J. Plant Biotechnology (2005) Vol. 7 (4). pp. 225~231

Journal of Plant Biotechnology

Assessment of Factors Influencing *Agrobacterium* Mediated Transformation in Cucumber (*Cucumis sativus* L)

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Abstract

Five day old cotyledon explants of Cucumber (*Cucumis sativus* L) cv Poinsett 76 were cocultivated with two *Agrobacterium* strains (EHA105 and LBA 4404) each carrying GUS as the reporter gene and *npt-II* as the selection marker gene in the T-DNA region of the vector. Transformed shoots were selected at 150 mg/L kanamycin. A two day cocultivation coupled with 20 μM acetosyringone increased the frequency (8.2 and 15.4 shoots) of GUS expression in the shoots of transformed plant. Among the two *Agrobacterium* strains, EHA 105 performed better than LBA 4404 in bringing two-fold increase in transformation efficiency (14%) than LBA 4404 (7.4%). PCR analysis was done to confirm the integration of T-DNA into cucumber genome.

Key words: Acetosyringone, Co-cultivation period, GUS expression, Kanamycin

Introduction

Cucumber (*Cucumis sativus* L) an important horticultural plant, is mainly cultivated for its fruits, which are used for slicing, pickling juice extraction and in cosmetics. The impetus to produce disease resistant stock is essential since agricultural yield decreases considerably due to various diseases caused by phytopathogenic fungi, bacteria and viruses (Tabei et al. 1998). The need to breed disesase resistant variety in cucumber has become a subject of commercial importance. However conventional crosses and transfer of desirable traits and particularly disease resistance have not been possible in *Cucumis sativus* (Esquinas-Alcazar and Gulick, 1983). Although procedures for transformation of cucumber (Trulson et al. 1986; Chee 1990; Tabei et al. 1994, 1998; Sarmento et al. 1992; Raharjo et al. 1996; Nishibayashi et al. 1996; Ganapathi and Perl -Treves 2000) have been established, still the transgenic techniques are not commercially exploited as for as cucumber is concerned. In addition, cucumber is a potential candidate for edible vaccine (Sharma et al. 1999). To accomplish these tasks, it is a prerequisite to develop a reliable protocol to achieve high frequency plant regeneration and genetic transformation in cucumber, hence the present work was undertaken to evolve a reliable protocol to produce transgenic cucumber taking into consideration of factors responsible for achieving high frequency transformation via *Agrobacterium tumefaciens*.

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Received Oct. 22, 2005; Accepted Dec. 25 2005

Materials and Methods

Plant material, regeneration and culture conditions

Seeds of *Cucumis sativus* cv Poinset 76 procured from Indo-American Hybrid Seeds Pvt Ltd, Bangalore, India were used for transformation experiments. The sterilized seeds were kept on moist cotton for 24 hrs. Then their coats were separated and aseptically removed without damaging the cotyledons. The cotyledons were carefully

separated from the embryonic axis. The distal end of the cotyledon was slightly injured and cotyledonary pieces (5 mm) were vertically inoculated in such a way that the distal end touches the MS medium (Murashige and Skoog 1962) amended with 3% sucrose (Himedia Laboratories Ltd, Mumbai, India), 0.8 % agar (Himedia Laboratories Ltd, Mumbai, India), BA (6.78 μM) and AdS ((67.8 μM) for shoot regeneration. The cultures were kept at 25±2℃ with a 16 hr (light) and 8 hr (dark) photo period under the light intensity of 120 μmol^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India).

Agrobacterium strains and culture media

Disarmed *Agrobacterium tumefaciens* strains EHA 105 and LBA 4404 were used to study the strain efficiency on genetic transformation. Both the strains ((kindly provided by Dr.Rafael Perl Treves, Bar Ilan University, Israel) containing a binary plasmid (pGA492GI) with *npt-II* (neomycin phosphotransferase), 35SGUS intron (β - glucuronidase) and bar (35S phosphinothricin) (Figure 1). The presence of chimeric gusA gene was useful to study the early events of transformation in plant tissue. *A. tumefaciens* strains were grown at 28℃ on LB (Luria Broth) medium (Himedia and co, Mumbai, India) containing casein hydrolysate (10 g/L), yeast extract (5 g/L), sodium chloride (5 g/L), kanamycin (50 g/L) (Sigma, St.Louis, USA) and 10mg/L tetracycline (Sigma, St. Louis, USA). For the co-cultivation, single colony of *A. tumefaciens* was inoculated in 50 ml of LB medium

