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Agrobacterium tumefaciens-mediated transformation of chili with CMV (Cucumber Mosaic Virus) coat protein gene

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ABSTRACT

The recombinant plasmid, pSNC3301 carrying the coat protein gene of cucumber mosaic virus (CpCMV) was used in the *Agrobacterium*-mediated transformation of chili (*Capsicum annuum* L.). The transformation protocol required 2 days co-cultivation, 2 days pre-selection in non-BASTA™ herbicide containing medium, 3-4 weeks selection in BASTA™ herbicide containing medium, 20-25 weeks for culturing of adventitious shoot in regeneration medium and finally 1 week in rooting medium. PCR analysis detected the presence of CpCMV gene with transformation efficiency of 1.5% (7/467) for hypocotyl and 0.7% (4/536) for shoot tip. Histochemical GUS assay of hypocotyls, showed only nine from 22 PCR positive transformants showed GUS expression. For shoot tip, three of 13 PCR-positive transformants expressed GUS. The transformation efficiency of coat protein using pSNC3301 was 1.5% (7/467) for hypocotyls and 0.7% (4/536) for shoot tip.

Key words: *Capsicum annuum*, shoot tip, hypocotyls, transformation, *Agrobacterium* Coat protein, CMV.

INTRODUCTION

Chili is an important fruit-vegetable crop in Malaysia. Diseases caused by viruses are the most difficult problems to control in chili cultivation. Five types of viruses have been reported to infect chili plants. They are the cucumber mosaic virus (CMV), chili veinal mottle virus (CVMV), tobacco mosaic virus (TMV), tomato spotted wilt virus (TSWV) and tobacco leaf curl virus (TLCV). CMV appears to be the most important virus with detrimental effects to chilli (Green, 1992).

There is no known effective control on viral disease although studies on the use of reflective mulches and barrier crops indicated some degrees of control although the economic gain through such practices leaves much to be desired. Considering the grave losses that we encounter with chilli, we propose to study the effectiveness of the viral coat protein genes via gene transfer technology through the application of genetic engineering. Genetic manipulation has been limited in peppers(*Capsicum annuum* L.) as chili is considered to be an extremely difficult recalcitrant species with respect to *in vitro* regeneration and genetic transformation (Liu *et al*. 1990). In addition, success of transformation is considerable inconsistent with low and variable transformation efficiencies.

One of the reasons postulated to explain this low efficiency and inconsistency is the interference of selection agents used in the cultivation media particularly the antibiotic kanamycin (Brasileiro & Aragao 2001; Estopa *et al.* 2001). The unsuccessful generation of explants from shoot-like structures formed on chili explants following transformation with kanamycin-containing pBI121-CMVCP plasmid (Ahmad *et al.* unpublished data). This therefore prodded us to construct a new transformation plasmid carrying a different selection marker.

In recent years, the bar gene which encodes for phosphinothricin-N-acetyltransferase enzyme, and commonly known as glucofosinate,

has been used for selection of transformants in several crop plants (Toldi *et al.* 2000). The transfer and expression of the *bar* gene via appropriate transformation vectors and protocols enable the plant cells to withstand the application of the selective agent, thereby allowing the growth of resistant tissues and regeneration of plantlets (Cajacob *et al.* 2004). In this study we constructed the pSNC3301 plasmid vector and applied it generate transgenic chili plants carrying the CpCMV gene.

MATERIAL AND METHODS

Plant materials

Seeds of Cilibangi 4 were surface sterilized in absolute ethanol for 3 minutes and subsequently in 20% (v/v) sodium hypochlorite for 20 minutes with a final rinse in several changes of sterile water. Seeds were germinated on MS medium (Murashige and Skoog, 1962) with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.7 solidified with 0.8% (w/v) Plant agar (Duchefa, Harlem, The Netherlands). Seeds were incubated in a growth room at 25± 2°C with a photoperiod of 16h/day. Shoot tips and hypocotyls explants was taken from 10-12 day aseptically grown seedlings.

