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Production of Genetically Modified Peach (*Prunus persica* L. Batsch) El-Sheikh Zewaied Cultivar Plants

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Abstract: Peach crop is considered to be one of the most important crops for farmers in North Sinai. Peach trees are actually plagued by so many different pests and diseases before and after harvest and about 20% or more of peach fruits were destroyed through packaging and transportation processes, because it has soft skin and juicy flesh. Therefore, using of breeding and genetics research programmes is very essential to improve peach quality. The transformation systems were established for the local peach (El-Sheikh Zewaied) cultivar. *Agrobacterium*-mediated and microprojectile bombardment transformation systems (using 1100 &1350 psi) were successfully used to introduce *gus* and *bar* genes as reporter and selectable genes, respectively. Transformation efficiency was 3% by using *Agrobacterium*-mediated transformation system. Effect of mannitol and bombardment pressure treatments on transformation efficiency were tested through a number of experiments. The previous treatments had a positive effect on transformation. The expression of the introduced genes was detected using histochemical assay and confirmed by PCR analysis for both genes. Southern blotting technique was carried out to confirm the integration of *bar* gene in the transformed tissues.

Keywords: Peach (*Prunus persica* L. Batsch), Genetically Modified

INTRODUCTION

The peach [*Prunus persica* L. (Batsch)] is a member of the family *Rosaceae*, all commercial cultivars belong to [*P. persica* (L.) Batsch], and are primarily grown in temperate zones between; Latitudes 30° and 45° N and S.

Peach crop is considered to be one of the most important fruit crops for farmers in North Sinai. Peach acreage reached about 59257 feddans in North Sinai, these areas produces about 14000 ton / yearly from fresh fruits (Agri. Statistics, July 2003). Peach trees are actually plagued by many different pests and diseases before and after harvest and about 20% or more from peach fruits were damaged through packaging and transportation processes, due to its soft skin and juicy flesh. Therefore, it needs more using of breeding and genetics research to improve peach quality (delay softening, retard overall ripening and extended shelf life), productivity and value of peach crop.

Gene transfer offers the peach breeder on opportunity to transfer specific genes into peach germplasm. Initial work in peach transformation indicated that peach tissue was susceptible to *A. tumefaciens* infection[8]. Scorza *et al.*,[17] demonstrated that transgenic callus could be obtained from peach leaves, stems, sexual embryos, and somatic embryos using an *Agrobacterium* vector. Smigocki and Hammerschlag[18] reported the development of transgenic peach plants having the *ipt* gene through *A. tumefaciens*. These plants were regenerated from seed-derived somatic embryos.

The impacts of transformation are depended on the isolation of useful genes and the ability to transfer these genes into peach cultivars. Genes of potential benefit to plants have been isolated from microorganisms. Such genes include those that alter growth habit isolated from *Agrobacterium rhizogenes*[14,19], insecticidal protein genes from *Bacillus thuringiensis*[6], or coat protein genes from plant viruses[1]. Genes active during peach fruit development, as in the ripening and softening stages have been isolated and partially or fully sequenced. Genes that have been identified include those for ethylene odxidase[2,3,4] and endopolygalacturonase[11]. The use of these genes and other fruit-ripening genes in antisense transformation studies could allow for the direct manipulation of fruit maturity and softening[3,4,5]. Studies are aimed to develop an *Agrobacterium* – *mediated* gene transfer system for peach as reported by Hammerschlag *et al.,*[8].

The ultimate goal of this work is to establish a reliable and stable transformation system for peach plant cultivar (El- El-Sheikh Zewaied) grown in North Saini. The first step to achieve this goal was to introduced *bar* and *gus* genes into plant expression

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vector downstream of 35S CaMV promoter and to carry out the transformation by *Agrobacterium*-mediated technique and biolistic bombardment gene transfer methods. Genomic Southern blot hybridization and PCR strategy were performed to confirm the integration of the genes into peach genome.

MATERIALS AND METHODS

Transformation System of Peach [(*Prunus persica*(L.) Batsch]: The herbicide resistance of non-transformed peach tissues was determined, by planting embryonic axes slices on Murashige and Skoog[13] MS salts basal medium with different concentrations, i.e., 0, 1, 2, 3, 4 and 5 mg/l of Bialaphos phosphinothricin (PPT), 5 explant for each concentration. The embryonic axis medium also contained 20 µM benzylaminopurine (BAP) and 0.25 µM naphthalene acetic acid (NAA) to stimulate cell proliferation. Bialaphos was sterilized by filtration through disposable filters (0.22µn) and incorporated into pre-cooled (45-50˚C) autoclaved medium.

