Virus-Host Interactions of Tomato Yellow Ring Virus, a New Tospovirus from Iran

Afshin Hassani-Mehraban

Promotor:
Prof. dr. ir. R.W. Goldbach
Hoogleraar Virologie
Wageningen Universiteit

Co-promotor:
Dr. ir. R.J.M. Kormelink
Universitair Docent, Labratorium voor Virologie
Wageningen Universiteit

Samenstelling promotiecommissie:
Prof. dr. ir. P.J.G.M. de Wit (Wageningen Universiteit)
Prof. dr. ir. J. Bakker (Wageningen Universiteit)
Dr. ir. R.A.A. van der Vlugt (Plant Research International)
Dr. ir. J.F.J.M. van der Heuvel (De Ruiter Seeds)

Dit onderzoek is uitgevoerd binnen de onderzoekschool Experimentele Plantwetenschappen

Virus-Host Interactions of Tomato Yellow Ring Virus, a New Tospovirus from Iran

Afshin Hassani-Mehraban

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. M.J. Kropff,
in het openbaar te verdedigen
op maandag 8 September 2008
des namiddags te vier uur in de Aula

A. Hassani-Mehraban (2008)
Virus-Host Interactions of Tomato Yellow Ring Virus, a New Tospovirus from Iran
PhD thesis Wageningen University, The Netherlands
With references - summaries in English and Dutch - appendix
ISBN: 978-90-8504-939-5
Subject headings: virus-host interactions, tomato yellow ring virus, tospovirus, transgenic resistance, RNAi

Contents

Chapter 1	**General Introduction**	1
Chapter 2	**A new tomato-infecting tospovirus from Iran**	13
Chapter 3	**Molecular and biological comparison of two *Tomato yellow ring virus* (TYRV) isolates: challenging the Tospovirus species concept**	33
Chapter 4	**RNAi-mediated resistance to the tomato strain of *Tomato yellow ring virus* broken by co-infection with a different strain of this virus**	49
Chapter 5	**Assessing the intergenic hairpin structure of *Tomato yellow ring virus* S RNA as potential target and inducer for RNAi**	67
Chapter 6	**General discussion**	85
References		93
Summary		111
Samenvatting		115
Acknowledgements		119
About the author		123
Graduate School Certificate		125
Appendix (colour figures)		127

This thesis is dedicated to my dear parents, my dear wife and my lovely sons: Borna, Shayan and Noyan.

Chapter 1

General introduction

The genus *Tospovirus*

Tospoviruses represent the genus of plant-pathogenic viruses within the *Bunyaviridae*, a large family of enveloped viruses with a tripartite RNA genome, members of which are further restricted to animals (Elliott, 1990; Goldbach & Peters, 1996). While for a long time *Tomato spotted wilt virus* (TSWV) was thought to be the sole member of this genus, the availability of more powerful diagnostics and improved surveys have revealed the existence of an increasing number of additional species. Currently, 14 different tospovirus species are being recognised of which the International Committee on Taxonomy of Viruses (ICTV) has listed 8 established and 6 tentative (Fauquet *et al*., 2005) (Table 1-1). Tospoviruses are transmitted by a limited number of thrips species in a persistent manner. Host range and vector species are important biological denominators for distinction of tospovirus species, in addition to the obvious molecular and serological parameters as used in modern virus taxonomy. Currently the nucleocapsid (N) protein sequence divergence represents the main tospoviral species demarcation criterion, using a 90% identity threshold (de Ávila *et al*., 1993; Goldbach & Kuo, 1996).

In Europe, TSWV was first identified in 1931 (Smith, 1932) but has for long not been prevalent. It was until the 80's that due to the introduction of the Western Flower Thrips, *Frankliniella occidentalis* (Pergande), the virus became widely distributed in Western Europe. In Asia, TSWV was first reported from Malaysia in 1989 (Roselló *et al*., 1996) and in 1992 from the Middle-East (Antignus *et al*., 1994). The annual crop loss of TSWV is estimated over 1 billion US$ and the virus ranks among the top ten of economically most important plant viruses worldwide (Goldbach & Peters, 1994). TSWV has a very broad host range that spans more than 1100 different plant species from within more than 80 families, monocots as well as dicots (Peters, 2003). The economic impact of the other tospovirus species is probably smaller, as these viruses (except for *Impatiens necrotic spot virus* [INSV]) have a narrower host range and most of them are not (yet) worldwide distributed.

Table 1-1*: Tospovirus species with their thrips vector species and geographical distribution

Tospovirus species	Vector species	Geographical distribution
Capsicum chlorosis virus (CaCV) [1]	*Ceratothripoides claratris* [42]	Australia, Thailand
Chrysanthemum stem necrosis virus (CSNV) [2]	*Frankliniella occidentalis* [24]	Brazil
Groundnut bud necrosis virus (GBNV) [3,4]	*Thrips palmi* [25,26,27] *F. schultzei* [25,28]	India [4], South-east Asia
Groundnut ringspot virus (GRSV) [5]	*F. gemina* [43] *F. occidentalis* [29] *F. schultzei* [29]	South America [15], South Africa
Impatiens necrotic spot virus (INSV)	*F. occidentalis* [30,31]	USA [7], West and South Europe[6,16,17,18]
Iris yellow spot virus (IYSV) [8]	*T. tabaci* [32]	Brazil [2], Israel [19], The Netherlands[8], USA
Melon yellow spot virus (MYSV) [9]	*T. palmi* [32]	Taiwan [9], Japan [20]
Peanut chlorotic fan-spot virus (PCFV) [10]	*Scirtothrips dorsalis* [10]	Taiwan
Peanut yellow spot virus (PYSV) [11]	?[#]	India [11], Thailand [11]
Tomato chlorotic spot virus (TCSV) [5]	*F. occidentalis* [29] *F. intonsa* [29] *F. schultzei* [29]	South America [12, 21]
Tomato spotted wilt virus (TSWV) [12,13]	*F. bispinosa* [34] *F. cephalica*[44] *F. gemina*[43] *F. fusca* [33] *F. intonsa* [29] *F. occidentalis* [35] *F. schultzei* [29,36] *F. setosus* [37,38] *T. tabaci* [39]	Worldwide [22]
Watermelon bud necrosis virus (WBNV)	*T. palmi* [40]	India
Watermelon silver mottle virus (WSMoV) [14]	*T. palmi* [40]	Japan [23], Taiwan [14]
Zucchini lethal chlorotic virus (ZLCV) [2]	*F. zucchini* [41]	Brazil [2]

Virion structure and genomic organisation

Like other bunyaviruses, tospovirus particles are spherical, being enveloped by a lipid membrane containing spike proteins that consist of two types of viral glycoproteins (G_N and G_C) (Figure 1-1A-B). The core consists of the viral genome tightly encapsidated by the nucleocapsid (N) protein and 10 to 20 copies of the viral RNA-dependent RNA polymerase forming infectious ribonucleoproteins (RNPs) (Figure 1-1C) (van Poelwijk *et al*., 1993).

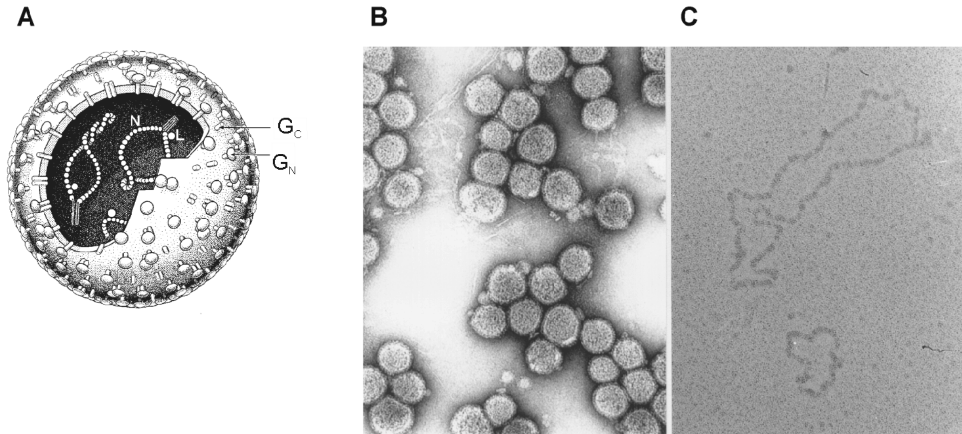


Figure 1-1: *Tomato Spotted Wilt Virus*. Panel A, Schematic representation of a virus particle. The membrane envelope contains the viral glycoproteins (G_N and G_C). Inside the envelope are the ribonucleoproteins (RNPs), consisting of viral RNA encapsidated by nucleoprotein (N) and polymerase (L). Panel B, electron micrograph of purified virus particles. Panel C, electron micrograph of purified RNPs.

Table 1-1

[*]The Table presented here is modified version from I. Wijkamp, PhD thesis, 1995. Tentative tospovirus species are indicated in grey box.

[1]McMichael *et al*., 2002, [2]Resende *et al*., 1996; [3]Reddy *et al*., 1992; [4]Satyanarayana *et al*., 1996; [5]de Ávila *et al*., 1993; [6]de Ávila *et al*., 1992b; [7]Law & Moyer, 1990; [8]Cortês *et al*., 1996; [9]Kato & Hanada, 2000; [10]Chen & Chiu, 1996; [11]Reddy *et al*., 1991; [12]Francki *et al*., 1991; [13]de Haan, 1991; [14]Yeh & Chang, 1995; [15]de Ávila *et al*., 1990; [16]Vaira *et al*., 1993; [17]Marchoux *et al*., 1991; [18]Louro, 1996; [19]Gera *et al*., 1998a; [20]Takeuchi *et al*., 2001; [21]Granval de Millan *et al*., 1998; [22]Goldbach & Peters, 1994; [23]Kameya-Iwaki *et al*., 1984; [24]Nagata & de Ávila, 2000; [25]Lakshmi *et al*., 1995; [26]Palmer *et al*., 1990; [27]Vijayalakshmi, 1994; [28]Amin *et al*., 1981; [29]Wijkamp *et al*., 1995a; [30]DeAngellis *et al*., 1993; [31]Wijkamp & Peters, 1993; [32]Gera *et al*., 1998b; [33]Sakimura, 1963; [34]Webb *et al*., 1998; [35]Gardner *et al*., 1935; [36]Samuel *et al*., 1930; [37]Fujisawa *et al*., 1988; [38]Tsuda *et al*., 1996; [39]Pittman, 1927; [40]Yeh *et al*., 1992; [41]Nakahara & Monteiro, 1999; [42]Premachandra *et al*., 2005; [43]Borbón *et al*., 2006; [44]Ohnishi *et al*., 2006; # : unknown.

The genome is composed of three single-stranded (ss) RNA segments of negative (L RNA) or ambisense (M and S RNAs) polarity (Figure 1-2). Due to complementarity at their 5′ and 3′ ends, these segments form a panhandle structure and appear as pseudo-circular structures in the electron microscope (Figure 1-1C) (de Haan *et al*., 1989; Mohamed, 1981; van den Hurk *et al*., 1977). The L RNA encodes the viral RNA-dependent RNA polymerase (RdRp) (de Haan *et al*., 1991) involved in virus transcription and replication (Adkins *et al*., 1995; Chapman *et al*., 2003). The ambisense M and S RNAs both contain two open reading frames (ORFs) separated by an intergenic region (IGR). The viral strand (v sense) of the M RNA codes for a non-structural protein (NS_M) which represents the viral movement protein involved in tubule-guided cell-to-cell transport of nucleocapsids (Kormelink *et al*., 1994; Storms *et al*., 1995). The viral complementary (vc) strand of the M RNA codes for the glycoprotein precursor which is post-translationally cleaved into two glycoproteins, G_N and G_C (n and c refer to the amino and carboxyl terminal topology within the precursor). The glycoproteins are major determinants for thrips transmission and specificity (Wijkamp, *et al*., 1995; Sin *et al*., 2005; Ulman *et al*., 2005) and dictate the site of particle assembly (Kikkert *et al*., 1999, 2001; Ribeiro, 2007). The S RNA encodes a non-structural protein (NS_S) in v sense that has been identified as a suppressor of RNA silencing (Bucher *et al*., 2003; Takeda *et al*., 2002), and the viral nucleocapsid (N) protein in vc sense (de Haan *et al*., 1990). The latter protein is multifunctional, being involved in transcription/replication regulation and in particle structure (Snippe *et al*., 2007). All ORFs in the M and S RNA become expressed via the synthesis of subgenomic-length mRNA molecules (Figure 1-2). Typical for segmented negative-strand RNA viruses, initiation of tospoviral genome transcription takes place through cap-snatching, as best studied for TSWV. During this process, the virus snatches capped leader sequences, usually 12-18 nucleotides in length, from cellular mRNAs to prime transcription of its own genome (Duijsings *et al*., 1999, 2001). The intergenic regions (IGRs) in the M and S RNA contain A/U-rich stretches and are predicted to form a stable hairpin structure (de Haan *et al*., 1990; Kormelink *et al*., 1992).

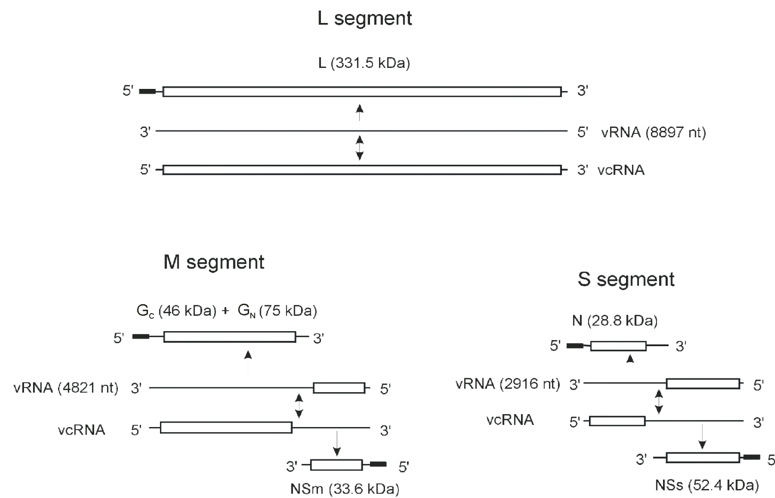


Figure 1-2: Genomic organisation and expression strategy of TSWV. vRNA is viral sense RNA, vcRNA is viral complementary RNA. Open reading frames (ORFs) are indicated by white boxes, non-templated leader sequences by black bars.

Similar foldings have been predicted for the S RNA IGR of two phleboviruses, *Punta toro virus* (PTV) and *Uukuniemi virus* (UUKV), co-inciding with sites of transcription termination (Emery and Bishop, 1987; Simons and Pettersson 1991). Also for Arenaviruses (family *Arenaviridae)*, a hairpin structure within the S RNA IGR has been shown to be involved in transcription termination (López & Franze-Fernández, 2007). As the 3′ terminal ends of TSWV S RNA transcripts have been shown to include the predicted hairpin structure, also for tospoviruses the IGR-located hairpin structure has been proposed to fulfil this role (van Knippenberg *et al.*, 2005).

Vector transmission

Tospoviruses are transmitted by thrips (Thysanoptera: *Thripidae*) in a persistent and propagative manner. Until now, 13 vectoring species have been identified (Premachandra *et al.*, 2005; Whitfield *et al.* 2005; de Borbón *et al.*, 2006; Ohnishi *et al.*, 2006). TSWV utilises the largest number of vector species and is most efficiently transmitted by *F. occidentalis* (Daughtrey *et al.*, 1997;

Ullman *et al*., 1997; Wijkamp *et al*., 1996). The thrips life cycle from egg to adult involves two larval and two pupal stages (Figure 1-3). Adult thrips that feed on infected plants do not become viruliferous even after prolonged feeding on infected plants (Ulman *et al*., 1992; Ullman *et al*., 1989; van de Wetering *et al*., 1996). Virus acquisition can only take place during the first and second larval stages while transmission can be observed with late second stage larvae but occurs most efficiently by viruliferous adults (van de Wetering *et al*., 1996; Wijkamp and Peters, 1993; Wijkamp *et al*., 1993).

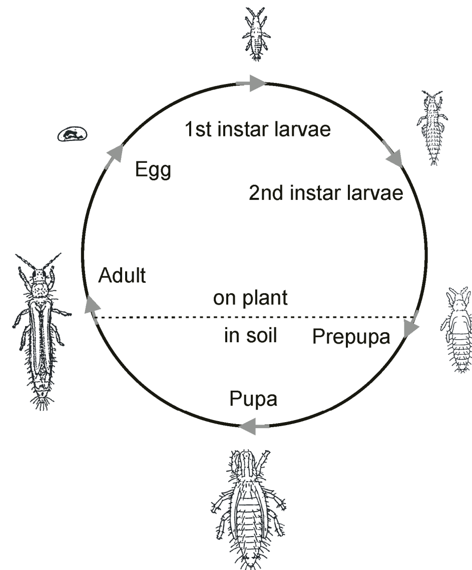


Figure 1-3: Schematic representation of thrips life cycle. TSWV is acquired and replicates during larval stages and transmitted by adults to new plants.

After acquisition, the virus replicates in the midgut epithelium of the first section (Mg1) and from there spreads into visceral and longitudinal muscular cells. Subsequently, the next two midgut sections (Mg2 and Mg3) become infected followed by entering salivary glands (Ullman *et al*., 1993). Several models for midgut-to-salivary gland virus transfer have been suggested of which that of Moritz *et al*., (2004), by direct contact between these organs during first and early second larval stage appears the most likely. During next

developmental stages these tissues disconnect explaining lack of virus transmissibility by thrips acquiring the virus in these later stages.

Natural and engineered resistance to tospoviruses

Of all tospoviruses, TSWV has the largest economic impact worldwide. The difficulties in the management of disease caused by tospoviruses are due to 1) the broad host range of the virus, 2) the cosmopolitan nature of its vector(s), 3) difficulties to control these vectors chemically (Brødsgaad, 1994; Robb *et al.*, 1995; Zhao *et al.*, 1995) or biologically (Morse & Hoddle, 2006) and, last but not least, the limited availability of resistance genes for commercial breeding purposes.

Sofar, two single dominant TSWV resistance genes in host plants have been well described. The first one has been identified in *Lycopersicon peruvianum* for which high resistance levels to various TSWV isolates were observed (Stevens *et al.*, 1992). This gene has been introgressed into the tomato variety 'Stevens' that showed resistance against two additional tospoviruses, GRSV and TCSV (Boiteux & Giordano, 1993). The resistance has been mapped to a single gene, i.e. the *Sw*-5b copy (Folkerstma *et al.*, 1999; Spassova *et al.*, 2001; Brommonschenkel *et al.*, 2000) within the *Sw*-5 gene cluster, and is also functional in other host plants (Spassova *et al.*, 2001). The *Sw*-5 resistance appears not absolutely durable, as TSWV variants overcoming this resistance have already been reported (Latham & Jones, 1998). The second resistance gene, denoted *Tsw,* was identified in *Capsicum chinense* 'PI' accessions in which resistance displayed by a hypersensitive reaction (HR) to a broad range of TSWV isolates. Like *Sw*-5, the resistance is governed by a single dominant gene (Black *et al.*, 1991; Boiteux, 1995) and also for this trait resistance-breaking variants have been identified (Roggero *et al.*, 2002; Aramburu & Marti, 2003).

Given the limited resources of natural host resistance extensive studies have been done on developing engineered resistance (Goldbach & de Haan, 1993; Goldbach *et al.*, 2003). Transgenic expression of the viral N protein

rendered resistance to TSWV in tobacco (Gielen *et al.*, 1991, MacKenzie & Ellis 1992; Pang *et al.*, 1992) and tomato plants (Ultzen *et al.*, 1995). In addition, expression of untranslatable N gene versions were shown to confer the same level of resistance, indicating a pivotal role for the transgenically expressed viral RNA to induce resistance, leading to the concept of RNA-mediated resistance (de Haan, 1992). Further studies revealed that the resistance is based, like in many other cases of transgenic virus resistance, on a process called post-transcriptional gene silencing (PTGS) or, shortly, RNA silencing (Baulcombe, 1996). RNA silencing (in animals often referred to as RNAi, from RNA interference [Fire *et al.*, 1998]), is an evolutionary conserved process involving the sequence-specific breakdown of RNA into small regulatory RNA molecules. While in animals RNAi seems to only serve a gene (and hence developmental biologically) regulatory function, in plants it also has an antiviral role. Actually, in plants RNA silencing is the innate immunity mechanism against viral invaders, in analogy to the interferon pathway in mammals (Pedersen *et al.*, 2007). In infected plants, or in plants transgenic for a viral sequence, viral ssRNA is copied into dsRNA through respectively the viral replicase or a host-encoded RNA-dependent RNA polymerase (RDR) activity and then cleaved by RNase III-like DICER enzymes into small interfering (si) RNA of 21-24 nucleotides (Baulcombe, 2004; Dunoyer & Voinnet, 2005). In the next step, one of the siRNA strands i.e. the guide strand is incorporated into a ribonucleoprotein complex termed RNA-induced silencing complex (RISC). In the transgenic virus-resistant plant, siRNA guide the assembled RISC molecules to degrade single-stranded cognate RNA sequences of the invading homologous virus in the cytoplasm. As a counter defence to overcome RNA silencing plant viruses have evolved through encoding silencing suppressor proteins e.g. the NS_S protein in the case of tospoviruses (Bucher *et al.*, 2003; Takeda *et al.*, 2002; Voinnet, 2005). Since the primary trigger of RNA silencing is dsRNA, it is beneficial to use inverted repeat transgene cassettes which are expressed as ds hairpin RNA.

A potential drawback of RNA-mediated virus resistance is its high sequence specificity (Prins *et al.*, 1996). The initial experiments in which TSWV N gene

sequences conferred resistance were therefore later extended with the aim to generate resistance against a set of three tospovirus species (Prins *et al*, 1995). Also other tospoviral gene sequences have been tested for their potential to induce transgenic resistance, but only N and NS_M gene sequences appeared to have this capacity (Prins *et al.*, 1996). As for N gene-mediated resistance, Jan *et al.* (2000) showed that a minimum length of 236-387 base pairs of N transgene sequence is sufficient to induce resistance or even shorter length (59-110 base pairs) provided that the latter sequence is fused to a (non-target) carrier sequence e.g. green fluorescent protein (GFP).

Previous results on transgenic TSWV-resistant plants using NS_M gene sequences support the view that tospoviruses are not silenced at the genomic level but at the transcript level. Indeed the genomic RNA segments are fully encapsidated by N protein and seem less attractive to serve as RNA silencing target. To obtain broad resistance against several tospovirus species, Bucher *et al.* (2006) designed an inverted-repeat construct (ds-construct) containing short pieces of the N genes from 4 different tospoviruses which indeed provided full resistance against all four viruses in *Nicotiana benthamiana* in more than 80% of the transgenic lines tested. This kind of constructs is therefore particularly attractive for transformation of plant species that suffer from low transformation/regeneration efficiencies.

Outline of the thesis

At the onset of the research described in this thesis, only limited data was available on the possible occurrence and distribution of tospoviruses in Iran. In 1998, a tospovirus-like disease was reported in thrips-infested tomato in Varamin, one of the major production areas for vegetables and ornamentals in Iran. The symptoms included necrotic lesions on the foliage and chlorotic ring spots on the fruits resembling the symptoms induced by TSWV. Serological assays initially suggested the presence of TSWV (Bananej *et al.*, 1998). From then onwards, similar reports appeared on the possible prevalence of TSWV in both vegetables and ornamentals from different areas in Iran (Golnaraghi *et al.*,

2001; Moeini *et al.*, 2000; Pourrahim *et al.*, 2001), soon followed by reports claiming the occurrence of *Peanut bud necrosis virus* (PBNV) (Golnaraghi *et al.*, 2002), INSV (Shahraeen *et al.*, 2002) and *Iris yellow spot virus* (IYSV) (Shahraeen & Ghotbi 2003) from different crops in different areas (Figure 1-4), but since only serological data were presented these data should be considered as rather preliminary.

The research described in this thesis set out to characterise the virus initially identified as a Varamin isolate of TSWV and four additional putative tospovirus isolates collected from chrysanthemum, gazania, potato and soybean, as to obtain a first picture concerning the occurrence of known or possibly new tospoviruses in Iran. A next aim was to investigate how transgenic resistance, based on RNAi, could be developed against these viruses.

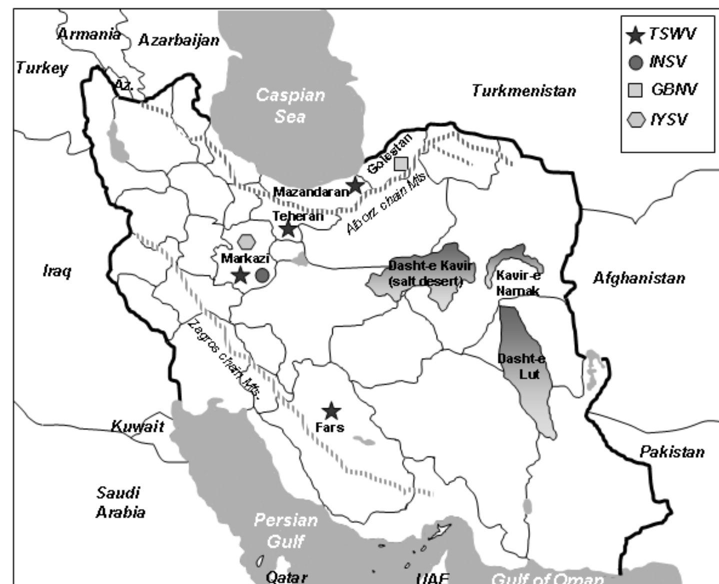


Figure 1-4: Distribution of Tospovirus species preliminarily identified based on serological tests during 1998-2003 in Iran.

Chapter 2 describes the serological and molecular characterisation of the tospovirus isolate from Varamin, revealing that this represents a new tospovirus

species and which was named *Tomato yellow ring virus* (TYRV). In chapter 3, the occurrence of distinct strains of this virus in different crops (tomato, potato, and soybean) has been investigated. In chapter 4, options for effective transgenic resistance to TYRV, and other tomato-infecting tospoviruses have been investigated using inverted repeat transgene constructs.

As the transgenic resistance developed in Chapter 4 was based on RNA silencing, next a long standing enigma has been resolved: are viral siRNAs also produced during the natural tospovirus infection process? The results of this study (chapter 5) complement the studies sofar limited to transgenic resistant host plants. Of special interest in this study has been the presumed hairpin structure at the 3' end of tospoviral transcripts as these could potentially act as potent target and inducer of RNA silencing.

Finally, in Chapter 6 the results obtained in the experimental chapters are discussed in a broader context and an updated model for tospovirus induced RNA silencing and suppression is presented.

Chapter 2

A new tomato-infecting tospovirus from Iran

This chapter has been published in a slightly modified version as:
Hassani-Mehraban, A., Saaijer, J., Peters, D., Goldbach, R. & Kormelink, R. (2005). A new tomato-infecting tospovirus from Iran. *Phytopathology* **95:**852-858.

Summary

A new tospovirus species serologically distinct from all other established tospoviruses was found in tomato in Iran. Typical disease symptoms observed include necrotic lesions on the leaves and yellow ring spots on the fruits, hence the name *Tomato yellow ring virus* (TYRV) was proposed. The S RNA of this virus was cloned and its 3,061 nucleotide sequence showed features characteristic for tospoviral S RNA segments. The nucleocapsid (N) protein with a predicted Mr of 30.0 kDa showed closest relationship to the N protein of *Iris yellow spot virus* (74% sequence identity).

Introduction

Tospoviruses represent the plant-infecting viruses within the family *Bunyaviridae*, a virus family further restricted to animals (Fauquet *et al.*, 2005). They are propagatively transmitted by a limited number of phytophagous thrips (Goldbach & Kuo, 1996). *Tomato spotted wilt virus* (TSWV), type species of the genus *Tospovirus*, has an extremely broad host range and has so far economically the greatest impact of all (Goldbach & Kuo, 1996; Goldbach & Peters 1994). Most other tospoviruses, e.g., Iris yellow spot virus (IYSV) (Cortês *et al.*, 1998) and *Peanut yellow spot virus* (PYSV) (Reddy *et al.*, 1991), have narrow host ranges or, like *Impatiens necrotic spot virus* (INSV), are mainly restricted to ornamental plants (Law & Moyer, 1990). Tospoviral particles are quasi-spherical, enveloped and contain three single-stranded (ss)RNA segments designated small (S), medium (M), and large (L) RNA. Each RNA segment is tightly packaged by copies of nucleocapsid (N) protein and small amounts of the viral RNA-dependent RNA polymerase (RdRp) (van Poelwijk *et al.*,1993) forming infectious ribonucleocapsid proteins (RNPs). Due to the presence of inverted complementary repeat sequences at the termini of all tospoviral RNA segments, the RNPs have a pseudo-circular appearance (van den Hurk *et al.*, 1977). As far as investigated, all tospoviruses have ambisense S and M RNAs, only the L RNA being of complete negative polarity. The genomic RNA encodes in viral (v) sense for a suppressor of RNA silencing (NS_S) (Bucher *et al.*, 2003; Takeda *et*

al., 2002) and in viral complementary (vc) sense for the nucleocapsid (N) protein, while the M RNA encodes the cell-to-cell movement protein (NS_M) in v sense and the precursor to the glycoproteins (G_N and G_C) in vc sense (de Haan *et al*., 1998; de Haan *et al*., 1990; Kormelink *et al*., 1992). The L RNA encodes the putative viral RdRp, also referred to as L protein (de Haan *et al*., 1991). To date, 14 established tospovirus species have been identified based on both biological and molecular (N protein sequence) properties (Cortês *et al*., 1998; McMichael *et al*., 2002). A few have worldwide distribution, e.g., TSWV and INSV, whereas most others remain restricted to the Eurasian or American continents. So far, the largest diversity of tospoviruses is observed in the eastern part of Asia where nine species can be found, i.e., TSWV, *Peanut bud necrosis virus* (PBNV), *Watermelon silver mottle virus* (WSMoV), *Watermelon bud necrosis virus* (WBNV), PYSV, *Peanut chlorotic fan-spot virus* (PCFV), *Melon yellow spot virus* (MYSV), IYSV, and a tentative species reported from Australia as *Capsicum chlorosis virus* (CaCV) (McMichael *et al*., 2002). TSWV was the first tospovirus reported to occur in tomato cv. Pito Early in Iran, in the Varamin area of Teheran province (Bananej *et al*., 1998), soon followed by reports of INSV (Shahraeen *et al*., 2002), PBNV (Golnaraghi *et al*., 2002) and IYSV (Shahraeen & Ghotbi, 2003). Large scale surveys on tospovirus infections have so far not been made and therefore no estimates can be given about the economic impact of tospoviral diseases in Iran. Moreover, the possible occurrence of other tospovirus species in Iran, and even new ones, cannot be excluded. In light of this, a tospo-like virus has very recently been isolated from tomato in the Varamin area during a period coinciding with large thrips infestations. The symptoms on tomato consisted of systemic chlorotic and necrotic spots on leaves and yellow rings on fruits, and the plants generally showed a growth reduction. In a preliminary study, the virus was shown to induce necrotic local lesions on petunia leaves, indicative for the presence of a tospovirus, and subsequent serological data provided evidence that it concerned TSWV (Bananej *et al*., 1998). Here we describe a more detailed characterisation

of this virus, which indicates that it represents a novel tospovirus for which the name *Tomato yellow ring virus* (acronym TYRV) is proposed.

Materials and Methods

Virus isolates and plants

The virus isolate was originally collected from diseased tomato in the Varamin area of Iran in 2002. The virus was transferred from fruits onto *Petunia hybrida* by mechanical inoculation using 0.01 M phosphate buffer, pH 7.0, containing 0.1% sodium sulfite. After two passages a single local lesion was isolated and inoculated on *Nicotiana benthamiana* and maintained by mechanical inoculation. Tospovirus isolates TSWV BR-01 (de Ávila *et al.*, 1992b), *Tomato chlorotic spot virus* (TCSV) BR-03 (de Ávila *et al.*, 1992b), *Groundnut ringspot virus* (GRSV) SA-05 (de Ávila *et al.*, 1992b), INSV NL-07 (de Ávila *et al.*, 1992a), WSMoV (Tospo-to) (Heinze *et al.*, 1995), and IYSV-NL (Cortês *et al.*, 1998) used in serological studies, were also maintained on *N. benthamiana*. For determination of the experimental host range, leaf tissue of TYRV-infected *N. benthamiana* was mechanically inoculated on different host species to compare with those hosts tested for TSWV (Table 2-1). The plants were kept in the greenhouse under normal day-light conditions or in a light/dark regime of 16/8 h and monitored for 3 to 4 weeks for symptom expression.

