academicJournals

Vol. 14(46), pp. 3112-3123, 18 November, 2015
DOI: 10.5897/AJB2015.14983
Article Number: E69D18956235
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro transformation of pearl millet (P*ennisetum glaucum* (L). R. BR.): Selection of chlorsulfuron-resistant plants and long term expression of the *gus* gene under the control of the *emu* promoter

Kouakou Tiécoura[1], Abou Bakari Kouassi[1], N'nan-Alla Oulo[1]*, Séry Gonedelé Bi[1], Monique Dinant[2] and Lucien Ledou[2]

[1]Laboratoire de Génétique, UFR de Biosciences, Université Félix Houphouët Boigny, 22 BP 582 Abidjan 22, Côte d'Ivoire.
[2]Laboratoire de Génétique Moléculaire, Département de Botanique, Université de Liège, Sart Tilman, B22, B-4000 Liège, Belgium.

Received 18 September, 2015; Accepted 5 November, 2015

The plasmids pULGU1 and pEmuGN were introduced by biolistics in embryogenic cell suspensions of pearl millet, *Pennisetum glaucum*. The plasmid pULGU1 contained the mutant acetolactate synthase (ALS) gene of *Arabidopsis thaliana*, responsible for resistance to the chlorsulfuron herbicide. The plasmid pEmuGN carried the reporter β-glucuronidase (GUS) gene of *Escherichia coli*. Two months after bombardment of the cells, transformed calli were selected on Murashige and Skoog medium containing 30 nM chlorsulfruron, a completely concentration inhibitory for non-transformed cells. A concentration of 200 nM chlorsufuron did not affect the growth of these selected resistant calli. Genomic DNA of resistant cells presented an electrophoretic pattern revealing the integration of the mutant ALS gene into the genome of *Pennisetum glaucum*. Expression of the GUS reporter gene was revealed by histochemical assay of the blue coloration of the calli in the presence of the substrate X-gluc (5-bromo-4-chloro-3-indolyl-β-D-Glucuronic acid). The presence of GUS gene was furthermore confirmed, by southern blot hybridization of non-radioactive probes, labelled with digoxigenin, on genomic DNA extracted from selected chlorsulfuron-resistant calli. GUS gene activity observed in all selected calli was high during the first 6 months after bombardment and then decreased. Pearl millet plants were regenerated from cell lines derived from chlorsulfuron resistant calli. Southern blot hybridization of non-radioactive probes with genomic DNA extracted from these regenerated plants showed the presence of the GUS and ALS transgenes, confirming the stable transformation of these plants.

Key words: *Pennisetum glaucum*, stable transformation, chlorsulfuron (ALS), β-glucuronidase (GUS), CaMV35S and Emu promoters.

INTRODUCTION

The Poaceae family includes important cereal crops which often need to be improved to face pathogens attacks and other environmental constrains such as drought, flooding and competition of weeds during cropping seasons. Crop plants are usually improved by crossing them with wild relatives. Unfortunately, this approach

has some limitations and sometimes genetic engineering methods are used to transfer genes of interest from wild plants to cultivated ones. As compared to dicotyledons plants, graminaceous are recalcitrant to transformation methods using the Ti plasmid as vector (Plaza-Wüthrich and Tadele, 2012; Hussain et al., 2013; Sah et al., 2014). The major obstacles to this gene delivery method were attributed to the limited host-range of Agrobacterium (Hwang et al., 2013; Qamar et al., 2015), and to the different responses of monocots (cell death) and dicots (cell division) to wounds (Potrykus, 1990; Hiei et al., 2014). A variety of techniques such as polyethylene glycol (PEG) method, electroporation method, mild ultra-sonication and biolistic have been proposed for genetic transformation of cereals. Some results have been obtained using the direct introduction of DNA into compe-tent protoplasts by polyethylene glycol (PEG) induced uptake (Tiécoura et al., 2001; Bajaj, 2012; Ji et al., 2013), electroporation (Vasil, 2012; Jordan et al., 2013), followed by the growth of calli and in some cases, the regeneration of transformed plants (Shimamoto et al., 1989; Bajaj and Mohanty, 2005; Bahieldin et al., 2005; Danilova, 2007).

Plant regeneration from protoplasts of cereals is difficult and far from routine (Jähne et al*.,* 1995; Vasil, 2012; Plaza-Wüthrich and Tadele, 2012; Tiécoura et al., 2015). However, several transformation systems leading to the introduction of DNA into monocotyledon intact tissues have been developed: for example electroporation into organized plant tissues (Dekeyser et al., 1990), silicon carbide fiber-mediated DNA transfer into maize suspension cells (Kaeppler et al., 1990; Sood et al., 2011) and the microprojectile bombardment or biolistic method (Klein et al., 1987; Prathibha and Sticklen, 2002; Martinez-Trujillo et al., 2003; Singh et al., 2013). Microprojectile bombardment is currently the most used method for *in vitro* transformation of graminaceous even if improvements of the Agrobacterium-mediated transfor-mation method have been achieved (Liu et al., 2005; Razzaq et al., 2010; Sharma et al., 2011; Plaza-Wüthrich and Tadele 2012). The biolistic method offers an effective alternative for the genetic transformation of cereal crops. Several successes, in transient expression of GUS and NPTII genes, have been indeed reported using this transformation method on *Triticum eastivum* (Jones, 2005; Tassy et al*.,* 2014), *Hordeum vulgare* (Ji et al., 2013), *Saccharum officinarum* (Singh et al., 2013), *Panicum virgatum* (King et al., 2014) and *Zea mays* (Que et al., 2014; Qamar et al., 2015).

Stable transformed calli have also been selected. Some examples include calli of *Sorghum bicolor* (Battraw and Hall, 1991; Girijashankar and Swathisree, 2009), *Lolium perenne* (Altpeter et al., 2000), *T. eastivum* (Sahrawat et al., 2003) and *H. vulgare* (Yadav et al., 2013) resistant to hygromycin or Kanamycin. This is also the case of calli of *Z. mays* (Nap et al., 2003) and *S. officinarum* (Singh et al., 2013) resistant to phosphinothricin. The biolistic technique has also allowed the regeneration of trans-genic fertile plants of cereal crops. For example, plants of *Z. mays* (Weymann et al.,1993; Que et al., 2014), *H. vulgare* (Hagio et al., 1995; Yadav et al., 2013) and *Oryza sativa* (Dai et al., 2001; Sah et al., 2014), resistant to hygromycin; plants of *Z. mays* (Nap et al., 2003) and *T. eastivum* (Tassy et al*.,* 2014) resistant to phosphinothricin and plants of *Z. mays* resistant to glyphosate (Qamar et al., 2015) have been obtained. Other examples include plants of *Allium sativum*, *Z. mays* and *T. eastivum* resistant to chlorsulfuron (Mee et al., 2002; Que et al., 2014); plants of *Sorghum bicolor* (Cass et al., 1997; Girijashankar and Swathisree, 2009) *H. vulgare* (Goedeke et al., 2007) and *S. officinarum* (Singh et al., 2013) resistant to kanamycin. Girijashankar et al. (2005) also obtained plants of *S. bicolo*r resistant to insects.

Concerning pearl millet, *Pennisetum glaucum*, the transient expression of the reporter ß-glucuronidase (GUS) gene in transformed calli has been reported (Vasil, 2008; Jha et al., 2011; Ramadevi et al., 2014). Stable calli have been selected and analysed by Lambé et al. (1995) after introducing the neomycin phosphotransferase gene (NPTII). Transgenic plants resistant to phosphinothricin and hygromycin (Goldman et al., 2003; O'Kennedy et al., 2004; Ceasar and Ignacimuthu, 2009; Ramadevi et al., 2014), kanamycin (Plaza-Wüthrich and Tadele, 2012) and mildew (Latha et al., 2006) have been studied. However, to date, no stable *P. glaucum* transformed calli and plant resistant to the chlorsulfuron herbicide have been reported yet. In the present study, the following were reported: 1) the transient and long term expression of the reporter β-glucuronidase gene (GUS) of *Escherichia coli* in *P. glaucum* embryogenic cell suspensions transformed using biolistic method; 2) The isolation and analysis of chlorsulfuron herbicide-resistant calli and plants of pearl millet regenerated after introduction of the mutant Acetolactase synthase (ALS) gene of *Arabidopsis thaliana* and the reporter GUS gene of *E. coli*.

MATERIALS AND METHODS

Embryogenic calli's of *P. glaucum* (variety NE of Côte d'Ivoire) were used to generate embryogenic cell suspensions (Tiécoura et al., 2003, 2014). Two sieves having respectively 250 and 630 µm in mesh diameter were used to release, from embryogenic cell

*Corresponding author. E-mail: nanoulo@yahoo.fr. Tel: 00 225 07 16 43 27.

