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Analysis of transgenic citrus (*Citrus aurantium* L.) plants expressing Citrus Tristeza Virus coat protein gene

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ABSTRACT

Due to high quality of fruits, resistance to various pathogens and abiotic stress, *Citrus aurantium* is widely used and considered as the most favorable rootstock worldwide. Genetic engineering approaches such as pathogen-derived resistance (PDR), is a common practice in citrus breeding. Analysis of transgenic plants requires reliable and quick methods for early screening of T_0 generation. In a PDR approach, a mosaic gene from Shiraz CTV strains was cloned and transferred into sour orange. Forty putative transformed shoots were isolated on selective medium, which were acclimatized and transferred to growth room. In the first screening method, leaf assay for Basta resistance was performed on liquid as well as solid selective medium. To further analyze the putative transgenic seedlings, PCR using *CTV* and *BAR* gene specific primers were performed and some of the PCR products were randomly chosen for sequencing. The transgene copy number(s) in individual genotypes was resolved using real-time PCR technique. Finally, the functionality of the transgene was provided using ELISA test, which confirmed that CTV amplification suppressed in five tested seedlings. In this experiment, the simple and economical screening methods were set up, which made unambiguous discrimination between transgenic and non-transgenic citrus possible.

Keywords: ELISA test, Herbicide resistance, Pathogen-derived resistance, Transgene copy number.

آنالیز گیاهان نارنج (*Citrus aurantium*) تراریخت حامل ژن پروتئین پوششی ویروس تریستیزای مرکبات

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چکیده

درخت نارنج دارای ویژگی‌های با ارزشِ یک پایه ایده‌آل در مرکبات است اما، این گیاه به بیماری ویروس تریستزای مرکبات (CTV) به شدت حساس است. بر این اساس، بذور نارنج در شرایط *in vitro* کشت و به مدت ٤ هفته درتاریکی و۱۰ روز در روشنایی رشد کردند. ریز نمونه ها از اپی‌کوتیل وهیپوکوتیل تهیه و با استفاده از *Agrobacterium tumefaciens* نژاد EHA105 حامل وکتور خاموشی pFGC5941 و بخشی از ژن کدکننده پوشش پروتئینی CTV، به مدت ۳روز هم‌کشت شدند. سپس ریزنمونه‌ها به محیط کشت انتخابی حاوی علفکش بستا و ترکیبی از تنظیم کننده‌های رشد BAP و NAA منتقل شدند. در اولین غربالگری تعدادی برگ از گیاهچه‌های تراریخت احتمالی در محیطهای MS مایع و همچنین MS جامد حاوی غلظت‌های مختلف علفکش بستا منتقل شدند. تعدادی از قطعات برگی در محیط انتخابی به رنگ سبز باقی ماندند و برگ گیاهان شاهد و غیرتراریخت‌ها سفید رنگ شدند. در مرحله بعد واکنش PCR با آغازگرهای اختصاصی ژن‌های *CTV* و *BAR* در میان گیاهان باقی مانده از غربالگری اولیه انجام و برخی از باندها توالی یابی شدند. تعداد نسخه‌های تراژن *CTV* با استفاده از تکنیک quantitative Real-Time در تعدادی از گیاهچه‌های نارنج محاسبه و تعداد آن‌ها بین ٤-۱ نسخه در ژنوم تعیین شد. تکثیر ویروس مطالعه و تست الایزا نشان داد که ویروس در گیاهان تراریخت تکثیر نشده است. در این تحقیق، روش‌های آسان و اقتصادی برای غربالگری اجرا شد که با استفاده از آن‌ها تمایز درست گیاهان تراریخت در مقابل غیرتراریخت نارنج امکان‌پذیر شد.

