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Comparison of Promoters and Target Materials in Development of Efficient *Agrobacterium*-mediated Transformation Method for Sugarcane

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Abstract: Investigation of transformation method for sugarcane was carried out by using *Agrobacterium tumefaciens* with two promoters (CaMV35S and RUBQ2). Embryogenic callus and suspension culture were sonicated and co-cultured with *Agrobacterium tumefaciens*. The transformed cells were analyzed for the distribution of GUS activity histochemically. The transformed calli derived from the NiF4, Ni9 and NCo310 cultivars had the blue coloration in its tissue. Thus, the gene for GUS appeared to have been transferred and to be expressed in the calli. The RUBQ2 promoter significantly enhanced the efficiency of sugarcane transformation. When using suspension culture, the proportion of the calli showing transient GUS expression was 4.7-fold greater with the RUBQ2 than with the CaMV35S promoter. Additionally, when transforming embryogenic callus with *Agrobacterium* harbored RUBQ2, we also successfully produced transformed calli with higher level of transient GUS expression, 12.1-fold greater with the RUBQ2 than with the CaMV35S promoter. This result showed that the embryogenic callus was more competent for transfer of T-DNA into sugarcane cells. Therefore, the results of GUS activity demonstrated that the RUBQ2 promoter can work for an effective regulatory element to generate strong expression in callus of sugarcane.

Key words: Transformation, sugarcane, promoters, suspension culture, embryogenic callus

INTRODUCTION

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. Nevertheless, 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 monocotyledonous plants was difficult. However, in recent years, several monocotyledonous plants (rice, maize, wheat, barley and onion) were successfully transformed (Hiei *et al*., 1994; Ishida *et al*., 1996; Cheng *et al*., 1997; Tingay *et al*., 1997; Eady *et al*., 2000). *Agrobacterium*-mediated transformation method and recovery of the first transgenic plants have been reported in sugarcane (Arencibia *et al*., 1998). Sugarcane cell was also successfully transformed using *Agrobacterium* tumefaciens-mediated transformation with vector pMLH7133 and pIG121 containing genes of GUS, NPTII and HPT (Arifin *et al*., 2002; Matsuoka *et al*., 2002).

Although, transformation of sugarcane has been carried out in many studies, 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* (De la Riva *et al*., 1998). However, in previous study, we successfully enhanced the efficiency of *Agrobacterium*-mediated transformation on sugarcane using the sonication-assisted *Agrobacterium*-mediated transformation (SAAT). The enhanced transformation rates using SAAT probably result from microwounding, where the energy released by cavitation causes small wounds both on the surface of and deep within the target tissue (Efendi *et al*., 2000; Arifin *et al*., 2002, 2004; Efendi, 2003).

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

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important for development of *Agrobacterium*-mediated transformation method for sugarcane. Therefore, we managed the RUBQ2 promoter in this study to enhance the efficiency of sugarcane transformation. Additionally, the target material such as embryogenic callus is another important factor that can improve the efficiency of *Agrobacterium*-mediated transformation for sugarcane. Hence, this study has reported the comparison of transient GUS gene expression in calli driven by RUBQ2 promoter of pCL4 and CaMV35S promoter of pMLH7133 via sonication-assisted *Agrobacterium*-mediated transformation in sugarcane embryogenic callus and aggregates cells of suspension culture.

MATERIALS AND METHODS

Suspension culture and embryogenic callus formation: Embryogenic callus and cell aggregates of suspension culture from 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 (Murashige and Skoog, 1962) containing 2 mg L^{-1} 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 containing 2 mg L^{-1} 2,4-D. The 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 and were culture onto MS medium containing 1.5 mg L^{-1} 2, 4-D for one month.

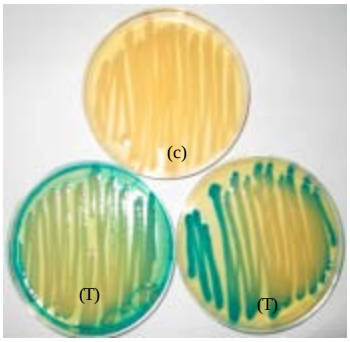
Transformation of *Agrobacterium tumefaciens*: Transformation of *Agrobacterium* was conducted by introducing pCL4 molecules into *E. coli* JM109. The construct was transferred into *Agrobacterium* LBA4404 by the freeze-thaw method using CaCl. Then, the *Agrobacterium* was constructed separately with 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, *Agrobacterium* were grown in LB medium containing 25 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin for 12-16 h. The bacterial cells were collected by centrifugation 5.000 rpm for 5 min.

