A Comparison Study for *Agrobacterium*-mediated transformation Method in Sugarcane (*Saccharum spp* L.)

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ABSTRACT

In order to compare transient expression of *gus* gene driven by CaMV 35S or rice ubiquitin RUBQ2 promoter, *Agrobacterium*-mediated transformation was conducted using embryogenic callus and suspension cultures of sugarcane. Histochemical observation of GUS activity after co-cultivation showed that rice ubiquitin promoter produced high level of clear blue spots both in embryogenic callus and suspension cultures, while the CaMV35S promoter was not detected. Regenerated shoots from the infected materials were found higher in suspension cultures than embryogenic callus. The results showed that the rice ubiquitin promoter as well as suspension cultures are more efficient for the *Agrobacterium*-mediated transformation. However, PCR analysis found there was no amplification of DNA neither for CaMV35 nor rice ubiquitin in genome DNA of regenerated shoot. Lacking of integration of the DNA into the genome showed that the regenerated shoots were not truly transformants might due to the presence of somaclonal variation that was common phenomenon in the regeneration from callus. To avoid the presence of somaclonal variation the transformation was then conduced using *in vitro* plant without intervening callus phase. Basal segment of *in vitro* plants prepared from axillary buds of sugarcane were used as the explants for the transformation. The histochemical observation of GUS activity showed that almost all of the infected materials partially exhibited blue color on the basal region. Infected *in vitro* plants showed rapidly grow and multiplied in the selection medium. Further investigation of the transformation using *in vitro* plants and *Agrobacterium* harboring *SoSUT1* DNA construct driven by rice actin promoter resulted in development of antibiotic resistant sugarcane shoots. Interestingly the PCR analysis found an insertion of the antibiotic DNA fragment into the sugarcane genome DNA. This result suggests that *in vitro* plant is an effective target tissue for *Agrobacterium*-mediated transformation in sugarcane.

Keywords: Sugarcane, genetic transformation, *Agrobacterium*, sucrose-transporter, protein

INTRODUCTION

Traditional plant breeding techniques have been widely used to enhance important traits in agronomic crops, but this approach is laborious and time-consuming, especially in species like sugarcane. Transformation of DNA in plant can serve an important function to introduce useful genes into sugarcane, where they would be difficult or impossible by standard procedure.

Recent research indicates that *Agrobacetrium*-mediated transformation is possible in monocots such as rice (Raineri *et al*. 1990, Park *et al*. 1996), maize (Ishida *et al*. 1996) and banana (May *et al*. 1995). This system offers several advantages, such as technical simplicity, minimal genome rearrangement in transformants and the ability to transfer long stretches of DNA. Although the *Agrobacterium*-mediated method has been applied also to sugarcane (Arencibia *et al*. 1998, Enriquez-Obregon *et al*. 1998), the lack of a reproducible result has been an obstacle to establish effective transformation protocol for routine genetic manipulation in the plant. The cells being traumatic due to *Agrobacterium* infection and poor survival rate. Oxidative burst, phenolization, and subsequent cell death are frequent phenomena after the infection (De la Riva *et al*. 1998).

The promoter is a key DNA regulatory element that directs appropriate strength and pattern of gene expression in a constitutive or specific manner, and therefore, plays a crucial role in successful transformation studies. There are some types of promoters that drive strong, constitutive, or organ specificity expression. For example, the viral Cauliflower Mosaic Virus 35S (CaMV 35S) promoter has been widely used in the transformation of many dicot and monocot. However, it has been demonstrated that activity of the promoter was low in sugarcane (Chowdhury *et al*. 1992, Gallo-Meagher *et al*. 1993). The rice actin 1

and the Emu elements have shown higher activity than CaMV 35 S in different sugarcane tissues (Gallo-Meagher & Ervine 1996) and it was recently reported that the rice ubiquitin promoter RUBQ2 has high transgene expression levels in sugarcane (Liu *et al*. 2003). Thus, it is an important issue to have comparative study on the type of promoters for sugarcane transformation.

Retaining desirable traits in the cell after transformation with novel genes is a major consideration for all transgenic crops improvement programs. The method of plant regeneration through callus cultures increases the risk of somaclonal variation, particularly in sugarcane (Lee 1987). Somaclonal variation has been reported in insect-resistant transgenic sugarcane plants produced by cell electrophoration of embryogenic callus (Arencibia *et al.* 1999). Direct regeneration from plants without an intervening callus phase has been reported for the transformation with shorter time required and transformation efficiencies as high as 50% (Manickavasagam *et al*. 2004).

