GENERATION OF TRANSGENIC MARKER-FREE CUCUMBER PLANTS BY CO-TRANSFORMATION STRATEGY

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Recombinant DNA technology has featured as a powerful tool for editing genes and genetic materials in plants to produce novel traits. Currently, genetic transformation in plants has become subroutine; up to 100 agricultural crops have been genetically modified around the global. The genetic modification of crop plants offers substantial progresses to agricultural practices, quality and quantity of the yield to meet the growing human needs. Upon plant transformation, selectable marker genes are commonly introduced in a linked position to the target gene or gene of interest in the same cassette. The most commonly used antibiotic and herbicide resistance genes are neomycin phosphotransferase (*nptII*) and phosphinothricin acetyltransferase (*bar/pat*) genes. Expression of selectable marker genes in transformed cells confer resistance to antibiotics or herbicide agents which allow them to detoxify lethal substances to the cells (Miki and McHugh, 2004) and enable efficient production and identification of transgenic cells. Despite that selectable marker genes are beneficial for the efficient recovery of transgenic regenerates; they often have no further function after generation of transgenic plant. Moreover, the existence of a selectable marker precludes the use of the same selectable marker gene for another round of transformation. Additionally, maintaining selectable markers which encode resistances to antibiotics is believed in some countries to be somehow risky (Kapusi *et al*., 2013). The safety concern is those genes may be transferred by outcrossing into weeds in the case of herbicide or, rarely, to other organisms through horizontal gene transfer and their continuous expression may interfere with normal plant growth and development (Ling *et al*., 2016). Therefore, transgenic plants that are grown at commercial level often required being free of those genes.

Different strategies have been developed to eliminate or remove marker gene from transgenic plants. One of such strategy is transferring the target gene and the selectable marker gene via co-transformation and segregation in the subsequent progeny (Puchta, 2000; Hare and Chua, 2002). Homologous recombination

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system (Ow, 2001; Zhang *et al.*, 2006), use of transposable elements (Cotsatifs *et al*., 2002) and site-specific recombination (Luo *et al*., 2000; Endo *et al*., 2002; Zhang *et al*., 2006) are the other strategies. This study aimed to develop selectable marker-free transgenic cucumber plants using the co-transformation method as a simple and cheap system. The study involves co-transformation of *gfp* gene as a gene of interest on a plasmid together with the *npt-II* as a selectable marker gene on another plasmid into plant cells. Both genes will possibly be integrated in unlinked genomic loci which can be separated from each other by segregation in the next generation.

MATERIAL AND METHODS

Plant material and seed sterilization

Seeds of cucumber (*Cucumis sativus* cv. Barracuda,) were provided by Agrotech for Modern Agriculture Co., Zamalek, Egypt. The research experiment was performed during 2014 to 2015 at Genetic Engineering and Biotechnology Institute, University of Sadat City. Seeds were surface-sterilized with 5% sodium hypochlorite (Clorox) for 20 min and rinsed three times with distilled water. The seed coats were removed, sterilized again with 1% sodium hypochlorite for 10 min, rinsed three times with distilled water and allowed to dry on autoclaved paper. Sterilized seeds (30 seeds) were incubated in vitro in darkness at 25±1°C for 10 days on MS medium (Murashige and Skoog, 1962) with 3% sucrose, 0.8% agar, pH at 5.8 and autoclaved at 121°C for 20 min.

Preparation of explants, callus induction and development of embryogenic calli

Cotyledons of the 30 *in vitro* grown cucumbers were divided longitudinally into two equal halves and transversely into four equal pieces which, producing eight explants/cotyledon with a total of 480 explants. For callus induction, the explants were distributed in 15 dishes, 8 explants each and four replications on MS medium supplemented with 2, 4-Dichlorophenoxyacetic acid (2, 4-D) at 1.0 mg/1 and kinetin at 0.1 mg/1. The 480 explants were cut into halves at second round of subculture (21 days) producing 960 explants. The explants were subcultured every three weeks on a fresh medium for three rounds.