Transformation of aggregates cell and embryogenic callus: The cell aggregates of sugarcane collected from two months suspension culture in liquid N6-2 medium were co-cultivated at 22°C, 150 rpm with *Agrobacterium* strain EHA101 contained binary vector pMLH7133 and strain LBA4404 contained pCL4. After co-cultivation of aggregates cell and embryogenic callus with Agrobacterium in liquid N6-2 containing 50 mg L^{-1} acetocyringon in five days, the inoculated calli were washed with sterilized water and five minutes sonication at 45 KHz to eliminate the overgrowth bacteria. Additionally, the cells were cultured in N6-2 medium containing 250 mg L^{-1} carbenicilin for two days to eliminate the remained bacterial contamination. The culture medium was replaced with MS solid medium containing 250 mg L^{-1} carbenicilin for a week. Continuously, embryogenic callus was co-cultured with *Agrobacterium* for five days on MS medium containing 2, 4-D 1.5 mg L^{-1} and acetocyringon 50 mg L^{-1} at 22°C. The cells were washed with sterilized water by shaking with Vortex and then cultured in N6-2 medium containing 250 mg L^{-1} carbenicilin for two days to eliminate bacterial contamination. Finally, screening of transformed callus was done four weeks with selective MS medium containing 50 mg L^{-1} geneticin and carbenicilin 250 mg L^{-1}. For regeneration, the calli were transferred to MS-R9s medium containing 1 mg L^{-1} BA, 0.2 mg L^{-1} IAA, 50 mg L^{-1} geneticin and 250 mg L^{-1} carbenicilin. The cultures were incubated in the dark for three days and then incubated under fluorescent light (26°C and 16 h light).

GUS assay: Histochemical localization of GUS activity was performed two weeks after transfer of the calli into the medium containing 50 mg L^{-1} geneticin and carbenicilin 250 mg L^{-1}. Calli were placed in a GUS assay mix (X-Gluc: 5-bromo-4-chloro-3-indolyl-d-glucuronic acid) and incubated overnight at 28°C according to the method of Jefferson *et al*. (1987). 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

Transformed *Agrobacterium tumefaciens*: Molecule of pCL4 contained RUBQ2 promoter was successfully introduced into *E. coli* JM109. The construct was



(a)

(b)

Fig. 1(a-b): (a) Transient expression of GUS gene in the transformed *Agrobacterium* LBA4404 by the freeze-thaw method using CaCl. The transformed colony was tested by spreading the cells on a LB agar plate containing 25 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin. GUS activity performed by incubating the bacteria with X-Gluc mix overnight at 28°C (C, control as non-transformed *Agrobacterium*; T, transformed *Agrobacterium* with vector pCL4). (b) GUS gene activity in transformed embryogenic calus of sugarcane cultivar NiF4 after *Agrobacterium*-mediated transformation and selection with selective MS medium containing geneticin 50 mg L^{-1} and carbenicilin 250 mg L^{-1} for two week. The GUS activity was driven by the binary vector pCL4 carried RUBQ2 promoter

Table 1: Frequency of GUS activity in transformed calli of sugarcane suspension culture and embryogenic callus after screening the inoculated callus with *Agrobacterium* harbored CaMV35S and RUBQ2 promoter during four weeks with selective MS medium containing 50 mg L^{-1} geneticin and 250 mg L^{-1} carbenicilin

		GUS positive of sugarcane cultivars (%)		
Promoters	Types of target materials	NCo310	Ni9	NiF4
CaMV35S: Cauli mosaic Virus 35S	Suspension culture	01.2	03.1	02.2
	Embryogenic callus	03.3	08.6	05.8
RUBQ2: Rice Ubi quitin 2	Suspension culture	04.5	09.1	10.3
	Embryogenic callus	20.8	10.3	70.4

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^{-1} rifampicin and 50 mg L^{-1} 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 the blue coloration (Fig. 1a). This result showed that the construct of pCL4 was integrated in the bacteria. Therefore, transient expression of GUS gene was successfully confirmed in the transformed *Agrobacterium*.

