THE EFFECTIVENES OF GENE TRANSFORMATION *SoSPS1* USING PLASMID *pSMAB-SoSPS1* CONSTRUCT IN SUGARCANE (*Saccharum officinarum* L.)

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ABSTRACT

SoSPS1 gene is a gene isolated from sugarcane plants. This gene plays an important role in the biosynthesis of sucrose and sucrose accumulation effect on the crop. Efforts to increase the sucrose content of the sugarcane can be done by using the transfomation SoSPS1 gene of plasmid constructs pSMAB-SoSPS1 through Agobacterium tumefaciens vector. This study aims to determine the effectiveness of SoSPS1 gene transformation using plasmid constructs pSMAB-SoSPS1 on sugarcane (Saccharum officinarum L.). The results showed that the effectiveness of SoSPS1 gene transformation using plasmid constructs pSMAB-SoSPS1 about 2% and PCR analysis amplified by the size of 462 bp.

Keywords: Gen *SoSPS1*, *pSMAB-SoSPS1*, gene transformation, Sugarcane (*Saccharum officinarum* L.)

INTRODUCTiON

Sugarcane (*Saccaharum officinarum* L.) is a major sugar-producing plant in Indonesia, but sugar production is still insufficient national. Based on data from Market & Trade Sugar Word 2004 / 2005, imports of sugar in the last 20 years to more than 50%. The development of techniques gene insertion of genetic material such as SPS, is expected to produce new varieties with high productivity and yield in a relatively quick time.

Sucrose phosphate synthase (SPS) plays an important role in the biosynthesis of sucrose and the affect to increased sucrose content (Huber and Huber, 1996; Laporte *et al*., 2001). Several studies have shown that overexpression of SPS gene can increase the sucrose content in tomatoes (Worrell *et al*., 1991; Nguyen-quoc *et al*., 1999), *Arabidopsis thaliana* (Signora *et al*., 1998), tobacco (Miswar *et al*., 2005) and sugarcane (Miswar *et al*., 2007), but the results still vary.

Some factors influencing the success of insertion of genetic material, among others, is a binary vector (An *et al*., 1986), varieties (Ling *et al*., 1998; Ellul *et al*., 2003; Santoso *et al*., 2009), explant type (Fillati *et al*., 1987), plant growth regulators (Pfitnzer *et al*., 1998; Cortina *et al*., 2004), the concentration of bacteria (Shahin *et al*., 1986), *Agrobacterium* strains (Roekel *et al*., 1993), concentration antibiotics (Hu and Phillips, 2001; Qiu *et al*., 2007) and promoter (Liu *et al*., [illegible] Yoo *et al*., 2005).

Promoter is a DNA regulator that aims to regulate gene expression patterns so that it can be seen that the presence and expression of the gene in plants. Promoter CaMV (*cauliflower mosaic virus*) are often used in plant transformation, but if used in the transformation of sugarcane (monocotyl) is low success rate (Chowdhury and Vasil., 1992). Promoter RUBQ2 (*rice polyubiquitin*) is a new constitutive promoter in rice plants and monocotyl plant groups (Wang and Oard, 2003), but for the transformation of the SPS gene in sugarcane effectiveness to be investigated.

Plasmid Constructs pSMAB-SoSPS1 (Sugiharto, 2009) which is controled by the promoter RUBQ2, never been tested in sugarcane. Therefore, genetic transformation using plasmid constructs pSMAB-SoSPS1 controlled by promoter RUBQ2 with lateral buds as explants needs to be done. According Manickavasagam *et al*, (2004), the use of lateral buds as explants to avoid somaclonal variation, faster regeneration time and can increase 50% the transformation efficiency.

EXPERIMENTAL DETAILS

Plant Material

Explants from axillary buds of sugarcane var. BL soaked in alcohol 70% for 5 seconds, then in 0.05% $HgCl_2$ solution for 60 seconds, rinsed 3 times with sterile distilled water and drained on a filter paper for 30 minutes. Explants grown on MS medium (Murashige and Skoog + 1.5 mgl^{-1} BAP and 0.1 mgl^{-1} GA_3 at 25°C under 16-h and 8-h cycle with a light intensity of 1600 lux illumination. Explant were sub cultured every 2 weeks and form plantlets for 10 weeks._Basal part of plantlet is used as a source for transformation.

Agrobacterium strain and plasmid vector

Agrobacterium tumefaciens strain LBA4404, harboring plasmid pSMAB, which contained bar gene as selectable marker, and *SoSPS1* gene construct, was utilized for *Agrobacterium*-mediated transformation. In this construct, the expression of *phosphinotricyn* gene was under the control of the nopaline synthase promoter (pNOS), pArbcs terminator and *SoSPS1* gene was driven by RUBQ2 (Figure 1).