واژه‌های کلیدی: **تست الایزا، تعداد نسخه تراژن، مقاومت مشتق از پاتوژن.مقاومت به علفکش.**

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Introduction

Citrus is the most extensively grown fruit tree crop, and its 115 million tons annual production represents 25% of the world's fruit production (FAOSTAT database results 2007: http://faostat3.fao.org/home/E). Sour orange (*Citrus aurantium* L.) was the predominant rootstock in most citrus-growing areas for many years because of its good agronomic attributes, particularly fruit yield, quality, rusticity, and tolerance to various pathogens and abiotic stresses. However, a major weakness is its sensitivity to decline caused by citrus tristeza virus (CTV). CTV, a member of genus *Closterovirus*, is the causal agent of the most economically important viral disease of this crop. CTV produces two main field diseases depending on the isolates: common isolates cause decline and death of most scion varieties grafted on sour orange (*Citrus aurantium* L.) rootstock, whereas highly virulent isolates additionally cause stem pitting on different scion varieties regardless of the rootstock, resulting in reduced vigour, yield and fruit quality. CTV is considered the most serious threat to the citrus industry worldwide (Rocha-Pe˜na *et al*, 1995). Genetic improvement of the sour orange rootstock to overcome its sensitivity to CTV would be desirable, and it remains an important objective for the citrus industry. The expression of coat protein (*CP*) genes in transgenic plants is an example of pathogen-derived resistance (PDR) and has been widely used to develop virus-resistant plants (Baulcombe 1996). This closterovirus affects all citrus species and varieties grafted on sour orange, with the exception of lemons (*C. limon* L. Burm.; Rocha-Pe˜na *et al*, 1995). This has led to the substitution of sour orange with other rootstocks that are resistant or tolerant to CTV.

Improvement of *Citrus* spp. by conventional breeding methods is hampered by various aspects of *Citrus* biology such as nucellar polyembryony, heterozygosity, sexual incompatibility and long juvenile period of many citrus cultivars (Grosser and Gmitter, 2005). With the recent advances in plant biotechnology, it is possible to introduce exogenous genes in plant genome, using gene transfer techniques. Biotechnological techniques such as cell and tissue cultures and molecular biology have helped breeders to overcome these difficulties. In addition, the hybridization through protoplast fusion and genetic transformation may contribute significantly to avoid these limitations (Mendes-da-Glória et al, 2000). Genetic transformation is a widely employed tool in both basic research and commercial plant breeding programs. Its application requires that transgenes be stably integrated and expressed in the plant genome. Transgenic plants must be characterized at the molecular level because the new DNA is randomly inserted into the plant genome. (Omar *et al*, 2008). Therefore, genetic transformation remains the main alternative in *Citrus* breeding programs such as development of CTV tolerant sour orange plants.

The premise behind DNA introduction into plant cells is the recovery of a phenotype from the activity of foreign gene(s). This phenotype is usually the ultimate goal of transformation but some phenotypes can be difficult to be determined; and additional means of confirming gene presence and function are necessary to determine whether a gene has been successfully introduced and if it is active.

In the present experiment, a number of putative transformed *C. aurantium* seedlings containing CP-25 were

generated. The success of genetic transformation process is monitored through the various methods in order to set up simple and effective analysis techniques in woody plants at early generation.

Materials and Methods

Plant material

Seeds were extracted from ripe fruits of sour orange (*Citrus aurantium* L.). The seed integument was removed and disinfestations were done in sodium hypochlorite solution (2.5% active chlorine) for 15 minutes followed by three rinses in distilled and sterilized water. The seeds were placed in test tubes (150 x 25 mm) containing 15 ml of MS medium (Murashige and Skoog, 1962) and maintained at 27 ± 2°C in the dark for four weeks followed by one week in a 16-hour photoperiod (40 µmol $m^{-2}s^{-1}$). Epicotyl segments approximately 1.0-cm-long were collected for the transformation experiments.

Transformation and regeneration

Transgenic plants of citrus containing the *CTV-CP* gene (*Citrus aurantium* L.) were produced by *Agrobacterium*-mediated transformation (Sohani *et al*, 2015). Briefly, *Agrobacterium tumefaciens* strain EHA105 carrying the plasmid of pFGC5941-*CTV* were cultured in solid YEP medium (10 $g^{-1}L^{-1}$ peptone, 10 $g^{-1}L^{-1}$ yeast extract, 5 $g^{-1}L^{-1}$ sodium chloride, 15 $g^{-1}L^{-1}$ agar) containing kanamycin (100 $mg^{-1}L^{-1}$) and rifampicin (50 $mg^{-1}L^{-1}$), for 48h. A single colony was transferred to a 250 ml Erlenmeyer flask with 50 ml of liquid YEP medium, supplemented with antibiotics and cultivated at 200 rpm at 28°C for 16h. The bacterial suspension was centrifuged at 4800 rpm (5°C/20 min) and resuspended in liquid MS medium (Murashige and Skoog, 1962). Epicotyl-originated explants were inoculated with *A. tumefaciens* for 20 minutes. Following incubation, explants were blotted dry and plated on co-cultivation MS medium containing 30 g L^{-1} sucrose at 27°C for 72 h at darkness. To induce regeneration the explants were transferred to medium supplemented with 0.25 $mg^{-1}L^{-1}$ NAA, 2.5 mg L^{-1} benzylaminopurine (BAP) and different concentrations of bialaphos (1-10 $mg^{-1}L^{-1}$), which is the main ingredient of Basta herbicide. To control the bacterial growth, 250 $mg^{-1}L^{-1}$ vancomycin and 500 $mg^{-1}L^{-1}$ cefotaxime were used. Explants were subcultured every 2 weeks.

