

AGROBACTERIUM-MEDIATED TRANSFORMATION AND EXPRESSION OF *Bt* GENE IN TRANSGENIC SUGARCANE

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ABSTRACT

Sugarcane borers are the major biotic pests of sugarcane. For the development of insect resistance in sugarcane an insect resistant gene *CryIA(b)*, also known as *Bt* gene was transferred to sugarcane through *Agrobacterium*-mediated transformation at the Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Thailand in 2015. The bacterium carries the binary vector pCAMBIA1302-Ab possessing *hygromycin phosphotransferase (hpt)* and *CryIA(b)*. Both genes are under the control of 35SCAMV promoter. For transformation, calli were induced from the most inner leaf whorl of 5-6 months old sugarcane shoot, of cultivar LK 92-11. The calli were submerged in *A. tumefaciens* suspension for 10 min and co-cultivated for 3 days in the dark on MS medium containing 100 μ M acetosyringone. The calli were transferred onto callus induction medium containing 30 mg/L hygromycin. After six weeks 20 calli were alive and 20 plants were regenerated. Out of 20 plants, 16 plants showed a PCR positive band of *hpt* gene at the size of 800 bp and 7 out of 16 plants showed PCR positive band of *CryIA(b)* gene at the size of 2500 bp. Southern blot analysis revealed that three transgenic plants had two copies of *CryIA(b)* gene and one transgenic plant had one copy of the transgene integrated in the genome. Expression analysis of the transgenic sugarcane plants was done by using real-time PCR. The four transgenic plants of three months old showed transcriptional expression of *CryIA(b)* gene. The transgenic sugarcane possessing a single copy of *CryIA(b)* showed about 6-19 times higher expression level compared to the two copies transgenic plants.

Key words: callus, real-time PCR, Southern blot, genetic transformation

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is very important to the agro-based industry and largely grown in tropical and sub-tropical region of the world. Sugarcane germplasms have a complex polyploidy level, with chromosome number varying from 80-120 (Joyce *et al.* 2010). Insect pests are a major problem for sugarcane yield loss all over the world. One of the significant pests of sugarcane

is Lepidopterous stem borers (Weng *et al.* 2011). The major Lepidopteran insect pests of sugarcane are stem borer (*Diatraea saccharalis*) (Rossato *et al.* 2010), root borer (*Emmalocera depressalis*), sugarcane top borer (*Chilo terrenellus*) (Goebel and Way 2003), pink borer (*Sesamia inferens*) and Maxican rice borer (*Eoreuma loftini*). These borers cause yield losses of nearly 25-30 percent (Kalunke *et al.* 2009), but insect resistant germplasm is not available in the collections all over the world.

Bacillus thuringiensis (*Bt*) is a gram-positive, spore-forming, soil bacterium. During the sporulation time *Bt* produces insecticidal proteins that paralyze the larvae of some harmful insects, including borers, all of which are common plant pests whose infestations produce considerable negative effects on important crops. The insecticidal toxins (*Cry* toxins) of *Bt*, is also known as δ -endotoxins. The *Cry* gene family code for this toxin. *Cry* genes express during the stationary phase of growth. *Cry* proteins constitute 20–30% of the cell dry weight which is usually accumulate in the mother cell. It starts in stage III of sporulation and continuing to stage VII. *Cry* toxin is a simple toxin and it is defined as a monomer or oligomer of a toxic simple protein on the basis of its mode of action (Ibrahim *et al.* 2010). Insect resistance has developed in many crops through the introduction of *B. thuringiensis* crystal protein (*Cry*) gene (Estruch *et al.* 1997). *Bt* gene is now used to develop transgenic insect resistance in many plant crops including brinjal or eggplant (Kumar *et al.* 1998), rice (Ye *et al.* 2003), cotton (Stewart *et al.* 2001), sugarcane (Braga *et al.* 2003), potato (Meiyalaghan *et al.* 2006), chickpea (Sanyal *et al.* 2005), white cabbage (Deng *et al.* 2011), mat rush (Ling *et al.* 2009), and pigeon pea (Sharma *et al.* 2006). Development of insect resistant crop varieties with resistance against insect pests is a very important advantage of genetic engineering in agriculture. The insect resistant crop plants with high productivity are also environmentally safe. For fully acceptance and adaption, insect resistant crops face so many challenges. But insect resistant crops can be commercialized by following biosafety guidelines. There are no strong evidences for any hazards of genetically modified crop plants. By using proper regulatory mechanism transgenic crops can be commercialize and improved (Bakhsh *et al.* 2015). Though there are so many restrictions to commercialize *Bt* transgenic crops, it is increasing rapidly and cultivated in more than 32 million hectares in all over the world. Some countries adopted genetically modified (GM) crops for insect resistance and many other countries are trying to introduce it in the future (Kumar *et al.* 2008). In 1996, transgenic *Bt* maize (corn) were first commercialized in North America (Shelton *et al.* 2002). It was developed to manage both corn borers and corn rootworms and created much interest for corn growers. At present, transgenic cotton and potatoes are available in USA and some other crops including rice, soybeans, broccoli, lettuce, walnuts, apples and alfalfa are being developed. A large number of *Bt* cotton areas were also found in India and China.