Agrobacterium strain and plasmids

Agrobacterium ATHV strain was used with pPNgus and pCATgfp plasmids. The pPNgus contained two genes, *gus* gene as a reporter gene controlled by *35S* promoter and *35S* terminator and *nptII* controlled by nos promoter and nos terminator. While, the plasmid pCATgfp included *gfp* gene which was considered as a gene of interest controlled by double *35S* promoter and *35S* terminator (Fig. 1).

Transformation of A. tumefactions by electroporation method

One µl of mixed plasmids DNA was added to 50 µl aliquot of thawed

electro-competent A. tumefactions cells, cells were transferred to cuvette, placed in the electroporator apparatus and electro-pulsed, followed by adding 450 µl LB media. The suspension was transferred to a 2 ml tube and incubated at 28°C for 1 h. Then, 250 µl of the bacterial culture were inoculated on solid LB media supplemented with 200 µl spectinomycin (spec) and streptomycin (strep) each and incubated 2 days at 28°C for transformation experiments (Khidr, 2007).

Agrobaterium-mediated transformation of embryogenic calli

The grown *Agrobacterium* colonies were suspended in liquid LB medium containing the same antibiotics concentration of spec/strep, and then incubated overnight at 28°C with shaking. The culture was centrifuged at 5000 rpm at room temperature for 10 min. *Agrobacterium* pellets were re-suspended in liquid MS medium and adjusted to optical density of 0.8. Five experiments were done for inoculation with *Agrobacterium*, fifty embryogenic calli (nine weeks from callus induction) were selected for genetic transformation per experiment. The embryogenic calli were mixed with *Agrobacterium* solution containing 100 µM acetosyringone for an hour. Then, the cultures were transferred onto a solid MS medium containing 1 mg/L 2,4-D for three days. Thereafter, embryogenic calli were transferred onto the same MS medium supplemented with 300 mg/L carbenicillin for inhibition of *Agrobacterium* growth and were transferred to selection and regeneration media after a week.

Analysis of β-glucuronidase activity and expression of gfp gene

Four weeks after co-cultivation with *Agrobacterium*, part of the putative embryogenic calli were immerged overnight at 37°C in *gus* staining buffer (0.1 M sodium phosphate pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.1% Triton X-100) containing 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-gluc) substrate (Jefferson, 1987). The solution was replaced with 70% ethanol to remove chlorophyll, and the blue spots appeared under binocular microscope. The embryogenic calli and regenerated plantlets were examined for the presence of the *gfp* gene by fluorescence microscope, the positive *gfp* plantlets were further tested for the presence of the *gus* gene and PCR analysis.

Selection and regeneration of putative transgenic plants

For selection and regeneration the putative transformed calli, they were transferred onto MS medium containing 500 mg/L carbenicillin, 200 mg/L kanamycin, 1 mg/L Benzylaminopurine (BAP), and 1 mg/L 1-Naphthaleneacetic acid (NAA) (Khidr *et al.*, 2012). The cultures were incubated at 25±1°C in darkness for six weeks. Upon conversion of somatic embryos to plantlets, the culture was transferred to the light (16 hour

light/8 dark) on a fresh medium for three subcultures every three weeks.

Segregation analysis of transgenic plants

The positive transgenic T_0 plantlets for the presence of the both constructs *gus-nptII* and *gfp* genes were transferred to *ex-vitro* onto small pots, covered with plastic bags for plant acclimatization for two weeks, transferred to bigger pots and were self-pollinated upon flowering to set T_1 seeds. Distribution of T_1 plants were tested against the expected ratios using the chi-square ($\chi 2$) test for segregation analysis of the transgenes in the progenies.

