Transformation of Citrus Plants with Constructs Related to Citrus Tristeza Virus (CTV) Sequences

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By

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ABSTRACT

Citrus is the most extensively grown fruit tree crop in the world and a major source of income and employment. *Citrus tristeza virus* (CTV) (genus *closterovirus* family *Closteroviridae*) is involved in a range of serious citrus diseases that limits production by causing decline of trees grafted on sour orange rootstocks and by stem-pitting that debilitates trees and reduces fruit quality regardless of rootstock. The aphid transmissible CTV virions are long, flexuous particles and contain the largest and most complex single stranded RNA genome among plant viruses. The CTV host range is mostly restricted to *Rutaceous* plants. Genetic improvement of citrus rootstocks to overcome their sensitivity to CTV remains an important objective for citrus industry. Recent developments in genetic engineering allows the expression of different viral genes in plants and resulted in transgenic plants with considerable levels of resistance to homologous virus infections. Citrus plants are recalcitrant to *Agrobacterium*-mediated genetic engineering and give only low *in vitro* regeneration efficiency. Therefore the task of transgenic improvement remains a most challenging objective. The effects of different conditions on the efficiency of the regeneration process and the ability to obtain transgenic citrus rootstocks were examined. The results of *Agrobacterium*-mediated transformation *in vitro* are reported for the five most economically important citrus rootstocks in etiolated epicotyl

stem segments of Troyer citrange (*Citrus sinensis x Poncirus trifoliata*), Sour orange (*C. aurantium*), Gou Thou (Chinese sour orange), Alemow (*C. macrophylla*), and Volkamer lemon (*C. limon*). *Agrobacterium*-mediated transformation was attempted with several CTV derived sequences including CTV p23 with 3' UTR (p23U, ORF 11), p61 (ORF 5) and the hairpin structure of p23U (p23UI). In addition to citrus transformation, we prepared transgenic *Nicotiana benthamiana* plants expressing the corresponding CTV sequences used for citrus transformation and used this system for the rapid analysis of construct and cloning efficiency. The availability of the *Grapevine Virus A* (GVA) based viral vector, infectious on *N. benthamiana* allowed the assessment of the transgenic *N. benthamiana* plants for PTGS-based resistance against a recombinant GVA vector containing the corresponding homologous CTV sequences. In total the citrus rootstocks transformation efforts resulted in more than 300 transgenic citrus rootstocks and 435 transgenic *N. benthamiana* plants that have tested positive for the presence of CTV specific sequences as indicated by a battery of tests including GUS, PCR and Southern hybridization with CTV- p61 and p23U gene specific DIG-labeled RNA probes. Hybridization of RNA extracts from either of these classes of transgenic plants with riboprobes specific to CTV-p61 and plus- and minus-strand p23U molecules demonstrated that most of the transgenes contained the expected size transcription

products as RNA and as dsRNA molecules. To test the effectiveness of CTV-p61, -p23U and -p23UI constructs to confer resistance when present in transgenic citrus and *N benthamiana* hosts, the plants were challenged with two types of virus inocula: (I) the authentic CTV and (II) an infectious GVA, harboring CTV-p23U and -p61. All tested transgenic p23UI *N. benthamiana* plants challenge-inoculated with the GVA vector harboring the p23U were highly resistant to the chimeric virus. In contrast, when experiments were conducted with transgenic citrus plants harboring similar cDNA constructs, none of the challenged plants were found to show durable resistance against the authentic CTV inoculum introduced by graft inoculation. We categorized the challenged transgenic citrus plants by their symptom development; those that showed a) severe symptoms like non transgenic plants b) short and/or long delay on symptom onset, c) recovered and d) non-visible symptoms. None of the CTV-derived sequences used in this study resulted in durable resistance. Both, transgenic and non transgenic citrus plants infected by CTV accumulated detectable levels of viral-specific siRNAs from various parts of the genome indicated that, irrespective of the presence or absence of viral-derived transgenes, CTV is the target of a PTGS in nature. Sequence analysis of CTV genome from authentic and recovered strains revealed differences in nucleotide composition and these findings will be useful in future studies aimed to locate the

"Seedling Yellows" pathogenicity determinant. The possible causes for the failure of the transgenes to confer durable resistance to CTV in transgenic citrus plants are discussed.

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1. INTRODUCTION

1.1 The Citrus Tristeza Virus (CTV)

Citrus tristeza virus (CTV) belongs to the *Closterovirus* genus and *Closteroviridae* family, and is considered the most economically damaging disease of *Citrus*, with millions of trees killed or rendered unproductive by the Tristeza disease in most citrus growing areas.

The origin of CTV infections is unknown, although in Asia the virus had existed for centuries unrecognized, possibly because the commonly grown citrus cultivars and rootstocks there were highly tolerant. *Citrus* was first introduced to Europe and the New World mainly in the form of fruits and seeds, and several phloem-associated pathogens, including CTV, did not spread to the new cultivation areas (Bar-Joseph *et al.*, 1989). The outbreak of *Phytophthora sp.,* the casual agent of root rot during the nineteen century led to the wide adaptation and use of *Phytophthora*-resistant sour orange rootstock. This decision had eventually dramatic effects on citrus production and was the main cause of the severe losses that occurred as a result of CTV pandemics throughout the world. The first tristeza disaster was reported in the 1930's in Argentina, where 90% of the citrus was planted on sour orange rootstock (Meneghini, 1946).

The host range of CTV is mostly restricted to *Rutaceous* plants (Bar-Joseph *et al.*, 1989) although experimentally it has been found also to infect *Nicotiana benthamiana* protoplasts (Navas-Castillo *et al.*, 1997). CTV causes different symptoms on citrus plants depending on the virus strain, the variety of citrus, and the scion-rootstock combination. In citrus-growing countries, various strains of CTV, generally referred to as seedling

yellows (CTV-SY), tristeza (CTV-T), stem pitting (CTV-SP). Also "mild type" symptomless isolates have been reported. Any of these strains may be present in most citrus, either singly or as a complex (Grant & Higgins, 1957; Moreno *et al.*, 1993; Broadbent *et al.*, 1996). CTV is considered as a continuous threat to existing plantings, especially of oranges and mandarin trees grafted on Sour orange rootstocks, and also of grapefruit varieties susceptible to stem pitting isolates.

1.1.2 Classification of Citrus Tristeza Virus

CTV belongs to genus *Closterovirus,* family *Closteroviridae* (Bar-Joseph *et al.*, 1979; Dolja *et al.*, 1994; Agranovsky, 1996; Martelli *et al.*, 2002). The *Closteroviridae* family contains more than 30 plant viruses with flexuous, filamentous virions and with either mono- or bipartite single-stranded, positive sense RNA genomes. A recent revision of taxonomy of the *Closteroviridae* based on vector transmission and on phylogenetic relationships using three proteins highly conserved among members of this family (a helicase, an RNA dependent RNA polymerase and a homologue of the HSP70 heat-shock proteins) (Karasev, 2000) has defined three genera: *Closterovirus*, including aphid-borne viruses with monopartite genome, *Ampelovirus*, comprising viruses with monopartite genome transmitted by mealybugs, and *Crinivirus*, that includes whitefly-borne viruses with bipartite or tripartite genomes (Martelli *et al.*, 2002).

1.1.3 Molecular characterization of CTV

CTV virions are long flexuous particles, 2000nm long × 10–12 nm in width (Bar-Joseph & Lee, 1989) with a positive-stranded RNA genome of ~19.3 kb organized into 12 open

reading frames (ORFs), and potentially encoding at least 19 protein products (Pappu *et al.*, 1994; Karasev *et al.*, 1995; Karasev & Hilf, 1997; Karasev, 2000). The CTV virions are polarly coated with two separate capsid proteins p25 and p27, designated as major and minor (ca. 3%) CPs, respectively (Febres *et al.*, 1996). The minor CP is associated with small amounts of at least two other structural proteins -p65, a homolog of cellular heat-shock proteins of the 70 kDa family (HSP70), and a large protein, p61 (Satyanarayana *et al.*, 2000).

In infected cells, the 12 ORFs of CTV (Fig. 1A and B) are expressed through a variety of mechanisms including; proteolytic processing of the polyprotein, translational frame shifting, and formation of up to 32 different 5'- and 3'-subgenomic RNAs (Che *et al.*, 2003; Gowda *et al.*, 2003). The first two mechanisms are used to express proteins encoded by the 5' -half of the genome, which contains ORFs 1a and 1b. A large, ~400 kDa polyprotein encoded by ORF1a is proteolytically processed by virus-encoded proteases (Karasev *et al.*, 1995; Mawassi *et al.*, 1996). ORF 1b, which encodes an RNA dependent RNA polymerase-like domain, is thought to be translated by a +1 frame-shift. The last mechanism is used to express the 3'-coterminal ORFs 2 to 11 (Hilf *et al.*, 1995).

CTV appears to be unusual as it also produces at least three distinct sizes of 5' sgRNAs designated as low-molecular-weight tristeza (LMT1 and LMT2) that make up major proportion of the total virus-associated RNAs in CTV-infected plants and large-molecular-weight tristeza (LaMT) (Che *et al.*, 2001; Gowda *et al.*, 2001; Che *et al.*, 2002).

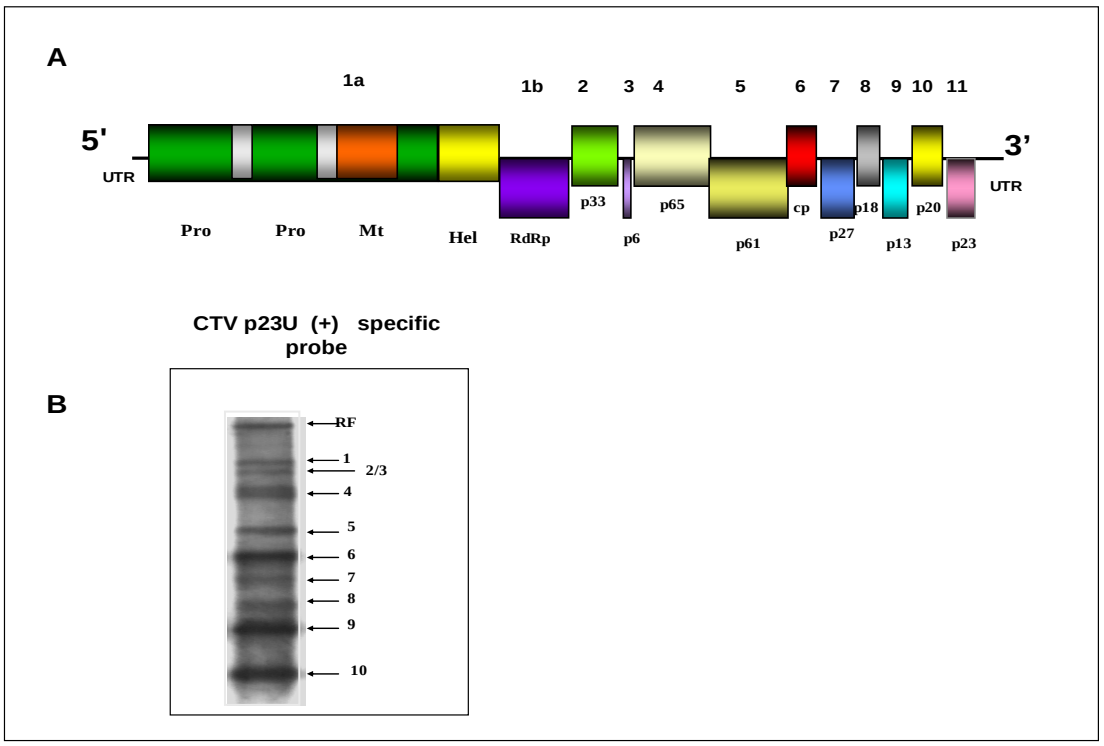

Figure 1. Genome organization and sgRNAs of CTV. **A** The 12 ORFs of CTV and their expected protein products with 5' and 3' un-translated region (UTR). **B** Northern blot hybridization of dsRNAs from CTV infected cells with CTV p23U plus (+) strand specific probe, indicating that the 3' half of the CTV genome is expressed as a nested set of 3'-coterminal subgenomic mRNAs. Pro= papain-like protease domain; Mt= methyltransferase-like domain; Hel= helicase-like domain; RdRp= RNA dependent RNA polymerase; RF= replicative form

Early genome characterization of CTV showed that defective (d)RNAs occur with almost all known CTV isolates. Most CTV dRNAs consist of the two genomic termini, with extensive internal deletions. CTV isolates have multiple dRNAs with various large sizes and different abundances (Mawassi *et al.*, 1995a; Mawassi *et al.*, 1995b; Yang *et al.*,

1997; Ayllon *et al.*, 1999; Yang *et al.*, 1999; Che *et al.*, 2003). Recently CTV dRNAs were categorized in six classes (Batuman *et al.*, 2004).

Different factors have been suggested to contribute to biological variability of CTV isolates, namely, genetic variation following super-infections with multiple isolates, homologous RNA recombination between sequence variants, the presence of different defective RNAs and top working to new varieties (Ayllon *et al.*, 1999; Vives *et al.*, 1999; Yang *et al.*, 1999; Rubio *et al.*, 2001; Roy & Brlansky, 2004; Vives *et al.*, 2005; Ayllon *et al.*, 2006).

1.1.4 Expressed proteins of CTV and their functions

The 5' half of the genome encompasses two ORFs encoding proteins associated with viral replication. ORF 1a encodes a large, ~400 kDa polyprotein, which includes two papain-like protease domains, a methyltransferase-like domain and a helicase-like domain. The ORF 1b encodes an RNA dependent RNA polymerase-like domain (Fig.1A) (Satyanarayana *et al.*, 1999).

The 3'-half of the genome encodes 10 genes that are not required for replication in protoplasts (Hilf *et al.*, 1995; Ayllon *et al.*, 2003) with five-gene block. The five-gene block is unique to closteroviruses, and encodes a small, 6 kDa hydrophobic protein (ORF3), a 65 kDa cellular heat-shock protein homolog (HSP70h, ORF4), a 61 kDa protein (ORF5), and a tandem pair of structural proteins, a 27 kDa capsid protein (CPm, ORF6) duplicate followed by the 25 kDa (major CP ORF7) (Pappu *et al.*, 1994; Karasev *et al.*, 1995).

The small hydrophobic p6 is a single-span transmembrane protein not required for virus replication or assembly, that resides in ER, and functions in another closterovirus (beet yellows virus [BYV]), for cell to cell movement (Alzhanova *et al.*, 2000; Peremyslov *et al.*, 2004). p65 is a homologue of the HSP70 heat-shock proteins, which, together with p61 and the two capsid proteins, are required for virion assembly (Satyanarayana *et al.*, 2000; Satyanarayana *et al.*, 2004). Protein p20 accumulates in amorphous inclusion bodies of CTV-infected cells (Gowda *et al.*, 2000).

The product of the 3'-most ORF (ORF 11), p23, is a multifunctional protein with no homologue in other closteroviruses, that: (i) binds RNA molecules in a non-sequence-specific manner (Lopez *et al.*, 2000); (ii) contains a zinc finger domain that regulates the synthesis of the plus- and minus-strand molecules and controls the accumulation of plus-strand RNA during replication (Satyanarayana *et al.*, 2002); (iii) is an inducer of CTV-like symptoms in transgenic *C. aurantifolia* plants (Ghorbel *et al.*, 2001); and (iv) is a potent suppressor of intracellular RNA silencing in *Nicotiana tabacum* and *N. benthamiana* (Lu *et al.*, 2004). In addition to p23, the CTV genome contains two other suppressor genes: p25, which act as intercellular suppressor, and p20 which inhibits silencing both inter- and intra-cellularly (Lu *et al.*, 2004). The functions of proteins p33, p18 and p13 are presently unknown.

1.1.5 CTV host range and symptoms

CTV infects all species, cultivars and hybrids of *Citrus sp*. CTV also infects some citrus relatives such as *Aeglopsis chevalieri*, *Afraegle paniculata*, *Fortunella sp.* and *Pamburus missionis* and some intergeneric hybrids. Species of *Passiflora* have been infected

naturally and experimentally, and are the only non-*Rutaceous* hosts (Kitajima *et al.*, 1964; Bar-Joseph *et al.*, 1989).

The CTV decline strains are associated with the death of the phloem at the bud union, resulting to a girdling effect that may cause the overgrowth of the scion at the bud union, paucity of feeder roots, stunting, yellowing of leaves, reduced fruit size, poor growth, dieback, wilting, and death. However, other virulent and damaging CTV strains cause stem-pitting (SP, deep pits in the wood under depressed areas of bark) in scion cultivars and cause stunting and reduced production. The seedling-yellow reaction (SY, severe stunting and yellowing on seedlings of sour orange, lemon and grapefruit) is primarily a disease of experimentally inoculated plants but might also be encountered in the field in top-grafted plants (Bar-Joseph *et al.*, 1989).

1.1.6 CTV transmission

Mechanical inoculation by slashing of citrus plants with sap extracts is possible but only with difficulty under experimental conditions (Garnsey *et al.*, 1977; Satyanarayana *et al.*, 2001). Aphids are the main natural vectors of CTV. However, plant propagation material is the most important means of tristeza spread, especially from country to country.

Several aphid species including *Aphis gossypii*, *A. spiraecola*, *A. craccivora*, and *Toxoptera citricida* transmit CTV semi-persistently (Bar-Joseph *et al.*, 1989; Rocha-Pena *et al.*, 1995). Transmissibility is generally dependent on viral isolates (Bar-Joseph & Loebenstein, 1973; Roistacher, 1981; Yokomi & Garnsey, 1987). The brown citrus aphid (*T. citricida)* is the most efficient vector of CTV followed by *A. gossypii*. In side-by-side test, the single aphid transmission efficiencies of these two aphids were 16.0 and 1.4%

respectively (Yokomi *et al.*, 1994). Despite the less efficient transmission by *A. gossypii* and the absence of brown citrus aphid from many areas including the Mediterranean region, citrus decline and stem-pitting strains of CTV are often found to spread in Israel and California (Bar-Joseph & Loebenstein, 1973; Roistacher, 1981; Yokomi & Garnsey, 1987). Recently the brown citrus aphid had spread to Central America and Florida, and from the island of Madeira to Portugal (EPPO/CABI, 1996; Niblett *et al.*, 2000).

1.1.7 CTV control measures

Strategies to control CTV varied at different periods and geographic regions, including costly and ambitious eradication programs to prevent the spread of the virus, quarantine and budwood certification to prevent the introduction of CTV, the use of CTV-tolerant rootstocks, mild (or protective) strain cross protection, breeding for resistance, and attempts to obtain resistance by genetic engineering. Mild strain cross protection has been used in Australia, Brazil and South Africa (Costa & Muller, 1980; Bar-Joseph *et al.*, 1989). However, this type of protection is not possible when considering the sour orange as a rootstock. It is however widely used to control stem pitting symptoms in trees on tolerant rootstock varieties.

Resistance to CTV in *Poncirus trifoliata* has been mapped and shown to be controlled by a single dominant gene, *Ctv* (Gmitter *et al.*, 1996; Yoshida, 1996). The mechanism of action of this gene is currently unknown, since protoplasts of resistant plants support viral replication (Albiach-Marti *et al.*, 1999; Albiach-Marti *et al.*, 2004). Although mapping the location of *Ctv* was made (Deng *et al.*, 1996; Deng *et al.*, 2001; Yang *et al.,* 2001;

Yang *et al.*, 2003), the gene has still not been identified and the possibility to transfer the gene to other citrus species and obtain resistance is still waiting to be proved.

1.2 Genetic transformation of citrus

Citrus genetic improvement by conventional breeding has been limited mainly because some varieties show partial or complete pollen and ovule sterility, self and cross incompatibility (Gmitter *et al.*, 1992).

Application of genetic transformation to citrus has recently been reviewed by Moore *et al.* (2004). The earliest citrus transformation experiments involved direct uptake of DNA into embryogenic protoplasts (Kobayashi & Uchimiya, 1989; Vardi *et al.*, 1990; Niedz *et al.*, 1995). Particle bombardment was used to transform embryogenic suspension cultures of 'Page' tangelo (Yao *et al.*, 1996), and Hidaka *et al.* (1990) described *Agrobacterium tumefaciens*-mediated transformation of embryogenic cultures.

A plant transformation method based on *A. tumefaciens*-mediated gene transfer (*Agro*-transformation) is highly desirable since single copies of the transferred DNA can be integrated in the plant genome. The simplicity of the *Agro*-transformation method and its improvement by using acetosyringone further popularized the application of this technology for numerous plant systems (Horsch *et al.*, 1985; Gelvin, 2000; Gelvin, 2005; Tzfira & Citovsky, 2006).

The protocols for *Agro*-transformation for a number of citrus species and relatives have been reviewed by Pena *et al.* (2003). Several *Citrus sp.* and *Poncirus trifoliata* were transformed by Moore *et al*. (1992) and Kaneyoshi *et al.* (1994) with the *nptII* selection and *uidA* marker genes. Transformation of grapefruit plants has been described using *A.*

tumefaciens strain EHA101 with pGA482GG and strain C58C1 with pBin35SGUS both containing *nptII* and *uidA* were reported by Luth and Moore (1999) and Yang *et al.* (2000), respectively. Pena *et al.* (1995a; 1995b) reported efficient transformation of Carrizo citrange and sweet orange 'Pineapple' from internodal stem segments inoculated with *A. tumefaciens* strain EHA105 with 35SGUSINT (a *uid*A gene interrupted with an intron, so that it is only expressed in plant cells, controlled by the 35S Cauliflower mosaic virus promoter). In order to overcome poor rooting of transformed shoots, they were micro-grafted onto Troyer citrange. Bond and Roose (1998) transformed epicotyl segments of 'Washington' navel orange using *A. tumefaciens* strain C58C1 with p35SGUSINT. Transgenic key lime plants were recovered by Pena *et al.* (1997) using *A. tumefaciens* strain EHA105 with p35SGUSINT by co-culturing of stem pieces on feeder plates consisting of tomato cells on medium containing high auxin levels. Yu *et al.* (2002) refined *Agro*-transformation, and used epicotyl segments that were cut in half longitudinally to increase the wounded area of the explants. Ghorbel *et al.* (1999) transformed three citrus types using this procedure with *A. tumefaciens* strain EHA105 with pBin 19-sgfp, which contains a gene for the green fluorescent protein (GFP).

Recovery of transformed citrus in the mature phase has also been reported. Cervera *et al.* (1998a) partially rejuvenated mature sweet orange by grafting buds onto seedling rootstock. Internodal stem pieces were inoculated with *A. tumefaciens* strain EHA105 with p35SGUSINT, and then co-cultivated on tomato cell culture feeder plates or without feeder plates by Almeida *et al.* (2003). Some of the transformed regenerants flowered, and fruit were produced after 14 months. In another study, Carrizo citrange was transformed to express the *Arabidopsis* LEAFY (LFY) or the APETALA1 (AP1) genes,

which promote floral initiation (Pena *et al.*, 2001). The regenerants displayed an abnormal phenotype, but plants expressing AP1 had fertile flowers and bore fruit in the first year. Perez-Molphe-Balch and Ochoa-Alejo (1998) reported transformation of Mexican lime following inoculation of stem segments with a wild strain of *A. rhizogenes* containing a binary vector plasmid pESC4 that contained *nptII* and *uidA* genes. Shoots were regenerated directly or from hairy roots.

However, transformation is still relatively inefficient for most citrus species (Costa *et al.*, 2002) and several investigators reported a low frequency of stable transformants (Ghorbel *et al.*, 1999; Ghorbel *et al.*, 2000; Yang *et al.*, 2000). Improvement of citrus transformation to increase the number of regenerated transgenic plants through a variety of co-cultivation media and modification of conditions continued to be studied by several groups (Costa *et al.*, 2004; Pena *et al.*, 2004).

1.3 Genetic engineering for virus resistance

Two main transgenic strategies were previously adopted to obtain virus resistant plants. The first is based on pathogen-derived resistance (PDR), a concept pioneered by Sanford and Johnson (1985), where a partial or complete viral gene is introduced into the plant, which, subsequently, interferes with one or more essential steps in the life cycle of the virus. This concept was first illustrated in plants by introducing the coat protein gene of tobacco mosaic virus (TMV) into transgenic tobacco plants that were subsequently turned to show a delay of TMV infection (Powell-Abel *et al.*, 1986). The concept of PDR has been confirmed for several plant–virus systems (reviewed in Baulcombe, 1996; Dasgupta *et al.*, 2003).

Other PDR strategies utilize other virus genes e.g. replicase (Rep) protein and movement proteins (MP) (Golemboski *et al.*, 1990; Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993). Satellite RNA associated with certain viruses (Baulcombe, 1996), defective interfering (DI) DNA (Kollar *et al.*, 1993), and self-cleaving RNA (ribozymes) were also used to obtain resistance. Although these PDR strategies used efficiently, however in most of the cases ribozyme sequences were less effective (Lamb & Hay, 1990; Atkins *et al.*, 1995; De Feyter *et al.*, 1996).

The second transgenic strategy which holds promise is aiming to locate and identify host genes however, achieving this goal is far more difficult (reviewed in Lomonossoff, 1995; Prins & Goldbach, 1998; Prins, 2003; Soosaar *et al.*, 2005).

Table 1 summarizes published reports to introduce CTV resistance into citrus varieties, mainly through PDR. Integration and expression of the CP gene of CTV in transgenic Sour orange and Key lime plants, first reported by Gutierrez *et al.* (1997) was followed by several other groups (Dominguez *et al.*, 2000; Ghorbel *et al.*, 2000; Febres *et al.*, 2003; Piestun, 2003). Transformations with untranslatable versions of this gene were also reported (Yang *et al.*, 2000; Dominguez *et al.*, 2002b). In addition transgenic citrus plants harboring other CTV genomic sequences including full or truncated versions of p23 gene (Ghorbel *et al.*, 2001; Fagoaga *et al.*, 2005; Batuman *et al.*, 2006), RdRp, p27, p20, p61 and 3' UTR were also been reported (Piestun *et al.*, 1998; Febres *et al.*, 2003; Batuman *et al.* unpublished).

Ghorbel *et al.* (2001) and Fagoaga *et al.* (2005) showed that Mexican lime and Alemow plants expressing the p23 gene of CTV exhibited aberrations resembling CTV-induced leaf symptoms. Challenge-inoculation of these plants revealed an apparent immunity, as

indicated by ELISA and hybridization assays. The immunity of most of these propagations was however only temporary, and subsequently the plants showed symptoms. A small number of p23 transgenic Mexican lime plants showed resistance which however, was apparently inconsistent (Fagoaga *et al.*, 2006). Febres *et al.* (2003) and Dominuguez *et al.* (2002b) reported that transformation of grapefruit and Mexican lime plants with sequences of CTV-RdRp and CP also failed to confer CTV protection.

Table 1. Summarized literature reports on attempts to produce transgenic citrus plants with CTV-PDR.

Cultivar or variety	Citrus species	Gene/s introduced	Reference
Sour Orange, Key lime	*Citrus aurantium, Citrus aurantifolia*	CTV-CP (p25) Expressed	Gutierrez *et al.*, (1997)
Troyer Citrange	*[Citrus sinensis (L.) Osbeck] X [Poncirus trifoliate (L.) Raf.]*	Full & truncated CTV-RdRp	Piestun *et al.*, (1998)
Sour Orange	*Citrus aurantium* (Sour Orange)	CTV-CP (p25) Expressed	Ghorbel *et al.*, (2000)
Mexican Lime	*Citrus aurantifolia*	Full & truncated CTV-CP (p25)	Dominguez *et al.*, (2000; 2002a; 2002b; 2002c)
Mexican Lime	*Citrus aurantifolia*	Full & truncated CTV-p23	Ghorbel *et al.*, (2001)
Grapefruit	*Citrus paradisi*	Untranslatable CTV-CP, p25, RdRp, CTV 3'-end of genomic RNA	Febres *et al.*, (2003)
Mexican Lime Sour Orange Sweet Orange Troyer Citrange	*Citrus aurantifolia Citrus aurantium Citrus sinensis [Poncirus trifoliate (L.) Raf.]*	Full & truncated CTV-p23	Fagoago *et al.*, (2005; 2006)
Alemow	*Citrus macrophylla*	Intron spliced hair pin CTV-p23 + 3'UTR	Batuman *et al.*, (2006)

1.3.1 PDR strategies produce variable levels of resistance

Resistance obtained by using CP is conventionally called CP-mediated resistance. Replicase-mediated resistance has been pursued in a number of laboratories and in most of these cases, resistance has been shown to be due to an inherent plant response, known as post-transcriptional gene silencing (PTGS), described below. In general, these strategies produced highly variable levels of resistance. Contradictory to the PDR concept, resistance levels often did not correlate with protein expression levels. The discovery that in many cases the transcription of transgenic RNA, not the expression of viral proteins, was responsible for the observed resistance suggested on new means for resistance (Lindbo & Dougherty, 1992b). Sequence specific RNA-mediated virus resistance (RM-VR) proved far more potent in inducing high levels of resistance, often displaying complete immunity to the inoculated virus or RNA, with resistance against a narrow range of viral strains. Protein-mediated resistance generally resulted in lower levels of resistance, such as delay in symptom development and lower virus titers, but generally a broader resistance range (for reviews, see Dasgupta *et al.* 2003; Prins 2003; Lindbo & Dougherty, 2005).

1.4 Post-transcriptional gene silencing (PTGS) and its current mechanism

Post-transcriptional gene silencing (PTGS) is the mechanism that enables plants and other Eukaryote Kingdoms to specifically degrade viral and other invasive RNAs in a sequence-specific manner. Lindbo *et al.* (1993) were the first to present evidence that the observed resistance mediated by transgenic RNA was related to the previously observed co-suppression phenomenon of petunia plants (Napoli *et al.*, 1990; Van der Krol *et al.*,

1990). Baulcombe and associates suggested that RNA-mediated resistance was 'merely' preprogramming and thereby enhancing a previously unidentified antiviral strategy in plants, in a way reminiscent of genetic vaccination against viral sequences (Baulcombe, 1996). The discovery that some of the plant virus proteins are interfering with RNA silencing suggested a long interplay between the host defence and virus genomes (Brigneti *et al.*, 1998; Voinnet *et al.*, 1999).

Since small RNAs are repressors of gene expression, small RNA-mediated regulation is often referred to as RNA silencing, gene silencing, or RNA interference (RNAi). The same specific RNA degradation mechanism was reported in fungi, insects and vertebrates (Hannon, 2002). Such RNA degradation mechanisms have been lately implicated in the regulation of eukaryote developmental functions (Kasschau *et al.*, 2003). RNA silencing can be efficiently provoked by double stranded RNA (dsRNA) sequences: Smith *et al.* (2000) developed constructs that are able to produce transcripts folding into dsRNAs. Utilizing these constructs increased the success rates of transgenic RNA-mediated resistance by an order of magnitude (Wang & Waterhouse, 2002).

1.4.1 Post-transcriptional gene silencing

Post-transcriptional gene silencing (PTGS) is a specific RNA degradation mechanism functioning in a homology-dependent manner and is based on small RNAs of 20- to 27-nucleotide (nt) belonging to two classes, microRNAs (miRNAs) and short interfering RNAs (siRNAs). Interestingly small RNAs are found in four of the Eukaryote kingdoms (protists, fungi, plants, animals) but surprisingly were not found in the yeast *Saccharomyces cerevisiae*. PTGS activity is either present constitutively or induced as a

cellular defense against pathogens. The replicating form molecules are degraded to short siRNA of 21–25 nt. Gene silencing results from transcription inhibition (transcriptional gene silencing: TGS) or from RNA degradation (PTGS), and correlates with the accumulation of siRNAs corresponding to the silenced promoter or to the degraded RNA, respectively (Hamilton & Baulcombe, 1999; Mette *et al.*, 2000). A complex of host factors, including RNA-dependent RNA polymerase (RdRp) (Mourrain *et al.*, 2000), RNA-helicase (Dalmay *et al.*, 2001), translation elongation factor (Zou *et al.*, 1998), RNAse III (Ketting *et al.*, 1999), etc. along with small 21–25 nt RNA (from the elicitor RNA) acting as the guide RNA (Hammond *et al.*, 2001) to degrade RNA molecules bearing homology with the elicitor RNA. (Sivamani *et al.*, 2002; Qu *et al.*, 2003; MacDiarmid, 2005; Mourrain *et al.*, 2006; for review see Vaucheret, 2006).

When the viral RNA is either the elicitor or target of PTGS, the degradation mechanism is known as virus induced gene silencing (VIGS). VIGS is activated when plants recover from initial viral infection (viral recovery) or cross-protection when plants show resistance to super-infection of viruses with genomes bearing homology with those of the viruses used as primary inoculum (Angell & Baulcombe, 1999; Hamilton & Baulcombe, 1999). Infected plant mutants defective in PTGS components were found to be hyper-susceptible to virus infection (Mourrain *et al.*, 2000; Dalmay *et al.*, 2001; Qu *et al.*, 2005; Schwach *et al.*, 2005). Interestingly resistance involving PTGS applies also to DNA viruses (Kjemtrup *et al.*, 1998). Viruses can also induce silencing of host endogenes and transgenes that are similar in sequence to the inoculated virus (Jan *et al.*, 2000).

1.4.2 Current models of the RNAi mechanism

The current models of the RNAi mechanism include both initiation and effector steps (Hutvagner & Zamore, 2002). Initiation process starts with digestion of long dsRNAs into 21-23 nt siRNAs, called "guide RNAs" (Hammond *et al.*, 2001). The pathway involves RNase III enzymes, which dice dsRNAs into siRNAs, and Argonaute enzymes, which slice single-stranded RNAs complementary to siRNAs (for reviews, see Du & Zamore, 2005; Tomari & Zamore, 2005; Voinnet, 2005; Vaucheret, 2006). In the effector step, the siRNA bind to a nuclease complex to form the RNA-induced silencing complex (RISC). The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~21 nucleotides from the 3' terminus of the siRNA (Bernstein *et al.*, 2001; Matzke & Matzke, 2004). Because of the remarkable potency of RNAi, some organisms (fungi, worms, and plants) require RNA-dependent RNA polymerases (RdRp) to amplify RNAi. Amplification could occur by copying of the input dsRNAs, which would generate more siRNAs, or by replication of the siRNAs themselves. Alternatively or in addition, amplification could be effected by multiple turnover events of the RNA-induced silencing complex (RISC) (Sharp, 2001; Haley & Zamore, 2004).

The PTGS machinery participates in the RNA-based immune response against cucumber mosaic virus (CMV) infection (Mourrain *et al.*, 2000; Morel *et al.*, 2002; Boutet *et al.*, 2003). Apparently different viruses activate different responses and the *rdr6* mutants show susceptibility to CMV and *Potato virus X* (PVX), but not to several other viruses belonging to other families (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Dalmay *et al.*, 2001; Qu *et al.*, 2005; Schwach *et al.*, 2005). In contrast, *rdr1* mutants are impaired in a

paralog of RNA-Dependent RNA Polymerase 6 show susceptibility to TMV (Yu *et al.*, 2003). Given multiple genes in the model plant *Arabidopsis* involved in gene silencing (10 Argonaute [AGO], four dicer [DCL], and six RNA dependent RNA polymerase [RDR]) (Morel *et al.*, 2002; Schauer *et al.*, 2002; Yu *et al.*, 2003), the varying responses of different viruses to these mutations presumably reflect a diversification of siRNA pathways (Fig 2).

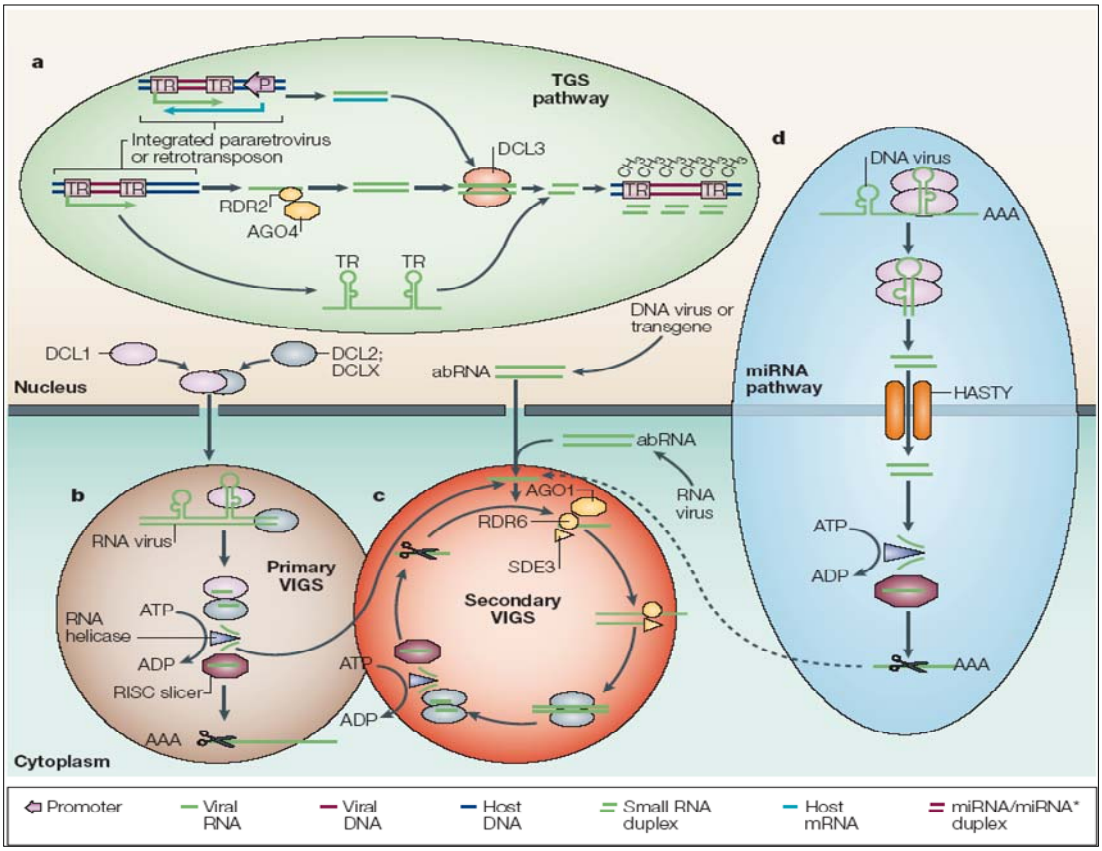

Figure 2. A scheme of antiviral RNA-silencing pathways in plants. An integrated scheme showing pathways that have been either experimentally demonstrated in plants (solid arrows) - inferred from work on other organisms - or purely speculative (dotted arrows).

