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*Full Length Research Paper*

# Regeneration and *Agrobacterium*-mediated transformation studies in carnation (*Dianthus caryophyllus* L. cv. Turbo)

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**Leaf explants of carnation (*Dianthus caryophyllus* L. cv. Turbo) were used for the transformation of gene performed by the EHA 105 strain of *Agrobacterium tumefaciens* harboring the binary vector, pGA482GG. This vector carries the marker genes, neomycin phosphotansferase II (*npt II*) that determine resistance to kanamycin and β-glucoronidase (GUS). Leaf segments of carnation plants were cultured on MS medium containing different combinations and concentrations of hormones. The best shoot regeneration was observed on MS medium with 1 mg/L BAP+ 0.05 mg/L NAA. Initiation of root formation was performed on MS medium containing 0.5 mg /L IBA. Transformation was done by the EHA 105 strain of *A. tumefaciens*, after determining the appropriate medium and the plant tissue for the transformation. Plant tissues, selected on MS medium containing kanamycin, were tested by isolating them from transgenic plant tissues and shoots regenerated from these transgenic plants. PCR were done to indicate transformed GUS gene; 660 bp specific DNA bands of GUS were observed. In this work, an appropriate regeneration system for later studies on carnation and an efficient technique for the transformation of important genes that can resist diseases, using *A. tumefaciens* were developed.**

**Key words:** *Agrobacterium tumefaciens*, carnation (*Dianthus caryophyllus* L. cv. Turbo), gene transformation, plant regeneration.

## INTRODUCTION

Turkey is one of the countries with climatic and geographical structure that is very suitable for cut flower. The total area of hothouses allocated for decorative plant production was around 1500 ha, and in 53% of this total area cut flowers are produced. Izmir is the number one city producing cut flowers by 28%, followed by Antalya, Istanbul and Yalova in order of importance. Two thirds of the land allocated for cut-flower production, exceeding 950 ha, is covered. 12% of the hothouses are covered with glass, while the remaining 88% are made of plastic materials. 30% of the cut-flower production in Turkey is grown in uncovered hothouses. The carnation is the top item with 52%, followed by the gladiolus (17%), the rose (10%) and the marigold (8%). 97% of carnation production is maintained in plastic hothouses in Izmir and Antalya (Anonymous, 2003; Taşcıoğlu and Sayın, 2005; AIB, 2005). Moreover, production of carnation for export started in 2002, in Isparta, which is expected to become a serious rival to Antalya in the near future (AIB, 2003). It is possible to produce and sell the rose and the carnation in all the seasons of the year. Carnation (*Dianthus caryophyllus* L.) is attacked by a number of pathogens, including fungi, bacteria and viruses. Vascular wilt caused by the fungus, *Fusarium oxysporum f.sp. dianthi* is considered to be the most serious and severe disease affecting carnation (Lahdenpera, 1987; Melero-Vara et al., 2002). Attempts to eradicate the pathogen have not been successful (Cuellar et al., 1999). Due to the continued

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**Abbreviations: BA,** benzyladenine; **GUS,** β-glucuronidase; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog nutrient medium; **NAA,** naftalin acedic acid, **npt II,** neomycin phosphotansferase II, **PCR,** polymerase chain reaction; **SPSS,** Statistical Package for the Social Sciences, **X-gluc,** 5-bromo-4-chloro-3-indolyl-D-glucuronide.

lack of success in eradicating this disease, it is necessary to search for resistant cultivars, carnation breeders aim at producing new varieties with improved horticultural traits such as disease and pest resistance and increased vase life. Being highly heterozygous, conventional breeding procedures in carnation have some restrictions. Attempts have been made to induce diseases resistance through the crossing of susceptible cultivars with resistant stocks, using conventional breed-ing approaches. However, the development and detection of resistant varieties and clones is a time-consuming and expensive operation. It is, therefore, imperative that novel methods be applied to introduce the variability needed for inducing specific resistance traits in the available germplasm. In ornamental plants, biotechnological approaches such as selection of somaclonal variations in cell and tissue culture, somatic hybridization through protoplast fusion and genetic transformation by direct and in direct gene transfer techniques have been recognized as useful strategies for the breeding besides conventional cross hybridization method. Consequently, regeneration system widely used for transformation studies have been restricted to the explants based on direct shoot regeneration system such as stem (Zuker et al., 1999; Zucker et al., 2001), leaf (Estopa et al., 2001), node (Nontaswatsri et al., 2004) and petal explants (Van Altovarst et al., 1995a). The logical gene transfer is choices for developing diseases resistant cultivars with-out altering other desirable attributes.

