An Efficient *Agrobacterium*-mediated Transformation Method for Sugarcane (*Saccharum officinarum* L.)

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Abstract. Investigation of transformation method for sugarcane was carried out by using *Agrobacterium tumefaciens* with a vectors pMLH7133 that contained CaMV35S promoter and marker genes. Embryogenic callus and cell aggregates of suspension culture were used as the target materials for transformation. The aggregates cell was sonicated, and co-cultured with *Agrobacterium tumefaciens*. The cells were cultured in N6-2 medium containing an appropriate antibiotic to eliminate bacterial contamination. Embryogenic callus was co-cultured with *Agrobacterium*, and was also cultured in N6-2 medium as same as suspension culture. Selection was carried out by culturing the treated suspension culture and embryogenic callus with MS medium containing geneticin. The transformed calli were transferred to MS-R9s for shoot formation. The transformed cells were analyzed for the distribution of GUS activity histochemically. After histohemical staining with X-glux, light microscopy observation revealed that the transformed calli derived from the NiF4, Ni9, and NCo310 cultivars had blue coloration in its tissue. Thus, the gene for GUS appeared to have been transferred and to be expressed in the calli. The construct of pCL4 was also integrated in the bacteria. Transient expression of GUS gene was successfully confirmed in the transformed *Agrobacterium*. When we use suspension culture, the proportion of the calli showing transient GUS expression was 4.7-fold greater with the vector in pMLH7133. We also successfully produced transformed calli with higher level of transient GUS expression. The percentage of the calli showing the best transient GUS expression is pCL4. The embryogenic callus was more competent for transfer of T-DNA into sugarcane cells. Analysis of GUS activity indicated that the gene was expressed into the calli of sugarcane. The Results indicate that the promoter can serve as an effective regulatory element to produce strong expression in callus of sugarcane. When we inoculated embryogenic callus with *Agrobacterium* harbored binary vector pCL4, we also successfully produced transformed calli with higher level of transient GUS expression. Thus, the gene for GUS appeared to have been transferred and to be expressed into the calli. Putative transformed plants were tested by performing PCR and Southern Hybridization to confirm the integration and expression of the introduced genes.

Key words: Transformation, sugarcane, *Agrobacterium*, embryogenic callus.

Introduction

Conventional plant breeding techniques have been extensively employed by selecting improved varieties. However, some important traits such as low resistance to insect and to herbicides appear to be limited for yield increasing. The *Agrobacterium*-mediated transformation of plants has become important in efforts aimed at the improvement of crops and may have an important impact on sugarcane yields in future. Plant transformation mediated by *Agrobacterium tumefaciens* has to be the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Efendi et al., 1996, Efendi et al., 1998).

However, the lack of a reproducible methodology for stable transformation of sugarcane was an important obstacle for genetic manipulation during many years. *Agrobacterium*-mediated gene transfer into monocotyle-donous plants was difficult. However, in recent years, several monocotyledonous plants (rice, maize, wheat, barley, onion, soybean) were successfully transformed (Hiei et al., 1994; Ishida *at al*., 1996; Cheng *at al.*., 1997; Tingay *at al*., 1997; Eady *at al.*., 2000; Efendi et al., 2000; Efendi, 2001). *Agrobacterium*-mediated transformation method and recovery of the first transgenic plants have been reported in sugarcane (Arencibia *at al.*, 1998). Recently, sugarcane cell cv. NiF4 and NiF9 was also successfully transformed using *Agrobacterium* tumefaciens-mediated transformati-on with vector pMLH7133 and pIG121 containing genes of GUS, NPTII, and HPT (Arifin et al., 2002 and Matsuoka et al., 2002; Efendi, 2003; Efendi and Matsuoka, 2003).

