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Comparison of β-glucuronidase expression and anatomical localization in bombarded immature embryos of banana cultivar Mas *via* biolistic transformation

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Abstract. To obtain stable expression of a foreign gene, it is important to have optimized conditions for plant genetic transformation. Here, a GUS intron-containing gene driven by CaMV 35S promoter was used to optimize the conditions of biolistic transformation of banana immature embryos and also for the study of GUS localization in the transformed cells. The GUS activity detected histochemically and fluorometrically were further analysed by microscopy. This histological study confirmed the results in the histochemical and fluorometric assay, where highest expression of GUS was achieved when the immature embryos bombarded with the helium pressure of 1350 psi and placed at the target distance of 6 cm. The observation of strong GUS staining in the deep layers of the cell structure were produced by higher acceleration pressure and shorter target distance, whereas weak GUS staining in the plant epidermis layer were observed in most lower acceleration pressure and higher target distance. The study of GUS localization on biolistic transformation provided more reliable parameters for transformation. It may also indicate the locality of the foreign gene expression area in the transgenic plant that can provide more understanding of the nature of transgene and its integration.

Keywords. Somatic embryogenesis, Plant transformation, Biolistic bombardment, GUS localization, transgenic banana.

Abbreviations:6-BA: 6-Benzyladenine, 2,4-D: 2,4-Dichlorophenoxyacetic AcidNAA:1-Naphthaleneaceticacid, 2ip:*N*^{6}-(2isopentenyl)adenine, SCV: settled cell volume, GUS: β-glucuronidase.

INTRODUCTION

The delivery of foreign DNA into any plant species through biolistic bombardment or microprojectile-mediated transformation is based on the acceleration of DNA (or RNA) coated microparticle (gold or tungsten) which could penetrate into the plant cell wall and membrane, and subsequently incorporate the DNA of interest into plant nuclear genome. The transformed plant cells are then selected and regenerated into complete plants. With this technique, a successful banana transformation and regeneration sytem was established by Sagi *et al.* (1995) and Becker *et al.* (2000).

In routine biolistic bombardment experiments, reliable optimized conditions for biolistic transformation greatly influence the efficiencies of stable integration of foreign genes. Therefore, screenable markers that do not exist naturally in the host plant such as β-glucuronidase (GUS), chloramphenicol acetyl transferase (CAT), luciferase (LUC) and green fluorescent protein (GFP) are always used for transient expression experiment as indication of the integration of a transgene. The GUS reporter gene is one of the most widely used reporter gene in transgenic plant research (Jefferson *et al.,* 1987). It is an ideal plant reporter gene because of the convenient, high sensitivity and specific enzyme assay for the reporter gene product. In this investigation, we studied the correlation between β-glucuronidase expression and anatomical GUS localization in biolistic bombarded immature embryos of banana cultivar Mas derived from embryogenic

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cell suspension. Currently there is no biolistic transformation protocol available from this culture. The comparative analysis of GUS transient assay provides insight on GUS expression in the different layers of the cell structure upon penetration of microparticle-coated DNA driven by high acceleration pressure in biolistic transformation.

MATERIALS & METHODS

Plant materials. Cell suspensions of *Musa acuminata* cv. Mas (AA) were established from embryogenic callus derived from immature male flower clusters cultured for six months according to the method developed by Mahanom *et al.* (2003). The embryogenic cell suspension cultures were subcultured every two weeks interval in M2 medium containing Murashige and Skoog (MS) marconutrients and micronutrients supplemented with 2.0 mg/L glycine, 0.40 mg/L thiamine-HCl, 0.50 mg/L nicotinic acid, 0.50 mg/L pyridoxine-HCl, 100.0 mg/L myo-inositol, 10.0 mg/L ascorbic acid, 1.1 mg/L 2,4-D (Sigma), 250.0 µg/L trans-zeatin (Sigma) and 20.0 g/L sucrose. The pH was adjusted to 5.7 prior to autoclaving. Throughout the experiment, approximately 2.0 ml of settled cell volumes (SCV) of the cell aggregates were inoculated into 50.0 ml M2 media in 250.0 ml Erlenmeyer flask. The cultures were maintained in an orbital shaker at 70 rpm, 25±1°C, 16 hours light: 8 hours dark photoperiod with a light intensity of 31.4 µmol $m^{-2}s^{-1}$. For plant transformation, the suspension cells maintained in M2 medium were sieved through a 450 µm mesh. The filtrate was left to settle down in a 50.0 ml Falcon tube followed by the adjustment of SCV to approximately 1:5 (SCV: liquid M2 medium). Cell aggregates were then resuspended and 200.0 µl aliquots dispensed into each 20 ml hormone-free MS liquid medium. The optimum tissue type of immature embryos for biolistic bombardment was obtained within three to four weeks of culture.