Virus purification, antiserum production and RNA extraction

Nucleocapsids of TYRV were purified from systemically infected *N. benthamiana* as described by de Ávila *et al.* (1990) but subsequently applied on 25 to 45% $CsSO_4$ gradients for further purification. However, a partially purified preparation of the virus was used for electron microscopic observation of the virions in which the specimen was fixed with 1% glutaraldehyde and stained with 2% uranyl acetate. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the N proteins (Laemmli *et al.*, 1970), nucleocapsids of IYSV and TSWV were likewise purified. Nucleocapsid material

of TYRV purified from CsSO4 gradients was used to produce a polyclonal antiserum to the N protein. Rabbits were intradermally immunised by two injections at an interval of 2 weeks with 50 to 100 µg of nucleocapsid preparation after emulsification with incomplete Freund's adjuvant (1:1, wt/vol). Blood was collected 2 weeks after the last injection and serum was prepared after overnight incubation of the blood at 4℃. Viral RNA of TYRV wa s extracted from either a semipurified preparation, obtained after centrifugation on a 30% sucrose cushion, or from a purified nucleocapsid preparation obtained after $CsSO_4$ gradient centrifugation. The RNA was isolated by treatment of nucleocapsids with 1% SDS followed by phenol/chloroform extraction and ethanol precipitation.

Serological analyses

TYRV was serologically compared with other tospovirus species by a double-antibody sandwich, enzyme-linked immunosorbent assay (DAS-ELISA) (Clark & Adams 1977) using polyclonal antisera directed against the N protein of each virus. The antisera for TSWV, TCSV, GRSV, and INSV were previously prepared by de Ávila *et al.* (1992a) and for IYSV by Cortês *et al.* (1998). Polyclonal anti-N serum for WSMoV (Tospo-to) was supplied by G. Adam (University of Hamburg).

Reverse transcription-polymerase chain reaction cloning and sequence determination

To obtain S RNA-specific clones of TYRV, reverse transcription was performed on purified nucleocapsid RNA using oligonucleotide "Asian Termini" (AT; 5′ dCCCGGATCC**AGAGCAATCGAGG** 3′) which (in bold) is complementary to the first 8 terminal nucleotides of the 3′ end conserved for all tospoviruses (de Haan *et al.*, 1989), extended with 5 additional nucleotides as found conserved for all Asian tospoviral S RNA segments (data not shown). Reverse transcription (RT) was carried out using Superscript RT (Invitrogen, Carlsbad, CA) or Enhanced Avian RT (Sigma-Aldrich, St. Louis, MO). First-strand cDNA primed by AT was subsequently polymerase chain reaction (PCR)-

amplified with primer AT only or in combination with primer UHP (dCACTGGATCC**TTTTGTTTTTGTTTTTTG**) (Cortês *et al.*, 2001) or P1 (dTCCCGGATCC**CYTCATTYCTBCC**), complementary to nucleotide 246 to 259 numbered from the 5′ end of the TYRV vRNA strand and containing a conserved sequence from the start codon region of the NS_S open reading frame (ORF). In a second approach, newly obtained sequences were used to design additional primers that were used in combination with primer AT or UHP to obtain RT-PCR fragments covering remaining parts of the S RNA segment. RT-PCR fragments covering the intergenic region were obtained by immunocapture (IC)-RT-PCR (Mumford & Seal 1997). To this end, Eppendorf tubes were coated with 50 µl of TYRV antisera (1:1,000 of a 1-mg/ml stock) in coating buffer (0.05 M Na carbonate, pH 9.6) for 2 h at 37℃, washed with phosphate-buffered saline containing 0.05% (vol/vol) Tween 20 (PBS-T), and subsequently incubated with 50 µl of TYRV RNPs for 2 h at 37℃. After final removal of the contents, the tubes were washed and immediately used for RT according to the circumstances as described previously. Amplification was done using the Expand Long Template PCR System (Roche Diagnostics, Penzberg, Germany) as previously described by Cortês *et al.* (1998, 2001). Fragments obtained after PCR were blunt-end cloned into pGEM-T vector (Promega Corp., Madison, WI) and used for nucleotide sequence determination. DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) on an automatic sequence machine (Applied Biosystems, Foster City, CA). Nucleotide and amino acid sequences were compiled and analysed using BLAST and CLUSTAL W (Thomson *et al.*, 1994). Data from CLUSTAL W were used as input for the construction of a phylogenetic tree using PAUP 3.1.1 package (Illinois Natural History Survey, Champaign, IL) based on 100 replicates and using midpoint rooting (Swofford, 1993). RNA secondary structures were predicted by Mfold (Zuker, 2003; Mathews *et al.*, 1999; Rensselaer Polytechnic Institute; http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). The panhandle and hairpin structures were predicted on the input of 98 nucleotides (nts) of both terminal ends and 208 nts of the intergenic region, respectively. The nucleotide sequence

for the full-length S RNA of TYRV is accessible as GenBank accession no. AY686718.

Expression of viral N protein in *Escherichia coli*

To confirm whether the vcORF of TYRV S RNA codes for the nucleocapsid (N) protein, the vcORF was cloned for expression in *E. coli*. To this end, a set of specific primers, N1 (dCCCGGATCC**ATGGCTACCGCACGAGTG**) containing an *Nco*I site and N2 (dCCCGGATCC**GCACTCATTAAAATGCATC**) with a *Bam*HI cloning site, was used to RT-PCR amplify the vcORF. The fragment obtained was cloned as an *Nco*I-*Bam*HI fragment in plasmid pET-11t (modified from pET-11d; Novagen, Madison, WI) and transformed into BL21 *E. coli* cells. Positive clones were induced with isopropyl-β-thiogalactopyranoside (IPTG) as described by Kormelink *et al*. (1994). Total proteins of induced and non-induced *E. coli* cells were analysed on 15% SDS polyacrylamide gel (Laemmli, 1970), followed by western immunoblot analysis on Immobilon-P membrane (Millipore Corp., Bedford, MA) using polyclonal anti-TYRV N serum.

Results

Ultrastructure, host range and symptomatology

During the tomato-growing season in the Varamin area a number of tomato fields were infected by a putative virus causing necrotic and yellow rings on leaves and fruits, respectively (Figure 2-1A). As the symptoms were reminiscent to those of TSWV and thrips were abundant, infected material was serologically tested for the presence of TSWV. Although these tests were negative (data not shown), electron microscopical studies revealed the presence of tospovirus-like particles when partially purified preparations of infected *N. benthamiana* were analysed (Figure 2-1B).

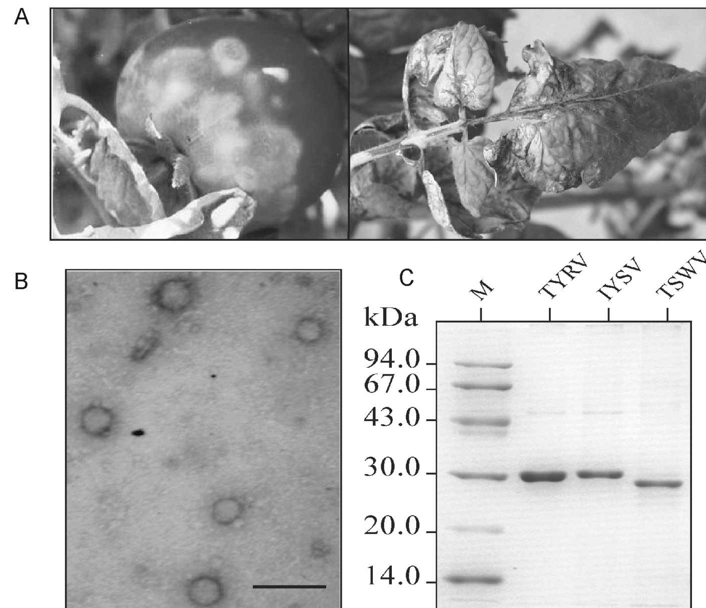


Figure 2-1: Symptoms, virus particles and N protein analysis of Tomato yellow ring virus (TYRV). A, Picture shows yellow rings on tomato fruit, chlorosis and necrosis on leaves with leaf stem necrosis; B, Electron micrograph of partially purified TYRV stained with 2% uranyl acetate, bar represents 200nm. C, Comparison of nucleocapsid protein of TYRV with those of Iris yellow spot virus (IYSV) and *Tomato spotted wilt virus* (TSWV) resolved on 15% sodium dodecyl sulfate polyacryamide gel and stained with Coomassie Brilliant Blue. Low molecular weight size markers (M) are indicated at the left of the gel.

As a further step to characterise this potentially new tospovirus, the experimental host range was determined. In approximately half of the hosts tested, a systemic viral infection was observed often initiated after the appearance of chlorotic and/or necrotic local lesions on the inoculated leaves (Table 2-1). Typical tospovirus symptoms were observed on *Petunia hybrida* and *N. benthamiana* plants on which necrotic local lesions and chlorotic spots with leaf deformation could be observed, respectively. The virus induced local symptoms on *N. rustica* and *Capsicum annuum*. No local or systemic symptoms were observed on *Zinnia elegans*, *Arachis hypogea*, *Phaseolus vulgaris*, and *Vicia faba*. Mechanical back-inoculation on *Lycopersicon esculentum* cv. Pito Early induced chlorosis followed by necrosis on leaves, leaf stem, and top necrosis but plants could recover.

Table 2-1: Response of several host palnts to *Tomato yellow ring virus* (TYRV) and *Tomato spotted wilt virus* (TSWV)[a]

Host plants	TYRV		TSWV	
	Local reaction	Systemic reaction	Local reaction	Systemic reaction
Amaranthaceae				
Gomphrena globosa	NL	-	NL	-
Balsaminaceae				
Impatiens spp.	NL	NS	NL	M,MO,CL,NL
Chenopodiaceae				
Chenopodium amaranthicolor	NL	-	NL	-
Chenopodium quinoa	NL	-	NL	-
Compositae				
Chrysanthemum sp.	-	-	NS	CS,NS,SN-
Emilia sonchifolia	CS	MO	NL	M,MO,LD
Gazania sp.	CS	LD,GR	NL	MO
Zinnia elegans	-	-	NL	-
Cucurbitaceae				
Cucumis sativus	CL	-	CL	-
Leguminosae				
Arachis hypogea	-	-	CS	MO,NS
Physalis vulgaris	-	-	CL	-
Pisum sativum	NL	VC,NS,LD	-	LN,SN
Vicia vaba	-	-	-	LN
Vigna unguiculata	NL	VC,M	NL	MO,LD
Liliaceae				
Alstroemeria	CS	CS,C	CS	MO,VC,NS
Solanaceae				
Capsicum annum	CL	-	NR	MO
Datura metel	CS	VC,LDCS	CS	M,MO
Datura stramonium	CS	NS,LD	CS,NS	M,MO
Lycopersicon esculentum cv. Pito Early	CS	CS,NS,TN,GR	CS,NS	MO,LBP,TN
Nicotiana benthamiana	CS	M,LD	CS	VC,M,MO,LD
Nicotiana clevelandii	CS,NL	VC,LD,PD	CS,NL	M,MO,LD
Nicotiana glutinosa	NL	LD,PD	NL	M,MO,NS
Nicotiana rustica	NL	-	NL	M,VN
Nicotiana tabacum Samsun	NR	NR,LD	NL	VC
Nicotiana tabacum NN	CL	-	NR	-
Petunia hybrida	NL	-	NL	-
Tropaeolaceae				
Tropaaeolum majus	CS	CS	-	YS,GS

[a]C = leaf curling, CL = chlorotic lesions, CS = chlorotic spots, GR = growth reduction, GS = green spots, LBP = leaf bronzing or purpling, LD = leaf deformation, M = mosaic, LN = leaf necrosis, MO = mottling, NL = necrotic lesions, NR = necrotic rings, NS = necrotic spots, SN =srtem necrosis, PD = plant death, TN = tip necrosis, VC = veinal chlorosis, YS = yellow spots, and - = no symptoms.

After some time, fruits developed symptoms that resembled those initially observed on diseased tomato collected from Iran (Figure 2-1A). These data indicated that the pathogen was a putative tospovirus causing somewhat distinct symptoms compared to those of TSWV described so far (Table 2-1).

Serological relationship to other tospoviruses

As a first step towards the production of a polyclonal antiserum against TYRV which would allow serological comparison with other tospovirus species, RNPs were purified, dissociated and analysed by SDS-PAGE (Figure 2-1C). These analyses showed that TYRV N protein (more or less) co-migrated with the N protein of IYSV and was estimated to be approximately 30 kDa. Rabbits were immunised with purified RNPs and serum was collected for the preparation of anti-N immunoglobulin G. Subsequent serological comparison of TYRV with six different tospovirus species in a DAS-ELISA format revealed positive reactions for all homologous combinations (Figure 2-2).

As expected, additional cross-reactions were observed between TSWV, TCSV, and GRSV, whereas only homologous reactions were observed for INSV, WSMoV, and IYSV. No significant cross-reaction was observed for TYRV with antisera of other tospoviruses and vice versa suggesting that TYRV was serologically distinct from the other tospoviruses tested.

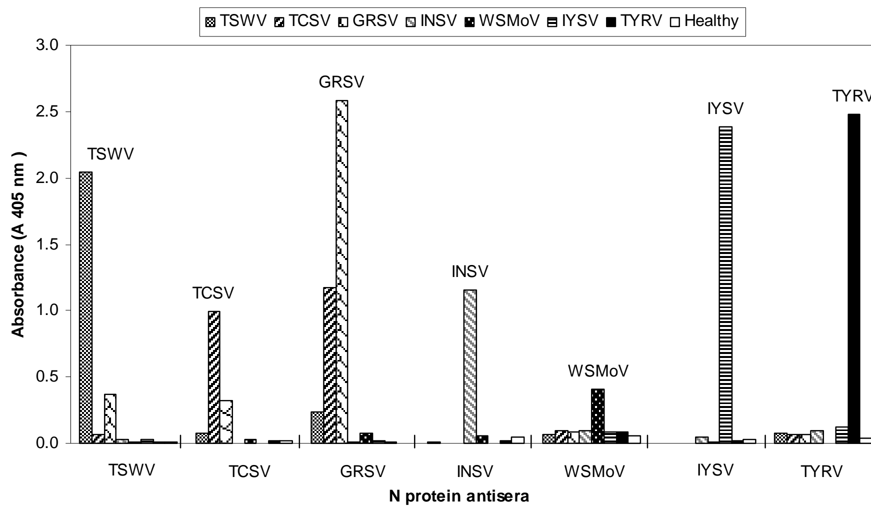


Figure 2-2: Serological differentiation between *Tomato yellow ring virus* (TYRV) and six established tospovirus species in double-antibody sandwich enzyme-linked immunosorbent assay format using polyclonal antisera raised against respective N proteins and the extracts from infected plants as antigen source.

RT-PCR and sequence analysis of the S RNA

In order to obtain the entire S RNA nucleotide sequence several approaches were used. The first RT-PCR reaction was carried out using the primers AT and P1 (described previously), which resulted in a fragment of 259 nts containing 71 nts of the 5′ untranslated region (UTR) and 188 nts of the NS_S ORF. When the primer combination AT and UHP was used a fragment of 1,152 nts was obtained representing 71 nts of the 3′ UTR sequence, the entire N ORF (825 nts), and 256 nts of the intergenic region. Using newly designed primers, the remaining part of the S RNA was amplified resulting in a full set of RT-PCR clones encompassing the entire (3,061 nts) S RNA segment (Figure 2-3A).

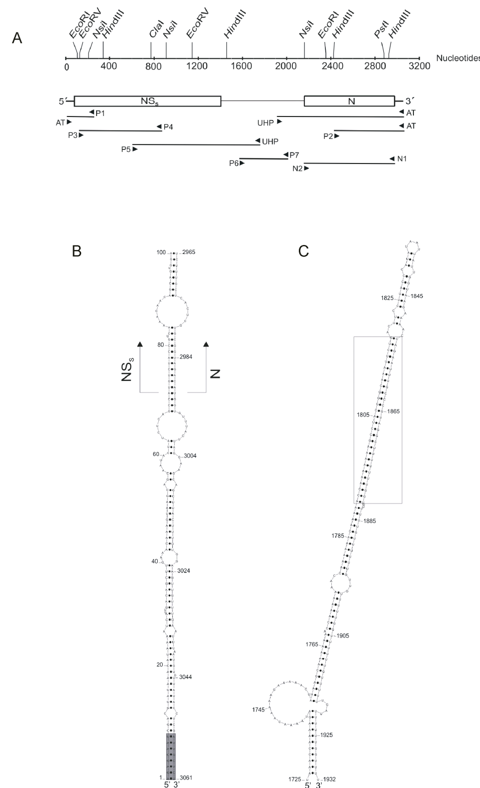


Figure 2-3: Cloning strategy of the *Tomato yellow ring virus* (TYRV) S RNA segment. Schematic representation of the S RNA along a scale bar with a 6 base restriction map (A). Primers (arrowhead) used and DNA fragments (straight lines) obtained from reverse transcription-polymerase chain reaction cloning are indicated. Predicted folding of the panhandle (B) and intergenic hairpin structure of TYRV S RNA (C). The eight conserved terminal nucleotides are shown in gray. A stretch of 28 nucleotides within the hairpin structure, showing full complementarity, is marked with a box.

Since primer AT was only 13 nts in size and could potentially cross-anneal to different positions along all three genomic viral RNAs, the specificity of amplified fragments was verified by restriction enzyme analysis using either the *Bam*HI site within the primers or other restriction sites present in the overlapping sequences. The S RNA sequence obtained was complete, demonstrated by the presence of 5′ and 3′ UTRs containing at least eight residues which are conserved between all tospoviral RNAs (de Haan *et al.*, 1989). The terminal sequences showed long stretches of full complementarity within the first 100 nts potentially involved in pseudo-circularisation of the genome segment to form a so-called panhandle structure (Figure 2-3B). The viral strand of TYRV S RNA contained an ORF starting with an AUG at nucleotide position 72 and terminating with a UGA codon at position 1403, coding for the NS_S protein with a predicted Mr of 50.2 kDa. The vcORF coding for the N protein started with an AUG at nucleotide position 2990 and terminated with a UAA stop codon at nucleotide position 2164. The N protein sequence was determined to be 274 residues long with a predicted molecular mass of 30.0 kDa. The non-coding intergenic region runs from nucleotide position 1404 to 2165 and possesses a high AU rich content enabling the formation of a stable hairpin (Figure 2-3C). Within this structure a perfect double-stranded RNA (dsRNA) region extending over 28 nts was observed, involving nucleotide residues running from nucleotide positions 1791 to 1818 and 1853 to 1880.

Overall comparison of TYRV S RNA with those of IYSV, MYSV, PBNV, WSMoV, and TSWV revealed that Eurasian tospoviruses contained a 5′ and 3′ UTR with a size between 65 to 71 nts. For TSWV, 5′ and 3′ UTRs of 88 and 153 nts were observed, respectively (Figure 2-4). Sequence alignment of the 5′ UTRs showed first of all a consensus sequence of 8 nts (AGAGCAAU) but, moreover, a conserved sequence motif around nucleotide positions 56 to 71 (AGNAAUACUA(N)2UCAGNC) just upstream of the NS_S start codon. Alignment of the 3′ UTRs showed that, apart from the first conserved terminal residues, less sequence conservation was observed in comparison to the respective 5′ UTRs (data not shown).

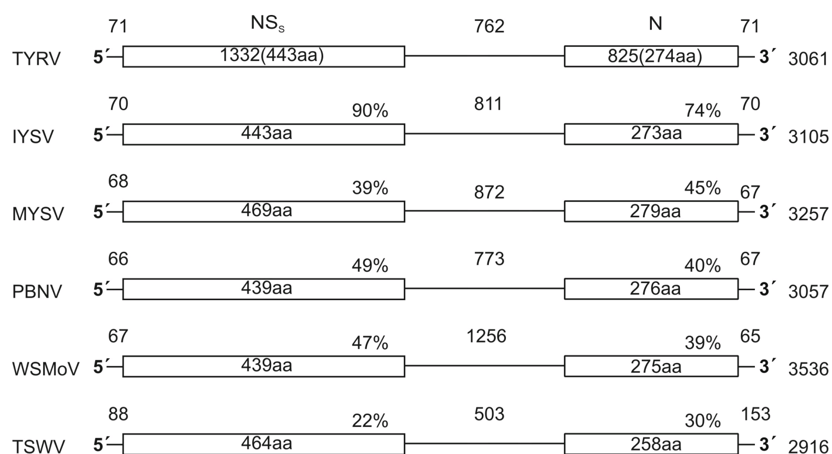


Figure 2-4: Topological comparison of the S RNA segment of *Tomato yellow ring virus* (TYRV) to those of five other tospovirus species. The nucleotide lengths are indicated. The sizes of the 5´ and 3´ untranslated regions and the intergenic regions (in nucleotides) and proteins (in amino acids) are indicated.

Multiple sequence alignment

To clarify the taxonomic position of TYRV, its N protein was analysed by multiple sequence alignment to other tospoviral N proteins (Table 2-2). These analyses revealed the highest homology between the TYRV and IYSV N proteins (74% identity), and much lower homology to the other Eurasian species amongst others, PBNV, WSMoV, WBNV, MYSV, and CaCV (39 to 45% identity). Whereas the N proteins showed an overall low homology, a conserved stretch of amino acids was observed around residues 153 and 206 that was restricted to Eurasian tospoviral N sequences (data not shown). Data from multiple sequence alignments were used as input for the construction of a phylogenetic tree (Figure 2-5). The results clearly showed two major clusters, i.e., one containing all (de Ávila *et al.*, 1992b) tospovirus species that were isolated and primarily distribute in the Americas (Figure 2-5, upper branch), and one containing all (de Haan *et al.*, 1989) species that were isolated and primarily distribute in Eurasia (Figure 2-5, lower branch). The analyses, furthermore, seemed to point towards a separate clustering of IYSV and TYRV, diverging from the major one consisting of PBNV, WSMoV, WBNV, and MYSV.

Table 2-2: Tospoviral N protein sequence identities (%)

Virus	TSWV	TCSV	GRSV	INSV	CSNV	ZLCV	PBNV	WSMoV	WBNV	MYSV	PCFV	PYSV	CaCV	IYSV	TYRV
TSWV[a]	**100**	77	78	53	75	72	25	28	26	26	18	19	29	30	30
TCSV	...	**100**	81	52	72	71	27	27	26	27	19	20	28	29	31
GRSV	...	...	**100**	52	73	75	27	29	28	27	19	19	29	29	31
INSV	...	...	...	**100**	53	50	27	27	26	24	21	21	27	26	29
CSNV	...	...	...	...	**100**	80	26	28	24	29	20	20	28	31	29
ZLCV	...	...	...	...	...	**100**	26	26	26	26	19	19	27	29	29
PBNV	...	...	...	...	...	...	**100**	86	85	60	21	20	84	42	40
WSMoV	...	...	...	...	...	...	...	**100**	86	58	20	20	86	41	39
WBNV	...	...	...	...	...	...	...	...	**100**	58	19	20	82	42	39
MYSV	...	...	...	...	...	...	...	...	...	**100**	19	19	59	47	45
PCFV	...	...	...	...	...	...	...	...	...	...	**100**	59	20	18	17
PYSV	...	...	...	...	...	...	...	...	...	...	...	**100**	20	18	20
CaCV	...	...	...	...	...	...	...	...	...	...	...	...	**100**	43	40
IYSV	...	...	...	...	...	...	...	...	...	...	...	...	...	**100**	74
TYRV	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**100**

[a] The tospovirus referred are the following: TSWV(D00645); TCSV(S54325); GRSV(S54327); INSV(S40057); CSNV(AF067068); ZLCV(AF067069); PBNV(U27809); WSMoV(Z46419); WBNV(AF045067); MYSV(AF067151); PCFV(AF080526); PYSV(AF013994); CaCV(AY036058); IYSV(AF001387); TYRV in this study.

The identities (%) of the N protein have been calculated from the sequence data using the vector NTI Suite 6 program (gap opening penalty 10 and gap extension penalty 0.1).

In conclusion, TYRV was shown to be distinct from all other established tospovirus species and hence should be regarded as a new species.

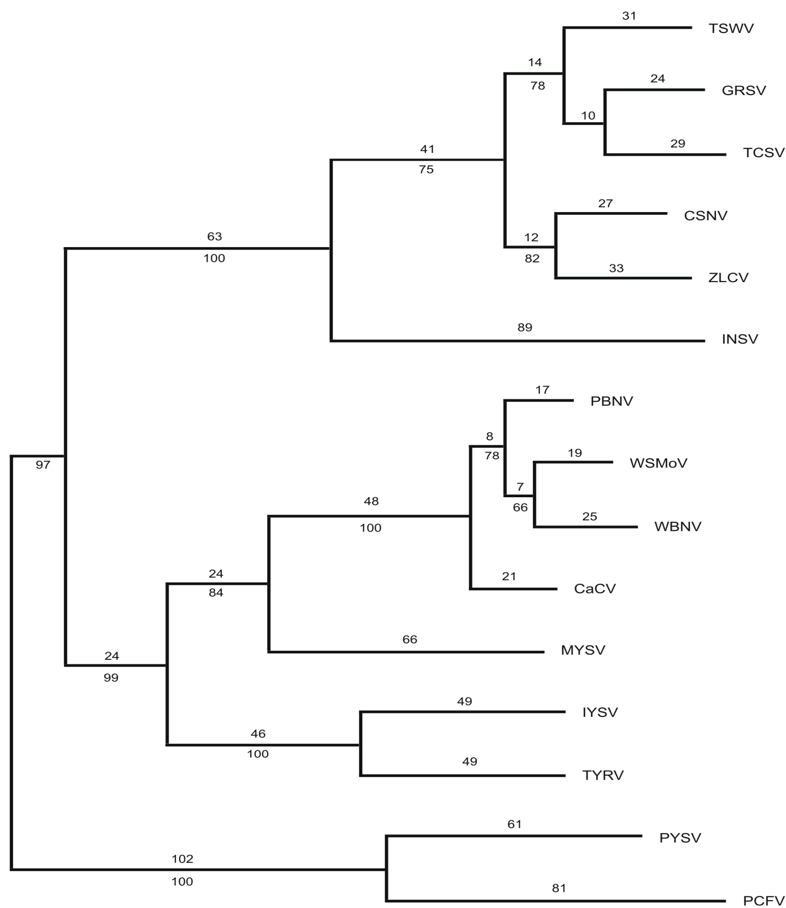


Figure 2-5: Phylogenetic tree of different tospovirus species based on N protein sequence data. The phenogram was constructed using PAUP 3.1.1 (Illinois Natural History Survey, Champaign, IL) from PileUp (Genetics Computer Group, Madison, WI) as input based on 100 replicates using midpoint rooting. Sources of the sequences referred are as follows: *Tomato spotted wilt virus* (TSWV) (D00645); *Tomato chlorotic spot virus* (TCSV) (S54325); *Groundnut ringsport virus* (GRSV) (S54327); *Impatiens necrotic spot virus* (INSV) (S40057); *Chrysanthemum stem necrosis virus* (CSNV) (AF067068); *Zucchini lethal chlorosis virus* (ZLCV) (AF067069); *Peanut bud necrosis virus* (PBNV) (U27809); *Watermelon silver mottle virus* (WSMoV) (Z46419); *Watermelon bud necrosis virus* (WBNV) (AF045067); *Melon yellow spot virus* (MYSV) (AF067151); *Peanut chlorotic fan-spot virus* (PCFV) (AF080526); *Peanut yellow spot virus* (PYSV) (AF013994); *Capsicum chlorosis virus* (CaCV) (AY036058*); Iris yellow spot virus* (IYSV) (AF001387); and *Tomato yellow ring virus* (TYRV) in this study.

Expression of the viral protein in *E. coli*

To confirm that the vcORF of TYRV S RNA segment indeed coded for the N protein, the vcORF was RT-PCR-amplified with primers N1 and N2, cloned in pET-11t, and subsequently transformed to BL21 *E. coli* cells for protein induction. The vcORF-encoded protein produced was estimated to be approximately 30 kDa. The expressed product specifically reacted with the anti-TYRV serum and co-migrated with N protein from TYRV RNP preparations (Figure 2-6), confirming the N protein identity of the vcORF.

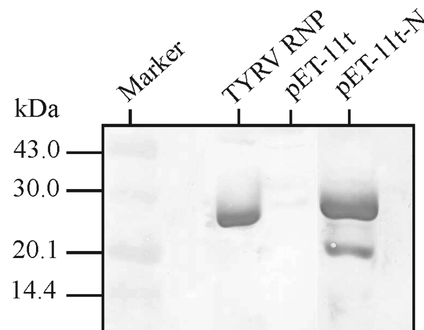


Figure 2-6: Expression of *Tomato yellow ring virus* (TYRV) nucleoprotein in *Escherichia coli*. Purified TYRV RNP and pET-11t–transformed BL-21 cells were included as positive and negative controls, respectively. Low molecular weight size markers (Amersham Pharmacia Biotech, Uppsala, Sweden) are indicated at the left.

Discussion

Based on host range, symptomatology, ultrastructure, serology, and genomic sequence data, the occurrence of a novel tospovirus in tomato cultivations in Varamin, Iran, has been demonstrated. In view of its disease symptoms in tomato, which were confirmed by back-inoculation experiments on tomato cv. Pito Early, the name *Tomato yellow ring virus* (TYRV) is proposed. Although the symptoms of TYRV on infected tomato leaves and fruits were similar to those already described for TSWV in the same region (Bananej *et al.*, 1998), no mixed infections with TSWV have been found in collected samples. Next to tomato, TYRV was also detected in naturally infected chrysanthemum (Varamin) and gazania (Teheran) plants as confirmed by DAS-ELISA and

nucleotide sequence analysis of the N gene (data not shown). These results altogether indicate that TYRV has a (experimental/natural) host range that includes agricultural and ornamental crops. Due to the presence of *Thrips tabaci* in tomato crops during the moment of sample collection, this thrips species may represent a potential vector species of TYRV. Several transmission experiments have been performed in which a range of different thrips species amongst others populations of *Frankliniella occidentalis*, *T. tabaci*, and *T. palmi* have been tested as vector of the virus. However, these analyses have so far failed to identify *T. tabaci* or other species as vector (data not shown).

The nucleotide sequence of TYRV S RNA showed, as expected, complementarity of the 5′ and 3′ terminal ends allowing formation of a panhandle structure typical for all tospoviral RNA segments (van den Hurk *et al.*, 1977). The complementarity is 100% for the first 11 nts with more mismatches between nucleotide 12 up to nucleotide position 100 where the panhandle formation more or less ends (Figure 2-3B). Analysis of the hairpin structure showed a region of dsRNA of 28 nts, which extended to 42 nts when two mismatches were included (Figure 2-3C). Recently the 3′ terminal ends of TSWV S RNA specific transcripts have been mapped and showed the presence of the hairpin structure in viral transcripts. This suggested that the hairpin may have a function in transcription termination of the viral messenger RNAs (van Knippenberg *et al.*, 2005). The presence of this hairpin and in specific the presence of long stretches of full complementary sequences extending over 28 nts in viral RNA transcripts is interesting in light of dsRNAs triggering the RNA silencing machinery (Meister *et al.*, 2004). Whether these sequences indeed are the target for the silencing remains to be investigated. Alignment of the N protein sequence of TYRV with those of 14 other tospoviruses has indicated closest relation to IYSV (74% identity) and lowest to PCFV (17% identity) (Table 2-2). However, alignment of the NS_S protein sequences of TYRV, IYSV, MYSV, PBNV, WSMoV, and TSWV revealed a greater divergence between the NS_S proteins (22 to 90% identity) than those of the respective N protein sequences (30 to 74% identity) (Figure 2-4).

The phylogenetic analysis (Figure 2-5) revealed that tospoviruses, excluding TSWV and IYSV which have apparently further spread by international trading, can be assigned in an American cluster (TSWV, GRSV, TCSV, INSV *Chrysanthemum stem necrosis virus* [CSNV] and *Zucchini lethal chlorosis virus* [ZLCV]) and a Eurasian cluster (PBNV, WSMoV, WBNV, CaCV, MYSV, IYSV, TYRV, PYSV, and PCFV). Since TYRV and IYSV are closely related, and the former virus seems indigenous to Iran, a country that does not play a major role in worldwide agricultural trading yet, it is well possible that both viruses have their origin in the Middle East where IYSV has started to spread all over the world (Cortês *et al*., 1998; Gera *et al*., 1998; Schwarz *et al*., 2002). The existence of a Middle East (sub) clustering of tospovirus species within the large Eurasian cluster would support such a hypothesis. To find this, a more detailed tospovirus survey in the Middle East area is required. In conclusion, based on the present data TYRV represents the first new tospovirus species isolated from the Middle East. Given the fact that plant virology in this area is still in its infancy, it might be expected that it will not be the last one.

Note added

A highly similar N protein gene sequence from a tospovirus referred to as Tomato yellow fruit ring virus has been submitted to GenBank by Dr. Stephan Winter and colleagues and is accessible at accession number AJ493270.

Acknowledgements

We thank Dr. N. Shahraeen and T. Ghotbi (Plant Pests and Diseases Research Institute, Teheran, Iran) for supplying several virus samples and helpful discussions, Mr. D. Shahriary (Agricultural Research Station, Varamin, Iran) for providing pictures of symptoms on tomato, Dr. J. van Lent for his assistance in EM assay, Mr. J. Vink (Plant Research International, Wageningen University, The Netherlands) and the Laboratory of Phytopathology (Wageningen University, The Netherlands) for assisting in the production of TYRV antiserum and providing Vector NTI Suite 6 Program, respectively. This

work was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality.

