New insights into geminivirus complexes from Vietnam and Thailand

Der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des akademischen Grades

Doktorin der Gartenbauwissenschaften
Dr. rer. hort.

genehmigte Dissertation von
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Hannover, 2008

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Tag der Promotion: 20.02.2008

Para o amorzinho da minha vida

„In short, more is not just more
it is sometimes ***other*** *than less“*
Daniel Gilbert

ABSTRACT

Begomoviruses are single-stranded circular DNA viruses, which replicate in the nucleus of their host cells and are classified in monopartite or bipartite viruses depending on their genome organization. *Tomato yellow leaf curl Thailand virus* –[Asian Institute of Technology] (TYLCTHV-[AIT]) is a bipartite begomovirus, which genome constitutes of two DNA molecules (components A and B).

In the first chapter of the present work, the relationship and interactions between begomoviruses occurring in Thailand and Vietnam are presented. Five DNA molecules were sequenced from tomato infecting begomoviruses complexes in Vietnam. Multimeric clones of the A components and also from a ß satellite were generated and used in infectivity studies and transreplication assays involving two different begomoviruses occurring in Thailand. The B component of TYLCTHV-[AIT] was transreplicated by different A components of viruses from Vietnam and Thailand in *Nicotiana benthamiana* and *Solanum lycopersicum*. Moreover, the TYLCTHV-[AIT] B component facilitated the mechanical transmission of monopartite viruses either by rub-inoculation as well by particle bombardment in *N. benthamiana* and tomato plants. Finally, defective DNAs ranging from 735 to 1457 nucleotides were generated in *N. benthamiana* from those combinations containing TYLCTHV-[AIT] B component.

The second part of this work consisted in generating transgenic *N. benthamiana* plants harboring six different inverted-repeat constructs of the TYLCTHV-[AIT] genome. Disease expression was monitored by calculating the disease index of each line. A total of 94 transgenic lines out of 114 generated putative lines were tested for virus resistance in the T_1 generation. Here we reported for the first time, recovery symptoms from *N. benthamiana* plants harboring an inverted-repeat construct of the AC2/C3 region of TYLCTHV-[AIT]. Moreover, delayed symptom expression of transgenic T_1 lines was obtained 30 days post-inoculation (dpi) for some lines as well as 90 dpi for one line harboring the IR/CP construct. Seeds from selected transgenic T_1 lines were employed in a T_2 resistance test. Three selected T_2 lines (BC4_1, BC8_8 and BC8_12) harboring partial sequences of the intergenic region and the gene coding for the movement protein of the DNA B showed resistance response with a delayed symptom expression obtained 29 dpi.

Finally, we were interested to know if TYLCTHV-[AIT] can be engineered to function as an expression and VIGS vector system. To this extent, the AV1 gene of TYLCTHV-[AIT] was replaced by the gene coding for the green fluorescent protein

(*gfp*) tagged or not with the hemagglutinin epitope (HA) at its C-terminus or by the entire sequence coding for the coat protein (CP) of *Vicia cryptic virus* (VCV). Confocal laser scanning microscopy (CLSM) and western blots were used to monitor the expressed proteins. The integration of the 1464 bp coding sequence of the entire VCV CP led to deletion mutants in the DNA A, while expressing GFP and/or GFP-HA did not interfere with the TYLCTHV-[AIT] replication process. Therefore, only a part of the VCV CP tagged with the HA sequence (ΔCP_VCV_HA) was used to replace the AV1 gene of TYLCTHV-[AIT]. Both GFP_HA and ΔCP_VCV_HA were detected in locally agroinoculated leaves at 15 dpi using anti-HA antibodies in immunoblot assays. Moreover, the multimeric clone expressing GFP from the TYLCTHV-[AIT] A component alone or in combination with its B component was used to agroinoculate transgenic *N. benthamiana* 16c plants. The progression of *gfp* silencing signs was monitored by CLSM and under UV light at 5, 14, 19 and 35 dpi. Systemic silencing was observed at 14 dpi in 30 out of 30 inoculated 16c plants harboring the A multimeric clone together with the B component of TYLCTHV-[AIT], while only two transgenic 16c plants out of 30 plants harboring solely the A multimeric clone showed first silencing signs at 35 dpi. In addition, geminivirus symptoms were observed in five out of 30 transgenic 16c plants at 19 dpi in both combinations, suggesting that probably already a small amount of viral DNA molecules were able to generate symptoms. Taken together, TYLCTHV-[AIT] could be useful either as an expression system as well as a silencing vector.

Keywords: *Tomato yellow leaf curl Thailand virus*, transgenic plants, virus-induced gene silencing

ZUSAMMENFASSUNG

Begomoviren sind zirkuläre Einzelstrang-DNA-Viren, die sich im Kern ihrer Wirtszellen reproduzieren. Abhängig von ihrer Genomorganisation (ein- oder zweiteilig) werden sie in mono- oder bipartite Viren eingeteilt. Das *Tomato yellow leaf curl Thailand Virus* - [Asian Institute of Technology] ist ein bipartites Begomovirus, dessen Genom aus zwei DNA Molekülen besteht (Komponente A und B).

Im ersten Kapitel dieser Arbeit werden die Verwandtschaft und Wechselwirkung zwischen Begomoviren untersucht, die in Thailand und Vietnam vorkommen. Fünf DNA-Moleküle von Begomoviruskomplexen, die in Vietnam Tomaten infizieren, wurden sequenziert. Multimere Klone der A Komponenten und ebenfalls eines ß Satelliten wurden konstruiert und für Infektionsstudien und Transreplikationsuntersuchungen mit zwei unterschiedlichen Begomoviren aus Thailand verwendet. Die B Komponente von TYLCTHV-[AIT] wurde durch unterschiedliche A Komponenten von Viren aus Vietnam und Thailand in *Nicotiana benthamiana* und *Solanum lycopersicum* transrepliziert. Überdies vermittelt die TYLCTHV-[AIT] B Komponente die mechanische Übertragung von monopartiten Viren auf *N. benthamiana* und Tomatenpflanzen sowohl durch Inokulation als auch durch Partikelbeschuss. In Viruskombinationen, welche die TYLCTHV-[AIT] B Komponente enthielten, wurden in *N. benthamiana* defekte DNA Moleküle mit Längen zwischen 735 und 1457 Nukleotiden erzeugt.

Im zweiten Teil dieser Arbeit wurden transgene *N. benthamiana* Pflanzen mit sechs unterschiedlichen Konstrukten, abgeleitet aus dem TYLCTHV-[AIT] Genom, erzeugt. Die Virusinfektion wurde mit Hilfe eines Krankheitsindexes bewertet. Aus 114 mutmaßliche transgenen Linien wurden insgesamt 94 Linien auf Virenresistenz in der T_1 Generation getestet. Erstmals konnte gezeigt werden, dass sich Pflanzen von einer Infektion wieder erholten (Recovery-Effekt), die mit einem Konstrukt aus invertiert wiederholten Abschnitten der AC2/C3 Region von TYLCTHV-[AIT] transformiert worden waren. Für andere transgene Linien traten in der T_1 Generation Krankheitssymptome erst verzögert 30 Tage nach Inokulation (TnI) auf und im Falle einer Linie, transformiert mit dem IR/CP Konstrukt, sogar erst nach 90 TnI. Die Samen ausgewählter transgener Linien in der T_1 Generation wurden verwendet für Resistenztests in der T_2 Generation. Drei ausgewählte T_2 Linien (BC4_1, BC8_8 und BC8_12), transformiert mit Sequenzabschnitten der intergenen Region und des Genes, welches für die virale Ausbreitung in der Pflanze verantwortlich ist, zeigten

Resistenzreaktionen mit verzögerten Symptome bis zu 29 TnI.

Im letzten Teil der Arbeit wurde untersucht, ob TYLCTHV-[AIT] als Expressionsvektor fremder Gene und als VIGS (virus induced gene silencing) System benutzt werden kann. Zu diesem Zweck wurde das AV1 Gen von TYLCTHV-[AIT] ersetzt durch das Gen für das Green Fluorescent Protein (*gfp*) sowohl mit als auch ohne Hemagglutin (HA) Tag bzw. durch die Sequenz des Hüll-Proteins (CP) von *Vicia cryptic virus* (VCV). Die GFP-Expression wurde mittels konfokaler Laser-Scanning-Mikroskopie (CLSM) und Western-Blot-Analyse kontrolliert. Die Integration der 1464 bp langen Sequenz, welche das gesamte Hüll-Protein von VCV kodiert, führte zu Deletionsmutanten in der DNA A Komponente, während die GFP- oder GFP-HA-Expression den Replikationsprozess von TYLCTHV-[AIT] nicht beeinflusste. Daher wurde nur eine Teilsequenz des VCV CP mit HA Tag (ΔCP_VCV_HA) verwendet, um das AV1 Gen von TYLCTHV-[AIT] zu ersetzen. Beide Proteine, GFP_HA und ΔCP_VCV_HA, konnten 15 TnI in lokal inokulierten Blättern mit Hilfe von anti-HA Antikörpern im Immunoblot nachgewiesen werden. Außerdem wurde der multimere GFP-Klon der TYLCTHV-[AIT] A Komponente allein oder in Kombination mit der B Komponente auf transgene *N. benthamiana* 16c Pflanzen agroinokuliert. Der Verlauf des *gfp*-Silencing wurde mit Hilfe von CLSM und unter UV Licht 5, 14, 19 und 35 TnI kontrolliert. Für transgene 16c Pflanzen, die sowohl mit den multimeren GFP-Klon als auch mit der B-Komponente von TYLCTHV-[AIT] inokuliert worden waren, konnte systemisches Silencing bereits 14 TnI in allen 30 inokulierten Pflanzen beobachtet werden, während in Abwesenheit der B Komponente nur zwei von insgesamt 30 inokulierten Pflanzen 35 TnI ein erstes Silencing aufwiesen. Darüber hinaus wurden Symptome einer Geminiviren Infektion in fünf von 30 16c Pflanzen nach 19 TnI beobachtet, sowohl in An- als auch in Abwesenheit der TYLCTHV-[AIT] B Komponente. Dies legt nahe, dass wahrscheinlich bereits eine kleine Menge viraler DNA Moleküle ausreicht, um Krankheitssymptome zu erzeugen. Zusammenfassend konnte gezeigt werden, dass TYLCTHV-[AIT] sowohl als Expressionsvektor fremder Gene als auch als VIGS System verwendet werden kann.

Keywords: *Tomato yellow leaf curl Thailand virus*, Transgene Pflanzen, virus-induced gene silencing

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ABBREVIATIONS

Δ	Delta: representing the symbol for deletion
%	Percentage
°C	Degree Celsius
ß	Beta
ßC1	DNA ß transcript, ßC1 gene
µl	Microliter, microliters
µg	Microgram
µM	Micromolar
aa	Amino acid(s)
AC1/C1	Replication protein
AC2/C2/TrAP/AL2	Transcription transactivator protein
AC3/C3/AL3/REn	Replication enhancer protein
AC4/C4	Begomovirus protein
ADK	Adenosine kinase
ago1-27	Argonaute mutant
AIT	Asian Institute of technology
ATPase	Adenosine triphosphatase
AtSKeta	*Arabidopsis thaliana* shaggy-related protein kinase
AV1/V1	Coat protein
AV2/V2/V1	"Precoat" found in Old World viruses
BC1/BL1	Movement protein
bp	Base pairs
BV1/BR1	Nuclear shuttle protein
CaMV	Cauliflower mosaic virus
ccc	Covalently closed circular
CLSM	Confocal laser scanning microscopy
CP	Coat protein
CR	Common region
C-terminus / termini	Carboxyl terminus / termini
ddm1/som8	Deficient in DNA methylation 1
Def-DNA(s)	Defective-DNA(s)
DI	Disease index
dpi	Days post inoculation
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dsRNA(s)	Double-stranded RNA(s)
DX1, DX2	Dang Xa1, Dang Xa2
E2F	Transcription factor E2F

e35S	Enhanced 35S promoter
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay(s)
ER	Endoplasmic reticulum
g	Gram
GFP	Green fluorescent protein
GUS	β-Glucuronidase
h	Hours
HA tag	Hemagglutinin tag
hpRNA	Hairpin RNA
ICTV	International Committee on Taxonomy of Viruses
IR	Intergenic Region
IRD	Iteron-related domain
kb	Kilobases / kilobase pairs
Kg/ha	Kilogram per hectare
L	Liter
LB	Left border
LB-media	Luria Broth-media
M	Marker
MES	2-(N-morpholino)ethanesulfonic acid
min	Minute(s)
miRNA	Micro RNA
ml	Milliliter / milliliters
mM	Millimolar
mom1	Morpheus' molecule 1 mutant
MP	Movement protein
mRNA	Messenger RNA
MS-medium	Murashige and Skoog-medium
Na-DIECA	Natrium-Diethyldithiocarbamic acid
NES	Nuclear export signal
NLS	Nuclear localization signal
nm	Nanometers
nptII	Neomycin phosphotransferase II
NSP	Nuclear shuttle protein
nt	Nucleotides
N-terminal / terminus	Amino-terminal / terminus
NTP	Nucleoside triphosphate
OC	Open circular
OD_{600}	Optical density at a specific wavelength (600 nm)
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
poly-A signal	Polyadenylation signal

pRBR	Retinoblastoma-related protein
PTGS	Posttranscriptional gene silencing
RB	Right border
RC	Rolling circle
RCA	Rolling circle amplification
RCR	Rolling circle replication
RDR	Recombination-dependent replication
RdRp	RNA-dependent RNA polymerase
Rep	Replication protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotations per minute
SCR	Satellite-conserved region
SEL	Size-exclusion limit
sgs1, sgs2, sgs3	Suppressor of gene silencing mutants
SINAC1	*Solanum lycopersicum* NAC1
siRNA(s)	Small interfering RNA(s)
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
su	Sulfur allele
T	Triangulation numbers
T_0	First regenerated plants obtained from plant transformation
T_1	Progeny of T_0
T_2	Progeny of T_1
TAS-ELISA	Triple-antibody sandwich-enzyme-linked immunosorbent assay
TGS	Transcriptional gene silencing
U-test	Mann-Whitney test
UV	Ultraviolet
VIGS	Virus-induced gene silencing
VRQ, VR2	Vien Rau Qua, Vien Rau Qua2
wpi	Weeks post-inoculation

1.0 General Introduction and Objectives

1.1 The genus *Begomovirus*

The family *Geminiviridae* is constituted of four genera: *Begomovirus*, *Mastrevirus*, *Curtovirus* and *Topocuvirus*. Begomoviruses belong to the circular single-stranded DNA viruses, which replicate in the host cell nucleus. The genus *Begomovirus* comprises more than 200 known sequences deposited in the GenBank and has a total of 131 species of the 148 officially recognized within the family *Geminiviridae*. Due to the growing number of sequences, the new species criteria demarcation is based on the identity of 89% threshold of the complete DNA A component nucleotide sequence (Fauquet and Stanley, 2005). Geminiviruses have a geminate shape (bisegmented), consisting of two incomplete icosahedra (T=1), no envelope and the virion has a diameter of approximately 20 nm and a length of 30 nm with a groove running around the middle axis at 90° (Harrison and Robinson, 1999). The 110 monomers of the coat protein (CP) are arranged in 22 pentameric capsomers constituting the geminate particle morphology (Francki et al., 1979).

The history of geminiviruses begins in the middle-end of the seventies, where first reports showed the existence of virus particles with a very characteristic geminate morphology (Figure 1). *Maize streak virus* (MSV) and *Beet curly top virus* (BCTV) were the first to be isolated showing the quasi-isometric twinned particles morphology of the virion (Bock et al., 1974; Mumford, 1974). Therefore, the word geminivirus originates from 'Gemini', which means twins. In 1976, Gálvez and Castaño were the first to identify the paired particles for the begomovirus *Bean golden mosaic virus* (BGMV). In 1977 the name geminiviruses were first used by Harrison BD et al. and recognized by the ICTV in 1979 (Mathews REF). Few years later, beginning of the 80's, first insights were given on the nucleic acid contained in those paired particles, which consist of one or two circular single-stranded DNA components. Also, the infectivity of such molecules could be demonstrated. Haber et al. (1981) showed the presence of only one DNA molecule of BGMV in each paired particle as well as the evidence for requiring both DNA molecules for infectivity. At the same time Bisaro et al. and Hamilton et al. (1982) reported the cloning and identification of the two components of the *Tomato golden mosaic virus* (TGMV) genome.

Begomoviruses are the only one within the family *Geminiviridae* to be transmitted by whiteflies. The vector is *Bemisia tabaci* occurring in the tropic and subtropic regions causing many economically important diseases on dicotyledonous plants specially solanaceous species such as tomato, pepper and potato plants, legumes and cassava.

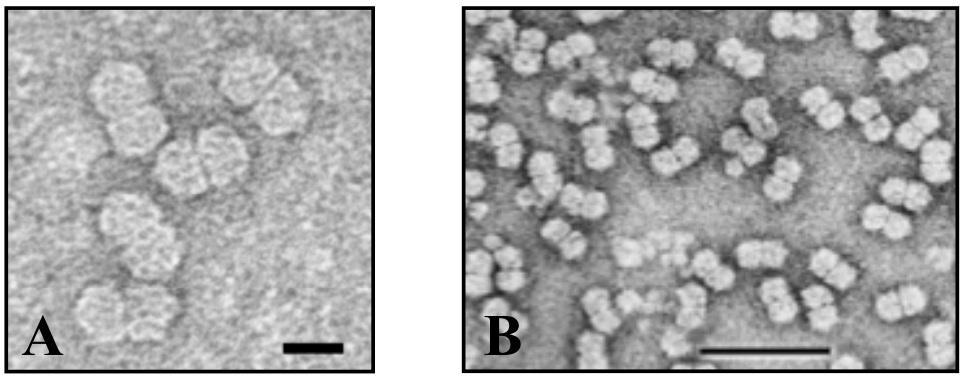


Figure 1. Purified *African cassava mosaic virus* (A) negatively stained with uranyl acetate and (B) *Tomato yellow leaf curl virus*. Bars at the right side indicate (A) 20 nm and (B) 100 nm. (Pictures taken from Tidona and Darai, 2001 and Gafni, 2003)

1.2 Genome organization of Begomoviruses from the Old World

Begomoviruses may be classified according to their genome organization in bipartite or monopartite viruses. Bipartite viruses are composed of two circular single-stranded DNA molecules, so-called A and B, while monopartite viruses are composed only of one DNA A molecule, which may become associated to half-sized molecules so called ß DNA and/or DNA-1 (Figure 2). The genome varies in size from 2500 to 2800 bp. Although most of the sequences placed in GenBank are derived from the A component, many begomoviruses have two components. This demonstrates the difficulties in cloning the less conserved B components. Therefore, it has been shown that it is much easier to amplify and recognize sequences from the A component, either by using degenerative primers, such as Homer and Krusty primers designed to amplify 580 nt of the begomovirus coat protein (Revill et al., 2003), or by using hybridization approaches, respectively.

The A component of bipartite viruses comprises four open reading frames (ORFs), coding for the coat protein (AV1), the replication-associated protein (Rep), the transcription transactivator protein (AC2) and finally the replication enhancer protein

(AC3). Moreover, two additional ORFs may be found associated to some begomoviruses, the AV2/V1/V2 and AC4/C4, named depending on the virus. The AV2/V2 gene is found only in bipartite and monopartite begomoviruses from the Old World and is known to participate in the process of movement and cell-cycle control (Briddon and Stanley, 2006) while AC4 has been reported to be involved in the silencing suppression pathway of bipartite viruses. Further functions of proteins coded by the sense and antisense viral strand on the A component will be described at chapters 1.3 and 1.4 respectively.

The B component has two ORFs, one coding for the nuclear shuttle protein (NSP), BV1 protein, and the other for the movement protein (MP), the BC1 protein. Functions of proteins coded by the sense and antisense viral strand on the B component will be described at chapter 1.5. Component A and B share a common region (CR) of around 200 bp, which is very conserved and contains motifs (CA, TATA and G-box) involved in the virus replication process. In addition, the CR has a putative stem-loop structure containing the nonanucleotide TAATATTAC, which is highly conserved and functions in the initiation of the rolling circle replication.

Finally, bipartite begomoviruses may be associated with defective molecules originated from the A and/or B components (Mansoor et al., 2003; Figure 2), which have been connected with delay and attenuation of symptoms (Stanley et al., 1990; Frischmuth and Stanley, 1991, 1994; Ndunguru et al., 2006). Patil et al. (2007) reported the formation of defective DNAs (def-DNAs) when cloned DNA A and B components of Indian cassava-infecting geminivirus were coinoculated into the experimental host *N. benthamiana*. All cloned def-DNAs contained the CR harboring all *cis*-elements necessary for replication and partly regions of the DNA A and/or B molecules.

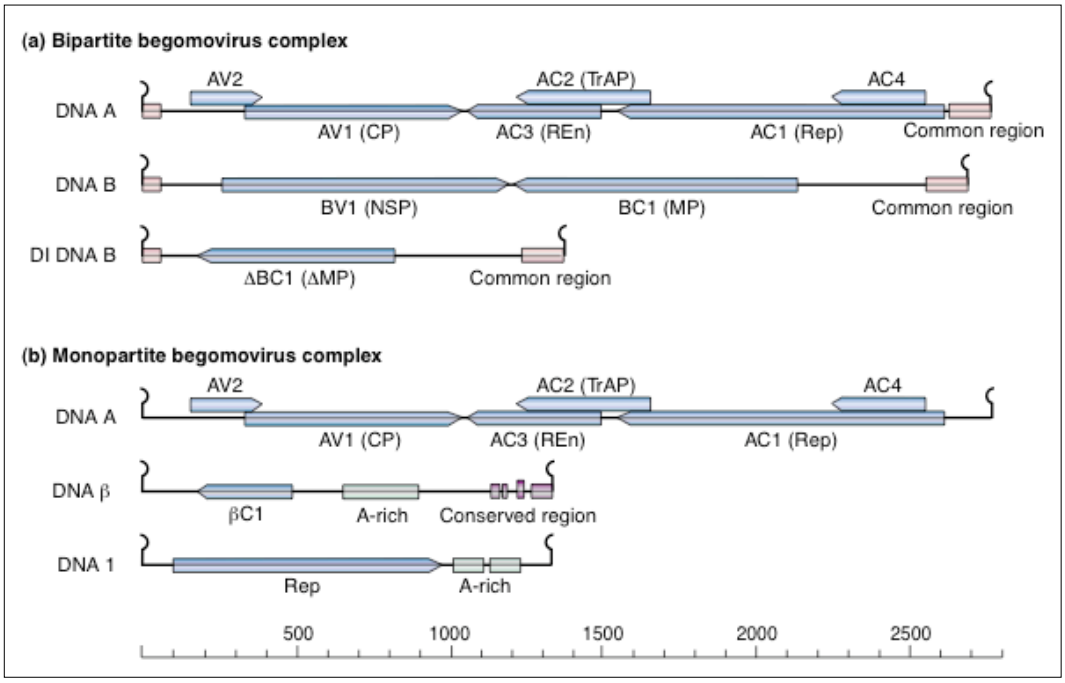


Figure 2. Genome organization of bipartite (a) and monopartite (b) begomoviruses occurring in the Old World. (a) Bipartite begomoviruses may be associated to def-DNAs. These components are half-sized molecules; normally retaining the entire CR. (b) Monopartite viruses may be associated to beta satellites and/or DNA-1 components. Beta satellites depend on its helper virus for replication and encapsidation, may influence the host range infectivity, while DNA-1 are autonomously replicating resembling nanoviruses. (Picture taken from Mansoor et al., 2003).

1.3 Function of proteins coded by the sense viral strand on the A component

The first gene of the sense viral strand is the AV1 or V1 gene, named depending on the geminivirus. It is translated into the coat protein (CP). The CP of geminiviruses is the only known structural component (Lazarowitz, 1992). The CP of the monopartite *Tomato yellow leaf curl virus* (TYLCV) is essential for virus infection and insect transmission. Noris et al. (1998) studied the transmissibility of a constructed *Tomato yellow leaf curl Sardinia virus* (TYLCSV) containing different amino acid sequences in the CP. A combination of eight possible amino acid variations at position 129, 134 and 152 of the TYLCSV CP affected its infectivity and transmissibility. Sequences of the CP, which have been shown to be important for virus transmission, are mapped to the central part of the protein (Höhnle et al., 2001; Kheyr-Pour et al., 2000).

While the CP of monopartite geminiviruses is required for virus spread and symptom development (Boulton et al., 1989; Lazarowitz et al., 1989; Liu et al., 1999), the CP of

bipartite viruses is not (Gardiner et al., 1988; Padidam et al., 1995; Pooma et al., 1996). However, mutations in the CP of bipartite viruses do influence whitefly transmissibility. Azzam et al. (1994) studied the whitefly transmissibility of the BGMV by mutating its CP. The mutated BGMV produced systemic infection of *Phaseolus vulgaris* but could not be sap transmitted. In addition, reduced levels of viral ssDNA but not of dsDNA were observed in plants or protoplasts containing the CP mutant. Further confirmation that the CP of bipartite viruses influences virus transmission was given by Höhnle et al. (2001). They exchanged the CP of an *Abutilon mosaic virus* (AbMV) isolate (not whitefly transmissible) by the CP of *Sida golden mosaic virus* (SiGMV-[Hoyv]), a whitefly transmitted virus. Pseudorecombinants were obtained and transmitted only in those combinations containing SiGMV-[Hoyv] CP. Moreover, Höhnle et al. showed that an exchange of two amino acids of the AbMV CP at positions 124 and 149 were sufficient to reestablish whitefly transmission.

Finally, the CP of geminiviruses showed to be multifunctional by also being involved in the viral transport. The CP of the monopartite TYLCV is localized to the nucleus and nucleolus and functions as a nuclear shuttle protein, playing a role in import and export of the viral DNA (Rojas et al., 2001). Sequences, which may be related to nuclear localization and nuclear export signals (NLS and NES), have been identified in the CP of mono- and bipartite geminiviruses (Unseld et al., 2001; 2004). Moreover, geminivirus CP binds to ssDNA and dsDNA *in vitro* in a sequence nonspecific manner (Liu et al., 1997; Padidam et al., 1996; Palanichelvam et al., 1998).

The second gene of the viral sense strand of begomoviruses is the AV2, V2 or V1 gene named according to the virus. It is known as the ‘precoat protein’. AV2/V2/V1 protein is present only in viruses from the Old World. Padidam et al. (1996) gave first evidence that the AV2 protein is involved in viral movement by using protoplast inoculation. Deletions in AV2 influenced the symptom pattern and accumulation of both single-stranded and double-stranded viral DNA. Further evidences were given by Rojas and coworkers in 2001. Rojas et al. (2001) investigated the functional properties of the TYLCV V1 protein. The V1 protein is localized around the nucleus and at the cell periphery. They proposed a model for TYLCV movement where V1 is involved by enhancing the nuclear export of viral DNA at the nuclear periphery and then by helping the delivery of DNA from the nuclear periphery to the cell periphery. Rojas and coworkers speculated that possible interactions with the endoplasmic reticulum (ER) are

involved in the viral DNA transport process. Finally, very recently Zrachya et al. (2007b) identified the V2 protein of *Tomato yellow leaf curl Israel virus* (TYLCV-[IL]) as a suppressor of gene silencing. Silencing assays showed that V2 inhibited RNA silencing of the reporter green fluorescent protein (GFP). The amount of GFP transcript and protein levels increased but not the amount of GFP-specific short interfering RNAs, suggesting that V2 is involved in suppression of the RNA silencing pathway probably subsequent to the Dicer-mediated cleavage of dsRNA. Moreover, TYLCV-[IL] V2 was localized in protoplasts and tissues and showed to be associated with cytoplasmic strands and inclusion bodies in the cortical regions of the cell and not in the nucleus as shown for phosphorylated forms of AC2 silencing suppressor.

1.4 Functions of proteins coded by the complementary viral strand on the A component

Four genes are located on the viral complementary strand of the begomovirus genome (AC1/C1, AC4/C4, AC2/C2 and AC3/C3), named depending on the geminivirus. The AC1/C1 gene codes for the replication associated protein (Rep), which has been shown to be multifunctional. As long known, the N-terminal domain (aa 1-120) of the replication protein of the geminiviruses is involved in the initiation of the rolling circle DNA replication (Laufs et al., 1995a; Koonin and Ilyina, 1992), which uses double-stranded DNA intermediates as templates (Rogers et al., 1986; Saunders et al., 1991). The Rep protein cleaves and ligates single-stranded DNA. Laufs et al. (1995b) used the Rep protein of the TYLCV for *in vitro* and *in vivo* analysis. They identified the amino acid of Rep (tyrosine-103), which mediates cleavage and also ligation of the viral DNA. Further proof was provided by Orozco and Hanley-Bowdoin (1998), who studied the binding and cleavage domains of the TGMV by generating a series of site-directed mutations. Further details concerning the rolling-circle amplification are given in Chapter 1.7.

Additional functions have been associated to the Rep protein. In 1995, Desbiez et al. showed that purified Rep protein of the TYLCV expressed in *Escherichia coli* has an ATPase activity *in vitro*. The NTP binding consensus motif (P-loop) was mutated resulting in a decrease or loss of the ATPase activity (ATPase domain aa 181-130). In addition, Pant et al. (2001) demonstrated that nicking activity of Rep is not only

modulated by ATP in *Mungbean yellow mosaic virus* (MYMV) but also Rep can act as a site-specific type-I topoisomerase. Moreover, based on the fact that the Rep protein has a NTP binding motif, it has been suggested that it also may have a helicase activity (Choudhury et al., 2006; Gorbalenya et al., 1990), which may depend on the oligomeric state of the protein (Clérot and Bernardi, 2006). Rep interacts with itself due to the oligomization domain (aa 121 to 180) (Orozco et al., 1997; Orozco et al., 2000), with the AC3 protein (Settlage et al., 1996) and with the retinoblastoma-related protein (pRBR) (Ach et al., 1997; Castillo et al., 2003). Argüello-Astorga et al. (2004) showed that the conserved leucine residue located in helix 4 of TGMV Rep and helix 4 itself are part of the pRBR-binding interface. Rep binds to the host factor pRBR and this interaction influences the expression pattern of proliferating cell nuclear antigen (PCNA) accumulation during cell infection. The pRBR interacts with the transcription factor (E2F), playing a central role in regulating cell growth and death. Therefore, Rep induces the expression of the host DNA synthesis protein, the PCNA, acting as a transcriptional regulator to repress its own synthesis and activates host gene expression. Finally, it has been suggested that probably the Rep geminivirus protein binds to other host proteins and transcription factors playing an important role in virus replication and host interaction (Egelkrout et al., 2001).

The second protein is encoded by AC4/C4, which is located entirely within the gene coding for the Rep protein but in a different frame. In monopartite geminiviruses the C4 protein has been reported to be involved in pathogenesis. Mutations in the C4 coding sequence of the curtovirus BCTV resulted in loss of disease severity suggesting its role as a pathogenesis determinant in the host plants (Stanley et al., 1992) and also its role in induction of cell proliferation (Latham et al., 1997). Also for monopartite begomoviruses C4 protein has been related to pathogenesis and shown to be a multifunctional protein. Disruption of the C4 ORF in monopartite begomoviruses such as *Tomato leaf curl virus* (ToLCV) and TYLCV showed plants with attenuated symptoms and a lower infectivity of the virus (Jupin et al., 1994; Rigden et al., 1994) as found for curtoviruses. However, the disruption of ORF AC4 in bipartite begomoviruses had no effect on the infectivity and symptom development in *African cassava mosaic virus* (ACMV) (Etessami et al., 1991), in BGMV (Hoogstraten et al., 1996), in TGMV (Pooma and Petty, 1996) and *Potato yellow mosaic virus* (PYMV) (Sung and Coutts, 1995a).

However, also *African cassava mosaic virus* (ACMV-[CM]) and *East African cassava mosaic Cameroon virus* (EACMCV) AC4 proteins have been shown to suppress gene silencing in transient assays. ACMV-[CM] AC4 driven by the 35S promoter increased the accumulation of EACMCV DNA in protoplasts by 8 times (Vanitharani et al., 2004). In 2005 Chellappan et al. demonstrated the ability of AC4 of ACMV-[CM] to bind single-stranded forms of micro RNAs (miRNAs) and short interfering RNAs (siRNAs). Fondong et al. (2007) showed that the AC4 protein of the *East African cassava mosaic virus* (EACMV) is a major determinant in pathogenicity and it is also involved in silencing suppression. Fondong and coworkers suggested that posttranslational N-myristoylation of the AC4 protein might play a role in EACMV pathogenicity. On the other hand, C4 of *Tomato leaf curl Java virus* (ToLCJV) was unable to suppress posttranscriptional gene silencing induced by GFP in *N. benthamiana* 16c transgenic line (Kon et al., 2007).

Beside acting as silencing suppressor for some begomoviruses, the AC4/C4 protein has been reported to be involved in TYLCV movement (Jupin et al., 1994) and also even to counter the mechanism of plant defense together with Rep in ACMV and *Tomato yellow leaf curl China virus* (TYLCCNV). The expression of AC4 or C4 in plants, which were expressing the Rep protein, resulted in severe necrosis and cell death (Van Wezel et al., 2002). Finally, the AC4/C4 protein of TGMV and BCTV has been shown to interact with *Arabidopsis thaliana* shaggy-related protein kinase (AtSKeta) either using *in vitro* assays or in a yeast two-hybrid system (Piroux et al., 2007). Piroux et al. suggested that AC4-AtSKeta interaction influence the brassinosteroid signalling pathway.

The third protein coded by begomoviruses is known as TrAP, AL2, AC2 or C2 depending on the virus. AL2 is commonly used with New World viruses such TGMV, while AC2 and C2 with Old World bipartite and monopartite viruses, respectively. In the following, AC2 ORF or protein will be used instead of AL2 or TrAP.

Sunter and Bisaro (1991) gave first insight that the AC2 gene functions as a transcriptional activator protein. They replaced the coat protein of the TGMV by the reporter gene (*gus*) containing a mutated ORF of the AC2 sequence. No GUS expression was observed in the tobacco protoplasts containing the mutated AC2 ORF, suggesting its role as an activator of transcription. Moreover, the AC2 protein of the

TGMV has been shown to be a transcriptional activator protein, necessary for efficient transcription of the coat protein (AV1) and the movement protein (BC1) (Gröning et al., 1994; Sunter and Bisaro, 1991; 1992). Later reports have shown that also AC2 protein of ACMV and PYMV activates the expression of CP, BC1 and BV1 genes (Haley et al., 1992; Sung and Coutts, 1995a).