Bacterial strain and genetic construct

Disarmed *A. tumefaciens* strain LBA4404 harboring plasmid pCAMBIA containing the CMV-CP (coat protein gene) driven by CaMV35S promoter was constructed in this study. This plasmid was named pSNC3301 and carries the *Bar* selectable marker gene and the *uid*A reporter gene controlled by the CaMV35S promoter.

Construction of pSNC3301 vector

The CMV-CP gene was amplified sequence was amplified by PCR using specific designed primers. The ~750bp gene fragment was amplified with a restriction enzyme sites for *Xba*I and *Hind*III were incorporated into the forward primer and the *BamH*I site was incorporated into a reverse primer amplifying the gene. This PCR product was digested with *Xba*I and *BamH*I restriction enzymes and then ligated into the *Xba*I/*BamH*I site of pBluscript KS-(Strategene). The PCR was conducted using a Robocycler gradient 96 (Stratagene). Plasmid DNA(20 ng) was mixed with PCR buffer (10 mM Tris-HCl (pH8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, GibcoBRL)), 5 ìM dNTPs (Sigma), 100 pM primers (Sigma), and 1 unit of Deep Vent *Taq* polymerase(BioLAB). The PCR reaction was conducted under conditions of pre-denaturation at 95 °C for 5 min for 1 cycle, and 30 cycles for denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min 30 sec, and elongation at 72 °C for 1 min.

The GUS coding sequence of ~750bp was amplified by PCR from pBI121. The forward primer of the GUS coding sequence contains a *Bgl*II restriction site. The PCR product was digested with *Bgl*II and *Nsi*I, and then ligated to the *BamH*I and *Pst*I sites of pBluescript KS- to obtain the construct CMV-CP/TNos/P35S/GUS/TNos. The P35s promoter was amplified from vector The CMV-CP/TNos/P35S/GUS/TNos sequence coding sequence was cloned into the pCAMBIA 1301 vector for the first step of this experiment.

Agrobacterium transformation

Transformed *Agrobacterium* was grown at 28°C in Luria broth (LB) medium supplemented with 100mgl^{-1} streptomycin and 50mgl^{-1} kanamycin for selection of the pSNC3301 vector. The cultures were collected in the log phase, when the absorbance at 600nm was between 0.5 and 0.6.

Media

The compositions of media were based on the work of Li *et al.* (2003), and these were DM1, DM2, DM3, EM and RM. These media were used for differentiation, elongation and rooting of the shoots, respectively. Media DM1 and EM consist of MS basal medium [MS salts (Murashige-Skoog (1962), Gamborg B5 vitamins (Gamborg *et al.* 1968) and 30g/l sucrose] supplemented with a combination of 1.0mg/L IAA + 5.0mg/L BAP (DM1, 2, 3) and 1.0mg/L + 5.0mg/L BAP (EM). RM is an MS basal medium supplemented with a hormone combination of 0.2mg/L NAA + 0.1mg/L IAA. All of the media were solidified by agar (0.8 %), and the pH was fixed at 5.8 before autoclaving at 121°C for 20 min.

DJ nutrients

To obtain the DJ nutrients, 15 g (fresh weight) of chili seedlings (Cilibangi 4) was ground

in 3-4 ml water. The slurry was then transferred to a 50 ml centrifuge tube containing 40 ml distilled water, mixed well and the centrifuge at 8 000 rpm for 3 min.