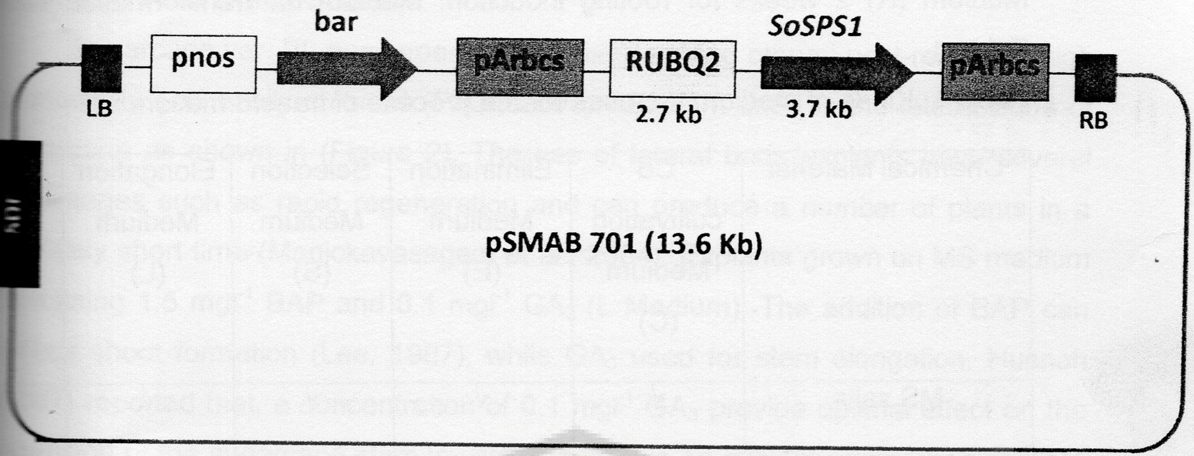

Figure 1. Map of pSMAB-SoSPS1 construct. cDNA-*SoSPS1* under the control of RUBQ2 promoter and resistance gene (*bar*) for *phosphinotricyn* (Sugiharto *et al*., 2009).

Agrobacterium tumefaciens preparation for Transformation

Single colonies of *Agrobacterium tumefaciens* LBA4404, harboring plasmid pSMAB-SoSPS1 on Luria Betani (LB) medium plate (10 gl^{-1} pepton, 10 gl^{-1} yeast extract, 5 gl^{-1} NaCl, 14 gl^{-1} agar, pH 7.0) were inoculated in 2 ml liquid Yeast Extract Peptone (YEP) medium supplemented with 50mgl^{-1} kanamycin, 30 mgl^{-1} Streptomicyin and 50 mgl^{-1} refamicyin and incubated overnight at 28^0C with continuous shaking at 150 rpm. *Agrobacterium* resubcultured into 50 ml liquid YEP medium containing 50 mgl^{-1}kanamycin, 30 mgl^{-1} Streptomicyin and 50 mgl^{-} refamicyin and incubated 4-h at 28^0C to a final density of 0.3-0.4 OD_{600}. *Agrobacterium* cells were harvested by centrifugation at 5.000 rpm for 10 min and resuspended in liquid MS medium.

Transformation mediated by *Agrobacterium tumefaciens*.

The explants were infected by immersing in *Agrobacterium* suspension for 30 min with gentle shaking three to five times during the infection process. Subsequently, the infected explants were dried on a sterile filter paper and transferred onto co cultivation medium (C) for 2-d in dark at 25°C. After co cultivation, the infected explants were washed with 50 ml of liquid MS containing cefotaxime 500 mgl^{-1}, drained on sterile filter paper and transferred onto Elimination medium (E) at 25°C under 16-h light, 8-h dark cycle for 5 days. Explants were cultured subsequently on Selection medium (S) 2 weeks for shoot induction with 3 times subculture, elongation medium (L) 2 weeks for shoot enlargement and rooting medium (R) 2 weeks for rooting induction. Medium for transformation shown in Table 1.