Sugarcane is a polyploid crop with a complex genetic make-up. Due to its genetic complexity and long breeding cycle, transgenic technology could be the best method to develop borer resistance in sugarcane. Genetic transformation using *Bt* genes is regarded as an effective method to develop insect-resistant transgenic plants (Valderrama *et al.* 2007; Weng *et al.* 2011). For transformation of the *Bt* gene, the *Agrobacterium*-mediated transformation method was used in this study. Plant transformation by the soil-borne plant pathogen, *Agrobacterium tumefaciens*, has become the most commonly used method for plant transformation (Tripathi 2005). *Agrobacterium* – mediated transgenic plants are generally fertile and the foreign genes are often transmitted to the progeny in a Mendelian manner (Rhodora and Thomas 1996). The development of transgenic crops that produce *Bt* *Cry* proteins has been a major breakthrough in the substitution of costly and laborious spraying of chemical insecticides with environmental friendly alternatives (Bravo *et al.* 2007).

The present work was carried out through the transfer of an insect resistant *Bt* or *CryIA(b)* gene into a commercial sugarcane cultivar to develop transgenic sugarcane plants using the *Agrobacterium*-mediated transformation method. To determine and compare the expression level of

the transgene in each transgenic sugarcane plants, real-time PCR technique was used. This will be important data for green house bio-assay in the future.

MATERIALS AND METHODS

Callus induction and determination of hygromycin concentration for selection

The experiment was conducted in 2015 at the Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Thailand.

Unexpanded shoot apices of 5-6 months old sugarcane cultivar LK 92-11 were used as the explants for callus induction (Fig. 1A). The explants were surface sterilized by using 15% commercial bleach for 15 minutes. These were washed with sterile water three times and the most inner young leaves were cut to approximately 0.5 cm each (Fig. 1B) and cultured on MS medium supplemented with 20 g/L sucrose, 7 g/L agar, 3 mg/L 2,4-D and 10% (V/V) coconut water (CW), at 26±1°C in the dark. Calli were sub-cultured at 30 day intervals onto the same medium. After 60 days, embryogenic calli were transferred onto the same medium containing different concentrations of hygromycin (0, 10, 20 and 30 mg/L) to find out the appropriate concentration for the selection of the resistant calli. The calli were sub-cultured on the new selective medium every two weeks. After six weeks of cultivation the appropriate concentration of hygromycin was selected.

Agrobacterium-mediated transformation

Before transformation all big calli were divided into small pieces and cultured on callus induction medium for five days. Each big callus was divided into 6-7 pieces and a total of 100 small pieces of calli were precultured for transformation.

Agrobacterium culture

Agrobacterium tumefaciens strain EHA105 carries the binary vector pCAMBIA1302-Ab possesses *hygromycin phosphotransferase (hpt)* and *CryIA(b)* kindly provided by Dr. S. Chanprame was used for transformation. This *CryIA(b)* was isolated and modified from native bacterium found in Thailand. Both genes are under the control of 35SCAMV promoter. The bacterium was cultured in 20 mL liquid LB medium containing 50 mg/L kanamycin (Thai Meiji Pharmaceuticals Ltd., Bangkok, Thailand) at 28° C and shaken overnight at 180 rpm. From this culture again 2 mL of suspension was transferred to 20 mL liquid LB medium containing 50 mg/L kanamycin and 100 µM acetosyringone (SIGMA-ALDRICH, USA) and shaken overnight at 200 rpm.

