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High efficiency *Agrobacterium*-mediated transformation of sour orange (*Citrus aurantium* L.) using gene encoding Citrus Tristeza Virus coat protein

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Abstract

Citrus trees are widely grown in tropical and subtropical climates due to their luscious taste, nutritional and medical benefits. Citrus fruits are native to southeastern Asia and are among the oldest fruit crops domesticated by humans. Breeding programs including the incorporation of genetic resistance to insect pests and diseases are necessary in this crop. Citrus tristeza virus (CTV) is of particular importance due to its rapid epidemic resulting in severe plant damage. The present research aimed to transform *Citrus aurantinum* with a gene encoding coat protein of CTV through *Agrobacterium*-mediated transformation. The *p25* coat protein gene was isolated from two native CTV isolates. Two conserved regions from the two isolates, were identified and subcloned as a single chimer into a pFGC5941 silencing vector. Epicotyls-originated explants of *C. aurantium* were transformed by EHA105 strain of *Agrobacterium tumefaciens*. Some of the effective factors in gene transformation were examined by inoculation methods with *Agrobacterium* such as Acetosyringon effect (0, 50, and 100 µM), inoculation time (5, 10, 15, 20, and 25 min), and co-cultivation period (1, 2, 3 and 4 days). Based on our results, maximum number of transformed plants (13.7%) were obtained under combined treatment of 50 µM acetosyringone after 15 min inoculation time and 2 days of co-cultivation with *Agrobacterium*. One of the advantages of the current protocol is regeneration of explants through direct organogenesis which avoid callus phase and consequently somaclonal variation.

Key words: Acetosyringon, *vir* gene induction, virus-induced gene silencing, Citrus tristeza virus

Introduction

Citrus, belonging to the family Rutaceae, is one of the world's most important fruit crops. Regarding the fact that world-wide production of citrus fruits is about 115 million tons (FAO, 2007) breeding programs are important for developing superior varieties for increasing the production and quality of the fruit. However, conventional breeding in Citrus species faces several limitations such as high heterozygosity, apomixis, self-incompatibility, and long juvenile period (Grosser and Gmitter, 2005). As a result, *Agrobacterium*-mediated transformation has been considered by various researchers. Hidaka *et al*. (1990) were the first to produce Washington and Trovita transformed orange using *A. tumifaciens*. This method was then used for transformation of Carrizo citrange (Moore *et al*., 1992; Kaneyoshi *et al*., 1994). This approach was later improved using changes in some factors such as inducing longitudinal incision (Yu *et al*., 2003), using GFP marker (Ghorbel *et al*., 1999) and Acetosyringone (AS; Gelvin, 2000). Yet, the efficiency of citrus transformation has always been reported low. Some of the underlying reasons reported are: low rate of transformed plants rooting, adverse effects following transformation (Gutierrez *et al*., 1997), and gene escape. Among citrus, *Citrus aurantium* is taken to be one of the species, resistant against genetic transformation and manipulation.

Citrus tristeza virus (CTV) is the main viral diseases of citrus. This disease, so far, has ruined millions of citrus trees (Whiteside *et al*., 1988). *C. aurantium* stalk and the presence of *Aphis gossypii* – CTV vector – are the potential factors of outbreak of this disease in citrus areas of Iran (Barzegar *et al*., 2006). Due to its potential growth in saline and limy soils and to create desirable taste and flavor in fruits, *C. aurantium* is among suitable rootstocks for citrus cultivation. Nevertheless, this rootstock is especially susceptible to *Citrus tristeza virus*. It has been shown that substituting other rootstocks for *C. aurantium* results in the reduction of fruit yield and quality (Ghorbel *et al*., 2000). Accordingly, *C. aurantium* genetic transformation for further resistance against CTV becomes considerably important in breeding programs. Some citrus genotypes are successfully transformed to induce resistance against CTV including, *C. aurantium* (Gutierrez *et al*., 1997; Ghorbel *et al*., 2000), *C. aurrantifolia* (Dominguez *et al*., 2000), *C. paradisi* (Febres *et al*., 2003), *C. sinensis* (L.) (Muniz *et al*., 2012), and *poncirus* (Zou *et al*., 2008).

