# The *Sw-5* gene cluster

## Analysis of tomato resistance against tospoviruses

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# The *Sw-5* gene cluster

## Analysis of tomato resistance against tospoviruses

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# Chapter 1

## General Introduction

Athos Silva de Oliveira

**Introductory overview**

Tomato (*Solanum lycopersicum* L.) is one of the most consumed vegetables worldwide, with an annual world production of 161 million tons in 2012 according to the Food and Agriculture Organization of the United Nations (FAO). Nevertheless, it is predicted that a significant part of cultivated tomato is annually lost due to pathogenic diseases. The spotted wilt disease caused by *Tomato spotted wilt virus* (TSWV) is one of the major threats among viral diseases (Scholthof et al., 2011). The development of TSWV-infected tomatoes (Figure 1) compromises tomato fruit production in both quantitative and qualitative ways, and sometimes leads to plant death (Pappu et al., 2009).

TSWV is the type species of the genus *Tospovirus,* the only genus that harbors plant-infecting viruses within the family *Bunyaviridae* (King et al., 2012a). Tospoviruses are transmitted by thrips (order Thysanoptera) in a propagative and circulative manner (Whitfield et al., 2005). The virus particles are spherical and membrane bound (80-120 nm in diameter) and contain a tripartite single stranded (ss)-RNA genome of ambisense/ negative polarity (King et al., 2012a). Tospoviruses are found worldwide and some species have been reported in all continents (Pappu et al., 2009). Besides TSWV, tospovirus species such as *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), and *Chrysanthemum stem necrosis virus* (CSNV) may also cause significant damage on tomato production (Dianese et al., 2011). Tospovirus infections have been reported to cause yield losses in many other important crops as well, such as pepper, lettuce, peanut, soybean, potato (Pappu et al., 2009).

So far there are two known natural genetic sources that provide resistance genes suitable for commercial resistance breeding against TSWV. One of these, named Sw-5, was first observed in a wild tomato species from Peru, *Solanum peruvianum* L., and has allowed the development of commercial tomato cultivars resistant to TSWV for more than one decade (Boiteux and de B. Giordano, 1993; Stevens et al., 1991). Meanwhile, *Sw-5* has been cloned from *S. peruvianum* and presents a gene cluster consisting of at least five different paralogs named *Sw-5a* to *Sw-5e.* From those, only the *Sw-5b* copy has been experimentally proven to be functional against TSWV (Folkertsma et al., 1999; Spassova et al., 2001). The predicted proteins of *Sw-5* genes share conserved domains with other plant and animal innate immunity proteins. In plants these proteins are generally referred to as "nucleotide-binding domain and leucine-rich repeat" (NLR) proteins or also Resistance (R) proteins (de Ronde et al., 2014a; Medzhitov, 2001; van der Biezen and Jones, 1998). This class of proteins mostly comprises cytoplasmic innate immunity receptors that are able to (in)directly perceive the presence of pathogen-derived molecules. Their triggering often leads to a hypersensitive cell death response (HR), as visualized by necrotic spots on the leaves at the site of entry, which prevents the pathogen from systemic spread in the host tissue (Jones

and Dangl, 2006).

In this thesis, research is described that aimed to further unravel the mechanism of Sw-5-mediated resistance against tospoviruses at a cellular and molecular level. In the following paragraphs first a bibliographic overview will be presented on the plant immune system, followed by a state of the art description on Sw-5-mediated resistance against tospoviruses as known at the onset of this thesis research. At the end of this chapter the scope of this thesis is presented.

**Figure 1. TSWV-infected tomatoes.** (From Whitney Cranshaw, Colorado State University).

**General overview on the plant immune system**

Plants have developed a complex immune system to defend themselves against biotic invasions. Unlike animals, plants do not have a somatic and mobile cell immune system. Instead, they rely for their protection on the immune basis of each single cell and systemic signals emitted from sites of infection. At first sight the plant immune system appears less complex, but the truth is that plants use different immune strategies to achieve and build an immune response, which in the end is as sophisticated as observed in vertebrates.

One of the first barriers that phytopathogens have to overcome is the plant cell wall. Necrotrophic pathogens, those that feed from dead tissue, secrete hydrolytic enzymes to degrade the cell wall. Biotrophic and hemibiotrophic pathogens have to establish a different type of interaction, since they cannot kill their host cells during all or part of their

life cycles, as observed for haustorium-forming fungal and oomycete mildews (Underwood, 2012). Rapid deposition of structural compounds like callose (a polysaccharide) is observed as an early strategy of the plant host to reinforce the cell wall at sites of infection. Although those depositions have been correlated to plant resistance against fungal pathogens, it is not understood how many haustorium-forming pathogens suppress this mechanism and manage to establish an infection (Underwood, 2012).

After the cell wall has been passed, pathogenic invaders encounter "heavy molecular artillery" before them. To this end, each plant cell is equipped with an arsenal of receptors that are able to (in)directly sense the presence of pathogens and, once detected, trigger resistance mechanisms to halt their growth and/or dissemination. Successful pathogens are those able to interfere with or escape from this cellular surveillance. The plant immune receptors are usually divided in two main classes (Figure 2). The first class is constituted by Pattern Recognition Receptors (PRR) that consist of transmembrane proteins able to recognize conserved and slowly-evolving microbial molecules from pathogens, termed as pathogen- or microbial-associated molecular patterns, acronym PAMPs/MAMPs (Zipfel, 2014). A well-known example of a PRR is FLS2 that recognizes the active epitope flg22 from bacterial flagellin (Gomez-Gomez and Boller, 2002). Other examples of PAMPs recognized by plant PRRs are the bacterial elongation factor Tu, type-I secreted quorum-sensing peptide Ax21, bacterial lipopolysaccharides and fungal chitin (Bahar et al., 2014; Erbs and Newman, 2012; Kaku et al., 2006; Zipfel et al., 2006). Pathogens that managed to establish an infection evade and suppress this PAMP-triggered immunity (PTI) by secreting molecules, termed effectors (Abramovitch et al., 2006).

The second class of plant immune receptors, the R proteins, recognize those effectors and lead to effector-triggered immunity (ETI) (Figure 2) (Jones and Dangl, 2006). These effectors are also often referred to as avirulence (Avr) determinants. Due to the natural selection pressure conferred by R proteins, pathogens arise that are able to evade ETI, which in turn drives the evolvement of new *R* genes to trigger yet another ETI response. This constant "arms race" can be applied to all pathogen-host interactions. But how do those R proteins trigger resistance? What happens locally and systemically upon pathogen recognition by those receptors?

Since the cloning of the first genes for PRRs and R proteins, efforts have been made to unravel the downstream signaling pathways upon PAMP and effector recognition. So far, most attempts have ended up unsuccessful, probably due to the nature of those molecular interactions, which are mostly thought to be transient, and the redundancy of signaling pathways (Qi and Innes, 2013). However, the exploration of different plant immune receptors has revealed multiple molecular strategies to resistance triggering. Drawing attention to similarities rather than discrepancies, overlapping molecular and cellular events are

observed for PTI and ETI, such as intracellular signaling by mitogen-activated protein kinase (MAPK) cascades, transcriptional reprogramming that leads to production of antimicrobial cargo, accumulation of reactive oxygen species ($O_2^-$ and $H_2O_2$) and changes in intracellular calcium levels (Coll et al., 2011; Panstruga et al., 2009). On the other hand, ETI usually involves a Hypersensitive Response (HR), in which the initially invaded and neighboring cells are killed via a programmed cell death mechanism that prevents the pathogen from spreading in the plant tissue. ETI is a more accelerated and amplified response than PTI and those quantitative differences seem to account for HR induction (Coll et al., 2011).

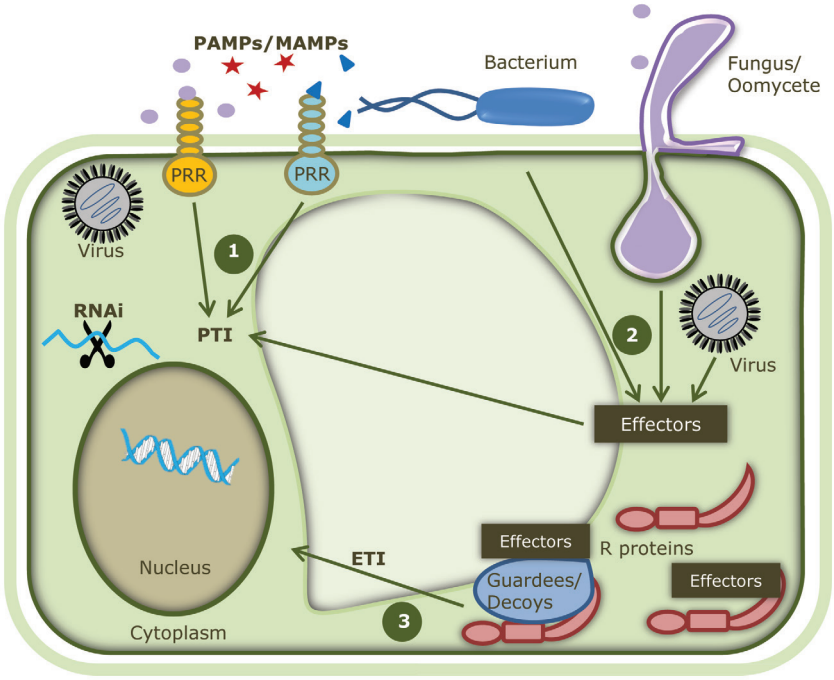


**Figure 2. PAMP- and effector-triggered immunity in plants.** Pathogens like bacteria, nematodes, fungi and oomycetes contain conserved molecules (chitin, flagellin, etc.) termed as Pathogen/Microbial-Associated Molecular Patterns (PAMPs/MAMPs) that are sensed by Pattern Recognition Receptors (PRRs) at the cell surface (1). No PRRs have been reported against viral pathogens, for which the (intracellular) RNAi machinery is regarded one of the first biochemical lines of defence. Detection of PAMPs leads to PAMP-Triggered Immunity (PTI), preventing pathogen growth and spread. Adapted pathogens, however, deliver effectors in the plant cell that suppress PTI and RNAi (2). Plants, as a result, evolved another class of receptors, the Resistance (R) proteins that recognize those effectors, leading to Effector-Triggered Immunity (ETI) (3). Usually, ETI involves triggering of programmed cell death in infected and neighbouring cells, a process called Hypersensitive Response (HR). The detection of effectors can be either direct or indirect. In the latter, modification in other plant proteins (Guardees/Decoys) by effectors are sensed by R proteins. Figure adapted from (Dangl et al., 2013).

Following the local cellular responses, plants can achieve a systemic immune status that protects them against future infections by the same or even other pathogens, a phenomenon named systemic acquired resistance (SAR) (Durrant and Dong, 2004; Fu and Dong, 2013). Many efforts have been made to identify mobile signals from infection sites responsible for SAR induction and some studies indicate that those signals are mostly composed of multiple proteins, and lipid-derived and hormone-like molecules (Spoel and Dong, 2012). The release of these signals and subsequent transport through the vascular plant system result in salicylic acid accumulation in the phloem. Altogether, these signals induce a transcriptional reprogramming of cells that, amongst others, leads to up-regulation of pathogenesis-related (PR) genes. Some of the proteins encoded by these genes are secreted and have antimicrobial properties (Wang et al., 2006). SAR can also induce accumulation of immune signaling components like mitogen-protein kinases and transcription co-factors in the cells (Beckers et al., 2009; Mou et al., 2003). In the study on kinases, these proteins were only active after a secondary pathogen attack, indicating that long-lasting memory in plants may rely on cell priming. It means that plant cells may remain in a sensitized state, keeping and accumulating components that will allow them to respond faster to a secondary pathogen infection. Thus, a primary infection is likely to trigger genetic and epigenetic reprogramming that leads to increase of those components.

**The plant immune system against viral pathogens**

Metagenomic studies have allowed the discovery and sequencing of hundreds of new viral genomes in plants (Roossinck, 2012). These data have indicated an impressive viral diversity and interestingly showed that most “infected” plants were asymptomatic. Thus, disease seems to represent an exception rather than a rule in host-virus interactions. There are even reports of mutualistic viruses that increase plant tolerance to heat, cold and drought (Roossinck, 2013; Xu et al., 2008). Besides those ecological aspects towards adaptation, plants do evolve their immune system to control viruses.

Differently from many fungi, oomycetes, and bacteria, plant viruses only have an intracellular “life cycle”. Once actively introduced into the cell, mostly by invertebrate vectors, they start their infection cycles. Plants have adapted their immune system to defeat viral pathogens in a different manner, at least in the first biochemical line of defense. So far, no PAMPs and PRRs have been identified for viruses. Instead, it is believed that RNA silencing is the first line of innate immune response (Figure 2) and regarded a PTI against viruses (de Ronde et al., 2014a; Zvereva and Pooggin, 2012). This evolutionary-conserved mechanism is responsible for the regulation of gene expression mediated by micro(mi)RNAs but also acts as a defense mechanism against invasive nucleic acids such as transposons and viruses. The latter response is mediated by a class of so-called small-interfering(si)RNAs (Baulcombe,

2004). Apart from their function, miRNAs and siRNAs also differ in biogenesis. While miRNAs are processed from host-encoded transcripts that fold in imperfect double-stranded (ds) RNA stem-loop structures, siRNAs are generally processed from exogenous dsRNA molecules that arise from e.g. replicative intermediates or secondary folding structures of viral RNAs. Plant Dicer-like enzymes (DCL) cleave these dsRNAs into siRNAs of 21-24 nucleotides (nt) in size. One strand of the siRNA molecule, called guide strand, is uploaded in a protein complex named RNA-induced silencing complex (RISC) and turns this into an activated (antiviral) RISC. This complex surveils the cell for target (viral) RNA molecules with sequence complementarity to the guide strand. Target strands become degraded by Argonaut, the core slicer component of RISC. To counteract antiviral RNAi, most viruses encode proteins that are able to interfere with this mechanism, so-called RNA silencing suppressors (RSS) (Roth et al., 2004). Some RSS proteins also act as effectors and trigger an ETI (de Ronde et al., 2014a; de Ronde et al., 2013). Successful viruses are those that are able to evade PTI and ETI.

**Structure, expression, and activation of R proteins**

The second line of defense (ETI) is mediated by single dominant *R* genes. Most R proteins belong to the NLRs, which are usually divided into two main classes based on their N-terminal ends, however other variations have been reported. The first class contains NLRs with a Toll/interleukin-1 receptor domain (TIR) at their N-terminus. Animal proteins of this type have been shown to be involved in activation of antimicrobial responses (Jenkins and Mansell, 2010; Radhakrishnan and Splitter, 2010). The second class is composed of NLRs with secondary coiled-coil (CC) structures at the N-terminus. The CC and TIR crystal structures from barley Mla10 and flax L6, respectively, have recently been solved (Bernoux et al., 2011; Maekawa et al., 2011). The first one shows a rod-shaped homodimer and implies a self-association of Mla10 proteins. Such association has been shown essential for cell death induction. The crystal structure of L6 TIR domain also shows a homodimer and leads to self-association of L6, which is required for immune signaling.

The TIR and CC domains are mostly followed by an NB-ARC domain (acronym for Nucleotide-Binding adaptor shared by ApaF-1, Resistance proteins, and CED-4,) and a LRR domain (acronym for Leucine-Rich-Repeat, at the C-terminus) (Takken and Goverse, 2012). The NB-ARC is a conserved domain shared by NLRs from plants and animals. Although the crystal structure of a plant NB-ARC has not been resolved yet, the one from human ApaF-1 (apoptotic protease-activating factor-1) has been solved (Riedl et al., 2005). It is postulated that the NB-ARC works as a switch since “on” and “off” states are observed. In the “on” state, the NB-ARC domain takes on an “open” structural conformation in which ATP (adenosine triphosphate) is preferentially bound. In the “off” state, a “closed” structural

conformation is adopted and ADP (adenosine diphosphate) becomes preferentially bound. The ability to switch from active and inactive states is assumed to control the protein status in triggering downstream signaling that leads to pathogen resistance. The tomato R proteins I-2 and Mi-1 are capable of binding ATP and exhibit ATPase activity (Tameling et al., 2002). Depending on the R protein, either NB-ARC, CC or TIR domains have been experimentally shown as the protein unit responsible for the immune signaling that leads to cell death. For barley Mla10 and flax L6, overexpression of CC and TIR, respectively, leads to HR induction (Bernoux et al., 2011; Maekawa et al., 2011), while for potato Rx the NB-ARC is able to induce HR (Rairdan et al., 2008). For *Arabidopsis* RPS5, on the other hand, HR is triggered only by overexpression of both CC and NB-ARC domains in *cis* (covalently bounded) but not in *trans* (from independent constructs) (Ade et al., 2007).

The LRR domain is commonly involved in protein-protein and protein-ligand interactions. Besides NLRs, PRRs also contain an LRR domain, which is involved in PAMP recognition (Boller and Felix, 2009). In spite of being responsible for sensing pathogens, the LRR domain from most R proteins does not interact directly with Avr determinants, which strengthens the "Guard" and "Decoy" models (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). According to the Guard model, R proteins (the guards) indirectly perceive the presence of pathogens by changes of plant proteins (the guardees) once these are targeted by the pathogen effector. After perceiving a modification in the guardee, the R protein becomes activated and triggers disease resistance in the host. This model supports the idea of a single *R* gene to perceive multiple effectors, which is likely considering the presence of only a small repertoire of plant *R* genes versus a high number of pathogens. However, many guardees are indispensable for pathogen virulence in plants without a correlated *R* gene. To accommodate for this, the "Decoy model" has been proposed in which the host proteins targeted by effectors just act as "decoys". They evolved to perceive pathogens by mimicking effector targets, but do not have any other cellular function. Most studied R proteins and corresponding Avr determinants involve indirect interactions, but a few examples exist of a direct interaction: (I) rice Pi-ta and its corresponding Avr from the fungus *Magnaporthe grisea*; (II) *Arabidopsis* RRS1 and the bacterial Pop2 protein from *Ralstonia solanacearum*; (III) tobacco N protein and the helicase domain of *Tobacco mosaic virus* p50 (Deslandes et al., 2003; Jia et al., 2000; Ueda et al., 2006).

*NLR* genes are mostly arranged in pairs or clusters, indicating a source of structural plasticity (Leister, 2004). In addition, polymorphisms exist within a population or between species, which results in more genetic variation (Shen et al., 2006). The LRR region, especially, presents high ratios of nonsynonymous to synonymous amino acid changes, which indicate that they have evolved to detect variation in effectors, guardees, and/or decoys (Michelmore and Meyers, 1998). Recent *in silico* data suggest that diploidization following duplication is the major source of *R* gene structural variation (Zhang et al., 2014). The same study also

indicated that miRNAs play an important role in regulating *R* gene expression, preferentially targeting duplicated *R* genes. It seems that when retained after diploidization, redundant *R* genes require modification or specialization in their expression. The screening of miRNA libraries from plants identified candidate miRNAs that target messenger (m)RNA sequences from genes of plant innate immune receptors (Li et al., 2012; Shivaprasad et al., 2012). In general, most miRNAs seem to downregulate the expression of *R* genes in the absence of their cognate pathogens, resulting from miRNA-mediated cleavage of their mRNAs and enhanced silencing after subsequent production of secondary siRNAs by plant RNA-dependent RNA polymerase 6 (Li et al., 2012; Shivaprasad et al., 2012). In the presence of viruses or bacteria the miRNA-based silencing machinery is suppressed by viral respectively bacterial RSSs (Li et al., 2012; Shivaprasad et al., 2012). Unregulated expression of *R* genes regarding both levels and time usually ends in autoimmunity. This is easily observed experimentally by overexpression of *R* genes via *Agrobacterium tumefaciens*. Usually, auto-HR is triggered when the levels of expression are too high due to the promoters used or the simultaneous presence of RNA silencing suppressors in the expression vectors (Gabriels et al., 2007; Zhang et al., 2004).

During their activation, most NLRs localize in both cytoplasm and nucleus, indicating that the signaling pathways involved are located in both compartments. The barley Mla10 protein, for example, interacts with the nuclear transcriptional repressor WRKY and activator MYB6 in the presence of its cognate powdery mildew effector (Chang et al., 2013; Shen et al., 2007). The potato Rx also shows a nucleocytoplasmic distribution but recognition of the Potato virus X viral coat protein (CP), that serves as Avr, and the resistance signaling seem to occur in the cytoplasm (Slootweg et al., 2010; Tameling et al., 2010). In a different way, RPS5 and RPM1 from *Arabidopsis* require plasma membrane localization, likely reflecting the location of their guardees and the cognate effectors from *Pseudomonas syringae* (Chung et al., 2011; Dowen et al., 2009; Gao et al., 2011; Liu et al., 2011; Shao et al., 2003).

So far still little is known about the signal transduction pathway leading to resistance activation. Although genetic screens have revealed some interactors, they do not seem to be required for all NB-LRRs (Qi and Innes, 2013). The EDS1 protein from *Arabidopsis*, for example, is required by those presenting the TIR domain, but not CC (Aarts et al., 1998). On the other hand, chaperones like HSP90, SGT1, and RAR1 seem essential for stability and correct conformational changes of R proteins during pathogen recognition and signal transduction for resistance (Liu et al., 2004; Shirasu, 2009). Despite some divergences, the signaling components for activation of R proteins seem conserved in plants as supported by the observation that R proteins from different plant species manage to trigger cell death in heterologous plant species as well. In many cases this happens to be one of the widely used experimental plant hosts *Nicotiana benthamiana* L. and *N. tabacum* L. (Bai et al., 2012; Hallwass et al., 2014; Zhang et al., 2004). The *R* genes RPS4 and RRS1 from *Arabidopsis*, for

example, still trigger resistance against fungi and bacteria when transformed into *Brassicaceae* (*Brassica rapa* and *B. napus*) and *Solanaceae* (*N. benthamiana* and *S. lycopersicum*) (Narusaka et al., 2013).

**The *Sw-5* gene cluster**

The *Sw-5* gene cluster on chromosome 9 from *S. peruvianum* contains five paralogs, named *Sw-5a* to *Sw-5e* (Spassova et al., 2001). Their encoded proteins belong to the CC-NB-LRR group (Brommonschenkel et al., 2000; Spassova et al., 2001). Transgenic tobacco plants transformed with the paralogs *Sw-5a* and *Sw-5b*, separate or combined, demonstrated that the *b* copy is the functional one and sufficient to halt TSWV infection (Spassova et al., 2001). Despite the latter observation, the functionality or involvement of the other *Sw-5* paralogs remains to be investigated. Interestingly, and rather unique for a dominant resistance gene, *Sw-5b* not only confers resistance to TSWV but also to isolates of GRSV and TCSV (Boiteux and Giordano, 1993; Gubba et al., 2002). Screening of *S. peruvianum* accessions has also revealed resistance against a fourth tospovirus species, CSNV (Dianese et al., 2011). All those tospovirus species are geographically and phylogenetically related, and present an "American" origin (Pappu et al., 2009). "Eurasian" tospoviruses, like *Capsicum chlorosis virus* (CaCV), are likely to overcome the Sw-5 resistance (McMichael et al., 2002). It is the broad-spectrum tospovirus resistance conferred by Sw-5 that has encouraged breeders to introgress this resistance into commercial tomato cultivars (*S. lycopersicum*) for more than a decade (Ferraz et al., 2003; Langella et al., 2004). Although the *Sw-5* gene cluster was already cloned in 2001, within the last decade most studies have focused on development of new molecular markers to assist in breeding (Dianese et al., 2010; Langella et al., 2004). In addition, the fitness of Sw-5 resistance-breaking (RB) TSWV isolates was investigated (Aramburu et al., 2011; Latham and Jones, 1998; Lopez et al., 2011) and although these isolates replicated, they appeared to exhibit a lower fitness. Transmission by mechanical inoculation mostly failed for resistance-breakers, but the transmission efficiency by their natural thrips vector *Frankliniella occidentalis* was as good as observed for Sw-5 resistance-inducing TSWV isolates.

Although resistance-breaking isolates have emerged, the *Sw-5* gene cluster in general provides high levels of resistance and reduced virus disease incidence in most areas where bred tomatoes are cultivated, especially when susceptible and Sw-5-resistant cultivars are interchanged (Gordillo et al., 2008). Resistance breaking isolates seem to come up in areas where the virus pressure is extremely high and only Sw-5 resistant cultivars are continuously grown (Gordillo et al., 2008). The Sw-5 resistance is also overcome during mixed infections of TSWV with *Tomato chlorosis virus* (ToCV), a phloem-limited and positive single-stranded RNA virus belonging to the genus *Crinivirus* (Family *Closteroviridae*) (Garcia-

Cano et al., 2006). However, this only occurs in case of a pre-infection with ToCV and not when both viruses are co-inoculated at the same time. How ToCV compromises Sw-5 resistance remains to be investigated. Another study interestingly showed that SPRYSEC-19, an effector of the nematode *Globodera rostochiensis,* interacts with an orthologous Sw-5 protein from *S. lycopersicum*, named Sw-5f (Rehman et al., 2009) and inhibits HR triggered by an auto-active Sw-5b protein (Postma et al., 2012; Rehman et al., 2009).

**Tospoviruses**

*Tospovirus* is the only genus classified in the family *Bunyaviridae* that contains plant-infecting members. The other four genera, i.e. *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, and *Phlebovirus,* all contain animal infecting viruses amongst which some, like *Crimean Congo Hemorhagic Fever virus* (CCHFV) and *Rift Valley Fever virus* (RVFV), are listed as biological threats (King et al., 2012a). Tospoviruses cause significant damage on vegetables and agricultural crops. Their transmission is carried out by a limited number of thrips species (order Thysanoptera) in a circulative and propagative fashion (Mound, 2002; Ullman et al., 1993; Wijkamp et al., 1993). Although more than 5000 thrips species have been reported, only 15 are listed as vectors for tospoviruses, suggesting an intrinsic and specific thrips-tospovirus co-evolution (Pappu et al., 2009; Riley et al., 2011; Zhou and Tzanetakis, 2013). Tospoviruses present spherical particles with a diameter of 80-120 nm and a single-stranded tripartite RNA genome tightly encapsidated by multiple copies of the nucleocapsid (N) protein. Also, tospovirus particles have RNA-dependent RNA polymerase (L protein) unities since the viral genome has both ambisense and negative polarities (Van poelwijk et al., 1993). Particles contain a Golgi-derived lipid envelope with spike proteins that consist of two viral glycoproteins Gn and Gc (Kikkert et al., 1999).