Chapter 3

Molecular and biological comparison of two *Tomato yellow ring virus* (TYRV) isolates: challenging the *Tospovirus* species concept

This chapter has been published in a slightly modified version as:
Hassani-Mehraban, A., Saaijer, J., Peters, D., Goldbach, R. & Kormelink, R. (2007). Molecular and biological comparison of two *Tomato yellow ring virus* (TYRV) isolates: challenging the *Tospovirus* species concept. *Archives of Virology* **152**:85-96.

Summary

Two strains of *Tomato yellow ring virus* (TYRV, genus *Tospovirus*), one from tomato (referred to as TYRV-t) and the other from soybean and potato (denoted TYRV-s), collected from different geographical regions in Iran, were compared. Their genomic S RNA segments differed in size by 55 nucleotides. Comparison of the S RNA intergenic regions revealed the absence of a stretch of 115 nucleotides within the S RNA segment of TYRV-s and, conversely, of 56 nts in that of TYRV-t, apparently a stable genetic difference as it was also found in another isolate of TYRV-s collected from potato. Sequence comparison revealed an identity of 92% between the N proteins of both strains, and the observed strong cross-reaction of TYRV-s in DAS-ELISA with a polyclonal antiserum directed against the TYRV-t N protein confirmed this high identity. Host range analysis revealed several differences, e.g. TYRV-s, but not TYRV-t, being able to systemically infect *Nicotiana* species. The observed molecular and biological differences of both viruses call into question the currently used criteria for *Tospovirus* species demarcation.

Introduction

Species of the genus *Tospovirus* (family *Bunyaviridae*) have enveloped and quasi-spherical virions, 80-120 nm in diameter, and are transmitted by thrips (Thysanoptera; *Thripidae*) in a persistent manner (Fauquet *et al.*, 2005). Currently, 13 different thrips species have been reported as tospovirus vectors, the western flower thrips, *Frankliniella occidentalis*, being the most important one (de Borbón *et al.*, 2006; Ohnishi *et al.*, 2006; Premachandra *et al.*, 2005; Whitfield *et al.*, 2005). The tripartite tospoviral genome consists of ambisense S and M RNA segments and a negative-stranded L RNA (de Haan *et al.*, 1990 and 1991; Kormelink *et al.*, 1992). The genome encodes 6 mature proteins: the RNA-dependent RNA polymerase protein (RdRp or L protein) by the L RNA, the cell-to-cell movement protein (NS_M) and the suppressor of silencing (NS_S) protein in viral sense by the M and S RNA, and the two membrane glycoproteins (G_N and G_C) and the nucleoprotein (N) in viral complementary sense by the M and S

RNAs, respectively (Bucher *et al.*, 2003; de Haan *et al.*, 1990 and 1991; Kormelink *et al.*, 1992; Kormelink *et al.*, 1994; Takeda *et al.*, 2002).The open reading frames (ORFs) in the M and S RNA segments are separated by large AU-rich intergenic regions (IGR), which form a stable hairpin structure assumed to be involved in transcription termination (van Knippenberg *et al.*, 2005).

Serological distinction among tospovirus species is based on double-antibody sandwich (DAS)-ELISA using the N protein, which is the least, conserved tospoviral protein, as antigen (de Ávila *et al.*, 1990; Wang & Gonsalves, 1990). Indeed, the identification of new tospovirus species was initially based on ELISA and resulted in the classification of tospoviruses into serogroups (de Ávila *et al.*, 1990 and 1993b). However, serological differentiation soon appeared insufficient as a taxonomic criterion, and nowadays the N protein sequence in combination with biological characters such as thrips vector species and host range represent the main classification criteria for the establishment of a new tospovirus species, with the N protein sequence identity threshold set at 90% (de Ávila *et al.*, 1993a; Goldbach & Kuo, 1996). Based on these criteria, 16 tospovirus species have been recognised with the largest diversity being observed within the Asian continent (Hassani-Mehraban *et al.*, 2005; Lin *et al.*, 2005). With its worldwide distribution and wide host range *Tomato spotted wilt virus* (TSWV) is the most prominent member of all tospoviruses.

As described in Chapter 2, recently, an isolate of a new tentative tospovirus species, named *Tomato yellow ring virus* (TYRV), was collected from tomato in Iran, and this virus was most closely related (by N protein sequence identity) to *Iris yellow spot virus* (IYSV) (Cortês *et al.*, 1998; Hassani-Mehraban *et al.*, 2005). In a limited field survey, mainly in Teheran province, four additional tospovirus isolates were collected from chrysanthemum, gazania, potato, and soybean during 2000–2002. Upon further analysis, the first two isolates were found to represent TYRV isolates (Hassani-Mehraban *et al.*, 2005), whereas preliminary RT-PCR analyses of their N gene suggested that the isolates from soybean and potato represented a different tospovirus. Here we report the

further characterisation of the Iranian tospovirus isolates from soybean and potato and show that they belong to a distinct strain of TYRV. The degree of difference in molecular and biological characters between the two strains of TYRV may urge revision of the *Tospovirus* species concept.

Materials and Methods

Virus isolates and host range

Tospovirus isolates collected in Iran, i.e. TYRV isolates from tomato, chrysanthemum, gazania, and potato (Teheran province) and one isolate from soybean (Mazandaran province), were maintained and propagated on *Nicotiana benthamiana*. To avoid formation of defective interfering (DI) isolates due to serial mechanical passages, new virus inocula were prepared from liquid nitrogen stocks every 4–5 passages (Inoue-Nagata *et al.*, 1997). TYRV and the soybean isolate were inoculated on a large range of plant species including those tested for TYRV (Hassani-Mehraban *et al.*, 2005) and different *Nicotiana* species and *N. tabacum* cultivars. The plants were monitored for the expression of symptoms during several weeks while being kept at 22-25℃ and 12 h light/dark regime under greenhouse conditions. To confirm systemic infections in infected plants, both symptomatic and asymptomatic leaf samples, next to uninfected leaf material as negative control, were tested by DAS-ELISA using polyclonal antiserum directed to the N protein of TYRV (de Ávila *et al.*, 1990; Hassani-Mehraban *et al.*, 2005).

To investigate whether distinct TYRV isolates could mix-infect host plants one isolate was inoculated on young tomato seedlings (cultivars Pito Early and Moneymaker) and, 4 days later, with the second one on the same leaf. Leaves showing systemic symptoms were harvested and compared to single infections as controls.

Serological analysis

Serological analysis was performed by DAS-ELISA using polyclonal anti-N sera of TYRV (Hassani-Mehraban *et al.*, 2005), TSWV (de Ávila *et al.*, 1990), and IYSV (Cortês *et al.*, 1998). In this test, crude extracts from infected *N. benthamiana* and healthy plants were diluted 1:30 and were used as antigen source and negative control, respectively.

Purification of viral N protein, viral RNA and total RNA

Systemically infected *N. benthamiana* leaves were used to purify nucleocapsids of both the soybean and potato tospovirus isolates as described by de Ávila *et al.* (1990). Viral RNA was extracted from purified nucleocapsid preparations obtained after $CsSO_4$ gradient centrifugation by treating the extract with 1% SDS, followed by phenol/chloroform extraction and ethanol precipitation (Cortês *et al.*, 1998). Total plant RNA was isolated from infected *N. benthamiana* plants according to Kormelink *et al.* (1992).

Cloning of the S RNA segment and sequence analysis

For cloning, reverse transcriptase polymerase chain reaction (RT-PCR) was performed to amplify fragments representing the entire S RNA sequence of the soybean isolate. As a first step, partial sequences of the N and NS_S ORFs were amplified using primer "Asian Termini" (AT; 5´-dCCCGGATCC**AGAGCAATCGAGG**-3´containing (in bold) 13 terminal nucleotides conserved in all Asian tospoviral S RNA segments in combination with a universal hairpin primer (UHP; dCACTGGATCCTTTTGTTTTTGTTTTTTG) (Cortês *et al.*, 2001). To amplify and clone the remaining sequences, specific primers were designed based on sequences obtained from S RNA-derived clones. For most of the cloning procedure, RT-PCR was carried out using Superscript RT (Invitrogen, Carlsbad, CA) and the Expand Long Template PCR System (Roche Diagnostics, Penzberg, Germany), with the exception of IGR, which was amplified by immunocapture RT-PCR (Mumford & Seal, 1997). PCR products were cloned

into pGEM-T Easy vector (Promega Corp. Madison, WI) and sequenced by the dideoxynucleotide chain termination method (Sanger *et al*., 1977) using an automatic sequence machine (Applied Biosystems, Foster City, CA). Sequences obtained were analysed using BLAST. RNA folding structures were predicted using Mfold (Zuker, 2003; Mathews *et al*., 1999; Rensselaer Polytechnic Institute; http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). Multiple sequence alignment was carried out using Vector NTI (Invitrogen). Phylogenetic trees were constructed from ClustalW (Thompson *et al*., 1994) data in PAUP 3.1 with a heuristic search, based on 100 replicates, using midpoint rooting (Swofford, 1993). The nucleotide sequence for the complete S RNA of TYRV-s has been submitted to Genbank under accession number DQ462163.

Results

RT-PCR amplification and cloning of the Iranian soybean and potato tospoviral N genes

During the identification and characterisation of a new tomato-infecting tospovirus in Iran (See Chapter 2 and Hassani-Mehraban *et al*., 2005), denoted *Tomato yellow ring virus* (TYRV), a primer set was designed to allow rapid amplification and cloning of the N gene of this virus. This set was tested here on four recently collected tospovirus isolates from Iran, sampled from chrysanthemum, gazania, soybean, and potato, as these were suspected to also belong to this species. RT-PCR amplification of total RNA from infected *N. benthamiana* resulted, for the chrysanthemum and gazania isolates, in a clear DNA fragment co-migrating with the amplified N gene from TYRV (Figure 3-1). Upon sequence analysis, these isolates were shown to be almost identical to TYRV from tomato (further referred to as TYRV-t) with only 0.7–1.5% sequence divergence in their N protein sequence (data not shown). However, no DNA fragments could be amplified for the soybean and potato isolates, indicating that these isolates might be distinct. Also, amplification using a multiplex primer set

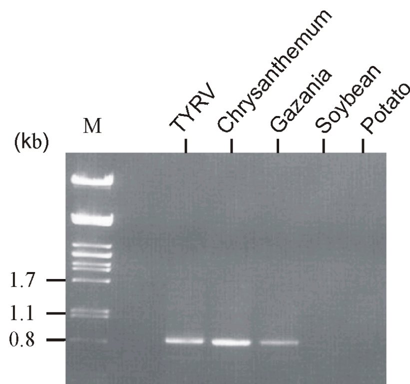


Figure 3-1: RT-PCR-based differentiation of Iranian TYRV isolates. *M*, molecular size marker λ DNA x *Pst*I. Primers for amplification of the N gene were deduced from the TYRV-t S RNA sequence.

that recognises the N genes of TSWV, INSV, GRSV, and TCSV remained negative.

N protein serology

As PCR amplification remained negative for the soybean and potato isolates, their serological relationship to TYRV-t and other tospoviruses was determined using DAS-ELISA. No reaction was observed with polyclonal antisera against TSWV or IYSV, but a strong positive reaction was obtained with anti-TYRV serum (Figure 3-2). Repeated analysis showed that the ELISA values were always consistently lower compared to the homologous TYRV-t signal. This provided evidence that, despite the negative RT-PCR outcome, both soybean and potato isolates had a close taxonomic relationship to TYRV-t.

Comparative analysis of the S RNA segment

To elucidate the taxonomic position of the soybean isolate, its entire S RNA was cloned and sequenced and subsequently compared to that of TYRV-t S RNA. As is typical for tospoviruses, the S RNA of this isolate contained, in ambisense arrangement, ORFs for NS_S and N proteins, with a predicted Mr of 50.0 and 30.0 kDa, respectively. The NS_S and N proteins of the soybean isolate and TYRV-t showed 94 and 92% amino acid (aa) sequence identity,

respectively. This still falls, albeit marginally, inside the range allowed within the International Committee om Taxonomy of Viruses (ICTV) species concept for tospoviruses (Goldbach & Kuo 1996), and in view of the marked differences, the soybean isolate was further referred to as TYRV-s (*versus* TRYV-t for the tomato isolate). TYRV-s and TYRV-t also displayed clear differences in their IGRs, the IGR of TYRV-s lacking 115 nts present in TYRV-t and, conversely, that of TYRV-t lacking 56 nts present in TYRV-s (Figure 3-3).

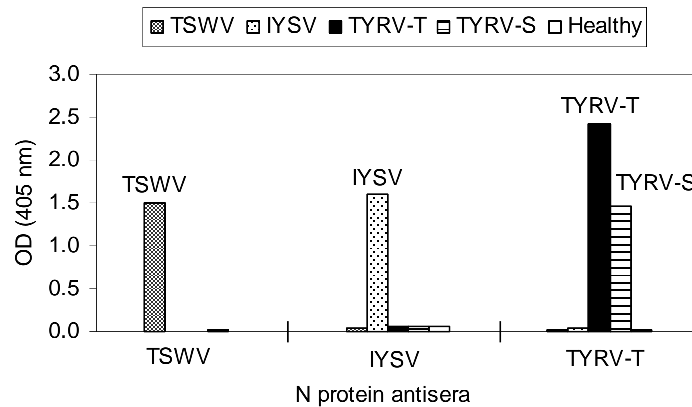


Figure 3-2: Serological differentiation between TYRV-t (tomato isolate) and the soybean isolate (TYRV-s). The chart shows A405 values (y-axis) from a double-antibody-sandwich enzyme-linked-immunosorbent-assay (DAS-ELISA) performed using polyclonal anti-N sera from different tospoviruses (shown at the x-axis) and extracts from infected plants as antigen source (represented by patterned bars).

The central part of both IGRs can be folded in a secondary structure which has a longer stretch of ds-RNA (34 nts) in TYRV-s than in TYRV-t (28 nts) (Chapter 2 and Hassani-Mehraban *et al.*, 2005). The 5´ and 3´ -terminal untranslated regions (UTR) were of similar length (71–72 nts), with a high potential tendency to form a genomic panhandle pseudo-circle.

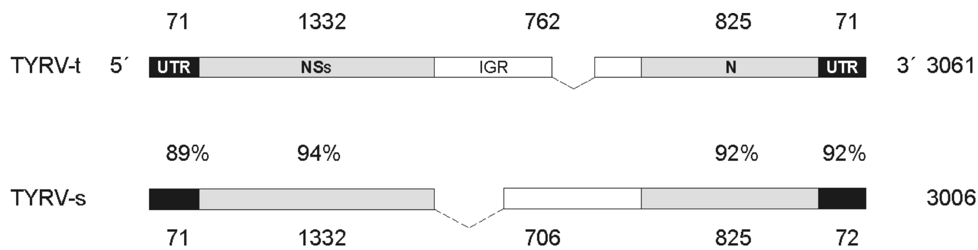


Figure 3-3: Comparative analysis of the S RNA segments of TYRV-t and TYRV-s. Untranslated regions (UTR) at the termini are marked in black, open reading frames (ORFs) in gray, and the intergenic region (IGR) in white. Only major deletions within the IGR are indicated by interruption of the IGR-box. The sizes of the N and NS_S ORFs are indicated in nucleotides. The level of identity (%) between the UTRs (at the nucleotide level) and between the ORFs (amino acid level) of both viruses is shown between the S RNA segments.

Multiple sequence alignment and phylogenetic relationship

To establish the taxonomic position of TYRV-s (and TYRV-t) within the genus *Tospovirus*, the N protein sequences were compared with those of members of established tospovirus species. This analysis showed that TYRV-s (from soybean and potato) and TYRV-t (from tomato, chrysanthemum, and gazania) are most closely related (72.4–73.1%) to (the NL isolate of) IYSV (Table 3-1).

Data from a multiple sequence alignment were used as input to construct a phylogenetic tree, revealing a close clustering of TYRV-t, TYRV-s, and IYSV-NL within the large Eurasian tospovirus cluster (Figure 3-4).

Comparative host range analysis

In view of the clear molecular difference between TYRV-t and TYRV-s (i.e. 8% divergence in their N protein and deletions in their IGRs), and in view of their different crop origin, it was next investigated whether the two strains would exhibit differences in their host range (Table 3-2). Upon testing a large number of plant species, it was found that on some hosts both TYRV isolates induced only local symptoms, whereas on others, systemic symptoms were observed.

Table 3-1: Tospoviral N protein sequence identities (%)

Virus [a]	TSWV	TCSV	GRSV	INSV	CSNV	ZLCV	PBNV	WSMoV	WBNV	MYSV	PCFV	PYSV	CCSV	CaCV-au	CaCV-th	IYSV-NL	IYSV-BR	TYRV-t	TYRV-s
TSWV	100	77	78	53	75	72	25	28	26	26	18	19	24	29	29	30	31	30	30
TCSV	…	100	81	52	72	71	27	27	26	27	19	20	23	28	28	29	29	31	30
GRSV	…	…	100	52	73	75	27	29	28	27	19	19	25	29	29	29	30	31	31
INSV	…	…	…	100	53	50	27	27	26	24	21	21	23	27	28	26	26	29	27
CSNV	…	…	…	…	100	80	26	28	24	29	20	20	24	28	27	31	29	29	29
ZLCV	…	…	…	…	…	100	26	26	26	26	19	19	24	27	27	29	28	29	30
PBNV	…	…	…	…	…	…	100	86	85	60	21	20	66	84	85	42	44	40	43
WSMoV	…	…	…	…	…	…	…	100	86	58	20	20	65	86	86	41	44	39	43
WBNV	…	…	…	…	…	…	…	…	100	58	19	20	66	82	82	42	43	39	42
MYSV	…	…	…	…	…	…	…	…	…	100	19	19	59	59	60	47	49	45	46
PCFV	…	…	…	…	…	…	…	…	…	…	100	59	18	20	21	18	18	17	18
PYSV	…	…	…	…	…	…	…	…	…	…	…	100	17	20	22	18	21	20	19
CCSV	…	…	…	…	…	…	…	…	…	…	…	…	100	64	64	45	44	44	45
CaCV-au	…	…	…	…	…	…	…	…	…	…	…	…	…	100	92	43	44	40	43
CaC-th	…	…	…	…	…	…	…	…	…	…	…	…	…	…	100	45	45	44	44
IYSV-NL	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	100	91	75	74
IYSV-BR	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	100	74	72
TYRV-t	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	100	92
TYRV-s	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	…	100

[a] The tospovirus referred are the following: TSWV(D00645); TCSV(S54325); GRSV(S54327); INSV(S40057); CSNV(AF067068); ZLCV(AF067069); PBNV(U27809); WSMoV(Z46419); WBNV(AF045067); MYSV(AF067151); PCFV(AF080526); PYSV(AF013994); CCSV(AY867502); CaCV-au (AY036058); CaCV-th(AF134400); IYSV-NL(AF001387); IYSV-BR(AF067070); TYRV-t(AY686718); TYRV-s in this study.

The identities (%) of the N protein have been calculated from the sequence data using the vector NTI Suite 6 program (gap opening penalty 10 and gap extension penalty 0.1).

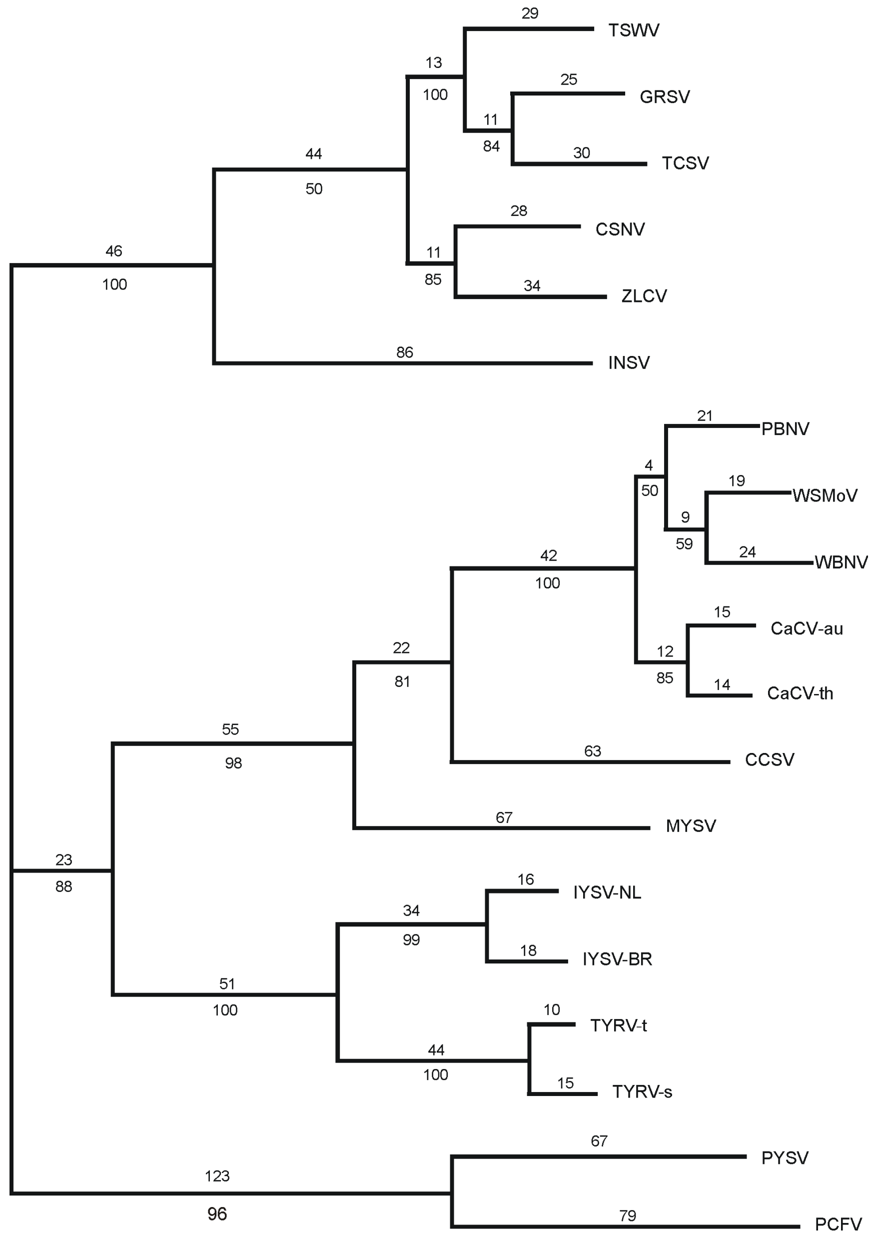


Figure 3-4: Evolutionary relationship of tospovirus species. The phylogenetic tree was constructed using PAUP 3.1, with the multiple alignment of tospoviral N protein sequences from ClustalW as input data. Genbank accessions for the tospovirus N gene sequences used are listed in Table 3-2. The tree was based on bootstrap analysis (100 replicates) of heuristic search, using midpoint rooting.

Table 3-2: Host range symptomatology after infection with TYRV-t and TYRV-s

Plant species	TYRV-t		TYRV-s	
	Local	Systemic	Local	Systemic
Leguminosae				
Glycine max cv. Sahar	CLL, NLL	CS, MO	CLL, NLL	CS, MO
Pisum sativum	CLL	MO, NS	CLL	–
Solanaceae				
Capsicum annum	CS	M, LD	CS	M, LD
Datura metel	CS	M, SN	CS	M, SN
D. stramonium	CS, NLL	C, LD	CS, NLL	C, LD
Lycopersicon esculentum cv. Pito Early	CS	CS, NS, TN, GR	CS	CS,NS,
Nicotiana benthamiana	CS		CS	VC,M,MO,LD
N. christi	NLL	VC,M,MO,LD	NLL	M, LD
N. clevelandii	M	M, LD	M	PN
N. edwardsoni	NLL	PN	GS, NLL	PN
N.glutinosa	CLL	M, LD	NLL	GS, LD
N. kawakamii	NLL	–	–	–
N. roselata	CS	–	CS	MO, LN, TN
N. tabacum Av	GS, LL	MO, LN, TN	GS	CS,VC
N. tabacum B2/7	GS	–	GS	GS, LD
N. tabacum B84/61	GS, NLL	–	–	GS, VC, NS
N. tabacum D25	–	–	NLL	M, N
N. tabacum Sr1	–	–	M, LD	M, LD
N. tabacum Samsun	LL	–	NLL	CS
N. tabacum van Hieko	LL	–	GS, NS	GS,VC
N. tabacum White burley	–	–	CS	Y
N. tabacum Xanthi	NLL	–	NLL	NS, LD
Petunia hybrida	NLL	– –	NLL	–

C chlorosis; *CLL* chlorotic local lesion; *CS* chlorotic spots; *GS* green spots; *GR* growth reduction; *LD* leaf deformation; *LN* leaf necrosis; *M* mosaic; *MO* mottling; *N* necrosis; *NLL* necrotic local lesion; *NS* necrotic spots; *PN* plant necrosis; *TN* top necrosis; *SN* stem necrosis; *VC* veinal chlorosis; *Y* yellowing and – no symptoms.

Symptoms varied from only necrotic lesions up to plant necrosis. In total, 10 out of 22 plant species tested became systemically infected by TYRV-s but not by TYRV-t. On the other hand, TYRV-t induced mosaics and systemic necrotic spots on *Pisum sativum* (Table 3-2). These results showed that TYRV-s and TYRV-t exhibit striking differences in their host range and symptomatology.

Cross-protection between TYRV-s and TYRV-t

Tomato seedlings singly inoculated with TYRV-s or TYRV-t showed differences in symptom severity. Compared to TYRV-t, strain TYRV-s induced significantly milder symptoms on both tomato cultivars tested (Figure 3-5A; Pito Early and Moneymaker cultivars). Tomato plants first inoculated with TYRV-s

and after 4 days super-inoculated with TYRV-t developed only the mild symptoms of the former, and RT-PCR analysis confirmed that only the first inoculated strain was present (Figure 3-5B). Also in the reverse scenario the first inoculated strain (e.g. TYRV-t) was able to exclude the second strain inoculated.

Discussion

Limited reports have appeared on the occurrence and distribution of plant viruses in Iran, a few of these describing the presence of tospoviruses such as TSWV, INSV, and PBNV (Bananej *et al.*, 1998; Ghotbi *et al.*, 2005; Golnaraghi *et al.*, 2002; Golnaraghi *et al.*, 2004; Shahraeen *et al.*, 2002). However, large-scale surveys on tospovirus infections from that area are scarce, and so is information on the presence of hitherto unidentified new tospoviruses. Hence, the economical impact of tospoviral diseases in Iran (and the further Middle-East area) remains unclear. During the last few years we have investigated the identity of five tospovirus isolates from tomato, chrysanthemum, gazania, soybean, and potato collected from different regions in Iran. These analyses identified the presence of a new tospovirus species in tomato cultivations in the Varamin area (Hassani-Mehraban *et al.*, 2005), denoted *Tomato yellow ring virus* (TYRV) (Chapter 2) and also referred to as Tomato yellow fruit ring virus (TYFRV) or Tomato varamin virus (ToVV) by others (Ghotbi *et al.*, 2005, Genbank AJ493270). For this tospovirus, *Thrips tabaci* and *Microcephallothrips abdominalis* were reported as potential vectors (Ghotbi *et al.*, 2005).

From chapter 2 it is evident that this virus also occurs in chrysanthemum (Varamin) and gazania (Teheran) as confirmed by RT-PCR cloning and sequence analysis of the N gene. Whereas the N gene from the chrysanthemum and gazania isolates could readily be amplified by TYRV-t N-gene-specific primers, similar analysis of the soybean and potato isolates rendered negative results. DAS-ELISA, on the other hand, confirmed a clear serological relationship of the latter two isolates with TYRV, so to elucidate their taxonomic

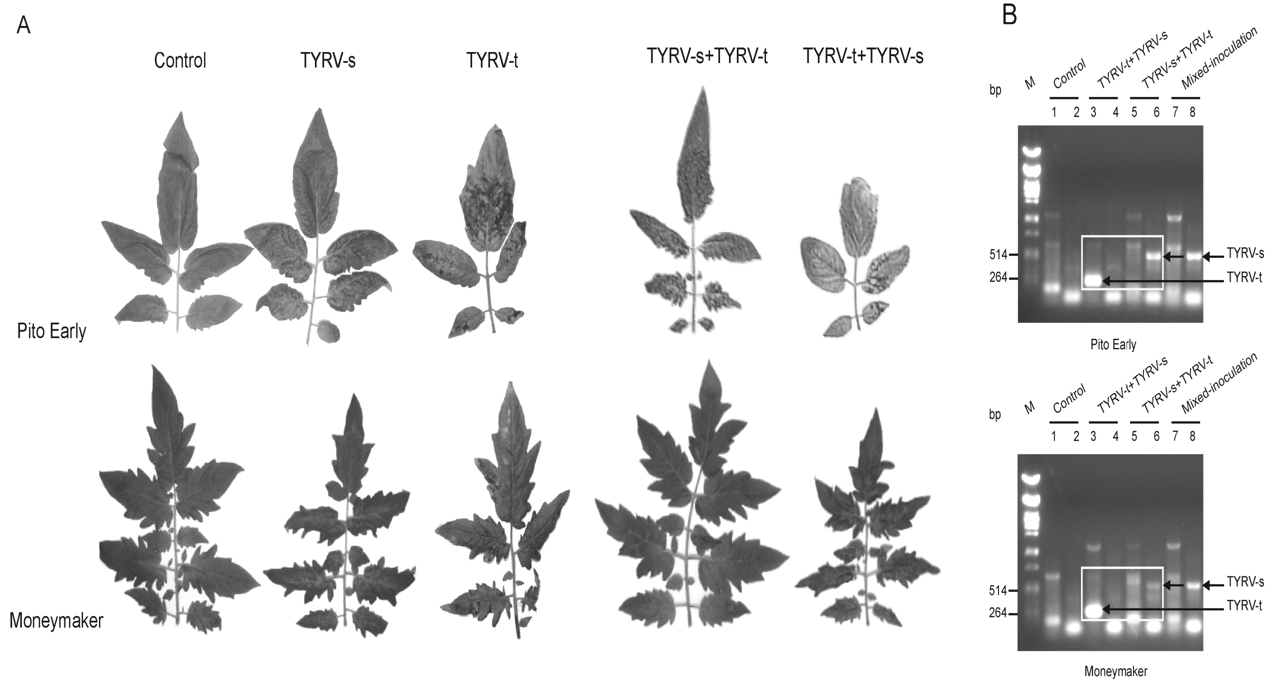


Figure 3-5: Mechanical inoculation of TYRV-s and TYRV-t on tomato cultivars Pito Early and Moneymaker. Panel A, single inoculation of each virus strain and/or followed by the other strain. Panel B, RT-PCR on total RNA of the cultivars infected with the second strain after 4 dpi of the first challenged strain using strain specific primer sets; lanes 1, 3 and 5 and 2, 4 and 6 are samples used as templates for TYRV-t and TYRV-s primers, respectively.

relationship to TYRV, the nucleotide sequence of the S RNA was determined. Comparison of the soybean isolate to TYRV-t did reveal 92% identity between their N genes, which is just above the threshold value of 90% used for species demarcation.

The soybean isolate thus may be best regarded as a distinct strain of TYRV, even though the presumed TYRV-specific primers allowed the soybean isolate to escape RT-PCR detection, and host range analysis of both revealed some clear differences (Table 3-1). In view of their differences, the strain found in soybean and potato was further referred to as TYRV-s, and the originally sequenced TYRV isolate from tomato (and now also found in gazania and chrysanthemum) was denoted TYRV-t. Further comparative analysis of the S RNA sequences from TYRV and the soybean isolate revealed two clear differences in the IGR.

Sequence analysis of the corresponding IGR and the N genes from the potato isolate of TYRV-s revealed almost (99%) completely identical sequences to the isolate from soybean, demonstrating that the difference in IGR sequence is a genetically stable difference between TYRV-t on one hand and TYRV-s on the other. Differences between the IGRs of (closely) related tospoviruses have been reported earlier (Heinze *et al*., 2001; Heinze *et al*., 2003; Okuda *et al*., 2003; Pappu *et al*., 2000). In one study (Qui *et al*., 1998) on genome reassortants from closely related viruses, a correlation was observed between sequences within the IGR and competitiveness of S RNA segments during mixed infections, and in another, the S RNA was suggested to be involved in symptom differences (Okuda *et al*., 2003). Whether differences in the IGR would contribute to the observed host range differences between TYRV-t and TYRV-s remains to be investigated. In light of this, it is interesting to note that both strains out-compete each other during consecutive inoculations on tomato, during which TYRV-s cross-protected against the more virulent TYRV-t strain. Whereas the N gene of TYRV-t and TYRV-s diverge up to 8% at the amino acid level, and even more (9%) when TYRV-s is compared to the gazania isolate of TYRV-t, sequence comparison of the N proteins of 52 TSWV isolates and 47

PBNV isolates deposited in Genbank revealed an intra-species diversity worldwide of only 4% for TSWV and 5% for PBNV (data not shown). A similarly large divergence as found for TYRV can be observed only for two other tospoviruses (Table 3-2, Figure 3-4), i.e. for IYSV (Cortês *et al.*, 1998; Pozzer *et al.*, 1999) and for CaCV (McMichael *et al.*, 2002; the Thai isolate sequence data are accessible from Genbank only). However, for these viruses, only N-gene derived data are available.

