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Agrobacterium-mediated transformation of grapefruit with the wild-type and mutant RNA-dependent RNA polymerase genes of *Citrus tristeza virus*

Bayram ÇEVİK[1,*], Richard F. LEE[2], Charles L. NIBLETT[3]

[1]Department of Plant Protection, Faculty of Agriculture, Süleyman Demirel University, 32260 Isparta - TURKEY

[2]USD-ARS National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA 92521 - USA

[3]Venganza, Inc., Raleigh, NC 27606 - USA

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Abstract: *Citrus paradisi* Macfad. 'Duncan' was transformed with constructs coding for the wild-type and mutant RNA-dependent RNA polymerase (RdRp) of *Citrus tristeza virus* (CTV) for exploring replicase-mediated pathogen-derived resistance (RM-PDR). The RdRp gene was amplified from a CTV genome and used to generate the wild-type and 2 mutant RdRp constructs for plant transformation. One mutant had the key amino acids GDD changed to AAA (RdRp-mGDD), and the second mutant had a deletion encompassing the GDD domain (RdRp-ΔGDD). Etiolated epicotyl segments of Duncan grapefruit (*Citrus paradisi* Macfad. 'Duncan') were transformed with each of these constructs using the *Agrobacterium*-mediated transformation method. From 4540 transformed epicotyl segments, 1402 kanamycin-resistant shoots were regenerated. After testing for expression of green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter genes by fluorescence microscopy and histochemical staining, respectively, 146 GUS-positive plants were rooted and 97 surviving plants were established in soil in pots. A total of 70 plants were tested for the presence of the GUS gene and CTV RdRp transgenes by polymerase chain reaction (PCR). A total of 51 GUS and CTV transgene-positive transgenic plants (15 with RdRp, 21 with RdRp-mGDD, and 15 with RdRp-ΔGDD) were identified.

Key words: *Citrus tristeza virus*, plant transformation, replicase-mediated resistance, RNA-dependent RNA polymerase

Turunçgil tristeza virüsünün (*Citrus tristeza virus*) normal ve mutant RNA-bağımlı RNA polimeraz genlerinin *Agrobacterium* aracılığıyla altıntopa aktarılması

Özet: *Citrus paradisi* Macfad. 'Duncan' turunçgil tristeza virüsünün (*Citrus tristeza virus* = CTV) normal ve mutant RNA-bağımlı RNA polimerazını (RdRp) kodlayan gen yapılarıyla transforme edilerek turunçgillerde replikaza dayalı etmenden elde edilen dayanıklılık (RD-EED) araştırılmıştır. RdRp geni CTV genomundan çoğaltılarak bitki transformasyonunda kullanılmak üzere bir normal ve 2 mutant RdRp oluşturulmuştur. Mutantlardan birinde RdRp'lerin anahtar amino asitleri GDD AAA'ya (RdRp-mGDD) dönüştürülürken diğer mutantta ise RdRp'nin GDD motifini içine alan bir kısmı çıkartılmıştır (RdRp-ΔGDD). Duncan altıntop (*Citrus paradisi* Macfad. 'Duncan') bitkisinin etiole edilmiş epikotil parçaları hazırlanan bu gen yapılarıyla transforme edilmiştir. Transformasyon yapılan 4540 epikotil parçasından 1402 kanamisine dayanıklı sürgün elde edilmiştir. Yeşil floresan ve β-glukuronidaz (GUS) raportör

* E-mail: bayramcevik@sdu.edu.tr

genlerinin floresan mikroskop ve histokimyasal boyama yöntemiyle test edilmesi sonucunda belirlenen 146 GUS pozitif bitki, köklendirilerek bunlardan canlı kalan 97 tanesi topraklı saksılara aktarılmıştır. Toplam 70 bitkinin raportör GUS geni ve CTV RdRp transgenlerini içerip içermedikleri polimeraz zincir reaksiyon (PCR) yöntemiyle test edilmiştir. Bu testler sonucunda 15 RdRp, 21 RdRp-mGDD ve 15 RdRp-ΔGDD olmak üzere toplam 51 GUS ve CTV RdRp transgen-pozitif özellikte potansiyel transgenik bitki belirlenmiştir.

Anahtar sözcükler: Bitki transformasyonu, replikaza-dayalı dayanıklılık, RNA-bağımlı RNA polimeraz, turunçgil tristeza virüsü