Transformation *via Agrobacterium*: Embryonic axes extracted from mature seeds were slices (0.5-1 mm) and collected in sterile petri dishes under aseptic conditions. Slices were immersed for 1 hr in resuspended culture of *Agrobacterium tumefaciens* strain AgL0 containing the PCGP1258 plasmid (DNA plasmid has been kindly offered by ICARDA which contains *bar* and *gus*-intron genes Fig. 1). After incubation, the excess bacteria was blotted on sterile filter paper and the slices were spread out onto the co-cultivation medium [Shoot Regeneration Medium (SRM) without antibiotics]. After 2 days at 28±1°C in the dark, the explants were washed in a sterile solution of half strength MS medium with 250 mg/l cefotaxime and blotted briefly on sterile filter paper. Five slices were planted on SRM medium consisted of the basic MS medium supplement with 3 % sucrose, 1ml of MS vitamin mixture, 100 mg/l myo-inositol and 2 g/l Phytagel. The growth regulators used to induce shoot regeneration from mature embryo slices were 20 µM benzylaminopurine (BAP), 0.25 µM NAA and 3 mg/l Bialaphos and 250 mg/l cefotaxime sodium salt. Incubation was carried out at 25±1°C in the dark for 30 to 45 days till shoots were formed and then transferred into fresh Shoot Regeneration Medium (SRM) containing 3 mg/l bialaphos and 250 mg/l cefotaxime sodium salt. The cultures were kept in the growth chamber at 26±1°C.

Transformation *Via* Biolistic Bombardment System: Explants Preparation: The embryo axes from mature seeds were longitudinal dissected into two sections and cultured into the middle of plats containing the regeneration medium. In order to develop the optimum bombardment protocol, the distance of explants in relation to carrier membrane (6 and 9 cm) and the helium pressure (1100 and 1350 psi) were studied. Each treatment has 5 replicates, the number of explants in each treatment was 50 embryo discs per plate. Osmotic treatment as another factor was studied by adding the mannitol at two concentrations of 0.2 and 0.4 M. The explants were cultured on the mannitol medium and mannitol-free medium used as a control and incubated for two days or for one week in other treatment.

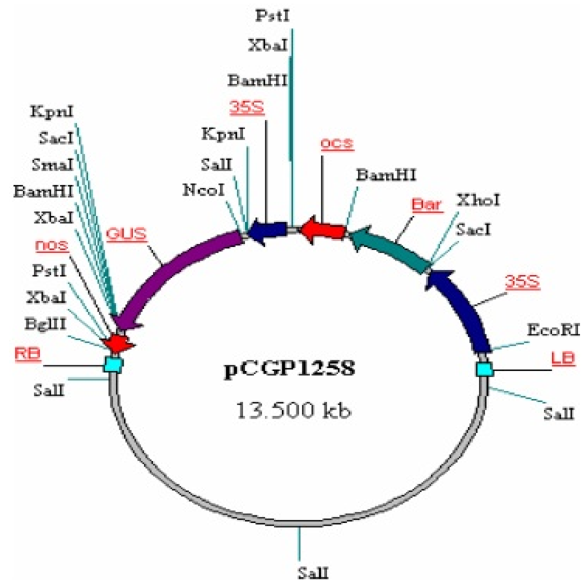


Fig. 1: pCGP1258 binary vector, used in peach transformation, containing the bar and gus genes.

Coating of Gold Particles with DNA: Half ml absolute ethanol was added to thirty mg of gold particles (1.0 µM) and vortexed at high speed for 1-2 min. Spin down at 10,000 rpm for 10 sec was performed. Previous step was repeated 3 times. The supernatant was removed and 0.5 mL of sterile water was added.

Under sterile conditions, 5 µl of DNA (1µg/µl) of the plasmid pCG1258 was added to 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. The mixture was vortexed for three min and spun down 10 sec. The supernatant was then removed as much as possible followed by adding a volume of 250 µl of absolute ethanol, vortexing, spinning down and removing the supernatant. Thereafter, the gold coated with DNA was re-suspended again in 70 µl of absolut ethanol. Ten µl of this mixture per shot were used for transformation.