A In the nucleus, viruses and sub-viral pathogens that are integrated in the host genome can be subject to transcriptional gene silencing (TGS). In the situation in the upper part, read through transcription leads to the production of dsRNA that is complementary to viral sequences, whereas in the situation shown in the middle, dsRNA is produced *de novo* through the activity of the argonaute protein AGO4 and the RNA-dependent RNA polymerase RDR2. Finally, the situation in the lower part shows dsRNA that is produced by intramolecular pairing of an RNA that contains terminal repeat (TR) sequences. In all cases, the dsRNA is recognized by DCL3, which results in the production of viral siRNAs. These then interact with the corresponding regions of the viral DNA within the host genome, directing epigenetic modifications (shown as methylation (CH3)) to this region, which results in the silencing of gene expression. **B** In the cytoplasm, silencing is initiated through the process of virus-induced gene silencing (VIGS). DCL2 is shown here as potentially interacting with DCL1 to promote its nuclear export and to facilitate processing of imperfect stem-loops that are found in RNA virus and viroid genomes, although this has not yet been tested. The resulting viral small interfering RNAs are unwound by an ATP-dependent RNA helicase and then incorporated into the RNA induced silencing complex. The RISC complex is then directed to the corresponding viral mRNA, which is degraded. **C** The primary signal can be amplified in the secondary VIGS pathway. Viral small RNAs produced in primary VIGS, or aberrant RNA (abRNA; for example, expressed from a transgene, or produced by a virus) are converted into dsRNA by the combined actions of the RNA-dependent RNA polymerase RDR6 (also known as SDE1), the AGO1 protein and SDE3, which might be an RNA helicase. In the same process that occurs in primary VIGS, these dsRNAs are then processed and lead to

degradation of the corresponding viral or transgene mRNA. **D** The miRNA pathway might also be involved in VIGS. There is evidence, from experiments in human cells, to suggest that viral dsRNAs can be processed in the nucleus by DCL1 and subsequently exported to the cytoplasm, where they enter the antiviral RNA silencing pathway. HASTY, the exportin 5 homologue of *Arabidopsis thaliana*. Figure 2 is from Voinnet (2005).

1.5 Viral suppression of RNA-silencing

Viruses have evolved a range of mechanisms to overcome their destruction by RNA-silencing. Pruss *et al.* (1997) reported that the potyvirus-encoded helper component proteinase (HC-Pro) enhances the replication of many unrelated viruses. This finding prompted the idea that HC-Pro acts as silencing suppressor (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998). The CMV-encoded 2b protein was also found to suppress silencing (Brigneti *et al.*, 1998) and both HC-Pro and 2b also act as pathogenicity determinants (Voinnet *et al.*, 1999). Several distinct silencing suppressors were found to reside in a large CTV genome (Lu *et al.*, 2004).

1.5.1 Molecular basis of silencing suppression.

The strategies of viral silencing suppression include (i) direct inhibition of silencing-effector molecules, (ii) recruitment of endogenous pathways that negatively control RNA silencing and (iii) modification of the host transcriptome (Moissiard & Voinnet, 2004; Roth *et al.*, 2004; reviewed in Voinnet, 2005).

Inhibition of key components of RNA silencing pathways were shown for the tombusviral p19 protein. The p19 specifically binds the short siRNA duplexes to interfere with the incorporation of siRNA into the RISC. This was demonstrated by crystallization of p19 homodimers directly bound to siRNA duplexes (Vargason *et al.*, 2003; Lakatos *et al.*, 2004) (Fig. 3A). Using a yeast two-hybrid system (Anandalakshmi *et al.*, 2000), identified a calmodulin-related protein (termed rgs-CaM) that interacts with HC-Pro and reported that rgs-CaM, like HC-Pro itself, suppresses gene silencing as an endogenous silencing suppressor through an as yet uncharacterized calcium-dependent pathway (Fig. 3B).

A third strategy of host transcriptome modifications was shown when the geminivirus transcriptional-activator proteins (TrAPs) were identified as silencing suppressors. The nuclear localization and zinc- and DNA-binding activities of TrAPs are all required for their suppressor function, indicating that TrAPs function at the host-DNA level (Hartitz *et al.*, 1999; van Wezel *et al.*, 2002; van Wezel *et al.*, 2003) (Fig. 3C).

Silencing suppression could also be RNA- rather than protein-mediated and, paradoxically, this could involve viral small (vs) RNAs. A novel mechanism of RNAi suppression by *Red clover necrotic mosaic virus* (RCNMV) was reported to suppress RNAi, with multiple viral components, which include viral RNAs and putative RNA replicase proteins. A close relationship between the RNA elements required for negative-strand RNA synthesis and RNAi suppression suggests a strong link between the viral RNA replication machinery and the RNAi machinery and proposed a model in which, to replicate, RCNMV deprives the RNAi machinery of the Dicer-like enzymes involved in both siRNA and miRNA biogenesis (Takeda *et al.*, 2005). Indeed, some vsRNAs might

not necessarily promote effective cleavage once loaded into the RISC complex if they are derived from portions of the pathogen's genome that are in-accessible to this complex. Considering the large amount of vsRNA in plant- and insect-infected cells (Hamilton & Baulcombe, 1999; Szittya *et al.*, 2002), it is therefore plausible that many of them are non-productive bait for the RISC complex (Fig. 3D). Abundant vsRNAs might also out-compete endogenous small RNAs for the RISC, and therefore interfere with host biology - a possible cause of some of the symptoms of viral infection.

Viral suppression of RNA silencing often - although not always - has adverse effects on host biology, and forms the basis of some of the cytopathic symptoms associated with virus infections in plants. This is, at least partly, an incidental consequence of the primary suppression of VIGS at an intermediate step that is shared with the miRNA pathway. Viruses can also evade RNA silencing through a range of means that include sub-cellular compartmentalization and loss of silencing-target sequences due to high mutation rates. Viruses might also deliberately hijack their host silencing pathways to establish optimal infection conditions.

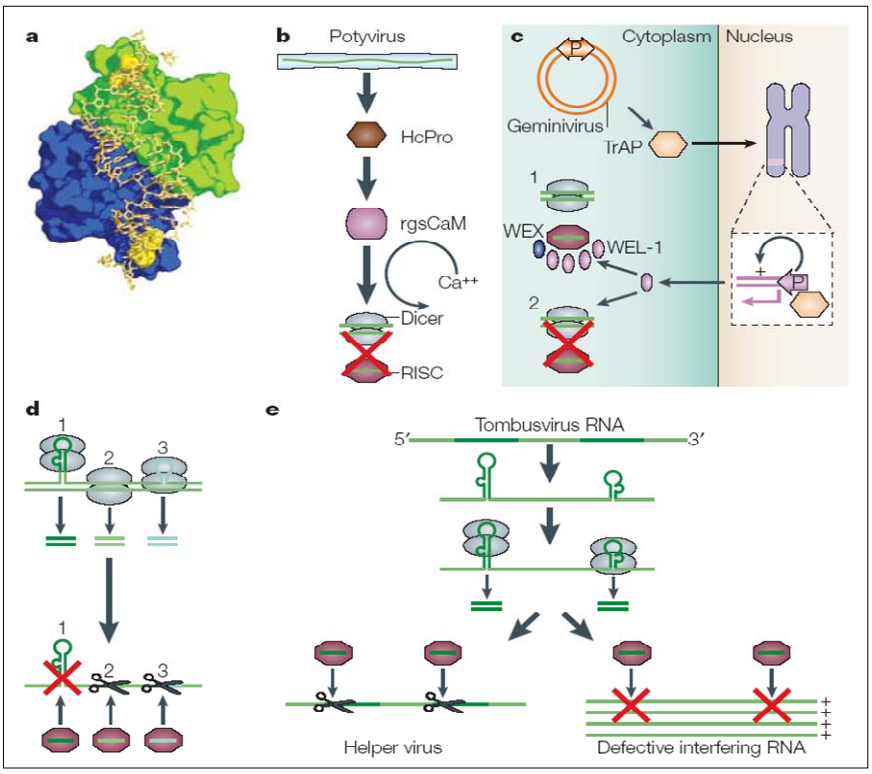

Figure 3. Viral strategies for suppression and evasion of RNA-silencing. **A** Direct interference with silencing-effector molecules is illustrated by the tombusviral p19 protein. The head-to-tail organization of p19 homodimers (blue and green) allows binding to small interfering RNA (siRNA) duplexes (yellow). Two sets of tryptophan residues (yellow) bind to the last set of base pairs on either end of the siRNA, leading to effective measurement of the duplex length, such that p19 selects siRNAs of 21nt for binding. The sequestered siRNA is prevented from entering the RNA induced silencing complex and is therefore inactivated. **B** Recruitment of endogenous negative regulators of RNA silencing is illustrated by the potyviral helper component proteinase (HC-Pro). HC-Pro interacts with the calmodulin-like protein rgsCaM (regulator of gene silencing CaM) to inactivate the RNA-silencing pathway through an unknown mechanism at an

intermediate step that involves both RISC and Dicer. **C** Geminiviral transactivator proteins (TrAPs) suppress RNA silencing by altering the host transcriptome so that proteins such as Werner Exonuclease-Like 1 (WEL1) that are homologues of components of the silencing pathway are produced at excessive levels. This can lead to dominant-negative effects by competing with positive effectors of silencing - in this case, Werner Syndrome like Exonuclease (WEX) - for interaction with the core silencing machinery (case 1). The TrAP-induced factors might also directly inhibit the silencing machinery (case 2). **D** Out-competition of RISC by unproductive viral-derived small RNA (vsRNA) could be a common feature of plant and insect virus infections. Stem-loop regions of the genome that are accessible to Dicer-like enzymes (DCLs) but inaccessible to RISC might generate unproductive vsRNA (case 1). If such regions are favored as DCL substrates, as with tombusviruses, the resulting vsRNA could then out-compete productive vsRNA (2 and 3) for loading into RISC. **E** Evasion of silencing by loss of silencing target sequences is illustrated by the generation of defective interfering RNA molecules from tombusviruses. Defective interfering RNA molecules result from skipping of the viral replicase at the junctions of stem-loop structures that are normally potent silencing inducers. Defective interfering RNA molecules are therefore devoid of silencing targets (bottom right) and have a strong selective advantage over the helper virus (bottom left). P, promoter. Figure 3 is from Voinnet (2005).

1.6 The objectives of the present study

The particular objectives of this PhD. study included:

- Improvement of the *in vitro* culture and regeneration system for citrus rootstocks.
- Optimization of the *Agro*-transformation protocol for different citrus rootstocks.
- Cloning of cDNAs from CTV genomic sequences and insertion into appropriate binary vectors.
- *Agro*-transformation of different citrus rootstocks and *N. benthamiana* with CTV-derived constructs.
- *In vitro* and greenhouse grafting of transformed citrus plants.
- Molecular analyses of the resulting transgenic plants.
- Challenge inoculations and testing for durable resistance among the resulting transgenic citrus plants.

2. MATERIALS AND METHODS

2.1 Plasmid construction and bacterial strains

Standard techniques for gel electrophoresis, clone manipulations and amplifications in *Escherichia coli* strain DH5α were followed (Sambrook *et al.*, 1989). The coding sequence of the p61 (ORF5; 13685-15295) and p23U (ORF11 plus 3'UTR; 18325-19226) of CTV-VT strain was synthesized from dsRNA extracts of CTV-infected bark by reverse transcription and PCR amplification with primer sequences listed in Table 2. The sequence and nucleotide numbering are according to CTV-VT described by Mawassi et al. (1996) (GenBank accession No. U56902). The first strand cDNAs of CTV were prepared with primers VT16 and VT14 using Superscript II RnaseH$^-$ reverse transcriptase (Invitrogene). The p61 sequence was synthesized with primers VT15-VT16, sense p23U with VT13-VT14 and anti-sense p23U with VT25-VT26 using *Pfu* DNA polymerase (Stratagene) by PCR. The Castorbean catalase (CAT) intron was isolated from the binary vector pCambia2301 (CAMBIA, Canberra, Australia) (accession number AF234316) by PCR using primers INT1-INT2. The CTV-cDNA fragments were separated by electrophoresis on 1% agarose gel, excised from the gel and digested at restriction enzyme sites located within the 5' primer end. The 1.6 kb p61 S*tu*I/E*co*RI fragment was ligated into similarly digested pUC57 (MBI Fermentas). The 900 bp sense p23U K*pn*I/E*co*RI, 200 bp intron E*co*RI/B*am*HI and 900 bp anti-sense p23U B*am*HI/E*cl*136II fragments were step-wise ligated into similarly digested pBluescript II KS + (Stratagene), to obtain pKS23, pKS23i and pKS23i23 clones, respectively. The p61 and sense p23U were amplified with primers (XB21-XB22 and XB19-XB20) from pUC61 and pKS23 plasmids to regenerate N*ot*I/A*pa*I restriction sites on both 5'-ends, respectively. To obtain

the GVA-CTV chimeric virus vector the 1.6 kb p61 and 900 bp p23U N*ot*I/A*pa*I fragments were inserted between the duplicated movement protein (MP) sub genomic RNA (sgRNA) promoters of the GVA vector (Haviv *et al.,* 2006), resulting in infectious clones GVA-p61 and GVA-p23U.

Truncated CTV-p23U (50, 100, 200 and 400 bp) fragments were generated by XB19 as forward primer paired with VT44, VT45, VT46 and VT47 reverse primers (listed in Table 2) and cloned between MP sgRNA promoters of GVA vector, respectively (Fig. 17). The 1.6 kb S*tu*I/E*co*RI p61 fragment, 900 bp K*pn*I/E*co*RI p23U fragment and the 2 kp K*pn*I/E*cl*136II fragment encompassing the sense-intron-anti-sense clone was isolated from pUC61, pKS23 and pKS23UI23, subcloned between the enhanced cauliflower mosaic virus (CaMV) 35S promoter and terminator of S*tu*I/E*co*RI, K*pn*I/E*co*RI and K*pn*I and T4-filled E*co*RI sites of pCaas2 (Shi *et al.*, 1997), respectively. Complete cassettes were isolated by H*ind*III/P*vu*II from pCassEN35Sp61T, pCassEN35Sp23UT and pCassEN35S23UI23T, and inserted in H*ind*III/S*ma*I digested pCambia2301 vector's multiple cloning site to obtain final binary vectors pCamEN35Sp61T, pCamEN35S23UT and pCamEN35S23UI23T, respectively (Fig 4). The *uidA* (GUS) gene driven by the CaMV 35S promoter and nopoline synthase (NOS) terminator sequences served as a reporter gene. The presence of the intron in the *uidA* gene of pCambia2301 blocked its direct expression in *A. tumefaciens*. The neomycin phosphotransferase II (*nptII*) gene driven by the CaMV 35S promoter and terminator sequences was used as a selectable marker. The resulting binary vectors were electroporated into *A. tumefaciens* strain EHA 105 (Hood *et al.*, 1993) as described by Singh *et al.* (1993).

Bacteria were cultivated in YEP solid medium (10 g l^{-1} peptone, 10 g l^{-1}, 10 g l^{-1} yeast extract, 5 g l^{-1} sodium chloride, 15 g l^{-1} agar, pH 7.0) containing 100 mg l^{-1} kanamycin and 30mg l^{-1} rifampicin for 24 h. Single bacterial colonies were transferred to 250 ml Erlenmeyer flask, with 50 ml YEP liquid medium, supplemented with half concentration of antibiotics. The cultures were grown overnight in a shaker (220 rpm) at 28 ^{o}C. Bacterial suspensions were centrifuged at 6000 rpm (room temperature, 15 min) and resuspended in hormone-free MS medium for use in *N. benthamiana* or in the induction medium (IM) (Gelvin & Liu, 1994) for citrus transformation. The MS medium consisted of MS salts (Murashige & Skoog, 1962) supplemented with Gamborg's B5 vitamins (Gamborg *et al.*, 1968), 100 µM acetosyringone, pH 5.5.

The IM medium consisted of 1x AB salts, 2 mM $NaPO_4$, 50 mM MES, 0.5% glucose and 200 µM acetosyringone, pH 5.5. Bacterial suspensions were further cultured 1, 3, 5 hours and overnight to induce virulence for recalcitrant citrus transformation. Virulence-induced bacteria suspensions were centrifuged at 6000 rpm (room temperature, 15 min), resuspended in hormone-free MS medium with 200 µM acetosyringone. Final bacterial concentration was adjusted to approximately $5x10^8$ CFU/ml. The antibiotics were filter-sterilized and added to the autoclaved medium.

Table 2. List of primers used for preparing CTV specific cDNA fragments.

Primer Code	Rest. enzyme site	Sequence (5' to 3')	Position on template sequence
VT13	K*pn*I	*GGTACC*ATGGACGATACTAGCGGAC	CTV-VT 18325
VT14	E*co*RI	*GAATTC*TGGACCTATGTTGGCCCCC	CTV-VT 19226
VT25	B*am*HI	*GGATCC*TGGACCTATGTTGGCCCCCCATA	CTV-VT 18325
VT26	E*cl*136II	*GAGCTC*ATGGACGATACTAGCGGAC	CTV-VT 19226
VT15	S*tu*I	*AGGCCT*ATGTCGTCTCATCACGTATGG	CTV-VT 13685
VT16	E*co*RI	*GAATTC*TTAGGAAGCATCGTGTAACCT	CTV-VT 15295
Int1	E*co*RI	*GAATTC*GTAAATTTCTAGTTTTTCTCCTTCATT	CAT intron 1
Int2	B*am*HI	*GGATCC*CTGTAACTATCATCATCATAGACA	CAT intron 190
XB19	N*ot*I	*GCGGCCG*CATGGACGATACTAGCGGAC	CTV-VT 18325
XB20	A*pa*I	*GGGCCC*TGGACCTATGTTGGCCCCC	CTV-VT 19226
XB21	N*ot*I	*GCGGCCG*CATGTCGTCTCATCACGTATGG	CTV-VT 13685
XB22	A*pa*I	*GGGCCC*TTAGGAAGCATCGTGTAACCT	CTV-VT 15295
VT44	A*pa*I	*GGGCCC*CAGAAAAGTTCACAGAAATG	CTV-VT 18375
VT45	A*pa*I	*GGGCCC*CGCTTCCAAACTTACGGT	CTV-VT 18425
VT46	A*pa*I	*GGGCCC*CTATTATTCTCGCGCGAA	CTV-VT 18525
VT47	A*pa*I	*GGGCCC*TTCTTTGGTATGCATAAAC	CTV-VT 18725

Italic letters represents the added restriction enzyme sequences.

2.2 Citrus transformation experiments

These were based on a protocol for *Agro*-transformation of citrus epicotyl explants described in Luth and Moore (1999), and modified by Piestun (2003). In addition, different modes of epicotyl explant preparation from etiolated seedlings of citrus rootstocks, optimization of *Agro*-transformation, and co-cultivation parameters on efficiency of transformation were extensively studied.

2.2.1 Plant materials

Cold-stored seeds of Sour orange (*C. aurantium*), Gou Thou (also named the Chinese sour orange), Alemow (*C. macrophylla*), Volkamer lemon (*C. limon*) and Troyer citrange (*C. sinensis x Poncirus trifoliate*) were peeled to remove the seed coats, disinfected for 10 min in a 0.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) Tween 20, and rinsed three times with sterile distilled water. Seeds were sown individually in 25×150 mm culture tubes containing 25 ml of germination medium consisting of MS (Murashige & Skoog, 1962) salt solution and 3 % sucrose, solidified with 2.3 g l^{-1} gelrite, pH 5.7. The cultures were maintained in darkness at 27 ^{o}C for 1-5 weeks.

Leaves of 4- to 6-week-old glasshouse grown *N. benthamiana* plants were used for *Agrobacterium* transformation and inoculations with the GVA vectors.

2.2.2 Explant preparation and preculture

Etiolated 1- to 5-week-old seedlings of different citrus rootstocks were used as a source of tissue for transformation. Explants were prepared by excising most of the roots,

cotyledons and axillary buds. Two explants per seedling were prepared as follows; the first explant (RootHE) comprised of the top of the root (1cm), 2-3mm hypicotyl, 2-3mm of the epicotyl. The second explant was 0.5 - 2 cm long epicotyl from most proximal to the cotyledonary node (Fig. 9). Later in the experiments, other types of epicotyl explants from distal cotyledonary node were also used. Five to six explants were precultured horizontally on preculture medium (PM) for 0 - 4 days. Petri plates were wrapped with saran wrap and cultures maintained at 22-26 ^{o}C under a variety of light and/or dark combinations. PM consists of MS salts with Gamborg's B5 vitamins, 5 % sucrose, with concentrations of 0 - 44 μM 6-benzyladenine (BA) or 0 - 46 μM kinetin (Kin), 0 - 5.4 μM α-naphthalene acetic acid, and 0 – 18.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D), pH5.7. Filter-sterilized 2.5 g l^{-1} polyvinilpirrolidone (PVP) and 50 mg l^{-1} malt extract was added to the autoclaved medium.

2.2.3 Transformation and coculture

Precultured explants in groups of 15 to 20 were placed for incubation into 50 mm disposable Petri plates containing 10 ml of induced bacteria suspension cultures in MS solution, pH 5.5. Batches of plates with *Agrobacterium* were vacuum infiltrated for 5 - 10 min connected to vacuum pump with a 2.6 m^3/h vacuum capacity. After vacuum release, plates were placed on orbital shaker at 50 rpm, for 5 - 60 min. Treated explants were blotted dry on sterile filter paper and placed on PM plates supplemented with different concentrations of BA. Petri plates were wrapped with saran wrap and cocultured at 26 ^{o}C for 1 - 5 days in darkness.

2.2.4 Selection and shoot tip grafting (STG)

After 1- to -5 days of co-cultivation, explants were washed with liquid MS supplemented 500 mg l^{-1} cefotaxime and 250 mg l^{-1} vancomycin to reduce excess *Agrobacterium*. Explants were blotted dry on sterile filter paper, transferred to selection medium (SM) and maintained for 3-6 weeks at 26 ^{o}C under 16 h photoperiod, 45 µE m^{-2} s^{-1} illumination and 60% relative humidity. Explants were subcultured to fresh medium every 3 weeks. The SM is equivalent of PM but supplemented with 100-300 mg l^{-1} kanamycin for selection and 500 mg l^{-1} cefotaxime and 250 mg l^{-1} vancomycin to control bacterial growth.

To recover whole putative transgenic plants, well-developed shoots (2-4 mm) were separated from the explants, the basal portions were tested histochemically for β-glucuronidase (GUS) activity, and the decapitated shoots were placed on hormone-free solid MS medium with 5 % sucrose for 2 days to reduce any carry-over effect of hormones. After confirmation of the GUS activity of basal portions, the shoot tips were *in vitro* grafted onto etiolated, ca. two week old Troyer citrange seedlings. Shoot-tip grafting (STG) was according Pena *et al.* (1995b) and Piestun *et al.* (1998) but shoots were grafted in to a rectangular incision on top of decapitated rootstock, with the same size of the excised shoot tip. After 4-5 weeks, the epicotyl parts of shoot-tip grafted plants were grafted on vigorous seedlings of Volkamer lemon (*C. limon*) growing in the greenhouse at 24-28 ^{o}C.

2.3 *N. benthamiana* transformation, selection and rooting

A. tumefaciens containing the pCamEN35Sp61T, pCamEN35S23UT and pCamEN35S23UI23T binary vectors were used to transform *N. benthamiana* leaf discs as described by Horsch *et al.* (1985) with minor modifications. Briefly: surface-sterilized leaf discs were transformed by dipping into *Agrobacterium* solution. The treated discs were blotted dry onto sterile filter paper and placed on a coculture medium and kept at 26 ^{o}C in darkness. After 2-3 days the cocultured explants were transferred to selection medium with 300 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin, and kept to induce callus or/and differentiate into shoots. Resistant shoots were collected and placed for rooting in the presence of 100 mg l^{-1} kanamycin, 500 mg l^{-1} carbenicillin. Whole plants were regenerated at 26 ^{o}C, 16 h photoperiod, 45 μE m^{-2} s^{-1} illumination, 60% relative humidity. Plant sections were used both for histochemical GUS activity and for genomic DNA (gDNA) extraction. Transformant lines which were positive for GUS activity and the contained the transgene in the genomic DNA were selected and potted first into pre-moistened soil medium in a Magenta box, and then, into containers with regular turf which were placed in plastic bags for 5-7 days, until their adaptation to ambient humidity by gradual removal of the bags. Seeds were harvested when completely dried at about 6-8 weeks from potting.

2.4 Histochemical GUS assay

Histochemical staining of plant materials was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as described by Jefferson (1987). Plant pieces were vacuum infiltrated with a solution containing 0.5 mg ml^{-1} X-gluc, 50 mM sodium phosphate, pH 7,

1 mM EDTA and 0.5% TritonX-100 and incubated for 16-18 h at 37 ^{o}C before clearing the tissue with ethanol. Explants were observed under a stereomicroscope and each blue spot was considered as an independent transformation event. Plant materials showing blue color after GUS analysis were considered putatively transgenic.

2.5 PCR and Southern blot analyses

For detection of specific DNA sequences, the genomic (g)DNA was extracted from young leaves of *in vitro* and *in vivo* plantlets with DNAzol ES (genomic DNA isolation kit, MRC, Inc., Ohio) according to manufacturer's instructions. PCR amplification was performed using 50-100ng of genomic DNA, 200 µM of each dNTP, 2.5 mM $MgCl_2$, 2U Taq DNA polymerase (MBI Fermentas) and 0.25 µM primers. Primers (Table 2) VT13 and VT14 were used to amplify a 900 bp specific fragment of the p23U gene. Primers VT15 and VT16 (Table 2) amplified a 1.6 kb specific fragment of the p61 gene. The PCR reactions were performed as follows: samples were heated to 94 ^{o}C for 4 min followed by 30 cycles of 40 sec at 94 ^{o}C, 30 sec at 58 ^{o}C, and 90 sec at 72 ^{o}C with terminal elongation step of 5 min at 72 ^{o}C.

For Southern analysis, 20 µg of genomic DNA samples digested by either H*ind*III (a single site cutter in the pCambia binary vector) or B*gl*II (which excises the introduced expression cassettes from T-DNA) were separated on 1% (w/v) agarose gels (Sambrook *et al.*, 1989). The genomic DNA was blotted onto positively charged Hybond N+ nylon membranes (Roche, Germany) and the membranes were fixed by UV irradiation. Membranes were hybridized with non radioactive DIG-labeled (Roche, Germany) riboprobes according to manufacturer's instructions. Restriction endonuclease

linearization of the DNA templates, allowed creation of "run off" transcripts of uniform length. The RNA probes were labeled with digoxigenin according to manufacturer's instructions (Roche, Germany). The p61 and p23U of CTV-VT genes cloned into pGEM-T vector (Promega) were used to synthesize riboprobes specific to positive- or negative-stranded-RNA with T7 or SP6 RNA polymerase as described in Che *et al.* (2001; 2002).

2.6 Northern blot analyses

Total single-stranded RNAs (ssRNA) were extracted from leaves and/or young bark of plants, using Tri-Reagent solution, according to the manufacturer's instructions (Molecular Research Center, Inc.).

Enriched double-stranded (ds) RNAs preparations were obtained as reported by Dodds and Bar-Joseph (1983). For rapid mini scale preparation of dsRNAs from small amounts of tissue the following modifications were used. Samples of fresh or frozen leaf or bark tissue (0.5-1 *g*) were ground to powder in liquid nitrogen with a mortar and pestle and added to a suspension of 0.5 ml STE X 2 (0.05 M Tris, 0.1 M NaCL, and 10mM EDTA), 0.01 ml SDS 10%, 2 µl ß-mercapto ethanol, 0.3 ml phenol, and 0.3 ml chloroform, and shaken for 20 min at room temperature. The extracts were centrifuged for 20 min at 10,000 *g*, the supernatants collected, adjusted to 16.5% (v/v) ethanol, and stored overnight 4°C or –80°C for 1h. The solution was centrifuged (10,000 *g* for 20 min), the supernatant was subjected to two cycles of chromatography on small CF-11 cellulose columns (0.1g CF-11 dry powder, wetted by 2.4 ml buffer: 1 x STE and 16.5% ethanol) (Whatmann, Clifton, NJ 07014) designed for dsRNA isolation. Each elution cycle consisted of loading the column with approximately 1 ml of sample in 83.5% STE buffer:

16.5% ethanol (v/v) (STE-ET), washing the column with STE-ET buffer, and finally eluting the column with 0.4 ml of 1 x STE buffer. The eluted solutions were adjusted to 70% ethanol (v/v), ~3% 3 M sodium acetate, stored at –20°C overnight or –80°C for 1 hour and centrifuged for 20 min at 10,000 *g* at 4°C. The pellets were washed with 75% ethanol, vacuum dried and resuspended in 10 µl sterile water. Resulting RNAs were denatured with methyl mercury hydroxide, separated by electrophoresis in formamide-formaldehyde denaturing 1.1% agarose gels in 1 x MOPS buffer (10 x MOPS: 50mM sodium Acetate, 10mM EDTA, and 0.2M Morpholinopropansulphonate), blotted onto positively charged Hybond N+ nylon membranes (Roche, Germany) and fixed by UV irradiation.

For analysis of siRNAs, 50 µg of total ssRNA were denatured at 65°C for 10 min, separated on 15% polyacrylamide gel containing urea 8 M and transferred onto Hybond N+ membranes (Roche, Germany) using a EC120 mini vertical gel system according to the manufacturer's instructions (Thermo Electron Corp., USA) and fixed by UV irradiation. The membranes were hybridized with non-radioactive DIG-labeled riboprobes, reacted with anti-digoxigenin-alkaline phosphatase Fab fragment (antibody) and developed using CSPD chemiluminescent substrate according to the manufacturer's instructions (Roche, Germany).

The cDNAs of different CTV-VT sequences cloned into pGEM-T vectors (Promega) were used to synthesize riboprobes specific to positive- or negative-stranded-RNA with T7 or SP6 RNA polymerase as described in Che *et al.* (2001; 2002). The DIG-labeled 5' end specific riboprobes was prepared by cloning the 5' part of the GVA vector into pGEM-T vector (Promega). Riboprobes specific to positive- or negative-stranded-RNA

were synthesize with T7 or SP6 RNA polymerase as described in Galiakparov *et al.* (2003).

2.7 *In vitro* transcription of GVA for *N. benthamiana* inoculation

The CTV cDNAs were inserted downstream of a single copy T7 RNA promoter in GVA vectors assembled in pCambia2301. *In vitro* transcription was carried out in 25µl of a mixture consisting of 1.5 µg S*al*I- linearized plasmids, 40 mM Tris-HCL pH 7.9, 20mM DTT, 8.5 mM $MgCl_2$, 1.2 mM of each of ATP, CTP, UTP and Cap analog (m^7G[5']ppp[5']G) (Epicenter Technologies), 0.048 mM GTP, 20 U rRNasin ribonuclease inhibitor (Takara Biomedicals, Shiga, Japan) and 20 U T7 RNA polymerase (MBI Fermentas). The reaction mix was incubated in 37 ^oC; the GTP concentration was increased to 0.5 mM and reaction was further incubated for an additional 1h and 45 min. Freshly prepared *in vitro* transcripts without additional purification were used directly for inoculation of wild type and transgenic *N. benthamiana* plants. For virus challenge experiments, 4-to 6-week-old wild type and F1/F2 progenies of transgenic *N. benthamiana* plants were grown under glasshouse condition. Plants that tested positive for GUS activity and control plants with two fully expanded leaves were rubbed with RNA transcript solution supplemented with carborundum. The treated plants were washed with tap water and maintained at 26-28^oC for up to two months.

2.8 Virus sources, propagation and inoculation

The biological, serological and genomic characteristics of CTV-VT and Morasha (Mor-T) isolates were described previously (Ben-Zeev *et al.*, 1989; Mawassi *et al.*, 1993; Yang

et al., 1999). The CTV isolates were propagated annually by re-grafting on Alemow seedlings, and inoculated plants were maintained in insect-proof screen houses.

For challenge inoculation experiments of the transgenic citrus rootstocks, we prepared at least two siblings plants of each transgenic line. Buds of each GUS-positive sample plant were grafted onto commercial nursery-grown Volkamer lemon (*C. limon*). Challenge inoculations were conducted by chip-bud grafting of the severe CTV-VT-type isolate Mor-T. To ensure the maximal exposure of the transgenic plants to the challenging virus, the chip-buds were not placed directly on the transgenic part, but on the CTV-sensitive Volkamer lemon rootstocks and the infected Alemow buds used as the inoculum tissue were continuously maintained on the grafted plants (Fig.21).

2.9 CTV genome cloning and sequencing

The dsRNA extracts from Alemow citrus inoculated with two parental strains (Mor-T= MA and MB) and the "recovered" isolate (Mor-T/R= RA and RB) were used for cDNA synthesis and cloning into the pGEM-T Easy Vector (Promega) system and for sequence analysis. To obtain a set of overlapping reverse transcription (RT)-PCR cDNA clones covering the entire genome of CTV isolates, VT-specific forward and reverse primers (Table 3) were designed based on the sequence of the closely related VT isolate (Mawassi *et al.*, 1996). The sequence and nucleotide numbering are according to the CTV-VT strain of Mawassi *et al.* (1996) (Gene Bank Accession Number U56902) and sequenced by a commercial facility (HyLabs, Rehovot, Israel).

The program DNAMAN (Lynnon Corporation, Canada) to edit sequences, BioEdit (Ibis Therapeutics, Carlsbad, CA) to compare nucleotide and amino acid sequences, and

translate to obtain amino acid sequences were used for sequence analysis. Multiple sequence alignments were performed using the ClustalW program (Thompson *et al.*, 1994), and sequences were assembled using the BioEdit program.

Table 3. List of RT-PCR primers used to generate specific CTV cDNA clones for sequencing.

Forward Primer	Location	Reverse Primer	Location
VT-1	1-18	VT-2	2982-3000
VT-3	2850-2868	VT-4	5832-5850
VT-5	5700-5718	VT-6	8682-8700
VT-7	8550-8568	VT-8	11532-11550
VT-9	11400-11418	VT-10	14382-14400
VT-11	14250-14268	VT-12	17232-17250
VT-13	17100-17118	VT-14	19208-19226

3. RESULTS

3.1 Section 1: *Agrobacterium tumefaciens* mediated gene transformation of citrus rootstocks and *Nicotiana benthamiana* plants

3.1.1 Plasmid constructs

The cDNA sequences from CTV-p23 plus its downstream 3' UTR (p23U) and of p61 (ORF 5) were cloned. The p23U sequence was modified to yield an intron-spliced RNA with a hairpin structure (p23UI). The constructs were inserted into pCambia 2301 binary vectors to yield three plasmids designated as pCamEN35Sp61T, pCamEN35S23UT, and pCamEN35S23UI23T, respectively (Fig 4). These plasmids were used for *Agro*-transformation of a selection of citrus rootstocks including Sour Orange, Alemow, Volkamer lemon, Gou Tou and Troyer. The viability of plasmids was first tested by transformation of *N. benthamiana* plants.

3.1.2 Development of transgenic *N. benthamiana* plants

Axenically cut *N. benthamiana* leaf explants were *Agro*-infected with *A. tumefaciens*, EHA 105 inocula containing the three cloned binary constructs. Transformed tissue was selected for kanamycin-resistance and regenerated into plants. To reduce the possibility of selecting chimeric plants, shoots were taken from kanamycin-resistant calluses and rooted in the presence of kanamycin, and whole plants were regenerated under continuous selection pressure *in vitro* (Fig 5).

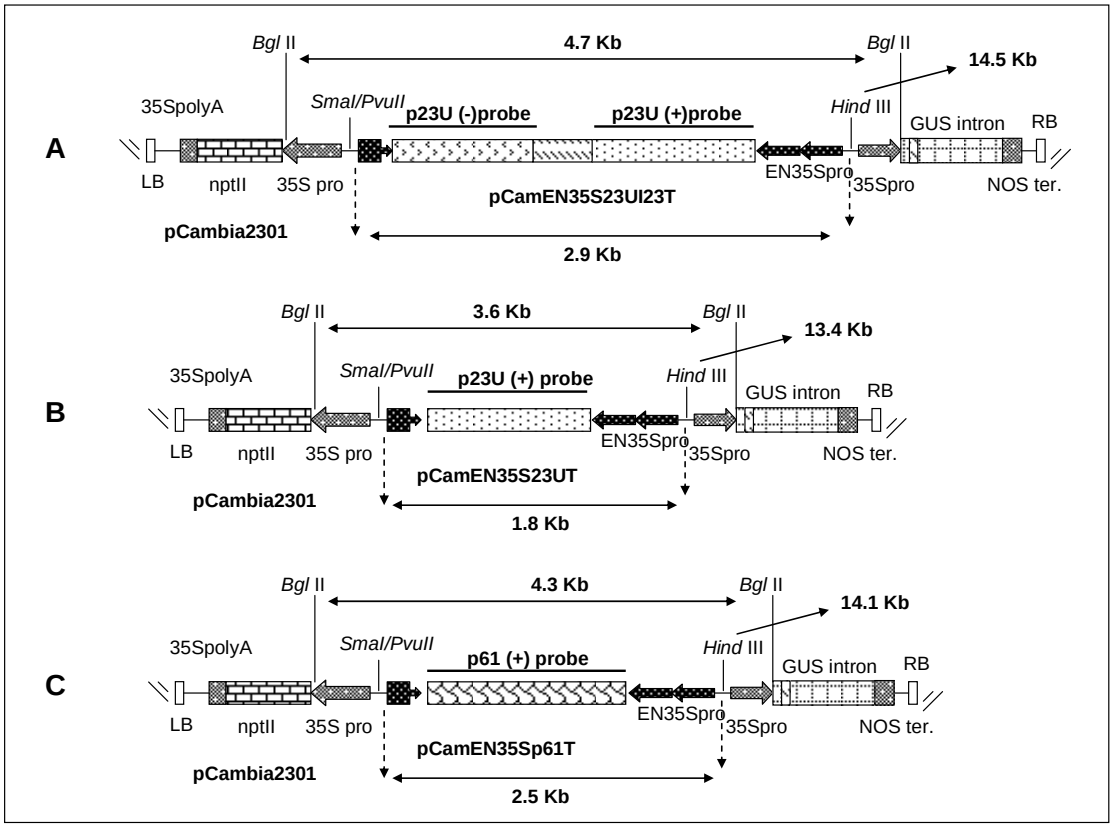

Figure 4. A schematic presentation of plasmid constructs used for *Agrobacterium*-mediated transformation of citrus rootstocks and *N. benthamiana* plants. **A, B and C,** the construction of CTV-p23UI, CTV-p23U and CTV-p61 genes and their insertions into the final pCambia 2301 binary vector, respectively. Arrow headed bars indicate fragment size of inserts and their corresponding plasmid fragment sizes when digested with B*gl*II and H*ind*III restriction enzymes. Bars on inserts show position of probes used for hybridizations. 35S pro and EN35S= the *Cauliflower mosaic virus* 35S promoter and enhanced 35S promoter, respectively; NOS ter = nopaline synthase terminator; *nptII* = neomycin phosphotransferase II gene; LB and RB = Left and right T-DNA border, respectively.