AC2 is capable of binding zinc ions, which is required for interaction with ssDNA, and has a C-terminal acid domain and a N-terminal basic domain, which has been related to the transcriptional activation domain in TGMV. AC2 activation domain promotes transcription by binding and recruiting factors of the transcriptional machinery, such as transcriptional factors, TATA binding-protein associated factors, to the promoter (Hartitz et al., 1999). In addition, the homolog C2 (aa 31-104) protein of the monopartite TYLCV binds preferentially to single-stranded DNA in a sequence-nonspecific manner than to double-stranded DNA (Noris et al., 1996b).

AC2 protein can restore infectivity in mixed infections, meaning that the protein is not virus specific and allows complementation (Saunders and Stanley 1995; Sung and Coutts, 1995b). Saunders and Stanley (1995) demonstrated that dysfunctional AC2 was complemented by AC2 of TGMV homologue. In addition, AC2 is required for coat protein accumulation (Sunter et al., 1990). The C2 protein of ToLCV has been involved in transactivation of the virion-sense gene expression (Dry et al., 2000). It has been suggested that C2 protein plays a role in the systemic spread of the monopartite TYLCV in a host-specific manner (Wartig et al., 1997). Van Wezel et al. (2001) demonstrated that TYLCCNV C2 protein contributes to viral pathogenicity and is nuclear localized. They expressed in *N. benthamiana* the fused C2-GFP using the *Potato virus X* (PVX) expression system. Plants inoculated with C2-GFP showed necrotic ringspots on inoculated leaves and necrotic vein banding and severe necrosis on systemically infected leaves. Therefore, Van Wezel and coworkers speculated the involvement of TYLCCNV C2 protein in silencing suppression. Finally, C2 protein of TYLCCNV has been reported to possess a functional arginine-rich nuclear localization signal and to act as a suppressor of posttranscriptional gene silencing (PTGS) (Dong et al., 2003). However, phosphorylation of TGMV AC2 seems to influence its localization. Wang et al. (2003) demonstrated that non-phosphorylated AC2 is located in both cytoplasm and nucleus of insect cells, while phosphorylated forms accumulate in the nucleus.

Finally, suppression of gene silencing activity has been reported for AC2 of TGMV, BCTV, MYMV, ToLCJV, EACMV and *Indian cassava mosaic virus* (ICMV) (Kon et al., 2007; Trinks et al., 2005; Vanitharani et al., 2004; Wang et al., 2005). Trinks et al. (2005) demonstrated the requirement of the nuclear localization signal (NLS) and an intact zing finger (transcriptional activation domain) for PTGS using the C2 of MYMV. On the other hand, at the same time in 2005, Wang et al. reported the ability of AC2/C2 of TGMV (although lacking the transcriptional activation) and of BCTV to suppress PTGS. Wang and coworkers found the ability of AC2 to interact with and inactivate adenosine kinase (ADK), which play an important role supporting the methyl cycle and S-adenosylmethionine-dependent methyltransferase activity. Therefore, evidence that AC2 can suppress silencing in a transcriptional-independent manner was for the first time put forward.

The fourth protein coded from the complementary strand is the replication enhancer protein known as REn, AC3, C3 or AL3, named depending on the virus. In the following, AL3 and Ren will be used as AC3 or C3. Disruption of AC3 TGMV protein causes delay and attenuation of symptoms in transgenic *N. benthamiana* plants carrying the integrated tandem copies of the TGMV B component (Elmer et al., 1988). Amelioration and reduction of virus symptoms caused by mutated AC3/C3 has been also reported for ACMV (Morris et al., 1991), PYMV (Sung and Coutts, 1995a) and BCTV (Stanley et al., 1992). In addition, reduced levels of single-stranded and double-stranded DNA were observed from TGMV AC3 mutants (Sunter et al., 1990). AC3/C3 has a nuclear localization signal (Pedersen and Hanley-Bowdoin, 1994; Nagar et al., 1995), is able to interact with Rep (Settlage et al., 1996) and can form homo-oligomers (Settlage et al., 2005). Moreover, Castillo et al. (2003) showed the ability in case of C3 of TYLCSV to interact with tomato PCNA using the two-hybrid system and a pull-down assay. The binding domain was located at amino acids 132 to 187. PCNA keeps the polymerase associated with DNA during replication. Therefore, Castillo and coworkers suggested that PCNA, Rep and C3 interaction promotes the machinery necessary to replicate the viral DNA by facilitating the recruitment of the host replication machinery for viral replication or by avoiding PCNA to interact with other cell-cycle regulators. In 2005, Settlage et al. examined the impact of mutated C3 protein of TYLCV on the interaction with itself, C1, PCNA and pRBR. TYLCV C3 consists of 134 amino acids containing three hydrophobic clusters. Hydrophobic residues located in the middle of the C3 protein were important for oligomerization (aa 28 to 95) and for

binding of C1 (aa 28 to 128) and PCNA (aa 7 to 95), while polar residues at both N (aa 1 to 13) and C (aa 125 to 134) termini were important for C3-pRBR interaction. They suggested that C3-pRBR interaction is not a prerequisite for viral replication in cycling cells but may be important during infection of differentiated cells. Finally, TYLCV C3 is able to induce SINAC1 expression and therefore substantial accumulation in viral DNA. SINAC1 is a new protein of the NAC family, a family of genes of the Arabidopsis genome involved in many diverse processes of development, senescence and embryo formation in the plant (Selth et al., 2005).

1.5 Functions of proteins coded by the B component

Two proteins are coded by the B component of bipartite viruses, the BC1 or BL1 and BV1 or BR1 proteins known to be involved in the viral movement process. The BC1/BL1 protein is the movement protein, while BV1/BR1 is the nuclear shuttle protein (NSP) in begomoviruses. The BC1 protein expression has been shown to be a major symptom determinant in transgenic tobacco plants expressing the BC1 gene of *Tomato mottle virus* (ToMoV), in tomato expressing the BC1 or BV1 of *Bean dwarf mosaic virus* (BDMV), in *N. benthamiana* expressing the BR1 and BL1 genes of *Squash leaf curl virus* (SqLCV) (Duan et al., 1997a; Hou et al., 2000; Ingham et al., 1995; Pascal et al., 1993). However, expression of BC1 protein was not a pathogenicity determinant in transgenic plants expressing the BC1 protein of *Tomato leaf curl New Delhi virus* (ToLCNDV). Only when expressing the BV1 protein of ToLCNDV in *N. benthamiana* plants the typical leaf curl symptom appeared (Hussain et al., 2005).

Two different models have been proposed for bipartite begomovirus movement. The first one begins with the transfer of viral dsDNA from the nucleus to the cytoplasm mediated by the BV1 protein. The NSP delivers the viral DNA to the BC1 protein, which guides crossing through the plasmodesmata (Gilbertson et al., 2003; Rojas et al. 2001). The second model states that an NSP-complex shuttles the bound-ssDNA from the nucleus to the cytoplasm, where the NSP-complex then interacts with the MP to move from cell to cell. The whole complex is transported from cell to cell in this case (Hehnle et al., 2004; Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995). Hehnle et al. (2004) gave first evidence for interaction between BV1, BC1 and CP by observing conspicuous structures via electron microscopy. They visualized the assembly of

dsDNA with BV1 and BC1 proteins supporting the latter proposed model. Moreover, additional evidences were recently given by Frischmuth et al. (2007). They monitored the interaction and cellular localization of BC1 and BV1 of AbMV *in situ* using electron microscopy. The expressed BV1 proteins in yeast showed to be accumulated within the nuclei, while the BC1 protein was targeted to the plasma membrane.

1.6 Beta satellites and DNA-1

Monopartite begomoviruses may be associated with half sized molecules termed as ß satellite and/or DNA-1. The first satellite described is the ToLCV-sat, which was isolated from tomato plants and comprises 682 nucleotides (Dry et al., 1997). Begomoviruses satellites depend on the helper begomovirus for its replication and encapsidation and have no or little sequence similarity to its helper virus. However, they also contain the conserved nonanucleotide TA(A/G)TATTAC motif, which is by analogy the site of recognition of the Rep protein.

While infectivity with ToLCV-sat is not required for ToLCV infectivity, the satellite found associated with *Ageratum yellow vein virus* (AYVVß) is required for symptomatic infection in Ageratum but not in *N. benthamiana* plants (Stanley, 2004; Stanley et al., 1997). In cotton leaf curl disease, also a half sized molecule (DNA-1), which encoded Rep and replicate autonomously but depends on the helper virus for encapsidation and movement has been identified (Briddon et al., 2004; Mansoor et al., 1999; Stanley, 2004). DNA-1 molecules are related to the nanovirus Rep component. DNA-1 probably originates from mixed infections of nanoviruses and begomoviruses and in order to be encapsidated an increase in genomic size has occurred. Since then, these small sized satellite molecules have gained more importance concerning the elucidation of complex begomoviruses diseases.

Basically, beta satellites can be classified in two major classes: Those occurring in the family *Malvaceae* and those occurring in other plant families (Briddon et al., 2003). However, there are some exceptions due to host range adaptation. Saunders et al. (2002) demonstrated that *Sri Lankan cassava mosaic virus* (SLCMV) A DNA can change its ability to infect another host by inoculating the A component together with the beta satellite of *Ageratum yellow vein virus* (AYVV). DNA ß has an A-rich region, a highly

conserved structure referred to as satellite-conserved region (SCR), which is located close to the putative stem loop TAATATTAC motif and contains the ßC1 gene. Saunders et al. (2004) mapped a DNA beta transcript (ßC1), which has been shown to be a major determinant in pathogenicity. In addition, the expression of ßC1 either in transgenic plants or by using expression vectors showed plants with severe symptoms (Cui et al., 2004; 2005a; Qazi et al., 2007). Alberter et al. (2005) identified intermediates of rolling circle replication (RCR) and recombination-dependent replication (RDR) of ToLCV and also from the DNA ß associated to *Cotton leaf curl Multan virus* (CLCuMV). Alberter and coworkers suggested that replication strategies of satellites depend on the helper virus. Moreover, ßC1 was shown to function as a silencing suppressor to bind ssDNA and dsDNA in a sequence nonspecific manner and to posses either a putative nuclear localization signal or nuclear export signal (Cui et al., 2005a, 2005b; Gopal et al., 2007; Kumar et al., 2006). Beta satellites share no significant homology to the helper virus and depend on them for replication, encapsidation and movement as well as transmission between plants (Briddon et al., 2003).

Last, very recently, Saeed et al. (2007) showed that cotton leaf curl satellite virus (CLCuVß) could substitute the B component of ToLCNDV to permit systemic infection. Saeed and coworkers demonstrated that inoculation of ToLCNDV A component alone produced a local but not a systemic infection. However, when ToLCNDV A component was coinfected with CLCuVß the systemic infection was reestablished. In addition, ßC1 fused to GFP was localized to the cell periphery in association with punctate bodies. They emphasized that ßC1 has a role in movement, by transporting DNA A from the nuclear site of replication to the plasmodesmatal sites.

1.7 Replication of begomoviruses

Geminiviruses replicate via RCR and via RDR (Jeske et al., 2001, Figure 3). In the RCR mechanism, the fist step consists of binding the Rep to the origin of replication. Rep nicks and binds covalently to the 5'-end of DNA following the release of Rep and formation of new viral ssDNA. RDR is based on homologous replication, which initiates replication. The RDR mechanism was first described for AbMV. First, the incomplete ssDNA interacts with covalently closed circular DNA at homologous sites.

Homologous recombination takes place generating the elongation of ssDNA molecules and the synthesis of the complementary strand originating new dsDNA molecules (Figure 3). Preiss and Jeske (2003) analyzed further geminiviruses such as ACMV, BCTV, TGMV and TYLCV concerning the alternative RDR route. They showed the production of RDR intermediates (mainly linear and closed circular dsDNA) upon agroinoculation on *N. benthamiana* plants.

Moreover, geminiviruses that are complemented to form double-stranded circular intermediates in nuclei can form minichromosomes in infected cells (Pilartz and Jeske, 1992; 2003). Pilartz and Jeske (2003) described AbMV minichromosomes, which have a nucleosome-free region in the intergenic region and a second minichromosomal region allowing viral DNA to interact with host factors. They found viral minichromosomes in at least two defined structures covered with 11 or 12 nucleosomes. Pilartz and Jeske suggested that the chromatin structure might play a role as major determinant in gene regulation of begomoviruses and therefore minichromosomes should have an important implication when considering the construction of expression vectors, for explaining size selection of viral DNA in the absence of the CP and finally for the optimization of defective vectors to induce resistance.

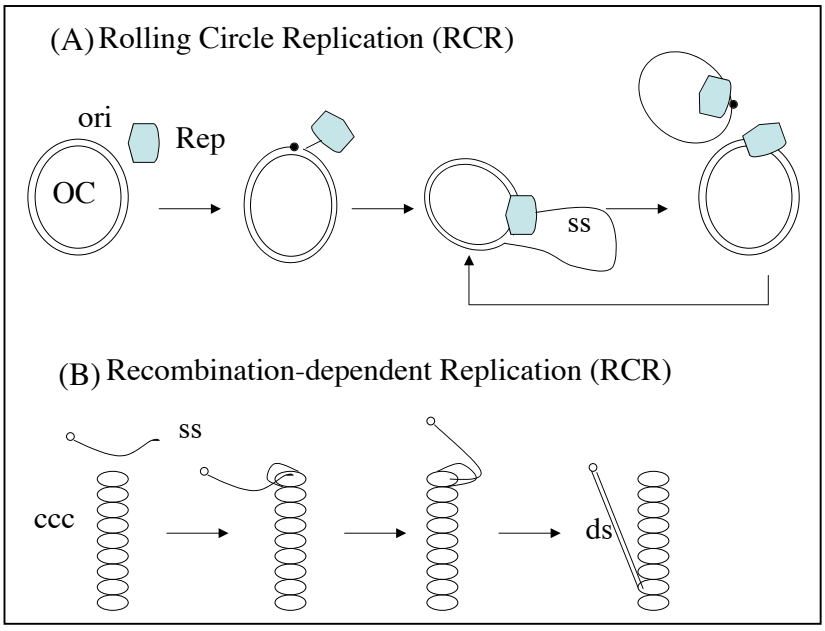


Figure 3. Geminiviruses replication. (A) Rolling circle replication and (B) Recombination-dependent replication. OC: open circular DNA; ss: single-stranded DNA; ds: double-stranded DNA. ccc: covalently closed circular. Modified from Jeske et al., 2001.

Transcription of geminiviruses occur bidirectionally and the origin of replication is located at the intergenic region (IR). As already mentioned Rep binds in a sequence-specific manner to motifs (iterons) located on the IR. Iterons are tandemly repeated motifs located at different distances from the conserved nonameric motif (TAATATTAC). Argüello-Astorga and Ruiz-Medrano (2001) proposed a model of potential Rep-iteron contacts. They identified the iteron-related domain (IRD), which is adjacent to a conserved motif (motif 1-FLTYP) characteristic of a superfamily of RCR initiator proteins. Argüello-Astorga and Ruiz-Medrano suggested that IRD and motif 1 might be components of the same Rep functional domain.

1.8 Pseudorecombination and defective molecules

Some begomoviruses have the ability to pseudorecombine by reassortment of genomic components from different begomoviruses. There have been two explanations for those begomoviruses where pseudorecombination failed. The first explanation is based on the inability of the A component to transreplicate the heterologous B component due to the tight specificity of the Rep proteins to its own binding site (Argüello-Astorga et al., 1994a; Fontes et al., 1994; Ramos et al., 2003). The second one is based on the inability of the B component to promote movement of the heterologous DNA A component. Frischmuth et al. (1993) studied the ability of ACMV and ICMV, ACMV and TGMV and TGMV and AbMV to form pseudorecombinants. They were not infectious in *N. benthamiana* and did not produce pseudorecombinants. Frischmuth and coworkers suggested that part of the results was due to the inability of DNA A to replicate heterogenomic DNA B. In 1998, Hill et al. investigated the ability of SqLCV and *Cabbage leaf curl virus* (CabLCuV) to form pseudorecombinants in different hosts. The pseudorecombinant was systemically infectious in *N. benthamiana* but not in pumpkin or Arabidopsis. Hill and coworkers suggested that replication alone is not the only factor responsible to cause systemic infection in distinct hosts because although the replication origins and AL1 proteins were similar to generate infectious pseudorecombinants, systemic infection in pumpkin and Arabidopsis could not be established. Probably movement proteins are responsible for this difference in host range.

Nevertheless, there are few examples where pseudorecombinants occur with viruses having a different Rep binding site (Andrade et al., 2006; Garrido-Ramirez et al., 2000). Andrade et al. (2006) showed the ability of TGMV DNA A and *Tomato yellow spot virus* (ToYSV) to form an infectious pseudorecombinant. Also Garrido-Ramirez and co-workers showed the ability of BGMV B DNA and tomato leaf crumple virus (TLCrV) A component to infect beans but not *N. benthamiana* plants, although, no infectious pseudorecombinant in beans was observed using the A DNA of BGMV and B DNA of TLCrV. Garrido-Ramirez and coworkers suggested that BGMV B component is probably extremely well adapted to common bean and therefore this could be the reason for the host specificity.

Finally, geminiviruses can produce a significant amount of viral DNA with deletions, which is correlated with amelioration of symptoms and decrease of the replicated viral DNA. These molecules may be derived from DNA A (Ndunguru et al., 2006) and/or DNA B components and are called defective interfering DNAs (def-DNAs). They vary in size and are encapsidated (Patil and Dasgupta, 2006). Def-DNAs have been reported in higher amounts in experimental hosts rather than in their natural host. Patil et al. (2007) sequenced defective molecules ranging in size from 549 to 1555 nucleotides, originated from ICMV and SLCMV. They identified for the first time defective molecules that were formed by recombination between A and B components suggesting that they might represent accumulated replication by-products resulting from recombination-dependent replication.

Moreover, def-DNAs may serve as expression vectors being transreplicated by the Rep of the helper virus because they contain all necessary motifs important for the viral replication. Behjatnia et al. (2007) isolated def-DNAs of infected ToLCV including the origin of replication. When def-DNAs were coinoculated with tandem repeat constructs of the viral DNA they could be replicated by the viral DNA although did not moved systemically. The use of geminiviruses as expression vectors has been exploited mainly from viruses belonging to the *Mastrevirus* genus including MSV, *Wheat dwarf virus* (WDV), *Bean yellow dwarf virus* (BeYDV) as well as in curtoviruses as BCTV (Hefferon and Fan, 2004; Hefferon et al., 2004; Matzeit et al., 1991; Palmer and Rybicki, 2001; Zhang and Mason, 2006). Moreover, few members of the genus *Begomovirus* as TGMV and CabLCuV have been used to express foreign genes (Kjemtrup et al., 1998; Muangsan et al., 2004; Peele et al., 2001). Protein expression

level has been monitored by replacing the CP of monopartite and some bipartite geminiviruses by reporter genes as *gfp* and *gus*. Moreover, increase in protein expression has been obtained by including the CaMV 35S promoter sequence in the replicating virus (Hayes et al., 1989; Kim et al., 2007). Kim et al. improved the expression of the recombinant GFP by including different promoters (CaMV 35S, *Cassava vein mosaic virus*, CsVMV) within the replicating vector of BCTV in *Nicotiana benthamiana*.

1.9 Transmission and occurrence of tomato infecting begomoviruses

Begomoviruses are transmitted by the whitefly vector *Bemisia tabaci* (Gennadius) (Homoptera/Hemiptera: Aleyrodidae) in a circulative manner. Whiteflies have been the cause of many geminivirus out-breaks in tropical and subtropical areas (Morales and Jones, 2004; Brown, 2000; Figure 4). The introduction of the B type of *Bemisia tabaci* from the Eastern Hemisphere to the Western Hemisphere starts late in the 80's and beginning of the 90's. Today cultivated plants such as cotton, cassava, legumes, tomato and other Solanaceous crops are frequently affected by begomoviruses.

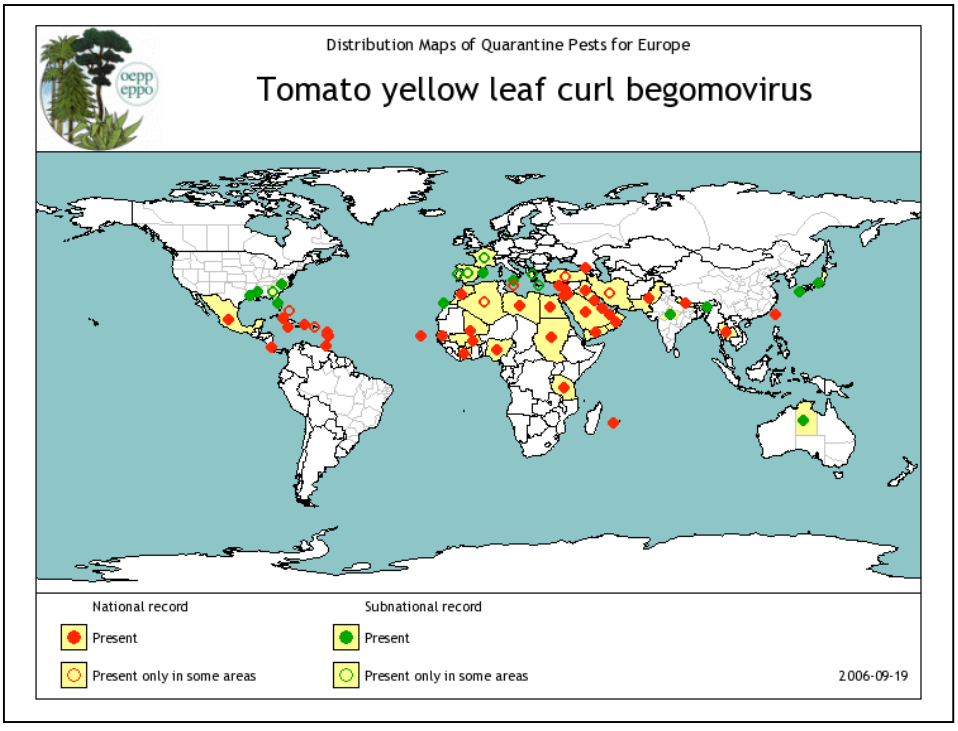


Figure 4. World-wide distribution of Tomato yellow leaf curl begomoviruses according to EPPO (2006). http://www.eppo.org/QUARANTINE/listA2.htm

For instance, TYLCV was first reported in Israel in the late 30's also in association with whiteflies out-breaks. Today the disease has spread throughout the Mediterranean area, the Middle East, tropical regions of Africa, Central America, Australia, USA and Japan (Figure 4).

Most of the genome constitution of TYLCV is found in nature as monopartite (Mediterranean, Central America and US isolates) but the virus occurs also in a bipartite form (Thailand viruses) (Gafni, 2003). In Thailand, bipartite TYLCV isolates have been found infecting tomatoes (Rochester et al., 1990; Sawangjit et al., 2005). Rochester et al. (1990) cloned the two different components of A and B of *Tomato yellow leaf curl Thailand virus* (TYLCTHV). In addition, the A component of TYLCTHV solely proved to be infectious when introduced in tomato and *N. benthamiana* plants via agroinoculation.

Ghanim and Czosnek, (2000) investigated the transmission of TYLCV-[IL] using whiteflies. They suggested that the virus could be sexually transmitted from insect to insect and that the hemolymph was partially responsible for the circulative transmission. Moreover, differential transcriptional activity of begomoviruses in *Bemisia tabaci* has been reported. Sinisterra et al. (2005) showed the differences in accumulation of viral DNA and transcripts in the plant and insect. They suggested that this difference might be part of a strategy to assure a virus titre inside the vector sufficient to promote transmission to other plant species.

1.10 Silencing and VIGS

RNA silencing is a process, which was initially thought to be triggered only in plants, activating part of the defense mechanism against viruses. The formation of double-stranded RNAs (dsRNA), which could be originated from a viral replicative form, are recognized by processing enzymes (DICERS) resulting in RNA cleavage and production of small interfering RNAs (siRNAs). The siRNAs are recognized and incorporated into a complex known as the RNA induced silencing complex (RISC) leading to a specific mRNA target and degradation.

Today similar silencing mechanism is known to be also present in all eukaryotes including animals, protozoa and plants. In plants, RNA silencing works in three levels. The first is known as PTGS, which is a cytoplasmic silencing triggered by dsRNA resulting in mRNA cleavage. The second involves the formation of miRNAs, which may base-paired to specific mRNA resulting in RNA degradation or blocking protein translation. Finally the third mechanism is associated to sequence-specific methylation of the DNA leading to suppression of transcription (transcriptional gene silencing).

In Geminiviruses two silencing pathways have been described. The first is a posttranscriptional gene silencing (cytoplasmic RNA silencing) leading to mRNA degradation and the second one leads to transcriptional gene silencing (TGS) through a siRNA-directed methylation. The replicative form, which is generated in the nucleus during geminivirus replication, is a potential target of methyltransferases. Methyltransferases can modify DNA and histone proteins (methylation of histone H3 at lysine 9, Bender, 2004) in viral minichromosomes. Seemanpillai et al. (2003) described the transcriptional silencing of a tobacco transgene containing promoter sequences derived from ToLCV. Systemic infection and silencing of plants was associated with hypermethylation of the promoter of ToLCV transgene. On the other hand, the role of DNA methylation in transgenic plants expressing ToLCV C2 gene as an inverted-repeat construct have been analyzed by Bian et al. (2006). Those transgenic plants were able to escape the silencing mechanism even if the replicative forms (RF) of ToLCV DNA were highly methylated. The authors suggested that complete inhibition of methylation caused by C2 is not present in infected cells and probably a small pool of unmethylated ToLCV RF is sufficient for viral transcription.

Gene silencing in Geminiviruses can also be triggered from chromosomal cassettes. Atkinson et al. (1998) produced transgenic *Petunia hybrida* plants containing an integrated chromosomal T-DNA copy of *Tobacco yellow dwarf virus* (TbYDV) with a CaMV 35S promoter-driven chalcone synthase A. Transgenic plants produced white-spotted flowers that was inherited through meiosis. In addition, Tao and Zhou (2004) modified the beta satellite of TYLCCNV into a gene silencing vector by replacing the ßC1 ORF with a multiple cloning site. The modified vector did not produce typical begomovirus symptoms when associated to TYLCCNV and the silencing effect was easily visualized.

Muangsan et al. (2004) gave further elucidation on the requirements for geminivirus-induced gene silencing. Arabidopsis mutants for PTGS (*sgs*1, *sgs*2, *sgs*3, *ago*1-27) and for TGS (*ddm*1/*som*8, *mom*1) were inoculated with a CabLCuV-derived vector carrying the *chlorata*42 gene instead the coat protein. Muangsan and coworkers showed that DNA viruses do not share the same silencing pathway as RNA viruses and that the ability of the geminiviral vector to initiate endogenous gene silencing was compromised in two of the PTGS mutants, but not in the mutants of TGS. Only in some PTGS mutants silencing could be effectively initiated. Therefore, they proposed a model explaining DNA VIGS of chromosomal genes. They suggested that (i) CabLCuV induce gene silencing occurs due to the fact that considerable amounts of single-stranded RNA are produced; (ii) CabLCuV induce gene silencing is not caused by TGS mediated by proteins MOM1 and DDM1/SOM8; (iii) AGO1 and SGS1 have only a modest effect on silencing of *chlorata*42 and may modify the action of important silencing components such as SGS2/SDE1 and SGS3; (iv) both SGS2/SDE1 and SGS3 have a similar effect on DNA VIGS and on sense transgene mediated PTGS and finally (v) dsRNA is also in geminiviruses the main element induced in the silencing pathway.

Finally, Vanitharani et al. (2005) summarized the mechanism of geminivirus-induced RNA silencing in plants. First, siRNA might be produced from overlapping, abundant and folded forms of viral mRNAs; second, geminiviruses are unique due to the fact that different types of suppressor of PTGS (AC2, AC4) are able to interfere with silencing mechanism and third, geminiviruses may be even subject to transcriptional gene silencing if their promoters are integrated in the genome of the plant.

Geminiviruses have been used as virus-induced gene silencing (VIGS) vectors, where the expression of genes in the host can be knocked down due to the silencing mechanism (Turnage et al., 2002). To date four different viruses and a beta satellite have been used as VIGS vectors (Carrillo-Tripp et al., 2006). Kjemtrup et al. (1998) were the first to explore the genome of the bipartite TGMV as an expression vector to cause silencing. The replicating episomal DNA could cause silencing of homologous, chromosomal genes. To that purpose TGMV was engineered by replacing its CP either by the sulfur allele (*su*) of magnesium chelatase or by the firefly luciferase gene and delivered to *N. benthamiana* via bombardment. Moreover, silencing was propagated through tissue culture, but not in the progeny of the plants. Later the same group, (Peele et al., 2001) showed the ability of TGMV B DNA with an insertion equal or smaller

than 154 bp without deletion of the CP to be able to induce gene silencing. They also demonstrated that silencing of an endogenous gene could occur in meristematic apical cells even in the absence of the geminivirus. Also CabLCuV, ACMV and *Pepper huasteco yellow vein virus* (PHYVV) have been used to construct gene silencing vectors (Carrillo-Tripp et al., 2006; Fofana et al., 2004; Muangsan et al., 2004). Moreover, Seemanpillai et al. (2003) observed that transgenic plants containing ToLCV promoter-driven *gus* became silenced under ToLCV infection, but silencing did not prevent the replication of the homologous virus.

1.11 Objectives

Tomato yellow leaf curl begomoviruses have been causing huge yield losses varying from 10% to 100% in tomato plants in Asia. Therefore, with the intention of understanding the begomovirus complex disease in those occurring areas of Thailand and Vietnam and with the intention of avoiding future begomoviruses out-breaks, this work has been divided mainly in three parts.

The first objective consisted of verifying whether tomato infecting begomoviruses in Vietnam are able to transreplicate the TYLCTHV-[AIT] DNA B component. TYLCTHV isolates are the only one within the tomato yellow leaf begomoviruses diseases to have a bipartite genome. All other tomato yellow infecting viruses in the Old World have a monopartite genome. Normally bipartite viruses do not transreplicate heterologous DNA molecules due to the high specificity of the replication protein to its own binding site (iterons). Therefore, we were interested to known if TYLCTHV isolate AIT could be transreplicate by other monopartite viruses infecting tomato plants occurring in the Old World, especially in Vietnam. Transreplication assays were performed using introduction of multimeric clones via agroinoculation, via biolistics and/or via mechanical inoculation.

The second part of this work consisted of generating transgenic *Nicotiana benthamiana* plants resistant to TYLCTHV-[AIT]. Geminiviruses can also trigger the silencing mechanism that has been observed in RNA viruses, although the detailed mechanism is still unknown. In RNA viruses, the use of inverted-repeated constructs to confer resistance has been successfully employed. Therefore, we were interested to known if TYLCTHV-[AIT] can be target of post-transcriptional gene silencing and thereby which part of the genome could be used as inverted-repeat to generate plants with the best resistance response. To this purpose, four inverted-repeated constructs were generated harboring different parts of the TYLCTHV A genome and additionally two constructs of the TYLCTHV B component.

Finally the third part of this study consisted of verifying the possibility of TYLCTHV-[AIT] to be engineered as an expression vector. Therefore, the AV1 gene was replaced by *gfp* and also by the sequence coding for the coat protein of the *Vicia cryptic virus* and expression was monitored by either using the confocal laser

microscope and/or by immunoblotting assays. Moreover, TYLCTHV-[AIT] is considered to be a facultative bipartite virus because the A component alone is able to systemically replicate inside the plant without its B component. Therefore, agroinoculation of TYLCTHV-[AIT] multimeric clone harboring GFP in transgenic *Nicotiana benthamiana* 16c plants represents the first tentative to monitor the progression of silencing signs *in vivo* in the presence or absence of the begomovirus TYLCTHV-[AIT] DNA B component.

All together the studies may help when considering new strategies of resistance against TYLCTHV-[AIT] in future as well as considering the relationships to other begomoviruses infecting tomato plants in Vietnam.

2.0 Transreplication of a *Tomato yellow leaf curl Thailand virus* DNA B and replication of a ß DNA component by *Tomato leaf curl Vietnam virus* and tomato yellow leaf curl Vietnam virus

2.1 Abstract

The genomes of two tomato-infecting plant DNA viruses from Vietnam were cloned and sequenced. A new strain of *Tomato leaf curl Vietnam virus* (ToLCVV) consisting of a DNA A component and associated with a ß molecule as well as an additional begomovirus tentatively named tomato yellow leaf curl Vietnam virus (TYLCVV) consisting also of a DNA A molecule were identified. To study the relationship between viruses occurring in Vietnam and Thailand, infectivity assays were performed using tobacco leaf curl Thailand virus (TbLCTHV), *Tomato yellow leaf curl Thailand virus* (TYLCTHV), TYLCVV, ToLCVV and ToLCVVß components. Multimeric clones of the above mentioned viruses proved to be infectious when introduced either by biolistics or agroinoculation in *Nicotiana benthamiana* and *Solanum lycopersicum* depending on the combination employed.

The ability of TYLCVV, ToLCVV and TbLCTHV to transreplicate the DNA B component from *Tomato yellow leaf curl Thailand virus* with or without ß molecules was investigated. In addition, defective DNAs ranging from 735 to 1457 nucleotides, generated in *N. benthamiana* from those combinations containing TYLCTHV B were cloned and sequenced. Finally, TYLCVV, ToLCVV, TYLCTHV and TbLCTHV were mechanically transmitted to *N. benthamiana* and TYLCTHV and ToLCVV to tomato only in the presence of TYLCTHV B molecules.

Keywords: Tomato yellow leaf curl virus, begomovirus, transreplication, recombination, defective-DNAs

2.2 Introduction

The family *Geminiviridae* has been divided into four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus* and *Begomovirus*) based on their vector transmission, host range and genome organization (Fauquet and Stanley, 2005; Stanley, 2004). Begomoviruses are transmitted by *Bemisia tabaci* (Gennadius) in a circulative and persistent manner (Rosell et al., 1998). They have a high economical impact on the yield of many important agricultural products especially of tomato where losses may vary from 40% to 100% (Czosnek and Laterrot, 1997).

