Molecular characterization of Capsicum chlorosis virus, *Tomato yellow leaf curl Thailand virus*, Tobacco leaf curl Thailand virus and RNA-mediated virus resistance in *Nicotiana benthamiana* Domin.

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

Doktors der Gartenbauwissenschaften
-Dr. rer. hort.-

genehmigte Dissertation
von

Dipl.-Ing.agr. Dennis Knierim
geboren am 21.11.1974 in Lich

Angefertigt am
Institut für Pflanzenkrankheiten und Pflanzenschutz

Hannover, Februar 2007

Referent: Prof. Dr. Edgar Maiß

Koreferent: Prof. Dr. Günter Adam

Tag der Promotion: 05.02.2007

Abstract

Production of vegetables in the tropics and subtropics demands special requirements for cultivation. One reason is the occurrence of pests like insects, mites and nematodes, whereas the direct damage caused by pathogens is often not a factor for yield reduction. More serious is the possibility of virus transmission and the quantitative and qualitative yield losses triggered by virus infection. The cultivation of virus resistant plants offers the possibility to circumvent frequent pesticide applications. However, not for every crop and for every viral disease adequate natural resistant sources are available and therefore, the application of pathogen-mediated transgenic resistant may be an appropriate alternative.

As a part of a larger project in Thailand tomato plants were grown under protected cultivation. Here, disease symptoms occurred on tomato plants, which were partly caused by phytopathogenic viruses. This study presented here the molecular characterization of these tomato infecting viruses and the production of transgenic virus resistance plants by using the strategy of RNA-silencing.

By using different antisera one virus was identified as a Tospovirus with relationship to *Watermelon silver mottle virus* and *Groundnut bud necrosis virus*. For the molecular characterization, the complete genome sequence was determined. Phylogenetic analysis revealed the highest identity to Capsicum chlorosis virus (CaCV) isolates described in Australia. Beside the CaCV isolate collected at the Asian Institute of Technology in Bangkok [CaCV-(AIT)], several other Thai tospovirus isolates were identified after multiple sequence analyses as CaCV isolates. For a biological characterization, the host range of CaCV-(AIT) revealed difference to the host range described for the Australian CaCV isolates.

Moreover, two begomoviruses were identified as causal agents in diseased tomato plants. The complete genome sequences of these both viruses were determined by applying rolling circle amplification (RCA) with Phi29 DNA polymerase. Sequence analysis exhibited the presence of the bipartite *Tomato yellow leaf curl Thailand virus* (THLCTHV), as well as the infection with an unknown monopartite recombinant begomovirus, tentatively named as Tobacco leaf curl Thailand virus (TbLCTHV). The infectivity of TYLCTHV and TbLCTHV full-length clones was verified by inoculation of *N. benthamian* plants. For this the cloned DNA-A and DNA-B

components of TYLCTHV were excised by restriction enzyme digest from the respective plasmids, recircularized by ligation and subsequently amplified by Phi29 DNA polymerase. The concatameric viral copies were inoculated by particle bombardment on *Nicotiana benthamiana* plants and proved to be at least as effective as partial repeat construct inoculation, but being less laborious.

For generation of transgenic virus resistance a post-transcriptional gene silencing (PTGS) approach was used. For this purpose viral CP or N gene sequences of 423 to 548 nts were arranged as inverted repeats joined by the ST-LS1 intron. The sequences were chosen from four RNA viruses, which can infect tomato plants in South East Asia: CaCV, *Tomato spotted wilt virus* (TSWV), *Cucumber mosaic virus* (CMV) und *Tomato mosaic virus* (ToMV). For all four constructs a uniform cloning strategy was applied. After transformeation of *N. benthamiana* plants via *Agrobacterium tumefaciens* 35 independent transgenic T_0 plants were regenerated. The progeny of 23 plants was resistant and 13 CaCV, 6 TSWV, 3 CMV and 1 ToMV resistant line was selected. On selected homozygous T_2 lines the stability of virus resistance was proven.

Key words: Tospovirus, Begomovirus, RNA-mediated virus resistance, Capsicum chlorosis virus (CaCV), *Tomato yellow leaf curl Thailand virus* (TYLCTHV), *Tobacco leaf curl Thailand virus* (TbLCTHV)

Zusammenfassung

Die Produktion von Gemüse unter tropischen und subtropischen Bedingungen stellt eine besondere Herausforderung dar. Eine Ursache ist das Auftreten von Schadinsekten, wobei die direkt an den Pflanzen verursachten Schäden meist nicht der entscheidende Faktor für Ertragsverluste sind. Gravierender ins Gewicht fallen die Möglichkeiten der Virusübertragung und die durch Virusinfektionen ausgelösten quantitativen und qualitativen Ertragseinbußen. Der Anbau von virusresistenten Sorten bietet hier eine vielversprechende Maßnahme, die indirekt auch zu einer Reduktion in der Anwendung von Pflanzenschutzmitteln beitragen könnte. Da nicht für jede Kultur und nicht gegen jede Viruserkrankung ausreichend natürliche Resistenzquellen zur Verfügung stehen, kann der Einsatz einer pathogen-vermittelten transgenen Resistenz eine sinnvolle Alternative darstellen.

In Thailand wurden im Rahmen eines Verbundprojektes Tomaten unter geschützten Bedingungen in Foliengewächshäusern kultiviert. Hierbei traten Symptome auf, die unter anderem durch die Infektion mit phytopathogenen Viren ausgelöst wurden. In der hier vorgelegten Arbeit wurden diese Viren näher molekular charakterisiert, sowie die Strategie des RNA-Silencings zur Erzeugung von Virusresistenzen angewandt.

Ein Virus wurde zunächst mit Hilfe verschiedener Antiseren als ein Tospovirus mit Verwandtschaft zum *Watermelon silver mottle virus* und *Groundnut bud necrosis virus* identifiziert. Für die vollständige molekulare Charakterisierung wurde die komplette Genomsequenz bestimmt. Die phylogenetischen Analysen ergaben die größte Ähnlichkeit zu den in Australien beschriebenen Capsicum chlorosis virus (CaCV) Isolaten, so dass das thailändische Virusisolat, nach seiner Herkunft vom Asian Institute of Technology in Bangkok, als CaCV-(AIT) benannt wurde. Weitere multiple Sequenzvergleiche erlaubte die Einordnung vermeintlich verschiedener in Thailand vorkommender Tospoviren als Isolate des CaCV. Zur Bestimmung des Wirtspflanzenspektrums wurden Infektionsversuche mit CaCV-(AIT) an einem Testpflanzensortiment durchgeführt. Hier zeigten sich Unterschiede des thailändischen Virusisolates zum publizierten Wirtspflanzenkreis der bekannten australischen CaCV Isolate.

Ferner konnten zwei Begomoviren als weitere Ursache für die Erkrankung von Tomatenpflanzen identifiziert werden. Aus Blattproben wurden die kompletten

Genome der beiden unterschiedlicher Begomoviren durch „Rolling circle amplification“ mit Phi29 DNA-Polymerase zunächst angereichert und nachfolgend kloniert. Die Sequenzanalysen zeigten das Vorliegen des erwarteten bipartiten *Tomato yellow leaf curl Thailand virus* (TYLCTHV), sowie die Infektion der Tomaten mit einem bislang nicht beschriebenen neuen monopartiten rekombinanten *Begomovirus*, welches vorläufig als Tobacco leaf curl Thailand virus (TbLCTHV) benannt wurde. Die Infektiösität von TYLCTHV und TbLCTHV konnte durch Inokulation von *N. benthamiana* Pflanzen mit Volllängenklonen gezeigt werden. Hierzu wurden die klonierten DNA Komponenten von TYLCTHV, DNA-A und DNA-B, isoliert, durch Ligation rezirkularisiert und anschließend mit Hilfe der Phi29 DNA-Polymerase vermehrt. Die konkatemeren viralen Kopien wurden mittels Partikelbombardment zur Inokulation von *N. benthamiana* Pflanzen eingesetzt und führten genauso erfolgreich zur Infektion der Pflanzen wie die bislang verwendeten multimere Kopien der Begomoviren-Genome, jedoch mit dem Vorteil eines geringeren Arbeitsaufwandes.

Zur Erzeugung von Virusresistenzen wurde der Mechanismus des „Post-transkriptionalen Gene Silencings (PTGS)“ genutzt. Dazu wurden invers angeordnete virale Sequenzen des CP- bzw. N-Gens mit 423-548 Nukleotiden die durch das ST-LS1 Intron verbunden wurden, eingesetzt. Ausgewählt wurden vier RNA Viren, die im südostasiatischen Raum Tomatenpflanzen infizieren können: CaCV, *Tomato spotted wilt virus* (TSWV), *Cucumber mosaic virus* (CMV) und *Tomato mosaic virus* (ToMV). Vier nach einer einheitlichen Klonierungsstrategie entwickelte Konstrukte wurden mittels *Agrobakterium*-vermitteltem Gentransfer in *N. benthamina* transformiert. Insgesamt wurden 35 unabhängige transgene T_0-Pflanzen erhalten. Die Nachkommen von 23 Pflanzen erwiesen sich als resistent, wobei für das CaCV 13 Linien, das TSWV 6 Linien, das CMV 3 Linien sowie für das ToMV 1 Linie selektiert wurden. An ausgewählten homozygoten T_2 Linien konnte die Stabilität der Virusresistenz bestätigt werden.

Schlagworte: Tospovirus, Begomovirus, RNA-vermittelte Virusresistenz, Capsicum chlorosis virus (CaCV), *Tomato yellow leaf curl Thailand virus* (TYLCTHV), *Tobacco leaf curl Thailand virus* (TbLCTHV)

Contents

1 General introduction 1

2 The complete nucleotide sequence of a capsicum chlorosis virus isolate from *Lycopersicum esculentum* in Thailand 16

2.1 Abstract 16

2.2 Introduction 16

2.3 Material and Methods 18

2.4 Results 23

2.5 Discussion 31

3 Application of Phi29 DNA polymerase in identification and full length clone inoculation of Tomato yellow leaf curl Thailand virus and Tobacco leaf curl Thailand virus 36

3.1 Abstract 36

3.2 Introduction 37

3.3 Material and Methods 38

3.4 Results 43

3.5 Discussion 50

4 RNA-mediated virus resistance in *Nicotiana benthamiana* against four important RNA-viruses infecting *Solanum lycopersicum* in Asia 54

4.1 Abstract 54

4.2 Introduction 55

4.3 Material and Methods 57

4.4 Results 62

4.5 Discussion 67

5 General discussion 71

6 References 80

Abbreviations

%	Percent
°C	Degree Celsius
βC1	ORF 1 on the complementary-sense strand of DNA-β
χ^2	Statistical chi-square test
35S	CaMV 35S promoter
aa	Amino acids
A	Adenin
AC1	ORF 1 on the complementary-sense strand of DNA-A
AC2	ORF 2 on the complementary-sense strand of DNA-A
AC3	ORF 3 on the complementary-sense strand of DNA-A
AC4	ORF 4 on the complementary-sense strand of DNA-A
AC5	ORF 5 on the complementary-sense strand of DNA-A
AIT	Asian Institute of Technology
amiRNAs	Artificial miRNAs
A. tumefaciens	*Agrobacterium tumefaciens*
AV1	ORF 1 on the viron-sense strand of DNA-A
AV2	ORF 2 on the viron-sense strand of DNA-A
Avr gene	Avirulence gene products
BAR-gene	Resistance gene against PPT
BC1	ORF 1 on the complementary-sense strand of DNA-B
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BV1	ORF 1 on the viron-sense strand of DNA-B
C	Cytosin
cDNA	Complementary DNA
CP	Coat protein
CRA	Common region of DNA-A
CRB	Common region of DNA-B
cv.	Cultivar
DAS-ELISA	Double-Antibody-Sandwich ELISA
DNA	Deoxyribonucleic acid

dNTPs	Mix of the four deoxynucleotide triphosphates
dpi	Days past inoculation
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
et al.	Et alii
FAST RED	4-chloro-2-2-methybenzenediazonium salt
G	Guanin
Gc	Glycoprotein C-terminal
GMOs	Genetically modified organisms
Gn	Glycoprotein N-terminal
Gn/Gc	Glycoprotein precursors
ha	Hectare
HC-Pro	Helper component-proteinase
HR	Hypersensitive response
IC	Immunocapture
IGR	Intergenic region
IPM	Integrated pest management
IR	Intergenic region
IR	Inverted repeat
IRD	Iteron-related domain
kb	Kilobase
kDa	Kilodalton
LBA4404	Strain of *A. tumefaciens*
LB media	Luria-Bertoni-Medium
L protein	Large protein codes on the L RNA
L RNA	Large RNA
MES	Morpholinoethansulfonacid
mg	Milligram
miRNA	Micro RNA
ml	Milliliter
mM	Millimolar
µl	Microliter

M.M.	Molecular mass
mRNA	Messenger RNA
M RNA	Medium RNA
MS	Murashige and Skoog media
NBS-LLR	Nucleotide-binding site - leucin-rich repeat
NCBI	National Center for Biotechnology Information
ng	Nanogram
N protein	Nucleocapsid protein
npt II	Neomycin phototransferase II
NSm	Non-structural protein on M RNA
NSs	Non-structural protein on S RNA
nt	Nucleotide
n.t.	Not tested
OD_{600}	Optical density measured at 600 nm
ORF	Open reading frame
P	Statistical probability value
P25	PVX suppressor of gene silencing
pH	Negative decade logarithm of hydrogen ion concentration
PDR	Pathogen-derived resistance
Pfu	*Pyrococcus furiosus*
Phi29	DNA polymerase of the *Bacillus subtilis* bacteriophage ϕ 29
PIG	Particle inflow gun
PLACE	Plant cis-acting regulatory DNA elements
pmol	Picomolar
PPT	Phospinothricin
PTGS	Post-transcriptional gene silencing
RCA	Rolling circle amplification
RdDM	RNA-depending DNA methylation
RDP	Recombination Detection Program
RdRp	RNA-dependent RNA polymerase
Rep protein	Replication-associated protein
R gene	Resistance gene
RISC	RNA-induced silencing complex

RITS	RNAi-induced transcriptional gene silencing
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcription
siRNA	Short interfering RNA
S RNA	Small RNA
ss	Single stranded
ST-LS1 IV2	Intron 2 from the ST-LS1 gene of potato
T	Thymin
TAS-ELISA	Triple-Antibody-Sandwich
T-DNA	Transfer DNA
TGS	Transcriptional gene silencing
TPIA	Tissue-print-immuno-assay
U	Unit
UTR	Untranslated regions
VPI	Vascular puncture inoculation

Virus acronyms

AEV	*Ageratum enation virus*
AMV	*Avian myeloblastosis virus*
AYVCNV	*Ageratum yellow vein China virus*
AYVSLV	*Ageratum yellow vein Sri Lanka virus*
AYVV	*Ageratum yellow vein virus*
BYVMV	*Bhendi yellow vein mosaic virus*
CaCV	Capsicum chlorosis virus
CaMV	*Cauliflower mosaic virus*
CCSV	Calla lily chlorotic spot virus
ChiLCuV	*Chilli leaf curl virus*
CMV	*Cucumber mosaic virus*
CLCuAV	*Cotton leaf curl Alabad virus*
CLCuKV	*Cotton leaf curl Kokhran virus*
CLCuMV	*Cotton leaf curl Multan virus*
CLCuRV	*Cotton leaf curl Rajasthan virus*

CSNV	Chrysanthemum stem necrosis virus
CYVMV	*Croton yellow vein mosaic virus*
BYDV	*Barley yellow dwarf virus*
EpYVV	*Eupatorium yellow vein virus*
GBNV	*Groundnut bud necrosis virus*
GRSV	*Groundnut ringspot virus*
HLFPV	*Hibiscus latent Fort Pierce virus*
ICMV	*Indian cassava mosaic virus*
INSV	*Impatiens necrotic spot virus*
IYSV	Iris yellow spot virus
MYSV	Melon yellow spot virus
MYVV	*Malvastrum yellow vein virus*
ORSV	*Odontoglossum ringspot virus*
OYVMV	*Okra yellow vein mosaic virus*
PaLCuCNV	*Papaya leaf curl China virus*
PaLCuV	*Papaya leaf curl virus*
PBNV	*Peanut bud necrosis virus*
PCFV	Peanut chlorotic fan-spot virus
PepLCBV	*Pepper leaf curl Bangladesh virus*
PepLCV	*Pepper leaf curl virus*
PepMV	*Pepino mosaic virus*
PPV	*Plum pox virus*
PSMV	Physalis severe mottle virus
PVX	*Potato virus X*
PVY	*Potato virus Y*
PYSV	*Peanut yellow spot virus*
SbCLV	*Soybean crinkle leaf virus*
SbDV	*Soybean dwarf virus*
SLCCNV	*Squash leaf curl China virus*
SLCMV	*Sri Lankan cassava mosaic virus*
SLCYNV	*Squash leaf curl Yunnan virus*
StaLCuV	*Stachytarpheta leaf curl virus*
TbCSV	*Tobacco curly shoot virus*
TbLCJV	*Tobacco leaf curl Japan virus*

TbLCKoV	*Tobacco leaf curl Kochi virus*
TbLCTHV	Tobacco leaf curl Thailand virus
TbLCYNV	*Tobacco leaf curl Yunnan virus*
TCSV	*Tomato chlorotic spot virus*
TMV	*Tobacco mosaic virus*
ToLCBDV	*Tomato leaf curl Bangladesh virus*
ToLCBV	*Tomato leaf curl Bangalore virus*
ToLCGV	*Tomato leaf curl Gujarat virus*
ToLCKV	*Tomato leaf curl Karnataka virus*
ToLCLV	*Tomato leaf curl Laos virus*
ToLCMV	*Tomato leaf curl Malaysia virus*
ToLCNdV	*Tomato leaf curl New Delhi virus*
ToLCPV	*Tomato leaf curl Philippines virus*
ToLCSLV	*Tomato leaf curl Sri Lanka virus*
ToLCTWV	*Tomato leaf curl Taiwan virus*
ToLCV	*Tomato leaf curl virus*
ToLCVV	*Tomato leaf curl Vietnam virus*
ToMV	*Tomato mosaic virus*
TSWV	*Tomato spotted wilt virus*
TNSV	Tomato necrotic spot virus
TuMV	*Turnip mosaic virus*
TYFRV	Tomato yellow fruit ring virus
TYLCCNV	*Tomato yellow leaf curl China virus*
TYLCTHV	*Tomato yellow leaf curl Thailand virus*
TYLCV	*Tomato yellow leaf curl virus*
TYMV	*Turnip yellow mosaic virus*
TYRV	Tomato yellow ring virus
WBNV	Watermelon bud necrosis virus
WSMoV	*Watermelon silver mottle virus*
ZLCV	*Zucchini lethal chlorosis virus*

1 General introduction

Tomato plants, *Solanum lycopersicum* L. (Solanaceae), were grown in Thailand under protected cultivation. Here, the aspects of viral diseases were evaluated.
The climate in Thailand is described as tropical monsoons with daily average temperatures from 20-37°C. Over the year three seasons are distinguished: hot season from March to June, rainy season from July to October and cold season from November to February [306].

Production of vegetables under tropical and subtropical climatic conditions demands in general special requirements in the cultivation. One reason is the pressure of pests like insects, mites and nematodes, whereas the direct damage is most of the time not the crucial factor, more the potential to act as vectors for plant viruses [64, 151, 249, 261, 275, 284, 335]. When acting as virus-vectors, the threshold level for insects are drastically lower [275], because plant viruses can only be indirectly controlled by elimination of their vectors, whereas a direct control e.g. by the application of chemicals is not possible [214, 315, 335]. In conventional plant protection this results often in prophylactic application of pesticides with all the negative effects on the environment, like on the population of beneficial organisms or the development of pesticide resistant insects [33, 91, 200, 256]. In addition, there is a possible direct impact on growers and consumers health [33, 54, 91, 149, 153, 231, 306].
Beside the ecological and social effects, the application of pesticides results in external costs for the society, which were estimated up to 200% of the private costs [91]. Here, the application of integrated pest management (IPM) strategies seem to be suitable, which lead to a reduction of chemical pest control, because IPM combines biological control, host plant resistance and appropriate farming practices [13, 20, 64, 82, 149, 153, 196, 256]. The holistic view of IPM includes all classes of pests like viruses, fungi, bacteria, insects, vertebrates and weeds in a multiple pest management strategy [82]. Hereby this knowledge-intensive approach results in very complex handling [196], which is often not completely practicable for farmers and is more academically [82]. This is also demonstrated by more than 65 different definitions of IPM [82], and the continuous integration of new techniques in the IPM strategy like

biotechnology for diagnostics and the production of transgenic plants [20, 113, 196, 256].

The aspects of viral diseases in IPM are the knowledge about the virus, the understanding of the ecology and epidemiology and the evaluation of resistant cultivars [214]. When the sequence data from a virus is available, molecular detection methods like PCR and microarray techniques allow a more accurate and quick detection [74, 113, 199]. Moreover sequence data disclose the population structure and the degree of recombination of the pathogens. On the basis of the evolutionary potential it is possible to conclude on the durability of virus resistant plants [101]. From the ecological and epidemiological aspects like virus transmission routes from plant-to-plant of the respective virus different treatments are deduced, which also depend on the crop and the type of culture (protected/unprotected) [153, 214]. For instance, the traditional spraying against vectors of non-persistently transmitted viruses is ineffective for field-grown crops, whereas for the same virus, when infecting plants in greenhouses, this control strategy changes to an overall reduction of vectors [64, 214, 275]. In general, the use of virus free seeds and the immediate elimination of infected plants are advisable especially in greenhouses [96, 214]. The knowledge about the population dynamics of a plant virus vector is important, for instance when the vector does not occur in one seasonal period on the crop, a control is not necessary and ineffective spraying may be prevented [251]. Other possibilities for plant protection is simply to exclude plant virus vectors, like it can be done for greenhouse production with installation of insect-proof nets, but the efficiency depends on the type of vector [122]. However, the best strategy is to work with virus resistant cultivars [158, 161, 174, 214], because this requires no specific action in virus control by farmers [161]. In general, most of the insecticides are applied to control viral diseases, for instance in the UK 67% of insecticide sprays was expected only to control viruses [20]. But farmers will not use even virus resistant cultivars, when the yield is not as high as with conventional cultivars [153].

The plant resistance against a pathogen is common, because most plant species are resistant against most pathogens; moreover the susceptibility is the exception [67, 75, 158, 297]. Non-host resistance describes the resistance at the level of a whole plant species against a particular pathogen [75, 92, 158]. Here, two types of non-host resistance were distinguished: Type I results in no visible cell death, whereas type II is

the localized programmed cell death referred to as hypersensitive response (HR) [230]. Hence, host resistance describes the reaction of individual genotypes in a species, which is heritable [158]. Here, two reactions were distinguished, resistance to the pathogen and resistance to the disease [158]. Resistance to the pathogen, which includes indeed resistance to the disease, means that the pathogen is not able to move or establish in the host, but this might result to necrotic reactions or being even lethal induced by a HR [158, 174, 325]. However, resistance to disease symptoms or tolerance to the disease, means that the host is fully susceptible to the pathogen, here, the virus may move through the host like in a susceptible host, whereas no or only weak symptoms occur [158, 174, 325].
For the generation of virus resistant and tolerant cultivars two major antiviral strategies are used [261]. One is based on the application of natural plant resistance genes (*R* genes), whereas the other is an engineered resistance derived from the pathogen itself, hence named pathogen-derived resistance (PDR) strategy [105, 261].

R genes were introduced in cultivars from plants showing resistance. This is done by classical breeding or more rapidly with the help of marker selection techniques [105]. In addition, well-known *R* genes can be as well transferred by genetic engineering, necessary when classical breeding is not possible or to cumbersome for this plant species [105, 136, 342]. The sources of *R* genes are genotypes of the cultivated species, old varieties, land races or from related wild type species [161, 174, 315]. In addition, *R* genes are distinguished as dominant and recessive [75, 161, 261]. Here, the dominant monogenic *R* genes are simpler in screening and breeding for the protection against viruses as the recessive genes [261].

The dominant R proteins are distinguished on the basis of their predicted protein domains. All known viral dominant *R* genes belong to the NBS-LLR class (nucleotide-binding site, leucin-rich repeat), which are subdivided on the basis of their N-terminal domain [105, 119, 136, 261]. However, the exact pathway how the gene-for-gene interaction of the *R* gene and the pathogene avirulence (*Avr*) gene products interact and results in a HR is still under investigation [105, 136, 261].

Recessive resistance genes as well as dominant *R* genes are used widely in crop protection [75]. However, the recessive resistance led not to a HR, here, specific cell

factors of the host are affected, which are required by the virus to complete its replication cycle [75].
For tomato several *R* genes are described and introduced in commercial cultivars. The two *R* genes against *Tomato mosaic virus* (ToMV) (*Tm*-2^2) and *Tomato spotted wilt virus* (TSWV) (*Sw*-5) are both dominant, which results in a HR reaction [101, 261], whereas against *Tomato yellow leaf curl virus* (TYLCV) both dominant and recessive genes are described [325].

The durability of resistance or tolerance of plants against viruses is more common than resistance breaking [101] because plant viral genomes are often small, genes are tightly packed, overlapping, multifunctional and even non-coding regions have important regulatory roles and an even small change may result in a lower fitness [101]. However, resistance breaking seems to depend more on the evolutionary potential of the virus like population size and genetic exchange between them, than on the genetic nature of resistance like dominant, recessive, monogenetic or polygenetic and the expression of resistance like HR, decreased virus accumulation or tolerance [101]. Hence, a virus resistance is durable, when based on a region of the viral genome, where a genetic exchange results in a lower fitness of the strain, here, this regions indicated with a lower rate of genetic exchange and lower population variability [101].

The PDR strategy gives the opportunity to produce resistance in virtually every crop and at least theoretically to each virus. Here, the pathogen itself delivers the genetic information for the resistance [274]. The concept of PDR was first proposed from Sanford and Johnson (1985) [274]. The engineered resistance was possible since the pioneering work of plant transformation was carried out [307, 353]. The PDR is important especially for cultivars, where less natural *R* genes are available or the introduction from wild forms in cultivars is to cumbersome [261]. However, the PDR strategy is subdivided into two categories: protein-mediated and RNA-mediated resistance [261]. The first engineered resistance was protein-mediated by the expression of a viral coat protein in plants [2] and was later demonstrated for several viruses and with different viral proteins [105]. However, beside the success of the strategy, the protein-mediated strategy was not used widely in commercial crops due to biosafety concerns, because viral genome parts are introduced in the plant genome

and complete viral proteins are expressed, which may result in viral recombination or heterologous encapsidation [1, 105, 305]. Another protein-mediated strategy is the production of antibodies or antibody fragments referred to as plantibodies, here the disadvantage of introduction of viral sequences in the genome is circumvented [105]. The production of antibodies recognizing the viral polymerase or other catalytically acting viral proteins is promising, which may lead to a broad resistance [254]. However, the mechanism how the resistance is mediated is not explained till now in detail, limited progress appears and the disadvantage of transgenic protein expression still remains [105].

The RNA-mediated virus resistance overcomes these major drawbacks; here small non-coding viral sequences are sufficient to achieve resistance [105, 289]. The RNA-activated sequence-specific RNA degradation phenomenon is referred to as post-transcriptional gene silencing (PTGS), RNA silencing or RNA interference (RNAi) [178] and was first discovered as co-suppression in transgenic petunia plants [223, 313]. The first virus resistant transgenic plant using the RNA-mediated mechanism was created by Lindbo et al. [179]. The successive discovery of the RNA silencing showed that this is a general eukaryotic mechanism present in animals and plants [7, 23, 120, 194, 202, 244, 334, 355]. In addition, beside the general similarities some differences between animal and plant RNA silencing pathways were observed [23, 194, 322]. However, at least three natural pathways of the RNA silencing were distinguished [23]. Here, for all pathways small RNAs of 21-26 nt length were involved in the sequence specific recognition, whereas the sources of small RNAs, the targets and effects may differ or being the same [23, 29, 79, 118, 194, 302, 326].

In the cytoplasmic short interfering RNA (siRNA) pathway the siRNAs derives from dsRNA by Dicer cleavage, which has different sources like exogenous origin (for instance viral), endogenous origin, annealed overlapping transcripts of opposite polarity, RNA being partially double stranded, aberrant RNA amplified by RNA-dependent RNA polymerases (RdRps) to a double strand or normal RNA amplified by RdRps because being primed by siRNAs [23, 137, 186, 194, 213, 326]. The silencing of RNA in the siRNA pathway results from the binding of the siRNA to RNA, here a perfect pairing mediates a cleavage of the RNA by the RNA-induced silencing complex (RISC), whereas an imperfect pairing results in translational repression of

mRNAs by the non-cleaving RISC [194, 302]. However, the described siRNA primed amplification by RdRps of RNAs results as well in silencing due to Dicer cleavage [23, 194, 302]. In addition, siRNAs were proposed to be the mobile signal of RNA silencing in plants [28, 105, 118, 326].

The micro RNA (miRNA) pathway is very similar to the siRNA pathway, here, in analogy to the siRNA either a perfect or imperfect pairing of miRNAs (about 21-23 nt in length) results in silencing, however, it differs in the enzymes involved and the source of dsRNA from which the miRNAs derive [7, 194, 202, 302]. The short miRNAs derived from endogenous non-coding transcripts, which are referred to as miRNA genes, in two steps [194, 202, 302]. The primary miRNA is processed in the nucleus to the precursor miRNA by Drosha and Pacha in the microprocessor complex [202, 302]. This precursor miRNA of about 70 nt is than exported to the cytoplasm, where Dicer cleaves the precursor into short-lived double-stranded miRNA intermediates from which one strand is matured to the single stranded miRNA [194, 202, 302].

The third pathway describes the RNA-depending DNA methylation (RdDM) by small RNAs [23, 185, 332, 333]. Here, the small RNAs were introduced in the RNAi-induced transcriptional gene silencing (RITS) complex, which mediates sequence specific DNA methylation [86, 139, 185, 242, 288, 334]. In addition, a hallmark of RdDM is the high level of DNA methylation of asymmetric CpNpN residues [139, 144, 287]. The DNA methylation of promoter sequences results in transcriptional gene silencing (TGS) [83, 139, 144, 152, 206, 334]. In addition, DNA methylation was also proposed to inhibit elongation of transcription and results in aberrant mRNA production [21, 86, 271], but other experiments showed a proceeding of transcription downstream methylated sequences, here, the methylation of transcribed sequences was co-incident with the PTGS, but not essential for it [139, 332, 333]. In addition, the mechanism of RdDM is associated with the histone modification and heterochromatic silencing [185, 242, 329, 334, 358].

However, the RNA silencing pathways are a general effective viral defense strategy of the hosts [23, 244]. Hence, infecting viruses adapted to these RNA silencing with different counter-defensive strategies like the evasion of the RNA silencing by the

loss of being silencing-target (compartmentalization or high mutation rates), the exploit of the host silencing response by the establishment of optimal infection conditions with the help of the silencing pathways or with a direct strategy by coding for suppressors of silencing [212, 322, 326, 328]. Here, the respective viral suppressors of silencing are diverse and inhibit different steps of the pathways [23, 105, 212, 326, 328] and moreover, one virus may encoded several distinct suppressors [326]. Because of the similarity between these three pathways and the importance of RNA silencing in the host development, it was shown that the suppression of silencing causes many of the plant virus symptoms [23, 212, 326].

The currently engineered RNA-meditated virus resistance against plant viruses utilizes mainly the cytoplasmatic siRNA pathway. Here, the sequence specific viral siRNAs, which were produced from the transgenic plants bearing the resistance construct, are able to attack directly the viral RNA genome, so that the viruses failed to establish in the host, whereas in cases of DNA viruses only the viral mRNA can be degraded by this pathway. In addition, DNA viruses might be restricted as well by RNA silencing at the transcriptional level using the third pathway, the promoter silencing [326].

However, optimization essays showed that inverted repeat constructs spaced by intron or spacer sequences, mediate the highest chance to receive a virus resistance [289]. The success of these hairpin constructs could be demonstrated for different virus species and plants, for an overview see chapter 4.

However, one drawback of this strategy is the sequence specificity required for RNA degradation. It is proposed that the resistance construct should have an overall sequence identity of at least 90%, else no resistance occurs [261]. A second drawback of this technique is that the resistance can be overcome easily from other viruses or isolates, which are not recognized from the produced siRNAs, because the respective viral suppressor neutralizes the complete RNA-mediated virus resistance [209, 210]. Moreover, a double infection is possible although the plant bears a resistance construct against one of these viruses (see chapter 4). Hence, it is important to have a good knowledge about viruses occurring in a crop growing region, where this strategy should be applied.

For future virus resistance constructs it is thought to use the miRNA pathway, here, natural miRNA genes may be modified in the way, that they can recognize viral RNAs [261], which was shown very recently for the first time [227]. In addition,

another study proposed to use direct dsRNA for an exogenously application to protect plants against viruses [304].

The conclusion of all the different virus resistance strategies, if natural or PDR derived, is that in the near future most probably natural *R* genes are preferred to receive resistance, because of the biosafety discussion on transgenic plants especially in Europe [105], whereas for instance in Asia the acceptance level of genetically modified organisms (GMOs) is quite promising [4, 46]. However, well-defined natural *R* genes are able to be introduced quickly by marker based breeding techniques and the complete pathway how they interact in the plant will be unraveled as well in the next years [105]. In addition, the PTGS as a natural virus resistance strategy may be applied after further exploitation [105]. However, when no natural resistances exist, the use of transgenic virus resistant plants should be taken into consideration and putative risks when viral sequences were introduced in plants should be balanced against risks resulting from conventional practices in agriculture like the excessive use of pesticides [305].