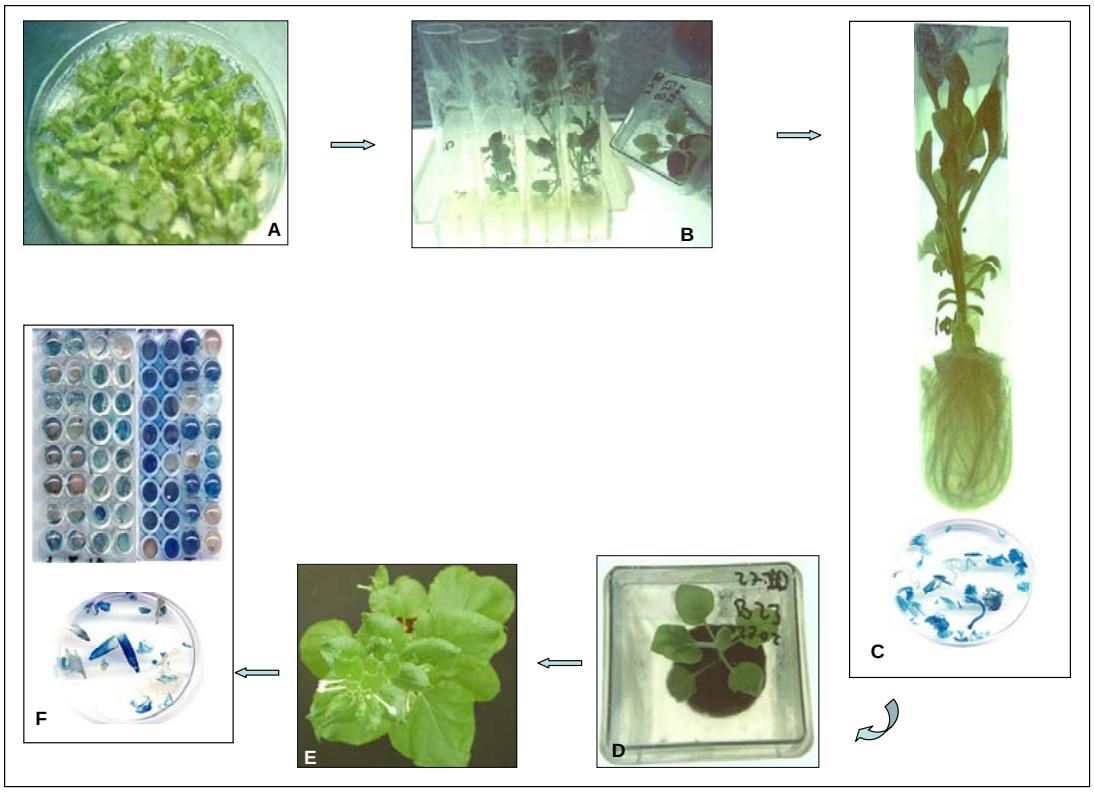

Figure 5. Flow-chart presentation of the transgenic *N. benthamiana* selection process. The transgenic shoots were separated from kanamycin-resistant calluses (**A**) and rooted in the presence of kanamycin (**B** and **C**). GUS-positive rooted plantlets were acclimated (**D**) and transferred to greenhouse conditions (**E**). Greenhouse-grown transgenic plants and their offsprings were routinely checked for GUS expression (**F**) and their seeds were collected.

All the transgenic *N. benthamiana* plants (n=435) obtained from these transformation experiments morphologically resembled the wild type and displayed normal growth. Plants were first confirmed to be putatively transgenic by PCR analysis using primers specific for corresponding genes (Table 2). Later Southern hybridization with gene-

specific riboprobes of genomic DNAs from transgenic *N. benthamiana* plants confirmed that in total we obtained 435 lines, of which 135 were p61-bearing plants, 142 p23U-bearing plants and 176 p23UI-bearing plants, respectively (Figs. 6 and 7). Most of the transgenic plants (96%) expressed the GUS gene (Fig. 5).

A group of the transgenic plant lines, positive for PCR, Southern, and GUS assays were analyzed by northern hybridization to examine the accumulation of RNA transcripts in the transgenes. Hybridization of total RNA and of dsRNA extracts with cDNA specific riboprobes for p61, and for positive- or negative-strand-specific p23U sequences showed the accumulation of significant amounts of RNA molecules in 40 out of 50 (ca. 80%) of the tested transgenic plant lines (Fig 8A and B). Figures 8C and D show the accumulation of significant amounts of dsRNA molecules, as indicated by their reactions with both strand-specific probes in all tested transgenic plants. The molecular mass of the hybridization signals were approximately the same as the denaturated dsRNA molecules of the corresponding sgRNAs of CTV –p61 and of -p23. None of the non-transformed wild type plants showed similar RNA molecules (Fig 8).

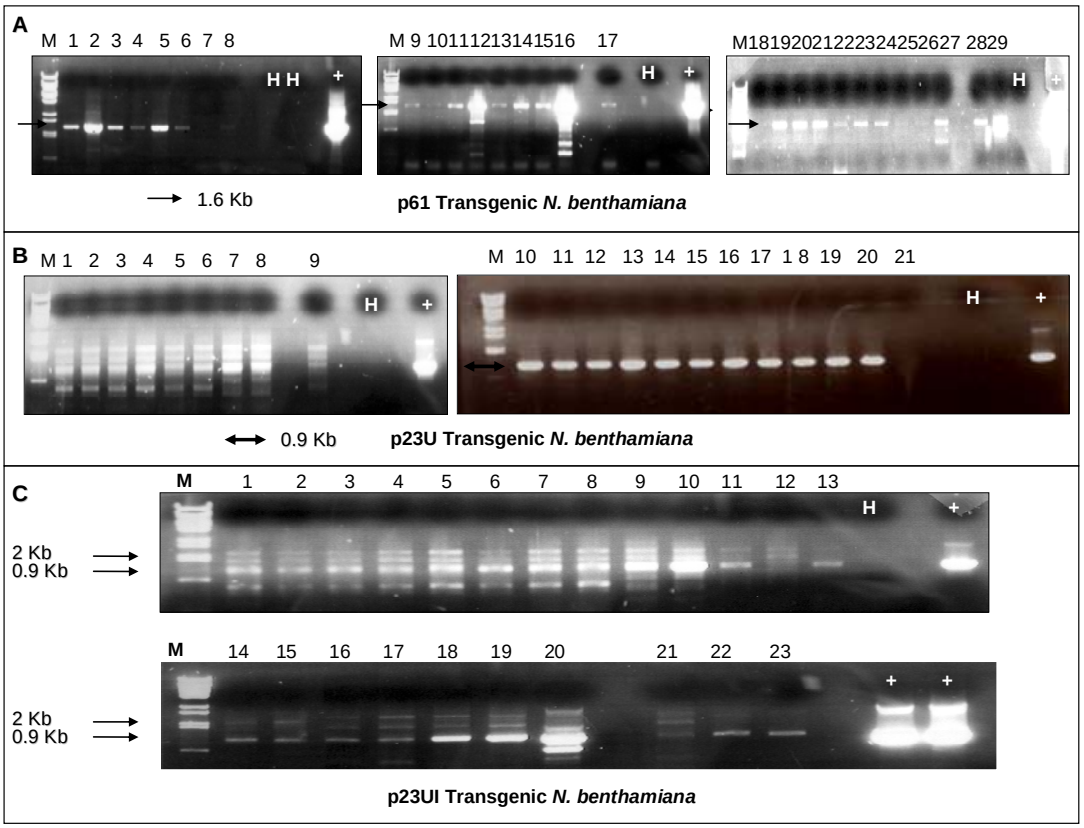

Figure 6. PCR analysis of F1 transgenic *N. benthamiana* plants using primers specific for CTV -p23U and -p61 genes. **A** Lanes 1 - 29: p61 transgenic lines p61nb 1 to 29, respectively. **B** Lanes 1 - 21: p23U transgenic lines p23Unb 1 to 21, respectively. **C** Lanes 1 - 23: p23UI transgenic lines p23UInb 1 to 23, respectively. Lanes M: lambda DNA marker (Fermentas, MBI). Lanes H and +: non transgenic plants and a sample of positive control from plasmids harboring the corresponding CTV genes.

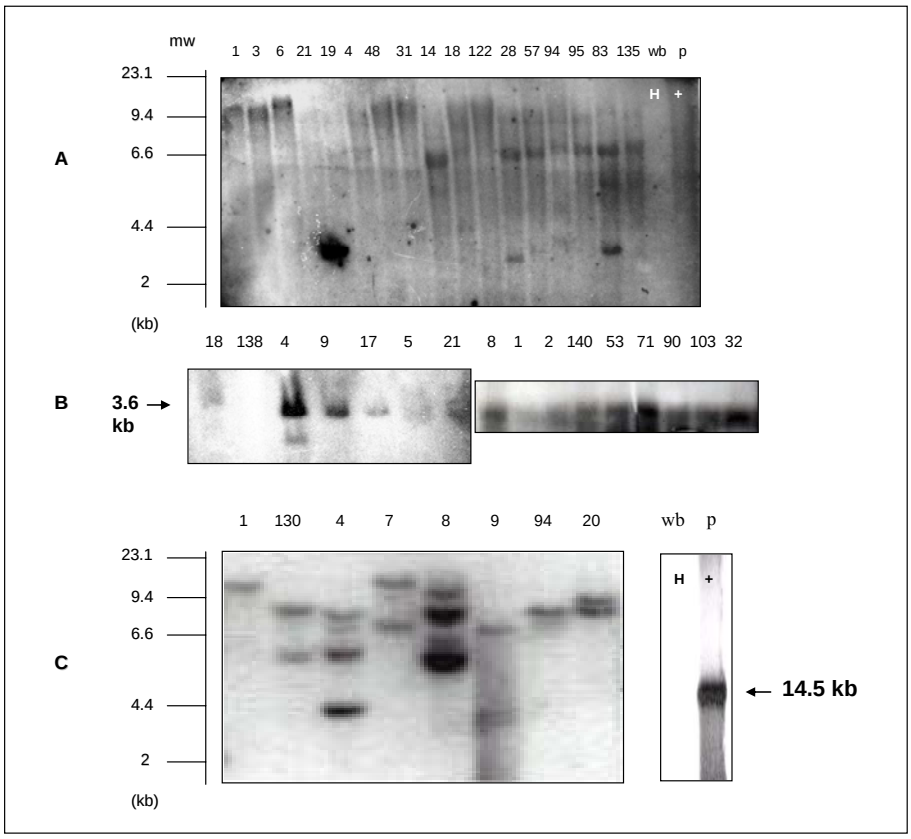

Figure 7. Southern hybridization of transgenic *N. benthamiana* plants using CTV -p61 and -p23U gene specific DIG-labeled RNA probes. **A** Genomic DNAs restricted with H*ind*III from p61 transgenic lines; p61nb 1, 3, 6, 21, 19, 4, 48, 31, 14, 18, 122, 28, 57, 94, 95, 83 and 135 respectively. Lanes wb and p: wild type *N. benthamiana* used as a negative (H) control and pCamEN35Sp61T plasmid restricted with H*ind*III as a positive (+) control, respectively. **B** Genomic DNAs restricted with B*gl*II from p23U transgenic lines; p23Unb 18, 138, 4, 9, 17, 5, 21, 8, 1, 2, 140, 53, 71, 90, 103 and 32, respectively. **C** Genomic DNAs restricted with H*ind*III from p23UI transgenic lines; p23UInb 1, 130, 4, 7, 8, 9, 94 and 20, respectively. Lanes wb and p: wild type *N. benthamiana* as a negative (H) control and pCamEN35S23UI23T plasmid restricted with H*ind*III as a positive (+) control, respectively.

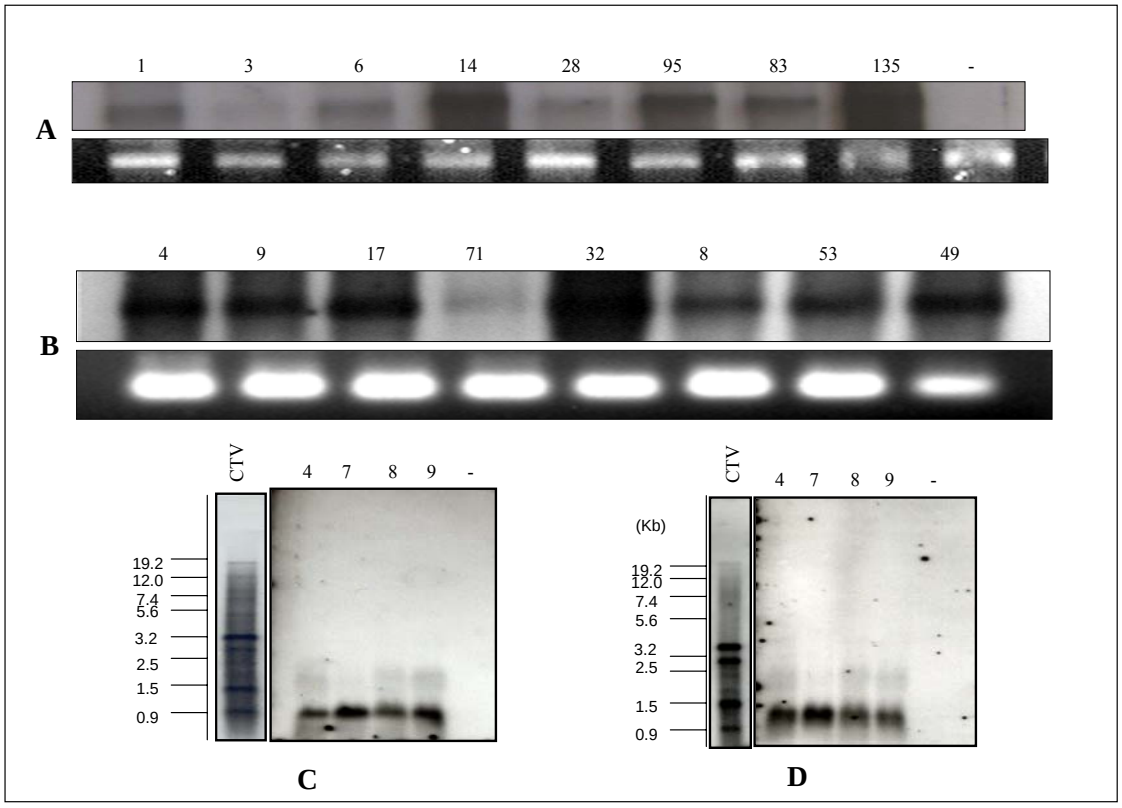

Figure 8. Northern blot hybridizations with DIG-labeled riboprobes of CTV p61 and p23U with fractionated RNAs from non-transgenic and from transgenic *N. benthamiana* plants. **A** Hybridization with p61 specific riboprobe of total RNA extracts from p61 transgenic *N. benthamiana* lines. Ethidium bromide stained bands of tRNA from these samples are shown below. **B** Hybridization with p23U plus (+) specific riboprobe of total RNA extracts from p23U transgenic *N. benthamiana* lines. Ethidium bromide stained bands of tRNA from these samples shown below. **C** Hybridization with p23U plus (+) strand specific riboprobe of dsRNAs from p23UI transgenic *N. benthamiana* lines and wild type (-) *N. benthamiana*, respectively. **D** Shows duplicate membrane of **C** hybridized with p23U minus (-) strand specific riboprobe. CTV= Sample of dsRNA from CTV-infected non transgenic Alemow plant as a marker.

3.1.3 Attempts to optimize conditions for *A. tumefaciens* transformation of citrus rootstocks

3.1.3.1 Selecting conditions for optimal plantlet preparation prior to transformation

One of the main problems of citrus transformation is the low rate of regeneration of transgenic plants. Despite numerous attempts and literature reports, citrus transformation remains a difficult and erratic technology. This thesis includes amendments to the numerous variations of citrus transformation procedures that were previously described from our laboratory (Piestun *et al.* 1998; Piestun, 2003).

The optimal "plant growth regulation" (PGR) requirements for regenerating adventitious shoot from epicotyl cuttings (explants) of different citrus rootstocks was found to be dependent on: a) source of plant material b) age and position of explant, c) the pre-culture schedule, d) co-cultivation method and period, e) selection pressure, and f) environmental conditions.

Dark-grown, one to five week etiolated old seedlings of Troyer citrange (*C. sinensis x P. trifoliata*), Sour orange (*C. aurantium*), Gou Thou (Chinese sour orange), Alemow (*C. macrophylla*), and Volkamer lemon (*C. limon*), were used to test the effects of different regeneration conditions. Following previous experiments (Piestun, 2003), different methods of (1) etiolated seedling preparation, (2) placement on tissue culture media (3) composition of culture media and (4) inoculation, incubation and environmental conditions were tested and adjusted for each citrus rootstock species. The regeneration response differed mainly between two groups, those of Troyer citrange and Gou Tou (grouped as TG rootstocks) and those of Sour orange, Alemow and Volkamer lemon

(grouped as SAV rootstocks). The transformation and regeneration of the TG group was found to be more convenient and faster than for the SAV group which remained relatively recalcitrant.

3.1.3.2 Source, age and cutting methods of plant material for optimal regeneration

The age of etiolated seedlings used for explant preparation, explant size and the cutting position were found to affect regeneration frequency (Tables 4, 5 and 6). The competence for regeneration and transformation were found to differ for each citrus rootstock species. Regeneration was high when younger seedlings of Troyer (Try) and Gou Tou (Gto) were used. The highest regeneration capacities (4-6 shoots per explant) were obtained with explants from two-week-old Try and Gto. For Sour Orange (So), Volkamer Lemon (Vol) and Alemow (Alm) three to four-week-old seedlings gave the best results, however, only 2-4 shoots per explant were produced. Irrespective of the age of seedlings, the number of shoots formed decreased markedly with the distance of the explant from the cotyledonary node (data not shown). Thus, all following transformation experiments were conducted with explant tissues that originated from cotyledonary nodes (Fig. 9 and Table 4).

Explants size was found to be critical for regeneration ability of So, Gto and Vol (minimum 1 cm) but not for Try and Alm (minimum 0.5 cm) rootstocks. Based on these results 1 cm long explants were used for all the rootstocks.

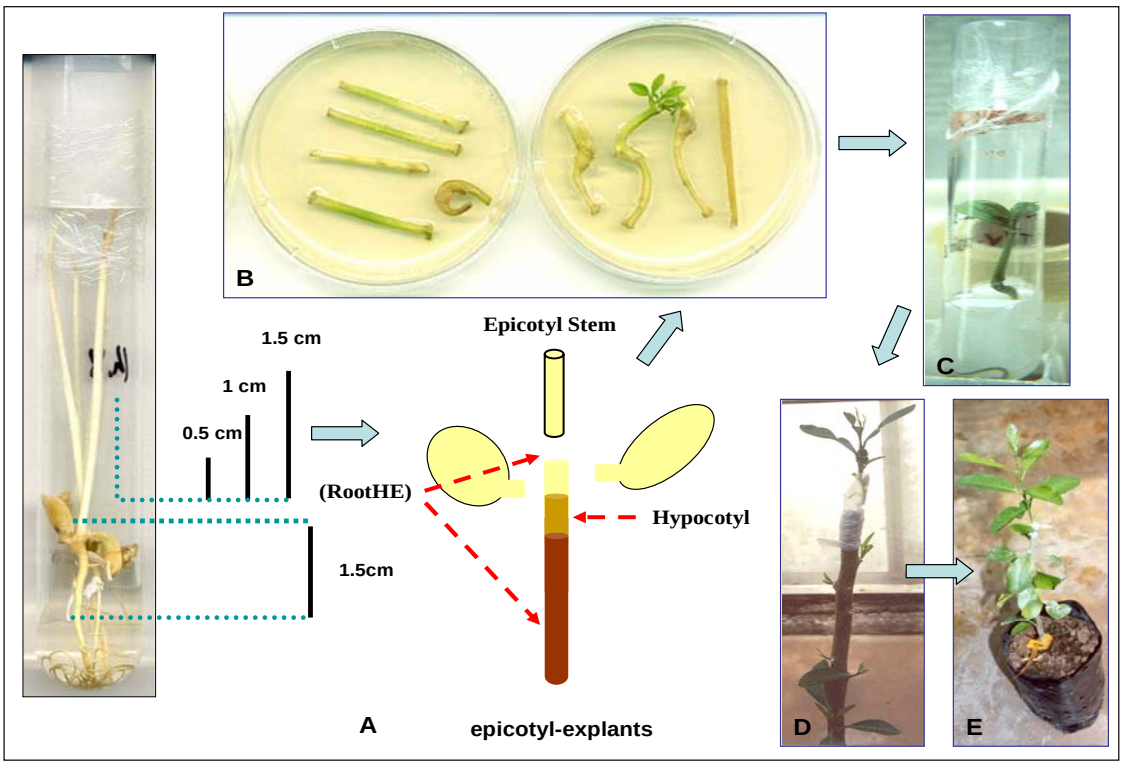

Figure 9. A schematic presentation of explant preparation and obtaining transgenic whole plant. **A** One to five week old etiolated seedlings were used to prepare explants. The cotyledons were removed, and two different explants were prepared from each seedling. An epicotyl segment was excised proximal to the cotyledonary node (0.5-1.5 cm long), cut 0.2-0.3 cm above the cotyledonary node. The second explant (RootHE) consisted of the top 1cm of the root, the hypocotyl, the cotyledonary node and the lowest 0.2-0.3 cm of the epicotyl. **B** Alemow explants on selection medium after about three weeks (left) and six weeks (right) after transformation. **C** Transgenic shoots *in vitro* STG grafted onto etiolated seedlings of Troyer citrange. **D** STG plantlets re-grafted onto Volkamer lemon in greenhouse. **E** Transgenic whole plants obtained at about four months after transformation.

Table 4. Regeneration efficiency of explants from etiolated citrus rootstock seedlings of different ages.

Rootstock sp. and age	No. of explants	Regenerated explants (%)	No. of Shoots produced	Shoots per regenerating explant
Troyer citrange				
1week	10	7 (70)	12	1.7
2 week	50	48 (96)	298	6.2
3 week	50	46 (92)	240	5.2
4 week	50	33 (66)	109	3.3
Gou Tou				
1week	10	3 (30)	4	1.3
2 week	50	43 (86)	182	4.2
3 week	50	38 (76)	162	4.2
4 week	50	23 (46)	56	2.4
Sour orange				
1week	NA	NA	NA	NA
2 week	50	23 (46)	33	1.4
3 week	50	32 (64)	106	3.3
4 week	50	29 (58)	90	3.1
Alemow				
1week	10	2 (20)	2	1
2 week	50	39 (78)	92	2.3
3 week	50	42 (84)	173	4.1
4 week	50	44 (88)	142	3.2
Volkamer lemon				
1week	NA	NA	NA	NA
2 week	50	21 (42)	29	1.3
3 week	50	32 (64)	107	3.3
4 week	50	30 (60)	96	3.2

NA= Not Applicable

The frequency of regeneration increased when the apical part of explants were sliced at an angle to increase wound surface. This practice allowed the convenient location whether shoots developed from basal or apical explant ends. When all explants were placed onto medium horizontally with the basal end dipped into medium, explant regeneration frequency increased two fold. Most of the regenerated shoots were of basal origin (data not shown). Based on these results all further experiments were conducted in this way for all the citrus rootstock species.

3.1.3.3 Optimization of added plant growth regulators and preculture medium

Shoot regeneration frequencies of explants of Try, Gto, So, Alm and Vol rootstocks as affected by different concentrations of Kinetin (Kin) and 6-benzylaminopurine (BA) (cytokinins) 2,4-dichlorophenoxyacetic acid (2,4 D) and naphtaleneacitic acid (auxin) were examined. The effect of plant growth regulator (PGR) on regeneration frequency was observed two weeks after starting the treatments (Fig. 10).

The frequencies of shoot regeneration were similar with use of Kin and BA for all tested rootstocks. Both cytokinins were effective for regeneration and shoot formation but Kin was slightly better for shoot elongation. However, due to solid callus production on BA treatment the use of BA was preferred in further experiments. The addition of 0.54 μM NAA into media significantly improved organogenesis via callus formation and increased the number of transformed shoots regenerated. The optimal cytokinin-auxin concentration was determined for each rootstock genotype (Tables 5 and 6).

In experiments to determine the optimal concentration of BA for shoot regeneration from citrus epicotyl segments we found that regeneration frequency and number of shoots per

explant increased by using 0.54 µM NAA with BA at 4.4 µM for TG group and 22.2 µM for the SAV group, and decreased in both groups with BA concentrations higher than 44 µM (Tables 5 and 6).

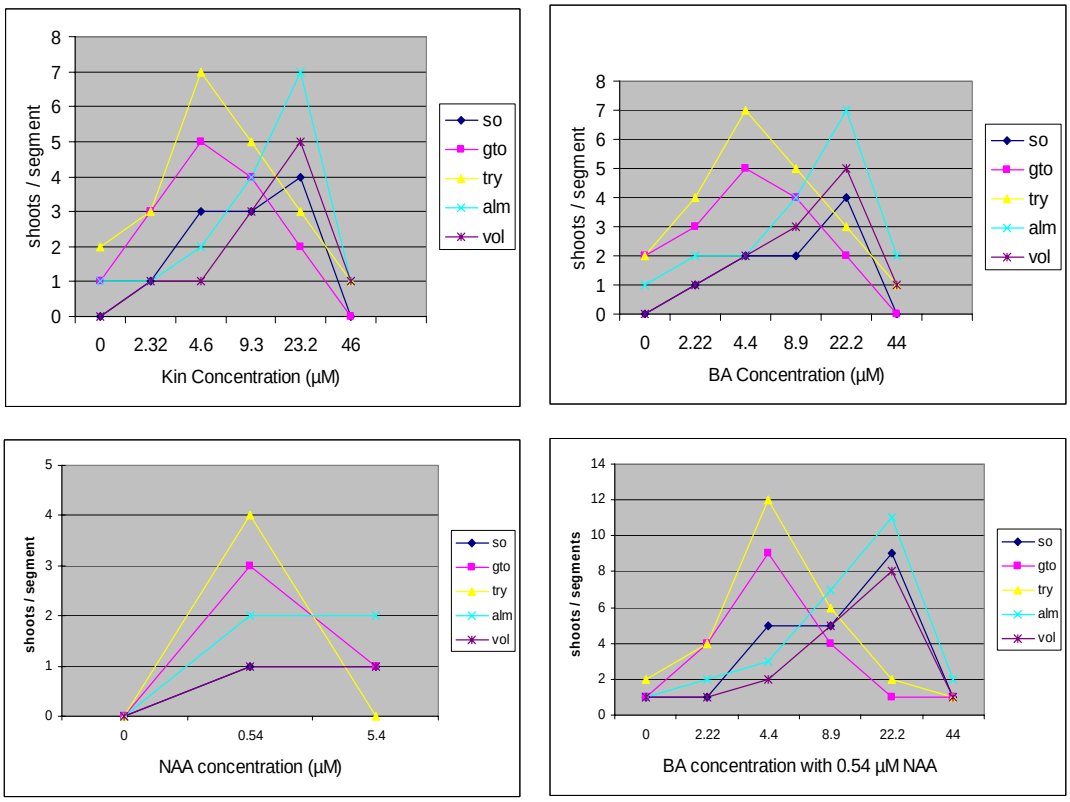

Figure 10. The effect of BA, Kin and NAA on shoot organogenesis from citrus epicotyl explants.

Pre-culturing explants in cytokinin-auxin rich media prior to inoculation and cocultivation with *Agrobacterium* significantly increased the frequency of regeneration, shoot formation and genetic transformation especially for the SAV group plantlets, and also for the TG group (Tables 5 and 6). The SAV group was consequently pre-cultured at least for four days prior to *Agrobacterium* inoculation step, while two days were sufficient for TG group.

Table 5. Effects of different factors on transformation efficiency of TG group plantlets (*SD* standard deviation).

Factor evaluated	Treatment[a]	Number of explants[b]	Explants (%) producing shoots	Shoots produced	GUS^+ shoots (%)	Explants (%) with GUS^+ shoots
Preculture period	0	58	4 (6.9)	4	2 (50)	1 (1.7)
	2 day	55	9 (16.3)	13	8 (61.5)	4 (7.3)
	3 day	59	12 (20.3)	26	21(80.8)	11 (18.6)
Incubation period with *Agrobacterium*	5 min	49	16 (32.7)	19	2 (10.5)	1 (2.0)
	15 min	47	13(27.7)	18	2 (11.1)	1 (2.1)
	30 min	48	9 (18.8)	11	9 (81.8)	8 (16.7)
	60 min	48	6 (12.5)	7	6 (85.7)	6 (12.5)
Coculture medium with 2,4-D	0 µM	68	6 (8.8)	7	2 (28.6)	2 (2.9)
	0.44 µM	67	24 (35.8)	29	12 (41.4)	12 (17.9)
	2.26 µM	67	17 (25.4)	19	4 (21.1)	4 (6.0)
	4.52 µM	69	10 (14.5)	11	2 (18.2)	2 (2.9)
	18.10 µM	67	2 (3.0)	2	1 (50.0)	1 (1.5)
Coculture period	1 day	47	3 (6.3)	3	0	0
	2 day	48	13 (27.1)	16	9 (56.3)	9 (18.8)
	3 day	46	18 (39.1)	18	11 (61.1)	9 (19.6)
	5 day	46	9 (19.5)	9	8 (88.9)	8 (17.4)
BA concentration in selective medium (with 0.54 µM NAA)	0 µM	68	6 (8.8)	6	0	0
	2.22 µM	67	12 (17.9)	21	10 (47.6)	6 (9.0)
	4.40 µM	69	25 (36.2)	43	22 (51.2)	13 (18.8)
	8.90 µM	69	23 (33.3)	39	18 (46.2)	9 (13.0)
	13.3 µM	68	6 (8.8)	8	2 (25.0)	2 (2.9)
	22.2 µM	68	4 (5.9)	4	1 (25.0)	1 (1.5)

[a] Explants inoculated with pCamEN35S23UT

[b] Data are the summary of two independent experiments

Table 6. Effects of different factors on transformation efficiency of SAV group plantlets (*SD* standard deviation).

Factor evaluated	Treatment[a]	Number of explants[b]	Explants (%) producing shoots	Shoots produced	GUS^+ shoots (%)	Explants (%) with GUS^+ shoots
Preculture period	0	58	0	0	0	0
	2 day	58	3 (5.1)	3	1 (16.7)	1 (0.9)
	4 day	59	7 (11.8)	7	2 (28.6)	2 (3.4)
Incubation period with *Agrobacterium*	5 min	47	7 (14.9)	8	0	0
	15 min	47	6 (12.7)	7	2 (28.6)	1 (2.1)
	30 min	48	6 (12.3)	7	4 (57.1)	3 (6.3)
	60 min	46	5 (10.8)	6	4 (66.7)	4 (8.7)
Coculture medium with 2,4-D	0 µM	68	4 (5.9)	4	1 (25.0)	1 (1.5)
	0.44 µM	67	7 (10.4)	9	5 (55.6)	3 (4.5)
	2.26 µM	67	7 (10.4)	10	6 (60.0)	3 (4.5)
	4.52 µM	66	18 (27.3)	23	11 (47.8)	6 (9.1)
	18.10 µM	67	1 (1.5)	1	0	0
Coculture period	1 day	45	2 (4.4)	2	0	0
	2 day	48	3 (6.2)	4	1 (25.0)	1 (2.1)
	3 day	46	3 (6.5)	4	2 (50.0)	2 (4.3)
	5 day	47	4 (8.5)	4	3 (75.0)	3 (6.4)
BA concentration in selective medium (with 0.54 µM NAA)	0 µM	66	3 (4.5)	3	0	0
	2.22 µM	67	3 (4.5)	3	1 (33.3)	1 (1.5)
	4.40 µM	69	5 (7.2)	6	2 (33.3)	2 (2.9)
	8.90 µM	69	7 (10.1)	8	4 (50.0)	3 (4.3)
	22.2 µM	68	10 (14.7)	15	9 (60.0)	7 (10.3)
	44.0 µM	68	1 (1.5)	1	0	0

[a] Explants inoculated with pCamEN35S23UT

[b] Data are the summary of two independent experiments

The effect of different dark/light regimes were tested by placing explants in different conditions. The dark pre-cultured explants showed improved callus formation and subsequently the number of elongated shoots increased (data not shown). These results suggested that rapid cell division and callus formation improved *Agrobacterium* penetration and T-DNA delivery in dark cultured explants. In the following experiments explants were incubated in darkness during the preculture period.

3.1.3.4 Induction of *A. tumefaciens* cells for efficient transformation of citrus

A. tumefaciens induction was achieved by culturing the explants in recalcitrant plant induction medium (IM) at 26 ^{o}C. Acetosyringone addition (200 μM) to IM increased transformation frequency. Induction of *Agrobacterium* for 24 hours was significantly more effective than shorter periods of 1-4 hours (data not shown). However, extending the induction period of *Agrobacterium* cells in IM resulted in the accumulation of large masses (2-3 mm) of bacterial aggregates and altered the pH of the medium. Inoculation of explants with IM solution had toxic effects on the regeneration of explants. The induced *Agrobacterium* cells were collected by centrifugation, resuspended in MS solution pH5.5, supplemented with 200 μM acetosyringone, prior to explant inoculation.

To increase *Agrobacterium* penetration into explants and transformation frequency of citrus, vacuum infiltration and the inoculation period were examined. A 10 min vacuum infiltration and 30 - 60 min inoculation period resulted in an increased number of GUS spots compared with explants inoculated without vacuum and/or shorter inoculation periods. Explants inoculated with *Agrobacterium*, by vacuum infiltration and/or longer inoculation periods suffered of bacterial over-growth due to excessive penetration of bacteria. In many instances the bacterial over-growth resulted in a necrotic response at

the cut ends of the epicotyl explants and considerably reduced shoot regeneration. Therefore although maximum regeneration of shoots expressing the GUS gene was obtained with 60 min treatment with *Agrobacterium*, the inoculation period was reduced to 30 min followed with 10 min vacuum infiltration (Tables 5 and 6).

3.1.3.5 Optimization of coculture medium and environmental conditions

Etiolated epicotyl explants were incubated for 30 - 60 min in bacterial suspension, blotted dry on sterile filter paper, and placed horizontally on preculture plates specific for each rootstock genotype. The explants were co-cultivated for different periods (1 - 5 days), dark /light, and temperatures (22 - 26 ^{o}C) (Tables 5 and 6).

The presence of 2,4-D in pre- or co-culture media improved shoot regeneration and transformation frequency in citrus (Pena *et al.,* 2004). Likewise, we found that pre-treating explants in media containing 2,4-D for 3 h before inoculation increased the frequency of regenerated shoots expressing the GUS marker gene, compared with those pretreated with water, medium without 2,4-D, or without pretreatment. However, overnight pretreatment with 2,4-D decreased the transformation frequency (data no shown). 2,4-D treatments increased the transformation frequency of all tested rootstocks explants (Tables 5 and 6). Positive effects of 2,4-D on transformation were dependent on concentration and differed for SAV and TG genotypes. The optimum concentration of 2,4-D was 4.52 µM for the SAV group, resulting in a transformation frequency of 9% (vs. 1% for control). For TG rootstocks 0.44 µM 2,4-D resulted in a transformation frequency of 18% (vs. 3% for control) (Tables 5 and 6). The 2,4-D treatments had positive effects on the frequency of regenerated shoots expressing the GUS gene, but not on the

regeneration frequency (Tables 5 and 6). Concentrations higher than optimum and/or a longer period of 2,4-D treatment promoted the production of unorganized callus, and reduced shoot regeneration significantly. Optimal cocultivation periods were adopted for each rootstock genotype to prevent bacterial over-growth (Tables 5 and 6).

We compared transformation frequencies following cocultivation and selection at 26 ºC with cocultivation and first month of selection at 22 ºC. Cocultivation or selection at 22 ºC did not significantly alter transformation frequency compared with cocultivation or selection at 26 ºC (data not shown). A temperature of 26 ºC was used for cocultivation and selection in further experiments.

The optimal PGR addenda for adventitious shoot regeneration in epicotyl explants of rootstocks varied with the illumination condition. The response to illumination and to PGR differed for direct and indirect (through callus) regeneration pathways. Shoot formation through the direct organogenic pathway decreased as concentrations of BA in the medium increased, when explants were incubated in darkness or in 16 h photoperiod.

Preculture in darkness visibly improved callus and shoot induction compared with in light precultured etiolated epicotyl explants. Maintaining explants during preculture and coculture with *Agrobacterium* in darkness before exposing to light for 1 - 3 weeks, improved callus formation and consequent regeneration (data not shown).

3.1.3.6 Optimization of selection media and regeneration conditions

Optimal concentrations of kanamycin, as an antibiotic for the selection of transgenic explants were evaluated. Concentrations of 200 or 300 μM kanamycin completely inhibited shoot formation in non-inoculated control explants. The strong selection

pressure with 300 μM kanamycin applied on *Agrobacterium* inoculated explants reduced the number of non-transgenic Try shoots but was deleterious on the other genotypes. Frequency of regeneration among other rootstocks increased with the use of only 200 μM kanamycin. Thus, for optimal selection antibiotic concentrations of 300 μM and 200 μM kanamycin were needed for Try and So, Alm, Vol and Gto, respectively (data not shown). When explants were inoculated with *Agrobacterium* and co-cultivated in optimized media, the regeneration process was similar to that described above but cell division and callus formation proceeded more slowly, and organogenesis observed several weeks later. This could be attributed to the negative effect of the bacteria on citrus cells and tissues. After 4 weeks incubation in selection medium, histological examination of explants revealed that a variable proportion of callus cells showed GUS activity (Fig. 12).