Pre-culture and inoculation with *Agrobacterium*

Shoot tip and hypocotyl explant transformations were carried out as described by Ahmad *et al.* (2002). A single clone of bacteria was grown overnight in LB medium with 100mgl^{-1} streptomycin and 100mgl^{-1} kanamycin, diluted to optical density of 0.1(OD_{600}=0.1) and grown to OD_{600} 0.4-0.6 for 3 hours at 28°C. Shoot tip and hypocotyl explants were cut from 10-12 day old seedlings. Following preculture on DM1 medium supplemented with 15, 000 mg/L DJ nutrient for 2 days, the explants were inoculated with the culture of bacteria for 15 min, followed by co-culture on DM1 medium with 15, 000 mg/L DJ nutrients for 2 days and culture of DM2 medium with 15, 000 mg/L DJ and 500 mg/L Carb for 2 days of delay selection. The explants were then transferred to DM3 medium with 15, 000 mg/L DJ nutrients, 10 mg/L $AgNO_3$, 5.0 mg/L BAP, 1.0 mg/L IAA, 500 mg/L Carb and 2.0 mg/L Basta for selection. After 3-4 weeks, the regenerated buds were transferred to EM medium with 15, 000 mg/L DJ nutrients, 10 mg/L $AgNO_3$, 2 mg/L GA_3, 3.0 mg/L BAP, 1.0 mg/L IAA and 500 mg/L Carb for bud elongation and development. The buds were excised from the explants and subcultured on the respective media, which were EM for a period between 15-20 weeks, followed by RM for rooting.

Transformant analysis

β-Glucuronidase (GUS) activity assays

The histochemical GUS assay substrate was performed as describe by Jefferson *et al.* (1987). Thin sections of leaf were immersed in an X-Gluc solution (Kosugi *et al.* 1990). The GUS assay buffer consists of 0.2 M $NaPO_4$ buffer (pH 7.0), 0.1 mM $K_3[Fe(CN)_6]$, 0.1 mM $K_4[Fe(CN)_6]$, 0.5 M EDTA, 0.1% Triton^{Ò} X-100, 20% (v/v) methanol and 100 mg X-Gluc. Tissues were stained overnight in the dark at 37°C. The tissues were subsequently fixed and cleared in FAA solution (85% ethanol 50% (v/v); 5% glacial acetic acid; 10% formalin) prior to visualization.

Polymerase chain reaction analysis

Chili Genomic DNA was extracted from young leaf tissues of transgenic and non-transgenic plants essentially according to the CTAB method of Doyle & Doyle (1990). The primer sequences used in the PCR analyses for detection of the CMV-CP gene insertion were P1: 5'-GTGGAGCACGACA CACTTGTCTAC-3' and P2: 5'-CGGACTGTCACCC ACACGGTAG-3'. The expected amplified fragment DNA would be ~ 0.750 kb. The GUS gene insertion into the plant genome was amplified using primer sequences P1: 5'-CGCCGATGCAGATATTCGTA-3'

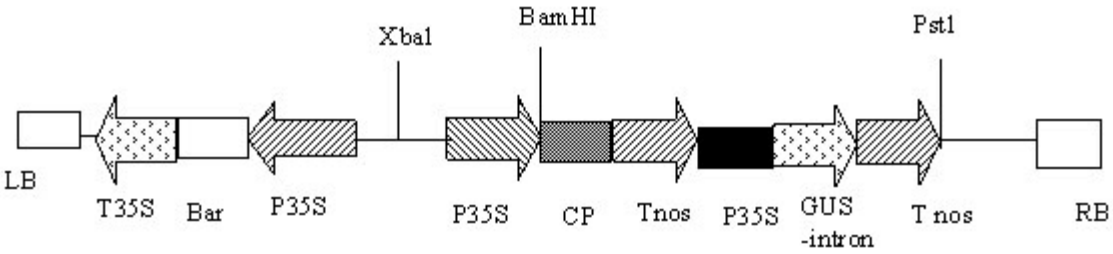

Fig. 1. : Linear map of pSNC3301-TDNA cassette. LB/RB- left/right T-DNA border sequences; P35S/T35S-CaMV 35S promoter/terminator; *bar*-coding region of the phosphinotricin resistance gene; T*nos*- nopaline synthase terminator; gus-intron-*gus*A gene coding region with intron sequence.

