Turk J Agric For
30 (2006) 173-182

Genetic Transformation of *Citrus paradisi* with Antisense and Untranslatable RNA-dependent RNA Polymerase Genes of *Citrus tristeza closterovirus*

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Received: 26.07.2005

Abstract: Protein and RNA-mediated forms of pathogen-derived resistance (PDR) have been developed against many viruses in different plants. However, no resistance has been reported against *Citrus tristeza virus* (CTV), a closterovirus, in *Citrus* species transformed with coat protein genes or other sequences of CTV. The successful use of replication-associated genes in RNA-mediated resistance in other crops prompted the use of the RNA-dependent RNA polymerase (RdRp) gene of CTV for the development of RNA-mediated PDR in *Citrus*. The RdRP gene was amplified from CTV isolate DPI3800 from Florida and used to generate antisense (RdRp-AS) and untranslatable (RdRp-UT) constructs with point mutation consecutive stop codons in the 5' end of the RdRp gene for use in plant transformation. A total of 3120 etiolated epicotyl segments of Duncan grapefruit (*Citrus paradisi* Macf. cv. Duncan) were transformed with these constructs using *Agrobacterium tumefaciens*-mediated transformation. From these segments 1040 kanamycin-resistant shoots were regenerated, and a total of 131 putative transgenic shoots were identified by fluorescent microscopy and histochemical β-glucuronidase (GUS) assays. One hundred GUS positive plants were rooted and 66 plants survived and were established on soil. A total of 41 plants were tested by polymerase chain reaction (PCR) for the presence of the GUS gene and for the transgenes. Eighteen GUS-positive and transgene-positive plants (8 with RdRp-AS, and 10 with RdRp-UT) were identified.

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

Citrus Tristeza Closterovirüsü'nün Tersine Çevrilmiş ve Protein Sentezleyemeyen RNA-Bağımlı RNA Polimeraz Genleriyle *Citrus paradisi*'in Genetik Transformasyonu

Özet: Bir hastalık etmeninden elde edilen dayanıklığın (EED) protein ve RNA'ya dayalı şekli farklı bitkilerde bir çok virüse karşı geliştirilmiştir. Ancak citrus tristeza virüsünün (CTV) kılıf proteinini kodlayan yapısal genler ve diğer genleri ile transform edilen turunçgil türlerinde henüz bir dayanıklılık geliştirilememiştir. Replikasyonla ilgili genlerin farklı bitkilerde bir çok virüse karşı RNA'ya dayalı EED geliştirmede başarılı bir şekilde kullanıldığı için bu çalışmada CTV'ün RNA-bağımlı RNA polimeraz (RdRp) geni turuçgillerde CTV ye karşı RNA'ya dayalı dayanıklık geliştirmek amacıyla kullanıldı. RdRp geni CTV'nün DP3800 izolatının genomundan polimeraz zincir reksiyon yöntemiyle çoğaltılarak klonlandı ve bitki transformasyonu için tersine çevrilmiş (RdRp-AS) ve 5' ucunda ardarda beş tane translasyonu durdur kodonu içerdiği için protein sentezleyemeyen (untranslatable, RdRp-UT) formları oluşturuldu. Hazırlanan RdRp-AS ve RdRp-UT genleri *Agrobacterium tumefaciens*'e dayalı transformasyon yöntemi kullanılarak Duncan greyfurt (*Citrus paradisi* Macf. cv. Duncan) çeşidinden elde edilen toplam 3120 epikotil parçasına aktarıldı. Bunlardan 1040 tane kanamicine dayanıklı sürgün çoğaltıldı ve elde edilen sürgünlerden floresan mikroskop ve β-glukronidaz (GUS) testiyle toplam 131 potansiyel transgenik sürgün belirlendi. GUS pozitif sürgünlerin 100 tanesi köklendirilerek bitki oluşturuldu. Bu bitkilerden sağlıklı kalarak yaşamını sürdüren 66 tanesi toprak ile doldurulmuş saksılara dikilip seraya aktarılarak büyütüldü. Toplam 41 bitki PCR yöntemiyle test edilerek raportör GUS genini ve CTV'un RdRp genlerini taşıyıp taşımadıkları araştırıldı. Sonuçta GUS geni ve RdRp genlerinden birini taşıyan 18 transgenik greyfurt bitkisi (8 GUS ve RdRp-AS pozitif, 10 GUS ve RdRp-UT pozitif) belirlendi.