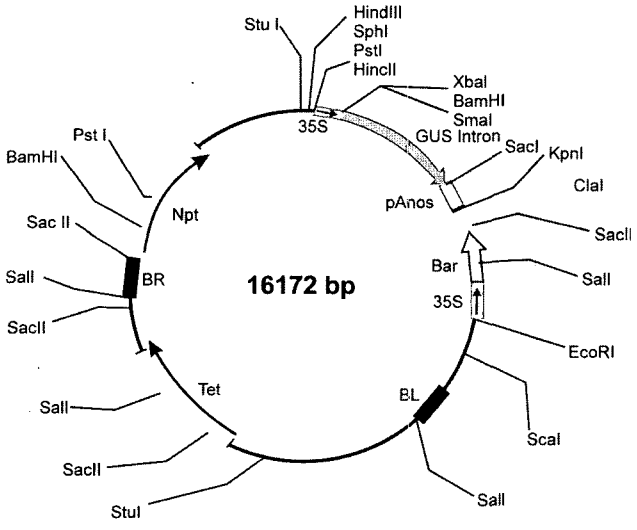

Figure 1. Schematic map of plasmid pGA-492 showing the T-DNA and non T-DNA regions. BR-Border right, BL-border left, NPT-Neomycin phosphotransferase gene, Cauliflower mosaic virus (CaMV) 35S promoter, GUS β-glucuronidase, Bar-phosphinothricin acetyl transferase gene driven by 35S CaMV promoter

containing 50 mg/L kanamycin and 10 mg/L tetracycline and incubated for 6 hr. After 6 hr, the culture was transferred to 50 ml of AB minimal medium with pH 7.0 (Chilton et al. 1974) and incubated at 28℃ on a rotary shaker (Orbitek, India) for 24 hr. The *Agrobacterium* suspension at 1.0 OD (600 nm = 1.0 OD) was pelleted at 5000 rpm at 28℃ using a refrigerated centrifuge (Plasto Crafts, India). The pellet was resuspended in half strength MS liquid medium (1:50) containing 50 mg/L kanamycin. One hour before the co-cultivation of explants, acetosyringone (Sigma, USA) (10 -50 μM) was supplemented. The proximal end of the cotyledon was pricked with a sterile needle to induce agro-infection. The explants (110 per treatment) were dipped in bacterial suspension for 10 min (each of the two strains were evaluated as separate experiments).

Kanamycin sensitivity

The response of cotyledon explants to kanamycin was determined by culturing the cotyledon explants in MS medium augmented with BA (6.78 μM) and AdS (67.8 μM) along with different concentrations of kanamycin (0, 50, 75, 100, 150, 200 and 250 mg/L). Selective concentration of kanamycin was used throughout the selection procedures. A positive control without kanamycin was maintained.

Preculture and infection

After 5 days of preculturing of cotyledon explants on MS medium supplemented with BA (6.78 μM) and AdS (67.8 μM), the cotyledon enlarges to about four times from its original size and the proximal end of the cotyledon explants was pricked with sterile needle of size 0.63 x 25 mm (Dispovan, Hindustan Syringes & medical Ltd., Faridabad, India) and immersed in an *Agrobacterial* suspension (1.0 OD = 600 nm) containing 20 μM of acetosyringone with gentle shaking for 10 min to induce agro- infection.

Cocultivation and selection

Infected explants were blotted dry on sterile Whatmann No. 1 filter paper and co-cultivated for 1-3 days under dark at 25±2℃. After co-cultivation, infected explants were washed three times with sterile distilled water containing 300 mg/L cefotaxime followed by washing with MS basal liquid medium containing 300 mg/L cefotaxime for three times with vigorous stirring using sterile forceps and blotted dry on sterile filter paper. Later the co-cultivated explants (after washing) were cultured on MS medium containing BA (6.78 μM), AdS (67.8 μM), kanamycin (150 mg/L) and cefotaxime (300 mg/L) (selection medium). The newly

initiated shoots from the cotyledon explants on the selection medium were later transferred to elongation medium containing BA (6.78 μM), GA_3 (1.44 μM) and kanamycin (150 mg/L). The elongated shoots (5-7 cm length) produced prominent roots on half strength MS medium supplemented with IBA (2.46 μM) on selection medium. The rooted plantlets were successfully hardened in closed green house.

