AGROBACTERIUM-MEDIATED) TRANSFORMATION OF CUCUMBER *(Cucumis sativus* L.) AND PLANT REGENERATION

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ABSTRACT
Cucumber plant (Cucumis sativus L.) was transformed by EHA101 strain of A. tumefaciens harboring the binary vector pGA482GG plasmid carrying the marker genes the neomycin phosphotransferase II (npt II) determining resistance to kanamycin and p-ghicuronidase (GUS). Cotyledon and hipocotyl segments of in vitro grown seedlings were used as explants. These tissue segments were cultured on MS medium containing different hormone combinations and concentrations to identify optimum shoot regeneration condition. The highest number of shoot regeneration was observed on MS medium containing 2 mg/1 zeatin. In the tested explant tissues, the highest shoot formation was obtained from the proxiroal ends of cotyledon leaves which were cut transversely into two halves. Plant tissue segments were co-cultivated with A. tumefaciens for 2 days. Tissues, selected on the MS medium containing kanamycin (50 mg/1) were tested by histochemical GUS assay. Shoots regenerated from the transgenic tissue were cultured on the basal MS medium to induce root formation for four to six weeks. The rooted plants with 10 cm height were transferred to the soil for their adaptation to the natural environment. It was observed that transgenic plants had generally sterile flowers. To confirm presence of the transgenes, DNA from GUS $^{+}$ transgenic cucumber plants was analysed by PCR. Thus an effective protocol for Agrobacterium-mediated genetic transformation of cucumber was optimised.

Introduction

Cucumbers *(Cucumis sativus* L.) is one of the most important vegetable crops that are widely cultivated throughout the world. Different diseases and pests cause significant crop losses in cucumber (18). Cultural, physical and chemical methods are commonly used to control diseases and pests, hence decrease the yield losses of crops. The chemical control is one of the most widely used method resulting many problems such as environmental pollution and increased production costs. Therefore recent researches are widely focused on developing diseases and pest tolerant or resistant plant varieties. Recent advances in *Agrobacterium-mediated* transformation have made it possible to introduce foreign genes into various plant species. Conventional plant breeding technique is used generally to obtain a high yielding new plant variety, which is resistant to some diseases and pests. The introduction of desirable genes by conventional plant breeding technique requires much time and is often accompanied by changes in other desirable characteristic traits and it has o-

ther problems such as sterility barriers between species. This problem is especially acute in cucumber (C. *sativus* L.) where successful crosses can be made only between *C. sativus* and the closely related C. *hardwickii*, but not other species (20).

Different plant tissue culture techniques are useful for propagation of important plants and for the production of transgenic plants (20, 16, 10). We need some given systems for each plant to do transformation with various foreign genes, selection of transgenic cells and plant regeneration. There is no report on successful plantlet regeneration from different explants via somatic embryogenesis in cucumber (20, 1,2, 12, 13, 10).

The transformation of cucumber was first achieved by Trulson et al. (1986) and then the transgenic cucumber plants were regenerated from roots, cotyledons and leaves that were induced by the inoculation of inverted ones with *Agrobacterium tumefaciens* and *A. rhizogenes*. Hence several transgenic plants were obtained (2, 4, 21, 16, 15, 19, 6). In these studies, only a few varieties and a few transformation systems were performed to express the marker genes in cucumber. Similar studies are commonly carried out to obtain transgenic plants and recently performed a transgenic system for each variety.

In this study, we have established an efficient method for high frequency shoot production via organogenesis from cucumber and an efficient method for transformation.

Materials and Methods

Plant Material

The seed coats of cucumber cultivar "Çengel Köy" were removed manually and sterilized in 70% ethanol for 1 min and 15% (v/v) Clorox (Commercial bleach containing 5.25% sodium hypochlorite) with a drop of Tween 20 for 15 min and then rinsed 4 times in sterile, distilled water. The seeds were placed in sterile petri plates (approx. 30 per plate) on filter paper and cultured on half strength of MS medium (14) at different periods (3,5 and 10 days) at 25 ±1 °C in the dark to assure uniform and rapid germination. All media used in the experiments were supplemented with 30% sucrose, 0.2% phytogel and different hormone combinations and concentrations. MS medium was sterilized at 121 ° C at 1 atm for 15 min by autoclaving. The different concentrations of zeatin (1, 2 and 3 mg/1) were filter sterilized and added the medium after autoclaving.