The three genomic segments of tospoviruses are denominated according to their sizes in Small (S), Medium (M), and Large (L) RNAs (Figure 3). For TSWV, their average size in nucleotides is 2.9 kb, 4.8 kb, and 8.9 kb, respectively. The S RNA has an ambisense gene arrangement and encodes the non-structural RNA silencing suppressor protein ($NS_S$) on the v-strand and the N protein on the vc-strand (Dehaan et al., 1990; Takeda et al., 2002). The M RNA, likewise, encodes the non-structural, cell-to-cell movement protein ($NS_M$) in v-sense and the precursor to the envelope glycoproteins Gn and Gc in vc-sense (Kormelink et al., 1992; Kormelink et al., 1994). The L RNA is entirely of negative polarity and encodes the L protein, the putative RNA-dependent RNA polymerase (RdRp) (Dehaan et al., 1991). The L protein is generally assumed to contain several enzymatic functions/activities such as replicase, transcriptase, nuclease, helicase, cap-binding and NTPase as supported by a multiple sequence alignment with other viral RdRPs (Adkins et al., 1995; Chapman et al., 2003; Tordo et al., 1992). The M- and S-RNA encoded proteins are expressed from

subgenomic mRNAs that are transcribed from both v- and vc-strands, and the RdRP/L protein from a near genomic length mRNA of the L RNA (Figure 3). Viral transcription is initiated by cap-snatching, a process in which host mRNAs are recruited and cleaved by the L protein 12$^{th}$-18$^{th}$ nucleotides after the 5'cap (Duijsings et al., 1999; Duijsings et al., 2001). These capped leader sequences are then used as primers for synthesis of viral mRNAs. Unlike host mRNAs, tospoviral mRNAs do not contain a common eukaryotic poly-A tail. The vcRNA strands, besides presenting a template for transcription in case of the ambisense segments, function as templates for synthesis of progeny viral genomic RNAs that are then encapsidated (Snippe et al., 2005). For particle maturation, the RNPs are surrounded by cisterns from the Golgi complex, generating double enveloped particles. Then, they fuse with each other along with membranes of the endoplasmic reticulum, to accumulate as single enveloped particles in large cytoplasmic vesicles (Elliott et al., 1996; Kikkert et al., 1999). There they remain until thrips acquire the entire content of a cell, including virus particles upon feeding. Particle maturation in thrips cells shows similarities to that observed for vertebrate-infecting bunyaviruses (Kikkert et al., 2001). After budding of RNPs into the lumen of the Golgi complex, tospovirus particles are secreted by the cell (Wijkamp et al., 1993). The cell-to-cell movement of tospoviruses in plants involves infectious RNPs and is facilitated by the $NS_M$ protein. This protein forms tubule structures through plasmodesmata and enlarges their size exclusion limit to allow passage of $NS_M$-RNP complexes (Kormelink et al., 1994; Storms et al., 1995).

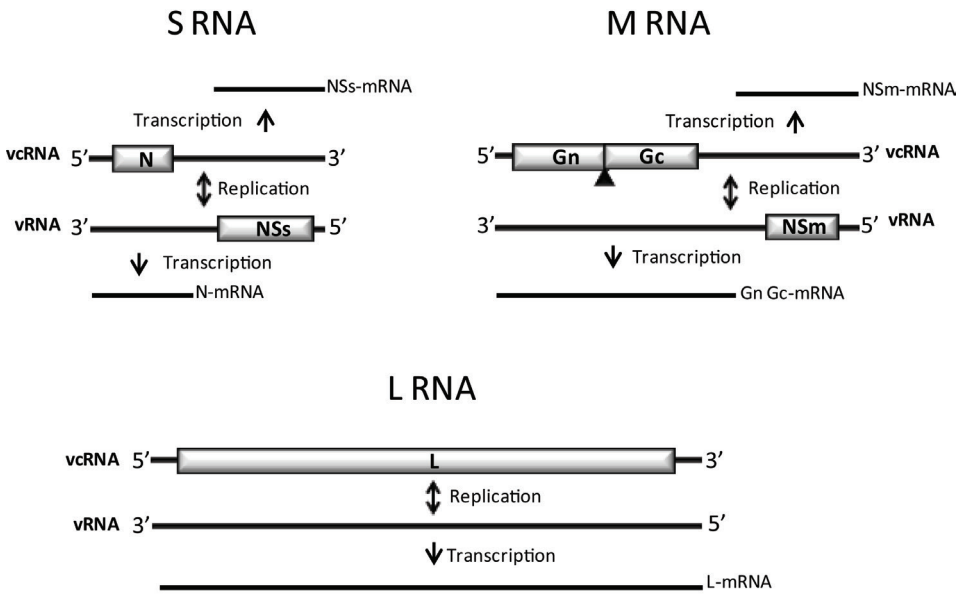


**Figure 3. Genome organization and expression strategy of tospoviruses.** Blocks represent open reading frames and are located in both viral (v) and viral complementary (cv) RNAs for the S and M segments or just in the vcRNA for the L segment.

**Tospovirus species and their control**

Tospoviruses are spread worldwide, of which TSWV, *Impatiens necrotic spot virus* (INSV), and *Iris yellow spot virus* (IYSV) have been reported in all continents (Pappu et al., 2009). With the availability of serological and molecular tools a large number of new tospovirus species have been identified and characterized during the last two decades. Host-range, symptomatology, thrips transmission, serology, and the N protein sequence are commonly used for characterization, differentiation, and taxonomic classification of new tospovirus species (King et al., 2012a). However, the most important criterion for demarcation of a new tospovirus species is the amino acid sequence identity of the N protein to that of already established species, which should be lower than 90% (King et al., 2012a). The evolutionary history of the genus *Tospovirus* and its members, though, is reflected in all viral proteins (Bertran et al., 2011; Lovato et al., 2004; Silva et al., 2001).

So far control of tospovirus diseases remains difficult for various reasons: cosmopolitan distribution of thrips vectors and the difficulty to manage/limit thrips infestations by biological or chemical means, broad host range of the viruses and limited availability of natural resistance sources for commercial breeding programs (Bielza, 2008; Morse and Hoddle, 2006; Zhao et al., 1995). Besides Sw-5, a second source of resistance is available from pepper (*Capsicum chinense*), denominated Tsw. This resistance gene has allowed the generation of pepper cultivars resistant against isolates of TSWV (Boiteux, 1995; Jahn et al., 2000). Transgenically engineered resistance strategies have also been developed to combat tospovirus infections. Many of these are based on the transformation of plant hosts with tospoviral gene cassettes to introduce an RNAi-based resistance. The viral gene cassettes are designed in such a way that they produce hairpin structures, which lead to the production of siRNAs and *a priori* activate the antiviral RISC complex to immediately degrade incoming viral genomes (Bucher et al., 2006; Dehaan et al., 1992; Peng et al., 2014; Prins et al., 1997).

**Thesis outline**

At the onset of this thesis research experimental evidence for the identity of the cognate Avr determinant of Sw-5b protein was not available and knowledge on the signal transduction route leading to Sw-5-mediated resistance and HR was lacking. The focus of this thesis was to identify the Avr determinant and to investigate the Sw-5-mediated resistance in further detail. In **Chapter 2** the identification of the Avr determinant of the Sw-5b-mediated resistance is described and demonstrated by HR induction in both *Sw-5b*-transformed *N. benthamiana* and Sw-5-resistant tomato isolines. The broad resistance spectrum of *Sw-5b*-transgenic *N. benthamiana* plants is shown and extended to another

tospovirus species. In **Chapter 3**, this Avr determinant is dissected to determine protein regions required for the triggering of the Sw-5b-mediated resistance. In **Chapter 4** a new tospovirus, tentatively named Bean necrotic spot virus (BeNMV), is characterized in respect to its serological and molecular features to indicate its evolutionary relation towards other established tospovirus species. The BeNMV *Avr* homolog is tested for HR-induction upon co-infiltration with Sw-5b in wild type *N. benthamiana*. In **Chapter 5** the Sw-5b protein has been dissected and the ability of each of the domains CC, NB-ARC, and LRR tested singly or in a concerted (*cis/trans*) manner to trigger HR. In a similar approach the most highly conserved paralogous protein to Sw-5b (from *S. peruvianum*), Sw-5a, as well as an orthologous protein from *S. lycopersicum*, named here as Sw-5a$^{S}$, have been analyzed. In **Chapter 6** experimental data is presented on the subcellular localization of the Sw-5b protein and the (type of) interactions between Sw-5b and the TSWV $NS_M$ protein are investigated. In the general discussion, **Chapter 7**, all findings of this thesis are integrated and discussed in relation to susceptibility or a resistance response of plants containing one of the *Sw-5* homologs upon challenge with TSWV.

# Chapter 2

# The $NS_M$ protein of TSWV is the Avr determinant of the Sw-5b-mediated resistance

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**ABSTRACT**

Although the *Sw-5* gene cluster has already been cloned, and *Sw-5b* identified as the functional gene copy that confers resistance to *Tomato spotted wilt virus* (TSWV), its avirulence (Avr) determinant still has not been identified. Whereas *Nicotiana tabacum* 'SR1' plants transformed with a copy of *Sw-5b* gene were immune without producing a clear visual response upon challenging with TSWV, it is here shown that *N. bethamiana* transformed with *Sw-5b* gives a rapid and conspicuous hypersensitive like response (HR). Using these plants, from all structural and non-structural TSWV proteins tested, the TSWV cell-to-cell movement protein ($NS_M$) was confirmed as the Avr determinant using a PVX-replicon or a non-replicative, pEAQ-HT expression vector system. HR was induced in *Sw-5b*-transgenic *N. benthamiana* as well as in resistant tomato near-isogenic lines after agroinfiltration with a functional cell-to-cell movement protein ($NS_M$) from a resistance inducing (RI) TSWV strain (BR-01), but not with $NS_M$ from a Sw-5-resistance breaking (RB) strain (GRAU). This is the first biological demonstration that Sw-5 mediated-resistance is triggered by the TSWV $NS_M$ cell-to-cell movement protein.

**INTRODUCTION**

Tospoviruses are amongst the most destructive pathogens known for tomatoes and are a limiting factor in vegetable production worldwide (Aramburu and Marti, 2003; Boiteux and Giordano, 1993; Finetti Sialer et al., 2002; Pappu et al., 2009; Williams et al., 2001). *Tomato spotted wilt virus* (TSWV) is the best studied member of the genus *Tospovirus* and produces a great burden to many economically important agricultural and ornamental crops in (sub)tropical and temperate climate regions. The virus is transmitted in a propagative manner by various species of thrips (*Thripidae*, main vector - *Frankliniella occidentalis*), and currently ranks second on the list of most important plant viruses in the world (Scholthof et al., 2011).

Ever since TSWV has been identified and classified as the first phytopathogenic virus in the *Bunyaviridae*, a large family of mostly arthropod-borne animal-infecting RNA viruses, more than 20 new tospovirus species have been identified and distinguished based on the nucleoprotein (N) gene sequence and vector specificity (King et al., 2011). Based on phylogenetic analysis of the N gene, all tospovirus species are grouped in two major clusters that correlate with their geographical distribution, i.e. the New World and Old World tospoviruses. Like all members of the *Bunyaviridae*, tospoviruses consist of enveloped virus particles (∅ 80-120 nm) and contain three RNA segments that according to their size are denoted S (small), M (medium) and L (large) RNA. The S RNA encodes the N protein and the RNA silencing suppressor protein $NS_S$ (Bucher et al., 2003; Kormelink et al., 1991; Takeda et

al., 2002). The M RNA encodes the precursor (GP) to the glycoproteins $G_N$ and $G_C$ (N and C refer to the amino- and carboxy-terminal position in the precursor) and the cell-to-cell movement protein $NS_M$ (Kormelink et al., 1992; Kormelink et al., 1994; Storms et al., 1995) and the L RNA encodes the viral polymerase (de Haan et al., 1991).

Several strategies for chemical and cultural control of tospoviruses and their vectors have shown to be ineffective. The most successful, however, is the cultivation of resistant cultivars, which are usually developed by introgression of resistance genes in commercial genotypes. Some tomato accessions are sources of natural resistance to TSWV, and are being used in breeding programs throughout the world (Finlay, 1953; Price et al., 2007; Saidi and Warade, 2008). In the last decade, two single dominant resistance genes have received most attention due to their applicability for commercial resistance breeding against tospoviruses, i.e. *Sw-5* and *Tsw*. The first one, *Sw-5* (Aramburu et al., 2011; Cho et al., 1995; Stevens et al., 1991), is the most interesting one as it confers a broad tospovirus resistance against TSWV, *Chrysanthemum stem necrosis virus* (CSNV), *Tomato chlorotic spot virus* (TCSV) and *Groundnut ringspot virus* (GRSV) (Boiteux and Giordano, 1993; Stevens et al., 1995; Stevens et al., 1991; Stevens et al., 1994). The resistance derives from a tomato cultivar (Stevens), which has been obtained in South Africa by a cross between *Solanum peruvianum* and *S. lycopersicum* (Van Zijl et al., 1985) and protects against virus invasion by induction of a hypersensitive response (HR). Little is yet known on the mechanism of defense driven by this resistance gene. The second, single dominant, resistance gene is *Tsw* (Boiteux, 1994). This gene originates from distinct *Capsicum chinense* accessions and is highly specific as it only confers resistance against TSWV isolates (Boiteux, 1995).

Single dominant resistance (*R*) genes generally make up the second line of defense of the plant immune system against pathogens, a battle that is generally illustrated with the zig-zag model (Jones and Dangl, 2006) and involves RNA silencing as one of the first lines of defense strategies against plant viruses. The first lines of defense are triggered by the so-called microbial- or pathogen-associated molecular patterns (MAMPs respectively PAMPs) (Nürnberger and Brunner, 2002), which are being perceived by pattern recognition receptors (PRRs) (Postel and Kemmerling, 2009) and lead to the onset of PAMP-triggered immunity (PTI) (Chisholm et al., 2006). Viruses encode virulence factors (effectors) that counteract PTI, and thereby enable them to achieve a successful infection. In a next phase these same effectors are specifically recognized directly or indirectly by protein products from resistance (*R*) genes and are also referred to as effector-triggered immunity (ETI) (Chapter 1). This recognition generally leads to a rapid HR, and involves a programmed cell death (PCD) at the site of infection. Recently, the RNA silencing suppressor $NS_S$ has been identified as the effector triggering *Tsw*–mediated resistance (de Ronde et al., 2013), but the effector that triggers Sw-5b-governed resistance so far has remained unknown.

Proteins encoded by single dominant *R* genes typically contain a nucleotide-binding-leucine rich repeat (NB-LRR) domain (Dangl and Jones, 2001). In plants, the NB-LRR type of *R* genes are further divided into two groups based on the structure of the conserved N-terminal domain: TIR-NB-LRRs, that share homology to Toll/Interleukin receptors; and the coiled coil (CC)-NB-LRRs (Pan et al., 2000). *Sw-5* belongs to the CC-NB-LRR group and its locus contains at least six homologs, denoted *Sw-5a* through *Sw-5f* (Folkertsma et al., 1999; Rehman et al., 2009). The *Sw-5b* gene represents the functional resistance gene copy and is able to provide broad tospovirus resistance as demonstrated in transgenic tobacco plants expressing Sw-5b (Spassova et al., 2001).

The possible occurrence of genome rearrangements and ongoing evolution of new TSWV variants by mutations poses a constant threat to the broad resistance against tospoviruses conferred by the *Sw-5b* gene. Some natural field isolates of TSWV able to breakdown Sw-5 resistance have already been reported in tomato crops in Hawaii, Australia, South Africa (JF1 isolate), Spain (GRAU isolate) and Italy (Aramburu and Marti, 2003; Cho et al., 1995; Ciuffo et al., 2005; Latham and Jones, 1998; Thompson and Van Zijl, 1995). Studies on TSWV reassortants indicated that the genetic determinant involved in overcoming the resistance is associated with the M RNA (Hoffmann et al., 2001). More recently, Lopez et al. (2011) proposed, by *in silico* analysis, that two amino acid substitutions in the $NS_M$ protein of TSWV could be responsible for overcoming the resistance of Sw-5b, but no experimental evidence was provided to support this assumption.

In the present study, the TSWV cell-to-cell movement protein ($NS_M$) has been identified as Avr determinant for the *Sw-5* resistance gene in *Nicotiana benthamiana* transformed with the functional *Sw-5b* copy and in resistant tomato near-isogenic lines. In addition, a tospovirus challenging assay has shown that Sw-5b-mediated resistance also protects against the more distinct *Impatiens necrotic spot virus* (INSV), demonstrating its broad resistance profile against several tospovirus species.

## RESULTS

### *N. benthamiana* transformed with *Sw-5b* show HR upon challenge with a TSWV RI isolate

Earlier, the *Sw-5b* resistance gene copy was identified as the functional resistance gene since *Nicotiana tabacum* 'SR1' transformed with this copy conferred resistance against TSWV (Spassova et al., 2001). These transgenic lines, however, did not reveal a clear HR and appeared totally immune, which hampered the identification of the TSWV Avr determinant of Sw-5b. To determine whether transformation of another host with *Sw-5b* would provide similar results or, in contrast, would reveal a visual HR and thereby allow identification of the

TSWV Avr determinant, *N. benthamiana* was transformed and subsequently analyzed for an HR. While TSWV BR-01 (RI – resistance inducing) and GRAU (RB – resistance breaking) both gave a systemic infection on untransformed *N. benthamiana* (Figure 1A and B), isolate BR-01 (RI) triggered an HR within three to four days as visualized by necrotic lesions only on inoculated leaves of transformed *N. benthamiana* (Figure 1C-E), indicative for an Sw-5b induced resistance response. A challenge with TSWV RB isolate GRAU did not trigger an HR like response but instead resulted in a systemic infection, similarly as in untransformed *N. benthamiana* (Figure 1F and G). Transformed *N. benthamiana* were additionally challenged with INSV, belonging to the same phylogenetic clade of American tospoviruses, and *Melon yellow spot virus* (MYSV, Physalis severe mottle virus (PSMV) isolate), another more distinct tospovirus from the EurAsian clade. While MYSV systemically infected *Sw-5b* transformed *N. benthamiana*, INSV surprisingly triggered an HR as observed with TSWV-RI, suggesting that the spectrum of Sw-5b-mediated resistance covers a large range of members from the "American" tospovirus clade (Figure S1). Although no systemic symptoms were observed in *Sw-5b* transformed *N. benthamiana* upon challenge with TSWV-RI and INSV, upper leaves (non-inoculated) from wild type (positive controls) and transformed *N. benthamiana* were checked for systemic tospovirus replication at 10 d.p.i. As expected, viral RNAs were just detected in wild type plants or in *Sw-5b* transformed *N. benthamiana* inoculated with TSWV RB or MYSV (Figure 1H).

**Transient expression of TSWV genes from a non-replicative pEAQ-HT or from a PVX replicon identifies $NS_M$ as the TSWV Avr determinant of Sw-5b**

Earlier studies to identify the TSWV Avr determinant that triggered *Tsw*-governed resistance resulted in different and conflicting reports (Lovato et al., 2008; Margaria et al., 2007). Recent analysis unambiguously identified the TSWV RNA silencing suppressor $NS_S$ as the Avr determinant (de Ronde et al., 2013), but this was only successful due to the use of a non-replicative transient pEAQ-HT expression vector (Sainsbury et al., 2009), and not when using PVX. To not exclude any possibility, genes coded from TSWV M RNA ($NS_M$ and GP) and candidates of being the Avr determinant of Sw-5b, were cloned into both PVX (pGR107) and pEAQ-HT (Figure 2) for agroinfiltration into *Sw-5b* transformed *N. benthamiana.* Cloning of the glycoprotein precursor GP into pGR107 failed, likely due to size constrains and stability. As controls, constructs for the S RNA encoded N and $NS_S$ genes were included (Figure 2; de Ronde et al., 2013). All gene constructs were verified for their translatability from pEAQ-HT or PVX during agroinfiltration of the constructs into untransformed *N. benthamiana* and subsequent western immunoblot analysis. While all $NS_M$ (Figure 3C), $NS_S$ and N gene constructs (de Ronde et al., 2013) were well expressed, the viral glycoproteins $G_N$ and $G_C$ processed from GP could not be detected (data not shown), likely due to low transient

expression levels as earlier witnessed (unpublished results).

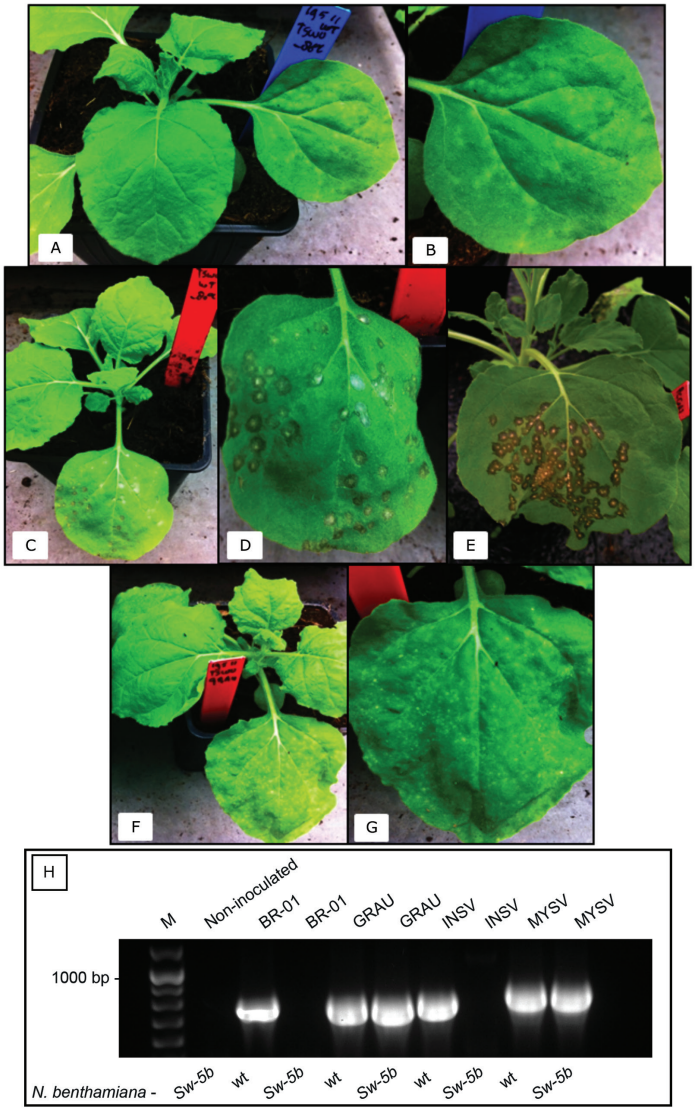


**Figure 1: Challenging of untransformed and Sw-5b transformed *Nicotiana benthamiana* with resistance-inducing TSWV BR-01 and resistance-breaking TSWV GRAU isolates. Pictures were taken 5-7 days post inoculation (p.i.).** (A) untransformed *N. benthamiana* plant challenged with TSWV-BR-01 and showing typical chlorotic lesions on locally infected leaves, (B) enlarged view of the locally infected leaf from figure 1A; (C) *N. benthamiana*-Sw-5b challenged with TSWV BR-01; (D) enlarged view of the virus challenged leaf from figure 1C; (E) *N. benthamiana*-Sw-5b challenged with TSWV BR-01 showing the challenged leaf 15 days p.i.; (F) *N. benthamiana*-Sw-5b challenged with TSWV GRAU; (G) enlarged view of the virus challenged leaf from figure 1F. Round necrotic local lesions typical of a hypersensitive resistance response (HR) can be observed on the enlarged views of a leaf from *N. benthamiana*-Sw-5b challenged with TSWV BR-01. (H) RT-PCR for systemic infection monitoring of wild type (wt) and Sw-5b *N. benthamiana* plants inoculated with TSWV (BR-01 and GRAU), INSV, and MYSV. Total RNA of non-inoculated upper leaves were used as template for N (nucleocapsid) gene amplification at 10 d.p.i. M: GeneRuler 100bp Plus DNA ladder.

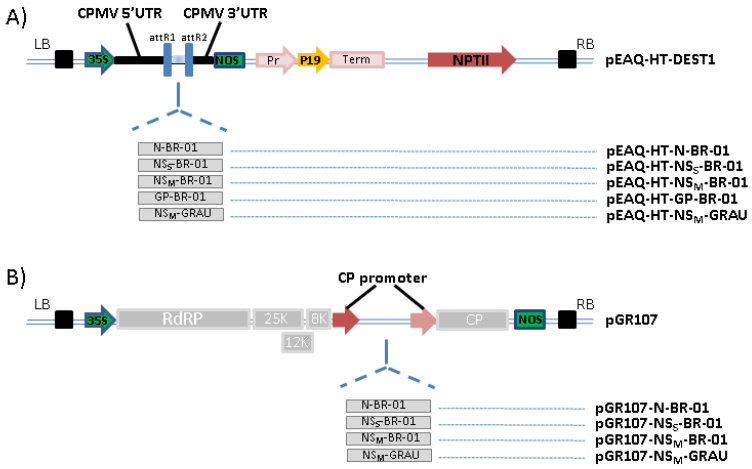


**Figure 2: Schematic representation of the expression vector constructs used to challenge *N. benthamiana* transformants containing *Sw-5b*. (A)** *Tomato spotted wilt virus* (TSWV) genes (*NS$_M$* of BR-01 and GRAU isolates; *N*, *NS$_S$* and the Glycoprotein precursor (GP) from BR-01) cloned individually in the pEAQ-HT vector. Attr= Gateway recombination sites. **(B)** *Tomato spotted wilt virus* (TSWV) genes (*NS$_M$* of BR-01 and GRAU isolate, *N*, *NS$_S$*) cloned individually in the appropriated restriction sites (*ClaI* and *SalI*) of the pGR107 vector (Lovato *et al*., 2008). LB = left border; RB = right border; 35S = *Cauliflower mosaic virus* promoter region; RdRp = PVX RNA dependent RNA polymerase; 25K, 12K and 8K = PVX movement proteins (triple-gene-block); CP promoter = PVX coat protein subgenomic RNA promoter; CP = PVX coat protein; nos = nopaline synthase terminator.

Whereas, as expected, a visual HR was observed upon challenge of *Sw-5b* transformed *N. benthamiana* with TSWV BR-01 (RI) and a systemic infection with TSWV GRAU (RB) (Figure 1), repeated analysis consistently revealed a dark brownish necrosis, typical of an (upcoming) HR-like response in leaves infiltrated only with pEAQ-HT- NS$_{M\ BR\text{-}01}$ or pGR107+ NS$_{M\ BR\text{-}01}$ (Figure 3). Although pictures were taken after 5 and 6 dpi for better digital images, the HR-like response was already observed after 3 dpi. This necrosis became more distinct later, but was not observed with pEAQ-HT- NS$_{M\ GRAU}$ or pGR107+ NS$_{M\ GRAU}$ (Figure 3A and B). The latter two constructs just revealed a weak chlorosis after agroinfiltration of *Sw-5b*-transgenic *N. benthamiana*, similar to those caused by pEAQ-HT or pGR107 without any insert (Figure 3A and B), or containing GP, N or NS$_S$ from the resistance inducing BR-01 strain, or just plain *A. tumefaciens*. Upon chlorophyll removal of agroinfiltrated leaves, the HR-response after infiltration with NS$_{M\ BR\text{-}01}$ was even more distinct (Figure 3A and B). Infiltration of NS$_{M\ BR\text{-}01}$ and NS$_{M\ GRAU}$ constructs on leaves from untransformed *N. bethamiana* only revealed a weak interveinal chlorosis (Figure S2).