Anahtar Sözcükler: *Citrus tristeza* virüsü, RNA'ya dayalı dayanıklılık ve replikaz, bitki transformasyonu, RNA-bağımlı RNA polimeraz

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Introduction

The pathogen-derived resistance (PDR) concept was first proposed by Sanford and Johnston (1985) and demonstrated by Powell-Abel et al. (1986) in tobacco transformed with the coat protein (CP) gene of *Tobacco mosaic virus* (TMV). Since then many crop plants, including vegetables, fruits, cereals and forage crops, have been transformed with genes encoding structural and non-structural proteins as well as antisense sequences from viruses to develop PDR against different viruses (Baulcombe, 1994, 1996; Hackland et al., 1994; Beachy 1997; Fuchs and Gonsalves, 1997). Although the results have been somewhat variable in different plant-virus systems, the use of non-structural genes, especially those encoding movement proteins and replication-associated proteins, such as the RNA-dependent RNA polymerase (RdRp), have shown promising results for PDR in transgenic plants (Beachy 1997; Palukaitis and Zaitlin, 1997).

PDR induced by expression of wild type or mutants of replication-associated genes, such as the RdRp of plant viruses, is called replicase-mediated resistance (RMR). The first RMR was developed against bacteriophage Qβ in a bacterium where the expression of a modified replicase of bacteriophage Qβ induced resistance to the phage in bacteria (Inokuchi and Hirashima, 1987). The RMR to plant viruses was first reported by Golemboski et al. (1990), who transformed tobacco with the 54 kDa RdRp of TMV to determine the function of this protein. Transgenic plants expressing the 54 kDa RdRp were highly resistant to TMV infection. Since then RMR has been extensively explored for a number of other plant RNA viruses using full-length and defective constructs, including antisense and untranslatable replicase genes. RMR has been successfully developed against a number of plant viruses from different genera including *Alfamovirus*, *Bromovirus*, *Comovirus*, *Cucumovirus*, *Luteovirus*, *Potexvirus*, *Potyvirus*, *Tobamaovirus*, *Tombusvirus* and *Tobravirus* (Palukaitis and Zaitlin, 1997).

Citrus tristeza virus (CTV) is one of the most destructive pathogens, causing significant economic losses in many citrus-producing regions worldwide (Bar-Joseph et al., 1989), including Turkey (Yilmaz and Baloğlu, 1998). Recent improvements in citrus transformation techniques and the characterization of CTV genome now enable the application of PDR in citrus against CTV. While several different *Citrus* species including the sour orange, Mexican lime and grapefruit have been transformed with the minor or major coat protein genes of CTV (Gutierrez et al., 1997; Dominguez et al., 2000; Febres et al., 2003), no resistance has been reported. Expression of translatable and untranslatable forms of the non-structural p23 gene of CTV in Mexican lime not only failed to develop resistance but the transgenic plants expressing showed symptoms similar to CTV-induced symptoms in the trangenic Mexican lime (Ghorbel et al., 2001). Because of the failure of citrus plants transformed with the coat protein genes of CTV and as the non-structural p23 gene did not result in expression of resistance to CTV, we decided to develop transgenic citrus with the RdRp gene from CTV as an alternative strategy for development of PDR to CTV. Therefore, wild type and mutant constructs of RdRp gene were transferred to grapefruit (Cevik et al., 2000) to see if overexpression of functional or expression of dysfunctional RdRp would interfere with the viral RdRp and induce protein-mediated resistance by inhibiting or reducing the virus replication in transgenic plants. In this study, grapefruit epicotyl segments were transformed with antisense and untranslatable constructs of the RdRp gene of CTV using an *Agrobacterium tumefaciens*-mediated transformation method to explore the possibilities of RNA-mediated resistance against CTV.

Materials and Methods

Cloning of Plant Transformation Constructs

The RdRp gene was amplified from a cDNA clone containing this region of the CTV isolate DPI 3800 from Florida (provided by the Florida Division of Plant Industry) by PCR using primers CN356 and CN357 (Table 1). Since the RdRp gene does not contain a translation initiation codon, an ATG codon and a translational enhancer sequence (ACC) from *Cucumber mosaic virus* were incorporated into the 5' end of the RdRp sequence during PCR amplification. In addition, a *Not* I restriction site was integrated into both the 5' and 3' ends to facilitate cloning (Table 1). This sequence was designated as RdRp (Figure 1) and used as a template for the generation of an antisense (AS) construct.

