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DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

Transfer of citrus tristeza virus (CTV)-derived resistance candidate sequences to four grapefruit cultivars through *Agrobacterium*-mediated genetic transformation

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Abstract Transgenic plants of grapefruit (*Citrus paradisi* Macf.) *cvs*. 'Duncan', 'Flame', 'Marsh', and 'Ruby Red' were obtained using *Agrobacterium tumefaciens*-mediated transformation of seedling epicotyl tissue. Two citrus tristeza virus (CTV)-derived candidate resistance genes: '392' (3′ region of the p23 ORF plus 3′ untranslated region—UTR) and 'p23 hairpin' (sense-p23 ORF plus UTR plus antisense-p23 ORF) were introduced into grapefruit using *Agrobacterium* strains EHA105 and EHA101, respectively. Epicotyl explants from 1-mo.-old *in vitro* etiolated seedlings were incubated in bacterial suspension. Green shoots that formed on explants after 4–5 wk after bacterial incubation were tested for the presence of the GUS gene by histochemical analysis. The percentage of GUS-positive shoots and transformation efficiency was 30.3±3.3% and 3.5% for treatment with EHA101 and 15.3±1.7% and 1.3% for treatment with EHA105. GUS-positive shoots were micrografted onto Carrizo citrange (*Citrus sinensis* L. Osbeck×*Poncirus trifoliata* L. Raf.) seedling rootstocks, and the presence of transgene sequences in these plants was confirmed by polymerase chain reaction (PCR), Southern blot, and reverse transcription PCR analyses. Resulting transgenic grapefruit plants were challenged with CTV and tobacco mosaic virus using a protoplast challenge assay as an initial screen to determine the effects of the transgenes on virus replication. Although complete RNA-mediated resistance was not achieved, preliminary results showed that 5.2% of the recovered transgenic plants containing the '392' CTV-derived sequence repeatedly exhibited reduced CTV replication in protoplasts. These plants are being further evaluated using the traditional method of virus inoculation followed by enzyme-linked immunosorbent assay.

Keywords Citrus variety improvement · Organogenesis · Protoplast challenge assay · RNA interference (RNAi) · RNA-mediated resistance

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Introduction

Citrus tristeza virus (CTV) is a single-stranded RNA *Closterovirus* that is transmitted by aphids or by grafting. CTV causes economically important diseases of citrus trees worldwide including "quick decline" of trees grafted on sour orange rootstock (*Citrus aurantium* L.), a fatal disease, and "stem pitting" that can significantly reduce productivity and fruit quality (Bar-Joseph et al. 1989). The strategy for controlling CTV employs the utilization of resistant rootstocks (such as Carrizo citrange). However, these resistant rootstocks are effective in protection of the scion only against "quick decline." In addition, the available resistant rootstocks are limited by poor soil adaptation and/or production of fruit with poor quality, and efforts to develop improved rootstocks that overcome these problems are underway (Grosser et al. 2004; Ananthakrishnan et al. 2006). "Stem-pitting" disease caused by specific isolates of CTV (not yet widespread in Florida) affects all sweet orange [*Citrus sinensis* (L.) Osbeck] and grapefruit cultivars, regardless of rootstock. Research on isolation of the gene(s) responsible for the resistance of trifoliate orange (*Poncirus trifoliata* Raf.) to virtually all CTV isolates is

almost complete and could lead to production of transgenic plants resistant to CTV (Deng et al. 2001). High susceptibility to CTV infection of economically important sweet orange and grapefruit cultivars has led researchers to focus on genetically engineering tolerant scions in most citrus growing areas.

Citrus species can be genetically transformed using various methods, including the direct uptake of DNA by protoplasts (Kobayashi and Uchimiya 1989; Fleming et al. 2000; Niedz et al. 2003; Olivares-Fuster et al. 2003), *Agrobacterium*-mediated transformation of embryogenic suspension cultured cells (Hidaka et al. 1990), and particle bombardment of similar tissue (Yao et al. 1996). At present, *Agrobacterium*-mediated transformation using juvenile explants 1–2 cm in length is the most popular and efficient method for producing transgenic citrus plants (Peña et al. 1995). Using this protocol, transgenic plants of Carrizo citrange (Moore et al. 1992), sweet orange (Bond and Roose 1998), sour orange (Gutiérrez et al. 1997), grapefruit (Luth and Moore 1999; Febres et al. 2003; Yang et al. 2005), and 'Mexican' lime (*Citrus aurantiifolia* (L.) Swingle; Domínguez et al. 2000) have been obtained. Our approach to engineer CTV resistance in susceptible citrus cultivars has been to exploit pathogen-derived resistance by introducing selected segments of the CTV genome into plants. Progress in using this approach has been reported by other researchers (Fagoaga et al. 2006), especially with the CTV coat protein gene (Domínguez et al. 2000; Febres et al. 2003). Previously, protoplasts isolated from ten sweet orange callus lines genetically transformed with the CTV-392 sequence from the CTV genome exhibited different CTV replication levels after the introduction of CTV virions (Olivares-Fuster et al. 2003). One callus clone showed resistance to CTV, and two other clones showed a reduction of virus replication, suggesting potential for this sequence.

We have used p23 ORF as it has been shown earlier that the p23 subgenomic mRNA is highly expressed in infected plants as well as in protoplasts (Hilf et al. 1995; Navas-Castillo et al. 1997; Satyanarayana et al. 1999, 2002) and is known to induce RNA-mediated silencing. This approach, also known as an RNAi approach, was used previously to induce resistance to barley yellow dwarf virus (Wang et al. 2000).