GUS assay and histochemical localization

Leaf tissues from the regenerated shoots were assayed for the expression of *uid A* (GUS INT) genes following the histochemical procedure described by Jefferson *et al.*, (1987), using the substrate 5 bromo-4chloro-3 indolyl β-D glucuronide (X-gluc) (Sigma, St. Louis, USA). Assays to score transient GUS expression in cotyledon explants were carried out in 7day post co-cultivation to study the effect of strain specificity and efficiency. Transient GUS expression of explants was measured at 21st day after co-cultivation. Further the leaf tissues were cleared and fixed in 95% (v/v) ethanol : 1% acetic acid. The leaves collected from the selected transformants were microscopically analyzed. For microscopic analysis 100 μm of tissue sections of leaves of transgenic plants were stained for 8 hrs at 37℃ in 2 mM X-Glu. The tissues were further observed as described by Pichon et al. (1992). The sections were documented with Nikon SM2T & E-400 with H-III Camera (Nikon Co., Tokyo, Japan).

PCR analysis

For PCR analysis, DNA samples (co-cultivated with EHA 105 and LBA 4404) from putative transformants were isolated according to Murray and Thompson (1980). The *npt-II* gene fragment (0.69 kb) was amplified using primers 5' GCC GCT TGG GTG GAG AGG CTAT 3' and 5' GAG GAA GCG GTC AGC CCA TTCG 3' All PCR reactions were carried out using a Peltier effect Thermal cycler (M J Research, Waltham, Mass). Samples containing 100 ng of genomic DNA were first heated at 94℃ for 5 min followed by 30 cycles at 94℃ for 30 sec, primer annealing at 55℃ for 30 sec, primer extension at 72℃ for 30 sec, followed by final extension at 72℃ for 5 min. Hundred nanogram of plasmid DNA was used as positive control. The PCR reactions contained 10 pmol each primer, 10 mM dNTPs mix, 15mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% (v/v) Triton X-100, 2 U *Taq* DNA polymerase and 100 ng template DNA in 1 X reaction buffer. The amplified DNA was analyzed by 1 % agarose gel electrophoresis at 100 V for 90 min followed by staining in sterile distilled water containing 1 $\mu g\ l^{-1}$ ethidium bromide (Sigma, USA) for 15 min.

Statistical analysis

The experiments consisted of 20 explants per treatment and the values represent the mean $\pm$ standard error, means followed by same letter within the column or not significantly different according to Duncan's multiple range test (DMRT) and the significance was determined at the 5% level (Gomez and Gomez 1976).

Results and Discussion

Tissue culture system

Direct regeneration system was adopted using cotyledon explants for transformation studies. This system has already been successfully adopted to recover transgenic plants (Tabei et al. 1998; Ganapathi and Perl- Treves 2000; Suresh Kumar 2002). In addition, direct regeneration system has been considered as an ideal system to get true-to-true type regenerants (Burza and Plader 1995; Plader et al. 1998).

Sensitivity of cotyledon explants to kanamycin

Co-cultivated cotyledon segments swelled and shoot buds initiated on the proximal end after 5-7day on selection medium containing BA (6.78 μM), AdS (67.8 μM), kanamycin (150 mg/L) and cefotaxime (300 mg/L). Further increase in the kanamycin concentration reduced the regeneration frequency as the shoots became pale yellowish and failed to regenerate. Although reduced concentration of kanamycin (100 mg/L) produced shoots, but most of them were escapes (data not shown). At an optimum concentration of 150mg/L of kanamycin, about 36 shoots were produced out of which 8% were chimeric, 2% showed true to true type with escapes of about 2% (data not shown). Hence this concentration (150mg/L) was used for selecting the transformants. A strong selection agent is an important factor for the transformation of cucumber particularly the one which regenerates via direct organogenesis from cotyledon explants (Gaba et al. 1995).