Effect of Explants Source on Shoot Regeneration

Explants were prepared from cotyledons and hypocotyls of *in vitro* grown seedlings. The cotyledons cut transversely and longitudinally into two halves were used as explants. The hypocotyls were cut 0.5-1 and 2 cm in length. The cotyledonary hypocotyls were cultured on the plant regeneration medium. The hypocotyl and cotyledon derived explants were cultured on MS medium containing different concentrations and combinations of plant growth regulators such as BAP (6-Benzyl amino purine), NAA (1-Naphthalene acetic acid), IAA (Indole-3- acetic acid), zeatin, kinetin and 2,4-D (2,4-Dichlorophenoxy acetic acid) to induce shoot regeneration. Only cotyledon-derived explants were cultured abaxial side down and up in the petri dish (10 cm) containing shoot regeneration medium.

Bacterial Strain and Plasmid

The *Agrobacterium* strain was disarmed EHA 101 harboring vector pGA482GG plasmid carrying the marker genes, the neomycin phosphotransferase II *(nptII)* determining resistance to kanamycin and reportable marker β-glucuronidase (GUS).

Transformation, Selection and Plant Regeneration

The *Agrobacterium* strain was grown on solid LB medium with 50 mg/1 kanamycin, 10 mg/1 tetracycline and 60 mg/1 gentamicin. A single colony of bacterium was inoculated into 2 ml of liquid LB medium including same antibiotics as mentioned above and incubated at 28°C on a shaker at 200 rpm for 16 h. After 16 h, 500 µl of this culture is transferred to 20 ml of fresh medium supplemented with 50 mg/1 kanamycin, 10 mg/1 tetracycline and 60 mg/1 gentamicin and 100 µM acetosyringone and incubated at 200 rpm for 6 h at 28° C. Prior to inoculation of explants, the bacteria were centrifuged at 2000 rpm for 20 min in a 10 ml centrifuge tubes. The bacterial pellet was diluted (OD_{600} = 0.8-1.0) with MS medium including 2 mg/1 zeatin, 100 µM acetosyringone. The explants were dipped in bacterial suspension for 15-20 min and blotted on sterile filter papers. Then they were transferred to co-cultivation medium (MS medium with 2 mg/1 zeatin and 0.5 g/1 MES (2-[N-Morpholino] ethanesulfonic acid) and incubated at 25 ±1 ° C in the dark for 2 days.

The selection medium was comprised of the shoot regeneration medium supplemented with 50 mg/1 kanamycin to select transformed tissue and 300 mg/1 carbenicillin to suppress *Agrobacterium* growth. Every two weeks, the explants were sub-cultured on the fresh selection medium and observed for shoot regeneration.

Plantlets regenerated on explants were transferred into MS medium containing 0.1 mg/1 NAA, 0.1 mg/1 IAA and basal MS medium to induction of root regeneration for four to six weeks. Subsequently, the regenerated plants were potted in a mixture of 1 soil: 1 perlite (v/v) and grown in a greenhouse for their adaptation to natural environment.

Histochemical GUS Assay

β-glucuronidase (GUS) activity in the leaves and shoots were performed according to Jefforson et al. (7) with some modifications (8). For this aim two days after *Agrobacterium* infection of explants transformed plants and putative transgenic shoots were incubated in X-gluc (5- bromo- 4- chloro 3-indolyl β-D glucuronide) solution at 37°C for 24 h. Transformation efficiency was calculated as the total number of GUS^+ shoots/total number of *Agrobacterium* inoculated explants X 100.

Isolation of DNA and PCR Analysis

Total nucleic acids were extracted from GUS^+ shoots following the procedure as described by Sharp et al. (17).

