

Comparison between two transformation systems for introducing CMV coat protein construct into local hot peppers

Zarina Zainuddin¹, Norzulaani Khalid² and Tan Chong Seng³

^{1,2}Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

³Biotechnology Centre, MARDI, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia

ABSTRACT Two transformation systems, via *Agrobacterium tumefaciens* and particle bombardment, for two varieties of chilli pepper (*Capsicum annuum* L.) were determined. The plasmid pCMV.CP was used for the transformation. After transformation, buds were selected on the regeneration medium containing kanamycin (50 mg/l). Based on PCR analysis, transformation via *A. tumefaciens* was the best approach for introducing a novel chimeric gene into chilli tissues.

ABSTRAK Dua sistem transformasi, menggunakan *Agrobacterium tumefaciens* dan 'particle bombardment' bagi 2 varieti cili ditentukan di dalam kajian ini. Plasmid pCMV.CP digunakan bagi transformasi. Selepas proses transformasi pucuk dipilih di atas media regenerasi yang mengandungi 50 mg/l kanamisin. Berdasarkan kepada analisis PCR, didapati transformasi menggunakan *A. tumefaciens* adalah kaedah terbaik bagi memperkenalkan gen kimerik ke dalam tisu cili.

(*Capsicum annuum*, *Agrobacterium tumefaciens*, particle bombardment, coat protein)

INTRODUCTION

Chilli pepper ranks as one of the most popular fruit vegetable in this country. Malaysians consumed over RM 90 million worth of chilli in 1991 [1] and in the year 1998, commercially growing of chilli covered about 2735 hectares in Peninsular Malaysia [2]. The occurrence of virus disease has become a major production constraint in chilli, causing yield loss as high as 80%. Five types of viruses have been reported to infect chilli plants, namely cucumber mosaic virus (CMV), chilli veinal mottle virus (CVMV), tobacco mosaic virus (TMV), tomato spotted wilt virus (TSWV) and tobacco leaf curl virus (TLCV) [3]. Among these, CMV and CVMV appear to be the most important viruses in terms of incidence and yield loss [4] causing yield losses ranging from 10-15% if infection comes at the later stage and may reach up to 60%, if plants are infected at an early stage [5]. Thus, chilli should be a prime target to receive viral coat protein genes via gene transfer technology.

The introduction of genes into plants by means of genetic engineering provides a powerful tool for

crop improvement. In the last two decades there have been a significant development in plant transformation technology, due to the development of a range of *Agrobacterium*-mediated and direct DNA delivery techniques, plus an appropriate tissue culture technique in a large number of species. Although chilli belongs to the family Solanaceae, whose members are easily amenable to tissue culture and transformation practices, it is highly recalcitrant [6].

There have been only a few reports on genetic transformation of pepper via *A. tumefaciens*. Liu *et al.* [7] had produced transformed shoot buds but failed to regenerate. Lee *et al.* [8] and Zhu *et al.* [9] reported the production of transgenic hot pepper and sweet pepper, respectively. However in both cases the transformation efficiencies were low. So far there is no report on reliable and stable genetic transformation of the local chilli pepper. In this study our aim is to develop a reliable protocol for transformation by two approaches, *A. tumefaciens*-mediated transformation and particle bombardment. Here we managed to develop an efficient and stable

transformation system for introducing a chimeric gene consisting CMV construct in chilli.

MATERIALS AND METHODS

Establishment of aseptic plants

Seeds of two chilli varieties, MC12 and Kulai, obtained from the Malaysian Agricultural Research Development Institute (MARDI) were imbibed overnight. The following day, the seeds were surface sterilised with 70% (v/v) ethanol for 30 seconds followed by a 10 minutes treatment with 25% (v/v) commercially prepared sodium hypochlorite (Clorox) and a few drops of surfactant, Tween 20. The surface sterilised seeds were thoroughly washed with sterilised water. Seeds were germinated for 2 weeks on solidified MS media to obtain explant source. The pH of the media was adjusted to 5.8 prior to autoclaving. All cultures were incubated at 25°C under continuous light.

Bacterial strain and vector

Disarmed *A. tumefaciens* strain LBA 4404 harboring plasmid pCambia containing the CMV coat protein gene driven by 35S promoter (Figure 1) was provided by Dr. Tan Chong Seng from MARDI. The bacterial strain was grown overnight in yeast extract broth (YEB) medium with appropriate antibiotics and collected in the log phase.

