Diversity and host interactions of emerging tomato begomoviruses in Brazil

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O valor das coisas não está no tempo em que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis.

The value of things is not in the time they last, but in the intensity in which they happen. That is why there are unforgettable moments, inexplicable things, and incomparable people.

Fernando Pessoa

Para meus pais,
Geraldina e Horácio

Contents

Chapter 1

General Introduction

Tomato (*Lycopersicon esculentum Mill.*) is one of the economically most important vegetable crops and is widely cultivated year-round in tropical and subtropical regions of the world. In Brazil, about sixty thousand hectares are cultivated annually for both fresh market and processing industry with a total production of 3.5 million tons. Processing tomato is the one of the most important Brazilian agribusiness. In 2004, the Brazilian production of processing tomato was approximately 1,200,000 tons, with a mean productivity of 72 t/ha (http://www.CNPH.embrapa.br). The production and processing of tomato involves a vast human labor and generates direct and indirect job opportunities in the producing areas.

Over the last three decades, insects belonging to the genus *Bemisia* (*Homoptera: Aleyrodidae*), commonly known as whiteflies, became one of the major pests in the worldwide agricultural systems (Jones, 2003; Naranjo & Ellsworth, 2001; Oliveira *et al.*, 2001). A major impact of the increase in the population of whiteflies was the proliferation of disease problems caused by viruses transmitted by these insects. The most widespread and important are those associated with viruses classified in the family *Geminiviridae,* more specifically to those belonging to the genus *Begomovirus*, which have emerged as a restriction to the cultivation of tomato and other vegetable and fiber crops in various parts of the world (Morales & Anderson, 2001; Moriones & Navas-Castillo, 2000; Polston & Anderson, 1997; Ribeiro *et al.*, 1998b; Varma & Malathi, 2003).

Geminiviruses: taxonomy and genomic organization

Geminiviruses are small single-stranded circular DNA plant viruses with distinctive twinned isometric particle morphology of approximately 20 x 30 nm (Fig. 1.1). The family *Geminiviridae* is divided into four genera, *Mastrevirus, Curtovirus, Topocuvirus* and *Begomovirus*, classified based on genome structure, insect vector and host range (Fauquet & Stanley, 2003; Rybicki *et al.*, 2000; Stanley *et al.*, 2005) (Fig. 1.2 and 1.3).

These four genera infect a broad range of plants and cause substantial crop losses worldwide (Mansoor *et al.*, 2003; Morales & Anderson, 2001). Having a small genome, geminiviruses utilize a strategy of overlapping genes in different frames to efficiently code the proteins needed for replication, control of gene expression, encapsidation and movement (Fig. 1.3). Mastreviruses have a single component encoding four proteins. They are transmitted by leafhoppers in a persistent circulative manner, i.e. once the insect feeds on an infected plant and acquires the virus, transmission can occur within hours and continue for the entire life of the insect (Harrison, 1985).

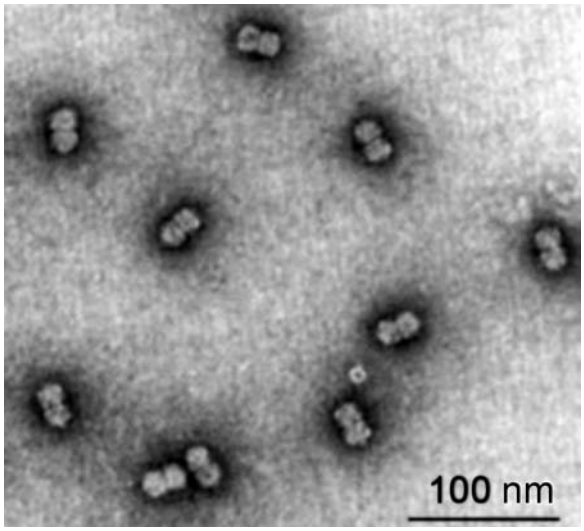

Figure 1.1: Electron micrograph of purified geminivirus particles

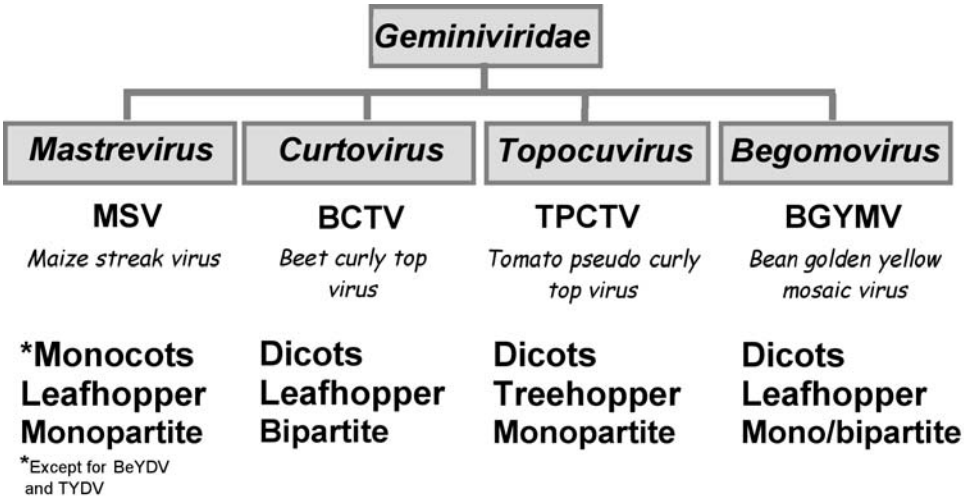

Figure 1.2: The taxonomy of the family Geminiviridae: Type species, genome organization, host plants and insect vectors. BeYDV: Bean yellow dwarf virus, TYDV: Tobacco yellow dwarf virus

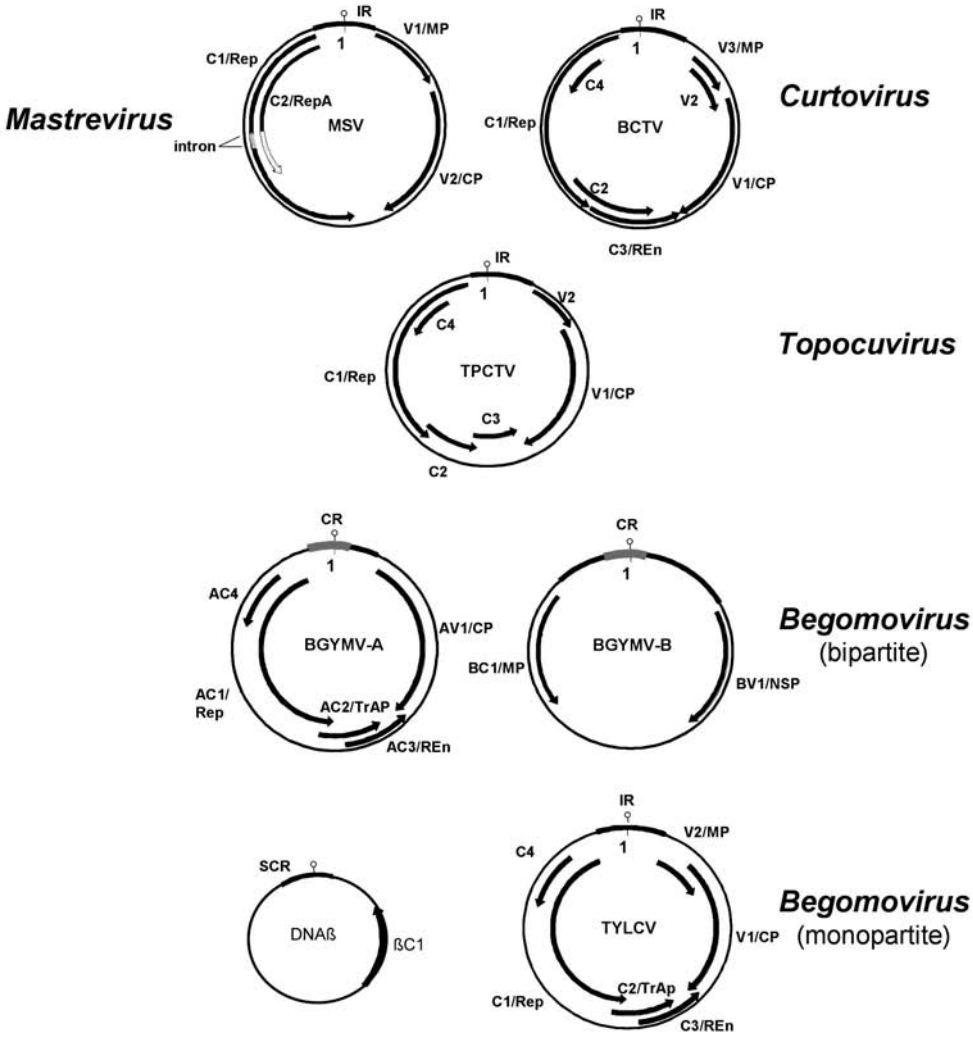

Figure 1.3: Genome organization of the genera belonging to the Geminiviridae. Arrows represent open reading frames. CP: coat protein, Rep: replication initiator protein, TrAP: transcriptional activator protein, REn: replication enhancer protein, MP: movement protein, NSP: nuclear shuttle protein, IR: intergenic region, CR: common region, SCR: satellite conserved region

Mastreviruses mainly infect monocotyledonous plants, but a few have dicotyledonous hosts. The Curtoviruses also have a monopartite genome, encoding seven proteins, and are transmitted in a persistent circulative manner by leafhoppers to dicotyledonous plants. The genus *Topocuvirus* has only one species, *Tomato pseudo-curly top virus*, with a monopartite genome encoding six proteins; it infects dicotyledonous plants and is transmitted by a treehopper. *Begomovirus* constitute the largest and economically most important genus. Virus species in this genus are transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a persistent, circulative manner. They infect dicotyledonous plants and cause several of the most devastating and important plant diseases including cassava mosaic, cotton and tomato leaf curl and bean golden mosaic with losses estimated up to several billions of dollars (Harrison & Robinson, 2002; Varma & Malathi, 2003). Begomoviruses can either have a bipartite genome, with the A component encoding five or six proteins and the B component encoding two proteins, each component being 2.5-2.8 kb in size, or a monopartite genome encoding six proteins (~ 3 kb).

Begomovirus DNA-A codes for proteins responsible for particle encapsidation (AV1), viral replication (AC1 and AC3), regulation of gene expression and for some viruses suppression of gene silencing (AC2). Movement and modulation of symptom development was attributed to the protein encoded by C4 gene of monopartite begomoviruses while AC4 of some bipartite viruses acts as suppressor of gene silence. The gene AV2 is only present in begomoviruses originated in the Old World. The genes BV1 and BC1 located in DNA-B encode two proteins involved in intracellular (nuclear shuttle protein – NSP) and intercellular (movement protein – MP) virus movement (Lazarowitz, 1992). The DNA-A component is capable of autonomous replication and can produce virus particles but requires DNA-B for efficient systemic infection. The two components of a begomovirus have little sequence homology, except for an intergenic region (IR), that includes the common region (CR) of approximately 200 nucleotides. The CR contains a hairpin structure with the characteristic geminiviral nonanucleotide sequence TAATATT↓AC in the loop at the expected origin of virion strand DNA replication (Hanley-Bowdoin *et al.*, 1999), the binding sequences recognized by the AC1 (Rep) protein (Arguello-Astorga *et al.*, 1994) and regulatory regions for bidirectional transcription (Hanley-Bowdoin *et al.*, 1999).

Most of the monopartite begomoviruses are associated with a satellite DNA molecule of ~1.4 kb referred to as DNA-ß which encodes a single protein (ßC1) (Briddon *et al.*, 2003; Briddon *et al.*, 2001; Saunders *et al.*, 2000)(Fig 1.3). Other subviral ssDNA components associated with some begomoviruses are the nanovirus-like Rep-encoding DNA1 and the Tomato leaf curl virus-satellite non coding molecule (Dry *et al.*, 1997; Mansoor *et al.*, 1999; Saunders & Stanley, 1999).

Geminivirus evolution and diversity

Geminiviruses are thought to have evolved as episomal DNA replicons of primeval prokaryotic organisms that later adapted to primitive eukaryotic progenitors of modern plants (Rojas *et al.*, 2005). Several evidences such as the conserved features of the replication initiator proteins of contemporary eukaryotic and prokaryotic DNA replicons (Campos-Olivas *et al.*, 2002; Ilyina & Koonin, 1992), polycistronic mRNAs and ability of diverse geminivirus types to replicate in *Agrobacterium tumefaciens* (Frischmuth *et al.*, 1990; Selth *et al.*, 2002) support this hypothesis. During co-evolution with their hosts, these DNA replicons acquired new properties by recombination with host genome or other replicons (Rojas *et al.*, 2005). Phylogenetic studies (Padidam *et al.*, 1995; Rybicki, 1994) suggest that it is possible that the ancestral geminivirus had a single component, infected monocotyledoneous hosts and was leafhopper-transmitted. Further changes facilitated the transmission by whiteflies and infection of dicotyledoneous plants resulting in the ancestral Old World begomoviruses. The acquisition of a second component is likely to have occurred later on the evolutionary scale, although this might have happened before the separation of the continents, as bipartite begomoviruses are found in both Old and New World (Rojas *et al.*, 2005). Association of the monopartite begomoviruses with satellite molecules like DNA-ß may possibly have opened new possibilities to adapt and colonize new hosts (Briddon *et al.*, 2003; Rojas *et al.*, 2005). The genus *Curtovirus* might have originated from ancient recombination events between a mastrevirus and a begomovirus (Harrison & Robinson, 1999). Another recombination event possibly generated the genus *Topocuvirus* which seems to have emerged after the recombination of an ancient curtovirus and probably another virus not related with the modern geminiviruses belonging to the other two genera (Briddon *et al.*, 1996). Recombination has always been fundamental to geminivirus diversity. This mechanism may have been facilitated by the recombination-dependent replication strategy (Preiss & Jeske, 2003) by frequent natural mixed infections (Harrison *et al.*, 1997; Pita *et al.*, 2001; Ribeiro *et al.*, 2003; Sanz *et al.*, 2000; Torres-Pacheco *et al.*, 1996) and by the unusual feature among plant viruses, that two begomoviruses can infect the same cell nucleus (Morilla *et al.*, 2004). This mechanism might have contributed to the high number of virus species in the family *Geminiviridae*. Figure 1.4 shows a phylogenetic tree representing more than one hundred species belonging to the four genera of the family the *Geminiviridae*. Begomovirus geographical repartition can also be observed.

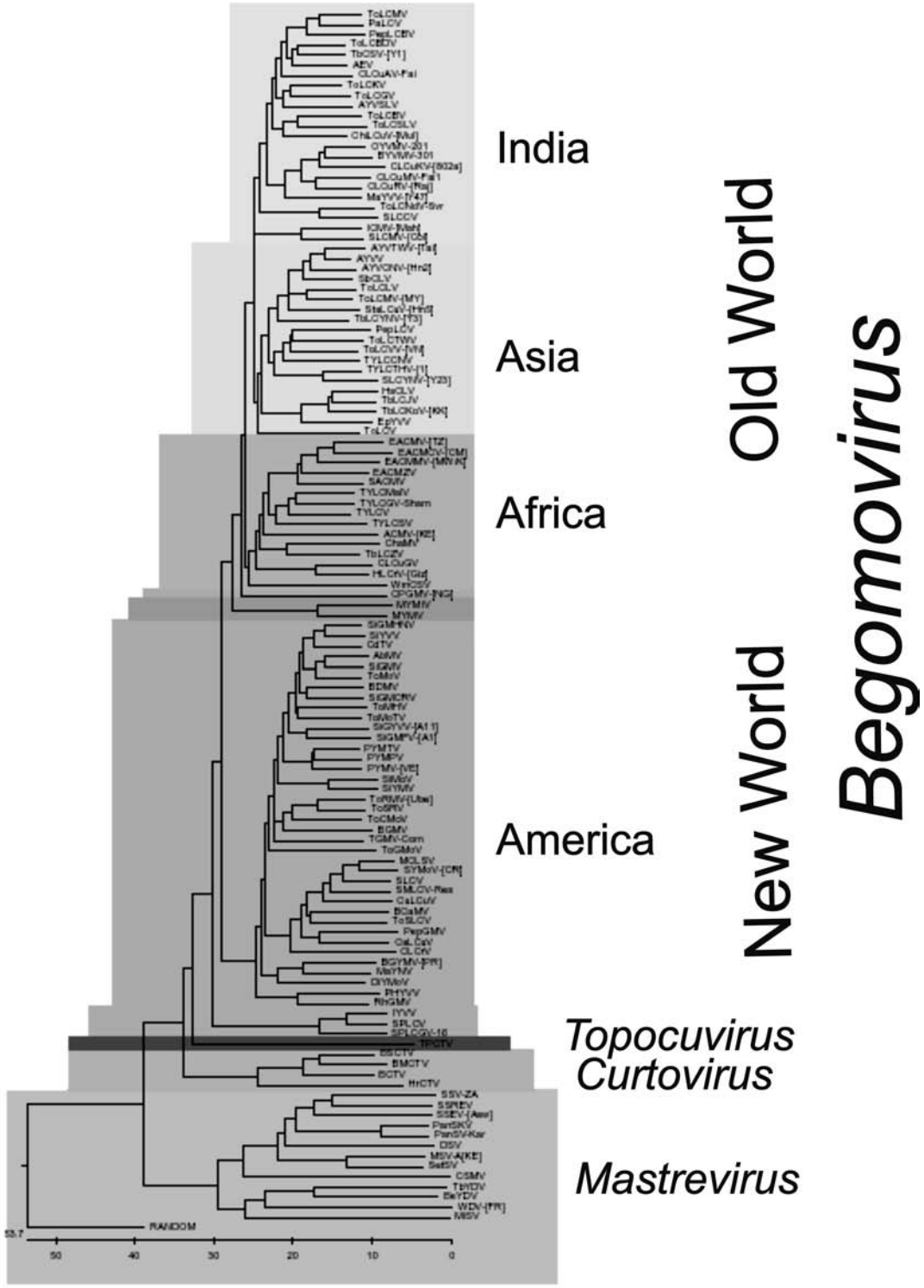

Figure 1.4. Phylogenetic tree based on the complete DNA-A (for bipartite begomoviruses) nucleotide sequences representing 119 species of geminiviruses. Modified from www.danforthe-center.org/ILTAB/geminiviridae/quantax.HTLM

Begomovirus genome expression and protein function

Geminiviruses replicate within the cell nucleus and depend on host machinery to amplify their DNA genomes which is accomplished through double-stranded DNA intermediates using a combination of rolling circle and recombination strategies (Gutierrez, 1999; Hanley Bowdoin *et al.*, 1999; Jeske *et al.*, 2001; Preiss & Jeske, 2003). Transcription also takes place inside the nucleus and is bidirectional from promoter sequences located in the intergenic region. Transcription can be quite complex, frequently giving rise to multiple overlapping polycistronic mRNAs. The convergent transcripts overlap for several nucleotides at their 3' ends (Hanley-Bowdoin *et al.*, 1989; Hanley-Bowdoin *et al.*, 1999; Shivaprasad *et al.*, 2005; Sunter & Bisaro, 1989)

Begomoviruses code for two conserved proteins, AC1 and AC3, implicated in virus replication. AC1 is the replication-associated protein (Rep) and mediates the initiation of rolling circle replication (Elmer *et al.*, 1988; Hanley-Bowdoin *et al.*, 1990). AC1 is constituted by several structural domains which are responsible for a range of different functions during viral DNA replication. It binds to highly specific viral DNA sequences (referred to as iterons) which are located at the conserved common region (Fontes *et al.*, 1994), represses its own promoter (Eagle *et al.*, 1994; Sunter *et al.*, 1993), cleaves and ligates DNA (Laufs *et al.*, 1995), and also plays a role as a DNA helicase (Pant *et al.*, 2001). This protein can interact with itself (Orozco *et al.*, 1997), with AC3 (Settlage *et al.*, 1996), and a number of host proteins (Ach *et al.*, 1997; Castillo *et al.*, 2003; Castillo *et al.*, 2004; Kong & Hanley-Bowdoin, 2002; Luque *et al.*, 2002). The most studied host protein interacting with AC1 is a plant retinoblastoma homologue, which regulates the cell cycle and differentiation (Arguello-Astorga *et al.*, 2004; Kong *et al.*, 2000). This interaction provides the necessary requirements by reprogramming mature plant cells to replicate viral DNA allowing infection to take place (Kong *et al.*, 2000). AC3 is an auxiliary replication enhancing protein (Ren) that increases viral DNA accumulation (Sunter *et al.*, 1990). AC3 forms homo-oligomers and interacts with AC1 and host factors (Castillo *et al.*, 2003; Selth *et al.*, 2005; Settlage *et al.*, 2001; Settlage *et al.*, 1996; Settlage *et al.*, 2005).

The AC2 gene codes for a multifunctional regulatory protein and is also termed transcriptional activator protein, TrAP (Hartitz *et al.*, 1999; Sunter & Bisaro, 1991). This protein is required for efficient transcription of the late viral sense genes, AV1 and BV1 (Sunter & Bisaro, 1992, 1997) and has a modular structure consisting of three conserved domains: a basic domain with a nuclear localization signal at the N terminus, a central DNA-binding Zn-finger motif and C terminal acidic activator domain (Hartitz *et al.*, 1999). It binds to ssDNA in a non specific way and only weakly to dsDNA, suggesting that it is not a canonical transcriptional factor, but probably interacts with host plant

cellular proteins to trigger transcriptional activation (Hartitz *et al.*, 1999). Furthermore, AC2 interacts and inactivates SNF1 and adenosine kinases, enzymes which appear to be involved in defense response (Hao *et al.*, 2003; Wang *et al.*, 2003), and finally it also has been implicated as a suppressor of RNA silencing (Selth *et al.*, 2004; Trinks *et al.*, 2005; van Wezel *et al.*, 2001; Vanitharani *et al.*, 2004; Voinnet *et al.*, 1999; Wang *et al.*, 2005).

The ORF AC4 is completely embedded within AC1 coding region but in a different frame (Hanley-Bowdoin *et al.*, 1999). AC4 proteins of bipartite begoviruses are highly variable and no general role has been ascribed to this gene product. Mutations in the AC4 of Tomato golden mosaic virus (TGMV) (Elmer *et al.*, 1988), African cassava mosaic virus (ACMV) (Etessami *et al.*, 1991) and Bean golden mosaic virus (BGMV) (Hoogstraten *et al.*, 1996) did not reveal any effect on viral replication or symptom development. However, recently the bipartite ACMV-[CM]-AC4 and Sri Lankan cassava mosaic virus (SLCMV)-AC4 were reported to have the capacity of suppression of gene silence (Vanitharani *et al.*, 2005; Vanitharani *et al.*, 2004). In monopartite begomoviruses the analogous protein was implicated in virus movement, since a Tomato yellow leaf curl (TYLCV) AC4 mutant was unable to move systemically in tomato plants (Jupin *et al.*, 1994) and was also reported as virulence factor for Tomato leaf curl virus (TLCV) (Krake *et al.*, 1998; Selth *et al.*, 2004).

The coat protein (CP), the sole geminivirus structural protein, encoded by the AV1 gene, is also a multifunctional protein (Harrison *et al.*, 2002). It forms multimeres (Hallan & Gafni, 2001) and encapsidates the viral ssDNA resulting into the characteristic geminate particles (Unseld *et al.*, 2004; Zhang *et al.*, 2001). The coat protein is responsible for vector specificity (Briddon *et al.*, 1990) and although it is not necessary for bipartite begomovius movement, it is indispensable for systemic movement of monopartite begomoviruses (Noris *et al.*, 1998; Wartig *et al.*, 1997) and for efficient accumulation of viral ssDNA (Harrison *et al.*, 2002; Qin *et al.*, 1998). CP interacts with importin α and might use this pathway to dock virus particles/nucleoproteins to the nucleus in initially infected cells (Gafni & Epel, 2002; Guerra-Peraza *et al.*, 2005). It was also reported to interact and down regulate AC1, thereby it my have a role in controlling geminiviral DNA replication (Malik *et al.*, 2005).

The ORF AV2 (pre-coat) is only present in Old World begomoviruses (Harrison & Robinson, 1999). In the monopartite TLCV it has been implicated in ssDNA accumulation (Rigden *et al.*, 1993). In TYLCV it was shown to mediate viral DNA export from the nucleus to the plasmodesmata (Rojas *et al.*, 2001) and for Tomato leaf curl New Delhi virus (also know as Tomato leaf curl virus – India) it was shown to be involved in systemic movement (Padidam *et al.*, 1996).

The genes encoded by the B component of bipartite begomoviruses, BV1 and BC1, provide functions required for virus movement. BV1, the nuclear shuttle protein

(NSP) and BC1, the cell-to cell movement protein (MP) coordinate the movement of the viral DNA from the nucleus and across the cell boundaries (Gafni & Epel, 2002; Noueiry *et al.*, 1994; Sanderfoot & Lazarowitz, 1995, 1996). Though it is not precisely known if single stranded or double stranded form is transported, BV1 packages the viral DNA, interacts with BC1 in the cytoplasm to be transported through the plasmodesmata into the neighboring cell (Hehnle *et al.*, 2004; Lazarowitz & Beachy, 1999). Both BC1 and BV1 movement proteins of different bipartite begomoviruses are reported as virulence determinants in different host plants (Carvalho & Lazarowitz, 2004; Duan *et al.*, 1997a; Fontes *et al.*, 2004; Hou *et al.*, 2000; Hussain *et al.*, 2005; Ingham *et al.*, 1995; Pascal *et al.*, 1993; von Arnim & Stanley, 1992).

Several small subviral ssDNA components have been reported to be associated with monopartite begomoviruses. Tomato leaf curl virus-satellite is a non-coding molecule which is dependent on the helper virus for replication and is neither required for infectivity nor interferes with symptoms (Dry *et al.*, 1997). DNA-1 is a nanovirus-like Rep-encoding molecule which replicates autonomously but is dependent on the helper begomovirus for encapsidation, movement and transmission. In most cases, DNA-1 appears not to contribute in the disease induction by the helper virus (Briddon & Stanley, 2006; Mansoor *et al.*, 1999; Saunders & Stanley, 1999; Stanley, 2004). Nearly all monopartite begomoviruses were found to be associated with a satellite molecule, denoted DNA-ß which is involved in host range determination and is essential for the induction of the typical disease symptoms (Briddon *et al.*, 2003; Briddon & Stanley, 2006). This satellite is completely dependent on the helper virus for replication, movement and dissemination. DNA-ß encodes a single protein (ßC1) which has a nuclear localization and functions as a suppressor of RNA silencing (Briddon *et al.*, 2003; Cui *et al.*, 2005; Mansoor *et al.*, 2003; Stanley, 2004). Nearly all begomovirus-DNA-ß complexes are also associated with a DNA-1 subviral component (Briddon *et al.*, 2004; Briddon & Stanley, 2006).

Tomato Begomoviruses in Brazil: economic importance and distribution

The first report of a geminivirus infecting tomato plants in Brazil, was in the State of São Paulo in 1960 (Flores *et al.*, 1960). The virus was named Tomato golden mosaic virus (TGMV) (Costa *et al.*, 1975; Maytis *et al.*, 1975). TGMV was considered of low economical relevance and indeed, only after more than 20 years it was reported again in Brazil, in the State of Rio de Janeiro (Afenas *et al.*, 1998).

The occurrence of tomato-infecting geminiviruses was very limited until the mid 1990s when geminivirus-like diseases were reported in different parts of the coun-

try causing important economical losses (Faria *et al.*, 2000). Those reports were associated with the presence of the B biotype of *B. tabaci*, which was probably introduced via imported ornamental plants coming from Europe or the USA (Melo, 1992). From the initial site of introduction in São Paulo (Lourenção & Nagai, 1994), this new vector biotype invaded agricultural regions in the Southeastern states of Minas Gerais, Rio de Janeiro and Espirito Santo (Peixoto *et al.*, 1996). Then, it quickly disseminated to the Federal District and other states in Central Brazil (França *et al.*, 1996) and to the Northeastern states of Bahia and Pernambuco, where heavy infestation in vegetable and fruit crops were recorded (Haji *et al.*, 1996). Later, the new whitefly biotype spread to the other neighboring Northeastern states (Haji *et al.*, 2004).

The spread of tomato-infecting geminiviruses followed the same track as the newly introduced vector. Symptoms of begomovirus in tomato were reported initially in the Federal District, (Ribeiro *et al.*, 1994), several regions of Minas Gerais (Rezende *et al.*, 1996; Zerbini *et al.*, 1996), São Paulo (Faria *et al.*, 1997), Rio de Janeiro and then tomato-geminiviruses were reported widely disseminated in the states of Bahia (Ribeiro *et al.*, 1996) and Pernambuco (Bezerra *et al.*, 1997; Lima *et al.*, 2001), the most important area for production of processing tomato at that time. Losses in the production ranged from 40 to 100% depending on the cultivar and age of the plant when infection initiated (Faria *et al.*, 2000). A preliminary molecular characterization of these viruses indicated the presence of several putative new species (Ribeiro *et al.*, 1998b) and in many cases, the viruses were present in mixed infections (Ribeiro *et al.*, 1998a).

Resistance to begomoviruses

The control of begomovirus-induced diseases is difficult, costly, and with limited alternatives. In some places, the viruses are managed through cultural practices and intensive use of insecticides, but these practices are often not very efficient. The development of insecticide resistance and the loss of natural enemies due to successive insecticide applications contribute to inefficient control and environmental problems. The most effective measure for begomovirus disease control is the use of integrated management with resistant cultivars combined with rational use of pesticides and cultural practices aiming to at reducing the virus inoculum load. Therefore breeding begomovirus resistant plants is a paramount long-term goal.

A variety of approaches have been used to achieve geminivirus resistance, including classical breeding and biotechnological strategies (Freitas-Astua *et al.*, 2002; Lapidot & Friedmann, 2002). Due to the damage caused by geminiviruses on tomato cultivation, search for resistant germplasm to be used in breeding programs has been intensified in many countries. Most of the work accomplished to date involves the search

for resistance to TYLCV, an important and widespread monopartite begomovirus. The genetic base of the resistance seems to vary with the studied species. The wild species *L. pimpinellifolium* and *L. hirsutum* show partial resistance in the field, while *L. chilense* presents high resistance levels (Zakay *et al.*, 1991). The introgression of the gene Ty-1 from *L. chilense* into cultivated tomato resulted in TYLCV tolerant hybrids (Zamir *et al.*, 1994). Some TYLCV tolerant genotypes are also tolerant to the virus present the Federal District area in Brazil (Ferreira *et al.*, 1999; Santana *et al.*, 2001).

Various biotechnological strategies have been tested to obtain resistance against geminiviruses. Arabidopsis plants expressing an artificial zinc finger protein, which associates with the AC1 binding sites induced resistance to a curtovirus, but the system was not tested for begomoviruses (Sera, 2005). Transgenic plants expressing dianthin (a ribosome inactivating protein) using a virus induced promoter exhibited resistance (Hong et al., 1996), however the safety of this plant toxin for use in food crops is not known.

Transgenic resistance based on the concept of pathogen-derived resistance (Sanford & Johnston, 1985) is the most used approach (Prins, 2003; Ritzenthaler, 2005; Soosaar *et al.*, 2005). Regarding the resistance to tomato geminiviruses, the transformation of tobacco plants (*Nicotiana tabacum* or *N. benthamiana*) with antisense or truncated forms of the AC1 gene (from TGMV or TYLCV), truncated forms of the AV1 gene or mutated BC1 gene (from Tomato Mottle virus), generated plants with attenuated and delayed symptoms, decreased viral replication or suppression of symptoms in non inoculated leaves (Day *et al.*, 1991; Duan *et al.*, 1997b; Noris *et al.*, 1996; Sinisterra *et al.*, 1999). Transgenic tomato plants expressing the coat protein gene of TYLCV initially presented symptoms upon virus infection, but later recovered (Kunik *et al.*, 1994). AC1-derived transgenes can confer resistance to begomovirus infection by RNA silencing or by interfering with the protein function. RNA silencing has been implicated in the resistance phenotype of transgenic tomato plants carrying truncated versions of the TYLCV Rep coding region (Yang *et al.*, 2004). Transgene expression was also suppressed in tomato plants carrying a truncated TYLCSV AC1, suggesting involvement of RNA silencing, but these plants were susceptible to infection by the homologous virus nonetheless (Noris *et al.*, 2004). Tomato plants expressing and accumulating high levels of truncated AC1 protein were resistant to TYLCSV (Brunetti *et al.*, 2001; Lucioli *et al.*, 2003). Transgenic resistance associated with RNA silencing was successfully achieved against other begomoviruses such as *Bean golden mosaic virus* (Aragão *et al.*, 1998) *Cotton leaf curl leaf virus* (Asad *et al.*, 2003) and even broad resistance against cassava-infecting begomoviruses (Chellappan *et al.*, 2004). Using an inverted repeat construct containing the common region of the begomovirus Vigna mungo yellow mosaic virus (VMYMV) in a transient assay, Pooggin *et al.* (2003) obtained recovery from virus infection.

Outline of this thesis

The increasing occurrence and the great diversity of begomoviruses found in tomato plants in Brazil demanded the development of strategies to minimize losses and to pursue the control through resistant cultivars. This PhD thesis is part of an effort aiming to better understand and to control the tomato begomovirus epidemics in Brazil. A first important step to achieve is the identification of the viruses involved in the disease epidemics and their further characterization at both molecular and biological levels. This is essential to direct breeding programs aiming to develop resistant cultivars. A better understanding of the components involved in the host-virus interaction may help to design new strategies that will contribute to the development of more effective integrated control management.

In chapter 2 an investigation was conducted to establish the identity and genetic diversity of begomoviruses associated with disease outbreaks in tomato fields throughout Brazil. During these studies a novel begomovirus species named *Tomato chlorotic mottle virus* was found to be widespread in the country. Chapter 3 covers the biological characterization, cloning and sequencing of the complete genome of the isolate Tomato chlorotic mottle virus-[Bahia-Seabra1] (ToCMoV-[BA-Se1]). Extensive sequence comparisons with other New World begomoviruses were performed to elucidate the role of recombination in the emergence of these viruses. In chapter 4 the RNA silencing response in plants upon infection with ToCMoV-[BA-Se1] was investigated in terms of production and origin of virus–specific siRNAs. Furthermore, the potential to generate transgenic begomovirus resistance by using the most targeted sequences from the virus genome was explored. To better understand the complexity of interactions of ToCMoV-[BA-Se1] with different plant hosts, chapter 5 investigates the contribution of individual viral genes encoded by ToCMoV-[BA-Se1] in eliciting disease symptoms in local and systemic host plants. As suppression of RNA silencing is often implicated in virulence and symptom induction, the potential role of these ToCMoV proteins in counteracting RNA silencing was also investigated. Finally chapter 6 presents a general discussion on the emergence of tomato begomoviruses in Brazil, the role of RNA silencing in geminivirus infection and the perspectives of using biotechnological strategies for begomovirus resistance.

Chapter 2

Distribution and Genetic Diversity of Tomato-Infecting Begomoviruses in Brazil

Summary

Tomato-infecting begomoviruses have been reported throughout Brazil since the introduction of the B biotype of *Bemisia tabaci*. Here, we report a large scale survey on the distribution and genetic diversity of tomato-infecting begomoviruses. Tomato samples with typical begomovirus symptoms were collected in seven different states, comprising the major tomato growing areas of the country. Viruses were detected by polymerase chain reaction (PCR) using universal primers for the genus *Begomovirus*. PCR-amplified fragments were cloned and sequenced. Based on sequence comparisons and phylogenetic analyses, at least seven previously undescribed species of begomoviruses were found. Four of the new viruses were found exclusively in the Southeastern states, two exclusively in the Northeastern states, and one was found in both regions. Sequence comparisons reveal strong evidence of recombination among the Brazilian begomoviruses. Together, the results indicate the existence of a high degree of pre-existing genetic diversity among tomato-infecting begomoviruses in Brazil and suggest that these viruses have emerged after being transferred from natural hosts to tomatoes, due to the introduction in Brazil of a novel polyfagous biotype of the whitefly vector.

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Introduction

Geminiviruses are small, single-stranded DNA plant viruses with unique particle morphology of twinned incomplete icosahedra. The family *Geminiviridae* is divided into four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*), based on genome structure, type of insect vector and host range (Hanley Bowdoin *et al.*, 1999; Pringle, 1999). Begomoviruses are transmitted by whiteflies (*Bemisia tabaci*) to dicotyledonous plants. Most begomoviruses have bipartite genomes consisting of two ssDNA molecules of approximately 2.6 kb, referred to as DNA-A and DNA-B, which are responsible for different functions in the infection process. DNA-A encodes genes responsible for viral replication (AC1 and AC3), regulation of gene expression (AC2) and particle encapsidation (AV1). DNA B encodes for two proteins, BC1 and BV1, involved in cell-to-cell movement within the plant, host range and symptom modulation (Hanley Bowdoin *et al.*, 1999). The two DNA components of a given begomovirus have little sequence identity, except for a common region (CR) of approximately 200 nucleotides. This region contains the origin of replication and *cis*-acting elements recognized by the AC1 (Rep) protein (Arguello Astorga *et al.*, 1994; Fontes *et al.*, 1994).

In the last two decades the incidence and severity of diseases caused by geminiviruses has increased rapidly in many areas of the world (Brown & Bird, 1992; Polston & Anderson, 1997). The increase on the incidence of begomoviruses is specifically associated with the explosion of *Bemisia* populations (Polston & Anderson, 1997).

In Brazil, the occurrence of six whitefly-transmitted begomoviruses in tomato, including *Tomato golden mosaic virus* (TGMV), was first reported in 1975 in the state of São Paulo (Maytis *et al.*, 1975). Despite this report, the occurrence of begomoviruses in tomato had been of little economical relevance until recently. In the last seven years, a sharp increase in the incidence of begomovirus diseases has been observed in tomato fields in the states of Minas Gerais, São Paulo, Bahia, Pernambuco, and in the Federal District, with yield losses ranging from 40 to 100% (Faria *et al.*, 1997; Ribeiro *et al.*, 1998b). The spread of these viruses has been associated to the introduction and rapid dissemination of the B biotype of *Bemisia tabaci* (França *et al.*, 1996; Ribeiro *et al.*, 1998b). Begomoviruses are currently the main limiting factor to tomato production in many areas of Brazil. However, characterization of these new viruses had not been carried out so far. These studies are essential if a control strategy based on host resistance is to be attempted.