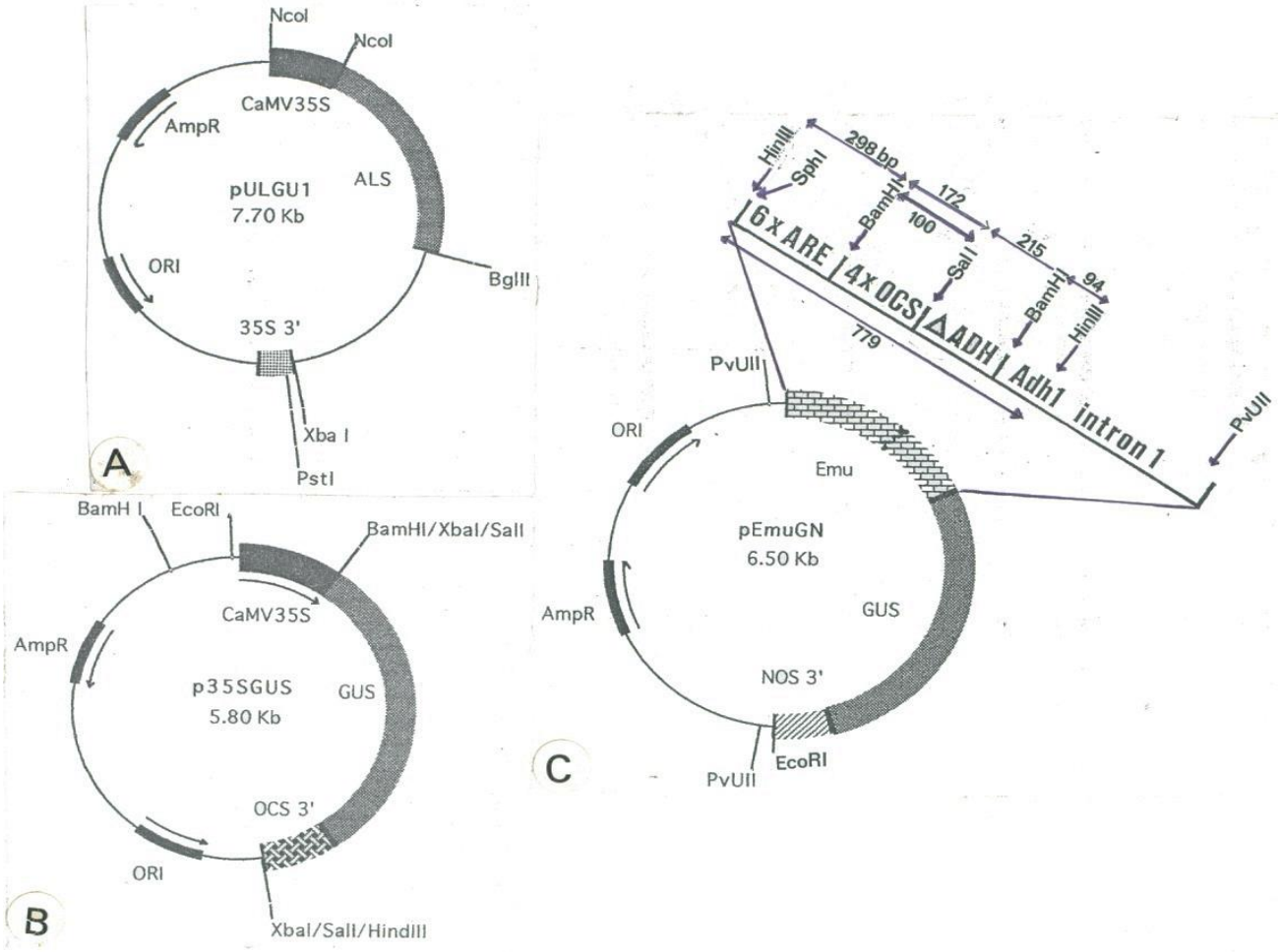

Figure 1. Different plasmids used for the bombardment of *P. glaucum* embryogenic calli. **A)** pULGU1 plasmid carrying the gene for resistance to sulfonylurea herbicides (ALS) controlled by the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and terminator. **B)** p35SGUS plasmid carrying the β-glucuronidase (GUS) gene controlled by the CaMV35S promoter and the Octopine Synthase (OCS) terminator. **C)** pEmuGN plasmid carrying the β-glucuronidase (GUS) gene controlled by the Emu promoter and the NOS terminator.

suspensions, two types of calli whose sizes were respectively less than 250 and 630 µm. 5 ml of each type of calli at a concentration of 60 to 100 mg/ml were dispersed on a nitrocellulose membrane by vacuum filtration and bombarded with plasmids DNA.

Plasmid DNA

Three types of plasmids (Figure 1) were used to transform the embryogenic cells of *P. glaucum*.

pULGU1 plasmid

The plasmid pULGU1 has a size of 7.7 kbp (Figure 1A). It contains the NCol-XbI fragment carrying the mutant acetolactate synthase (ALS) gene of *Arabidopsis thaliana* controlled by the Cauliflower Mosaic Virus (CaMV) 35S promoter and terminator (Haughn and Somerville, 1986). The mutant ALS gene was isolated from *A. thaliana* plant resistant to chlorsulfuron, a sulfonylurea herbicide. Resistant cells carrying the mutant ALS genes can grow normally in a medium containing the chlorsulfuron herbicide, whereas susceptible cells necrose.

p35SGUS plasmid

The plasmid p35SGUS (Szabados et al., 1995) has a size of 5.8 kbp (Figure 1B). It carries the reporter β-glucuronidase (GUS) gene of *E. coli* controlled by the CaMV 35S promoter and the *Agrobacterium tumefaciens* octopine synthase (OCS) terminator.

pEmuGN plasmid

The plasmid pEmuGN has a size of 6.504 kb (Figure 1C). It contains also the reporter GUS gene controlled by the Emu promoter and the Nopaline Synthetase (NOS) terminator. The Emu promoter is a recombinant promoter based on truncated maize Adh1 promoter, with multiple copies of the Anaerobic Responsive Element from the maize Adh1 gene and OCS-elements from the octopine synthase gene of *A. tumefaciens* (Last et al., 1991).

Preparation of DNA-coated tungsten micro-projectiles

10 mg of tungsten micro-projectiles (1.2 µm in diameter) were put in 1 ml of ethanol 95%, sonicated for 2 min and let to stand for 2 h. The microprojectiles were then centrifuged, subjected to two rinses of 2 h each and stored in 200 µl of glycerol 50%. The DNA/microprojectiles mixture was prepared by adding to 20 µl of microprojectiles, 4 µl of 1 µg/µl plasmid DNA, 20 µl of 2.5 M $CaCl_2$ and 8 µl of 0.1 M spermidine. The mixture was then vortexed for 20 s, incubated for 10 min on ice and 30 µl of the supernatant were removed. DNA of plasmid p35SGUS or plasmid pEmuGN was used to transform the cells for transient GUS gene expression. A DNA mixture of the plasmids pULGU1 and pEmuGN (1/1) was used to bombard the cells for their stable transformation.

Assessing the transient expression of GUS gene

Embryogenic calli of less than 250 and 630 µm in diameter were respectively transferred to twenty nitrocellulose membranes and bombarded with the plasmid pEmuGN or the plasmid p35SGUS. Control membranes were bombarded without plasmid. 48 h after bombardment, the nitrocellulose membranes were incubated at 37°C for 24 h in a GUS histochemical assay buffer containing 0.1 M $NaPO_4$ pH 7.0, Triton X-100 1% and 0.5 mg/ml X-GLUC. GUS activity was measured by counting GUS positive spots (GUPS) due to cleavage of the X-GLUC substrate which leads to a blue-coloured precipitate (Figure 3A).

Selection of stable transformed calli

Eight nitrocellulose membranes carrying calli of less than 250 µm in diameter were bombarded with a mixture of plasmids pEmuGN and pULGU1. After bombardment, nitrocellulose membranes were transferred to the MS 2.5, 1, 1 medium, that is, Murashige and Skoog medium, supplement with 2.5 mg/l 2,4-dichlorophenoxyacetic acid, 1 mg/l napthalene acetic acid, 1 mg/l 6-benzylaminopurine. The MS 2.5, 1,1 medium was also supplemented with 30 nM chlorsulfuron, a lethal concentration for non-transformed-cells of *Pennisetum glaucum* (Tiécoura and Ahoussou, 2001). Two months after bombardment, four calli selected in these conditions and whose growth rate was not affected by a concentration of up to 200 nM chlorsulfuron were considered chlorsulfuron-resistant calli. They were labelled CAL1, CAL2, CAL3 and CAL4 and used for GUS activity assay 2, 6 and 9 months after bombardment.