Begomoviruses can be classified as bipartite or monopartite viruses depending on their genomic components. Bipartite viruses consist of two DNA molecules, A and B component, whereas monopartite viruses have only one DNA component which may be associated with half size molecules, so called ß molecules. Usually bipartite viruses need both components to infect their natural host to cause systemic infection. Nevertheless, there are some exceptions. Although *Tomato yellow leaf curl Thailand virus* (TYLCTHV) (Rochester et al., 1990; Rochester et al., 1994), *Sri Lankan cassava mosaic virus* (SLCMV) and *Tomato chlorotic mottle virus* (ToCMoV-[MG-Bt1]) (Fontenelle et al., 2007; Galvão et al., 2003) are found in nature as bipartite viruses, the A component solely is able to systemically infect *Nicotiana benthamiana* plants. For this reason, SLCMV has been suggested as an intermediate in the evolution from monopartite to bipartite geminiviruses (Patil et al., 2007; Saunders et al., 2002).

Most of the tomato infecting geminiviruses found in the Old World are classified as monopartite viruses, with exception of the Thailand strains such as, TYLCTHV-[1], TYLCTHV-[2], TYLCTHV-[AIT], TYLCTHV-[CM], TYLCTHV-[NK], TYLCTHV-[SK] and *Tomato yellow leaf curl Kanchanaburi virus* –[Thailand Kanchanaburi 2]. TYLCTHV is a bipartite virus (Sawangjit et al., 2005). The DNA A component possessing two open reading frames (ORFs) on the viral-strand, one coding for the pre-coat (AV2) and the other for the coat protein (AV1) and four ORFs on the viral-complementary strand (AC4, AC1, AC2, AC3). AV2 protein is coded by begomoviruses of the Old World, but not by those from the New World and has been recently related to gene silencing suppression (Zrachya et al., 2007a). AC1 codes for the protein (Rep) involved in replication of the viral DNA. C2 protein has been demonstrated to be involved in gene silencing for *Tomato yellow leaf curl China virus* (TYLCCNV) (Dong et al., 2003), whereas C3 codes for a protein that function as enhancer of transcription, thereby promoting viral replication (Settlage et al., 2005). C4

protein has been involved in movement of TYLCV. It localizes to the host cell periphery and interacts with plasmodesmata (Jupin et al., 1994; Rojas et al., 2001; Rojas et al., 2005). In addition, AC4 product has been related to gene silencing in *African cassava mosaic virus* (ACMV-[CM]) and SLCMV (Vanitharani et al., 2004). The B component has two ORFs, one coding for the nuclear shuttle protein (BV1) on the viral strand and the other for the movement protein on the complementary strand (BC1). DNA A and DNA B share a common region (CR) of ~200 bp, containing several regulatory *cis*-elements, including TATA and CA motifs, probably involved in the initiation of transcription of AV1/AV2 and AC1/AC4, and also the nonanucleotide sequence (TAATATTAC), which is known as recognition site for the Rep protein. Geminiviruses replicate via a double-stranded DNA intermediate through a rolling-circle mechanism (RC) and via a recombination-dependent replication mechanism (Alberter et al., 2005; Jeske et al., 2001).

Tomato leaf curl virus is known as a monopartite virus and comprises six ORFs, two are encoded on the sense virion strand and four on the complementary virion strand. Monopartite viruses may be associated with ß molecules. The first ß molecule to be sequenced and characterized was a satellite found in tomato plants associated with tomato leaf curl geminivirus from northern Australia (Dry et al., 1997). There are many recent reports about monopartite viruses that are associated with a DNA ß satellite (Amin et al., 2006; Briddon and Stanley, 2006; Guo and Zhou, 2006; Tao and Zhou, 2004); however ß satellites share no significant homology with their helper virus, on which they depend for replication, encapsidation and movement inside plants (Saunders et al., 2000).

Geminiviruses are able to acquire extra DNA components, to pseudorecombine and to produce defective DNAs (def-DNAs) in their process of replication. Defective DNAs have been reported and are associated to both bipartite and monopartite viruses (Patil et al., 2007; Patil and Dasgupta, 2006). Molecules of half the size of the genomic DNA component are reported to cause a decrease in accumulation of genomic DNA and attenuation of symptoms (Frischmuth and Stanley, 1994; Stanley et al., 1990). However, whether they occur at high levels in naturally infected plants or have a functional role in other DNA viruses infecting crops under field conditions is still not clear. The only exception of high levels of def-DNAs in natural hosts has been reported for *Ageratum conyzoides* infected with *Ageratum yellow vein virus* (Stanley et al., 1997).

Intermolecular reassortment experiments have been reported for several bipartite viruses (Andrade et al., 2006; Brown et al., 2002; Frischmuth et al., 1993; Frischmuth et al., 1997; Hill et al., 1998; Höfer et al., 1997; Hou and Gilbertson, 1996; Hou et al., 1998; Levy and Czosnek, 2003; Ramos et al., 2003; Unseld et al., 2000a); however one report describe the facultative monopartite SLCMV that evolved by acquisition of a B component from a different bipartite virus: *Indian cassava mosaic virus* (ICMV) (Saunders et al., 2002). Normally bipartite viruses do not transreplicate heterologous B components. It is known that the intergenic region (IR), which is highly conserved between both components, has *cis*-acting elements responsible for the specificity of the rep-iteron binding and therefore is involved in determining virus replication specificity. Here we present the genome organization and phylogenetic relationships of *Tomato leaf curl Vietnam virus* (ToLCVV-[DX2]) and tomato yellow leaf curl Vietnam virus (TYLCVV-[DX1]) infecting tomato plants in Vietnam. In addition, reassortment assays using TYLCTHV B component and a ToLCVVß satellite were performed. Furthermore, virus transmission through rub-inoculation was successful only when TYLCTHV B DNA was coinoculated. The fact that TYLCTHV-[AIT] B component can be transreplicated by three different monopartite viruses occurring in Asia (ToLCVV-[DX2], TYLCVV-[DX1] and TbLCTHV) in the presence or absence of ToLCVVß satellite will be discussed.

2.3 Materials and Methods

Plant material, cloning of viral DNAs, particle bombardment and sap transmission

Infected tomato leaves were collected from different field locations situated in Dang Xa (DX1, DX2) and Vien Rau Qua (VRQ, VR2) in the district of Gia Lam-Hanoi (Vietnam) showing typical geminivirus symptoms. DNA was isolated according to Crespi et al., (1991). After DNA extraction, the rolling circle amplification (RCA) was performed according to Knierim and Maiss, (2007). RCA derived multimeric products were used as template for PCR to amplify the different geminiviral components. To this extent, primers TYLCV_Vts 5'-GGTGTCTTATTTATATGTGGACACC-3' and TYLCV_Vtas 5'-CGATAATGCCATTTGGTGTCCACAT-3' were employed. ß molecules were amplified from the different DNA samples using the primers (beta01/beta02) described by Briddon et al. (2002). The obtained PCR fragments (around 3 kb and 1.3 kb) were

cloned into a *Eco*RV digested pBluescript vector and sequenced by MWG-Biotech, Martinsried, Germany.

The cloned single copy of the viral DNA genome was excised from their plasmid vector, religated and used as template for RCA. Multimeric products were inoculated onto *N. benthamiana* via particle bombardment according to Knierim and Maiss, (2007). Symptomatic leaves were used as inoculum for the mechanical transmission experiments. Leaves were ground in phosphate-Na buffer (0.05 M Na/K phosphate, pH 7.0, containing 1 mM EDTA and 5 mM Na-DIECA) together with charcoal. The plant sap was used to inoculate *N. benthamiana* using Celite 545 as abrasive. Infection of plants was confirmed by PCR and/or by TAS-ELISA using monoclonal antibody AS-0588-546/2, (DSMZ). The mechanical transmission experiments involving *Solanum lycopersicum* were performed using symptomatic sink leaves originated from agroinoculated tomato plants. Inoculation was performed as described above for *N. benthamiana* plants. Also systemic infection was confirmed by PCR using specific primers.

Construction of infectious multimeric clones and agroinoculation

The multimeric clones (1.4x, 1.5x and 1.6x copy of the genome) of the ToLCVVß satellite, ToLCVV-[DX2] and TYLCVV-[DX1] A components were firstly constructed in pDrive (Qiagen, Germany), giving pToLCVV_0.4ß, pToLCVV_0.5A and pTYLCVV_0.6A. Subsequently, either multimeric products obtained by RCA or plasmids containing a single copy of the viral genome were digested with the appropriated restriction enzyme *Xba*I, *Nco*I or *Hind*III and the excised genomic full-length was cloned into pToLCVV_0.4ß, pToLCVV_0.5A, pTYLCVV_0.6A resulting in pToLCVV_1.4ß, pToLCVV_1.5A and pTYLCVV_1.6A, respectively. The multimeric copies were cloned into a modified binary vector (pBIN 19). TYLCTHV-[AIT] and TbLCTHV full-length clones used in these work were described by Knierim and Maiss, (2007).

For agroinoculation experiments, the multimeric constructs were transferred to *Agrobacterium tumefaciens* strain C58C1 via electroporation and used for inoculation onto *N. benthamiana* and *S. lycopersicum* plants. Agrobacteria were grown overnight in LB media containing its respective antibiotic (28°C and 250 rpm) to OD_{600} of 1.7. 1 ml of each bacterial suspension containing the respective multimeric copies (A and B

components) were centrifuged and resuspended in 1-2 ml of agroinoculation buffer (10 mM $MgSO_4$, 10 mM MES and 100 µM acetosyringone). A dilution of 1:100 (using agroinoculation buffer) was used only when inoculating *N. benthamiana*. The suspension was incubated for 3-4 hours before agroinoculation at room temperature.

Detection of viral, defective DNA and southern blot

DNA was extracted from agroinoculated leaves from infected *N. benthamiana* and *S. lycopersicum* plants 20 days after inoculation (dpi). PCR was performed either using abutting primers TYLCV_E5s 5'-AAATGATATCTTTAACAACTTAGAACAAGATG-3' and TYLCV_E5as 5'-TAAAGATATCATTTTGAGATATTTGTCCAA -3' to search for a putative viral B DNA of TYLCTHV, recombinant or defective molecules or using primers IR01 5'-GTTAGTTCCTGACTCCATTATTGATGA-3' and IR02 5'-ATCACTGTTAAAGCCAAAAGGTCGT-3' to identify only the intergenic region of the viral B DNA. Amplified fragments were cloned and sequenced. In addition, Southern blot hybridization was also used to detect viral B DNA from TYLCTHV. For this purpose, 0.2 µg of total extracted DNA from *N. benthamiana* and 3 µg of DNA extracted from tomato were separated in a 1% agarose gel for 2 h. The genomic and defective DNAs were transferred to a Hybond-N nylon membrane (Amersham Pharmacia, Germany) using a vacuum blotter. The probe was DIG-labeled using the PCR Labeling Mix kit (Roche, Germany) and hybridization was performed according to the manufacturer.

Recombination events and sequence analysis

Attempting to identify recombinant sequences and recombination breakpoints, the viral sequences were analyzed using RDP v.2 Beta 08 (Martin and Rybicki, 2000; Martin et al., 2005) with following settings: window size 10, highest acceptable probability 0.001, internal reference sequences. In addition, recombinant sequences were also confirmed employing Simplot (Lole et al., 1999). Multiple sequence alignments were performed by ClustalX v1.8 (Thompson et al., 1994) and graphics were generated by TreeView X (v.0.4.1). The following Begomovirus DNA A sequences were retrieved from GenBank for multiple sequence analysis: AbMV - X15983; ACMV-[CM] - AF112352; AEV - AJ437618; ALCuV-[G52] - AJ851005; AYVCNV-[Hn2] - AJ495813;

AYVSLV - AF314144; AYVTV-[TW] - AF307861; AYVV - X74516; BcaMV - AF110189; BDMV - M88179; BGMV-[BR] - M88686; BGYMV-[CU] - AJ544531; BYVMV-[301] - AJ002453; CabLCuV - U65529; CdTV-[H6] - AF226665; ChaYMV - AJ223191; ChiLCuV-[Mul] - AF336806; CLCrV - AF480940; CLCuAV-[802a] - AJ002455; CLCuKV-[72b] - AJ002448; CLCuMV-[26] - AJ002458; CLCuRV - AF363011; CPGMV-[BR] - AF88708; CuLCrV - AF224760; CYVMV - AJ507777; CYVVV-[Hoa] - AY727903; DoYMV - AY309241; EACMCV-[CM] - AF112354; EACMMV-[K] - AJ006460; EACMV-[UG2] - Z83257; EACMZV - AF422174; EpYVMV-[SOJ3] - AJ438937; EpYVV - AB007990; EuLCV-[G35] - AJ558121; HoLCrV-[Giza] - AF014881; HYVMV - AB020781; ICMV - Z24758; IYVV - AJ132548; MYMIV-[Ako] - AY271893; MYMV-[TH1] - D14703; MYVV-[Y47] - AJ457824; OYVMV-[201] - AJ002451; PaLCuCNV-[G2] - AJ558123; PaLCuCNV-[G30] - AJ558117; PaLCuGuV-[GD2] - AJ558122; PaLCuV - Y15934; PepLCBV - AF314531; PepLCV - AF134484; PYMPV - Y15034; SiYMCNV-[Hn8] - AJ810096; SLCCNV - AB027465; SLCMV-[Col] - AJ314737; SLCYNV-[Y23] - AJ420319; StaLCuV-[Hn5] - AJ495814; TbCSV-[Y1] - AF240675; TbCSV-[Y35] - AJ420318; TbCSV-[Y41] - AJ457986;; TbLCKoV-[KK] - AB055009; TbLCTHV – DQ871221; TbLCYNV-[Y136] - AJ512761; TbLCYNV-[Y143] - AJ512762; TbLCYNV-[Y161] - AJ566744; TbLCYNV-[Y3] - AF240674; TGMV-[Com] - M73794; ToLCBDV - AF188481; ToLCBV - Z48182; ToLCCNV-[G16] - AJ704602; ToLCCNV-[G32] - AJ558118; ToLCGuV-[G2] - AY602165; ToLCGV-[Kel] - AF449999; ToLCKV - U38239; ToLCLV - AF195782; ToLCMYV - AF327436; ToLCNDV-[Mild] - TLU15016; ToLCPV - AB050597; ToLCSLV - AF274349; ToLCTWV - U88692; ToLCV-[AU] - S53251; ToLCVV - AF264063; ToLCVV-[DX2] - EU189149, TYLCCNV-[Y10] - AJ319675; TYLCCNV - AF311734; TYLCGuV-[G3] - AY602166; TYLCKaV-[TH:Kan2] - AF511530; TYLCSV - X61153; TYLCTHV-[1] - X63015; TYLCTHV-[2] - AF141922; TYLCTHV-[AIT] - DQ871222; TYLCTHV-[CM] - AY514630; TYLCTHV-[MM] - AF206674; TYLCTHV-[NK] - AY514631; TYLCTHV-[SK] - AY514632; TYLCTHV-[Y72] - AJ495812; TYLCV-[Alm]-AJ489258, TYLCVV-[DX1] - EU189150.

The following Begomovirus DNA ß sequences were retrieved from GenBank for multiple sequence analyses: ALCuVß - NC_005046; AYVCNVß - NC_007067; AYVVß - NC_003403; BYVMVß - NC_003405; ChiLCuVß - NC_005048; CLCuBVß - NC_007219; CLCuGVß - NC_006935; CLCuRVß - RCO421678; CPSLCVß - NC_006952; EpYVVß - NC_004515; ErYMVß - DQ641713; HYVMVß - NC_005052; LuYVVß - NC_007212; LYMVß - NC_008031; MYMIVß - DQ118862; MYVVß - NC_004733; OkLCuVß-[DNA10] - NC_004093; OkYLVVß - NC_005051; PaLCuVß - NC_004706; SiLCuVß - NC_007639; SiYMCNVß - NC_006267; SiYVVß - NC_007213; TbCSVß - NC_004546; TbLCYNVß - NC_005030; ToLCCNVß - NC_006289; ToLCJVß - NC_005497; ToLCNDVß - NC_005359; ToLCVVß-[DX2] - EU189146; ToLCVVß-[VR2] - EU189147; ToLCVVß-[VRQ] - EU189148; ToLCVß - NC_004715; TYLCCNVß-[G102] - AM050556; TYLCCNVß-[Y10] - AJ781298; TYLCCNVß-[Y36] - AJ506791; TYLCCNVß-[Y64] - AJ421483; TYLCCNVß - AJ781297; TYLCMLVß - NC_007485; TYLCTHVß - NC_004903; TYLCVVß - DQ641714; ZiLCVß - NC_005047.

2.4 Results

Sequence analysis

ToLCVV and ß satellite

ToLCVV comprises 2745 nucleotides in length and has six ORFs as found in viruses from the Old World. Attempts to find a B component failed as described also by Green et al., (2001). Blastn search performed with the complete A DNA component revealed the highest identity of 98% to the A DNA component of *Tomato leaf curl Vietnam virus* (ToLCVV) and secondly to *Tomato leaf curl China virus* (ToLCCNV-[G32]) with an identity of 88%. Therefore, the new strain was called ToLCVV-[DX2], because it was isolated from the Dang Xa location in Vietnam. The IR consists of 274 bp and revealed the highest nucleotide sequence identity of 99% to ToLCVV and secondly of 83% to the IR found in TYLCTHV-[ChMai]. In addition, several *cis*-acting elements were present including four CAA, four CAAA and two CAAAA repeats and a TATA box, probably all involved in the replication process. The core sequence, GGKGT, was identified as being the nucleotide sequence for binding of the Rep protein. When comparing the TYLCTHV-[AIT] to the ToLCVV-[DX2] genome, the highest nucleotide identity was obtained for the AC4/C4 ORF. ToLCVV-[DX2] exhibit the C4 ORF inside the Rep gene as found in TYLCTHV-[AIT], TYLCVV-[DX1] and TbLCTHV. Analysis using pairwise comparisons of nucleotide sequence revealed that C4 ORF is highly conserved (91 to 95%) among the sequenced Chinese and Vietnamese viruses found at GenBank (Table 1).

Table 1. Pairwise comparisons of the nucleotide sequence identities of A components from begomoviruses, ORFs and intergenic regions. Upper values represent alignment performed with TYLCVV-[DX1] and lower values with ToLCVV-[DX2]

Virus isolates	DNA A	AV2 (V2)	AV1 (V1)	AC3 (C3)	AC2 (C2)	AC1 (C1)	AC4 (C4)	IR
TYLCVV-[DX1]- EU189150 vs. ToLCVV-[DX2]- EU189149	88	90	90	77	76	85	92	89
ALCuV-[G52]-AJ851005	89	95	89	87	90	93	85	94
	89	90	89	74	75	80	80	96
AYVCNV-[Hn2]-AJ495813	85	82	82	84	81	92	94	87
	79	81	79	76	74	84	92	88
PaLCuCNV-[G10]-AJ558125	90	97	90	84	82	94	95	90
	84	90	91	76	74	84	91	89
PaLCuCNV-[G30]-AJ558117	87	95	89	85	82	90	92	84
	83	90	90	75	74	83	92	82
PaLCuCNV-[G43]-AJ876548	90	97	90	84	82	94	96	98
	85	90	90	76	74	85	92	89
PaLCuCNV-[GD2]-AJ558122	87	93	88	84	81	90	94	78
	83	90	90	74	72	83	92	77
TbLCTHV-DQ871221	84	84	77	82	80	86	94	79
	83	82	75	77	75	85	93	79
ToLCCNV-[G32]-AJ558118	82	89	78	76	76	86	94	88
	88	85	77	97	98	94	95	92
ToLCGuV-[G2]-AY602165	85	91	90	87	89	80	75	86
	90	92	91	77	76	77	73	84
ToLCTWV-U88692	86	83	87	88	88	88	93	88
	84	84	87	74	76	84	92	84
ToLCVV-AF264063	84	90	90	75	75	86	93	89
	98	98	99	98	98	98	98	98
TYLCCNV-[Y10]-AJ319675	86	89	74	86	87	86	95	90
	82	88	74	78	78	87	93	90
TYLCGuV-[G3]-AY602166	88	89	89	88	90	89	92	86
	91	91	91	76	76	83	89	82
TYLCTHV-[AIT]-DQ871222	88	76	76	84	85	86	96	87
	82	78	75	76	75	86	94	88

ß satellite molecules were found in DX2, VR2 and VRQ samples but not in DX1 samples. The ToLCVVß-[DX2] satellite (1357 bp) showed the highest identity of 97% to the ß satellite associated with Tomato yellow leaf curl Vietnam (acc. number DQ641714). ToLCVVß-[VR2] and ToLCVVß-[VRQ] consist of 1348 and 1346 bp, respectively. All three molecules showed an ORF coding to the ßC1 protein of 118 amino acids (identity of 98.3%). With blastp the highest identity of 99% was found to the ßC1 protein from tomato yellow curl Vietnam virus ß satellite and secondly of 80% to the ß satellite DNA present in one of the major weeds from Southeast Asia, *Erechtites valerianifolia* (acc. number DQ641713). Figure 1 shows that ToLCVVß clusters mainly with ß components isolated from tomato plants in Vietnam and China.

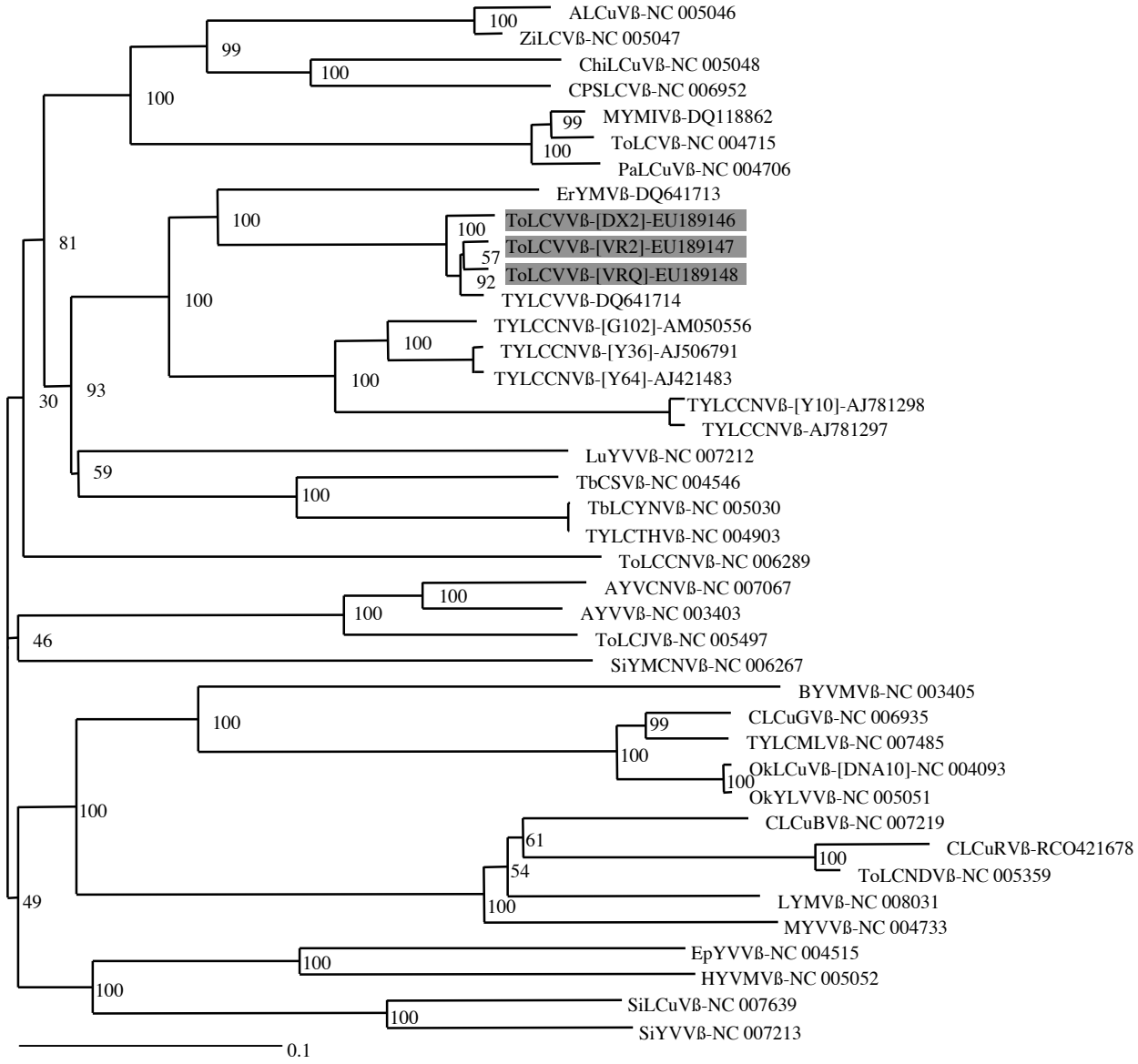


Figure 1. Phylogenetic tree of the complete nucleotide sequence of the ß component of representative begomoviruses. Isolates analysed in this study are shaded grey. Bootstrap scores in percent are shown at nodes (100 replicates).

TYLCVV

TYLCVV consists of 2748 bp and has also six ORFs. Blastn search revealed the highest nucleotide identity of 90% to the A components of *Papaya leaf curl China virus* (PaLCuCNV-[G43] and PaLCuCNV-[G10]) and 88% to the A component of *Tomato yellow leaf curl Guangdong virus* (TYLCGuV-[G3]).

The new virus was found only in tomato samples collected from DX1 location. Therefore, we tentatively named the virus as tomato yellow leaf curl Vietnam virus (TYLCVV-[DX1]). TYLCVV-[DX1] has the V2 ORF, also found in ToLCVV-[DX2], TYLCTHV-[AIT] and TbLCTHV, typical of monopartite viruses from the Old World. However, the nucleotide sequence coding for the V2 protein showed the highest identity of 97% to the V2 sequence found in *Papaya leaf curl virus* (Table 1). The IR consists of 277 bp and revealed the highest nucleotide identity of 84% to TYLCTHV-[ChMai] and secondly of 83% to TYLCTHV-[NoK] (identities established employing blastn search). Several *cis*-acting elements were also identified in the IR including three CAA, four CAAA motifs and one CAAAA motif. The TATA box and an identical nucleotide core, GGKGT, were found in TYLCTHV-[AIT] and TYLCVV-[DX2], but differ from that of TbLCTHV (GGGTM). Analysis of the iteron-related domain in the rep protein revealed "MAPPNKFRIN" in ToLCVV-[DX2], TYLCTHV-[AIT], TYLCVV-[DX1]. A different domain "MPQPKKFFIN" was identified for TbLCTHV.

Recombination analysis performed either using Simplot as RDP software indicated that the A component of TYLCVV-[DX1] is probably a recombinant between TYLCGuV-[G3] and PaLCuCNV-[G43]. In addition, comparison of nucleotide sequence identities of single ORFs performed by blastn showed differences in the similarity of the genome. The region covering C1, C4 and IR showed high similarity to PaLCuCNV-[G43] with identities of 94%, 96% and 98%, respectively, whereas the regions C2 and C3 showed only relative low identities of 82% and 84% (Table 1). The phylogenetic tree in Figure 2 shows the clustering of TYLCVV-[DX1] to geminiviruses found in papaya plants.

Attempts to amplify a B component associated with the DX1 samples using degenerate primer pairs PCRc1/PBL1v2040 (Rojas et al., 1993) and CR01/CR02 (Fondong et al., 2000) failed. In addition, digestion of rolling circle products from DNA isolated from infected tomato using different enzymes also did not reveal the presence of a second component (data not shown).

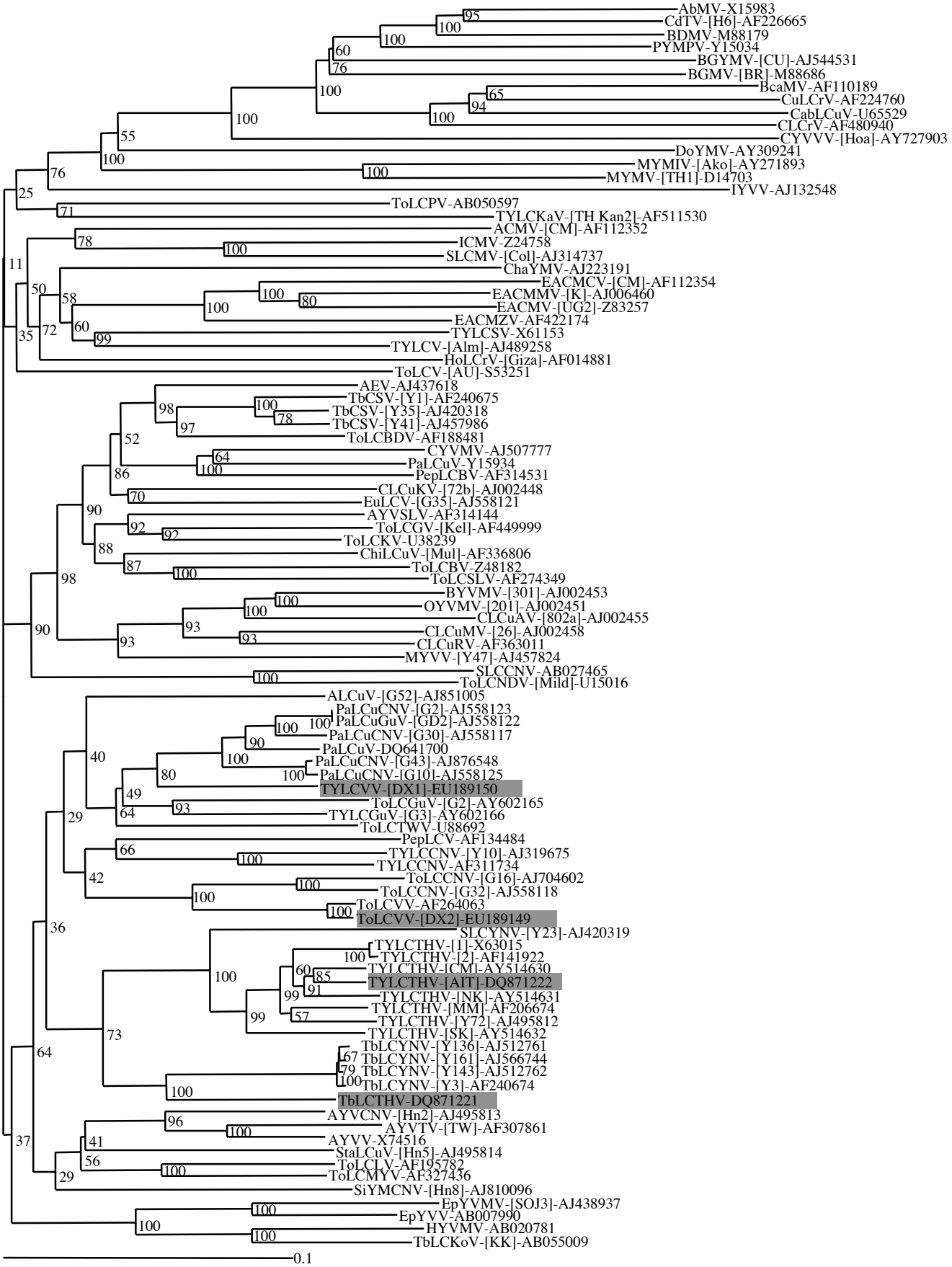


Figure 2. Phylogenetic tree of the complete nucleotide sequence of the DNA A component of representative begomoviruses assigned in the VIII report of viruses. Isolates analyzed in this study are shaded grey. Bootstrap scores in percent are shown at nodes (100 replicates).

Transreplication of DNA B component of TYLCTHV by ToLCVV, TYLCVV and TbLCTHV

The multimeric copies derived from the A component of TYLCTHV-[AIT], TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV proofed to be highly infectious when agroinoculated onto the experimental host *N. benthamiana* (Table 2), which developed severe symptoms 3 weeks post-inoculation (wpi). In addition, also the natural host, *S. lycopersicum*, showed typical geminivirus infections 4 wpi when agroinoculated with A components of the above mentioned viruses. Thus, Kochs's postulates were fulfilled. Inoculation of multimeric copies derived from the A component solely showed always the typical symptom of upward curling of the young leaves on *N. benthamiana*. A predominant leaf upward curling symptom was also observed on *S. lycopersicum*. Only when *S. lycopersicum* was inoculated with TYLCVV-[DX1] young leaves reacted with a more downward curling symptom. This suggests that TYLCVV-[DX1] and ToLCVV-[DX2] are monopartite viruses and need only the DNA A component to infect tomato plants while TYLCTHV-[AIT] is a facultative bipartite virus as reported earlier for TYLCTHV.

The occurrence of different symptoms in the transreplication assays (Table 2) depended on the combination of DNAs. Symptoms observed on *N. benthamiana* and/or tomato plants included yellow mottling, mosaic/chlorosis up and downward curling of leaves and stunting. When mixing the A component of ToLCVV-[DX2], TYLCVV-[DX1] and TbLCTHV, respectively, each together with the B component of TYLCTHV, very characteristic mosaic/chlorosis symptoms become visible after particle bombardment or agroinoculation in *N. benthamiana* and also in tomato plants. Initially, small yellow spots appeared on young leaves, which later developed into mosaic yellowing and necrosis. In these cases, fragments of TYLCTHV B DNA were reliably detected by PCR. Moreover, when agroinoculating multimeric copies of the A component of ToLCVV-[DX2], TYLCVV-[DX1] and TbLCTHV, respectively, together with the ToLCVVß copies on *N. benthamiana* or tomato, upward curling of young leaves disappeared. The presence of the ß component changed the symptoms from upward curling to severe downward leaf curling. It was always possible to amplify the ß satellite from the agroinoculated tomato and *N. benthamiana* plants. However, it was never possible to amplify the ß satellite from the mechanically inoculated plants.

Multimeric products originated from A and/or ß components by RCA were inoculated via particle bombardment into *N. benthamiana* plants. In almost all

combinations, no systemic infection was observed and detection of viral DNA by PCR failed. The only exception where a systemic infection was observed was in case of biolistic inoculation with the combination of ToLCVVß satellite multimeric products and TbLCTHV products. TYLCTHV-[AIT], TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV were mechanically transmitted to *N. benthamiana* and TYLCTHV-[AIT] and ToLCVV-[DX2] additionally to tomato only in mixed infections involving TYLCTHV B molecules (Table 2).