The primary objective of this study was to produce transgenic grapefruit plants carrying the CTV-392 sequence, as no plants were recovered from the previously reported resistant transgenic callus (Olivares-Fuster et al. 2003). A second objective of this work was to produce grapefruit plants carrying a CTV-derived p23 hairpin construct. Resulting transgenic plants were tested for CTV resistance using our previously developed protoplast challenge assay as an initial screen (Olivares-Fuster et al. 2003; Albiach-Marti et al. 2004).

Materials and Methods

Plasmid vector construction, bacterial strains, and plant materials Schematic diagram of the CTV genome is presented in Fig. 1. The p23 hairpin construct contained the p23 ORF, the 3′ untranslated region (UTR) of CTV in the sense orientation, and a second copy of the p23 ORF in the antisense orientation (Fig. 2). The p23 ORF and the 3′ UTR of CTV under the full-length promoter of figwort mosaic virus (FMV; Maiti et al. 1997) were amplified by overlap polymerase chain reaction (Ho et al. 1989) using an infectious cDNA clone of CTV (Satyanarayana et al. 1999) and FMV promoter clone (Gowda et al. 1989) as templates. The amplified product containing *Eco*RI and *Xho*I restriction sites at the 5′ end of the FMV promoter and at the 3′ end of the CTV 3′ UTR, respectively, was digested with *Eco*RI and *Xho*I and cloned into corresponding sites of bacterial plasmid pUC119. Next, the p23 ORF in the antisense orientation with the Rubisco terminator in the sense orientation was generated by overlap polymerase chain reaction (PCR). This product was digested with *Xho*I and *Hin*dIII restriction enzymes and cloned into the pUC119 bacterial plasmid already containing the FMV promoter, p23 ORF, and the 3′ end of CTV in sense orientation. The resulting fragment coding for hairpin structure was cut out and ligated into pGA482GG binary vector (Febres et al. 2003) to obtain pTLAB10.

The plasmid pCTV392 contained the 3′ region of the p23 ORF of CTV (nts 18558–19293) together with the 273 bp 3′ UTR (Fig. 1). Sense (5′-ACATACCGGTTATCAGGGCGC TCGCTTCGCGCGA-3′, *Age*I restriction site—underlined) and antisense (5′-AGATGCGGCCGCTGGACCTAT GTTGGCCCCCCATAGGGA-3′, *Not*I restriction site—

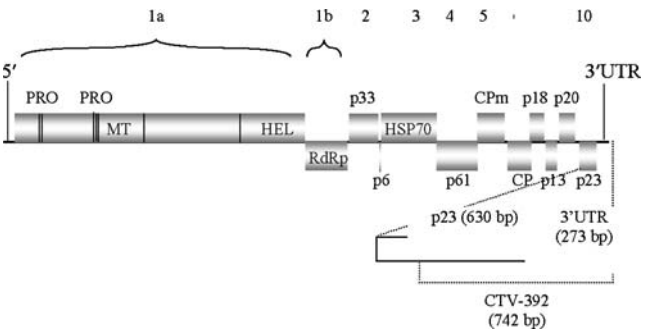

Figure 1. Schematic presentation of the open reading frames (ORFs) in the genomic CTV RNA. The 5′ ORFs *1a* and *1b* produce a fusion protein with two papain-like protease (*PRO*), plus methyltransferase (*MT*), helicase (*HEL*), and the RNA-dependent RNA polymerase (*RdRp*) domains. The 10 ORFs of the 3′ half of the genome encode a 6-kDa hydrophobic protein, a 65-kDa homologue of the HSP70 heat-shock proteins, the 25- and 27-kDa major and minor coat proteins (*CP* and *CPm*, respectively), and other proteins of 33, 61, 18, 13, 20, and 23 kDa. The 3′ p23 ORF, and the 3′ UTR used for construction of CTV-392 are enlarged in the figure.

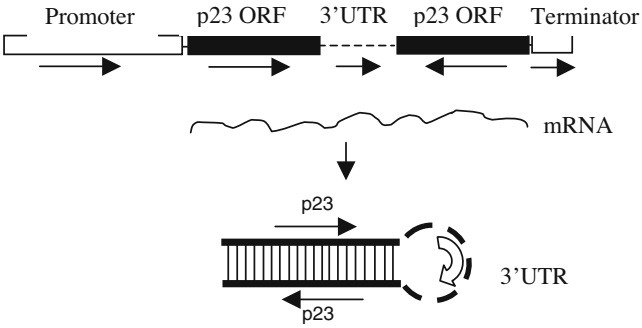

Figure 2. Schematic presentation of the CTV p23 hairpin structure. Orientation of the p23 ORFs and 3′ UTR are indicated by the *arrows*. The *wavy line* represents the CTV-specific mRNA transcribed that can potentially fold into a hairpin structure.

underlined) primers were used to amplify the 742-bp region using an infectious cDNA clone as template (Satyanarayana et al. 1999). The amplified product was cloned into pGEM-T Easy vector (Promega Corp., Madison, WI), subsequently excised as an *Age*I/*Not*I fragment and inserted into the plasmid containing the FMV promoter and Nopaline synthase (NOS) terminator (Gowda et al. 1989). The resulting fragment containing the CTV-392 sequence was excised and ligated into binary vector pCAMBIA2301 (CAMBIA, Canberra, Australia) to obtain pTLAB14. Both binary vectors contained the GUS gene as a reporter. The freeze–thaw method (Walkerpeach and Velten 1994) was used to incorporate pTLAB10 into *Agrobacterium* strain EHA101 and pTLAB14 into EHA105. Standard techniques for the manipulation and cloning into plasmids were as described by Sambrook and Russell (2001).

