Analysis of

Tomato spotted wilt virus

effector-triggered immunity

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Voor mijn ouders.....

“Charles Darwin had a big idea, arguably the most powerful idea ever.
And like all the best ideas it is beguilingly simple”

Richard Dawkins

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“Nobody said it was easy”

The scientist
Coldplay (2002)

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Chapter 1

Dominant resistance against plant viruses:

a review

Abstract

To establish a successful infection plant viruses have to overcome a defence system composed of several layers. This chapter will overview the various strategies plants employ to combat viral infections with main emphasis and a state of the art description on single dominant resistance (*R*)-genes against plant viruses and the corresponding avirulence (*Avr*)-genes identified so far. The most common models to explain the mode of action of dominant *R*-genes will be presented. Finally, the hypersensitive response (HR) and extreme resistance (ER), both often triggered after induction of dominant *R*-genes but not the cause for resistance, will be described. In light of the scope of this thesis, the functional and structural similarity of *R*-genes of plants to sensors of innate immunity from animal cell systems will briefly be described.

I. Introduction

When looking around in nature, it is quite obvious to see that most plants are healthy and do not seem to suffer from any serious infection. This can only be true if plants, like all other organisms, have an advanced defence system. In past decades, scientists have shown that indeed plants have a unique and complex defence system that consists of several layers, which enables them to avoid, suppress or actively defend against pathogens from all kingdoms like fungi, bacteria, nematodes and viruses. Of all plant viruses known, only a few cause serious diseases and, if so, mostly limited to a very small number of crops. In general, most viruses have a limited (natural) host range and the number of so-called non-hosts exceeds those of hosts. In those plants that are hosts, viruses encounter different mechanisms of defence. Some plants act general against all viruses and this response is part of the innate immune system, while other plants are virus-specific hosts. In the latter case resistance genes of the host are involved that leads to necrosis at the site of virus entry upon activation, and prevents further infection of the entire host plant. In several cases resistance genes do not confer absolute resistance against a virus and low levels of virus replication can still be observed. In those cases the genes are also referred to as partial resistance genes or tolerance genes.

While throughout the years reviews on resistance genes have appeared with regular intervals, these mostly had their main focus on fungal and bacterial resistance genes, primarily due to the large amount of data available. This chapter aims to present an overview on the state of the art on resistance genes against plant viruses, with emphasis on single dominant resistance genes. Prior to this, a brief introduction will be given on the versatile ways plants try to combat viruses to prevent the establishment of an infection.

The very basis of the fact that plant viruses cannot infect all plants is due to a mechanism called non-host resistance (NHR) (For an extensive review on this, see Uma *et al.* (2011)). NHR holds for all plant pathogens and is a generic, nonspecific resistance against pathogens which is divided into two main types, distinguished by the mechanism and mode of recognition (Mysore and Ryu, 2004). Type 1 is the most pre-dominant type of NHR and presents a basic defence mechanism that prevents pathogen invasion, *e.g.* thickening of the cell-wall, secondary metabolite production, etc. This type of resistance usually is symptomless. In contrast, type 2 NHR is associated with induction of necrosis at the site of infection, and is induced when pathogens overcome type 1 resistance. Here, the pathogen is recognised through specific structures or proteins that are associated with the pathogen. The recognition of these structures/proteins, so called microbe associated molecular patterns (MAMPs) or PAMPS (Pathogen), takes place by pattern recognition receptors (PRRs) on plant plasma membranes. These PRRs recognise conserved structures of pathogens, like flaggelin from the flagella of bacteria or chitin from the cell wall of fungi, and induce a so called PAMP triggered immunity (PTI) response (Jones and Dangl, 2006).

Since plant viruses need to overcome the physical barrier of a cell wall, they enter their host cells either via mechanical inoculation or the infection is mediated by vectors like insects, nematodes or even fungi. Therefore, recognition by PRRs on the plasma membrane does not apply here. One of the first innate immune responses all plant viruses encounter when invading a host consists of antiviral RNA silencing (also called RNA interference (RNAi) and in the very early days post-transcriptional gene silencing (PTSG)). RNA silencing is a host response triggered by double stranded (ds)RNA. These molecules thus act as a MAMP/PAMP and in which RNAi can be regarded as PTI. The main difference with pathogens such as fungi and bacteria is that recognition of viral MAMPs/PAMPs occur intracellularly

(Ding and Voinnet, 2007).

RNA silencing mainly consists of two major 'branches'; the first one is that of small-interfering (si)RNAs, and one of the hallmarks for antiviral RNAi, and the second one is that of (host-gene encoded) micro (mi)RNAs involved in gene regulation. MicroRNAs and siRNAs share structural and functional similarities, but differ by their biogenesis pathways. Although dsDNA viruses have also been shown to encode miRNAs that are involved in the modulation of the hosts' innate immune responses, these will not be discussed here (for an extensive description of RNAi, see reviews on this by Ding (2010) and Sharma *et al.* (2012)). The antiviral RNAi response is induced by viral double stranded (ds)RNA molecules that arise from replicative intermediates or secondary RNA folding structures. These structures are sensed by a host RNase type III-like enzyme called Dicer-like (DCL) protein and cleaved into short interfering (si)RNA of 21-24 nucleotides (nt) in size (Sharma *et al.*, 2012). The siRNAs generated are unwound and only one strand, the so-called guide-strand, is uploaded into a functional protein complex termed RNA-induced silencing complex (RISC). This activated complex next surveils and subsequently degrades (viral) RNA target molecules with sequence complementarity to the guide-strand. Degradation of double stranded RNA is mediated by slicer, the core component of RISC, which is represented by a member of the Argonaut (AGO) family of proteins (Vaucheret, 2008; Sharma *et al.*, 2012). After primary siRNAs have been generated, in plants an amplification of siRNAs follows, which is required to mount an RNAi response that is effective to combat virus infections locally and systemically. This amplification involves host RNA dependent RNA polymerases (RDRs) that are able to convert (aberrant) viral (m)RNAs into dsRNA in a siRNA-dependent and -independent manner (Csorba *et al.*, 2009). Their subsequent processing by DCL leads to the generation of secondary siRNAs that correspond to sequences outside the primary target sequence, a process also called transitive silencing (Sijen *et al.*, 2001). The antiviral RNAi response acts against all RNA and DNA viruses (Incarbone and Dunoyer, 2013), but in general is a relatively slow process that does not lead to complete clearance of viral infections.

Besides RNAi, viruses may also run into another layer of defence that involves resistance genes. These are triggered by and confer resistance to a specific virus only. The major class of these genes represent single dominant resistance genes (described further below), while others are recessive, tolerance or partial resistance

genes. A very nice example of the latter case has recently been described with the cloning and characterisation of the *Ty-1* resistance gene from tomato against *Tomato yellow leaf curl* geminivirus (TYLCV) (Verlaan *et al.*, 2013). This gene encodes an RNA-dependent RNA polymerase (RdRp) that amplifies the RNAi signal, causing enhanced methylation of the viral DNA genome (siRNA directed DNA methylation: RdDM) and thereby leading to transcriptional silencing of geminivirus genes. Tomato plants containing *Ty-1* do not show symptoms upon challenge with TYLCV, but low levels of virus can still be detected (Verlaan, PhD-thesis 2013).

Recessive resistance (Truniger and Aranda, 2009) so far has mainly been described for viruses, and relies on the observation that viruses require host factors (also called susceptibility factors) to enable an infection. The inability of interaction between such host factor and the virus leads to resistance. Since susceptibility factors are dominant, a resistance based on these requires all gene copies to be in the (resistant) recessive state. This explains why such resistance is generally termed recessive resistance. The majority of the recessive resistance genes known against plant viruses have been reported for potyviruses (Kang *et al.*, 2005) and encode translation initiation factors of the 4E or 4G family (eIF4E/eIF4G) (Truniger and Aranda, 2009). The latter proteins need to interact with the cap-structure on (viral) transcripts, to allow for translation. Potyviral transcripts do not contain a cap structure, but provide a VPg (Virus-protein genome linked) to render their transcripts translatable in a cap-independent manner. Potyvirus infection leads to host shut off of cap-dependent transcripts, but only allow the cap-independent transcripts to be translated mediated by a subgroup of translation initiation factors; eIF(iso)4E/G. Viruses that encode their own cap-like structure (like potyviruses: VPg) require interaction with the translation initiation factors eIF4E/eIF4G for translation, this in turn induces a selection pressure on the host to escape the interaction between VPg and eIF4e, leading to recessive resistance. Recessive resistance towards other pathogens, such as fungi and bacteria have only limited been described and these susceptibility factors (*S*-gene) are proposed to provide a more durable resistance than dominant *R*-genes. However due to their functions they may cause pleiotropic effects when knocked out from the host genome (Gawehns *et al.*, 2013).

II. Dominant resistance

II.1 Effector-mediated triggering of single dominant resistance genes

Plant pathogens need to evade or suppress the PTI response in plants and do this by encoding effector proteins that can interfere with the recognition by PRRs, usually by binding to the substrate that PRRs would otherwise recognise. This process allows the pathogen to establish a successful infection, and is referred to as Effector Triggered Susceptibility (ETS) (Figure 1.1): a strategy that also applies to antiviral RNAi. One of the most common strategies plant viruses use to counteract RNAi is to encode RNA silencing suppressors (RSS), viral proteins that interfere with a specific part of the RNAi pathway and thereby reduce its effectiveness against plant viruses (Burgyan and Havelda, 2011). The majority of plant virus RSS proteins exert this activity through binding of small interfering (si)RNAs, or sometimes (also) long dsRNA, and thereby prevent their uploading into RISC and Dicer-cleavage, respectively (Lakatos *et al.*, 2006). In recent years some RSS have also been discovered to inhibit the RNAi pathway in other ways, *e.g.* by binding directly to key-enzymes proteins like AGO1, the core component of RISC during the antiviral RNAi response (Zhang *et al.*, 2006; Giner *et al.*, 2010). Viral suppression of RNAi leads to a stage of effector triggered susceptibility (ETS) during which viruses are able to establish a successful infection.

Single dominant resistance (*R*) gene products (in)directly sense the presence of a specific pathogen by their effector, termed avirulence factors (Avr), as a counter defence against ETS, leading to a stage called Effector-Triggered Immunity (ETI) (Figure 1.1). Triggering of *R*-genes is generally associated with a (concomitant) induction of a programmed cell death response, as visualised by the rapid appearance of necrotic lesions (a hypersensitive response, HR) or in rare occasions extreme resistance (ER) during which no necrosis is observed at all. However, more and more evidence is presented, that there is an uncoupling of the resistance response from the programmed cell death response, although both can work in concert. Due to these responses, viruses (and other pathogens) are confined to the site of entry/invasion where infections are prevented. In contrast to the mostly slow onset of antiviral RNAi the *R*-gene response generally is rapid, within ~3/4 days. The major class of *R*-genes encode proteins that, irrespective of the pathogen they recognise, consist of three domains; 1) the Nucleotide Binding Domain (NBS) in the

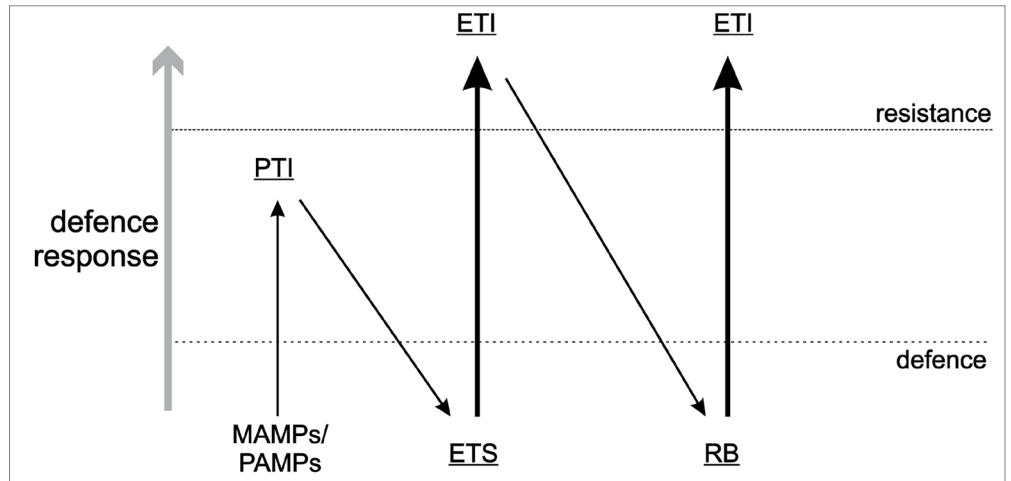


Figure 1.1 Zig-zag-model. The arms race between pathogen and host as illustrated in the 'Zig-zag-model' of Dangl and Jones (2006). Here, a modified version of that model shows the sequential responses as described in this chapter. MAMPs/PAMPs: Microbe/Pathogen associated molecular patterns, PTI: PAMP triggered immunity, ETS: Effector triggered susceptibility, ETI: Effector-triggered immunity, RB: resistance breaker.

centre of the protein, 2) a Leucine Rich Repeat (LRR) at the C-terminal end, and 3) a Coiled-coil (CC) or Toll and Interleukin-1 Receptor (TIR) domain at the N-terminal end of the resistance gene product (Moffett, 2009). The LRR determines the specificity of the target protein and is the most variable part of the protein, therefore considered to be under selection pressure to evolve for recognition of (new) target proteins. The NBS is composed of a conserved part that contains the Nucleotide Binding site (NB) and an ARC-domain, both required to bind and hydrolyse ATP. *R*-genes that contain an N-terminal TIR domain are only found in dicotyledonous plant species, and through this domain share homology to Toll-like receptor (TLR) proteins, that act as PRRs in the innate immunity response in animal systems. Those with a CC-domain, which has no predicted structure at its N-terminus, are also termed non-TIR group. All three domains are involved in an interaction with each other and change conformationally upon activation to subsequently induce the resistance response (Lukasik and Takken, 2009; Slootweg *et al.*, 2010).

Only a few cases have been described in which the dominant *R*-gene product recognises an Avr-protein through direct interaction, one of which is a viral Avr-protein (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006; Ueda *et al.*, 2006; Cesari *et al.*, 2013). In the majority of known *R*-genes recognition of the pathogen occurs indirectly and involves host proteins, which are considered guardees, decoys or baits, depending on the model, as further discussed below (II.3) (van der Biezen and Jones, 1998; Jones and Dangl, 2006; van der Hoorn and Kamoun, 2008; Collier and Moffett, 2009).

II.2 Cloned *R*-genes and their known Avr-determinants

While for fungi and bacteria many resistance genes have been cloned and characterised, resistance genes against plant viruses have received growing interest during the last two decades, but still only few have been cloned so far. Table S1.1 gives an up-to-date summary of all *R*-genes against plant viruses, known or under investigation thus far. For some of these genes the viral *Avr*-determinant has been identified. From this large, extensive list of *R*-genes, only 22 have been cloned and characterised. Some *R*-genes have functional alleles in other plant species, often showing a similar Avr-recognition. The majority of the known *R*-gene products are of the CC-NB-LRR type, whereas only a small group belongs to the TIR-NB-LRR group (Table 1.1).

Dominant *R*-genes against viruses have been described that do not belong to the NB-LRR type of genes, *i.e.* the *RTM1*, *RTM2* and *RTM3* resistance genes have been identified from *A. thaliana*, which prevent the systemic spread of several potyviruses. In those cases the virus is not able to upload into the phloem to systemically disseminate into the host. In addition, there is also no induction of HR or production of salicylic acid (SA), as commonly observed with NB-LRR mediated resistance responses (Cosson *et al.*, 2012). No direct interaction occurs between the RTM proteins with the potyvirus CP (Avr) protein. Another type of *R*-gene is *Tm-1*, found in the wild tomato species *S. hirsutum*, encoding a protein that contains a TIM-barrel. This barrel binds the replication proteins of *Tomato mosaic virus* (ToMV) and thereby inhibits RNA replication (Ishibashi *et al.*, 2007). Also here, no typical NB-LRR type-associated response, like HR, is induced. Many homologs of *Tm-1* are found in other organisms like fungi, archae and bacteria, suggesting that this gene

(originally) presents a more common household gene (Ishibashi *et al.*, 2012). Both *RTM* and *Tm-1* seem to play a role in the inhibition of a specific step required for successful infection by the virus. Whether these present a new class of dominant resistance genes remains to be determined.

From only a ⅓ of the total number of *R*-genes directed against plant viruses, the virus Avr-determinant is identified (Table 1.1 and S1.1). Interestingly, functionally quite different viral proteins act as Avr-determinants. Several *R*-genes belonging to the same locus (for instance the L-proteins in *Capsicum* spec. and Rx1 and Rx2 from *S. tuberosum*) recognise the same Avr-protein from overlapping virus species, indicating that these conserved R-proteins are able to recognise similar structures but with an adapted spectrum. For several viruses, their corresponding *R*-genes have not been identified yet, but their single dominant nature is deduced from the observation that an HR is being triggered. In some of these cases, the viral gene responsible for the induction of resistance, as indirectly monitored by HR, has been identified.

As described before and clear from Table 1.1, many different viral proteins can act as Avr-determinants; whether it is the coat protein (*e.g. L*-locus from *Capsicum* against Tobamoviruses), the movement protein (*e.g. Tm-2*/*Tm-2²* from tomato against Tobamoviruses), the replicase protein (*e.g. Tm-1* from tomato against *Tobacco mosaic virus* [TMV]) or the RNAi suppressor protein (*e.g. HRT* from *A. thaliana* against *Turnip crinkle virus* [TCV]), all potentially can act as elicitor of resistance (Meshi *et al.*, 1989; Ishibashi *et al.*, 2012; Moury and Verdin, 2012). Interestingly, for a majority of cases the ability to induce the resistance, as monitored by visual HR, could be uncoupled from the endogenous function of the viral protein, but exceptions exist.

While the function of a viral protein is not a selective criterium to act as Avr-determinant, the 'Zig-zag-model' by Jones and Dangl (2006) (Figure 1.1) implies that ETI (*R*-gene mediated resistance) is a response to ETS and governed by effector molecules. If this also applies to plant viruses, all virus proteins acting as Avr would thus have to aid in overcoming PTI (initiating ETS). For viral proteins that act as RSS this might be easily explained as these directly interfere with the PTI response. For viral cell-to-cell movement proteins this seems less logical, but also here an example exists that can position these proteins in the 'Zig-zag-model'; the movement protein of TMV was shown to stimulate the spread of small RNAs, thereby tempering

Table 1.1 Cloned antiviral *R*-genes and their corresponding Avr-determinants:

Plant host	R-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Arabidopsis thaliana Mouse ear cress	*HRT*	Yes: CC-NB-LRR [HR]	TCV [*Turnip crinkle virus*]	*Carmovirus*	CP	1, 2
	RCY1	Yes: CC-NB-LRR [HR]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	CP	3-6
	RTM1 *RTM2* *RTM3*	Yes: Jacalin-like [prev. syst. mov.] [RTM3 not cloned]	TEV [*Tobacco etch virus*] PPV [*Plum pox virus*] LMV [*Lettuce mosaic virus*]	*Potyvirus*	CP CP CP	7-9
Brassica campestris Field mustard	*BcTuR3*	Yes: TIR-NBS-LRR	TuMV [*Turnip mosaic virus*]	*Potyvirus*	unknown	17, 18
Capsicum annuum frutescens chinense chacoense Pepper	*L*-locus*:* *L*^{1} *L*^{2} *L*^{3} *L*^{4}	Yes All: CC-NB-LRR	TMV [*Tobacco mosaic virus*] by L^{1234} ToMV [*Tomato mosaic virus*] by L^{1234} TMGMV [*Tobacco mild green mosaic virus*] by L^{1234} BPeMV [*Bell pepper mottle virus*] by L^{1234} PaMMV [*Paprika mild mottle virus*] by L^{234} ObPV [*Obuda pepper virus*] by L^{234} PMMoV [*Pepper mild mottle virus*] by L^{34}	*Tobamovirus*	CP (all)	25, 31-34, 43-45
Glycine max Soybean	*Rsv1* (locus)	Yes: CC-NB-LRR [ER/HR]	SMV [*Soybean mosaic virus*]	*Potyvirus*	P3+ HC-Pro	65-69
Nicotiana glutinosa Tobacco	*N*	Yes: TIR-NB-LRR [cell-cell mov.]	TMV [*Tobacco mosaic virus*]	*Tobamovirus*	p50 [Helicase]	105-111
Phaseolus vulgaris Kidney bean	*PvVTT1*	Yes: TIR-NB-LRR [HR]	BDMV [*Bean dwarf mosaic virus*]	*Begomovirus*	BV1 (NSP)	134-139
	PvCMR1 (RT4-4)	Yes: TIR-NB-LRR [syst. necrosis]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	2a	156
Phaseolus vulgaris Kidney bean	*I* (locus)	Yes: TIR-NB-LRR [ER/HR/phloem necr.]	BCMV [*Bean common mosaic virus*] BNMV [*Bean necrotic mosaic virus*] BICMV [*Blackeye cowpea mosaic virus*] AzMV [*Azuki mosaic virus*] CABMV [*Cowpea aphid-borne mosaic virus*] PWV [*Passionfruit woodiness virus*] SMV [*Soybean mosaic virus*] ThPV [*Thailand passiflora virus*] WMV [*Watermelon mosaic virus*] ZYMV [*Zucchini yellow mosaic virus*]	*Potyvirus*	unknown	127-133
Poncirus trifoliate Trifoliate orange	*Ctv* (locus)	Yes: CC-NB-LRR	CTV [*Citrus tristeza virus*]	*Closterovirus*	unknown	158-160
Solanum hirsutum Tomato	*Tm-1*	Yes: TIM-barrel-like domain protein [ER/Replication]	ToMV [*Tomato mosaic virus*]	*Tobamovirus*	Replicase Helicase-domain	169-174
Solanum peruvianum Tomato	*Sw5b*	Yes: CC-NB-LRR [HR]	TSWV [*Tomato spotted wilt virus*] and other tospoviruses	*Tospovirus*	NSm	179-183
	Tm-2	Yes: CC-NB-LRR [HR]	TMV [*Tobacco mosaic virus*] ToMV [*Tomato mosaic virus*] and other tobamoviruses	*Tobamovirus*	30kD MP.	171, 188, 189
	Tm-2^{2}	Yes: CC-NB-LRR [HR]	ToMV [*Tomato mosaic virus*] TMV [*Tobacco mosaic virus*] and other tobamoviruses	*Tobamovirus*	30kD MP.	171, 190-193
Solanum tuberosum Potato	*Rx1*	Yes: CC-NB-LRR [ER/HR]	PVX [*Potato virus X*] and other potex viruses	*Potexvirus*	CP	195, 198, 199, 230-234
	Rx2	Yes: CC-NB-LRR	PVX [*Potato virus X*]	*Potexvirus*	CP	138, 232
	Y-1	Yes: TIR-NB-LRR.	PVY [*Potato virus Y*]	*Potyvirus*	unknown	237, 238
Vigna mungo Black gram	*CYR1*	Yes: CC-NB-LRR	MYMV [*Mungbean yellow mosaic virus*]	*Begomovirus*	CP	256, 257

1. Cooley *et al.*, 2000; 2. Ren *et al.*, 2000; 3. Takahashi *et al.*, 2001; 4. Takahashi *et al.*, 2002; 5. Takahashi *et al.*, 2004; 6. Sekine *et al.*, 2006; 7. Chisholm *et al.*, 2000; 8. Whitham *et al.*, 2000; 9. Decroocq *et al.*, 2009; 17. Provvidenti and Hampton, 1992; 18. Ma *et al.*, 2010; 25. Moury and Verdin, 2012; 31. Tomita *et al.*, 2008; 32. Tomita *et al.*, 2011; 33. Matsumoto *et al.*, 2008; 34. Sawada *et al.*, 2004; 43. de la Cruz *et al.*, 1997; 44. Holmes, 1937; 45. Berzal-Herranz *et al.*, 1995; 65. Hayes *et al.*, 2004; 66. Hajimorad and Hill, 2001; 67. Hajimorad *et al.*, 2005a; 68. Wen *et al.*, 2013; 69. Eggenberger *et al.*, 2008; 105. Whitham *et al.*, 1994; 106. Erickson *et al.*, 1999; 107. Baker *et al.*, 1995; 108. Dinesh-Kumar *et al.*, 1995; 109. Dinesh-Kumar *et al.*, 2000; 110. Padgett and Beachy, 1993; 111. Padgett *et al.*, 1997; 127. Vallejos *et al.*, 2006; 128. Ariyarathne *et al.*, 1999; 129. Collmer *et al.*, 2000; 130. Kelly *et al.*, 1995; 131. Kyle *et al.*, 1986; 132. Fisher and Kyle, 1994; 133. Fisher and Kyle, 1996; 134. Zhou *et al.*, 2007; 135. Garrido-Ramirez *et al.*, 2000; 136. Seo *et al.*, 2004; 137. Seo *et al.*, 2007; 138. Wang *et al.*, 1999; 139. Gururani *et al.*, 2012; 156. Seo *et al.*, 2006; 158. Yang *et al.*, 2003; 159. Rai, 2006; 160. Harper *et al.*, 2010; 169. Ishibashi *et al.*, 2007; 170. Ishibashi *et al.*, 2012; 171. Pelham, 1966; 172. Yamafuji *et al.*, 1991; 173. Meshi *et al.*, 1988; 174. Kato *et al.*, 2013; 179. Finlay, 1953; 180. Holmes, 1948; 181. Brommonschenkel *et al.*, 2000; 182. Hallwasser *et al.*, Submitted for publication; 183. Hoffmann *et al.*, 2001; 188. Hall, 1980; 189. Meshi *et al.*, 1989; 190. Weber *et al.*, 1993; 191. Lanfermeijer *et al.*, 2003; 192. Lanfermeijer *et al.*, 2005; 193. Tanksley *et al.*, 1998; 195. Cockerham, 1970; 198. Solomon-Blackburn and Barker, 2001b; 199. Solomon-Blackburn and Barker, 2001a; 230. Bendahmane *et al.*, 1995; 231. Bendahmane *et al.*, 1999; 232. Bendahmane *et al.*, 2000; 233. Baures *et al.*, 2008; 234. Querci *et al.*, 1995; 237. Vidal *et al.*, 2002; 238. Zvereva and Pooggin, 2012; 256. Maiti *et al.*, 2012; 257. Pal *et al.*, 1991.

the virulence of the virus during systemic infection (Vogler *et al.*, 2008). For viral proteins with other functions (*e.g.* coat protein) their position as effector is more difficult to envision, unless another innate immune response is being counteracted.

II.3 Model of *R*-gene recognition

Although the mode of action of resistance genes still remains a matter of debate, one of the most commonly accepted models is the 'guard hypothesis' (van der Biezen and Jones, 1998; Jones and Dangl, 2006). In this model the resistance gene product guards a certain host protein, the 'guardee', and perceives alterations of this protein upon interaction with the Avr determinant through which a resistance response is initiated. It is possible that multiple *R*-genes guard the same guardee, possibly *vice versa* as well, which thereby broadens the resistance spectrum of (a limited number of) *R*-genes to a wide range of various pathogens; *e.g.* Rx1 and GPa2 both interact with the same guardee RanGAP2 (Tameling and Baulcombe, 2007; Moffett, 2009). Unfortunately, this model does not explain how resistance breaking virus isolates maintain their virulence. For this reason, alternative models have been postulated. According to the 'decoy model' (van der Hoorn and Kamoun, 2008), a (proteinaceous) decoy evolved to act as a molecular sensor to only detect

a pathogen without having any other role in the household machinery of the host. The 'bait and switch model' and the similar 'mousetrap model' have been more recently postulated and proposes that the *R*-gene product in an 'OFF' state forms a complex together with the guardee/decoy protein, that upon interaction of the Avr-protein with the complex, subsequently causes it to conformationally switch ('ON') and activates a downstream signalling pathway leading to resistance (Collier and Moffett, 2009).

The mechanism through which the resistance is induced still remains unclear. However, one described case of the *R*-gene from tobacco, the *N*-gene, has revealed some of the downstream ways of controlling virus replication and obtaining resistance. The *N*-gene encodes a TIR-NB-LRR protein and confers resistance against TMV and, upon transient co-expression with the p50 elicitor (helicase), an HR is induced in *N. tabacum*, a response that does not occur in *N. benthamiana*. Bhattacharjee *et al.* (2009) employed this observation in a series of experiments to dissect and assign downstream signalling of defence responses, related to the *R*-gene. The studies indicated that the *N*-gene based antiviral response leads to a translational arrest of viral transcripts by a process that involves Argonaute 4 (AGO4). As a result, synthesis of viral proteins is inhibited, ultimately preventing virus accumulation and spread. Whether this mechanism is generic to all *R*-genes against plant-viruses remains to be investigated.

Two independent studies were published in 2012 that showed that the translation of *R*-genes is tightly controlled through the activity of miRNAs. One study showed the miR482/2118 superfamily negatively controlled the translation of NB-LRR proteins by targeting its P-loop motif (Shivaprasad *et al.*, 2012), while Li *et al.* (2012) showed that other miRNA families controlled the translation of NB-LRR proteins as well, with the TIR-NB-LRR protein N as example. Interestingly, in the on-going 'arms race' between virus and host, a viral infection thus can supress the miRNA induced silencing of *R*-genes, leading to enhanced expression of the *R*-genes and induction of ETI. Additionally, the expression levels of *R*-genes have to be carefully regulated as high expression levels of *R*-genes can lead to auto-immunity (Xia *et al.*, 2013).

III. Downstream defence responses

Dominant *R*-genes trigger a hypersensitive response (HR) or an extreme response (ER) in case the reaction occurs in a single cell. Both involve a programmed cell death (PCD) response that rapidly kills infected cells and prevents systemic spread of the (virus) pathogen. An induced HR is quite characteristic and involves the activation and expression of salicylic acid (SA), jasmonic acid (JA), nitride oxide (NO), ethylene, reactive oxygen species (ROS) and Ca^{2+}, and expression of Pathogenesis Related (*PR*)-genes. While each component has a specificity towards certain pathogens, only SA, ROS and Ca^{2+} seem to be effective against viruses (Loebenstein, 2009; Carr *et al.*, 2010).

In the past, an HR was considered to be part of the resistance response, however, recent insights into R-protein downstream signalling indicate that programmed cell death (HR) and resistance are distinct physiological pathways (Bendahmane *et al.*, 1999; Bai *et al.*, 2012). One of the best examples in support of this comes from studies on Rx-based resistance against PVX. The *Rx*-gene product is a CC-NB-LRR protein from potato that is triggered by the PVX structural CP protein. The Rx-protein localises in the cytoplasm while shuttling to and from the nucleus thereby triggering resistance (Slootweg *et al.*, 2010). Although an HR is monitored, this response can be knocked out without Rx-mediated resistance against PVX (Bendahmane *et al.*, 1999), another example is the *N*-gene mediated resistance against TMV as described above in section II.3 (Bhattacharjee *et al.*, 2009). Similar observations have been made by others (Cole *et al.*, 2001; Cawly *et al.*, 2005; Genger *et al.*, 2008; Bulgarelli *et al.*, 2010; Bai *et al.*, 2012) and indicate that the actual resistance response is different from an HR, although both mostly are triggered and may act in concert to clear viral invasions. Whether both are triggered by a pathogens' Avr determinant or whether HR is sequentially triggered following the *R*-gene response is not clear.

While several interacting proteins have been identified that control R-protein activity in the absence of pathogens (RAR1, SGT1, WRKY1, TPR1), more recently it has been found that there are also proteins that modulate the strength of defence responses (RanGAP, EDS1-PAD4) (Wiermer *et al.*, 2005; Sacco *et al.*, 2009). The benefit for the plant in a modulated fine-tuning of the ETI response to specific pathogens lies in improved effector sensing and minimizing the fitness costs involved with certain defence responses (free radical production, defence

protein synthesis, cell death) (Padmanabhan and Dinesh-Kumar, 2010). While *R*-gene mediated defence is taking place locally at the site of entry, it is also able to induce defence signalling responses in distally located tissues, known as systemic acquired resistance (SAR) (Vlot *et al.*, 2008). For both the *N*-gene in tobacco and *Rx1* in potato, SAR has been demonstrated (Delaney *et al.*, 1994; Liu *et al.*, 2010) and in both cases this response is mediated by the SA-dependent pathway as a mobile signal. SAR also prevents infection by other pathogens in the host by activating *PR*-genes in the systemic tissue, which are used as a hallmark of SAR and were shown to have antimicrobial activity, although a direct inhibition on virus replication has not been shown (Durrant and Dong, 2004; Loebenstein, 2009; Carr *et al.*, 2010).

IV. Functional and structural homology of plant- and animal-sensors of innate immunity

Viruses are pathogens to many different organisms and, irrespective of the host species they infect, often share similarities in genome organisation and functions of encoded proteins. A good example of this is exemplified by viruses from the *Bunyaviridae* family where all members infect animals with the exception of those from the *Tospovirus* genus (see V) that, besides infecting their thrips vector, are plant pathogenic and are postulated to have evolved from a common ancestor. Likewise, as a result of co-evolution driven by host-pathogen interactions, plants and animals show some similarities in their innate immune sensory systems. While in plants the aforementioned *R*-genes are important in mounting an ETI response, in animals two major classes are distinguished that (partially) share similarity to these *R*-genes, however both function as PRRs in the PTI response. The first major class present the 'nucleotide-binding domain and leucine-rich repeat'-proteins (NLRs) and the second class is that of Toll like receptors (TLRs), which are all found to function as PRRs in the PTI response. Both are immune receptors aimed at detecting 'foreign' structures and activating downstream defence responses. The family of NLRs share the most homology, as evidenced when looking at *R*-genes from plants and NACHT-LRR encoding genes from the animal kingdom (NAIP – CIITA - HET-E - TP1 domain) (Leipe *et al.*, 2004; Takken *et al.*, 2006; Maekawa *et al.*, 2011). They both contain a nucleotide binding domain and a leucine rich repeat (Maekawa *et al.*, 2011)

(Figure 1.2). Additionally, plant R-proteins also share homology at their N-termini with animal Toll-like receptors (TLRs), membrane-bound immune receptors that function as sensors in pathogen recognition across membranes.

The NLRs of both plant and animal kingdom share homology through the presence of the Leucine-rich repeats (LRR) in these proteins. As discussed in previous chapters, the most prevalent type of R-proteins in plants belonging to the NB-LRR protein structural class, from which the central nuclear binding domain (NBS) exhibits similarity to the nucleotide binding domain in several metazoan apoptosis regulating proteins: Apaf-1 from mammals and CED-4 from *C. elegans*. Due to the latter this domain is also often referred to as NB-ARC domain (from Apaf1 – R-protein - CED4) (van der Biezen and Jones, 1998; Takken *et al.*, 2006). The N-terminal domain furthermore separates different classes of *R*-genes; TIR-NB-LRRs harbour a Toll/Interleukin-1 Receptor domain with similarity to metazoan Toll-like receptors (TLR) (Burch-Smith *et al.*, 2007). CC-NB-LRRs contain coiled coil domain forming the more irregular shaped intertwined alpha-helices (Lupas, 1997). Parallel to the discovery of many NB-LRR encoding *R*-genes in plants in the recent years, the search for homology to Apaf-1 and CED-4 resulted in the recognition of the NACHT-LRR protein family in vertebrates (Koonin and Aravind, 2000; Leipe *et al.*, 2004). Animal NLRs activate caspase-1 leading to activation and release of the cytokine interleukin-1 beta (Case, 2011), which subsequently induces local and systemic immune reactions. Similar to plant NB-LRR proteins, NLRs were found to act as higher-order active complexes, *e.g.* NLRP1-3 and NLRC4 form a complex often termed the inflammasome.

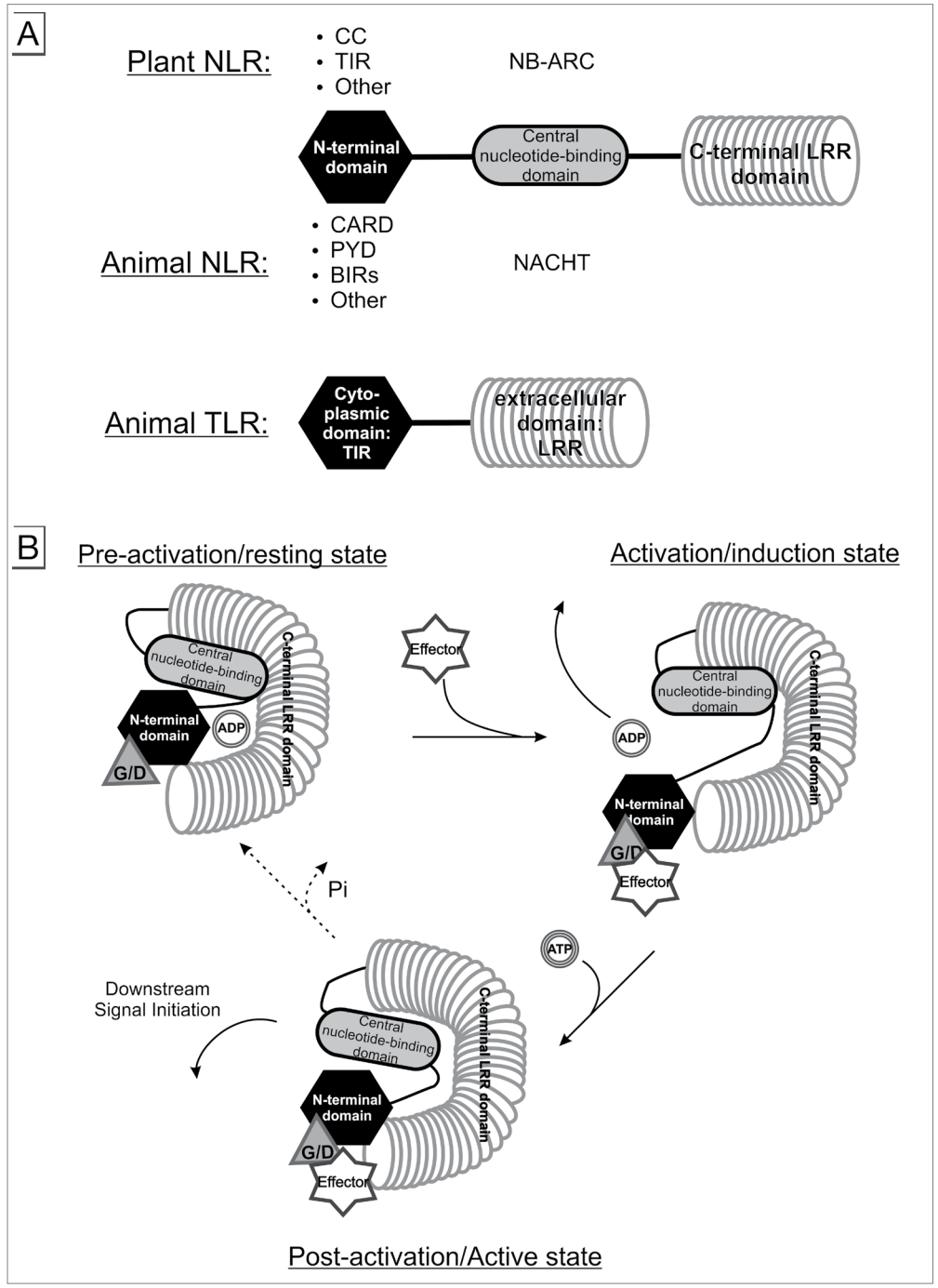
TLRs represent the best studied family of PRRs in mammals so far. They are transmembrane glycoprotein receptors with an extracellular PAMP-binding domain consisting of multiple leucine rich repeats (LRR) that fold into a 'horseshoe' structure. Additionally, it possesses intracellular signalling regions that have similarity to the intracellular domain of the Interleukin-I receptor ((IL-1R), which is referred to as Toll/IL-1R (TIR) domain that mediates downstream signalling upon activation of the receptor. TLRs initiate signal cascades involving the activation of nuclear factor kappa b (NF-κB), mitogen-activated protein kinase (MAPK) and interferon regulatory factors (IRFs). This subsequently leads to a concerted expression of interferons, cytokines and chemokines. Finally, inflammatory processes, cell cycle arrest and cell death are induced (Honda *et al.*, 2005; Kaisho and Akira, 2006).

In humans, 10 TLRs have been identified of which TLR2, -3, -4, -7 and -8 are involved in sensing structural components of RNA viruses like double-stranded RNA, single-strand RNA and viral glycoproteins (Bowie and Unterholzner, 2008). While most TLRs are involved in extracellular recognition of PAMPs, TLR3, -7 and -8 are primarily restricted to intracellular compartments (endoplasmic-reticulum (ER), endosomes etc.) where they sense structural components of viral RNA. Besides TLRs cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs; RIG-I, MDA5 and LSP2) have been identified as sensors of RNA viruses and are involved in the very early response of some RNA viruses (Bowie and Unterholzner, 2008; Gerlier and Lyles, 2011; Jensen and Thomsen, 2012).

Pathogen recognition in both animal and plant kingdoms involves the LRR domain, which binds the ligand in its horseshoe shape, often followed by activation of a signalling cascade through kinase phosphorylation. Structural similarities between animal TLR/NLR and plant NB-LRR proteins point to a convergent evolution of these defence-related pathways. However innate immunity in animals and plants differs substantially in their downstream defence response, with interleukin/interferon-activated inflammatory responses combined with activating the adaptive immune system in mammalian systems and a resistance response (as explained before) often seen as a programmed cell death response in form of HR in plants.

V. Tomato spotted wilt virus

Tomato spotted wilt virus (TSWV) currently ranks 2nd on the list of scientifically/economically most important plant viruses worldwide (Scholthof *et al.*, 2011). TWSV is the representative of the *Tospovirus* genus within the family of arthropod-borne *Bunyaviridae*. Besides this genus, the family comprises the genera *Orthobunya-*, *Hanta-*, *Nairo-* and *Phlebovirus*, which all contain members that infect animals. Like all members of the *Bunyaviridae* family TSWV has a spherical morphology, contains a host-derived membrane (from either plant or insect vector) and has a diameter between 80 and 120 nm. Its genome consists of three segmented RNA molecules, of which the large (L)-segment is of negative polarity and encodes the RNA-dependent RNA polymerase (RdRp) (Figure 1.3). The medium (M)-segment contains an ambisense gene arrangement and encodes the movement protein



← Figure 1.2 Comparison between the structure of plant and animal NLRs.

A. The structure of 'Nucleotide binding and leucine rich repeat proteins' (NLRs) from the animal and plant kingdom share highest homology, as all proteins belonging to this class have a C-terminal leucine rich repeat (LRR), a central nucleotide binding domain and a varying N-terminal domain (modified from Maekawa (2011)). Animal TLRs also contain an (extracellular) LRR domain and possess a TIR-domain, they do however, lack a nucleotide binding domain. CC: Coiled-coil, TIR: Toll-interleukin receptor, CARD: Caspase-activation and recruitment domain, PYR: Pyrin domain, BIR: Baculovirus inhibitor-of-apoptosis repeats, NB-ARC: Nucleotide binding and Apaf1-R protein-CED4 domain, NACHT: NAIP – CIITA - HET-E - TP1 domain. **B.** A model of NB-LRR R-protein recognising a specific Avr-protein through a guardee or decoy host protein. Upon interaction with the Avr-protein the R-protein conformationally changes and the ADP can be exchanged for ATP, leading to a second conformational change triggering downstream resistance (Modified from Lukasik (2009)). Whether the R-protein returns to its resting state is not known yet. G/D: Guardee/Decoy.

(NSm) on the viral strand and the precursor to the glycoproteins (Gn and Gc) on the viral complementary strand (Figure 1.3). The small (S)-segment encodes, in similar arrangement as the M-RNA, the RNA silencing suppressor (NSs) on the viral strand and the nucleocapsid protein (N) on the viral complementary strand (Figure 1.3) (Kormelink *et al.*, 2011). All genomic RNA segments form a panhandle structure by interaction of their 5' and 3' inverted, complementary UTR sequences.