Source of vectors and constructs. The plasmid pCAMBIA1301 (CAMBIA, Australia, Figure 1) was used for the biolistic bombardment experiment. The reporter gene is an intron-containing *gusA* gene under the control of 35S promoter of Cauliflower Mosaic Virus (CaMV35S).

Biolistic-mediated transformation. Immature embryos were bombarded by using Biolistic PDS-1000/He ™ System (Bio-Rad, USA). The Bio-Rad's recommended settings were used as starting parameters for bombardment. These parameters included the target distances (6.0, 9.0, 12.0 cm), helium pressure (900, 1100, 1350 psi) and microparticles (1.0 µm gold). For optimization of biolistic transformation, the immature embryos were bombarded with the plasmid pCAMBIA1301. The optimal target distances and helium pressure were evaluated by using GUS histochemical and fluorometric assay as described by Jefferson (1987).

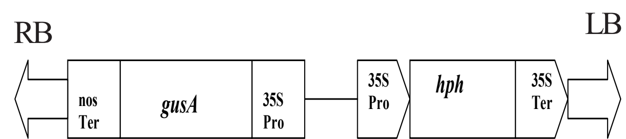


Figure 1. Linear map of plasmid vector pCAMBIA 1301

Histochemical analysis of GUS expression. After bombardment of the cell, the tissues were incubated in the dark at 24±1°C for at least two days to allow cell repair and DNA intergration (Jefferson, 1987). In this experiment, bombarded immature embryos were incubated for a week in order to differentiate the viable embryos from those damage tissues. The viable embryos were transferred into histochemical reagent containing 0.1 M phosphate buffer, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.1% triton X-100, 10.0 mM EDTA, 20% methanol and 1.0 mM 5-bromo-3-indolyl-glucuronide (X-gluc) (Clontech). The samples were incubated for 18 to 24 hours at 37°C, at which time the tissues were evaluated for their level of GUS expression . After staining, the samples were cleared with 70% ethanol and fixed in FAA solution (45% absolute ethanol, 5% glacial acetic acid, 5% formaldehyde). The samples were examined under contrast phase light microscope (Zeiss).

Fluorometric assay. The fluorometric assay for specific GUS enzyme activity was quantified by measuring the hydrolysis rate of the fluorogenic substrate 4-methylumbelliferyl â-D-glucuronide (MUG) (Sigma) as described by Jefferson (1987). Three replicates for each sample were measured with a DyNA Quant 200 Fluorometer (Hoefer Scientific Instruments). The protein concentration of each sample was determined with a spectrophotometer (Novo) at OD_{595} by using Quick Start Bradford Protein Assay kit (Bio-Rad). All data were analysed using analysis of variances (MINITAB version 11.12, MINITAB Inc). The testing of paired differences between treatments was done using paired *t*-test at $P = 0.05$.

Analysis of anatomical GUS localization. The anatomical localization of GUS expression in transformed immature embryos were identified using microscopy technique. The GUS staining products were fixed for 24 hours in FAA solution (45 % v/v absolute ethanol, 5% v/v glacial acetic acid, 5 % v/v formaldehyde) and dehydrated in increasing concentration of ethanol (30% and 50% for 2 hours, 70% for overnight, 80%, 90%, 95% and 100% for 1 hour). After infiltration, specimens were embedded in LKB resin and cut into 3.5µm sections. The images of tissues localized with GUS stain were captured prior to Schiff staining. After

Table 1. Influence of helium pressure and target distance on transformation of banana immature embryos