Nucellar seedlings of the following four grapefruit cultivars were utilized: 'Flame', 'Marsh', 'Ruby Red' (low-seeded commercially important cultivars), and 'Duncan' (a cultivar of low commercial importance but popular in transformation experiments because of its seediness). The seeds were extracted from fruit, peeled to remove the seed coat, and surface-sterilized for 20 min in a 1.5% (w/v) sodium hypochlorite solution. After sterilization, seeds were rinsed with sterile distilled water and germinated in culture tubes containing 20 ml of solid MS medium [consisting of MS salts and vitamins (Murashige and Skoog 1962), sucrose 25 gl^{-1}, agar 8 gl^{-1}, pH 5.8]. Cultures were kept in the dark for 4–5 wk at 26°C. Epicotyls of the seedlings obtained this way were cut into 15- to 20-mm segments, incubated in suspension of appropriate *Agrobacterium* strain (OD 0.5–0.6), and left on solid cocultivation medium for 2 d after which they were transferred to selective regeneration medium for 4–5 wk. After this period of time, those shoots that sprouted from treated explants and were at least 4–5 mm in length were removed from explants and transferred onto growth medium (GM) for an additional 4 wk. All media are as described by Orbović and Grosser (2006).

Screening of transgenic shoots and plants After 2–3 wk, basal cross-sections of shoots growing on GM medium were used for GUS assay (Jefferson et al. 1987). Only those shoots that exhibited blue staining in the GUS assay were micrografted *in vitro* (Peña et al. 1995) onto Carrizo seedlings growing in liquid MS medium with 7% sucrose. All grafted plants were transferred to potting mix and maintained under a controlled environment (light intensity=40 μmol m^{-2} s^{-1}, relative humidity ~80–90%) for acclimatization. These GUS "positive" plants were designated as transgenic if there was an appropriate amplification product in PCR reaction using genomic DNA and p23 specific primers (Fig. 3, data not shown). Transformation efficiency was calculated as a ratio of the number of transgenic plants produced to the number of shoots that sprouted on explants treated with *Agrobacterium*.

Polymerase chain reaction analysis Genomic DNA from transgenic and nontransgenic plants was isolated using the CTAB method (modified by Dr. Erik Mirkov, personal communication). Leaf tissue (1.5 g) was ground to a fine powder in liquid nitrogen. CTAB buffer (10% CTAB in 0.7 M NaCl) and extraction buffer (0.78 M NaCl, 11.2 mM ethylenediamine tetraacetic acid pH 8.0, 112.3 mM Tris-HCl pH 7.5, 1.25% 2-mercaptoethanol), 1.5 and 8 ml, respectively, both prewarmed to 70°C, were added to the ground leaf samples. The samples were subsequently incubated in a water bath at 70°C for 1.5 h. Chloroform/Octanol mix (24:1) was added to the samples, which were shaken well before centrifuging at 5,000 rpm for 10 min. This step was repeated twice. An equal volume of isopropanol was

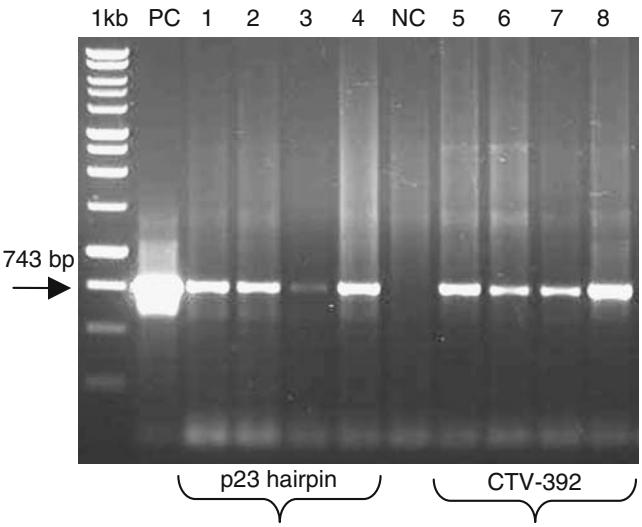

Figure 3. PCR analysis of the regenerated CTV gene positive grapefruit plants. A 743-bp CTV gene fragment (indicated by an *arrow*) in transgenic plants, PC-positive plasmid control, and NC-nontransformed grapefruit plant. The transgenic plant *numbers* are indicated *above* each *lane*. Extreme *left* lane contains a 1-kb molecular marker.