Selection and Regeneration of Transgenic Shoots: The bombarded explants were incubated for 2 days, there after; they were transferred to regeneration medium containing Bialaphos compound. The active ingredient of bialaphos is glufosinate ammonium. Plates were kept in the dark for 3 days, and then transferred into the light condition for 4 weeks. Subsequently, produced shoots were transferred to the elongation medium for another 4 weeks.

Detection of *bar* gene:

PCR Confirmation: DNA of transformed as well as non-transformed plant materials was extracted following the methods reported by Xu, *et al.*[21]. The target DNA sequence was detected using primers (*bar* 1), as forward primer 5` GCAGGAACCGCAGGAGTGG A 3` and reverse primer of (*bar* 2) 5` AGCCCGATGACAGCGACCAC 3`. Thirty five cycles of amplification were carried out under the following conditions, denaturation at 95°C for 30 sec, Annealing at 66°C for 90 sec and extinction at 72°C for 2 minute. An aliquot solution of 10 ml PCR product were analyzed on 1.3% agarose gel. The *gus* gene was detected by the same approach using *gus* primer (1) 5` CCTGTAGAAACCCCAACCCG 3` as a forward primer and *gus* primer (2) 5` TGGCTGTGACGCACAGTTCA 3`as a reverse primer.

Southern Blot Hybridization: PCR was run on 1% agarose gel in TBE buffer. After fractionation, gels were soaked in 25M NaOH/1.5M NaCl for 20 minutes. DNA was blotted into Nylon membranes (Boehringer Mannhein) for 3 hours using 0.25M NaOH/1.5M NaCl as transfer buffer. After blotting, filters were pre hybridized and hybridized using a random labeled *Bar* gene probe labeled with DIG-random primer labeling (Boehringer Mannhein) after the manufacturer's protocol.

Histochemical *Gus* Assay: Transformation was conformed by the *gus* assay in different stages of the experiment according to Jefferson[10]. The 5-bromo-4-chloro-3-indolyl β-D-glucuronide (x-Gluc, Sigma) was used as the chromogenic agent. The transient expression of the *gus* gene was detected in the bombardment mature embryos by performing the *gus* histochemical assay on 10 embryos (chosen at random) from each bombardment plate, after 48 hr of each bombardment experiment. The total number of embryos tested was 270 embryos, the embryos were incubated with 500 µl GUS assay buffer in darkness at 37˚C for 20 hours and the number of transient signals (blue spots) was determined under the binocular-stereomicroscope.

RESULTS AND DISCUSSIONS

Herbicide Bialaphos Sensitivity: Prior to transformation it is necessary to study the sensitivity of growth and differentiation of *P.persica* tissues to bialaphos (glufosinate ammonium), which was used in the establishment of transformation through selection of transformed shoots. To determine the optimum concentration of bialaphos for the selection of transformed peach shoots, a kill curve experiment was carried out using non-transformed embryonic axes of El-Shiekh Zewaied peach cv.

Embryonic axes explants were cultured under the same conditions which were used for regeneration. Six selective media were prepared by adding filter sterilized 1mg/ml (active ingredient) stock solution of glufosinate ammonium to autoclaved Shoot Regeneration Medium (SRM), to reach a final concentration of 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0mg/l (Table 1 and Fig. 2). Results showed that increasing in the Bialaphos concentrations were accompanied by decrease in the percentage of the survival explants. The lethal dose of Bialaphos was estimated at 3mg/l. Where, 0.0, 1.0 and 2.0mg/l of Bialaphos were recorded 33.3, 13.3 and 6.6%, respectively, and no survival was observed when 3, 4 and 5mg/l Bialaphos were used (Table 1) and Fig. 2). The concentration of 3mg/l Bialaphos was then chosen as a selection marker for transformed tissues in the El-Sheikh Zewaied cv.

Transformation *via Agrobacterium*: *Agrobacterium tumefaciens* AgL0 harboring the *bar* and *gus* genes was co-cultivated with embryonic axes tissues. The transformed embryonic cells were transferred into selective medium supplemented with 3mg/l Bialaphos (selective herbicide) and 250mg/l cefotaxime to inhabit *Agrobacterium* over growth on the medium. Herbicide resistant transformed embryonic cells with *bar* gene (herbicide resistant gene) were capable to survive by detoxifying bialaphos and were able to grow and maintained on the selective medium. Shoot initials developed along the edges of the embryonic cells after 6-7 weeks. The transformation efficiency based on the number of transgenic regenerates per inoculated explant was 3% (Table 2). The number of shoots surviving seven weeks post-co-cultivation swas 12 shoots. Table (2), showed the survival percentage of El-Sheikh Zewaied peach cultivar before and after treatment with *Agrobacterium* on the regeneration medium. It was obvious that El-Sheikh Zewaied has a higher number of survival tissue (71%) on the control medium compared to medium supplemented with bialaphos as a selectable agent (3%), it was also clear that the co-cultivation with the bacterium considerably decrease the survival rate of the tissue on the medium regardless of

Table 1: Determination of the lethal dose of herbicide Bialaphos.