All PCR analyses were performed as described by M. Kayim (9). GUS+ transgenic plants were screened for the presence of marker genes *(uidA.* and *npt II)* by PCR. Primers for the *uidA* and *npt II* genes were5'-GTGGGCATTCAGTCTGGATCG-3', 5'-CATAGAGATAACCTTCACCCGG-3' (GUS-R),5'-CACGCAGGTTCTCCG-GCCGC-3' *(npt II-F)* and 5'-TGGCGCT-GCGAATCGGGAG- CG-3' *(npt II-R)* respectively. All reactions were performed in 25 µl containing 100 ng DNA, 200 µM dNTPs, 3 mM Mg PCR buffer (Idaho Technology, Idaho Falls, ID), 1 µM of each primer and 1.0 Unit of *Taq* polymerase. Each PCR reaction was incubated at 9 ° C for 1 min before amplification cycle. The temperature cycling for the PCR was follows: 29 cycles of 94° C for 1 min, 64 ° C for 30 s and 72 ° C for 2 min. The 30^{th} cycle was the same with the exception that DNA extension at 72° C was carried out for 10 min. PCR products *of uidA* and *npt II* genes were observed on 8% of polyacrylamide gels (PAGE) in buffer TAB, stained with ethidium bromide and viewed over a UV light source and the photos were taken with a digital camera.

Results and Discussion

Direct Shoot Regeneration From Explants

Shoots developed from the proximal end of the cotyledon leaves were cut transversely into two halves within six weeks (**Fig 2.A**). Low concentration of BAP (0.1 mg/1) produced more shoots than high concentration of BAP (**Fig 2.F**). High concentrations of BAP (2, 3, 4 and 5 mg/1) induced only calli production in the previous weeks, however, following weeks this calli turned brownish form and died (results not shown). Similar observation was reported by Ganapathi et al. (6).

The highest number of embryo formation in cultures resulted in MS medium containing 2 mg/1 zeatin from proximal ends of cotyledon explants and this result was followed by 3 mg/1 zeatin and 0.1 mg/1 BAP respectively. The results of shoot regeneration from transversely cut cotyledon explants were shown in **Fig. 1**.

A higher shoot regeneration was reported in a previous work (10) when they used 2 mg/1 zeatin in the same medium than obtained in this study (**Fig. E**). The reason of these differences could be different plant variety used in the experiments. Therefore lower number of shoot regeneration occurred from explants. The similar result was reported by Kujiper et al. (11).

The shoot induction rate in explants of 5-day-old was seedlings higher than in explants of 3- and 10-day-old seedlings. The shoot regeneration rate decreased in older seedlings. However, 3 days after germination, the shoot induction rate in cotyledons and hypocotyls dramatically decreased. Therefore, 5-day-old seedlings have a high competence for shoot organogenesis. These results are similar to the reports of Trulson et al. (20), Fang et al. (5), Chee et al. (1) and Kim et al. (10). The best adventitious shoots induction was obtained from longitudinally cut cotyledon explants and 1 cm long hypocotyls (**Fig. 2.A**). The other explants were cultured on MS medium containing different plant growth regulators and induced calli 5 days after culturing. When the explants were cultured the abaxial side down, shoot regeneration was induced. While explants were cultured the abaxial side up calli were induced on the same medium. The same result was

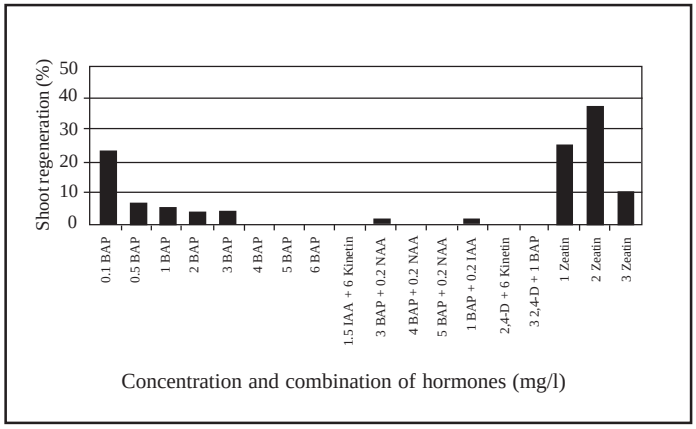

Fig. 1. The effect of different combination and concentration of hormones on adventitous shoot formation from cotyledon explants.