Putative transformed calli: It was produced transformed calli after screening the inoculated callus during four weeks with selective MS medium containing 50 mg L^{-1} geneticin and 250 mg L^{-1} carbenicilin. The results showed a genotypic variation in GUS activity of sugarcane variety. After GUS assay, light microscopy observation revealed that the inoculated calli derived from the variety of NiF4, Ni9 and NCo310 had the blue coloration in its tissue (Fig. 1b) with variation in the rate of transformation, 1.2 to 70.4%. The variation was depended on promoter, target material and variety.

Generally, the inoculated calli with *Agrobacterium* harbored the CaMV35S promoter showed the lower rate of transformation than the RUBQ2 promoter (Table 1). When using the CaMV35S promoter, the lowest rate of transformation (1.2%) found at the transformed suspension culture of NCo310 cultivar and the highest rate of transformation (8.6%) discovered at the transformed embryogenic calus of Ni9 cultivar. In contrast, when using RUBQ2 promoter, we got many selected sugarcane calli and found that the RUBQ2 promoter significantly enhanced the efficiency of transient GUS expression in sugarcane transformation. The results also showed the genotypic variation in the rate of transformation. When using suspension culture, the proportion of the calli showing transient GUS expression was 10.3% or 4.7-fold greater with the pCL4 containing the RUBQ2 promoter than with the CaMV35S promoter (Table 1).

Additionally, when transforming embryogenic callus with *Agrobacterium* harbored RUBQ2 promoter, we successfully produced transformed calli with higher level of transient GUS expression. The percentage of the calli showing transient GUS expression was 70.4% or 12.1-fold greater with the RUBQ2 than with the CaMV35S promoter

(Table 1). Thus, the gene for GUS appeared to have been transferred and to be expressed into the calli of sugarcane. According to the results, it could be considered that significant differences existed among the sugarcane cultivars in transformation. Thus, transformation of embryogenic callus in sugarcane is highly genotype dependent.

DISCUSSION

When using *Agrobacterium* harbored RUBQ2 promoter in transformation of embryogenic callus, the percentage of calli showing transient GUS expression was 12.1-fold greater with the RUBQ2 promoter than with the CaMV35S promoter (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. Bower and Birch (1992) successfully recovered transgenic sugarcane plants from cell suspensions and embryogenic calli transformed by particle bombardment. Nieves *et al.* (2003) proved that embryogenic callus has more soluble proteins, free proline, proteolytic activity and soluble sugars. Consequently, the embryogenic callus was more competent for transformation of sugarcane. Moreover, successful somatic embryogenesis and regeneration was further studied in sugarcane using different explants and medium composition (Taylor *et al*., 1992; Falco *et al*., 2001; Khan *et al*., 2004; Asad *et al*., 2009). Therefore, the development of somatic embryogenesis was a turning point in sugarcane biotechnology (Ming *et al*., 2010). Gandonou *et al.* (2005) also discovered that embryogenic callus production and regeneration ability in sugarcane is highly genotype dependent.

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 improved the efficiency of embryogenic callus transformation of rice.

The result shows, analysis of GUS activity proved that the gene was expressed into the calli of sugarcane. Results from this GUS activity showed 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. High GUS gene expression levels driven by RUBQ2 in sugarcane described in this report suggests that the rice polyubiquitin promoter would function efficiently in other monocotyledonous plants as well. This results 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 of sugarcane showed many calli stained blue color after co-cultivation with *Agrobacterium* containing binary vector pCL4 harbored RUBQ2 promoter. However, stable GUS expression levels by RUBQ2 were increased only 1.6-fold. Recently, Joyce *et al.* (2010) demonstrated plant regeneration at a frequency of 0.8-4.8% occurred when callus was transformed with the maize ubiquitin (*ubi-1*) promoter.

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 dicotyledone and monocotyledone 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) but they have not been widely utilized in subsequent studies. In other hand, 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). Otherwise, Wang *et al.* (2000) successfully identified a rice RUBQ2 polyubiquitin promoter containing 5` upstream and intron regions from a rice Bacterial Artificial Chromosome (BAC) genomic library. Liu *et al.* (2003) found that transient GUS activity driven by RUBQ2 in rice suspension cells was two to three times higher than the maize *Ubi-1* promoter. Although Agrobacterium-mediated transformation of calli with the pCL4 resulted in significantly improve the efficiency of embryogenic callus transformation, to obtain whole transformed plant, we need further experiments for regeneration and selection of transformed plants. Moreover, 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, more 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.

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