In this article, we reported a comparative study of *Agrobacterium*-mediated transformation of *gus* gene driven by CaMV 35S or rice ubiquitin RUBQ2 with different target tissues in sugarcane.

METHOD

Plant materials

Apical portions of healthy stalk of sugarcane were collected from the field and used for callus induction and *in vitro* propagation. Leaf segment (around 20 cm) containing the apical meristem were cut and wiped with 70% ethanol. The outer leaves were aseptically removed until the inner leaf was exposed. Transverse segment 3-5 mm wide were excised just above the apical meristem and placed on callus induction medium containing Murashige-Skoog (MS) basal with 3 mg l^{-1} 2,4 D (MS1) in the dark at 26^{o}C for 3 weeks.

Mature axillary buds remaining in the stalk were aseptically isolated and after rinsed with 70% were placed on shoot formation medium containing MS basal with 0.1 mg l^{-1} 6-benzyladenin (BA) in the light at 26^{o}C for 3 weeks to establish *in vitro* grown plants.

Preparation of explants

The induced callus were subcultured into fresh MS1 medium and incubated under the same condition for 3 weeks interval. Embryogenic callus (EC) was selected based on their nodular, compact and yellowish morphological characteristic (Matsuoka *et al*. 2002). The cultured EC was transferred to liquid MS1 medium to develop suspension culture (SC) according to the method described by Arencibia *et al*. (1998).

The primary shoots developed from axillary buds were sub-cultured on secondary shoots formation under the same condition for another 3 weeks. Green and healthy (around 3 cm in height) of the secondary shoots were separated and cultured in MS basal (hormone free) medium to induce root formation of *in vitro* plants for 2 weeks. Basal segment of the shoots were excised and used as explants for the transformation.

Plasmid vectors and *Agrobacterium* culture

Transformation was performed using *Agrobacterium tumefaciens* strain LBA4404 harboring pBI121, pCL4 or pAct plasmid vector. Plasmid pBI121 and pCL4 contain *gus* gene driven by CaMV35S promoter (Toyobo, Inc.) and rice ubiquitin RUBQ2 (Liu *et al*. 2003), respectively, while plasmid pAct contains sugarcane *SoSUT1* gene (Sugiharto, 2010 unpublished result) driven by rice actin promoter (provided by Dr. Matsuoka).

A single colony of *Agrobacterium* containing each plasmid was inoculated in 3 mL liquid YEP medium containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifamphicine and incubated at 28^{o}C on shaker for 2 days. One ml of the culture was added to 50 ml of liquid YEP medium containing the antibiotic and incubated in same condition until the culture reached an OD_{600} 0.8-1.0. The culture was centrifuged at 4000 x *g* for 10 min and suspended in fresh 2 ml LB medium.

***Agrobacterium* infection and co-cultivation**

Approximately 2 g of EC was collected and around same weight of SC was aseptically filtered and brief dried treatment under laminar flow condition for 30 min. Both EC and SC were placed in flask containing 50 ml LB and subjected to sonication for 5 min. *Agrobacterium* infection was conducted by immersion into the *Agrobacterium* suspension adjusted to OD_{600} 0.8 containing acetosyringone (100 mg l^{-1}) and incubated at 28^{o}C for 30 min. Before co-cultivation, infected materials were washed once with sterile water and then brief dried treatment in laminar flow condition. Co-cultivation treatment was conducted by inoculation of the infected callus on solid MS1 medium containing acetosyringone in the dark at 28^{o}C for 3 days.

For infection of *in vitro* plants, the basal segments of *in vitro* plants were injured slightly by pricking 4-5 times with a sterile needle and injured plants were immersed in *Agrobacterium* suspension (OD_{600} 1.0) containing acetosyringone (100 mg l^{-1}). The mixture was then incubated on shaker (150 rpm) at 28^{o}C for 30 min. The infected explants were blot dried using sterile Whatman filter paper and inoculated onto basal MS solid medium containing

acetosyringone. The co-cultivation was performed for 3 days in the dark at 28°C.