In present study, *C. aurantium* was transformed by a silencing vector, encoding part of 2 *CTV*-P25-CP genes to generate transgenic sour orange with induced constitutive resistance against CTV isolates.

Materials and methods

CTV isolates and bioinformatics analysis: The nucleotide sequences of *CTV-CP-p25* gene from two native isolate (provided by Plant Virology Research Center; Shiraz, Iran) were used in this study. For this purpose, 10 *CTV-CP-p25* sequences, which were obtained from different native isolates in this center were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and further analysed by clustalW2 phylogenic program (http://

www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). This was aimed to find 2 sequences with maximum difference in this set. At the same time, Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied to search for *CTV* sequences in database similar to Shiraz sequences. It was intended to determine sequences from Shiraz set with maximum homology with *CTV* identified sequences in database. As a result, among 10 *CTV* isolates collected from southern Iran, two sequences (*CTV1* and *CTV2*) were selected. These 2 sequences, cloned in PTZ57R/T plasmid, had minimum homology with each other and maximum homology with the existing data in gene bank.

CTV-1 and *CTV-2* sequences were amplified using specific forward and reverse primers (Table 1). Forward CTV1 and reverse CTV2 primers carried *Xho*I restriction site. For *CTV-CP-P25* amplification, reactions were carried out at: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, for 30 cycles, plus a final extension at 72 ºC for 2 min. Amplified DNA was detected by ultraviolet illumination after electrophoresis on 2% (w/v) agarose–ethidium bromide gels. PCR fragments were digested by *Xho*I enzyme according to manufacturer's manual (Thermo Scientific). Two pieces were ligated using T4 enzyme (Thermo Scientific) based on Sambrook and Russell (2001) instruction and and a DNA fragment of about 400 bp was resulted. After purifying the respective (CTV1 and CTV2 CP genes) mosaic fragment, it was used as template in further PCR reactions (Zamani, 2009).

Synthesis of a mosaic inserts (P1 and P2): The resulting mosaic fragment was used as a DNA template for producing 2 inserts and their cloning into the silencing vector. In this section, a 340 bp fragment of DNA template was amplified using F-mosaic and R-mosaic primers (Table 1). Each primer contained 2 different restriction sites, which allowed directional cloning yet opposite insertion into the silencing vector. The inverted repeat is assembled directly in a binary vector by a 2-step cloning process using the introduced restriction enzyme sites. In the first cloning step, the PCR product is cleaved at the inner restriction sites, *Asc*I and SwaI, and ligated to cleaved *Asc*I and *Swa*I sites in pFGC5941. The cloned fragment was sequenced using gene specific primers to confirm their identity. In the second step, the resulting PCR product is cleaved with *BamH*I and *Xba*I and inserted into the *BamH*I and *Xba*I-cleaved template plasmid based on manufacturer manual (Thermo Scientific) and named as P1 and P2, respectively. This yields an inverted repeat separated by the *ChsA* intron.

Silencing vector characteristics: pFGC5941 vector (GenBank: AY310901.1) was a gift from Arabidopsis Biological Research center (ABRC; CD3-447). It is a binary vector specific to dicotyledonous plants, and gene transfer is based on ligase and restriction enzymes. Selection markers for bacteria as well as plant are kanamycin and basta (glufosinate-ammonium), respectively. Its dsRNA promoter is CaMV35S and its spacer is *ChsA* intron with repetitive reverse sequence (Fig. 1).