3.1.3.7 Whole transgenic plants obtained via shoot tip grafting.

To recover whole transgenic plants, emerging shoots were assayed for GUS activity and shoot-tip grafted (STG) *in vitro* onto etiolated Troyer citrange seedlings. Grafting the *in-vitro*-grown plants on vigorous rootstocks allowed rapid acclimatization and development of regenerated plants in greenhouse conditions.

The size of shoot for grafting and the sucrose concentration of seed germination medium for Try seedlings used as rootstocks for STG, were critical for success of *in vitro* grafting. Transgenic shoots were prepared as large as possible for grafting onto Try seedlings. Increasing sucrose concentration of the seed germination medium from 2.5 % to 5 % improved grafting success. Furthermore, we found that placing excised transgenic shoots on hormone-free culture medium for 2-3 days prior to STG increased the success rate of

grafting to 94 % compared with 72 % when the medium was left unchanged (data not shown).

3.1.3.8 Improved protocol for citrus rootstocks transformation

All results from these studies were combined to produce an optimized protocol for transformation of citrus rootstocks. The protocol includes (I) preculture for 2-5 days, (II) incubation of explants for 30 min in *Agrobacterium* solution, (III) 3 days cocultivation on medium supplemented with BA and NAA, (IV) transfer of the explants to selective regeneration medium, (V) *in vitro* STG of transgenic shoots, and (VI) greenhouse grafting of transgenic shoots (Fig. 11 and Table 7). Using this protocol in later experiments increased transformation efficiency of citrus rootstocks compared with the previous method (Piestun, 2003). For fast recovery of transgenic plants, a slightly modified STG method (Pena *et al.* 1995b) for the GUS positive shoots was used.

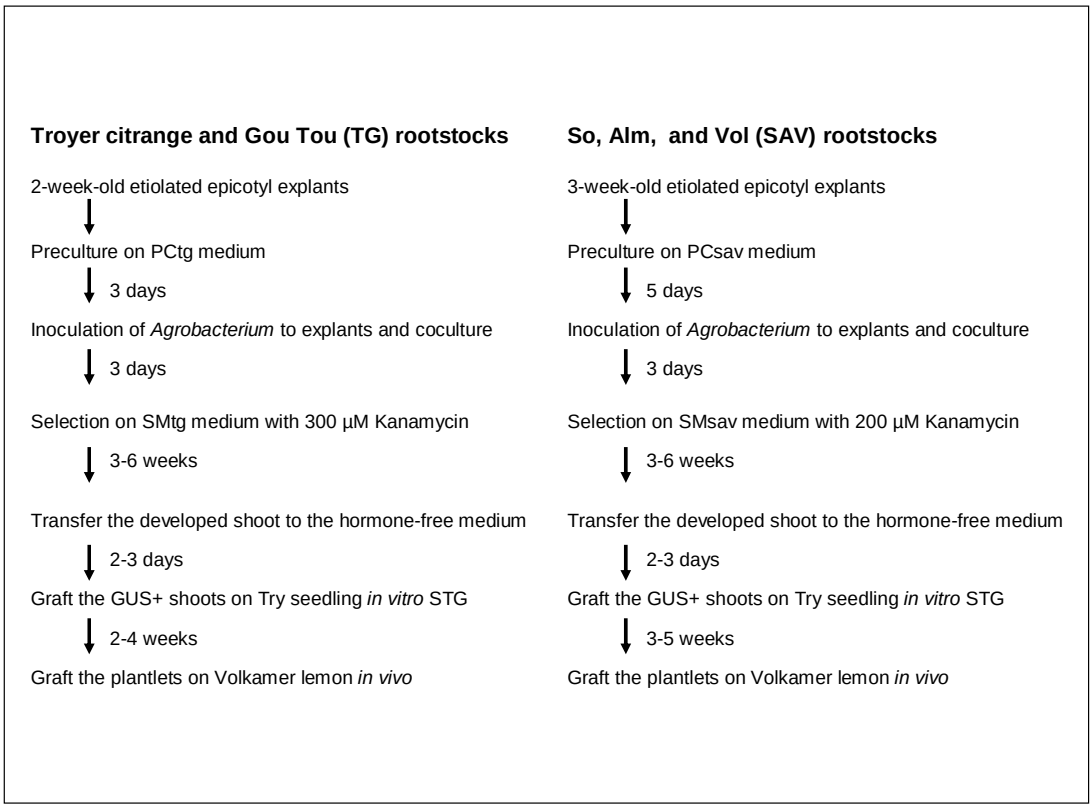

Figure 11. Schematic presentation of our improved protocol to obtain transgenic citrus plants. The period for each step is indicated.

Table 7. Summary of the improved protocol for citrus rootstocks transformation (next two pages).

Targeted Plant			**PGR and media**				**Period**			
citrus species	donor plant	explant	preculture	*Agro*-inoculation	coculture	selection	pre-culture	*Agro*-inoculation	*Agro*-plant coculture	selection
Sour orange (SO)	3 week old etiolated seedling	1 cm epicotyl and 1.5 cm RootHE	22.2 µM BA 0.54 µM NAA PCsav + *2 µM 2,4-D	Induced in IM inoculated with MS pH5.5	22.2 µM BA 0.54 µM NAA PCsav	22.2 µM BA 0.54 µM NAA SMsav + 200 µM Kanamycin	3-5d	1h	3d	60 d
Alemow (Alm)	2-3 week old etiolated seedling	1 cm epicotyl and 1.5 cm RootHE	22.2 µM BA 0.54 µM NAA PCsav + *2 µM 2,4-D	Induced in IM inoculated with MS pH5.5	22.2 µM BA 0.54 µM NAA PCsav	22.2 µM BA 0.54 µM NAA SMsav + 200 µM Kanamycin	3-5 d	1h	3d	60 d
Volkamer Lemon (Vol)	3 week old etiolated seedling	1 cm epicotyl and 1.5 cm RootHE	22.2 µM BA 0.54 µM NAA PCsav + *2 µM 2,4-D	Induced in IM inoculated with MS pH5.5	22.2 µM BA 0.54 µM NAA PCsav	22.2 µM BA 0.54 µM NAA SMsav + 200 µM Kanamycin	3-5 d	1h	3d	60 d

Targeted Plant			**PGR and media**				**Period**			
citrus species	donor plant	explant	preculture	*Agro*-inoculation	coculture	selection	pre-culture	*Agro*-inoculation	*Agro*-plant coculture	selection
Gou Tou (Gto)	2 week old etiolated seedling	1 cm epicotyl and 1.5 cm RootHE	4.4 µM BA 0.54 µM NAA PCtg + *0.4 µM 2,4-D	Induced in IM inoculated with MS pH5.5	4.4 µM BA 0.54 µM NAA PCtg	4.4 µM BA 0.54 µM NAA SMtg + 200 µM Kanamycin	2d	1h	3d	45 d
Troyer citrange (Try)	2 week old etiolated seedling	1 cm epicotyl and 1.5 cm RootHE	4.4 µM BA 0.54 µM NAA PCtg + *0.4 µM 2,4-D	Induced in IM inoculated with MS pH5.5	4.4 µM BA 0.54 µM NAA PCtg	4.4 µM BA 0.54 µM NAA SMtg + 300 µM Kanamycin	2d	1h	3d	45 d

Abbreviations: PGR - plant growth regulator; *Agro* - *Agrobacterium tumefaciens*; RootHE - explant consist of root, hypocotyl and epicotyl; BA - 6-benzylaminopurine; NAA - naphthalene acetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; IM - induction medium; PCsav and PCtg - preculture medium for the SAV and TG groups, respectively; SMsav and SMtg - selection medium for the SAV and TG groups, respectively; d - days; h - hours.

*pre-cultured on medium supplemented with 2,4-D for 3h prior to *Agrobacterium*-inoculation

3.1.4 Analysis of transgenic citrus rootstocks

3.1.4.1 GUS staining

The histochemical GUS assay was used for locating transgenic shoots. The assay was conducted by cutting thin sections from the bases and leaves of regenerated shoots and staining the sections according to Jefferson (1987). Many of the sections that stained positively for GUS was sectored (~80%), implying that an origin from sectorial transgenic chimeras (Fig. 12). The number of shoots expressing the GUS gene varied between 16% and 75% for SAV rootstocks (average 35%), and for the TG rootstocks between 10% and 89% (average 45%) (Tables 5 and 6). The GUS positive shoots were *in vitro* grafted, and later the epicotyl part of these STG plants was further grafted on large container-grown seedlings of Volkamer lemon rootstocks (*C. limon*) in the greenhouse.

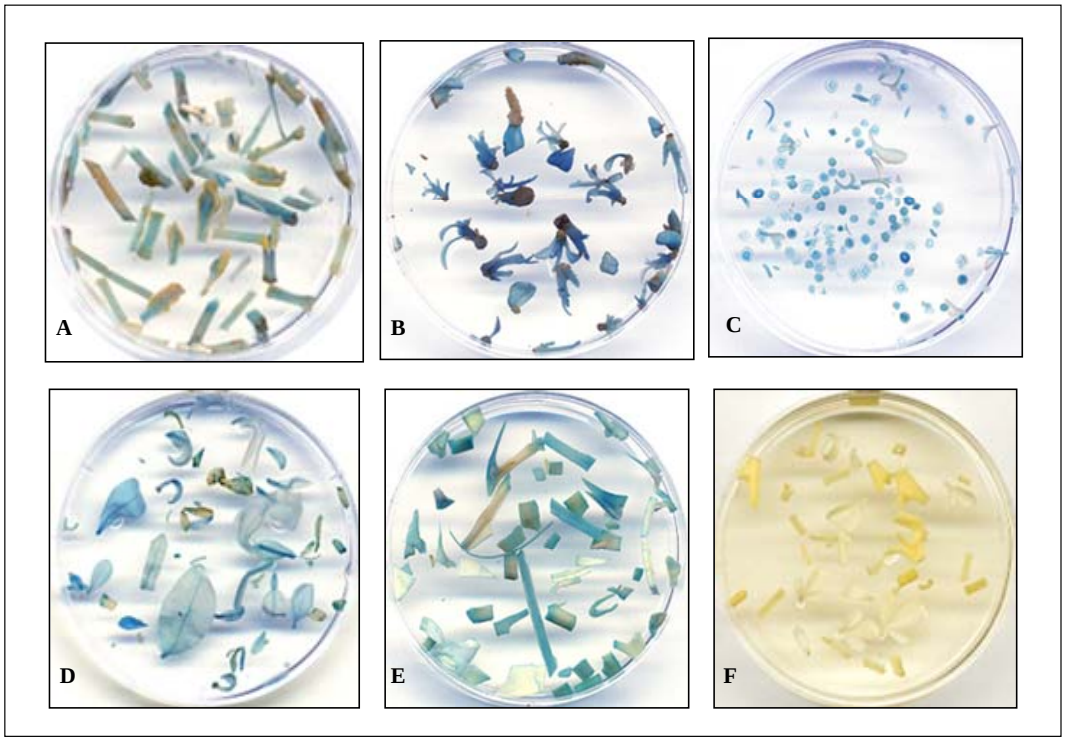

Figure 12. The histochemical assay used for GUS expression in transgenic citrus rootstocks. **A** Citrus epicotyl stem segments were assayed a week after inoculation with *Agrobacterium.* GUS expression was detected mainly in the callus, but frequently also in the whole explant. **B** Some transgenic citrus shoots were stained to explore the extent of sectorial expression of GUS activity. **C** GUS staining was conducted mainly on thin sections from the bases and leaves of regenerated citrus rootstocks shoots used for *in vitro* STG grafting. **D** Tissue pieces and leaf sections from *in vitro* grafted shoots showing GUS expression. **E** Staining of tissues from citrus plants that were grown for >1year in a glasshouse. **F** The absence of GUS staining from non-transgenic Alemow and Troyer seedlings grown in similar conditions.

3.1.4.2 Analysis of T-DNA integration and transcription by Southern and northern blot hybridization

After selection and regeneration about three hundred putative transgenic citrus lines gave positive reactions by the GUS-histochemical assay (Table 8). The presence of inserts among GUS reacting plants was verified by Southern hybridization, and some of these plants were also analyzed by northern hybridization. Southern analyses of B*gl*II digested genomic DNA blots from transgenic rootstocks probed with the p61 and p23U genes show hybridization of a single band of the predicted size for p61, p23U and p23UI (Figs. 13, 14 and 15 give examples). These results confirmed the presence of the introduced CTV-derived genes in different transgenic plants. The H*ind*III-digested genomic DNA from p23U transgenic rootstocks (Fig. 14C) showed multiple hybridizations bands of relatively high molecular weight, indicating multiple integration sites in these plants (1 to 5). Several samples expressing transcripts of the corresponding CTV transgenes *in vivo* are shown by northern blots of total RNAs and of CF-11 enriched dsRNAs (Fig. 16).

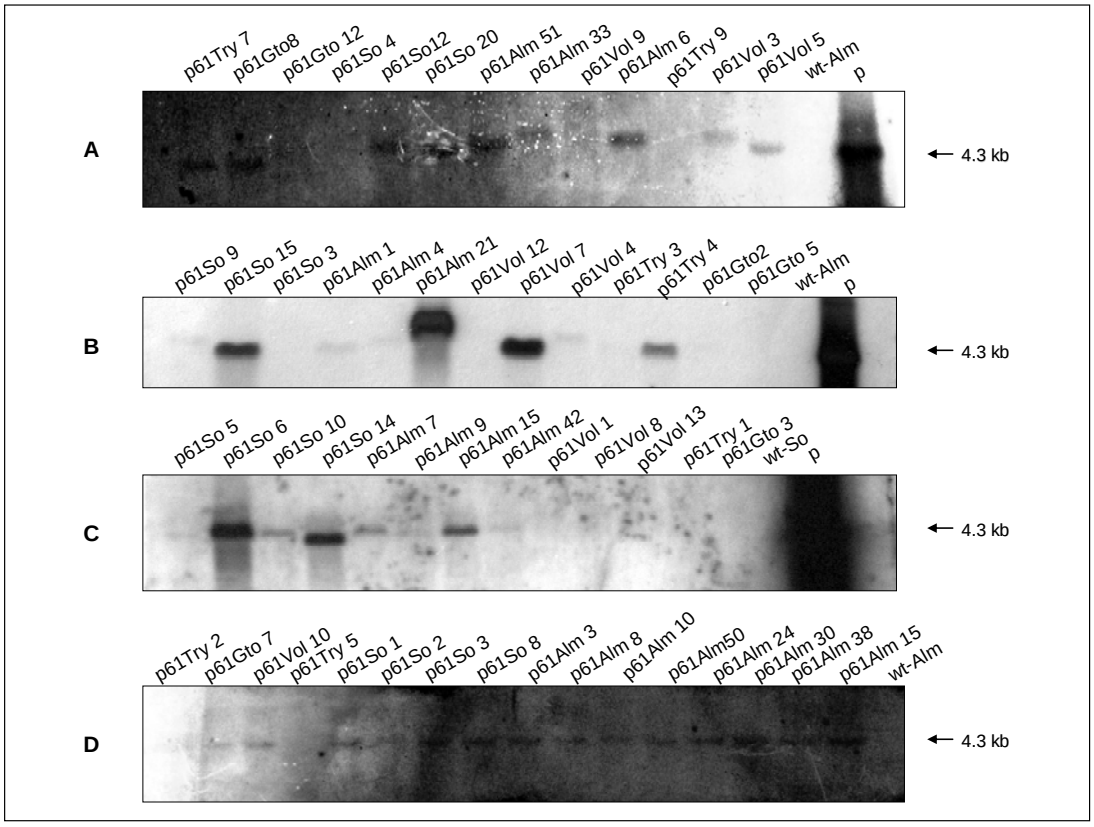

Figure 13. Southern blot hybridization of genomic DNAs extracts from citrus rootstocks transformed with the CTV p61 gene. **A-D**, The genomic DNA samples digested with B*gl*II were electrophoretically separated, transferred to nylon membranes and probed with p61 specific DIG-labeled riboprobe. Wt-Alm and Wt-So: non transgenic Alemow and Sour orange genomic DNA and p: pCamEN35Sp61T plasmid digested with B*gl*II as negative and positive controls, respectively. Arrow indicates predicted size (4.3 kb) for B*gl*II fragment from pCamEN35Sp61T plasmid. Try: Troyer citrange, Gto: Gou Tou, So: Sour orange, Alm: Alemow, Vol: Volkamer lemon.

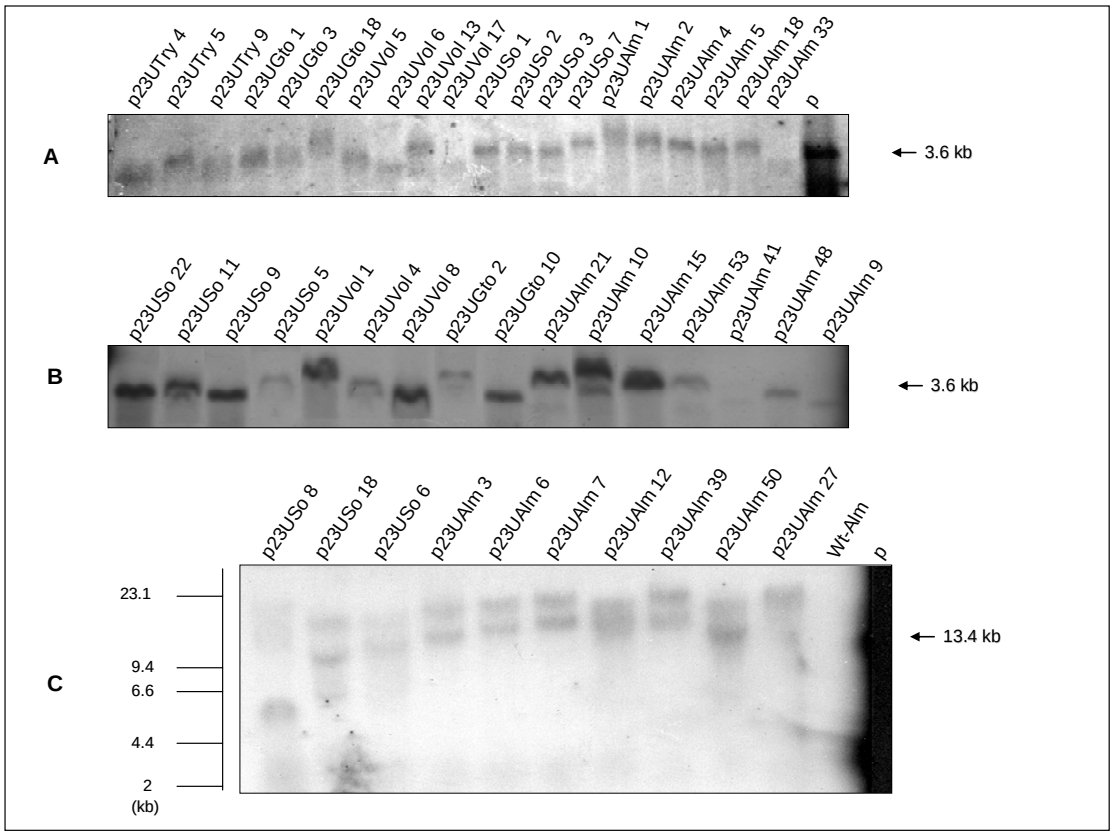

Figure 14. Southern blot hybridization of genomic DNAs extracts from citrus rootstocks transformed with the CTV p23U gene. **A and B**, Genomic DNAs digested with B*gl*II or (**C**) genomic DNAs digested with H*ind*III, were electrophoretically separated, then transferred to nylon membranes and hybridized with p23U specific DIG-labeled riboprobe. Wt-Alm and p: non transgenic Alemow genomic DNA and pCamEN35S23UT plasmid digested with H*ind*III as negative and positive controls, respectively. Arrow indicates H*ind*III digested linear pCamEN35S23UT plasmid with predicted size of 13.4 kb. Try: Troyer citrange, Gto: Gou Tou, So: Sour orange, Alm: Alemow, Vol: Volkamer lemon.

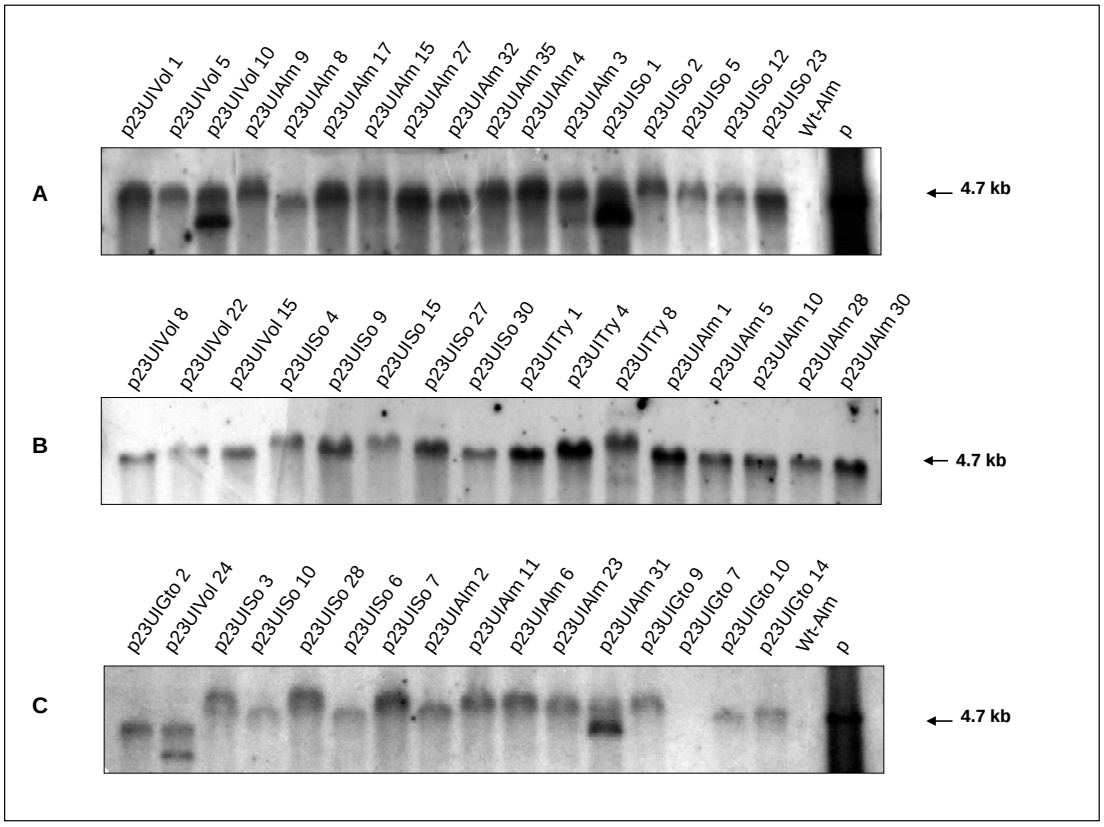

Figure 15. Southern blot hybridization of genomic DNAs extracts from citrus rootstocks transformed with the CTV p23UI gene. **A-C**, Genomic DNAs digested with B*gl*II and electrophoretically separated, were transferred to nylon membranes and hybridized with CTV- p23U specific DIG-labeled riboprobe. Wt-Alm and p: non transgenic Alemow genomic DNA and pCamEN35S23UI23T plasmid digested with B*gl*II as negative and positive controls, respectively. Try: Troyer citrange, Gto: Gou Tou, So: Sour orange, Alm: Alemow, Vol: Volkamer lemon.

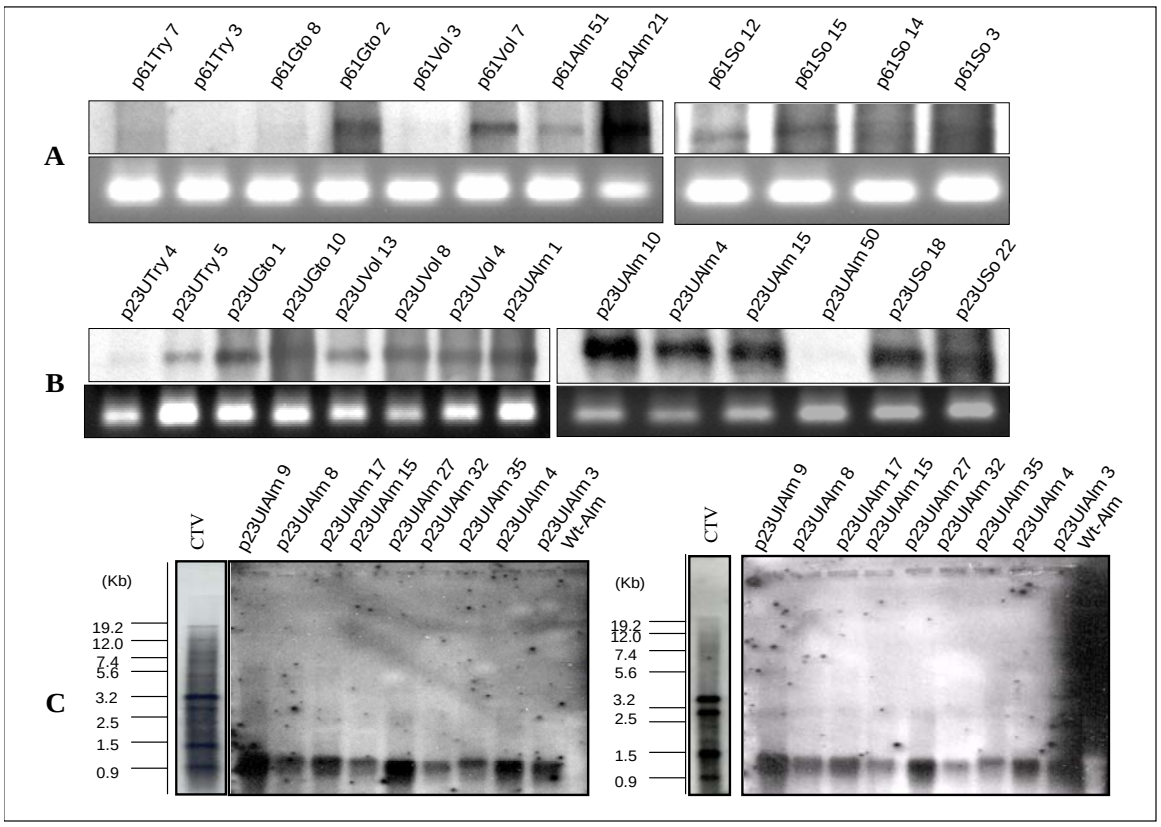

Figure 16. Northern blots of RNAs from non-transgenic and transgenic citrus rootstocks hybridized with DIG-labeled riboprobes of CTV -p61 and -p23U. **A** Northern blot hybridization with p61 riboprobe of total RNA extracts from p61 transgenic citrus rootstocks. Ethidium bromide staining of tRNA shown below indicates loading of apparently equal quantities of total RNAs. **B** Northern blot hybridization with p23U plus (+) specific riboprobe of total RNA extracts from p23U transgenic citrus rootstocks. Loading of apparently equal quantities of total RNAs is indicated bellow by ethidium bromide staining. **C** Northern blot hybridization with p23U plus (+) specific riboprobe (left) and minus (-) specific riboprobe (right) of dsRNAs from p23UI transgenic Alemow lines and non-transgenic Alemow plant (Wt-Alm), respectively. CTV= dsRNA from CTV-infected non-transgenic Alemow plant serving as a marker. Try: Troyer citrange, Gto: Gou Tou, So: Sour orange, Alm: Alemow, Vol: Volkamer lemon.

3.1.4.3 Characterization of transgenic citrus rootstocks

All GUS-positive shoots were shoot tip grafted (STG) *in vitro* and 1-2 months later the epicotyl parts of the supporting rootstocks were re-grafted onto vigorous wild type Volkamer lemon seedlings in the greenhouse.

Most of the transgenic citrus plants selected were morphologically similar to the wild type plants and displayed normal growth and development. However, some of the plants showed severe stunting and die back when grafted on wild type Volkamer lemon plants in the greenhouse. In total we obtained 145 transgenic Alemow plants, 73 transgenic Sour Orange plants, 47 transgenic Volkamer lemon plants, 45 transgenic Gou Tou plants, and 495 transgenic Troyer plants. All the plants mentioned tested positive for the presence of corresponding genes by PCR and by Southern analysis (Table 8).

Table 8. A break down count of the number of transgenic citrus rootstocks produced and tested by GUS staining, PCR and Southern analysis with the corresponding CTV probes.

Citrus Rootstocks	**Transgenes**			
	p61	**p23U**	**p23UI**	**Total**
Sour orange	21	22	30	73
Alemow	52	58	35	145
Volkamer lemon	13	19	25	47
Gou-Tou	12	18	15	45
Troyer citrange	135	170	190	*495
Total no of transgenic plants not including Try				**310**

*****Only 10 of each category of the Troyer citrange transgenes were thoroughly analyzed.

3.2 Section 2: Challenge inoculations of transgenic plants and reaction to viral infections.

3.2.1 The reactions of transgenic *N. benthamiana* to a chimeric GVA vector harboring CTV genes

To investigate the ability of CTV sequences to induce post-transcriptional gene silencing (PTGS) in transgenic *N. benthamiana* plants, we challenged the plants with a chimeric *Grapevine virus A* (GVA) viral vector harboring the corresponding CTV genes. The sense p23U and p61 genes of CTV were cloned into the multiple cloning sites located between two copies of the movement protein (MP) promoters of GVA viral vector (Fig. 17). Sequence analyses of chimeric GVA demonstrated the correct integration of the CTV genes (data not shown).

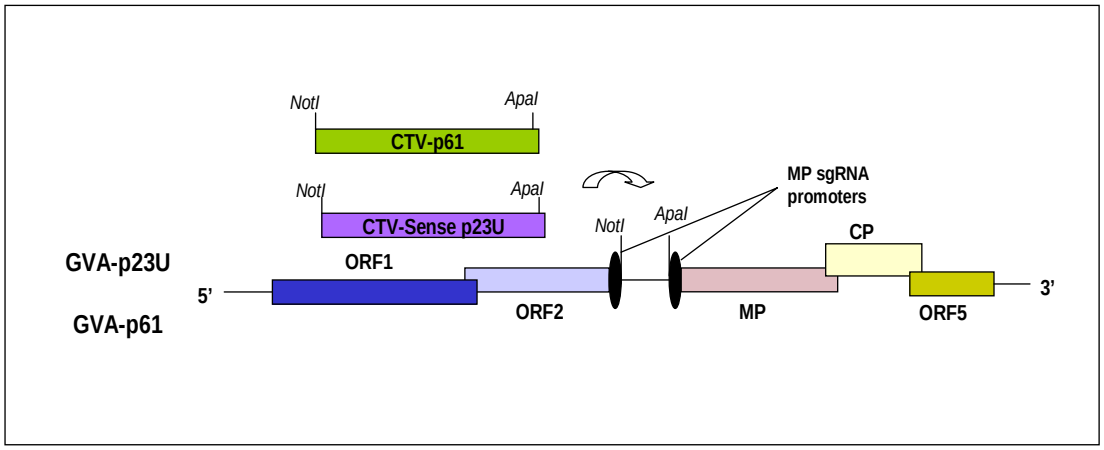

Figure 17. A schematic presentation of the genome organization and insertion sites of the infectious *Grapevine Virus A* (GVA) vector. The N*ot*I and A*pa*I restricted CTV p61 and p23U cDNAs were inserted between duplicates of the movement protein promoters of GVA to construct infectious clones GVA-p61 and GVA-p23U, respectively.

3.2.1.1 Transgenic *N. benthamiana* with p23UI inserts are resistant to GVA harboring p23U

The RNA transcripts from infectious GVA clones harboring the sense-orientated p61 and p23U sequences were used to inoculate transgenic *N. benthamiana* plants expressing p61, p23U and p23UI (Fig. 17). Inoculations were conducted on 4 to 6-weeks-old glasshouse grown GUS positive transgenic *N. benthamiana* F_1/F_2 plants. Transgenic and the control non-transgenic *N. benthamiana* plants were grown and maintained at similar conditions. Both symptom expression and RNA accumulation in the inoculated plants were followed for two months post inoculation.

None of the inoculated p23UI transgenic plants (three plants for each of the four tested transgenic lines), exhibited the symptoms expected for GVA, whereas all the p23U, p61 transgenic and non-transgenic control plants showed disease symptoms including severe stunting 9-14 days post inoculation (dpi) (Fig. 18).

Northern hybridization of dsRNA extracts from plants transgenic for p23U and p61, and non transgenic controls challenged with GVA-p23U and GVA-p61, with riboprobes directed to the CTV -p61 and -p23U and the GVA 5'-terminus, showed strong hybridization signals (data not shown). In contrast, northern hybridization of dsRNA extracts from p23UI transgenic plants, challenged with GVA-p23U, using riboprobes directed to CTV-p23U and the 5'-terminus of GVA, did not detect RNAs of the challenging chimeric GVA-p23U virus. Non-transgenic plants infected with chimeric GVA virus showed strong hybridization signals (Fig. 19A and B). These results, repeated in six experiments showed that all tested p23UI transgenic *N. benthamiana* plants were highly resistant to infection with the chimeric GVA-p23U virus (Batuman *et al.,* 2006).

3.2.1.2 PDR in p23UI transgenic *N. benthamiana* plants.

To elucidate possible mechanism of resistance we inoculated p23UI transgenic and non-transgenic *N. benthamiana* plants with authentic infectious GVA transcripts. All inoculated plants displayed characteristic symptoms of GVA nine days post inoculation (dpi) (Fig. 18E). Northern hybridization of dsRNA extracts from p23UI transgenic and non-transgenic plants challenged with authentic GVA, using riboprobes directed to the GVA 5'-terminus, showed accumulation of apparently similar levels of GVA RNA in both transgenic and non-transgenic *N. benthamiana* plants (Fig. 19C).

Although initially all p23UI transgenic *N. benthamiana* plants showed resistance to chimeric GVA-p23U, a change of this situation was observed eventually and a few of the resistant plants turned symptomatic with new leaves showing typical symptoms at about 30-40 dpi (data not shown). To assess the possible reasons for the breakdown of resistance, the dsRNA from symptomatic leaves was extracted and hybridized with CTV -p23U and GVA 5'-terminus directed riboprobes. Only the GVA riboprobe, but not the p23U probe, reacted with dsRNA from plants that reverted from resistant to symptomatic, suggesting that the chimeric virus used as inoculum lost its CTV component, and a result the native GVA could overcome PDR induced by CTV sequence (data not shown).

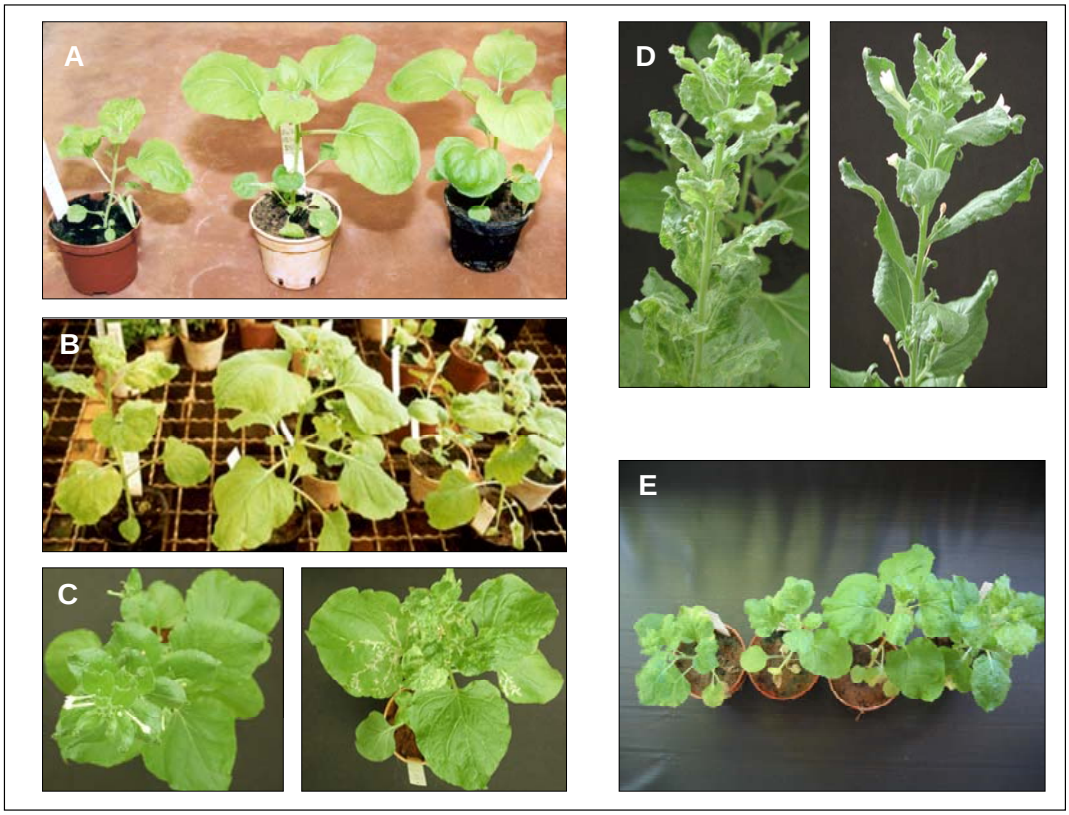

Figure 18. Symptoms caused by an infectious *Grapevine Virus A* (GVA) vector used for challenge inoculation of transgenic and control non-transgenic *N. benthamiana* plants. **A** A non transgenic *N. benthamiana* plant inoculated with GVA-p23U showing severe symptoms 9 dpi (left), whereas the plants carrying the p23UInb 4 and p23UInb 7 transgenes (middle and right, respectively) remained symptomless. **B** Transgenic *N. benthamiana* plants p23Unb line 9, p23UInb line 8 and p61nb line 94 challenge inoculated with GVA-p23U and –p61 plants at 14 dpi, respectively. Note the severe symptoms on p23Unb line 9 and p61nb line 94 transgenic *N. benthamiana* plants (left and right) and their absence on p23UInb line 8 (middle). **C** The resistant p23UInb line 7 transgenic *N. benthamiana* plant flowered at 30 dpi and remained symptomless for two months, while **D** the corresponding non transgenic plants were stunted and never

flowered. **E** Resistant plants p23UInb lines 7 to 9, along with a non transgenic *N. benthamiana* plant (left to right, respectively) inoculated with authentic GVA. Symptom severity following GVA inoculation did not differ among all tested plants regardless of their transgenic or non-transgenic background.