Selection and regeneration of transformants

Co-cultivated materials were washed three times with 500 mg l^{-1} cefotaxime and then blot dried treatment on sterile Whatman filter paper in laminar flow condition. The infected EC and SC were inoculated on MS1 medium containing cefoxatime (500 mg l^{-1}) and keep in dark at 26°C for a week, while infected *in vitro* plants were inoculated on MS containing cefoxatime (500 mg l^{-1}) and incubated under light at 26°C also for a week. The cultures were then transferred to selective medium (same medium) containing cefotaxime and geneticin (50 mg l^{-1}) and incubated in the same condition for addition of 2-3 weeks.

Resistant callus generated from EC and SC were subcultured in the selective regeneration medium (MS2) containing cefotaxime and geneticin for additional 3 weeks. The regenerated shoots were then subcultured in same selective regeneration medium and after 3 successive cycles subcultures the shoots were subcultured in selective rooting medium (MSR) for 3 weeks. Plants from a single callus were considered as a clone of putative transformant.

Regenerated resistant *in vitro* plants were subcultured in the same selective medium for additional 2 weeks and after 5 successive cycles in the selective medium, the plant were subjected to PCR analysis.

GUS assay

The infected materials were assayed for expression of *gus* gene following the histochemical procedure described by Jefferson *et al.* (1987) with some modification. For EC and SC, the infected calli were assayed a week after co-cultivation, and *in vitro* plant was after co-cultivation. Samples were washed once with 0.1 M potassium phosphate buffer (pH 7.0) and then incubated with same buffer containing 2% methanol, 0.3% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucoronide at 37°C for overnight. GUS assay for putative transformant was conducted with same method using shoot of the putative transformants.

PCR analysis

Total genomic DNA was isolated from leaves of putative transformant plants using a DNeasy Plant Mini Kit (Qiagen) according to the manual protocol. PCR analysis was performed with the isolated genome DNA (0.5 µg), TaKaRa Ex Taq polymerase (Takara Bio Inc) and a set primer designed either from CaMV, *hpt*II or GUS DNA sequences. The PCR condition was 30 cycles at 98°C for 10 seconds, 55°C for 30 second, 72°C for 1 min, followed by 5 min final extension at 72°C. The amplified DNA were analyzed by 1% agarose gel electrophoresis and photographed.

RESULTS AND DISCUSSION

Transformation using explants embryogenic callus and suspention culture

In order to compare the transient expression of *gus* gene driven by CaMV or RUBQ2 promoter the transformation was conducted using EC and SC. Histochemical observation of GUS activity after co-cultivation showed that rice ubiquitin RUBQ2 promoter produced high level of clear blue spots both in EC and SC, while the CaMV35S promoter was not detected (Figure 1). The observation with at least one blue spot taken into account resulted in slightly increase transient GUS gene expression in SC compared to EC (Table 1). These results suggest that RUBQ2 can serve as a regulatory element to provide high levels of transgene expression.

To allow multiplication of the transformed single cells, the infected callus from EC and SC were cultured a week after co-cultivation without selection pressure, then transferred to selective callus induction medium. The antibiotic resistant callus with embryogenic appearance were obtained, and then sub-cultured to selective regeneration medium. Non-transformant callus did not show continues growth and turned brown, but the transformant callus regenerated shoots. Among the different explants, higher percentage of shoots regeneration from the callus was obtained in SC than EC (Table 1). This suggest that replacement of culture medium each two days in the SC resulted in a better nutrient availability and increase a number of meristematic cell population. The regenerated shoots were sub-cultured to the same selective medium and after 3 successive cycles were sub-cultured on selective rooting medium. Some of the antibiotic resistant shoots developed roots and vigorously grown, named as putative transformants (Figure 2).