The purpose of this investigation was to establish the identity and genetic diversity among begomoviruses associated with the recent disease outbreaks in tomato fields throughout Brazil. A preliminary report has been published (Ribeiro *et al.*, 1998b).

Methods

Collection of plant samples

Tomato (*Lycopersicon esculentum*) plants showing begomovirus-like symptoms such as chlorosis, yellow or golden mosaic, mottling, leaf curling and distortion, were collected in fields located in several states of Brazil, from 1994 to 1999 (Table 2.1, Fig. 2.1). Fields located in the Northeastern states consisted predominantly of processing tomatoes, while those located in the Southeast were predominantly for fresh market fruits. Individual leaf samples were allowed to dry at room temperature pressed between sheets of paper towel, and stored at -80°C.

Table 2.1 Locations where tomato samples were obtained, dates of collection and designation of viral clones.

Location*	Date of collection	Clone
Brasília, DF	1994	DF-Br1
Bicas, MG	1999	MG-Bi1
Igarapé, MG	1996	MG-Ig1
Igarapé, MG	1999	MG-Ig2
Uberlândia, MG	1996	MG-Ub1
São Fidélis, RJ	1999	RJ-Sf1
Juazeiro, BA	1999	BA-Ju1
Juazeiro, BA	1999	BA-Ju2
Seabra, BA	1996	BA-Se1
Campina Grande, PB	1999	PB-Cg1
Campina Grande, PB	1999	PB-Cg2
Campina Grande, PB	1999	PB-Cg3
Belmonte, PE	1998	PE-Be1
Pesqueira, PE	1998	PE-Pq1
Pesqueira, PE	1997	PE-Pq2†
Pesqueira, PE	1997	PE-Pq3†
Petrolina, PE	1997	PE-Pt1‡
Petrolina, PE	1997	PE-Pt2‡
Petrolina, PE	1997	PE-Pt3‡
Petrolina, PE	1998	PE-Pt4
Petrolina, PE	1999	PE-Pt5
Petrolina, PE	1999	PE-Pt6
Petrolina, PE	1999	PE-Pt7
Mossoró, RN	1999	RN-Mo1

*DF, Federal District; MG, Minas Gerais; RJ, Rio de Janeiro; BA, Bahia; PB, Paraíba; PE, Pernambuco; and RN, Rio Grande do Norte.
†Clones PE-Pq2 and PE-Pq3 were obtained from the same plant sample.
‡Clones PE-Pt1, PE-Pt2 and PE-Pt3 were obtained from the same plant sample.

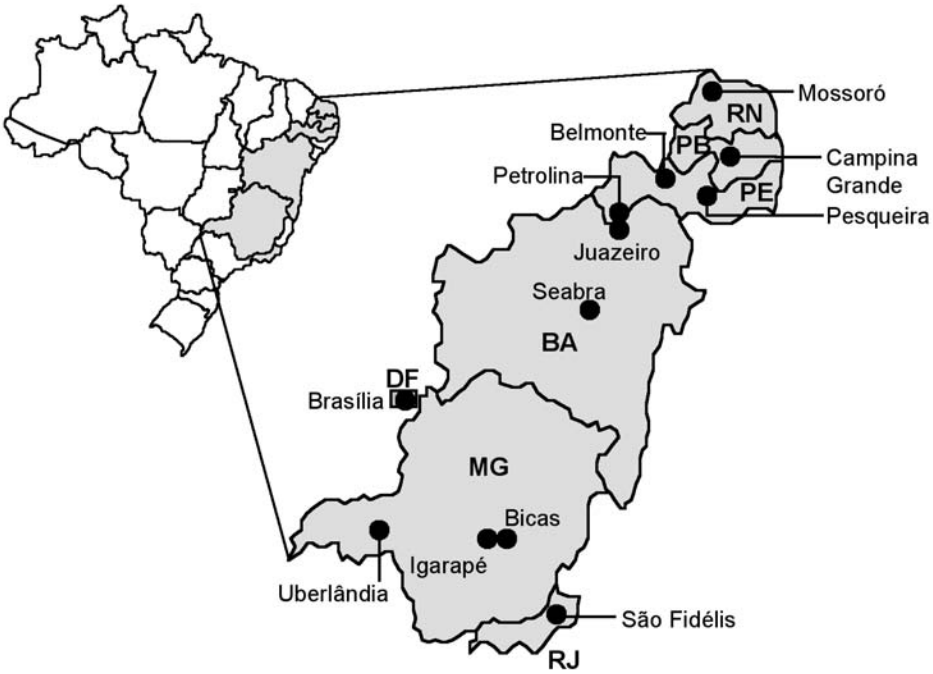


Figure. 2.1. Geographical map of Brazil indicating the locations where tomato samples were collected. DF, Federal District; SP, São Paulo; MG, Minas Gerais; RJ, Rio de Janeiro; BA, Bahia; PB, Paraíba; PE, Pernambuco; RN, Rio Grande do Norte.

DNA extraction, hybridization and PCR

Total DNA was extracted from leaf samples following the procedure described by Dellaporta et al. (1983). DNA pellets were resuspended in water and stored at -20°C.

Two microliters of total DNA were spotted onto nylon membranes (Hybond N+). For some samples, 1 cm leaf disks were directly ground onto the membranes. Membranes were hybridized with the full-length A components of Bean golden mosaic virus (BGMV), Bean golden yellow mosaic virus (BGYMV), Tomato leaf crumple virus (ToLCrV) or Tomato mottle virus (ToMoV), labeled with ^{32}PdCTP by random priming. Hybridizations were carried out at medium stringency (55°C) following standard protocols (Sambrook *et al.*, 1989).

Viral DNA was amplified using primer pairs PAL1v1978/PAR1c715, PAL1v1978/PAR1c496 or PAL1v1978/CP2 (Inoue-Nagata *et al.*, 1999; Rojas *et al.*, 1993). These primer pairs direct the amplification of a fragment of the DNA-A of begomoviruses comprising the 5'-region of the AC1 gene, the entire common region (CR) and the 5'-region or the entire AV1 gene. PCR conditions were as described by Rojas *et al.* (1993). Amplified fragments were visualized in agarose gels stained with ethidium bromide.

Cloning and sequence analysis

PCR-amplified products were either eluted from agarose gels using the GeneClean kit or ethanol-precipitated directly from the PCR reactions, and ligated into the pGEM-T, pGEM-T easy or pCR-script plasmid vectors. One to five clones harboring the expected size inserts for each PCR product were selected for sequencing. Selected clones were completely sequenced in automated ABI sequencers models 310 or 377 using dye terminator cycle sequencing.

Sequences were assembled using the software package from the Genetics Computer Group, Inc. (Devereux *et al.*, 1984). Multiple sequence alignments of nucleotide sequences of the entire cloned fragments as well as of nucleotide and derived amino acid sequences of the N-terminal regions of the AC1 (Rep) and AV1 (CP) proteins were performed with the DNAMAN software package version 4.0 for Windows (Lynnon BioSoft, Quebec, Canada) and Clustal W (www.ebi.ac.uk/clustalw). Phylogenetic trees were generated with the MEGA program (www.megasoftware.net), using UPGMA for nucleotide sequences and neighbor-joining with Poisson correction for amino acid sequences. Tree branches were bootstrapped with 2000 replications (Felsenstein, 1985). The determination of putative recombination events was carried out using the GENECONV program (Padidam *et al.*, 1995). The program was applied to multiple sequence alignments generated by Clustal W, using the command line "geneconv <alignment_file_name>/n0/mkg0.05/g1", where /n0 means to suppress the calculation of permutation-test *P* values, /mkg0.05 means to list all fragments whose BLAST-like *P* value is ≤ 0.05, and /g1 means to set *g* scale = 1. The following begomovirus sequences were used in the phylogenetic and recombination analyses: Abutilon mosaic virus (AbMV, GenBank accession number X15983), African cassava mosaic virus (ACMV, J02057), Bean dwarf mosaic virus (BDMV, M88179), Bean golden mosaic virus (BGMV, M88686), Bean golden yellow mosaic virus (BGYMV, M10070), Leonurus mosaic virus (LeMV, U92532), Pepper huasteco virus (PHV, X70418), Potato yellow mosaic virus (PYMV, D00940), Sida golden mosaic Costa Rica virus (SGMCRV, X99550), Sida golden mosaic Florida virus (SGMFV, AF049336), Squash leaf curl virus (SqLCV, M38183), Tomato golden mosaic virus (TGMV, K02029), Tomato mottle virus (ToMoV, L14460), Tomato leaf crumple virus (ToLCrV, AF101476), Tomato leaf curl virus (ToLCV, S53251), Tomato leaf curl New Delhi virus (ToLCNDV, U15016), Tomato yellow leaf curl virus (TYLCV, AF105975), Tomato yellow leaf curl Sardinia virus (TYLCSV, X61153), and Tomato yellow vein streak virus (TYVSV, U79998).

Results

Tomato samples with typical begomovirus symptoms were collected in several states of Brazil's Southeastern and Northeastern regions from 1994 to 1999 (Fig. 2.1). A total of 323 samples were collected, and 185 tested positive for the presence of begomoviruses. All samples that tested positive by hybridization also yielded PCR fragments of expected sizes.

The selection of samples for cloning viral fragments was based primarily on the locations where samples were collected. When more than one sample from a given location tested positive by PCR, fragments from at least two samples were cloned. For some locations with a large number of positive samples, PCR-amplified fragments were directly sequenced using the PAR1c496 primer. Fragments were retained or discarded based on the identity of sequences obtained. Using these criteria, a final number of 24 clones, comprising samples from seven Brazilian states, were selected for subsequent phylogenetic analyses (Table 2.1). In two cases, different sequences were obtained from clones from the same plant sample (Table 2.1), indicating the occurrence of mixed infections in the field. Analyses were carried out using a 1152 nt fragment, from which an N-terminal sequence of the AC1 (Rep) protein with 221 amino acids, and an N-terminal sequence of the capsid protein with 47 aa, were deduced. The fragment also included the entire intergenic region of the DNA-A, including the origin of replication.

The sequences of the intergenic regions from all 24 clones were aligned using Clustal W (data not shown). This region contains a series of *cis*-acting elements involved in DNA replication and transcription of the AC1 gene (Arguello Astorga *et al.*, 1994). All such elements are present in the sequences of the 24 tomato begomovirus clones, including (i) an inverted repeat and two direct repeats (binding sites for the AC1 (Rep) protein), (ii) the TATA box for the AC1 gene, and (iii) a conserved stem-loop motif which includes the nonanucleotide sequence nicked by the AC1 (Rep) protein to initiate DNA replication. Although the sequences of the inverted and direct repeats show significant variation among the clones, the stem-loop and the nonanucleotide sequences are completely conserved.

The nucleotide sequence identities for the 1152 nt fragments from all 24 clones ranged from 66 to 100% (sequence identity tables can be examined at <www.ufv.br/dfp/virologia/gemini/tables.html>). Similar values were obtained when only the CP amino acid sequence, which has been used to establish taxonomical relationships among begomoviruses (Brown *et al.*, 2001; Padidam *et al.*, 1995; Rybicki, 1994), was analyzed. When the 1152 nt fragment was submitted to a BLAST search, identity values were always below 80%, except for clone MG-Bi1 (85% identical to BGMV) (data not shown). When the CP sequences of the 24 tomato begomovirus clones were

submitted to a BLAST search, identities were always below 90%, except for clones MG-Ig2 (95% identical to LeMV), and MG-Bi1 (93% identical to BGMV) (data not shown). Phylogenetic trees were prepared based on the nt sequence of the entire fragment, or the aa sequence of the CP (Fig. 2.2).

When the entire fragment was analyzed, clones DF-Br1 and PE-Pq3 were less than 82% identical to all other tomato clones, as well as to previously characterized species. Similar values were found when their AC1 (Rep) or AV1 (CP) aa sequences were used for the analyses. Moreover, these two clones were placed in distinct branches in both phylogenetic trees (Fig. 2.2a). These results suggest that these two clones represent two distinct, new begomovirus species.

A third new species is represented by clones PE-Be1, PE-Pq1, PE-Pt4, RJ-Sf1, BA-Ju2, BA-Se1 and MG-Ig1. The complete DNA-A nucleotide sequences of clones BA-Se1 and MG-Ig1 have been determined (GenBank accession numbers AF490004 and AY049206, respectively), and the name Tomato chlorotic mottle virus (ToCMoV) is proposed for this species. Phylogenetic analysis revealed three subgroups of ToCMV isolates (Fig. 2.2a). The first is comprised of clones PE-Be1, PE-Pq1 and PE-Pt4 (which are 99.9% identical to each other in terms of sequence). The second is comprised of clones RJ-Sf1 and BA-Ju2, with nt sequence identities ranging from 82 to 90% for the entire fragment. The third subgroup is comprised of clones MG-Ig1 and BA-Se1. These two clones are closely related to each other, with nt sequence identities greater than 89% for either their entire fragments or their AC1, CR and CP regions. The nt sequences of these clones, and the aa sequences of their AV1, are 84-86% and 85-89% identical, respectively, to the other five clones representing ToCMV. However, their AC1 (Rep) and CR sequences are only 80-86% and 77-78% identical at the aa and nt levels, respectively, to those clones. The GENECONV program failed to detect potential recombination events between these clones. Therefore, although these seven clones are certainly related based both on nucleotide sequence comparisons and phylogenetic analysis, it is difficult to conclude, based solely on these analyses, whether they actually comprise a single species or should be further subdivided into distinct species. Infectious clones of isolates BA-Se1 and MG-Ig1 have been obtained (S.G. Ribeiro, E.C. Andrade and F.M. Zerbini, manuscript in preparation), and further genetic studies using these clones should clarify their taxonomical relationships.

A fourth new species is represented by clones BA-Ju1, PB-Cg1, PB-Cg2, PB-Cg3, PE-Pq2, PE-Pt1, PE-Pt2, PE-Pt3, PE-Pt5, PE-Pt6, PE-Pt7, and RN-Mo1. Identities between any two of these sequences are never below 87% for the entire fragment (nt) or 83% for the CP (aa). These clones were also placed in the same branch of both phylogenetic trees, although the genetic distances were higher in the tree based on the entire fragment when compared to the CP tree (Fig. 2.2a). Within the clones that

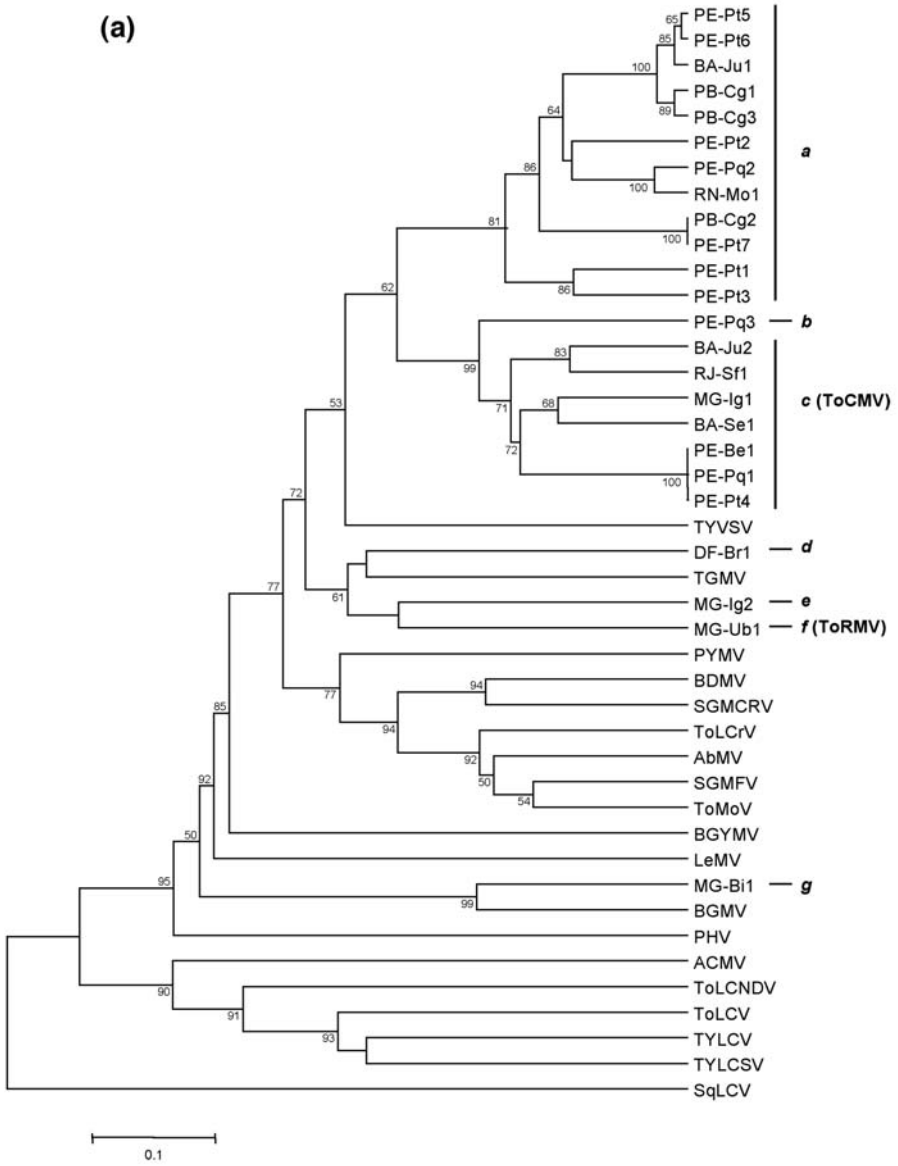
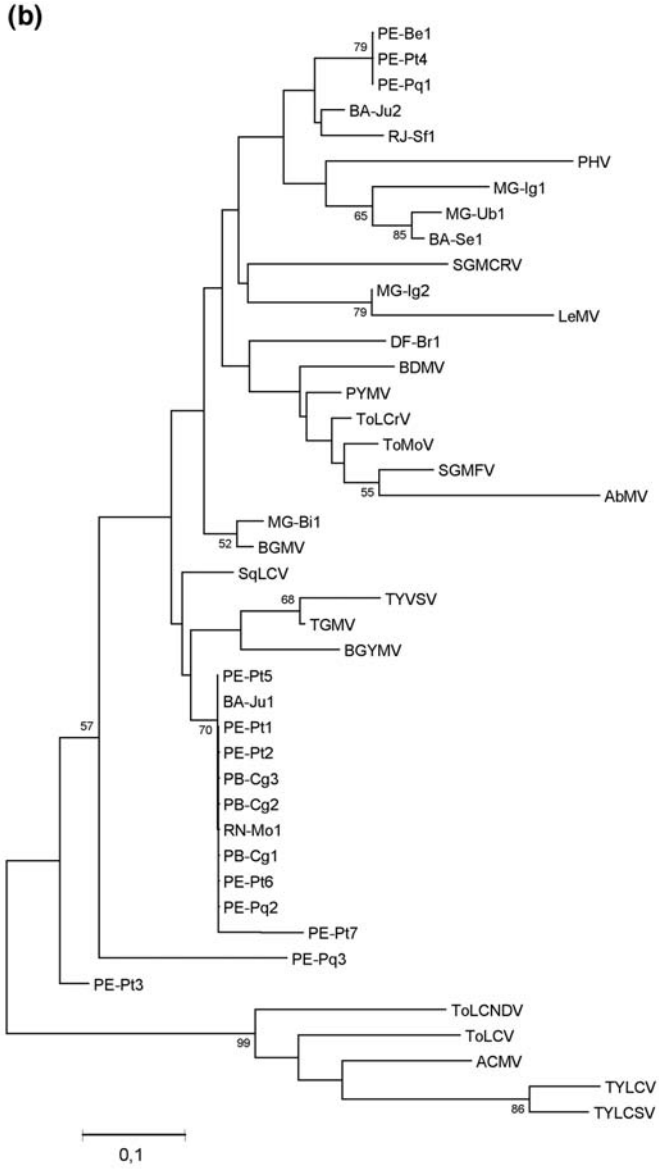


Figure 2.2. Phylogenetic trees constructed based on the nucleotide sequence of the entire fragment sequenced (a), or the deduced 47 amino acid sequence of the capsid protein (b). Trees were generated with the MEGA program (www.megasoftware.net), using UPGMA for nucleotide sequences and neighbor-joining with Poisson correction for amino acid sequences. Tree branches were bootstrapped with 2000 replications. The scale bar represents Nei's genetic distance. The seven new begomovirus species proposed are indicated to the right of tree based on the entire fragment.


(b)
PE-Be1
PE-Pt4
PE-Pq1
BA-Ju2
RJ-Sf1
PHV
MG-Ig1
MG-Ub1
BA-Se1
SGMCRV
MG-Ig2
LeMV
DF-Br1
BDMV
PYMV
ToLCrV
ToMoV
SGMFV
AbMV
MG-Bi1
BGMV
SqLCV
TYVSV
TGMV
BGYMV
PE-Pt5
BA-Ju1
PE-Pt1
PE-Pt2
PB-Cg3
PB-Cg2
RN-Mo1
PB-Cg1
PE-Pt6
PE-Pq2
PE-Pt7
PE-Pq3
PE-Pt3
ToLCNDV
ToLCV
ACMV
TYLCV
TYLCSV
79
65
85
79
55
52
68
57
70
99
86
0,1

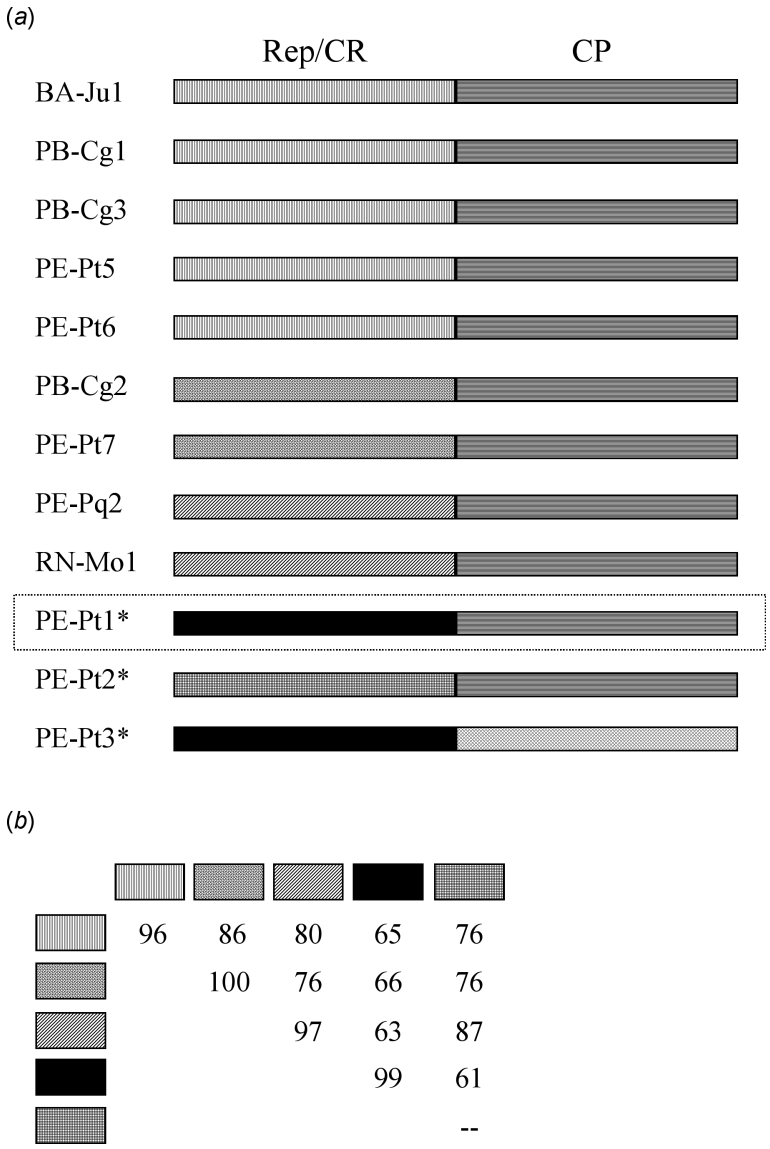


Figure 2.3. (a) Schematic representation of the Rep/CR and CP regions from the 12 related isolates represented by clone BA-Ju1. Each type of Rep/CR and CP sequence is indicated by a distinct pattern: five patterns for Rep/Cr and two patterns for the CP. Clones marked with an asterisk were obtained from the same plant sample. The putative recombinant isolate PE-Pt1, which shares the same Rep/CR with isolate PE-Pt3 and the same CP with isolate PE-Pt2, is indicated by the dotted box. (b) Average nucleotide sequence identities between the five distinct types of Rep/CR regions.

represent this species, three clusters of highly identical clones can be identified: clones PB-Cg2 and PE-Pt7 are 100% identical, clones PE-Pq2 and RN-Mo1 are 97% identical, and clones BA-Ju1, PB-Cg1, PB-Cg3, PE-Pt5 and PE-Pt6 are at least 95% identical to each other. Clones PE-Pt1, PE-Pt2 and PE-Pt3 were obtained from the same plant sample. When the AC1, RC and AV1 regions of these clones are compared separately, it is clear that clones PE-Pt1 and PE-Pt3 share the same AC1 and CR (99% identity in both cases, aa for AC1 and nt for CR), while clones PE-Pt1 and PE-Pt2 share the same CP (100% aa identity). Therefore, clone PE-Pt1 seems to be a recombinant between PE-Pt2 and PE-Pt3. This hypothesis was reinforced by gene conversion analysis using the GENECONV program, which detected potential recombination events between these clones (the output from the GENECONV analysis can be found at <www.ufv.br/dfp/virologia/gemini/ geneconv_res.html>). In fact, when the AC1, CR and AV1 regions of all clones representing this fourth species are compared separately, a complex picture of distinct AC1/CR and AV1 sequences arises (Fig. 2.3a). All clones, except PE-Pt3, share the same type of AV1. However, five distinct types of AC1/CR sequences can be identified. The nt sequence identity between these distinct types of AC1/CR sequences can be as low as 61% (Fig. 2.3b), suggesting that trans-replication may not occur between such viruses. In agreement with the results of sequence comparisons, clones PB-Cg2 and PE-Pt7 have the same types of AC1/CR and AV1 sequences. The same is true for clones PE-Pq2 and RN-Mo1, and for clones BA-Ju1, PB-Cg1, PB-Cg3, PE-Pt5 and PE-Pt6. Together, these results indicate that this species comprises a number of distinct strains, which probably originated via recombination between distinct, unidentified parent viruses.

The nt sequence identity for the entire fragment of clone MG-Ub1 was always below 80% with all other tomato clones or previously characterized species. However, its CP is 96% identical (aa) to the one from clone BA-Se1. Clone MG-Ub1 was placed in the same branch of clone BA-Se1 in the tree based on the CP sequence (Fig. 2.2b). However, these two clones were placed in completely distinct branches of the tree based on the sequence of entire fragment (Fig. 2.2a). The possibility of MG-Ub1 being a recombinant was tested with the GENECONV program. However, this analysis failed to identify potential recombination events between MG-Ub1 and BA-Se1, or with any other clone or previously characterized species. Thus, clone MG-Ub1 represents a fifth new species. This clone has been completely sequenced (GenBank accession numbers AF291075 and AF291706), and the name Tomato rugose mosaic virus (ToRMV) is proposed.

The nt sequence of the entire fragment from clone MG-Bi1 is 85% identical to BGMV. The AC1 and AV1 proteins from these viruses share 82 and 94% identity (aa), respectively, and their CRs are 77% identical (nt). Clone MG-Bi1 was placed in the same

branch of BGMV in both trees (Fig. 2.2). However, since they have distinct host ranges (BGMV does not infect tomatoes) and probably do not *trans*-replicate, BGMV and MG-Bi1 are probably better considered to be distinct, albeit related, species. Similarly, the nucleotide sequence of clone MG-Ig2 is 74% identical to LeMV, and their AC1 and AV1 proteins share 66 and 95% identity, respectively. Clone MG-Ig2 was placed in the same branch of LeMV in the CP tree, but in distinct branches in the tree based on the entire fragment. Since LeMV does not infect tomatoes (J.C. Faria, *personal communication*) and the two viruses probably do not *trans*-replicate, clone MG-Ig2 might also represent a distinct, new species.

Together, our results confirm the widespread occurrence of tomato-infecting begomoviruses in Brazil, and indicate the existence of at least seven new begomovirus species in the country.

Discussion

Begomoviruses have been present in Brazil for a long time, although they were not recognized as a distinct group of plant viruses until the 1970's. Reports of whitefly-transmitted viral diseases of *Abutilon* and other species of the Malvaceae date as far back as the 1950's (Costa, 1965a; Costa & Bennet, 1950). Bean golden mosaic virus (BGMV) was first described in Brazil in the 1960's (Costa, 1965b). The incidence of BGMV increased dramatically in Brazil during the 1970's, due to the increasing acreage occupied by soybeans, an excellent host of the whitefly (Costa, 1975). The first report of tomato-infecting begomoviruses in Brazil (including TGMV) appeared at this time (Maytis *et al.*, 1975). Interestingly, tomato-infecting begomoviruses did not become an economical problem in the country, most likely because the A biotype of *Bemisia tabaci*, which was prevalent at the time, does not normally colonize tomatoes. The B biotype of *B. tabaci*, which readily colonizes tomato plants (Brown *et al.*, 1995), was observed in Brazil for the first time in 1994, and quickly disseminated throughout the warm and dry areas of the country (França *et al.*, 1996).

The emergence of tomato-infecting begomoviruses in Brazil displayed a remarkable correlation to the introduction and dissemination of the B biotype of *B. tabaci* throughout the country. A preliminary sequence analysis of PCR fragments from these viruses indicated that they represented several new begomovirus species (Ribeiro *et al.*, 1998), suggesting that the vector was transferring indigenous viruses from weed and/or wild hosts into the tomato.

Although symptoms like yellow or golden mosaic, leaf crumpling, epinasty, down cupping, and dwarfing were commonly associated with begomovirus infection, it

was difficult to establish a precise relationship between a particular type of symptom and a given viral species. Symptoms are highly dependent on the host genotype, environmental conditions, or the occurrence of mixed infections. Infectious clones of some of these new begomoviruses have been recently obtained (S.G. Ribeiro, E.C. Andrade, J.J. Fernandes and F.M. Zerbini, *unpublished results*), which should allow for a better correlation between viral species and symptoms to be determined. However, this correlation might be of little practical use, since mixed infections will continue to occur in the field. In view of these facts, our approach to viral characterization was based mainly on molecular data.

Detailed analysis of the sequences of all 24 clones demonstrates the existence of previously undescribed begomovirus species infecting tomatoes in Brazil. At least seven putative new species were identified. Phylogenetic analysis placed these viruses in distinct clades, apart from all the previously characterized New World begomoviruses, but more closely related to begomoviruses from Brazil (TYVSV, TGMV and BGMV). This suggests that these viruses are indigenous to Brazil, and have not been introduced from other regions. A likely scenario for such rapid emergence would be the transferring of these viruses from local wild hosts to tomatoes, followed by rapid adaptation to the new host via recombination or pseudorecombination. The new viruses are then disseminated by the insect vector or due to the movement of infected transplants. Detection of ToCMoV in Belmonte (PE), Igarapé (MG), Juazeiro (BA), Pesqueira (PE), Petrolina (PE), São Fidélis (RJ) and Seabra (BA), which are up to 2000 km apart (Fig. 2.1), suggests that dissemination of these viruses within the country is rapidly taking place. Alternatively, the fact that samples infected by ToCMoV were collected at both Igarapé and Seabra in 1996 (Table 2.1) suggests that the same virus could have emerged simultaneously in these regions.

The analysis of the 12 related clones BA-Ju1, PB-Cg1, PB-Cg2, PB-Cg3, PE-Pq2, PE-Pt1, PE-Pt2, PE-Pt3, PE-Pt5, PE-Pt6, PE-Pt7 and RN-Mo1 illustrates the hypothesis that new begomoviruses evolve by recombination or pseudorecombination between previously existing species (Padidam *et al.*, 1999; Rybicki, 1994; Torres Pacheco *et al.*, 1993; Zhou *et al.*, 1997). Comparisons between distinct regions of the amplified DNA-A fragments present a strong indication that clone PE-Pt1 is a recombinant between PE-Pt2 and PE-Pt3: it contains the CP sequence of PE-Pt2 and the AC1/CR sequence of PE-Pt3. The fact that these three clones were obtained from the same plant sample further supports this hypothesis. There are several well-documented cases of recombination among begomoviruses, in many cases leading to the emergence of more virulent or better-adapted viruses in specific host plants. Such is the case of *Pepper huaste-*

co virus in Mexico, which is a recombinant between a New World and an Old World begomovirus (Torres Pacheco *et al.*, 1993). Also, an unusually virulent isolate of African cassava mosaic virus (ACMV) from Uganda was shown to be a recombinant between ACMV and Eastern African cassava mosaic virus (EACMV) (Zhou *et al.*, 1997).

A different perspective on the genetic diversity of tomato-infecting begomoviruses in Brazil is the existence of at least four distinct species in the state of Minas Gerais, and three distinct species in the state of Pernambuco. Three distinct species were found in Bicas and Igarapé (MG), located less than 30 km apart from each other. Two distinct species were found in Petrolina (PE), and also in Pesqueira (PE). All of these cities are located in major tomato-growing areas. The fact that distinct viruses were detected at such close proximity is further evidence of the simultaneous transfer of pre-existing indigenous viruses from wild hosts into tomatoes.

It is possible that, as these new viruses adapt to their new host, one or two viral species will start to predominate and, in the medium to long term, will displace less adapted species, similar to the situation that has been occurring in Mexico over the last two decades (Polston & Anderson, 1997). Several begomoviruses were described in that country over this period of time. Over the years, the incidence of some viruses, such as ToLCrV, has been reported to have decreased, accompanied by a simultaneous increase in the incidence of other species, such as PHV (Polston & Anderson, 1997; Torres Pacheco *et al.*, 1996). The realization that such scenario could be taking place in Brazil is of great importance if a management strategy based on host resistance is to be attempted to control these viruses. Unless a source of resistance with a broad spectrum is identified, the identification of species-specific resistance genes might lead to a quick breakdown of resistance, due to the frequent emergence of new species and the displacement of species less adapted to tomato by fitter ones. It is interesting to note the contrast between the high degree of genetic variability of tomato-infecting begomoviruses in Brazil with the low variability found among *Phaseolus*-infecting begomoviruses (Faria & Maxwell, 1999). In this case, it would seem the virus and its hosts have been co-evolving for a significantly greater period of time, which probably allowed selection of better adapted viruses to predominate in the field, resulting in lower genetic diversity.

The introduction of the B biotype of *Bemisia tabaci* and the subsequent emergence of tomato-infecting begomoviruses in Brazil has resulted in increased cost of tomato production as well as additional environmental costs due to pollution by increased insecticide use. Clearly, management strategies based on host resistance would be highly preferable. However, these can only be successfully implemented if the complex diversity and evolutionary aspects of the viruses involved is fully recognized.

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Chapter 3

Molecular and biological characterization of *Tomato chlorotic mottle virus* suggests that recombination underlies the evolution and diversity of Brazilian tomato begomoviruses

Summary

Tomato chlorotic mottle virus (ToCMoV) is a new begomovirus species currently emerging throughout tomato growing areas of Brazil. We have determined the first complete genome sequence of an infectious ToCMoV genome. The ToCMoV isolate BA-Se1 (ToCMoV–[BA-Se1]), is also the first begomovirus isolated in the northeast of Brazil. When introduced as infectious clone by particle bombardment into tomato, the resulting replicating ToCMoV–[BA-Se1] DNA-A and DNA-B components caused typical chlorotic mottle symptoms. The cloned virus was whitefly transmissible and while it was infectious in hosts such as *Nicotiana benthamiana,* pepper, tobacco and *Nicandra physaloides*, it was unable to infect *Arabidopsis thaliana*, bean, *N. glutinosa* and *Datura metel*. Sequence and biological analyses indicate that ToCMoV–[BA-Se1] is a typical New World begomovirus requiring both DNA-A and DNA-B components to establish systemic infections. Although evidence of multiple recombination events was detected within the ToCMoV–[BA-Se1] DNA-A, they apparently occurred relatively long ago implying that recombination has probably not contributed to the recent emergence of this species.

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Introduction

The genus *Begomovirus* encompasses geminiviruses that are transmitted by the whitefly *Bemisia tabaci* and exclusively infect dicotyledonous plants. Begomoviruses have emerged everywhere in the world where environmental conditions support large *B. tabaci* populations, and have become a major constraint in the production of food and fiber crops such as cassava, tomato, cucurbits, pepper, beans and cotton (Morales & Anderson, 2001; Varma & Malathi, 2003).

Begomoviruses indigenous to the Western hemisphere (New World begomoviruses) have bipartite genomes with components referred to as DNA-A and DNA-B. DNA-A encodes genes responsible for viral replication, regulation of gene expression, suppression of gene silencing and particle encapsidation. DNA-B encodes proteins involved in virus movement, host range determination and symptom development (Lazarowitz, 1992; Rojas *et al.*, 2005).

On the American continent tomato represents the vegetable crop currently suffering most seriously from begomovirus infection. Infestations that can result in up to 100% yield losses often have severe economic and social consequences (Faria *et al.*, 2000; Morales & Anderson, 2001; Polston & Anderson, 1997; Varma & Malathi, 2003). Although tomato is the most common host from which New World begomoviruses have been isolated (Fauquet & Stanley, 2003; Varma & Malathi, 2003), there is no reason to suppose that all begomovirus species threatening tomato have yet been sampled. Considering the current rate at which new begomovirus species are being discovered, it is probable that many more hitherto undescribed species are present in nature yet to emerge as a threat to tomato cultivation in the Americas.

Before the 1990's the only known begomovirus disease of tomatoes in Brazil was that caused by Tomato golden mosaic virus (TGMV). However, the introduction and rapid dispersal of a new *Bemisia tabaci* biotype (referred to as biotype B) throughout Brazil in the mid 1990's coincided with a massive increase in the prevalence and severity of tomato-infecting begomoviruses, and associated disease problems in tomatoes have escalated to epidemic proportions (Faria *et al.*, 2000; Ribeiro *et al.*, 1998b). Partial characterization of begomoviruses collected in the main tomato producing areas of Brazil during these outbreaks has revealed that they have an extraordinarily high degree of genetic diversity, with at least nine new emergent species believed to be present in the country (Faria *et al.*, 2000; Ribeiro *et al.*, 2003). Although there is ample information available on TGMV, very little is known about the other recently discovered tomato-infecting begomovirus species.