GUS fluorometric assay

β-glucuronidase extraction from P. glaucum cells

300 mg of *P. glaucum* calli or leaves was pounded, in 500 µl of an extraction buffer composed of 50 mM $NaPO_4$ (pH7.0), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosyl, Triton X-100 0.1%, and the supernatant was collected after centrifugation for 3 min.

GUS activity monitoring

0.5 ml of GUS assay buffer, composed of extraction buffer, methanol 20% (v/v) and 1.25 mM 4-MUG (4-methylumbelliferyl β-D-galactopyranoside), was mixed with 50 µl of β-glucuronidase extracted from *P. glaucum* cells. The mixture was incubated in a double boiler at 37°C and every 30 min, 100 µl were aliquoted and added to 900 µl of a stop buffer consisting of 0.2 M Na_2CO_3. The fluorescence of MU (4-methyl umbelliferyl), due to the cleavage of the substrate 4-MUG, was measured with a « KONTRON SFM 25 » fluorometer (excitation at 365 nm, emission at 455 nm). The fluorometer was preliminarily calibrated with MU solutions (100 nM up to 1 µM)

GUS protein assay

100 µl of β-glucuronidase, extracted from *P. glaucum* cells, were added to 3 ml of reagent containing 100 mg/l Coomassie Brilliant Blue G-250, 50 ml/l ethanol 95%, 100 ml/l phosphoric acid 85%. The optical density (OD) of the mixture was measured at 595 nm after incubation for 5 min. A calibration curve was constructed using bovine serum albumin. GUS activity was assessed on the basis of the optical density of the mixture which depends on the quantity in nanomole of MUG cleaved per minute and per mg of GUS protein.

DNA analysis of chlorsulfuron-resistant calli and plants

Six months after bombardment, plants were regenerated from resistant calli. In order to verify the presence of the GUS gene of *E. coli* and the mutant ALS gene of *A. thaliana* in the transformed cells of pearl millet, total genomic DNA of chlorsulfuron-resistant calli and plants (Figure 3B) were analysed respectively three and six months after bombardment, by Southern blotting. Genomic DNA extracted from non-transformed calli was used as control. The mutant ALS gene of *A. thaliana* was detected using a probe of 1.7 kbp obtained by digesting the plasmid pULGU1 with the enzymes *NcoI* and *BglII*. The probe contains the 5'coding region of the mutant ALS gene of *A. thaliana* (Figure 5). The presence of the GUS gene was tested using a probe of 2.797 kbp-long obtained by digesting the plasmid pEmuGN with the restriction enzymes *EcoRI* and *BamHI*. This fragment contains the intron 1, the coding region of the GUS gene and the NOS terminator (Figure 6). In each case, the genomic DNA of chlorsulfuron-resistant calli and plants was also digested with the restriction enzymes used to isolate the probes. The probes were labelled with digoxigenin.

Genomic DNA was extracted from 1 g of non-transformed and transformed-cells of *P. glaucum* calli or plants. 10 µg of each DNA sample, non-digested or digested with the appropriate restriction enzymes, was submitted to electrophoresis on 0.8% agar gel for 2 h. DNA fragments were then transferred to Nytran nylon membrane. Non-radioactive probes labelled with digoxigenin were used to detect the target fragments of GUS and ALS genes.

RESULTS

Transient expression of the reporter GUS gene

Results of GUS activity, 48 h after bombardment of the calli, are presented in Figure 2. No blue spot was observed on control nitrocellulose membranes. Blue-coloured GUS positive spots (GUPS) were observed on nitrocellulose membranes bombarded with plasmids p35SGUS and pEmuGN. The average number of GUPS was higher on nitrocellulose membranes carrying calli of less than 250 µm in diameter; 100 and 250 spots were recorded after bombardment with plasmids p35SGUS and pEmuGN, respectively. In the case of nitrocellulose membranes carrying calli of less than 630 µm in diameter, an average of 20 and 25 GUPS were recorded when the bombardment was performed with plamsids

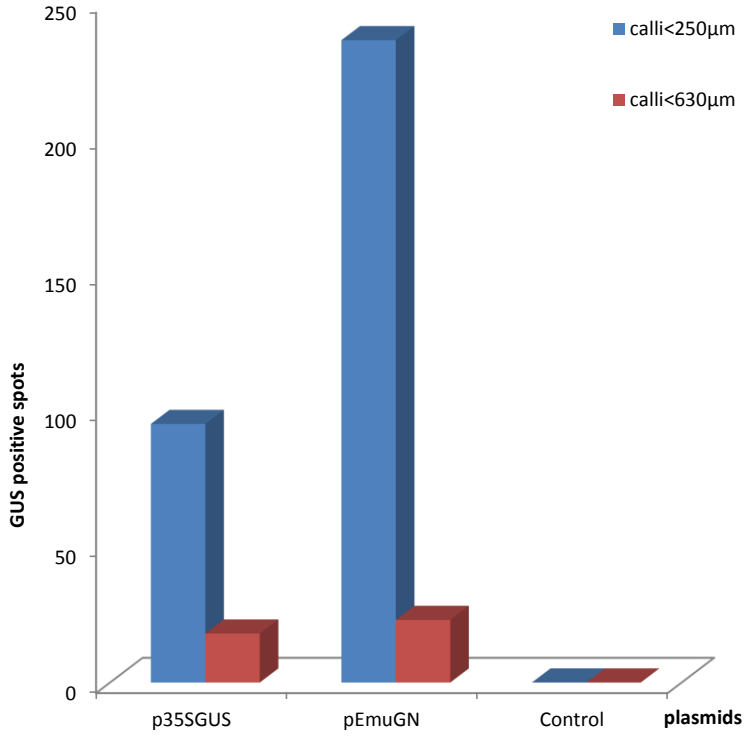

Figure 2. Histochemical assay of the β-glucuronidase (GUS) gene activity in *P. glaucum* transformed-calli, 48 h after bombardment. GUPS = number of blue-coloured GUS positive spots; calli<250 µm and calli<630 µm = respectively calli size.

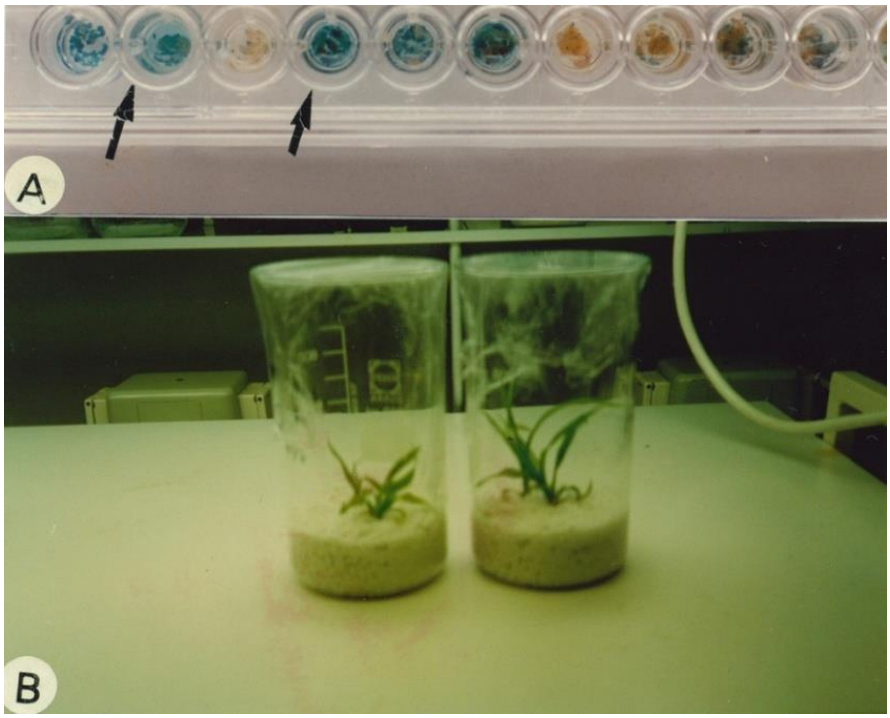

Figure 3. Stable *P. glaucum* transformation using microprojectile bombardment. **A)** Monitoring GUS activity in chlorsulfuron-resistant calli by X-GLUC staining. **B)** Pearl millet plants regenerated from stable chlorsulfuron-resistant calli.