Concentration of (Bialaphos) PPT mg/l	No. of Explant Used / each treatment	No. of Explants shooted after 4 weeks	Survival % after 4 weeks
0.0	15	5	33.3
1.0	15	2	13.3
2.0	15	1	6.6
3.0	15	0	0
4.0	15	0	0
5.0	15	0	0

Table 2: Regeneration and transformation percentages of El-Sheikh Zewaied peach cultivar.

Cultivar	Treatment	Embryo Survival %	Shoot %	Transformation efficiency %
EL-Sheikh	Control	71	79.86	-
Zewaied	*Agrobacterium* Strain AgL0	3	3.75	3

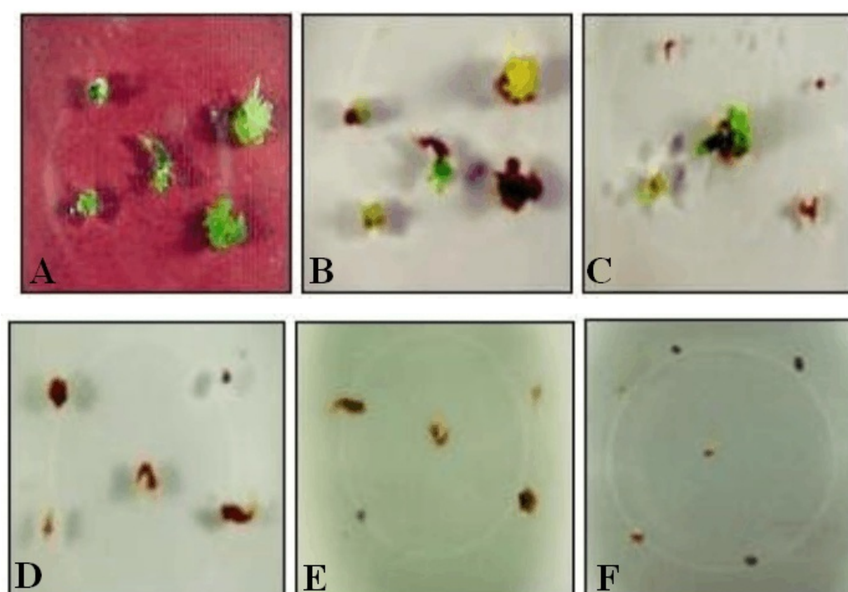


Fig. 2: Shoots proliferation of the peach mature embryo explants on different concentration of Bialaphos (PPT). A= MS+0mg/l PPT, B= MS+1.0mg/l PPT, C= MS+2.0mg/l PPT, D= MS+3.0mg/l PPT, E= MS+4.0mg/l PPT and F= MS+5.0mg/l PPT

the genotype of the plant. From previous results, it was concluded that regeneration percentage was high in El-Sheikh Zewaied peach cultivar (79.86%). while, the transformation efficiency (3%) was low. Transformation percentage was similar to the results obtained by Gonzalez *et al.*[7] in plum plants. Results indicated that *gus* activity in transformed peach explants (embryonic axes slices) which co-cultivated with *Agrobacterium* could be detected histchemicaly for *gus* expression after 2 days as the first evidence of transformation. Most of the tissues turned to deep blue color compared with control (Fig. 3). Transformed embryonic cells were incubated at 37°C in the *gus* buffer with β-glucuronidase for 24 hr. These results are in agreement with other reports by different researchers [12].

Effect of Mannitol on Transformation Efficiency: This experiment was performed to determine the effects of different concentrations of mannitol (0, 0.2 and 0.4M), as an osmotic reagent during bombardment on the transformation efficiency and stable expression of *gus* and *bar* genes of El-Shiekh Zewaied peach cv. The mature embryos were incubated for 4h before and 16h after bombardment on osmotic containing medium which consisted of Shoot Regeneration Medium (SRM) containing different concentrations of mannitol.