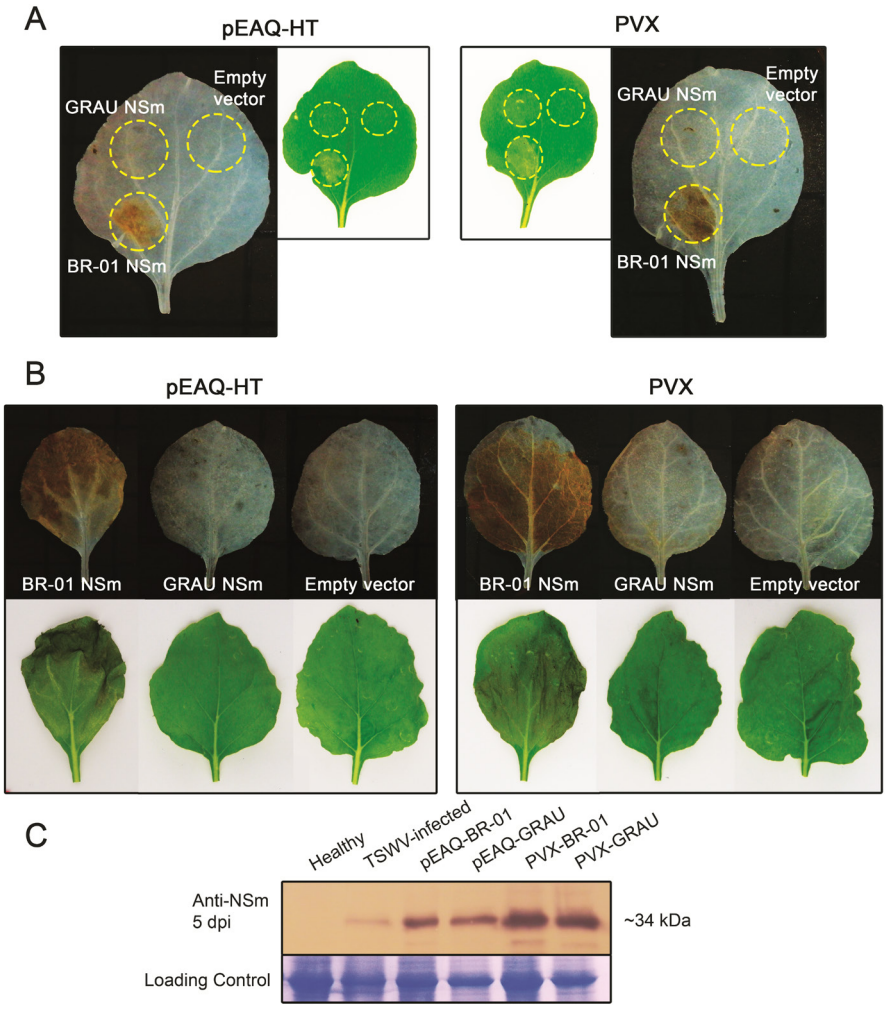


**Figure 3: Response of transgenic *N. benthamiana* (*Sw-5b*) leaves infiltrated with pEAQ-HT and PVX-constructs expressing $NS_M$ from isolates BR-01 and GRAU.** Pictures of leaves shown in panels A and B were taken 5-6 days post infiltration (d.p.i.). In both panels leaves were submitted to treatment with ethanol (destaining) for removal of chlorophyll to facilitate HR visualization; (A) Individual leaf simultaneously infiltrated with three constructs pEAQ-HT + $NS_M$ BR-01, pEAQ-HT + $NS_M$ GRAU and pEAQ-HT Empty vector (left side) and with PVX-Gw + $NS_M$ BR-01, PVX-Gw + $NS_M$ GRAU and PVX-Gw-Empty (right side) showing the visual response upon infiltration; (B) Whole leaves infiltrated with pEAQ-HT + $NS_M$ BR-01, pEAQ-HT + $NS_M$ GRAU and pEAQ-HT Empty vector (left side) and with PVX-Gw + $NS_M$ BR-01, PVX-Gw + $NS_M$ GRAU and PVX-Gw-Empty (right side) showing the visual response upon infiltration (C) Western immunoblot analysis with specific polyclonal antiserum against NSm protein (34 kDa) of TWSV. kDa = molecular weight marker protein; Samples were taken from *N. benthamiana* (*Sw-5b*) plants inoculated with the wild type TSWV virus and infiltrated with the constructs pEAQ-HT or PVX-Gw containing the $NS_M$ BR-01 and $NS_M$ GRAU. Extract from healthy plants was used as negative control.

**Induction of HR in *Sw-5* near isogenic tomato lines**

To further substantiate the observation that $NS_M$ presented the Avr determinant for *Sw-5b*-mediated resistance in a natural and commercial host fashion, a tomato breeding isoline (CNPH 'LAM 147') harboring a functional *Sw*-5 gene against tospovirus was agroinfiltrated with pEAQ-HT constructs of $NS_M^{RI}$ and $NS_M^{RB}$. After 4 days p.i. leaves infiltrated with $NS_M^{RI}$ showed a clear HR, while those with $NS_M^{RB}$ did not, as was more evident after chlorophyll removal from the leaves (Figure 4).

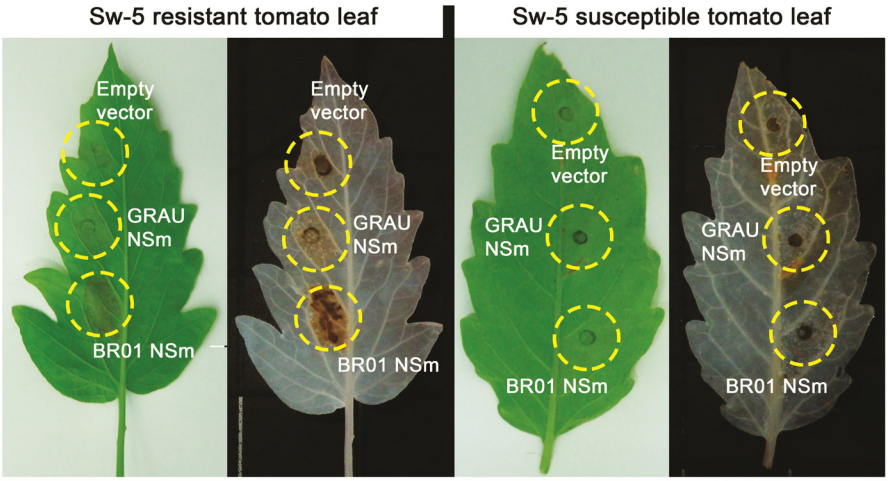


**Figure 4: Response of susceptible and resistant (harboring the *Sw*-5 gene) tomato near-isogenic lines leaves infiltrated with individual pEAQ-HT constructs expressing $NS_M$ from isolate BR-01 or GRAU**. Leaves shown in the panel were taken 4 days post infiltration (d.p.i.). In both cases leaves were submitted to treatment with ethanol (destaining) for removal of chlorophyll to facilitate HR visualization. Individual leaf from the resistant line (left side) and susceptible isoline (right side) were simultaneously infected with three constructs pEAQ-HT + $NS_M$ BR-01, pEAQ-HT + $NS_M$ GRAU and pEAQ-HT Empty vector. Leaves of the resistant line showed the visual necrotic response (HR lesions) upon infiltration.

## DISCUSSION

During earlier studies in which *N. tabacum* 'SR1' were transformed with *Sw-5* resistance gene candidates, *Sw-5b* was identified as the functional resistance gene copy (Spassova et al., 2001). Although, in those transgenic lines no clear HR was observed upon challenging with TSWV, transgenic plants were totally immune. Here we have successfully generated transgenic *N. benthamiana* containing *Sw-5b* that responded with an HR upon challenging with the resistance inducing TSWV BR-01 (wild type reference strain) but not

with the resistance breaker TSWV GRAU isolate (Aramburu and Marti, 2003). Although initially not reported for the transgenic *N. tabacum* (Spassova et al., 2001), challenging of the *Sw-5b*-transgenic *N. benthamiana* also exhibited resistance to GRSV and TCSV (data not shown), two tospovirus species phylogenetically close to TSWV and already reported to induce *Sw-5* resistance (Boiteux and Giordano, 1993). Surprisingly, protection was also observed against INSV, but not against MYSV which belongs to a distant phylogenetic lineage of EurAsian tospoviruses (de Oliveira et al., 2012), and indicate that *Sw-5b* resistance acts against a broad spectrum of related tospoviruses belonging to the American tospovirus clade.

Using the *Sw-5b*-transformed *N. benthamiana*, and confirmed with a near isogenic tomato line, it was shown that *Sw-5b*-governed resistance is triggered by the $NS_M$ cell-to-cell movement protein and not by the (precursor to the) glycoproteins, both candidates for the avirulence determinant (Hoffmann et al., 2001), as visualized by the induction of HR after expression of $NS_M$ from the resistance-inducing TSWV BR-01 isolate, but not from the resistance-breaker TSWV GRAU isolate. Both genes did not trigger such response when agroinfiltrated into untransformed *N. benthamiana* (Figure S1). In contrast to earlier work on the identification of the $NS_S$ RNA silencing suppressor as Avr determinant of the single dominant *Tsw* resistance gene (de Ronde et al., 2013), $NS_M$ was able to induce Sw-5b-mediated HR when expressed not only from the non-replicative pEAQ-HT vector system (Sainsbury et al., 2009) but also from a PVX-replicon (pGR107).

An HR response is not restricted to *R*-gene-mediated defense, as it can also result from basal defense and non-host resistance (Somssich and Hahlbrock, 1998) and can be uncoupled from the resistance response conferred by the *R* gene (Bendahmane et al., 1999), but in general is activated after *R* genes are being triggered and thereby used as an indirect indicator for *R* gene activation. The latter is assumed to involve direct or indirect interactions between the plant resistance gene product (*R*) and the pathogen avirulence factor (Avr) (Morel and Dangl, 1997). So far, only a small number of single dominant resistance genes against plant viruses have been cloned, and for those as well others of which the *R* genes have not been cloned yet, the polymerase protein (Kim and Palukaitis, 1997; Les Erickson et al., 1999), the movement protein (Weber et al., 1993; Yoshikawa et al., 2006), the coat protein (Asurmendi et al., 2004; Bendahmane et al., 2002) or the RNA silencing suppressor (Li et al., 1999; Malcuit et al., 1999; Oh, 1995; Román et al., 2011; de Ronde et al., 2013) have been identified as Avr determinant.

Nowadays, RNA silencing is generally accepted as a virus triggered immunity mechanism in plants and suppressed by viral RNA silencing suppressor proteins (RSS), alternatively referred to as effectors. *R*-gene mediated immunity is a second line of defense that is triggered by effectors. The resulting arms race, nicely illustrated by the zig-zag model

(Dangl and Jones, 2001), thereby implies a link between RNAi and *R*-gene mediated immunity for viral pathogens with a key role for viral RSS as effectors. Having identified the $NS_M$ movement protein as the Avr determinant for Sw-5b raises the question as to how this protein should be projected as an effector in this same Zig-Zag model. Only few cases are reported in which a plant viral cell-to-cell movement protein has been identified as effector and simultaneously shown to modulate the antiviral RNAi response (Lanfermeijer et al., 2003; Vogler et al., 2008). Whether TSWV $NS_M$ also contains such modulating activities remains to be investigated. Earlier, TSWV $NS_M$ has already been reported to provoke depositions of 1,3-ß-D-glucan (GLU) or callose in mesophyll plasmodesmata (Pd) and resembling GLU depositions observed during an HR upon viral infections (Rinne et al., 2005).

A recent bioinformatics analysis (Lopez et al., 2011) on $NS_M$ sequences available from databases, indicated that a few amino acid substitutions are responsible for the breakdown of resistance in tomato lines carrying the *Sw-5* gene. Although no experimental data was provided in support, the authors suggested that the ability of TSWV to infect resistant plants was related to replacement of a cysteine (C) to tyrosine (Y) at position 118 (C118Y) or a threonine (T) to asparagine (N) at position 120 (T120N). The resistance breaking isolate TSWV GRAU used in this study contained a C and N at positions 118 and 120, respectively, while the resistance inducing isolate TSWV BR-01 contained a C and T at those positions that are also found in TCSV and GRSV $NS_M$ sequences. INSV has only the T conserved as seen in the figure S3. Whether these residues indeed are responsible to overcome the resistance conferred by the dominant *Sw-5* gene can now be tested by mutating these residues within $NS_M^{RI}$ and in a complementing assay, reverting the corresponding residues in $NS_M^{RB}$ into those of the $NS_M^{RI}$ context.

While the *Sw-5b* gene has already been cloned a decade ago (Folkertsma et al., 1999; Spassova et al., 2001) and, similarly, the Avr determinant of *Sw-5b*-governed resistance mapped to the M RNA (Hoffmann et al., 2001), we here have provided evidence that identifies $NS_M$ as the trigger for Sw-5 resistance. Unraveling the mechanism by which Sw-5b is being triggered will now become a challenge and not only contribute to insight into dominant resistance genes, but may also help to understand the broad spectrum resistance of Sw-5b (against TSWV, TCSV, GRSV and INSV), a feature that is quite uncommon for a single dominant resistance gene, and which will be essential in light of developing broad-spectrum resistance strategies.

## SUPPORTING INFORMATION

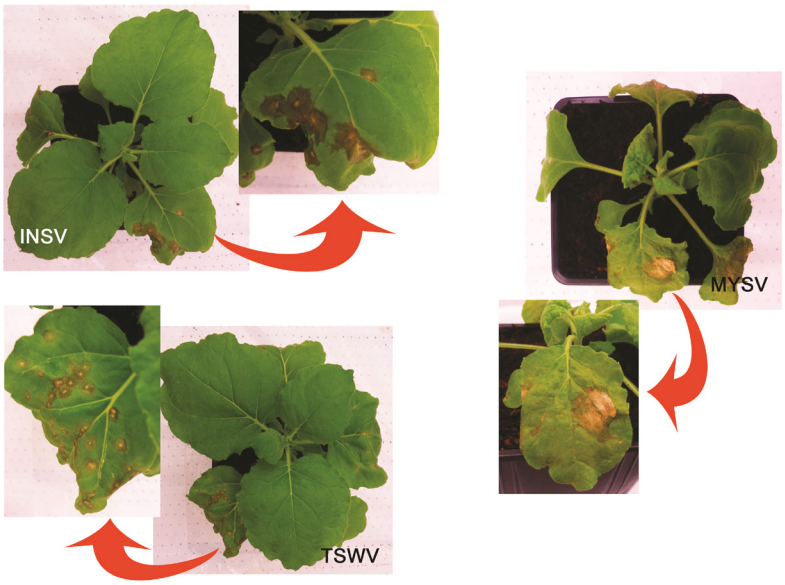


**Figure S1: Response of *Sw-5b* transgenic *N. benthamiana* on challenging with TSWV, INSV and MYSV.** While TSWV and INSV induce a clear local HR, MYSV inoculation leads to a local and systemic infection.

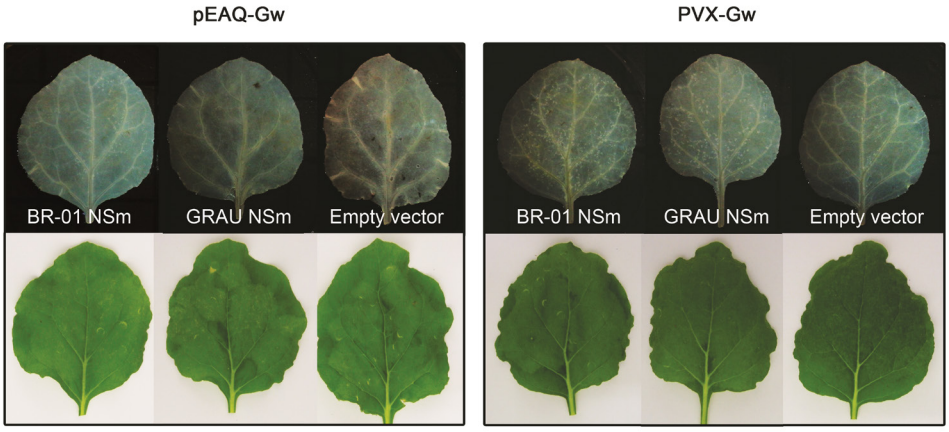


**Figure S2: Response of untransformed, wild type *N. benthamiana* leaves infiltrated with pEAQ-HT and PVX-constructs expressing $NS_M$ from isolates BR-01 and GRAU.**

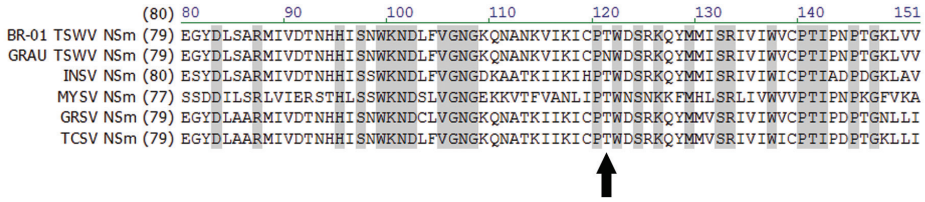


**Figure S3: Alignment of the cell-to-cell movement proteins ($NS_M$) discussed in this study. Identical amino acid residues are shaded in grey.** The arrow indicates position 120 for *Tomato spotted wilt virus* (TSWV) $NS_M$. GenBank accession numbers of the sequences: S58512 (TSWV BR-01), FM163370 (TSWV GRAU), AF513220 (*Groundnut ringspot virus*, GRSV), AF213674 (*Tomato chlorotic spot virus*, TCSV), M74904 (*Impatiens necrotic spot virus*, INSV) and AB061773 (Melon yellow spot virus, MYSV).

## MATERIAL AND METHODS

### Virus and plant material

Two TSWV isolates were used in this study, i.e. the Sw-5b resistance-inducing (RI) reference strain BR-01 (de Avila *et al*., 1993) and the resistance-breaking (RB) isolate GRAU (Aramburu and Marti, 2003). Isolates were maintained by mechanical inoculation on *N. benthamiana* and grown under greenhouse conditions (24 °C with a 16h light/8h dark regime).

### *N. benthamiana* transformation (*Sw-5b* gene)

The construct pBIN+Sw-5b containing the functional resistance gene copy *Sw-5b* was introduced into *Agrobacterium tumefaciens* strain LBA4404 and used to generate *N. benthamiana* transformants similar to the procedure earlier described to generate transgenic *N. tabacum* SR1 containing various *Sw-5* gene copies (Spassova et al., 2001).

### Development of tomato near-isogenic lines harboring the *Sw-5* gene

The *S. lycopersicum* cultivar 'Santa Clara' (highly susceptible to *Tospovirus* species) was crossed (as female parent) with the resistant cultivar 'Viradoro', a germplasm source of the *Sw-5* locus derived from the tomato cultivar 'Stevens' (Dianese et al., 2011). After seven generations of backcross breeding (using 'Santa Clara' as a recurrent parent) a *Tospovirus*-

resistant near-isogenic line was selected from a segregating $F_8$ population. This inbred line (named as 'CNPH LAM 147') was phenotypically very similar to 'Santa Clara', differing, however, for its resistant response to *Tospovirus* due to the presence of the genomic segment encompassing the locus with *Sw*-5 gene cluster (Dianese et al., 2010). Progeny tests were carried out with 'Santa Clara' and 'CNPH LAM 147' in order to confirm the homozygous condition of both inbred lines. Seeds of these two genotypes were sown in 5L pots filled with sterile soil and maintained in a greenhouse. Mechanical inoculation with TSWV (BR-01 isolate) was done in 20 plants of 'Santa Clara' and 20 plants of the 'CNPH LAM 147' line, following standard procedures (Boiteux and Giordano, 1993). A co-dominant molecular marker system (Dianese et al., 2010) able to discriminate heterozygous resistant (*Sw*-5/*sw*-5) from homozygous resistant (*Sw*-5/*Sw*-5) plants was employed in conjunction with the inoculation assays aiming to obtain two pure and contrasting near-isogenic lines. Total genomic DNA was extracted from asymptomatic 'CNPH LAM 147' and also from the mock-inoculated 'Santa Clara' plants. A single dominant homozygous resistant (*Sw*-5/*Sw*-5) plant of the line 'CNPH LAM 147' and a single recessive homozygous susceptible (*sw*-5/*sw*-5) 'Santa Clara' plant were selected. Seeds of these two contrasting genotypes were multiplied under greenhouse conditions and used in all subsequent assays.

**Cloning of N, $NS_M$, $NS_S$ and the glycoprotein precursor (GP) genes into binary vectors**

The TSWV BR-01 glycoprotein precursor (GP) gene was excised with *Bam*HI from pBIN19-GP (Bucher et al., 2003), and after agarose gel-electrophoresis purified and treated with T4 DNA polymerase. The pENTR11 entry vector was digested with *Eco*RI and after agarose gel electrophoresis purified and religated to remove the *ccdB* region. The resulting pENTR11 vector, lacking the *ccdB* region was digested with *Eco*RV and after dephosphorylation used for cloning of the blunt-ended GP gene, yielding the vector pENTR11 + $GP_{BR\text{-}01}$. The TSWV BR-01 $NS_M$ gene was amplified by PCR from pGR107+$NS_{M\text{-}BR\text{-}01}$ (Lovato et al., 2008), while the GRAU $NS_M$ gene was amplified by RT-PCR from total RNA of TSWV GRAU-infected *N. benthamiana* extracted with Trizol (Invitrogen). For the cDNA synthesis, M-MLV reverse transcriptase was used (Promega). The PCRs were performed with primers (listed below) containing attB sites (Gateway technology, Invitrogen) and the resulting PCR products were recombined into pDONR207 entry vector using BP Clonase (Invitrogen), yielding pDONR207 + $NS_M$-$_{BR\text{-}01}$ and pDONR207 + $NS_{M\text{-}GRAU}$. Primers for BR-01 and GRAU $NS_M$ amplification: $NS_M$-BR01-pD1 (GGGGACAAG TTTGTACAAA AAAGCAGGCT TCGAAGGAG ATAGAACCAT GTTGACTCTT TTCGGTAACAA), $NS_M$-BR01-pD2 (GGGGACCACT TTGTACAAGA AAGCTGGGTC CTATATTTCA TCAAAGGATA ACTG), $NS_M$-GRAU-pD1 (GGGGACAAGT TTGTACAAAA AAGCAGGCTT CGAAGGAGAT AGAACCATGT TGACTTTTTT CAGCAATAAG) and $NS_M$-GRAU-pD2 (GGGGACCACT TTGTACAAGA AAGCTGGGTC CTATATTTCA TCAAAAGATA ACTG).

The entry vectors pENTR11 + $GP_{BR-01}$, pDONR207 + $NS_M$-$_{BR-01}$, and pDONR207 + $NS_M$-$_{GRAU}$ were recombined into Gateway-compatible pEAQ-HT and pGR107 (Lacorte et al., 2010) by LR Clonase (Invitrogen) yielding pEAQ-HT-$GP_{BR-01}$, pEAQ-HT-$NS_{M-BR01}$, pEAQ-HT-$NS_{M-GRAU}$, pGR107-$NS_{M-BR01}$, and pGR107-$_{GRAU}$. The binary vectors pEAQ-HT harboring TSWV BR-01 N and $NS_S$ genes were previously built and used as described (de Ronde et al., 2013). All constructs were transformed into *A. tumefaciens* strain Cor308 or LBA4044. All standard procedures were performed following the manufactures' recommendations and Green et al. (2012).

**Agroinfiltration and monitoring of HR**

*Agrobacterium* infiltration assays were performed according to the protocol of Bucher et al. 2003, with slight modifications. To this end, *A. tumefaciens* Cor308 or LBA4044 (Ooms et al., 1982) harboring single constructs were grown overnight at 28 °C in LB3 medium containing 2 µg/ml tetracycline and 100 µg/ml kanamycin selection pressure. From this culture, 600 µl was freshly inoculated into 3 ml induction medium and grown overnight. Leaves were agroinfiltrated with a suspension containing a final $O.D._{600nm}$ 1.0 per construct. Development of HR was monitored daily up to 12 days after agroinfiltration. For easy monitoring and confirmation of HR, chlorophyll was removed from infiltrated leaves with ethanol and acetic acid 3:1 (v/v), respectively.

**RNA extraction and RT-PCR**

RNA extraction of wild type and *Sw-5b*-transformed *N. benthamiana* leaves were carried out using Trizol Reagent (Invitrogen). Amplification of N genes via RT-PCR were performed using M-MLV reverse transcriptase (Promega) and Go Taq DNA polymerase (Promega) with the specific primers: TSWV-N-F (ATGTCTAAGGTTAAGCTCACTA), TSWV-N-R (TCAAGCAAGTTCTGCGAGTTTT), INSV-N-F (ATGAACAAAGCAAA GATTACCA), INSV-N-R (TTAAATAGAATCATTTTTCCCA), MYSV-N-F (ATGTCTACCGTTGCTAAGCTGA), and MYSV-N-R (TTAAACTTCAATGGACTTAGAT). All procedures followed the manufacturers' instructions.

**Western immunoblot detection of viral proteins**

For western immunoblot detection of TSWV proteins, four leaf discs from *N. benthamiana* leaves were macerated in 400 µL of phosphate buffer (0.01 M, pH 7.0). Supernatant was collected and supplemented with 2X Laemmli buffer (Sigma-Aldrich, USA).

Denatured proteins were resolved on 12.5% SDS-PAGE and after electrophoresis were transferred to immobilon-P membrane (Millipore-USA). Blotted membranes were blocked with PBS (50mM sodium phosphate buffer; 10mM NaCl) containing 2% non-fat milk, after that the blots were incubated using polyclonal antibodies (1µg/mL) specific for TSWV N, $NS_S$, $NS_M$ and GP proteins (de Avila et al., 1990; Kormelink et al., 1991 and 1994; Kikkert et al., 1999).

# Chapter 3

# The $NS_M$ protein lacking viral movement motifs still triggers Sw-5b-mediated resistance

Athos Silva de Oliveira, Renato O. Resende and Richard Kormelink

**ABSTRACT**

*Tomato spotted wilt virus* (TSWV) is a tospovirus species belonging to the family *Bunyaviridae*. In recent years, this virus has gained attention for causing damage to numerous crops worldwide. Two natural resistance sources, however, have been found against TSWV and allowed the breeding of tomato and sweet pepper. For tomatoes, the source of resistance is the *Sw-5b* gene which codes an NB-LRR receptor that perceives the presence of the cell-to-cell movement protein $NS_M$ of TSWV and activates disease resistance which includes hypersensitive cell death response (HR). Apart from that, a considerable gap in knowledge exists between $NS_M$ and Sw-5b interaction. In the present study, we have investigated whether truncated versions of $NS_M$ lacking motifs associated with tubule formation, cell-to-cell or systemic viral movement still activate Sw-5b-mediated HR like the full length $NS_M$ protein. Upon co-expression of Sw-5b and $NS_M$ truncations in *Nicotiana benthamiana* leaves, HR was still triggered when 50 amino acids were deleted from the amino- or carboxy-terminal ends suggesting that motifs involved in viral movement are not required for $NS_M$ recognition by the Sw-5b protein.

