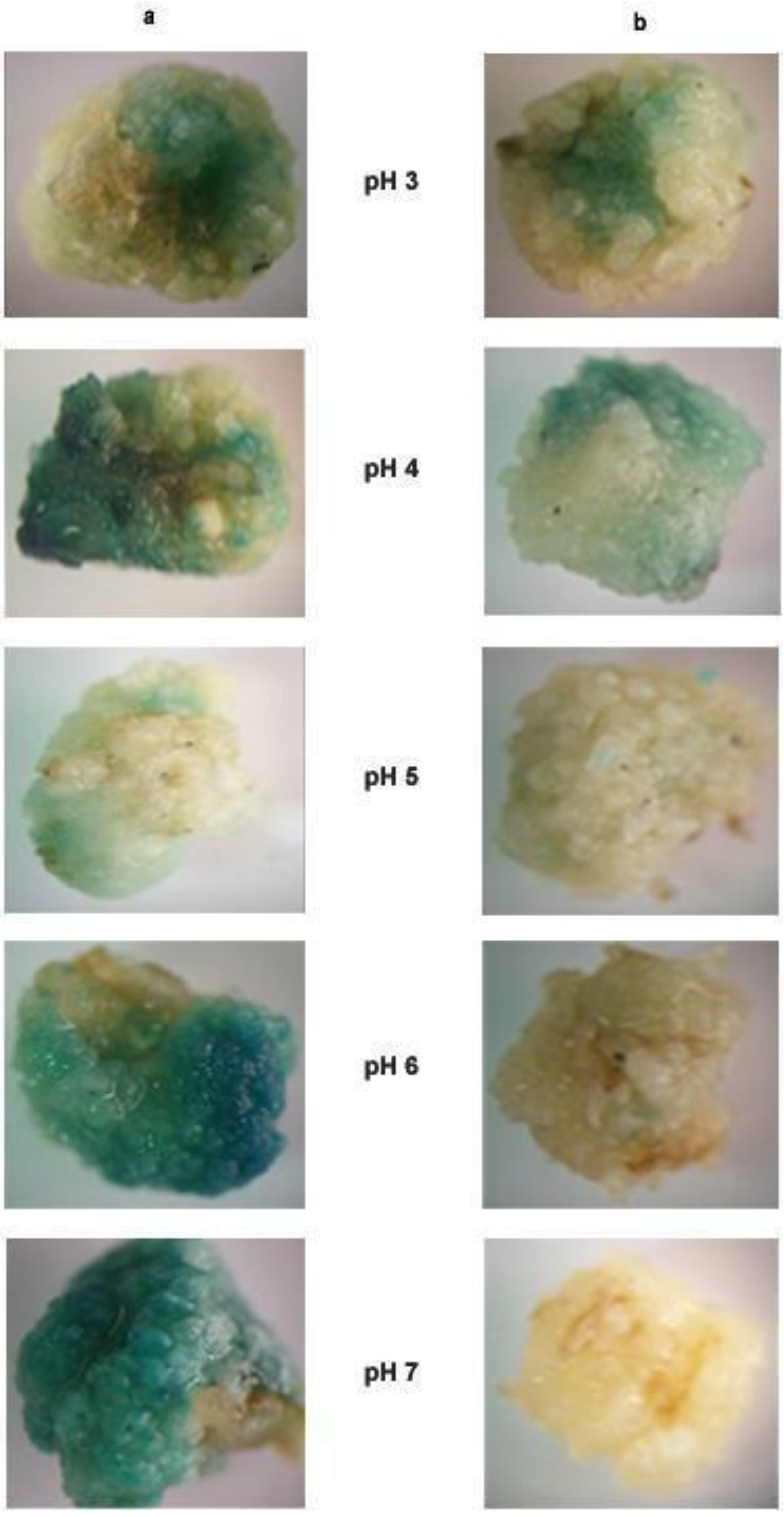

Figure 3. Transgenic rice (a) and non-transgenic rice (b) calli showing differential GUS activity between pH 3 and 7.