Infection

After overnight culture, 20 mL of *Agrobacterium* suspension was centrifuged at 6000 rpm for 5 min and the supernatant was discarded. The pellet was re-suspended (by gently pipetting) in 5 mL liquid callus induction medium. Five mL of suspension were transferred into a bottle containing 15 mL liquid callus induction medium (MS+ 20 g/L sucrose, 3 mg/L 2,4-D + 10% (V/V) coconut water (CW) and 100 µM acetosyringone). The precultured calli were immersed in the bacterial suspension and shaken at 120 rpm for 15 min at 26° C. The calli were placed on tissue paper to remove the excess medium and later placed on co-cultivation medium.

Co- cultivation and selection

The infected calli were transferred onto co-cultivation medium (MS+20 g/L sucrose, 7 g/L agar, 3 mg/L 2,4-D, 10% (V/V) CW + 100 µM acetosyringone, pH: 5.7) and kept in the dark for three days. After three days, the calli were washed with liquid MS callus induction medium + 200 mg/L cefotaxime three times to remove most of the bacteria. Then the calli were placed on sterile tissue paper and placed in selective medium (MS+20 g/L sucrose, 7 g/L agar, 3 mg/L 2,4-D, 10% (V/V) CW + 30 mg/L hygromycin, 200 mg/L cefotaxime (Siam Pharmaceuticals Co. Ltd., Bangkok, Thailand), pH: 5.7). Every two weeks, the calli were washed one time with liquid MS+300mg/L cefotaxime and subcultured on the same medium. Then, after six weeks of selection the live calli were transferred onto regeneration medium.

Plant regeneration

The putative transformed calli were transferred to regeneration medium (MS+20 g/L sucrose, 7 g/L agar + 30 mg/L hygromycin, 200 mg/L cefotaxime, pH: 5.7) until the shoots were visible. After 60 days of sub-culturing the putative transgenic shoots were transferred to the rooting medium (MS+20 g/L sucrose, 7 g/L agar, 5 mg/L NAA+ 30 mg/L hygromycin, 200 mg/L cefotaxime, pH: 5.7). After 60 days of growth in the culture medium, the plants were transferred into pots with a soil-pertile mixture (1:1 ratio).

Confirmation of the existence of the transgenes by PCR technique

Transgenic plants were confirmed by using the polymerase chain reaction (PCR). Phire Plant Direct PCR Master Mix (Thermo Scientific, Lithuania) was used as the PCR master mixture. This master mix is designed to perform PCR directly from different plant material without prior DNA purification. A small piece of three months old putative transgenic sugarcane leaf (approximately 2 mm in size) was taken and placed in 20 µL of dilution buffer (supplied by manufacturer), the leaf sample was crushed with a pipette tip, by pressing briefly. The plant material was centrifuged and used 0.5 µL of the supernatant as a template. In a 20 µL PCR reaction mixture containing 10 µL 2X Phire Plant Direct PCR Mix, 0.2 µl (10 µM) of each *hpt* (*hygromycin phosphotransferase*) forward primer (5'-CCTGAACTCACCGCGACG-3') and reverse primer (5'-AAGACCAATGCGGAGCATA TA -3'), 0.5 µL of template and 9.1 µL distilled water. The same master mix was also made with 10 µM *CryIA(b)* for both forward (5'-CATGGACAACAACCCAAACATCAACG-3') and reverse primer (5'-GTCACCTTGCTACCGAAAGTCCTCGTT-3'). For both sets, primer PCR conditions were one cycle of initial denaturation at 98° C for 5 min, followed by 40 cycles of denaturation at 98° C for 5 sec, annealing at 62° C for 5 sec and an extension at 72° C for 20 sec, followed by a final extension at 72° C for 1 min. This analysis was conducted by using BIO-RAD T100™ Thermal Cycler, USA. After that the PCR amplified products were electrophoresis on 1% agarose gel at 50 V for 60 min.