Like all segmented, negative-strand RNA viruses, bunyaviruses do not cap their own messengers, as a prerequisite for translation, but instead steal this structure from host cellular mRNAs by a process called cap-snatching (Duijsings *et al.*, 1999). Viral transcripts do not contain a poly-A tail, like common eukaryotic mRNAs, but instead contain a predicted hairpin structure (HP) at their 3'-ends, suggested to be involved in transcription termination (van Knippenberg *et al.*, 2005). *Tospovirus* cell-to-cell movement is enabled by the non-structural NSm protein that facilitates the movement of viral ribonucleoproteins (RNPs) through plasmodesmata in a tubule-guided manner (Kormelink *et al.*, 1994; Storms *et al.*, 1995, 1998). Further spread by virus loading into the phloem tissue allows systemic infection of the whole plant.

TSWV is transmitted by thrips in a propagative manner, meaning that the virus also replicates in the thrips vector (Wijkamp *et al.*, 1993). TSWV has an extremely

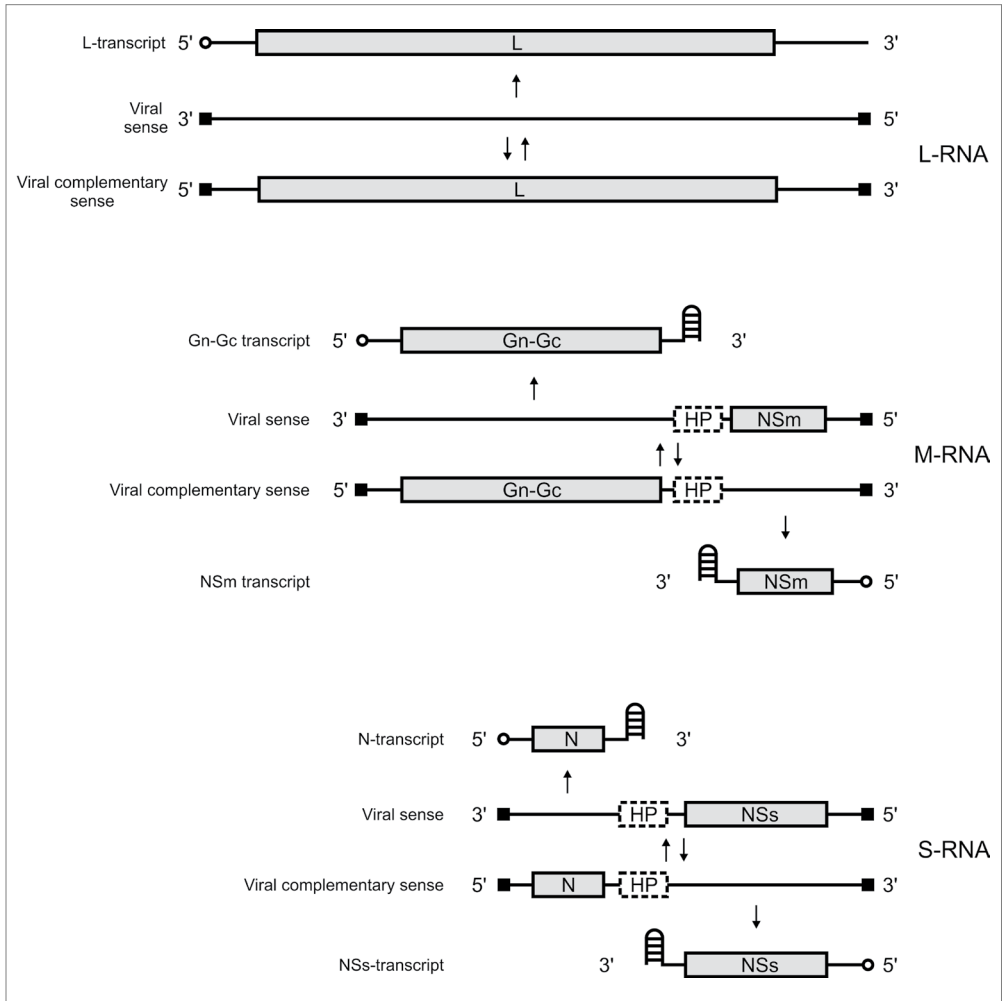


Figure 1.3 Genomic organisation of *Tomato spotted wilt virus*. The three segments of TSWV are presented, of which the L-segment is of negative polarity and M- and S-RNA are ambisense. The viral sense strand is predominantly present in the virus particle, while the viral complementary strand is made upon replication. The predicted hairpin structures (HP) in the M and S-segment are indicated and assumed to act as bi-directional transcription terminator for the ambisense encoded genes; For the L-segment a putative, smaller hairpin is predicted in the 3'-untranslated region (UTR).

large host range, and infects over 1000 different plant species from more than 80 different families, including dicots and monocots (Parrella *et al.*, 2003). Among these are economically important crops like pepper (*Capsicum spec*.), tomato and potato (*Solanum spec*.). Natural sources of resistance in these crops have been found but for commercial breeding is limited to two dominant resistances against TSWV. In tomato (*Solanum peruvianum*), the *Sw5* resistance gene has been identified, that encodes a CC-NB-LRR protein. The *Sw5*-gene confers resistance to TSWV, but also to the closely related, but distinct tospoviruses *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) (Folkertsma *et al.*, 1999; Brommonschenkel *et al.*, 2000). The second dominant *R*-gene, *Tsw* (Jahn *et al.*, 2000) has been described from pepper (*Capsicum chinense*), but is, in contrast to *Sw5*, specific against TSWV only. Both *R*-genes have been introgressed into commercial cultivars and are used in production fields. Meanwhile, resistance breaker isolates against each resistance gene have already been indentified and described (Moury *et al.*, 1997; Roggero *et al.*, 2002; Aramburu and Martí, 2003; Margaria *et al.*, 2004; Sharman and Persley, 2006) stressing the importance of a search for new resistance genes against tospoviruses and/or development of alternative resistance strategies.

Scope of the thesis

At the onset of the research as described in this thesis, *Tsw*-breaking tospoviruses were becoming a major problem in *Capsicum annuum* growing areas like Spain and South America, both production areas and important export markets for Dutch seed companies. At that time, also two contradicting papers had appeared that claimed either the TSWV *N*-gene (Lovato *et al.*, 2008) or the *NSs*-gene (Margaria *et al.*, 2007) as the avirulence gene triggering *Tsw* resistance from *C. annuum*. The major objective of this thesis was to unambiguously identify and characterise the viral protein triggering *Tsw* resistance, since knowledge on its identity would notably contribute to a further understanding of *Tsw*-mediated resistance, but also assist in improved marker-selected breeding and development of diagnostic markers for *Tsw*-breaking pathotypes.

To this end, both S-RNA encoded *N*- and *NSs*-genes, previously identified as *Avr* candidate genes from reassortant studies by Jahn *et al.* (2000), were cloned

and transiently expressed via agroinfiltration in *Tsw+ C. annuum* plants to monitor the induction of HR, indicative for the triggering of a single dominant resistance gene. This led to the identification of the Avr-determinant as described in Chapter 2. In a next step the *Avr*-gene from the TSWV resistance-inducing (RI) isolate BR-01 was subjected to alanine substitution analysis, deletion analysis, and domain swapping with corresponding domains from the TSWV resistance breaking (RB) isolates 160 and 171 to identify amino acids/domains required for Avr-activity as well as investigate this activity in view of its 'primary' function in the natural infection cycle (Chapter 3). During the mutational screen of the Avr-determinant indications were obtained for interactions with an important host protein involved in innate immunity. Using Co-IP efforts were made to demonstrate the actual interaction between the Avr and this host protein (Chapter 4). Besides the RI and RB isolates used in these studies, several other isolates were available that showed a temperature-dependent behaviour during their triggering of *Tsw*-mediated resistance. While under standard greenhouse conditions these isolates were able to trigger HR, they lost this ability at elevated temperatures (28 °C) at which temperature the RI isolates could still trigger *Tsw* resistance. In Chapter 5 these isolates have been analysed and characterised, their *Avr*-gene has been cloned to test i) its ability to trigger HR after transient expression, and ii) its primary function, both at standard greenhouse and elevated temperature conditions. Based on the results from Chapters 2, 3 and 5, it was clear that RB isolates were not generated just by a single amino acids mutation, although one amino acid residue seemed to be relatively important. Based on this mutation, an RT-PCR test was developed to investigate its potential to distinguish between RI and RB isolates to be used for detection of RB isolates in the field (Chapter 5). In Chapter 6, the results have been summarised and together with known information on TSWV assembled into a model to position the TSWV Avr-protein as an effector in the 'Zig-zag-model' (Dangl and Jones (2006) (Figure 1)) and –although speculative- to explain the possible mode of action of *Tsw* resistance. Finally, with respect to the TSWV Avr-protein, a brief comparison is made between the innate immune responses encountered by the plant-infecting versus the animal-infecting bunyaviruses.

Table S1.1 Complete overview of dominant resistances known against plant viruses.

Plant host	*R*-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Arabidopsis thaliana Mouse ear cress	*HRT*	Yes: CC-NB-LRR [HR]	TCV [*Turnip crinkle virus*]	*Carmovirus*	CP	1, 2
	RCY1	Yes: CC-NB-LRR [HR]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	CP	3-6
	RTM1 *RTM2* *RTM3*	Yes: Jacalin-like [prev. Syst. Mov.] [RTM3 not cloned]	TEV [*Tobacco etch virus*] PPV [*Plum pox virus*] LMV [*Lettuce mosaic virus*]	*Potyvirus*	CP CP CP	7-9
	TuNI	No [syst. HR]	TuMV [*Turnip mosaic virus*]	*Potyvirus*	P3	10, 11
Beta vulgaris Common beet	*Bm*	No	BtMV [*Beet mosaic virus*]	*Potyvirus*	unknown	12
	Rz1 *Rz2* *Rz3*	No [partial resistance]	BNYVV [*Beet necrotic yellow vein virus*]	*Benyvirus*	unknown	13, 14
Brachypodium distachyon Purple false brome	*Bsr1*	No 'CC-NB-LRR'	BSMV [*Barley stripe mosaic virus*]	*Hordeivirus*	TGB1 (MP)	15, 16
Brassica campestris Field mustard	*BcTuR3*	Yes: TIR-NB-LRR	TuMV [*Turnip mosaic virus*]	*Potyvirus*	unknown	17, 18
	Unknown gene	No			unknown	17
Brassica napus Rapeseed	*TuRB01,* *TuRB01b,* *TuRB02,* *TuRB03,* *TuRB04,* *TuRB05*	No [ER] No No [Partial Res.] No [ER] No [ER] No [HR]	TuMV [*Turnip mosaic virus*]	*Potyvirus*	TuRB01: CI TuRB01b: CI Unknown TuRB03: P3 TuRB04: P3 TuRB05: CI	19-22
	Tum	No	TuMV [*Turnip mosaic virus*]	*Potyvirus*	Unknown	17
	Monogenic	No[ER]	TuMV [*Turnip mosaic virus*]	*Potyvirus*	Unknown	
Brassica rapa Turnip	*TuRB01b*	No [ER]	TuMV [*Turnip mosaic virus*]	*Potyvirus*	CI	19, 22-24
Capsicum annuum Pepper	*Pvr4* (locus)	No [ER]	PVY [*Potato virus Y*] PepMoV [*Pepper mottle virus*] PepYMV [*Pepper yellow mosaic virus*] PepSMV [*Pepper severe mosaic virus*] ERV [*Ecuadorian rocoto virus*] PTV [*Peru tomato mosaic virus*]	*Potyvirus*	Nlb (RdRp)	25-30
	L^1 (locus) L^{1o5} [temp insens.] (locus)	Yes: CC-NB-LRR	TMV [*Tobacco mosaic virus*] ToMV [*Tomato mosaic virus*] TMGMV [*Tobacco mild green mosaic virus*] BPeMV [*Bell pepper mottle virus*]	*Tobamovirus*	CP CP CP CP	25, 31-34
	Cmr1	No [blocks syst. Mov.]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	Helicase (1a)	25, 30, 35-39
	HK	No [Incomp. Dom.]	PaMMV [*Paprika mild mottle virus*]	*Tobamovirus*	Methyl transferase	40, 41
Capsicum chacoense Pepper	L^4 (locus)	Yes: CC-NB-LRR	TMV [*Tobacco mosaic virus*] ToMV [*Tomato mosaic virus*] TMGMV [*Tobacco mild green mosaic virus*] BPeMV [*Bell pepper mottle virus*] PaMMV [*Paprika mild mottle virus*] ObPV [*Obuda pepper virus*] PMMoV [*Pepper mild mottle virus*]	*Tobamovirus*	CP CP CP CP CP CP CP	25, 31, 32
Capsicum chinense Pepper	*Tsw*	No [HR]	TSWV [*Tomato spotted wilt virus*]	*Tospovirus*	NSs	42
	L^3 (locus)	Yes: CC-NB-LRR	TMV [*Tobacco mosaic virus*] ToMV [*Tomato mosaic virus*] TMGMV [*Tobacco mild green mosaic virus*] BPeMV [*Bell pepper mottle virus*] PaMMV [*Paprika mild mottle virus*] ObPV [*Obuda pepper virus*] PMMoV [*Pepper mild mottle virus*]	*Tobamovirus*	CP CP CP CP CP CP CP	31, 32, 43, 44
	Pvr7	No	PepMoV [*Pepper mottle virus*]	*Potyvirus*	Unknown	30

Plant host	*R*-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Capsicum frutescens Pepper	*L*[2] (locus)	Yes: CC-NB-LRR	TMV [*Tobacco mosaic virus*] ToMV [*Tomato mosaic virus*] TMGMV [*Tobacco mild green mosaic virus*] BPeMV [*Bell pepper mottle virus*] PaMMV [*Paprika mild mottle virus*] ObPV [*Obuda pepper virus*]	*Tobamovirus*	CP CP CP CP CP CP	25, 31, 32, 44, 45
Chenopodium amaranticolor Goosefoot	Unknown	No	CaMV [*Cauliflower mosaic virus*]	*Caulimovirus*	Gene VI product	46, 47
Cucurbita moschata Squash, Pumpkin	*Cmv*	No	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	Unknown	48
	Wmv	No [No sympt.]	MWMV [*Watermelon mosaic virus*]	*Potyvirus*	Unknown	48, 49
	Zym	No [No sympt]	ZYMV [*Zucchini yellow mosaic virus*]	*Potyvirus*	Unknown	48, 50-53
	Slc	No	SLCV [*Squash leaf curl virus*]	*Begomovirus*	RNAs 2 and 3	54-56
Cucumis melo Melon	*Mnr1, Mnr2*	No [cell to cell move.]	MNSV [*Melon necrotic spot virus*]	*Carmovirus*	Unknown	57
	Pvr1, Pvr2	No No	PRSV [*Papaya ringspot virus*]	*Potyvirus*	Unknown Unknown	50
	Monogenic polygenic	No [poly: partial res]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	Unknown Unknown	58, 59
	Zym	No [No sympt]	ZYMV [*Zucchini yellow mosaic virus*]	*Potyvirus*	CP	50-53
	Wmr	No	MWMV [*Watermelon mosaic virus*]	*Potyvirus*	Unknown	50, 57, 60
	Wmv-1, Wmv-1[2]	No	MWMV [*Watermelon mosaic virus*]	*Potyvirus*	Unknown Unknown	61
Cucumis sativus Garden cucumber	*Wmv1-1, Prsv-2*	No [tol., no sympt, high titres]	PRSV [*Papaya ringspot virus*]	*Potyvirus*	Unknown Unknown	62, 63
Dioscorea rotundata White yam	*Ymv-1* (locus)	No [resistance]	YMV [*Yam mosaic virus*]	*Potyvirus*	Unknown	64
Glycine max Soybean	*Rsv1* (locus)	Yes: CC-NB-LRR [ER/HR]	SMV [*Soybean mosaic virus*]	*Potyvirus*	P3+ HC-Pro	65-69

Glycine max Soybean [cont.]	*Rsv3* (sim *Rsv1*) *Rsv4* (not sim.)	No [ER/HR/stem-tip necrosis] 'CC-NB-LRR' No [broad resistance: delay repl + move.]	SMV [*Soybean mosaic virus*]	*Potyvirus*	CI P3	68, 70-74
	Rcv	No [HR]	CCMV [*Cowpea chlorotic mottle virus*]	*Bromovirus*	unknown	75
	Rpv1, Prmv	No No	PeMoV [*Peanut mottle virus*]	*Potyvirus*	Unknown unknown	76, 77
	Rav1	No	AMV [*Alfalfa mosaic virus*]	*Alfamovirus*	Unknown	78
	Unknown	No	TSV [*Tobacco streak virus*]	*Ilarvirus*	Unknown	79, 80
Hordeum bulbosum Bulbous barley	*Rym14, Rym16 Rym17*	No	BaYMV [*Barley yellow mosaic virus*]	*Bymovirus*	Unknown	81-85
Hordeum vulgare Barley	*Rrs-1*	No [Replication]	BSMV [*Barley stripe mosaic virus*]	*Hordeivirus*	unknown	86, 87
	Ryd2/Yd2, Ryd3	No [Yd2: tol. Ryd3: resist.]	BYDV [*Barley yellow dwarf virus*]	*Luteovirus*	Unknown	88-97
Lactuca saligna Willowleaf lettuce	*Rsv, Rsv2*	No [syst. res.]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	Unknown	98, 99
Lactuca sativa Garden lettuce	*Tu*	No	TuMV [*Turnip mosaic virus*]	*Potyvirus*	Unknown	100, 101
	Tvr1	No [partial resist.]	LNSV [*Lettuce necrotic stunt virus*]	*Tombusvirus*	Unknown	102, 103
Nicotiana edwardsonii	Unknown	No	CaMV [*Cauliflower mosaic virus*]	*Caulimovirus*	P6	104
Nicotiana glutinosa Tobacco	*N*	Yes: TIR-NB-LRR [cell-cell mov.]	TMV [*Tobacco mosaic virus*]	*Tobamovirus*	p50 [Helicase]	105-111
	Unknown	No	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	1a	112, 113
Nicotiana sylvestris Wood tobacco	*N'*	No [HR] 'NB-LRR'	TMV [*Tobacco mosaic virus*]	*Tobamovirus*	CP	114-116
Nicotiana tabacum Common tobacco	Unknown	No	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	1a	112, 113, 117

Plant host	*R*-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Nicotiana tabacum Common tobacco [cont.]	Unknown	No	ToLCNDV [*Tomato leaf curl New Delhi virus*]	*Begomovirus*	NSP (BV1)	118, 119
	Unknown	No [HR]	PaLCuV [*Papaya leaf curl virus*] CLCuKV [*Cotton leaf curl Kokhran virus*]	*Begomovirus*	V2	120
	Unknown	No	TAV [*Tomato aspermy virus*]	*Cucumovirus*	2b	121, 122
Nicotiana spec.	Polygenic	No [HR]	TBSV [*Tomato bushy stunt virus*] CymRSV [*Cymbidium ringspot virus*] CNV [*Cucumber necrosis virus*]	*Tombusvirus*	P19/P22/P41 P19/P22 P20/P21	123, 124
Pisum sativum Pea	*En*	No	PEMV [*Pea enation mosaic virus*]	*Enamovirus*	Unknown	125, 126
Phaseolus vulgaris Kidney bean	*I* (locus)	Yes: TIR-NB-LRR [ER/HR/phloem necr.]	BCMV [*Bean common mosaic virus*] BNMV [*Bean necrotic mosaic virus*] BICMV [*Blackeye cowpea mosaic virus*] AzMV [*Azuki mosaic virus*] CABMV [*Cowpea aphid-borne mosaic virus*] PWV [*Passionfruit woodiness virus*] SMV [*Soybean mosaic virus*] ThPV [*Thailand passiflora virus*] WMV [*Watermelon mosaic virus*] ZYMV [*Zucchini yellow mosaic virus*]	*Potyvirus*	unknown	127-133
	PvVTT1	Yes: TIR-NB-LRR [HR]	BDMV [*Bean dwarf mosaic virus*]	*Begomovirus*	BV1 (NSP)	134-139
	Amv *Amv-2*	No	AMV [*Alfalfa mosaic virus*]	*Alfamovirus*	Unknown	140, 141
	Bgp-1	No [Norm. pod form.]	BGMV [*Bean golden mosaic virus*]	*Begomovirus*	Unknown	142
	Bgp-2	No			Unknown	143, 144
	By, *By-2*	No	BYMV [*Bean yellow mosaic virus*]	*Potyvirus*	Unknown	145, 146
	Bcm	No	BICMV [*Blackeye cowpea mosaic virus*]	*Potyvirus*	Unknown	140, 147

Phaseolus vulgaris Kidney bean [cont.]	Monogenic	No	BBWV [*Broad bean wilt virus*]	*Fabavirus*	Unknown	148
	Cam *Cam2*	No	CABMV [*Cowpea aphid-borne mosaic virus*]	*Potyvirus*	Unknown	149, 150
	Pwv	No [Syst. res.]	PWV [*Passion fruit woodiness virus*]	*Potyvirus*	Unknown	151
	Smv *Hss*	No [Syst. res.] No	SMV [*Soybean mosaic virus*]	*Potyvirus*	Unknown Unknown	152, 153
	Wmv *Hsw*	No [syst. spread] No [full resis.]	WMV [*Watermelon mosaic virus*]	*Potyvirus*	Unknown Unknown	154, 155
	PvCMR1 *(RT4-4)*	Yes: TIR-NB-LRR [syst. necrosis]	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	2a	156
	Bct	No	BCTV [*Beet curly top virus*]	*Curtovirus*	Unknown	157
	Azm1 *Azm2*	No	AzMV [*Azuki mosaic virus*]	*Potyvirus*	Unknown	133
Poncirus trifoliate Trifoliate orange	*Ctv* (locus)	Yes: CC-NB-LRR	CTV [*Citrus tristeza virus*]	*Closterovirus*	unknown	158-160
Rubus idaeus Raspberry	*Bu*	No	RBDV [*Raspberry bushy dwarf virus*]	*Idaeovirus*	Unknown	161-164
	2 genes	No	RcRSV [*Raspberry ringspot virus*]	*Nepovirus*	Unknown	165
	2 genes	No	TBRV [*Tomato black ring virus*]	*Nepovirus*	Unknown	165
Saccharum spontaneum Wild sugarcane	Monogenic	No	SCMV [*Sugarcane mosaic virus*]	*Potyvirus*	Unknown	17
Solanum chilense Tomato	*Ty-1* *Ty-3*	Yes: RDR [Tol.]	TYLCV [*Tomato yellow leaf curl virus*]	*Begomovirus*	No	30, 166, 167
Solanum esculentum Tomato	Unknown	No	ToLCNDV [*Tomato leaf curl New Delhi virus*]	*Begomovirus*	NSP	118, 119
	Unknown	No	ToLCJV [*Tomato leaf curl Java virus*]		V2	119
Solanum habrochaites Tomato	*Ty-2*	No [Tol.]	TYLCV [*Tomato yellow leaf curl virus*]	*Begomovirus*	Unknown	167

Plant host	*R*-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Solanum hirsutum Tomato	Monogenic	No [Tol.]	PTV [*Peru tomato mosaic virus*]	*Potyvirus*	Unknown	168
	Tm-1	Yes: TIM-barrel-like domain protein [ER] [Replication]	ToMV [*Tomato mosaic virus*]	*Tobamovirus*	Replicase: Helicase-domain	169-174
Solanum lycopersicum Tomato	*Am*	No [ER]	AMV [*Alfalfa mosaic virus*]	*Alfamovirus*	Unknown	175
	Unknown	No [ER: immune]	PVY [*Potato virus Y*]	*Potyvirus*	Unknown	176, 177
	Cmr	No	CMV [*Cucumber mosaic virus*]	*Cucumovirus*	Unknown	178
Solanum peruvianum Tomato	*Sw5b*	Yes: CC-NB-LRR [HR]	TSWV [*Tomato spotted wilt virus*] and other tospoviruses	*Tospovirus*	NSm	179-183
	Unknown	No [ER]	TYTV [*Tomato yellow top virus*]	*Luteovirus*	Unknown	184-187
	Tm-2	Yes: CC-NB-LRR [HR]	TMV [*Tobacco mosaic virus*] ToMV [*Tomato mosaic virus*] and other tobamoviruses	*Tobamovirus*	30kD MP.	171, 188, 189
	Tm-2²	Yes: CC-NB-LRR [HR]	ToMV [*Tomato mosaic virus*] TMV [*Tobacco mosaic virus*] and other tobamoviruses	*Tobamovirus*	30kD MP.	171, 190-193
Solanum acaule Potato	*Rxacl*	No [ER]	PVX [*Potato virus X*]	*Potexvirus*	Unknown	194-196
	Xᵃ	No [ER]	PVX [*Potato virus X*]	*Potexvirus*	Unknown	194-196
	Rx_{acl}^{n} ($X^n = Nx_{acl}$)	No [HR]	PVX [*Potato virus X*]	*Potexvirus*	Unknown	194-196
Solanum chacoense Potato	Ny_{chc}	No [HR]	PVY [*Potato virus Y*] PVA [*Potato virus A*]	*Potyvirus*	Unknown	194, 195
Solanum demissum Potato	Ny_{dms} ($= N^y$)	No [ER]	PVY [*Potato virus Y*] PVA [*Potato virus A*]	*Potyvirus*	Unknown	195-199
	Ry_{dms}^{a} ($Na_{dms}=N^a$)	No [HR]	PVA [*Potato virus A*]		Unknown	

Solanum etuberosum Potato	Rlr_{etb}	No	PLRV [*Potato leafroll virus*]	*Polerovirus*	Unknown	196, 200-203
Solanum gourlayi Potato	*Gm*	No	PVM [*Potato virus M*]	*Carlavirus*	Unknown	196, 204, 205
Solanum hougasii Potato	Ry_{hou}	No [ER]	PVY [*Potato virus Y*] PVA [*Potato virus A*]	*Potyvirus*	Unknown	195, 198, 199
Solanum megistracrolobum Potato	*Nm*	No [HR]	PVM [*Potato virus M*]	*Carlavirus*	Unknown	196, 205-208
	Rm	No [HR]			Unknown	
Solanum sparsipilum Potato	Nc_{spl}	No [HR]	PVY [*Potato virus Y*]	*Potyvirus*	HcPro	196, 209
	Nx_{tbr}^{spl}	No [HR]	PVX [*Potato virus X*]	*Potexvirus*	Unknown	195, 196
Solanum stoloniferum Potato	Ry_{sto} ($Ry=R^1$)	No [ER] 'NB-LRR'	PVA [*Potato virus A*]	*Potyvirus*	Unknown	30, 195, 196 198, 199, 210-213
			PVV [*Potato virus V*]		Unknown	
			PVY [*Potato virus Y*]		NlaPro	
	Ry_{sto}^{na} ($=R2$)	No [HR]	PVA [*Potato virus A*]	*Potyvirus*	Unknown	195, 196, 214, 215
		No [HR/ER]	PVV [*Potato virus V*]			
		No [ER]	PVY [*Potato virus Y*]			
	Ry_{sto}^{rna} ($= R3$)	No [HR/ER]	PVA [*Potato virus A*] PVY [*Potato virus Y*]	*Potyvirus*	Unknown	195, 196
	Ry_{sto}^{n1} ($Ryn = Ny_{sto}^{1}$)	No [HR]	PVY [*Potato virus Y*]	*Potyvirus*	Unknown	194-196
	Ry_{sto}^{n2} ($R^2 = Ny_{sto}^{2}$)	No [ER]	PVY [*Potato virus Y*]	*Potyvirus*	Unknown	195, 196
	Na_{sto} ($R^0 = Rym$)	No [HR]	PVA [*Potato virus A*]	*Potyvirus*	Unknown	194-196
	Ra ($=Ra_{sto}$)	No [HR]	PVA [*Potato virus A*]	*Potyvirus*	Unknown	196, 216
Solanum tuberosum Potato	Na_{kE} ($=Na_{kEtbr}$)	No [HR]	PVA [*Potato virus A*]	*Potyvirus*	unknown	196, 217

Plant host	*R*-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Solanum tuberosum Potato [cont.]	*Na_{tbr} (=Na)*	No [HR]	PVA [*Potato virus A*] PVY [*Potato virus Y*]	*Potyvirus*	Unknown	195, 196, 198, 199, 218
	Nb (=Nb_{tbr})	No [HR]	PVX [*Potato virus X*]	*Potexvirus*	25K	195, 196, 219, 220
	Nc_{tbr} (=Nc)	No [HR]	PVY [*Potato virus Y*]	*Potyvirus*	HcPro	195, 196, 209, 220
	Ns	No [HR]	PVS [*Potato virus S*]	*Carlavirus*	Unknown	196, 204, 206, 221-223
	Nv_{tbr} (=Nv)	No [HR]	PVV [*Potato virus V*]	*Potyvirus*	unknown	196, 198, 199, 214, 224
	Nx (=Nx_{tbr})	No [HR]	PVX [*Potato virus X*]	*Potexvirus*	CP	195, 196, 198, 199, 225
	Ny_{adg}	No [HR]	PVY [*Potato virus Y*]	*Potyvirus*	Unknown	196, 226
	Nytbr (=Ny)	No [HR]	PVY [*Potato virus Y*]	*Potyvirus*	HcPro	195, 196, 198, 199, 209, 227
	Ny-1	No [HR]	PVY [*Potato virus Y*]	*Potyvirus*	Unknown	196, 228
	Ra_{adg}	No [ER]/[HR]	PVA [*Potato virus A*]	*Potyvirus*	Unknown	196, 229
	Rx1	Yes: CC-NB-LRR [ER/HR]	PVX [*Potato virus X*] and other potex viruses	*Potexvirus*	CP	195, 198, 199, 230-234
	Rx2	Yes: CC-NB-LRR	PVX [*Potato virus X*]	*Potexvirus*	CP	138, 232
	Ry_{adg}	No [No sympt/ER]	PVY [*Potato virus Y*]	*Potyvirus*	Unknown	196, 198, 199 235, 226
	ss_{tbr} (=s)	No [ER]	PVS [*Potato virus S*]	*Carlavirus*	Unknown	196, 236
	Y-1	Yes: TIR-NB-LRR.	PVY [*Potato virus Y*]	*Potyvirus*	unknown	237, 238
	Polygenic	No [ER/HR]	TRV [*Tobacco rattle virus*]	*Tobravirus*	MP [29K]	239

Sorghum bicolor Sorghum	*'Krish'*	No	MDMV [*Maize dwarf mosaic virus*] JGMV [*Johnsongrass mosaic virus*] SCMV [*Sugarcane mosaic virus*]	*Potyvirus*	Unknown	17, 240
Thinopyrum intermedium Intermediate wheatgrass	*Bdv2 Bdv3 Bdv4*	No [resistance]	BYDV [*Barley yellow dwarf virus*]	*Luteovirus*	unknown	241-247
	Wsm1	No	WSMV [*Wheat streak mosaic virus*]	*Tritimovirus*	unknown	248-250
Triticum aestivum Common wheat	*Bdv1*	No [Tol.]	BYDV [*Barley yellow dwarf virus*]	*Luteovirus*	unknown	247, 251
	Wss1	No	WSSMV [*Wheat spindle streak mosaic virus*]	*Bymovirus*	Unknown	252, 253
	Sbm [locus]	No	SBWMV [*Soil-borne wheat mosaic virus*] SBCMV [*Soil-borne cereal mosaic virus*]	*Furovirus*	Unknown	254, 255
Vicia faba Broad bean	*Bym-1, Bym-2*	No	BYMV [*Bean yellow mosaic virus*]	*Potyvirus*	Unknown	17
Vigna mungo Black gram	*CYR1*	Yes: CC-NB-LRR	MYMV [*Mungbean yellow mosaic virus*]	*Begomovirus*	CP	256, 257
Vigna unguiculata Cowpea	Monogenic	No [ER]	CCMV [*Cowpea chlorotic mottle virus*]	*Bromovirus*	Unknown	258, 259
	Cry	No [HR]	CMV [*Cucumber mosaic virus*]	*Cucomovirus*	2a polymerase	260-265
	Monogenic	No [partial Dom/ER]	SBMV [*Southern bean mosaic virus*]	*Sobemovirus*	Unknown	266, 267
	Monogenic	No	TRSV [*Tobacco ringspot virus*]	*Nepovirus*	Unknown	259, 268, 269
	Monogenic	No	CABMV [*Cowpea aphid-borne mosaic virus*]	*Potyvirus*	Unknown	150,270, 271
	Cpa	No [ER]	CPMV [*Cowpea mosaic virus*]	*Comovirus*	Protease, 24K Pro	272, 273
Zea mays Maize/corn	*Mdm-1 (Rdm-1)*	No	MDMV [*Maize dwarf mosaic virus*]	*Potyvirus*	Unknown	17, 274
	Msv1	No [Tol./Partial Dom]	MSV [*Maize streak virus*]	*Mastrevirus*	Unknown	275, 276
	Scmv1 Scmv2 Rscmv1 Rscmv2	No (all)	SCMV [*Sugarcane mosaic virus*]	*Potyvirus*	Unknown Unknown Unknown Unknown	243, 277-282

Plant host	*R*-gene	Cloned/type	Recognises	Virus genus	AVR	Reference
Zea mays Maize/corn [cont.]	*Wsm1* *Wsm2* *Wsm3*	No	WSMV [*Wheat streak mosaic virus*] MDMV [*Maize dwarf mosaic virus*] SCMV [*Sugarcane mosaic virus*] SrMV [*Sorghum mosaic virus*] JGMV [*Johnsongrass mosaic virus*]	*Tritimovirus* *Potyvirus*	Unknown	17, 283, 284

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Chapter 2

Tsw gene-based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*

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Abstract

Due to contradictory reports, the avirulence (Avr) determinant that triggers *Tsw*-gene based resistance in *Capsicum annuum* against the *Tomato spotted wilt virus* (TSWV), is still unresolved. Here, the *N* and *NSs* genes of resistance inducing (RI) and resistance breaking (RB) isolates were cloned and transiently expressed in resistant *Capsicum* plants to determine the identity of the Avr-protein. It is shown that the NSs^{RI} protein triggers a hypersensitive response (HR) in *Tsw* containing *Capsicum* plants –and not on susceptible *Capsicum*- while no HR was discerned after expression of the $N^{RI/RB}$ protein, or when NSs^{RB} was expressed. Whereas NSs^{RI} was able to suppress silencing of a functional GFP construct during *Agrobacterium tumefaciens* transient assays on *Nicotiana benthamiana*, NSs^{RB} had lost this capacity. The observation that RB isolates suppressed local GFP silencing during an infection indicated a recovery of RNA silencing suppressor (RSS) activity for the NSs protein, as none of the other TSWV proteins are shown to aid in this. The role of NSs as RNA silencing suppressor and Avr-determinant will be discussed in light of a putative interplay between RNAi and the natural *Tsw* resistance gene.

Introduction

The 'Zig-zag-model' (Jones and Dangl, 2006) is commonly accepted to illustrate the arms race between the plant immune system and plant pathogens. Within this model, the first line of defence is triggered by so-called microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively). These molecules are being recognised by pattern recognition receptors (PRRs) and lead to the (slow) onset of PAMP-triggered immunity (PTI) (Chisholm *et al.*, 2006). Well known PAMPs are bacterial flagellin and fungal chitin (Nicaise *et al.*, 2009). Pathogens encode virulence factors (effectors) that interfere with PTI, and thereby enable them to achieve a successful colonisation/infection. In a third phase, these same effectors are specifically recognised by a second branch of the plant immune system that involves protein products from *resistance* (*R*) genes, and is called effector-triggered immunity (ETI). This recognition generally leads to a rapid hypersensitive response (HR), and involves a programmed cell death (PCD) at the infection site.

Although plant viruses are obligate parasites and replicate intracellular, they too are subject to PTI. RNA silencing can be regarded as a PTI mechanism which enables plants and insects to clear viral infections (Ding and Voinnet, 2007). In this case viral double-stranded (ds) RNA molecules, either from replicative intermediates or folding structures, act as PAMP and their recognition leads to the induction of RNA silencing, also referred to as RNA interference (RNAi) (Ding and Voinnet, 2007). This defence mechanism involves dsRNA cleavage by an RNaseIII-like enzyme, called dicer-like (DCL) protein, into small short-interfering (si)RNA duplex molecules. One strand of this duplex molecule, the so-called guide-strand, is being uploaded into an RNA-induced silencing complex (RISC) which then starts to surveil and sense target (viral) RNA molecules with complementarity to the guide strand, leading to their degradation (Ding and Voinnet, 2007). Plant viruses have evolved different ways to counteract this antiviral defence mechanism. One of the most commonly used strategies is to encode RNA silencing suppressor (RSS) proteins (Díaz-Pendón and Ding, 2008). For many plant viruses RSS proteins have been identified and these have been shown to exert this function in diverse manners, *e.g.* some RSS sequester long or short dsRNAs and thereby prevent their cleavage by DCL or upload into RISC, respectively (Vargason *et al.*, 2003; Lakatos *et al.*, 2006; Alvarado and Scholthof, 2009; Csorba *et al.*, 2009; Giner *et al.*, 2010; Schnettler *et al.*, 2010). In some other cases, the RSS protein prevents maturation of the RISC complex or cleavage of RNA target sequences (Ding and Voinnet, 2007).

Due to the large economic impact of *Tospovirus* diseases, ranking second on the list of most important plant viruses worldwide (Scholthof *et al.*, 2011), the search for natural resistance genes in breeding programs receives a growing interest. So far, only two single dominant resistance genes, *Sw5b* and *Tsw*, have been well described and are available for commercial resistance breeding against tospoviruses. *Sw5b* has been identified in *Solanum peruvianum* and provides high resistance levels to TSWV, *Groundnut ringspot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) (Stevens *et al.*, 1992; Boiteux and de B. Giordano, 1993). The *Tsw* gene (Black *et al.*, 1991; Boiteux, 1995; Jahn *et al.*, 2000) originates from *Capsicum chinense* 'PI' accessions and meanwhile has been introgressed into *C. annuum* cultivars. Resistance is displayed by an HR, like with *Sw5b*, that prevents systemic spread of the virus and eventually leads to leaf abscission (Boiteux and de Avila, 1994). The resistance only holds to isolates belonging to the species TSWV, and not to GRSV,

TCSV or more distantly related tospoviruses (Boiteux and de Avila, 1994). Like in the case of *Sw5b*, resistance-breaking variants of *Tsw* have been identified (Boiteux and de B. Giordano, 1993; Roggero *et al.*, 2002; Aramburu and Martí, 2003; Margaria *et al.*, 2004).

The viral gene product that triggers the *Tsw* resistance has been mapped to the S RNA segment of TSWV (Jahn *et al.*, 2000), pointing towards either *N* or *NSs* as the *avirulence* (*Avr*) gene. In recent years, two reports have been published by Lovato *et al.* (2008) and Margaria *et al.* (2007), which reported contradictory results on the identification of the *Avr*-gene, leaving the identity of the Avr-determinant unsolved.

Here, we have identified the NSs protein as the Avr-determinant of the *Tsw*-based resistance, using a highly-translatable transient expression vector construct.

Results

Characterisation of different TSWV isolates

To identify the *N* or *NSs* gene as Avr-determinant of *Tsw*-gene based resistance, several TSWV RI and RB isolates were collected from different regions in Europe (Table 2.1). Prior to cloning and sequence analysis of the *N* and *NSs* genes, the TSWV isolates were verified for their phenotype on *Tsw* resistant *Capsicum* plants by mechanical inoculation on *Capsicum* species (*C. annuum Tsw+, C. annuum Tsw-* and *C. chinense*) and on *N. benthamiana* as a positive control. Resistant *Capsicum* plants inoculated with RI TSWV isolates Vir127 and Vir129 showed an HR 3-4 days post inoculation (dpi) (Figure 2.1) as necrotic lesions on the inoculated leaf. The small necrotic lesions appeared after 3 dpi (1-2 mm diameter) and expanded over time to large necrotic lesions (4-5 mm diameter) at ± 7 dpi, after which the whole leaf abscised. On these plants, no systemic symptoms (≥7 days) could be discerned, nor could the virus be detected by double antibody sandwich (DAS)-ELISA in these leaves. The resistance breaking isolates Vir160 and Vir171 did not induce HR on the resistant *Capsicum* plants (Figure 2.1) but gave clear systemic symptoms at 10-12 dpi, and detectable levels of virus presence by DAS-ELISA. Susceptible *Capsicum* plants, challenged with all four isolates, showed typical TSWV symptoms, including local and systemic leaf chlorosis, vein yellowing, mottling and overall plant stunting at 10-12 dpi. The presence of virus was confirmed by DAS-ELISA.

These results confirmed the resistance inducing (RI) phenotype of isolates Vir127 and Vir129, and resistance breaking (RB) phenotype of isolates Vir160 and Vir171.

Nucleotide sequence analysis of the *N* and *NSs* genes

To identify differences within the amino acid sequences of the N and NSs proteins of TSWV RI and RB isolates that could point towards the *Avr* candidate gene, these genes were cloned and their nucleotide sequence determined. The amino acid sequences deduced from the *N* and *NSs* genes were used in a multiple sequence alignment to identify differences between the RI and RB isolates. To exclude sequence divergence due to polymorphism, the TSWV reference isolate Br01 was included in the alignments (de Avila *et al.*, 1990). In both alignments several mutations were observed that only showed up in the sequences of the N (Figure 2.2A) and NSs (Figure 2.2B) proteins of the RB isolates and not in the RI isolates. Although some of these mutations were conserved (boxed black), differences were found in both N and NSs amino acid sequence of the RB and RI isolates. These data did not provide support for one of the two genes as the candidate *Avr*-gene.

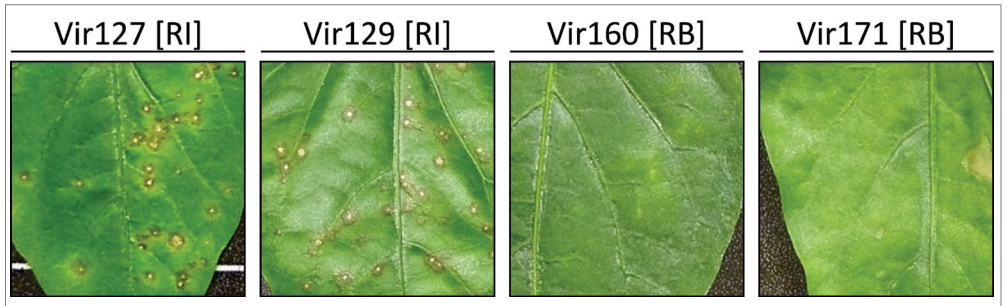


Figure 2.1 Local symptoms on resistant *C. annuum* leaves at 5 dpi with different TSWV isolates. TSWV Vir127, Vir129, Vir160 and Vir171 (from left to right, respectively), were mechanically inoculated on resistant *C. annuum* leaves. Vir127 and Vir129 induce an HR (necrotic lesions), while Vir160 and Vir171 only induce chlorosis. Similar symptoms were observed with these isolates on *C. chinense* (not shown). Pictures were taken at 5 dpi.

Table 2.1 The TSWV isolates used in this study.