Influence of explant age on transformation

Age of the explants is a critical factor which influences transformation efficiency (De Bondt et al.1994). One to five day-old cotyledons were analyzed for GUS expression to assess transformation efficiency and it was found that five day old explants showed high percentage of GUS intensity; 75% with EHA 105 and 55% with LBA 4404 (Table.1). However in contrast to our findings Cao et al. (1998) observed

Table 1. Effect of age of cotyledon explants on GUS expression with LBA4404 and EHA105 cultured on MS selection medium containing BA (6.78 μM) + Ads (67.8 μM) + Kan (150 mg/L)

Explant age (days)	Percentage of GUS expression	
	LBA 4404	EHA 105
1	4.0 ± 0.16^{e}	12.0 ± 0.58^{d}
2	18.0 ± 0.14^{cd}	32.0 ± 0.44^{c}
3	28.0 ± 1.41^{c}	42.0 ± 0.44b^{c}
4	42.0 ± 0.20^{b}	54.0 ± 0.47^{b}
5	55.0 ± 0.35^{a}	75.0 ± 0.35^{a}
6	48.0 ± 0.37^{a}	68.0 ± 0.48^{ab}

The experiments consisted of 20 explants per treatment and the values represent the mean±standard error, means followed by same letter within the column or not significantly different according to Duncan's multiple range test (DMRT) and the significance was determined at the 5% level

Table 2. Effect of *Agrobacterium* strain, co-cultivation period and acetosyringone (20 μM) on transformation efficiency in cotyledon explants of Cucumber cv Poinsett 76.

Agrobacterium Strains	Cocultivation period (days)	No. of explants forming GUS positive shoots		
		With out acetosyringone	With acetosyringone (20 μM)	T.E ^{z} (%)
LBA 4404	1	1.2e	3.4de	3.0
	2	3.2cd	8.2c	7.4
	3	2.6d	4.6d	4.2
EHA105	1	3.4c	8.2bc	7.4
	2	6.2a	15.4a	14.0
	3	4.4b	10.2b	9.2

^{z} Transformation efficiency = No of GUS positive shoots produced/ total no of explants cocultivated with *Agrobacterium* after treatment with acetosyringone (20 μM) X 100

β- *Glucuronidase* (GUS) assay was conducted in shoots formed after 21 days of cocultivation.

Each value represents the treatment means of five replicates with 110 explants per treatment. Means followed by same letter within the column or not significantly different according to Duncan's multiple range test (DMRT) and the significance was determined at the 5% level.

in blue berry that age of explants did not have any significant effect on GUS expression. But the studies on cucumber proved that age of the explants played a vital role in transformation (Chee 1990; Chee and Slightom 1991; Raharjo et al. 1996; Nishibayashi et al. 1996; Vasudevan et al. 2002).

Influence of acetosyringone and co-cultivation period on transformation

The most commonly used techniques in transformation of dicots were the addition of phenolic compounds such as acetosyringone to *Agrobacterium* cultures (Van Wordragen and Dons 1992). Acetosyringone is a phenolic compound produced during wounding of plant cell that induces the transcription of the virulence genes of *A. tumefaciens*. In the present study, 550 explants were infected with *Agrobacterium* strains LBA 4404 and EHA 105 in the presence (10-50 μM) and absence of acetosyringone. After 1-3 days of cocultivation, they were transferred to shoot regeneration medium containing 150 mg/L of kanamycin. It was observed that acetosyringone at 20 μM concentration and 2-day cocultivation period resulted in the production of maximum number of GUS positive shoots in both the strains (8.2 shoots in LBA 4404 and 15.4 shoots in EHA 105 as against 3.2 and 6.2 GUS positive shoots in the absence of acetosyringone respectively) (Table 2). Use of acetosyringone beyond 20 μM, enhanced bacterial leaching and was difficult to control the growth of *Agrobacterium*. Enhanced transformation efficiency using acetosyringone in cucumber had been reported earlier (Nishibayashi et al. 1996; Mohinuddin et al. 2000; Vasudevan et al. 2002). Prolonged co-cultivation period of more than 2 days has been successfully used for transformation of certain plants (DeBondt et al. 1994; Cao et al. 1998; Cervera et al. 1998; Mourgues et al. 1996). In earlier studies on cucumber transformation, the co-cultivation period varied from 2-5 days for different explants (Chee 1990; Chee and Slighton 1991; Raharjoet al. 1996; Nishibayashi

et al. 1996). In the present study acetosyringone (20 μM) coupled with 2 day cocultivation increased the transformation efficiency to 2 fold (7.4% in LBA 4404 and 14% in EHA 105 respectively) (Table.2).