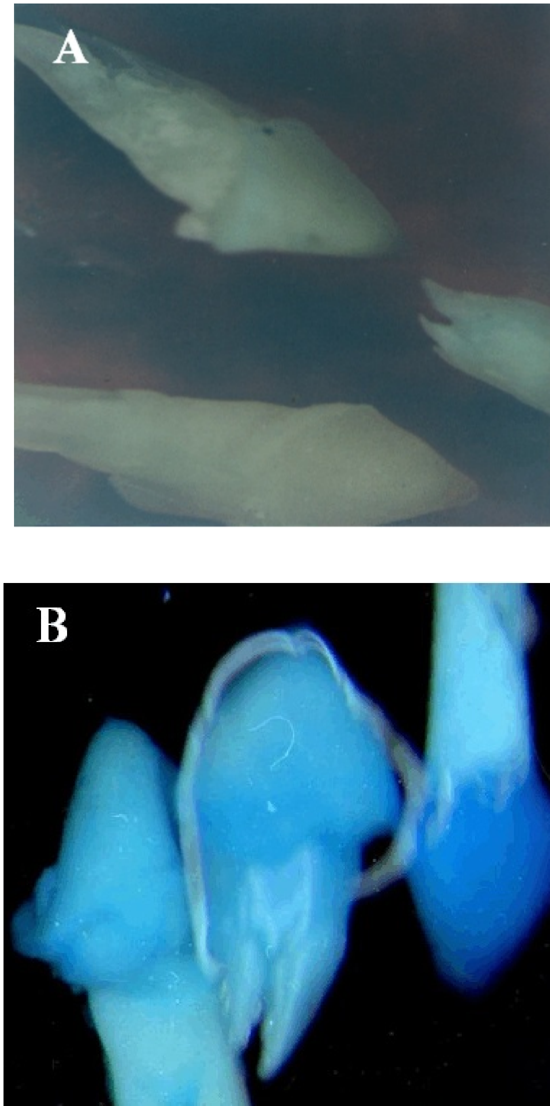


Fig. 3: Histochemical assay of ß-glucuronidase (*gus*) gene in embryonic axes explants of El-Sheikh Zewaied peach cultivar transformed via Agro bacterium tumefactions.
A) None transformed explants
B) Transformed explant of El-Sheikh Zewaied cv. showing blue color

Comparisons between treatments showed that, there are significant differences across osmotic pressures and days after bombardment. As the concentration of osmotic increases, the number of blue cells increases, until it reached at 0.4 M of mannitol, at this concentration, the number of blue cells was doubled compared to the control Table (3). Also, there are significantly different regard to number of days after

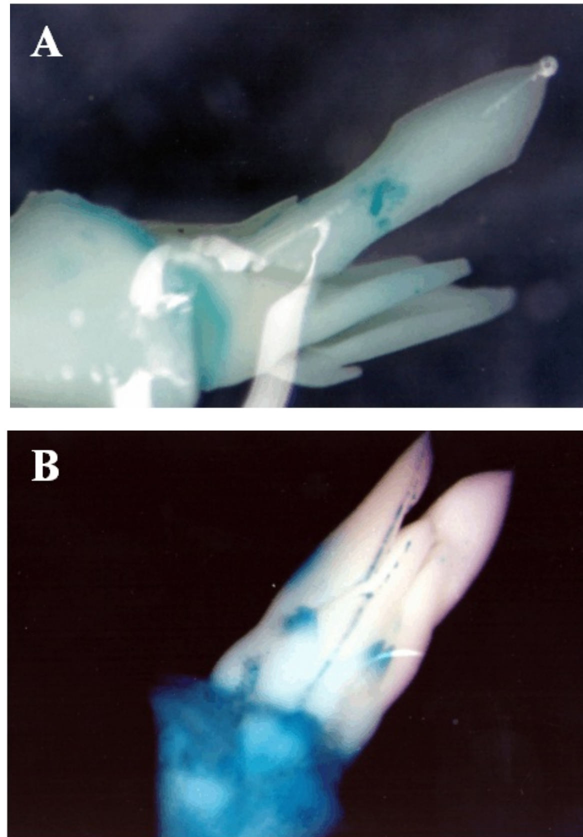


Fig. 4: Transient gus gene expression in transformed mature embryos.
A: Without osmotic treatment
B: With osmotic treatment.

bombardment in their number of blue spots /embryo, it was shown that number of blue cells/embryo after two days was significantly higher than that after seven days across osmotic pressures. The comparison within different osmotic pressures indicated that number of significant difference between days after bombardment. In other wards, it can be concluded that osmotic treatment resulted in higher cell competence to foreign DNA (*gus* -containing plasmid) (Fig. 4). Xiaojian *et al.*[20] reported that the number of blue cells increased when 0.25 or 0.5 M mannitol was added to the medium.