TSWV isolate	Origin	Location	Collection date	Phenotype
Vir127	Romania	Unknown	1998	Resistance Inducing
Vir129	The Netherlands	Wageningen University	2002	Resistance Inducing
Vir160	Spain	Field Isolate Almeria	2006	Resistance Breaking
Vir171	Spain	Field Isolate Almeria	2008	Resistance Breaking

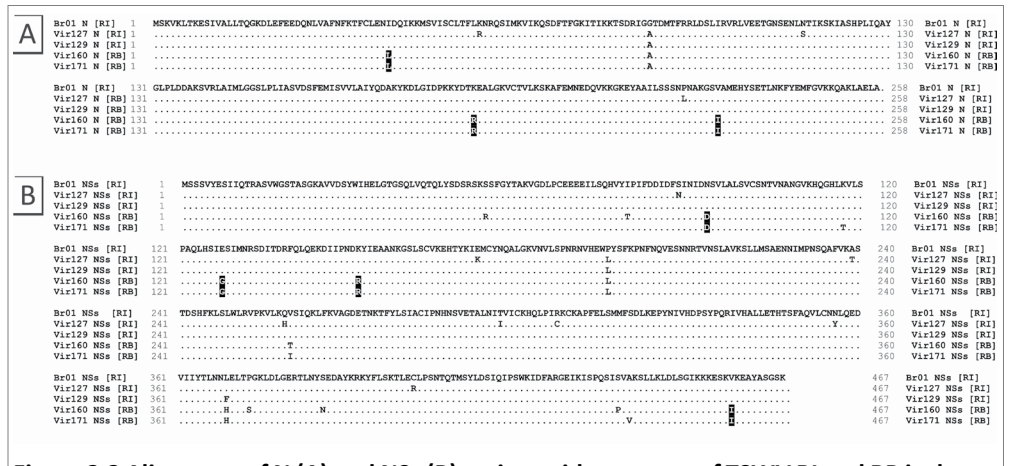


Figure 2.2 Alignment of N (A) and NSs (B) amino acid sequence of TSWV RI and RB isolates. The N and NSs amino acid sequences of the TSWV isolates Vir127, Vir129, Vir160 and Vir171 were aligned with TSWV Br01 isolate included as a reference. Highlighted in bold are the differences in amino acid residues between the RI and RB isolates. Since both N and NSs amino acids show differences, no candidate Avr-protein could be determined.

Expression of TSWV NSs protein by PVX-replicon in resistant *Capsicum* plants was unsuccessful in triggering HR

Avr-determinants of dominant resistance genes are commonly identified by (transient) expression of candidate genes and subsequent visual observation of a resistance response, *i.e.* induction of HR. Here a similar approach was applied to identify the TSWV protein Avr protein for *Tsw* resistance. To this end, the *N* and *NSs* genes of RI and RB isolates were expressed in *Capsicum* plants carrying the *Tsw* resistance gene using the PVX-expression vector pGR106. At 7 dpi, resistant *Capsicum* plants showed local necrosis with all PVX constructs, including the empty

negative control (Figure 2.3). Furthermore, the systemic symptoms were equal in all plants tested, irrespective of the PVX construct used. Similar results were obtained when, instead of the PVX replicon, a TMV replicon was used to express the *N* and *NSs* genes (data not shown).

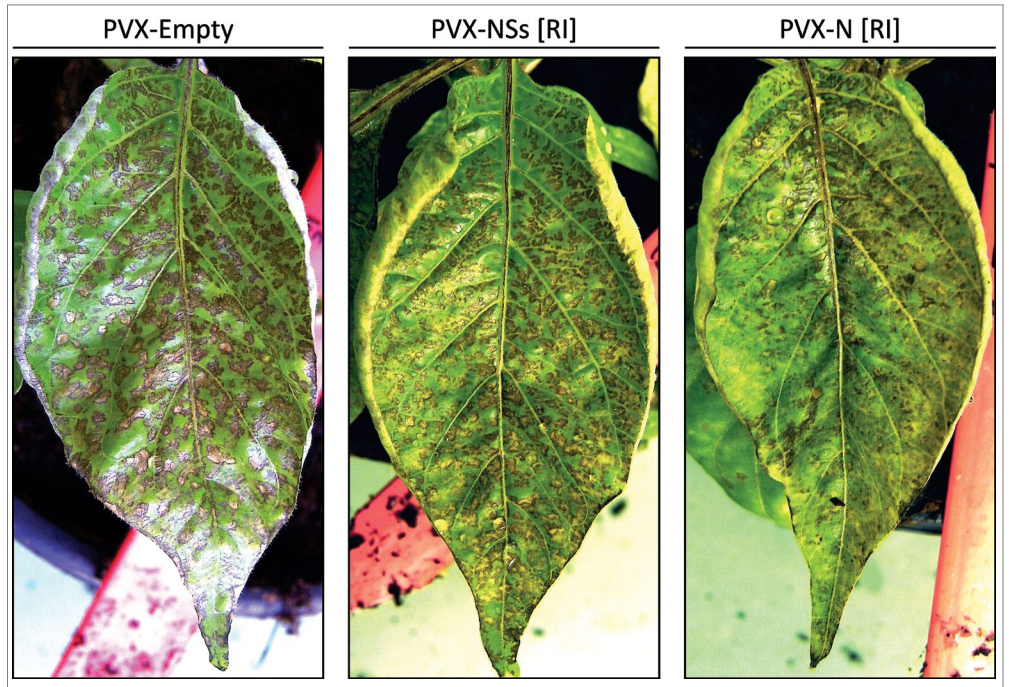


Figure 2.3 Systemic infection of *Capsicum chinense* by PVX recombinants. Recombinant PVX constructs carrying the N^{RI} or the NSs^{RI} gene, were inoculated on resistant *Capsicum chinense* plants using an empty PVX vector as a negative control. As visible in all three panels, at 7 dpi PVX induced necrosis is covering most of the locally infected leaves, irrespective of the gene insertion.

Transient expression of TSWV NSs protein triggers an HR in resistant *Capsicum* plants

Since the viral replicon system appeared unsuitable for the identification of the *Avr*-gene, an *Agrobacterium*-based transient expression vector system was employed. At first various *A. tumefaciens* strains (1D1249, AGLO, AGL1, COR308, GV3101 and LBA4404) equipped with the highly translatable binary expression vector pEAQ-HT

were tested for transformation efficiency and symptom expression in *Capsicum* plants. For easy monitoring the vector contained a copy of the green fluorescence protein gene (GFP). All *A. tumefaciens* strains except 1D1249 induced necrosis starting from 5 dpi. Strain 1D1249 only showed mild chlorosis upon extended (>7 dpi) incubation. Furthermore, the presence of helper plasmid pCH32 significantly increased transformation efficiency of *Capsicum* leaves. Subsequently, the *N* and *NSs* genes from the TSWV-RI [Vir129] and -RB [Vir171] isolates were cloned into pEAQ-HT and transformed into *Agrobacterium* 1D1249 (+ pCH32). With these constructs an *Agrobacterium tumefaciens* transient transformation assay (ATTA) was performed and protein expression was verified and confirmed by Western immunoblot analysis of infiltrated leaf samples (Figure 2.4). Transient expression of the *NSs* gene of the RI isolate induced a clear necrosis of the infiltrated area on *Tsw* containing *Capsicum* plants, visual from 3 dpi (Figure 2.5A and B). The NSs from the RB isolate, and the N derived from RI and RB isolates only caused mild chlorosis of the infiltrated area, similar to leaves infiltrated with the negative control (empty *Agrobacterium* 1D1249 + pCH32; data not shown). A similar chlorosis was observed for all constructs, including NSs from the RI isolate, on susceptible *Capsicum* plants (Figure 5A and B). These results were repeated and confirmed with the NSs from another RI isolate [Vir127] and another RB isolate [Vir160]. To exclude that the presence of the P19 (RNA silencing suppressor from *Tombusvirus*) protein from the pEAQ-HT vector interfered with HR induction, the Avr-protein activity of NSs was also tested after expression from a standard 35S promoting plasmid (pBin19), without the P19 protein. Also in this case an HR was clearly induced 3-5 dpi on the resistant *Capsicum* plants (data not shown).

NSs^{RB} lost its function to trigger HR and its ability to act as RSS

Previously, it was shown that the TSWV NSs protein has RSS activity, and recently Schnettler *et al.* (2010) showed that this protein was able to exert this activity most likely by sequestering long dsRNA and short (si and mi)RNAs, thereby preventing cleavage by dicer and uploading into RISC, respectively. The finding that the NSs protein also triggers *Tsw*-induced HR raised the question whether NSs from the RB isolate still retained the capacity to suppress RNA silencing. To answer this question, the NSs^{RB} was tested in a co-ATTA with a sense GFP-construct on *N. benthamiana*

(Voinnet *et al.*, 1999). As the pEAQ-HT expression vector contains P19, the *NSs* genes were re-cloned into the binary expression vector pK2GW7 (Karimi *et al.*, 2002) using the Gateway technology and verified for protein expression by Western immunoblot analysis (data not shown). In co-ATTAs of the *NSs* genes from the RI and RB isolates and a sense GFP construct, silencing of GFP was suppressed by the NSs^{RI} at 5 dpi as was indicated by an increase in fluorescence (Figure 2.6A). Leaves expressing the NSs^{RB} from isolates Vir160 and Vir171 did not show increase in fluorescence and the protein apparently had lost the RSS capability (Figure 2.6A). RSS activity was also quantitatively estimated by measuring the number of fluorescent units from infiltrated leaves (Figure 2.6B). Controls consisted of untreated leaves and leaves co-infiltrated with GFP and the negative control Maltose Binding Protein (MBP) (Schnettler *et al.*, 2010). The amount of fluorescence in leaves infiltrated with NSs^{RI} was approximately 4 times higher than that recorded in leaves infiltrated with MBP (control) showing RSS activity of the NSs^{RI} protein. In leaves infiltrated with NSs^{RB} constructs fluorescence levels were similar to those in the MBP control, indicating that the NSs from both RB isolates had lost the RSS capacity (Figure 6B).

The resistance breaking viruses are able to suppress local silencing of GFP

As the NSs^{RB} protein had lost its RSS activity, the question arose whether the RB viruses had a reduced fitness in comparison to RI viruses. To address this question, *N. benthamiana* plants were inoculated with two RI and two RB isolates of TSWV and systemically infected leaves were analysed by DAS-ELISA to determine the virus titres. In addition, an antigen coated plate (ACP)-ELISA was performed on the same leaf material to measure the amount of NSs protein. Surprisingly, no difference in virus and NSs titres was found between the different RI and RB isolates (Figure 2.7A and 2.7B), and next to the earlier observation that NSs from the RB virus was not able to suppress GFP silencing in a leaf patch assay raised the question whether during a natural infection the RB virus was still able either to counteract RNAi or evade from it in another way. To test this hypothesis, a local RNA silencing suppression assay was performed, but this time in the presence of a viral infection. During repeated experiments, the results consistently showed that, in contrast to the inability of the NSs^{RB} protein to suppress GFP silencing under transient conditions, the TSWV RB isolates, like the RI isolates, were still able to suppress GFP silencing (Figure 2.7C).

The absence of a clear GFP silencing suppression in the presence of PVX (Figure 2.7C), known to encode a weak RSS (P25), strengthened to indicate that TSWV (RB) virus somehow was still able to suppress RNA silencing.

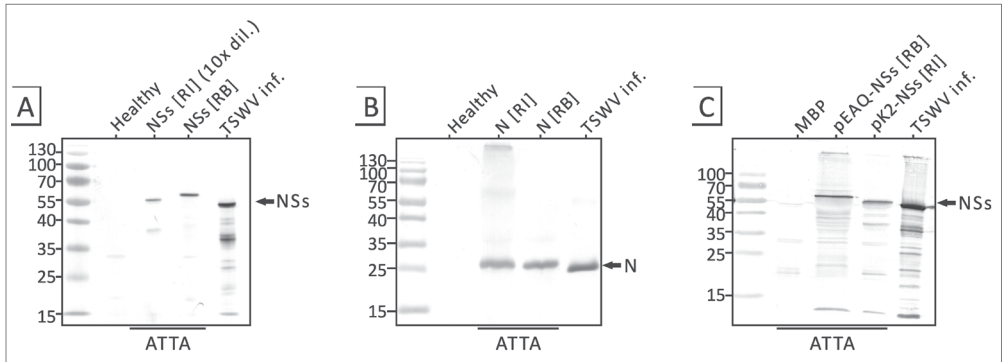
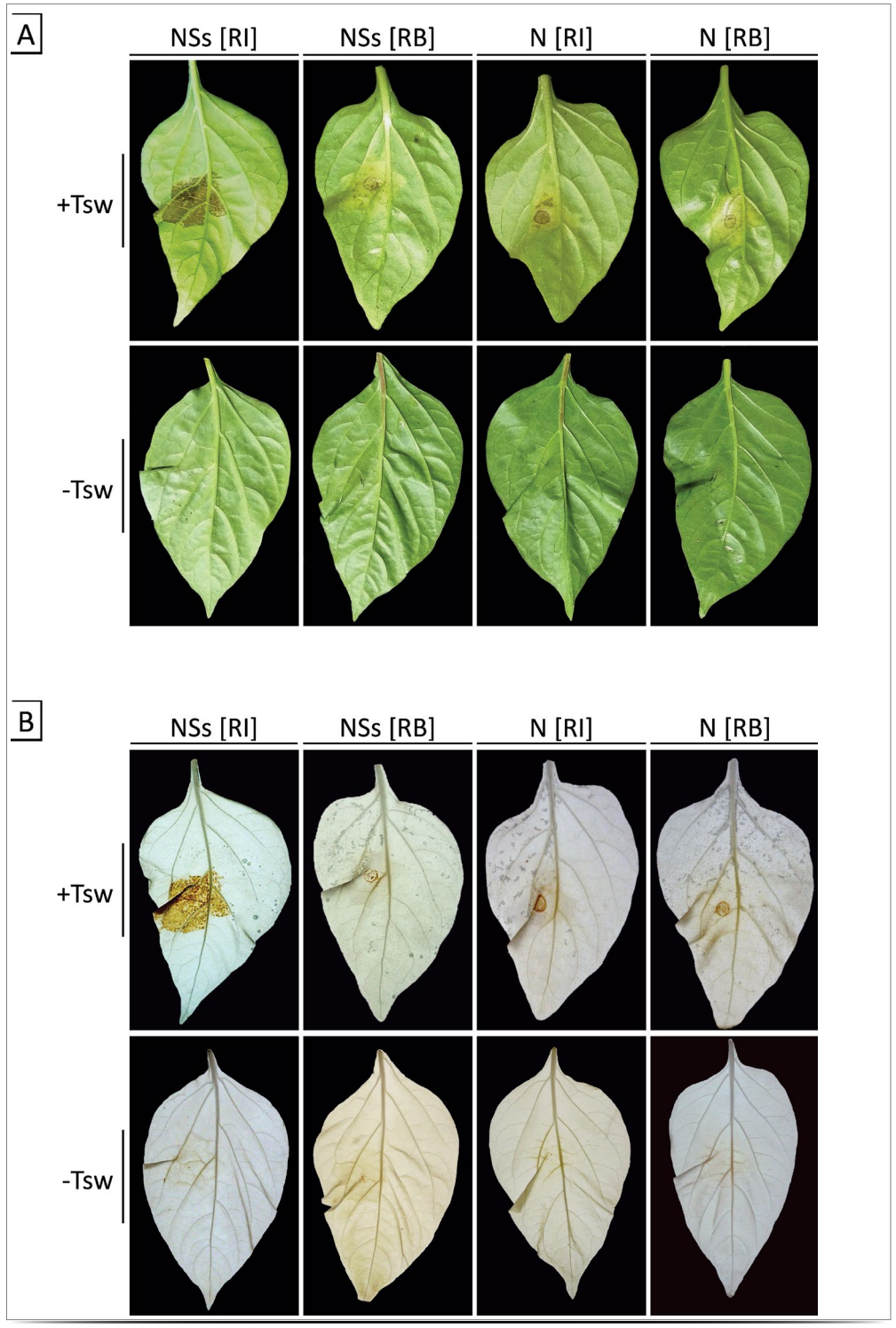


Figure 2.4 Western immunoblot detection of N and NSs proteins expressed from pEAQ-HT in *N. benthamiana*. Infiltrated *N. benthamiana* leaves were collected at 5 dpi. **A.** Westernblot result of the pEAQ-NSs samples, NSs^{RI} was diluted 10x in comparison to the NSs^{RB}. The positive control here is the TSWV extract (Vir129) and the detection was performed with αNSs. **B.** Westernblot result of the pEAQ-N samples, where same amounts of sample were loaded in each lane. Also here, TSWV extract is used as a positive control, detection was done using αN. **C.** Western blot analysis of the NSs^{RB171} expressed from the pEAQ-vector, compared with the NSs^{RI} from the pK2GW7-vector. Similar expression levels can be observed. As positive control, also here TSWV extract is used, detection here was performed with αNSs.

→ Figure 2.5 Symptoms on resistant and susceptible *C. annuum* leaves infiltrated with various pEAQ-HT constructs. The *N* and *NSs* genes derived from RI and RB isolates were transiently expressed in the resistant and susceptible *Capsicum* plants using the pEAQ-HT vector. **A.** Leaves were collected at 5 dpi from the resistant *Capsicum* leaves (upper row) and susceptible *Capsicum* leaves (bottom row). An HR was only observed with a NSs^{RI} protein gene construct on the resistant *Capsicum* plants, but not on the susceptible plants. **B.** The leaves from panel A were destained to better visualise the necrotic area (HR). Clearly, only the entire infiltrated area of the NSs^{RI} is completely necrotic, while in the other leaves some local wounding of the leaf epidermis (allowing easy infiltration of the *Agrobacterium* culture) can be seen.



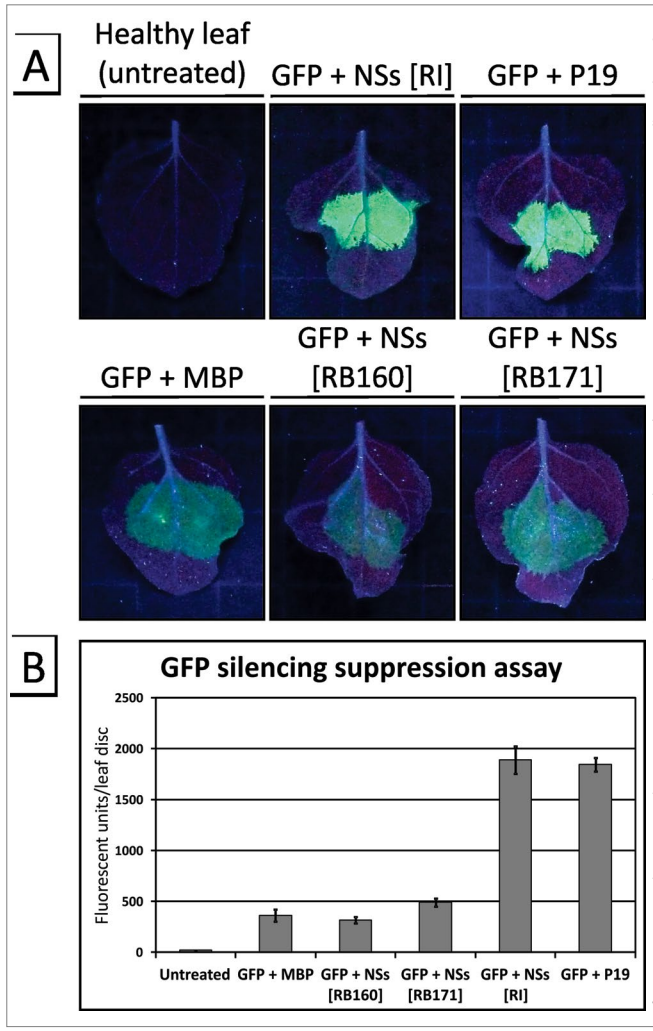


Figure 2.6 GFP silencing suppression assay in *N. benthamiana* leaves. A co-infiltration of a GFP-expressing construct with pK2GW7-NSs^{RI} (from Vir129) or NSs^{RB} was performed on *N. benthamiana* and leaves were collected at 5 dpi for GFP monitoring. **A.** Images of GFP fluorescence in infiltrated leaves, showing that NSs^{RI} is able to suppress RNA silencing, as compared to the positive control P19, while both the NSs^{RB} are not able to suppress the silencing, as compared to the negative control MBP. **B.** The number of fluorescent units measured in a leaf disc (1 cm^2) collected from the agroinfiltrated leaf area. Error bars are calculated from an average of six measurements. This is in support of panel A.

Additional TSWV proteins do not compensate or rescue the loss of RSS activity from NSs^{RB}

The observed difference in RSS activity of the NSs^{RB} when expressed transiently versus the RB-virus, indicated that either the presence of additional TSWV proteins during a viral infection could potentially aid the NSs^{RB} in its RSS activity or, alternatively, another viral protein possesses RSS activity. Previously, other TSWV proteins were already shown to not possess RSS activity (Takeda *et al.*, 2002; Bucher *et al.*, 2003), however, these studies did not include the large polymerase

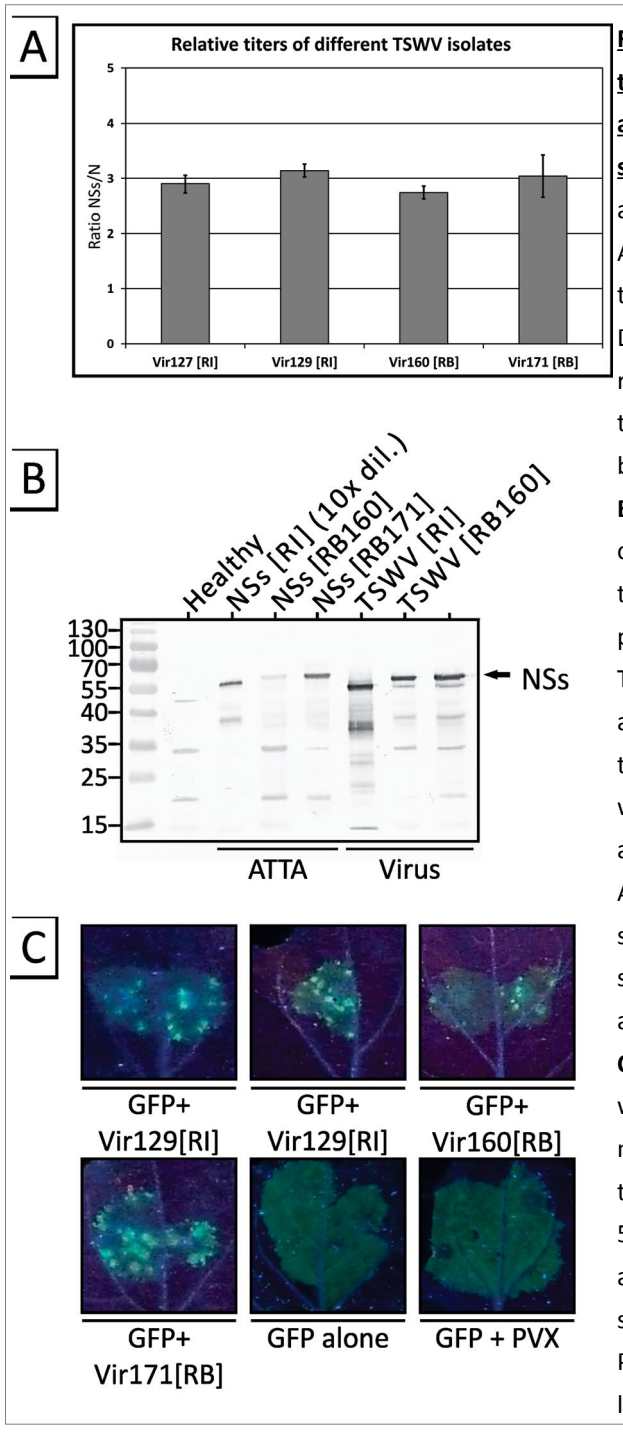


Figure 2.7 ELISA measuring virus titres of different TSWV isolates and their ability to suppress GFP silencing. A. NSs titres of TSWV RI and RB isolates were measured by ACP-ELISA and are shown relative to the virus titres measured by DAS-ELISA. The average of four repetitions is shown, showing that there is no significant difference between these isolates. **B.** Western immunoblot detection of NSs in *N. benthamiana*, transiently expressed from pEAQ-HT or after virus infection. Transiently expressed NSs shows a difference in amount between the RI (from Vir129) and the RB, while in the virus setting similar amounts can be observed. Additionally, in both the transient setting and the viral setting the size difference between the RI and the RB can be observed. **C.** Local transient GFP expression was superimposed with a mechanical TSWV inoculation on the same leaf. Pictures were taken 5 dpi, and show that all isolates are able to suppress local GFP silencing. As a negative control PVX was used, that does not show local suppression of GFP silencing.

(L: RdRp) protein. Therefore, a GFP silencing assay was performed in which the L-protein, expressed from a full-length translatable gene construct (unpublished data) in pK2GW7, or NSs^{RB} protein in the presence of other TSWV proteins, were tested for their ability to suppress RNA silencing. Since the structural viral glycoproteins do not play a role in plants, as they are primarily required for vector transmission, these proteins were not included in this assay. Different combinations of TSWV proteins (N, NSm and L) were mixed with NSs^{RB}, but none of the additional viral proteins aided in or (completely) restored the RSS activity of NSs^{RB} to the level of NSs^{RI} (Figure 2.8).

Discussion

Here we unambiguously show that the NSs protein of TSWV is the Avr-determinant of the *Tsw*-gene based resistance. While the NSs of TSWV resistance inducing (RI) isolates triggered an HR in resistant *Capsicum* 3-5 dpi, the NSs of resistance breaking (RB) isolates and the N protein of RI and RB isolates did not. Interestingly, loss of Avr-activity of the NSs^{RB} coincided with a loss of RNA silencing suppressor (RSS) activity. Although plant virus RSS have been reported previously as Avr-determinant (Li *et al.*, 1999; Moffett, 2009), the present research shows for the first time that the *Avr*-corresponding gene from a natural occurring resistance breaking isolate has entirely lost both its Avr- and RSS activity. In the past a similar situation was reported for resistance breaking isolates of *Tobacco mild green mosaic virus* (TMGMV) against the *Tm-1* resistance gene from tomato, but in those cases the isolates had not completely lost their ability to suppress RNA silencing (Ishibashi *et al.*, 2011). These results not only indicate a putative link between the *Tsw* resistance mechanism and the RNAi pathway but also positions TSWV NSs as effector protein in the 'Zig-zag-model' (Jones and Dangl, 2006).

In two previous papers, the identification of the TSWV *Avr*-gene for *Tsw*-based resistance has been addressed, with conflicting outcome. Margaria and co-workers (2007) suggested the *NSs* as the *Avr*-gene, while Lovato *et al.* (2008) identified the TSWV *N* gene as Avr-determinant. Margaria and co-workers based their conclusion merely on the exclusion of other TSWV proteins as candidate for the Avr-determinant, because attempts to provide the actual experimental proof,

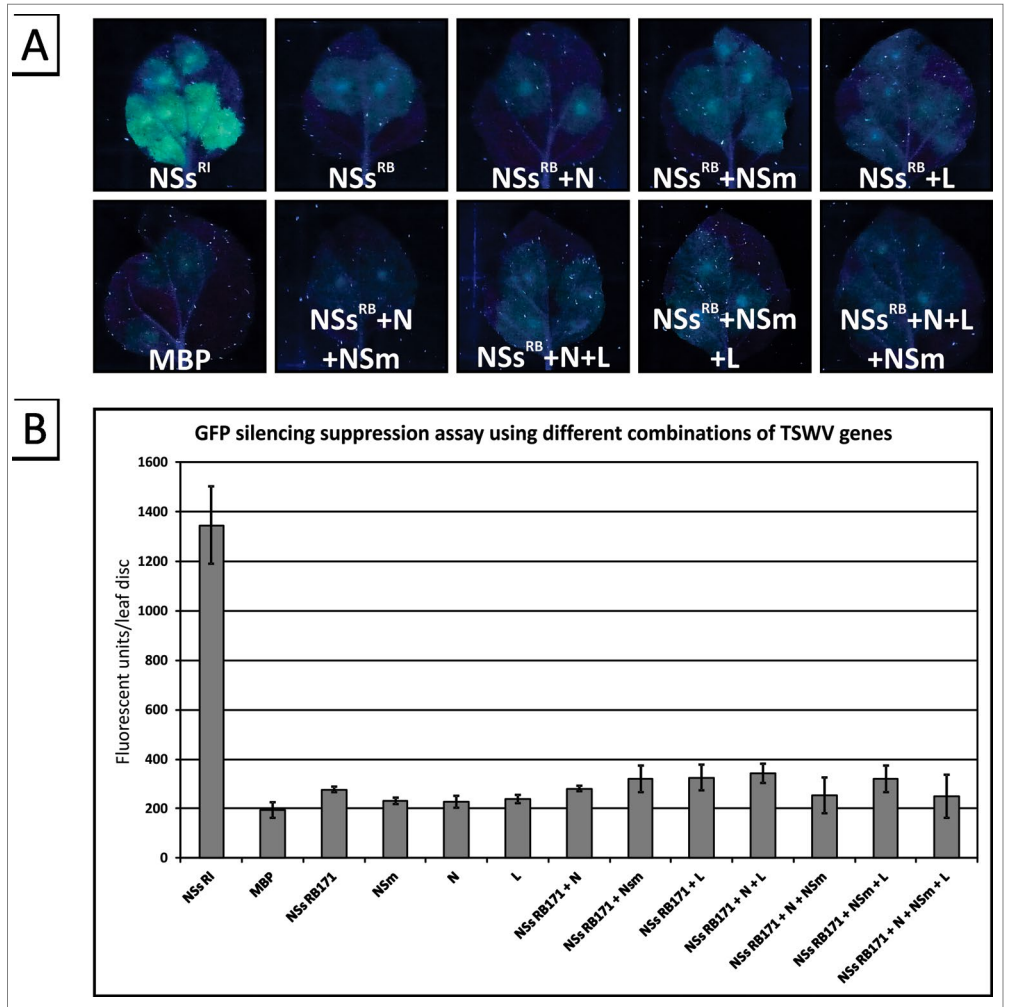


Figure 2.8 GFP silencing suppression assay using additional TSWV proteins in a co-ATTA with NSs^{RB}. TSWV proteins N, NSm and L were co-expressed with NSs^{RB} and GFP to identify a possible rescue of RSS activity from NSs^{RB}. **A.** Panel of co-ATTA pictures showing GFP fluorescence after expression of different combinations of TSWV proteins. As positive and negative controls, NSs^{RI} and MBP, respectively, were included. **B.** The GFP from the ATTA with single constructs and the pictures shown in panel A were quantified by spectrometry and shown as an average out of four replicates per sample, with standard deviations shown as error bars.

i.e. show HR-induction by NSs, failed. Lovato and co-workers used a PVX-replicon in their assays to express the viral proteins, and as we showed here, such viral replicons induce necrotic lesions on *Capsicum*, which can be mistaken for an HR response. A third, more recent paper described a phylogenetic analysis of different TSWV isolates derived from *Capsicum* plants, in which the authors claim that some mutations observed in the *NSs* gene were positively selected by the *Tsw*-gene (Tentchev *et al.*, 2011). Although the latter paper hints towards the NSs as being the Avr-determinant of *Tsw*-based resistance, no experimental proof was provided in support of this.

An additional interesting finding from our study was the observation that the NSs protein from the resistant breaking isolate lost its RSS activity. Considering that a loss of RSS activity affects a virus' counter defence against RNAi and as a consequence would lead to a reduction in virus titres, it was surprising to see that for the RI and RB isolates similar virus titres were detected during an infection in *N. benthamiana*, concomitant with the presence of RNA silencing suppression activity as observed during the GFP silencing in a leaf patch assay. These data suggest that NSs RSS activity is somehow recovered during viral infection, as none of the other viral proteins are shown to aid in this. Since NSs expression levels from RB isolates during a natural infection were always higher when compared to transient NSs^{RB} expression, but similar to those from RI isolates, the possibility that NSs^{RB} still contains some residual RSS activity that is only observed upon enhanced expression levels (during virus infection) and not during lower expression levels (transient), cannot be entirely ruled out. However, as transient expression levels of NSs^{RB} were consistently lower, as also observed with other non-functional viral RSS proteins (Díaz-Pendón and Ding, 2008; Schnettler *et al.*, 2008), even from the high expression vector pEAQ-HT, this not only indicated that the protein was affected in its RSS functionality but also further hampered its transient expression to high levels to analyse for residual RSS activity. Additionally, other TSWV proteins do not seem to aid in the restoration of transient RSS activity of the NSs^{RB}, including the previously untested L-protein (331 kDa), indicating that the NSs^{RB} somehow is RSS active during virus infection. The observation that PVX did not give a clear GFP silencing suppression, while its suppressor of RNAi, P25, earlier showed transient RSS activity in this assay (Bayne *et al.*, 2005), supported the idea that the observed RSS activity with TSWV (RB) virus is not just an artefact.

To analyse whether the loss of RSS activity of the NSs^{RB} proteins was due to loss of affinity for siRNAs, an electrophoretic mobility shift assay (EMSA) was performed on transiently expressed NSs^{RI} and NSs^{RB} from pEAQ, with N as negative control, which showed that NSs^{RI} is able to shift siRNAs, while NSs^{RB} cannot (Figure 2.9A). However, since the expression levels of NSs^{RB} are much lower compared to NSs^{RI} and the presence of P19 protein strongly competes for the majority of the siRNAs, the absence of a shift does not rule out that NSs^{RB} might still possess some (residual) affinity to small RNAs. This was supported by the observation that EMSAs performed with virus extracts from TSWV-RI and –RB clearly showed a shift of siRNAs with both isolates (Figure 2.9B).

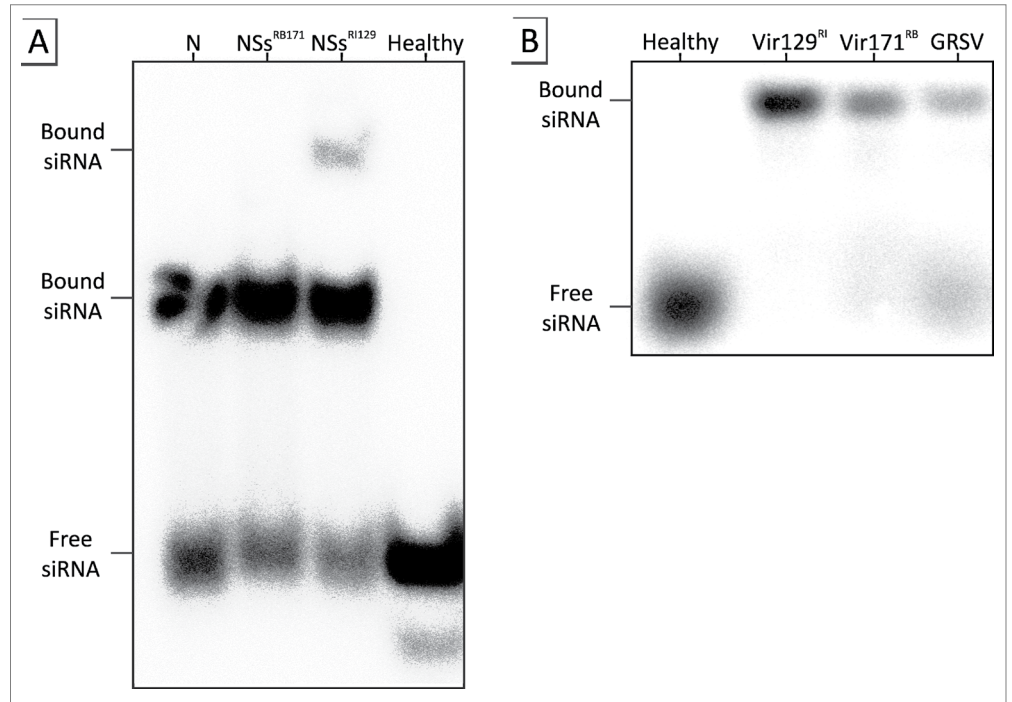


Figure 2.9 Electro mobility shift assay (EMSA) with siRNAs performed on TSWV extracts and transiently expressed N and NSs proteins. An EMSA was performed using leaf material transiently expressing the NSs constructs (**A**) and systemically infected leaf material of TSWV isolates (**B**) used in this study. A mobility shift in siRNAs upward in the gel reflects NSs binding of siRNAs. The additional shift lower in the gel is due to the P19 protein present in the construct.

Nowadays, RNA silencing is well accepted as a virus triggered immunity mechanism in plants and suppressed by viral RNA silencing suppressor proteins (RSS), which could alternatively be referred to as effectors. *R*-gene mediated immunity is a second line of defence that is triggered by effectors. The resulting arms race, nicely illustrated by the 'Zig-zag-model' (Jones and Dangl, 2006), thereby implies a link between RNAi and *R*-gene mediated immunity for viral pathogens with a key role for viral RSS as effectors but experimental evidence for this so far has been scarce. Only a few cases have been reported in which viral RSS are also reported as effectors (Avr-determinant), and these are limited to *Tomato bushy stunt virus* (TBSV) P19, *Tomato aspermy virus* (TAV) 2b, *Potato virus X* (PVX) 25K and *Turnip crinkle virus* (TCV) Coat protein (Oh *et al.*, 1995; Scholthof *et al.*, 1995; Li *et al.*, 1999; Malcuit *et al.*, 1999; Angel *et al.*, 2011). Only for the Tav2b protein a clear link was found between the Avr and RSS activity (Li *et al.*, 1999; Chen *et al.*, 2008), but not for the TBSV P19 (Hsieh *et al.*, 2009) and TCV CP (Choi *et al.*, 2004).

Although the *Tsw* resistance gene has not been cloned yet, it has been shown to be a single dominant resistance gene (Jahn *et al.*, 2000) and thus most likely of a common NB-LRR type. Whereas the TSWV NSs protein is not the first example of an RSS protein that also acts as Avr-determinant, it so far is the first virus of which natural resistance breaking isolates have been collected from the field and their respective *NSs* gene copy was shown to lack both RSS- and Avr activity. Even though their functional domains have not been mapped yet, these observations were confirmed for two different RB isolates which strengthens the idea that RSS and Avr activity within NSs are tightly linked, similar as for TAV 2b. Although these findings support the idea that the corresponding *R*-genes may be triggered by a functional (RSS) aspect of the Avr-determinant, the possibility that this only requires a minimal secondary structural feature cannot be excluded. However, solving this issue might be hampered by the difficulty to separate functions in – multifunctional - viral proteins and as a consequence only functional viral proteins may be recognised by *R*-gene products. Another example in support of this is the inability to obtain PVY NIaPro mutants that had lost protease activity but still retained the ability to trigger HR by the *Ry*-resistance gene (Mestre *et al.*, 2000; 2003).

In past and current literature, examples have been described in which the necrotic response (cell death) induced by effector proteins and the resistance against the pathogen are physiological processes that can be separated

(Bendahmane *et al.*, 1999; Bai *et al.*, 2012). Still and up till now, HR is generally accepted as a clear output of activated resistance, also in the case of a systemic HR (SHR) and the invasion of the pathogen is not entirely prevented. The latter is supported by studies on *e.g.* PVX and the *Rx* resistance gene, where insufficient or partial recognition of the Avr-determinant (CP) leads to a SHR (Farnham and Baulcombe, 2006; Tameling and Baulcombe, 2007), and other examples, like work of Dinesh-Kumar on the *N* gene from Tobacco against TMV (Dinesh-Kumar *et al.*, 2000) and the study of *Plantago asiatica mosaic virus* (PlAMV) in *N. benthamiana* (Komatsu *et al.*, 2011). In our study we have clearly shown that NSs^{RI} only induces necrosis on *Tsw*-containing plants and not on susceptible plants, which indicates that induction of HR is directly linked to the presence of the resistance gene and identifies NSs as Avr-determinant. Although Margaria *et al.* (2007) have reported on TSWV RB isolates that induce systemic HR, these observations are likely to be explained as a result of a partial recognition of the Avr-determinant as described above for PVX and *Rx*. The induction of HR is the dominant response, as a co-ATTA with NSs^{RI} and either NSs^{RB160} or NSs^{RB171} from the pEAQ-vector on resistant *Capsicum* plants showed an HR (Figure 2.10).

Interestingly, on SDS-PAGE NSs^{RI} was consistently running at a higher molecular weight compared to $NSs^{RB160/171}$, and this was irrespective of transient or viral expression. A closer look at the aa-sequence of both did not give any indications for possible post-translational modifications in NSs^{RI} that could cause for this. Predictive tools on several possible post translational modifications were used (Phosphorylation, Sumolation, Acetylation, [N/O]-Glycosylation, Ubiquitination) with NSs^{RI} and NSs^{RB} sequences as input, but none gave significant differences between the sequences, thus the reason for the migration difference remains unsolved so far.

Based on all data a model is presented (modified from Chisholm *et al.* (2006) and Moffett *et al.* (2009)) for the TSWV-*Tsw* pathosystem in which the dual role of NSs as suppressor of the innate (RNAi) immune system and Avr-determinant for *Tsw*-induced HR is presented (Figure 2.11). Whether the RSS function of NSs is coupled to Avr activity just because of an (overlapping) structural conformation, or truly functionally coincides with Avr activity will have to be solved by future analysis of additional RB isolates and NSs domain mapping studies. Although the mode of action of resistance genes still remains a matter

of debate, one of the most commonly accepted models is the guard-hypothesis (van der Biezen and Jones, 1998; Jones and Dangl, 2006). In this model, the resistance gene product is guarding a certain host protein, and is able to perceive alterations of its 'guardee' target upon interaction with the Avr-determinant, which leads to an induction of HR. Unfortunately, this model does not explain how resistance breaking virus isolates preserve their virulence, as present in our described case of TSWV. Besides the guard model, and to explain the preservation of virulence, other models were proposed, *e.g.* the decoy model (van der Hoorn and Kamoun, 2008) or the more recently proposed broader resistance model, the bait and switch model (Collier and Moffett, 2009). Regardless of the model for *Tsw* resistance, the identification of possible host protein target(s) for NSs, whether guardee, decoy or bait, will become a next challenge and contribute to a further unravelling of resistance gene mechanisms.

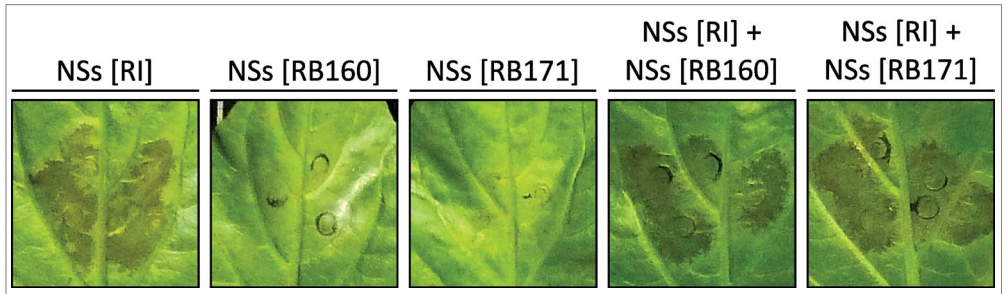


Figure 2.10 Triggering of HR upon co-ATTA of NSs^{RI} with NSs$^{RB160/171}$ on resistant *Capsicum* plants. Leaves of resistant *Capsicum annuum* plants were infiltrated with the NSs^{RI}, NSs^{RB160} and NSs^{RB171} alone and in a co-ATTA setting. Pictures were taken at 5 dpi and show an HR in those leaves where NSs^{RI} was expressed.

Materials and Methods

Virus and Plant material

Four different virus isolates of TSWV were included in this study, *i.e.* Vir127 [Romania, 1998], Vir129 [The Netherlands; WUR, 2002], Vir160 [Spain; Almeria, 2006] and Vir171 [Spain; Almeria, 2008] (Table 1). Virus isolates were maintained

on *Nicotiana benthamiana* by serial passaging (maximal 5 times) using mechanical inoculation (de Avila *et al.*, 1993) and stocked as frozen leaves at -80 °C. To confirm the phenotypes of the isolates, two genotypes of *C. annuum* were used: HK0004, a TSWV susceptible cultivar (*Tsw*-), and YF0009, a TSWV resistant cultivar (*Tsw+*). *C. chinense* PI 152225 was included as the original *Tsw* source host. All plants were grown and maintained under greenhouse conditions (24 °C with a 16h light/8h dark regime).