Table 1. Kinds of medium are used for the process of transformation

Chemical Material	Co cultivation Medium (C)	Elimination Medium (E)	Selection Medium (S)	Elongation Medium (L)	Rooting Medium (R)
MS salts	√	√	√	√	√
IAA (mgl^{-1})	0.2	0.2	0.2	-	-
BAP (mgl^{-1})	2	2	2	0.25	-
GA3 (mgl^{-1})	-	-	-	0.25	-
NAA (mgl^{-1})	-	-	-	-	0.15
Acetosyringone (mgl^{-1})	50	-	-	-	-
Cefotaxim (mgl^{-1})	-	500	500	500	500
Phosphinotricyn (mgl^{-1})	-	-	5	5	5
Phytagel (gl^{-1})	3	3	3	3	3
Sucrose (gl^{-1})	30	30	30	30	30
Ph	5.8	5.8	5.8	5.8	5.8

DNA isolation and PCR analysis

Genomic DNA was isolated from non-transformed (control) and transforme leaves. PCR analysis were carried out to detect the presence of the *bar* gene using primer bar-F/R, 462 bp: 5-ATC GTC AAC CAC TAC ATC GAG AC-3 dan 5-CCA GCT GCC AGA AAC CCA CGT C-3. Linkage analysis of these genes in transgenic plants was conducted using PCR amplification.

RESULTS AND DISCUSSSION

Sugarcane var. BL has superior properties, among others: pest-resistant, hig productivity and easily cultured (P3GI, 2009). Explant used is the lateral buds o sugarcane as shown in (Figure 2). The use of lateral buds explants have severa advantages such as rapid regeneration and can produce a number of plants in relatively short time (Manickavasagam *et al.*, 2004). Explants grown on MS mediu containing 1.5 mgl^{-1} BAP and 0.1 mgl^{-1} GA$_3$ (L Medium). The addition of BAP ca induce shoot formation (Lee, 1987), while GA$_3$ used for stem elongation. Husna (2007) reported that, a concentration of 0.1 mgl^{-1} GA$_3$ provide optimal effect on th extension of the sugarcane stem in vitro. Figure 2 is an explant preparation proces to form sugarcane plantlets. Plantlets age of 10 weeks (Figure 2E) ready to be use for the transformation process.

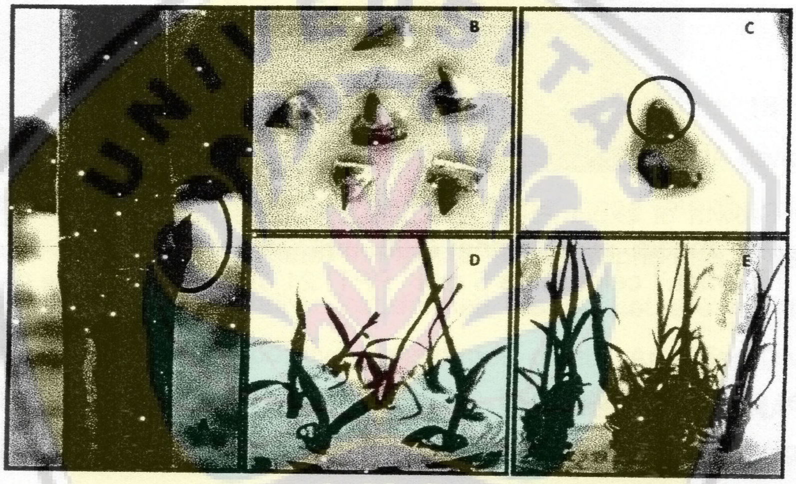

Figure 2. Culture of sugarcane lateral shoots. Lateral shoots of sugarcane (A), lateral bud explant isolation (B), the growth of shoots (C), 8 weeks (D), 10 weeks (E).

SoSPS1 gene transformation in sugarcane explants with *A. tumefaciens* lateral buds have several stages of co-cultivation, elimination and selection. The result of the transformation that has been done using pSMAB-SoSPS1 shown in (Figure 2). In Table 2 it can be seen the number of explants of co-cultivation and the elimination of 50 explants. At the time of selection 1 (Figure 2 C) decrease the number of explants that are resistant to the selection agent. Explants were resistant to the selection agent for 31 explants. End of Selection 2 (Figure 2 D), from 31 explants who passed 1, only 23 explants that pass the selection. At the time of selection 3 (Figure 2 E), a putative transformed plant obtained a total of 18 plants. The results of the final stage of the selection of 18 plants indicated on the plant resistance *phosphinotricyn* (PPT) media (S Medium). Miki and McHugh, 2003 states transformed plants will have a tolerance to PPT, PPT is an inhibitor of glutamine synthetize. Glutamine synthetize is the only enzyme that can catalyze the assimilation of ammonia to glutamic acid. Therefore, the existence of this inhibition in the end the accumulation of ammonia which can lead to cell death (Toki et al., 1992). It is characterized by non-transformed plants will experience death (Song and Douches, 2009).