To produce an untranslatable construct of CTV RdRp that can potentially be used for RNA mediated resistance, the RdRp gene was amplified from a cDNA clone containing the RdRp region of the CTV isolate DPI3800

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	Anti-sense
CN309	TGTTTTGTACCGGACCCTTA	Sense
CN310	GTACTCGCCTTCCATCCA	Anti-sense
CN355	AAA*GCGGCCGC*A*T*GAGAC**T**CTG**C**CCCT**AGT**GACTCCGGTAACTTA**G**ACGAACC	Sense
CN356	*AAAGCGGCCGCACC**ATG**G*AGACACTGCCCCTCCCGACTCC	Sense
CN357	AAA*GCGGCCGC*TCAGCCGGTCGCTAAGTCGTCCG	Anti-sense

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

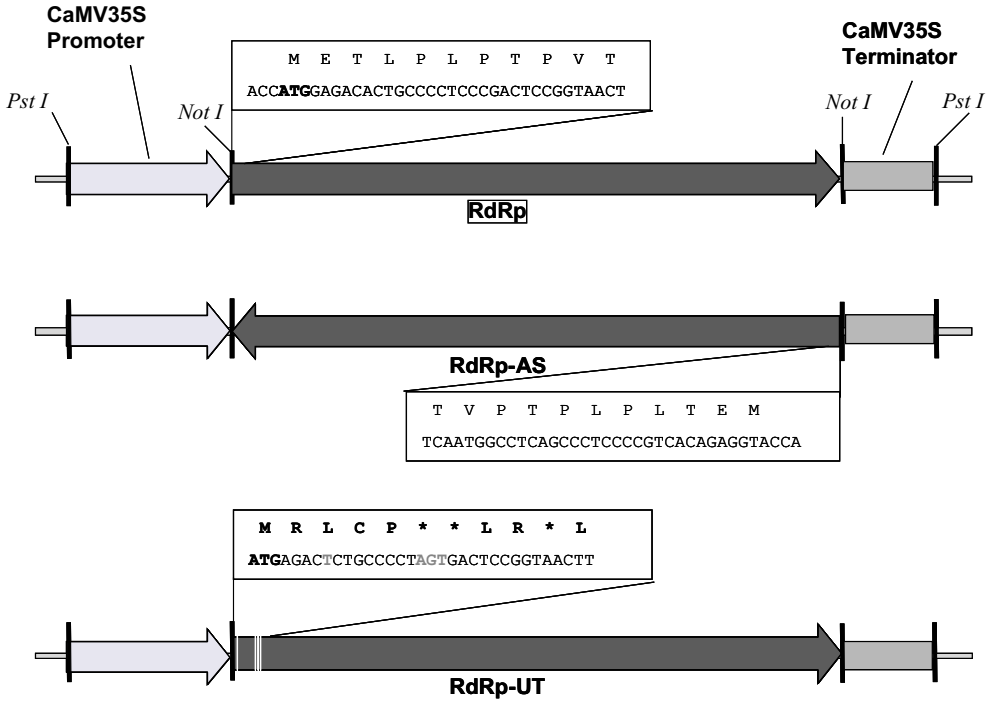

Figure 1. Mutagenesis and cloning of antisense and untranslatable constructs of CTV RdRp used for plant transformation. Partial T-DNA map of RdRp plant transformation constructs showing site-directed mutagenesis in the 5' end of the RdRp gene. The parts of the constructs are indicated above the first construct. The nucleotide and amino acid sequence of the 5' end of the RdRp constructs are shown in the box above or below the partial map of each construct. Point mutations and added bases in the 5' end are indicated in gray and bold letters, respectively, in the sequence of each construct. Asterisk in the protein sequence indicates stop codons.

genome by PCR using primers CN355 and CN357 (Table 1). Two nucleotides, A and T, were added to the 5' end of the original RdRp sequence to form an out-of-frame translation initiation codon, ATG, and several point mutations also were made to introduce 3 stop codons close to the 5' end of the RdRp sequence by using the oligonucleotide primer CN355 during PCR amplification. This resulted in an untranslatable sequence with *Not* I site at both the 3' and 5' ends, which was designated as RdRp-UT (Figure 1).