Region Amplified	Primers	Direction
P35S	5'CTAGTCTAGAAAGCTTGATCAAGATCGCCGCG3'	Forward
	5'CGCGGATCCCGCAAATGAGGCAGAAA3'	Reverse
Tnos	5'GAAAACTGCAGATAAGTATGAACTAAAATGCA3'	Forward
	5'CCGGCGGTACCACTAGTAGATCTATCATAACAT3'	Reverse

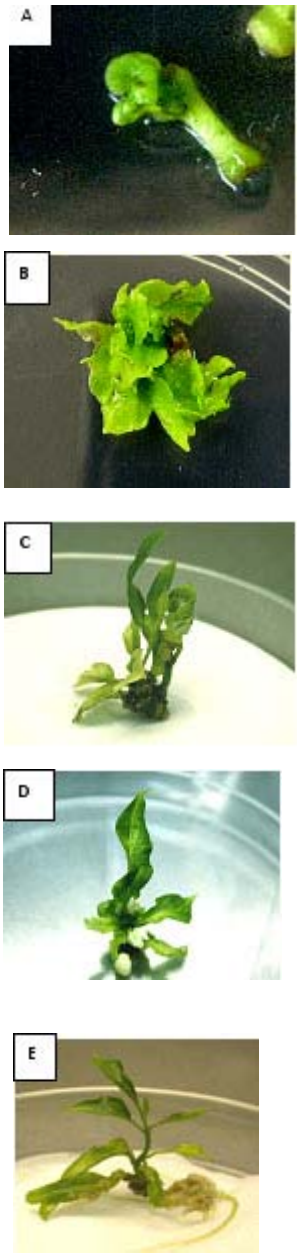

Fig. 2 a-e: Development of direct bud adventitious bud following co-culture for hypocotyl explants and incubated on different media. A: Adventitious bud formation (4 weeks on BASTA selection medium). B: Multi-shoot formation (7 weeks on EM medium). C: Multi shoot elongation (15 weeks on EM). D: Single-shoot development and elongation from multi shoot (19 weeks on EM medium). E: Root formation (20-25 weeks on RM)

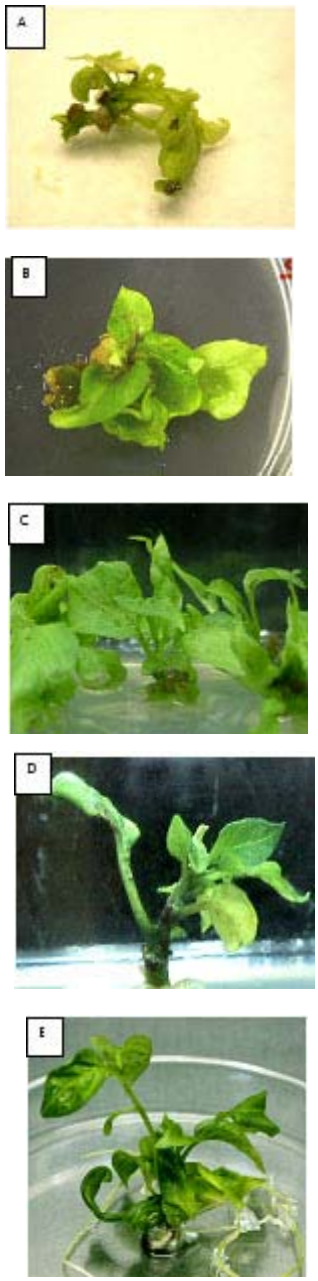

Fig. 3a-e: Development of direct bud adventitious bud following co-culture for shoot tip explants. (A) adventitious bud formation (3 weeks on BASTA selection medium). (B) multi-shoot formation (7 weeks on EM medium). (C) Multi shoot elongation (15 weeks on EM medium . (D) single-shoot development and elongation from multi shoot (19 weeks on EM medium). (E) root formation (20-25 weeks on RM medium). Black lines represent 1 cm.