To adapt with environmental condition, the regenerated shoots were transferred to pots containing 1:1:1 ratio of vermicompost, peat moss, and vermiculite, kept under 28°C for 16 h photoperiod.

Citrus leaf assay with basta

Three healthy green leaves about 1 cm long were excised from each plant and embedded into media. The medium was composed of MS salts (Murashige and Skoog, 1962), 0.5 $mg^{-1}L^{-1}$ 6-BA (6-benzylaminopurine), 1-10 $mg^{-1}L^{-1}$ bialaphos, which is the active ingredient of Basta herbicide (Bayer, crop science), and 8 $g^{-1}L^{-1}$ agar. The assays were kept at 24°C under a 16 h/8 h light/dark regime. In liquid culture, MS medium with the same Basta concentrations was used. Citrus regenerants were assayed with different concentrations of bialaphos (1-10 $mg^{-1}L^{-1}$), which is the active ingredient of Basta herbicide.

PCR analyses

Genomic DNA was isolated from the leaves of control and putative transgenic plants following the method described by Dellaporta *et al* (1983), with required modifications.

Integration of transgenes in the putative transformants was confirmed by PCR of *CTV-CP* and *BAR* genes.

Amplifications were performed in 25 µl reactions containing 10 mM Tris–HCl (pH 9.0), 50 mM potassium chloride, 1.5 mM magnesium chloride, 200 mM of each dNTPs, 75 ng upstream 5′- TGC TGC TGA GTC TTC TTT CG-3′ and downstream 5′- GCT CTA GAA GTC TCT GTT CAA TGC CGC G-3′ *CTV-CP* gene specific primers, 25 ng of template DNA and 2 units of Taq DNA polymerase, following standard protocols. For *BAR* gene amplification the forward 5′- GAC TTC AGC AGG TGG GTG TA-3′ and reverse 5′- CAA CGC CTA CGA CTG GAC G-3, gene specific primers were used in similar reactions. Reactions were carried out in a Bio-Rad cycler. The PCR reactions were set up at the following temperature profile: pre-incubation at 94 °C for 3 min, leading to 35 cycles of melting at 94°C for 1 min, annealing at 55°C for 1 min and synthesis at 72°C for 1 min, followed by an extension at 72°C for 5 min. Two µl of the amplified product was analyzed on a 0.8% agarose gel.

Inoculation of the citrus plants with CTV

Two leaf discs which contained contain phloem were used as inoculum tissue and grafted in a way that cut surfaces of phloem tissue of donor and receptor plants were in a good contact (Roistacher, 1991). The greenhouse temperatures were set at the maximum of 24-28°C during the day and the minimum of 17-21°C at night for all tristeza and seedling-yellows indexing. Temperature control is especially important when checking for mild isolates. Relative humidity was kept between 60-80%. First symptoms were observed three to five weeks later (the end of first or second growth flush)

ELISA analyses

Immunodetection was performed using double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISAs), which reacted with polyclonal antibody against *CTV-CP* (Bioreba, Switzerland) as the primary antibody, and goat anti-mouse IgGs (Boehringer-Mannheim) which conjugated with alkaline phosphatase as the secondary antibody. Virus accumulation was estimated by Indirect ELISA (Converse and Martin, 1990). Absorbance was measured at 490 nm. The extracts from transgenic limes carrying the *CTV-CP* gene inoculated with SD6 and Lan virus isolates (CTV gene bank, Ramsar Citrus Institute, Ramsar, Iran). The extracts from negative non-transformed plants and positive control were included in the experiment as well. A plant was considered infected when the absorbance values at 405 nm were at least twice those of non-inoculated control plants.