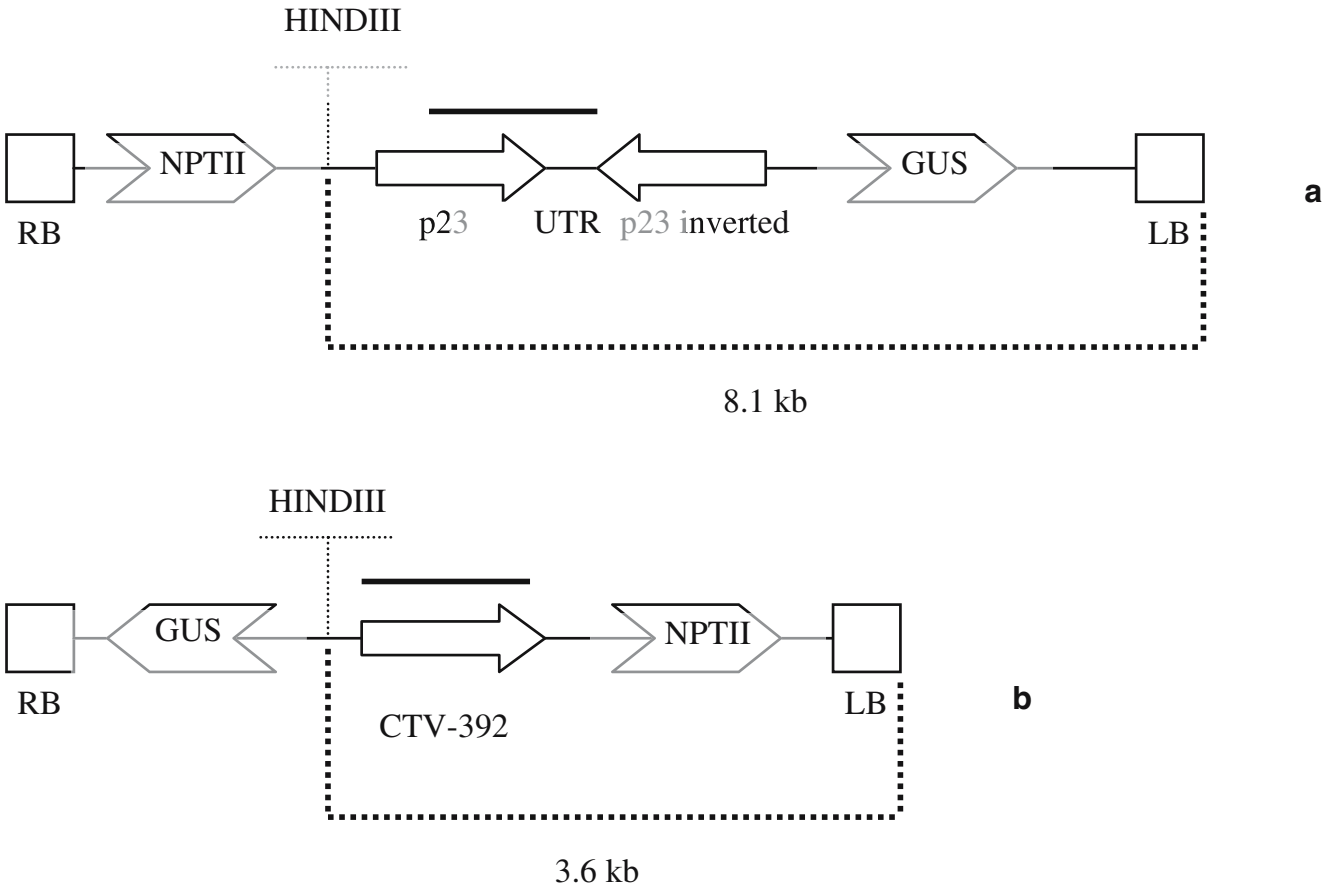

Figure 4. Schematic presentation of T-DNAs from the binary vector *a* pTLAB10 and *b* pTLAB14. For Southern blot hybridizations, DNA was digested with *Hin*dIII, which cuts once in the T-DNAs of both plasmids. The digoxigenin-labeled probe prepared by PCR of 743 bp and designed on CTV-392 coding region is visualized in the figure as a *black bar*.

added to the amount of supernatant recovered and shaken briefly. The mixture was stored at −20°C for 30 min and pelleted at 8,000 rpm for 5 min at 4°C. The pellet was treated with 3 ml of 0.2 M sodium acetate in 76% EtOH, incubated at −20°C for 20 min, and centrifuged at 8,000 rpm for 5 min at 4°C. Then the pellet was rinsed with 10 mM ammonium acetate in 76% EtOH and pelleted at 8,000 rpm for 5 min at 4° C. Subsequently, the pellet was resuspended in TE buffer, treated with RNAse, and pelleted with 3 M sodium acetate pH 5.2 and isopropanol. Finally, the pellet was washed with 70% EtOH, air-dried, and used as a template for the amplification of the transgenes by PCR and for Southern blot hybridization. PCR was carried out in a thermal cycler (MJ Research, Watertown, MA) with an initial denaturing at 94°C for 2 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 2.5 min; and a final extension at 72°C for 5 min. Amplified DNA fragments were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. All photographs were digitized. The primers for the amplification of both CTV p23 hairpin structure and CTV-392

Table 1. Shoot morphogenesis indices, percentage of GUS positive shoots, and efficiency of transformation with CTV-392 sequence (EHA105+ pTLAB14) for different cultivars of grapefruit

Cultivar	Number of explants	Number of shoots	SMI±SE	Percent of GUS positive shoots±SE	Transformation efficiency (%)	Whole plant recovery
Flame	790	814	1.030±na	13.3±na	0.49	4
Marsh	4,220	3,470	0.818±0.070	14.0±1.4	1.35	47
Ruby red	1,845	661	0.360±0.078	21.5±3.5	3.78	25
Duncan	1,838	1,722	0.977±0.260	10.3±3.4	0.64	11
All	8,693	6,667	0.736±0.091	15.3±1.7	1.30	87