Southern blot analysis

Genomic DNA was extracted from young leaves of transgenic and untransformed control sugarcane plants by using the modified DNA extraction methods described by Aljanabi *et al.* (1999). Then genomic DNA (50 µg per reaction) from each plant was digested with *Nco*I restriction enzyme for 16 hr. Next the DNA was transferred, by capillary transfer (Sambrook and Russell, 2001) onto a positively charged membrane (Amersham Hybond™ -N⁺, GE Healthcare, UK). The PCR amplified gene probe (900 bp) of *CryIA(b)* labeled with DIG-dUTP (Roche Diagnostics, Germany) was used in the hybridization. Pre-hybridization and hybridization was done by using the standard protocol (Sambrook and Russell, 2001). After 16 hr of hybridization the membrane was washed with different

concentrations of saline sodium citrate (SSC) buffer (2x, 1x and 0.5x SSC). The membrane was transferred into a plastic bag containing 15 ml blocking solution (10x blocking reagent:maleic acid buffer=1:9) with 1 µL (0.75U/µL) of anti Digoxigenin-AP Fab fragments (Roche Diagnostics, Germany), sealed and shaken for 30 min. Then the membrane was washed twice with washing buffer (1x maleic acid and 0.3% tween 20) for 30 min. After washing the membrane was exposed to X-ray film (Kodak medical X-ray film, USA) for 15 min. The film was transferred to a box containing developing solution for 1-2 min. When the DNA band was visible the film was immediately transferred to a box containing fixing solution for 1-2 min and air dry the film for 10-15 min at room temperature.

Determination of expression levels of *CryIA(b)* in transgenic sugarcane

Total RNA was extracted from young leaves (0.1 g) of 3 months old sugarcane by using the method described by Laksana and Chanprame (2015). The concentration of total RNA was measured by using Nanodrop™ and adjusted to 1500 ng/µL. The extracted total RNA was used for synthesis of 1st strand cDNA. The reaction mixture contained 1 µg of total RNA, 2 µg Oligo (dT) primer (IDT, Singapore), 0.8 mM dNTP (Thermo Scientific, Lithuania). RNase free water was added to make the volume 12.5 µL and mixed gently. The reaction was incubated at 65° C for 5 min and then cooled at 4° C for at least 2 min. Then 1x reaction buffer, 0.5 unit RiboRock RNase inhibitor (Fermentas, Lithuania), 1mM dNTP and 1µL Revert Aid M-Mul VRT (Fermentas, Lithuania) was added to the reaction mixture tube and mixed gently, incubated at 42° C for 1 h and the reaction was stopped at 70° C for 10 min, then cooled at 4° C for at least 5 min. RNaseH (0.2 µL) was added for removal of the remaining total RNA. The specific primers for *CryIA(b)* gene (AY742219.1) were designed by using the primer3 program ([http:// simgene.com/Primer3](http://simgene.com/Primer3)) (Table 1). The product size was about 200 bp. For real-time PCR, in 20 µL reaction mixture containing 100 ng of cDNA, 10 µL 2X SensiFAST SYBR No-ROX mix buffer (Bioline Reagent Ltd. USA) and 0.8 µL of 10 µM forward and reverse primers specific to *CryIA(b)* and *Actin* genes (Table 1). The amplification of both genes was performed by using the following condition: preliminary denaturation at 95° C for 2 min, 45 cycles of denaturing at 94° C for 15 sec, annealing at 58° C for 15 sec and an extension at 72° C for 20 sec. This analysis was conducted by using Mastercycler® ep realplex4 (Eppendorf). The expressions of *CryIA(b)* in transgenic sugarcane were compared with the control sugarcane (non-transgenic). *Actin* was used as a reference gene. Three replications of each sample were used in this experiment.

Table 1. Primers for amplifying *CryIA(b)* gene and *Actin*

Remarks	Primers	Sequence
For amplifying partial <i>CryIA(b)</i>	Partial <i>CryIA(b)</i> F	5'-CTCCACAACAACGCATCGTC-3'
	Partial <i>CryIA(b)</i> R	5'-GTAGGCGAACTCAGTGCCAT-3'
For amplifying <i>Actin</i>	<i>Actin</i> F	5'-GAGAGGGGTTACTCCTTC-3'
	<i>Actin</i> R	5' - CTCTTTTCAACTGAGGAGCT-3'

RESULTS AND DISCUSSION

Plant transformation and regeneration

In this study, sugarcane callus was used as transformation material for the *Agrobacterium*-mediated transformation (Fig. 1C). Prior to transformation, the calli were subjected to different concentrations (0, 10, 20 and 30 mg/L) of hygromycin (Fig. 2A). At 30 mg/L of hygromycin all the non-transformed sugarcane calli died within 6 weeks and this concentration of hygromycin was selected and added in the growth medium (Fig. 2B). Dongting *et al.* (2007) used 30 mg/L

hygromycin in the selection media during *Agrobacterium*-mediated transformation in sugarcane. Arencibia *et al.* (1998) used 25 mg/L hygromycin as the selection pressure in sugarcane transformation. Subramanyam *et al.* (2011) used a 30 mg/L hygromycin in banana, as well as in oil palm (Surrerat and Sompong 2012).