Tomato plants *Solanum lycopersicum* L. (Solanaceae) (synonym *Lycopersicon esculentum* Mill.) are native in Central and South America [6, 18, 303]. The cherry tomato *S. lycopersicum* var. *cerasiforme* is the ancestor of the modern cultivated forms, which was selected for larger-fruited forms, however both are still extremely closely related and are free crossable [303]. The tomato crop is cultivated worldwide particularly in warm climates or in greenhouses [6]. The world production is estimated with 122 million metric tonnes (2005) on an acreage of 4 million ha (2002) [87]. In Thailand the tomato production is about 270 000 metric tonnes (2005) on 10100 ha (2002) [87]. Here, the tomato crop is cultivated in all regions of the country, whereas the major production is focused in central and northeastern Thailand [11]. The fruit is produced for fresh market and for export as canned fruit, concentrated juice, sugar preserved or dried fruit [11]. The export achieved a value of 4.62 million US $, however also tomatoes were imported for 3.52 million US $ (2004) [87].

For the production of vegetables in Thailand the use of pesticides is much more intensive compared to other agricultural products [156], for instance on only 0.36 % of the total agricultural land vegetables were grown, but the market for insecticides

used for vegetable production has a share of 18% (1993) [156]. This results in an intensive insecticide application of 29.55 kg/ha for vegetable production, and especially tomato production has an outstanding insecticide application of 42.36 kg/ha (1993) [156]. In addition, vegetables get sprayed often even one day before they are harvested [9, 22], which results in high residues, hence 27% of Thailand vegetables were described to be polluted by pesticides [306]. Often the farmers mix different pesticides without knowledge of risks of the combinations and neither apply the recommended dosages nor do an adequate self protection during application of pesticides [45, 54, 149, 156]. Thus, this inappropriate handling of pesticides is harmful directly for the sprayers and also for the consumer [15, 45, 306]. In the year 1996 a morbidity rate of 3175 patients was recorded from government hospitals leading to death for 31 persons caused by pesticides poisoning, whereas 46% originated from occupational exposure [306]. However, it is expected that only some of the workers with poisoning incidents consult hospitals, hence the cases of pesticide poisoning were expected to 39,600 a year [156].

In 2004 Thailand imported pesticides for 257.8 million US$ and exported pesticides for 37.3 million US$ [87]. The consumption of fungicides, bactericides, herbicides and insecticides has an accumulated volume of 19,382 tonnes in the year 2000 [87]. However, the side effects of the pesticide use causes external costs like current market value loss due to residues, resistance and resurgence of pests, human health damage and governmental regulation [91, 156] and polluted the environment [135]. Here, an estimation for the year 1994 calculates a volume of 228.8 million US$ external costs, whereas the pesticide market value was 247 million US$ [91, 156]. The pesticide policy in Thailand still changes, like the implementation of IPM strategies and the overall educational advertising and sensitisation of pesticide use [15, 22, 45, 148, 149, 156]. For instance since 1989 the cases of pesticide poisoning decreases continuous over the years [306]. In addition, it was shown that farmers, which apply IPM methods to control pests, were exposed only to a fifth part of pesticides than traditional farmers [149].

The major common viral diseases, which were expected to infect tomato plants in greenhouses, are from the following virus genera and the respective virus species: *Tospovirus* with the type species *Tomato spotted wilt virus* (TSWV), *Begomovirus* with the species *Tomato yellow leaf curl virus* (TYLCV), *Cucumovirus* with the type

species *Cucumber mosaic virus* (CMV) and *Tobamovirus* with type species *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) [96, 214]. In addition, the emergence of other viral diseases damaging worldwide tomato plants in greenhouses like the infections with Pepino mosaic virus (PepMV), which starts in 1999 in the Netherlands [314], is still possible [182]. However, till now this virus has not been found in Thailand. PepMV is reported to be more widespread in Europe [190, 323], North [93, 190] and South America [181, 290].

The genus *Tospovirus*, which is the unique genus in the family *Bunyaviridae* containing viruses infecting plants, comprises currently eight assigned and eight tentative species [184, 225]. The species are actually ordered in three major serogroups (TSWV, WSMoV and IYSV) and four tospovirus species have no serological relationship to any other group (INSV, PYSV, PCFV and MYSV) [58, 184, 216]. The genome comprises of three molecules of negative or ambisense ssRNAs, which exhibit totally five open reading frames (ORFs) [225].

Tospoviruses occur worldwide and are transmitted by thrips in a persistent, propagative manner [151, 214]. They cause significant yield losses in many important crops in temperate and subtropical regions [56, 341]. Reports described losses caused by tospoviruses for peanut of 50% and in some individual fields up to 100% [64]. In addition, the tomato production in Hawaii in the 1940s was limited by TSWV, because of plant losses of 75-100%. However, the losses could be reduced by crop management strategies to 10% [55].

The control of tospoviruses is difficult, because the typical vector control by using insecticides is ineffective due to the wide host range of both, the viruses and the vectors [64, 214]. Hence, the best practice is to work with resistant cultivars [64, 214]. For tomato resistant cultivars exist, which are based on the *Sw*-5 gene [41, 158, 295, 296]. Another *R* gene conferring resistance against TSWV like the *Tsw* gene is described for pepper [147]. However, also efforts in the PDR resistance strategy against TSWV in tomato plants were made (protein and RNA mediated) [44, 56, 71, 145, 146, 175, 238, 239, 252, 253, 254, 272, see chapter 4].

In Thailand three tospovirus species were currently described to infect plants. In addition, two of them, Capsicum chlorosis virus (CaCV) and *Peanut bud necrosis virus* (PBNV), were described to infect *S. lycopersicum* plants, here both species belong to the WSMoV serogroup.

CaCV isolate AIT was found in tomato plants grown in greenhouses [163, 251, see chapter 2]. Three other isolates of CaCV in Thailand were found as well in tomato (AY647437, AF134400, AY626762) and one isolate in peanut (DQ022745). CaCV-AIT causes necrotic spots on tomato leaves and necrotic ringspots on fruits [163, see chapter 2]. Here, the thrips species *Ceratothripoides claratris* (Shumsher) (Thysanoptera: Thripidae) was identified as one vector of CaCV-AIT [251]. The CaCV isolate 958, which was found in Australia, was observed to overcome the *Sw*-5 TSWV resistance gene in tomato [131, 201]. Hence, it is important to evaluate other resistance strategies against CaCV like PDR (see chapter 4). The possibility to exclude the thrips vectors with nets of different mesh sizes were tested, but seem to have application limitations in greenhouses in tropical regions [122].
The other tospovirus species like PBNV was detected once on a not protected tomato field and on many other crop plants in Thailand [347]. Whereas the third tospovirus species Physalis severe mottle virus (PSMV), which was later renamed as an isolate of Melon yellow spot virus (MYSV), was found only once in the weed *Physalis minima* L., and seems not to be common in crop plants [63].

The genus *Begomovirus* is described in the family *Geminiviridae* with the genera *Mastrevirus*, *Curtovirus* and *Topocuvirus*. The different genera are distinguished on the basis of their vector specificity, host range and genome structure [293]. *Begomoviruses* are transmitted from whiteflies in a circulative manner, infecting dicotyledonous plants and have either two or one genomic component [88, 265, 293]. The genome of begomoviruses consists of circular single-stranded DNAs of 2.5-3.0 kb in lengths, which are separately packed in the typical twin-shaped particles [265, 293]. However, most begomovirus species possess a bipartite genome, whereas in some cases satellite DNAs were found to be disease associated with monopartite viruses, which have about half of the normal DNA size [36, 293]. In total currently over 117 begomoviruses are assigned [293].
Plant viruses of the family *Geminiviridae* cause enormous yield losses in tropical and subtropical agro ecosystems [31, 42, 96, 221, 265]. Here, reports point out losses up to 95% for a tomato harvest in the Dominican Republic by a geminivirus infection [211] and in the seventies of the 20th century TYLCV infection got a serious economic problem in the Middle East, because of losses up to 100% [66].

Chemical control of vectors and avoidance of virus sources are appropriate crop cultivation methods in greenhouses, however, when the crop is cultivated under suboptimal semi-protected conditions the chemical control is ineffective [214]. In addition, with nets it is possible to exclude vectors from greenhouses as well in tropical climates, here the mesh sizes allowed enough air exchange [122]. Another possibility is to work with virus resistant or tolerant cultivars [214]. The F_1 hybrid TY20 is a commercial tomato cultivar showing a tolerant reaction [243, 267], similar to the *Ty-1* gene which mediates TYLCV tolerance [207, 354], however, even resistance genes have been described [121, 325].

Against geminiviruses the PDR strategy was applied as well, here for instance as protein mediated resistance by the expression of a truncated replication associated protein [12, 228] or the capsid protein [168]. However, several groups attempted to apply the RNA-mediated resistance [247, 317], which has recently been documented in transient assays by using the first pathway the cytoplasmatic mRNA degeneration [3, 316] and by using the third pathway the promoter silencing of the geminivirus by the RdDM mechanism [248]. In addition, transgenic geminivirus resistant plants were described which harbor a sense construct of the replication-associated protein (Rep) [16, 50] and now the first intron-hairpin RNA construct transferred to tomato plants were described, which confers TYLCV resistance [95].

In Thailand three Begomovirus species were described infecting tomato plants. These are *Tomato yellow leaf curl Thailand virus* (TYLCTHV) with several isolates [262, 280, see chapter 3], Tobacco leaf curl Thailand virus (TbLCTHV) [see chapter 3] and *Tomato yellow leaf curl Kanchanaburi virus* [293].

The virus symptoms on tomato from TYLCTHV infections are an overall stunting of the plant whereas the youngest leaves developed distorted, yellowed and curled [280].

The genus *Cucumovirus* ordered in the family *Bromoviridae* is described with the type species CMV together with two other species [268]. The CMV isolates are divided into two serogroups, whereas serogroup 1 is subdivided in subgroup 1a and 1b [74, 269]. The tripartite genome of the positive-sense RNAs with 5´-terminal cap structures has a size of about 8.8 kb and codes for 5 ORFs [268]. The host range of CMV is extremely broad. Approximately 1,000 plant species of 85 distinct plant families can be infected with CMV, including many important vegetable crops [214, 268]. CMV is transmitted by aphids in a non-persistent manner [268] and occurs

worldwide, manly in temperate regions, but is also present in tropical countries [97, 214]. It is one of the most economically important plant viruses [183, 309, 310]. For instance the yield losses reported for tomato range from 30 to 60% [309, 335].
The symptoms on tomato depend on the virus strain. The plants can be stunted and leaves may show mild, green mottling or more severe fern leaf symptoms and the leaves may twist, fail to unfold and curl up or down [214, 336]. In addition the fruit set is reduced and the fruits could be non-marketable [214].
The control of CMV by reduction of aphid vectors is not sufficient, because of the rapid transmission and the wide host range from CMV [97, 214]. Control measures like the elimination of infected plants and alternative hosts in and around the crop are recommended, whereas in protected cultivation the use of aphid-proof nets is reasonable [97, 214, 336]. The use of resistant crops based on *R* genes is limited; here only resistant or tolerant cultivars are available for some cucumber and melon species [97, 158, 174, 214] and *Arabidopsis thaliana* [300]. However, for tomato no *R* gene has been utilized [96, 349], because the CMV resistance found in wild type tomato species seems to have a polygenic nature, which is not usable for breeding [349]. Hence, special efforts were made with the PDR strategy against CMV for different plant species. First protein mediated resistance strategies have been often applied [48, 65, 103, 107, 108, 142, 159, 220, 222, 233, 234, 255, 344, 345, 349, 352] and now a RNA mediated resistance strategy of hairpin producing RNAs is used [53, 157, see chapter 4].
In Thailand CMV is described with four isolates found on cucumber and chilli [74], as well as on pepper plants (AY560555, AY560556). All isolates belong to the serotype 1b [74]. However till now no reports of infected tomato plants in Thailand exist, but it is still possible because of the wide host range of CMV.

The *Tobamovirus* genus with the type member TMV is currently described with 22 species and one tentative species [176]. The genome codes at least for 4 proteins on one linear positive sense ssRNA molecule of about 6.3-6.6 kb, which is packaged in very stable elongated rigid cylinders of 300-310 nm length, whereas possible shorter virons contains sgRNAs [176]. The natural host ranges of tobamovirus species are usually quite narrow but under experimental conditions moderate to wide [176]. The viruses are easily mechanically transmissible by contact between plants, hence handling of the crop is one important source of spread especially in greenhouse

production [176, 214]. However, no natural vectors are known, but in certain cases virons are seed-transmitted [176, 214]. Tobamoviruses occur worldwide, whereas in tomato production only isolates of ToMV and TMV contribute to yield losses [214, 336]. The virus symptoms on tomato fruits like mottling, bronzing, yellow mosaic, necrotic streaks or rings depend on the isolate, whereas the plants are generally stunted and the leaves show mild mottling to serve leaf distortion [336]. However, the importance of ToMV in protected tomato cultivation has been decreased for years, due to the development of resistant cultivars [214]. From the three R genes described against ToMV for tomato plants (*Tm*-1, *Tm*-2 and *Tm*-2^2), the Tm-2^2 R-gene is used widely in tomato breeding, because it confers a remarkable stable resistance over the years [117, 158, 170, 205]. It was shown, that the *Tm*-2^2 resistance gene compared to the *Tm*-2 genes recognizes more than one domain of the viral movement protein [337], which is the avirulence gene for both resistance genes to induce the hypersensitive response [47, 338]. However, few resistance breaking strains were observed against the *Tm*-2^2 R-gene [47, 338], but the isolates were less fit and virulent than the wild-types [158, 170].
In Thailand three tobamovirus species are currently described to infect plants. One is the type species TMV (AY633749) found in tobacco plants, which is also able to infect capsicum. The two other species *Hibiscus latent Fort Pierce virus* (HLFPV) (AY560554, AY560557) and *Odontoglossum ringspot virus* (ORSV) (AY376394) are found in ornamental plants like hibiscus and orchids, respectively.

The presented work was carried out as part of a larger study, which aims to establish an approach to sustainable vegetable production in the humid tropics. In this study the aspect of plant viral diseases was evaluated. The overall objective was to clarify the status of plant viruses, which occur in the protected cultivation of tomato plants in the year 2001 to 2004 in Thailand at the Asian Institute of Technology (AIT) and the evaluation of the PDR resistance strategy as a tool for virus defence.
The objectives of the chapter 2 were the determination of the complete nucleotide sequence of the CaCV-[AIT], which causes many problems in the tomato cultivation, and the evaluation of its host spectrum. Now the first complete genome sequence of a CaCV isolate is available and with this data it is the fifth complete determined genome of a tospovirus species. In addition, this effort may be helpful for the

production of an infectious tospovirus full-length clone in the future for application in reverse genetic analysis.

The chapter 3 describes the analysis of DNA viruses, which infect tomato plants at the AIT beside the tospoviruses. Here, the complete genome of the expected bipartite TYLCTHV and a proposed new monopartite geminivirus referred to as TbLCTHV were determined. For both geminivirus species infectious full-length clones were established. In addition, for the sequence analysis and infection tests rolling circle amplification by Phi29 DNA-polymerase was applied, which allows now a more rapid analysis of geminiviruses. Here, the full-length clones will be needed for further virus resistance tests, because geminiviruses are not easy mechanical transmittable.

The main objective of chapter 4 was the application of the RNA mediated virus resistance by using the inverted repeat technique against possible and occurring viruses infecting tomato plants in Thailand. Here, four different RNA viruses out of three virus genera were selected. For each of the viruses the resistance constructs were tested on transgenic homozygous *Nicotiana benthamiana* T_2 lines, which showed virus resistance against the respective virus. The single resistance constructs could be combined in further experiments in one construct and transformed into tomato plants [44, 90, 264].

2 The complete nucleotide sequence of a capsicum chlorosis virus isolate from *Lycopersicum esculentum* in Thailand[1]

2.1 Abstract

The complete nucleotide sequence of a tospovirus isolated from *Lycopersicum esculentum* in Thailand was determined. The L RNA comprises of 8912 nt and codes for the RNA-dependent RNA-polymerase (RdRp) (2877 aa). Two ORFs are located on the M RNA (4823 nt) encoding the non-structural (NSm) protein (308 aa) and the viral glycoprotein precursors (Gn/Gc) (1121 aa) separated by an intergenic region of 433 nt. ORFs coding for the non-structural (NSs) and nucleocapsid (N) protein, 439 aa and 275 aa, respectively, were identified on the S RNA (3477 nt) separated by an intergenic region of 1202 nt. The N protein of the Thailand isolate was most closely related to that of capsicum chlorosis virus (CaCV), sharing an amino acid sequence identity of 92.7%. Additionally, multiple sequence analyses revealed significant similarities to tospoviruses of the species *Watermelon silver mottle virus* and to several putative tospovirus entries in GenBank. Based on these alignments it is proposed to refer to all these different viruses as isolates of CaCV.

2.2 Introduction

Plant viruses belonging to the genus *Tospovirus* occur worldwide and cause significant yield reductions in many important crops [341]. Tospoviruses form a unique genus in the family *Bunyaviridae*, having a tripartite genome of ssRNA molecules comprising a total of five open reading frames (ORFs) [62, 225]. The large (L) RNA with a negative polarity encodes the RdRp (L protein) [72]. Both the medium (M) RNA and small (S) RNA have two ORFs in ambisense orientation separated by an intergenic region (IGR) [164]. The ORF in the viral sense strand of the M RNA codes for a non-structural protein (NSm) involved in cell-to-cell movement of the virus [165], whereas the ORF in the viral complementary strand

[1] Published as Knierim D, Blawid R, Maiss E (2006) in Arch Virol 151: 1761-1782 [163]; The nucleotide sequence data reported in this chapter are available in the GenBank database under accession numbers: DQ256123 (S RNA), DQ256125 (M RNA) and DQ256124 (L RNA)

codes for the glycoprotein precursor (Gn/Gc) [164]. The glycoproteins Gn and Gc are located on the virus particle membrane and are required for the circulative propagative transmission of tospoviruses by various thrips species (Thysanoptera) [151, 286, 340]. The ORF in the viral sense strand of the S RNA codes for a non-structural (NSs) protein, identified as a suppressor of gene silencing [301], and the ORF in the viral complementary strand encodes the nucleocapsid (N) protein [73].

The tospovirus RNAs share another characteristic structural feature in that their eight 5′ terminal nucleotides (AGAGCAAU…) are perfectly conserved and reverse complementary to the 3′ end (UCUCGUUA…) [57, 160, 225]. These reverse complementary ends lead to non-covalently closed, pseudo-circularized RNAs (panhandle structures), which are encapsidated by the N protein and packaged together with the L protein into quasi-spherical enveloped particles [225].

The genus *Tospovirus* currently includes eight assigned and eight tentative species [184, 225]: *Tomato spotted wilt virus* (TSWV) [38], *Tomato chlorotic spot virus* (TCSV) [69], *Groundnut ringspot virus* (GRSV) [69], *Impatiens necrotic spot virus* (INSV) [172], *Groundnut bud necrosis virus* (GBNV) [258], *Watermelon silver mottle virus* (WSMoV) [350], *Peanut yellow spot virus* (PYSV) [276], *Zucchini lethal chlorosis virus* (ZLCV) [27], Chrysanthemum stem necrosis virus (CSNV) [27], Iris yellow spot virus (IYSV) [61], Peanut chlorotic fan-spot virus (PCFV) [51, 58], Melon yellow spot virus (MYSV) [160], Watermelon bud necrosis virus (WBNV) [143, 285], Tomato yellow fruit ring virus (TYFRV) [346] {synonym: Tomato yellow ring virus (TYRV) [123]}, Calla lily chlorotic spot virus (CCSV) [52, 184] and Capsicum chlorosis virus (CaCV) [201].

A major criterion for tospovirus species demarcation is that the N protein amino acid sequence should have less than 90% identity with that of any other known tospovirus species [106]. Whereas tospoviruses were formerly subdivided into serogroups I to V, this system has recently been replaced with a system that refers to a type species in a serogroup [58, 216]. Currently, there are three major serogroups: TSWV (TSWV, GRSV, TCSV, CSNV and ZLCV), WSMoV and IYSV (IYSV and TYFRV/TYRV). In addition, there are four tospovirus species (INSV, PYSV, PCFV and MYSV) whose members are not serologically related to one another or to those of any other species and are thus regarded as individual serotypes [184]. The major serogroup WSMoV includes five tospovirus species, all of them occurring in Asia: GBNV, WBNV (both in India), WSMoV (Japan, Taiwan), CCSV (Taiwan) and CaCV

(Australia, Thailand). A WSMoV serogroup tospovirus infecting capsicum and tomato in Queensland, Australia, was described as CaCV [201]. In addition, a CaCV isolate infecting tomato in Thailand was transmitted by the thrips species *Ceratothripoides claratris* (Shumsher) (Thysanoptera: Thripidae), which had previously not been shown to be a tospovirus vector [251].

Complete genome sequences have been reported for members of five tospovirus species: TSWV [72, 73, 164], INSV [70, 173, 319], GBNV [109, 277, 278], WSMoV [57, 59, 351] and MYSV [160, 232, AB061774]. For CaCV only partial genome sequence information is available [201, 251].

In this paper, we report on the host range and the complete nucleotide sequence of a WSMoV serogroup tospovirus isolate from tomato in Thailand. This isolate was identified here as a CaCV isolate (referred to here as CaCV-AIT). In addition, analyses of the nucleotide sequences of CaCV and several putative tospovirus isolates available from GenBank revealed striking similarities, suggesting that they all be considered isolates of CaCV.

2.3 Materials and methods

Plant material, virus inoculation and host range study

Tomato plants (*L. esculentum* cv. King Kong 2), which were cultivated under protected conditions in greenhouses at the Asian Institute of Technology (AIT) in Bangkok, Thailand, showed necrotic spots on leaves and necrotic ringspots on fruits. Leaves of symptomatic plants were ground in buffer (0.05 M Na/K phosphate, pH 7.0, containing 1 mM EDTA and 5 mM Na-DIECA) and a spatula tip of charcoal. The plant sap was used for mechanical inoculation of *Nicotiana benthamiana* Domin. using Celite 545 as an abrasive. Subsequently, the virus was maintained in *N. benthamiana* by a series of mechanical transmissions.

In host range studies plants were mechanically inoculated as described above by using leaf extracts from *N. benthamiana*. Four seedlings of each test plant species were tested twice, whereby virus-inoculated *N. benthamiana* plants served as positive controls and mock-inoculated plants as negative controls.

The CaCV isolate AIT is available from “Deutsche Sammlung von Mikroorganismen und Zellkulturen” (DSMZ, Braunschweig, Germany; PV-0864).

ELISA

The TospoBroadRange antiserum (LOEWE, Cat. No. 07507) was used for detection of TSWV and INSV serogroup isolates and the compound direct ELISA test kit (AGDIA®, Cat. No. SRA 61500) for detection of the WSMoV serogroup isolates. They were both used in DAS-ELISA formats following the supplier's protocols.

RNA extraction

Three different RNA purification protocols were performed to obtain RNAs covering the whole genome of CaCV. First, total RNA was extracted from original infected tomato leaves (250 mg) according to Verwoerd et al. [324], including a DNA digest [294] and an additional purification step using the RNeasy MinElute Cleanup system (Qiagen). This RNA extract was used in reverse transcriptase (RT)-PCR to detect CaCV-AIT in tomato leaves.

Subsequently, CaCV-AIT was maintained in *N. benthamiana*, and RNA was extracted using silica particles as binding matrix [204] for RT-PCR experiments. This quick method permitted amplification of long CaCV fragments from infected *N. benthamiana* plants.

As a third method an imunocapture (IC)-RNA extraction from infected *N. benthamiana* was used, mainly for amplification of the CaCV-AIT RNA termini. For this purpose, 5 µl of polyclonal antibodies (AGDIA® Kit) were diluted in 1 ml of coating buffer (AGDIA®) and bound to 0.12 g of CF11 cellulose (Whatman) in a 1.5-ml reaction tube for 4 h at room temperature, followed by three washing steps (wash buffer, AGDIA®) and an overnight blocking step at 4°C in 1 ml of PBS containing 0.2% bovine albumin (Sigma, Fraction V). Plant material of mechanically inoculated *N. benthamiana* (0.5 g) was ground in General Extract buffer (2.5 ml) according to AGDIA's protocol. The sap was centrifuged for 90 sec (12,000 g) and 1 ml of the supernatant was added to the antibody-coated CF11 cellulose. After overnight incubation (4°C) and washing (4x), the IC-RNA extraction was continued at the step of the DNA digest of the modified Verwoerd method using a volume of 300 µl [294].

Amplification conditions

Both one-step and two-step RT-PCRs were performed for amplification of the RNA genome. For one-step RT-PCR, 2 U of avian myeloblastosis virus reverse transcriptase (AMV-RT) and a heat stable DNA polymerase purified according to Pluthero [245] were used. In the two-step procedure, cDNA synthesis was done using Expand Reverse Transcriptase (Roche) with an antisense primer according to the manufacturer's protocol in a 20 µl reaction volume. For PCR, 0.4 µl of the cDNA was incubated in a 20 µl reaction volume with Phusion™ high-fidelity DNA polymerase (Finnzymes, 0.4 U) using an extension time of 35 sec per 1 kb of PCR fragment. The reactions were carried out according to the manufacturer's recommendations. If necessary, an unspecific annealing step at 37°C was included and repeated 5 times before the optimal annealing temperature was chosen (Thermocycler T3, Biometra). Prior to re-amplification of PCR fragments, DNA bands were purified from agarose gels (Qiagen; Gel Extraction Kit).

Amplification strategy, cloning and sequencing

After serological assignment of the isolate CaCV-AIT to the WSMoV serogroup, a one-step RT-PCR was performed using primers generated from internal regions of the known N gene sequences of WSMoV and CaCV. Fragments 1 and 2 (Fig. 1) were amplified using total RNA extracts from the original infected tomato plants. Subsequently, a strategy was designed to amplify the whole genome of CaCV-AIT in two main steps.

In a first step the 5' and 3' termini of the S, M and L RNAs were amplified using primer J13 [63], which contains the eight nucleotides that are highly conserved in all known tospoviruses. Six different fragments were amplified using primer J13 in combination with one of six primers derived from conserved regions in tospovirus RNAs at a distance of 400 to 800 nt from each terminus. The following primers were used: TTCCCTTGAGATGTTCTCTCTCCA for the S RNA 5′ end (fragment 3), CACTGTTCCATGTAGGCATTAT for the M RNA 5′ end (fragment 5), GGTTACC ATCGAGTTCTTCAGG for the M RNA 3′ end (fragment 6), GTACAAGAGCCAT TTAAAGCAG for the L RNA 5′ end (fragment 7) and GCATCTACTGAAACTTT CCAATC for the L RNA 3′ end (fragment 8) (Fig. 1). The S RNA 3′ end (fragment

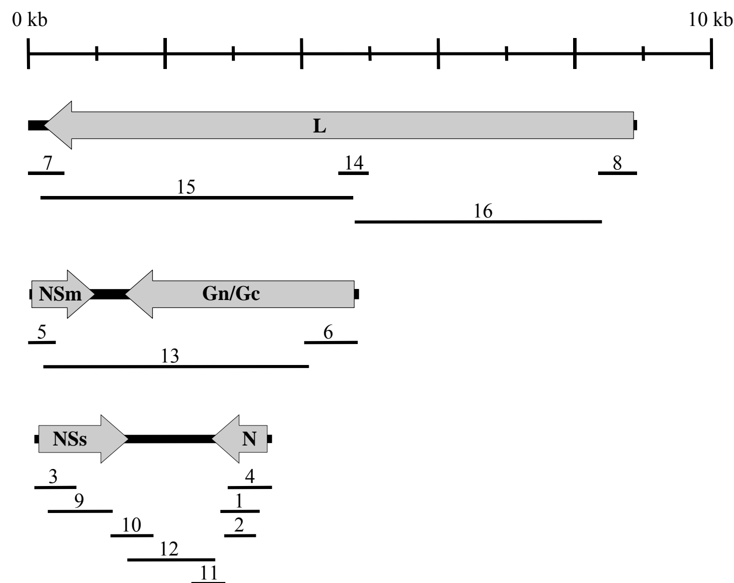


Fig. 1. Scheme of the CaCV-AIT genome and positions of the cloned fragments

4) was generated using a specific primer designed from the obtained N gene sequence and primer J13 (Fig. 1). All fragments were amplified in a one-step RT-PCR procedure using IC-RNA from infected *N. benthamiana*.

In a second step, the interior missing sequences between the 5’ and 3’ ends were amplified by two-step RT-PCR. Total RNA was extracted from infected *N. benthamiana* applying the silica-based method. Three internal fragments were generated from the S RNA. From each new sequenced fragment a specific primer was derived. In this way, fragments 9, 10 and 11 (Fig. 1) were obtained by ‘primer walking’. The primer (ACTTTCCCAATTTGTTCATGCATA) for fragment 9 was used together with the corresponding primer (TTTGAACACAATGATCGGCTTCT) generated from conserved regions of tospoviruses, whereas for fragment 10 the specific primer (GATTGTAAAGGAACTATTGGGTGTCC) was used with primer UHP [63], and for fragment 11 the specific primer (GCTAAGGTTCTTTCTGCTT CTCA) was used with a poly T primer. The missing sequence (fragment 12, Fig. 1) between fragment 10 and 11 was amplified using two specific primers.

The major part of the M RNA (fragment 13; Fig. 1) was amplified using two specific primers derived from the M RNA terminal sequences. To determine the L RNA sequence, a small fragment was amplified initially from the middle of this RNA segment using a one-step RT-PCR and primers derived from conserved regions of WSMoV serogroup viruses (CATAGACACTGGGAATGTGTT and GACAAAAAT

GGATAGAGAGAT). The two long fragments 15 and 16 were obtained using a two-step RT-PCR and specific primer pairs (Fig. 1).
Amplified PCR-fragments were cloned into standard cloning vectors. Purified plasmid DNA was sequenced by MWG-Biotech AG (Ebersberg, Germany).

Sequence analyses

Contigs of L, M and S RNA were assembled using the SeqMan^{TM}II software (V.4.05). The 5´ and 3´ ends of the contigs contain the primer-derived, conserved 8 nt. Alignments were performed by ClustalX [308]. Graphics and phylogenetic trees were generated using Genedoc 2.5.002 [224] and TreeView 1.5.2 [236], respectively. Peptide cleavage sites, transmembrane domains, N- and O-linked glycosylation sites were predicted using SignalP 3.0 [24, 226], TMHMM Server v. 2.0 [166, 291], NetNGlyc 1.0 Server [114] and the NetOGlyc 3.1 Server [155], respectively. RNA secondary structures were predicted via the GeneBee-Molecular Biology Server [39, 40].

Virus sequences used for multiple sequence analyses

Tospovirus sequences were retrieved from GenBank for multiple sequence analyses. Accession numbers of tospovirus S RNAs: CaCV-1043 - AY036058; CaCV-958 - AY036057; CaCV-AIT - DQ256123; CaCV-To-Kalasin - AY626762; calla lily chlorotic spot virus (CCSV) [52, 184] - AY867502; CSNV - AF067068; GBNV - NC_003619; gloxinia ringspot virus - AY312061 (referred to here as GRS); Gloxinia tospovirus HT-1 [134] - AF059577 and AF059578 (referred to here as HT-1); GRSV - L12048; INSV - NC_003624; IYSV - AF001387; MYSV - AB038343; PCFV [58] - AF080526; PYSV - AF013994; TCSV - S54325; Thailand tomato tospovirus - AF134400 (referred to here as ThTT); tomato necrosis virus TD8 - AY647437 (referred to here as TD8); tomato yellow fruit ring virus (TYFRV) [346] - AJ493270; tomato yellow ring virus (TYRV) [123] – AY684718; TSWV - NC_002051; WBNV - AF045067; WSMoV - NC_003843; ZLCV - AF067069.
Accession numbers of tospovirus M RNAs: CaCV-AIT - DQ256125; CSNV - AF213675; GBNV - NC_003620; Gloxinia tospovirus HT-1 [134] - AF023172 (referred to here as HT-1); GRSV - AF213673 and AY574055; INSV - NC_003616;

IYSV - AF214014; MYSV - AB061773; TCSV - AF213674 and AY574054; TSWV - NC_002050 and AAP13904 for NSm; WSMoV - NC_00384; ZLCV - AF213676.
Accession numbers of tospovirus L RNAs: CaCV-AIT - DQ256124; GBNV - NC_003614; INSV - NC_003625; MYSV - AB061774; TSWV - NC_002052; WSMoV - NC_003832.

2.4 Results

ELISA, host range study and symptomatology

Since tomato plants growing in greenhouses at the AIT in Bangkok, Thailand, showed symptoms reminiscent of a tospovirus infection, they were analysed by ELISA. The TospoBroadRange antiserum gave no ELISA reactions. However, antiserum specific for WSMoV serogroup tospoviruses reacted strongly, indicating the occurrence of a WSMoV serogroup tospoviruses (results not shown).
To further characterize this tospovirus, its host range and symptomatology were determined (Table 1). Whereas all *N. benthamiana* plants were readily infected, only a small number of the inoculated tomato plants, irrespective of the tomato cultivar used, reacted with clearly visible virus symptoms. Infected tomato plants showed necrotic leaf spots on systemically infected leaves (Table 1). In addition, necrotic ringspots appeared on fruits of the tomato cultivar King Kong 2. *Nicotiana rustica* plants showed necrotic ringspots only on inoculated leaves but developed no systemic symptoms. However, systemic infections were detected in young leaves by ELISA. Infected *Capsicum annum* cv. Mazurka RZ reacted with severe systemic mosaic symptoms and later crippled fruits were produced. Gloxinia plants (*Sinningia speciosa*) showed asymmetric necrotic ringspots on inoculated and systemically infected leaves. Necrotic local lesions were observed on inoculated leaves of *Chenopodium quinoa* and *Petunia hybrida* cv. Burgundy, but not on those of mock-inoculated plants. Moreover, inoculated *C. quinoa* leaves gave ELISA reactions. Neither symptoms nor ELISA reactions were observed for *Cucumis sativus* cv. Chinesische Schlange, *Cucurbita pepo* cv. Kaempe Melon, *Lactuca sativa* cv. Viktoria and *Phaseolus vulgaris var. nanus* cv. Maxi (Table 1).