DNA isolation and polymerase chain reaction analysis

About 100 mg of the tissues was ground in liquid nitrogen using a pestle and mortar to a fine powder. The ground samples were used for DNA isolation using the DNA extraction kit (iNtRON Biotech., Inc.) according to their manufacturer instructions and the concentration was adjusted at 25 ng/µl. Primers used for analyzing putative transgenic plants were nptII-F: 5´acaagatggattgcacgcagg3´, nptII-R: 5´aactcgtcaagaaggcgatag3´ and GFP-F: 5´gagggagagggtgatgctac3´, GFP-R: 5´tgtactccagcttgtgtcca3´. Expected sizes of the amplified DNA fragments were 800 bp for the *nptII* gene and 467 bp for *gfp* gene. PCR reactions were performed in a total volume of 25 µl as following: 12 pmol (0.25 µl) of each specific primers, 1 µl of a mixture of four deoxyribonucleoside triphosphates (dNTPs); 0.25 µl of Dream Taq DNA polymerase, 2.5 µl of 10X Taq buffer and 20.5 µl of nuclease-free water. The mixture was transferred in a thermal cycler PCR in a 0.2 ml tubes, denatured at 94°C for 4 min followed by 35 cycles at 94°C for 30 second, 58°C for 30 second and 72°C for 1 min. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide using standard procedures as described by Sambrook *et al.* (1989).

RESULTS AND DISCUSSIONS

Callus induction and embryogenic callus formation

The callus induction initiated after three weeks from culturing the cotyledon explants on solid MS medium with 1 mg/L 2,4-D and 0.1 mg/L kinetin. The results in Table (1) from eight experiments with a total of 480 explants indicated that number of induced calli ranged from 28 to 61 with a total of 438 out of 480 explants. The percentage of callus induction ranged from 87.5% to 95.3% with an average of 91.25% (Table 1). Embryogenic calli was achieved from cotyledon explants on the same MS medium after 7 weeks from the initiation of callus induction. The number of embryogenic calli ranged from 15 to 31 with a total of 184 embryogenic calli and from the eight experiments (Table 1). The percentage of embryogenic calli ranged from 25% to 48.4% with an average of 38.3% (Table 1 and Fig. 2a). Our results in the callus induction was similar to re-

sults obtained by Khidr and Nasr (2012) who found highest percentage of 92% and 94% induction of callus formation in squash and cucumber, respectively, using cotyledon explants on MS medium with 1 mg/L 2,4-D. El-Absawy *et al*. (2012) found a maximum callus induction (94% and 92%) in cotyledons and mature seeds of cucumber, respectively, on MS medium supplemented with 1 mg/L 2,4-D. Furthermore, Jesmin and Main (2016) reported that the highest proportion of callus induction (74.43%) was obtained from cotyledon explants of cucumber on MS medium supplemented with 0.5 mg/L BAP and 1.0 mg/L NAA. On the same sequence, Lou and Kako (1994) obtained 90% of embryogenic callus formation on cotyledon explants. Moreover, the results were in harmony with those obtained by Abu-Romman *et al*. (2013), who achieved callus induction frequency with 82.8% on MS media having 2, 4-D at 1.0 and 1.5 mg/l in cucumber. Furthermore, Ju *et al*. (2014) obtained 90% of embryos which were converted to normal plantlets but without transformation in gherkin (*Cucumis anguria* L.). In contrast, Jesmin and Main (2016) reported low frequency of callus induction (13.33%) using cotyledon explants on MS medium with 0.5 mg/L BAP. The highest percentage was only 27% through cotyledon on MS medium with 0.5 mg/L BAP and 1.0 mg/L NAA (Mazlan *et al*., 2014). On the other hand, The maximum frequency of somatic embryos (33.5%) was observed on MS medium supplemented with 2.0 μM 2,4-D for three weeks of culture, whereas it increased to 44.5% on MS liquid medium with 2.0 μM 2,4-D and 0.5 μM L-glutamine in gherkin (*Cucumis anguria* L.), (Thiruvengadam *et al*., 2013). The differences among the percentage of callus formation may refer to the genotypes, type of the explants, growth stage of the plant, incubation condition, kind and concentration of the plant growth regulators. Generally, incorporation of auxins in the medium enhances callus induction and embryogenic callus formation compared to cytokinins.