Quantitative gene expression of CTV gene by quantitative real time-PCR

Transcription levels of *CTV-CP* gene were monitored using quantitative real time-PCR (RT-qPCR; Kariola *et al*, 2005). 1 µl of 10X diluted cDNA derived from samples was amplified in each PCR reaction, which was comprised of 10 µl of SYBR® Green RT-PCR reagent (Bioneer), 3 µl of forward and reverse primers (4 µM concentration) and 3 µl water. The amplification was performed in a CFX cycler system (Bio-Rad). The thermal cycler was programmed as follows: Initial activation step for 15 min at 95ºC followed by 45 cycles of 30s at 95ºC, 30s at 60ºC and 30s at 72ºC. The melting curve was used to obtain optimal temperature for data acquisition. Sequencing was done from both directions to confirm the size and identity of the PCR product. A dilution series of 10^{-7} to 10^{-1} copies per µl was prepared from original solutions and used as standard. In each qPCR experiment three replicates of each of seven standard

concentrations together with two "no template" control ("no SYBER" and "no DNA") were included.

The Primer3 (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the gene specific primer for control and *CTV-CP* gene. A pairs of primers for *Actin* gene including 5'- GAG TTC TTC ACG CGA TAC CTC CA-3' and 5'-GAC CAC CTT TAT TAA CCC CAT TTA CCA-3' were used as internal control as a forward and reverse, respectively, in order to calculate an RT-PCR normalization factor (NFn, n = 3; Vandesompele *et al*, 2002).

Transgene copy number determination by real-time PCR

To analyze the integrity of the p25 CP expression cassette and to estimate the copy numbers of the *CTV-CP* transgene, Real-time PCR assays were performed.

A serial dilution of plasmid DNA of the known quantity was prepared. The entire mass of a single plasmid molecule (plasmid plus insert) was calculated using equation 1:

$$m = (n) \quad \left(1.096e - 21\frac{g}{bp}\right)$$

where, n= entire plasmid size; m= mass. A standard curve was generated, with the logarithm of the initial copy number of the standard plotted along x-axis and their respective CT values plotted along y-axis (Figure 6).

The starting quantity of citrus gDNA concentration (A) in each unknown PCR reaction was calculated by interpolation in to a standard curve. Based on equation for linear regression: [y = mx + b, or C_T= m (log quantity) + b], equation A was used to determine the quantity of an unknown sample; A: Quantity (ng)= e (C_T-b)/m); Where, b =intercept; m=Slop. Since one set of citrus genome quantity is equal to 4.033211558×10^{-4} ng (B), the total number of starting sour orange chromosome set (C) in a template DNA is equal to: A/B. The starting quantity of fragment that amplified in PCR cycles was calculated using equation 2:

$$DNA(ng) = 10^{\frac{CT-b}{m}}$$

$N_0 (1+E)^{CT+1}$; $XCT= X_0(1+E)^{CT}$.

It is crucial in this technique to amplify the standards in parallel with the samples every time the experiment is performed.

Results and Discussion

Determination of transformation efficiency

As a result of transformation the highest percentage of responsive explants was 90% that obtained from regeneration medium supplemented with 2.0 mg L^{-1} 6-benzylaminopurine (BAP) in combination with the 0.25 $mg^{-1}L^{-1}$ Naphthaleneacetic acid (NAA) treatment in MS selection medium (Figure 1B), which confirm the previous results presented by Sohani *et al*. (2015) and Rezadoost *et al.* (2013). One of the advantages of the current protocol is regeneration of explants through direct organogenesis which avoid callus phase and consequently somaclonal variation.

To adapt with environmental condition, the regenerated shoots were transferred to the growth chamber to further analyze the transgene integration (Figure 1C).

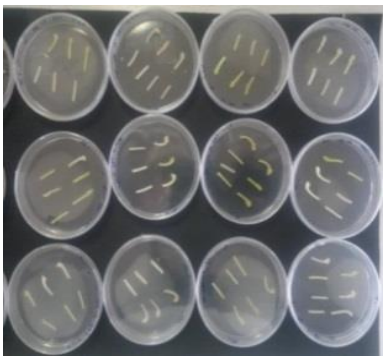
After 4 months growing in the growth chamber, all putative transgenic plants showed a normal phenotype, identical to that of control non-transgenic sour orange plants.