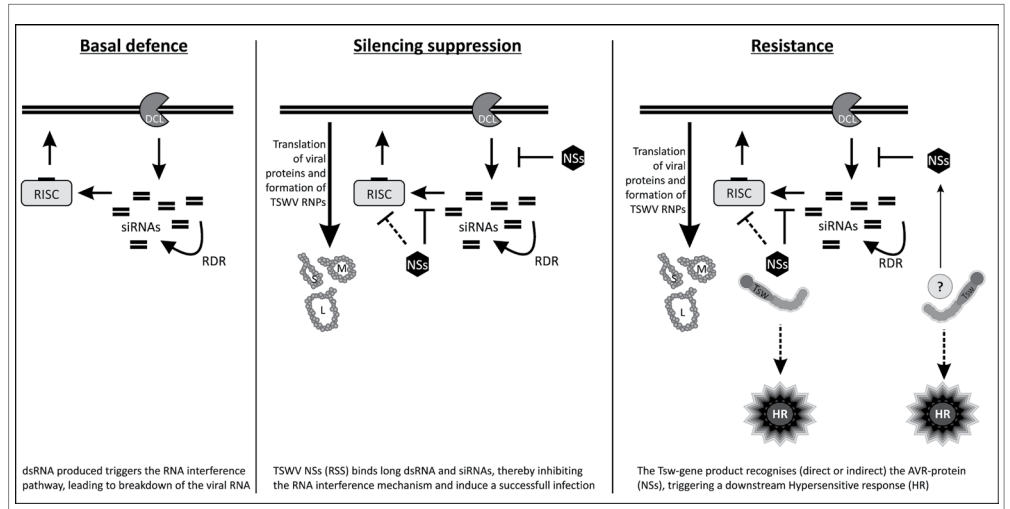


Figure 2.11 Illustration of the Zig-zag-model for the TSWV pathosystem and the *Tsw* resistance. Representation of the arms race between TSWV and plants containing the *Tsw* resistance gene (modified from Moffett (2009) and Chisholm (2006)) in which the roles of NSs as RSS and Avr-protein are indicated. The left panel shows the stage of PAMP-triggered immunity (PTI), represented by the RNAi response against plant virus infection. The middle panel depicts the stage of a successful infection during which a functional viral RNAi suppressor protein, *in casu* TSWV NSs, blocks the RNA silencing pathway (preventing cleavage of dsRNA by DCL and uploading of siRNAs into RISC). The right panel represents the stage of effector-triggered-immunity (ETI) during which the NSs is recognised as Avr-determinant and triggers *Tsw*-induced HR.

Amplification and sequence verification of *N* and *NSs* genes

Total RNA was isolated from (infected) leaves using Trizol (Invitrogen). From the total RNA, 0.5 µg was used as a template for first strand cDNA synthesis and subsequent polymerase chain reaction (PCR) amplification of the *N* and *NSs* genes using the following primer sets at an annealing temperature of 55 °C:

N-Fw (5'-dG**GC GGC CGC** ATG TCT AAG GTT AAG-3') and
N-Rv (5'-dCC**G TCG AC**T CAA GCA AGT TCT GC-3'),
NSs-Fw (5'-dG**GC GGC CGC** ATG TCT TCA AGT GTT-3') and
NSs-Rv (5'-dCC**G TCG AC**T TAT TTT GAT CCT GAA-3').

For feasible cloning, all forward (Fw) primers additionally contained a *Not*I restriction site, and the reverse (Rv) primers a *Sal*I restriction site, both at the 5'-end (highlighted in bold). PCR amplification was performed using Phusion high-fidelity Taq polymerase according to the manufacturers' procedures (Finnzymes). Amplified DNA products were resolved on a 1% agarose gel and fragments corresponding in size to the *N* and *NSs* genes were gel-purified and subsequently cloned into pJET vector (Thermo Scientific). Positive clones were selected and verified by sequence analysis. Nucleotide and amino acid sequences from the *N* and *NSs* genes of the TSWV isolates in this study were analysed by multiple sequence alignment using the ClustalW algorithm. Alignments were edited using the BioEdit program (Hall, 1999). The sequence of TSWV BR01 (Genbank accession D00645) was included as reference isolate.

Cloning procedures

To express the *N* and *NSs* genes from a PVX replicon, the corresponding genes were excised by *Not*I and *Sal*I from pJET plasmid and subsequently cloned into *Not*I/*Sal*I digested pGR106 (Lu *et al.*, 2003). Positive clones were selected and transformed into *Agrobacterium* strain GV3101 (Holsters *et al.*, 1980) containing helper plasmid pSoup (Hellens *et al.*, 2000). An ATTA was performed and the PVX replicon was expressed. Expression of *N* and *NSs* from the PVX replicon was verified by SDS-PAGE and Western immunoblot analysis of *N. benthamiana* leaf samples collected from local and systemic leaves, 5 dpi and 7 dpi, infiltrated with *Agrobacterium* containing the PVX replicon constructs. Systemic infected leaves of *N. benthamiana* that scored positive for N and NSs expression were used as an inoculum source for challenging *Capsicum* plants. For transient expression of TSWV *N* and *NSs*, the highly

translatable pEAQ-HT vector system was used (Sainsbury *et al.*, 2009). To this end, coding sequences for *N* and *NSs* were re-cloned by *Not*I excision from pJET vector constructs into *Not*I digested pEntr11-*ccdB* (from which the *ccdB* gene was removed by *Eco*RI digestion). Positive clones were selected and verified by sequence analysis and subsequently used for transfer of the *N*/*NSs* gene inserts via an LR-reaction into a Gateway (Invitrogen) compatible pEAQ-HT-pDest1 destination vector (Sainsbury *et al.*, 2009). Obtained clones were transformed into *A. tumefaciens* 1D1249 cells, containing helper plasmid pCH32. An ATTA was performed to express the transgenes and the expression was verified by Western immunoblot analysis of leaf samples infiltrated and collected at 5 dpi. Leaves were destained in ethanol and acetic acid solution (3:1 v/v) for 5-6 days to visualise the necrotic tissue after induction of the HR.

Agrobacterium Transient Transformation Assay (ATTA)

The ATTA assay was performed according to the protocol of Bucher *et al*. (2003), with slight modifications. In brief: *Agrobacterium tumefaciens* was grown overnight at 28 °C in LB3 medium containing proper antibiotic selection pressure. From this culture, 600 µl was freshly inoculated into 3 ml induction medium and grown overnight. Strain *A. tumefaciens* 1D1249 (Wroblewski *et al.*, 2005) with helper plasmid pCH32 (Hamilton *et al.*, 1996) was grown under 1.25 µg/ml tetracycline selection pressure, while *A. tumefaciens* LBA4044 (Ooms *et al.*, 1982) was grown under 20 µg/ml rifampicin selection pressure. Additional strains used during this study were COR308 (Hamilton *et al.*, 1996) selected with 2 µg/ml tetracycline, AGL0 and AGL1 (Lazo *et al.*, 1991) selected with 20 µg/ml rifampicin and 100 µg/ml carbenicillin, respectively.

Serological detection of virus and proteins

TSWV virus was detected, and titres determined by dotblot and enzyme-linked immunosorbent assay (ELISA) analysis, respectively. Dotblot analysis was performed on leaf samples from systemically infected *N. benthamiana* leaves (7 dpi) ground in PBS-Tween (0.05 % v/v) and spotted on nitrocellulose in a dilution series of 0, 5, 25 and 125 times. The filter was blocked with 2 % ELK + PBS-Tween (0.05 % v/v), washed with 0.25 % ELK + PBS-Tween and subsequently incubated with antiserum against TSWV (de Avila *et al.*, 1993). Antigen-antibody complexes were detected

with goat anti-rabbit IgG conjugated to alkaline phosphatase (Dako) NBT/BCIP as a substrate (Roche). Virus titres were analysed by ELISA using antiserum against TSWV. ELISA was performed on systemically infected leaf extracts from *Capsicum* species (10 dpi) and *N. benthamiana* plants (7 dpi) in PBS-Tween buffer (1:3 w/v) in a double-antibody-sandwich (DAS) format according to de Avila *et al.* (1993). Absorbance values were measured at 405 nm using the Fluorstar plate reader (BMG Labtech) 30 and 50 minutes after addition of the substrate. Antigen-coated plate (ACP-)ELISA was used to measure the NSs titres using the αNSs as a primary antibody. ACP-ELISA was performed similar to DAS-ELISA, except that the plates were coated with extracts from systemically infected leaves ground in 2x coating buffer (1 L: 3.18 g Na_2CO_3 + 5.86 g $NaHCO_3$). Expression of TSWV N and NSs proteins was analysed by SDS-PAGE and subsequent Western immunoblot analysis using polyclonal antisera against TSWV N and NSs as previously described (de Avila *et al.*, 1993).

GFP silencing suppression assay

Leaves were agroinfiltrated with a functional GFP construct (Tsien, 1998) as described above, with a final $O.D._{600nm}$ 0.5 per construct. A construct expressing MBP was used as a negative control (Schnettler *et al.*, 2010). Infiltrated leaves were monitored for GFP expression at 5 and 10 dpi using a hand UV-lamp. For quantification of GFP fluorescence, leaf discs with a diameter of 1 cm were taken from the infiltrated leaf area and the number of fluorescent units was measured using the Fluorstar Optima (BMG Labtech). Suppression of local RNA silencing by virus infection was analysed after agroinfiltration of a functional GFP construct and subsequent mechanical inoculation of the same leaf area with each of the TSWV isolates and PVX, as described earlier. The results were observed 5 dpi.

Electrophoretic mobility shift assays (EMSA)

The affinity of NSs for siRNA duplex molecules was analysed according to Schnettler *et al.* (2010). In brief: 0.6 grams of systemically infected leaf or local inoculated leaf was ground in liquid nitrogen and suspended in 1.5 ml binding buffer (20 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT). Radio-labelled siRNAs (0.5 µM) were incubated with ± 2 µg total protein amount derived from virus-infected or local inoculated leaf extracts per 20 µl reaction and incubated for 20 minutes at room temperature in binding buffer. As negative controls, siRNAs alone or incubated with

healthy plant extracts, were included. Complexes were resolved on 0.5x TBE native 8% PAGE gel. After electrophoresis, gels were vacuum dried at 80 °C for 30 minutes and exposed o/n to a phosphor screen and scanned (Molecular Dynamics Typhoon Phosphor imager, Amersham Biosciences).

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Chapter 3

Analysis of *Tomato spotted wilt virus* NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression

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Abstract

Recently, TSWV NSs protein has unambiguously been identified as Avr-determinant for *Tsw*-based resistance. The observation that NSs from two natural resistance breaking isolates had lost RNA silencing suppressor activity and avirulence suggested a link between both functions. To test this, a large set of NSs mutants was generated by alanine substitutions in NSs from resistance-inducing wild type isolate (NSs^{RI}), amino acid reversions in NSs from resistance-breaking isolates (NSs^{RB}), domain deletions and – swapping. Testing these mutants for their ability to suppress GFP silencing and trigger *Tsw*-mediated HR revealed that both functions can be separated. Changes in the N-terminal domain revealed to be detrimental for both activities and indicated the importance of this domain, additionally supported by domain swapping between NSs^{RI} and NSs^{RB}. Swapping domains between the closely related *Tospovirus* GRSV NSs and TSWV NSs^{RI} showed that Avr-functionality cannot simply be transferred between species. Whereas deletion of the C-terminal domain converted NSs completely dysfunctional, only few single amino acid mutations in the C-terminus affected both functions. Mutation of a GW/WG motif (position 17/18) rendered NSs completely dysfunctional for RSS and Avr-activity and indicated a putative interaction between NSs and AGO1 and its importance in TSWV virulence and viral counter defence against RNAi.

Introduction

RNA silencing (also named RNA interference, RNAi) is part of the innate immune response in plants against viral invasion. It is triggered by viral double stranded (ds)RNA, derived from secondary folding structures or replicative intermediates, that is being cleaved in the cytoplasm by a host Dicer-like (DCL)-protein into small interfering (si)RNAs of 21-24 nucleotides in length (Ding and Voinnet, 2007). Upon unwinding of siRNAs, one of the strands (guide strand) is being uploaded in the RNA induced silencing complex (RISC), while the other strand is destroyed. Activated RISC surveils the cytoplasm for the presence of complementary (viral) RNA target molecules, and degrades these using the Argonaute (AGO) slicing activity. Viruses evade or suppress this pathway to achieve a successful infection in the plant

host. The most widespread known way of doing this is by encoding RNA silencing suppressors (RSSs), proteins that interfere at various steps in the RNA silencing pathway (Díaz-Pendón and Ding, 2008). Many plant RNA viruses code for an RSS that exert its function by sequestering long dsRNA or siRNAs and thereby prevent their cleavage by DCL and their uploading into RISC, respectively. Although other modes of interference (at the level of AGO1, DCL and RdR) are also reported (Zhang *et al.*, 2006; Giner *et al.*, 2010; Incarbone and Dunoyer, 2013), all prevent viral RNA target molecules from becoming degraded by the RISC complex. For *Tomato spotted wilt virus* (TSWV), representative of the plant-infecting bunyaviruses (Kormelink *et al.*, 2011), the NSs protein has been identified as RSS (Takeda *et al.*, 2002; Bucher *et al.*, 2003) and most likely exerts this activity by sequestering long dsRNA and small short interfering-(si)RNAs (Schnettler *et al.*, 2010). Besides its RSS activity, NSs enhances translation of viral transcripts, which appears most strongly in the additional presence of the viral nucleoprotein (N). It is postulated that NSs is able to do this as a kind of functional equivalence of a poly(A) tail binding protein (PABP), by binding to the dsRNA hairpin structure predicted from the 3'-untranslated region of viral transcripts and encoded by the intergenic region of the ambisense RNA elements (Geerts-Dimitriadou *et al.*, 2012).

TSWV is also prone to a second line of defence in plants that is based on dominant resistance (*R*-)genes, and is also referred to as Effector-Triggered Immunity (ETI) (Jones and Dangl, 2006). During this defence mechanism, a host *R*-gene product (in-) directly perceives the virus by one of its (effector) proteins, which activates a programmed cell death response visualised by a Hypersensitive Response (HR). The resistance model explaining indirect recognition of avirulence (Avr) proteins is often referred to as the 'guard-model' or 'decoy-model', in which the *R*-gene product guards a host factor (functional or decoy) that upon interaction with the Avr-protein triggers the *R*-gene mediated resistance (Moffett, 2009). Two dominant *R*-genes are available for commercial breeding against TSWV, namely the *SW5b*-gene from tomato and the *Tsw*-gene from pepper. Whilst in the past conflicting papers have appeared on the identification of the *Avr*-gene from TSWV upon recognition by the *Tsw*-gene product causing an HR in pepper (Margaria *et al.*, 2007; Lovato *et al.*, 2008), recently, we unambiguously identified NSs as the Avr-determinant for *Tsw*-based resistance (Chapter 2). Analysis of two natural resistance breaking field isolates and characterisation of their NSs proteins, showed that for these isolates

the NSs protein lacked both RSS and Avr-activity which indicated a putative link between both activities (Chapter 2).

Here, we have generated and tested a large set of NSs mutants for their ability to suppress GFP silencing and trigger *Tsw*-mediated HR. The results indicate that both functions can be individually disrupted, although the N-terminal part of NSs seems to hold the most important sequences for both functions.

Results

Generation of NSs mutants

To test whether the RSS activity and elicitation of *Tsw*-mediated HR were indeed tightly linked, and furthermore identify the role of certain (conserved) amino acids (aa), motifs and domains in NSs for either one of those functions, a large NSs mutant screen was performed. To this end, three series of mutants were generated and tested for their ability to 1) suppress GFP silencing in a leaf patch assay on *Nicotiana benthamiana* and 2) elicit *Tsw*-mediated HR on *Capsicum annuum* (*Tsw*+). Therefore, the NSs sequence obtained from a TSWV resistance inducing (RI) isolate (Vir127) and a resistance breaking (RB) isolate (Vir160) and referred to as NSs^{RI} and NSs^{RB}, respectively (Chapter 2), was used to generate mutants from.

The earlier identified affinity of NSs to long- and small dsRNA implied an important role for RNA binding domains in RSS activity (Schnettler *et al.*, 2010). For this reason, the first set of mutants was generated from NSs^{RI} in which amino acids from within 3 predicted RNA binding domains (Figure 3.1A), according to BindN (Wang and Brown, 2006), were substituted for alanines, as described in the Materials and Methods, resulting in mutants designated S48A, S48A/R51A, K53A/G57A, R211A and KKK452/K457A (Table 3.1).

A second set of mutants was generated from NSs^{RI} based on multiple sequence alignment of different *Tospovirus* NSs sequences (Figure 3.1A). Amino acid (aa) residues within conserved domains were selected and substituted for alanines to test the importance of these domains for RSS and Avr-activity. The resulting mutants were denoted: S29A/Y30A, S74A/Q75A, Q113A/G114A, G160A/S161A, Y197A/S198A, N288A/S289A, N355A/N356A, P373A, L396A/S397A and S411A/Y412A (Table 3.1).

To identify the aa residue(s) within NSs^{RI} that are important for the appearance of resistance breaking isolates, a third set of mutants was generated in which single aa residues within NSs^{RB160}, unique and different from the NSs^{RI} sequence (Chapter 2), were reverted into aa from the NSs^{RI} isolate (Figure 3.1B). The constructs obtained, denoted P48S, T79I, T260V, D384N, N386S, D384N/N386S and P438S, were tested for a gain of RSS and/or Avr-function. Additionally, the corresponding aa residues within the NSs^{RI} sequence were substituted for alanines resulting in mutants and denoted: S48A, I79A, V260A, N384A, S386A, N384A/S386A and S438A (Table 3.1). The latter were tested for a loss of RSS and/or Avr-activity.

In addition to these three sets of mutants a fourth mutant was constructed in which a GW/WG motif in the N-terminal domain of NSs was substituted for alanines, and denoted W17A/G18A. The GW/WG motif has recently been reported to be involved in the interaction of several RSS proteins with AGO1, the slicer component from the RISC complex (Giner *et al.*, 2010).

All NSs mutants generated (Table 3.1) were confirmed by sequence analysis, and their translatability verified by SDS-PAGE and western immunoblot analysis of *N. benthamiana* leaves agroinfiltrated with these constructs. NSs constructs that lost their ability to supress RNA silencing showed very low expression with the pK2GW7 vector, while the same constructs expressed through pEAQ-HT were well detectable and of similar levels as RSS active NSs (Figure 3.S1).

Essential amino acid sequences for Avr-activity primarily map to the N-terminus

To test the ability of NSs^{RI} mutants and NSs^{RB160} revertants to trigger an HR, constructs were cloned into pEAQ-HT and subsequently transiently expressed via infiltration of *Agrobacterium tumefaciens* 1D1249 (containing helper plasmid pCH32) on resistant *C. annuum* plants (Chapter 2). Plants were monitored for the appearance of HR at 5 days post agroinfiltration (dpa). The NSs mutants were expressed from vector pEAQ-HT to compensate (via the additional expression of the RNA silencing suppressor P19) for low expression levels in case the NSs mutants were non-functional for RSS activity. Whereas infiltration with pEAQ-HT-NSs^{RI} (control), but not with pEAQ-HT-N (Nucleocapsid protein of TSWV) or the pEAQ-HT-NSs^{RB160}, lead to a clear HR after 5 days, quite a number of NSs^{RI}-mutants lost their ability to trigger the HR

Table 3.1 NSs mutants designed in this study and ordered according to amino acid numbering from the amino-terminal end and tested on their functionality;
Minus (-): No or loss of activity. Plus (+): activity (gained). Plus/minus (+/-): Partial activity.

#	Mutant	Mutant target	HR-induction	RSS-activity
1	NSs^{RI}-W17A/G18A	Putative AGO1 interaction domain	-	-
2	NSs^{RI}-S29A/Y30A	Conserved NSs domain	-	-
3	NSs^{RI}-S48A	Predicted RNA binding domain	-	+/-
4	NSs^{RI}-S48A/R51A	Predicted RNA binding domain	-	-
5	NSs^{RI}-K53A/G57A	Predicted RNA binding domain	-	+
6	NSs^{RI}-S74A/Q75A	Conserved NSs domain	-	+
7	NSs^{RI}-I79A	RB mutation	-	-
8	NSs^{RI}-Q113A/G114A	Conserved NSs domain	-	-
9	NSs^{RI}-G160A/S161A	Conserved NSs domain	+	+
10	NSs^{RI}-Y197A/S198A	Conserved NSs domain	-	+/-
11	NSs^{RI}-R211A	Predicted RNA binding domain	+	-
12	NSs^{RI}-V260A	RB mutation	-	+
13	NSs^{RI}-N288A/S289A	Conserved NSs domain	-	+
14	NSs^{RI}-N355A/N356A	Conserved NSs domain	-	-
15	NSs^{RI}-P373A	RB mutation	+	+
16	NSs^{RI}-N384A	RB mutation	+	+
17	NSs^{RI}-S386A	RB mutation	+	+
18	NSs^{RI}-N384A/S386A	RB mutation	-	+
19	NSs^{RI}-L396A/S397A	Conserved NSs domain	-	+/-
20	NSs^{RI}-S411A/Y412A	Conserved NSs domain	-	+
21	NSs^{RI}-S438A	RB mutation	+	+
22	NSs^{RI}-KKK452AAA/K457A	Predicted RNA binding domain	-	+
23	NSs^{RB160}-P48S	Reversion mutation	-	-
24	NSs^{RB160}-T79I	Reversion mutation	-	-
25	NSs^{RB160}-T260V	Reversion mutation	-	-
26	NSs^{RB160}-D384N	Reversion mutation	-	-
27	NSs^{RB160}-N386S	Reversion mutation	-	-
28	NSs^{RB160}-D384N/N386S	Reversion mutation	-	-
29	NSs^{RB160}-P438S	Reversion mutation	-	-

(Figure 3.2 and 3.3, Table 3.1). A further look showed that all mutations in the N-terminal domain (aa 1-133) led to a loss of the ability to elicit HR, while only half of the other mutants in the remaining internal or C-terminal domain led to such loss. These results indicated the importance of the N-terminal domain of NSs function for Avr-activity. Mutant N384A/S386A had lost the ability to trigger HR while the individual single mutants N384A and S386A were still functional. Furthermore, and interestingly, mutation of the WG-motif also led to a loss of Avr-activity, indicating its putative biological relevance. From all NSs^{RB160}-single aa reversion mutants generated, none had regained the ability to trigger HR. As control, all mutant NSs constructs were additionally tested on susceptible *Capsicum* plants, and none showed to trigger an aspecific necrotic response (Figure 3.4).

Essential sequences for RSS activity overlap with those for Avr-activity

To test NSs mutants (NSs^{RI} alanine substitutions and NSs^{RB160}-revertants) for their ability to suppress RNA silencing, constructs were cloned in pK2GW7 and by means of *A. tumefaciens* LBA4404 (Chapter 2) co-infiltrated with a functional GFP construct in *N. benthamiana*. At five dpa, levels of GFP expression were monitored visually by UV-light, and fluorescence units were quantified by spectrometry. Whereas agroinfiltration of only GFP showed clear levels of silencing, which was suppressed in the additional presence of NSs^{RI}, a variety of GFP silencing suppression levels were observed when the NSs^{RI} mutants were co-infiltrated (Figure 3.2, Table 3.1). Similar to the results from the mutant screen on HR-induction/elicitation, loss of RSS activity was mainly found with mutations that resided in the N-terminal domain, while only fewer mutations in the remainder part of NSs had such effect. Mutations made in the first two predicted RNA binding domains (aa 48 and aa 211) showed a negative effect on RSS-function, while those made in the third predicted RNA binding domain (aa 452) did not show any effect on RSS functioning. Some NSs^{RI}-mutants, *i.e.* S48A, Y197A/S198A and L396A/S397A, only partially lost their RSS-activity, while the double mutant S48A/R51A had completely lost its RSS-activity. Mutation of the WG-motif, besides Avr-activity, also abrogated the RSS activity of NSs. Furthermore, as with the HR-induction assay, none of the single NSs^{RB160} reversion mutants re-gained RSS activity.

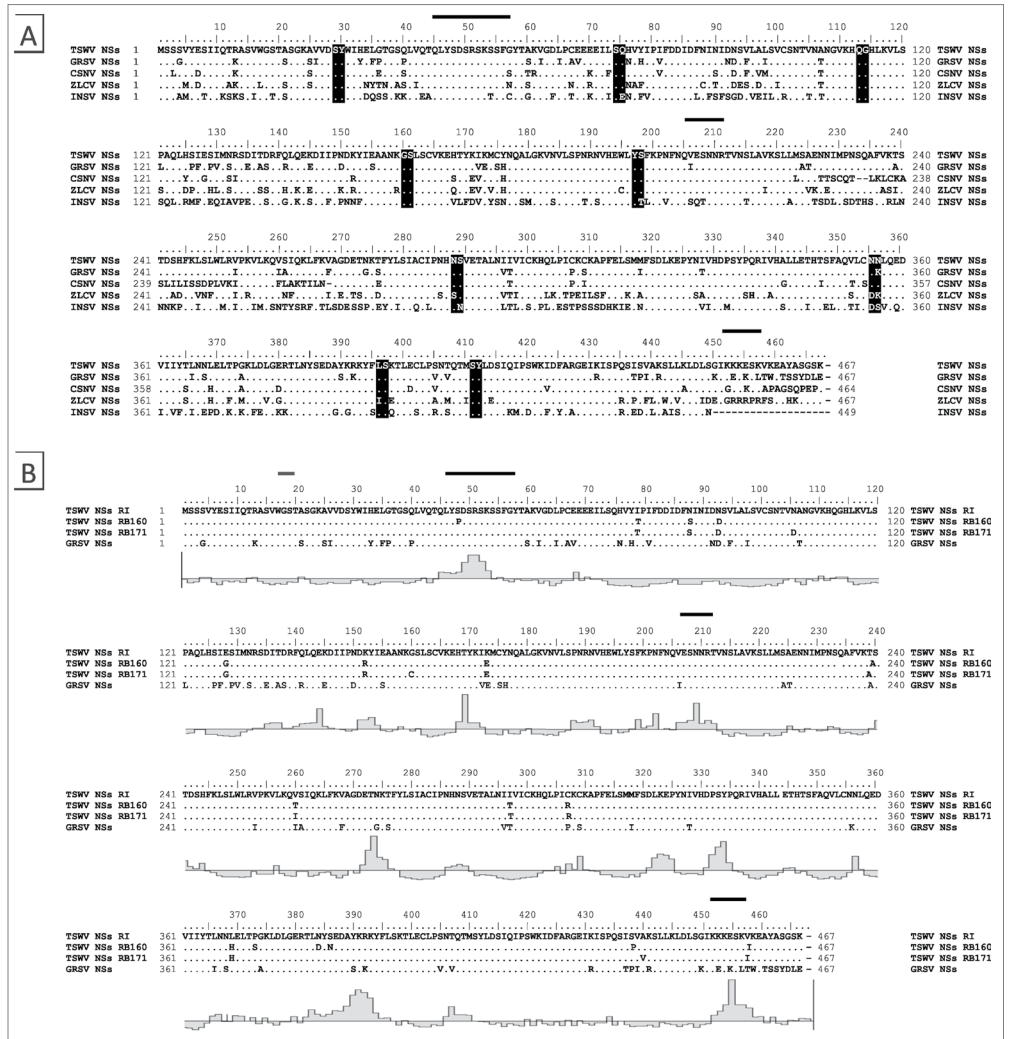


Figure 3.1 Multiple sequence alignment of different TSWV NSs proteins with GRSV NSs and an alignment with other tospoviral NSs sequences. **A.** The NSs amino acid sequences from different tospoviruses, belonging to the American clade, were aligned to identify conserved and diverse regions within the NSs protein. Conserved amino acids highlighted in black were selected for alanine substitutions. Black horizontal bars represent predicted RNA binding domains. TSWV: *Tomato spotted wilt virus*, GRSV: *Groundnut ringspot virus*, CSNV: *Chrysanthemum stem necrosis virus,* ZLCV: *Zucchini lethal chlorosis virus* and INSV: *Impatiens necrotic spot virus*. **B.** A multiple sequence alignment of TSWV NSs^{RI} and TSWV NSs^{RB} from isolates 160 and 171 is shown and differences are highlighted. The NSs from a

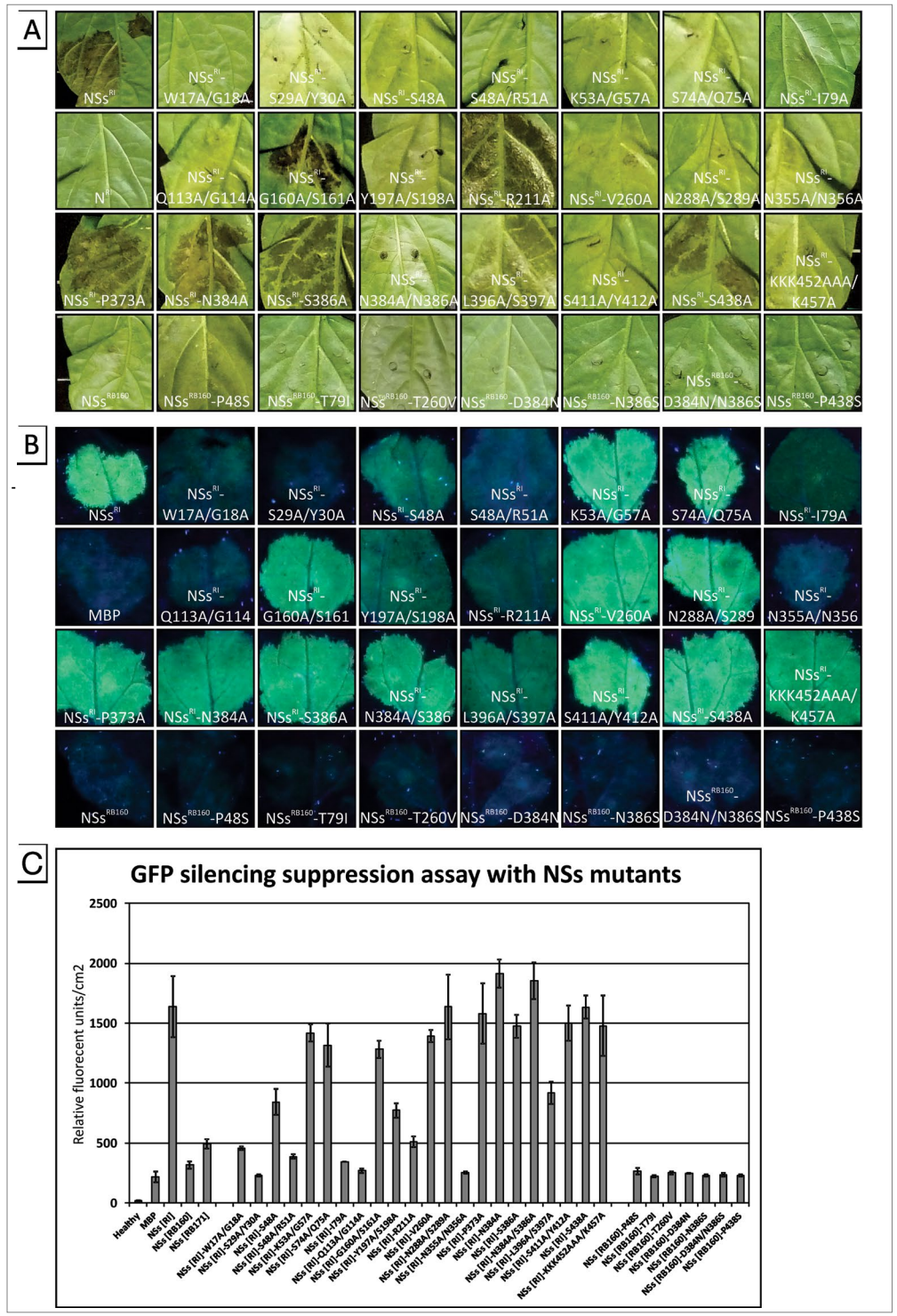
(←) closely related but distinct *Tospovirus* GRSV is included. Predicted (three) RNA binding domains (black bars), a WG/GW-motif (grey bar) and a hydrophobicity plot are indicated.

Elicitation of HR and RSS-activity of NSs-chimera and additional mutants

The results from the NSs^{RB160} revertants indicated that a single nucleotide reversion was apparently not sufficient to restore either one or both of the RSS and Avr-activities. Interestingly, a further look at the N-terminal aa sequences of NSs^{RB} from the 160 and 171 resistance breaker isolates in comparison to NSs^{RI} (Figure 3.1B) showed that the NSs^{RB171} only deviated from the NSs^{RI} at one position namely aa 79 (T79). Considering the importance of the N-terminal domain for both RSS and Avr-activity, we analysed next whether T79 represented one of the key aa-residues for the generation of the TSWV RB phenotype/isolates. To this end, T79 in NSs^{RB171} was reverted into isoleucine (T79I) and after cloning into the appropriate vectors subsequently tested for HR-induction and RSS-activity. Interestingly, the NSs^{RB171}-T79I mutant gained RSS activity again (Figure 3.5), but not the ability to elicit HR. To analyse whether reversion of two aa in the N terminal domain of NSs^{RB160}, and deviating from NSs^{RI}, restored full functionality of RSS and Avr-activity, the double mutant P48S/T79I was generated. In contrast to the results with the single reversion mutant NSs^{RB171}-T79I, which shared a similar N terminal domain as the double reversion mutant NSs^{RB160}-P48S/T79I, the latter mutant showed a gain of function for both activities (Figure 3.5).

To further substantiate the importance of the N-terminus of NSs in both activities, additional NSs chimera were made. To this end, the first 133 aa (N-terminal domain) or the remainder part of NSs (referred to as C-terminal domain) were swapped between the NSs^{RI} and the NSs^{RB160} or NSs^{RB171}, leading to the construction of four NSs-chimera (Figure 3.6). All NSs chimera constructs were tested for their ability to suppress GFP silencing in a leaf patch assay and elicit a *Tsw*-gene based HR. The results showed that NSs chimera containing the N-terminal domain of NSs^{RI} and theremainder C-terminal part of NSs^{RB} (160 or 171) regained RSS- and Avr functionality again (Figure 3.5).

Adversely, NSs chimera containing the N-terminal domain of NSs^{RB} and the remainder C-terminal part of NSs^{RI} showed no Avr-activity. Interestingly, residual/ low levels of RSS activity were observed with chimera containing the N terminal



← Figure 3.2 HR and RSS activity of NSs mutants. NSs mutants as listed in Table 1, were tested for their Avr-activity by triggering of HR (necrosis) on *Tsw+ Capsicum* plants. The presence of RSS activity was analysed in a GFP-silencing leaf patch assay on *N. benthamiana*. **A.** Visual monitoring of the Avr-activity from all the mutants tested and ordered from upper left to right bottom, according to the mutations positioned in NSs from N-terminal to C-terminal end. **B.** Suppression of GFP silencing by the NSs mutants used and presented in the order as in panel A. The presence of Avr-activity and (full/intermediate/loss) of RSS activity is summarised and presented in Table 2. **C.** The GFP units obtained in the RSS-assay were quantified by fluorescence spectrometry. Measurements of leaf discs (Ø 1cm) of each infiltration were repeated at least three times and the average amount is shown with the standard deviations as error bars.

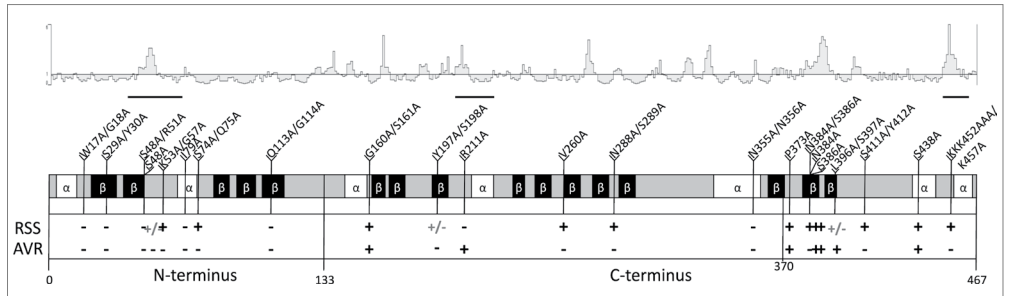


Figure 3.3 Graphical overview of the NSs mutant analysis. The outcome of Avr and RSS functionality from the NSs^{RI} mutants are depicted in a schematical drawing including the predicted secondary NSs structure (α-helix in white boxes and β-sheets in black boxes), the hydrophobicity plot on top of the figure and predicted RNA binding domains are shown as horizontal black bars.

domain of NSs^{RB171}, but not with the corresponding domain from NSs^{RB160}. Since only few mutations in the C-terminus of the NSs^{RI} led to a loss of either RSS or Avr-activity, a C-terminal deletion mutant of NSs^{RI} was made in which the last 97 aa of the NSs^{RI} coding region were deleted and two stop codons were introduced. Analysis of this construct, referred to as NSs^{RI}-ΔC, showed that both RSS and Avr-activity were abolished and indicating that the C-terminus of NSs is required to retain a functional protein.

Figure 3.4 Agroinfiltration of NSs mutants on susceptible *Capsicum* plants. All NSs mutants generated in this study (listed in tables 1 and 3) were, as control, agroinfiltrated in susceptible *Capsicum* plants to analyse their phenotype and to confirm the induction of *Tsw*-mediated HR triggering in *Tsw*+ *Capsicum* plants (Figures 2 and 5). Agroinfiltrated leaves are presented from all mutants tested and ordered from upper left to right bottom according to the mutations positioned in NSs from N-terminal to C-terminal end, followed by the reversion- and chimera/deletion mutants, respectively.

Inability of transferring the Avr-activity to a closely related but virulent *Tospovirus* species

The observation that the N terminus (133 aa) of NSs^{RI} seemed most essential for RSS and Avr-activity tempted us to analyse whether avirulence could be transferred from a TSWV-RI isolate to *Groundnut ringspot virus* (GRSV). The latter represents a distinct *Tospovirus* species that is not able to elicit *Tsw*-mediated HR but whose NSs shares high sequence similarity to TSWV NSs (89%). To this end, and in analogy

to the approach as described above, the first 133 aa of GRSV NSs was exchanged with the corresponding part of NSs^{RI} of TSWV, and *vice versa*. All NSs chimera generated (Table 3.2) were confirmed by sequence analysis, and verified for their translatability as earlier described (data not shown). During a GFP-silencing assay on *N. benthamiana*, the chimera with the N-terminal domain of the TSWV NSs showed the ability to suppress RNA silencing to the same level as the complete NSs^{RI}, but the chimera carrying the N-terminal domain of the GRSV NSs partially had lost RSS activity (intermediate RSS activity). However, when both chimera were tested for avirulence, neither chimera was able to elicit *Tsw* mediated HR.

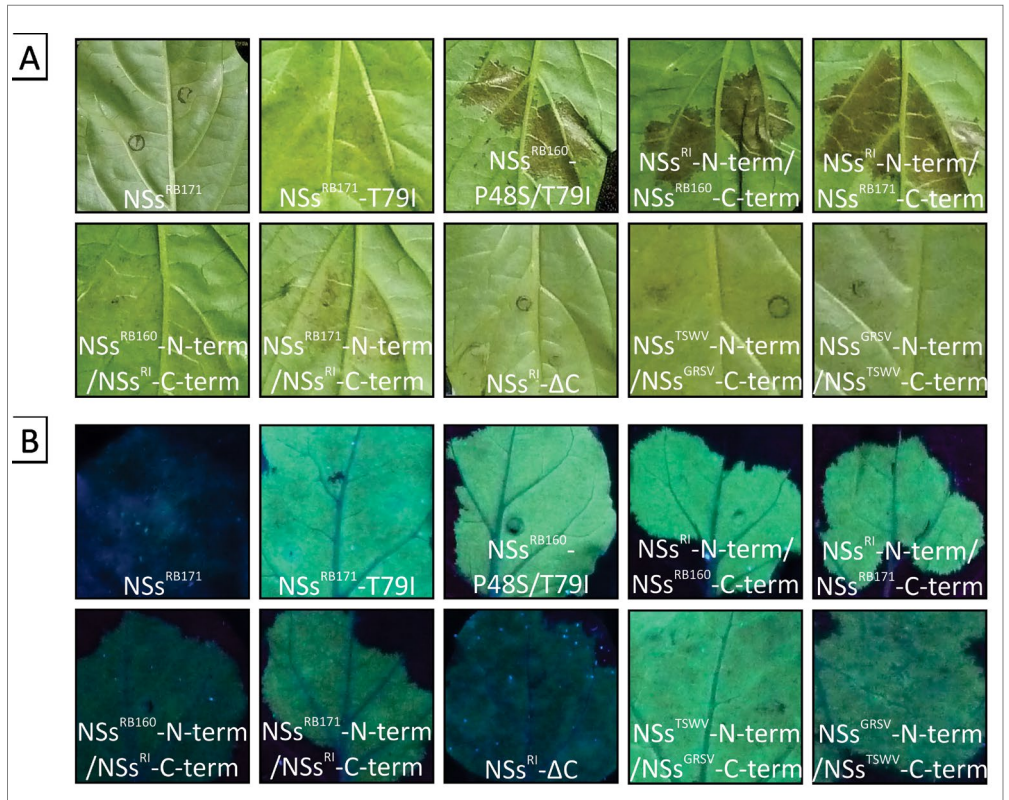


Figure 3.5 HR-induction and RSS-activity of the NSs domain swapping chimera. Leaves in the upper row show the results of the HR assay of the NSs chimera tested, similar to panel A in Figure 2. Leaves in the bottom row show the results of the GFP silencing suppression assay of mutants as shown in panel B of figure 2. The presence of Avr-activity and (full/intermediate/loss) RSS activity of the mutants is summarised and presented in Table 3.

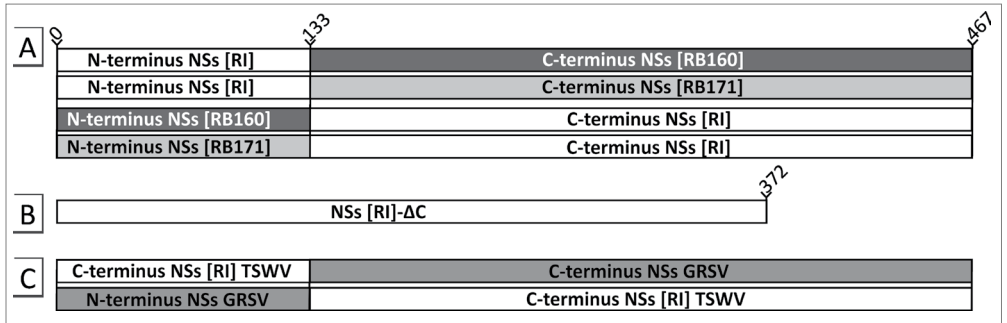


Figure 3.6 Schematical presentation of additional NSs chimera and the NSsRI-ΔC mutant construct. A. Presentation of four NSs chimera in which the first 133 aa between the NSs^{RI} and the NSs^{RB160} and NSs^{RB171} were swapped. **B.** Presentation of NSs-ΔC, an NSs^{RI} deletion mutant from which the last 95 amino acids on the C-terminal end were deleted. **C.** Presentation of NSs chimera made from GRSV NSs and TSWV NSs^{RI}, similarly as with the constructs in panel A, in which the first 133 amino acids between the NSs^{GRSV} and NSs^{RI} from TSWV have been swapped.

Table 3.2 Additional NSs mutants and chimera made and tested on their functionality; Minus (-): No or loss of activity. Plus (+): activity (gained). Plus/minus (+/-): Partial activity.