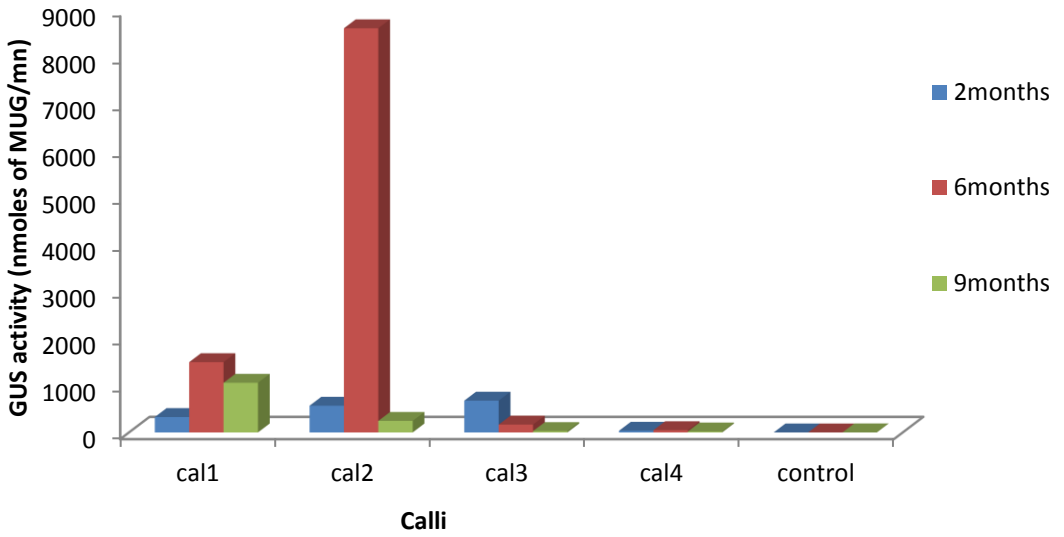

Figure 4. Fluorometric GUS assay for assessing the β-glucuronidase (GUS) gene activity in *P. glaucum* transformed-calli, 2, 6 and 9 months after bombardment. GUS activity = Activity of the β-glucuronidase (GUS) gene assessed on the basis of the optical density which depends on the quantity (in nanomole) of the substrate MUG (4-methylumbelliferyl β-D-glucuronide) cleaved per minute. CAL1, CAL2, CAL3 AND CAL4 = chlorsulfuron-resistant calli selected 2 months after bombardment; Control = non-transformed callus.

p35SGUS and pEmuGN, respectively.

Long term expression of the reporter GUS gene in selected chlorsulfuron-resistant calli

Figure 4 presents the evolution of GUS activity in four selected chlorsulfuron-resistant calli during nine months after bombardment. Apart from CAL4 (50 nmoles of MU/min/mg GUS Prot.), GUS activity was high in transformed-calli. In CAL1 and CAL2, GUS activity increased during the six first months after bombardment, from 400 to 1500 nmoles of MU/min/mg GUS Prot. and 500 to 900 nmoles of MU/min/mg GUS Prot, respectively. GUS activity decreased thereafter and reached 1100 and 300 nmoles of MU/min in CAL1 and CAL2, respectively, nine months after bombardment. GUS activity was high in CAL3, (1000 nmoles of MU/min/mg GUS Prot.) two months after bombardment and decreased up to 30 nmoles of MU/min/mg GUS Prot., nine months after bombardment.

Detection of the mutant ALS gene in chlorsulfuron-resistant calli and plants

Figure 5A-C present the results of DNA analysis of chlorsulfuron-resistant calli, (CAL1, CAL2, CAL3 and CAL4), by southern blotting, three and six months after bombardment. Three months after bombardment (Figures 5A and B), the probe did not hybridize with the control DNA (lane 1). In contrast, it hybridized with non-digested DNA (lanes 2, 3, 4, and 5) and digested DNA (lanes 6, 7, 8 and 9) of CAL1, CAL2, CAL3 and CAL4, respectively. The digested genomic DNA of these calli harbours a 1.7 Kbp fragment equalling the size of the probe obtained from the digestion of the plasmid pULGU1 by the restriction enzymes *BglII* and *PstI* (lane 13). Six months post bombardment (Figure 5C), similar results were obtained for non-digested DNA (line 2 and 3) and digested DNA (lanes 4 and 5) of pearl millet plants regenerated from CAL1 and CAL2

Luminescence intensities of the 1.7 Kbp DNA fragments revealed in CAL1, CAL2 CAL3 and CAL4 (Figure 5B: lanes 6, 7, 8 and 12) correspond to the luminescence intensities of 5 to 10 copies of the ALS gene (Figure 5B: lanes 11 and 12).

A DNA fragment of 7.7 Kbp long, corresponding to the linearized plasmid pULGU1, was observed three months after bombardment (Figure 5A) in the genomic DNA of CAL1 and CAL2, non-digested (lanes 2 and 3) or digested by the restriction enzymes *BglII* and *PstI* (lanes 10 and 11). And 7.7 Kbp DNA fragment was no longer observed six months after bombardment (Figure 5B: lanes 2 and 3).

Detection of the reporter GUS gene in chlorsulfuron-resistant calli and plants

Results of detection of the reporter GUS gene, by southern blotting, in genomic DNA of chlorsulfuron- resistant