Confirmation of CTV mosaic insertion in silencing vector: First, the enzyme double digestion of pFGC5941 silencing vector (ABRC) was carried out using *BamH*I and *Xba*I restriction enzymes based on manufacturer manual (Thermo Scientific) to link P2 gene. In the next stage, silencing vector containing P2 fragment underwent enzymatic digestion by *Asc*I and *Xba*I and P1 fragment ligated as described for P2 fragment based on Sambrook & Russell (2001). Finally, the construct was transformed into *E. coli* cells using electroporation method (Bio-Rad). Evaluating and examining colonies and transformed bacteria cultures were conducted by the aid of PCR. Plasmid was minipreped from positive colonies

Table 1. Characteristics and sequence of the primers used in cloning and PCR analysis of the transformants

Primers	Sequences	Restriction sites
F-CTV1	5'- CGCCTCGAGGCTAAACGATACAACATCATAGC- 3'	*Xho*I
R-CTV1	5'- GCTAAACGATACAACATCATAGC -3'	-
F-CTV2	5'- CCACTTCAATACCCTCCCG -3'	*Xho*I
R-CTV2	5'- CGCCTCGAGCGACTCTGATAGCGATGAACG- 3'	-
F-mosaic	5'- GCTCTAGAGGCGCGCCTGCTGCTGAGTCTTCTTTCG -3'	*Xba*I, *Asc*I
R-mosaic	5'- GCGGATCCATTTAAATCCGTGGTGTCATCATCACTT -3'	*Swa*I, *BamH*I
BAR-F	5'-GAAGTCCAGCTGCCAGAAAC-3'	-
BAR-R	5'-AGTCGACCGTGTACGTCTCC-3'	-
CTV-F	5'-CGCCATGGACGAAACAAAG-3'	-
CTV-R	5'-CCACTTCAATACCCTCCCG-3'	-
KAN-F	5'-ATGTTGCTGTCTCCCAGGTC-3'	-
KAN-R	5'-GAAAGCTGCCTGTTCCAAAG-'	-

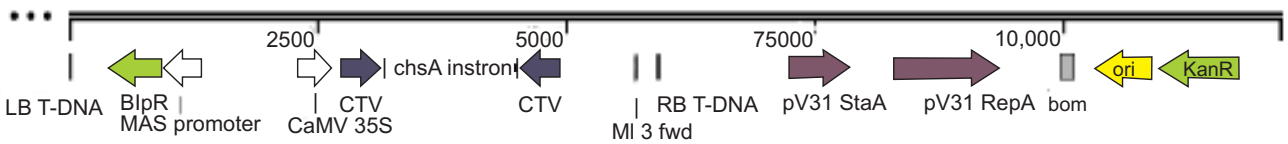

Fig. 1. Schematic representation of the T-DNA from the binary Plasmid pFGC5941, engineered to carry double copies of the gene encoding coat protein from CTV: LB: left border repeat from nopaline C58 T-DNA; a kanamycin resistance (kan^R) gene for bacterial selection, a basta resistance (*BAR*) gene for plant selection, a CaMV 35S promoter to drive the expression of the inverted repeat target sequence, and a 1,352 bp *Chs*A intron (from the petunia *Chalcone Synthase A* gene) to stabilize the inverted repeat of the target gene fragment. RB: right border repeat from nopaline C58 T-DNA (the map was created by SnapGene).

(Bioneer). Accordingly, 2 viral DNA mosaic fragment with 340 bp length and similar sequences were integrated in 2 different parts of silencing vector and in 2 opposite directions. Chalcone synthase intron (~1400bp) is located between these 2 pieces (Fig. 1).

To further confirm the cloning of P1 and P2 inserts, double enzymatic digestion reactions was performed. Namely, extracted silencing plasmid containing respective pieces underwent enzymatic digestion either by *Asc*I and SwaI or *BamH*I and *Xba*I in separate reactions. In both reactions, it was expected that a piece of destination silencing vector with ~400bp length to get separated and observed on gel.

To further confirm the cloning mosaic fragment in destination vector, the same pFGC591 silencing construct was digested by *Asc*I and *Xba*I. As a result, it was expected to detect a 2200bp piece including chalcone synthase intron plus 2 ~400 bp mosaic fragment on gel.