Transformation via *A. tumefaciens*

The shoot tip explants were precultured in the regeneration medium containing Murashige and Skoog's (MS) medium, 2 mg/l 6-benzylaminopurine (BAP) and 0.25 mg/l indo-3-acetic acid (IAA) for 3 days. Then shoot tips were soaked for 1 minute with *Agrobacterium* suspension, blotted dry on a sterile paper towel and returned to the regeneration medium. After 2 days, the explants were placed on regeneration media complemented with 300 mg/l carbenicillin and cefotaxime to kill the *Agrobacterium*. The explants were then transferred to selection medium containing 50 mg/l kanamycin.

Transformation via particle bombardment

The shoot tips were placed in the center of petri plates containing the regeneration medium. Shoot tips were bombarded with the plasmid pCambia 1301 which contained the GUS gene expressed from the Cauliflower mosaic virus 35S promoter for the GUS expression study and for optimizing biolistic transformation. After optimization of the

system, the plasmid pCMV.CP was used for transformation. Both plasmids were prepared by minipreparations method described by Sambrook *et al.* [10] and were coated with M17 tungsten microparticles. The particle bombardment was carried out with a Biolistic® PDS-1000He particle delivery system (Bio Rad) and 2 parameters were evaluated: helium pressure (900 and 1100 psi) and target distances (6,9 and 12 cm).

Histochemical GUS assay

The location of GUS expressing cells was visualized using a histochemical reaction based on Jefferson *et al.* [11]. Shoot tips were transferred to incubation solution (0.1M phosphate buffer, 3% (w/v) Triton X-100, 5mM ferricyanide, 5mM ferrocyanide, 0.3% (w/v) X-gluc and sodium phosphate buffer pH 7.0) at 37°C and kept overnight. The shoot tips were then washed with sterile distilled water and were fixed in formaldehyde acetic acid (FAA) solution at 37°C overnight before being stored in 70% (v/v) ethanol. In this experiment, transformed and untransformed shoot tips were tested for the presence of GUS activity.

Polymerase chain reaction (PCR)

For PCR analysis, genomic DNA was extracted from transformed and untransformed plants using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle [12]. The regime comprised an initial denaturation at 94°C (3 minutes), with 25 cycles at 94°C denaturation (45 seconds), 50°C annealing (45 seconds), 72°C extension (1 minute), followed by a final extension at 72°C (4 minutes). Two primers of the CMV coat protein gene were used: 5'-ATG GAC AAA TCT GAA TCA ACC-3' and 5'-TCT AGA AAC TGG GAG CAC CCC AGA TG-3'. Expected size of the fragment was 700 bp. PCR amplification was carried out in 50 µl containing 5 µl of the DNA solution, 10mM dNTPs, 25 mM MgCl₂, 25 pmol of each primer and 1 µl of Taq DNA polymerase. Amplified DNA fragments were electrophoresed on 1% (w/v) agarose ethidium bromide gel and observed under ultraviolet.

RESULTS AND DISCUSSION

GUS assay

An example of chilli sections transformed with the plasmid pCambia 1301 and stained for GUS expressing cells is shown in Figure 2.

Histochemical GUS assay showed a positive GUS expression on transformed tissues while non-transformed shoot tips did not stain blue. Results to determine the effects of helium pressure and target distance are shown in Table 1. Shoot tip sections bombarded at 9 cm target distance were transformed more efficiently than sections bombarded at 12 cm target distance. A higher level of GUS expression was observed when shoot tips were bombarded at a pressure of 1100 psi compared to 900 psi. Cauliflower tissue

was reported to give high transformation efficiency at a shorter target distance and changing the helium pressure between 1100 and 1500 psi had little effect on GUS expression [13]. Based on the results above, three combinations of helium pressure and target distance: 900 psi and 6 cm (combination 1), 900 psi and 9 cm (combination 2) and 1100 psi and 9 cm (combination 3) were chosen for introducing the plasmid pCMV.CP into chilli tissues.