Citrus leaf assay with bialaphos

A few days after placing leaf segments in liquid medium containing 1-10 $mg.l^{-1}$ bialaphos, non-transgenic leaves showed yellowing. The whole tissue of the explants became uniformly bleached after three and seven days with 10 and 3 $mg^{-1}L^{-1}$ bialaphos, respectively (Figure

2). The higher herbicide concentration the sooner leaf segments bleached. Lower concentrations of bialaphos, even the 1 $mg^{-1}L^{-1}$ concentration, also resulted in yellowing and bleaching of the non-transgenic leaves after two weeks, but a concentration above 3 $mg^{-1}L^{-1}$ appeared to be optimal for the assay. Transgenic leaf segments remained completely healthy and green in the first week (Figure 2), and largely or visibly green for up to four weeks.

A)



B)

C)

Figure 1. Sour orange (*C. aurantium*) transformation procedures. Epicotyl-originated explants (A); adventitious shoot formation (B) and acclimatized putative transgenic seedlings (C).

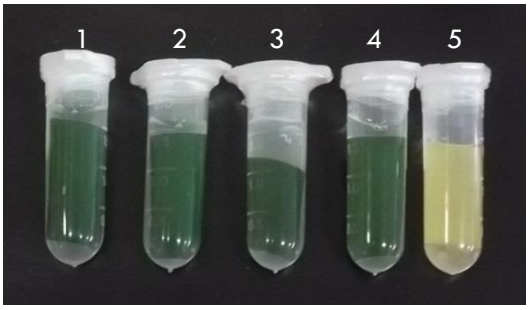


Figure 2. Leaf assay for bialaphos resistance in liquid medium. Citrus leaves, after seven days on 5 $mg^{-1}L^{-1}$ bialaphos (active ingredient of Basta herbicide) in liquid medium. The samples 1-4 are defined as transgenic plants and sample 5 is defined as non-transgenic plant. Yellow and bleached non-transformed leaves were observed seven days after incubation in the liquid selective medium.

The reactions of citrus leaves in solid MS selection medium which contained different concentrations of bialaphos were similar to cultures in liquid medium (Figure 3).

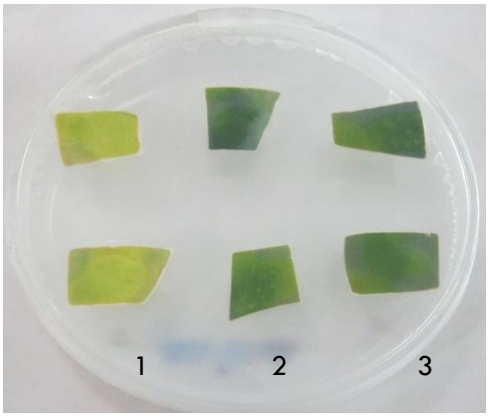


Figure 3. Leaf assay for bialaphos resistance in solid medium. Citrus leaves, after seven days on 5 $mg^{-1}L^{-1}$ bialaphos in solid MS selection medium. Non-transgenic controls (1) showed yellowing and Transgenic leaves (2 and 3) remained green during the course of the experiment.

As a result of leaf assay in liquid and solid selective media, some of the genotypes including A, B, C, Q, and H showed resistance and were chosen for further analysis.

Identification of transgenic plants instantly after the transformation process

usually involves time-consuming, laborious and often expensive procedures, such as Southern or Northern hybridization, dot blot analysis, enzymatic assays or polymerase chain reaction (PCR). Leaf assay is a simple method for early and quick screening of a large transgenic population of citrus plants expressing the phosphinothricin acetyl transferase (PAT) resistance gene, *BAR* (Murakami *et al*, 1986). The leaf assay method described above clearly discriminates between transgenic (with the selectable marker genes *bar*) and non-transgenic plants. This method does not require sterile technique. Instead, it requires minimal use of chemicals and plant tissues, and causes no lasting harm to the plants. It is therefore mostly appropriate for initial screening of large primary transformants and dramatically reduces the numbers of segregating population. A similar method could be applicable to other plant species transformed using *BAR* as the selectable marker genes.

