Tomato yellow leaf curl virus resistance in *Solanum lycopersicum* through transgenic approaches

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Akademischen Grades

Doktorin der Naturwissenschaften
- Dr. rer. nat. -

genehmigte Dissertation von
Master of Science in Agriculture

Dang Thi Van
geboren am 31.07.1964 in NamDinh, Vietnam

2009

Referent: Prof. Dr. Hans-Jörg Jacobsen

Korreferent: Prof. Edgar Maiß

Tag der Promotion: 07.12.2009

ABSTRACT

Tomato yellow leaf curl virus (TYLCV), belonging to the *Geminiviridae* (Genus: *Begomovirus*), constitutes a serious constraint to tomato production worldwide and leads, especially in the tropics and subtropics, to large economical losses. Resistant tomato varieties are powerful tool to control TYLCV disease. However, nearly all commercially available tomato varieties are susceptible to TYLCV and resistance genes are mainly present in wild type tomato. Genetic engineering can provide a potential solution for the introduction of beneficial traits including virus resistance. This study was conducted to develop a transformation system for *Solanum lycopersicum* to create transgenic tomato plants resistant to TYLCV via a gene silencing (RNA interference, RNAi) approach.

The study focused first on optimization of a transformation protocol using *Agrobacterium tumefaciens* EHA105 harbouring the helper plasmid pSoup and pGreenII as a vector for the delivery of genes into expanding leaves of different commercial tomato cultivars from Vietnam. As an efficient transformation system depends on both an efficient regeneration system as well as an efficient method for the introduction of foreign genes into the plant cells, optimization of media and conditions for shoot regeneration from expanding leaves of four tomato cultivars was performed using glucuronidase (*gus*) as a marker gene. The experiments showed phytohormones (*trans*-zeatin and indolacetic acid) have an effect to induce competent cells for transformation. Supplement of *trans*-zeatin in combination with indolacetic acid into pre-treatment, inoculation, as well as co-culture media resulted in a higher frequency of transformation and a stronger *gus* expression. As a wide variety of inoculation and co-culture conditions have been shown to be important for the transformation, the results of the study showed that the temperature during the inoculation and co-culture as well as the concentration of *A. tumefaciens* had the highest influence on the transformation efficiency. In addition, the experiments also showed that *Agrobacterium* inoculation was an additional stress to the explants, resulting in a more sophisticated glufosinate selection scheme, leading to an optimized protocol for tomato transformation using pSoup / pGreenII.

Two inverted-repeat transgenes derived from different regions of *Tomato yellow leaf curl Thailand virus* (TYLCTHV) DNA-A were used to transform and regenerate *Solanum*

lycopersicum var. FM372C plants that can trigger RNAi to induce TYLCV resistance. The first construct derived from the intergenic region included a part of the gene coding for the replication-associated protein (IR/Rep), while the second construct incorporated parts of the pre-coat protein and coat protein (Pre/Cp). The independent transgenic (To) plants were screened for the presence of the transgenes by PCR and Southern blot analyses. The T_1 transgenic plants in the 5-7 leaf stage were verified by PCR for IR/Rep and Pre/Cp, respectively, before agroinoculation either with TYLCTHV DNA-A and DNA-B or *Tomato yellow leaf curl Vietnam virus* (TYLCVV). The disease development was recorded and presence of the viruses was determined by PCR and ELISA. Early symptoms, like yellowing and curling of leaves in non-transgenic and susceptible transformed plants occurred 3 weeks after inoculation and progressed into severe symptoms, characteristic of TYLCV disease, in the following weeks. Resistance to TYLCV was ranged form tolerance, typical in several Pre/CP transgenic lines to immunity of one IR/Rep transgenic line. In addition, IR/Rep transgenic plants were able to resist TYLCTHV as well as TYLCVV, while Pre/CP transgenic plants were only tolerant to the cognate virus, the TYLCTHV. The results of the study indicate that inverted repeat constructs are able to confer resistance to geminiviruses.

Keywords: Transformation, *Solanum lycopersicum*, TYLCV, RNAi, resistance.

Zusammenfassung

Das *Tomato yellow leaf curl virus* (TYLCV), Familie *Geminiviridae* (Gattung: Begomovirus), stellt weltweit, vor allem aber in den Tropen und Subtropen, ein ernsthaftes Problem in der Tomatenproduktion dar, wobei es erhebliche wirtschaftliche Verluste verursachen kann. Eine Möglichkeit, um TYLCV wirkungsvoll zu bekämpfen, stellen resistente Tomatensorten dar. Fast alle im Handel erhältlichen Tomatensorten sind jedoch anfällig für TYLCV und Resistenzgene für Züchtungsprogramme finden sich hauptsächlich in Wildtyp-Tomaten. Gentechnische Ansätze könnten eine mögliche Lösung für die Etablierung von Resistenzen gegenüber Viren liefern. Diese Arbeit hatte zum Ziel ein Transformationssystem für *Solanum lycopersicum* zu optimieren, um damit transgene Tomatenpflanzen mit einer Resistenz gegen TYLCV über ein Gen-Silencing-Konzept (RNA-Interferenz, RNAi) zu entwickeln.

Die Arbeiten konzentrierten sich zunächst auf die Optimierung des Transformationsprotokolls von Blattmaterial verschiedener kommerzieller Tomatensorten aus Vietnam unter Verwendung von *Agrobacterium tumefaciens* EHA105 mit dem Helferplasmid pSoup und pGreenII als Vektor für das zu transformierende Gen. Ein effizientes System zur Transformation hängt von der effektiven Regeneration und einer effektiven Methode für die Einführung fremder Gene in die Pflanzenzellen ab. Die Optimierung der Nährmedien und der Bedingungen für die Regeneration von vier Tomatensorten erfolgte mit Glucuronidase (*gus*) als Markergen. Die Versuche zeigten, dass Phytohormone (trans-Zeatin und Indolylessigsäure; IAA) einen Effekt auf die Kompetenz der Zellen für die Transformation ausübten. Die Zugabe von trans-Zeatin und IAA in die Vorkulturmedien, während der Inokulationsphase und in die Co-Kultur Medien führte zu einer höheren Transformationsfrequenz und eine stärkeren GUS-Expression. Auf die Transformation hatten die Temperatur während der Inokulation und der Co-Kultur sowie die Konzentration von *A. tumefaciens* die stärksten Einflüsse. Darüber hinaus zeigten die Versuche auch, dass die Agrobacterium-Inokulation eine zusätzliche Belastung für die Regeneration der Explantate darstellte, so dass eine Verbesserung der Glufosinat-Selektion nötig wurde, um zu einem optimierten Protokoll für die Tomatentransformation mittels pSoup / pGreenII zu gelangen.

Zwei als inverted-repeat angeordnete Regionen der DNA-A des *Tomato yellow leaf curl Thailand virus* (TYLCTHV) wurden zur Transformation und Regeneration von *Solanum lycopersicum* var. FM372C verwendet, um RNAi gegen das TYLCV zu erzielen. Das erste Konstrukt umfasst die sogenannte „Intergenic region“ einschließlich eines Teils des Gens für das replikationassoziierte Protein (IR/Rep), während das zweite Konstrukt Teile des Pre-Hüllprotein- und Hüllproteingens (Pre/Cp) enthält. Die unabhängigen transgenen (To) Pflanzen wurden auf das Vorhandensein des jeweiligen Transgens mittels PCR und Southern-Blot-Analysen überprüft. Die T1-transgenen Pflanzen wurden im 5-7 Blatt-Stadium erneut durch PCR auf die Präsenz von IR/ Rep bzw. auf Pre/Cp geprüft, bevor die Pflanzen entweder mit TYLCTHV DNA-A und DNA-B bzw. mit *Tomato yellow leaf curl Vietnam virus* (TYLCVV) agroinokuliert wurden. Die Symptome wurden bonitiert und das Auftreten der Viren durch PCR und ELISA bestimmt. Frühe Symptome, wie Gelbfärbung der Blätter und Blattrollen in nicht-transgenen und anfällig reagierenden transformierten Pflanzen traten 3 Wochen nach Inokulation auf. Mit Fortschreiten der Erkrankung kam es zu schweren Symptomen, die charakteristisch für die TYLCV Krankheit waren. In mehreren Pre/Cp transgenen Linien wurde eine Toleranz gegen das TYLCTHV, nicht aber gegen das TYLCVV gefunden. Eine Linie der IR/Rep transgenen Pflanzen reagierte mit Immunität auf die Inokulation mit TYLCTHV und TYLCVV. Die Ergebnisse zeigen, dass mit inverted-repeat Konstrukten Toleranz bzw. Resistenz auch gegen Geminiviren erzielt werden kann.

Stichworte: Transformation, *Solanum lycopersicum*, TYLCV, RNAi, Resistenz

TABLE OF CONTENTS

ABSTRACT……………………………………………………………………………I

ZUSAMMENFASSUNG……………………………………………………………….III

TABLE OF CONTENTS……………………………………………………………..V

ABBREVIATIONS…………………………………………………………………..IX

CHAPTER 1

General information

1.1 General introduction…………………………………………………………………1

1.2 Literature review………………………………………………………………………..5

1.2.1 Tomato yellow leaf curl virus – Taxonomy……………………………………..5

1.2.2 Begomoviruses-genome structure……………………………………………….....6

1.2.2.1 The intergenic region - promoters and transcription………………………………..8

1.2.3 Viral proteins……………………………………………………………………….....9

1.2.3.1 The coat protein…………………………………………………………………..9

1.2.3.2 The precoat protein………………………………………………………………...10

1.2.3.3 The replication associated protein (REP) …………………………………………10

1.2.3.4 The replication enhancer protein (REn)……………………………………………...11

1.2.3.5 The transcriptional activator protein (TrAP)………………………………………11

1.2.3.6 The AC4/C4 protein……………………………………………………………….12

1.2.3.7 The movement proteins (BC1 and BV1)…………………………………………..12

1.2.3.8 Beta satellites and the βC1 protein………………………………………………...12

1.2.4 Infection cycle of begomovirus…………………………………………………...13

1.2.4.1 Begomovirus transmission………………………………………………………...13

1.2.4.2 Infection cycle in plants…………………………………………………………...14

1.2.5 Resistance breeding through transgenic approaches………………………………...16

1.2.5.1 Pathogen-derived resistance through the expression of viral proteins…………….17

1.2.5.1.1 REP-mediated resistance……………………………..……………………….17

1.2.5.1.2 Coat protein-mediated resistance…………………………………………………..18

1.2.5.1.3 Movement protein-mediated resistance……………………………………….19

1.2.5.2 RNA/DNA-mediated resistance……………………………………………………...19

1.2.5.2.1 Post-transcriptional gene silencing (PTGS)..…………………………………….19

1.2.5.2.2 Antisense RNA……………….…………………………………………………21
1.2.5.2.3 Defective interfering DNA (DI)……….……………………………………..22
1.2.5.3 Expression of non-pathogen derived antiviral agents……………………………..23
1.2.5.3.1 Trans-activation of a toxic protein………..……………………………………23
1.2.5.3.2 Expression of DNA binding proteins…..………………………………………23
1.2.5.3.3 A Chaperonin (GroEL)...……………………………………………………..24
1.2.5.3.4 Peptide aptamers……………………………………………………………...24
1.2.6 Gene silencing via RNAi…………………………………………………………25
1.2.7 Tomato transformation……………………………………………………………28
1.3 Aims and significance of the study………………………………………………31

CHAPTER 2

Development of a simple and effective protocol for leaf disc transformation of commercial tomato cultivars via *Agrobacterium tumefaciens*

2.1 Introduction………………………………………………………………………33
2.2 Materials and methods…………………………………………………………...34
2.2.1 Materials…………………………………………………………………………..34
2.2.2 Method of optimising for shoot regeneration ……………………………………….35
2.2.3 Methods of optimising conditions for transformation……………………………….35
2.2.4 Development of the transformation process…………………………………………36
2.2.5 Experimental design and data analysis………………………………………………37
2.3 Results ……………………………………………………………………………...37
2.3.1 Optimising shoot induction from leaf explants……………………………………...37
2.3.2 Effect of Agrobacterium cell density on transformation frequencies……………….38
2.3.3 Effect of temperature during inoculation and co-culture on transformation frequencies……………………………………………………………………………...40
2.3.4 Effect of plant phytohormones during inoculation and co-cultivation on transformation frequencies…………………………………………………………...41
2.3.5 Determining the critical concentration of glufosinate on callus and root induction...43
2.3.6 Establishment of a full transformation process ……………………………………..46
2.4 Discussion…………………………………………………………………………..47

CHAPTER 3

The inverted-repeat hairpinRNA derived from intergenic region and *Rep* gene of TYLCTHV confers resistance to homologous and heterologous viruses

3.1 Introduction…………………………………………………………………………54
3.2 Materials and methods………………………………………………………………...55
3.2.1 Transformation of plants…………………………………………………………….55
3.2.1.1 Bacterial system and vectors………………………………………………………55
3.2.1.2 RNAi constructs (*s*elf-complementary hairpin RNA constructs)…………………55
3.2.1.3 Plant transformation procedure and anlayses of transgenic plants………………...56
3.2.1.4 Plant DNA isolation………………………………………………………………….56
3.2.1.5 Polymerase chain reaction (PCR)…………………………………………………….57
3.2.1.6 Southern hybridization……………………………………………………………….58
3.2.2 Evaluation of plants resistance in transgenic plants………………………………....59
3.2.2.1 Plant material………………………………………………………………………....59
3.2.2.2 Virus agroinoculation………………………………………………………………...59
3.2.2.3 Evaluation of virus symptoms………………………………………………………..60
3.2.2.4 Confirmation of virus presence by PCR……………………………………………...62
3.3 Results………………………………………………………………………………….63
3.3.1 Confirmation of successful transformation via PCR………………………………...63
3.3.2 Seed production from To plants……………………………………………………..64
3.3.3 Identification of transgene copy number in transformed plants……………………..64
3.3.4 TYLCTHV resistance tests in T_1 plants transformed with the IR/Rep-hpRNA construct…………………………………………………………………………………...68
3.3.4.1 Agroinoculation of *Nicotiana benthamiana* with TYLCTHV and TYLCVV…….68
3.3.4.2 Agroinoculation of transgenic tomato plants with TYLCTHV……………………69
3.3.4.3 TYLCTHV detection by PCR………………………………………………………..72
3.3.4.4 Molecular characterization of transgene in immunity plants by Southern hybridization…………………………………………………………………………………74
3.3.4.5 Agroinoculation of transgenic tomato plants with TYLCVV……………………..75
3.4 Discussion..……………………………………………………………………………77

CHAPTER 4

Inverted-repeat hairpinRNA derived from a truncated pre-coat/coat-protein gene of TYLCTHV confers resistance in transgenic tomato plants

4.1 Introduction……………………………………………………………………………80
4.2 Materials and methods……………………………………………………………....81
4.2.1 RNAi construct ……………………………………………………………………...81
4.2.2 Evaluation of virus resistance in transgenic tomato…………………………………82
4.2.3 Triple antibody sandwich (TAS) ELISA for detection of TYLCV…………………83
4.3 Results…………………………………………………………………………………84
4.3.1 Results of transformation……………………………………………………………84
4.3.1.1 Confirmation of successful transformation via PCR………………………........84
4.3.1.2 To seed production…………………………………………………………...86
4.3.1.3 Detection of transgene copy number by Southern Blot analyses…………………86
4.3.2 Evaluation of TYLCTHV and TYLCVV resistance……………………………...91
4.3.2.1 Resistance tests for *Tomato yellow leaf curl Thailand virus*..…………………………91
4.3.2.2 TYLCTHV detection by PCR …………………………………………………......95
4.3.2.3 TYLCTHV coat protein detection by ELISA………..…………………………......96
4.3.3 Resistance test for *Tomato yellow leaf curl Vietnam virus*..………………………….97
4.4 Discussion………………………………………………………………………………..98

GENERALDISCUSSION…………………………………………………………..102

REFERENCES…………………………………………………………………...111

APPENDIX…………………………………………………………………….....137

ACKNOWLEDGEMENT………………………………………………………….…139

CURRICULUM VITAE………………………………………………………….…...141

STATEMENT……………………………………………………………………….....145

ABBREVIATIONS

g	Gram
h	Hours
mg	Milligram
ml	Milliliter
mM	Millimolar
µM	Micromolar
µl	Microliter
ppm	Part per million
L	Liter
%	Percent
°C	Degree Celsius
aa	Amino acid
bp	Base pair
BCM	Basic culture medium
CP	Coat protein
cp	Gene encoding coat protein
CR	Common region of geminivirus genome
cv.	Cultivar
dpi	Days past inoculation
DNA	Deoxyribonucleic acid
dNTPs	Mix of the four deoxynucleotide triphosphates
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DMSO	Dimethylsulfoxid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
e35S CaMV	Enhanced 35S CaMV promoter

ER	Endoplasmic reticulum
GUS	β-Glucuronidase
hpRNA	Hairpin RNA
IAA	Indolacetic acid
IR	Intergenic region
Kb	Kilobase
LB	Left border
MES	2-(N-morpholino)ethanesulfonic acid
MP	Movement protein
miRNA	Micro RNA
mRNA	Messenger RNA
MS	Murashige and Skoog media
NES	Nuclear export signal
NLS	Nuclear localization signal
nptI	Bacterial *kanamycin* resistance gene
nt	Nucleotide
nd	Not ditermined
NTP	Nucleoside triphophate
PD	Plasmodesmata
NPC	Nuclear pore complex
OD_{600}	Optical density measured at 600 nm
ORF	Open reading frame
P	Statistical probability value
PAZ-domain	Binding domain in Argonaute and Dicer family protein
bar	Basta resistance gene
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDR	Pathogen-derived resistance
pH	Negative decade logarithm of hydrogen ion concentration
PIWI-domain	A domain of Argonaute protein
Pmol	Picomolar
PTGS	Post-transcriptional gene silencing

RAPD	Random amplification polymorphic DNA
RB	Right border
RC	Rolling circle
RdDM	RNA-directed DNA methylation
RdRp	RNA-dependent RNA polymerase
REP	Replication-associated protein
Rep	Gene encoding replication-associated protein
RISC	RNA-induced silencing complex
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
ssDNA	Single strand DNA
ssRNA	Single strand RNA
AZPs	Artificial zinc-finger proteins
siRNA	Short interfering RNA
ST-LS1	Intron from the ST-LS1 gene of potato
TAE	Tris-acetate-EDTA
TAS-ELISA	Triple-Antibody-Sandwich ELISA
T_{DNA}	Transferring DNA
TGS	Transcriptional gene silencing
T-*Rep*	Truncated *Rep* gene
To	First regeneration of transformed plants obtained from transformation
T_1	Progenies of To
vir gene	Virulence genes of *Agrobacterium tumefaciens*
wt	Wild type
X-Gluc	5-bromo-4-chloro-3-indoly-glucoronide
Zea	Trans-zeatin

CHAPTER 1

General information

1.1 General introduction

Vegetables cultivated in tropical and subtropical regions are commonly influenced by different diseases including virus diseases. Currently, viruses from three important genera, including *Potyvirus, Begomovirus,* and *Tospovirus*, cause a severe decrease in crop yields worldwide (Rybicky et al., 1999). One important affected vegetable is cultivated tomato (*Solanum lycopersicum,* formerly known as *Lycopersicum esculentum)* which belongs to the *Solanaceae* family (Rick, 1960).

Among the geminiviruses, *Tomato yellow leaf curl virus* (TYLCV), which belongs to the genus *Begomovirus*, influences tomato production in many tropical and subtropical regions and causes yield reduction up to total loss of the crop (Pico et al., 1996; Czosnek and Laterrot, 1997). Tomato yellow leaf curl disease has long been known in the Middle East, North, and Central Africa, as well as in Southeast Asia. The disease has spread to Southern Europe, the Caribbean region and the United States resulting in a worldwide distribution (Figure 1). Therefore, the disease causes economically important problems for tomato production around the world (Pico et al., 1996; Czosnek and Laterrot, 1997; Moriones et al., 2000).

The traditional management methods to prevent TYLCV diseases depend on controlling the vector transmitting the viruses (whiteflies). However, control is difficult due to the very wide host range and the complex interrelationships among virus, host, vector, virus source and environment. To date, insecticidal spraying is the most frequently used method to control the vectors. Nevertheless, chemical treatments are very often only partially effective and can cause adverse environmental effects. Thus, one of the best ways to eliminate the yield losses due to viruses is to develop tomato varieties that are resistant or tolerant to a given virus.

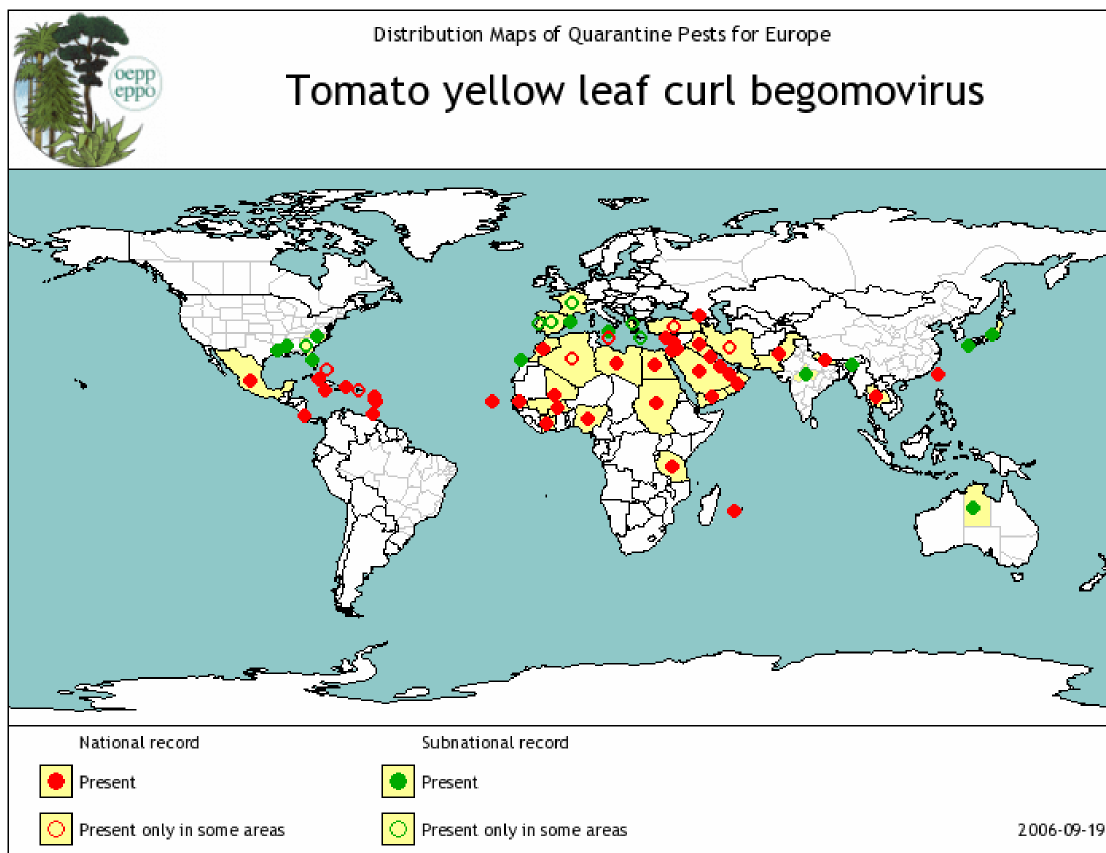

Figure 1: Distribution map of Tomato yellow leaf curl virus according to EPPO report, 2006 (Source: www.eppo.org/QUARANTINE/virus/TYLC_virus/TYLCV_map.htm).

In principle, resistance traits can be incorperated into commercial tomato varieties by crossing with a virus resistant variety. However, all commercial tomato cultivars have been found to be completely susceptible to TYLCV, urging breeders to screen wild tomato accessions for potential resistance traits (Pilowsky and Cohen, 1990; Pilowsky and Cohen, 2000; Friedmann et al., 1998; Vidavsky et al., 1998a, Vidavsky et al., 1998b; Zamir et al., 1994; Kasrawi et al., 1988; Pico et al., 1999). However, so far only a few resistance genes were mapped. The resistance gene TY-1 to TYLCV, on chromosome 6 of *L. chilense*, has been identified. Two more resistance modifier genes were mapped to chromosome 3 and 7 of *L. chilense* (Zamir et al., 1994). Another TYLCV-resistance gene, originating from *L. pimpinellifolium* had been mapped using RAPD PCR-based markers to chromosome 6, but to a different locus from TY-1 (Chague et al., 1997). In addition, a resistance gene against the *Tomato leaf curl Taiwan virus* was mapped to chromosomes 8 and 11 of *L. hirsutum* (Hanson et al., 2000). The first TYLCV-resistant commercial cultivar resulting from breeding programmes is TY-20, which carries a resistance derived from *L. peruvianum*,

which shows a delay both in symptom development and viral accumulation (Pilowsky and Cohen, 1990; Rom et al., 1993). In most cases, the sources of TYLCV resistance appeared to be controlled by multiple genes (Zakay et al., 1991; Pico et al., 1996; Pico et al., 1999). Examples of the different resistant lines are given in the review by Lapidot and Friedmann (2002). Nevertheless, after 20 years of breeding only a few commercial genotypes with increased levels of TYLCV resistance are on the market.

There are several problems to be overcome in breeding of resistant varieties by crossing between cultivated *Solanum lycopersicum* and wild type tomatoes. The first are breeding barriers between these species, which restrict breeders access to these gene pools. The use of *in vitro* embryo culture or embryo rescue for zygote survival is needed, but plantlet recovery through embryo culture from the cross between cultivated *Solanum lycopersicum* and wild types is usually very low. The second is that undesired traits are being transferred with the resistance traits. Furthermore, quite often the resistance trait is controlled by multiple genes. Consequently, it takes a very long time to obtain a commercial variety using a back crossing program. An example of this work was reported by Vidavsky et al. (1998b), which showed that after more than 20 years of work the best cultivars and breeding lines were only tolerant to the virus rather than immune. The third disadvantage is that resistant gene pools are limited and usually confer specific resistances. These resistances will soon be overcome by the virus due to genetic diversity and the high mutation rate. Therefore, it is necessary to find a durable solution to overcome the disadvantages of conventional breeding.

Genetic engineering has the potential to provide an abundant source of beneficial plant traits, including virus resistance. Different approaches have been considered in the development of transgenic resistance to geminiviruses due to the expression of either pathogen derived resistance (PDR) or non pathogen derived resistance. Pathogen derived resistance is mediated either by protein or by gene silencing including DNA methylation or RNA interference (RNA-mediated). During the last two decades, different strategies have been applied in the development of transgenic resistance against viruses including antisense RNA, the use of coat protein genes, intact or truncated replication associated proteins, defective interfering DNA and viral activated antiviral proteins. In protein-mediated resistance, proteins encoded by the transgenes interfere in some manner with the virus function or act as dominant negative inhibitors to block virus replication,

accumulation, and systemic infection (Beachy, 1997; Goldbach et al., 2003). For geminiviruses, expression of viral coat proteins, truncated or mutant viral replicase, and movement proteins have been investigated and succeeded to enhance virus resistance in different plants (Kunik et al., 1994; Hong and Stanley, 1996; Noris et al., 1996b; Brunetti et al., 1997; Hanson and Maxwell, 1999; Sangare et al., 1999; Hou et al., 2000; Chatterji et al., 2001; Lucioli et al., 2003; Antignus et al., 2004; Shivaprasad et al., 2006). Another approach is to express antisense transgenes that are complementary to a target mRNA to inhibit expression of homologous genes by preventing translation or promoting degradation. This technology has been successfully applied to engineer resistance to geminiviruses (Day et al., 1991; Bejarano and Lichtenstein, 1994; Aragão et al., 1998; Bendahmane and Gronenborn, 1997; Praveen et al., 2005). Recently, RNA silencing has been found to be a robust technology for silencing genes by either suppressing transcription (transcriptional gene silencing [TGS]) or by activating a sequence-specific RNA degradation process (Poogin et al., 2003). RNA silencing has been successfully used to develop resistance against RNA viruses (Bucher et al., 2006; Tougou et al., 2006; Di Nicola-Negri et al., 2005; Missiou et al., 2004; Mitter et al., 2003; Pandolfini et al., 2003; Kalantidis et al., 2002; Smith et al., 2000). For DNA viruses, Pooggin et al. (2003) demonstrated that transient expression of both sense and antisense *Vigna mungo yellow mosaic virus* (VMYMV) promoter sequences in an inverted-repeat resulted in complete recovery of infected VMYMV plants. The recovery of the whole plant from VMYMV infection indicated that the interfering signal spread throughout the plant. They proposed that RNA interference, as has been described for RNA viruses, is also possible for a DNA virus. A RNA-based strategy to control geminiviruses was demonstrated when tobacco and tomato plants were transformed with constructs derived from the AC1 gene of *African cassava mosaic virus* (ACMV) or transgenes developed from the *Rep* gene of TYLCV. These plants were highly resistant to either *Cotton leaf curl virus* or TYLCV, respectively (Asad et al., 2003; Yang et al., 2004). It has been shown that PTGS in plants can be triggered at high efficiency by the presence of an inverted-repeat in the transcribed region of a transgene (Chuang and Meyerowitz, 2000; Hamilton et al., 1998; Levin et al., 2000). An intron-hairpin structure could enhance the stability and efficiency of duplex RNA formation inducing the PTGS response in such a way that the plant could become immune to a RNA virus infection (Smith et al., 2000). The present research followed this strategy, consisting in the design of a construct arranged in a way that, when transcribed, renders

intron-hpRNA directed against the TYLCV C1-gene and V1-gene to interfere with TYLCV replication and produces tomato plants resistant to two isolates of TYLCV such as *Tomato yellow leaf curl Thailand virus* (TYLCTHV) as well as *Tomato yellow leaf curl Vietnam virus* (TYLCVV).

1.2 Literature review

1.2.1 Tomato yellow leaf curl virus – Taxonomy

Tomato yellow leaf curl virus (TYLCV) is a true ssDNA plant virus, a member of the family *Geminiviridae*, of the genus *Begomovirus*. *Geminiviridae* is a large plant-infecting virus family, divided into four genera: *Curtovirus*, *Topocuvirus*, *Mastrevirus* and *Begomovirus* (Fauquet et al., 2008). The division is based on host range, symptom phenotype, insect vector, coat protein characteristics and nucleotide sequence identity. The morphology of *Geminiviridae* is unique, two incomplete icosahedra, with a T=1 surface lattice, (approx. 20 nm diameter and 30 nm length) form a virion. TYLCV, like all members of *Geminiviridae*, has geminate (twinned) particles, 18-20 nm in diameter, 30 nm long, apparently consisting of two incomplete icosahedra joined together in a structure with 22 pentameric capsomers and 110 identical protein subunits (Figure 2).

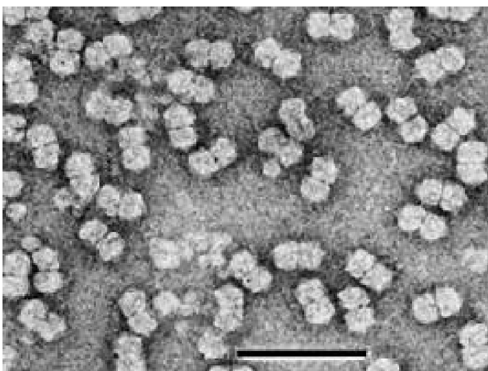

Figure 2: Particles of *Tomato yellow leaf curl virus*. Electron micrograph of purified, negatively stained TYLCV particles. Bar = 100 nm (picture taken from Gafni, 2003).

All members of *Geminiviridae* possess single stranded DNA genomes consisting of one or two components and are therefore called monopartites or bipartites, respectively. The genomic components are transcribed, replicated and encapsidated in the nuclei of infected plant cells and are able to move within and between the cells.

Three species currently belong to the genus *Curtovirus* (type species: *Beet curly top virus*) along with one tentative species. The genus includes viruses with monopartite genomes, encoding six to seven proteins, which are transmitted by leafhoppers (*Hemiptera: Cicadellidae*) and prominently infect dicotyledonous plants (sugar beet, melon and tomato).

The *Mastrevirus* genus include the type species *Maize streak virus*, 12 species and six tentative species, which have a monopartite genome encoding four proteins. The infection of this genus is found on monocotyledonous plants, transmitted through leafhoppers (*Hemiptera: Cicadellidae*) in a persistent, circulative and non-propagative manner.

The genus *Topocuvirus* has only one representative (*Tomato pseudo-curly top virus*) and the differences of this virus to other *Geminiviridae* are based on the use of other host organisms, the treehoppers (*Hemiptera: Micrutalis malleifera*) and on the fact that this particular virus has evolved by recombination between unknown viruses belonging to different genera (Briddon et al., 1996). The *Topocuvirus* genus has a monopartite genome encoding six proteins. On the virion sense strand, two proteins are encoded: the movement and the coat protein (MP and CP, respectively).

Begomovirus is the only genus in the *Geminiviridae* family, which is either monopartite or bipartite, composed of one ssDNA (DNA A-like) on which all of the six genes are residing or of two genomic components encoding five to six (DNA-A) and two proteins (DNA-B), respectively (Stanley et al., 2005). It is the most important genus, not only because it covers more than 80% (117 of 133) of all known geminiviruses (Stanley et al., 2005), but also due to its heavy impact on agriculture, causing up to 100% yield losses in different important crops. These viruses are transmitted by whiteflies (*Bemisia tabaci*) and infect dicotyledonous plants; every year the number of species discoved belonging to this genus is increasing (Fauquet et al., 2008).

1.2.2 Begomoviruses-genome structure

Begomoviruses can be divided according to the number of mono- and bipartite virus genomic components. Monopartite viruses consist only of the DNA-A component, while bipartite begomoviruses consist of two different DNA molecules: the A and B component. The A component of begomoviruses typically consists of six genes, which are organized bidirectionally (Figure 3).

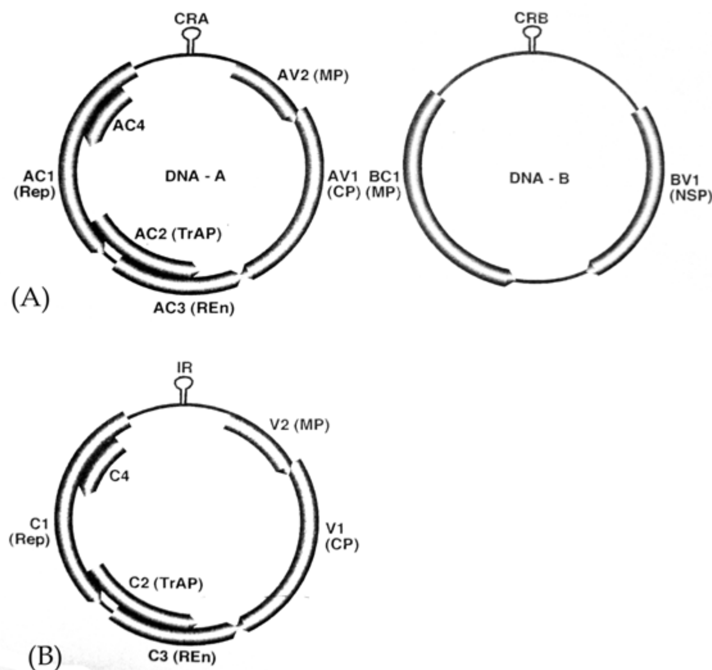

Figure 3: Genomic organisation of begomoviruses. (A) Bipartite begomoviruses; (B) Monopartite begomoviruses. ORFs are denoted as belonging to either the complementary strand (C), or the virion strand (V) (Stanley et al., 2005).

Four genes (AC1/C1, AC2/C2, AC3/C3, and AC4/C4) are arranged in complementary direction. AC1 encodes a replication-associated protein (REP; Elmer et al., 1988) which is essential for viral DNA replication in association with host factors (Arguello-Astorga et al., 2004). AC2 encodes a transcriptional activator protein (TrAP) that transactivates the expression of the coat protein gene and the BV1 movement gene of the B component (Sunter and Bisaro, 1991; Sunter and Bisaro, 1992). AC3 encodes the replication enhancer protein (REn) that regulates the virus replication rate, possibly via the activation of an early gene (AV1/V1) required for DNA synthesis (Azzam et al., 1994; Settlage et al., 2005). In sense direction, AV1/V1 and AV2/V2 encode coat and movement proteins respectively (Padidam et al., 1996). The B part, which can not replicate in the absence of the A component, consists of a BV1 gene encoding a nuclear-shuttle protein (NSP) and BC1 protein directly involved in movement, which contribute functions involved in virus movement and symptom development (Sanderfoot and Lazarowitz, 1995; Gafni and Epel, 2002; Hehnle et al., 2004).

The A and B components in bipartite begomoviruses share a common region (CR)/intergenic region (IR), which consists of a block of approximately 200 bps (Sunter and Bisaro, 1991; Lazarowitz, 1992; Stanley et al., 2005). The CRs are virtually identical in sequence in a given bipartite begomovirus, but are completely different in sequence among the other geminiviruses. The CR contains a GC-rich inverted repeat sequence that has the potential to form a stem-loop structure. The inverted repeats flank an 11 to 16 base AT-rich sequence that is hypothesised to be the origin of the rolling circle replication (Lazarowitz et al., 1992; Heyraud-Nitschke et al., 1995; Stanley et al., 2005).

Monopartite begomoviruses, such as isolates of *Tomato yellow leaf curl virus* from the Old World and *Tomato golden mosaic virus* (TGMV), only have a single genomic component of about 2.7 kb designated as DNA-A (Kheyr-pour et al., 1991; Navot et al., 1991; Yin et al., 2001). The ssDNA genome contains six open reading frames (ORFs). The arrangement of TYLCV ORFs is similar to that of the DNA-A component of bipartite begomoviruses. The ORFs encoding REP, TrAP, and REn partially overlap, and a small ORF (C4) is located within the Rep ORF, but in a different reading frame (Dry et al., 1993; Noris et al., 1994; Ha et al., 2008). AC4 encodes an important symptom determinant (Rigden et al., 1994; van Wezel et al., 2002; Selth et al., 2004). In addition, the satellite DNA-ß molecules associated with monopartite begomoviruses are involved in symptom enhancement (Mansoor et al., 2003; Cui et al., 2004; Saeed et al., 2007).

1.2.2.1 The intergenic region - promoters and transcription

The CR contains a hairpin structure with the characteristic geminiviral nonanucleotide sequence TAATATT/AC in the loop at the expected origin of virion strand DNA replication (Hanley-Bowdoin et al., 1999) and binding sequences, which are recognized by the AC1 (REP) protein (Arguello-Astorga et al., 1994) as well as regulatory regions for bidirectional promoters for transcription of the viral-sense genes (V2 and V1) and the complementary sense genes C1 and C4 (Hanley-Bowdoin et al., 1999). Most of the transcription data on begomoviruses came from analyses using *Tomato golden mosaic virus* (TGMV; Hanley-Bowdoin et al., 1988; Sunter et al., 1989), ACMV (Zhan et al., 1991) or *Tomato leaf curl virus* (ToLCV; Mullineaux et al., 1993). Mostly, but not exclusively, at the 5′-end of the inverted repeat/nonanucleotide sequence, short (8-12 nucleotides) direct repeat sequences, so called “iteron sequences”, are found (Argüello-Astorga et al., 1994).

These are recognised and bound by the REP, and are assumed to act specificity as determinants for interaction of a given REP with its coding DNA (Eagle et al., 1994; Fontes et al., 1994a; Fontes et al., 1994b). Additional evidence for such sequence-specific origin recognition was also derived by using the two species TYLCV and *Tomato yellow leaf curl Sardinia virus* (TYLCSV; Jupin et al., 1995). The results have led to a model for specificity of geminivirus REP-origin recognition in general (Argüello-Astorga and Ruiz-Medrano, 2001). However, biochemical data on the direct binding of REP to such sequences remain limited (Behjatnia et al., 1998; Chatterji et al., 1999; Chatterji et al., 2000). The potential importance of intergenic region sequences for virus-host interactions was increased by the finding of Poogin et al. (2003) that these sequences, in a so far unexplained fashion, may contribute to silencing of geminivirus gene expression.

1.2.3 Viral proteins

1.2.3.1 The coat protein

The coat protein (CP) of TYLCV is encoded by the V1 gene on the viral sense strand. The main role of the CP is to form particles which encapsidate the DNA. It is the only known structural component of the viral capsid in TYLCV (Lazarowitz, 1992). Here, the coat protein is essential for the infection, (Boulton et al., 1989; Lazarowitz et al., 1989), systemic movement of the virus into the host cell nucleus (Wartig et al., 1997), and insect transmission (Briddon et al., 1990; Azzam et al., 1994; Höfer et al., 1997; Noris et al., 1998; Morin et al., 1999). An intact CP is necessary for the spread of *Tomato leaf curl virus* (TLCV) from Australia (Rigden et al., 1993) and other related monopartite geminiviruses (Boulton et al., 1989; Briddon et al., 1989), and therefore suggests that within the plant, the monopartite virus moves in the form of complete encapsidated particles (Noris et al., 1998). Noris et al. (1998) studied two defective genomic DNAs of the TYLCV and in comparison with a wild type *Tomato yellow leaf curl Sardinia virus* (TYLCSV). They found that single amino acid variations in the CP at positions 129, 134 and 152 can affect its transmissibility and infectivity.

The CP is localised in the nucleus and functions as a nuclear shuttle protein (Rojas et al., 2001). Latter research confirmed that the CP of bipartite and monopartite begomoviruses contains sequences which may be related to nuclear localisation and nuclear export signals

(NLS and NES; Unseld et al., 2001; Unseld et al., 2004). Recently, Zrachya et al. (2007b) showed that siRNA targeted against the CP of TYLCV can confer virus resistance in transgenic tomato plants.

In bipartite geminiviruses the CP is not required for virus spread and symptom development (Gardiner et al., 1988; Padidam et al., 1996). However, mutations in the CP do influence the transmissibility by the vector. Höhnle et al. (2001) exchanged the CP in a *Abutilon mosaic virus* (AbMV) isolate, which is not whitefly transmissible, with the CP of *Sida golden mosaic virus* (SiGMV-[Hoyv]), a vector transmissible virus. Only the recombinants containing (SiGMV-[Hoyv]) CP were transmitted by the whitefly. Moreover, Höhnle et al. (2001) were able to re-establish the transmission of AbMV by the exchange of two amino acids at positions 124 and 149.

1.2.3.2 The precoat protein

The tomato infecting viruses differ in their number of open reading frames (ORFs). In the Old World viruses, either bipartite or monopartite, two overlapping ORFs (CP and AV2) on the A component can be found. In the New World viruses, like TGMV and *Tomato leaf crumple virus* (TLCrV), only the ORF for the coat protein is present. The AV2/V2 or MP genes are named according to the particular begomovirus, and encode the "precoat" protein (Padidam et al., 1996). This protein may be involved in the particle movement of monopartite viruses. In bipartite begomoviruses the precoat protein may improve the fitness of the virus and may be dispensable for movement (Rothenstein et al., 2007). Recently, Zrachya et al. (2007a) identified a functional V2 protein of *Tomato yellow leaf curl Israel virus* (TYLCV-[IL]). In silencing assays, V2 inhibited the RNA silencing of a reporter gene (*GFP*) construct. In contrast with the increasing of transcript and protein levels, the accumulation of GFP-specific short interfering RNAs were not found. This suggests that V2 is involved in suppression of the RNA silencing pathway, probably subsequent to the Dicer-mediated cleavage of dsRNA.

1.2.3.3 The replication associated protein (REP)

The replication associated protein is encoded by the AC1/AL1 (C1/L1) gene on the complementary viral strand of the A component. The N-terminal domain of the REP is involved in initiation of the DNA replication (Koonin and Ilyina, 1992; Laufs et al.,

1995a). It binds to highly specific viral DNA sequences (referred to as iterons) which are located at the conserved common region (Fontes et al., 1994b), represses its own promoter (Eagle et al., 1994; Sunter et al., 1993) and cleaves and ligates DNA (Laufs et al., 1995a). This is identified by *in vitro* and *in vivo* analysis that the tyrosine T103 initiated the cleavage and is the physical link between the REP and its origin DNA (Laufs et al., 1995b). It also plays a role as a DNA helicase (Clerot and Bernardi, 2006). Another biochemical activity of REP is its capacity to hydrolyse nucleoside triphosphates. Mutants of TYLCSV REP impaired in this function were found to be replication deficient (Desbiez et al., 1995). REP protein can interact with a number of host proteins (Ach et al., 1997; Castillo et al., 2003; Castillo et al., 2004; Kong and Hanley-Bowdoin, 2002; Luque et al., 2002) and with a plant retinoblastoma homologue, which regulates the cell cycle and differentiation (Arguello-Astorga et al., 2004; Kong et al., 2000). This interaction provides the necessary requirements by reprogramming mature plant cells to replicate viral DNA, thus promoting infection (Kong et al., 2000). TYLCSV REP has been shown to directly interact with the proliferating cell nuclear antigen [PCNA], possibly to recruit this "sliding clamp" to the viral origin and the replisome (Castillo et al., 2003).