Plant material: Ripe citrus fruits were provided from Ramsar Citrus Research Center (Ramsar, Iran). Seeds were collected and disinfected using sodium hypochlorite solution (2.5%) and rinsed 3 times by sterilized distilled water. The disinfected seeds were cultured in Murashige and Skoog (MS; Duchefa) medium and were kept for 4 weeks in darkness followed by 10 days under 16 h-daylength at 28 °C (Almedia *et al*., 2002).

To prepare explants and carry out transformation, *in vitro* grown epicotyls were cut in 1 cm pieces. A 1 mm longitudinal incision was made in both ends of the explants. A 1-mm longitudinal incision was made at both ends of the explants and then the explants were transferred to liquid pre-culture medium containing MS salts, B5 vitamin, 500 mg/L malt extract, 2.5 mg/L BAP, 0.05 mg/L 1-Naphthaleneacetic acid (NAA; Sigma), 4.7% w/v sucrose (Merck), and pH was adjusted to 5.8. They were slowly stirred for 2 days in darkness at 28 °C.

***Agrobacterium* preparation:** *Agrobacterium tumefaciens* (EHA105 strain) was transformed by electroporation to receive recombinant plasmids. *A. tumefaciens* EHA 105 is a disarmed derivative of *A. tumefaciens* A281, which is supervirulent in citrus (Cervera *et al*. 1998b). To select transformed bacteria, they were cultured on solid yeast extract peptone dextrose (YEP) medium containing 100 mg/L kanamycin, 50 mg/L rifampicin, 7 g/L agar and pH 6.8 at 28 °C for 48 h. A colony was cultured in liquid YEP medium with the same concentrations of antibiotic for 16 h at 28 °C. Bacterium suspension with an OD_{600} = 1 was centrifuged at 6000 rpm for 5 min. Its pellet was dissolved in liquid MS medium containing 0, 50, and 100 µM Acetosyringone (AS; 3,5-methoxy-4-hydroxyacetophenone; Sigma). The same concentration of AS were also applied in co-cultivation period.

Transformation, selection and regeneration: Epicotyl-originated explants were submersed in MS inoculation liquid containing *Agrobacterium* for 5, 10, 15, 20, and 25 min at 28 °C. Subsequently, they were dried on filter paper so that extra bacteria are removed from explants. After inoculation, explants were cultured in co-cultivation medium including MS salts, B5 vitamin, 500 mg/L malt extract, 2.5 mg/L BAP, 0.05 mg/L NAA, 0, 50, and 100 µM AS, 4.7% w/v sucrose, 8 g/L agar, at pH 5.8. They were kept for 1, 2, 3 and 4 days in darkness at 28 °C.

After the end of co-cultivation period, explants were transferred to regeneration medium containing MS salts, B5 vitamin, 500 mg/L malt extract, 2.5 mg/L BAP, 0.05 mg/L NAA, 4.7% w/v sucrose, 8 g/L agar, and pH 5.8. To select transformed plant cells, 50 µM BASTA herbicide (Bayer Crop Science) and to remove *Agrobacterium* 500 mg/L cefotaxime were added to the medium. Media were placed in 16-h photoperiod for 5 weeks at 28 °C till 1 mm seedlings were observed on explants.

Explants were transferred to a shoot growth medium. This medium was applied to accelerate shoots longitudinal growth. It contained salts, MS vitamin, 3% w/v sucrose, 0.5 mg/L Gibberellic acid (GA_3), 8 g/L agar with pH 5.8. When shoots further grew, sampling was carried out for DNA extraction.