Evaluation of transformation and regeneration efficiency

Fifty embryogenic calli per experiment were co-cultivated with *Agrobacteium* harbouring pPNgug and pCATgfp plasmids. The transformation experiment (50 calli) was repeated five times with the same conditions. The presence of blue *gus* spots and green *gfp* fluorescent genes was observed on putative transgenic calli two weeks from cocultivation on kanamycin-containing selection medium (Fig. 3a and b). The transgenic calli were maintained on selection medium until conversion to plantlets and then were screened for presence of the *gfp* gene again (Fig. 3c and d as control). Stable transgenic lines started to develop after 6 to 8 weeks on selection media. A number of 18 transgenic plants were generated and ranged from 2 to 5 per experiment. Expression analysis of *gus* and *gfp* genes by Gus staining under binocular microscope and fluorescence microscope revealed that 11 out of 18 transgenic lines were positive for *gus-nptII*

genes and the remaining seven were co-transgenic with both the *gus-nptII* and *gfp* genes (Table 2). The results indicated that total number of transformation events were 25 and ranged from 3 to 7 per experiment. The transformation efficiency for *gus-nptII* genes ranged from 4% to 10% with an average of 7.2% while, co-transformation efficiency of *gus-nptII* and *gfp* genes ranged from 2% to 4% with a total of 14% and an average of 2.8% (Table 2). The general transformation events ranged from 6% to 14% with a total of 50% and an average of 10% (Table 2). The percentage of co-transformation frequency of *gus-nptII* and *gfp* in comparison to the total number of transformation with *gus-nptII* and co-transformation with *gus-nptII* and *gfp* ranged from 33.3% to 50% with total of 38.89% and an average of 39.32%. The seven co-transgenic lines containing both the *gus-nptII* genes and *gfp* gene were taken into consideration and transferred into pots containing soil to set seeds of T_1 for segregation possibility of the *gfp* gene in T_1 generation and elimination of *nptII* gene to produce *npt*-free transgenic cucumber lines. The percentage of the transformation efficiency in this study was much higher than those obtained by Gupta *et al*. (2012) who produced marker-free transgenic cucumber (*Cucumis sativus* L.) cv. Poinsett 76 SR plants. They observed a transformation efficiency of 1.62 with *Agrobacterium tumefaciens* strain LBA4404 harbouring Arabidopsis *cbf1* gene. On the other hand, the percentage of transformation efficiency was similar to those obtained by Nanasato *et al*. (2013) who produced 7 transgenic cucumbers with an average efficiency of 11.9 %. Furthermore, Kose and Koç (2003) reported a transformation efficiency of 16% using EHA101 strain of *A. tumefaciens* harboring *gus* gene in the cotyledon explants and without regeneration in cucumber, however, our obtained percentages are resulted from the entire transgenic plants. Alternatively, it was lower than those found by Gupta and Rajam (2013) who achieved high co-transformation frequency (24%) using two *Agrobacterium* strains cultures in tomato. The percentage plant regeneration was similar to that obtained by Usman *et al*. (2011) who regenerated shoots without transformation with an average of 12% and 14%. Moreover, Mazlan *et al*. (2014) found low proportion of the germinating explants less than 10%. In general, Yin *et al*. (2005) mentioned in a review on transformation methods in cucumber that the frequency of *Agrobacterium*-mediated transformation ranged from 0.8 to 10% and was influenced by the selection agent, the regeneration efficiency, activation of *vir* genes expression, the explant size, bacterial cell density, the length of exposure and the co-cultivation period. Consequently, co-transformation is greatly influenced by the state of the plant material, the tissue culture conditions, and the *Agrobacterium* strain.