Genetic transformation enables the introduction of genes of interest into the plant genome, which results in transgenic plants with desirable traits. Recently, the most transformation protocol used for selectable marker gene is neomycin phosphotrensferase II (*npt II*) that confers the resistance to aminoglycoside antibiotics, such as kanamycin, neomycin and geneticin and reportable marker, *GUS* gene. Recent advances in *Agrobacterium* - mediated transformation have made it possible to introduce foreign genes into carnation species (Estopa et al., 2001). Successfully developing transgenic carnation plants were obtained only from a few varieties with a few transformation systems (Nontaswastri et al., 2005). This study was conducted to establish an efficient method for regeneration from leaf tissue and the conditions of transformation to give a high yield of transformants and generated carnation cv. Turbo plants

## MATERIALS AND METHODS

### Plant materials

Carnation (*Dianthus caryophyllus* L. cv. Turbo) leaf explants were used for regeneration and transformation. Young leaves explants were cut small (1-2 cm) and surface sterilized for 10 min in 3% hypocloride solution, rinsed three times in sterile distilled water and used for regeneration and transformation. The leaf explants was cultured into MS medium (Murashige and Skoog, 1962) supplemented BA (1, 2, 3 and 4 mg/L) + 0.05 NAA+30 g/L sucrose+ 7 g/L agar in Petri dishes under 16 h photoperiod at 25°C. Each treatment was replicated with four Petri dishes. Recovered shoots (2 - 3 cm) were cultured for rooting into MS+ 0.5 IBA, pH: 5.7.

### Agrobacterial infection and regeneration

The *A. tumefaciens* strain, EHA 105 harbours the binary vector, pGA482GG carrying the marker genes, the neomycin phosphortansferase II (*npt II*) that determines resistance to kanamycin and GUS genes, controlling the expression of ß-glucuronidase. This strain was cultured overnight at 28°C in YEP medium with 300 µg/ml carbenicillin. The bacteria were centrifuged at 2000 rpm for 15 min and re-suspended in the MS liquid medium with 1 mg/L IAA, 200 µM Asetosyringone and 4 mM Betain; the density was adjusted to 1 x $10^5$ spore/ml. Leaf explants were wounded, suspended in inoculums for 15 min, blotted dry on sterile blotting paper, placed with abaxial side in contact on the regeneration medium (hormone-free) and incubated at 25°C in the dark for 48 h. Fifteen explants per treatment were used for transformation assay. After co-cultivation with *Agrobacterium*, the explants were washed overnight in liquid MS medium (hormone-free), blotted dry and placed over regeneration medium containing 1 mg/L BAP+ 0.05 mg/L NAA+ 300 µg/ml carbenicillin, 50 µg/ml kanamycin. Two weeks later, the leaf explants were transferred to the fresh identical medium. Green, putative-transformed shoots were repeatedly transferred to fresh MS medium. The same selection procedure was used for leaf explants without *Agrobacterium*, control group. Plantlets regenerated on explants were transferred into MS medium containing 0.5 IBA for 4-6 weeks to induce root regeneration. The regenerated plants from leaf explants were washed carefully with running water to remove the agar and were transferred into plastic pots containing an autoclaved mixture of soil and perlite (1:1 v/v). Each pot was covered with a polyethylene bag to maintain humidity at 25°C under 16 h photoperiod conditions. After 10 days, the plastic bag was removed and the plants were cultured in the same conditions for 2 - 3 weeks. Acclimatized plants were transferred to be grown in the glasshouse.