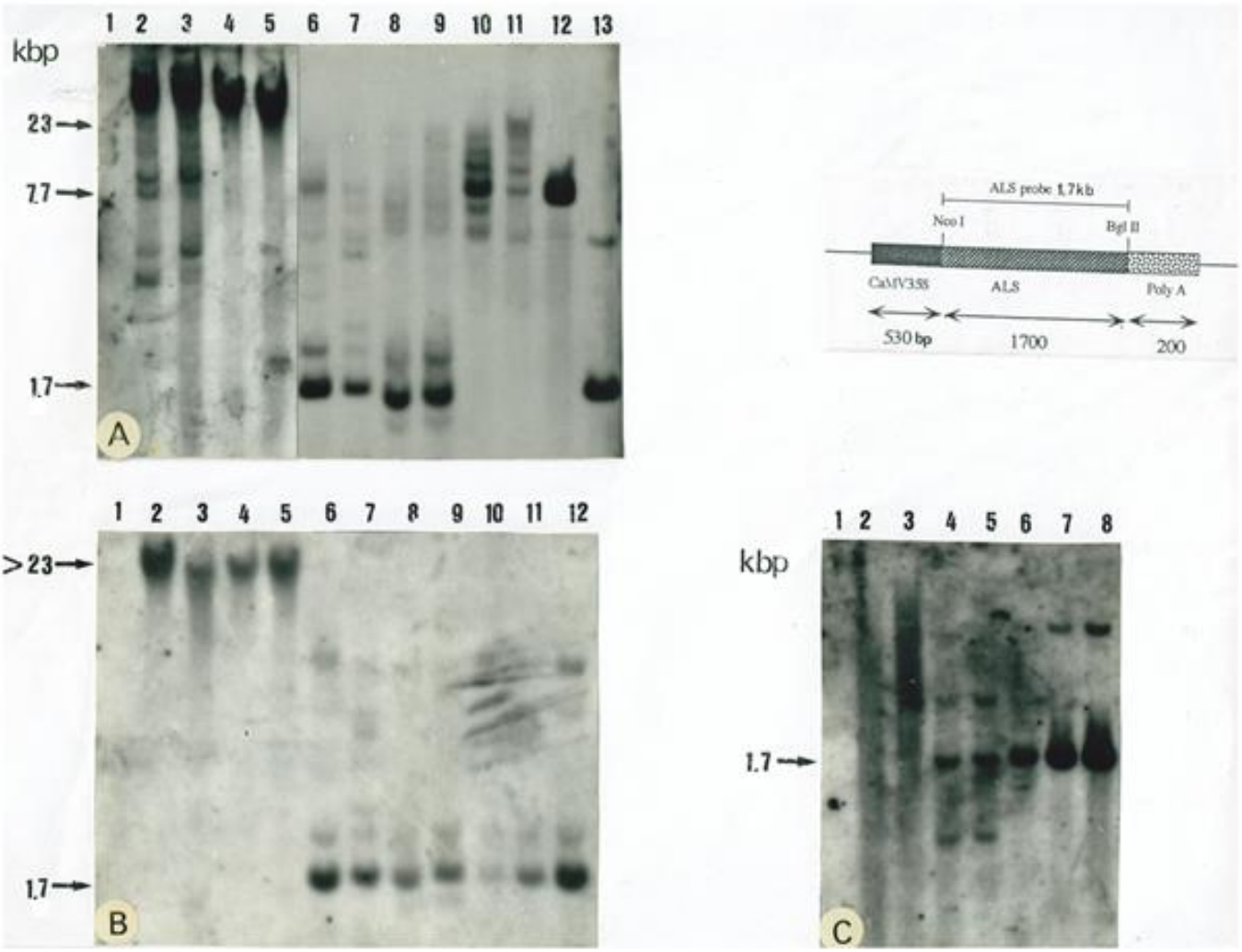

Figure 5. Southern blot analysis of genomic DNA of pearl millet chlorsulfuron-resistant calli (A-B) and plants (C) for detection of the mutant acetolactate synthase (ALS) gene, respectively 3 and 6 months after bombardment. The presence of the ALS gene was checked using a probe of 1.7 kbp-long obtained by digesting the plasmid pULGU1 with the restriction enzymes NCoII and BglII. The plasmid pULGU1 linearized by digestion with the restriction enzyme PstI or BglII has a size of 7.7 kbp. **A.** DNA analysis of chlorsulfuron-resistant calli (CAL1, CAL2, CAL3 and CAL4) three months after bombardment. lane 1: Non digested control DNA of non-transformed callus; lanes 2, 3, 4 and 5: Non-digested DNA of CAL1, CAL2, CAL3 and CAL4, respectively; lanes 6, 7, 8 and 9: DNA of CAL1, CAL2, CAL3 and CAL4, respectively, digested with the restriction enzymes NCoII and BglII; Lane 10: DNA of CAL1 digested with BglII; lane 11: DNA of CAL2 digested with PstI; lane 12: 34 pg DNA of plasmid pULGU1 digested with PstI; lane 13: 34 pg DNA of plasmid pULGU1 digested with NCoII and BglII. **B.** DNA analysis of chlorsulfuron-resistant calli (CAL1, CAL2, CAL3 and CAL4) six months after bombardment. lane 1: Non digested control DNA of non-transformed callus; lanes 2, 3, 4 and 5: non-digested DNA of CAL1, CAL2, CAL3 and CAL4, respectively; lanes 6, 7, 8 and 9: DNA of CAL1, CAL2, CAL3 and CAL4, respectively, digested with the restriction enzymes NCoII and BglII; lanes 10, 11 and 12: Respectively 1, 5 and 10 copies of plasmid pULGU1 digested with NCoII and BglII. **C.** DNA analysis of pearl millet plants regenerated from two chlorsulfuron-resistant calli (CAL1 and CAL2), six months after bombardment. lane 1: Non digested control DNA of non-transformed callus; Lanes 2 and 3: Non-digested DNA of plants regenerated from CAL1 and CAL2, respectively; lanes 4 and 5: DNA of plants regenerated from CAL1 and CAL2, respectively, digested with NCoII and BglII; lanes 6, 7 and 8: respectively 1, 5 and 10 copies of plasmid pULGU1 were digested with NCoII and BglII.

calli and plants are presented in Figure 6. Figure 6A presents results of DNA analysis of chlorsulfuron-resistant calli, (CAL1, CAL2, CAL3 and CAL4) three months after bombardment. The probe did not hybridize with the control DNA (lane 1). The probe hybridized to non-digested DNA (lanes 2, 3, 4, and 5) and digested DNA (lanes 6, 7, 8 and 9) of CAL1, CAL2, CAL3 and CAL4, respectively. The digested DNA of these calli revealed a 2.797 Kbp fragment equal to the size of the probe obtained from the digestion of plasmid pEmuGN by the restriction enzymes *BamHI* and *EcoRI* (lanes 10, 11 and 12). Luminescence intensities of the 2.797 Kbp DNA

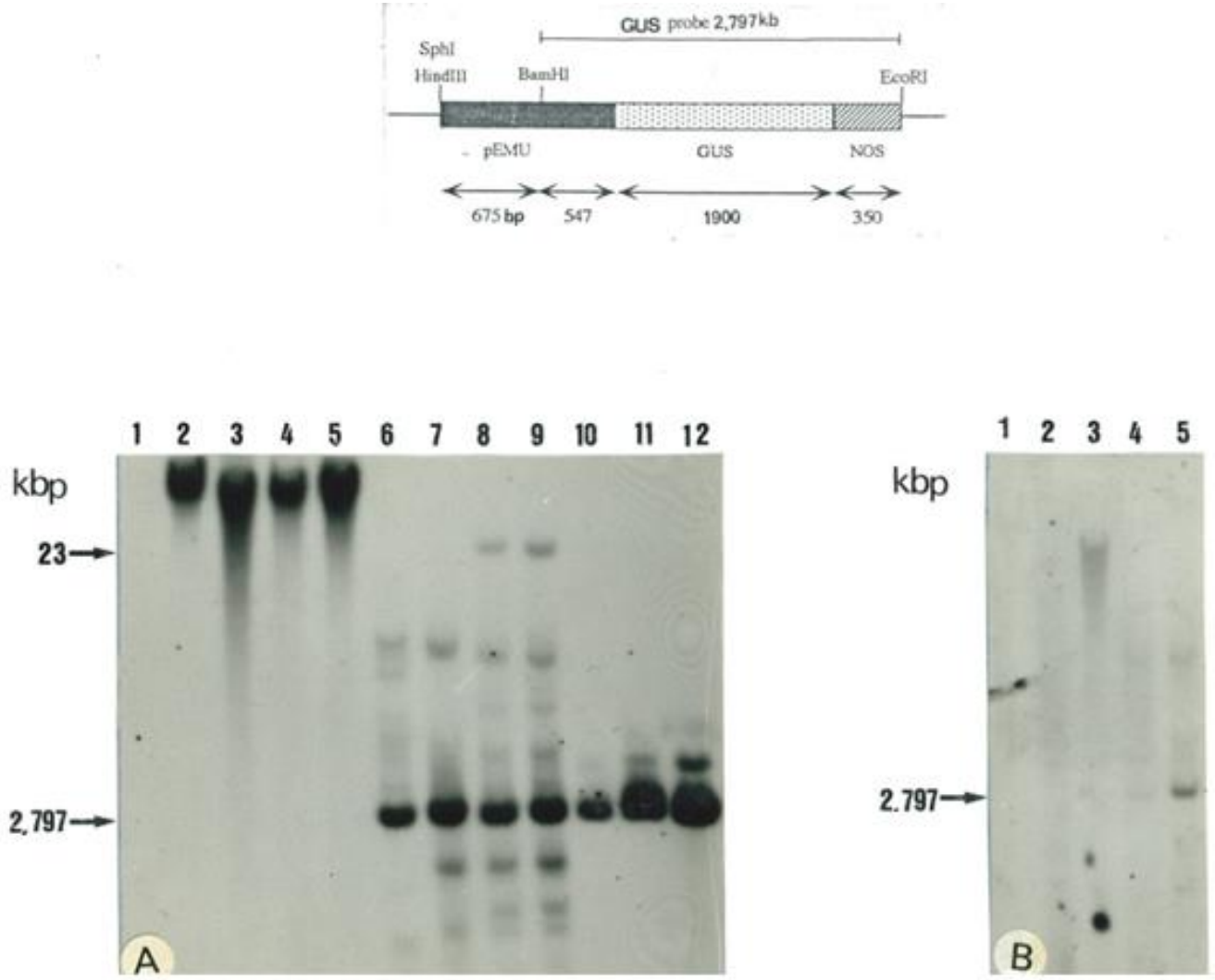

Figure 6. Southern blot analysis of genomic DNA of pearl millet chlorsulfuron-resistant calli (A) and plants (B) for detection of the β-glucuronidase (GUS) gene, respectively three and six months after bombardment. The presence of the GUS gene was checked using a probe of 2.797 kbp-long obtained by digesting the plasmid pEmuGN with the restriction enzymes ECoRI and BamHI. A: DNA analysis of chlorsulfuron-resistant calli (CAL1, CAL2, CAL3 and CAL4) three months after bombardment. Lane 1: Non digested control DNA of non-transformed callus; lanes 2, 3, 4 and 5: non-digested DNA of CAL1, CAL2, CAL3 and CAL4, respectively; lanes 6, 7, 8 and 9: DNA of CAL1, CAL2, CAL3 and CAL4, respectively, digested with the restriction enzymes ECoRI and BamHI; lanes 10, 11 and 12: respectively 1, 5 and 10 copies of plasmid pEmuGN digested with ECoRI and BamHI. **B.** DNA analysis of pearl millet plants regenerated from two chlorsulfuron-resistant calli (CAL1 and CAL2), six months after bombardment. Lane 1: non digested control DNA of non-transformed callus; lanes 2 and 3: Non-digested DNA of plants regenerated from CAL1 and CAL2, respectively; lanes 4 and 5: DNA of plants regenerated from CAL1 and CAL2, respectively, digested with ECoRI and BamHI.

fragments detected in CAL1, CAL2 CAL3 and CAL4 (Lanes 6, 7, 8 and 9), corresponds to 1 to 2 folds the luminescence intensity of one copy of the GUS gene (lane 10).

Six months post bombardment (Figure 6B), the probe did not hybridize with non-digested DNA (Line 2) and digested DNA (Line 4) of pearl millet plants regenerated from CAL1. The GUS gene was detected in DNA of plants regenerated from CAL2 but the luminescence intensity was weak (Lanes 3 and 5).