One of these new species is Tomato chlorotic mottle virus (ToCMoV). While ToCMoV was the first begomovirus species isolated in the northeastern part of Brazil (Faria *et al.*, 2000; Ribeiro *et al.*, 2003), it appears to be widely distributed and is also

present in several states in the southeastern region of the country (Ambrozevicius *et al.*, 2002; Ribeiro *et al.*, 2003). The DNA-A component of a ToCMoV isolate (Mg-Bt1) from the southeastern state Minas Gerais was previously isolated and reported to systemically infect *Nicotiana benthamiana* plants, but not tomato, its natural host (Galvão *et al.*, 2003). This chapter reports the biological characterization and cloning and sequencing of the complete ToCMoV-[BA-Se1] genome. Extensive sequence comparisons with other New World begomoviruses were performed to elucidate the role of recombination in the emergence of these viruses.

METHODS

Virus isolate

In 1996 tomato leaf samples with yellow mottle symptoms were collected in Seabra, Bahia, Brazil. Total DNA was isolated from the infected leaves (Dellaporta *et al.*, 1983) and evaluated for the presence of begomovirus by PCR using the degenerate primer pair PAL1v1978 and PAR1c715 for DNA-A and CRC2 and PBL1v2040 for DNA-B (Rojas *et al.*, 1993). For propagation of the isolate, total DNA was precipitated onto tungsten particles and delivered to healthy seedlings of *Nicotiana benthamiana* by particle bombardment (Aragão *et al.*, 1995).

Cloning and sequencing of the viral genome

Total DNA was isolated from *N. benthamiana* infected leaf, digested with several restriction enzymes and analyzed by Southern blot, using DNA-A and DNA-B PCR fragments (labeled by random priming with ^{32}P), as probes to identify the enzymes with single cutting sites in each component. From this analysis, *Kpn* I and *Hind* III appeared to linearize DNA-A and DNA-B, respectively. Hence, these enzymes were used to digest total DNA. *Kpn* I and *Hind* III digested DNA was fractionated in agarose gel and fragments ranging from 2.0 to 3.0 kb were excised from the gel and ligated into either *Kpn* I or *Hind* III digested pBS KS^+. Recombinant clones were identified by colony hybridization with DNA-A or DNA-B probes. Full-length DNA-A and DNA-B clones were completely sequenced. Open reading frame (ORF) determination, sequence analysis and comparisons were performed using the ORF finder and BLAST web servers and DNAMAN Version 4.0 (Lynnon Biosoft, Quebeck, Canada) software package.

Infectivity of cloned ToCMoV-[BA-Se1] genome components

To test the infectivity of cloned ToCMoV-[BA-Se1] genome components, DNA-A and DNA-B were cut from their vector with *Kpn* I or *Hind* III, respectively, and self-

ligated to form closed circular dsDNA molecules. Approximately 5 µg of each component separately, or a mixture of 2.5 µg of each component, were precipitated onto tungsten particles and inoculated by particle bombardment into *Lycopersicon esculentum* 'Viradoro' seedlings. Plants bombarded only with pBS KS^+ were included as negative controls. Plants were monitored for symptom development and infection was confirmed by squash blot hybridization using probes specific for ToCMoV-[BA-Se1] DNA-A and DNA-B.

Whitefly transmission

To test whether the progeny of cloned ToCMoV-[BA-Se1] components were whitefly transmissible, symptomatic tomato plants inoculated by particle bombardment were used in transmission experiments. Individuals of *B. tabaci* biotype B were reared on squash plants, transferred to an infected tomato plant and allowed an acquisition access period of 48 hours. Five adult flies were subsequently transferred to a clip cage and placed in contact with the abaxial leaf surface of a healthy tomato plant for an inoculation access period of 48 hours. Two cages were placed on each test plant. The plants were observed for symptom development and analyzed for the presence of virus by squash hybridization.

Experimental host range of ToCMoV-[BA-Se1]

During host range experiments with ToCMoV-[BA-Se1], we inoculated potential hosts both mechanically and by particle bombardment. Seedlings of *L. esculentum* ('Santa Clara' and 'Viradoro'), *Nicotiana tabacum* ('TNN'), *N. benthamiana*, *Nicandra physaloides, Datura stramonium, D. metel, Capsicum annuum* ('Margarita' and 'AG10') and *Glycine max* ('Celeste' and 'Conquista') were inoculated by particle bombardment as described above. Mechanical inoculations were conducted by grinding *N. benthamiana* infected leaf tissue in phosphate buffer pH 8.0, containing 25 mM EDTA and 0.01% sodium sulfite, and rubbing the extract onto carborundum dusted leaves of *Arabidopsis thaliana* var. Columbia, *L. esculentum* ('Money maker' and 'Tiny Tim'), *C. annuum* ('Pikante reuzen'), *N. tabacum* ('White burley'), *N. rustica, N. rosellata, N. clevelandii, N. benthamiana, D. stramonium, Petunia hybrida* and *Phaseolus vulgaris* ('Pinto'). Mock inoculated plants were used as negative controls. Plants were monitored and scored for symptoms. Virus infection was confirmed by squash blot and/or PCR using degenerated primers.

Reassortment experiments

To test if ToCMoV-[BA-Se1] and the closely related virus, Tomato rugose mosaic virus (ToRMV) (Fernandes, 2002) were able to form viable pseudorecombinants, infectious DNA-A and DNA-B clones of both viruses were mixed and inoculated, in all

possible combinations, into *N. benthamiana* seedlings by particle bombardment. Results were scored by visual observation of symptom development and squash blot using virus-specific probes.

Phylogenetic analysis

Multiple alignments of complete New World begomovirus DNA-A and DNA-B components were prepared using ClustalX (Thompson *et al.*, 1997). Names, acronyms and accession numbers for these viruses are presented in Table 3.1. Phylogenetic trees were constructed using the maximum parsimony method implemented in PAUP version

Table 3.1: Begomoviruses* used in comparative analysis with their respective acronym and accession numbers.

		Accession numbers	
Geminivirus	**Acronym**	A component	B component
Abutilon mosaic virus	AbMV	X15983	X15984
Abutilon mosaic virus- HW	AbMV-HW	U51137	U51138
African cassava mosaic virus- [Cameroon]	ACMV-[CM]	NI†	AF112353
Bean calico mosaic virus	BCaMV	AF110189	AF110190
Bean dwarf mosaic virus	BDMV	M88179	M88180
Bean golden mosaic virus - [Brazil]	BGMV-[BR]	M88686	M88687
Bean golden yellow mosaic virus [Dominican Republic]	BGYMV-[DO]	L01635	L01636
Bean golden yellow mosaic virus [Guatemala]	BGYMV-[GT]	M91604	M91605
Bean golden yellow mosaic virus- [Mexico]	BGYMV-[MX]	AF173555	AF173556
Bean golden yellow mosaic virus [Puerto Rico]	BGYMV-[PR]	M10070	M10080
Cabbage leaf curl virus	CaLCuV	U65529	U65530
Chino del tomate virus-[H6]	CdTV-[H6]	AF226665	NA‡
Chino del tomate virus-[H8]	CdTV-[H8]	AF226664	NA
Chino del tomate virus-[IC]	CdTV-[IC]	AF101476	AF101472
Cotton leaf crumple virus	CLCrV	AF480940	NI
Cucurbit leaf curl virus	CuLCuV	AF224760	AF224761
Cucurbit leaf curl virus-[Arizona]	CuLCuV-[AZ]	AF256200	AF327556
Dicliptera yellow mottle virus	DiYMV	AF139168	AF170101
Macroptilium mosaic Puerto Rico virus	MaMPRV	AY044133	AY044134

Macroptilium yellow mosaic Florida virus	MaYMFV	AY044135	AY044136
Macroptilium yellow mosaic virus	MaYMV	AJ344452	NA
Melon chlorotic leaf curl virus	MCLCuV	AF325497	NA
Nicandra deforming necrosis virus	NDNV	unpublished	NA
Pepper huasteco yellow vein vairus	PHYVV	X70418	X70419
Potato yellow mosaic Panama virus	PYMPV	Y15034	Y15033
Potato yellow mosaic Trinidad virus	PYMTV	AF039031	AF039032
Potato yellow mosaic virus-[Venezuela]	PYMV-[VE]	D00940	D00941
Potato yellow mosaic virus-[Gadalupe]	PYMV-[GP]	AY120882	NI
Rhyncosia golden mosaic virus	RhGMV	AF239671	NA
Sida golden mosaic Costa Rica virus	SiGMCRV	X99550	X99551
Sida golden mosaic Honduras virus	SiGMHV	Y11097	Y11098
Sida golden mosaic virus	SiGMV	AF049336	AF039841
Sida micrantha mosaic virus -[A1B3]	SimMV-[A1B3]	AJ557450	AJ557454
Sida micrantha mosaic virus -[A2B2]	SimMV-[A2B2]	AJ557451	AJ557453
Sida mottle virus	SiMoV	AY090555	NA
Sida yellow mosaic virus	SiYMV	AY090558	NA
Sida yellow vein virus	SiYVV	Y11099	Y11100
Squash leaf curl virus	SLCV	M38183	M38182
Squash mild leaf curl virus	SMLCV	AF421552	AF421553
Squash yellow mild mottle virus-[Costa Rica]	SYMMoV-[CR]	AY064391	AF440790
Sweet potato leaf curl Georgia virus	SPLCGV	AF326775	NA
Sweet potato leaf curl virus	SPLCV	AF104036	NA
Tomato chlorotic mottle virus-[BA-Se1]	ToCMoV-[BA-Se1]	AF490004	AF491306
Tomato chlorotic mottle virus-[MG-Bt-1]	ToCMoV-[MG-Bt1]	AY090557	NA
Tomato crinkle leaf yellow virus	ToCrLYV	NA	AY090556
Tomato golden mosaic virus-[Yellow vein]	TGMV-[YV]	K02029	K02030
Tomato mosaic Havana virus-[Quivican]	ToMHV-[Qui]	Y14874	Y14875
Tomato mosaic leaf curl virus	ToMLCV	AY508991	NI
Tomato mottle Taino virus	ToMoTV	AF012300	AF012301
Tomato mottle virus-[Florida]	ToMoV-[FL]	L14460	L14461
Tomato rugose mosaic virus	ToRMV	AF291705	AF291706
Tomato severe leaf curl virus-[Guatemala 96-1]	ToSLCV-[GT96-1]	AF130415	NA
Tomato severe rugose virus	ToSRV	AY029750	NA
Tomato yellow margin leaf curl virus	ToYMLCV	AY508993	NI
Tomato yellow leaf curl virus-[Dominican Republic]	TYLCV-[DO]	AF024715	M§

*Begomoiviruses are named according to ICTV (Stanley et al., 2005)
† Not included
‡ Not available
§ Monopartite virus

4.0b10 (Swofford, 1998). A most parsimonious tree was searched for using the heuristic method with stepwise addition and the tree bisection-reconnection branch-swapping options. Bootstrap analysis was performed using 1000 replicates. Tomato yellow leaf curl virus (TYLCV-[DO]) and African cassava mosaic virus (ACMV-[CM]) were used as outgroups for DNA-A and DNA-B phylogenetic trees, respectively.

Recombination analysis

An alignment of 55 New World begomovirus DNA-A sequences was analyzed for recombination events involving either of the two ToCMoV isolates, [BA-Se1] and [MG-Bt1] using the RDP (Martin & Rybicki, 2000), GENECONV (Padidam *et al.*, 1999), BOOTSCAN (Martin *et al.*, 2005a), MAXIMUM CHI SQUARE (Maynard Smith, 1992), CHIMAERA (Martin *et al.*, 2005b) and SISTER SCAN (Gibbs *et al.*, 2000) detection methods as implemented in RDP2 (Martin *et al.*, 2005b). A multiple comparison corrected P-value cutoff of 0.05 and default settings were used throughout and only events detectable with two or more different methods were retained for further analysis. For each event involving either of the two ToCMoV isolates as donor or acceptor sequences, two neighbor joining trees were constructed using portions of the alignment bounded by the two detected recombination breakpoints. The minimum number of branch transplants needed to convert the tree topology of one tree into that of the other was used to infer which of the sequences used to detect recombination events were donors or acceptors (McLeod *et al.*, 2005). In the case of ties between two sequences, a distance based measurement based on that used in the PHYLPRO method (Weiller, 1998) was used as a tie-breaker. Both methods are implemented in the beta version of RDP3, which is available on request from DPM. An alignment of 33 New World begomovirus DNA-B sequences was examined for evidence of recombination events involving ToCMoV [BA-Se1] in a manner identical to that described for the DNA- A sequences.

Results

Cloning and infectivity of cloned viral components

Begomovirus infections were initially detected by PCR in field-infected tomato plants collected in Seabra, Bahia, Brazil, using degenerate primers. Primer pair PAL1v1978 and PAR1c715 yielded a ~1400 nt fragment of DNA-A sequence, whereas CRC2 and PBL1v2040 yielded a ~400 nt fragment of DNA-B sequence (Ribeiro *et al.*, 2003).

Several potentially full-length clones were obtained from total DNA extracted from *N. benthamiana* infected by particle bombardement with DNA from diseased tomato. Recombinant plasmids harboring inserts of ~2600 nt at *Kpn* I or *Hind* III sites

were identified as DNA-A or DNA-B, respectively, by Southern hybridization with PCR-derived component-specific probes. Two clones representing DNA-A (pK56 and pK133) and two DNA-B clones (pH81 and pH2) were chosen for infectivity tests. About 14 days post inoculation (dpi), tomato seedlings 'Viradoro' bombarded with all combinations of cloned DNA-A and DNA-B components developed symptoms of vein chlorosis, chlorotic spots and yellow mottle similar to those observed in the field (Fig.3.1b). Systemic infection was confirmed by squash blot of newly emerged symptomatic leaves using both DNA-A and DNA-B probes. None of the plants inoculated either with DNA-A, DNA-B or pBS KS^+ alone developed any symptoms and no virus was detectable by Southern blot analysis. Since all combinations of DNA-A and DNA-B were infectious and induced indistinguishable symptoms in tomato plants, clones pK56 and pH81 were chosen as being representative of ToCMoV-[BA-Se1] DNA-A and DNA-B components, respectively, and were used throughout this study.

Host range and whitefly transmission

ToCMoV-[BA-Se1] could infect a range of different host species when inoculated either by particle bombardment with cloned DNA-A and DNA-B or by mechanical inoculation using infected *N. benthamiana* as an inoculum source (Table 3.2, Fig. 3.1). All inoculated tomato cultivars displayed chlorotic spots, vein chlorosis and typical yellow mottling about 14 dpi, regardless of the inoculation procedure (Fig. 3.1a and 1b). Infected *N. benthamiana* plants were stunted and developed vein clearing, crumpling, epinasty, and systemic mottling (Fig. 3.1c). However, no symptoms were observed and viral DNA was undetectable when either plant species was bombarded with only ToCMoV-[BA-Se1] DNA-A or DNA-B alone, demonstrating again the requirement of both components for the establishment of a systemic infection. Tobacco plants 'TNN' and 'White Burley' presented systemic mottling (Fig. 3.1d) and *P. hybrida* exhibited vein chlorosis, chlorotic spots and chlorotic mottle (Fig. 3.1e). Pepper cultivars displayed chlorotic spots and leaf deformation (Fig. 3.1h). In the weed *N. physaloides,* ToCMoV-[BA-Se1] caused mosaic, leaf deformation and very severe stunting (Fig. 3.1g). Only one soybean plant from the cultivar Celeste became infected and showed vein chlorosis, chlorotic spots, leaf mottle and deformation (Fig. 3.1f). While virus could be detected in all symptomatic plants by either squash blot or PCR, mock inoculated control plants always gave negative detection results. Although the virus did not spread systemically in *D. stramonium*, it could be detected by PCR within localized chlorotic spots at the site of inoculation. One out of four inoculated *N. clevelandii* and *N. rustica* plants developed a systemic asymptomatic infection. Neither symptomatic nor asymptomatic infections (as determined by negative squash blot and PCR results) were observed in *G. max* 'Conquista', *D. metel*, *N. rosellata, N. glutinosa*, *P. vulgaris* 'Olathe Pinto' and *A. thaliana var*. Columbia.

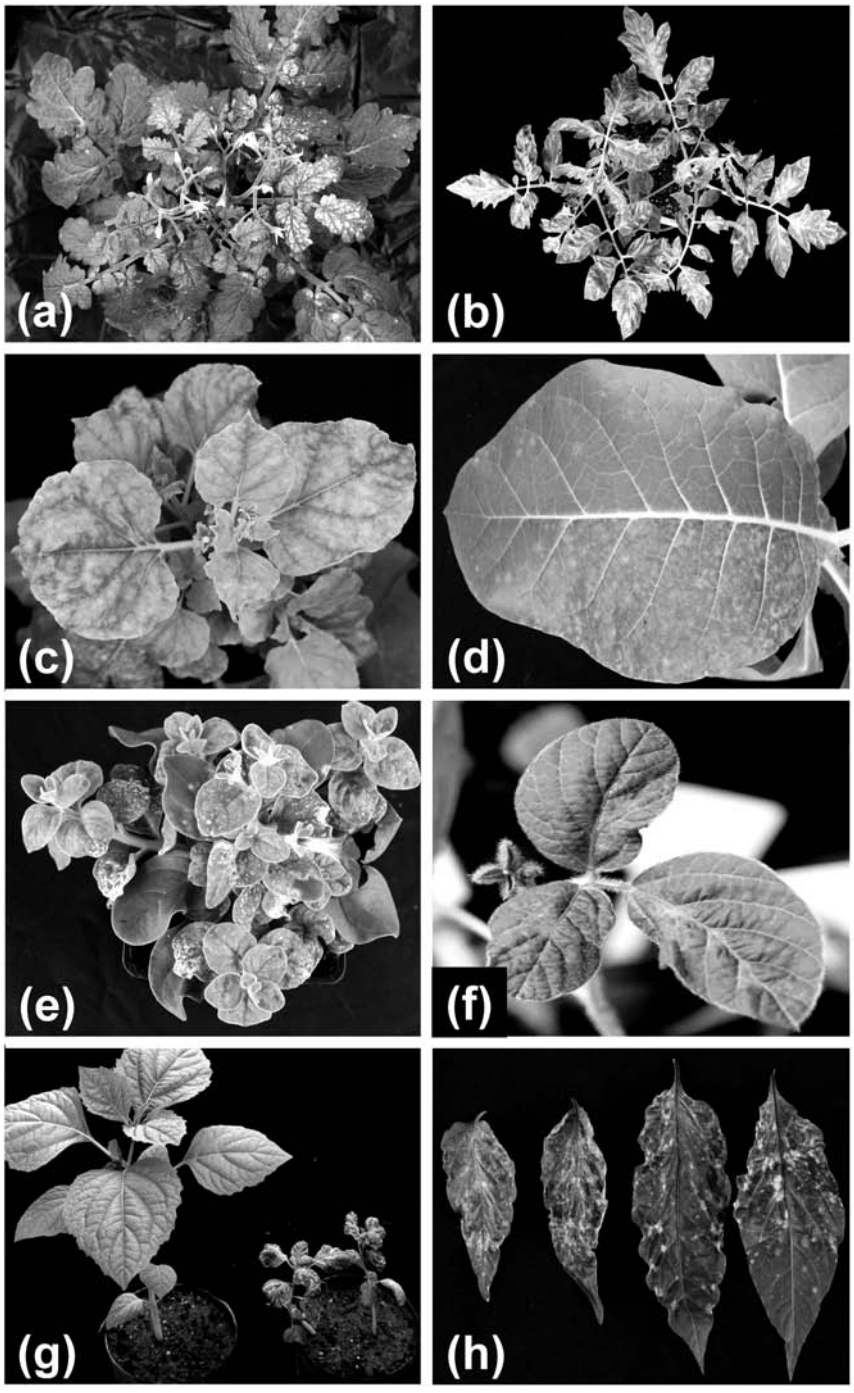


Figure 3.1. Symptoms induced by Tomato chlorotic mottle virus–[BA-Se1]. (a) Tomato 'Tiny Tim' and (b) 'Viradoro' showing typical yellow mottle. (c) Mottling in *N. benthamiana*, (d) tobacco 'White Burley' (e) Petunia and (f) soybean 'Celeste'. (g) *Nicandra physaloides* showing mosaic, leaf deformation and severe stunting. (h) Chlorotic spots and leaf deformation in pepper 'Pikante Reuzen'

Table 3.2. Experimental host range and symptoms displayed by test species inoculated by particle bombardment or mechanically with Tomato chlorotic mottle virus –[BA-Se1]

Host plant	Infectivity*	Symptoms†
Biolistic inoculation		
Nicotiana benthamiana	9/10	E, VC, Cr, Mo
Nicotiana tabacum 'TNN'	10/11	Mo
Capsicum annuum 'Margarita'	4/7	CS
Capsicum annuum 'AG 10'	9/12	CS
Glycine max 'Conquista'	0/7	-
Glycine max 'Celeste'	1/11	VC, CS, LD, Mo
Datura metel	0/9	-
Lycopersicon esculentum 'Viradoro'	8/8	VC, Mo, CS
Lycopersicon esculentum 'Santa Clara'	8/9	VC, Mo, CS
Nicandra physaloides	5/6	M, LD, St,
Sap inoculation		
Lycopersicon esculentum 'Money maker'	5/5	VC, Mo, Cr, CS
Lycopersicon esculentum 'Tiny Tim'	6/6	VC, Mo, CS
Capsicum annuum 'Pikante Reuzen'	6/6	CS, LD
Nicotiana tabacum 'White burley'	5/5	Mo
Nicotiana rustica	1/4	-
Nicotiana rosellata	0/4	-
Nicotiana clevelandii	1/4	-
Nicotiana benthamiana	20/20	Cr, E, VC, Mo, St
Nicotiana glutinosa	0/5	-
Datura stramonium	5/5	LCS
Petunia hybrida	8/8	CS, VC, Mo
Phaseolus vulgaris 'Pinto'	0/5	-
Arabdopsis thaliana var. Columbia	0/19	-

* Number of plants with symptoms and/or positive detection of infection by squash blot or PCR /number of plants inoculated

† CS= chlorotic spots, Cr= crumpling, E= epinasty, LCS= local chlorotic spots, LD= leaf deformation, M=Mosaic, Mo=mottle, VC= veinal chlorosis, St= stunting.

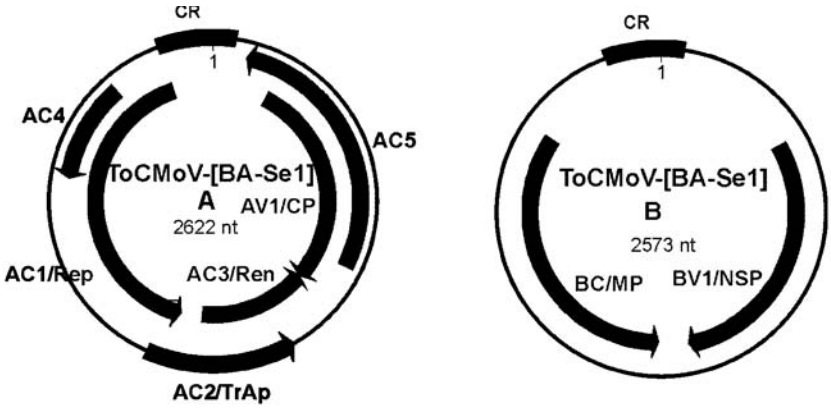


Figure 3.2. Genomic organization of Tomato chlorotic mottle virus–[BA-Se1]. Arrows indicate the ORFs in virion (V) or complementary (C) sense orientation. Coordinates indicate the start and stop condon of each ORF. Nucleotide number one is the A 3' of the nicking site (↓) in the nonanucleotide TAATATT↓AC, the origin of virion strand replication.

ToCMoV-[BA-Se1] was whitefly transmissible and whitefly inoculated tomato plants developed typical mottle symptoms about 2 weeks after inoculation. Ten whiteflies per plant were sufficient to achieve 100% transmission.

Genome organization

The ToCMoV-[BA-Se1] DNA-A and DNA-B components are 2622 nt and 2573 nt in length, respectively. The genomic organization of these components is characteristic of New World begomoviruses. DNA-A potentially possesses the virion-sense gene (AV1) and four complementary-sense genes (AC1, AC2, AC3 and AC4) that are common to all New World begomoviruses. An additional large complementary-sense ORF (AC5) between nucleotide positions 849 and 97 potentially encoding a 250 amino acid (aa) protein is also present (Fig. 3.2). DNA-B potentially possesses the two genes, one virion-sense (BV1) and one complementary-sense (BC1), characteristic of the DNA-B components of all bipartite begomoviruses (Fig. 3.2). In both components, the virion and complementary strand ORFs are separated by an intergenic region. Within this region, ToCMoV-[BA-Se1] DNA-A and DNA-B components share a 225 nt common region (CR) (Fig. 3.2) with 98% sequence identity, indicating that the two components are very likely the cognate pair of the same begomovirus species. The CR contains a hairpin structure with the characteristic geminiviral nonanucleotide sequence TAATATT?AC in the loop at the expected origin of virion strand DNA replication (Hanley Bowdoin *et al.*, 1999; Laufs *et al.*, 1995). A direct repeat "iteron" sequence GGGGACTGGGGT, the presumed Rep (AC1 protein product) binding site (Arguello Astorga *et al.*, 1994;

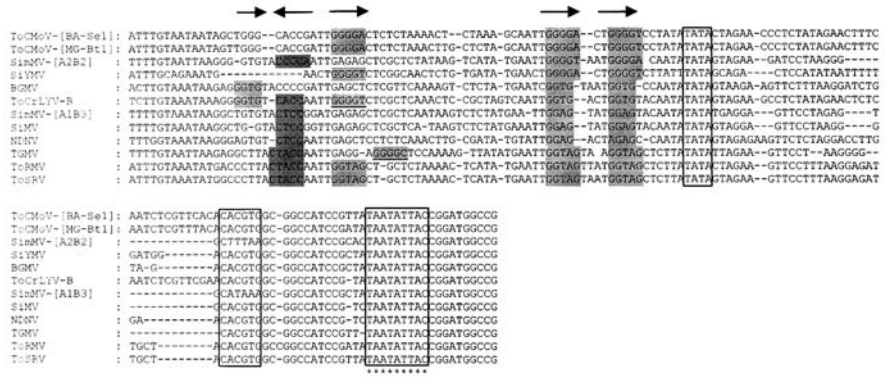


Figure 3.3. Multiple alignment of the common regions of a selection of Brazilian begomoviruses. The TATA box, G-Box and conserved nanonucleotide are boxed. Iterated direct repeats (iterons) are in light gray whereas inverted repeats are in dark gray. The arrows indicate the direction of the repeat. GGGGT in TYLCrV and GGGGC sequences in TGMV-[YV] are underlined. All sequences are derived from the A component, except for ToCrLYV.

Fontes *et al.*, 1994), is located just upstream of a TATA box that is potentially involved in complementary sense gene transcription. A third repeat of this iteron is located 27nt downstream from the AC1 initiation codon (Fig. 3.3).

Phylogenetic analysis

A BLASTn search of public databases indicated that the DNA components of ToCMoV-[BA-Se1] shared greatest identity with New World begomoviruses, and was particularly similar to various other Brazillian begomoviruses. A maximum parsimony tree constructed using a multiple alignment of 48 New World begomovirus DNA-A sequences (Fig. 3.4a) indicated that ToCMoV-[BA-Se1] and ToCMoV-[MG-Bt1] are sister taxa in a group of crop-infecting viruses within a larger Brazilian cluster of viruses. They share 92% overall identity in the whole DNA-A component and, in accordance with the ICTV guidelines for species demarcation in the *Geminiviridae* (Stanley *et al.*, 2005) they should therefore be regarded as isolates/strains of the same new begomovirus species, *Tomato chlorotic mottle virus*. ToCMoV-[BA-Se1] is also closely related to ToRMV and Tomato severe rugose virus (ToSRV).

While fewer DNA-B sequences are available, phylogenetic analysis of this component also placed most of the Brazilian begomoviruses within a distinct cluster (Fig. 3.4b). However, the division of crop and weed infecting viruses was not as clearly defined as it was for the DNA-A component. ToCMoV-[BA-Se1] DNA-B was more closely related to

Table 3.3. Percent nucleotide and amino acid identities of ToCMoV-[Ba-Se1] as compared with other Brazilian begomoviruses

Virus	A	B	CR†	AV1	AC1	AC2	AC3	AC4	AC5	BV1	BC1
ToCMV-[MG-Bt1]	92	NA	95	91/96	93/93	95/90	94/94	95/86	90/76	NA	NA
ToRMV	84	63	75	92/99	77/80	95/91	94/94	71/43	92/80	71/67	73/78
ToSRV	78	NA	76	82/90	75/75	82/75	85/81	70/43	84/62	NA	NA
TGMV-[YN]	76	62	71	82/88	74/74	79/70	83/78	73/54	81/52	70/66	76/84
BGMV-[BR]	76	62	71	83/92	73/72	84/76	83/81	67/32	84/59	70/71	73/78
NDMV	75	NA	66	84/91	72/71	83/76	83/80	63/30	90/76	NA	NA
SiMV	75	NA	73	84/91	72/64	81/69	82/78	77/53	87/70	NA	NA
SiYMoV	78	NA	78	83/84	78/76	80/59	83/64	86/71	87/64	NA	NA
SimMV-[A1B3]	75	63	68	83/91	73/71	78/72	82/77	69/42	88/70	71/71	72/80
SimMV-[A2B2]	75	62	72	83/90	70/72	82/65	83/81	73/47	88/70	70/70	71/78
ToCrLYV	NA	76	81	NA	NA	NA	NA	NA	NA	81/81	86/91

*Nucleotide sequence/deduced amino acid sequence identities

†CR sequence is from the A component, except for ToCrLYV

Tomato crinkle leaf yellow virus (ToCrLYV) and Bean golden mosaic virus (BGMV-[BR]) whereas ToRMV was more closely related to a group of viruses isolated from sida.

Detailed nucleotide and deduced amino acid (aa) sequence comparisons between ToCMoV-[BA-Se1] and all available Brazilian begomovirus sequences are presented in Table 3.3. For DNA-A, the AV1 ORF was the most conserved among Brazilian begomoviruses both in nucleotide and deduced amino acid sequence. The similarity in aa sequence ranged from 84% with *Sida* yellow mosaic virus (SiYMV) to 99% with ToRMV. The least conserved ORF was AC4 showing a maximum degree of aa similarity with ToCMoV-[MG-Bt1] (86%) and least with *Nicandra* deforming necrosis virus (NDNV) (30%). As expected ToCMoV-[BA-Se1] generally shared the greatest degree of identity with ToCMoV-[MG-Bt1], their AV1, AC1, AC2 and AC3 nucleotide and putative amino acid sequences being greater than 90% identical. Despite nucleotide sequence identities in excess of 90% for the AC4 and AC5 ORFs, amino acid identities for these ORFs were 86 and 76%, respectively. Although the ToCMoV-[BA-Se1] and ToRMV AV1, AC2, and AC3 sequences share greater than 90% nucleotide and deduced amino acid sequence identity, their AC1 and AC4 sequences share only 77% and 71% nucleotide sequence identity and 80% and 43% deduced amino acid sequence identity respectively.

The DNA-B nucleotide sequence of ToCMoV-[BA-Se1] is most similar to that of ToCrLYV with which it shares 76% identity. The aa similarities of putative BV1 and BC1 proteins of ToCMoV-[BA-Se1] and ToCrLYV, were 81% and 91%, respectively.

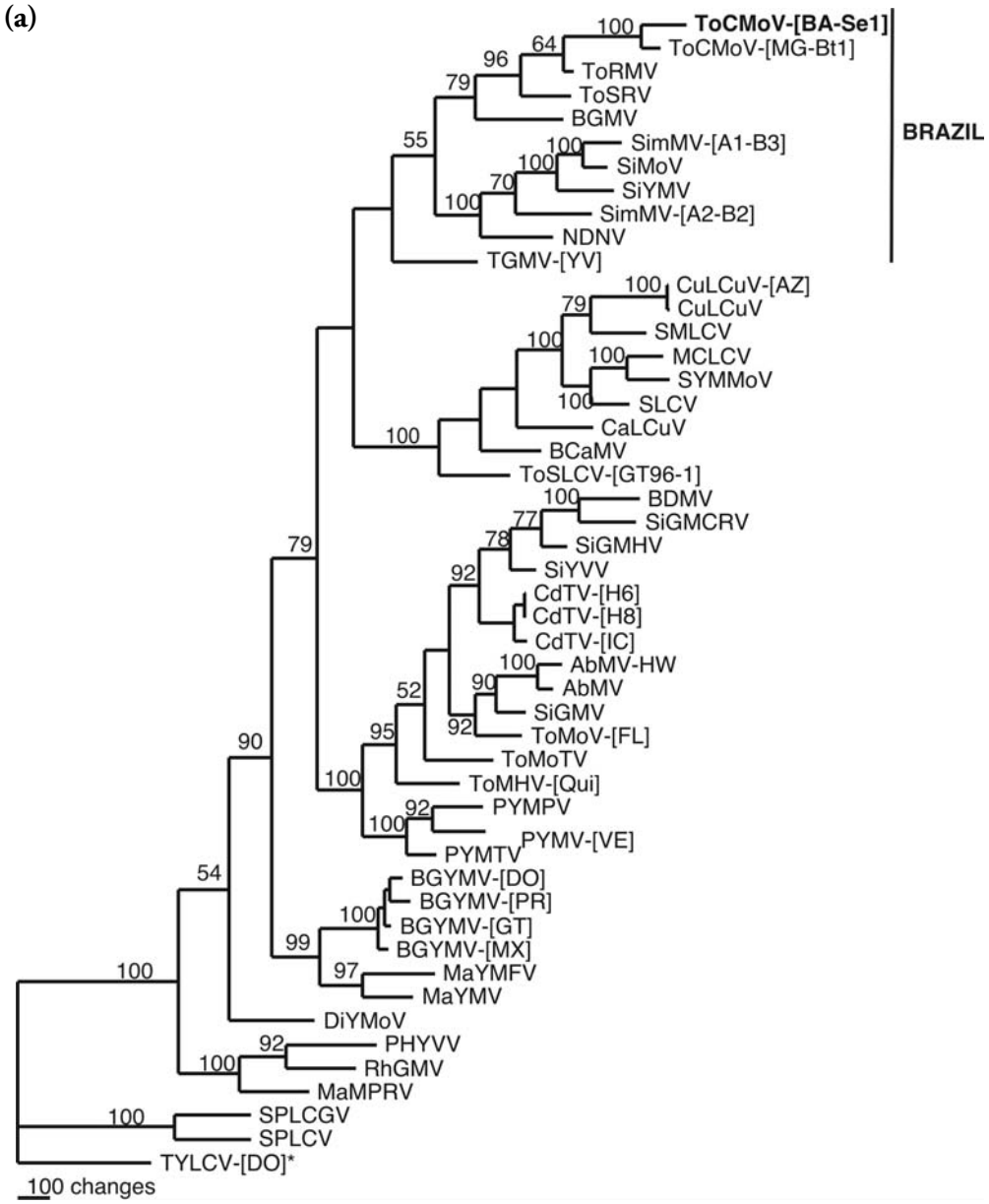
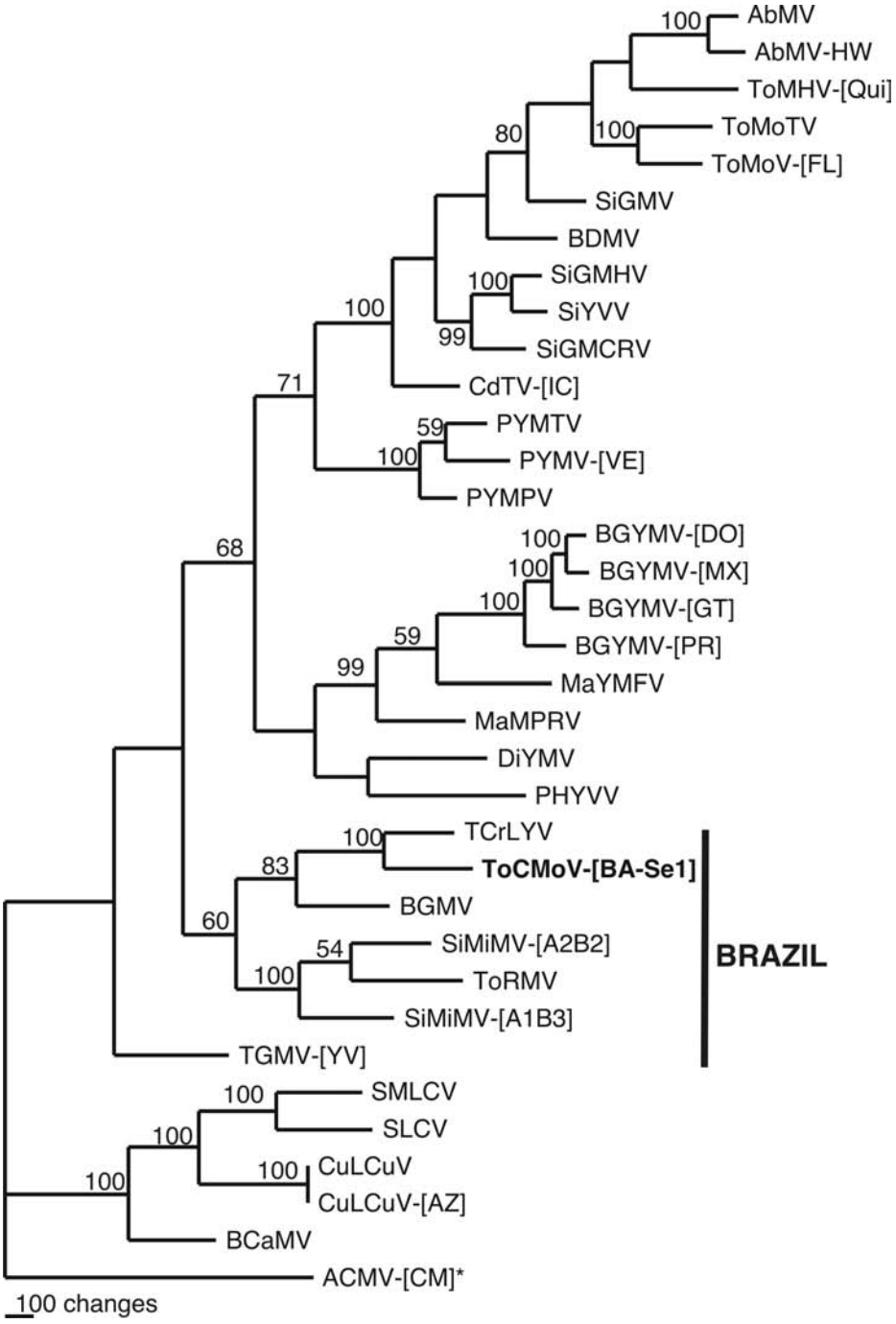


Figure 3.4. Phylogenetic trees illustrating predicted relationships between (a) ToCMoV-[BA-Se1] DNA-A and (b) ToCMoV-[BA-Se1] DNA-B sequences with those of other New World begomoviruses. Trees were constructed using maximum parsimony after bootstrapping for 1000 replicates. Bootstrap values greater than 50% are indicated at the nodes. Acronyms and accession numbers are supplied in Table 3.1. Viruses used as outgroups are indicated by an asterisk.