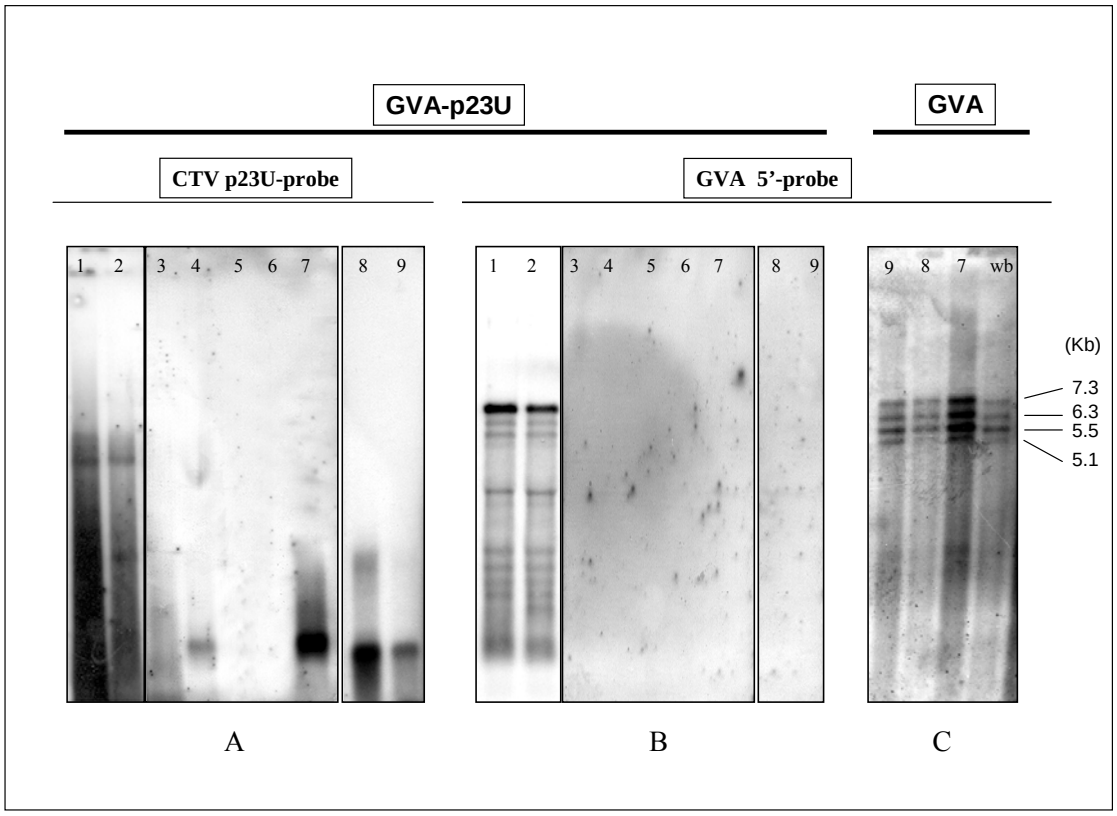

Figure 19. Northern blot hybridizations with DIG-labeled plus (+) strand specific riboprobe of p23U and the GVA 5'- terminus with fractionated dsRNAs from transgenic and non-transgenic *N. benthamiana* plants, 14 days after inoculation with GVA-p23U and authentic GVA. **A** Strong hybridization of large RNAs from two non-transgenic plants (lanes 1 and 2) and the absence of hybridization signals from p23UI transgenic *N. benthamiana* p23UInb lines 1, 4, 130, 94, 7, 8 and 9, respectively (lanes 3 to 9). Note considerable differences in the intensities of signals from resistant lines inoculated with GVA-p23U transcript, in lanes 3, 5 and 6 (barely visible) and their abundance in lanes 4, 7, 8 and 9. **B** Duplicated membranes of (**A)** probed with 5'- terminus plus (+) specific GVA riboprobe. Note the GVA accumulation in non-transgenic *N. benthamiana* plants (lanes 1 and 2) and the absence of GVA from resistant transgenic plants (lanes 3 to 9). **C**

Accumulation of authentic GVA vector subgenomic (sg) RNAs in infected transgenic (lanes 9, 8 and 7) and non-transgenic (lane wb) *N. benthamiana* plants and their molecular weight marker indicated. The probe is the same used in **B**.

These results indicated that resistance was the result of the presence of the CTV sequences in the transgenic plants. Resistance was observed only when the same CTV sequences were present in both transgenic plants and the chimeric virus used for challenge inoculation. These results demonstrated that CTV sequences are able to induce PDR. All tested p23UI transgenic *N. benthamiana* plants showed resistance to chimeric GVA-p23U in challenge inoculation experiments.

3.2.1.3 The size of truncated CTV-p23 gene inserted into GVA vector and its effect on resistance of transgenic *N. benthamiana* plants.

To investigate the required sequence position and/or size of the CTV-p23 gene that is capable to induce the resistance mechanism of p23UI transgenic *N. benthamiana* plants we synthesized and tested a series of chimeric GVA constructs harboring a range of 50, 100, 200 and 400 bp, 5' co-terminal CTV-p23 sequences (Fig. 20A). None of the p23UI transgenic *N. benthamiana* plants inoculated with transcripts of the truncated-p23-GVA vectors was able to induce complete resistance at the level observed for the full length p23U. However, symptom severity was reduced and the leaf size increased in correlation with the increase of the CTV insert size (Fig. 20B). The GVA vector harboring the shortest p23 sequence (50 bp) gave the most intense symptoms. Symptom severity decreased with the increase of insert size in the order of 50>100>200≥400. Durable resistance in p23UI-bearing *N. benthamiana* plants were only observed when using the GVA-p23U for challenge inoculation. Therefore, increase of the homologous sequence in the challenging virus acted better as a PTGS trigger.

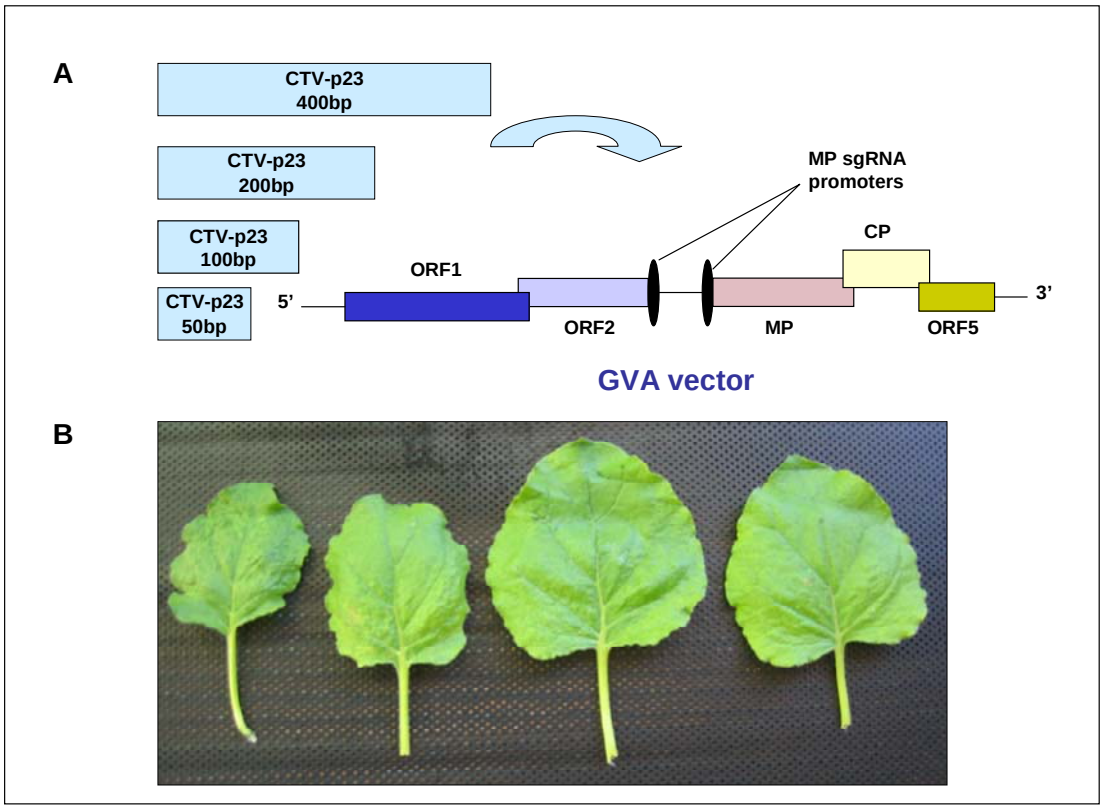

Figure 20. A A schematic presentation of the different-sized inserts of CTV p23 into infectious *Grapevine Virus A* (GVA) vector and **B** differences in symptom severity and leaf size at 14 dpi from plants inoculated with these chimeric vectors. Note symptom severity and consequent small leaf size of the transgenic *N. benthamiana* p23UInb line 9 plants inoculated with the GVA-p23 50, 100, 200 and 400 bp vector (left to right), respectively.

3.2.2 Challenge inoculations of transgenic citrus rootstocks with CTV

At least two sibling plants were prepared from each transgenic line by grafting on commercial nursery-grown Volkamer lemon seedlings. The grafted plants were challenge inoculated with the severe VT-type isolate Mor-T by chip-bud grafting. To ensure the maximal exposure of the transgenic tissues to the challenging virus, the chip graft buds used for inoculation were placed on the CTV-sensitive rootstock, and the inoculum buds were maintained continuously on the inoculated plants (Fig. 21).

Most of the challenged transgenic citrus plants and the non-transgenic plants started to show symptoms at the first flush about 6-8 weeks post inoculation. However, differences in the type of symptom onset were observed among different transgenic plants. The challenged transgenic plants were categorized by symptoms: a) lines that showed severe symptoms like non transgenic plants; b) lines that showed short and/or long delay on symptom onset; c) lines showing severe symptom onset in their first flush and normal growth in subsequent flushes (recovery); and d) plants without visible symptoms.

Symptoms of typical CTV were observed on 126 out of 290 tested transgenic Alemow plants at the first growth flush, about 6 weeks after inoculation. In 16 of the 35 challenged transgenic lines (p61Alm lines 6, 21 and 51, p23UAlm lines 1, 4, 15 and 50, p23UIAlm lines 3, 4, 8, 9, 15, 17, 27, 32 and 35) a delay of symptom appearance was noted. In these lines symptoms appeared only at the second flush cycle, three or more months post inoculation. In three of these lines (transgenic p23UIAlm lines 8, 9 and 17) symptom appearance was delayed in both challenged sibling plants. These results suggest that the expression of p23UI in citrus conferred only an initial delay in symptom appearance and did not provide durable protection against the expression of CTV

symptoms (Batuman *et al.*, 2006). Figure 22 shows an example of different CTV leaf symptoms on challenge-inoculated transgenic Alemow rootstocks.

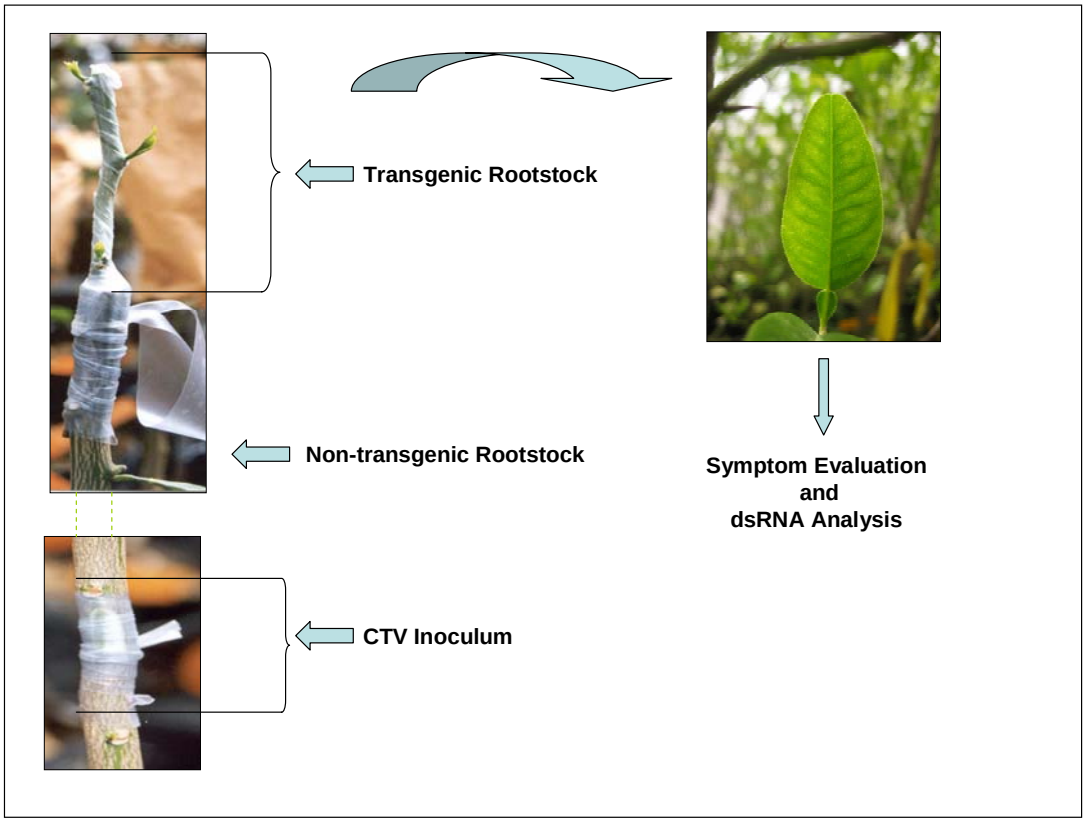

Figure 21. The CTV challenge inoculation system for transgenic and non transgenic citrus rootstocks. Note our inoculation was aggressive and aimed to imitate natural conditions, where CTV-sensitive varieties will be grafted onto transgenic rootstocks. Inoculum of the severe VT-type isolate Mor-T CTV was placed on the sensitive rootstock.

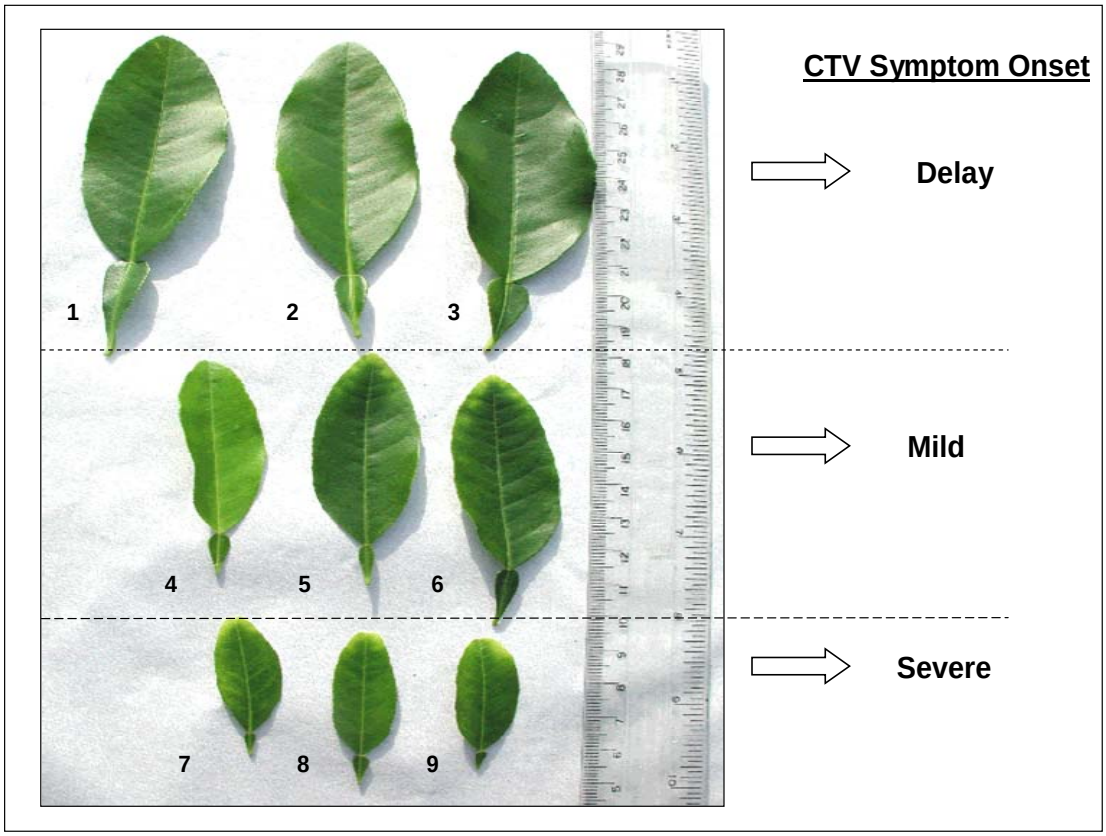

Figure 22. Transgenic Alemow plants showing different symptoms following challenge inoculation with CTV. Typical leaf symptoms of VT-type isolate Mor-T in Alemow plants including vein-clearing, leaf curling and reduced leaf size (leaves of lines 4 to 9). However, the leaves of some of the plants showed barely visible symptoms. Numbers represent plant lines from which leaves were collected: 1= non-inoculated Alemow plant, 2= p23UIAlm line 17, 3= p23UAlm line 4, 4= p23UIAlm line 7, 5= p23UAlm line 23, 6= p61Alm line 40, 7= p23UIAlm line 34, 8= p23UAlm line 19, and 9= CTV inoculated wild type Alemow plant.

Six weeks after challenge inoculation, the viral dsRNA from transgenic and control non transgenic plants were separated by agarose gel electrophoresis and hybridized with DIG-riboprobes plus (+) strand specific to the p23U of CTV. Northern blot analysis indicated that most of the transgenic and non transgenic rootstocks were CTV-infected and contained similar amounts of viral RNAs (Figs. 23, 24 and 25 give examples). Overall, relative virus levels of dsRNA accumulation differed between plants, both among the non transgenic and transgenic lines (Figs. 23, 24 and 25). However differences in signal intensities could not be related to the transgenic nature of the plants (Batuman *et al.*, 2006). For example; while transgenic Alemow line p23UIAlm 17, p61Alm 40 and p23UAlm 19 showed delay, mild and severe symptoms, respectively their signal intensities to hybridization did not show significant differences (Figs. 23, 24 and 25).

The CTV symptoms were not expected on the transgenic Gou Tou and Volkamer lemon leaves as the non transgenic infected leaves are symptomless and their reaction to challenge inoculation was only followed by dsRNA extraction and northern blot hybridization assays (Figs. 23 and 24).

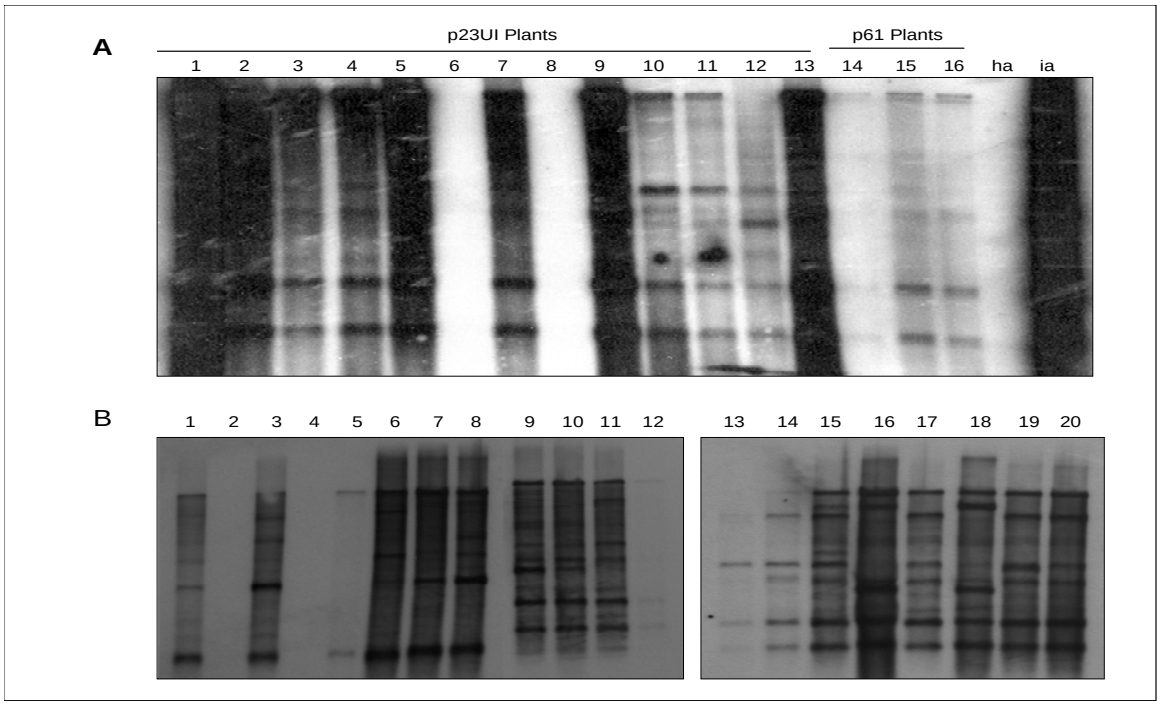

Figure 23. A and **B**, northern blots derived from dsRNA extracts of CTV-challenged transgenic citrus rootstocks, 6-8 weeks after the first inoculation. Hybridization with riboprobes specific to the plus (+) strand of p23U showed that most plants contained replicative CTV regardless of whether their appearance was symptomatic or symptomless. **A** Lanes 1-16: transgenic lines of 1= p23UIAlm 1, 2= p23UIAlm 2, 3= p23UIVol 18, 4= p23UIVol 24, 5= p23UIAlm 5, 6= p23UIAlm 15, 7= p23UISo 29, 8= p23UIAlm 9, 9= p23UIAlm 16, 10= p23UIAlm 7, 11= p23UIAlm 10, 12= p23UIAlm 21, 13= p23UIGto 4, 14= p61So 12, 15= p61So 15, and 16= p61So 3, respectively. Lanes ha and ia: healthy and CTV-infected non-transgenic wild type Alemow plants, respectively. **B** Lanes 1-19: transgenic lines of 1= p23UVol 18, 2= p23UVol 13, 3= p61Vol 3, 4= p23UAlm 4, 5= p23UAlm 10, 6= p23UGto 1, 7= p61Gto 8, 8= p23UIGto 5, 9= p61Alm 21, 10= p61Alm 51, 11= p23UIAlm 30, 12= p23UIAlm 27, 13= p61So 21, 14= p23UISo 7, 15= p23USo 2, 16= p23UIAlm 18, 17= p23UAlm 19, 18= p23UIAlm 24, and 19= p23UIAlm 33, respectively. Lane 20: CTV-infected non transgenic Alemow plant.

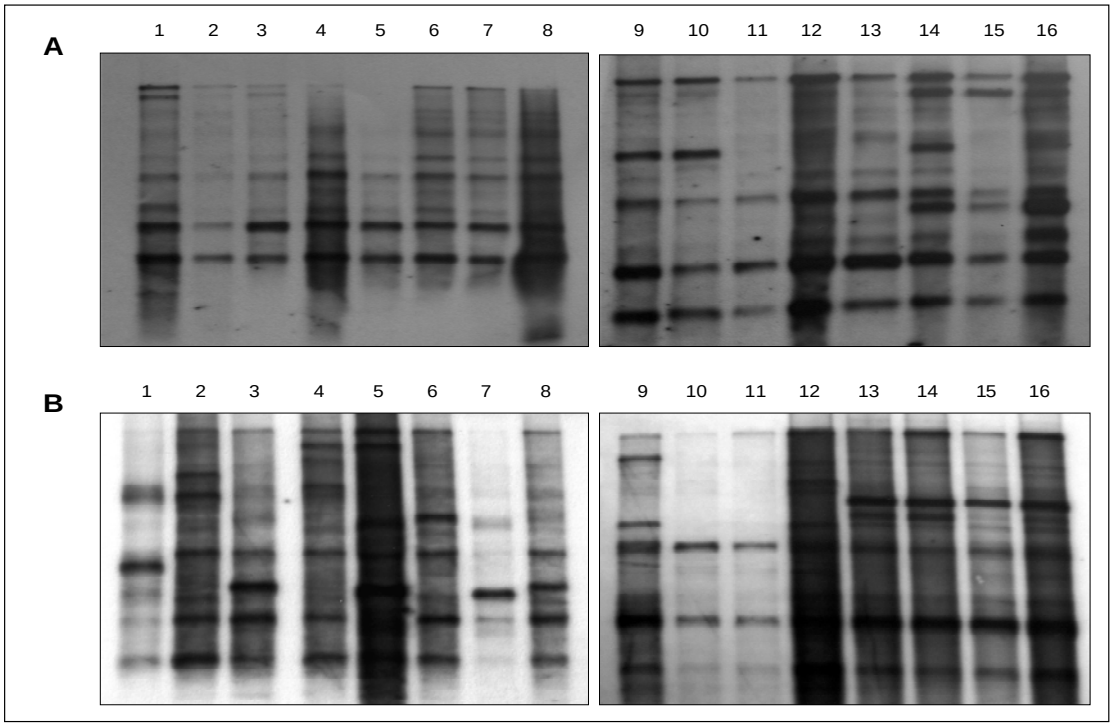

Figure 24. A and **B**, northern blots of dsRNA extracts from challenge inoculated transgenic lines at their second cycle of flush about three months after inoculation. Hybridization with riboprobes directed to plus (+) strand p23U showed that all plants were infected. Increased hybridization signal intensities of some lines indicated increased accumulation of CTV dsRNAs over time. **A** Lanes 1-15: transgenic lines of 1= p23UAlm 2, 2= p23UIAlm 9, 3= p61Alm 42, 4= p23UIAlm 11, 5= p23UIAlm 15, 6= p23UVol 2, 7= p23UIVol 20, 8= p61Vol 13, 9= p23USo 9, 10= p23UISo 27, 11= p61So 12, 12= p23UGto 18 13= p61Alm 40, 14= p23USo 4 and 15= p23UITry9, respectively. Lane 16: CTV-infected non-transgenic Alemow plant. **B** Lanes 1-15: transgenic lines of 1= p23UAlm 4, 2= p61Alm 7, 3= p61Vol 9, 4= p23UIAlm 12, 5= p23UAlm 41, 6= p23UIVol 25, 7= p23UVol 13, 8= p61Alm 30, 9= p23UISo 14, 10= p23UAlm 10, 11= p23UAlm 27, 12= p23UIGto 13, 13= p61Alm 52, 14= p61Alm 4 and 15= p23UAlm 3, respectively. Lane 16: CTV-infected non-transgenic Alemow plant.

Levels of dsRNA in some of the p23UI transgenic Alemow lines with delayed symptom expression were as high as or even higher than those of lines without a delay of symptom expression. Few transgenic plants showed significant reduction in the levels of CTV RNAs compared with non-transgenic plants (Fig. 25A and B). For example, the dsRNA level in line p23UIAlm 17, which showed a delay of symptom appearance, was even higher than those found in lines p23UIAlm 32 and p23UIAlm 35, which did not exhibit a delay of symptom appearance (Fig. 25A and B, lanes 17, 32 and 35, respectively). None of the transgenic plants showed a significant reduction of viral RNA accumulation compared to non-transgenic control plants (Fig. 25A). The dsRNA of lines p23UIAlm 8, 9 and 17, which exhibited a delay symptom appearance for both siblings, differed considerably (Fig. 25A and B). ELISA assays also failed to associate the delay of symptom appearance with a significant reduction of CTV antigen levels (data not shown). The effects of CTV inoculation in these plants was followed for a period of 2 to 3 years. No apparent differences in stem pitting symptom intensities were observed among transgenic and non-transgenic lines.

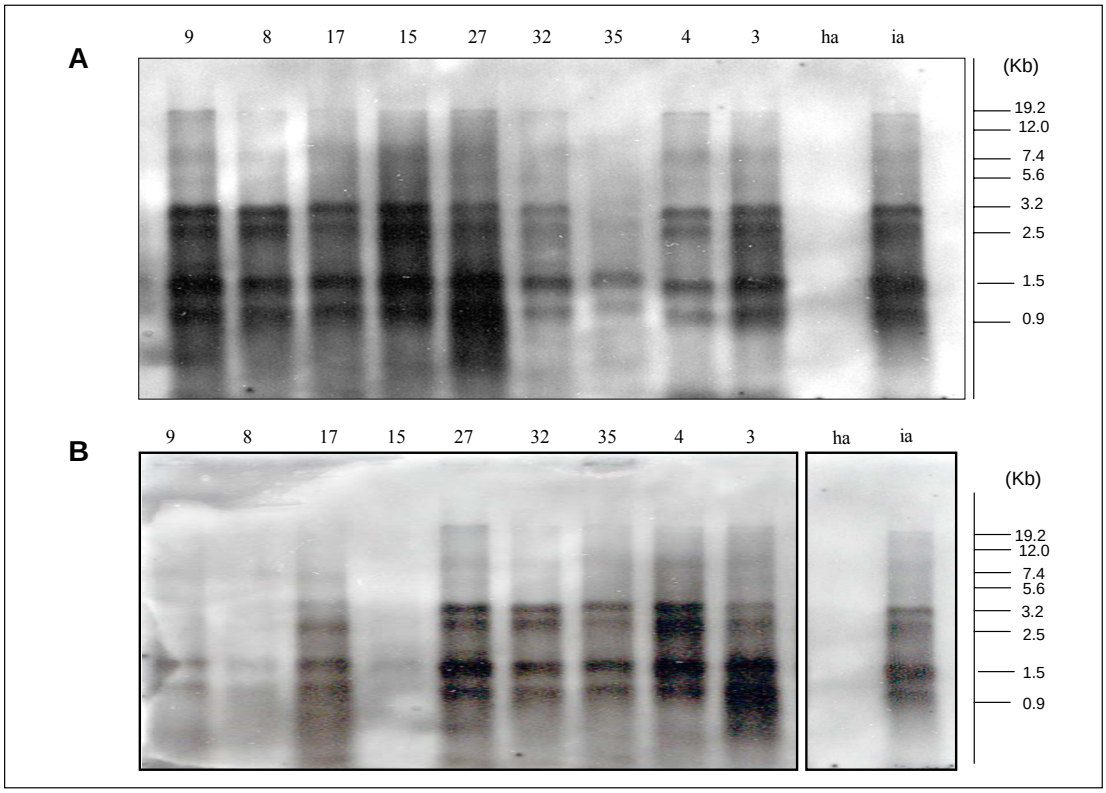

Figure 25. A and **B**, northern blot hybridizations of dsRNA enriched extracts from group of two siblings of p23UI transgenic Alemow plants challenge inoculated with CTV-Mor-T. Hybridization with riboprobes specific to the plus (+) p23U of CTV showed all plants to be infected with CTV. Note: (i) differences in the intensities of hybridization signals among siblings (compare **A** to **B**); (ii) differences in the intensities of hybridization signals, with strong (lanes **A** 9, 15, 27, 3; lanes **B** 27, 4, 3), intermediate (lanes **A** 8, 17; lanes **B** 17, 32, 35) and weak (lanes **A** 32, 35, 4; lanes **B** 9, 8, 15) signals, respectively. None of these differences was associated with noticeable differences in symptom appearance (not shown). Lanes correspond to p23UI transgenic Alemow lines; ia = non-transgenic Alemow plant infected with CTV-Mor-T; ha = healthy Alemow plant.

3.2.2.1 Accumulation of siRNA in CTV challenged plants

We examined the occurrence of CTV siRNA molecules in the p23UI transgenic plants associated with plant virus resistance through PTGS. When the total RNAs were analyzed by northern-blot hybridization, the p23U-specific siRNAs were undetectable in the non-infected p23UI-transgenic plants (data not shown). To evaluate the occurrence of siRNAs in infected plants, total RNA extracts from infected leaves of transgenic and non-transgenic plants were separated by 15% denaturing polyacrylamide gel electrophoresis (PAGE), blotted, and hybridized with DIG-labeled riboprobes representing the different parts of the virus genome. The challenged inoculated transgenic and non transgenic plants accumulated detectable levels of viral-specific siRNAs of 21–24 nt (Fig. 26). Thus indicating that, irrespective of the transgenic nature of the host, CTV RNAs are the target of PTGS (Fig. 26).

Total RNA extracts from CTV infected transgenic plants of lines p23UIAlm 9, p23UAlm 4, p61Alm 30 and non transgenic Alemow plants ((Fig. 26A, B, C and D lanes 1, 2, 4 and 5, respectively) with mild symptoms showed similar levels of siRNA accumulation when hybridized with riboprobes plus (+) strand specific to the pRdRp, p61, p20 and 5'-terminus of CTV. No siRNA were detected in non inoculated Alemow plants (lane 3). The levels of siRNA accumulation in some of the p23UI transgenic Alemow lines with delayed symptoms were higher than those from lines which expressed symptoms normally. Only a few of the transgenic plants showed a significant increase of viral-specific siRNAs compared with those of non transgenic control plants (Fig. 26E and F).

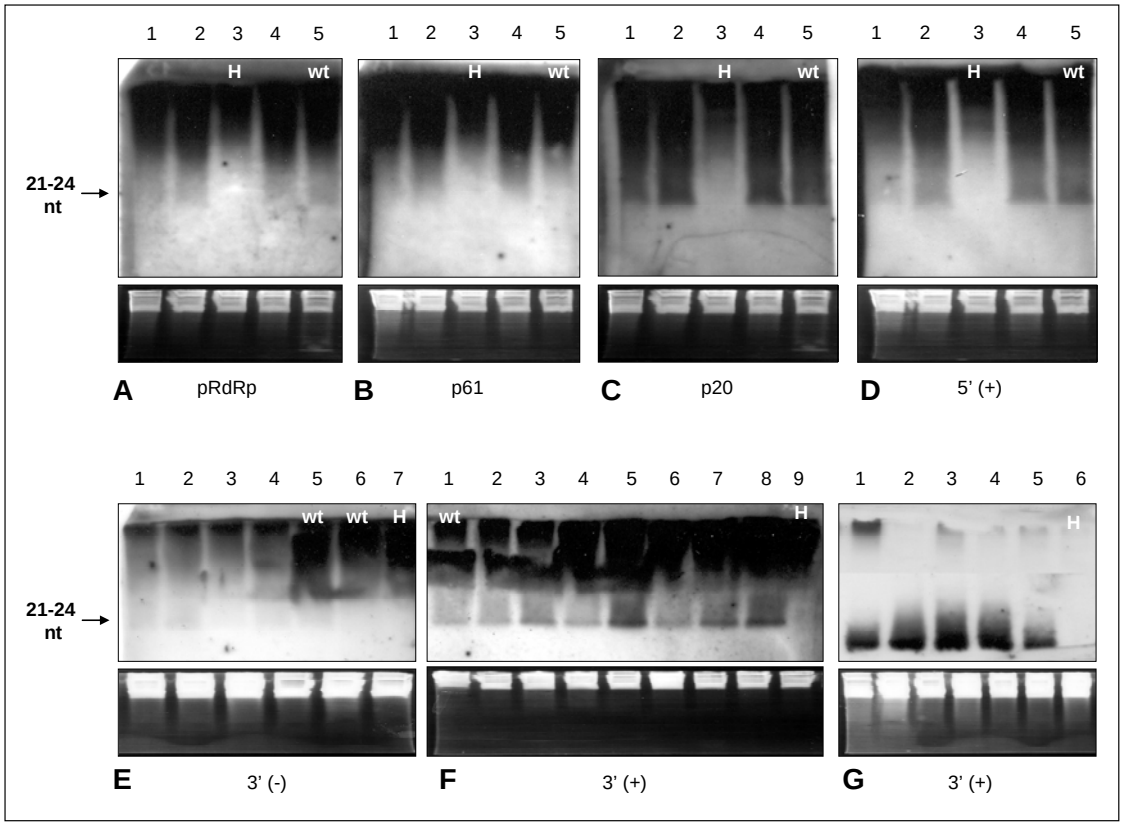

Figure 26. Accumulation of siRNAs in CTV challenged transgenic and non transgenic plants. Northern blot analysis of total RNAs (50 µg) from CTV inoculated and non-inoculated control plants. **A** The presence of siRNAs in total RNA extracts from CTV infected transgenic lines p23UIAlm 9 and p23UAlm 4 (lanes 1 and 2), non inoculated Alemow plant (lane 3), transgenic line p61Alm 30 and non transgenic Alemow plants (lanes 4 and 5) hybridized with plus (+) strand pRdRp riboprobe. **B, C** and **D** duplicate membranes from **A** hybridized with plus (+) strand specific riboprobes of p61, p20 and 5'- terminus of CTV, respectively. **E** The presence of siRNA in CTV infected transgenic lines p23UAlm 2, p23UIAlm 17a, p23UIAlm 17b, p61Alm 51 (lane 1-4), non transgenic Alemow and Sour orange plants (lanes 5 and 6), respectively and non inoculated Alemow plant (lane 7) with a minus (-) strand specific riboprobe from the 3'- terminus of CTV. **F**

The presence of siRNA in CTV infected non transgenic (lane 1), transgenic Alemow lines of p23UAlm10, p23UAlm27, p61Alm21, p23UIAlm9, p23UIAlm32, p23UIAlm35 and p23UIAlm8 (lanes 2-8), and non inoculated Alemow plant (lane 9), respectively with plus (+) specific riboprobe of the 3'-terminus of CTV. **G** The presence of siRNA in CTV infected non-transgenic Sour orange plants (lanes 1-5) and in non inoculated Sour orange plant (lane 6) with riboprobe specific to plus (+) strand 3'-terminus of CTV.

3.2.2.2 The "Recovery" phenomena among some challenged transgenic plants

Challenge inoculations of the p61 transgenic Sour Orange plants resulted in sensitive reactions, with plants showing the expected severe Seedling Yellows (SY) symptoms. Subsequently some of these SY plants reverted to almost normal growth. These results suggested that the expression of the transgene had apparently no effect on the early stages of symptom expression. However, later on, the new leaves accumulated increased levels of viral dsRNAs (compared with the non-recovered plants), and developed apparently symptomless leaves and stem growth (Fig. 27, shows an example). The p61 transgenic Sour orange plants exhibiting this response were said to have "recovered" (Fig. 27). It should be noted however, that similar phenomena were recorded in the past for non-transgenic plants. Furthermore the number of control non-transgenic plants in this experiment did not allow us to conclude if the presence of the p61 transgene significantly increased the recovery rates.