Tissue Sample	Helium Pressure, (psi)	Particle	Target Distance, (cm)	GUS Score* Experiment, 1	2	3
Immature Embryo	900	1.0 µm Gold	6	++	++	++
Immature Embryo	900	1.0 µm Gold	9	++	++	++
Immature Embryo	900	1.0 µm Gold	12	-	-	-
Immature Embryo	1100	1.0 µm Gold	6	+++	+++	+++
Immature Embryo	1100	1.0 µm Gold	9	++	++	++
Immature Embryo	1100	1.0 µm Gold	12	-	-	-
Immature Embryo	1350	1.0 µm Gold	6	+++	+++	+++
Immature Embryo	1350	1.0 µm Gold	9	++	++	++
Immature Embryo	1350	1.0 µm Gold	12	-	-	-

*GUS Score: **-** no blue spots; **+** low; **++** intermediate; **+++** high density of the blue spot

staining, the sections were mounted with Surgipath mounting medium with cover slips and again the same location of GUS stained tissues were visualized under a phase contrast light microscope (Zeiss). The images were captured using a microscope adapted Nikon digital camera (COOLPIX995).

RESULTS & DISCUSSION

To monitor GUS reporter gene transformation efficiencies, plasmid pCAMBIA1301 was used to transform immature embryos of banana cultivar Mas via biolistic bombardment transformation. The tested physical parameters included helium pressure (900, 1100 and 1350 psi) and target distance (6, 9 and 12 cm). Unlike routine transformation studies, the evaluation of the optimized conditions for biolistic bombardment was based on both the histochemical and fluorometric assay analysis of GUS expression. In this study we further verified the anatomical GUS localization of bombarded immature embryos using microscopy techniques.

Histochemical GUS assay. Consistent with the histochemical analysis of GUS expression, the influence of helium pressure and target distance on the early events in biolistic transformation of banana immature embryos had been determined (Table 1). These results indicated that optimum transient expression was obtained when immature embryos were placed at a target distance of 6 cm and bombarded at a helium pressure of either 1100 or 1350 psi with 1.0 µm gold particles. The GUS score is a qualitative assessment of the level of GUS expression in the bombarded immature embryos (Figure 3). There are numerous variables that greatly influence the result of the GUS histochemical analysis, including the substrate penetration and diffusion of the product as well as the permeability of the fixative. Besides that, the localized peroxidase activity may also enhance the oxidative dimerization of X-gluc product into insoluble indigo, which may vary the accuracy of the final results of GUS activity. Therefore, the GUS expression results were further verified by using quantitative fluorometric assay and microscopy technique.

Fluorometric GUS analysis. In this study, the immature embryos were bombarded with plasmid pCAMBIA1301 coated onto 1.0 µm gold microparticles. The fluorometric assays of GUS activity demonstrated that the transcript level of GUS intron-containing gene in the putative transformed