DISCUSSION

GUS positive spots (GUPS) observed on bombarded nitrocellulose membranes indicate that plasmids p35SGUS and pEmuGN have been successfully introduced in *P. glaucum* cells. The low average number of GUPS (20 and 25, respectively for plasmid p35SGUS and plasmid pEmuGN) observed on nitrocellulose membranes carrying calli of less than 630 µm in diameter could be explained on the bases of calli size. It is possible

that, because of their relatively larger size, they hardly stick to the nitrocellulose membrane, and they have probably been scattered by the micro-projectiles.

Regardless of the size of the calli carried by the bombarded nitrocellulose membranes, average numbers of GUPS increased when the bombardment was performed with plasmid pEmuGN (Figure 1). This result is due to the fact that the *Emu* promoter is stronger than the 35S promoter (Last et al., 1991; Li et al., 1997; Chowdhury et al., 1997; Tiecoura et al.; 2001). It has also been observed that other promoters are stronger than the 35S promoter when used for *in vitro* transformation of different monocot and dicot plants. Indeed, the Cauliflower Mosaic Virus (CaMV) 35S promoter was less effective in driving the GUS gene expression in tomato (dicot) and maize (monocot) plant tissues as compared to the maize promoters Gos-2, Enolase, Actin-2 (Shireen et al., 2002) and Actin-1 (Prakash et al., 2008). By using immature and mature embryos, shoot tips and embryogenic calli for *in vitro* transformation of *S. bicolor*, Tadesse et al. (2003) observed that the strength of the CaMV 35S promoter ranked after those of the *ubi*1, *act*1*D*, *adh*1 promoters. Two switchgrass (*Panicum virgatum* L.) ubiquitin genes promoters PvUbi1 and PvUbi2, showed strong expression of the GUS gene in switchgrass and rice calli, equaling or surpassing the expression levels of the CaMV 35S promoter (Mann et al., 2011). Furthermore, in these studies, GUS expression levels driven by the different promoters vary according to the plant species and the type of explants used for *in vitro* transformations. Our results confirmed these between-plant species and between-promoters variabilities of GUS gene expression. They are also consistent with previous *in vitro* transformation experiments which showed that the *Emu* promoter is a strong promoter for transient and stable transgene expression in monocot (Singh et al., 2013).

By co-bombarding embryogenic cells of pearl millet with a mixture of plasmids pULGU1 and pEmuGN carrying respectively, the mutant Acetolactate Synthetase gene of *A. thaliana* and the reporter GUS gene of *E. coli*, we have obtained chlorsulfuron-resistant calli in which GUS activity was detectable during nine months post bombardment. However, the evolution of GUS activity presented different patterns in four selected chlorsulfuron-resistant calli. Indeed, in two calli, GUS activity increased during the six first months post bombardment and decreased thereafter. In contrast, GUS activity only decreased in two other calli. The variability of GUS activity in transformed tissues is a common feature reported by different authors. For instance, Peng et al. (1990) introduced the GUS gene in protoplasts of different rice (*O. sativa*) genotypes by polyethylene glycol-mediated transformation and noted that evolution of GUS activity varied according to the rice genotype. Lambé et al. (1995) transformed calli of pearl millet using biolistic and observed between and within- calli variations of long term expression of the GUS gene. At intra calli was longer in meristematic cells than in elongated differentiated cells. These different patterns of GUS activity can be explained by the fact that the used transformation methods do not target any particular region of the genome to be transformed. Thus, expression of the transgene depends on the part of the genome in which it is integrated and regulatory mechanisms to which it will be subjected (Latham et al., 2006). So, whatever the power of the promoters used for *in vitro* transformations, expression of the transgenes also depends on the genetic backgrounds, that is, the genotypes of the transformed plants (King et al., 2014). On the other hand, when considering the same plant genotype, a high physiological cell activity can lead to a strong expression of the GUS gene in some explants (Jha et al., 2011). In addition, the increase of GUS activity in some calli during the first 6 months post bombardment may be due to the increase of the number of GUS gene copies when transformed-cells undergo mitotic divisions. Whatever the variations of GUS activity, it decreases as the time after the bombardment increases and eventually disappears.

DNA analysis with isoschizomeric enzymes responding differentially to methylation of restriction sites and gene reactivation after treatment with 5-azacytidine, showed that this gradual decrease of the GUS activity is related to a progressive methylation of the transgene in transformed plants. This process is supposedly much higher in monocots (Lambé et al., 1995; Sood et al., 2011). However, in the current study, in contrast to GUS activity, resistance to chlorosulfuron was stably expressed, at least during the first nine months post bombardment, by pearl millet plants regenerated from transformed calli. Thus, since the GUS gene originates from *E. coli* (a bacteria) and the mutant ALS gene was isolated from *A. thaliana* (a higher plant), this result illustrates the fact that the origin of the transgène is a parameter determining its more or less rapid inactivation in transformed plants (Stam et al., 1997; Iyer et al., 2000). In addition, six months after the bombardment, the GUS gene was hardly detectable by southern blotting analysis in the genomic DNA of pearl millet plants regenerated from transformed calli. Thus, this suggests that the GUS gene is less stable in the genome of pearl mill than the mutant ALS gene of *A. thaliana*

Genomic DNA analysis of selected chlorsulfuron-resistant calli, by southern blotting (Figure 5A), revealed the presence of the *A. thaliana* ALS transgene in the genome of pearl millet's transformed cells. This result confirms the fact that the resistance of selected calli to chlorsulfuron is due to the expression of the ALS transgene. Despite the progress and the new interest in using Agrobacterium-mediated methods for *in vitro* transformation of graminaceous (Jha et al., 2011; Sharma et al., 2011; Plaza-Wüthrich and Tadele, 2012; Hiei et al., 2014), the particles bombardment is still an effective technique for the introduction of transgenes in monocot plant cells with an average transformation frequency of more than 50%

(Goldman et al., 2003). Thus, our results indicate that the biolistic method is suitable for *in vitro* transformation of *P. glaucum* varieties of Côte d'Ivoire because these varieties are recalcitrant to plants regeneration from protoplasts (Tiécoura et al., 2015).

The probe used for southern blotting analysis should not have any homologous sequence in the target genome in order to allow an effective identification of transformed plant materials (Li et al., 1992). The authors observed no cross-hybridization between the probe, consisting of the 5'-half segment of the mutant *A. thaliana* ALS coding sequences, and the genomic DNA extracted from non-transformed pearl millet calli and plants. This result is in agreement with previous studies which showed that the 5'-half segments of ALS coding sequences are sufficiently different between monocot and *A. thaliana* to eliminate cross-hybridization with non-transformed material when performing southern blotting analyses (Park et al., 2002; Miki and McHugh, 2004).

Three months post bombardment, a DNA fragment of 7.7 Kbp long, equalling the size of the plasmid pULGU1, was observed in the genomic DNA of two chlorsulfuron-resistant calli, non-digested or digested by the restriction enzymes BglII and Pst1 (Figure 5A). However, this fragment of 7.7 Kbp long was no longer observed six months post bombardment. These results indicate that, at least up to three months after bombardment, some plasmids remained free, that is, not integrated in the genomic DNA of the transformed cells. Free plasmid DNA has also been observed in transformed cells of plants such as wheat, barley, maize and Colza (Langridge et al., 1992; Cheng et al., 2004, 2010). Langridge et al. (1992) stated, first, that endophytic microorganisms, such as mycoplasma associated with cereals cells, are responsible for the free plasmid-DNA observed in transgenic cells. Then, Cheng et al. (2004, 2010) indicated that the presence of free plasmid DNA may be due to the fact that the stable transformation of plant cells is progressive and, cell organelles such as chloroplasts and mitochondria are also transformed. So, in these organelles which are very close to the prokaryotes, the plasmid DNA can be free for a long time before being digested by plant endonucleases. Finally, Rivera et al. (2012) and Sah et al. (2014) supported these two hypotheses indicating that other organelles or cellular mico-organisms can harbor plasmid DNA outside the nucleus of transformed cells.

Six months after bombardment, southern blot analysis revealed the presence of the mutant ALS gene of *A. thaliana* in the genomic DNA of pearl millet plants regenerated from chlrosulfuron-resistant calli (Figure 5C). This result indicates that the regenerated plants have been stably transformed. Pearl millet transformation has been performed for resistance to some herbicides and some pathogens (Girgi et al., 2002; Goldman et al., 2003; Ceasar and Ignacimuthu, 2009). However, to the knowledge of the authors, the first transgenic plants of *P. glaucum* resistant to the chlorsulfuron herbicide were obtained in the current study.