Table 1. Local and systemic symptoms induced by CaCV-AIT on different plant species

Test plants	Local reaction[a]	Systemic symptoms	No. of symptomatic plants / plants inoculated
Capsicum annum L.			
cv. Mazurka RZ	-	Mo, LD, St	7[b]/8
Chenopodium quinoa Willd.	NL	-	16[b]/16
Cucumis sativus L.			
cv. Chinesische Schlange	-	-	0/8
Cucurbita pepo L.			
cv. Kaempe Melon	-	-	0/8
Latuca sativa L.			
cv. Viktoria	-	-	0/8
Lycopersicum esculentum Mill.			
cv. Lizzy	-	NS	2[b]/11
cv. King Kong 2	-	NS	2[b]/8
cv. Vollendung	-	NS	1[b]/8
cv. Hildares F1	-	-	0/8
Nicotiana benthamiana Domin.	-	VY, W	12[b]/12
Nicotiana glutinosa L.	NR	NV, LD	4[b]/4
Nicotiana rustica L.	NR	-	8[b]/8
Nicotiana tabacum L.			
cv. Xanthi-nc	NR	NS, NV, St	6[b]/8
Petunia hybrida Vilm.			
cv. Burgundy	NL	-	8/8
Phaseolus vulgaris L.			
var. nanus (L.) Asch. cv. Maxi	-	-	0/8
Sinningia speciosa Lodd.	-	NR	3[b]/3

[a]LD, leaf deformation; Mo, mottle; NL, necrotic lesions; NS, necrotic spots; NR, necrotic ringspots; NV, necrotic veins; St, stunting; VY, vein yellowing; W, wilt; -, no symptoms
[b]Positive ELISA readings; in general only top leaves of inoculated plants were analysed by ELISA. However, when no systemic symptoms were visible, the inoculated leaves showing local lesions were used for ELISA analysis

Sequence analysis of the S RNA

The S RNA of CaCV-AIT is 3477 nt in length and has two non-overlapping ORFs in ambisense orientation. The ORF (nt 67-1383) on the viral sense strand RNA encodes the NSs protein of 439 amino acids and a predicted molecular mass of 49.7 kDa (Table 2). So far, all WSMoV serogroup tospoviruses, with the exception of CCSV, have the same number of amino acid residues for their NSs proteins (Table 2). The ORF (nt 3410-2586) on the viral complementary strand codes for the N protein (275 aa) with a predicted molecular mass of 30.6 kDa, which is very similar in size compared to the other WSMoV serogroup tospoviruses (Table 2).

Table 2. RNA and protein characteristics of tospoviruses

Species	S-RNA length (nt)	5´-UTR length (nt)	3´-UTR length (nt)	IGR length (nt)	NSs protein		N protein	
					No. aa	M.M. kDa	No. aa	M.M. kDa
CaCV-AIT	3477	66	67	1202	439	49.6	275	30.6
Isolate HT-1	-	-	-	-	439	49.8	277	30.6
GBNV	3057	66	67	779	439	49.5	276	30.6
WSMoV	3534	66	65	1261	439	49.7	275	30.6
CCSV	3172	66	64	831	460	51.8	277	30.4
MYSV	3232	68	67	853	469	53.1	279	31.0
IYSV	3105	70	70	817	443	50.1	273	30.5
TYRV	3061	71	71	768	443	50.2	274	29.9
PYSV	2970	57	76	659	480	53.5	246	28.1
PCFV	2833	67	79	461	461	52.5	270	31.1
TSWV	2916	88	153	509	464	52.4	258	28.8
GRSV	3049	87	151	636	467	52.5	258	28.7
INSV	2992	62	149	648	449	51.2	262	28.7
Species	M-RNA length (nt)	5´-UTR length (nt)	3´-UTR length (nt)	IGR length (nt)	NSm protein		Gn/Gc protein	
					No. aa	M.M. kDa	No. aa	M.M. kDa
CaCV-AIT	4823	56	47	433	308	34.2	1121	127.2
Isolate HT-1	-	-	-	441	308	34.3	1122	127.7
GBNV	4801	56	47	414	307	34.3	1121	127.3
WSMoV	4880	55	47	479	312	35.0	1121	127.6
MYSV	4815	58	48	404	308	34.4	1127	128.3
IYSV	4838	63	49	385	311	34.8	1136	128.6
TSWV	4821	100	84	326	302	33.6	1135	127.3
TCSV	4882	100	87	357	303	34.0	1141	128.5
GRSV	4855	100	84	342	303	33.8	1138	128.2
INSV	4972	85	169	479	303	34.1	1110	124.9
Species	L-RNA length (nt)	5´UTR length (nt)	3´UTR length (nt)		L protein			
					No. aa	M.M. kDa		
CaCV-AIT	8912	250	31		2877	331.3		
GBNV	8911	245	32		2877	330.9		
WSMoV	8917	248	32		2878	331.8		
MYSV	8918	276	32		2870	330.2		
TSWV	8897	236	33		2875	331.5		
INSV	8776	150	31		2865	330.3		

For abbreviations see Materials and Methods

The two ORFs are separated by an extremely long A/U-rich (77.7%) intergenic region (IGR) of 1202 nt. This is only 59 nt shorter than the IGR of WSMoV (Table 2). The IGR sequence is predicted to form a stable hairpin structure, showing several loops and two major branches (data not shown). Similar secondary structures of the larger WSMoV IGR and the shorter GBNV IGR were identified. The 5´ and 3´ untranslated regions (UTR) of the S RNA are able to form a panhandle structure (data not shown). Base pairing of the RNA termini is facilitated because of complementary sequences,

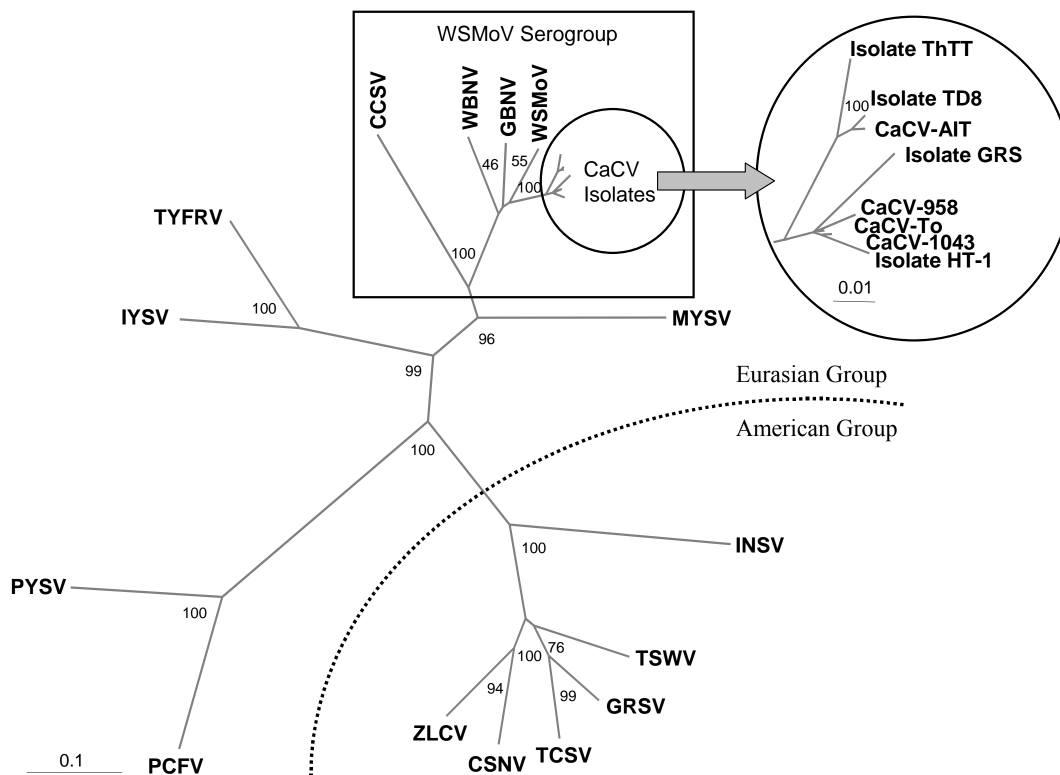


Fig. 2. Phylogenetic tree of tospoviruses based on N protein sequences. Bootstrap values in percent are shown at nodes

whereas the 14 nucleotides at the very 5' and 3' termini are nearly perfect inverted repeats forming a double strand with only one mismatch.

In N protein sequence alignment, CaCV-AIT clusters with isolates of the WSMoV serogroup (Fig. 2; Table 3A). Highest N protein amino acid sequence identities were observed for CaCV isolates (958, 1043, To) and four unassigned tospovirus isolates (HT-1, GRS, ThTT and TD8; Table 3A). Like the phylogenetic tree of the N protein, the NSs protein of CaCV-AIT also formed a cluster with WSMoV serogroup tospoviruses, with that of isolate HT-1 being most closely related (Fig. 3A).

Table 3. Tospovirus protein sequence identities in percent. **A.** N and NSs protein. **B.** NSm and Gn/Gc protein. **C.** L protein (RdRp)

A

% aa identity N / NSs % aa identity	TSWV	GRSV	TCSV	CSNV	ZLCV	INSV	PCFV	PYSV	IYSV	TYFRV	MYSV	CCSV	WSMoV	GBNV	WBNV	CaCV-958	CaCV-1043	CaCV-To	Isolate HT-1	Isolate GRS	Isolate ThTT	Isolate TD8	CaCV-AIT
TSWV	...	79	77	76	73	54	18	18	33	32	27	26	30	30	29	31	31	31	31	32	31	32	32
GRSV	78	...	84	74	77	55	18	19	32	35	28	27	31	30	30	31	31	31	31	31	30	31	31
TCSV			...	72	72	53	17	17	31	33	28	25	28	28	28	29	29	29	29	29	29	30	30
CSNV				...	80	55	19	20	31	31	30	26	28	28	27	28	29	29	28	28	29	29	29
ZLCV					...	52	18	18	30	32	28	26	29	29	27	29	30	30	30	29	29	29	29
INSV	52	54				...	18	19	29	30	27	25	30	29	28	29	30	30	30	30	30	29	29
PCFV	11	12				13	...	67	18	18	19	19	21	22	21	22	22	23	23	21	22	21	22
PYSV	12	15				13	55	...	21	20	21	18	21	20	21	22	22	23	23	22	23	24	24
IYSV	18	21				20	16	15	...	74	50	48	43	45	44	46	46	46	46	47	47	47	47
TYRV	19	23				21	16	15	90	...	49	46	43	44	44	45	45	46	45	45	45	45	45
MYSV	18	18				19	14	13	40	39	...	58	59	60	60	59	60	60	59	59	60	60	60
CCSV	17	20				18	17	15	52	51	47	...	64	65	65	63	64	64	64	64	63	63	63
WSMoV	17	19				20	17	13	49	49	50	64	...	86	85	85	86	85	85	87	85	85	85
GBNV	17	20				19	17	13	49	49	50	66	85	...	85	84	85	84	84	85	85	85	85
WBNV															...	81	81	81	80	82	81	82	82
CaCV-958																...	99	99	97	93	91	92	92
CaCV-1043																	...	99	99	94	92	93	93
CaCV-To																		...	99	94	91	92	93
Isolate HT-1	17	19				19	17	13	51	51	48	62	78	81					...	93	90	91	92
Isolate GRS																				...	92	93	94
Isolate ThTT																					...	98	97
Isolate TD8																						...	99
CaCV-AIT	17	19				19	17	13	52	52	49	65	81	81					86				...

B

% aa identity NSm / Gn/Gc % aa identity	TSWV	GRSV	TCSV	CSNV	ZLCV	INSV	IYSV	MYSV	WSMoV	GBNV	Isolate HT-1	CaCV-AIT
TSWV	…	86	85	86	70	69	38	37	38	40	38	38
GRSV	80	…	97	85	71	69	40	40	40	41	39	39
TCSV	81	93	…	84	70	70	39	39	39	41	39	39
CSNV				…	71	69	39	40	38	42	40	39
ZLCV					…	61	34	36	35	36	35	34
INSV	64	61	62			…	37	36	38	39	38	38
IYSV	34	34	35			34	…	59	67	70	68	69
MYSV	33	33	34			35	56	…	63	63	65	63
WSMoV	34	34	34			35	62	64	…	83	81	81
GBNV	34	34	35			35	63	65	89	…	88	89
Isolate HT-1	34	34	34			34	62	64	84	87	…	91
CaCV-AIT	34	34	35			34	62	66	84	87	90	…

C

% aa identity L / L % aa similary	TSWV	INSV	MYSV	WSMoV	GBNV	CaCV-AIT
TSWV	…	69	44	44	44	44
INSV	84	…	45	46	46	46
MYSV	63	65	…	76	76	76
WSMoV	64	65	88	…	91	91
GBNV	64	65	88	96	…	90
CaCV-AIT	64	65	89	96	96	…

CaCV isolates are shown in bold frames. For abbreviations see Materials and Methods

Sequence analysis of the M RNA

The M RNA of CaCV-AIT has a length of 4823 nt and a similar arrangement of two non-overlapping ORFs like the S RNA. The ORF (nt 57-980) on the viral sense strand of the M RNA encodes the NSm protein with a predicted molecular mass of 34.2 kDa (308 aa). The ORF (nt 4776-1414) on the viral complementary strand codes for the Gn/Gc precursor glycoprotein with a predicted molecular mass of 127.1 kDa (1121 aa). There is almost no variation in Gn/Gc precursor glycoprotein size among WSMoV serogroup tospoviruses (Table 2). Different amino acid motifs were recognized within this protein (Fig. 4). The CaCV-AIT motifs resembled those of WSMoV serogroup tospoviruses and, moreover, MYSV in the arrangement of the five predicted transmembrane domains and in the location of N-glycosylation sites (Fig. 4). However, CaCV-AIT differs in the number of N- and O-glycosylation sites. With

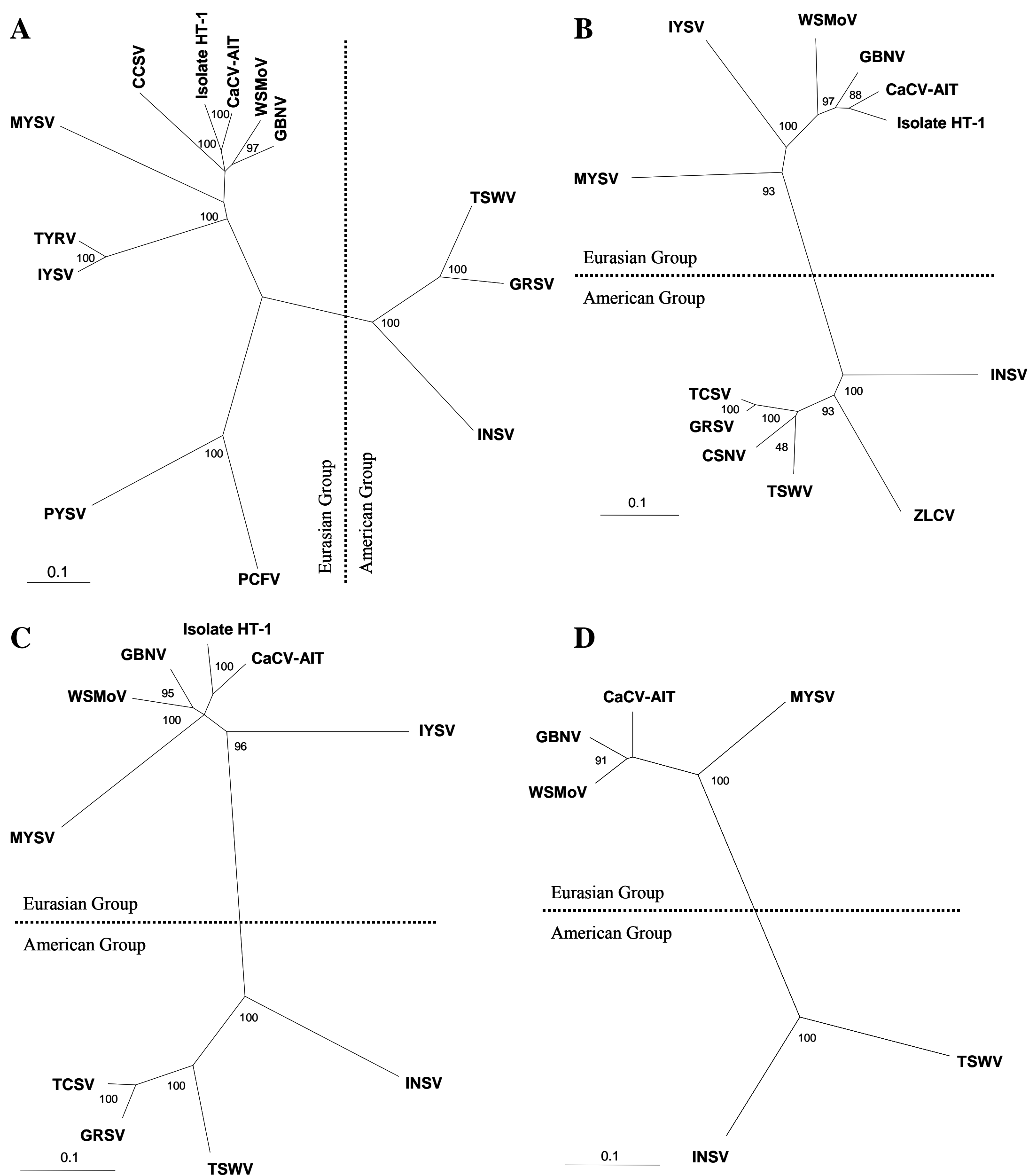


Fig. 3. Phylogenetic trees of tospoviruses based on amino acid sequences of NSs protein (**A**), NSm protein (**B**), Gn/Gc precursor protein (**C**) and L protein (RdRp) (**D**). Bootstrap values in percent are shown at nodes

only three N-glycosylation sites CaCV-AIT is exceptional among all tospovirus species. In contrast, two O-glycosylation sites were predicted for CaCV-AIT, whereas they seem to be absent from any other tospovirus of the WSMoV serogroup (Fig. 4).
CaCV-AIT M RNA is very similar to WSMoV serogroup tospoviruses in terms of lengths of the UTRs (Table 2). These regions at the 5′ and 3′ terminal ends consist of 56 and 47 nt, respectively, and can form a panhandle with the extreme termini leading to 13 perfectly base paired nucleotides and one mismatch. An A/U-rich (77.8%) IGR

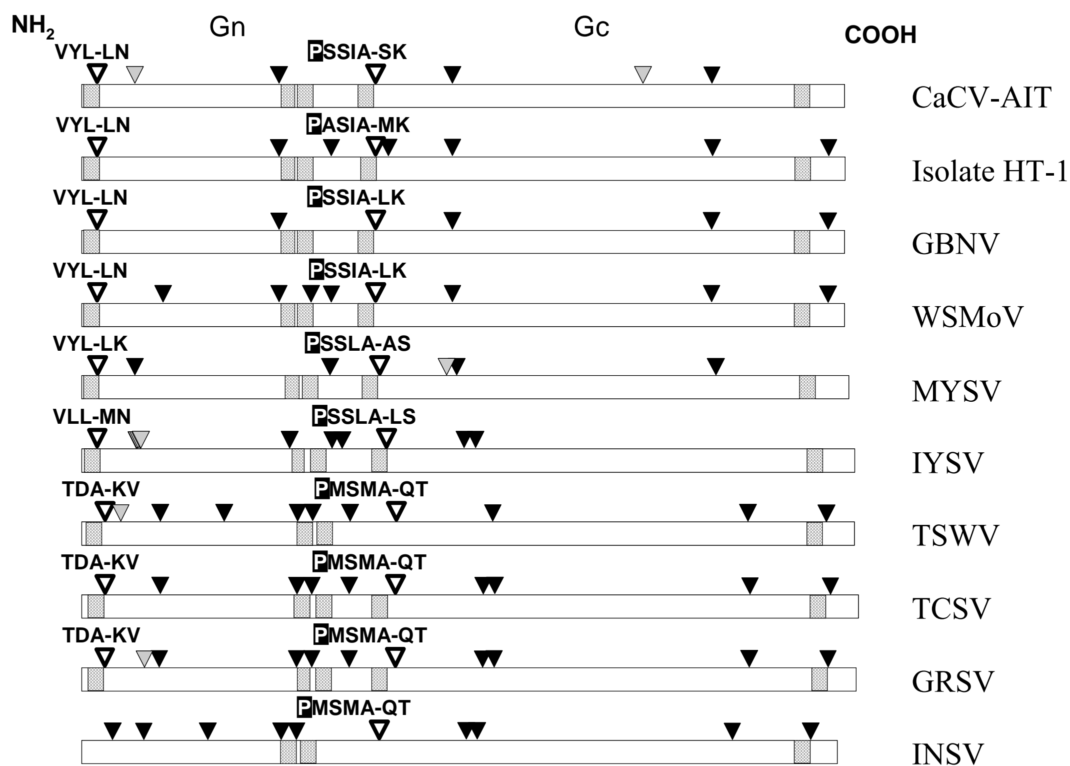


Fig. 4. Topology of the glycoprotein precursors of ten tospovirus species. Hatched boxes represent transmembrane domains. Predicted N-glycosylation sites are indicated as black triangles and predicted O-glycosylation sites as gray triangles. Open triangles below the amino acids denote the potential peptide cleavage sites. The conserved proline five amino acids upstream of the predicted cleavage site between Gn and Gc is indicated

of 433 nt separates the two ORFs. Compared to the IGR length variation of the S RNAs among tospovirus species, this variation is considerably lower for the M RNAs IGRs (Table 2). A stable hairpin structure can be predicted for the IGR sequence of the M RNA (data not shown).

Phylogenetic trees on the basis of NSm and Gn/Gc proteins alignments revealed a clustering of CaCV-AIT to the WSMoV serogroup (Fig. 3B and C). CaCV-AIT shared amino acid sequence identities ranging from 81% to 89% with other established members of the WSMoV serogroup (Table 3B) and 90 to 91% identity with isolate HT-1, a proposed CaCV isolate (Table 3B, bold frame).

Sequence analysis of the L RNA

The largest genome segment of CaCV-AIT (8912 nt) possesses only one single ORF (nt 8881-251) in the viral complementary strand. This ORF potentially encodes the RdRp protein (or L protein) with a predicted molecular mass of 331.3 kDa (2877 aa) (Table 2). The eight-amino-acid motifs characteristic for the *Bunyaviridae* RdRps are also conserved in the CaCV-AIT sequence (Fig. 5). Therefore, on the basis of six available tospovirus RdRp sequences, revised consensus motifs of the tospovirus RdRps are now proposed (Fig. 5). The 5′- and 3′-UTRs of 250 and 31 nt, respectively, are complementary also in the L RNA and result in a panhandle structure consisting of 18 base-paired nucleotides and one mismatch. Phylogenetic analysis of L protein sequences showed a clustering of CaCV-AIT with tospoviruses of the

Motif	A	B	C
TSWV	L S A D Q S K W S A S G L T	M N W L Q G N L N Y L S S V Y H S C A M K	W I V H S D D N A T
INSV	L S A D Q S K W S A S D L T	M N W L Q G N L N Y L S S V Y H S C A M K	W I V H S D D N A T
MYSV	L S A D Q S K W S A S D L T	M N W L Q G N L N Y L S S V Y H S C A M L	W M V H S D D N A T
WSMoV	L S A D Q S K W S A S D L T	M N W L Q G N L N Y L S S V Y H S C A M I	W M V H S D D N A T
GBNV	L S A D Q S K W S A S D L T	M N W L Q G N L N Y L S S V Y H S C A M M	W M V H S D D N A T
CaCV-AIT	L S A D Q S K W S A S D L T	M N W L Q G N L N Y L S S V Y H S C A M M	W M V H S D D N A T
Rice stripe	T S D D A S K W N - Q G H Y	T G M M Q G I L H Y T S S L F H A I F L D	N M E S S D D S S F
Uukuniemi	T S D D A A K W N - Q C H H	T G M M Q G I L H Y T S S L L H T L L Q E	V L Q S S D D S G M
Bunyamwera	I N A D M S K W S A Q D V F	R N W L Q G N F N Y I S S Y V H S C A M L	S M V H S D D N Q T
Hantaan	V S A D A T K W S P G D N S	G N W L Q G N L N K C S S L F G V A M S L	F A H H S D D A L F
La Crosse	I N A D M S K W S A Q D V F	R N W L Q G N F N Y T S S Y V H S C A M S	S L V H S D D N Q T
Consensus A	D x x K W S x Q x H	Q G x x x Y x S S L L H S	S D D
Consensus B	D x x K W S x S x L	Q G x x x Y x S S V Y H S	S D D

Motif	D	E	F1	F2 / F3
TSWV	F C I T L N P K K S Y	S S E V E F I S E R I S K	V T G S V D F L V S V F E	K M Q R T K T D R E I Y L M
INSV	F C I T L N P K K S Y	S S E V E F I S E R I V N	A S D A I D F L V S V F E	K M Q R T K T D R E I Y L M
MYSV	Y C I T L N P K K S Y	E S E V E F I S E R I I N	N A G N T N F L V S V F E	K M Q R T K M O R E I Y L M
WSMoV	Y C I T L N P K K S Y	E S E V E F I S E R I I N	N A G N T D F L V S V F E	K M Q R T K M D R E I Y L M
GBNV	Y C I T L N P K K S Y	E S E V E F I S E R I I N	N A G N T D F L V S V F E	K M Q R T K M D R E I Y L M
CaCV-AIT	Y C I T L N P K K S Y	E S E V E F I S E R I I N	N A G N T D F L V S V F E	K M Q R T K M D R E I Y L M
Rice stripe	L G I Y K S P K S T T	L F V M E F N S E F F F S	L N K N E C M H I C I F K	K N Q H G G - L R E I Y V L
Uukuniemi	L G I Y S S V K S T N	L H L L E F N S E F F F H	V E S Q G C M H V C L F K	K P Q H G G - L R E I Y V L
Bunyamwera	F G C Q A N M K K T Y	H T C K E F V S L F N L H	M K N H K E F S F T F F N	K G Q K T A K D R E I F V G
Hantaan	G S I K I S P K K T T	P T N A E F L S T F F E G	E T R E Q K A M A R I V R	K Y Q R T E A D R G F F I T
La Crosse	F G C Q A N M K K T Y	N C I K E F V S L F N L Y	M V D H K K F Y F T F F N	K G Q K T S K D R E I F V G
Consensus A	Y x x x K	E F x S E	K E V x 4-10	K x Q R T G x D R E I Y
Consensus B	T x x x K	E F x S E	T D F x 6	K x Q R T K x D R E I Y

Fig. 5. Conserved motifs of *Bunyaviridae* RdRps. The *Bunyaviridae* consensus sequence according to Bruenn [43] is shown as Consensus A and the tospovirus consensus sequence derived here is shown as Consensus B. The tospovirus related RdRps were those of the tenuivirus *Rice stripe virus* (D31879), the phlebovirus *Uukuniemi virus* (D10759), the bunyaviruses *Bunyamwera virus* (X14383) and La Crosse virus (U12396), and the hantavirus *Hantaan virus* (X55901). The source of the six tospovirus sequences is given in Materials and Methods. Similar or identical residues are shaded in gray according to Sambrook [273].

WSMoV serogroup (Fig. 3D). CaCV-AIT shared amino acid sequence identities of 90-91% with WSMoV and GBNV, the two other WSMoV serogroup tospoviruses for which L RNA sequences are available and revealed the RdRp as the most highly conserved protein of the tospoviruses (Table 3).

2.5 Discussion

Here, we describe the sequence analysis of the complete RNA genome of a WSMoV serogroup tospovirus isolate infecting tomato plants in Thailand. Besides vector specificity, which was reported earlier [251], the experimental host range of CaCV-AIT was determined, contributing to its taxonomic assignment (Table 1). Ringspots on gloxinia plants, as found after inoculation with isolates HT-1 and GRS [49], were also observed on CaCV-AIT infected plants although they were necrotic rather than chlorotic. Since CaCV isolates 958 and 1043 were not tested on gloxinia plants [201], no general statement with respect to the symptomatology of CaCV isolates on gloxinia plants can be made. In addition, together with the CaCV isolates 958 [201] and HT-1 [134], AIT failed to induce systemic infections in cucurbits, indicating a relationship of CaCV-AIT to these virus isolates. All other WSMoV serogroup tospoviruses (WSMoV, GBNV [347] and WBNV) infect cucurbits. Similar symptoms (necrotic spots) were observed on tomato and pepper plants for CaCV isolate 958 [201] and CaCV-AIT. Despite the similar host spectrum described so far for the CaCV isolate 958 and CaCV-AIT, they differed in their ability to cause systemic infections in *N. glutinosa* and *N. tabacum* cv. Xanthi-nc. CaCV-AIT caused conspicuous systemic symptoms in the present study, whereas no symptoms were observed for isolate 958 in these test plants [201]. However, it should be taken into consideration that virus symptoms may differ not only due to the use of different isolates but also to differences in test conditions, e.g. environmental factors [188]. Necrotic local lesions were observed in *C. quinoa* and *P. hybrida* cv. Burgundy following CaCV-AIT inoculation. However, the samples taken from local lesions on *P. hybrida* cv. Burgundy tested negative in ELISA, probably due to very low virus titers and/or inhibitory substances in the plant sap.

On the basis of the molecular criteria for tospovirus species demarcation, N protein sequence identities were used to assign the isolate AIT to CaCV [251]. It is likely that CaCV is the predominant tospovirus species in Thailand, because there have been

reports on the occurrence of WSMoV serogroup tospoviruses in northeastern Thailand [246]. These reports are supported by our S RNA sequence analysis (Fig. 2 and Table 3A). Another tospovirus isolated in Thailand initially referred to as Physalis severe mottle virus (PSMV) [63] was later renamed as isolate of MYSV [160]. This virus is not serologically related to WSMoV serogroup viruses [63] and shares N and NSs protein sequence identities of 60% and 49%, respectively, with the corresponding proteins of CaCV-AIT (Table 3A). However, this virus was found only once in the weed *Physalis minima* L. and does not seem to be common in crop plants [63].

Three N protein sequences from tospovirus isolates in Thailand appeared to be closely related to that of CaCV. The phylogenetic tree (Fig. 2) and the identity matrix (Table 3A) revealed that CaCV-AIT clusters with the two Thai isolates ThTT and TD8, (N protein sequence identities of 97-99%) and the Gloxinia isolate HT-1 forms a group with the Thai isolate CaCV-To and the two Australian isolates 958 and 1043 (identities of 97-99%). The N protein sequence identities between the two branches were in the range of 90-93%, warranting the classification of these isolates as CaCV isolates (bold frame in Table 3A). The CaCV isolate Peanut-Khon Kaen (DQ022745) from Thailand is not listed separately in this report, because of an N protein amino acid sequence identity of 100% to the CaCV-To. Since no sequence information on genes other than the N gene is available for recognized CaCV isolates, further sequence analyses were made only with those of HT-1 and AIT. The NSs protein sequence identity between these two isolates is 86% (Table 3A), which is in agreement with the observation that the NSs protein is the least conserved protein of the tospoviruses [106].

Analysis of the S RNA IGR sequences of different tospovirus species indicated a high variation in length, ranging from 461 nt (PCFV) to 1261 nt (WSMoV) (Table 2) [240]. A similar variation was found in WSMoV serogroup tospoviruses (Table 2). Although the IGR length can greatly vary from 554 to 969 nt even within one species (TSWV), the biological function of the IGR size differences is yet unknown [128]. Pappu et al. [240] and Heinze et al. [127] have shown that the geographical origin of TSWV isolates correlates with the S RNA IGR sequences, regardless of the host origin [240]. A comparison of the S RNA IGR sequences of various CaCV isolates, especially the Australian and Thai isolates, might provide clues on their geographical origin and subsequent spread. Unfortunately, no sequence data are available for the S RNA IGR of the Australian CaCV isolates.

Sequence analysis of the M RNA could offer valuable clues to thrips transmission of CaCV. Reassortment based experiments with thrips transmissible and thrips non-transmissible TSWV isolates have demonstrated that the M RNA encodes transmission determinants, namely the glycoproteins Gn and Gc [286]. By using a soluble form of TSWV Gn glycoprotein, a specific binding to the midgut epithelial cells of larval thrips was shown, which was demonstrated without the presence of any other TSWV protein [340]. A single mutation of proline to threonine at position 459 in the Gn/Gc precursor protein of TSWV did not influence the processing of this glycoprotein but impeded transmission by thrips [286]. All analyzed glycoproteins shown in Fig. 4 had a proline five amino acids upstream of the predicted peptide cleavage site of Gn and Gc. For CaCV-AIT the proline is located at amino acid position 429 of the precursor glycoprotein. Another motif, described as RGD motif, was proposed to be involved in the attachment of the virus to thrips tissue [162]. Tospoviruses belonging to the "American" group possess this motif [62], which is absent from "Eurasian" tospovirus species like CaCV.

The type and role of Gn/Gc glycosylation is still under discussion [341]. Although no N- but O-glycosylation was reported for TSWV Gn [340], Naidu et al. [219] found no evidence for O-glycosylation, but extensive N-glycosylation on Gc compared to Gn. It is well accepted that N-glycosylation is accomplished in the lumen of the ER. The glycosylation acceptor sites must have a distance of at least 12-15 residues from the transmembrane domains [226]. According to this rule, some tospovirus species were predicted to have no N-glycosylation in the Gn protein [62]. This is in agreement with the prediction of transmembrane domains and the location of the intervening loop regions calculated with TMHMM2.0 [166, 291]. With the exception of TSWV and INSV, all tospovirus species shown in Fig. 4 have two long intervening loop regions of Gn. For CaCV-AIT the Gn is predicted to lack N-glycosylation and two N-glycosylation sites are predicted for Gc, in line with findings of Naidu et al. [219] proposing extensive glycosylation for Gc in comparison to Gn of TSWV. Gc is predicted to play an important role in the life cycle of TSWV [219].