The PCR amplified RdRp and RdRp-UT sequences were cloned into a *Not* I site between the *Cauliflower mosaic virus* (CaMV) 35S promoter and the termination signal in the pUC118 CaMVP-T vector. The clones were screened for orientation; one RdRp-UT clone in forward orientation and one RdRp clone in reverse orientation (RdRp-AS) were selected. The selected pUC118 CaMVP-T with RdRp-UT and RdRp-AS constructs were then digested with *Pst*I restriction endonuclease to release the CTV RdRp sequences with the CaMV 35S promoter and the termination signal. These fragments were cloned into the *Pst* I site in the binary plant transformation vector pCambia 2203 with Npt II (kanamycine resistance) gene for selectable marker and GUS and GFP as the bi-functional reporter gene (Figure 1). The pCambia 2203 binary vector with RdRp constructs was then introduced into *A. tumefaciens* strain Agl I.

Transformation of Grapefruit

An *A. tumefaciens*-mediated transformation protocol originally developed for epicotyl segments from rootstock cultivars Carrizo citrange or Swingle citrumelo (Moore et al., 1992, 1993) and later improved and applied to the etiolated seedlings of *Citrus paradisi* Macf. cv. Duncan (Luth and Moore, 1999) was used for the transformation and regeneration of transgenic plants. The steps of this transformation method are briefly described.

Seeds from *Citrus paradisi* cv. Duncan were peeled and sterilized with 70% ethanol for 5 min and 0.525% hypochlorite solution plus 0.05% Tween-20 for 10 min and rinsed thoroughly with sterile distilled water 5 times. The seeds were germinated in 150 x 25 mm tubes containing half-strength MS medium (2.13 g l^{-1} MS salt, 50 mg l^{-1} myo-inositol, 15 g l^{-1} sucrose and pH 5.7) with 7 g l^{-1} agar in the dark at 28 ºC or at room temperature for 4-6 weeks (Figure 2A). The epicotyl portions of the etiolated seedlings were cut into 1-cm segments (Figure 2B) and soaked in 5 x 10^8 cfu ml^{-1} *A. tumefaciens* strain AGL 1 with either RdRp-AS or RdRp-UT constructs in MS medium with 100 µM acetosyringone for 1 min (Figure 2C). The inoculated segments were placed horizontally on petri dishes containing co-cultivation medium (MS medium plus 7 g l^{-1} agar, and 100 µM acetosyringone) (Figure 2D) and incubated in the dark at room temperature for 2-3 days for transformation.

Co-cultivated epicotyl segments were transferred to a shooting medium (MS medium with 0.5 mg l^{-1} or 1.5 mg l^{-1}, for initial experiments with RdRp-AS, benzyl adenine (BA) and 7 g l^{-1} Bacto-agar supplemented with 500 mg l^{-1} Claforan to inhibit further growth of *A. tumefaciens* and with 75 mg l^{-1} kanamycin sulfate. These were maintained at 28 ºC with a 16/8 h photoperiod provided by cool-white fluorescent light for 4-5 weeks for selection of transgenic shoots (Figure 2E and F). Shoots, 5-10 mm in length, were excised from the explants and placed on rooting medium (MS medium with 0.5 mg l^{-1} naphthalene acetic acid (NAA) (Figure 2G) after removing a small segment from their basal ends for histochemical GUS staining. Shoots remained on rooting medium for 3-5 weeks or until they produced roots (Figure 2H and I).

When shoots formed 1-3 roots and the root was at least 1-cm long, the plants were transferred to sealed culture jars containing sterilized potting mix and half-strength MS (Figure 2J), which were placed in a growth chamber at 28 ºC with a 16 h light and 8 h dark photoperiod or at room temperature (Figure 2K). Once the plants outgrew the culture jars, they were removed, transferred into pots containing potting mix, and they were placed either in a high humidity growth room at 30 ºC with 16 h light and 8 h dark photoperiod or in the greenhouse. The plants initially placed in the high humidity growth room were later moved to the greenhouse and maintained (Figure 2L).