Segregation of progeny (T1) plants and PCR analysis of transgenic lines

The seven regenerated transgenic lines having both the *gus-nptII* and *gfp* genes in each of T_0 plants were self-pollinated to obtain T_1 seeds. The pres-

ence of *gus* gene is an indicator for the presence of *nptII* gene where both are present in one T-DNA construct. The seeds resulted from each of self-pollinated line were allowed to germinate in pots. The segregation ratios were compared with the expected values in the $\chi 2$ test table for the T_1 progenies of the seven transgenic lines. The segregation ratio ranged from 1.3:1 to 4:1 for the *nptII* gene and the total $\chi 2$ value was 5.14 and ranged from 0.03 to 3.0. Whereas, the segregation ratio of marker-free transgenic plants ranged from 1.5:1 to 7:1 for the *gfp* gene and the total $\chi 2$ value was 1.587 and ranged from 0.023 to 1.2 (Table 3). The percentage of marker-free transgenic cucumber plants in the T_1 generation ranged from 20% to 43.75% with a total percentage of 35.7% (Table 3). These results were higher than that obtained by Gupta and Rajam (2013) who reported that segregation frequency of marker-free transgenics was 22 - 24% in tomato. The results of the statistical $\chi 2$ for segregation of T_1 plants were compared to the critical value at 0.05% and were not significantly different from a 3:1 ratio in all lines tested and were inherited in a Mendelian manner. Further investigation was carried out by PCR analysis. The presence of the transferred gene sequences was test by PCR with genomic DNA as template using the *nptII* and *gfp* primers. The PCR amplification revealed a presence of the *nptII* and *gfp* genes which resulted in amplification products of 800 and 467 bp, respectively, in the parents (the seven T_0 plants) and in their progenies (T_1 plants) (Figs. 4 and 5). Furthermore, detection of the *gus* and *gfp* genes was applied using Gus staining and fluorescence microscope at embryogenic calli stage and at adult plants. The results clearly indicated visible expression of both genes (Fig. 3). This demonstrated that the co-transformation strategy we performed was efficient and feasible in eliminating *nptII* marker gene.

SUMMARY

Generating of selectable marker-free transgenic plants is desirable in such countries around the world including Egypt. In this study, thirty selectable marker-free transgenic T_1 lines of cucumber were generated through embryogenic calli-driven cotyledon explants and *Agrobacterium*-mediated transformation. Eighteen T_0 transgenic lines were obtained, seven of them were positive for the presence of both constructs (the *gus-nptII* genes and *gfp* gene). Whereas, a total number of 11 plants were positive for the presence of the *gus-nptII* genes. The co-transformation efficiency of the *gus-nptII* genes and *gfp* gene ranged from 2% to 4% with a total percentage of 14% and an average of 2.8%. While, the total percentage of transformation efficiency for the *gus-nptII* genes ranged from 4% to 10% with a total of 36% and an average of 7.2%. The percentage of co-transformation frequency of the *gus-nptII-gfp* genes in comparison to the percentage of *gus-nptII* and *gus-nptII-gfp* genes together ranged from 33.3% to 40% with a total ratio of 38.89 and a main of 39.32%. The seven T_0 transgenic lines generated a total of 84 transgenic plants in the T_1 progeny. Thirty of these transgenic plants

were selectable marker- free having only the *gfp* gene. In addition to 26 transgenic plants have only the both, *gus* and antibiotic resistant gene (*nptII*) and the remaining 28 transgenic lines have both the *gus-nptII* and *gfp* genes. The total number of progeny generated from T_0 plants per line ranged from 5 to 16 plants. On the other hand, regeneration frequency of the total transgenic plants having *gus-nptII* and those having *gus-nptII-gfp* ranged from 4% to 10% with an average of 7.2%. The percentage of marker-free transgenic cucumber plants in the T_1 generation ranged from 20% to 43.75% with a total percentage of 35.7%. The *gfp* segregation ratio ranged from 1.5: 1 to 7: 1 and the total $\chi 2$ value was 1.587 and ranged from 0.023 to 1.2. Whereas, the *nptII* segregation ratio ranged from 1.3: 1 to 4: 1 and the total $\chi 2$ value was 5.14 and ranged from 0.03 to 3.0. Comparison of the statistic $\chi 2$ value with the tabled value at 0.05 % significance revealed that there is no significant difference from 3:1 segregation ratio. Fluorescence microscope, Gus staining, PCR and $\chi 2$ analysis proved the presence and inheritance of the transgenes and that T_1 progeny plants segregated in a Mendelian manner. Our study successfully resulted in generation of marker-free transgenic cucumber with higher percentage through co-transformation strategy.