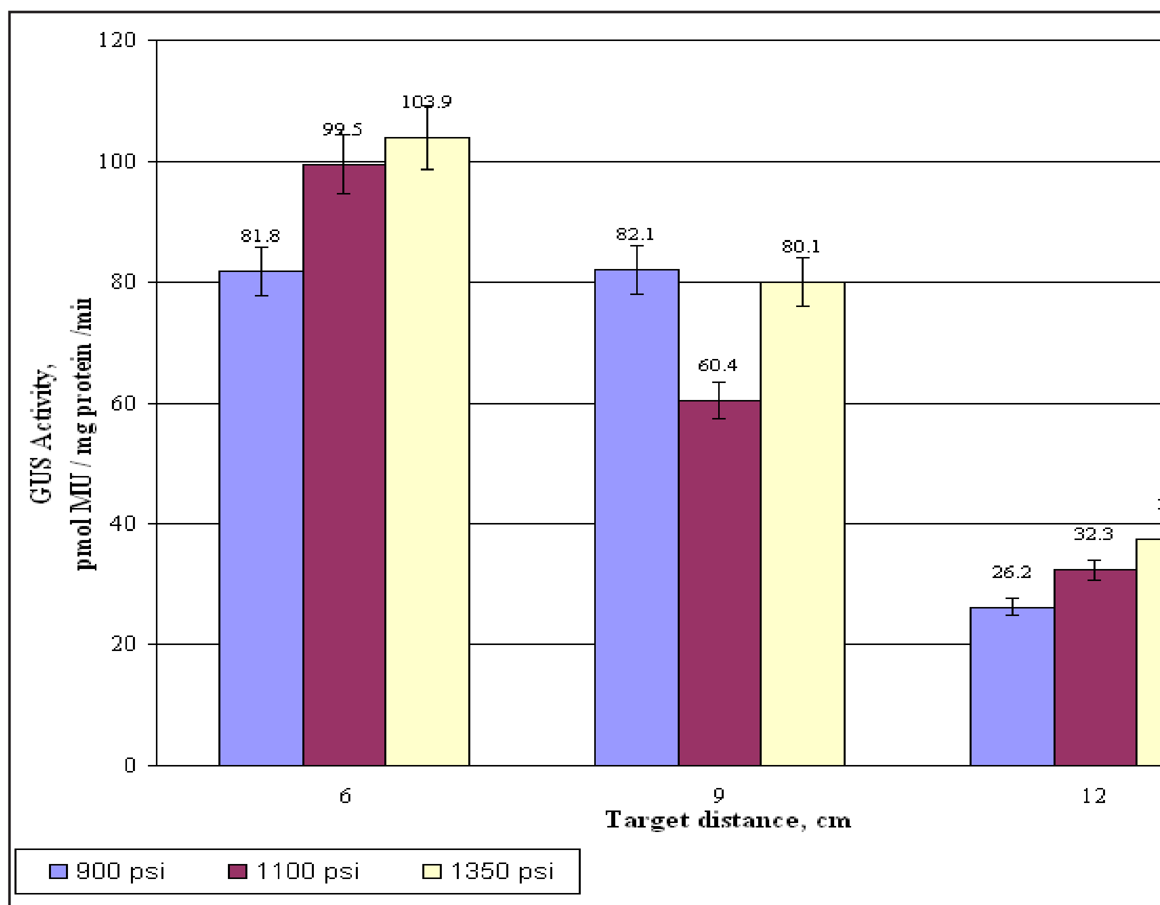


Figure 2. The influence of helium pressure and target distance on GUS activity. Fluorometric GUS activity assays were performed two weeks after bombardment.

Table 2. Statistical analysis of variances between GUS activity in putative transformed immature embryos. The testing of paired differences between parameters of bombardment were done using paired *t*-test at $P = 0.05$ (MINITAB version 11.12, MINITAB Inc).

Helium pressure, distances Psi	*P*-value of GUS activity achieved at different target		
	6 vs. 9 cm	**9 vs. 12 cm**	**6 vs. 12 cm**
900			
1100	0.21	0.0340	0.0035
1350	($P > 0.05$)	($P < 0.05$)	($P < 0.05$)