Northern blots of dsRNA extracts were made from CTV challenged transgenic citrus rootstocks 6-8 weeks after inoculation. The dsRNA level in leaves from some of the p61 transgenic Sour orange lines that later "recovered", were higher than the levels of leaves from lines that did not exhibit the recovery phenotype (Fig. 27C). The "recovered" p61 transgenic p61So 12, p61So 15 and p61So 21 plants showed a significant increase of virus RNAs compared with the SY reacting leaves, however these were still low compared with the signals obtained with different Alemow lines (Fig. 23A and B). Moreover, when tissue from recovered leaves was used for graft inoculation of non-transgenic Sour orange plants not all plants were symptomless and some of the inoculated

plants showed SY reaction. Alemow plants inoculated with recovered and the authentic Mor-T isolate were found to contain similar levels of dsRNAs (data not shown).

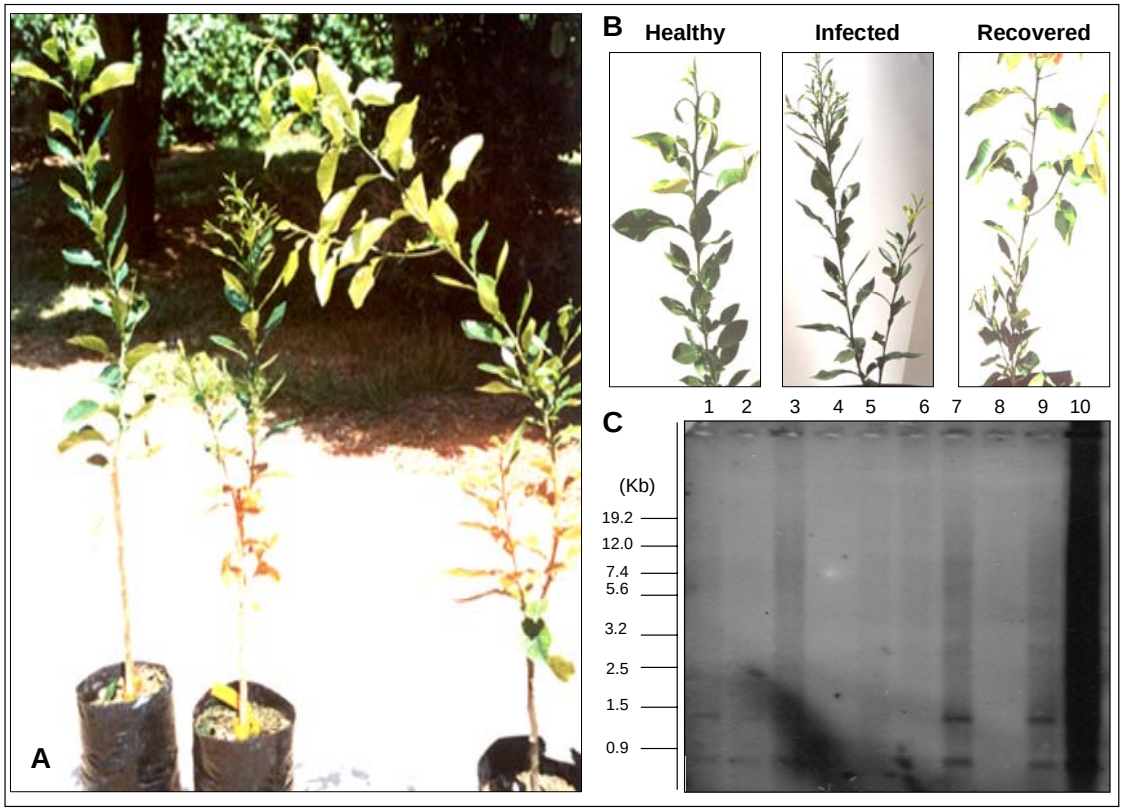

Figure 27. The p61 transgenic and non-transgenic Sour orange plants challenge inoculated with CTV-Mor-T. **A** Non-inoculated (left) and inoculated (middle) non-transgenic Sour orange plants, and a recovered (right) Sour orange plant. **B** Close-up presentations of plants shown in **A**. Note reduced leaf size considered as a severe SY symptom of CTV in infected non recovered plant and also in the lower part of the recovered plant. The upper part of recovered Sour orange plants is apparently symptomless. **C** Northern blots of dsRNAs extracts from recovered Sour orange plants. Hybridization with riboprobes specific to the plus (+) strand from the 3' end of CTV

showed that in the upper leaves (without symptoms) (lanes 1, 2, 3, 7, 9) accumulated higher levels of the viral dsRNA than in lower symptomatic leaves (lanes 4, 5, 6, 8). However, levels of dsRNAs in SO plants were lower than in Alemow plant used as control (lane 10) that graft inoculated with recovered SO leaves. Lanes 1-3: dsRNA extracts from symptomless leaves of recovered p61So 12, 15 and 21; lanes 4-6: dsRNA extracts from symptomatic leaves of recovered p61So 12, 15 and 21; lanes 7 and 9: dsRNA extracts from symptomless leaves of recovered p61So 12A and its sibling 12B and from symptomatic leaves of recovered p61So 12B (lane 8); Lane 10: dsRNA extract from symptomless leaves of non-transgenic Alemow plants graft inoculated with symptomless leaves from recovered p61So 12.

3.2.2.3 Sequence analysis of recovered and authentic strains of CTV

Recovery phenomena of virus-infected plants were recently attributed to reduced virus accumulation due to the silencing mechanism. Since our initial experiments did not show a direct correlation between siRNA accumulation and symptom reduction, we explored a different possibility: a change in the virus genome to a mild isolate by possible mutations and/or recombination mechanisms. To test this possibility, we compared the genomic sequences of the original virus isolates used for inoculation with those of the recovered Sour orange plants virus, after graft inoculation on Alemow plants.

The dsRNAs extracts from Alemow plants inoculated with either the parent strain (Mor-T= MA and MB) or the recovered isolate (Mor-T/R= RA and RB) were used for cDNA synthesis, cloning into pGEM-T Easy Vector (Promega) system and for sequence analysis.

Analysis of the resulting complete isolates sequences revealed a genome organization (Fig. 1A) identical to that reported for the CTV-VT sequence (Mawassi *et al.,* 1995). The homology between the complete sequences of CTV-VT, and the original and "recovered" Mor-T isolates was high as expected (96.7-99.7%, data not shown). Nucleotide sequence alignment of the genome of VT with those of the original Mor-T (MA and MB) and the "recovered" (RA and RB) and VT genomes revealed close identity between the MA MB and the RA RB sequences (data not shown). The calculated genome sizes of both recovered (RA and RB) isolates were nearly of the same size (19,301 and 19,298 nucleotides, nt), and their mean nucleotide identity was 99.1%. The cDNAs of both Mor-T (MA and MB) isolates were also nearly identical in size (19,268 and 19,263 nt), and their mean nucleotide identity was 99.8%. The identity between the Recovered and Mor-

T isolates was however lower (97.1%). The identities between the different ORFs ranged from 91.2% to 100%. Their 3' UTRs were 99.2- 100% identical, and their 5' UTRs were 100% identical (data not shown).

The homology between the p61 and p23 gene sequences of analyzed isolates was greater than expected (Figs. 28 and 29). The identities between the p23 and p61 genes were 99.6 % and 99.7 %, respectively. There was no consistent nucleotide change at the same positions between all isolates, indicated absence of recombination but random mutations through out p23 and p61 genes (Figs. 28 and 29).

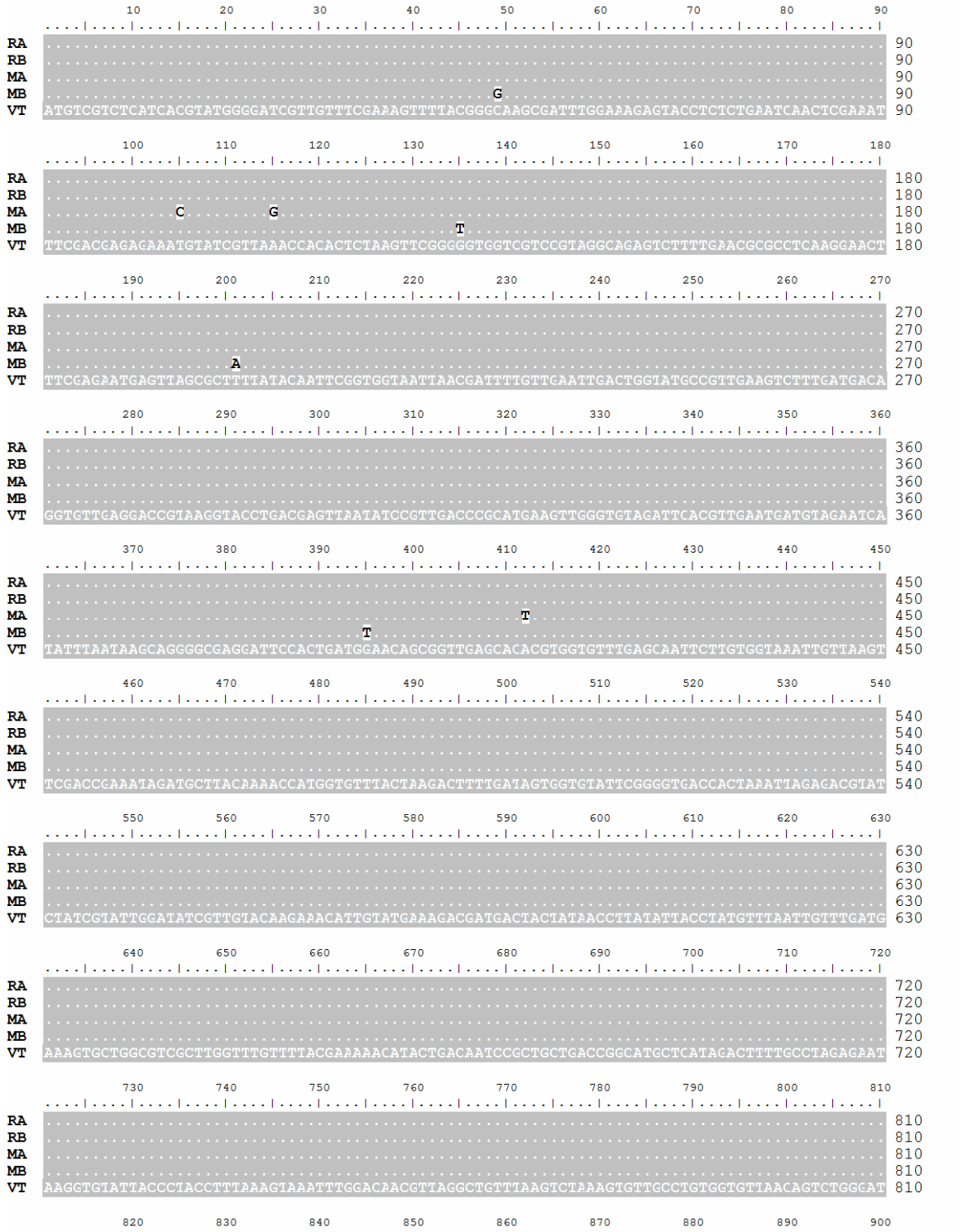

Figure 28. Nucleotide sequence alignments of p61 genes of CTV-VT with those of the original Mor-T (MA and MB) and the "recovered" (RA and RB) isolates. Nucleotide differences in aligned sequences are shown in boldface (next two pages).

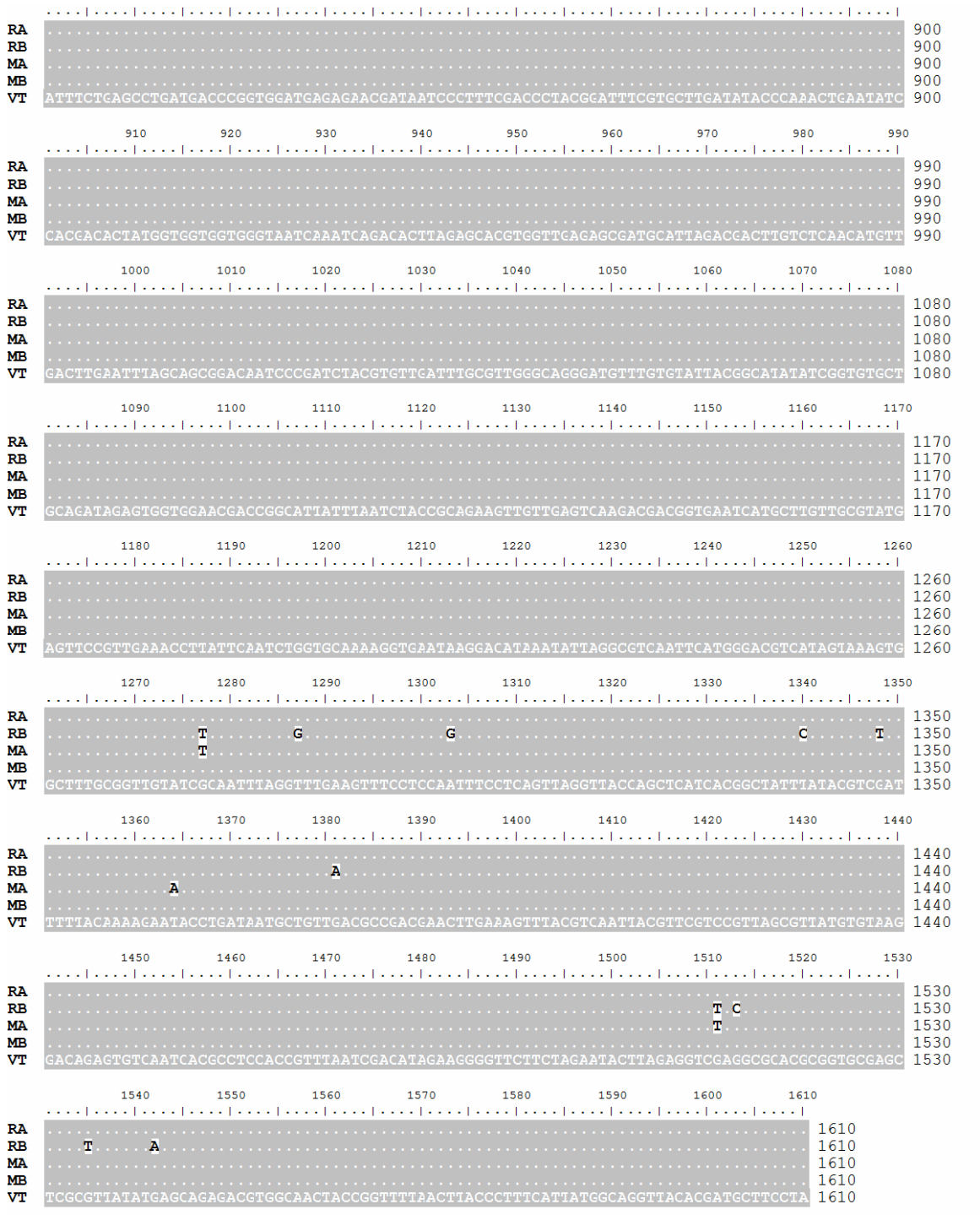

Figure 28. -*Continued*

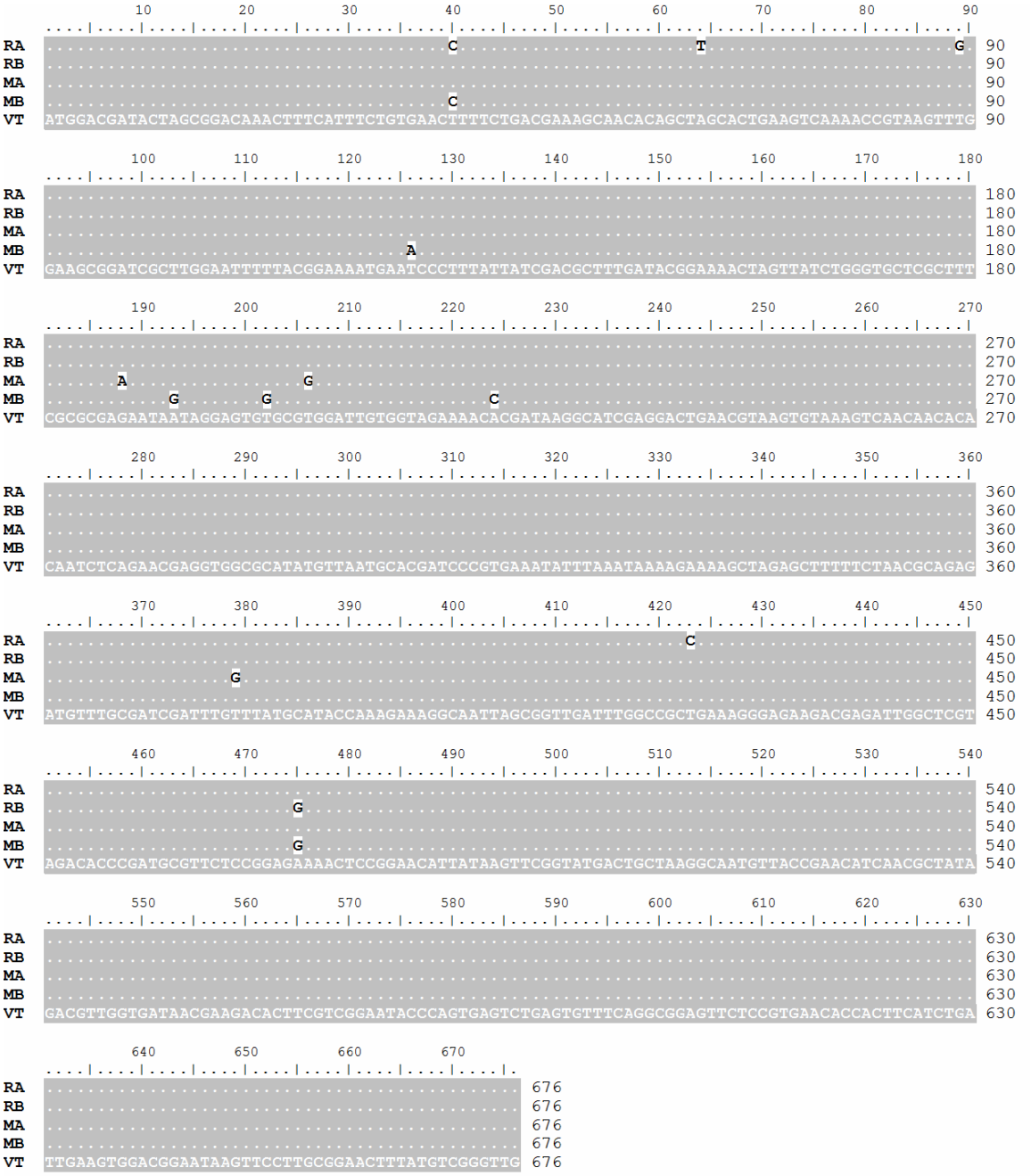

Figure 29. Nucleotide sequence alignments of p23 genes of CTV-VT with those of the original Mor-T (MA and MB) and the "recovered" (RA and RB) isolates. Nucleotide differences in aligned sequences are shown in boldface.

4. DISCUSSION

4.1 Selecting CTV sequences for PDR

Pathogen derived resistance (PDR) of transgenic plants can be induced by a range of viral sequences and is now commonly applied to prevent plant viral infections (Sanford & Johnson, 1985; Baulcombe, 1996; Beachy, 1997; Ravelonandro *et al.*, 2000; Dominguez *et al.*, 2002a). Previous attempts to utilize transgenic PDR to control disease manifestations of CTV one of the most destructive viruses affecting citrus, did not confer durable and stable resistance (Gutierrez *et al.,* 1997; Piestun *et al.,* 1998; Dominguez *et al.,* 2000; Ghorbel *et al.,* 2000; Ghorbel *et al.,* 2001; Dominguez *et al.,* 2002a; Febres *et al.,* 2003; Fagoaga *et al.,* 2005 and Batuman *et al.,* 2006 as part of this dissertation work) (Table 1). The present study was aimed to test the effectiveness of several CTV sequences including -p61, -p23U and -p23UI to confer resistance to a range of transgenic hosts inoculated with two virus systems: the authentic CTV and an infectious GVA clone harboring CTV-p23U and -p61 sequences.

Our primarily aim was to achieve practical results in the form of CTV tolerant or resistant citrus rootstocks. We reasoned that transgenic rootstocks will not meet the considerably objection expected when cultivating a transgenic fruiting scion.

The product of CTV-p23 gene, expressed from ORF11 was found to be multifunctional (Lopez *et al.,* 2000; Ghorbel *et al.,* 2001; Satyanarayana *et al.,* 2002; Lu *et al.,* 2004; Fagoaga *et al.,* 2005 and 2006). We expected that inserting this sequence will interrupt early phase of CTV replication and other essential viral functions for CTV replication and/or accumulation by means of PDR. Previous attempts to use p23 to control CTV however, unexpectedly revealed its role in CTV pathogenicity (Ghorbel *et al.,* 2001;

Fagoaga *et al.,* 2005). It should be noted that the sequence of p23 from different isolates is highly conserved and it was expected therefore to provide broad range of resistance against the numerous CTV isolates known to infect citrus trees (Bar-Joseph *et al.,* 1989).

The CTV-p61, is the product of ORF5, encoding the p61 protein, and its analogues are universally found in *Closteroviridae.* The function of p61 in virus assembly and cell-to-cell movement (Satyanarayana *et al.,* 2000) also suggested a possible use in PDR to control of CTV.

The CTV-p23UI construct consisting of intron-spliced RNA with hairpin structure from ORF 11 and 3' UTR produced the p23U sense and anti-sense sequences flanked by Castorbean catalase intron, as expected p23UI in dsRNA molecules. Similar inverted repeats were reported to efficiently cause PDR due to the folding of transgene-derived RNAs into long, stable dsRNA structures (Smith *et al.,* 2000; Fagoaga *et al.,* 2006).

4.2 Citrus rootstocks

The implementation of genetic engineering in food plants has led to considerable fear regarding their stability and safety in the environment and therefore, it is a matter of considerable commercial concern (reviewed in Singh *et al.*, 2006). Thus, as indicated above by choosing citrus rootstocks as targets for transformation rather than edible varieties we were mainly aiming to prevent the opposition of consumers, not willing to utilize the resulting genetically modified product. The economically important citrus rootstocks that were tested in this study differ however considerably in their reactions to CTV infection as indicated in Table 13.

Table 9. The reaction of the selected citrus rootstocks to CTV infections.

Rootstock	Quick Decline (QD)	Decline (D)	Stem Pitting (SP)	Seedling Yellow (SY)	Virus Titers
Sour orange *Citrus aurantium*	+	-	+/-	+	+/-
Gou Tou Chinese sour orange	-	+/-	+/-	-	+[2]
Alemow *C. macrophylla*	-	+	+	-	+[3]
Volkamer lemon *C. limon*	-	-	-	-	+[2]
Troyer citrange *C. sinensis* x *P. trifoliata*	-	-	-	-	+/- or -

- None, + Yes, +[2] Moderate levels, +[3] High levels

The table suggests on considerable improvement expected from effective PDR of CTV for the Sour orange and Alemow rootstocks and probably also for Gou Tou.

4.3 Transgenic *N. benthamiana* plants – a useful model for testing the effectiveness of cloning and transformation steps

The main aim of producing transgenic *N. benthamiana* plants expressing specific CTV sequences was as a system for rapid testing of the viability of plasmid constructs before attempting their transformation of the more difficult citrus plants. The availability of the infectious *Grapevine Virus A* (GVA)-based viral vector in our laboratory (Haviv *et al.,* 2006) facilitated the use of transgenic *N. benthamiana* plants to assess the possibility of obtaining resistance against chimeric GVA vectors harboring CTV sequences. Although *N. benthamiana* plants are not hosts of CTV, this approach simplified the early evaluation process of PTGS-based resistance against CTV. *N. benthamiana* is an herbaceous plant and is more amenable than are woody citrus plants to rapid analysis of transgenic progenies and, as was shown in this study, different viruses and transgenes were rapidly evaluated (Batuman *et al.,* 2006). Indeed our results demonstrated that the p23UI transgenic *N. benthamiana* plants displayed resistance to chimeric GVA vector harboring CTV-p23U, but were highly sensitive to GVA (Figs. 18 and 19).

Inoculating the transgenic *N. benthamiana* plants bearing p23U and p61 genes with GVA-containing homologous sequences showed susceptible phenotypes whereas F1 and F2 progenies of the p23UI-bearing transgenic plants showed a durable resistance (Figs. 18 and 19). The reason for the lack of resistance in transgenic p23U and p61 plants when challenged with chimeric GVA is not known. Recently, similar experiments with the PVX vector demonstrated that CTV-CP sequence transgenic *N. benthamiana* plants failed to show resistance to PVX-containing homologous sequences while plants bearing other CTV sequences (p23, p20 and 3' UTR) were resistant (Roy *et al.*, 2006). Thus

while, Roy *et al.* (2006) reported on the resistance of p23 transgenic *N. benthamiana* to infection by PVX-p23 our study with GVA-p23 was different. Among the possible causes of the noted differences between the two systems are (1) PVX is largely a mesophyll-infecting virus whereas GVA is mainly a phloem-limited virus, and (2) the silencing suppressor/s of the virus vectors might differ in their targets.

4.4 Optimization of the transformation process of citrus rootstocks

During the last decade several laboratories including ours, were attempting to establish genetic transformation systems for economically important citrus genotypes, mainly to elucidate functional genomics and for genetic improvement. In spite of the considerable efforts invested, transformation efficiencies, for most of the important citrus genotypes were usually low. This was probably due to the lack of information on the important events that turn citrus cells competent for both transformation and regeneration. In this study, cell competence and the role of plant growth regulators (PGR) for increasing efficiency of transformation and regeneration of shoots from citrus epicotyl explants were extensively studied for citrus rootstocks.

We characterized the *in vitro* responses of epicotyl explants from five citrus rootstocks to different concentrations of BA and Kin. Both cytokinins stimulated bud and shoot regeneration in different concentrations, in all tested genotypes (Fig. 10, Tables 5 and 6). Similarly, at low concentrations a promotive effect of BA was observed for explants from different citrus genotypes, whereas a toxic effect has been described at higher BA concentrations (Sim *et al.*, 1989; Goh *et al.*, 1995; Maggon & Singh, 1995; Gutierrez *et al.*, 1997; Costa *et al.*, 2002; Costa *et al.*, 2004).

The pathway of regeneration at the cut edges of the explants in the five rootstocks tested was dependent on concentration of BA. Epicotyl explants cultured on medium containing lower concentrations of BA produced shoots by direct organogenesis. However, when higher BA concentrations were applied in the culture medium, shoots were produced by indirect organogenesis. Such a response has been observed in some citrus genotypes, when epicotyl explants were placed horizontally on the culture medium (Sim *et al.*, 1989; Goh *et al.*, 1995; Maggon & Singh, 1995).

A morphogenic gradient along the epicotyl axis was observed with reduced organogenic response with increasing distance of the explants from the cotyledonary node. A gradient of expression was influenced by genotype, concentration of cytokinins, illumination conditions, and seedlings age. Our results indicated that BA concentration was one of the most important factors affecting the morphogenic gradient along the epicotyl axis.

There are conflicting reports on the expression pattern of the morphogenic gradient along the epicotyl axis of citrus. Some reports suggested that maximal response was obtained in regions nearest to cotyledons (Burger & Hackett, 1986; Garcia-Luis *et al.*, 1999; Moreira-Dias *et al.*, 2001), others indicate the opposite (Sim *et al.*, 1989; Goh *et al.*, 1995; Costa *et al.*, 2004). Costa *et al.* (2004) suggested the differences in results are due to (1) variation in plant material (i.e., genotype, age of explants), (2) composition of the culture medium (i.e. type and concentration of PGR), or, (3) incubation conditions (i.e. explant orientation, light regime). In our study, greater organogenic responses were observed in explants from cotyledonary regions, irrespective of the citrus genotype, when culture medium containing both BA and NAA combinations were used.

4.4.1 Preculture

Preculture of citrus epicotyl explants on cocultivation medium prior to incubation in *Agrobacterium* solution significantly increased the numbers and percentages of shoot producing explants, total shoots produced, total number of shoots displaying GUS activity, and fraction of explants with shoots displaying GUS activity (Tables 5 and 6). Different results were described by Costa *et al.* (2002) for transformation of Duncan grapefruit, where preculture treatment drastically reduced the transformation efficiency. Cervera *et al.* (1998b) also reported that a 1-day preculture of Carrizo citrange stem segments on cocultivation medium reduced the number of GUS expression sites on explants to less than half of the number obtained without preculture. Ghorbel *et al.* (2000) reported that preculture of sour orange explants resulted in a stress response and was detrimental to transformation.

The cellular basis for the promotion of *Agro*-transformation after preculturing explants has not been completely elucidated. It has been proposed that the production of *vir*-inducing compounds by metabolically active cells plays an important role (Stachel *et al.*, 1985). Villemont *et al.,* (1997) reported that pre-cultivation of petunia leaf explants in a medium with 2,4-D and BA for 2-3 d prior to co-cultivation with *Agrobacterium* drastically enhanced genetic transformation. During the preculture, cells enter active cell division (M-phase) and duplicate (S-phase) DNA, and at the moment of bacterial inoculation PGR-activated cell nuclei were recruited into S-phase. Similar results were now reported for citrus (Pena *et al.*, 2004). In our study, preculture and a short exposure of explants to a medium rich in auxin (2,4-D) gave similar results. This was however influenced by the genetic composition. Thus, while SAV group rootstocks showed

improved results only after four days of preculture, two days were sufficient for TG group rootstocks.

4.4.2 Induction and inoculation of *A. tumefaciens*

The Ti plasmid virulence (*vir*) genes of *A. tumefaciens* are involved in increased transformation frequency. In our study we observed an increase in transformation frequencies and in subsequent regeneration using an induction medium (IM) previously developed for recalcitrant crop transformation (Gelvin & Liu, 1994). IM was however toxic to the incubated citrus explants due to low pH and composition. To overcome this draw-back we replaced the IM prior to explant inoculation with MS solution, supplemented with acetosyringone (200µM), a *vir* inducing a phenolic component. Sunilkumar *et al.,* (1999) reported that the preculture step of tobacco leaf disc transformation, could be bypassed if acetosyringone is provided to the freshly cut explants. In our experiments, bypassing preculture drastically reduced transformation efficiency, therefore acetosyringone was only supplied into *Agrobacterium* suspension and the preculture step was included for all citrus genotypes, irrespective of the treatment. Different investigators incubated the citrus explants in *Agrobacterium* suspensions for short periods of 10 min to 30 min (Kaneyoshi *et al.*, 1994; Pena *et al.*, 1997; Bond & Roose, 1998; Cervera *et al.*, 1998b; Luth & Moore, 1999; Yang *et al.*, 2000; Ghorbel *et al.*, 2001), although only Costa *et al.* (2002) run these experiments side by side. Bond and Roose (1998) observed that an incubation period longer than 10 min, significantly decreased transformation efficiency of epicotyl explants from Washington navel orange (*C. sinensis*). Costa *et al.* (2002) found that 20 min incubation was optimal for Duncan

grapefruits. We observed similar trends among explants that were inoculated with *Agrobacterium* by vacuum infiltrations and/or longer inoculation periods. These treatments often caused serious problems with bacterial over-growth on explants during the cocultivation period, mainly because of excessively penetrated bacteria. Maximum GUS activity in regenerated shoots was obtained after 60 min inoculation. However, a 30 min inoculation period followed by 10 min vacuum infiltration increased transformation efficiency and was thereafter used routinely for all citrus genotypes (Tables 5 and 6).

4.4.3 Coculture medium (CM)

Auxins, especially 2,4-D, increased transformation frequencies when added to the preculture and/or cocultivation medium. Pena *et al.* (1997) obtained improved transformation of citrus by using tomato cell feeder layers on an auxin-rich medium during the cocultivation period (Dominguez *et al.*, 2000). Reevaluation of the effects of the feeder plate medium, the filter paper layer, and the tomato cell suspensions, showed that highest rates of transformation were obtained with auxin-rich medium alone (Cervera *et al.*, 1998b). Pena *et al.* (2004) reported that cocultivation on an auxin-rich medium caused a significant increase in the fraction of actively dividing cells in S-phase, the stage in which cells are more prone to integrate foreign DNA. Our study showed that exposing explants to 2,4-D for 3h prior to *Agrobacterium* inoculation improved transformation results, consistent with the report of Yu *et al.* (2002). The positive effect of 2,4-D treatments contributed to an increase in the frequency of GUS-positive, but did not increase regeneration frequency (Tables 5 and 6). Above optimal 2,4-D concentrations (4.52 μM for SAV and 0.44 μM for TG) and/or a long treatment with 2,4-D promoted the

production of unorganized callus, and significantly reduced shoot regeneration. Thus, the cocultivation step of explants was carried on PM for 3-5 days in the absence of 2,4-D.

4.4.4 Selection media and regeneration conditions

The ineffectiveness of kanamycin use for selection has been suggested as one of the drawbacks of citrus transformation (Moore *et al.*, 1992; Pena *et al.*, 1995a; Pena *et al.*, 1995b; Gutierrez *et al.*, 1997; Pena *et al.*, 1997). Attempts to improve selection of citrus on kanamycin-containing medium include the continuous exposure of the explants to the antibiotic (Pena *et al.,* 1995b), addition of a liquid medium overlay containing kanamycin to culture plates (Gutierrez *et al.,* 1997; Yang *et al.,* 2000), and replacement of kanamycin with a different antibiotic (geneticin), which could also be detoxified by *nptII* activity (Pena *et al.,* 1997). However, most of these techniques are laborious and caused to the overgrowth of bacteria, or were not sufficiently effective in preventing the emergence of un-transformed escapee shoots. Hygromycin was reported to be less efficient than kanamycin (Costa *et al.,* 2002). In the present study, kanamycin was mainly used as a selective agent, although we also obtained hygromycin-resistant citrus plants (Piestun, 2003; Batuman *et al.,* unpublished). We found that selection medium (SM) containing 200 μM kanamycin did not alter regeneration of all the tested citrus rootstock genotypes and it effectively reduced the growth of GUS-negative shoots without resulting in *Agrobacterium* overgrowth. This strong selection pressure was also found to be sufficiently effective in reducing escaped shoots (Fig. 28).

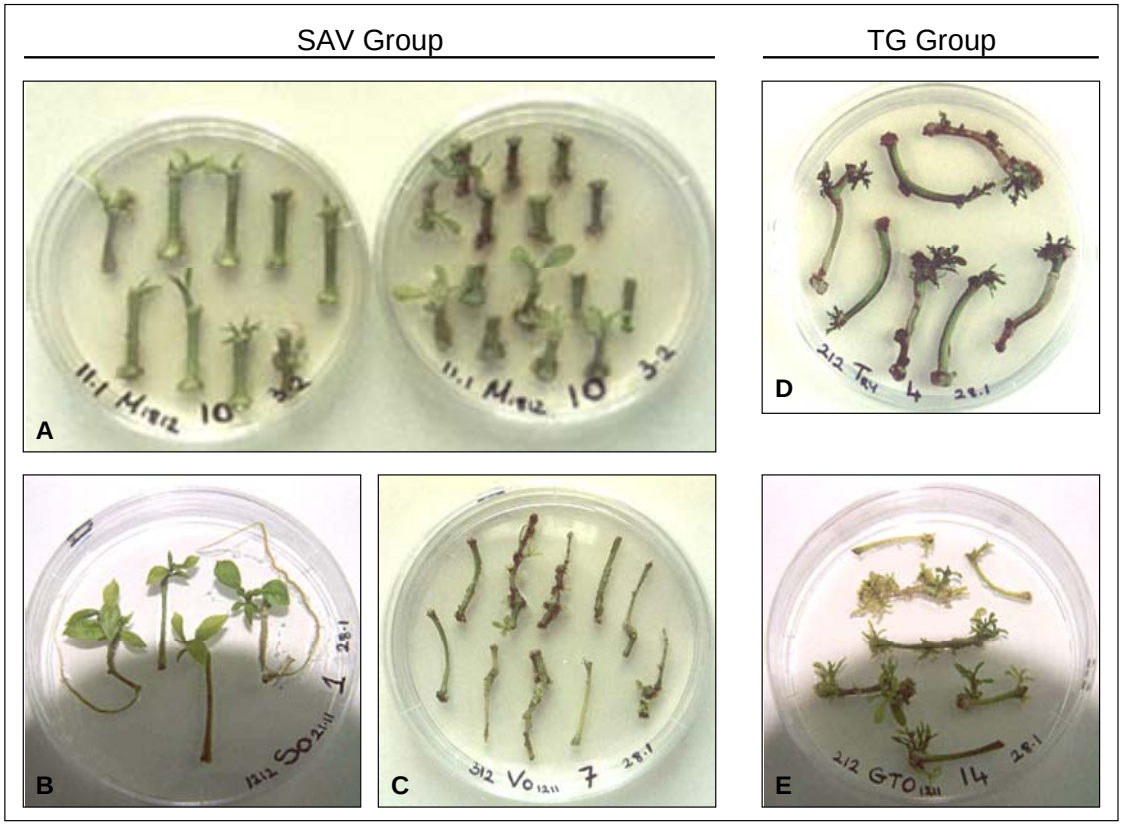

Figure 30. Transgenic citrus shoots, 3-6 weeks after transformation under high selection pressure. **A-C** Transgenic shoots produced from epicotyl segments of SAV group plants. (**A**) Alemow (**B**) Sour orange and (**C**) Volkamer lemon on selection medium with 200 µM kanamycin. **D-E** The TG group transgenic shoots produced from epicotyl segments of (**D**) Troyer citrange and (**E**) Gou Tou on selection medium with 300µM kanamycin.