DISCUSSION

β-Glucuronidase (GUS) is a glycosyl hydrolase and well known to the plant biologists because of the use of uidA from *E. coli* for gene expression studies (Jefferson et al., 1987). Most β-glucuronidases characterized so far have been classified under glycosyl hydrolase family-2. How-ever, a small number of β-glucuronidases are categorized

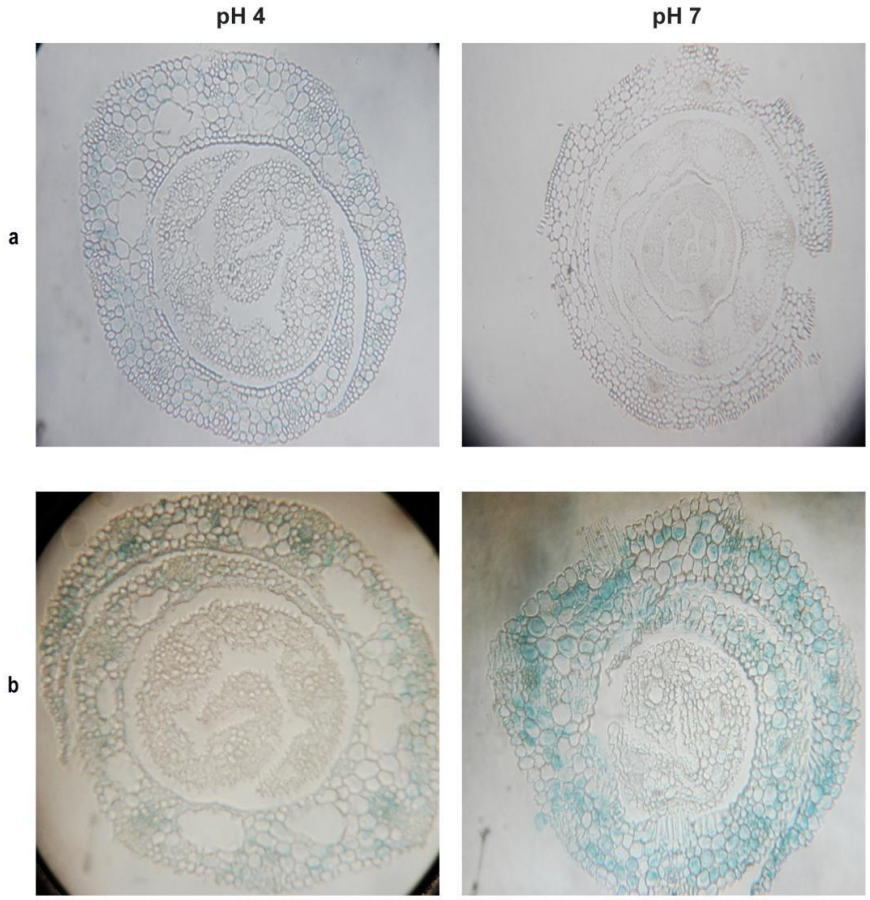

Figure 4. Transverse section of the roots of non-transgenic rice (a) and transgenic rice (b) showing differential GUS activity staining at pH 3 to 7, respectively. Lack of blue colour was observed in the shoots of non-transgenic rice at pH 7.

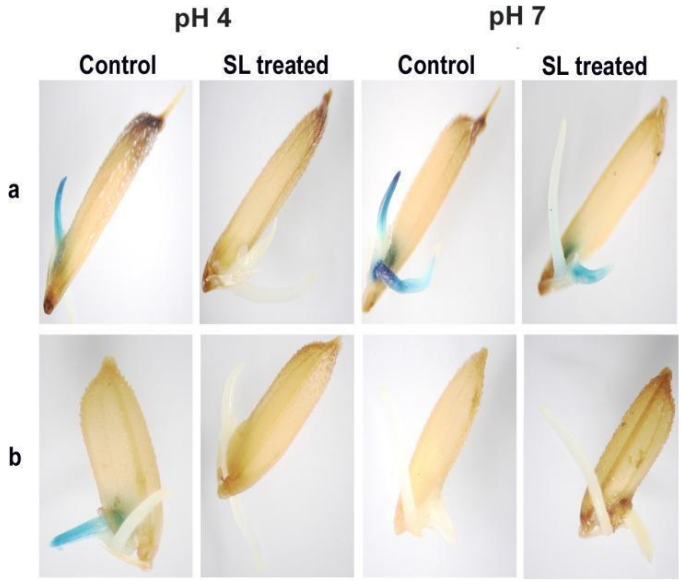

Figure 5. Saccharic acid 1,4-lactone (SL) treated and untreated (control) transgenic rice (a) and non-transgenic rice (b) plumules showing differential GUS activity at pH 4 to 7, respectively.