(b)


AbMV
AbMV-HW
ToMHV-[Qui]
ToMoTV
ToMoV-[FL]
SiGMV
BDMV
SiGMHV
SiYVV
SiGMCRV
CdTV-[IC]
PYMTV
PYMV-[VE]
PYMPV
BGYMV-[DO]
BGYMV-[MX]
BGYMV-[GT]
BGYMV-[PR]
MaYMFV
MaMPRV
DiYMV
PHYVV
TCrLYV
ToCMoV-[BA-Se1]
BGMV
SiMiMV-[A2B2]
ToRMV
SiMiMV-[A1B3]
BRAZIL
TGMV-[YV]
SMLCV
SLCV
CuLCuV
CuLCuV-[AZ]
BCaMV
ACMV-[CM]*
100 changes

Analysis of the common region and reassortment experiments

The common region identities of all described cognate Brazilian begomovirus DNA-A/DNA-B pairs are greater than 93%. For this reason, in the multiple alignment of CRs depicted in Fig. 3.3 (with the exception of ToCrLYV for which only the DNA-B sequence is available) only DNA-A CR sequences are considered. In this region all *cis*-acting elements involved in viral DNA replication and transcription of the AC1 ORF are present (Fig. 3.3) (Hanley-Bowdoin *et al.*, 1999). The sequence and number of inverted and direct repeats vary among different viruses. ToCMoV-[BA-Se1], ToCMoV-[MG-Bt1], SiYMV and Sida micrantha mosaic virus-[A2B2] (SimMV-[A2B2]) have the same GGGGA iteron. However, instead of a third direct repeat, SimMV-[A2B2] has an inverted repeat. The sequence identity of these elements suggests that reassortment among components of these viruses might be possible. It is interesting to point out that although the iteron sequences for ToCrLYV and TGMV-[YV]) are GGTG and GGTAG, respectively, both viruses also have a GGGGT (GGGGC for TGMV DNA-A) sequence upstream of their 5' iteron (Fig. 3.3) which might facilitate the interaction of genome components of these two viruses with the Reps of viruses possessing GGGGA iteron sequences.

Although the frequency of component reassortment in nature is unknown, experimental reassortment can sometimes yield viable pseudorecombinants (Gilbertson *et al.*, 1993; Ramos *et al.*, 2003). For example, Galvão *et al.* (2003) obtained viable pseudorecombinants between the Brazilian begomoviruses ToCMoV-[MG-Bt1] DNA-A and ToCrLYV DNA-B despite these viruses having different iteron sequences. Although ToCMoV-[MG-Bt1] DNA-A (with GGGGA iterons) and ToCrLYV DNA-B (with GGTG iterons) could systemically infect *N. benthamiana* and tomato, in tomato no symptoms developed. To determine whether viable reassortments between ToCMoV-[BA-Se1] and ToRMV were possible, cognate and reassorted components were introduced into *N. benthamiana* seedlings by particle bombardment. While high infection rates were obtained with cognate ToCMoV-[BA-Se1] components (15/17 inoculated plants) and ToRMV components (10/14 plants) none of the reassortants between these viruses were viable as evidenced by the absence of both symptoms and detectable viral DNA in inoculated plants.

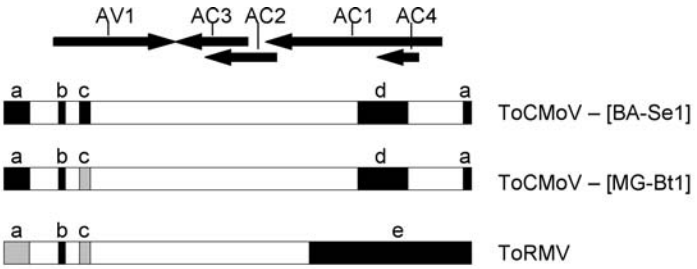
Recombination

In an analysis of recombination between New World begomovirus DNA-A components, we detected significant evidence of 49 unique events involving either of the two ToCMoV isolates as potential donors or acceptors of sequence. It was, however, in most cases very difficult to discern whether the ToCMoV isolates were donors or acceptors of sequence during these events. Each detected recombination event split the alignment into two fractions – each corresponding to the portions of sequence derived from either of two

parental viruses. We called the portion of the alignment corresponding to the larger fraction the *major region* and the fraction of sequence corresponding to the smaller fraction the *minor region*. Starting with the most obvious recombination events (those yielding the lowest P-value scores) we progressively examined every event, constructing two bootstrapped (1000 replicates) neighbor joining trees for the major and minor regions indicated by each event. From these trees the minimum number of tree modifications (branch cutting and grafting) required to convert each tree into the other, was determined using a method similar to that described by MacLeod *et al*. (2005). Given both the minimum number of cuts and grafts required to convert each tree into the other and the 49 sets of potential parental and recombinant sequences identified for the different recombination events, in 34 cases we inferred that the sequences moved during pruning and grafting were recombinants. In the 15 remaining cases however, pruning and grafting also involved moving either one or both parents. This probably indicated that the parental viruses used to detect the events were themselves recombinants. In such cases we used the PHYLPRO method (Weiller, 1998) to choose the most likely recombinant from amongst the two or three sequences that the pruning and grafting method had indicated were possible recombinants. The PHYLPRO method examines the pairwise Jukes-Cantor distances between a potential recombinant and all the other sequences in the alignment and linearly regresses the distances determined for the major region of the alignment against those determined for the minor region. The comparison yielding the lowest correlation coefficient indicates which of the sequences is most likely to be recombinant.

Of the 49 events involving the ToCMoV isolates as either donor or acceptor sequences we identified only four events in which it is very likely that the ToCMoV isolates were sequence acceptors (events a-d in Fig. 3.5). Although both isolates bear evidence of all four events, event c was only detectable with more than one recombination detection method in ToCMoV-[BA-Se1]. Since all of these events are detectable in both sequences it is likely that they occurred before the isolates diverged and are therefore reasonably old events. Interestingly, the results of our recombination analysis contradict that of a previous study (Galvão *et al.*, 2003) which indicated that ToCMoV-[MG-Bt1] could be a ToCMoV-[BA-Se1]–ToRMV recombinant. Since both the prune-graft and PHYLPRO methods indicate that ToRMV is an acceptor sequence, we propose here that it is in fact ToRMV that is a ToCMoV-[MG-Bt1]-ToSRV recombinant (event e in Fig. 3.5). ToRMV is more closely related to ToCMoV-[MG-Bt1] between genome positions 1 and 1700 than it is to ToCMoV-[BA-Se1] implying that this recombination event is reasonably recent and occurred after [MG-Bt1] and [BA-Se1] diverged. Accordingly, ToRMV also bears evidence of three of the more ancient recombination events detected in this region in the ToCMoV isolates.

No evidence of recombination was detected in the DNA-B sequences of ToCMoV-[BA-Se1].



Region	Breakpoints Begin	End	Parents* Major	Minor	Detection Method RDP	GENECONV	BOOTSCAN	MAXCHI	CHIMAERA	SISCAN
a	2578	93	BGMV-[BR]†	ToMoV-[FL]†	7.89×10^{-7}	2.02×10^{-4}	2.80×10^{-5}	8.76×10^{-3}	NS‡	NS
b	232	272	ToSRV	Unknown§	2.25×10^{-5}	8.80×10^{-3}	NS	1.32×10^{-3}	5.27×10^{-3}	2.29×10^{-11}
c	361	424	BGMV-[BR]†	MaMPRV†	5.47×10^{-3}	NS	NS	NS	NS	3.53×10^{-4}
d	1973	2283	BGMV-[BR]†	PYMPV†	1.34×10^{-6}	2.97×10^{-2}	2.14×10^{-5}	3.40×10^{-8}	1.56×10^{-7}	8.20×10^{-2}
e	1701	2932	ToCMoV-[MG-Bt1]	ToSRV	5.36×10^{-52}	2.33×10^{-45}	9.29×10^{-60}	6.28×10^{-33}	2.50×10^{-16}	1.70×10^{-8}

*The major parent contributed most of the recombinant's sequence and the minor parent contributed the smaller fraction of sequence.
†Identified parent is only a distant relative of the actual parent.
‡NS = No significant P-value (P > 0.1) obtained with this test.
§Given adequate sampling, a recombinant sequence can be detected even if only one of the parental sequences has been sampled.

Figure 3.5. Five recombination events detected within DNA-A of the ToCMoV isolates, [BA-Se1] and [MG-Bt1], and the closely related virus, ToRMV, using six different recombination detection methods. Arrows at the top of the figure indicate the positions of five conserved begomovirus genes. White boxes represent the DNA-A sequences of the three isolates and black or hatched boxes represent the regions of sequence with a potentially recombinant origin. Black boxes indicate that a recombinant region was detected with a high probability (P <0.05) by at least two methods. Hatched boxes indicate regions where, although recombination was detected with only one method, the same ancestral recombination event was detected in another sequence by at least two different methods. The events are labeled (a-e) and details of approximate breakpoints, probable parents and probability scores for the different analysis methods are given at the bottom of the figure.

Discussion

We have undertaken a comprehensive molecular and biological characterization of a new begomovirus species involved in tomato disease outbreaks in Northeastern Brazil. It is the first identified begomovirus from this region of South America (chapter 3; Ribeiro *et al.*, 2003) and although related viruses have been partially characterized elsewhere, this study is the first to report the full sequence of cloned genome components capable of inducing symptomatic infections in tomato indistinguishable from those seen in the field.

We have demonstrated that cloned ToCMoV–[BA-Se1] was both whitefly transmissible and infectious using different methods of artificial inoculation. The molecular and biological characteristics of the two genome components were those of a typical bipartite begomovirus with infection only being achievable when both components were inoculated into a susceptible plant.

The very high DNA-A sequence identity shared by ToCMoV–[BA-Se1] and ToCMoV-[MG-Bt1] is surprising in that these two viruses apparently have vastly different infection capabilities. ToCMoV-[MG-Bt1] DNA-A can systemically infect and induce symptoms in *N. benthamiana* in the absence of a DNA-B (Galvão *et al.*, 2003). Despite several attempts to infect *N. benthamiana* and tomato with ToCMoV–[BA-Se1] DNA-A on its own, neither symptomatic nor asymptomatic systemic infections were achievable in these hosts in the absence of DNA-B. The biological significance in nature of ToCMoV-[MG-Bt1] DNA-A being able to symptomatically infect *N. benthamiana* without a DNA-B is unknown. The DNA-A components of other usually bipartite viruses such as ACMV, *Abutilon mosaic virus*, *Tomato mottle virus*, *Bean dwarf mosaic virus*, *Sri Lankan cassava mosaic virus*, *Tomato leaf curl Thailand virus* and Tomato leaf curl Guajarat virus-[Var] also have the capacity to infect *N. benthamiana,* in the absence of a DNA-B. However, the symptoms induced in these exclusively DNA-A infections are invariably milder than when DNA-A components are co-inoculated with their cognate DNA-B components. Moreover, all of these viruses, including ToCMoV, are always found accompanied by a cognate DNA-B in nature (Chakraborty *et al.*, 2003; Evans & Jeske, 1993; Galvão *et al.*, 2003; Hou *et al.*, 1998; Klinkenberg & Stanley, 1990; Ribeiro *et al.*, 2003; Rochester *et al.*, 1990; Saunders *et al.*, 2002). In addition to improving the capacity of bipartite viruses to move within and between infected cells (Hou *et al.*, 1998; Unseld *et al.*, 2000) a DNA-B component may increase the whitefly transmissibility of viruses (Liu *et al.*, 1997). In a natural setting, either new opportunities (such as that provided by a more promiscuous vector biotype) or environmental pressures to infect new host species may also favor the maintenance of DNA-B.

Host range information is an important prerequisite for understanding the epidemiology of viruses and can also be useful for their characterization and effective management (Polston & Anderson, 1997). The experimental host range of ToCMoV-[BA-Se1] includes agricultural, ornamental and weed species commonly found in the Northeastern parts of Brazil where the virus was isolated. Our data imply both that ToCMoV can infect and become a problem in crops like tobacco, soybean and pepper, and that these crops along with weed species such as *N. physaloides* might provide an inoculum source for the infection of tomato crops. We have previously detected begomovirus infected pepper plants in the state of Pernambuco (Lima *et al.*, 2001) at the same time and area where other ToCMoV variants were detected from tomato (Ribeiro *et al.*, 2003). Although the exact identity of this pepper-infecting begomovirus is unknown, the ability of ToCMoV-[BA-Se1] to infect all of the pepper genotypes tested indicates that the virus is a potential threat to pepper cultivation.

ToCMoV and other emerging tomato-infecting begomovirus species are apparently indigenous Brazilian viruses that, probably due to the broad host preference of the newly introduced B biotype of *B. tabaci* (Bedford *et al.*, 1994; Lourenção & Nagai,

1994), have only recently become transmissible between their original hosts and tomato. *N. physaloides* is a common weed found in tomato fields. Even though ToCMoV-[BA-Se1] experimentally infected this host and induced similar symptoms to those induced by NDNV, a begomovirus isolated from this host (A.K. Inoue-Nagata & S.G. Ribeiro, *unpublished*), ToCMoV-[BA-Se1] and NDNV share only 75% sequence identity. Whereas alternative natural hosts have been identified for ToRMV (Fernandes, 2002; Jovel *et al.*, 2004) and Tomato yellow vein streak virus (ToYVSV) (Ribeiro *et al.*, 2005), recent surveys have thus far failed to detect ToCMoV in any host other than tomato (Ambrozevicius *et al.*, 2002; A.K. Inoue-Nagata, *unpublished*). One possible reason for this is that sampling has generally focused on the collection of symptomatic plants. We have shown here that, as is the case with other Brazilian tomato begomoviruses (Fernandes, 2002; Santos *et al.*, 2004), ToCMoV-[BA-Se1] is capable of asymptomatically infecting some host species. A case for the importance of asymptomatic infections in the spread of begomoviruses has been made with TYLCV-[DO]. In the Dominican Republic, this virus asymptomatically infects several weed species and it has been demonstrated that viruses transmitted from these weeds play an important role in the primary infection of tomato plants early in the season (Salati *et al.*, 2002). Surveys of symptomless weed plants within the vicinity of infected tomato fields might indicate whether a similar situation is occurring with ToCMoV and other Brazilian tomato begomoviruses.

Phylogenetic analyses based on New World begomovirus DNA-A sequences support recently published results (Rojas *et al.*, 2005) indicating that the Brazilian begomoviruses form a distinct monophyletic group. Within this group, the viruses are apparently segregated into crop and weed-infecting clades, although additional sampling may blur this distinction. ToCMoV–[BA-Se1] clusters together with ToCMoV-[MG-Bt1] within the crop-infecting group, indicating that both virus isolates, despite being isolated more than 1,000 km apart, belong to the same new species.

Pseudorecombination and recombination are apparently common features of begomovirus evolution (Harrison & Robinson, 1999; Padidam *et al.*, 1999; Rojas *et al.*, 2005) and might contribute to the emergence of novel highly pathogenic virus variants such as the one causing severe cassava mosaic disease in Uganda (Pita *et al.*, 2001). Although experimental reassortment between components of Brazilian begomoviruses has been demonstrated (Galvão *et al.*, 2003) we have found here that ToCMoV-[BA-Se1] and the closely related virus, ToRMV, do not form viable pseudorecombinants. This is probably due to the high degree of divergence in both their iteron and AC1-binding domain sequences.

Our recombination analysis indicates a possible reason for ToRMV having an apparently incongruent B-component. Whereas ToRMV and ToCMoV isolates are very similar from their origins of virion strand replication to the end of their AC1 ORFs, most

of the ToRMV AC1 and the common region containing the iteron sequences and Rep binding domain have been derived from a sequence resembling ToSRV. It is probable that following a relatively recent ToSRV-ToCMoV recombination event, the nascent ToRMV selected the ToSRV B-component with which it shared appropriate iteron and Rep binding sequences. Properly testing this hypothesis would first require the isolation of, and pseudorecombination studies with, a ToSRV DNA-B sequence.

Our recombination analysis also suggests that ToCMoV has a long history of recombination. Four recombination events were detected within the DNA-A sequences of the ToCMoV isolates, [BA-Se1] and [MG-Bt1]. Although only distant relatives of the actual parental viruses involved in these events were identified, further sampling and characterization of both crop and weed-infecting begomoviruses might lead to the identification of a virus more closely resembling ToCMoV's parents. Whereas ToRMV is apparently the product of a quite recent ToCMoV-ToSRV recombination event, the four events detected in ToCMoV (three of which are also identifiable in ToRMV) are considerably older and precede the divergence of ToCMoV [BA-Se1] and [MG-Bt1]. Therefore, whereas recombination may have contributed to the recent emergence of ToRMV, it is unlikely that it has contributed to the emergence of ToCMoV.

Of the new begomoviruses believed to be involved in Brazilian tomato disease epidemics (Faria *et al.*, 1997; Ribeiro *et al.*, 2003), ToCMoV, ToRMV, ToSRV, ToYVSV and Tomato mottle leaf curl virus seem to have expanded their initial geographic ranges in the last 5 years. ToCMoV was recently detected in central Brazil infecting tomatoes in the Federal District (A.K. Inoue-Nagata, *unpublished*) and in Paty dos Alferes, RJ, the largest fresh market tomato producing area in the country (F.M. Zerbini, *unpublished*). Interestingly, tomatoes in the latter area were begomovirus free until 2004. In both cases, ToCMoV was found in mixed infections with at least one other begomovirus, and novel recombinants were detected. Although it seems that these five species are currently dominating the begomovirus epidemic in tomato, it appears that the situation is far from static. While inbred or transgenic resistance in tomatoes is going to be difficult enough to achieve against all these viruses, there is a perpetual risk that resistance breaking or ultra pathogenic recombinant variants and possibly even new species could emerge at any time.

Acknowledgments

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Chapter 4

Tomato chlorotic mottle virus as target of RNA silencing in transgenic and non-transgenic host plants

Summary

Tomato chlorotic mottle virus (ToCMoV) is a begomovirus found widespread in tomato fields in Brazil. ToCMoV isolate BA-Se1 (ToCMoV–[BA-Se1]) was shown to trigger the plant RNA silencing surveillance in different host plants, coinciding with a decrease in viral DNA levels and accumulation of siRNAs specific to ToCMoV-[BA-Se1]. Though not homogeneously distributed, the siRNA population in both infected *Nicotiana benthamiana* and tomato plants represented the entire DNA-A and DNA-B genomes. We determined that in *N. benthamiana,* the primary targets corresponded to the 5' end of AC1 and the embedded AC4, the intergenic region and 5' end of AV1 and overlapping middle part of AC5. Subsequently, transgenic *N. benthamiana* plants were generated that were preprogrammed to express dsRNA corresponding to this most targeted portion of the viral genome by using an intron-hairpin construct. These plants were shown to indeed produce transgene-specific siRNAs. When challenge-inoculated, most transgenic lines showed significant delays in symptom development and two lines even displayed highly resistant and immune plants. Interestingly, the levels of transgene-produced siRNAs were similar in resistant and susceptible siblings of the same line. This indicates that, in contrast with RNA viruses, the mere presence of transgene siRNAs corresponding to DNA virus sequences does not guarantee virus resistance and that other factors may play a role in determining RNA-mediated resistance to DNA viruses.

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Simone G. Ribeiro, Dick Lohuis, Rob Goldbach, and Marcel Prins. Tomato chlorotic mottle virus is a target of RNA silencing but the presence of specific siRNAs does not guarantee resistance in transgenic plants.

Introduction

The geminiviruses infect a broad range of plants and cause important crop losses worldwide (Morales & Anderson, 2001; 2003). They are named after their typical twin icosahedral capsids and carry single-stranded circular DNA genomes. The genomic DNA replicates in the nuclei of infected cells through double-stranded DNA intermediates, using a combination of rolling circle and recombination-dependent replication (Gutierrez, 1999; Hanley-Bowdoin *et al.*, 1999; Jeske *et al.*, 2001; Preiss & Jeske, 2003). The family *Geminiviridae* is divided into four genera based on genome structure, insect vector and host plant. Members of the largest genus, *Begomovirus*, are all transmitted by the whitefly *Bemisia tabaci* (Gennadius) and infect dicotyledonous plants. Most begomoviruses, among which all New World begomoviruses, have their genome divided into two components, denoted DNA-A and DNA-B, which respectively encode genes involved in expression/replication/encapsidation and movement. In both components, the viral strand and its complement encode open reading frames (ORFs), separated by an intergenic region (IR). This region includes the common region (CR), the only part of the genome highly identical between DNA-A and DNA-B of the same virus species. The IR harbors the origin of replication and promoter sequences for the transcription of viral genes (Hanley Bowdoin *et al.*, 1999). DNA-A encodes the AV1 (coat protein, CP) in the viral sense and four genes in the complementary sense. AC1 (Rep) encodes the only protein strictly essential for virus replication (Elmer *et al.*, 1988; Hanley Bowdoin *et al.*, 1999), AC2 (TrAp) is a transcriptional factor acting on the promoters of viral sense genes (Sunter & Bisaro, 1992) and has been shown to act as silencing suppressor for a number of begomoviruses (Trinks *et al.*, 2005; van Wezel *et al.*, 2002; Vanitharani *et al.*, 2004; Voinnet *et al.*, 1999; Wang *et al.*, 2005), AC3 (REn) is a replication enhancing factor (Morris *et al.*, 1991; Sunter *et al.*, 1990). For the AC4 protein no function had been assigned until a recent report implied it as an RNA silencing suppression for two cassava-infecting begomoviruses (Vanitharani *et al.*, 2004). DNA-B encodes two genes involved in virus movement. BV1 is the nuclear shuttle protein (NSP) engaged in viral DNA transport from the nucleus to the cytoplasm, while BC1 (MP) is responsible for cell-to-cell and systemic movement (Gafni & Epel, 2002; Noueiry *et al.*, 1994; Sanderfoot & Lazarowitz, 1995, 1996). Geminivirus transcription is bidirectional and can be quite complex, frequently giving rise to convergent multiple polycistronic RNAs, that overlap at their 3' ends (Hanley Bowdoin *et al.*, 1989; Hanley Bowdoin *et al.*, 1999; Shivaprasad *et al.*, 2005; Sunter & Bisaro, 1989).

RNA silencing is an evolutionary conserved process that is active in a wide variety of eukaryotic organisms and can lead to inhibition of transcription or translation of a target gene in a sequence-specific manner (Baulcombe, 1999; Ding, 2000). A key role in this process is played by short interfering RNA molecules of 21-26 nt (siRNAs)

(Hamilton & Baulcombe, 1999) which are the result of the cleavage of longer dsRNAs by Dicer (Bernstein *et al.*, 2001). The strands of the siRNAs are unwound and one of the strands is retained in the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000) where it guides the RISC to a complementary mRNA target (Meister & Tuschl, 2004; Tang *et al.*, 2003). Transcriptional silencing as a result of methylation of DNA sequences homologous to siRNAs has also been observed, suggesting siRNAs may be active in the nucleus (Cerutti, 2003; Lippman & Martienssen, 2004; Wassenegger, 2000). Biological functions of RNA silencing in plants include regulation of endogenous gene expression, heterochromatin formation, repression of transposable elements and defense against virus infection (Baulcombe, 2005; Waterhouse *et al.*, 2001).

Plant cells infected with RNA viruses have been shown to produce virus-specific siRNAs, which were suggested to be originated from the breakdown of dsRNA replicative forms or from secondary structures of the viral RNA (Hamilton & Baulcombe, 1999; Molnar *et al.*, 2005). DNA plant viruses like caulimoviruses and geminiviruses are also targets of RNA silencing (Al-Kaff *et al.*, 1998; Chellappan *et al.*, 2004b; Lucioli *et al.*, 2003). In some cases this response can lead to recovery of the plants from the virus symptoms (Al-Kaff *et al.*, 1998; Chellappan *et al.*, 2004b; Covey *et al.*, 1997), therefore it was suggested that RNA silencing is a natural defense mechanism that protect plants from viral invasion (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). Indeed, harnessing this RNA silencing by generating siRNA-producing transgenic plants has proven to be a potent means to engineer resistance to RNA viruses (Baulcombe, 1996; Goldbach *et al.*, 2003; Prins, 2003; Ritzenthaler, 2005). Also for DNA viruses such as geminiviruses, biotechnological approaches using transgenic plants expressing sense and anti-sense RNA have been employed with relative success (Freitas-Astua *et al.*, 2002; Lapidot & Friedmann, 2002).

Tomato chlorotic mottle virus is a typical New World begomovirus which has been found widespread in tomato fields in Brazil (Ribeiro *et al.*, 2003, chapters 2 and 3). In this chapter we have first investigated the RNA silencing response generated in plants upon Tomato chlorotic mottle virus – [Bahia-Seabra1] (ToCMoV-[BA-Se1]) infection in terms of production and origin of virus–specific siRNAs. Subsequently, the potential to transgenically generate begomovirus resistance in plants by using the most targeted sequences from the virus genome was explored.

Methods

Plant inoculation

For virus inoculation, carborundum dusted leaves of *Nicotiana benthamiana*, tomato (*Lycopersicon esculentum*) and petunia (*Petunia hybrida*) were rubbed with the

extract of ToCMoV-[BA-Se1]-infected *N. benthamiana* plants prepared by grinding infected leaf tissue (1:10 w/v) in phosphate buffer, pH 8.0, containing 25 mM EDTA and 0.01% sodium sulfite.

Preparation of ToCMoV-[BA-Se1]-specific PCR fragments and in vitro transcripts

The ToCMoV-[BA-Se1] DNA-A and DNA-B components were PCR-amplified in a series of 6 fragments for each component (A1-A6 and B1-B6) using the appropriate primer pairs (Table 4.1) and full-length infectious clones as templates (chapter 3).

ToCMoV-[BA-Se1]-derived PCR fragments A3, A4 and A6 (Table 4.1) were cloned into the pGEMT-easy vector (Promega) and viral and complementary sense *in vitro* transcripts were prepared by T7 or SP6 RNA polymerases using the m-message m-machine kit (Ambion).

The PCR fragments and transcripts were respectively separated on native agarose gel or a denaturing formaldehyde gel. The gels were blotted onto positively charged nylon membrane (Hybond N^+, Amersham) and probed with ^{32}P-labeled purified (Szittya *et al.*, 2002) low-molecular mass RNA isolated from infected *N. benthamiana* or tomato plants.

Extraction of total nucleic acids and isolation of low-molecular-mass RNA

The extraction of total nucleic acid from plant leaves was done according to Bucher *et al.* (2004). Briefly, leaf material was ground in liquid nitrogen and mixed with extraction buffer (2% Sarkosyl/5 M NaCl). After phenol extraction, polysaccharide contaminants were precipitated by adding 3 M sodium acetate. The supernatant was ethanol precipitated and resuspended in Tris-EDTA (TE) buffer. To separate DNA and larger RNA molecules from low-molecular mass RNAs, a PEG precipitation step was performed (Hamilton & Baulcombe, 1999). The pellet containing DNA and longer RNA was resuspended in TE buffer. The supernatant containing the low-molecular-mass RNAs was precipitated with ethanol and resuspended in TE buffer.

Preparation and labeling of siRNAs

For purification of small RNAs from infected *N. benthamiana* and tomato plants, about 30 µg of low-molecular-mass RNAs were fractionated in a 15% denaturing polyacrylamide gel containing 8M urea. After staining with ethidium bromide, the region containing the small RNAs was excised from the gel, cut in small pieces and incubated in 3 M NaCl overnight at 4 °C to allow diffusion. After centrifugation, the super-

Table 4.1. Primers used to amplify the ToCMoV-[BA-Se1] - derived fragments.

Fragment name	Primer name	Primer sequence* (5'→3')	PCR fragment size (bp)
A1	A1F	GGGGAGTGGCATATTTG	379
	A1R	GGGGCMTCMCKCTTAGRCAT	
A2	A2F	CCCGTCGACATGYCTAAGMGKGAKGCCCC	360
	A2R	GATTTCTGCAGTTDATRTTYTCRTCCATCCA	
A3	A3F	GCATCTGCAGGGATGGANGANAANATNAA	420
	A3R	CGCAACAGACAGACAATATG	
A4	A4F	TGCGAATCGAACAGCTCTAAT	440
	A4R	ATGCGCAATTCATCT T	
A5	A5F	GCGCATTGTGCTTTGTCCT	320
	A5R	GCATCTGCAGACNGGRAAGACRATGTGGGC	
A6	A6F	GCATCTGCAGGCCCACATYGTCTTYCCNGT	731
	A6R	TAGGATCCATGCCACTCCCCAGACATTTC	
B1	B1F	GGAGGAACAACCAACTGAGAA	392
	B1R	TAGGATCCATGGCCGCCACGTGTGT	
B2	B2F	CTCGTTCACACACGTGG	464
	B2R	CGACGCTTGTTACCATTGAA	
B3	B3F	ATGTATTTCAATGGTAACAAGCGTCG	435
	B3R	ATGTGTACAGACTGCCGGAA	
B4	B4F	CGGCAGTCTGTACACATTCG	346
	B4R	ATTATCCAATATAGTCAAGGTC	
B5	B5F	ACTGGTCGACGCGGCCGCAYCTBGAYTATDTYGG	464
	B5R	GTAGGTTATGGGTCATGGGA	
B6	B6F	CCTACATGAACGAAATCGATATCC	526
	B6R	ATGGTACCGCGGCCGCATGRRDTCTCAGYTDG	
RC	GWAttb 1-AC1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGACGTCTGAGGAG CTCTTAG	938
	GWAttb 2-AV1R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTAATACCGTTAC CACGTGT	

*N=A, C, G or T; M=A or C; Y=C or T; B=C, G or T; D=A, G or T; K=G or T; R=A or G

natant was precipitated by ethanol (Chellappan *et al.*, 2004b). The small RNAs (approximately 1 µg) were dephosphorylated with alkaline phosphatase and labeled with ^{32}P by T4 polynucleotide kinase using [γ-^{32}P] ATP according to the manufacturer's instructions (Invitrogen).

Construction of plant expression vectors

To evaluate the potential of RNA silencing for the control of ToCMoV-[BA-Se1], an intron-hairpin construct was generated containing those sequences of the virus which were most highly targeted by RNA silencing during virus infection in *N. benthamiana*. A fragment of 938 bp (denoted RC) was amplified by PCR from ToCMoV-[BA-Se1] DNA-A (chapter 3) with *Pfu* DNA polymerase using the primers GWAttb1-AC1F and GWAttb2-AV1R (Table 4.1). The RC fragment consists of 300 nt of the 5' end of the AV1 gene (including the end of the overlapping AC5 ORF) the entire com-

mon region and 300nt of the 5' end of the AC1 gene (including a part of the nested AC4 gene). The recombinant plasmids were obtained using the GATEWAY™ system (Invitrogen) following the manufacturer's instructions. The RC fragment was introduced by BP recombination into pDonr 207 and subsequently into the destination binary vector pK7GWIWG2 (Karimi *et al.*, 2002) by LR recombination by which it was inserted downstream of a CaMV 35S promoter in an inverted repeat array, separated by an intron, producing the binary plant expression vector pIR-RC. After confirming the presence of the sense and the antisense arms of the inverted repeat and the orientation of the intron by restriction enzyme digestion, pIR-RC was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Plant transformation and evaluation

Leaf discs of *Nicotiana benthamiana* plants were transformed using standard protocols (Topping, 1998). Kanamycin resistant regenerated shoots were rooted and transferred to soil. Transgene integration in the T0 plants was verified by PCR. PCR positive plants were self-fertilized and the progenies of these plants (T1) were sown on selective MS medium containing kanamycin (100 mg/l). Green seedlings with well developed roots were potted and evaluated for virus resistance.

Eleven transgenic lines were tested for virus resistance. Ten seedlings from each transgenic line were mechanically inoculated as described above. Wild type *N. benthamiana* plants were inoculated as positive controls. Plants were monitored regularly and scored for symptoms. Virus infection was confirmed by squash or tissue blot analysis at 20 days post inoculation (dpi) and 45 dpi, using PCR fragment A3 (Table 4.1) labeled by random priming with ^{33}P as a probe.

Accumulation of viral DNA forms was detected by Southern blot analysis. Approximately 3 µg of total DNA was fractionated on a 1% agarose gel and blotted onto Nylon membrane. Viral DNA was detected by Southern hybridization with ^{33}P labeled whole ToCMoV-[BA-Se1] DNA-A and DNA-B as probes.

Northern and Southern blot analysis

For Northern blotting, 12 µg of low-molecular-mass RNA was separated on a 15% denaturing polyacrylamide gel and probed with Digoxigenin-labeled A4 fragment (Table 4.1) for low-molecular-weight RNA molecules isolated from infected *N. benthamiana*, petunia and tomato plants or random primed ^{33}P dCTP labeled RC fragment (Table 4.1) for transgenic plants.

Fifteen micrograms of total RNA from transgenic plants were separated on a 1% denaturing formaldehyde–agarose gel and hybridized with a 35S terminator-derived dig-labeled probe and a ^{33}P-dCTP labeled RC fragment. For Southern blot, 10 µg genom-

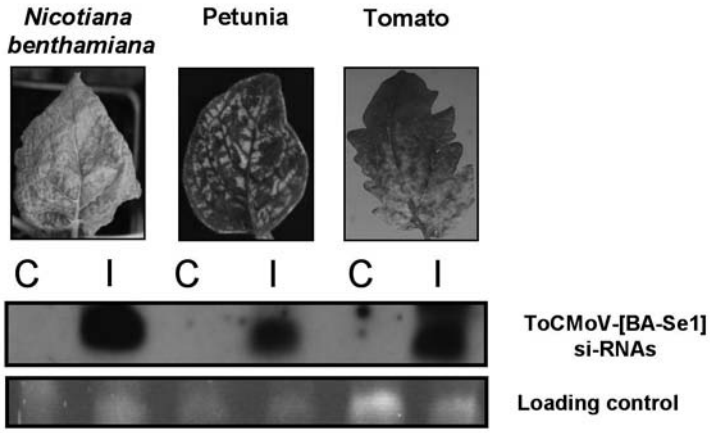

Figure 4.1. Symptoms of ToCMoV-[BA-Se1] in *N.benthamiana*, petunia and tomato, and Northern detection of virus-derived siRNAs in these hosts using a DNA-A specific probe. Low molecular RNAs were extracted from virus infected plant (I) or mock inoculated control (C) plants.

ic DNA from transgenic plants was digested with *Hind* III, separated on a 1% agarose gel, blotted to a nylon membrane and probed with ^{33}P labeled RC fragment. Hybridization using Church's buffer (Sambrook *et al.*, 1989) was carried out at 48°C and 65°C, for Northern or Southern blot, respectively.

RESULTS

ToCMoV-[BA-Se1] infection triggers RNA silencing in plants

Although in nature ToCMoV-[BA-Se1] has thus far been found only in tomato plants, experimentally this begomovirus infects a range of different host plants including *N. benthamiana* and petunia. Mechanically inoculated tomato plants show chlorotic spots, vein chlorosis and crumpling, followed by yellow mottling starting about 14 days post inoculation (dpi) (Fig. 4.1). *N. benthamiana* plants develop vein chlorosis, crumpling and epinasty starting at 7 to 10 dpi followed by systemic mottling and petunia exhibited vein chlorosis, chlorotic spots, concentric rings and chlorotic mottling (Fig. 4.1) (chapter 3).

Northern blot analysis of low molecular mass RNAs extracted from systemically infected leaves using a ToCMoV-[BA-Se1] DNA-A-derived probe showed accumulation of virus specific siRNAs indicating that ToCMoV-[BA-Se1] infection activates the

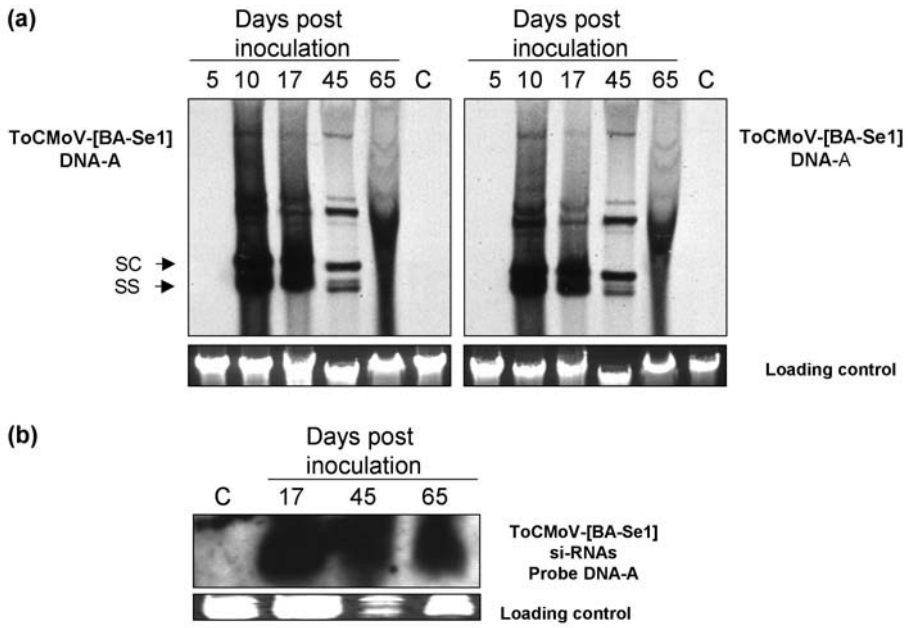

Figure 4.2. Time course of ToCMoV-[BA-Se1] DNA and virus-derived siRNA accumulation in infected *N. benthamiana* plants. (a) Viral DNA was extracted at different days post inoculation and detected by Southern hybridization using either DNA-A (left panel) or DNA-B-derived probe (right panel). ToCMoV-[BA-Se1] single stranded (SS) and supercoiled double stranded (SC) DNA forms are indicated. (b) Northern blot of ToCMoV-[BA-Se1]-specific siRNAs at different time points after inoculation. C: mock-inoculated control.