Here, we report a leaf assay method for screening transgenic citrus plants expressing the phosphinothricin acetyl transferase (PAT) resistance gene, *BAR*, which is one of the most widely used selectable marker genes for plant transformation (Miki and McHugh, 2004). Such a very simple leaf assay could rapidly and reliably identify any transgenic citrus plants expressing the *BAR* resistance gene.

Integration of transgene in putative transformants

The putative transgenic basta-resistant plants were tested for the presence of transgene, including marker genes, using PCR with gene specific primers. Integration of *CTV-CP* transgenes in the genome of putative citrus transformants was ascertained for some genotypes. The expected amplified product was ~450bp obtained from some; however, no product was detected in non-transformed control and no-template control samples (Figure 4).

Amplification of *BAR* transgene was confirmed by the production of a ~99bp size band for some of the genotypes (Figure 5).

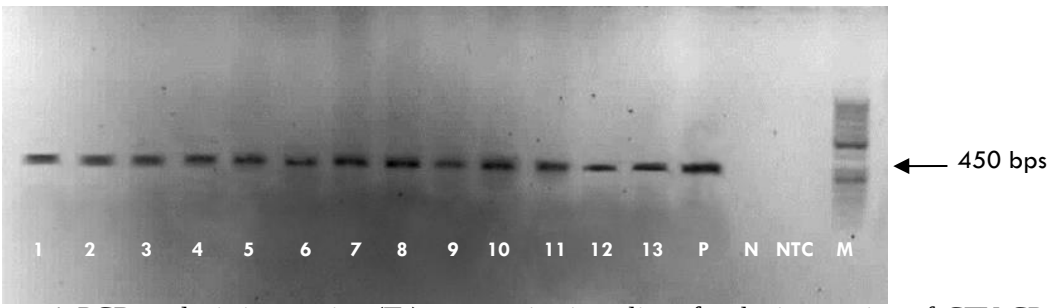


Figure 4. PCR analysis in putative (T_0) transgenic citrus lines for the integration of *CTV-CP* gene. 1-13: Putative transgenic genotypes; NTC: No template control; N: non-transformed control plant; M: molecular marker.

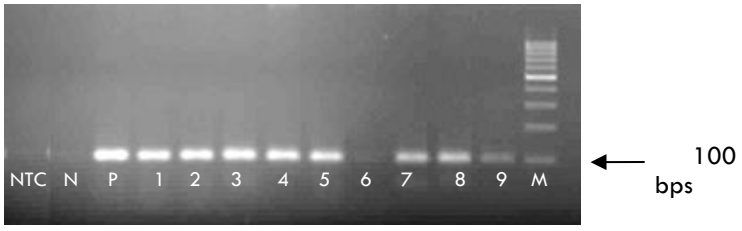


Figure 5. PCR analysis in putative (T_0) transgenic citrus lines for the integration of *BAR* gene. 1-9: Putative transgenic genotypes; NTC: No template control; N: non-transformed control plant; M: molecular marker.

Two samples from each *BAR* and *CTV-CP* PCR products were isolated and sequenced from both directions (Bioneer, South Korea). Blast search technique confirmed that the products were genuine. Moreover, Blast search technique showed that the mosaic *CTV-CP* gene has similarity with about 100 virus isolates, including 10 Iranian ones, in over 23 nucleotides.

Lack of *Agrobacterium* contamination in the putative transgenic regenerated shoots was confirmed by PCR using genes specific primers for Kanamycin gene that is located outside T-DNA region using 5'-ATG TTG CTG TCT CCC AGG TC-3'and 5'-GAA AGC TGC CTG TTC CAA AG-3'as forward and reverse primers, respectively.

Although PCR is a fast and sensitive method, it is susceptible to cross-contamination and the reaction conditions often need to be carefully optimized (Wong & Medrano, 2005).

Transgene copy number determination by Real-Time PCR

To quantify target DNA using real-time PCR, primers and PCR conditions were optimized. A standard curve was generated. The experiment was performed with known amount of diluted DNA from 10^{-1}-10^{-7} copies of CTV in order to generate a standard curve (Figure 6). The correlation coefficient (R^2) value was 1.000. The C_T values ranged from 25-32 cycles. The correlation between the amount of DNA used as a template in the beginning of PCR reaction and the C_T value during amplification was linear. As a result, the standard curve was used to calculate the amount of DNA in unknown transgenic samples (Figure 6).