Table 2. Infectivity of cloned DNA components on *Nicotiana benthamiana* and *Solanum lycopersicum*

DNA-A	DNA-B and/or DNA-ß	Agroinoculation[a]		Biolistic[a]	Rub-inoculation[a]	
		N. benthamiana	*S. lycopersicum*	*N. benthamiana*	*N. benthamiana*	*S. lycopersicum*
TYLCTHV-[AIT]	**__**	10/10	13/11	5/0	2/0	20/0
	TYLCTHV_DNA-B	10/10	13/10	10/8	11/11	20/3
	ToLCVV_DNA-ß	10/9	10/6	13/0	n.d	20/0
	TYLCTHV_DNA-B + ToLCVV_DNA-ß	10/10	8/8	5/5	10/10	20/6
TYLCVV-[DX1]	**__**	10/10	44/35	20/0	5/0	20/0
	TYLCTHV_DNA-B	10/10	45/41	10/5	5/5	20/0
	ToLCVV_DNA-ß	10/10	41/18	20/0	5/0	20/0
	TYLCTHV_DNA-B + ToLCVV_DNA-ß	10/10	8/7	10/6	5/5	20/0
ToLCVV-[DX2]	**__**	10/10	38/10	8/0	2/0	20/0
	TYLCTHV_DNA-B	10/10	47/15	13/9	10/10	20/1
	ToLCVV_DNA-ß	10/10	5/2	26/0	6/0	20/0
	TYLCTHV_DNA-B + ToLCVV_DNA-ß	10/10	8/3	5/5	10/10	15/3
TbLCTHV	**__**	10/10	33/6	5/0	n.d	n.d
	TYLCTHV_DNA-B	10/9	40/10	7/6	9/5	n.d
	ToLCVV_DNA-ß	9/9	39/12	11/4	n.d	n.d
	TYLCTHV_DNA-B + ToLCVV_DNA-ß	9/9	n.d	n.d	n.d	n.d

[a] Number of inoculated plants/number of symptomatic plants; n.d, indicates experiment not done
Rub-inoculation was performed from plants showing symptoms after particle bombardment for *N. benthamiana* and agroinoculation for *S. lycopersicum*.

B defective DNAs accumulated in infected plants

Genomic and defective DNAs occur often in plants infected with geminiviruses. To verify the presence of these geminiviral DNA forms, PCR and Southern blot analysis were performed with agroinoculated *N. benthamiana*. Eight different clones were generated and sequenced representing def-DNAs derived from TYLCTHV B DNA. Of these, seven clones were representing deletion derivatives of DNA B and only one clone was identified as a recombinant between TbLCTHV A component and TYLCTHV B DNA.

Def-DNAs ranged in size from 735 bp to 1457 bp and all clones retained the common region containing the *cis*-elements required for replication. It is known that def-DNAs contain normally the intergenic region and parts of the movement protein, the nuclear shuttle nucleotide sequence being absent. However, three defective clones contained 5'-stretches of BV1, whereas one of them contained the 5'-half of BC1 (Figure 3).

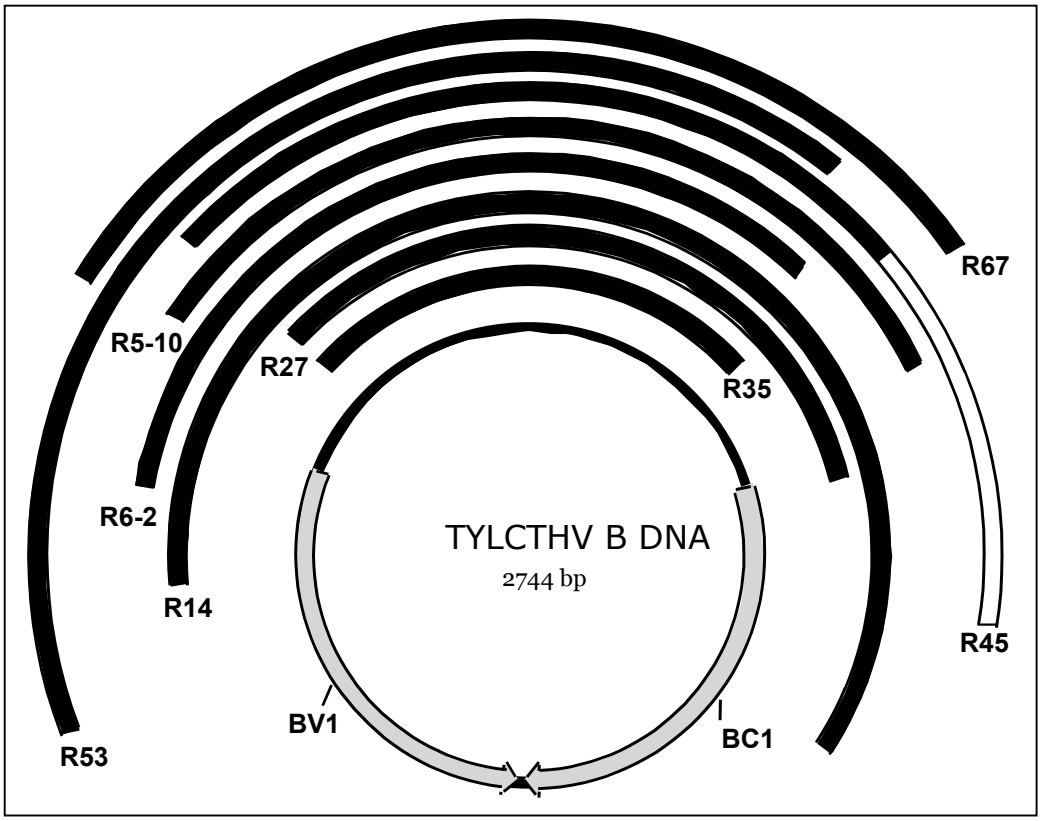


Figure 3. Schematic representation of the composition of the cloned defective-DNAs derived from the mix infection TYLCTHV B DNA. Black region indicates regions of progenitor DNA B retained while the white region of R45 the progenitor DNA A component of TbLCTHV.

Sequence analysis of the junctions between defective DNAs and TYLCTHV DNA B did not always revealed identical stretches of nucleotides as reported for def-DNAs derived from the B component from SLCMV and ICMV (Figure 4).

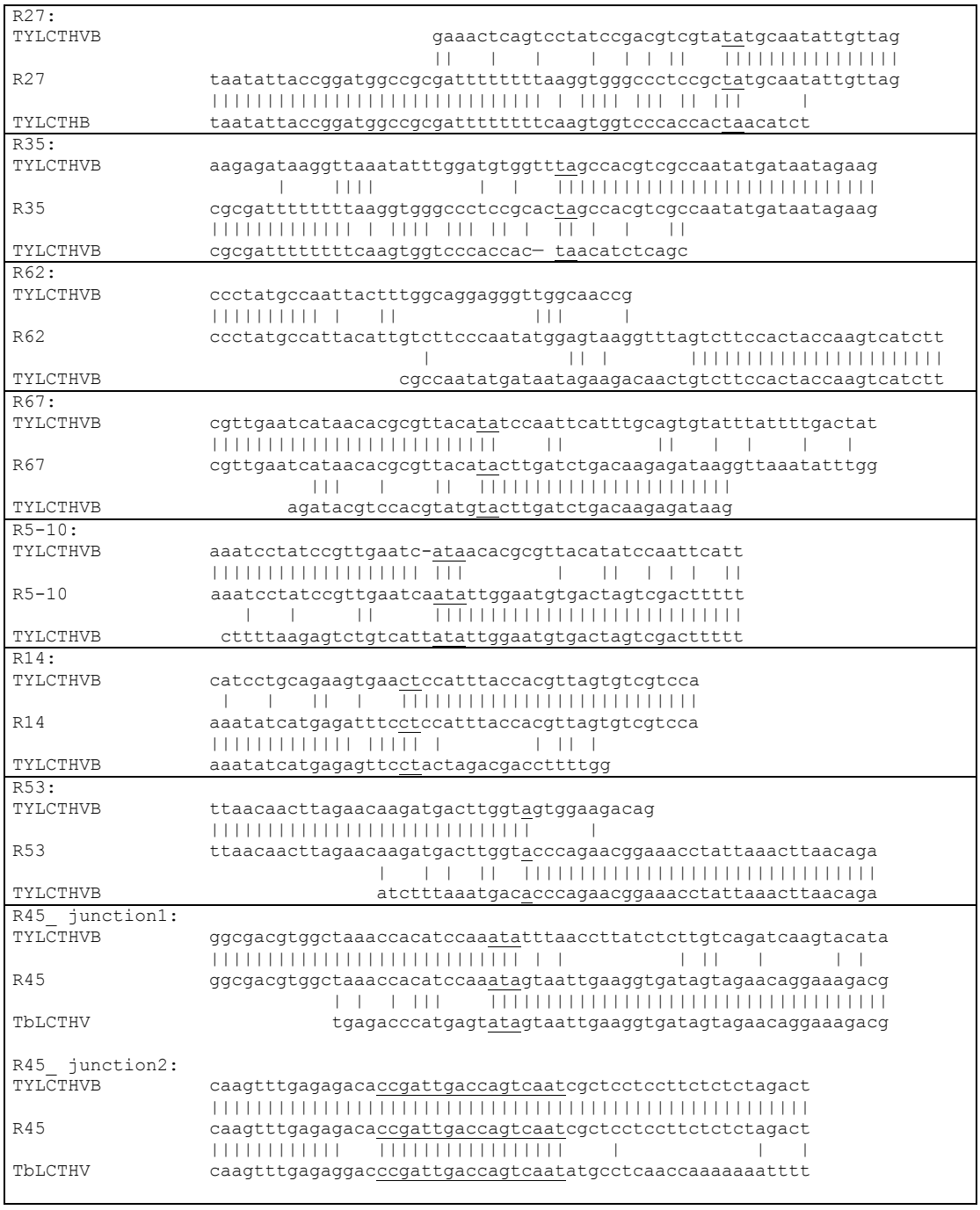


Figure 4. The junction sequences of def-DNA. Repeated sequences in donor and acceptor site are underlined. The defective DNA sequences are represented at the middle line in each box. Sequence R45 is the only sequence that showed a recombination event involving the respective A component. Repeated sequences in donor and acceptor site are underlined.

In addition, we also searched for defective molecules present in those combinations containing the ToLCVVß satellite. Only in combination with TYLCVV-[DX1], defective DNAs originated from ToLCVVß were formed (data not shown).

Def-DNAs were visualized in Southern blot hybridizations using 200 ng of DNA isolated after 20 dpi from *N. benthamiana* plants, but very few amounts were detected when using 3 µg of tomato DNA (Figure 5). These results confirm the suggestion that production of Def-DNAs is influenced by the host type and are often made in high levels in the experimental hosts but only in minute amounts in natural hosts.

In this work, ToLCVV-[DX2], TYLCVV-[DX1] and TbLCTHV A components were able to transreplicate the B component of TYLCTHV in *N. benthamiana* plants and additionally ToLCVV-[DX2], TYLCVV-[DX1] in tomato with or without ToLCVVß satellite virus. The infectivity data together with PCR results and Southern blot analysis confirmed the presence of TYLCTHV B DNA in all infected *N. benthamiana*.

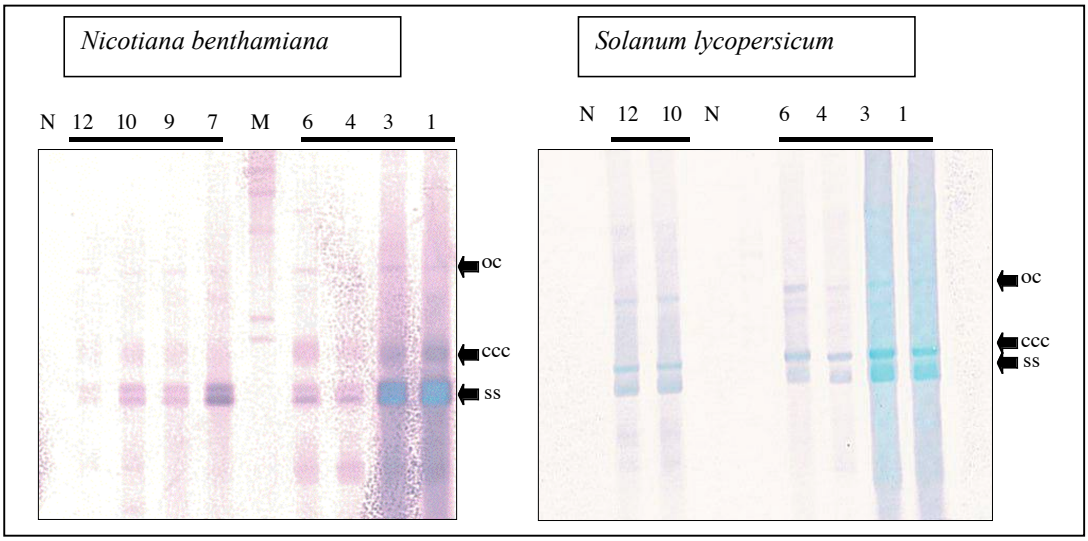


Figure 5. Southern blot analysis from total DNA extracted from systemically invaded leaves of agroinoculated *N. benthamiana* (left) and *S. lycopersicum* (right) leaves derived from different combinations of full-length clones presented on Table 2. Lane N: mock-inoculated plant; 12: ToLCVV-[DX2]+TYLCTHVB+ToLCVVß; 10: ToLCVV-[DX2]+TYLCTHVB; 9: TbLCTHV+TYLCTHVB+ToLCVVß; 7: TbLCTHV+ TYLCTHVB; M: Marker Roche (cat n. 1218590, base pair: 23130, 9416, 6557, 2322, 2027, 564); 6: TYLCVV-[DX1]+TYLCTHVB+ToLCVVß; 4: TYLCVV-[DX1]+TYLCTHVB; 3: TYLCTHV-[AIT]+ TYLCTHVB+ToLCVVß; 1: TYLCTHV-[AIT]+TYLCTHVB. OC: open circular dsDNA; CCC: covalently closed circular dsDNA; SS: single-stranded DNA.

2.5 Discussion

In this work, we cloned and sequenced the genome of two different geminiviruses infecting tomato plants originating from Vietnam (TYLCVV-[DX1] and ToLCVV-[DX2]). In addition, ß satellite molecules were only found to be present in samples containing ToLCVV. Comparison of the TYLCVV-[DX1] nucleotide sequence showed high identities to a group of viruses infecting papaya plants (Wang et al., 2004) while ToLCVV-[DX2] showed high identities to a previously reported sequence of ToLCVV (AF264063) (Green et al., 2001). To study a possible transreplication of geminivirus components occurring in Thailand (TYLCTHV-[AIT] and TbLCTHV; Knierim and Maiss, 2007) and those found in Vietnam, reassorted molecules were introduced into plants via biolistics and/or agroinoculation.

When TYLCVV-[DX1] and ToLCVV-[DX2] A components were inoculated via agrobacteria either in *N. benthamiana* or tomato, the typical geminivirus leaf curl symptoms were observed. Therefore, both viruses may be classified as monopartite viruses, because infectivity was shown and Koch's postulates were fulfilled solely by inoculation of the DNA A components. When TYLCTHV-[AIT], TYLCVV-[DX1] and ToLCVV-[DX2] A components were inoculated biolistically with or without ß satellite molecules, no symptoms could be observed in *N. benthamiana* plants. Only TbLCTHV A DNA together with ToLCVVß satellite produced visible symptoms. The fact that symptoms were observed only in mixed infections containing TbLCTHV molecules via particle bombardment suggests that an interaction of the ToLCVVß satellite and TbLCTHV facilitates the movement of the A component of TbLCTHV by a so far unknown mechanism. However, further experiments involving TbLCTHV are necessary to unreveal the function of the ß satellite.

Only when the B DNA of TYLCTHV was coinoculated biolistically together with the A components of TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV, respectively, a typical yellow mosaic symptom was observed. From those combinations, it was always possible to amplify TYLCTHV B DNA fragments by PCR. The experiments using biolistics as inoculation method suggest that TYLCVV-[DX1] and ToLCVV-[DX2] are phloem-limited viruses as previously reported for TYLCV (Morilla et al., 2004; Rojas et al., 2001) and ToLCV (Rasheed et al., 2006). The presence of TYLCTHV B component may help to overcome the phloem-limitation of TYLCVV-[DX1] and ToLCVV-[DX2]. Further evidence for a release of the phloem limitation is given by the mechanical transmission experiments performed with *N. benthamiana*

plants, where successful virus transmission occurred from all combinations containing the TYLCTHV B. In addition, rub-transmission experiments using tomato plants were also successfully conducted using TYLCTHV-[AIT] and ToLCVV-[DX2] A components only in the presence of TYLCTHV B component.

Lazarowitz (1991) gave the first report about transactivation and molecular characterization of two bipartite geminiviruses occurring in the Southwestern United States. Two Squash leaf curl viruses (SqLCVs) were found in a natural host and were designed as SqLCV-E (efficient replication in bean and squash) and SqLCV-R (restricted replication in bean and squash). In the field, plants containing two A components (A_E and A_R) in the presence of only one B_R component were found. Pseudorecombination may occur between geminiviruses strains (Frischmuth et al., 1997) or different species (Andrade et al., 2006; Brown et al., 2002; Hou et al., 1998; Levy and Czosnek, 2003; Ramos et al., 2003; Unseld et al., 2000b). The success to obtain viable pseudorecombinants between different geminiviruses may depend on the ability of an A component to transreplicate heterologous B components and on the ability of the DNA B encoded proteins to mediate the movement of the heterologous DNA A (Ramos et al., 2003). Most of the mentioned reports connect the ability of the Rep protein to bind to specific sites located within the intergenic region, to be responsible for successful replication of pseudorecombinant events. Reassortment experiments are usually performed between begomoviruses that have a bipartite or facultative bipartite genome (Saunders et al., 2002). Here we have demonstrated the possibility that the monopartite viruses, TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV transreplicate a heterologous B component of the facultative bipartite TYLCTHV-[AIT]. TYLCVV-[DX1] and ToLCVV-[DX2] share the same rep binding core (GGKGT) as found in TYLCTHV-[AIT], which could be one reason for the success in transreplicating TYLCTHV B DNA either in *N. benthamiana* or in tomato. Argüello-Astorga and Ruiz-Medrano developed in 2001 a model for the potential Rep-iteron contacts. They identified an iteron-related domain (IRD) mapped to the N terminal region of the Rep protein including the conserved 'motif 1'. The IRD in the rep protein of TYLCTHV-[AIT], TYLCVV-[DX1] and ToLCVV-[DX2] is identical and could be a determinant for binding of the rep protein to the DNA B component. However, TbLCTHV has a different core sequence and a different IRD but was able to facilitate transreplication of TYLCTHV B DNA. All begomoviruses are composed of an invariable GG sequence followed by three nucleotides (called N1, N2, and N3), which form the binding core sequence located in the intergenic region of the geminiviral

genome. According to Argüello-Astorga and Ruiz-Medrano (2001), the predicted amino-acid-nucleotide pairing occurs between X_{-4},and X_{-2} with the N3 nucleotide, N2 to X_1 and N1 to the last amino acid of the motif 1 named X_3. At the IRD in the Rep protein, amino acids X_{-2} (P) and X_3 (N) are identical in TYLCTHV-[AIT], TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV. These two amino acids could be sufficient to allow binding of the Rep protein to the DNA B component. Nevertheless, there are few examples (Andrade et al., 2006; Garrido-Ramirez et al., 2000) where infectious pseudorecombinants were formed between viruses, possessing Rep proteins containing different IRDs. Therefore, we cannot exclude that additional factors may contribute to transreplication of the TYLCTHV B DNA by heterologous tomato / tobacco leaf curl viruses.

The ability of geminiviruses to produce defective DNAs may play an important role not only in symptom modulation but also in evolution. Infectivity and the formation of defective molecules have been studied in different experimental hosts but also in natural hosts (Behjatnia et al., 2007; Patil et al., 2007). Defective DNAs were visualized from the Southern hybridization experiment and defective molecules were amplified using PCR from all combinations containing TYLCTHV B (data not shown). Sequencing revealed different types of molecules including the presence of deletions mutant containing almost the whole intergenic region with intact rep binding sites, and/or even recombinant molecules between A and B components, which might play a role in geminiviruses recombination and diversification. The intergenic region may serve as a source of heterologous DNA and therefore as donor sequences for other viruses. Interestingly, attempts to amplify defective DNAs derived from ToLCVVß DNA failed in almost all combinations used in the transreplication assays with exception from those performed with TYLCVV-[DX1] (data not shown). This may be due the fact that the presence of iterons in ß molecules are not essentially involved in virus replication specificity and/or to the higher preference of DNA A rep protein to bind more to DNA B molecules than to ß satellites.

The possibility that in future a new emerging virus could be originated in field due to the ability of TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV to transreplicate the B component from TYLCTHV-[AIT] cannot be excluded. New emerging viruses may be a product of different recombination events but also may result from the capture and reassortment of different DNA molecules. Severe yield losses of tomato may happen if mixture of viruses occurs with TYLCTHV B DNA. Furthermore, a special attention should be taken to the fact that TYLCTHV B DNA could overcome the phloem

limitation characteristic of TYLCVV-[DX1], ToLCVV-[DX2] in *N. benthamiana* and ToLCVV-[DX2] in tomato turning them into mechanical transmissible viruses. Finally, new biotechnological approaches for plant protection against geminivirus infection have to be developed. To date, the use of transgenic plants using inverted repeat constructs has been shown to confer only specific but not broad resistance. Because geminivirus infection are frequently reported as complex diseases, new studies e.g. identification of antibody-like molecules, which may confer broad-spectrum resistance to DNA viruses, might be important for resistance against geminiviruses.

3.0 Delayed symptom expression of transgenic *Nicotiana benthamiana* harboring six different inverted-repeat constructs of *Tomato yellow leaf curl Thailand virus* (TYLCTHV-[AIT])

3.1 Abstract

Tomato yellow leaf curl Thailand virus (TYLCTHV-[AIT]) is the only tomato yellow geminivirus found in the Old World, which has a bipartite genome constitution. Here we exploit the use of six different inverted-repeat constructs each containing the splicing intron ST-LS1 IV2 from potato for generating resistant *Nicotiana benthamiana* via *Agrobacterium tumefaciens* mediated transformation. Therefore, four inverted-repeat constructs were generated from the A component (AC2/C3, IR/CP, IR/Rep and Rep) and two from the B component (IR/BC and IR/BV) of TYLCTHV-[AIT].

A total of 114 putative transgenic *N. benthamiana* lines were regenerated from which 94 lines were tested for virus resistance in the T_1 generation and additional 9 lines in the T_2. Resistance tests were performed by inoculating a mixture of agrobacteria containing multimeric clones of the DNA A and DNA B genome. The symptom expression was monitored by calculating the disease index (DI) of each line. Transgenic *N. benthamiana* lines harboring sequences of AC2/C3, IR/CP, IR/BC showed significantly reduced DI values and therefore a resistance response. Delayed symptom expression of transgenic T_1 lines was obtained until 30 days post-inoculation (dpi) and even at 90 dpi for one line harboring the IR/CP construct. Moreover, it could be observed for the first time that plants harboring the inverted-repeat construct originating from the AC2/C3 region of the TYLCTHV-[AIT] genome show recovery symptoms. In general, transgenic lines showed lower calculated DI values in those experiments performed in the T_1 than in the T_2 generation. Three selected lines (BC4_1, BC8_8 and BC8_12) harboring partial sequences of the intergenic region and the gene coding for the movement protein of the DNA B showed a resistance response in the T_2 generation with a delayed symptom expression even at 29 dpi. The results obtained from resistance tests performed in the T_1 and T_2 generation will be presented and discussed.

Keywords: TYLCV, begomovirus, virus resistance, inverted-repeat constructs, RNAi

3.2 Introduction

The genus *Begomovirus* belongs to the family *Geminiviridae.* Begomoviruses consist of circular single-stranded molecules and are transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a circulative and propagative manner. They are classified as monopartite or bipartite viruses depending on their genome organization. Monopartite viruses consist of only one genomic DNA molecule, which may be associated with ß satellite viruses. Bipartite viruses have two genomic DNA molecules (called DNA A and DNA B).

Begomoviruses are responsible for extensive yield losses in many important crops throughout the world (America, the Caribbean basin, the Mediterranean basin, India and Southeast Asia). In Thailand, *Tomato yellow leaf curl Thailand virus* (TYLCTHV) has been described as a serious pathogen infecting *Solanum lycopersicum* plants, where yield losses may vary between 10% and 100% (Sawangjit et al., 2005). Therefore, different methods have been developed to generate transgenic plants, which are resistant against viruses. A common method in biotechnological approaches to generate transgenic plants that are resistant against plant RNA viruses is based on post-transcriptional gene silencing (PTGS) or RNA interference (RNAi), where small interfering RNAs (siRNAs) are generated *in planta*. The use of inverted-repeat constructs containing a viral gene, which is transcribed into dsRNA molecules, is today commonly used to confer virus resistance. These dsRNA molecules are targets for enzymes called Dicers resulting in siRNAs molecules (21 to 26 nt) having a 3'-protuding end. Once siRNAs are present inside the cells a complex with proteins (RNA-induced silencing complex (RISC)) is formed, which is guided to a complementary mRNA target.

Resistance approaches involving PTGS have been reported for plant RNA viruses (Chen et al., 2004; Goldbach et al., 2003; Missiou et al., 2004; Mitter et al., 2003; Pandolfini et al., 2003; Kalantidis et al., 2002; Wang et al., 2000; Smith et al., 2000) and also for plant DNA viruses (Chellappan et al., 2004a; Chellappan et al., 2004b; Vanitharani et al., 2005; Ribeiro et al., 2007). It has been suggested that although geminiviruses do not have a dsRNA phase in their replication cycle still PTGS is triggered in plants. Vanitharani et al. (2005) mentioned three different possibilities for formation of dsRNA molecules during geminivirus replication: (i) transcripts occurring from opposite polarity possibly formed from their bi-directional promoter, overlap at their 3'-ends forming dsRNAs (ii) abundant transcripts that serve as templates for the

host RdRp producing dsRNA molecules (iii) the possibility of secondary structures (dsRNA resembling structures) of geminivirus transcripts.

Only few reports exist on the use of RNAi-mediated virus resistance for the monopartite TYLCV (Zrachya et al., 2007b; Fuentes et al., 2006). Zrachya et al. (2007b) established transient assays involving agroinfiltration of a coat protein-silencing construct followed by infiltration of a green fluorescent protein (GFP)-fused - coat protein gene. The assays showed a down-regulation of the GFP expression in *Nicotiana benthamiana*. In addition, they produced transgenic tomato plants harboring an inverted-repeat construct targeting the CP. The transgenic plants did not show symptoms until seven weeks-post inoculation. Fuentes et al. (2006) developed resistant transgenic tomato plants, transformed with an intron-hairpin construct, which induced PTGS against the replication associated protein (Rep) gene of TYLCV-[Cuba].

TYLCTHV-[AIT] is a bipartite virus, consisting of six open reading frames (ORFs) in DNA A and two ORF in DNA B. DNA A component comprises two ORFs on the viral strand (AV1, AV2) and four ORFs on the viral-complementary strand (AC1, AC4, AC2, AC3) AV1 represents the coat protein and very recently the V2 protein has been shown to be involved in gene silencing suppression in TYLCV-[Israel] (Zrachya et al. 2007a). AC1 codes for the Rep protein involved in the viral DNA replication. AC2 and AC4/C4 proteins were demonstrated to be involved in gene silencing mechanism for TYLCV-[China] (Dong et al., 2003) and for *African cassava mosaic virus* (ACMV-[CM]) and *Sri Lankan cassava mosaic virus* (SLCMV) (Vanitharani et al., 2004), respectively. AC3/C3 protein functions as enhancer of transcription promoting viral replication (Settlage et al., 2005). The B component encodes two ORFs involved in virus movement. BV1 codes for the nuclear shuttle protein located on the viral strand and BC1 codes for the movement protein on the complementary strand. DNA A and DNA B share a common region (CR) of ~200 bps, containing several regulatory *cis*-elements and also the nonanucleotide sequence (TAATATTAC), which is known as recognition site for the Rep protein.

The main objective of this work is to assess, which region of the TYLCTHV-[AIT] genome is most suitable for generating inverted-repeat constructs leading to resistance via RNAi. To this extent, six intron-hairpin constructs containing different regions of the TYLCTHV-[AIT] genome were obtained. A total of 114 putative transgenic *N. benthamiana* lines were regenerated and 94 lines were tested for virus resistance. Resistance tests were performed by using a mixture of agrobacteria containing

multimeric clones of the DNA A and DNA B genome. The results obtained from the resistance tests performed in the T_1 and T_2 generation are presented and discussed.

3.3 Materials and Methods

Cloning of inverted-repeat hairpin constructs (intron-hpRNA constructs)

A general strategy was developed to construct the inverted-repeat cassettes. Three main steps were necessary for constructing the cassettes used to transform *Agrobacterium tumefaciens* LBA4404 or C58C1. The first step consisted of cloning PCR fragments of TYLCTHV-[AIT] DNA A and B blunt end into pBluescript or pGEM-T. The sense primers introduced the restriction sites *Asc*I and *Mlu*I and the antisense-primers introduced the restriction sites *Bam*HI and *Xba*I into the PCR fragments (Table 1). The *Asc*I/*Bam*HI digested fragments were subsequently cloned downstream of an enhanced (2X) 35S promoter of the *Cauliflower mosaic virus* (CaMV) and upstream of the CaMV poly-A-signal. Simultaneously fragments digested with *Mlu*I/*Xba*I were cloned into a plasmid containing the splicing intron ST-LS1 IV2 from potato. The remaining step consisted of joining both fragments residing in the plasmid containing the e35S promoter/poly-A-signal and in the plasmid containing the intron, respectively, resulting finally in plasmids containing the viral gene in sense and antisense orientation separated by the intron. The entire cassettes consisting of e35S/inverted repeat separated by ST-LS1 IV2 followed by the poly-A-signal were thereafter cloned into binary vectors (Figure 1). Cassettes originated from regions IR/CP, IR/Rep, AC2/C3, Rep and IR/BV of the TYLCTHV-[AIT] genome were cloned into pLX222 (Landsmann et al., 1988) and those originated from the IR/BC region was cloned into the modified binary vector pBIN 19 (Bevan, 1984).

Table 1. Primers used to amplify TYLCTHV sequences

Cassette	Primer name: sequence (5´- 3´)
IR/Rep	TYLCTHV_IR S: AA*GGCGCGCCACGCGT*ATGCGTCGTTGGCAGATTGG
	TYLCTHV_IR AS: AA*GGATCCTCTAGA*AAAAAAAATCGCGGCCATCC
Rep	TYLCTHV_Rep S: AA*GGATCCTCTAGA*ACTCTCCGTCGTCTGGTTGT
	TYLCTHV_Rep AS: AA*GGCGCGCCACGCGT*TAACTAACTAAGAACTTGAAGAATGGGCTTG
AC2/C3	TYLCTHV_AC2/C3 S: AA*GGCGCGCCACGCGT*GTTATTTCTATGACTCAGTGA
	TYLCTHV_AC2/C3 AS: AA*GGATCCTCTAGA*TAACTAACTAACCGCACTTAGAAACTGGGCT
IR/CP	TYLCTHV_CP S: AA*GGCGCGCCACGCGT*TAACTAACTAAGAGAAGACGTATTCCCCTGA
	TYLCTHV_CP AS: AA*GGATCCTCTAGA*ACCTGCTGAAAATCATAAGG
IR/BV	TYLCTHV_BV S: AA*GGCGCGCCACGCGT*TAACTAACTAATACATATCCAATTCATTTGC
	TYLCTHV_BV AS: AA*GGATCCTCTAGA*CAACAGCTGTGCAATATATG
IR/BC	TYLCTHV_BC S: AA*GGATCCTCTAGA*TCAATCTTTGGGGATTTAAA
	TYLCTHV_BC AS: AA*GGCGCGCCACGCGT*TAACTAACTAAGAGTTAGTGGTATTTAAAGG

Restriction sites are underlined and in italics

Six different constructs were made of the TYLCTHV-[AIT] genome. Figure 1 shows the schematic representation of the generated cassettes.

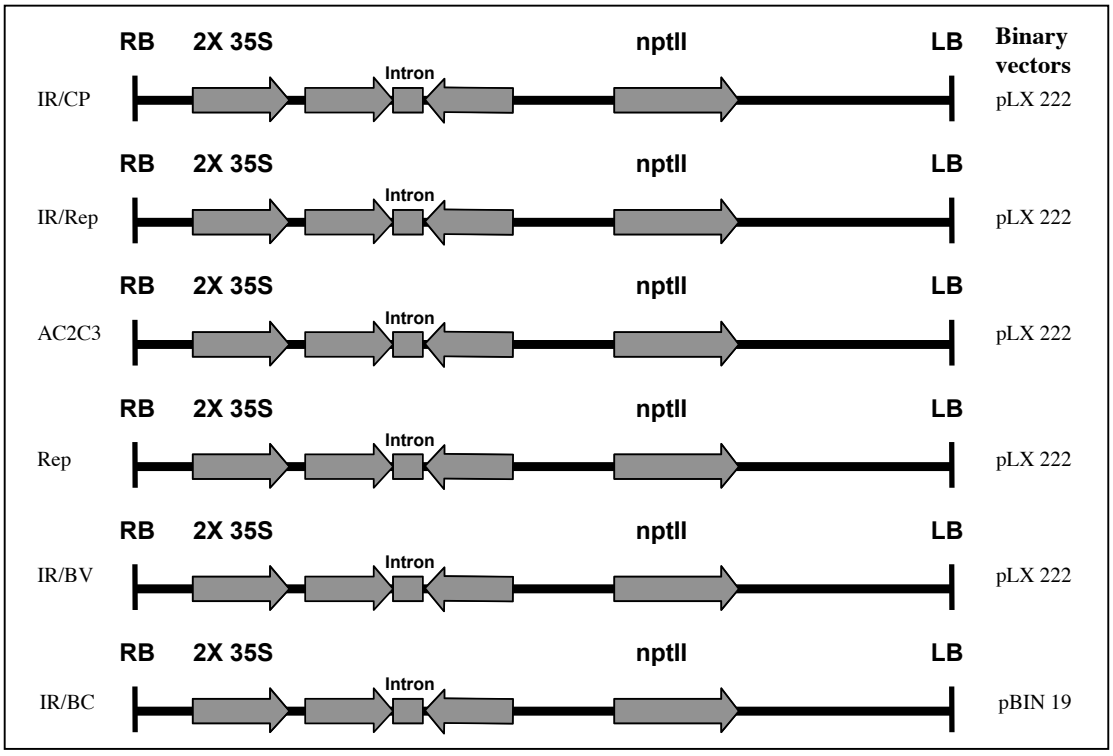


Figure 1. Physical map of the intron-hairpin RNA expression cassettes. 2X 35S: enhanced 35S promoter of CaMV; Intron: ST-LS1 IV2 from potato; RB: right border; LB: left border; nptII: neomycin phosphotransferase II; pLX222 and pBIN 19: binary vectors.