RNA silencing machinery and ToCMoV-[BA-Se1] mRNAs were targeted by RNA silencing in all three plant species tested (Fig. 4.1). Although over time, the accumulation of virus-specific siRNAs appeared to be relatively stable in *N. benthamiana*, the amount of viral DNA decreased dramatically. This may indicate that targeting ToCMoV-[BA-Se1] mRNAs interferes with viral replication and viral DNA accumulation, since both replicative dsDNA and ssDNA forms are reduced (Fig. 4.2). At 65 dpi, it was difficult to visualize viral DNA forms, probably also due to the senescent stage of the plant and presence of compounds that interfere with the gel electrophoresis (Fig. 4.2).

In infected plants siRNAs representing the entire genome of ToCMoV-[BA-Se1] are generated

To determine whether all genes and non-coding sequences in the bipartite genome were targeted by virus-induced silencing, PCR-amplified fragments covering the entire genome were prepared. Using appropriate primers, a series of six fragments was

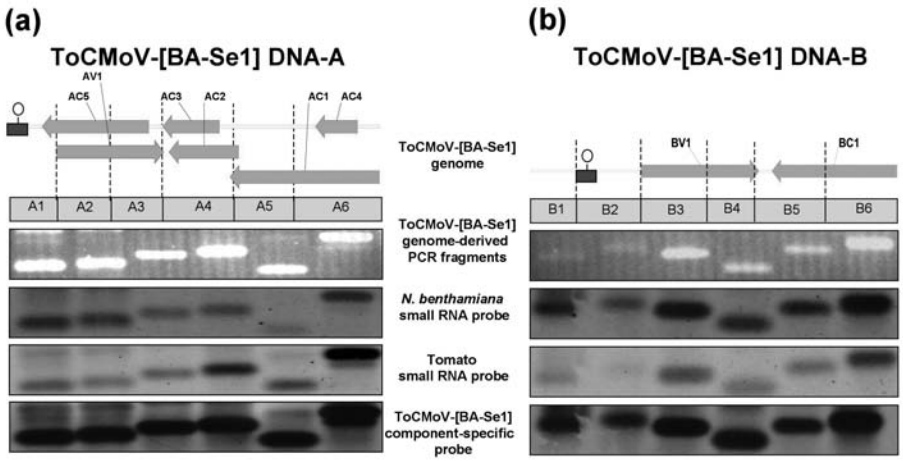

Figure 4.3. Origin and distribution of siRNAs from ToCMoV-[BA-Se1]-infected *Nicotiana benthamiana* and tomato plants. The bipartite genome of ToCMoV-[BA-Se1], consisting of DNA-A (a) and DNA-B (b), is schematically depicted. PCR fragments covering the viral genome were obtained and probed with 5'-labeled siRNAs extracted from *N. benthamiana* and tomato plants. As a control the same blots were also hybridized with probes derived from the complete DNA-A or DNA-B components of ToCMoV-[BA-Se1].

derived from each viral component (Fig. 4.3). Blotted fragments were probed with labeled small RNA molecules purified from ToCMoV-[BA-Se1]-infected *N. benthamiana* or tomato plants (Fig. 4.3). These analyses revealed that siRNAs populations isolated either from infected *N. benthamiana* or tomato plants represented the whole bipartite genome. Interestingly, these included both the coding and the intergenic regions. For the DNA-A, the fragments A6 (5' end of AC1 gene and the entire embedded AC4 gene), A1 (AC1 leader sequence, intergenic region including the common region, AV1 leader sequence and the overlapping 3' end of AC5) and A2 (5' end of AR1 and overlapping middle part of AC5) seemed to be the more abundantly targeted portions while segment A5 (3'end of AC1 gene) was under-represented in the siRNA population from *N. benthamiana* (Fig. 4.3a). Labeled siRNAs isolated from tomato hybridized predominantly to segments A6 and A4 (AC2 and AC3 sequences) while the differences among the rest of the segments were less pronounced (Fig 4.3a). For the B component, the siRNAs isolated from both plants seem to accumulate to high amounts and were spread about equally over the entire viral genomic sequence, except in the B2 (intergenic) region where siRNA production seemed lower (Fig.4.3b). As expected, strong hybridization signals for all fragments were obtained when the same blot was probed with either ToCMoV-[BA-Se1] DNA-A or DNA-B (Fig. 4.3).

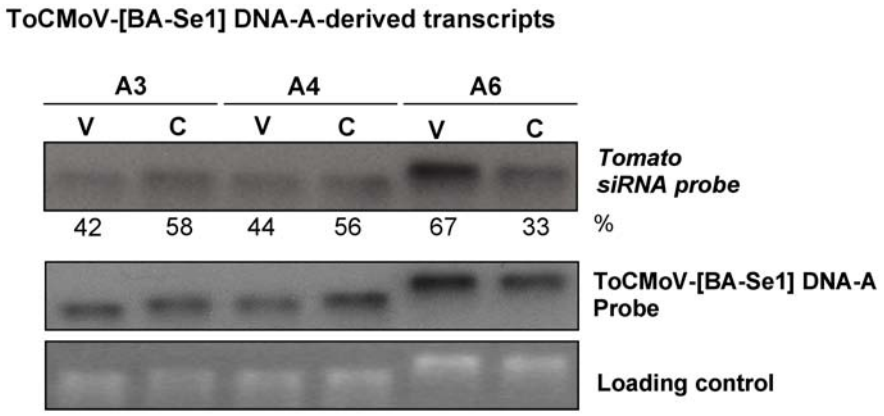

Fig. 4.4. Polarity of siRNAs accumulating in infected tomato plants corresponding to different ToCMoV-[BA-Se1] DNA-A-derived transcripts. Fragments A3, A4 and A6 were transcribed and equivalent amounts of transcripts in viral (V) and complementary sense (C) were blotted and probed with 5' labeled siRNAs extracted from ToCMoV-[BA-Se1] infected tomato plants. The blot was quantified using Gene Tools software and the numbers below each lane corresponds to the amount of siRNAs for the respective polarity in comparison to the total amount of siRNAs specific for that fragment and considering differences in loading (lower panels). As a control, the same blot was hybridized with a ToVMoV-[BA-Se1] DNA-A labeled probe.

ToCMoV-[BA-Se1]-specific siRNAs originate from diverse dsRNA sources

Geminivirus transcripts in opposite orientation are known to overlap, producing dsRNA at the 3' ends of the AV1 and AC3 genes (Chellappan *et al.*, 2004b). However it is not known if these overlapping transcripts can be elongated to some extent by the host RNA dependent RNA polymerase (RdRp) reported to be involved in RNA silencing (Dalmay *et al.*, 2000). To gain further information on this question, we have identified the polarity of ToCMoV-[BA-Se1]-specific siRNAs derived from DNA-A segments A3 and A4 that are known to overlap, as well as the more distal segment A6. *In vitro* transcripts in both viral and complementary sense were produced and probed with labeled low molecular mass RNAs from infected tomato plants. As could be anticipated, an approximately equal ratio between viral and complementary strand-derived siRNA was obtained for A3 and A4 (Fig. 4.4). However for the segment A6 a much higher proportion of siRNAs hybridized to the viral sense transcript (Fig 4.4), demonstrating that they are mainly – but not exclusively- derived from the complementary sense. This indicated that they may be primarily derived from the AC1/AC4 mRNA and not from RdRp activity.

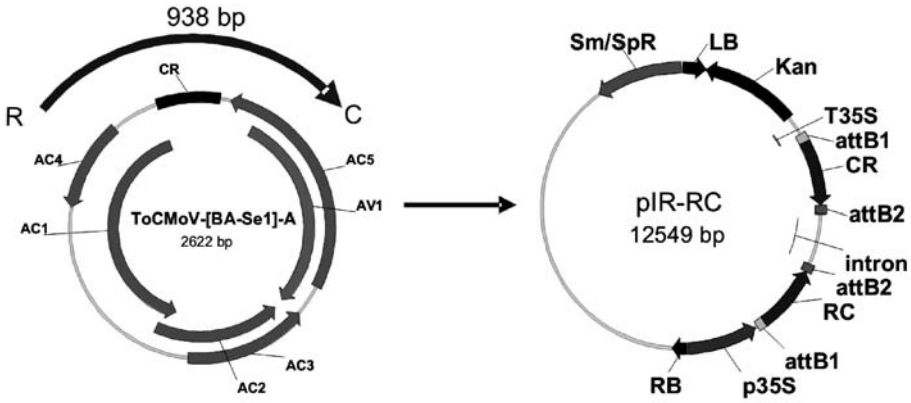

Figure 4.5. Genetic map of ToCMoV-[BA-Se1] DNA-A showing the origin of the fragment amplified and cloned into the plant expression vector pIR-RC used for *N. benthamiana* transformation.

Enhancing the plant RNA silencing system to increase virus resistance

From the experiments described above it could be concluded that as a response to infection with ToCMoV-[BA-Se1] the plant produces virus-specific siRNAs, the occurrence of these siRNAs coinciding with decreased viral DNA accumulation.

To enhance the plant's antiviral RNA silencing potential, transgenic *N. benthamiana* plants were produced expressing a virus-derived intron-hairpin construct. Thus, siRNAs would be produced prior to virus infection. This strategy has been demonstrated to be highly efficient and has been widely used to obtain host resistance against RNA viruses (Chen *et al.*, 2004; Smith *et al.*, 2000; Wang *et al.*, 2000; Waterhouse & Helliwell, 2003).

Segments from the ToCMoV-[BA-Se1] DNA-A that were shown to be more abundantly targeted by RNA silencing in *N. benthamiana* (Fig. 4.3) were cloned into the intron-hairpin binary destination vector pK7GWIWG2 (Karimi *et al.*, 2002) using the GATEWAY[a] technology. The inverted repeat construct, pIR-RC, comprised the 5' terminal 300nt of the AC1 gene (and part of the nested AC4 gene), the entire common region and the 300 nt 5' end of the AV1 gene (including the 3' end of the AC5 ORF) (Fig. 4.5).

This construct was used for plant transformation, resulting in forty-eight independent transgenic *N. benthamiana* lines. Proper insertion of the entire transgene sequence was confirmed by PCR analysis (not shown). All plants developed a normal phenotype.

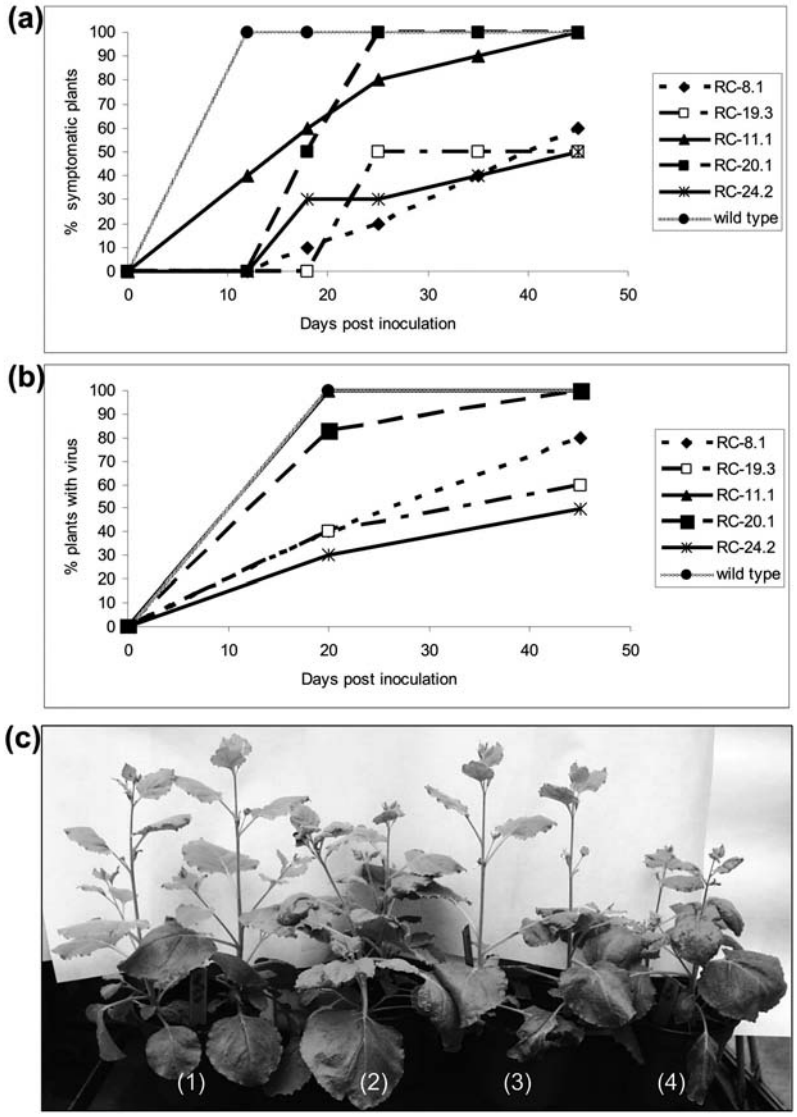

Figure 4.6. Systemic symptoms and virus accumulation in transgenic *N. benthamiana* plant lines challenge-inoculated with ToCMoV-[BA-Se1]. (a) Percentage of plants with symptoms evaluated visually in relation to time post inoculation (dpi). (b) Percentage of plants containing virus at 20 dpi and 45 dpi as evaluated by tissue blot hybridization. (c) Symptoms on plants from transgenic line RC-19.3 at 20 dpi: (1) Resistant inoculated transgenic plants; (2) Susceptible inoculated transgenic plants; (3) Transgenic mock-inoculated controls and (4) Wild type inoculated controls.

Performance of challenge-inoculated transgenic plants

Initial inoculation experiments were performed with the T1 progeny of 11 transgenic *N. benthamiana* lines. Northern blot analysis of low molecular weight RNA extracted from leaves from non inoculated transgenic plants of three lines hybridized with a probe derived from the transgene, indicating that the transgene had been transcribed and converted into siRNAs (not shown). Ten plants of each line were challenge-inoculated with ToCMoV-[BA-Se1] at the 5 to 6 leaf stage. In wild-type plants used as controls, symptoms of crumpling and chlorotic veins started to appear at 7-8 dpi. At 14 dpi all control plants were showing systemic symptoms. Most transgenic lines showed a delay in symptom expression of at least 8 days when compared to wild-type plants. Later, though, many transgenic plants started to show symptoms. By 45 dpi, five lines still had plants without symptoms, although virus could be detected in some of the symptomless plants by dot blot analysis (not shown).

The challenge experiment with the lines RC-8.1, RC-11.1, RC-19.3, RC-20.1 and RC-24.2, showing enhanced resistance, was repeated and similar results were obtained. At 12 dpi, all inoculated non-transgenic control plants exhibited systemic symptoms whereas the onset of the disease was delayed for the transgenic plants (Fig. 4.6a). At 20 dpi a large number of transgenic plants were still symptomless, although viral DNA could be detected in some these plants (Fig 4.6b). Symptoms displayed by infected susceptible transgenic plants were very similar to those displayed by wild type plants, though stunting was often less pronounced (Fig 4.6c). At 45 dpi, the best performing lines were RC-24.2 with 50% of the plants resistant to virus infection (no symptoms and no virus) and RC-19.3 with 50% of the plants symptomless including 30% entirely virus-free plants (Fig 4.6a and 4.6b).

siRNA levels in transgenic plants do not correlate with resistance

A more detailed analysis of symptomatic and symptomless siblings of lines RC-24.2 and RC-19.3, as judged by visual observation at 45 dpi, was performed including Northern analysis of transcripts and siRNA at 3 time points during the infection (0, 20 and 45 dpi). Southern analysis was performed to estimate the transgene copy number and viral DNA accumulation at 45 dpi. Southern blot analysis showed that siblings within a transgenic line had the same number and pattern of integrated copies, one in the case of RC-19.3 and two in RC-24.2 (not shown). Transgene specific siRNA was readily detected by Northern analysis in all transgenic plants before virus inoculation (0 dpi) (Fig 4.7a). Except for plant 17 of line RC-19.3 that clearly contained a higher amount of siRNA, all plants assayed displayed similar siRNA levels. The generation of siRNA from the double stranded transgenic mRNA in the plants seemed to be highly efficient,

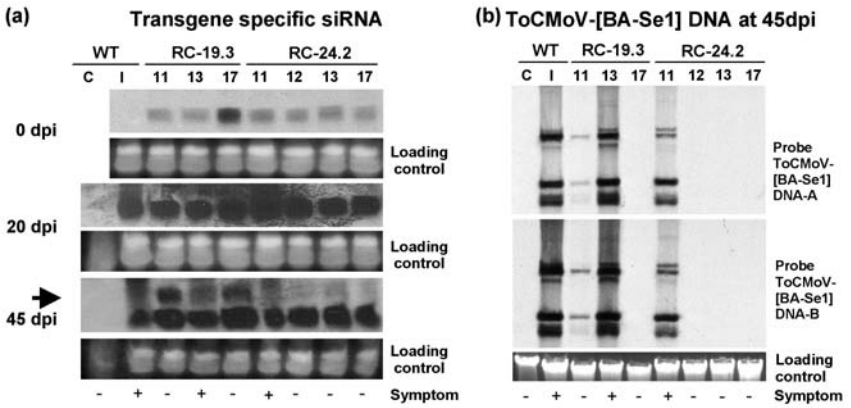

Figure 4.7. Analysis of resistant and susceptible S1 siblings of *N. benthamiana* lines RC-19-3 and RC-24-2. (a) Presence of transgene-derived siRNAs at 0, 20 and 45 days post inoculation (dpi) and (b) Accumulation of ToCMoV-[BA-Se1] DNA-A and DNA-B at 45 dpi. I: inoculated wild type control. C: mock-inoculated wild type control. The arrow indicates a higher molecular mass RNA species.

since transgene transcripts could not be detected by Northern analysis neither using a probe corresponding to the CaMV35S terminator nor a transgene-derived probe. However, transcripts could be amplified by RT-PCR after DNase treatment of the sample (not shown).

The amount of siRNA hybridizing to the RC probe increased greatly at 20 dpi (Fig 4.7a). The origin of the siRNAs can be from either the transgene or the virus, but since no virus is detected in several plants that nonetheless have elevated siRNA levels, this suggests the siRNAs arise from the transgene. At this time point only transgenic plant RC-24.2-11 and wild-type inoculated controls were virus-infected as determined by systemic symptoms and PCR using a virus specific primer pair outside the transgene sequence (not shown).

Figure 4.7a shows that there was no evident difference in the levels of transgene-derived siRNA among the analyzed transgenic plants, despite some of the plants showing symptoms, while others proved to be virus-free. At 45 dpi, the accumulation of siRNA corresponding to the RC sequence was still very high in all transgenic plants tested. A slightly higher amount of siRNA was present in resistant and immune plants in both transgenic lines when compared with a susceptible sibling of the same line (Fig 4.7a). Interestingly, a larger RNA species was visible in resistant and immune plants, especially in plants of line RC19.3 (Fig 4.7a).

Southern blot analysis at 45 dpi showed that plants RC-19.3-11 and 19.3-13 became infected and viral DNA accumulation in 19.3-13 was comparable to the inoculated wild-type control. Plant RC-19.3-11 remained symptomless, yet became infected albeit that viral DNA accumulation was extremely reduced (Fig 4.7b). Plants RC-24.2-

12, RC-24.2-13 and RC-24.2-17 remained symptomless and viral DNA could not be detected by Southern blot (Fig 4.7b) or PCR (not shown).

Discussion

Upon virus infection, plants undergo complex metabolic changes to accommodate the host requirements for defense but also the needs of the virus for replication and movement. RNA silencing is an important defense response to virus infection in plants. In a normal infection by a RNA or DNA virus or by a viroid, it has been observed that the silencing machinery in the plant can be activated with specific siRNAs directed to the invading pathogen being produced (Chellappan *et al.*, 2004b; Denti *et al.*, 2004; Hamilton & Baulcombe, 1999; Lucioli *et al.*, 2003; Molnar *et al.*, 2005; Papaefthimiou *et al.*, 2001; Yoo *et al.*, 2004).

In this chapter it is shown that also the begomovirus ToCMoV-[BA-Se1] triggers the plant RNA silencing machinery in different host plants. Upon infection, a population of siRNAs specific to ToCMoV-[BA-Se1] is generated. This reaction seems to interfere with the virus replication and/or accumulation in *N. benthamiana,* because following the acute symptoms of chlorotic veins, crumpling and epinasty, the plant develops a rather ameliorated systemic symptom of mottling which is accompanied by a decrease in the amount of viral DNA. These results are similar to those obtained from *N. benthamiana* and cassava (*Manihot esculenta*) plants infected by African cassava mosaic virus – [Cameroon] (ACMV-[CM]) and Sri-Lankan cassava mosaic virus (SLCMV), which are regarded as recovery phenotype viruses. Also in these viruses the remission of the symptoms is accompanied by an increase in virus-specific siRNAs and decrease in both viral mRNA and DNA (Chellappan *et al.*, 2004b).

Recent studies on siRNA induced by virus infection by RNA or DNA viruses in different hosts plants (Chellappan *et al.*, 2004b; Molnar *et al.*, 2005; Szittya *et al.*, 2002) have revealed that most viral genomes are not uniformly targeted. Although the siRNA population present in both *N. benthamiana* and tomato infected by ToCMoV-[BA-Se1] represented the entire DNA-A and DNA-B, it was not homogeneously distributed over the whole extent of the genome. Comparing the distribution of DNA-A derived siRNA from *N. benthamiana* and tomato, we observed a divergence in the preference for different parts of the genome in different hosts. In *N. benthamiana* the highest levels of siRNA accumulation corresponded to the 5' end of AC1 and embedded AC4 (fragment A6), IR (fragment A1 which includes AC1 leader sequence, CR, AV1 leader sequence and the overlapping 3' end of AC5) and 5' end of AV1 and overlapping middle part of AC5 (fragment A2). The precise reasons why the RNAs derived from these regions of the genome are preferentially targeted by the RNA silencing mechanism are

not known. The abundance of the transcript, the secondary structure of the mRNA or accessibility to Dicer may influence the targeting (Bernstein *et al.*, 2001; Szittya *et al.*, 2002). The transcript correspondent to AC1 is probably abundant early in the infection cycle (Chellappan *et al.*, 2004b). The ToCMoV-[BA-Se1] AC1 transcript has not yet been mapped. However, by using the program Mfold (Zucker, 2003) to analyze the secondary structure of the RNA sequence predicted from the AC1 ORF, several high energy structures involving predicted stemloops were recovered (not shown). Similar structures were reported for the ACMV-[CM] AC1 transcript (Chellappan *et al.*, 2004b) and were targeted by the RNA silencing machinery in plants infected by *Cymbidium ringspot virus* (Molnar *et al.*, 2005). Since AC1 is the main protein involved in the viral DNA replication and paramount for virus replication (Hanley-Bowdoin *et al.*, 1999), targeting AC1 mRNA would impact viral replication by reducing viral DNA accumulation over time in this host. AV1 codes for the coat protein which is a late expressed gene and the correspondent mRNA was shown to accumulate in high amounts (Shivaprasad *et al.*, 2005). The coat protein is involved in ssDNA accumulation (Azzam *et al.*, 1994), and therefore downregulating CP production would result in further decrease in ssDNA accumulation.

Similar to *N. benthamiana*, the majority of siRNAs in infected tomato is targeted to the 5' end of the AC1 gene. However, also a large amount of siRNAs preferentially hybridized to the fragment corresponding to AC2 and the overlapping AC3 (Fragment 4). Since AC2 is a transcription activator for the late genes AV1 and BV1, an indirect down regulation of these two genes would lead to reduced amounts of viral DNA accumulation and movement. The AC1 and AC2 sequences are also the preferred target of RNA silencing directed to different viruses involved in cassava mosaic disease (Chellappan *et al.*, 2004b; Chellappan *et al.*, 2005). The relative discrepancy between the major target sequences in different hosts infected by ToCMoV-[BA-Se1] might reflect different composition of the silencing machinery in the two host plants leading to different interactions with the virus or maybe due to differential accumulation/transcription of complementary sense RNA. This is similar to what has been found for *East African cassava mosaic virus*, which is also targeted differently in *N. benthamiana* and cassava (Chellappan *et al.*, 2004b; Chellappan *et al.*, 2005).

An intriguing and unresolved issue is how geminiviruses, with no dsRNA step in their replication cycle, can induce RNA silencing in infected plants. Vanitharani and coworkers (2005) suggested three possibilities including induction by overlapping transcripts in opposite direction, by abundant early transcripts converted into dsRNA by a host RdRp, or by strong secondary structures of transcripts perceived as dsRNA. We have addressed these possibilities by looking at the polarity of the siRNAs from ToCMoV-[BA-Se1]-infected tomato. For siRNAs binding to fragments that include the region

where transcripts are known to overlap, we found that siRNAs of viral and complementary sense are present in almost equal amounts. This is similar to results of Chellapan *et al.* (2004b), who used strand-specific probes to detect the overlapping transcripts. However, for the 5' end of AC1 a higher proportion (2:1) of complementary sense polarity was present suggesting that the majority of the siRNAs from this region possibly originated from the AC1 mRNA fold-back structure directly being the substrate for a Dicer-like protein as described for *Cymbidium ringspot virus*, *Potato virus X* and *Tobacco mosaic virus* (Molnar *et al.*, 2005). An alternative explanation would be the preferential incorporation of the complementary sense-strand into the RISC (Hutvagner, 2005). This question would be unequivocally resolved by sequencing the siRNAs from the virus-infected plant (Molnar *et al.*, 2005).

RNA-mediated virus resistance (RMVR) has been used as a potent strategy to generate host resistance against RNA viruses (Prins, 2003; Ritzenthaler, 2005). Also for the ssDNA geminiviruses, sense and anti-sense versions mainly involving the AC1 gene have been employed though with varying success rates (Aragão *et al.*, 1998; Bejarano & Lichtenstein, 1994; Bendahmane & Gronenborn, 1997; Day *et al.*, 1991; Yang *et al.*, 2004). The realization that RNA silencing was responsible for the RMVR mechanism (Baulcombe, 1996) and the recognition that dsRNA and siRNAs played a major role in the process (Hamilton & Baulcombe, 1999; Waterhouse *et al.*, 1998) led to the introduction of intron-hairpin constructs which directly produce self complementary dsRNAs that efficiently induce targeted gene silencing and virus resistance (Smith *et al.*, 2000). Using this type of construct containing the common region of the begomovirus Vigna mungo yellow mosaic virus (VMYMV) in a transient assay, Pooggin *et al.* (2003) obtained recovery from virus infection.

In this chapter, we have explored the natural RNA silencing response to begomovirus ToCMoV-[BA-Se1] infection in *N. benthamiana* by preprogramming transgenic plants to express dsRNA corresponding to the most targeted portions of the viral genome. This was achieved by using an intron-hairpin construct comprising the 300 nt of the 5' end of the AV1 gene (including the end of the overlapping AC5 ORF) the entire common region and 300nt of the 5' end of the AC1 gene (including a part of the nested AC4 gene). Indeed, transgenic plants were shown to contain transgene-specific siRNAs. In contrast to experiments with RNA viruses (Chen *et al.*, 2004; Hily *et al.*, 2005; Missiou *et al.*, 2004; Nomura *et al.*, 2004) completely immune lines were not observed. Most transgenic lines, however, did show significant delays in symptom development and two lines encompassed highly resistant and even immune plants. In siblings of these lines, RC-19.3 and RC-24.2, siRNAs corresponding to the transgene sequence were readily detected and the amount increased at least 3 to 6 fold after virus inoculation, although virus infection could not be detected by PCR, a feature also observed

upon infection of resistant plants expressing tospovirus siRNAs with the corresponding virus (E. Bucher and M. Prins, *unpublished*). At 45 dpi, both lines had susceptible and resistant plants though resistant plants at this stage contained a slightly higher amount of siRNAs. Interestingly, a somewhat larger RNA species was visible in resistant and immune plants of line RC-19.3. This fragment might represent an incompletely cleaved RNA product, but why it specifically occurs in resistant plants remains puzzling. Interestingly, Boutla and co-workers (2002) reported that an RNA species of ~ 80 nt purified from GFP silenced plants was a very potent inducer of silencing when injected in GFP-expressing *Caenorhabditis elegans*, suggesting the presence of functional RNA species of slightly larger size. Indeed, such RNA species were also observed by the same researchers in *Cucumber mosaic virus* resistant plants expressing inverted repeat transgenes (K. Kalantidis, *personal communication*). Our results collectively indicate that the presence of transgene siRNAs corresponding to viral sequences prior to virus inoculation does not guarantee virus resistance. Noris *et al.* (2004) reported similar results for *Tomato yellow leaf curl Sardinia virus*, where the virus was able to infect tomato plants expressing siRNAs specific to AC1. Both cases contrast with RNA silencing mediated resistance to RNA viruses, where the presence of virus-specific siRNAs in transgenic plants strongly correlates with virus resistance (Chen *et al.*, 2004; Hily *et al.*, 2005; Missiou *et al.*, 2004; Nomura *et al.*, 2004), indicating that RNA viruses are more susceptible to RNA silencing, possibly because both mRNAs and genomic RNAs can be target of the silencing machinery.

When plants infected with VMYMV were bombarded with a hairpin construct containing virus-specific common region sequences they recovered from infection (Pooggin *et al.*, 2003). The authors suggested that the viral DNA is targeted and methylated possibly by an RNA dependent DNA methylase, thus resulting in remission of the symptoms. This observation contrasts with results obtained with *Tomato leaf curl virus* (Seemanpillai *et al.*, 2003) where a transgene encoding a viral promoter becomes methylated after virus infection, while the virus itself is not affected. We have not been able to prove if or to what extent the transgene or the viral genomic DNA is affected by methylation. We favor the suggestion by Noris and colleagues (Noris *et al.*, 2004) that a threshold of siRNA expression exists below which the virus can bypass the resistance and build up a critical mass of virus in the primary infected cell from which it can subsequently spread over the plant.

The results reported in this chapter together with those published by others (Chellappan *et al.*, 2004b; Chellappan *et al.*, 2005; Pooggin & Hohn, 2004; Trinks *et al.*, 2005; Vanitharani *et al.*, 2005; Vanitharani *et al.*, 2004) add to the conclusion that there is a significant but highly complex relationship between begomoviruses and the host plant RNA silencing plant defense process. Even though transgenic resistance associated

with gene silencing can be achieved successfully (Asad *et al.*, 2003; Chellappan *et al.*, 2004a) it is dependent on the virus-host combination, possibly the strength of the RNA silencing suppressor of the virus (Chellappan *et al.*, 2004a), and environnemental factors (Chellappan *et al.*, 2005; Szittya *et al.*, 2003).

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Chapter 5

Involvement of the individual gene products of *Tomato chlorotic mottle virus* in symptom induction and suppression of RNA silencing

Summary

Proteins encoded by all open reading frames of both DNA-A and DNA-B of the begomovirus Tomato chlorotic mottle virus – [Bahia-Seabra1] (ToCMoV-[BA-Se1]) were expressed using a Potato virus X (PVX) vector and evaluated for eliciting disease symptoms in local and systemic host plants. All ToCMoV-[BA-Se1] genes, with the exception of AC3 and AC5, induced enhanced symptoms depending on the host plant. The most striking reactions were induced by AC2, which caused systemic necrotic lesions, necrotic veins and petioles leading to leaf collapse and subsequent severe stunting of *Nicotiana benthamiana* plants. Since several genes were found to augment the virulence of PVX, the silencing suppression potential of all ToCMoV-[BA-Se1] genes was studied. Applying the two most commonly used strategies, i.e. the local *Agrobacterium* transient assay and the reversal of transgene silencing assay, none of the ToCMoV-[BA-Se1] gene products was identified as RNA silencing suppression protein. Our results indicate that although several ToCMoV-[BA-Se1] proteins can be regarded as virulence determinants, none encodes a RNA silencing suppressor that could be detected in the commonly used assays.

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Introduction

Geminiviruses (family *Geminiviridae*) are plant viruses which have small circular single-stranded DNA genome and geminate isometric particle morphology. These viruses are found in tropical to warm temperate geographical zones and can infect a wide range of plants including crops and native weeds and wild plants (Morales & Jones, 2004). Virus species belonging to the largest genus of geminiviruses, *Begomovirus,* are transmitted by the whitefly *Bemisia tabaci* (Gennadius) and represent a major threat to many cultivated dicotyledonous plants. Begomoviruses can either have a monopartite or a bipartite genome. The New World begomoviruses are all bipartite.

In the bipartite begomoviruses the genomic DNAs are termed DNA-A and DNA-B. DNA-A encodes genes responsible for (a) viral functions related to replication (AC1 and AC3), (b) regulation of gene expression and sometimes suppression of RNA silencing (AC2) and (c) for particle encapsidation (AV1) (Hanley-Bowdoin *et al.*, 1999). Until recently, no function could be assigned to the ORF AC4, however, a recent report has demonstrated its involvement in RNA silencing suppression by two cassava-infecting begomoviruses (Vanitharani *et al.*, 2004). In some begomoviruses an additional ORF (AC5) has been observed, with hitherto unresolved function (Fernandes *et al.*, 2006; Galvão *et al.*, 2003; Hong *et al.*, 1993; Kheyr Pour *et al.*, 2000; Torres-Pacheco *et al.*, 1993). The DNA-B of the bipartite begomoviruses encodes two genes, BC1 and BV1, involved in the virus cell-to-cell and systemic movement through the plant (Gafni & Kunik, 1997; Lazarowitz, 1992).

The development of disease symptoms depends on many different factors, which, besides environmental influences, are derived from the virus as well as the host plant. The interplay between the latter factors can lead to the establishment of a successful infection or prevent it. The involvement of different plant viral genes on the disease process related to symptomatology and virulence have been studied employing different experimental systems. Viruses containing mutations in genes of interest, chimeric viruses with exchanged homologous genes, pseudorecombinants and transgenic plants expressing viral proteins are the most commonly used techniques for begomoviruses and have implicated the involvement of genes from both DNA-A and DNA-B in the development of symptoms induced by different viruses in different hosts (Duan *et al.*, 1997; Garrido-Ramirez *et al.*, 2000; Hou *et al.*, 2000; Rigden *et al.*, 1994; Sunter *et al.*, 2001; von Arnim & Stanley, 1992).

Viral vectors are alternative tools that can be used to explore the contribution of specific individual genes in viral pathogenesis, bypassing some difficulties which might arise when using the above mentioned methods. Examples of these problems are the lethality of mutated essential genes and poor recovery of transgenic plants expressing toxic proteins.

The most commonly used viral vectors for this purpose are those derived from Potato virus X (PVX) (Chapman *et al.*, 1992) and Tobacco mosaic virus (TMV)

(Shivprasad *et al.*, 1999). Both vectors permit high expression of foreign proteins in plants and have been employed in diverse interaction studies between plants and specific viral gene products (Canto *et al.*, 2004; Hong-Wei *et al.*, 1999; Hussain *et al.*, 2005; Lukhovitskaya *et al.*, 2005; Scholthof *et al.*, 1995; Selth *et al.*, 2004; van Wezel *et al.*, 2002a; van Wezel *et al.*, 2001).

RNA silencing is a process that involves the degradation of RNA in a sequence-specific manner (Baulcombe, 1999). This process is active in most eukaryotic organisms and is characterized by the cleavage of dsRNA templates by an enzyme called Dicer generating small interfering RNAs (siRNAs) of 21-26 nt in length (Bernstein *et al.*, 2001; Hamilton & Baulcombe, 1999). These siRNAs target complementary RNAs for degradation via a multiprotein RNA inducing silencing complex (RISC) (Hammond *et al.*, 2000). RNA silencing in plants is involved in several key pathways such as virus resistance, the regulation of endogenous gene expression, heterochromatin formation and repression of transposable element activity (Baulcombe, 2005; Waterhouse *et al.*, 2001). Since plant viruses are both triggers and targets of RNA silencing (Chellappan *et al.*, 2004; Denti *et al.*, 2004; Hamilton & Baulcombe, 1999; Lucioli *et al.*, 2003; Molnar *et al.*, 2005; Papaefthimiou *et al.*, 2001; Yoo *et al.*, 2004) they have evolved a self defense mechanism encoding proteins that operate as suppressors of gene silencing (Voinnet, 2005; Voinnet *et al.*, 1999). Silencing suppressors from different viruses are unrelated both in their sequence homology and function during the virus life cycle and affect different steps in the RNA silencing pathway (Moissiard & Voinnet, 2004; Roth *et al.*, 2004). Despite divergent functions during the viral infection cycle, many of these RNA silencing suppressor proteins have previously been implicated in virulence (Brigneti *et al.*, 1998; Moissiard & Voinnet, 2004; Voinnet *et al.*, 1999).

Tomato chlorotic mottle virus is a bipartite begomovirus which has been found naturally infecting tomato plants in Brazil (chapters 2 and 3). The strain studied in this thesis, Tomato chlorotic mottle virus–[Bahia-Seabra1] (ToCMoV-[BA-Se1]) has the genomic organization characteristic of the New World begomoviruses, except that DNA-A contains a large extra complementary sense ORF (AC5). Like all bipartite begomoviruses, DNA-A and DNA-B are necessary for systemic infection. Besides tomato, ToCMoV-[BA-Se1] can infect a range of other hosts including vegetable, ornamental and weed species causing symptoms like chlorotic spots, vein chlorosis, crumpling, epinasty, leaf deformation, stunting and mottling, depending on the host (chapter 3).

To better understand the complexity of interactions of ToCMoV-[BA-Se1] with different plant hosts, we analyzed the contribution of all individual viral genes encoded by both DNA-A and DNA-B in eliciting disease symptoms in local and systemic host plants. Since virulence determinants have often been implicated in counteracting RNA silencing, we also investigated the potential role of the ToCMoV-[BA-Se1] gene products in suppressing RNA silencing. This was achieved by transient expression of these proteins from *Agrobacterium*-infiltrated binary vectors and PVX-derived vectors.