Introduction

Pathogen-derived resistance (PDR) was first proposed as a strategy to confer resistance against one specific pathogen or a range of pathogens by transforming host cells with genes or sequences derived from the genome of the pathogen (Sanford and Johnston 1985). PDR was first demonstrated against *Tobacco mosaic virus* (TMV) in transgenic tobacco plants that expressed the coat protein (CP) gene of TMV (Abel et al. 1986). Since then, CP-mediated resistance (CPMR) has been developed successfully against a wide number of economically important viruses belonging to many genera in a number of crop plants including vegetables, fruits, cereals, and forage crops (Baulcombe 1994; Hackland et al. 1994; Beachy 1997; Fuchs and Gonsalves 1997). However, attempts to develop CPMR failed for some plant viruses. This promoted the use of nonstructural viral genes to develop resistance. Plants were transformed with noncoding sequences from the 5' and/or 3' untranslated regions (UTRs) of viral genomes (Nelson et al. 1993; Zaccomer et al. 1993), satellite RNAs (Harrison et al. 1987), defective interfering RNAs (Kollar et al. 1993), and a wide range of nonstructural genes encoding proteases (Maiti et al. 1993), cell-to-cell movement proteins (Malyshenko et al. 1993), and RNA replicase and replication-associated proteins (Carr and Zaitlin 1991; Palukaitis and Zaitlin 1997) to produce transgenic plants resistant to one or more viruses. Although results varied in different plant-virus systems, the use of nonstructural genes, especially movement proteins and replication-associated proteins such as the RNA-dependent RNA polymerase (RdRp), was shown to be an effective and promising strategy to develop virus resistance in transgenic plants (Beachy 1997; Palukaitis and Zaitlin 1997).

Citrus tristeza virus (CTV) is one of the most important pathogens of citrus. It causes significant economic losses in most citrus-producing regions of the world. PDR against CTV has not been explored in citrus due to limitations in available transformation methods and the complex genome of CTV. Recent improvements in citrus transformation techniques and characterization of the CTV genome have enabled the possible application of PDR in citrus. The CP gene of CTV has been introduced into sour orange (Gutiérrez et al. 1997), Mexican lime (Dominguez et al. 2000), and grapefruit (Febres et al. 2003) to develop CPMR against CTV. In addition, transgenic Duncan grapefruit plants expressing the CP and other sequences from the CTV genome have been produced (Febres et al. 2003). Recently, transgenic Mexican lime (*Citrus aurantifolia*) plants expressing translatable and untranslatable forms of the p23 gene of CTV were also produced (Ghorbel et al. 2001). It was reported that transgenic plants expressing the translatable p23 gene had symptoms similar to CTV-infected Mexican lime (Ghorbel et al. 2001). This indicates that the p23 gene is involved in the pathogenicity of CTV and symptom formation in citrus (Ghorbel et al. 2001). However, RNA-mediated resistance was recently developed by the expression of the untranslated p23 gene of CTV in Mexican lime plants (Fagoaga et al. 2006). Although the untranslated form of the p23 gene conferred resistance to CTV, no protein-mediated resistance was reported in transgenic plants expressing the CP or other genes of CTV. Therefore, it appears to be useful to explore the use of other genes, including those coding for replication-associated proteins, as a strategy for developing PDR to CTV.

Replicase-mediated (RM) PDR against many RNA viruses has been developed using the wild-type, untranslatable, and defective or mutant constructs of their RdRp genes in many annual crops. While

resistance to some viruses was developed by expressing a full-length (wild-type) RdRp gene (Golemboski et al. 1990; MacFarlane and Davies 1992; Rubino et al. 1993; Rubino and Russo 1995; Kaido et al. 1995; Sijen et al. 1995; Huet et al. 1999; Thomas et al. 2000), defective RdRp genes with deletions of the 3' or 5' terminus, or of the highly conserved GDD motif or point mutations in the GDD motif, were used to engineer RM-PDR against other viruses in annual crops such as tobacco, tomato, potato, and pepper (Anderson et al. 1992; Audy et al. 1994; Donson et al. 1993; Longstaff et al. 1993; Brederode et al. 1995; Guo and Garcia 1997). However, RM-PDR has not been explored in woody perennial crops due to difficulties associated with transformation and regeneration of these plants as well as the difficulty of in vivo testing for viral resistance in these plants. Constructs coding only for full-length RdRp (Febres et al. 2003) and untranslated and antisense constructs (Çevik et al. 2006) of the RdRp gene of CTV have been introduced into citrus; however, to date, no RM-PDR against CTV or any other virus has been reported in citrus.

In this study, epicotyl segments of Duncan grapefruit seedlings were transformed using an *Agrobacterium*-mediated transformation protocol with the wild-type and 2 modified constructs of the RdRp gene of coding for mutant RdRp proteins in order to explore the potential of RM-PDR in *Citrus*, a woody perennial plant. Transgenic grapefruit plants carrying all 3 constructs of the CTV RdRp gene were regenerated in vitro and established in the greenhouse.

Materials and methods

Mutagenesis and cloning of the CTV RdRp gene for transformation

The RdRp gene of stem-pitting isolate 3800 of CTV isolated from a grapefruit tree in Florida was amplified from a cDNA clone by reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers CN356 and CN357 (Table 1). Since the RdRp gene is expressed by a +1 translational frameshift and does not possess a translation initiation codon, an ATG codon and a translational enhancer sequence (ACC) from *Cucumber mosaic virus* (CMV) were incorporated into the 5' end of the RdRp sequence during RT-PCR amplification. In addition, a *Not* I restriction site was integrated into both the 5' and 3' ends of the sequence to facilitate cloning (Table 1). This sequence was designated as RdRp (Figure 1) and used as the template to generate the other constructs.

Table 1. Primers used for PCR amplification, mutagenesis, and sequencing of the RdRp constructs and PCR amplification of the GUS gene.