#	Mutant	Mutant target	HR-induction	RSS-activity
30	NSs^{RB171}-T79I	Reversion mutation	-	+
31	NSs^{RB160}-P48S/T79I	Reversion mutation	+	+
32	NSs^{RI}-N-term/NSs^{RB160}-C-term	Replacement N-term	+	+
33	NSs^{RI}-N-term/NSs^{RB171}-C-term	Replacement N-term	+	+
34	NSs^{RB160}-N-term/NSs^{RI}-C-term	Replacement N-term	-	-
35	NSs^{RB171}-N-term/NSs^{RI}-C-term	Replacement N-term	-	+/-
36	NSs^{RI}-ΔC-term	C-terminal deletion	-	-
37	NSs^{TSWV}-N-term/NSs^{GRSV}-C-term	Replacement N-term	-	+
38	NSs^{GRSV}-N-term/NSs^{TSWV}-C-term	Replacement N-term	-	+/-

Discussion

In this study a mutant screen was performed on the NSs protein of TSWV to identify and map essential domains required for RSS and Avr-activity and investigate their potential tight functional linkage. The results have strongly indicated the importance of the N-terminal domain of NSs for both functions since mutations introduced in the first 133 aa of NSs^{RI} most often led to a functional loss of both RSS and Avr-activity, while this was observed only occasionally and to a lesser extent with mutations in the remaining C-terminal part of the protein. A few mutants were affected only in the RSS- or Avr-activity. This observation dismissed the idea of a tight functional linkage. This was further supported by data from another study (Margaria *et al.*, 2007) in which the NSs protein of some resistance breaking isolates, although only partially characterised, still seemed to exhibit RSS activity.

The importance of the N-terminal domain for both functions was further strengthened by additional domain swaps between NSs^{RI} and NSs^{RB}, in which the RSS/Avr-activity of the NSs$^{RI/RB}$ chimera could be restored when provided with the N-terminal part of NSs^{RI}. In addition, only mutants of NSs$^{RB160-171}$ containing a reversion in the N-terminal domain, but not in the C-terminal part, were (partially) restored in RSS and/or Avr-activity. Although the mutant screen indicated a lower importance of the C-terminal domain of TSWV NSs for both functions, deletion of this part (mutant NSs^{RI}-ΔC) rendered NSs non-functional. This suggests that the C-terminal domain likely plays a structural role, rather than possessing domains required for the tested functions. Furthermore, a chimeric GRSV NSs containing the N-terminal domain of TSWV NSs^{RI}, could not be provided Avr functionality but retained its RSS activity, which strengthens the requirement for the C-terminal domain of NSs for proper functioning, likely in structural folding. Mutation of the WG-motif in the N-terminus of NSs and concomitant loss of RSS and Avr-activity pointed to its biological relevance, likely for interaction with AGO1 as earlier demonstrated with *Sweet potato mild mottle virus* P1 protein (Giner *et al.*, 2010). From all amino acid (aa) mutations in the NSs protein analysed, the residue at position 79 also seems to play a key role, as mutant NSs^{RI}-I79A lost both activities, while reversion mutants of the resistance breakers, *i.e.* NSs^{RB171}-T79I and NSs^{RB160}-P48S/T79I, both had gained RSS ability and the latter in addition Avr-activity. Clearly, aa residue 79 is directly involved in RSS activity, but affects Avr-activity as well.

Of the known viral Avr-proteins, only a small number of cases have been reported in which the viral Avr also had RSS activity. These are limited to *Tomato bushy stunt virus* (TBSV) P19, *Tomato aspermy virus* (TAV) 2b, *Potato virus X* (PVX) 25K, *Turnip crinkle virus* CP and *Potato virus Y* (PVY) Hc-Pro (Oh *et al.*, 1995; Scholthof *et al.*, 1995; Li *et al.*, 1999; Malcuit *et al.*, 1999; Angel *et al.*, 2011; Tian and Valkonen, 2013). Although for most of these cases the corresponding resistance genes have not been cloned yet (TBSV, PVX, TAV and PVY), they are single dominant and therefore likely of the NBS-LRR type. The most well-known viral Avr and RSS *R*-gene model is the one from *Turnip crinkle virus* (TCV). Like TSWV NSs, the TCV CP protein binds dsRNA and prevents systemic silencing (Qu *et al.*, 2003). The TCV coat protein (CP) is the Avr-determinant of the *HRT* resistance gene in *Arabidopsis thaliana*, where *HRT* is an NBS-LRR type resistance gene (Ren *et al.*, 2000). For TCV CP, mutants were made that lost the ability to induce HR, but remained RSS active, suggesting that the suppressor and resistance-elicitor functions are not tightly linked, most likely because these reside in different domains, which tempted the authors to propose that TCV CP interferes with multiple host basal defence pathways (Choi *et al.*, 2004; Jeong *et al.*, 2008). Similar findings were obtained for TBSV, where P19 mutants that lost siRNA binding properties were still able to trigger an effective resistance response (HR) in *N. tabacum* (Hsieh *et al.*, 2009). Recently, the TAV 2b crystal structure was elucidated and two separate studies showed the importance of the same arginine residue at position 28 from TAV 2b in binding of siRNAs and thus for RSS activity (Chen *et al.*, 2008) and Avr-activity (Li *et al.*, 1999), indicating that RSS and Avr-activity in case of TAV 2b are tightly linked. Most of these studies support the idea that triggering of the corresponding *R*-genes does not require a functional RSS aspect of the Avr-determinant, like in case of TSWV NSs, but rather a (minimal secondary) structural feature that is shared by both, and in case of TSWV NSs could be the N-terminal domain. Support for this also comes from another recent study on the *Cucumber mosaic virus* (CMV), where the replicase function of 2a could be uncoupled from its ability to trigger an HR on Cowpea leaves (unknown *R*-gene) (Hu *et al.*, 2012). However, for some viruses solving this issue might be hampered by the difficulty to separate functions in – multifunctional - viral proteins and as a consequence only functional viral RSS proteins may be recognised by R-proteins, which could explain the observations made on TAV 2b.

Recently TSWV NSs has been shown to bind small RNAs (mi/si), and long dsRNA, and this characteristic has been proposed as the mode by which this protein interferes in (suppressing) RNA silencing (Schnettler *et al.*, 2010). Alanine substitutions in three predicted RNA binding domains showed that only two of these led to a loss in RSS-activity. The third one around aa452, in which even four lysines were substituted, did not lead to a loss of RSS activity and suggested that this sequence was not required. In contrast, a similar sequence with predicted RNA binding capacity was earlier shown to be involved in small RNA binding by the *Tenuivirus Rice hoja blanca virus* (RHBV) NS3 and its mutation leading to a loss of RSS activity (Hemmes *et al.*, 2009). Whether the predicted RNA binding domains genuinely are involved in binding small and/or large dsRNA, so far has not been solved. In light of this, it is important to point out that attempts have been made to perform electrophoretic mobility shift assays (EMSA) using transiently expressed NSs, but mostly only weak shifts were observed when using NSs^{RI}, while no shift was observed using NSs^{RB} (data not shown). Considering that NSs^{RI} was well detectable on Western immunoblots, but the amount of NSs^{RB}, and of most mutant NSs proteins, was at least 10 fold lower, already indicated that the absence of a shift could also be due to low expression levels, and the outcome of the EMSA analysis would thus not be conclusive at all.

The same predicted RNA binding domains are also present in other closely related *Tospovirus* sequences (Figure 3.1A), and indicate that these could be a conserved and shared feature throughout the genus. Among all the NSs mutants analysed, one (R211A) had lost RSS activity, but was still able to trigger HR. Considering that the R211A mutant expressed from pEAQ-HT is still able to trigger HR, while its expression is 10x lower when compared to NSs^{RI} from pEAQ-HT, indicates that induction of HR apparently requires only low amounts of the Avr-protein.

The crystal structure of the TSWV NSs protein or other *Tospovirus* NSs proteins has not been elucidated and therefore the 3D-structure of the NSs protein is still unknown. This clearly hampers the predictability of the outcome of the mutations in the NSs protein sequence and we cannot rule out that a loss of function due to some mutations resulted from an effect on the structure of the protein, rather than disrupting a functional domain. Elucidating the structure of NSs might also give clues as to how and why mutation of the WG motif affects the NSs functioning as Avr.

Recently, a publication appeared on the biochemical function of NSs from *Groundnut bud necrosis virus* (GBNV), a distinct *Tospovirus* (Lokesh *et al.*, 2010). It was shown that GBNV NSs contains RNA stimulated NTPase activity and dATPase activity, which was supported by the presence of two Walker motifs (A and B) in the NSs amino acid sequence. In addition, the NSs showed 5' RNA/DNA phosphatase activity. Although the Walker domains do not show up in the TSWV NSs sequence, the presence of similar biochemical functions for this protein cannot be excluded nor their involvement in the RSS/Avr-activity of the NSs protein. Unfortunately, the NSs mutants made in the GBNV study were not tested for their biological relevance, *e.g.* RNAi suppressor activity.

In another, more recent study (Geerts-Dimitriadou *et al.*, 2012) TSWV NSs has been shown to enhance translation of viral transcripts. It is clear from all this that NSs represents a multifunctional protein, as observed with many other viral proteins (Díaz-Pendón and Ding, 2008; Hu *et al.*, 2012). Whether any of these functions overlap or somehow crosstalk/interplay with RSS/Avr-activity remains to be analysed.

In conclusion, here we have performed a first mutant screen of TSWV NSs that provides a first glimpse on the topology of NSs in relation to its roles as RSS and Avr-determinant, which shows that the most essential sequences required for both functions map to the N-terminal part of the protein and closely overlap, although both functions can be separated. While the mode of action of dominant resistance genes, to which *Tsw* classifies, still remains a matter of debate, one of the most commonly accepted models is the guard-hypothesis (van der Biezen and Jones, 1998; Jones and Dangl, 2006). In this model, the resistance gene product is guarding a certain host protein, and is able to perceive alterations of its 'guardee' target by interaction with the Avr-determinant, which leads to an induction of HR. Whether AGO1 indeed interacts with NSs, and presents a candidate protein for the guardee will be an hypothesis to be tested in the near future.

Materials and Methods

Plant material

Two genotypes of *Capsicum annuum* were used for the HR-induction assay: HK0004, a TSWV-susceptible cultivar (*Tsw-*), and YF0009, a TSWV-resistant cultivar (*Tsw+*). Additionally, *Nicotiana benthamiana* plants were used for the RNA silencing suppression assay. All plants were grown and maintained under glasshouse conditions (24 °C with a 16 h light/8 h dark regime).

Multiple sequence alignment

The obtained sequences were aligned using the CLUSTALW algorithm. Sequences of NSs^{RI}, NSs^{RB160} and NSs^{RB171} were previously described (Chapter 2). Sequences of NSs derived from other tospoviruses were retrieved from Genbank; TSWV-NSs: Genbank accession number D00645, ZLCV-NSs: accession number JN572104, GRSV-NSs: accession number JN571117, INSV-NSs: accession number GU112504, CNSV-NSs: accession number AB600873. Alignments were edited using the BioEdit program (Hall, 1999).

PCR-mutagenesis and subcloning of constructs

NSs mutants were made by PCR-mutagenesis, using primers to introduce the mutation on a gateway-compatible entry vector pEntr11, carrying either the NSs^{RI}, the NSs^{RB160} or the NSs^{RB171} sequence. Chimera mutants were made by amplifying fragments by PCR with introduced restriction sites for subsequent restriction and ligation into the appropriate backbone sequence. Plasmids obtained were sequence verified and subsequently cloned by Gateway cloning in their destination vectors pK2GW7 (Karimi *et al.*, 2002) for the GFP-silencing suppression assay, and into pEAQ-HT (Sainsbury *et al.*, 2009) for the Avr-assay. The obtained binary vectors were transformed into Agrobacterium 1D1249 + pCH32 (pEAQ-HT) and LBA4404 (pK2GW7) (Ooms *et al.*, 1982; Hamilton *et al.*, 1996; Wroblewski *et al.*, 2005).

Agrobacterium Transient Transformation Assay (ATTA)

The ATTA assay was performed according to Bucher *et al.* (2003), with slight modifications. In brief: Agrobacteria were grown overnight at 28 °C in LB3 medium containing proper antibiotic selection pressure. From this culture, 600 µl was freshly

inoculated into 3 ml induction medium and grown overnight. Plants were watered in excess 1 hour before the infiltration, using a needleless syringe, was performed. Strain *A. tumefaciens* 1D1249 with helper plasmid pCH32 was grown under 1.25 µg/ml tetracycline selection pressure, while *A. tumefaciens* LBA4044 was grown under 20 µg/ml rifampicin selection pressure.

Serological detection of NSs proteins

Expression of the different mutants of the TSWV NSs protein was analysed by SDS-PAGE and Western immunoblot analysis using polyclonal antisera against TSWV NSs as previously described (Kormelink *et al.*, 1991).

HR-induction assay and GFP silencing suppression assay

For the HR-induction, ATTAs (see above) were performed of the pEAQ-NSs mutant constructs on resistant *Capsicum* plants (+*Tsw*) and susceptible *Capsicum* plants (-*Tsw*), and plants scored for the presence or absence of HR. As positive control NSs^{RI} was included and as negative controls NSs^{RB160} and the nucleocapsid (N) protein of TSWV, respectively (Chapter 2). The GFP silencing assay was performed by agroinfiltration of *Nicotiana benthamiana* leaves with a functional GFP construct (Tsien, 1998) mixed with one of the NSs constructs, as described above in the ATTA section, using a final $O.D._{600nm}$ 0.25 per construct. A construct expressing the Maltose binding protein (MBP) was used as a negative control (Schnettler *et al.*, 2010). Infiltrated leaves were monitored for GFP expression at 5 dpa using a hand-held UV-lamp. For quantification of GFP fluorescence, leaf discs with a diameter of 1 cm were taken from the infiltrated leaf area and the number of fluorescent units was measured using a Fluorstar Optima (BMG Labtech).

Acknowledgements

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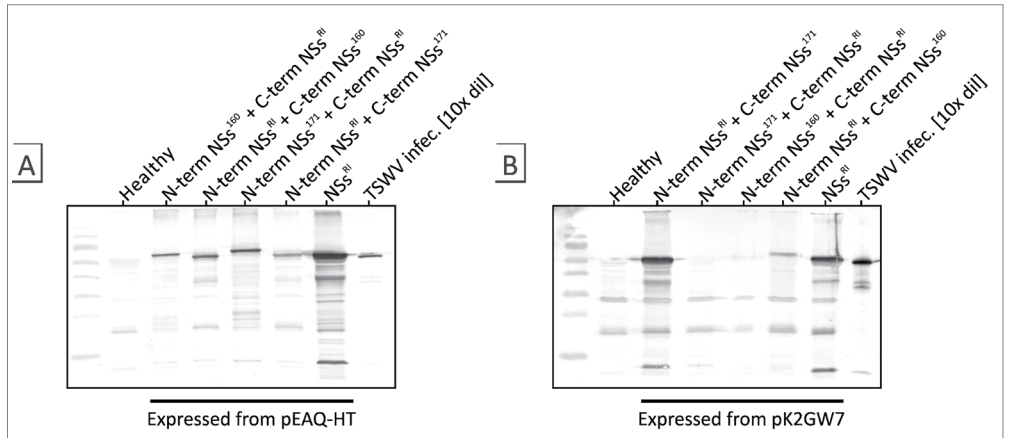


Figure 3.S1 Westernblot analysis of chimeric-NSs expressed from pEAQ-HT and pK2GW7. NSs chimera constructs made between NSs^{RI} and $NSs^{RB160/171}$ were expressed in *N. benthamiana* and samples were made using Trizol for westernblot analysis as described in chapter 2, with αNSs as antiserum. Constructs tested were expressed from the pEAQ-HT vector (**A**) and from pK2GW7 (**B**).

Chapter 4

Analysis of a putative interaction between AGO1 and the *Tomato spotted wilt virus* NSs protein

Abstract

The NSs protein of *Tomato spotted wilt virus* (TSWV) represents the suppressor of RNAi (RSS) and is able to sequester small interfering (si)RNA and long double stranded (ds)RNA. A recent mutant study performed on the NSs protein has shown that alanine substitutions of a GW/WG-motif disrupted its RSS activity (Chapter 3). This motif has earlier been demonstrated in other RSS-proteins to enable binding to AGO1, the core component of the RNA induced silencing complex (RISC) and essential for the RSS activity of viral proteins containing this motif. In this study, the putative interaction of the TSWV NSs protein and AGO1 was examined in *Nicotiana benthamiana* leaf material agroinfiltrated with binary constructs of the *NSs* gene and a FLAG-tagged AGO1 construct. Using a co-immunoprecipitation approach, NSs showed to co-immunoprecipitate with AGO1, although the reciprocal assay remained inconclusive.

Introduction

In plants, a basic defence mechanism against virus infections exists, which is based on small RNAs and is often referred to as RNA interference (RNAi), or RNA silencing. This mechanism is triggered by the generation of double stranded (ds)RNA structures that arise during replication or result from secondary viral RNA structures (Agius *et al.*, 2012). The dsRNA is recognised by a host Dicer-like (DCL) protein that cleaves it into small interfering (si-) RNAs of 21-24 nt long (Ding and Voinnet, 2007). The siRNA duplex molecules are subsequently unwound, one strand being destroyed, while the other so-called guide strand is uploaded in a protein complex called RNA-induced silencing complex (RISC). The complex uses the guide strand as a template to surveil for the presence of target sequences with complementarity to the guide strand. Once these (viral) RNA target molecules are found, they are cleaved by the Argonaute protein (AGO) of RISC, leading to their degradation. In plants, the siRNA signal is amplified by host RNA dependent RNA polymerases (RdRs), a process in which siRNAs are used as a primer to convert single stranded RNA target molecules into dsRNA. Their processing ultimately leads to the generation of secondary siRNAs, and to the spreading of the siRNA signal to neighbouring sequences of the initial

dsRNA source, also referred to as transitive silencing (Sijen *et al.*, 2001). Besides priming by siRNAs, host RdRp can additionally convert RNA target molecules into dsRNA in an unprimed manner (Agius *et al.*, 2012).

Viruses inhibit the RNAi machinery to establish a successful infection, and the most common way in doing this is by encoding RNA silencing suppressor proteins (RSS) (Díaz-Pendón and Ding, 2008). These proteins exert RSS activity in various ways, *e.g.* by sequestering long dsRNA and thereby prevent their cleavage by DCL, or sequestering of siRNAs and prevent their uploading into RISC (Lakatos *et al.*, 2006). Another way of inhibiting RNAi is by association of RSS to one of the main enzymatic key players in the pathway. Although in plants no RSS proteins directly associating with DCL proteins have been described, in insects the B2 protein of an Alphanodavirus (Wuhan nodavirus) binds and blocks DCL-2 activity (Qi *et al.*, 2012). More recently, several RSS from different plant viruses have been shown to interact with AGO1 and inhibit its activity, like P1 from *Sweet potato mild mottle virus* (SPMMV), P38 from *Turnip crinkle virus* (TCV) and 2b from *Cucumber mosaic virus* (CMV: fny-strain) (Zhang *et al.*, 2006; Azevedo *et al.*, 2010; Giner *et al.*, 2010). Alternatively, P0 from *Polerovirus* possesses a F-box motif which is involved in targeting AGO1 for degradation (Baumberger *et al.*, 2007), as does the P25 from Potato virus X (PVX), but whether this involves a direct or indirect interaction is not exactly known yet (Chiu *et al.*, 2010). While it is still unclear how 2b from CMV^{fny} binds to AGO1, both SPMMV-P1 and TCV-P38 bind via a GW/WG motifs in their sequence, earlier found in host proteins and required for their association to AGO1 (Azevedo *et al.*, 2010; Giner *et al.*, 2010).

The *Tomato spotted wilt virus* (TSWV) encodes an NSs protein that is active as an RSS by binding long dsRNA and siRNAs (Takeda *et al.*, 2002; Bucher *et al.*, 2003; Schnettler *et al.*, 2010). A recent alanine substitution analysis of NSs has shown the importance of the N-terminal domain for RSS and triggering the *Tsw*-gene based resistance in *Capsicum annuum* (Avr). A loss of RSS activity was not only observed after targeting predicted RNA binding domains but also of a GW/WG motif found at amino acid position 17-18, which indicated its putative biological relevance (Chapter 3). In this study, co-immunoprecipitation studies were employed using tagged proteins to confirm a putative interaction between TSWV-NSs and AGO1. First indications for this interaction have been obtained after immunoprecipitation of AGO1, but future complementing assays are needed for confirmation.

Results

RNA silencing suppression assay

Constructs used in this study are summarised in table 4.1. Prior to their use in co-immunoprecipitation, the RNA silencing suppressor activity of the RSS proteins used in this study was confirmed. To this end RSS encoding- binary gene constructs were agroinfiltrated together with a functional GFP construct in *N. benthamiana* plants. At 5 days post-infiltration (dpi), plants were visually monitored for GFP expression to assess for RSS activity relative to Maltose binding protein (MBP) and $P1_{mut}$ (a mutant of P1 that had lost RSS activity) as negative controls. While GFP was silenced in the presence of MBP and $P1_{mut}$, silencing showed clear suppression in the presence of P19, NSs^{RI} and His-tagged NSs^{RI} (N-terminal His-fusion) (Figure 4.1). $CMV^{fny\text{-}2b}$ and $P1_{wt}$ also suppressed silencing of GFP, but to a lesser degree compared to P19 and NSs (Figure 4.1). These results confirmed the functionality of the RSS gene constructs for their use in the following experiments.

Time course of transient protein expression

To identify the most optimal time point for harvesting leaf samples, infiltrated with various gene constructs and to be used for (co-)immunoprecipitation analysis, a time course experiment on protein expression was performed. Earlier, NSs constructs were observed to express well at 5 dpi (Chapter 2), but for pBA-6Myc-AGO1 and pBA-CMVfny-2b-3HA constructs this was not well reported (Zhang *et al.*, 2006; Giner *et al.*, 2010). Therefore, leaves were infiltrated with these constructs and sampled at 3, 4, 5, 6, and 7 days post infiltration (dpi). As negative control, a leaf sample agroinoculated with a MBP construct was included. Samples were prepared using Trizol (Materials and Methods) and subsequently resolved on SDS-PAGE, followed by Western immunoblot to analyse protein quantities (Figure 4.2A and B). While myc-AGO1 was present in leaves harvested at all time points (~150 kDa), expression seemed highest at 5 dpi. For 2b-HA (~20 kDa) expression was relatively high at all dpi samples tested. Based on these results, leaf samples were collected 5 dpi to be used in following experiments.

Table 4.1 Constructs used in this study including their tags.

Constructs used (incl. promoter)	Tag
35S::NSs[1]	No tag
35S::His-NSs	N-terminal 6xHis-tag
35S::2b-HA[2]	C-terminal 3xHA-tag
35S::HA-P1wt[3]	N-terminal HA-tag
35S::HA-P1mut[3]	N-terminal HA-tag
35S::Myc-AGO1[2]	N-terminal 6xMyc-tag
35S::FLAG-AGO1[4]	N-terminal FLAG-tag
35S::MBP[1]	No tag

[1](Chapter 2). [2](Zhang *et al.*, 2006). [3](Giner *et al.*, 2010). [4](Baumberger *et al.*, 2007).

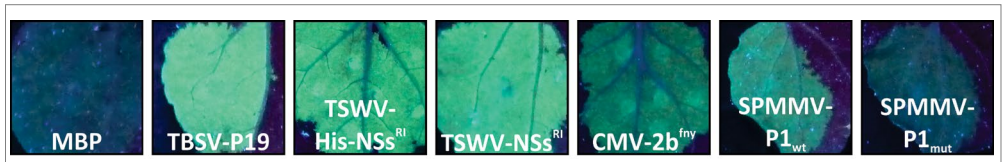


Figure 4.1 RNA silencing suppression activity of constructs used in this study. Constructs used in this study were verified for their ability to suppress RNA silencing of a GFP construct, images were taken at 5 dpi using a UV-lamp. MBP: Maltose binding protein, TBSV: *Tomato bushy stunt virus*, TSWV: *Tomato spotted wilt virus*, CMV: *Cucumber mosaic virus*, SPMMV: *Sweet potato mild mottle virus*.

Immunoprecipitation assays

The feasibility of a protocol employing Fe-beads for (co-)immunoprecipitation was first tested on the nucleocapsid (N) protein of *Tomato spotted wilt virus* (TSWV). Using beads coated with antibodies against N (de Avila *et al.*, 1990), this protein was specifically pulled down from infected leaf samples (data not shown), demonstrating the usefulness of the approach.

In a next step, the occurrence of in planta interactions, between Myc-AGO1 with $CMV^{fny\text{-}2b\text{-}HA}$ and NSs were analysed. Firstly, Fe-beads were coated with rat-αHA and tested to see whether $CMV^{fny\text{-}2b}$ was specifically pulled down from leaf extracts collected 5 days post agroininfiltration with pBA-CMVfny-2b-3HA. While 2b-HA was well detected in input leaf extracts (Figure 4.2C, Lane 6), a band of expected size and co-migrating with 2b-HA from the positive sample (Trizol isolated 2b-HA) was

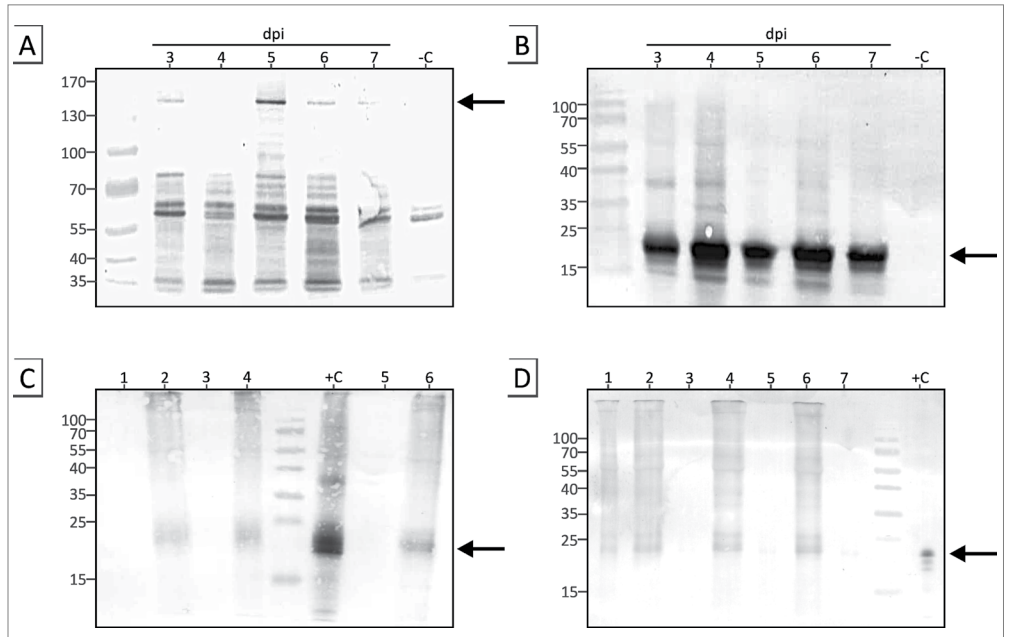


Figure 4.2 Time series and IP of ATTA expressed Myc-AGO1 and CMV$^{fny\text{-}2b\text{-}HA}$. **A.** Westernblot analysis of ATTA samples of Myc-AGO1 harvested at different days post infiltration (dpi) and detected by αMyc. **B.** Westernblot analysis of ATTA samples of CMV$^{fny\text{-}2b\text{-}HA}$ harvested at different days post infiltration (dpi), detected by αHA. In both cases the –C (negative control) was a leaf extract from an ATTA sample expressing MBP. **C.** An immunoprecipitation using Fe-beads was performed on ATTA samples from CMV$^{fny\text{-}2b\text{-}HA}$ and MBP using rat-αHA antibody for detection. 1; Elution after IP of MBP with αN. 2; Elution after IP of 2b-HA with αHA. 3; Elution after IP of MBP with αHa. 4; Elution after IP of 2b-HA with αN. 5; Input MBP. 6; Input 2b-HA. Positive control here (+C) for the westernblot analysis is Trizol isolated 2b-HA from ATTA. **D.** Beads were blocked after coating with the HA-antibody, then used for immunoprecipitation using Fe-beads on ATTA samples from CMV$^{fny\text{-}2b\text{-}HA}$ using rat-αHA antibody for detection (similar to A). 1; Input 2b-HA. 2; Supernatant after IP with empty Fe-beads. 3; Elution after IP of with empty Fe-beads. 4; Supernatant after IP with αN. 5; Elution after IP with αN. 6; Supernatant after IP with rat-αHA. 7; Elution after IP with αHA. Positive control here (+C) for the westernblot analysis is Trizol isolated 2b-HA from ATTA.

detected in the elution sample after IP (Figure 4.2C, lane 2). However, a protein band of similar size and intensity was obtained after an IP with beads carrying antibodies against the N-protein of TSWV and suggested some aspecificity in 2b-HA binding. To reduce and overcome a possible aspecific binding to the beads, an additional blocking step with milk powder was added during the coating protocol of the beads. Although using these beads the aspecific binding to beads this time was reduced, unfortunately, hardly any 2b-HA was observed in the elution sample collected after IP (Figure 4.2D, lane 7).

Since the strategy using Fe-beads appeared unsuccessful, an alternative protocol using protein-G Dynabeads was employed (Materials and Methods). Again, its applicability was first tested on a TSWV infected *N. benthamiana* sample using beads coated with αN directed against the N protein of TSWV. As a negative control, antibodies against VP2 of *Chicken anaemia virus* (CAV) were used (rabbit-αVP2). The results showed that N was specifically and efficiently immunoprecipitated from infected leaf samples when beads were coated using αN, but hardly with αVP2 (data not shown).

In a next step, this approach was used to immunoprecipitate His-, Myc- or HA-tagged NSs, AGO1 and 2b, respectively, to verify the specificity of the antibodies. When using αHis during an IP on leaf material containing transiently expressed His-NSs (input and IP-samples), a large smear was observed during detection of His-NSs using αNSs, but the elution sample clearly showed a large enrichment for NSs as deduced from the removal of Rubisco (Figure 4.3A, lane 3). Hardly any His-NSs was detected when the IP was performed using αMyc (Figure 4.3A, lane 5). In an analogous way, the specific precipitation of 2b-HA was tested using αHA on extracts of leaves infiltrated with a binary CMV$^{fny\text{-}2b\text{-}HA}$ construct, and using αMyc as a negative control. While 2b-HA clearly could be detected in the positive control (Trizol isolated 2b-HA), only a weak smear was detected in the input sample, likely due to low expression levels of the protein (Figure 4.3B, lane 1). Analysis of the IP-elute using αHA revealed the presence of two strong bands of which the lower one co-migrated with 2b-HA from the positive control. However, both bands also showed up at similar ratios when αMyc-AGO1 was applied during the IP, and indicated that the bands most likely presented the light (~25K) and heavy chains (~55K) of immunoglobulins. During IP analysis of extracts from leaves infiltrated with Myc-AGO1 constructs, the absence of a clear positive signal from the positive control and input samples

already indicated its low expression levels (Figure 4.3C, lanes 1 and C+). In contrast, a strong (smearing) signal was observed in the elution sample using αMyc, and indicated that an IP using the αMyc antibodies seemed to successfully enrich for AGO1 (Figure 4.3C, lane 3). Unexpectedly, the Myc-AGO1 protein (smearing) signals also showed up when using αHis, though less strong when compared to αMyc, likely due to an aspecific cross-reaction (Figure 4.3C, lane 5). The use of αHA, in contrast, hardly or not gave any signals after IP (Figure 4.3C, lane 7). Altogether, the results indicated that some of the antibodies used gave unwanted/undesired cross-reactions and therefore had to be used with caution in follow up experiments.

Co-immunoprecipitation assays

Having selected the protein-G Dynabeads as IP strategy and verified the ability of antibodies to precipitate His-, HA- and Myc-tagged proteins and their cross-reactions, extracts from agroinfiltrated leaves with a tagged-AGO1 construct in combination with various tagged RSS protein gene constructs were subjected to a Co-IP analysis.

A Co-IP on plant extracts from a co-ATTA expressing Myc-AGO1 and His-NSs was performed, using different antisera. The presence of the His-NSs protein in the elution sample after IP with αHis showed an effective IP (Figure 4.4A, lane 7), but when analysed with αMyc, did not show the presence of a distinct band of AGO1-size (Figure 4.4B, lane 7). Instead, an increased smear was observed, similarly as in the AGO1 IP-control, and pointed to the possible presence of AGO1. The reciprocal Co-IP using αMyc to precipitate AGO1, and next analysed using αNSs, showed the presence of a (thin) band after elution and of expected NSs size, suggesting a successful Co-IP (Figure 4.4A, lane 5).

As a positive control for the Co-IP experiment a binary construct of $CMV^{fny\text{-}2b\text{-}HA}$ was co-infiltrated with Myc-AGO1 and immunoprecipitated using different antisera. Unfortunately, the Co-IPs using either αHA or αMyc as primary antibody and subsequently analysed for the presence of the Co-IP target protein were not successful (Figure S4.1, panel A and B). This was likely due to low expression levels of both proteins, as neither could be well detected in the input or positive control sample. The bands that did show up after detection with αHA, again most likely presented the light (~25K) and heavy chains (~55K) of immunoglobulins.

Since $CMV^{fny\text{-}2b\text{-}HA}$ did not express well under the conditions applied, the P1 wildtype ($P1_{wt}$) RRS gene construct from *Sweet potato mild mottle virus* (SPMMV), earlier shown to interact with AGO1, was used as positive control for the Co-IP (kindly supplied by Giner *et al.* (2010)). A mutant P1 ($P1_{mut}$), containing 3 disrupted GW-motifs and unable to interact with AGO1 was used as negative control. Due to relatively low expression levels of the Myc-AGO1 construct, also here another AGO1 construct was used (described by Baumberger and colleagues (2007)), but this time containing a FLAG-tag at its N-terminal end. The expression of $P1_{wt}$ and FLAG-AGO1 was confirmed, although $P1_{mut}$ was not detectable on westernblot. The latter was likely due to mutation of the WG-motifs and subsequent abolishment of its RSS activity (Figure 4.1), rendering this protein unable to suppress its own silencing. A Co-IP experiment was performed on ATTA samples of FLAG-AGO1 with $P1_{wt}$, but rendered a strong signal at the height of P1 (Figure S4.1, panel C and D) that was also observed in the negative IP control.

Anti-FLAG affinity gel purification of FLAG-AGO1

To further substantiate the Co-IP results and compensate for the low levels of AGO1 expression, an affinity gel purification using anti-FLAG was performed. An IP using FLAG-beads on FLAG-AGO1 expressing plant tissue was successfully performed and a Co-IP procedure on Co-ATTA expressed FLAG-AGO1 + His-NSs using these beads did co-purify His-NSs. However, His-NSs was also detected in a similar co-IP setup when FLAG-AGO1 was not co-infiltrated (Figure 4.5), indicating that the co-purification was likely aspecific.

Discussion

Nowadays it is commonly known that viral RNA silencing suppressors (RSS) interfere in the RNAi machinery at various and sometimes multiple steps. A more recently identified interference mechanism is by direct binding of RSS proteins to AGO1, the core component of RISC, and thereby inhibiting its slicer activity (Zhang *et al.*, 2006; Giner *et al.*, 2010). Alanine substitution of residues W17/G18 present in the TSWV NSs RSS protein, previously identified as a motif required for binding of host

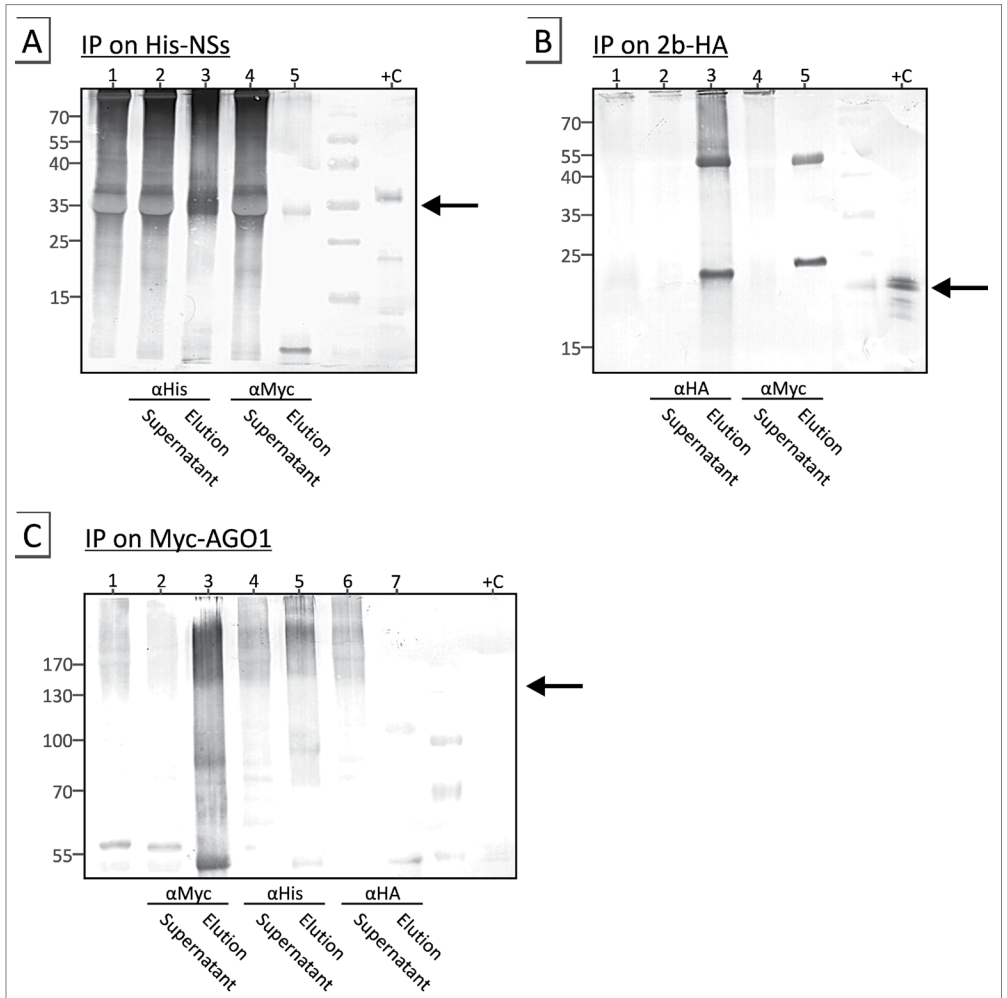


Figure 4.3 IP using protein G-beads on Myc-AGO1, CMV$^{fny\text{-}2b\text{-}HA}$ and His-NSs. IPs were done on ATTA samples expressing AGO1, CMV-2b and NSs by the corresponding tags. **A.** His-NSs was expressed by ATTA and IPs using different antisera were performed, detected by αNSs. 1; His-NSs input for IP. 2; supernatant after IP with αHis. 3; Elution after IP with αHis. 4; supernatant after IP with αMyc. 5; Elution after IP with αMyc. Positive control here (+C) for the westernblot analysis was Trizol isolated His-NSs. **B.** CMV$^{fny\text{-}2b\text{-}HA}$ was expressed by ATTA and IPs were done using different antisera, here detected with αHA. 1; 2b-HA input for IP. 2; supernatant after IP with αHA. 3; Elution after IP with αHA. 4; supernatant after IP with αMyc. 5; Elution after IP with αMyc. Positive control here (+C) for the westernblot analysis

(←) was Trizol isolated CMV$^{fny\text{-}2b\text{-}HA}$. **C.** AGO1-Myc was expressed by ATTA and IPs were done using different antisera, here detected with αMyc. 1; Myc-AGO1 input for IP. 2; Supernatant after IP with αMyc. 3; Elution after IP with αMyc. 4; Supernatant after IP with αHis. 5; Elution after IP with αHis. 6; Supernatant after IP with αHA. 7; Elution after IP with αHa. Positive control here (+C) for the westernblot analysis was Trizol isolated Myc-AGO1.

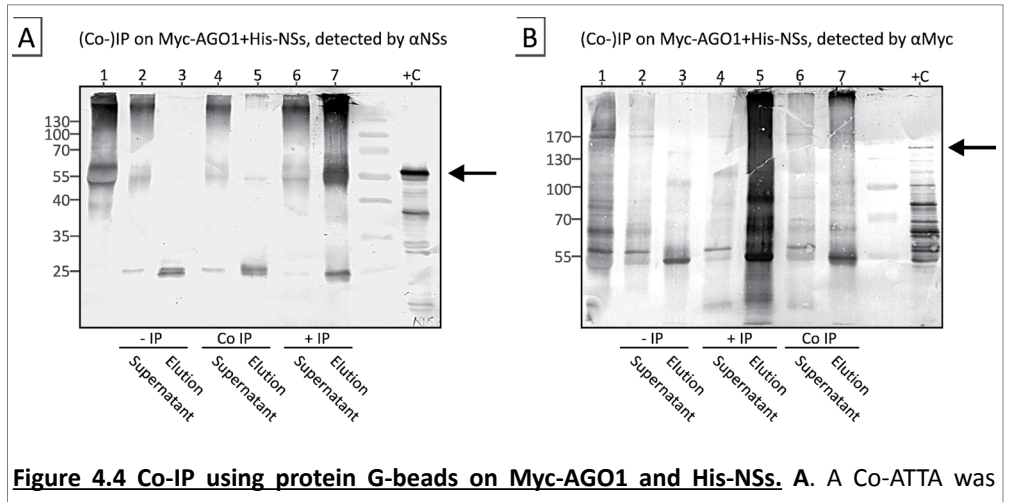


Figure 4.4 Co-IP using protein G-beads on Myc-AGO1 and His-NSs. A. A Co-ATTA was performed with His-NSs and Myc-AGO1, detected here with αNSs. 1; input Co-ATTA for IP. 2; Supernatant after IP with αHA. 3; Elution after IP with αHA. 4; Supernatant after IP with αMyc. 5; Elution after IP with αMyc. 6; Supernatant after IP with αHis. 7; Elution after IP with αHis. Positive control here (+C) for the westernblot analysis was Trizol isolated co-ATTA sample of His-NSs and Myc-AGO1. **B.** Same Co-ATTA sample as in panel A, but now detected with αMyc. 1; input Co-ATTA for IP. 2; Supernatant after IP with αHA. 3; Elution after IP with αHA. 4; Supernatant after IP with αMyc. 5; Elution after IP with αMyc. 6; Supernatant after IP with αHis. 7; Elution after IP with αHis. Positive control here (+C) for the westernblot analysis was Trizol isolated co-ATTA sample of His-NSs and Myc-AGO1.

proteins and viral RSS to AGO1 (Zhang *et al.*, 2006; Giner *et al.*, 2010), recently has demonstrated the biological relevance of this motif for NSs RSS activity (Chapter 3). Using Co-immunoprecipitation, we have provided here the first preliminary indications that AGO1 and NSs do interact, but results from complementary assays are needed to further support this observation.

Previously, TSWV NSs was shown to exhibit affinity to siRNAs, long dsRNAs and miRNAs (Schnettler *et al.*, 2010), a feature common to the majority of viral RSS proteins (Lakatos *et al.*, 2006). In contrast, only a minority has been to shown to act differently, or act in multiple ways, *e.g.* by means of direct or indirect interaction with one of the RNAi enzymatic key players or affecting their expression levels/ degradation. A number of viral RSS interact with AGO1, and include SPMMV-P1, TCV-P38, $CMV^{fny\text{-}2b}$, PVX-P25 and the P0 of *Polero*- and *Enamovirus*, but of which only the first two do this by means of a WG/GW motif. For the other cases it remains unknown how the binding is established (Zhang *et al.*, 2006; Baumberger *et al.*, 2007; Azevedo *et al.*, 2010; Chiu *et al.*, 2010; Giner *et al.*, 2010; Fusaro *et al.*, 2012). Interestingly, from all these, P0 from *Polero*- and *Enamovirus* and P25 from PVX destabilise AGO1 ultimately leading to its degradation (Baumberger *et al.*, 2007; Chiu *et al.*, 2010; Fusaro *et al.*, 2012). Valleray and colleagues (2010) described another mode of action to inhibit AGO1, which involved the action of the miRNA pathway. They showed that the silencing suppressor from a number of different viruses, *i.e. Cymbidum ringspot virus*, *Tobacco mosaic virus* and *Potato virus X*, were able to reduce AGO1 protein levels by specifically stimulating miR168 and not other (unrelated) miRNAs, causing translational arrest of AGO1 mRNA. The latter was supported by detection of increasing levels of AGO1 mRNA concomitant with decreasing levels of AGO1 protein synthesis. Whether TSWV NSs interacts with AGO1, to prevent its functionality or trigger its degradation similar to *Polero*-/ *Enamovirus* P0 and PVX, and/or (additionally) stimulates miR168 expression to silence AGO1, similar to *Tombusvirus* P19 and other viral RSSs, remains to be investigated.