**INTRODUCTION**

*Tomato spotted wilt virus* (TSWV) is notorious for being one of the most devastating plant viruses worldwide, causing severe damage to many economically important agronomical crops. TSWV was first reported in Australia by Brittlebank in 1919 (Stevens et al., 1991) but has re-emerged since the 1990's due to the worldwide expansion of its most important thrips vector *Frankliniella occidentalis* (Western flower thrips). Annual outbreaks are nowadays common in tropical and temperate climate zones, where new susceptible host species have been recorded (Pappu et al., 2009). TSWV belongs to the genus *Tospovirus*, which constitutes the only genus with plant-infecting members within the family *Bunyaviridae* (King et al., 2012a). TSWV has a tripartite RNA genome of negative and ambisense polarity that contains five open reading frames (ORFs) coding for 4 structural (L, Gn, Gc, N) and 2 non-structural ($NS_M$ and $NS_s$) proteins. Current efforts for TSWV disease management involves the implementation of cultural, phytosanitary, and chemical tactics (Pappu et al., 2009). To ease the financial and environmental constraints associated with insecticide abuse, greater focus has been put on obtaining genetically resistant cultivars as an integral component of disease management strategies.

The *Sw-5* gene cluster from *Solanum peruvianum* is the only known resistance source available for commercial breeding of tomato against TSWV (Boiteux and Giordano, 1993; Stevens et al., 1991). Although five *Sw-5* paralogs, named *Sw-5a* to *Sw-5e,* have been cloned from this gene cluster from resistant tomato, only *Sw-5b* has been shown to confer

resistance against TSWV (Spassova et al., 2001). The *Sw-5b* gene codes for a member of the NB-LRR receptor protein family with a coiled-coil (CC) N-terminal domain (Spassova et al., 2001). Recently, the $NS_M$ of TSWV has been reported as the avirulence determinant (Avr) of Sw-5b, inducing a hypersensitive cell death response (HR) in both *Sw-5b*-transformed *N. benthamiana* and in *Sw-5*-resistant tomato isolines (Chapter 2). The $NS_M$ protein is a non-structural protein that facilitates the spread of tospoviruses in plants by cell-to-cell movement via plasmodemata (Kormelink et al., 1994; Storms et al., 1995). To this end, the protein forms tubules protruding from plasmodesmata from the infected- into the neighboring healthy cells and modifies their size exclusion limit. The TSWV $NS_M$ protein consists of 301 amino acids (for TSWV) and domains required for tubule formation, cell-to-cell movement and long distance movement have been mapped by earlier studies (Figure 1).

During the last two decades, resistance-breaking TSWV isolates have emerged, most likely due to high selection pressure on the virus, thereby threatening the durability of the Sw-5b-resistance (Gordillo et al., 2008). While this has provoked increased efforts to screen germplasms and breeding lines for resistance, understanding the underlying molecular mechanisms linked to resistance breakdown has thus far proven being important. Using 18 different TSWV isolates, Lopez et al. (2011) reported that resistance conferred by Sw-5b can be overcome by single-amino acid substitutions in the $NS_M$ protein as a result of positive selection as opposed to recombination. It remains to be established, however, whether the Sw-5b and $NS_M$ proteins directly interact or if there are host intermediates. Furthermore, in light of the broad resistance spectrum of Sw-5b, it is not known yet whether avirulence is correlated to any of the (conserved) domains in $NS_M$ required for cell-to-cell-movement functionality. In this study, truncated versions of the $NS_M$ protein lacking viral moment motifs have been made and co-expressed with Sw-5b in *N. benthamiana* leaves for HR monitoring. The results show that truncations at the amino- and carboxy terminus of the $NS_M$ protein, that affect its functionality as movement protein, do not abrogate avirulence.

## RESULTS

To test whether Sw-5b-mediated HR triggering by $NS_M$ required cell-to-cell movement functionality, six truncations of the $NS_M$ protein from the resistance-inducing TSWV isolate BR-01 were made (Figure 1). Upon their co-expression with Sw-5b in *N. benthamiana* leaves the onset of HR was monitored up to 4 days post agroinfiltration (dpa). While the full-length $NS_M$ protein from the resistance inducing (RI) BR-01 isolate (positive control), but not from the resistance-breaking TSWV isolate GRAU (negative control), led to a strong HR-response, many of the $NS_M$ truncations still triggered an HR. Results of the screening at 4 dpa are summarized in Table 1. Constructs with truncations of 25-50 amino

acids (aa) from the C-terminus gave HR responses comparable to the BR-01 positive control, but a deletion of 100 aa at the C-terminus led to a notably weaker HR induction relative to the control (Fig. 3A). Similar observations were made with N-terminal deletion constructs, in which $NS_M$ lacking the first 25 or 50 aa still induced an HR response that did not differ from the BR-01 positive control. However, no detectable HR was observed when 100 aa were removed from the N-terminal region (Fig. 3B). To rule out that the absence of HR triggering was not due to the lack of protein expression, the synthesis of $NS_M$ truncations was verified by western immunoblot analysis (Fig.2C). Altogether, the results indicate the importance of the central 51-252 aa region in triggering Sw-5b-mediated cell death.

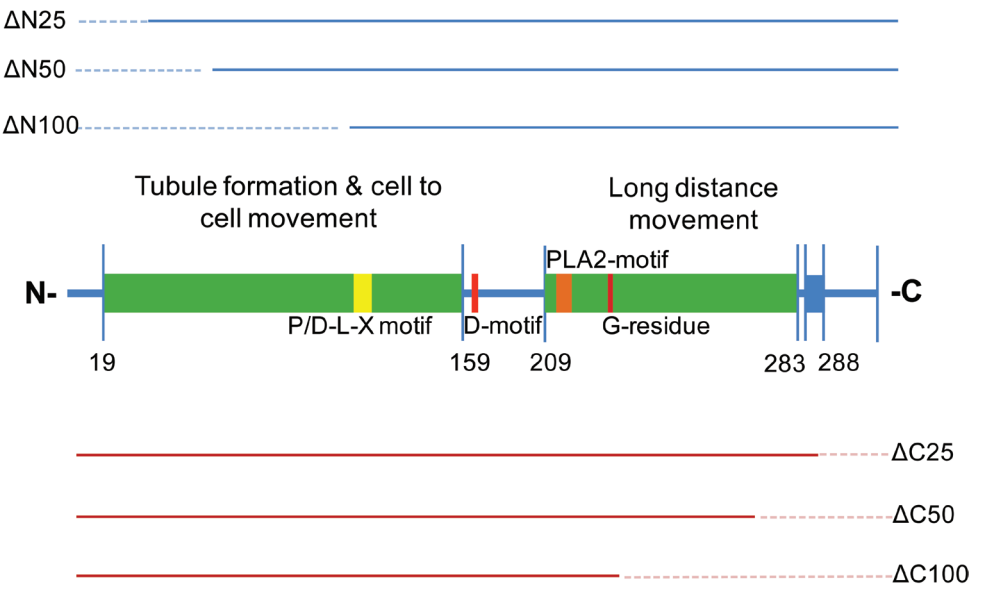


**Figure 1. Schematic diagram of the $NS_M$ protein (301 aa) highlighting major motifs and functional regions.** In green are regions of the protein reported to be necessary for tubule formation and cell-to-cell movement (Li et al., 2009). In blue is a region involved in long distance movement. Interspersed throughout the entire length of the protein are additional motifs. Specifically, the P/D-L-X motif, G-residue and D-motifs are conserved among members of the '30K superfamily' of virus movement proteins (Silva et. al, 2001). In addition to the motifs typical for the 30K superfamily, TSWV $NS_M$ has a region homologous to the phospholipase 2 (PLA2) catalytic site that is often observed in tospoviruses with a broad host range. This scheme was made in reference to http://www.uniprot.org/uniprot/P36292. The lines alongside the $NS_M$ scheme depict the six deletion constructs of the $NS_M$ protein, successively removing 25, 50 and 100 amino acids either from the N-terminus (blue) or the C-terminus (red). Each construct was co-infiltrated with *Sw-5b* into wild type *N. benthamiana* leaves.

**Table 1**. Screening of cell death responses at 4 dpa upon the co-expression of truncated TSWV BR-01 $NS_M$ versions and Sw-5b in *N. benthamiana* leaves.

| | -25 aa | -50 aa | -100 aa |
|---|---|---|---|
| C-terminus | ++ | ++ | +/- |
| N-terminus | +++ | +++ | - |

Key: +: weak HR response; ++: strong HR response; +++: very strong HR response. Note, severity was deduced relative to the BR-01 positive (+) control. The $NS_M$ from GRAU was included as negative (-) control.

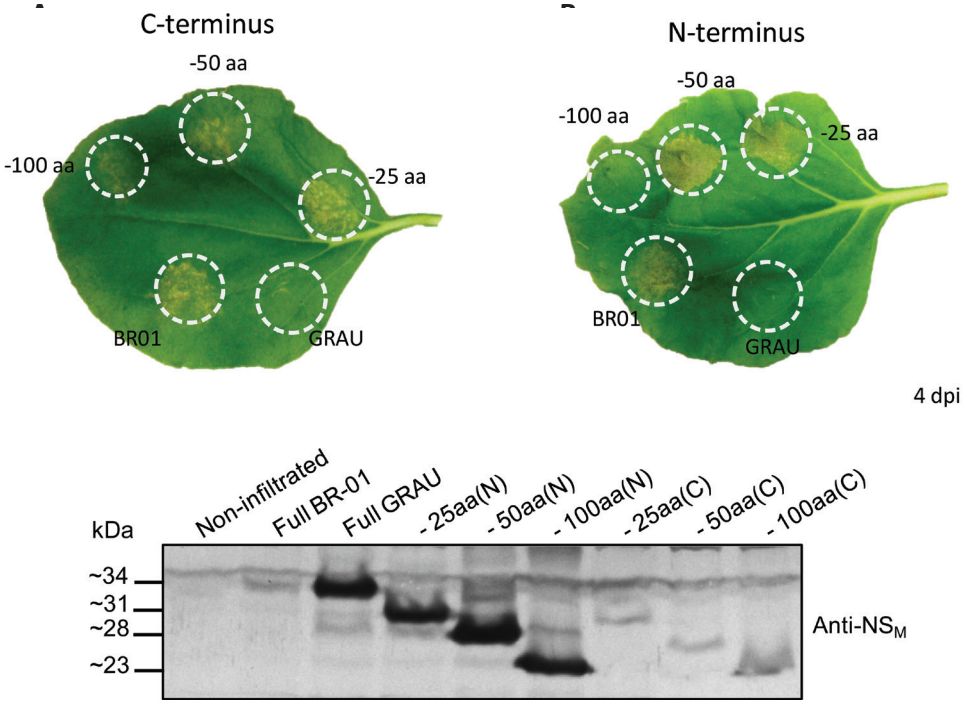


**Figure 2: Co-expression of truncated $NS_M$ proteins and Sw-5b in *N. benthamiana* leaves.** (A) Co-expression of the 25 and 50 aa C-terminal deletion constructs gave clear cell death responses on infiltrated sites, whereas removing 100 aa from the C-terminus gave comparatively weaker HR response. (B) No evident cell death response was observed following co-agroinfiltration with the 100 aa N-terminal deletion construct. Both the 25 and 50 aa N-terminal deletion constructs gave similar HR responses to the positive BR-01 control. (C) Western immunoblot detection of full-length and truncated $NS_M$ proteins in extracts of agroinfiltrated *N. benthamiana* leaves. Anti-$NS_M$ was used as primary antibody. Pictures and samples for Western analysis were taken at 4 dpa.

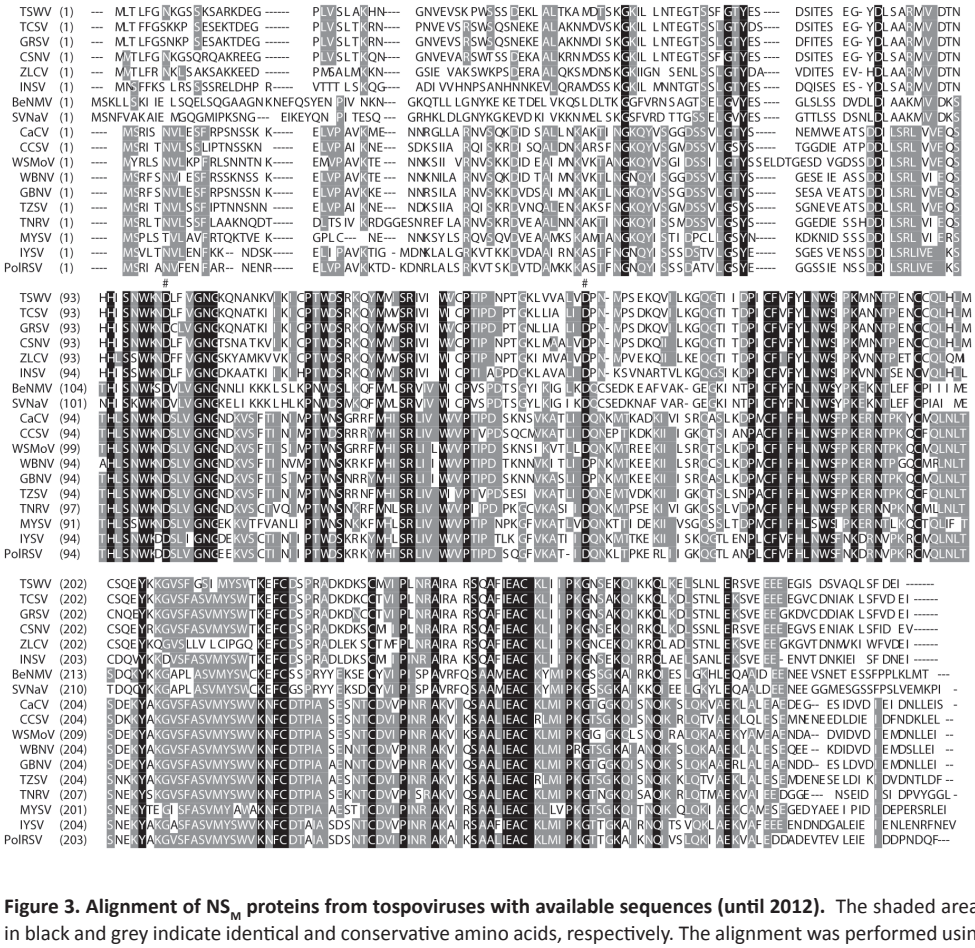

**Figure 3. Alignment of $NS_M$ proteins from tospoviruses with available sequences (until 2012).** The shaded areas in black and grey indicate identical and conservative amino acids, respectively. The alignment was performed using the software ClustalW. # indicates conserved aspartate residues found in "30K-superfamily". Full names and GenBank accession numbers of tospoviruses used: *Tomato spotted wilt virus* TSWV (NC_002050), *Tomato chlorotic spot virus* TCSV (AF213674), *Groundnut ringspot virus* GRSV (AF513220), Chrysanthemum stem necrosis virus CSNV (AF213675), *Zucchini lethal chlorosis virus* ZLCV (AF213676), *Impatiens necrotic spot virus* INSV (NC_003616), Bean necrotic mosaic virus BeNMV (JN587269), Soybean vein necrosis associated virus SVNaV (HQ728386), Capsicum chlorosis virus CaCV (DQ256125), Calla lily chlorotic virus CCSV (FJ822961), *Watermelon silver mottle virus* WSMoV (NC_003841), Watermelon bud necrosis virus WBNV (GU584185), *Groundnut bud necrosis virus* GBNV (NC_003620), Tomato zonate spot virus TZSV (NC_010490), Tomato necrotic ringspot virus TNRV (FJ947152), Melon yellow spot virus MYSV (NC_008307), Iris yellow spot virus IYSV (AF214014), and Poligonum ringspot virus PolRSV (EU271753).

## DISCUSSION

The TSWV $NS_M$ cell-to-cell movement protein gene represents the main genetic adaptation of bunyaviruses to infect plant hosts. The protein associates with viral ribonucleoproteins (RNPs) to assist their movement through $NS_M$-derived tubules in plasmodesmata (Kormelink et al., 1994; Storms et al., 1995). In recent years, domains have

been mapped in the TSWV $NS_M$ protein related to tubule formation, movement, and plant host-symptomatology (Li et al., 2009). Here, a small study has been performed on the requirement of some of these sequences in the elicitation of Sw-5b resistance against tospoviruses (Chapter 2). It is shown that $NS_M$ containing truncations of up to 50 aa at both ends do not affect its avirulence to trigger Sw-5 mediated HR. Considering that these truncations do disrupt (some of) its cell-to-cell movement functionality (references), the results altogether indicate that avirulence and cell-to-cell movement are not functionally linked, as recently also observed for TSWV NSs RNA silencing suppression activity and avirulence-determinance of the *Tsw* resistance gene (de Ronde et al., 2014b).

Although removal of the N- and C-terminal aa sequences from $NS_M$ leads to a loss of tubule formation and virus movement (Figure 1), they show some diversity among tospovirus $NS_M$ proteins, especially at the N terminus (Figure 3). A more detailed look at the C-terminus also shows the presence of a stretch of Glutamate (E) residues that are negatively charged. A similar stretch can be observed in the C-terminus of the RdRP (L) protein and could reflect a binding domain for (positively charged) viral RNPs. If so, removal of the $NS_M$ C-terminal domain would lead to the inability to bind to RNPs and targeting them to the plasmodesmata, but apparently not required for triggering of Sw-5b. In contrast to the N- and C-terminal ends, the central core of $NS_M$ shows a highly conserved nature. In light of this, it is tempting to speculate that the central domain of the protein reflects the most essential part for cell-to-cell movement while the termini have a more supporting role in proper folding, targeting and as scaffold for tubule formation and RNP ligand. Whether this core directly interacts with Sw-5b or is being indirectly perceived, remains to be investigated. In case of an indirect interaction, the core domain could be used in a CoIP approach to identify a potential guardee/decoy, but prior to this a BiFC approach might be followed first to test for a potential direct interaction between Sw-5b and $NS_M$.

## MATERIALS AND METHODS

### Molecular Cloning

pDONR207 harboring the $NS_M$ gene from the TSWV isolate BR-01 (Chapter 2) was used as template for amplification of truncated gene versions by PCR. Primers annealing at the beginning (for C-terminal deletion constructs) or at the end (for N-terminal deletion constructs) of the $NS_M$ gene (Chapter 2) were combined with the following primers to truncate the coding sequence: N-25aa-F GGGGACAAGT TTGTACAAA AAAGCAGGCT TCGAAGGAGA TAGAACCATG CATAATGGCA GTGTTGAAGTC; N-50aa-F GGGGACAAGT

TTGTACAAAA AAGCAGGCTT CGAAGGAGAT AGAACCATGG CATCCAAAGG AAAGATACTG; N-100aa-F GGGGACAAGT TTGTACAAAA AAGCAGGCTT CGAAGGAGAT AGAACCATGC TTTTTGTTGG CAACGGAAAG; C-25aa-R GGGGACCACT TTGTACAAGA AAGCTGGGTC CTATGAGCTC AGTTCTTTAA GCTG; C-50aa-R GGGGACCACT TTGTACAAGA AAGCTGGGTC CTAAATGAAT GCCTGAGATC TAGC; C-100aa-R GGGGACCACT TTGTACAAGA AAGCTGGGTC CTAACACATC AAATGCAGCT GACA. The PCR products were then recombined with pDONR207 by BP clonase (Invitrogen). Finally, the resulting entry vectors were recombined with pK2GW7 (Karimi et al., 2002) by LR clonase (Invitrogen) and used to transform the *Agrobacterium tumefaciens* strain Core308.

**Agroinfiltration, protein extraction and western blotting**

The agroinfiltration assays were performed as previously described (Bucher et al., 2003). After harvesting and resuspending the bacteria in MS-MES buffer, *N. benthamiana* leaves were infiltrated with combinations of bacterial suspensions containing a final $OD_{600}$ of 1.0 per construct. Pictures were taken at 4 dpa. For protein extraction leaves were harvested 3 dpa. To this end, about 100 mg of leaf material per sample was ground in liquid nitrogen and subsequently mixed with 250 µl of 95ºC pre-heated Berger-buffer (Berger et al., 1989). Samples were incubated for 10 minutes at 95ºC and centrifuged at 12000 x g for 10 minutes. Supernatants were transferred to new tubes and mixed with a similar volume of 2x SDS-loading (Laemmli loading buffer) buffer. Samples were incubated at 95ºC for 3 minutes. A volume of 15 µl per sample was loaded onto 10% SDS-PAGE gels. Western immunoblotting was performed as previously described (Chapter 2).

**Acknowledgments**

We would like to thank Octavina C.A. Sukarta and Sytske Drost for performing the agroinfiltration experiments.

# Chapter 4

# Characterization of Bean Necrotic mosaic virus: a new “American” tospovirus species that does not trigger the Sw-5b-mediated HR

This chapter has been published in a slightly modified version as:

Athos Silva de Oliveira, Fernando Lucas Melo, Alice Kazuko Inoue Nagata, Tatsuya Nagata, Elliot Watanabe Kitajima and Renato Oliveira Resende (2012). Characterization of Bean necrotic mosaic virus: a member of a novel evolutionary lineage within the genus *Tospovirus*. PLoS ONE. DOI: 10.1371/journal.pone.0038634

**ABSTRACT**

Tospoviruses (Genus *Tospovirus*, Family *Bunyaviridae*) are phytopathogens responsible for significant crop losses worldwide. They have a tripartite negative and ambisense RNA genome with segments termed S (Small), M (Medium) and L (Large) RNA. The vector-transmission is mediated by thrips in a circulative-propagative manner. For acceptance of new tospovirus species by the International Committee on Taxonomy of Viruses, several analyses are needed, e.g., the determination of the viral protein sequences for enlightenment of their evolutionary history. Biological (host range and symptomatology), serological, and molecular (S and M RNA sequencing and evolutionary studies) experiments were performed to characterize and differentiate a new tospovirus, Bean necrotic mosaic virus (BeNMV), which naturally infects common beans in Brazil. Based upon the results, BeNMV can be classified as a novel species and, together with Soybean vein necrosis-associated virus (SVNaV), is a member of a new evolutionary lineage within the genus *Tospovirus* circulating in the American continent. Co-expression of the BeNMV-$NS_M$ protein with Sw-5b in wild type *Nicotiana benthamiana* leaves did not trigger a hypersensitive response (HR) in contrast to the *Tomato spotted wilt virus* (TSWV) $NS_M$ protein.

**INTRODUCTION**

*Tospovirus* is the genus with plant-infecting viruses of the family *Bunyaviridae* and its members are responsible for significant quality and yield losses to crops worldwide (Pappu et al., 2009). The tospoviruses have enveloped quasi-spherical particles and a tripartite negative and ambisense RNA genome containing five open reading frames (King et al., 2012b). The genomic segments are denominated according to their size, as S (Small), M (Medium) and L (Large). The S RNA encodes a non-structural RNA-silencing suppressor protein ($NS_S$) and the nucleocapsid protein (N) (Dehaan et al., 1990; Takeda et al., 2002). The M RNA encodes a cell-to-cell movement protein ($NS_M$) and the precursor to the envelope glycoproteins (GP) (Kormelink et al., 1992; Storms et al., 1998). The L RNA encodes an RNA-dependent RNA polymerase (RdRp), also called L protein (Dehaan et al., 1991).

Tospoviruses are transmitted by thrips insects (Order Thysanoptera) in a circulative-propagative manner (Ullman et al., 1993; Wijkamp et al., 1993). Despite the existence of more than 5,000 thrips species, only 14 species are known as potential tospovirus vectors and most of them belong to the genera *Frankliniella* and *Thrips* (Riley et al., 2011). The *Frankliniella* genus is neotropical with all but seven vector species considered endemic to the New World (Nakahara, 1997), while the worldwide distributed genus *Thrips* has no vector species native to South America (Mound, 2002). Interestingly, the natural distribution of these vector species is somewhere reflected in the tospovirus phylogenetic relationships,

with *Fankliniella*-transmitted tospoviruses clustering in an "American lineage" and *Thrips*-transmitted tospoviruses clustering in an "Eurasian lineage" (Pappu et al., 2009). Another evolutionary lineage is formed by two tospoviruses isolated from peanut and transmitted by thrips from genus *Scirtothrips* (Inoue and Sakurai, 2007).

Recently, two new tospoviruses were described infecting soybean (*Glycine max* (L.) Merr.) (Zhou et al., 2011) and common bean (*Phaseolus vulgaris* L.) (de Oliveira et al., 2011), both of them clustered together and apart from the other tospovirus lineages. While the genome of the soybean-infecting tospovirus Soybean vein necrosis-associated virus (SVNaV) has been completely sequenced (Zhou et al., 2011), only the RNA-dependent RNA polymerase gene of the common bean tospovirus (Bean necrotic mosaic virus, BeNMV) has been determined (de Oliveira et al., 2011). No biological characterization has been reported for either virus. Therefore, we carried out an extensive analysis of a BeNMV isolate, including the study of its host range, symptomatology, serological differentiation, and genome sequencing which identified this virus as a representative of a new evolutionary lineage within the genus *Tospovirus*.

## RESULTS AND DISCUSSION

### Polyclonal antibodies against the nucleoprotein discriminate BeNMV from other Brazilian tospoviruses

After nucleocapsid (N) purification from BeNMV-infected *Physalis pubescens* L., a protein of approximately 29 kDa was visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). Polyclonal antibodies against this protein were produced in rabbits. The serological differentiation performed through a dot enzyme-linked immunosorbent assay (DOT-ELISA) revealed the presence of an N protein distinct from the N protein of 4 other Brazilian tospoviruses: *Tomato spotted wilt virus* (TSWV) (de Avila et al., 1993), *Tomato chlorotic spot virus* (TCSV) (de Avila et al., 1993), *Groundnut ringspot virus* (GRSV) (de Avila et al., 1993) and *Zucchini lethal chlorosis virus* (ZLCV) (Bezerra et al., 1999) (Figure 1). A negligible cross-reaction was observed between BeNMV and TSWV and GRSV, strengthening the idea that BeNMV is a new Brazilian tospovirus. Usually, when polyclonal antibodies against the N proteins are utilized, a strong cross-reaction is visualized between phylogenetically close species (Chu et al., 2001; Hassani-Mehraban et al., 2010), challenging the serological diagnosis.