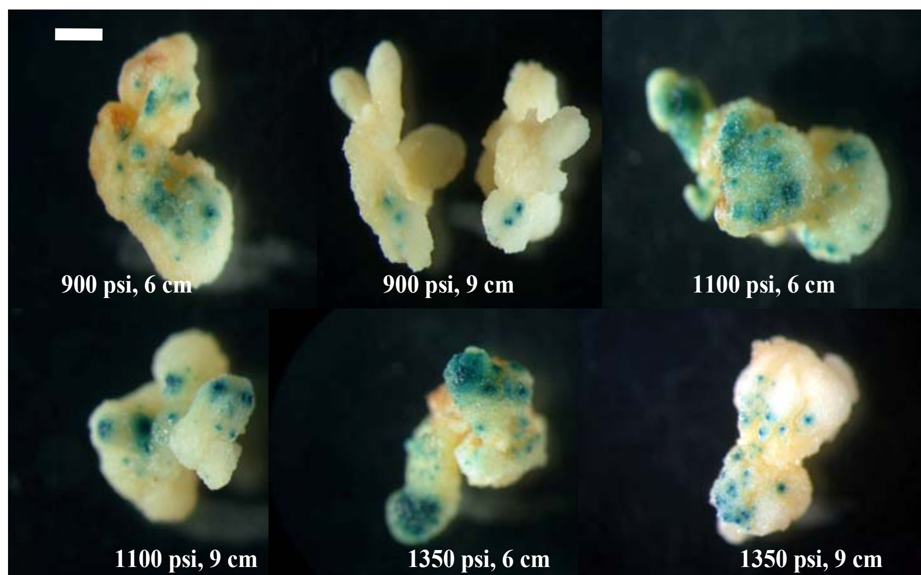


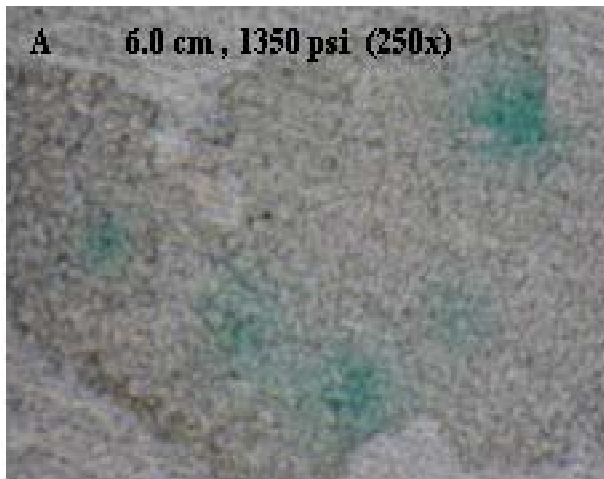
Figure 3. Transient expression of the GUS gene in banana immature embryos (*bar*: 1.0 mm). The value given in each figure represents the helium pressure and target distance of the bombardment.

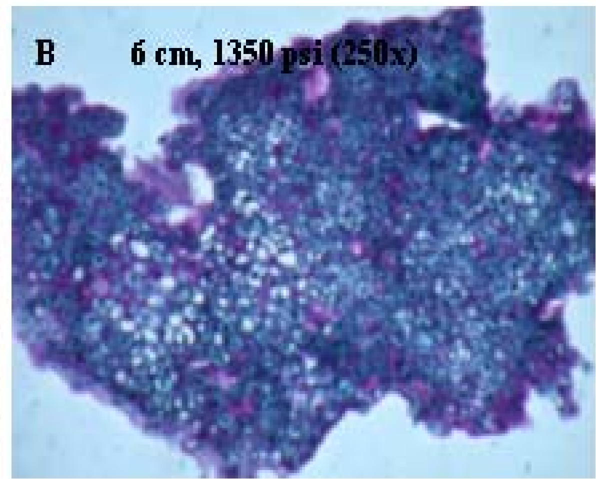
immature embryos. The presence of β-glucuronidase enzyme will cleave the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (MUG) to produce fluorescent 4-methylumbelliferone (4-MU). The bombarded immature embryos were incubated for two weeks to allow cell repair and stable *gusA* gene integration before fluorometric assay was performed. Figure 2 indicates that the transient gene expression level was always detected fluorometrically in tissue extracts of immature embryos prepared two weeks after bombardment experiment, regardless of the helium pressure and target distance used. In comparison of the bombarded immature embryos, high GUS activity was always achieved at 6 and 9 cm target distance with 900, 1100 and 1350 psi helium pressure respectively. However, the GUS activity for the putative transformed immature embryos revealed no significant ($P > 0.05$) differences between 6 and 9 cm target distance of bombardment (Table 2). Therefore, it was a necessary to determine the GUS localization on the bombarded tissues, in order to gain more understanding on using the transient expression as a guide to develop system for stable transformation of banana.

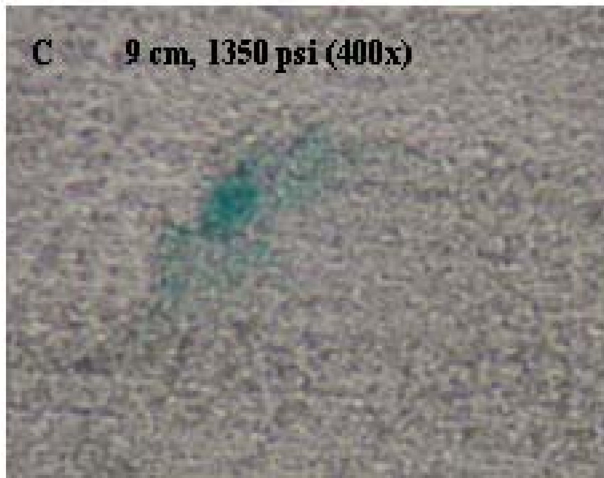
Anatomical GUS localization. Histological study for localization of histochemical GUS staining was carried out on bombarded immature embryos with plasmid pCAMBIA1301 containing *gusA* gene. Figure 4A, C, E, G, I and K displayed GUS staining in sections of immature embryos; subsequent counterstaining revealed the surrounding cell structure (Figure 4B, D, F, H, J and L). The cell walls and storage proteins were stained with periodic acid Schiff and Naphthol Blue Black respectively. Strong GUS staining was observed on immature embryo sections bombarded with 1350 psi helium pressure and placed at a target distance of 6 cm.The internal GUS stained cell structure shown in Figure 4A and 4B indicated the high efficiency of the penetration of 1.0 μm gold particle coated with plasmid pCAMBIA1301. Even though, the shorter target distance and higher helium pressure were causing significant damage to the bombarded cells upon penetration, the expression level of the *gusA* gene was much more promising. Whereas the other parameters showed weak GUS stained sections in the epidermic layer of the tissues, where there is no differentiation of plant organs. This analysis confirmed the previous results of histochemical and fluorometric GUS analysis. The helium pressure of 1350 psi and a target distance of 6 cm were most suitable for use in biolistic transformation of banana immature embryos.