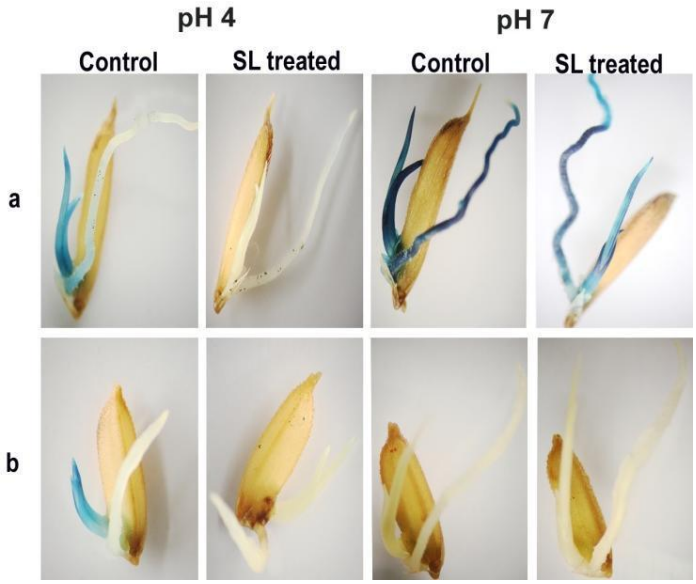

Figure 6. Saccharic acid 1,4-lactone (SL) treated and untreated (control) transgenic rice (a) and non-transgenic rice (b) shoots showing differential GUS activity staining at pH 4 to 7, respectively.

under family-1 and 79 glycosyl hydrolases, but their properties and biological functions have not yet been extensively investigated.

The family-79 β-glucuronidase is also referred to as heparanase, in human, it catalyzes the hydrolysis of heparan sulfate by cleaving the β-1,4-glycosidic bond between D-glucuronate and D-glucosamine in heparan sulfate (a sulfated polysaccharide that is found on the surface of most mammalian cells as part of proteo-glycans). Besides vertebrates, heparanase orthologous genes have been reported in a few microorganisms (*Bradyrhizobium japonicum*, *Burkholderia mallei, Burkholderia pseudomallei, Marinomonas* sp., *Novosphingobium aromaticivorans, Saccharophagus degradans, Solibacter usitatus*) and in plants as well (*Scutellaria baicalensis*, *Arabidopsis thaliana*, *Oryza sativa*, *Hordeum vulgare, Zea mays* and *Medicago truncatula*). The occurrence of endogenous GUS (family-79) activity in plants was reported more than half a century ago by Levvy (1954). The only plant β-glucu-ronidase which has been thoroughly characterized till date is sGUS from *S. baicalensis* Georgi (Sasaki et al., 2000). Three *AtGUS* genes showing high similarity with *sGUS* were cloned from *A. thaliana*, the *AtGUS1*/2 encoded proteins were predicted to be family-79 β-glucuronidase and showed appreciable similarity to heparanase of human, mouse and rat (Woo et al., 2007). In our study, we have chosen rice, a model crop to ascertain endogenous β-glucuronidase (family-79) activity and to differentiate the same from *E. coli* based β-glucuronidase (family-2). Histochemical assay with X-gluc was done along with/without a specific competitive inhibitor saccharic acid 1,4-lactone (SL) which interfered with the GUS activity. The investigation dwells on characterizing endogenous GUS activity during the early stages of seed germination and also from rice callus. Also, similar stu-dies were made from homozygous transgenic rice line (T_4-generation) expressing *E. coli* GUS under the control of glyoxalase I promoter. Histochemical assay was carried out in rice plumule, shoots and calli. The assay was done between pH 3.0 and pH 7.0 for transgenic and non-transgenic rice, concurrently. The pH of the assay buffer turned out to be very critical for the detection of GUS activity in plants. Non-transgenic rice was found to display differential GUS staining when assayed at varying pH conditions. The plumule, shoots and calli of the non-transgenic rice showed blue stain only between pH 3.0 and 5.0 demonstrating the presence of endogenous β-glucuronidase activity at a lower pH range. Earlier, Sasaki et al. (2000) had reported that family-79 β-glucu-ronidase (sGUS) from *Scutellaria* catalyzed the cleavage of glucuronic acid from baicalein 7-O-β-D-glucuronide in an H_2O_2 induced acidic cellular environment. Further, Woo et al. (2007) reported the presence of endogenous GUS activity in vegetative and floral organs of *A. thaliana* at pH 5.0. Alwen et al. (1992) also reported endogenous GUS activity at pH 5.0 in a wide variety of plant species. It has also been reported that endogenous GUS activity in rye, potato, apple and almonds is optimal between pH 4.0 and 5.0 (Hodal et al., 1992). Besides, heparanase in animals (vertebrates and invertebrates) are known to have an acidic pH optimum for its activity. The human heparanase has a pH optimum between 3.5 and 5.0 (Levvy and Marsh, 1959; Dutton, 1980). In *Caenorhabditis elegans*, the GUS has its optimum activity at pH 5.0 (Sebastiano et al., 1986) and between pH 3.0 and 5.5 in