### GUS histochemical analysis

GUS (β glucuronidase) activity was tested histochemically in various plant parts like leaf, stem of different transgenic plants as well as their subsequent progenies. The histochemical determination was carried out according to the procedure of Jeferson et al. (1987). Tissue sections or organs were incubated in dark for 24 h at 37°C in x-gluc solution made up of 10 mM EDTA, 100 mM sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (w/v) x-gluc. Chlorophyll was removed by treating the tissue in 1:3 aceto-ethanol mixtures. A blue color was observed in 70% ethanol in transformed shoots in comparison to white color in untransformed control plant.

### DNA extraction and PCR analysis

PCR amplification of the gene for GUS using specific primers was carried out to check the presence of a transgene in the plant genome. Young leaves from carnations were picked and extracted according to the method of Sharp et al. (1988). The presence of transgene (GUS) was detected through PCR analysis. PCR was conducted in a reaction containing 1 µl of the genomic DNA ( about 25 - 50 ng), 2.5 µl each of the upstream and downstream primers, 2.5 µl dNTP and 0.2 µl Taq polymerase (Fermentas), 2.5 µl PCR buffer, 2.5 µl $MgCl_2$ in a total volume of 25 µl. The primers used in this experiment were GUS (5′- GGT GGG AAAGCG CGT TAC

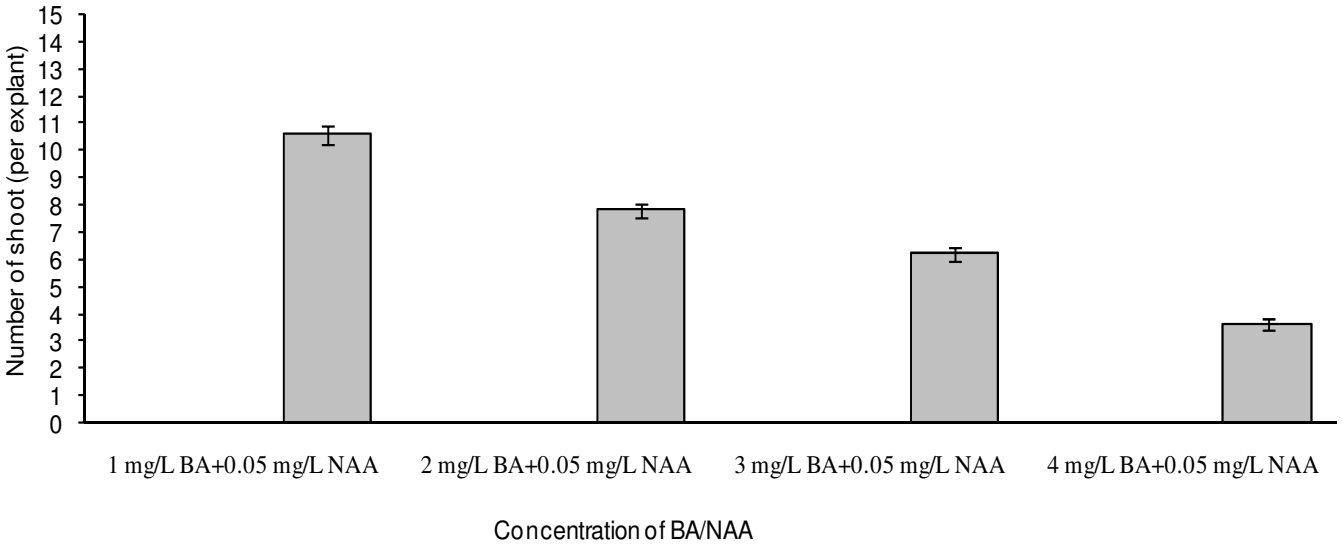


**Figure 1.** Effect of combinations and concentrations of hormones on shoot formation from carnation leaves.

AAG 3′ and 5′-GTT TAC GCG TTG CTT CCG CCA-3′). One non-transgenic plants of cv. Turbo was used as negative control and 15-20 ng of the plasmid as positive control. PCR was carried out for 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 54°C, 1 min at 72°C and supplemental incubation for 5 min at 72°C with an automatic thermal cycler (Techne). PCR amplificates were separated on a 1.5% (w/v) agarose gel and ethidium bromide staining.