Transgenic shoot analysis: To verify transformation and evaluate its efficiency, DNA was extracted from young leaves and PCR was performed using 3 pairs of primers. Primers were related to herbicide-resistant genes (*BAR*) and *CTV* mosaic, which are located at T-DNA region of pFGC5941 Vector (Table 1). Besides, Kanamycin resistance gene (*kan*), located at the outside of T-DNA of the vector, was used in PCR to examine and determine whether positive results of PCR on tested genes were originated from *Agrobacterium* contamination of explants and shoots or from real transformed plant genome. Some PCR products were chosen randomly and sequenced using gene specific primers.

Seedlings DNA extraction: DNA extraction was carried out based on Edward *et al*. (1991) protocol. First, 20 mg of upper leaves were collected and grinded in 1.5 mL tube by liquid nitrogen. Then, 1 mL Edward buffer (200 mM Tris, 250 mM NaCl, 0.5% w/v SDS, 25 mM EDTA) was added to the grinded tissue. After 5s vortex, it was placed at room temperature for 2 min centrifugation for 2 min at 13000 rpm. 500 µL supernatant was transferred to new 1.5 mL tube, containing 500 µL cold isopropanol and kept at room temperature for 2 min before tube was centrifuged at 14000 rpm for 5min. When pellets dried, 50 µL TE buffer (10 mM Tris, 1 mM EDTA) was added to pellets. Samples were kept in freezer at -20 °C till being used.

Statistical Analysis: Only shoots positively identified by two PCR reactions to contain *CTV mosaic* and *BAR* genes along with negatively to *kan* gene were considered as transformed plant.

Statistical analysis was carried out in complete randomised block design in 3 replications (each in Petri dish with 6-8 explants). The experiment was carried out in an independent and stepwise approach. In each test, only 1 factor was examined independently. Actually, the best treatment was applied to next experiment. Data was analyzed using SAS 9.2 software. Means were compared by Duncan's Multiple Range Test.

Results and discussion

Confirming double integration of CTV Mosaic fragment in pFGC5941 Vector: As a result of performing PCR reactions by means of mosaic specific primers, 2 piece of ~200 bp from genes encoding *CTV1* and *CTV2* amplified, respectively (Fig. 2). Following amplification of the PCR products, double digestion and ligation performed. A piece of ~400bp length chimer was obtained when the ligation product used as a DNA template in PCR reaction. It proved mosaic fragment synthesis as well as ligation of *CTV-1* and *CTV-2* fragments (Fig. 3).

Further, recombinant silencing plasmid extracted from *E. coli* was double digested by *Asc*I and SwaI for P1 isolation as well

as *Xba*I and *BamH*I for P2 isolation in separate reactions. After electrophoresis of enzymatic digestions products, 400bp fragments (P1 and P2) observed from each reaction (Fig. 4).

In addition, enzymatic double digestion of recombinant silencing vector was performed by *Asc*I and *Xba*I. As a result, ~2200bp fragment was observed in the electrophoresis. This fragment included sum of 2 400bp mosaic from CTV and 1400bp pieces from chalcone synthase intron (data not shown).

The effect of acetosyringone concentration on transformation efficiency: In this test, applying 50 µM AS in inoculation and co-cultivation media resulted in 28 % shoot regeneration (14 out of 50 explants) from which, 9.3 % of them proved to be transformed. Applying 100 µM AS led to the bacterium overgrowth and consequently explants death. However, at this AS concentration, 1 out of 22 regenerated seedlings found to be transformed. There was no significant differences between 100 µM and 0 µM AS treatment regarding transformed shoots (Table 2).

Seedlings considered as transformed if only the PCR results of *CTV* and *BAR* genes were positive (Fig. 5A and 5B). A PCR reaction from each gene were chosen randomly and sequenced in order to prove the genuinity of PCR products.

The effect of inoculation time on transformation efficiency: In present study, 2-day co-cultivation led to the best results in producing transformed seedlings (13.7 %). In 3-day co-cultivation, number of transformed shoots versus total shoots resulted in the slightly lower figure compare to 2 days co-cultivation, which had a significant difference with 1- and 4-day periods (Table 4).