Apparently, the diversity within TYRV, even though only isolates originating from a single country are considered, is significantly greater than the intra-species diversity observed for TSWV and PBNV, and this molecular divergence coincides with differences in biological characters, specifically host range and symptomatology. It will be of great interest to search for further variants of this virus in Iran and its neighbouring countries, which may deliver further data that, will challenge the current ICTV-authorized tospovirus species demarcation criteria. Until that time, the isolates described here can best be referred to as strain TYRV-t (in tomato, gazania and chrysanthemum) and strain TYRV-s (in soybean and potato).

Acknowledgements

The authors are grateful to Dr. N. Shahraeen and T. Ghotbi (Plant Pests and Diseases Research Institute, Teheran, Iran) for supplying some of the tospovirus isolates. This work was supported by the Dutch Ministry of Agriculture, Nature and Food Quality.

Chapter 4

RNAi-mediated resistance to the tomato strain of *Tomato yellow ring virus* broken by co-infection with a different strain of this virus

This chapter has been submitted in a slightly modified version as:
Hassani-Mehraban, A. Brenkman, A. B., van den Broek, N. J. F., Goldbach, R. & Kormelink, R. " RNAi-mediated resistance to the tomato strain of *Tomato yellow ring virus* broken by co-infection with a different strain of this virus".

Summary

Previously it has been shown that an inverted repeat transgene cassette containing partial nucleoprotein (N) gene sequences from four different tomato-infecting tospovirus species and expressed as hairpin RNA confers resistance against all these viruses. It is now shown that extension of this transgene construct with a partial N gene sequence from the tomato strain of *Tomato yellow ring virus* (TYRV-t), renders transgenic *Nicotiana benthamiana* plants resistant to this strain but not to the soybean strain of this tospovirus species (TYRV-s), both strains exhibiting 14.4% sequence divergence in their N genes. Surprisingly, co-inoculation of the TYRV-t resistant transgenic lines with both TYRV-t and TYRV-s resulted in rescue of the former. Mass-spectrometric analysis of the viral ribonucleocapsids accumulating in the transgenic plants showed the presence of the N proteins of both strains excluding hetero-encapsidation as rescue mechanism and indicating suppression of TYRV-t N gene transcript breakdown by RNAi. Co-inoculation of resistant transgenic lines with TYRV-t and a PVX vector expressing the TYRV-s silencing suppressor (NS_S) gene also resulted in TYRV-t infection. It is therefore concluded that TYRV-t is rescued from transgenic resistant lines by trans-complementation with the silencing suppressor protein of TYRV-s.

Introduction

Sources of natural resistance to tospoviruses, suitable for commercial breeding, are very limited and therefore extensive studies have been made to create engineered forms of host resistance against these pathogens (Goldbach *et al.*, 2003). Among these are the use of plantibodies or aptamers (Prins *et al.*, 2005; Rudolph *et al.*, 2003), but sofar the most successful and widely studied strategy employs transgenic expression of partial or intact nucleoprotein (N) gene sequences which has lead to heritable immunity against homologous tospoviruses (Gielen *et al.*, 1991, Jan *et al.*, 2000; Prins *et al.*, 1996, Stoeva *et al.*, 1998). It has been demonstrated that this form of resistance is based on RNA silencing (Baulcombe, 1999) i.e. transgenically expressed viral RNA

sequences provoke the antiviral RNA silencing mechanism in the plant. Analysis of transgenic plants containing sequences from different parts of the viral genome has shown that in case of tospoviruses antiviral RNA silencing targets the viral transcripts rather than the (N protein-encapsidated) viral genome (Prins *et al.*, 1996 and 1997). To induce RNA silencing against tospoviruses, a minimal length of the N transgene sequence (236-387 bp) is required but when fused to a non-target gene a shorter sequence (59-110 bp) is sufficient (Pang *et al.*, 1997; Jan *et al.*, 2000).

A major drawback of RNA-mediated virus resistance is its high specificity. For instance, RNAi-mediated resistance against *Tomato spotted wilt virus* (TSWV) does not hold against *Groundnut ring spot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) despite 78-80% genome sequence homology to TSWV (Prins *et al.*, 1996). To circumvent this problem, broad tospovirus resistance has recently been created by designing a chimaeric transgene cassette containing multiple (partial) N gene sequences derived from different (tomato-infecting) tospoviruses and triggering the silencing of all tospoviral targets simultaneously (Bucher *et al.*, 2006). Moreover, since this transgene cassette, due to its inverted repeat topology, is expressed as ds hairpin RNA molecule, resistant lines were obtained at very high efficiency.

Recently, we have identified and characterised a new tomato-infecting tospovirus species from Iran named *Tomato yellow ring virus* (Hassani-Mehraban *et al.*, 2005). Further studies on this novel species have revealed the presence of two distinct strains, i.e. a tomato strain (TYRV-t) and a soybean strain exhibiting 20 % sequence divergence in their S RNA segment (TYRV-s; Chapter 3 and Hassani-Mehraban *et al.*, 2007).

To further improve the existing (Bucher *et al*, 2006) transgene cassette, directed against 4 different tomato-infecting tospoviruses, i.e. TSWV, TCSV, GRSV and *Watermelon silver mottle virus* (WSMoV), this cassette has now been extended with a partial N gene sequence of the fifth tomato-infecting tospovirus*Tomato yellow ring virus* from tomato (TYRV-t), and tested for its performance in *Nicotiana benthamiana*. It is shown that transgenic resistance

against TYRV-t does not hold against the TYRV-s strain and, moreover is broken by TYRV-t when co-inoculated with TYRV-s. Further investigations indicate a role of the viral silencing suppressor protein in this phenomenon.

Materials and Methods

Virus sources and maintenance

TSWV, GRSV, TCSV, WSMoV (de Ávila *et al.*, 1992) and the tomato and soybean strains from *Tomato yellow ring virus* (TYRV), referred to as TYRV-t and TYRV-s, respectively, (Hassani-Mehraban *et al.*, 2007) were maintained on *N. benthamiana* by mechanical inoculation. For inoculation, infected leaf material was ground in 0.01 M phosphate buffer, pH 7.0 containing 0.01 M Na_2SO_3 and subsequently rubbed on carborundum dusted leaves of wild type (wt) or *Agrobacterium tumefaciens* transformed *N. benthamiana*. Plants were kept under greenhouse conditions at a 16 h light/8 h dark regime.

Cloning and construction of inverted-repeat constructs

For the construction of a gene cassette containing partial N gene sequences from 5 different tospovirus species, an earlier described N gene cassette containing fragments of approximately 150 nucleotides (nts) of the N genes from 4 different tospoviruses (Bucher *et al.*, 2006) was used and extended by PCR fusion with a 159 nt fragment of the TYRV-t N gene using TYRV-IR-F and N-Stop primers (Table 4-1). This fragment mapped to nt positions 667-825 of the N gene and was specifically selected with no homology to the (partial) N gene sequences within the gene cassette to avoid instability, due to intra-molecular recombination either in bacteria or plants, of the construct. Clones in pGEM-T Easy vector (Promega, Corp. Madison, WI) were selected for both orientations using M13F and N-Stop primers (Table 4-1). To obtain a hairpin RNA construct of this gene cassette, two copies of the N gene cassette were cloned as an inverted repeat by using the GATEWAY^{TM} Cloning Technology according to the manufacturer's instructions (Invitrogen, Carlsbad,

CA). To this end, the N gene cassette was first flanked with recombination adapter sequences by PCR using PGateEasyF and PGateEasyR primers (Table 4-1) prior to introduction into donor vector pDONR207 by BP clonase. The cassette was subsequently transferred to destination vector pK7GWIWG2 by LR clonase to give rise to an inverted repeat construct in which the N gene cassette repeats were separated by an *Arabidopsis* intron sequence (Karimi *et al*. 2002). Clones were selected containing an inward (IN) or outward (OUT) directed inverted repeat construct with a sense or antisense intron (INTs or INTas) sequence. Construct fedility was verified by restriction enzyme digestion and sequence analysis prior to transformation into *Agrobacterium tumefaciens* strain GV3101.

Table 4-1: List of primers used in this study

Primer	Sequence* 5' to 3'
TYRV-IR-F	CCC*GGATCC*CTAAATGACTGTACTCCAGGG
N-Stop	CCC*GGATCC*GCACTCATTAAAATGCATC
FP01	CCC*GCGGCCGC*TCTAACGTTAAGCAGCTCACAG
M13F	TGTAAAACGACGGCCAGT
PGateEasyF	*GGGGACAAGTTTGTACAAAAAAGCAGGCT*ACGACGGCCAGTGAATTGTA
PGateEasyR	*GGGGACCACTTTGTACAAGAAAGCTGGGTG*ACCATGATTACGCC AAGCTA
FP6	*CATTGTTTGCAGTCTCTTCTATGA* GCTTAACTCTTATCATGCTATC
FP2	*GAGATAACTTTCACAAACTGC* TTGACTTTCCTGAAAAATCGCC
TYRV-t ND	GAATTCACTTTCACTGGG
TYRV-s ND	CCC*GGATCC*TGTGGAAAATGTGGCTG
IY1	CCC*GAGGATCCATCC* ATGGCTACCGTTAGGG
IY2	CCC*GAGGATCC*AAATTAATTAT ATCTATCTTTCTTGG
J060	CAT *GGATCCTGCAG*AGCAATTGTGTCA
J064	CTTTGCTTTTCAGCACAGTGCA
CMVCP-5	CCCC*GGATCC*TGGTCTCCTT
CMVCP-1	CCCC*GGATCC*ACATCAYAGTTTTRAGRTTCAATTC
NS_SF	CCCC*GCGGCCGC*ATGTCTACCGTCAAAACA
NS_SR	CCCC*GCGGCCGC*TCACTGCACCTCTTCTAC

*Non-viral sequences (restriction enzymes, a*tt*b1-2 recombination sites and fusion-PCR overlapping stretches) included in the primers are shown in italics.

Transformation and regeneration of *N. benthamiana*

Transformation and regeneration of *in vitro* grown *N. benthamiana* were performed by the leaf disk method according to Horsch *et al.* (1995). Transgenic tobacco shoots, selected for resistance to kanamycin (100µg/ml), were rooted, potted in soil and transferred to the greenhouse. Forty lines of each construct were generated and from these, fifteen lines were challenged with TYRV-t by mechanical inoculation. Those lines showing a resistance phenotype were selected to produce seeds for the F_2 generation.

Testing transgenic lines for tospovirus resistance

Seeds of the F_2 progeny were plated on selective MS medium (4.4 g/l Murashige and Skoog including vitamins, 3% sucrose (w/v), pH 5.7-5.8 and 6 g/l agarose (Bucher *et al.*, 2006) containing kanamycin selection pressure (100 µg/ml). Young seedlings were transferred to pots and challenged by mechanical inoculation with TYRV-t, TYRV-s, TSWV, GRSV, TCSV or WSMoV individually. In co-inoculation studies, seedlings were challenged with TYRV-t in a combination with TYRV-s, *Iris yellow spot virus* (IYSV), *Impatiens necrotic spot virus* (INSV), *Cucumber mosaic virus* (CMV) or *Potato virus Y* (PVY). Plants were monitored for symptom expression on a daily basis. After 3-4 weeks plants were checked for the presence of tospoviruses by DAS-ELISA.

Infections with TYRV-t and/or TYRV-s were verified by RT-PCR on total RNA extracted from systemically infected leaves 14 days post-inoculation using Trizol agent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription-PCR was performed on 2 µg RNA using AMV Reverse Transcriptase (Promega, Corp. Madison, WI) and primers TYRV-t ND, TYRV-s ND, specific for TYRV-t and TYRV-s respectively, in combination with N-Stop (Table 4-1) rendering an N gene fragment of 220 nucleotides for TYRV-t and 496 nucleotides for TYRV-s respectively. IYSV, INSV and CMV infections were verified by RT-PCR in an analogous way, using species-specific primers as described in Table 4-1.

Detection of small interfering RNA

Total RNA was isolated from transgenic line 31.5 challenged with TYRV-t, followed by PEG precipitation (Bucher *et al*., 2004). Approximately 20 μg of total RNA was resolved on a 12% polyacrylamide gel containing 7 M urea and 0.5 M TBE buffer and subsequently transferred to Hybond N+ membrane (Amersham Biosciences UK Limited). The RNA was screened with probes specific for detection of TCSV and TYRV-t. Probes were about 150-160 bp in length, and digoxiginine labelled during PCR amplification using primers FP6 and FP2, and TYRV-IR-F and N-Stop, respectively, (Table 4-1) in the presence of NTP-digoxiginine (Roche Dignostics GmBH, Manheim, Germany). Pre-hybridisation and hybridisation was carried out in 5x SSC at 48℃. After overnight hybridisation, membranes were finally washed twice with 2x SSC and 0.2 % SDS at 48℃. Small interfering (si)RNA molecules were detected by chemiluminescence as described by Webster and Barker (1998).

PVX vector constructs

The NS_S gene of TYRV-s was RT-PCR amplified using primers NS_SF and NS_SR (Table 4-1), and subsequently cloned in the pGR106-I binary vector (kindly provided by Dr. David Baulcombe) containing a PVX replicon. Clones containing the sense orientation were transformed to *A. tumefaciens* strain GV3110 containing the pSoup helper plasmid. *A. tumefaciens* was infiltrated on *N. benthamiana* plants for transient expression of TYRV-s NS_S. Samples were taken from infiltrated leaves after 3-8 days post-infiltration (dpi) and analysed for the expression of NS_S by western immunoblotting using WSMoV NS_S monoclonal antibody (kindly provided by Drs. Shyi-Dong Yeh and Tsung-Chi Chen, Taiwan).

A. tumefaciens clone expressing TYRV-s NS_S protein was selected and used in *Agrobacterium tumefaciens* transient expression assay (ATTA) on TYRV-t resistant, transgenic lines prior to inoculation with TYRV-t at 2 dpi. Plants were monitored for symptom expression on a daily basis, and analysed by RT-PCR for the rescuing and systemic spread of TYRV-t.

Tandem Mass Spectrometry of RNP-derived nucleoprotein

Mass spectrometry (MS) was performed on tospoviral N protein excised from Coomassie Brilliant Blue stained SDS-PAGE gels loaded with purified ribonucleoprotein (RNP)-preparations (de Ávila *et al.*, 1990). In-gel proteolytic digestion of simply-blue-stained protein was performed essentially as described (Lebbink *et al.*, 2006) using trypsin (Roche Diagnostics, Penzberg, Germany). Samples were subjected to nanoflow liquid (LC) chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100 µm ID, 2 cm). Peptides were separated on an analytical column (75 µm ID, 20 cm) at a flow rate of 200 nl/min with a 60 min. linear acetonitrile gradient from 0 to 80%. The LC system was directly coupled to a QTOF Micro tandem mass spectrometer (Micromass Waters, UK). A survey scan was performed from 400-1200 amu s^{-1} and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches using Proteinlynx Global Server version 2.1 (Micromass, UK) or MASCOT software (Matrixscience) against SWISSPROT and the NCBI non-redundant database, with a 0.5 Da mass tolerance for both precursor ion and fragment ion. The identified peptides were confirmed by manual interpretation of the spectra.

Results

Performance of an extended tospoviral transgene cassette

Previously chimaeric inverted repeat transgene constructs with N gene sequences from four different tomato-infecting tospoviruses have been generated and shown to confer resistance to these viruses in *N. benthamiana* (Bucher *et al.*, 2006). The resistance was shown to be based on RNA silencing of incoming viral RNA target sequences, induced by hairpin RNA molecules *in planta* transcribed from these transgene cassettes. To extend the resistance spectrum to a fifth tomato-infecting tospovirus, TYRV (Chapter 2 and Hassani-Mehraban *et al.* 2005), the chimaeric N gene cassettes were provided with a

partial N gene sequence of TYRV-t. Four constructs were made in which the 5 N gene sequences were cloned in different orientations (IN *versus* OUT) as inverted repeats separated by an intron sequence in sense (INTs) or antisense (INTas) polarity (Figure 4-1). For each of these 4 constructs at least 15 independent transgenic *N. benthamiana* lines were generated and tested for resistance by mechanical inoculation with TYRV-t. Transformed lines containing the N gene RNAi cassette with a sense intron sequence (IR-OUT-INTs and IR-IN-INTs; Figure 4-1 and Table 4-2) already showed high levels (up to 67-73%) of resistance in the F_1 generation, whereas no resistance levels were obtained when the intron sequence was present in antisense orientation. From the lines containing an N gene RNAi cassette with a sense oriented intron sequence, the F_2 progeny showed 100% resistance to all 5 tospoviruses (Table 4-2). Of these, line 31, containing an IR-IN-INTs construct, was used for further analysis.

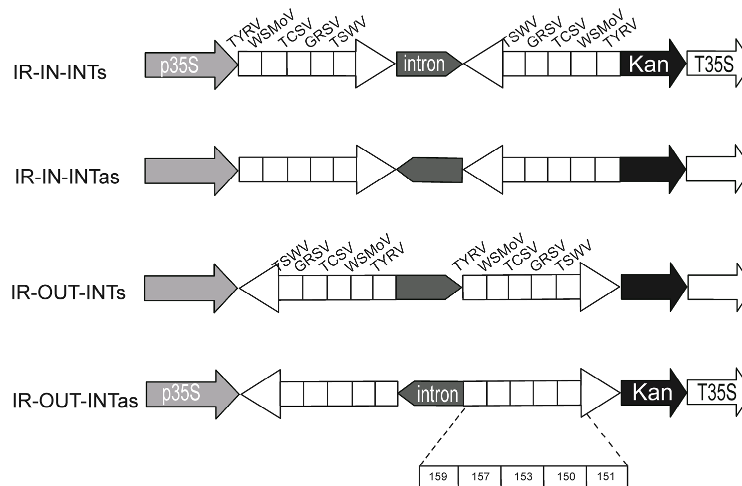


Figure 4-1: Schematic presentation of the inverted repeat N gene cassette constructs used for transformation of *N. benthamiana*. Fragments approximately 150 nts in length, specific for the N gene of TSWV (nt position 477-627 relative to the ATG start codon at +1), GRSV (nt position 327-476), TCSV (nt position 157-311), WSMoV (nt position 4-160) and TYRV-t (nt position 667-835) were PCR fused and cloned as inverted repeat (IR) flanking an intron (IN) sequence in various orientations as depicted.

Table 4-2: TYRV-t resistance levels in F_1 and F_2 progeny of transgenic *Nicotiana benthamiana* lines

Line (Construct)	#Resistant F1 lines* / #F1 Lines tested	Resistant Plants (%)	
		F1	F2
IR-OUT-INTs	11/15	73	100
IR-IN-INTs	10/15	67	100
IR-IN-INTas	0/15	0	0
IR-OUT-INTas	0/15	0	0

* Indicating that all 10 plants for each line tested were resistant to TYRV-t.

The transgenic resistance is based on RNA silencing

Analysis of siRNAs in transgenic lines containing a multiple N gene cassette demonstrated that the resistance was based on RNA silencing (Bucher *et al*, 2006). To confirm that this also applied for TYRV-t in transgenic plants containing the extended tospoviral N gene cassette, transgenic TYRV-resistant plants were tested for the presence of TYRV-t specific siRNAs. To this end, total RNA was purified from leaves nonchallenged of F_2 line 31.5 and leaves inoculated with TYRV-t and/or TYRV-s, and subsequently examined by Northern blot analysis for the presence of siRNAs. Indeed, TYRV N gene specific siRNAs were present in line 31.5, co-migrating with those derived from TCSV and with a 21-nt siRNA size marker (Figure 4-2), while no siRNA was detectable in non-transgenic *N. benthamiana*. This indicated that the obtained resistant phenotype was also for TYRV based on RNA silencing.

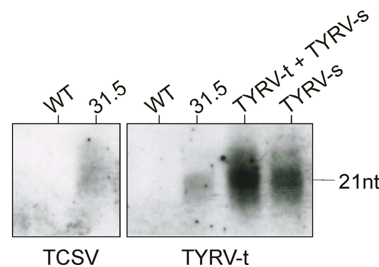


Figure 4-2: Northern blot analysis of siRNAs purified from transgenic resistant line 31.5 prior to, or after challenging with TYRV-t. Hybridisation was performed with a digoxiginine labelled RT-PCR probe specific for TCSV (left panel) or TYRV-t (right panel). The position of a 21nt siRNA size marker is indicated at the right.

Rescuing of TYRV-t by mix-inoculation with TYRV-s

Previous studies have shown that transgenic resistant plants expressing (partial) TSWV N gene sequences are only resistant to strains and isolates of the homologous virus but not to other, closely related tospoviruses (de Haan *et al*. 1992; Gielen *et al*. 1991; Prins *et al*., 1995). Recently, we have reported on the occurrence of two distinct strains of TYRV in Iran, i.e. TYRV-s in soybean along with TYRV-t in tomato, differing in several biological parameters and showing 14.4% sequence diversity of their N gene (Chapter 3 and Hassani-Mehraban *et al*., 2006). TYRV-t resistant line 31.5 appeared susceptible to TYRV-s (Figure 4-3), indicating that a sequence divergence of 14.4% is sufficient to overcome the RNAi-mediated resistance.

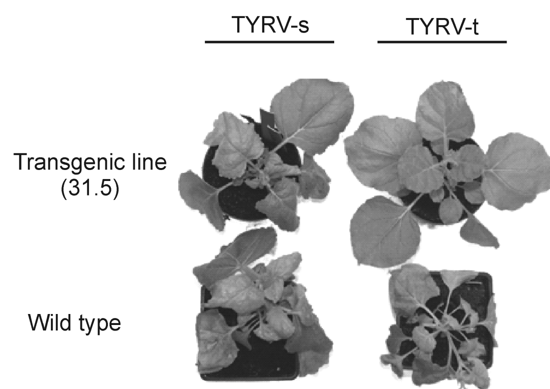


Figure 4-3: Symptomatology on wild type and transgenic, TYRV-t resistant *N. benthamiana* plants upon mechanical inoculation with TYRV-s and TYRV-t.

Even more surprisingly, when co-inoculated with TYRV-s, TYRV-t was able to systemically infect line 31.5 as demonstrated by RT-PCR detection (Figure 4-4A). This rescue phenomenon was not observed when TRYV-t was co-inoculated with two more distantly related tospoviruses, i.e. IYSV (24.3% N gene sequence divergence to TYRV-t) or INSV (52.1%) (Figure 4-4B and C), or with non-related viruses CMV (Figure 4-4D) and PVY (data not shown).

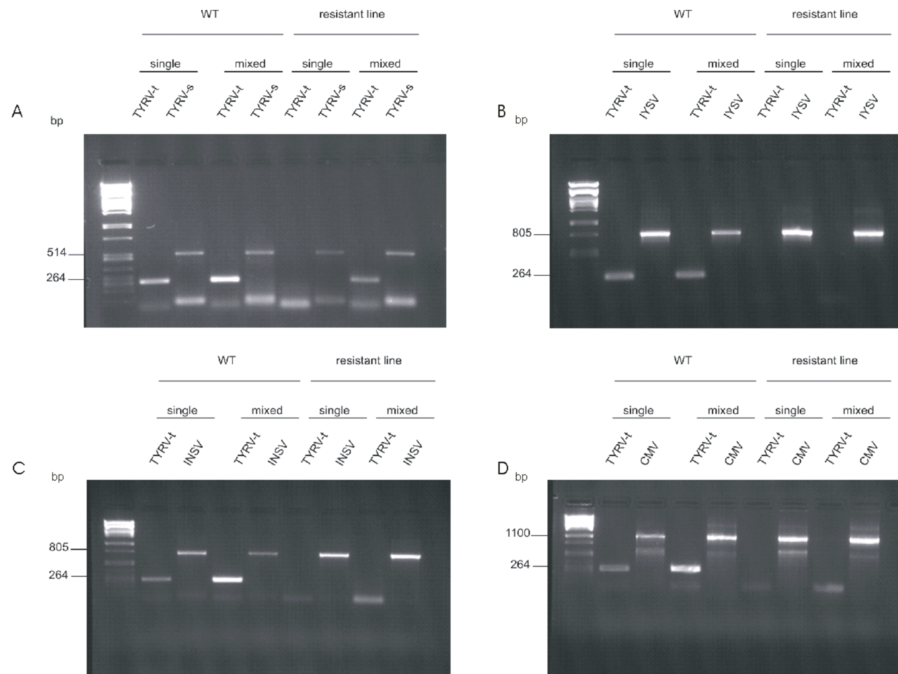


Figure 4-4: RT-PCR detection of TYRV-t in transgenic resistant lines upon single and mixed inoculation with other *Tospovirus* species or CMV. As size marker *Pst*I-digested λ DNA is included at the left of each gel.

Rescuing of TYRV-t by TYRV-s is not based on hetero-encapsidation

The observation that TYRV-t could be rescued in transgenic resistant lines by TYRV-s implied a trans-complementation event involving a protein from TYRV-s. Since the N protein gene of TYRV-s was not silenced in line 31.5, it was tempting to assume that the TYRV-s N protein was able to hetero-encapsidate the TYRV-t genome, thus supporting its replication and systemic spread.

To test this possibility, non-transformed and resistant (line 31.5) *N. benthamiana* were inoculated singly or mixed with TYRV-s and TYRV-t and analysed for the presence of N protein of either strain. Once leaves were systemically infected, these were harvested for the purification of viral ribonucleoproteins (RNPs), after first having verified the presence of both strains

by RT-PCR. Purified RNP preparations were resolved on SDS-PAGE, N protein excised from the gels, trypsin cleaved and subsequently analysed by Mass Spectrometry (MS). Analysis of TYRV-t and TYRV-s N protein from the non-transformed, singly infected control plants showed distinct peptide profiles and thus allowing the use of MS for distinguishing TYRV-t and -s N proteins, even in mixtures (Table 4-3).

Table 4-3: Mass spectrometry data obtained from RNP-derived N protein purified from TYRV-s and TYRV-t (singly) infected wild type *Nicotiana benthamiana* and a mixed infection of transgenic line 31.5

RNP sample	Unique peptide*	Position	m/z	Charge state [M+H]+	Mascot score	TYRV-t + TYRV-s
TYRV-s	K.MFMELIAVENV**A**EQQK.M	104-119	942.35	2+	96	x
	K.LCELPMV**S**AYGLKPS**S**K.F	124-140	639.6	3+	101	x
	K.ILNDCTPG**A**AGAASLNK.F	222-238	851.79	2+	108	x
	K.ALES**T**FGK.I	245-252	411.68	2+	45	x
TYRV-t	K.MFMELIAVENA**S**EQQK.M	104-119	948.34	2+	101	x
	K.LCELPMV**N**AYGLKPS**P**K.F	124-140	627.25	3+	97	x
	K.ILNDCTPG**T**AGAASLNK.F	222-238	836.85	2+	126	x
	K.ALES**A**FGK.I	245-252	426.68	2+	47	x

* The tomato and soybean strain-specific peptides, their charge state, mass and MASCOT score (see methods) as identified by Tandem MS/MS are shown with the distinguishing amino acids indicated in bold.

Nucleoprotein purified from RNP preparations of a mixed infection of line 31.5 demonstrated the presence of TYRV-t specific N protein peptides along with those of TYRV-t N peptides (Table 4-3 last column, Figure 4-5), ruling out hetero-encapsidation as rescue mechanism.

TYRV-t is rescued by TYRV-s NS_S protein

The presence of TYRV-t N protein during a mixed infection of line 31.5 with TYRV-s indicated that the TYRV-t N gene transcript was not silenced anymore. This suggested that the presence of the TYRV-s encoded silencing suppressor (NS_S) was crucial for rescuing TYRV-t. To test this possibility the resistant line 31.5 was co-inoculated with TYRV-t and a PVX vector expressing the TYRV-s NS_s protein. As the timing of TYRV-s NS_S expression from PVX was thought to be quite important, a time course experiment was performed in which line 31.5 plants were infiltrated with PVX-NS_S (from TYRV-s) at day 0, and infected with TYRV-t two days later. Using RT-PCR, the presence of TYRV-t was monitored in

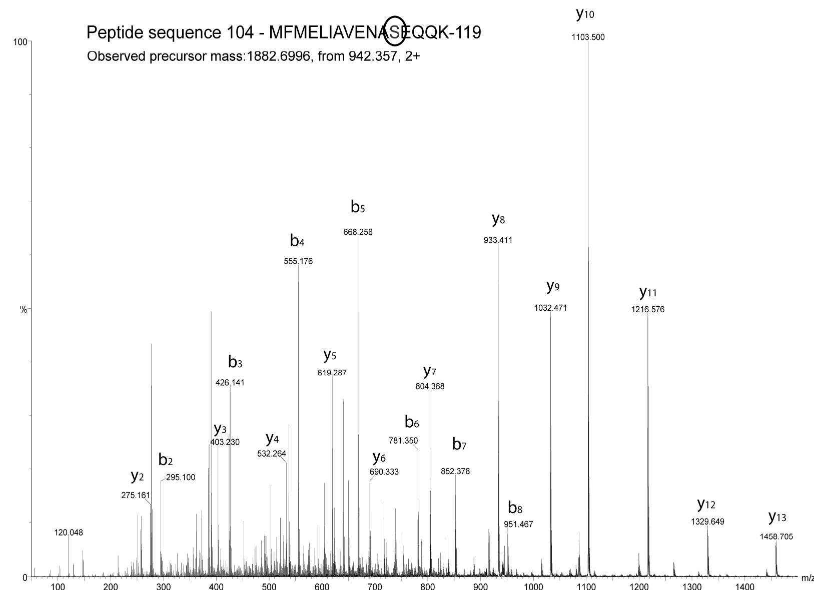


Figure 4-5: Mass Spectrometric analysis of RNP-derived N protein from TYRV-t resistant transgenic line 31.5 co-infected with TYRV-t and TYRV-s. Representative MS/MS sequence of a TYRV-t specific peptide spanning amino acids 222-238, as identified by MASCOT software. b and y ions are shown. The distinctive t-strain specific Serine at position 230 is shown within the circle.

the primary and systemically-infected leaves. In repeated inoculation experiments it could thus be demonstrated that TYRV-t was rescued when plants were pre-inoculated with PVX-NS_S.

Time course analysis showed that in this case the rescuing of TYRV-t occurred transiently, between 4-6 dpi or 2-4 days post-inoculation (Figure 4-6A). Western blot analysis of inoculated and systemic leaves revealed the absence of NS_S from the systemic leaves (data not shown), and its transient presence in the inoculated leaf during the timespan that TYRV-t was rescued (Figure 4-6B). Control experiments in which TYRV-t and empty PVX vector were co-inoculated on 31.5 were negative, as expected (data not shown).

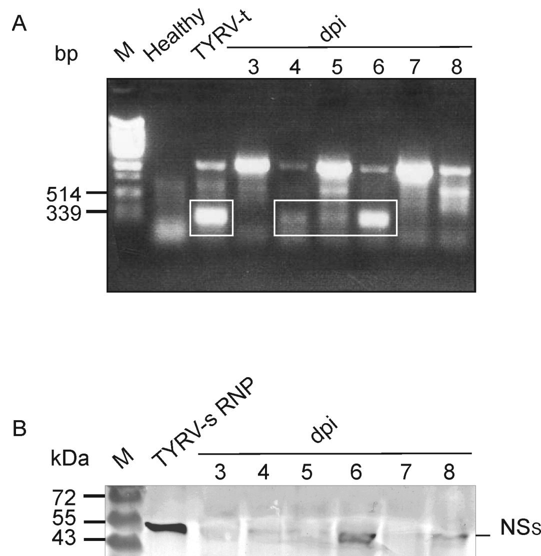


Figure 4-6. Time-course RT-PCR detection of TYRV-t during local infection of transgenic line 31.5 trans-complemented with TYRV-s NS_S protein expressed from PVX (A). As controls, total RNA purified from mock-infected (lane Healthy) and TYRV-t infected *N. benthamiana* (lane TYRV-t) were included. In the first lane (M) Lambda DNA digested with *Pst*I has been included as size marker. Leaves were infiltrated with pGR106-I-NS_S at day 0, and inoculated with TYRV-t 2 days later. At day 3 post-infiltration, local leaves were harvested each day for analysis up to 8 days post-infiltration (dpi). B) Leaf material from the samples analysed in A, were simultaneously processed for Western blot analysis to screen for the presence of NS_S. Western blots were probed with a monoclonal antiserum specific to NS_S from the Eurasian tospovirus species.