Effect of *Agrobacterium* strain

Comparing the two *A. tumefaciens* strains, EHA 105 consistently produced a significantly higher transformation response than LBA 4404 at 1.0 OD concentration (14% Vs 7.4%), a difference commonly noted by Hood *et al.*, 1986; 1993) who reported strains EHA 101 and EHA 105 were more effective than strain LBA 4404.

Gus assay

Transformed shoots (5-7 cm) were assayed histochemically for GUS expression. The non-transformed control shoots did not show blue color staining, whereas the putative transgenic shoots exhibited blue coloration (Figure 2C). Visible GUS expression was observed at the meristematic zone of the cotyledon explants (Figure 2A) in each strain 24 hrs after transferring to a selection medium containing kanamycin (150 mg/L) and cefotaxime (300 mg/L). In contrast to the findings of Cao et al. (1998) in Blue Berry and Cervera et al. (1998) in citrange, where no GUS expression was observed immediately after co-cultivation. EHA 105 co-cultivated explants showed dense blue colour (Figure 2D) evoking better GUS expression than LBA 4404. Leaves from the putatively transformed shoots (21 days old) were assayed. The entire leaf was stained blue indicating the integration and expression of GUS gene in the transformants. The appearance of dense blue coloration in our study revealed strong GUS expression and stable integration unlike pale coloration in leaf tissues. It may be due to lower GUS activity in cells that divide less frequently (Nagata et al. 1987). The cross section of leaves from the putatively transformed shoots revealed GUS activity in vascular bundles (mid rib region) following selection on kanamycin (150 mg/L) (Figure 2E). The localized GUS expression in actively dividing cells by CaMV 35S constitutive promoter was well envisaged (Jefferson et al. 1987; Valles and Lasa 1994).

PCR analysis

DNA isolated from transformed plants, non-transformed plants and plasmid pGA492GI (isolated from bacterial culture) was used as template DNA for PCR amplification of the *npt-II* gene. The presence of a band of 0.69 kb in samples loaded in lane 4-6 from transformed shoots of

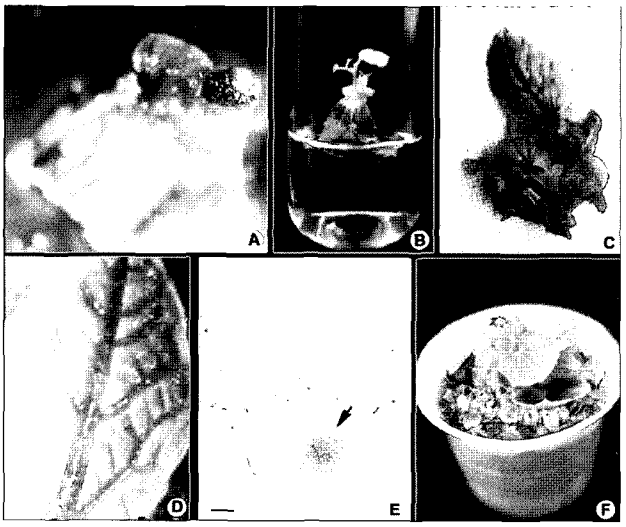

Figure 2. *Agrobacterium* mediated transformation in cotyledon explants using EHA 105 and LBA 4404 strain. A: Proximal end of the cotyledon showing GUS expression, B: Regeneration of shoots from the proximal end of the cotyledon in the selection medium [MS + BA (6.78 μM) +AdS (67.8 μM) + Kan (150 mg/L], C: GUS expression of entire transformed plants, D: Single leaf showing GUS expression, E: Histological section of leaf showing GUS expression (Arrow indicates cross section of midrib region of leaves showing strong GUS expression. bar = 50 μM), F: Hardened transgenic plants.