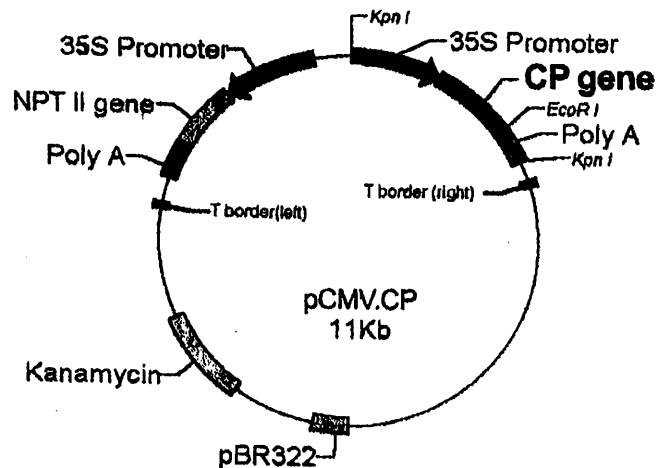


Figure 1. Structure of pCMV.CP, a vector carrying CMV coat protein gene under the control of the CaMV 35S promoter and kanamycin resistance (NPT II).

Table 1. Effects of helium pressure and target distance on transformation of chilli tissue

No. of explants	Helium pressure ^a	Target distance ^b	GUS score ^c
30	900	6	+++
30	900	9	+++
30	1100	9	++++
30	900	12	+
30	1100	12	++

^a Burst pressure of rupture disk in psi

^b Approximate distance (in cm) from stopping screen to samples

^c The GUS score in semi-quantitative estimation of the level of GUS expression. Each chilli section was given a low (+), intermediate (++,+++), or high (++++), value based on the number and density of the blue spot which were visible on the surface of the tissue

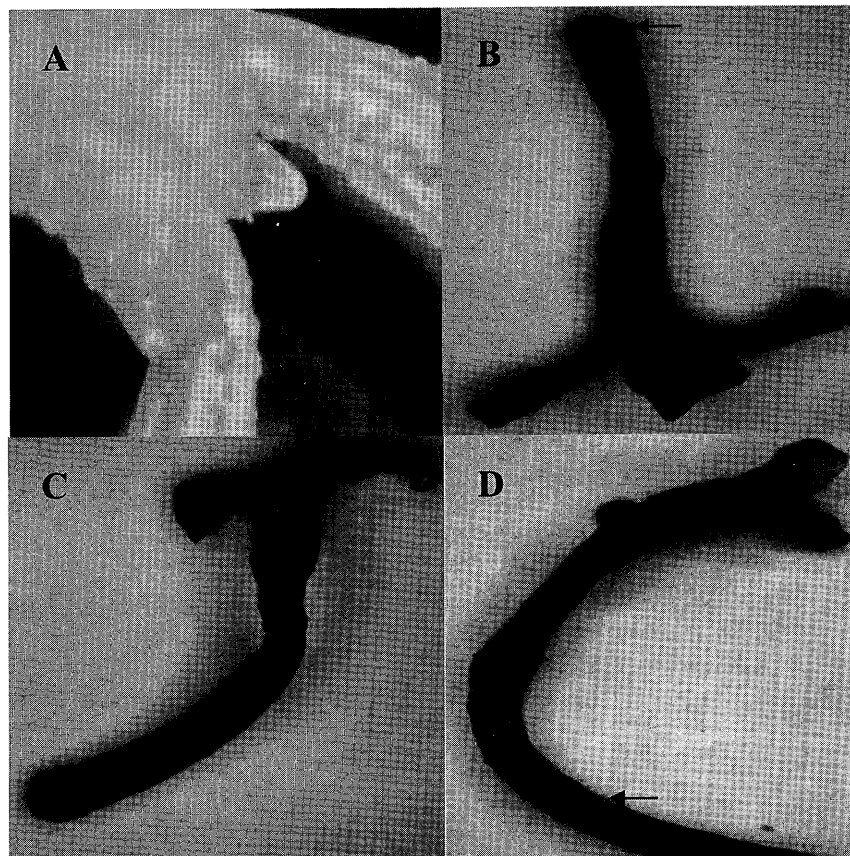


Figure 2. Transient expression of GUS in chilli tissues. A: Non-transformed shoot tip B: Transformed tissue (using 900 psi and 12 cm target distance) C: Transformed tissue (using 900 psi and 6 cm target distance) D: Transformed tissue (using 900 psi and 9 cm target distance)

Transformation

For transformation, shoot bud induction was observed on the selection medium containing 50 mg/l kanamycin. Plantlets that survived the selection were tested by PCR to determine the presence of the CMV coat protein transgenes. Two specific primers derived from CMV gene sequences were used to detect a 700 bp fragment. The amplified DNA samples from both varieties were run on electrophoresis 1% (w/v) agarose gel.