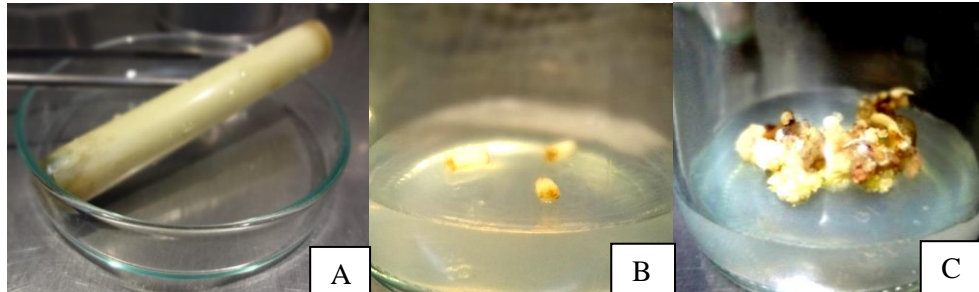


Fig. 1. Callus induction from sugarcane leaf. A) A section of unexpanded young leaf B) Pieces of inner most leaf C) Callus initiated from a piece of inner most leaf

During infection, 100 μ M acetosyringone was added to the infection medium, because it increases the transformation efficiency. *Agrobacterium* culture supplemented with an appropriate concentration of antibiotic and 100 μ M acetosyringone increased the transformation efficiency in monocot (Southgate *et al.* 1996).

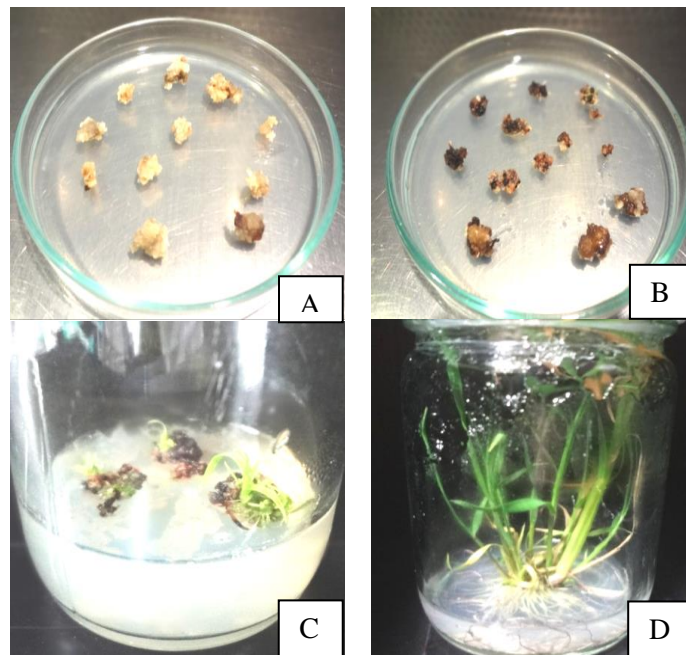


Fig. 2. Selection of putative transgenic sugarcane. A) Sugarcane calli on selective medium, B) After six weeks of selection, C) Putative transformed calli and shoots on shooting medium, D) Regenerated plants on rooting medium.

After transformation, regeneration efficiency was good in sugarcane. All of the selected calli developed shoots after 60 days on shoot induction medium (Fig. 2C). The plantlets were transferred to rooting medium and within 30 days these produced roots (Fig. 2D). Twenty plants were regenerated from 20 transformed calli giving a 100% regeneration. Among these, 16 plantlets showed PCR positive for *hpt*. The transformation efficiency for *hpt* was 80%. Seven out of 16 plantlets showed PCR positive for both *hpt* and *CryIA(b)* gene, with a co-transformation efficiency of both genes of about 43%. These were transferred to soil and finally four plants survived (Fig. 3).