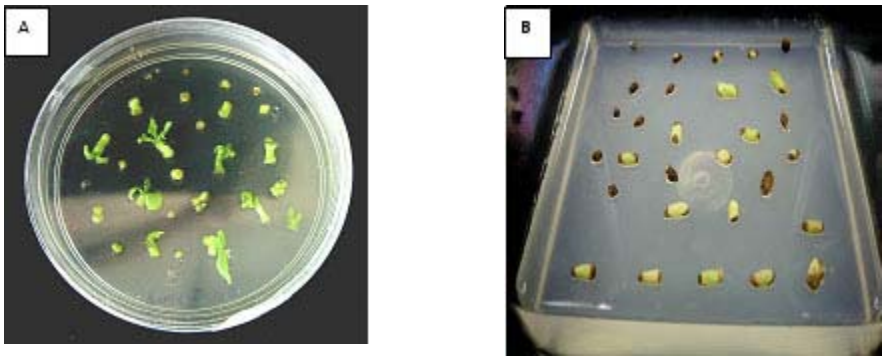

Fig. 4: (A) Hypocotyl explants resistant after 4 weeks in selection medium (2.0 mg/L Basta). (B) Basta-sensitive control explants

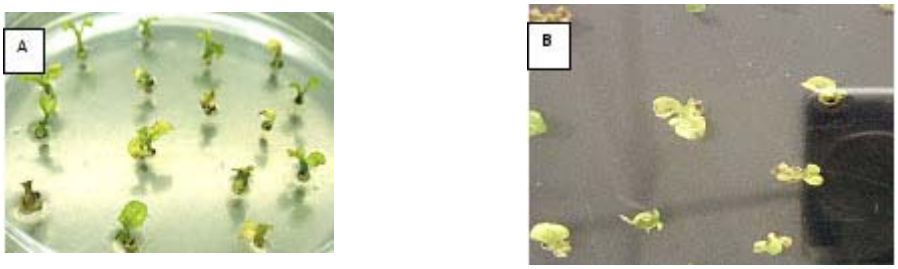

Fig. 5(A): Shoot tip explants resistant after 4 weeks in selection medium (2.0 mg/ Basta). (B) Basta-sensitive control explants