4.4.5 Shoot tip grafting (STG)

It has been reported that relatively high concentrations of BA in the selective regeneration medium, although necessary for shooting, reduced the ability of regenerated shoots to root subsequently (Gutierrez *et al.,* 1997). For this reason, we routinely used the STG method as described by Pena *et al.* (1995b) and modified by Piestun *et al.* (1998) to obtain grafted transgenic plants. Using the STG method we could use relatively high concentration of PGR and consequently the transgenic plants developed faster (in 2 to 5 weeks) compared with the rooting method. Minor modifications of the STG method improved the rates of the grafting success to almost 100%. The main modifications included the use of larger shoots for grafting, the increase of sucrose concentration for germinating Try seeds and pretreatment of excised transgenic buds by placing them on a hormone free culture medium for 2-3 days prior to STG.

4.5 Transgenic citrus rootstocks

Dominguez *et al.* (2004) reported that the actual frequency of transformation is underestimated when it is based on the expression of marker genes and suggested that in many cases the transgenes are only expressed at low or null levels. Furthermore it was reported that regeneration under selective conditions limited the recovery of silenced lines, because more than 30% of the transgenic limes regenerated under non-selective conditions had all the transgenes silenced (Dominguez *et al.*, 2002c). Based on this reports we did not discard the transgenic citrus rootstocks prior to PCR or Southern based analysis. As a consequence, the protocol for the transformation of citrus rootstocks allowed us to obtain >300 transgenic plants with at least one copy of the cDNA integrated in plants genome (Table 8).

Most of the transgenic citrus plants obtained were morphologically similar to the wild type plants and displayed normal growth and development. The ready and stable folding of p23UI transcripts into dsRNA molecules, that should not be translated in to a protein product, possibly explains why p23UI citrus and *N. benthamiana* transgenic plants showed normal phenotypes, whereas p23 transgenic Mexican lime (*C. aurantifolia*) showed CTV-like symptoms (Ghorbel *et al.*, 2001; Fagoaga *et al.*, 2005). However, none of our p23U transgenic citrus and *N. benthamiana* plants showed the CTV-like symptoms. The reasons for these differences between our Alemow plants and Pena's groups Mexican lime plants remain unsolved. We may speculate that p23 along with its 3' UTR accumulates in different compartments/or specific type of cells or their accumulation did not reach a certain threshold necessary to incites CTV-like symptoms (Fagoaga *et al.*, 2005). It should be noted that some of transgenic shoots regardless of their genetic background were stunted or suffered an apparent die back-problem (results not shown) when grafted on wild type Vol plants. Although we interpreted this problem to unsuccessful grafting or/and wrong shoot stage, timing and maintenance, it may however be part of the CTV-like symptomatology.

4.6 Challenge inoculation experiments

It was previously suggested that the PDR strategies, using virus-encoded genes, may not provide virus resistance to trees such as citrus, since they only slow down the spread of the virus (Beachy, 1997; Gutierrez *et al.*, 1997). For several crops, transgenic resistance has developed against different viruses by introducing a variety of viral sequences by

genetic transformation. Many of the virus-resistant transgenic crops were developed by introducing either the viral CP or the replicase gene sequences.

The p23 gene functions as a suppressor of RNA silencing of CTV, and its conserved 3' UTR (p23U), p61 and an intron-spliced hairpin structure of p23U (p23UI) were used for producing transgenic *N. benthamiana* and citrus rootstocks. Hybridization of RNAs from these transgenic plants with riboprobes specific to CTV-p61 and to plus- and minus-strand p23U molecules showed that most of the transgenes contained the expected transcription product either as RNA or as dsRNA molecules (Figs. 8 and 16).

Irrespective of the mechanism, delivery of RNAs with the potential to form duplexes has been an important strategy for virus resistance and gene silencing in transgenic plants as pioneered by Waterhouse et al. (1998) with the protease (Pro) gene of potato virus Y (PVY) in transgenic tobacco plants. Effective PTGS-mediated resistance against CTV may be somewhat more problematic than it has been for *N. benthamiana* plants. However, effective PTGS-mediated resistance has been developed against some phloem-limited viruses such as *Barley yellow dwarf virus* (Wang et al. 2000), suggesting that phloem-limited viruses, as such, can be targeted by PTGS. Recently, Roy et al. (2006) reported similar results by using PVX based vector for challenge inoculation of transgenic *N. benthamiana* plants with CTV derived genes and showed that while p23, p20 and 3' UTR provided durable resistance, CP gene failed to show resistance to recombinant PVX vector.

All p23UI-transgenic *N. benthamiana* plants (n = 40) were highly resistant to local and systemic infections with GVA-p23U, whereas inoculation of these plants with GVA resulted in typical symptoms of GVA-infected wild-type plants (Fig. 19). These results

clearly established that the p23UI transcripts when folded into dsRNA (Fig. 8) were effectively protecting these plants against the p23U-harboring virus used for challenge inoculation, whereas p61 and p23U transgenes failed to provide protection against chimeric GVA vectors with corresponding CTV sequences (Fig. 18).

In contrast, when the transgenic p23UI Alemow plants (n = 70) were graft-inoculated with CTV, only nine of the 35 tested lines exhibited delayed appearance of symptoms. In 16 of the 35 challenged transgenic Alemow lines (p61Alm 6, 21, 51; p23UAlm 1, 4, 15, 50; p23UIAlm 3, 4, 8, 9, 15, 17, 27, 32, 35) symptom appearance started only at the second flush cycle, three or more months after inoculation. However, in only three of these lines symptom delay was observed among both sibling plants from the same bud-line (transgenic lines p23UIAlm 8, 9 and 17). Moreover, even these lines did not show durable resistance, as indicated by similar northern hybridization signals (Fig. 25) and ELISA readings for the transgenic and non-transgenic citrus plants (not shown). Thus, similarly to previous attempts to produce resistant transgenic Alemow with the CTV-derived sequences of CP and truncated and non-truncated RdRp constructs (Piestun *et al.,* 1998; Batuman *et al.,* unpublished), citrus plants transgenic for p61, p23U and p23UI also failed to exhibit durable protection against CTV infection.

CTV infection sometimes remains symptomless or expresses differently among citrus species. Since CTV leaf symptom evaluation in challenged transgenic and non-transgenic Gou Tou and Volkamer lemon rootstocks was difficult or impossible because of their healthy plant appearance, the dsRNA contents of these plants were regularly analyzed by northern blot hybridization (Figs. 23 and 24). Overall, levels of viral dsRNA accumulation varied over time between individual plants from both the non- and

transgenic lines (Figs. 23, 24 and 25). However the differences in signal intensities as revealed by northern hybridization were not correlated with the delay of symptom appearance in transgenic plants.

Both transgenic and non-transgenic citrus plants infected by CTV accumulated detectable levels of viral-specific siRNAs corresponding to different parts of the CTV genome indicating that CTV RNAs, irrespective of the presence or absence of viral-derived transgenes, is the target of PTGS reaction (Fig. 26). The amount of siRNAs in some of the p23UI transgenic Alemow lines with delayed symptoms was higher than those accumulated in other lines (Fig. 26E and F). However few transgenic plants showed a significant increase of viral-specific siRNAs compared with the non transgenic control plants (Fig. 26F). These results indicated that p23UI transgene altered accumulation of replicating virus to some extent and consequently resulted in lower levels of genomic dsRNAs (Fig. 25). It should be noted that although we could not detect siRNA in non inoculated p23UI transgenic citrus and *N. benthamiana* plants, precursor dsRNAs of the expected size were easily detected (Fig. 8C and D, Fig. 16C). These results differ from what was expected to be found if transgene dsRNA molecules would have been targets of PTGS mechanisms. Possibly the dsRNAs of the transgenic plants are folding structures that are inaccessible to DICER-like enzymes and hence protected from its endocatalytic processing.

Ghorbel *et al.* (2001) and Fagoaga *et al.* (2005, 2006) showed that most Mexican lime (*C. aurantifolia*) and Alemow plants expressing the p23 gene of CTV exhibited aberrations resembling CTV-induced leaf symptoms. A few of the transgenic lines that remained symptom-free were found to display characteristics expected for post-transcriptional gene

silencing (PTGS). These included multiple copies of the transgenes, low levels of corresponding mRNA, methylation of the silenced transgene, and accumulation of p23-specific small (interfering?) RNAs. Challenge-inoculation of these plants revealed an apparent immunity, as indicated by ELISA and hybridization assays. The immunity of some of these propagations was only temporary, and the plants subsequently showed attenuated symptoms. Interestingly, the symptom-exhibiting plants accumulated p23-specific siRNAs at significantly higher levels than the immune and the non-inoculated transgenes, which suggests that the virus replication machinery was able to override the p23-activated silencing machinery (Fagoaga *et al.,* 2006). It should, however, be noted that the number of resistant p23-transgenic Mexican lime lines was limited, and the resistance was inconsistent, when the challenge inoculation was made directly to the transgenic scions (Fagoaga *et al.,* 2006; Pena, L. 2005, personal communication). Our challenge inoculations were administered by grafting the inocula into the CTV-sensitive Vol rootstock, thus allowing a much strong inocula pressure toward the transgenic scions. Febres *et al.* (2003) reported that transformation of grapefruit plants with sequences of CTV-RdRp and CP failed to confer protection to CTV. These negative results were suggested to result of either: (1) the CP-transgenic plants did not confer resistance against the virus, or (2) the virus challenge was so great that it overwhelmed any resistance that might have been present, or (3) the virus did nor replicate in the transgenic material but was merely translocated from the rootstock.

4.6.1 Recovery of p61 transgenic Sour orange plants after SY infection

The term "recovery" implies that CTV infected plants that initially show strong seedling yellowing (SY) symptoms typical of the Mor-T isolate infection, but after several months, new shoots were produced that were almost symptom-free. This type of recovery was first observed by Wallace (1965) and later studied by Yang *et al.* (1999), who showed that in the VT isolate it is associated with a 4kb 5'-end RNAs. Interestingly, the VT "recovery" observed differed from the "recovery" described by Lindbo and Dougherty (1992a). Similarly in our study, recovered p61 transgenic SO plants show typical CTV symptoms, but the new leaves developed in subsequent flush cycles were symptomless (Fig. 27), and the level of virus accumulation was still low (Figs. 23 and 24) although higher than those from the SY-infected leaves (Fig. 27). When recovered leaves were graft-inoculated onto non-transgenic Alemow plants, normal levels of dsRNA were detected indicating that the recovered plants contained the virus. Savenkov and Valkonen (2002) reported that plants expressing HC-Pro cistron of Potato virus A (PVA) were initially susceptible to infection with the homologous virus but later exhibited strong silencing of both the transgene and the homologous virus, resulting in a recovery from infection. Recovery was dependent on sequence similarity between the virus and the transgene.

Our results are consistent with the hypothesis that a threshold model of RNA silencing, which predicts that the concentration of silencing target RNA is reduced to just below a threshold level in leaves where silencing has been initiated (Meins, 2000). It is interesting to note that in the recovered leaves dsRNAs accumulated to higher levels than in the non-recovered Sour orange leaves. However, the level was lower than in the sensitive

Alemow plants (Fig. 27C). These findings suggest a unique mechanism of recovery associated with the hypersensitive reaction of Sour orange plants (Yang *et al.,* 1999; Bar-Joseph *et al.,* 2000) to SY-inducing CTV isolates.

4.7 Sequence analysis of CTV from recovered and authentic strains

It is not known whether the symptoms induced by a CTV isolate in citrus plants are caused by the predominant genomic sequence, the viral population, a combination of genomic RNAs and defective (d)RNAs, or other factors. To examine whether different isolates inducing similar phenotypes might also have similar sequences, the complete sequence of the two recovered (RA and RB) and two Mor-T CTV isolates were determined and compared with the sequence of the Israeli CTV isolate VT.

The dsRNAs from Alemow citrus inoculated with the parent strain (Mor-T) and the recovered isolate (Mor-T/R) were used for cDNA synthesis, cloning into pGEM-T Easy Vector (Promega) system and sequencing.

Analysis of complete nucleotide sequences of isolates did not reveal major differences through out the genome (data not shown). Moreover, the detailed analyses of p23 and p61 sequences showed high nucleotide identities (99.6 % and 99.7 %, respectively) and inconsistent nucleotide mutations among the isolates (Figs. 28 and 29). The absence of proofreading in the viral RNA polymerases (Drake and Holland, 1999) is thought to generate random mutations thus mutant genomes making up the viral quasispecies distribution (Domingo *et al.,* 1995). Pairwise comparisons between independent cDNA sequences from the VT population provided an estimate of 0.5% nucleotide variability (data not shown). This degree of nucleotide variability was supported by similar

measurements within other CTV populations (Ayllon *et al.,* 1999) and between CTV dRNAs and their helper (Mawassi *et al.,* 1995).

Elucidating the exact change(s) that was or were involved in the alteration of CTV-Mor-T pathogenicity is a lengthy and complicated task for a large single stranded (ss) viral RNA such as CTV and was not dealt within the scope and time frame of this thesis.

5. CONCLUSIONS

The present study was aimed to test the effectiveness of the CTV-p61, -p23U and -p23UI genes to confer resistance against natural CTV infection of citrus plants. The concept to generate of transgenic citrus rootstocks rather than edible varieties was aimed to prevent consumer rejection of the expected transformed products. Our experiments followed preliminary testing of transgenic *N. benthamiana* for evaluating PTGS-based resistance against CTV derived-gene products. The *N. benthamiana* plants with the p23UI transgene displayed considerable resistance to CTV-p23U delivered by the chimeric GVA vector.

Among the five plants selected for transformation, two citrus rootstocks Sour orange and Alemow were highly CTV sensitive. Obtaining transgenic CTV resistance could have considerably benefited citrus growers, as most of the immune and tolerant rootstocks suffer of varying types of inadequacies, such as inadaptability to the calcareous soils prevailing in the Negev area of this country.

We developed improved transformation protocols for citrus rootstocks, cloned several CTV genes and inserted them into the citrus genomes and showed that a high concentration of kanamycin can be used as an effective selective agent in the transformation of citrus. We demonstrated that there are differences in the morphogenic potential along the epicotyl axis of citrus rootstocks, and that this gradient may be influenced by factors related to genotype, age, composition of the culture medium and incubation conditions. The cotyledonary regions of the epicotyls produced the greatest *in vitro* responses, and these can be used in future genetic transformation experiments for citrus improvement. Reducing residual hormone levels from transgenic shoots resulted in

increased STG success. Grafting *in-vitro*-grown transgenic shoots on vigorous rootstocks allowed the rapid acclimatization and development of plants under greenhouse conditions. We observed different a variety of symptom expressions among different transgenic lines when challenge-inoculated with severe VT strain Mor-T isolate. These were categorized as plants with: a) severe symptoms like non-transgenic plants b) short and/or long delay on symptom onset, c) recovery and d) non-visible symptoms. None of the CTV-derived sequences used in this study resulted in transgenic lines that could be considered as providing durable resistance. These results were consistent with recent reports of PDR attempts to obtain CTV resistance (Table 1), with the exception of Fagoaga *et al.* (2006) which reported inconsistent resistance.

The question thus remains: why dsRNAs that conferred resistance in *N. benthamiana* did not protect citrus plants challenged with CTV? Since we used different transgenic hosts and different virus systems for challenge inoculation, one of these differences should be a factor in causing these contrasting results. PDR was reported recently for transgenic citrus plants expressing the *Citrus psorosis virus* coat protein gene (Kayim *et al.*, 2005), therefore the hypothesis that PDR was not effective in the citrus host seems to be implausible, and the reason probably lies in some unique features of CTV.

Although CTV and GVA are plus-strand RNA viruses that preferentially infect phloem cells in their respective hosts, they differ considerably in their replication strategies and genetic contents (Rosciglione *et al.*, 1983; Karasev, 2000; Martelli *et al.*, 2002; Adams *et al.*, 2004). At least three distinct genes of the large CTV genome function as suppressors of the RNA-silencing antiviral defense (Lu *et al.,* 2004). These genes are the intracellular suppressor p23, the intercellular suppressor p25, and the p20, which inhibits the silencing

at both levels. Could the finding of three suppressors explain why transgenes harboring only the CP or the p23 sequences elicited only a temporary delay and did not provide resistance against the invasion of CTV virions harboring all three counter-defense genes? If so do these results indicate that transgenes aimed to disarm the sophisticated CTV counter-defense strategy should not be monogenic, but should include all three suppressor sequences? Would their transcription as a single molecule be sufficient to confer resistance or is it necessary that these genes act in concert or sequentially? The RNAi suppression of *Red clover necrotic mosaic virus* (RCNMV) recently was shown (Takeda *et al.,* 2005) to result from the concerted action of both the viral RNAs and the putative replicase proteins. Could it be that CTV also mobilizes some of its multiple RNA species in suppression of silencing? Such a mechanism could provide a possible clue to one of the present CTV enigmas: why the replicating mild CTV isolates offer PDR, as expressed by cross protection (Costa and Muller 1980; Vanvuuren et al. 1993), whereas transgenic plants harboring only parts of the viruses have so far not proved able to provide a durable protection against challenge inoculations.

The molecular basis of transgenic PDR to provide durable resistance against CTV and also other Closteroviridae (B. Falk, 2006; J. Valkonen, 2006; A. Gal-On and A. Zelcer 2006; personal communications) remains to be elucidated.

6. REFERENCES

Adams, M. J., J. F. Antoniw, M. Bar-Joseph, A. A. Brunt, T. Candresse, G. D. Foster, G. P. Martelli, R. G. Milne and C. M. Fauquet (2004). "The new plant virus family flexiviridae and assessment of molecular criteria for species demarcation." Archives of Virology 149: 1045-1060.

Agranovsky, A. A. (1996). "Principles of molecular organization, expression, and evolution of closteroviruses: Over the barriers." Advances in Virus Research, Vol 47 47: 119-158.

Albiach-Marti, M., J. W. Grosser, M. Hilf, S. Gowda, M. Mawassi, T. Satyanarayana, S. Garnsey and W. O. Dawson (1999). "CTV resistant plants are not immune at the cellular level." Abstract of the 18th Annual Meeting of the American Society for Virology University of Massachusetts, Amherst, Mass, July 10-14, 1999, pp 192.

Albiach-Marti, M. R., J. W. Grosser, S. Gowda, M. Mawassi, T. Satyanarayana, S. M. Garnsey and W. O. Dawson (2004). "Citrus tristeza virus replicates and forms infectious virions in protoplasts of resistant citrus relatives." Molecular Breeding 14: 117-128.

Almeida, W. A. B., F. A. A. Mourao, L. E. Pino, R. L. Boscariol, A. P. M. Rodriguez and B. M. J. Mendes (2003). "Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck." Plant Science 164: 203-211.

Alzhanova, D. V., Y. Hagiwara, V. V. Peremyslov and V. V. Dolja (2000). "Genetic analysis of the cell-to-cell movement of beet yellows closterovirus." Virology 268: 192-200.

Anandalakshmi, R., G. J. Pruss, X. Ge, R. Marathe, A. C. Mallory, T. H. Smith and V. B. Vance (1998). "A viral suppressor of gene silencing in plants." Proceedings of the National Academy of Sciences of the United States of America 95: 13079-13084.

Anandalakshmi, R., R. Marathe, X. Ge, J. M. Herr, C. Mau, A. Mallory, G. Pruss, L. Bowman and V. B. Vance (2000). "A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants." Science 290: 142-144.

Angell, S. M. and D. C. Baulcombe (1999). "Technical advance: Potato Virus X amplicon-mediated silencing of nuclear genes." Plant J 20: 357-62.

Atkins, D., M. Young, S. Uzzell, L. Kelly, J. Fillatti and W. L. Gerlach (1995). "The expression of antisense and ribozyme genes targeting citrus exocortis viroid in transgenic plants." Journal of General Virology 76: 1781-1790.

Ayllon, M. A., C. Lopez, J. Navas-Castillo, M. Mawassi, W. O. Dawson, J. Guerri, R. Flores and P. Moreno (1999). "New defective RNAs from citrus tristeza virus: Evidence for a replicase-driven template switching mechanism in their generation." J Gen Virol 80: 817-21.

Ayllon, M. A., S. Gowda, T. Satyanarayana, A. V. Karasev, S. Adkins, M. Mawassi, J. Guerri, P. Moreno and W. O. Dawson (2003). "Effects of modification of the transcription initiation site context on citrus tristeza virus subgenomic RNA synthesis." J Virol 77: 9232-43.

Ayllon, M. A., L. Rubio, V. Sentandreu, A. Moya, J. Guerri and P. Moreno (2006). "Variations in two gene sequences of citrus tristeza virus after host passage." Virus Genes 32: 119-128.

Bar-Joseph, M. and G. Loebenstein (1973). "Effects of strain, source plant, and temperature on the transmissibility of citrus tristeza virus by the melon aphid." Phytopathology 63: 716-720.

Bar-Joseph, M., S. M. Garnsey and D. Gonsalves (1979). "The closteroviruses: A distinct group of elongated plant viruses." Adv Virus Res 25: 93-168.

Bar-Joseph, M. and R. F. Lee (1989). "Citrus tristeza virus." *CMI/AAB Descriptions of Plant Viruses* No 353 Wellesbourne, Warwick, U K.

Bar-Joseph, M., R. Marcus and R. F. Lee (1989). "The continuous challenge of citrus tristeza virus control." Annual Review of Phytopathology 27: 291-316.

Bar-Joseph, M., Che, X., Piestun, D., Batuman, O., Gofman, R., Ben Shalom, Y., Yang, G., and Mawassi, M., 2000. Citrus Tristeza Virus Biology Revisited: Quick Decline and Seedling Yellows-The Cost of Sour Orange Resistance Gene(s). Proc. Intl. Soc. Citricult. IX Congr. 2000. 963-965

Batuman, O., X. Che, Y. Moskowits, O. Cohen, M. Mawassi and M. Bar-Joseph (2004). "Variation in composition and biology of *citrus tristeza virus* defective RNAs." APS Annual Meeting July 31-August 4, 2004, Anaheim, California.

Batuman, O., M. Mawassi and M. Bar-Joseph (2006). "Transgenes consisting of a dsRNA of an RNAi suppressor plus the 3' UTR provide resistance to citrus tristeza virus sequences in *Nicotiana benthamiana* but not in citrus." Virus Genes 33: 319-327.

Baulcombe, D. C. (1996). "Mechanisms of pathogen-derived resistance to viruses in transgenic plants." Plant Cell 8: 1833-1844.

Beachy, R. N. (1997). "Mechanisms and applications of pathogen-derived resistance in transgenic plants." *Curr Opin Biotechnol*: 8:215-20.

Ben-Zeev, I. S., M. Barjoseph, Y. Nitzan and R. Marcus (1989). "A severe citrus tristeza virus isolate causing the collapse of trees of sour orange before virus is detectable throughout the canopy." Annals of Applied Biology 114: 293-300.

Bernstein, E., A. A. Caudy, S. M. Hammond and G. J. Hannon (2001). "Role for a bidentate ribonuclease in the initiation step of RNA interference." Nature 409: 363-366.

Bond, J. E. and M. L. Roose (1998). "*Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington Navel orange." Plant Cell Reports 18: 229-234.

Boutet, S., F. Vazquez, J. Liu, C. Beclin, M. Fagard, A. Gratias, J. B. Morel, P. Crete, X. M. Chen and H. Vaucheret (2003). "*Arabidopsis* hen1: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance." Current Biology 13: 843-848.

Brigneti, G., O. Voinnet, W. X. Li, L. H. Ji, S. W. Ding and D. C. Baulcombe (1998). "Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*." Embo J 17: 6739-46.

Broadbent, P., R. H. Brlansky and J. Indsto (1996). "Biological characterization of Australian isolates of citrus tristeza virus and separation of subisolates by single aphid transmissions." Plant Disease 80: 329-333.

Burger, D. W. and W. P. Hackett (1986). "Gradients of adventitious bud formation on excised epicotyl and root sections of citrus." Plant Science 43: 229-232.

Cervera, M., J. Juarez, A. Navarro, J. A. Pina, N. Duran-Vila, L. Navarro and L. Pena (1998a). "Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage." Transgenic Research 7: 51-59.

Cervera, M., J. A. Pina, J. Juarez, L. Navarro and L. Pena (1998b). "*Agrobacterium*-mediated transformation of citrange: Factors affecting transformation and regeneration." Plant Cell Reports 18: 271-278.

Che, X., D. Piestun, M. Mawassi, G. Yang, T. Satyanarayana, S. Gowda, W. O. Dawson and M. Bar-Joseph (2001). "5'-coterminal subgenomic RNAs in citrus tristeza virus-infected cells." Virology 283: 374-81.

Che, X., M. Mawassi and M. Bar-Joseph (2002). "A novel class of large and infectious defective RNAs of citrus tristeza virus." Virology 298: 133-45.

Che, X., W. O. Dawson and M. Bar-Joseph (2003). "Defective RNAs of citrus tristeza virus analogous to crinivirus genomic RNAs." Virology 310: 298-309.

Costa, A. S. and G. W. Muller (1980). "Tristeza controlled by cross protection, a U.S.-Brazil cooperative success." *Plant Dis* 64: 538-41.

Costa, M. G. C., W. C. Otoni and G. A. Moore (2002). "An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradisi* (macf.) and production of transgenic plants containing carotenoid biosynthetic genes." Plant Cell Reports 21: 365-373.

Costa, M. G. C., V. S. Alves, E. R. G. Lani, P. R. Mosquim, C. R. Carvalho and W. C. Otoni (2004). "Morphogenic gradients of adventitious bud and shoot regeneration in epicotyl explants of citrus." Scientia Horticulturae 100: 63-74.

Dalmay, T., A. Hamilton, S. Rudd, S. Angell and D. C. Baulcombe (2000). "An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus." Cell 101: 543-553.

Dalmay, T., R. Horsefield, T. H. Braunstein and D. C. Baulcombe (2001). "Sde3 encodes an RNA helicase required for posttranscriptional gene silencing in *Arabidopsis*." Embo Journal 20: 2069-2077.

Dasgupta, I., V. G. Malathi and S. K. Mukherjee (2003). "Genetic engineering for virus resistance." Current Science 84:341-354.

De Feyter, R., M. Young, K. Schroeder, E. S. Dennis and W. Gerlach (1996). "A ribozyme gene and an antisense gene are equally effective in conferring resistance to tobacco mosaic virus on transgenic tobacco." Molecular & General Genetics 250: 329-338.

Deng, Z., S. Huang, S. Xiao and F. G. Gmitter (1996). "Development and characterization of scar markers linked to the citrus tristeza virus resistance gene from *Poncirus trifoliata*." Genome 40:697-704.

Deng, Z., Q. Tao, Y. L. Chang, S. Huang, P. Ling, C. Yu, C. Chen, F. G. Gmitter and H. B. Zhang (2001). "Construction of a bacterial artificial chromosome (BAC) library for citrus and identification of BAC contigs containing resistance gene candidates." Theoretical and Applied Genetics 102: 1177-1184.

Dodds, J. A. and M. Bar-Joseph (1983). "Double-stranded RNA from plants infected with closteroviruses." *Phytopathology*: 73:419-23.

Dolja, V. V., A. V. Karasev and E. V. Koonin (1994). "Molecular-biology and evolution of closteroviruses - sophisticated buildup of large RNA genomes." Annual Review of Phytopathology 32: 261-285.

Dominguez, A., J. Guerri, M. Cambra, L. Navarro, P. Moreno and L. Pena (2000). "Efficient production of transgenic citrus plants expressing the coat protein gene of citrus tristeza virus." Plant Cell Reports 19: 427-433.

Dominguez, A., A. H. de Mendoza, J. Guerri, M. Cambra, L. Navarro, P. Moreno and L. Pena (2002a). "Pathogen-derived resistance to citrus tristeza virus (CTV) in transgenic Mexican lime (*Citrus aurantifolia* (Christ.) Swing.) plants expressing its p25 coat protein gene." Molecular Breeding 10: 1-10.

Dominguez, A., C. Fagoaga, L. Navarro, P. Moreno and L. Pena (2002b). Constitutive expression of untranslatable versions of the p25 coat protein gene in Mexican lime does not confer resistance to citrus tristeza virus (CTV). Proc. Conf. Int. Organ Citrus Virol. 15th, IOCV, Riverside, CA.

Dominguez, A., C. Fagoaga, L. Navarro, P. Moreno and L. Pena (2002c). "Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes." Mol Genet Genomics 267: 544-56.

Dominguez, A., M. Cervera, R. M. Perez, J. Romero, C. Fagoaga, J. Cubero, M. M. Lopez, J. A. Juarez, L. Navarro and L. Pena (2004). "Characterization of regenerants obtained under selective conditions after *Agrobacterium*-mediated transformation of citrus explants reveals production of silenced and chimeric plants at unexpected high frequencies." Molecular Breeding 14: 171-183.

Du, T. T. and P. D. Zamore (2005). "Microprimer: The biogenesis and function of microRNA." Development 132: 4645-4652.

EPPO/CABI (1996). *Toxoptera citricidus*. In: Quarantine pests for Europe. 2nd edition (Ed. by Smith, I.M.; McNamara, D.G.; Scott, P.R.; Holderness, M.). CAB INTERNATIONAL, Wallingford, UK.

Fagoaga, C., C. Lopez, P. Moreno, L. Navarro, R. Flores and L. Pena (2005). "Viral-like symptoms induced by the ectopic expression of the p23 gene of citrus tristeza virus are citrus specific and do not correlate with the pathogenicity of the virus strain." Mol Plant Microbe Interact 18: 435-45.

Fagoaga, C., C. Lopez, A. H. de Mendoza, P. Moreno, L. Navarro, R. Flores and L. Pena (2006). "Post-transcriptional gene silencing of the p23 silencing suppressor of citrus tristeza virus confers resistance to the virus in transgenic Mexican lime." Plant Mol Biol 60: 153-65.

Febres, V. J., L. Ashoulin, M. Mawassi, A. Frank, M. BarJoseph, K. L. Manjunath, R. F. Lee and C. L. Niblett (1996). "The p27 protein is present at one end of citrus tristeza virus particles." Phytopathology 86: 1331-1335.

Febres, V. J., C. L. Niblett, R. F. Lee and G. A. Moore (2003). "Characterization of grapefruit plants (*Citrus paradisi* Macf.) transformed with citrus tristeza closterovirus genes." Plant Cell Rep 21: 421-8.

Galiakparov, N., D. E. Goszczynski, X. Che, O. Batuman, M. Bar-Joseph and M. Mawassi (2003). "Two classes of subgenomic RNA of *Grapevine Virus A* produced by internal controller elements." Virology 312: 434-48.

Gamborg, O., R. Miller and K. Ojima (1968). "Nutrient requirements of suspension cultures of soybean root cells." Exp Cell Res 50: 151-158.

Garcia-Luis, A., Y. Bordon, J. M. Moreira-Dias, R. V. Molina and J. L. Guardiola (1999). "Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange." Annals of Botany 84: 715-723.

Garnsey, S. M., D. Gonsalves and D. E. Purcifull (1977). "Mechanical transmission of citrus tristeza virus." Phytopathology 67:965-968.

Gelvin, S. B. and C. N. Liu (1994). Genetic manipulation of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant plant species. Dordrecht, The Netherlands, Kluwer Academic Publishers.

Gelvin, S. B. (2000). "*Agrobacterium* and plant genes involved in T-DNA transfer and integration." Annu Rev Plant Physiol Plant Mol Biol 51: 223-256.

Gelvin, S. B. (2005). "Agricultural biotechnology: Gene exchange by design." Nature 433: 583-4.

Ghorbel, R., J. Juarez, L. Navarro and L. Pena (1999). "Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants." Theoretical and Applied Genetics 99: 350-358.

Ghorbel, R., A. Dominguez, L. Navarro and L. Penna (2000). "High efficiency genetic transformation of Sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus." Tree Physiol 20: 1183-1189.

Ghorbel, R., C. Lopez, C. Fagoaga, P. Moreno, L. Navarro, R. Flores and L. Pena (2001). "Transgenic citrus plants expressing the citrus tristeza virus p23 protein exhibit viral-like symptoms." *Mol Plant Pathol* 2:27-36.

Gmitter, F. G., J. Grosser and G. Moore (1992). "Citrus." In: Biotechnology of perennial fruit and nut crops (Hamerschlag, F, and Litz, R E, eds) pp 550 CABI Wallingford, Oxon.

Gmitter, F. G., S. Y. Xiao, S. Huang, X. L. Hu, S. M. Garnsey and Z. Deng (1996). "A localized linkage map of the citrus tristeza virus resistance gene region." Theoretical and Applied Genetics 92: 688-695.

Goh, C. J., G. E. Sim, C. L. Morales and C. S. Loh (1995). "Plantlet regeneration through different morphogenic pathways in Pommelo tissue culture." Plant Cell Tissue and Organ Culture 43: 301-303.

Golemboski, D. B., G. P. Lomonossoff and M. Zaitlin (1990). "Plants transformed with a tobacco mosaic virus non-structural gene sequence are resistant to the virus." Proceedings of the National Academy of Sciences of the United States of America 87: 6311-6315.

Gowda, S., T. Satyanarayana, C. L. Davis, J. Navas-Castillo, M. R. Albiach-Marti, M. Mawassi, N. Valkov, M. Bar-Joseph, P. Moreno and W. O. Dawson (2000). "The p20 gene product of citrus tristeza virus accumulates in the amorphous inclusion bodies." Virology 274: 246-254.

Gowda, S., T. Satyanarayana, M. A. Ayllon, M. R. Albiach-Marti, M. Mawassi, S. Rabindran, S. M. Garnsey and W. O. Dawson (2001). "Characterization of the *cis*-acting elements controlling subgenomic mRNAs of citrus tristeza virus:

Production of positive- and negative-stranded 3'-terminal and positive-stranded 5'-terminal RNAs." Virology 286: 134-51.

Gowda, S., M. A. Ayllon, T. Satyanarayana, M. Bar-Joseph and W. O. Dawson (2003). "Transcription strategy in a closterovirus: A novel 5'-proximal controller element of citrus tristeza virus produces 5'- and 3'-terminal subgenomic RNAs and differs from 3' open reading frame controller elements." J Virol 77: 340-52.

Grant, T. J. and R. P. Higgins (1957). "Occurrence of mixtures of tristeza virus strains in citrus." Phytopathology 47:272-76.

Gutierrez, E. M. A., D. Luth and G. A. Moore (1997). "Factors affecting *Agrobacterium*-mediated transformation in citrus and production of Sour orange (*Citrus aurantium*) plants expressing the coat protein gene of citrus tristeza virus." Plant Cell Reports 16: 745-753.

Haley, B. and P. D. Zamore (2004). "Kinetic analysis of the RNAi enzyme complex." Nature Structural & Molecular Biology 11: 599-606.

Hamilton, A. J. and D. C. Baulcombe (1999). "A species of small antisense RNA in posttranscriptional gene silencing in plants." Science 286: 950-952.

Hammond, S. M., A. A. Caudy and G. J. Hannon (2001). "Post-transcriptional gene silencing by double-stranded RNA." Nature Reviews Genetics 2: 110-119.

Hannon, G. J. (2002). "RNA interference." Nature 418: 244-251.

Hartitz, M. D., G. Sunter and D. M. Bisaro (1999). "The tomato golden mosaic virus transactivator (TRAP) is a single-stranded DNA and zinc-binding phosphoprotein with an acidic activation domain." Virology 263: 1-14.

Haviv, S., Galiakparov, N., Goszczynski, D. E., Batuman, O., Czosnek, H. and M. Mawassi (2006). "Engineering the genome of Grapevine virus A into a vector for expression of proteins in herbaceous plants." Journal of Virological Methods 132: 227-231.

Hidaka, T., M. Omura, M. Ugaki and M. Tomiyama (1990). "*Agrobacterium*-mediated transformation and regeneration of *Citrus spp* from suspension cells." Japanese Journal of Breeding 40: 199-207.

Hilf, M. E., A. V. Karasev, H. R. Pappu, D. J. Gumpf, C. L. Niblett and S. M. Garnsey (1995). "Characterization of citrus tristeza virus subgenomic RNAs in infected tissue." Virology 208: 576-82.

Hood, E. E., S. B. Gelvin, L. S. Melchers and A. Hoekema (1993). "New *Agrobacterium* helper plasmids for gene transfer to plants." *Transgenic Res* 2:208-218.

Horsch, R. G., J. E. Fry, N. L. Hoffman, D. A. Eicholtz, S. G. Rogers and R. T. Fraley (1985). "A simple and general method for transferring genes into plants." *Science* 227:1229-1231.

Hutvagner, G. and P. D. Zamore (2002). "A microRNA in a multiple-turnover RNAi enzyme complex." Science 297: 2056-2060.

Jan, F. J., S. Z. Pang, D. M. Tricoli and D. Gonsalves (2000). "Evidence that resistance in squash mosaic comovirus coat protein-transgenic plants is affected by plant developmental stage and enhanced by combination of transgenes from different lines." Journal of General Virology 81: 2299-2306.

Jefferson, R. A. (1987). "Assaying chimeric genes in plants: The GUS gene fusion system." *Plant Mol Biol Rep* 5: 387-405.

Kaneyoshi, J., S. Kobayashi, Y. Nakamura, N. Shigemoto and Y. Doi (1994). "A simple and efficient gene-transfer system of Trifoliate orange (*Poncirus-trifoliata* raf)." Plant Cell Reports 13: 541-545.

Karasev, A. V., V. P. Boyko, S. Gowda, O. V. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. Cline, D. J. Gumpf, R. F. Lee and et al. (1995). "Complete sequence of the citrus tristeza virus RNA genome." Virology 208: 511-20.

Karasev, A. V. and M. E. Hilf (1997). "Molecular biology of the citrus tristeza virus." *In* "Filamentous Viruses of Woody Plants" (Monette, P L, Eds) Trivandrum, India: Research Signpost.

Karasev, A. V. (2000). "Genetic diversity and evolution of closteroviruses." Annual Review of Phytopathology 38: 293-324.

Kasschau, K. D. and J. C. Carrington (1998). "A counter defensive strategy of plant viruses: Suppression of posttranscriptional gene silencing." Cell 95: 461-470.

Kasschau, K. D., Z. X. Xie, E. Allen, C. Llave, E. J. Chapman, K. A. Krizan and J. C. Carrington (2003). "P1/Hc-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function." Developmental Cell 4: 205-217.

Kayim, M., G. A. Barthe, J. M. G. Beretta and K. S. Derrick (2005). Transgenic citrus plants expressing the coat protein gene of citrus psorosis virus. Plant & Animal Genomes XIII Conference January 15-19, Town & Country Convention Center, San Diego, CA.

Ketting, R. F., T. H. A. Haverkamp, H. G. A. M. van Luenen and R. H. A. Plasterk (1999). "Mut-7 of *C. elegans*, required for transposon silencing and RNA

interference, is a homolog of Werner syndrome helicase and RNaseD." Cell 99: 133-141.