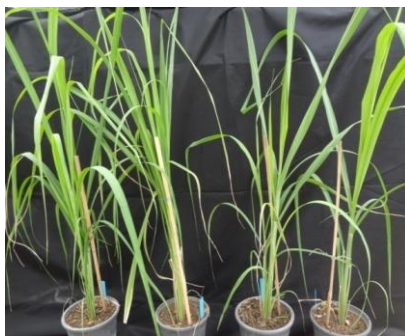


Fig. 3. The 3 months old transgenic sugarcane plants in pot

PCR for confirmation of transgenic plants

Putative transgenic sugarcanes were confirmed by using polymerase chain reaction (PCR). The 0.5 μ L of Thermo Scientific Phire Plant Direct PCR Master Mix containing crude extract of sugarcane leaves was used as the template for determination of *CryIA(b)* gene. The expected size of the positive PCR product is 2500 bp. It was found that all plants showed 2500 bp of *CryIA(b)* gene (Fig. 4). Wang *et al.* (2005) reported that the efficiency of *Agrobacterium*-mediated transformation to produce PPT-resistant sugarcane was 30% as detected by PCR.

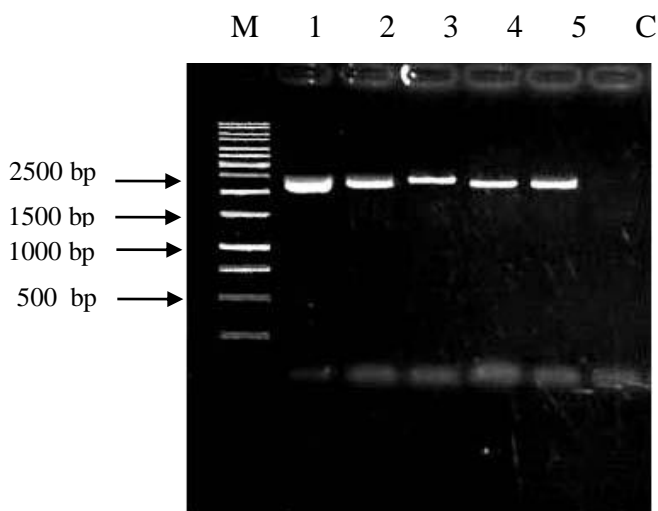


Fig. 4. PCR analysis of putative transgenic sugarcane plants. Lane M: GeneRular™ 1 Kb DNA ladder (Fermentas), lane 1: positive control, pCAMBIA1302-Ab, lane 2-5: *CryIA(b)* gene from 4 independent transgenic sugarcane plants and lane c: non-transgenic sugarcane plants.

Southern blot analysis

Southern blot analysis was done in 4 *CryIA(b)* PCR positive plants. The genomic DNA (50 µg) of 4 PCR positive plants, untransformed control plant and pCAMBIA1302-Ab were digested with *Nco* I restriction enzyme. A *CryIA(b)* probe (900 bp) was used for detection of the transgene. It was found that three transgenic plants (lane 1-3) showed two copies of the gene integration and one transgenic plant (lane 4) showed a single copy of gene integration (Fig. 5).

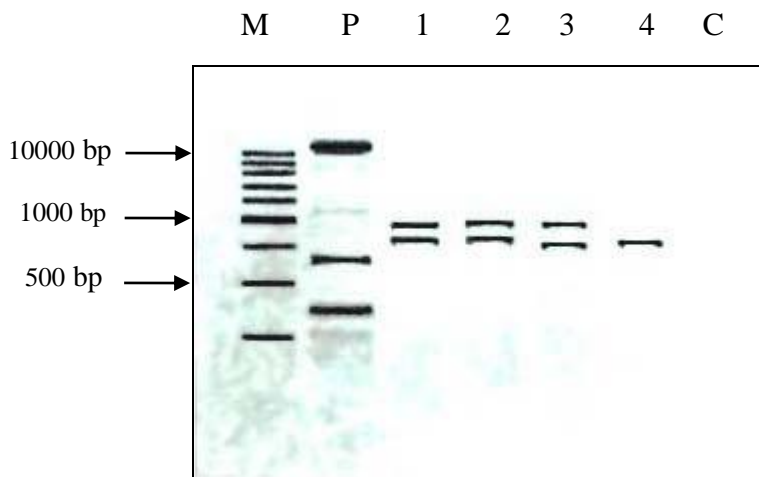


Fig. 5. Southern blot analysis of transgenic sugarcane plants. Lane M: GeneRular™ 1 Kb DNA ladder (Fermentas), lane P: positive control (pCAMBIA1302-Ab), lane 1-4: 4 independent transgenic sugarcane plants, lane C: Untransformed plants.

