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Callogenesis and *Agrobacterium*-mediated genetic transformation of sugarcane (*Saccharum officinarum* L.) with a chitinase gene

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Abstract. The low cane and sugar yields due to fungal diseases have raised a very serious problem for sugarcane growers. Development of sugarcane plants with fungal resistant traits through genetic engineering is one way to overcome this problem. This study was therefore carried out to establish the *Agrobacterium*-mediated chitinase gene transformation system in sugarcane. Sugarcane calli formation were performed by inducing young leaf sheath of commercial sugarcane plants on Murashige and Skoog (MS) solid mediums supplemented with 3 mg/L 2,4-D and 10% coconut water. Calli obtained were then transferred to the same mediums supplemented with different concentrations of 6-benzylaminopurine (BAP) combined with indole-3-butyric acid (IBA) for shoot regeneration, and the maximum shoot regeneration was observed on mediums amended with 3 mg/L BAP combined with 0.5 mg/L IBA. *Agrobacterium tumefaciens* strain LBA 4404 (PBI 121), carrying a chitinase gene, 35S promoter and NOS terminator, was employed for the genetic transformation. Successful genetic transformation of sugarcane could be achieved by cocultivating sugarcane calli with A. tumefaciens for 30 min, followed by selection of transformed calli with 1,250 mg/L kanamycin. PCR and RT-PCR analyses confirmed the integration of the transgene.
Key Words: *Agrobacterium*-mediated transformation, chitinase gene, regeneration, sugarcane.

Introduction. Sugarcane (*Saccharum officinarum* L.) is a C_4 grass, which is commercially grown in many regions of the world. It accounts for approximately 80% of the world's sugar and is cultivated on a large scale in tropical and subtropical regions (Grof & Campbell 2001; Raza et al 2012). The low cane and sugar yields are attributed to several factors where salinity, drought, pests and diseases constitute major constraints (Nasir et al 2000; Khaliq et al 2005). The major pests and diseases that cause losses in sugarcane production include canegrubs, feral pigs, ratoon stunting diseases (RSD), sugarcane rusts, chlorotic streak and soil-borne diseases caused by fungi, bacteria, viruses and insect pest (McLeod et al 1999; Kumer et al 2004; Kalunke et al 2009). Approximately 100 diseases of sugarcane have been reported from different parts of the world which hinder sugarcane growth (Khurana & Singh 1975), thus lowering sugar production. Fungal diseases in sugarcane are the most predominant diseases appearing as spots on the leaves. These spots prevent the vital process of photosynthesis to take place, thus affecting growth and consequently the yield (Patil & Bodhe 2011). The applications of smokes and fungicide are among the most common methods to control fungal diseases in Thailand; however, these methods are laborious and the chemicals used may endanger the environment for many years. Development of sugarcane plants with fungal resistance ability through biotechnological tools could overcome such problems as, by this means, genes of interest can be introduced into plant genomes, and the genes will encode proteins which function as antifungal substances, thus enhancing sugarcane plants to protect themselves against fungal attack (Logemann & Schell 1993).

The enzyme chitinase, e.g., poly [1,4-*N*-acetyl-D-glucosaminid] glycan hydrolase (EC 3.2.1.14), is a low molecular weight pathogenesis-related (PR) protein that is often extracellular, acid soluble and protease resistant (Collinge et al 1993; Graham & Sticklen 1994). It catalyzes the hydrolysis of chitin (ß-1,4 linked polymer of *N*-acetyl-D-glucosamine), which is a major component of the cell wall of most fungi (Bartnicki-Garcia 1968). The introduction of a chitinase gene into plants has been shown to enhance resistance to fungal pathogens in several crops (Asoa et al 1997; Kramer & Muthukrishnan 1997; Nishizawa et al 1999; Punja 2001; Radjacommare et al 2004; Tohidfar et al 2005).

The present study reports on the establishment of the *Agrobacterium*-mediated chitinase gene transformation system in sugarcane.

Material and Method

Plant Materials. Young leaf rolls of field grown sugarcane plants collected from the Mitr Phol Sugarcane Research Center and brought to the laboratory. The outermost hard leaves were removed and 10 cm long leaf rolls were washed thoroughly in running tap water for 30 min. After that they were washed with detergent to remove all the traces of dust particles and disinfected in 70% alcohol for 3 min, soaked in 10% sodium hypochlorite solution supplemented with Tween 20 for 20 min, and then washed five times in sterile distilled water. After being dried on sterile papers, leaf rolls were transversely sliced into 2-3 mm thick slices and cultured on the Murashige and Skoog (MS) solid medium supplemented with 3 mg/L of 2,4-D, 10% coconut water (v/v), 30 g/L sucrose, pH to 5.7, before autoclaving for 20 min. The explants were cultured in the dark at 25±2°C for 4 weeks. After that, calli were monthly subcultured and transferred onto fresh medium for further callus proliferation. For plantlet regeneration, calli were transferred onto the MS solid medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (Table 1), 30 g/L sucrose, and 500 mg/L casein hydrolysate, pH 5.7. The cultures were kept at 25±2°C under a 16h light/8 dark photoperiod. After 8 weeks, number of shoots per callus was recorded.