1.2.3.4 The replication enhancer protein (REn)

AC3 is an auxiliary replication enhancing protein that increases viral DNA accumulation (Gutierrez, 1999; Settlage et al., 2005; Sunter et al., 1990). AC3 forms homo-oligomers and interacts with AC1 and host factors (Castillo et al., 2003; Selth et al., 2005; Settlage et al., 1996; Settlage et al., 2001; Settlage et al., 2005). TYLCSV REn has been shown to interact with both Rep and PCNA (Castillo et al., 2003), the sliding clamp of the replisome. Thus, it can be predicted that when REP, REn, and PCNA of the replisome act in a balanced and concerted way will result in efficient geminivirus DNA replication.

1.2.3.5 The transcriptional activator protein (TrAP)

The TrAP is encoded by the AC2/C2 gene. It is a multifunctional regulatory protein. TrAP N-terminus includes a nuclear localisation sequence (van Wezel et al., 2001), a central core with a zinc finger-like region (Noris et al., 1996a) and a distinct acidic C-terminal activation domain (Hartitz et al., 1999). TrAP enhances transcription of the virion-sense

promoter of DNA-A as well as the BV1 and BC1 promoters of DNA-B in bipartite begomoviruses (Haley et al., 1992; Sunter and Bisaro, 1992). It also has been implicated as a suppressor of RNA silencing (Selth et al., 2004; Trinks et al., 2005; van Wezel et al., 2001; Vanitharani et al., 2004; Voinnet et al., 1999; Wang et al., 2005).

1.2.3.6 The AC4/C4 protein

The AC4 gene is located within the AC1 coding region but in a different reading frame. Experiments with TGMV showed that C4 protein is not essential for infectivity (Elmer et al., 1988). However, for TLCV it was reported as a virulence factor (Krake et al., 1998; Selth et al., 2004) and a TYLCV C4 mutant was unable to move systemically in tomato plants (Jupin et al., 1994). Recently, ACMV-[CM]-C4 and *Sri Lankan cassava mosaic virus* (SLCMV)-C4 were reported to have the capacity for suppression of gene silencing (Vanitharani et al., 2004; Vanitharani et al., 2005).

1.2.3.7 The movement proteins (BC1 and BV1)

The genes encoded by the B component of bipartite begomoviruses, BV1 and BC1, provide functions required for virus movement. BV1, the nuclear shuttle protein (NSP) and BC1, the cell-to cell movement protein (MP), coordinate the movement of the viral DNA from the nucleus and across the cell wall to a contiguous cell (Noueiry et al., 1994; Sanderfoot and Lazarowitz, 1995; Sanderfoot and Lazarowitz, 1996; Gafni and Epel, 2002). However, it is not precisely known if a single stranded or double stranded DNA form is transported. BV1 packages the viral DNA and interacts with BC1 in the cytoplasm to be transported through the plasmodesmata into the neighbouring cell (Lazarowitz and Beachy, 1999; Hehnle et al., 2004). Both BC1 and BV1 movement proteins of different bipartite begomoviruses are reported as virulence determinants in different host plants (von Arnim and Stanley, 1992; Pascal et al., 1993; Ingham et al., 1995; Duan et al., 1997a; Hou et al., 2000; Carvalho and Lazarowitz, 2004; Hussain et al., 2005).

1.2.3.8 Beta satellites and the βC1 protein

A strange class of DNA molecules has been found associated with certain Old World begomoviruses (for a review see Briddon and Stanley, 2006). The search for potentially missing DNA components in monopartite viruses led to the discovery of an additional circular ssDNA molecule of about 1,350 bases, named DNA-β. DNA-β encodes a single

protein (βC1) which has a nuclear localization and functions as a suppressor of RNA silencing (Mansoor et al., 2003; Briddon et al., 2003; Stanley, 2004; Cui et al., 2005).

DNA-β molecules are required for infection of hosts *Ageratum conyzoides* or cotton. Expression of the βC1 protein results in an increase in symptom severity of the respective begomovirus (Saeed et al., 2005; Saunders et al., 2004). This is also true for the TYLCVs, where βDNAs accompany *Tomato leaf curl China virus* (ToLCCNV) (Zhou et al., 2003) and TYLCTHV (Cui et al., 2004). So-called DNA-1 molecules were found closely connected to the discovery of the DNA-β satellite-like molecules, yet they are another class of small DNAs associated with certain Old World monopartite begomoviruses (Mansoor et al., 1999). They share an A-rich sequence with DNA-β and encode a nanovirus Rep-related protein. Nothing at all is currently known about their function for begomovirus biology (Briddon et al., 2004).

1.2.4 Infection cycle of begomovirus

1.2.4.1 Begomovirus transmission

Begomoviruses are transmitted by whitefly (*Bemisia tabaci* [*B.tabaci*], Homoptera: *Aleyrodidae*) and have a circulative mode of transmission (Cohen et al., 1989), requiring an average of 6-12 h prior to a transmission event (Fargette et al., 1996). The transmission experiments conducted by Zeidan and Czosnek (1991) of TYLCV showed that whitefly feeding periods of 4 h or longer were necessary to achieve TYLCV transmission rates near to 90%. The whiteflies were able to pass the virus 8 h after the start of the acquisition access period (AAP) in the research of Ghanim et al. (2001a). It has been reported that the efficiency of transmission is gender-dependent and females were proved as a more efficient vector of TYLCV and ToLCBV than males (Muniyappa et al., 2000; Ghanim et al., 2001a). Although for long time TYLCV was not supposed to be transmissible to the progeny, since it was though only adults or larvae could acquire the virus. However, Ghanim et al. (1998) noted that TYLCV-Mld could be transmitted through the egg for at least two generations. It was also reported that TYLCV could be sexually transmitted among whiteflies in the same biotype (from viruliferous males to non viruliferous females) and the recipient insects were able to efficiently inoculate tomato test plants (Ghanim and Czosnek, 2000; Ghanim et al., 2007).

Hunter et al. (1998) proposed a model for the movement of begomoviruses in the whitefly vector carrying *Tomato mottle begomovirus* (ToMoV) and *Cabbage leaf curl begomovirus* (CaLCV) in various tissues of *B. tabaci* B biotype by immunfluorescent labelling of viral coat protein in freshly dissected whiteflies. According to his model, in the vector *B. tabaci* virus particles are ingested along with plant fluids into the whitefly oesophagus and foregut, after which nutrients and begomoviruses are concentrated in the filter-chamber of the whitefly. Begomovirus particles are absorbed to specific sites on the alimentary membrane or to sites along the anterior region of the midgut, and then move out of these tissues into the hemolymph, eventually invading the salivary glands. A microscopic analysis of the morphology and ultrastructure of the digestive, salivary, and reproductive systems of adult *B. tabaci* B type from Ghanim et al. (2001b) confirmed the prior findings. While feeding on a plant, the virus particles are introduced into a plant cell by the vector. Whiteflies feed on the phloem by inserting their stylets into plant tissue and locating the vascular tissue. The phloem tissue transports carbohydrates produced as a result of photosynthesis and other substances throughout the plant, which increases rapidly the virus infection in all the plant parts.

1.2.4.2 Infection cycle in plants

After being delivered by the insect vector into the phloem of susceptible host plants, the virus particles find their way into permissive cells and subsequently into the nucleus of these cells. To infect the plant, the virus begins to replicate and spreads from cell-to-cell. In most plant cell nuclei, begomovirus DNA replication is accomplished through a rolling circle mechanism with a dsDNA intermediate. This process can be divided into two steps (Figure 4):

a) Conversion of single-stranded virion DNA into a double-stranded form that serves as the template for transcription of the viral genes;

b) Production of single-stranded virion DNA from the double-stranded intermediate.

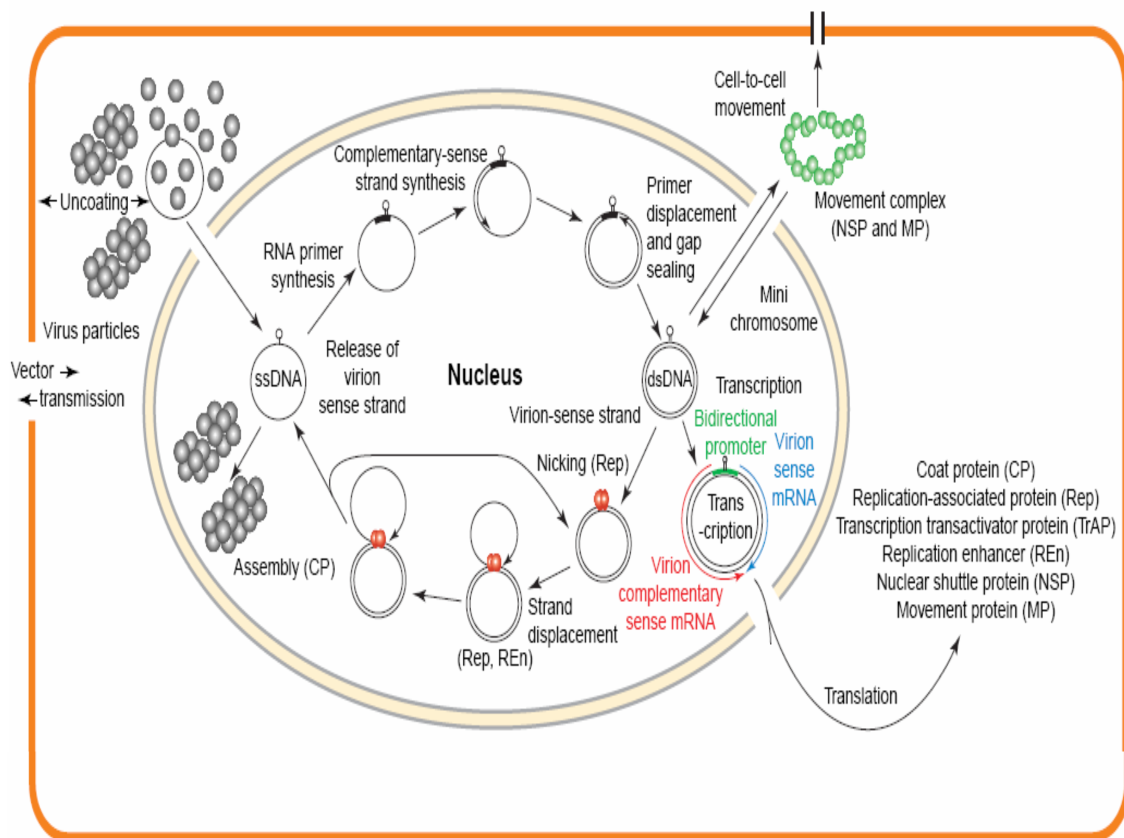

Figure 4: A model of *Geminivirus* replication and cell-to-cell movement in plants. (Modified from Vanitharani et al., 2005).

Begomoviruses have a small genome and do not encode their own DNA polymerases. Therefore, the viruses depend on host cell factors for replication in order to amplify their genome, as well as transcription factors. The replication takes place in nuclei of mature cells, which are not competent for DNA replication, so an early step in geminivirus infection may be the induction of host DNA replication enzymes (Nagar et al., 1995; Nagar et al., 2002; Egelkrout et al., 2001). At the early step, the single-stranded circular DNA is converted to a double-stranded circular intermediate. This step is still not fully understood in molecular terms, but the use of host factors must be involved as well as using the viral plus-sense DNA strand as a template to produce a complementary negative-sense strand. The following step is the creation of an intermediate single-stranded virion DNA from the double-strand. First REP, TrAP and other proteins are synthesized in the cytoplasm, then the double-stranded DNA intermediates serve as a template for rolling circle replication. A new ssDNA is syntheszied from the dsDNA template by a rolling circle mechanism

involving REP and REn of virus in association with host factors (Hanley-Bowdoin et al., 2004; Castillo et al., 2004; Settlage et al., 2005; Selth et al., 2005; Morilla et al., 2006).

Geminiviruses manage the transport of their DNA within plants with the help of three proteins, the coat protein (CP), the nuclear shuttle protein (NSP), and the movement protein (MP). CP and NSP revealed a sequence-independent affinity for both double-stranded and single-stranded DNA (Hehnle et al., 2004). In the current model for bipartite begomovirus cell-to-cell movement, BV1 coordinates the movement of viral DNA from the nucleus to the cytoplasm through the nuclear pore complex (NPC) and BC1 mediates cell-to-cell movement across the cell wall via plasmodesmata (PD) (Gafni and Epel, 2002; Lazarowitz and Beachy, 1999; Noueiry et al., 1994; Rojas et al., 2005; Sanderfoot and Lazarowitz, 1995). In case of the monopartite viruses, CP mediates nuclear export of ds-DNA RF for cell-to-cell and long distance movement within the plant (Rojas et al., 2001). They proposed a model that at the nuclear periphery, V1 serves to enhance nuclear export of viral DNA and then mediates the delivery of viral DNA to the cell periphery, possibly through an interaction with the endoplasmic reticulum (ER). The C4, through a putative N-terminal myristoylation domain, acts in the delivery of the viral DNA to the PD and mediates cell-to-cell transport. Upon entry into an adjacent uninfected phloem cell, the viral DNA moves across the nuclear pore complex to repeat the infection cycle. To initiate a systemic infection, the viral DNA or virions must cross the specialized PD of the companion cell-sieve element (CC-SE) to enter the SE for delivery to sink tissues (Rojas et al., 2001).

1.2.5 Resistance breeding through transgenic approaches

Multiple approaches to the engineering of resistance to geminiviruses are currently being evaluated for the development of crops resistant to geminiviruses. Most of these have involved pathogen-derived resistance strategies. The pathogen derived resistance (PDR) was at first proposed by Sanford and Johnson (1985) and reported by Abel et al. (1986), suggesting the resistance by transforming a susceptible plant with DNA sequences derived from the pathogen itself. The authors proposed that the expression of certain gene products during infection could interfere with the pathogene. Many advances have been made during the last years covering several virus-plant combinations. Even for geminiviruses,

there also have been some successful approaches reported although it seems more difficult to cope with DNA-, than with RNA-viruses.

In general, the transgenic resistance strategies (including PDR and non-PDR) can be classified into three categories; (1) protein mediated-resistance, (2) gene silencing known as RNA/DNA-mediated resistance, and (3) resistance due to the expression of non-pathogen derived antiviral agents.

1.2.5.1 Pathogen-derived resistance through the expression of viral proteins

While begomoviruses have six open reading frames, most of the attention on the development of resistance has been focused on the replication-associated protein (REP), movement proteins (MPs), and coat protein (CP) genes.

1.2.5.1.1 REP-mediated resistance

The multifunctionality of REP and the central role this protein plays in geminivirus replication have made it a favoured target of pathogen derived resistance strategies. A wide variety of *Rep* constructs have been used to produce virus resistance with a vast array of results. A number of reports indicate that full-length *Rep* constructs result in few or no transformants or produce transgenic plants with altered phenotypes due to phytotoxic effects (Bendahmane and Gronenborn, 1997; Hanley-Bowdoin et al., 1990; Nagar et al., 1995). Thus, researchers have used various truncated or mutated *Rep* constructs to overcome the phytotoxic effects of expressed REP in transgenic plants.

The repression of virus replication was observed in *N.benthamiana* protoplasts expressing N-terminally truncated REP (T-*Rep*) (Hong and Stanley, 1995; Brunetti et al., 2001) and T-*Rep* transgenic plants showed a certain level of resistance (Noris et al., 1996b). Expression of the N-terminal region of *Tomato leaf curl New Delhi virus* is sufficient to interfere with binding and oligomerisation of ToLCV REP as well as REPs of different geminivirus origin. This led to a decrease of more than 70% in DNA accumulation of the homologous virus and also decreases a 20-50% in DNA accumulation of heterologous ACMV, *Huasteco yellow vein virus* and *Potato yellow mosaic virus* (Chatterji et al., 2001). Similarily, studies by Lucioli et al. (2003) showed that over-expression of T-*Rep* of a *Tomato yellow leaf curl Sardinia virus* also conferred resistance to the homologous and

heterologous viruses. However, in this case the resistance is due to different mechanisms. Homologous virus resistance was shown to occur as a result of truncated REP binding to the intergenic region (IR) and tightly repressing the viral *Rep* promoter, whereas it affected a heterologous geminivirus by the formation of dysfunctional complexes with the REP of the heterologous virus. In both cases, however, resistance was eventually overcome by virus-mediated post-transcriptional homology-dependent gene silencing.

In addition to truncated REPs, over-expression of REP containing function-abolishing mutations in conserved motifs with key roles in viral replication has also shown potential to confer resistance to geminiviruses. Hanson and Maxwell (1999) over-expressed REP containing a mutation in the tyrosine kinase phosphorylation site, which is believed to play a role in nicking (Laufs et al., 1995a; Laufs et al., 1995b), and resulted in interfering with BGMV replication in a tobacco cell suspension system. Similar mutants of REP from ACMV were used in research of Sangare et al. (1999). The *N. benthamiana* transgenic plants exhibited tolerance to infection consisting in a delay of symptom appearance and/or the presence of mild symptoms.

1.2.5.1.2 Coat protein-mediated resistance

Coat protein-mediated resistance (CP-MR) refers to the resistance of transgenic plants that produce CP to the virus from which the CP gene is derived (Abel et al., 1986). CP is required for systemic infection by monopartite geminiviruses (Briddon et al., 1989; Rojas et al., 2001). The tomato plants expressing the CP of the monopartite begomovirus *Tomato yellow leaf curl virus* exhibited delayed symptom development, which was dependent on the expression levels of transgenic CP (Kunik et al., 1994). In contrast, the CP of bipartite geminiviruses is not absolutely necessary for the systemic spread of the virus, as NSP can substitute for the function of CP in transport (Ingham et al., 1995; Pooma et al., 1996). Therefore, it has been assumed that a CP-mediated strategy against bipartite geminiviruses will not produce a high level of resistance. Nevertheless, geminivirus CPs may have the potential for transgenic interference as they control specific interactions with the virus vector (Briddon et al., 1990; Azzam et al., 1994; Höfer et al., 1997; Noris et al., 1998; Morin et al., 1999).

1.2.5.1.3 Movement protein-mediated resistance

Geminivirus movement proteins (MPs) are required for their cell-to-cell and long distance systemic spread and they have been used to engineer resistance to various begomoviruses. It was first found that the expression of TGMV movement protein had a deleterious effect on systemic infection of ACMV DNA-A in *N. benthamiana* plants (von Arnim and Stanley, 1992). Tobacco plants expressing a mutated version of *Tomato mottle geminivirus* (TMoV) MP were also resistant to TMoV and CaLCuV, whose movement proteins share 80% amino acid sequence identity (Duan et al., 1997b). Tomato plants transformed with a mutated *Bean dwarf mosaic virus* (BDMV) movement protein gene showed resistance to ToMoV, which has a movement protein sharing 93% amino acid sequence identity with that of BDMV (Hou et al., 2000).

While it is promising that the resistance in these examples appears quite broad, the transgenic plants expressing the geminivirus NSP and MP genes were reported to be phenotypically abnormal (von Arnim and Stanley, 1992; Hou et al., 2000). The use of MP transgene is constrained by the fact that they are often toxic when over-expressed in plant cells, and in the case of begomoviruses, these genes are known as pathogenicity determinants. Their uncontrolled expression can therefore have many undesirable effects on various aspects of plant development (Hou et al., 2000). Similar with the use of *Rep* transgenes, regeneration of phenotypically normal plants may necessitate the expression of defective mutant or truncated movement proteins.

1.2.5.2 RNA/DNA-mediated resistance

1.2.5.2.1 Post-transcriptional gene silencing (PTGS)

More recently, it was discovered that in most cases where PDR was being aimed, the observed transgenic resistance was caused by transcriptional rather than translational expression of the viral transgene sequences (Sinisterra et al., 1999; Lucioli et al., 2003; Vanitharani et al., 2004). The mechanism behind these cases turned out to be RNA silencing or RNA interference (RNAi), a sequence-specific breakdown mechanism in plants which represents a natural antiviral defense mechanism (Voinnet, 2001; Vanitharani et al., 2003; Chellappan et al., 2004a). RNA interference can occur either through repression of transcription (transcriptional gene silencing), which is usually induced by DNA methylation (Rountree and Selker, 1997; Mette et al., 1999; Mette et al., 2000) or by

mRNA degradation based on dsRNAs homologous to viral coding sequences (Baulcombe and English, 1996; van Blokland et al., 1994) (for more detailed description of mechanism see section 1.2.6). The PTGS pathway is initiated by the generation of dsRNAs that are then digested into small, 21-26 nts RNA fragments. The small RNA causes the suppression of gene expression by complementary base pairing and destruction of targeted mRNA molecules in cytoplasm (Elbashir et al., 2001a). Geminiviruses are able to both induce PTGS as well as serve as a target for PTGS. This is unusual because geminiviruses do not contain a dsRNA intermediate during their replication cycle. However, recently it has been shown that transcripts initiated from the bidirectional promoter within the intergenic region may overlap to generate dsRNA, which serve as a target for PTGS (Vanitharani et al., 2005). In addition, any dsRNAs homologous to viral coding sequences may enter both known RNAi pathways (Baulcombe, 2004). On the one hand, they may act in TGS complexes as sequence-specific mediators for the methylation of homologous viral DNA sequences in the nucleus. On the other hand, they may serve as mediators for sequence-specific PTGS, i.e. degradation of viral transcripts and/or inhibition of translation. As described for the intergenic region, siRNA directed methylation may also affect coding regions and thereby cause reduced transcription.

As the *Rep* gene is strictly required for replication (Hanley-Bowdoin et al., 1999), it has been considered the most promising RNAi target. Vanitharani et al. (2003) observed a strong decrease in *Rep* mRNA accumulation and reduced viral replication in tobacco BY2 protoplasts transiently expressing the siRNAs homologous to *Rep* of ACMV. An siRNA construct designed to target the mRNA encoding the replication associated protein (AC1) of the ACMV from Cameroon blocked AC1 mRNA accumulation by 90-92% and inhibited accumulation of the ACMV genomic DNA by 65-68% at 36 and 48 h after transfection. The accumulated siRNAs in cassava plants recovering from infection by ACMV-CM were derived from the *Rep* genomic region (Chellappan et al., 2004a).

Methylation of a TLCV-derived transgene promoter resulting in transgene silencing has been observed on TLCV infection (Seemanpillai et al., 2003). This group observed that all *gus* transgenes expression driven by all six TLCV promoters was silenced. GUS plants (V2:GUS_C) were characterized in more detail and bisulphite sequencing showed that silencing was associated with cytosine hypermethylation of the TLCV-derived promoter sequences of the V2:GUS_C transgene. Recovery from *Vigna mungo yellow mosaic virus*-

infected plants has been reported after bombardment with DNA constructs expressing dsRNAs homologous to the bidirectional viral promoter (Pooggin et al., 2003). Akbergenov et al., (2006) detected 21, 22 and 24 nts siRNAs of both polarities, derived from both the coding and the intergenic regions of *Cabbage leaf curl virus* in *Arabidopsis* and ACMV in *N. benthamiana* and cassava. Genetic evidence showed that all the 24 nts and a substantial fraction of the 22 nts viral siRNAs are generated by the dicer-like proteins DCL3 and DCL2, respectively. The viral siRNAs were 5´-end phosphorylated, as shown by phosphatase treatments, and methylated at the 3´-nucleotide. These results indicated that the double strand small RNA-directed methylation of geminivirus bidirectional promoters may down-regulate the transcription of viral genes, resulting in inefficient virus replication. Triggering TGS of geminivius promoters by pre-expression or induced expression of specific dsRNAs may therefore constitute a promising strategy for interfering with virus replication.

So far, PTGS has been put to use, in the development of resistance against the geminiviruses: TYLCV (Fuentes et al., 2006; Zrachya et al., 2007b), ToLCV (Ramesh et al., 2007), *Bean golden mosaic virus* (BGMV; Bonfim et al., 2007), ACMV (Chellappan et al., 2004; Vanderschuren et al., 2007). Although only in its early stages, research utilizing this process to achieve geminivirus resistance is very promising in that any viral coding or non-coding sequences can be targeted.

1.2.5.2.2 Antisense RNA

An ‘‘antisense’’ RNA molecule that is complementary to a particular mRNA will base-pair with it and prevent the mRNA from being translated if both molecules are transcribed in the same cell. Antisense RNA strategies have been successfully exploited since 1991 to target and selectively suppress the expression of geminivirus genes. Day et al. (1991) successfully used asRNA technology to engineer geminivirus resistance in tobacco plants. TGMV replication was reduced in transgenic plants expressing a *Rep* asRNA sequence, and one transgenic line showed more than 90% symptomless plants after infection. Mubin et al., (2007) reported transgenic resistance against a bipartite begomovirus obtained by targeting a virion-sense of AV2 gene *Tomato leaf curl New Delhi virus*. *Rep* asRNA-mediated resistance was also engineered against the monopartite TYLCV in *N.benthamiana* (Bendahmane and Gronenborn, 1997) and tomato (Yang et al., 2004).

Zhang et al. (2005) demonstrated that resistance to ACMV infection of cassava can be achieved with high efficacy by expressing asRNAs against viral mRNAs encoding essential non-structural proteins. Asad et al. (2003) achieved CLCuV resistance in tobacco with a similar anti-sense approach against *Rep*, *REn* and *Trap*.

It is still unclear whether or how asRNA molecules enter the RNAi pathway to contribute to geminivirus resistance in transgenic plants. The suppression of gene expression by anti-sense RNA (asRNA) sequences was used before the discovery of gene silencing mechanisms. Later on, Asad et al. (2003) found small RNA with 21-23 nts long that suggested a mechanism might more or less be linked to PTGS. The duplex RNA resulting when the mRNA and its complement pair might also induce PTGS by the formation of siRNAs. However, Zhang et al. (2005) found no siRNAs in asRNA transgenic cassava plants prior to infection, suggesting that resistance is achieved by sense-antisense interactions after infection and not by the constitutive production of siRNAs from the transgene.

While many of these studies have achieved varying degrees of geminivirus resistance, there are also some reports of failure with this approach. For example, truncated antisense *Reps* totally failed to inhibit *Maize streak virus* (MSV) replication in cultured maize cells (Shepherd et al., 2007), and *Mungbean yellow mosaic virus* (MYMV) (Shivaprasad et al., 2006) antisense *Reps* (respectively in *N.benthamiana* and *N. tabacum*) failed to provide resistance against these viruses.

1.2.5.2.3 Defective interfering DNA (DI)

Defective circular single-stranded DNA molecules about half size of virus genomic DNA have been detected with some begomovirus infections (Stanley and Townsend, 1985; Stanley et al., 1997; Liu et al., 1998). Effectivity of defective DNA in delaying of symptoms have been shown in different studies: *N. benthamiana* plants transformed with a tandem repeat of subgenomic defective ACMV DNA B showed reduced symptoms compared with untransformed plants on ACMV infection (Stanley et al., 1990). Biolistic inoculation of *N. benthamiana* with infectious defective DNA-A-15 clone and *East African cassava mosaic Cameroon virus* (EACMCV) resulted in symptom amelioration as compared with EACMCV singly inoculated plants and there was an accumulation of defective DNA-A-15 in systemically infected leaves (Ndunguru et al., 2006). The

transformed *N. benthamiana* plants with a tandem repeat of subgenomic defective *Beet curly top virus* (BCTV) DNA-B showed symptom amelioration when challenged with the virus (Stenger, 1994). However, the mechanism has not been reported. Whether the integration of several DI sequences isolated from different cassava geminiviruses in cassava could protect against the infection by these viruses is still unknown.

1.2.5.3 Expression of non-pathogen derived antiviral agents

Recently, non pathogen-derived resistance has been investigated. The investigation includes the use of geminivirus-inducible toxic proteins to kill infected cells, and the expression of DNA binding proteins, peptide aptamers, or molecular Chaperonin (GroEL) homologues that either disrupt geminivirus infections or lessen their harmful effects.

1.2.5.3.1 Trans-activation of a toxic protein

Infected plants often have an innate defensive hypersensitive reaction that limits virus movement to the site of infection by inducing death in infected cells and their neighbours. An approach to engineer resistance to ACMV in transgenic cassava using Dianthin, the ribosome-inactivating protein (RIP), was described by Hong et al. (1996). Expression of Dianthin under this promoter in transgenic *N. benthamiana* plants reduced the susceptibility to infection by ACMV isolates originating from widely separated locations (Hong et al., 1996). However, this approach would only be of agronomic usefulness if residual transgene expression in the absence of infection did not cause any detrimental effects on plant performance. Such a reaction can be artificially induced (Zhang et al., 2003; Trink et al., 2005) to provide geminivirus resistance in transgenic plants, therefore a virus-induced cell death strategy may be particularly useful for engineering geminivirus resistance.

1.2.5.3.2 Expression of DNA binding proteins

The use of transgenically expressed DNA binding proteins to provide virus resistance relies on the identification of virus sequence-specific binding proteins that will not bind host DNA sequences. The sequence-specific dsDNA binding activities of geminivirus REP have a role in origin recognition and transcriptional repression, whereas the ssDNA binding activity of REP is involved in DNA cleavage (Hanley-Bowdoin et al., 1999). This sequence specific activity has been exploited by designing artificial zinc-finger proteins

with high affinity for the REP-specific direct repeats in the v-ori of different geminiviruses (Sera and Uranga, 2002), based on the idea that the artificial zinc-finger proteins (AZPs) will competitively block the binding of REP due to the higher affinity of the artificial zinc-finger protein-dsDNA interaction, thereby inhibiting viral replication. The utility of this approach was successfully demonstrated in *A. thaliana* against *Beet severe curly top virus* (BSCTV). Expression of AZPs with a nuclear localization signal (NLS) under the control of a *Cestrum yellow leaf curling virus* promoter in *A. thaliana* produced transgenic lines with reduced or no replication of BSCTV (Sera, 2005).

Antibodies against geminivirus viral proteins may be efficient factors for the impairment of key functions of these proteins when they target their active sites. Safarnejad et al. (2009) reported the expression of a single-chain variable fragment (scFv) antibody that protected *N. benthamiana* plants from a prevalent Iranian isolate of the virus (TYLCV-Ir). They expressed two recombinant antibodies (scFv-ScRep1 and scFv-ScRep2) that interact with the multifunctional replication initiator protein in *N. benthamiana*. Transgenic plants challenged with TYLCV-Ir showed that the scFv-ScRep1 were able to suppress TYLCV-Ir replication.

1.2.5.3.3 A Chaperonin (GroEL)

Morin et al. (1999) observed that a homologue of GroEL, which is produced by endosymbiotic bacteria from the whitefly vector *B. tabaci*, was able to bind with high affinity to the coat protein of TYLCV. Therefore, it may protect the virus from destruction during its passage through the insect's haemolymph. This idea was proven by Akad et al. (2007). The *B. tabaci* GroEL gene, which is expressed in transgenic tomatoes under the control of a phloem-specific promoter, protected the plants from infection with TYLCV (which is phloem limited in tomatoes). Plants infected with TYLCV were either asymptomatic or only mildly symptomatic and the GroEL formed complexes with the virus as expected (Akad et al., 2007).

1.2.5.3.4 Peptide aptamers

Peptide aptamers are proteinaceous agents which are selected for specific binding to a given target protein under intracellular conditions. Typically, peptide aptamers consist of a short variable peptide domain presented in the context of a supporting protein scaffold (Colas et al., 1996). Thus, in principle peptide aptamers act as recombinant proteins that

bind to inactivate a protein of interest (Colas et al., 1996; Hoppe-Seyler and Butz, 2000; Hoppe-Seyler et al., 2004). Peptide aptamers were first applied to engineering virus resistance in transgenic *N. benthamiana*, targeting the nucleoprotein (N) of the *tospovirus-Tomato spotted wilt virus* (Rudolph et al., 2003). To engineer geminivirus resistance using a similar strategy, *Rep* specific aptamers of geminiviruses have been identified by Lopez-Ochoa et al. (2006).

Due to the heavy impact of geminivirus infection in agriculture and the difficulty of controlling viral diseases, a variety of strategies have been studied to develop geminivirus resistance. The present study focusses on a RNA interference strategy.

1.2.6 Gene silencing via RNAi

Gene silencing via RNAi (namely post transcriptional gene silencing-PTGS) has been discovered in plants as their response to viral infections and other exogenous RNAs. While further examples of PTGS in plants continued to accumulate (Baulcombe, 1996; Metzlaff et al., 1997; Waterhouse et al., 1998), the RNA silencing phenomenon was independently observed in other eukaryotic organisms such as fungi (here termed “quelling”). It is a highly conserved phenomenon closely related to RNA interference (RNAi), occurring in different species such as protozoa, fungi, and mammals (Elbashir et al., 2001a; Fire et al., 1998; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Tuschl et al., 1999). RNAi is mediated by small interfring RNAs (siRNAs; 21-26 nucleotides), double-stranded RNA molecules with two to three nucleotide overhangs (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Elbashir et al., 2001b). Recently, new kinds of small RNAs have been revealed to be associated with RNA silencing in plants: tasiRNAs (*trans*-acting siRNAs) and nat-siRNAs (natural antisense transcript-derived siRNAs) (Vazquez et al., 2004; Allen et al., 2005; Adenot et al., 2006).

The silencing machinery consists of two protein complexes, Dicers and RNA-induced silencing complexes (RISC) are leading to sequence-specific RNA degradation and thus to a knockdown of the corresponding gene (reviewed in Aronin, 2006; Collins and Cheng, 2005; Dykxhoorn et al., 2003; Hammond, 2005; Hannon, 2002). The Dicer complex consists of RNAseIII-type enzymes responsible for processing small RNA duplices from double-stranded RNA molecules. Human, mice, nematode and yeast each possess only one

Dicer gene, insects and fungi have two Dicer like proteins (DCLs), (Tomari and Zamore, 2005; Catalanotto et al., 2004), while plants have even more DCL genes: *A. thaliana* four, poplar five and rice six (Gasciolli et al., 2005; Margisa et al., 2006). In *Arabidopsis*, one of the DCL genes (DCL1) was identified by sequence homology as AtDCL1. The other members of the gene family were identified by the same means (Schauer et al., 2002; Xie et al., 2004; Gasciolli et al., 2005). However, there are six non-DCL RNAseIII enzymes in the *Arabidopsis* genome (Bouche et al., 2006). RISC, after joining one of the sRNA strands, leads to sequence-specific cleavage of the target mRNA. To the RISC complex belong members of the Argonaute (Ago) protein family, which have a sRNA-binding PAZ-domain and also a PIWI-domain. They possess an endonuclease activity, known as the “slicer” activity, directed against complementary mRNA strands bound to the siRNA fragment. Silencing can be triggered in plants by replicating viruses, double-stranded RNA molecules, and foreign genes (transgenes) that allow the production of high levels of normal or “aberrant” messenger RNAs.

The majority of plant-infecting viruses have RNA genomes, except caulimoviruses, nanoviruses and geminiviruses. Caulimoviruses posses a double-stranded DNA (dsDNA) genome, which replicates through a RNA-intermediate using reverse transcription (Hull and Covey, 1986), therefore, this RNA strand can be a target for PTGS. The *Geminiviridae* are true DNA viruses that replicate their genomes in the nucleus by a rolling-circle (RC) mechanism that employs host replication machinery (Jeske et al., 2001; Preiss and Jeske, 2003). The double-stranded DNA (dsDNA) intermediates that mediate both viral replication and transcription associate with cellular histone proteins to form “minichromosomes” (Pilartz and Jeske, 1992; Pilartz and Jeske, 2003). Transcripts produced from these “minichromosomes” are subject to PTGS. In addition, given the role of RNA-directed methylation in silencing endogenous invasive DNAs, it is possible that plants might also use methylation as a means to repress transcription and/or replication from a viral “minichromosome” (Bisaro, 2006; Ding and Voinnet, 2007).

The key of RNA-based gene silencing is the long dsRNA that will be cleaved by DCL enzymes into small RNA, 21-26 nts in size. Based on this feature, Smith et al. (2000) designed gene constructs encoding intron-spliced RNA with a hairpin structure that can induce PTGS with almost 100% efficiency when directed against viruses or endogenous genes. Similarily, the present study used inverted repeat transgene constructs arranged in a

way that, when transcribed, render intron-hpRNA directed against invading TYLCV gene translation as well as TYLCV replication. The hypothesis of the study is summarized in Figure 5.

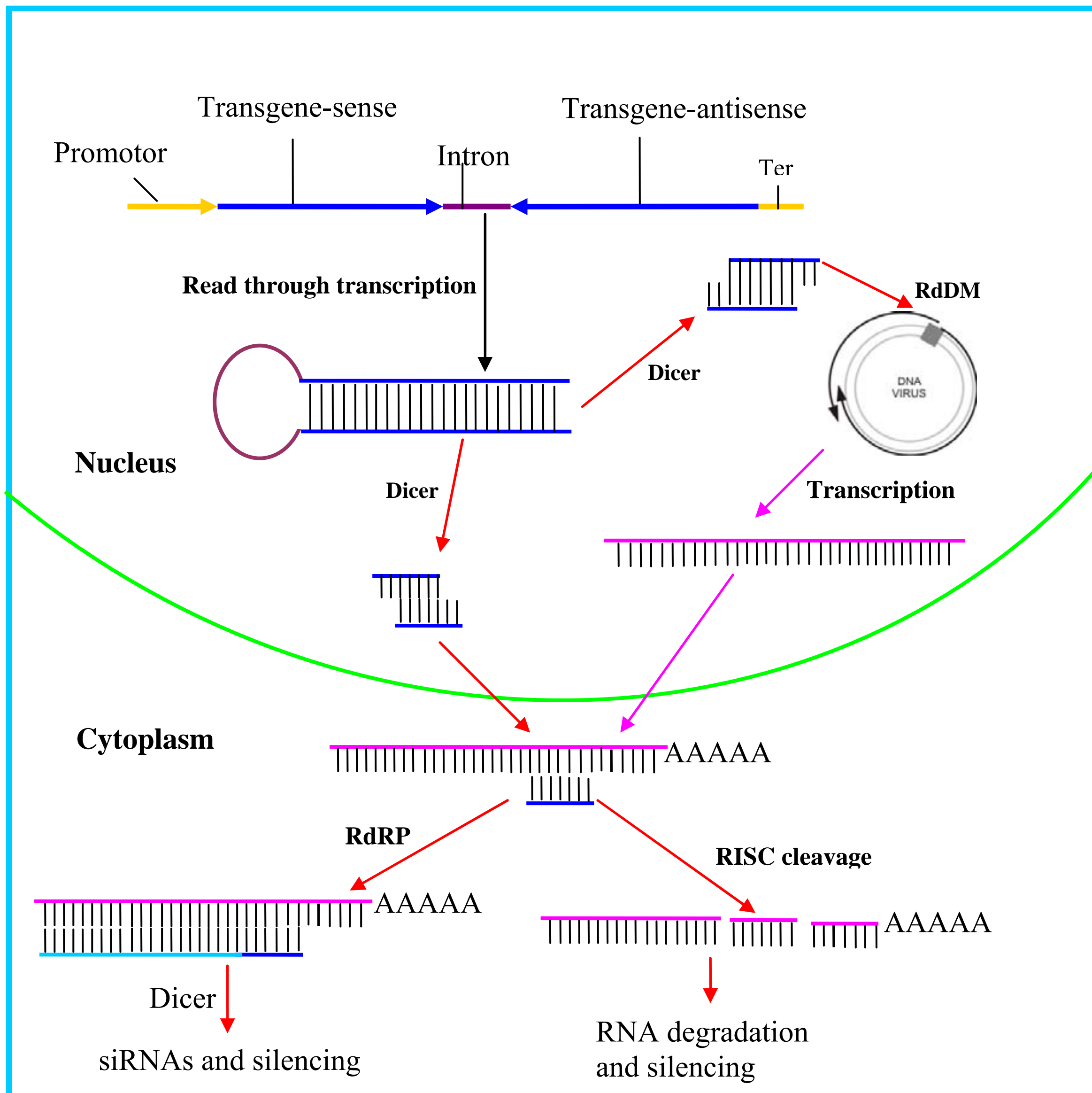

Figure 5: Inverted-repeat transgenes induced gene silencing (information ref. from Smith et al., 2000; Poogin et al., 2003; Dogar, 2006 and Biraso, 2006).

Legend: - RdDM: RNA-directed DNA methylation.

- RISC: RNA-induced silencing complexes.
- RdRp: RNA dependent RNA polymerase.

After the inverted transgene has been transcribed, the mRNA will automatically form a double strand by complementarity between sense and antisense. Then they are cleaved into small RNAs (21 to 26 nts in length) by dsRNA-specific Drosha-like nucleases or Dicer. On the one hand, these small RNAs are perfectly complementarity to the target mRNA of viruses. They guide RISC (RNA-induced silencing complex) to cleave target mRNA of viruses (Hammond et al., 2000), or these small RNAs are probably used as primers for RdRP to synthesize the secondary dsRNA, then the secondary dsRNA molecules are recognized and cleaved by Dicer into small RNAs. On the other hand, the dsRNA (24-26 bps in length) trigger transcription or replication of virus through RdDM (Dogar, 2006). The virus proteins can not be synthesised and virus can not replicate or move from cell-to-cell. The disease can be delayed or stopped.

1.2.7 Tomato transformation

Agrobacterium-mediated genetic transformation has been widely used as a low-cost, effective transformation method for both dicotyledonous and monocotyledonous plants. *A. tumefaciens* is used for genetic transformation of plants due to its natural ability to transfer foreign DNA into the host plant genome. The transfer of DNA from the soil bacterium *A. tumefaciens* into plant cells is an efficient process utilizing both bacterial and host machineries. First of all, phenolic compounds, which are released from wounded plant tissues, lead to recognition and induction of the bacterial virulence (vir) machinery. Vir proteins are responsible for the excision of the single-stranded transfer DNA (T_{DNA}). T_{DNA} delivery to the host cell cytoplasm occurs in complex with a single molecule of VirD2 at the 5'-end. The T-strand is encased by numerous VirE2 proteins to form a transfer complex (T-complex), which is then imported into the nucleus of host cells. During the transformation process, other bacterial proteins and host factors are involved in genomic integration and expression of the encoded genes (for details see Eckardt, 2004; Gelvin, 2003; McCullen and Binns, 2006).

Since both bacterial and host machineries are required for the DNA transfer from *Agrobacterium* into plant cells, a wide range of factors, such as pH, cocultivation media, temperature and period , *Agrobacterium* density, as well as genotype, explant types, can influence the gene transfer efficiency. In tomato, various factors that affect the efficiency of *Agrobacterium*-mediated transformation have been investigated so far. These factors

include co-cultivation temperature (Dillen et al., 1997), explant types (Frary and Earle, 1996; Ellul et al., 2003; Park et al., 2003) addition of phenolic compounds (Cortina and Culianez-Macia, 2004; Sun et al., 2006), vector constructs (van Roekel et al., 1993; Qiu et al., 2007), *Agrobacterium* concentration (Ellul et al., 2003; Qiu et al., 2007) and composition of the medium (Hamza and Chupeau, 1993; Frary and Earle, 1996; Ling et al., 1998; Krasnyanski et al., 2001; Pozueta-Romero et al., 2001; Park et al., 2003; Cortina and Culianez-Macia, 2004). Nevertheless, different aspects in tomato transformation that need to be considered are:

(1) Type of explants is correlated to ploidy level

In vitro plant regeneration from cell or tissue explants frequently results in chromosome variation (Karp et al., 1982; Karp et al., 1984; Pramanik and Datta, 1986; Sree Ramulu et al., 1986). In tomato, Koornneef et al. (1989) showed that diploid materials used in regeneration predominantly resulted in diploid plants. Tomato plant tissues are reported to be mixed populations of cells at different ploidy levels (van den Bulk et al., 1990; Smulders et al., 1995). Among the three types of tomato tissues, hypocotyls have proved to possess the highest, while the leaf tissues have the lowest polyploidy. Van den Bulk et al. (1990) and Smulders et al. (1995) observed that in the leaf tissue of tomato the content of diploid cells was about 70-93 %, whereas in cotyledons it was 39-60 %. In hypocotyls, only 19-40 % of the cells were diploid. A similar correlation was observed by Sigareva et al. (2004) who both transformed and regenerated three different genotypes of *S. lycopersicum*. Regenerants from hypocotyl explants of three different genotypes (“SG048”, “00-5223-1” and “00-0498-B”) were 25%, 36%, and 27% diploid, while regenerants from leaves were 85%, 82%, and 100% diploid.

(2) Roles of genotypes

The genotype response to tissue culture conditions is believed to drive the frequency of regeneration of transgenic plants. From an experiment using ten tomato cultivars, El-Bakry (2002) reported that shoot induction from aseptically grown cotyledons showed significant effects of both genotype and growth regulator with a non significant interaction between the two factors. The effect of genotype on the regeneration of tomato tissues was also reported in other studies (McComick et al., 1986; Tan et al., 1987; Moghaieb et al., 1999 etc.). For tomato transformation, only some cultivars have been intensively used thus far. The cultivar “UC 82b”, well known for its regenerating capacity, has been transformed by

McCormick et al. (1986), Fillatti et al. (1987), Hamza and Chupeau (1993), Pozueta-Romero et al. (2001), Gubis at el. (2003), Cortina and Culianez-Macia (2004). The cv. "Moneymaker" has been used in the researches of Tan et al. (1987), van Roekel et al. (1993), Smulders et al. (1995), Frary et al. (1996) and Ling et al. (1998). Another cv. "Aisla Craig" has been transformed by Bird et al. (1988), Lipp-Joao and Brown (1993) and cv. "PusaRuby" has been used by Patil et al. (2002), Roy et al. (2006), Sharma et al. (2009), and Afroz et al. (2009). The transformation protocols have been developed for several model varieties such as miniature cultivar "Micro-Tom" and "Micro-MsK" (Sun et al., 2006; Qiu et al., 2007; Mamidala and Nanna, 2009).