Primer	Sequence (5' to 3')	Orientation
CN216*	CAACGAACTGAACTGGCAG	Sense
CN217*	CATCACCACGCTTGGGTG	Antisense
CN309	TGTTTTGTACCGGACCCTTA	Sense
CN310	GTACTCGCCTTCCATCCA	Antisense
CN356	*AAAGCGGCCGCACC**ATG**G*AGACACTGCCCCTCCCGACTCC	Sense
CN357	*AAAGCGGCCGC*TCAGCCGGTCGCTAAGTCGTCCG	Antisense
CN358	GTCCG**C**TG**C**TG**CTAGC**TTGATTTACTCCAAAAAAGG	Sense
CN359	CAA**GCTAGC**A**G**CA**G**CGACACGAGAAGTAACTCG	Antisense

Italic letters are non-CTV sequences.
Underlined letters indicate specific restriction sites incorporated into the primers.
Bold letters describe the mutations.
Asterisks indicate GUS gene-specific primers.

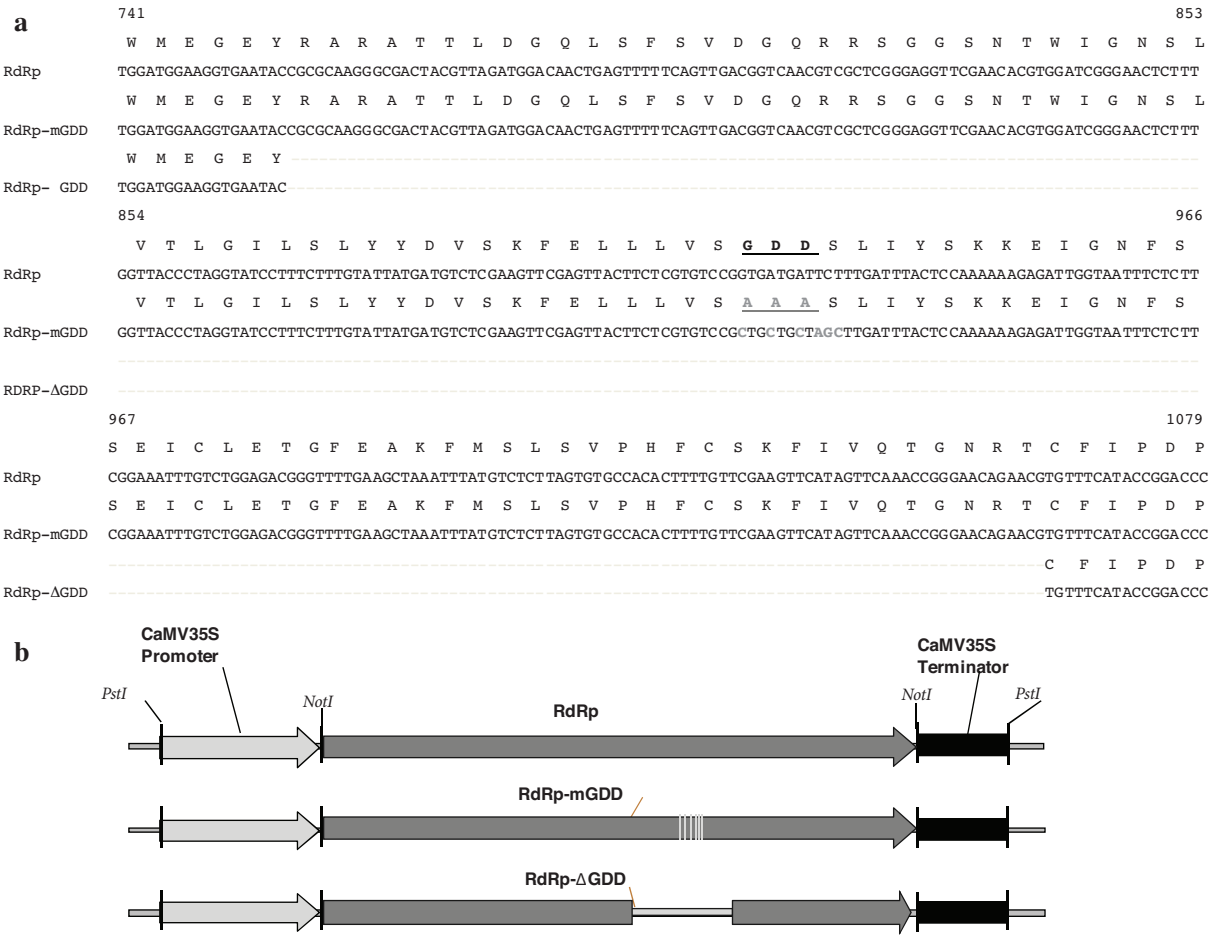

Figure 1. Cloning and mutagenesis of wild-type and mutant CTV RdRp constructs for plant transformation. a) Partial sequence alignments of wild-type and mutant CTV RdRp plant transformation constructs showing site-directed mutations in the GDD motif and the 300-bp deletion in that region. Wild-type CTV sequences are shown in black; site-directed mutations are displayed in gray letters. Dashes indicate deleted sequences. b) Partial T-DNA map of RdRp plant transformation constructs showing site-directed mutagenesis and deletions. The parts of the constructs are indicated above the first construct, and the mutations and deletions in the RdRp are shown in gray vertical and horizontal lines, respectively.