Effect of Different Bombardment Pressures on Transformation Efficiency: Results in Table (4), indicated that the influences of different acceleration pressures, on the number of blue spots / embryos. Pressure1100psi gave the highest numbers of blue spots compared with 1350psi. The number of blue spots after 2 and 7 days from shooting by 1100psi pressure was high compared with 1350psi. It could be suggested that

Table 3: Effect of different concentrations of mannitol and time after bombardment on transformation efficiency.

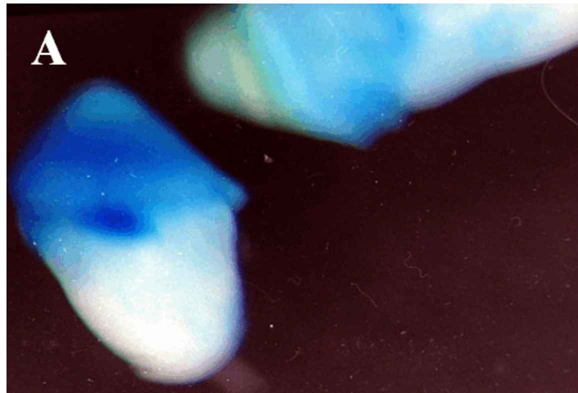
Days after bombardment	Osmotic treatments(Mannitol concentrations)			Pressure means
	0 mg	0.2 mg	0.4 mg	
2	23.66c	31.33b	46.6a	33.86
7	14.66c	19.00b	25.00a	19.55
Day means	19.16	25.16	35.8	

Means having the same alphabetical letters within each column are not significantly different at the 0.05 level, according to Duncan's multiple range test.

Table 4: Effect of different bombardment pressures and time after bombardment on transformation efficiency of peach.

Days after bombardment	Pressure (psi)		Pressure means
	1100	1350	
2	48.32a	46.10b	47.21
7	49.66a	45.72b	47.69
Days means	48.99	45.91	

Means having the same alphabetical letters within each column are not significantly different at the 0.05 level, according to Duncan's multiple range test.



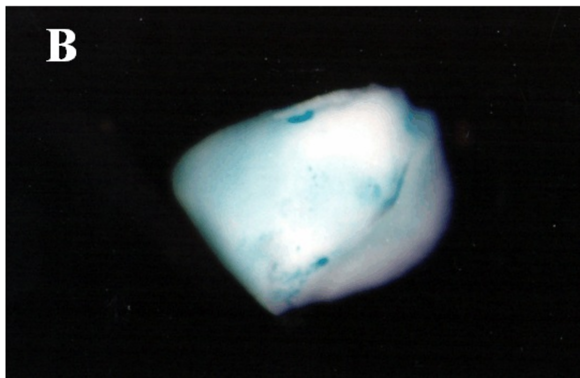


Fig. 5: Transient gus activity 24 hours post-bombardment of mature peach embryos under different bombardment pressures.
A: *gus* pressure 1100 psi
B: gus pressure 1350 psi

the decrease in the number of blue spots after 7 days under 1350psi from bombardment, resulted from a higher penetration of the DNA coated particles which damaged a large number of cells.

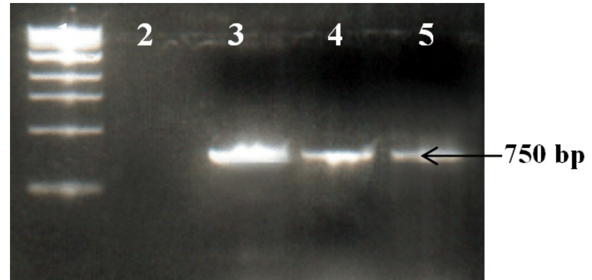


Fig. 6: PCR product of gus gene amplifying partial length (750bp) in putative peach embryonic axis tissues transformed *via Agrobacterium*.
Lane 1: 1 Kb DNA ladder (Marker)
Lane 2: Non transformed peach lines (negative control)
Lane 3: plasmid pCGP1258 (positive control)
Lanes 4 to5: putative transformed embryos tissue

From this experiment we may concluded that, it was very important to avoid tissue damage from bombardment by reducing the pressure as much as possible. Our results showed that the use of 1100 psi and 1350 psi with two shots gave the highest number of transient *gus* signals (Fig. 5).