In transformation, genotype-dependence has been reported (McCormick et al., 1986; Agharbaoui et al., 1995; Gubis et al., 2003; Ellul et al., 2003; Shahriari et al., 2006; etc). Davis et al. (1991) reported that the effect of bacterial concentration on transformation efficiency may be due to different genotypes. McCormick et al., (1986) showed that the different genotypes had varying ability to form shoots from transformed leaf pieces as well as the length of time required for culture before shoots could be established in soil. They expected that most commercial cultivars are amenable to transformation. However, modifications of hormone levels or other culture conditions might be required. Agharbaoui et al. (1995) reported that the two genotypes "LA2747" and "LA1930", showed a distinct difference in their aptitude to transformation. Shahriari et al. (2006) archived the transformation frequency 17% for cv. "Kal-early" and 35% for cv. "KalG".

(3) Research in improvement of transformation frequency

Agrobaterium-mediated transformation requires *S* phase of cells for T_{DNA} integration (Villemont et al., 1997). Phytohormones have effects in cell division, thus they could affect *Agrobacterium* transformation. There are evidences about the effect of phytohormones inducing the competence of cells for transformation. For transformation of *A. thaliana,* Sangwan et al. (1992) found that competent cells in cotyledon, leaf, and root explants were induced only after phytohormone pre-treatment. De Kathen and Jacobsen (1995) proved that the induction of competence by auxins was concentration-dependent. Preculture of explants with phytohormone enhanced competence of cells has been reported in transformation of different plants such as in *A.thaliana* (Chateau et al., 2000), hybrid cottonwoods (Han et al., 2000), carnation (Nontaswatsri et al., 2004), cucumber

(Vasudevan et al., 2007), tomato leaf discs transformation (Patil et al., 2002) and in leaf segment transformation of *Saintpaulia ionantha* (Kushika, 2002).

The use of tobacco feeder-layer cell suspensions in tomato transformation experiments as reported by Fillati et al. (1987). Van Roekel et al. (1993) showed that the use of feeder layers combined with overnight pre-incubation appears to be an essential step in the transformation. Latter on, the use of a feeder layer of cell suspensions during pre-culture and *Agrobacterium* co-cultivation was reported in tomato transformations of different groups (Hamza and Chupeau, 1993; Frary and Earle, 1996; Ling et al., 1998; Zhang and Blumwald, 2001; Frary and van Eck, 2005; Hussain et al., 2008, etc). However, the use of a feeder layer makes the transformation procedure more complicated to carry out as well as the requiring of a tobacco suspension culture system.

(4) Selection of transformed cells

Most all tomato transformation protocols have been developed using an antibiotic resistance as selectable marker gene that is probably not accepted in commercially grown crops due to the law of European Union. Thus non-antibiotic selection marker should be taken into account in plant transformation.

1.3 Aims and significance of the study

The study aims to applying RNAi technology using inverted-repeat transgenes to produce tomato plants, which resist to TYLCV.

An efficient protocol for tomato transformation and its subsequent regeneration is a prerequisite for the production of transgenic plants. Due to the lack of a tomato transformation system in the Plant Biotechnology Laboratory (Hannover University) and the genotype dependence of tomato transformation via *A. tumefaciens*, the first aim of this study is the development of an efficient protocol of *Agrobacterium*-mediated transformation for different tomato varieties. Subsequently, the transformation with different intron-hairpin RNA constructs will be carried out. The transformed plants will be inoculated with TYLCTHV as well as TYLCVV for virus resistance evaluation.

Since no efficient methods to control TYLCV disease have been developed thus far, transgenic approaches are highly promising for achieving resistant varieties. Currently, many different strategies are being studied for produce resistant plants. The results of this

research might reveal evidence for controlling TYLCV towards the RNA silencing strategy.

CHAPTER 2
Development of a simple and effective protocol for leaf disc transformation of commercial tomato cultivars via *Agrobacterium tumefaciens*

2.1 Introduction

The transformation by *Agrobacterium tumefaciens (A.tumefaciens)* includes different steps: attachment of the bacterium to the plant cell wall, activation of the *vir*-operon, excision of single strand T_{DNA} and formation of the T_{DNA}-protein complex, targeting of the T_{DNA}-protein complex into the plant cell nucleus and finally, T_{DNA} integration into the plant genome. Thus the efficiency of genetic transformation could be affected by many factors. In summary, successful plant transformation demands (1) a target plant tissue competent both for transformation and regeneration, (2) an efficient DNA delivery method, (3) procedures to select for transgenic tissues, (4) the ability to recover fertile plants while avoiding somaclonal variation in transgenic plants, and (5) a simple, efficient, reproducible, genotype-independent and cost-effective regeneration protocol (Hansen and Wright, 1999). Depending on the regeneration capacity of tissue in different plant species, different explant types are being selected for transformation. For tomato, three types of tissues including hypocotyls, cotyledons and leaves have been used as explant material for *Agrobacterium*-mediated transformation. Among those, hypocotyls had the highest regeneration capacity and leaves had the lowest (Plastira and Perdikaris, 1997; Gubis et al., 2003; Park et al., 2003; Sigareva et al., 2004). However, *in vitro* plant regeneration from cell or tissue explants frequently results in chromosome variation (Karp et al., 1982; Karp and Maddock, 1984; Pramanik and Datta, 1986; Sree Ramulu et al., 1986). Moreover, tomato plant tissue has been reported to be a mixed population of cells at different ploidy levels (Van den Bulk et al., 1990; Smulders et al., 1995). Among the three types of tomato tissues mentioned, hypocotyls had the highest polyploidy levels and the leaf tissues had the lowest (Van den Bulk et al., 1990; Smulders et al., 1995; Sigareva et al., 2004). Interestingly, the research of Koorneef et al. (1989) showed that the plants, which were regenerated from

leaf explants of diploids, were predominantly diploid. This result indicated that ploidy levels of transformants depend preferably on the original ploidy levels of the tissues, which were used as material for transformation.

Beside the polyploidy effect, the integration of T_{DNA} into the plant genome occurs randomly, and frequently in two or more copies. Negative effects follow, such as low or no expression of the introduced transgene due to silencing (Matzke and Matzke, 1998; Assaad et al., 1993; Chalfun-Junior et al., 2003). Hence, the ratio of transformed plants with stable desired traits might be low. For those reasons, an effective transformation protocol based on a merely diploid explant source like expanding leaves can increase the number of transformed plants with stable and inherited transgene expression.

On the other hand, tomato regeneration and transformation quite often was found to be genotype dependent (McCormick et al., 1986; Tan et al., 1987; Agharbaoui et al., 1995; El-Bakry, 2002; Gubis et al., 2003; Ellul et al., 2003). Further more, most tomato transformation protocols have been developed using antibiotic resistance as a selectable marker which is not accepted in the Europian Union. Thus a non-antibiotic resistance system should incoporated in plant transformation. The present study analyses a number of parameters and propose a simple protocol for leaf disc transformation using glufosinate selection for three commercial tomato varieties, which have not been used for transformation experiments before. The protocol needs only one step of pre-treatment of explants with phytohormone without using pre-culture media.

2.2 Materials and methods

2.2.1 Materials

Expanding leaves of 4 different tomato varieties (MTS, DM8 and FM 372C and PT18) were used as explant source for experiments.

A. tumefaciens strain EHA105 harbouring the helper plasmid pSoup and a plasmid vector pGII0229 containing the *gus*-gene with the selection marker *bar* gene was used for transformation.

Basic culture medium (BCM), which contained MS inorganic basal salts (Murashige and Skoog, 1962) plus Gamborg B5 vitamins (Gamborg et al., 1968) supplemented with 30 g/l

sucrose and 0.5 g/l MES [2-(N-morpholino) ethanesulfonic acid], was used throughout the research.

YEP liquid medium (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, pH 7.0) was used for culture of *A.tumefaciens*.

2.2.2 Method of optimising for shoot regeneration

The expanding leaves from 4 weeks in vitro seedling plants were cut into small pieces with sizes of about 0.5x0.7cm. For each treatment, 4 Petri dishes were used. A total of 15 leaf explants were cultured in each plastic 90-mm Petri dish. The treatment differed from each other with regard to the addition of 11 different concentrations of *trans*-zeatin (0.4; 0.9; 1.3; 1.8; 2.3; 2.7; 3.2; 4.5; 7.0; 9.0; 13.5 µM) to an auxin concentration of 1 µM indolacetic acid (IAA). The explants were transferred to fresh medium every 2 weeks. Results (percentage of organogenic explants) were recorded after 6 weeks of culture.

2.2.3 Methods of optimising conditions for transformation

Four experiments were carried out including (1) the effect of *A.tumefaciens* concentration which was accomplished by comparing three optical densities (at 600 nm) of *A.tumefaciens* suspension, 0.3, 0.5 and 0.9 respectively; (2) the effect of temperature during inoculation and co-culture was carried by comparing four temperatures at 21, 24, 26, and 28˚C; (3) the effect of phytohormone supplemented into pre-treatment, inoculation and co-culture media was evaluated with four different combinations of zeatin and IAA, 4 µM zeatin/2 µM IAA, 4 µM zeatin/4 µM IAA, 8 µM zeatin/5 µM IAA and 8 µM zeatin/8 µM IAA ; and (4) the evaluation of glufosinate concentration for selection.

Procedure of experiment No. 1, 2 and 3*: Agrobacteria* were grown overnight in liquid YEP medium (with content 5 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl, pH 7.0) containing 50 mg/l of kanamycin and 5 mg/l tetracycline. For the bacterial concentration and temperature experiments, *Agrobacteria* from overnight cultures was collected by centrifugation at 4.000 rpm for 10 min at 18°C and re-suspended in liquid BCM-media, pH 5.5, plus 4 µM zeatin /2 µM IAA and 100 µM acetosyringone. While in the phytohormone experiment, the bacteria was re-suspended in the media with four different combinations of zeatin and IAA as description above. The *Agrobacterium* suspension was prepared at least 3 hours before inoculation. Leaves of tomato were cut in Petri dishes containing the same

used for re-suspending *Agrobacteria*. After the cutting was completed, the liquid media were discarded and the Petri dishes were kept in darkness for at least 20 hours before inoculation. In the temperature and phytohormone experiments, *Agrobacterium* concentration at an OD_{600}=0.5 was inoculated for explants. While the temperatureused used for *A.tumefaciens* concentration and phytohormone experiments was 24±1°C. After 60 min of inoculation, the explants were transferred into co-culture medium (the solid inoculation medium without acetosyringone). After 4 days of co-culture in darkness the same temperature as inoculation, the explants were transferred into elimination medium (co-culture medium containing ticarcilin 100 mg/l and sulbactam 100 mg/l, pH 5.8). They were then maintained in growth culture-room with 16h light/8h dark photoperiod, at 24˚C±1˚C. GUS-assays were carried out at day 7 after co-culture.

Optimising of glufosinate concentration for selection: In order to identify the most suitable glufosinate concentration for selecting transformants during callus induction and shoot regeneration, leaf tissues were directly cultured in solid BCM media containing 4 µM zeatin/4 µM IAA supplemented with either 1.5 ppm or 3.0 ppm of glufosinate. To determine an appropriate glufosinate concentration for rooting, two types of shoots were used: (1) shoots (1-2 cm) derived from calluses, and (2) shoot tips with 3 leaves of one month seedling plants. Five different concentrations of glufosinate, 1.5, 2.5, 3.5, 4.5, and 5.5 ppm, were supplemented into BCM medium plus 0.2 µM IAA for growing of type (2) shoots, while the shoots derived from calluses (type 1) were tested at 1.5 ppm and 3.0 ppm glufosinate, respectively. Three varieties were included in this experiment. The subculture was carried out for every two weeks. Morphogenesis was rated after 4 weeks of culture.

2.2.4 Development of the transformation procedure

Based on the results of all above experiments, the best conditions were selected for carrying out the final transformation experiment using 3 varieties including DM8, MTS and FM372C.

Histology and histochemical analysis of GUS-expression

The X-Gluc (5-bromo-4-chloro-3-indoly-glucoronide) was completely dissolved by DMSO (1 µl DMSO/0.1 mg X-Gluc), then mixed well with the staining buffer (100 mM phosphate buffer pH 7.0) at a concentration of 0.5 mg X-Gluc/1 ml of buffer. Explants

were washed with distilled water, submerged in X-Gluc solution, and kept in an incubator at 37˚C for 20 hours in darkness, and then the solution was discarded. The explants were then stored in 70% ethanol until blue spots appeared clearly.

2.2.5 Experimental design and data analysis

The regeneration as well as glufosinate concentration experiments were carried out with 60 explants per treatment without replication. Three other experiments were Completely Randomised Design (CRD). Each treatment was repeated three times. The transformation frequency was calculated as the total number of explants with at least one zone of GUS-expression (blue spot) produced relative to the total number of explants infected by *A.tumefaciens.*

$$\text{Transformation frequency (\%)} = \frac{\Sigma \text{ explants with blue spot}}{\Sigma \text{ inoculated explants}} \times 100$$

The GLM procedure of Statistical Analysis System version 9.2 (SAS Institute, Cary, NC) was used for statistical analysis. One-way analysis of variance (ANOVA) was used to determine mean separation between treatments. Two-way ANOVA was used to evaluate the interaction between treatments and genotypes. P values <0.05 were considered significant.

2.3 Results

2.3.1 Optimising shoot induction from leaf explants

Preliminary experiments conducted in our laboratory (data not shown) clearly showed that IAA and *trans*-zeatin were the most promising auxins and cytokinins for tomato regeneration. Therefore IAA was used in combination with 11 concentrations of *trans*-zeatin (Table 1).

Table 1: Effects of different zeatin concentrations for shoot induction in tomato varieties with a standard IAA concentration of 1 µM

Phytohormone concentration (µM)	Capacity of shoot regeneration			
	MTS	DM8	FM372C	PT18
0.4 Zeatin+1IAA	-	-	+-	-
0.9 Zeatin+1IAA	-	-	+	-
1.3 Zeatin+1IAA	-	-	+	+-
1.8 Zeatin+1IAA	+-	+-	++	+
2.3 Zeatin+1IAA	+-	+-	++	+
2.7 Zeatin+1IAA	+-	+-	++	++
3.2 Zeatin+1IAA	+	+	++	++
4.5 Zeatin+1IAA	+	+	++	++
7.0 Zeatin+1IAA	++	++	++	++
9.0 Zeatin+1IAA	++	++	++	++
13.5 Zeatin+1IAA	+	+	+	+-

Legend: (-): shoot regeneration 0 %; (+-): shoot regeneration <40 %; (+): shoot regeneration from 40-60%; (++): shoot regeneration >60 %.

Although the 4 varieties tested exhibited quantitative response differences, zeatin showed its effects on shoot regeneration in all 4 varieties. Sufficient shoot induction occurred with cv. FM372C and PT18 in a very wide range of zeatin concentrations (1.8 to 9.0 µM and from 2.7 to 9.0, respectively), while cv. MTS and DM8 formed shoots in a narrower range (from 7.0 to 9.0 µM) (Table 1). In general, the optimal concentration range for zeatin was from 7.0 to 9.0 µM in all varieties.

2.3.2 Effect of Agrobacterium cell density on transformation frequencies

The effect of the *Agrobacterium* cell density on transient transformation of tomato leaf tissue was determined using three different densities as shown in Table 2.

Table 2: Effect of *Agrobacterium* density on the transient expression of four tomato varieties

Variety	OD_{600}	Number of inoculated explants	Number of necrotic explants	Number of GUS-expressing explants	Frequency (%)	Ratio highest/lowest frequency
FM372C	0.2	318		3	0.94c	2.04
	0.5	312		6	1.92a	
	0.9	320	5	5	1.56b	
DM8	0.2	304		3	0.98c	2.32
	0.5	306		7	2.28a	
	0.9	298	8	5	1.67b	
MTS	0.2	345		3	0.87c	2.32
	0.5	346		7	2.02a	
	0.9	337	20	4	1.18b	
PT18	0.2	378		3	0.79c	2.37
	0.5	372		7	1.88a	
	0.9	366	5	6	1.78a	

Legend: Means in each variety followed by the same letter were not significant different at $P<0.05$

The results showed that the low bacterial density of 0.2 resulted in lower percentage of transient expression in all varieties (from 0.79 in PT18 to 0.98% in DM8 variety). It showed a trend towards an increase to the maximum frequency of transiently transformed explants at an $OD_{600}=0.5$ (1.88, 1.92%, 2.02%, 2.28% in PT18, FM372C, MTS and DM8, respectively) and there was a tendency towards a decrease at higher concentrations at $OD_{600}=0.9$ (1.18%, 1.56%, 1.67%, 1.78 in MTS, FM372C, DM8 and PT18, respectively) (Table 2). The effect of bacterial densities on transformation was significantly different ($P<0.05$) between an $OD_{600}=0.2$ and an $OD_{600}=0.5$. An increase in the density from $OD_{600}=0.2$ to $OD_{600}=0.5$, resulted in a twofold higher transformation frequency and the transformation frequency was significantly decreased at $OD_{600}=0.9$ except in the PT18 variety. Interestingly, no apparent interaction between the tomato genotypes and the respective concentrations of *Agrobacteria* was found. However, at $OD_{600}=0.9$ apparently

caused bacterial overgrowth resulting in a number of necrotic tissues after only 4 days of inoculation, as 20 of 337 (5.93%) inoculated cv MTS explants were necrotic. With the other varieties (PT18, FM372C and DM8), the rates were lower.

2.3.3 Effect of temperature during inoculation and co-culture on transformation frequencies

In order to optimize the temperature for transformation, four different temperatures were examined during inoculation and co-cultivation cultivation with the optimal density of *A.tumefaciens* as found in the previous part (OD_{600}= 0.5).

Table 3: Effects of temperature during inoculation and co-cultivation of tomato explants with *Agrobacterium tumefaciens*

Variety	Temperature (˚C)	Number of inoculated explants	Number of GUS-expression explants	Frequency (%)
FM372C	21	121	1	0.90a
	24	128	4	3.12b
	26	125	4	3.20b
	28	123	0	0a
DM8	21	140	2	1.42a
	24	126	8	6.34b
	26	128	9	7.03b
	28	145	0	0a
MTS	21	120	1	0.83a
	24	126	2	1.58b
	26	121	3	2.47b
	28	121	0	0a
PT18	21	143	0	0a
	24	123	4	3.25b
	26	128	4	3.12b
	28	129	0	0a

Legend: Means in each variety followed by the same letter were not significantly different (P <0.05).

The results (Table 3) indicate that transformation efficiency was influenced by temperature. In all of the 4 varieties, the percentage of transformation frequency at 24°C and 26°C are higher than those at 21°C ($P<0.05$). The frequency at 26°C was a little higher than at 24°C, except with PT18. At 21°C the transformation frequency was very low, even no blue spot could be observed in variety PT18. No explants with blue spots in all four varieties were recorded when inoculation and co-cultivation were carried out at 28°C. Between 24°C and 26°C, there was a slightly higher level of transformation frequency at 26°C in three varieties (FM372C, DM8, and MTS) with insignificant decrease in variety PT18.

2.3.4 Effect of plant phytohormones during inoculation and co-cultivation on transformation frequencies

Based on previously published data on the enhancement of transformation frequencies through auxin in pea (de Kathen and Jacobsen, 1995), four different combinations of the phytohormones zeatin and IAA at different concentrations were used for investigating the affect of zeatin and IAA on transformation frequencies in tomato. The results are presented in Table 4.

Generally, transformation efficiencies increased with an increase in phytohormone concentrations. In all varieties, the transformation frequencies were higher if media were supplemented with 8 µM of zeatin in combination with 5 µM of IAA or 8 µM zeatin/8 µM IAA. The highest ratio reached 7.07 for cv. DM8 and the lowest was 2.41 for cv. PT18. The percentage of transformation between high concentrations and to low concentrations of phytohormone was significant ($P<0.001$).

Table 4: Effect of IAA- and zeatin during pre-treatment, inoculation and co-culture on transformation efficiency

Variety	Concentration of plant phytohormone (µM)	Number of inoculated explants	Number of GUS-expressing explants	Frequency (%)	Ratio highest/lowest frequency
FM 372C	4 zeatin/2 IAA	317	15	4.73a	
	4 zeatin/4 IAA	323	11	3.40a	
	8 zeatin/5 IAA	334	37	11.07b	3.44
	8 zeatin/8 IAA	330	26	7.87 b	
DM8	4 zeatin/2 IAA	268	14	5.22a	
	4 zeatin/4 IAA	258	11	4.26a	
	8 zeatin/5 IAA	282	85	30.14b	7.07
	8 zeatin/8 IAA	289	73	25.25b	
MTS	4 zeatin/2 IAA	201	8	3.98a	
	4 zeatin/4 IAA	226	7	3.09a	
	8 zeatin/5 IAA	211	30	14.21b	4.59
	8 zeatin/8 IAA	227	25	11.01b	
PT18	4 zeatin/2 IAA	305	10	3.27a	
	4 zeatin/4 IAA	311	10	3.21a	
	8 zeatin/5 IAA	311	24	7.71b	
	8 zeatin/8 IAA	310	24	7.74b	2.41

Legend: Means in each variety followed by the same letter were not significantly different (P <0.001).

There was effective interaction between genotype and the concentration of phytohormon ($P<0.001$). The four varieties with three levels based on the transformation efficiency. DM8 had highest transformation frequency (25.25% and 30.14% in media containing 8 µM zeatin/8 µM IAA and 8 µM zeatin/5µM IAA, respectively), while two varieties, FM372C and PT18, had the lowest transformation frequency, and MTS had a transformation frequency between two first groups. The results also showed the dependence of transgene-expression on the concentration of phytohormones. An increase in phytohormone concentrations also resulted in stronger GUS-expression. At high concentrations of phytohormones (8 µM zeatin/5µM IAA), the explants with strong GUS-expression presented more GUS stained blue areas than those with lower phytohormone concentrations (Figure 6b and 6c).

Figure 6: Effect of phytohormones on GUS-expression (cv.372C). a) No pre-culture, inoculation and co-cultivation at 4 µM zeatin/2 µM IAA; b) Pre-treated 24 hours, inoculation and co-cultivation in 4 µM zeatin/2 µM IAA; c) Pre-treated 24 hours, inoculation and co-cultivation in 8 µM zeatin/5 µM IAA.

2.3.5 Determining the critical concentration of glufosinate for callus and root induction

The purpose of this experiment was to identify the minimal glufosinate concentration that eliminates untransformed cells without resulting in severe growth inhibition of surviving transgenic cells, and minimizes the risk of the escape of non-transformed plants prior to rooting.

Table 5: Effect of glufosinate concentration on inducing of calluses and rooting of shoots

Variety	Rate (%) of leaf explants forming callus at glufosinate concentrations		Root formation from two types of shoots at glufosinate concentrations						
			Type 1		Type 2				
	1.5 ppm	3.0 ppm	1.5 ppm	3.0 ppm	1.5 ppm	2.5 ppm	3.5 ppm	4.5 ppm	5.5 ppm
FM372C	60	0	31.81	0.00	100.00	41.66	0.00	0.00	0.00
DM8	40	0	40.00	0.00	90.00	50.00	10.00	10.00	0.00
MTS	40	0	26.92	0.00	87.50	75.00	10.00	0.00	0.00

Legend: - Callus induction of leaf tissue was observed after 4 weeks cultured in glufosinate media.
- Root formations were observed from two types of shoots. Type 1: shoots derived from calluses with size 1.0-2.0 cm; type 2: shoot tips derived from 1 month old seedling plants with 3 expanding leaves in glufosinate media.

After four weeks in culture on media with 3.0 ppm glufosinate, non-transformed leaf explants did not form any callus. They became chlorotic after four weeks of culture (Figure 7a). With 1.5 ppm of glufosinate in the media, 40-60 % of the explants slightly induced callus. From this result it can be concluded that a glufosinate concentration of 3.0 ppm could be applied for selection during callus induction and shoot regeneration. However, when this concentration was applied for the first inoculation with *Agrobacteria* at OD_{600}=0.5 (cv. MTS), after four weeks all leaf explants turned brown and died before the emergence of calli (Figure 7b). At a reduced concentration of glufosinate (2.0 ppm), several explants slightly formed calli after four weeks of incubation but all of them were also brown with out any recovery of transformed cells. Therefore, the concentration of glufosinate for selection at these stages must be < 2.0 ppm. When the concentration of 1.5 ppm was used, dead areas and freshly formed callus were scattered on the explants (Figure 7c).

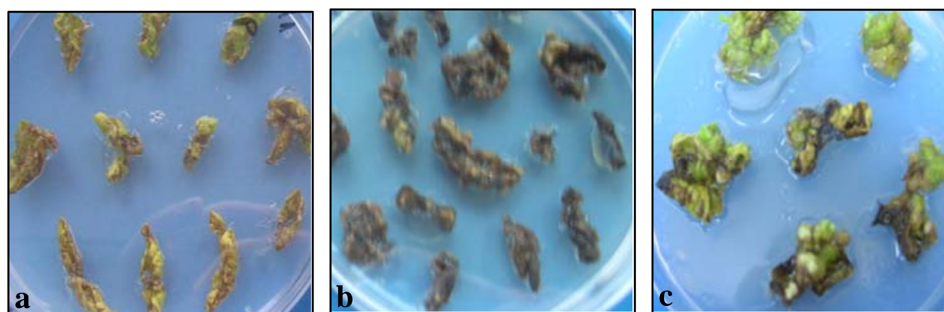

Figure 7: Effect of glufosinate inducing callus on leaf tissues. a) leaf tissue without *Agrobacterium* inoculation in medium at 3 ppm of glufosinate; b) leaf tissue with *Agrobacterium* inoculation in medium at 3 ppm of glufosinate; c) leaf tissue with *Agrobacterium* inoculation in medium at 1.5 ppm of glufosinate.

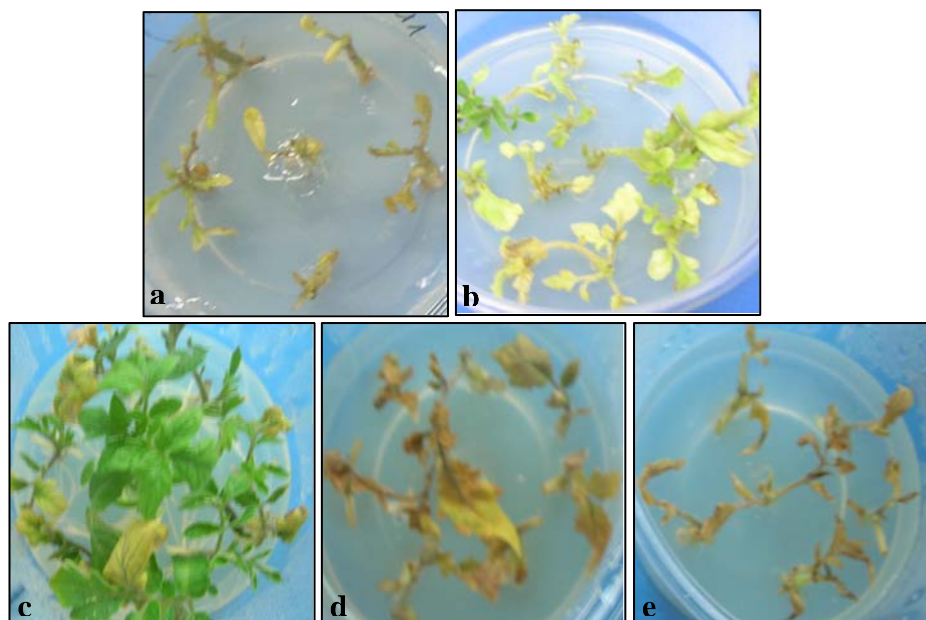

Figure 8: Effect of glufosinate in rooting of shoots: a) shoots derived from callus at 3.0 ppm glufosinate; b) shoots derived from callus at 1.5 ppm glufosinate; c) shoots derived from seedlings at 1.5 ppm glufosinate; d) shoots derived from seedlings at 3.5 ppm glufosinate; e) shoots derived from seedling plants at 5.5 ppm glufosinate.

During rooting, the shoots derived from callus appeared to be very sensitive to glufosinate and at a concentration of 3 ppm all shoots were dead after 2 weeks (Figure 8a). At 1.5 ppm, yellow leaves were observed in most of the shoots (Figure 8b). When the surviving plants were transferred to medium with 3.0 ppm of glufosinate, all shoots died.

The shoots derived from seedling plants were found to be more tolerant to glufosinate. At 1.5 ppm of glufosinate, most of plants survived (Figure 8c). With increasing glufosinate up to 3.5 ppm, only some shoots of DM8 and PT18 were viable but they did not show any rooting and at 5.5 ppm glufosinate all of tested plants were dead (Figure 8d). In this experiment, it was not possible to compare the effects of glufosinate between two shoot types because the shoots derived from calluses were smaller than seedling derived shoots. However, the result obtained from seedling-derived shoots may be a good reference for a further selection step to minimize the escape of non-transformed plants in the rooting media.

2.3.6 Establishment of a full transformation process

From the results of the experiments carried out, the best conditions were combined to transform for 3 varieties (DM8, MTS and FM372C) with a GUS construct. GUS staining was carried out in monthly. The results are shown in Table 6.

Table 6: Compilation of results using optimal conditions in transformation
Legend: GUS-expression as parameter

Parameter	Time after inoculation	Variety		
		MTS	DM8	FM372C
Number of explants with GUS expression	26 days	1	2	3
	2 months	2	4	4
	3 months	3	3	3
	4 months	3	3	3
	5 months	5	4	3
	6 months	3	5	6
∑explants showing GUS-expression.		17	21	26
∑ inoculated explants		200	200	200
Frequency (%)		8.50	10.50	13.00

The maximum percentage of transient expression was observed in the variety DM8 (see Table 4), but here, the transformation rate of DM8 is only 10.5 %. A similar trend was found for variety MTS (8.5 %- Table 6 compared to 14.21 %- Table 4). Interestingly, FM372C exhibited the highest frequency of transformation in all four studied varieties. The stable transformation of this variety was even a little higher than the maximum frequency of transient transformation (13.00 % compared to 11.07 % transient transformants-Table 4). According to the results, it was possible to conclude that all tested varieties were able to be transformed by *Agrobacterium* using the protocol developed.

2.4 Discussion

An efficient transformation system depends on both an efficient regeneration system as well as an efficient method for the introduction of foreign genes into the plant cells. A superior regenerating potential is important for successful leaf disc transformation mediated by *A. tumefaciens* (Koornneef et al., 1986). McCormick et al. (1986) also noticed variation in the regeneration response of leaf discs in different commercial tomato lines, with the best regenerable lines producing the highest number of transgenics. Chyi and Phillips (1987) developed a highly efficient *Agrobacterium*-mediated transformation for *S. lycopersicum* based on conditions favourable for regeneration. The information about genotype effects on tomato regeneration has been reported by various authors (Tan et al., 1987; El-Bakry, 2002; Gubis et al., 2003; Ellul et al., 2003). As a main outcome of transformation, it can be noted that an almost genotype-neutral regeneration system can be applied for the 4 varieties, using zeatin at 8 µM in combination with IAA at 1 µM for shoot induction.

The bacterial cell density used for transformation was found to be a very important factor influencing the efficiency of the process. In some species, it was found that increasing the bacterial cell density during inoculation improved transformation frequency (De Bondt et al., 1994; Cheng et al., 1997; De Clercq et al., 2002) and a too low concentration of *Agrobacteria* resulted in no transformation (Davis et al., 1991). However, higher bacterial cell densities or longer co-cultivation periods frequently lead to *Agrobacteria* overgrowth followed by explant necrosis, and/or failure to control *Agrobacteria* growth in subsequent cultures (Humara et al., 1999). In general, an increase in explant survival frequencies at optimum bacterial cell densities could be attributed to recognition of specific signal

molecules from the invading pathogen which facilitated the process of T_{DNA} transfer in explants whereas too high densities of *Agrobacterial* suspension resulted in rapid tissue necrosis and cell death around the infection site. The consequence is lower recovery that ultimately reduces growth; also the intensive growth of bacteria causes an inhibition of callus production and organogenesis (Fedorowicz et al., 2000). Since basically the interaction of *Agrobacteria* and the host plant is a pathogenic one, a defense response i.e. the hypersensitive reaction (Ciccarelli et al., 2005), can be expected and may explain the results obtained at different densities of *Agrobacterial* suspension. In tomato transformation, various *Agrobacterium* cell densities for inoculation with plant tissue have been reported: while Park et al. (2003), Ahsan et al. (2007) and Cortina et al. (2004) used high densities of *Agrobacteria* (up to OD_{600}=1.0), very low densities (OD_{600} from 0.1 to 0.3) were applied by Ling et al. (1998), van Roekel et al. (1993), Krasnyanski et al.(2001), Ellul et al. (2003) and Qiu et al. (2007). The present study results showed that the optical density of *Agrobacteria* optimal for transformation is OD_{600}=0.5. This concentration is similar to previous recommendations made by different authors (Frary and Earle, 1996; Agharbaoui et al., 1995, etc.). In agreement with another report (Davis et al., 1991), the present study found that high concentrations of *Agrobacteria* (OD_{600}=0.9) resulted in some of necrotic tissue development due to rapid bacterial overgrowth and the plants defense reactions. After 4 days of inoculation, twenty explants (5.93%) out of 337 inoculated explants for cv. MTS were necrosis with 5/366 (1.36%), 5/320 (1.56%), 8/298(2.68%) for PT18, FM372C and DM8, respectively.

The success of *Agrobacterium*-mediated transformation depends on T_{DNA} delivery and its transfer from the bacterium to the plant cell and finally on T_{DNA} integration into the host genome. The efficiency of T_{DNA} transfer depends largely on how efficiently *vir* genes are induced by wound factors secreted by plant cells. These factors include specific classes of plant phenolic compounds that are released by wounding, such as acetosyringone and monosaccharides such as sugars (Cangelosi et al., 1990; Peng et al., 1998) and an acidic pH (Turk et al., 1991; Holford et al., 1992). Further more, temperature has been found to influence the transformation process. Early studies on *A. tumefaciens* mediated tumorigenesis showed that high temperatures were detrimental to tumor development (Braun, 1947; Braun, 1958). Currently, scientists can explain the effect of temperature in *Agrobacterium*-mediated transformation at the molecular level. The activities of *vir* proteins of *Agrobacterium*, which are essential for excision and transport of T_{DNA} from the

bacterial cell to the nucleus of a plant cell, are sensitive to temperature (Alt-Mörbe et al., 1989; Jin et al., 1993). Fuller et al. (1996), Fuller and Nester (1996) and Baron et al. (2001) also found that temperature effects the T_{DNA} transfer machinery. It has an effect on the ability to assemble a functional T-pillus, required for the T_{DNA} and protein transfer to recipient cells.

In the present research, the optimal temperature for inoculation and co-culture as found to range from 24 to 26°C. In contrast, Dillen et al. (1997) reported an optimal temperature of 22°C for T_{DNA} delivery to *Phaseolus acutifolius* callus and tobacco leaves. The number of delivery events decreased at ≥25°C. In a report of Uranbey et al. (2005) on tobacco transformation, the highest transformation frequency of tobacco leaf discs was achieved between 22°C and 24°C and the frequency of transformation was significantly decreased at 26°C. Nevertheless, our result is at least partially consistent with several previous studies. For example, co-culture at 25°C led to the highest number of transformed plants in tobacco (Salas et al., 2001). In a recent report of Ahsan et al. (2007), the highest frequency of transformation in 3 tomato cultivars ("Koma", "Seokwang" and "Green Grape") was achieved at 24°C. These results indicate that the optimal temperature for T_{DNA} delivery and transformation depends on species and type of explants. Therefore, the optimal temperature for stable transformation should be evaluated with each specific explant and the respective *Agrobacterium* strain involved.

Also, the cell cycle plays an important role in transformation efficiency. De Kathen and Jacobsen (1995) applied cell cycle inhibitors leading to a reduction of the number of transformation competent cells in pea. Research of Villemont et al. (1997) demonstrated the absolute requirement of S-phase cells for transfer and/or integration of the T_{DNA}. Auxins and cytokinins act synergistically to stimulate cell division in cultured cells through regulation subsets of cell-cycle genes such as cyclins, and cyclin dependent kinases (*CDKs*) (for review see Horvath et al., 2003). In addition, the exogenous cytokinin supplement in the media presumably minimized changes in plant cell cycle control even when the photoperiod changed. This is involved in cyclin homeostasis to prevent rapid changes in cyclin gene expression in plants undergoing rapid changes of photoperiod. Exogenous cytokinins replaced the role of light in the induction of de-etiolation (Golan et al., 1996). In the dark, cytokinins induce the expression of genes that are usually induced by light and are partially involved in chloroplast development (Chory et al., 1991). Once

kinetin was added to the media, the transcript levels of the cyclin genes did not change when the 15-day-old seedlings were transferred to continuous dark or light for 24 hours (Lee et al., 2006). It is important that transformation by *Agrobacteria* as co-culture is best during darkness (Mendes et al., 2002). In transformation, an explant becomes more susceptible to *Agrobacterium* when it is pre-cultured on medium containing phytohormones. Several studies showed that phytohormone induced competent cell for transformation. In the transformation of *A. thaliana,* Sangwan et al. (1992) found that competent cells in cotyledon, leaf and root explants were induced only after phytohormone pre-treatment. In transformation of pea, De Kathen and Jacobsen (1995) proved that the induction of competence by auxins was concentration-dependent. Currently, preculture of explants with phytohormone-enhanced competence of cells has been reported in transformation of different plants: *A. thaliana* (Chateau et al., 2000), hybrid cottonwoods (Han et al., 2000), carnation (Nontaswatsri et al., 2004), cucumber (Vasudevan et al., 2007), tomato leaf discs (Patil et al., 2002), leaf segment transformation of *Saintpaulia ionantha* (Kushika, 2002); etc. The period of preculture has ranged from 2 days to a week (Patil et al., 2002; Han et al., 2000, etc), or even 2 weeks (Kushika, 2002). However, preculture had no effect on transformation in other report (Ahsan et al., 2007), and the explants had also been used directly for inoculation without pre-incubation in a medium containing phytohormones (Wang-Pruski and Szalay, 2002; Sigareva et al., 2004; Banerjee et al., 2006). These results are not surprising, considering of genotype factor.

In the present study, the effects of preculture was investigated (with 4 µM zeatin/2 µM IAA) for 48 and 72 hours in the varieties FM372C and PT18 (data not showed). The frequency of transient GUS-expression with precultured explants for 48 hours was less than that of non-precultured explants, even though there were no blue spot in precultured explants for 72 hours. A very short period of pre-treatment (24h) resulted in no changes in transformation frequency but increased levels of GUS-expression were found (Figure 6a and 6b). It can be assumed that tomato explants pre-treated with 4 µM zeatin/2 µM IAA for 24 hours before inoculation with *A.tumafaciens* enhance their respective transformation competence. Therefore, in all following experiments the pre-treatment of explants for 24 hours with phytohormone was used (see: Method). The role of phytohormones on tomato transformation was more appearent when higher concentrations phytohormones were applied. The transformation not only resulted in an increased number of explants with blue

spots (see: Table 4), but also exhibited more blue spots per explant (Figure 6b, 6c). It is likely that high concentrations of exogenously applied phytohormones induced more cells to enter into the cell cycle. On the other hand, exogenous cytokinins and auxins were found to induce stomata opening in darkness (She and Song, 2006) that might enable better entry of *Agrobacterium* into deeper tissue layers in the leaf explants. The results also showed that a high frequency of shoot regeneration was achieved in cv. FM372C in a very wide range of zeatin concentrations (1.8 to 9 µM zeatin in comparison with 7-9 µM in cv. MTS and DM8, Table 1), which might relate to the higher rate of stable transformants in that variety.

As only a few cells of an explant are usually transformed after inoculation/co-culture with *Agrobacteria*, leading to a chimeric tissue consisting of transformed/untransformed cells, the selection procedure that favours the growth of transformed cells over untransformed cells is a critical step. However, selection agents significantly decrease the relative density of viable cells by killing untransformed cells, usualy resulting in severe growth inhibition of the surviving transgenic cells. The appropriete dose of selection agent was found to be dependent on the plant species. In each species the concentration of selection agent also depends on the stage of plant development and its viability. The present transformation system with tomato used glufosinate and the *bar*-gene for selection. The respective threshold concentrations had to be determined and should be appropriate to maintain the recovery capacity of transformed cells and minimize the development of non-transformed cells. In addition, the level of glufosinate that eliminates non-transformed regenerants should be chosen for selection at the critical rooting stage. Currently, there are only a few references for tomato, where the *bar*-gene has been used as a selection marker. Most authors identified the concentration of glufosinate from 4 to 6 ppm as suitable for inhibiting the tomato shoot regeneration (Saker and Rady, 1999; Fuentes et al., 2008; Hussain et al., 2008). In contrast, Chen et al. (2006) reported that glufosinate at 20mg/l inhibited shoot regeneration of hypocotyls and cotyledon of cv. “Money-Maker”. However in all of those studies, cotyledons or hypocotyls were used as the source material for transformation. In the present study it can be shown that tomato leaf tissues were very sensitive to glufosinate. Here, even 3 ppm glufosinate prevented callus induction from expanding leaf tissues. It can be surmised that the toxicity of glufosinate depends on genotype and specific tissues. As glufosinate is toxic to all plants tissues, it is considered

fundamental for selection of *A.tumefaciens* transformed plants. Here, the data also showed that inoculation with *Agrobacteria* induces as an additional stress, a hypersensitive response, suggesting that different harmful factors affect the plant tissue at the same time, which should be considered for assessing success of transformation. Without *Agrobacterium*-stress, a concentration of glufosinate at 1.5 ppm did not totally inhibit callus proliferation of tomato leaf tissue, but under *Agrobacterium*-stress, the same concentration was definitely suitable for the selection of transgenic shoots from leaf discs for all three varieties. Although at this concentration a number of non-transformed cells still survived, the recovery capacity of transformed cells could be maintained. Also, shoot regeneration could be induced (see Figure 7c, 9a, 9b, 9c). Therefore, this concentration was used throughout the study. For the rooting stage, single shoots (without any callus) were cultured for 2 weeks on medium with 1.5 ppm glufosinate. The transgenic shoots developed into green rooted plantlets, whereas the non-transformed shoots almost turned yellow-white without any rooting (Figure 9d, 9e).

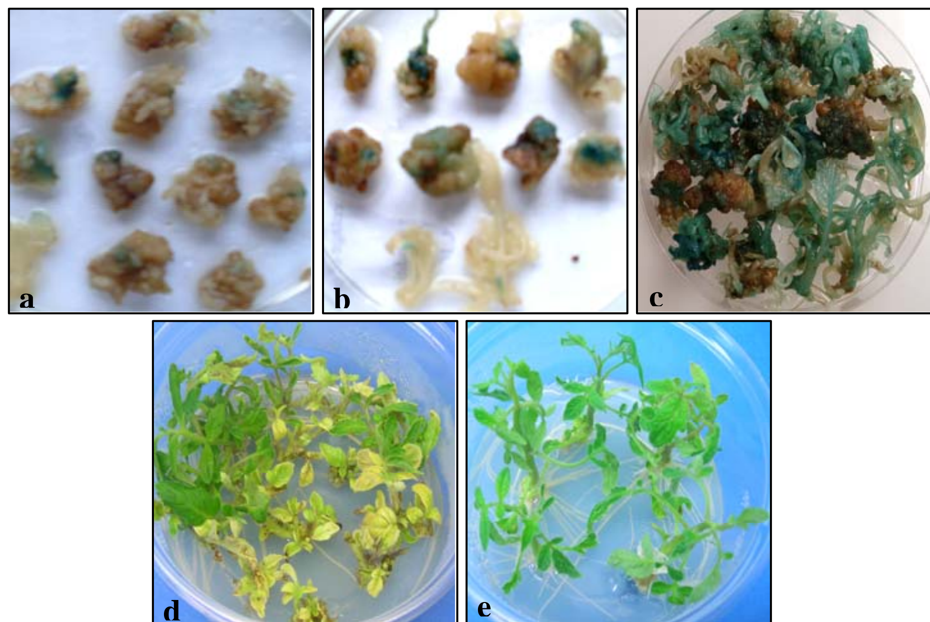

Figure 9: Leaf disc transformation with *gus* gene using 1.5 ppm glufosinate for selection. a) 2 months after inoculation; b) 3 months after inoculation; c) 6 months after inoculation; d) single shoots in rooting medium on medium containing 1.5 ppm glufosinate; e) the shoots of rooting-plants from (d) in rooting medium with 3 ppm of glufosinate.