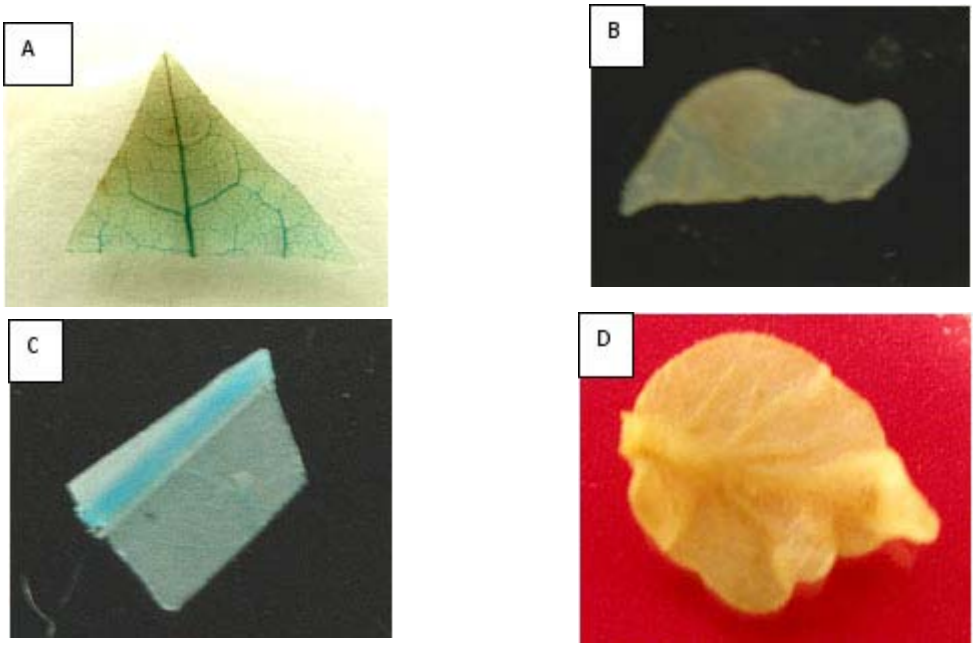

Fig. 6: Histochemical localization of *GUS* activity in putative *GUS* transformants. Staining observed in the vascular tissue of leaf. (A): *GUS* expression in hypocotyls explants. (B): *GUS* expression in shoot tip explants

and P2: 5'-ATTAATGCGTGGTCGTGCAC-3'. The estimated amplified fragment was ~ 0.750 kb. PCR analysis was performed in 25-ml final volumes containing 20 mM primers, 10 mM dNTP mix, 5 U/ml *Taq* DNA polymerase and 25 mM $MgCl_2$. Amplification of the coat protein gene involved 30 cycles of 95°C (1 min), 58°C (1 min, 30 sec) and 72°C (2 min). For GUS gene, amplification program consisted 30 cycles of 95°C (30 sec), 60 °C (1 min) and 72°C (2 min). Amplification was carried out in a DNA Master Cycler Gradient (Eppendorf).

RESULTS AND DISCUSSION

The general patterns of chilli shoot formation were identified. A shoot or multiple shoots formed directly from the wound or cut region of the shoot tip and hypocotyl explants (direct regeneration) (Fig. 2). Five stages of shoot development were observed: direct shoot formation, multi-shoot, elongation, single-shoot elongation, and root formation. In general, 20-25 weeks of shoot development after co-culture was required before acclimation treatment. The patterns of development for hypocotyls are as in Fig. 2 and those exhibited by shoot tip explants is as shown in Fig. 3.

Frequencies of adventitious bud induction

In order to determine the adventitious bud formation rate after transformation, 467 explants from hypocotyls and 536 explants from shoot tips were transformed with the pSNC3301 vector. The number of hypocotyls surviving after 3 weeks in selection medium (2.0 mg/L Basta) was 44.5% (208/467). The rate of developing adventitious bud after 4 weeks in selection medium was 28.3% (59/208) (Fig. 4). The number of shoot tips surviving after 2 weeks in selection medium (2.0 mg/L) was 43.7% (234/536). The rate of developing adventitious buds after 3 weeks in selection medium was 25.2% (59/234) (Fig. 5). Non- transformed explants of hypocotyls and shoot tips, survived the selection medium (2.0 mg/L Basta).

GUS expression and molecular characterization of transformed plants

Leaves from hypocotyls and shoot tip from 30-day old explants were stained histochemically to monitor GUS activity in both control and transformed explants. The non-transformants of both explants failed to exhibit a blue coloration normally associated with GUS activity (Fig. 6-b & d). Out of 95 hypocotyls, 9.5% (9/95) explants showed GUS expression and out of 89 shoot tips, 3.6% (3/83) showed a positive result for GUS staining. The transformed explants exhibited a blue colorations at the vascular tissue of leaf (Fig. 6-a & c). This study shows that the CaMV35S promoter, which is the promoter most commonly used to express transgenes in dicotyledon, functions well in chilli (Hauptmann *et al.* 1987). The GUS expression was highly expressed in vascular bundle. In transgenic rubber tree (*Havea brasiliensis*), the CaMV35S promoter directs GUS expression in the phloem tissue and laticifers (Arokiaraj *et al.* 1998). The 35S-GUS-intron construct ensures that the GUS expression arises from transformed plants tissue and not from bacterial contaminant (Vancanneyt *et al.* 1990).