Discussion

Previously it was shown that plants, transformed with a hairpin construct containing partial N gene sequences from 4 tospovirus species (TSWV, GRSV, TCSV and WSMoV) are resistant to all of them, and that the underlying mechanism is based on Post Transcriptional Gene Silencing (PTGS, also referred to as RNAi) (Bucher *et al.* 2006). In the present work this chimaeric N gene cassette has been extended with a partial N gene sequence of a fifth, tomato-infecting tospovirus from Iran, TYRV, and successfully shown to broaden the resistance spectrum to 5 different tospoviruses after transformation to *N. benthamiana*. Four different inverted repeat constructs, spaced by an intron sequence, were made and - in case of a sense intron insert - shown to generate

a high frequency of resistance, up to 73%, already in the F_1 progeny. Resistance was shown to be based on RNA silencing of target sequences. When this N gene cassette was provided with the intron insert in reverse orientation the resistance frequency of the transgenic plants obtained was dramatically lower, implying that intron orientation did matter and contrasting earlier data from Heilersig *et al.* (2006). Interestingly, the resistance against TYRV-t did not hold against another strain of TYRV (TYRV-s), found in soybean and potato, again stressing the high sequence specificity of RNA-mediated resistance (Baulcombe, 1999; de Haan *et al*., 1992; Gielen *et al*., 1991; Goodwin *et al*., 1996; Prins *et al*., 1996). More surprisingly, however, was the observation that during a mixed (TYRV-t and TYRV-s) inoculation also strain TYRV-t was able to systemically infect the transgenic, resistant plants. The presence of TYRV-t N protein in these plants, as confirmed by mass spectrometry, indicated that TYRV-t N gene transcripts were not silenced anymore and that TYRV-t genome replication and systemic spread in the resistant transgenics was not due to hetero-encapsidation by TYRV-s N protein. Based on these observations it was most likely that suppression of silencing was caused by the NS_S protein expressed from TYRV-s. This was confirmed by the observation that TYRV-t also became rescued from resistant plants by trans-complementation with the NS_S protein of TYRV-s expressed from a PVX replicon. Since TSWV NS_S has recently been shown to exhibit affinity for siRNAs and longer dsRNAs (precursors to miRNAs; Hemmes, 2007) it is likely that the rescuing of TYRV-t is due to a reduction in TYRV-t sensing RISC complexes in the transgenics. Rescuing of TYRV-t was only transient and restricted to the inoculated leaf when NS_s was provided using a PVX vector, the absence of systemic rescue being probably due to the intrinsic instability of the PVX-vector construct (Lacorte, 2006; Uhde *et al*., 2005; Ziegler *et al*., 2000). Indeed, western immunoblot experiments to detect NS_S systemically were always negative, whereas the protein was easily detected in the inoculated leaf (results not shown). Furthermore, a PVX-based vector expressing NS_S of TSWV was shown to render a mixture of PVX molecules with intact, partially or lost NS_S ORF in the progeny (Sonoda & Tsumuki 2004), a

phenomenon also assumed to result from vector instability (Avesani *et al.*, 2007). Next to this, a decrease of NS_S expression may have been caused by silencing of the PVX replicon through siRNAs generated after TYRV-t replication.

RNA-mediated resistance is highly sequence specific and as a result limited to certain virus species and their strains (de Haan *et al.*, 1992; Gielen *et al.*, 1991; Prins *et al.*, 2008). Here we have successfully exploited this strategy and extended an available RNAi N gene cassette with another tospovirus N gene sequence to generate operational resistance against 5 different tomato-infecting tospoviruses. However, for TYRV it is also shown that the transgenic resistance is limited to the tomato strain, which, moreover, can overcome the resistance when the soybean strain would co-infect the host plant. Although this is a rather unlikely scenario in practice, as both strains seem to exclude each other (Chapter 3), our experiments indicate that care should be taken for the application of RNAi-mediated transgenic resistance when significant intraspecific sequence variation occurs.

Acknowledgements

The authors wish to thank Dr. Etienne Bucher (University of Geneva, Geneva, Switzerland) and Dick Lohuis for their help in the construction of the N gene cassettes and siRNA purification, respectively, Janneke Saaijer, Henk Smid and Bert Essenstam for their assistance in the inoculation and maintenance of plants in the greenhouse. We thank Dr. David Baulcombe for providing PVX vector. WSMoV NS_S monoclonal antibody was kindly provided by Drs Shyi-Dong Yeh and Tsung-Chi Chen, National Chung Hsing University, Taiwan. We also wish to thank Dr. René van der Vlugt (Wageningen University and Research Centre, Wageningen, The Netherlands) for supplying PVY inoculum. This work was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality and carried out according to Dutch law under license GGO 02-104.ab.

Chapter 5

Assessing the intergenic hairpin structure of *Tomato yellow ring virus* S RNA as target and inducer for RNAi

This chapter will be submitted in a slightly modified version as:
Hassani-Mehraban, A., Lohuis, D., Hemmes, H., Goldbach, R. & Kormelink, R. " Assessing the intergenic hairpin structure of *Tomato yellow ring virus* S RNA as target and inducer for RNAi".

Summary

Tomato yellow ring virus (TYRV) infected *Nicotiana benthamiana* plants produce virus-derived siRNAs which map to almost all regions within the viral S RNA segment but particularly to the start of the NS_S open reading frame where a short stem-loop structure is predicted. Remarkably, hardly any siRNA molecules were generated from the intergenic region (IGR) in the S RNA that is predicted to fold into a stable hairpin structure with long stretches of double stranded RNA. Synthetic transcripts containing the viral IGR hairpin were *in vitro* efficiently cleaved into small RNA molecules by the RNase-III enzyme dicer from *Drosophila melanogaster* embryo extract. Furthermore, *Agrobacterium tumefaciens* mediated transient expression assays (ATTAs) of an YFP gene in *Nicotiana benthamiana* showed a stronger silencing of YFP fluorescence when this gene was provided with the TYRV IGR hairpin sequence. Suppression of YFP gene silencing by the tospoviral RNAi suppressor NS_S was also more difficult when the hairpin-containing sequence was present. Hence, although the long IGR hairpin of TYRV S RNA is a potent inducer of RNA silencing in several assays, this is not the case during a genuine virus infection of the host plant. Reasons for this apparent discrepancy and the occurrence of an siRNA-inducing hotspot near the NS_s gene in S RNA will be discussed.

Introduction

RNA silencing, also named post transcriptional gene silencing (PTGS) or RNA interference (RNAi), is a conserved cellular mechanism in plants and animals in which double-stranded (ds)RNA and imperfect hairpin RNAs trigger a chain of processes leading to sequence-specific RNA degradation. During this process, dsRNA is processed into small interfering RNAs (siRNAs) or microRNAs (miRNAs) of 21-26 nucleotides in length by RNase-III-type enzymes called Dicer (DCL) (Fire *et al*, 1998; Bernstein *et al*., 2001; Lee *et al*., 2003). One strand of the RNA duplex, the so-called guide strand, is incorporated into the RNA-induced silencing complex (RISC) based on thermodynamic stabilities at the two ends (Schwarz *et al*., 2003; Khvorova *et al*., 2003). The RISC complex,

being activated with the guide strand and a member of the Argonaute (Ago) protein family, continuously mediates recognition and subsequent cleavage of (m)RNA target sequences with complementarity to the guide RNA, leading to endogenous- or transgene silencing.

Plant viruses also induce RNA silencing often referred to as Virus-Induced Gene Silencing (VIGS), as can be observed by the generation of viral specific siRNA molecules during the infection process. To escape from this antiviral defence mechanism viruses have developed ways to counteract or evade from this. One way that has been postulated for viruses to evade from RNA silencing is by inducing membrane cavities to replicate in (e.g. *Bromo Mosaic virus*) and thereby avoiding exposure of viral dsRNA molecules to dicer (Schwartz *et al.*, 2002; Tijsterman & Plasterk, 2004; Voinnet, 2005). Many plant viruses, though, encode proteins that are able to suppress RNA silencing by direct interference in the cascade of reactions that eventually leads to viral RNA degradation. Some suppressors have been shown to inhibit silencing by sequestering siRNAs (NS3, NS_S, P19) thus preventing their incorporation into RISC, whereas others avoid cleavage of dsRNA into siRNAs (HC-Pro), systemic transport of siRNAs (2b) or combinations of these (Diaz-Pendon *et al.*, 2007; Goto *et al.*, 2007; Hemmes *et al.*, 2007; Hui & Shou, 2002; Lakatos *et al.*, 2004 and 2006; Llave *et al.*, 2000; Mallory *et al.*, 2002; Zhang *et al.*, 2006).

In contrast to the increasing insight into the working mechanisms of plant viral suppressor proteins, hardly any information is available on the origin of dsRNA molecules that induce VIGS. For RNA viruses it is generally assumed that ds replicative intermediates play a role in this. Cloning and sequence analysis of siRNAs from *Cymbidium ringspot tombusvirus* infections have revealed more siRNAs from the (+) strand than the (-) strand and that many of these mapped to a region within the genomic RNA containing intramolecular hairpin structures (Szittya *et al.*, 2002; Molnár *et al.*, 2005).

In plants silencing requires an amplification step involving a host RNA-dependent RNA polymerase (RDR) and this may occur in two ways. In the first way, primary siRNAs recruit RDR to homologous RNA molecules that serve as

template for the generation of complementary RNA, and thereby generate dsRNA from which secondary siRNAs are synthesised. In the second way, aberrant RNA molecules that arise as incomplete viral transcripts or resulting from RISC-mediated RNA target cleavage are recognised by RDR independent from primary siRNAs, and used as template to generate dsRNA. The amplification not only results in the production of secondary siRNAs identical to the dsRNA inducer sequence but also to the adjacent regions of target mRNA. This phenomenon of silencing spreading along the entire mRNA target sequence is referred to as transitive RNA silencing (Sijen *et al.*, 2001).

Tospoviruses are the plant-infecting members of the arthropod-borne *Bunyaviridae* that primarily consists of animal infecting viruses (Elliott, 1991). They are transmitted by a limited number of thrips species (Thysanoptera: *Thripidae*) in a propagative manner (Wijkamp *et al.*, 1993). Tospoviruses have a tripartite ssRNA genome, of which the S and M RNA segment are ambisense and the L RNA negative-stranded. The S RNA segment codes for the suppressor of silencing (NS_S) in viral (v) sense and nucleocapsid protein (N) in viral complementary (vc) sense. The M RNA codes for the movement protein (NS_M) in (v) sense and precursor to the glycoproteins (G_N and G_C) in vc sense. The L RNA codes for the viral RNA-dependent RNA polymerase protein (L) (de Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992a, 1994; Storms *et al.*, 1995; Bucher *et al.*, 2003; Takeda 2002). The open reading frames (ORFs) within the S and M RNA segments are separated by a non-coding intergenic region (IGR) predicted to fold into a stable hairpin structure. Both ORFs become expressed by the synthesis of subgenomic length mRNA molecules that do not possess a poly(A)-tail (Kormelink *et al.*, 1992b) but contain the hairpin-encoding sequence at their 3'-end (van Knippenberg *et al.*, 2005).

Here, the IGR hairpin sequence of the tospoviral S RNA has been further investigated as a potential target and inducer of RNA silencing. Whilst it is known form previous work (Prins *et al.* 1996 and 1997), that the tospoviral transcripts rather than the (N protein encapsidated) genome is targeted by antiviral RNA silencing, the inclusion of the IGR hairpin in the viral transcript (van

Knippenberg *et al*., 2005) makes this structure extremely relevant in relation to the RNA silencing machinery. The experiments reported in this chapter were performed on the IGR hairpin of *Tomato yellow ring virus* (TYRV), whose S RNA segment has been sequenced (Chapter 2) and contains a predicted IGR hairpin structure with long stretches (30-40 nts) of almost full complementarity (Figure 5-1A; and Chapter 2). A similar structure, but with smaller stretches of full complementarity, has been predicted for the S RNA IGR sequence of *Tomato spotted wilt virus* (TSWV) S RNA (Figure 5-1B). The results obtained show that although in several assays the TYRV IGR hairpin represents a potent inducer of RNA silencing, this is not reflected by the population of S RNA derived siRNAs produced in virus-infected plants.

Materials and Methods

Purification and mapping of siRNAs

Low-molecular-weight (LMW) RNA molecules were purified by PEG precipitation from total RNA preparations of healthy and TYRV-t infected *N. benthamiana* plants (Bucher *et al*, 2004; Ribeiro *et al*., 2006). For the purification of siRNAs, thirty µg of LMW RNA was resolved on a 15% denaturing polyacrylamide gel containing 8M urea. After ethidium bromide staining, the region containing siRNAs was excised from the gel, ground to small pieces and incubated in 3 M NaCl overnight at 4˚C to extract the siRNAs by diffusion. After centrifugation, the supernatant was collected and the siRNAs precipitated by ethanol. Small interfering RNA molecules were dephosphorylated with alkaline phosphatase and subsequently labelled with γ-^{32}P ATP by T4 polynucleotide kinase according to the manufacture's instructions (PerkinElmer Inc., UK).

The S RNA segment of TYRV-t was PCR-amplified in 7 fragments spanning the entire S RNA segment using specific primer sets (Table 5-1). The fragments obtained were cloned in pGEM-T Easy (Promega Corp., Madison, WI) and verified by sequence analysis. Equimolar amounts of DNA from these fragments were resolved on 1% agarose gel and subsequently blotted to Hybond-N

membrane (Amersham Biosciences Limited UK) by top-down blotting. Filters were subsequently hybridised overnight to γ-^{32}P-labelled siRNAs in Church buffer at 48˚C (Sambrook *et al.*, 1989). After filters were washed, radioactive signals were exposed to a phosphor screen and visualised by phospoimaging (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences).

Table 5-1: Primers used to amplify the TYRV-t S RNA segment, TYRV-t and TSWV intergenic hairpin transcripts and YFP and YFP-Hp fragments

Primer	Sequence* 5´ to 3´	Nucleotide position of fragment / fragment size (bp)
VT-2	AGAGCAATCGACGTATAAAC	1-577
NS-1R	GGCTGAACCCAGAAACATCAGC	/ -
NS-1F	GCTGATGTTTCTGGGTTCAGCC	577-1097
NS-2R	GCGCAATTATCTTGTTGTAGTTCGG	/ -
NS-2F	CCGAACTACAACAAGATAATTGCGC	1097-1642
NS-3R	CTGACTTACATACCTTAC	/ -
IR-1	GTAAGGTATGTAAGTCAG	1642-2020
IR-2	GTGTGCCATATAGTTTATCAG	/ -
N-3F	CTGATAAACTATATGGCACAC	2020-2363
N-3R	CACTTTCACTGGGGTCTCC	/ -
N-2F	GGAGACCCCAGTGAAAGTG	2363-2702
N-2R	CAGGAGGTTGGAAGGCTTC	/ -
N-1F	GAAGCCTTCCAACCTCCTG	2702-3061
N-1R	AGAGCAATCGAGGTATAACAC	/ -
VT-2	AGAGCAATCGACGTATAAAC	1-142
NS-1aR	CTAGAACGAGAGTCACAGG	/-
NS-1aF	CAACGACTGCTATAGGA	142-284
NS-2aR	AATCTCTGCTTCATTAGC	/-
NS-2aF	ATTGATGAGCATCACTATT	284-426
NS-3aR	CAAATATCTGATTGTGC	/-
NS-3aF	AACCTTCAACACAACTG	426-588
NS-1R	GGCTGAACCCAGAAACATCAGC	/-
T7-TS-IR	TAATACGACTCACTATAGGGT CTCTGTTTGTCATCTCTTTC	-
TS-IR-2	GCTTTCTGTGTGTTGTGC	/342
T7-TY-IR	TAATACGACTCACTATAGGGT AAGGTATGTAAGTCAG	-
TY-IR-2	GTGCCATATAGTTTATCAG	/ 418
P-Gate-YFP-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTGAGCAAGGGCGAG	-
P-Gate-YFP-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTGTACAGCTCGTC	/720
P-Gate-YFP-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTGAGCAAGGGCGAG	-
P-Gate-YFP-Hp	GGGGACCACTTTGTACAAGAAAGCTGGGTGTAAGGTATGTAAGTCAG	/1117

* The sequences corresponding to T7 promoter and *att*B1-2 recombination sites are underlined.

Folding predictions for the S RNA intergenic hairpin sequence

Folding predictions were performed at 37˚C, using Mfold (Zuker, 2003; Mathews *et al.*, 1999; Rensselaer Polytechnic Institute; http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). The TYRV-t S RNA sequence (Chapter 2 and Hassani-Mehraban *et al.*, 2005) from residues 1634 to 2030, spanning the core region of the non-coding IGR, was used as input for Mfold. For TSWV S RNA (de Haan *et al.*, 1990), the analogous region, running from residue 1549 to 1869 was used.

Synthesis of ^{32}P-radiolabelled hairpin RNA substrate

DNA templates of the hairpin encoding sequence containing the T7 promotor sequence were made by RT-PCR amplification using primers T7-IR-1 and IR-2 (Table 5-1). Prior to transcription, PCR fragments were purified by phenol-chloroform extraction and ethanol precipitation. Radiolabelled RNA transcripts were made by T7 RNA polymerase (Promega Corp., Madison, WI) and α^{-32}P rCTP (PerkinElmer Inc., UK) according to protocol using a gel purified (High Pure PCR purification kit; Roche, Germany) PCR template. The RNA molecules were allowed to fold into a hairpin structure by heating for 10min. at 85°C and slowly allowing the RNA to cool down to room temperature. Products from the *in vitro* transcription reaction were resolved on an 8% denaturing acrylamide gel and the radiolabelled hairpin-sequence encoding RNA molecules excised from the gel. RNA was extracted by diffusion into 2x PK buffer (200 mM Tris pH 7.5, 300 mM NaCl, 5 mM EDTA, 2% SDS) followed by phenol-chloroform and ethanol precipitation. The concentration of the transcript was measured prior to use in Dicer cleavage assays.

Drosophila melanogaster based-dicer cleavage assays

A *Drosophila* RNA silencing system (Benjamin *et al.* 2003) was used for the cleavage of dsRNA templates by RNase-III like enzyme Dicer. To this end, embryo extracts were prepared according to Haley *et al.* (2003). Dicer cleavage assays were performed according to Lakatos *et al.* (2006) in a final volume of

10 µl containing 5 µl *Drosophila* embryo extract, 3 µl reaction mix (mix consisted of 40 µl 5x lysis buffer (LB) without KAc (5x LB: 500 mM KAc, 10 mM MgAc, 150 mM HEPES-KOH pH 7.5) , 2 µl 1 M DDT, 4 µl 100 mM ATP, 0.6 µl creatine kinase (20 mg/ml stock), 20 µl creatine phosphate (0.012 g/100 µl), 40 µl 100% glycerol, 4 µl RNase inhibitor [Promega Corp., Madison, WI] and 9.4 µl H_2O) 1 µl H_2O and 1 µl ^{32}P-labelled hairpin transcript (approximately 50 pmol/µl) and incubated for 2-3 hours at 30˚C. After incubation, 200 µl 2x PK buffer (200 mM Tris-HCl pH7.5, 300 mM NaCl, 5 mM EDTA, 2% SDS), 1 µl glycogen (10 mg/ml) and 0.3 µl proteinase K (10 mg/ml) were added and the mixture incubated for 10 minutes at 65˚C. After phenol-chloroform-isoamyl-alcohol extraction the RNA was precipitated by ethanol, washed and subsequently resuspended in 1x FDE loading buffer (2x FDE: 10 ml de-ionised formamide, 10 mg xylene cyanol, 10 mg bromphenolblue, 200 µl 0.5 mM EDTA pH 8.0) followed by denaturing for 5 minutes at 65˚C. Green fluoerescent protein (GFP) small interfering RNAs (21 nucleotides) were radiolabelled by T4 polynucleotide kinase using γ-^{32}P ATP (PerkinsElmer Inc., UK) and used as a size marker for the analysis of Dicer cleavage products. The sample was resolved on an 8% denaturing acrylamide gel. After electrophoresis, the gel was dried for 30 minutes at 80˚C using a SGD 4050 slab gel dryer (Savant, Farmingdale, NY, USA), followed by overnight exposure to a phosphor imager screen.

Electrophorectic mobility shift assay

Protein samples for use in gel retardation of the RNA hairpin sequence were prepared from infected plant extracts according to Mérai and co-workers (2006) with some modifications (Hemmes, 2007). In brief, one gram of systemically-infected plant material was ground in liquid nitrogen and 2 ml extraction buffer (20mM Tris-HCl pH 7.5, 5mM MgCl2, 50mM KCl and 1mM DDT), followed by two centrifugation steps at 15,000 g for 15 min. at 4℃ to remove crude cellular material. Supernatant was transferred to a new tube and stocked in small aliquots at -80°C prior to use. Total protein concentration of plant extracts was estimated by means of the Bio-Rad protein assay. Synthetic

RNA transcripts (0.5 ng/µl) and folded into dsRNA molecules were incubated with plant extracts (approx. 2 µg) from either healthy, TYRV, PVX-NS_S- or PVX-infected *N. benthamiana* in a final volume of 10 µl for 20 min. at room temperature. Reaction products were loaded on a 5% non-denaturing acryl/bisacrylamide gel and run at 150V at 4^oC for 2h. Labelled GFP siRNAs (21 nucleotides) were included as a size marker. After electrophoresis, the gel was dried and exposed to a phosphor-imager screen.

Agrobacterium tumefaciens mediated transient expression of YFP-hairpin constructs *in planta*

The inducer of silencing capacity of functional gene constructs was analysed using the *Agrobacterium tumefaciens* transient expression assay (ATTA) system as described by Johansen and Carrington (2001). To monitor the effect of the hairpin sequence on the induction of silencing of a functional yellow fluorescent protein (YFP) gene construct, the hairpin-encoding sequence was fused to the C terminus of YFP and subsequently amplified by primers P-GATE-YFP-F/ P-GATE-YFP-Hp. YFP was amplified using P-GATE-YFP-F/ P-GATE-YFP-R (Table 5-1). Amplified YFP-Hp and YFP fragments were cloned in binary vector pK2GW7 (Karimi *et al.*, 2002) using the Gateway^{TM} Cloning Technology (Invitrogen). For suppression of silencing the NS_S of TYRV-t was cloned in pK2GW7. Binary vector constructs were transformed to *Agrobacterium tumefaciens* strain GV3101 and cultured in 3 ml LB3 medium containing antibiotics (Spectinomycin 250 µg/ml; Rifampicin 20 µg/ml) at 28˚C overnight in the dark. From the overnight culture 600 µl was added to 3 ml induction medium (K_2HPO_4 10.5 g; KH_2PO_4 4.5 g; $(NH_4)_2SO_4$ 1.0 g; Na-citrate 0.5 g; mM $MgSO_4$; 0.2 % (w/v) glucose; 0.5 % (v/v) glycerol; 150 µM acetosyringone; 10 mM MES pH 5.6) and grown at 28˚C overnight. The induced culture was centrifuged for 10 minutes at 3300 rpm and resuspended in MS (30g/l sucrose; 40g/l MS pH 5.7) containing 150 µM acetosyringone and 10 mM MES to an OD_{600} of 0.5. This suspension was used to infiltrate leaves of *N. benthamiana* plants at the 5-6 leaf stage. Silencing of YFP and suppression by NS_S proteins was assessed by UV

light and western blot analysis, respectively. To suppress silencing, NS_S constructs were provided in a co-ATTA with YFP constructs. To this end, induced *Agrobacterium* suspensions were mixed at a final OD_{600} of 0.5 prior to infiltration. Plants infiltrated with and without NS_S were kept at 25˚C and monitored for YFP fluorescence during a 5-day period using a GFP fluorescence-stereo-microscope.

Results

Non-uniform production of siRNAs along S RNA

Nucleotide sequence determination of the genomic S RNA segment of TYRV-t (Chapter 2) has revealed the presence of an intergenic region (IGR) containing long stretches of A-rich and U-rich sequences and predicted to fold into a stable hairpin structure (Figure 5-1A).

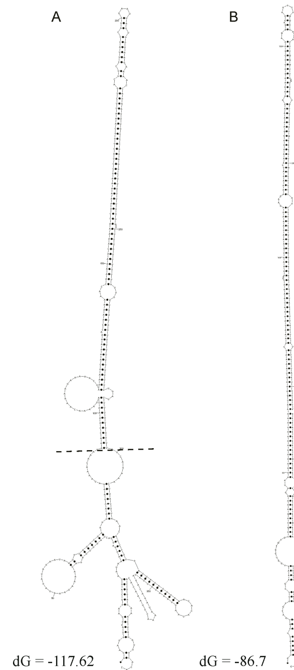


Figure 5-1: Schematic presentation of the intergenic region of the S RNA of TYRV-t (A) and TSWV (B) showing the predicted hairpin structure (Mfold). The flanking sequences of TYRV-t hairpin are shown below the dashed line.

A similar folding has been reported for the S RNAs of other tospoviruses and appears a common feature to all tospoviruses (van Knippenberg *et al.*, 2005). Interestingly, the hairpin structure of the TYRV-t S RNA segment showed long stretches of full complementarity up to 30-40 nucleotides, which therefore may act as potent inducer and target of antiviral RNAi. To verify this, total small RNA molecules from TYRV-infected *N. benthamiana* leaf material was analysed for the presence of IGR-specific small RNAs. Figure 5-2A indicates that upon virus infection the total amount of siRNAs significantly increased in the plant, and Northern blot analysis of these (radiolabelled) siRNAs indicates that the viral siRNAs mapped to all three genomic fragments with lowest amounts for L RNA specific ones (Figure 5-2B).

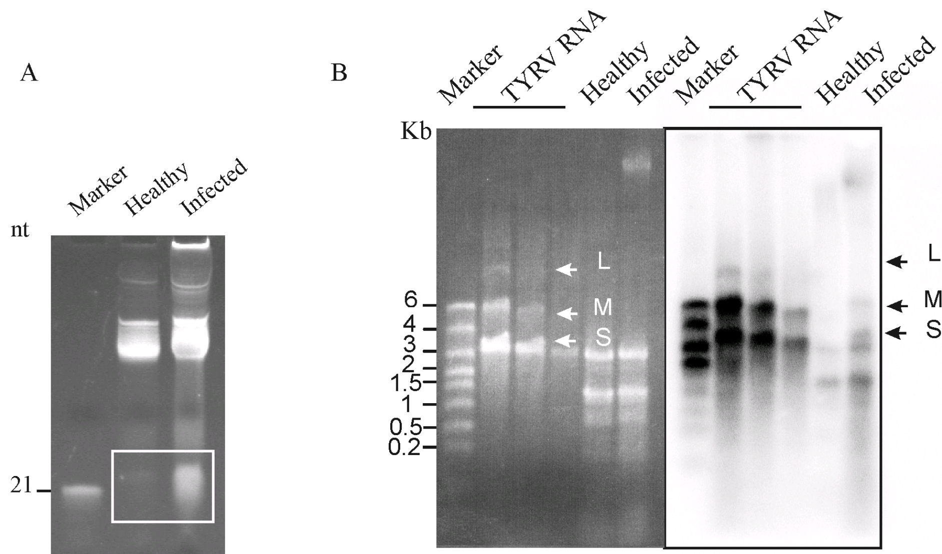


Figure 5-2: Selected area of small RNA molecules of healthy and infected plants followed by northern blotting using radio-labeled small RNAs on total RNA of healthy and infected *N. benthamiana*. The small RNAs cut from 8% polyacrylamide gel shown in the box (panel A) co-migrated with Luciferase GL3 siRNA size marker (Qiagen, Benelux). The viral RNA and total RNA of healthy and infected plant material were loaded on 1% agarose gel with an RNA size marker (RNA ladder-high range, Fermentas Inc.) shown as loading control (B-left). The blot hybridised with small RNA purified from infected plant (B-right).

For further fine mapping within the S RNA sequence the radiolabelled siRNAs were hybridised to PCR fragments spanning this genome segment, the

outcome indicating that the population of S RNA-specific siRNA molecules unequally distributes over the S RNA segment. A high amount of siRNAs was generated from the first 588 nucleotides of the NS_S transcript, and, rather unexpectedly, hardly any siRNAs originating from the IGR hairpin sequence were found (Figure 5-3A). Fine mapping of the sequence around the start of the NS_S ORF revealed that siRNAs specifically derived from the sequence at nt position 142 to 284 (Figure 5-3B). Structure folding of the RNA sequence by Mfold revealed the presence of a secondary structure at nucleotide position 105 to 177 (data not shown). No signals were observed when siRNAs from healthy *N. benthamiana* were used as probe (data not shown).

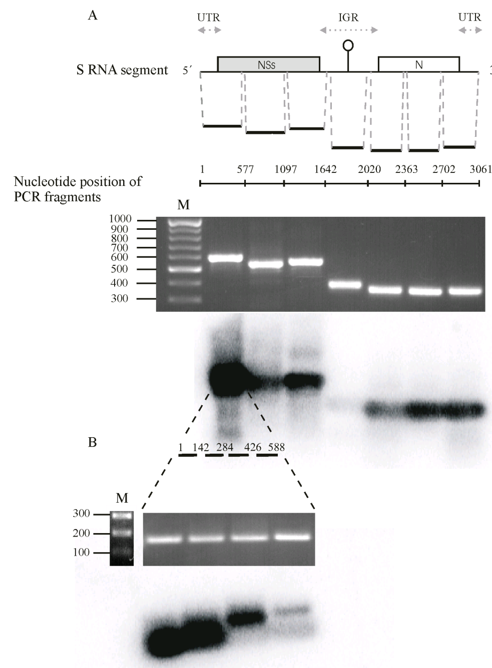


Figure 5-3. Origin and distribution of siRNAs from TYRV-t -infected *Nicotiana benthamiana* plant mapped on the TYRV-t S RNA. Schematic representation of the ambisense S RNA segment encoding the NS_S (suppressor of silencing) and N (nucleocapsid) genes. PCR fragments spanning the entire viral S RNA segment, indicated by their nucleotide positions along the S RNA(panel A, top), were PCR amplified and after Southern blotting probed with 5´- labeled siRNAs extracted from *N. benthamiana* (panel A, bottom). The siRNA hotspot within the first 577 nt of the S RNA was further fine mapped (panel B). 100bp DNA ladder Plus (Fermentas Inc., UK) used as size markers (M) is indicated.

Hairpin-encoding sequence transcripts are cleaved by Dicer

While only few IGR hairpin-derived siRNAs were found in virus-infected host plants, this region was further investigated as potential inducer or target of antiviral RNAi in a dicer assay. To this end, transcripts of the hairpin-encoding sequence were allowed to fold into a dsRNA hairpin structure and subsequently offered to RNAi-induced *Drosophila* embryo extracts containing Dicer-1 and Dicer-2 (Lee *et al.*, 2004). Analysis of the product on non-denaturing acrylamide gels showed that the hairpin transcript was cleaved into small RNAs, co-migrating with siRNAs (21 nucleotides) cleaved from a 114 nt dsRNA transcript and with the siRNA size marker (Figure 5-4A). This result indicated that the IGR hairpin structure may potentially act as an RNAi inducer molecule. Likewise, the TSWV S RNA IGR hairpin was readily cleaved into si RNAs in this assay (Figure 5-4B).

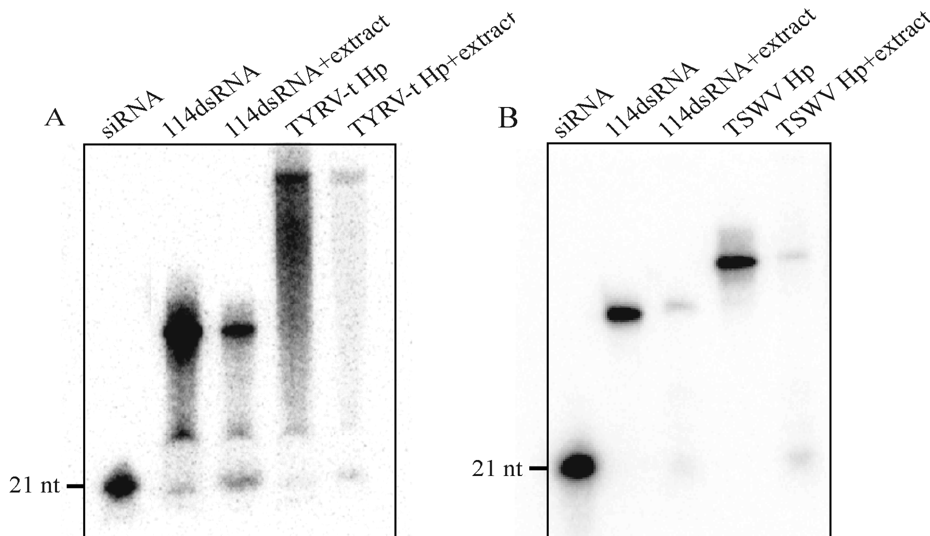


Figure 5-4. Dicer-mediated dsRNA cleavage assay using *Drosophila melanogaster* embryo extract. The hairpin sequence predicted by Mfold program for TYRV-t and TSWV tospoviruses and 114 nt dsRNA were mixed with the extract, incubated for 2-3 hours at 30℃ and loaded onto a 8% denaturing gel. Lanes 1 and 2: untreated 21 nt siRNA and 114 nt dsRNA as size markers, lane 3: 114 nt dsRNA treated with the extract, lane 4: TYRV-t (panel A) or TSWV(panel B) hairpin sequences, lane 5: TYRV-t (panel A) and TSWV (panel B) hairpin sequences treated with the extract.