In the end, transgenic shoots (Fig. 5C and Fig. 5D) were propagated by bud grafting onto sour orange seedlings as rootstocks since sour orange is vigorous and thus, ensures fast growth of the transgenic scion (Fig. 5E). Transplanted explants were kept in liquid MS medium at 28 °C for 16 h photoperiod. To adapt with environmental condition, they were transformed to pots containing 1:1:1 ratio of vermicompost, peat moss, and vermiculite. Then, they were kept under 28 °C for 16 h photoperiod.

The effect of co-cultivation period on transformation efficiency: In present study, 2-day co-cultivation led to the best results in producing transformed seedlings (13.7%). In 3-day co-cultivation, number of transformed shoots versus total shoots resulted in the slightly lower figure compare to 2 days co-cultivation, which had a significant difference with 1- and 4-day periods (Table 4).

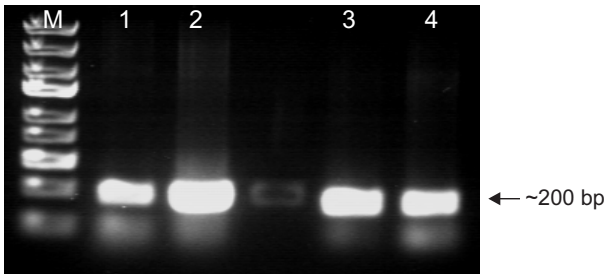

Fig. 2. Amplification of genes encoding *CTV*1 and *CTV2*. M = ladder 100 bp.; Lane 1 and 2 = *CTV1*; Lane 3 and 4 = *CTV2*.

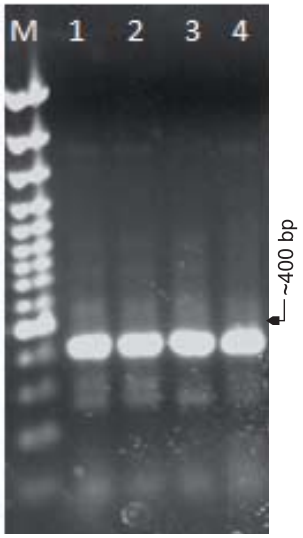

Fig. 3. Confirmation of mosaic fragment synthesis using R- and F-mosaic primers. PCR products indicates ligation of 2 *CTV1* & *CTV2* fragments (lane 1-4), molecular weight marker (M).

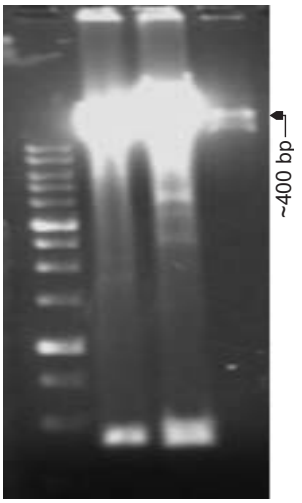

Fig. 4. Confirmation regarding insertion of 2 copies of *CTV* fragments in pFGC5941 destination vector. 2 types of products in lane 1 and 2 are the result of double digestions of silencing vector using SwaI - *Asc*I and *Xba*I - *BamH*I, respectively. Lane 3: undigested plasmid along with M: marker.

Table 2. Effects of acetosyringon concentration on transformation efficiency of epicotyl-originated explants

Acetosyringone conc. (µM)	Responsive explants/ total explants	PCR^+ shoots/total shoots (transformation efficiency)
0	8/50 (b)	1/18 (5.55%)
50	14/50 (a)	3.32 (9.37%)
100	10/50 (b)	1.22 (4.54%)

* Lowercase letters indicates significant differences (P≤ 0.01%) in response to each treatment

Table 3. Effects of inoculation time on transformation efficiency of epicotyl-originated explants. Positive PCR shoots regarding *CTV* and *BAR* genes plus negative PCR results relating to *kan* considered as transformed