Although, transformation of sugarcane have been carried out in many studies, but only a few successful studies of stable *Agrobacterium*-mediated transformation of sugarcane have been reported. The problem of transferring *Agrobacterium*-mediated gene to plants is related with poor survival rate of the target cells or necrosis. The inoculated

cells were being traumatic due to the infection of *Agrobacterium*. Oxidative burst, phenolization and subsequent cell death have been described as frequent phenomena during the interaction of *Agrobacterium* with monocotyledonous plant cells (De la Riva et al., 1998). Thus, we need an improve method of transformation to solve the problem of phenolization.

Recently, we reported that the use of ultrasound enhanced the efficiency of *Agrobacterium*-mediated transformation on sugarcane. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) consists of subjecting the target tissue to ultrasound before immersing in an *Agrobacterium* suspension. The enhanced transformation rates using SAAT result from microwounding, where the energy released by cavitation causes small wounds both on the surface of and deep within the target tissue (Efendi and Matsuoka, 2004). Unfortunately, the use of ultrasound was not enough to establish a reproducible transformation method in sugarcane. Some improvements such as the use of different types of promoters and target materials are important for development of *Agrobacterium*-mediated transformation method for sugarcane.

The promoter is a key DNA regulatory element that directs appropriate strength and patterns of gene expression in a constitutive or specific manner, and therefore, plays a crucial role in successful transformation studies. Moreover, the number and types of promoters that drive strong and constitutive expression of transgenes are relatively few in sugarcane. The viral Cauliflower Mosaic Virus 35S (CaMV35S) promoter has been widely used in the transformation of many dicot and monocot crops, but activity in sugarcane has been low as demonstrated in various studies (Elliott et al., 1998). The rice actin 1 and the Emu elements have shown higher activity than CaMV35S in different sugarcane tissues (Gallo-Meagher and Irvine, 1993), however, they have not been widely utilized in subsequent studies. Two sugarcane ubiquitin promoters, ub4 and ub9, were recently used to drive transient β-glucuronidase (GUS) expression in sugarcane calli, but GUS expression was not detected in regenerated plant tissue (Wei et al., 2001). The maize ubiquitin promoter Ubi-1 has been used to drive constitutive transgenes expression in sugarcane studies (Fako et al., 2000). The rice RUBQ2 polyubiquitin promoter, containing 5`upstream and intron regions, was identified from a rice bacterial artificial chromosome (BAC) genomic library (Wang et al., 2000). Transient GUS activity driven by RUBQ2 in rice suspension cells was ten to 15 times higher than with constructs containing the CaMV35S, and two to three times higher than the maize Ubi-1 promoter (Liu et al., 2003).

In other hand, the target material such as embryogenic callus is another important factor that can improve the efficiency of *Agrobacterium*-mediated transformation for sugarcane. Liu et al. (2003) reported that the use of embryogenic callus showed many calli stained blue color after co-cultivation with *Agrobacterium* containing binary vector pCL4 harbored RUBQ2 promoter. *Agrobacterium*-mediated transformation of calli with the pCL4 resulted in significantly improve the efficiency of embryogenic callus transformation. Zhang et al. (2004) also successfully transformed embryogenic callus of sugarcane using *Agrobacterium*-mediated transformation.

In this paper, we reported comparison of transient GUS gene expression in calli driven by RUBQ2 promoter of pCL4 and CaMV35S promoter of pMLH7133 via *Agrobacterium*-mediated transformation in sugarcane embryogenic callus and aggregates cells of suspension culture. The aggregates cell was sonicated, and co-cultured with the bacteria of *Agrobacterium tumefaciens*.