Analysis of Transgenic Shoots and Plants

Fluorescent Microscopy and β-glucuronidase (GUS) Assays: The epicotyl segments transformed with *A. tumefaciens* containing either the RdRp-UT or RdRp-AS constructs as well as regenerated shoots were periodically examined for the expression of green fluorescent protein (GFP) using a fluorescent dissecting microscope (Zeiss). Regenerated shoots were removed from epicotyl segments while they were growing in the selection medium, and small (1-2 mm) sections were cut from the basal end of each shoot. They were placed in

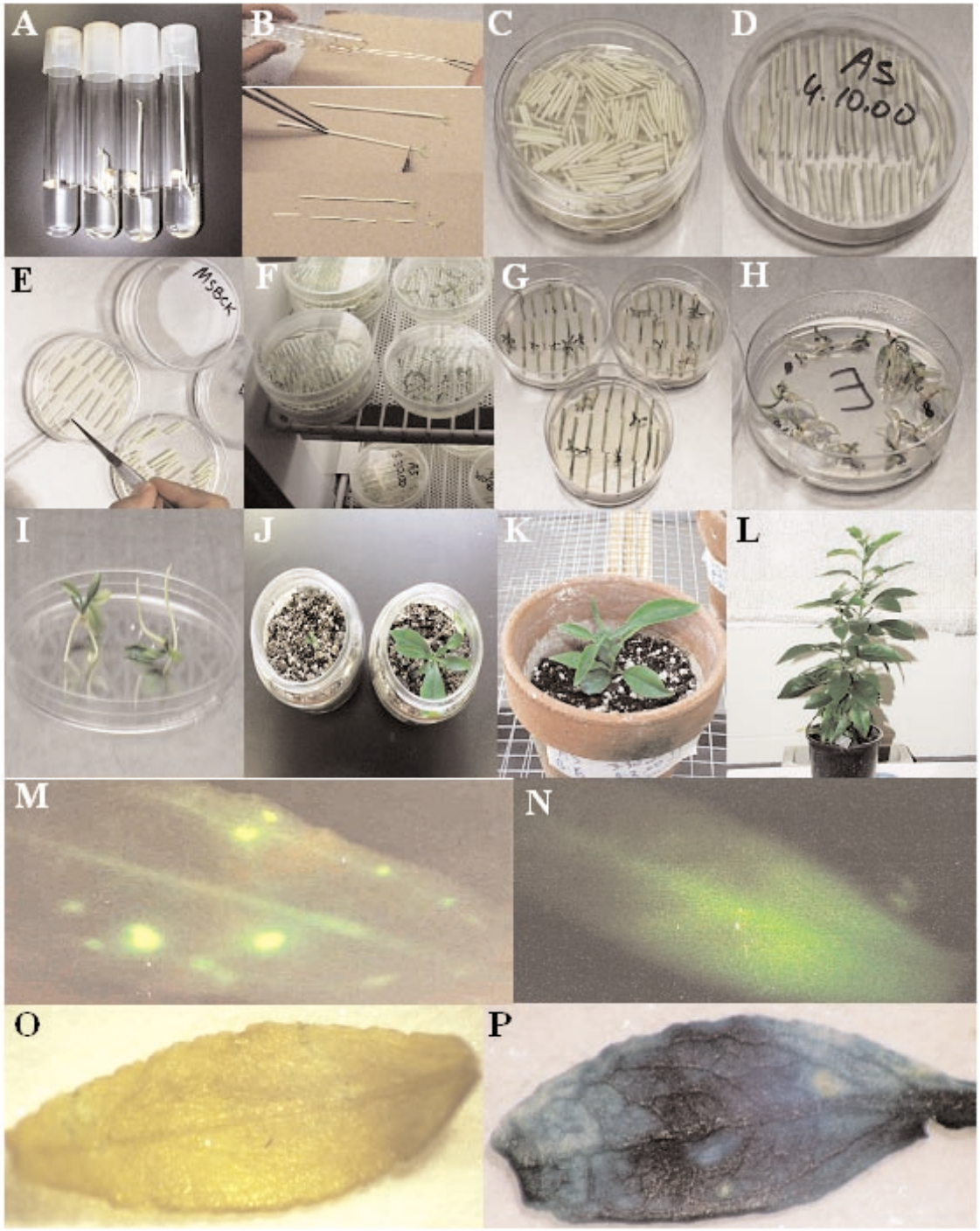

Figure 2. Production and analysis of transgenic plants from epicotyl segments of grapefruit using an *Agrobacterium*-mediated transformation method. A) In vitro production of etiolated seedlings. B) Preparation of etiolated seedling segments for transformation. C) Inoculation of epicotyl segments with *Agrobacterium* with CTV-RdRp constructs. D) Co-cultivation of epicotyl segments with *Agrobacterium* strain with CTV-RdRp constructs. E-G) Regeneration and selection of transgenic shoots using benzyl adenine and kanamycin sulfate. H-I) Rooting of transgenic shoots in a naphthalene acetic acid-containing medium. J) Transfer and maintenance of rooted transgenic shoots in soil culture jars. K-L) Establishment of transgenic plants in pots in the greenhouse. M-N) Fluorescent microscopy of leaves from a transgenic shoot transformed with the RdRp construct. O-P) Histochemical GUS staining of leaves from non-transgenic (O) and transgenic (P) plants with the CTV RdRp construct.