Only one thrips species (*C. claratris*) has been described so far as a vector of CaCV [251]. *C. claratris*, which was previously not known as a tospovirus vector, seems to be the predominant thrips species in central Thailand [217, 250]. Since the vector-virus interaction was demonstrated only for the CaCV isolate AIT, there is a need to verify thrips transmission of the other CaCV isolates, especially the isolates 958 and

1094 from Australia [201]. On the other hand, the fact that *C. claratris* has not been reported from Australia [171] suggests that there might be other thrips species capable of transmitting CaCV.

The amino acid alignment of the tospoviral NSm proteins, which are involved in cell-to-cell movement [165], was analyzed for the occurrence of common motifs (data not shown). Only a few motifs of the “30 K” superfamily of viral movement proteins were conserved [203]. The well-established $LXDX_{50-70}G$ motif [203] was found in the NSm protein of the isolate AIT and all other tospoviruses whereby the G residue was located 55 amino acids downstream of the D residue. The P/D-L-X motif [203] was recognized on CaCV-AIT, but as described by Silva et al. [283], the leucine was exchanged with serine in all the ‘Eurasian’ tospoviruses.

The IGR sequences of the tospovirus M RNAs are shorter (Table 2) and less heterogeneous than those of the S RNAs [240]. Pappu et al. [240] found identities of 84-94% for isolates of one tospovirus species, whereas they were in the range of 46-59% for distinct tospovirus species. The only available sequences for the M RNA IGR of CaCV isolates are those of the isolates AIT (this report) and HT-1. Interestingly, AIT shares an M RNA IGR sequence identity of only 51% with HT-1, suggesting the existence of two distinct tospovirus species. However, Pappu et al. [240] also observed S RNA IGR sequence identities of 49-57% between TSWV-B and other TSWV isolates. Although TSWV-B was later re-classified as a separate species (GRSV), the usefulness of IGR sequence identities as a criterion for tospovirus species demarcation should be examined further, as this has been considered only for the differentiation of TSWV isolates. IGR comparisons might permit the discrimination of geographically diverse isolates [127, 240], as CaCV-AIT was isolated in Thailand [251] and the Gloxinia isolate HT-1 originated from the US [134]. Determination of the M RNA IGR sequences of the Australian CaCV isolates might contribute to establishing a correlation between IGR sequences and the geographical origin of the different CaCV isolates.

For L and M RNA cloning, an improved amplification strategy was performed using a high-fidelity polymerase to generate large DNA fragments of about 4500 nt from total RNA preparations without the need of nucleocapsid purification (Fig. 1). Sequencing of the fragments revealed a high quality sequence without frame shifts.

Several of our attempts failed to amplify the complete S RNA as described for PSMV [63]. One reason could be the use of a total RNA extract as template instead of purified viral RNA. Another explanation could be the secondary structure of the long IGR, whereas the IGR length seems not to be the limiting factor, as shown for the fragments generated from the M and L RNA.

The synopsis of the tospovirus genome characteristics (Table 2) and the phylogenetic trees illustrating the relationships among tospoviruses (Figs. 3 and 4) indicate that CaCV is a member of the WSMoV serogroup and support the proposed assignment of the WSMoV serogroup to the ‘Eurasian’ group of tospoviruses [192, 283]. The ‘American’ and ‘Eurasian’ groups are distinguished on the basis of obvious differences in geographic prevalence, N and NSm protein sequences [283] and 3′-UTR length of the M RNA [192]. Furthermore, our data suggest that the two geographic groups can also be discriminated on the basis of differences in 3′-UTR length of the S RNA, 5′-UTR length of the M RNA as well as in NSs, Gn/Gc and L protein sequences (Table 2 and Fig. 3). It is noteworthy that the N and NSs protein sequences of PYSV and PCFV formed a separate cluster in phylogenetic trees (Figs. 2 and 3A). However, the S RNA 5′- and 3′-UTR lengths (Table 2) and the geographical origin of PCFV (Taiwan) and PYSV (India) support the assignment of these two tospoviruses to the ‘Eurasian’ group.

Based strictly on criteria of N protein amino acid sequence similarities, the prevalent tospovirus infecting tomatoes in Thailand was classified as an isolate of CaCV. Overall sequence similarities and serological relationship clearly provide evidence for its assignment to the WSMoV serogroup of tospoviruses. This serogroup now includes three entirely sequenced species and thus is the best-described tospovirus serogroup. In addition, comparisons of the CaCV-AIT sequences with different tospoviral sequences available from GenBank allowed all of these isolates to be combined under one (proposed) species name: Capsicum chlorosis virus. The results of this study will contribute to the classification of CaCV as assigned member of the genus *Tospovirus*. In addition, it will facilitate the development of diagnostic tools and control measures for CaCV. Such tools and measures are required for an integrated pest management system consisting, for instance, of transgenic plants and improved strategies of thrips vector control.

3 Application of Phi29 DNA polymerase in identification and full-length clone inoculation of *Tomato yellow leaf curl Thailand virus* and Tobacco leaf curl Thailand virus[2]

3.1 Abstract

Tomato plants grown in greenhouses in Thailand developed typical symptoms of a *Tomato yellow leaf curl Thailand virus* (TYLCTHV) infection. After confirmation by ELISA a Phi29 DNA polymerase approach was chosen for further molecular analysis of TYLCTHV. Total DNA purified from infected tomato leaves was subjected to rolling circle amplification (RCA) of DNA-A and DNA-B of TYLCVTHV. In addition, a new monopartite geminivirus with a putative recombinant background was identified by RCA and tentatively named Tobacco leaf curl Thailand virus (TbLCTHV). To confirm the composition of both geminiviruses full-length clones were established and used for inoculation of *Nicotiana benthamiana* by particle bombardment or agroinfection. When TYLCTHV DNA-A and DNA-B were applied together by particle bombardment or agroinfection severe stunting, yellowing and leaf curling was observed. Whereas TYLCTHV DNA-A and TbLCTHV revealed no infection after particle bombardment, similar symptoms in *N. benthamiana* like leaf upward curling and yellowing were observed following agroinfection.

DNA components of TYLCTHV DNA-A and DNA-B were excised from respective plasmids, ligated and amplified by Phi29 DNA polymerase. The ability of viral concatamere inoculation was evaluated in particle co-bombardment experiments on *N. benthamiana*. Thus, particle bombardment of RCA derived multimeric products proved to be at least as effective as inoculation with a partial repeat construct and tenfold as effective as inoculation with excised unit-lengths of DNA-A and DNA-B of TYLCVTHV when using each DNA component in an amount of 5 ng.

[2] Published as Knierim D, Maiss E (2006) in Arch Virol [Epub ahead of print]; The nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers: DQ871222 (TYLCTHV-A-[AIT]), DQ871220 (TYLCTHV-B-[AIT]) and DQ871221 (TbLCTHV)

3.2 Introduction

Plant viruses of the family *Geminiviridae* cause economically losses in important crops of tropical and subtropical and nowadays also in temperate agroecosystems [31, 42, 211, 265]. The circular single-stranded DNA geminiviruses are classified into four genera depending on vector specificity, host range and genome structure: *Mastrevirus* (leafhoppers, monocots and a few dicots), *Curtovirus* (leafhoppers, dicots) and *Topocuvirus* (treehopper, dicots). All of theses have a single genomic component, whereas viruses of the genus *Begomovirus* (whiteflies, dicots) have either one or two components [88, 265, 293]. The typical twin-shaped particles contain a single genomic component of 2.5-3.0 kb in length [265, 293]. In same cases satellite DNAs were found to be disease associated [36, 293].

For analysis of circular DNA viruses rolling circle amplification (RCA) with Phi29 DNA polymerase has a tremendous potential, because this high-fidelity enzyme can be used without sequence information on a random hexamer primed template [68, 115, 140, 257]. Consequently it is possible to detect so far uncharacterized and new components of geminiviruses. In addition, components like DNA-B can be easier amplified than with common methods used so far [140]. Also for routine diagnosis of geminiviruses Phi29 DNA polymerase might be useful in future [115].

To analyze the complete genome of viruses and to fulfill Koch's postulates full-length clones are valuable tools [34, 270]. Different inoculation methods were established for full-length clones in geminivirus research, depending on the objective of investigation and properties of the respective virus.

Particle bombardment of full-length clones of non phloem-limited geminiviruses has been developed [102, 104], revealing a high percentage of infected plants. Non phloem-limited geminiviruses belong generally to the group of bipartite begomoviruses, encoding two major proteins on DNA-B (BV1 and BC1) involved in virus movement and two proteins on component A (AV1 and AC4) with a limited movement function [266]. DNA is introduced by particle bomardment to the outer layer cells and rarely to phloem-associated cells [292, 298], depending on the inoculated host plant [292]. Phloem limited viruses can be introduced with vascular puncture inoculation (VPI), which delivers the full-length clones directly to the vascular bundles [191, 259, 260]. Finally, another common artificial inoculation

method depends on the transfer of full-length clones integrated as T-DNA in *Agrobacterium tumefaciens* plasmids (agroinoculation) [19, 35, 85, 111, 112, 215].
Major decision criteria for the choice of a full-length clone inoculation method are effectiveness and handling complexity. Mechanical inoculation of excised geminivirus unit-length DNA is the simplest way [312], but not applicable for each geminivirus / host combination [104, 270]. In addition, the efficacy is reported to be lower than inoculation by particle bombardment [115]. Particle bombardment is easy to perform and viral DNA can be applied as plasmid DNA with partial or dimer repeats of the genome [32, 104, 154, 292], or as excised unit-length DNA, which reduces extra cloning steps [32, 34, 35, 102, 104, 270, 292]. However, when using linear DNA unit-lengths for inoculation the relative start and end point of the genome [32] and the sensitivity of linear DNA to nuclease degradation before re-circularisation in planta limits infection efficiency [102, 312]. At last, agroinoculation of full-length clones is highly effective but cumbersome [32, 102, 104, 259] and some multimeric constructs, which are used to increase infection effectiveness, are unstable in *A. tumefaciens* [292].
Here we report the application of Phi29 DNA polymerase for characterization of two geminivirus species infecting tomato plants in Thailand as well as its suitability for infectious full-length clone production. A new isolate of *Tomato yellow leaf curl Thailand virus* (TYLCTHV), named AIT, and the detection of a proposed new monopartite geminivirus tentatively named Tobacco leaf curl Thailand virus (TbLCTHV) are described. Furthermore, an optimized inoculation method of full-length clones for the non-phloem limited TYLCTHV-[AIT] was developed using concatameric copies of the viral genome produced with Phi29 DNA polymerase. The method combines the advantages of minor cloning efforts and high infection efficiency.

3.3 Materials and methods

Plant material, ELISA and DNA isolation

Tomato plants (*Solanum lycopersicum* cv. King Kong 2) cultivated under protected conditions in greenhouses at the Asian Institute of Technology (AIT) in Bangkok,

Thailand, showed typical symptoms of a geminivirus infection: stunted growth with yellow and curled leaves.
The TAS-ELISA Kit (AS-0588-546/2 form the DSMZ) was used for the serological detection of geminiviruses according to the suppliers protocol.
DNA isolation was based on the method of Accotto et al. [5, 10]. The DNA extract was further purified with the plasmid isolation kit from Qiagen according to the suppliers protocol.

Full-length genome amplification, cloning and sequencing

Circular geminiviral DNA was amplified by RCA using Phi29 DNA polymerase for different applications. In a first approach total DNA isolated from infected tomato leaves was used for RCA followed by restriction enzyme digest and cloning into standard cloning vectors. In a second approach cloned full-length DNA components were excised from the plasmid, agarose gel purified, recircularized in a 20 µl standard ligation mixture and subjected to RCA. A standard RCA reaction was made of 2.5 µl DNA, 1.25 µl dNTPs (25 mM) and 1.25 µl random hexamer primer (10 pmol/µl; phosphorylated at the 3 primed end; Carl Roth, Germany) and adjusted to 25 µl with A. bidest. The mix was heated for 3 minutes at 95°C and immediately cooled on ice. A master mix of 25 µl consisting of 0.25 µl Phi29 DNA polymerase (Fermentas GmbH), 5 µl 10X reaction buffer and 19.75 µl A. bidest was added and the reaction was incubated for 18 hours at 30°C followed by an heat inactivation step of 10 minutes at 65°C.
The products were used for construction of multimeric clones and as concatameres for particle bombardment.
Both TYLCTHV-[AIT] genome components were cloned with *Eco*RI, whereas the TbLCTHV genome was cloned with *Bam*HI in standard cloning vectors. Sequencing of purified plasmid DNA containing the geminivirus inserts was done by MWG-Biotech AG (Ebersberg, Germany) using standard or specific oligonucleotides.
The DNA sample from which the TbLCTHV genome has been amplified was also checked for the presence of a ß-component with the conserved primers Beta01 (ACTACGCTACGCAGCAGCC) and Beta02 (TACCCTCCCAGGGGTACAC) in a standard PCR reaction [60].

Construction of multimeric clones

Multimeric clones of TYLCTHV-[AIT] A, B and TbLCTHV were build up in pDrive (Qiagen) and subsequently transferred into the binary vector pBIN19 [26]. Briefly, multimeric components were generated by using different single restriction enzyme recognition sites in the respective DNAs. DNA A was assembled as a 1.4 multimeric form starting at the *Bam*HI site located 5'-end of the AV2 gene and ending at the *Bsp*120I site. The B component was cloned as a 1.7-mer construct, starting at the *Mlu*I site located in the intergenic region (IR) and ending at the *Sal*I site. The 1.6-mer construct of TbLCTHV starts at the *Pst*I site located in V2 and ends at the *Sal*I site (see thin arrow lines in Figs. 1 A and 1 B).

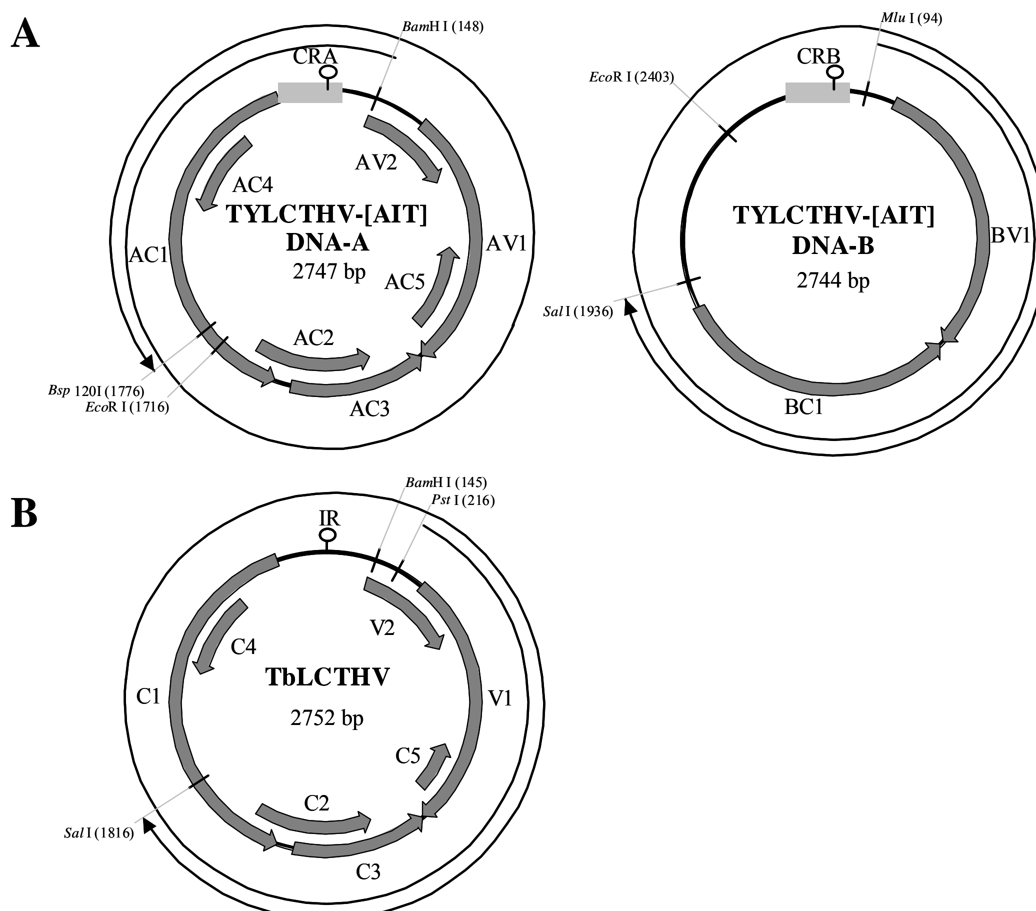


Fig. 1. Map of the bipartite TYLCTHV-[AIT] (**A**) and the monopartite TbLCTHV genome (**B**) analyzed in this study. Large arrows show ORFs in viral sense or complementary orientation. The loop symbol presents the position of the stem-loop, containing the conserved TAATATTAC sequence. Thin arrows around genomic segments show the start and end point of the multimeric copies. Indicated restriction sites were used in cloning procedures.

Full-length clone inoculation

Full-length clone inoculation was either performed by particle bombardment or agroinoculation.

For biolistic delivery DNA was precipitated on tungsten microprojectiles (BIO-RAD) and introduced into plants with a particle inflow gun (PIG) [110] using an air pressure of 3 bar and a vacuum of 200 mbar. Three sources of full-length viral DNA were used with TYLCTHV-[AIT] in co-bombardment experiments (A and B component). Firstly, full-length viral genomes were excised from plasmids with the respective restriction enzyme (for TYLCTHV *Eco*RI and TbLCTHV *Bam*HI); secondly, DNA was delivered as a multimeric copy and thirdly, concatameric copies of Phi29 DNA polymerase amplified DNA were used for inoculation. DNA was purified in all cases with SureClean (Bioline GmbH) and concentration was determined spectrophotometrically. For bombardment experiments each component was adjusted to 5 ng of viral DNA if not stated otherwise. Single geminiviral components were applied as RCA products for TYLCTHV-[AIT] A and B, respectively, as well as TbLCTHV, which was additionally tested with DNA-B of TYLCTHV-[AIT] in co-bombardment experiments.

For agroinoculation multimeric constructs were transferred to *A. tumefaciens* strain C58C1. Recombinant agrobacteria were streaked out and subsequently 5 single colonies were transferred and grown overnight in 30 ml LB media (OD_{600} of 1, 28°C and 250 rpm). For infection with a single geminiviral component 2 ml of the bacteria suspension were pelleted and resuspended in the same volume of agroinoculation buffer (10 mM $MgSO_4$, 10 mM MES and 100 µM acetosyringone), whereas for co-inoculation of A and B components 2 ml of each bacteria were pelleted in the same tube and resuspended in 2 ml agroinoculation buffer. After 3 to 4 hours incubation at room temperature two leaves of each plant were injected into the abaxial intercellular leaf space. Plants were left for 1 day at 26°C in a climate chamber before transfer to the greenhouse.

Sequence analysis

Sequence analysis was done with NCBI/BLAST, alignments were performed with ClustalX [308] and promoter analysis was done with PLACE [130]. Iteron sequences

and the iteron-related domain (IRD) of Rep were analysed according to Arguello-Astorga and Ruiz-Medrano 2001 [14], graphics were generated with TreeView 1.5.2 [236] and SimPlot Version 3.5.1 [189]. Recombination analysis was done with the RDP method [197] included in the RDP-V2 Beta 08 software 2 [198] with following settings: window size 10, highest acceptable probability 0.001, internal reference sequences [19, 25, 281]. The following Begomovirus DNA A sequences were retrieved from GenBank for multiple sequence analyses: AEV - AJ437618; AYVCNV-[Hn2] - AJ495813; AYVSLV - AF314144; AYVV - X74516; BYVMV-[301] - AJ002453; ChiLCuV-[Mul] - AF336806; CLCuAV-[802a] - AJ002455; CLCuKV-[72b] - AJ002448; CLCuMV-[26] - AJ002458; CLCuRV - AF363011; CYVMV - AJ507777; EpYVV - AB007990; ICMV - Z24758; MYVV-[Y47] - AJ457824; OYVMV-[201] - AJ002451; PaLCuCNV-[G2] - AJ558123; PaLCuCNV-[G30] - AJ558117; PaLCuV - Y15934; PepLCBV - AF314531; PepLCV - AF134484; SbCLV-[JP] - AB050781; SLCCNV - AB027465; SLCMV-[Col] - AJ314737; SLCYNV - AJ420319, StaLCuV-[Hn5] - AJ495814; TbCSV-[Y1] - AF240675; TbCSV-[Y35] - AJ420318; TbCSV-[Y41] - AJ457986; TbLCJV - AB028604; TbLCKoV-[KK] - AB055009; TbLCTHV - DQ871221; TbLCYNV-[Y136] - AJ512761; TbLCYNV-[Y143] - AJ512762; TbLCYNV-[Y161] - AJ566744; TbLCYNV-[Y283] - AJ971267; TbLCYNV-[Y3] - AF240674; Tobacco leaf curl virus isolate Kamphaensaen - AY633750 (referred to here as Isolate-[K]); Tobacco leaf curl virus isolate Tak Province - AY633751 (referred to here as Isolate-[TP]); ToLCBDV - AF188481; ToLCBV - Z48182; ToLCGV-[Kel] - AF449999; ToLCKV - U38239; ToLCLV - AF195782; ToLCMV - AF327436; ToLCNdV-[Luc] - Y16421; ToLCPV - AB050597; ToLCSLV - AF274349; ToLCTWV - U88692; ToLCV - S53251; ToLCVV - AF264063; Tomato yellow leaf curl Thailand virus (host tobacco) - AY639605 (referred to here as Isolate-[CM]); TYLCCNV - AF311734; TYLCTHV-[1] - X63015; TYLCTHV-[2] - AF141922; TYLCTHV-[AIT] - DQ871222; TYLCTHV-[CM] - AY514630; TYLCTHV-[MM] - AF206674; TYLCTHV-[NK] - AY514631; TYLCTHV-[SK] - AY514632; TYLCTHV-[Y72] - AJ495812.

The following Begomovirus DNA B sequences were retrieved from GenBank for multiple sequence analyses: TYLCTHV-[1] - X63016; TYLCTHV-[2] - AF141897; TYLCTHV-[AIT] - DQ871220; TYLCTHV-[CM] - AY514633; TYLCTHV-[NK] - AY514634; TYLCTHV-[SK] - AY514635.

3.4 Results

Detection, amplification and cloning of geminiviral genomes

Leaf samples of tomato plants grown in greenhouses at the AIT in Bangkok, Thailand, which showed typical symptoms of a geminivirus infection, were positively tested by ELISA with Anti-TYLCV antiserum. Total DNA was extracted and Phi29 polymerase amplification was applied and verified by visualization of high molecular weight DNA on agarose gels. RCA products were digested with a restriction enzyme putatively cutting only once in the TYLCTHV genome (*Eco*RI). DNA bands of about 2.7 kb where identified for two samples, whereas a third sample delivered a band of about 2.6 kb. All fragments were subsequently cloned and sequenced, revealing complete sequences of a putative DNA-A and DNA-B components of TYLCTHV. In addition an incomplete DNA component of a so far unknown geminivirus was sequenced (2.6 kb), Completion of the unknown geminivirus sequence was achieved by additional cloning steps of *Bam*HI digested RCA products. Attempts to gain supplemental geminiviral sequences from this plant sample by RCA and PCR approaches to identify a putative DNA-B component and/or a beta satellite DNA were not successful, indicating a single infection.

Sequence analysis of TYLCTHV DNA-A and DNA-B component

Complete sequencing of the TYLCVTHV DNA-A and DNA-B components revealed lengths of 2747 bp and 2744 bp, respectively. The typical geminivirus GC-rich stem-loop structure was identified along with the conserved nonanucleotide sequence. The common region of DNA-A (CRA) is located from nt 2615 to 41 and shares 97% sequence identity to the CRB (nt position 2612-41) of DNA-B. Both components possess the same iteron sequences "ATY**GGKGT**T" (core sequences in bold) for DNA-A from nt position 2620-2627 and for DNA-B from nt position 2617-2624, which is in accordance with the IRD "FRIN" of the Rep protein of TYLCTHV-[AIT] DNA-A. Additionally, 9 ORFs were identified on DNA-A and DNA-B as already described for TYLCTHV (Fig. 1A). However, the first ATG codon of AV2 is unlikely to act as a start codon, because a CAAT-Box (61-64 nt) and two possible TATA-Boxes (88-93 nt: TTATTT; 103-109 nt: TATTTAA) precede the more likely start

codon at nt position 142. In addition, a reading frame, named AC5, is predicted to be transcribed based on a CAAT-Box at nt position 1115-1112 and a TATA-Box at nt position 1074-1068 (TATTAAT).

The DNA-B of the putative TYLCTHV revealed sequence identities from 94% to 95% to all described B components of this species ([1], [2], [CM], [SK] and [NK]), whereas no higher identities were found to any other geminiviral B component with a BLAST search. Furthermore the GC-rich stem-loop structure with the conserved nonamer sequence was present in the CRB. On DNA-B the two typical major ORFs for BV1 in sense and BC1 on the complementary strand were detected. The corresponding transcription starts were predicted from the sequence by existence of preceding CAAT- and TATA-Boxes.

According to criteria for geminivirus species demarcation the nucleotide identity of DNA-A was used. Alignments of DNA-A with selected geminiviruses showed the highest identity to all other described TYLCTHV strains ranging from 93%-97%, exceeding clearly the species separation threshold of 89% (Table 1). The similarity of TYLCTHV-[AIT] to other isolates of this species was underlined by a separate cluster in the phylogenetic tree with geminiviruses from Asia and India (Fig. 2). The identity was continuously high over the complete DNA-A segment (Table 1), indicating a non recombinant component. Nevertheless, to test if TYLCTHV-[AIT] results from an intra-isolate recombination all available TYLCTHV isolates (1, 2, CM, MM, NK, SK, Y72) were analysed with the RDP method. For TYLCTHV-[AIT] no significant recombination event was detected. All together the sequence analysis of the cloned A and B components revealed all typical geminivirus sequence characteristics and lead to the proposal of the new strain TYLCTHV-[AIT].

Table 1. Nucleotide identities between TYLCTHV-[AIT] and TbLCTHV first row and 16 other begomoviruses. Viruses with highest nucleotide identities were found with a BLAST search using the complete DNA-A sequence, individual ORFs and IR. Upper value represents alignment with TYLCTHV-[AIT], lower value with TbLCTHV

Virus isolates	A segment	AV2 (V2)	AV1 (V1)	AC3 (C3)	AC2 (C2)	AC1 (C1)	AC4 (C4)	IR
TYLCTHV-[AIT] vs. TbLCTHV	85	78	92	79	80	87	95	76
TYLCTHV-[1]	96	96	98	99	98	95	96	88
	85	79	91	79	80	87	95	82
TYLCTHV-[MM]	95	95	94	97	96	96	98	93
	84	78	90	80	80	87	93	77
TYLCTHV-[Y72]	93	96	94	97	96	92	95	86
	85	79	89	80	80	87	95	81
TYLCTHV-[CM]	97	96	97	99	100	96	99	94
	85	78	91	79	80	87	94	76
TYLCTHV-[NK]	96	97	97	97	96	94	97	94
	85	78	92	79	81	86	93	77
TYLCTHV-[SK]	93	88	93	99	100	91	93	91
	87	87	97	79	80	88	98	74
TbLCYNV-[143]	81	76	91	79	80	78	71	76
	88	97	98	97	96	81	72	74
TbLCYNV-[Y3]	81	75	90	79	80	77	71	75
	88	96	97	97	95	81	72	74
TbLCYNV-[283]	80	74	87	79	80	78	71	75
	87	96	92	97	96	81	72	74
TbCSV-[Y35]	80	76	76	75	78	87	95	81
	85	96	79	77	74	92	100	84
TbCSV-[Y41]	82	76	76	75	78	90	98	87
	83	96	80	76	74	89	93	79
TbCSV-[Y1]	80	76	76	74	78	86	95	81
	85	96	80	76	73	92	100	83
AEV	76	75	76	73	77	78	75	71
	78	96	80	75	73	78	74	64
AYVCNV-[Hn2]	79	70	75	80	81	84	93	78
	84	75	77	96	94	87	95	82
PaLCuCNV- [G30]	79	76	75	81	81	83	93	76
	86	84	76	96	93	91	98	86
PaLCuCNV-[G2]	79	75	76	80	82	83	93	73
	86	83	76	95	92	90	98	85

Sequence analysis of a putative new geminivirus species occurring in tomato

Sequence analysis revealed one genome component of a putative new geminivirus of 2752 bp. Additionally, efforts to detect other geminiviral components failed. The intergenic region (IR) presented a typical geminiviral GC-rich stem-loop structure with the conserved nonanucleotide sequence. The iteron sequence “WTY**GGGTM**C” (core sequence in bold) found from nt position 2619-2627 with the corresponding Rep IRD “KKFFIN” differs clearly from those of TYLCTHV-[AIT]. However, ORFs were organized like those of TYLCTHV DNA-A (Fig. 1B). In addition, a nested start

codon at nt position 2449 was predicted for the ORF C4, concluded from homology to geminiviral C4 proteins and a separate putative internal transcription start (CAAT-Box at nt position 2610-2607 nt and TATA-Box at nt 2583-2577 for TATAAAT).

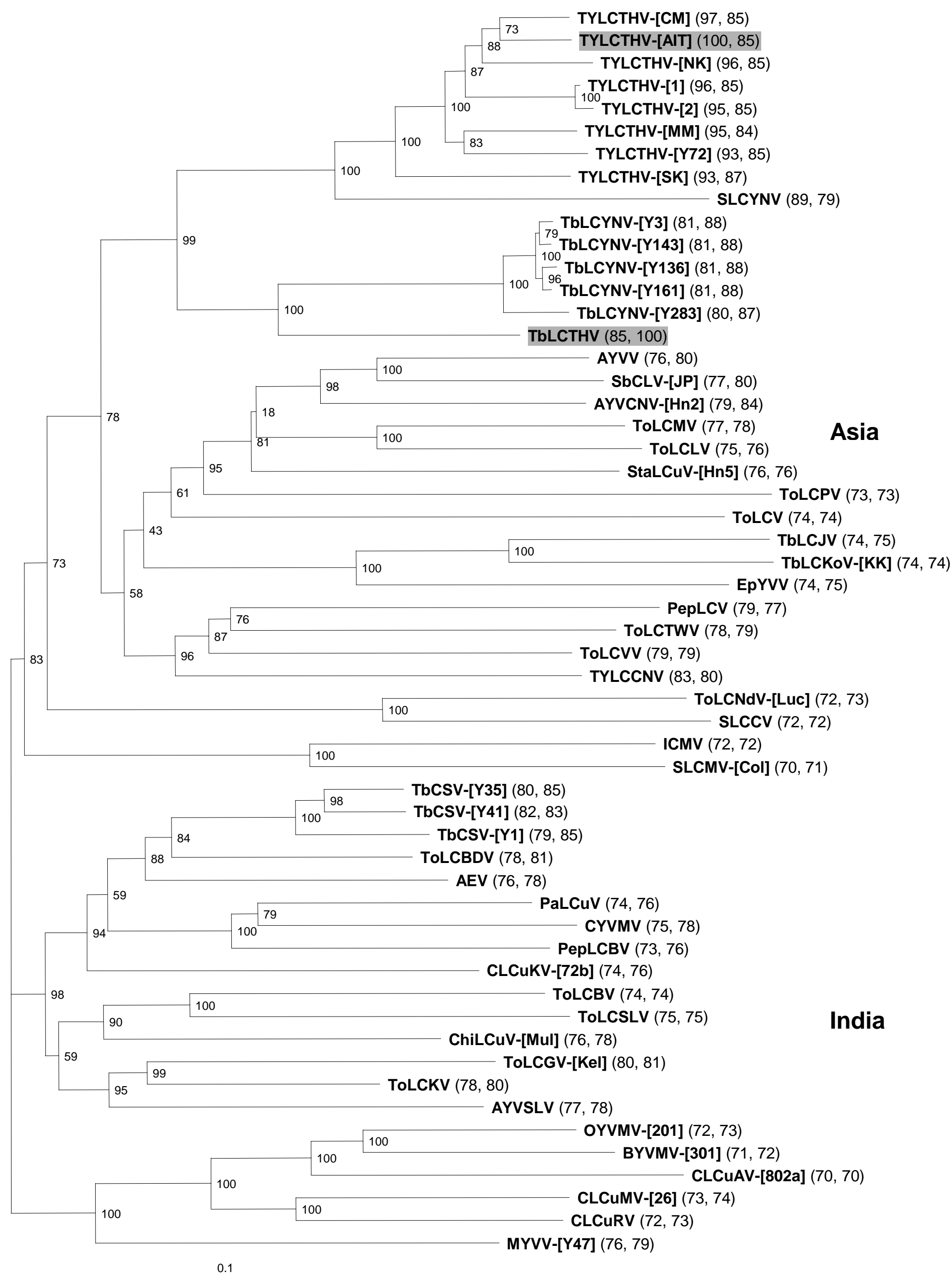


Fig. 2. Phylogenetic tree of the complete nucleotide sequence of the DNA A component of representative begomoviruses limited on species of the Old World, which are assign to the Asia and India group in the VIII report of viruses [293]. Isolates analysed in this study shaded grey. Percent nucleotide identity to TYLCTHV-[AIT] and TbLCTHV presented in brackets. Bootstrap scores in percent are shown at nodes (100 replicates)

However, even if C5 showed an identical start position it revealed only 56 amino acids residues (aa) compared to the predicted TYLCTHV-AIT AC5 with 99 aa.