Out of 60 explants used in the transformation experiments via *Agrobacterium*, 21.7% variety MC12 and 16.7% variety Kulai were successful in producing shoots on the selective regeneration media containing 50 mg/l kanamycin. The PCR amplification results showed that 8 out of 13 and 2 out of 10 putative resistant chilli varieties

MC12 and Kulai, respectively contained CMV coat protein gene and were regarded as transgenic. A 700 bp fragment co-running with the amplified product from pCMV.CP could be detected from the transgenic plantlets but not from untransformed plant (Figure 3).

For introducing the CMV coat protein gene in chilli tissues by particle bombardment, three physical biolistic parameter combinations that have been optimized in the GUS assay study were used. For chilli variety MC12, explants survived on the selection media at 0%, 25% and 31.8%, respectively whilst for variety Kulai explants produced shoots on the selective regeneration media containing 50 mg/l kanamycin at frequencies 0%, 22% and 27.7%, respectively when combinations 1, 2 and 3 biolistic parameters were employed. However no

band was amplified as the PCR product from the genomic DNA of the surviving shoots produced. This shows that the T-DNA of CMV coat protein

gene was not successfully integrated into the plant genome when shoot tips were transformed using particle bombardment.

Error!

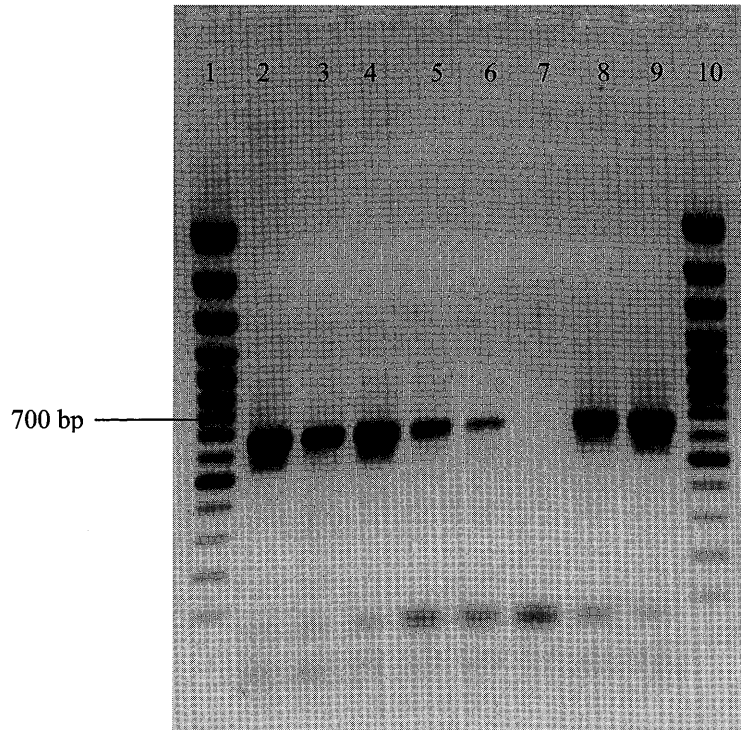


Figure 3. PCR analysis of kanamycin resistant transgenic plants showing the presence of an expected 700 bp DNA fragment of CMV coat protein gene. Lane 1: GeneRuler™ 100 bp DNA Ladder Plus; Lane 2: Positive control (pCMV.CP); Lane 3-6: Transgenic MC12 plants; Lane 7: Negative control (untransformed); Lane 8-9: Transgenic Kulai plants and Lane 10: GeneRuler™ 100 bp DNA Ladder Plus

From the results above, we found that one important constraint in the transformation system based on the bombardment of the shoot tip tissue of chilli was the difficulty of selecting transgenic plantlets because of the contradicting results from the kanamycin selection media and PCR analysis. On the contrary, cocultivation method with *Agrobacterium* showed consistent results where some shoots selected from the kanamycin selection media produced positive PCR analysis. Kartha *et al.* [14] and Aragao *et al.* [15,16] reported similar observations in the transformation system of meristematic tissue of embryonic axes of soybean. The difference in these results could be due to the fact that in particle bombardment, multiple copies of DNA

were introduced which may recombine or be fragmented [17] whereas *Agrobacterium*-mediated transformation has the advantage where a lower number of DNA copies is normally integrated into the plant genome [18].