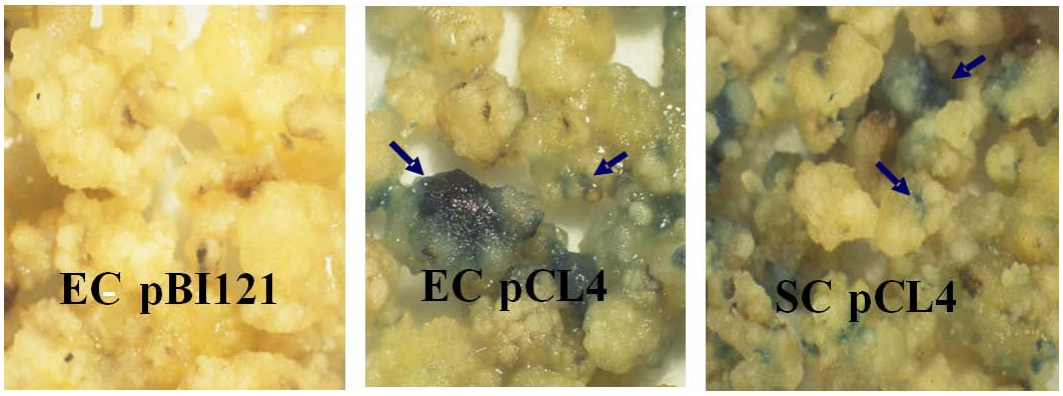


Figure 1. Different expression levels of *gus* gene driven by CaMV 35S (pBI121) and RUBQ2 (pCL4) promoters in the infected EC and SC (Blue spots represent the GUS expression (arrows) and were photographed using a stereoscopic microscopy. A visible clear blue spot in each clump was taken into account and the data is presented in Table 1).

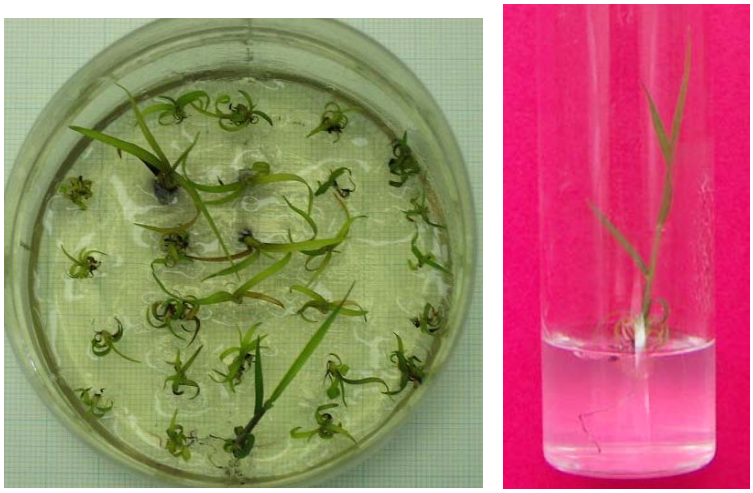
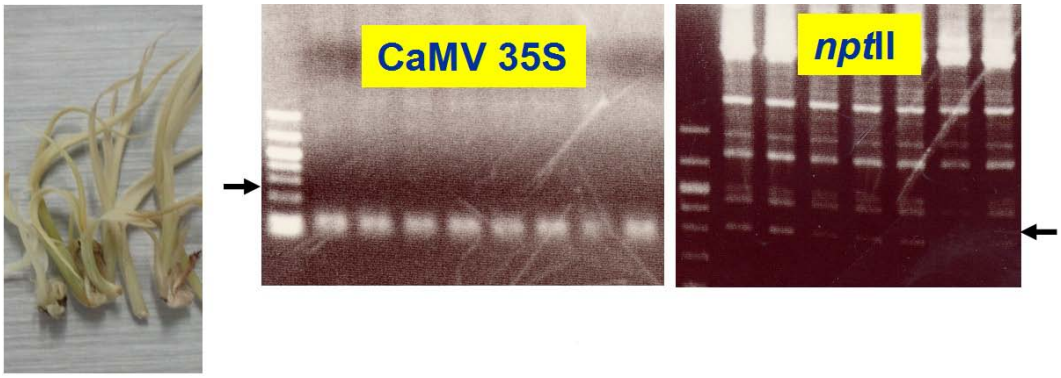


Figure 2. Growth of the putative transformants in selective rooting medium (left) containing antibiotic and after sub-cultured (right) in the same medium for additional 3 weeks.

Table 1 Comparison of Agrobacterium-mediated transformation with differences in explants and plasmid vectors.

Explants	Plasmids	GUS assay (%)*	Regenerated plantlet (%)	Putative transformants	GUS assay**
EC	pBI121	ND	3.1	0	0
	pCL4	54	1.5	0	0
SC	pBI121	ND	17.8	0	0
	pCL4	61.5	17.5	0	0
EC	pBI121	ND	29.4	6	0
	pCL4	73	25.1	2	0
SC	pBI121	ND	69.6	2	0
	pCL4	85.5	71.4	5	0

Data represent means from two separated experiments.
* After co-cultivation, ** on leaves of putative transformants



(a) (b)

Figure. 3 (a) Histochemical assay of *gus* gene expression in putative transformant of sugarcane shoots. The blue spots were not found, (b) Agarose gel (1%) electrophoresis of the PCR amplification of DNA for CaMV 35S (left) and *npt*II (right) in putative transformants of sugarcane. Total genome DNA was isolated from the putative transformants and used as template for the PCR. The corresponding DNA bands for CaMV (0.4 kb) and *npt*II (0.5 kb) genes were not detected (arrows).