Plant transformation

N. benthamiana plants were transformed according to standard protocols. Briefly, leaf disks were incubated in 20 ml of liquid Murashige and Skoog-medium (MS) containing 200 µl of agrobacteria. The suspension was incubated at 26-28°C in a climate chamber for three days. Leaf discs were washed in water containing 500 mg/L of Claforan and transferred to solid MS-medium containing hormones. The kanamycin-resistant regenerated plantlets were transferred to soil and kept under greenhouse conditions. Transgene integration in T_0 plants was confirmed by PCR. The plants were self-pollinated and seeds were used to generate T_1 plants for the first resistance test. Those plants showing a recovery or delay in symptoms were used for seed production. Their progenies (T_2 plants) were used for the second resistance test. The number of transgene insertions was verified by placing selected T_1 or T_2 seeds on 400 mg/L of kanamycin MS-medium. The numbers of surviving and dead plantlets were counted and statistical analysis (χ^2-test) was employed to determine the numbers of insertion.

Resistance test using Agrobacterium tumefaciens and evaluation of symptoms

The resistance screening was performed using multimeric clones originated from TYLCTHV-[AIT] A and B components (Knierim and Maiss, 2007). Each multimeric clone was grown separately in LB-media until OD_{600} of around 1.7. 1 ml of agrobacteria culture containing each of the multimeric clone of TYLCTHV-[AIT] A and B was centrifuged and resuspended in a final volume of 2 ml of agroinoculation buffer (10 mM $MgCl_2$, 10 mM MES, 100 µM Acetosyringon) and incubated for 3 to 4 hours. 1 ml of a 1:500 dilution was used to agroinoculate each transgenic plant in a 4 to 5 leaf stage. Fifteen plants originating from seeds of each transgenic T_0 line and also 15 non-transgenic plants were agroinoculated for each experiment. Symptoms severity was assessed for each plant and graded on a scale of 0 to 4. Disease index (DI) was calculated using the grade of infection: (a) grade of 0 for symptomless; (b) 1 for very mild yellowish mosaic and very mild downward leaf curling; (c) 2 for mild yellowish mosaic and mild downward leaf curling; (d) 3 for yellow spots and downward leaf curling and (e) 4 for severe yellowish mosaic, severe stunting and severe downward leaf curling.

DI were calculated according to the formula below,

$$DI=\frac{(0xa)+(1xb)+(2xc)+(3xd)+(4xe)}{(a+b+c+d+e)x4}$$

where (a, b, c, d, and e) represents the number of plants showing the respective symptoms. DI values were meaning as follows: (0) plants are considered to be immune; (0-0.3) plants are highly resistant; (0.3-0.6) plants are resistant; (0.6-0.8) plants are mildly resistant and (0.8-1) plants are susceptible. These values were used according to Zhandong, et al. (2007), who classified the resistance level against *Turnip mosaic virus* in Chinese cabbage. The data obtained from the agroinoculation experiments were analyzed statistically using the Mann-Whitney test (U-test). The p=0.05 threshold was used throughout all statistical analyses.

3.4 Results

In this work we assessed the potential of six different intron-hpRNA constructs covering the whole genome of TYLCTHV-[AIT] for RNAi mediated resistance in *N. benthamiana*. Four cassettes are derived from TYLCTHV-[AIT] A component and two cassettes from the B component. To this purpose, different regions of the TYLCTHV-[AIT] genome were cloned in an inverted repeat orientation separated by the splicing intron ST-LS1 IV2 from potato and driven by the enhanced constitutive CaMV 35S promoter. *N. benthamiana* plants were transformed using *Agrobacterium tumefaciens* and resistance tests were performed using agroinoculation of multimeric clones derived from A and B components.

Evaluation of the AC2/C3 intron-hpRNA construct in the T_1 generation

The region 1185-1786 (601 bp) of TYLCTHV-[AIT] DNA A was used to generate the AC2/C3 intron hpRNA construct. Nine different PCR-positive transgenic lines were employed in the resistance test. Figure 2 shows the results of the calculated DI obtained in the T_1 generation. Symptom development was monitored until 76 dpi.

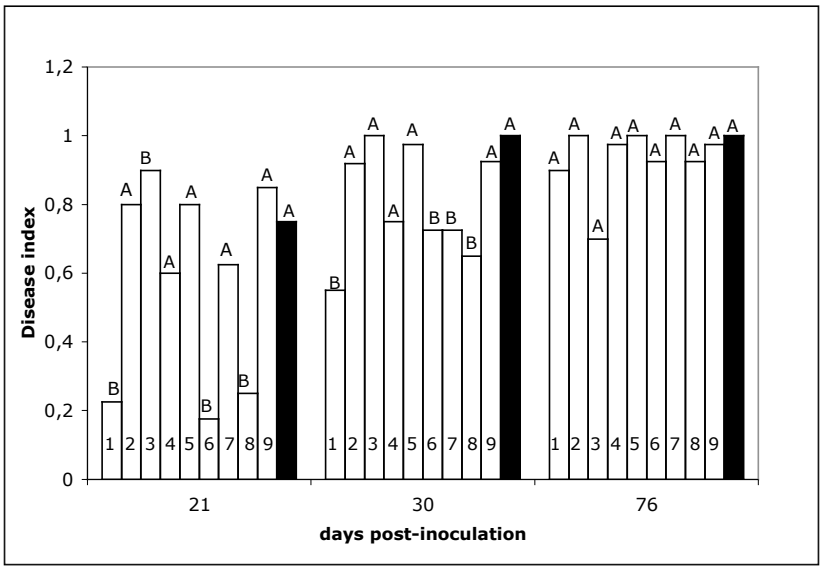


Figure 2. Disease index of lines transformed with AC2/C3 construct. Disease symptoms were scored 21, 30 and 76 days post-inoculation and the disease index was calculated for the transgenic plants containing the AC2/C3 intron-hpRNA construct. Bar numbers represent transgenic lines (1: AC2/C3_1; 2:AC2/C3_2; 3: AC2/C3_3; 4: AC2/C3_4; 5: AC2/C3_5; 6: AC2/C3_6; 7: AC2/C3_7; 8: AC2/C3_8; 9: AC2/C3_9 and black bars are non-transgenic plants agroinoculated with multimeric clones). Bars represented with the same letter per time point are not significantly different (p=0.05, Mann-Whitney test).

Lines AC2/C3_1, AC2/C3_6 and AC2/C3_8 were considered to be highly resistant at 21 dpi with calculated DI values varying from 0.175 to 0.25. The percentage of symptomatic plants was 60%, 40% and 70%, respectively at 21 dpi. After 30 dpi the percentage of symptomatic plants increased to 90%, 90% and 100%, respectively and DI values ranged from 0.55 to 0.725. Disease index values were statistically significant when compared to non-transgenic plants and transgenic lines AC2/C3_1, AC2/C3_6 and AC2/C3_8 were still considered to be resistant to mildly resistant. Figure 3 shows symptomatic plants from these lines at 30 and 68 dpi. At 76 dpi, all putative transgenic lines with the exception of one showed 100% of symptomatic plants and DI values did not differ statistically from the non-transgenic plants.

The transgenic line AC2/C3_3 was the only one generated in this work, which showed a recovery phenotype (Figure 3-A and B). 90% of the plants showed very severe symptoms until 21 dpi. However, after 51 dpi an amelioration of symptoms was observed in four plants. Newly emerging leaves did not show geminivirus symptoms. The obtained DI value was not statistically different from the DI valued obtained from the non-transgenic plants.

Finally, none of the generated lines showed to be immune after 76 days and only one plant belonging to the line AC2/C3_1 showed no symptoms (Figure 3-D). This plant was self-pollinated and the seeds were used for further experiments in the T_2 generation.

Figure 3. Symptoms of plants transformed with the AC2/C3 construct. A. Symptomatic plant from line AC2/C3_3 at 30 dpi; B. Plant from line AC2/C3_3 at 68 dpi showing a recovery phenotype; C. Non- transgenic plant at 30 dpi; D. Plant from line AC2/C3_1 at 68 dpi; E. Plant from line AC2/C3_8 at 68 dpi; F. Plant from line AC2/C3_6 at 30 dpi.

Evaluation of the IR/CP intron-hpRNA construct in the T_1 generation

The region 367-906 (540 bp) of TYLCTHV-[AIT] A DNA was used for constructing the IR/CP intron hpRNA cassette. A total of 37 transformed *N. benthamiana* plants were regenerated. From those, 26 different transgenic lines were selected using PCR. 19 lines were used for the resistance test performed in the T_1 generation.

The calculated DI values were obtained from two experiments, where disease scores were monitored until 90 dpi and 43 dpi, respectively (Figure 4). A third experiment was performed using additionally five lines (data not shown). However, none of the five lines showed delayed symptoms.

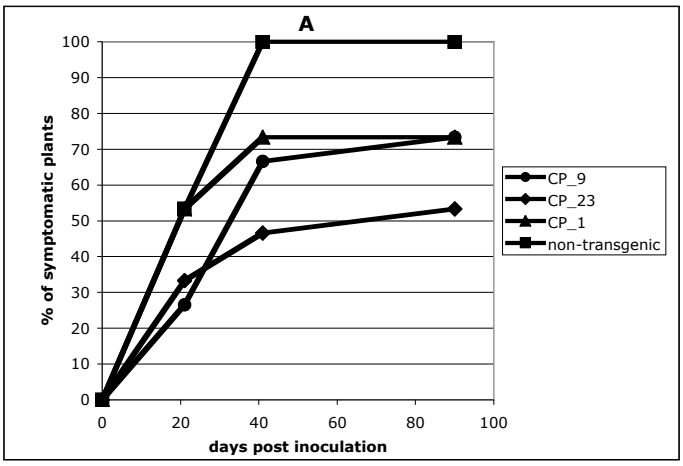
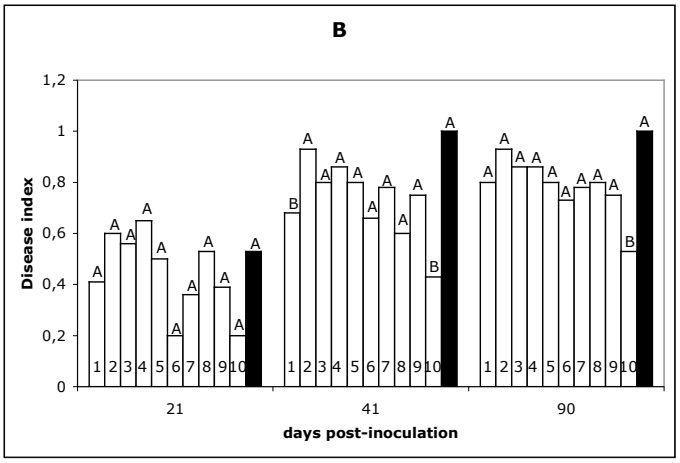
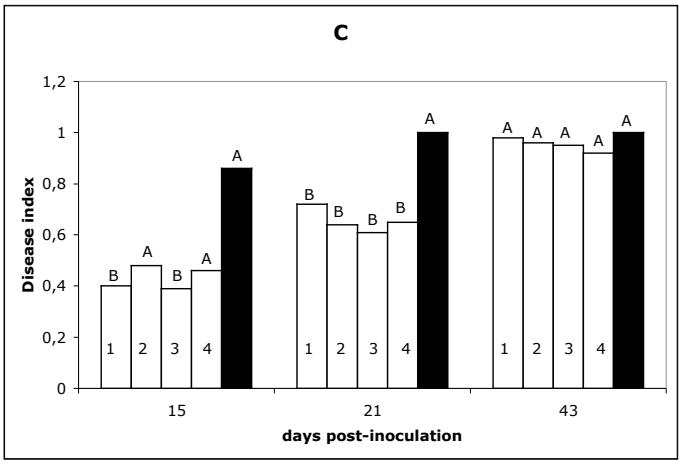


Figure 4. Resistance tests of IR/CP transgenic lines. A. Percentage of symptomatic plants obtained from selected coat protein inverted-repeat constructs (CP_9, CP_23 and CP_1) transgenic lines.



B. Disease index of lines transformed with IR/CP construct. Disease symptoms were scored at 21, 41 and 90 days post-inoculation for the first experiment. The disease index was calculated for transgenic plants containing the IR/CP intron-hpRNA construct. Bar numbers represent transgenic lines (1: CP_1; 2: CP_3; 3: CP_4; 4: CP_6; 5: CP_8; 6: CP_9; 7: CP_10; 8: CP_11; 9: CP_18; 10: CP_23).



C. Disease index of lines transformed with IR/CP construct. Disease symptoms were scored at 15, 21 and 43 dpi for the second experiment. Bar numbers represent transgenic lines (1: CP_20; 2: CP_29; 3: CP_32; 4: CP_33). In B. and C. black bars are non-transgenic plants agroinoculated with multimeric clones. Bars represented with the same letter per time point are not significantly different (p=0.05, Mann-Whitney test).

At 21 dpi transgenic lines CP_1, CP_9 and CP_23 showed 53%, 26% and 33%, symptomatic plants (Figure 4), whereas the non-transgenic plants revealed 53% symptomatic plants. At this early stage of the first experiment (Figure 4-A) transgenic plants showed DI values that did not differ significantly from non-transgenic plants. Nevertheless, at 41 dpi transgenic lines CP_1 and CP_23 had DI values of 0.68 and 0.43, respectively. Thus, transgenic lines CP_1, CP_23 and CP_9 showed disease resistance (Figure 5). The transgenic line CP_23 was highly resistant at 41 dpi when compared to non-transgenic plants (disease index 1) and even at 90 dpi, this line showed only 53% of symptomatic plants and a respective DI value of 0.46. On the other hand, DI values obtained from the second experiment were not statistically significant at 43 dpi, when compared each line to the control (Figure 4-B). Therefore, asymptomatic plants of line CP_23 were self-pollinated and the seeds were used for further experiments in the T_2 generation.

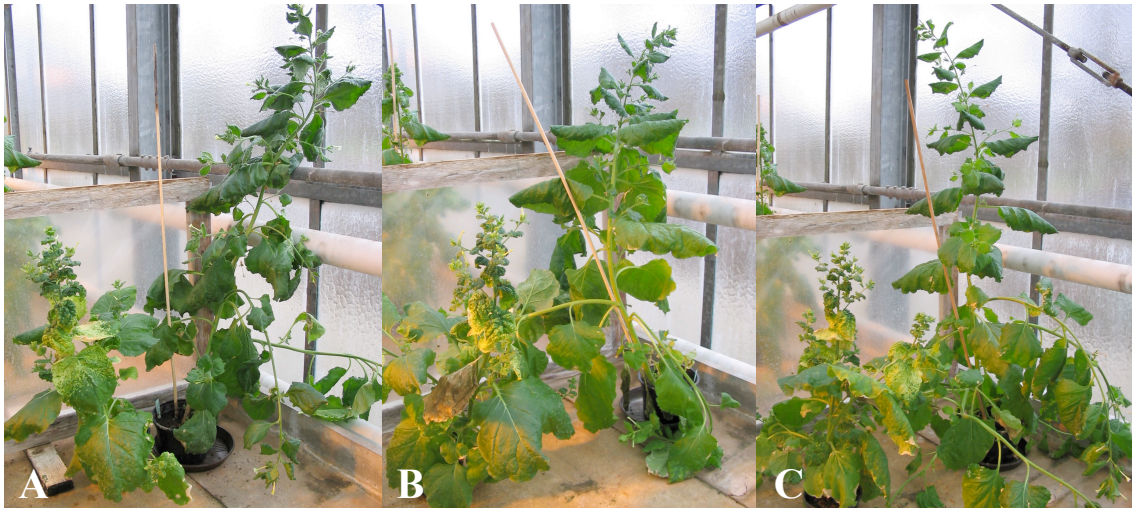


Figure 5. Symptoms of plants transformed with the CP construct. A. Plant from line CP_1 at 60 dpi; B. Plant from line CP_23 at 60 dpi; C. Plant from line CP_9 at 60 dpi. Plants on the left hand side are non-transgenic plants inoculated with multimeric copies of DNA A and B of TYLCTHV-[AIT].

Evaluation of IR/Rep and Rep intron-hpRNA constructs in the T_1 generation

The region 2348-171 (571 bp) was selected to construct the IR/Rep inverted repeat cassette, which includes the 5'-terminal sequence of the Rep. The region 1665-2165 (501 bp) was used for constructing the Rep intron-hpRNA. This region includes not

only the 3'-terminal part of the Rep sequence but also a part of the 5'-terminus of the AC2 gene.

Fifteen putative transgenic lines were generated containing the Rep intron-hpRNA construct. From those, 13 lines were tested for virus resistance in two experiments. Figure 6-A and B shows the calculated DI values.

In the first experiment only line Rep_5 was considered as a resistant one (DI of 0.6) until 21 dpi showing 86% of symptomatic plants. Transgenic lines Rep_2, Rep_4, Rep_7 and Rep_16 were considered as mildly resistant lines with DI values ranging from 0.63 to 0.78. At 41 dpi none of the lines showed a significant difference in DI values when compared to non-transgenic plants. Seven putative transgenic lines were employed in the second experiment and the disease score was monitored at 14 and 31 dpi, respectively. At 14 dpi only the line Rep_21 showed the lowest DI of 0.53 having 66% of symptomatic plants and thus was considered as a resistant line. However, at 31 dpi all lines revealed a similar DI not different from the DI of the non-transgenic plants. A total of 25 transgenic lines were generated containing the IR/Rep inverted-repeat construct. Twenty putative transgenic lines were tested in two experiments for virus resistance in the T_1 generation. The first experiment involved 13 transgenic lines. Those thirteen lines showed already at 23 dpi a DI value of 1, meaning that 100% of the plants were pursuing severe symptoms. All lines were considered to be susceptible and not employed in further experiments (data not shown). The second experiment involved seven different transgenic lines, with DI values plotted in figure 6-C. At 15 dpi lines IR/Rep_23 and IR/Rep_35 showed 80% and 64% of symptomatic plants, respectively and the lowest DI values of 0.58 and 0.43. However, after 21 dpi only the line IR/Rep_35 showed a statistically significant different DI value of 0.55 with 85% of the plants showing symptoms. Therefore, this line was classified as resistant. At 41 dpi all plants were susceptible to TYLCTHV-[AIT]. Finally, none of the lines IR/Rep or Rep showed to be resistant at 41 dpi.

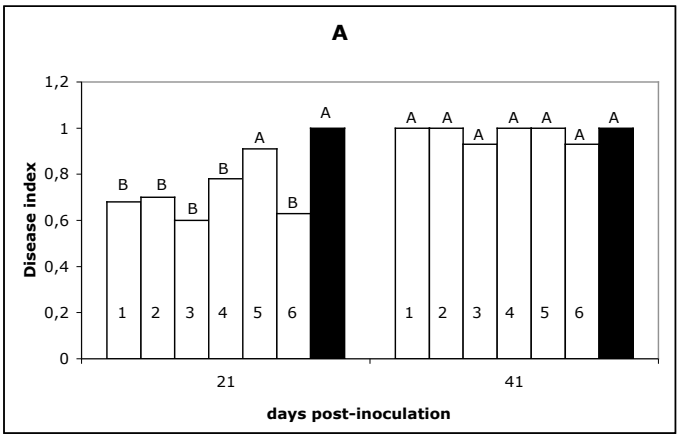
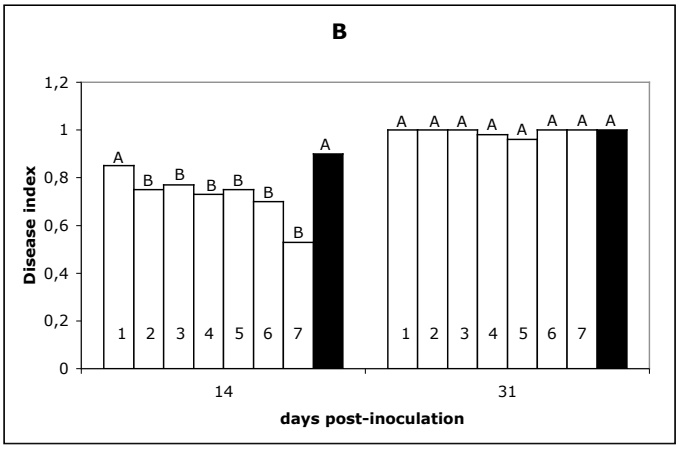
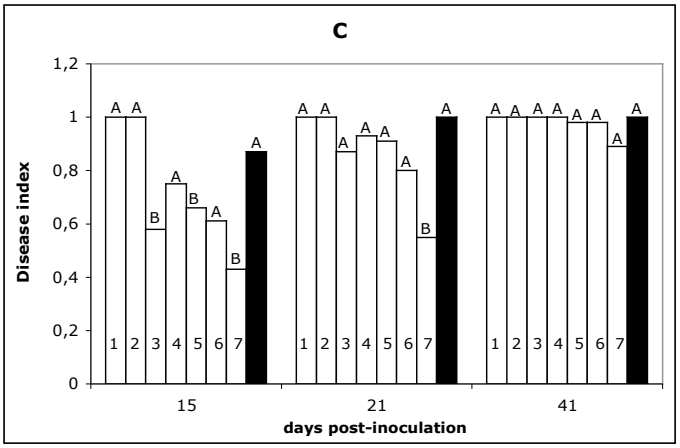


Figure 6. Resistance tests of Rep and IR/Rep transgenic lines. A. Disease index of lines transformed with Rep construct. Disease symptoms were scored at 21 and 41 dpi. Bar numbers represent transgenic lines (1: Rep_2; 2: Rep_4; 3: Rep_5; 4: Rep_7; 5: Rep_12; 6: Rep_16);



B. Disease index of lines transformed with Rep construct. Disease symptoms were scored at 14 and 31 dpi. Bar numbers represent transgenic lines (1: Rep_10; 2: Rep_11; 3: Rep_14; 4: Rep_15; 5: Rep_18; 6: Rep_20; 7: Rep_21);



C. Disease index of lines with IR/Rep construct. Disease symptoms were scored at 15, 21 and 41 dpi. Bar numbers represent transgenic lines (1: IR/Rep_11; 2: IR/Rep_20; 3: IR/Rep_23; 4: IR/Rep_24; 5: IR/Rep_33; 6: IR/Rep_34; 7: IR/Rep_35). In A., B. and C. black bars are non-transgenic plants agroinoculated with multimeric clones. Bars represented with the same letter per time point are not significantly different (p=0.05, Mann-Whitney test).

Evaluation of IR/BC intron-hpRNA constructs in the T_1 generation

The region 1729-2308 (580 bp) of TYLCTHV-[AIT] DNA B was used to create the respective IR/BC intron-hpRNA construct. Fifteen putative *N. benthamiana* transgenic lines were regenerated and 14 lines were tested in three experiments (Figure 7-A, B and C) for resistance. From those, 8 lines (BC_1, BC_10, BC_11, BC_17, BC_20, BC_22, BC_23, and BC_25,) were shown to be resistant until 21 dpi. The best resistance was obtained using BC_4, BC_8 and BC_13 lines. At 21 dpi, those respective lines showed 73%, 33% and 47% of symptomatic plants, which increased to 87%, 80% and 80%, respectively at 40 dpi (Figure 7-D). At 31 dpi, only the line BC_20 showed a DI value, which was significantly different from the non-transgenic plants. Thus, plants were considered as mildly resistant.

Finally, at 41 dpi none of the putative transgenic lines showed to be resistant against TYLCTHV-[AIT]. Because delayed symptom expression was obtained using plants of line BC_8 and BC_4, these were selected for the resistance test performed with T_2 plants.

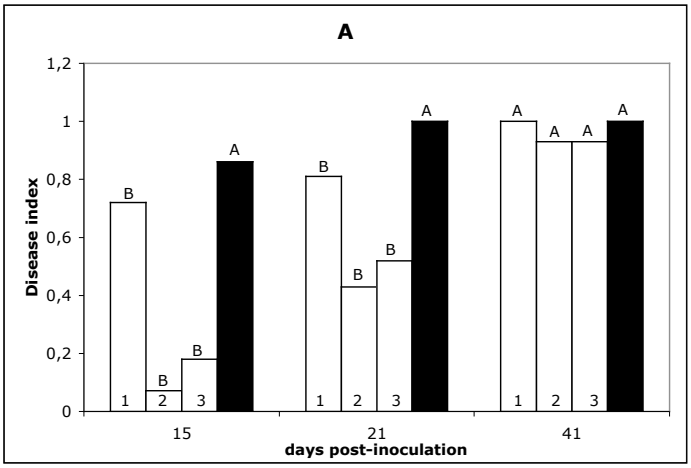
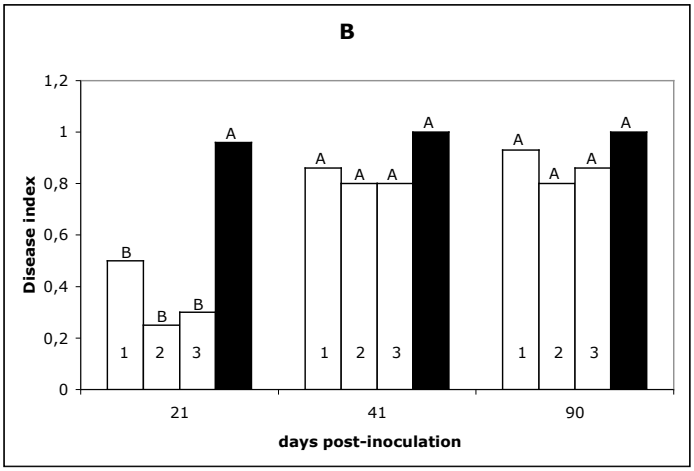
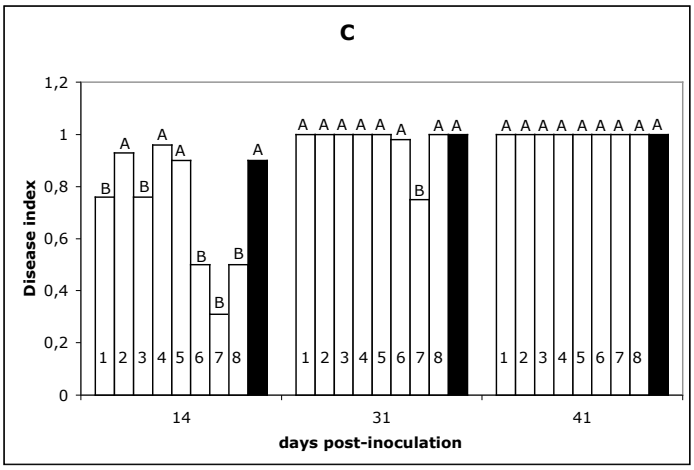


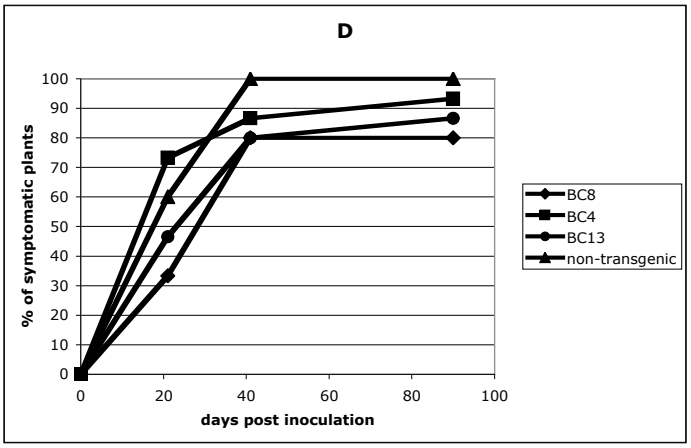
Figure 7. Resistance tests of IR/BC transgenic lines. A. Disease index of lines transformed with IR/BC construct. Disease symptoms were scored at 15, 21 and 41 dpi. Bar numbers represent transgenic lines (1: BC_11; 2: BC_23; 3: BC_25);



B. Disease index of lines transformed with IR/BC construct. Disease symptoms were scored at 21, 41 and 90 dpi. Bar numbers represent transgenic lines (1: BC_4; 2: BC_8; 3: BC_13);



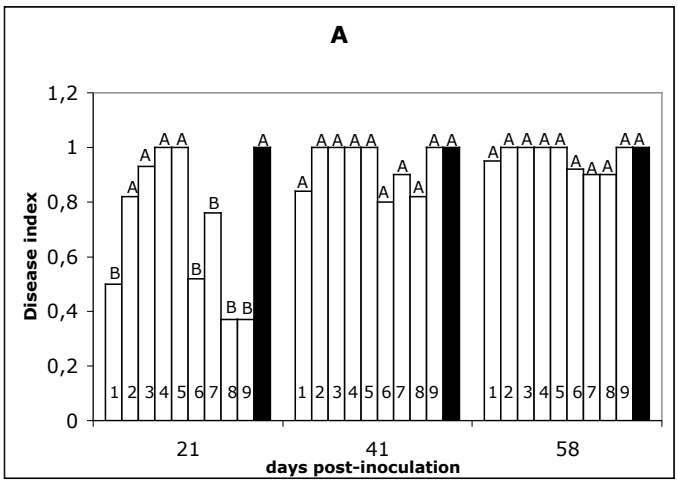
C. Disease index of lines transformed with IR/BC construct. Disease symptoms were scored at 14, 31 and 41 dpi. Bar numbers represent transgenic lines (1: BC_1; 2: BC_7; 3: BC_10; 4: BC_12; 5: BC_15; 6: BC_17; 7: BC_20; 8: BC_22). In A., B. and C. black bars are non-transgenic plants agroinoculated with multimeric clones. Bars represented with the same letter per time point are not significantly different (p=0.05, Mann-Whitney test).



D. Percentage of symptomatic plants from selected IR/BC lines.

Evaluation of IR/BV intron-hpRNA constructs in the T_1 generation

The region 441-981 (541 bp) of TYLCTHV-[AIT] A DNA was employed to generate the IR/BV inverted-repeat construct. Twenty-four transgenic lines were obtained, from which 19 lines were tested for resistance in two experiments (Figure 8-A and B).



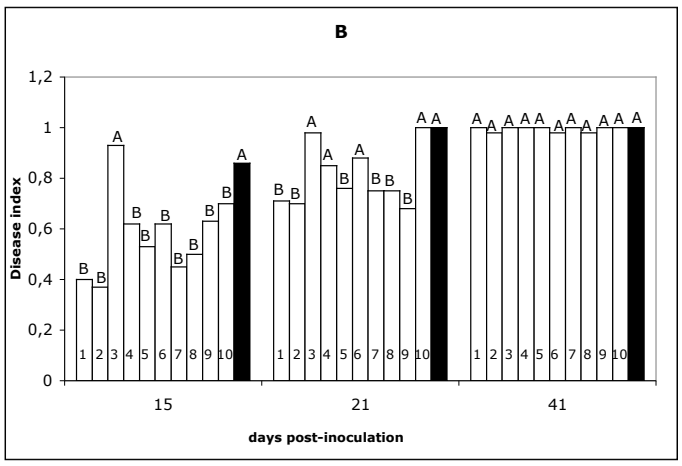


Figure 8. Resistance tests of IR/BV transgenic lines. A. Disease index of lines transformed with IR/BV construct. Disease symptoms were scored at 21, 41 and 58 dpi. Bar numbers represent transgenic lines (1: BV_1; 2: BV_3; 3: BV_4; 4: BV_5; 5: BV_8; 6: BV_11; 7: BV_12; 8: BV_14; 9: BV_24); B. Disease index of lines transformed with IR/BV construct. Disease symptoms were scored at 15, 21 and 41 dpi. Bar numbers represent transgenic lines (1: BV_13; 2: BV_16; 3: BV_17; 4: BV_18; 5: BV_21; 6: BV_23; 7: BV_27; 8: BV_30; 9: BV_34; 10: BV_35); In A. and B. black bars are non-transgenic plants agroinoculated with multimeric clones. Bars represented with the same letter per time point are not significantly different (p=0.05, Mann-Whitney test).

Eleven putative lines were shown to be highly resistant until 15 dpi according to the calculated DI values. Six days later, at 21 dpi, still 6 lines showed DI values, which were statistically different from the DI value calculated for non-transgenic plants. Therefore, four out those eleven putative lines (BV_1, BV_11, BV_14 and BV_24)

were considered as resistant. Disease index values were 0.5, 0.52, 0.37 and 0.37, respectively. Moreover, lines BV_14 and BV_24 showed a relative low percentage of symptomatic plants (60%) at 21 dpi when compared to all other lines (80%-100%). Finally, at 41 dpi all transgenic lines showed DI values of at least 0.80. Transgenic lines BV_1, BV_11, BV_14 and BV_12 were employed in the T_2 resistance test.

Evaluation of selected lines for the T_2 resistance test

After the resistance screening performed in the T_1 generation, nine transgenic lines were selected for the second (T_2) resistance test. For this purpose, seeds from asymptomatic plants and plants with delayed symptom expression in the T_1 generation were collected. In addition, eight out of these nine lines were tested for their segregation pattern of the nptII gene by placing selected T_2 seeds on MS kanamycin medium (Table 2). Five lines proved to be homozygous and 3 lines were heterozygous, whereas two of them (BC8_12 and BV14_1) have one insertion and BV11_15 has four insertions.

Table 2. Segregation analysis of transgenic *N. benthamiana* T_2 lines. Segregation pattern of the nptII gene in the T_2 generation of the selected plants containing four different intron-hairpin constructs was estimated by counting of surviving seedlings on kanamycin containing MS medium.

Transgenic lines	Number of seedlings (survived/dead)	χ^2 –test for segregation pattern	($\chi^2_{1;0.05} \leq 3,84$)
AC2/C3_1_1	517/0	Homozygous	-
CP23_1	174/0	Homozygous	-
BC8_8	276/0	Homozygous	-
BC4_1	270/0	Homozygous	-
BC8_12	144/38	3:1	1.648
BV14_11	47/13	3:1	0.355
BV1_5	66/0	Homozygous	-
BV11_15	393/2	255:1	0.1359

Transgenic plants containing the IR/BC inverted construct (BC8_8, BC8_12 and BC4_1) showed the lowest calculated DI value of 0.45 at 18 dpi and only 66% of symptomatic plants, respectively. Even at 29 dpi, these lines showed DI values (0.76 to

0.78), which were significantly different from the non-transgenic plants. At this stage, plants were classified as mildly resistant. The delayed symptoms were observed when compared to the non-transgenic plants. All other lines showed 100% of symptomatic plants after 29 dpi and a DI of 1 (Figure 9).