From the results of GUS transformation with 3 varieties, the transformation procedure is summarized in the flow chart shown in Figure 10.

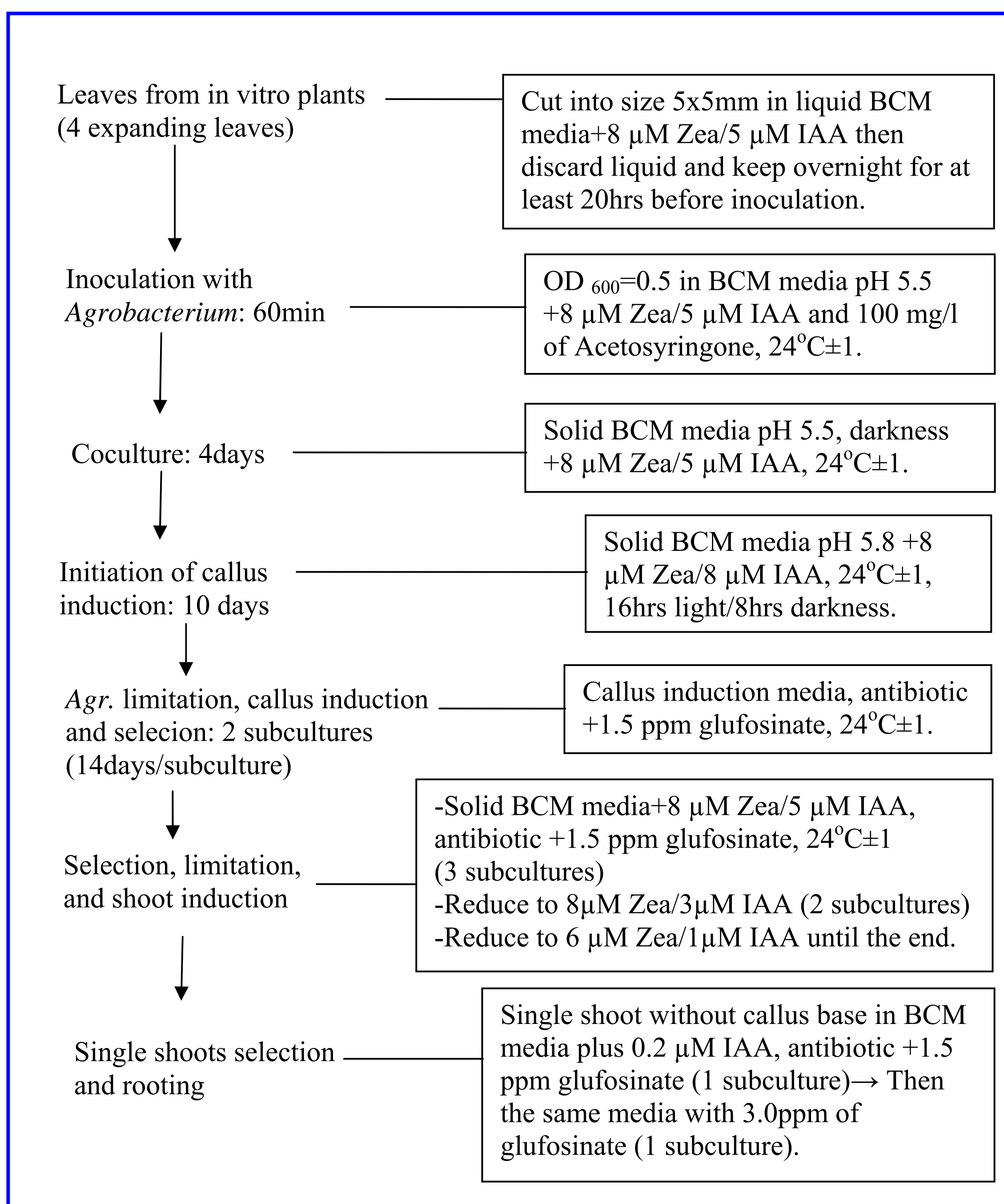

Figure 10: Flow diagram for tomato leaf disc transformation

The transformation prototcol for tomato leaf tissue developed presently is easy to be carried out and less time-consuming. Only a single step of pre-treatment with phytohormones is used with neither culture on solid media nor feeder layer. It resulted in a high transformation frequency (up to 19%, chapter 3), when used for transformation of variety FMT372C with other genes of interest.

CHAPTER 3

The inverted-repeat hairpinRNA derived from intergenic region and *Rep* gene of TYLCTHV confers resistance to homologous and heterologous viruses

3.1 Introduction

Post transcriptional gene silencing (PTGS) is a process in which double stranded RNA (dsRNA) triggers degradation of homologous RNAs in the cell. The dsRNA is diced into 21-25 nts long small interfering RNAs (siRNAs). The siRNAs then approach complementary RNAs and trigger their degradation. RNA silencing is a eukaryotic mechanism, which evolved in plants as a defence against viruses (Voinnet, 2001; Waterhouse et al., 2001). However, many viruses have evolved a strategy to overcome the defence of the host; they encode suppressors of RNA silencing (Moissiard et al., 2004; Roth et al., 2004). Transgenic expression of pathogen-derived sequences encoding hairpin RNAs has been considered as a sustainable strategy to obtain virus-resistant plants (Tenllado et al., 2004). This strategy has been successfully reported for plant RNA viruses (Tougou et al., 2006; Missiou et al., 2004; Mitter et al., 2003; Pandolfini et al., 2003; Kalantidis et al., 2002; Wang et al., 2000; Smith et al., 2000). For begomoviruses, the DNA viruses, there are only few reports, which describe the occurrence of PTGS after transforming plants with inverted-repeat constructs (Fuentes et al., 2006; Pooggin et al., 2003; Bonfim et al., 2007).

The present study shows that expression of self-complementary hairpin RNAs containing 397 bps of the 5´-terminus encoding the replication associated protein (Rep) and a 174 bps of the intergenic region (IR) of TYLCTHV is able to confer resistance to the cognate virus and a heterologous virus, TYLCVV. The regenerated T_1 transgenic plants are immune against TYLCTHV as well as TYLCVV under greenhouse conditions.

3.2 Materials and methods

3.2.1 Transformation of plants

3.2.1.1 Bacterial system and vectors

The transformation system EHA105/pSoup/PGII00229 developed with the *gus* gene (chapter 2) was used for transformation of *Solanum lycopersicum* var. FM372C. The only difference was the pGreenII0229 plasmid, which harboured the T_{DNA} containing the RNAi construct (see below).

3.2.1.2 RNAi constructs (*s*elf-complementary hairpin RNA constructs)

The transformation cassette was designed as an inverted repeat construct separated by an ST-LS1 intron (ST-LS1 intron IV2 from potato, Eckes et al., 1986) under control of an enhanced 35S promoter by Blawid (2008). Next to the left border the T_{DNA} contains a selection marker (*bar* gene) that is controlled by a nos-promoter and a nos-terminator. Inverted-repeat DNA fragments derived from the viral genome sequence are regulated by a 35S CaMV promoter and a CaMV terminator. A physical map of the construct is shown in Figure 11. The IR/Rep intron-hairpinRNA construct (IR/Rep-hpRNA) cassette contained 174 nts of the intergenic region (IR) plus 395 nts of the *Rep* gene (nucleotides 2.209 to 30 of GenBank accession no DQ871222). This region does not include only the 5′-terminal part of the *Rep* sequence but also a part of the 5'-terminus of the AC4 gene.

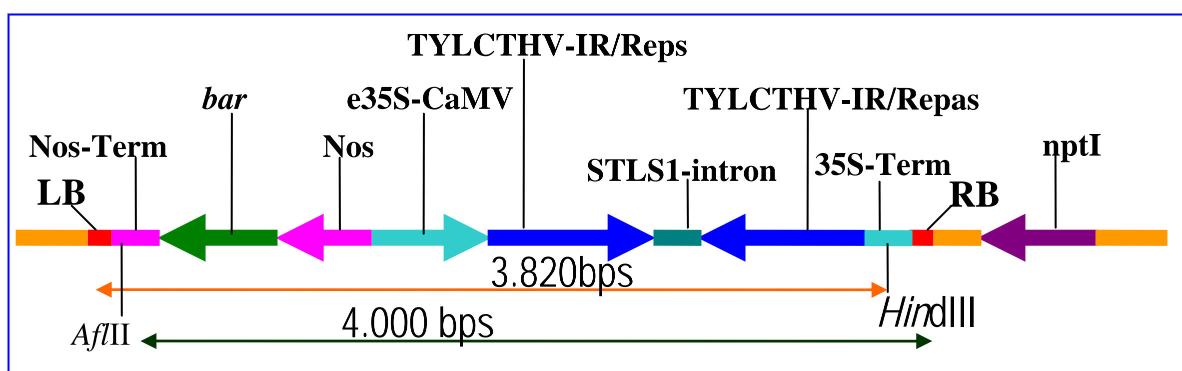

Figure 11: Physical map of IR/Rep-hpRNA constructs: inverted-repeat construct derived from the intergenic region (IR) combined with T-*Rep* of TYLCTHV. LB-left border, *nos*-terminator, *bar* gene, nos-promotor; 2x35S CaMV promoter (enhanced 35S promotor), IR/Repp_sense, STLS1 intron derived from potato, IR/Rep_antisense, CaMV-terminator; RB-Right border; *nptI* is located in the backbone sequence.

Table 7: Sequences of primers for detection of T_{DNA} integration and virus (TYLCTHV and TYLCVV) detection

Name	Sequence of the primer	Length of fragment (bp)
IR/Reps	5´- AAG GCG CGC CAC GCG TAT GCG TCG TTG GCA GAT TGG -3´	571
IR/Repas	5´- AAG GAT CCT CTA GAA AAA AAA ATC GCG GCC ATC C -3´	
bar-forward	5´- CGT CAA CCA CTA CAT CGA GAC -3´	423
bar-reversed	5´- TGC CAG AAA CCC ACG TCA TGC- 3´	
Reps	5´- ACT CTC CGT CGT CTG GTT GTC-3´	925
Repas	5´-TCCATCCGAACATTCAGGGAG-3´	
B-Ths	5´-GAGTTCCTACTAGACGACCTTTTGGC-3´	713
B-Thas	5´-GGGTCGAAAGGGAGCTGTTAACAA-3´	
Reps-VN	5´-TGGCCCACATTGTTTTACCCG-3´	593
Repas-VN	5´-ATTCTTCGACCTCACATCCCC-3´	

3.2.1.3 Plant transformation procedure and anlayses of transgenic plants

The transformation procedure developed with the *gus* gene (chapter 2) was used to transform *Solanum lycopersicum* var. FM372C. The surviving plantlets from rooting selection media containing 1.5 ppm glufosinate were considered as putative transformed plants. The presence of the transgene was confirmed by PCR before plants were transferred to the greenhouse to produce To seeds.

3.2.1.4 Plant DNA isolation

Plant genomic DNA for subsequent PCR and Southern blot analysis was isolated by a protocol modified from Dorokhov and Klocke (1998). Tissue from newly emerged leaves (0.1 g) was ground in liquid nitrogen. The homogenized leaf tissues were mixed with 400 µl of pre-heated (65°C) DNA extraction solution (200 mM Tris-HCl [pH 7.5], 250 mM Na_2 EDTA, 0.5% SDS) and incubated at 65°C for 15 min in a water bath, and mixed (by inverting) every 5 min during incubation. Next 200 µl of 5 M potassium acetate was added, mixed by inverting, and immediately placed on ice. After 10 min incubation, the samples were centrifuged at 13000 rpm and RT for 20 min.

The supernatant (500 µl) was transferred to new 15 ml microcentrifuge tubes. An equal volume of isopropanol (-20°C) was added to the supernatant and mixed gently. The samples were kept at -20°C for 10 min. The DNA was precipitated by centrifugation at 13000 rpm at RT for 10 min. The liquid phase was discarded and the pellet DNA was washed twice with 70% ethanol and dried by vacuum for 5 min or at 37˚C for 30 min. DNA samples were dissolved in RNase and DNase-free water and stored at -20°C. The concentration and puritiy of DNA samples were calculated by measuring the absorption ($Abs_{260/280nm}$) with an Ultrospec3000 spectrophotometer (Pharmacia Biotech).

3.2.1.5 Polymerase chain reaction (PCR)

Genomic DNA from plant tissue was extracted using the protocol above. Two primers were used to confirm the T_{DNA} integration (IR/Reps and IR/Repas). The amplified fragment (571 bps) confers to a part of the inverted-repeat intron hairpin region. Two other primers (bar-forward and bar-reverse) were designed to amplify a 423 bp fragment of the selectable marker gene (*bar*-gene).

<u>The PCR reaction mix contained (25µl):</u>

5.0 µl of GoTaq polymerase buffer (5x)

2 mM $MgCl_2$

250 µM dNTP's

1 µl of each primer (10pM)

2.5 U of Taq polymerase

and 100 ng of genomic DNA, then added ddH_2O up to 25 µl

The PCR reactions were carried out as follows:

	Step	Condition
	1 initial denaturation	4 min 94°C
30x	2 denaturation	1min 94°C
30x	3 annealing	30 s at 58°C (IR/Reps, IR/Repas) and 60°C (*bar* primers),
30x	4 extension	1 min at 72°C
	5 final extension	10 min at 72°C

All PCR reactions were carried out in a T3 thermocycler machine from Biometra. PCR products were separated on 1% agarose gels by electrophoresis (40 min at 120 volts) in TAE buffer (40 mM Tris-base, 20 mM acetic acid, 2 mM EDTA, pH 8.0). Gels were

stained with ethidium bromide (0.0015 mg/mL), and viewed with a UV transilluminator (Compact Imager).

3.2.1.6 Southern hybridization

The Southern hybridization was used for determining the number of T_{DNA} insertions into the plant genome. The methods used were based on the protocols described in the DIG Application Manual from Roche Applied Science.

Probe labelling: Plasmid DNA containing the inverted repeat construct was used as DNA template in PCR with Dig- labelling dUTP at a ratio of 1:6 or 1:3 and specific primer pairs for each fragment. The size of PCR products is shown in table 7.
A total of 30 µg genomic DNA was incubated with the restriction enzyme *Hin*dIII (Fermentas) for at least 16 hours. The restricted DNA was precipitated by absolute ethanol and dissolved by ddH_2O, then separated on 1.2 % agarose gel in 1X TAE buffer by electrophoresis at 80 V for 4 hours. The DIG-labeled Marker III was used as a standard ladder. All further procedures were carried out by shaking at RT. The depurination of DNA took place in 0.2 M HCl for 7 min. Then the gel was denaturated by incubation in 1.5 M NaCl + 0.1 M NaOH for 30 min. The neutralisation took 30 min in 0.5 M Tris-HCl + 3 M NaCl (pH =7.5). DNA was then transferred to a positively charged nylon membrane (cat number 11417240001-Roche-Applied-Sicence) and fixed by incubation to the membrane at 120ºC for 20 min.

DNA pre-hybridization was performed by incubating the membrane with 15 ml of hybridization buffer (2% blocking reagent; 5x SSC; 0.1% N-laurylsarcosine; 0.02% SDS and 50% formamide) at 42°C for 6 hours. Hybridisation followed with 5 ml hybridization buffer containing the DIG-labelled probe at the same temperature overnight. Unspecific fragments were removed by washing the membrane with low stringency buffer (2x SSC+0.1% SDS) at 42°C for 30 min, followed by high stringency buffer (0.1x SSC [1xSSC contains 0.15M NaCl, 0.15M Na-citrate] + 0.1% SDS) at 68°C for the same period. The membrane was blocked by 1 % blocking reagent in maleic buffer (0.1 M maleic acid + 0.15 M NaCl, pH 7.5). Furthermore, the membrane was incubated with Anti-DIG solution (12.5 µl Anti-DIG in 50 ml blocking solution). Washing the membrane for 30min with washing buffer (maleic buffer containing 0.3% Tween 20) at RT removed

unbound Anti-DIG. The membrane was incubated with detection buffer (0.1 M Tris-HCl + 0.1 M NaCl, pH 9.5) for 5 min at RT and subsequently supplemented with 1ml of CDP-Star solution (10 µl CDP-Star + 990 µl of detection buffer) and incubated for another 5 min at RT. The membrane was then transferred to a new nylon bag, CDP-Star solution was removed completely and the bag was sealed tightly. The nylon bag was placed in direct contact with an X-ray film (Kodak; cat.8761520) for at least 2 hours before the film was developed. The hybridized bands were visualized in developer (Tetenal, REF 103655) and fixed by fix stop solution (Tetenal, FX 103482).

3.2.2 Evaluation of virus resistance in transgenic plants

3.2.2.1 Plant material

Resistance studies were carried out with self-pollinated T_1 transgenic plants carrying the IR/Rep-hpRNA cassette. The T_1 plants were screened first by PCR to confirm the insertion of the gene. Fifteen independent transgenic lines with the IR/Rep-hpRNA construct were inoculated with tomato yellow leaf curl virus (TYLCV) by agroinoculation, when achieving the 5-7 leaf stage (Figure 12a).

3.2.2.2 Virus agroinoculation

Tomato yellow leaf curl Thailand virus (TYLCTHV-AIT; Knierim and Maiss, 2007) and *Tomato yellow leaf curl Vietnam virus* (TYLCVV; Blawid, 2008) were used for inoculation.

From TYLCTHV both viral DNA components (A and B) were agroinoculated. The bacteria carrying the A and B viral component, respectively, were grown separately for at least 16 hours at 28ºC in 300 ml of liquid YEP media supplemented with 50 mg/l of kanamycin up to an optical density OD_{600}=1.2. The bacteria were then centrifuged at 3.500 rpm for 10 min at 18°C. The pellet was carefully resuspended in 150 ml of agroinfiltration solution (10 mM $MgSO_4$; 10 mM MES and 100 µM acetosyringone). The bacteria suspension was then kept at room temperature for at least 3 hours. Before agroinfiltration, the bacterial suspension of A was mixed with an equal volume of the B component suspension. From TYLCVV only the A component was used for inoculation using the same procedure as for TYLCTHV. Each tomato plant was infiltrated with 1.5 ml of

bacteria suspension into 3 leaves (3 single leaves per stem with 4-8 infiltration points per leaf; see Figure 12b).

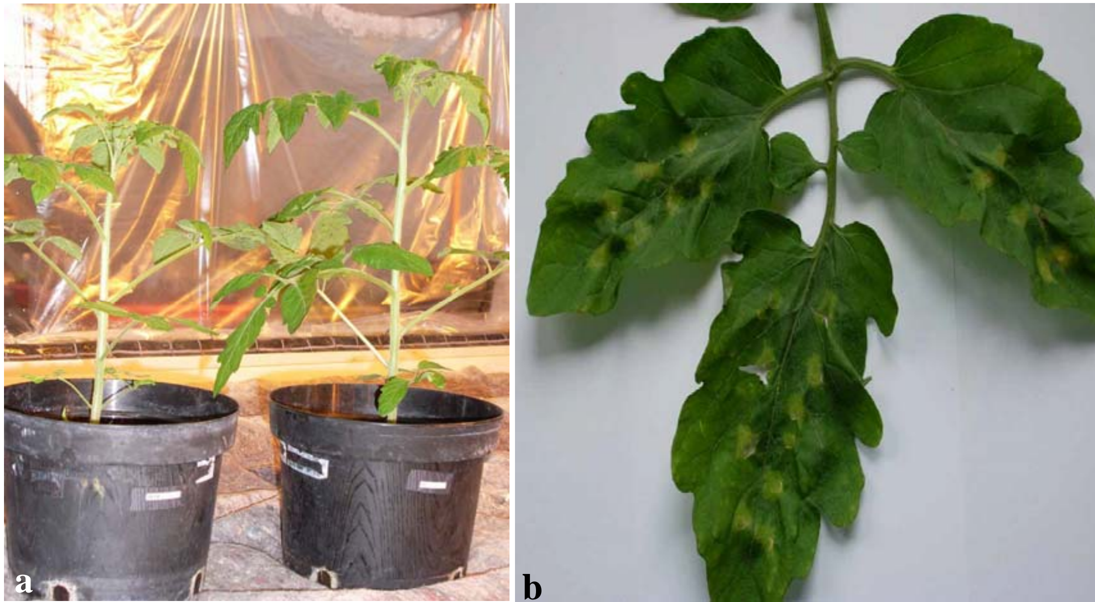

Figure 12: Agroinoculation of tomato plants. (a) Plant before agroinoculation; (b) Distribution of agroilfiltration points of TYLCV into tomato leaves 4 weeks after agroinoculation.

To confirm the infectivity of agroinoculated viruses and to check the morphological changes and symptoms occurring in *Nicotiana benthamiana* plants at 5-7 expanding leaf stage were inoculated. The agrobacteria containing full-length clones of the viruses were prepared as described above.

3.2.2.3 Evaluation of virus symptoms

The first step of resistance screening was carried out by observation of emerging disease symptoms. Tobacco plants first showed symptoms at 10 days past inoculation (dpi). The T_1 tomato plants were screened for symptoms, which started to emerge 3 weeks after inoculation. The observation and screening continued until plants were discarded after seed collection. The incidence of viral disease is given in percentage of the plants presenting disease symptoms. The transgenic lines with high disease incidence were discarded. Focusing on the symptomless lines, PCR tests were carried out to detect the virus. The

lines with positive PCR results were considered to be tolerant. Lines with negative PCR results were considered to be immune against the virus.

Phenotype of virus infected plants:

Figure 13: Symptom morphology at different times after inoculation.
Upper left: Shoot stunting in infected plants after 4 weeks past inoculation.
Upper right: Mosaic yellow leaves in infected plants after 4 weeks past inoculation.
Lower left: Infected plants after 120 days past inoculation in winter season.
Lower right: Infected plants after 70 days past inoculation in spring-summer season.

Infected plants were stunted or dwarfed. Newly developed leaves, produced after infection, were reduced in size. Leaflets rolled upwards and inwards. Young leaves were yellowish. Flowers dropped down and the plants showed prolonged flower abortion. Fruits, if produced at all, were small and dry.

3.2.2.4 Confirmation of virus presence by PCR

Genomic DNA was extracted from each sample using the protocol described. Two sets of primers were used for each component of the viral genome of TYLCTHV. With the set of primers Reps and Repas (for sequence see Table 7) we could amplify a 925 bp fragment belonging to the viral *Rep* gene, but it is located outside of the region used for the IR/Rep-hpRNA construct. With the primer pair Reps-VN and Repas-VN we could amplify a 593 bp fragment of TYLCVV-*Rep*. The amplified fragment is not identical with any part of the IR/Rep-hp RNA construct. The primer pair B-Ths/B-Thas was used to amplify an 813 bp fragment of B component of TYLCTHV.

The PCR reactions were carried out as follows:

1. initial denaturation 4 min 94°C
2. denaturation 1min 94°C
3. annealing 30 s at 61°C(Reps/Repas; Reps-VN/Repas-VN; 62°C for B-Ths/B-Thas)
4. extension 1 min at 72°C
5. final extension 10 min at 72°C.

30x (steps 2–4)

All the PCR reactions were carried out using a SENSOQUEST LabCycler. After performing the PCR reaction, the fragments were separated by electrophoresis (40 min, 120 volts) in 1% agarose gels in TAE buffer, pH 8. Gels were stained with ethidium bromide (0.0015 mg/mL) and DNA bands were viewed by a UV transilluminator (Compact Imager).

3.3 Results

Transformation success was determined by a polymerase chain reaction (PCR) using specific primers for the IR/Rep transgene region and for the selectable *bar* gene. Successfully transformed plants, confirmed by PCR, were transferred to the greenhouse for To seed production. The plants which were able to produce seeds were further tested for the copy number of the inserted transgene by Southern hybridization.

3.3.1 Confirmation of successful transformation via PCR

The plasmid containing the inverted repeat T_{DNA} was used as a positive control and the DNA samples of non-transformed plants as a negative control (wt). Electrophoresis results showed the predicted specific fragments for each primer pair. The amplified fragment using *bar* primers could be seen between 400 and 500 bps (Figure 14b), the fragments of IR/Rep-hpRNA were in the range of 600 bps (Figure 14a). There was no visible band from wt plants as well as in the water control. The size of the amplified fragments corresponded to the size of the positive controls.

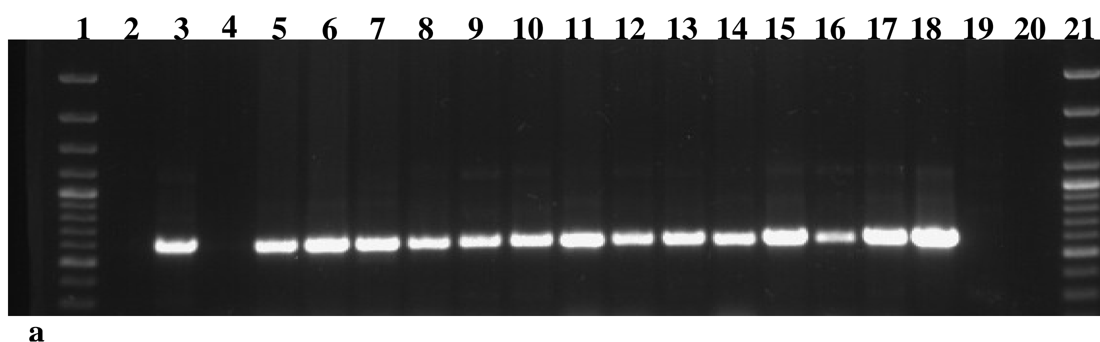

Figure 14a: PCR fragment amplified by IR/Reps and IR/Repas primers. Lane 1 and 21: DNA marker ladder 100bp (Fermentas). Lane2: IR/Rep 37-7; Line 3: IR/Rep 37-8; Lane 4: IR/Rep37-9; Lane 5: IR/Rep38-1; Lane 6: IR/Rep38-2; Lane 7: IR/Rep38-3; Lane 8: IR/Rep38-4; Lane 9: IR/Rep39-1; Lane 10: IR/Rep39-2; Lane11: IR/Rep39-3; Lane 12: IR/Rep 39-4; Lane13: IR/Rep40-1; Lane 14: IR/Rep40-2; Lane 15: IR/Rep 40-3; Lane16: IR/Rep 40-4; Lane 17: IR/Rep 41-1; Lane18: Possitive control (Plasmid DNA); Lane 19: Negative control; Lane 20: Water control .

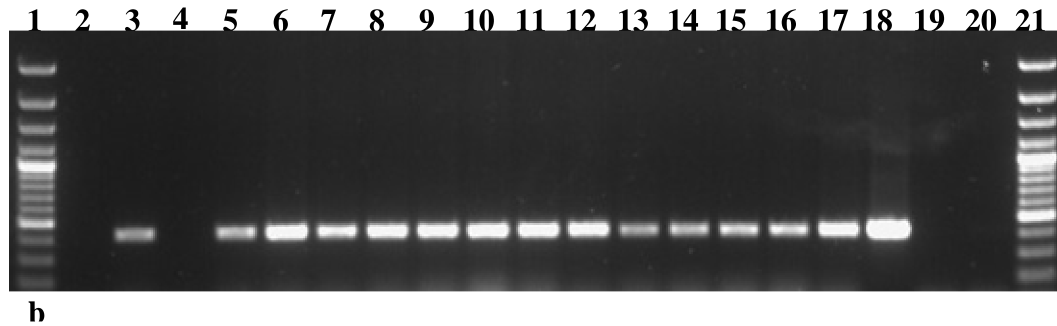

Figure 14b: Results of PCR by *bar* primers. The samples were placed as the same as Figure 14a.

From 210 leaf pieces which were inoculated with *Agrobacterium* containing IR/Rep region, 255 single plants originated and all of them showed positive results in PCR. These plants were derived from 40 calluses which originated from 40 inoculated leaf pieces, thus the transformation frequency using this construct is 19%.

3.3.2 Seed production from To plants

37 IR/Rep-hpRNA To plants were transferred to the greenhouse (one plant was randomly selected from each inoculated explant). Two plants died in nursery stage. Another 10 plants showed deviant leaf growth (thick and dark-green leaves); they had fruits but produced onlya few seeds. The remaining 25 plants exhibited the same habitus like non-transformed plants, produced many seeds. Five of them died in the stage of young fruits due to a fungal disease. However, these plants produced seeds and they were counted as seed producing plants. Therefore, the frequency of plants producing seeds was 67.6%.

3.3.3 Identification of transgene copy number in transformed plants

To identify the number of insertions in the remaining plants, Southern hybridization was performed. The recognition site for the restriction enzyme *Hin*dIII is located in the RB of T_{DNA}. This enzyme was used to digest genomic DNA of transformed plants. The results of hybridization with a DIG-labeled probe of IR/Rep and *bar* region are shown in Table 8.

Table 8: Southern hybridization with a DIG-labeled probe of the *Rep* and *bar* to DNA prepared from transgenic tomato lines

Plant line No.	Number of fragments hybridising with the *Rep* probe			Number of fragments hybridising with the *bar* probe		
	>5kb	3.5-4.9kb	<3.5kb	>5kb	3.5-4.9kb	<3.5kb
IR/Rep2-1	**2**	0	**1**	**3**	0	0
IR/Rep2-2	**2**	0	**1**	**3**	0	0
IR/Rep4-1	**2**	0	**1**	**2**	**1**	0
IR/Rep10-1	**2**	0	**1**	**3**	0	0
IR/Rep15-1	0	**2**	**2**	**1**	**1**	0
IR/Rep16-1	0	0	0	0	0	0
IR/Rep22-4	**2**	**1**	**1**	**2** (weak)	0	0
IR/Rep23-5	**2**	**2**	**2**	**2**	0	0
IR/Rep26-2	**1**	**1**	0	**1**	0	0
IR/Rep29-1	0	**2** (weak)	**3**	**2** (weak)	0	0
IR/Rep30-4	**2**	**1**	**1**	**1**	**1**	0
IR/Rep31-1	**1** (weak)	**3**	**3**	**1** (weak)	**3**	0
IR/Rep32-2	**1**	0	**1**	**1**	0	0
IR/Rep33-2	0	**2**	**2**	**1**	**1**	0
IR/Rep34-2	0	**1**	0	**1**	0	0
IR/Rep35-1	**1**	**1**	0	**1**	0	0
IR/Rep38-1	0	**2**	**3**	**1**	**1**	0
IR/Rep43-1	0	**2**	**2**	**1** (weak)	0	0
IR/Rep45-1	**1**	**1**	**3**	**1**	**1**	0
IR/Rep47-5	**1**	**1**	**3**	**1**	**1**	0
wt	0	0	0	0	0	0

There was no hybridization signal detected in DNA samples of the non-trans-formed plant (Figure 15), indicating that all other hybridisation signals derived from DNA samples of transgenic plants represent T_{DNA} insertions. The number of insertions in the IR/Rep-hpRNA transformants ranges from 1 to 7. Most of the plants showed 2-4 insertions (7 plants had 3; 5 plants had 4; and 5 plants had 2 insertions), while only 1 plant (5%) had a single T_{DNA} insertion. The line number 16-1 had neither a hybridization signal with the IR/Rep-hpRNA probe nor with the *bar*-probe, thus, DNA from this plant failed in the former PCR or was a chimeric type.

The size of the full-length T_{DNA} is 4299 bps. A *Hin*dIII restriction site is located near the RB, which leads after *Hin*dIII restriction digest to the appearance of a fragment with an expected minimum size of 3800 bps.

Based on the results of the hybridisation, both with the *Rep* probe and the *bar* probe, truncated and/or intact T_{DNA} insertions were indentified. If the hybridizing signal with *Rep* and *bar* probes of a plant are visualised at one position in the X-tray film that band will be an intact T_{DNA}. The results (Table 8) showed all transgenic lines contained a least one truncated insertion of the IR/Rep-hpRNA. Five out of 20 plants (25%) contained a truncated insertion of the *bar* gene (Table 8). The seven lines (IR/Rep47-5; IR/Rep45-1; IR/Rep33-2; IR/Rep30-4; IR/Rep10-1; IR/Rep4-1 and IR/Rep2-1) had 2 intact insertions. The eight lines IR/Rep43-1; IR/Rep38-1; IR/Rep35-1; IR/Rep32-2; IR/Rep26-2; IR/Rep23-5; IR/Rep22-4 and IR/Rep15-1 had 1 intact T_{DNA} insertion. The line IR/Rep31-1 contains 4 intact insertions and IR/Rep34-2; IR/Rep29-1 contained only truncated T_{DNA} insertions.

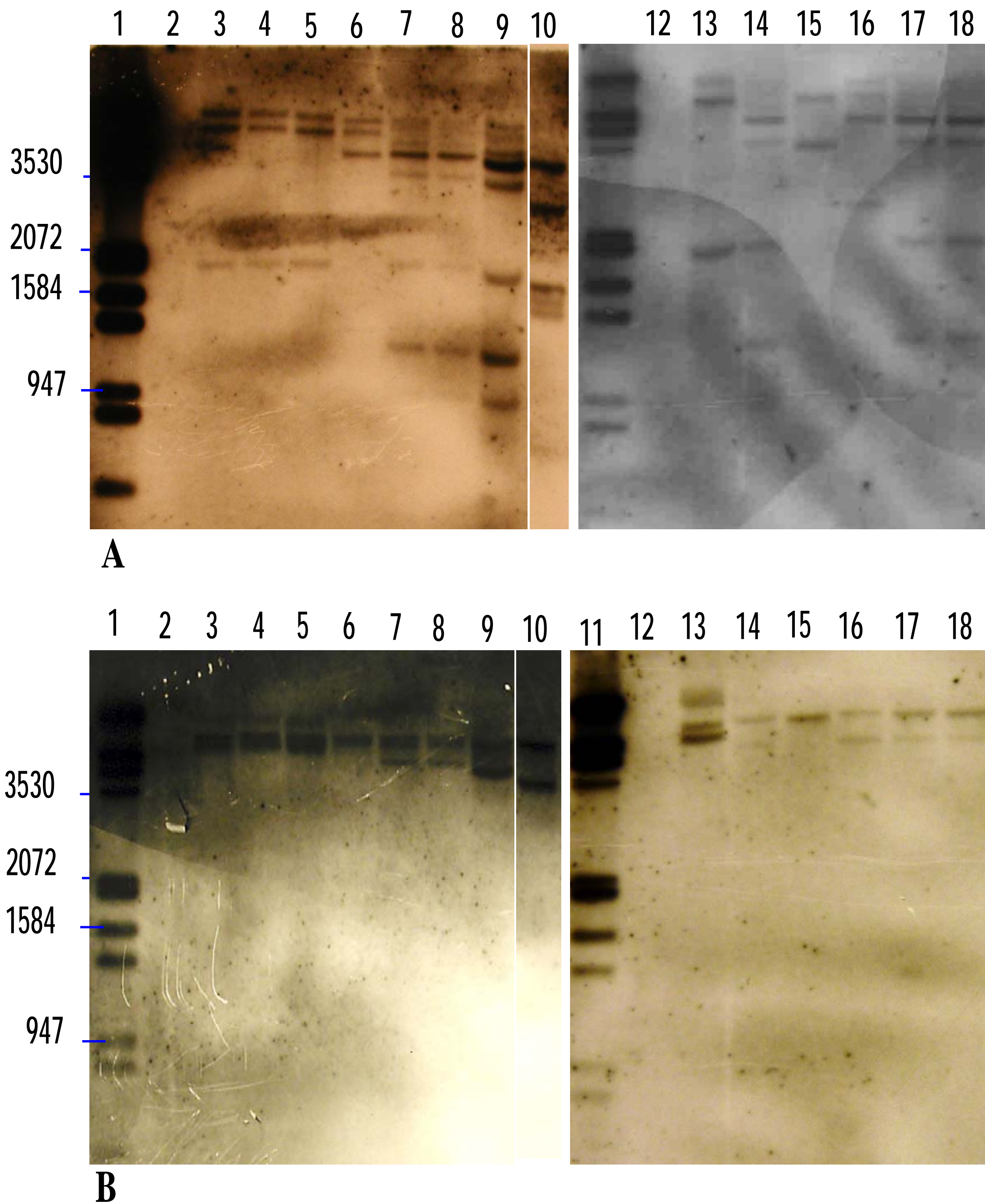

Figure 15: Hybridization of DNA from IR/Rep transgenic plants: **(A)** with *Rep* probe and (**B**) with *bar* probe. Lane 1: Marker III; Lane 2: Negative control (non-transformed plant); Lane 3: IR/Rep2-1; Lane 4: IR/Rep2-2; Lane 5: IR/Rep10-1; Lane 6: IR/Rep22-4; Lane 7: IR/Rep23-5; Lane 8: IR/Rep29-1; Lane 9: IR/Rep31-1; Lane 10: IR/Rep47-5; Lane 11: Marker III; Lane 12: Neg. control; Lane 13: IR/Rep4-1; Lane 14: IR/Rep15-1; Lane 15: IR/Rep26-2; Lane 16: IR/Rep30-4; Lane 17: IR/Rep33-2; Lane 18: IR/Rep38-1.

3.3.4 TYLCTHV resistance tests in T_1 plants transformed with the IR/Rep-hpRNA construct

3.3.4.1 Agroinoculation of *Nicotiana benthamiana* with TYLCTHV and TYLCVV

To confirm the infectivity of the plasmids carrying full-length TYLCTHV as well as TYLCVV before using agroinfiltration of transformed tomato plants, six *N. benthamiana* plants were agroinoculated. The results are shown in Table 9.

Table 9: Agroinoculation of *N.benthamiana*

Type of virus	Number of plants with virus symptoms (dpi)			
	10	15	20	25
TYLCTHV-A + B	6	6	6	6
TYLCTHV-A	0	1	5	6
TYLCVV-A	0	2	6	6

The suspension containing both A and B components was much more virulent than the A component alone. The plants exhibited symptoms in a very short time after inoculation. 10 days past inoculation 100% of the plants agroinoculated with the combination of A and B component of TYLCTHV showed yellowish colouring of young leaves- a specific symptom of TYLCVD. After 15 days, all young leaves in those plants were curled, yellow, and the shoots were stunted. After agroinoculation of the A component, only 1 out of 6 plants showed symptoms at 15 days past inoculation. The percentages reached up to 100% 25 days after inoculation. Similar results could be seen after inoculation with component A of TYLCVV. The symptoms started on the 15th day after inoculation and the infection rate reached 100% after 25 days (Table 9). However, a difference in the symptomatology could be observed: In comparison to the strong symptoms induced by inoculation with both viral components, plants agroinoculated only with the A component of TYLCTHV or TYLCVV

showed significantly more moderate symptoms. The plants displayed dark green crinkly leaves, were stunted, but show no yellowing (Figure 16).

Figure 16: Agroinoculation of TYLCTHV and TYLCVV in *N. benthamiana* plants. (a) Non-inoculated plants without symptoms; (b) Symptoms of TYLCTHV A+B component infected plants: curly leaves, yellow mosaic of the leaves and reduction of leaf; (c) curly leaves but no yellowing in plants infected with A component of TYLCTHV; (d) curly leaves but no yellowing in plants infected with A component of TYLCVV.

3.3.4.2 Agroinoculation of transgenic tomato plants with TYLCTHV

The first experiment for resistance evaluation through the IR/Re-hpRNA construct was carried out in the winter season. The temperatures in the greenhouse ranged from 23-28°C with 16 h light/8 h dark photoperiod. Six to ten plants of fifteen transgenic lines carrying the IR/Rep-hpRNA construct were agroinoculated with an infectious full-length clone of

the TYLCTHV (A and B component). In addition, non-transgenic tomato plants were agroinoculated as a control. Virus resistance was monitored by checking for morphological changes and appearance of viral symptoms (Table 10).

Table 10: Symptom development of transgenic IR/Rep-hpRNA tomato plants after agroinoculation with TYLCTHV

	Line No	Number of inoculated plants	Percentages of symptomatic plants (dpi)			
			21	30	40	70
1	IR/Rep2-1	10	0	0	0	0
2	IR/Rep4-1	10	0	0	60	60
3	IR/Rep10-1	9	0	0	33.3	33.3
4	IR/Rep15-1	8	0	37.5	37.5	37.5
5	IR/Rep16-1	8	50	62.5	75	75
6	IR/Rep23-5	10	0	20	40	40
7	IR/Rep26-2	10	10	20	50	60
8	IR/Rep29-1	10	20	30	50	60
9	IR/Rep30-4	10	20	30	50	50
10	IR/Rep31-1	10	10	30	50	50
11	IR/Rep33-2	5	20	40	40	60
12	IR/Rep34-2	8	0	12.5	50	50
13	IR/Rep38-1	6	0	16.7	37.5	37.5
14	IR/Rep43-1	9	11.1	22.2	55.6	55.6
15	IR/Rep47-5	10	70	80	90	90
	wt	10	70	90	90	90

Nearly all non-transgenic control plants became infected and showed typical yellow leaf curl symptoms and stunting. Based on the time symptoms appearance of and the number of symptomless plants, the TYLCTHV resistance level of transgenic plants was classified into four major categories.

The four lines IR/Rep 10-1, IR/Rep 15-1, IR/Rep 23-5 and IR/Rep 38-1 showed delayed symptoms as well as a lower percentage of symptomatic plants, the virus symptoms were

visible at 30-40 days after inoculation in comparison with 21 days past inoculation in the non-transgenic control plants. Seventy days past inoculation, these four lines revealed 33.3 – 37.5% plants with symptoms.

The eight lines numbered IR/Rep4-1, IR/Rep26-2, IR/Rep29-1, IR/Rep30-4, IR/Rep31-1, IR/Rep33-2, IR/Rep34-2, and IR/Rep43-1 were more susceptible to the virus as the symptoms appeared earlier. Symptoms become visible at the same time as in non-transgenic control plants (21 days after inoculation) and with a higher percentage of symptomatic plants at 70 days past inoculation, ranging from 50 to 60%.

The two lines IR/Rep16-1 and IR/Rep47-5 were nearly as susceptible to the virus as non-transgenic control plants.

One line - line IR/Rep2-1 displayed high level resistance to the virus. None of the ten inoculated plants of this line showed disease symptoms (Figure 17). This line was maintained until all fruits had ripened.

Figure 17: Resistance test of IR/Rep transgenic plants inoculated with TYLCTHV A+B component. Arrow (A) depicts plants of line IR/Rep 2-1 showing no symptoms; arrow (B) depticts non-transformed control plants with severe yellow leaf curl symptoms and stunting.

3.3.4.3 TYLCTHV detection by PCR

PCR was carried out only with plants of line IR/Rep2-1 and 6 asymptomous plants of line IR/Rep10-1 at 70 and 130 days past inoculation. Interestingly, no amplification product was obtained by using Reps and Repas primers (for A component), nor by using primers B-Ths and B-Thas (B-component), indicating that these plants were free of virus. Focusing on the line IR/Rep2-1, the experiment was repeated for 15 transgenic plants from the T_1-generation in the following spring season. The plants were grown in the same greenhouse but without control of temperature and photoperiod. Forty days past inoculation, mild symptoms of leaf curling was observed in one IR/Rep2-1 plant. The presence of viral DNA in this plant was shown by PCR. All other IR/Rep2-1 plants were free of disease symptoms and no viral DNA could be detected in these plants by PCR (see Table 11, Figure 18a and 18b). One symptomless plant out of the 16 non-transformed plants tested was found. In this non-symptom plant, the viral DNA was detected by PCR in the nearby inoculation place but not in the young leaves. All others plants displayed the specific symptoms of TYLCV and viral DNA were detected (Figure 18c).

Table 11: Symptom development of transgenic IR/Rep2-1 tomato plants after agroinoculation with TYLCTHV (second test).

Virus type	Plant type	Number of inoculated plants	Number of plants with virus symptom after inoculation (dpi)					PCR at dpi		
			21	30	40	70	90	30	70	90
TYLCTHV A+B component	IR/Rep 2-1	15	0	0	1	1	1	1	1	1
	wt	16	10	14	15	15	15	15	15	15

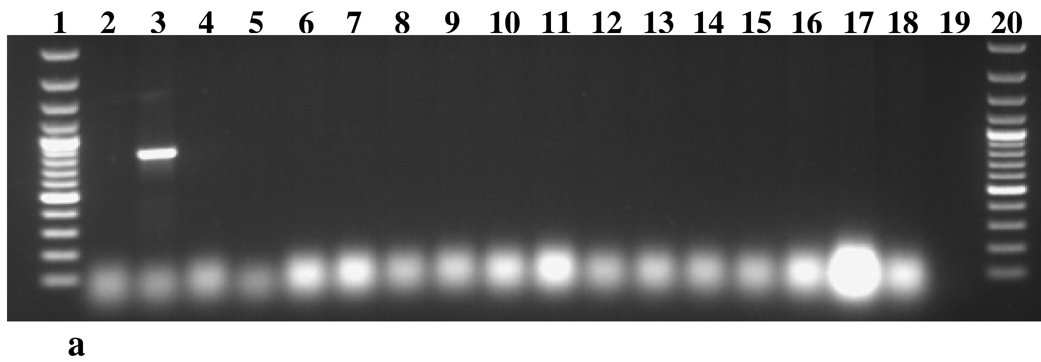

Figure 18a: PCR products amplified by Reps and Repas primers with DNA of transgenic plants of line IR/Rep2-1 (second test). Lane 1 and 20: DNA marker ladder 100bp (Fermentas); Lane 2 to 16: Plants of line IR/Rep2-1; Lane 17-18: Non-transformed non-infected plants; Lane 19: Water control.