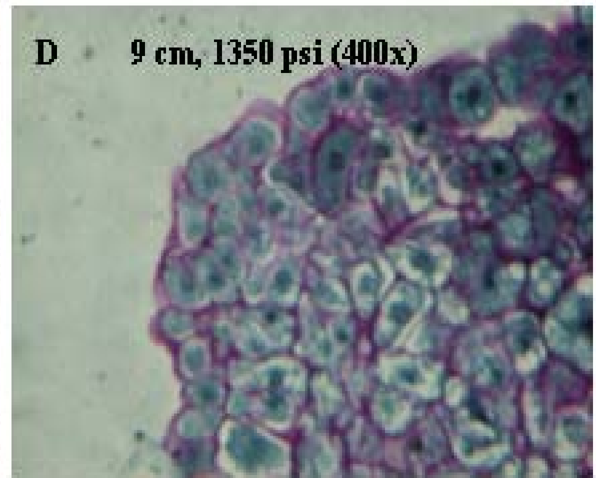
CONCLUSIONS

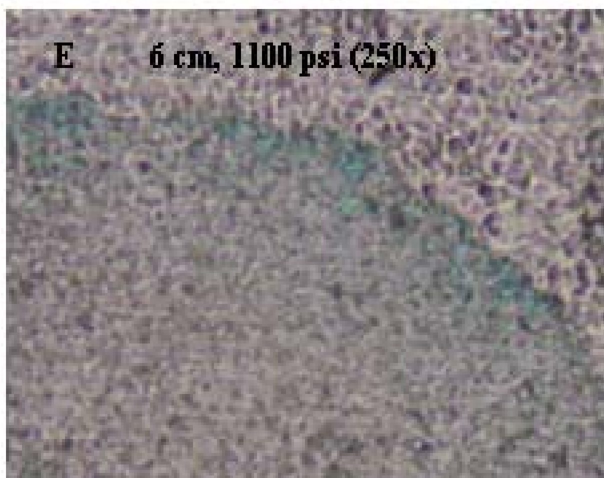
In this report, we have demonstrated GUS expression in bombarded immature embryos produced from biolistic bombardment experiment. The comparative GUS activity and localization indicated high GUS expression level was obtained using 1350 psi helium pressure and 6 cm target distance. The transient expression shown in this experiments provides the basic for developing stable transformation of banana cultivar Mas.

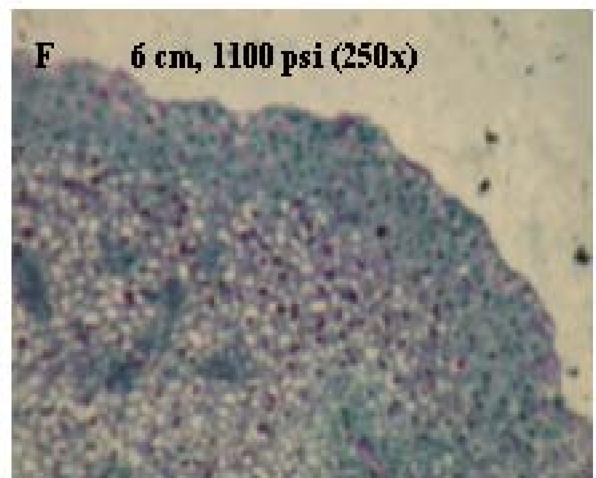

A 6.0 cm , 1350 psi (250x)


B 6 cm, 1350 psi (250x)


C 9 cm, 1350 psi (400x)


D 9 cm, 1350 psi (400x)


E 6 cm, 1100 psi (250x)