96-well assay plates containing β-glucuronidase assay solutions (50 mM $NaPO_4$, pH 7.0, 10 mM Na_2EDTA and 5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide). After the application of a mild vacuum for 5 min to infiltrate the substrate, the plates were sealed and were incubated overnight at 37 ºC. The shoot sections were then cleared in a solution of 70% ethanol and 30% acetic acid, and analyzed for GUS staining. The sections were examined under a stereomicroscope, scored for GUS staining, and classified as partial GUS positive having a few blue dots or solid GUS positives showing blue staining in whole section. Leaves from some of the GUS-positive plants were tested for GUS activity a second time before they were transplanted into pots. A small young leaf or a portion of a bigger young leaf was placed into a 1.5-ml microfuge tube containing the β-glucuronidase assay solutions, and a mild vacuum was applied. After overnight incubation at 37 ºC, leaves were cleared and evaluated for GUS histochemical straining as described previously.

Polymerase Chain Reaction (PCR): The putative transgenic plants were tested for presence of the GUS and CTV-RdRp gene using PCR with gene-specific primers (Figure 3A). First, the genomic DNA was isolated from about 1 cm^2 of leaf tissue from all putative transgenic plants using rapid genomic DNA extraction methods (Edward and Thompson, 1991) and/or citrus (Oliveira et al., 2000). The extracted DNA was tested by PCR using 2 sets of primers: one pair specific for the GUS gene (CN 216 and CN 217) and the other pair specific for the 5' half of the RdRp gene of CTV (Table 1). The amplification reaction was carried out in a thermocycler using a profile of 94 ºC for 3 min initial denaturation and 40 cycles of denaturation at 92 ºC for 30 min, primer annealing at 50 to 55 ºC for 1 min, and primer extension at 72 ºC for 1 min followed by final primer extension at 72 ºC for 5 min. The amplification products were separated in 1% agarose gels by electrophoresis in TAE buffer and analyzed by ethidium bromide staining.