The nucleotide identities to all described geminiviral DNA-A components were evaluated for the new sequence. The highest identity was found to *Tobacco leaf curl Yunnan virus* TbLCYNV-[Y143] with 88% and also to four other strains of TbLCYNV with 87-88% (Table 1). Following the criteria for geminivirus species demarcation, this will lead to a new geminivirus, tentatively named Tobacco leaf curl Thailand virus (TbLCTHV). The phylogenetic tree shows a distant relationship of TbLCTHV to TbLCYNV isolates, and also to TYLCTHV-[AIT] (Fig. 2).

Comparison of nucleotide sequence identities of single ORFs showed discontinuous similarities to one geminivirus (Table 1), indicating a recombinant nature of the genome. The overall nucleotide sequence identity to TbLCYNV-[Y143] with 88% results from high identities of ORFs V2, V1, C3 and C2 in a range of 96-98% and relatively low identities of C1 (81%), C4 (72%) and the IR (74%) (Table 1). For ORF C1, C4 and the IR the highest identities (92%, 99.7% and 84%, respectively) were found to *Tobacco curly shoot virus* (TbCSV) isolate Y35 (Table 1). To test if TbLCTHV results from an intra-species recombination event the RDP method was applied with all available TYLCTHV isolates, two TbLCYNV isolates (Y143 and Y3) and two TbCSV isolates (Y35 and Y1). For TbLCTHV one potential recombination event (breakpoints at nt 1935 and 2676) were detected with the probability (RDP: $P = 1.801 \times 10^{-26}$) that the indicated region do not have a recombinant origin (see Fig. 3B). TbLCYNV-[Y143] was identified as major parent and TbCSV-[Y35] as minor one (see Fig. 3B).

Further GenBank analysis identified three partial geminiviral sequences found in Thailand on tobacco plants referred here as isolate-[CM], -[K] and -[TP]. The nucleotide sequence identity of the complete V1 ORF (CP) revealed, that two of them (isolate-[CM] and -[TP]) have a continuous high identity to TbLCTHV and TbLCYNV-[Y143] CP (average 97-98%) (Fig. 3D). In contrast one isolate [K] has a continuous high identity to THLCTHV-[AIT] (98%) but only 91% to TbLCTHV. Both THLCTHV-[AIT] and Isolate-[K] show a typical divergence in den N-terminal part of the CP sequence compared to the TbLCTHV, TbLCYNV-[Y143], [CM] and [TP] (Fig. 3D).

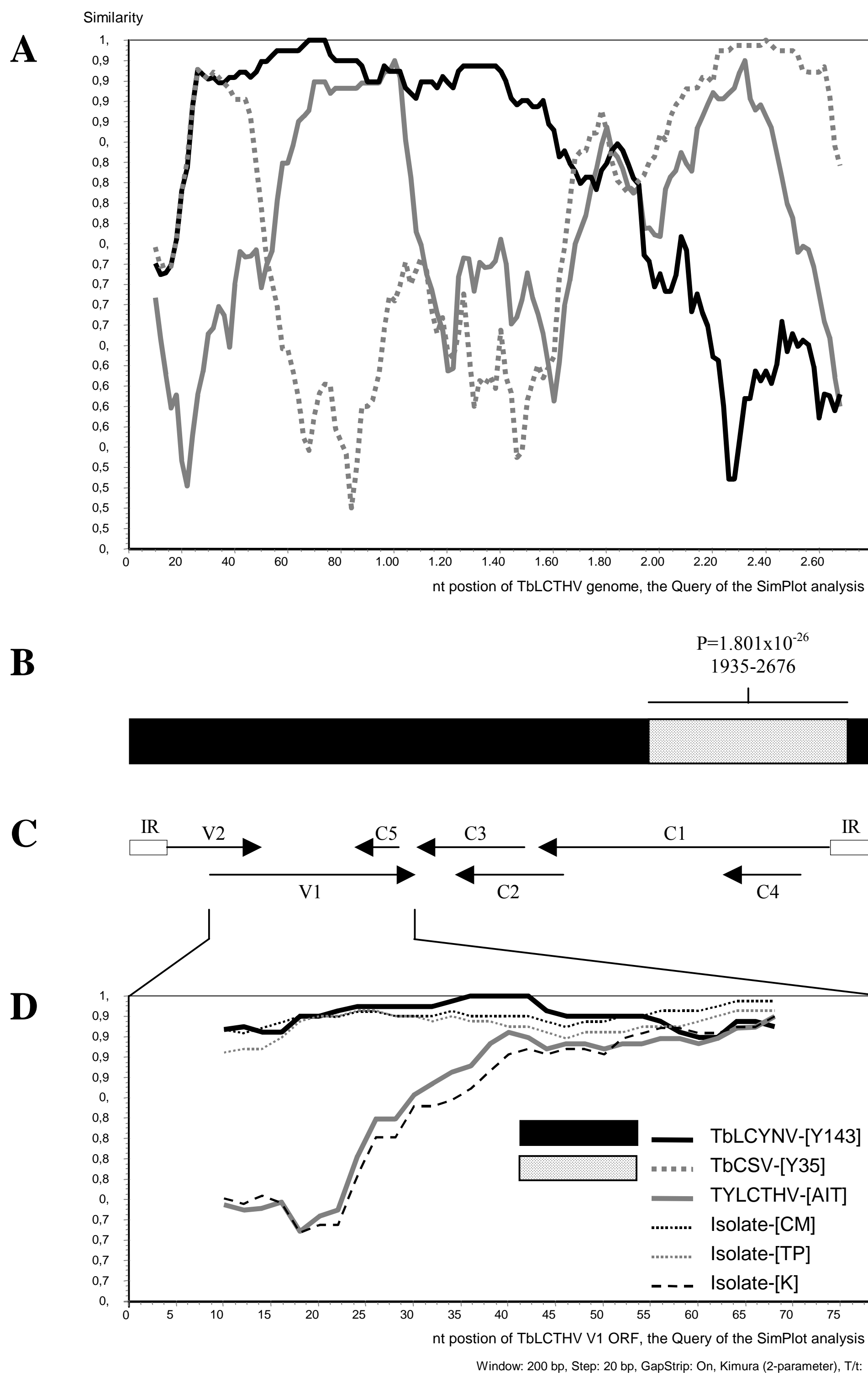


Fig. 3. Nucleotide sequence analysis of TbLCTHV. Plot of similarity of TbLCTHV genome as query to a set of three reference sequences [189] (**A**). Schematic representation of the recombinant region of TbLCTHV with the possible breakpoints and the probability (*P*) that the indicated regions do not have a recombinant origin [198] (**B**). Genome organisation of TbLCTHV drawn to scale (**C**). Plot of similarity of the nucleotide sequence of geminivirus V1 (CP). TbLCTHV was used as query to TBLCYNV-[Y143], TYLCTHV-[AIT] and three geminivirus CP sequences deposited in GenBank (**D**).

Infectivity of TYLCTHV-[AIT] and TbLCTHV full-length clones

To verify the infectivity of full-length clones of TYLCTHV-[AIT] and TbLCTHV and to confirm the distinct virus aetiology particle bombardment and agroinoculation experiments were carried out.

TYLCTHV-[AIT] DNA-A and DNA-B applied together with particle bombardment or agroinfection lead to severe stunting, yellowing and leaf curling on *N. benthamiana* plants (7 dpi). Virus symptoms correlated with positive ELISA readings (Table 2). If TYLCTHV DNA-A and DNA-B were introduced separately via particle bombardment neither visual virus symptoms nor virus titres were recorded (Table 2). However, TYLCTHV DNA-A applied via agroinoculation lead to leaf upward curling and yellowing of *N. benthamiana* plants and readily detectable virus titres (16 dpi).

The evaluation of RCA products in particle bombardment, tested by co-bombardment of TYLCTHV-[AIT] DNA-A and DNA-B at a concentration of 5 ng viral DNA each, revealed a high infection rate (91%), similar to results when viral DNA was applied via multimeric plasmids (87%), whereas the excised unit-length viral DNA showed an

Table 2. Infection of *N. benthamiana* after different geminivirus full-length clone inoculation methods. Samples were tested by TAS-ELISA

Inoculation method	component[a]	symptomatic plants/plants inoculated	ELISA test[c]
Particle bombardment			
RCA product	A	0/4	-
	B	0/4	-
	T	0/4	-
	TB	0/4	-
	AB	31/34	+
DNA excised	AB	3/30	n.t.
	AB[b]	5/5	n.t.
Plasmid multimeric	AB	26/30	n.t.
Agroinoculation			
Plasmid multimeric	A	28/29	+
	T	28/34	+
	AB	34/34	n.t.

[a] A, TYLCTHV-[AIT] DNA-A; B, TYLCTHV-[AIT] DNA-B; T, TbLCTHV
[b] 50 ng viral DNA per component
[c] -, negative ELISA readings; +, positive ELISA readings; n.t., not tested

infection rate of about one tenth (10%) (Table 2). Increasing the DNA concentration to 50 ng of viral DNAs for the excised unit-lengths, leads to an increase of the infection rate to 100% (5/5) (Table 2).

When TbLCTHV was introduced via particle bombardment neither visual virus symptoms were found nor positive ELISA readings were observed. However, in agroinoculation experiments TbLCTHV caused upward leaf curling and yellowing of *N. benthamiana* plants (16 dpi) similar to symptoms due to TYLCTHV DNA-A infection. In addition, TbLCTHV was detected by ELISA. To test a putative viability of pseudorecombinants between TbLCTHV and TYLCTHV DNA-B both genome components were introduced via particle bombardment, but no infection was found, whereas for a cobombardment experiment of TYLCTHV-[AIT] DNA-A and DNA-B a high infection rate (91%) was observed (Table 2).

3.5 Discussion

The RCA technique has been shown as highly efficient method in begomovirus analysis [140]. It has been also proposed as a practicable routine geminivirus diagnostic tool bearing advantages compared with antibody based and PCR techniques [115]. The results from our study confirm these advantages, leading to identification and cloning of DNA components of two different geminivirus species as well as construction of infectious full-length clones thereof.

TYLCTHV was expected to occur in Thailand tomato plants, whereas TbLCTHV, as a proposed new geminivirus species, would have been undiscovered with conventional ELISA or PCR analysis of the CP region only. A comprehensive sequence analysis of TbLCTHV including also three CP sequences of geminiviruses found in Thailand revealed that this virus was probably overlooked in the past. Isolates found in tobacco plants in Thailand were classified correctly on the basis of their CP data as TYLCTHV. Now, after our alignments with TbLCTHV two of them (CM and TP) should be reclassified as isolates of TbLCTHV, because of a higher homology to the new geminivirus species (Fig. 3D). Hence, it can be assumed that different geminivirus species occur in one crop in Thailand. Also in Chinas province Yunnan four different geminivirus species were found in one tomato crop growing region [187]. All plants show very similar symptoms and 16 monoclonal antibodies were necessary for the distinction of the geminiviruses [187], because some, although

belonging to a different species, have a high CP sequence identity as shown for TbLCYNV-[Y3] and TYLCTHV isolates [357]. For routine analyses complete sequencing of each virus sample is nearly impracticable, whereas RCA products made from viral DNA followed by restriction fragment length polymorphism analysis would allow the detection of virus variants as proposed by Haible et al. [115]. This fact could be important in view of development of new strategies using e.g. RNA mediated resistance strategies for control of geminiviruses, which threaten tomato production in Thailand [280].

TYLCTHV-[AIT] displays the typical genome organization of a bipartite geminivirus (Fig. 1). However, the necessity of both genomic components for a successful infection is not fully justified, because TYLCTHV-[Y72] was observed to occur without DNA-B just with a DNA-β, like a typical monopartite geminivirus [187]. To verify the monopartite or bipartite nature infectious full-length clones can be used [187]. In this study the TYLCTHV-[AIT] DNA-A inoculated by particle bombardment lead not to an infection, which might be due to a limitation of particle bombardment not suitable to introduce DNA efficiently into phloem associated tissues [292, 298]. Whereas when delivered by agroinoculation infection of *N. benthamiana* occurred with similar symptoms as shown for the proposed monopartite TbLCTHV from this study. Symptoms caused by TYLCTHV-[AIT] DNA-A component after agroinoculation developed about 9 days later (in total 16 dpi) than in agrocoinoculation experiments with component DNA-B (7 dpi). However, when only DNA-A of a typical bipartite begomovirus is agroinoculated, no symptoms and viral DNA were detectable [17, 25, 195]. As exceptions DNA-A component of the bipartite Sri Lankan cassava mosaic virus (SLCMV) [279] and also DNA-A component of TYLCTHV-[1] solely lead to symptom formation [262]. In addition, TYLCTHV exhibits an analogue of the V2 ORF that is typically involved in virus movement of monopartite begomoviruses [100]. In conclusion, exclusively particle bombardment experiments of full-length geminivirus clones do not substantiate the monopartite or bipartite nature of TYLCTHV as done for isolates CM, NK and SK [280]. Our results indicate a more facultative bipartite genome of TYLCTHV, because TYLCTHV-[AIT] DNA-A solely was infectious in agroinoculation, whereas when coinoculated with the DNA-B component the virus symptoms developed more rapidly and more severe on *N. benthamiana*. In addition, one isolate [Y72] of the TYLCTHV

species was observed in a natural infected tomato plants without a DNA-B component [187].

The proposed new geminivirus species TbLCTHV seems to be a monopartite geminivirus with a recombinant background. The experimental approach excluded other viral components, because the applied RCA method would have unrevealed other DNA components like DNA-B or ß satellites [115]. Moreover, a full-length clone of the single DNA component proved to be infectious when applied on *N. benthamiana*. Therefore, it is reasonable that the recombinant structure of the genome results form the monopartite ancestors TbLCYNV and TbCSV, because both occur in the same tomato growing area. In addition, some isolates of TbLCYNV and TbCSV occur even without a DNA-ß component [187, 348]. The potential breakpoints between the N-terminal part of C1 and the IR including ORF C4 are found in recombination hot spots of geminiviruses [19, 25] and covers two of six statistically significant hot spots of monopartite geminiviruses [89]. A viable pseudorecombinant of TbLCTHV and TYLCTHV DNA-B was not found in our study, probably because of different iteron sequences [14]. Nevertheless, examples exist, where replication of DNA-B components with different iterons were observed [8, 94].

Beside the useful application of Phi29 DNA polymerase for amplification and cloning of geminiviral DNA [115, 140], Phi29 DNA polymerase was used in this study to produce viral concatameric copies from DNA-A and DNA-B unit-length geminivirus clones. The RCA products were used on one hand for cloning procedures of partial multimeric copies and on the other for particle bombardment of concatameric copies, instead of reexcising full-length genomes from RCA products with respective restriction enzymes before inoculation [115]. In our experiments 5 ng of viral RCA DNA per component and plant was sufficient for a successful inoculation by particle bombardment. This is approximately three times less DNA described for a high infection rate of a bipartite geminivirus by particle bombardment [104] but five times more reported as limit for a high infection rate of a RNA virus full-length clone [98]. Despite a high infection rate of *N. benthamiana* plants with 5 ng viral RCA DNA or viral multimeric plasmid DNA this amount was insufficient when using viral excised unit-length DNAs. Here, a tenfold higher amount of viral excised unit-length DNA was necessary to obtain a high infection rate. The amount of DNA leading to a successful infection could be influenced by the start and end point of the unit-length DNA [32] and a possibly higher sensitivity of linear unit-length DNAs than

concatameric RCA DNA or circular multimeric DNA products to nuclease degradation [102, 312]. However, the amount of viral excised unit-length DNA leading to a high infection rate in our experiments, is around hundred fold less than reported in other studies [102, 115, 280].

Phi29 DNA polymerase was evaluated in this report for amplification, one step full-length cloning and inoculation purposes of two geminiviruses. In addition, TbLCTHV was proposed as a new geminivirus species containing a monopartite genome putatively originated from a recombination event. The application of viral RCA products for infection studies was improved in two steps. Firstly, concatameric viral copies were produced from recircularized reexcised cloned viral components instead of using original viral DNA isolated from infected leaves as a template [115], giving the advantage to use an accurately defined DNA. Secondly, a particle bombardment of RCA products was successfully developed without trimming concatameric DNA to unit lengths of viral genomic DNAs [115] with the advantage of high infection rates by applying minute amounts of DNA. Nevertheless, separate particle bombardment of viral RCA products with DNA components A and B revealed non-applicable to classify a geminivirus as bipartite. Therefore, agroinoculation is still demanding to elucidate the role of selected geminivirus DNA components in the infection process.

4 RNA-mediated virus resistance in *Nicotiana benthamiana* against four important RNA-viruses infecting *Solanum lycopersicum* in Asia[3]

4.1 Abstract

RNA silencing has been demonstrated to be a powerful tool to obtain virus resistant plants by using inverted repeat constructs. However, RNA mediated resistance is limited to specific isolates due to the demand of a high sequence specificity. In addition, in several studies RNA mediated resistance was applied against viruses by using different construct designs. Here, a uniform strategy is used against four different RNA viruses out of three virus genera known to infect tomato plants in Asia. The selected viruses are classified in the genera: (i) *Tospovirus* with Capsicum chlorosis virus (CaCV) and *Tomato spotted wilt virus* (TSWV); (ii) *Cucumovirus* with *Cucumber mosaic virus* (CMV) and (iii) *Tobamovirus* with *Tomato mosaic virus* (ToMV). Four different constructs were assembled as inverted repeats from fragments of the respective N or CP gene sequence of 423 to 548 nts spaced by the ST-LS1 intron and subsequently transformed via *Agrobacterium tumefaciens* to *Nicotiana benthamiana* plants. In total 35 independent transgenic lines were generated, which were tested as T_1 plants for resistance against the respective virus. Thereof 23 lines were found resistant, 4 lines showed a delayed symptom expression and 8 lines were susceptible. Selected homozygous lines were also tested in the T_2 generation and revealed resistance stability, indicated by 100% plants without symptoms.

The successfully tested inverted repeat constructs might be used in further experiments to obtain virus resistant tomato plants against the specific virus strains occurring in Asia.

[3] In preparation as Knierim D and Maiss E

4.2 Introduction

Production of vegetables under tropical and subtropical climatic conditions demands special requirements for cultivation, especially due to a high pressure of animal pests like insects, mites and nematodes. However, the direct damage is very often not a crucial factor, high losses occur mainly due to vectored viral diseases [151, 249, 335]. Hence, massive applications of pesticides to control viral vectors are done in conventional cultivation of crops, even of awareness causing side effects on the farmer, consumer and the environment, like on beneficial organisms [33, 91, 306]. Nowadays integrated pest management (IPM) strategies in combination with virus resistant cultivars are developed [64, 153, 200]. The cultivation of virus resistant plants offers the possibility to abandon frequent pesticide applications by farmers [161]. Thus the threshold level for control measures is high allowing a reduction of pesticide treatments [64, 153, 161, 275].

Two major antiviral strategies are used for crop protection. Firstly, use of naturally available resistance genes introduced into susceptible varieties by conventional breeding programs, secondly engineered transgenic resistances [105, 261].

In the first case natural resistance genes (*R* genes) are introduced in cultivars to pathogens or pests [158]. Sources of resistance are old varieties, land races or related wild type species [161, 315]. However, *R* genes have not been identified for all cultivars and each pathogen. In addition, the introduction of *R* genes by classical breeding programs can be cumbersome, especially from recessive genes [136]. For breeding approaches marker-assisted techniques or a direct *R* gene transfer might be helpful [105, 136]. Nevertheless, a more general method to establish virus resistance in virtually every crop and at least theoretically to each virus is a pathogen-derived resistance (PDR) strategy. Here the pathogen itself delivers the genetic information for the resistance [261, 274]. PDR was first applied as protein mediated resistance by expressing a functional viral protein [2]. Discovery of RNA interference (RNAi) reveals the fact that untranslatable sequences were also sufficient to achieve virus resistance [177, 178].

RNA-mediated virus resistance is based on the animal and plant inherent mechanism of RNAi [23, 202, 244, 334]. Currently at least three pathways are proposed for RNAi: (i) cytoplasmic RNA silencing triggered by small inferfering RNAs (siRNA); (ii) endogenous mediated RNA silencing triggered by micro RNAs (miRNA) and (iii)

silencing associated with DNA methylation and suppression of transcription [23]. However, in all three pathways small RNA molecules of 21-26 nucleotides in length, which results from degradation of dsRNA, are involved in the sequence specific recognition of RNA or DNA [23]. A perfect pairing in hybridization of small RNAs to e.g. messenger or viral RNAs results in their degradation, whereas an imperfect pairing leads to inhibition of translation [302]. Both mechanism act at the post-transcriptional level, referred to as post-transcriptional gene silencing (PTGS) [302], whereas DNA methylation of promoter regions results in transcriptional gene silencing (TGS) [23].

Engineered virus resistance involves the cytoplasmatic RNAi pathway. Transformation of inverted repeat (IR) constructs with respective viral sequences separated by either a spacer or an intron sequence revealed a high frequency of transgenic virus resistant plants [125, 289, 339]. The size and the sequence of the IR influenced the silencing efficiency in granule-bound starch synthase transgenic plants [126]. IR constructs with an intron or spacer sequence of at least 150 nt seem to be more stable, especially during cloning processes in prokaryotic cells, which tend to delete IR sequences [218]. In plants the effect of an intron or spacer seems to be less important [125, 339].

For DNA viruses like geminiviruses it is possible to use the TGS pathway [248]. Future resistance approaches might also use the miRNA-derived pathway as recently shown with artifical microRNAs conferring resistance to *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV) [227, 261].

One drawback of the RNA mediated virus resistance is its sequence specificity. It is proposed that at least an overall sequence identity of 90% is necessary to achieve resistance [261]. Moreover, a second drawback is, that other viruses are able to abolish the resistance with their respective suppressors of silencing [322, 328]. Therefore, it is important to know the precise crop production conditions, namely which virus species and isolates occur in a given crop-growing region, where transgenic plants with PDR are planned to be applied and released.

Virus resistant transgenic plants using IR approaches have been demonstrated for RNA containing viruses like *Cucumber mosaic virus* (CMV) [53, 157], *Plum pox virus* (PPV) [77, 237], *Potato virus Y* (PVY) [208, 210, 289], *Barley yellow dwarf virus* (BYDV) [331], *Soybean dwarf virus* (SbDV) [311] and *Tomato spotted wilt*

virus (TSWV) [44] and also for a DNA containing virus *Tomato yellow leaf curl virus* (TYLCV) [95].
In this study the RNAi-strategy was applied to produce virus resistant plants against CaCV, TSWV, CMV and ToMV occurring in crops of tropical and subtropical Asia. Four IR constructs with introns were generated from relatively short, simply deducible viral coat or nucleocapsid protein sequences and transformed via *Agrobacterium tumefaciens* into *Nicotiana benthamiana* plants.

4.3 Material and Methods

Virus isolates and plant inoculation

The virus isolates used in this study are available from the German Collection of Microorganisms and Cell Cultures (DSMZ): CaCV (PV-0864), TSWV (PV-0376), CMV (PV-0506), ToMV (PV-0137).
Infected leaf samples were grinded with buffer (0.05 M Na/K phosphate pH 7.0 with 1 mM EDTA and 5 mM Na-DIECA) and charcoal to produce sap, which was used for mechanical inoculation of *N. benthamiana* plants by using celite (Type 545 from Serva) as abrasive. Viruses were maintained in *N. benthamiana* plants.

Serological detection of viruses

The following antisera were used for serological detection of the four different viruses. CaCV was detected with *Watermelon silver mottle virus* and *Groundnut bud necrosis virus* antibodies from AGDIA® (Cat. No. SRA 61500); TSWV was detected with the TospoBroadRange antiserum from LOEWE (Cat. No. 07507); CMV was detected with the *Cucumber mosaic virus* DTL/ToRS antiserum from LOEWE (Cat. No. 07108) and ToMV was detected with the *Tomato mosaic virus* antiserum from LOEWE (Cat. No. 07047). Antisera were used in a DAS-ELISA according to the manufactures protocols. For the tissue print immuno assays (TPIA) only alkaline phosphatase labeled antibodies were used in a slighty modified method [133], changing to FAST RED (Sigma) for color development.

Cloning and molecular analyses of viral N- and CP-genes

The N-genes (TSWV and CaCV) and the CP-genes (CMV and ToMV) were amplified in a one-step RT-PCR reaction (AMV-RT and a heat stable DNA polymerase with 1/10 of Pfu). The primer pairs for each virus were designed from conserved regions of the respective species (Table 1.). As template a total RNA extraction was used, which were prepared from infected tomato leafs (250 mg) according to Verwoerd [324] and supplemented with a DNA digestion step [294]. The amplified fragments were subsequently cloned in standard cloning vectors and sequenced by MWG-Biotech AG (Ebersberg, Germany).

Table 1. Primers used to amplify viral sequences (first primer pair) and to generate the intron-hairpin constructs (second primer pair) of the respectively virus species

Virus	Primer name: sequence[a] (5´- 3´)
CaCV	WSMV-S: TTTGAATGATCACCATAATCA
	WSMV-AS: AGCAGATGTTGAAATTGAAAC
	CaCV_CAS-S: **AGCTAGCAGGATCC**AACAACTTATCTAAATCTTCT
	CaCV_CAS-AS: **ACTGCAGCCTAG**GAAATGACCTTCACAAACTGCTTGA
TSWV	TSWV-S: **AAGGATC**CACACTAAGCAAGCACAAGCAA
	TSWV-AS: **AAACTGCAG**ACATACGGTCAAAGCATATAACAA
	TSWV_CAS-S: **AAACGCGTACGGATCCTA**TAGTGTCATACTTCT
	TSWV_CAS-AS: **AAAGATCTGTCGACTAACTAAC**TAAGCTCACTAGGGAA
CMV	CMV-UP: **GGGGATC**CATGGACAAATCTGAA
	CMV_LOW: **AATTCTGCAGT**TCAAACTGGGAGCAC
	CMV_CAS-S: **AAAGATCTAGATAA**GGTTCCCGCTCCGCT
	CMV_CAS-AS: **AAACGCGTGTGGATCCTA**T ACTTTCTCATGTCA
ToMV	TMV1: **GGAAGCTTAT**TTAAGATGCAGGTGCAGAGGTC
	TMV2: **GGGGATCC**ATGGCTTACTCAATCACTTCTCCA
	ToMV_CAS-S: **ATAGATCTTAAGGTA**ACTTCTCCATCGCAAT
	ToMV_CAS-AS: **ACACGCGTGCTAGCTTATGCATTA**TTCAAAAGTATTCTG

[a]Engineered 5´overhang are shown in bold

Sequence analysis

Alignments were performed by ClustalX [308] and phylogenetic trees were generated with TreeView [236]. Sequences were retrieved from GenBank for multiple sequence analyses.

Accession numbers of CMV sequences: AS - AF013291; ChCu - X65017; DEL - AJ131626; DKRD - U10922; E5 - D42080; FC - D10544; Fny - NC_001440; Kin - Z12818; Mf - AJ276481; NT9 - D28780; PE - AF268597; S - AF063610; Syn -

U66094; TR15 - AJ810264; TrK7 - L15336; VAL90/1 - AJ829779; Xb - AF268598; Y - D12499; YN-B - AJ242585.

Accession numbers of ToMV sequences: camellia - AJ417701; Impatiens hawkeri - AY063743; K1 - AJ243571; Lisianthus - AY383730; OM - X02144; potato1 - AF260730; PV-0472 - AJ429086; Queensland - AF332868; S1 - AJ132845; SH 05 - DQ661035.

Accession numbers of tospovirus sequences: CaCV-(1043) - AY036058; CaCV-(958) - AY036057; CaCV-(AIT) - DQ256123; CaCV-(CP) - DQ355974; CaCV-(TD8) - AY647437; CaCV-(To-Kalasin) - AY626762; CCSV - AY867502; CSNV - AF067068; GBNV - NC_003619; GRSV-(B) - L12048; TCSV-(BR-03) - S54325; TSWV-(1439) - AY818321; TSWV-(CPNH9) - NC_002051; TSWV-(DH37) - AJ418779; TSWV-(KP) - AB175809; TSWV-(LE98/527) - AJ418781; TSWV-(NC-1) - AY744476; TSWV-(p170) - DQ431237; WBNV - AF045067; WSMoV - NC_003843; ZLCV - AF067069.

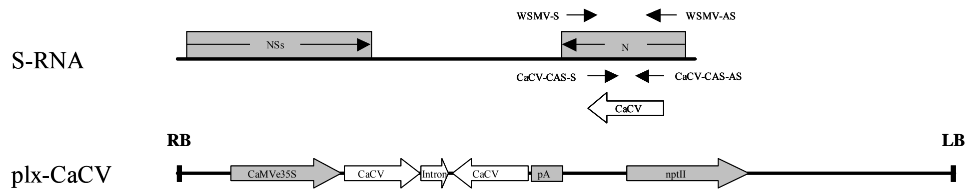
Construction of binary plasmids for plant transformation

The transformation cassettes were designed as inverted repeat constructs separated by an ST-LS1 intron under control of an enhanced 35S promoter (Fig. 1.). The virus sequences used for the transformation cassettes were amplified from sequenced plasmids with specific primers including appropriate restriction sites at the 5'-end of the genes (Table 1). After sequence confirmation the DNA fragments were assembled as IRs separated by intron ST-LS1 IV2 from potato [80], which was modified with restriction sites [150] and is spliced in *Nicotiana benthamiana* plants [356]. Cassettes consisting of CaCV, TSWV and CMV were cloned in the transformation vector pLX222 carrying the *npt*II gene as selection marker [169], whereas ToMV was cloned in pGREEN0229 carrying the BAR-gene as selection marker [129] (Fig. 1.).

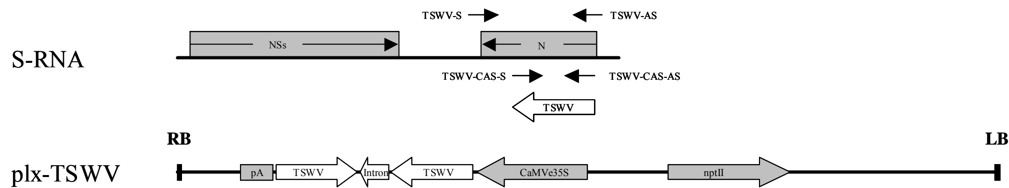
Plant transformation and analysis of the regenerated lines

Transformation vectors carrying the IR cassettes were introduced into *A. tumefaciens* LBA4404 and used for leaf disk transformation of *N. benthamiana* according to Dieterich 2000 [76, 132]. Selection of transformants was done on Murashige and

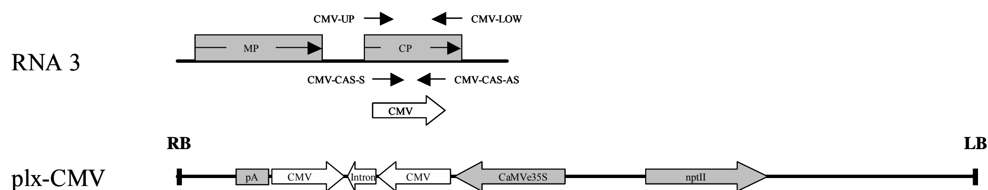
CaCV (PV-0864)



TSWV (PV-0376)



CMV (PV-0505)



ToMV (PV-0137)

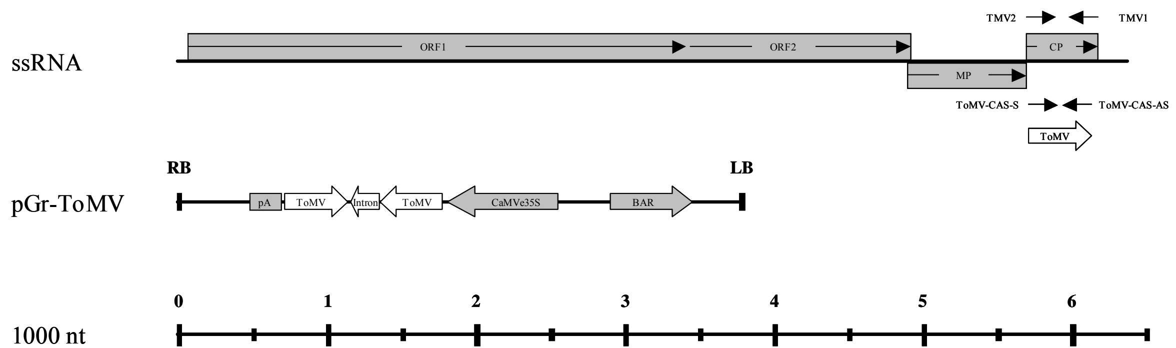


Fig. 1. Genomic map of the respective viral RNA with a schematic overview of the T-DNA fragment carrying the inverted repeat construct of CaCV, TSWV, CMV and ToMV, respectively

Skoog (MS) media with 50 mg/l kanamycin for pLX222 constructs and 4 mg/l DL-phospinothricin (PPT) for the pGREEN0229 construct.

Plants were regenerated from shoots (T_0 generation) and cultivated in the greenhouse to harvest the self-pollinated seeds from each line (T_1 generation). Surface sterilized T_1 seeds were placed on selective MS media 400 mg/l kanamycin for the pLX222 constructs and 15 mg/l DL-phospinothricin (PPT) for the pGREEN0229 construct to test the segregation pattern. The number of transgene insertions for each line was

calculated on the basis of the ratio of antibiotic resistant to sensitive seedlings, whereas a 3:1 segregation pattern was indicated as one, respectively a 15:1 and 63:1 as two and three insertions of the T-DNA on different chromosomes ($\chi^2_{1:0.05} \leq 3.84$). Besides the expression of the selection marker each line was tested for the presence of the transgene using standard PCR reactions. Here, the respective primers, which were used in construction of the plasmids (Table 1), were applied to DNA of the preselected T_1 plants. DNA extracts were purified according to Edwards et al. 1991 [81].