Materials and Methods

Suspension culture and callus formation

Embryogenic callus and cell aggregates of suspension culture of three sugarcane cultivars i.e. NiF4, Ni9, and NCo310, the leading commercial cultivar of sugarcane in Japan, were used as the target materials of *Agrobacterium*-mediated transformation in the present experiment. Spindle sections from healthy plant taken from field were used for callus induction. Explants sized 5-10 mm were cultured in MS-1 medium (Table 2) containing 2 mg/l 2,4-D and the formed calli were transplanted into a new medium every four weeks three times. The well growing and compact calli were chosen for preparation of suspension culture and embryogenic callus. Calli were suspended in a flask filled with 30 ml liquid N6-2 (Table2) containing 2 mg/l 2,4-D. Cultures were incubated on a rotary shaker at 150 rpm

and were maintained by transferring one ml volume of cell aggregates to a fresh N6-2 medium every week. Embryogenic callus was performed using aggregates cell. The cells were culture onto MS medium containing 1.5 mg/L 2, 4-D for one month.

Bacterial strain and vectors

We have investigated transformation method in sugarcane by using *Agrobacterium tumefaciens.* Transformation of *Agrobacterium* was conducted by introducing pCL4 molecule into *E. coli* JM109. The construct was transferred into *Agrobacterium* LBA4404 by the freeze-thaw method using CaCl. The *Agrobacterium* was constructed separately with two binary vectors (pMLH7133, pCL4). The vector of pMLH7133 was introduced in strain EHA101 of the *Agrobacterium*, the vector of pCL4 was transferred into strain LBA4404. The vector of pMLH7133 contained genes of GUS, NPTII, and HPT, and the vectors of pCL4 contained genes for NPTII and GUS. For inoculation of embryogenic callus and suspension culture, the bacteria *Agrobacterium* were grown in LB medium containing 25 mg/l rifampicin and 50 mg/l kanamycin for 12-16 hours. The bacterial cells were collected by centrifugation 5000 rpm for 5 minutes.

Transformation of aggregate cell

The cell aggregates of sugarcane collected from 2-3 months suspension culture in liquid N6-2 medium were co-cultivated at 22^{O}C, 150 rpm with *Agrobacterium* strain EHA101 contained binary vector pMLH7133 and strain LBA4404 contained pCL4. Elimination of the overgrowth bacteria was done in 5 day after co-cultivation in liquid N6-2 containing 50 mg/l acetocyringon by washing with sterilized water and 5 minutes sonication at 45 KHz. Then, the cells were cultured in N6-2 medium containing 250 mg/l carbenicilin for two days to eliminate bacterial contamination. The culture medium was replaced with MS solid medium containing 250 mg/l carbenicilin for a week.

Transformation of embryogenic callus

The Embryogenic callus was co-cultured with *Agrobacterium* for five days on MS medium containing 2, 4-D 1.5 mg/l and acetocyringon 50 mg/l at 22^{O}C. The cells were washed with sterilized water by shaking with Vortex and then cultured in N6-2 medium containing 250 mg/l carbenicilin for two days to eliminate bacterial contamination. Screening of transformed callus was done four weeks with selective MS medium containing 50 mg/l geneticin and carbenicilin 250 mg/l. For regeneration, the calli were transferred to MS-R9s medium containing 1 mg/l BA, 0.2 mg/l IAA, 50 mg/l geneticin and 250 mg/l carbenicilin. The cultures were incubated in the dark for 3 days, and then incubated under fluorescent light (26^{o}C and 16 h light). Histohemical GUS assays were performed 2 weeks after transfer of the calli into the medium containing 50 mg/l geneticin and carbenicilin 250 mg/l. Calli were placed in a GUS assay mix (Jefferson 1987) and incubated overnight at 28°C. The GUS assay mix was removed, and the tissue was rinsed twice with 70% ethanol to stop reaction. The number of GUS positive was observed by light microscope.

Results and Discussion

Molecule of pCL4 contained RUBQ2 promoter was successfully introduced into *E. coli* JM109. The construct was transferred into *Agrobacterium* LBA4404 by the freeze-thaw method using CaCl. A transformed colony of *Agrobacterium* was tested by spreading the cells on a LB agar plate containing appropriate antibiotic selection (25 mg/l rifampicin and 50mg/l kanamycin). Transient of GUS gene expression of the *Agrobacterium* was performed by incubating the bacteria with X-Gluc mix overnight at 28°C. This result showed that the transformed *Agrobacterium* had blue coloration (Figure 1.A) and indicated that the construct of pCL4 was integrated in the bacteria. Transient expression of GUS gene was successfully confirmed in the transformed *Agrobacterium*.