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Table (1): Percentage of induced calli and embryogenic callus formation.

Exp. No.	No. of ex-plants (NE)	No. of induced calli (NC)	% of induced calli (NC)/(NE)	No. of embryogenic calli (EC)	% of embryogenic calli (EC)/(NE)
1	64	58	90.60	16	25.00
2	64	57	89.06	22	34.38
3	64	60	93.75	23	35.94
4	64	60	93.75	30	46.88
5	64	57	89.06	30	46.88
6	64	61	95.31	17	26.56
7	64	57	89.06	31	48.44
8	32	28	87.50	15	46.88
Total	480	438	728.10	184	310.96
Mean	60	54.75	91.01	23	38.87

Table (2): Transformation efficiency and regeneration with *pPNgus* and *pCATgfp* in T_0 plants

Exp. no.	No. of inoc-ulated embryogenic calli (IC)	No. of *gus-nptII* positive plants (GP)	No. of cotransfored *gus-nptII* + *gfp* positive plants (GGP)	Total no.*gus-nptII* + *gus-nptII-gfp* plants (GP)+ (GGP)	Total no. of transfor-mation events (GP)+ (GGP x2)	*gus-nptII* transfor-mation effi-ciency (%) (GP)+(GGP)/ (IC)	*gus-nptII* + *gfp* Co-transfor-mation effi-ciency (%) (GGP)/(IC)	General transfor-mation events (%) (GP)+ (GGP x2)/(IC)	*gus-nptII* + *gfp* Co-transformation frequency of (%) (GGP)/ (GP)+ (GGP)
1	50	3	2	5	7	10	4	14	40.00
2	50	2	1	3	4	6	2	8	33.30
3	50	3	2	5	7	10	4	14	40.00
4	50	1	1	2	3	4	2	6	50.00
5	50	2	1	3	4	6	2	8	33.30
Total	250	11	7	18	25	36	14	50	38.89
Mean	50	2.2	1.4	3.6	5	7.2	2.8	10	39.32

Table (3): Segregation of T_1 transgenic lines and generation of *nptII* marker- free transgenic cucumber plants.

Line No.	*nptII*	*nptII-gfp*	*gfp*	total	*nptII* segregation ratio	χ^2 value for *nptII*	*gfp* segrega-tion ratio	χ^2 value for *gfp*	% of marker-free transgenic plants (*gfp*/total)
WT	0	0	0	10	-	-	-	-	-
1	4	3	3	10	2.30: 1	0.133*	1.50: 1	1.200*	30.00
2	5	4	6	15	1.50: 1	1.800*	2.00: 1	0.556*	40.00
3	4	5	6	15	1.50: 1	0.030*	2.75: 1	0.023*	40.00
4	2	2	1	5	4.00: 1	0.067*	1.50: 1	0.600*	20.00
5	1	4	3	8	1.70: 1	0.667*	7.00: 1	0.667*	37.50
6	5	6	4	15	2.75: 1	0.070*	2.00: 1	0.556*	26.70
7	5	4	7	16	1.30: 1	3.000*	2.20: 1	0.333*	43.75
Total	26	28	30	84	1.80: 1	5.140	2.20: 1	1.587*	35.70