T_1 lines showing virus resistance were selected for further seed production (T_2 generation). T_2 seeds were analyzed for segregation patterns on selective MS media. If the T_1 line previously determined with one insertion and its respective progeny (T_2) were segregating at rates 3:1 the T_1 lines were considered as heterozygous lines, whereas a 4:0 segregation indicates a homozygous T_1 line.

Resistance tests

Each independent transgenic plant line was first screened for virus resistance. Up to 15 plants of each line in the T_1 generation (Table 2) were mechanically inoculated (see *Virus isolates and plant inoculation*) with the respective virus, which was maintained in *N. benthamiana* plants. As controls 15 non transgenic *N. benthamiana* plants were inoculated with viruses and additional 15 plants were mock inoculated. Evaluation of symptom development was performed visually. In addition, random samples were tested with tissue prints and ELISA. For resistance tests of homozygous lines (T_2 generation) 5 plants were used.

4.4 Results

Analysis of virus isolates

Sets of oligonucleotides were designed to amplify entire or conserved N or CP regions of CaCV, TSWV, CMV and ToMV, respectively. All primer combinations revealed clearly visible DNA fragments on agarose gels, which were subsequently cloned and sequenced.

The phylogenetic analysis of each of the four selected isolates used for the transformation shows a clustering of the respective isolate to other isolates of the same virus occurring in Asia with nucleotide sequence identities clearly over 90% (Fig. 2.).

Analysis of transgenic plant lines

Overall 35 independent transgenic *N. benthamiana* lines were established for the four different IR constructs (CaCV and TSWV 13 lines each, CMV 6 lines and ToMV 3 lines) (Table 2 and Table 3). The independency of each line is assumable, because each one was derived from a different callus.

In the T_1 generation each line was positive tested for the respective insert by PCR. In addition, the segregation pattern for each T_1 line was evaluated and was significant either for one, two or three integrations. According to this 28 lines were assumed with one, 5 lines with two and 2 lines with three integrations (Table 2).

In total 32 T_2 lines with one putative insertion and virus resistance shown in the T_1 ancestor line (see below) were tested on selective media to detect possible homozygous lines. Here, 23 T_2 lines had a significant 3:1 segregation, which identified them to be heterozygous. However, 8 lines (T069-04, T075-10, T119-12, T149-02, T152-02, 153-13, T220-07 and T231-03) are homozygous indicated by an insensitivity of the seedlings to kanamycin or PPT (Table 2).

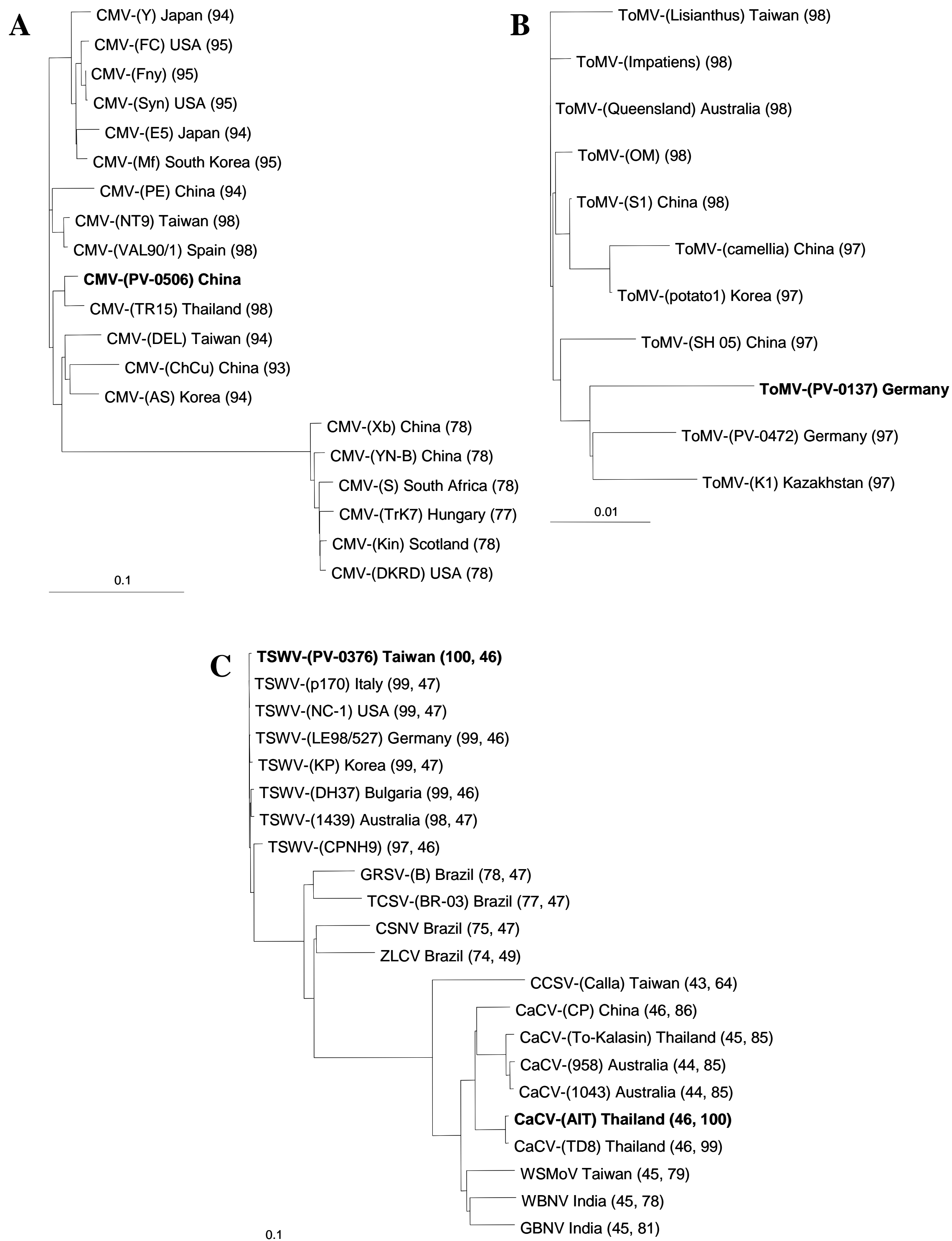


Fig. 2. Phylogenetic trees based on nucleotide sequences of CMV CP protein, percent nucleotide identity to CMV-(PV0506) presented in brackets (**A**), ToMV CP protein, percent nucleotide identity to ToMV-(PV0137) presented in brackets (**B**) and tospovirus N protein, percent nucleotide identity to TSWV-(PV0376) and CaCV-(AIT) presented in brackets (**C**). Isolates analyzed in this study bold

Table 2. Analysis of transgenic *N. benthamiana* lines. Resistance tests and segregation pattern of the marker gene in T_1 and T_2 generation of the four different intron-hairpin constructs are shown

No. of lines	Line	Gene-ration	Resistance/ plants tested	Segregation of marker	χ^2-test for segregation	($\chi^2_{1;0.05}\leq 3,84$)
CaCV						
1	T203	T_1	11/12	379:150	3:1	3.17
2	T205	T_1	5/11	418:26	15:1	0.11
3	T207	T_1	9/10	319:106	3:1	<0.01
4	T212	T_1	11/14	306:103	3:1	<0.01
	T212-04	T_2	-	93:24	3:1	1.25
	T212-13	T_2	-	68:19	3:1	0.46
	T212-15	T_2	-	56:18	3:1	0.01
5	T213	T_1	10/13	595:183	3:1	0.90
6	T218	T_1	8/10	312:2	63:1	1.74
7	T220	T_1	12/13	211:74	3:1	0.14
	T220-02	T_2	-	148:50	3:1	<0.01
	T220-04	T_2	-	67:20	3:1	0.18
	T220-07	T_2	5/5	357:0	-	-
	T220-08	T_2	-	77:26	3:1	<0.01
8	T229	T_1	7/13	193:75	3:1	1.27
	T229-05	T_2	-	189:59	3:1	0.19
9	T230	T_1	10/10	207:60	3:1	0.91
	T230-09	T_2	-	72:26	3:1	0.12
10	T231	T_1	12/15	208:69	3:1	<0,01
	T231-02	T_2	-	100:27	3:1	0.94
	T231-03	T_2	-	278:0	-	-
	T231-13	T_2	-	58:19	3:1	<0.01
11	T234	T_1	15/15	338:5	63:1	0.02
12	T238	T_1	9/12	368:125	3:1	0.03
13	T307	T_1	11/11	159:50	3:1	0.12
CMV						
1	T073	T_1	0/15	138:36	3:1	1.72
2	T074	T_1	0/14	324:96	3:1	1.02
3	T075	T_1	8/15	137:47	3:1	0.02
	T075-02	T_2	-	15:4	3:1	0.15
	T075-04	T_2	-	56:15	3:1	0.56
	T075-09	T_2	-	37:17	3:1	1.20
	T075-10	T_2	5/5	49:0	-	-
4	T106	T_1	15/15	228:22	15:1	2.77
	T106-09	T_2	-	144:1	-	-
	T106-10	T_2	-	134:0	-	-
	T106-13	T_2	-	177:0	-	-
5	T134	T_1	0/15	120:11	15:1	1.03
6	T149	T_1	9/15	179:62	3:1	0.06
	T149-02	T_2	5/5	107:0	-	-
ToMV						
1	T005	T_1	11[d]/15	193:73	3:1	0.84
2	T113	T_1	0/15	292:85	3:1	1.21
3	T119	T_1	10/15	183:68	3:1	0.58
	T119-03	T_2	6/10	369:92	3:1	6.25
	T119-12	T_2	-	312:0	-	-
TSWV						
1	T069	T_1	6/10	189:64	3:1	0.01
	T069-01	T_2	-	86:23	3:1	0.88
	T069-04	T_2	5/5	178:0	-	-
	T069-14	T_2	-	148:54	3:1	0.32
2	T078	T_1	9/11	171:72	3:1	2.77
	T078-07	T_2	-	86:27	3:1	0.07
	T078-11	T_2	-	132:36	3:1	1.14
	T078-13	T_2	-	71:32	3:1	2.02
3	T085	T_1	0/15	149:40	3:1	1.48
4	T089	T_1	13/14	357:17	15:1	1.85
5	T091	T_1	0/15	126:50	3:1	1.09
6	T096	T_1	10[d]/14	427:165	3:1	2.60
7	T101	T_1	6[d]/12	237:63	3:1	2.56
8	T124	T_1	0/14	42:20	3:1	1.74
9	T125	T_1	0/15	36:11	3:1	0.06
10	T129	T_1	13/15	440:129	3:1	1.64
11	T152	T_1	8/12	121:28	3:1	3.06
	T152-02	T_2	-	222:0	-	-
	T152-03	T_2	-	73:23	3:1	0.05
	T152-09	T_2	-	43:16	3:1	0.14
	T152-14	T_2	-	102:38	3:1	0.34
12	T153	T_1	12/15	129:48	3:1	0.42
	T153-01	T_2	-	65:26	3:1	0.61
	T153-04	T_2	-	36:7	3:1	1.74
	T153-10	T_2	-	27:10	3:1	0.08
	T153-13	T_2	5/5	115:0	-	-

[d] delayed symptom development

<0.01

Table 3. Overview of transgenic virus resistant plants generated with IRs separated by an intron or spacer sequence

References	Plant	Virus	Construct[a]				No. of resistent/ plants inoculated
			I/S	nt	Location of viral sequence	nt	
Bucher et al. 2006 [44]	*N. benthamiana*	tospo-viruses[c]	I	500	f N-gene	600	24/32[c]
Fuentes et al. 2006 [95]	*S. lycopersicum* var Campell-28	TYLCV	I	n.s.	3′ part of C1	727	1/11
Tougou et al. 2006 [311]	*Glycine max* cv. Jack	SbDV	S	670	CP	602	1/1
Di Nicola-Negri et al. 2005 [77]	*N. benthamiana*	PPV	I	707	5′UTR+ 5′ part P1	733	10/11[b]
			I	707	3′ part P1 + 5′ part HC-Pro	649	11/12[b]
			I	707	middle part HC-Pro	706	11/11[b]
			I	707	3′ part HC-Pro + 5′ part P3	678	6/6[b]
Chen et al. 2004 [53]	*N. benthamiana*	CMV	I	449	3′ part of RNA 2	1534	29/39
			Ias	449	3′ part of RNA 2	1534	24/32
			I	449	3′ part of RNA 2	490	8/25
			Ias	449	3′ part of RNA 2	490	4/12
			I	449	CP + 3′UTR	790	12/30
			Ias	449	CP + 3′UTR	790	6/12
Missiou et al. 2004 [208]	Potato	PVY	S	1250	3′ part of CP	605	13/15
Mitter et al. 2003 [210]	*N. tabacum*	PVY	S	362	NIa	735	1/1
Pandolfini et al. 2003 [237]	*N. benthamiana*	PPV	I	87	5′UTR+ 5′ part P1	197	3/5
Kalantidis et al. 2002 [157]	*N. tabacum*	CMV	S	1445	3′ part of CP	747	5/27
			S	1445	3′ part of CP	747	15/91
Wang et al. 2000 [331]	*Hordeum vulgare*	BYDV	S	860	ORF1 + 5′ part ORF2	1600	9/25
Smith et al. 2000 [289]	*N. tabacum*	PVY	I	741	Nia-protease (Pro) gene	730	22/23
			Ias	741	Nia-protease (Pro) gene	730	22/34
			S	800	Nia-protease (Pro) gene	730	25/43
This study	*N. benthamiana*	CaCV	I	190	f N-gene	509	13/13
		TSWV	I	190	f N-gene	548	6/13
		CMV	I	190	middle part of CP	491	3/6
		ToMV	I	190	f CP	423	1/3

[a] I, intron; Ias, intron antisense; S, spacer; n.s., not specified; f, fragment
[b] resistance test done with leaf discs
[c] TSWV, GRSV, TCSV and WSMoV; multiple resistant

Response of T_1 plants on virus inoculation

From each transgenic line up to 15 plants (T_1 generation) were mechanically inoculated with the respective virus and each plant was visually inspected for the presence of virus symptoms over a maximum period of 6 months. Plants exhibit three different reaction types following virus inoculation: susceptibility, delayed symptom expression and resistance. At first, susceptible T_1 plants revealed virus symptoms at the same time as non-transgenic *N. benthamiana* plants, which were simultaneously inoculated. Inoculated *N. benthamiana* plants showed typical virus symptoms 10 to 14 days past inoculation (dpi) depending on the respective virus and greenhouse conditions and were all tested positive by ELISA or TPIA, respectively. At second, transformed plants exhibit identical virus symptoms like non transgenic *N. benthamiana*, however there was a clear delay of at least 10 days in symptom development. At third, inoculated plants showed no virus symptoms and growth was not affected over a time period of 3 to 6 months. To exclude a symptomless infection, samples were tested by ELISA or TPIA substantiating virus free plants.
The resistance of a transgenic line was determined based on the response of plants (T_1 generation) descend from a single line (T_0 generation). It should be taken in consideration that individual plants of the T_1 generation can be homo- or heterozygous and consequently this generation can also include non-transgenic plants.
From each of the four viruses CaCV, TSWV, CMV and ToMV, transgenic virus resistant *N. benthamiana* lines were generated. All thirteen lines containing the CaCV IR construct showed CaCV resistance, whereas only six from the thirteen TSWV lines showed TSWV resistance. Three lines had a delayed response on TSWV inoculation (Table 2). Three out of six transgenic CMV lines showed resistance (Table 2), none of the other lines showed a delay in symptom expression. Only three independent transgenic ToMV lines were generated. From those, one line showed virus resistance, one had a response with delayed symptom expression and one line was susceptible (Table 2).

Virus resistance test of selected lines in the T_2 generation

Transgenic virus resistant lines were first screened with a segregating plant population (T_1). In an additional experiment selected lines T220-07 (CaCV), T075-10 (CMV), T149-02 (CMV), T069-04 (TSWV) and T153-13 (TSWV) with a homozygous transgenic background where tested for the stability of the resistance. The two tospovirus species caused symptoms in the non-transgenic control plants 14 dpi, whereas no CaCV was detectable in any plant of line T220-07 and no TSWV in lines T069-04 and T153-13, respectively (Table 2). The non-transgenic *N. benthamiana* plants inoculated with CMV developed clearly visible symptoms at 9 dpi. Again, neither CMV symptoms nor virus were detected in any plant of lines T075-10 and T149-02. Resistant plants developed no symptoms when analyzed over a period of 3 months.

4.5 Discussion

N. benthamiana plants were transformed with IR constructs consisting of N or CP gene fragments of different RNA viruses known to occur in tomato plants of subtropical and tropical regions in South East Asia. DNA fragments used for transformation revealed identities over 90% of the respective virus to virus isolates present in South East Asia. Transgenic lines were tested for the segregation pattern of the marker gene and the presence of the transgene followed by analysis of the response to mechanical virus inoculation with the respective isolate.

Expression of *npt* II in seedlings (T_1 generation) on selective media reveals the number of integrations of the marker gene on different chromosomes. One, two and three integrations were identified with a high significance using the χ^2-test. Although, concatameric inserts of T-DNA leading to multiple copies at the integration site can not be identified with this technique. However, T_0 transgenic plants, which were analyzed for the transgene copy number by Southern hybridization showing that the transgenic loci number matched with the copy number estimated from germination on kanamycin [157].

The initial resistance screening of transgenic lines was done with the respective virus isolate using the T_1 generation. Here, the genetic background concerning the inserts is

either homozygous or heterozygous. Both states should be able to confer resistance [77, 210]. This assumption is concluded from the fact that the segregation of the resistance tests exhibit as well a 3:1 pattern (resistant to susceptible plants) like the segregation of the marker gene when dealing with one integration site and the observation of a homogenous response for each transgenic plant of one line either reacting with susceptibility, delayed symptom expression or resistance (Table 2).

A single copy of a hairpin construct should be sufficient to achieve resistance [210, 331], but an increase of copies is proposed to improve the likeliness of siRNA generation and consequently virus resistance [157]. In addition, the overall length of the IR has a minor influence hence an extreme single short RNA can be sufficient [227].

For each of the four different constructs homozygous T_2 progenies already resistant in the T_1 generation with one integration site were selected. The subsequent resistance test verified the stability of resistance in the T_2 generation (Table 2), which was also shown for PVY and SbDV hairpin constructs [210, 311].

Other studies evaluated and compared different parts of the genome as a source of viral sequences for IR constructs. For PPV [77] and CMV [53] it was shown that all generated lines independent of the part chosen from the viral genome had a similar high level of resistance (Table 3). Nevertheless, there is a controversial discussion concerning the use of the 3´-UTR of CMV sequences, because the 3´-UTR can fold to tRNA-like structures and in this way might interfere with dsRNA formation [53]. This was circumvented in our study by using only fragments of the CP gene. However, a similar percentage of resistant lines of about 50% as with a CP sequence together with the 3´-UTR were achieved (Table 3) [53]. Experiments with bulges in the predicted dsRNA structure of a hairpin producing construct reveals no influence of those structures in a dsRNA to produce siRNAs [125]. In addition, the length used for the IR constructs in this study ranges from 423 to 548 nts, which is more than the proposed necessary length of over 300 nts [261], but less than the usual length of about 700 nts in other hairpin constructs (Table 3). Only one shorter viral sequence of 197 nts confers resistance to PPV [237].

The intron (ST-LS1 IV2), which was used for the IR constructs, was shown to be spliced in *N. benthamiana* [356], however, this was not experimentally tested in this study. Nevertheless, intron-spliced hairpin constructs were described to reveal the highest frequencies of resistant lines (90 to 100%) instead of using non-functional or

spacer sequences (50 to 58%) [289, 339]. Results for the IR constructs of TSWV, ToMV and CMV showed a frequency of 50% resistant lines, similar to hairpin and intron containing constructs [53, 237], whereas the CaCV IR construct pursuing the same intron lead to a 100% resistant lines. Here, our results are in concordance with other studies showing that an intron has no general increasing effect on efficiency of post-transcriptional gene silencing compared to spacer sequences in hairpin constructs [125].

In our study virus resistance was observed over three to six months, which is in concordance with others reports based on hairpin constructs [77, 237].

The PDR strategy by using spacer or intron-spliced hairpin RNAs has been shown as a highly efficient method to produce virus resistant plants and was shown for Cucumoviruses [53, 157] and resently Tospoviruses [44], whereas this strategy was not applied to Tobamoviruses till now. In addition for several other virus species different parts and lengths of the viral genome were used and tested (Table 3) [53, 77, 95, 157, 208, 210, 237, 289, 311, 331, 339]. Here, an uniform strategy was applied for four different virus species using N or CP sequences, respectively. This strategy has several advantages, like handy production of small DNA fragments bearing a high probability to confer resistance and limited putative environmental risks in the use of virus resistant plants. CP or N gene sequences are known from a multitude of viruses and are easily accessible. Hence, a simple amplification of these genes is possible by generic primers or with deduced ones from conserved regions of these genes. In addition, N and CP genes are usually conserved to a higher extent than other parts of the viral genome. Consequently, there is a high chance that constructs from this region might trigger virus resistance, not only against one respective isolate but also against many other isolates. It was proposed that an overall nucleotide sequence identity of at least 90% is able to mediate virus resistance to different isolates [261], which implies to use isolates occurring in the given crop-growing region. Inoculation of an IR carrying transgenic plant with a PVY isolate of 93% sequence identity confirmed this assumption [208]. Therefore, IR constructs from our selected N and CP regions could also permit a broad resistance against isolates of the respective species, as long as 90% sequence identity exists.

In general, the PDR by using inverted repeat constructs possesses a high biosafety, here the risk of transencapisdation or recombination of the transgene with an incoming virus is reduced, because the RNA is transcribed an folded to dsRNA,

which is subsequently processed by the natural cytoplasmic silencing pathway in tiny fragments (siRNAs) [23, 208, 210]. In addition, a possible suppression of silencing from a viral suppressor of any other virus appears to have no influence on the biosafety status, here, all described viral suppressors of silencing described till now, did not show to block the cleavage of dsRNAs [212].

In conclusion, our results showed the applicability to produce highly resistant *N. benthamiana* plants with IR constructs against four RNA virus species also infecting tomato. Further steps could be the integration of DNA viruses in the RNA mediated resistance strategy, underlined by the first promising results with TYLCV [95], so that against the main infecting virus on tomato plants in Asia virus resistance can be generated.

5 General discussion

In this study the virus status in a protected tomato cultivation system in Thailand has been evaluated during 2001 to 2004. Here, plant virus species from the genus *Tospovirus* and *Begomovirus* were detected, analyzed and sequenced to determine their respective complete genomic organisation. In addition, the RNA mediated virus resistance strategy was successfully applied against four important RNA viruses infecting tomato plants in Asia using the model plant *N. benthamiana*.

The detected tospovirus species was described as an isolates of CaCV, referred to as AIT isolate [163, 251, see chapter 2], on the basis of the current species demarcation criteria in the genus tospovirus, which discriminates a species when having less than 90% amino acid identity of the N protein to any other described species [225]. The AIT isolate shows with 92% the highest amino acid sequence identity to the Australian CaCV isolate 958 [163, see chapter 2]. However, a recent study proposed a revision of the tospovirus species demarcation concept, which is based on the analysis of three tospovirus species [124]. Here, for the species TYRV, IYSV and CaCV the divergence of some isolates in the N protein ranges from 8% to 9%, which is just below the threshold level of 10%. This is also in accordance with the CaCV isolate AIT from Thailand and the Australian CaCV isolate 958, whereas for TSWV and PBNV all isolates diverge only around 4% to 5% [124]. The second argument for a revision was the described divergence of the two TYRV isolates in their host range [124]. Our study showed also a clear difference in the host range of CaCV-[AIT] and substantiates the proposal of a revision [163, 201, see chapter 2]. In the case of reorganization of the genus the name Tomato necrotic spot virus (TNSV) is proposed for the CaCV-[AIT] isolate.

However, the determined host spectrum for CaCV-[AIT] includes till now no native wild plant species or weeds from Thailand. But nevertheless, it can be assumed that the seasonal infestation with CaCV-[AIT] results from a spread of a vector from infected plants beside the greenhouses, which might be important for further pest management strategies [249, 251]. Hence, a selective elimination of such plants, which may act as virus reservoir, could reduce the CaCV-[AIT] infestation [96]. This theory is concluded from the analysis of one CaCV-[AIT] vector, the thrips species

Ceratothripoides claratris (Shumsher) (Thysanoptera: Thripidae) in combination with the seasonal occurrence of the virus in the greenhouses [249, 250, 251]. Here, CaCV causes only losses in the tomato production in the dry season (October to March), which is the coldest season in Thailand and correlated with the temperature-dependant development of *C. claratris* [251]. However, *C. claratris* showed an adoption to the tropical climate, nevertheless the temperatures during the rainy period are obviously to high for this species, resulting in a low population and no infestation in greenhouses [250, 263]. The thrips species *C. claratris* seems to be the predominant in central Thailand [217, 250], but CaCV can also be vectored from other thrips species like *Thrips palmi* and *Frankliniella schultzii* [241], which may result in different vector-virus interactions.

In addition, the proposed vector depending seasonal spread from a natural virus reservoir is similar like it was described for TSWV in Tasmania [343]. However, a first survey of weeds near the greenhouses tested with the WSMoV serogroup tospovirus antisera was unsuccessful, but it was possible to infect the weed *Syndrella sp.* with CaCV-[AIT], which can be proposed as a potential natural host (data not shown). A natural infection of *Syndrella nodiflora* with the WSMoV serogroup tospovirus PBNV was also reported in Thailand [347].

However, CaCV seems to be the predominant tospovirus species in Thailand, because there have been reports on the occurrence of WSMoV serogroup tospoviruses in northeastern Thailand [246], which is supported from the N gene sequence analysis of this work [163, see chapter 2]. Furthermore, CaCV-[958] is described to break the *Sw-5* resistance gene [201], which necessitates an integration of CaCV in the RNA-mediated resistance strategy. Moreover, a RNA mediated resistance to CaCV contributed from the tospovirus TSWV construct is not expected, because of a great sequence divergence (46%). In addition, other IPM methods to control CaCV like the exclusion of thrips with nets from the greenhouses were less successful [122].

The complete genome sequence of CaCV-[AIT] presented in this study is the sixth complete genome sequence of a tospovirus species and will be helpful for further experiments like the construction of a CaCV-[AIT] full-length clone. Till now it was not possible to establish any infectious tospovirus full-length clone, however, such a reverse genetic tool for virus analysis would be of great improvement in tospovirus research. Reverse genetics has been shown for several RNA viruses, but rarely for

viruses of the family *Bunyaviridae* [138]. However, the established reverse genetic systems from virus species of the genus *Orthobunyavirus* [30, 37] and now recently *Phlebovirus* [138, 235], which are both members of the family *Bunyaviridae*, promise a chance for the production of infectious tospovirus full-length clones. In addition, the phleboviruses utilize, like tospoviruses, an ambisense coding strategy, which the other genera of the *Bunyaviridae* do not [225].

In addition, other viral diseases in the greenhouses were determined as begomovirus infections [see chapter 3]. Here, a new technique (rolling circle amplification) was applied to analyze these viruses and confirm the advantage of this new technology [115, see chapter 3]. Briefly, the rolling circle amplification (RCA) with Phi29 DNA polymerase is highly efficient, of high-fidelity and simple in handling, which consequently leads to the proposition of this method as a practicable routine geminivirus diagnostic tool [115]. All the geminivirus genome segments from this study were amplified in one step, with the aspected ORFs and by using a random hexamer primer, which makes sequence information of the viruses unnecessary [see chapter 3]. In this work two begomovirus species are described. One of them was the expected bipartite TYLCTHV and the other a new monopartite species, which is proposed as TbLCTHV with a recombinant genome [see chapter 3]. In addition, in Thailand only one other begomovirus species (*Tomato yellow leaf curl Kanchanaburi virus*) is described to infect tomato plants.
Moreover, the application of the Phi29 DNA polymerase was advanced in that way, to produce concatameric copies from the geminiviral genome from already cloned fragments, which were applied in a particle bombardment as full-length clones. However, the method showed the restriction of this technique to non phloem limited viruses like the bipartite TYLCTHV, whereas for phloem limited viruses the more laborious agroinoculation method seems to be necessary [see chapter 3].

In this study the geminiviruses were not integrated in the RNA-mediated resistance strategy, although transient experiments with hairpin constructs showed promising results [3, 248, 316]. However, this had several reasons. First of all geminviruses are DNA viruses, hence, the interference with the natural RNA silencing pathways differ from those of RNA viruses. For instance viral RNA genomes can be directly attacked by siRNAs following the theory of the cytoplasmatic silencing pathway, whereas the

geminivirus DNA genome components are not sensitive to cytoplasmic RNA silencing. Only their transcripts are exposed to this pathway [28]. Hence, the cytoplasmatic silencing of an essential geminiviral protein like the *Rep* protein, which is necessary for the viral replication, should result in resistance, but shows only a moderate success [28]. In addition, few cases are reported where *Rep* transgene silencing has been overcome, which indicates the existence of viral suppressors [193, 229], because geminiviruses provide a natural source of dsRNAs like the overlapping of the bidirectional transcribed mRNAs and the amplification of the host RdRps on aberrant or over-expressed viral transcripts [28, 317, 326]. In addition, the cytoplasmic RNA silencing is not the only component of hosts silencing response to geminivirus infection, because DNA viruses are as well targets of the third pathway the siRNA-directed epigenetic modification, which results in TGS [28, 317].

However, currently four different geminivirus proteins are described, which possesses suppressor activity [28, 359]. These are the proteins (here, all proteins are collectively described with the bipartite geminivirus nomenclature) AC2 [78, 321, 328, 330], AC4 [318], AV2 [359] and βC1, which is encoded by a satellite component [28, 60].

Interestingly, not every geminivirus species possess all suppressors, the functionality differs and depends sometimes on the interaction with the host [28, 318]. For instance AC2 is a transcription factor, which activates the expression of several cellular proteins, but as well a protein that may function as an endogenous negative regulator of the RNA silencing [28].

This might be an explanation why for some geminivirus-host combinations the *Rep* transgene silencing was successful [16, 50, 95]. Here, possibly AC4 and/or AC2 are silenced as well, which might be the only basis of suppression for this species or the suppressor is not active in this host. In addition, the synergism of mixed infections of different geminivirus species, which results in severe disease symptoms, seems to be correlated with the different suppressors of silencing of the respective virus [317, 318].

The diversity and the synergism effect of geminiviral suppressors in frequently described mixed infections [318], could make it difficult to establish a RNA mediated resistance strategy against geminiviruses either as PTGS and/or TGS [28], because if one suppressor is not silenced the complete RNA silencing can be overcome. Hence,

it is important to collect knowledge about the actual and exact conditions, which virus species and respective strains are present in a given geographical region, including information of their respective suppressors. In this study a first step was done. Two begomovirus species were described with their complete genomes [see chapter 3]. Moreover, the RCA technique, which has been applied for geminivirus analysis, showed how helpful it is. For instance the new TbLCTHV has probably been present in this crop growing region for many years but has been so far completely overlooked by conventional PCR approaches based on CP sequences or ELISA tests, which identifies a virus also on the basis of its CP. Because TbLCTHV has most propably a recombinant genome [see chapter 3] with a high nucleotide sequence identity to the CP of TYLCTHV (92%), both mentioned techniques will give not a precise result in identification of the virus species. However, other parts of both viruses like the AC2, which possesses suppressor activity [78, 321, 328, 330], differ more (80%) [see chapter 3]. Hence, the next step might be to investigate, if TYLCTHV and TbLCTHV possess different suppressors of gene silencing.
However, for the protected cultivation of tomato plants a first solution was found to control the whitefly transmitted *Begomoviruses* in an IPM strategy, which is to exclude their vectors with insect-proof nets from the greenhouses [122].

In this study the RNA mediated resistance strategy was first evaluated against four different RNA viruses infecting tomato plants in Asia (CaCV, TSWV, CMV and ToMV) [see chapter 4]. The respective inverted repeat constructs of each virus were successfully tested in the model plant *N. benthamiana* followed an *Agrobacterium tumefaciens* mediated gene transfer. At a rate of 50% to 100% transgenic virus resistant lines were identified [see chapter 4], which is in the range found with other inverted repeat constructs [53, 77, 208, 210, 237, 289, 311]. It should be pointed out, that once a line was described as resistant all plants of this line showed resistance in the homozygous T_2 generation. In addition, in this study the virus resistance was observed over three to six months, which is in concordance with others reports based on hairpin constructs [77, 237]. However, the transgenic plants were till now not analysed by RNA blotting for the presence of siRNAs, which would be one clear proof of the engendered virus resistance to be RNA-mediated.
A high frequency of generating resistant lines is important, when the inverted repeat construct are applied on the plant of interest like crops and ornamentals, which are in

many cases more cumbersome to transform than the model plant *N. benthamiana* for which well known transformation protocols exist [53, 77, 304]. Problems in tomato plant transformation are that the cultivars differ in transformation efficiency and the possible incidence of abnormal ploidy levels of transgenic tomato plants, which influences the efficiency of obtaining "useful" transformants [84, 141, 180, 282, 320]. A first test with a model plant system seems to be advisable, because the inverted repeat technique, which uses the cytoplasmatic silencing pathway, was further optimized in this study and it was shown in granule-bound starch synthase transgenic plants, that the size and the sequence of the IR influenced the silencing efficiency [126]. Here, an uniform strategy was applied for the different virus species by using fragments of about 423 to 548 nt in length from the viral protein which is used of the respective virus species for demarcation like the CP or N gene, whereas in other studies different parts and sizes (197 to 1600 nts) of the viral genome were applied [see chapter 4 Table 3]. In addition, against Tobamoviruses the inverted repeat resistance strategy has never been used before. This was done against Cucumoviruses (CMV) [53, 157] and a recent study shows now a multiple resistance against different tospovirus species by using one inverted repeat construct [44]. The advantages are that the viral CP or N sequences are usually higher conserved than other genome parts and most of the virus species are already described with their CP or N sequences and stored in GenBank. Therefore, they can simply be amplified with genus or species specific primers. This allows also a direct comparison to many other isolates to estimate if the respective construct may confer resistance, because it is proposed that at least an overall 90% nucleotide sequence identity is necessary [261]. If the RNA mediated resistance should be applied on the plant of interest, the transformation cassette should mediate resistance at least against the main virus species/isolates occurring in a certain crop growing region. Nevertheless, a nonhomologous virus with silencing suppression capabilities may reverse the complete PTGS, which results in loss of transgene-homologous virus resistance as well [304].