Inoculation period (min)	PCR^+ shoots/ Total shoots (transformation efficiency)	Responsive explants/ Total explants
5	0/7 (0.0%)	5/50 (d)
10	3/32 (9.37%)	14/50 (b)
15	4/45 (11.11%)	19/50 (a)
20	1/22 (4.5%)	10/50 (c)
25	1/16 (6.25%)	8/50 (c)

* Lowercase letters indicates significant differences (P≤ 0.01%) in response to each treatment

Table 4. Effects of co-cultivation period on transformation efficiency of epicotyl-originated explants. Highest transformation efficiency was obtained from 2 days co-cultivation. Shoots with positive PCR results regarding *CTV* and *BAR* genes and negative PCR results concerning *kan*- were considered as transformed

Days	Responsive explants/ Total explants	PCR^+ shoots/total shoots (Transformation efficiency)
1	6/50 (c)	0/6 (0.0%)
2	26/50 (a)	10/73 (13.7%)
3	19/50 (b)	5/45 (11.11%)
4	7/50 (c)	0/12 (0.0%)

* Lowercase letters indicates significant differences in response to each treatment

In the end, transgenic shoots (Fig. 5C and Fig. 5D) were propagated by bud grafting onto sour orange seedlings as rootstocks since sour orange is vigorous and thus, ensures fast growth of the transgenic scion (Fig. 5E). Transplanted explants were kept in liquid MS medium at 28 °C for 16 h photoperiod. To adapt with environmental condition, they were transformed to pots containing 1:1:1 ratio of vermicompost, peat moss, and vermiculite. Then, they were kept under 28 °C for 16 h photoperiod.

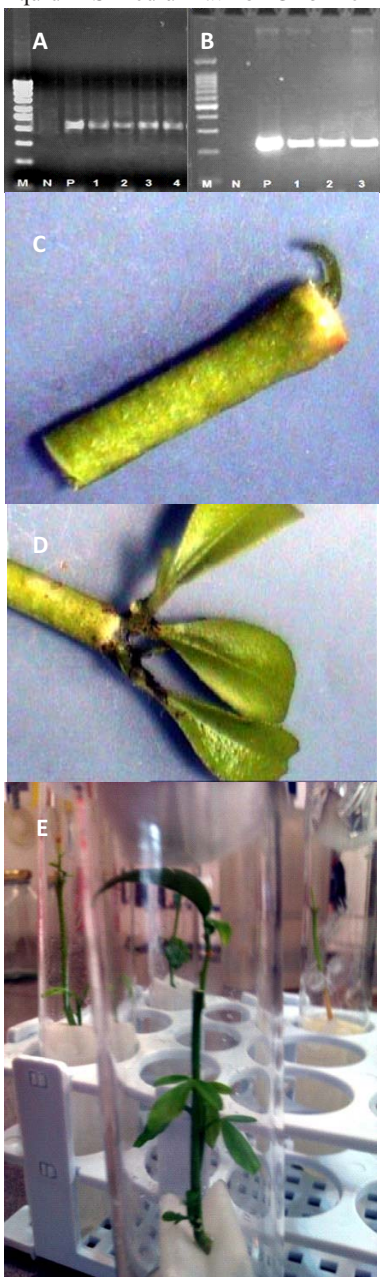

Fig. 5. Development and analysis of putative transformed seedlings. Confirmation of transgenic plants by PCR using genes located within T-DNA region including *CTV* (A) and *BAR* (B). N: non-transformed wild type plant; P: destination vector with insert, 1-3 putative transformed seedlings. Adventitious bud formation (C) and direct shoots organogenesis (D) from epicotyl-originated explants on selective medium in *C. aurantium*, Putative transgenic seedling grafted *in vitro* (E).

After 4 months growing in the growth chamber, all transgenic plants showed a normal phenotype, identical to that of control non-transgenic sour orange plants.