Hairpin structure processed into small RNAs by extract of infected plants

Recently, it was shown that TSWV NS_S exhibited affinity for both siRNAs and longer dsRNA precursors of miRNAs (Hemmes, 2007). Hence, the presence of only low amounts of siRNAs derived from the hairpin-encoding IGR sequence of the TYRV S RNA segment during a natural infection (Figure 5-3A) could have been caused by NS_S binding to this structure, thereby protecting the hairpin from becoming recognised and cleaved by plant DCL proteins. To test if NS_S indeed exhibited affinity for the hairpin-encoding RNA sequence, an electrophoretic mobility shift assay was performed. To this end, the hairpin transcript was incubated with plant extracts containing NS_S protein expressed after TYRV infection or from PVX replicons. The results, in different repeated experiments, showed that instead of the hairpin structure becoming protected by NS_S and leading to gel retardation, it became degraded into small RNAs (Figure 5-5). TYRV- or PVX-NS_s infected plant extracts showed higher activity in RNA cleavage than extracts from healthy plants, possibly due to lower (activated) state of RNAi in the latter.

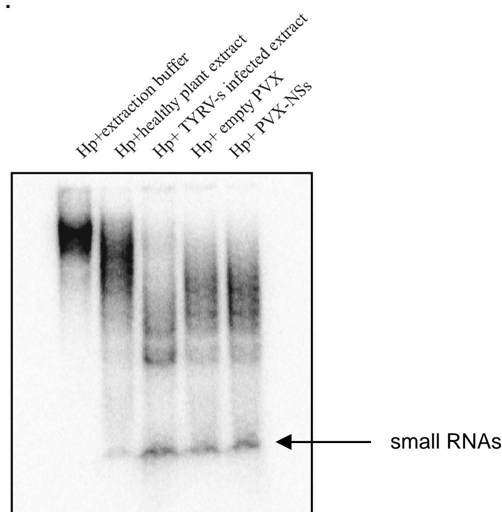


Figure 5-5. Gel shift mobility assay of hairpin dsRNA sequence in the presence or absence of NSs containing plant extracts. Hairpin + extraction buffer (lane 1), Hairpin + healthy plant extracts (lane 2), Hairpin + TYRV-s infected plant extract (lane 3), Hairpin + PVX-infected plant extract (lane 4) and Hairpin+PVX-NS_S infected plant extracts (lane 5).

Viral hairpin structure triggers stronger silencing of YFP transcripts *in planta*

To futher substantiate that the IGR hairpin structure potentially represents a target of RNAi, its role as an inducer of silencing was analysed by providing a functional YFP gene with this hairpin structure at the 3´- end and analyse the effect on silencing by ATTA on *N. benthamiana*. Plants infiltrated with an YFP-IGR hairpin fusion construct showed a significant higher level of YFP silencing 4 days post infiltration in comparison to infiltration with a functional YFP gene without this sequence. Repeated analysis showed no YFP signal in leaves infiltrated with YFP-IGR Hp (Figure 5-6, upper right panel), whereas small patches of YFP signal could still be discerned in leaves infiltrated with the original gene (Figure 5-6 upper left panel). Suppression of YFP silencing during a co-ATTA with TYRV or TSWV NS_S expressed from binary vectors, was slower in case of YFP constructs containing the hairpin structure (Figure 5-6, lower panels). Taken together these results indicate that the IGR hairpin is a potent inducer of RNA silencing, and that the absence of high amounts of hairpin-derived siRNAs during the infection process must be explained differently.

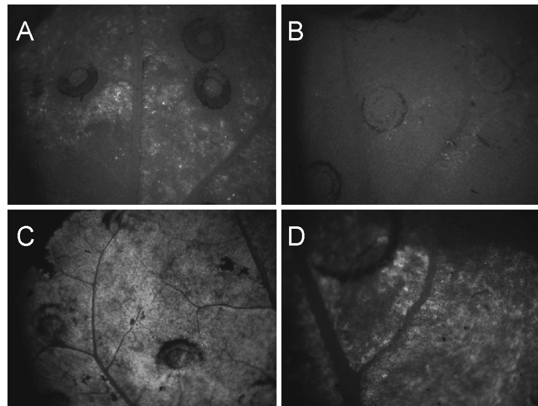


Figure 5-6: Effect of the presence of TYRV-t hairpin sequence on RNA silencing of YFP in *N. benthamiana* leaves. Upper panels show Agrobacterium infiltrated leaves with YFP (A) and YFP-hairpin (B) constructs. Lower panels show co-infiltration of the NS_S construct with YFP (C) and YFP-hairpin (D) constructs to suppress YFP RNA silencing 4 days post-infiltration.

Discussion

RNA silencing is an antiviral defence mechanism of plants that is being induced by dsRNA and imperfect hairpin RNAs. Here it is shown that the IGR hairpin within both TYRV and TSWV S RNA segments, able to fold into a hairpin structure, represent potent inducers and targets of antiviral RNAi in different assays. This sequence acts as target for DCR1 and DCR2 from Drosophila extracts (Lee *et al.*, 2004) and when flanking the 3'-end of a functional YFP gene induces a stronger silencing. From our earlier studies on transgenic resistant plants, it is known that not the (N protein-encapsidated) tospoviral genome is targeted by RNA silencing, but rather the viral trancripts (Prins *et al.*, 1996 and 1997). Tospoviral mRNAs are not encapsidated by the N protein (Kormelink *et al.*, 1992) and both (S RNA derived) N and NS_S mRNAs have been shown to contain the IGR hairpin sequence (van Knippenberg *et al.*, 2005). Hence, if the hairpin structure acts as RNA silencing target, it would do so on the viral transcription level. Our siRNA mapping results, though, indicate that the IGR hairpin structure is a very poor target of RNA silencing despite the assay results. A possible explanation could be that also in viral mRNAs the hairpin structure is masked, not necessarily by N protein but for instance by suppressor of RNA silencing protein i.e NS_S protein, which indeed has been shown to possess a high affinity for long dsRNA (Hemmes *et al.*, 2007). Further along this line, it could be imagined that the tospoviral NS_s protein has multiple functions by binding to dsRNA: firstly to counteract the antiviral RNAi (Bucher *et al.*, 2006), secondly to protect viral mRNAs form silencing through binding to the 3'-terminal hairpin, and thirdly - as the protein already binds to this terminus- by circularising the viral transcripts similar to the activity of PABP (poly-A binding protein) for poly-A tailed cellular mRNAs.

Most studies on viral siRNAs generated during plant infection with RNA or DNA viruses have shown that these siRNAs are not uniformly distributed along the viral genome (Ho *et al.*, 2006; Molnár *et al.*, 2005) and even may differ between host species (Ribeiro, 2006). Our results with an ambisense RNA virus model fits with these findings, as apparently a short hairpin structure at the

beginning of the NS_S gene within the tospoviral S RNA appears to act as such hotspot. The great surprise though of our siRNA mapping studies is that an even more prominent and potent inducer of RNA silencing, the long IGR hairpin, is apparently masked for being targeted during the viral infection process, even on the transcript level. The presence of high amounts of siRNAs mapping to the NS_S gene is of interest as this gene encodes the viral silencing suppressor which is an important virulence determinant (Bucher *et al.*, 2002, Kormelink *et al.*, 1991). Hence, though speculative, the presence of an RNAi target within the NS_S gene might be beneficial to the virus from the evolutionary point of view as it may tune the right balance between virus and host.

Acknowledgements

This work was financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality and carried out according to Dutch law under licence GGO 02-104.ab. The first author wishes to thank Agricultural Research and Education Organisation (AREO) of Iran for assisting his study in the Netherlands.

Chapter 6

General discussion

Tospovirus distribution in the Middle-East

When comparing the currently identified tospovirus species it is clear that some have a worldwide distribution (like TSWV and INSV), while others are more or less confined to certain geographic areas. Also, some areas/continents seem to host more tospoviruses than others. Considering the distribution of tospoviruses on the world map a gap seems to exist on the occurrence of tospoviruses in Africa and the Middle-East (Figure 6-1). Although TSWV and IYSV have been claimed to occur in the latter region (Gera *et al.*, 2000; Anfoka *et al.*, 2006) the analyses reported in this thesis demonstrate that at least part of the Iranian virus isolates reported to belong to 4 different, established tospovirus species, have been misidentified and appear now to belong to a new species (chapter 2 and 3). The reason for this is likely to be found in limited monitoring and tospovirus surveys within these regions and using antisera which often, to a certain extent, cross-react in ELISA assays. Furthermore, occurrence of tospovirus species presented in Figure 1 (Chapter 1) solely was based on serological assays without any N sequence data indicating probable misidentification by early 2003. At the onset of the research compelled in this thesis, 14 different tospovirus species were reported in the literature and in databases, of which 11 had received the formal species status by the International Committee on Taxonomy of Viruses (ICTV) (Fauquet *et al.*, 2005).

The work presented in this thesis aimed to analyse the occurrence of tospoviruses in Iran and started off with the identification and characterisation of tospovirus isolates from tomato, gazania, chrysanthemum, soybean and potato, collected from different regions in Iran. These studies have resulted in the identification of a new *Tospovirus* species, named *Tomato yellow ring virus* (TYRV) (Chapter 2) which together with the recent data, the total number of tospovirus species will increase up to 19 species (Figure 6-1).

In an independent study, Ghotbi *et al.* (2005), reported on the same virus which was referred to as Tomato varamin virus (ToVV) and Tomato yellow fruit ring virus (TYFRV). Further comparison of the Iranian tospovirus isolates from soybean, gazania, chrysanthemum and potato revealed that this novel species

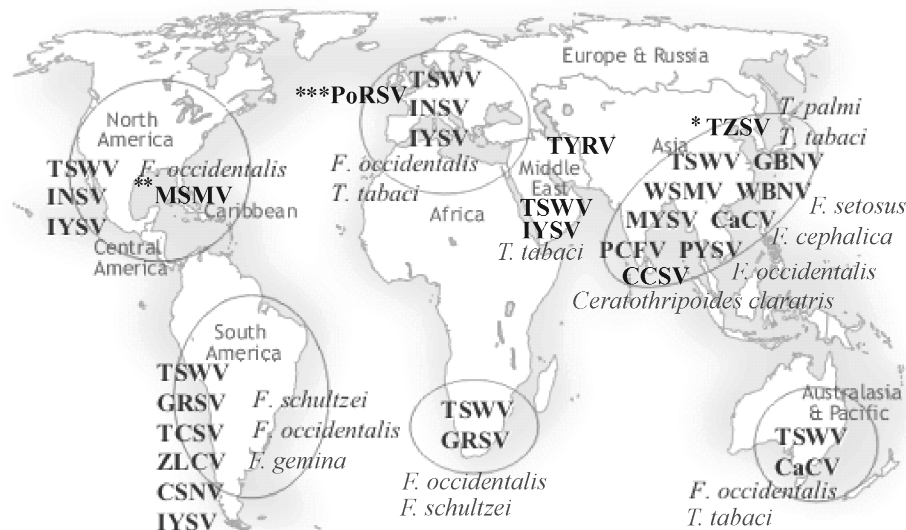


Figure 6-1: Geographical distribution of Tospovirus species and their (presumed) major vector species.
**Tomato zonate spot virus* (TZSV), Dong *et al.*, (2008); ***Melon severe mosaic virus* (MSMV) GenBank accession no. EU275149; ****Polygonum ringspot virus* (PoRSV); GenBank accession no. EF445397.

occurs in Iran in at least two distinct strains denoted TYRV-t and TYRV-s (Chapter 3).

Both strains differ considerably (8%) in their nucleoprotein (N) sequence, the main molecular descriptor used to distinguish tospovirus species. Also two other tospovirus species (IYSV and CaCV) may differ to the same extent in their N protein sequence. For instance, IYSV isolates from Brazil (IYSV-BR; Pozzer *et al.* 1999) and the Netherlands (IYSV-NL; Cortês *et al.*, 1998) show 9% sequence divergence in their N protein (Pappu *et al.*, 2006; pozzer *et al.*, 1999). Also for CaCV an 8% divergence has been reported between isolates from Australia (CaCV-au, McMichael *et al.*, 2002) and Thailand (CaCV-th, Premachandra *et al.*, 2005). However, TYRV is the first tospovirus where this remarkable level of divergence has been observed within the same region, *in casu* Iran, where apparently two genetically stable strains of this virus co-exist. In terms of virus evolution TYRV is therefore an interesting case as it seems that speciation is occurring without geographical separation. To achieve this, and to avoid

convergence of the two distinct strains, they should be isolated in a different way. Indeed, our data indicate that TYRV-t and TYRV-s have clear differences in their host range (Chapter 3). Moreover, data from our mixed-infection studies (Chapter 3) indicate that the two TYRV strains seem to mutually exclude each other, thus avoiding genome segment reassortment or recombination between the two. It has been shown that thrips species transmitting CaCV strains in Australia and Thailand are somehow different (Chiemsombat *et al*., 2008; Persley *et al*., 2006; Premachandra *et al*., 2005). Whether TYRV-t and TYRV-s use different thrips vectors remains to be determined, as information on TYRV vectoring is very poor yet. Rasoulpour & Izadpanah (2007) collected populations of *T. tabaci* from different hosts and showed that these were viruliferous for TYRV-CI (cineraria strain) and able to transmit this virus with rates ranging between 20-60%. Meanwhile, additional tospovirus surveys in Iran have revealed a widespread occurrence of TYRV in the southern part of Iran, both in vegetable and ornamental crops, with disease incidences sometimes up to 100% (Rasoulpour & Izadpanah, 2007). These data underscore the great impact of TYRV may have in the yield of several important crops in the country. Sofar, a routine ELISA testing of 400 suspected samples collected in Israel during 2007, ornamentals as well vegetables (tomato and pepper), has not shown the presence of TYRV (A. Gera, *personal communication*), and a first ELISA-based survey for the prevalence of TYRV in vegetable crops carried out in India and Thailand also were negative, suggesting that TYRV is (still) confined to the Middle-East (Dr. Thierry Jaunet, Marco Polo Seed Company, *personal communication*).

RNA-mediated resistance

To confer plants resistant to TYRV, post transcriptional gene silencing (PTSG) or RNA interference (RNAi) was exploited for development of RNA-mediated resistance. To this end, an existing transgene cassette for multiple tospovirus resistance (Bucher *et al*., 2006) was used as starting point to design different constructs. The starting construct contained partial N gene sequences

from 4 different tomato-infecting tospoviruses and was now extended with a partial N gene sequence of TYRV-t. Transforming *N. benthamiana* plants with the new constructs resulted in TYRV-t resistant phenotypes, but further tests indicated that these plants were still susceptible for TYRV-s. This finding again demonstrated the high sequence specificity of RNAi as already reported for transgenic plants resistant to TSWV but susceptible to TCSV and GRSV (de Haan *et al.*, 1992; Ultzen *et al.*, 1994). Our data confirm the limits of RNA-mediated resistance to be at approximately 10% RNA sequence divergence (Prins *et al.*, 1996). The limitations of the resistance even became more evident after the observation that TYRV-t could escape from RNAi in these resistant plants upon co-infection with TYRV-s (chapter 4). Also mixed inoculation of *Potato virus Y* with *Cucumber mosaic virus* results in breakage of dsRNA-induced transgenic immunity to PVY (Mitter *et al.*, 2003); however resistance breakage was only transiently as plants soon recovered from PVY infection. In the present case TYRV-t was able to systemically infect the resistant lines, and using Mass spectrometry and trans-complementation experiments, the rescuing could be attributed to the viral silencing suppressor protein, NS_S. While during mixed inoculation with both strains TYRV-t was able to spread and replicate systemically in the transgenic resistant plants, in the trans-complementation assays using PVX-NS_S vector constructs, TYRV-t was only detectable in locally-infiltrated leaves during a limited timespan. Since no NS_S protein was detected in the systemic leaves it can be deduced this is due to instability of the PVX vector, a phenomenon frequently encountered (Lacorte, 2006). Accumulation of TYRV-specific siRNAs was unaffected, similar to what was observed for the case of PVY (Mitter *et al.*, 2003). Although in the latter case the involvement of CMV 2b was likely, but not demonstrated, the absence of a systemic PVY infection could have been due to the characteristics of 2b. This protein only shows affinity for short (si and mi) RNA duplex molecules, whereas tospoviral NS_S can also bind longer dsRNA molecules (Hemmes, 2007). Hence, NS_S may already interfere with the RNAi pathway, at the dicer cleavage stage; whereas 2b only becomes active once siRNAs are produced. This idea is further supported with data from

ATTA assays using YFP silencing inducer constructs. Although resistance strategies exploiting the PTGS/RNAi machinery have been proven successfully, the associated RNA sequence specificity represents a limitation. With the observation that during a mixed-infection of closely related viruses such resistance can be broken, may have a major impact for the use of RNA-mediated resistance strategies during natural field situations, especially when such viruses have overlap in geographic range.

Tospoviral induction of RNAi and viral counter defence

The presence of extensive ds stretches within the predicted hairpin structure present in the intergenic region of the TYRV S RNA segment, lead to the question whether this structure would represent a potential target and inducer for RNAi. The observation that YFP constructs containing this predicted hairpin structure sequence at their 3' end, acted as a strong(er) inducer of silencing, and that this sequence *in vitro* was recognised as a potential target by *Drosophila melanogaster* DCR1 and DCR2 (Chapter 5), indicate that this secondary structure induces antiviral RNAi. Since plants transformed with TSWV NS_M (movement protein) gene sequences are resistant on tissue level but not in single cell level (Prins *et al.*, 1997) it is likely that the tospoviral mRNAs are targets for RNAi rather than the (tightly encapsidated) genomic RNA segments. Considering the available information a model for tospovirus induced RNA silencing and counter defence is proposed (Figure 6-2).

The model proposes that during the initial stages of infection, viral mRNAs are synthesised that terminate in a predicted hairpin structure (van Knippenberg *et al.*, 2005) instead of a poly(A)-tail. Whereas the interaction of the 5' and 3' terminal ends of eukaryotic mRNA molecules, as a pre-requisite for translation initiation (Gallie, 1998 and 2002), is mediated by the poly(A)-binding protein (PABP), the presence of a 3' hairpin structure suggests that the interaction between the 5' and 3' terminal regulatory elements of TSWV mRNAs is regulated differently. It is postulated that this interaction is mediated by binding of a PABP analogue to the hairpin structure.

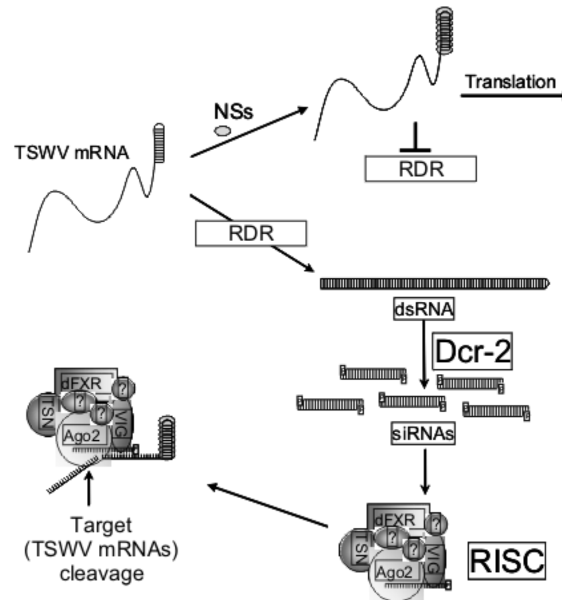


Figure 6-2: Model of Tospovirus-induced RNA silencing.

It has been observed that NS_S counterpart from the animal infecting bunyaviruses associate with 40S ribosomal units, and hence may be implicated in translation (Di Bonito *et al* 1999; Watkins & Jones, 1993; Simons *et al*., 1992). Recently, TSWV NS_S has been shown affinity to bind siRNAs and to longer dsRNAs that act as precursor to miRNAs (Hemmes, 2007). The first and likely target for this is the predicted hairpin structure at the 3' end which has similar structure for a miRNA molecule. Upon binding, NS_S avoids this structure being recognised by dicer. Furthermore, plants transformed with the intergenic region containing hairpin structure of TSWV are not resistant to TSWV (de Haan *et al*., 1991; Prins *et al*., 1996;) in which activated RISC harboring a guide RNA strand from the hairpin sequence is not able to target the corresponding, but apparently masked sequence in mature viral transcripts. Furthermore, it explains why during repeated analysis only very low levels of siRNAs mapped to the intergenic region containing the hairpin, whereas folded transcripts of this sequence can be readily cleaved by dicer(s) in *D. melanogaster* embryo extract (chapter 5).

Instead a hotspot present at the start of NS_S ORF and predicted to fold into another, though short hairpin structure still became recognised and cleaved, giving rise to high levels of siRNAs.

Why this siRNA-inducing hotspot is located at the NS_S of TYRV-t remains to be determined, but it is tempting to assume that it may be related to balancing virus-host interactions: since the NS_S protein suppresses the host's antiviral RNAi, targeting NS_S transcripts would immediately reduce viral virulence, thereby tuning virus-host relationship. A similar structure is also predicted in the IYSV NS_S transcript (data not shown) whether this is a generic property of tospoviruses remains to be investigated.

References:

Adkins, S., Quadt, R., Choi, T. -J., Ahlquist, P. & German, T. (1995). An RNA-dependent RNA polymerase activity associated with virions of tomato spotted wilt virus, a plant- and insect-infecting Bunyavirus. *Virology* **207**:308-311.

Albariño, C., Bird, B. H. & Nicol, S. T. (2007). A shared Transcription Termination Signal on Negative and Ambisense RNA Genome Segments of Rift Valley Fever, Sandfly Fever Sicilian, and Toscana Viruses. *Journal of Virology* **81**:5246-5256.

Amin, P. W., Reddy, D. V. R. & Ghanekar, A. M. (1981). Transmission of tomato spotted wilt virus, causal agent of bud necrosis of peanut, by *Scirtothrips dorsalis* and *Frankliniella schultzei*. *Plant Disease* **65**:663-665.

Anfoka, G. H., Abhary, M. & Stevens, M. R. (2006). Occurrence of *Tomato spotted wilt virus* (TSWV) in Jordan. *OEPP/EPPO Bulletin* **36**:517-522.

Antignus, Y., Gera, A., Pearlsman, M., Ben-Joseph, R., Ganaim, N., Raccah, B. & Cohen, S. (1994). Tomato spotted wilt, a new disease of flowers and vegetables in Israel. *Phytoparasitica* **22**:86-87 (Abstract).

Aramburu, J. & Marti, M., (2003). The occurrence in north-east Spain of a variant of tomato spotted wilt virus (TSWV) that breaks resistance in tomato (*Lycopersicon esculentum*) containing the Sw-5 gene. *Plant Pathology* **52**:407.

Avesani, L., Marconi, G., Morandini, F., Albertini, E., Bruschetta, M., Bortesi, L., Pezzotti, M. & Porceddu, A. (2007). Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment. *Transgenic Research* **16**:587-597.

Bananej, K., Shahraeen, N., Ahoonmanesh, A., Lesemann, D. E. & Shahryari, D. (1998). Identification of *Tomato spotted wilt virus* from tomato fields in Varamin area. *Iranian Journal of Plant Pathology* **34**:30-36 (Abstract).

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

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

Baulcombe, D. C. (1999). Gene silencing: RNA makes RNA makes no protein. *Current Biology* 9:R599-R601.

Baulcombe, D. C. & English, J. J. (1996). Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. *Current Opinion Biotechnology* **7**:173-180.

Black, L. L., Hobbs, H. A., & Gatti, J. M., Jr. (1991). Tomato spotted wilt virus resistance in *C. chinense* PI152225 and 159236. *Plant Disease* **75**:863.

Boiteux, L. S. (1995). Allelic relationships between genes for resistance to tomato spotted wilt tospovirus in *Capsicum. Chinese Theoritical Applied Genetics*. **90**:146-149.

Boiteux, L. S. & Giordano, L. de B., (1993). Genetic basis of resistance against two Tospovirus species in tomato (*Lycopersicon esculentum*). *Euphytica* **71**:151-154.

Brødsgaad, H. F. (1994). Insecticide resistance in European and African strains of western flower thrips (*Thysanoptera*: *Thripidae*) tested in a new residue-on-glass test. *Journal of Economic Entomology* **87**:1141-1146.

Brommonschenkel, S. H., Frary, A. & Tanksley, S. D. (2000). The broad-spectrum tospovirus resistance gene *Sw*-5 of tomato is a homolog of the root-knot nematode resistance gene Mi. *Molecular-Plant Microbe Interactions* **13**:1130-1138.

Browne, E. P., Li, J., Chong, M. & Littman, D. R. (2005). Virus-host interactions: new insights from the small RNA world. *Genome Biology* **6**:238.

Bucher, E. (2006). Antiviral RNA silencing and viral counter defence in plants. PhD thesis, Wageningen University, The Netherlands.

Bucher, E., Hemmes, H., de Haan, P., Goldbach, R. & Prins, M. (2004). The influenza A virus NS1 protein binds small interfering RNAs and suppress RNA silencing in Plants. *Journal of General Virology* **85**:983-991.

Bucher, E., Lohuis, D., van Poppel, P. M. J. A., Geerts-Dimitriadou, C., Goldbach, R. & Prins, M. (2006). Multiple virus resistance at a high frequency using a single transgene construct. *Journal of General Virology* **87**:3697-3701.

Bucher, E., Sijen, T., de Haan, P., Goldbach, R. & Prins, M. (2003). Negative-strand tospoviruses and tenuiviruses carry a gene for a suppressor of gene silencing at analogous genomic positions. *Journal of Virology* **77**:1329-1336.

Carr, J. P. & Zaitlin, M. (1993). Peplicase-mrdiated resistance. *Seminars in Virology* **4**:339-347.

Cernny, R. E., Qi, Y., Aydt, C. M., Huang, S., Listello, J. J., Fabbri, B. J., Conner, T. W., Crossland, L. & Huang, J. (2003). RNA-binding protein-mediated translational repression of transgene expression in plants. *Plant Molecular Biology* **52**:357-369.

Chapman, E. J., Hilson, P. & German, T. L. (2003). Association of the L protein and *in vitro Tomato spotted wilt virus* RNA-dependent RNA polymerase activity. *Intervirology* **46**:177-181.

Chen, C. C. & Chui, R. J. (1996). A tospovirus infecting peanut in Taiwan. *Acta Horticulturae* **431**:57-67.

Chen, S. L., Le, S. Y., Newton, D. L., Maizel, J. V., Rybak Jr., S. M. (2000). A gender-specific mRNA encoding a cytotoxic ribonuclease contains a 3´ UTR of unusual length and structure. *Nucleic Acids Research* **28**:2375-2382.

Chen, Y.-K., Lohuis, D., Goldbach, R. & Prins, M. (2004). High frequency induction of RNA-mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs. *Molecular Breeding* **14**:215-226.

Chiemsombat, P., Gajanandana, O., Warin, N., Hongprayoon, R., Bhunchoth, A. & Pongsapich, P. (2008). Biological and molecular characterisation of tospoviruses in Thailand. *Archives of Virology* **153**:571-577.

Clark, M. F. & Adams, A. N. (1977). Characterisation of the microplate method of enzyme linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**:475-483.

Cortês, I., Livieratos, I. C., Derks, A., Peters, D., & Kormelink, R. (1998). Molecular and serological characterisation of Iris yellow spot virus, a new and distinct tospovirus species. *Phytopathology* **88**:1276-1282.

Cortês, I., Saaijer, J., Wongjkaew, K. S., Pereira, A. -M., Goldbach, R., Peters, D. & Kormelink, R. (2001). Identification and characterisation of a novel tospovirus species using a new RT-PCR approach. *Archives of Virology* **146**:265-278.

Daughtrey, M. L., Jones, R. K., Moyer, J. W., Daub, M. E. & Baker, J. R. (1997). Tospoviruses strikes the greenhouse industry; INSV has become a major pathogen on flower crops. *Plant Disease* **81**:1220-1230.

DeAngelis, J. D., Sether, D. M. & Rossignol, P. A. (1993). Survival, development and reproduction in Western flower thrips (*Thysanoptera:Thripidae*) exposed to Impatiens necrotic spot virus. *Environmental Entomology* **22**:1308-1312.

de Ávila, A. C., de Haan, Kitajima, E. W., Kormelink, R., Resende, R. de O., Goldbach, R. W. & Peters, D. (1992a). Characterisation of a distinct isolate of tomato spotted wilt virus (TSWV) from Impatiens sp. in the Netherlands. *Journal of Phytopathology* **134**:133-151.

de Ávila, A. C., de Haan, P., Smeets, M. L. L., Resende, R. de O., Kormelink, R., Kitajima, E., Goldbach, R. & Peters, D. (1992b). Distinct levels of relationships between tospovirus isolates. *Archives of Virology* **128**:211-227.

de Ávila, A. C., de Haan, P., Kormelink, R., Resende, R. de O., Goldbach, R. W. & Peters, D. (1993a). Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. *Journal of General Virology* **74**:153-159.

de Ávila, A. C., de Haan, P., Smeets M. L. L., Resende, R. de O., Kormelink, R., Kitajima, E. W., Goldbach, R. W. & Peters, D. (1993b). Distinct levels of relationships between tospovirus isolates. *Archives of Virology* **128**:211-227.

de Ávila, A. C., Huguenot, C., Resende, R. de O., Kitajima, E. W., Goldbach, R. W. & Peters, D. (1990). Serological differentiation of 20 isolates of tomato spotted wilt virus. *Journal of General Virology* **71**:2801-2807.

de Borbón, C. M. ,Gracia, O. & Piccolo, R. (2000). Relationships between Tospovirus Incidence and Thrips Populations on Tomato in Mendoza, Argentina. *Journal of Phytopathology* **154**:93–99.

de Haan, P. (1991). Exploring and exploiting the RNA genome of tomato spotted wilt virus. PhD thesis Agricultural University Wageningen, The Netherlands.

de Haan, P., Gielen, J. J. L., Prins, M., Wijkamp, I. G., van Schepen, A., Peters, D., van Grinsven, M. Q. J. M. & Goldbach, R. (1992). Characterisation of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. *Bio/Technology* **10**:1133-1137.

de Haan, P., Kormelink, R., Resende, R. de O., van Poelwijk, F., Peters, D. & Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **72**:2207-2216.

de Haan, P., Wagemaker, L., Peters, D. & Goldbach, R. (1989). Molecular cloning and terminal sequence determination of the S and M RNAs of tomato spotted wilt virus. *Journal of General Virology* **70**:3469-3473.

de Haan, P., Wagemaker, L., Peters, D. & Goldbach, R. (1990). The S RNA segment of Tomato spotted wilt virus has an ambisense character. *Journal of General Virology* **71**:1001-1007.

Diaz-Pendon, J. A., Li, F., Li, W. -X. & Ding, S. W. (2007). Suppression of antiviral silencing by cucumber mosaic virus 2b protein in *Arabidopsis* is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. *Plant Cell* **19**:2053-2063.

Di Benito, P., Nicoletti, L., Mochi, S., Accardi, L., Marchi, A. & Giorgi, C. (1999). Immunological characterisation of Toscana virus proteins. *Archives of Virology* **144**:1947-1960.

Dong, J.-H., Cheng, X.-F., Yin, Y.-Y., Fang, Q., Ding, M., Li, T.-T., Zhang, L.-Z., Su, X.-X., McBeath, J. H. & Zhang, Z.-K. (2008). Characterisation of tomato zonate spot virus, a new tospovirus in China. *Archives of Virology* **153**:855-864.

Duijsings, D., Kormelink, R. & Goldbach, R. (1999). *Alfalfa mosaic virus* RNAs serve as cap donors for *Tomato spotted wilt virus* transcription during co-infection of *Nicotiana benthamiana*. *Journal of Virology* **73**:5172-5175.

Duijsings, D., Kormelink, R. & Goldbach, R. (2001). *In vivo* analysis of the TSWV cap snatching mechanism: single base complementarity and primer length requirements. *EMBO Journal* **20**:1-8.

Dunoyer, P. & Voinnet, O. (2005). The complex interplay between plant viruses and host RNA-silencing pathways. *Current Opinion in Plant Biology* **8**:415-423.

Elliot, R.M. (1990). Molecular biology of the Bunyaviridae. *Journal of General Virology* **71**:501-522.

Emery, V. C. & Bishop, D. H. (1987). Characterisation of *Punta toro* S mRNA species and identification of an inverted complementary sequence in the intergenic region of *Punta toro phlebovirus* ambisense S RNA that is involved in mRNA transcription termination. *Virology* **156**:1-11.

Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger U. & Ball, L. A. (2005). Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, San Diego, USA.