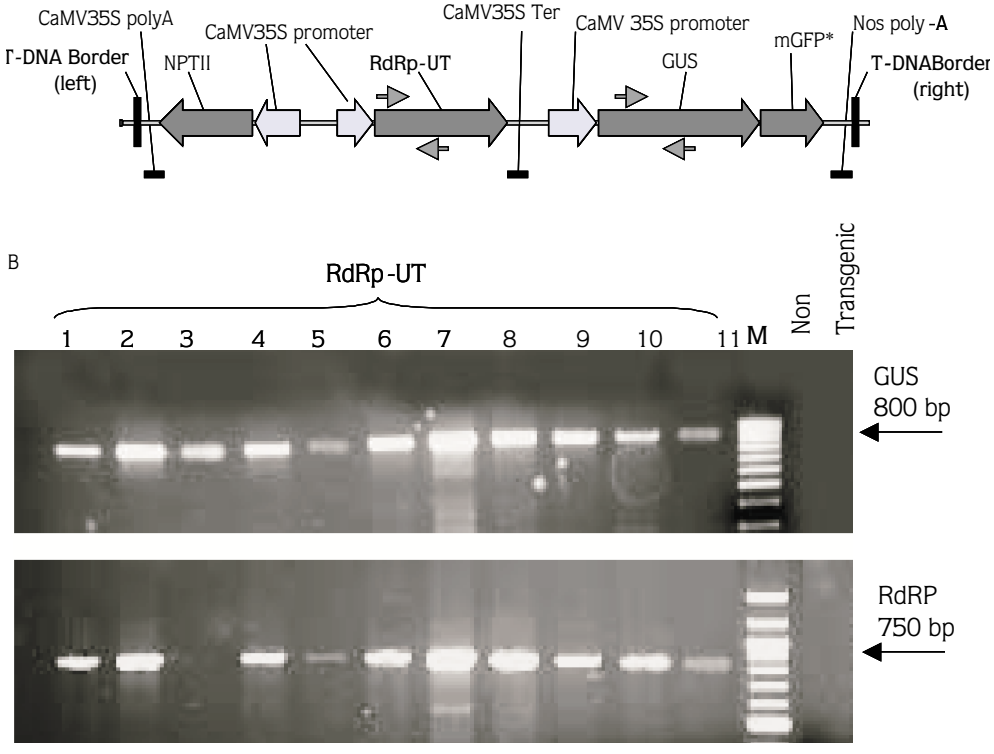

Figure 3. Analysis of the putative transgenic plants by polymerase chain reaction. A) The T-DNA map of the RdRp construct showing the location of the primers used for PCR analysis in the RdRp and the GUS regions. B) PCR amplification of part of the GUS and RdRp transgenes from genomic DNA isolated from plants transformed with the RdRp-UT construct.

Results and Discussion

Regeneration of Transgenic Plants:

A total of 3120 epicotyl segments were transformed with *A. tumefaciens* strain AGL I containing the pCambia 2303 binary plasmid with either the RdRp-AS or the RdRp-UT construct for the CTV RdRp gene. From these transformed segments, 1040 kanamycin resistance shoots were regenerated, indicating a regeneration efficiency of the epicotyl segments transformed by these constructs of greater than 30%. The average number of shoots per segment obtained in this study was similar to the number for grapefruit previously reported (Luth and Moore, 1999; Cevik et al., 2000). The percentage of shoot regeneration was 41% and 28% for RdRp-AS and RdRp-UT constructs, respectively. This obvious difference in the regeneration efficiency of these 2 constructs was probably due to the higher concentration (1.5 mg l^{-1}) of BA used for the initial regeneration experiments with the RdRp-AS construct. As we previously reported for the transformation of full-length RdRp, high concentrations of BA (1.5 mg l^{-1}) produce more shoots per segment but most of the shoots do not root even when they are maintained on antibiotic and hormone-free MS medium for a month before being transferred to the rooting medium. Because of this problem, 0.5 mg l^{-1} BA was established as the optimum rate for rooting and survival and used in all experiments with RdRp-UT constructs. A summary of the transformation experiments with individual RdRp constructs is given in Table 2. During regeneration, transformed segments and shoots were periodically examined for the expression of GFP using a fluorescent dissecting fluorescent microscope. The expression of GFP was detected in some segments and shoots (Figure 2O); however, the detection of GFP expression was not consistent and was masked by the strong autofluorescence in the citrus leaf (Figure 2N). Therefore, putative transgenic shoots were scored primarily by the histochemical GUS assay.

A section from the basal end of all 1040 regenerated shoots was tested for GUS activity before the shoots were transferred to the rooting media; 131 (12.6%) showed GUS staining (Table 2), ranging from a few blue dots to completely blue stained cuts or leaves (Figure 2R). These were considered putative transgenic shoots. Based on the degree of GUS staining, shoots were classified as solid GUS positive, showing complete blue staining, or partial GUS positive, with few blue stained cells. The majority of the shoots showed partial GUS staining and only 15.2% of 131 shoots rated as putative transgenic shoots showed solid blue staining. The results of histochemical staining for individual constructs are summarized in Table 2.

In the literature, the efficiency of citrus transformation was generally determined either by the number or percentage of GUS+ shoots from the total number of shoots regenerated from epicotyl segments

Table 2. Summary of transformation experiment results with 2 different constructs of the CTV-RdRp gene.

Construct	Number of segments	Regenerated shoots		Number of GUS positive shoots		%GUS positive shoots		Number of shoots	
		Total	%*	Total	Solid	Total	Solid	Rooted	Potted
RdRp-AS	1360	546	40.2	59	8	10.8	13.6	36	24
RdRp-UT	1760	494	28.1	72	12	14.6	16.7	64	42
Total	3120	1040	33.3	131	20	12.6	15.2	100	66

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

(Gutierrez et al., 1997; Luth and Moore, 1999) or by the percentage 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 calculated by the percentage of GUS+ shoots from the total shoots tested. The overall efficiency of transformation for this study was 12.6% and an almost 4% difference was observed between the transformation efficiency of RdRp-AS (10.8%) and RdRp-UT (14.6%) constructs (Table 2).