Table 2. Shoot morphogenesis indices, percentage of GUS positive shoots, and efficiency of transformation with CTV p23 (hairpin) sequence (EHA101+pTLAB10) for different cultivars of grapefruit

Cultivar	Number of explants	Number of shoots	SMI±SE	Percent of GUS positive shoots±SE	Transformation efficiency (%)	Whole plant recovery
Flame	2,647	787	0.282±0.087	35.2±4.7	2.67	21
Marsh	2,166	446	0.180±0.059	23.0±7.6	3.36	15
Ruby red	675	69	0.100±0.000	39.0±1.0	11.59	8
Duncan	2,186	928	0.483±0.106	29.0±5.6	3.66	34
All	7,674	2,230	0.272±0.050	30.3±3.3	3.50	78

sequence were 5′- CTTTGATACGGAAGAATAGTTAT CAGGG-3′ (C-1350-F) and 5′-GGGCGGCCGCTGGACC TATGTTGGCCCCCC-3′ (C-57-R), which amplified a 743-bp specific fragment.

Southern blot hybridization. Ten micrograms of genomic DNA were digested with 5 U of *Hin*dIII/μg of DNA. *Hin*dIII cuts the T-DNA once in both pTLAB10 and pTLAB14 (Fig. 4). The digested DNA was fractioned on a 1% (w/v) agarose gel (1 cm wide well), blotted onto charged nylon membranes (Roche, Indianapolis, IN), and cross-linked by UV irradiation. Hybridization was performed using a digoxigenin-labeled probe obtained by the amplification of 3′ end of CTV using specific primers C-1350-F and C-57-R as described by Sambrook and Russell (2001). The blots were washed twice for 15 min each in 0.1X SSC+0.1% SDS at 65°C. Hybridized DNA bands were visualized after the DIG chemiluminescent detection protocol provided by the manufacturer. X-ray films were exposed for 20–30 min, and resulting images were digitized.

RT-PCR analysis of the transgene. Total RNA was isolated from leaf tissues of transgenic and nontransgenic grapefruit plants using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from total RNA using SuperScript II RNaseH Reverse Transcriptase (Invitrogen) and a negative primer C-57 (5′GGGCGGCCGCTGGACCTA TGTTGGCCCCCC-3′) at 45°C for 1 h in a total volume of 20 μl. One-fourth volume of the cDNA product was used for PCR. Primers used to amplify CTV sequences were 5′-CGATGCGTTCTCCGGAAGAAAC-3′ (C-342-F) and (C-57-R). Amplified products were electrophoresed on 1% agarose gel, stained with ethidium bromide, and visualized under UV light. The images of RT-PCR products on the gel were digitized.

Protoplast challenge assay/Northern blot hybridization. Assays based on virus challenge of protoplasts were performed to test *in vitro* the resistance of transgenic plants to the virus. Tobacco mosaic virus (TMV) and CTV severe strain T36 virions were purified from infected bark tissue of *Citrus macrophylla* Wester using a 0.04 M phosphate buffer supplemented with 5% sucrose, pH 8.2. *In vitro* virus inoculations mediated by polyethyleneglycol (PEG; Olivares-Fuster et al. 2003) were performed on protoplasts isolated from young leaves of transgenic and control plants according to Grosser and Gmitter (1990). Protoplasts were resuspended at approximately 1.5×10^6 cells in 400 μl of 0.6 M BH3 (Grosser and Gmitter 1990) and divided into two aliquots of 200 μl each. Inoculation of each aliquot was carried out with 20 μl of virus suspension after which 0.4 ml of 30% PEG was immediately added. Forty-five s later, 4 ml of MMC medium (0.6 M mannitol, 0.25 mM MES) containing 10 mM CaCl2 was added. After 5 min of incubation at RT, protoplasts were washed once using light centrifugation in 4 ml of MMC medium [0.6 M mannitol, 6.25 mM MES (2-(4-morpholino)-ethane sulfonic acid)] and cultured with 1 ml of 0.6 M BH3 medium in the dark for 4 d.

For total RNA extraction, the RNeasy Kit (Qiagen, Inc., Valencia, CA) was used. Total RNA was separated by

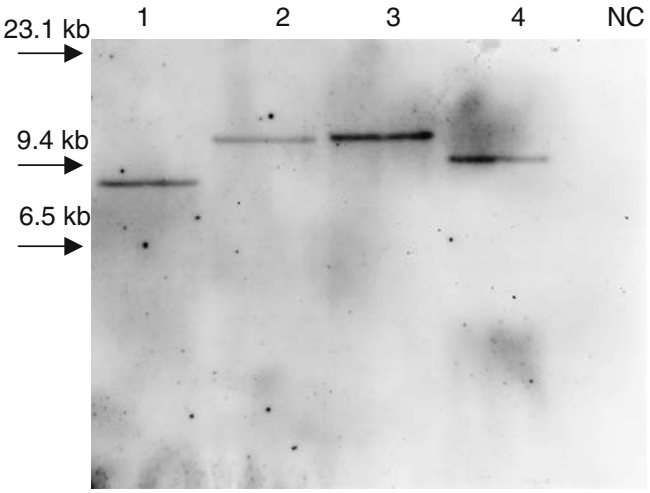

Figure 5. Southern blot analysis of transgenic grapefruit plants transformed with CTV p23 hairpin construct. The plant genomic DNA was digested with *Hin*dIII. *Lanes 1–4* represent independent transgenic plants and NC-nontransformed grapefruit plant. Molecular marker sizes are indicated by *arrows*.