F 6 cm, 1100 psi (250x)

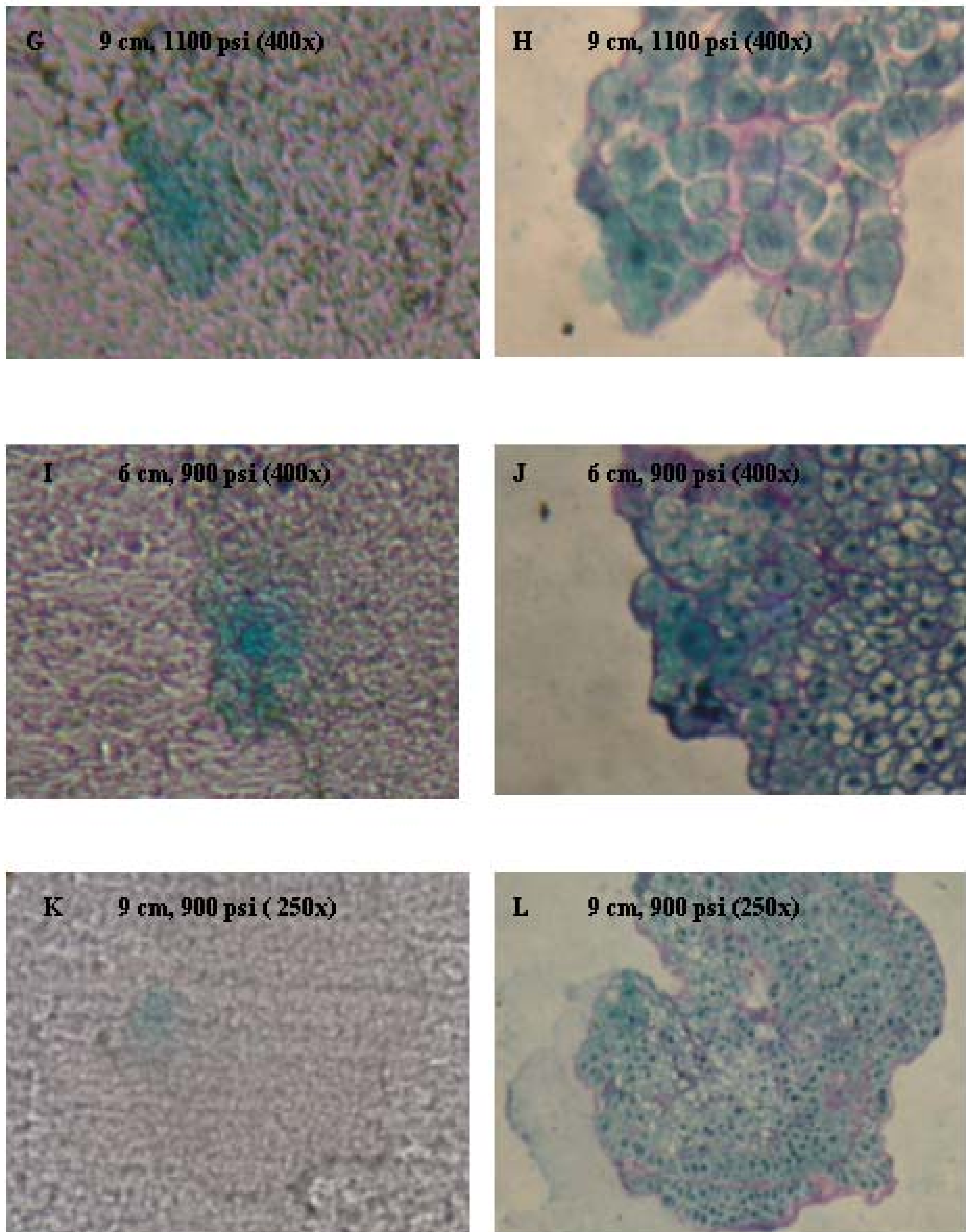


Figure 4A-L. Localization of GUS expression in bombarded immature embryos. **A, C, E, G, I and K** GUS-expressing immature embryos section before Schiff and Naphthol Blue Black Staining; **B, D, F, H, J and L** GUS-expressing immature embryos section after staining of Schiff and Naphthol Blue Black dye. The value given in each figure represents the helium pressure and target distance of the bombardment.

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