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

Folkertsma, R. T., Spassova, M. I., Prins, M., Stevens, M. R., Hille, J. & Goldbach, R. W. (1999). Construction of a bacterial artificial chromosome (BAC) library of *Lycopersicon esculentum* cv. Stevens and its application to physically map the *Sw-5* locus. *Molecular Breeding* **5**:197-207.

Francki, R. I. B., Fauquet, C. M., Knudson, D. L. & Brown, F. (1991). Classification and nomenclature of viruses: V report of international Committee on Taxonomy of Viruses. *Archives of Virology* Supplement **2**:1-450.

Fujisawa, I., Tanaka, K. & Ishili, M. (1988). Tomato spotted wilt virus transmission by three species of thrips, *Thrips setosus*, *Thrips tabaci* and *Thrips palmi*. *Annals of the Phytopathological Society of Japan* 54, Abstracts of papers presented at annual meeting of the society, Fuchu, Japan, 2-4 April: 392.

Gallie, D. R. (1998). A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* **216**:1-11.

Gallie, D. R. (2002). Protein-protein interactions required during translation. *Plant Molecular Biology* **50**:949-970.

Gardner, M. W., Tompkins, C. M. & Whipple, O. C. (1935). Spotted wilt of truck crops and ornamental plants. *Phytopathology* **25**:17.

Gera, A., Chen, J., Salomon, R. & Raccah, B. (1998a). Iris yellow spot tospovirus detected in onion (*Alium cepa*) in Israel. *Plant Disease* **82**:127.

Gera, A., Kritzman, A., Cohen, J. & Raccah, B. (1998b). Tospovirus infecting bulb crops in Israel. In: Peters, D. & Goldbach, R. (Editors). Recent progress in tospovirus and thrips research, Wageningen, the Netherlands :86-87.

Ghotbi, T., Shahraeen, N. & Winter, S. (2005). Occurrence of tospoviruses in ornamental and weed species in Markazi and Tehran provinces in Iran. *Plant Disease* **89**:425-429.

Gielen, J. J. L., de Haan, P., Kool, A. J., Peters, D., van Grinsven, M. Q. J. M. & Goldbach, R. W. (1991). Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. *Bio/Technology* **9**:1363-1367.

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

Goldbach, R. & de Haan, P. (1993). Prospects of engineered forms of resistance against tomato spotted wilt virus. *Seminars in Virology* **4**:381-387.

Goldbach, R. & Kuo, G. (1996). Introduction: Proceedings of the international symposium on tospovirus and thrips of floral and vegetable crops. *Acta Horticulturae* **431**:21-26.

Goldbach, R. & Peters, D. (1994). Possible causes of the emergence of tospovirus diseases. *Seminars in Virology* **5**:113-120.

Goldbach, R. & Peters, D. (1996). Molecular and biological aspects of tospoviruses. The Bunyaviridae. Elliot, R. M. New York, N. Y., Plenum Press: 129-157.

Golnaraghi, A. R., Shahraeen, N., Pourrahim, R. & Farzadfar, Sh. (2002). First report of *Groundnut bud necrosis virus* in Iran. *Plant Disease* **86**:561.

Golnaraghi, A. R., Shahraeen, N., Pourrahim, R., Ghorbani, Sh. & Farzadfar, Sh. (2001). First Report of Tomato spotted wilt virus on soybean in Iran. *Plant Disease* **85**:1290.

Golnaraghi, A. R., Shahraeen, N., Pourrahim, R., Farzadfar, Sh. & Ghasemi, A. (2004). Occurrence and relative incidence of viruses infecting soybeans in Iran. *Plant Disease* **88**:1069-1074.

Goodwin, J., Chapman, K., Swaney, S., Parks, T. D., Wernsman, E. A. & Dougherty, W. G. (1996). Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. *Plant Cell* **8**:95-105.

Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T. & Masuta, C. (2007). Characterisation of silencing suppressor 2b of Cucumber mosaic virus based on examination of its small RNA-binding abilities. *Plant Cell Physiology* **48**:1050-1060.

Granval de Millan, N., Piccolo, R. & Gracia, O. (1998). Potato (*Solanum tuberosum* L.) tuber transmission of tospoviruses (TSWV, GRSV and TCSV) in Mendoza province, Argentina. In: Peters, D. and Goldbach, R. (Editors), Recent progress in tospovirus and thrips research, Wageningen, the Netheralnds :46-47.

Haley, B., Tang, G. & Zamore, P. D. (2003). In vitro analysis of RNA interference in *Drosophila melanogaster*. *Methods* **30**:330-336.

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

Hassani-Mehraban, A., Saaijer, J., Peters, D., Goldbach, R. & Kormelink, R. (2007). Molecular and biological comparison of two Tomato yellow ring virus (TYRV) isolates: challenging the *Tospovirus* species concept. *Archives of Virology* **152**:85-96.

Heilersig, B. H. J. B., Loonen, A. E. H. M., Wolters, A. -M. A., & Visser, R. G. F. (2006). Presence of an intron in inverted repeat constructs does not necessarily have an effect on efficiency of post-transcriptional gene silencing. *Molecular Breeding* **17**:307-316.

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). *Microbiologica* **24**:175-187.

Heinze, C., Maiss, E., Adam, G. & Caspar, R. (1995). The complete nucleotide sequence of the S RNA of a new *Tospovirus* species, representing serogroup IV. *Phytopathology* **85**:683-690.

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. *Archives of Virology* **148**:199-205.

Hemmes, H. (2007). Strategies underlying RNA silencing suppression by negative strand RNA viruses. PhD thesis, Wageningen University, The Netherlands.

Ho, T., Pallett, D., Rusholme, R., Dalmay, T. & Wang, H. (2006). A simplified method for cloning of short interfering RNAs from Brassica juncea infected with Turnip mosaic potyvirus and Turnip crinkle carmovirus. *Journal of Virological Methods* **136**:217-223.

Hoffmann, K., Qiu, W. P. & Moyer, J. W. (2001). Overcoming host- and pathogen-mediated resistance in tomato and tobacco maps to the M RNA of *Tomato spotted wilt virus. Molecular Plant-Microbe Interactions* **14**:242-249.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G. & Fraley, R. T. (1985). A simple method for transferring genes into plants. *Science* **227**:1229-1231.

Ikegami, T., Won, S., Peters, C. J. & Makino, S. (2007). Characterisation of Rift Valley Fever Virus transcriptional terminations. *Journal of Virology* **81**:8421-8438.

Inoue-Nagata, A. K., Kormelink, R., Nagata, T., Kitajima, E. W., Goldbach, R. & Peters, D. (1997). Effects of temperature and host on the generation of Tomato spotted wilt virus defective interfering RNAs. *Phytopathology* **87**:1168-1173.

Jan, F. -J., Fagoaga, C., Pang, S. -Z. & Gonsalves, D. (2000). A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance. *Journal of General Virology* **81**:235-242.

Johansen, L. K. & Carrington, J. C. (2001). Silencing on the spot. Induction and suppression of RNA silencing in the Agrobacterium-mediated transient expression system. *Plant Physiology* **126**:930-938

Jones, R. A. C. & Sharman, M. (2005). Capsicum chlorosis virus infecting *Capsicum annuum* in the East Kimberley region of Western Australia. *Australasian Plant Pathology* **43**:397-399.

Kameya-Iwaki, M., Honda, Y., Hanada, K., Tochihara, H., Yonaha, T. Hokama, K. & Yokoyama, T. (1984). Silver mottle disease of watermelon caused by tomato spotted wilt virus. *Plant Disease* **68**:1006-1008.

Karimi, M., Inzé, D. & Depicker, A. (2002). Gateway™ vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science* **7**:193-195.

Kato, K. & Hanada, K. (2000). Characterisation of the S RNA segment of melon yellow spot virus. *Japanese Journal of Phytopathology* **66**:252-254.

Khvorova, A., Reynolds, A. & Jeyasena, S. D. (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**:209-216.

Kikkert, M., van Lent, J., Storms, M., Bodegom, P., Kormelink, R. & Goldbach, R. (1999). Tomato spotted wilt virus particle morphogenesis in plant cells. *Journal of Virology* **73**:2288-2297.

Kikkert, M., Verschoor, A., Kormelink, R., Rottier, P. & Goldbach, R. (2001). Tomato spotted wilt virus glycoproteins exhibit trafficking and localization signals that are functional in mammalian cells. *Journal of Virology* **75**:1004-1012.

Kormelink, R., de Haan, P., Meurs, C., Peters, D. & Goldbach, R. (1992a). The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with ambisense RNA segment. *Journal of General Virology* **73**:2795-2804.

Kormelink, R., de Haan, P., Peters, D. & Goldbach, R. (1992b). Viral-RNA synthesis in *Tomato spotted wilt virus*-infected *Nicotiana rustica* plants. *Journal of General Virology* **73**:687–693.

Kormelink, R., Peters, D. & Goldbach, R. (1998). Tospovirus genus. In*: AAB Description of Plant Viruses.*

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

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

Lacorte, C. (2006). Improving and assessing viral vectors for recombinant protein production in plants. PhD thesis, Wageningen University, The Netherlands.

Lakshmi, K. V., Wightman, J. A., Reddy, D. V. R., Ranga Rao, G. V., Buiel, A. A. M. & Reddy, D. D. R. (1995). Transmission of peanut bud necrosis virus by *Thrips palmi* in India. In: Parker, B. L., Skinner, M. & Lewis, T. (Editors). Thrips biology and management, Plenum Press, New York: 170-184.

Latham, J. R. & Wilson, A. K. (2008). Transcomplementation and synergism in plants: implications for viral transgenes? *Molecular Plant Pathology* **9**:85-103.

Latham, L. J. & Jones, R. A. C. (1998). Selection of resistance breaking strains of tomato spotted wilt tospovirus. *Annals of applied biology* **133**:385-402.

Law, M. D. & Moyer, J. W. (1990). A tomato spotted wilt-like virus with a serologically distinct N protein. *Journal of General Virology* **71**:933-938.

Le, S. -Y. & Maizel Jr., J. V. (2007). Data mining of imperfect double-stranded RNA in 3´ untranslated regions of eukaryotic mRNAs. *Biomolecular Engineering* **24**:351-359.

Lebbink, R. J., de Ruiter, T., Adelmeijer, J., Brenkman, A.B., van Helvoort, J. M., Koch, M., Farndale, R. W., Lisman, T., Sonnenberg, A., Lenting, P.J. & Meyaard, L. (2006). Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *The Journal of Experimental Medicine* **203**:1419-1425.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J. & Carthew, R. W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**:69-81

Lin Y. H., Chen, T. C., Hsu, H. T., Liu, F. L., Chu, F. H., Chen, C. C., Lin, Y. Z. & Yeh, S. D. (2005). Serological comparison and molecular characterisation for verification of Calla lily chlorotic spot virus as a new tospovirus species belonging to Watermelon silver mottle virus serogroup. *Phytopathology* **95**:1482-1488.

Llave, C., Kasschau, K. D. & Carrington, J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of National Academy of Sciences of the USA* **97**:13401-13406.

López, N. & Franze-Fernández, M. T. (2007). A single stem-loop structure in Tacaribe arenavirus intergenic region is essential for transcription termination but not required for a correct initiation of transcription and replication. *Virus Research* **124**:237-244.

Louro, D. (1996). Detection and identification of tomato spotted wilt virus and impatiens necrotic spot virus in Portugal. *Acta Horticulturae* **431**:99-105.

MacKenzie, D. J. & Ellis, P. J. (1992). Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene. *Molecular Plant-Microbe Interactions* **5**:34-40.

Mallory, A. C., Reinhart, B. J., Bartel, D., Vance, V. B. & Bowman, L. H. (2002). A viral suppressor of RNA silencing differentiallt regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proceedings of National Academy of Sciences of the USA* **99**:15228-15233.

Marchoux, G., Gebreselassie, K. & Villevieille, M. (1991). Detection of tomato spotted wilt virus and transmission by *Frankliniella occidentalis* in France. *Plant Pathology* **40**:347-380.

Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. (1999). Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure. *Journal of Molecular Biology* **288**:911-940.

McMichael, A. L., Perley, D. M. & Thomas, J., E. (2002). A new tospovirus serogroup IV species infecting capsicum and tomato in Queensland, Australia. *Australasian Plant Pathology* **31**:231-239.

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

Mérai, Z., Kerényi, Z., Kertész, S., Magna, M., Lakatos, L. & Silhavy, D. (2006). Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA Silencing. *Journal of Virology* **80**:5747-5756.

Mitter N, **Sulistyowati E. & Dietzgen R. G. (2003).** *Cucumber mosaic virus* infection transiently breaks dsRNA-induced transgenic immunity to *potato virus Y* in tobacco. *Molecular Plant-Microbe Interactions* **16**:936-944.

Mitter, N., Sulistyowati, E., Graham, M. W. & Dietzgen, R. G. (2001). Suppression of gene silencing: a threat to virus-resistant transgenic plants? *Trends in Plant Science* **6**:246-247.

Moeini, A. A., Sahandpour, A. & Izadpanah, K. (1998). Identification of Tomato spotted wilt virus (TSWV) in ornamental plants and tomato in Shiraz, In: *Proceedings of 13th Iranian Plant Protection Congress*, Karaj, Iran. p.199.

Mohamed, N. A. (1981). Isolation and characterisation of subviral structures from tomato spotted wilt virus. *Journal of General Virology* **53**:197-206.

Molnár, A., Csorba, T., Lakatos, L., Várallyay, E., Lacomme, C. & Burgyán, J. (2005). Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *Journal of Virology* **79**:7812-7818.

Moritz, G., Kumm, S. & Mound, L. (2004). Tospovirus transmission depends on thrips ontogeny. *Virus Research* **100**:143-149.

Morse, J. G. & Hoddle, M. S. (2006). Invasion biology of thrips. *Annual Review of Entomology* **51**:67-89.

Mumford, R. A. & Seal, S. E. (1997). Rapid single immunocapture RT-PCR for the detection of two yam potyviruses. *Journal of Virological Methods* **69**:73-79.

Nagata, T. & de Ávila, A. C. (2000). Transmission of chrysanthemum stem necrosis virus, a recently discovered Tospovirus, by two thrips species. *Journal of Phytopathology* **148**:123-125.

Nakahara S. & Monteiro R. C. (1999). *Frankliniella zucchini* (Thysanoptera: Thripidae), a new species and vector of tospovirus in Brazil. *Proceedings of the Entomological Society of Washington* **101**:290-294.

Ohnishi, J., Katsuzaki, H., Tsuda, S., Sakurai, T., Akutsu, K. & Murai, T. (2006). *Frankliniella cephalica*, a new vector for Tomato spotted wilt virus. *Plant Disease* **90**:685.

Okuda, M., Taba, S. & Hanada, K. (2003). The S RNA segment determines symptom differences on *Tetragonia expansa* between two Watermelon silver mottle virus isolates. *Physiological & Molecular Plant Pathology* **62**:327-332.

Palmer, J. M., Reddy, D. V. R., Wightman, J. A., & Ranga Rao, G. V. (1990). New information on the thrips vectors of tomato spotted wilt virus in groundnut crops in India. *Inernational Arachis Newsletter* **7**:24-25.

Pang, S. Z., Nagpala, P., Wang, M., Slightom, J. L. & Gonsalves, D. (1992). Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene. *Molecular Plant Pathology* **82**:1223-1229.

Pappu, H. R., du Toit, L. J., Schwarz, H. F. & Mohan, S. K. (2006). Sequence diversity of the nucleoprotein gene of iris yellow spot virus (genus *Tospovirus*, family *Bunyaviridae*) isolates from the western region of the United States. *Archives of Virology* **151**:1015-1023.

Pappu, S. S., Bhat, A. I., Pappu, H. R., Deom, C. M. & Culbreath, A. K. (2000). Phylogenetic studies of tospoviruses (Family: Bunyaviridae) based on intergenic region sequence of small and medium genomic RNAs. *Archives of Virology* **145**:1035-1045.

Pedersen, I. M., Cheng, G., Wieland, S., Volinia, S., Croce, C. M., Chisari, F. V. & David, M. (2007). Interferon modulation of cellular microRNAs as an antiviral mechanism. *Nature* 449:919-923.

Persley, D. M., Thomas, J. E. & Sharman, M. (2006). Tospoviruses - an Australian perspective. *Australasian Plant Pathology* **35**:161-180.

Peters, D. (2003). Tospoviruses: a threat for the intensive agriculture in tropics. In: Loebenstein, G. & Thottappilly, G. (Editors). Virus and virus-like diseases of major crops in developing countries. Kluwer Academic Publishers, Dordrecht, the Netherlands: 719-742.

Pittman, H. A. (1927). Spotted wilt of tomatoes. Preliminary note concerning the transmission of the “spotted wilt” of tomatoes by an insect vector (*Thrips tabaci* Lind.). Commonwealth of Australia, *Council of Science and Industrial Research Bulletin* **1**:74-77.

Pourrahim, R., Farzadfar, Sh., Moeini, A. A., Shahraeen, N. & Ahoonmanesh, A. (2001). First report of Tomato spotted wilt virus on potatoes in Iran. *Plant Disease* **85**:442.

Pozzer, L., Bezzerra, I. C., Kormelink, R., Prins, M., Peters, D., Resende, R. de O. & de Ávila, A. C. (1999). Characterisation of a tospovirus isolate of Iris yellow spot virus associated with a disease in onion fields in Brazil. *Plant Disease* **83**:345-350.

Premachandra, W. T. S. D., Borgemeister, C., Maiss, E., Knierim, D. & Poehling, H. - M. (2005). *Ceratothripoides claratris*, a New Vector of a Capsicum chlorosis virus Isolate Infecting Tomato in Thailand. *Phytopathology* **95**:659-663.

Prins, M., de Haan, P., Luyten, R., van Veller, M., van Grinsven, M. Q. J. M. & Goldbach, R. (1995). Broad resistance to tospoviruses in transgenic tobacco plants expressing three tospoviral nucleoprotein gene sequences. *Molecular Plant-Microbe Interactions* **8**:85-91.

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

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M. & Tepfer, M. (2008). Strategies for antiviral resistance in transgenic plants. *Molecular Plant Pathology* **9**:73-83.

Prins, M., Lohuis, D., Schot, A. & Goldbach, R. (2005). Phage display-selected single-chain antibodies confer high levels of resistance against *Tomato spotted wilt virus*. *Journal of General Virology* **86**:2107-2113.

Prins, M., Resende, de O., Anker, Corine, van Schepen, de Haan, P. & Goldbach, R. (1996). Engineered RNA-mediated resistance to tomato spotted wilt virus is sequence specific. *Molecular Plant-Microbe Interactions* **9**:416-418.

Prins, M. (2005). Broad virus resistance in transgenic plants. *Trends in Biotechnology* **21**:373-375.

Qui, W. P., Geske, S. M., Hickey, C. M. & Moyer, J. W. (1998). Tomato spotted wilt tospovirus genome reassortment and genome segment-specific adaptation. *Virology* **244**:186-194.

Rasoulpour, R. & Izadpanah, K. (2007). Characterisation of cineraria strain of Tomato yellow ring virus from Iran. *Australasian Plant Pathology* **36**:286-294.

Reddy, D. V. R., Ratna, A. S., Sudarshana, M. R., Poul, F. & Kumar, I. K. (1992). Serological relationships and purification of bud necrosis virus, a tospovirus occurring in peanut (*Arachis hypogaea* L.) in India. *Annals of Applied Biology* **120**:279-286.

Reddy, D. V. R., Sudarshana, M. R, Ratna, A. S., Reddy, A. S., Amin, P. W., Kumar, I. K. & Murthy, A. K. (1991). The occurrence of yellow spot virus, a member of tomato spotted wilt group, on peanut (*Arachis hypogaea* L.) in India. In: Hsu, H. -T. & Lawson, R. H. (Editors). Virus-thrips-plant interactions of TSWV, *Proceedings USDA workshop, Beltsville*, April 18-19, 1990. USA ARS-87, National Technical Information Service, Springfield: 77-78.

Resende, R. de O., Posser, L., Nagata, I., Bezzera, C., Lima, M. I., Kitajima, E. W. & de Ávila, A. C. (1996). New tospoviruses found in Brazil. *Acta Horticulturae* **431**:78-79.

Ribeiro, D. (2007). Towards understanding TSWV particle assembly: Analysis of the interacellular behaviour of the viral structural proteins. PhD thesis, Wageningen University, The Netherlands.

Ribeiro, S. G. (2006). Diversity and host interactions of emerging tomato begomoviruses in Brazil. PhD thesis, Wageningen University, The Netheralnds.

Robb, K. L., Newman, J., Virzi, J. K. & Parrella, M. P. (1995). Insecticide resistance in western flower thrips. In: B. L. Parker, M. Skinner, and T. Lewis (Editors). *Thrips Biology and Management*. Plenum Press, New York: 33-40.

Roggero, P., Masenga, V. & Tavella, L. (2002). Field isolates of *Tomato spotted wilt virus* overcoming resistance in pepper and their spread to other hosts in Italy. *Plant Disease* **86**:950-954.

Roselló, S., Díez, M. J. & Nuez, F. (1996). Viral diseases causing the greatest economic losses to the tomato crop I. The Tomato spotted wilt virus - a review. *Scientia Horticulturae* **67**:117-150.

Rudolph, C., Schreier, P. H. & Uhrig, J. F. (2003). Peptide-mediated broad spectrum plant resistance to tospoviruses. *Proceedings of the National Academy of Sciences of the USA* **100**:4429-4434.

Sabariegos, R., Giménez-Barcons, M., Tàpia, N., Clotet, B. & Martínez, M. A. (2006). Sequence homology required by human immunodeficiency virus type1 to escape from short interfering RNAs. *Journal of Virology* **80**:571-577.

Sakimura, K. (1963). *Frankliniella fusca*, an additional vector for the tomato spotted wilt virus, with notes on *Thrips tabaci*, another vector. *Phytopathology* **53**:412-415.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory Press.

Samuel, G., Bald, J. G. & Pitman, H. A., (1930). Investigations of “spotted wilt” of tomatoes. Commonwealth of Australia, *Council of Science and Industrial Research Bulletin* 44.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the USA* **74**:5463-5467.

Satyanarayana, T., Mitchel, S. E., Reddy, D. V. R., Brown, S., Kresovich, S., Jarret, R., Nidu, R. A. & Demski, J. W. (1996). Peanut bud necrosis tospovirus S RNA: complete nucleotide sequence, genome organisation and homology to other tospoviruses. *Archives of Virology* **141**:85-98.

Schwartz, H. F., Brown, W. M., Blunt, T., Jr. & Gent, D. H. (2002). *Iris yellow spot virus* on onion in Colorado. *Plant Disease* **86**:560.

Schwarz, D. S., Hutvágner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**:199-208.

Shahraeen, N. & Ghotbi, T. (2003). Natural occurrence of different Tospovirus species infecting ornamentals and other agricultural crops in Iran. In ‘8th International Congress of Plant Pathology, Christchurch, New Zealand’. 23.26.

Shahraeen, N., Ghotbi, T. & Mehraban, A. H. (2002). Occurrence of *Impatiens necrotic spot virus* in ornamentals in Mahallat and Tehran provinces in Iran. *Plant Disease* **86**:694.

Stevens, M. R., Scott, S. J. & Gergerich, R. C. (1992). Inheritance of a gene for resistance to tomato spotted wilt virus (TSWV) from Lycopersicon peruvianum Mill. *Euphytica* **59**:9-17.

Simons, J. F., Persson, R., Pettersson, R.F. (1992). Association of the nonstructural protein NSs of *Uukuniemi virus* with the 40S ribosomal subunit. *Journal of Virology* **66**: 4233–4241.

Simons, J. F. & Pettersson, R. F. (1991). Host-derived 5′ ends and overlapping complementary 3′ ends of the two messenger RNAs transcribed from the ambisense S segment of *Uukuniemi virus*. *Journal of Virology* **65**: 4741-4748.

Sijen. T., Fleenor, J., Simmer, F., Thijssen, K. L., Parrish, S., Timmons, L., Plasterk, R. H. & Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. *Cell* **107**:465-476.

Sin, S. -H., McNalty, B. C., Kennedy, G. G. & Moyer, J. (2005). Viral genetic determinants for thrips transmission of Tomato spotted wilt virus. *Proceedings of the National Academy of Sciences of the USA* **102**:5168-5173.

Smith, K. M., (1932). Studies on plant virus diseases. XI. Further experiments with a ringspot virus. Its identification of spotted wilt of the tomato. *Annals Applied Biology* **19**:305-330.

Smith, N. A, Singh, S. P., Wang, M. -B., Stoutjesdijk, P. A., Green, A. G. & Waterhouse, P. M. 2000. Total silencing by intron-spliced hairpin RNAs. *Nature* **407**:319-320.

Snippe, M., Willem Borst, L., Goldbach, R. & Kormelink, R. (2007). Tomato spotted wilt virus G_C and N proteins interact in vivo. *Virology* **357**:115-123.

Sonoda, S. & Tsumuki, H. (2004). Analysis of RNA-mediated virus resistance by NS_S and NS_M gene sequences from *Tomato spotted wilt virus*. *Plant Science* **166**:771-778.

Spassova, M. I., Prins, T. W., Folkertsma, R. T., Klein-Lankhorst, R. M., Hille, J., Goldbach, R. W. & Prins, M. (2001). The tomato gene *Sw-5* is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco. *Molecular Breeding* **7**:151-161.

Stoeva, P., Yankulova, M., Nicolaeva, V., Bachvraova, R., Ivanova, L., Maiss, E., Adam, G., Vulkov, V., Guelemerov, S. & Atanassov, A. (1998). Long-term resistance to tomato spotted wilt virus in transgenic tobacco cultivars expressing the viral nucleoprotein gene: greenhouse and field tests. *Molecular Breeding* **4**:155-164.

Storms, M. M. H., Kormelink, R., Peters, D., van Lent, J. W. M. & Goldbach, R. (1995). The non-structural NS_M protein of Tomato spotted wilt virus induces tubular structures in plant and insect cells. *Virology* **214**:485-493.

Swofford, D. L. (1993). PAUP: Phylogenetic analysis using parsimony. Version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, IL.

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

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

Takeuchi, S., Okuda, M., Hanada, K., Kawada, Y. & Kameya-Iwaki, M. (2001). Spotted wilt disease of cucumber (*Cucumis sativus*) caused by melon yellow spot virus. *Japanese Journal of Phytopathology* **67**:46-51.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**:4673-4680.

Tsuda, S. Fujisawa, I., Ohnishi, J., Hosokawa, D. & Tomary, K. (1996). Localization of tomato spotted wilt tospovirus in larvae and pupae of the insect vector *Thrips setosus*. *Phytopathology* **86**:1199-1203.

Uhde, K., Fischer, R. & Commandeur, U. (2005). Expression of multiple foeign epitopes presented as synthetic antigens on the surface of Potato virus X particles. *Archives of Virology* **150**:327-340.

Ullman, D. E., Cho, J. J., Mau, R. F. L., Hunter, W. B., Westcot & Custer D. M., (1992). Thrips-tomato spotted wilt virus interactions: morphological, behavioural and cellular components influencing thrips transmission. In: *Advances in Disease Vector Research*, Editor. K.F. Harris, 9:195-240. New York: Springer-Verlag.

Ullman, D. E., German, T. L., Sherwood, J. L., Westcot, D. M. & Cantone, F. A. (1993). Tospovirus replication in insect vector cells: immuncytochemical evidence that the non-structural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. *Phytopathology* **83**:456-463.

Ullman, D. E., Sherwood, J. L. & German, T. L. (1997). Thrips as vectors pf plant pathogen. In: T. Lewis (Editor). *Thrips as Crop Pests*. CAB International, Oxon, U.K. 539-565.

Ullman, D. E., Westcot, D. M., Hunter, W. B. & Mau, R. F. L. (1989). Internal anatomy and morphology of *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) with special reference to interactions between thrips and tomato spotted wilt virus. *International Journal of Insect Morphology and Embryology* **18**:289-310.

Ullman, D. E., Whitfield, A. E. & German, T. (2005). Thrips and tospoviruses come of age. Mapping determinants of insect transmission. *Proceedings of the National Academy of Sciences of the USA* **102**:4931-4932.

Ultzen, T., Gielen, J. J. L., Venema, F., Westerbroek, A., de Haan, P., Tan, M. -L., Schram, A., van Grinsven, M. Q. J. M. & Goldbach, R. (1995). Resistance to tomato spotted wilt virus in tomato hybrids. *Euphytica* **85**:159-168.

Vaira, A. M., Roggero, P., Luisoni, E., Milne, R. G. & Lisa, V. (1993). Characterisation of two tospoviruses in Italy: tomato spotted wilt virus and impatiens necrotic spot virus. *Plant Pathology* **42**:530-542.

van den Hurk, J., Tas, P. W. L. & Peters, D. (1977). The ribonucleic acid of tomato spotted wilt virus. *Journal of General Virology* **36**:81-91.

van de Wetering, F., Goldbach, R. & Peters, D. (1996). Tomato spotted wilt tospovirus ingestion by first instar larvae of *Frankliniella occidentalis* is a prerequisite for transmission. *Phytopathology* **86**:900-905.

van Knippenberg, I., Goldbach, R. & Kormelink, R. (2005). Tomato spotted wilt virus S-segment mRNAs have overlapping 3´-ends containing a predicted stem-loop structure and conserved sequence motif. *Virus Research* **110**:125-131.

van Poelwijk, F., Boye, K., Oosterling, R., Peters, D. & Goldbach, R. (1993). Detection of the L protein of tomato spotted wilt virus. *Virology* **197**:468-470.

Voinnet, O. (2005). Induction and suppression of RNA silencing: insights from viral infections. *Nature Reviews Genetics* **6**:206-220.

Vijayalakshmi, K. (1994). Transmission and ecology of *Thrips palmi* Karny, the vector of peanut bud necrosis virus. PhD thesis, Andrha Pradesh Agricultural University, Hyderabad, 99 pp.

Wang, M. & Gonsalves, D. (1990). ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. *Plant Disease* **74**:154–158.

Wassenegger M. & Krczal, G. (2006). Nomenclature and functions of RNA-directed RNA polymerases. *Trends in Plant Science* **11**:142-151.

Watkins, C.A. and Jones, I.M., (1993). Association of the 40S ribosomal subunit with the NSs nonstructural protein of Punta Toro virus. In: Proceedings of the International Congress of Virology, Glasgow, p. 136.

Webb, S., Tsai, J. & Mitchell, F. (1998). Biomics of *Frankliniella bispinosa* and its transmission of tomato spotted wilt virus. In: Peters, D. & Goldbach, R. (Editors). *Recent Progress in Tospovirus and Thrips Research*, Wageningen, the Netherlands: 67.

Webster, K. D. & Barker, H. (1998). Detection and quantification of transcript RNA in transgenic plants using digoxigenin-labelled cDNA probes. In: Foster G. D., (Editor) In *Methods in Molecular Biology: Plant Virology*. Vol. 20. Totowa, NJ: Humana Press; pp. 437-445.

Whitfield, A. E., Ullman, D. E. & German, T. L. (2005). Tospovirus-Thrips Interactions. *Annual Review of Phytopathology* **43**:459-489.

Wijkamp, I., Almarza, R., Goldbach, R. & Peters, D. (1995). Distinct levels of specificity in thrips transmission of tospoviruses. *Phytopathology* **85**:1069-1074.

Wijkamp, I. & Peters, D. (1993). Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. *Phytopathology* **83**:986-991.

Wijkamp, I., van de Wetering, F., Goldbach, R. & Peters, D. (1996). Transmission of tomato spotted wilt virus by *Frankliniella occidentalis*; median acquisition and inoculation access period. *Annals of applied biology* **129**:303-313.

Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R. & Peters, D. (1993). Multiplication of tomato spotted wilt virus in its vector, *Frankliniella occidentalis*. *Journal of General Virology* **74**:341-349.

Yeh, S. -D. & Chang, T. F. (1995). Nucleotide sequence of the N gene of watermelon silver mottle virus, a proposed new member of the genus Tospovirus. *Phytopathology* **85**:58-64.

Yeh, S. -D., Lin, Y. -C., Cheng, Y., -H., Jih, C. -L., Chen, M. -J., & Chen, C. -C. (1992). Identification of tomato spotted wilt-like virus on watermelon in Taiwan. *Plant Disease* **76**:835-840.

Zhang, X., Yuan, Y. -R., Pei, Y., Lin, S. -S., Tuscl, T., Patel, D. J. & Chua, N. -H. (2006). Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute 1

cleavage activity to counter plant defense. *Genes and Development* **20**:3255-3268.

Zhao, G., Liu, W., Brown, J. M. & Knowles, O. (1995). Insecticide resistance in field and laboratory strains of western flower thrips (*Thysanoptera: Thripidae*). *Journal of Economic Entomology* **88**:1164-1170.

Ziegler, A., Cowan, G. H., Torrance, L. Ross, H. A. & Davis, H. V. (2000). Facile assessment of cDNA constructs for expression of functional antibodies in plants using the potato virus X vector. *Molecular Breeding* **6**:327-335.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridisation prediction. *Nucleic Acids Research* **31**:3406-3415.