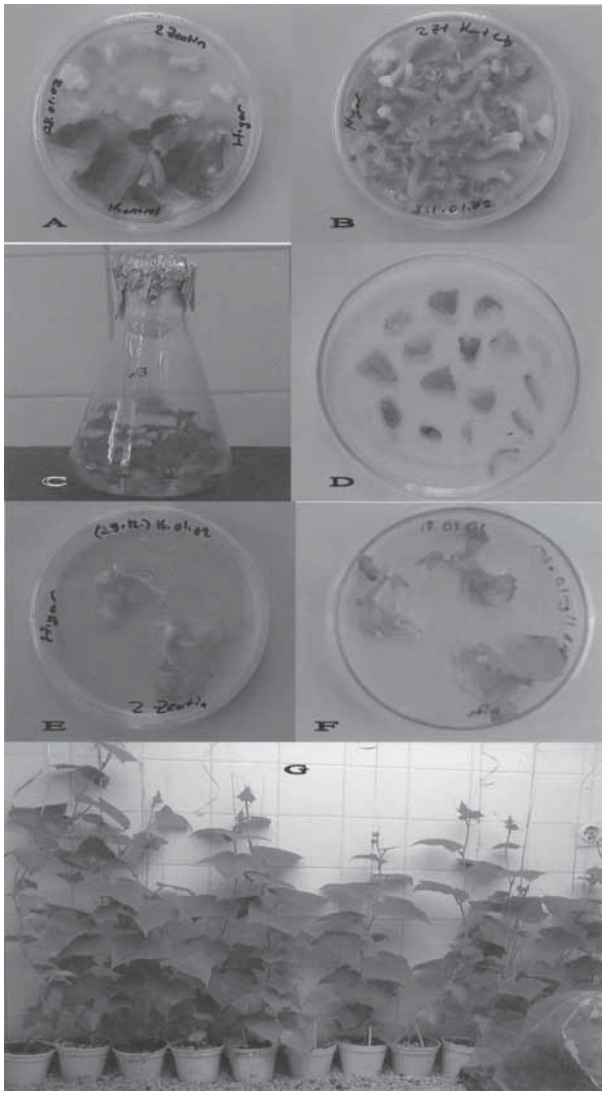

Fig. 2. A: Plantlets regeneration and calli formation from cotyledons and hypocotyls cultured on MS medium containing 2 mg/1 zeatin, **B:** After Agrobacterium infection, shoot regeneration from cotyledonary hypocotyls explants on MS medium containing 2 mg/1 zeatin and 50 mg/1 kanamycin after *Agrobacterium-mediated* transformation, **C:** Shoot elongation in longitudinally cut of cotyledon explants cultured on MS medium containing 2 mg/1 zeatin, **D:** Histochemical GUS assay of explants two days after transformation, **E-F**: Shoots regenerated from longitudinally cut cotyledon explants were cultured on MS medium containing 2 mg/1 zeatin and 0.1 mg/1 BAP respectively after 4 weeks, **G:** Two months old transgenic cucumber plants were grown in growth chamber.

reported by Kim et al. (10). On the other hand when the cotyledonary hypocotyl explants were cultured on the plant regeneration medium only one shoot was appeared (**Fig 2. B**). When over fifteen explants were cultured in one plate the shoot regeneration was performed slowly. When ten explants were cultured in one plate the shoot regeneration was optimum.