To construct a dysfunctional RdRp, which may act as a negative dominant mutation, the conserved GDD motif in CTV RdRp was mutated to AAA by site-directed mutagenesis using an overlap extension PCR method (Urban et al. 1997). First, overlapping sense and antisense internal oligonucleotide primers CN358 and CN359 (Table 1) with the desired mutation were designed. They were used with an external primer, CN356 or CN357, specific to the 5' and 3' ends of the RdRp gene for PCR amplification of 2 overlapping DNA fragments from the RdRp gene. These DNA fragments were then mixed and used as a template for a second round of PCR amplification using only the external primers (CN356 and CN357) to produce a complete RdRp open reading frame (ORF) with the desired mutations, designated as RdRp-mGDD (Figure 1).

A 300-bp nucleotide sequence containing the GDD coding region was deleted using a ligation-mediated PCR method (Guilfoyle et al. 1997) to produce another dysfunctional RdRp, which may function as a negative dominant mutant. First, one antisense (CN310) and one sense (CN309) in-frame internal primers, located up- and downstream of the GDD coding region, respectively, were selected. They were used with an external primer, CN356 or CN357,

specific to the 5' and 3' ends of the RdRp gene for PCR amplification of 2 DNA fragments of the CTV-RdRp. These DNA fragments were mixed and ligated using T4 DNA ligase. The ligation mixture was used as the template for a second round of PCR amplification using the external primers (CN356 and CN357) to amplify the approximately 1200-bp RdRp gene with the desired 300-bp deletion spanning around the GDD coding region. This construct was designated as RdRp-ΔGDD (Figure 1).

A pUC118-based plasmid vector containing the *Cauliflower mosaic virus* (CaMV) 35S promoter and termination signal (pUC118 CaMVP-T), kindly provided by Dr Vicente Febres, was used to generate the RdRp transgene constructs. First, the PCR-amplified and modified CTV RdRp sequences were cloned into a *Not* I site between the CaMV 35S promoter and the termination signal. The resulting pUC118 CaMVP-T clones with RdRp constructs were digested with *Pst* I to release the CTV RdRp sequences with the CaMV 35S promoter and termination signal. These fragments were cloned into the *Pst* I site in the binary plant transformation vector pCambia 2203 (Cambia, Australia) with the Npt II gene as a selectable marker and β-glucuronidase (GUS) and green fluorescent protein (GFP) genes as bifunctional reporter genes. The plasmids were sequenced to ensure the integrity of all constructs. The pCambia 2203 binary vectors with RdRp constructs were introduced into *Agrobacterium tumefaciens* strain AGL I using the freeze-thaw transformation method (Höfgen and Willmitzer 1988).

Transformation of citrus with wild-type or mutant RdRp genes of CTV

An *Agrobacterium*-mediated transformation protocol, previously developed for epicotyl segments of etiolated seedlings of Duncan grapefruit by Luth and Moore (1999), was used with antisense and untranslatable RdRp genes of CTV (Çevik et al. 2006) for all stages of transformation and regeneration of transgenic plants in this study. Briefly, Duncan grapefruit seeds were germinated in 150 × 25 mm tubes containing half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (2.13 g L^{-1} MS salt, 50 mg L^{-1} myo-inositol, and 15 g L^{-1} sucrose at pH 5.7) with 7 g L^{-1} agar in the dark at 28 °C (Figure 2a). The epicotyl portions of the etiolated seedlings were cut into 1-cm segments (Figure 2b) and soaked in the *Agrobacterium* inoculum with a final concentration of 5×10^8 cfu mL^{-1} in MS medium with 100 mM acetosyringone for 1 min (Figure 2c). The inoculated segments were placed horizontally on petri plate medium (MS medium plus 7 g L^{-1} Bacto agar and 100 mM acetosyringone) and cocultivated with *Agrobacterium* in the dark at room temperature for 2-3 days (Figure 2d). After 2-3 days of cocultivation, the epicotyl segments were transferred into a shooting medium [MS medium with 0.5-2.0 mg L^{-1} benzyl adenine (BA) and 7 g L^{-1} Bacto agar supplemented with 500 mg L^{-1} Claforan® (cefotaxime sodium) and 75 mg L^{-1} kanamycin sulfate]. Potentially transgenic shoots were then selected (Figures 2e and 2f). Kanamycin-resistant shoots of 5-10 mm in length were removed from the explants, and a small section was cut from the basal end for histochemical GUS staining. The shoots were then transferred onto rooting medium [MS medium with 0.5 mg L^{-1} naphthalene acetic acid (NAA)] (Figure 2g) for development of roots for 3-5 weeks or longer (Figures 2h and 2i). Rooted plants were transferred to sealed culture jars containing sterilized soil and half-strength MS (Figure 2j) and maintained in a growth chamber at 28 °C with a 16-h photoperiod (Figure 2k). Surviving plants were transferred into pots with soil and maintained in the greenhouse (Figure 2l).