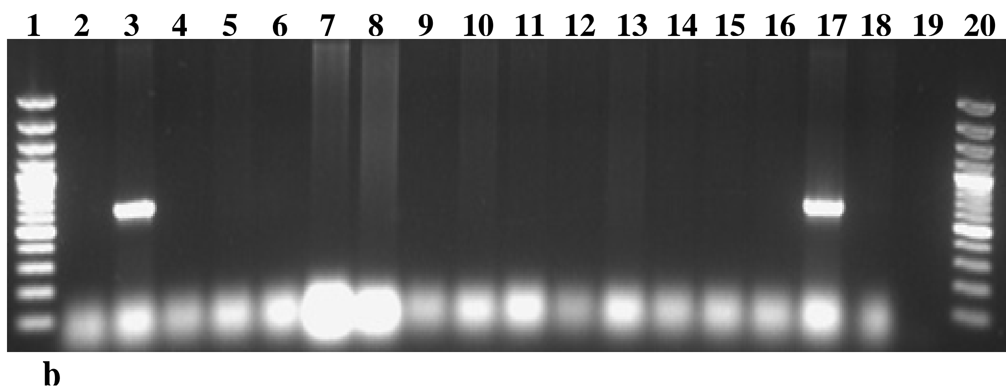

Figure 18b: PCR products amplified by B-Ths and B-Thas primers with genomic DNA of transgenic plants (second test). Lane 1 and 20: DNA marker ladder 100bp (Fermentas); Lane 2 to 16: plants of line IR/Rep2-1; Lane 17: non-transformation infected plant; Lane 18: non-transformed non-infected plant; Lane 19: Water control.

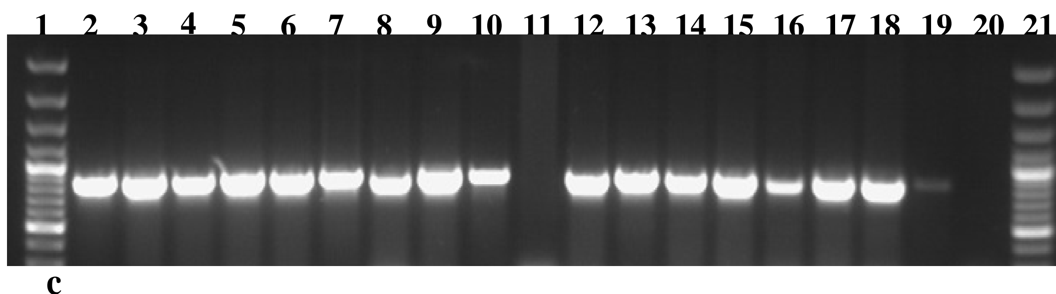

Figure 18c: PCR products amplified by Reps and Repas primers with DNA of non-transformed plants (second test). Lane 1 and 21: DNA marker ladder 100bp (Fermentas); Lane 2 to17: non-transformation infected plants; Lane 18: agrobacterium carried virus DNA; Lane 19: non-transformation non-infected plant; Lane 20: Water control.

3.3.4.3 Molecular characterization of transgenes in immunity plants by Southern hybridization

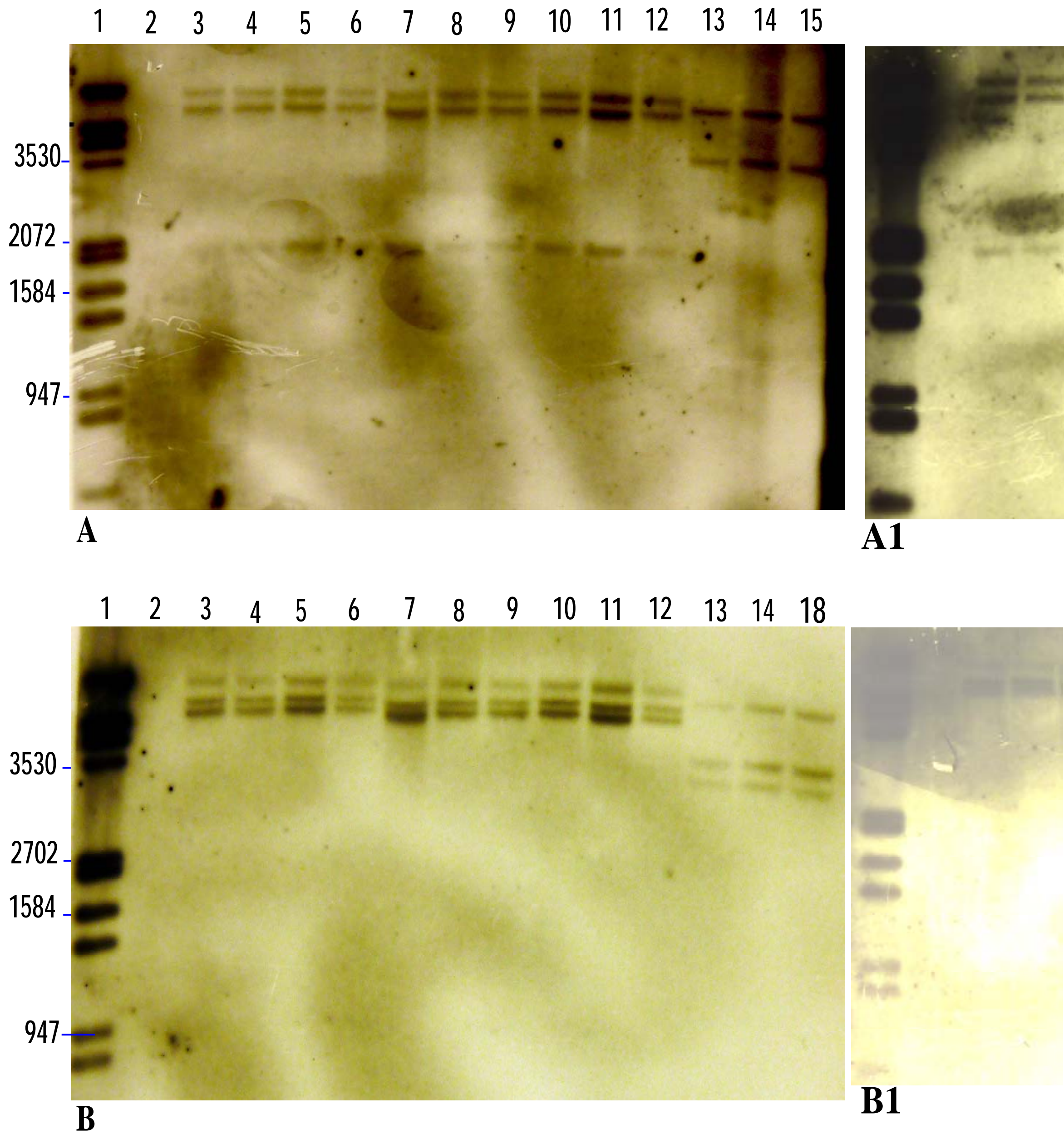

Figure 19: Hybridization of DNA from immunity IR/Rep2-1plants: (A) with *Rep* probe and (B) with *bar* probe. Lane 1: Marker III.
- *Hin*dIII cutter: Lane 2: Non-transformed plant; Lane 3: 2-1-3; Lane 4: 2-1-4; Lane 5: 2-1-5; Lane 6: 2-1-6; Lane 7: 2-1-7; Lane 8: 2-1-8; Lane 9: 2-1-10; Lane 10: 2-1-11; Lane 11: 2-1-13; Lane 12: 2-1-14
- *Afl*II cutter: Lane 13: 2-1-3; Lane 14: 2-1-4; Lane 15: 2-1-5.

Figure 19A shows a Southern hybridisation of DNA digested with *Hin*d III. Hybridization with the *rep*-probe indicated 3 T-DNA insertions. Digestion with *Afl*II revelead only 2 hybridizing bands. These results indicated that three IR/Rep insertions were located in 2 different positions of the plant genome. In combination with the hybridization result of the *bar*-probe (19B), all transgenic plants contain one truncated IR/Rep region and one truncated *bar*-region. These results are in agreement with the results of the Southern hybridization experiment of the T_0 plant (19A1, 19B1).

3.3.4.5 Agroinoculation of transgenic tomato plants with TYLCVV

Ten plants of the line IR/Rep2-1 as well as ten non-transformed plants were infected with TYLCVV by agroinfiltration. Symptom development was recorded up to 90 days after inoculation and PCR was performed to confirm viral accumulation 30, 70 and 90 days post-inoculation. The results are shown in Table 12.

Table 12: TYLCVV resistance assays in transgenic IR/Rep2-1 tomato plants

Virus type	Plant type	Number of inoculated plants	Number of symptomatic plants after inoculation (dpi)					PCR at dpi		
			21	30	40	70	90	30	70	90
TYLCVV A component	IR/Rep 2-1	10	0	0	0	0	0	0	0	0
	wt	10	1	1	6	1	1	6	10	10

Only one of the non-transformed plants presented typical symptoms of TYLCV infection at 21 days after inoculation. These symptoms remained until the end of the experiment. Five other plants showed very slight yellow colouring 35 days after of inoculation, but only for a very short time and then symptoms disappeared. Four other plants were totally symptomless. However, the viral DNA was detected in all ten plants (Figure 20b), while in

all 10 plants of the IR/Rep2-1 line symptoms were not observed nor viral DNA detected (Figure 20a).

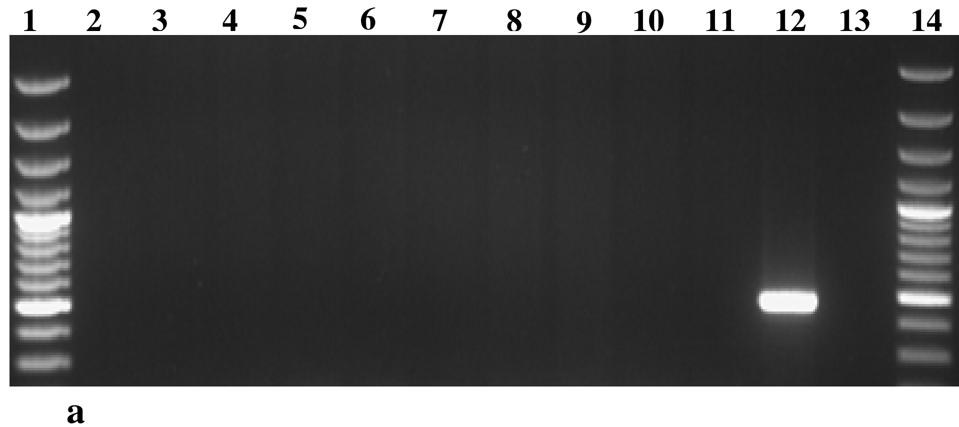

Figure 20a: PCR fragments amplified by Reps-VN/Repas-VN with DNA of transgenic plants. Lane 1 and 14: DNA marker ladder 100bp (Fermentas); Lane 2 to 11: Plants of line IR/Rep2-1; Lane 12: Non-transformed infected plant; Lane 13: Water control.

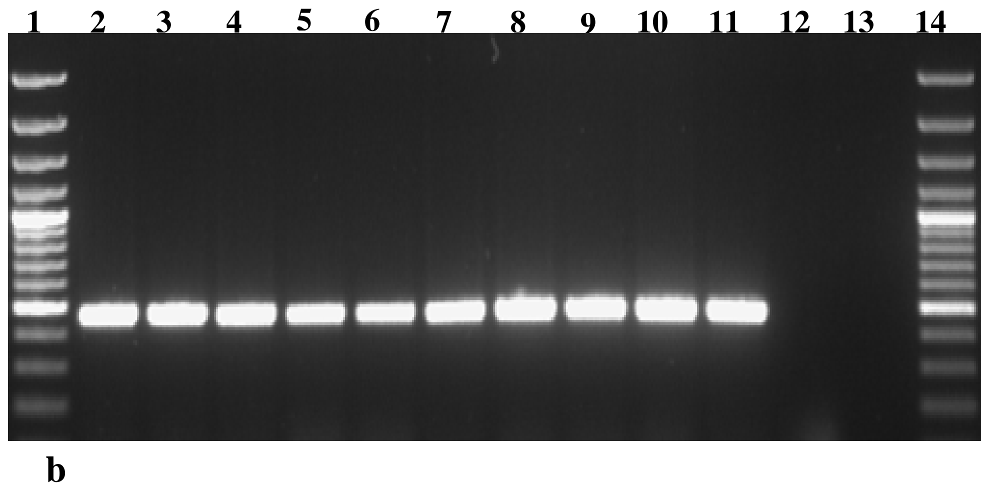

Figure 20b: PCR fragments amplified by Reps-VN/Repas-VN with DNA from non-transformed plants. From left to right: Lane 1 and 14: DNA Marker ladder 100bp (Fermentas); Lane 2 to 11: Non-transformed infected plant; Lane 12: Non-transformed non-infected plant; Lane 13: Water control.

3.4 Discussion

After delivery into plant cells, geminiviruses enter the replication cycle followed by DNA accumulation, assembly of particles, and spreading in the host. In most plant cell nuclei, geminiviruses replicate through the rolling circle replication (RCR) mechanism. Recently, geminiviruses have been shown to utilize two strategies, the RCR, as well as a recombination-dependent replication (RDR) (Jeske et al., 2001; Preiss and Jeske, 2003). For viral DNA replication, the "Replication-associated protein" is necessary. The N-terminus of the protein harbours activities for specific DNA binding, nicking, and joining, whereas the C-terminus is responsible for ATPase and helicase activity (Desbiez et al., 1995; Orozco et al., 1997; Orozco and Hanley-Bowdoin, 1998; Pant et al., 2001; Choudhury et al., 2006; Clerot and Bernardi, 2006). REP is a multifunctional protein fulfilling tasks of specific nicking and joining of DNA, autorepression of its own transcription, reprogramming the cell cycle to induce DNA-dependent DNA polymerase expression in differentiated cells, as well as ATP hydrolysis. All of these functions are an inevitable prerequisite for geminivirus replication. REP recognizes the origin by binding to a specific DNA sequence and catalyzes DNA cleavage and ligation and so begins and ends the rolling circle replication (Fontes et al., 1994b; Laufs et al., 1995b; Orozco and Hanley-Bowdoin, 1996; Orozco and Hanley-Bowdoin, 1998; Orozco et al., 1997). It also actively represses its own transcription in a virus-specific manner (Eagle et al., 1994; Gladfelter et al., 1997; Sunter et al., 1993; Eagle and Hanley-Bowdoin, 1997) and is reprogramming the cell cycle by interaction with a host derived protein to induce the expression of a host DNA synthesis protein, the PCNA, in non-dividing plant cells (see review Hanley-Bowdoin et al., 2004). REP binds to the viral replication enhancer protein, which in turn binds to PCNA, the processivity factor for DNA polymerase δ. It also interacts with components of the host replication apparatus, like PCNA and the replication factor C complex, the clamp loader that transfers PCNA to the replication fork. These interactions are likely to represent early steps in the assembly of a DNA replication complex of the geminivirus origin (Luque et al., 2002; Castillo et al., 2003; Settlage et al., 2005; Selth et al., 2005; Morilla et al., 2006).

Because the REP is involved in many different pathways, it has been mostly employed in different strategies to confer geminivirus resistance. A number of reports achieved virus

resistance by expressing either the truncated REP ACMV (Hong and Stanley, 1996; Sangare et al., 1999); TYLCVs (Noris et al., 1996b; Brunetti et al., 1997; Lucioli et al., 2003; Antignus et al., 2004; Chatterji et al., 2001) and BGMV (Hanson and Maxwel, 1999). Alternatively, full-length of *Rep* was used for transformation (Hong and Stanley, 1996; Shivaprasad et al., 2006). However, in all of the publications the typical effect seems to be tolerance rather than immunity. Expression of REP (full-length, truncated or mutant) resulted in only reducing viral accumulation in infected tissue and in symptom attenuation. Only Antignus et al. (2004) reported three lines that seemed to be immune to the virus. When using whitefly inoculation, the plants did not show disease symptoms and viral DNA was detected by dot-hybridization. However, those lines became susceptible to virus by agroinoculation.

Different results were obtained when the *Rep* gene was used to engineer resistance against begomovirus based on a RNA-mediated resistance pathway. So far there are only few reports of successful begomoviruses resistance development using the *Rep* sequence (Asad et al., 2003; Yang et al., 2004; Ramesh et al., 2007; Fuentes et al., 2006; Bonfim et al., 2007). In most cases, the plants could be immunized against the viruses. Asad et al. (2003) showed that the resistant tobacco plants neither developed symptoms nor contained detectable amounts of DNA of CLCuV. Yang et al. (2004) tested eight different *Rep* constructs of an isolate of TYLCV from Florida (TYLCV-[FL]). No symptoms were observed and no TYLCV-DNA was detected by PCR or hybridization in resistant plants. Fuentes et al. (2006) demonstrated immunity to TYLCV in tomato plants transformed with a cassette consisting of 726 nts of the 3′-end of the *Rep* gene (sense and anti-sense orientation) functioning as arms of the hairpin. Young plants (four-leaf stage), were exposed to hundreds of viruliferous whiteflies for 60 days. Afterwards, no TYLCV DNA could be detected in these plants. Bonfim et al. (2007) achieved one line which seemed to be immune. In this line, a semiquantitative polymerase chain reaction analysis revealed the presence of viral DNA in transgenic plants exposed to viruliferous whiteflies for a period of 6 days, and when insects were removed, no viral DNA could be detected after an additional 6 days.

The results of the present study are consistent with the previous research. Out of 15 independent transgenic lines transformed with an IR/Rep-hpRNA construct, at least two lines were observed to be resistant to TYLCTHV. All plants from the line IR/Rep2-1, and

6 of 9 plants from IR/Rep10-1 showed no disease symptoms 10 weeks after groinoculation and viral DNA was not detected by PCR. IR/Rep2-1 plants were maintained until fruits ripened (130 days). Even after this long time, no indication of disease could be found. Focussing on this particular line, virus inoculation was repeated. Only one plant out of 15 showed symptoms 3 weeks after inoculation. All other plants were healthy, showing no symptoms as well as no viral DNA detected by PCR.

Interestingly, the line IR/Rep2-1 also showed resistance to a heterologous virus, TYLCVV. Although the non-transformed plants did not present high incidence of the disease, the PCR results showed the presence of viral DNA in all of them. However, neither the presence of symptoms nor of viral DNA in all IR/Rep2-1 plants was detectable through the whole growth period until fruits were ripened.

Different studies show that transgenic plants expressing the viral REP are able to resist only a specific virus. For example, none of transgenic plants of Hong and Stanley (1996) were resistant to the distantly related viruses TGMV and *Beet curly top virus* (BCTV). Similarly, the expression of a truncated TYLCSV REP interfered with cognate viral infection in transgenic plants (Noris et al., 1996b; Brunetti et al., 1997), but it did not protect against the closely related virus strain, TYLCSV-ES, and the closely related species, *Tomato leaf curl virus* (ToLCV-Au), (Brunetti et al., 1997). Similarly, a truncated *Rep* gene from the *Tomato yellow leaf curl virus-Israel* (TYLCV-Is) mild strain conferred resistance in transgenic tomato to the cognate strain but not to the TYLCV-Is severe strain (Antignus et al., 2004). However, the broad-spectrum resistance to begomoviruses viruses associated with transgene-induced gene silencing has been also reported by different authors. Abhary et al. (2006) designed a chimera intron-hairpin to generate resistance to TYLCV as well as other strains and monopartite begomoviruses. They achieved transformed plants resistant to TYLCV, TYLCV-Mld and TYLCSV-ES by whitefly inoculation and TYLCSV-(Sar) by agroinfiltration. In research of Chellappan et al. (2004a), the transgenic plants resistant to ACMV were also challenged with isolates of *East African cassava mosaic Cameroon virus* (EACMCV) and *Sri Lankan cassava mosaic virus* (SLCMV). Those results, as well as our results, are interesting because in principle RNA-mediated resistance is homology-dependent thus it usually results in narrow-resistance.

CHAPTER 4

Inverted-repeat hairpinRNA derived from a truncated pre-coat/coat-protein gene of TYLCTHV confers resistance in transgenic tomato plants

4.1 Introduction

Since the first demonstration that a virus coat protein expressed in plants provides some level of resistance (Abel et al., 1986), pathogen-derived resistance has been applied to RNA viruses (Beachy, 1990; Lomonossoff, 1995). Various transgenic plants that accumulate a viral coat protein acquire resistance against cognate viruses, for instance, tobacco plant resistance to *Tobacco mosaic virus* (TMV; Abel et al., 1986), *Cucumber mosaic virus* (CMV; Cuozzo et al., 1988) and *Potato virus Y* (PVY; Hemenway et al., 1988). The sense CP gene had mainly been used as a transgene conferring resistance until the mechanism of viral resistance via RNA silencing in transgenic plants was demonstrated. The resistance mechanism is through initiation of RNA silencing via an accidental formation of dsRNA or over-expression of aberrant RNA. The introduction of inverted-repeat viral genomic sequences expressed as hairpin dsRNA in host plants is an efficient method for inducing RNA silencing and conferring viral resistance (Waterhouse et al., 1998). The strategy of expressing a gene encoding intron-spliced RNA can induce PTGS with almost 100% efficiency (Smith et al., 2000). Previous studies have shown that resistance can be acquired in many plants, for instance, tobacco resistant to PVY (Smith et al., 2000) and CMV (Kalantidis et al., 2002), barley resistant to *Barley yellow dwarf virus* (BYDV; Wang et al., 2000), potato resistant to PVY (Missiou et al., 2004) and soybean resistant to *Soybean dwarf virus* (SbDV; Tongou et al., 2006) and to *Soybean mosaic virus* (SMV, Furutani et al., 2007). Zrachya et al. (2007b) designed intron-hairpin RNA constructs in order to analyze their effects on the accumulation of the only known part of the TYLCV virus capsid, the coat protein (CP). The siRNAs derived from them targeted the V1 gene product. A co-agroinfiltration with a GFP-CP fusion construct showed a

down-regulation of GFP in tobacco. In one of the tomato varieties (cv. “Micro-Tom”), an inhibiting affect of the ihpRNA construct on CP production and subsequently on the disease symptoms could be observed. Whereas non-transgenic control plants were symptomatic 2 weeks post inoculation, the transgenic tomato plants needed 7 weeks to exhibit symptoms.

In this study, plants were transformed with an intron-hairpin RNA construct derived from the precoat/coat protein region of TYLCTHV. The transformed plants confer tolerance to the homologous virus up to 120 days past inoculation by agroinfiltration under greenhouse conditions.

4.2 Materials and methods

All methods for transformation as well as the detection of transgenic plants and TYLCTHV were carried out essentially as described in chapter 3, except for the hairpin RNAi construct, which was derived from the pre-coat/coat protein region of TYLCTHV (see description below). Therefore, the primers used for detection of the transgene were different (Table 13).

Table 13: Primers used for detection of TYLCTHV T_{DNA} integration

Name	Primer sequence	Length of fragment (bp)
Pre/Cps	5´- AAG GCG CGC CAC GCG TTA ACT AAC TAA GAG AAG ACG TAT TCC CCT GA- 3´	595
Pre/Cpas	5´-AAG GAT CCT CTA GAA CCT GCT GAA AAT CAT AAG G-3´	

4.2.1 RNAi construct

A physical map of the RNAi construct designed by Blawid (2008) is shown in figure 21. The precoat/coat protein intron-hairpin RNA construct (Pre/Cp-hpRNA) contains 540 nts

(225 to 765 of GenBank accession no. DQ871222). The region includes a 3´-part of the AV2 gene and a 5'-part of the AV1 gene.

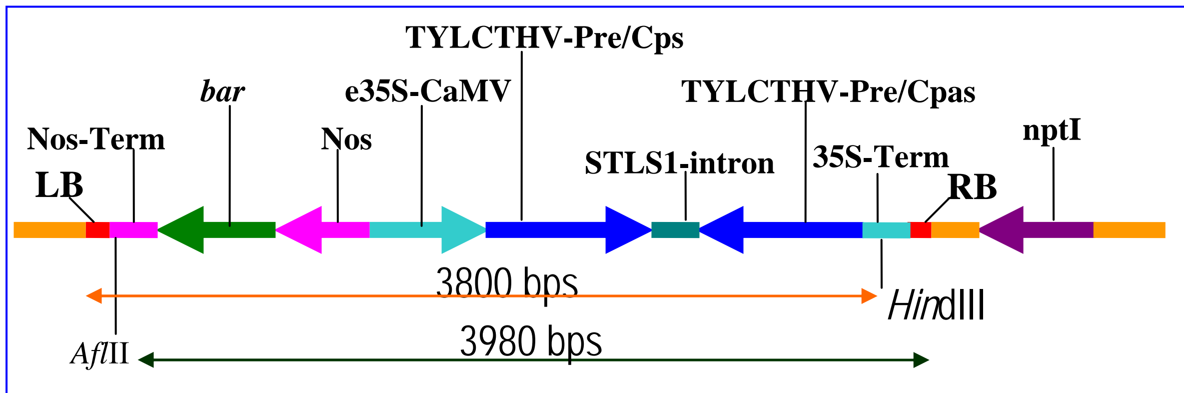

Figure 21: Physical map of hairpin-RNAi constructs. Inverted-repeat transgene derived from precoat and coat protein region of TYLCTHV. LB-left border, *nos*-terminator, *bar* gene, nos-promotor; 2x35S CaMV promoter (enhanced 35S promotor), Pre/Cp_sense, STLS1 intron derived from potato, Pre/Cp_antisense, CaMV-terminator; RB-Right border; *nptI* is located in the backbone sequence.

4.2.2 Evaluation of virus resistance in transgenic tomato

Agroinoculation was carried out according to the protocols described in chapter 3. The first step of virus resistance screening was done by observating of disease symptoms. Plants were screened for the presence or absence of TYLCD symptoms.

The T_1 tomato plants were screened for disease symptoms beginning 3 weeks after inoculation and continually until seed collection. The incidence of disease was evaluated as the percentage of plants exhibiting symptoms. Lines showing no symptoms were subjected to PCR and ELISA tests to detect TYLCTHV infection. Based on the results of PCR screening, virus positive transgenic lines without symptoms were considered tolerant. Transgenic lines free of symptoms and with negative PCR results were considered immune.

4.2.3 Triple antibody sandwich (TAS) ELISA for detection of TYLCV

Buffers:

Extraction buffer: 0.05 M Tris-HCl, 0.06 M Na_2SO_3, pH was adjusted by HCl to 8,5.

Carbonate coating buffer (pH 9.6): 15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 3 mM NaN_3, the solution was autoclaved and stored at 4°C.

10×phosphate buffered saline (PBS, pH 7.4): 1,4 M NaCl, 15 mM KH_2PO_4, 80 mM Na_2HPO_4, 27 mM KCl , 30 mM NaN_3 (pH was adjusted by NaOH or HCl, the solution was autoclaved and stored at room temperature.

Phosphate buffered saline-Tween (PBS-T) pH 7.4: 100 ml 10 × PBS, 0.5 ml Tween 20, 900 ml water; pH was adjusted once more and the solution was stored at room temperature.

Antibody buffer (PBS-TPO): 5 g PVP was dissolved in 250 ml PBS-T buffer, pH was controlled at 7.4 and the solution stored at 4°C.

Blocking solution: 2 g skim milk in 100 ml of PBS-T buffer.

Substrate buffer (diethanolamine buffer): 1 M Diethanolamine, adjust pH 9.8 with concentrated HCl; 5 mM $MgCl_2$. The solution was stored at 4°C.

Sample preparation

Newly expanded young leaves present in the uppermost parts of the plants were collected, carefully avoiding cross-contamination by punching a piece of leaf directly into a microcentrifuge tube, using the lid as a cutting instrument. Five leaf discs were stamped out using one microcentrifuge tube for each sample. The samples were immediately incubated on ice. Protein extraction and preparation was carried out. ELISA (enzyme-linked immunosorbent assay) was used for the determination of TYLCV-capsid protein according to the protocol below:

The crude IgG (DSMZ AS-0588) antibody was diluted 1:1000 in the coating buffer. The microtitre plates were coated by pipetting 100 µl of the solution into each well. The microtitre plate was covered by a plastic bag and incubated for 2-4 hours at 37°C. Afterwards the solution was discarded and the wells were washed three times with PBS-Tween, with 3 minute soaks between washes. The wells were dried before blocking them with 100 µl 2% skim milk per well and incubating the plates at 37°C for 30 min. The microtitre plates were washed three more times with PBS-Tween, as described above. The

samples were centrifuged for 90 sec at 13.000 rpm at room temperature. Each well was loaded with 100 µl of the supernatant. The plates were covered by a plastic bag and incubated at 4°C overnight. Another washing step with PBS-Tween followed. Then 100 µl of the monoclonal antibody (AS-0546/2 at 1:1000) was loaded into each well. The plates were covered by a plastic bag and incubated at 37°C for 2-4 hours. Again, a washing step was done. 100 µl of the rat-anti-mouse antibody coupled with alkaline phosphatase (RaM-ap at 1:1000) was loaded into each well. The plates were covered and incubated at 37°C for 2 hours. The substrate buffer was prepared; 1 mg of p-nitrophenyl phosphate (LOEWE-Biochemical) was added to 1 ml of substrate buffer. The microtitre plates were washed with PBS-Tween as before. 100µl of substrate solution was added into each well and incubated at room temperature. 1.5 h after the addition of the substrate p- nitrophenyl phosphate in 9.5% diethanolamine (Roth) buffer (pH 9.8), the absorbance at OD_{415nm} was measured on automated microplate reader BiO-RAD 550.

Plants were considered as TYLCV infected when the corresponding OD_{415nm} values were at least the double the control values obtained from material of healthy non-inoculated plants.

4.3 Results

4.3.1 Results of transformation

The transformed *Solanum lycopersicum* var. FM372C plants were identified by PCR using a specific primer pair, amplifying a fragment of $T_{DNA,}$ containing the RNAi construct (Figure 21, Table 7 and 13). Successfully transformed plants, confirmed by PCR were transferred to the greenhouse to produce To seeds. The plants which were able to produce seeds were further tested for the copy number of transgene insertions by Southern hybridization (see chapter 3).

4.3.1.1 Confirmation of successful transformation via PCR

The plasmid containing inverted repeat T_{DNA} was taken as a positive control; the DNA samples of non-transformed plants (wt) were used as a negative control. The fragments amplified using *bar* primers were visible between 400-500 bps; the size of fragments amplified using *cp* primers (Pre/Cps and Pre/Cpas) was between 500-600 bps. There were

no bands visible in wild type (non transformed plants; negative control) and in the water control. All fragments amplified from a DNA template of putative transgenic plants had the same size as the positive control (Figure 22a, 22b).

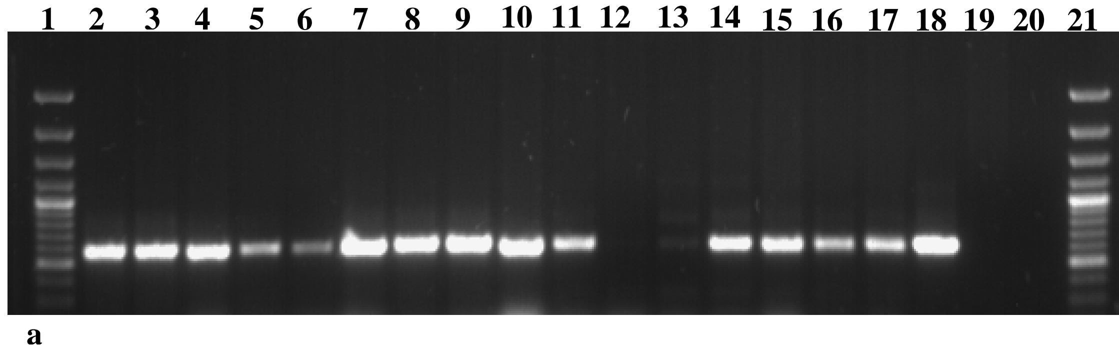

Figure 22a: PCR results amplified by Pre/Cps and Pre/Cpas from plants transformed with Pre/Cp-hpRNA construct. Lane 1 and 21: DNA marker ladder 100bp (Fermentas). Lane 2: Pre/Cp2-1; Lane 3: Pre/Cp2-2; Lane 4: Pre/Cp3-1; Lane 5: Pre/Cp3-2; Lane 6: Pre/Cp4-1; Lane 7: Pre/Cp4-2; Lane 8: Pre/Cp6-1; Lane 9: Pre/Cp6-2; Lane 10: Pre/Cp8-1; Lane 11: Pre/Cp8-2; Lane 12: Pre/Cp8-3; Lane 13: Pre/Cp8-4; Lane 14: Pre/Cp11-1; Lane 15: Pre/Cp11-2; Lane 16: Pre/Cp11-3; Lane 17: Pre/Cp14-1; Lane 18: Possitive control (Plasmid DNA); Lane 19: Negative control (non-transformed plant); Lane 20: Water control.

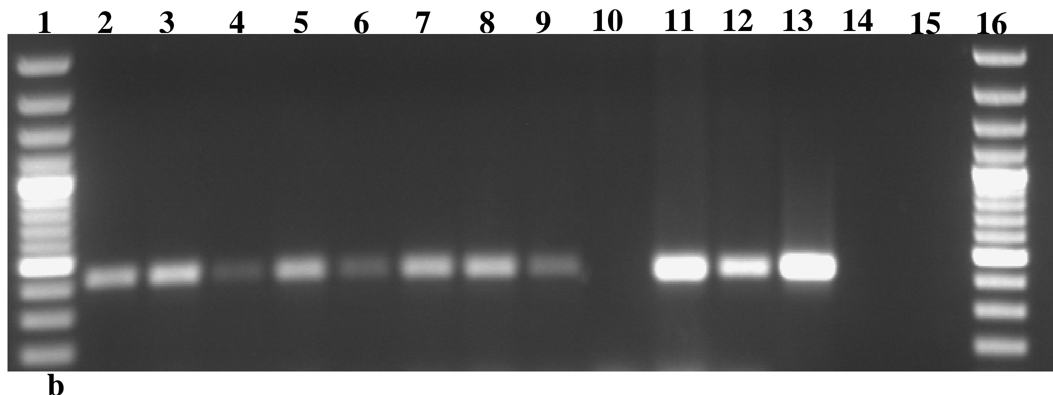

Figure 22b: PCR produce amplified by bar-primers of plants transformed with Pre/CP-hpRNA construct. Lane 1 and 16: DNA marker ladder 100bp (Fermentas). Lane 2: Pre/Cp 3-1; Lane 3: Pre/Cp 3-2; Lane 4:Pre/Cp 4-1; Lane 5: Pre/Cp 4-2; Lane 6: Pre/Cp 6-1; Lane 7: Pre/Cp 6-2; Lane 8: Pre/Cp 8-1; Lane 9: Pre/Cp 8-2; Lane 10: Pre/Cp 8-3; Lane 11: Pre/Cp 8-4; Lane 12: Pre/Cp 11-1; Lane 13: Positive control (Plasmid DNA); Lane 14: Negative control (Non-transformed plant); Lane 15: Water control.

Ninety one plants obtained from 410 leaf pieces were transformed with *Agrobacterium* and showed positive PCR. These plants were originally derived from 37 calluses (original from 37 inoculated leaf pieces), thus the transformation frequency of this construct is 9.8%.

4.3.1.2 To seed production

T_0 plants containing *cp*-TYLCV hairpin-transgene were transferred to the greenhouse. Fifteen plants showed abnormal form, they did not produce fruit or fruited with low seed production. Seventy six plants grew like the non-transformed plants. Among of these plants, fifteen plants died in a latter development stage because of a fungal disease. However, young fruits grown on some of these plants produced a sufficient amount of seeds. Finally, 61 plants (derived from 33 calluses) were able to produce fruits, and subsequently, also seeds. The frequency of plants that produced seeds was 83.5%.

4.3.1.3 Detection of transgene copy number by Southern Blot analyses

DNA samples of To plants which produced seeds, were hybridised with a DIG-labelled probe of TYLCTHV *cp* as well as with a probe of the *bar* gene for identification of the copy number of T_{DNA} insertions. The results are shown in Table 14a and Figure 23.

The results of Southern hybridisation (Table 14a) showed that 5 of 58 plants failed to hybridize with the *cp* probe as well as with the *bar* probe. Selection in the rooting stage was only done once using 1.5 ppm of glufosinate. Thus, it is possible that those plants were either chimeras or escapes of the non-transformed form. There was one plant (31-2) which only showed a hybridization band with the *bar* probe, and accordingly it contained a truncated T_{DNA} insertion.

Identification of independent transformed lines was based on the copy number and the size of hybridization signals, as well as the original explants that transformed plants derived from. If the plants that were regenerated from the same inoculated explant event showed the same size and number of insertions, therefore they should be adopted in the one transformed line.

Table 14a: Results of Southern hybridization with c*p*-TYLCTH and *bar* probe

Plant No.	Number of signals hybridising with		Independent line	Plant No.	Number of signals hybridising with		Independent line
	cp probe	*bar* probe			*cp* probe	*bar* probe	
2-1	1	1	1	25-1	1	nd*	1
3-1	1	1	1	25-2	1	nd*	
4-1	1	1	1	29-1	2	1	1
4-2	1	1		29-2	2	1	1
5-5	1	1	1	32-1	2	1	1
6-1	1	1	1	32-4	2	1	
6-2	1	1		33-1	2	1	1
7-2	1	1	1	33-2	2	1	
8-1	1	1	1	33-3	2	1	
8-2	1	1		33-4	2	1	
10-2	1	1	1	34-1	2	1	1
10-4	1	1		34-2	3	1	1
11-1	1	1	1	38-1	2	1	1
11-2	1	1		38-2	3	1	1
11-3	1	1		40-1	3	1	1
14-1	2	1	1	40-2	3	1	
14-2	2	1		44-1	2	1	1
14-3	2	1		45-1	2	1	1
15-2	2	1	1	45-3	2	1	
15-3	2	1		48-1	1	1	1
15-4	2	1		48-2	1	1	
17-1	1	nd*	1	48-3	1	1	
18	2	1	1	48-4	1	1	
19	2	1	1	50-3	2	1	1
20	0	0		50-4	2	1	
21-1	0	0		26-1	0	0	
21-2	1	1	1	26-2	0	0	
23-1	1	nd*	1	31-2	0	1	1
30	2	1	1	31-3	0	0	0
wt	0	0	0				

nd*: Not determinded

Most of the inoculated explants led to only 1 transformed line. However, there were three inoculated explants that produced 2 different transformed lines. Explant 29 produced 2 surviving shoots and both of them had 2 copies of insertion but the size of insertion was different. Explant 34 and 38 produced transformed shoots with different copy numbers of insertion (34-1 carried 2 copies, 34-2 carried 3 copies; 38-1 had 2 and 38-2 had 3 copies) thus they are different independent lines. In summary, there were 32 independent lines regenerated from transformation with the Pre/Cp-hpRNA construct.

The number of insertions ranged from 1 to 3. Single insertions were observed in 34.4 % of the plants (11 of 32 independent lines, of them one line, 31-2, contained single insertion of only bar gene), 2 bands could be observed in 15 of 32 plants (46.9%), and three insertions were found in 3 plants (9.34%). The results for three lines (17, 23 and 25; equal 9.4%) were not conclusive, because there was one very weak band hybridising with the Pre/Cp probe and the hybridisation with *bar* probe was not performed.

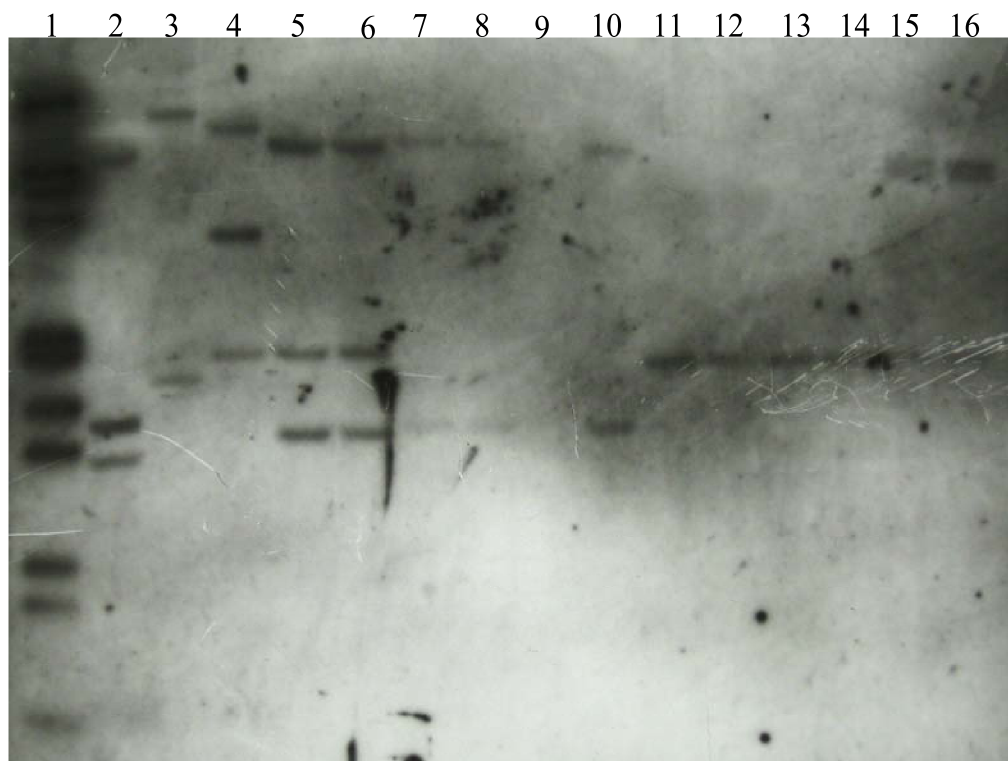

Figure 23A: Southern hybidization of *cp* probe. Lane 1- Marker III; Lane 2: Pre/Cp34-2; Lane 3: Pre/Cp38-1; Lane 4: Pre/Cp38-2; Lane 5: Pre/Cp40-1; Lane 6: Pre/Cp40-2; Lane 7: Pre/Cp44-1; Lane 8: Pre/Cp45-1; Lane 9: Pre/Cp45-2; Lane 10: Pre/Cp45-3; Lane 11: Pre/Cp48-1; Lane 11: Pre/Cp48-2; Lane 11: Pre/Cp48-3; Lane 14: Pre/Cp48-4; Lane 15: Pre/Cp50-3; Lane 16: Pre/Cp50-4.

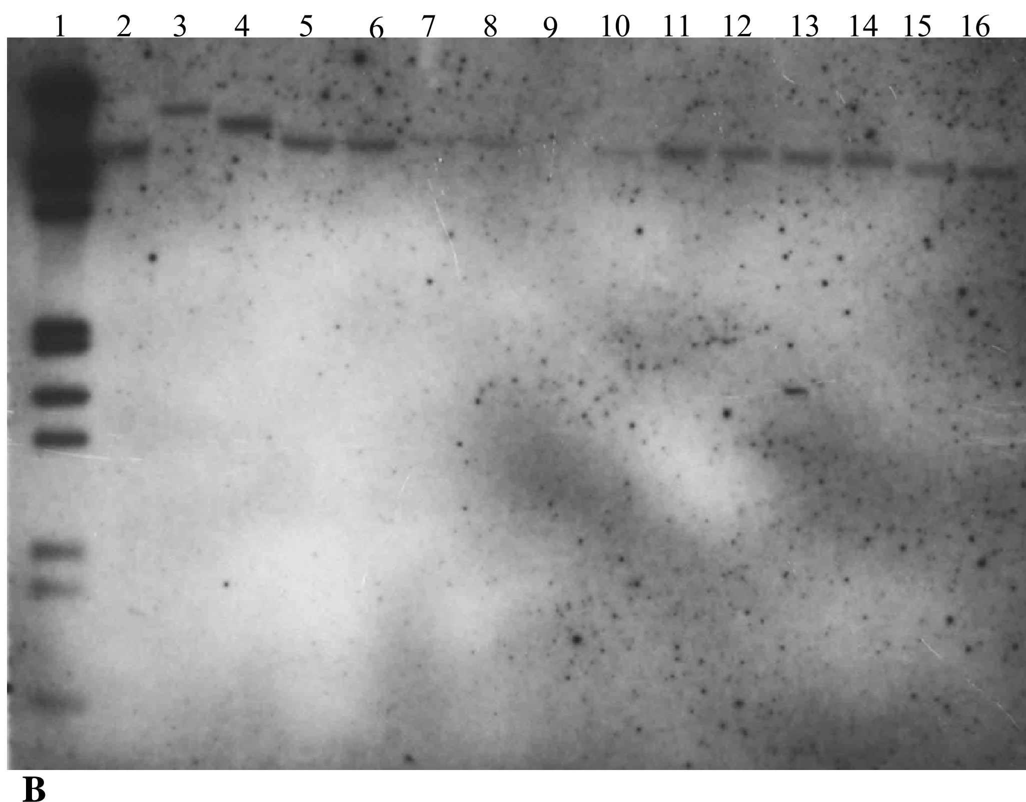

Figure 23B: Southern hybidization of *bar* probe. Lane 1- Marker III; Lane 2: Pre/Cp34-2; Lane 3: Pre/Cp38-1; Lane 4: Pre/Cp38-2; Lane 5: Pre/Cp40-1; Lane 6: Pre/Cp40-2; Lane 7: Pre/Cp44-1; Lane 8: Pre/Cp45-1; Lane 9: Pre/Cp45-2; Lane 10: Pre/Cp45-3; Lane 11: Pre/Cp48-1; Lane 11: Pre/Cp48-2; Lane 11: Pre/Cp48-3; Lane 14: Pre/Cp48-4; Lane 15: Pre/Cp50-3; Lane 16: Pre/Cp50-4.