Transformation, Selection, Whole Plant Regeneration

The effect of acetosyringone treatment during the co-cultivation was investigated and acetosyringone treatment increased the transgenic shoot regeneration (data not shown). This result was the same as reported by Raharjo et al. (16) and Nishibayashi et al. (15). When the explants were inoculated with *A.*

tumefaciens for longer than 15 min and co-cultured for 2 days, the explants did not survive due to overgrowth of*Agrobacterium.* 15 min soaks and 2 days co-culture of the explants were routinely used in these experiments. Two days of co-cultivation resulted in the highest percentage of shoot regeneration on the selected medium. However, when co-cultivation period was prolonged, it was not possible to completely eliminate *Agrobacterium* from explants.

When the explants, non-transformed by *A. tumefaciens* were cultured on plant regeneration medium containing antibiotics, their appearance were normal for a few days and the following days they turned brown and finally died.

The transgenic tissues were selected on shoot regeneration medium containing 50 mg/1 kanamycin. Shoot regeneration from this tissue occurred about 4-6 weeks later from the calli which was derived from the hypocotyl and cotyledon explants. Ninety-eight plantlets were regenerated from calli of 474 cotyledon explants and four plantlets were regenerated from the calli of 650 hypocotyl explants (**Table 1**).

Transgenic shoots were transferred onto rooting medium and the best rooting of the plant was observed on hormone-free MS

TABLE 1

Frequency of responding explants, number of shoots formed on selection medium, and number of transformed plants assayed by GUS+ test *ofCucumis sotivus*

	# explants	#shoot regeneration	Shoot regeneration (%)	#GUS$^+$ shoots (%)	Transformation efficiency (%)
Cotyledon	474	98	20.6	75.8	16
Hypocotyl	650	4	0.6	3.25	0.5

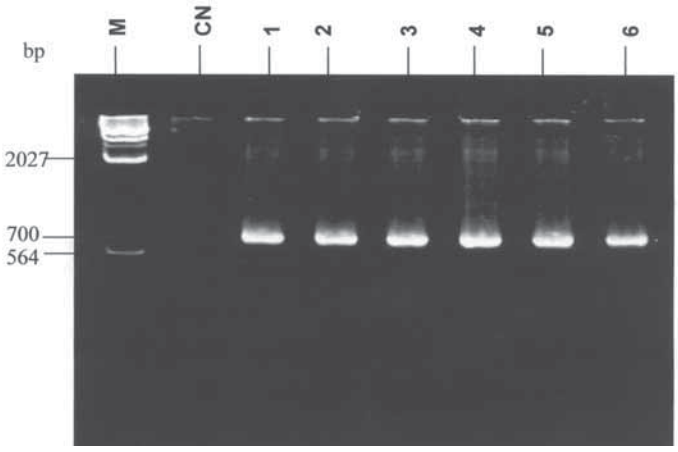

Fig. 3. PCR analysis of transgenic cucumber plants for a 700 bp *npt II* gene fragment. CN; non-transformed cucumber plants, 1-6; transgenic plants, M; λ DNA Hind III marker is indicated in basepare.

medium (**Fig 2.C**). The rooted plants with 10 cm height were transferred to soil for their adaptation to the natural environment (**Fig 2. G**).

Histochemical GUS Assay and PCR Analysis

The control plants (negative) did not display GUS expression in the cells of roots, cotyledons and hypocotyls, but the transgenic plants which were selected on the shoot regeneration medium containing antibiotics, the plant tissues turned into blue colour within 6 h after GUS staining (**Fig 2. D)**. We observed 75.8% GUS^+ from cotyledons used in this experiment.

In order to determine the plants after regeneration, each plant was tested for the presence of *npt II* by PCR. Results are shown **Fig. 3.** All transgenic cucumber lines (1, 2, 3, 4, 5 and 6) amplified a 700 bp DNA fragment for an internal *npt II* gene except non-transformed control plant. Same transgenic plants lines also amplified a 660 bp DNA fragment for an internal *uidA* gene (GUS).

Conclusions

We reported this study thus an effective protocol for *Agrobacterium-mediated* genetic transformation of cucumber variety of Cengel Koy. We studied in this work only one variety but in)the future will study with another variety and optimised an effective method transformation systems for cucumber.

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