Analysis of transgenic shoots and plants

Fluorescent microscopy: Epicotyl segments transformed with *Agrobacterium* containing the CTV RdRp constructs, and shoots regenerated from these segments, were examined periodically for the expression of GFP using a dissecting microscope (Zeiss) as previously described (Çevik et al. 2006).

β-Glucuronidase (GUS) assay: Basal sections of regenerated shoots or small leaves of whole plants were removed and placed in 96-well assay plates or 2-mL Eppendorf tubes containing GUS assay solutions (50 mM Na_2PO_4, pH 7.0, 10 mM Na_2EDTA, and 5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide). Histochemical GUS staining was performed and GUS activity was scored as partial and solid, as previously described (Çevik et al. 2006).

Polymerase chain reaction: Putative transgenic plants were tested for the presence of GUS and wild-type and mutant CTV RdRp gene sequences

using PCR with gene-specific primers. Genomic DNA was isolated using 1 cm^2 of leaf tissue from all putative transgenic plants with a rapid genomic DNA extraction method as reported for *Arabidopsis* (Edward et al. 1991) and/or citrus (Oliveira et al. 2000) and tested for the presence of the GUS and the RdRp gene by PCR with 2 sets of primers, the first set specific to the GUS gene (CN216 and CN217) and the other (CN309 and CN310) specific to the 5' half of the RdRp gene of CTV (Table 1), as described previously (Çevik et al. 2006).

Results

Regeneration of transgenic plants

A total of 4540 epicotyl segments were transformed with *A. tumefaciens* strain AGL I, carrying the binary plasmid pCambia 2203 with 3 different constructs of the RdRp gene of CTV. About 30% of these segments produced kanamycin-resistant shoots, resulting in 1402 total shoots for all 3 constructs. The results for transformation experiments with individual RdRp constructs are summarized in Table 2. During regeneration, transformed segments and shoots regenerated on these segments were examined weekly for the expression of GFP using a dissecting fluorescent microscope. The expression of GFP was detected in some segments (Figure 2m) during the regeneration process. While no GFP was detected in nontransgenic control shoots (Figure 2n), it was clearly detected in the potentially transgenic shoots (Figure 2o). Even though GFP was clearly detected in the budding segments when they were etiolated, detection of GFP was hindered by intense chlorophyll in the green shoots, and GFP expression was not detected consistently in some transgenic shoots. Therefore, fluorescent microscopy was not used for routine scoring of transgenic plants, and putative transgenic shoots were primarily scored using the histochemical GUS assay.

Before the shoots were transferred to the rooting media, a section from the basal end of all 1402 regenerated potential transgenic shoots from nontransgenic controls (Figure 2p) was tested for GUS activity using histochemical staining. From the 1402 shoots, 201 (14.3%) showed GUS staining (Table 2), ranging from a few blue dots (Figure 2q) to completely blue-stained cuts (Figure 2r). These were considered to be putative transgenic shoots. Sections from the remaining shoots did not show any visible GUS staining under the dissecting microscope, and no GUS staining was observed on the cuts from untransformed shoots. Based on the degree of the GUS staining, shoots were classified as solid GUS positives showing complete blue staining (Figure 2r), or as partial GUS positives with only a few blue-stained cells. The majority of the shoots tested showed partial GUS staining, and only 15% of total GUS positive shoots showed solid blue staining. The results of the histochemical staining for the individual constructs are summarized in Table 2. The overall efficiency of transformation for this study was 14.3%, ranging from 12.5% (RdRp) to 15.7% (RdRp-ΔGDD) for individual constructs.

The regenerated shoots were rooted in MS media containing 0.5 mg mL^{-1} NAA, and rooted GUS-positive (GUS+) plants were transferred into soil in sterile small glass jars with lids. A total of 201 GUS+ shoots were placed onto rooting media, and 67.6% of them rooted and were transferred into soil jars. The remaining shoots did not survive or did not root. The number of rooted GUS+ plants was higher for constructs RdRp-mGDD and RdRp-ΔGDD than for the wild-type RdRp construct. The results are summarized in Table 2. The difference was due to the higher concentration of BA used for shoot regeneration in some initial experiments with the RdRp wild-type construct, which reduced the rooting efficiency of transgenic shoots, and some GUS+ shoots from these constructs did not root. In 1-3 months, or when the surviving plants grew out of the jars, they were transplanted into nonsterile pots containing soil mixture and maintained in the greenhouse. A total of 97 plants were potted and transferred to the greenhouse (Table 2). Most of these plants survived and are still maintained in the greenhouse. There were more than 10 putative transgenic lines established and maintained in the greenhouse for each CTV-RdRp construct.