According to luminescence intensities of the DNA fragments revealed by the probes in the genomic DNA of chlorsulfuron-resistant calli, respectively 1 to 2 copies of the GUS gene and 5 to 10 copies of the mutant ALS gene of *A. thaliana* were integrated in the genomic DNA of pearl millet. Integration of many copies of the trans-gene, even in the same site or in different chromosomal locations, is an important factor associated with gene silencing in transformed plants (Stam et al., 1997). An increase of single copy insertion has been obtained by optimizing the factors influencing microprojectile bombardment (Jagga-Chugh et al., 2012; Tassy et al., 2014). Application of optimization conditions for further bombardments should thus allow us to obtain pearl millet transformants harboring single copy of the mutant ALS gene of *A. thaliana.*

In conclusion, in the present study, transgenic plants of Côte d'Ivoire's *P. glaucum* NE variety, resistant to chlorsulfuron, by co-bombardment of embryogenic calli have been obtained with the mutant ALS gene of *A. thaliana* and the reporter GUS gene of *E. coli.* Latha et al. (2006) used a similar approach to develop transgenic pearl millet plants resistant to mildew. So, since several genes of interest can be introduced into the genomic DNA of pearl millet without interfering with their activities, co-transformation could be a helpful means to improve *P. glaucum* varieties of Côte d'Ivoire.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Altpeter F, Xu J, Salahuddin A (2000). Generation of large numbers of independently transformed fertile perennial ryegrass (*Lolium perenne* L.) plants of forage- and turf-type cultivars. Mol. Breed. 6(5):519-528.

Bahieldin A, Eissa HF, Mahfouz HT, Dyer WE, Madkour MA, Qu RD (2005). Evidence for non-proteinaceous inhibitor(s) of beta-glucuronidase in wheat (*Triticum aestivum* L.) leaf and root tissues. Plant Cell Tissue Org. Cult. 82(1):11–17.

Bajaj S, Mohanty A (2005). Recent advances in rice biotechnology – towards genetically superior transgenic rice. Plant Biotechnol. J. 3(3):275–307.

Bajaj YPS (2012). Biotechnology in Agriculture and Forestry 9: Plant Protoplasts and Genetic Engineering II. Springer Science & Business Media. 499p.

Battraw M, Hall TC (1991). Stable transformation of Sorghum bicolor protoplasts with chimeric neomycin phosphotransferase II and b-glucuronidase genes. Theor. Appl. Genet. 82:161-168.

Cass AM, Kononowicz AK, Haan TG, Tomes DT, Bressan RA, Hasegawa PM (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. *In Vitro* Cell Dev. Biol. Plant 33:92-100.

Ceasar SA, Ignacimuthu S (2009). Genetic engineering of millets: Current status and future prospects. Biotechnol. Lett. 31:779-788.

Cheng L, Li HP,Qu B, Huang T, Tu JX, Fu TD, Liao YC (2010). Chloroplast transformation of rapeseed (*Brassica napus*) by particles bombardment of cotyledons. Plant Cell Rep. 29:371-381.

Cheng M, Lowe BA, Spencer TM, Ye XD, Armstrong CL (2004). Factors

influencing Agrobacterium-mediated transformation of monocotyledonous species. In Vitro Cell. Plant Biol. Dev. 40:31-45.
Chowdhury MKU, Parveez GKA, Saleh NM (1997). Evaluation of five promoters for use in transformation of oil palm *(Elaeis guineensis* Jacq.). Plant Cell Rep. 16:277 281.
Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001). Comparative analysis of transgenic rice plants obtained by Agrobacterium-mediated transformation and particle bombardment. Mol. Breed. 7(1):25-33.
Danilova SA (2007). The technologies for genetic transformation of cereals. Russ. J. Plant Physiol. 54:569–581.
Dekeyser RA, Claes B, De Kycke RMU, Habets ME, Van Montagu MC, Caplan AB (1990). Transient gene expression I intact and organized rice tissues. Plant Cell 2:591-602.
Girgi M, O'Kennedy MM, Morgenstern A, Mayer G, Lörz H, Oldach KH (2002). Trasgenic and herbicide resistant pearl millet (*Pennisetum glaucum* L.) R.Br. via microprojectile bombardment of scutellar tissue. Mol. Breed. 10:243-252.
Girijashankar V, Sharma HC, Sharma KK, Swathisree V, Sivarama PL, Bhat BV, Royer M, San Secundo B, Lakshmi NM, Altosaar I, Seetharama N (2005). Development of transgenic *Sorghum* for insect resistance against the spotted stem borer (*Chilo partellus*). Plant Cell Rep. 24(9):513-522.
Girijashankar V, Swathisree V (2009). Genetic transformation of *Sorghum bicolor* Physiol. Mol. Biol. Plants 15(4):287-302.
Goedeke S, Hensel G, Kapusi E, Gahrtz M, Kumlehn J (2007). Transgenic Barley in Fundamental Research and Biotechnology. Transgenic Plant J. 1(1):104-117
Goldman JJ, Hanna WW, Fleming G (2003). Fertile transgenic pearl millet (*Pennisetum glaucum* (L.) R. Br.) plants recovered through microprojectile bombardment and phosphinothricin selection of apical meristem-, inflorescence-, and immature embryo-derived embryogenic tissues. Plant Cell Rep. 21:999-1009.
Hagio T, Hirabayashi T, Machii H, Tomotsune H (1995). Production of fertile transgenic barley (*Hordeum vulgare* L.) plant using the hygromycin-resitance marker. Plant Cell Rep. 14:329-334.
Haughn GW, Somerville C (1986). Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol. Gene Genet. 204:430-434.
Hiei Y, Ishida Y, Komari T (2014). Progress of cereal transformation technology mediated by *Agrobacterium tumefaciens*. Front. Plant Sci. 5: 628.
Hussain J, Manan S, Ahmad R, Ahmed T, Shah MM (2013). Biotechnologies used in genetic transformation of *triticum aestivum*: A mini overview. FUUAST J. Biol. 3(2):105-109.
Hwang H-H, Wu E T, Liu S-Y, Chang S-C, Tzeng K-C, Kado CI (2013) Characterization and host range of five tumorigenic *Agrobacterium tumefaciens* strains and possible application in plant transient transformation assays. Plant Pathol. 62(6):1384–1397.
Iyer LM, Kumpatla SP, Chandrasekharan MB, Hall TC (2000). Transgene silencing in monocots. In: Plant gene silencing; Springer Science and Business Media Dordrecht. pp. 203-226.
Jagga-Chugh S, Kachhawaha S, Sharma M, Kothari-Chajer A, Kothari SL (2012). Optimization of factors influencing microprojectile bombardment-mediated genetic transformation of seed-derived callus and regeneration of transgenic plants in *Eleusine coracana* (L.) Gaertn. Plant Cell Tissue Organ Cult. 109:401–410.
Jähne A, Becker D, Lötz H (1995). Genetic engineering of cereal crop plants: a review. Euphitica 85:35-44.
Jha P, Shashi RA, Agnihotri PK, Kulkami VM, Bhat V (2011). Efficient Agrobacterium-mediated transformation of *Pennisetum glaucum* (L)R. Br. Using shoot apies as explants sources. Plant Cell Tissue Organ Cult. 107:501-512.
Ji Q, Xu X, Wang K (2013). Genetic transformation of major cereal crops. Int. J. Dev. Biol. 57:495-508.
Jones HD (2005). Wheat transformation: current technology and application to grain development and composition. J. Cereal Sci. 41: 137-147.
Jordan CA, Neumann E, Sowers AE (2013). Electroporation and Electrofusion in Cell Biology. Springer Science & Business Media. 436 p.
Kaeppler HF, Gu W, Somers DA, Rines HW Cockburn AF (1990). Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Rep. 9:415-418.
King ZR, Bray AL, LaFayette PR, Parrott WA (2014). Biolistic transformation of elite genotypes of switchgrass (*Panicum virgatum* L.). Plant Cell Rep. 33:313–322.
Klein TM, Wolf ED, Wu R. Sanford JC (1987). High-velocity microprojectiles for delivering nucleic acid into living cells. Nature 327:70-73.
Lambé P, Dinant M, Matagne R (1995). Differential long-term expression and methylation of the hygromycin phosphotransferase (hph) and B-glucuronidase (GUS) genes in transgenic pearl millet (*Pennisetum glaucum*) callus. Plant Sci. 108:51-62.
Langridge P, Brettschneider R, Lazzeri P, Lörz H (1992). Transformation of cereals via Agrobacterium and the pollen pathway a critical assessment. Plant J. 2:631-638.
Last DI, Brettell RIS, Camberlain DA, Chaudhury AM, Larking PJ, Marsh EI, Peacock WJ, Dennis ES (1991). pEmu: an improved promoter for gene expression in cereal cells. Theor. Appl. Genet. 81: 581-588.
Latha AM, Rao LV, Reddy TP (2006). Development of transgenic pearl millet (*Pennisetum glaucum* (L.)R. Br.) plants resistant to mildew. Plant Cell Rep. 25:927-935.
Latham JR, Wilson AK, Steinbrecher RA (2006). The Mutational Consequences of Plant Transformation. J. Biomed. Biotechnol. 2006(2):25376.
Li Z, Hayashimoto A, Murai N (1992). A Sulfonylurea Herbicide Resistance Gene from *Arabidopsis thaliana* as a New Selectable Marker for Production of Fertile Transgenic Rice Plants. Plant Physiol. 100:662 – 668.
Li Z, Upadhyaya NM, Meena Selva, Gibbs AJ, Waterhouse PM (1997). Comparison of promoters and selectable marker genes for use in Indica rice. Mol. Breed. 3:1–14.
Liu YH, Yu JJ, Zhao Q, Ao GM (2005). Genetic transformation of millet (*Setaria italic*) by Agrobacterium-mediated. Agric. Biotechnol. J. 13: 32-37.
Mann DGJ, King ZR, Liu W, Joyce BL, Percifield RJ, Hawkins JS, Lafayette PR, Artelt BJ, Burris JN, Mazarei M, Bennetzen JL, Parrott WA, Stewart Jr CN (2011). Switchgrass (*Panicum virgatum* L.) polyubiquitin gene (PvUbi1 and PvUbi2) promoters for use in plant transformation. BMC Biotechnol. 11:74-87.
Martinez-Trujillo M, Cabrera-Ponce JL, Herrera-Estrella L (2003). Improvement of rice transformation using bombardment of scutellum-derived calli. Plant Mol. Biol. Rep. 21:429-437.
Mee YP, Yi NR, Lee HY, Kim ST, Kim M, Park J-H, Kim J-K, Lee JS, Cheong J-J, Choi YD (2002). Generation of chlorsulfuron-resistant transgenic garlic plants (*Allium sativum* L.) by particle bombardment. Mol. Breed. 9(3):171-181.
Miki B, McHugh S (2004). Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J. Biotechnol. 107(3):193–232.
Nap J-P, Metz PLJ, Escaler M, Conner AJ (2003). The release of genetically modified crops into the environment Part I. Overview of current status and regulations. Plant J. 33:1–18.
O'Kennedy MM, Burger JT Bosha FC (2004). Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. Plant Cell Rep. 22:684-690.
Park MY, Yi NR, Lee HY, Kim ST, Kim M, Park JH, Kim J-K, Lee JS, Cheong J-J, Choi YD (2002). Generation of chlorsulfuron-resistant transgenic garlic plants (*Allium sativum* L.) by particle bombardment. Mol. Breed. 9(3):171-181.
Peng J, Lyznik LA, Lee L, Hodges TK (1990). Co-transformation of rice protoplasts with gusA and neo genes. Plant Cell Rep. 9:168-172.
Plaza-Wüthrich S, Tadele Z (2012). Millet improvement through regeneration and transformation. Biotechnol. Mol. Biol. Rev. 7(2):48-61.
Potrykus I (1990). Gene transfer to cereals: An assessment. Nat. Biotechnol. 8:535-542.
Prakash NS, Prasad V, Chidambram TP, Cherian S, Jayaprakash TL, Dasgupta S, Wang Q, Mann MT, Spencer TM, Boddupalli RS (2008). Effect of promoter driving selectable marker on corn transformation. Transgenic Res. 17:695-704.
Prathibha D, Sticklen M (2002). Culturing shoot-tip clumps of pearl millet (*Pennisetum glaucum* (L) R. Br.) and optimal microprojectile bombardment parameters for transient expression. Euphytica 125:45-50.