METHODS

Construction of expression vectors

The eight different genes of ToCMoV-[BA-Se1] (Fig. 5.1) were PCR-amplified using proof-reading Pfu DNA polymerase, appropriate primer pairs for individual ORFs (Table 5.1) and infectious full-length DNA-A and DNA-B clones as templates (chapter 3). Primers were designed such that the sequences downstream of the translation initiation codon maintained the viral coding context and a purine (A/G) is on the -3 position (Kozak, 1999). The recombinant plasmids were obtained using the GATEWAY™ system (Invitrogen) following the manufacturer's instructions, except that the reaction mixture was scaled down to one fourth of the recommended quantity. The PCR fragments were introduced by BP recombination into pDonr207. Sequence analysis was performed to verify the correct nucleotide sequence of each ORF. Subsequently, they were introduced either into PVX-GW, a PVX-derived vector (Jones *et al.*, 1999) modified to contain the GATEWAY™ recombination site, or inserted into the destination binary vector pK7WG2 (Karimi *et al.*, 2002), downstream of the 35S promoter and *Tobacco mosaic virus* leader sequence by LR recombination. PVX vectors containing the AC2, AV1, BC1 and BV1 genes in antisense, to be used as negative controls, were obtained by placing the attB2 site in the forward primer and attB1 and the reverse primer (Table 5.1). The resulting expression vectors were named after each gene as pGWAC1, pGWAC2 etc., or PVX-AC1, PVX-AC2, and PVX-AC2-AS, PVX-AV1-AS etc. for the antisense versions. The expression binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 and PVX-derived vectors were electroporated into the same *A. tumefaciens* strain containing the pSoup helper plasmid (Hellens *et al.*, 2000).

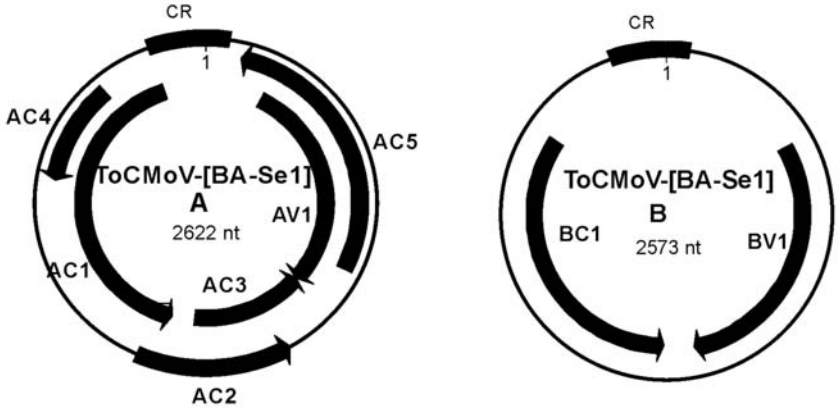


Figure 5.1. Organization of the bipartite genome of Tomato chlorotic mottle virus–[BA-Se1]. Arrows indicate the ORFs in virion (V) or complementary (C) sense orientation. CR is part of the intergenic region shared between the two genomic components.

Table 5.1. Primers used to PCR amplify the ToCMoV-[BA-Se1] genes, PVX coat protein gene and GFP.

Primer name	Primer sequence* (5'→ 3')	Fragment size (bp†)
GW-AC1-F	attB1- **ATG**CCACTCCCCAGACATTT	1056
GW-AC1-R	attB2- CTTTTTA<u>GCAGCCCGATGTTG</u>	
GW-AC2-F	attB1- **ATG**CGCAATTCATCTTTCTC	399
GW-AC2-R	attB2- <u>CTA</u>TTTAAATATGTCATCCCAGAAGC	
GW-AC3-F	attB1- **ATG**GATTCACGCACAGGGGA	390
GW-AC3-R	attB2- GATTCGATAACAAA<u>TTA</u>ATAA AG	
GW-AC4-F	attB1- AAA**ATG**GGGAACCTCATCTCCAC	294
GW-AC4-R	attB2- <u>TTA</u>ATATATTGAGTGCTTCCATGAC	
GW-AC5-F	attB1- **ATG**ATTCTCGTACTTCCCGGCT	753
GW-AC5-R	attB2- <u>TCA</u>GATTACGTTTGACGAGC	
GW-AV1-F	attB1- AAA**ATG**CCTAAGCGCGAT	756
GW-AV1-R	attB2- CT<u>TTA</u>TTAATTTGTTATCGAATC	
GW-BC1-F	attB1- **ATG**AGTTCTCAGTTGGTTGTT CC	882
GW-BC1-R	attB2- GCATCTGTAAT<u>TTA</u>TTTC AAAGAC	
GW-BV1-F	attB1- **ATG**TATTTCAATGGTAACAAGCGTC	828
GW-BV1-R	attB2- AA<u>TTA</u>TCCAATATAGTCAAGGTC	
PVX-CP-F	**ATG**TCAGCACCAGCTAGCAC	695
PVX-CP-R	G<u>TTA</u>TGGTGGTGGTAGAGTG	
GW-GFP-F	attB1- C**ATG**GGCAAAGGAGAAGAACTT	720
GW-GFP-R	attB2- <u>TTA</u>TTTGTATAGTTCATCCATGC	

*Nucleotides in bold and underlined indicate start and stop codons, respectively. attB1 (-5'GGGGACAAGTTTGTACAAAAAAGCAGgCT3') and attB2 (-5'GGGGACCACTTTGTACAA-GAAAGCTGGGT3') are recombination sequences for BP recombination reactions with pDonr plasmid; lower case g in attB1 is a preferred purine at -3 position relative to the initiation ATG.
† Fragment size is given in base pairs excluding the nucleotides from the attB sites.

The binary vector phpGFP harboring GFP in an inverted repeat array was obtained by recombining a PCR product amplified with appropriated primers (Table 5.1) into pDonr207 and subsequently into pK7GWIWG2 (Karimi *et al.*, 2002) using the GATEWAY™ system as described above.

An agro-infectious ToCMoV-[BA-Se1] DNA-A clone was obtained by digesting the genomic infectious DNA-A clone (chapter 3) with *Eco*RI and *Nco*I and ligating into pCAMBIA 0380 digested with the corresponding enzymes. The plasmid was then digested with *Kpn*I and a full-length *Kpn*I digested DNA-A component was ligated generating

pCAMBIA-DNA-A which contains 1.8 copies the viral genome.

Binary vectors harboring *Tomato spotted wilt virus* NS_S (pBinNS_S), *Carnation Italian Ringspot virus* p19 (pBinp19) and GFP (pBinGFP) and the PVX vector containing the NS1 gene of *Influenza A virus* (PVX-NS1) have been described previously (Bucher *et al.*, 2004; Bucher *et al.*, 2003; Vargason *et al.*, 2003)

Virus inoculation to plants

For virus inoculation, carborundum dusted leaves of *Nicotiana benthamiana* and *Datura stramonium* plants were rubbed with the extract of ToCMoV-[BA-Se1]-infected *N. benthamiana* plants prepared by grinding infected leaf tissue (1:10 w/v) in phosphate buffer, pH 8.0, containing 25 mM EDTA and 0.01% Na_2SO_3.

Agroinoculation and agroinfiltration

N. benthamiana plants were inoculated with PVX constructs by the toothpick piercing agro-delivery method. *A. tumefaciens* carrying the construct was grown for two days in solid medium and a tip of a sterile toothpick was dipped in a bacterium colony and used to pierce a leaf six times (Bucher *et al.*, 2004).

For PVX inoculation of *Datura stramonium* and tomato (*Lycopersicon esculentum*) 'Moneymaker' as well as for the experiments to assess whether any of the ToCMoV-[BA-Se1] genes functioned as silencing suppressors, agroinfiltration was performed as described by Bucher *et al.* (2003). In the case of PVX inoculation, a bacterial suspension containing the appropriate vectors was infiltrated once in one leaf of *D.stramonium* and three times in two leaves of tomato plants. Empty PVX vector (pGR107) (Jones *et al.*, 1999) was used as control. Inoculated plants were kept at 25°C and monitored for symptom development.

Derivatives of two commonly used procedures for identification of viral silencing suppressors were employed to assess the potential silencing suppressor of ToCMoV-[BA-Se1]: i) *Agrobacterium*-mediated transient assay (Voinnet *et al.*, 2000) and ii) reversal of transgene-induced gene silencing in whole plants using the wild-type ToCMoV-[BA-Se1] and chimeric PVX containing ToCMoV-[BA-Se1] genes (Voinnet *et al.*, 1999).

The local silencing assay was performed as described by Bucher *et al.* (2004). Wild type *N. benthamiana* leaves were infiltrated with a mixture of pBinGFP combined with the binary vectors containing each different ToCMoV-[BA-Se1] gene, the infectious pCAMBIA-DNA-A or a mixture with all eight binary vectors containing ToCMoV-[BA-Se1] genes in a ratio of 8:1 of each of the individual genes. Plants were monitored for GFP expression and photographed under UV light at the fifth day after infiltration.

For the reversal of transgene silencing assay, GFP-transgenic *N. benthamiana* plants; line 16C (obtained from the Baulcombe lab) were silenced by agroinfiltrating phpGFP into the lower leaves of three week old seedlings. After 18 days the plants were inoculated with ToCMoV-[BA-Se1] or agroinoculated with PVX containing ToCMoV-[BA-Se1] genes or PVX-NS1. By this time the GFP expression in these plants was silenced except for the very small top leaves. The plants were monitored for viral symptom development and reversal of silencing of the GFP transgene.

RNA extraction and analysis

RNA was extracted from systemic PVX infected *N. benthamiana* leaves 15 days post infiltration (dpi), and from local GFP infiltrated patches 5 dpi as described by Bucher *et al.* (2004). Enrichment of siRNAs was essentially performed according to Hamilton & Baulcombe (1999).

For viral (10 µg) or mRNA (15 µg) detection, total RNA was separated in denaturing formaldehyde-agarose gel and transferred to nylon membrane Hybond N^+ (Amersham). Twelve micrograms of siRNAs were separated in 15% denaturing polyacrylamide gel containing 8M urea and electro-transferred to Hybond N^+.

In the case of PVX detection, blots were hybridized with ToCMoV-[BA-Se1] DNA-A or DNA-B probes labeled with ^{33}P-dCTP by random priming using the Rediprime II kit (Amersham). For GFP mRNA and siRNA detection, a Dig-labeled GFP-specific probe was used (Bucher *et al.*, 2003).

The Superscript One-step RT-PCR kit (Invitogen) was used to detect ToCMoV-[BA-Se1] transcripts in PVX-infected and agroinfiltrated tissues following the manufacturer's recommendations. In the case of RNA extracted from agroinfiltrated tissues a DNase I treatment was performed prior to cDNA preparation. First strand cDNA was synthesized from total RNA using an oligo-dT primer and 1/10 of the reaction was used together with the gene-specific primers described for the PCR step (Table 5.1). As a negative control, the RNA after DNase I treatment was used in an identical PCR reaction without reverse transcription.

Protein analysis

Protein expression was analyzed on Western blots. One hundred milligrams of leaf material infected by PVX, PVX-AC1, infiltrated with pBinGFP, pBinGFP plus pGW-AC1 or mock inoculated plant was directly ground in 200 µl of protein loading buffer. For immunodetection, polyclonal antiserum against ToCMoV-[BA-Se1] AC1 (prepared in our lab from recombinant protein) or GFP (Molecular Probes) were used. Chemoluminescent detection was performed using CSPD (Roche).

RESULTS

Expression of individual ToCMoV-[BA-Se1] genes from a PVX-based vector

To identify potential virulence factors encoded by ToCMoV-[BA-Se1], individual genes expressed from a virus vector were inoculated in local and systemic host plants. *D.stramonium*, *N. benthamiana* and tomato plants were agroinoculated with PVX or PVX-derived vectors containing the different ToCMoV-[BA-Se1] genes. Systemic symptoms started to appear about one week after inoculation, depending on the plant and on the cloned gene. Regardless of the plant used, systemic symptoms were delayed for PVX viruses containing ToCMoV-[BA-Se1] sequences, as compared to the parent virus.

The expression of the cloned ToCMoV-[BA-Se1] genes was analyzed in systemically infected *N. benthamiana* leaves at 15 dpi. Northern blots of total RNA extracted from symptomatic leaf tissues were hybridized to probes of either ToCMoV-[BA-Se1] DNA-A or DNA-B to identify genomic and sub-genomic RNA species. Whereas no signal could be detected by the DNA-A or DNA-B probes in the samples from plants infected by PVX alone or a mock-inoculated control, ToCMoV-specific RNA sequences were detected for every recombinant construct (Fig. 5.2 a). RT-PCR analysis were used to confirm the Northern blot results and indicated the occurrence of gene specific bands with sizes corresponding to the respective ToCMoV inserts when using appropriate primer pairs (Table 5.1) (Fig. 5.2 b). As antibodies were only available for the AC1 protein, only the expression of this protein could be confirmed in western blot analysis (Fig 5.2 c).

Deviating phenotypes produced by PVX vectors carrying ToCMoV genes in the local host D. stramonium

When inoculated with the entire ToCMoV-[BA-Se1] virus, *D. stramonium* plants developed localized chlorotic lesions at the site of inoculation, which sometimes became necrotic (Fig. 5.3). The sites showing this reaction seemed to restrict the virus at these localized lesions and the virus could not be detected in the upper non-inoculated leaves (chapter 3).

Agro-inoculation of *D. stramonium* plants with PVX containing most of the ToCMoV-[BA-Se1] genes did not evidently alter the mild mosaic symptoms displayed by this plant species when infected by PVX, as exemplified by PVX-BC1 (Fig. 5.3). Systemic mosaic symptoms started at 7-10 dpi and were always mild and almost unnoticeable by 30 dpi (Fig. 5.3). Inoculation with PVX-AC1, PVX-AC2 and PVX-AC4, however, produced a considerable change in the PVX disease phenotype. Plants inoculated with PVX-AC1 remained symptom-free for about 18 days after which systemic necrotic spots started to appear. The spots were distributed throughout the leaf but did

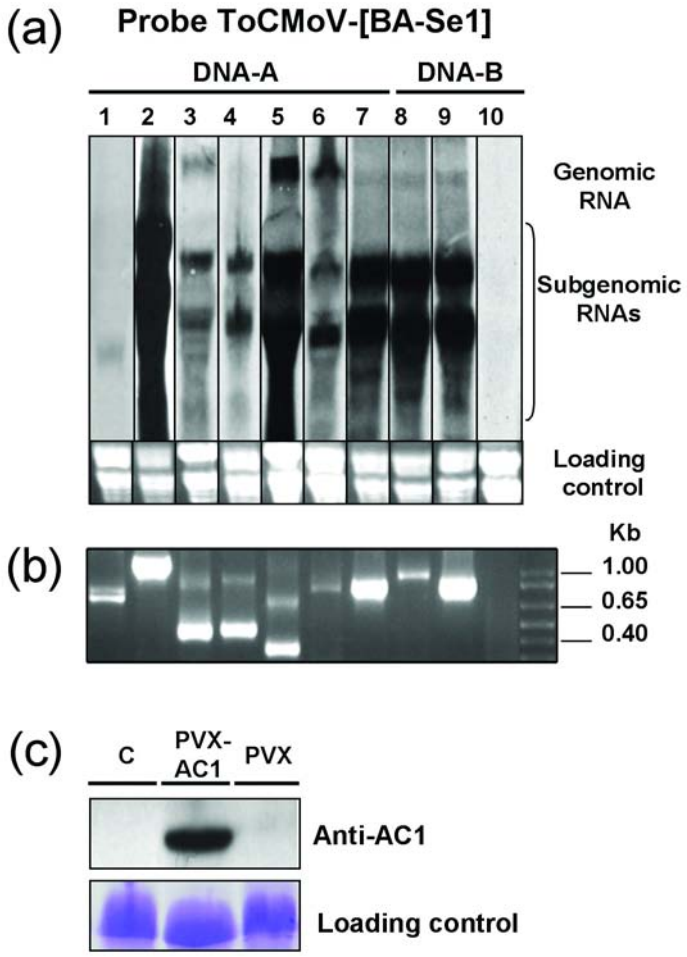


Figure 5.2. Expression of ToCMoV-[BA-Se1] genes from PVX-based vectors. (a) Northern blot analysis of viral RNAs extracted from systemically infected *N. benthamiana* leaves 15 dpi and hybridized with ToCMoV-[BA-Se1] DNA-A or DNA-B probe as indicated. Lane 1, parental PVX; lane 2, PVX-AC1; lane 3, PVX-AC2; lane 4, PVX-AC3; lane 5, PVX-AC4; lane 6, PVX-AC5; lane 7, PVX-AV1; lane 8, PVX-BC1; lane 9, PVX-BV1 and lane 10 mock inoculated control. Viral genomic and sub-genomic RNAs are indicated. (b) RT-PCR analysis of the same RNAs described above. RNA was reversed transcribed using an oligo-dT primer, followed by PCR with specific primers for PVX-coat protein or ToCMoV-[BA-Se1] genes. PVX-CP primers were also used in the reaction with mock inoculated control. (c) Western blot analysis of total protein extracted from leaves inoculated with PVX-AC1 and PVX with ToCMoV-[BA-Se1]-AC1 specific polyclonal antibody. C is mock-inoculated control.

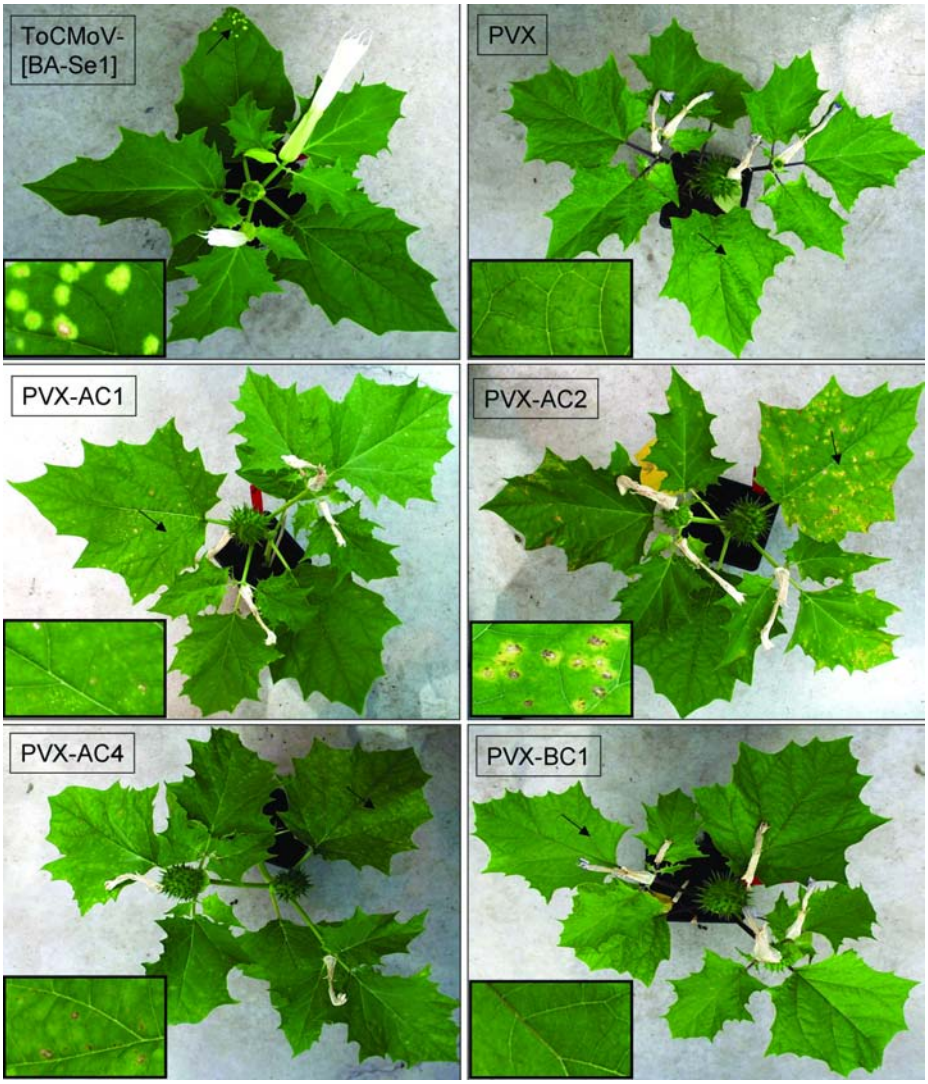


Figure 5.3. Symptoms induced in *Datura stramonium* infected plants by ToCMoV-[BA-Se1] or by PVX, PVX-AC1, PVX-AC2, PVX-AC4 or PVX-BC1 30 dpi. Arrows point to the leaf region displayed in the enlarged insert.

not enlarge with time (Fig. 5.3). PVX-AC4 plants developed numerous chlorotic lesions lacking a defined periphery. With time, some of those lesions became necrotic (Fig. 5.3). Plants infected by PVX-AC2 displayed the most striking phenotype as abundant chlorotic lesions developed about 20 dpi. The lesions expanded, coalesced and the center of the lesions became necrotic (Fig. 5.3). Sometimes the lesions expanded so much that they took over most of the leaf lamina. Although these results indicate that AC1, AC4 and

AC2 act as symptom determinants in *Datura stramonium* and some of the lesions and spots resemble the symptoms incited by ToCMoV-[BA-Se1] (Fig. 5.3), the chlorotic and necrotic lesions provoked by PVX-AC1, PVX-AC2 or PVX-AC4 were not able to restrict the spread of the chimeric PVX. This suggests that the observed response is not a gene-for-gene type hypersensivity that may be related to systemic resistance in this host.

Reaction of PVX-ToCMoV chimeric constructs in systemic hosts tomato and *N. benthamiana*

Two weeks old tomato 'Moneymaker' seedlings were infiltrated with PVX and PVX-ToCMoV chimeric constructs in two lower leaves. After two weeks, wild type PVX infected plants developed mild vein chlorosis, followed by mosaic symptoms (Fig. 5.4 a). All PVX-ToCMoV-[BA-Se1] constructs and also the PVX-antisense constructs for AC2, AV1, BC1 and BV1 developed types of symptoms comparable to the PVX control, though in plants infiltrated with chimeric PVX, the symptoms were initially somewhat stronger. The only exception were the PVX-AC2 infected plants which presented additional chlorotic spots in the lower leaves, especially on or close to the veins (Fig. 5.4 b). In newly developing leaves, however, the symptoms changed to a mosaic similar to that in plants inoculated with the other constructs and parental PVX.

The plant species most severely affected by PVX-ToCMoV chimeric viruses was *N. benthamiana*, where most PVX-ToCMoV-[BA-Se1] constructs induced more severe symptoms. ToCMoV-[BA-Se1] infection in this host usually is characterized by the development of veinal chlorosis, crumpling, epinasty, and systemic mottling (chapter 3). However, when a concentrated inoculum (1:2 w/v) is used for inoculation, these symptoms appear exacerbated and a fine necrosis develops in the small veins, small scattered bleached spots appear and epinasty and leaf curling are more pronounced (Fig. 5.4 c). Necrotizing symptoms, but much more severe than in wild-type ToCMoV-[BA-Se1]-infected plants, were often associated with the infection of chimeric PVX-ToCMoV-[BA-Se1] genes in this plant species. PVX-AC1 induced bleached and necrotic lesions, veinal necrosis and leaf curling (Fig. 5.4 d and e). Again, the most aggravated symptoms appeared in plants agroinoculated with PVX-AC2. Numerous chlorotic and necrotic spots developed in the leaves and sometimes a dark halo became visible around these lesions while the leaves became curled (Fig. 5.4 f and g). Moreover, the necrosis extended to the veins and petioles (Fig. 5.4 h and i) leading to collapse of the leaf (Fig. 5.4 j). As a result, the infected plants appeared severely stunted when compared with plants inoculated with the parent PVX or with PVX-AC2AS (Fig. 5.4 j). In PVX-AV1 infected plants, small chlorotic spots evolved to larger necrotic spots, with the necrosis reaching the veins (Fig. 5.4 k and l). Necrotic vein banding and necrotic concentric ringspots were also commonly associated with PVX-AV1 (Fig. 5.4 m). Plants infected with PVX-BC1

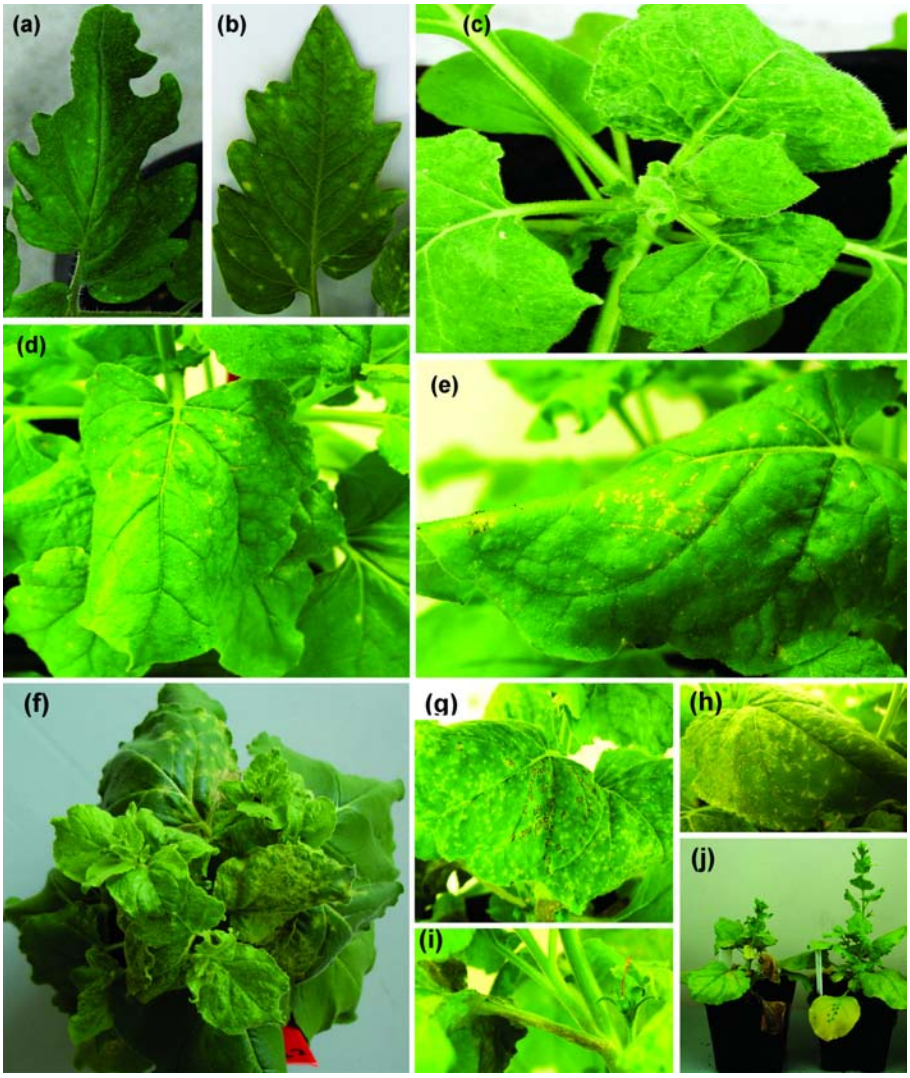
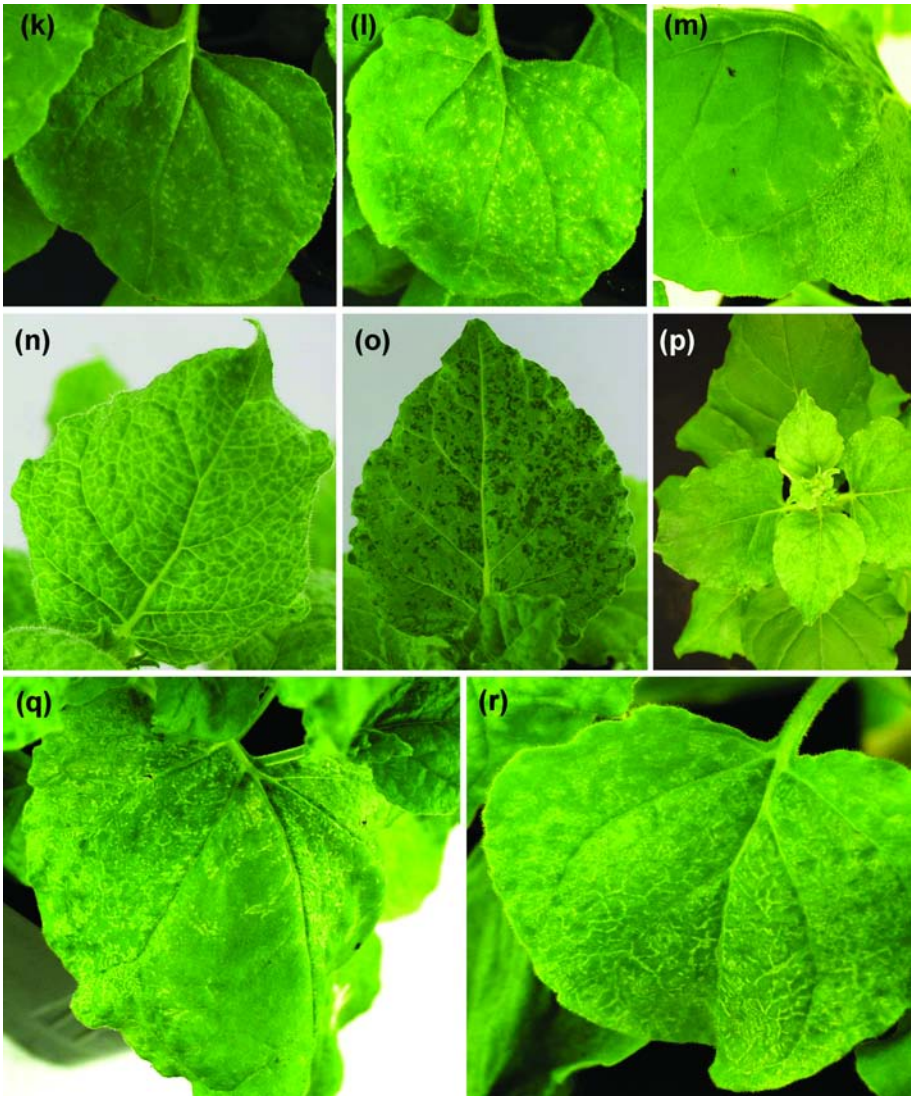


Figure 5.4. Symptoms of tomato and *N. benthamiana* plants inoculated with ToCMoV-[BA-Se1], PVX-chimeric constructs and parental PVX. Tomato leaflet from a plant infected by PVX (a) or PVX-AC2 (b) at 15 dpi. *N. benthamiana* plants inoculated with ToCMoV-[BA-Se1] using a concentrated inoculum (c). Systemic necrotic symptoms in *N. benthamiana* infected by PVX-AC1 (d and e). PVX-AC2 infected plants develop numerous chlorotic and necrotic systemic lesions and spots and leaves become curled (f and g), also the veins (h) and the petioles (i) developed necrosis. The leaves collapsed and the plant was stunted comparing with a plant inoculated with PVX-AC2-AS (j). Necrotic symptoms induced by PVX-AV1 (k, l and m). PVX-BC1 induces vein chlorosis and severe mosaic with dark green islands (n and o) while PVX-BV1 induces different types of necrotic reaction


(k)
(l)
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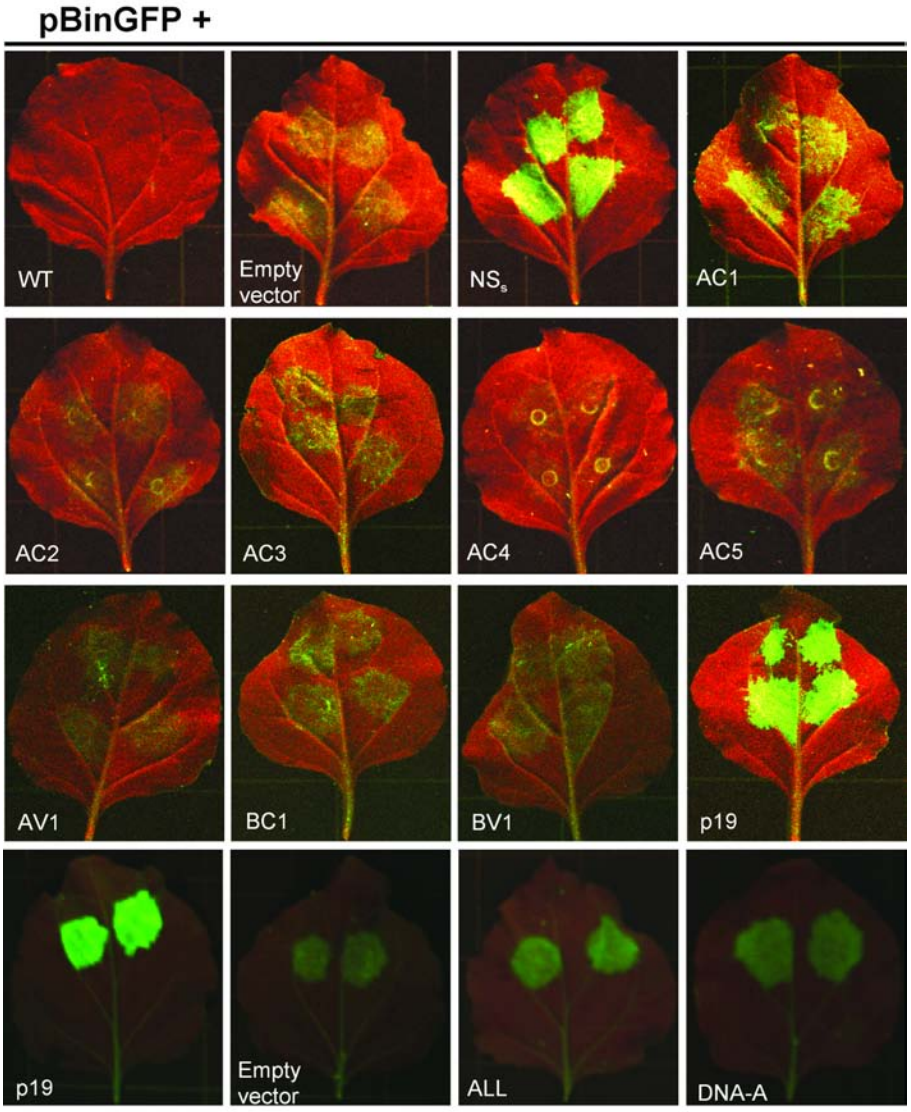

Figure 5.5. Assessment of local silencing suppression by ToCMoV-[BA-Se1] genes. *N. benthamiana* leaf tissues were coinfiltrated with A. tumefaciens cultures containing pBinGFP mixed with empty pBin vector, binary vectors containing ToCMoV-[BA-Se1] genes separately or all mixed toghether (ALL), agroinfectious ToCMoV-[BA-Se1] DNA-A clone and as positive controls pBinNSS and pBinp19. WT is non-infiltrated leaf.

also presented an altered phenotype. Leaves had an extensive vein chlorosis and a severe mosaic-with-green-islands pattern (Fig. 5.4 n and o). Plants infected with PVX-BV1 presented necrotic lesions, necrotic ringspots, necrotic veins and necrotic vein banding (Fig. 5.4 q and r). The symptoms induced by PVX-AC3, PVX-AC4 and PVX-AC5 were mild, similar to the PVX without insert (Fig. 5.4 p).

Assessment of silencing suppression activity of ToCMoV-[BA-Se1] gene products

Since many different ToCMoV-[BA-Se1] genes influenced symptom development and PVX virulence in different plants, the silencing suppression potential of all these genes was studied. To investigate their ability to suppress RNA silencing, several

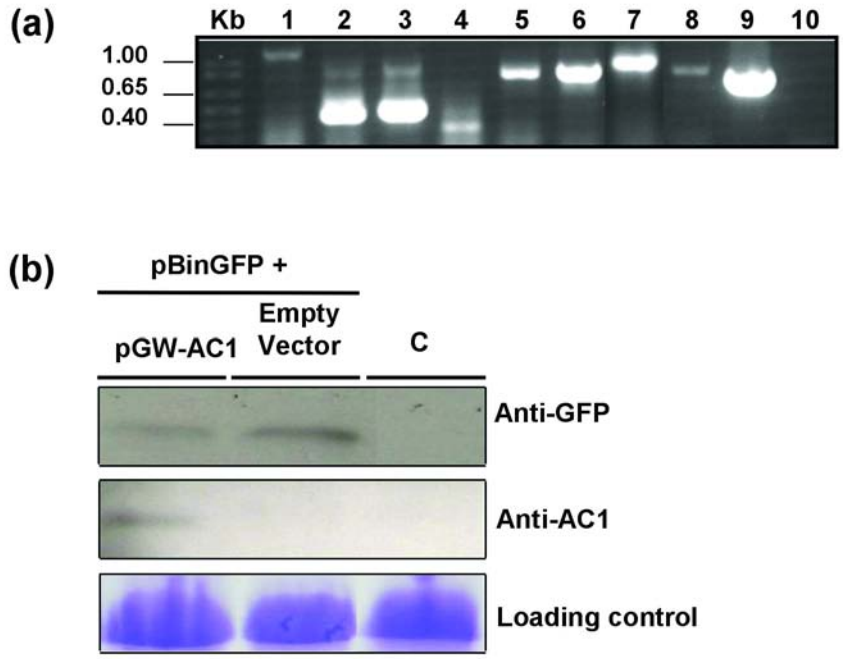

Figure 5.6. Expression of ToCMoV-[BA-Se1] genes coinfiltrated with pBinGFP into *N. benthamiana* leaves (a) RT-PCR analysis of the RNAs extracted from infiltrated patches. After DNAse treatment, RNA was reverse transcribed using an oligo-dT primer, followed by PCR with specific primers for ToCMoV-[BA-Se1] genes or GFP. Lanes 1 to 10 correspond to pBinGFP mixed with pGW-AC1, PGW-AC2, PGW-AC3, PGW-AC4, PGW-AC5, PGW-AV1, PGW-BC1, PGW-BV1, empty pBin and non-infiltrated control. (b) Western blot analysis of total protein extracted from leaves co-infiltrated with pBinGFP/pGW-AC1 and with pBinGFP/empty pBin. Immunodetection was done with GFP or ToCMoV-[BA-Se1]-AC1 specific polyclonal antibody. C is non-infiltrated control.