#### Statistic analyses

Fifteen leaves were inoculated with $1x10^5$ spore/ml *A. tumefaciens* in each Petri dish with four Petri dishes as replicates in each treatment. The total number of regenerated shoots divided by the total number of regenerated explants was calculated as shoot number per regenerated plants. Differences in the hormone concentrations for plant regeneration were tested for each explant using one-way ANOVAs. Turkey's multiple range tests were applied when the one-way ANOVA showed significant ($P < 0.05$) differences. All the statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, Illinois).

## RESULTS

### Shoot regeneration

Five combinations of BAP with NAA regenerated from leaf explants were tested. The numbers of adventitious shoots from leaf explants were generated when cultured into the MS medium with plant regulator. The best hormone combination was found in 1 mg/L BA and in 0.05 NAAmg/L (10.6 shoots per leaf explants) (Figure 1). Maximum of 14 shoots were regenerated from leaf explants. It was observed that there was a considerable in-crease in shoot number in lower BA levels. High level of BA was less effective in shoot regenerated from leaf explants than in low levels. Few of the plant regeneration was observed in 4 mg/L BA, and 0.05 mg/L NAA was observed in very few plants that were regenerated. Adventitious shoot formation was observed from the entire leaf surface. When leaf explants were cultured on BA–NAA supplemented medium, shoots were clearly visible arising in clumps from the leaf surface in 10 days of culturing. Callus formation was also observed on NAA–BA supplemented medium but it did not inhibit the shooting stimulus. Shoots formed on MS medium containing different BA-NAA concentration were maintained through 4 weekly subcultures on MS medium 1 mg /l BA and 0.05 mg/L NAA (Figure 2a, b).

### Agrobacterial infection and regeneration

Asetocyringone increased shoot regeneration of carnation (Table 1). When carnation leaf explants were suspended in inoculums for longer than 30 min and co-cultured for 48 h, the explants did survive due to over-growth of *Agrobacterium.* 15 min soaking and 48 h co-culturing of the explants were routinely used because they resulted in the highest percentage of shoot regeneration on MS +1 mg/L BAP+ 0.05 mg/L NAA + 50 µg/ml kanamycin. It was impossible to completely eliminate *A. tumefaciens* from explants if co-cultivation period was prolonged. When the untransformed by *A. tumefaciens* explants were cultured on regeneration medium containing antibiotics, they turned brown, were completely inhibited and finally died for a few days (Figure 3). The transgenic plants were selected on shoot regeneration medium containing 50 mg/L kanamycin. Shoot regeneration from leaf explants 3-4 weeks were transferred to the regeneration medium.

The ability of leaf explants to regenerate shoots was significantly reduced by infection with *A. tumefaciens*.