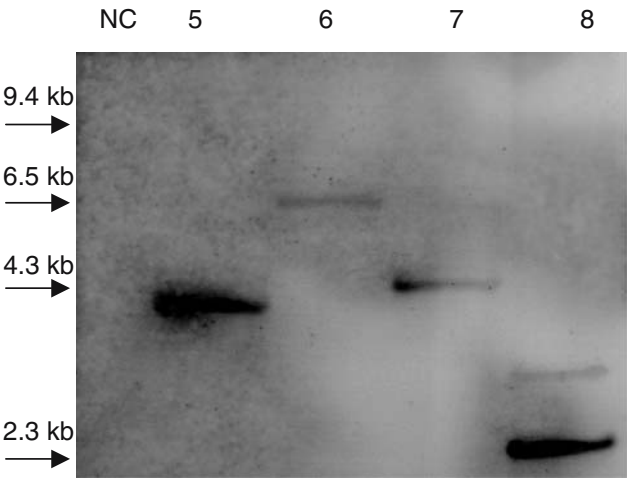

Figure 6. Southern blot analysis of transgenic grapefruit plants transformed with the CTV-392 sequence. The plant genomic DNA was digested with *Hin*dIII. *Lanes 5–8* represent independent transgenic plants and NC-nontransformed grapefruit plant. Molecular marker sizes are indicated by *arrows*.

electrophoresis in a formaldehyde-containing agarose gel and transferred to nylon membrane. Northern blot hybridizations were performed using a 3′CTV negative sense and 3′ TMV negative sense digoxygenin-labeled RNA riboprobes.

Results and Discussion

In the present study, we transformed four grapefruit cultivars with two selected CTV sequences in an attempt to generate CTV resistant citrus plants. Shoot Morphogenesis Index (SMI) as a ratio of the number of shoots and the number of explants on which shoots were found was calculated for all four cultivars of grapefruit. The lowest SMI of 0.10 was recorded for Ruby Red and the highest for Flame explants (1.03). In general, SMI was higher for explants treated with *Agrobacterium* strain EHA105 when compared to explants treated with EHA101 (Tables 1 and 2). Percentage of GUS positive shoots for CTV-392 was very similar for all four cultivars, and it ranged from 10.3 to 21.5% (Table 1). Percentage of GUS positive shoots for CTV p23 hairpin sequence ranged from 23.0% to 39.0% (Table 2). The transformation efficiency varied with *Agrobacterium* strain and grapefruit cultivar. Recorded efficiency was higher for

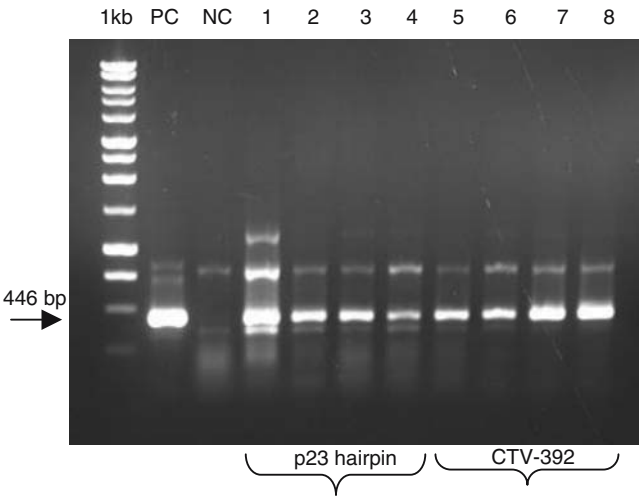

Figure 7. Analysis of transgene transcription by RT-PCR. cDNA prepared from leaves of individual transformants (numbered *1–8*) were used as templates for RT-PCR using a combination of the CTV primer and the adaptor sequence primer used for cDNA synthesis. Ethidium bromide staining of RT-PCR products (446 bp indicated by an *arrow*) separated by agarose gel electrophoresis. NC-nontransgenic control grapefruit plant exhibiting no RT-PCR amplification. *PC*, positive control consisting of the plasmid DNA.