Table 1

Concentrations of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) contained in the Murashige and Skoog (MS) medium used for plant regeneration

Hormone	*Medium*									
(mg/L)	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9	MS10
BAP	0	0.5	1.0	2.0	3.0	4.0	0.5	0.5	0.5	0.5
IBA	0	0.5	0.5	0.5	0.5	0.5	1.0	2.0	3.0	4.0

Effect of Antibiotics. Optimal concentration of kanamycin required to inhibit growth of calli was determined. Calli were cultured on the MS solid medium supplemented with 3 mg/L 2,4-D, 10% coconut water and 0, 250, 500, 750, 1,000, 1,250 and 1,500 mg/L kanamycin. The cultures were then kept at 25±2°C with 16/8 h light/dark photoperiod for 4 weeks. The optimal concentration of the antibiotic required to select transformed-calli was determined.

Agrobacterium-Mediated Transformation. *Agrobacterium tumefaciens* strain LBA 4404 was used for establishment of the transformation. The plasmid PBI 121 contained a chitinase gene and kanamycin resistance (*hpt*) gene, each of which was expressed under CaMV 35S promoter. For the transformation, a single bacterial colony was suspended in a 25 ml of LB liquid medium containing 50 mg/L kanamycin and incubated at 28^{o}C on a shaker at 150 rpm and cell suspension in the late log phase A_{600} at 1 was harvested for cocultivation with calli. After calli were immersed in the *Agrobacterium* suspension for 0, 10, 20, 30, 40, 50, 60 and 90 min, they were blotted on sterilized tissue papers and further cocultivated on the MS solid medium supplemented with 3 mg/L 2,4-D and 10% coconut water for 2 days. After that, *A. tumefaciens* was eliminated from the calli by washing the calli with sterile distilled water supplemented with 300 mg/L cefotaxime for

30 min. The calli were subsequently transferred to the MS solid medium for 7 days and then transferred onto the selection medium supplemented with 3 mg/L 2,4-D, 10% coconut water and 300 mg/L cefotaxime and 1,250 mg/L kanamycin. The cultures were kept under fluorescent illumination with 16/8 h light/dark at 25±2°C. After four weeks, the survival percentage of calli was recorded.

Polymerase Chain Reaction (PCR) Analysis. Total DNA was extracted from transformed and nontransformed control calli using the CTAB method (Doyle & Doyle 1987). The primer sequences for PCR were as follows: 35S forward sequence (F) 5' GCT CCT ACA AAT GCC ATC A 3', reverse sequence (R) 5' GAT AGT GGG ATT GTG CGT CA 3'; NOS (F) 5' GAA TCC TGT TGC CGG TCT TG 3', (R) 5' TTA TCC TAG TTT GCG CGC TA 3'; chi (F) 5' ACG CAA ACG CCG TCT ACT TCA CCA A 3', (R) 5' GCA TCC CAG AAC ATG CTG CCT CCC A 3'. Reactions were performed with the standard program which was comprised of denaturation for 5 min at 94^{o}C for 35S and NOS, and at 95^{o}C for chi, followed by amplification [30 sec at 94^{o}C; 30 sec at 55^{o}C for 35S, 40 sec at 60^{o}C for NOS, and 1 min at 60^{o}C for chi; 1 min at 72^{o}C] and final extension for 10 min at 72^{o}C. PCR products were size-separated by electrophoresis on a 1.5% agarose gel with ethidium bromide at 50 V for 90 min.