Summary

During the past decades, an increasing number of new tospovirus species occurring in various agricultural and horticultural crops have been reported. The emergence of new tospoviruses may be attributed to intensified international trading, to increasing problems to control their thrips vectors, but certainly also by better recognition based on new diagnostic tools.

The works presented in the thesis first focused on comprehensive characterisation and identification of a tospovirus species occurring in different crops in Iran, and next on transgenic approaches to control this virus.

In Chapter 2, five presumed Iranian tospovirus isolates from tomato, chrysanthemum, gazania, soybean and potato, collected in 2002, were analysed. All isolates induced necrotic local lesions on *Petunia hybrida*, indicative for tospoviruses. None of the available antisera against known tospoviruses reacted with the isolates, suggesting that if these were tospoviruses, they should belong to a novel species. As a next step the viral nucleoprotein (N) gene of the tomato isolate was cloned and sequenced and this information demonstrated that it represented a new tospovirus species for which the name *Tomato yellow ring virus* (TYRV) was proposed. Subsequently the N gene sequences of the chrysanthemum and gazania isolates were also obtained and showed these isolates to represent TYRV as well. Back-inoculation of the tomato isolate induced resembling chlorotic and necrotic spots on leaves and yellow rings on the tomato fruits. The complete S RNA sequence of this isolate revealed the generic topology of a tospoviral S RNA, containing both NS_S (suppressor of silencing) and N (nucleocapsid) genes separated by a long non-coding intergenic region including a predicted hairpin structure. Multiple sequence alignment of the N protein of TYRV with those of established tospovirus species revealed the closest relationship (74% identity) to *Iris yellow spot virus* (IYSV).

In chapter 3, the isolates from soybean and potato were analysed. Although in ELISA assays these isolates scored positive with antiserum raised against the TYRV-tomato isolate, they failed to amplify in RT-PCR when using primers

derived from the latter. The N gene sequences of these isolates indeed revealed a sequence divergence of 8% compared to that of TYRV-tomato, indicating these two belonged to different strains of TYRV (denoted TYRV-s, while the strain occurring in tomato was named TYRV-t). Additional differences between the two strains were found in their respective S RNA non-coding intergenic regions. Differences in their host range and symptom expression underscored the decision to treat them as separate strains. A preliminary cross-protection study indicated that TYRV-t and TYRV-s mutually exclude each other, indicating that the strains represent stable, isolated lineages which do not easily converge despite their geographical overlap.

In chapter 4, extended inverted repeat transgenic cassettes for broad tospoviral resistance were constructed and tested. These transgene cassettes contained partial N gene sequences from 5 different tomato-infecting tospoviruses, including TYRV-t from Iran, in such arrangement that transgenic expression would deliver a ds hairpin RNA. Using *Nicotiana benthamiana* as a model plant, transgenic lines harboring an inverted repeat construct interspaced with a sense-oriented intron were obtained with high frequencies of resistance up to 100% for all 5 tospoviruses in F2. By analysing the siRNA content of the transgenic plants it could be verified that the transgenic resistance was based on RNA silencing (or shortly RNAi). Whilst these transgenics were fully resistant to TYRV-t (whose N gene sequence was used in the transgene cassette), they were fully susceptible to TYRV-s, demonstrating again how narrow transgenic resistance based on RNAi is. Surprisingly upon co-inoculation with TYRV-s, TYRV-t also could overcome the transgenic resistance. Mass spectrometric analysis of viral ribonucleocapsid protein (RNP) purified from a mixed-infected transgenic line revealed that the N protein of both strains were present and hence hetero-encapsidation as possible mechanism to rescue TYRV-t from these plants could be excluded. Experiments involving the expression of the viral suppressor protein (NSs) from TYRV-s using a PVX vector, indicated that rescue of TYRV-t by TYRV-s was based on the expression of the TYRV-s RNAi suppressor.

Since most of the operational transgenic tospovirus resistance approaches are based on RNAi, involving the production of transgenic viral siRNAs, in chapter 5 the production and involvement of viral siRNA molecules during a natural tospovirus infection process was investigated. Special attention was given to the intergenic hairpin region of the S RNA segment. Total small RNA was isolated from TYRV-t infected *N. benthamiana* and mapped to the S RNA segment. These studies demonstrated the occurrence of a hot spot for siRNA induction within the S RNA, but surprisingly this hotspot was not mapped in the IGR hairpin but at the start of NS_S ORF where a much shorter hairpin structure was predicted. Surprisingly, fewest siRNAs mapped to the intergenic region which is predicted to fold into a long hairpin, despite additional experiments involving DICER cleavage by *Drosophila melanogaster* embryo extract and YFP (yellow fluorescent protein)-hairpin constructs indicating this region to be a potential inducer and target for RNAi.

In chapter 6, the observations done in the experimental chapters are discussed in a broader context and a model for tospovirus-induced RNA silencing presented.

Samenvatting

Gedurende de afgelopen decennia is een toenemend aantal nieuwe tospovirussen gerapporteerd in land- en tuinbouwgewassen. Het verschijnen van nieuwe tospovirussen kan enerzijds toegeschreven worden aan de toenemende internationale handel en de problemen om de tripsvectoren te beheersen, en anderzijds aan verbeterde detectie gebruikmakend van nieuwe diagnostische technieken.

Het in dit proefschrift beschreven onderzoek richtte zich eerst op de identificatie en karakterisering van een nog onbekend tospovirus dat in verschillende gewassen in Iran voorkomt, en vervolgens op de ontwikkeling van transgene strategiëen om dit virus te beheersen.

In hoofdstuk 2 worden 5 Iraanse tospovirus isolaten uit tomaat, chrysant, “gazania”, soja en aardappel, allen verzameld in 2002, geanalyseerd. Alle isolaten induceerden lokale, necrotische lesies op *Petunia hybrida*, indicatief voor een tospovirusinfectie. Echter, geen van de beschikbare tospovirus antisera reageerde met deze isolaten, en dit wees er op dat het hier om een nieuwe tospovirus soort zou kunnen gaan. In een volgende stap werd van het tomatenisolaat het gen coderend voor het nucleocapside eiwit (N) gekloneerd en de nucleotidenvolgorde bepaald. Daarmee kon definitief worden vastgesteld dat het inderdaad een nieuw tospovirus betrof waarvoor de naam *Tomato yellow ring virus* (TYRV) werd voorgesteld. Bepaling van de N gen sequenties van de chrysanten- en gazania-isolaten liet zien dat ook deze isolaten tot TYRV behoren. De volledige nucleotidenvolgorde van het S RNA van het tomatenisolaat toonde aan dat dit genoomsegment, kenmerkend voor tospovirussen, ambisense is, voorzien van twee genen, coderend voor NS_S (de virale RNAi suppressor) en N (Nucleocapside eiwit), en onderling gescheiden door een lange, niet-coderende intergene regio met daarin een voorspelde dubbelstrengs haarspeldstructuur. Een vergelijkende analyse op basis van de aminozuurvolgorden van de N eiwitten toonde aan dat TYRV de grootste verwantschap (74% identiteit in N eiwit) vertoont met *Iris yellow spot virus* (IYSV).

In hoofdstuk 3 werden vervolgens ook de virusisolaten uit soja en aardappel geanalyseerd. Hoewel beide isolaten positief reageerden in een DAS-ELISA met antiserum opgewekt tegen het TYRV-tomatenisolaat, trad er geen amplificatie op tijdens een RT-PCR met primers specifiek voor de laatste. Nader onderzoek liet zien dat de nucleotidenvolgorde van hun N genen maar liefst 8% verschilde met dat van het TYRV-tomaat isolaat. Gezien de overige biologische (waardplantbereik en symptomen) en moleculaire (in S RNA) verschillen rechtvaardigde dit de beslissing om hier van verschillende TYRV stammen te spreken, te weten TYRV-t voor het isolaat in tomaat, en TYRV-s voor de isolaten in soja en aardappel. Een pilot studie naar cross-protectie wees uit dat TYRV-t en TYRV-s elkaar in tomaat wederzijds uitsluiten, hetgeen er opnieuw op wijst dat beide stammen stabiele, geisoleerde lijnen vormen die ondanks hun geografische overlap niet gemakkelijk samen voorkomen.

In hoofdstuk 4 zijn chimaere N-transgencasettes voor het verkrijgen van brede tospovirusresistentie gemaakt en getest. De casettes bevatten korte delen van de N genen van 5 verschillende tomaat-infecterende tospovirussen, waaronder TYRV-t, in een geinverteerde repetitie zodat transgene expressie zou leiden tot synthese van een dubbel-strengs haarspeld RNA molecuul. Trangene *Nicotiana benthamiana* planten, voorzien van een dergelijk transgenconstruct waarin bovendien een “sense”-georiënteerd intron was aangebracht, bleken in de F2 generatie een hoge resistentiefrequentie (tot 100%) te hebben tegen alle 5 tospovirussen. Door analyse van het siRNA profiel van de transgene planten kon vervolgens worden vastgesteld dat de resistentie ook voor TYRV gebaseerd was op “RNA silencing” (kortweg RNAi). Terwijl de transgene lijnen volledig resistent waren tegen TYRV-t (waarvan de N gen sequentie was gebruikt in de transgencasette), bleken ze volledig vatbaar voor infectie met TYRV-s, waarmee nogmaals de hoge sequentiespecificiteit van transgene resistentie werd bevestigd. Nog verrassender was dat co-incolatie met TYRV-s leidde tot resistentiedoorbreking door TYRV-t. Massa-spectrometrische analyse van een gezuiverde virale ribonucleoproteïne (RNP) fractie uit deze dubbel-geinoculeerde transgene lijn liet zien dat beide N eiwitten aanwezig waren en

hiermee werd hetero-encapsidatie als mogelijk mechanisme voor resistentiedoorbreking uitgesloten. Gebruikmakend van een PVX expressievector kon worden aangetoond dat het door TYRV-s gecodeerde NSs eiwit (de RNA silencing suppressor) verantwoordelijk was voor het doorbrekingseffect.

Aangezien transgene vormen van tospovirusresistentie meestal zijn gebaseerd op RNAi, waarin transgene virale siRNAs worden geproduceerd, werd in hoofdstuk 5 de produktie en de betrokkenheid van virale siRNAs gedurende het natuurlijke tospovirus infectieproces nader bestudeerd. Hierbij werd speciale aandacht besteed aan de intergene hairpinregio van het S RNA segment. Deze analyses wezen uit dat er weliswaar een hot-spot voor siRNA inductie in het S RNA segment voorkomt, maar dat deze niet samenvalt met de intergene hairpin regio maar met de start van het NSs open leesraam waar een veel kleinere hairpinstructuur werd voorspeld. Het was zelfs zo dat de intergene hairpinregio het minst vertegenwoordigd was onder de gekarteerde siRNAs, ondanks de bevindingen in verschillende assays dat deze regio potentieel een sterke inductor van RNAi kan zijn.

In hoofdstuk 6 worden de bevindingen van de experimentele hoofdstukken in een bredere context besproken en een model voor interactie tussen tospovirussen en het RNAi mechanisme van de waardplant gepresenteerd.

Acknowledgements

Now looking back at my last 5 years, I realise that I have made many achievements and I have gained both professional and social experiences. I am grateful to many persons and with the risk that I will forget some of them I want to acknowledge them here. First of all I would like to thank the Dutch Ministry of Agriculture, Nature and Food Quality for financially supporting my PhD study at Wageningen University, in particularly *Mr. T. Breimer* for his efforts. Also I am grateful to my home institution, *Mr. Ahmadreza Abbasifar and Mr. Mojtaba Ghalandar*, Agricultural Research Centre of Markazi province and *Dr. Behzad Gharehyazie*, the Ex-director of Agricultural Research and Eductaion Organisation (AREO) of Iran, for allowing me my long-term leave to the Netherlands.

I am deeply indebted to my promoter, *Prof. Rob Goldbach*, and co-promoter, *Dr. Richard Kormelink*, for their continuous support both socially and scientifically.

Dear *Rob*, you gave me the opportunity to join your research group, writing all those formal letters as nowadays required when hosting an Iranian scientist. I would like to thank you for all your supports, encouragement and enthusiasm as well as your great advices how to proceed my experiments, how to improve my conceptual thinking and how to finalise the chapters of my thesis. Indeed I would never forget your support during my accident, not to work for almost one year, and afterwards. You have been both kind and very serious during our work discussions and I got used to this Dutch habit to be direct, which helped me a lot to be more efficient, Harstikke bedankt voor alles.

Now I have to appreciate my co-promoter and daily supervisor that I learned a lot from him. Dear *Richard*, I just say to myself that it was my lucky fortune to have such a supervisor like you. You also supported me at any time during failure of certain experiments and in periods when results seemed hard to obtain. You always were available for even my simple questions without any complain easily because you always say “Vragen kost niks”. I know how long you spent your time to guide me for improving experimental set-ups,

interpretations of results and preparing our manuscripts. Well, like your Ex-PhD students I never forget the special day “I love Richard day” we spent with one another and I should honestly say that you have been my best teacher, thank you so much.

Meantime it was my pleasure to meet and work with *Dr. Dick Peters*, a man always on the move, even after his retirement. Dear *Dick*, we spent a lot of time to set up thrips transmission experiments, and although these were not so fruitful, I am very happy to say I learned a lot from you also for your help in analysing host range data, thank you very much.

Then I would like to thank *Dr. Marcel Prins* who was helpful for me in our bi-weekly lunch discussions and during my talks in the colloquium about transgenic resistance. Dear *Marcel*, I also would like to appreciate you for inviting me next to your Ex-PhD students for outdoor activities.

Well, I should thank two great technicians in plant lab, *Janneke Saaijer* and *Dick Lohuis*. Indeed you gave me your great hands to do several experiments at the same time. Dear *Janneke*, I started my work with purifying tospoviral RNP, and to practicise serology and plant experiments; well you showed me how to be precise and patient to master such and many other techniques. I will never forget your great support and sympathy to me and my family during unexpected events which happened to us. Dear *Dick*, your expertise in molecular techniques has been useful not only for me but also for everybody in the lab. Your great experience in siRNA purification, blotting and so on was significant for me to finish very crucial experiments, dank je wel.

I also wish to thank my fellow PhD students, *Hans Hemmes* for his guidance in performing those biochemical experiments ending up with nice publishable results, and *Esther Schnettler*, the most precise PhD student I met and always so helpful to my work in isotope lab and also during work discussions. Dear *Esther*, you also kindly accepted to be one of my paranimphs, Danke sehr und mit besten Wünschen.

It was a great pleasure for me to share my office with *Ingeborg van Knippenberg*, *Etienne Bucher* and *Christina Geerts-Dimitriadou*. I learned a lot

from all of you, also humorwise. Dear *Christina*, you always had a good attitude to me and my family, thank you very much for your supports. *Marjolein Snippe*, you kindly allowed me to share your computer during the first month after my arrival, thank you.

I also had a chance to get acquainted to other PhD students in the plant lab: *Simone Ribeiro*, *Cristiano Lacorte, Daniela Ribeiro*, dear friends we often ended working just before the alarm would be activated and also mostly all weekends. I learned a lot from you except Portuguese which was the second major language in our lab for several years, thank you all and also *Erico Dianese* who recently joined to the Plant lab.

I also want to extend my gratitude to our helpful secretary *Thea van Bemmel*. Dear *Thea*, I don’t know exactly how many times I asked you to read and translate those letters in Dutch or to fill my data in different forms.

I also would like to thank *Wout Rozeboom* for his help to order and support chemicals as soon as possible.

I wish to thank all colleagues, PhD and MSc students worked or working in Baculo-, Whispo- and Plant labs for their nice friendship and attentions to me.

Well, Mojgan and I have found also very good Dutch and Iranian friends during our study at Wageningen University. Herewith, I would like to thank all of you: *Nel* and *Ate de Heij-Wiltenburg*, you were very kind and helpful for the whole family and always follow-up our future. Thank you very much for your great attention. We also knew *Margreet and Klaas Zwart-Klomp*, you and your daughters were kind and helpful to the whole family. I have to thank *Ms. Heleen van Haaften* for her support, showing different places in the Netherlands and providing great hospitality for almost one year in her house. Then, I should thank all Iranians I met in Wageningen, especially *Ms. Fatemeh Nouri* who was very kind and helpful to my family. I am pleased to thank our young and talent Iranian students *Sina Salim* and *Kamyar Pournazari* for their unforgettable helps.

I also had the opportunity to know around 30 Iranian PhD students with their respected families: *Reza Aghnoum*, you and your family were helpful to my family and you kindly accepted to be one of my paranimphs, thank you very

much for everything. *Mahmoud Tabib Ghaffary*, you, your wife and your daughter were very supportive for us and honestly I gained many experiences from you, especial thanks to you and your respected family. I am also proud of having friends like *Hedayat Bagheri*, *Mohammad Balali*, *Mohammadali Nematollahi*, *Arash Azarfar*, *Rahim Mehrabi*, *Hossein Jafary*, *Mostafa Karbasioun*, *Benyamin Houshyani Hassanzadeh*, *Alireza Seifi*, *Mahmoud Otroshy*, *Bouda Vosough Ahmadi* and others with their families.

Last but not least, I should express my appreciation to my parents and family. I owe my life and whatever I have learned to my parents. Thank you for all your sacrifices you have done for me. To my wife, dear *Mojgan*, you did a great job to study here and meantime to treat our lovely sons. I am very happy that you understood my situation and allowed me to work longer in the lab even during weekends. I also would like to express my appreciation to my parents-in-law who have accepted my financial commitment to the AREO and helped my family especially with taking care of my twins, *Shayan* and *Noyan*, and *Borna* during finalising my thesis book. Finally, I wish to thank my sisters, *Afsoun* and *Afra*, who always encourage me to further pursue my education.

About the author

Afshin Hassani-Mehraban was born on 25th of February 1966 in Teheran, Iran. After finishing high school in Biological Sciences in 1984 in Teheran, he started his academic education towards ASc degree in the field of Agronomy at Buali Sina University in Hamedan, Iran in 1987. He continued his study to obtain his BSc degree in the field of Plant Protection in 1990 at Isfahan University of Technology in Isfahan, Iran. Then he worked at the Ministry of Agriculture of Iran, Deputy of Horticulture, as a plant protectionist for 1.5 years. In 1994 he was awarded a scholarship from Agricultural Research & Education Organisation, Iran, to continue his MSc degree in the field of Plant Pathology at Ferdowsi University of Mashhad, Mashhad, Iran. His MSc dissertation was focused on the inverstigation and distribution of *lettuce mosaic virus* in the lettuce fields of Mashhad region. After finishing his MSc study, he worked for 5 years as a researcher at Agricultural Research Centre of Markazi Province, Arak, Iran. In November 2002, he strated his PhD program at Wageningen University and Research centre, laboratory of Virology, that financially supported by the Dutch Ministry of Agriculture, Nature and Food Quality. Finally, he was involved in an eight-month postdoc fellowship during the last months of his PhD study at the laboratory of Virology.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: **Afshin Hassani-Mehraban**
Date: **September 08, 2008**
Group: **Laboratory of Virology, Wageningen University**

1) Start-up phase	date
First presentation of your project	
Characterisation of Tomato spotted wilt virus and other tospoviruses from Iran	Apr 11, 2003
Writing or rewriting a project proposal	
Writing a review or book chapter	
MSc courses	
MOB-20304 Genetechnology	Jun 27, 2003
PHP 30806 Molecular Aspects of Bio-Interactions	Jan 10 , 2004
Laboratory use of isotopes	
Safe handling of radioactive materials and sources, level 5B	Aug 01, 2004
Subtotal Start-up Phase	9.0 credits*

2) Scientific Exposure	date
EPS PhD student days	
EPS PhD Student Day, Nijmegen University	Jun 02, 2005
EPS PhD Student Day, Wageningen University	Sep 19, 2006
EPS theme symposia	
Theme 2 Symposium "Interactions between Plants and Biotic Agents" Wageningen University	Sep 17, 2004
Theme 2 Symposium "Interactions between Plants and Biotic Agents" Leiden University	Jun 23, 2005
Theme 2 Symposium "Interactions between Plants and Biotic Agents", University of Amsterdam	Feb 02, 2007
NWO Lunteren days and other National Platforms	
Dutch meeting of Plant Virologists, Wageningen, The Netherlands	2003
NWO-ALW Lunteren meeting	Apr 07-08, 2003
Dutch Annual Virology Symposium 2003, Amsterdam, The Netherlands	Mar 07, 2003
NWO-ALW Lunteren meeting	Apr 06-07, 2004
Dutch Annual Virology Symposium 2004, Amsterdam, The Netherlands	Mar 12, 2004
NWO-ALW Lunteren meeting	Apr 04-05, 2005
Dutch Annual Virology Symposium 2005, Amsterdam, The Netherlands	Mar 04, 2005
NWO-ALW Lunteren meeting	Apr 03-04, 2008
Dutch Annual Virology Symposium 2006, Amsterdam, The Netherlands	Mar 10, 2006
NWO-ALW Lunteren meeting	Apr 02-03, 2007
Dutch Annual Virology Symposium 2007, Amsterdam, The Netherlands	Mar 09, 2007
NWO-ALW Lunteren meeting	Apr 07-08, 2008
Seminars (series), workshops and symposia:	
4th joint meeting of Dutch and German plant virologists, Wageningen, The Netherlands	Mar 10-11, 2005
Flying Seminar Prof. dr. James C. Carrington:"Diversification and evolution of small RNA pathways in plants" Wageningen University, The Netherlands	May 2006 Mar 25, 2007
Seminar plus	
Flying Seminar Plus Prof. dr. James C. Carrington, Wageningen University, The Netherlands	Mar 25, 2007
International symposia and congresses:	
Plant Biotechnology Symposium, Bonn, Germany	Oct 05, 2005
XIII International Congress on Molecular Plant-Microbe Interactions, Sorrento, Italy	Jul 21-27, 2007
Presentations:	
(Oral) Dutch meeting of Plant Virologists, Plant Research International, Wagenigen, The Netherlands	2003
(Poster) 4th joint meeting of Dutch and German plant virologists, Wageningen, The Netherlands	Mar 10-11, 2005
(Poster) EPS PhD Student Day, Nijmegen University, The Netherlands	Jun 2, 2005
(Poster) EPS PhD Student Day, Wageningen University, The Netherlands	Sep 19, 2006
(Poster) XIII International Congress on Molecular Plant-Microbe Interactions, Sorrento, Italy	Jul 21-27, 2007
(Oral) ALW meeting Experimental Plant Sciences, Lunteren, The Netherlands	Apr 07-08, 2008
IAB interview	Sep 18, 2006
Excursions	
Subtotal Scientific Exposure	14.3 credits*

3) In-Depth Studies	date
EPS courses or other PhD courses	
Proteomics, Wageningen University	Feb 27-Mar 02, 2006
Signaling in Plant Developments and Plant Defence; towards system biology, Wageningen University	Jun 19-21, 2006
Molecular Phylogenies: Reconstruction & Interpretation, Wageningen University	Oct 16-20, 2006
Journal club	
participated in literature discussion group ' Virology '	2002 - 2006
Individual research training	
Subtotal In-Depth Studies	6.9 credits*

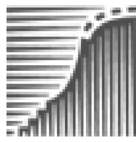
4) Personal development	date
Skill training courses	
Information Literacy	Oct 11-12, 2005
Techniques for presenting and writing a scientific paper	Feb 07-10, 2006
Organisation of PhD students day, course or conference	
Memberschip of Board, Committee or PhD council	
Subtotal Personal Development	1.8 credits*

TOTAL NUMBER OF CREDIT POINTS*	**32**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

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

The work described in this thesis was performed at the Laboratory of Virology of Wageningen University and was financially supported by the
Dutch Ministry of Agriculture, Nature and Food Quality.



**agriculture, nature
and food quality**

Agricutural Research and Education Organisation (AREO) of Iran for assisting PhD candidate's study in the Netherlands.

Layout and design by the author:

Front page: wild type *Nicotiana benthamiana* leaf infected with TYRV-t (left), tospoviral enveloped particle (centre - from Wageningen University, Laboratory of Virology), transgenic resistant *N. benthamiana* leaf upon TYRV-t mechanical inoculation (right).

Back page: Effect of the presence of TYRV-t hairpin sequence on RNA silencing of YFP in *N. benthamiana* leaves. (see chapter 5); framed with TYRV-predicted intergenic hairpin sequence.

Printed by: Propress Grafiko, Nudepark 144, 6702 DX Wageningen.

Appendix

Colour Figures

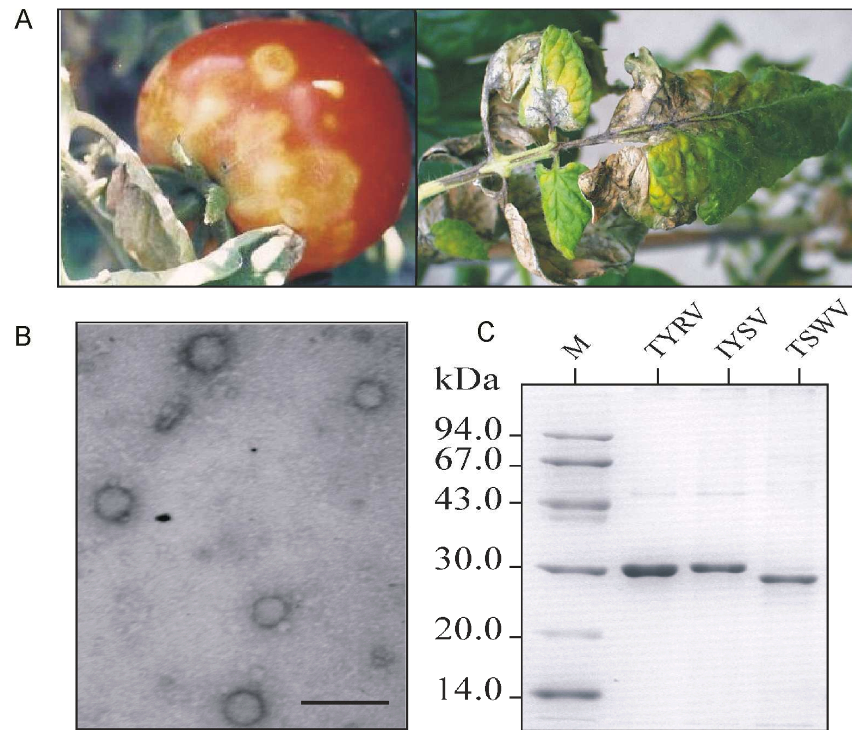


Figure 2-1

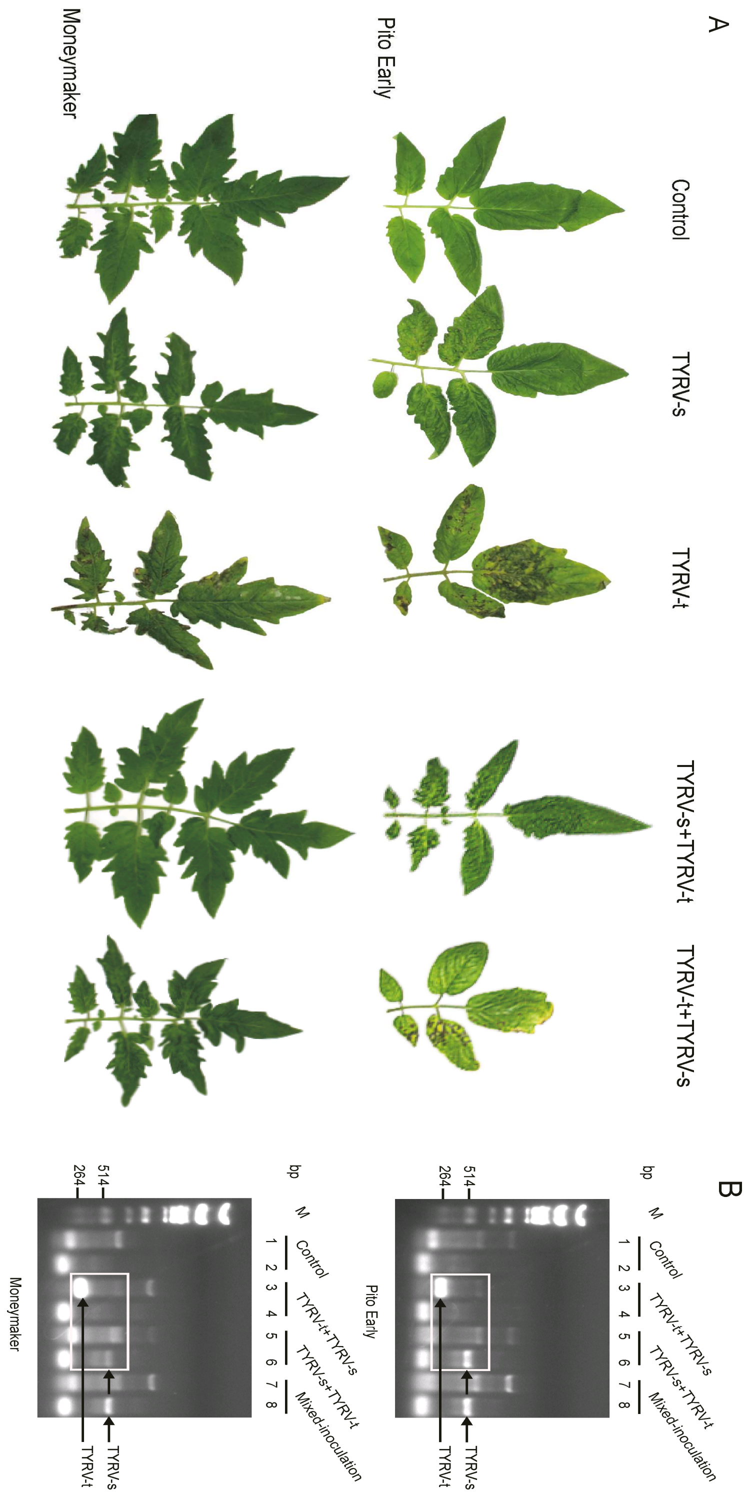


Figure 3-5

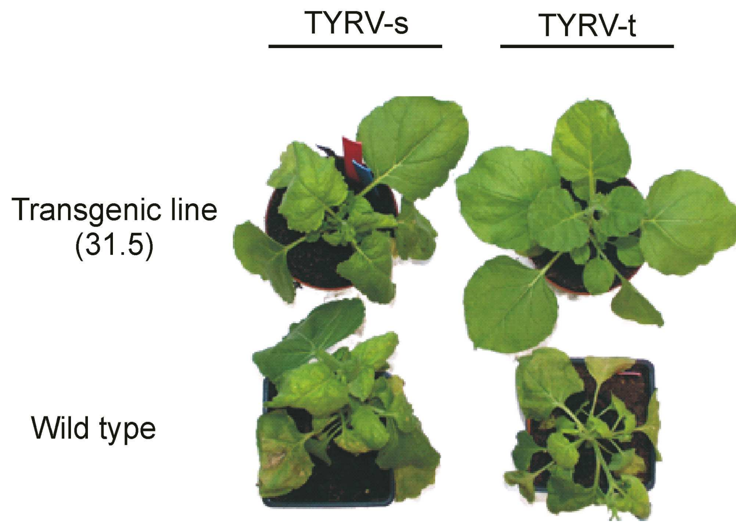


Figure 4-3

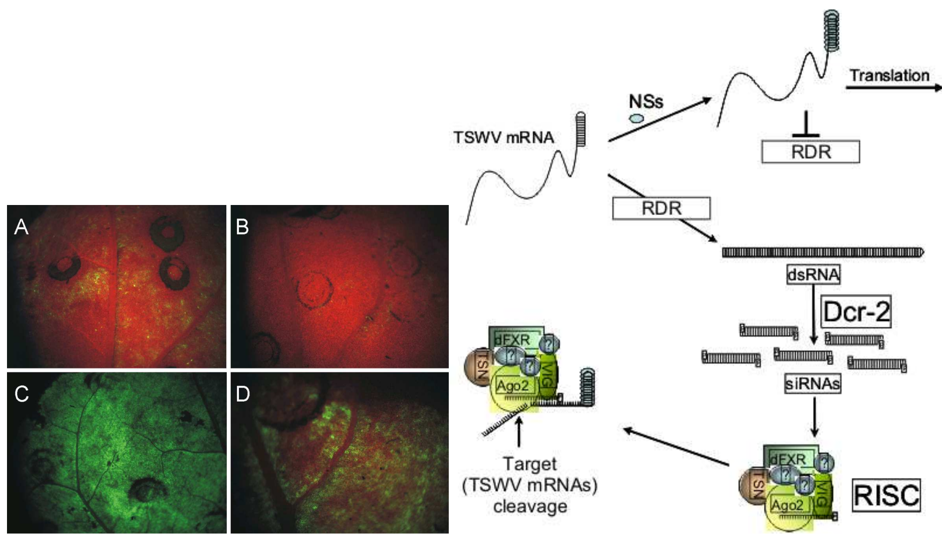


Figure 5-6

Figure 6-2