Knowledge that the T-DNA could be transferred from the Ti plasmid of *A. tumefaciens* into plant cells and integrated into the nuclear DNA certainly raised the possibility that it could be used as a vector to transfer genes of interest into plant cells and, ultimately, into fertile plants that could transmit the genes to their progeny. Several findings emerged that were crucial in the endeavor to generate transgenic plants. The first of these were studies on tumors induced by T-DNA variants, either naturally occurring or isolated through mutagenesis.

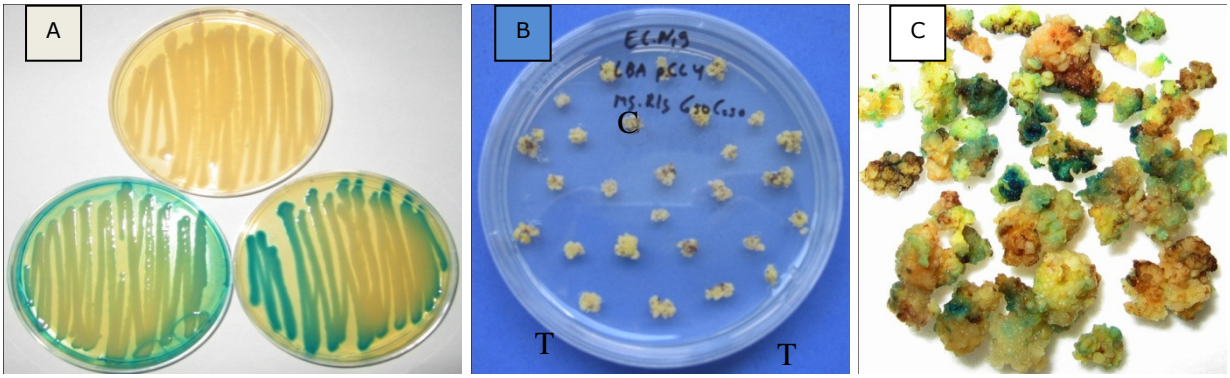

Figure 1. A. Transient expression of GUS gene in the transformed colony of *Agrobacterium*. C, control, non-transformed *Agrobacterium;* T, transformed *Agrobacterium* with vector pCL4. B. Selected calli of embryogenic callus cultivar Ni9 after screening the inoculated callus during four weeks with selective MS medium containing 50 mg/l geneticin and 250 mg/l carbenicilin. C. GUS gene activity in inoculated callus of sugarcane cultivar NiF4 after the *Agrobacterium*-mediated transformation and selection with selective MS medium containing geneticin 50 mg/l and carbenicilin 250 mg/l for two week.

The results showed that it was produced many selected calli after screening the inoculated callus during four weeks with selective MS medium containing 50 mg/l geneticin and 250 mg/l carbenicilin (Figure 1.B). After GUS assay, light microscopy observation revealed that the inoculated calli derived from NiF4, Ni9, and NCo310 had blue coloration in its tissue (Figure 1.C). In consequence, we got many selected sugarcane calli, and found that the binary vector pCL4 carried RUBQ2 promoter significantly enhanced the efficiency of sugarcane transformation, especially in cultivar NiF4. When we use suspension culture of the agregates cell, the proportion of the calli showing transient GUS expression was 4.7-fold greater with the pCL4 containing the RUBQ2 promoter than with the CaMV35S promoter in pMLH7133 (Table 1). When we inoculated the embryogenic callus with *Agrobacterium* harbored binary vector pCL4, we also successfully produced transformed calli with higher level of transient GUS expression. Thus, the gene for GUS appeared to have been transferred and to be expressed into the calli.