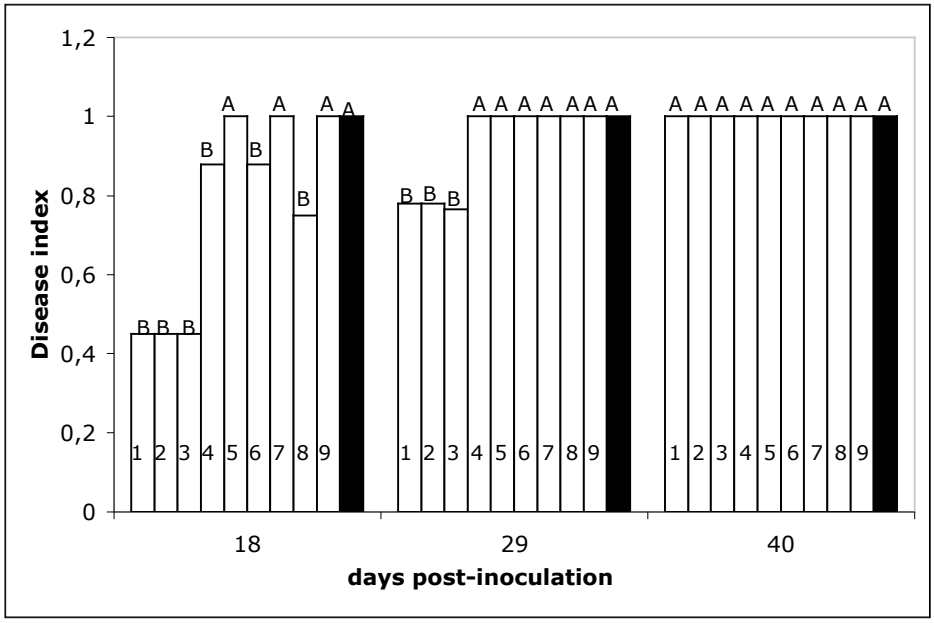


Figure 9. Disease index of selected transgenic T_2 lines. Disease symptoms were scored at 18, 29 and 40 dpi. Bar numbers represent transgenic lines (1: BC8_8; 2: BC8_12; 3: BC4_1; 4: AC2/C31_1; 5: BV14_11; 6: BV12_4; 7: BV1_5; 8: BV11_15; 9: CP23_1); Bars represented with the same letter per time point are not significantly different (p=0.05, Mann-Whitney test).

3.5 Discussion

Geminiviruses have caused much damage in crop plants, especially in tomato since the beginnings of the 1960's. In Asia, areas as Thailand and Vietnam have been serious compromised and losses have been reported between 40% and 100% (Czosnek and Laterrot, 1997). Begomoviruses as tomato leaf curl viruses and beta satellites have been found not only in tomato plants but are also present in weeds in this area. They are transmitted by the whitefly *Bemisia tabaci* (Gennadius) and many important measures of insect and weed control have been used to obtain plants that are free or almost free of viruses. The use of high amounts of insecticides to control the vector in Asia is expensive and led to development of resistance in the insect population (Morales, 2006). Therefore, different biotechnological approaches have been studied to confer resistance against geminiviruses.

Expression of viral proteins can mediate resistance in transgenic plants (protein-mediated resistance). The expression of truncated proteins has been susccessfully employed against geminiviruses (Brunetti et al., 2001; Kunik et al., 1994; Lucioli et al., 2003; Noris et al., 1996a). The main protein employed in protein-mediated resistance is the Rep protein. The geminivirus Rep protein regulates transcription of sense and complementary viral genes and also mediates origin recognition and DNA cleavage/ligation to begin and end rolling circle replication. Rep protein acts as a transcriptional regulator to repress its own synthesis and activates host gene expression, for instance the proliferating cell nuclear antigene (PCNA). In addition, the Rep protein is involved in several protein interactions, including oligomerization, interaction with the retinoblastoma-related protein (pRBR) and binding to the viral replication enhancer AC3. pRBR interacts with transcription factors (E2F), playing a central role in regulating cell growth and death. Probably the Rep geminivirus protein binds as well to other host proteins and transcription factors playing an important role in virus replication and host interaction. Because the Rep protein is involved in many different pathways, it has been from all geminivirus proteins the mostly employed in different strategies to confer geminivirus resistance (Bendahmane and Gronenborn, 1997; Bonfim et al., 2007; Brunetti et al., 2001; Lucioli et al., 2003; Noris et al., 1996a; Shivaprasad et al., 2006). Lucioli et al. (2003) reported two distinct molecular mechanisms involved in establishing resistance against *Tomato yellow leaf curl Sardinia virus*. The expression of the N-terminal 210 amino acids (Rep-210) conferred resistance to homologous and heterologous viruses either via the ability of the Rep-210

to inhibit Rep transcription or via protein interaction, respectively. Kunik et al. (1994) used the gene that encodes the CP of the monopartite *Tomato yellow leaf curl virus* placed under the control of the 35S promoter to transform tomato plants. Transformed tomato plants exhibit delayed symptom expression up to one month and recovered phenotypes (after 4 months). Reinoculation in recovered plants resulted in appearance of the disease after 2.5 months followed again by recovery symptoms. Delayed symptoms expression was explained by the fact that only few virions would enter the nucleus due to overexpression of the CP. Moreover, the B component of bipartite viruses, which encodes two proteins, the nuclear shuttle protein (BV1) and the movement protein (BC1), has been used to confer resistance against geminiviruses (Duan et al., 1997b). In 1997b, Duan et al. transformed tobacco plants with sense and antisense constructs of BC1 and BV1 genes of *Tomato mottle virus* (ToMoV), a bipartite virus. Transgenic plants expressing high levels of BC1 showed phenotypic differences, which were associated to transgene mutations. Three selected T_1 lines out of 19 expressing the BC1 protein showed plants with different response to virus inoculation including symptomatic, asymptomatic and recovery patterns. Both single and multiple-copy BC1 transgenic tobacco showed the same level of resistance. However, in the present work we were interested to know if TYLCTHV can be a successful target of PTGS by using RNAi and not by viral protein expression. Therefore, inverted-repeat constructs from the TYLCTHV-[AIT] genome, which contain stop codons placed in different frames to avoid the generation of possibly truncated proteins, were generated.

Inverted repeat constructs to confer resistance against RNA viruses have been successfully used by different research groups (Bucher et al., 2006; Tougou et al., 2006; Di Nicola-Negri et al., 2005; Chen et al., 2004; Missiou et al., 2004; Mitter et al., 2003; Pandolfini et al., 2003; Kalantidis et al., 2002; Smith et al., 2000). Even DNA viruses have been successfully shown to be a target of PTGS by transforming plants with inverted-repeat constructs (Fuentes et al., 2006; Pooggin et al., 2003; Bonfim et al., 2007; Zrachya et al., 2007b) or employing antisense RNA (Asad et al. 2003; Bejarano et al., 1994; Bendahmane and Gronenborn, 1997; Mubin et al., 2007; Yang et al., 2004b). However, complete immune transgenic lines were not obtained using intron-hpRNA constructs for *Tomato chlorotic mottle virus* (Ribeiro et al., 2007) or employing sense-antisense sequences of *Tomato yellow leaf curl Sardinia virus* (Noris et al., 2004). Moreover, silencing escape has been shown for *Tomato leaf curl virus* (Bian et al., 2006).

Different regions of the begomovirus genome have been successfully used to trigger PTGS. The AC2/C2 protein has been associated to suppression of gene silencing in TYLCV-[China] (Dong et al., 2003), in *Mungbean yellow mosaic virus*-Vigna (MYMV) (Trinks et al., 2005), in ACMV-[CM] and SLCMV (Vanitharani et al., 2004). Therefore, transforming plants with inverted-repeat constructs harboring the begomoviral AC2 sequence may be very promising. In 2003, Asad et al. produced resistant transgenic tobacco plants expressing sense and antisense RNAs from regions -5' and -3' of the Rep sequence and also from the overlapping genes AC2 and AC3 of the *Cotton leaf curl virus*. Asad and coworkers generated 20-25 independent T_0 transgenic lines for each construct (3 sense and 3 antisense constructs), where 12 T_1 lines per construct were scored for disease expression. Lines were scored as resistant if more than 70% of plants showed no symptoms. Only two out of 12 tobacco antisense lines and four out of 12 tobacco sense lines did not developed symptoms for the AC2/C3 construct for 120 days. However, the authors could not exclude the possibility that resistance was due to production of trans-dominant defective geminiviral proteins encoded by the constructs. In opposite to our results, Asad and coworkers did not observe a recovery phenomenon in any of the susceptible AC2AC3 transgenic lines. Moreover, when tobacco T_2 and T_3 lines were tested to know if the resistance trait was stably inherited, it was found that only 25% of 72 tested tobacco transgenic lines showed heritable resistance (T_1 to T_3 generation). Also in the present work only 33% of the tested lines showed heritable resistance in the T_2 generation.

Also the sequences coding for the coat and pre-coat protein of TYLCTHV-[AIT] were employed in the present work for constructing the IR/CP hairpin-construct. The coat protein of many RNA viruses has been used successfully in constructs to engineer transgenic resistance plants since the end of the 1980's (Abel et al., 1986). Recently, resistant transformed plants, harboring the CP of RNA viruses as an inverted-repeat construct, have been successfully obtained (Krubphachaya et al., 2007; Tougou et al., 2006). However, only few reports are known concerning geminivirus resistance employing constructs expressing CP and only one report for using CP as an inverted-repeat construct (Zrachya et al., 2007b). Zrachya et al. (2007b) analyzed the effect of siRNAs derived from an intron-hpRNA construct targeting the V1 (CP) gene product of TYLCV in tomato plants. These transgenic plants expressing siRNA against the CP did show no symptoms until seven weeks post-inoculation but after this period disease symptoms were observed. In addition, no immune lines were obtained. Percentage of resistance to the virus ranged from 12% to 20% in T_1 and 41% to 55% in T_2 progeny.

Interestingly, when comparing these results to the results obtained in the present work, *N. benthamiana* transgenic plants containing the IR/CP hairpin construct did not show symptoms even at 9 weeks post-inoculation and they showed a higher percentage of disease resistance to the virus ranging from 27% to 47% in the T_1 progeny. One cannot exclude that these differences may be due to the different transformed hosts and/or to the effect related to the transgene insertion position in the host genome. Moreover, the success in obtaining resistant transgenic plants using the IR/CP hairpin in the present work may be also attributed to the fact that TYLCTHV is a facultative bipartite virus. Normally the CP of bipartite viruses is not essential for systemic infection and viral movement and transport are performed by proteins encoded by the B component. However, the TYLCTHV A component can infect its natural host alone (Rochester et al., 1990) and therefore TYLCTHV is considered as a facultative bipartite virus. Because the CP of TYLCTHV may also be involved in cell to cell movement as found for monopartite viruses, we used partially sequences of the CP and AV2 for constructing one of the inverted-repeat cassettes. Moreover, the AV2/V2 (pre-coat) sequence is well conserved among viruses from the Old World and has been recently related also to gene silencing in *Tomato yellow leaf curl virus* (Zrachya et al., 2007a; Rojas et al., 2001).

Even if the sequence coding for the Rep protein is one of the mostly employed for generating geminivirus resistant transgenic plants, the use of inverted-repeat constructs resulting in target of Rep transcripts have been not much exploited yet and only few reports are known (Bonfim et al., 2007; Fuentes et al., 2006). Only very recently, Bonfim et al. (2007) generated 18 transgenic common bean lines using an intron-hairpin construct from the Rep gene. Only one out of the 18 lines presented no symptoms until 30 dpi when inoculated with whiteflies in the T_1 generation. All other plants were susceptible to *Bean golden mosaic virus* (BGMV). Interestingly, progenies of the resistant line produced homozygous as well as heterozygous lines, which were susceptible to BGMV although the percentage number of susceptible plants per line in heterozygous lines was higher as in homozygous lines. In the present work we cannot exclude that the amount of transgenic Rep lines generated was not sufficient to obtain resistant T_1 transgenic lines.

In this work, we cloned different regions of the TYLCTHV-[AIT] sequence to generate a total of six inverted-repeat constructs covering the whole genome of the TYLCTHV-[AIT]. As result, transgenic plants expressing delayed symptoms were observed mainly in plants harboring the AC2/C3, IR/CP and IR/BC constructs. From all

transgenic lines generated in the present work, those lines containing sequences of the movement protein of TYLCTHV-[AIT] showed resistance not only in the T_1 but also in the T_2 generation even at 29 dpi. Moreover, transgenic plants harboring the IR/CP hairpin construct showed delayed symptoms expression until 90 dpi in the T_1 generation. In addition, the DI values of lines originated from the same construct did not statistically differ between homozygous or heterozygous lines. Because we did not verify the number of transgene copies inserted in the genome of the transgenic plants, we cannot exclude that not only the insertion position but also the copy number may play a role in TYLCTHV disease response in transgenic *N. benthamiana* plants.

Although highly resistant lines harboring inverted-repeat constructs have been reported showing no symptoms during a period varying from 30 to 70 days maximum (Bonfim et al., 2007; Fuentes et al., 2006; Zrachya et al., 2007b), completely immune lines against geminiviruses have not been shown yet. Nevertheless, it is clear that geminiviruses are targets of PTGS and transgenic plants expressing delayed response have been successfully reported for different begomoviruses. In this work we reported for the first time transgenic plants with a recovery symptom pattern harboring the AC2/C3 inverted-repeat construct. Until now, recovery symptom expression has been only reported for transgenic plants expressing the geminiviral protein (CP) and/or mutated forms (BC). Therefore, it will be interesting to investigate further the AC2/C3 transgenic lines. Moreover, inverted-repeat constructs harboring the three regions of the TYLCTHV-[AIT] (AC2/C3, IR/CP and IR/BC) resulted in resistant transgenic plants. One should not forget that the use of agroinoculation for the resistance test delivers continuous amounts of viral DNA. In this work we were able to generate 100% of symptomatic non-transgenic plants even using agrobacterial dilutions up to 1:10.000 (data not shown) and we cannot exclude the possibility that a dilution of 1:500 delivers higher amounts of viral DNA in the host than viruliferous whiteflies do.

Finally, further studies should be taken to understand the mechanism of geminivirus resistance. Geminivirus resistance has been shown to be dependent not only on the virus itself but also on the balance between initial titers of replicating virus and the silencing response. From our results it is clear that the use of intron-hairpin constructs to prevent TYLCTHV-[AIT] infection can not solely guarantee transgenic plants free of the virus and it will be interesting to verify if TYLCTHV-[AIT] has evolved different means of circumventing the silencing pathways.

4.0 Engineering of *Tomato yellow leaf curl Thailand virus* as expression and VIGS vector system

4.1 Abstract

Tomato yellow leaf curl Thailand virus (TYLCTHV-[AIT]) is a bipartite begomovirus causing severe losses in tomato plants. Here we exploited the use of the TYLCTHV-[AIT] genome as an expression and VIGS vector system. For this purpose, the coat protein (AV1 gene) of TYLCTHV-[AIT] was replaced by the GFP/GFP_HA (Hemagglutinin-tag), the entire coat protein (CP) of *Vicia cryptic virus* (VCV) or a HA-tagged part of the VCV CP. Proteins expressed in *Nicotiana benthamiana* were monitored either using the confocal laser scanning microscope (CSLM) and/or by Western blot. While expressing the complete gene of GFP/GFP_HA did not interfere with the replication of the TYLCTHV-[AIT], integration of the 1464 bp coding sequence of the entire CP of VCV led to deletion mutants in the DNA A. Therefore, only a part of the VCV CP gene tagged with the sequence of the HA epitope (ΔCP_VCV_HA) was inserted. Both GFP_HA and ΔCP_VCV_HA were detected in locally agroinoculated leaves at 15 dpi using anti-HA antibodies in immunoblot assays.

Moreover, transgenic *N. benthamiana* 16c plants were agroinoculated with the multimeric clone expressing GFP from the TYLCTHV-[AIT] A component or in combination with its B component. The progression of *gfp* silencing was monitored by CLSM and under UV light at 5, 14, 19 and 35 days post inoculation. All 16c plants (30 out of 30) inoculated together with the B component showed systemic silencing as early as 14 dpi, while only two out of 30 plants containing the TYLCTHV-[AIT] A component harboring *gfp* solely showed silencing at 35 dpi. In addition, in both combinations five out 30 inoculated 16c plants showed geminivirus symptoms at 19 dpi, suggesting that probably few viral DNA molecules were able to generate symptoms. Taken together, TYLCTHV-[AIT] could be useful as a tool to express heterologous proteins in *N. benthamiana* plants as well as silencing vector.

Keywords: Begomovirus, expression vector, VIGS, silencing

4.2 Introduction

The four genera *Mastrevirus*, *Topocuvirus*, *Begomovirus* and *Curtovirus* compose the family *Geminiviridae*. Begomoviruses are constituted of single-stranded circular DNA and are classified as bipartite or monopartite viruses according to their genome organization. *Tomato yellow leaf curl Thailand virus* TYLCTHV-[AIT] is a facultative bipartite virus (A and B component), which causes yield losses varying between 10% and 100% in Thailand (Sawangjit et al., 2005). The A component of the TYLCTHV comprises six different open reading frames (ORFs) namely AV1 and AV2 on the viral strand and AC1, AC4, AC2 and AC3 on the complementary strand. AV1 codes for the coat protein (CP) and AV2 for the pre-coat, which has been recently related to be involved in gene silencing mechanism (Zrachya et al., 2007a). AC1 gene codes for the replication protein (Rep) involved in the replication process. AC4 and AC2 have been involved in silencing suppression for *African cassava mosaic virus* (ACMV), *Sri Lankan cassava mosaic virus* (SLCMV) and *Tomato yellow leaf curl China virus* (TYLCCNV) (Dong et al., 2003; Vanitharani et al., 2004). AC2 is also known to function as transcriptional activator and AC3 serves as a replication enhancer in begomoviruses.

The coat protein (CP) of monopartite begomoviruses is necessary for systemic infection, but not the CP of bipartite viruses. Engineering begomoviruses as expression vectors have already begun at end of the 80's and beginning of the 90's by replacing the geminivirus coat protein for a foreign gene (Timmermans et al., 1994). Hayes et al. (1989) were the first to develop a geminivirus vector by replacing the coat protein gene with a heterologous gene. Hayes and coworkers transformed *Nicotiana tabacum* plants containing the *Tomato golden mosaic virus* (TGMV) viral vector, where the AV1 gene was replaced either with the beta-glucuronidase (*gus*) or neomycin phosphotransferase (*neo*) gene. Studies showing the increase of viral DNA suggested already that time the potential use of geminiviruses as an expression system (Hayes et al., 1988). Since then, the use of geminiviruses as replicating based expression vector has been recognized.

Replicating viruses in plant cells activate silencing mechanisms. The use of virus-induced gene silencing (VIGS) to suppress host-gene expression has been commonly achieved by using RNA viruses (*Tobacco ecth virus*, *Tobacco rattle virus*, *Tobacco mosaic virus*, *Potato virus X* and *Potato virus A*). Also DNA viruses have been exploited as VIGS vectors showing many advantages compared to VIGS-based on RNA viruses (Carrilo-Tripp et al., 2006). The genus *Mastrevirus* including *Maize streak virus*

(MSV), *Wheat dwarf virus* (WDV), *Bean yellow dwarf virus* (BeYDV) and also the curtovirus *Beet curly top virus* (BCTV) have been used as expression vectors (Hefferon et al., 2004a; Hefferon et al., 2004b; Matzeit et al., 1991; Palmer and Rybicki, 2001; Zhang and Mason, 2006). However, few members of the genus *Begomovirus* namely TGMV and *Cabbage leaf curl virus* (CabLCuV) have been used to express genes (Kjemtrup et al., 1998; Muangsan et al., 2004; Peele et al., 2001). Thus, the coat protein of monopartite and some bipartite geminiviruses has been replaced by reporter genes, like green fluorescent protein (GFP) and *gus*, aiming to study their expression level. Increase in protein expression has been obtained by including the *Cauliflower mosaic virus* (CaMV) 35S promoter sequence in the replicating virus (Hayes et al., 1989; Kim et al., 2007). In 2007, Kim et al. improved the expression of the recombinant GFP by including different promoters (CaMV 35S, *Cassava vein mosaic virus* (CsVMV)) within the replicating vector of BCTV in *Nicotiana benthamiana*. The inclusion of the 35S promoter increased three times the expression of recombinant GFP than those from a non-replicating vector and 320% of the *gfp* transcript level when using the CsVMV promoter.

Here we investigated the capability of the genomic DNA A of TYLCTHV-[AIT] to function as an expression system in the presence or absence of its DNA B component. Therefore, the AV1 gene was replaced by the entire coat protein gene of *Vicia cryptic virus* (VCV) or by a truncated version of the VCV CP tagged with the Hemagglutinin (HA) epitope. *Vicia cryptic virus* belongs to the family *Partitiviridae*. In general, genomes of cryptic viruses consist of two separate monocistronic dsRNA segments, whereas the smaller dsRNA codes for the CP and the larger one for the RdRp. The linear dsRNA segments are encapsidated in non-enveloped isometric particles of 29-38 nm in diameter. In addition, the AV1 gene was replaced by the GFP reporter gene to monitor gene expression tagged or not at its C-terminus with HA.

Finally, the multimeric copies of the TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_VCV_CP were introduced into *N. benthamiana* plants via agroinoculation solely or together with a DNA B multimeric clone. Moreover, *gfp* silencing was monitored under UV light of a hand lamp and by CLSM.

4.3 Materials and Methods

Cloning of vector viruses (TYLCV-[AIT]_GFP; TYLCV-[AIT]_VCV_CP; TYLCV-[AIT]_GFP_HA; TYLCV-[AIT]_VCV_CP_HA and TYLCV-[AIT]_Express_VCV_CP_HA)

A clone containing the 1.4x copy of the TYLCTHV-[AIT] DNA A genome (Knierim and Maiss, 2007) was used to replace the coat protein gene by: (i) the GFP or GFP tagged HA, (ii) the complete VCV CP and (iii) a partial sequence of the C-terminus of the CP of VCV tagged with HA.

The first step consisted in performing a mutagenesis PCR using primers Mut_DNA1 and Mut_DNA2 for deletion of the AV1 gene and simultaneous introduction of the restriction sites *Age*I and *Xho*I. Primers were designed for VCV and GFP (VCV_gem1/VCV_gem2 and GFP_DNA1/GFP_DNA2) and used to amplify the coding region of the CP of VCV and also GFP (*gfp*, originating from *N. benthamiana* 16c plants, Ruiz et al., 1998), respectively, resulting in plasmids TYLCV-[AIT]_VCV_CP and TYLCV-[AIT]_GFP. Additional mutagenesis reactions primers HA_GFP2/HA_AS2 and HA_VCV1/HA_AS1 were used to introduced the HA tag at the C-terminus of GFP and the CP of VCV resulting in TYLCV-[AIT]_GFP_HA and TYLCV-[AIT]_VCV_CP_HA, respectively. Finally, a mutagenesis reaction was performed using primers HA_VCV_Express1 and HA_VCV_Express2 to create a deletion mutant of only the last 741 bp of the VCV coat protein gene to get TYLCV-[AIT]_Express_VCV_CP_HA.

Table 1. Primers used to amplify *gfp*, VCV CP and HA sequences

Primer name: sequence (5´- 3´)
Mut_DNA1: AAA*CTCGAG*AATTAATAAATATTAAATTTTATATCATGT
Mut_DNA2: AAA*ACCGGT*CATAATTCTTCGCACGAATTACACTGA
VCV_gem1: AAA*ACCGGT*ATGGAAGCTCATACTCCCGCTGCTGATG
VCV_gem2: AAA*CTCGAG*TCAGTCGACACGGTTGGCGGCAAGTTG
VCV_both2: CCTATCCAGTCAAAAGGGCCATTG
GFP_DNA1: AAA*ACCGGT*ATGAGTAAAGGAGAAGAACTTTTC
GFP_DNA2: AAA*CTCGAG*CTATTTGTATAGTTCATCCATGCCATG
HA_GFP2: AACATCGTATGGGTATTTGTATAGTTCATCC
HA_AS2: CCAGATTACGCTTAGCTCGAGAATTAATA
HA_VCV1: AACATCGTATGGGTAGTCGACACGGTTGGCGGCAAGTT
HA_AS1: CCAGATTACGCTTAATAAATATTAAATTTTATATC
HA_VCV_Express1: ATGCAAGGCGACCTCTACCCTACCTTC
HA_VCV_Express2: AATTCTTCGCACGAATTACACTGATAAG

Restriction sites are represented by underlined and cohesive characters

Cassettes containing the *gfp* or VCV CP gene instead the CP of TYLCTHV-[AIT] were cloned into a derivative of pBIN 19 and used to transform *Agrobacterium tumefaciens* C58C1. All primers used in this study are listed in Table 1.

Agroinoculation and CLSM

Agrobacterium tumefaciens C58C1 containing the modified multimeric copy of TYLCTHV-[AIT] were grown overnight at 28°C until OD_{600} around of 1.7. 1 ml of each agrobacteria suspension was centrifuged and resuspended in a final volume of 1 to 2 ml of agroinoculation buffer (10 mM $MgCl_2$, 10 mM MES, 100 µM Acetosyringon) depending on the combination used and incubated for 3 to 4 hours. 1 ml of the agrobacteria suspension was used to agroinoculate either the wild type *N. benthamiana* or the transgenic line 16c of *N. benthamiana*, which shows strong constitutive GFP expression (Ruiz et al., 1998). For each viral vector ten *N. benthamiana* and 30 transgenic *N. benthamiana* 16c plants were agroinoculated at the 4 to 5 leaves stage.

Plants were analyzed under the confocal laser scanning microscope (Leica TCS SP2) at 9, 14, 21 and 40 days post-inoculation (dpi) using the Ar/ArKr laser, which excites the GFP at 488 nm. In addition, plants were monitored under a long-wavelength UV lamp (Black Ray model B 100 AP) and photographed at 5, 14, 19 and 35 dpi with a Canon Powershot A60 digital camera using a yellow filter HOYA K2 HMC, 52 mm, Tokina, Japan.

DNA extraction

DNA was extracted from young leaves at 9, 14, 21 dpi from wild type *N. benthamiana* plants agroinoculated with TYLCV-[AIT]_GFP and TYLCV-[AIT]_VCV solely or together with the multimeric clone of the DNA B component of TYLCTHV-[AIT]. Moreover, DNA was extracted at 15 dpi from *N. benthamiana* plants agroinoculated with TYLCV-[AIT]_GFP_HA and TYLCV-[AIT]_VCV_HA together with TYLCTHV B component. DNA was extracted at 35 dpi from the transgenic line 16c of *N. benthamiana*. All DNA extractions were performed according to Crespi et al. (1991). Inserts in the DNA A were confirmed by using specific primers described in Table 1 or specific primers designed from the AC2/C3 region of the TYLCTHV-[AIT].

Western blot

Western blot was performed according to standard protocols. Briefly, 1 g of plant materials was collected at 15 dpi and ground in liquid nitrogen and transferred to an eppendorf tube containing 500 µl of heated buffer: 750 mM Tris-HCl, pH 8.8, 4% SDS, 4% ß-Mercaptoethanol; 40% Saccharose. Tubes were incubated at 95°C for 10 min and centrifuged at 13.000 rpm for 10 min. The supernatant was collected and used for the immunoblot assays using a standard protocol. The detection step was based on the oxidation of a luminol-based substrate by horseradish peroxidase resulting in light-emitting reaction (SuperSignal West Femto Kit (Pierce Biotechnology)). For this purpose, immunoblot assays were performed using an anti-HA antibodies (Roche) or anti-GFP antibodies (MBL International) in a 1:4000 dilution as the first antibodies followed by incubation with horseradish peroxidase-conjugated anti-rat or anti-rabbit F(ab')2 fragment (Jackson ImmunoResearch) diluted 1:6600 to detect the HA tagged and GFP proteins, respectively.

4.4 Results

Agroinoculation of TYLCTHV-[AIT] _GFP solely or together with TYLCTHV-[AIT] B component

The viral vector TYLCTHV-[AIT]_GFP was agroinoculated either alone or together with the TYLCTHV-[AIT] DNA B multimeric clone in *N. benthamiana* plants. To monitor the replication of the viral vector TYLCTHV-[AIT]_GFP, DNA was extracted at 9, 14 and 21 dpi from new emerging leaves and PCR was performed. Primers GFP_DNA1/GFP_DNA2 were used to amplify a 717 bp fragment confirming the presence of *gfp*. Moreover, systemic infection was monitored until 21 dpi in both combinations inoculated with and without the DNA B component of TYLCTHV-[AIT].

When agroinoculating the multimeric clones TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT] B simultaneously, mosaic symptoms were observed in eight out of 10 plants at 21 dpi. Symptoms were more attenuated when compared to symptoms caused by wild type TYLCTHV-[AIT] multimeric clones in *N. benthamiana* (Figure 1-B). In addition, it was always possible to amplify *gfp* fragments from the new emerging young leaves of different plants at 9, 14 and 21 dpi in the presence of TYLCTHV-[AIT]

B component. An increase of amplified viral DNA was observed from 9 to 21 dpi, probably indicating an increase in viral replication (Figure 2, lanes 1-4 and 9-12).

Although it was also possible to amplify the *gfp* fragment from agroinoculated plants using the multimeric clone TYLCTHV-[AIT]_GFP solely, no systemic symptoms were observed in those plants (Figure 1-A). Young as well as old leaves did not show any symptoms. In addition, an increase of viral DNA was not observed as for those plants containing TYLCTHV-[AIT]_GFP together with the B component.

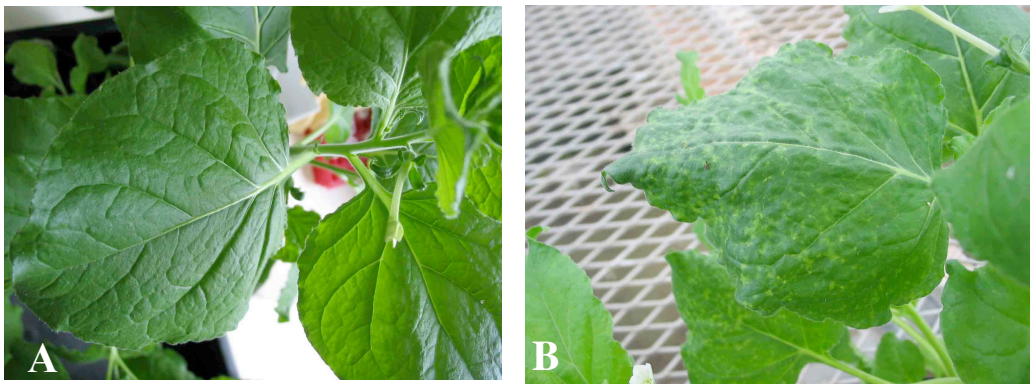


Figure 1. A. *N. benthamiana* leaves infected systemically with the multimeric clone TYLCTHV-[AIT]_GFP at 21 dpi and B. Systemic infection observed on agroinoculated *N. benthamiana* plants harboring multimeric clones TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT] B at 21 dpi.

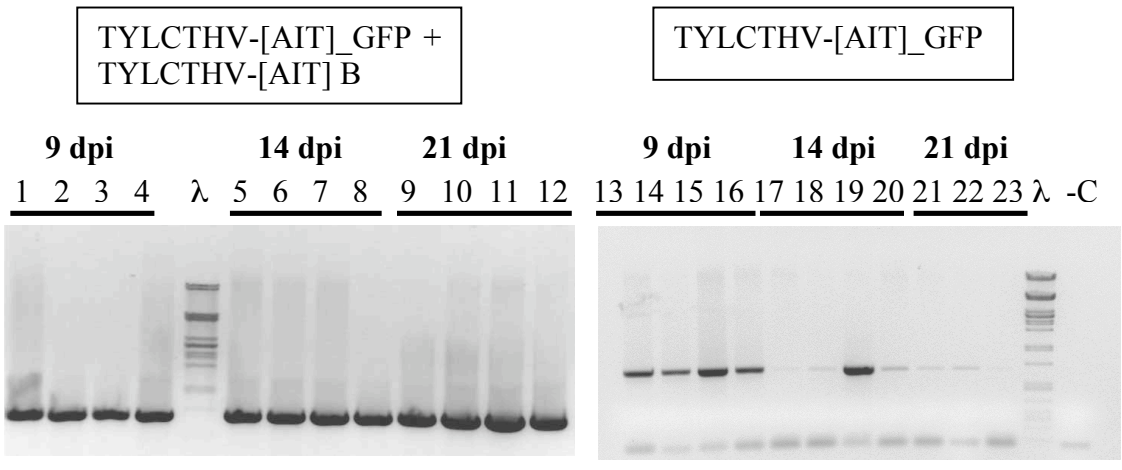


Figure 2. PCR fragments amplified from agroinoculated *N. benthamiana* plants. PCR amplified fragments from *N. benthamiana* plants agroinoculated with TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B at 9 dpi (lanes 1-4), at 14 dpi (lanes 5-8), at 21 dpi (lanes 9-12); PCR amplified fragments from *N. benthamiana* plants agroinoculated solely with TYLCTHV-[AIT]_GFP at 9 dpi (lanes 13-16), at 14 dpi (lanes 17-20) and at 21 dpi (lanes 21-23). λ: λ-DNA digested with *Pst* I and –C: negative control from a mock-inoculated plant. PCR was performed using GFP_DNA1 and GFP_DNA2 primers and DNA extracted from young emerging leaves.

Agroinoculation of the multimeric clone TYLCTHV-[AIT]_VCV_CP solely or together with TYLCTHV-[AIT] B component

The viral vector TYLCTHV-[AIT]_VCV_CP was agroinoculated either alone or together with the TYLCTHV-[AIT] B multimeric clone. Replication of the viral vector TYLCTHV-[AIT]_VCV_CP was monitored by PCR. DNA was extracted from young emerging leaves at 9, 14 and 21 dpi and primers VCV_gem1/VCV_gem2 were used to amplify a 1464 bp fragment of the VCV CP by PCR.

Systemic infection was monitored until 21 dpi and clearly visible symptoms were only observed in combination with TYLCTHV-[AIT] B (Figure 3-B). Similar to the results obtained when agroinoculating TYLCTHV-[AIT]_GFP solely, no symptoms were observed in those plants agroinoculated exclusively with the TYLCTHV-[AIT]_VCV_CP multimeric clone (Figure 3-A). In contrast to agroinoculated TYLCTHV-[AIT]_GFP plants no VCV_CP PCR fragment was amplified either from the agroinoculated *N. benthamiana* plants containing the multimeric clone TYLCTHV-[AIT]_VCV_CP solely or from plants inoculated with TYLCTHV-[AIT]_VCV_CP and the multimeric clone TYLCTHV-[AIT] B up to 14 dpi. Only at 21 dpi fragments with deletions of VCV CP were amplified using extracted DNA from new young emerging leaves containing the B component of TYLCTHV-[AIT] and particularly only at 21 dpi symptoms could be observed in five out of ten agroinoculated plants. Symptoms included small yellow spots visualized on old leaves, while young leaves were free of viral symptoms (Figure 3-B).