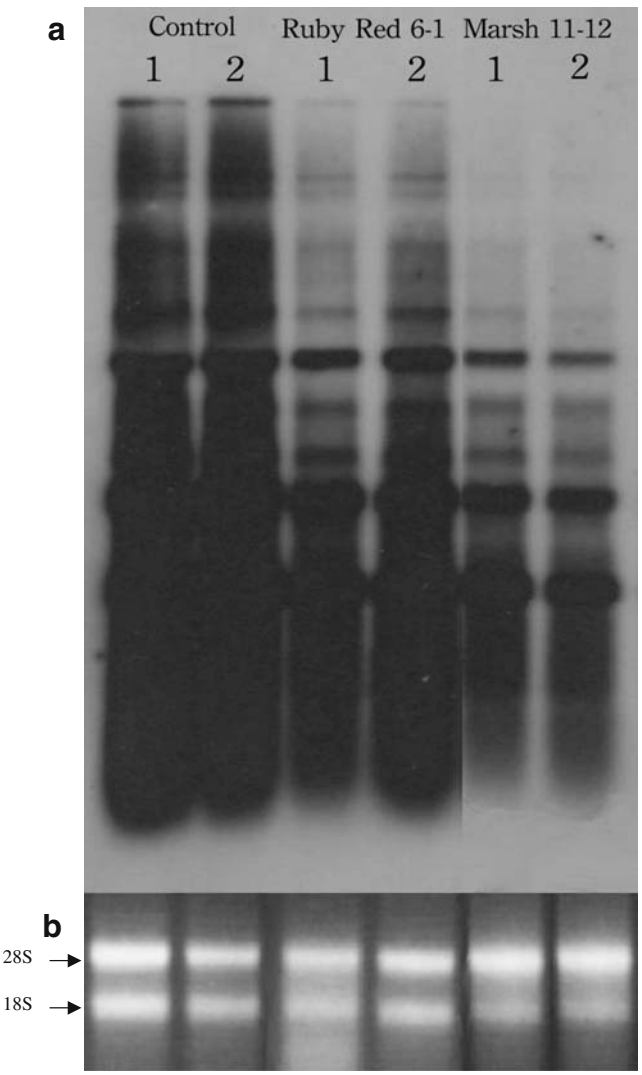

Figure 8. Northern blot analysis of RNA extracted from infected control and transgenic pTLAB14 grapefruit protoplasts after 4 d of incubation. (*a*) Different CTV replication levels are shown following the infection of protoplasts with CTV virions; (*b*) ethidium bromide staining of RNA in a denaturing gel shows that equivalent amounts of RNA were loaded into each lane. *Lanes 1* and *2* represent two treatments on same batch of protoplasts.

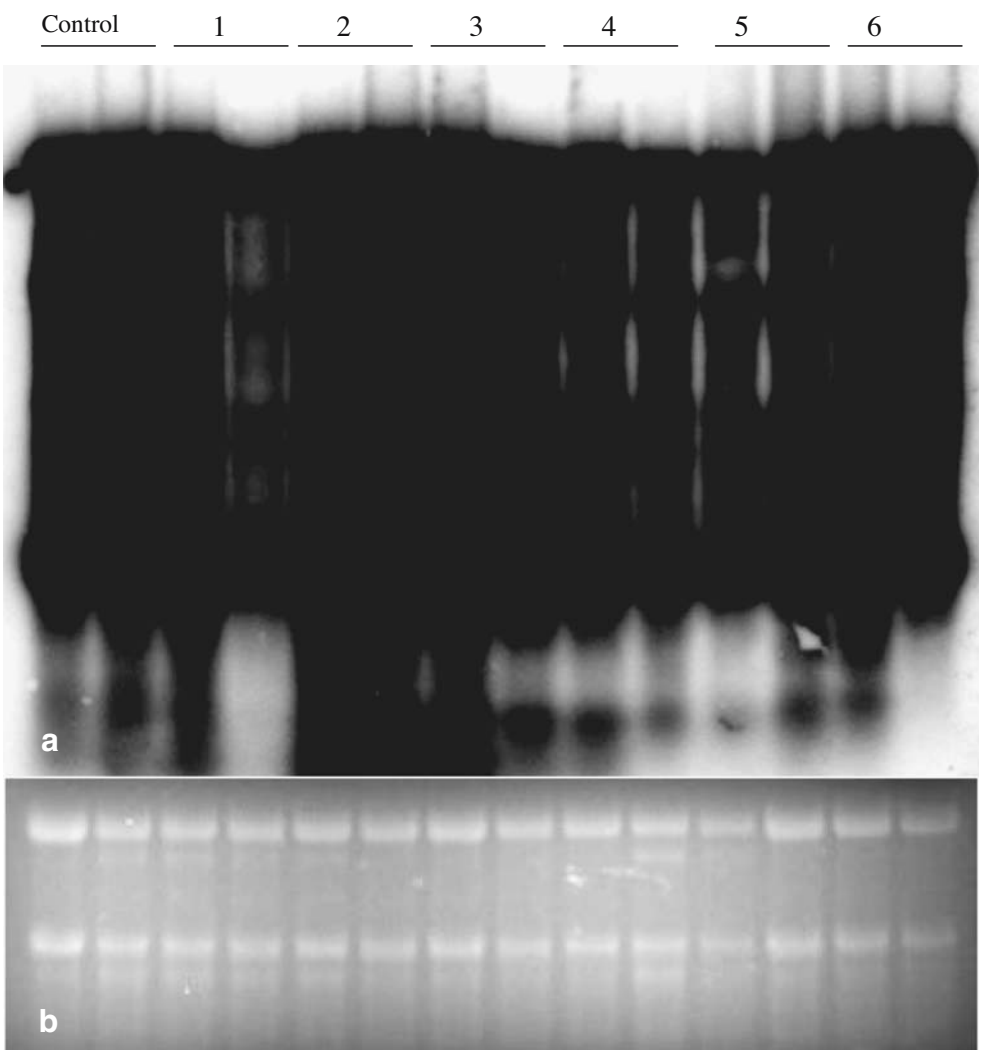

Figure 9. Northern blot analysis of RNA extracted from infected control and transgenic pTLAB14 citrus (numbers *1–6*) protoplasts after 4 d of incubation. *a* TMV replication levels are shown following the infection of protoplasts with TMV virions; *b* ethidium bromide staining of RNA in a denaturing gel shows that equivalent amounts of RNA were loaded into each lane. Control: non-transgenic 'Duncan' grapefruit; *1*: 'Marsh' 10–20; *2*: 'Valencia' sweet orange 23–1; *3*: 'Duncan' 13–8; *4*: 'Marsh' 11–12; *5*: 'Marsh' 13–21; and *6*: 'Marsh' 13–23.

explants treated with *Agrobacterium* strain EHA101 when compared to explants treated with EHA105 (Tables 1 and 2). It has been previously reported that EHA101 strain was more efficient than A518 strain for transformation of sour orange (Gutiérrez et al. 1997). On the other hand, EHA105 strain was more efficient in transformation of sweet orange than Ach5 and C58 strains (Cervera et al. 1998).