Analysis of transgenic plants by PCR

Initially, 10 putative transgenic plants that repeatedly showed solid GUS staining were selected for PCR analysis. Their genomic DNA was isolated and tested for the presence of GUS and the RdRp genes using a

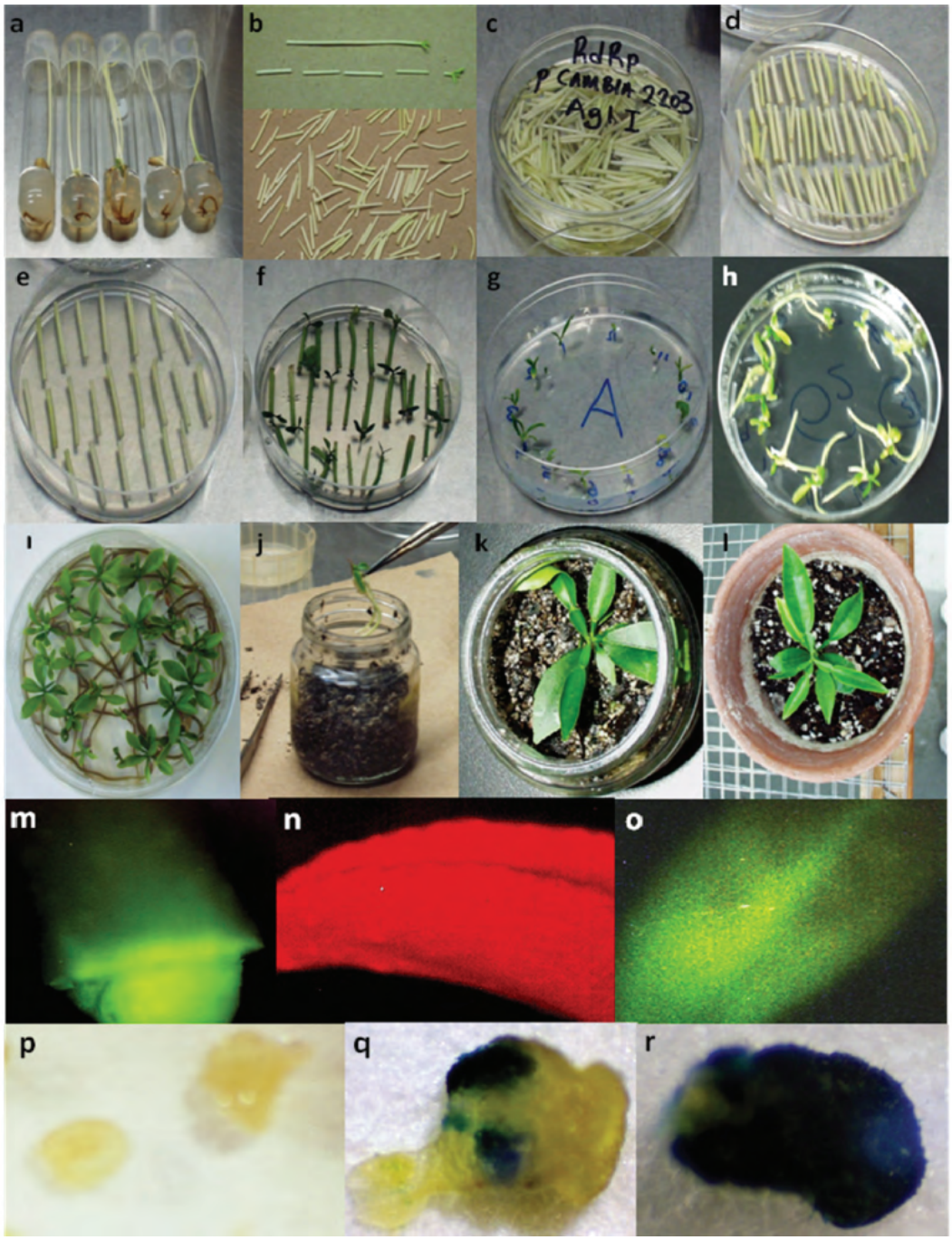

Figure 2. Production and analysis of transgenic plants from epicotyl segments of grapefruit using an *Agrobacterium*-mediated transformation method. a) *In vitro* production of etiolated seedlings. b) Preparation of etiolated seedling segments for transformation. c) Inoculation and d) cocultivation of epicotyl segments with *Agrobacterium* strain AGL I containing the binary plasmid with CTV-RdRp constructs. e) Early and f) late stages of regeneration and selection of transgenic shoots in medium containing benzyl adenine and kanamycin sulfate. g) Early, h) middle, and i) late stages of rooting transgenic shoots in a naphthalene acetic acid-containing medium. j) Transfer and k) maintenance of rooted transgenic shoots in the soil jars. l) Establishment of transgenic plants in soil pots in the greenhouse. Fluorescent microscopy of m) a transformed stem segment, n) leaves from a nontransgenic control shoot, and o) a leaf of a transgenic shoot transformed with the RdRp-mGDD construct. Histochemical GUS staining of the basal end of p) nontransgenic shoots, and putative transgenic shoots showing q) partial and r) solid staining.

Table 2. Summary of transformation experiments with 3 different CTV-RdRp gene constructs.