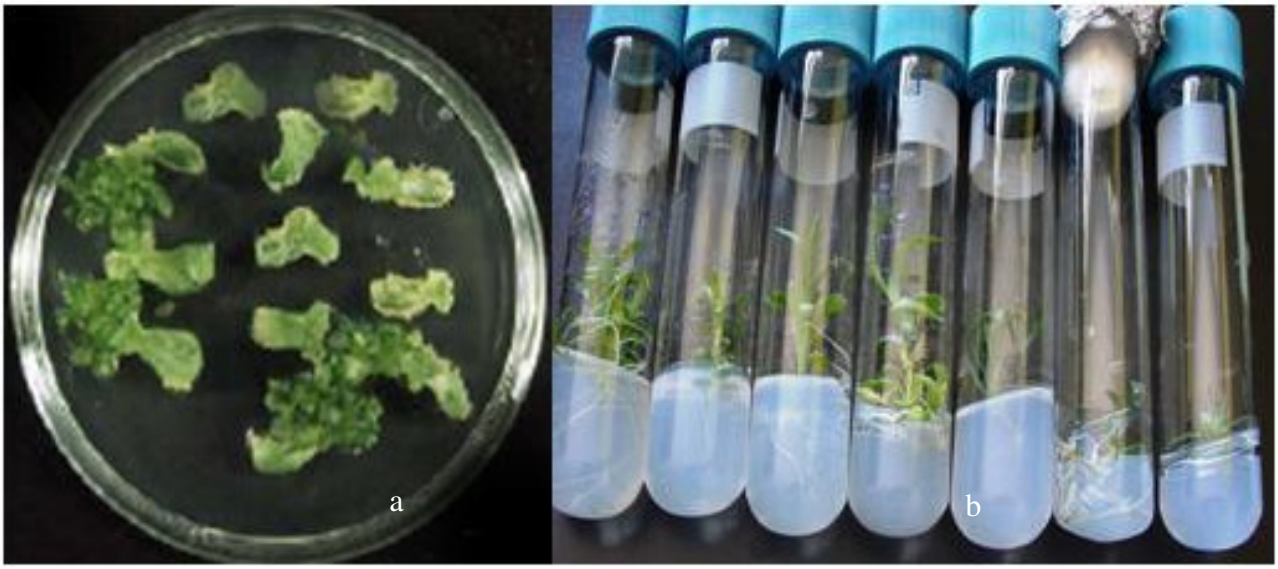


**Figure 2.** Transformation of carnation explants by using *A. tumefaciens* and plant regeneration. (a) Plantlet regeneration from leaf explants cultured on MS medium containing1 mg/L BAP+ 0.05 mg/L NAA; (b) shoot regeneration from leaves on MS medium containing containing1 mg/L BAP+ 0.05 mg/L NAA and 50 mg/l kanamycin after *Agrobacterium* -mediated transformation.

**Table 1.** Effect of acetocyringone (As) and IAA application on shoot regeneration, and transformation efficiency (%).

| Concentration and combination | | No. of explants | Transgenic shoot regeneration | Transformation efficiency (%) |
|---|---|---|---|---|
| As (µM) | IAA (mg/L) | | | |
| 0 | 1 | 20 | 5 | 25 |
| 50 | 1 | 20 | 8 | 40 |
| 100 | 1 | 20 | 14 | 70 |
| 150 | 1 | 20 | 11 | 55 |

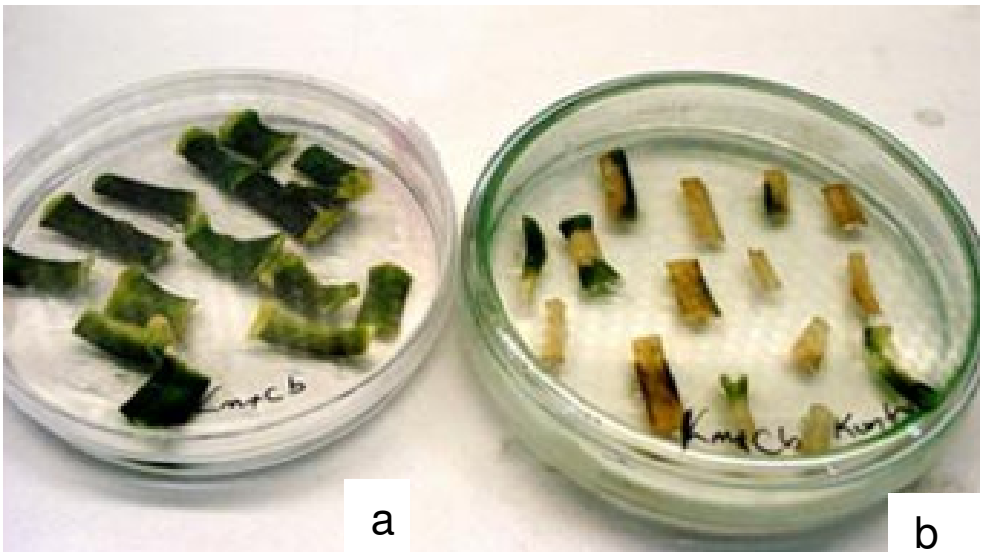


**Figure 3.** Leaves from transformated (a) and untransformated (b) by *A. tumefaciens* explants on antibiotics selective medium.