In the present study, regenerated plants were prescreened by using a thin section of the basal portion of the shoots for GUS histochemical analysis, so that transgenic plants could be saved without further damage. In earlier reports, many shoots recovered on selective medium were found to be escapes, i.e., not GUS positive (Moore et al. 1992), and the ineffectiveness of kanamycin selection has also been previously reported in citrus (Kobayashi and Uchimiya 1989; Hidaka et al. 1990; Moore et al. 1992; Peña et al. 1995). Some researchers have found that the number of escapes from citrus stem segments was decreased by prolonged continuous exposure to kanamycin, e.g., for 5–6 mo. (Peña et al. 1995). In our experiments, prolonged exposure to kanamycin did not promote any new shoots and eventually retarded growth. To facilitate a rapid whole plant recovery, GUS-positive transgenic shoots were micrografted onto Carrizo seedlings (Peña et al. 1995).

Amplification of incorporated DNA in transgenic plants by polymerase chain reaction was performed to confirm the presence of the CTV p23 hairpin/CTV-392 sequence. PCR analysis showed the predicted size of amplification product for all transgenic plants tested (data not shown). In Fig. 3, results of PCR analysis for eight randomly selected plants are presented, including four containing the p23-hairpin sequence and four with the CTV-392 sequence.

DNA isolated from selected transgenic plants and a nontransformed control plant was digested with *Hin*dIII and analyzed by Southern blot. The CTV-specific probe was used to confirm the presence of the transgene. A single copy insertion event was identified with CTV p23 hairpin transgenic plants, and both single and double copy insertion events were identified with CTV-392 transgenic plants (Figs. 5 and 6). As expected, no hybridization signal was

detected in the nontransformed control plant. These results demonstrate that we have obtained stably transformed grapefruit plants containing both CTV-derived target genes. In lane 8 of Fig. 6, there are two bands, and both of them are smaller in size than the smallest expected band of 3.6 kb (Fig. 4*b*), which suggests incomplete integration of the T-DNA. Integration of incomplete T-DNAs has been suggested as a possible factor contributing to the absence of transgenes in transgenic plants (Dominguez et al. 2002).

The same transgenic plants that were used for PCR and Southern analysis were also tested with RT-PCR for the presence of desired transcript. The results (Fig. 7) confirm that the transgene is transcribed in the transgenic grapefruit plants. As expected, no amplification was found in the nontransgenic grapefruit plant. Other unexpected bands in Fig. 7 were regarded as genomic artifacts.

Unfortunately, the preliminary screen of transgenic grapefruit plants using the protoplast challenge assay showed that the goal of complete RNA-mediated resistance was not achieved in transgenic plants tested. However, banding patterns from repeated Northern blot analyses suggested lower levels of CTV virion replication in a few of the plants containing the CTV-392 sequence (3 of 57 plants tested). Northern blot analyses (Fig. 8*a*) confirmed high replication levels of the virus after infection and 4 d of incubation in control protoplasts ('Duncan' wild-type) and in transgenic 'Ruby Red' 6–1. Under same conditions, lower replication levels were found in 'Marsh' 11–12 protoplasts (Fig. 8*a*). All three plants repeatedly showing lower CTV replication levels were from the cultivar 'Marsh'. To confirm that the protoplasts of plants challenged with CTV virions do not have decreased capacity to respond to virus infection in general, we performed challenge test with TMV virions. It is clear that the replication levels of TMV attained in the protoplasts obtained from all sources, including those that showed decreased levels of CTV replication ('Marsh' 11–12, 'Marsh' 13–21, and 'Marsh' 13–23), are similar (Fig. 9*a*). Although the meaning of this result is unclear at present, it suggests the possibility of CTV resistance in these transgenic 'Marsh' plants. An alternative explanation could be a differential uptake of CTV virions by grapefruit protoplasts, but this was never observed in numerous control runs. As mentioned, the protoplast challenge assay is a preliminary test that still requires validation by standard greenhouse CTV inoculation followed by enzyme-linked immunosorbent assay (ELISA). Moreover, CTV replication in grapefruit is known to be inconsistent and sometimes sectored in field-grown grapefruit trees (Steve Garnsey, personal communication). None of the plants containing the CTV-hairpin p23 sequence exhibited the ability to limit the replication of CTV virions in protoplasts (69 plants tested). Therefore, work is ongoing to correlate the protoplast challenge assay results with standard whole plant CTV challenge methods in the greenhouse and monitoring of virus replication via ELISA (Bar-Joseph et al. 1979).

This is the first report of successful transgenic plant production for 'Marsh' and 'Flame' grapefruits, whereas transgenic plants of 'Ruby Red' have been recently reported (Gonzalez et al. 2005). Efforts are underway to improve citrus transformation efficiency and to produce more transgenic plants, especially with sweet oranges using the CTV-392 sequence. If identified, resistant plants of the commercially important cultivars must be field-tested for cultivar integrity and yield and could be of direct utility to the Florida citrus industry, assuming consumer acceptance of genetically modified fruit.

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