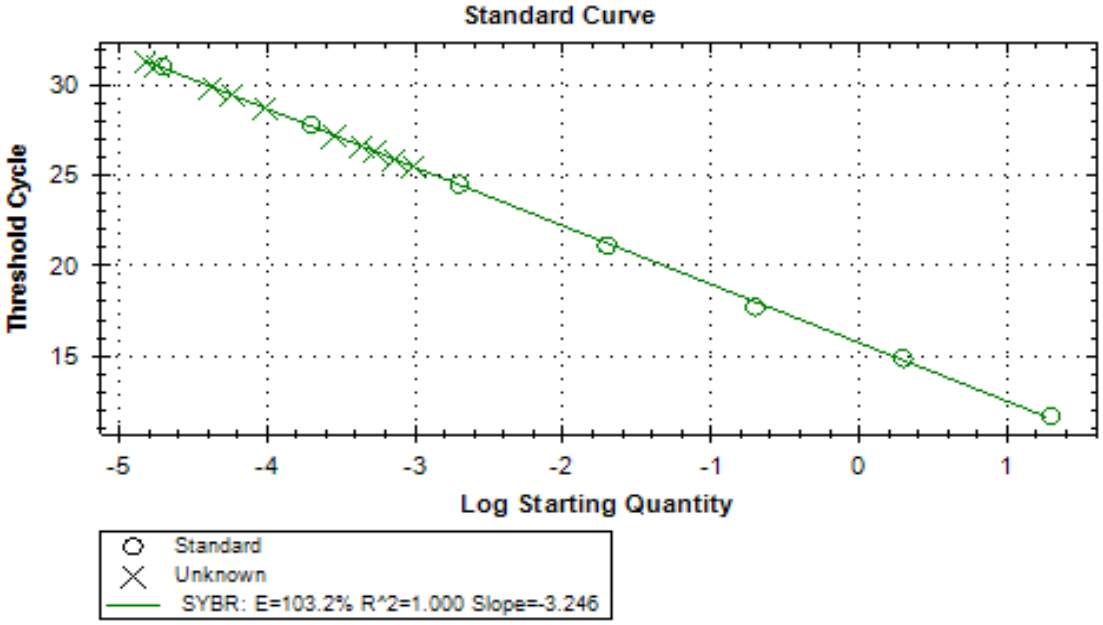


Figure 6. Absolute quantification using a standard curve. To determine the copy number of *CTV-CP* in unknown samples of A, B, C, H and Q; 10-fold dilution of a known concentration of template (10^2–10^7 copies of plasmid plus insert) were assayed with the five unknown samples in the same run using a SYBR Green I assay specific to *CTV-CP*. The regression line from the dilution curve was used to determine the copy number of the unknown samples. All standard dilutions and unknown samples were assayed in triplicate Bio-Rad real-time PCR detection system. Standard curve with the logarithm of the initial copy number of the standards are plotted along the X-axis and their respective C_T values are plotted along the Y-axis.

A dissociation curve was generated by Bio-Rad software and has been used for product differentiation following PCR reactions. A single strong and consistent melting temperature was observed in almost all samples except NTC and wild type (non-transgenic) DNA samples.

The C_T values and the calculated copy numbers using standard curve are given for a limited number of genotypes in Table 1. There were 1-4 copies of *CTV-CP* in transgenic citrus plants. No amplification was recorded in the wild type citrus plant DNA.

The Southern blot analysis requires relatively large amounts of DNA, and thus a large amount of transgenic plant material, which is not available at the early seedling stage. Furthermore, in the case of restriction site loss or concatamers the number of bands does not correspond to the copy number (Miyao *et al.*, 2003). Unlike the traditional hybridization-based methods, qPCR assay allows more samples to be analyzed in a shorter time (Huang *et al.*, 2011).

While multiple copies of the transgene are useful for over-expression experiments, multiple copies of DNA integrated into one or more chromosomal locations affects the level and stability of gene expression sometimes resulting in transgene silencing (Iyer et al., 2000; James et al., 2002). Tang et al. (2007) reported post-transcriptional gene silencing in transgenic lines with more than three copies of T-DNA, but not in transgenic lines with only one copy of T-DNA. However, Craig et al. (2005) found no relationship between the number of transgene insertions and the expression level, suggesting that multiple insertions had little or no effect on transgene expression.