Considering the biological relevance of the GW/WG motif in TSWV NSs it is noted that P1 from *Sweet potato feathery mottle virus* (SPFMV), closely related to P1 of SPMMV, does not exhibit RSS activity but like TSWV NSs contains a single GW/WG site. However, when two additional GW/WG domains were introduced to resemble SPMMV-P1, the protein acquired RSS activity (Szabo *et al.*, 2012). Besides SPMMV-P1, also TCV-P38 minimally requires two of these domains to be a functional RSS (Azevedo *et al.*, 2010; Giner *et al.*, 2010). Although alanine substitution of the GW/WG motif turned TSWV NSs into a dysfunctional RSS, confirmation of a genuine AGO1 interaction will be interesting to indicate whether a single domain suffices to support interaction. NSs is a strong RSS, and about similar compared to one of

the strongest RSS known, *i.e.* P19 from a *Tombusvirus* (Figure 4.1). Whether the strong RSS activity of NSs is caused by its ability to interfere at various stages of the RNA silencing pathway, besides its affinity to small and long dsRNAs, remains to be investigated.

The data presented on the interaction of AGO1 and NSs remain inconclusive, mainly because of the low expression levels of some of our constructs, specifically the AGO1 constructs, used in the Co-IP experiments. This also accounts for the positive control samples for the Co-IP study, namely $CMV^{fny\text{-}2b\text{-}HA}$ and $SPMMV\text{-}P1_{wt}$. In addition, the cross reactions of immuno-globulins (IgG) used in this study to perform the (Co-)IP with the heavy chain of IgG (±55 kDa) and the light chain of IgG (±25 kDa), at times also hampered the analysis since the NSs protein (±55 kDa) respectively the $CMV^{fny\text{-}2b}$ (±20 kDa) more or less co-migrated at exactly similar positions. Data from alternative strategies will have to be provided to proof the genuine existence of NSs-AGO1 interactions, *e.g.* by Yeast-two hybrid screen or bimolecular fluorescence complementation (BiFC).

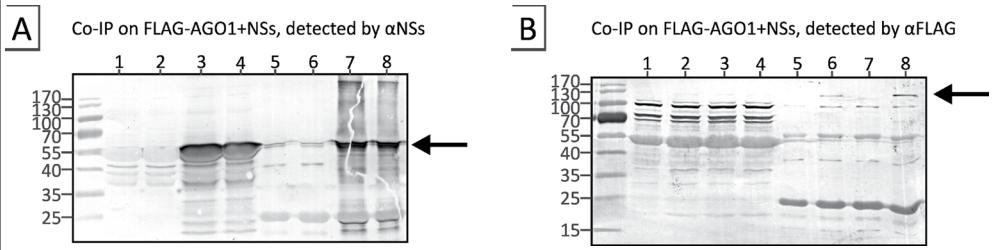


Figure 4.5 FLAG affinity gel precipitation of FLAG-AGO1+NSs. FLAG-affinity beads were used to perform Co-IP experiments on co-ATTA expressed FLAG-AGO1+NSs. **A.** Co-IP results using the FLAG-affinity beads of different (Co-)ATTA expressed constructs, detected here with αNSs. 1; supernatant after IP on MBP. 2; supernatant after IP on AGO1+MBP. 3; supernatant after IP on NSs. 4; supernatant after IP on AGO1+NSs. 5. Elution after IP on MBP. 6; Elution after IP on AGO1+MBP. 7; Elution after IP on NSs. 8; Elution after IP on AGO1+NSs. **B.** Same samples as in panel A, but now detected with αFLAG.

Material and Methods

Constructs and bacteria

The constructs pK2GW7-MBP, pK2GW7-NSs^{RI}, pBin-GFP have been previously described (Chapter 2). The construct pK2GW7-His-NSs was made by quick change PCR on the gateway compatible entry vector pEntr11-NSs^{RI} introducing a 6x His-tag at the N-terminal side of the *NSs* gene and subsequent recombination into the pK2GW7 vector (Karimi *et al.*, 2002). The pBA-6Myc-AGO1 construct and the pBA-CMVfny-2b-3HA were described in Zhang *et al.* (2006). The other construct containing the *Ago1*-gene was the pBin-FLAG-AGO1 construct from Baumberger and co-workers (2007). The constructs encoding the *P1* gene were the pBin-HA-P1$_{wt}$ and pBin-HA-P1$_{mut}$, which were described in Giner *et al.* (2010). All constructs were driven by the 35S promoter from the *Cauliflower mosaic virus* (CaMV). Constructs were transformed to *Agrobacterium tumefaciens* LBA4404 strain (Ooms *et al.*, 1982).

Agrobacterium tumefaciens transient transformation assay (ATTA)

The ATTA assay was performed according to Bucher *et al.* (2003), with slight modifications as described in Chapter 2. In brief: Agrobacteria were grown overnight at 28 °C in LB3 medium containing proper antibiotic selection pressure. From this culture, 600 µl was freshly inoculated into 3 ml induction medium and grown overnight. *Nicotiana benthamiana* plants were watered in excess 1 hour before the infiltration, which was done using a needleless syringe. Strain *A. tumefaciens* LBA4044 was grown under 20 µg/ml rifampicin selection pressure.

RNA silencing suppression assay

The RNA silencing suppression assay was performed by agroinfiltration of *Nicotiana benthamiana* leaves with a functional GFP construct (Tsien, 1998) as described above, using a final O.D. at 600nm of 0.25 per construct. A construct expressing the Maltose binding protein was used as a negative control (Schnettler *et al.*, 2010). Infiltrated leaves were monitored for GFP expression at 5 dpa using a hand-held UV-lamp.

Serological detection of expressed proteins

Expression of the different constructs were analysed by SDS-PAGE and subsequent Western immunoblot analysis using specific antisera against the constructs tested as previously described (Kormelink *et al.*, 1991). Most constructs tested were run on a 15 % gel, while the detection of the AGO1 protein ran on a 10 % gel. For the detection of the Myc-AGO1 construct a rabbit-αMyc antibody was used, while for the detection of the FLAG-AGO1 construct rabbit-αFLAG was used. The constructs CMV$^{fny\text{-}2b\text{-}HA}$ and both HA-P1 constructs were detected with rat-αHA, and finally the His-NSs construct was detected by mouse-αHis.

Immunoprecipitation

Two protocols were used to perform the (Co-)immunoprecipitation (IP) studies. At first, the protocol using iron (Fe-)beads was used. These were made by mixing 2.7 gr. $FeSO_4 \cdot 7H_2O$ in 10 ml of dH_2O with 5.7 gr. $FeCL_3 \cdot 6H_2O$ in 10 ml of dH_2O and adding 123 ml of concentrated ammonia. Sample was heated to 80 °C for 20 minutes after which the sample was centrifuged for 3 minutes at 3300 rpm and finally washed 5 times with dH_2O. The pellet was dissolved in 200 ml TE pH 8.0. The beads were coated with the appropriate antibody by mixing 25 µg of antibody to 1 ml of Fe-beads in a 1x coating buffer and incubated for 2 hours at Room temperature (RT). The plant sample was prepared by grinding the infiltrated leaf at 5 dpi in PBS-Tween (9 ml per gram leaf) using a mortar on ice. The sample was then centrifuged (5 min at 5000 rpm) and supernatant was added to the beads; 1 ml of sample with 100 µl beads, which were mixed and incubated for 3 hours at 4 °C. The supernatant was removed in a next step and the beads were washed with PBS-Tween, transferred to a clean tube and washed again. The pellet was resuspended in 100 µl stripbuffer (0.05M Tris pH6.8, 2 % SDS and 10 mM β-mercaptoethanol) and was heated to 53 °C (on vortex). The supernatant was transferred to a clean tube and used for SDS-PAGE and Westernblot analysis, after adding 35 µl 50 % glycerol with bromophenolblue, heated to 95 °C for 5 minutes.

The second (Co-)IP protocol made use of the protein-G Dynabeads. This protein is able to bind to specific immuno-globulins (IgG) that were used in this study. In this protocol, the ATTA samples were ground in liquid nitrogen and pre-cooled lysis buffer was added (5 ml/gr leaf). The sample was centrifuged twice (first; 15 min at 5000 rpm, second; 15 min 13000 rpm), and 5 ml of supernatant was used per

IP reaction. The sample was pre-cleared by adding 10 µl/ml protein-G Dynabeads for 20 minutes at 4 °C. The supernatant was transferred to a new tube and the appropriate antibody was added (10 µg/ml) and was incubated for 1-2 hours at 4 °C. Subsequently, the Dynabeads were added (75 µl/ml) and incubated o/n at 4 °C. The last steps are as described above for the first IP protocol, but washing was done with lysis buffer. To optimise detection Trizol purification was performed on these beads.

The third immunoprecipitation protocol that was performed used the FLAG-affinity gel-beads (Sigma-Aldrich). FLAG-AGO1 and NSs were expressed in a co-ATTA setting, and leaf samples were taken at 5 dpi. Leaves were ground in a 3x Volume extraction-buffer (50 mM Tris, pH 8.0 + 1.5 % Pvp + 30 % Glycerol + 0.01 M DTT + protein inhibiter cocktail) and passed through miracloth to remove plant debris; samples from here onwards were kept at 4 °C. Samples were incubated and rotated for 15 minutes before centrifugation for 10 minutes at 10K rpm. The supernatant was transferred to a new tube and the samples were precleared by adding 0.1 gr of (empty) beads (in extraction buffer). Samples were incubated and rotated again for 10 minutes and subsequently centrifuged for 5 minutes at 10K rpm. The supernatant was passed through a 0.45 µm filter and put in a new tube. The FLAG-beads (washed 3x with extraction buffer and 1x with 1 % Elk in TBS) were added to the sample and incubated (rotated) for 2 hours. Samples were centrifuged for 30 seconds at 10K rpm and the pellet was washed 5x with extraction buffer and 2x with TBS. A 4x denaturation solution was added to the beads, heated to 95 °C for 5 minutes and subsequently spin down for 1 minute at 10K rpm. The supernatant was transferred to a new tube and used to load on a SDS-PAGE gel and subsequent western blotting procedure.

Acknowledgements

Many thanks to drs. Ana Giner, Juan José López-Moya and prof. József Burgyán for their help with both the SPMMV-P1$_{wt}$ and P1$_{mut}$ constructs. Also thanks to drs. Nicolas Baumberger, Laura Taylor and prof. David Baulcombe for providing the FLAG-AGO1 construct. Finally, we would like to thank drs. Xiuren Zhang, Jessica Pash and Prof. Nam-Hai Chua for their help with the CMV$^{fny\text{-}2b\text{-}3HA}$ and 6Myc-AGO1 constructs.

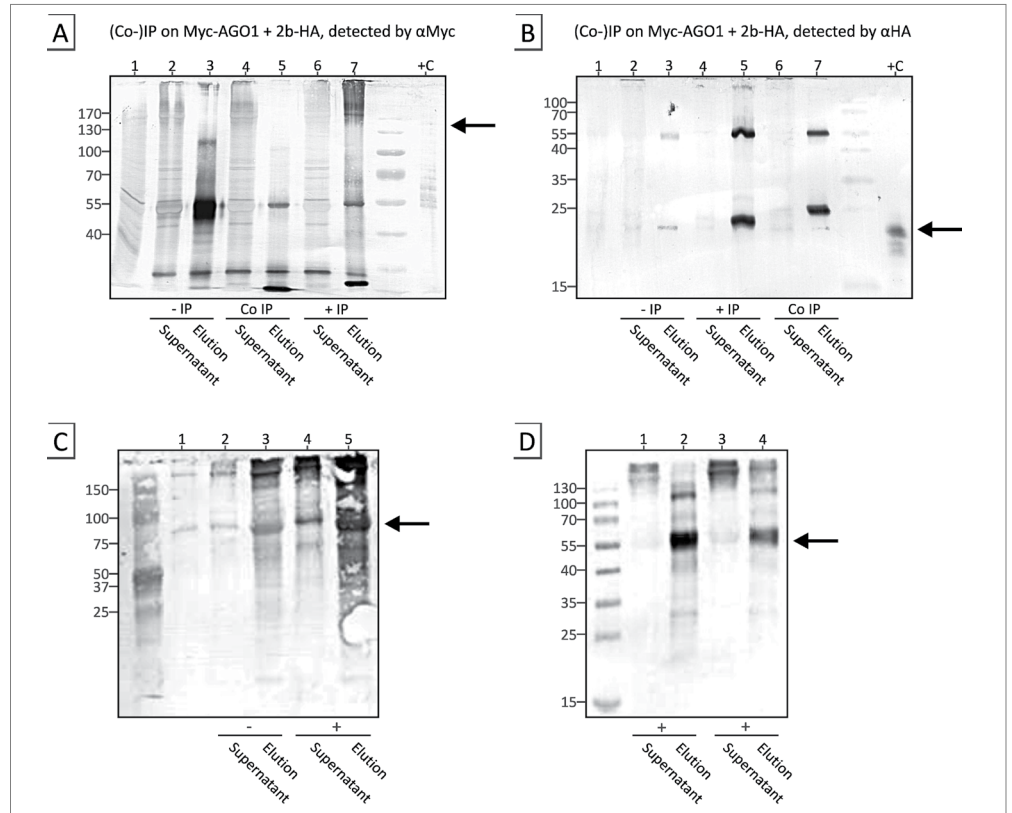


Figure S4.1 Westernblot analysis of different Co-IP analysis. A. A Co-IP was performed on Co-ATTA expressed CMV$^{fny\text{-}2b\text{-}HA}$ + Myc-AGO1, detected here with αMyc. 1; input Co-ATTA. 2; Supernatant after IP with αN. 3; Elution after IP with αN. 4; Supernatant after IP with αHA. 5; Elution after IP with αHA. 6; Supernatant after IP with αMyc. 7; Elution after IP with αMyc. Positive control here (+C) for the westernblot analysis was Trizol isolated co-ATTA sample of CMV$^{fny\text{-}2b}$ + AGO1. **B.** Same Co-ATTA sample as in panel A, but now detected with αHA. Positive control here (+C) for the westernblot analysis was Trizol isolated co-ATTA sample of CMV$^{fny\text{-}2b}$ and AGO1. **C.** Co-IP experiment were performed using αFLAG beads on co-ATTA expressed FLAG-AGO1 with either P1$_{wt}$ or P1$_{mut}$, detected with αHA. 1; MBP. 2; supernatant after Co-IP of FLAG-AGO1 + P1$_{mut}$. 3; Elution after Co-IP of FLAG-AGO1+ P1$_{mut}$. 4; supernatant after Co-IP of FLAG-AGO1 + P1$_{wt}$. 5; Elution after Co-IP of FLAG-AGO1 + P1$_{wt}$. **D.** Co-IP experiment were performed using αFLAG beads on co-ATTA expressed FLAG-AGO1 with either His-NSs or NSs, detected with αNSs. 1; supernatant after Co-IP of FLAG-AGO1 + NSs. 2; Elution after Co-IP of FLAG-AGO1 + NSs. 3; supernatant after Co-IP of FLAG-AGO1 + His-NSs. 4; Elution after Co-IP of FLAG-AGO1 + His-NSs.

Chapter 5

Identification and characterisation of a new class of temperature-dependent *Tomato spotted wilt virus* resistance breaking isolates of *Tsw*-based resistance and the development of a diagnostic tool

This chapter will be submitted in a slightly modified version as:

"Identification and characterisation of a new class of temperature-dependent *Tomato spotted wilt virus* resistance breakers isolates of *Tsw*-based resistance".

Abstract

The single dominant *Tsw* resistance (*R*-)gene from *Capsicum chinense* against the *Tomato spotted wilt virus* (TSWV) has been described as being temperature sensitive; at 32 °C the resistance does not hold against resistance inducing isolates. Here, we have described a new class of temperature-sensitive TSWV isolates that depending on the temperature either induce (at T <28 °C) or break (at T ≥ 28 °C) *Tsw*-mediated resistance. The *NSs* genes from these isolates were cloned and upon transient expression analysed for RNA silencing suppressor (RSS) activity or the ability to induce *Tsw*-mediated HR. In contrast to the virus-setting some NSs proteins surprisingly did not induce *Tsw*-mediated HR when transiently expressed at standard temperatures (22 °C). Concomitantly, varying degrees of RSS activity were observed among the NSs proteins from this class of resistance breakers. Attempts expressing and testing the NSs proteins for functionality at an elevated temperature using *Agrobacterium* remained unsuccessful. Multiple sequence alignment of the *NSs* genes from TSWV resistance inducing (RI) and resistance breaker (RB) isolates revealed the importance of two amino acid residues in RNA silencing suppression and avirulence, features that were lost from several TSWV RB isolates analysed. One amino acid residue was found to be critical for both functions tested. To detect the presence of this altered codon sequence in *NSs* genes, and thereby identify and distinguish TSWV RB isolates from RI isolates, a primer set was designed and tested using RT-PCR on a small collection of TSWV RI and RB isolates. Although RB isolates were detected and distinguished from RI isolates, some RB isolates escaped from detection.

Introduction

Tomato spotted wilt virus (TSWV) is the type species of the *Tospovirus* genus, and represents the plant infecting members within the family of arthropod-born *Bunyaviridae*. In mammals, the animal-infecting bunyaviruses encounter interferon-induced innate immune responses and an adaptive immune response that eventually leads to their clearance from the host. In plants, tospoviruses encounter an immune system that also involves two main layers. The first one involves the (relatively

slow) onset of antiviral RNA silencing (RNA interference, RNAi), and a second layer of effector-triggered immunity (ETI), mediated by single dominant resistance (*R*)-genes. While RNA silencing acts against all plant viruses, *R*-genes generally confer resistance to specific virus species only. *R*-genes encode NBS-LRR proteins that directly or indirectly perceive a specific viral protein, named avirulence (Avr) determinant or effector, and upon recognition triggers a hypersensitive response (HR). The latter is basically a programmed cell death response (PCD; necrosis) and easily visualised by the formation of necrotic lesions on leaves at the site of virus entry (Moffett, 2009).

TSWV currently ranks second on the list of economically most important plant viruses worldwide (Scholthof *et al.*, 2011). So far, only two single dominant resistance genes are available for commercial resistance breeding against this virus, *i.e. Sw-5* from tomato and *Tsw* from *Capsicum* (Brommonschenkel *et al.*, 2000; Jahn *et al.*, 2000). The *Sw5b* gene has been cloned and encodes a CC-NB-LRR protein that just recently was shown to be triggered by the TSWV cell-to-cell movement protein (NSm) (Spassova *et al.*, 2001; Hallwasser *et al.*, Submitted for publication). The *Tsw* gene has not been cloned, but has been shown to be single dominant and therefore likely of the NBS-LRR type. Recently, the NSs RNA silencing suppressor (RSS) protein was identified as the Avr-determinant (Chapter 2). Triggering of *Tsw*-based resistance in *Capsicum* requires a functional RNA silencing suppressor (RSS), since NSs from natural resistance breaker isolates of TSWV lost avirulence and RSS-activity. However, a more recent NSs mutant screen has shown that both functions can be separated (Chapter 3).

Dominant resistance proteins against different plant pathogens display temperature sensitivity and do not provide resistance above a certain temperature. This threshold temperature differs between R-proteins (Zhu *et al.*, 2010). For instance, the *N* resistance gene product from tobacco only provides resistance against *Tobacco mosaic virus* (TMV) at temperatures below 28 °C, while Rx1 from potato still provides resistance up to 30 °C (Wang *et al.*, 2009). Basal defence genes of dominant resistance by the class of TIR-NB-LRR, EDS1 and PAD4 are also indirectly affected by temperature. Regulators of these are down regulated at 28 °C in comparison to 22 °C, and so is the defence related hormone salicylic acid (SA), involved with systemic required resistance (Wang *et al.*, 2009).

The *Tsw* resistance gene is no longer able to provide resistance at a temperature of 32 °C or higher (Moury *et al.*, 1998). In contrast, the antiviral RNAi pathway seems to be up-regulated at elevated temperatures (Zhang *et al.*, 2012). While some plant viruses are less fit at elevated temperatures, an increase in the rate of infection and higher virus titres are observed for TSWV (Soler *et al.*, 1998).

In relation to the *Tsw R*-gene, most TSWV isolates are either classified as a typical wild type, also called resistance-inducer (RI), or resistance-breaker (RB) isolate. However, virus challenging assays on *Tsw+ Capsicum* lines have revealed the existence of a third class of isolates that has not been described and well characterised yet, but whose ability to trigger HR (or not) deviates from members belonging to the first two classes. Here, we typified these TSWV isolates as temperature-dependent resistance breaker isolates of *Tsw*-based resistance. Members of this group are able to break resistance at 28 °C, a condition at which *Tsw* is still functional, but require *de novo* Avr protein synthesis at temperature conditions below 28 °C to induce HR.

Results

Phenotyping TSWV isolates in relation to *Tsw*-based resistance

TSWV isolates tested for their ability to induce or break *Tsw*-mediated resistance and included in this study are listed in Table 5.1. Under standard greenhouse conditions (22 °C) some of these isolates did not trigger an HR, but caused a systemic infection of resistant *Capsicum*, as observed with Vir131, Vir169, p272 and Ve427 (Figure 5.1A). Other isolates clearly induced an HR (necrotic lesions) at these conditions, as shown for isolates Br01, Vir128, Vir130, Vir164, It98 and p166 (Figure 5.1A). TSWV isolate p166 exhibited a rather unique phenotype as this virus was able to cause a systemic HR, although this was not consistently observed. After symptoms were recorded (Figure 5.1A), systemically infected/top leaves were collected and used to determine virus titres by DAS-ELISA. While top leaves from *Tsw*+ plants were completely free from virus in case local HR was observed, TSWV isolates were consistently detected in top leaves that showed a systemic infection (Figure 5.2).

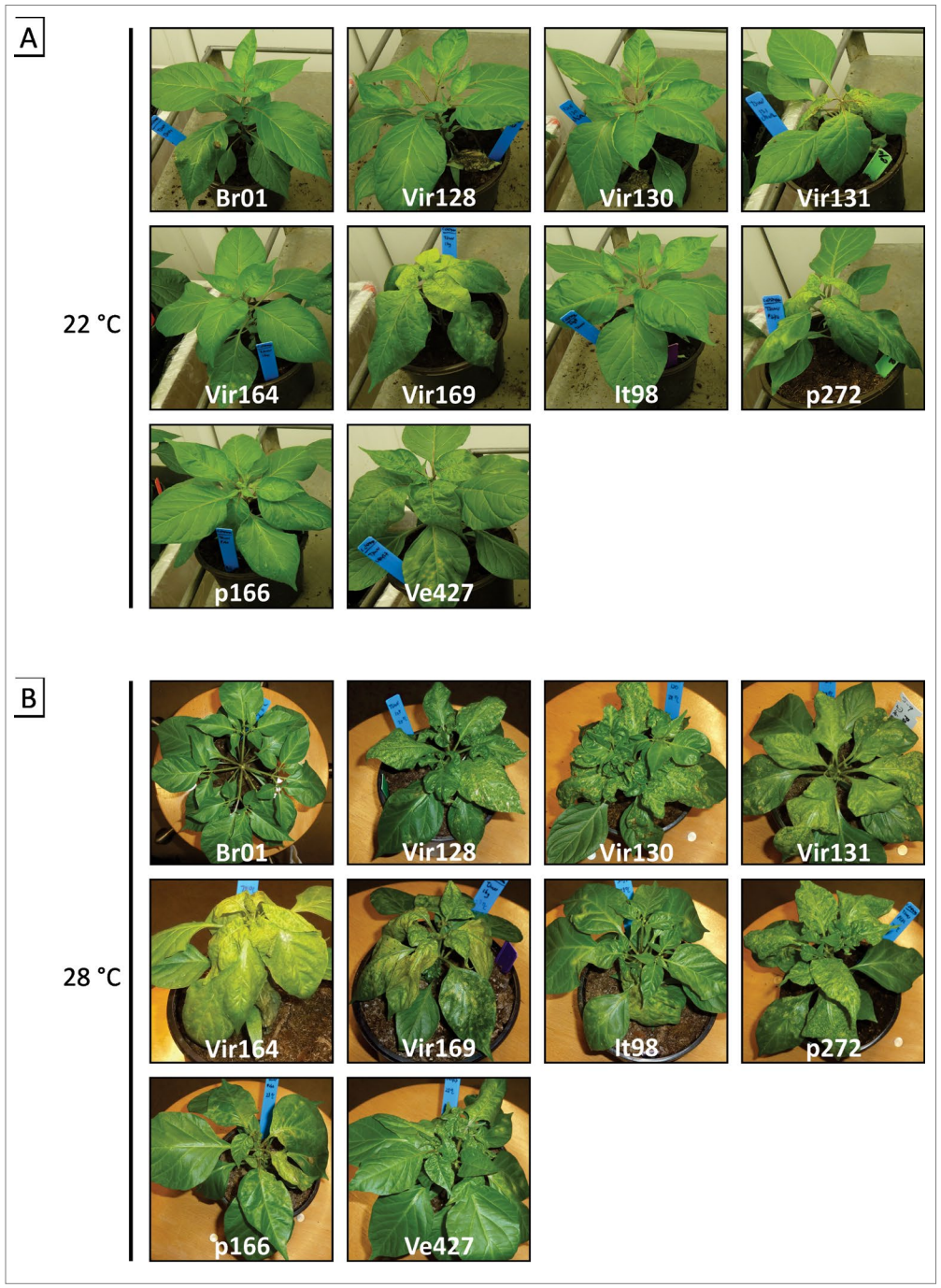
Table 5.1 Description of TSWV isolates tested in this study. RI: resistance inducer. AbsRB: Absolute resistance breaker. TempRB: Temperature dependent resistance breaker.

Virus isolate	Phenotype	Location origin	Year
Br01	RI	Brazil	1996
Vir171	AbsRB	Almeria (Spain)	2008
Vir128	TempRB	Spain	2000
Vir130	TempRB	Netherlands	2002/2003
Vir131	AbsRB	Spain	2002/2003
Vir164	TempRB	Netherlands	2005
Vir169	AbsRB	Spain	2006
It98	TempRB	Italy	1998
p272*	AbsRB	Albenga (Italian Riviera)	2002
p166*	TempRB	Albenga (Italian Riviera)	1998
Ve427*	AbsRB	Almeria (Spain)	2003

*From Margaria (2007)

Temperature-dependent phenotype of a subset of TSWV isolates

Isolates Vir128, Vir130 and Vir164 used in this study were collected from cultivations of TSWV resistant *Capsicum* plants but, unexpectedly, triggered an HR on resistant plants at greenhouse conditions. Since field conditions might have differed from our greenhouse conditions, the possible effect of temperature on the resistance response was further analysed. To this end, *Tsw+ Capsicum* plants were challenged with all virus isolates and incubated at different temperatures (23, 25, 28, 30 and 32 °C). The resistance inducing (RI) reference isolate Br01 was included as positive control for *Tsw*-mediated HR (Chapter 2). While some isolates consistently revealed a resistance-breaker phenotype regardless of the temperature tested (Vir131, Vir169, p272, Ve427, Vir171 and Br01) other isolates surprisingly showed a resistance-breaker phenotype only at elevated temperatures (≥28 °C) (Figure 5.1B and Table 5.2). The observation that Br01 was still able to trigger an HR at the temperatures tested, confirmed the functionality of the *Tsw* resistance gene (Figure 5.1B).



← Figure 5.1 Symptoms induced on *Capsicum* after challenging with different TSWV isolates at different temperatures. **A.** *Capsicum chinense* (*Tsw*+) plants infected with different TSWV isolates and incubated at 22 °C. Pictures were taken at 12 dpi. **B.** Similar to panel A, *Capsicum chinense* plants infected with TSWV isolates, but now incubated at 28 °C. Pictures were taken at 12 dpi.

Table 5.2 The ability of each of the three identified phenotypes to infect the resistant (*Tsw+*) *Capsicum* plant at different temperatures. RI: resistance inducer. AbsRB: Absolute resistance breaker. TempRB: Temperature dependent resistance breaker.

Temperature (in °C)	23	25	28	30	32
RI	Resistant				
TempRB				Susceptible	
AbsRB					

These analyses indicated that the resistance-breaker phenotype of this distinct class of TSWV isolates involved a temperature sensitive feature of these viruses, and from now onwards referred to as temperature-dependent resistance breakers (TempRB). TSWV isolates that systemically infected *Tsw+* resistant *Capsicum* plants, regardless of temperature used, hence were renamed as Absolute resistance breaker (AbsRB). As expected, at 32 °C *Tsw*-resistance was no longer functional and all virus isolates rendered a systemic infection of *Tsw+ Capsicum* (Moury *et al.*, 1998).

Triggering of HR by TSWV TempRB isolates requires *de novo* synthesis of NSs

To further investigate the temperature dependency of TSWV-TempRB isolates, experiments were performed in which plants were challenged with these viruses at high temperature conditions (32 °C) and later on replaced to a lower temperature (22 °C). Resistant *Capsicum* plants challenged with a resistant inducing isolate of TSWV (Br01) and incubated at the elevated temperature of 32 °C, at which the

Tsw-R-gene (product) normally is inactive became systemically infected. In addition, local HR was still induced (Figure 5.3A), but this was apparently insufficient to prevent the virus from systemically infecting the host. At 12 days post infection (dpi), plants showed clear symptoms of systemic infection. At this point, plants were replaced from 32 °C to greenhouse conditions (22 °C) and monitored for several days. While initially no response was observed during the first few hours after replacement, a severe necrosis emerged in the days following in the top leaves of the plants (Figure 5.3A). The systemically infected leaves became necrotic and after 5 days the plants were completely dead and the remainder of the plant tilted over due to stem necrosis (Figure 5.3A).

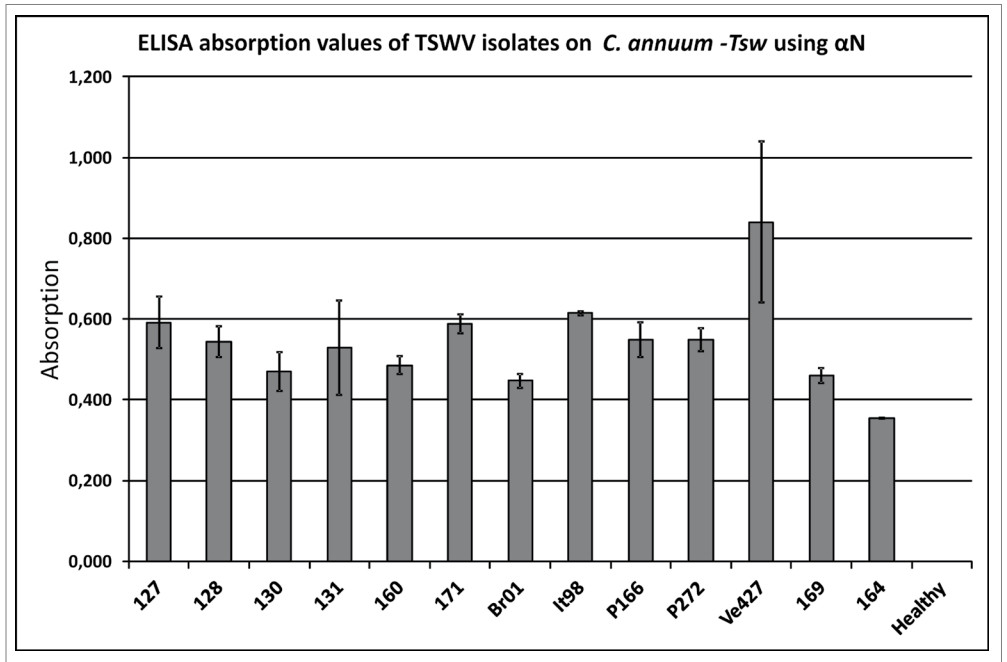


Figure 5.2 TSWV titres on susceptible *C. annuum* plants show similar values. Susceptible *C. annuum* plants (*Tsw*-) were infected with all TSWV isolates described in this study and virus titres were measured by DAS-ELISA using αN antiserum. Values were measured after 50 minutes post substrate addition. The error bars represent standard deviations of 3 repetitions.

A similar experiment was performed using TSWV TempRB isolates, including AbsRB and RI isolates as control, but this time resistant *Capsicum* plants were challenged with the virus at 28 °C, a temperature at which the *Tsw* resistance gene was still functional. Except for those plants challenged with the Br01 RI isolate (HR-induction), all TempRB and AbsRB isolates (Vir128, Vir130, It98, Vir171) caused a systemic infection of *Tsw+ Capsicum* plants. At 12 dpi, plants were replaced to a lower temperature greenhouse condition (22 °C) and monitored for another two weeks (Figure 5.3B). During the first few days after replacement no changes were observed at all, in contrast to the first temperature shift experiment (32 °C to 22 °C) with the TSWV RI isolate. However, at 9 days post replacement, necrosis was observed at the top of the plants. In the next following days, this completely killed the top meristem, and no new young leaves emerged from these plants (Figure 5.3B). Systemically infected leaves below the top meristem, remained seemingly unchanged throughout this time.

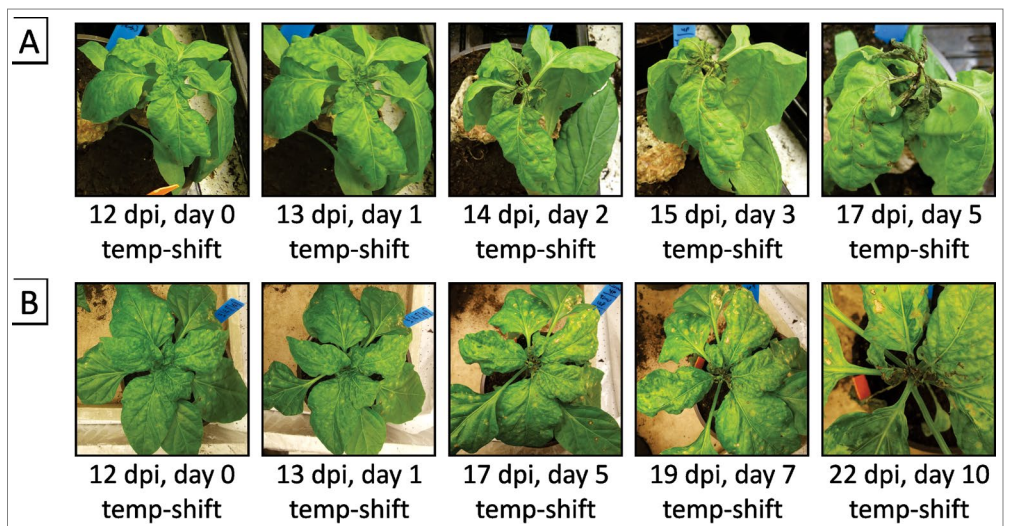


Figure 5.3 Effect of temperature shift after systemic infection of resistant *Capsicum* plants. A. *Capsicum annuum* plants infected with a TSWV-RI isolate at 32 °C, were replaced after systemic infection at 12 dpi, to 22 °C. HR was induced on the local leaves and finally led to leaf abscission. Pictures were taken at several stages during the experiment, as indicated. **B.** *Capsicum annuum* plants infected with TSWV-TempRB Vir128 (and RI/AbsRB as controls) at 28 °C, were showing a systemic infection at 12 dpi, and were transferred to 22 °C. Pictures were taken on each day.

Not all TSWV TempRB NSs proteins induce *Tsw*-mediated HR upon transient expression

NSs genes amplified from TSWV TempRB and AbsRB isolates were cloned into the highly translatable plant expression vector pEAQ-HT earlier used for induction of *Tsw*-mediated HR (Chapter 2), and after transformation into *Agrobacterium tumefaciens* 1D1249 cells, agro-infiltrated in *Nicotiana benthamiana* to verify translatability of the constructs. All constructs showed expression of the NSs protein of expected size (data not shown), and next were infiltrated on *Capsicum Tsw+* plants at standard temperature greenhouse conditions (22 °C), to analyse their ability to trigger HR at 5 dpi. Surprisingly, NSs constructs made from Vir128, Vir130, Vir164 and p166, designated NSs^{128}, NSs^{130}, NSs^{164} and NSs^{p166}, respectively, did not trigger HR while their corresponding virus isolates, like the positive control TSWV RI (Br01), did (Figure 5.4A and Table 5.3). On the other hand, the NSs from AbsRB isolate Vir169 (NSs^{169}) unexpectedly triggered an HR (Figure 5.4A and Table 5.3), while the corresponding virus did not. These results were consistently obtained during several repetitions of the experiment.

Table 5.3 Summarizing overview on scores for avirulence (Avr) and RSS-activity of the NSs proteins tested at greenhouse conditions (22 °C).

Minus (-): No or loss of activity. Plus (+): activity (gained). Plus/minus (+/-): Partial activity.

Phenotype virus	NSs construct	AVR-activity	RSS activity
RI	NSs^{RI}	+	+
AbsRB	NSs^{171}	-	-
TempRB	NSs^{128}	-	+
TempRB	NSs^{130}	-	+/-
AbsRB	NSs^{131}	-	+
TempRB	NSs^{164}	-	+
AbsRB	NSs^{169}	+	-
TempRB	NSs^{It98}	+	+/-
AbsRB	NSs^{p272}	-	+
TempRB	NSs^{p166}	-	-
AbsRB	NSs^{Ve427}	-	+

Not all TSWV TempRB NSs proteins suppress RNA silencing upon transient expression

To verify whether NSs from the various TSWV isolates was able to suppress RNA silencing, the *NSs* genes were cloned in the expression vector pK2GW7 and transformed to *Agrobacterium tumefaciens* LBA4404, as described in chapter 2. *Nicotiana benthamiana* plants were co-infiltrated with *Agrobacterium* carrying the *NSs* construct and *Agrobacterium* carrying a functional *GFP* construct (Chapter 2), in a 1:1 ratio. At 5 dpi, the plants were monitored for GFP expression by a hand-held UV-lamp and fluorescent units were measured by spectrometry (Figure 5.4C). Similar to the outcome of the AVR-assays, some of the results were unexpected (Figure 5.3B and table 5.3). NSs from the AbsRB Vir169 (NSs^{169}) had lost the ability to suppress RNA silencing, like those from resistance breakers reported earlier (Chapter 2), while the NSs from AbsRB Vir131 (NSs^{131}) surprisingly still maintained RSS activity. From the group of TempRB isolates, the NSs proteins from isolates Vir128 and Vir164 (NSs^{128} and NSs^{164}) still showed RSS activity. On the other hand, NSs^{130} and NSs^{It98} from the respective isolates had only partially lost their RSS activity while NSs^{p166} completely was non-functional on this point (Figure 5.4B and table 5.3). Interestingly, all virus isolates from this study showed to possess RSS activity (data not shown).

Functional analysis of NSs proteins from TempRB-isolates at elevated temperatures

To analyse the TempRB NSs proteins for RSS activity and their ability to induce *Tsw*-mediated HR at 28 °C, experiments as describe above were repeated but this time performed at 28 °C. *NSs* gene constructs from the RI (NSs^{RI}) and AbsRB (NSs^{RB171}) isolates and Maltose binding protein (MBP) were included as positive and negative controls. Five days post agroinfiltration, plants were monitored for the presence of HR and suppression of GFP silencing. However, at 28 °C no HR was observed on *Capsicum Tsw+* using the positive control NSs^{RI} (Figure 5.5A). Even during experiments in which plants were first agroinfiltrated at 22 °C and kept for 8, 16 and 24 hours before being moved to 28 °C, no HR was observed with NSs^{RI} (data not shown).

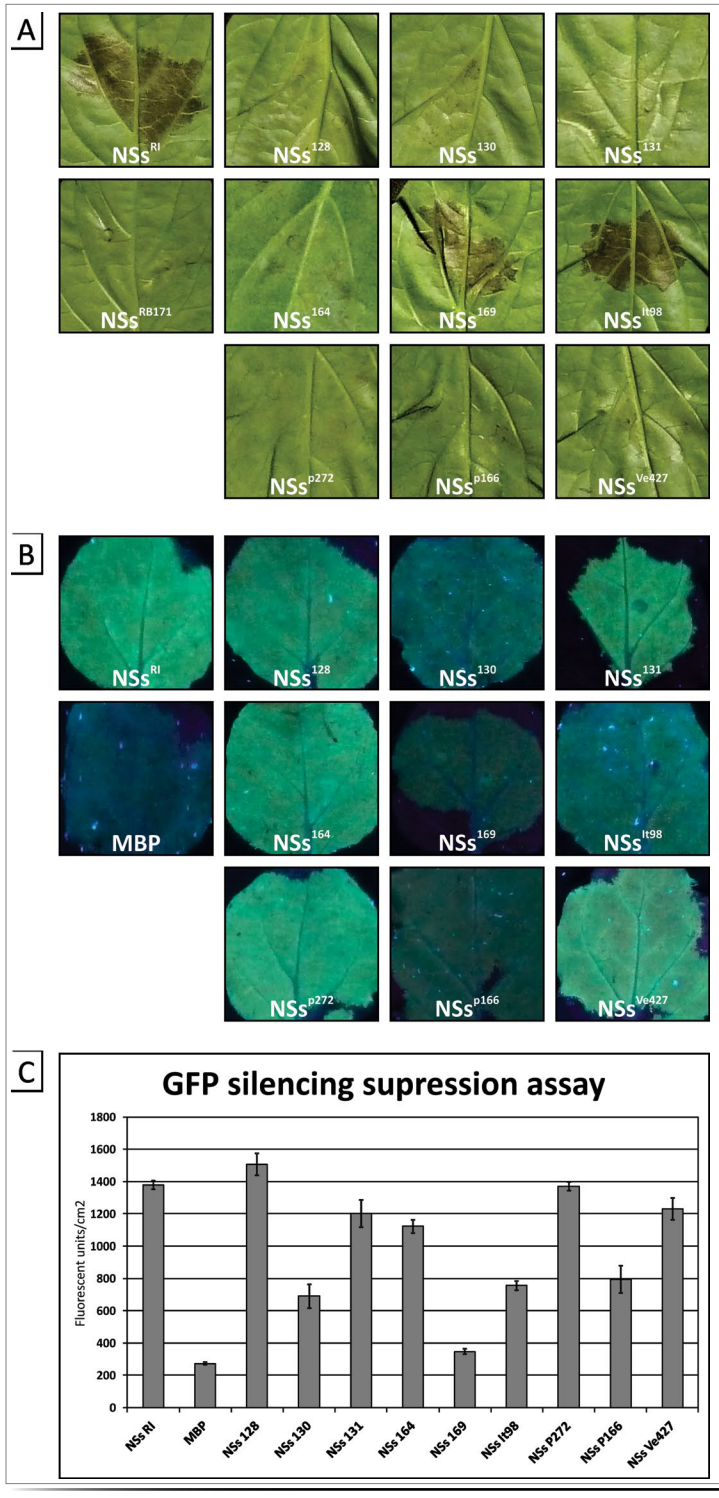


Figure 5.4 Avr- and RSS activity of cloned NSs constructs.

A. Avirulence of different NSs expressing constructs on resistant *Capsicum* plants, as visualised by *Tsw*-mediated HR induction. **B.** Suppression of GFP silencing by various NSs constructs as indicated. **C.** Quantification of GFP fluorescence measured from the leaves shown in panel B. Each construct was repeated four times, indicated as standard deviations.