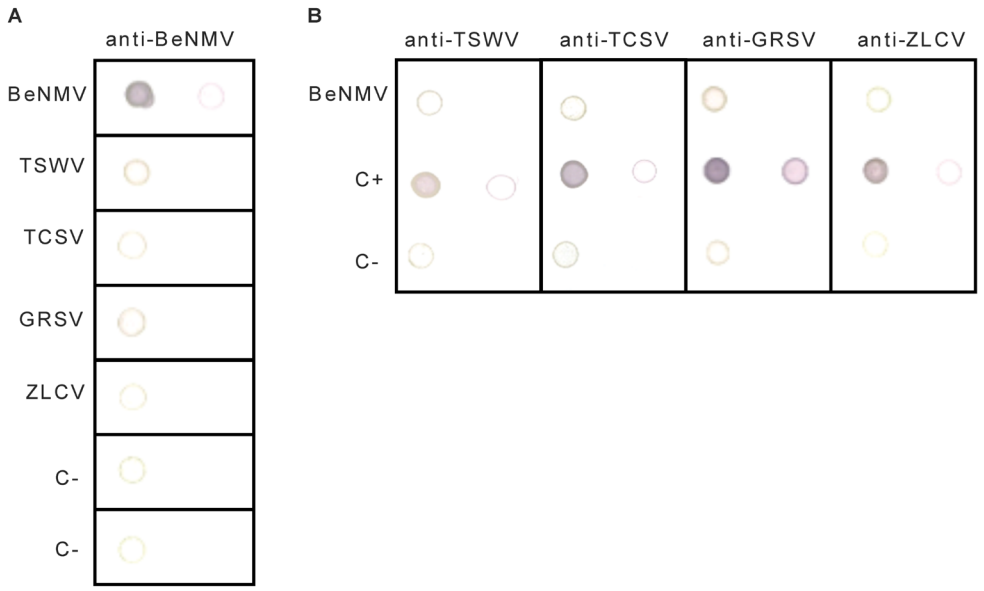


**Figure 1. Serological differentiation between BeNMV and other Brazilian tospovirus species.** A) Tospovirus-infected plant extracts were dot blotted on nitrocellulose and incubated with anti-BeNMV polyclonal serum. As plant hosts, *Physalis pubescens* was used for BeNMV and *Datura stramonium* for the others. Healthy *P. pubescens* and *D. stramonium*, respectively, were included as negative (C-) controls. B) Screening of BeNMV infected samples for cross-reaction with polyclonal antisera directed to the other tospovirus species (Confirmation of the virus presence in the samples utilized and evaluation of cross-reactions). The dots in the first column represent 1:100 dilutions of infected leaf material (leaf mass per volume of 0.5X PBS (g/ml), while the second column shows 1:1000 dilutions.

### BeNMV has a very limited host range by mechanical inoculation

Twenty distinct plants, including various common test-plants and some fabaceous plants, were inoculated with the BeNMV isolate to assess for transmission by mechanical inoculation, host range and symptomatology (Table 1). Interestingly, only *Phaseolus vulgaris* cv. Santana, *Datura stramonium* L. and *P. pubescens* exhibited systemic symptoms post-inoculation (Table 1 and Figure 2). Specifically, foliar deformation, interveinal chlorosis and stunting were seen in *P. vulgaris* (Figure 2B). In *D. stramonium* the symptoms consisted of mottling, necrotic lesions, foliar deformation and stunting (Figure 2C), while *P. pubescens* plants exhibited mottling and stunting (Figure 2D). To further confirm that BeNMV did not replicate in the other *P. vulgaris* varieties, a DOT-ELISA to detect the N protein was performed to evaluate the degree of systemic infection, using both inoculated and upper leaves. Only

**Table 1. Host range of BeNMV determined via mechanical inoculation 4 days post-inoculation.**

| Plant host | Local symptoms | Systemic symptoms |
|---|---|---|
| **Chenopodiaceae** | | |
| *Chenopodium amaranticolor* | NL | - |
| *Chenopodium quinoa* | NL | - |
| *Chenopodium murale* | NL | - |
| **Cucurbitaceae** | | |
| *Cucurbita pepo* | CS | - |
| **Fabaceae** | | |
| *Phaseolus vulgaris* "Manteiga" | - | - |
| *Phaseolus vulgaris* "Santana" | - | St, IC, FD |
| *Phaseolus vulgaris* "BT2" | - | - |
| *Vigna angularis* | - | - |
| *Vigna unguiculata* | - | - |
| **Solanaceae** | | |
| *Capsicum annuum* | - | - |
| *Capsicum chinense* | - | - |
| *Datura metel* | NL | - |
| *Datura stramonium* | - | NL, Mo, FD, St |
| *Solanum lycopersicum* | - | - |
| *Nicandra physaloides* | - | - |
| *Nicotiana benthamiana* | - | - |
| *Nicotiana rustica* | - | - |
| *Nicotiana tabacum* Samsun | NL, VC | - |
| *Nicotiana tabacum* TNN | NL | - |
| *Physalis pubescens* | CS | Mo, St |

*CS* chlorotic spots; *FD* foliar deformation; *IC* interveinal chlorosis; *Mo* mottling; *NL* necrotic lesion; *St* Stunting; *VC* vein chlorosis

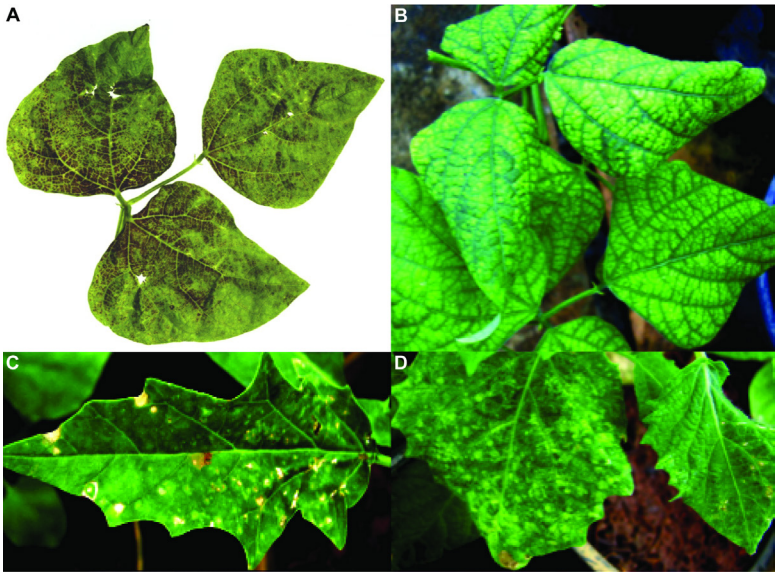


**Figure 2. Symptoms caused by BeNMV under field and greenhouse conditions.** A) *Phaseolus vulgaris* showing mosaic necrotic symptoms under field conditions. B), C) and D) *P. vulgaris* cv "Santana", *Datura stramonium*, and *Physalis pubescens* presenting systemic symptoms under greenhouse conditions 4 days post-inoculation, respectively.

*P. vulgaris* cv. Santana was found positive (Supplementary Figure 1). Seven out of twenty tested plants reacted with local symptoms, which later did not evolve to systemic infection (Table 1). So far, no such limited host range has been observed with any other known tospovirus species.

The transmission by mechanical inoculation of BeNMV proved difficult, noticeable from its narrow host range. Despite the transmission to *P. vulgaris*, its natural host, the field-observed symptoms (Figure 2A) were not totally reproducible in the greenhouse. After repeated attempts, only one out of three common bean varieties was susceptible. Similar observations where made with the tospovirus Alstroemeria necrotic streak virus (ANSV), for which mechanical inoculation to its natural host also failed (Hassani-Mehraban et al., 2010). For BeNMV, a more efficient transmission might be achieved using thrips species known to serve as tospovirus vectors. However, it cannot be excluded that like ZLCV, which has a distinct host range and exclusive vector (Bezerra et al., 1999; Nakahara and Monteiro, 1999), BeNMV is also being transmitted by a new thrips species, different from all those identified for tospoviruses present in Brazil so far.

The difference between field symptoms and those observed under greenhouse conditions may have originated from genotypic differences between *P. vulgaris* collected in

the field (cv. unknown) and the cultivar "Santana". On the other hand, environmental interferences cannot be discarded as having played a part in the difference between symptoms. Furthermore, the allocated period for observation (4 weeks) may not have been sufficient for the development of necrotic lesions.

**The S and M RNA of BeNMV share a similar genetic organization with those of other tospoviruses**

The S and M RNA sequences of BeNMV were assembled from the nucleotide sequences determined from viral-specific clones obtained from a cDNA library and by using degenerate and specific primers in reverse transcription coupled with polymerase chain reaction (RT-PCR) (Figure 3).. The S RNA of BeNMV contains 2,584 nucleotides (nt) and two open reading frames (ORFs) in ambisense gene arrangement like in all other tospoviruses. The viral strand contains an ORF of 1,320 nt that starts at nucleotide position 61 and terminates at position 1,380 and codes for the $NS_S$ protein of 439 aa with a predicted molecular mass of 49.2 kDa. The viral complementary strand contains an ORF of 813 nt that starts (as numbered from the viral strand) at nucleotide position 2,508 and terminates at nucleotide 1,696 and codes for the N protein of 270 amino acids (aa) with a predicted molecular mass of 29.8 kDa. The S RNA contains a 5' untranslated region (UTR) and a 3'UTR of 60 and 76 nt, respectively. The two ORFs are separated by an A/U-rich (79.4%) intergenic region (IR) of 315 nt, the second smallest IR from any tospovirus S RNA sequence elucidated so far (Supplementary Table 1). The nucleotide and amino acid sequences are available in GenBank under the accession number JN587268.

The M RNA contains 4,886 nts and two ORFs in ambisense arrangement, like the S RNA segment, encoding the GP and $NS_M$ proteins. The $NS_M$ protein gene starts at position nt 65 and terminates at position nt 1,018, resulting in an ORF of 954 nt and coding for a protein of 317 aa with a predicted molecular mass of 35.4 kDa. The size of this $NS_M$ protein is the largest among all tospoviruses analyzed so far (Supplementary Table 1). In the viral complementary strand, the GP gene starts (numbering from the viral strand) at position nt 4,803 and terminates at nt 1,318, generating an ORF of 3,486 nts, coding for a protein of 1,161 aa with a predicted molecular mass of 130.7 kDa. Both ORFs are separated by A/U-rich (82.6%) IR with a size of 299 nt, and a 5'- and 3'UTR of 64 respectively 83 nt. The nucleotide and amino acid sequences are available in GenBank under the accession number JN587269.

Like with all tospoviruses (King et al., 2012b), the S and M genomic segments of BeNMV contain an ambisense gene configuration. Both molecules showed the highest similarity to those of SVNaV (Zhou et al., 2011). Structural characteristics such as size of the

segments, ORFs, and non-coding sequences, as well as predicted molecular mass of their proteins are similar (Supplementary Table 1). For all BeNMV S and M RNA encoded proteins, highest identity was observed with those of SVNaV (Supplementary Table 2).

The topology of GP revealed six putative N-glycosylation sites ($Asn_{123}$, $Asn_{207}$, $Asn_{320}$, $Asn_{360}$, $Asn_{521}$, and $Asn_{1048}$), but no O-glycosylation sites. Similar to other tospoviral GP proteins, two putative cleavage sites were predicted. The first one localizes in the N-terminal region between $Leu_{20}$ and $Asp_{21}$, immediately following a putative signal peptide sequence. The second one between $Ala_{468}$ and $Met_{469}$, which most likely presented the cleavage site for proper GP processing into the individual Gn and Gc proteins. Three transmembrane domains were predicted (from 297 aa to 319 aa, 326 aa to 348 aa, and 1075 aa to 1097 aa), while other programs sometimes additionally predicted two more domains (from 6 aa to 24 aa and 442 aa to 460 aa). Since all features of the BeNMV GP protein resembles those of other tospoviruses (Knierim et al., 2006), functions related to particle assemble and tospovirus-thrips interactions performed by the glycoproteins can therefore be extrapolated to this new virus (Ribeiro et al., 2008; Snippe et al., 2007; Whitfield et al., 2005).

Recently, features of the L RNA segment of BeNMV have been described (de Oliveira et al., 2011) and shown to resemble those of RNA-dependent-RNA polymerase genes from other tospovirus species. With 2,932 aa and a predicted molecular mass of 335.9 kDa, the BeNMV L protein is the largest RdRp for this genus at the present date.

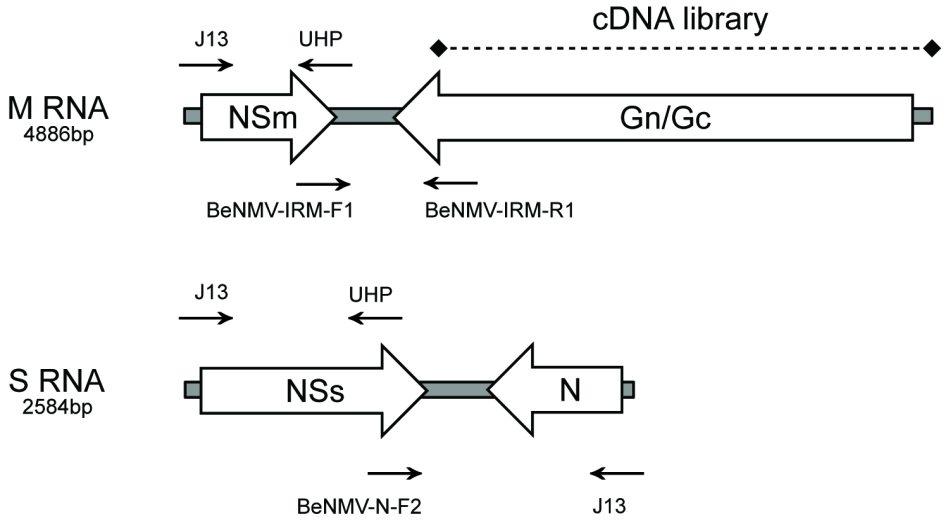


**Figure 3. Cloning strategy for S and M RNA segments.** Arrows indicate primers and their annealing positions. The dotted line marks the genomic region from which the sequence was obtained by the cDNA library methodology.

**Phylogenetic analysis confirmed that BeNMV is, indeed, a distinct tospovirus species**

The nucleoprotein (N) is commonly used for taxonomic classification (Cortez et al., 2001; Dong et al., 2008; Seepiban et al., 2011) and new species should exhibit no more than 89% amino acid sequence identity to another member of the genus (King et al., 2012b). Therefore, to clarify the evolutionary relationship between BeNMV and other tospoviruses, a data set composed of N protein sequences from distinct tospoviruses was collected. A pairwise comparison showed that BeNMV N differs from those of other tospoviruses, with identities ranging from 17.2% to 52.2% (Supplementary Table 2). This degree of identity is much lower than the established threshold for new species acceptance (<90% identity), confirming that BeNMV is a new tospovirus. Although we did not include all known tospoviruses in this analysis, the lack of serological cross-reactivity in DOT-ELISA experiments (Figure 1) also supports this idea.

The maximum likelihood tree based on the N protein is shown in Figure 4. As previously observed (de Oliveira et al., 2011; Inoue and Sakurai, 2007), the American and Eurasian clades form distinct monophyletic groups (bootstrap values of 98 and 98, respectively), while Peanut chlorotic fan-spot virus (PCFSV) (Chen and Chiu, 1995) and *Peanut yellow spot virus* (PYSV) (Satyanarayana et al., 1998) formed a monophyletic basal clade among tospoviruses. Crucially, the BeNMV was related to SVNaV, and formed a monophyletic clade (bootstrap value of 93%), consistent with their pairwise distances (Supplementary Table 2). The phylogenetic trees generated from $NS_S$, $NS_M$ and GP data sets are shown in Figure 4. Furthermore, a phylogenetic tree inferred from the concatenated protein sequences (RdRp, N, $NS_S$, $NS_M$ and GP) robustly supports the observed clades (Figure 5), except for the PCFSV-PYSV clade, which was not included in this latter analysis. Within all these trees BeNMV consistently clustered with SVNaV, suggesting that reassortments were not involved in the origin of this BeNMV isolate.

The results confirmed that BeNMV is distinct from other characterized tospovirus species, as previously suggested based on multiple sequence alignments with the RNA-dependent RNA polymerase (RdRp) protein (de Oliveira et al., 2011). The correspondence between N and RdRp protein phylogeny suggests that both genes can be used to understand the phylogenetic relationships among tospoviruses, with the advantage of using a more conserved region for RdRp, which consequently, simplifies PCR-based strategies for detection of highly divergent new viruses.

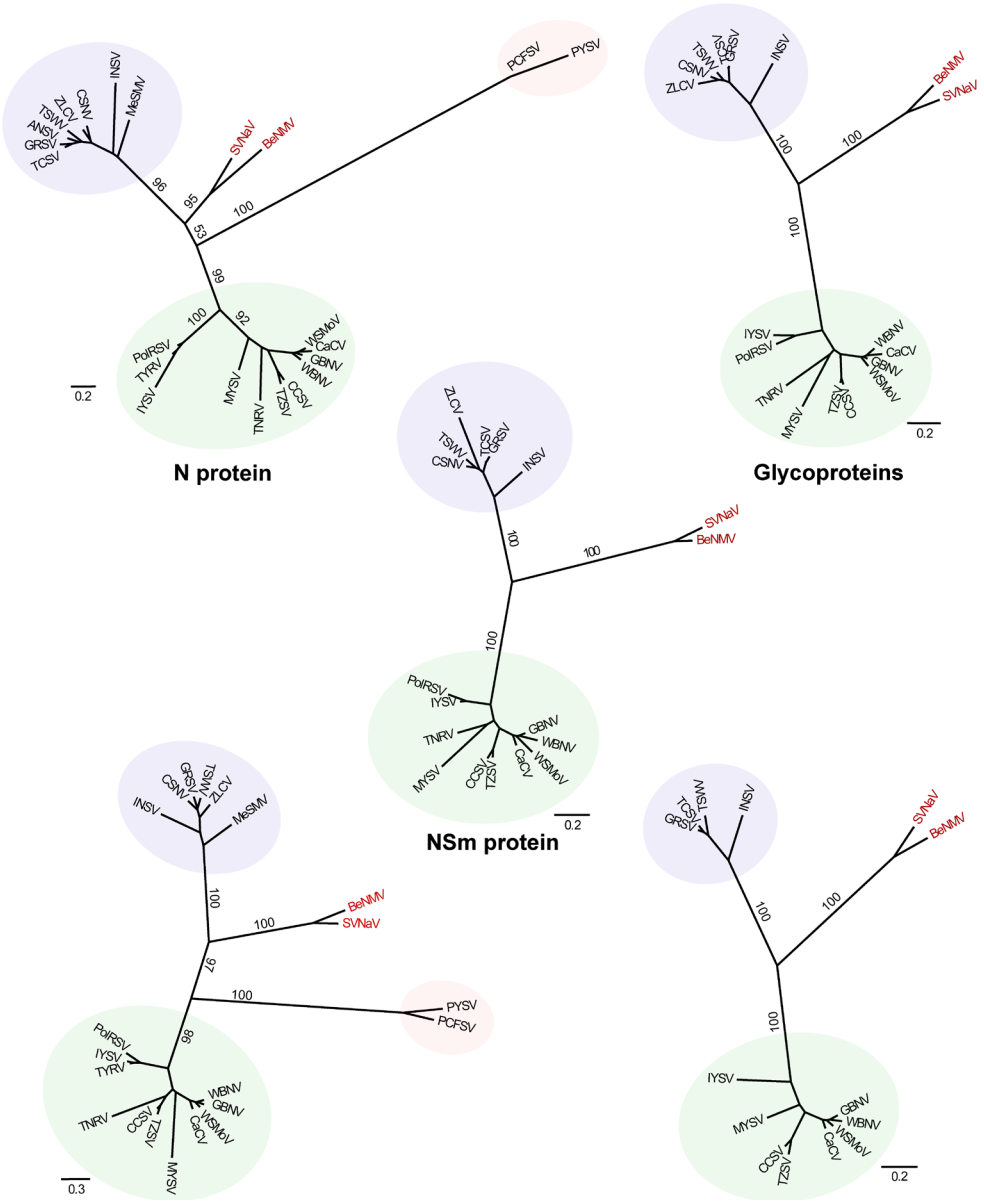


**Figure 4. Phylogenetic relationships of *Tospovirus* species inferred using S and M RNA-encoded proteins.** The trees were inferred with the maximum likelihood criterion implemented in the program RAxML. Node support was evaluated using non-parametric bootstrap resampling (500 replicates) and values are shown for key nodes. The BeNMV-SVNaV clade is shown in red. The shaded areas in purple, green and red represent the "Eurasian", "American" and PCFSV-PYSV clades, respectively. Tree inferred using **N protein** alignment: 23 taxa and 217 amino acids. Tree inferred using the **$NS_M$ protein** alignment: 18 taxa and 291 amino acids. Tree inferred using the **$NS_S$ protein** alignment: 21 taxa and 391 amino acids. Tree inferred using the **GP** alignment: 18 taxa and 1007 amino acids. Tree inferred using the **RdRp protein** alignment: 14 taxa and 2811 amino acids. The complete viral names are found in the Supplementary Table 2.

**The BeNMV-SVNaV clade is a novel evolutionary lineage within the genus *Tospovirus***

The phylogenetic analysis (Figure 4 and Figure 5) also showed that the BeNMV-SVNaV clade was almost equidistant between the American and Eurasian lineages, suggesting that this clade constitutes a novel evolutionary lineage within the tospoviruses. Importantly, when the PCFSV-PYSV clade was included in the analysis (N and $NS_S$ protein phylogenies) the BeNMV-SVNaV clade was most closely related to the American lineage (Figure 4). To further confirm this finding, the likelihood of the N protein best tree (lnL= -12018.58) (Figure 4) was compared to those estimated from alternative trees, constraining the BeNMV-SVNaV clade to be related to the Eurasian lineage (lnL= -12025.58) or to PCFSV-PYSV clade (lnL= -12024.78). Each of these alternative phylogenies was rejected with a Bayes factor above 5, further corroborating the suggested shared ancestry between the BeNMV-SVNaV clade and the American lineage. Altogether, these results strengthen the notion that the BeNMV-SVNaV clade is a distinct, fourth, clade of the genus *Tospovirus*.

This finding could have implications for tospovirus diagnosis and crop production. First, the available diagnostic reagents for tospoviruses, such as polyclonal serum and degenerated primers, might not detect viruses belonging to this new clade. Actually, several previously described primers designed for N gene amplification were not capable to detect BeNMV (data not shown), including those described for SVNaV (Zhou et al., 2011). Second, based on the diversity of the American and Eurasian clades, more species related to the BeNMV-SVNaV clade probably remain to be discovered. Along this line, the economic impact of tospoviruses from this clade on agriculture still remains to be estimated.

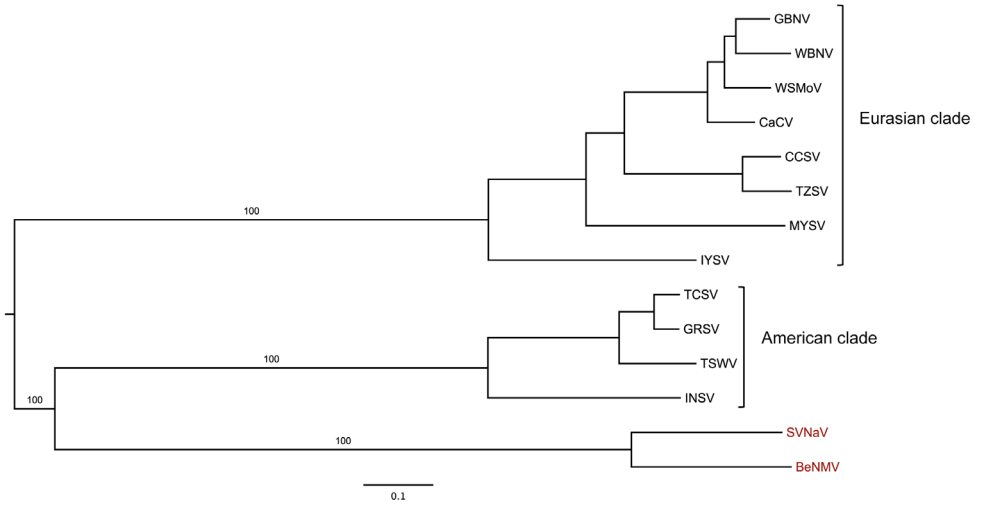


**Figure 5. Phylogenetic relationships of *Tospovirus* species inferred using a concatenated dataset (N, $NS_S$, $NS_M$, GP and RdRp).** The tree was inferred with the maximum likelihood criterion implemented in the program RAxML.

Node support was evaluated using non-parametric bootstrap resampling (500 replicates) and values are shown for key nodes. The tree is mid-point rooted for purposes of clarity. The BeNMV-SVNaV clade is shown in red and the previously described lineages are highlighted. The data set includes 14 taxa and 4721 amino acids.

**Evidence of episodic diversifying selection in the branches leading to the BeNMV-SVNaV clade and the American clade**

Intriguingly, BeNMV and SVNaV were isolated from common bean and soybean, respectively, which may indicate that viruses of this clade preferentially infect plants of the Fabaceae family, similar to the PCFSV-PYSV clade (Chen and Chiu, 1995; Satyanarayana et al., 1998). In fact, if the different viral proteins were evolving in response to plant or insect host specificity, an increase in the ratio of non-synonymous (Ka) to synonymous substitutions (Ks) would be expected on those nodes leading to the different lineages. Therefore, a random effects branch-site model (Pond et al., 2011) was implemented in order to detect lineages on which a proportion of sites has evolved under positive selection. We found evidence of episodic diversifying selection only in the $NS_M$ and RdRp datasets, along the branches leading to (*i*) the BeNMV-SVNaV and the American clade, to (*ii*) the BeNMV-SVNaV clade and to (*iii*) the American clade (Figure 6). It is hard to determine the biological constraints responsible for these events. Particularly, the occurrence of diversifying selection does not seem to be correlated with the broadness of the plant host range. However, when we analyzed the viral vector range it was possible to observe that the American clade is transmitted by several species of the *Frankliniella* genus (at least 7), while those viruses from the Eurasian clade are predominantly transmitted by only two species of the genus *Thrips* (Inoue and Sakurai, 2007)2007. It is important to stress that sequence divergence is expected to influence the power of the branch-site tests because many synonymous sites might be saturated (Yang and dos Reis, 2011). However, our results are reinforced by experimental data showing that both $NS_M$ and RdRp proteins are known to interact with the plant and insect restriction factors (Lopez et al., 2011; Medeiros et al., 2004), suggesting a classic evolutionary arms race. Actually, one of these studies showed that positive selection on the $NS_M$ protein was implicated in the tomato *Sw-5* gene resistance breaking by TSWV (Lopez et al., 2011).

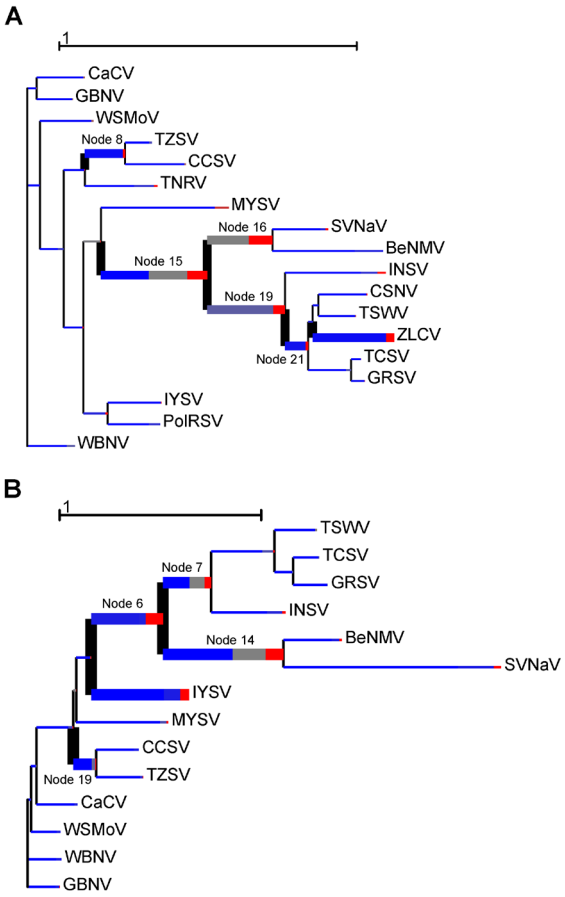


**Figure 6. Episodic diversifying selection detected on $NS_M$ and RdRp alignments.** Each tree is scaled on the expected number of substitutions/nucleotide. The hue of each color indicates strength of selection, with red corresponding to dn/ds >5, blue to dn/ds =0 and grey to dn/ds =1. The width of each color represents the proportion of sites in the corresponding class. Thicker branches have been classified as undergoing episodic diversifying selection (p>0.05).