The putative transformants developed from infected EC and SC were assayed histochemically for GUS expression (Figure 3a). It was observed that all of the tested leaves did not exhibit blue color spots in leaves of the putative transformant. To confirm the results, genomic DNA was isolated from putative transformant and used for PCR amplification of DNA for either CaMV 35S or *npt*II. The presence of corresponding bands of DNA for CaMV (0.4 kb) and *npt*II (0.5 kb) were not detected in the PCR analysis (Figure. 3b). The results indicated that the transformed gene did not integrate into plants genome of the putative transformants. The resistances to the antibiotic of the shoots were not because of insertion of the gene but might due to presence somaclonal variation during callus stage. It was reported that plant regeneration through callus cultures increases the risk of somaclonal variation, particularly in sugarcane (Lee 1987).

Transformation using explants *in vitro* plants

To minimize the risk of somaclonal variation the transformation was conducted using *in vitro* plants. Shoot tips were isolated from axillary buds and apical portion of sugarcane and inoculated in the medium to generate *in vitro* plants (Figure 4 left). The *in vitro* plants were then micro-propagated in liquid MS media without hormone addition. Base segments of *in vitro* plants was separated from the plants and used as a target tissue for the transformation (Figure 4 right)

The infected *in vitro* plants were assayed histochemically for GUS expression. After co-cultivation the infected materials were washed 3 times with sterile water containing cefotaxime and subjected to GUS staining. The histochemical observation showed that almost all of the infected materials partially exhibited blue color in the basal region of both i*n vitro* shoots and plants (Figure 5). Observation of infected *in vitro* plants showed that they rapidly grown and multiplied in the selection medium. The results suggest that *in vitro* plants are efficient to be used as target tissues for *Agrobacterium*-mediated transformation in sugarcane.

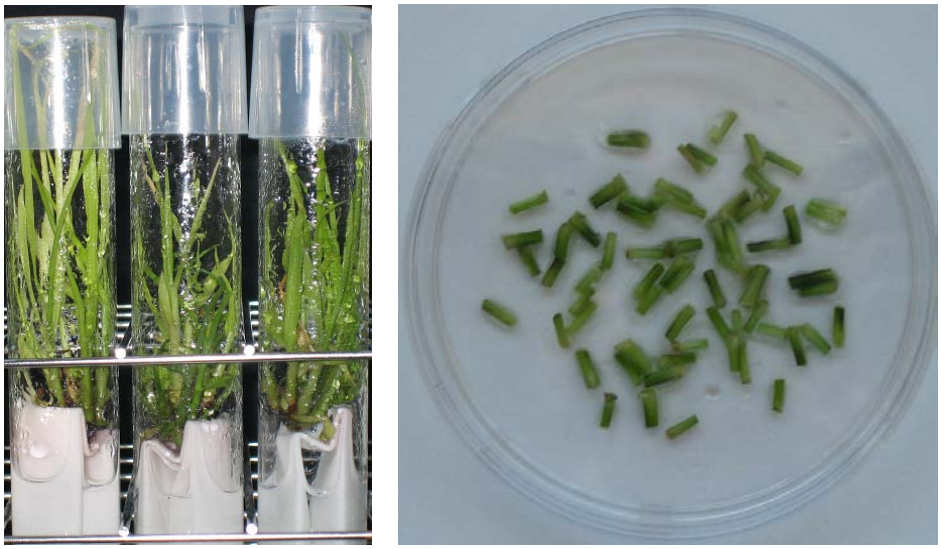


Figure 4. Shoots propagation of in vitro plants in liquid medium (left) and base segment of the in vitro plants that are used for the transformation.

Figure 5 . Histochemical assay of *gus* gene expression in the *in vitro* plants (left) and multiple shoot (right) after co-cultivation (The blue spots were photographed using a stereoscopic microscopy. Arrows represent blue spots of GUS expression).