Discussion

The explants were culturepHd on solid regeneration medium after co-cultivation in order to induce shoot. Many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinin and auxin concentrations (van Staden *et al*., 2008). With this regard, shoot formation could be induced predictably using relatively low levels of auxin and a high level of cytokinin in the growth medium. The main intention in this experiment was to generate a balance between 2 growth regulators to induce adventive shoots on explants without going through a callus phase. These so called "direct organogenesis" helps to eliminate risk of somaclonal variation (Rezadoost *et al*., 2013).

Almeida *et al*. (2003) reported that transformation efficiency decreased when AS was applied. This difference between our finding and Almeida's is due to secretion of polyphenolic compounds from cut ends of explants to pre-culture medium. Accordingly, due to the lack of polyphenolic compounds in co-cultivation medium, application of an external phenolic compound like AS, might be the reason for further *vir* genes induction.

Cervera *et al*. (1998a) found 100 µM AS to be the best possible amount for Washington navel orange transformation. In the transformation of *Poncirus triliantus* epicotyls, Zou *et al*. (2008) noted that 50 µM AS had the best results. Luth and Moore (1999) also noted that 100 µM AS is appropriate for the transformation of *Citrus paradisi* explants. Bond and Roos (1998) also reported that 200 µM AS was the best in Washington navel (*Citrus sinensis* L. Osbeck) transformation. Reports on the use of AS concentrations are varied. Apparently, the finding figures depend on transformation method to a great extent.

In the bacterial cell culture protocol, which was adopted from Gelvin (2006), 3 steps of culture (culture in YEP medium, AB medium, and induction medium) were used to induce *vir* genes of *Agrobacterium.* The *vir* region includes 6 operons, which have been designated *virA*, *virB*, *virC*, *virD*, *virE*, and *virG*. The VIR gene products act in trans to mobilize the transferred DNA (T-DNA) element from the bacterial Ti plasmid to the plant genome (Stachel *et al*., 1986). The expression of the virulence genes except for *virA* is specifically induced by phenolic compounds, including AS, which are released from the wounded plant cells (Das *et al*., 1986). AS also acts as a strong inducer for *vir* gene expression between pH 5.0 and pH 5.5. The optimal induction of vir gene was attained at acidic level (~5.2-6.0) (Yuan *et al.*, 2008). The temperature optimum for *vir* gene induction (~25 °C) is generally lower than that optimal for vegetative growth of *Agrobacterium* (28-30 °C; Alt-Moerbe *et al.*, 1988).

Bond and Roose (1998) showed that transformation efficiency decreased over 10 min. Cervera *et al*. (1998) introduced 15 min as the optimum time for the inoculation of *Carrizo citrange* explants. Many researchers suggested 20 min as optimum inoculation time for transformation (Ghorbel *et al*., 2000; Pena *et al*., 2004; Zou *et al*., 2008; Silva *et al*., 2010). However, in this study, the number of PCR^+ shoots mainly decreased after 15 min due to *Agrobacterium* over growth. Perez-Molphe and Alejo (1998) found maximum time for citrus explants inoculation was 45 min. in *Citrus aurantifolia* internodes transformation via co-cultivation by *Agrobacterium rhizogenes*.

Production of shoots in selection medium does not necessarily mean that they are transformed. In our experiments many of the shoots that regenerated on Basta selective medium were "escapes", *i.e.*, were not CTV^+. The insensitivity to selective agent may be due to protection of nontransformed cells from the selective agent by the surrounding transformed cells (Gutierrez *et al*., 1997). In previous reports, escape is also introduced as the most critical challenge in citrus transformation (Moore *et al*., 1992; Pena *et al*., 1995). However, attempt was made here to minimize the use of these materials to control the level of error.

One of the advantages of the current protocol is regeneration of explants through direct organogenesis which avoid callus phase and consequently somaclonal variation and reduces

the risk of genetic deterioration of genetic stocks. *Agrobacterium*-mediated transformation of mature sour orange embryos is a promising approach to make a shortcut to generate transgenic citrus in future experiments.

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