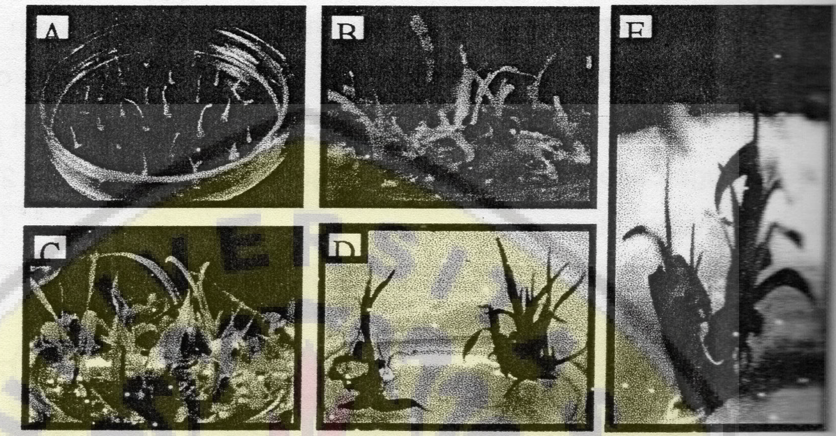

Figure 3. Growth of explants on co-cultivation medium (100 mgl^{-1} acetosyringone) (A), media eliminations (500 mgl^{-1} cefotaxime) (B), media selection (500mgL^{-1} cefotaxime 5 mgl^{-1} PPT) 21 days (C), 42 days (D) and 63 days (E).

After the end of the third selection, putative transformed plants moved into MS0 media with the addition of 500 mgl^{-1} cefotaxim and 0.5 mgl^{-1} Naphtalene cefotaxime Acetic Acid (NAA) which serves to induce root. After the complete plant morphology, plantlets acclimatized to genomic DNA isolated from the leaves of plants.

Table 2. number of putative transformed plant in co cultivation, selection and rooting medium

	Number of explant
Cocultivation	50 (100%)
Elimination	50 (100%)
Selection 1	31 (62%)
Selection 2	23 (46%)
Selection 3	18 (36%)
Rooting	18 (36%)
Acclimatization	12 (24%)
Effectiveness Transformation	1 (2%)

The success of acclimatization by 70% because of the 18 plants were successfully acclimatized only 12 plants that can be isolated DNA genome. PCR is used to see the successful integration of the target gene into the plant genome SoSPS1. In (Figure 3) can be seen the results of electrophoresis of genomic DNA are amplified. Of the 12 putative transformed plants that are resistant to PPT analyzed using PCR, only 1 positive transformed plants. Amplified DNA indicated the presence of DNA bands or band size 462 bp, the presence of DNA bands were seen indicates that SoSPS1 gene has been integrated into the plant genome. While 11 other plants are not a band that signifies not the integration of T-DNA into the plant genome.

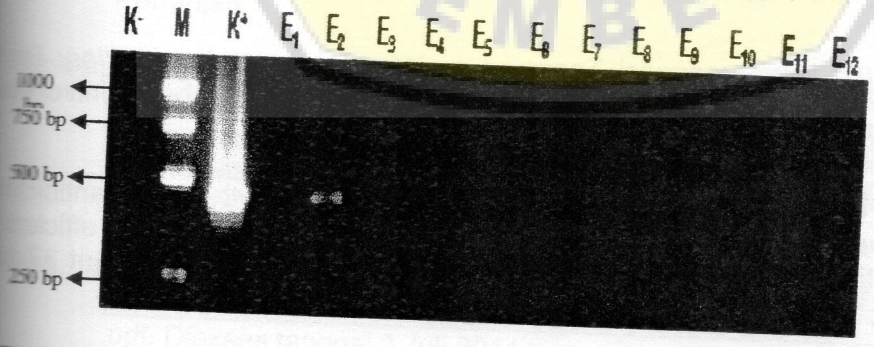

Figure 3. Analysis PCR of plant genomic results with primer F / R. K +: pSMAB plasmid-SoSPS1, K-: control plants (wild type), M: 1 kb DNA ladder, E1-12: plant 1-12.

Percentage of successful transformation obtained by 2% (1 of 50 explant transformed plant) indicates the efficiency is still low compared to the use of plasmids and different promoters, because SoSPS1 gene transformation research has been done using the plasmid with the promoter CaMV 35S pKYS (Miswar et al. 2007) and plasmid pCl4 with promoter RUBQ2 (Baskoro, 2012) demonstrated the success of 4% and 6%.

CONCLUSION

The results showed that the effectiveness of *SoSPS1* gene transformation using plasmid constructs pSMAB-SoSPS1 about 2% and PCR analysis amplified by the size of 462 bp.

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