The results showed that all of the transformed plants presented only one hybidisation signal with the *bar* probe. While in hybidisation with the *cp* probe*,* many plants contained more than one band. These results indicated that there were also truncated forms of T_{DNA} which do not contain the *bar* gene. Furthermore, table 14b shows that the line Pre/Cp48- and Pre/Cp21-2 has one hybridizing band with the *cp* probe as well as one hybridizing band with the *bar* probe but they were in different size. In these plants, the *cp*-hybridizing band was around 2000 bps, while the hybridization with the *bar* probe was presented in a band larger than 5000 bps. It is likely that 2 different truncated T_{DNAs} were inserted into the plant. The first one is probably a piece of T_{DNA} containing only the Pre/Cp fragment; the second is a truncated insertion with only the left part of the $T_{DNA,}$ which contains only the

bar-fragment. Both T_{DNAs} were integrated into the plant genome at different loci. One other case, the line Pre/Cp31-2, had only ome hybridising signal with the *bar* probe. Thus this insertion contains only the left region of the T_{DNA}. Generally, there were 20 out of 32 (62.5%) independent lines containing truncated insertions. 19 of them had truncated insertions of the Pre/Cp region, and 3 lines had a truncated *bar* gene (Table 14b).

Table 14b: Size of hybridising signals with *cp*-TYLCTH and *bar* probe

Line No.	Hybridising with *cp* probe			Hybridising with *bar* probe		
	>5kb	>3.5-4.9kb	<3.5kb	>5kb	>3.5-4.9kb	<3.5kb
Pre/Cp-14-2	**1**	0	**1**	**1**	0	0
Pre/Cp 15-4	**1**	0	**1**	**1**	0	0
Pre/Cp 18	**1**	0	**1**	**1**	0	0
Pre/Cp 19	**1**	0	**1**	**1**	0	0
Pre/Cp 21-2	0	0	**1**	**1**	0	0
Pre/Cp 30	0	**1**	**1**	0	**1**	0
Pre/Cp 29-1	**1**	**1**	0	**1**	0	0
Pre/Cp 29-2	**1**	0	**1**	**1**	0	0
Pre/Cp 31-2	0	0	0	**1**	0	0
Pre/Cp 32-1	**1**	0	**1**	**1**	0	0
Pre/Cp 33-3	**1**	0	**1**	**1**	0	0
Pre/Cp 34-1	**1**	0	**1**	**1**	0	0
Pre/Cp 34-2	**1**	0	**2**	**1**	0	0
Pre/Cp 38-1	**1**	0	**1**	**1**	0	0
Pre/Cp 38-2	**1**	0	**2**	**1**	0	0
Pre/Cp 40-2	**1**	0	**2**	**1**	0	0
Pre/Cp 44-1	**1**	0	**1**	**1**	0	0
Pre/Cp 45-1	**1**	0	**1**	**1**	0	0
Pre/Cp 48-1	0	0	**1**	**1**	0	0
Pre/Cp 50-3	**1**	**1**	0	**1**	0	0

4.3.2 Evaluation of TYLCTHV and TYLCVV resistance

4.3.2.1 Resistance tests for *Tomato yellow leaf curl Thailand virus*

Transgenic (18 lines) and non-transgenic tomato plants were agroinoculated with an infective full-length clone of TYLCTHV including both the A and B components (Figure 24a). All non-transformed plants exhibited severe symptoms of TYLCD. Typical yellowing and curling of young leaves appeared about 3-4 weeks after agroinoculation (Figure 24b).

Figure 24a: Overview of agroinfiltration experiment with transformed plants from Pre/Cp-hpRNA construct.

Figure 24b: 4 weeks after agroinoculation of TYLCTHV in transformed plants of Pre/Cp-hpRNA construct.

Virus resistance evaluation result obtained by observing morphological changes and appearance of symptoms are given in Table 15.

Table 15: Symptoms of TYLCTHV in T_1 plants transformed with the Pre/Cp-hpRNA construct.

Line No.	Number of inoculated plants	Percentages of symptomatic plants after inoculation (dpi)					
		20	26	34	42	55	120
Pre/Cp2-1	7	28.6	71.4	100	100	100	
Pre/Cp3-1	10	70	90	100	100	100	
Pre/Cp4-1	8	50	87.5	100	100	100	
Pre/Cp5-1	8	37.5	100	100	100	100	
Pre/Cp6-1	5	40	60	60	100	100	
Pre/Cp7-2	7	28.6	57.1	71.4	71.4	100	
Pre/Cp8-1	10	10	40	50	60	70	
Pre/Cp10-2	10	10	10	10	10	10	10
Pre/Cp14-2	10	20	40	60	80	100	
Pre/Cp15-4	10	10	40	40	60	80	
Pre/Cp17-1	10	50	70	90	90	100	
Pre/Cp23-1	6	0	0	0	0	0	0
Pre/Cp29-1	8	37.5	50	87.5	100	100	
Pre/Cp45-3	3	66.7	100	100	100	100	
Pre/Cp30	4	0	0	0	0	0	50
Pre/Cp32-1	4	0	0	0	0	0	0
Pre/Cp38-1	10	10	70	70	70	70	
Pre/Cp40-2	4	25	25	50	100	100	
wt	10	70	100	100	100	100	

Disease symptoms appeared in almost all plants 3 to 5 weeks after inoculation. 66% of the non-transformed plants were showed symptoms of yellowing in the young leaves followed by curling. Apical shoots of plants were stunted. In non-transformed plants, the virus incidence reached 100% at 26 days after inoculation. Some transgenic lines, Pre/Cp3-1, Pre/Cp4-1, Pre/Cp5-1, and Pre/Cp45-3, were as susceptible to the virus as non-transgenic plants. The disease symptoms were observed with frequencies from 87 to 100% 26 days past inoculation. Fourteen other lines showed delayed symptoms. Seven lines, Pre/Cp2-1, Pre/Cp6-1, Pre/Cp7-2, Pre/Cp14-2, Pre/Cp17-1, Pre/Cp29-1, and Pre/Cp40-2 had 100% virus incidence in the sixth or the seventh week after inoculation. The lines Pre/Cp38-1,

Pre/Cp15-4 and Pre/Cp8-1 showed disease symptoms in 70-80% of plants in the 8th week after inoculation. Finally, there were 4 lines which showed resistance including 90% of plants in line Pre/Cp10-2 and 100% in lines: Pre/Cp23-1, Pre/Cp30, and Pre/Cp32-1. Plants showing no symptoms were maintained until fruits were harvested. 120 days past inoculation, two plants of line IR/Cp30 presented mild symptoms. After fruits were harvested, 9 plants of the line Pre/Cp10-2 were decapitated and further maintained up to 160 days in order to observe symptom occurrence in the newly developing shoots, symptoms appeared in one plant.

Table 16: Symptom development in plants expressing Pre/Cp-hpRNA construct

Virus types	Line No.	Number of inoculated plants.	Number of symptomatic plants after inoculation (dpi)				
			21	28	35	42	70
TYLCTH A+B component	Pre/Cp 10-2	9	0	0	0	0	0
	Pre/Cp 23-1	4	0	0	0	0	0
	Pre/Cp 30	6	0	1	1	1	1
	Pre/Cp 32-1	8	0	1	1	1	1
	wt	12	8	10	10	10	10

The resistance test was repeated with 4 lines showing no symptoms (Table 16). Based on the results of the first test, where disease symptoms occurred, at lates, 60-70 days after inoculation, the plants were maintained only until 70 days past inoculation during the second test. Only 1 out of 6 plants from line Pre/Cp30 and 1 of 8 plants from line Pre/Cp32-1 showed symptoms. All plants from the other two lines (Pre/Cp10-2 and Pre/Cp23-1) did not show symptoms at all. In the second test, 83% of non-transformed plants showed symptoms.

4.3.2.2 TYLCTHV detection by PCR

To confirm the resistance, PCR was carried out 70 days and 120 days past inoculation. Samples from different parts of the plant were collected for DNA isolation. All DNA samples were mixed together, thus only 1 PCR reaction was performed for each plant.

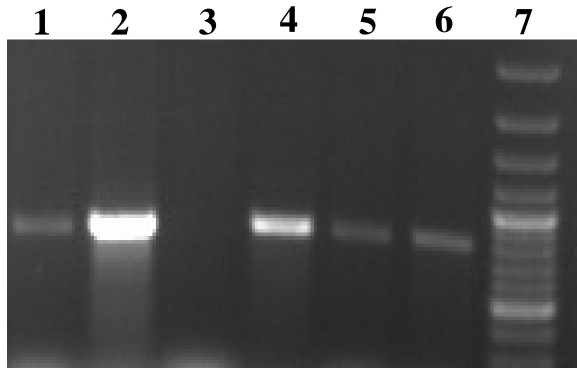

Figure 25a: Electrophoresis of PCR products amplified by Reps/Repas primers Lane 1-6: Asymptomatic plants line Pre/Cp10-2; Land 7: DNA marker ladder 100bp (Fermentas).

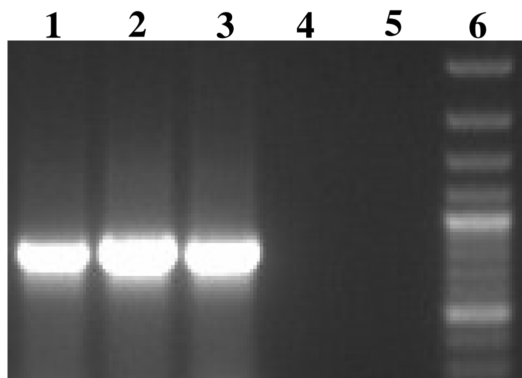

Figure 25b: Electrophoresis of PCR products amplified by Reps/Repas primers from non-transformed plants. Lane 1-3: Non-transformed infected plants; Lane 4: Non-transformed non-infected plant; Lane5: Water control; Lane 6: Marker 100bp ladder.

The TYLCTHV DNA A and B were found in both groups of plants (Figure 25a and 25b). PCR results showed bands of the predicted size for plants showing no symptoms and for the non-transgenic control. No bands could be observed in non-transgenic plants, which were not inoculated or in the water control. Primers Reps and Repas (see table 13) amplify a 925 bps fragment, a part of the *Rep* gene not included in the Pre/Cp-hpRNA. Even though the disease symptoms were not observed, the results of PCR indicated the presence of the virus in all transformed plants from lines Pre/Cp 10-2, Pre/Cp23-1, Pre/Cp30, and Pre/Cp32-1. The properties of transgenic plants listed above are characteristic of tolerance against the virus, but not immunity.

4.3.2.3 TYLCTHV coat protein detection by ELISA

Most of the detectable virus is present in young leaves in the uppermost regions of the plant; therefore these leaves were used for coat protein isolation. Non-transformed infected plants (showing symptoms) were used as positive controls in ELISA tests. Healthy leaf material of non-transformed, non-infected plants was used as negative control. Leaves were collected carefully, avoiding cross-contamination. A piece of leaf was transferred directly into a microcentrifuge tube using the lid as a cutting instrument. Five leaf discs were stamped out using one microcentrifuge tube for each sample. The protein extraction was carried out by the protocol of Triple-antibody-sandwich ELISA. The results are depicted in Figure 26.

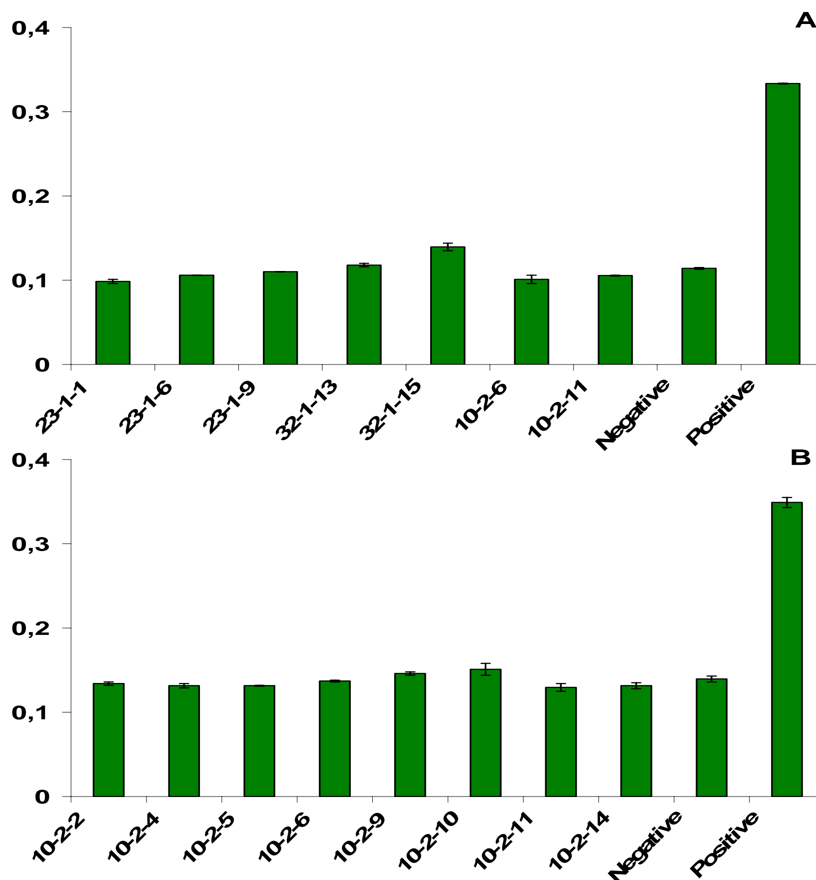

Figure 26A: Results of ELISA test with different tomato lines. **B:** Results of ELISA test with the tomato line Pre/Cp10-2. The error bars represent standard deviations or are within the column size.

The absorbance values of transformed plants showing no symptoms were not different from those of healthy plants (non-transformed as well as non-inoculated with virus), while the absorbance value in the non-transformed but infected plant were more than 2-fold higher (Figure 26). These results indicate that no viral coat protein was detectable by this ELISA in the asymptomatic plants.

4.3.3 Resistance test for *Tomato yellow leaf curl Vietnam virus*

Due to unsuccessful seed germination of line Pre/Cp23-1, only three lines (Pre/Cp10-2; Pre/Cp30; Pre/Cp32-1) were inoculated with TYLCVV, 8 non-transformed plants were used as controls. Symptoms appeared in three from eight non-transformed tomato plants 5 weeks past inoculation. However, the results of PCR were positive throughout, i.e. all plants possessed viral DNA. In the transformed plants, symptoms were observed in two plants from the line Pre/Cp10-2 and PCR also showed positive results in all symptomatic and non-symptomatic plants (Figure 27 and Table 17).

Table 17: Symptom development in plants expressing the Pre/Cp-hpRNA construct

Virus types	Line No.	Number of inoculated plants.	Number of symptomatic plants after inoculation (dpi)					PCR
			21	28	35	42	70	
TYLCVV A component	IR/Cp 10-2	8	0	0	2	2	2	8
	IR/Cp 30	7	0	0	0	0	0	7
	IR/Cp 32-1	6	0	0	0	0	0	6
	wt	8	0	0	3	3	3	8

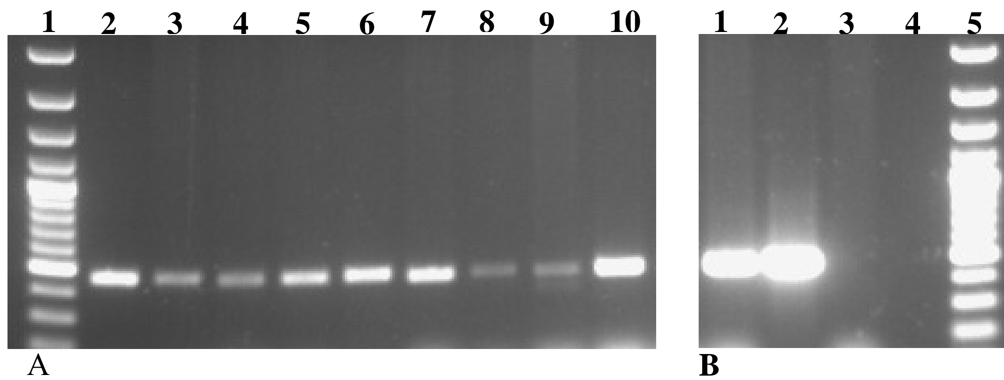

Figure 27: Electrophoresis of PCR products amplified by Reps-VN/Repas-VN primer at 120 days past inoculation with TYLCVV:
(**A**) Lane 1: DNA marker ladder 100bp (Fermentas); lane 2 to 9: From plants of the line Pre/Cp10-2; Lane 10: non-transformed infected plants.
(**B**): Lane 1-2: non-transformed infected plants; Lane 3: non-transformed non-infected plants; Lane 4: Water control; Lane 5: DNA marker ladder 100bp (Fermentas).

4.4 Discussion

To establish systemic infection in a plant, a geminivirus must move from the infection site into the plant cell nuclei to replicate its genome as well as translocate the replicated DNA to uninfected cells. The process of viral movement includes import, export of viral DNA into/or out of the plant nucleus and long-distance movement of viral DNA. Previous studies have shown that geminiviral coat protein (CP) plays an important role in directing viral nucleic acids into and out of the nucleus (Kunik et al., 1998; Kotlitzky et al., 2000; Rhee et al., 2000). However, the role of CP in virus infection is different between monopartite and bipartite geminiviruses. In a monopartite TYLCV, the CP carries functional nuclear localization signals (NLS) (Kunik et al., 1998) which were shown to be essential for translocation of the viral CP into plant nuclei. Furthermore, Palanichevam et al. (1998) found that the CP binds cooperatively to single-stranded DNA in a non sequence-specific manner. It has been suggested that TYLCV CP interacts with tomato karyopherin-α1, mediating its nuclear import, thus it was proposed that TYLCV CP functions as a transporter of the viral genome into the host cell nucleus. (Kunik et al., 1998, 1999; Palanichelvam et al., 1998). Results of Noris et al. (1998) indicated that capsid

protein of two isolates, TYLCVSic (from Sicily) and TYLCV-Sar (from Sardinia) is crucial for systemic infection, particle formation, and insect transmission. In their studies they have found that the CP region between amino acids 129-134 is essential for both the correct assembly of virions and transmission by the insect vector. Rojas et al. (2001) found that the TYLCV CP is localized to the nucleus and nucleolus and acts as a nuclear shuttle, mediating the import and export of DNA. At least two distinct functions were unravelled, nuclear export of the infectious form of the virus, and encapsidation of ss-DNA into virions. CP mediates nuclear export of ds-DNA, cell-to-cell and long distance movement within the plant and encapsidates ss-DNA within the nucleus to form virions that are required for plant-to-plant spread via the whitefly vector. Therefore, for monopartite geminiviruses, the CP is absolutely neccessary for systemic infection as well as for particle formation and insect transmission**.** In contrast, bipartite geminiviruses have genomes composed of two circular 2.5- to 2.8-kb *ss*-DNA molecules (designated DNA-A and DNA-B). DNA B component encodes two MPs (BV1 and BC1), which are required for virus movement (Lazarowitz, 1992; Jeffrey et al., 1996; Sudarshana et al., 1998). The BV1 protein has been shown to increase the size exclusion limit of plasmodesmata (Noueiry et al., 1994) whereas the BC1 protein traffics ssDNA (Pascal et al., 1994) or dsDNA (Noueiry et al., 1994) into and out of the nucleus. Thus BC1 and BV1 have distinct but essential roles in cell-to-cell movement. Therefore, for bipartite begomoviruses, a CP is not required for either local or systemic viral spread (Gardiner et al., 1988; Pooma et al., 1996; Padidam et al., 1995; Sudarshana et al., 1998). Nevertheless, the coat protein plays an essential role in the transmission process of *B. tabaci* (Höfer et al., 1997; Briddon et al., 1990; Azzam et al., 1994).

The begomovirus coat protein was initially thought to offer the best target for engineering resistance to different tomato-infecting begomoviruses. Disruption of the TYLCSV *cp* gene performed symptom development and accumulation of viral DNA in tobacco and tomato plants (Wartig et al., 1997). In the tomato V1 mutants, symptoms did not occur (Rigden et al., 1993). However, very few reports have shown successful CP-mediated resistance (Kunik et al., 1994; Raj et al., 2005) or RNA-mediated resistance (Sinisterra et al., 1999; Zrachya et al., 2007b). Kunik et al. (1994) showed that tomato plants expressing the V1 *(cp*) gene were resistant to TYLCV infection. The resistance was associated with high levels of expressed CP. The resistance presented itself as a delay in symptoms

development and a recovery phenotype. Raj et al. (2005) expressed TYLCV coat protein in tomato. T1-generation transgenic plants were challenged by TLCV through whiteflies, which showed variable degrees of disease resistance/tolerance compared to the untransformed control. Sinisterra et al. (1999) transformed tobacco with a modified coat protein of ToMoV, but they could not detect the protein product of the transgene in any of the resistant lines. Thus, they assumed that the resistance may be due to a RNA-mediated mechanism. Only one report exists (Zrachya et al., 2007b), where the use of inverted repeat constructs to confer resistance against TYLCV is described. They produced transgenic tomato plants harbouring an inverted-repeat construct targeting the CP. The transgenic plants first showed symptoms seven weeks past inoculation, with less accumulation of the virus than in non-transformed infected plants.

Interestingly, when comparing these results with the results obtained in the present work, the resistant lines preservered much longer. No symptoms were present even at 120 days past inoculation in all of plant lines Pre/Cp10-2, Pre/Cp23-1, and Pre/Cp32-1. The mild symptoms were only present in 2 plants of line IR/Cp 30. Although the viral DNA was detectable (Figure 24, 25) in all asymptomatic plants, the coat protein was not (Figure 26). Thus, the absence of symptoms in lines Pre/Cp10-2, Pre/Cp23-1 and Pre/Cp32-1 could result from the absence of the coat protein. This is an interesting result, because TYLCTHV is a bipartite begomovirus. Normally, the CP of bipartite viruses is not essential for systemic infection because viral movement and transport are performed by proteins encoded by the B component. The Pre/Cp-hpRNA transgene can only induce dsRNA that is complementary to cognate mRNA of coat protein but not other proteins. Thus, viral DNA can be replicated as usual while the movement and spread to whole plant is still provided by MPs (MP and NSP), which are encoded on the B component. It seems that the TYLCTHV coat protein has some influence on the presence of symptoms. This effect can be explained due to the characteristics of the TYLCTHV A component, the A component can infect its natural host alone, has been showed by Rochester et al. (1990) as well as in the present research (see Figure 16c chapter 3).

In the test involving *Tomato yellow leaf curl Vietnam virus*, the plants were inoculated with only the A component of the monopartite TYLCVV. Here, we were not able to produce high levels of infected control plants. Although the A component of TYLCVV

should replicate as well as be transported to whole plant, the symptoms were present only in 30% of inoculated plants.

TYLCVV is a monopartite begomovirus, thus the coat protein is absolutely required for virus systemic infection. The viral DNA was detectable in each transformed plant. This fact indicated that dsRNA from Pre/Cp-hpRNA did not affect cleavage/degradation mRNA of the TYLCVV coat protein; the coat protein of TYLCVV was still synthesized and supported the systemic infection. Even though the symptoms were not present, it is assumed that the plants resistant to TYLCTHV are not resistant to TYLCVV infection due to the negative results of infection of some control plants, as well as the presence of viral DNA in transformed plants.

GENERAL DISCUSSION

Plant transformation is an important research tool for producing genetically modified commercial crops. *A. tumefaciens* has been used for genetic transformation of plants by its natural ability to transfer foreign DNA into the host plant genome. This process is carried out by utilizing both bacterial and host machineries. Therefore, transformation frequency is influenced by many factors. Those factors have been investigated and elucidated in previous researches (Gelvin, 2003; Opabode, 2006) and a wide variety of inoculation and co-culture conditions have been shown to be important for the transformation. Temperature is a factor that affects activation of *vir* genes of *A. tumefaciens* as well as regeneration of plant tissues; hence, it influences the efficiency of transformation (Alt-Mörbe et al., 1989; Jin et al., 1993; Salas et al., 2001; Uranbey et al., 2005). The influence of *Agrobacterium* concentration on transformation has been shown (Davis et al., 1991; De Bondt et al., 1994; Cheng et al., 1997; Humara et al., 1999; De Clercq et al., 2002). In addition, the data from the present study shows that *A. tumefaciens* is an additional harmful factor affecting the plant tissues, besides the selection chemical (glufosinate) during transformation. Thus, the effective concentration of glufosinate must be identified under pressure of *A. tumefaciens*. The factors stimulating plant cell division and T_{DNA} integration may have increased transformation efficiency in different reports (Sangwan et al., 1992; de Kathen and Jacobsen, 1995). In the current research, pre-treament of explants with phytormones (cytokinin and auxin) and complement phytohormones in the inoculation medium resulted in significantly increasing the frequency of transformation. In agreement with Park et al. (2003), by comparing shoot regeneration media and optimising different parameters which influenced the transformation process, the present study has developed an efficient protocol for leaf disc transformation of three commercial varieties (DM8, MTS, FM372C). The developed protocol, when applied for transformation with RNAi constructs of cv. FM372C, achived frequencies of transformation ranging from 9 to 19%, while it was 13% in the transformation with *gus* gene. It seems that plant tissues are mixed populations of cells with competence for many different responses including competence for T_{DNA} transformation and shoot regeneration. Tissues containing the most cells with competence for both T_{DNA} transformation and shoot regeneration will be able to afford the higher frequency of transformation.

Genetic engineering has the potential to provide an abundant source of beneficial plant traits including virus-resistance. Different approaches have been considered for the development of transgenic resistance to geminiviruses by the expression of either pathogen derived resistance (PDR) or non pathogen derived resistance, as described in the first chapter. Silencing pathways are complex and partially overlapping, but at least three basic classes can be distinguished: cytoplasmic RNA silencing (or post-transcriptional gene silencing; PTGS) mediated by small interfering RNAs (siRNAs), silencing mediated by microRNAs (miRNAs), and transcriptional gene silencing (TGS) mediated by siRNA-directed methylation of DNA and histone proteins (Bisaro, 2006). The *Geminiviridae* are true DNA viruses that replicate circular, single-stranded DNA genomes in the nucleus by a rolling-circle mechanism that employs host replication machinery (Jeske et al., 2001; Preiss and Jeske, 2003). The double-stranded DNA (dsDNA) intermediates that mediate both viral replication and transcription associate with cellular histone proteins to form “minichromosomes” (Pilartz and Jeske, 1992; Pilartz and Jeske, 2003). Transcripts produced from these “minichromosomes” are subject to PTGS. In addition, given the role of RNA-directed methylation in silencing endogenous invasive DNAs, it is possible that plants might also use methylation as a mean to repress transcription and/or replication of a viral “minichromosome” (Bisaro, 2006; Ding and Voinnet, 2007).

Different regions of the begomovirus genome have been successfully used to trigger silencing. The AC2/C2 protein has been associated with the suppression of gene silencing in *Mungbean yellow mosaic virus* -Vigna (MYMV; Trinks et al., 2005), in ACMV-[CM] and SLCMV (Vanitharani et al., 2004). The AC2/C3 protein of *Cotton leaf curl virus* was successfully used by Asad et al. (2003). Ribeiro et al. (2007) used a fragment consisting of 300 nts of the 5´-end of the AV1 gene (including the end of the overlapping AC5 gene) the entire common region and 300 nts of the 5´-end of the AC1 gene (including a part of AC4 gene) of *Tomato chlorotic mottle virus* (ToCMoV). Forty five days post inoculation they achieved two best-resistant lines: “RC-24.2” with 50% of plants resistant to virus infection (no symptoms and no virus present), and “RC-19.3”, with 50% of the plants symptomless, including 30% entirely virus free plants. Research of Gopal et al. (2007) showed strong suppression of gene silencing activities for C4 and ßC1 of *Bhendi yellow vein mosaic virus* in *N. benthamiana*. Even though, the *Rep* gene has been mostly employed in different strategies to confer geminivirus resistance, at present, there are only a few reports of

successful begomovirus resistance development through *Rep* sequence expression to triggering PTGS (Asad et al., 2003; Yang et al., 2004; Ramesh et al., 2007; Fuentes et al., 2006; Bonfim et al., 2007). The resistance could be triggered by using a truncated *Rep* gene either from the 3´-end (Bonfim, 2007; Asad et al., 2003; Fuentes et al., 2006) or from the 5´-end (Asad et al., 2003; Yang et al., 2004). The short (81 nts) intergenic region in the transgenic construct of Yang et al. (2004) increased the frequency and quality of the resistance obtained with a partial TYLCV *Rep* gene and could act as a trigger for PTGS. This result could be due to following the RNA-directed DNA methylation (RdDM) pathway. Methylation of a TLCV-derived transgene promoter and consequent transgene silencing has been observed on TLCV infection (Seemanpillai et al., 2003). RNA-directed methylation of geminivirus bidirectional promoters may down-regulate the transcription of viral genes, resulting in inefficient virus replication (Pooggin et al., 2003; Dogar, 2006). Alternatively, the dsRNAs derived from a bidirectional promoter region might interfere with the rolling cycle replication of the virus or target viral single strand-DNA (Pooggin et al., 2003).

Regarding the use of a non-coding region triggering PTGS, Abhary et al. (2006) used three non-coding fragments of the virus genome denoted C1C2, C2C3, and V1V2 of TYLCV in transformation. They achieved transformed plants resistant to TYLCV, TYLCV-Mld and TYLCSV-ES after whitefly inoculation and tomato yellow leaf curl virus-Sardinia TYLCSV-(Sar) after agroinfiltration. Although largely unexplored, intergenic regions may prove useful in the development of resistance. Recovery of *Vigna mungo yellow mosaic virus*-infected plants has been reported after bombardment with DNA constructs expressing dsRNAs homologous to the bidirectional viral promoter (Pooggin et al., 2003). Dogar (2006) used the 360 nucleotide fragment corresponding to the intergenic region of ACMV DNA-A to construct the intron-hpRNA for triggering PTGS. The author proposed that during DNA virus infection the mRNAs transcribed from the geminivirus genome are subjected to degradation by 21-22 nts small RNAs. On the other hand, the geminiviral genomic DNA seems to be subject to RdDM by 24-25 nts small RNAs. Corresponding to the results published by Dogar (2006), in the same virus (ACMV-KE), Vanderschunren et al. (2007) used an intron-hairpin construct from 256 bps of the common region for transformation. In their construct, the 256 bps from the common region contained a bidirectional promoter of ACMV-KE. In two of three independent transgenic lines,

accelerated plant recovery from ACMV-NOg infection was observed, which correlates with the presence of transgene-derived siRNAs 21-24 nts in length. Their result suggested that a natural RNA silencing mechanism targeting DNA viruses through production of virus derived siRNAs is turned on earlier and more efficiently in transgenic plants expressing dsRNA cognate to the viral promoter and common region. Research on methylation levels of the CaLCuV intergenic region from Raja et al. (2008) showed a greater proportion of non-CG methylation than CG methylation in the CaLCuV IR. Furthermore, cytosine residues in the vicinity of the conserved hairpin and AL1 binding sites were the most frequently methylated. Very recently, results of Rodriguez-Negrete et al. (2009) suggested that *Pepper golden mosaic virus* (PepGMV) was targeted by both posttranscriptional and transcriptional gene silencing mechanisms. In their research, two types of virus-related small interfering RNAs (siRNAs) were detected: siRNAs of 21 to 22 nts in size that are related to the coding regions (*Rep*, *Trap*, *REn*, and *MPs* gene) and a 24-nts population primarily associated to the intergenic regions. They observed an inverse correlation between the methylation status of the intergenic region and the concentration of viral DNA and symptom severity. The intergenic regions also showed a methylation profile which was conserved in all analysis. Conversely, the *cp* region did not show a defined profile and its methylation density was significantly lower than the one found on the intergenic region. The double small RNA-directed methylation of geminivirus bidirectional promoters may down-regulate the transcription of viral genes, resulting in inefficient virus replication. The viral promoter and CR may undergo siRNA-directed DNA methylation and histone modifications that reduce both the transcriptional activity of the promoter and/or impair the recruitment of DNA polymerase necessary for replication, via altered Rep-binding site properties. Therefore, triggering TGS of geminivirus promoters by pre-expression or induced expression of specific dsRNAs may constitute a promising strategy for interfere with virus replication.

In the present research, the intron-hairpin IR/Rep construct that led to TYLCV resistance contained 397 nts from the 5′-end of the *Rep* gene (included truncated AC4 from the 5′-end) and 174 nts of the IR. The IR harboured the sequence upstream of the expected transcription start of the *Rep* gene as well as the nonameric motif 5′-TAATATT/AC-3′. The upstream sequence contains sequence specific elements (iterons) for REP binding during the rolling circle replication of begomoviruses (Argüello-Astorga and Ruiz-

Medrano, 2001) and the nonameric motif 5´-TAATATT/AC-3´ invariably located at the loop of a conserved "hairpin" element, where REP introduces a site-specific nicks to initiate virus replication via a RC mechanism (Laufs et al., 1995a). In the present study, we did not detect siRNAs that were possibly derived from an intron-hpRNA transgene as well as the mRNA of *Rep* from the viruses; therefore the mechanism of resistance in line IR/Rep2-1 is not clear. However, from different previous experiments as described above, it can be concluded that the 397 bps from the 5´-end of *Rep* also included truncated AC4 from the 5´-end that can produce siRNAs, which is able to trigger PTGS of both AC1 as well as AC4. The 174 bps sequence of IR, after transcription, could produce siRNAs, which can trigger the methylation of viral DNA by RdDM.

The use of the coat protein gene under the intron-hairpin construct in order to trigger PTGS has been successful in RNA viruses of different plants, for instance, in tobacco plants resistant to PVY (Smith et al., 2000) and CMV (Kalantidis et al., 2002), barley resistant to BYDV (Wang et al., 2000), potato resistant to PVY (Missiou et al., 2004; Vargas et al., 2008), soybean resistant to SbDV (Tongou et al., 2006) and *Soybean mosaic virus* (SMV; Furutani et al., 2007). However, with tomato yellow leaf curl virus, there has only been one report used an inverted repeat construct of the coat protein gene to confer resistance against TYLCV from Zrachya et al. (2007b). They produced transgenic tomato plants harbouring an inverted-repeat construct targeting the *cp.* The transgenic plants did not show symptoms until seven weeks past-inoculation and the virus accumulation was less than that of non-transformed infected plants. However, those plants then showed disease symptoms 7 weeks after inoculation. In constrast to their results, in this research there was no symptom even at 120 days after inoculation in 9/10 plants of the line Pre/Cp10-2 as well as no symptom in all plants from line Pre/Cp23-1 and Pre/Cp32-1. Even though the viral DNA was detectable in all plants, no coat protein was detectable. It seems that the Pre/Cp-hpRNA transgene triggers silencing of the coat protein gene. However, the construct contained 540 nts in length that included truncated 464 nts from the 5´-end of the *cp* and 255 nts from the 3´-end of the pre-coat. Thus, this construct can only trigger the silencing of translation of the mRNA-coat protein and pre-coat, but not of other genes. Both components of the virus still can replicate. Then MPs (BV1, BC1) can support viral DNAs (both single and double stranded movement, In this case, it is still a question why

plants did not show symptoms while viral DNA was present in whole plants event until 130 days past inoculation.

At present, there is a lack of clear understanding on the mechanisms that determine the gene silencing efficiency of a given siRNA in begomoviruses. However, the previous and presented results as well as recent studies show that the gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. To further test the relationship between silencing efficiency and targeted region of mRNA, work needs to be done on these aspects. For example, in the present study the IR/Rep-hpRNA construct confers immunity, while the plants of the Pre/Cp-hpRNA were tolerant to the virus. However, it is not clear whether the immunity was achieved by degradation of mRNA of transcriptional silencing of the *Rep* gene by RdDM, which could prevent the *Rep* transcription and/or direct rolling circle replication of the virus.

Further more, TYLCV disease is a complex infection, which can be caused by different viruses. There are many tomato-infecting begomoviruses and some of these occur in mixed infections with TYLCV (Abhary et al., 2007). Broad spectrum resistance against TYLCV and other tomato-infecting begomoviruses would be very useful and economically desirable (Freitas-Astua et al., 2002). Broad-spectrum resistance based on RNA-mediated virus resistance has been described in Abhary et al. (2006). By using the silencing construct from the conserved region of V1V2, C1C2 and C2C3, Abhary et al. (2006) successfully developed tomato and *N. benthamiana* plants resistant to TYLCV-[EG], TYLCV, TYLCV-Mld and TYLCSV-ES[2]. Chellappan et al. (2004) achieved transgenic plant lines resistant to ACMV that were challenged with isolates of EACMCV and *Sri Lankan cassava mosaic virus* (SLCMV). However, it was not clear wherther the resistance by the AC1 transgene caused by protein-based or RNA-based mechanisms, or a combination of both within the different transgenic plant lines.

The IR/Rep2-1 line developed in the present research confers resistance to TYLCTHV and TYLCVV. Here again, the Pre/Cp-hpRNA construct does not confer resistant to the TYLCVV. Assuming that in case of the Pre/Cp-hpRNA transgenic plants, the resistance mechanism is based on RNAi, the susceptibility of the lines to TYLCVV infection could be due to the relatively low sequence similarity level of the Cp region between the two viruses. Whereas the sequence similarity between the construct derived from the IR/Rep

region and that of TYLCVV is 92%, while it is 75% in the Pre/Cp region (see appendix in page 137-138).

The intron-hairpinRNA construct has been considered as being highly effective for inducing PTGS. In principle, the inverted-repeat intron hairpin transgene can induce dsRNA, the key trigger for the process that leads to degradation of homologous RNAs (Voinnet et al., 1999; Bass, 2000; Vaucheret and Fagard, 2001). This strategy of expressing a gene encoding intron-spliced RNA can induce PTGS with almost 100% efficiency, when directed against viruses, leading to plants which are immune to the virus (Smith et al., 2000). Begomoviruses have been successfully shown as a target for PTGS by transforming plants with inverted-repeat constructs (Fuentes et al., 2006; Pooggin et al., 2003; Bonfim et al., 2007; Zrachya et al., 2007b). However, the transformed plants which carried the same intron-hpRNA induced variation resistance levels. Some plants were still fully susceptible to infection and only a few of them were resistant/tolerant or immune. Bonfim at al. (2007) achieved 1 immune line from 18 independent transgenic lines. Completed immune transgenic lines were not obtained using intron-hpRNA constructs for ToCMoV (Ribeiro et al., 2007) or plants only delayed symptoms of *Tomato yellow leaf curl virus* infection for 7 weeks past inoculation (Zrachya et al., 2007b). Silencing escape has also been shown for TLCV (Bian et al., 2006).

In the present research, the transformation with an IR/Rep-hpRNA construct resulted in very different levels of resistance. The levels of resistance were ranging from immunity (line IR/Rep2-1), or delay (IR/Rep10-1, IR/Rep15-1, IR/Rep23-5 and IR/Rep38-1) to as susceptibe as non-transformed plants (IR/Rep16-1 and IR/Rep47-5). Nevertheless, the frequency of immune lines in this research was very low; only one line out of 17 IR/Rep-hpRNA transgenic lines was found to be immune. Similarily, only 4 tolerant lines were obtained out of 18 lines tested with the Pre/Cp-hpRNA construct. Those results indicate that resistant responses depend not only on the presence of the transgene but also on the interactions between the transgene and the plant genome. Although many different factors might combined to activate silencing inducing transgenes, the variability of transgene expression can be attributed to several factors. The insertion of T_{DNA} is random within the plant genome and the activity of the introduced genes may be affected by adjacent plant DNA. For example, if an endogenous gene and the transgene are orientated in opposite directions, reduced expression could result from production of antisense RNA, potentially

forming double stranded (ds) RNA with sense mRNA, leading to RNAi (position, orientation effect; Matzke and Matzke, 1998; Kooter et al., 1999). Tandemly repeated transgenes at the same locus are often silenced in plants, a phenomenon named repeat-induced gene silencing (Assaad et al., 1993). For example, the research of Chalfun-Junior et al. (2003) showed that all plants containing more than a single T_{DNA} insertion showed methylation of the 35S enhancer and revealed a dramatic decrease in 35S enhancer activity. The effect of copy number on transgene expression is described as being a consequence of DNA methylation (Kooter et al., 1999; Selker, 1999; Mette et al., 2000; Wassenegger, 2000; Sijen et al., 2001).

In the present transformation work, all most all transformed plants with the IR/Rep-hpRNA construct contained multible-insertions (1 line had 7 T_{DNA} insertions, 7 lines had 3; 5 lines had 4; and 5 lines had 2 insertions). However, not all insertions were intact $T_{DNA.}$ Finally, there were eight lines containing a single intact T_{DNA}, seven lines contain 2 intact insertions, one line (IR/Rep31-1) contains 4 intact insertions and two lines (IR/Rep34-1; IR/Rep29-1) contain only truncated T_{DNA} insertions.

Linked to results of the resistance test, the IR/Rep2-1 line, which was immune line, contained 2 intact and 1 truncated T_{DNA} insertions. The IR/Rep10-1 line, with a delay of symptom development, also contained 2 intact and 1 truncated insertions. In this line, the symptoms were observed in 33% of plants at 40 dpi remaining until 70 dpi to the end of the experiment. The line IR/Rep47-5 containing 2 intact and 3 truncated insertions was as susceptible as non-transgenic control plants. Several lines with single intact insertions also slightly delayed virus disease. In the lines IR/Rep38-1 (1 intact and 4 truncated insertions) and IR/Rep15-1 (1 intact and 3 truncated insertions) symptoms were observed at 30 dpi and at 70 dpi the symptom were present in 37.5% of the plants. The IR/Rep34-2 line contained only 1 truncated insertion showed a delayed symptom expression at 30 dpi; however the incidence of diseaese was 50% of the tested plants at 40 dpi.

In the transformation with the Pre/Cp-hpRNA construct, all 32 transformed lines contained a single intact T_{DNA} insertion. In addition, truncated insertions were observed in 20 out of 32 (62.5%) independent lines. Only the Pre/Cp10-1 line without a truncated insertion showed no symptoms. Two other lines, Pre/Cp 30 and Pre/Cp32-1, also carried one intact and one truncated T_{DNA} insertion and showed no symptoms.

Generally, it is not clear why the frequency of resistant lines is very low. It seems that several factors have an influence on silencing. In both transformations, either with the IR/Rep or with the Pre/Cp-hpRNA construct, it looks like that transgenes were transcriptionally silenced, probably due to their position in the plant genome, resulting in virus susceptibility of the plants. Anyway, the resistance tests were carried out with T_1 transformed plants. Thus, the inheritance of resistance has to be evaluated in subsequent progenies. Most of the resistant lines were observed carrying the transgene in multiple copies so it can be expected that segregation of T_{DNA} insertions will take place in later propagations.

Several attempts have been made to engineer tomato plants resistant to TYLCV via a gene silencing strategy. In some cases the resistance has been overcome when silenced plants were challenged with other strains of the virus that can silence the homologous transgene. The result of the present work showed that the IR/Rep2-1 line was able to trigger a high level of resistance in tomato plants against two viruses belonging to the TYLCV complex (TYLCTHV and TYLCVV) by agroinoculation. Although the three lines, Pre/Cp10-2, Pre/Cp30-1, and Pre/Cp32-1 were not able to resist one isolate of TYLCVV, however, we were successful in detecting 3 different isolates that cause the type of TYLCV symptoms in the disease samples of Vietnam tomato (unpublished data). Therefore, to shed more light on the efficiency and stability of the resistance developed in this study, transgenic tomato plants expressing the IR/Rep2-1, Pre/Cp10-2, Pre/Cp23-1, Pre/Cp32-1 and Pre/Cp30-1 need to be evaluated under field conditions where high virus pressure occurs (e.g. Vietnam).

REFERENCES

Abel PP, Nelson R, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232: 738-743.

Abhary M, Patil BL, Fauquet CM (2007) Molecular biodiversity, taxonomy and nomenclature of tomato yellow leaf curl-like viruses. In Czosnek (ed.), *Tomato Yellow Leaf Curl Virus Disease*. *Springer:* 85-118.

Abhary MK, Anfoka GH, Nakhla MK, Maxwell DP (2006) Post-transcriptional gene silencing in controlling viruses of the Tomato yellow leaf curl virus complex. Arch Virol 151: 2349-2363.