**Figure 4.** Transformation of carnation explants by using *A. tumefaciens* and plant regeneration (a) rooted transgenic carnation plants on modified MS medium containing 0.5 mg/l IBA in glass tubes; (b) two-months-old transgenic carnation plants.

Presence of kanamycin (50µg/ml) in the selective medium had adverse effect on shoot induction. The shoot number per regenerated explants was higher in control than infected with EHA 105 (Not data shown). The growth of transgenic shoots in kanamycin containing media was similar to that obtained with control shoots in kanamycin free medium. There was not a determined difference between control and transgenic plants. Transgenic shoots were transferred to rooting medium (Figure 4a). The rooted 10-cm-high plants were planted to soil (Figure 4b).

## GUS and PCR analyses

GUS activity was tested histochemically in various plant parts such as leaf, stem of different transgenic plants as well as their subsequent progenies. Histochemical assay for presence of *gus* gene was carried out in all the putative transformants; small pieces of leaves from each plant were used for the assay. A blue colour was observed in transformed shoots in comparison to white colors in untransformed control plant. The few constitutively expressing GUS-positive plants were tested for the presence of GUS gene in different plant parts (Figure 5). Transformed plants grown in pot showed high GUS activities in shoots. This observation confirmed successful genetic transformation and stable gene expression in the transformed plants.

Each plant was tested by PCR for the presence of the GUS gene after regeneration. The PCR results of selected plants showed a band of the corrected expected size in the GUS (bp) genes. Figure 6 shows amplification of the GUS gene of nine transformants and for the positive control (pGA482GG). No amplification was observed in negative control (cv. Turbo). Nine plantlets from leaf explants were regenerated after gene transformation. It was observed that almost all transgenic carnation lines amplified a 660 bp specific DNA bands for an inserted GUS gene.

## DISCUSSION

Shoot formation from leaf explants is a preferable technique for clonal propagation as it prevents somaclonal variations in the cultures. The type of plants explants used for clonal propagation affects the genetic variation. Callus leads to more frequent genetic deviations particularly with prolonged subculturing in shoot regeneration. The regeneration response declined towards the third pair of leaves. Leaf explants have been most widely studied for adventitious shoot formation in carnation (Sankhla et al., 1995; Yantcheva et al., 1998). We focused on the shoot from leaf explants. Shoot growth was observed by 3-4 weeks on regeneration medium supplemented with BA-NAA. Adventitious shoot formation from leaf has also been reported by Kakehi (1979), Gimelli et al. (1984), Van Altvorst et al. (1995b) and Kathia and Kothari (2002). All the reports conformed on regeneration through proximal part. In an earlier report, organogenesis was recorded from the callus cultures of *Dianthus chinensis a*nd no direct adventitious shoot formation was observed possibly due to varietals difference (Jethwani and Kothari, 1996). Combination of auxin with cytokinin proved to be excellent for shoot regeneration. Shoot regeneration from leaf explants is most preferred if *Agrobacterium*-mediated gene transfer is to be achieved and leaf explants are best suited for both adventitious shoot formation and *Agrobacterium*-mediated gene transfer experiments (Estopa et al., 2001; Nontaswastri and Fukai, 2005; Sankhla et al., 1995; Yantcheva et al., 1998).

We also found that acetosyringone was of vital importance to induce transformation and to regenerate transgenic shoots from carnation leaf explants. Acetosyringone was identified as a plant signal molecule,

**Figure 5.** Histochemical GUS assay of kanamycin-resistant plants parts after transformation with the EHA $10^5$ strain of *A. tumefaciens* harboring the binary vector pGA482GG carrying the marker genes, the neomycin phosphotansferase II (*npt II*) determining resistance to kanamycin and GUS gene which controls the expression of ß-glucuronidase.