* Non-significant difference from 3:1 segregation ratio at $p \leq 0.05$, $\chi 2$ for P = 5% is 3.84. $\chi 2$ value indicates a good fit to the expected 3:1 Mendelian ratio at 0.05 % significance, WT = control

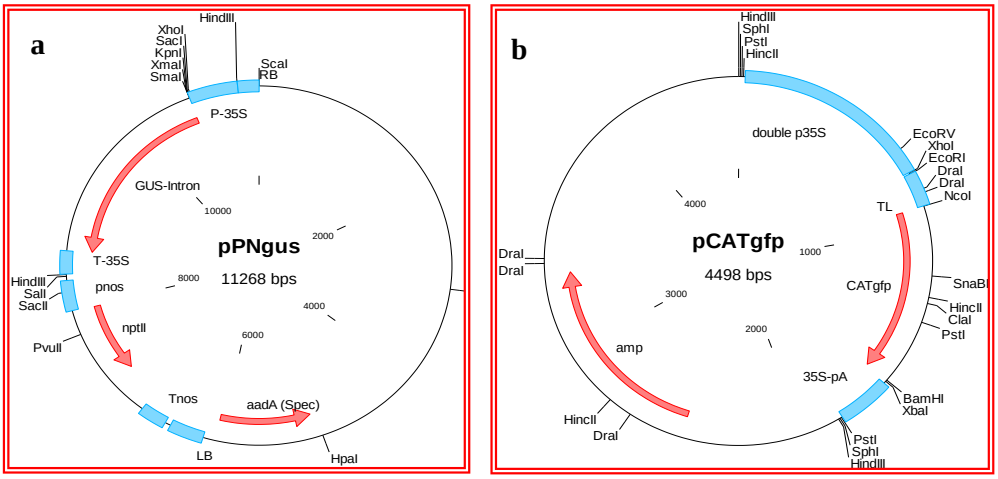

Fig. (1): Schematic representation of the transformation constructs pPNgus (a) and pCATgfp (b). The T-DNA of pPNgus (a) contained *gus* gene controlled by P-35S and T-35S, and *nptII* gene controlled by P-*nos* and T-*nos*. The T-DNA of pCATgfp(b) contained CATgfp gene controlled by *double* P-*35S* and 35S-pA.

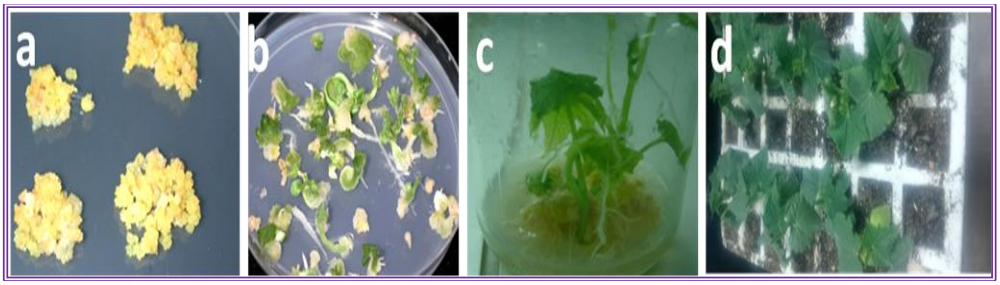

Fig. (2): Conversion of somatic embryos to plantlets. a: embryogenic calli, 10 weeks from culturing the explants; b: conversion of somatic embryos to plantlets, three weeks from transferring to light on hormone free medium; c: regenerated plant 5 weeks from transferring the plantlets to gars and d: acclimatized plants.