The bands indicate the integration of *CryIA(b)* gene into the genome of the transformed sugarcane plants, whereas the untransformed plant did not showed any integration of the transgene. In sugarcane, using the *Agrobacterium*-mediated transformation method one to three copies of transgene integration were described by Kalunke *et al.* (2009).

***CryIA(b)* expression analysis in transgenic sugarcane plants**

The transcriptional expression levels of *CryIA(b)* gene in four transgenic sugarcane plants were determined by using real-time PCR. It was found that the transcriptional levels of the transgene were higher in all transgenic plants compared to the control sugarcane plant (non-transgenic) (Fig. 6). Real-time PCR is a high-throughput method for quantifying transgene expression in transformed plants as Toplak *et al.* (2004) who reported that some transgenic lines of potato and tobacco showed high transgene expression in real-time PCR analysis.

Among the 4 transgenic plants, plant no. 4 showed 6-19 times higher *CryIA(b)* gene expression compared with the others. The expression of the gene in this plant was about 160 times higher than the untransformed plant. In Southern blot analysis, this plant showed a single gene insertion. Gene expression level is negatively correlated with copy number of the inserted gene. More than one copy of gene insertion creates co-suppression during gene expression. In tobacco, gene expression reduced with the increment of copy number of the integrated gene (Allen *et al.*, 1996; Gang *et al.*, 2002). Rao *et al.* (2013) also studied the copy number of transgene in transgenic tobacco and its impact on expression level. Expression in line QCC11 having two copies of transgenes was

high while the least expression was seen in lines QCC10 showing three copies of gene. The *Agrobacterium*-mediated transformation process is suitable for sugarcane. *Agrobacterium* transformation of plants has a high frequency of single gene insertion. The insertion of fewer gene copies targeted into transcriptionally active regions in the plant genome and with fewer truncated gene introductions currently makes *Agrobacterium*-mediated gene transfer a promising method for introducing agronomic genes into sugarcane (Grevelding *et al.* 1993).

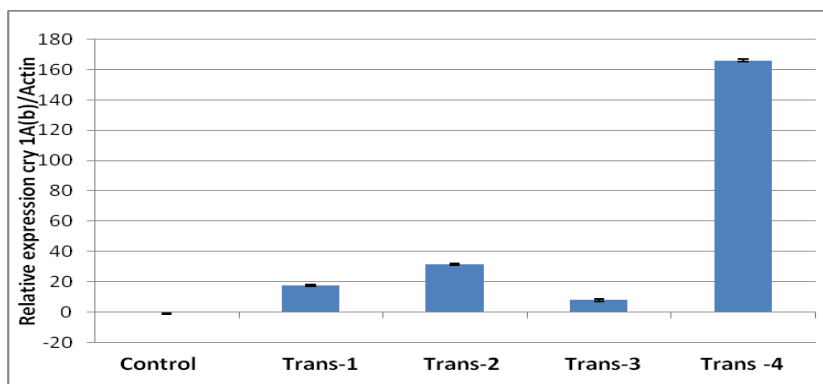


Fig. 6. Expression levels of *CryIA(b)* gene in 4 transgenic sugarcane plants (3 months old) compared with control (untransformed) sugarcane plant.

CONCLUSION

Sugarcane is a very important economic crop in tropical and subtropical regions of the world. The transformation of the *CryIA(b)* gene will be one of the efficient approaches to control the stem borer infestation. *Agrobacterium*-mediated transformation method was found suitable for sugarcane cultivar LK 92-11. During transformation 100 μ M acetosyringone was used. As a selection pressure 30 mg/L hygromycin was used. This concentration may be used as suitable selection pressure for further transformation of sugarcane. We found 80% transformation efficiency of the *hpt* gene and 43% for co-transformation of *hpt* and *CryIA(b)* gene. The transformed plants also showed a high expression of the transgene compared to the control, in real-time PCR analysis. Southern blot analysis showed one to two copies of gene integration in the selected transgenic plants. The method described here could be used as an efficient approach for gene transformation in sugarcane. Due to the *CryIA(b)* gene was isolated from native bacterium, this will make *CryIA(b)* gene is more specific to insect pest of sugarcane in Thailand. This data will be important for bio-safety procedure in greenhouse bio-assay in the future.

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