Reverse Transcription-PCR (RT-PCR) Analysis. The expression of a chitinase gene in transformed and nontransformed control calli was determined by RT-PCR analysis. Total RNA was isolated from sugarcane calli with RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase-treated RNA was reverse-transcribed into first-strand cDNA with the use of reagents supplied from Promega Corporation, Madison, USA. Afterwards, cDNA was used as a template for the amplification of chitinase and TPI genes by using chitinase and TPI primers, respectively. In reverse transcriptase-PCR (RT-PCR), normalization was performed using a TPI gene as a housekeeping gene as reference against the expression level of a gene under investigation. Control reaction or a template without reverse-transcribed RNA (-RT) was performed to rule out possible amplification from contaminating genomic DNA. The primer sequences were as follows: chi forward sequence (F) 5' ACG CAA ACG CCG TCT ACT TCA CCA A 3', reverse sequence (R) 5' GCA TCC CAG AAC ATG CTG CCT CCC A 3'; TPI (F) 5' CAA TGA CTG GAG CAA CGT AG 3', (R) 5' GTA ACA GAG CCT CCG TAG AT 3'. Amplified cDNAs were resolved on 1.5% agarose gel, stained with ethidium bromide (EtBr) and documented.

Results and Discussion

Plantlet Regeneration. The effect of various concentrations of BAP and IBA on shoot regeneration was evaluated after 8 weeks of culture. Appearance of green spots on each callus clump was observed in the first week of the culture. The maximum number of shoots per callus of 20, and the highest shoot regeneration capacity of 100% were observed when calli were cultured on the MS solid medium supplemented with 3.0 mg/L BAP and 0.5 mg/L IBA (Table 2). The current study was in good agreement with one previous study which reported that shoot regeneration of sugarcane cultivar CO 671 from calli could be achieved by the application of 1.0 mg/L BAP combined with 0.5 mg/L IBA (Gopitha et al 2010). Shoot initiation and multiplication of sugarcane varieties Isd-16 and Isd-28 were successful using 1.0 mg/L BAP combined with 0.5 mg/L IBA (Karim et al 2002). Another study supported that the combination of BAP and IBA promoted shoot formation in sugarcane (Islam et al 1982). In addition to the use of BAP and IBA, the combination of BAP and NAA has also been reported to have a positive effect on shoot formation, but with inferior effect. MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA performed the best for shoot formation in sugarcane cultivar Nayana (Behera & Sahoo 2009). It was noticed that combination of high level of cytokinin and low level of auxin was essential for shoot differentiation in sugarcane calli obtained from young leaf sheaths. It could be concluded that callus regeneration capacity was specific and genotype dependent phenomenon and at the same time it is in parallel with hormonal concentration and combination (Maretzki 1987). The results demonstrated that casein hydrolysate could be used as a source of calcium, phosphate, several microelements, vitamins and most importantly a mixture of up to 18 amino acids (George et al 2008).

Several previous studies indicated that casein hydrolysate itself is more effective for plant culture than the addition of major amino acids (Molnar et al 2011). Evidently, the application of 500 mg/L casein hydrolysate as a natural complex nutrient source in the MS medium for shoot regeneration in different varieties of sugarcane has been reported in previous studies (Aftab et al 1996; Gandonou et al 2005).

Table 2

Effect of different concentrations of BAP and IBA on shoot regeneration induced from sugarcane calli

Medium	*Shoot regeneration capacity (%)*	*Number of shoots per callus ±SE*
MS1	100	14±0.8718
MS2	100	13±2.8914
MS3	100	10.4±2.9428
MS4	100	19.6±1.0770
MS5	100	20.0±1.7029
MS6	80	2.6±0.8124
MS7	60	2.6±1.6613
MS8	100	15.2±3.2156
MS9	100	15.0±2.2136
MS10	40	15.0±2.2136

Effect of Antibiotics. The optimal concentration of kanamycin was determined for screening transformants after the transformation. The results showed that the calli could survive under the concentrations of 0, 250 and 500 mg/L kanamycin, with the survival percentage of 100. However, the lowest concentration of 1,250 mg/L was found to completely inhibit callus growth (Fig. 1). All the calli turned brown and eventually died within 4 weeks and no escapes were found at this concentration. Therefore, the concentration of 1,250 mg/L was considered optimal for selection of transformants. The results demonstrated that sugarcane calli possess high natural resistance to kanamycin as other monocotyledonous plants reported in many previous studies. Kanamycin concentrations of above 500 mg/L were found to be crucial to inhibit the growth of rice calli (Colby & Meredith 1990). Furthermore, kanamycin concentrations of more than 800 mg/L were found to be required for inhibiting the growth of several Gramineae cell cultures incubated in the dark (Hauptmann et al 1988).