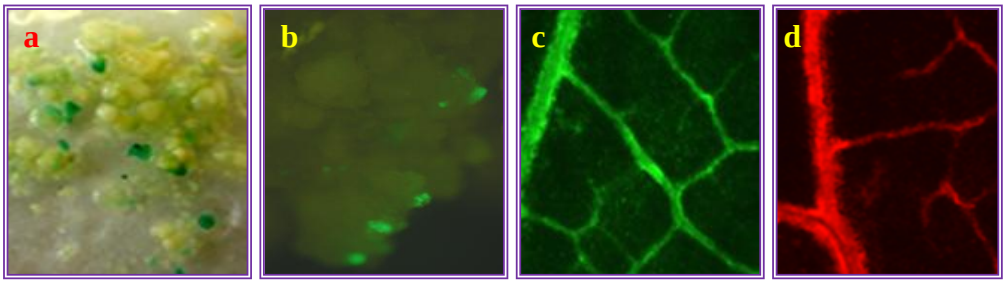

Fig. (3): Embryogenic calli of T_0 and leaves of cucumber plants expressing the *gus* and *gfp* genes. a) Embryogenic calli on selective media, four weeks from co-cultivation with *Agrobacterium* (a and b, respectively) and leaf of T_0 transgenic lines expressing *gfp* gene (c), and non-transgenic control (d).

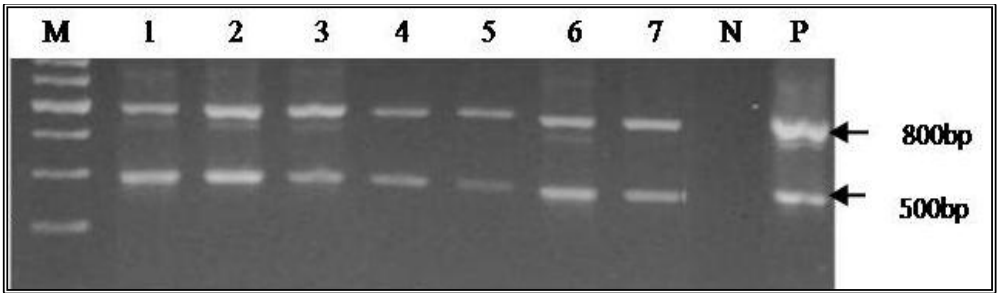

Fig. (4): PCR analysis from the DNA of leaves of the seven T_0 Transgenic plants showing amplification of the *nptII* gene (800 bp) and and *gfp* gene (467 bp). M: DNA ladder (100 bp); 1-7: transgenic lines positive for both the *nptII* and *gfp* genes; N: negative control (non-transgenic); P: positive control for both the *nptII* and *gfp* genes together, respectively.

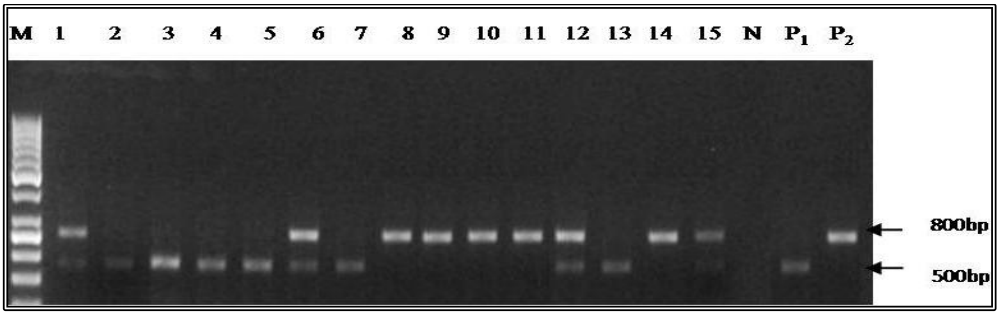

Fig. (5): PCR screening of 15 transgenic lines of T_1 progeny of line number 2. M: 1Kb DNA ladder; 1, 6, 12 and 15: transgenic lines with both the *nptII* and *gfp* genes; 2-5, 7, and 13: transgenic lines with the *gfp* gene; 8-11 and 14: transgenic lines with the *nptII* gene; N: negative control (non-transgenic cucumber); P_1 and P_2: positive control for the *gfp* and *nptII* genes, respectively.