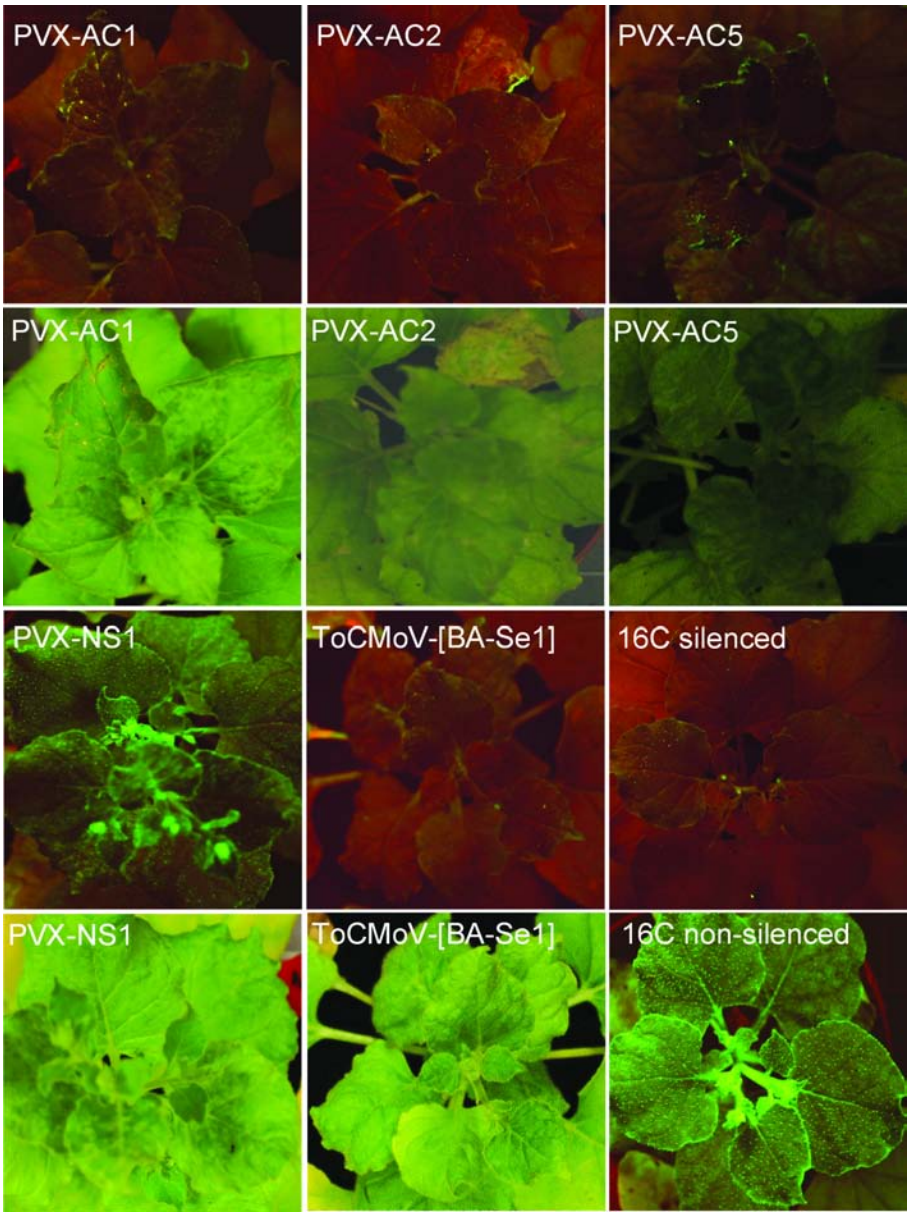

Figure 5.8. ToCMoV-[BA-Se1] and PVX containing ToCMoV-[BA-Se1]-derived genes do not recover GFP fluorescence in silenced *N. benthamiana* line 16C. GFP-transgenic line 16C plants were silenced and subsequently inoculated with the indicated virus. Pictures were taken 25 days after inoculation either under UV light (upper panel) or normal light to evidence virus symptoms (lower panel). Pictures of 16C silenced and non-silenced plants were taken under UV light. PVX-NS1 was used as positive control.

approaches were used. The first approach used represented a local transient assay involving coinfiltration in *N. benthamiana* leaves of two *A. tumefaciens* strains respectively containing a binary vector expressing GFP reporter gene and a vector harboring the candidate viral RNA silencing suppressor protein (Voinnet *et al.*, 2000). In this approach, the leaf patch infiltrated with *Agrobacterium* culture containing pBinGFP initially expresses high levels of GFP. However after four to five days, local RNA silencing is triggered and the GFP fluorescence fades. In the presence of a strong local silencing suppressor this RNA silencing will be suppressed leading to greatly intensified fluorescence (Roth *et al.*, 2004). As positive controls, the established viral silencing suppressors TSWV-NS_S and CaIRSV-p19 (Bucher *et al.*, 2003) were used. GFP expression in infiltrated leaves was monitored and photographed under UV light 5 days after infiltration.

While, as anticipated, NS_S and p19 greatly enhanced the GFP fluorescence, none of the ToCMoV-[BA-Se1] genes had an effect on GFP expression when compared to the empty vector control (Fig. 5.5), suggesting that none of the ToCMoV gene products has a strong silencing inhibition function. To verify if a combination of viral gene products or viral replication was necessary for suppression activity, a combination of all viral gene expression vectors was infiltrated as well as GFP-infiltration combined with an agroinfectious ToCMoV-[BA-Se1] DNA-A clone. However, both experiments showed no enhanced GFP fluorescence in the infiltration sites (Fig. 5.5). To confirm the expression of ToCMoV-[BA-Se1] genes in infiltrated leaf patches, RT-PCR was performed in DNase treated RNA extracted from these tissues using primers described in Table 5.1. Bands with expected sizes corresponding to each gene were obtained, suggesting active transcription of the genes (Fig. 5.6 a). Western blot analysis using specific antibodies con-

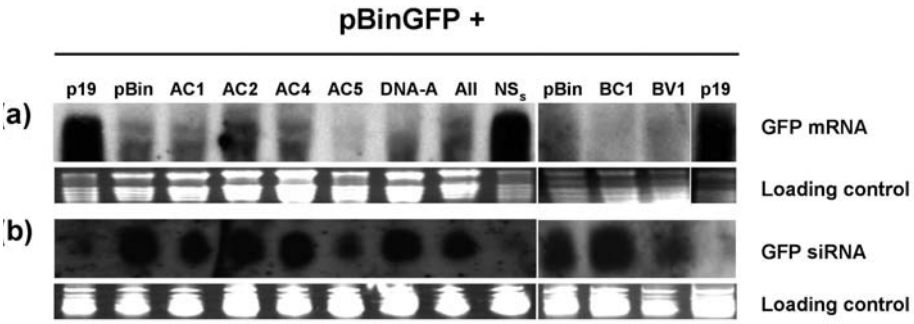

Figure 5.7. Northern analyses of GFP mRNA and siRNA from tissues co-infiltrated with pBinGFP combined with binary vector containing ToCMoV-[BA-Se1] genes as indicated, agroinfectious ToCMoV-[BA-Se1] DNA-A clone or binary vectors containing all ToCMoV-[BA-Se1] combined together (All) (a) Northern blot of mRNA extracted from infiltrated leaf patches probed with a Dig-labeled GFP-specific probe. pBin is pBin19 without an insert. (b) GFP siRNAs extracted from infiltrated leaf patches probed with a Dig-labeled GFP-specific probe.

firmed protein expression of AC1 and GFP (Fig. 5.6 b). Southern blot analysis, performed as described in chapters 3 and 4, confirmed ToCMoV-[BA-Se1] replication in leaves infiltrated with agroinfectious ToCMoV DNA-A. Single and double stranded DNA forms, characteristic of a replicating geminivirus, were detected by hybridization with virus-specific probe (results not shown), implying that all proteins coded by pCAMBIA-DNA-A were successfully expressed and resulted in DNA-A replication and viral DNA accumulation. The described results suggest that neither individual genes, nor the combination of all genes from separate vectors nor the replicating viral DNA-A were able to enhance GFP fluorescence. This would indicate that ToCMoV did not suppress RNA silencing of the GFP reporter gene. Visual observations were confirmed by Northern analysis of GFP-derived transcripts and siRNAs. Whereas both p19 and NS_S supported high accumulation of GFP mRNA by suppressing RNA silencing, comparable highly reduced levels of mRNA were detected in the tissues infiltrated with GFP combined either with the empty pBin vector or with ToCMoV-[BA-Se1]-derived constructs (Fig. 5.7 a). Conversely, drastically reduced GFP siRNA levels were detect in tissues infiltrated with the combination of GFP and p19 or NS_S, while comparable high levels of GFP-specific siRNAs accumulated in leaf patches infiltrated with GFP combined either with the empty vector or with ToCMoV-[BA-Se1]-derived constructs (Fig. 5.7 b). Surprisingly though, despite the lack of visually enhanced GFP expression, the expression of AC5 caused a considerable reduction in the levels of GFP siRNAs, nonetheless it appeared not to protect GFP mRNA from degradation by the RNA silencing machinery (Fig. 5.7 a and b).

To exclude a possible influence of the agroinfiltration assay for a putative ToCMoV silencing suppressor protein, a second assay was used the reversal of transgene silencing (Voinnet *et al.*, 2000). In this assay, the transgenic plant line 16C, which expresses GFP is systemically silenced using a local infiltration of an *Agrobacterium* strain carrying a hairpin construct of the GFP gene. Upon induction of silencing the plant is inoculated with a virus. If the subsequent virus infection would result in reappearance of GFP, the infecting virus encodes a suppressor of silencing. PVX carries only a weak systemic suppressor and hence this virus is ideal to be used as vector for expressing putative suppressor proteins in this assay (Roth *et al.*, 2004; Voinnet *et al.*, 1999). Silenced 16C *N. benthamiana* plants were inoculated with ToCMoV-[BA-Se1], the aforementioned PVX-chimeras containing ToCMoV-[BA-Se1] genes and PVX-NS1. All plants developed similar symptoms to those observed in infected wild-type plants (Fig. 5.8). PVX-NS1 (Bucher *et al.*, 2004) was used as a positive control. In the plants infected with the PVX-NS1 positive control a full recovery of GFP expression was observed, whereas in none of the GFP-silenced plants inoculated either with ToCMoV-[BA-Se1] or PVX-ToCMoV-[BA-Se1] chimeras the recovery of GFP expression was observed. Some examples of the results obtained in this experiment are shown in Fig. 5.8.

Discussion

In this study, the phenotypes induced by the genes encoded by both DNA-A and DNA-B of the bipartite begomovirus ToCMoV-[BA-Se1], expressed from a heterologous viral vector in different hosts, were examined and provided insight into ToCMoV-[BA-Se1]-host interactions. A response characterized by increased symptoms and virulence was observed with chimeric PVX vector viruses harboring ToCMoV-[BA-Se1] genes in different hosts, with the exception of PVX-AC3 and PVX-AC5.

D. stramonium inoculated with ToCMoV-[BA-Se1] develops local chlorotic and occasionally necrotic lesions at the site of inoculation and these lesions arrest the spread of the virus. Although necrosis is not always present, this reaction resembles the hypersensitive response (HR) which is characterized by specific interaction of R/Avr genes resulting in the death of the cells accompanied by a series of systemic reactions in the plant and pathogen containment (Dangl *et al.*, 1996). Expression of PVX-AC1, PVX-AC2 and PVX-AC4 was associated with chlorotic and/or necrotic lesions in this host. The ORF AC1 includes the embedded ORF AC4 and the overlapping 5' end of ORF AC2 which are encoded in a different frame. Though unlikely, we can not exclude that leaky scanning or internal initiation from the same transcript could lead to translation of these two proteins and influence the effect of AC1 or even have an undesirable effect on AC1 translation. Expressing a similar construct derived from African cassava mosaic virus (ACMV) and Tomato yellow leaf curl China virus (TYLCCV) in *N. benthamiana,* van Wezel *et al.* (2002a) reported that AC4 influenced AC1 response by increasing the severity of the symptoms. In our study, on the contrary, PVX-AC4 by itself elicits a more severe phenotype than the PVX-AC1 construct and therefore AC4 most likely does not contribute to the phenotype observed in the PVX-AC1 infected plants. In a similar way, the AC2 ORF includes a large portion of the overlapping ORF AC3 in another reading frame. Since AC3 has no influence in the symptoms displayed by PVX, it is most likely that it does not contribute to the PVX-AC2 phenotype. Taken together, our inoculation results show that ToCMoV-[BA-Se1] AC1, AC2 and AC4 alter the symptomatology of the hybrid PVX virus, invoking chlorotic/necrotic lesions which sometimes mimic the original local lesion symptoms induced by the wild type ToCMoV-[BA-Se1]. Although the symptom development is much delayed in comparison with the parental-type PVX or the chimeric PVX harboring other ToCMoV-[BA-Se1] genes, these lesions fail to prevent the spread of the chimeric PVX, indicating that none of these genes is an avirulence determinant (Avr) (Dangl *et al.*, 1996). Furthermore, these results suggest that a combination of genes, rather than a single viral gene might be responsible for the local lesion phenotype in *D. stramonium* inoculated with ToCMoV-[BA-Se1] as is the case of *Barley stripe mosaic virus* phenotype on a local lesion host (Petty *et al.*, 1994). Therefore the local lesion response induced by ToCMoV-[BA-Se1] in *D. stramonium* is not likely to be a hypersensitive response.

From the two ToCMoV-[BA-Se1] systemic hosts used in this study, *N. benthamiana* showed the most striking effects when infected by chimeric PVX constructs. Expression of most ToCMoV-[BA-Se1] genes resulted in the induction of necrotizing reactions. Despite PVX expressing ToCMoV-[BA-Se1] AC1 inducing systemic bleached and necrotic lesions and veinal necrosis, these symptoms were less severe than those reported for the PVX expressing AC1 from ACMV and C1 of TYLCCV (van Wezel *et al.*, 2002a). For these two viruses an HR-like local response was observed that could not prevent systemic viral infection resulting in severe systemic necrosis and collapse of developing tissues. On the other hand, Tomato leaf curl virus (TLCV)-C1 expressed from a TMV vector in *N. benthamiana* seemed to induce a rapid HR response restricting the infection in the initially infected cells without a visual necrotic response (Selth *et al.*, 2004). These rather distinct results with different viruses using viral vectors were also observed in stably transformed plants with AC1 genes from different viruses. While AC1 proteins from ACMV (Hong & Stanley, 1996) and Tomato golden mosaic virus (TGMV) (Hanley-Bowdoin *et al.*, 1990) have been expressed in *N. benthamiana* without producing any adverse phenotypic effect, recovery of transgenic tobacco plants expressing C1 from TLCV was not possible (Selth *et al.*, 2004). These divergent results might reflect differences in the interaction between AC1 (C1) proteins of individual viruses with their host factors (Selth *et al.*, 2004).

Also the PVX-AV1 elicited different types of necrotic symptoms. This is the first time that the ectopic expression of AV1 of a begomovirus is reported to induce an enhanced virulence phenotype. Although AV1 is involved in viral ssDNA accumulation (Qin *et al.*, 1998) and some AV1 mutants can generate attenuated and delayed symptoms (Unseld *et al.*, 2004), AV1 is not essential for systemic infection of bipartite begomoviruses (Gardiner *et al.*, 1988) and seems to have little influence on symptom type in host plants (Harrison *et al.*, 2002). Indeed previous studies have shown that the expression of the AV1 either from a viral vector (Selth *et al.*, 2004) or in transgenic plants (Frischmuth & Stanley, 1998; Kunik *et al.*, 1994) induced no altered phenotype in the plants.

In the present study, it was shown that both movement proteins BV1 and BC1 encoded by DNA-B influence the symptoms in *N. benthamiana* when expressed from chimeric PVX constructs. These results are consistent with numerous reports which have shown that both movement proteins of bipartite begomoviruses are symptom-inducing determinants (Carvalho & Lazarowitz, 2004; Duan *et al.*, 1997; Fontes *et al.*, 2004; Hou *et al.*, 2000a; Hussain *et al.*, 2005; Ingham *et al.*, 1995; Pascal *et al.*, 1993; von Arnim & Stanley, 1992). ToCMoV-[BA-Se1] BV1 induced different types of necrotic symptoms. Tomato leaf curl New Delhi virus (ToLCNDV) BV1 induces necrosis typical of HR in tobacco and in tomato indicating that it is an avirulence determinant (Hussain *et al.*, 2005) which is also the case for BV1 from Bean dwarf mosaic virus (BDMV) in some

bean genotypes (Garrido-Ramirez *et al.*, 2000). However, in *N. benthamiana,* ToLCNDV BV1 induces virus-like symptoms when expressed from chimeric PVX. Recently, different host factors that interact with BV1 from different begomoviruses have been identified, including a transmembrane receptor kinase (Fontes *et al.*, 2004; Mariano *et al.*, 2004) and an *Arabidopsis* acetyltransferase (Carvalho & Lazarowitz, 2004; McGarry *et al.*, 2003). These factors were suggested to play an important role in begomovirus infection and pathogenicity. Begomovirus BC1 proteins are reported as major symptom determinants inducing disease-like symptoms (Gafni & Epel, 2002). ToCMoV-[BA-Se1] BC1 when expressed from PVX induced extensive vein chlorosis and more severe mosaic symptoms when compared with PVX induced mild mosaic. A similar effect was observed with PVX or transgenic plants expressing BDMV BC1 (Duan *et al.*, 1997) or transgenic plants transformed with BC1 from *Squash leaf curl virus* or *Tomato mottle virus* (Duan *et al.*, 1997; Hou *et al.*, 2000a; Pascal *et al.*, 1993). These virus disease-like phenotypes might be related, at least to some extent, with the interference of BC1 with normal macromolecular intercellular trafficking (Gafni & Epel, 2002).

The gene product of ToCMoV-[BA-Se1] AC2 expressed from PVX had the most extreme effect in the systemic hosts, especially in *N. benthamiana.* A range of severe necrotic effects were observed including spots, lesions, complete collapse of leaves and severe stunting. AC2/C2 genes of both bipartite and monopartite begomoviruses such as ACMV (Hong *et al.*, 1997; Voinnet *et al.*, 1999), TYLCCV (van Wezel *et al.*, 2001) and TLCV (Selth *et al.*, 2004) also induce necrotic reactions of different intensities, but less severe than those of ToCMoV-[BA-Se1] AC2. Although the response incited by AC2/C2 of these begomoviruses were attributed to the involvement of these proteins in suppression of RNA silencing, the phenotypic variation was suggested to be due to different experimental systems used or functional diversity among the AC2 protein of the different viruses (Selth *et al.*, 2004).

It is worth mentioning that the host plant reactions observed in the current study are most probably the result of a high expression of the ToCMoV-[BA-Se1] genes, combined with the heterologous PVX background. Although mild necrotic reactions were observed in *N. benthamiana* when a concentrated ToCMoV-[BA-Se1] inoculum (1:2 w/v) was used, suggesting it might be due to a higher number of infection foci and an overall higher level of viral protein products, in a genuine geminivirus infection as with ToCMoV-[BA-Se1], the transcription and expression of the different genes is temporally and spatially controlled and coordinated for the maximal profit of the virus (Gutierrez, 2002; Hanley-Bowdoin *et al.*, 1999; Hanley-Bowdoin *et al.*, 2004).

In conclusion, depending on the host plant, all ToCMoV-[BA-Se1] genes except AC3 and AC5 induced enhanced symptom phenotypes when expressed from a PVX vector. Since it is possible that Avr gene products are virulence factors until a corresponding R

gene evolves in a specific host (Hong-Wei *et al.*, 1999), it can be conceived that ToCMoV-[BA-Se1] 'virulence' proteins expressed *in planta* by heterologous expression could be used in future experiments to either identify a host genotype containing specific resistance genes or to study the mechanism or the underlying resistance in a resistant plant.

One of the biological functions of RNA silencing in plants is an antiviral defense response (Baulcombe, 2004; Waterhouse *et al.*, 2001). As a counter defense strategy viruses have evolved proteins that suppress RNA silencing (Voinnet, 2005; Voinnet *et al.*, 1999). In recent years, a growing number of silencing suppressor proteins from diverse taxonomical groups of plant viruses have been identified (Moissiard & Voinnet, 2004; Roth *et al.*, 2004). Most of those suppressors had been previously implicated in virulence and reported as symptom determinants (Brigneti *et al.*, 1998; Bucher *et al.*, 2003; Pruss *et al.*, 1997; Voinnet *et al.*, 1999; Yelina *et al.*, 2002).

For geminiviruses, more specifically for those belonging to the genus *Begomovirus,* two different gene products, AC2 and AC4 have been identified as RNA silencing suppressors for different viruses (Selth *et al.*, 2004; Trinks *et al.*, 2005; van Wezel *et al.*, 2002b; Vanitharani *et al.*, 2005; Vanitharani *et al.*, 2004; Wang *et al.*, 2005). The present study demonstrated that all genes of ToCMoV-[BA-Se1] except for AC3 and AC5 induced enhanced symptom severity of PVX. The involvement of these ToCMoV-[BA-Se1] gene products in suppressing RNA silencing was investigated using several approaches. First, the co-infiltration transient assay was used. Here binary vectors containing individual ToCMoV-[BA-Se1] genes were co-expressed with a vector containing GFP in *N. benthamiana* leaves. To our surprise, in this assay none of the genes induced enhanced GFP expression implying that none of ToCMoV-[BA-Se1] genes had RNA silencing suppressor activity. Expression of ToCMoV-[BA-Se1] genes was confirmed by RT-PCR and in the case of AC1, by immunodetection. Since for *Red clover necrotic mosaic virus* (Takeda *et al.*, 2005) it was shown that suppression of RNA silencing required multiple viral components and viral replication, a mixture of all binary vectors harboring ToCMoV-[BA-Se1] genes was co-infiltrated, as well as an agro-infectious ToCMoV-[BA-Se1]-DNA-A. However, also in these cases, similar results were obtained, thus demonstrating that also in the context of viral DNA-A replication none of the gene products exerted RNA silencing suppression activity. In addition, also in a second assay, i.e, infiltration of pBinGFP in infected plants at different stages of infection, also in a lack of enhancement of GFP expression (results not shown). Northern analysis confirmed the silencing of GFP since almost no mRNA was detected whereas high levels of GFP-specific siRNA were produced. Curiously, despite the lack of visually enhanced GFP expression and apparent GFP mRNA degradation by the RNA silencing machinery, the expression of ToCMoV-[BA-Se1] AC5 caused a considerable reduction in the levels of GFP-siRNAs. Whether this reduction reflects any biological significance remains to be deter-

mined. The presence of a large ORF such as ToCMoV-[BA-Se1] AC5 is not a general characteristic among begomoviruses and its expression and function in ToCMoV-[BA-Se1] infection is not yet established. Mutations in the AC5 ORF of *Watermelon chlorotic stunt virus* did not abolish infection or altered symptomatology and expression of the protein was not assayed (Kheyr Pour *et al.*, 2000). However, the immunolocalization of AC5 protein in the cytoplasm of *N. benthamiana* mesophyll cells infected by *Tomato rugose mosaic virus* (F.M. Zerbini, *unpublished results*) demonstrates that this ORF is expressed in this species, which is closely related to ToCMoV (chapter 3).

The negative results obtained in the reversal of transgene silencing assay (Voinnet *et al.*, 2000), where silenced line 16C GFP plants inoculated with ToCMoV-[BA-Se1] or chimeric PVX carrying ToCMoV-[BA-Se1] genes displayed no recovery of GFP expression, supports the results from the local silencing assay.

When comparing results reported elsewhere for some begomoviral silencing suppressors, a number of interesting observations can be made. For example, for ACMV-[CM], AC4 was identified as RNA silencing suppressor (Vanitharani *et al.*, 2004), whereas for ACMV-[KE], AC2 was reported to be the RNA suppressor protein (Voinnet *et al.*, 1999). Also the TGMV AC2 appears to be active as suppressor of RNA silencing, but only when assayed with the co-infiltration assay using a binary vector expressing GFP in *N. benthamiana* leaves (Wang *et al.*, 2005), but not in transfected protoplasts (Qi *et al.*, 2004). In part these discrepancies could be explained by the different assays used in the different studies (Roth *et al.*, 2004), but they also imply complications in determining RNA silencing suppressor activity for these DNA viruses, possibly due to their relative weakness.

Taken all together, our results indicate that several ToCMoV-[BA-Se1] genes can be regarded as virulence determinants, but nonetheless ToCMoV-[BA-Se1] does not seem to encode an RNA silencing suppressor that could be identified in the commonly used assays. It can, however, not be excluded that it is too very weak be unequivocally identified by the procedures used. A final alternative could be that the suppressor exerts a novel silencing action that can not be detected in the current assays. Beyond doubt the findings in this chapter add yet another puzzling factor to the already complex and intriguing interaction between begomovirus infection and RNA silencing in host plants.

Acknowledgments

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Chapter 6

GENERAL DISCUSSION

EMERGENCE OF TOMATO BEGOMOVIRUSES IN BRAZIL

Geminiviruses (family *Geminiviridae*) are etiological agents of the most devastating diseases of vegetable, grain and fiber crops particularly in tropical and sub-tropical regions of the world (Varma & Malathi, 2003). Whitefly-transmitted geminiviruses that infect dicotyledonous plants belong to the genus *Begomovirus,* the largest genus to date comprising 117 different recognized species (Stanley *et al.*, 2005).

Human activity and the way modern agriculture is managed are key factors in the emergence of geminiviruses in various parts of the world. Changing crop systems by introducing new crops to accommodate needs for internal consumers as well as for export, cultivation of extensive areas with susceptible genotypes, massive use of insecticides, global movement of plant material and introduction of exotic viruses and their vectoring insects into new environments have been implicated in the outbreaks of geminiviruses in different crops (Morales & Anderson, 2001; Varma & Malathi, 2003). Also intrinsic variability features characteristic of begomoviruses such as their evolutionary capabilities through recombination and pseudorecombination, can give rise to new species or variants which can start new disease epidemics in novel niches (Harrison & Robinson, 1999).

Several whitefly-transmitted diseases were described in Brazil during the 1940's and 1950's infecting both cultivated and wild malvaceous species (Costa, 1954, 1955; Silberschmidt, 1943) and plants belonging to the *Euphorbiaceae* (Costa & Carvalho, 1960). Bean golden mosaic virus (BGMV) was first described in the 1960's (Costa, 1965) as a minor disease of common bean (*Phaseolus vulgaris*) and not considered of economical importance. However starting a decade later, and until today, this virus has become prevalent throughout the bean cultivation areas and has proven to be a limiting factor for bean production in Brazil and other Latin American countries (Faria *et al.*, 2000; Morales & Anderson, 2001). The crucial factor for this change was a striking rise in the populations of the whitefly vector, attributed to the increase of the area cultivated with soybeans, the export commodity of choice and an excellent host for the whitefly (Costa, 1975). A survey comprising samples collected in the early 1990's representing the main bean-growing areas in Brazil was carried out. The results revealed a very low variability among the isolates indicating that BGMV was present as an homogeneous population and was the only representative species infecting common beans in the country (Faria & Maxwell, 1999).

Table 6.1. Begomoviruses infecting tomato in Brazil†

Begomovirus species	**Acronym**
Tomato chlorotic mottle virus	ToCMoV
Tomato golden mosaic virus	TGMV
Tomato rugose mosaic virus	ToRMV
Tomato severe rugose virus	ToSRV
Begomovirus tentative species	**Acronym**
Tomato chlorotic vein virus	ToCVV
Tomato crinkle yellow leaf virus	ToCrYLV
Tomato crinkle virus	ToCrV
Tomato infectious yellows virus	ToIYV
Tomato mottle leaf curl virus	ToMoLCV
Tomato severe mosaic virus	ToSMV
Tomato yellow vein streak virus	TYVSV
Sida micrantha mosaic virus	SimMV

† Based on Ribeiro *et al.* (2003), Faria *et al.* (1997) and Zerbini *et al.* (2005)

The first reported tomato-infecting begomovirus, *Tomato golden mosaic virus,* was characterized in the early 1970's (Maytis *et al.*, 1975). However, tomato-infecting begomoviruses did not become an economical concern, most probably because the A biotype of *Bemisia tabaci*, the only type of whitefly present at the time, does not feed and reproduce successfully in tomatoes (Bedford *et al.*, 1994).

The emergence of tomato-infecting begomoviruses in Brazil started after the introduction, in the beginning of the 1990's, of the new biotype B of *Bemisia tabaci* (Melo, 1992). This *B. tabaci* biotype rapidly spread throughout the agricultural areas of Brazil, including soybean farms and green belts around the towns where a variety of vegetables including tomatoes are cultivated year-round. The B biotype is polyphagous and colonizes a higher number of plant species, has higher fecundity and shorter life cycle, thus reaching very high populations and becoming a plague in a short time (Bedford *et al.*, 1994). The dispersal of the new whitefly vector coincided with a dramatic increase in the occurrence and severity of tomato-infecting begomoviruses and these disease problems in tomatoes rapidly reached epidemic status leading to serious economic losses (Faria *et al.*, 2000; Ribeiro *et al.*, 1998). This situation was very similar to the one described for other Latin America countries after the introduction of the whitefly biotype B (Morales & Anderson, 2001; Polston & Anderson, 1997). In chapter 2, the analysis of the distribution and genetic diversity of the viruses present in Brazil revealed a situation

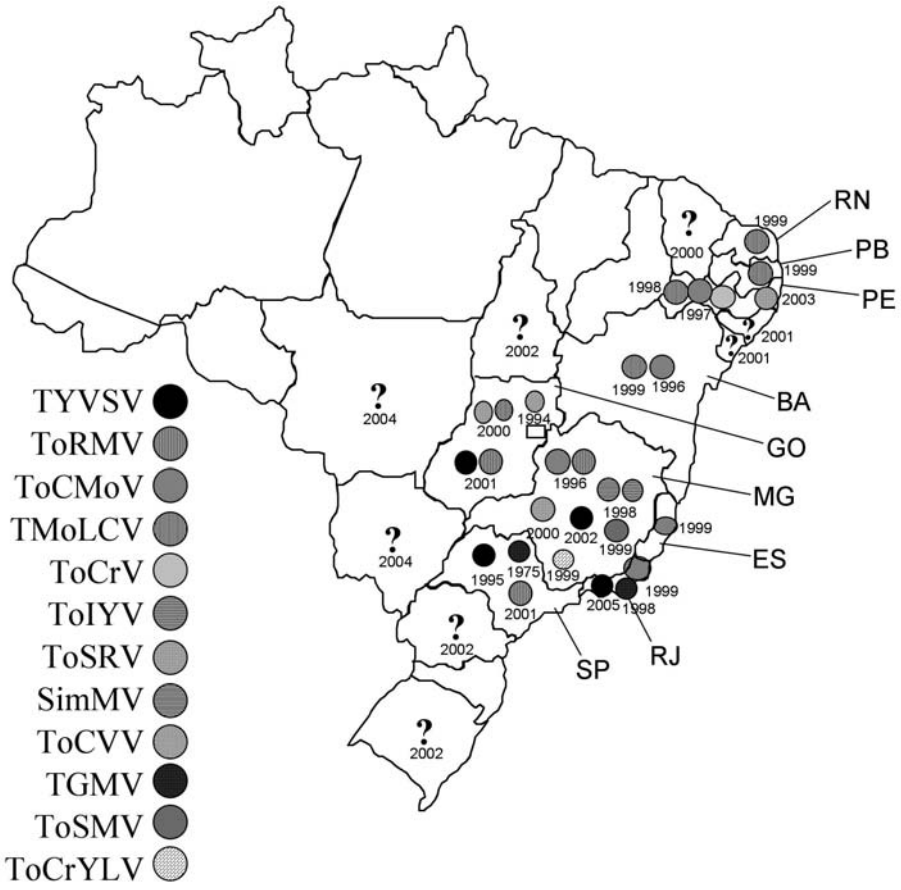

Figure 6.1. Distribution of tomato-infecting geminiviruses in Brazil as of July 2005. The year in which a given species was first reported in each state is indicated. (?) indicates geminivirus detection without species determination. States are indicated by their abbreviations: SP, São Paulo; RJ, Rio de Janeiro; ES, Espírito Santo; MG, Minas Gerais; GO, Goiás; BA, Bahia; PE, Pernambuco; PB, Paraíba; RN, Rio Grande do Norte. Modified from Zerbini et al. (2005)

completely different from what was observed for bean-infecting begomovirus. This study established the existence of several previously unreported begomovirus species infecting tomatoes in Brazil. At least seven putative new species were identified. Together with the tomato-infecting begomoviruses described over the same period by others (Ambrozevicius *et al.*, 2002; Faria *et al.*, 1997; Galvao *et al.*, 2003) a total of 11 putative new species were reported to be involved in the tomato-begomovirus epidemics during the late 1990's (Table 6.1, Fig. 6.1). This high diversity suggested that these viruses were indigenous to Brazil, which indeed was supported by phylogenetic studies (chapters 2 and 3). Thus it appears that the introduced vector biotype facilitated the transfer of

indigenous viruses occurring in wild plant species to tomato, where mixed infections are common, permitting mechanisms like recombination and pseudorecombination to happen, possibly generating novel viruses, which could adapt to the new host and flourish. The discrepancy between the variability of bean and tomato-infecting begomoviruses could be explained by the fact that in the case of the bean-infecting begomovirus, it seems that the virus and its hosts have been co-evolving for a significantly longer time, which probably allowed selection of a better adapted virus to predominate in the field. The biotype B of *B. tabaci* is now predominant in most, if not all, agroecosystems of the country and has displaced biotype A (G. L. Villas Boas, *personal communication*). The B biotype is also well adapted to common bean (Barbosa *et al.*, 2004) and there is a possibility of transmission of begomoviruses infecting other plants, for example tomato, to bean plants increasing the variability of begomoviruses in this crop. Also the chance of mixed infection with BGMV could increase the chances of recombination in this virus species. It would be interesting at this stage to repeat the begomovirus survey in bean plants to access the effect of the new whitefly vector biotype in the begomovirus disease evolution.

Pseudorecombination and recombination are major driving forces contributing to begomovirus evolution and diversity (Harrison & Robinson, 1999; Molfat, 1999; Padidam *et al.*, 1999; Rojas *et al.*, 2005) and might add to the emergence of new extremely virulent virus variants. Such events have been reported to be responsible for a severe cassava mosaic disease in Uganda (Pita *et al.*, 2001; Zhou *et al.*, 1997) and of a recombinant between Tomato yellow leaf curl virus (TYLCV) and Tomato yellow leaf curl Sardinia virus (TYLCSV) with a novel pathogenic phenotype in Southern Spain (Monci *et al.*, 2001; Monci *et al.*, 2002). Although Tomato chlorotic mottle virus (ToCMoV) described in this thesis appears to be a recombinant species (chapter 3), the recombinant parts of the genome seem to have originated from events that happened before the two characterised isolates ToCMoV-[BA-Se1] and ToCMoV-[MG-Bt1] diverged, most likely before the outbreak of this virus. Therefore, recombination probably did not constitute a major contribution for the emergence of this novel virus species. On the other hand, another novel begomovirus, Tomato rugose mosaic virus (ToRMV), seems to be a recent recombinant between ToCMoV-[MG-Bt1] and Tomato severe rugose virus (ToSRV) (chapter 3) and its emergence could indeed (in part) be related to this characteristic. Recently, additional full-length virus sequences have been obtained, representing both new begomovirus species and new isolates of already identified species from crop plants and weeds (A. Inoue-Nagata and F. M. Zerbini, *unpublished*). A broader recombination study including 18 Brazilian DNA-A sequences corresponding to 14 different species and other New World begomovirus DNA-A sequences is under way in an attempt to reveal overlooked evolutionary relationships among these begomoviruses (A. Inoue-Nagata, F. M. Zerbini, S. Ribeiro & D. Martin, *unpublished*).

Introduction of exotic begomoviruses, especially the Old World monopartite begomoviruses TYLCV and TYLCSV, has always been a great concern for growers and researchers in Brazil. Their introduction would impose even a greater threat to tomato cultivation, similar to what occurred in other countries on the American continent (McGlashan *et al.*, 1994; Polston *et al.*, 1999; Salati *et al.*, 2002) and elsewhere (Accotto *et al.*, 2000; Delatte *et al.*, 2005; Moriones & Navas-Castillo, 2000). Auspiciously, a survey in São Paulo State failed to detect a possible introduction of those viruses (Inoue-Nagata *et al.*, 2004b). Since Brazil does not import tomato transplants or tomato fruits, which can be the primary carriers of these viruses (Delatte *et al.*, 2003; Polston & Anderson, 1997), the risk is somewhat reduced. Nevertheless there will always be a danger of an unintentional import of infected alternative hosts, such as ornamental plants, or plants infested with viruliferous whiteflies.

New, yet undescribed begomoviruses are still being found in the field (Inoue-Nagata *et al.*, 2004a; Pires *et al.*, 2004). However, considering the begomoviruses thought to be involved in Brazilian tomato disease epidemics in the late 1990's (chapters 2 and 3) (Faria *et al.*, 1997; Ribeiro *et al.*, 2003) (Fig. 6.1), the latest surveys indicate that five viruses, namely ToRMV, ToCMoV, ToSRV, Tomato mottle leaf curl virus (ToMoLCV), and Tomato yellow vein streak virus (TYVSV) are predominating and are responsible for most of the recent epidemics (Avila *et al.*, 2005; Carrijo *et al.*, 2005; Ferreira *et al.*, 2005). It is possible that, these viruses became better adapted to their new host and are better fit regarding to replication efficiency, movement or vector transmissibility and are displacing the less adapted species. Even though it appears that these five species are prevailing in the main tomato producing areas, it seems that the situation continues to evolve and there is an incessant prospect that even more virulent recombinant variants or new species could emerge at any time. Continued sampling will have to be carried out to monitor the evolution and epidemiology of the tomato-infecting begomoviruses in the producing areas a well as to aid ongoing breeding programs.

GEMINIVIRUSES AND RNA SILENCING

RNA silencing is a regulatory mechanism participating in a number of essential eukaryotic cellular processes. This mechanism involves the processing of an inducer dsRNA into siRNAs of 21-26 nts. These siRNAs associate with specific proteins to from the RISC complex and direct the degradation of homologous RNAs or methylation of DNA (Baulcombe, 2004; Voinnet, 2005).

The relevance of RNA silencing for viruses having a DNA genome, which replicate in the plant cell nucleus and lack dsRNA replicative forms/intermediates seems limited at first glance. Yet, despite their genomic and replicative features, geminiviruses were

shown to trigger the host RNA silencing mechanism and their mRNAs are targeted in infected plants (chapter 4) (Chellappan *et al.*, 2004b; Lucioli *et al.*, 2003). In chapters 4 and 5 it is shown that siRNA corresponding to all regions of the bipartite genome are generated, during the infection process, including the intergenic region, containing promoter sequences and the origin of replication, which are not expected to be transcribed (chapter 4) (Chellappan *et al.*, 2004b). The identification of siRNAs matching this region might be due to the size of the PCR fragments used in the experiments, including surrounding transcribed regions; therefore a finer mapping of this region using smaller PCR fragments would more clearly indicate if the promoter and origin of replication sequences are actually targeted by the RNA silencing machinery.