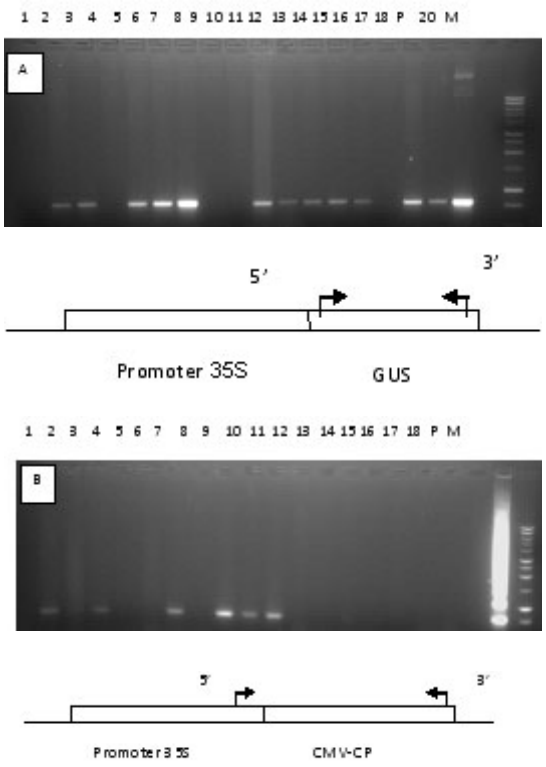

Fig. 7: PCR amplification detect the presence of GUS (a) and coat protein (CMV-CP) genes in hypocotyl and shoot tip transformants. The pSNC3301 plasmid was used as positive control.

The degree of GUS expression was not uniform in different line, some show strong expression and others weak expression. This may due to individual plants having a different number of transgene copies (Hobbs et al. 1990). Prols and Meyer (1992) also reported that different expression intensity could be due to methylation of the chromosomal integration region. The variations of GUS activity was most likely the random integration of the T-DNA into the genome and reflective of the effect of adjacent genomic sequences on the expression of the GUS gene (Igasaki 2000). Non-expression of the GUS gene could be due to incomplete insertion of the GUS gene, DNA methylation of the promoter region or chromosome, positional effects or because of transgenic silencing (Kilby *et al.* 1992; Ohta 1986; Prols & Meyer 1992).

Transformation rate

In total, 95 Basta-resistant shoots were regenerated from hypocotyls explants and 83 Basta-resistant shoots were regenerated from shoot tip explants. When the genomic DNA of the 95 hypocotyl-derived regenerants and 83 shoot tip-derived regenerants were analysed by PCR, both the 0.748 kb GUS (Fig. 7 a) and the 0.751 kb CMV-CP (Fig. 7b) proteins were detected. The transformation efficiencies obtained were 1.5% (7/467) for hypocotyl explants and 0.7% (4/536) for shoot tip explants.

PCR analysis detected 22 GUS-positive transformants. However, the histochemical GUS assay for hypocotyl, revealed that of the 22 tested, nine were positives. Thus in total, only 9.5% (9/95) showed GUS expression. All 73 PCR negative putative transformants did not express GUS. For shoot tip, three out of 13 or 3.6% (3/83) PCR-positive transformants expressed GUS, while 70 negative putative transformants tested, did not express GUS. The transformation efficiency of coat protein gene carried in the pSNC3301 vector was 1.5% (7/467) for hypocotyl and 0.7% (4/536) for shoot tip. The transformation efficiency of GUS gene using pSNC3301 was 4.7% (22/467) for hypocotyl and 2.4% (13/536) for shoot tip.

Although the number of putative transformants obtained in this study may be low, this study has been successful in generating transgenic plant via the *Agrobacterium*-mediated gene transfer methodology (Dong & McHugen 1993; James *et al.* 1990; Schmulling & Schell 1993). Dong & McHughen (1993) suggested that the types of chimeric transgenic plants varied depending upon the number and position of transformed cells in the developing tissues. This transformed cells divided and elongate sectorally or periclinally, forming clear boundaries with non-transformed cell. Hence, leaves that develop from chimeric plants may be fully transformed, non-transformed, or chimeric.

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