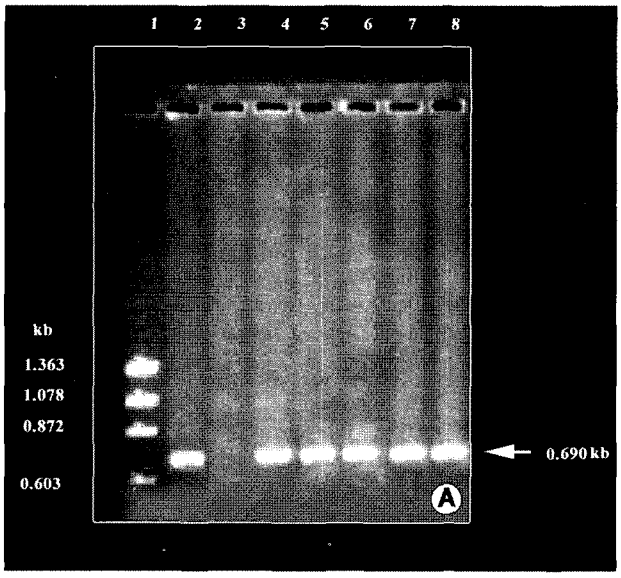

Figure 3. PCR amplification of the *npt-II* gene fragment in transformed Plants (Arrow indicate the amplification of *nptII* fragement at 0.69 kb). Lane 1: Marker (Lambda DNA Hae III digest), Lane 2: Positive control (plasmid DNA vector pGA492), Lane 3: Negative control (control plants), Lane 4-6: DNA samples from putative transformants co-cultivated with EHA 105, Lane 7-8: DNA samples from putative transformants co-cultivated with LBA4404

EHA105 and lane 7 and 8 in LBA 4404 revealed the stable integration of *npt-II* gene in the cucumber genome. A positive control (plasmid DNA vector pGA492GI) in lane 2 also showed similar banding pattern. Amplification of this fragment (0.690 kb) was not observed in non-transformed control plants in lane 3 (Figure 3A)

In conclusion we report the establishment of a simple and efficient method of *Agrobacterium* mediated gene transfer in cucumber. The transformants were stringently selected on kanamycin at 150 mg/L concentration thereby reducing escapes and the use of optimum concentration of acetosyringone (20 μM) increased the transformation efficiency by two fold. The strain efficiency studies revealed that EHA 105 performed better than LBA 4404. In future, this methodology may be adopted for introducing any character traits into cucumber genome.

Acknowledgments

The authors are gratefully acknowledge to Department of Science and Technology (DST), Government of India (under Indo-Israel collaborative program 8979-1-97) for the financial support.

References

Burza W, Plader S (1995) Direct plant regeneration from leaf explant in Cucumber (*Cucumis sativus L.*) is free of stable genetic variation. Plant Breed 12: 341-345

Cao X, Liu Q, Rowland LJ, Hammerschlag FA (1998) GUS expression in blueberry (*Vaccium* spp.) factors influencing *Agrobacterium*-mediated gene transfer efficiency. Plant Cell Rep 18: 266-270

Cervera M, Pina JA, Juarez J, Navarro L, Pena L (1998) *Agrobacterium*-mediated transformation of Citrange : factors affecting transformation and regeneration. Plant Cell Rep 18: 271-278

Chee PP. (1990) Transformation of *Cucumis sativus* tissue by *Agrobacterium tumefaciens* and the regulations of transformed plants. Plant Cell Rep 9: 245-248

Chee PP, Slightom, JL (1991) Transfer and expression of Cucumber Mosaic Virus Coat protein gene in the genome of *Cucumis sativus*. J. Amer Soc Sci Hortic 116: 1098-1102

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proct Natl Acad Sci USA 71:3672-3676

De Bondt A (1994) *Agrobacterium* mediated transformation of apple (*Malus* x *domestica* Borkh): An Assessment of factors affecting gene transfer efficiency during early transformation steps. Plant Cell Rep13: 587-593

Esquinas-Alcazer JT, Gulick PJ (1983) Genetic resources of Cucurbitaceae. IBPGR

Gaba V, Feldmesser E, Amit Gal-on, Antigus KY (1995) Genetic transformation of a recalcitrant melon (*Cucumis melo* L.) variety. In: Lester G, Dunlap et al. (eds), Cucurbitaceae' 94. Gateway printing, USA, pp 188-190