Ach RA, Durfee T, Miller AB, Taranto P, Hanley-Bowdoin L, Zambriski PC, Gruissem W (1997) RRB1 and RRB2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. Mol Cell Biol 17: 5077-5086.

Adenot X, Elmayan T, Lauressergues D, Boutet S, Bouche N, Gasciolli V, Vaucheret H (2006) DRB4-dependent TAS3 transacting siRNAs control leaf morphology through AGO7. Curr Biol 16: 927-932.

Afroz A, Chaudhry Z, Khan R, Rashid H, Khan SA (2009) Efect of GA_3 on regeneration response of three tomato cultivars (Lycopersicon esculentum). Pak J Bot 41: 143-151.

Agharbaoui Z, Greer AF, Tabaeizadeh Z (1995) Transformation of the wild tomato *Lycopersicon chilense* Dun. by *Agrobacterium tumefaciens.* Plant Cell Rep 15: 102-105.

Ahsan N, Lee SH, Lee DG, Anisuzzaman M, Alam MF, Yoon SH, Choi MS, Yang JK, Lee BH (2007) The effects of wounding type, preculture, infection method and ocultivation temperature on the *Agrobacterium*-mediated gene transfer in tomatoes. Ann Appl Biol 151: 363-372.

Akad F, Eybishtz A, Edelbaum D, Gorovits R, Dar-Issa O, Iraki N, Czosnek H (2007) Making a friend from a foe: expressing a GroEL gene from the whitefly *Bemisia tab*aci in the phloem of tomato plants confers resistance to Tomato yellow leaf curl virus. Arch Virol 152: 1323-1339.

Akbergenov R, Si-Ammour A, Blevins T, Amin I, Kutter C, Vanderschuren H, Zhang P, Gruissem W, Meins F Jr, Hohn T, Pooggin MM (2006) Molecular characterization of geminivirus derived small RNAs in different plant species. Nucleic Acids Res 34: 462-471.

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell 121: 207-221.

Alt-Mörbe J, Kühlmann H, Schröder J (1989) Differences in induction of Ti plasmid virulence genes *vir*G and *vir*D, and continued control of *vir*D expression by four external factors. Mol Plant-Microbe Interact 2: 301-308.

Antignus Y, Vunsh R, Lachman O, Pearlsman M, Maslenin L, Hananya U, Rosner A (2004) Truncated Rep gene originated from Tomato yellow leaf curl virus-Israel [Mild] confers strain-specific resistance in transgenic tomato. Ann Appl Biol 144: 39-44.

Aragão FJL, Ribeiro SG, Barros LMG, Brasileiro ACM, Maxwell DP, Rech EL, Faria JC (1998) Transgenic beans (Phaseolus vulgaris L) engineered to express viral antisense RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. Mol Breed 4: 491-499.

Argüello-Astorga GR, Guevara-Gonzalez RG, Herrera-Estrella LR, Rivera-Bustamante RF (1994) Geminivirus replication origins have a group-specific organization of iterative elements: a model for replication. Virology 203: 90-100.

Arguello-Astorga G, Lopez-Ochoa L, Kong LJ, Orozco BM, Settlage SB, Hanley-Bowdoin, L (2004) A novel motif in geminivirus replication proteins interacts with the plant retinoblastoma homolog RBR. J Virol 78: 4817-4826.

Arguello-Astorga GR, Ruiz-Mendoza R (2001) An iteron-related domain is associated to Motif 1 in the replication proteins of geminiviruses: Identification of potential interacting amino acid-base pairs by a comparative approach. Arch Virol 146: 1465-1485.

Aronin N (2006) Target selectivity in mRNA silencing. Gene Ther 13: 509-516.

Asad S, Haris WA, Bashir A, Zafar Y, Malik KA, Malik NN, Lichtenstein CP (2003) Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. Arch Virol 148: 2341-2352.

Assaad FF, Tucker KL, Signer ER (1993) Epigenetic repeat-induced gene silencing (RIGS) in Arabidopsis. Plant Mol Biol 22: 1067-1085.

Azzam O, Fraser J, De La Rosa D, Beaver JS, Ahlquist P, Maxwell DP (1994) Whitefly transmission and efficient ssDNA accumulation of bean golden mosaic geminivirus require functional coat protein. Virology 204: 289-296.

Banerjee AK, Prat S, Hannapel DJ (2006) Efficient production of transgenic potato (S. tuberosum L. ssp. andigena) plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci 170: 732-738.

Baron C, Domke N, Beinhofer M, Hapfelmeier S (2001) Elevated temperature differentially affects virulence, VirB protein accumlation, and T-pilus formation in different *Agrobacterium tumefaciens* and *Agrobacterium vitis* strains. J Bacteriol 183: 6852-6861.

Bass BL (2000) Double-stranded RNA as a template for gene silencing. Cell 101: 235-238.

Baulcombe D (1996) RNA as a target and an initiator of post-transcriptional gene silencing in trangenic plants. Plant Molecular Biology 32: 79-88.

Baulcombe D (2004) RNA silencing in plants. Nature 431: 356-363.

Baulcombe DC, English JJ (1996) Ectopic pairing of homologous DNA and posttranscriptional gene silencing in transgenic plants. Curr Opin Biotechnol 7: 173-180.

Beachy RN (1997) Mechanisms and applications of pathogen derived resistance in transgenic plants. Curr Opin Biotechnol 8: 215-220.

Beachy RN, Loesch-Fries S, Tumer NE (1990) Coat protein resistance against virus infection. Annu Rev Phytopathol 28: 451-474.

Behjatnia SAA, Dry IB, Ali Rezaian M (1998) Identification of the replication associated protein binding domain within the intergenic region of tomato leaf curl geminivirus. Nucleic Acids Res 26: 925-931.

Bejarano ER, Lichtenstein CP (1994) Expression of TGMV antisense RNA in transgenic tobacco inhibits replication of BCTV but not ACMV geminiviruses. Plant Mol Biol 24: 241-248.

Bendahmane M, Gronenborn B (1997) Engineering resistance against Tomato yellow leaf curl virus (TYLCV) using antisense RNA. Plant Mol Biol 33: 351-357.

Bian XY, Rasheed MS, Seemanpillai MJ, Ali Rezaian M (2006) Analysis of silencing escape of Tomato leaf curl virus: an evaluation of the role of DNA methylation. Mol Plant- Microbe Interact 19: 614-624.

Bird CR, Smith CJS, Ray JA, Moureau P, Bevan MJ, Birds AS, Hughes S, Morris PC, Grierson D, Schuch W (1988) The tomato polygalacturonase gene and ripening specific expression in transgenic plants. Plant Mol Biol 11: 651-662.

Bisaro DM (2006) Silencing suppression by geminivirus proteins. Virology 344: 158-168.

Bisaro DM, Hamilton WDO, Coutts RHA, Buck KW (1982) Molecular cloning and characterisation of the two components of *Tomato golden mosaic virus*. Nucleic Acids Res 10: 4913-4922.

Blawid R (2008) New insight into Geminivirus complexes from Vietnam and Thailand. PhD. thesis, Hannover University.

Bonfim K, Faria JC, Nogueira EOPL, Mendes EA, Aragao FJL (2007) RNAi-mediated resistance to *Bean golden mosaic virus* in genetically engineered common bean (Phaseolus vulgaris). Mol Plant-Microbe Interact 20: 717-726.

Bouche N, Lauressergues D, Gasciolli V, Vaucheret H (2006) An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. EMBO J 25: 3347-3356.

Boulton MI, Steinkellner H, Donson J, Markham PG, King DI, Davies JW (1989) Mutational analysis of the virion-sense genes of *Maize streak virus*. J Gen Virol 70: 2309-2323.

Braun AC (1947) Thermal studies on the factors responsible for tumor initiation in crown gall. Am J Bot 34: 234-240.

Braun AC (1958) A physiological basis for autonomous growth of the crown-gall tumor cell. Proc Natl Acad Sci USA 44: 344-349.

Briddon RW, Bedford ID, Tsai JH, Markham PG (1996) Analysis of the nucleotide sequence of the treehopper-transmitted geminivirus, tomato pseudo-curly top virus, suggests a recombinant origin. Virology 219: 387-394.

Briddon RW, Bull SE, Amin I, Mansoor S, Bedford ID, Rishi N, Siwatch SS, Zafar Y, Abdel-Salam AM, Markham PG (2004) Diversity of DNA 1: a satellite-like molecule associated with monopartite begomovirus-DNA beta complexes. Virology 324: 462-474.

Briddon RW, Bull-Simon E, Amin I, Idris AM, Mansoor S, Bedford ID, Dhawan P, Rishi N, Siwatch SS, Abdel-Salam AM, Brown JK, Zafar Y, Markham PG (2003) Diversity of DNA

beta, a satellite molecule associated with some monopartite begomoviruses. Virology 312: 106-121.

Briddon RW, Pinner MS, Stanley J, Makham PG (1989) The coat protein of beet curly top virus is essential for infectivity.Virology 172: 628-633.

Briddon RW, Pinner MS, Stanley J, Markham PG (1990) Geminivirus coat protein gene replacement alters insect specificity. Virology 177: 85-94.

Briddon RW, Stanley J (2006) Subviral agents associated with plant single-stranded DNA viruses. Virology 344: 198-210.

Brunetti A, Tavazza R, Noris E, Lucioli A, Accotto G P, Tavazza M (2001) Transgenically expressed T-Rep of Tomato yellow leaf curl Sardinia virus acts as a trans -dominant - negative, mutant, inhibiting viral transcription and replication. J Virol 75: 10573-10581.

Brunetti A, Tavazza M, Noris E, Tavazza R, Caciagli P, Ancora G, Crespi S, Accotto GP (1997) High expression of truncated viral Rep protein confers resistance to *Tomato yellow leaf curl virus* in transgenic tomato plants. Mol Plant-Microbe Interact 10: 571-579.

Bucher E, Lohuis D, van Poppel PMJA, Geerts-Dimitriadou C, Goldbach R, Prins M (2006) Multiple virus resistance at a high frequency using a single transgene construct. J. Gen Virol 87: 3697-3701.

Cangelosi GA, Ankenbauer RG, Nester EW (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl Acad Sci USA 87: 6708-6712.

Carvalho MF, Lazarowitz SG (2004) Interaction of the movement protein NSP and the *Arabidopsis* acetyltransferase AtNSI is necessary for cabbage leaf curl geminivirus infection and pathogenicity. J Virol 78: 11161-11171.

Castillo AG, Collinet D, Deret S, Kashoggi A, Bejarano ER (2003) Dual interaction of plant PCNA with geminivirus replication accessory protein (REn) and viral replication protein (Rep). Virology 312: 381-394.

Castillo AG, Kong LJ, Hanley-Bowdoin L, Bejarano ER (2004) Interaction between a geminivirus replication protein and the plant sumoylation system. J Virol 78: 2758-2769.

Catalanotto C, Pallota M, ReFalo P, Sach MS, Vaysie L, Macino G, Cogoni C (2004) Redundancy of the two *dicer* genes in transgene induced posttranscripcional gene silencing in *Neurospora crassa*. Mol Cell Biol 24: 2536-2545.

Chague V, Mercier JC, Guenard M, de Courcel A, Vedel F (1997) Identification of RAPD markers linked to a locus involved in quantitative resistance to TYLCV in tomato by bulked segregant analysis. Theor Appl Genet 95: 671-677.

Chalfun-Junior A, Mes JJ, Mlynarova L, Aarts MGM, Angenent GC (2003) Low frequency of T-DNA based activation tagging in *Arabidopsis* is correlated with methylation of CaMV 35S enhancer sequences. FEBS Lett 555: 459-463.

Chateau S, Sangwan RS, Sangwan-Norreel B (2000) Competence of Arabidopsis thaliana genotypes and mutants for *Agrobacterium tumefaciens*-mediated gene transfer: role of phytohormones. J Exp Bot 51: 1961-1968.

Chatterji A, Beachy RN, Fauquet CM (2001) Expression of the oligomerization domain of the replication-associated protein (Rep) of Tomato leaf curl New Delhi virus interferes with DNA accumulation of heterologous geminiviruses. J Biol Chem 276: 25631-25638.

Chatterji A, Chatterji U, Beachy RN, Fauquet CM (2000) Sequence parameters that determine specificity of binding of the replication-associated protein to its cognate site in two strains of tomato leaf curl virus-New Delhi. Virology 273: 341-350.

Chatterji A, Padidam M, Beachy RN, Fauquet CM (1999) Identification of replication specificity determinants in two strains of *Tomato leaf curl virus* from New Delhi. J Virol 73: 5481-5489.

Chellappan P, Masona MV, Vanitharani R, Taylor NJ, Fauquet CM (2004a) Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. Plant Mol Biol 56: 601-611.

Chen T, Zhou S, Sauve R, Bhatti S, Powell WA, Allen RD (2006) Genetic transformation of tomato with the wheat oxalate oxidase gene. In Plant Breeding and Evaluation. Ranney TG Editor and Moderator. SNA Research conference 51: 560-564.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115: 971-980.

Chory J, Aguilar N, Peto CA (1991) The phenotype of *Arabidopsis thaliana* det1 mutants suggests a role for cytokinins in greening. Symp Soc Exp Biol 45: 21-29.

Choudhury NR, Malik PS, Singh DK, Islam MN, Kaliappan K, Mukherjee SK (2006) The oligomeric Rep protein of *Mungbean yellow mosaic* India virus (MYMIV) is a likely replicative helicase. Nucleic Acids Res 34: 6362-6377.

Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana.* Proc Natl Acad Sci USA 97: 4985-4990.

Chyi YS, Phillips GC (1987) High efficiency *Agrobacterium-mediated* transformation of *Lycopersicon* based on conditions favorable for regeneration. Plant Cell Rep 6: 105-108.

Ciccarelli FD, Bork P (2005) The why domain mediates the response to desiccation in plants and bacteria. Bioinformatic 21: 1304-1307.

Clerot D, Bernardi F (2006) DNA helicase activity is associated with the replication initiator protein rep of *Tomato yellow leaf curl* geminivirus. J Virol 80: 11322-11330.

Cohen S, Duffus, EJ, Liu H (1989). Acquistion, interference and retention of C*ucurbit leaf curl viruses* in whiteflies. Phytopathology 79: 109-113.

Colas P, Cohen B, Jessen T, Grishina I, McCoy J, Brent R (1996) Genetic selection of peptide aptamers that recognize and inhibit cyclin dependent kinase 2. Nature 380: 548-550.

Collins R, Cheng X (2005) Structural domains in RNAi. FEBS Lett 579: 5841-5849.

Cortina C, Culianez-Macia FA (2004) Tomato transformation and transgenic plant production. Plant Cell Tiss Org Cult 76: 269-275.

Cui X, Li G, Wang D, Hu D, Zhou X (2005) A begomovirus DNAβ encoded protein binds DNA, functions as a supressor of RNA silencing, and targets the cell nucleus. J Virol 79: 10764-10775.

Cui X, Tao X, Xie Y, Fauquet CM, Zhou X (2004) A DNAβ associated with *Tomato yellow leaf curl* China virus is required for symptom induction. J Virol 78: 13966-13974.

Cuozzo M, O'Connell KM, Kaniewski W, Fang RX, Chua NH, Tumer NE (1988) Viral protection in transgenic plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Nat Biotechnology 6: 549-557.

Czosnek H, Laterrot H (1997) A worldwide survey of *Tomato yellow leaf curl viruses*. Arch Virol 147: 1391-1406.

Davis ME, Lineberger RD, Miller AR (1991) Effects of tomato cultivar, leaf age, and bacterial strain on transformation by *Agrobacterium tumefaciens.* Plant Cell Tiss Org Cult 24: 115-121.

Day AG, Bejarano ER, Buck KW, Burrell M, Lichtenstein CP (1991) Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus *Tomato golden mosaic virus*. Proc Natl Acad Sci USA 88: 6721-6725.

De Bondt A, Eggermont K, Druart P, De Vil M, Goderis I, Vanderleyden J, Broekaert WF (1994) *Agrobacterium*-mediated transformation of apple (*Malus domestica* Borkh): an assessment of factors affecting gene transfer efficiency during early transformation steps. Plant Cell Rep 13: 587-593.

De Clercq J, Zambre M, Van Montagu M, Dillen W, Angenon G (2002) An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* A. Gray. Plant Cell Rep 21: 333-340.

De Kathen A, Jacobsen H-J (1995) Cell competence for *Agrobacterium*-mediated DNA transfer in *Pisum sativum* L. Trans Res 4: 184-191.

Desbiez C, David C, Mettouchi A, Laufs J, Gronenborn B (1995) Rep protein of tomato yellow leaf curl geminivirus has an ATPase activity required for viral DNA replication. Proc Natl Acad Sci USA 92: 5640-5644.

Di Nicola-Negri E, Brunetti A, Tavazza M, Ilardi V (2005) Hairpin RNA-mediated silencing of Plum pox virus P1 and HC-Pro genes for efficient and predictable resistance to the virus. Transgenic Res 14: 989-994.

Dillen W, De Clercq J, Kapila J, Zambre M, Van Montagu M, Angenon G (1997) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J 12: 1459-1463.

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. Cell 130: 413-426.

Dogar AM (2006) RNAi dependent epigenetic marks on a geminivirus promoter. Virology J 3: 5.

Dorokhov BD, Klocke E (1997) A rapid and economic technique for RAPD analysis of plant genomic. Russ J Genet 33: 358-365.

Dry IB, Rigden JE, Krake LR, Mullineaux PM, Rezaian MA (1993) Nucleotide sequence and genome organization of tomato leaf curl geminivirus. J Gen Virol 74: 147-151.

Duan YP, Powell CA, Purcifull DE, Broglio P, Hiebert E (1997a) Phenotypic variation in transgenic tobacco expressing mutated geminivirus movement/pathogenicity (BC1) proteins. Mol PlantMicrobe Interact 10: 1065-1074.

Duan YP, Powell CA, Webb SE, Purcifull DE, Hiebert H (1997b) Geminivirus resistance in transgenic tobacco expressing mutated BC1 protein. Mol Plant-Microbe Interact 10: 617-623.

Dykxhoorn DM, Novina CD, Sharp PA (2003) Killing the messenger: Short RNAs that silence gene expression. Nat. Rev Mol Cell Biol 4: 457-467.

Eagle PA, Hanley-Bowdoin L (1997) Cis-Elements that contribute to geminivirus transcriptional regulation and efficiency of DNA replication. J Virol 71: 6947-6955.

Eagle PA, Orozco BM, Hanley-Bowdoin L (1994) A DNA sequence required for geminivirus replication also mediates transcriptional regulation. Plant Cell 6: 1157-1170.

Eckes P, Rosahl S, Schell J, Willmitzer L (1986) Isolation and characterization of a light-inducible, organ-specific gene from potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. Mol Gen Genet 205: 14-22.

Eckardt NA (2004) Host proteins guide Agrobacterium-mediated plant transformation. The Plant Cell 16: 2837-2839.

Egelkrout EM, Robertson D, Hanley-Bowdoin L (2001) Proliferating cell nuclear antigen transcription is repressed through an E2F consensus element and activated by geminivirus infection in mature leaves. Plant Cell 13: 1437-1452.

El-Bakry AA (2002) Effect of genotype, growth regulators, carbon source and pH on shoot induction and plant regeneration in tomato. In vitro Cell Dev Biol Plant 38: 501-507.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-498.

Elbashir SM, Lendeckel W, Tuschl T (2001b) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15: 188-200.

Ellul P, Garcia-Sogo B, Pineda B, Rios G, Roig LA, Moreno V (2003) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L.Mill.) is genotype and procedure dependent. Theor Appl Genet 106: 231-238.

Elmer JS, Brand L, Sunter G, Gardiner WE, Bisaro DM, Rogers SG (1988) Genetic analysis of the *Tomato golden mosaic virus*. II. The product of the AL1 coding sequence is required for replication. Nucleic Acids Res. 16: 7043-7060.

EPPO report (2006). www.eppo.org/QUARANTINE/virus/TYLC_virus/TYLCV00_map.htm).

Fargette D, Colon LT, Bouveau R, Fauquet C (1996) Components of resistance of cassava to African cassava mosaic virus. J Plant Pathol 102: 645-654.

Fauquet CM, Briddon RW, Brown JK, Moriones E, Stanley J, Zerbini M, Zhou X (2008) Geminivirus strain demarcation and nomenclature. Arch Virol 153: 783-821.

Fedorowicz O,Bartoszewski G, Stoeva P, Niemirowicz-Szczytt K (2000) *Agrobacterium*-mediated transformation of cultivated tomato with construct carrying the nucleoprotein (N) gene from tomato spotted wilt virus (TSWV). Acta physiol plant 22: 277-281.

Fillatti JJ, Kiser J, Rose R, Comai L (1987) Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technol 5: 726-730.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SA, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811.

Fontes EPB, Eagle PA, Sipe PS, Lucknow VA, Hanley-Bowdoin L (1994a) Interaction between a geminivirus replication protein and origin DNA is essential for viral replication. J Biol Chem 269: 8459-8465.

Fontes EPB, Gladfelter HJ, Schaffer RL, Petty ITD, Hanley-Bowdoin L (1994b) Geminivirus replication origins have a modular organization. Plant Cell 6: 405-416.

Frary A, Earle ED (1996) An examination of factors affecting the efficiency of *Agrobacterium* -mediated transformation of tomato. Plant Cell Rep 16: 235-240.

Frary A, Van Eck J (2005) Organogenesis from transformed tomato explants. Methods Mol Biol 286: 141-150.

Freitas-Astua J, Purcifull DE, Polston JE, and Hiebert E (2002) Traditional and transgenic strategies for controlling tomato-infecting begomoviruses. Fitopatol Bras 27: 437-449.

Friedmann M, Lapidot M, Cohen S, Pilowski M (1998) A novel source of resistance to tomato yellow leaf curl virus exhibiting a symptomless reaction to virus infection. J Am Soc Hortic Sci 123: 1004-1006.

Fuentes A, Ramos PL, Fiallo E, Callard D, Sanchez Y, Peral R, Rodriguez R, Pujol M (2006) Intron-hairpin RNA derived from replication associated protein C1 gene confers immunity to *Tomato yellow leaf curl virus* infection in transgenic tomato plants. Trans Res 15: 291-304.

Fuentes AD, Ramos PL, Sanchez Y, Callard D, Ferreira A, Tiel K, Cobas K, Rodriguez R, Borroto C, Doreste V, Pujol M (2008) A transformation procedure for recalcitrant tomato by addressing transgenic plant-recovery limiting factors. Biotechnol. J 3: 1088-1093.

Fullner KJ, Lara JC, Nester EW (1996) Pilus assembly by *Agrobacterium* T-DNA transfer genes. Science 273: 1107-1109.

Fullner KJ, Nester EW (1996) Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J Bacteriol 178: 1498-1504.

Furutani N, Yamagishi N, Hidaka S, Shizukawa Y, Kanematsu S, Kosaka Y (2007*) Soybean mosaic virus r*esistance in transgenic soybean caused by post-transcriptional gene silencing. Breed Sci 57: 123-128.

Gafni Y (2003) Tomato yellow leaf curl virus, the intracellular dynamics of a plant DNA virus. Mol plant Pathol 4: 9-15.

Gafni Y, Epel BL (2002) The role of host and viral proteins in intra- and inter-cellular trafficking of geminiviruses. Physiol Mol Plant Pathol 60: 231-241.

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

Gardiner WE, Sunter G, Brand L, Elmer JS, Rogers SG, Bisaro DM (1988) Genetic analysis of tomato golden mosaic virus: The coat protein is not required for systemic spread or symptom development. EMBO (Eur Mol Biol Organ) J 7: 899-904.

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. Curr Biol 15: 1494-1500.

Gelvin SB (2003) Agrobacterium-mediated plant transformation: the Biology behind the "Gene-Jockeying" Tool. Rev Microb Mol Biol 67: 16-37.

Ghanim M, Morin S, Czosnek H (2001a) Rate of *Tomato yellow leaf curl virus* translocation in thecCirculative transmission pathway of its vector, the whitefly *Bemisia tabaci*. Virology 91: 188-196.

Ghanim M, Morin S, Zeidan M, Czosnek H (1998) Evidence for transovarial transmission of tomato yellow leaf curl virus by its vector the whitefly *Bemisia tabaci*. Virology 240: 295-303.

Ghanim M, Rosell RC, Campbell LR, Czosnek H, Brown JK, Ullman DE (2001b) Digestive, salivary and reproductive organs of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) Biotype B. J Morphol 248: 22-40.

Ghanim M, Sobol I, Ghanim M, Czosnek H (2007) Horizontal transmission of begomoviruses between Bemisia tabaci biotypes. Arthropod-Plant Interact 1: 195-204.

Ghanim M., Czosnek H (2000) *Tomato yellow leaf curl geminivirus* (TYLCV-Is) is transmitted among whiteflies (*Bemisia tabaci*) in a sexrelated manner. J Virol 74: 4738-4745.

Gladfelter HJ, Eagle PA, Fontes EPB, Batts LA, Hanley-Bowdoin L (1997) Two domains of the AL1 protein mediate geminivirus origin recognition. Virology 239: 186-197.

Golan A, Tepper M, Soudry E, Horwitz BA, Gepstein S (1996) Cytokinin, acting through ethylene, restores gravitropism to *Arabidopsis* seedlings grown under red light. Plant Physiol 112: 901-904.

Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. Virus Res 92: 207-212.

Gopal P, Kumar PP, Sinilal B, Jose J, Yadunandam AK, Usha R (2007) Differential roles of C4 and ßC1 in mediating suppression of post-transcriptional gene silencing: Evidence for

transactivation by the C2 of *Bhendi yellow vein mosaic virus*, a monopartite begomovirus. Virus Res 123: 9-18.

Gubis J, Laichova Z, Farago J, Jurekova Z (2003) Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) in vitro. Czech J Genet Plant Breed 39: 9-14.

Gutierrez C (1999) Geminivirus DNA replication. Cellular Mol Life Sci 56: 313-329.

Ha C, Coombs S, Revill P, Harding R, Vu M, Dale J (2008) Molecular characterization of begomoviruses and DNA satellites from Vietnam: additional evidence that the New World geminiviruses were present in the Old World prior to continental separation. J Gen Virol 89: 312-326.

Haley A, Zhan X, Richardson K, Head K, Morris B (1992) Regulation of the activities of African cassava mosaic virus promoters by the AC1, AC2, and AC3 gene products. Virology 188: 905-909.

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286: 950-952.

Hamilton AJ, Brown S, Yuanhai H, Ishizuka M, Lowe A, Solis AGA, Grierson D (1998) A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. Plant J 15: 737-746.

Hammond SM (2005) Dicing and slicing: The core machinery of the RNA interference pathway. FEBS Lett 579: 5822-5829.

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404: 293-296.

Hamza S, Chupeau Y (1993) Re-evaluation of conditions for plant regeneration and *Agrobacterium*-mediated transformation from tomato (*Lycopersicon esculentum*). J Exp Bot 44: 1837-1845.

Han KH, Meilan R, Ma C, Strauss SH. (2000) An *Agrobacterium tumefaciens* transformation protocol effective on a variety of cottonwood hybrids (genus *Populus*). Plant Cell Rep 19: 315-320.

Hanley-Bowdoin L, Elmer JS, Rogers SG (1988) Transient expression of heterologous RNAs using *Tomato golden mosaic virus*. Nucleic Acids Res 16: 10511-10528.

Hanley-Bowdoin L, Elmer JS, Rogers SG (1990) Expression of functional replication protein from *Tomato golden mosaic virus* in transgenic tobacco plants. Proc Natl Acad Sci USA 87: 1446-1450.

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagor S, Robertson D (1999) *Geminiviruses*: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Plant Sci 18: 71-106.

Hanley-Bowdoin L, Settlage SB, Robertson D (2004) Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. Mol Plant Pathol 5: 149-156.

Hannon GJ (2002) RNA interference. Nature 418: 244-251.

Hansen G, Wright MS (1999) Recent advances in the transformation of plants. Trends Plant Sci 6: 226-231.

Hanson SF, Maxwell DP (1999) Trans-dominant inhibition of geminiviral DNA replication by *Bean golden mosaic geminivirus* rep gene mutants. Phytopathology 89: 480-486.

Hanson PM, Bernacchi D, Green SK, Tanksley SD, Muniyappa V, Padmaja AS, Chen HM, Kuo G, Fang D, Chen JT (2000) Mapping a wild tomato introgression associated with tomato yellow leafcurl virus resistance in a cultivated tomato line. J Am Soc Hortic Sci 125: 15-20.

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

Hehnle S, Wege C, Jeske H (2004) Interaction of DNA with the movement proteins of geminiviruses revisited. J Virol 78: 7698-7706.

Hemenway C, Fang RX, Kaniewski WK, Chua NH, Tumer NE (1988) Analysis of the mechanism of protection in transgenic plants expressing the *potato virus X* coat protein or its antisense RNA. EMBO J 7: 1273-1280.

Heyraud-Nitschke F, Shumacher S, Laufs J, Schaefer S, Schell J, Gronenborn B (1995) Determination of the origin cleavage and joining domain of geminivirus Rep proteins. Nucleic Acids Research 23: 910-916.

Höfer P, Bedford ID, Markham PG, Jeske H, Frischmuth T (1997) Coat protein gene replacement results in whitefly transmission of an insect nontransmissible geminivirus isolate. Virology 236: 288-295.

Höhnle M, Höfer P, Bedford ID, Briddon RW, Markham PG, Frischmuth T (2001) Exchange of three amino acids in the coat protein results in efficient whitefly transmission of a nontransmissible *Abutilon mosaic virus* isolate. Virology 290: 164-171.

Holford P, Hernandez N, Newbury HX (1992) Factors influencing the efficiency of T-DNA transfer during co-cultivation of *Antirrhinum majus* with *Agrobacterium tumefaciens.* Plant Cell Rep 11: 196-199.

Hong Y, Saunders K, Hartley MR,Stanley J (1996) Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants. Virology 220: 119-127.

Hong Y, Stanley J (1996) Virus resistance in *Nicotiana benthamiana* conferred by African cassava mosaic virus replication-associated protein (AC1) transgene. Mol Plant-Microbe Interact 9: 219-225.

Hong Y, Stanley J (1995) Regulation of African cassava mosaic virus complementary-sense gene expression by N-terminal sequences of the replication- associated protein AC1. J Gen Virol 76: 2415-2422.

Hoppe-Seyler F, Butz K (2000) Peptide aptamers: powerful new tools for molecular medicine. J Mol Med 78: 426-430.

Hoppe-Seyler F, Crnkovic-Mertens I, Tomai E, Butz K (2004) Peptide aptamers: specific inhibitors of protein function. Curr Mol Med 4: 529-538.

Horvath DP, Anderson JV, Chao WS, Foley ME (2003) Knowing when to grow: signals regulating bud dormancy. Trends in Plant Sci 8: 534-540.

Hou YM, Sanders R, Ursin VM, Gilbertson RL (2000) Transgenic plants expressing geminivirus movement proteins: abnormal phenotypes and delayed infection by *Tomato mottle virus* in transgenic tomatoes expressing the *Bean dwarf mosaicvirus* BV1 or BC1 proteins. Mol Plant-Microbe Interact 13: 297-308.

Hull R, Covey S (1986) Genome organization and expression of reverse transcribing elements: Variations and a theme. J Gen Virol 67: 1751-1758.

Humara JM, Lopez M, Ordas RJ (1999) *Agrobacterium tumefaciens*-mediated transformation of *Pinus pinea* L. cotyledons: an assessment of factors influencing the efficiency of uidA gene transfer. Plant Cell Rep 19: 51-58.

Hunter WB, Hiebert E, Webb SE, Tsai JK, Polston JE (1998) Location of geminiviruses in the whitefly *Bemisia tabaci* (Homoptera: Aleyrodidae). Plant Dis. 82: 1147-1151.

Hussain AF, Anfoka GH, Hassawi DS (2008) Transformation of tomato with TYLCV gene silencing construct using optimized *Agrobacterium*-mediated protocol. Biotech 7: 537-543.

Hussain M, Mansoor S, Iram S, Fatima AN, Zafar Y (2005) The nuclear shuttle protein of *Tomato leaf curl New Delhi virus* is a pathogenicity determinant. J Virol 79: 4434-4439.

Ingham DJ, Pascal E, Lazarowitz SG (1995) Both bipartite geminivirus movement proteins define viral hostrange, but only BL1 determines viral pathogenicity. Virology 207: 191-204.

Jeffrey JL, Pooma W, Petty ITD (1996) Genetic requirements for local and systemic movement of *Tomato golden mosaic virus* in infected plants. Virology 223: 208-218.

Jeske H, Lütgemeier M, Preiss W (2001) Distinct DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic geminivirus. EMBO J 20: 6158-6167.

Jin S, Song YN, Deng WY, Gordon MP, Nester EW (1993) The regulatory *vir*A protein of *Agrobacterium tumefaciens* does not function at elevated temperatures. J Bacteriol 175: 6830-6835.

Jupin I, De Kouchkovsky F, Jouanneau F, Gronenborn B (1994) Movement of *Tomato yellow leaf curl geminivirus* (TYLCV): involvement of the protein encoded by ORF C4 Virology 204: 82-90.

Jupin I, Hericourt F, Benz B, Gronenborn B (1995) DNA replication specificity of TYLCV geminivirus is mediated by the amino-terminal 116 amino acids of the Rep protein. FEBS Lett 362: 116-120.

Kalantidis K, Psaradakis S, Tabler M, Tsagris M (2002) The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. Mol Plant-Microbe Interact 15: 826-833.

Karp A, Maddock SE (1984) Chromosome variation in wheat plants regenerated from cultured immature embryos. Theor Appl Genet 67: 249-255.

Karp A, Nelson RS, Thomas E, Bright SWJ (1982) Chromosome variation in protoplast-derived potato plants. Theor Appl Genet 63: 265-272.

Kasrawi MA, Suwwan MA, Mansour A (1988) Sources of resistance to *Tomato yellow leaf curl virus* (TYLCV) in *Lycopersicon* species. Euphytica 37: 61-64.

Kheyr-Pour A, Bendahmane M, Matzeit V, Accotto GP, Crespi S, Gronenborn B (1991) *Tomato yellow leaf curl virus* from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Res 19: 6763-6769.

Knierim D, Maiss E. (2007) Application of Phi29 DNA polymerase in identification and full-length clone inculation of *Tomato yellow leaf curl Thailand* virus and *Tobacco leaf curl Thailand virus*. Arch Virol 152: 941-954.

Kong L, Hanley-Bowdoin L (2002) A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. Plant Cell 14: 1817-1832.

Kong LJ, Orozco BM, Roe JL, Nagar S, Ou S, Feiler HS, Durfee T, Miller AB, Gruissem W, Robertson D, Hanley-Bowdoin L (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. EMBO J 19: 3485-3495.

Koonin EV, Ilyina TV (1992) Geminivirus replication proteins are related to prokaryotic plasmid rolling circle DNAreplication initiator proteins. J Gen Virol 73: 2763-2766.

Koornneef M, Hanhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J (1986) Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci 45: 201-208.

Koornneef M, van Diepen JAM, Hanhart CJ, Kieboom-de Waart AC, Martinelli L, Schoenmakers HCH, Wijbrandi J (1989) Chromosomal instability in cell- and tissue cultures of tomato haploids and diploids. Euphyt 43: 179-186.

Kooter JM, Matzke MA, Meyer P (1999) Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci 4: 340-347.

Kotlitzky G, Boulton MI, Pitaksutheepong C, Davies JW, Epel B (2000) Intracellular and intercellular movement of maize streak geminivirus V1 and V2 proteins transiently expressed as green fluorescent protein fusions. Virology 274: 32-38.

Krake L, Rezaian MA, Dry IB (1998) Expression of the tomato leaf curl geminivirus C4 gene produces viruslike symptoms in transgenic plants. Mol Plant-Microb Interact 11: 413-417.

Krasnyanski S, Sandhu J, Domier LL, Buetow DE, Korban S (2001) Effect of an enhanced CaMV 35S promoter and a fruit-specific promoter on uidA gene expression in transgenic tomato plants. In Vitro Cell Dev Biol-Plant 37: 427-433.

Kunik T, Salomon R, Zamir D, Navot N, Zeidan M, Michelson I, Gafni Y, Czosnek H (1994) Transgenic tomato plants expressing the *Tomato yellow leaf curl* capsid protein are resistant to the virus. Bio/Technology 12: 500-504.

Kunik T, Mizrachy L, Citovsky V, Gafni Y (1999) Characterization of a tomato karyopherin α that interacts with the *Tomato yellow leaf curl virus* (TYLCV) coat protein. J Exp Bot 50: 731-732.

Kunik T, Palanichelvam K, Czosnek H, Citovsky V, Gafni Y (1998) Nuclear import of the capsid protein of *Tomato yellow leaf curl virus* (TYLCV) in plant and insect cells. Plant J 13: 393-399.

Kushika S (2002). Pre-culture treatment enhances transient GUS gene expression in leaf segment of *Saintpaulia ionantha* Wendl. after inoculation with *Agrobacterium tumefaciens*. Plant Biotech 19: 149-152.

Lapidot M, Friedmann M (2002) Breeding for resistance to whitefly-transmitted geminiviruses. Ann Appl Biol 140: 109-127.

Laufs J, Schumacher S, Geisler N, Jupina I, Gronenborn B (1995b) Identification of the nicking tyrosine of geminivirus Rep protein. FEBS Letters 377: 258-262.

Laufs J, Traut W, Heyraud F, Matzeit V, Rogers SG, Schell J, Gronenborn B (1995a) In vitro cleavage and joining at the viral origin of replication by the replication initiator protein of tomato yellow leaf curl virus. Proc Natl Acad Sci USA 92: 3879-3883.

Lazarowitz SG (1992) *Geminiviruses*: genome structure and genome function. Crit Rev Plant Sci 11: 327-349.

Lazarowitz SG, Wu LC, Rogers SG, Elmer JS (1992) Sequence-Specific lnteraction with the Vira1 ALI Protein ldentifies a Geminivirus DNA Replication Origin. The Plant Cell 4: 799-809.

Lazarowitz SG, Beachy RN (1999) Viral movement proteins as probes for intracellular and intercellular trafficking in plants. Plant Cell 11: 535-548.

Lazarowitz SG, Pinder AJ, Damsteegt D, Rogers SG (1989) *Maize streak virus* genes essential for systemic spread and symptom development. EMBO J 8: 1023-1032.

Lee H, Auh CK, Kim D, Lee TK, Lee S (2006) Exogenous cytokinin treatment maintains cyclin homeostasis in rice seedlings that show changes of cyclin expression when the photoperiod is rapidly changed. Plant Physiol Biochem 44: 248-252.

Levin JZ, de Framond AJ, Tuttle A, Bauer MW, Heifetz PB (2000) Methods of double-stranded RNA-mediated gene inactivation in *Arabidopsis* and their use to define an essential gene in methionine biosynthesis. Plant Mol Biol 44: 759-775.

Ling HQ, Kriseleit D, Ganal MW (1998) Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon* esculentum Mill). Plant Cell Rep 17: 843-847.

Lipp-Joao KH, Brown TA (1993) Enhanced transformation of tomato co-cultivated with *Agrobacterium tumefaciens* C58C1Rifr: pGSFR1161 in the presence of acetosyringone. Plant Cell Rep 12: 422-425.

Liu Y, Robinson DJ, Harrison BD (1998) Defective forms of cotton leaf curl virus DNA-A that have different combinations of sequence deletion, duplication, inversion and rearrangement. Journal of General Virology 79: 1501-1508.

Lomonossoff GP (1995) Pathogen derived resistance to plant viruses. Annu Rev Phytopathol 33: 323-343.

Lopez-Ochoa L, Ramirez-Prado J, Hanley-Bowdoin L (2006) Peptide aptamers that bind to a geminivirus replication protein interfere with viral replication in plant cells. J Virol 80: 5841-5853.

Lucioli A, Noris E, Brunetti A, Tavazza R, Ruzza V, Castillo AG, Bejarano ER, Accotto GP, Tavazza M (2003) *Tomato yellow leaf curl Sardinia virus* rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. J Virol 77: 6785-6798.

Luque A, Sanz-Burgos AP, Ramirez-Parra E, Castellano MM, Gutierrez C (2002) Interaction of geminivirus Rep protein with replication factor C and its potential role during geminivirus DNA replication. Virology 302: 83-94.

Mamidala P, Nanna RS (2009) Efficient *in vitro* plant regeneration, flowering and fruiting of dwarf Tomato cv. Micro-Msk. Plant Omics J 2: 98-102.

Mansoor S, Briddon RW, Zafar Y, Stanley J (2003) Geminivirus disease complexes: an emerging threat. Trends Plant Sci 8: 128-134.

Mansoor S, Khan SH, Bashir A, Saeed M, Zafar Y, Malik KA, Briddon R, Stanley J, Markham PG (1999) Identification of a novel circular single-stranded DNA associated with cotton leaf curl disease in Pakistan. Virology 259: 190-199.

Margisa R, Fusaroa AF, Smitha NA, Curtina SJ, Watsona JM, Finnegana EJ, Waterhouse PM (2006) The evolution and diversification of Dicers in plants. FEBS Letters 580: 2442-2450.

Matzke AJ, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1: 142-148.

McCormic S, Niedermeyer J, Fry B, Barnason A, Horch R, Farley R (1986) Leaf disk transformation of cultivated tomato *(L.esculentum*) using *Agrobacterium tumefaciens.* Plant Cell Rep 5: 81-84.

McCullen CA, Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu Rev Cell Dev Biol 22: 101-27.

Mendes BMJ, Boscariol RL, Mourao Filho FAA, Almeida WAB (2002) Agrobacterium-mediated genetic transformation of “Hamlin” sweet orange. Pesq Agropec Bras 37: 955-961.

Mette MF, Augsatz W, van der Winden J, Matzke MA, Matzke AJM (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO J 19: 5194-5201.

Mette MF, van der Winden J, Matzke MA, Matzke AJM (1999) Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. EMBO J 18: 241-248.

Metzlaff M, O’Dell M, Cluster PD, Flavell RB (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in *Petunia*. Cell 88: 845-854.

Missiou A, Kalantidis K, Boutla A, Tzortzakaki S, Tabler M, Tsagris M (2004) Generation of transgenic potato plants highly resistant to *Potato virus Y* (PVY) through RNA silencing. Mol Breed 14: 185-197.

Mitter N, Sulistyowati E, Dietzgen RG (2003) *Cucumber mosaic virus* infection transiently breaks dsRNA-induced transgenic immunity to Potato virus Y in tobacco. Mol Plant-Microbe Interact 16: 936-944.

Moghaieb REA, Saneka H, Fujiita K (1999) Plant regeneration from hypocotyl and cotyledon explant of tomato (*Lycopersicon* esculentum Mill.). Soil Sci Plant Nutr 45: 639-646.

Moissiard G, Voinnet O (2004) Viral suppression of RNA silencing in plants. Mol Plant Pathol 5: 71-82.

Morilla G, Castillo AG, Preiss W, Jeske H, Bejarano ET (2006) A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. J Virol 80: 3624-3633.

Morin S, Ghanim M, Zeidan M, Czosnek H, Verbeek M, van den Heuvel JFJM (1999) A GroEL homologue from endosymbiotic bacteria of the whitefly *Bemisia tabaci i*s implicated in the circulative transmission of *Tomato yellow leaf curl virus*. Virology 256: 75-84.

Moriones E, Navas-Castillo J (2000) *Tomato yellow leaf curl virus*, an emerging virus complex causing epidemics worldwide. Virus Res 71: 123-134.

Mubin M, Mansoor S, Hussain M, Zafar Y (2007) Silencing of the AV2 gene by antisense RNA protects transgenic plants against a bipartite begomovirus. Virol J 4:10.

Mullineaux PM, Rigden JE, Dry IB, Krake LR, Rezaian MA (1993) Mapping of the polycistronic RNAs of *Tomato leaf curl geminivirus*. Virology 193: 414-423.

Muniyappa V, Venkatesh HM, Ramappa HK, Kulkarni RS, Zeidan M, Tarba CY, Ghanim M, Czosnek H (2000) *Tomato leaf curl virus* from Bangalore (ToLCV-Ban4): sequence comparison with Indian ToLCV isolates, detection in plants and insects, and vector relationships. Arch Virol 145: 1583-1598.

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

Nagar S, Hanley-Bowdoin L, Robertson D (2002) Host DNA replication is induced by geminivirus infection of differentiated plant cells. Plant Cell 14: 2995-3007.

Nagar S, Pedersen TJ, Carrick KM, Hanley-Bowdoin L, Robertson D (1995) A geminivirus induces expression of a host DNA synthesis protein in terminally differentiated plant cells. Plant Cell 7: 705-719.

Navot N, Pichersky E, Zeidan M, Zamir D, Czosnek H (1991) *Tomato yellow leaf curl virus*: A whitefly-transmitted geminivirus with a single genome component. Virology 185: 151-161.

Ndunguru J, Legg JP, Fofana IBF, Aveling TAS, Thompson G, Fauquet CM (2006) Identification of a defective molecule derived from DNA-A of the bipartite begomovirus of *East African cassava mosaic virus.* Plant Pathology 55: 2-10

Nontaswatsri C, Fukai S, Goi M (2004) Revised cocultivation conditions produce effective *Agrobacterium*-mediated genetic transformation of carnation (*Dianthus caryophyllus* L.). Plant Sci166: 59-68.