Higher transformation efficiencies have been reported for *Agrobacterium*-mediated transformation of other citrus types including citrange (Gutierrez et al., 1997; Cervera et al., 1998) Mexican lime (Gutierrez et al., 1997; Pena et al., 1997), and sweet orange (Bond and Roose, 1998). Since it has been reported that different *Citrus* species, or even different cultivars of the same *Citrus* species, are transformed with different efficiencies, the variation observed in the transformation efficiency of grapefruit in this study and previous reports on citrange, Mexican lime and sweet orange (Gutierrez et al., 1997; Pena et al., 1997; Cervera et al., 1998) is probably due to differences in citrus variety (type) used in each study. Although the percentage of solid GUS+ shoots in this study (15.7%) was higher than that previous reported for *A. tumefaciens*-mediated transformation of Duncan grapefruit (Luth and Moore, 1999), the total number of GUS+ shoots was lower. While the same citrus cultivar and transformation/regeneration techniques were used in both studies, the *A. tumefaciens* strain and the binary plasmids were different. Therefore, the variation in the transformation frequency of grapefruit between this study and the previous one may be due to the use of a different *A. tumefaciens* strain. It has been reported previously that a wide spectrum of variation occurs in transformation efficiency with different stains of *A. tumefaciens* in citrus (Gutierrez et al., 1997; Pena et al., 1997; Bond and Roose, 1998; Cervera et al., 1998).

Of the 131 GUS+ shoots placed in rooting media, 100 (76.3%) rooted and were transferred into soil culture jars (Table 2). The number of rooted GUS+ plants and the rooting efficiency of the RdRp-UT constructs (88.8%) were much higher than those of the RdRp-AS construct (61%). This variation may have been due to the higher concentration of BA used for some of the initial experiments with RdRp-AS constructs. When the surviving plants outgrew the culture jars, they were transplanted into a non-sterile potting mix and maintained in the greenhouse (Figure 2L). From 100 GUS+ rooted plants in culture jars, 66 survived and were transferred to the greenhouse.

Analysis of Transgenic Plants by PCR

Genomic DNA was extracted from the 41 GUS+ plants in the greenhouse and tested for the presence of the GUS and RdRp genes by PCR (Figure 3). The 800 bp fragment of the GUS gene was present in 26 plants while the 750 bp fragment of the CTV RdRp gene was present in 24 plants (Figure 3 and Table 2). Both the GUS and the RdRp genes were present in 18 plants, while 8 plants had neither the GUS nor the RdRp (Table 2), indicating these 8 plants were false positives. The remaining 15 plants showed the presence of either the GUS or RdRp gene, but not both; 10 plants contained the GUS gene only, while 5 plants had only the RdRp gene (Table 2). PCR testing of these greenhouse plants was performed at least twice to

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

Constructs	Number of Potted Plants	PCR Analysis						
		Tested GUS+	Total RdRp+	Total RdRp+	GUS + RdRp –	GUS + RdRp –	GUS – RdRp +	GUS – RdRp –
RdRp-AS	24	18	11	12	8	4	4	2
RdRp-UT	42	23	15	12	10	6	1	6
Total	66	41	26	24	18	10	5	8

confirm the presence or absence of the genes. The number of plants tested by PCR for each construct and the results of PCR analysis are summarized in Table 3. The differential integration of the GUS and RdRp genes in the genome of the transgenic plants may have resulted due to incomplete integration of the T-DNA into the plant genome. The transfer of T-DNA from *Agrobacterium* to the plant cell starts at the right border (RB) and continues toward the left border (LB); any interruption during the T-DNA transfer may result in incomplete transfer of the T-DNA region close to the RB where the GUS gene of pCambia 2203 is located. In addition, polarity has been reported during T-DNA integration into the plant genome, leading to the insertion of only part of T-DNA into the plant genome (Sheng and Citovsky, 1996).

Conclusions

Transgenic grapefruit plants with untranslatable and antisense constructs of the CTV RdRp gene were developed using an *A. tumefaciences*-mediated transformation method. Putative transgenic plants were first determined by fluorescent microscopy and a histochemical GUS assay. These plants were then analyzed by PCR for the presence of the GUS and the RdRp transgenes. A total of 18 transgenic plants, 10 transformed with RdRp-UT and 8 transformed with RdRp-AS constructs, were identified and established in the greenhouse. Some of these plants were micro-grafted onto sour orange rootstocks for propagation, and future testing for resistance to CTV is underway. The transgenic plants will be more thoroughly evaluated and analyzed for the expression of transgenes.

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