Construct	No. of segments	No. of regenerated shoots		No. of GUS+ shoots		% GUS+ shoots		No. of shoots	
		Total	%*	Total	Solid	Total	Solid	Rooted	Potted
RdRp	1220	511	41.9	64	9	12.5	14.1	31	22
RdRp-mGDD	1640	459	28.0	69	10	15.0	14.5	51	37
RdRp-ΔGDD	1680	432	25.7	68	11	15.7	16.2	54	38
Total	4540	1402	30.8	201	30	14.3	14.9	136	97

*The shoot percentage was calculated using the total number of shoots regenerated from the total number of segments.

specific primer whose approximate location is shown in the map of the transfer DNA (T-DNA) region of the binary plasmid (pCambia 2203) used for genetic transformation (Figure 3a). An 800-bp fragment was amplified from all 10 putative transgenic plants and pCambia 2203 plasmid DNA using primers specific to the GUS gene, confirming that they contained the GUS gene in their genome (Figure 3b). A 750-bp DNA fragment was amplified from the genomic DNA from all 10 plants and DNA from the pCambia 2203 plasmid with the RdRp gene using CTV-RdRp specific primers (Figure 3b), indicating that these plants contained the RdRp gene of CTV introduced by the *Agrobacterium*-mediated transformation method. Since no amplification was detected in the nontransformed samples (Figure 3b), these fragments were gene-specific.

Once the PCR was optimized, genomic DNA from all GUS+ plants (solid or partial) was isolated and tested for amplification of the GUS and RdRp genes using specific primers. An additional 60 plants were tested for the presence of the GUS and RdRp genes. Among 70 GUS+ plants tested, the 800-bp fragment of the GUS gene and the 750-bp fragment of the CTV RdRp gene were amplified from 56 and 54 individual plants, respectively. Both the GUS and the RdRp genes were amplified from 51 plants and neither the GUS nor the RdRp gene was amplified from 7 plants, indicating that the plants were false positives or their DNA was not good for PCR. The remaining 12 plants showed differential amplification of the GUS and RdRp genes, meaning that only the GUS gene was amplified from 5 plants and only the RdRp gene was amplified from the other 7 plants. These results were confirmed by 2 or more PCR amplifications from these plants. The number of plants analyzed by PCR for each of the constructs and the results of the PCR analysis are summarized in Table 3.

Discussion

Various citrus species and hybrids have been transformed with structural and nonstructural genes from the CTV genome for the development of transgenic resistance against CTV. Although no transgenic plant resistant to CTV has been developed yet, potential strategies for transgenic resistance to CTV have been explored and useful data about the transformation and regeneration efficiency of various citrus cultivars and rootstocks have been determined in the last 10 years. The average number of shoots per segment in this study was similar to previously reported numbers for grapefruit (Luth and Moore 1999; Çevik et al. 2006). The percentage of shoot regeneration for RdRp constructs (41%) was higher than that of the other constructs (25% for RdRp-ΔGDD and 28% for RdRp-mGDD), because of the use of 1.5 mg L^{-1} of BA for shoot regeneration in the initial experiments with RdRp construct. Although a higher concentration of BA (1.5 mg L^{-1}) produced more shoots per segment, most of the regenerated shoots did not root even when they were kept on antibiotic and hormone-free MS medium for 1 month and then transferred to the rooting medium. Therefore, the BA concentration was reduced to 0.5 mg L^{-1} in the later experiments with all 3 constructs to improve the rooting efficiency.