Qamar Z, Aaliya K, Nasir IA, Farooq AM, Tabassum B, Ali Q, Awan MF, Ali A, Tariq M, Husnain T(2015) An overview of genetic transformation of glyphosate resistant gene in *Zea mays*. Nat. Sci. 13(3):80-90.

Que Q, Elumalai S, Li X, Zhong H, Nalapalli S, Schweiner M, Fei X, Nuccio M, Kelliher T, Gu W, Chen Z, Chilton M-DM (2014). Maize transformation technology development for commercial event generation. Front. Plant Sci. 5:379.

Ramadevi R, Rao KV Reddy VD (2014). Agrobacterium tumefaciens-mediated genetic transformation and production of stable transgenic pearl millet (*Pennisetum glaucum* (L)R. Br.). *In Vitro* Cell Dev. Biol. Plant 50:392-400.

Razzaq A, Hafiz AI, Mahmood I, Hussain A (2010). Development of in planta transformation protocol for wheat. Afr. J. Biotechnol. 10:740-750.

Rivera AL, Gomez-Lim M, Fernandez F, Loske AM (2012). Physical methods for genetic plant transformation. Phys. Life Rev. 9:308-345.

Sah SK, Kaur A, Kaur G, Cheema GS (2014). Genetic transformation of rice: Problems, Progress and Prospects. J. Rice Res. 3:132.

Sahrawat AK, Becker D, Lütticke S, Lörz H (2003). Genetic improvement of wheat via alien gene transfer, an assessment. Plant Sci. 165:1147-1168.

Sharma M, Kathari-Chajer A, Jagga-Chugh S, Kathari SL (2011). Factor influencing *Agrobacterium tumefasciens*-mediated genetic transformation of *Eleucine coracana* (L) Gaertn. Plant Cell. Tissue Organ. Cult. 105:93-104.

Shimamoto K, Terada R, Tzawa T, Fujimoto H (1989). Fertile transgenic rice plants regenerated from transformed protoplasts. Nature 338: 274-276.

Shireen KA, El-Itriby HA, Hussein EHA, Saad ME, Madkour MA (2002). Comparison of the efficiency of some novel maize promoters in monocot and dicot plants. Arab J. Biotech. 5(1):57-66.

Singh RK, Kumar P, Tiwari NN, Rastogi J, Singh SP (2013). Current Status of Sugarcane Transgenic: an Overview. Adv. Genet. Eng. 2:112.

Sood P, Bhattacharya A, Sood A (2011). Problems and possibilities of monocot transformation. Biol. Plant. 55(1):1-15.

Stam M, Mol JNM, Kooter JM (1997). The Silence of Genes in Transgenic. Plants Ann. Bot. 79:3-12.

Szabados L, Charrier B, Kondorosi A, Bruijn FJ, Ratet P (1995). New plant promoter and enhancer testing vectors. Mol. Breed. 1:419-423.

Tadesse Y, Sagi L, Swennen R, Jacobs M (2003). Optimisation of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) via microparticle bombardment. Plant Cell Tissue Org. Cult. 75:1-18.

Tassy C, Partier A, Beckert M, Feuillet C, Barret P (2014). Biolistic transformation of wheat: increased production of plants with simple insertions and heritable transgene expression. Plant Cell Tissue Organ Cult. 119:171–181.

Tiécoura K, Ahoussou N (2001). New selection agents for pearl millet (*Pennisetum glaucum* (L) R.). Rev. Iv. Sci. Tech. 2:119-129.

Tiécoura K, Kouassi AB, N'nan-Alla O, Dinant M, Ledoux L (2014). Optimisation des conditions d'établissement de suspensions cellulaires embryogènes à partir de cals d'apex caulinaire de mil (*Pennisetum glaucum* (L.) R.). Agron. Afr. 26 (3):205-215.

Tiécoura K, Kouassi AB, N'nan-Alla O, Gonedelé Bi S, Dinant M, Ledoux L (2015). Isolation and culture of protoplasts of Côte d'Ivoire's pearl millet (*Pennisetum glaucum* (L.) R.) varieties. J. Appl. Biosci. 92:8620-8929.

Tiécoura K, Ledoux L, Dinant M (2001). Expression transitoire du gène de la ß-glucuronidase (GUS) dans les protoplastes de mil (*Pennisetum glaucum* (L.) R.) : étude comparative de deux promoteurs, CaMV35S et Emu. Sci. Et Technique, Sci. Nat. Et Agron. 25(2):87-97.

Tiécoura K, Ledoux L, Dinant M (2003). Plant regeneration through tissue culture of pearl millet (*Pennisetum glaucum* (L) R.). Agron. Afr. 15(3):105-121.

Vasil IK (2012). Molecular improvement of cereal crops. Springer Science and Business Media. 402p.

Vasil IK (2008). A short history of plant biotechnology. Phytochem. Rev. 7:387–394.

Weymann K, Urban K, Ellis DM, Novitzky R, Dunker E, Jayne S, Page G (1993). Isolation of transgenic progeny of maize by embryo rescue under selective conditions. In Vitro Cell. Div. Biol. 29:33-37.

Yadav T, Kachhwaha S, Kothari SL (2013). Efficient *in vitro* plant regeneration and generation of transgenic plants in barley (*Hordeum vulgare* L.) using particle bombardment. J. Plant Biochem. Biotechnol. 22(2):202–213.