Regardless of the entire genome being targeted, siRNAs are differentially directed to parts of the genome depending on the virus-host combination as exemplified by ToCMoV-[BA-Se1] (chapter 4) and *East African cassava mosaic virus* (Chellappan *et al.*, 2004b; Chellappan *et al.*, 2005b). In general, a hotspot seems to correspond to the AC1 gene region in DNA-A of begomoviruses of which the severity of the disease decreases with time. The exact reason why this genome region is a preferred target is not known, but the structure of the AC1 mRNA might be important, as a high proportion of AC1 specific complementary sense versus viral sense siRNA was observed (chapter 4). In any case, and not surprisingly, the targeting of ToCMoV-[BA-Se1] mRNAs and accumulation of viral siRNAs probably results in the down regulation of AC1 and likely other viral proteins. This hinders virus replication and corresponds to a decrease in the viral DNA accumulation and reduction in disease symptoms (chapter 4) (Chellappan *et al.*, 2004b). Taken all together, RNA silencing does seem to influence the development of diseases caused by geminiviruses although the precise mechanism by which geminiviruses induce RNA silencing in infected plants is still enigmatic. Some lines of evidence indicate that a combination of mechanisms might be occurring. For instance, overlapping divergent mRNAs have been identified in infected plants and could be implicated in the induction (Chellappan *et al.*, 2004b). Indeed such overlapping transcripts could be extended by the action of a host RdRp generating dsRNA. In addition, or in stead, other "aberrant" or highly expressed transcripts could have their complementary strands synthesized in a similar way. This mechanism seems be an important step since detailed analysis of *Arabidopsis* mutants revealed that the RdRp encoding RDR6 gene is required for successful geminivirus-induced gene silencing (VIGS) of endogenous genes (Muangsan *et al.*, 2004). In addition, Dicer might be directed to process secondary structure of viral transcripts. This still open issue could be solved by cloning and sequencing the virus-derived siRNAs accumulating in infected plants, similar to experiments performed on siRNAs in plants infected with the (RNA) tombusviruses (Molnar *et al.*, 2005). This approach would reveal the origin, polarity and molecular nature of the viral-derived siRNAs molecules.

Plant-virus interactions can be influenced by environmental factors such as

temperature. It has indeed been shown that for *Cymbidium ring spot virus* (an RNA virus) and for several cassava-infecting geminiviruses that increasing temperature significantly boosted RNA silencing and increased virus-derived siRNA accumulation with consequent development of milder symptoms (Chellappan *et al.*, 2005b; Havelda *et al.*, 2005; Szittya *et al.*, 2003). Raising the temperature to 30ºC for different cassava begomoviruses viruses resulted in an increase in siRNA levels. Moreover, there was a change in the distribution of the siRNA along the genome for some of them and for all the viruses tested, the levels of viral DNA as well as the severity of symptoms decreased (Chellappan *et al.*, 2005b). This is a very interesting observation because it seems to contrast with the fact that geminiviruses, more specifically begomoviruses, are most important and cause devastating diseases in (sub)tropical areas, where the day time temperatures in long periods of the year are well above 30º C. Nevertheless, symptoms are very severe and viral DNA can be isolated in abundance from infected tissues. The disease situation in a natural setting in the field is influenced by other factors such as plant genotype, age of infection, environmental factors such as temperature, water stress, exposure to chemicals and to mechanical injury and by the continuous exposure to viruliferous whiteflies resulting in frequent virus (re-)inoculation. It would be interesting to set up experiments in which the contribution of these factors on the development of the disease and the RNA silencing mechanism under field conditions are studied. In these studies, also the role of putative virus-encoded RNA silencing suppressor proteins should be considered.

To interfere with the plant defense RNA silencing mechanism, most plant viruses express proteins that suppress RNA silencing (Vance & Vaucheret, 2001; Voinnet *et al.*, 1999). Also geminiviruses have been demonstrated to encode such silencing suppressors, which seem to function through multiple and diverse mechanisms. AC2 and AC4 have been identified for different geminiviruses. Although AC2 proteins from different viruses share high sequence identity, there is a difference in the way they operate. AC2 from Mungbean Yellow mosaic virus (MYMV) and Tomato yellow leaf curl China virus (TYLCCV), require the nuclear localization, an active zinc finger and in the case of MYMV, also the activation domain for the silencing suppression activity (Trinks *et al.*, 2005; van Wezel *et al.*, 2002; Van Wezel *et al.*, 2003). Therefore, they seem to be active in the nucleus and are dependent on mechanisms related to DNA binding and transcription activation. Studies with MYMV and ACMV-[KE] suggested that their AC2 proteins might suppress silencing in an indirect way by inducing the transcription of genes that control the host RNA silencing system (Trinks *et al.*, 2005). On the other hand the silencing suppression function of TGMV-AC2 seems to be transcription-independent because this domain is dispensable for the silencing suppression activity (Bisaro, 2005; Wang *et al.*, 2005). TGMV-AC2 also appears to act indirectly by binding and inactivating adenosine kinase (ADK). ADK plays a role in sustaining the methylation pathway and might be important in RNA silencing (Wang *et al.*, 2003). By inactivating ADK,

TGMV-AC2 could interfere in a mechanism involving siRNA-directed methylation, inhibiting epigenetic change of the geminiviral genome (Bisaro, 2005). This would imply that geminivirus genome is targeted by the host siRNA silencing-inducing DNA methylation pathway. However, experimental evidence for *in vivo* methylation of the geminivirus genome in infected plants still awaits confirmation. The AC4 protein was determined to be the silencing suppressor protein of ACMV-[CM] and Sri Lankan cassava mosaic virus (SCMV). Expression of ACMV-[CM]-AC4 in transgenic *Arabidopsis* induces developmental defects as it binds single stranded siRNAs and microRNAs resulting in increased levels of the corresponding target mRNA. Thus, ACMV-[CM]-AC4 seems to interfere with the cytoplasmic siRNA silencing pathway and at the same time with the miRNA pathway (Chellappan *et al.*, 2005a). Interestingly, mixed infections in cassava with African cassava mosaic virus-[Cameroon] and *East African cassava Cameroon virus* result in seriously enhanced disease symptoms, associated with the synergism of functionally different silencing suppressors (AC4 and AC2) (Vanitharani *et al.*, 2004). This confirms that the RNA silencing mechanism impacts geminivirus life cycle and that the interaction with the host is very complex and diverse.

The results described in chapter 5 add yet another piece to the already complex begomovirus-host RNA silencing interaction puzzle. Would it be possible that there are begomoviruses which do not encode a functional RNA silencing suppressor protein at all or one not strong enough to be detectable by the most commonly used experimental settings? This indeed seems to be the case for ToCMoV-[BA-Se1]. When first described using the "silencing reversion" assay, *Tomato bushy stunt virus* p19 was considered a weak suppressor (Voinnet *et al.*, 1999). However, using *Agrobacterium* transient assay, it was shown to be one of the strongest viral silencing suppressors (Hamilton *et al.*, 2002; Voinnet *et al.*, 2003). Also using the silencing reversion assay, only three of five potexviruses suppressed RNA silencing of GFP, PVX being one of the two not showing any suppressor activity at all (Voinnet *et al.*, 1999). Subsequently, p25 was shown to be a silencing suppressor using a modified *Agrobacterium* transient assay (Voinnet *et al.*, 2000). Therefore different assays may lead to different results suggesting prudence when interpreting the results from these popular assays (Vance & Vaucheret, 2001).

A way to study the contribution of RNA silencing to ToCMoV-[BA-Se1] would be to assess the interaction with components of RNA silencing by using knockout mutant plants for different genes involved in RNA silencing, for example RDR6, SGS3, DDM1, etc. New insights could be gained by inoculating the virus and evaluating the influence in the disease phenotype, presence and targeting of siRNAs and viral DNA load. Studying the genetics requirements for DNA VIGS using *Arabidopsis* mutants and a *Cabbage leaf curl virus*-derived vector, Muangsan *et al.* (2004) demonstrated that effective endogenous gene silencing required RDR6 and SGS3 but not SGS1 and AGO1, even though all of these gene products are involved in different steps in cytoplasmic RNA

silencing. MOM1 and DDM1/SOM8, genes engaged in transcriptional gene silencing maintenance, were not required.

In conclusion, ToCMoV-[BA-Se1] is targeted by the host RNA silencing machinery and the viral replication/DNA accumulation process is affected (chapter4) but a (strong) silencing suppressor to protect the virus against this plant defense mechanism could not be identified by the tests used (chapter 5). However there seems to be a balance between replication and silencing and the timing of these events allow the virus to replicate, spread over the entire plant and be transmitted by the vector allowing the propagation of the disease cycle in the field.

PERSPECTIVES FOR BEGOMOVIRUS CONTROL

Shortly after the outbreaks of tomato-infecting begomoviruses in Brazil were established, a breeding program was initiated in 1995 by EMBRAPA-Vegetables aiming to select genotypes with good resistance to Brazilian tomato begomoviruses. The initial screening studies were performed using inbred *Lycopersicon esculentum* lines and other wild *Lycopersicon* spp genotypes. Although the begomovirus species used in these pioneer works was not characterized, a promising resistance source was discovered characterized by absence of symptoms and very low viral DNA accumulation (Ferreira *et al.*, 1999; Santana *et al.*, 2001). The virus species studies carried out during this thesis (chapter 2 and 3) (Faria *et al.*, 1997; Ribeiro *et al.*, 2003) has been instrumental to aid the breeding programs for the selection and introgression of the identified resistance trait into tomato cultivars and hybrids (Giordano *et al.*, 2005). The genetic basis of the resistance to ToCMoV in the accession *L. esculentum* line 'TX 468-RG' is characteristic of a single recessive gene (locus), which was named *tcm-1*. The effectiveness of the resistance in the line 'TX 468-RG' to individual species of other Brazilian tomato infecting begomoviruses is not yet known, however field tests to this end with selected lines carrying the *tcm1* locus were initiated. Anticipating a possible introduction of the Old World monopartite begomovirus TYLCV in Brazil, the resistance genotypes were evaluated and the same resistance reaction was observed (Resende, 2005). Therefore, tomato plants carrying the *tcm1* locus seem to have broad-spectrum begomovirus resistance. The situation in Brazil is complex and at the moment, with at least five different begomovirus species predominating in the tomato crop and chances of new species to emerge seem significant. Since a broad and durable resistance is the ultimate goal, a large scale evaluation of advanced bred tomato families carrying this locus should be tested in different locations.

Additional to introgression of natural sources of resistance, alternative biotechnological approaches for resistance in transgenic plants have been investigated to improve begomovirus resistance. The most commonly employed strategies deal with the over

expression or silencing of the AC1 gene which seems to be the gene of choice because of its pivotal requirement for viral replication.

Truncated or mutant AC1 proteins have proven effective in interfering with begomovirus replication. For example, transgenic T3 generation of common bean plants expressing a mutant NTP binding site of BGMV AC1 protein were resistant to the virus (J.C. Faria & F.J.L. Aragão, *unpublished results*). In another study, transgenic *N. benthamiana* and tomato plants expressing high levels of a truncated form of AC1 of TYLCSV, lacking the NTP binding domain and the associated ATPase activity but retaining other functional motifs, are resistant to the homologous and a heterologous virus. Study on the mechanisms underlying this resistance showed that it acts as a trans-dominant-negative protein. The resistance, however, was eventually overcome by silencing of the transgene due to the presence of the homologous virus (Lucioli *et al.*, 2003). To avoid the silencing problem, a synthetic transgene with alternative nucleotide sequence with reduced identity to the original virus gene, but maintaining the protein amino acid sequence could be introduced (Pooggin & Hohn, 2004). An alternative strategy could be expressing a truncated AC1 lacking the pRBR (retinoblastoma) binding domain in tomato plants. Resistance was reported for plants expressing TYLCV-Mld mutant AC1, however, it was overcome by another strain of the virus (Antignus *et al.*, 2004). Expression of a bicistronic vector containing AC1 and AC3 mutants in transgenic plants could confer an effective resistance to TGMV. AC3 mutants interference with virus infection seems to be non specific and to enhance AC1-derived resistance suggesting that it should be possible to achieve a broader resistance (Dallas *et al.*, 2004).

The expression of complete antibodies (Hiatt *et al.*, 1989) and single-chain variable antibody fragments (scFvs) (Owen *et al.*, 1992) in transgenic plants opened new possibilities for their production and use as immunotherapeuticals, specific inhibition of physiological functions or pathogen inactivation (Tavladoraki *et al.*, 1993). The expression of scFvs has been used to obtain high levels of resistance to *Tomato spotted wilt virus* (Prins *et al.*, 2005) and *Tomato bushy stunt virus* (Boonrod *et al.*, 2004). These examples demonstrate the potential use of "plantibodies" (as highly expressed immunoglobulins in plants are often referred to) as an alternative strategy also for resistance to geminiviruses. The expression of recombinant antibodies directed for example to full-length or conserved functional domains of AC1 or movement proteins or even to a conserved sequence of DNA, such as the origin of replication of geminiviruses are unexplored potential to engineer geminivirus resistance in transgenic plants. In the course of this thesis we have used a DNA oligonucleotide representing the stem loop sequence that harbors the conserved origin of replication of geminiviruses. This oligonucleotide was used to screen a single-chain antibody phage display library. Several phages that bind to this oligonucleotide were select. However, the effectiveness of these scFvs to protected transgenic plants against geminivirus infection remains to be determined.

A recently reported alternative resistance strategy is the use of artificial zinc finger proteins (AZP) (Sera, 2005). These proteins can be designed and selected for specific DNA sequence binding, such as the AC1 binding site at the origin of replication of geminiviruses. The principle and usefulness of the strategy was demonstrated in transgenic *Arabidopsis* expressing an AZP and presenting a very high resistance to *Beet severe curly top virus,* a curtovirus. The system has not yet been tested for begomoviruses. One issue of this approach is that it is specific for each AC1/virus binding site; therefore it is of limited use in areas where a high variability of geminiviruses is present (Sera, 2005).

Exploiting RNA silencing by producing siRNA in transgenic plants has proven to be a powerful way to engineer resistance to RNA viruses (Baulcombe, 1996; Goldbach *et al.*, 2003; Prins, 2003; Ritzenthaler, 2005). Results from transgenic resistance associated with RNA silencing in begomovirus, however, are very inconsistent. Even when sequences derived from the same gene are used, as the case AC1, the reported cases range from complete susceptibility (TYLCV-tomato) (Noris *et al.*, 2004) to cases of very high and broad levels of resistance (ACMV/EACMV/SLCMV-cassava) (Chellappan *et al.*, 2004a). In both cases, the AC1 gene is a target sequence in natural infections (Chellappan *et al.*, 2004b; Lucioli *et al.*, 2003). In chapter 4 of this thesis, transgenic *N. benthamiana* plants were produced that express dsRNA corresponding to most targeted portion of the ToCMoV-[BA-Se1] genome. These transgenic plant lines were shown to present an activated RNA silencing mechanism and to produce transgene-specific siRNAs. However, the levels of transgene-produced siRNAs were similar in resistant and susceptible siblings of the same line and therefore factors other than the simple occurrence of siRNAs seem to contribute for the resistance phenotype. This is contrary to RNA silencing mediated resistance to RNA viruses where the presence of siRNAs can be regarded as a molecular marker of resistance (Chen *et al.*, 2004; Kalantidis *et al.*, 2002). This highlights that virus-host plant interplay in RNA silencing in the case of geminiviruses is very complex and the infection outcome most likely depends on particular features of a specific virus/host species and efficacy and strength of the RNA suppressor in that particular host. Despite the variable results, RNA silencing based resistance in transgenic plants has a potential application for controlling begomoviruses and perhaps it is a matter of determining the right combination of virus/target sequence/host plant. It is clear that further work should focus directly on specific virus/crop plant combination.

In the future, a well performing transgenic line can be crossed with selected resistant lines/cultivars expressing natural resistance genes such as *tcm-1* to pyramid transgenic and conventional resistances, thereby expanding the strength and broadness of the resistance and increase durability. The use of resistant cultivars coupled with cultural practices such as a crop free period aiming at reducing initial virus load, and a rational use of pesticides should lead to a reduction of virus incidence and eventually to a satisfactory control of these severe tomato diseases caused by begomoviruses in Brazil.

Summary

Summary

Over the last decade, the prevalence and severity of tomato infecting begomoviruses have increased to epidemic proportions and consequently, begomoviruses became one of the major limitations for tomato production throughout Brazil. The sudden emergence of these whitefly-transmitted geminiviruses followed the introduction and rapid dispersal of the B biotype of *Bemisia tabaci.* As limited sources of resistance were available, this situation required the development of novel strategies for the disease control including the production of resistant cultivars which should be part of an integrated management approach. These management strategies should be based on a more detailed understanding of the pathosystems involved. Therefore, this PhD research focused on a comprehensive identification and characterization of the begomovirus species involved in the epidemics. Also a study of different aspects implicated in the complex virus-host interactions involving virulence determinants encoded by the virus and the RNA silencing response generated by begomovirus infection in plants was carried out.

A countrywide survey of tomato-infecting begomoviruses was conducted in chapter 2. The molecular characterization of 24 begomoviruses isolated throughout the country revealed the panorama of their distribution and genetic diversity. Samples with typical begomovirus symptoms were collected in seven different states, including the most important fresh-market and processing tomato growing areas of the country. Detailed analysis of these isolates established the existence of previously undescribed begomovirus species. At least seven putative novel species were identified. Phylogenetic analysis placed these viruses in separate clades, distant from all the previously characterized New World begomoviruses. Interestingly, the most closely related established virus species were *Bean golden mosaic virus* and *Tomato golden mosaic virus*, begomoviruses identified in Brazil over a decade ago. Further detailed sequence comparisons provided strong evidence of recombination among the Brazilian tomato-infecting begomoviruses.

During these studies a species named *Tomato chlorotic mottle virus* was found to be widely dispersed in both Southeastern and Northeastern tomato producing regions of the country. The molecular and biological properties of a representative isolate of this virus from the northeast of Brazil, Tomato chlorotic mottle virus-[Bahia-Seabra1] (ToCMoV-[BA-Se1]), were further investigated in chapter 3. The full-length genome of ToCMoV-[BA-Se1] was cloned and sequenced. The cloned ToCMoV–[BA-Se1] DNA-A and DNA-B components were fully infectious and caused typical chlorotic mottle symptoms when inoculated by particle bombardment into tomato plants. The progeny of cloned ToCMoV–[BA-Se1] components was also proven to be whitefly transmissible. The experimental host range of this virus includes vegetable and ornamental crops and

some weed species, predominantly, but not exclusively, in the family *Solanacea*, which are widely disseminated throughout the country. Sequence analyses showed that ToCMoV–[BA-Se1] is a typical New World begomovirus which requires both DNA-A and DNA-B to establish systemic infection. Phylogenetic studies based on the DNA-A sequences of New World begomoviruses placed ToCMoV–[BA-Se1] together with 10 other Brazilian begomoviruses in a monophyletic group. ToCMoV–[BA-Se1] shares a 92% sequence identity with an isolate reported from the state of Minas Gerais, and referred to as ToCMoV-[MG-Bt1]. Extensive sequence comparisons suggest that both ToCMoV isolates bear evidence of four recombination events indicating that these events have occurred long before the divergence of ToCMoV [BA-Se1] and [MG-Bt1] and the recent emergence of this species. Taken all together our observations suggest that the newly identified begomoviruses are native to Brazil and were transmitted from weeds and wild plants into crops such as tomato by the introduced whitefly biotype, which, in contrast to the local whitefly populations, effectively colonize this crop.

RNA silencing is a regulatory mechanism that involves the degradation of RNA in a sequence-specific manner and in plants is involved in antiviral response. Upon infection by different viruses, plants have been shown to produce virus-specific siRNAs, sometimes leading to recovery from the disease. Hence, RNA silencing has been used as a biotechnological approach in transgenic plants to interfere with viral infection. In chapter 4 the RNA silencing response generated in plants infected by ToCMoV-[BA-Se1] was examined. Upon infection with ToCMoV–[BA-Se1], the RNA silencing machinery in host plants such as tomato, *N. benthamiana* and petunia was triggered and a population of siRNAs specific to ToCMoV-[BA-Se1] was generated. Concomitantly, a decrease in the viral DNA levels was observed, thus suggesting that indeed DNA viruses are sensitive to RNA silencing, possible by targeting the viral mRNAs. The siRNA population isolated from in infected *N. benthamiana* and tomato plants represented the complete bipartite genome however, it was not uniformly distributed over the entire extent of the DNA-A and DNA-B. Moreover, comparing the distribution of DNA-A derived siRNA from *N. benthamiana* and tomato, a divergence in the preference for different parts of the genome in different hosts was observed. In *N. benthamiana,* the primary targets corresponded to the 5' end of AC1 and the embedded AC4, the intergenic region and 5' end of AV1 and part of overlapping AC5 ORF. Aiming to generate virus resistant plants, the natural RNA silencing reaction to ToCMoV-[BA-Se1] infection in *N. benthamiana* was exploited. For this purpose, transgenic plants with an intron-hairpin construct expressing dsRNA corresponding to the most targeted portions of the viral genome were produced. As intended transgenic *N. benthamiana* lines indeed expressed transgene-specific siRNAs prior to infection with the virus. Underscoring the feasibility of the strategy, most transgenic lines showed a considerable delay in symptom development and two

lines proved to contain highly resistant and immune plants. Curiously, the levels of transgene-produced siRNAs were similar in resistant and susceptible siblings of the same line. In contrast to many RNA viruses, where the presence of transgene siRNAs corresponding to viral sequences guarantees virus resistance, RNA-mediated resistance to DNA viruses is more complex being not strictly related to the mere presence of siRNAs.

In chapter 5, all genes encoded by both DNA-A and DNA-B of ToCMoV-[BA-Se1] were expressed using a Potato virus X (PVX) vector and evaluated for distinct symptom phenotypes in local and systemic host plants. Depending on the host plant, all PVX viruses harboring ToCMoV-[BA-Se1] genes had increased symptom reactions, with the exception of AC3 and AC5. In all host plants tested, the most conspicuous responses were induced by PVX-AC2. In *Nicotiana benthamiana,* this chimeric construct caused severe systemic necrotic lesions, necrotic veins and petioles leading to leaf collapse and pronounced stunting.

One of the important biological functions of RNA silencing in plants is antiviral defense. As a logical consequence, plant viruses express proteins that counteract this defense mechanism. Most established RNA silencing suppressors proteins have previously been implicated in virulence and reported as symptom determinants. Therefore, the silencing suppression potential of all ToCMoV-[BA-Se1] genes was investigated. To this end, the two most commonly used tests, i.e. the local *Agrobacterium* transient assay and the transgene silencing reversal assay were employed. Using either assay, it was found that, despite contributing to virulence, none of the ToCMoV-[BA-Se1] gene products appear to possess an RNA silencing suppression activity. One explanation could be that for ToCMoV-[BA-Se1] a possible silencing suppressor is too weak to be identified by these commonly used assays.

Finally in chapter 6, the emergence of tomato begomoviruses in Brazil, the possible role of RNA silencing in begomovirus infection and the perspectives of their control by using biotechnological strategies are discussed.

Samenvatting

SAMENVATTING

Gedurende de afgelopen 10 tot 15 jaren is de verspreiding van begomovirussen en de daarmee samenhangende schade in de tomatenteelt in Brazilië tot epidemische proporties toegenomen. Als gevolg daarvan zijn begomovirussen tegenwoordig één van de belangrijkste beperkingen voor tomatenproductie geworden. Deze plotselinge ontwikkeling van deze door wittevliegen (*Bemisia tabaci*) overgebrachte virusziekte volgde onmiddellijk na de introductie en de snelle verspreiding van het agressieve biotype B van deze vector. Omdat er maar beperkte bronnen van natuurlijke resistentie beschikbaar waren, vereiste deze situatie de ontwikkeling van nieuwe strategieën voor bestrijding van begomovirusziekten met inbegrip van transgene productie van resistente cultivars, die deel zouden moeten uitmaken van een geïntegreerde beheersbenadering. Het succes van dergelijke beheersstrategieën is uiteraard afhankelijk van een voldoende gedetailleerd inzicht in het betreffende pathosysteem. Daarom concentreerde het in dit proefschrift beschreven onderzoek zich allereerst op een gedetailleerde identificatie en karakterisering van de begomovirussoorten die betrokken zijn bij de huidige epidemie. Ook werd een studie naar verschillende aspecten van de complexe virus-gastheer interacties gedaan in het bijzonder waar het de virulentiedeterminanten betreft en het effect van de betrokken virusgecodeerde eiwitten op het onderdrukken van RNA silencing.

Een landelijk onderzoek naar de tomateninfecterende begomovirussen in Brazilië werd uitgevoerd en is beschreven in hoofdstuk 2. De moleculaire karakterisering van 24 in Brazilië aangetroffen begomovirussen toonde het spectrum van hun verspreiding en genetische diversiteit. De steekproeven van planten met typische begomovirussymptomen werden verzameld in zeven verschillende staten, met inbegrip van de belangrijkste tomatenproductiearealen van het land. De gedetailleerde analyse van de verzamelde virusisolaten bevestigde het bestaan van niet eerder beschreven soorten. Aldus werden tenminste zeven nieuwe begomovirussen geïdentificeerd. Phylogenetische analyse plaatste deze virussen in afzonderlijke groepen, ver van al eerder gekarakteriseerde nearctische begomovirussen. Het meest nauw verwant aan deze nieuwe virussoorten bleken *Bean golden mosaic virus* en *Tomato golden mosaic virus* te zijn, die beiden al een decennium geleden in Brazilië aangetroffen werden. Nadere genoomanalyses leverden sterke aanwijzingen op voor recombinaties tussen de nieuwe tomateninfecterende begomovirussen.

Een van de nieuwe begomovirussen, waarvoor de naam *Tomato chlorotic mottle virus* (ToCMoV) werd bedacht, bleek wijdverbreid in tomaten voor te komen, zowel in het zuidoosten als het noordoosten van het land. De moleculaire en biologische eigenschappen van een representatief isolaat van dit virus uit het noordoosten van Brazilië, *Tomato chlorotic mottle virus* -[Bahia-Seabra1] (ToCMoV-[BA-Se1]), werden verder onderzocht in hoofdstuk 3. Daartoe werd de volledige nucleotidenvolgorde van het genoom opgehelderd. De gekloneerde componenten DNA-A en DNA-B van ToCMoV-[BA-Se1] bleken volledig infectieus en veroorzaakten in tomatenplanten induceerden na

kunstmatige inoculatie in tomaat de typische chlorotische symptomen van het oorspronkelijke virus. De op deze wijze uit gekloonde componenten geregenereerd ToCMoV-[BA-Se1] bleek ook overdraagbaar door wittevlieg te zijn. De experimentele waardplantenreeks van dit virus omvat groente- en siergewassen en sommige onkruidsoorten, hoofdzakelijk, maar niet uitsluitend behorend tot de familie *Solanacea*, die wijdverbreid in Brazilië voorkomen. Genoomanalyses toonden verder aan dat ToCMoV-[BA-Se1] een typisch nearctisch begomovirus is, dat beide (A en B) DNA componenten nodig heeft om een systemische infectie van de plant te bewerkstelligen. Phylogenetische studies gebaseerd op de sequenties van DNA-A van Noord- en Zuid-Amerikaanse begomovirussen, plaatsten ToCMoV-[BA-Se1] samen met 10 andere Braziliaanse begomovirussen in een monophyletische groep. Het isolaat ToCMoV-[BA-Se1] blijkt 92% sequentieovereenkomst te hebben met een ToCMV isolaat uit de staat Minas Gerais (ToCMoV-[MG-Bt1]). Onderlinge vergelijking lijkt er op te wijzen dat beide ToCMoV isolaten maar liefst vier recombinatiegebeurtenissen hebben ondergaan, voor het scheiden van de twee isolaten en de recente opkomst van de soort. Samenvattend kan voorzichtig geconcludeerd worden dat de in hoofdstuk 2 en 3 geïdentificeerde begomovirussen inheems zijn aan Brazilië en vanuit wilde plantensoorten zijn overgebracht naar gewassen zoals tomaat, en dat dit op zijn beurt weer samenhangt met de opkomst van het recent geïntroduceerde *B. tabaci* biotype B dat, in tegenstelling tot de oorspronkelijke wittevliegenpopulatie, dit gewas effectief koloniseert.

RNA silencing is een regulatiemechanisme dat op een sequentiespecifieke wijze betrokken is bij de afbraak van RNA en, althans in planten, betrokken is bij de antivirale afweer. Na infectie door een plantenvirus kan in veel gevallen korte, virusspecifieke RNA moleculen ("small interfering" RNAs, ofwel siRNAs) in de plant worden aangetoond, dat vervolgens kan leiden tot het onderdrukken of verdwijnen van de infectie. Vandaar dat RNA silencing vaak gebruikt wordt als biotechnologische benadering om virusresistente planten te verkrijgen middels transgenese met virale genoomsequenties. Voor begomovirussen, met hun DNA genoom, was nog niet vastgesteld of deze ook herkend worden door het RNA silencing mechanisme. Dit is in hoofdstuk 4 nader onderzocht en hieruit blijkt dat tijdens infectie met ToCMoV-[BA-Se1] waardplanten zoals tomaat, *Nicotiana benthamiana* en petunia inderdaad hun RNA silencing mechanisme inzetten, waarbij een populatie van ToCMoV-[BA-Se1]-specifieke siRNAs werd gevormd. Omdat gelijktijdig een daling van de virale DNA hoeveelheden optrad, kan geconcludeerd worden dat begomovirussen, ondanks hun DNA genoom, inderdaad gevoelig zijn voor RNA silencing, mogelijk doordat hun mRNAs doelwit zijn. De populatie siRNAs die uit besmette *N. benthamiana* en tomatenplanten werd gevormd, bleek het volledige (bipartite) genoom te vertegenwoordigen. Desondanks was de afkomst van de siRNAs niet uniform verdeeld over DNA-A en DNA-B. In *N. benthamiana* en tomaat werden verschillen waargenomen m.b.t. frequentie waarin de verschillende delen van het genoom in siRNAs vertegenwoordigd waren. De natuurlijke RNA silencing reactie in *N. benthamiana* op ToCMoV-[BA-Se1] infectie werd gebruikt als mogelijk uitgangspunt

voor transgene resistentie in deze plant. Om dit te bewerkstelligen werden transgene planten geproduceerd met intron-haarspeld constructen die dsRNA aanmaken dat overeen komt met die gedeelten van het virale genoom die het meest gevoelig zijn voor RNA silencing. Zoals bedoeld produceerden transgene *N. benthamiana* lijnen, voorafgaand aan besmetting met het virus, inderdaad siRNAs specifiek voor het virale transgen. De meeste transgene lijnen toonden een aanzienlijke vertraging in symptoomontwikkeling en twee lijnen bleken zelfs immune planten te bevatten, waarmee de haalbaarheid van de strategie wordt onderstreept. Opmerkelijk was dat de niveaus van de door het transgen geproduceerde siRNAs gelijkwaardig waren in resistente en vatbare planten van een zelfde lijn. Dit is in tegenstelling tot wat gevonden is voor RNA virussen, waar de aanwezigheid van transgen siRNAs virusresistentie zo goed als garandeert. Blijkbaar is het fenomeen van *RNA-mediated* resistentie tegen DNA virussen (nog) complexer dan voor RNA virussen en is de aanwezigheid van virusspecifieke siRNAs niet kritisch wat betreft resistent phenotype.

In hoofdstuk 5, werden alle (zowel van DNA-A als DNA-B afkomstige genen van ToCMoV-[BA-Se1] apart gekloneerd en *in planta* tot expressie gebracht met behulp van een aardappel virus X (PVX) vector. Doel was enerzijds inzicht te verkrijgen in de betrokkenheid van de individuele virale genproducten in ziektesymptoominductie en anderzijds aanwijzingen te krijgen voor het bestaan van een RNA silencing onderdrukkend eiwit. De resulterende symptoomkenmerken van de diverse PVX/ToCMoV chimeren werden geëvalueerd in lokale en systemische waardplanten. De meeste genen van ToCMoV-[BA-Se1] veroorzaakten versterkte symptomen. Uitzondering waren AC3 en AC5. In alle geteste waardplanten werden de heftigste symptomen veroorzaakt door PVX-AC2. In *Nicotiana benthamiana* veroorzaakte dit chimere construct sterke systemische necrotische lesies, inclusief necrose van nerven en bladstelen, resulterend in groeiremming en vervolgens bladsterfte.

Een van de belangrijke biologische functies van RNA silencing in planten is antivirale afweer. Als logisch gevolg produceren plantinfecterende RNA virussen eiwitten die dit defensiemechanisme tegengaan. De meeste onderzochte RNA silencing onderdrukkingseiwitten zijn tevens determinanten van virulentie en betrokken bij symptoomontwikkeling. Daarom werd de RNA silencing onderdrukkingsactiviteit van alle genen van ToCMoV-[BA-Se1] onderzocht. Daarvoor werden de twee het meest gebruikte tests gebruikt: de "*Agrobacterium* transfectie" toets en de "transgen silencing reversie" toets. Ondanks hun bijdragen aan ziektesymptoominductie, bleek geen van de ToCMoV-[BA-Se1] genproducten in staat RNA silencing significant te onderdrukken. Een mogelijke verklaring zou kunnen zijn dat de onderdrukkende activiteit bij ToCMoV-[BA-Se1] te zwak is om in de gebruikte toetsten te worden opgemerkt.

Tot slot worden in hoofdstuk 6 de opkomst van tomateninfecterende begomovirussen in Brazilië, de mogelijke rol van RNA silencing tijdens begomovirusinfecties, en de (mede daaruit voortkomende) vooruitzichten op de ontwikkeling en toepassing van biotechnologische beheersstrategieën bediscussieerd.

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THE AUTHOR

Simone da Graça Ribeiro was born in 6th of August, 1962, in Ribeirão Preto, São Paulo state, Brazil. She studied biology at the Universidade de Brasilia and got her degree in 1985 with a major in Zoology. During her undergraduate study years, she worked at the laboratory of electron microscopy, mainly with immuno-specific electron microscopy applied to plant virus detection. She also worked at the ornithology lab with parasitism among birds in the cerrado ecosystem. Since her biological clock and those of the birds did not quite match, she decided to stick with plant parasites and got her MSc degree in Plant Pathology in 1988 also at the Universidade de Brasilia, under the supervision of Prof. Elliot Kitajima. Her work dealt with the characterization of a tobacco isolate of Eggplant mosaic virus, a tymovirus. At the same year she was granted a fellowship to work with viroid diseases and carried out the work at Embrapa Hortaliças and Embrapa Recursos Genéticos e Biotecnologia (Cenargen) for one year. In 1989 she got a permanent position as a researcher scientist at Embrapa Cenargen and worked with geminivirus diseases in beans and, later, in tomatoes. In November 2000 she started her PhD studies at the Laboratory of Virology, in Wageningen, under supervision of Dr. Marcel Prins and Prof. Rob Goldbach. She worked within the research project that resulted in this thesis. In September 2005 she resumed her duties as a researcher at Embrapa Cenargen.

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Simone da Graça Ribeiro
Date: 1 December 2006
Group: Laboratory of Virology, Wageningen University

1) Start-up phase	date
First presentation of your project	
Distribution and genetic diversity of tomato-infecting geminiviruses in Brazil	Jan 2001
Writing or rewriting a project proposal	
Engineering geminivirus resistance in tomato using plantibodies	Jan 2002
2) Scientific Exposure	**date**
EPS PhD student days	
Wageningen University	Jan 2001
Wageningen University	Jan 2002
Utrecht University	Mar 2003
Vrije Universiteit Amsterdam	Jun 2004
EPS theme symposia	
Theme 2 'Interactions between Plants and Biotic Agents', Utrecht University	Dec 2000
Theme 2 'Interactions between Plants and Biotic Agents', Leiden University	Dec 2001
Theme 4 'Genome Plasticity', Radboud University	Dec 2002
Theme 2 'Interactions between Plants and Biotic Agents', University of Amsterdam	Jan 2003
Theme 2 'Interactions between Plants and Biotic Agents', Wageningen University	Dec 2003
NWO Lunteren days and other National Platforms	
Studiegroep nucleinezuren NWO-CW Lunteren	Dec 2001
Studiegroep nucleinezuren NWO-CW Lunteren	Dec 2002
Studiegroep nucleinezuren NWO-CW Lunteren	Dec 2003
Dutch annual virology symposium	Mar 2004
Seminars (series), workshops and symposia	
Symposium Bionanotechnology: Assembly and Nanostructures	May 2005
Symposium current themes in ecology: Global Ecology	April 2003
Symposium current themes in ecology: Genomics for Nature	Nov 2001
Seminar Frontiers in Plant science: Cell cycle control and plant development	Feb 2003
International symposia and congresses	
3rd International geminivirus symposium, JIC, Norwich (UK)	Jul 2001
7th International congress of plant molecular biology, Barcelona (Spain)	Jun 2003
International Congress of Virology - IUMS, Paris (France)	Jul-Aug 2002
XXXV Annual meeting of the Brazilian Phytopathological Society, Recife (Brazil)	Aug 2002
Plant derived vaccines and antibodies: potential and limitations, Veyrier-du-Lac (France)	Mar 2004

Presentations	
DPG Arbeitskreis Viruskrankheiten der Pflanzen and the Nederlandse Kring voor Plantevirologie (oral)	Mar 2001
ssDNA viruses of plants, birds, pigs and primates - comparative virology (oral and poster)	Sep 2001
7th International congress of plant molecular biology (poster)	Jun 2003
4th international geminivirus symposium (oral)	Feb 2004
Nederlandse Kring voor Plantevirologie and the DPG Arbeitskreis Viruskrankheiten der Pflanzen (oral)	Mar 2005
Biologishes Kolloquium Sommeresemester 2005- Biologishes Institut der Universitat Stuttgart (oral)	Apr 2005
IAB interview	Mar 2003
3) In-Depth Studies	**date**
EPS courses or other PhD courses	
Summer school Environmental signaling: Arabidopsis as a model	Aug 2001
PhD course Bioinformatics-1	Apr 2001
PhD course Molecular phylogenies: reconstruction and interpretation	Nov 2003
Journal club	
Member of a literature discussion group at Virology	2000-2003
Individual research training	
Host range of ToCMoV inoculated by particle bombardment - Embrapa-Cenargen-Brasilia, Brazil	Aug 2002
4) Personal development	**date**
Skill training courses	
Dutch Courses	2001-2002
TOTAL NUMBER OF CREDIT POINTS* 39,5	

* A credit represents a normative study load of 28 hours of study

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