In this study ToMV and TSWV were integrated in the RNA mediated resistance strategy although well characterized and durable *R* genes for the tomato crop were described against these viruses species like the *Tm*-2^2 gene [158, 170, 337] and the *Sw*-5 gene [41, 158, 295]. Moreover natural *R* genes were proposed to be more preferable in the future compared to transgenic virus resistance approaches [105].

However, the ToMV and TSWV constructs were tested for the purpose to combine different, individually successful, resistance constructs in one transformation cassette for further experiments.

Overcoming of RNA mediated virus resistance was observed in one experiment (data not shown), where an accidental ToMV infection suppressed the CaCV silencing and both viruses were able to replicate in transgenic *N. benthamiana* plants. The suppressor of silencing from ToMV the 130K replicase is known to block utilization of siRNAs in the RISC formation [167]. However, not each virus species is able to suppress the silencing of virus resistant transgenic plants for another virus. It depends on the respective suppressor of silencing the virus codes for. For instance, a CMV/PVY mixed infection was described to overcome a transgenic PVY resistance and PVY becomes detectable [210], whereas Potato virus X (PVX) was unable to break PVY resistance in a similar experiment [208]. The inability of PVX to suppress the silencing for PVY is explained by the specific function of the PVX suppressor of gene silencing (P25) [208], which is described to prevent a spread of the systemic silencing signal [327], but this has no effect, because each cell of the transgenic plant produces dsRNA [208].

However, RNA mediated virus resistance has now been demonstrated with constructs using the miRNA pathway by creating artificial miRNAs (amiRNAs) [227]. The advantages are a reduction of viral gene sequences needed for the construct (21 nt) [227], the maintenance of the resistance at a low temperature (15°C), at which siRNA generated RNA silencing is sensitive [227, 299], and the possibility to combine different miRNAs in one transformation cassette [227]. However, for this strategy selection of very conserved sequences are suitable, because it has been shown for viral genes coding for the suppressor of gene silencing, like the HC-pro, that a mutation in this gene may not result in a viable or resistance breaking strain [227]. The similar is described for natural *R* genes, which are durable of many years. For instance the *Tm*-2^2 gene is described to be durable over 25 years [101], because the virus isolates which have overcome the resistance [47, 338], where not competitive as the wild types and play no role under natural conditions, because the movement protein, which acts as an avirulence product, has to be changed e.g. by mutation of its corresponding gene so that a loss of important functions will arise [170, 158].

In general, the engineered virus resistance via PDR strategy (protein or RNA mediated) possesses the possibility to produce theoretically a resistance against every virus and will play a key role in the future for modern agriculture [261]. The overall aim to reduce or avoid pesticide application, which is currently done only to control viral diseases [20], may be achieved. However, the PDR strategy has application drawbacks independent of the biological actability, mainly due to the acceptance of transgenic approachs and biosafety concerns of the public, which could result in a significant limitation of its use [105, 261]. In addition, the major drawbacks of the first PDR virus resistant plants, which used the protein mediated strategy, are circumvented with the RNA mediated approach. Here, no viral proteins are expressed (e.g. no heterologous encapsidation possibilities exist) and an almost low recombination probability due to expression of very short viral RNAs (21 nt) produced from the constructs [227].

However, RNA mediated virus resistance has as well drawbacks. For instance the described differences when applied against DNA viruses (the viral genome is not attacked directly) and the required sequence specifity (at least 90% sequence identity) results in the possibility to be overcome from other viruses for which the constructs do not code for and may reverse the complete PTGS [227, 261]. Hence, when the RNA mediated resistance should be applied for a plant species, a good overview of the respective virus species and strains, which can infect the plant of interest and are occurring in the crop growing region is necessary. In this study a first step has been done for the tomato production in the region of Bangkok, Thailand. The complete genome sequences of the CaCV, TYLCTHV and the newly discovered TbLCTHV were determined.

Nevertheless, the advantage of the RNA mediated resistance strategy, which was improved with the amiRNA technique [227], is beside the fact to confer resistance a higher biosafety level in comparison to a protein-mediated resistance strategy [105, 227]. However, acceptance problems and the possibitity that the transgene may spread with pollen to wild type plants, if the transgenic plant is not sterile or used only as rootstock, still remain [99, 116, 227]. In addition, the argument, that viruses play a role in the ecosystem in regulating plant populations in natural communities, will remain for every kind of transgenic virus resistance independent of its construct design, which may outcross to wild type plants [116]. But the possibilities and

benefits of RNA mediated resistance provide a powerful tool for a more environmental safe production of plants by reducing the application of pesticides used for protection against insect vectors of viral pathogens [116].

4 References

1. Aaziz R, Tepfer M (1999) Recombination in RNA viruses and in virus-resistant transgenic plants. J Gen Virol 80: 1339-1346
2. Abel PP, Nelson RS, 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
3. 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
4. Abu Bakar UK, Pillai V, Hashim M, Daud HM (2005) Sharing Malaysian experience with the development of biotechnology-derived food crops. Food Nutr Bull 26: 432-435
5. Accotto GP, Navas-Castillo J, Noris E, Moriones E, Louro D (2000) Typing of Tomato yellow leaf curl viruses in Europe. Eur J Plant Pathol 106: 179-186
6. Aichele D, Schwegler HW (2004) Die Blütenpflanzen Mitteleuropas 4. Franckh-Kosmos Verlag, Stuttgart
7. Ambros V (2004) The functions of animal microRNAs. Nature 431: 350-355
8. Andrade EC, Manhani GG, Alfenas PF, Calegario RF, Fontes EPB, Zerbini FM (2006) Tomato yellow spot virus, a tomato-infecting begomovirus from Brazil with a closer relationship to viruses from Sida sp., forms pseudorecombinants with begomoviruses from tomato but not from Sida. J Gen Virol 87: 3687-3696
9. Anonymous (2001) Toxic Trail. Broadcast on BBC World as part of the 'Earth Report' series. Transcript: http://www.toxictrail.org/Documents/ToxicTrail-Transcripts.pdf Accessed on 14.09.2006
10. Anonymus (2005) Diagnostics. Tomato yellow leaf curl and Tomato mottle begomoviruses OEPP/EPPO Bulletin 35: 319-325
11. Anonymous (2006) Thailand information. Agriculture SUNSITE Thailand. http://sunsite.au.ac.th/thailand/agriculture/Crop.html Accessed on 14.09.2006
12. Antignus Y, Vunsh R, Lachman O, Pearlsman M, Maslenin L, Hanaya U, Rosna 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

13. Anton A, Castells F, Montero JI, Huijbregts M (2004) Comparison of toxicological impacts of integrated and chemical pest management in Mediterranean greenhouses. Chemosphere 54: 1225-1235
14. Arguello-Astorga GR, Ruiz-Medrano 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
15. Arphorn S, Brooks R, Permsirivanich P (2006) Chainat: A case study in occupational health and safety promotion for farmers. Ind Health 44: 98-100
16. Asad S, Haris WAA, 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
17. Balaji V, Vanitharani R, Karthikeyan AS, Anbalagan S, Veluthambi K (2004) Infectivity analysis of two variable DNA B components of *Mungbean yellow mosaic virus-Vigna* in *Vigna mungo* and *Vigna radiata*. J Biosci 29: 297-308
18. Baltisberger M (2003) Systematische Botanik: einheimische Farn- und Samenpflanzen. vdf Hochschulverlag AG, Zürich
19. Bananej K, Kheyr-Pour A, Salekdeh GH, Ahoonmanesh A (2004) Complete nucleotide sequence of Iranian tomato yellow leaf curl virus isolate: further evidence for natural recombination amongst begomoviruses. Arch Virol 149: 1435-1443
20. Barker I, Henry CM, Thomas MR, Stratford R (1998) Potential Benefits of the transgenic control of plant viruses in the United Kingdom. In: Foster GD, Taylor SC (eds) Plant Virology Protocols: From Virus Isolation to Transgenic Resistance, Humana Press, Totowa, New Jersey, pp 557-566
21. Barry C, Faugeron G, Rossignol JL (1993) Methylation induced premeiotically in Ascobolus: Coextension with DNA repeat lengths and effect on transcript elongation. Proc Natl Acad Sci USA 90: 4557-4561
22. Bartlett A, Bijlmakers H (2003) Did you take your poison today? IPM DANIDA. www.ipmthailand.org Accessed on 14.09.2006
23. Baulcombe D (2004) RNA silencing in plants. Nature 431: 356-363

24. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides - SignalP 3.0. J Mol Biol 340: 783-795
25. Berrie LC, Rybicki EP, Rey MEC (2001) Complete nucleotide sequence and host range of South African cassava mosaic virus: further evidence for recombination amongst begomoviruses. J Gen Virol 82: 53-58
26. Bevan M (1984) Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res 12: 8711-8721
27. Bezerra IC, Resende RO, Pozzer L, Nagata T, Kormelink R, de Avila AC (1999) Increase of tospoviral diversity in Brazil, with the identification of two new tospovirus species, one from chrysanthemum and another from zucchini. Phytopathology 89: 823-830
28. Bisaro DM (2006) Silencing suppression by geminivirus proteins. Virology 344: 158-168
29. Bitko V, Barik S (2001) Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiol 1: 34
30. Blakqori G, Weber F (2005) Efficient cDNA-based rescue of La Crosse bunyaviruses expressing or lacking the nonstructural protein NSs. J Virol 79: 10420-10428
31. Bock KR (1982) Geminivirus diseases in tropical crops. Plant Dis 66: 266-270
32. Bonilla-Ramírez GM, Guevara-González RG, Garzón-Tiznado JA, Ascencio-Ibánez JT, Torres-Pacheco I, Rivera-Bustamante RF (1997) Analysis of the infectivity of monomeric clones of Pepper huasteco virus. J Gen Virol 78: 947-951
33. Bosch R. (1976) Public health advantages of biological insect controls. Environ Health Perspect 14: 161-163
34. Briddon RW, Liu S, Pinner MS, Markham PG (1998) Infectivity of African cassava mosaic virus clones to cassava by biolistic inoculation. Arch Virol 143: 2487-2492
35. Briddon RW, Mansoor S, Bedford ID, Pinner MS, Markham PG (2000) Clones of Cotton Leaf Curl Geminivirus Induce Symptoms Atypical of Cotton Leaf Curl Disease. Virus Genes 20: 19-26

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

37. Bridgen A, Elliott RM (1996) Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. Proc Natl Acad Sci USA 93: 15400-15404

38. Brittlebank CC (1919) Tomato diseases. J Agric Victoria 17: 231-235

39. Brodsky LI, Ivanov VV, Kalaydzidis YL, Leontovich AM, Nikolaev VK, Feranchuk SI, Drachev VA (1995) GeneBee-NET: internet-based server for analysing biopolymers structure. Biochemistry (Moscow) 60: 923-928

40. Brodsky LI,VasilyevAV, KalaydzidisYL, OsipovYS,Tatuzov ARL, Feranchuk SI (1992) GeneBee: the program package for biopolymer structure analysis. Dimacs 8: 127-139

41. Brommonschenkel SH, Frary A, Frary A, Tanksley SD (2000) The broad-spectrum tospovirus resistance gene Sw-5 of tomato is a homolog of the root-knot nematode resistance gene Mi. Mol Plant Microbe Interact 13: 1130-1138

42. Brown JK (1994) The status of Bemisia tabaci Genn. as a pest and vector in world agroecosystems. FAO Plant Prot Bull 42: 3-32

43. Bruenn JA (2003) A structural and primary sequence comparison of the viral RNA dependent RNA polymerases. Nucleic Acids Res 31: 1821-1829

44. 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

45. Buranatrevedh S, Sweatsriskul P (2005) Model Development for Health Promotion and Control of Agricultural Occupational Health Hazards and Accidents in Pathumthani, Thailand. Ind Health 43: 669-676

46. Cairns G (2005) Food biotechnology and consumer perceptions in Asia. Food Nutr Bull 26: 443-444

47. Calder VL, Palukaitis P (1992) Nucleotide sequence analysis of the movement genes of resistance breaking strains of tomato mosaic virus. J Gen Virol 73: 165-168

48. Carr JP, Gal-On A, Palukaitis P, Zaitlin M (1994) Replicase-mediated resistance to cucumber mosaic virus in transgenic plants involves suppression of both virus replication in the inoculated leaves and long-distance movement. Virology 199: 439-447

49. Chang CA, Chen CC, Ueng PP, Maroon-Lango CJ, Hsu HT (2005) Three tospovirus isolates reported in the United States, Taiwan and Australia are closely related cultures of a virus belonging to the watermelon silver mottle serogroup. Abstracts of the XIII International Congress of Virology: 14-15

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

51. Chen CC, Chiu RJ (1996) A tospovirus infecting peanut in Taiwan. Acta Hortic 431: 57-67

52. Chen CC, Chen TC, Lin YH, Yeh SD, Hsu HT (2005) A chlorotic spot disease on calla lilies (Zantedeschia spp.) is caused by a tospovirus serologically but distantly related to Watermelon silver mottle virus. Plant Dis 89: 440-445

53. Chen YK, Lohuis H, Goldbach R, Prins M (2004) High frequency induction of RNA-mediated resistance against Cucumber mosaic virus using inverted repeat constructs. Mol Breed 14: 215-226

54. Chitra GA, Muraleedharan VR, Swaminathan T, Veeraraghavan D (2006) Use of pesticides and its impact on health of farmers in south India. Int J Occup Environ Health 12: 228-233

55. Cho JJ, Mau RFL, German TL, Hartmann RW, Yudin LS (1989) A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Dis 73: 375-383

56. Cho JJ, Mau RFL, Pang SZ, Wang M, Gonsalves C, Watterson J, Custer DM, Gonsalves D (1998) Approaches for controlling tomato spotted wilt virus. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 547-564

57. Chu FH, Chao CH, Chung MH, Chen CC, Yeh SD (2001) Completion of the genome sequence of Watermelon silver mottle virus and utilization of degenerate primers for detecting tospoviruses in five serogroups. Phytopathology 91: 361-368

58. Chu FH, Chao CH, Peng YC, Lin SS, Chen CC, Yeh SD (2001) Serological and molecular characterization of Peanut chlorotic fan-spot virus, a newspecies of the genus Tospovirus. Phytopathology 91: 856-863

59. Chu FH, Yeh SD (1998) Comparison of ambisense M RNA of Watermelon silver mottle virus with other tospoviruses. Phytopathology 88: 351-358

60. 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
61. Cortês I, Livieratos J, Derks A, Peters D, Kormelink R (1998) Molecular and serological characterization of iris yellow spot virus, a new and distinct tospovirus species. Phytopathology 88: 1276-1282
62. Cortez I, Aires A, Pereira AM, Goldbach R, Peters D, Kormelink R (2002) Genetic organisation of *iris yellow spot virus* M RNA: indications for functional homology between the $G_{(C)}$ glycoproteins of tospoviruses and animal-infecting bunyaviruses. Arch Virol 147: 2313-2325
63. Cortez I, Saaijer J, Wongjkaew KS, Pereira AM, Goldbach R, Peters D, Kormelink R (2001) Identification and characterization of a novel tospovirus species using a new RT-PCR approach. Arch Virol 146: 265-278
64. Culbreath AK, Todd JW, Brown SL (2003) Epidemiology and management of tomato spotted wilt in peanut. Annu Rev Phytopathol. 41: 53-75
65. Cuozzo M, O'Connell KM, Kaniewski W, Fang RX, Chua NH, Tumer NE (1988) Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. Biotechnology (N Y) 6: 549-557
66. Czosnek H, Laterrot H (1997) A worldwide survey of tomato yellow leaf curl viruses. Arch Virol 142: 1391-1406
67. Dangl JL, Jones JDC (2001) Plant pathogens and integrated defence responses to infection. Nature 411: 826-833
68. Dean FB, Nelson JR, Gesler TL, Lasken RS (2001) Rapid amplification of plasmid and phage DNA using phi29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res 11: 1095-1099
69. De Avila AC, Huguenot C, Resende RO, Kitajima EW, Goldbach RW, Peters D (1990) Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. J Gen Virol 71: 2801-2807
70. De Haan P, de Avila AC, Kormelink R, Westerbroek A, Gielen JJL, Peters D, Goldbach R (1992) The nucleotide sequence of the S RNA of impatiens necrotic spot virus, a novel tospovirus. FEBS Lett 306: 27-32
71. De Haan P, Gielen JJ, Prins M, Wijkamp IG, van Schepen A, Peters D, van Grinsven MQ, Goldbach R (1992) Characterization of RNA-mediated

resistance to tomato spotted wilt virus in transgenic tobacco plants. Biotechnology (NY) 10: 1133-1137

72. De Haan P, Kormelink R, de Oliveira Resende R, van Poelwijk F, Peters D, Goldbach R (1991) Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J Gen Virol 71: 2207-2216
73. De Haan P, Wagemarker L, Peters D, Goldbach R (1990) The S RNA segment of tomato spotted wilt virus has an ambisense character. J Gen Virol 71: 1001-1007
74. Deyong Z, Willingmann P, Heinze C, Adam G, Pfunder M, Frey B, Frey JE (2005) Differentiation of Cucumber mosaic virus isolates by hybridization to oligonucleotides in a microarray format. J Virol Methods 123: 101-108
75. Diaz-Pendon JA, Truniger V, Nieto C, Garcia-Mas J, Bendahmane A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223-233
76. Dieterich G (2000) Molekularbiologische Untersuchungen zur subzellulären Lokalisierung des putativen Transportproteins - P19,5k - des beet western yellows virus (BWYV) und Erarbeitung der Grundlagen für eine gentechnisch zu erzeugende Resistenz gegen das BWYV. Dissertation von der Gemeinsamen Naturwissenschaftlichen Fakultät der Technischen Universität Carolo-Wilhelmina zu Braunschweig
77. 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
78. Dong X, van Wezel R, Stanley J, Hong Y (2003) Functional characterization of the nuclear localization signal for a suppressor of posttranscriptional gene silencing. J Virol 77: 7026-7033
79. Dunoyer P, Voinnet O (2005) The complex interplay between plant viruses and host RNA-silencing pathways. Curr Opin Plant Biol 8: 415-423
80. 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

81. Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19: 1349

82. Ehler LE (2006) Perspective Integrated pest management (IPM): definition, historical development and implementation, and the other IPM. Pest Manag Sci 62: 787-789

83. Eike MC, Mercy IS, Aalen RB (2005) Transgene silencing may be mediated by aberrant sense promoter sequence transcripts generated from cryptic promoters. Cell Mol Life Sci 62: 3080-3091

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

85. Elmer JS, Sunter G, Gardiner WE, Brand L, Browning CK, Bisaro DM, Rogers SG (1988) Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAs. Plant Mol Biol 10: 225-234

86. English JJ, Mueller E, Baulcombe DC (1996) Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell 8: 179-188

87. FAO (2006) FAOSTAT-Agriculture. http://faostat.fao.org Accessed on 4.9.2006

88. Fauquet CM, Bisaro DM, Briddon RW, Brown JK, Harrison BD, Rybicki EP, Stenger DC, Stanley J (2003) Revision of taxonomic criteria for species demarcation in the family Geminiviridae, and an updated list of begomovirus species. Arch Virol 148: 405-421

89. Fauquet CM, Sawyer S, Idris AM, Brown JK (2005) Sequence Analysis and Classification of Apparent Recombinant Begomoviruses Infecting Tomato in the Nile and Mediterranean Basins. Phytopathology 95: 549-555

90. Fitzgerald A, Van Kan JA, Plummer KM (2004) Simultaneous silencing of multiple genes in the apple scab fungus, Venturia inaequalis, by expression of RNA with chimeric inverted repeats. Fungal Genet Biol 41: 963-971

91. Fleischer G, Waibel H (2003) Pesticide policy and integrated pest management. In: Maredia KM, Dakouo D, Mota-Sanchez D (eds) Integrated

pest management in the global arena, CABI Publishing, Wallingford, Oxon, UK, pp 49-63

92. Fraser RSS (1998) Biochemistry of resistance to plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 56-64

93. French CJ, Bouthillier M, Bernardy M, Ferguson G, Sabourin M, Johnson RC, Masters C, Godkin S and Mumford R (2001) First report of Pepino mosaic virus in Canada and the United States. Plant Dis 85: 1121

94. Frischmuth T, Engel M, Lauster S, Jeske H (1997) Nucleotide sequence evidence for the occurrence of three distinct whitefly-transmitted, Sida-infecting bipartite geminiviruses in Central America. J Gen Virol 78: 2675-2682

95. 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. Transgenic Res 15: 291-304

96. Gabarra R, Besri M (1999) Tomatoes. In: Albajes R, Gullino ML, Van Lenteren JC, ELAD Y (eds) Integrated pest and disease management in greenhouse crops, Kluwer academic publishers, Dordrecht , Boston, London pp 420-434

97. Gallitelli D (1998) Present status of controlling cucumber mosaic virus. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 507-523

98. Gal-On A, Meiri E, Huet H, Hua WJ, Raccah B, Gaba V (1995) Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus. J Gen Virol 76: 3223-3227

99. Gal-On A, Wolf D, Antignus Y, Patlis L, Ryu KH, Min BE, Pearlsman M, Lachman O, Gaba V, Wang Y, Shiboleth YM, Yang J, Zelcer A (2005) Transgenic cucumbers harboring the 54-kDa putative gene of *Cucumber fruit mottle mosaic tobamovirus* are highly resistant to viral infection and protect non-transgenic scions from soil infection. Transgenic Res 14: 81-93

100. Galvao RM, Mariano AC, Luz DF, Alfenas PF, Andrade EC, Zerbini FM, Almeida MR, Fontes EP (2003) A naturally occurring recombinant DNA-A of

a typical bipartite begomovirus does not require the cognate DNA-B to infect *Nicotiana benthamiana* systemically. J Gen Virol 84: 715-726

101. García-Arenal F, McDonald BA (2003) An Analysis of the Durability of Resistance to Plant Viruses. Phytopathology 93: 941-952

102. Garzón-Tiznado JA, Torres-Pacheco I, Ascencio-Ibanez JT, Herrera-Estrella L, Rivera-Bustamante RF (1993) Inoculation of peppers with infectious clones of a new geminivirus by a biolistic procedure. Phytopathology 83: 514-521

103. Gielen J, Ultzen T, Bontems S, Loots W, van Schepen A, Westerbroek A, de Haan P, van Grinsven M (1996) Coat protein-mediated protection to cucumber mosaic virus infections in cultivated tomato. Euphytica 88: 139-149

104. Gilbertson RL, Faria JC, Hanson SF, Morales FJ, Ahlquist P, Maxwell DP, Russell DR (1991) Cloning of the complete DNA genomes of four bean-infecting geminiviruses and determining their infectivity by electric discharge particle acceleration. Phytopathology 81: 980-985

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

106. Goldbach R, Kuo G (1996) Introduction. In: International symposium on tospoviruses and thrips of floral and vegetable crops. Acta Hortic 431: 21-26

107. Gonsalves C, Xue B, Yepes M, Fuches M, Ling K, Namba S, Chee P, Slightom JL, Gonsalves D (1994) Transferring cucumber mosaic virus white leaf strain coat protein gene into Cucumis melo L. and evaluating transgenic plants for protection against infections. J Amer Soc Hort Sci 119: 345-355

108. Gonsalves D, Chee P, Provvidenti R, Seem R, Slightom JL (1992) Comparison of coat protein-mediated and genetically-derived resistance in cucumbers to infections of cucumber mosaic virus under field conditions with natural challenge inoculations by vectors. Biotechnology (N Y) 10: 1561-1570

109. Gowda S, Satyanarayana T, Naidu RA, Mushegian A, Dawson WO, Reddy DVR (1998) Characterization of the large (L) RNA of peanut bud necrosis tospovirus. Arch Virol 143: 2381-2390

110. Gray DJ, Hiebert E, Lin CM, Compton ME, McColley DW, Harrison RJ, Gaba VP (1994) Simplified construction and performance of a device for particle bombardment. Plant Cell Tissue Organ Cult 37: 179-184

111. Grimsley N, Hohn B, Hohn T, Walden R (1986) ``Agroinfection,'' an alternative route for viral infection of plants by using the Ti plasmid. Proc Natl Acad Sci USA 83: 3282-3286

112. Grimsley N, Hohn T, Davies JW, Hohn B (1987) Agrobacterium-mediated delivery of infectious Maize streak virus into maize plants. Nature 325: 177-179

113. Gullino MI, Albajes R, van Lenteren JC (1999) Setting the stage: Characteristics of protected cultivation and tools for sustainable crop protection. In: Albajes R, Gullino ML, Van Lenteren JC, ELAD Y (eds) Integrated pest and disease management in greenhouse crops, Kluwer academic publishers, Dordrecht , Boston, London pp 1-15

114. Gupta R, Jung E, Brunak S (2004) Prediction of N-glycosylation sites in human proteins. In preparation

115. Haible D, Kober S, Jeske H (2006) Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. J Virol Methods 135: 9-16

116. Hails RS (2000) Genetically modified plants - the debate continues. Trends Ecol Evol 15: 14-18

117. Hall TJ (1980) Resistance at the Tm-2 locus in the tomato to tomato mosaic virus. Euphytica 29: 189-197

118. Hamilton A, Voinnet O, Chappell L, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. EMBO J 21: 4671-4679

119. Hammond-Kosack KE, Parker JE (2003) Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding. Curr Opin in Biotechnol 14: 177-193

120. Hannon GJ, Conklin DS (2004) RNA interference by short hairpin RNAs expressed in vertebrate cells. Methods Mol Biol 257: 255-266

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

122. Harmanto IME, Tantau HJ, Salokhe VM (2006) Microclimate and Air Exchange Rates in Greenhouses covered with Different Nets in the Humid Tropics. Biosystems Engineering 94: 239-253

123. Hassani-Mehraban A, Saaijer J, Peters D, Goldbach R, Kormelink R (2005). A new tomato-infecting tospovirus in Iran. Phytopathology 95: 852-858

124. Hassani-Mehraban A, Saaijer J, Peters D, Goldbach R, Kormelink R (2006) Molecular and biological comparison of two Tomato yellow ring virus (TYRV) isolates: challenging the Tospovirus species concept. Arch Virol Published online: 21 August 2006 [Epub ahead of print]

125. Heilersig BHJB, Loonen AEHM, Wolters AA, Visser RGF (2006) Presence of an intron in inverted repeat constructs does not necessarily have an effect on efficiency of post-transcriptional gene silencing. Molecular Breeding 60: 647-62

126. Heilersig HJB, Loonen A, Bergervoet M, Wolters AMA, Visser RGF (2006) Post-transcriptional gene silencing of GBSSI in potato: effects of size and sequence of the inverted repeats. Plant Mol Biol 60: 647-662

127. Heinze C, Letschert B, Hristova D, Yankulova M, Kauadjouor O, Willingmann P, Atanassov A, Adam G (2001) Variability of the N-protein and the intergenic region of the S RNA of tomato spotted wilt tospovirus (TSWV). New Microbiol 24: 175-187

128. Heinze C, Willingmann P, Schwach F, Adam G (2003) An unusual large intergenic region in the S-RNA of a Bulgarian tomato spotted wilt virus isolate. Arch Virol 148: 199-205

129. Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol Biol. 42: 819-32

130. Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27: 297-300

131. Hoffmann K, Qiu WP, Moyer JW (2001) Overcoming host- and pathogen-mediated resistance in tomato and tobacco maps to the M RNA of Tomato spotted wilt virus. Mol Plant Microbe Interact 14: 242-249

132. Horsch RB, Fry JE, Hofman NL, Eicholtz D, Rogers SD, Fraley R T (1985) A simple and general method for transferring genes into plants. Science 227: 1229-1231

133. Hsu HT, Lawson RH (1991) Direct tissue blotting for detection of tomato spotted wilt virus in Impatiens. Plant Dis 75: 292-295

134. Hsu HT, Ueng PP, Chu FH, HUZ, Ye Z, Yeh SD (2000) Serological and molecular characterization of a high temperature-recovered virus belonging to tospovirus serogroup IV. J Gen Plant Pathol 66: 167-175

135. Hudak PF, Thapinta A (2005) Agricultural Pesticides in Groundwater of Kanchana Buri, Ratcha Buri, and Suphan Buri Provinces, Thailand. Bull Environ Contam Toxicol 74: 631-636

136. Hulbert SH, Webb CA, Smith SM, Sun Q (2001) Resistance gene complexes: Evolution and Utilization. Annu Rev Phytopathol 39: 285-312

137. Hutvagner G, Mlynarova L, Nap J (2000) Detailed characterization of the posttranscriptional gene-silencing-related small RNA in a GUS gene-silenced tobacco. RNA 6: 1445-1454

138. Ikegami T, Won S, Peters CJ, Makino S (2006) Rescue of infectious rift valley fever virus entirely from cDNA, analysis of virus lacking the NSs gene, and expression of a foreign gene. J Virol 80: 2933-2940

139. Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A (1994) Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc Natl Acad Sci USA. 91: 10502-10506

140. Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T (2004) A simple method for cloning the complete begomovirus genome using the bacteriophage ϕ 29 DNA polymerase. J Virol Methods 116: 209-211

141. Jacobs JP, Yoder JI (1989) Ploidy levels in transgenic plants determined by chloroplast number. Plant Cell Rep 7: 662-664

142. Jacquemond M, Teycheney P, Carrere I, Navas-Castillo J, Tepfer M (2001) Resistance phenotypes of transgenic tobacco plants expressing different cucumber mosaic virus (CMV) coat protein genes. Mol Breed 8: 85-94

143. Jain RK, Pappu HR, Pappu SS, Reddy MK, Vaini A (1998) Watermelon bud necrosis tospovirus is a distinct virus species belonging to serogroup IV. Arch Virol 143: 1637-1644

144. Jana S, Chakraborty C, Nandi S (2004) Mechanisms and roles of the RNA-based gene silencing. Electron J Biotechnol 7: 324-335

145. Jan FJ, Fagoaga C, Pang SZ, Gonsalves D (2000) A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. J Gen Virol 81: 235-242

146. Jan FJ, Fagoaga C, Pang SZ, Gonsalves D (2000) A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing. J Gen Virol 81: 2103-2109

147. Jahn M, Paran I, Hoffmann K, Radwanski ER, Livingstone KD, Grube RC, Aftergoot E, Lapidot M, Moyer J (2000) Genetic mapping of the Tsw locus for resistance to the Tospovirus Tomato spotted wilt virus in Capsicum spp. and its relationship to the Sw-5 gene for resistance to the same pathogen in tomato. Mol Plant Microbe Interact 13: 673-682

148. Janhong K, Lohachit C, Butraporn P, Pansuwan P (2005) Health promotion program for the safe use of pesticides in Thai farmers. Southeast Asian J Trop Med Public Health 36: 258-261

149. Jirachaiyabhas V, Visuthismajarn P, Hore P, Robson MG (2004) Organophosphate Pesticide Exposures of Traditional and Integrated Pest Management Farmers from Working Air Conditions: A Case Study in Thailand. Int J occup environ health 10: 289-295

150. Johansen IE (1996) Intron insertion facilitates amplification of cloned viral cDNA in Escherichia coli while biological activity is re-established after transcription in vivo. Proc Natl Acad Sci USA 93: 12400-12405

151. Jones DR (2005) Plant viruses transmitted by thrips. Eur J Plant Pathol 113: 119-157

152. Jones L, Ratcliff F, Baulcombe DC (2001) RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Curr Biol 11: 747-757

153. Jones RCA (2004) Using epidemiological information to develop effective integrated virus disease management strategies. Virus Res 100: 5-30

154. Jovel J, Reski G, Rothenstein D, Ringel M, Frischmuth T, Jeske H (2004) Sida micrantha mosaic is associated with a complex infection of begomoviruses different from Abutilon mosaic virus. Arch Virol 149: 829-841

155. Julenius K, Mølgaard A, Gupta R, Brunak S (2005) Prediction, conservation analysis and structural characterization of mammalian mucin-type O-glycosylation sites. Glycobiology 15: 153-164

156. Jungbluth F (1996) Crop protection policy in Thailand: Economic and political factors influencing pesticide use. Pesticide Policy Project, Publication Series No. 5, Uni Druck, Hannover

157. 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

158. Kang B, Yeam I, Jahn MM (2005) Genetics of plant virus resistance. Annu Rev Phytopathol 43: 581-621

159. Kaniewski W, Ilardi V, Tomassoli L, Mitsky T, Layton J, Barba M (1999) Extreme resistance to cucumber mosaic virus (CMV) in transgenic tomato expressing one or two coat proteins. Mol Breed 5: 111-119

160. Kato K, Hanada K, Kameya-Iwaki M (2000) *Melon yellow spot virus*: A distinct species of the genus *Tospovirus* isolated from melon. Phytopathology 90: 422-426

161. Khetarpal RK, Maisonneuve B, Maury Y, Chalhoub B, Dinant S, Lecoq H, Varma A (1998) Breeding for resistance to plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 14-32

162. Kikkert M, Meurs C, van de Wetering F, Dorfmüller S, Peters D, Kormelink R, Goldbach R (1998) Binding of tomato spotted wilt virus to a 94-kDa thrips protein. Virology 88: 63-69

163. Knierim D, Blawid R, Maiss E (2006) The complete nucleotide sequence of a capsicum chlorosis virus isolate from Lycopersicum esculentum in Thailand. Arch Virol 151: 1761-1782