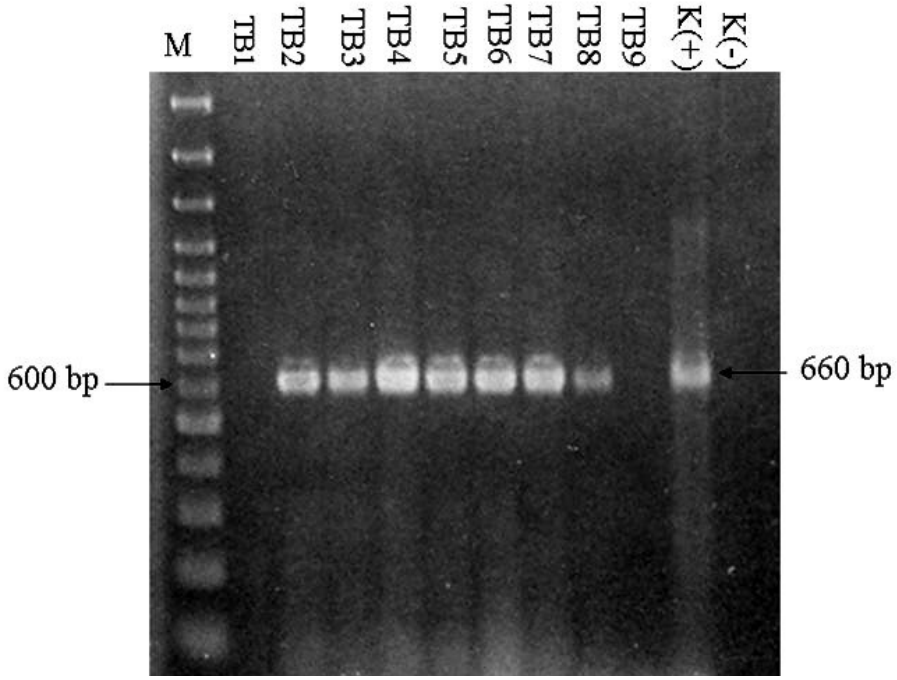


**Figure 6.** PCR analysis of transgenic carnation plants probed for a 660-bp GUS gene fragment. K, EHA 105 strain of *A. tumefaciens* Harboring pGA482GG plasmid; TB1, nontransformed carnation plants; TB2–TB9, transgenic plants; M, 100-bp DNA Ladder marker.

which induced the activation of the *vir* genes in *A. umefaciens* and can be used to increase transformation frequencies. The beneficial effect was similar to that reported by Raineri et al. (1990).

PCR showed that transgenic *D. caryophyllus* L. cv. Turbo lines contained the GUS and the *nptII* gene for kanamycin resistance. Although transgenic plants were selected in media with kanamycin, some escapes occurred based on PCR results. When leaf explants were placed in culture media, they elongated after 4-5 days, some of them curling in such a way that the regeneration area localized at the base of the leaf did not remain in contact with the culture medium. Therefore, in these curled explants, the concentration of available kanamycin probably decreases, which could partially explain the presence of escapes. Similar results were found by Estopa et al. (2001) and Nontaswatstri and Fukai (2005).

Transformation rate is very low, and this result was found by other authors (Estopa et al., 2001; Van Altovarst et al., 1995a). The addition of kanamycin decreased the number of shoots/explant. The kanamycin prevented the regeneration shoots during the transformation process. The selection procedure is one of the most important factors affecting transformation efficiency, because only a limited number of cells are transformed during transformation. When the selection pressure is too high, the transformed cell or tissue will not grow, or even die. On the other hand, when the selection pressure is too low, more escapes will be regenerated due to low concentrations of selective agent.

Agrobacterium-mediated gene transfer is mostly used for ornamentals (Bellarmino and Mii, 2000; Clarke et al., 2008; Suziki and Nakano, 2002). Gene transfer is generally a single cell event and requires a high frequency regeneration protocol. Leaves of plants are most commonly used in transformation expression of carnation due to its ability for regenerating shoots (Estopa et al., 2001; Nontaswatsri et al., 2004; Nontaswastri and Fukai, 2005; Lu et al., 1991). The protocol reported here for *D. caryophyllus* L. cv. Turbo is efficient, reproducible and could be used for genetic transformation experiments such as disease and pest resistance and increased vase life.

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