Similar, negative results were obtained when NSs^{RI} was tested for its ability to suppress GFP silencing in *N. benthamiana* at 28 °C. At 5 dpi, only very low levels of GFP were observed for the positive control NSs^{RI}, relative to the negative control MBP (Figure 5B). All attempts to improve the outcome by applying different temperature conditions during the agroinfiltrations also failed here (Figure 5.5C). Leaves co-infiltrated with GFP and NSs^{RI} and incubated for 5 days at lower temperature (22 °C) before being transferred to elevated temperatures remained fluorescent for at least 5 days more (data not shown), suggesting that GFP itself remained stable. These data altogether indicated that the transient transformation using *Agrobacterium*, rather than expression of the transgene, seemed to be the cause of failure. The application of other *Agrobacterium* strains (Chapter 2) for agroinfiltration, did not solve this problem.

Earlier the use of a viral, PVX-replicon hampered the identification of the Avr-determinant (Chapter 2). However, NSs has meanwhile been identified as Avr-determinant and, in addition, the *Tobacco rattle virus* (TRV) induces less symptoms on *Capsicum* compared to PVX. Therefore, and as an alternative to agroinfiltration, TempRB *NSs* genes were cloned and expressed from a TRV-replicon in the *Capsicum* host to test their ability to induce the resistance response at different temperatures. The *NSs* genes that were tested in the HR- and RSS assay above, and available in pEntr11-vector (Materials and Methods), were transferred to a TRV gateway compatible vector. TRV clones were PCR selected but upon sequence analysis shown to lack the *NSs* genes. Currently, attempts are being made to clone the NSs genes in TRV, to test for their RSS and Avr-activity at elevated temperatures.

NSs proteins from TSWV RI, AbsRB and TempRB isolates are highly homologues

As earlier described (Chapter 2 and 3), no amino acid(s) were identified that gave a 100% correlation with the initial (Abs)RB phenotype. Furthermore, the *NSs* genes from the two RB isolates, analysed in chapter 2, lacked both Avr- and RSS activity. Besides the identification of a new class of TempRB isolates, however, new RB isolates have been characterised from which the NSs still contained one or the other function. To identify potential SNPs involved in the generation of TSWV Abs- and TempRB isolates the amino acid sequence of the cloned *NSs* genes was determined and aligned to those of the TSWV RI and AbsRB isolates from chapter 2.

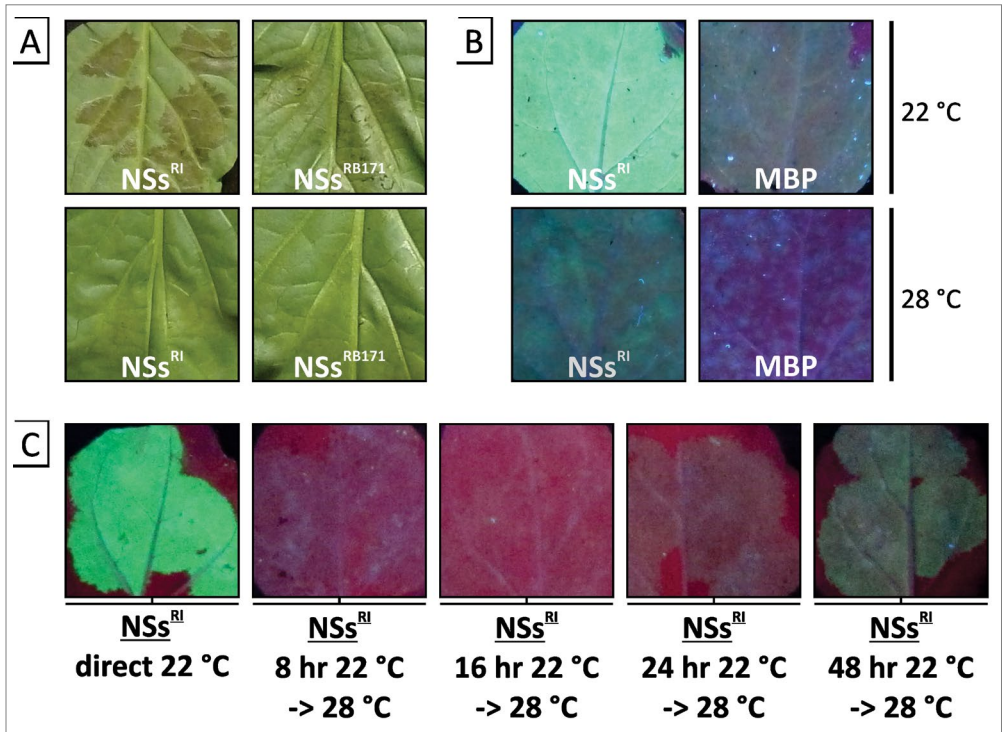


Figure 5.5 Effect of temperature on transformation efficiency of *Agrobacterium tumefaciens*. **A.** Expression of two control constructs on resistant *Capsicum* leaves incubated at 22 °C and 28 °C. Pictures were taken at 5 dpi. **B.** Expression of two control constructs with a functional GFP on *N. benthamiana* incubated at 22 °C and 28 °C. Pictures were taken at 5 dpi. **C.** Expression of NSs^{RI} together with a functional GFP construct, incubated either 0, 8, 16, 24 and 48 hours at 22 °C before being transferred to 28 °C. Pictures were taken at 5 dpi.

Additional TSWV-NSs sequences were retrieved from Genbank randomly and taken along in the alignment to observe whether certain mutations would be present in these sequences as well. For the majority of their corresponding virus isolates, the phenotype in relation to *Tsw*-mediated resistance has not been reported. In addition, NSs sequences were included from virus isolates that were earlier described by Margaria and colleagues (2007) and that were phenotyped for their ability to induce or break *Tsw*-mediated resistance. The NSs amino acid sequences were ordered according to the presence or absence of Avr-activity. Amino acids previously identified to be of importance in light of the evolvement of TSWV RB

isolates (Chapter 3) were highlighted. As expected, a high level of identity between the NSs sequences was observed, with only a few single nucleotide polymorphisms between the isolates (Figure 5.6). From the new RB isolates that were classified here as AbsRB, none of the transiently expressed NSs lacked both Avr- and RSS-activity, like the two AbsRB-NSs' described in chapter 2, but instead rather retained one or both functions. One NSs protein (NSs^{169}), derived from an AbsRB-isolate, contained a change at amino acid position 79 from isoleucine (I) to threonine (T), as earlier observed for RB isolates described in chapter 2, which appears to correspond to RSS-activity (Figure 5.6). Only one exception existed, *i.e.* the *NSs* gene from TSWV RB-isolate 166, which still contained I79, but was RSS inactive. From those AbsRB isolates of which NSs still exhibited Avr and/or RSS activity (NSs^{Ve427}, NSs^{p272}, NSs^{169} and NSs^{131}), all show (different) mutations in the N-terminal domain of their corresponding NSs and also throughout the rest of the sequence, but no essential common amino acids could be identified that was shared by all and distinct from NSs^{RI} (Figure 5.6).

For the TempRB NSs proteins (NSs^{It98}, NSs^{128}, NSs^{130}, NSs^{p166} and NSs^{164}), the same holds true as for the AbsRB-NSs proteins; mutations throughout the sequence were found, of which many are not shared by all. This class of isolates contained NSs proteins that exhibited (partial) RSS activity (NSs^{It98}, NSs^{130}, NSs^{128} and NSs^{164}). From these, NSs^{130} showed mutations in the N-terminal part of the protein, while NSs^{It98} showed mutations in the C-terminal part. In contrast to this, only NSs^{p166} is inactive as RSS; this protein showed mutations in the C-terminal end. With the exception of NSs^{It98}, all NSs proteins of this class of isolates are Avr-active.

Data from the ClustalW multiple sequence alignment was used as input for the assembly of a phylogenetic tree (Figure 5.7). Interestingly, all isolates analysed ended up in three clades of which each one seemed to correspond to a phenotype. The first one contained the majority of the (randomly selected) NSs amino acid sequences from Genbank. The second clade contained NSs^{127}, NSs^{129} and NSs^{Br01} together with another RI Br20, and reflected an Avr+ out-group. Those NSs-proteins from all other RB and AbsRB isolates, including few from RI, clustered in a third clade. The latter one could be distinguished in two sub-clades that corresponded to the geographical origin of the isolates, *i.e.* Spain or Italy (Figure 5.7)(Margaria *et al.*, 2007).

Towards a diagnostic tool for detection of RB-isolates

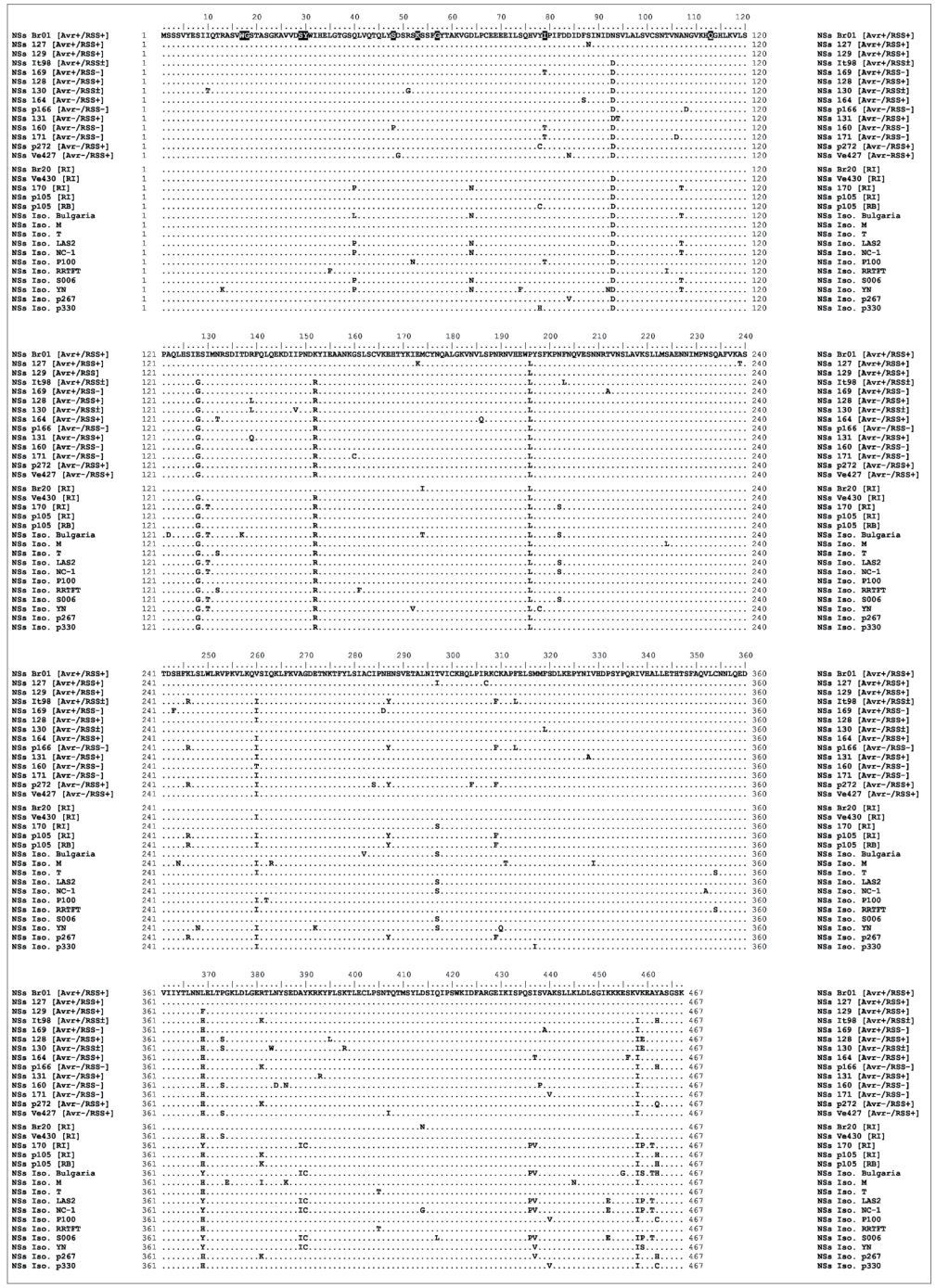
The amino acid at position 79 showed to be important for RSS-activity, and was shared by three AbsRB-NSs proteins, while this residue also appeared quite important for both RSS and Avr-activity, deduced from the NSs mutagenesis study described in Chapter 3. Therefore, primers were designed that aimed to distinguish (most) (Abs)RB isolates from RI isolates based on the detection of this SNP. Although such primer set would likely not detect all (Abs)RB isolates, a relative high positive score on well characterised (Abs)RB isolates would indicate its potential use in future diagnostics, *i.e.* for early (RT-PCR) detection of (harmful) RB isolates. Primers were designed in such way as to allow proper annealing and subsequent extension of the 'forward' primer only in the presence of a T (threonine) encoding ACC coding for aa position 79, and indicative for an RB isolate, but not in the presence of an I (isoleucine) encoding ATC codon (RI isolate). The reverse primer was designed 300 nt downstream and complementary to a conserved stretch of sequence present in both (Abs)RB and RI isolates. Initial tests with the primers during a PCR on purified DNA plasmids containing NSs^{RI} and NSs^{RB} genes, showed that these could discriminate the RI from the RB isolates (data not shown). To test the usefulness of these primers, *N. benthamiana* plants were infected with a selection of different TSWV RI and (Abs)RB isolates and RNA extracted from systemically infected leaves for RT-PCR analysis. Whereas the primers were able to amplify a fragment of expected size for a number of RB (Abs and Temp) isolates (55 % of the samples testes), none of the RI isolates gave a positive RT-PCR result (Figure 5.8). However, a few RB isolates escaped from detection and did not render a positive PCR signal of expected size.

Discussion

Here, we have identified and characterised a new, second type of resistance breaker of *Tsw*, besides the earlier described absolute resistance breaker (AbsRB; Chapter 2), that exhibits a temperature-dependent resistance breaking (TempRB) phenotype. Isolates that match this phenotype trigger an HR at temperatures below 28 °C, but above are able to systemically infect *Tsw*+ *Capsicum* plants. Intriguingly, upon transient expression of their NSs proteins the ability to elicit the *Tsw*-mediated HR

on *Capsicum* does not always correspond to the phenotype of the corresponding virus isolates. Surprisingly, the NSs^{169} derived from an AbsRB transiently triggers *Tsw*-mediated HR, while from the TempRB NSs clones, only NSs^{It98} triggers the resistance. The ability to (transiently) suppress RNA silencing also shows quite some variability; while the NSs^{169} from an AbsRB isolate lacks RSS activity, like two other previously described AbsRB-NSs clones (Chapter 2), a few other AbsRB derived NSs clones exhibit RSS activity (NSs^{131}, NSs^{P272} and NSs^{Ve427}). The TempRB-NSs clones all show either full RSS activity (NSs^{128} and NSs^{164}) or partial RSS activity (NSs^{It98}, NSs^{130} and NSs^{P166}). So far, attempts to further analyse the NSs proteins on RSS and Avr activities at elevated temperatures (28 °C) have failed due to technical problems of the expression system. Based on the importance of I79 for development of RB isolates (Chapter 2 and 3) and supported by the extended alignment studies described in this chapter, an RT-PCR detection tool has been developed that targets the altered codon from (Abs)RB isolates. Although the tool has not given false positives when applied on RI isolates from infected leaf material, it rendered a positive score on 55% of RB isolates.

Virus challenge of *Capsicum Tsw*+ with TSWV RI at 32 °C followed by a shift to 22 °C nicely has shown that at the elevated temperature the *R*-gene is at an 'OFF' mode and not able to prevent a systemic infection. A shift to the lower temperature turns the *R*-gene 'ON' and enables its gene product to perceive the Avr protein (NSs^{RI}), from an established TSWV-RI infection at the elevated temperature. As a consequence, an HR is observed in all (systemic) leaves where the virus is present. A similar TSWV-RI challenging experiment performed at 28 °C quickly reveals an HR, indicative that at this temperature the *R*-gene is 'ON' and able to perceive the Avr, unless plants are challenged with TSWV TempRB isolates. This clearly suggests that the NSs from the latter are not perceived, likely due to altered protein-folding allowing an escape of TSWV TempRB. A shift to the lower temperature does not induce an HR in the infected leaves as rapidly with TSWV RI. Instead, necrosis is observed in the top meristem after 9 days, a time that the virus normally takes to systemically infect meristematic tissues. These results clearly suggest that *Tsw*-mediated resistance is only triggered by *de novo* NSs synthesis from TSWV TempRB isolates at lower temperatures. NSs already synthesised at higher temperatures (28 °C) either is not able to refold into a functional Avr (irreversible), or is prevented from doing so due to it being part of a larger protein complex.



← Figure 5.6 Multiple amino acid sequence alignment of NSs. All NSs amino acid sequences used in this study were analysed in a multiple amino acid sequence alignment. NSs proteins positively tested for the ability to induce *Tsw*-mediated HR were ordered on top of the alignment (indicated by Avr+), followed by those lacking this ability (indicated Avr-). In addition, the presence (+), absence (-) or intermediate (±) RSS activity of each tested NSs construct is indicated. The phenotype of NSs (RI or RB) from the virus isolates as described by Margaria and co-workers (2007), is shown in square brackets.

Unravelling the crystal structure of NSs^{RI} and NSs$^{(Temp)RB}$ could provide further insight into this. The NSs from TempRB isolates exhibits a temperature-dependent behaviour in a viral setting, although this could not be tested in a transient setting yet. So far, temperature sensitivity has not been reported for *Tospovirus* NSs proteins. A study on the NSs of *Rift Valley fever virus* (RVFV) has shown that its NSs expression is not temperature sensitive (Vaughn *et al.*, 2010). Instead, the L and M segments of RVFV may carry temperature sensitive mutations (Vialat *et al.*, 1997). Temperature sensitive mutants have been described for various other viruses as well, in which many different processes during viral replication are affected and in general mostly lead to lower virus titres at the non-permissive conditions. Examples are; the assembly of the replicase complex by the p33 and p92 proteins of *Cucumber necrosis virus* (CNV) (Pathak *et al.*, 2013), polyprotein processing by the nsp5 protein of the *Murine hepatitis virus* (Stobart *et al.*, 2012), virus core assembly of mammalian reoviruses (Lemay and Bisaillon, 2012) and DNA replication and encapsidation of the tsm5 mutant of *Murine cytomegalovirus* (Al-Ali *et al.*, 2012). Therefore, the effect of temperature sensitivity of NSs is not entirely unique, however further studies are needed to identify the mutations involved in temperature sensitivity and to gain further understanding.

The multiple sequence alignment that was performed on NSs sequences did not provide clear evidence to support certain amino acids to be essential for either RSS-activity or the ability to induce the *Tsw*-mediated HR. However, the codon at position 79 appeared to be of high importance and correlated to RSS- and AVR-activity (Chapter 3). Cases on virus resistance are reported in which only 1 or 2 mutations within the Avr-sequence suffice to break the resistance (Bendahmane *et al.*, 1995; Moffett, 2009).

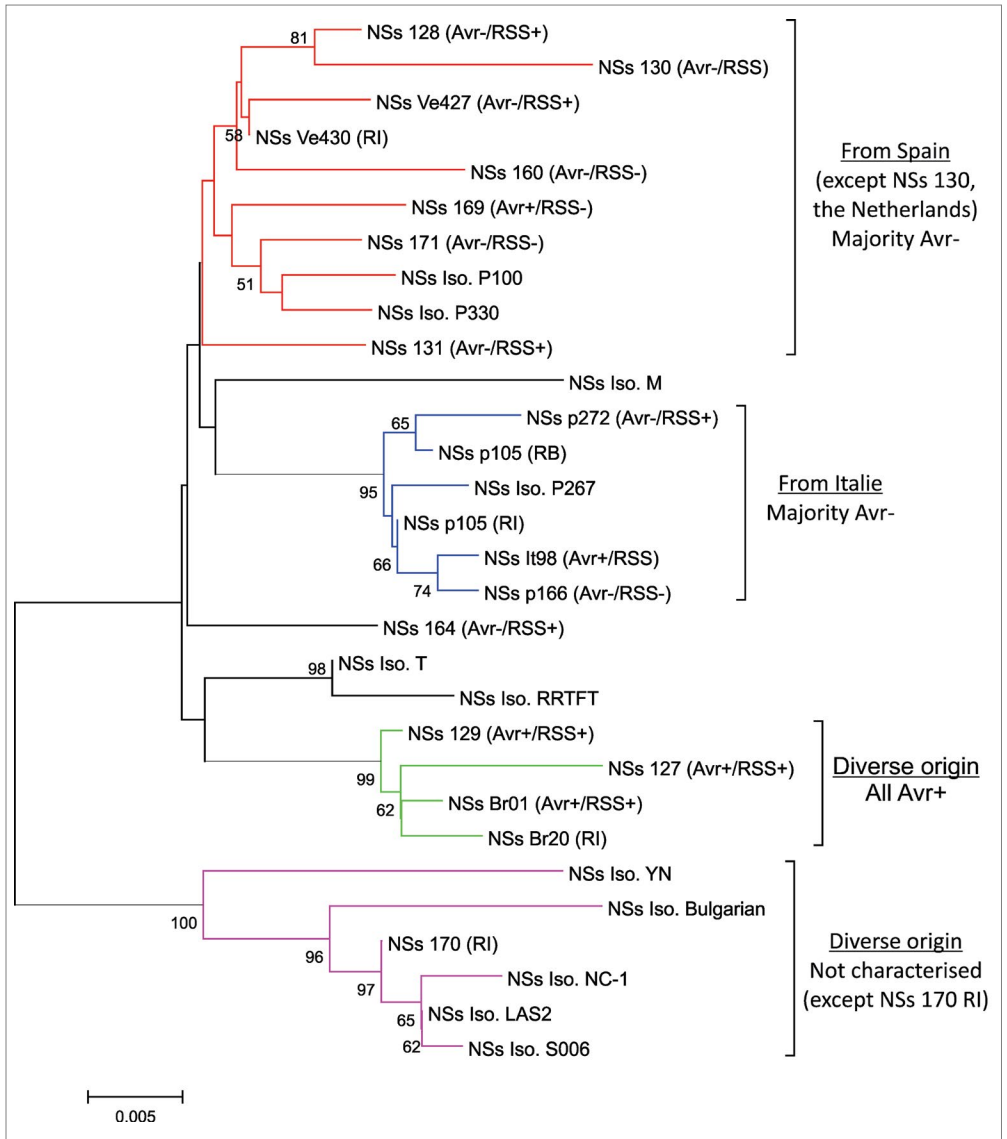


Figure 5.7 Phylogeny of NSs from TSWV RI, RB and AbsRB isolates. A phylogenetic tree was created with Mega5.1 software (Tamura *et al.*, 2011) and using data from the multiple sequence alignment of ClustalW as input. The tree was constructed using the Neighbour joining method and based on bootstrap analysis (500 replicates) with midpoint rooting. Numbers at the branches indicate the percentage of times that branch of the tree was constructed in the same way. Only values above 50% are depicted in the tree.

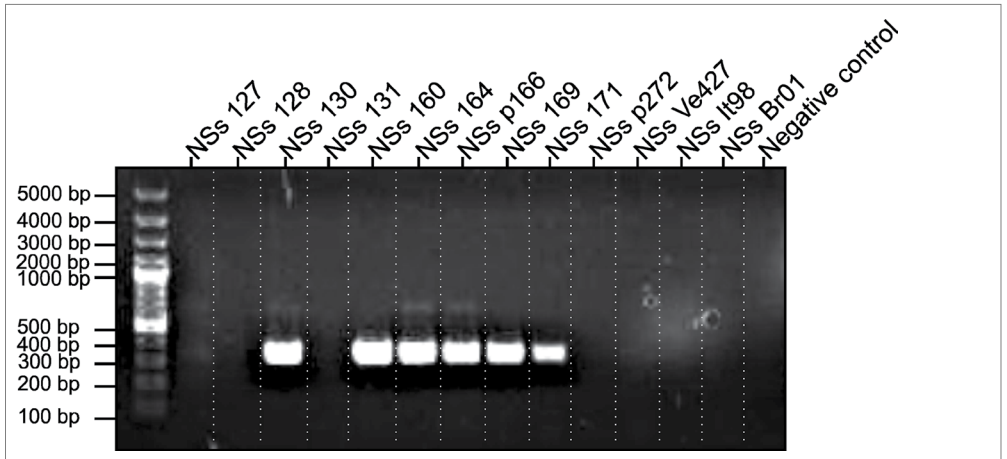


Figure 5.8 RT-PCR detection of TSWV RB isolates. A cDNA library produced from an RT-PCR on total RNA extracts from systemically infected leaves of *N. benthamiana* with various TSWV RI and (Abs)RB isolates were tested using primers '79-Fw' and '79-Rv'. Samples of NSs^{130}, NSs^{160}, NSs^{164}, NSs^{p166}, NSs^{169} and NSs^{171} are from (Abs/Temp)RB isolates while NSs^{127} and NSs^{Br01} are from RI isolates. A DNA size marker is included at the left hand side of the gel-picture.

A recent bioinformatics study on NSs sequences derived from TSWV infections in resistant *Capsicum* fields has shown that the *Tsw*-resistance gene positively selected for certain amino acids in the TSWV NSs sequence (Tentchev *et al.*, 2011). In two out of three different algorithms used to analyse NSs sequences, the amino acid residue at position 79 has been identified as a positive target for selection. Together with experimental data from Chapter 3, this strengthened the importance of I79 in triggering *Tsw*-gene mediated defence (and concomitant RNA silencing suppression). The study of Tentchev and colleagues (2011) furthermore proposed that the appearance of a resistance breaking phenotype is likely the result of multiple independent evolutionary events. This is supported by our studies, in which I79 was not the only and single amino acid that defined resistance breaking phenotypes. In those (Abs)RB isolates that still contained I79, the phenotype was caused by (a combination) of other important residues, although these are not found at the position of other important residues identified by Tentchev and co-workers (2011).

Phylogenetic analysis of the TSWV RB isolates analysed here clustered all Avr-NSs proteins into one part of the tree, while the Avr+ ones clustered together in

another part of the tree. Interestingly, the Avr- NSs proteins subdivided based on geography, into an Italian branch and a Spanish branch. Although TSWV isolate Vir130 has been isolated in the Netherlands, it clustered in the Spanish branch, likely due to the possibility that it originates from Spain as well. Most of the random Genbank NSs sequences cluster as an out-group and distinct from all characterised NSs sequences from this study, suggesting that the latter evolved under the selection pressure of the *Tsw*-resistance gene. However, no clear differentiation between the AbsRB isolates and TempRB isolates is observed from the phylogenetic analysis.

It is well known that temperature plays a modulating part in all aspects of plant life cycle. Temperature sensitivity has been described for at least a few dominant *R*-genes of different plant pathogens (de Jong *et al.*, 2002; Wang *et al.*, 2009; Zhu *et al.*, 2010), and confirmed here for the *Tsw* resistance protein. Studies performed on the temperature sensitive *R*-gene *N* from Tobacco, providing resistance against *Tobacco mosaic virus* (TMV), showed that mutations in the *R*-gene sequence provides some levels of temperature insensitivity, indicating that in this case the R-protein itself is the critical temperature sensitive part of the resistance mechanism and not the downstream signalling pathway. This is supported by the observation that the N-protein is also exhibiting temperature sensitivity when transferred to another host (tomato) (Zhu *et al.*, 2010). In addition, the R-protein no longer moves from the cytoplasm to the nucleus to signal the downstream resistance pathway at elevated temperatures, which is required for the induction of resistance and programmed cell death (Slootweg *et al.*, 2010; Zhu *et al.*, 2010). The authors hypothesised that a conformational shift of the R-protein from 'OFF' to 'ON' status is inhibited at high temperatures, and nuclear localisation probably takes place during the 'ON' status (Zhu *et al.*, 2010). Besides the temperature sensitivity of R-proteins, other defence related mechanisms are also temperature dependent. The genes *EDS1* and *PAD4*, associated with the *R*-gene class of TIR-NB-LRR, are down regulated in their expression upon increase of temperature (Zhu *et al.*, 2010). Also, the plant hormone Salicylic acid (SA), involved in systemic acquired resistance (SAR), is affected by temperature (Wang *et al.*, 2009).

Agrobacterium does not transform well at elevated temperatures (Baron *et al.*, 2001), because several Vir-proteins required for an efficient transformation of transgenes by the type IV secretion machinery are unstable at elevated temperatures (Baron *et al.*, 2001). For this reason, two additional wild *Agrobacterium* strains, able

to form tumours at 28 °C, have been used in this study: Ach5 and Chry5 (Baron *et al.*, 2001). However, when these strains were transformed with a functional GFP and NSs^{RI}, these strains did not transform the plants tested (data not shown). Furthermore, several cases have been reported in which 35S-promotor driven expression appeared temperature sensitive as well and reduces at elevated temperatures (Wang *et al.*, 2009). Both reasons could have caused failure of expression of the transgenes at 28 °C in neither *N. benthamiana* nor *C. annuum*. Although temperature seems to have a modulating effect on many biological processes, not all defence related pathways in plants are down regulated upon temperature increase. The antiviral RNA interference pathway in plants is up regulated at elevated temperatures, most likely through the modulation of DCL and RDR6 (Szittya and Burgyan, 2001; Qu *et al.*, 2005; Zhang *et al.*, 2012). The miRNA pathway, on the other hand, does not seem to be influenced by temperature (Szittya and Burgyan, 2001).

The identification of a temperature dependent resistance breaking isolates raises questions about the evolution and generation of resistance breaking isolates. Although speculative, it is very well possible that temperature adaptation generated the first resistance breaking isolates (TempRB), which later evolved into temperature independent breaker isolates (AbsRB), as the collected resistance breaking isolates from this study are all derived from subtropical regions, where high temperatures are more regular. Whether or not AbsRB isolates evolved from TempRB, knowledge about all these isolates and their evolvement will contribute to insight on the durability of the *Tsw*-gene based resistance. The availability of a diagnostic tool aids to (partially) detect the presence of resistant breaker isolates in an early stage of infection and will be of great benefit to growers that cultivate resistant *Capsicum* plants.

Materials and methods

Virus and plant material

Eleven different virus isolates of TSWV were used this study, *i.e.* Br01, Vir171, Vir128, Vir130, Vir131, Vir164, Vir169, It98, p272, p166 and Ve427 (Table 5.1). These last three isolates were described in the paper of Margaria and colleagues (2007). Virus

isolates were maintained on *Nicotiana benthamiana* by serial passaging (maximum of five times) using mechanical inoculation (de Avila *et al.*, 1993) and systemically infected leaves were stocked and frozen at -80 °C. The plants used in this study were two genotypes of *Capsicum annuum*: HK0004, a TSWV-susceptible cultivar (*Tsw*-), and YF0009, a TSWV-resistant cultivar (*Tsw+*). *N. benthamiana* was used as the host for the RNA silencing suppression assay. All plants were grown and maintained either under greenhouse conditions (24 °C with a 16 h light/8 h dark regime) or at 28 °C (16 h light/8 h dark regime) or at 32 °C (16 h light/8 h dark regime).

Amplification and sequence verification of *NSs* genes

Total RNA was isolated from (systemically infected) leaves using Trizol (Invitrogen). From the total RNA, 0.5 µg was used as a template for first-strand cDNA synthesis and subsequent polymerase chain reaction (PCR) amplification of the *NSs* gene, employing the following primer sets at an annealing temperature of 55 °C: NSs-Fw (5'-d CCGTCGACATGTCTTCAAGTGTT-3') and NSs-Rv (5'-d GGCGGCCGCTTATTTTGATCCTGAA-3'). For feasible cloning, the forward (Fw) primer contained a *Sal*I restriction site, and the reverse (Rv) primer contained a *Not*I restriction site, both at the 5' end (highlighted in italics). PCR amplification was performed using Phusion high-fidelity Taq polymerase, according to the manufacturer's procedures (Finnzymes; Thermo Scientific). Amplified DNA products were resolved on a 1% agarose gel and fragments corresponding in size to the *NSs* gene were gel purified and subsequently cloned into pJet-vector (Thermo Scientific). Positive clones were selected and verified by sequence analysis.

Cloning of NSs in destination vectors

For transient expression of TSWV NSs, the highly translatable pEAQ-HT vector system was used (Sainsbury *et al.*, 2009). To this end, coding sequences for *NSs* were re-cloned by *Sal*I/*Not*I excision from the pJet vector constructs into *Sal*I/*Not*I-digested pEntr11-ccdB (from which the *ccdB* gene was removed by *Eco*RI digestion). Positive clones were selected and verified by sequence analysis, and subsequently used for transfer of the *NSs* gene insert via an LR reaction into a Gateway (Invitrogen)-compatible pEAQ-HT-pDest1 destination vector (Sainsbury *et al.*, 2009) for the HR-induction assay and into the Gateway compatible pK2GW7 destination vector

(Karimi *et al.*, 2002) for the RNA silencing suppression assay. The clones obtained were transformed into *A. tumefaciens* 1D1249 cells (pEAQ-HT), containing helper plasmid pCH32 (Hamilton *et al.*, 1996), and into the *A. tumefaciens* LBA4404 cells (pK2GW7). An ATTA was performed to express the transgenes and the expression was verified by Western immunoblot analysis of leaf samples infiltrated on *N. benthamiana* leaves and collected at 5 dpi. The entry vector carrying the *NSs* genes were also used in an LR reaction to clone the genes into the TRV expression system (Liu *et al.*, 2002), which were subsequently transformed into *A. tumefaciens* LBA4404 cells.

Agrobacterium transient transformation assay (ATTA)

ATTA was performed according to the protocol of Bucher *et al.* (2003), with slight modifications. In brief, *A. tumefaciens* was grown overnight at 28 °C in LB3 medium containing appropriate antibiotic selection pressure. From this culture, 600 µL were freshly inoculated into 3 mL of induction medium and grown overnight. Strain *A. tumefaciens* 1D1249 (Wroblewski *et al.*, 2005) with helper plasmid pCH32 (Hamilton *et al.*, 1996) was grown under 1.25 µg/mL tetracycline selection pressure, whereas *A. tumefaciens* LBA4044 (Ooms *et al.*, 1982) was grown under 20 µg/mL rifampicin selection pressure. Additional strains used during this study were COR308 (Hamilton *et al.*, 1996), selected with 2 µg/mL tetracycline, and AGLO and AGL1 (Lazo *et al.*, 1991), selected with 20 µg/mL rifampicin and 100 µg/mL carbenicillin, respectively.

Serological detection of virus and proteins

TSWV virus was detected and titres were determined by ELISA analysis. ELISA was performed on systemically infected leaf extracts from *Capsicum* species (10 dpi) and *N. benthamiana* plants (7 dpi) in PBS-Tween buffer (1:3, w/v) in a DAS format, according to de Avila *et al.* (1993).

HR-induction assay

To test the cloned NSs constructs for their ability to elicit the *Tsw*-mediated HR response, an ATTA was performed with *A. tumefaciens* carrying the pEAQ-HT vector containing the *NSs* gene. A suspension with a final optical density at 600 nm of 1.0 was infiltrated in the leaves of the *Capsicum* plants using a needleless syringe. The results were observed at 5 dpi, with the TSWV NSs derived from the resistance

inducing isolate (NSs^{RI}) as a positive control and the TSWV NSs from the resistance breaking isolate (NSs^{RB171}) as a negative control (Chapter 2).

GFP silencing suppression assay

N. benthamiana leaves were agroinfiltrated with a functional GFP construct (Tsien, 1998) as described above, with a final optical density at 600 nm ($OD_{600\,nm}$) of 0.5 per construct. A construct expressing MBP was used as a negative control (Schnettler *et al.*, 2010), while the TSWV NSs from the Resistance inducing isolate (NSs^{RI}) was used as positive control (Chapter 2). Infiltrated leaves were monitored for GFP expression at 5 using a hand-held UV lamp. For the quantification of GFP fluorescence, leaf discs with a diameter of 1 cm were taken from the infiltrated leaf area and the number of fluorescence units was measured using a Fluorstar Optima (BMG Labtech).

TRV-infections

An ATTA was performed on *N. benthamiana* with *A. tumefaciens* carrying the TRV-replicon containing the *NSs* or *PDS* genes (Liu *et al.*, 2002). The local and systemic infected material was used at 6 dpi to mechanically inoculate the resistant *Capsicum* plants using carborundum powder. The challenged plants were incubated at 22 °C and 28 °C and were observed daily for two weeks.

Multiple sequence alignment

The sequences of the *NSs* clones that were obtained were aligned using the CLUSTALW algorithm. The NSs sequences described by Margaria and colleagues (2007) in relation to *Tsw*-mediated resistance (NSs Br20-RI, NSs Ve430-RI, NSs 170-RI, NSs p105-RI and -RB) were included, together with a set of random NSs sequences, derived from Genbank: Br20-RI-NSs: accession number DQ915948, Ve430-RI-NSs: accession number DQ376184, p170-RI-NSs: accession number DQ431237, p105-RI-NSs: accession number DQ376178, p105-RB-NSs: accession number DQ915946, Bulgaria-NSs: Genbank accession number P26003, M-NSs: accession number AY870391, T-NSs: accession number AY870392, LAS2-NSs: accession number FR692831, NC-1-NSs: accession number AY744476, P100-NSs: accession number FR692840, RRTFT-NSs: accession number FR693030, S006-NSs: accession number FR693032, YN-NSs: accession number JF960235, P267-NSs: accession number DQ376180, P330-NSs: accession number HE600702.

Alignments of NSs were edited using the BioEdit program (Hall, 1999).

Phylogeny

The NSs sequences were used to create a phylogenetic tree using the Mega5.1 software, in which the Neighbour joining method was used to create the tree. Tree was constructed with the P-distance model and was bootstrapped 500 times, of which the values in the tree represents the number of times (in %) that branch of the tree was reconstructed in the same way, only values above 50 % are depicted in the tree.

RNA isolation, RT-PCR and subsequent PCR-screen

TSWV isolates used in this study were challenged on *Nicotiana benthamiana* plants and at 10 days post infection (dpi), systemically infected leaf material was collected and RNA was extracted using Trizol (Invitrogen). From the total RNA, 0.5 µg was used as a template for first-strand cDNA synthesis using a reverse primer designed on the 3'-end of the *NSs* gene: NSs-Rv 5'-dTTATTTTGATCCTGAAGCATACGC-3'. The quality of the cDNA template was initially checked by performing a PCR using NSs-start and –stop primers: NSs-Fw 5'-dATGTCTTCAAGTGTTTATGAGTCG-3; NSs-Rv 5'-dTTATTTTGATCCTGAAGCATACGC-3'. The PCR-screen was performed by using the following designed primers: Screening-residue-79-Fw 5'-dTCTCAGCATGTGTATAC-3' and Screening-residue-79-Rv 5'-dGAACATTCACTTTGCCT3'. An annealing temperature of 59 °C was used to get the most optimal result. The amplified DNA was loaded on a 1 % agarose gel to visualize the product size.

Acknowledgements

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Chapter 6

General discussion

The work presented in this thesis aimed at the identification of the TSWV Avr determinant that triggers the single dominant *Tsw* resistance (*R*) gene. Conflicting papers on this issue had appeared (Margaria *et al.*, 2007; Lovato *et al.*, 2008), leaving the issue unsettled. The identification of the *Avr* gene would allow its characterisation in view of *R*-gene triggering and its viral function(s), but also would assist in development of diagnostic markers to monitor resistance breaking (RB) isolates and detect the presence of yet undiscovered new *Tsw*-breaking pathotypes.

The results obtained during this research unambiguously identified TSWV-NSs as Avr-determinant of *Tsw*-mediated resistance. This protein was earlier identified as viral RNA silencing suppressor (Takeda *et al.*, 2002; Bucher *et al.*, 2003) and the absence of this functionality in NSs from TSWV RB isolates suggested a tight link between both. However, a large NSs mutant screen indicated that these two functions could be uncoupled and furthermore indicated a putative interaction with AGO1 via a GW/WG motif in the N-terminal part of NSs. Based on these results combined with information reported in literature, a model is proposed to position NSs in the 'Zig-zag-model' of Dangl and Jones (2006), indicating the multifunctional modes by which NSs is able to counteract and modulate host defence responses (Figure 6.1).

According to this model the first response of the host as an attempt to clear viral invasion consists of RNA silencing that is triggered by viral dsRNA molecules. The latter may originate from dsRNA replicative intermediates or secondary RNA folding structures of the viral RNA. After processing of these into siRNAs by the RNAi machinery the siRNAs, after being uploaded into RISC, assist in the sensing and subsequent degradation of viral RNA target molecules by AGO1, the core component of RISC. The TSWV NSs protein is able to bind long dsRNA as well as small (si- and mi-) RNAs and thereby prevents their cleavage by DCL and uploading into RISC, respectively (Schnettler *et al.*, 2010). In view of this observation, it is most likely that NSs initially binds and protects the dsRNA hairpin structure within the 3'- untranslated regions (UTRs) of the M- and S-RNA derived subgenomic mRNA molecules (van Knippenberg *et al.*, 2005) to prevent these from cleavage by DCL. This is in agreement with the observation that siRNAs were found to originate from most of the S-RNA sequence of *Tomato yellow ring virus* (TYRV) *Tospovirus*, with the exception of the hairpin encoding intergenic region from where hardly siRNAs were found (Hassani-Mehraban, PhD thesis 2008). Further support for NSs binding to the

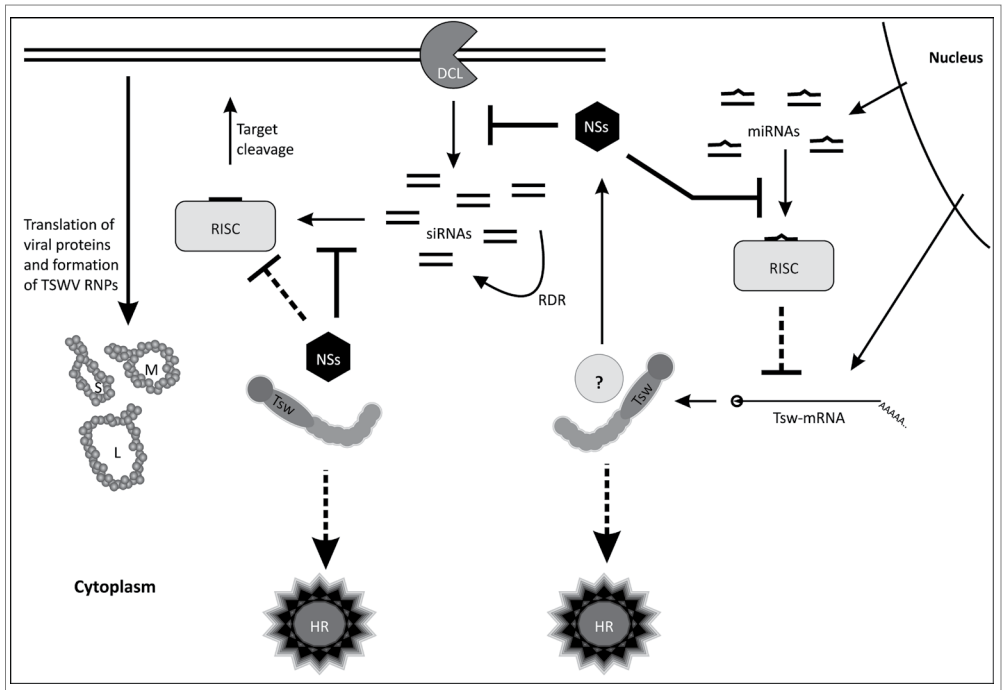

Figure 6.1 Model of NSs-functioning in its role as RSS and Avr-determinant. The model depicts TSWV-NSs as RSS; through its affinity to bind long dsRNA; thereby blocking the cleavage by DCL, its affinity for siRNAs; blocking their upload into RISC. Also, the putative interaction with AGO1 (RISC) directly is shown (dashed-inhibition line), which acts as a guardee in this model, triggering *Tsw*-mediated resistance leading to downstream HR. Additionally, the affinity of NSs for miRNAs is shown, blocking their upload into RISC, possibly inhibiting AGO-mediated translational arrest of *Tsw*-mRNA, leading to enhanced expression of the *R*-gene. DCL: Dicer-like protein, dsRNA: double stranded RNA, HR: Hypersensitive response, miRNAs: microRNAs, mRNA: messenger RNA, RDR: RNA dependent RNA polymerase, RISC: RNA-induced silencing complex, RNP: Ribonucleocapsid protein, siRNA: small interfering RNA, TSWV: *Tomato spotted wilt virus*.

predicted hairpin structure has been obtained from *in vivo* reporter translation studies, in which NSs was shown to enhance translation of a luciferase reporter gene containing the hairpin structure encoding 3' UTR (Geerts-Dimitriadou *et al.*, 2012). Binding of NSs to the predicted hairpin thus may serve two purposes, the first one is to protect viral mRNAs from degradation and the second one is to act as

a functional analogue of poly-A-tail binding protein (PABP) and stimulate translation of the viral mRNAs. Short-interfering RNAs that are generated from viral mRNAs will also be sequestered by NSs to prevent their uploading into RISC and reduce their involvement in secondary siRNA amplification (and transitive silencing).