The percentage of calli showing transient GUS expression was 12.1-fold greater with the pCL4 than with the pMLH7133 (Table 1). This result showed that the embryogenic callus was more competent for transfer of T-DNA into sugarcane cells. It is suggested that the embryogenic callus was less sensitive to necrosis, oxidative burst, or phenolization that caused by bacterial infection. Analysis of GUS activity indicated that the gene was expressed into the calli of sugarcane. Results from this GUS activity indicate that RUBQ2 can serve as an effective regulatory element to produce strong expression in callus of sugarcane, especially for cultivar NiF4. DNA constructs containing RUBQ2 promoter produced higher levels of transient GUS expression by *Agrobacterium*-mediated transformation in calli of sugarcane.

Table 1. Frequency of GUS activity in transformed calli of sugarcane that using CaMV35S & RUBQ2 promoters

Types of materials	Promoters	GUS positive of sugarcane cultivars (%)		
		NiF4	Ni9	NCo310
Suspension culture	CaMV35S	2.2	3.1	1.2
Embryogenic callus	CaMV35S	5.8	8.6	3.3
Suspension culture	RUBQ2	10.3	9.1	4.5
Embryogenic callus	RUBQ2	70.4	10.3	20.8

CaMV35S: Cauli Mosaic Virus 35S promoter. RUBQ2 : Rice Ubi Quitin2 promoter

High GUS gene expression levels driven by RUBQ2 in sugarcane described in this report suggest that the rice polyubiquitin promoter would function efficiently in other monocotyledonous plants as well. This result showed that the use of RUBQ2 promoter could improve the efficiency of *Agrobacterium*-mediated transformation for sugarcane, especially in the calli of sugarcane. Liu et al. (2003) reported that the use of embryogenic callus showed many calli stained blue color after co-cultivation with *Agrobacterium* containing binary vector pCL4 harbored RUBQ2 promoter. The method of *Agrobacterium*-mediated transformation of calli with the pCL4 resulted in significantly improve the efficiency of embryogenic callus transformation. Zhang et al. (2004) also successfully transformed embryogenic callus of sugarcane using *Agrobacterium*-mediated transformation.

However, to obtain whole transformed plant, we need further experiments for regeneration and selection of transformed plants. Continuous analysis of putative transformed plants should be carried out by performing PCR and Southern Hybridization to confirm the integration and expression of the introduced genes from intact transgenic plants. The use of RUBQ2 promoter was effective to enhance the transient GUS expression in calli of sugarcane and could contribute to set up an efficient transformation method for sugarcane. However, further investigation of detailed conditions, such as variations of pH, temperature, and period of co-culture should be carried out to establish an efficient and reproducible protocol. Introduction of useful genes also needed to be studied to make the method practical.

Conclusions

Embryogenic callus and cell aggregates were used for sugarcane transformation. The materials was sonicated, and co-cultured with *Agrobacterium tumefaciens*. Selection was carried out by culturing the treated materials with MS medium containing geneticin. The transformed calli were transferred to MS-R9s for shoot formation. The transformed cells were analyzed for the distribution of GUS activity histochemically. Light microscopy observation revealed that the transformed calli derived from the NiF4, Ni9, and NCo310 cultivars had blue coloration in its tissue. Tthe gene for GUS appeared to have been expressed in the calli. When we use suspension culture, the proportion of the calli showing transient GUS expression was 4.7-fold greater with the vector in pMLH7133. We also successfully produced transformed calli with higher level of transient GUS expression. The percentage of the calli showing the best transient GUS expression. This result showed that the embryogenic callus was more competent for transfer of T-DNA into sugarcane cells. Analysis of GUS activity indicated that the gene was expressed into the calli of sugarcane. Results from this results indicate that the promoter can serve as an effective regulatory element to produce strong expression in callus of sugarcane.

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