## Co-expression of Sw-5b and BeNMV-$NS_M$ proteins in *N. benthamiana* does not lead to HR triggering

Recent evidence identified the $NS_M$ protein of TSWV is the Avr determinant of Sw-5b-mediated resistance (Chapter 2). Transient expression of this viral protein in *Sw-5b*-transformed *N. benthamiana* and Sw-5-resistant tomato isolines leads to HR triggering. Furthermore, INSV inoculation of *Sw-5b*-transformed *N. benthamiana* also resulted in HR triggering, indicating that the Sw-5b-mediated resistance works against "American"

tospoviruses. To test whether Sw-5b resistance also holds against members of this newly proposed, second evolutionary lineage circulating in the American continent, the BeNMV $NS_M$ protein was co-expressed with Sw-5b in wild type *N. benthamiana*. Since no antibodies were available for this protein, GFP was fused to either ends. In contrast to the $NS_M$ proteins from TSWV (including fusions to GFP (not shown)) and INSV, the expression of BeNMV-$NS_M$ protein did not trigger Sw-5b-mediated HR (Figure 7). Although Sw-5 originates from tomato, which does not present a host for BeNMV (Table 1), its resistance does not seem to be triggered by BeNMV. These results thereby further strengthens the position of this virus in a separate, distinct clade from the other American tospovirus species. As a consequence, the episodic diversifying selection leading to the BeNMV-SVNaV clade does not include Sw-5b selection pressure.

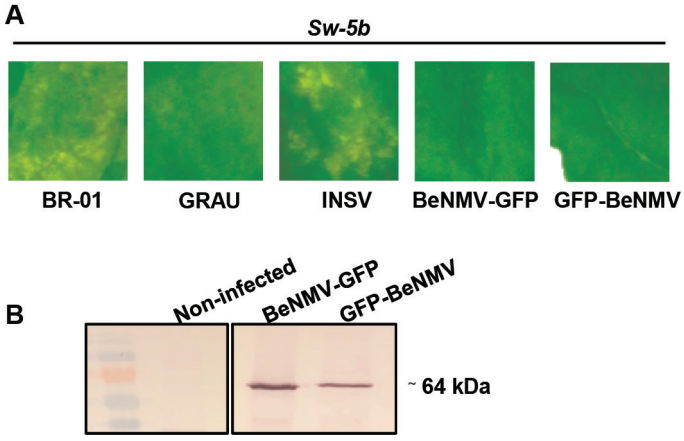


**Figure 7. Co-expression of $NS_M$ and Sw-5b proteins in *N. benthamiana*.** (A) Co-expression of $NS_M$ proteins from the resistance-inducing TSWV isolate (BR-01), the resistance-breaking TSWV isolate (GRAU), INSV and BeNMV with Sw-5b in *N. benthamiana*. GFP was fused at the N- or C-terminus of BeNMV-$NS_M$. (B) Western immunoblot detection of BeNMV $NS_M$ protein fused to GFP. Anti-GFP was used as primary antibody.

## SUPPORTING INFORMATION

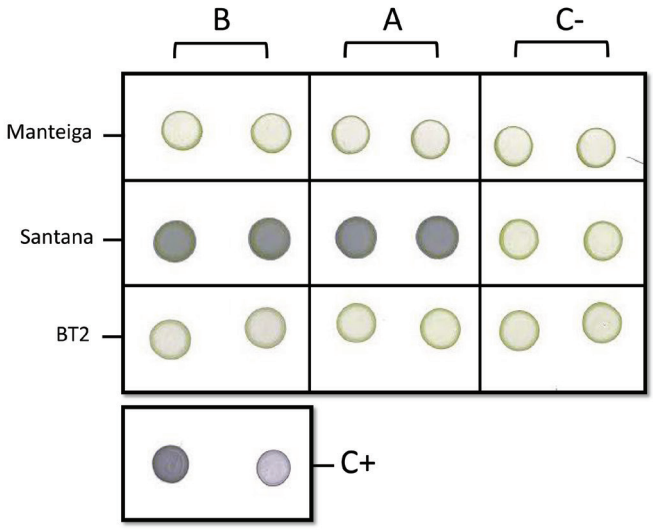


**Figure S1. DOT-spot immunodetection of BeNMV nucleoprotein detection in extracts from *Phaseolus vulgaris* varieties.** Polyclonal antibody against BeNMV nucleoprotein was used. A. Extract of inoculated leaves. B. Extract of systemic leaves. Uninfected *P. vulgaris* was used as negative control (C−). Infected *Physalis pubescens* was used as positive control (C+). The dots are 1:100 dilutions (leaf mass per volume of 0.5× PBS (g/ml).

**Supplementary Table 1.** Characteristics of the S and M RNA for the available tospoviruses.

| Virus | S RNA length (nt) | 5'UTR (nt) | 3'UTR (nt) | Intergenic region (nt) | N protein (aa) | N protein mass (kDa) | NSs protein (aa) | NSs protein mass (kDa) |
|---|---|---|---|---|---|---|---|---|
| ANSV | - | - | - | - | 258 | 28.7 | - | - |
| **BeNMV** | 2,584 | 60 | 76 | 315 | 270 | 29.8 | 439 | 49.2 |
| CaCV | 3,477 | 66 | 67 | 1,196 | 275 | 30.6 | 439 | 49.6 |
| CCSV | 3,172 | 66 | 64 | 825 | 277 | 30.4 | 460 | 51.8 |
| CSNV | 2,940 | 79 | 152 | 531 | 260 | 29.2 | 464 | 51.7 |
| GBNV | 3,057 | 66 | 67 | 773 | 276 | 30.6 | 439 | 49.5 |
| GRSV | - | - | - | - | 258 | 28.7 | - | - |
| INSV | 2,992 | 62 | 149 | 642 | 262 | 28.7 | 449 | 51.1 |
| IYSV | 3,105 | 70 | 70 | 811 | 273 | 30.3 | 443 | 50 |
| MeSMV | 3,283 | 80 | 159 | 887 | 262 | 29.5 | 455 | 51 |
| MYSV | 3,232 | 68 | 67 | 847 | 279 | 31 | 469 | 53.1 |
| PCFSV | 2,833 | 67 | 79 | 455 | 270 | 31 | 472 | 52.3 |
| PolRSV | 2,484 | 72 | 72 | 183 | 274 | 30.1 | 443 | 50.1 |
| PYSV | 2,970 | 57 | 76 | 653 | 246 | 28.1 | 480 | 53.4 |
| SVNaV | 2,603 | 58 | 70 | 318 | 277 | 30.7 | 440 | 49.8 |
| TCSV | - | - | - | - | 258 | 28.5 | - | - |
| TNRV | 3,023 | 65 | 66 | 690 | 281 | 30.9 | 451 | 51.3 |
| TSWV | 2,916 | 88 | 153 | 503 | 258 | 28.8 | 464 | 52.4 |
| TYRV | 3,061 | 71 | 71 | 762 | 274 | 29.9 | 443 | 50.2 |
| TZSV | 3,279 | 64 | 64 | 934 | 278 | 30.6 | 459 | 51.9 |
| WBNV | 3,401 | 66 | 67 | 1,120 | 275 | 30.5 | 439 | 49.6 |
| WSMoV | 3,534 | 66 | 65 | 1,255 | 275 | 30.6 | 439 | 49.7 |
| ZLCV | - | - | - | - | 260 | 29 | - | - |

| Virus | M RNA length (nt) | 5'UTR (nt) | 3'UTR (nt) | Intergenic region (nt) | GP (aa) | GP mass (kDa) | NSm protein (aa) | NSm protein mass (kDa) |
|---|---|---|---|---|---|---|---|---|
| **BeNMV** | 4,886 | 64 | 83 | 299 | 1,161 | 130.7 | 317 | 35.4 |
| CaCV | 4,823 | 56 | 47 | 427 | 1,121 | 127.2 | 308 | 34.1 |
| CCSV | 4,704 | 54 | 45 | 303 | 1,123 | 127.5 | 309 | 34.6 |
| CSNV | - | - | - | - | 1,135 | 127.5 | 303 | 34 |
| GBNV | 4,801 | 56 | 47 | 408 | 1,121 | 127.3 | 307 | 34.2 |
| GRSV | - | - | - | - | 1,133 | 127.5 | 303 | 33.8 |
| INSV | 4,972 | 85 | 169 | 473 | 1,110 | 124.8 | 303 | 34.1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| IYSV | 4,838 | 63 | 49 | 379 | 1,136 | 128.5 | 311 | 34.7 |
| MYSV | 4,815 | 58 | 48 | 398 | 1,127 | 128.2 | 308 | 34.3 |
| PolRSV | 4,689 | 62 | 28 | 267 | 1,135 | 128.5 | 307 | 34.1 |
| SVNaV | 4,955 | 57 | 92 | 267 | 1,195 | 134.3 | 316 | 35.3 |
| TCSV | - | - | - | - | 1,134 | 127.7 | 303 | 33.9 |
| TNRV | 4,716 | 59 | 48 | 307 | 1,122 | 128 | 310 | 34.2 |
| TSWV | 4,821 | 100 | 84 | 320 | 1,135 | 127.3 | 302 | 33.7 |
| TZSV | 4,945 | 54 | 46 | 546 | 1,122 | 127.5 | 309 | 34.5 |
| WBNV | 4,794 | 55 | 47 | 402 | 1,121 | 127.1 | 307 | 34.3 |
| WSMoV | 4,880 | 55 | 47 | 473 | 1,121 | 127.6 | 312 | 35 |
| ZLCV | - | - | - | - | 1,135 | 127.5 | 303 | 34.1 |

**Supplementary Table 2.** Sequence identity comparison (%) of BeNMV proteins from S and M RNA.

| | N | NSs | GPp | NSm |
|---|---|---|---|---|
| BeNMV | 100 | 100 | 100 | 100 |
| SVNaV | 52.2 | 61.6 | 64.5 | 75.2 |
| ANSV | 34.4 | - | - | - |
| CSNV | 35.5 | 18.8 | 36.0 | 37.6 |
| GRSV | 32.2 | - | 35.1 | 39.7 |
| INSV | 32.3 | 20.7 | 32.7 | 39.1 |
| MeSMV | 35.9 | 20.0 | - | - |
| TCSV | 34.1 | - | 35.3 | 38.9 |
| TSWV | 32.2 | 18.4 | 35.4 | 36.6 |
| ZLCV | 35.9 | - | 35.6 | 33.8 |
| CaCV | 27.1 | 18.2 | 31.5 | 36.5 |
| CCSV | 30.1 | 15.8 | 31.9 | 34.2 |
| GBNV | 28.5 | 17.6 | 32.2 | 36.8 |
| IYSV | 30.7 | 18.2 | 31.4 | 34.9 |
| MYSV | 28.5 | 16.2 | 30.6 | 34.3 |
| PolRSV | 31.6 | 18.7 | 30.3 | 35.7 |
| TNRV | 30.9 | 18.3 | 32.3 | 37.5 |
| TYRV | 30.5 | 18.9 | - | - |
| TZSV | 27.1 | 16.3 | 31.8 | 34.8 |
| WBNV | 27.9 | 17.3 | 31.6 | 36.2 |
| WSMoV | 28.2 | 18.0 | 31.9 | 36.3 |
| PCFSV | 18.0 | 14.9 | - | - |
| PYSV | 17.2 | 15.4 | - | - |

Acronyms: Bean necrotic mosaic virus (BeNMV), Soybean vein necrosis associated virus (SVNaV), Alstroemeria necrotic streak virus (ANSV), Chrysanthemum stem necrosis virus (CSNV), *Groundnut ringspot virus* (GRSV), *Impatiens necrotic spot virus* (INSV), Melon severe mosaic virus (MeSMV), *Tomato chlorotic spot virus* (TCSV), *Tomato spotted wilt virus* (TSWV), *Zucchini lethal chlorosis virus* (ZLCV), Capsicum chlorosis virus (CaCV), Calla lily chlorotic virus (CCSV), *Groundnut bud necrosis virus* (GBNV), Iris yellow spot virus (IYSV), Melon yellow spot virus (MYSV), Poligonum ringspot virus (PolRSV), Tomato necrotic ringspot virus (TNRV), Tomato yellow ring virus (TYRV), Tomato zonate spot virus (TZSV), Watermelon bud necrosis virus (WBNV), *Watermelon silver mottle virus* (WSMoV), Peanut chlorotic fan-spot virus (PCFSV), and *Peanut yellow spot virus* (PYSV). (-) Indicate the sequences are not available in GenBank database.

## MATERIALS AND METHODS

### Virus isolates, host range and symptomatology

The BeNMV isolate was isolated in São Paulo State in Brazil (de Oliveira et al., 2011) and maintained in *P. pubescens* by mechanical inoculation (Deavila et al., 1993). The other tospovirus isolates (TSWV, TCSV, GRSV and ZLCV) were maintained in *D. stramonium*. In order to determine host range and symptoms, several plant species (Table 1) were mechanically inoculated with BeNMV. The plants were maintained in the greenhouse and the onset of symptoms was observed up to four weeks post-inoculation.

### Virus purification and serology

Three weeks post mechanical inoculation, ribonucleocapsids were purified from 100 g of infected *P. pubescens* leaves following the protocol described by De Àvila et al. (Deavila et al., 1993). The purified ribonucleocapsids were injected in rabbits for polyclonal antibody production against the BeNMV N protein as described (Deavila et al., 1993). The serological differentiation was performed through DOT-ELISA between BeNMV and four tospovirus species found in Brazil (TSWV, TCSV, GRSV, and ZLCV) (Bezerra et al., 1999; de Avila et al., 1993). The antisera to other tospoviruses were kindly supplied by Embrapa Vegetables (Brazil).

### RNA extraction and sequencing

Genomic RNA was extracted from BeNMV ribonucleocapsids. For each 250 µl of sample 750 µL of Trizol LS (Invitrogen) was used and RNA purification was performed following the manufacturer's recommendations. To determine the S and M RNA sequences two procedures were adopted. The first was the construction of a cDNA library using the Universal Riboclone cDNA Synthesis System Kit (Promega). By this method, only the BeNMV RNA-dependent RNA polymerase (de Oliveira et al., 2011) and a truncated version of the GP nucleotide sequences were obtained. Then, RT-PCR was performed to clone the remaining parts of the genome. Initially, J13 and UHP primers were utilized as described (Cortez et al., 2001), where the latter was used for first-strand cDNA synthesis. Specific primers were then designed to complete the S and M RNA nucleotide sequences. The primer BeNMV-N-F2 (5'CTTCTGATGACAAGCTGCAAGGTA3') and J13 were used to amplify the end of $NS_s$ open reading frame (ORF) and the remainder of S RNA. The primers BeNMV-IRM-F1

(5'GGCTGCAATAGATGAAGAGAATGAA3') and BeNMV-IRM-R1 (5'GCCCTTTTGATTCTGTTAT GACTTG3') were used to amplify the end of $GP_p$ ORF and M RNA intergenic region. The Figure 3 illustrates the cloning strategies for S and M RNA segments. M-MLV Reverse Transcriptase (Promega) was used for RT reactions and Platinum Taq DNA Polymerase (Invitrogen) was used for PCR. All procedures followed the manufacturer's instructions. All cDNA fragments were cloned in pGEM-T easy (Promega) and sequenced by the chain-termination method using an automatic sequencer by Macrogen Corporation, Seoul, Korea. Sequence data were edited and assembled with Staden Package program (Staden, 1996)1996.

**Molecular cloning, protein extraction and western blotting (Sw-5b and $NS_M$ assay)**

The $NS_M$ gene from BeNMV was amplified with the following primers: BeNMV-pD-F: GGGGACAAGT TTGTACAAAA AAGCAGGCTT CGAAGGAG ATAGAACCAT GTCGAAGCTG TTGTCAAA (forward); BeNMV-pD-R: GGGGACCAC TTTGTACAAG AAAGCTGGGT CCTATGTCAT GAGTTTCAAA GGAG (reverse); BeNMV-pD-Rws: GGGGACCACT TTGTACAAGA AAGCTGGGTC TGTCATGAGT TTCAAAGGAG (reverse without stop codon). PCR products were then recombined into pDONR207 by BP clonase (Invitrogen). The pDONR207 vectors harboring the $NS_M$ genes were finally recombined into the binary vectors pK7FWG2 and pK7WGF2 by LR clonase (Invitrogen). After 4 days post infiltration in wild type *N. benthamiana* proteins were extracted and western blot analysis was performed as described in chapter 2. The cloning procedure of *Sw-5b* in pK7WGF2 is described in chapter 4.

***In silico* analysis**

N-glycosylation, O-glycosylation, and cleavage sites predictions were performed with the programs NetNGlyc 1.0 Server, NetOGlyc 3.1 Server (Julenius et al., 2005), and SignalP 3.0 (Emanuelsson et al., 2007), respectively. Transmembrane domain predictions were performed according to the TMHMM Server 2.0 program.

**Evolutionary Analyses**

All available tospovirus genome sequences were downloaded from GenBank: SVNaV (HQ728387, HQ728386), ANSV (GQ478668), CSNV (AF067068, AB600873, AF213675, AB274026), GRSV (AF513219, AY574055, AF513220), INSV (NC_003624, NC_003616), MeSMV (EU275149), TCSV (AF282982, AY574054, AF213674), TSWV (NC_002051,

NC_002050), ZLCV (AF067069, AB274027, AF213676), CaCV (DQ256133, DQ256125), CCSV (AY867502, FJ822961), GBNV (NC_003619, NC_003620), IYSV (AF001387, AF214014), MYSV (NC_008300, NC_008307), PolRSV (EF445397, EU271753), TNRV (FJ489600, FJ947152), TYRV (AY686718), TZSV (NC_010489, NC_010490), WBNV (GU584184, GU584185), WSMoV (NC_003843, NC_003841), PCFSV (AF080526), and PYSV (AF013994). The complete viral names are available in Supplementary Table 2.

The RNA coding sequences were aligned based on their corresponding amino acid sequence using the software Muscle (Edgar, 2004)2004 implemented in TranslatorX web server (Abascal et al., 2005). The resulting alignments were inspected by eye and manually edited using Se-Al (v2.0a11 Carbon, http://tree.bio.ed.ac.uk/software/seal/), and all gap-containing sites were excluded. The level of substitution saturation was checked using Xia et al. (2003) (Xia et al., 2003) method implemented in DAMBE (Xia and Xie, 2001). The third codon positions of all alignments were saturated or nearly saturated (data not shown), therefore, phylogenetic trees were based on protein sequences alignments. The resulting data sets (available upon request) of 23 taxa for the N protein (217 amino acids, aa), 21 taxa for the $NS_S$ protein (391 aa), 18 taxa for the $NS_M$ protein (291 aa), 18 taxa for GP (1007 aa), 14 taxa for the RdRp protein (2811 aa). We also constructed a concatenated alignment of all proteins (N, $NS_S$, $NS_M$, GP and RdRp) with 14 taxa (4721 aa). The phylogenetic relationships among the tospoviruses were inferred using maximum likelihood (ML) criterion implemented in RAxML (Stamatakis et al., 2008), using the RAxML BlackBox web-server at CIPRES [http://www.phylo.org] [43]. The most appropriate model of protein evolution was selected with the software ProTest [44]. Node support was determined using non-parametric bootstrap resampling (500 replicates). The marginal likelihood of alternative topologies estimated using MrBayes 3.2 [45] were compared using Bayes factor. A log difference in the range of 3–5 is typically considered strong evidence in favor of the better model, while a log difference above 5 is considered very strong evidence [46].

The selection analyses were performed using the random effects branch-site model [47] available in www.datamonkey.org [48]. This method can identify branches in a tree with evidence of episodic diversifying selection. Therefore, each nucleotide alignment (N, $NS_S$, $NS_M$, GP and RdRp) was submitted to the Datamonkey webserver.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the Embrapa Vegetables technicians Lúcio Flávio and Hamilton for assistance in the greenhouse and Fernanda Ferreira and Rosana Blawid for critical reading of the manuscript.

# Chapter 5

# Cell death triggering and effector recognition by Sw-5 CC-NB-ARC-LRR proteins from *Tomato spotted wilt virus* resistant and susceptible tomato isolines

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Athos Silva de Oliveira; Ivo Koolhaas; Octav Caldararu; Andrei-Jose Petrescu; Renato de Oliveira Resende & Richard Kormelink. Cell death triggering and effector recognition by Sw-5 CC-NB-ARC-LRR proteins from *Tomato spotted wilt virus* resistant and susceptible tomato isolines.

**ABSTRACT**

The *Sw-5b* resistance gene encodes a CC-NB-ARC-LRR protein able to halt tospovirus infections in *Solanum peruvianum* L. and bred *S. lycopersicum* L., wild and commercial tomato species, respectively. Recently, the cell-to-cell moment protein ($NS_M$) of *Tomato spotted wilt virus* (TSWV), the type species of the genus *Tospovirus*, has been identified as the avirulence determinant (Avr) of the Sw-5b-mediated resistance. The transient expression of the $NS_M$ protein triggers a hypersensitive cell death response (HR) in tomato and transformed *Nicotiana benthamiana* L. harboring the *Sw-5b* gene. Here, it is shown that a high accumulation of the Sw-5b protein in *N. benthamiana* leaves, achieved by co-expression of the Sw-5b protein with RNA silencing suppressors (RSS), leads to auto-HR in the absence of $NS_M$. In a similar approach Sw-5a, the highest conserved paralog of Sw-5b from *S. peruvianum*, also triggered auto-HR while the highest conserved ortholog from susceptible *S. lycopersicum*, named Sw-$5a^S$, did not. However, none of those last two homologs were able to trigger a $NS_M$-dependent HR. Truncated and mutated versions of those Sw-5 proteins revealed that the NB-ARC domain is sufficient for HR-triggering and seems to be suppressed by the CC domain. Furthermore, a single mutation was sufficient to restore auto-HR activity within the NB-ARC domain of Sw-$5a^S$. When the latter domain was fused to the Sw-5b LRR domain, $NS_M$-dependent HR triggering was regained, but not in the presence of its own Sw-$5a^S$ LRR domain. Based on these findings a mechanistic model for the activation of Sw-5 proteins is discussed.

**INTRODUCTION**

*Tomato spotted wilt virus* (TSWV) is a member of the genus *Tospovirus* (family *Bunyaviridae*) and responsible for significant damages in crop production around the world (Pappu et al., 2009). From this perspective, TSWV has been ranked as the runner-up of most important plant viruses (Scholthof et al., 2011). In this context, two natural single dominant resistance sources have been found and are being used for commercial resistance breeding against TSWV: *Tsw* and *Sw-5*. The first one was identified in *Capsicum chinense* Jacq. "PI" and has been commercially introgressed into sweet pepper (*Capsicum annuum* L.) cultivars (Boiteux, 1995; Jahn et al., 2000). The second one, *Sw-5*, was originally identified in *Solanum peruvianum* L. and presents a gene cluster in which five paralogs, named *Sw-5a* to *-5e*, have been described (Brommonschenkel et al., 2000; Spassova et al., 2001). From this cluster only the copy "*b"* has been shown to be functionally active and to confer resistance not only against TSWV, but also to the more distantly related tospovirus species *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), and *Impatiens necrotic spot virus* (INSV) (Hallwass et al., 2014; Spassova et al., 2001). This broad resistance spectrum is rather unique for a dominant resistance (*R*) gene and turns *Sw-5* into the most interesting target for

resistance breeding against tospoviruses.

Dominant *R* genes hold an important position in the arms race between pathogen and host, a battle that is most commonly presented by the "zigzag" model (Jones and Dangl, 2006) and in which the plant immune system consists of two main biochemical branches with overlapping signaling pathways (Chapter 1). The first one relies on Pattern Recognition Receptors (PRRs), transmembrane proteins that are able to identify highly conserved and slowly evolving microbial- or pathogen-associated molecular patterns (MAMPs/PAMPs) such as chitin from fungi, flagellin and elongation factor Tu from bacteria and β-glucans from oomycetes. The second branch encompasses the *R* proteins, also designated as an intracellular class of PRRs by some authors, that are able to perceive the presence of pathogens that have overcome the primary physical and biochemical barriers of plant immunity (de Ronde et al., 2014a; Jones and Dangl, 2006; Qi and Innes, 2013). These receptors are activated by pathogen-encoded effectors also referred to as avirulence determinants (Avr), which are species/race/strain specific molecules that contribute to pathogen virulence in susceptible hosts. However, the differentiation between PAMPs and effectors and, as a result, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) remains ambiguous and confusable since there are narrowly conserved PAMPs and effectors that are widely distributed (Thomma et al., 2011).

Viruses have an intracellular infection cycle and as such are only being perceived by (intracellular) R proteins. A single viral protein is usually the Avr responsible for triggering an *R* gene-mediated resistance (de Ronde et al., 2013; Hallwass et al., 2014; Takahashi et al., 2001; Wen et al., 2013). For the Sw-5b-mediated resistance, the non-structural cell-to-cell movement protein ($NS_M$) of TSWV has been identified as Avr (Chapter 2). The most commonly accepted model to explain *R* gene-mediated resistance involves indirect sensing of Avr determinants as presented by the Guard and Decoy hypotheses. In the first hypothesis the *R* gene product guards a specific host protein (called the guardee) and is able to sense changes of this protein upon interaction with the Avr determinant, which triggers the resistance response (Dangl and Jones, 2001). The 'Decoy hypothesis' mainly questions the function of the 'guardees', seeing them as 'decoys' of effectors without any other roles in the cell, just evolving for this purpose (van der Hoorn and Kamoun, 2008). Although most resistance responses still remain an enigma, the activation of *R* genes (proteins) usually comes with an additional triggering of a Hypersensitive Response (HR), a programmed cell death mechanism that leads to the formation of necrotic spots and prevents further spread of the pathogen from the primary infection site (Soosaar et al., 2005). Dominant *R* genes can be grouped into two classes, i.e. those encoding NB-LRRs presenting the major class and all others (de Ronde et al., 2014). The NB-LRRs share structural homology with innate immunity-sensors from animal cell systems (Medzhitov, 2001; van der Biezen and Jones, 1998) and commonly have at their N-terminal end either a Toll and Interleukin-1 Receptor (TIR) domain

or a Coiled Coil (CC) domain (Meyers et al., 2003). All *Sw-5* genes encode putative *R* proteins of the type CC-NB-ARC-LRR (Meyers et al., 2003; Spassova et al., 2001), in which the N-terminal CC domain is followed by a NB-ARC, acronym for Nucleotide-Binding adaptor shared by Apaf-1 (from humans), R proteins and CED-4 (from nematodes), and a Leucine Rich Repeat (LRR) domain at the C-terminus (Leipe et al., 2004). Although functions for the three major domains have been explored for some R proteins and mechanistic models were proposed (Takken and Goverse, 2012), many differences are seen that makes it difficult to present a single activation model that applies to all *R* genes.