In light of its affinity to (short and long) dsRNA, NSs may also be able to bind to the panhandle structure, which is formed by complementarity of the 3' and 5' UTRs of each segment. This is being supported by the observation that purified RNPs, in comparison to purified virus particles, were enriched for NSs (van Knippenberg, PhD thesis 2005). The presence of NSs at the genomic RNA panhandle structure would not only prevent it from becoming recognised by the DCL, but possibly could also mask recognition of its 5' terminal-end. TSWV genomic RNAs contain a 5'-ppp that in mammals, as shown with several animal-infecting RNA viruses (*e.g. Influenza A virus* (FLUAV), *Vesicular stomatitis virus* (VSV), *Rift Valley fever virus* (RVFV) (Rehwinkel and Reis e Sousa, 2013)) is recognised as a PAMP by RIG-I, a cytosolic NLR receptor. Activation of RIG-I leads to a downstream signalling and interferon production to mount an antiviral immune response (Bowie and Unterholzner, 2008). In contrast to this observation, it has been shown that several negative strand RNA viruses (*e.g. Hantaan virus* (HTNV), *Crimean-Congo hemorrhagic fever virus* (CCHFV), *Borna disease virus* (BDV)), including some members of the *Bunyaviridae* family, actively remove part of the 5'-ppp group, leaving a mono-phosphate group behind that can no longer be bound by RIG-I, thereby escaping recognition (Habjan *et al.*, 2008).

In this respect, it is interesting to note that the NSs from *Groundnut bud necrosis virus* (GBNV), another distinct *Tospovirus*, possesses NTPase, dATPase and 5' RNA/DNA phosphatase activity (Lokesh *et al.*, 2010). The catalytic Walker motifs (A and B) observed in GBNV are indicative for these activities. However, these motifs are not present in TSWV-NSs. Besides a removal of the 5'-ppp from genomic RNAs, the phosphatase activity of NSs could also be involved in the removal of phosphate groups from small siRNAs, thereby inactivating these for further processing and uploading into RISC (Zamore, 2004; Lokesh *et al.*, 2010).

In addition to the affinity of NSs for RNA molecules, NSs may likely exert RSS activity at the level of proteins as well (*i.e.* targeting key-enzymes in the RNAi machinery). The observed loss of RSS and Avr-activity after mutation of a GW/WG motif in NSs, in other viral proteins shown to be required for binding to AGO1, strongly supports the biological relevance of this motif. Whether binding of NSs to

the core component AGO1 prevents uploading of siRNAs and/or further maturation of RISC, as described for CMV-2b and SPMMV-P1 (Zhang *et al.*, 2006; Giner *et al.*, 2010), or triggers its degradation, as shown for P0 from *Polerovirus* and *Enamovirus* and PVX-P25 (Baumberger *et al.*, 2007; Chiu *et al.*, 2010; Fusaro *et al.*, 2012), remains to be further investigated. Interestingly, the GW/WG-motif is only found in the NSs sequence of members within the American *Tospovirus* clade, but not in those belonging to the Eurasian clade.

Studies have shown that *R*-genes are under translational control of miRNAs families (Li *et al.*, 2012; Shivaprasad *et al.*, 2012). TSWV NSs has affinity for miRNAs, suggesting that during a TSWV infection, NSs could relieve the translational arrest of miRNAs on *R*-gene translation, leading to enhanced *R*-gene product and subsequent HR and resistance upon Avr-recognition. Additionally, a recent study on *R*-genes showed that enhanced *R*-gene expression can lead to auto-immunity (Xia *et al.*, 2013).

From the animal-infecting bunyaviruses, members of the *Phlebovirus* and *Orthobunyavirus* genus also encode an NSs protein from the S-RNA. In mammals, the NSs proteins from the orthobunyaviruses *Bunyamwera virus* (BUNV) and *La Crosse virus* (LACV) and the *Phlebovirus Rift valley fever virus* (RVFV) are shown inhibit the type I IFN response by blocking RNA polymerase II transcription in order to shut off the antiviral defence genes, although they do this in different ways (Weber *et al.*, 2002; Billecocq *et al.*, 2004; Thomas *et al.*, 2004; Hollidge *et al.*, 2011). The RVFV NSs additionally induces specific degradation of dsRNA-dependent protein kinase (PKR; (Habjan *et al.*, 2009)), a process that occurs independently from the NSs-mediated blocking of host gene transcription (Kalveram *et al.*, 2011). Recently, several hantaviruses were also reported to contain an open reading frame (ORF), like the orthobunyaviruses, and overlapping the *N*-gene that encodes an NSs protein with weak IFN antagonistic properties (Jääskeläinen *et al.*, 2007). A closer look at NSs from the animal infecting bunyaviruses did not reveal the presence of a GW/WG-motif, although studies from viruses infecting members of different kingdoms showed that interaction with AGO proteins not necessarily requires a GW/WG-motif (*e.g. Cucumber mosaic virus* (CMV) 2b-protein, *Noravirus* VP1-protein and *Cricket paralysis virus* (CrPV) 1A-protein (Zhang *et al.*, 2006; van Mierlo *et al.*, 2012)). Since all bunyaviruses are arthropod borne, with the exception of hantaviruses, and replicate in the insect vector, they all encounter antiviral RNAi.

For this reason, bunyaviruses, irrespective of plant- or animal infecting, likely suppress antiviral RNAi in insects as well. However, studies performed on the insect vectors of bunyaviruses showed that while infected none suffered a fitness penalty, in contrast to the animal/plant host. Recent work done on RVFV in three different insects cell lines showed that these are capable of mounting a potent RNAi response against the virus, leading to a persistent infection (Léger *et al.*, 2013), while another study on this virus in whole mosquitoes showed an important role of both non-structural proteins NSs and NSm (Crabtree *et al.*, 2012). In addition, the requirement of *Bunyawera* (BUNV) NSs for a successful infection in both mosquito cell lines and whole mosquitoes was also recently confirmed (Szemiel *et al.*, 2012). On this point it is interesting to note that contradicting reports on the RSS activity of *La Crosse virus* (LACV) NSs in insect cells have appeared (Soldan *et al.*, 2005; Blakqori *et al.*, 2007). Future studies are needed to resolve the mode of action by which animal- and plant-infecting bunyavirus NSs proteins suppress innate immune signalling pathways in their shared insect vectors.

TSWV resistance breaking isolates

Besides TSWV RI and RB isolates a third class of so called temperature-dependent resistant breakers (TempRB) has been identified and characterised to a limited extent (Chapter 5). Although these viruses triggered *Tsw*-mediated HR in *Capsicum* at standard temperature conditions, transient expression of their NSs proteins surprisingly did not induce *Tsw*-mediated HR. Similarly, varying degrees of RSS activity were observed between these proteins. A temperature shift assay using TSWV isolates indicated that *de novo* synthesised NSs triggers the resistance, rather than available NSs. This implied that protein folding could play a role, and mature NSs already folded/involved in a complex is hindered and not able to properly (re-)fold into a functional structure. In terms of the resistance model proposed it could be imagined that NSs from the TempRB resistance breaker isolates still interacts with AGO1, due to the presence of the GW/WG motif, but due to a subtle change in its folding structure somehow is unable to release or revert in a functional format to induce *Tsw*-mediated resistance. In support for this folding-based hypothesis is the observation that RSS and avirulence within NSs are not functionally linked (Chapter 3), but likely have overlapping structural requirements.

As such, NSs affected in RSS activity may exhibit an (partially) altered Avr-phenotype.

The thrips transmissibility of all resistance breaker isolates used in this study have not been analysed. However, considering that these isolates have been collected from the (*Capsicum*) field, indicates that these isolates can be successfully acquired and spread by thrips. It is not unlikely that the resistance, combined with thrips transmissibility, may have contributed to the evolvement and selection of TSWV resistance breaker isolates without losing their fitness to infect the host. After all, if the mutations required to overcome the resistance would lead to a reduced fitness, such isolates would rapidly be outcompeted by other (wild type/RI) isolates. All RB isolates used in this study were shown to be similarly fit as the resistance inducer isolate (under non *R*-gene conditions; Chapters 2 and 5). It remains to be investigated whether, and if so, how these (resistance breaking) isolates counteract antiviral innate immune responses (amongst others RNAi) in their thrips vector.

To prevent mayor crop loss due to resistance breaking viruses it is of critical importance to identify the presence of resistance breaking viruses at an early stage. With the knowledge obtained from the RI and (Temp)RB isolates analysed in this study a diagnostic RT-PCR tool was developed based on amino acid residues identified to be of importance in light of resistance breaking. Although this tool did not score positive on any of the TSWV RI-isolates, a few RB isolates still escaped from detection. This requires not only further improvement on the quality of the diagnostic tool, but also if there is a genetic basis of the temperature-dependent behaviour of RB-isolates or not.

Outlook

The data collected in this thesis described the role of the TSWV NSs protein in triggering *Tsw*-mediated HR and the suppression of RNAi. The antiviral response in plants against viruses consist of RNAi and *R*-gene mediated defence and although the two mechanisms seem to be separate layers of response, evidence linking the two pathways have been described (Bhattacharjee *et al.*, 2009; Li *et al.*, 2012). In future work the multi-functionality of NSs need to remain the focus of research, identifying additional roles of NSs in either the suppression of defence or its role in (enhanced viral) translation, as suggested by Geerts-Dimitriadou (2012). Also, the role of NSs in its vector will be of interest, as studies on the function of NSs during

the infection in its thrips-vector have hardly been performed. As all bunyaviruses are spread by arthropods and all are able to systemically infect their vector, it will be interesting to find out if NSs from all these viruses (plant- and animal-infecting) counteract the innate immune response in a similar way, which could be anticipated due to a common ancestor. This will be one of the challenging questions to resolve for the future.

Appendices

I. References

II. List of abbreviations

III. Summary

IV. Samenvatting

V. Nawoord

VI. About the author

VII. My publications

VIII. EPS certificate

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List of abbreviations

Aa:	Amino acids
AbsRB:	Absolute resistance breaker
AGO-protein:	Argonaut protein
ARC:	Apaf1 – R-protein – CED4
ATP:	Adenosine triphosphate
ATTA:	*Agrobacterium* transient transformation assay
Avr-determinant:	Avirulence determinant
BDV:	*Borna disease virus*
BiFC:	Bimolecular fluorescence complementation
BIR:	Baculovirus inhibitor-of-apoptosis repeat
bp:	base-pairs
BUNV:	*Bunyamwera virus*
C-terminus:	Carboxyl-terminus
CaMV:	*Cauliflower mosaic virus*
CARD:	Caspase-activation and recruitment domain
CAV:	*Chicken anaemia virus*
CC:	Coiled-coil
CCHFV:	*Crimean-Congo hemorrhagic fever virus*
CMV:	*Cucumber mosaic virus*
CNV:	*Cucumber necrosis virus*
Co-IP:	Co-immunoprecipitation
CymRSV:	*Cymbidium ringspot virus*
DCL-protein:	Dicer-like protein
DNA:	Deoxyrobonucleic acid
dpa:	days-post agroinfiltration
dpi:	days-post infection
dsDNA:	double stranded DNA
dsRNA:	double stranded RNA
e.g.:	*exempli gratia* (Latin for 'for example')
eIF:	eukaryotic translation initiation factor
ELISA:	Enzyme-linked immunosorbent assay
EMSA:	Electrophoretic mobility shift assay
ER:	Extreme resistance
et al.:	*et alii* (Latin for 'and others')
ETI:	Effector-triggered immunity
ETS:	Effector triggered susceptibility
FLIM:	Fluorescence-lifetime imaging microscopy
FLUAV:	*Influenza A virus*
FRET:	Förster resonance energy transfer
Fw:	Forward
G/D:	Guardee/Decoy
GBNV:	*Groundnut bud necrosis virus*
GFP:	Green fluorescent protein
GRSV:	*Groundnut ringspot virus*
HR:	Hypersensitive response
HTNV:	*Hantaan virus*
i.e.:	*id est* (Latin for 'that is')
JA:	Jasmonic acid
LACV:	*La Cross virus*
LRR:	Leucine rich repeat
MAMPs:	Microbe associated molecular patterns
MAPK:	Mitogen-activated protein kinase
MBP:	Maltose binding protein
MHV:	*Murine hepatitis virus*
miRNA:	micro-RNA
mRNA:	messenger-RNA
N-protein:	nucleocapsid protein
N-terminus:	amino-terminus
NACHT:	NAIP – CIITA - HET-E - TP1 domain
NB:	nucleotide binding domain
NF-κB:	nuclear factor kappa B
NHR:	Non-host resistance
NLR:	Nucleotide binding and leucine rich repeat proteins
NO:	Nitride oxide
NSm-protein:	Non-structural protein from medium segment

NSs-protein:	Non-structural protein from small segment
nt:	nucleotides
$O.D._{600nm}$:	Optical density at 600 nm
PABP:	Poly(A)-tail binding protein
PAMPs:	Pathogen associated molecular patterns
PCD:	Programmed cell death
PlAMV:	*Plantago asiatica mosaic virus*
PR-genes:	pathogenesis related genes
PRR:	Pattern recognition receptors
PTGS:	Post transcriptional gene silencing
PTI:	PAMP triggered immunity
PVX:	*Potato virus X*
PVY:	*Potato virus Y*
PYR:	Pyrin domain
R-genes:	Resistance genes
RB:	Resistance breaker
RdDM:	RNA directed DNA methylation
RDR:	RNA dependent RNA polymerase
RHBV:	*Rice hoja blanca virus*
RI:	Resistance inducer
RIG-I:	retinoic acid-inducible gene I
RISC:	RNA-induced silencing complex
RLRs:	RIG-I like receptors
RNA:	Ribonucleic acid
RNAi:	RNA interference
RNPs:	Ribonucleocapsid proteins
ROS:	reactive oxygen species
RSS:	RNA silencing suppressor
RT-PCR:	Reverse transcriptase – polymerase chain reaction
Rv:	Reverse
RVFV:	*Rift Valley fever virus*
S-gene:	Susceptibility gene
SA:	Salicylic acid
SAR:	Systemic acquired resistance

SDS-PAGE:	Sodium dodecyl sulfate –polyacrylamide gel electrophoresis
SHR:	Systemic HR
siRNA:	small interfering-RNA
SPFMV:	*Sweet potato feathery mottle virus*
SPMMV:	*Sweet potato mild mottle virus*
TAV:	*Tomato aspermy virus*
TBSV:	*Tomato bushy stunt virus*
TCSV:	*Tomato chlorotic spot virus*
TCV:	*Turnip crinkle virus*
TempRB:	Temperature dependent resistance breaker
TIR:	Toll and interleukin-1 receptor
TLR:	Toll-like receptors
TMGMV:	*Tobacco mild green mosaic virus*
TMV:	*Tobacco mosaic virus*
TRV:	*Tobacco rattle virus*
TSWV:	*Tomato spotted wilt virus*
TYLCV:	*Tomato yellow leaf curl virus*
TYRV:	*Tomato yellow ring virus*
UTR:	Untranslated region
UV:	Ultraviolet light
VPg:	Virus-protein genome-linked
VSV:	*Versicular stomatitis virus*
Y2H:	Yeast Two-hybrid

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English Summary

Resistance in *Capsicum* against the *Tomato spotted wilt virus* (TSWV), type species of the *Tospovirus* genus within the *Bunyaviridae* family, employs the single dominant resistance gene *Tsw*. This resistance has meanwhile been broken by resistance breaking (RB) TSWV isolates and is causing increasing problems in many different (*Capsicum* cultivating) countries. The research described here aimed to identify and characterise the viral protein triggering *Tsw* resistance and provide further insight into the mechanism of *Tsw*-mediated resistance. Knowledge gained from the genetic and phenotypic characterisation of *Tsw*-resistance breaking isolates was used to develop diagnostic markers for detection of *Tsw*-breaking pathotypes in field cultivations.

The NSs RNA silencing suppressor (RSS) protein was identified as the avirulence determinant of *Tsw*-mediated resistance (Chapter 2). While the NSs protein from the TSWV resistance inducer (RI) isolate was active as RNA silencing suppressor and avirulence determinant, the NSs protein from two different TSWV RB isolates lacked both functions as evidenced from transient assays. Surprisingly, the corresponding resistance breaking virus isolates still exhibited RNAi suppressor activity. None of the other viral proteins were able to aid in the transient recovery of RSS activity. Electrophoretic mobility shift assays (EMSAs) using plant extracts containing transiently expressed NSs proteins showed a shift of siRNAs with NSs^{RI}, indicative for binding, but not with NSs^{RB}. In agreement with the local leaf RSS assays using a virus infection, plant extracts of virus infected leaves were able to shift the siRNAs, showing recovery of the RSS activity during virus infection.

The linkage of RNAi suppression and avirulence in NSs was further investigated by mutational analysis (Chapter 3). A large set of NSs mutants was generated using alanine substitutions of authentic TSWV NSs amino acids and was tested for their ability to trigger *Tsw*-mediated HR and ability to suppress RNAi. These assays showed that the N-terminal domain of NSs carried most important residues involved with both activities. However, single mutations could be introduced that disrupted one function, while maintaining the other one and vice versa indicating that RSS activity and avirulence were not functionally linked. Swapping of domains between NSs^{RI} and NSs^{RB} not only confirmed the importance of the N-terminal domain but also the specificity within the TSWV species, since domain swaps between NSs^{RI} and NSs

from GRSV, a related but distinct *Tospovirus*, could not transfer the Avr phenotype to GRSV. Mutation of a GW/WG-motif in the N terminal region of NSs^{RI} lead to a loss of both functions and indicated that this motif, known to be involved in AGO1 interaction of other viral RSS, was of biological relevance for TSWV NSs.

The putative interaction of AGO1 and NSs was investigated by using different approaches to co-immuno precipitate (Co-IP) on transiently co-expressed tagged-AGO1 and (His-)NSs (Chapter 4). Initial indications for such interaction were obtained, however further support for this putative interaction will have to come from complementary experiments, *e.g.* Yeast-2-hybrid (Y2H), FRET-FLIM or BiFC.

Several additional TSWV isolates were analysed that besides the known resistance inducing- and resistance breaking-phenotype showed a temperature-dependent phenotype (Chapter 5). Isolates classified to this type exhibited an RI phenotype at standard greenhouse conditions (~22 °C) while at elevated temperatures (≥28 °C), but still below temperatures that inactivated the *R*-gene product (≥31 °C), were able to break the resistance. Virus challenging assays at various conditions indicated that induction of *Tsw* resistance at a lower temperature by these so called temperature dependent resistance breaking isolates (TempRB) involved de novo synthesis of the avirulence protein, *i.e.* NSs, and that protein folding might play a role. NSs proteins cloned and expressed from this additional new set of TSWV resistance inducing, resistance breaking and temperature dependent resistance breaking isolates revealed variable results regardless of their corresponding virus phenotype, when tested for their ability to induce Tsw-mediated HR and suppress RNAi at normal greenhouse conditions (22 °C). However, similar assays to analyse their activity at the elevated temperature (28 °C) failed when using *Agrobacterium* mediated transient assays. So far, the mechanism of temperature dependency has not been clarified yet and needs further investigation. Using the information obtained, a diagnostic tool was developed to screen for the potential presence of resistance breaking isolates of TSWV using reverse transcription-polymerase chain reaction amplification (RT-PCR). A primer set was designed targeting an important codon at aa position 79 and showed to be able to distinguish RB-isolates from RI-isolates. However, a few RB-isolates still escaped from detection indicating the limited and conditional use of this tool.

In summary, NSs has been identified as Avr-determinant of *Tsw*-mediated resistance, but this function is not tightly linked to its RNAi suppressor-activity.

Preliminary data indicate a putative interaction between AGO1 and NSs. Besides the typical RI and RB phenotypes, a third phenotypic class of TSWV isolates has been identified that exhibits a temperature dependency on triggering Tsw-mediated resistance and possibly involves an altered protein folding of NSs. A diagnostic tool has been developed to detect resistance breaking isolates in the field based on RT-PCR, but this tool still allows for escapes of RB isolates. The results on NSs are discussed in light of its role as effector within the 'Zig-zag-model' of plant host defence responses. Finally, TSWV NSs is briefly discussed and compared to the animal-infecting (NSs) paralogs of the Bunyaviridae family, also in light of functional and structural homologies between the sensors of innate immunity in plant (*R*-genes) and animal (NLRs/TLRs) cell systems.

Samenvatting

Resistentie in *Capsicum annuum* (Paprika) tegen het tomatenbronsvlekkenvirus [Engels: *Tomato spotted wilt virus* (TSWV)] verloopt via het dominante gen *Tsw*. Deze resistentie is inmiddels doorbroken door verschillende TSWV-isolaten en die veroorzaken ernstige schade aan paprika planten in de hele wereld. Het onderzoek dat hier is uitgevoerd beschrijft het virale activator-eiwit, dat de resistentie aanschakelt, en verschaft nieuwe inzichten in het mechanisme van *Tsw*-resistentie. De opgedane genetische en fenotypische kennis van verschillende *Tsw*-doorbrekende TSWV-isolaten is gebruikt voor de ontwikkeling van een diagnostische toets om doorbrekende isolaten in het veld te kunnen identificeren.

De basale afweer in planten tegen virussen verloopt via RNAi (RNA-interferentie) en in het verleden was reeds aangetoond dat het NSs-eiwit van TSWV die afweer remt door o.a. binding van kleine RNA moleculen. In de huidige studie is aangetoond dat TSWV-NSs de activator van *Tsw*-resistentie is (avirulentie-factor, Hoofdstuk 2). Lokale expressie van NSs-eiwitten in planten van een resistentie-inducerend TSWV-isolaat liet actieve remming van RNAi en activering van resistentie zien, terwijl de NSs eiwitten van twee verschillende resistentie-doorbrekende TSWV-isolaten beide activiteiten niet bezaten. Daarentegen waren de resistentie-doorbrekende TSWV-isolaten zelf wel actief als remmer van RNAi. Van geen van de andere virale eiwitten is aangetoond dat ze konden bijdragen aan de RNAi-remmings activiteit van NSs uit de resistentie-doorbrekende TSWV isolaten. Een ‘Electrophoretic mobility shift assay (EMSA)’ is uitgevoerd welke liet zien dat in de plant lokaal aangemaakte NSs eiwit van de resistentie-inducerende TSWV-isolaten nog in staat is tot het binden van siRNA (‘short-interfering RNA’), terwijl de NSs eiwitten van resistentie-doorbrekende isolaten dit niet bleken te kunnen. Daarentegen, en verassenderwijs, waren diezelfde NSs eiwitten na aanmaak tijdens een reguliere infectie met de resistentie-doorbrekende isolaten wel in staat tot het binden van siRNAs, hetgeen hun RNAi-remmer-activiteit verklaart.

Een mutatie-analyse van NSs werd uitgevoerd om te onderzoeken of er een koppeling bestaat tussen de RNAi-remmings-activiteit en de activering van *Tsw*-resistentie (Hoofdstuk 3). Er werd een grote hoeveelheid NSs-mutanten gemaakt, welke zijn getoetst op de mogelijkheid tot het activeren van *Tsw*-resistentie in paprika en het remmen van de RNAi response. Deze experimenten hebben

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laten zien dat het amino-terminale deel van het NSs-eiwit betrokken is bij beide functies. Omdat het mogelijk was om één functie uit te schakelen terwijl de andere functie intact bleef, en *vice versa*, kon worden aangetoond dat de twee functies niet functioneel gekoppeld zijn. Ook de uitwisseling van domeinen van een NSs uit een resistentie-inducerend TSWV-isolaat met domeinen van een NSs uit een resistentie-doorbrekend isolaat liet zien dat het amino-terminale deel van het NSs-eiwit belangrijk is voor beide functies. Daarnaast liet de uitwisseling van domeinen tussen verschillende soorten virussen binnen het genus *Tospovirus* zien dat de activiteit niet kan worden overgedragen aan een NSs van een ander *Tospovirus*. Mutatie van een specifiek domein (WG/GW) in het amino-terminale deel van TSWV NSs liet zien dat dit domein mogelijk een rol speelt in de interactie met het AGO1-eiwit, zoals ook voor andere RNAi remmers is aangetoond.

De mogelijke interactie tussen TSWV NSs en AGO1 werd verder onderzocht door middel van immunoprecipitatie-studies, gebruikmakende van antilichamen tegen specifieke eiwitten (Hoofdstuk 4). Er zijn indicaties gevonden voor een mogelijke interactie, maar toepassing van andere technieken zal moeten uitwijzen of deze interactie inderdaad plaatsvindt.

Additionele TSWV-isolaten zijn geanalyseerd, welke een temperatuurs-afhankelijkheid lieten zien met betrekking tot het activeren van *Tsw*-resistentie (Hoofdstuk 5). Deze groep TSWV-isolaten gedroegen zich als resistentie-inducerende isolaten bij standaard kastemperaturen (22 °C), maar hadden het fenotype van een doorbrekend isolaat bij hogere temperaturen (28 °C), waarbij de *Tsw*-resistentie nog steeds actief was (≤31 °C). Virus infecties bij verschillende temperaturen lieten zien dat de resistentie bij kastemperaturen geactiveerd kon worden door *de novo* (nieuw geproduceerd) NSs-eiwit, en dat de vouwing van het NSs eiwit een essentiële rol speelt in de herkenning. Gekloneerde en getoetste NSs uit de verschillende TSWV isolaten lieten variabele resultaten zien met betrekking tot de RNAi-remmingsactiviteit en de activering van *Tsw*-resistentie. De activiteit van de verschillende NSs-eiwitten bij een hogere temperatuur (28 °C) kon niet worden getoetst vanwege technische beperkingen van het expressie-systeem. Het onderliggende mechanisme van de temperatuurgevoeligheid is nog niet opgehelderd en behoeft verder onderzoek. Met behulp van de verkregen informatie van de verschillende gekloneerde TSWV-*NSs*-genen is een diagnostische toets ontwikkeld om resistentie-doorbrekende isolaten te kunnen identificeren in het veld door middel van RT-PCR. Een specifieke

set van primers (korte DNA-sequenties) werd ontwikkeld, welke een essentiële sequentie in het *NSs*-gen kon detecteren, en waarmee de resistentie-doorbrekende isolaten onderscheiden konden worden van de resistentie-inducerende isolaten. Sommige resistentie-doorbrekende isolaten werden echter (nog) niet opgepikt.

Samenvattend, TSWV-NSs is geïdentificeerd als avirulentie factor van *Tsw*-resistentie en lijkt te zijn ontkoppeld van de RNAi-remmings-functie van TSWV-NSs. De eerste aanwijzingen zijn gevonden dat NSs en AGO1 een interactie met elkaar aangaan. Naast de resistentie-inducerende en de resistentie-doorbrekende isolaten is er een derde fenotype geïdentificeerd, dat afhankelijk is van de temperatuur om de resistentie te doorbreken, mogelijk door een andere eiwitvouwing. Een diagnostische toets is ontwikkeld die het mogelijk maakt om in het veld resistentie-doorbrekende isolaten te kunnen identificeren. TSWV-NSs kan als een 'effector'-eiwit worden geplaatst in het 'Zig-zag-model' dat de wapenwedloop tussen gastheer en pathogeen beschrijft. Tenslotte werd TSWV NSs vergeleken met andere leden van de virusfamilie *Bunyaviridae*, die ook een NSs eiwit coderen, en werden vergelijkingen getrokken tussen het dierlijke immuunsysteem en de antivirale afweer in planten.

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Dick L. De man met de gouden handen. Alles, alles wat ik je gaf om te doen in het lab lukte. Zelfs als ik, gek van frustratie, maar aan jou vroeg om een construct te maken, lukte het je. Gelukkig lukte het meeste wat ik zelf wilde doen wél, maar zonder jouw inzet had ik nooit zoveel data kunnen verzamelen. Zeker in de laatste

jaren heb je ontzettend veel werk voor mij verzet en daar ben ik je zeer dankbaar voor. Met je droge humor en je tomeloze inzet en energie ben je een voorbeeld voor iedereen in het lab. Ze komen niet voor niets allemaal bij jou voor hulp. Nog een paar jaar en dan mag je van je pensioen genieten, maar het lab zal je missen. Bedankt!

Jan. Als onderdeel van de STW-groep was je er altijd bij. Bedankt voor je input tijdens werkbesprekingen en project meetings. Jouw frisse blik op de zaak was af en toe wel nodig.

Ook de overige stafleden van Virologie wil ik graag bedanken; Gorben, bedankt voor je tips and tricks m.b.t. de mutanten creatie en de ontspanning tijdens borrels en feestjes. Monique, ook bedankt voor je hulp door de jaren heen. Nu sta jij aan het roer van Virologie en ik hoop dat Virologie de diverse, unieke groep blijft die het altijd is geweest. Succes beiden!

De samenwerking met de heren van Monsanto (voorheen De Ruiter Seeds) was altijd aangenaam en daarbij wil ik graag Rolf Folkertsma en Pieter van Poppel bedanken voor hun input, en de fijne samenwerking. Ook Ton Allersma bedankt voor de samenwerking, voor de levering van de zaden (soms op korte termijn) en de virus isolaten die we hebben gebruikt in een aantal studies.

I also need to acknowledge the students I got to supervise in the last years. Without them I would not have so much data. Adrien, Lan, Su, Jikke, Aranka and Bart. Adrien, you were my first student and we had to set-up a system of generating mutants, which worked really well. Thanks for all the help and good luck in Strasbourg. I hope we stay in touch! Also Su, thanks for that pile of work you performed; really a lot of the data from chapter 3 is your work. Our work together with Adrien's was recently published as well! Thanks, both of you! Good luck in the future! Aranka, ondanks dat je teleurgesteld was dat het maar niet lukte met die Co-IPs heb je wel ontzettend veel gedaan. Ik maak me dan ook geen zorgen voor je toekomst. Succes met je stage en het vinden van een uitdagende baan!

Ik wil ook alle mede-AIOs, post-docs en analisten bedanken voor de fijne tijd in het lab en daarbuiten. De Arbo-groep; Stefan, Corinne, Mia en Jelke, een gezellige club mensen die wel van een feestje op z'n tijd houden, en daardoor mij de hoognodige ontspanning bezorgde. Thanks! De baculo-dames, Stineke en Vera. Vera, bedankt voor je hulp op het laatst met de phylogenie en de gezellige tijd op de kamer. Stineke, met wie ik op dezelfde dag binnen kwam lopen op het

oude Viro op de Binnenhaven! Onze trip naar Cambridge was superleuk en de laatste maanden op de kamer was altijd gezellig; zeker vanwege jouw gevoel voor humor lag ik regelmatig in een deuk! Succes met het afronden van je proefschrift en de toekomst in Engeland! Paulus, als mede-plantenviroloog hebben we vaak wetenschappelijke zaken besproken, wat altijd wel nuttig was, en daarnaast kende we ook veel ontspanning met vogels kijken, muziek luisteren en blèren in het lab. Succes met je laatste loodjes van je proefschrift, en bedankt dat je naast me komt te staan als mijn paranimf bij de verdediging van mijn proefschrift. Marcio, we also started at the same day at virology and because our topics were so closely related, a collaboration wasn't surprising. Thanks for the experiments that you did for me and I hope a nice story will be published soon using your system and some of my constructs. Good luck in the future! Maarten, ook wij hebben dezelfde tijdslijn gevolgd, met alle ups and down van beide projecten. Jij ben inmiddels zeer succesvol gepromoveerd, en ik hoop dat je snel een leuke baan vindt. Bedankt voor je hulp de afgelopen jaren. Martin, ten tijde van dit schrijven moet je promotie nog plaatsvinden, maar daar heb ik alle vertrouwen in. Onze korte samenwerking leidde helaas niet tot het gewenste resultaat, maar er zijn nog ideeën genoeg. Ook bedankt voor de afgelopen tijd en wellicht tot ziens! De analisten Janneke, Hanke en Els. Janneke, bedankt voor je hulp in het met lab met ELISAs. Met zulke getrainde handen wist ik altijd wel dat het goed zou gaan, en bedank ik je voor je steun. Je kwam altijd vragen hoe het was en hoe het ging, dat was fijn! Hanke en Els, zo door de jaren heen hebben we regelmatig contact gehad over zaken in en om het lab, bedankt voor alle hulp!

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Of course, there are many people who came and left during my time at Virology. Esther, my project had quite a few overlaps with your work and I could always ask you for help. That was great. And now you have your own group in Scotland; no surprise that you would do well! Good luck there and with the family expansion. Christina, also under the wings of Richard, I remember vivid discussions and as my first roommate in the old building we talked about all kinds of stuff. Thanks for that

and I hope all the best for you and your family. Afshin, although we didn't work together on the same project, we were always involved in each other's work, which was nice. Thanks for all the tips and tricks, especially in the beginning working with pEAQ-HT. I hope you will find a nice job that fits your skills soon! People I'd like to thank that I've met during my PhD-time; Ke, Magda, Amaya, Marcel, Adriaan, Tiny, Geert, Marleen B, Athos, Dick P, Marleen H, Mariana, Thomas, Edze, Simon, Carlijn, Joël and others I might forget, it was nice meeting you all, and thanks for the good times.

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In Wageningen ben ik uiteraard ook veel mensen tegengekomen die ik inmiddels tot mijn vrienden reken; en daar mogen mijn vaste woensdagavond-vrienden niet bij ontbreken; Jos, Marleen, Margot, Tim en ook Arne. Dankzij jullie, was de woensdagavond altijd een feestje en jullie interesse in mij en mijn werk waardeerde ik erg. Wie weet waar de toekomst ons brengt, maar we zullen altijd contact blijven houden (als het aan mij ligt). Vincent S, oud-studiegenoot, oud-mede-DnD-er en ook vriend. Ondanks dat jij ook niet meer in Wageningen woont, hebben we gelukkig nog regelmatig contact. Bedankt voor de steun en de ontspanning; ik kan altijd enorm met je lachen. Ik hoop dat je AIO-tijd voorspoedig gaat en ik zal zeker bij je promotie aanwezig zijn! Succes en ook Lisa bedankt en succes! Ik hoop dat er nog menig DnD-weekend gepland gaat worden in te toekomst.

Door mijn jaren op de Haarweg (Kaal 239) heb ik ook een leuke club mensen leren kennen, in het bijzonder Marieke. Een goede vriendin, me wie ik over veel zaken kan praten. En soms was dat wel nodig, aangezien we allebei in het AIO-schuitje zitten(/zaten). Bedankt voor je steun en ik hoop dat jouw AIO-schap ook een voorspoedig einde mag kennen. Succes ermee! Natuurlijk ook Vincent R bedankt voor je hulp, zeker aan het einde met Indesign. Justin, ook met jou heb ik regelmatig kunnen

praten over het werk en je was altijd geïnteresseerd, bedankt daarvoor. Succes in Schotland. Peter van G. Al snel verliet je Wageningen om je PhD te doen in the US, wat je erg graag wilde, en ondanks dat het contact niet altijd zo sterk is wens ik je al het beste toe daar! Tot ooit!

There is a big group of people that I met through virology which I'm happy to know; Cheryl and Juriaan, Nikolina, Vasso, Raoul and Thomas, Ola and Slawek, Iohanna and Paschalis, and Jeroen; thanks for all the good times, whether it was the wedding in Poland or the 1920-party at Cheryl's place, it was a blast, thanks all!! Even though we're spread out over the country and even Europe, we'll meet again!

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About the author

Dryas de Ronde was born on the August 23, 1984 in Lelystad, the Netherlands. He got his VWO diploma at O.S.G. de Rietlanden in 2003, after which he moved to Wageningen to study Biology at the Wageningen University and Research centre. He specialised in plant biology during his Bachelor (BSc) and graduated in 2006. He continued there with his Master (MSc) in biology and performed his Master thesis at the Laboratory of Plant cell biology under the supervision of Jan W. Vos. There he studied the role of a Microtubule associated protein TPX2 from *Arabidopsis thaliana* involved in cell divsion, by investigating T-DNA knock-out lines of this protein in *A. thaliana*. For his internship he moved to Strassbourg in France, to continue working on homologs of TPX2 and the characterisation of two new Microtubule associated proteins (MAPs). In 2008 he received his MSc-diploma from Wageningen University. In 2009 he started his PhD at the laboratory of Virology where he focussed on *Tomato spotted wilt virus* and the resistant host *Capsicum annuum*. With a strong background in plant biology and a PhD in plant-virology, he wants to pursue a carreer in phytopathology and expand his field to other pathogens as well.

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My publications:

D. de Ronde, P. Butterbach, D. Lohuis and R. Kormelink. "Identification and characterisation of a new class of temperature-dependent *Tomato spotted wilt virus* resistance breaking isolates of *Tsw*-based resistance". (Manuscript in prepration).

D. de Ronde, P. Butterbach and R. Kormelink. "Dominant resistance against plant viruses: a review". (To be submitted).

D. de Ronde, A. Pasquier, S. Ying, P. Butterbach, D. Lohuis and R. Kormelink (2013). "Analysis of *Tomato spotted wilt virus* NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression". Molecular Plant Pathology. (Accepted for publication, doi: 10.1111/mpp.12082).

D. de Ronde, P. Butterbach, D. Lohuis, M. Hedil, J. W. M. van Lent and R. Kormelink (2013). "*Tsw* gene-based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*" Molecular Plant Pathology 14(4): 405-415.

J. W. Vos, L. Pieuchot, J. L. Evrard, N. Janski, M. Bergdoll, D. de Ronde, L. H. Perez, T. Sardon, I. Vernos and A. C. Schmit (2008). "The plant TPX2 protein regulates prospindle assembly before nuclear envelope breakdown". Plant Cell 20(10): 2783-2797.

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Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Dryas de Ronde**
Date: **8 November 2013**
Group: **Virology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Towards durable Tospovirus resistance in Capsicum chinense	Mar 30, 2009
► **Writing or rewriting a project proposal**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Course "Veilig werken met radioactieve stoffen en bronnen"	May 26-28, 2009
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Students Day 2009, Leiden University	Feb 26, 2009
EPS PhD Students Day 2010, Utrecht University	Jun 01, 2010
EPS PhD Students Day 2011, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS theme 2 symposium and Willie Commelin Scholten Day: "Interactions between plants and biotic agents", Utrecht University	Jan 22, 2009
EPS theme 2 symposium and Willie Commelin Scholten Day: "Interactions between plants and biotic agents", Utrecht University	Jan 15, 2010
EPS theme 2 symposium and Willie Commelin Scholten Day: "Interactions between plants and biotic agents", University of Amsterdam	Feb 03, 2011
EPS theme 2 symposium and Willie Commelin Scholten Day: "Interactions between plants and biotic agents", Wageningen University	Feb 10, 2012
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 06-07, 2009
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 19-20, 2010
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 04-05, 2011
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 02-03, 2012
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
DAVS symposium, Amsterdam	Mar 05, 2009
DAVS symposium, Amsterdam	Mar 06, 2010
DAVS symposium, Amsterdam	Mar 18, 2011
DAVS symposium, Amsterdam	Mar 02, 2012
DAVS symposium, Amsterdam	Mar 08, 2013
"Evolution, a Matter of Facts", KNAW symposium, Utrecht	Sep 15, 2009
Santiago Elena "Mechanisms of genetic robustness in RNA virusses", WEES	Nov 19, 2009
Peter Moffett "Constitutive and R gene-induced defences against plant viruses", Wageningen	Feb 05, 2010
Denis Fargette "Evolution and adaption of *Rice yellow mottle virus* , an RNA plant virus", Wageningen	Mar 31, 2010
Andreas Suhrbier "A model of *Chikungnya virus* arthritis in immunologically intact adult mice", Wageningen	May 10, 2010
"Recent Developments in Crop Protection" KNPV, Hof van Wageningen	Jun 16, 2010
Beccie Ambrose "NS4A and regulation of cellular responses during *West Nile virus* infection", Wageningen	Jun 09, 2011
Richard Elliot "Engineering bunyaviruses: fluorescent viruses and rearranged genomes", Wageningen	Jun 27, 2011
Javier Palatnik 'Biogenesis and function of plant microRNAs', Wageningen	Aug 25, 2011
James Dale "Bananas for the 21st Century: pushing back the threat of extinction"	Sep 20, 2012
David C. Baulcombe, Plant versus virus: defense, counter defense and counter counter defense', Rob Goldbach Memorial Lecture	Oct 10, 2012
Patrick Forterre, New concepts on the origin and nature of viruses: their major role in both ancient and recent biological evolution, WEES-lectures	Oct 18, 2012
Gabino Sanchez-Perez, Is your Research becoming Digital? Time to call the BioinforMagician! Wageningen	Mar 12, 2013
Seminar "Viruses transmited by mosquitoes" by Gorben Pijlman and Jolanda Smit, Wageningen	May 22, 2013
► **Seminar plus**	
► **International symposia and congresses**	
German-Dutch Virology meeting in Hamburg	Apr 08-09, 2009
"International Advances in Virology", Arnhem, the Netherlands	Sep 05-07, 2010
"Advances in Plant Virology", AAB-meeting, Dublin, Ireland	Mar 28-30, 2012
XV International congress on Molecular Plant-Microbe Interactions, Kyoto, Japan	Jul 29-Aug 02, 2012
► **Presentations**	
Towards durable tospovirus resistance in *Capsicum annuum* : Identifying the avirulence gene in TSWV against *Tsw* resistance	Sep 05-07, 2010
The single dominant resistance gene *Tsw* is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*	Nov 01-03, 2011
The single dominant resistance gene *Tsw* is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*	Feb 10, 2012
The resistance gene *Tsw* is triggered by the RNA silencing suppressor protein of the *Tomato spotted wilt virus*	Mar 02, 2012
The single dominant resistance gene *Tsw* is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*	Mar 28, 2012
The single dominant resistance gene *Tsw* is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*	Apr 03, 2012
Tsw gene based resistance is triggered by a functional RNA silencing suppressor protein of the *Tomato spotted wilt virus*	Jul 31, 2012
► **IAB interview**	Feb 18, 2011
► **Excursions**	
Subtotal Scientific Exposure	*20.0 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Course "A Dip into EBI Resources"	Oct 19-22, 2009
Springschool "RNAi & the world of small RNA molecules"	Apr 14, 15, 16, 2010
Course "Introduction to Electron Microscopy"	Jun 21-25, 2010
Autumnschool "Host-microbe interactomics"	Nov 01-03, 2011
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*4.5 credits**

4) Personal development	*date*
► **Skill training courses**	
Course "Scientific writing"	Oct-Dec 2009
Course "Information Literacy, including Introduction Endnote"	Jun 09-10, 2009
Course "PhD competence assessement"	Jan 19 & Feb 16, 2010
Course "Presentation Skills"	May 13-Jun 03, 2011
Course "Career assessement"	Oct 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**31,5**

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 ECTS

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

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