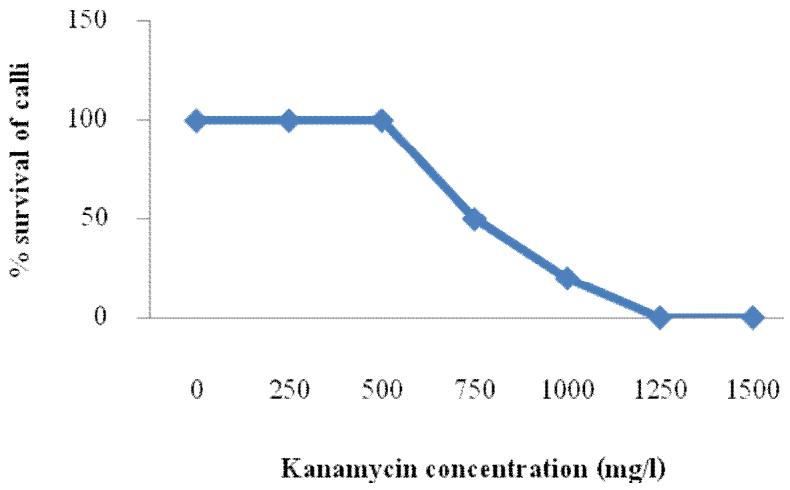

Fig. 1 Effect of kanamycin on sugarcane callus growth.

Agrobacterium-Mediated Transformation. Cocultivation time is considered crucial in the *Agrobacterium*-mediated transformation as it determines gene expression level and transformation efficiency. In the present study, the results showed that the highest transformation efficiency of 72% was achieved by cocultivating sugarcane calli in *Agrobacterium* suspension for 30 min (Table 3). Previous studies suggested that different types of tissues from different plant species require different cocultivation time for maximum transformation efficiency. The highest transformation efficiency of rice was observed in from 7-day-old seed-derived calli cocultivated in *Agrobacterium* suspension for 30 min (Maneewan et al 2005).

Table 3

Effect of cocultivation time on number of infected calli and survival percentage of the calli

Cocultivation time (min)	*Number of infected calli*	*Transformation efficiency (%)*
0	50	0±0.0000
10	50	36±0.6782
20	50	46±1.2083
30	50	72±0.7348
40	50	54±1.0295
50	50	22±0.5831
60	50	16±0.2449
90	50	8±0.3741

PCR Analysis. Successful introduction of the three genes (35S, NOS and chitinase) was confirmed by PCR using specific primers for each gene. The results showed expected fragments of 195, 180 and 1,200 bp of 35S, NOS and chitinase genes, respectively (Fig. 2). The results suggested that target genes were successfully introduced into plant tissues by the function of a very strong constitutive promoter CaMV35S. The current study was well supported by previous studies reporting that a CaMV35S promoter is widely used as promoter in transgenic wheat and rice (Lin et al 1995; Chen et al 1998).

RT-PCR Analysis. The expression of the target gene in the transformed calli was analyzed by RT-PCR. The target gene chitinase with the band 1200 bp in length at the transgenic calli were detected, which indicated that the chitinase gene successfully expressed in transgenic sugarcane calli at RNA level (Fig. 3). Non-transformed calli were used as negative controls and no PCR products were detected. The TPI gene was used as a housekeeping gene as references against the expression level of a chitinase gene and our results showed that transformed calli had higher expression level of a chitinase gene than the TPI gene. In RT-PCR analysis, fewer than 10 copies of target DNA are required, and it has been successful when the RNA was isolated from a single cell (Razin et al 1991). It is well known for many previous studies that RT-PCR technique is widely used to detect gene expression in many plant species, e.g., olive (Jafarzadeh-Bajestani et al 2011) and potato (Kashani et al 2012).

Conclusions. A protocol for successful genetic transformation of sugarcane was achieved. Factors such as explants, cocultivation time and concentrations of selective agents have a profound effect on successful genetic transformation. The genetic transformation system used in this study is very easy to handle, cost-effective, and non-laborious.