Detection of the Genes (*Gus* and *bar* genes):
PCR analysis:
Transformed Tissues by *Agrobacterium*: As shown in Fig. (6) The amplified fragments for *gus* in lane (3-4) have the expected size of coding region of *gus* gene (750bp), lane (1) positive control reflected amplified plasmid pCGP1258 and lane (2) negative control of non-transformed line. Fig. (7) shows the presence of *bar*-DNA fragment at expected molecular weight (264bp) in lanes (3-4), lane (1) positive control and lane (2) negative control of non-transgenic peach tissues.

Transformed Tissues by Bombardment: Results in Fig. (8) revealed the amplified fragments for *gus* in lane (3-4) which have the expected size of coding

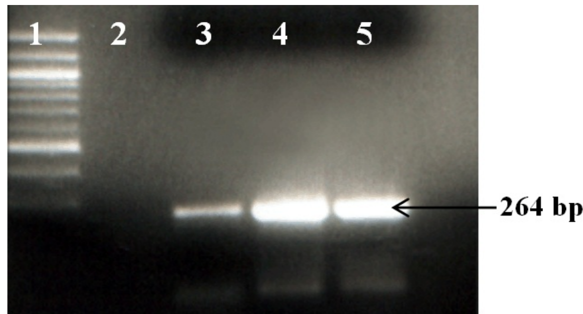


Fig. 7: PCR product of bar gene amplifying partial length (264bp) in putative peach tissues transformed *via Agrobacterium*.
Lane 1: 100 bp DNA ladder (Marker)
Lane 2: Non transformed peach lines (negative control)
Lane 3: plasmid pCGP1258 (positive control)
Lanes 4 to 5: putative transformed embryos tissue

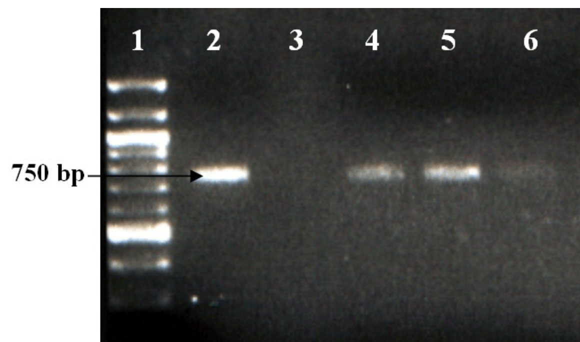


Fig. 8: PCR product of *gus* gene amplifying partial length (750 bp) in putative peach embryonic cells transformed *via* Biolistic gene gun. The arrow indicates the amplified for fragment.
Lane: 100 bp DNA ladder (Marker)
Lane 2: plasmid pCGP1258 (positive control)
Lane 3: Non transformed peach lines (negative control)
Lanes 4 to 6: putative transformed embryos tissue

region of *gus* gene (750bp), lane (1) positive control resulted in amplified plasmid pCGP1258 and lane (2) negative control (non-transformed lines). Fig. (9) shows the presence of *bar*-DNA fragment at expected molecular weight (264bp) in lanes 3-4, lane (1) positive control (plasmid pCGP1258) and lane (2) negative control (non-transgenic lines).

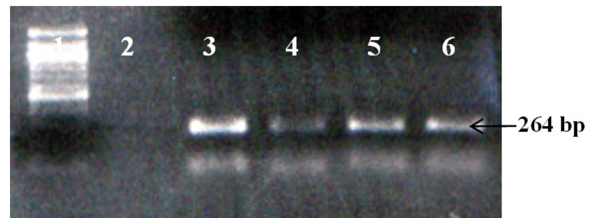


Fig. 9: PCR product of *bar* gene amplifying partial length (264 bp) in putative peach embryonic axis tissues transformed via Biolistic gene gun. The arrow indicates the amplified for fragment.
Lane 1: 100 bp DNA ladder (Marker)
Lane 2: Non transformed peach lines (negative control)
Lane 3: plasmid pCGP1258 (positive control)
Lanes 4 to 6: putative transformed embryos tissue