In this study the genetic basis for resistance or susceptibility to TSWV was investigated with three homologs of *Sw-5,* i.e the known functional resistance gene copy (*Sw-5b*), its highest conserved paralog from *S. peruvianum* (*Sw-5a*) and their highest conserved ortholog from susceptible *S. lycopersicum* (*Sw-5a*$^{S}$). It is shown that the Sw-5b protein is able to trigger HR in a $NS_{M}$-dependent and -independent manner (auto-HR) while Sw-5a is only able to do this independently and Sw-5a$^{S}$ completely fails in both ways. The CC, NB-ARC and LRR domains have each been further investigated for those abilities and the results are discussed in light of the *Sw*-5 proteins to confer resistance against tospoviruses.

## RESULTS

### High expression levels of Sw-5b induce auto-HR

So far only Sw-5b has been shown to halt tospovirus infections, although its paralog Sw-5a, that shares almost 95.1% amino acid sequence identity, does not. To study the genetic basis for resistance or susceptibility to TSWV, the Sw-5a and Sw-5b were further analyzed and dissected into their respective CC-, NB-ARC- and LRR-domains for their role in Sw-5-mediated HR. Using a specific primer set for the amplification of *Sw-5a/b*, an additional homolog was amplified from susceptible tomato plants, from here onwards referred to as *Sw-5a*$^{S}$. This homolog is seen in the Heinz 1706 tomato genome sequence available on NCBI (Sato et al., 2012). Even though there are other *Sw-5* paralogs in the Heinz 1706 genome (Andolfo et al., 2014), Sw-5a$^{S}$ is the closest homologous protein to Sw5-a/b with a slightly higher amino acid identity to Sw-5a (94.7%) than to Sw-5b (94.2%).

To further dissect the genetic basis for resistance or susceptibility from different Sw-5 proteins, an alternative to the time-consuming use of transgenic *Nicotiana benthamiana* lines (Chapter 2) had to be developed. To this end, and as a first step, the functional resistance gene copy *Sw-5b* was cloned in two different binary vectors, i.e. pK2GW7 and

pEAQ-HT (Karimi et al., 2002; Peyret and Lomonossoff, 2013) and tested in transient settings in wild type *N. benthamiana*.

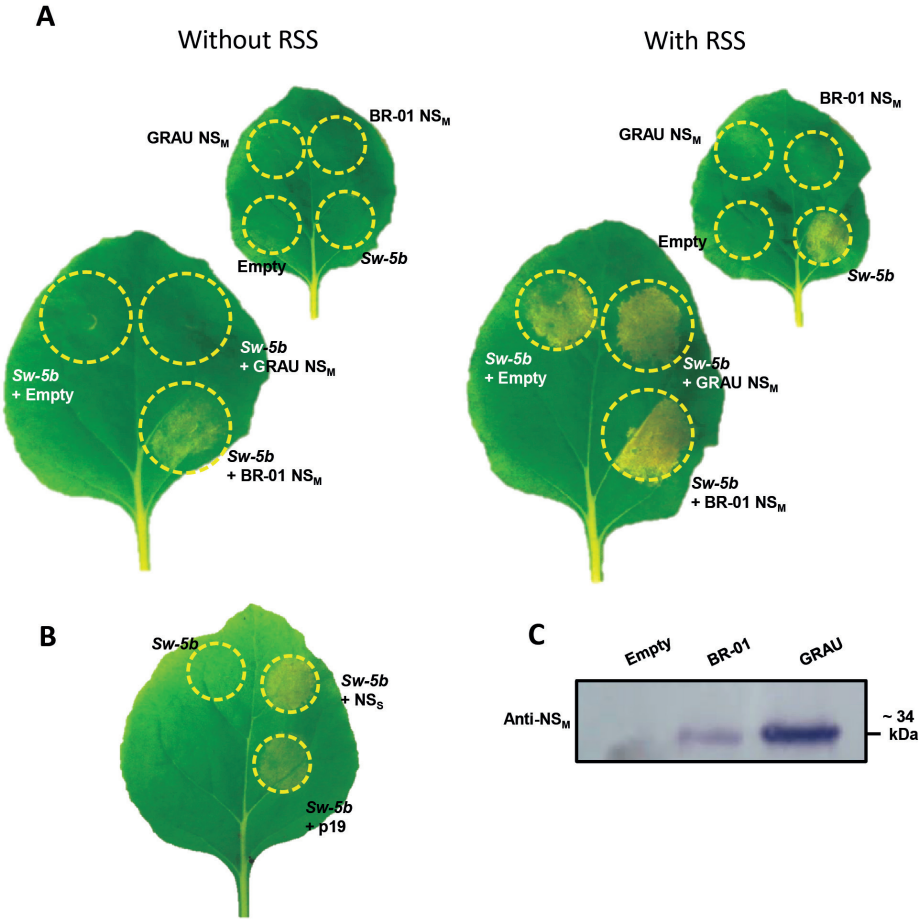


**Figure 1. NS$_M$ (in)dependent induction of Sw-5b-mediated HR.** (A) The two large leaves of *N. benthamiana* were agroinfiltrated with constructs of BR-01- or GRAU-NS$_M$ and *Sw-5b*, cloned into pK2GW7 (left leaf) or pEAQ-HT (right leaf; pEAQ-HT additionally contains the RSS p19 gene in its backbone). As negative controls all constructs were singly infiltrated (small leaves) with Sw-5b expressed from pK2GW7 again on the left one and expressed from pEAQ-HT on the right one. (B) Co-infiltration of agrobacteria harboring the *Sw-5b* gene (cloned into pK2GW7) and NS$_S$ or p19 (cloned into pK2GW7 respectively pBIN). (C) Western immunoblot analysis of NS$_M$ proteins expressed from pK2GW7. The expression of NS$_M$ constructs from pEAQ-HT was earlier confirmed and described (Hallwass et al., 2014). Pictures were taken 4 days post agroinfiltration (dpa).

While the expression level of genes cloned into pK2GW7 relies on the 35S promoter and in general is sufficient for most research purposes, those obtained from pEAQ-HT are generally higher due to the enhancement by flanking Cowpea mosaic virus RNA2 leader/trailer sequences and suppression of transgene silencing by the presence of the

*Tombusvirus*-P19 RNA silencing suppressor (RSS) gene in the vector backbone (Peyret and Lomonossoff, 2013). When constructs of *Sw-5b* were co-agroinfiltrated with TSWV $NS_M$ constructs from either the Resistance Breaking (RB) GRAU isolate or the Resistance Inducing (RI) BR-01 isolate (Hallwass et al., 2014; Lopez et al., 2011) a specific Avr triggered HR was only observed for pK2GW7-based constructs (Figure 1A). On the other hand, and interestingly, when expressed from pEAQ-HT-based constructs Sw-5b was able to trigger HR in the absence of the $NS_M$ (RI) protein (Figure 1A). In the control samples, no cell death response was triggered by the 'empty' pEAQ-HT vector, nor when this vector was infiltrated into *Sw-5b*-transgenic *N. benthamiana* or in *Sw-5*-containing tomato lines (Hallwass et al., 2014). These data suggested that the auto-HR by Sw-5b was likely induced by higher expression levels obtained from pEAQ-HT. To further support this idea a similar experiment was performed in which Sw-5b was expressed from pK2GW7 in the absence or additional presence of RSS proteins (P19 or $NS_S$). While in the absence of RSS proteins no Avr independent HR was triggered, in the presence of both p19 or $NS_S$, auto-HR was observed (Figure 1B).

**Sw-5a protein also triggers auto-HR but fails to recognize $NS_M$ as Avr while Sw-5a$^S$ lacks both functions**

Having established a transient expression system that discriminated between $NS_M$-dependent and $NS_M$-independent HR triggering (by co-infiltration with RSS), we set out to separately analyze effector recognition and HR activation of other Sw-5 homologs. Initial tests using the functional *Sw-5b* resistance gene copy already demonstrated that GFP and His-Tag fusions at both N- and C-terminal ends did not affect its HR triggering activity. Since no antibodies against Sw-5 proteins are available, and to be able to verify the translatability of constructs in case of a negative outcome, all Sw-5 proteins and their derived constructs used from this points onwards were also fused at their N-terminus either to GFP (pK7WGF2) or to a His-Tag (pEAQ-HT-DEST2).

Sw-5a and Sw-5a$^S$ proteins were tested on the ability to trigger HR in a $NS_M$-dependent and $NS_M$-independent manner. Whereas Sw-5a, like the (GFP-fused) Sw-5b, triggered an auto-HR when co-expressed with p19, in contrast, it failed to trigger HR when co-expressed with BR-01 $NS_M$ (Figure 2A). In both ways Sw-5a$^S$, on the other hand, was not able to trigger HR at all (Figure 2A). These data altogether indicated that Sw-5a still maintained the ability to trigger a downstream pathway leading to HR, but failed to (in) directly sense the viral Avr determinant $NS_M$ while Sw-5a$^S$ appeared nonfunctional on both features.

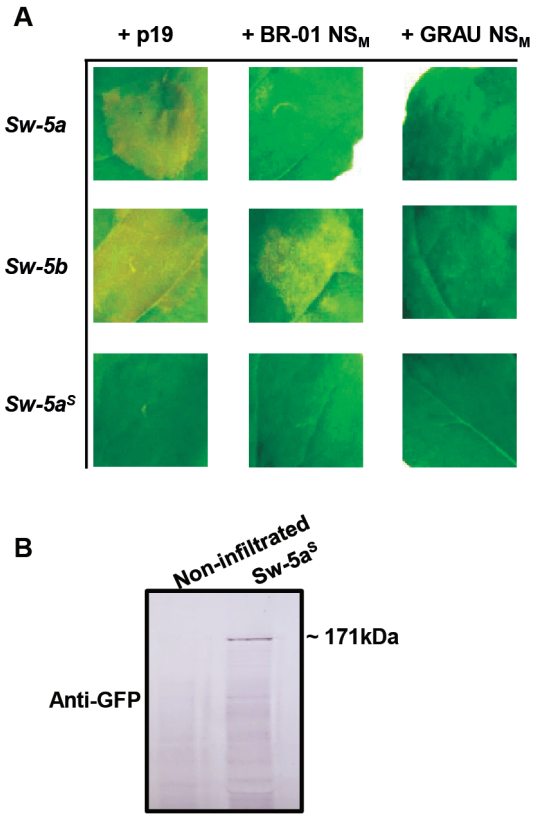


**Figure 2. NS$_M$ (in)dependent HR induction by Sw-5 orthologs.** (A) Analysis of HR induction by Sw-5a and Sw-5a$^S$ was done in analogy to the experiment as described in Figure 1 and using the functional Sw-5b as a positive control. In the first column of (square) images, *N. benthamiana* leaves were co-infiltrated with agrobacteria harboring *Sw-5a*, *Sw-5b*, or *Sw-5a$^S$* (cloned into pK7WGF2) and p19 for auto-HR triggering. In the next columns images are shown from leaf areas co-infiltrated with agrobacteria harboring the same *Sw-5* constructs and BR-01-NS$_M$ (second column) or GRAU-NS$_M$ (third column), all constructs cloned in pK2GW7. Images were taken 4dpa. (B) Western blot of Sw-5a$^S$ that presented a negative outcome for HR triggering. Western blot was screened with anti-GFP as primary antibody.

### The Sw-5b NB-ARC domain is sufficient to trigger HR

In order to assign the ability to trigger HR or sense the Avr to any of the CC-, NB-ARC- or LRR-domains, truncated constructs were made that covered each of these individual domains or in combination (CC-NB-ARC, NB-ARC-LRR) from Sw-5b (Figure 3A). After having verified their translatability (Figure 3C), all Sw-5b-based proteins were expressed in the presence of p19, to test for auto-HR, respectively NS$_M$, to test for Avr recognition (Figure 3A). Besides the full-length Sw-5b protein (positive control), auto-HR was also triggered by the

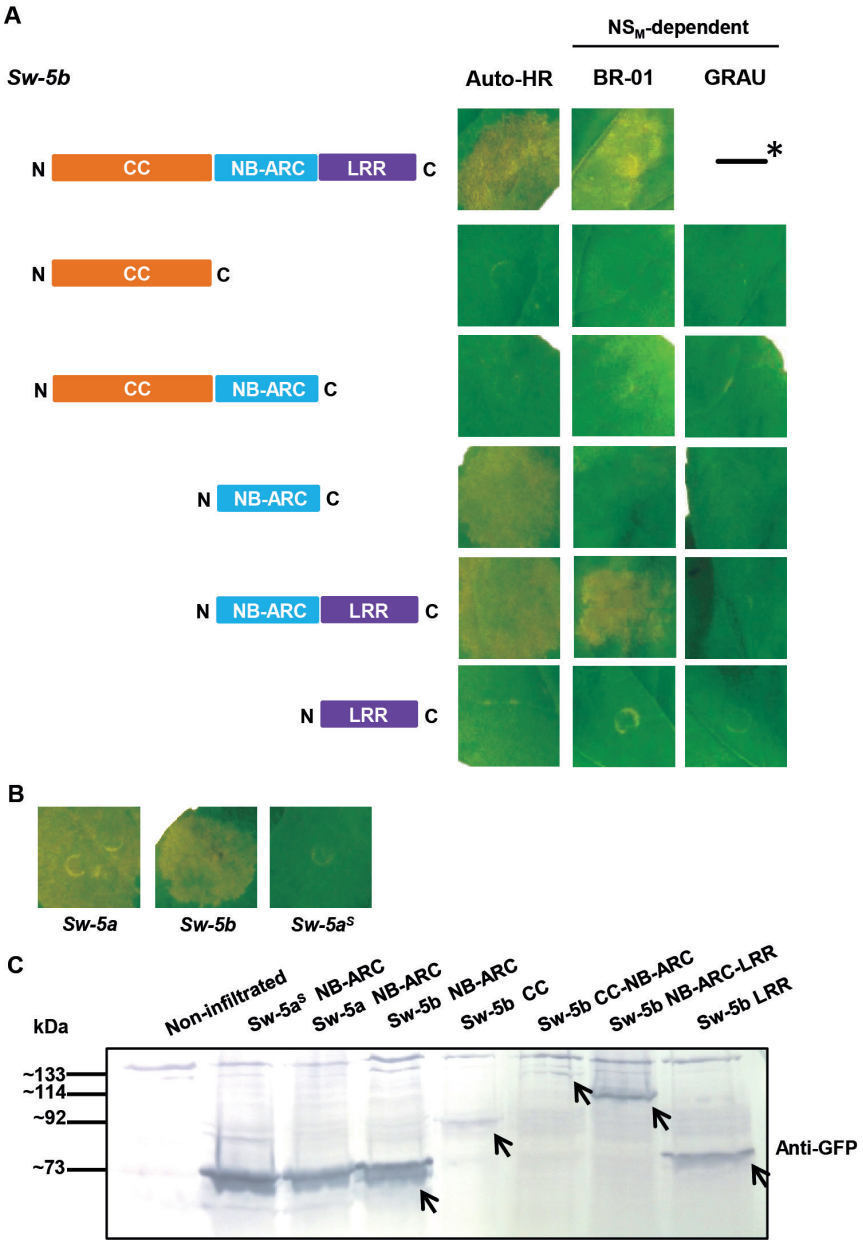


**Figure 3. Analysis of NS$_M$ (in)dependent HR induction by domains from Sw-5 orthologs.** (A) Co-infiltration of agrobacteria harboring *Sw-5b* gene-based derivatives (cloned into pK7WGF2) as depicted, with p19 (first column), BR-01 NS$_M$ (second column), or GRAU NS$_M$ (third column). All constructs were always infiltrated in the same leaf per assay. Pictures were taken 4dpa. *As a positive control on (auto)HR triggering, the *Sw-5b* full-length gene was co-infiltrated with p19 (first column; auto-HR) or BR-01-NS$_M$ (second column; NS$_M$-dependent). (B) Co-infiltration of agrobacteria harboring the NB-ARC domains from *Sw-5a*, *Sw-5b*, or *Sw-5a$^S$* and p19 for auto-HR triggering; pictures were taken 4dpa. (C) Western blot of the Sw-5b-truncated proteins expressed via pK7WGF2-based constructs. Anti-GFP was used as primary antibody.

NB-ARC and NB-ARC-LRR domains, but not with CC-NB-ARC (Figure 3A). When NB-ARC was co-expressed with the CC domain from a separate construct, auto-HR was suppressed like in the case of the CC-NB-ARC construct (Figure 4). These findings indicated that the NB-ARC domain is sufficient for triggering HR, a process that can be suppressed by CC in *cis* (Figure 3A) as well as in *trans* (Figure 4). Upon expression of all Sw-5b truncations and individual domain constructs in the presence of BR-01 $NS_M$, Avr dependent HR triggering was only observed with NB-ARC-LRR (Figure 3A), suggesting that the LRR domain is responsible for Avr recognition. In addition, expression of the NB-ARC domain from Sw-5a also triggered auto-HR while the one from Sw-5a$^S$ did not (Figure 3B), in agreement with the results obtained when using their full length genes (Figure 2).

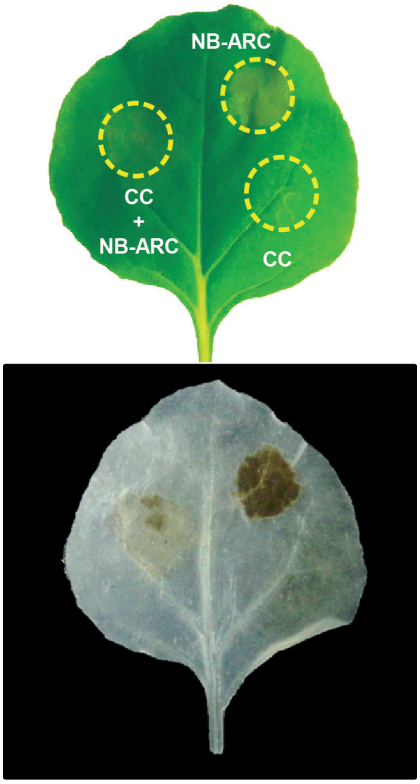


**Figure 4. Analysis of NB-ARC-triggered HR trans-complemented with the CC domain.** *N. benthamiana* leaf was co-infiltrated with agrobacteria harboring the NB-ARC (pK7WGF2) and CC (pEAQ-DEST2) domains and monitored for HR at 4 dpa. p19 (from pBIN) was added in combination with pK7WGF2-NB-ARC and -CC for auto-HR triggering. Pictures were taken 4dpa and chlorophyll was removed in the lower leaf for a better visualization of HR.

**The non-functional Sw-5a$^S$ NB-ARC domain regains the ability to trigger auto-HR upon a single amino acid reversion**

Since the NB-ARC domain was sufficient to trigger HR, a multiple amino acid (aa) sequence alignment was performed from the NB-ARC domains of Sw-5 homologs to identify potential aa residues that could be responsible for the 'loss-of-fuction' (auto-HR induction) of Sw-5a$^S$ from susceptible tomato. Based on the alignment, three putative amino acids residues were identified in the NB-ARC domain sequence of Sw-5a$^S$ that clearly differed from those of the others (Figure 5A). These were reversed one by one into the corresponding aa residue as present in the Sw-5b NB-ARC domain (Figure 5A). Binary constructs (pEAQ-HT-DEST2) of the three revertants were infiltrated into *N. benthamiana*, next to positive and negative controls, and tested for auto-HR. Interestingly, a gain-of-function was observed with the mutant Q599R (Figure 5B), but not with the others. This mutation localizes outside of the ATP/ADP binding site (Figure 6). A similar approach was considered for the Sw-5a$^S$ LRR domain, to investigate its inability to recognize $NS_M$. However, in comparison to the low number of variable amino-acids residues observed in the NB-ARC domains, a larger number of point mutations was observed in the LRR domains that hampered this approach.

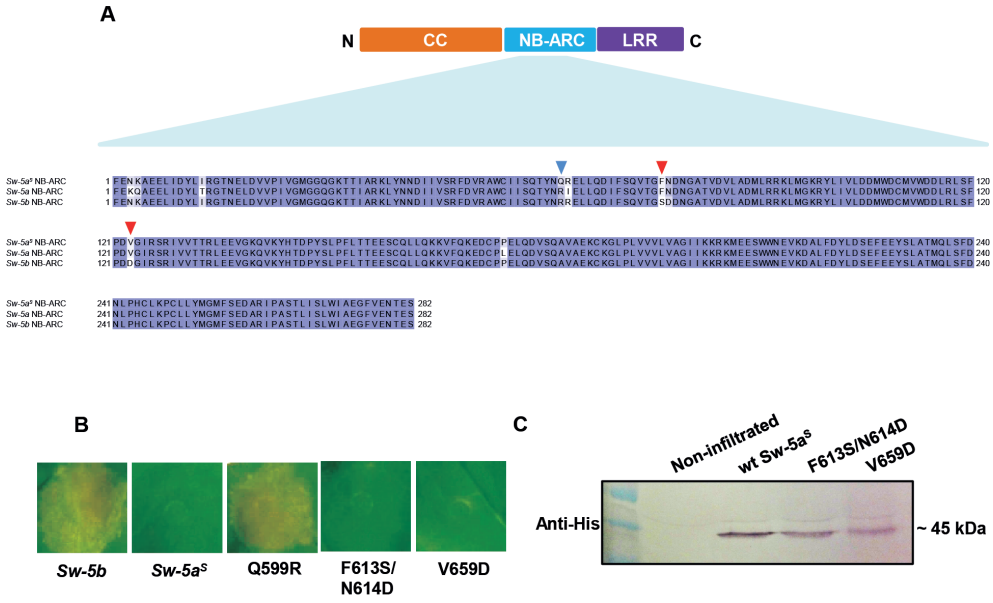


**Figure 5. Analyses of mutant Sw-5a$^S$ NB-ARC on auto-HR triggering.** (A) Multiple sequence alignment of NB-ARC domains from different Sw-5 homologs. The blue inverted triangle indicates a mismatch of Sw-5a$^S$ with both Sw-5a and Sw-5b, and red inverted triangles indicate mismatches only seen with Sw-5b. (B) *N. benthamiana* leaves agroinfiltrated with pEAQ-DEST2 constructs (which also contain p19) of NB-ARC domains from wild type Sw-5b, Sw-5a$^S$ and mutants Q599R, F613S/N614D, and V659D made from the Sw-5a$^S$ NB-ARC domain. Pictures were taken 4dpa. (C) Western immunoblot analysis to verify the expression of NB-ARC constructs that negatively tested on HR-triggering in panel B. Anti-His was used as primary antibody.

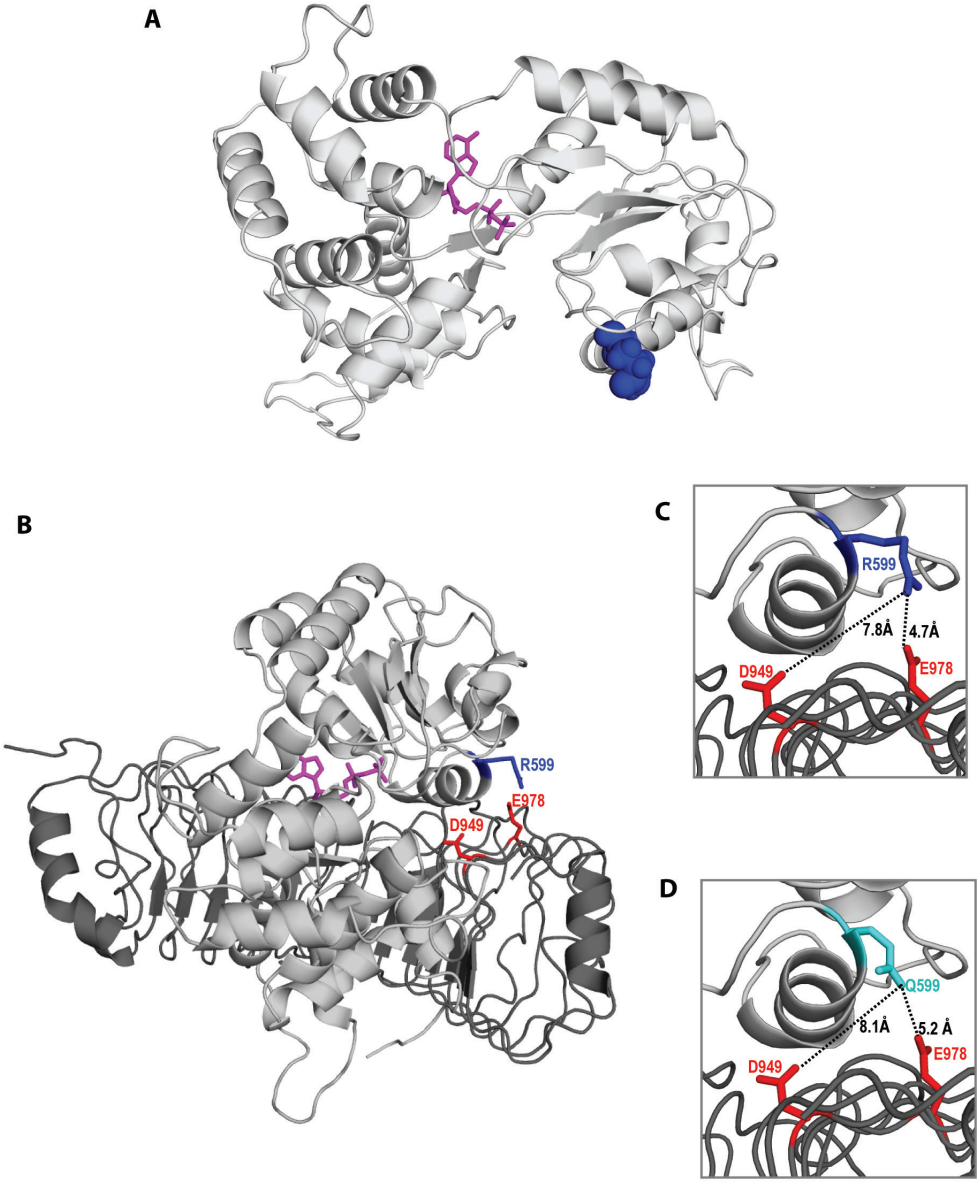


**Figure 6. 3D structure models of Sw-5aS NB-ARC domain and Sw-5b NB-ARC-LRR domains interface.** (A) The 3D structure of the Sw-5aS NB-ARC domain is depicted, with the NB subdomain on the right, and the ARC1 and ARC2 subdomains on the left. The location of the R599Q mutation on the surface of the NB subdomain is represented in blue. The bound ADP is also shown in magenta. (B) 3D structure of the NB-ARC domain of Sw-5b interacting with the LRR domain of Sw-5b. The location of the R mutated for a Q on the surface of the NB-ARC domain in Sw-5aS, close to the LRR domain, is represented in blue. Two acidic residues (D949 and E978, shown in red) on the surface of the LRR are close in space to the R599 and are interacting in the model; (C) Position and distances between R599 and D949/E978 in Sw-5b; (D) Position and distances between Q599 (Sw-5aS) and D949/E978.