Ganapathi A, Perl-Treves R (2000) *Agrobacterium* mediated transformation in *Cucumis sativus* L. via direct organogenesis. Acta Horticult 510: 405-407

Gomez KA,Gomez KA (1976) Statistical Procedure for Agricultural research, John Wiley and Sons, New Delhi

Hood E.E, Gelvin S.B, Melchers, Hoekema A (1993) New *Agrobacterium* vectors for plant transformation. Transgenic Research 2:208-218

Hood E.E, Helmer G.C, Fraley RT, Chilton M.D (1986) The hypovirulence of *Agrobacterium tumefaciens* A281 is encoded in the region of PtiB0542 outside the T-DNA. J Bactriol 168:1291-1301

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901-3907

Mohinuddin AKM, Harikrishna K, Chowdhury MKU, Abdillah ZC, Napis S (2000) Influence of Acetosyringone on *Agrobacterium*-mediated transformation of cucumber (*Cucumis sativus* L.). Plant Tissue Cult 10: 167-173

Mourgues F, Chevreau E, Lambert C, De Bondt R.(1996) Efficient *Agrobacterium* mediated transformation and recovery of transgenic plants from pear (*Pyrus communis* L.). Plant Cell Rep 15: 809-814

Muraskige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant 15: 473-497

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321-4325

Nagata T, Okada K, Kawazu T, Takebe I (1987) Cauliflower mosaic virus 35S promoter directs S phase specific expression in plant cells. Mol Gen Genet 207:242-244

Nishibayashi S, Kaneko H, Hayakawa T (1996) Transformation of Cucumber (*Cucumis sativus* L.) plants using *Agrobacterium tumefaciens* and regeneration from hypocotyl explants. Plant Cell Rep 15: 809-814

Pichon M, Journet EP, Dedieu A, Billy F de, Truchet G, Barker, DG (1992) *Rhizobium melitoli* elicitis transient expression of the early nodulin gene Enod12 in the differentiating toot epidermis of transgenic *alfafa*. Plant Cell Rep 4: 1199-1211

Plader W, Burza W, Rusinowski Z (1998) The relationship between the regeneration system and genetic variability in the cucumber (*Cucumis sativus* L.). Euphytica 103: 9-15

Raharjo SHT, Hernandey MO, Zhang YY, Punja, ZK (1996) Transformation of pickling Cucumber with chitinase - encoding genes using *Agrobacterium tumefaciens*. Plant Cell Rep 15: 591-596

Sarmento GG, Alpert K, Tang FA, Punja ZK (1992) Factors

influencing *Agrobacterium tumefaciens*-mediated transformation. Plant Cell Tiss Org Cult 31: 185-193

Sharma AK, Mohanty A, Singh Y, Tyagi AK (1999) Transgenic plants for the production of edible vaccines and antibodies for immunotherapy. Curr. Sci 77: 524-529

Suresh kumar, P (2002) In vitro studies and *Agrobacterium* mediated gene transformation in *Cucumis sativus* . Ph.D Thesis

Tabei Y, Nishio T, Kurihara K, Kanuo T(1994). Selection of transformed callus in a liquid medium and regeneration of transgenic plants in cucumber (*Cucumis sativus* L.). Breed Sci 44:47-51

Tabei Y, Kitade S, Nisihizawa Y, Kikuchi N, Kayano T, Hibi, T, Akutsu K (1998) Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). Plant Cell Rep 17: 159-164.

Trulson, AJ, Shahin EA (1986). *In vitro* plant regeneration in the genus *Cucumis*. Plant Sci 47: 35-43

Valles MP, Lasa JM (1994). *Agrobacterium*-mediated transformation of commercial melon (*Cucumis melo* L, cv. *Amarillo Oro*) Plant Cell Rep 13: 145-148.

Van Wordragen MF, Dons HJM (1992). *Agrobacterium tumefaciens* mediated transformation of recalcirant crops. Plant Mol Biol Rep 10:12-36.

Vasudevan A, Ganapathi A, Selvaraj N, Vengadesan G (2002). Factors influencing GUS expression in Cucumber (*Cucumis sativus* Linn.) Indian Journal of Biotechnology Vol 1, 344-349.