Therefore, it is evident that both *gus* and *bar* genes were physically present in the genomic background of the transformed peach tissues. Using of PCR as indicator for the presence of foreign genes into transformed peach tissues has been reported by many investigators[18,20]. They reported that PCR analysis confirmed in most of the putative transformats, that the chimeric *gus*/*npt II* gene had been incorporated into the genome of peach cells transformed by particle bombardment

Gonzalez *et al*.[7] reported the stable integration of the *npt II* and *gus* genes into the genome of plum plants transformed with plasmid pBISNI, pGA482GG or pGA428GGi and tested by PCR analysis.

To confirm the integration of the *bar* gene (Fig. 10, A), the positive PCR products for *bar* gene obtained from the transformed peach tissues (lanes 4-5 transformed by Biolistic and lanes 6-8 by *Agrobacterium*), were subjected to Southern blot hybridization using a probe which prepared by digesting the recombinant plasmid pCGP1258 with *BamHI* and *XhoI* restriction enzymes to releases the *BamHI* fragment (1.91 Kb) of pCGP1258 which containing *bar* gene cassette, this fragment was labeled with the digoxigenin non-radioactive labeling and detection kit.

As shown in Fig. (10, B) DNA hybridization analysis revealed that the *bar* cassette probe hybridized with the PCR products lane 3-7, as well as the positive control (pCGP1258 plasmid), while no hybridization occurred with the negative non-transformed tissues. These results indicated that the *bar* gene integrated into the five positive *bar* events.

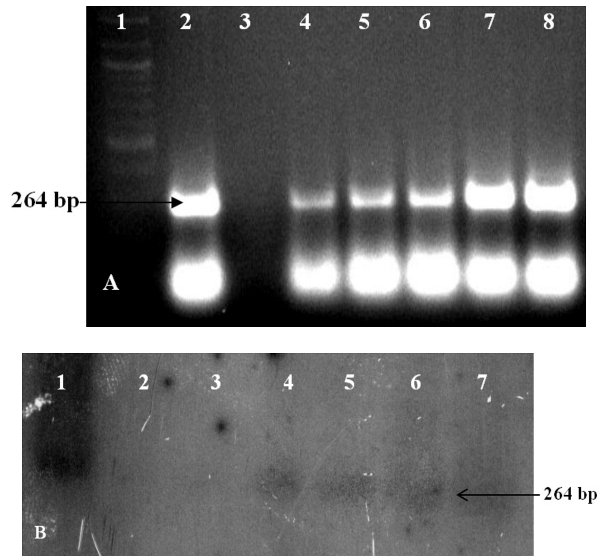


Fig. 10A: PCR involving the *bar* gene (264bp) of the five transformed EL-Sheikh Zewaied peach tissues (4-5 transformed by biolistic and 6-8 transformed by *Agrobacterium*).
Lane 1: Marker 100bp DNA
Lane 2: pCGP1258 plasmid (positive control)
Lane 3: Non transformed peach line (negative control)
Lanes 4-5: peach embryonic tissues transformed by biolistic
Lanes 6-8: putatively transformed peach shoots by *Agrobacterium*

B: Southern blot analysis of PCR involving the bar gene (264bp) of the five transformed EL-Sheikh Zewaied peach tissues indicating the integration of the bar gene in peach genome (1-2 transformed by biolistic & 3-5 putatively transformed peach shoots by *Agrobacterium*) against a 1900 bp bar fragment used as a probe.
Lane 1: pCGP1258 plasmid (positive control)
Lane 2: Non transformed peach line (negative control)
Lanes 3-4: peach embryonic tissues transformed by biolistic
Lanes 5-7: putatively transformed peach shoots by *Agrobacterium*

Results were in agreements with the reports of many investigators who studied the integration of the transgenes in the plant genomes using Southern analysis. Hauptmann *et al.* [9] reported that the Southern blot hybridization confirmed stable integration of DNA plasmid into *T. monococcum* using hygromicin vectors and *P. maximum* using the methotrexate vectors. Gonzalez *et al.*[7] reported the stable integration of the *npt II* and *gus* genes into the genome of plum plants transformed with plasmid pBISNI, pGA482GG or pGA428GGi using Southern blot analysis.

The success of marker gene transformation carried out through this work could offer an opportunity to transfer specific genes related to quality enhancement into peach germplasm.

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