Kitajima, E. W., D. M. Silva, A. R. Oliveira, G. W. Mueller and A. S. Costa (1964). "Thread-like particles associated with tristeza disease of citrus." Nature 201: 1011-2.

Kjemtrup, S., K. S. Sampson, C. G. Peele, L. V. Nguyen, M. A. Conkling, W. F. Thompson and D. Robertson (1998). "Gene silencing from plant DNA carried by a geminivirus." Plant Journal 14: 91-100.

Kobayashi, S. and H. Uchimiya (1989). "Expression and integration of a foreign gene in orange (*Citrus sinensis* Osb.) protoplasts by direct DNA transfer." Jap J Genet 64:91-97.

Kollar, A., T. Dalmay and J. Burgyan (1993). "Defective interfering RNA-mediated resistance against cymbidium ringspot tombusvirus in transgenic plants." Virology 193: 313-8.

Lakatos, L., G. Szittya, D. Silhavy and J. Burgyan (2004). "Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses." Embo Journal 23: 876-884.

Lamb, J. W. and R. T. Hay (1990). "Ribozymes that cleave potato leaf roll virus-RNA within the coat protein and polymerase genes." Journal of General Virology 71: 2257-2264.

Lapidot, M., R. Gafny, B. Ding, S. Wolf, W. J. Lucas and R. N. Beachy (1993). "A dysfunctional movement protein of tobacco mosaic-virus that partially modifies

the plasmodesmata and limits virus spread in transgenic plants." Plant Journal 4: 959-970.

Lindbo, J. A. and W. G. Dougherty (1992a). "Pathogen-derived resistance to a potyvirus - immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide-sequence." Molecular Plant-Microbe Interactions 5: 144-153.

Lindbo, J. A. and W. G. Dougherty (1992b). "Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus-replication in transgenic plants and protoplasts." Virology 189: 725-733.

Lindbo, J. A., L. Silvarosales, W. M. Proebsting and W. G. Dougherty (1993). "Induction of a highly specific antiviral state in transgenic plants - implications for regulation of gene-expression and virus-resistance." Plant Cell 5: 1749-1759.

Lindbo, J. A. and W. G. Dougherty (2005). "Plant pathology and RNAi: A Brief History" *Annu Rev Phytopathol* 43: 191-204

Lomonossoff, G. P. (1995). “Pathogen-Derived Resistance to Plant Viruses.” *Annu Rev Phytopathol* 33: 323-343.

Lopez, C., J. Navas-Castillo, S. Gowda, P. Moreno and R. Flores (2000). "The 23-kda protein coded by the 3'-terminal gene of citrus tristeza virus is an RNA-binding protein." Virology 269: 462-70.

Lu, R., A. Folimonov, M. Shintaku, W. X. Li, B. W. Falk, W. O. Dawson and S. W. Ding (2004). "Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome." Proceedings of the National Academy of Sciences of the United States of America 101: 15742-15747.

Luth, D. and G. Moore (1999). "Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation." Plant Cell Tissue and Organ Culture 57: 219-222.

MacDiarmid, R. (2005). "RNA silencing in productive virus infections." Annual Review of Phytopathology 43: 523-544.

Maggon, R. and B. D. Singh (1995). "Promotion of adventitious bud regeneration by ABA in combination with BAP in epicotyl and hypocotyl explants of Sweet orange (*Citrus sinensis* L. Osbeck)." Scientia Horticulturae 63: 123-128.

Malyshenko, S. I., O. A. Kondakova, J. V. Nazarova, I. B. Kaplan, M. E. Taliansky and J. G. Atabekov (1993). "Reduction of tobacco mosaic virus accumulation in transgenic plants producing nonfunctional viral transport proteins." Journal of General Virology 74: 1149-1156.

Martelli, G. P., A. A. Agranovsky, M. Bar-Joseph, D. Boscia, T. Candresse, R. H. Coutts, V. V. Dolja, B. W. Falk, D. Gonsalves, W. Jelkmann, A. V. Karasev, A. Minafra, S. Namba, H. J. Vetten, G. C. Wisler and N. Yoshikawa (2002). "The family closteroviridae revised." Arch Virol 147: 2039-44.

Matzke, M. A. and A. J. M. Matzke (2004). "Planting the seeds of a new paradigm." Plos Biology 2: 582-586.

Mawassi, M., R. Gafny and M. Bar-Joseph (1993). "Nucleotide sequence of the coat protein gene of citrus tristeza virus: Comparison of biologically diverse isolates collected in Israel." Virus Genes 7: 265-75.

Mawassi, M., R. Gafny, D. Gagliardi and M. Bar-Joseph (1995a). "Populations of citrus tristeza virus contain smaller-than-full-length particles which encapsidate sub-genomic RNA molecules." J Gen Virol 76 (Pt 3): 651-9.

Mawassi, M., A. V. Karasev, E. Mietkiewska, R. Gafny, R. F. Lee, W. O. Dawson and M. Bar-Joseph (1995b). "Defective RNA molecules associated with citrus tristeza virus." Virology 208: 383-7.

Mawassi, M., E. Mietkiewska, R. Gofman, G. Yang and M. Bar-Joseph (1996). "Unusual sequence relationships between two isolates of citrus tristeza virus." J Gen Virol 77 (Pt 9): 2359-64.

Meins, F., Jr. (2000). "RNA degradation and models for post-transcriptional gene-silencing." Plant Mol Biol 43: 261-73.

Meneghini, M. (1946). "Sobre a naturaleza e transmissibilidade de doenca "tristeza" dos citrus." O Biologico 12:285-287.

Mette, M. F., W. Aufsatz, J. van der Winden, M. A. Matzke and A. J. M. Matzke (2000). "Transcriptional silencing and promoter methylation triggered by double-stranded RNA." Embo Journal 19: 5194-5201.

Moissiard, G. and O. Voinnet (2004). "Viral suppression of RNA silencing in plants." Molecular Plant Pathology 5: 71-82.

Moore, G. A., C. C. Jacono, J. L. Neidigh, S. D. Lawrence and K. Cline (1992). "*Agrobacterium*-mediated transformation of citrus stem segments and regeneration of transgenic plants." Plant Cell Reports 11: 238-242.

Moore, G. A., J. W. Grosser and F. G. J. Gmitter (2004). "*Rutaceae*." In: Biotechnology of perennial fruit and nut crops (Litz, R E, ed), pp550 CABI, Wallingford, Oxon.

Moreira-Dias, J. M., R. V. Molina, J. L. Guardiola and A. Garcia-Luis (2001). "Day-length and photon flux density influence the growth regulator effects on morphogenesis in epicotyl segments of Troyer citrange." Scientia Horticulturae 87: 275-290.

Morel, J. B., C. Godon, P. Mourrain, C. Beclin, S. Boutet, F. Feuerbach, F. Proux and H. Vaucheret (2002). "Fertile hypomorphic argonaute (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance." Plant Cell 14: 629-639.

Moreno, P., J. Guerri, J. F. Ballesterolmos, R. Albiach and M. E. Martinez (1993). "Separation and interference of strains from a citrus tristeza virus isolate evidenced by biological-activity and double stranded RNA (dsRNA) analysis." Plant Pathology 42: 35-41.

Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo and H. Vaucheret (2000). "*Arabidopsis* sgs2 and sgs3 genes are required for posttranscriptional gene silencing and natural virus resistance." Cell 101: 533-542.

Mourrain, P., R. van Blokland, J. M. Kooter and H. Vaucheret (2006). "A single transgene locus triggers both transcriptional and post-transcriptional silencing through double stranded RNA production." Planta 2006 Aug 19 *in press*.

Murashige, T. and F. Skoog (1962). "A revised medium for rapid growth and bioassays with tobacco tissue cultures." *Physiol Plant* 15:473-497.

Napoli, C., C. Lemieux and R. Jorgensen (1990). "Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans." Plant Cell 2: 279-289.

Navas-Castillo, J., M. R. Albiach-Marti, S. Gowda, M. E. Hilf, S. M. Garnsey and W. O. Dawson (1997). "Kinetics of accumulation of citrus tristeza virus RNAs." Virology 228: 92-7.

Niblett, C. L., H. Genc, B. Cevik, S. Halbert, L. Brown, G. Nolasco, B. Bonacalza, K. L. Manjunath, V. J. Febres, H. R. Pappu and R. F. Lee (2000). "Progress on strain differentiation of citrus tristeza virus and its application to the epidemiology of citrus tristeza disease." Virus Res 71: 97-106.

Niedz, R. P., M. R. Sussman and J. S. Satterlee (1995). "Green fluorescent protein: An in vivo reporter of plant gene expression." Plant Cell Rep 14:403-406.

Pappu, H. R., A. V. Karasev, E. J. Anderson, S. S. Pappu, M. E. Hilf, V. J. Febres, R. M. Eckloff, M. McCaffery, V. Boyko, S. Gowda and et al. (1994). "Nucleotide sequence and organization of eight 3' open reading frames of the citrus tristeza closterovirus genome." Virology 199: 35-46.

Pena, L., M. Cervera, J. Juarez, A. Navarro, J. A. Pina, N. Duranvila and L. Navarro (1995a). "*Agrobacterium*-mediated transformation of Sweet orange and regeneration of transgenic plants." Plant Cell Reports 14: 616-619.

Pena, L., M. Cervera, J. Juarez, C. Ortega, J. A. Pina, N. Duranvila and L. Navarro (1995b). "High-efficiency *Agrobacterium*-mediated transformation and regeneration of citrus." Plant Science 104: 183-191.

Pena, L., M. Cervera, J. Juarez, A. Navarro, J. A. Pina and L. Navarro (1997). "Genetic transformation of lime (*Citrus aurantifolia* Swing): Factors affecting transformation and regeneration." Plant Cell Reports 16: 731-737.

Pena, L., M. Martin-Trillo, J. Juarez, J. A. Pina, L. Navarro and J. M. Martinez-Zapater (2001). "Constitutive expression of *Arabidopsis* LEAFY or APETALA1 genes in citrus reduces their generation time." Nat Biotechnol 19: 263-7.

Pena, L., M. Cervera, R. Ghorbel, A. DomoAnguez, C. Fagoaga, J. Juarez, J. A. Pina and L. Navarro (2003). "Transgenic citrus." In: Jaiwal PK, Singh RP, eds Plant genetic engineering Vol 3 Improvement of commercial plants Houston, TX: Sci-Tech Publishing, 261±282.

Pena, L., R. M. Perez, M. Cervera, J. A. Juarez and L. Navarro (2004). "Early events in *Agrobacterium*-mediated genetic transformation of citrus explants." Ann Bot (Lond) 94: 67-74.

Peremyslov, V. V., I. A. Andreev, A. I. Prokhnevsky, G. H. Duncan, M. E. Taliansky and V. V. Dolja (2004). "Complex molecular architecture of beet yellows virus particles." Proc Natl Acad Sci U S A 101: 5030-5.

Perez-Molphe-Balch, E. and N. Ochoa-Alejo (1998). "Regeneration of transgenic plants of Mexican lime from *Agrobacterium rhizogenes* transformed tissues." Plant Cell Reports 17: 591-596.

Piestun, D., O. Batuman, X. Che, R. Gofman, V. Filatov, S. Zypman, R. Gafny and M. Bar-Joseph (1998). “Truncated versions of the citrus tristeza virus (CTV) replicase and basta resistance genes incorporated in transgenic Troyer citrange.” Proceedings of the First International Symposium on Citrus Biotechnology, Eilat, Israel, Acta-Horticulturae.

Piestun, D. (2003). Transformation of citrus with citrus tristeza virus (CTV) cDNA sequences and their influence on tristeza disease. The Hebrew University of Jerusalem, Rehovot, PhD**:** 148pp.

Powell-Abel, P., R. S. Nelson, B. De, N. Hoffman, S. G. Rogers, R. T. Fraley and R. N. Beachy (1986). "Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene." Science 236:738-743.

Prins, M. and R. Goldbach (1998). The emerging problem of tospovirus infection and nonconventional methods of control. Trends Microbiol **6**: 31-35.

Prins, M. (2003). "Broad virus resistance in transgenic plants." Trends Biotechnol 21: 373-5.

Pruss, G., X. Ge, X. M. Shi, J. C. Carrington and V. Bowman Vance (1997). "Plant viral synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses." Plant Cell 9: 859-68.

Qu, F., T. Ren and T. J. Morris (2003). "The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step." Journal of Virology 77: 511-522.

Qu, F., X. H. Ye, G. C. Hou, S. Sato, T. E. Clemente and T. J. Morris (2005). "RdR6 has a broad-spectrum but temperature-dependent antiviral defense role in *Nicotiana benthamiana*." Journal of Virology 79: 15209-15217.

Ravelonandro, M., R. Scorza, A. Callahan, L. Levy, C. Jacquet, M. Monsion and V. Damsteegt (2000). "The use of transgenic fruit trees as a resistance strategy for virus epidemics: The plum pox (sharka) model." Virus Research 71: 63-69.

Rocha-Pena, M. A., R. F. Lee, R. Lastra, C. L. Niblett, F. M. Ochoacorona, S. M. Garnsey and R. K. Yokomi (1995). "Citrus-tristeza-virus and its aphid vector *Toxoptera citricida* - threats to citrus production in the Caribbean and Central and North-America." Plant Disease 79: 437-445.

Roistacher, C. N. (1981). "A blueprint for disaster. Part 2: Changes in transmissibility of seedling yellows." Calif Citrogr 67: 28-32.

Rosciglione, B., M. A. Castellano, G. P. Martelli, V. Savino and G. Cannizzaro (1983). "Mealybug transmission of *Grapevine Virus A*." *Vitis* 22: 331-347.

Roth, B. M., G. J. Pruss and V. B. Vance (2004). "Plant viral suppressors of RNA silencing." Virus Research 102: 97-108.

Roy, A. and R. H. Brlansky (2004). "Genotype classification and molecular evidence for the presence of mixed infections in Indian citrus tristeza virus isolates." Archives of Virology 149: 1911-1929.

Roy, G., M. R. Sudarshana, D. E. Ullman, S. W. Ding, A. M. Dandekar and B. W. Falk (2006). "Chimeric cDNA sequences from citrus tristeza virus confer RNA silencing-mediated resistance in transgenic *Nicotiana benthamiana* plants." Phytopathology 96: 819-827.

Rubio, L., M. A. Ayllon, P. Kong, A. Fernandez, M. Polek, J. Guerri, P. Moreno and B. W. Falk (2001). "Genetic variation of citrus tristeza virus isolates from California and Spain: Evidence for mixed infections and recombination." J Virol 75: 8054-62.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Sanford, J. C. and S. A. Johnson (1985). "The concept of parasite-derived resistance: Deriving resistance genes from the parasites own genome." *J Theor Biol*: 115:395-405 75.

Satyanarayana, T., S. Gowda, V. P. Boyko, M. R. Albiach-Marti, M. Mawassi, J. Navas-Castillo, A. V. Karasev, V. Dolja, M. E. Hilf, D. J. Lewandowski, P. Moreno, M. Bar-Joseph, S. M. Garnsey and W. O. Dawson (1999). "An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication." Proc Natl Acad Sci U S A 96: 7433-8.

Satyanarayana, T., S. Gowda, M. Mawassi, M. R. Albiach-Marti, M. A. Ayllon, C. Robertson, S. M. Garnsey and W. O. Dawson (2000). "Closterovirus encoded HSP70 homolog and p61 in addition to both coat proteins function in efficient virion assembly." Virology 278: 253-65.

Satyanarayana, T., M. Bar-Joseph, M. Mawassi, M. R. Albiach-Marti, M. A. Ayllon, S. Gowda, M. E. Hilf, P. Moreno, S. M. Garnsey and W. O. Dawson (2001). "Amplification of citrus tristeza virus from a cDNA clone and infection of citrus trees." Virology 280: 87-96.

Satyanarayana, T., S. Gowda, M. A. Ayllon, M. R. Albiach-Marti, S. Rabindran and W. O. Dawson (2002). "The p23 protein of citrus tristeza virus controls asymmetrical RNA accumulation." J Virol 76: 473-83.

Satyanarayana, T., S. Gowda, M. A. Ayllon and W. O. Dawson (2004). "Closterovirus bipolar virion: Evidence for initiation of assembly by minor coat protein and its restriction to the genomic RNA 5' region." Proceedings of the National Academy of Sciences of the United States of America 101: 799-804.

Savenkov, E. I. and J. P. T. Valkonen (2002). "Silencing of a viral RNA silencing suppressor in transgenic plants." Journal of General Virology 83: 2325-2335.

Schauer, S. E., S. E. Jacobsen, D. W. Meinke and A. Ray (2002). "Dicer-like1: Blind men and elephants in *Arabidopsis* development." Trends in Plant Science 7: 487-491.

Schwach, F., F. E. Vaistij, L. Jones and D. C. Baulcombe (2005). "An RNA-dependent RNA polymerase prevents meristem invasion by Potato Virus X and is required for the activity but not the production of a systemic silencing signal." Plant Physiology 138: 1842-1852.

Sharp, P. A. (2001). "RNA interference - 2001." Genes & Development 15: 485-490.

Shi, B. J., S. W. Ding and R. H. Symons (1997). "Plasmid vector for cloning infectious cDNAs from plant RNA viruses: High infectivity of cDNA clones of tomato aspermy cucumovirus." J Gen Virol 78 (Pt 5): 1181-5.

Sim, G. E., C. J. Goh and C. S. Loh (1989). "Micropropagation of *Citrus mitis* Blanco multiple bud formation from shoot and root explants in the presence of 6-benzylaminopurine." Plant Science 59: 203-210.

Singh, A., T. H. Kao and J. J. Lin (1993). "Transformation of *Agrobacterium tumefaciens* with t-DNA vectors using high-voltage electroporation." *Focus* 15:84-87.

Singh, O. V., S. Ghai, D. Paul and R. K. Jain (2006). "Genetically modified crops: Success, safety assessment, and public concern." Applied Microbiology and Biotechnology 71: 598-607.

Sivamani, E., C. W. Brey, L. E. Talbert, M. A. Young, W. E. Dyer, W. K. Kaniewski and R. D. Qu (2002). "Resistance to wheat streak mosaic virus in transgenic wheat engineered with the viral coat protein gene." Transgenic Research 11: 31-41.

Smith, N. A., S. P. Singh, M. B. Wang, P. A. Stoutjesdijk, A. G. Green and P. M. Waterhouse (2000). "Gene expression - total silencing by intron-spliced hairpin RNAs." Nature 407: 319-320.

Soosaar, J. L. M., T. M. Burch-Smith and S. P. Dinesh-Kumar (2005). "Mechanisms of plant resistance to viruses." Nature Reviews Microbiology 3: 789-798.

Stachel, S. E., E. Messens, M. Vanmontagu and P. Zambryski (1985). "Identification of the signal molecules produced by wounded plant-cells that activate T-DNA transfer in *Agrobacterium tumefaciens*." Nature 318: 624-629.

Sunilkumar, G., K. Vijayachandra and K. Veluthambi (1999). "Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction." Plant Science 141: 51-58.

Szittya, G., A. Molnar, D. Silhavy, C. Hornyik and J. Burgyan (2002). "Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus." Plant Cell 14: 359-372.

Takeda, A., M. Tsukuda, H. Mizumoto, K. Okamoto, M. Kaido, K. Mise and T. Okuno (2005). "A plant RNA virus suppresses RNA silencing through viral RNA replication." *The EMBO J*: 24:3147-3157.

Thompson, J. D., D. G. Higgins and T. J. Gibson (1994). "Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence

weighting, position-specific gap penalties and weight matrix choice." Nucleic Acids Res 22: 4673-80.

Tomari, Y. and P. D. Zamore (2005). "Perspective: Machines for RNAi." Genes & Development 19: 517-529.

Tzfira, T. and V. Citovsky (2006). "*Agrobacterium*-mediated genetic transformation of plants: Biology and biotechnology." Curr Opin Biotechnol 17: 147-54.

Van der Krol, A. R., L. A. Mur, M. Beld, J. N. M. Mol and A. R. Stuitje (1990). "Flavonoid genes in petunia - addition of a limited number of gene copies may lead to a suppression of gene-expression." Plant Cell 2: 291-299.

Van Wezel, R., X. L. Dong, H. T. Liu, P. Tien, J. Stanley and Y. G. Hong (2002). "Mutation of three cysteine residues in tomato yellow leaf curl virus-china c2 protein causes dysfunction in pathogenesis and posttranscriptional gene-silencing suppression." Molecular Plant-Microbe Interactions 15: 203-208.

Van Wezel, R., H. T. Liu, Z. R. Wu, J. Stanley and Y. H. Hong (2003). "Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing." Journal of Virology 77: 696-700.

Vardi, A., S. Bleichman and D. Aviv (1990). "Genetic-transformation of citrus protoplasts and regeneration of transgenic plants." Plant Science 69: 199-206.

Vargason, J. M., G. Szittya, J. Burgyan and T. M. Tanaka Hall (2003). "Size selective recognition of siRNA by an RNA silencing suppressor." Cell 115: 799-811.

Vaucheret, H. (2006). "Post-transcriptional small RNA pathways in plants: Mechanisms and regulations." Genes & Development 20: 759-771.

Villemont, E., F. Dubois, R. S. Sangwan, G. Vasseur, Y. Bourgeois and B. S. SangwanNorreel (1997). "Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of petunia: Evidence of an S-phase control mechanism for T-DNA transfer." Planta 201: 160-172.

Vives, M. C., L. Rubio, C. Lopez, J. Navas-Castillo, M. R. Albiach-Marti, W. O. Dawson, J. Guerri, R. Flores and P. Moreno (1999). "The complete genome sequence of the major component of a mild citrus tristeza virus isolate." J Gen Virol 80 (Pt 3): 811-6.

Vives, M. C., L. Rubio, A. Sambade, T. E. Mirkov, P. Moreno and J. Guerri (2005). "Evidence of multiple recombination events between two RNA sequence variants within a citrus tristeza virus isolate." Virology 331: 232-237.

Voinnet, O., Y. M. Pinto and D. C. Baulcombe (1999). "Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants." Proc Natl Acad Sci U S A 96: 14147-52.

Voinnet, O. (2005). "Induction and suppression of RNA silencing: Insights from viral infections." Nat Rev Genet 6: 206-20.

Wallace, J. M. (1965). "Protection in citrus after recovery from seedling yellows and loss of yellows-inducing virus." Phytopathology 55: 1081-&.

Wang, M. B., Abbott, A. C., and Waterhouse, P. M. (2000). “A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus.” Mol. Plant Pathol. 1:347-356.

Wang, M. B. and P. M. Waterhouse (2002). "Application of gene silencing in plants." Current Opinion in Plant Biology 5: 146-150.

Waterhouse, P. M., Graham, M. W. and M. B. Wang (1998). “Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA.” Proc. Natl. Acad. Sci. USA 95: 13959–13964.

Yang, G., M. Mawassi, R. Gofman, R. Gafny and M. Bar-Joseph (1997). "Involvement of a subgenomic mRNA in the generation of a variable population of defective citrus tristeza virus molecules." J Virol 71: 9800-2.

Yang, G. A., X. B. Che, R. Gofman, Y. Ben-Shalom, D. Piestun, R. Gafny, M. Mawassi and M. Bar-Joseph (1999). "D-RNA molecules associated with subisolates of the VT strain of citrus tristeza virus which induce different seedling-yellows reactions." Virus Genes 19: 5-13.

Yang, Z. N., I. L. Ingelbrecht, E. Louzada, M. Skaria and T. E. Mirkov (2000). "*Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.)." Plant Cell Reports 19: 1203-1211.

Yang, Z. N., Ye, X. R., Choi, S., Molina, J., Moonan, F., Wing, R. A., Roose, M. L. and T. E. Mirkov (2001). Construction of a 1.2-Mb contig including the citrus tristeza virus resistance gene locus using a bacterial artificial chromosome library of *Poncirus trifoliata* (L.) Raf. Genome. 44: 382-93.

Yang, Z. N., Ye, X. R., Molina, J., Roose, M. L. and T. E. Mirkov (2003). Sequence analysis of a 282-kilobase region surrounding the citrus Tristeza virus resistance gene (*Ctv*) locus in *Poncirus trifoliata* L. Raf. Plant Physiol.; 131: 482-92.

Yao, J. L., J. H. Wu, A. P. Gleave and B. A. M. Morris (1996). "Transformation of citrus embryogenic cells using particle bombardment and production of transgenic embryos." Plant Science 113: 175-183.

Yokomi, R. K. and S. M. Garnsey (1987). "Transmission of citrus tristeza virus by *Aphis gossypii* and *Aphis citricola* in Florida." Phytophylactica 19: 169-172.

Yokomi, R. K., R. Lastra, M. B. Stoetzel, V. D. Damsteegt, R. F. Lee, S. M. Garnsey, T. R. Gottwald, M. A. Rochapena and C. L. Niblett (1994). "Establishment of the brown citrus aphid (Homoptera, Aphididae) in Central America and the Caribbean basin and transmission of citrus tristeza virus." Journal of Economic Entomology 87: 1078-1085.

Yoshida, T. (1996). "Graft compatibility of citrus with plants in the *Aurantioideae* and their susceptibility to citrus tristeza virus." Plant Disease 80: 414-417.

Yu, C. H., S. Huang, C. X. Chen, Z. N. Deng, P. Ling and F. G. Gmitter (2002). "Factors affecting *Agrobacterium*-mediated transformation and regeneration of Sweet orange and citrange." Plant Cell Tissue and Organ Culture 71: 147-155.

Yu, D. Q., B. F. Fan, S. A. MacFarlane and Z. X. Chen (2003). "Analysis of the involvement of an inducible *Arabidopsis* RNA-dependent RNA polymerase in antiviral defense." Molecular Plant-Microbe Interactions 16: 206-216.

Zou, C., Z. L. Zhang, S. Y. Wu and J. C. Osterman (1998). "Molecular cloning and characterization of a rabbit eif2c protein." Gene 211: 187-194.

7. APPENDIX

LIST OF PUBLICATIONS

Papers in reviewed journals

***Batuman, O.**, Mawassi, M. and Bar-Joseph, M. 2006. Transgenes consisting of a dsRNA of an RNAi suppressor plus the 3' UTR provide resistance to Citrus tristeza virus sequences in *Nicotiana benthamiana* but not in citrus. Virus Genes 33: 319-327

Cohen, O., **Batuman**, **O.,** Stanbekova, G., Sano, T., Mawassi, M. and Bar-Joseph, M. 2006. Construction of a multiprobe for the simultaneous detection of viroids infecting fruit trees. Virus Genes 33: 287-292

*Haviv, S., Galiakparov, N., Goszczynski, D. E., **Batuman, O.,** Czosnek, H., Mawassi, M. 2006. Engineering the genome of *Grapevine Virus A* into a vector for expression of proteins in herbaceous plants. J. Virological Methods 132: 227-231

Cohen, O., **Batuman**, **O.,** Moskowits, Y., Gootwine, E., Mawassi, M. and Bar-Joseph, M. 2004. Goat Horns, Platforms for Viroid Transmission to Fruit Trees? Phytoparasitica 33:141-148

Galiakparov, N., Goszczynski, D. E., Che, X., **Batuman, O.,** Bar-Joseph, M., Mawassi, M., 2003. Two classes of sub genomic RNA of grapevine virus A produced by internal controller elements. Virology 312: 434-438.

Wang, Q.C., Li, P., **Batuman, O.,** Gafny, R. and Mawassi, M., 2003. Effect of Benzyladenine on Recovery of Cryopreserved Shoot Tips of Grapevine and Citrus Cultured *in vitro.* CryoLetters 24: 293-302

Wang, Q.C., **Batuman**, **O.,** Li, P., Bar-Joseph, M., Gafny, R., 2002. Cryopreservation of in vitro grown shoot tips of Troyer Citrange (*Poncirus trifoliate* Raf. x *Citrus sinensis* (L.) Osbeck.) by encapsulation-dehydration. Plant Cell Rep., 20: 901-906.

Wang, Q.C., **Batuman**, **O.,** Li, P., Bar-Joseph, M., Gafny, R., 2002. A simple and efficient cryopreservation of in vitro-grown shoot tips of 'Troyer' citrange (*Poncirus trifoliate* Raf. x *Citrus sinensis* (L.) Osbeck.) by encapsulation-vitrification. Euphytica 128: 135-142.

Abstracts and Proceedings

***Batuman, O**., Mawassi, M. and Bar-Joseph, M., 2006. Transgenes consisting of a dsRNA of an RNAi suppressor plus the 3' UTR provide resistance to Citrus tristeza virus sequences in *Nicotiana benthamiana* but not in citrus. EMBO Workshop in Plant Virology 2006. Suppression and Circumvention of Host Defence by Plant Viruses, July 1-5, 2006, Finland

***Batuman, O.,** Che, X., Moskowits, Y., Mawassi, M. and Bar-Joseph, M., 2004. Interference or insurance? On the possible roles of different classes of Citrus tristeza virus defective RNA's. XVIth Conference of International Organization of Citrus Virologists, Monterrey, Mexico November 7-13, 2004

***Batuman, O.,** Che, X., Moskowits, Y., Cohen, O., Mawassi, M. and Bar-Joseph, M., 2004. Variation in composition and biology of *Citrus tristeza virus* defective RNAs. Invited presentation at The 2004 Annual Meeting of The American Phytopathology Society. APS 2004 Annual Meeting July 31–August 4, 2004 Anaheim, California.

*Bar-Joseph, M., Che, X., Piestun, D., **Batuman**, **O.,** Gofman, R., Ben Shalom, Y., Yang, G., and Mawassi, M., 2000. Citrus Tristeza Virus Biology Revisited: Quick Decline and Seedling Yellows-The Cost of Sour Orange Resistance Gene(s). Proc. Intl. Soc. Citricult. IX Congr. 2000. 963-965

Piestun, D., **Batuman, O**., Che, X., Gofman, R., Filatov, V., Zypman, S., Gafny, R., Bar-Joseph, M., Goren, R. (ed.), Goldschmidt, E.E., 2000. Truncated versions of the citrus tristeza virus (CTV) replicase and basta resistance genes incorporated in transgenic Troyer citrange. Proceedings of the First International Symposium on Citrus Biotechnology, Eilat, Israel, 29 November to 3 December 1998. Acta Horticulturae. 2000, 535: 223-230

Papers and abstracts relevant to this thesis have been marked with an asterix (*).

תקציר

ענף ההדרים הוא ענף המטעים הנפוץ ביותר בעולם וגידול הדרים לשיווק כרכז וכפרי טרי בעל תרומה נכבדת לכלכלת ישראל וארצות אחרות בעולם

נגיף הטריסטזה של ההדר *Citrus tristeza virus* (CTV) השייך לסוג *closterovirus*, ולמשפחת *Closteroviridae* מעורב בכמה סוגי מחלות הדרים הגורמות לנזקים כלכליים קשים, בעקבות פגיעתם הקשה ברמת היבולים ובאיכות הפרות של זני הדר רגישים דוגמת האשכוליות ובעיקר בגלל הניוון המהיר של עצי הדר המורכבים על כנת החושחש ועל כנת המקרופילה.

הנגיף מועבר מעצים נגועים לבריאים על ידי כנימות עלה והוא בעל חלקיקים חוטיים ארוכים המכילים את גנום ה-RNA החד גדילי הגדול ביותר מבין הווירוסים הצמחיים המוכרים.

טווח הפונדקאים של הנגיף מוגבל לצמחי הדר ולמיני פסיפלורה אך בתנאי מעבדה הנגיף מדביק גם פרוטופלסטים של צמחי *Nicotiana benthamiana* .

בעבר נוסו שיטות השבחה שונות לקבלת כנות וזני הדרים עמידים לנגיף אך חסרה עדיין כנת הדרים שתחליף את כנת החושחש שמלבד רגישותה לטריסטזה היא מצטיינת ככנה המקנה עמידות בפני מחלות קריפטוגמיות ומותאמת לתנאי קרקע קשים דוגמת אלה השוררים בנגב הצפוני איזור הנטיעה העתידי העיקרי בארץ.

התפתחויות בהנדסה גנטית של צמחים מאפשרים היום קבלת עמידות כנגד שורה של נגיפי צמחים על ידי שימוש בצמחים מותמרים המבטאים מקטעי רצף של הנגיף.

ניסיונות דומים שנערכו בעבר לקבלת עמידות טרנסגנית לטריסטזה על ידי שתילת הגן לחלבון המעטפת לא צלחו.

העבודה הנוכחית עסקה בניסויים שנועדו לשפר את יעילות ההתמרה של כנות הדרים ובחינת העמידות של כנות ההדרים שהותמרו במבנים שהכילו את הגנים p61 שהוא מרכיב חיוני להיעטפות הנגיף ו- p23 מעורב בשכפול הנגיף, ובביטוי המחלה ומשמש כאחד משלושת הגנים המעורבים בעיכוב מנגנון ההשתקה הטבעי של הנגיף על ידי הפונדקאי.

הגן p23 הוחדר לצמחים באמצעות מבנים שיועדו לבטאו כמולקולה חד ו- דו-גדילית (p23UI).

שיפור מהלך ההתמרה של כנות הדרים הושג לאחר בדיקת השפעתם של מגוון גדול של משתנים להכנת צמחוני המוצא וכן להכנת מצעי הגידול של הבקטריות ושל הצמחונים שהותמרו. פרוטוקולים משופרים שפיתחנו שימשו אותנו לקבלת כ 300 כנות הדרים מהסוגים מקרופילה, חושחש וולקמריאנה וגו-טו מותמרות ברצפים שמקורם בנגיף הטריסטזה וכן של כ 400 צמחוני טרוייר שהיא כנה עמידה וקלת התמרה.

במקביל לניסויי ההתמרה של כנות ההדרים בחנו את יעילות כל המבנים שסונתזו על ידי התמרת צמחי *N. benthamiana* המערכת הזו נוצלה לבחינה מהירה של התנהגות מקטעי הטריסטזה בתנאי הדבקת אתגר שבוצעו באמצעות ווקטור ויראלי שהתבסס על *Grapevine Virus A* (GVA) שהונדס לנשיאת מקטעי רצף שמקורם בנגיף הטריסטזה.

התברר כי התמרת צמחי *N. benthamiana* במקטעי רצף של טריסטזה שבוטאו כמולקולות RNA דו – גדיליות(p23UI) הקנתה עמידות לנגיף GVA שנשא את הגן p23U, בעוד שצמחים שהותמרו בתבניות שבוטאו כמולקולות חד גדיליות לא הראו עמידות דומה.

שורה של ניסויים לאימות תופעת העמידות הראו כי היא נשמרה כל זמן שהנגיף GVA המהונדס שמר על מקטע הרצף של הטריסטזה.

בעקבות תוצאות אלה בהם הוכחנו כי מקטע הרצף הדו-גדילי של נגיף הטריסטזה מסוגל לעורר עמידות כנגד נגיף המכיל את המקטע הזה, עברנו לבחינת עמידותם של כנות ההדר שהותמרו ברצף הזה וברצפים האחרים. לבדיקת העמידות הוכנו מכל קו מותמר לפחות 2 צמחים מורכבים שהודבקו בתבדיד טריסטזה אלים במיוחד Mor-T. את המדבק הרכבנו על גבי הכנות הבלתי מותמרות ששימשו לנשיאת הרכב המותמר. התברר כי למרות שצמחי ההדר המותמרים ביטאו את מקטעי הרצף ששימשו להתמרה בריכוזים שניתן היה לגלותם על ידי היברידיזציה בגלאים לא רדיואקטיביים ולמרות שחלק מהצמחים שהודבקו איחרו להראות סימפטומים, עמידות צמחי ההדר המותמרים לא הייתה יציבה וממושכת. בשלבי בדיקה מאוחרים יותר כל צמחי ההדר המותמרים והלא מותמרים כאחד הכילו כמויות זהות של נגיף ושל מולקולות RNA דו-גדילי , מצב שהוכיח שבניגוד לעמידות הטובה שהתקבלה במערכת העשבונית, מנגנון ההשתקה שהותמר לצמחי הדר נבלם על ידי מערך הגנים בולמי ההשתקה של נגיף הטריסטזה.

בבחינת נוכחות מולקולות siRNAs המעידות על פעילות תהליך ההשתקה של הנגיף על ידי מנגנוני ההשתקה של הפונדקאי התברר כי הן צמחי הדר המותמרים וגם אלה שלא הותמרו הכילו רמות גבוהות של תוצרי ההשתקה ממקטעי רצף שונים של גנום הנגיף. נראה לכן כי אף שהנגיף הזה משמש יעד להשתקה על ידי מנגנון ההגנה הטבעי המושרה בצמחי הדר , הנגיף נושא עימו אמצעי הגנה מספיקים על מנת לבלום את השתקתו. יתירה מכך מנגנון בלימת ההשתקה של הנגיף יעיל דיו גם כדי לבלום השתקה שמקורה במקטעי רצף שהושתלו לצמחים המותמרים.

מחקרים שנערכו במהלך הניסויים האלה הוכיחו כי נגיף הטריסטזה ייחודי והוא נושא לפחות שלושה גנים שונים הפועלים לבלימת השתקתו על ידי הפונדקאי.

בפרק האחרון עסקנו בניסיון לאפיין את הרצף המולקולארי של תבדידי טריסטזה שמקורם בצמחי חושחש שעברו תהליך הבראה מתופעת צהבון עלים. בהשוואת רצפים מנגיפים שהופקו מצמחים שהבריאו ממחלת צהבון העלים לאלה שהודבקו בגזע Mor-T המקורי נמצאו כ 300 שינויים המפוזרים באופן אקראי לאורך הגנום של התבדידים שנבחנו ותידרש עבודה נוספת לפענח מי מקרב הרצפים השונים מעורב בתופעת ההבראה.

בסיכום העבודה זו תרמה לשיפור פרוטוקול ההתמרה של כנות הדרים והוכיחה כי מקטעי רצף של הנגיף המקנים עמידות בצמחים אחרים אינם עומדים אל מול מנגנון עיכוב ההשתקה של נגיף הטריסטזה.

טרנספורמציה של הדרים עם קונסטרקט המכיל רצף של *Citrus Tristeza Virus (CTV)*

חיבור לשם קבלת תואר דוקטורט לפילוסופיה

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דצמבר 2006

עבודה זו נעשתה תחת הנחייתו של

פרופ׳ משה בר יוסף

המעבדה לחקר מחלות הדרים ע״ש טולקובסקי

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