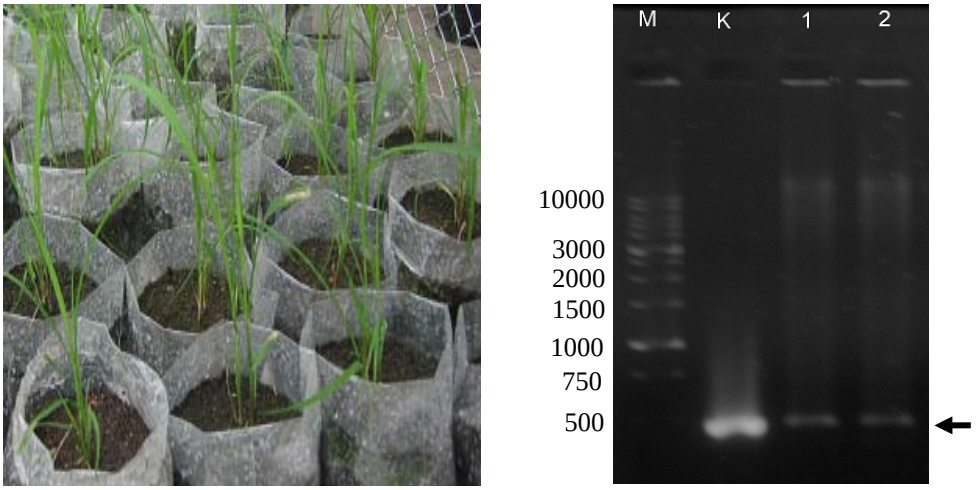


Figure 6. Growth of acclimated putative transgenic sugarcane in green house (left) and agarose gel (1%) electrophoresis of the PCR amplification of DNA for *hpt*II (right). Total genome DNA was isolated from the putative transgenic sugarcane and used as template for the PCR. The corresponding DNA bands for *hpt*II are indicated by arrow in line 1 and 2. Line K is amplified *hpt*II DNA from control plasmid pAct-SoSUT1 DNA construct and line M is marker DNA 1 kb ladder (Fermentas).

Transformation of *in vitro* sugarcane with *Agrobacterium* harboring pAct-*SoSUT1*

To confirm efficiency of *Agrobacterium*-mediated transformation using *in vitro* plant as the target tissue, the transformation was conducted with *Agrobacterium* harboring pAct-*SoSUT1* DNA construct. The construct containing *SoSUT1*-DNA encoding sucrose transporter protein driven by rice actin promoter and hygromicin resistant gene (*hpt*II). Around 200 base segment of *in vitro* plants were infected with the *Agrobacterium* and then incubated in selection media containing hygromicin antibiotic. The infected *in vitro* plants were rapidly grown in the selection media, and 13 *in vitro* plants were developed as antibiotic resistant plantlets after 5 successive cycles in same media. The resistant plantlets were then acclimated in pots containing soil media under green house condition (Figure 6). Analysis of PCR using genome DNA isolated from the resistant plantlets and set primers designed for *hpt*II gene showed clear 470 bp DNA band for *hpt*II DNA after separation in agarose gel electrophoresis (Figure 6). This result indicated that Agrobacterium-mediated transformation using *in vitro* plants for is an efficient method for sugarcane.

The high transient expression of *gus* gene driven by promoter rice ubiquitin in the infected callus of sugarcane confirmed previous studies (Liu *et al*. 2003). However, presence of the *gus* expression was not detected by histochemical analysis on the putative transformants regenerated from both embryogenic callus and suspension cell culture (Figure 3A). The absence of integrated *gus* gene in genome DNA of the putative transformants was further confirmed by PCR analysis using primer designed from DNA sequences of CaMV and *npt*II.

Direct regeneration from explants without an intervening callus phase causes minimal genetic changes and is routinely used for mass multiplication of plants, including sugarcane. Manickavasagam *et al* (2004) successfully reported the *Agrobacterium*-mediated transformation using axillary buds in sugarcane. These reports lead to an idea for the transformation using *in vitro* multiple shoots and plants of sugarcane. Histochemical observation of infected *in vitro* shoots and plants after co-cultivation showed the presence of clear blue spots in the basal region of the plants (Figure 5). Although the *gus* gene expression was not analysis in leaves the *in*

vitro plants after 3 successive cycles on selective medium, the transformation system is prospective to be further investigated. The explants were rapidly grown and multiplied in the selective medium without intervening callus, and thus minimize long- term culture and somaclonal variation. The conditions for the *Agrobacterium* infection using *in vitro* plants were successfully applied for insertion of *SoSUT1* gene in transgenic sugarcane. This is first report for the *Agrobacterium*-mediated transformation using *in vitro* plants for sugarcane.

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