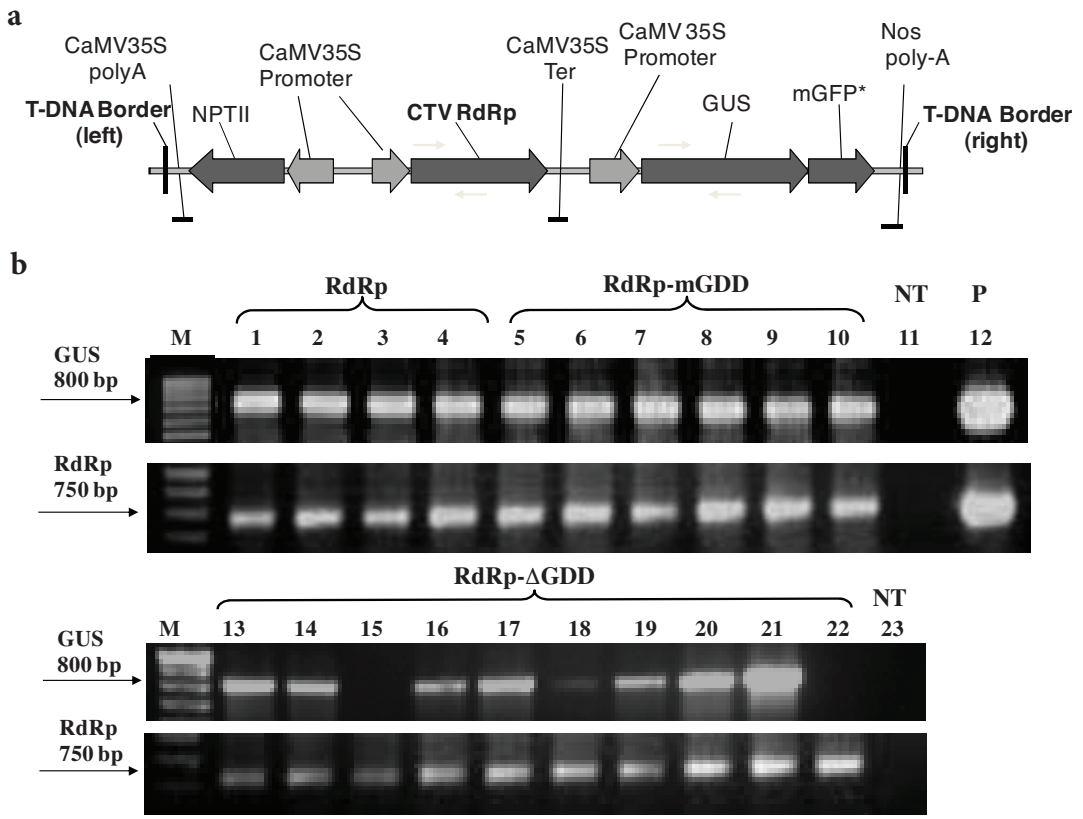

Figure 3. Analysis of the putative transgenic plants by polymerase chain reaction. a) The T-DNA map of the RdRp construct showing the location of the primers used for PCR analysis in the RdRp and the GUS regions. b) PCR amplification of the part of the GUS and RdRp transgene from genomic DNA isolated from plants transformed with the RdRp, RdRp-mGDD, and RdRp-ΔGDD constructs. M: DNA ladder used as molecular weight marker, NT: nontransgenic grapefruit plant used as a negative control, P: pCambia 2203 plasmid with CTV RdRp gene used as a positive control.

Table 3. Summary of the PCR analysis of the transgenic plants with different constructs of the CTV RdRp gene.

Constructs	No. of plants potted	PCR analysis						
		No. tested	GUS+	RdRp+	GUS+ RdRp+	GUS+ RdRp-	GUS- RdRp+	GUS- RdRp-
RdRp	22	18	16	16	15	1	1	1
RdRp-mGDD	37	31	23	24	21	2	3	5
RdRp-ΔGDD	38	21	17	18	15	2	3	1
Total	97	70	56	58	51	5	7	7

The efficiency of transformation in citrus was generally determined by the number of GUS+ shoots divided by the total number of shoots regenerated from epicotyl segments (Gutiérrez et al. 1997; Luth and Moore 1999) or the number of epicotyl segments producing GUS+ shoots (Bond and Roose 1998; Cervera et al. 1998). Since a large number of epicotyl segments were used in this study, the transformation efficiency was determined by the percentage of GUS+ shoots from the total shoots tested. While the percentage of GUS+ shoots was lower in this study than in previous reports on transformation

of other citrus including citrange (Gutiérrez et al. 1997; Cervera et al. 1998), Mexican lime (Gutiérrez et al. 1997; Pena et al. 1997), and sweet orange (Bond and Roose 1998), it was similar to the findings of a previous report on the percentage of GUS+ shoots in grapefruit (Çevik et al. 2006). Since it has been reported that different citrus species and even cultivars of citrus are transformed with different efficiencies, the variation in the transformation frequency of grapefruit in this study and in previous reports on citrange, Mexican lime, and sweet orange (Gutiérrez et al. 1997; Pena et al. 1997; Cervera et al. 1998) may be due to differences in the type of citrus and the transformation and regeneration techniques used in each study. The total number of GUS+ shoots regenerated in this study was also lower than in a previous report on *Agrobacterium*-mediated transformation of Duncan grapefruit (Luth and Moore 1999), which was the same variety used in this study. However, the percentage of solid GUS+ in this study (15.7%) was higher than in the previous report by Luth and Moore (1999). The same citrus cultivar and transformation techniques were used in both studies; however, a different *Agrobacterium* strain and binary plasmid were used in the previous report of grapefruit transformation. It has been reported that a wide range of variations can be observed during transformation with different strains of *Agrobacterium* in citrus and other plants (Gutiérrez et al. 1997; Pena et al. 1997; Cervera et al. 1998; Luth and Moore 1999). Conflicting results on the transformation frequency of grapefruit may be due to the use of different *Agrobacterium* strains.

Differential detection of the GUS and RdRp genes may simply be due to false positive amplification of one of the genes or the result of partial integration of T-DNA into the plant genome. It was reported that the transfer of T-DNA from *Agrobacterium* to the plant cell starts at the right border (RB) and continues toward the left border (LB) (Sheng and Citovsky 1996). Any interruption during the T-DNA transfer may result in partial transfer of the T-DNA region close to the RB where the GUS gene of pCambia 2203 is located. A polarity may also be observed during T-DNA integration into the plant genome, leading to insertion of only part of the T-DNA into the plant genome. Although partial integration may explain the differential amplification of the GUS and RdRp genes in some transgenic plants, PCR amplification by itself does not conclusively prove the integration of the GUS and RdRp genes into the plant genome.

Here we reported the transformation of grapefruit with wild-type and mutant constructs of the RdRp gene of CTV and the regeneration of transgenic plants carrying these constructs. We also compared the transformation and regeneration results of this study with the results of previously published transformation studies on the other *Citrus* species with different constructs. We demonstrated that grapefruit is transformed efficiently with wild-type and mutant constructs of the RdRp gene of CTV. This study provides the plant material for development of replicase-mediated resistance, and more studies are needed to reveal the mechanism of potential CTV resistance in *Citrus*.

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