To date, qPCR technology has been applied to analyze copy number of transgenic plants, including soybean and peanut (Schmidt and Parrott, 2001), tomato (Mason *et al*, 2002), maize Shou *et al*, 2004), rapeseed (Weng *et al*, 2004), wheat (Doshi *et al*, 2007), tobacco (Subr *et al*, 2006), cotton (Yi *et al*, 2008), citrus (Omar *et al*, 2008), grape (Costa *et al*, 2009) and cassava (Beltran *et al*, 2009).

Transgenic sour orange plants showed different levels of *CTV-CP* expression

The expression of *CTV-CP* gene was investigated by DAS-ELISA test. DAS-ELISA confirmed negligible expression of the *CTV-CP* in some transgenics (e. g. A, C, H and Q plants; Figure 7).

Variable levels of *CTV-CP* expression among the transgenic lines will allow studying the effect of CP accumulation in protection against CTV. Furthermore, lines without detectable protein expression could be of interest if they transcribe *CTV-CP* mRNA, since RNA mediated resistance is a highly efficient mechanism against viral infections (Baulcombe, 1996).

Table 1. Determination of the absolute transgene copy number of (*CTV-CP*) in unknown *C. aurantium* samples A, B, C, H and Q using the absolute quantification method. The equation of the linear regression line shown in Figure 6 was used to calculate the copy number of the unknown samples

D/C OR ~transgene copy No	D	C	A	C_T	Plant Samples
1	1.93×10^5	94688.80928	38.19	30.05	A
3	5.91×10^5	112689.3528	45.45	28.35	B
1	1.05×10^5	47865.08152	19.305	30.51	C
4	2.96×10^6	323315.5467	130.4	25.68	H
3	1.68×10^6	244718.1324	98.7	26.48	Q

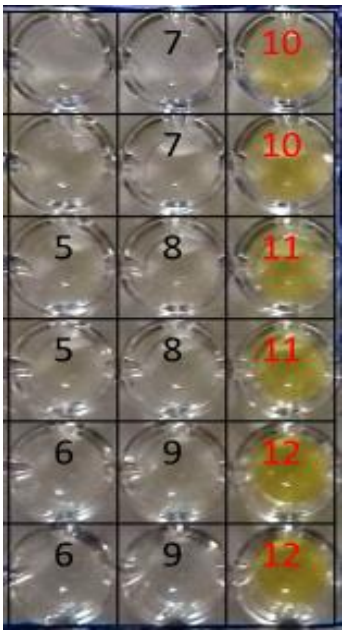


Figure 7. Protein expression analyses of 4 transgenic limes carrying the *CTV- CP* gene construct. DAS-ELISA reacted with polyclonal antibody against *CTV-CP* (Bioreba, Switzerland). Absorbance was measured at 490 nm. Wells 5-8 contained extracts from transgenic limes (A, C, H, Q, respectively) carrying the *CTV-CP* gene that was inoculated with SD6 and Lan virus isolates (CTV gene bank, Ramsar Citrus Institute, Ramsar, Iran). Well 9 had extract from negative non- transformed plant and wells 10-12 included positive control.

DAS-ELISAs combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration. The test is highly sensitive, which comes from the enzyme as a reporting group. By ELISA, a tracer of the antigen or antibody is achieved in the cell or subcellular level; also, antigen or antibody quantification can be done in the microgram or even nanogram levels. The test has a high specificity due to its ability to distinguish the target virus from other organisms, whether related or not (Chabirand *et al*., 2016).

It has been widely reported that engineered resistance to viruses requires the production of numerous independent transformants to be able to select at least one with the appropriate level of gene expression to achieve efficient resistance (Fuchs & Gonsalves, 1995; Scorza *et al*., 1994; Tricoli *et al.*, 1995).

Conclusions

The screening of putative transgenic citrus on liquid and solid selective medium containing Basta herbicide was founded to be a straightforward technique in discriminating between transgenics and non-transgenics individuals. In addition, Real-time PCR made it possible to determine transgene copy number(s) with a limited quantity of sample in a relatively short time.

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