Noris E, Accotto GP, Tavazza R, Brunetti A, Crespi S, Tavazza M (1996b) Resistance to tomato yellow leaf curl geminivirus in *Nicotiana benthamiana* plants transformed with a truncated viral C1 gene. Virology 224: 130-138.

Noris E, Hidalgo E, Accotto GP, Moriones E (1994) High similarity among the *Tomato yellow leaf curl virus* isolates from the West Mediterranean Basin: The nucleotide sequence of an infectious clone from Spain. Arch Virol 135: 165-170.

Noris E, Jupin I, Accotto GP, Gronenborn B (1996a) DNA-binding activity of the C2 protein of tomato yellow leaf curl geminivirus. Virology 217: 607-612.

Noris E, Vaira AM, Caciagli P, Masenga V, Gronenborn B, Accotto GP (1998) Amino acids in the capsid protein of *Tomato yellow leaf curl virus* that are crucial for systemic infection, particle formation, and insect transmission. J Virol 72: 10050-10057.

Noueiry AO, Lucas WJ, Gilbertson RL (1994) Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. Cell 76: 925-932.

Opabode JT (2006) Agrobacterium-mediated transformation of plants: emerging factors that influence efficiency. Biotech Mol Biol Rev 1: 12-20.

Orozco BM, Hanley-Bowdoin L (1996) A DNA structure is required for geminivirus replication origin function. J Virol 70: 148-158.

Orozco BM, Hanley-Bowdoin L (1998) Conserved sequence and structural motifs contribute to the DNA binding and cleavage activities of a geminivirus replication protein. J Biol Chem 273: 24448-24456.

Orozco BM, Miller AB, Settlage SB, Hanley-Bowdoin L (1997) Functional domains of a geminivirus replication protein. J Biol Chem 272: 9840-9846.

Padidam M, Beachy R, Fauquet CM (1996) The role of AV2 (precoat) and coat protein in viral replication and movement in *Tomato leaf curl geminivirus*. Virology 224: 390-404.

Padidam M, Beachy RN, Fauquet CM (1995) Tomato leaf curl geminivirus from India has a bipartite genome and coat protein is not essential for infectivity. J Gen Virol 76: 25-35.

Palanichelvam K, Kunik T, Citovsky V, Gafni Y (1998) The capsid protein of *Tomato yellow leaf curl virus* binds cooperatively to single-stranded DNA. J Gen Virol 79: 2829-2833.

Pandolfini T, Molesini B, Avesani L, Spena A, Polverari A (2003) Expression of self-complementary hairpin RNA under the control of the rolC promoter confers systemic disease resistance to *Plum pox virus* without preventing local infection. BMC Biotechnol 3:7.

Pant V, Gupta D, Choudhury NR, Malathi VG, Varma A, Mukherjee SK (2001) Molecular characterization of the Rep protein of the blackgram isolate of *Indian Mungbean yellow mosaic virus*. J Gen Virol 82: 2559-2567.

Park SH, Morris JL, Park JE, Hirchi KD, Smoth RH (2003) Efficient and genotype independent *Agrobacterium* -mediated tomato transformation. J Plant Physiol 160: 1253-1257.

Pascal E, Goodlove PE, Wu LC, Lazarowitz SG (1993). Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease. Plant Cell 5: 795-807.

Pascal E, Sanderfoot AA, Ward BM, Medville R, Turgeon R, Lazarowitz SG (1994) The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. Plant Cell 6: 995-1006.

Patil RS, Davey MR, Power JB, Cocking EC (2002) Effective protocol for *Agrobacterium*–mediated leaf disc transformation in tomato (*Lycopersicon esculentum* Mill.). Indian J Biotech 1: 339-344.

Peng WT, Lee Y-W, Nester EW (1998) The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein Are broadened by a high level of the sugar binding protein ChvE. J Bacteriol 180: 5632-5638.

Pico B, Dıez MJ, Nuez F (1996) Viral diseases causing the greatest economic losses to the tomato crop. II. The *Tomato yellow leaf curl virus*-a review. Sci Hortic 67: 151-196.

Pico B, Ferriol M, Dıez MJ, Nuez F (1999) Developing tomato breeding lines resistant to *Tomato yellow leaf curl virus*. Plant Breed 118: 537-542.

Pilartz M, Jeske H (2003) Mapping of Abutilon mosaic geminivirus minichromosomes. J Virol 77: 10808-10818.

Pilartz M, Jeske H (1992) Abutilon mosaic virus double-stranded DNA is packed into minichromosomes. Virology 189: 800-802.

Pilowsky M, Cohen S (1990) Tolerance to *Tomato yellow leaf curl virus* derived from *Lycopersicon peruvianum*. Plant Dis 74: 248-250.

Pilowsky M, Cohen S (2000) Screening additional wild tomatoes for resistance to the whitefly-borne *Tomato yellow leaf curl virus*. Acta Physiol Plant. 22: 351-353.

Plastira VA, Perdikaris AK (1997) Effect of genotype and explant type in regeneration frequency of tomato *in vitro*. Acta Hort 447: 231-234.

Pooggin M, Shivaprasad PV, Veluthambi K, Hohn T (2003) RNAi targeting of DNA virus in plants. Nat Biotechnol 21: 131-132.

Pooma W, Gillette WK, Jeffrey JL, Petty ITD (1996) Host and viral factors determine the dispensability of the coat protein for bipartite geminivirus systemic movement. Virology 218: 264-268.

Pozueta-Romero J, Houlne G, Canas L, Schantz R, Chamarro J (2001) Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium*-mediated transformation. Plant Cell Tiss Org Cult 67: 173-180.

Pramanik TK, Datta SK (1986) Plant regeneration and ploidy variation in culture derived plants *of Asclepias curassavica L.* Plant Cell Rep 3: 219- 222.

Praveen S, Mishra AK, Dasgupta A (2005) Antisense suppression of replicase gene expression recovers tomato plants from leaf curl virus infection. Plant Sci 168: 1011-1014.

Preiss W, Jeske H (2003) Multitasking in replication is common among geminiviruses. J Virol 77: 2972-2980.

Qiu D, Diretto G, Tavarza R, Giuliano G (2007) Improved protocol for *Agrobacterium*-mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene CsZCD. Sci Hort 112: 172-175.

Raj SK, Singh R, Pandey SK, Singh PB (2005) Agrobacterium-mediated tomato transformation and regeneration of transgenic lines expressing *Tomato leaf curl virus* coat protein gene for resistance against TLCV infection. Cur Sci 88: 1674-1679.

Raja P, Sanville BC, Buchmann RC and Bisaro DM (2008) Viral Genome Methylation as an Epigenetic Defense against Geminiviruses. J Virol 82: 8997-9007.

Ramesh SV, Mishra AK, Praveen S (2007) Hairpin RNA-Mediated Strategies for Silencing of Tomato Leaf Curl Virus AC1 and AC4 Genes for Effective Resistance in Plants. Oligo 17: 251-257.

Rhee Y, Gurel F, Gafni Y, Dingwall C, Citovsky V (2000) A genetic system for detection of protein nuclear import and export. Nature Biotech 18: 433-437.

Ribeiro SG, Lohuis H, Goldbach R, Prins M (2007) *Tomato chlorotic mottle virus* is a target of RNA silencing but the presence of specific short interfering RNAs does not guarantee resistance in transgenic plants. J Virol 81: 1563-1573.

Rick CM (1960) Hybridization between *Lycopersicon esculentum* and *solanum pennellii*: Phylogenetic and cytogenetic significane. Proc NAS 46: 78-82

Rigden JE, Dry IB, Mullineaux PM, Rezaian MA (1993) Mutagenesis of the virion-sense open reading frames of tomato leaf curl geminivirus. Virology 193: 1001-1005.

Rigden JE, Krake LR, Rezaian MA, Dry B (1994) ORF C4 of tomato leaf curl geminivirus is a determinant of symptom severity. Virology 204: 847-850.

Rochester DE, Kositratana W, Beachy RN (1990) Systemic movement and symptom production following agroinoculation with a single DNA of *Tomato yellow leaf curl geminivirus* (Thailand). Virology 178: 520-526.

Roche Molecular Biochemicals: DIG Application Manual for Filter Hybridization. Roche Dianostics GmbH 68298 Germany.

Rodriguez-Negrete EA, Carrillo-Tripp J and Rivera-Bustamante RF (2009) RNA Silencing against Geminivirus: Complementary action of posttranscriptional gene silencing and transcriptional gene silencing in Host Recovery. J Virology 83: 1332-1340.

Rojas MR, Hagen C, Lucas WJ, Gilbertson RL (2005) Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. Annu. Rev Phytopathol 43: 361-394.

Rojas MR, Jiang H, Salati R, Xoconostle-Cazares B, Sudarshana MR, Lucas WJ, Gilbertson RL (2001): Functional analysis of proteins involved in movement of the monopartite begomovirus, *Tomato yellow leaf curl virus*. Virology 291: 110-125.

Rom M, Antignus Y, Gidoni D, Pilowsky M, Cohen S (1993) Accumulation of tomato yellow leaf curl virus DNA in tolerant and susceptible tomato lines. Plant Dis 77: 253-257.

Roth BM, Pruss GJ, Vance VB (2004) Plant viral suppressors of RNA silencing. Virus Res 102: 97-108.

Rothenstein D, Krenz B, Selchow O, Jeske H (2007) Tissue and cell tropism of *Indian cassava mosaic virus* (ICMV) and its AV2 (precoat) gene product. Virology 359: 137-145.

Rountree MR, Selker EU (1997) DNA methylation inhibits elongation but not initiation of transcription in Neurospora crassa. Genes Dev 11: 2383-2395.

Roy R, Purty RS, Agrawal V, Gupta SC (2006) Transformation of tomato cultivar 'Pusa Ruby' with bspA gene from *Populus tremula* for drought tolerance. Plant Cell Tiss Organ Cult 84: 55-67.

Rudolph C, Schreier PH, Uhrig JF (2003) Peptide-mediated broad-spectrum plant resistance to tospoviruses. Proc Nat Acad Sci USA 100: 4429-4434.

Rybicki EP, Pietersen G (1999): Plant virus disease problems in the developing world Advances in Virus Research 53: 128-175.

Saeed M, Behjatnia SA, Mansoor S, Zafar Y, Hasnain S, Rezaian MA (2005) A single complementary-sense transcript of a geminiviral DNA beta satellite is determinant of pathogenicity. Mol Plant-Microbe Interact 18: 7-14.

Saeed M, Zafar Y, Randles JW, Rezaian MA (2007) A monopartite begomovirus- associated DNAβ satellite substitutes for the DNA B of a bipartite begomovirus to permit systemic infection. J Gen Virol 88: 2881-2889.

Safarnejad MR, Fischer R, Commandeur U (2009) Recombinant-antibody-mediated resistance against Tomato yellow leaf curl virus in Nicotiana benthamiana. Arch Virol 154: 457-467.

Saker MM, Rady MR (1999) Optimization of factors governing *Agrobacterium*-mediated transformation of the Egyptian tomato cultiva (Edkawy). Arb J Biotech 2: 53-62.

Salas MG, Park SH, Srivatanakul M, Smith RH (2001) Temperature influence on stable T-DNA integration in plant cells. Plant Cell Rep 20: 701-705.

Sanderfoot AA, Lazarowitz SG (1995) Cooperation in viral movement: The geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus of the cell periphery. Plant Cell 7: 1185-1194.

Sanderfoot AA, Lazarowitz SG (1996) Getting it together in plant virus movement cooperative interactions between bipartite geminivirus movement proteins. Trends Cell Biol 6: 353-358.

Sanford JC, Johnson SA (1985) The concept of parasite-derived resistance: deriving resistance genes from the parasites own genome. Journal of Theoretical Biology 115: 395-405.

Sangare A, Deng D, Fauquet C, Beachy R (1999) Resistance to *African cassava mosaic virus* conferred by a mutant of the putative NTP-binding domain of the Rep gene (AC1) in Nicotiana benthamiana. Mol Breed 5: 95-102.

Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan-Norreel B (1992) Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in Arabidopsis thaliana. Planta 188: 439-456.

Saunders K, Norman A, Gucciardo S, Stanley J (2004) The DNA beta satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity protein (betaC1). Virology 324: 37-47.

Schauer SE, Jacobsen SE, Meinke DW, Ray A (2002) DICER-LIKE1: blind men and elephants in Arabidopsis development. Trends Plant Sci 7: 487-491.

Seemanpillai M, Dry I, Randles J, Rezaian A (2003) Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. Mol Plant-Microbe Interact 16: 429-438.

Selker EU (1999) Gene silencing: repeats that count. Cell 97: 157-160.

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA (2005) A NAC domain protein interacts with *Tomato leaf curl virus* replication accessory protein and enhances viral replication. Plant Cell 17: 311-325.

Selth LA, Randles JW, Rezaian MA (2004) Host responses to transient expression of individual genes encoded by *Tomato leaf curl virus*. Mol Plant-Microbe Interact 17: 27-33.

Sera T (2005) Inhibition of virus DNA replication by artificial zinc finger proteins. J Virol 79: 2614-2619.

Sera T, Uranga C (2002) Rational design of artificial zinc-finger proteins using a nondegenerate recognition code table. Biochemistry 41: 7074-7081.

Settlage SB, Miller AB, Gruissem W, HanleyBowdoin L (2001) Dual interaction of a geminivirus replication accessory factor with a viral replication protein and a plant cell cycle regulator. Virology 279: 570-576.

Settlage SB, Miller B, Hanley-Bowdoin L (1996) Interactions between geminivirus replication proteins. J Virol 70: 6790-6795.

Settlage SB, See RG, Hanley-Bowdoin L (2005) Geminivirus C3 protein: replication enhancement and protein interactions. J Virol 79: 9885-9895.

Shahriari F, Hashemi H, Hosseini B (2006) Factor influencing regeneration and genetic transformation of three elite cultivars of tomato (Lycopersicon esculentum L.). Pak J Biol Sci. 9: 2729-2733.

Sharma MK, Solanke AU, Jani D, Singh Y, Sharma AK (2009) A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. J Biosci 34: 1-11.

She XP, Song XG (2006) Cytokinin- and auxin-induced stomatal opening is related to the change of nitric oxide levels in guard cells in broad bean. Physiol Plant 128: 569-579.

Shepherd DN, Mangwende T, Martin DP, Bezuidenhout M, Kloppers FJ , Carolissen CH, Monjane AL, Rybicki EP, Thomson JA (2007) *Maize streak virus*-resistant transgenic maize: a first for Africa. Plant Biotechnol J 5: 759-767.

Shivaprasad P, Thillaichidambaram P, Balaji V, Veluthambi K (2006) Expression of full-length and truncated Rep genes from *Mungbean yellow mosaic virus*-Vigna inhibits viral replication in transgenic tobacco. Virus Genes 33: 365-374.

Sigareva M, Spivey R, Willits MG, Kramer CM, Chang YF (2004) An efficient mannose selection protocol for tomato that has no adverse effect on the ploidy level of transgenic plants. Plant Cell Rep 23: 236-245.

SijenT, Vijn I, Rebocho A, van Blokland R, Roelofs D, Mol JNM, Kooter JM (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. Curr Biol 11: 436-440.

Sinisterra XH, Polston JE, Abouzid AM, Hiebert E (1999) Tobacco plants transformed with a modified coat protein of *Tomato mottle begomovirus* show resistance to virus infection. Phytopathology 89: 701-706.

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Gene expression–Total silencing by intron-spliced hairpin RNAs. Nature 407: 319-320.

Smulders MJM, Rus-Kortekaas W, Gilissen LJW (1995) Natural variation in patterns of polysomaty among individual tomato plants and their regenerated progeny. Plant Sci 106: 129-139.

Sree Ramulu K, Dijkhuis P, Roest S, Bokelmann GS, De Groot B (1986) Variation in phenotype and chromosome numher of plants regenerated from protoplasts of dihaploid and tetraploid potato. Plant Breed 97: 119-128.

Stanley J (2004) Subviral DNAs associated with geminivirus disease complexes. Vet Microbiol 98: 121-129.

Stanley J, Bisaro DM, Briddon RW, Brown JK, Fauquet CM, Harrison BD, Rybicki EP, Stenger DC (2005) Family Geminiviridae. In Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. Edited by Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. London: Elsevier Academic Press: 301-326.

Stanley J, Frischmuth T, Ellwood S (1990) Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. Proc Natl Acad Sci USA 87: 6291-6295.

Stanley J, Saunders K, Pinner MS, Wong SM (1997) Novel defective interfering DNAs associated with ageratum yellow vein geminivirus infection of *Ageratum conyzoides*. Virology 239: 87-96.

Stanley J, Townsend R (1985) Characterisation of DNA forms associated with cassava latent virus infection. Nucleic Acids Res 13: 2189-206.

Stenger DC (1994) Strain-specific mobilization and amplification of a transgenic defective-interfering DNA of the geminivirus beet curly top virus. Virology 203: 397-402.

Sudarshana MR, Wang HL, Lucas WJ, Gilbertson RL (1998) Dynamics of *Bean dwarf mosaic geminivirus* cell-to-cell and longdistance movement in Phaseolus vulgaris revealed, using the green fluorescent protein. Mol Plant-Microbe Interact 11: 277-291.

Sun H-J, Uchii S, Watanabe S, Ezura H (2006) A Highly Efficient Transformation Protocol for Micro-Tom, a Model Cultivar for Tomato Functional Genomics. Plant Cell Physiol. 47: 426-431.

Sunter G, Hartitz MD, Bisaro DM (1993) *Tomato golden mosaic virus* leftward gene expression: autoregulation of geminivirus replication protein. Virology 195: 275-280.

Sunter G, Bisaro DM (1991) Transactivation In A Geminivirus Al2 Gene Product Is Needed For Coat Protein Expression. Virology 180: 416-419.

Sunter G, Bisaro DM (1992) Transactivation of geminivirus AR1 and BR1 gene expression by the viral AL2 gene product occurs at the level of transcription. Plant Cell 4: 1321-1331.

Sunter G, Gardiner WE, Bisaro DM (1989) Identification of *Tomato golden mosaic virus*-specific RNAs in infected plants. Virology 170: 243-250.

Sunter G, Hartitz MD, Hormuzdi SG, Brough CL, Bisaro DM (1990) Genetic analysis of tomato golden mosaic virus ORF AL2 is required for coat protein accumulation while ORFAL3 is necessary for efficient DNA replication. Virology 179: 69-77.

Tan MMC, Colijn-Hooymans CM, Lindhout WH, Kool AJ (1987) A comparison of shoot regeneration from protoplasts and leaf discs of different genotypes of the cultivated tomato. Theor Appl Genet 75: 105-108.

Tenllado F, Llave C, Diaz-Ruiz JR (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. Virus Res 102: 85-96.

Tomari Y, Zamore PD (2005) Perspective: machines for RNAi. Genes Dev 19: 517-529.

Tougou M, Furutani N, Yamagishi N, Shizukawa Y, Takahata Y, Hidaka S (2006) Development of resistant transgenic soybeans with inverted repeat-coat protein genes of *Soybean dwarf virus*. Plant Cell Rep 25: 1213-1218.

Trinks D, Rajeswaran R, Shivaprasad PV, Akbergenov R, Oakeley EJ, Veluthambi K, Hohn T, Poogin MM (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. J Virol 79: 2517-2527.

Turk SCHJ, Melchers LS, den Dulk-Ras H, Regensburg-Tuink AJA, Hooykass PJJ (1991) Environmental conditions differentially affect *vir* gene induction in different *Agrobacterium* strains. Role of the VirA sensor protein. Plant Mol Biol 16: 1051-1059.

Tuschl T, Zamore PD, Lehmann R, Bartel DP, Sharp PA (1999) Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev 13: 3191-3197.

Unseld S, Frischmuth T, Jeske H (2004) Short deletions in nuclear targeting sequences of African cassava mosaic virus coat protein prevent geminivirus twinned particle formation. Virology 318: 89-100.

Unseld S, Höhnle M, Ringel M, Frischmuth T (2001) Subcellular targeting of the coat protein of *African cassava mosaic geminivirus*. Virology 286: 373-383.

Uranbey S, Sevimay CS, Kaya MD, Ipek A, Sancak C, Basalma D, Er C, Özcan S (2005) Influence of different co-cultivation temperatures, periods and media on *Agrobacterium tumefaciens*-mediated gene transfer. Biologia Plantarum 49: 53-57.

van Blokland R, van der Geest N, Mol JNM, Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. Plant J 6: 861-877.

van den Bulk RW, Löffler HJM, Lindhout WH, Koornneef M (1990) Somaclonal variation in tomato: effect of explant source and a comparison with chemical mutagenesis. Theor Appl Genet 80: 817-825.

van Roekel JSC, Damm B, Melchers LS, Hoekema A (1993) Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Rep 12: 644-647.

van Wezel RW, Liu H, Tien P, Stanley J, Hong Y (2001) Gene C2 of the monopartite geminivirus *Tomato yellow leaf curl virus*-China encodes a pathogenicity determinant that is localized in the nucleus. Mol. Plant Microbe Interact 14: 1125-1128.

van Wezel WR, Dong X, Liu H, Tien P, Stanley J, Hong Y (2002) Mutation of three cysteine residues in tomato yellow leaf curl virus-China C2 protein causes dysfunction in pathogenesis and posttranscriptional genesilencing suppression. Mol Plant-Microb Interact 15: 203-208.

Vanderschuren H, Akbergenov R, Pooggin MM, Hohn T, Gruissem EW, Zhang P (2007) Transgenic cassava resistance to *African cassava mosaic virus* is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. Plant Mol Biol 64: 549-557.

Vanitharani R, Chellappan P, Fauquet CM (2005) Geminiviruses and RNA silencing. Trends Plant Sci 10: 144-151.

Vanitharani R, Chellappan P, Fauquet CM (2003) Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. Proc Natl Acad Sci USA100: 9632-9636.

Vanitharani R, Chellappan P, Pita JS, Fauquet CM (2004) Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. J Virol 78: 9487-9498.

Vargas M, Martinez-Garcia B, Diaz-Ruiz JR, Tenllado F (2008) Transient expression of homologous hairpin RNA interferes with PVY transmission by aphids. Virology J 5: 42.

Vasudevan A, Selvaraj N, Ganapathi A, Choi CW (2007). *Agrobacterium*-mediated genetic transformation in cucumber (*Cucumis sativus* L.). Am J Biotech Biochem 3: 24-32.

Vaucheret H, Fagard M (2001) Transcriptional gene silencing in plants: targets, inducers and regulators Trends in Genet 7: 29-35.

Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert JL, Bartel DP, Crete P (2004) Endogenous *trans*-Acting siRNAs Regulate the Accumulation of *Arabidopsis* mRNAs. Mol Cell 16: 69-79.

Vidavsky F, Czosnek H (1998b) Tomato breeding lines immune and tolerant to *Tomato yellow leaf curl virus* (TYLCV) issued from *Lycopersicon hirsutum*. Phytopathology 88: 910-914.

Vidavsky F, Leviatov S, Milo J, Rabinowitch HD, Kedar N, Czosnek H (1998a) Response of tolerant breeding lines of tomato, *Lycopersicon esculentum* originating from three different sources *(L peruvianum, Lpimpinellifolium* and *Lchilense)* to early controlled inoculation by tomato yellow leaf curl virus (TYLCV). Plant Breed 117: 165-169.

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, Brigitte SS-N (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. Planta 201: 160-72.

Voinnet O (2001) RNA silencing as a plant immune system against viruses. Trends Genet 17: 449-459.

Voinnet O, Pinto YM, Baulcombe DC (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc Natl Acad Sci USA 96: 14147-14152.

von Arnim A, Stanley J (1992) Determinants of *Tomato golden mosaic virus* symptom development located on DNA B. Virology 186: 286-293.

Wang H, Buckley KJ, Yang XJ, Buchmann RC, Bisaro DM (2005) Adenosine kinase inhibition and suppression of RNA silencing by geminivirus AL2 and L2 proteins. J Virol 79: 7410-7418.

Wang MB, Abbott DC, Waterhouse PM (2000) A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to *Barley yellow dwarf virus*. Mol Plant Pathol 1: 347-356.

Wang-Pruski G, Szalay AA (2002) Transfer and expression of the genes of Bacillus branched chain alpha-oxo acid decarboxylase in *Lycopersicon esculentum*. Elec J Biotech 5: 141-153.

Wartig L, Kheyr-Pour A, Noris E, Kouchkovsky FD, Jouanneau F, Gronenborn B, Jupin I (1997) Genetic analysis of the monopartite tomato leaf curl geminivirus roles of V1, V2 andC2 ORFs in viral pathogenesis. Virology 228: 132-140.

Wassenegger M (2000) RNA-directed DNA methylation. Plant Mol Biol 43: 203-220.

Waterhouse PM, Graham MW, Wang MB (1998) Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA 95: 13959-13964.

Waterhouse PM, Wang MB, Lough T (2001) Gene silencing as an adaptive defence against viruses. Nature 411: 834-842.

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol 2: 642-652.

Yang Y, Sherwood TA, Patte CP, Hiebert E, Polston JE (2004) Use of *Tomato yellow leaf curl virus* (TYLCV) *rep* gene sequences to engineer TYLCV resistance in tomato. Phytopathology 94: 490-496.

Yin Q, Yang H, Gong Q, Wang H, Liu Y, Hong Y, Tien P (2001) *Tomato yellow curl China virus*: monopartite genome organization and agro-infection of plants. Virus Res 81: 69-76.

Zakay Y, Navot N, Zeidan M, Kedar N, Rabinowitch H, Czosnek H, Zamir D (1991) Screening *Lycopersicon* accessions for resistance to *Tomato yellow leaf curl virus*: presence of viral DNA and symptom development. Plant Disease 75: 279-281.

Zamir D, Ekstein-Michelson I, Zakay Y, Navot N, Zeidan M, Sarfatti M, Eshed Y, Harel E, Pleben T, van-Oss H, Kedar N, Rabinowitch HD, Czosnek H (1994) Mapping and introgression of a *Tomato yellow leaf curl virus* tolerance gene, TY-1. Theor Appl Genet 88: 141-146.

Zeidan M, Czosnek H (1991) Acquisition of tomato yellow leaf curl virus by the whitefly *Bemisia tabaci*. J Gen Virol 72: 2607-2614.

Zhan XC, Haley A, Richardson K, Morris B (1991) Analysis of the potential promoter sequences of African cassava mosaic virus by transient expression of the beta-glucuronidase gene. J Gen Virol 72: 2849-2852.

Zhang H, Blumwald W (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotech 19: 765-768.

Zhang P, Fütterer J, Frey P, Potrykus I, Puonti-Kaerlas J, Gruissem W (2003) Engineering virus-induced ACMV resistance by mimicking a hypersensitive reaction in transgenic cassava plants. In: Vasil IK (Ed.), Plant Biotechnology 2002 and Beyond: Proceedings of the 10th IAPTC&B Congress, Kluwer Academic Publishers: 143-146.

Zhang P, Vanderschuren H, Futterer J, Gruissem W (2005) Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. Plant Biotechnol J 3: 385-397.

Zhou X, Xie Y, Tao X, Zhang Z, Li Z, Fauquet CM (2003) Characterization of DNAβ associated with begomoviruses in China and evidence for co-evolution with their cognate viral DNA-A. J Gen Virol 84: 237-247.

Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V, Gafni Y (2007a) Suppressor of RNA silencing encoded by *Tomato yellow leaf curl virus-Israel*. Virology 358: 159-165.

Zrachya A, Kumar PP, Ramakrishnan U, Levy Y, Loyter A, Arazi T, Lapidot M, Gafni Y (2007b) Production of siRNA targeted against TYLCV coat protein transcripts leads to silencing of its expression and resistance to the virus. Trans Res 16: 385-398.

APPENDIX I: Similarity between IR/Rep sequence and TYLCVV sequence.

CLUSTAL W (1.81) multiple sequence alignment

Sequences (1:2) Aligned. Score: 92

```
IR/Reps         TGCGTCGTTGGCAGATTGGCAACCTCCTCTAGCCGATCTTCCATCGATCTGGAAAATTCC
TYLCVV          TGCGTCGTTGGCAGATTGGCAACCTCCTCTAGCCGATCTTCCATCGACCTGGAAAACTCC
                *********************************************** ******** ***

IR/Reps         ATTATCAAGCACGTCTCCGTCTTTTTCCATGTATGCTTTAACATCTGTTGAGCTTTTAGC
TYLCVV          ATGATCAAGCACGTCTCCGTCTTTTTCCATGTATGTTTTAACATCTGTTGAGCTTTTAGC
                ** ******************************** ************************

IR/Reps         TCCCTGAATGTTCGGATGGAAATGTGCTGACCTGGTTGGGGATGTGAGATCGAAGAATCT
TYLCVV          TCCCTGAATGTTCGGATGGAAATGTGCTGACCTGGTTGGGGATGTGAGGTCGAAGAATCT
                ************************************************ ***********

IR/Reps         TTGATTTTTACACTGGAATTTTCCTTCGAATTGGATGAGGACATGCAGGTGAGGAGACCC
TYLCVV          TTGATTTTTGCATTGGAATTTTCCTTCGAATTGGATGAGGACATGCAAGTGAGGAGTCCC
                ********* ** ********************************** ******** ***

IR/Reps         ATCTTCATGGAGTTCTCTGCAGATTCGGATGAATAATTTTTTAGTTGGTGTTTCTAGGGC
TYLCVV          ATCTTCGTGTAATTCCCTGCAGATTCGAATGAATAATTTATTAGTTGGGGTTTCTAAGGC
                ****** ** * *** *********** *********** ******** ******* ***

IR/Reps         TTGAATTTGTGAAAGTGCATCCTCTTTAGTTAGAGAGCAGTGTGGGTATGTGAGGAAATA
TYLCVV          TTTAATTTGGGAAAGTGCTTCTTCTTTGGTGAGAGAACAGTGTGGGTATGTGAGGAAATA
                ** ****** ******** ** ***** ** ***** ***********************

IR/Reps         GTTTTTGGCATTTATTCTGAATTTATTAGGAGGAGCCATTTTGACTTGGTCAATTGGTGT
TYLCVV          GTTTTTGGCATTTATTCTGAATTTATTTGGAGGAGCCAT--TGACT-GGTCAATCGGTGT
                *************************** ***********  ***** ******* *****

IR/Reps         CTCTCAAACTTGGCTATGCAATCGGTGTCTGGTGTCTTATTTATACCTGGACACCAAATG
TYLCVV          CTCTCAAACTTGGCTATGCAATTGGTGTCTGGGGTCTTATTTATATGTGGACACCAAATG
                ********************** ********* ************  *************

IR/Reps         GCATAATTGTAATTTATTAAATGTAATTCAAAATTCAAAATGCAATCGTGGCCATCCGTA
TYLCVV          GCATTATTGTAAATAATCATATGAAATTCAAAATTGAAATTGGTAAAGCGGCCATCCGTA
                **** ******* * ** * *** *********** *** **  *  * ***********
```

APPENDIX II: Similarity between IR/Rep sequence and TYLCVV sequence.

CLUSTAL W (1.81) multiple sequence alignment

```
Sequences (1:2) Aligned. Score:  75


Pre/Cp-hpRNA      TAAGAGACGACGTATTCCCCTGATACCTTGGGATTTGATCTCATCCGTGATCTTATCAGT
TYLCVV            GTAGAAAATACGTACTCTCCAGATACATTAGGGCACGATTTAATTCGCGATTTAATTTTA
                    *** *  ***** ** ** ***** ** **    *** * ** ** *** * **

Pre/Cp-hpRNA      GTAATTCGTGCGAAGAATTATGTCGAAGCGTCCAGCAGATATTCTCATTTCCACTCCCGT
TYLCVV            GTTATTCGTGCTAAAGATTATGTCGAAGCGTCCCGCCGATATAGTCATTTCCACTCCCGC
                  ** ******** **  ***************** ** *****  ***************

Pre/Cp-hpRNA      CTCGAAAGTACGTCGCCGTCTGAACTTCGACAGCCCATACAACAGCCGTGCTGCTGTCCC
TYLCVV            ATCCAAGGTGCGTCGCCGGCTGAATTTCGACAGCCCGTATGTCAGCCGTGCTGCTGCCCC
                   ** ** ** ******** ***** *********** **   ************** ***

Pre/Cp-hpRNA      CACTGTCCGCGCCACAAA---AGGGCAGATATGGAAGAACCGACCTGCATACAGAAAGCC
TYLCVV            CACTGTCCTCGTCACAAACAAAAGGAGGTCATGGGTGAATCGGCCCATGTACCGAAAGCC
                  ******** ** ******   * **  *  ****  *** ** **    *** *******

Pre/Cp-hpRNA      CAGGATCTACAGAATGTATAGAAGCCCTGATGTCCCTAAGGGATGTGAGGGTCCATGTAA
TYLCVV            CAGGATGTACAGAATGTACAGAAGCCCTGATGTCCCTCGTGGGTGTGAAGGCCCATGTAA
                  ****** *********** ******************   ** ***** ** ********

Pre/Cp-hpRNA      GGTCCAATCTTTCGATGCGAAGAACGATATTGGACATATGGGCAAGGTAATCTGTTTGTC
TYLCVV            GGTCCAGTCTTTTGAACAGCGTCATGATATAGCCCATGTAGGTAAGGTCATTTGTGTCTC
                  ****** ***** **   *    * ***** *  *** * ** ***** ** *** * **

Pre/Cp-hpRNA      TGACGTTACCCGTGGTATTGGGCTTACCCATCGAGTTGGCAAGCGTTTCTGTGTGAAGTC
TYLCVV            TGATGTAACACGTGGTAATGGGCTTACCCATCGTGTTGGTAAGAGGTTCTGTGTGAAGTC
                  *** ** ** ******* *************** ***** *** * **************

Pre/Cp-hpRNA      ACTTTATTTTGTCGGGAAGATCTGGATGGATGAAAATATTAAGGTTAAGAATCACACTAA
TYLCVV            TGTTTATGTGTTGGGTAAGGTGTGGATGGATGAGAACATCAAGACGAAGAATCACACAAA
                    ***** *  * ** *** * *********** ** ** ***   *********** **

Pre/Cp-hpRNA      CACCGTTTTATTCTGGATAGTTAGGGATCGGCGTCCTACTGGAACGCCTTATGATTTTCA
TYLCVV            TACAGTTATGTTTTTTTTAGTTCGTGATAGGAGGCCCTTTGGCACTCCCCAGGATTTTGG
                   ** *** * ** *   ***** * *** ** * **   *** ** **  * ******

Pre/Cp-hpRNA      GCAGGTT
TYLCVV            GCAGGTG
                  ******
```

ACKNOWLEDGEMENTS

First of all I would like to express my deep gratitude to Prof. Dr. Hans-Jörg Jacobsen and Prof. Dr. Edgar Maiß, for giving me the opportunity to join their research groups, and their supervision, enthusiastic guidance, support and encouragement throughout the way of research. This dissertation was completed with their guidence and critical comments. Especially, their suggestions have also given me ideas about my future research in Vietnam.

My special thanks are extended to the Federal Ministry of Education and Research (BMBF) of Germany which provided financial support for my studies and to German Academic Exchange Service (DAAD) for providing me a fellowship during the final phase of this research.

My acknowledgements are expressed to doctors and their assistant group in NORDSTADT Hospital for Neurology of Hannover, whose gave me the invaluable treatment and care during my hospitalized time in the year 2005. Without their sophisticated surgery, I would not have recovered and my research would not be completed.

Further, I would like to express my gratefulness to Dr. Andre Frenzel for his enthusiation advice on cloning and sequencing of *Tomato yellow leaf curl Vietnam virus.* Also, my special thanks belong to Dr. Noel Ferro Diaz and to my friend, Pham Quoc Hung for their enthusiation helpful suggestions during my research.

I greatly thank Dr. Rosana Blawid for her help in gene construction used for transformation, and to Dr. Heiko Kiesecker, DMBZ, Braunschweig-Germany for providing me the GUS construct for this research.

My special thanks are sent to Dr. Nguyen Ba Tiep, Dr. Fathi Hassan, and my friend, Mrs. Livia Saleh for their help to read through parts of this thesis.

Also my special thanks belong to Mrs. Jutta Zimmerman for her help in cloning work and to Ms. Yvonne Koleczek for her help in Enzyme-linked Immunosorbent Assay, as well as to Ms. Ines Eikenberg and Ms.Maren Wichmann for their time and assistance.

I am very much obliged to Dr. Adrea Richter for her help in initiation step of my research and to Dr. Frank Schaarschmidt for his help in the use of “GLM procedure of Statistical Analysis System” for data analysis.

My many thanks are sent to Dr. Thomas Reinard for his hornest and effective ogarnisation during my research.

I would like to take this opportunity to thank to my colleagues at the Fruits and Vegetables Research Institute (FAVRI) of Vietnam for their help in collecting samples of TYLCV in Tomato as well as the tomato seeds for this work, to my colleagues and friends in Germany, Nicole, Igor, Till, Karsten, Sascha, Thaqif, Claudia, Philip, Emily, Bernardo and Thanh Trung for their help during my research.

It is a pleasure to acknowledge all the members of the Plant Biotechnology Division, Plant Genetics Institute-Leibniz University Hannover, the members of Biotechnology and Plant Protection group, Insititute for Plant Protection-Leibniz University Hannover, for their warm co-operation during my work, as well as the Technical Assistance group for their care after my tomato plants in the greenhouse.

Also my thanks go to all member of the Production Quality-Fruit Science Section, Institute of Biological Production system, for their support me in the use of equipment during my research.

My sincere thanks belong to Tuyet Le, Quang Huy, Nguyen Huyen, Thu Huong, Hai Hong, My Nguyet, Rehana, Isabel, Sandra and all other friends, for their encouragement during my residence here, especially, during my staying in the hospital.

Last but not the least, my thanks are expressed to my parents, my brothers and my sisters from whom I get love, encouragement and hope.

CURRICULUM VITAE

Personal details

Full name**:** Dang thi Van.

Sex: Female.

Date of birth: 31.07.1964

Place of birth: Namdinh- Vietnam.

Nationality: Vietnamese.

Marital status: Single.

Education

2005-2009: Ph.D. Student in Plant Biotechnology, Hannover-University, Germany (10 Semesters).

1995-1997: M.Sc. Hanoi Agricultural University No.1, Vietnam (4 Semesters).

1983-1988: B.Sc. Hanoi Agricultural University No.1, Vietnam (9 Semesters).

1978-1981: High School, Nghiahung, Namdinh, Vietnam (6 Semesters).

1975-1978: Secondary School, Nghiabinh-Nghiahung, Namdinh, Vietnam (6 Semesters).

1970-1975: Primary School, Nghiabinh-Nghiahung, Namdinh, Vietnam (10 Semesters).

Other training

16-07 to 27-07-2003: Participant of the International training course on Biotechnology funded by InWent-Germany.

16-09 to 27-09-2002: Participant in the training of second German-Vietnam workshop on Genetic Engineering and Bioinformatics.

17-08 to 15-12-2000: Participant in the training course on Biotechnology funded by DSE, Germany.

Work experiences

From 2/2003 to 2/2004: Plant transformation in Federal Centre for Breeding Research on Cultivated Plants (BAZ) in Quedlinburg, Germany.

Since 2001: Plant Tissue Culture and Molecular Biology

Since 1998: B.Sc. thesis supervisor for under-graduate students.

Since 1988: Plant Tissue Culture.

Employment

3/2004 to 6/2005: Deputy Head of General Laboratory, Leader of Plant Biotechnology Laboratory, Fruits and Vegetables Research Institute (FAVRI formerly known as RIFAV), Vietnam.

2/1997 to 2/2004: Head of plant Biotechnology Department, Fruits and Vegetables Research Institute (FAVRI), Vietnam.

4/1993 to 1/1997: Researcher on Plant Biotechnology, Fruits and Vegetables Research Institute (FAVRI), Vietnam.

4/1988 to 3/1993: Researcher in Plant Bio-physiology department (field of biotechnology), Hanoi Agricultural University No.1, Vietnam.

Publications

Article submited to GM Crops: **Dang thi Van**, Noel Ferro, Edgar Maiss, Hans-Jörg Jacobsen (2009): Development of a simple and effective protocol for leaf disc transformation of commercial tomato cultivars via *Agrobacterium tumefaciens.* GM Crops.

Blawid R, **Van DT,** Maiss E (2008): Transreplication of a Tomato yellow leaf curl Thailand virus DNA-B and replication of a DNAß component by Tomato leaf curl Vietnam virus and Tomato yellow leaf curl Vietnam virus. *Virus Res* 136: 107-117.

RadchukVV, **Van DT**, Klocke E (2005): Multiple gene co-integration in Arabidopsis thaliana predominantly occurs in the same genetic locus after simultaneous in planta

transformation with distinct *Agrobacterium tumefaciens* strains. *Plant Science.* 168: 1515-1523.

Dang thi Van, Vu Manh Hai, Mark von Stackelberg, Bui Quang Dang, Nguyen thi Bich Hong, La thi Nguyet (2004): Evaluation genetic diversity of Longan tree in Vietnam by RAPD and AFLP method. *Vietnam Journal of Science, Technology and Economic Management* 8. (English abstract).

Dang thi Van, Vu Manh Hai, Mark von Stackelberg, Hans-Jörg Jacobsen, Bui Quang Dang, Le Viet Hung (2004): Initiation results of research for identification the linking between Isozymes and seedless characterization of Grape-fruit (Pomelo) in Vietnam. *Vietnam Journal Science and Technology* 8: 1082-1084. (English abstract).

Dang thi Van et al. (2002): Research on the improvement of techniques for nursery of in vitro plant of Pineapple (smooth Cayen) under condition in the North of Vietnam. *Results of scientific research from 2000 to 2002. Research Institute of Fruits and Vegetables* (RIFAV) 12: 114-133.

Dang thi Van, Nguyen Quang Thach, Tran Khac Thi (1999) Research on improvement for system of producing disease-free for potato breeding by in vitro culture for Red River delta. *Vietnam Journal of Science, Technology and Economic Management* 4: 178-180.

Dang thi Van, Nguyen Quang Thach, Tran Khac Thi (1997): Initiation research results on nursery for in vitro plant of potato by applying the hydroponic technique. *Scientific and Technologycal information on Fruits and Vegetables* (RIFAV) 4: 4-7.

Dang thi Van, Pham Kim Thu (1997): Propagation of *Spathiphyllum Pantin* N.E.BR by tissue culture. *Scientific and Technologycal information on Fruits and Vegetables* (RIFAV) 8: 10-13.

Dang thi Van, Pham Kim Thu (1997): Efficiency of saccarose concentration for in vitro germination of orchid seed, the case of “Ngoc Diem Dai Chau” variety of Vietnam. *Scientific and technologycal information on Fruits and Vegetables* (RIFAV) 4: 9-11.

Thesis

Dang thi Van, Nguyen Quang Thach, Tran Khac Thi (1997) Research improvement of several techniques on breeding system for virus-free in potato for Red River delta; Master thesis, *Library of Hanoi Agr. University No.1*: 110 p.

Dang thi Van, Nguyen Quang Thach, Mai thi Kim Tan (1988) Research on propagation the virus-free material of potato by tissue culture; B.Sc. thesis, *Library of Hanoi Agri. University No.1*: 80 p.

BSc. thesis supervisor:

Vu xuan Hong, Nguyen thi Nhan, **Dang thi Van**: Research on in vitro propagation of Pomelo and evaluation the stability of the material after tissue culture by isozyme; BSc. thesis, *Library of Hanoi Agr. University No.1,* 2003, 56p.

Nguyen thi ngoc Anh, Nguyen thi Nhan, **Dang thi Van**: In vitro propagation of Pomelo for the purpose of preservation and development special varieties of Vietnam; BSc. thesis, *Library of Hanoi Agr. University No.1*, 2002, 46p.

Truong thi Hue, Vu manh Hai, Nguyen Quang Sang, **Dang thi Van**: Evaluation of genetic diversity of Longan tree in Vietnam by RAPD technique; BSc. thesis, *Library of Hanoi Agr. University No.1*, 2002, 45p.

Tran thi Tiec, Nguyen thi Kim Thanh, **Dang thi Van**: Research on development the protocol for micro-propagation of Grape (vitis) in Laboratary of RIFAV; BSc. thesis, *Library of Hanoi Agr. University No.1,* 2000, 58p.

Dinh xuan Sinh, Nguyen Ly Anh, **Dang thi Van**: Research on development the protocol for in vitro propagation of Strawberry in laboratory of RIFAV; BSc. thesis, *Library of Hanoi Agr. University No. 1*, 2000, 58p.

Tran thi thu Huong, Ngo Bich Hao, **Dang thi Van**: Research results about Bananas Bunchy top in Hanoi Area; BSc. thesis, *Library of Hanoi Agr. University No.1*, 1998, 53p.

STATEMENT

I declare that this thesis is my own work and has not been submitted in any form for another degree at any university or other institution of tertiary education. Other works have always been cited and acknowledged.

Hannover 20.10.2009

Dang thi Van