Drosophila (Langley et al., 1983). In contrast, *E. coli* β-glucuronidase belonging to family-2 glycosyl hydrolase has an optimal activity at pH of 7.0 (Jefferson, 1987). It is understandable that, family-79 β-glucuronidase (heparanase) is largely responsible for the histochemical staining that we observed at acidic pH (3.0 - 5.0) in non-transgenic rice. On the other hand, the lack of GUS staining at pH 7.0 indicates the absence of any β-glucuronidase activity on the physiological pH. However, in the case of transgenic rice carrying *E. coli* uidA under the control of rice glyoxalase I promoter, plumule, shoots and calli developed blue colour in almost all pH 3.0, 4.0, 5.0, 6.0 and 7.0. The wide spectrum of GUS activity observed in transgenic rice as compared to non-transgenic rice could be traced back to two different origins, partly it is due to the action of endogenous β-glucuronidase (family-79) between pH 3.0 and 5.0, complemented by the action of *E. coil* β-glucuronidase (family-2) between pH 6.0 and 7.0. These results are consistent with findings of previous researchers in which they used transiently expressed plant tissues (Sreenath and Naveen, 2004; Solís-Ramos et al., 2010; Abdollahi et al., 2011).

Further in this direction, we carried out GUS assay in the presence of saccharic acid 1,4-lactone (SL), a well known inhibitor of endogenous β-glucuronidase. There was a complete absence of blue staining at pH 4.0 and pH 7.0 in non-transgenic rice where as the control (untreated with SL) showed histochemical reaction at pH 4.0 but not at pH 7.0, indicating specific inhibition of endogenous GUS activity only at pH 4.0. Alwen et al. (1992) reported 50% inhibition with 5 mM SL and up to 75% inhibition of GUS activity with 25 mM of SL in plant species belonging to different families. In our study, we have observed 100% inhibition with 40 mM SL. In contrast, shoots of transgenic rice treated with SL gave rise to blue colour at pH 7.0, but showed no GUS staining at pH 4.0 indicating the suppression of only the endogenous GUS activity. This is suggestive of the fact that β-glucuronidase activity at pH 7.0 which is due to *E. coli* GUS (family-2) remains unaffected. On expected lines, transgenic shoots untreated for the inhibitor (SL) showed histochemical staining at pH 4.0 and 7.0 as well. Our study presents a comparative account on family-79 and family-2 β-glucuronidase in a stably transformed plant (T_4 generation) model system such as rice. We have demonstrated the occurrence of endogenous GUS activity in the early developmental stages of rice. The use of a specific inhibitor, saccharic acid 1,4-lactone, clearly demonstrates that family-2 and 79 β-glucuronidases are biochemically different from each other however, are involved in a common function, that is, cleavage of the glucuronide moiety from their respective substrates. Although this property is shared by the endogenous and *E. coli* β-glucuronidases, the two activities can be distinguished by: (i) their different pH optima (4.0) for the endogenous activity and close to neutral (7.0) for the *E. coli* beta glucuronidase and (ii) their different sensitivity to the specific inhibitor saccharic acid-1,4-lactone. The present study suggested that care should be taken to analyse the transgenic plants with GUS reporter system. Therefore, under appropriate experimental conditions such as maintaining pH of the GUS assay buffer to pH 7, it is possible to assay the *E. coli* β-glucuronidase in transgenic plants without interference from the endogenous plant activity.

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