PCR results suggested that a deletion has occurred in the inserted VCV_CP sequence, probably to adjust the genome size for proper encapsidation. Deletions were confirmed via PCR using specific primers VCV_gem1 and VCV_both2 to amplify the 5'-terminal 575 bp of the VCV CP sequence. Detection of the PCR fragment failed in all samples extracted from young leaves at 9, 14 and 21 dpi (Figure 4). However, it was possible to amplify a 575 bp fragment of VCV_CP from locally agroinoculated leaves (Figure 4, lane 10 and 16) as well as the entire VCV_CP fragment of 1464 bp (Figure 4, lane 26 and 32) using the VCV_gem1 and VCV_gem2 primers (Figure 4).

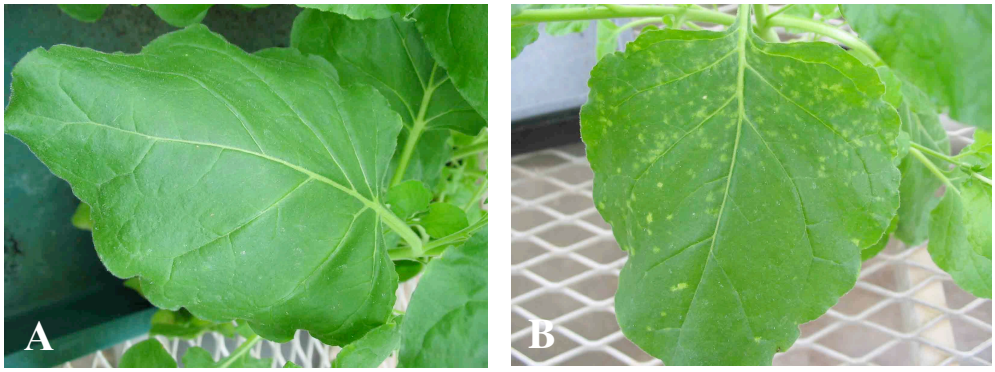


Figure 3. A. *N. benthamiana* leaves infected systemically with the multimeric clone TYLCTHV-[AIT]_VCV_CP at 21 dpi and B. Systemic infection observed on agroinoculated *N. benthamiana* plants harboring multimeric clones TYLCTHV-[AIT]_VCV_CP and TYLCTHV-[AIT] B at 21 dpi.

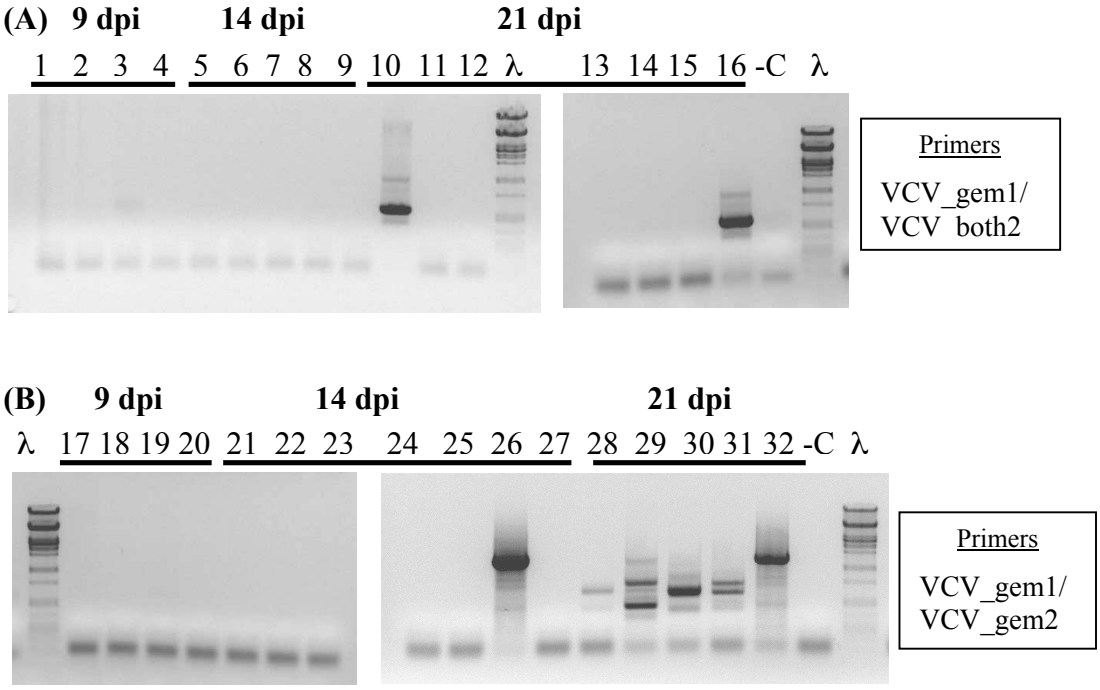


Figure 4. PCR fragments amplified from locally and systemically infected agroinoculated *N. benthamiana* plants with TYLCTHV-[AIT]_VCV and TYLCTHV-[AIT] B at 9, 14 and 21 dpi. In A. and B. PCR fragments in lanes 10, 16, 26 and 32 are derived from DNA samples extracted from locally agroinoculated leaves. All other lanes are derived from systemically infected leaves. A. PCR amplified fragments from newly emerged leaves at 9 dpi (lanes 1-4), at 14 dpi (lanes 5-9) and at 21 dpi (lanes 10-16) using primers VCV_gem1 and both2; B. PCR amplified fragments from newly emerged leaves at 9 dpi (lanes 17-20), at 14 dpi (lanes 21-27) and at 21 dpi (28-32) using VCV_gem1 and VCV_gem2 primers. λ: λ-DNA digested with *Pst* I and -C: negative control from a mock-inoculated plant.

Agroinoculation of multimeric clones TYLCTHV-[AIT]_Express_VCV_CP_HA and TYLCTHV-[AIT]_GFP_HA and Western Blot analyses

The viral vector TYLCTHV-[AIT]_GFP_HA was agroinoculated in *N. benthamiana* together with the multimeric clone of TYLCTHV-[AIT] B, while TYLCTHV-[AIT]_Express_VCV_CP_HA was agroinoculated solely or in combination with TYLCTHV-[AIT] B. To monitor viral DNA replication and movement inside the plant, DNA was extracted at 15 dpi from young emerging leaves and PCR was performed using specific primers GFP_DNA1 and HA_GFP2 to amplify the 738 bp coding for the tagged GFP and primers HA_VCV_Express1 and HA_VCV1 to amplify the 759 bp coding for the tagged C-terminus of the CP of VCV (Figure 5).

It was possible to amplify by PCR the expected 738 bp fragments from young leaves of all inoculated plants, although the amount of the amplified fragment differed from plant to plant when compared to those plants containing TYLCTHV-[AIT]_GFP without the HA tag (Figure 5).

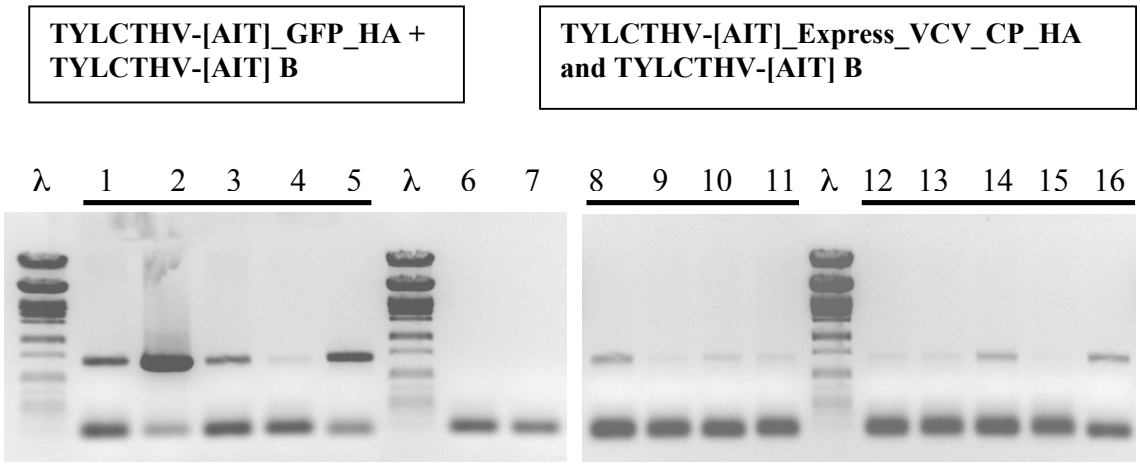


Figure 5. PCR fragments amplified from systemically infected agroinoculated *N. benthamiana* plants. PCR amplified fragments from agroinoculated *N. benthamiana* plants harboring TYLCTHV-[AIT]_GFP_HA together with TYLCTHV-[AIT] B using GFP_DNA1 and HA_GFP2 primers at 15 dpi (lanes 1-5); mock inoculated plants at 15 dpi (lanes 6-7); PCR amplified fragments from agroinoculated *N. benthamiana* plants harboring TYLCTHV-[AIT]_Express_VCV_CP_HA together with TYLCTHV-[AIT] B using HA_VCV_Express1 and HA_VCV1 primers at 15 dpi (lanes 8-11). PCR amplified fragments from agroinoculated *N. benthamiana* plants harboring only TYLCTHV-[AIT]_Express_VCV_CP_HA at 15 dpi using HA_VCV_Express1 and HA_VCV1 primers (lanes 12-16). λ: λ-DNA digested with *Pst* I.

In addition, it was already possible at 15 dpi to amplify the complete cloned C-terminus of the CP of VCV. No deletion fragments were observed by agarose gel electrophoresis as observed when expressing the complete VCV CP. Moreover, no symptoms were observed in *N. benthamiana* plants at 15 dpi harboring either TYLCTHV-[AIT]_GFP_HA or TYLCTHV-[AIT]_Express_VCV_CP_HA in the presence of the B component of TYLCTHV-[AIT].

Proteins were extracted from locally agroinoculated leaves as well as from young emerging leaves extracted from different plants and submitted to immunoblot assays (Figure 6).

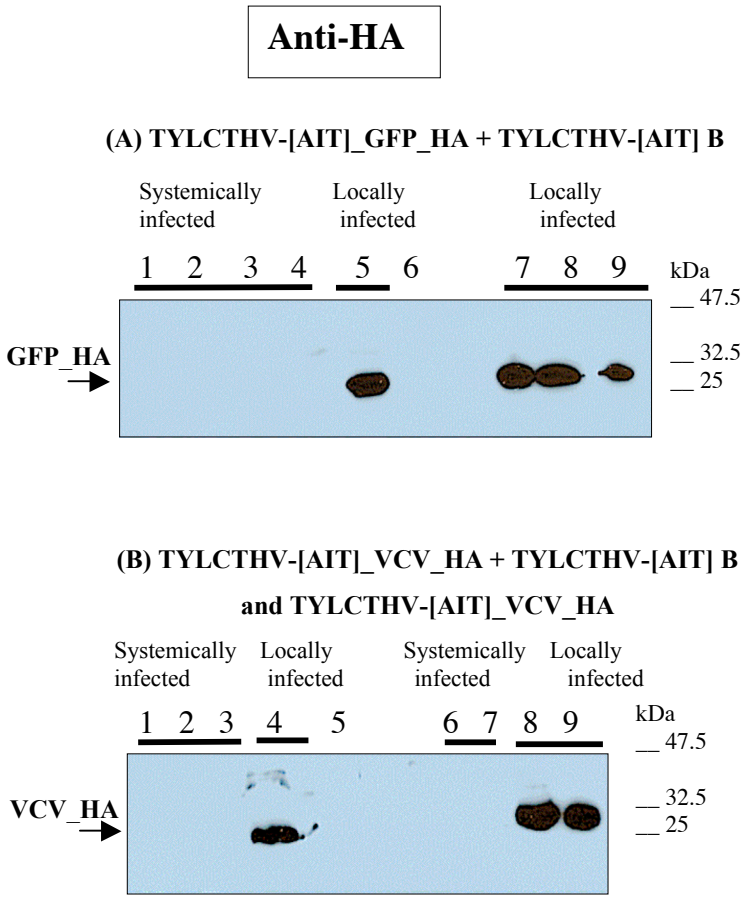


Figure 6. Detection of GFP_HA and VCV_CP_HA *in planta* using anti-HA antibodies. Immunoblot analyses of inoculated *N. benthamiana* leaves at 15 dpi. Upper panel: Assay using leaf material of systemically infected leaves (SI, lanes 1-4) agroinoculated with TYLCTHV-[AIT]_GFP_HA and TYLCTHV-[AIT] B component; Assay using leaf material of locally infected leaves (LI, lanes 5, 7-9) agroinoculated with TYLCTHV-[AIT]_GFP_HA and TYLCTHV-[AIT] B component; Leaf material of mock inoculated leaves (lane 6). Lower panel: Assay using leaf material of systemically infected leaves (SI, lanes 1-3) and locally infected leaves (LI, lane 4) agroinoculated with TYLCTHV-[AIT]_VCV_HA and TYLCTHV-[AIT] B component; Leaf material of mock inoculated leaves (lane 5); Assay using leaf material of systemically infected leaves (SI, lanes 6-7) and locally infected leaves (LI, lanes 8-9) agroinoculated with TYLCTHV-[AIT]_VCV_HA solely.

At 15 dpi, it was only possible to detect both GFP_HA and VCV_CP_HA expressed proteins from locally agroinoculated leaves of *N. benthamiana* and not from new emerging leaves using anti-HA antibodies (Figure 6), although the GFP and the C-terminus of VCV CP nucleotide sequences were amplified by PCR from young emerging leaves. Moreover, green fluorescence was detected in young leaves especially in veins of plants agroinoculated with TYLCTHV-[AIT]_GFP_HA using CLSM at 21 dpi (data not shown).

CLSM of N. benthamiana agroinoculated with TYLCTHV-[AIT]_GFP or agroinoculated with TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT] B component

Fluorescence of GFP was analysed in *N. benthamiana* plants agroinoculated with TYLCTHV-[AIT] containing the *gfp* instead of the AV1 gene using CLSM at 2, 7, 14, 21 and 40 dpi (Figure 7).

At 2 and 7 dpi single epidermal cells containing either TYLCTHV-[AIT]_GFP or TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B showed strong green fluorescence in the agroinoculated tissue. However, at 14 and 21 dpi the fluorescence disappeared from epidermal cells and it was detected only in mesophyll cells and vascular tissues from plants harboring both combinations. Therefore, to obtain a better visualization of infected cells, the epidermis was removed from leaves and mesophyll tissue was then exposed to the CLSM (Figure 7 E-F). Tissue containing the B component of TYLCTHV showed higher number of mesophyll cells with green fluorescence than those not containing the B component (Figure 7 G-H). This probably indicates that the B component could move the viral vector TYLCTHV-[AIT]_GFP out of site of the vascular tissue. On the other hand, plants infected only with TYLCTHV-[AIT]_GFP showed a higher number of vascular tissue expressing green fluorescence (Figure 7 I-J). Finally, only at 40 dpi a weak green fluorescence was detected in epidermal cells from young emerging leaves in plants agroinoculated with both combinations of multimeric clones (Figure 7 K-L).

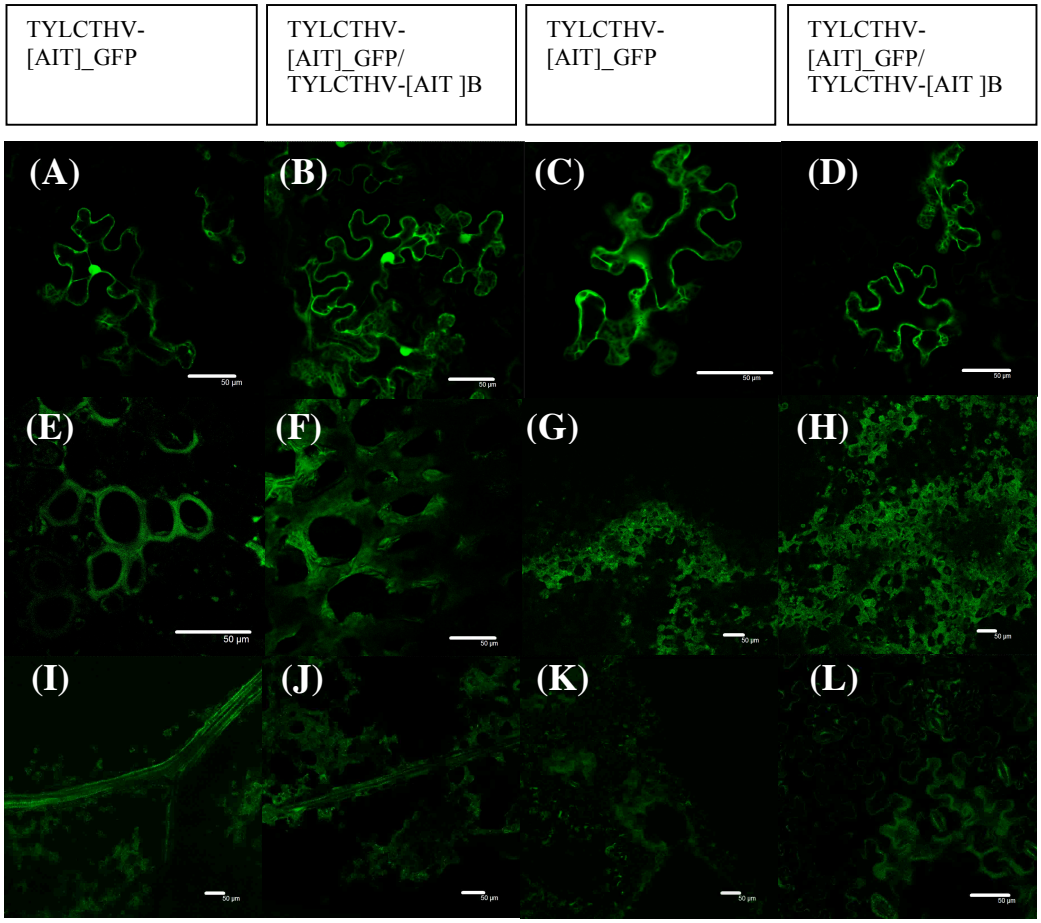


Figure 7. CLSM of *N. benthamiana* agroinoculated with TYLCTHV-[AIT]_GFP or agroinoculated with TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT] B component. Epidermal cells of agroinoculated *N. benthamiana* leaves expressing green fluorescence at 2 dpi from TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component, respectively (A-B); Epidermal cells of agroinoculated leaves harboring TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component at 7 dpi, respectively (C-D); Mesophyll cells showing green fluorescence expressed from TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component, respectively at 14 dpi (E-F), at 21 dpi (G-H), at 40 dpi (I-J); Epidermal cells showing weak green fluorescence expressed from TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component at 40 dpi (K-L); Scale bars represent 50 μm.

Comparison of gfp silencing using TYLCTHV-[AIT]_GFP or TYLCTHV-[AIT]_GFP/TYLCTHV-[AIT] B on transgenic Nicotiana benthamiana 16c plants

Silencing of *gfp* and geminivirus symptoms in transgenic *N. benthamiana* 16c plants were monitored under UV light at 5, 14, 19, 25 and 35 dpi (Figure 8).

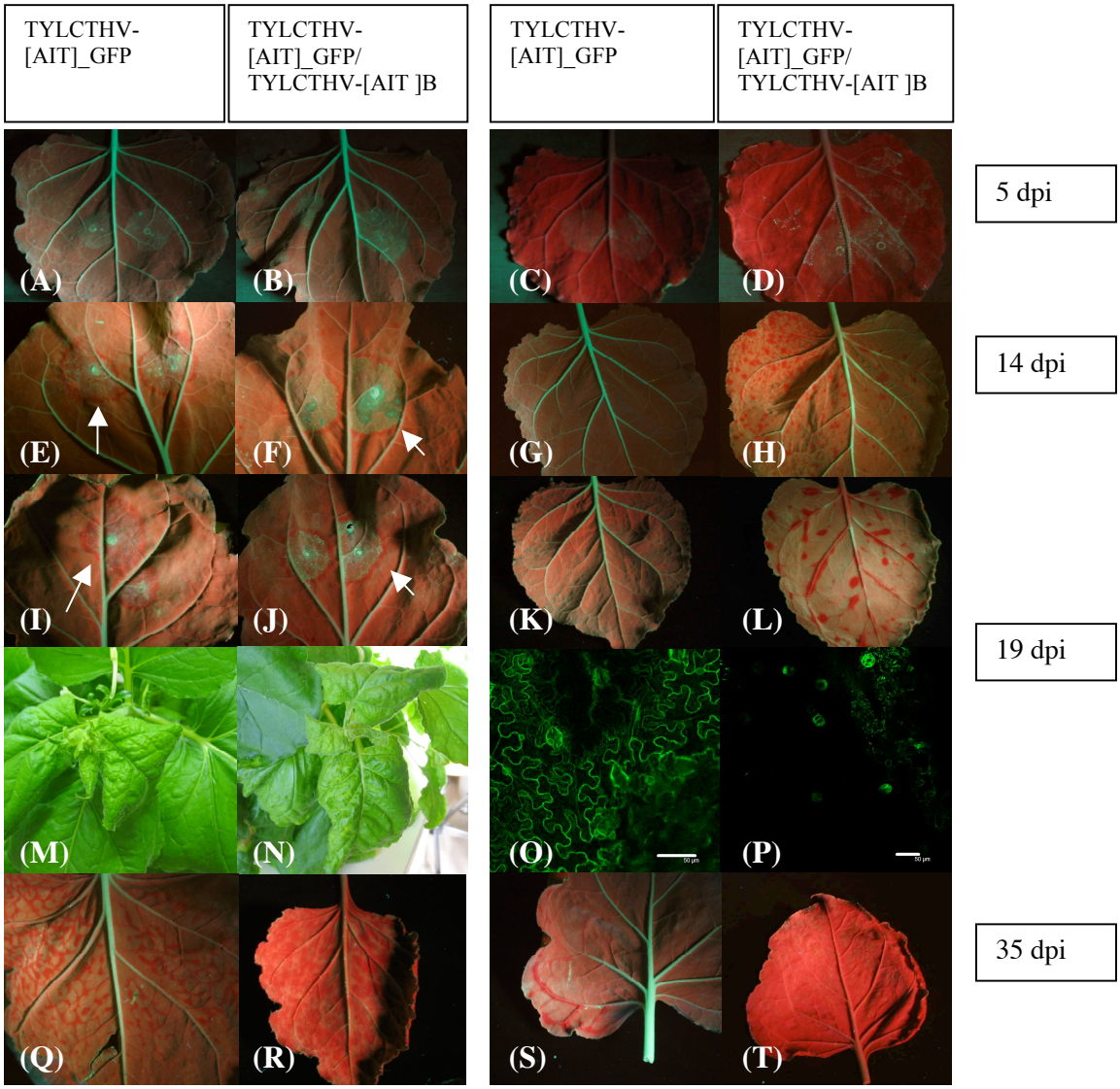


Figure 8. Silencing signs observed in *N. benthamiana* leaves harboring TYLCTHV-[AIT]_GFP or TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component monitored under the UV light and/or the CLSM. Agroinoculated transgenic 16c leaves monitored under UV light harboring TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component at 5 dpi, respectively (A-B); Agroinoculated non-transgenic leaves used as control, monitored under UV light harboring TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component at 5 dpi, respectively (C-D); Arrows indicate silencing signs (ring form) visualized on agroinoculated 16c leaves under UV light harboring TYLCTHV-[AIT]_GFP (E-I) and harboring TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component (F-J) at 14 and 19 dpi; Systemically infected transgenic 16c leaves harboring TYLCTHV-[AIT]_GFP (G-K) and silencing signs visualized on young sink 16c leaves harboring TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component (H-L) at 14 and 19 dpi; Symptoms of geminiviruses on agroinoculated 16c plants harboring TYLCTHV-[AIT]_GFP (M) and TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component (N) at 19 dpi; Transgenic 16c epidermal cells of a young sink leave visualized under the CLSM harboring TYLCTHV-[AIT]_GFP (O) and harboring TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B component (P) at 19 dpi; First silencing signs observed on mature 16c leaves harboring TYLCTHV-[AIT]_GFP (Q-S) and silenced tissue of 16c young sink leaves agroinoculated with TYLCTHV-[AIT]_GFP together with TYLCTHV-[AIT] B (R-T) at 35 dpi; Scale bars represent 50 μm.

At 5 dpi no *gfp* silencing was visualized at the site of agroinoculation either in plants agroinoculated with TYLCTHV-[AIT]_GFP solely or with TYLCTHV-[AIT]_GFP/TYLCTHV-[AIT] B multimeric clones (Figure 8 A-B). At 14 dpi first *gfp* silencing appeared at the local site of agroinoculation. The abaxial face of locally agroinoculated leaves showed typical silencing signs around the site of agroinoculation forming a red ring either when inoculating TYLCTHV-[AIT]_GFP solely or TYLCTHV-[AIT]_GFP/TYLCTHV-[AIT] B multimeric clones (Figure 8 E-F). Silencing signs developed to a darker red ring at 19 dpi (Figure 8 I-J).

At 14 dpi no systemic symptoms were observed on 16c plants either in plants inoculated with TYLCTHV-[AIT]_GFP solely or TYLCTHV-[AIT]/TYLCTHV-[AIT] B. Interestingly, systemic silencing signs (red tissues) were first observed in 16c plants containing both multimeric clones. Small red spots surrounding the vein tissue with green and red intervals between the veins were observed in young sink leaves under UV light due to suppression of *gfp* expression. At 14 dpi systemic silencing signs were only observed in new emerging leaves in all plants (30 out of 30 plants) agroinoculated with TYLCTHV-[AIT]_GFP and TYLCTHV-[AIT] B multimeric clones (Figure 8 H) and not in those plants agroinoculated with TYLCTHV-[AIT]_GFP solely .

At 19 dpi 16c plants inoculated with TYLCTHV-[AIT] _GFP multimeric clone solely did not show any systemic silencing signs neither in young sink leaves nor in mature leaves (Figure 8 K). Moreover, few plants (five out of 30 plants) showed downward leaf rolling symptoms, typical from a geminivirus infection (Figure 8 M). Transgenic 16c plants inoculated with both multimeric clones (TYLCTHV-[AIT]_GFP/TYLCTHV-[AIT] B) also showed a total of five out of 30 plants with downward leaf rolling symptoms (Figure 8 N) and an increase in red tissues on systemically infected leaves (young sink leaves) from the veins (silenced tissue) when visualized under UV light (Figure 8 L). The silenced tissues were analyzed under the CLSM and green fluorescence was detected only in stomata, indicating that silencing failed in these cells (Figure 8 P).

Finally at 35 dpi, first systemic signs of silencing were observed in leaves containing the multimeric clone TYLCTHV-[AIT]_GFP solely. In contrast to the results obtained when coinoculating the B component of TYLCTHV, silencing signs were observed only in mature leaves and only in two out of 30 inoculated 16c plants (Figure 8 Q-S). On the other hand, at 35 dpi almost all transgenic 16c plants inoculated with the B component and TYLCTHV-[AIT]_GFP showed practically a completely silenced tissue (Figure 8 R-T), whereas only the stomata were showing still green fluorescence.

Moreover, to verify if viral DNA could be amplified from systemically silenced tissue, DNA was extracted from 16c silenced plants and PCR was performed to amplify a fragment of 589 bp of the AC2 region of the TYLCTHV-[AIT] genome. Only very low amounts of the amplified fragment was obtained from 16c plants either inoculated with the TYLCTHV-[AIT]_GFP multimeric clone or in combination with its B component (data not shown).

4.5 Discussion

Here we investigated the ability of TYLCTHV-[AIT] to be engineered and used as an expression and VIGS vector system. This is the first report describing TYLCTHV as an expression vector. To this purpose, the AV1 gene of TYLCTHV-[AIT] was replaced by the coat protein gene of VCV (complete or partial sequences) and also with *gfp* with or without a HA tag. Once GFP was expressed from the TYLCTHV-[AIT] genome we used the mutated construct to understand the dynamics of gene silencing caused by TYLCTHV-[AIT]. For this purpose, the systemic progression of gene silencing was monitored using agroinoculated transgenic *N. benthamiana* 16c plants under UV light and also by CLSM.

The use of geminiviruses as expression systems may present several different advantages compared to RNA viruses: (i) a relatively small genomic size, facilitating cloning and sequencing; (ii) DNA is more stable than RNA and can be directly inoculated in plants via particle bombardment or even mechanically; (iii) the genome organization is very conserved, which may offer the discovery of new VIGS systems for different hosts (iv) geminiviruses induce a broad variety of symptoms varying from severe, mild to asymptomatic (Carrillo-Tripp et al., 2006). However, few disadvantages must be taken into consideration: (i) the strong genome packing size limitation and (ii) monopartite viruses need their CP for systemic infection (Morilla et al., 2006).

The use of GFP to monitor gene expression has been commonly used for RNA viruses and some reports are known to use geminivirus as expression vector (Cui et al., 2005b; Fofana et al., 2004; Kjemtrup et al., 1998; Kim et al., 2007; Mor et al., 2003; Morilla et al., 2006; Peele et al., 2001; Qian et al., 2006; Tao and Zhou, 2004; Sudarshana et al., 1998). However, few reposts have exploited the use of bipartite viruses as expression vectors and/or VIGS (Fofana et al., 2004; Kjemtrup et al., 1998; Peele et al., 2001; Sudarshana et al., 1998). Here we used TYLCTHV-[AIT] to express

the *gfp* as well as the CP of VCV in the presence or absence of its B component. When expressing the complete CP of VCV, no viral DNA was found to infect the plant systemically up to 14 dpi either in plants agroinoculated with TYLCTHV-[AIT] _VCV solely or in combination with the B component. However, small fragments, representing mutants with deletions in the VCV, were amplified using PCR. Probably the insertion of 1464 bps instead of the AV1 gene was exceeding the capacity of transport and packaging of DNA A. Gilbertson et al. (2003) examined the genome size limitation of the *Bean dwarf mosaic virus* (BDMV) genome. For that, they engineered a series of sized-increased DNA A components of the virus, which upon inoculation were reverted to the original size by homologous and non-homologous recombination as well as by template switching between the A and B components. Gilbertson and coworkers suggested that the BC1 protein is the primary factor responsible for genome reversion and not the BV1 protein. Our results confirm the findings of Gilbertson and coworkers. Only in *N. benthamiana* plants agroinoculated with the multimeric clone of the B component it was possible to amplify viral DNA A and not in those exclusively containing the TYLCTHV-[AIT]_VCV multimeric clone. Probably also here, BC1 protein, which is coded by the B component was responsible for the observed size reversion. Therefore, we constructed a new viral vector expressing only the C-terminus of the CP of VCV tagged with a hemagglutinin epitope. Although it was possible to amplify sequences coding for the entire proteins (GFP and Express_CP of VCV) from the upper young leaves by PCR, it was not possible to detect GFP or the truncated CP in immunoblot assays using anti-HA antibodies at 15 dpi. It was only possible to detect the HA-tagged expressed GFP and also HA-tagged truncated CP of VCV from locally agroinoculated leaves using immunoblot assays. However, green fluorescence emitted by TYLCTHV-[AIT]_GFP_HA expression was visualized from young tissues using the CLSM at 21 dpi (data not shown). One may question if the HA tagged proteins interfere either with the viral replication and/or with the virus movement inside the plant. The obtained results may suggest that either the amount of those proteins were produced in very low amounts in young emerging leaves at 15 dpi, which could not be detected via Western blot, or that a deletion occurred during viral replication and probably a loss of the HA tag.

Moreover, we showed the ability of TYLCTHV-[AIT] A component to induce gene silencing of a homologous transgene in *N. benthamiana*. However, the spread of the silencing signs were influenced by the presence of TYLCTHV B component. Transgenic *N. benthamiana* 16c plants showed systemic silencing signs already at 14

dpi in the presence of the TYLCTHV B component, while in plants agroinoculated only with TYLCTHV-[AIT]_GFP multimeric clone systemic silencing signs were observed only after 35 dpi. The presence of the B component not only influenced the time of appearance of the silencing signs but also its localization inside the plants. Transgenic *N. benthamiana* 16c plants agroinoculated with the TYLCTHV-[AIT]_GFP multimeric clone showed first silencing signs in old leaves, while those plants agroinoculated additionally with the TYLCTHV B multimeric clone showed first signs on upper young leaves. One might speculate that proteins encoded by the B component may play a role in transport of the silencing signals (siRNA) through the phloem and adjacent tissues, overall increasing the silencing effect.

Although agroinoculated *N. benthamiana* 16c plants containing only the modified A component of TYLCTHV-[AIT] did not show a complete systemic silencing, while plants containing in addition the B component were completely silenced, it was possible to amplify viral DNA from both transgenic plants but only in very low amounts at 35 dpi. This may suggest that the degree of silencing inside the plant does not correspond directly to the amount of replicated viral DNA. Moreover, symptoms of geminivirus infection including downward leaf curling appeared at 19 dpi in *N. benthamiana* 16c plants containing the viral vector of the A component as well as in those plants agroinoculated with both clones. Probably minimal amounts of viral DNA inside the plants, which were able to escape the silencing mechanism, were sufficient to generate geminivirus specific symptoms.

Moreover, silencing signs occurred first around the veins but not continuously through the veins. Green fluorescence intervals could be visualized scattered within the red tissue. Completely silenced tissue showed guard cells that escaped the silencing signals still at 48 dpi because stomata guard cells are symplastically isolated from neighbouring cells by plasmodesmata occlusion (Wille and Lucas, 1984). Characteristic silencing properties have been previously reported by Himber et al. (2003). They showed that silencing signals could spread independent of the presence of the homologous transcripts recruiting the same plasmodesmata channels for cell-to cell movements used for the silencing signals; that silencing first occurs around the veins, later invading the whole lamina and finally Himber and coworkers suggested that limited silencing movement is likely through plasmodesmata because stomata guard cells were unaffected showing green fluorescence.

Taken these results together, this study shows the ability of TYLCTHV-[AIT] to express a foreign protein and also induce gene silencing of a homologous host gene,

especially when coinoculated with its B component. TYLCTHV-[AIT] may be used as an expression for foreign genes but also used as VIGS vector for studying the function of endogenous host genes. Furthermore, this is the first report showing the influence of the B component in systemic gene silencing and its spread through the plant. Therefore, further studies including the B component of begomoviruses in elucidating the dynamics of silencing spread should be taken into consideration.