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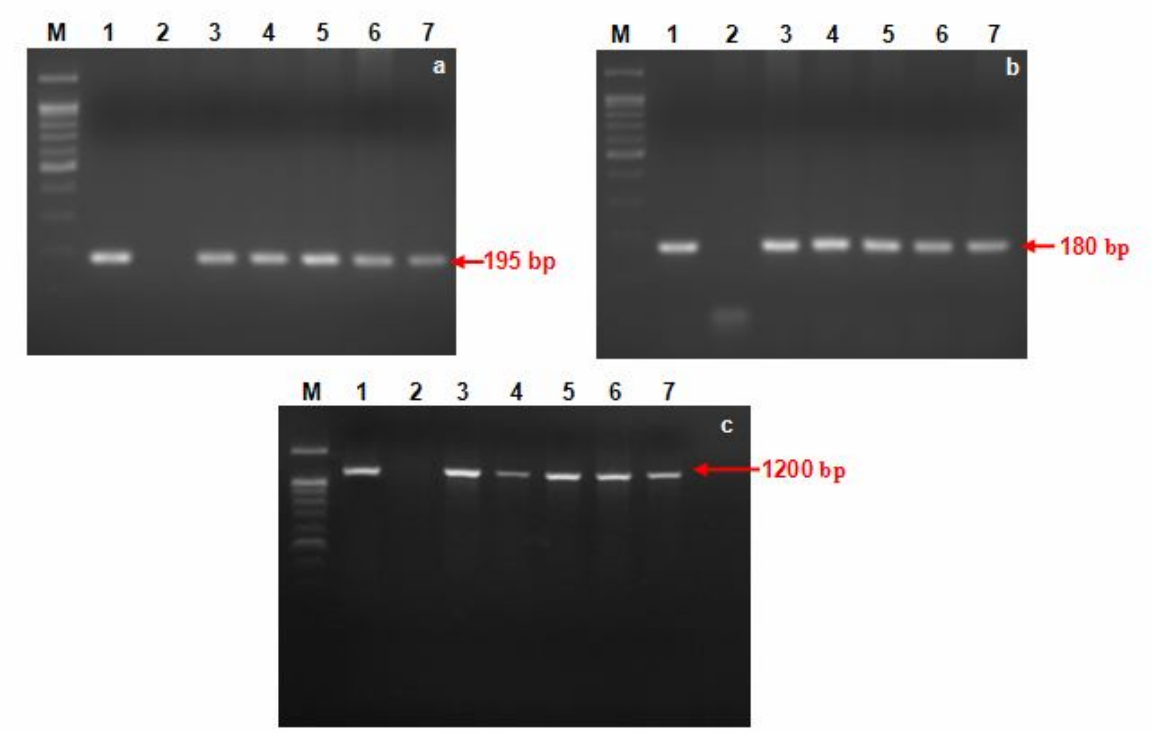

Fig. 2 PCR analysis of transformed sugarcane calli using primers to detect the 35S (A), NOS (B) and a chitinase gene (C); lane M: 100 bp DNA ladder, lane 1: positive control of the plasmid, lane 2: untransformed sugarcane calli, and lanes 3-7: transformed sugarcane calli.

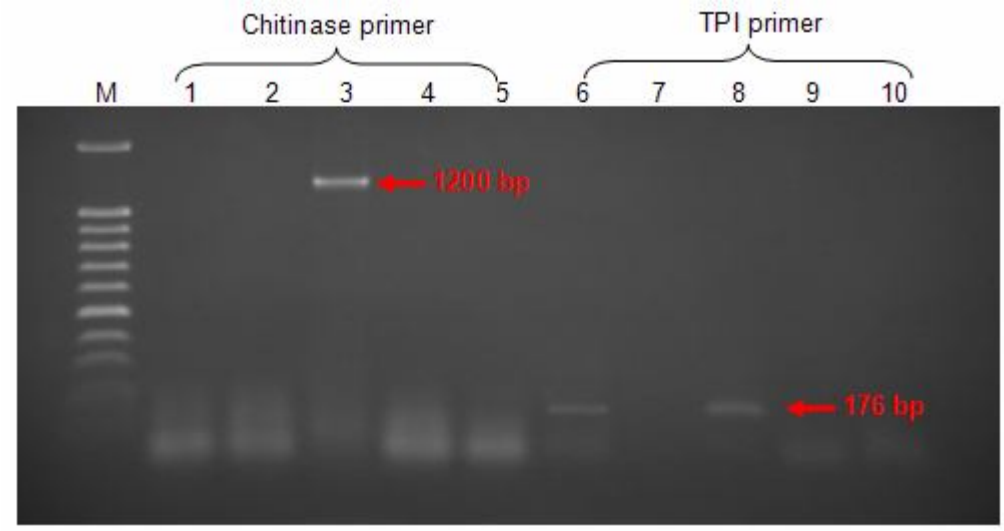

Fig. 3 RT-PCR analysis of the transformed sugarcane calli using chitinase and TPI primers; lane M: 100 bp DNA ladder; lanes 1 and 6: non-transformed sugarcane calli with reverse transcriptase enzyme; lanes 2 and 7: non-transformed sugarcane calli without reverse transcriptase enzyme; lanes 3 and 8: transformed sugarcane calli with reverse transcriptase enzyme; lanes 4 and 9: transformed sugarcane calli without reverse transcriptase enzyme; lanes 5 and 10: H_2O.

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