Our results have demonstrated that the *Agrobacterium*-mediated transformation system has made it possible for genes of economic importance and genes that contribute to basic research to be parted into chilli cultivar.

Acknowledgements One author, Zarina Zainuddin, was supported by NSF scholarship (funded by Ministry of Science, Technology and

Environment). The research was funded by IRPA grant 09-02-03 -0791.

REFERENCES

1. Anon (1992). Annual Report, Horticulture Division, MARDI, Serdang. In: Melor, R. (1995). Varieti Baru Cili MC 11 dan MC 12. *Tekno. Sayuran-sayuran*, **Jil. 11**. 6pp.
2. Department of Agriculture (1999). From the World Wide Web: <http://agrolink.moa.my/doa/english/mapstat/cili.html>.
3. Fujisawa, I., Hanada, T. and Anang, S.B. (1986). Virus disease occurring on some vegetable crops in West Malaysia. *Jpn. Agr. Res. Quarterly* **20**: 78-84
4. Green, S.K. (1992). Viruses in Asia Pacific Region. In: *Proceedings of The Conference on Chilli Pepper Production in the Tropics*: 98-129.
5. Ong, C.A., Varghese, G. and Ting, W.P. (1980). Aetiological investigations on a veinal mottle virus of chilli (*Capsicum annuum* L.) newly recorded from Peninsular Malaysia. *MARDI Res. Bull.* **7**: 78-88.
6. Manoharan, M., Sree Vidya, C.S. and Lakshmi Sita, G. (1998). *Agrobacterium*-mediated genetic transformation in hot chilli (*Capsicum annuum* L. var. *Pusa jwala*). *Plant Science* **131**: 77-83
7. Liu, W., Parrott, W.A., Hildebrand, D.F., Collins, G.B. and Williams, E.G. (1990). *Agrobacterium* induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot-like structures expressing introduced genes. *Plant Cell Reports* **9**: 360-364.
8. Lee, S.J., Kim, B. and Peak, K.H. (1993). *In vitro* plant regeneration and *Agrobacterium*-mediated transformation from cotyledon explants of hot pepper (*Capsicum annuum* cv. Golden Tower). *Korean J Plant Tiss Cult* **20**: 289-294.
9. Zhu, Y.X., Qu, Y., Wen, J., Zhang, Y. and Chen Z.L. (1996). Transgenic sweet pepper plants from *Agrobacterium*-mediated transformation. *Plant Cell Rep* **16**: 71-75.
10. Sambrook, J., Fritish, E. and Maniatis, T. (1989). *Molecular cloning: A laboratory manual*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York. pp. 545.
11. Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987). GUS fusion: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The Embo Journal* **6**:3901-3907.
12. Doyle, J.J. and Doyle, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus* **12**:13-15
13. Heiser, W. (2000). Optimization of Biolistic® transformation using the helium-driven PDS-1000/He system. *US/EG Bulletin* **1688**. 8 pp.
14. Kartha, K.K., Pahl, K., Leung, N.L. and Mroginski, L.A. (1981). Plant regeneration from meristems of grain legumes: soybean, cowpeas, peanut, chickpea and bean. *Canadian Journal of Botany* **59**: 1671-1679.
15. Aragao, F.J.L., Barros, L.M.G., Brasileiro, A.C.M., Ribeiro, S.G., Smith, F.D., Sanford, J.C., Faria, J.C. and Rech, E.L. (1996). Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed via particle bombardment. *Theor. Appl. Genet.* **93**: 142-150.
16. Aragao, F.J.L., Sarokin, L., Vianna, G.R. and Rech, E.L. (2000). Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean (*Glycine max* (L.) Merrill) plants at high frequency. *Theor. Appl. Genet.* **101**: 1-6.
17. Hadi, M.Z., McMullen, M.D. and Finer, J.J. (1986). Transformation of 12 different plasmids into soybean via particle bombardment. *Plant Cell Rep* **15**: 500-505
18. Tinland, B. and Horn, B. (1995). Recombination between prokaryotic and eukaryotic DNA: Integration of *Agrobacterium tumefaciens* T-DNA into the plant genome. In: Setlow, J.K. (ed), *Genetic engineering*, vol. 17. Plenum Press, New York. pp 209-229.