164. Kormelink R, de Haan P, Meurs C, Peters D, Goldbach R (1992) The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. J Gen Virol 73: 2795-2804

165. Kormelink R, Storms M, Van Lent J, Peters D, Goldbach R (1994) Expression and subcellular location of the NSM protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. Virology 200: 56-65

166. Krogh A, Larsson B, von Heijne G, Sonnhammer ELL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305: 567-580

167. Kubota K, Tsuda S, Tamai A, Meshi T (2003) Tomato Mosaic Virus Replication Protein Suppresses Virus-Targeted Posttranscriptional Gene Silencing. J Virol 77: 11016-11026

168. 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 virus capsid protein are resistant to the virus. Biotechnology (N Y) 12: 500-504

169. Landsmann J, Llewellyn D, Dennis ES, Peacock WJ (1988) Organ regulated expression of Parasponia andersonii haemoglobin gene in transgenic tobacco plants. Mol Gen Genet 214: 68-73

170. Lanfermeijer FC, Dijkhuis J, Sturre MJ, de Haan P, Hille J (2003) Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-2^2 from Lycopersicon esculentum. Plant Mol Biol 52:1037-1049

171. Laurence AM (2004) Australian thysanoptera - biological diversity and a diversity of studies. Aust J Entomol 43: 248-257

172. Law MD, Speck J, Moyer JW (1991) Nucleotide sequence of the 3´ non-coding region and N gene of the S RNA of a serologically distinct tospovirus. J Gen Virol 72: 2597-2601

173. Law MD, Speck J, Moyer JW (1992) The M RNA of impatiens necrotic spot *Tospovirus* (Bunyaviridae) has an ambisense genomic organization. Virology 188: 732-741

174. Lecoq H, Mourya B, Desbiez C, Palloix A, Pitrat M (2004) Durable virus resistance in plants through conventional approaches: a challenge. Virus Res 100: 31-39

175. Levin JS, Thompson WF, Csinos AS, Stephenson MG, Weissinger AK (2005) Matrix attachment regions increase the efficiency and stability of RNA-mediated resistance to Tomato spotted wilt virus in transgenic tobacco. Trans Res 14: 193-206

176. Lewandowski DJ (2005) Tobamovirus. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy, VIIIth Report of the ICTV Elsevier/Academic Press, San Diego, pp 1009-1014

177. Lindbo JA, Dougherty WG (1992) Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology 189: 725-733

178. Lindbo JA, Dougherty WG (2005) Plant pathology and RNAi: a brief history. Annu Rev Phytopathol 43: 191-204

179. Lindbo JA, Silva-Rosales L, Proebsting WM, Dougherty WG (1993) Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell 5: 1749-1759

180. 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

181. Ling KS (2006) Molecular characterization of two Pepino mosaic virus variants from imported tomato seed reveals high levels of sequence identity between Chilean and US isolates. Virus Genes Published online: 22 August 2006 [Epub ahead of print]

182. Ling KS, Carpenter L (2005) Pepino mosaic virus, an emerging disease in greenhouse tomato production worldwide: Is seed responsible? Acta Hortic 695: 43-49

183. Lin H, Rubio L, Smythe A, Jiminez M, Falk BW (2003) Genetic diversity and biological variation among California isolates of Cucumber mosaic virus. J Gen Virol 84: 249-258

184. Lin YH, Chen TC, Hsu HT, Liu FL, Chu FH, Chen CC, Lin YZ, Yeh SD (2005) Serological comparison and molecular characterization for verification of Calla lily chlorotic spot virus as a new tospovirus species belonging toWatermelon silver mottle virus serogroup. Phytopathology 95: 1482-1488

185. Lippman Z, Martienssen R (2004) The role of RNA interference in heterochromatic silencing. Nature 431: 364-370

186. Litiere K, van Eldik GJ, Jacobs JJMR, van Montagu M, Cornelissen M (1999) Posttranscriptional gene silencing of gn1in tobacco triggers accumulation of truncated gn1-derived RNA species. RNA 5: 1364-1373

187. Li ZH, Zhou XP, Zhang X, Xie Y (2004) Molecular characterization of tomato-infecting begomoviruses in Yunnan, China. Arch Virol 149: 1721-1732

188. Llamas-Llamas ME, Zavaleta-Mejia E, Gonzalez-Hernandez VA, Cervates-Diaz L, Santizo-Rincon JA, Ochoa-Martinez DL (1998) Effect of temperature

on symptom expression and accumulation of tomato spotted wilt virus in different host species. Plant Pathol 47: 341-347

189. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73: 152-160
190. Lopez C, Soler S, Nuez F (2005) Comparison of the complete sequences of three different isolates of Pepino mosaic virus: Size variability of the TGBp3 protein between tomato and L. peruvianum isolates. Arch Virol 150: 619-627
191. Louie R (1995) Vascular puncture of maize kernels for the mechanical transmission of maize white line mosaic virus and other viruses of maize. Phytopathology 85: 139-143
192. Lovato FA, Nagata T, Resende RO, de Ávila AC, Inoue-Nagata AK (2004) Sequence analysis of the glycoproteins of *Tomato chlorotic spot virus* and *Groundnut ringspot virus* and comparison with other tospoviruses. Virus Genes 29: 321-328
193. 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
194. Mallory AC, Vaucheret H (2004) MicroRNAs: something important between the genes. Current Opinion in Plant Biology 7: 120-125
195. Mandal B, Varma A, Malathi VG (1997) Systemic infection of Vigna mungo using the cloned DNAs of the blackgram isolate of mungbean yellow mosaic geminivirus through agroinoculation and transmission of the progeny virus by whiteflies. J Phytopathol 145: 505-510
196. Maredia (2003) Introduction and overview. In: Maredia KM, Dakouo D, Mota-Sanchez D (eds) Integrated pest management in the global arena, CABI Publishing, Wallingford, Oxon, UK, pp 1-8
197. Martin DP, Rybicki EP (2000) RDP: detection of recombination amongst aligned sequences. Bioinformatics 16: 562-563

198. Martin DP, Williamson C, Posada D (2005) RDP2: recombination detection and analysis from sequence alignments. Bioinformatics 21: 260-262

199. Martin RR (1998) Advanced diagnostic tools as an aid to controlling plant virus diseases. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 381-391

200. Maumbe B, Bernsten R, Norton G (2003) Social and economic considerations in the design and implementation of integrated pest management in developing countries. In: Maredia KM, Dakouo D, Mota-Sanchez D (eds) Integrated pest management in the global arena, CABI Publishing, Wallingford, Oxon, UK, pp 87-95

201. McMichael LA, Persley DM, Thomas JE (2002) A new tospovirus serogroup IV species infecting capsicum and tomato in Queensland, Australia. Australas Plant Pathol 31: 231-239

202. Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431: 343-349

203. Melcher U (2000) The '30K' superfamily of viral movement proteins. J Gen Virol 81: 257-266

204. Menzel W, Jelkmann W, Maiss E (2002) Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J Virol Methods 99: 81-92

205. Meshi T, Motoyoshi F, Maeda T,Yoshiwoka S,Watanabe H, OkadaY (1989) Mutations in the tobacco mosaic virus 30-kD protein gene overcome Tm-2 resistance in tomato. Plant Cell 1: 515-522

206. Mette MF, Aufsatz 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

207. Michelson I, Zamir D, Czosnek H (1994) Accumulation and translocation of tomato yellow leaf curl virus (TYLCV) in a *Lycopersicon esculentum* breeding line containing the *L. chilense* TYLCV tolerance gene Ty-1. Phytopathology 84: 928-933

208. 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

209. Mitter N, Sulistyowati E, Dietzgen RG (2001) Suppression of gene silencing: a threat to virus-resistant transgenic plants? Trends Plant Sci 6: 246-247

210. 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

211. Moffat AS (1999) Geminiviruses Emerge as Serious Crop Threat. Science 286: 1835

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

213. Molnar A, Csorba T, Lakatos L, Varallyay E, Lacomme C, Burgyan J (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J Virol 79: 7812-7818

214. Moriones E, Luis-Arteaga M (1999) Viral diseases. In: Albajes R, Gullino ML, Van Lenteren JC, ELAD Y (eds) Integrated pest and disease management in greenhouse crops, Kluwer academic publishers, Dordrecht , Boston, London pp 16-33

215. Morris BAM, Richardson KA, Andersen MT, Gardner RC (1988) Cassava latent virus infections mediated by the Ti plasmid of Agrobacterium tumefaciens containing either monomeric or dimeric viral DNA. Plant Mol Biol 11: 795-803

216. Moyer JW (1999). Tospoviruses (*Bunyaviridae*). In: Granoff A, Webster RG (eds) Encyclopedia of Virology, Academic Press, New York, USA, pp 1803-1807

217. Murai T, Kawai S, Chongratanameteekul W, Nakasuji F (2000) Damage to tomato by *Ceratothripoides claratris* (Shumsher) (Thysanoptera: Thripidae) in central Thailand and a note on its parasitoid, *Goethena shakespearei* Girault (Hymenoptera: Eulophidae). Appl Entomol Zool 35: 505-507

218. Muskens MW, Vissers AP, Mol JN, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Mol Biol 43: 243-260

219. Naidu RA, Ingle CJ, Deom CM, Sherwood JL (2004) The two envelope membrane glycoproteins of *Tomato spotted wilt virus* show differences in lectin-binding properties and sensitivities to glycosidases. Virology 319: 107-117

220. Nakajima M, Hayakawa T, Nakamura I, Suzuki M (1993) Protection against cucumber mosaic virus (CMV) strain O and Y and chrysanthemum mild mottle virus in transgenic tobacco plants expressing CMV-O coat protein. J Gen Virol 74: 319-322

221. Nakhla MK, Maxwell DP (1998) Epidemiology and management of tomato yellow leaf curl diseases. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 565-583

222. Namba S, Ling K, Gonsalves C, Gonsalves D, Slightom JL (1991) Expression of the gene encoding the coat protein of cucumber mosaic virus (CMV) strain WL appears to provide protection to tobacco plants against infection by several different CMV strains. Gene 107: 181-188

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

224. Nicholas KB, Nicholas HB (1997) GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the authors

225. Nichol ST, Beaty BJ, Elliott RM, Goldbach R, Plyusnin A, Schmaljohn CS, Tesh RB (2005) *Bunyaviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) *Virus taxonomy*, VIIIth Report of the ICTV Elsevier/Academic Press, San Diego, pp 695-716

226. Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 10: 1-6

227. Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH (2006) Expression of artifical microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. Nat Biotechnol 24: 1420-1428

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

229. Noris E, Lucioli A, Tavazza R, Caciagli P, Accotto GP, Tavazza M (2004) Tomato yellow leaf curl Sardinia virus can overcome transgene-mediated RNA silencing of two essential genes. J Gen Virol 85: 1745-1749

230. Oh S-K, Lee S, Chung E, Park JM, Yu SH, Ryu C-H, Choi D (2006) Insight into Types I and II nonhost resistance using expression patterns of defense-related genes in tobacco. Planta 223: 1101-1107

231. Oka IPGNJ (2003) Integrated Pest Management in Indonesia: IPM by Farmers. In: Maredia KM, Dakouo D, Mota-Sanchez D (eds) Integrated pest management in the global arena, CABI Publishing, Wallingford, Oxon, UK, pp 223-237

232. Okuda M, Kato K, Hanada K, Iwanami T (2006) Nucleotide sequence of melon yellow spot virus M RNA segment and characterization of non-viral sequences in subgenomic RNA. Arch Virol 151: 1-11

233. Okuno T, Nakayama M, Furusawa I (1993) Cucumber mosaic virus coat protein-mediated resistance. Semin Virol 4: 357-361

234. Okuno T, Nakayama M, Yoshida S, Furusawa I, Komiya T (1993) Comparative susceptibility of transgenic tobacco plants and protoplasts expressing the coat protein gene of cucumber mosaic virus to infection with virions and RNA. Phytopathology 83: 542-547

235. Overby AK, Popov V, Neve EP, Pettersson RF (2006) Generation and analysis of infectious virus-like particles of uukuniemi virus (bunyaviridae): a useful system for studying bunyaviral packaging and budding. J Virol 80: 10428-10435

236. Page RDM (1996) TreeView: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12: 357-358

237. 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

238. Pang SZ, Jan FJ, Carney K, Stout J, Tricoli DM, Quemada HD, Gonsalves D (1996) Post-transcriptional transgene silencing and consequent tospovirus resistance in transgenic lettuce are affected by transgene dosage and plant development. Plant J 9: 899-909

239. Pang SZ, Jan FJ, Gonsalves D (1997) Nontarget DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants. Proc Natl Acad Sci USA 94: 8261-8266

240. Pappu SS, Bhat AI, Pappu HR, Deom CM, Culbreath AK (2000) Phylogenetic studies of tospoviruses (family: *Bunyaviridae*) based on intergenic region sequences of small and medium genomic RNAs. Arch Virol 145: 1035-1045

241. Persley DM, Thomas JE, Sharman M (2006) Tospoviruses-an Australian perspective. Australas Plant Pathol 35: 161-180

242. Pickford AS, Cogoni C (2003) RNA-mediated gene silencing. Cell Mol Life Sci 60: 871-882

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

244. Plasterk RH (2002) RNA silencing: the genome’s immune system. Science 296: 1263-1265

245. Pluthero FG (1993) Rapid purification of high-activity *Taq* DNA polymerase. Nucleic Acids Res 21: 4850-4851

246. Pongsapich P, Chiemsombat P (2002) Characterization of tospovirus infecting tomatoes in Thailand revealed the presence of serogroup IV-tospovirus but not serogroup I-tomato spotted wilt virus. Page 92, Summary, First Int. Conf. Trop. Subtrop. Plant Dis. Chiang Mai, Thailand.

247. Pooggin MM, Hohn T (2004) Fighting geminiviruses by RNAi and vice versa. Plant Mol Biol 55: 149-152

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

249. Premachandra WTSD (2004) Biology, population dynamics, vector potential and management of Ceratothripoides claratris on tomato in central Thailand. PhD thesis, University of Hanover, Germany

250. Premachandra WTSD, Borgemeister C, Chabi-Olaye A, Poehling HM (2004) Influence of temperature on the development, reproduction and longevity of *Ceratothripoides claratris* (thysanoptera: thripidae) on tomatoes. Bull Entomol Res 94: 377-384

251. Premachandra WTSD, Borgemeister C, Maiss E, Knierim D, Poehling HM (2005) *Ceratothripoides claratris*, a new vector of a Capsicum chlorosis virus isolate infecting tomatoes in Thailand. Phytopathology 95: 659-663

252. Prins M, de Haan P, Luyten R, van Veller M, van Grinsven MQJM, Goldbach R (1995) Broad resistance to tospoviruses in transgenic tobacco plants

expressing three tospoviral nucleoprotein gene sequences. Mol Plant Microbe Interact 8: 85-91

253. Prins M, Kikkert M, Ismayadi C, de Graauw W, de Haan P, Goldbach R (1997) Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NS_M gene sequences. Plant Mol Biol 33: 235-243

254. Prins M, Lohuis D, Schots A, Goldbach R (2005) Phage display-selected single-chain antibodies confer high levels of resistance against Tomato spotted wilt virus. J Gen Virol 86: 2107-2113

255. Quemada HD, Gonsalves D, Slightom JL (1991) Expression of coat protein gene from cucumber mosaic virus strain C in tobacco: protection against infections by CMV strains transmitted mechanically or by aphids. Phytopathology 81: 794-802

256. Raman KV, Grafius E, Maredia KM (2003) The influence of biotechnology on integrated pest management in developing countries. In: Maredia KM, Dakouo D, Mota-Sanchez D (eds) Integrated pest management in the global arena, CABI Publishing, Wallingford, Oxon, UK, pp 31-47

257. Rector A, Tachezy R, Van Ranst M (2004) A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J Virol 78: 4993-4998

258. Reddy DVR, Ratna AS, Sudarshana MR, Poul F, Kumar IK (1992) Serological relationships and purification of bud necrosis virus, a tospovirus occurring in peanut (*Arachis hypogaea* L.) in India. Ann Appl Biol 120: 279-286

259. Redinbaugh MG (2003) Transmission of Maize streak virus by vascular puncture inoculation with unit-length genomic DNA. J Virol Methods 109: 95-98

260. Redinbaugh MG, Louie R, Ngwira P, Edema R, Gordon DT, Bisaro DM (2001) Transmission of viral RNA and DNA to maize kernels by vascular puncture inoculation. J Virol Methods 98: 135-143

261. Ritzenthaler C (2005) Resistance to plant viruses: old issue, news answers? Curr Opin Biotechnol 16: 118-122

262. 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

263. Rodmui P (2002) Population dynamics and biological control of thrips, Ceratothripoides claratris (Shumsher) (Thysanoptera: Thripidae), on tomato under protected cultivation in Thailand. MSc thesis, Kasetsart University, Bangkok

264. Rohr J, Sarkar N, Balenger S, Jeong BR, Cerutti H (2004) Tandem inverted repeat system for selection of effective transgenic RNAi strains in Chlamydomonas. Plant J 40: 611-621

265. 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

266. Rojas MR, Jiang H, Salati R, Xoconostle-Cázares 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

267. 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

268. Roossinck MJ, Bujarski J, Ding SW, Hajimorad R, Hanada K, Scott S, Tousignant M (2005) Bromoviridae. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy, VIIIth Report of the ICTV Elsevier/Academic Press, San Diego, pp 1049-1058

269. Roossinck MJ, Zhang L, Hellwald KH (1999) Rearrangements in the 5′ nontranslated region and phylogenetic analyses of Cucumber mosaic virus RNA 3 indicate radial evolution of three subgroups. J Virol 73: 6752-6758

270. Rothenstein D, Briddon RW, Haible D, Stanley J, Frischmuth T, Jeske H (2005) Biolistic infection of cassava using cloned components of Indian cassava mosaic virus. Arch Virol 150: 1669-1675

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

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

273. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Laboratory Press, New York

274. Sanford JC, Johnson SA (1985) The concept of parasite-derived resistance: Deriving resistance genes from the parasites own genome. J Theor Biol 115: 395-405

275. Satapathy MK (1998) Chemical control of insect and nematode vectors of plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 188-195

276. Satyanarayana T, Gowda S, Reddy KL, Mitchell SE, Dawson WO, Reddy DVR (1998) Peanut yellow spot virus is a member of a new serogroup of tospovirus genus based on small (S) RNA sequence and organization. Arch Virol 143: 353-364

277. Satyanarayana T, Mitchell SE, Reddy DVR, Brown S, Kresovich S, Jarret R, Naidu RA, Demski JW (1996) Peanut bud necrosis tospovirus S RNA: complete nucleotide sequence, genome organization and homology to other tospoviruses. Arch Virol 141: 85-98

278. Satyanarayana T, Mitchell SE, Reddy DVR, Kresovich S, Jarret R, Naidu RA, Gowda S, Demski JW (1996) The complete nucleotide sequence and genome organization of the M RNA segment of peanut bud necrosis tospovirus and comparison with other tospoviruses. J Gen Virol 77: 2347-2352

279. Saunders K, Salim N, Mali VR, Malathi VG, Briddon R, Markham PG, Stanley J (2002) Characterisation of Sri Lankan cassava mosaic virus and Indian cassava mosaic virus: evidence for acquisition of a DNA B component by a monopartite begomovirus. Virology 293: 63-74

280. Sawangjit S, Chatchawankanphanich O, Chiemsombat P, Attathom T, Dale J, Attathom S (2005) Molecular characterization of tomato-infecting begomoviruses in Thailand. Virus Res 109: 1-8

281. Sawangjit S, Chatchawankanphanich O, Chiemsombat P, Attathom T, Dale J, Attathom S (2005) Possible recombination of tomato-infecting begomoviruses in Thailand. J Gen Plant Pathol 71: 314-318

282. 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

283. Silva MS, Martins CRF, Bezerra IC, Nagata T, de Ávila AC, Resende RO (2001) Sequence diversity of NS_M movement protein of tospoviruses. Arch Virol 146: 1267-1281

284. Singh A, Singh S, Rao SN (2003) Integrated Pest Management in India. In: Maredia KM, Dakouo D, Mota-Sanchez D (eds) Integrated pest management in the global arena, CABI Publishing, Wallingford, Oxon, UK, pp 209-221

285. Singh SJ, Krishnareddy M (1996) Watermelon bud necrosis: a new tospovirus disease. Acta Hortic 431: 68-77

286. Sin SH, McNulty BC, Kennedy GG, Moyer JW (2005) Viral genetic determinants for thrips transmission of Tomato spotted wilt virus. Proc Natl Acad Sci USA 102: 5168-5173

287. Skarn M, Eike MC, Meza TJ, Mercy IS, Jakobsen KS, Aalen RB (2006) An inverted repeat transgene with a structure that cannot generate double-stranded RNA, suffers silencing independent of DNA methylation. Transgenic Res 15: 489-500

288. Smith HA, Swaney SL, Parks TD, Wernsman EA, Dougherty WG (1994) Transgenic plant virus resistance mediated by untranslatable sense RNAs: expression, regulation, and fate of nonessential RNAs. Plant Cell 6: 1441-1453

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

290. Soler S, Prohens J, Dıez MJ and Nuez F (2002) Natural occurrence of Pepino mosaic virus in Lycopersicon species in Central and Southern Peru. J Phytopathol 150: 49-53

291. Sonnhammer ELL, von Heijne G, Krogh A (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. In: Glasgow J, Littlejohn T, Major F, Lathrop R, Sankoff D, Sensen C (ed) Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology. AAAI Press, Menlo Park, CA, pp 175-182

292. Soto MJ, Chen LF, Seo YS, Gilbertson RL (2005) Identification of regions of the Beet mild curly top virus (family Geminiviridae) capsid protein involved in systemic infection, virion formation and leafhopper transmission. Virology 341: 257-270

293. Stanley J, Bisaro DM, Briddon RW, Brown JK, Fauquet CM, Harrison BD, Rybicki EP, Stenger DC (2005) *Geminiviridae*. In: Virus Taxonomy, Eight

Report of the ICTV. Academic Press, Amsterdam Boston Heidelberg London NewYork Oxford Paris San Diego San Francisco Singapore Sydney Tokyo, pp 301-326

294. Stephan D (2005) Molekulare Charakterisierung von *Beet mild yellowing virus* (BMYV) und *Beet chlorosis virus* (BChV) sowie Selektion von BMYV Amplicon-transgenen *Nicotiana benthamiana*. Ph.D. Thesis, University Hannover, Germany

295. Stevens MR, Lamb EM, Rhoads DD (1995) Mapping the Sw-5 locus for tomato spotted wilt virus resistance in tomatoes using RAPD and RFLP. Theor Appl Genet 90: 451-456

296. Stevens MR, Scott SJ, Gergerich RC (1992) Inheritance of a gene for resistance to tomato spotted wilt virus (TSWV) from *Lycopersicon peruvianun* Mill. Euphytica 59: 9-17

297. Stuiver MH, Custers JHHV (2001) Engineering disease resistance in plants. Nature 411: 865-868

298. Sudarshana R, Wang HL, Lucas WJ, Gilbertson RL (1998) Dynamics of Bean Dwarf Mosaic Geminivirus Cell-to-Cell and Long-Distance Movement in Phaseolus vulgaris Revealed, Using the Green Fluorescent Protein. Mol Plant Microbe Interact 4: 277-291

299. Szittya G, Silhavy D, Molnar A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. EMBO J 22: 633-640

300. Takahashi H, Miller J, Nozaki Y, Sukamto, Takeda M, Shah J, Hase S, Ikegami M, Ehara Y, Dinesh-Kumar SP (2002) RCY1, an Arabidopsis thaliana RPP8/HRT family resistance gene, conferring resistance to cucumber mosaic virus requires salicylic acid, ethylene and a novel signal transduction mechanism. Plant J 32: 655-667

301. Takeda A, Sugiyama K, Nagano H, Mori M, Kaido M, Mise K, Tsuda S, Okuno T (2002) Identification of a novel RNA silencing suppressor, NSs protein of *Tomato spotted wilt virus*. FEBS Lett 532: 75-79

302. Tang G (2005) siRNA and miRNA: an insight into RISCs. Trends Biochem Sci 30: 106-114

303. Taylor IB (1986) Biosystematics of the tomato. In: Atherton JG, Rudich J (eds) The tomato crop, Chapman & Hall, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp 1-34

304. 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

305. Tepfer M (2002) Risk assessment of virus-resistant transgenic plants. Annu Rev Phytopathol 40: 467-491

306. Thapinta A, Hudak PF (2000) Pesticide use and residual occurrence in Thailand. Environ Monit Assess 60: 103-114

307. Thomashow MF, Nutter R, Montoya AL, Gordon MP, Nester EW (1980) Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19: 729-739

308. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876-4882

309. Tien P, Wu G (1991) Satellite RNA for the biocontrol of plant disease. Adv Virus Res 39: 321-339

310. Tomlinson JA (1987) Epidemiology and control of virus diseases of vegetables. Ann Appl Biol 110: 661-681

311. 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

312. 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: 90-101

313. Van der Krol AR, Mur LA, Beld M, Mol JNM, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2: 291-299

314. Van der Vlugt RAA, Stijger CCMM, Verhoeven JTJ, Lesemann DE (2000) First report of pepino mosaic virus on tomato. Plant Dis 84: 103

315. Van der Want JPH, Dijkstra J (2006) A history of plant virology. Arch Virol 151: 1467-1498

316. 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 USA 100: 9632-9636

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

318. 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

319. Van Poelwijk F, Prins M, Goldbach R (1997) Completion of the impatiens necrotic spot virus genome sequence and genetic comparison of the L proteins within the family *Bunyaviridae*. J Gen Virol 78: 543-546

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

321. Van Wezel R, 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 gene-silencing suppression. Mol Plant Microb Interact 15: 203-208

322. Vaucheret H, Fagard M (2001) Transcriptional gene silencing in plants: Targets, inducers and regulators. Trends Genet 17: 29-35

323. Verhoeven JTJ, van der Vlugt RAA, Roenhorst JW (2003) High similarity between tomato isolates of pepino mosaic virus suggests a common origin. Eur J Plant Pathol 109: 419-425

324. Verwoerd TC, Dekker BMM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res 17: 2362

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

326. Voinnet O (2005) Induction and suppression of RNA silencing: insights from viral infections. Nat Rev Genet 6: 206-220

327. Voinnet O, Lederer C, Baulcombe DC (2000) A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell 103: 157-167

328. 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

329. Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297: 1833-1837

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

331. 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

332. Wang MB, Waterhouse PM (2000) High-efficiency silencing of a β-glucuronidase gene in rice is correlated with repetitive transgene structure but is independent of DNA methylation. Plant Mol Biol 43: 67-82

333. Wang MB, Wesley SV, Finnegan EJ, Smith NA (2001) Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. RNA 7: 16-28

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

335. Waterworth HE, Hadidi A (1998) Economic losses due to plant viruses. In: Hadidi A, Khetarpal RK, Koganezawa H (eds) Plant viral disease control, APS Press, St. Paul, Minnesota, pp 1-13

336. Watterson JC (1986) Diseases. In: Atherton JG, Rudich J (eds) The tomato crop, Chapman & Hall, London, Weinheim, New York, Tokyo, Melbourne, Madras, pp 443-484

337. Weber H, Ohnesorge S, Silber MV, Pfitzner AJP (2004) The Tomato mosaic virus 30 kDa movement protein interacts differentially with the resistance genes *Tm*-2 and *Tm*-2^2. Arch Virol 149: 1499-1514

338. Weber H, Schultze S, Pfitzner AJP (1993) Two amino acid substitutions in the tomato mosaic virus 30-kilodalton movement protein confer the ability to overcome the *Tm*-2^2 resistance gene in the tomato. J Virol 67: 6432-6438

339. Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27: 581-590

340. Whitfield AE, Ullman DE, German TL (2004) Expression and characterization of a soluble form of *Tomato spotted wilt virus* glycoprotein G_N. J Virol 78: 13197-13206

341. Whitfield AE, Ullman DE, German TL (2005) Tospovirus-thrips interactions. Annu Rev Phytopathol 43: 17.1-17.31

342. Whitham S, McCormick S, Baker B (1996) The N gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. Proc Natl Acad Sci USA 93: 8776-8781

343. Wilson CR (1998) Incidence of weed reservoirs and vectors of tomato spotted wilt tospovirus on southern Tasmanian lettuce farms. Plant Pathol 47: 171-187

344. Wintermantel WM, Banerjee N, Oliver JC, Paolillo DJ, Zaitlin M (1997) Cucumber mosaic virus is restricted from entering minor veins in transgenic tobacco exhibiting replicasemediated resistance. Virology 231: 248-257

345. Wintermantel WM, Zaitlin M (2000) Transgene translatability increases effectiveness of replicase-mediated resistance to Cucumber mosaic virus. J Gen Virol 81: 587-595

346. Winter S, Shahraeen N, Koerbler M, Lesemann DE (2005) Characterisation of Tomato fruit yellow ring virus: a new *Tospovirus* species infecting tomato in Iran. New Dis Rep 11: February-July

347. Wongkaew S (1995) Peanut bud necrosis disease in Thailand. Proc. Recent Studies on Peanut Bud Necrosis Disease, ICRISAT, Wageningen, pp 55-59

348. Xie Y, Jiang T, Zhou X (2006) Agroinoculation shows Tobacco leaf curl Yunnan virus is a monopartite begomovirus. Eur J Plant Pathol 115: 369-375

349. Xue B, Gonsalves C, Provvidenti R, Slightom JL, Fuches M, Gonsalves D (1994) Development of transgenic tomato expressing a high level of resistance to cucumber mosaic virus strains of subgroups I and II. Plant Dis 78: 1038-1041

350. Yeh SD, Chang TF (1995) Nucleotide sequence of the N gene of Watermelon silver mottle virus, a proposed new member of the genus *Tospovirus*. Phytopathology 85: 58-64

351. Yeh S, Sun I, Ho H, Chang T (1996) Molecular cloning and nucleotide sequence analysis of the S RNA of watermelon silver mottle tospovirus. Acta Hortic 431: 244-260

352. Yie Y, Zhao F, Zhao SZ, Liu YZ, Liu YL, Tien P (1992) High resistance to cucumber mosaic virus conferred by satellite RNA and coat protein in transgenic commercial tomato cultivar G-140. Mol Plant Microbe Interact 5: 460-465

353. Zambryski P, Holsters M, Kruger K, Depicker A, Schell J, Van Montagu M, Goodman HM (1980) Tumor DNA structure in plant cells transformed by A. tumefaciens. Science 209: 1385-1391

354. Zamir D, Ekstein-Michelson I, Zakay Y, Navot N, Zeidan N, Sarfatti M, Eshed Y, Harel E, Pleban 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

355. Zamore PD (2002) Ancient pathways programmed by small RNAs. Science 296: 1265-1269

356. Zhao Y, Owens RA, Hammond RW (2001) Use of a vector based on Potato virus X in a whole plant assay to demonstrate nuclear targeting of Potato spindle tuber viroid. J Gen Virol 82: 1491-1497

357. Zhou XP, Xie Y, Zhang ZK (2001) Molecular characterization of a distinct begomovirus infecting tobacco in Yunnan, China. Arch Virol 146: 1599-606

358. Zilberman D, Cao X, Jacobsen SE (2003) ARGONAUTE4 control of locus specific siRNA accumulation and DNA and histone methylation. Science 299: 716-719

359. Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V, Gafni Y (2006) Suppressor of RNA silencing encoded by Tomato yellow leaf curl virus-Israel. Virology Published online: 14 September 2006 [Epub ahead of print]

Danksagung

Mein besonderer Dank gilt Prof. Dr. Edgar Maiß für die hervorragende Betreuung und Unterstützung bei der Durchführung der Arbeit, sein großes Vertrauen in meine Arbeit und die ständige Erreichbarkeit bei Fragen.

Des Weiteren bedanke ich mich bei Herrn Prof. Dr. Günter Adam für die bereitwillige Übernahme des Ko-Referats.

Bei Herrn Prof. Dr. Michael Poehling bedanke ich mich für die Möglichkeit zur Durchführung dieser Arbeit am Institut für Pflanzenkrankheiten und Pflanzenschutz.

Allen derzeitigen und früheren Mitgliedern der Arbeitsgruppe Maiß und des Instituts sei für die Zusammenarbeit und das gute Arbeitsklima gedankt. Insbesondere möchte ich mich bedanken bei Dirk, Edgar, Rosana und Wulf für das Korrekturlesen meiner nicht immer leicht verständlichen Texte, sowie den Gärtnern Herrn Seelbinder und Herrn Arndt.

Ganz besonders danke ich meiner Familie auf deren Unterstützung ich mich immer verlassen kann.

Lebenslauf

Name:	Dennis Knierim
Geburtsdatum:	21. November 1974, Lich
Familienstand:	ledig
Anschrift:	Lindenallee 13, 31191 Bledeln

Schulbildung:

1987 - 1991	Privat-Gymnasium Pädagogium, Bad Schwartau (Abschluss: Realschule)
1991 - 1994	Leibniz-Gymnasium, Bad Schwartau (Abschluss: Abitur)

Zivildienst:

1995	Wilhelmine Possehl, Bad Schwartau, Alten und Pflegeheim

Berufsausbildung:

03/96 - 09/96	Praktikum, Gärtnerei Arndt, Stockelsdorf
10/96 - 09/01	Studium, Universität Hannover (Abschluss: Dipl.-Ing. agr. Fachrichtung Gartenbau)

Berufstätigkeit:

10/01 - 09/04	Wissenschaftl. Mitarbeiter, AG Virologie Institut für Pflanzenkrankheiten u. Pflanzenschutz Universität Hannover (Befristetes Arbeitsverhältnis)
10/04 – 09/05	Wissenschaftlicher Mitarbeiter, AG Virologie Institut für Pflanzenkrankheiten u. Pflanzenschutz Universität Hannover (Befristete Teilzeitarbeitsverträge)