5.0 General Discussion and Outlook

This study was undertaken to provide a better understanding of the replication and relationship of begomoviruses occurring in Thailand and Vietnam. The need to understand the factors, which are responsible for begomovirus-induced epidemics in the tropic and subtropic areas, as well as the application of novel methods to control begomoviruses diseases have lead further research on geminivirus dynamics. Therefore, this work approaches different fields and questions concerning geminiviruses transreplication, resistance and silencing dynamics. Moreover, we exploited the potential use of begomoviruses as expression vectors.

The first chapter explores the ability of TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV A component to transreplicate the B component of TYLCTHV-[AIT]. Pseudorecombinants have been reported for some begomoviruses (Andrade et al., 2006; Bull et al., 2007; Frischmuth et al., 1997; Gilbertson et al., 1993; Harrison and Robison, 1999; Hill et al., 1998; Hou and Gilbertson, 1996; Jovel et al., 2007; Levy and Czosnek, 2003; Pita et al., 2001; Sung and Coutts, 1995b; Unseld et al., 2000a). Viable pseudorecombinants may depend not only on a unique molecule but on the interaction of both A and B molecules. That means, the ability of an A component to transreplicate heterologous B components and form stable pseudorecombinants may also depend on the ability of proteins coded by the B component (movement and nuclear shuttle protein) to mediate movement of the heterologous A component (Ramos et al., 2003). Argüello-Astorga and Ruiz-Medrano (2001) proposed a model for the interaction between the domain located at the N-terminus of the Rep protein iteron-related domain and the binding sequences (iteron) located at the viral intergenic region (Argüello-Astorga et al., 1994a). The specificity of the Rep-iteron binding has been considered as one of the main explanations for the formation of viable pseudorecombinants.

However, some studies show that the Rep-iteron binding is not the only determinant in the formation of viable pseudorecombinants (Andrade et al., 2006; Garrido-Ramirez et al., 2000; Lin et al., 2003). In this study TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV could transreplicate the B component of TYLCTHV-[AIT] in both plants *N. benthamiana* and tomato. The IRD in the Rep protein of TYLCTHV-[AIT], TYLCVV-[DX1] and ToLCVV-[DX2] is identical and this could be the major reason for the formation of pseudorecombinants. However, TbLCTHV has a different iteron and IRD

and even though was able to facilitate transreplication of the TYLCTHV-[AIT] B component. In this context, one can not exclude that conserved amino acids in the IRD were sufficient to recognize and bind to the intergenic region of the TYLCTHV-[AIT] B component. Nevertheless, additional factors may have contributed to the transreplication of the TYLCTHV-[AIT] B DNA by TbLCTHV.

Furthermore, in the work presented here the transmission of pseudorecombinants was studied. Pseudorecombinants were transmitted to *N. benthamiana* and/or tomato by particle bombardment and also via mechanical means. The successful transmission probably indicates that the B component of TYLCTHV-[AIT] may overcome the phloem limitation of TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV. Symptoms included down/upward leaf curling, yellow mosaic pattern and severe stunting depending on the combination of multimeric clones used. The B component of TYLCTHV-[AIT] was the major determinant of yellow mosaic symptoms in *N. benthamiana* and tomato plants, while plants infected with ToLCVVß showed the typical downward leaf curling symptom. Interestingly, it was possible to detect in systemically infected leaves of both *N. benthamiana* and tomato plants DNA beta and the B component.

The emergence of new viral epidemics due to pseudorecombination events between viruses occurring in a determined region has been suggested in some studies. Pita et al. (2001) provided evidence that pseudorecombinants of EACMV were one of the causes for the cassava mosaic epidemics described in Uganda in 1988. From mixed infections viable pseudorecombinants, which might have a higher fitness in different hosts, might be selected. The new virus might have a higher replication rate; consequently accumulates more DNA and contribute to a more severe disease. Thailand and Vietnam are not far from each other and therefore one cannot exclude the possibility that the different tomato infecting begomoviruses occurring in the two countries meet each other and cause a new virus disease with severe yield losses of tomato.

The second part of the present work consisted in generating transgenic *N. benthamiana* plants resistant against TYLCTHV-[AIT]. Geminiviruses can be the target of PTGS and TGS. Vanitharani et al. (2005) summarized a model describing how viral dsRNA is formed inside the plant. DsRNA molecules may originate from abundant transcripts that might serve as template to host polymerases, from overlapping

transcripts originating from the bi-directional promoter and finally from secondary structures possibly formed by a strong fold-back of the viral transcript. Inverted-repeat constructs to generate transgenic plants resistant against RNA viruses have been successfully used (Bucher et al., 2006; Di Nicola-Negri et al., 2005; Chen et al., 2004; Missiou et al., 2004; Mitter et al., 2003; Pandolfini et al., 2003; Kalantidis et al., 2002; Kamachi et al., 2007; Smith et al., 2000). However, only few studies have shown the generation of resistant transgenic plants by using inverted-repeat constructs of geminiviruses (Fuentes et al., 2006; Pooggin et al., 2003; Bonfim et al., 2007; Zrachya et al., 2007b; Ribeiro et al., 2007).

The Rep nucleotide sequence is the most employed region for generating transgenic plants resistant to begomoviruses (Bendahmane and Gronenborn, 1997; Bonfim et al., 2007; Brunetti et al., 2001; Lucioli et al., 2003; Noris et al., 1996a). Rep is a multifunctional protein involved in different pathways inside the plant. It is not only involved directly in virus replication but also indirectly in regulation of cell growth and death. Therefore, the use of constructs harboring other parts of the begomovirus genome for plant resistant has been under-exploited and only some examples are known (Asad et al., 2003; Kunik et al., 1994; Zrachya et al., 2007b). However, in this study different regions of the TYLCTHV-[AIT] genome (AC2/C3, IR/Rep, Rep, IR/CP, IR/BV1 and IR/BC1) were chosen for generating inverted-repeat constructs driven by the 35S promoter of the CaMV and thereby constitutively expressed in *N. benthamiana* plants.

The use of inverted-repeat constructs to attempt the generation of transgenic plants, which are resistant to a begomovirus disease, are controversial. On one hand, the use of an inverted-repeat construct to promote resistance against ToCMoV did not guarantee immunity of *N. benthamiana* plants and accumulation of siRNA could not be correlated to timing of delayed symptoms (Ribeiro et al., 2007). In addition, Bian et al. (2006) showed susceptible plants harboring a hairpin construct of the ToLCV C2 sequence region. On the other hand, transformation of tomato plants harboring hairpin constructs generated immune plants until 70 dpi to TYLCV (Fuentes et al., 2006). Fuentes and coworkers observed that accumulation of siRNA was correlated to the resistance level. Moreover, tomato transgenic lines (126 and 100) harboring only a single copy of the transgene showed different resistance response. Plants were susceptible from line 100 and resistant from line 126. Therefore, they suggested that the difference in phenotype was not due the number of copies of the transgene but probably due to the site of

integration in the host genome, which plays a role in transcript production. Also, Bonfim et al. (2007) showed one common bean line out of 18 lines to be resistant against BGMV using an intron-hairpin construct harboring the sequence region of the viral AC1 gene.

Bian et al. (2006) provided first explanations for the delayed symptoms obtained when developing plants resistant against geminiviruses. Bian and coworkers transformed tomato plants harboring a hairpin construct from the ToLCV C2 gene, which resulted in delayed symptom expression of plants that could not prevent virus infection and DNA accumulation. Therefore, the group used transgenic plants harboring the C4 gene of ToLCV under the 35S promoter to monitor silencing upon virus infection. This was possible because transgenic plants containing 35S:C4 express a severe phenotype. They suggested that methylation of the virus replicative form and host plant DNA might be involved in the overcoming of the silencing mechanism by ToLCV. Although methylation of asymmetric and symmetric cytosines were observed in the viral DNA replicative form still ToLCV was able to replicate in silenced plants. The authors suggested the possibility that a small pool of unmethylated ToLCV replicative forms was sufficient for virus transcription. Both unmethylated replicative forms as well as the increase of asymmetric cytosine methylation upon virus inoculation probably contributed to facilitate ToLCV replication.

In this study, to generate plants resistant against TYLCTHV-[AIT] four inverted-repeat constructs containing sequences from the TYLCTHV-[AIT] A component and two constructs from the TYLCTHV-[AIT] B component were used. Delayed and/or recovered transgenic *N. benthamiana* T_1 plants harboring inverted-repeat constructs of the AC2/C3, IR/CP and IR/BC nucleotide sequence of the TYLCTHV-[AIT] could be generated. Delayed symptom expression of transgenic T_1 lines were obtained 30 days post-inoculation (dpi) and even at 90 dpi for the line harboring the IR/CP construct. Because no absolute resistance (immune lines) was verified in the T_1 generation it would be interesting to further investigate the obtained T_2 plant lines for begomovirus resistance.

The use of inverted-repeat constructs to generate resistant transgenic plants must be further exploited. As described, geminiviruses may be subject to two different silencing pathways (PTGS and TGS). The first is a posttranscriptional gene silencing

(cytoplasmic RNA silencing) leading to mRNA degradation and the second one leads to transcriptional gene silencing (TGS) through a siRNA-directed methylation. Maybe the ability to circumvent the silencing mechanism in begomoviruses is a virus-specificity issue. It has been shown that different begomoviruses proteins (AC2, AC4, AV2) may act as suppressor of gene silencing and have various functions in the silencing pathway. It is clear that the same protein may function as a suppressor of gene silencing in some begomoviruses but not in all. Therefore, more precise knowledge on the functions of begomoviruses proteins involved in the silencing pathway is needed. Moreover, it will be interesting to investigate if a construct generated to target at the same time different suppressors of gene silencing of begomoviruses would increase the probability of generating resistant transgenic plants.

Geminiviruses may be used as a tool for expressing foreign proteins, a topic investigated in the third part of the present work. The use of geminiviruses for production of foreign proteins shows many advantages when compared to RNA viruses. Small genomic size, an increasing number of available sequences, as well as the possibility of direct inoculation of the viral DNA makes geminiviruses an interesting tool to be not only used as an expression vector but also as VIGS vector. To exploit the potential of TYLCTHV-[AIT] as an expression vector, in this study the AV1 gene was replaced by the GFP/GFP-HA as well as by the entire sequence (or parts of it) coding for the CP of VCV with or without a HA tag. When expressing the entire CP of VCV, a delayed symptom expression accompanied by a reduction of the genomic size was observed. Genomic size limitations of geminiviruses have been observed also for BDMV (Etessami et al., 1989; Elmer and Rogers, 1990; Gilbertson et al., 2003). In addition, both GFP-HA and truncated VCV CP-HA resembling the original size of TYLCTHV-[AIT] were detected using HA-antibody only in the agroinoculated infected leaves and not in systemically infected leaves. However, systemic GFP fluorescence of mesophyll cells was detected using CLSM. Therefore, one could speculate about the level of protein expression, which might have been not sufficient to be detected using immunoblotting assays, or about a deletion of the HA tag, which may occur during viral replication. Recently, Schembri et al. (2007) discovered that the HA tag was cleaved during apoptosis from a chimera construct containing a N-terminally HA-tagged GFP. They gave proof that the active form of caspase 3/7 induced GFP release and HA cleavage in HeLa cells independent of the HA position in the tagged constructs. The cleavage of HA resulted in total loss of immunoreactivity. Plants caspases (1, 3 and/or

6-like) showing proteolytic activities have been found during induced plant program cell death (PCD) (San Martin et al., 2005; Woltering et al., 2002). It could be possible that the expression of GFP HA tagged from TYLCTHV-[AIT] was detected in agroinoculated leaves (expressed from integrated copies of host genome) but not in systemically infected cells because PCD is already activated in the latter as defense response.

Finally we were able to monitor the dynamics of gene silencing when TYLCTHV-[AIT] expressing GFP was agroinoculated alone or in combination with TYLCTHV-[AIT] B multimeric clone in *N. benthamiana* 16c transgenic plants. Silencing signs were monitored using UV light from 5 to 35 dpi. At 5 dpi first silencing signs were observed in locally agroinoculated leaves harboring both combinations. However, at 14 only leaves of agroinoculated leaves harboring the B component showed silencing signs in systemically infected leaves. At 35 dpi upper infected leaves were completely silenced. Interestingly, DNA isolation from silenced tissue amplified by PCR revealed low amounts of viral DNA. Probably, a small pool of replicating viruses was able to circumvent the silencing mechanism in inoculated *N. benthamiana* 16c transgenic plants. On the other hand, only very few 16c plants were showing first signs of systemically silencing at 35 dpi when inoculated with TYLCTHV-[AIT] expressing GFP solely. The observed difference in spread and localization of silencing signs in transgenic plants in the presence of B components of bipartite viruses should be further investigated. It will be interesting to know, if proteins coded from the B component may play a role in facilitating somehow the spread of silencing signals.

Taken all together, this study has contributed to a better understanding of the tomato infecting begomoviruses in Thailand. Tomato is grown in all regions in Thailand but mainly in the central and northeastern regions. It is not only consumed as table fruit, but also as concentrated juice and dried fruit. In 2006 in Thailand the cultivated harvest area of tomato was 11,500.00 ha with a yield of 17,189.74 Kg/ha, which is relatively low compared to other areas (FAO, 2007). Begomovirus infections contribute to the relative low yield. Moreover, very recently, TYLCTHV has been found infecting tomato plants containing also ToLCV in Taiwan (Jan et al., 2007). The fact that TYLCTHV could infect resistant tomato plants (H24), which carries the Ty-2 gene, may represent an additional problem in those areas. Therefore, not only the development of new strategies

to obtain resistance to begomoviruses but also the transreplication of B components and their evolutionary aspect in Asian begomoviruses call for further attention.

Outlook

Results obtained in the present study raise some questions about the epidemiological consequences of the particular ability of the studied begomoviruses to transreplicate the B component of TYLCTHV-[AIT], about the practical implications in producing transgenic plants resistant to geminiviruses and finally about the use of TYLCTHV as an expression system.

Genome variations of begomoviruses depend not only on the mutation rate but also on the particular characteristic of these viruses to acquire new molecules, to pseudorecombine and/or recombine. The possible occurrence of mixed infections in field conditions may lead to emergence of new complex diseases and an increase of virus spread (Lazarowitz et al., 1991; Harrison et al., 1997a, 1997b). Consequently, the fact that the A component of TYLCVV-[DX1], ToLCVV-[DX2] and TbLCTHV could transreplicate the B component from TYLCTHV-[AIT] may have epidemiological effects. Viral spread may be increased because, as shown in the present study, the B component of TYLCTHV-[AIT] facilitates mechanical transmission of the virus. In addition, results concerning the geminivirus-plant-whitefly association have been shown to be controversial. Sinisterrra et al. (2005) quantified the amounts of *Tomato mottle virus* (ToMoV) and *Tomato yellow leaf curl virus* (TYLCV) transcripts in whiteflies (*Bemisia tabaci* biotype B) when transferred to a non-host. Increase in level of TYLCV transcripts were detected in whiteflies while ToMoV transcripts were not detectable. Although increased amounts of TYLCV transcripts may be accumulated in whiteflies, it is still controversial if TYLCV replicates inside its whitefly vector. Hence it would be interesting to know if the level of possible pseudorecombinant transcripts increases in the whitefly and if yes, if this accumulation favors or not the virus replication and spread in different hosts.

Moreover, it has been shown that whiteflies containing tobacco curly shoot virus had an increase in fecundity by 36% and no effects on longevity while the presence of *Tomato yellow leaf curl China virus* reduced infectivity and longevity of the vector by 27% and 36%, respectively. (Jiu et al., 2007). Therefore, it will be interesting to know if

association of the pseudorecombinants with the *B. tabaci* biotype B would increase fecundity and longevity and thereby lead to an increase of virus spread.

Although nothing is known about the agronomic performance of resistant transgenic plants harboring geminiviruses constructs under field conditions, it is clear from other studies that transgenic plants (plum, papaya, peanut and wheat) engineered to confer virus resistance may be propagated over years without symptoms (Gonsalves, 2004; Hily et al., 2004; Sharp et al. 2002; Yang et al., 2004a). In Asia tomato plants are produced mainly in winter (November to February) due to absence of heat tolerant varieties. Virus infection should be avoided in the vegetative and early flowering period (50 to 60 days after planting) to assure high tomato yield. Therefore, if tomato transgenic plants harboring inverted-repeat constructs (as presented in this work) would be generated, probably a high performance in resistance against TYLCTHV-[AIT] as well as high yield of tomato production could be obtained. Tomato plants at seedling stage would contain none or very few viral DNA molecules, which is desirable for a high yield of tomato fruits.

The use of hybrid tomato resistant cultivars derived mainly from wild-type tomato species (*Lycopersicum chilense*, LA1969, LA1965, LA1932 LA1938*; L. hirsutum*, LA1777, LA380, LA1295; *L. pimpinellifolium*, LA121, LA1582 and *L. pennellii*, LA1275) (Czosnek et al., 1993; Pico et al. 1999, Zakay et al., 1991) are commonly used in the field. However, even breeding lines (UPV Ty 1, 3, 6, 9, 17 and 53) that are resistant to TYLCV still show under field condition 30% to 40% of yield loss (Pico et al., 1999). Therefore, it will be interesting to know if the generation of transgenic tomato harboring a resistance gene (e.g. TY-1, TY-20, TY-2) in combination with inverted-repeat constructs would generate plants, which upon field infection would be free of geminiviruses.

Finally, benefits of the use of geminiviruses as silencing and expression vector systems should be explored. The large-scale production of proteins by plants offers advantages like low cost and safety for agricultural, industrial and pharmaceutical applications (Hefferon and Fan, 2004). However, engineering geminiviruses for production of high levels of proteins may be advantageous in comparison to the use of transgenic plants. First, there is no need of plant transformation, which may be laborious and can lead to time delays concerning the protein production. Second, high

copy numbers within the plant's cell are generated (Hayes et al., 1988). In addition, a high production of proteins (constitutively expressed) may be toxic for the transgenic plants but not when expressed transiently from viruses. Moreover, mild geminiviral symptoms would probably not interfere with the quantity of produced protein.

On the other hand, symptoms are not desirable when engineering geminivirus as virus induced gene silencing vectors (VIGS). Therefore, it would be interesting to know if the mild symptoms, which appeared in *Nicotiana benthamiana* when agroinoculated with TYLCTHV-[AIT]_GFP will change when inoculated in *Solanum lycopersicum*. In addition, it would be interesting to assess how the engineered TYLCTHV as VIGS vector is affected by different environmental conditions. It has been shown that silencing of a phytoene desaturase gene maintained in inoculated tomato plants with *Tobacco rattle virus* (TRV) is affected by temperature and humidity (Fu et al., 2006).

6.0 Virus Taxonomy and Abbreviations

VIRUS TAXONOMY

AbMV	Abutilon mosaic virus
ACMV	African cassava mosaic virus
ACMV-[CM]	African cassava mosaic virus-[Cameroon]
AEV	Ageratum enation virus
ALCuV-[G52]	Ageratum leaf curl virus-[G52]
AYVCNV-[Hn2]	Ageratum yellow vein China virus-[Hainan 2]
AYVSLV	Ageratum yellow vein Sri Lanka virus
AYVTV-[TW]	Ageratum yellow vein Taiwan virus-[Taiwan]
AYVV	Ageratum yellow vein virus
BcaMV	Bean calico mosaic virus
BCTV	Beet curly top virus
BDMV	Bean dwarf mosaic virus
BeYDV	Bean yellow dwarf virus
BGMV	Bean golden mosaic virus
BGMV-[BR]	Bean golden mosaic virus-[Brazil]
BGYMV-[CU]	Bean golden yellow mosaic virus-[Cuba]
BYVMV-[301]	Bhendi yellow vein mosaic virus-[301]
CabLCuV	Cabbage leaf curl virus
CaMV	Cauliflower mosaic virus
CdTV-[H6] (TLCrV)	Chino del tomate virus-[H6]
ChaYMV	Chayote yellow mosaic virus
ChiLCuV-[Mul]	Chilli leaf curl virus-[Multan]
CLCrV	Cotton leaf crumple virus
CLCuAV-[802a]	Cotton leaf curl Alabad virus-[802a]
CLCuKV-[72b]	Cotton leaf curl Kokhran virus-[72b]
CLCuMV	Cotton leaf curl Multan virus
CLCuMV-[26]	Cotton leaf curl Multan virus-[26]
CLCuRV	Cotton leaf curl Rajasthan virus
CPGMV-[BR]	Cowpea golden mosaic virus-[Brazil]
CsVMV	Cassava vein mosaic virus

CuLCrV	Curcubit leaf crumple virus
CYVMV	Croton yellow vein mosaic virus
CYVVV-[Hoa]	Corchorus yellow vein virus-[Hoa]
DoYMV	Dolichos yellow mosaic virus
EACMCV-[CM]	East African cassava mosaic Cameroon virus-[Cameroon]
EACMMV-[K]	East African cassava mosaic Malawi virus-[K]
EACMV-[UG2]	East African cassava mosaic virus-[Uganda 2]
EACMV	East African cassava mosaic virus
EACMZV	East African cassava mosaic Zanzibar virus
EpYVMV-[SOJ3]	Eupatorium yellow vein mosaic virus-[SOJ3]
EpYVV	Eupatorium yellow vein virus
EuLCV-[G35]	Euphorbia leaf curl virus-[G35]
HoLCrV-[Giza]	Hollyhock leaf crumple virus-[Giza]
HYVMV	Honeysuckle yellow vein mosaic virus
ICMV	Indian cassava mosaic virus
IYVV	Ipomoea yellow vein virus
MSV	Maize streak virus
MYMV	Mungbean yellow mosaic virus
MYMIV-[Ako]	Mungbean yellow mosaic India virus-[Akola]
MYMV-[TH1]	Mungbean yellow mosaic virus-[Thailand 1]
MYVV-[Y47]	Malvastrum yellow vein virus-[Y47]
OYVMV-[201]	Okra yellow vein mosaic virus-[201]
PaLCuCNV-[G2]	Papaya leaf curl China virus-[G2]
PaLCuCNV-[G10]	Papaya leaf curl China virus-[G10]
PaLCuCNV-[G30]	Papaya leaf curl China virus-[G30]
PaLCuCNV-[G43]	Papaya leaf curl China virus-[G43]
PaLCuGuV-[GD2]	Papaya leaf curl Guandong virus-[GD2]
PaLCuV	Papaya leaf curl virus
PepLCBV	Pepper leaf curl Bangladesh virus
PepLCV	Pepper leaf curl virus
PHYVV	Pepper huasteco yellow vein virus
PVX	Potato virus X
PYMPV	Potato yellow mosaic Panama virus
PYMV	Potato yellow mosaic virus

SiGMV-[Hoyv]	Sida golden mosaic virus-[Honduras yellow vein]
SiYMCNV-[Hn8]	Sida yellow mosaic China virus-[Hainan 8]
SLCCNV	Squash leaf curl China virus
SLCMV	Sri Lankan cassava mosaic virus
SLCMV-[Col]	Sri Lankan cassava mosaic virus-[Colombo]
SLCYNV-[Y23]	Squash leaf curl Yunnan virus-[Yunnan 23]
SLCV (SqLCV)	Squash leaf curl virus
StaLCuV-[Hn5]	Stachytarpheta leaf curl virus-[Hainan 5]
TbCSV-[Y1]	Tobacco curly shoot virus-[Yunnan 1]
TbCSV-[Y35]	Tobacco curly shoot virus-[Yunnan 35]
TbCSV-[Y41]	Tobacco curly shoot virus-[Yunnan 41]
TbLCKoV-[KK]	Tobacco leaf curl Kochi virus-[KK]
TbLCTHV	Tobacco leaf curl Thailand virus
TbLCYNV-[Y3]	Tobacco leaf curl Yunnan virus-[Yunnan 3]
TbLCYNV-[Y136]	Tobacco leaf curl Yunnan virus-[Yunnan 136]
TbLCYNV-[Y143]	Tobacco leaf curl Yunnan virus-[Yunnan 143]
TbLCYNV-[Y161]	Tobacco leaf curl Yunnan virus-[Yunnan 161]
TbYDV	Tobacco yellow dwarf virus
TGMV	Tomato golden mosaic virus
TGMV-[Com]	Tomato golden mosaic virus-[Common]
ToCMoV	Tomato chlorotic mottle virus
ToCMoV-[MG-Bt1]	Tomato chlorotic mottle virus-[Minas Gerais-Betim1]
ToLCBDV	Tomato leaf curl Bangladesh virus
ToLCBV	Tomato leaf curl Bangalore virus
ToLCCNV-[G16]	Tomato leaf curl China virus-[G16]
ToLCCNV-[G32]	Tomato leaf curl China virus-[G32]
ToLCGuV-[G2]	Tomato leaf curl China virus-[G2]
ToLCGV-[Kel]	Tomato leaf curl Gujarat virus-[Kelloo]
ToLCJV	Tomato leaf curl Java virus
ToLCKV	Tomato leaf curl Karnataka virus
ToLCLV	Tomato leaf curl Laos virus
ToLCMYV	Tomato leaf curl Malaysia virus
ToLCNDV	Tomato leaf curl New Delhi virus

ToLCNDV-[Mild]	Tomato leaf curl New Delhi virus-[Mild]
ToLCPV	Tomato leaf curl Philippines virus
ToLCSLV	Tomato leaf curl Sri Lanka virus
ToLCTWV	Tomato leaf curl Taiwan virus
ToLCV	Tomato leaf curl virus
ToLCV-[AU]	Tomato leaf curl virus-[Australia]
ToLCVV	Tomato leaf curl Vietnam virus
ToLCVV-[DX2]	Tomato leaf curl Vietnam virus-[Dang Xa 2]
ToMoV	Tomato mottle virus
ToYSV	Tomato yellow spot virus
TYLCCNV (TYLCV-[China])	Tomato yellow leaf curl China virus
TYLCCNV-[Tb:Y10] (TYLCCNV-[Y10])	Tomato yellow leaf curl China virus-[Tobacco Y10]
TYLCGuV-[G3]	Tomato yellow leaf curl Guandong virus-[G3]
TYLCKaV-[TH:Kan2]	Tomato yellow leaf curl Kanchanaburi virus-[Thailand Kanchanaburi 2]
TYLCV	Tomato yellow leaf curl virus
TYLCV-[IL]	Tomato yellow leaf curl virus-[Israel]
TYLCSV	Tomato yellow leaf curl Sardinia virus
TYLCTHV	Tomato yellow leaf curl Thailand virus
TYLCTHV-[1]	Tomato yellow leaf curl Thailand virus-[1]
TYLCTHV-[2]	Tomato yellow leaf curl Thailand virus-[2]
TYLCTHV-[AIT]	Tomato yellow leaf curl Thailand virus-[Asian Institute of Technology]
TYLCTHV-[ChMai] (TYLCTHV-[CM])	Tomato yellow leaf curl Thailand virus-[Chiang Mai]
TYLCTHV-[MM]	Tomato yellow leaf curl Thailand virus-[Myanmar]
TYLCTHV-[NK]	Tomato yellow leaf curl Thailand virus-[Nong Khai]
TYLCTHV-[SK]	Tomato yellow leaf curl Thailand virus-[Sakon Nakhon]
TYLCTHV-[Y72]	Tomato yellow leaf curl Thailand virus-[Yunnan 72]
TYLCV	Tomato yellow leaf curl virus

TYLCV-[Alm]	Tomato yellow leaf curl virus-[Almeria]
TYLCVV-[DX1]	Tomato yellow leaf curl virus-[Dang Xa 1]
VCV	Vicia cryptic virus
WDV	Wheat dwarf virus

Satellites

ALCuVß	Ageratum leaf curl virus satellite DNA ß
AYVCNVß	Ageratum yellow vein China virus satellite DNA ß
AYVVß	Ageratum yellow vein virus satellite DNA ß
BYVMVß	Bean yellow vein mosaic virus satellite DNA ß
ChiLCuVß	Chilli leaf curl virus satellite DNA ß
CLCuBVß	Cotton leaf curl Bangalore virus satellite DNA ß
CLCuGVß	Cotton leaf curl Gezira virus satellite DNA ß
CLCuRVß	Cotton leaf curl Rajasthan virus satellite DNA ß
CLCuVß	Cotton leaf curl virus satellite DNA ß
CPSLCVß	Cowpea severe leaf curl virus satellite DNA ß
EpYVVß	Eupatorium yellow vein virus satellite DNA ß
ErYMVß	Erectites yellow mosaic virus satellite DNA ß
HYVMVß	Honeysuckle yellow vein mosaic virus satellite DNA ß
LuYVVß	Ludwigia yellow vein virus satellite DNA ß
LYMVß	Luffa yellow mosaic virus satellite DNA ß
MYMIVß	Mungbean yellow mosaic India virus satellite DNA ß
MYVVß	Malvastrum yellow vein virus satellite DNA ß
OkLCuVß-[DNA10]	Okra leaf curl virus-[DNA10] satellite DNA ß
OkYLVVß	Okra yellow leaf vein virus satellite DNA ß
PaLCuVß	Papaya leaf curl virus satellite DNA ß
SiLCuVß	Sida leaf curl virus satellite DNA ß
SiYMCNVß	Sida yellow mosaic China virus satellite DNA ß

SiYVVß	Sida yellow vein virus satellite DNA ß
TbCSVß	Tobacco curly shoot virus satellite DNA ß
TbLCYNVß	Tobacco leaf curl Yunnan virus satellite DNA ß
ToLCCNVß	Tomato leaf curl China virus satellite DNA ß
ToLCJVß	Tomato leaf curl Java virus satellite DNA ß
ToLCNDVß	Tomato leaf curl New Delhi virus satellite DNA ß
ToLCVVß-[DX2]	Tomato leaf curl Vietnam virus-[Dang Xa 2] satellite DNA ß
ToLCVVß-[VR2]	Tomato leaf curl Vietnam virus-[Vien Rau Qua 2] satellite DNA ß
ToLCVVß-[VRQ]	Tomato leaf curl Vietnam virus-[Vien Rau Qua] satellite DNA ß
ToLCVß	Tomato leaf curl virus satellite DNA ß
TYLCCNVß-[G102]	Tomato yellow leaf curl China virus-[G102] satellite DNA ß
TYLCCNVß-[Y10]	Tomato yellow leaf curl China virus-[Y10] satellite DNA ß
TYLCCNVß-[Y36]	Tomato yellow leaf curl China virus-[Y36] satellite DNA ß
TYLCCNVß-[Y64]	Tomato yellow leaf curl China virus-[Y64] satellite DNA ß
TYLCCNVß	Tomato yellow leaf curl China virus satellite DNA ß
TYLCMLVß	Tomato yellow leaf curl Mali virus satellite DNA ß
TYLCTHVß	Tomato yellow leaf curl Thailand virus satellite DNA ß
TYLCVVß	Tomato yellow leaf curl Vietnam virus satellite DNA ß
ZiLCVß	Zinnia leaf curl virus satellite DNA ß
ToLCV-sat	Tomato leaf curl virus-satellite

7.0 References

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PUBLICATIONS

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Transreplication of a tomato yellow leaf curl Thailand virus DNA B and replication of a ß DNA component by tomato leaf curl Vietnam virus and tomato yellow leaf curl Vietnam virus. Submitted (10/2007) to Virus Research.

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Blawid R, Maiss E.
Transreplication of a TYLCTHV DNA B component by geminiviruses from Vietnam and Thailand. Third European Congress of Virology, Nürnbeg, Germany, September 1-5, 2007.

Blawid R, Maiss E.
Transreplication of a TYLCTHV DNA B and replication of a ß DNA component by geminiviruses from Vietnam and Thailand. Deutsche Botanische Gesellschaft, Botanikertagung, Hamburg, Germany, September 3-7, 2007.

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Molecular characterization of *Vicia cryptic virus*. Joint meeting of the three divisions of the International Union of Microbiological Societies; XIIIth International Congress of Virology, San Francisco, USA, July 23-28, 2005.

Maiss E, Knierim D, Blawid R.
Sequence analysis of a serogroup IV tospovirus isolated from *Lycopersicum esculentum* in Thailand. Joint meeting of the three divisions of the International Union of Microbiological Societies; XIIIth International Congress of Virology, San Francisco, USA, July 23-28, 2005.

Eidesstattliche Erklärung zur Dissertation

Hierdurch erkläre ich an Eides statt, dass die Dissertation

New insights into geminiviruses complexes from Vietnam and Thailand

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

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Acknowledgments

First and foremost, I owe a very special debt of gratitude to Prof. Dr. Edgar Maiss, who has always a very motivated and enthusiastic mind. Thank you very much Edgar, for all these years. I learned how to think logically, how to approach new ideas and how to be persistent. You always said that you teach us for life. That is true and I am glad and lucky that I have a mentor like you.

Moreover, I wish to thank Prof. Dr. Christina Wege for accepting to act as external referee on very short notice and for the useful discussions and improvement of this work.

I wish to thank Prof. Dr. Hans-Michael Poehling for giving me the opportunity to carry out this work at the Institute of Plant Diseases and Plant Protection. Moreover, I would like to thank all members of the Institute especially Ms. Rothenhäuser and Ms. Ute Meisel for all support given.

I wish to thank Dr. Gisela Grunewaldt-Stöcker for introducing me in the CSLM and always being prompt for questions and excellent advise.

Furthermore, I want to thank the whole plant virology group. I am especially indebted to Jutta Zimmermann, Claudia Marx and Yvonne Koleczek for the kind and perfect technical assistance. Moreover, I want to thank Angelika Waltermann, who had a lot of patience for teaching me German words, for the nice and useful discussions not only about science but also about life. I would also like to thank Dennis Knierim and Stefan Flatken for nice discussions and help in the lab. I would like to thank Eva Scholz and Wulf Menzel for propagating a nice environment and also for support in the Lab. I wish to thank Christof Dietrich for useful discussions in the work.

I wish to thank the Gardeners Mr. Willi Arndt and Joachim Seelbinder for taking care of the plants and always being prompt for any other help.

I want to acknowledge the German Research Foundation for supporting this work.

Special thanks to my whole family: Gisela, Günter, Uschi, Potti, Rosa, Tia Lu, Lance and Antenor. To my sisters Renata and Roberta and my brother Lucas. Especially

thanks to my father Hugo Heredia, who promoted me in my career and supported me in all difficult moments of my life. Finally, I am grateful to have a very patient husband, who through all the years encouraged me with kind words and gave me always assistance.