## The LRR domain from Sw-5b fused to the Q599R NB-ARC revertant from Sw-5a$^S$ activates NS$_M$-dependent HR

Having identified Q599R as an important amino acid change within the Sw-5a$^S$ NB-ARC domain to restore its ability to induce HR, this mutation was next introduced in the full-length Sw-5a$^S$ (Figure 7). However, when this construct was expressed in *N. benthamiana* no auto- (from pEAQ-HT) nor NS$_M$-dependent (from pK2/pK7) HR was observed (Figure 7A). In light of the earlier described differences in the LRR domains, the LRR domain from the full-length Sw-5a$^S$ next was swapped for the one from Sw-5b. Considering that this chimeric Sw-5 contained a NB-ARC domain restored in its function to trigger auto-HR and a LRR domain functional in Avr recognition, it was surprising to see that only auto-HR was visualized upon infiltration of the construct into *N. benthiama* (Figure 7A). To find out whether this was due to the CC domain from Sw-5a$^S$, this domain was removed from the mutated (Q599R) full-lenght Sw-5a$^S$ and from the chimeric Sw-5a$^S$ /Sw-5b protein (Figure 7A). Interestingly, auto- and NS$_M$-dependent HR was observed for the chimeric NB-ARC(Q599R)-LRR construct, indicating that the Sw-5a$^S$ CC domain apparently prevented/hindered any functional activity.

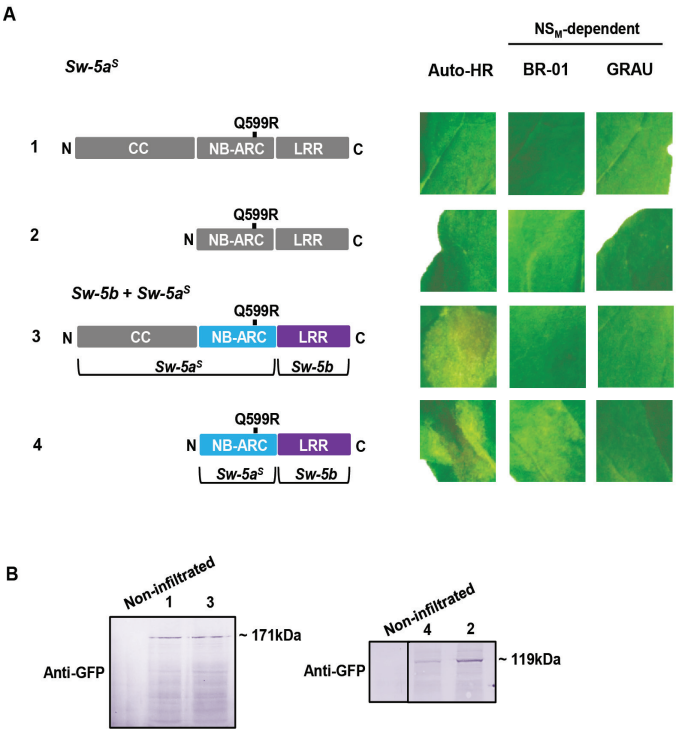


**Figure 7. NS$_M$ (in)dependent HR induction by Sw-5a$^S$-chimera.** (A) Co-agroinfiltration of (mutant) *Sw-5a$^S$* gene-based constructs (cloned into pK7WGF2) with p19 (first column; auto-HR), BR-01-NS$_M$ (second column; NS$_M$-dependent), or GRAU-NS$_M$ (third column; NS$_M$-dependent) in *N. benthamiana* leaves. Pictures were taken 4dpa. (B) Western immunoblot detection of (mutant/chimeric) Sw-5a$^S$ proteins from (A). Anti-GFP was used as primary antibody.

## DISCUSSION

The *Sw-5* gene cluster has been used for more than one decade in tomato resistance breeding against tospoviruses. Apart from the knowledge that the *Sw-5b* paralog isolated from *S. peruvianum* is the functional gene copy to confer resistance against TSWV (Spassova et al., 2001), not much is known on the other members of this gene cluster. Furthermore, in recent years the entire genome sequence of the Heinz tomato has been elucidated and annotated which has helped to map highly conserved homologs of *Sw-5* in susceptible tomatoes, like *S. lycopersicum*, that have not been crossed with *S. peruvianum* (Andolfo et al., 2014). Resistance genes often occur in gene clusters that result from tandem and segmental gene duplication or clusterization events leading to structural variations, making them one of the plant gene families with highest plasticity rates (Zhang et al., 2014). As a result many *R* genes contain deletions or truncations and till now, it is not clear whether all these resistance gene copies are still being expressed and play another role in host defense, development or homeostasis.

In this study, the functional Sw-5b resistance protein and its highly conserved paralog (Sw-5a) from *S. peruvianum* and ortholog (Sw-5a$^{S}$) from susceptible *S. lycopersicum* have been comparatively analyzed to identify the role of the CC, NB-ARC and LRR domains in HR-induction and $NS_M$-recognition as Avr. In this way we aimed to understand the genetic basis for the inability to confer resistance to TSWV by Sw-5a and Sw-5a$^{S}$. To circumvent the time-consuming generation of transgenic plants, a transient system to express Sw-5 homologs, their domains and chimeric versions was established. At higher levels of protein expression (via the additional presence of the RSS p19), the functional Sw-5b was able to trigger an auto-HR in the absence of the $NS_M$ Avr determinant, while at lower levels HR-triggering required $NS_M$ recognition. Using these assays auto-HR triggering and $NS_M$-recognition were investigated for the aforementioned three Sw-5 proteins. Sw-5a was still able to trigger (auto-)HR, but failed to do so in an $NS_M$-dependent fashion. This observation explains why *Sw-5a*-transformed tobacco plants still exhibited a TSWV-susceptible phenotype (Spassova et al., 2001). *Sw-5a$^{S}$*, on the other hand, failed to trigger HR at all.

The observation that high levels of Sw-5b accumulation in cells leads to an auto-immune scenario has earlier been reported for other R proteins such as RPS2, RPS4, and Pto (Day et al., 2005; Tang et al., 1999; Zhang et al., 2004), but cases have also been reported in which increased expression levels, instead of resulting in an auto-HR, lead to an increased (extreme) resistance response (Sato et al., 2014). Whether the triggering of HR at high protein expression levels ($NS_M$-independent) or at lower concentrations via Avr recognition ($NS_M$ -dependent) involves the same signaling pathway still remains to be investigated. The screening of *R* genes by HR remains another dispute among plant biologists because disease resistance and HR triggering have been demonstrated as two events that can be uncoupled

for potato Rx and barley Rrs1 (Coll et al., 2011; de Ronde et al., 2014a). For the *Sw-5b* gene and most other dominant resistance genes, however, HR is commonly observed upon resistance activation and therefore presents a reliable visual indicator for protein activation (de Ronde et al., 2014a; Hallwass et al., 2014).

Analysis of domains from the functional Sw-5b demonstrated that $NS_M$- recognition mapped, as expected, to the LRR domain while the induction of HR required the NB-ARC domain. Although the CC domain was not relevant for these functions, the in *cis* or *trans* presence of CC next to NB-ARC lacking LRR suppressed HR-induction. From the other two homologs, Sw-5a contained a functional NB-ARC domain but failed to recognize $NS_M$ by its LRR domain. On the other hand, all three domains of Sw-5a$^S$ from susceptible S. lycopersicum appeared hampered or changed in their ability to trigger auto-HR (NB-ARC) or $NS_M$-dependent HR (LRR). However, a single point mutation in its NB-ARC was sufficient for a gain-of-function to trigger auto-HR, which was not entirely surprising considering the very high protein sequence identity (95%) shared with Sw-5b. After alignment of the available Sw-5 protein sequences from *S. peruvianum* and *S. lycopersicum* Heinz (Figure S1 and Table S2), Sw-5aS is the only one that presents the point mutation R599Q related to loss-of-function. Whether the NB-ARC domain from other (non-tested) Sw-5 homologs lacking this mutation are able to trigger auto-HR still remains to be investigated, considering that more polymorphisms are seen among these. The NB-ARC domains are hypothesized to work as a switch with active and inactive states according to ATP and ADP binding, respectively. However, a structural folding prediction of Sw-5a$^S$ NB-ARC based on the Rx NB-ARC structure template revealed that the R599Q mutation is not located near the ADP/ATP binding site, but instead at the edge of it. This ruled out ATP binding as the cause for lack of auto-HR, and suggested that more likely the downstream signal transduction that leads to HR is affected. To investigate an additional link between R599 and the LRR domain, the structural folding of NB-ARC was also analyzed in the additional presence of the LRR domain. Although the Sw5-NB-ARC and LRR models were not properly docked but rather mapped onto the NB-ARC-LRR derived for Rx (Slootweg et al., 2013), it was striking to see how close the Sw5-NB-ARC-R599 and Sw5-LRR-D949/E978 came into space (Figure 6B). In this scenario, the occurrence of electrostatic interactions between the basic R599 and two acidic D949/E978 residues is highly attractive and very strong especially between R599 and E978 (4.7Å, Figure 6C). In case of the R599Q mutation (Sw-5a$^S$ NB-ARC) not only those distances slightly increased (from 4.7 to 5.2 Å and from 7.8 to 8.1 Å, Figure 6D) but a far weaker dipole-charge interaction (Q599-E978) is involved that altogether significantly weakens the contact between NB-ARC and LRR. Due to a very high sequence identity (>89%) between Sw-5a-LRR and Sw-5b-LRR it is unlikely that the small differences between these two domains alter the overall LRR fold. However, and interestingly, residue Sw-5b-E978, and possibly involved in an interaction with NB-ARC-R599, is replaced in Sw-5a with a basic Lysine (Sw-5a-K978). Whether this change (E978K) has led to a loss of $NS_M$-dependent HR triggering of Sw-5a NB-ARC-LRR and the

interaction between NB-ARC-R599 and LRR-D949/E978 is indeed required for functionality, remains to be investigated.

The sole requirement of the NB-ARC domain for HR triggering has also been reported previously for potato Rx (Rairdan et al. 2008). Nevertheless, CC and TIR domains have also been associated with downstream signaling that leads to HR-triggering for barley MLA10 and flax L6, respectively (Bernoux et al. 2011, Maekawa et al. 2011). For Sw-5b the CC domain suppressed HR, an observation that has earlier been made with the tomato Mi-1.2 resistance gene and specifically mapped to a so-called solanaceae domain (SD), a sort of first subdomain localized in the N-terminus of this protein (Lukasik-Shreepaathy et al. 2012). A closer look at the Sw-5 proteins also revealed the presence of this SD domain, but in this study the entire N-terminal region of Sw-5 was denominated as CC domain since coiled coil secondary structures are predicted to span the entire amino acid sequence until the beginning of the NB-ARC domain. Whether the HR suppression indeed involves this SD domain remains to be investigated by a further dissection of the Sw-5b CC domain. In general, the LRR domain seems to be most conserved in its role to activate R proteins but other domains have been reported interacting with effectors as well, e.g. the CC domain of the rice Pik protein (Kanzaki et al. 2012). Based on all those differences, it so far remains difficult to postulate one mechanistic model to explain the activation of all R proteins from the NB-LRR type.

The assembly of artificial R proteins seems very tricky since a very stable combination of domains is required. Swap of domains has ended up in inactivation for other NB-LRR proteins as well (Rairdan and Moffett, 2006; Sun et al., 2001)). In this study, for example, gain-of-function was just observed by introduction of the Q599R mutation in the Sw-5a$^{S}$ NB-ARC alone, but not in the full-length protein. Deletion of the CC domain, which suppresses auto-HR in Sw-5b, was not sufficient to retrieve the auto-HR in the reverted full length Sw-5a$^{S}$ protein. Only when its LRR domain was replaced by the one from Sw-5b and the CC domain deleted, the ability to trigger both auto- and $NS_{M}$-dependent HR was regained. Altogether our data support the idea that only changing a few amino acid residues might turn an apparently non-functional R protein into a functional one. With the recent availability of site-specific DNA editing tools like TALENs or CRISPR (Gaj et al., 2013), mapped gain-of-function mutations could be introduced into the genome of susceptible plants. Although no attempts have been reported on this point so far, if it turns out to be feasible it would enlarge the amount of genetic resources that can be exploited in future resistance breeding. Considering that *R* genes are plastic genetic sequences that are continuously shuffling during the course of evolution (Zhang et al., 2014), it is not unlikely that some *Sw-5* homologs are obsolete genes and temporarily needless in innate immunity but important for the “arms race” between tomatoes and pathogens.

## MATERIALS AND METHODS

### Plants

The seeds of the tomato near-isogenic isolines 'Santa Clara' and 'CNPH LAM 147' (Hallwass et al., 2014) were kindly supplied by Dr. Leonardo Boiteux (EMBRAPA Vegetables, Brazil). Both *Nicotiana benthamiana* and tomato plants were kept under glasshouse conditions (24ºC, 16h light/8h dark a day).

### Cloning into binary vectors

DNAZOL® reagent (Invitrogen, Carlsbad, CA, USA) was used for total DNA extraction of the tomato near-isogenic lines, which was then used as template for amplification of the *Sw-5* genes (*Sw-5a*, *Sw-5a*$^{S}$, *Sw-5b*) via PCR using Phusion Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA). The PCR products were recombined into pDONR207 entry vectors by BP Clonase Enzyme Mix (Invitrogen, Carlsbad, CA, USA). Truncated gene versions were PCR amplified from their full-length copies cloned in pDONR207 vectors and either ligated in pENTR11 (NcoI and XhoI restriction sites) or recombined again in pDONR207 as detailed in the supplemental table 1. The pDONR207 constucts harboring $NS_M$ from the resistance inducing BR-01 or resistance-breaking GRAU strains were made previously (Hallwass et al., 2014). All entry vectors were recombined with one or more of the following destination vectors by LR Clonase Enzyme Mix (Invitrogen, Carlsbad, CA, USA): pEAQ-DEST1, pEAQ-DEST2, pK2GW7, pK7WGF2 (Karimi et al., 2002; Peyret and Lomonossoff, 2013). A list of all primer sequences, entry and destination vectors of all constructs used in this study is shown in Supplemental Table S1. All procedures followed the manufacturers' instructions and Green et al (Green et al., 2012).

### Mutagenesis PCR and cloning

The pDONR207 vectors harboring the full- or truncated gene versions were used as template for overlapping PCR using primers to insert the desired mutations. After amplification with Phusion DNA polymerase, 1uL of DpnI restriction enzyme (NEB, Ipswich, MA, USA) was added for template DNA degradation. To facilitate a rapid and easy screening of mutants constructs, restrictions sites were introduced or removed via silent mutations. The chimeric *Sw-5* gene was made by fusion PCR, first amplifying the sequences separately and next fusing them during another PCR reaction. Fused PCR products were then cloned

into pDONR207. All constructs from the entry vectors were recombined with pEAQ-DEST2 or pK7 as listed in Supplemental Table S1.

**Agroinfiltration, protein extraction, and western blot analysis**

The agroinfiltration assays were performed following the protocol (Bucher et al., 2003). Thus, *A. tumefaciens* COR308 containing single constructs were grown overnight at 28 °C in LB3 medium with 2 µg/ml tetracycline and either 250 µg/ml spectinomycin for pK2 and pK7 vectors or 100 µg/ml kanamycin for pEAQ-HT vectors. Then, 600 µl LB3 inoculum was added into 3 ml induction medium and, once again, grown overnight. After harvesting and resuspending the bacteria with MS-MES buffer, *N. benthamiana* leaves were infiltrated with combinations of suspensions containing a final $OD_{600}$ 1.0 per construct. Pictures were taken 4 days post agroinfiltration (dpa). Chlorophyll was removed (Figure 4) with ethanol and acetic acid 3:1 (v/v).

For protein extraction leaves were harvested 3 dpa or 4 dpa for the full-length proteins. About 100 mg of leaf material per sample was ground in liquid nitrogen and then with 250 µL of 95ºC pre-heated Berger-Buffer (Berger et al., 1989). Samples were incubated for 10 minutes at 95ºC and centrifuged at 12000g for 10 minutes. Supernatants were transferred to new tubes and the same volume of 2x-SDS-loading buffer was added. Samples were incubated at 95ºC for 3 minutes. A volume of 15 µL per sample was loaded in 10% SDS-PAGE gels. The western blotting procedure was performed as previously described (Hallwass et al., 2014) and using anti-His and anti-GFP (Molecular Probes, Eugene, OR, USA) as primary antibodies.

***In Silico* analysis**

For structural folding predictions the Sw-5 proteins were virtually divided in three main portions (Sw-5b as reference): (01) CC 1-514aa; (02) NB-ARC 515-874aa; and (03) LRR 875-1246aa (For sequence see Supplemental Table 2). The nucleotide and amino acid alignments were performed by ClustalW (Larkin et al., 2007). The Coiled Coil (CC) secondary structures of the Sw-5 proteins were predicted by http://gpcr.biocomp.unibo.it/cgi/predictors/cc/pred_cchmm.cgi (Hochreiter et al., 2007). The NB-ARC and LRR domains were predicted by SMART (Letunic et al., 2012).

**3D modeling of the NB-ARC and LRR domains**

Domain delineation, sequence analysis and molecular modeling of the NB-ARC domains of Sw-5 proteins were performed as described in (Slootweg et al. 2013), starting from the crystal structure of APAF-1 (PDB code 1Z6T). The Sw-5b LRR domain was modeled using the Optimized Joint Fragment Remote Homology Modeling (OJFRHM) procedure described in (Sela et al. 2014, Sela et al. 2012, Slootweg et al. 2013). In the case of Sw-5b, the overall LRR frame was built piecewise for repeats: 1-2, 3-8, 9, 10-14 - starting from the closest four repeat templates found in our structural LRR database - PDB codes : 2A0Z-repeats 2-3 (aa 34-80), 2ID5 repeats 3-8 (aa 58-201), 2P1Q repeat 10 (aa 267-276) and 3BZ5 repeats 9-13 (aa 212-330). Simulated annealing with harmonic restraints on backbone atoms found in definite secondary structure states followed by model quality assessment with MetaMQAP (Pawlowski et al. 2013) was used to bring the models to a root mean squared deviation (RMSD) of less than 3Å from an optimal polypeptide path and an overall GDT_TS score of over 60. As Sw-5b and Rx1 are highly homologous sharing over 40% identity and over 50% similarity, the homology modeling procedure was considered appropriate to generate the structure of Sw-5b NB-ARC-LRR complex starting from Rx1 NB-ARC-LRR model described in (Slootweg et al. 2013). Consequently the Sequence Conserved Regions (SCR) were generated by coordinate transfer from Rx1 NB-ARC-LRR model and use as a scaffold to generate the Sequence Variable Regions (SVR) by standard modeling procedures. The overall structure of the NB-ARC-LRR complex was further optimized by repeated rounds of local and global energy minimization and simulated annealing.

**ACKNOWLEDGMENTS**

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## SUPPORTING INFORMATION


**Supplemental figure 1. Multiple sequence alignment of NB-ARC domains from available and full Sw-5 protein sequences from *S. peruvianum* and *S. lycopersicum* Heinz.** GenBank accessions: AY007366 (Sw-5a and b), AY007367 (Sw-5c, d, and e), and EF647603 (Sw-5a$^{S}$, Sw-5*, and Sw-5**). Only the *Sw-5* gene sequences from *S. lycopersicum* Heinz are found in Table S2. *This gene has been reported previously and referred as to *Sw-5f* (Rehman et al., 2009). However, this is not a paralog of *Sw-5b*, but an ortholog from *S. lycopersicum* as well as *Sw-5a*$^{S}$. **This gene is also from *S. lycopersicum*, being the highest conversed ortholog of *Sw-5d* from *S. peruvianum*.

**Supplemental table 1.** Primers and vectors used for building and expressing the full, truncated, and mutated *Sw-5* gene versions.

| Gene/Construct | Primers sequences | Entry vector | Destination vectors |
|---|---|---|---|
| Full *Sw-5a, Sw-5b*, and *Sw-5a*$^{s}$ genes and their mutated versions after overlapping and fusion PCRs | Sw5b-pD1: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCTGAAAATGAAA TTGAGG<br><br>Sw5b-pD2: GGGACCACTTTGTACAAGAAAGCTGGGTCCTAATCTGAGCGTTGTTTGACGAGG | pDONR207 | pK2GW7, pK7WGF2, pEAQ-DEST1 and -DEST2 |
| *Sw-5a-, Sw-5b-, Sw-5a*$^{s}$-NB-ARC domains and their mutated versions after overlapping PCR | NB-SW5b-F: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGCTGCTCCATTAAAACATCT<br><br>NB-SW5b-R: GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAAAGCATAAACTTTGCTTCTC | pDONR207 | pK2GW7, pK7WGF2, pEAQ-DEST1 and -DEST2 |
| *Sw-5b* CC domain | SW5N-ALL-F: CCATGGTCATGGCTGAAAATGAAATTGAGG<br><br>CC-SW5N-R: GCGGCCGCTCACACATAGTGAGGATTAAGAGG | pENTR11 | pK2GW7, pK7WGF2, pEAQ-DEST1 and -DEST2 |
| *Sw-5b* CC-NB-ARC domain | SW5N-ALL-F: CCATGGTCATGGCTGAAAATGAAATTGAGG<br><br>CC-NB-ARC-SW5N-R: GCGGCCGCTCAAAGCATAAACTTTGCTTCTC | pENTR11 | pK2GW7, pK7WGF2, pEAQ-DEST1 and -DEST2 |
| *Sw-5b* NB-ARC-LRR domain | NB-ARC-LRR-SW5N-F: CCATGGTCGCTGCTCCATTAAAACATCTGC<br><br>SW5N-ALL-R: GCGGCCGCTCAATCTGAGCGTTGTTTGAC | pENTR11 | pK2GW7, pK7WGF2, pEAQ-DEST1 and -DEST2 |
| *Sw-5b* LRR domain | LRR-SW5N-F: CCATGGTCGCAGTGAAGGGTCAATATATCCA<br><br>SW5N-ALL-R: GCGGCCGCTCAATCTGAGCGTTGTTTGAC | pENTR11 | pK2GW7, pK7WGF2, pEAQ-DEST1 and -DEST2 |
| *Q599R NB-ARC (*Sw-5a*$^{s}$) | Sw5S-Q599R-1: TCTCAAACGTATAATCGGAGAGAGTTATTA<br><br>Sw5S-Q599R-2: AAAGATATCTTGTAATAACTCTCTCCGATT | pDONR207 | pEAQ-DEST2 |
| *F613S/N614D NB-ARC (*Sw-5a*$^{s}$) | Sw5S-FN613SD-1: AGTCAAGTTACAGGTTCCGACGACAATGGA<br><br>Sw5S-FN613SD-2: GGAACCTGTAACTTGACTAAAGATATCTTG | pDONR207 | pEAQ-DEST2 |
| * V659D NB-ARC (*Sw-5a*$^{s}$) | Sw5S-V659D-1: GATGGAATTCGAAGCAGAATAGTCGTAACA<br><br>Sw5S-V659D-2: TCTGCTTCGAATTCCATCATCTGGAAAAGA | pDONR207 | pEAQ-DEST2 |

| | | | |
|---|---|---|---|
| **Q599R-*Sw-5a*$^{S}$ (CC-NB) + *Sw-5b* (LRR) | P2-Sw5S-CC-NB-R: CTTCACTGCAAGCA-TAAACTTTGCTTC<br><br>P3-Sw5b-LRR-F: GCAGTGAAGGGTCAATATA-TCCATTTTC | pDONR207 | pK7WGF2 |
| *Sw-5a*$^{S}$ CC | CC-pD-F: GGGGACAAGTTTGTACAAAAAAG-CAGGCTTCATGGCTGAAAATGAAATTGA<br><br>CC-pD-R: GGGGACCACTTTGTA-CAAGAAAGCTGGGTCCTACACATAATGAG-GATTAAGAG | pDONR207 | pK7WGF2 |
| Q599R-*Sw-5a* NB-LRR | Sw-5aS-NB-LRR-pD-F: GGGGACAAGTTTG-TACAAAAAAGCAGGCTTCATGGCTGCTCCAT-TAAAACATCT<br><br>NB-LRR-pD-R: GGGGACCACTTTGTACAA-GAAAGCTGGGTCCTAATCTGAGCGTTGTTT-GACGA | pDONR207 | pK7WGF2 |

*Primers used for overlapping PCR (insertion of mutations);

**Primers used for fusion PCR (combined with primers with att sites also described above).

**Supplemental table 2.** *Sw-5* gene sequences from *S. lycopersicum* Heinz.

>Sw-5a$^{S}$

atggctgaaaatgaaattgaggaaatgttagagcacctgagaaggatcaagagtggaggtgatctggattggttcgacatattgcgaatt-
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>Sw-5*

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>Sw-5**

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# Chapter 6

# Subcellular localization and protein interactions of Sw5b subdomains

Athos Silva de Oliveira, Renato O. Resende and Richard Kormelink

**ABSTRACT**

NB-LRR proteins are intracellular plant receptors that (in)directly perceive the presence of effectors secreted by pathogens. This perception generally leads to plant resistance activation which usually involves an additional hypersensitive, programmed cell death response (HR) in infected and neighboring cells. The distribution of NB-LRR proteins in the cell seems to match with the location of their corresponding effectors or other plant proteins "manipulated" by effectors. Usually, NB-LRR proteins also localize to the nucleus during a particular stage of pathogen invasion, inducing genetic reprogramming of infected cells. The *Sw-5b* gene encodes a NB-LRR receptor that activates resistance against *Tomato spotted wilt virus* (TSWV) and other related tospovirus species (Family *Bunyaviridae*) after perception of their cell-to-cell movement ($NS_M$) protein. In this study the subcellular localization of full-length and truncated Sw-5b proteins has been determined. First preliminary data indicate that Sw-5b and its individual domains have a nucleocytoplasmic distribution, in which the CC domain seems to be required for a nuclear localization. Moreover, a possible direct interaction between the subdomains NB-ARC and LRR with $NS_M$ has been investigated by bimolecular fluorescence complementation (BiFC). Unexpectedly, the first data from these experiments have indicated a direct interaction of NB-ARC with $NS_M$, but not between LRR and $NS_M$.

**INTRODUCTION**

Plants contain an arsenal of receptors capable of recognizing pathogens and activating downstream signaling that end up in disease resistance. These receptors localize throughout the cell in the plasma membrane and cytoplasm. Those exposed on the plant cell membrane are often classified as Pattern Recognition Receptors (PRR) and usually sense Pathogen-Associated Molecular Patterns (PAMPs) from extracellular pathogens (Zipfel, 2014). PAMPs are conserved and slowly-evolving molecules such as chitin and flagellin from fungi and bacteria, respectively. Most receptors located in the cytoplasm are classified as Resistance (R) proteins and perceive pathogens via their proteins referred to as effectors (Jones and Dangl, 2006). These effectors, usually less conserved among pathogens, are thought to inhibit primary biochemical defense lines such as those triggered by transmembrane PRRs and RNAi. An effector that activates a corresponding R protein is commonly denominated as avirulence (Avr) determinant.

As signature, most (dominant) R proteins are from the type class NB-LRR, referring to two domains that are acronyms for Nucleotide-binding respectively Leucine-Rich-Repeat. Coiled-coil (CC) secondary structures or Toll-like/interleukin-1 receptor domains often precede the NB-LRR domains at the N-terminus (Takken and Goverse, 2012). Besides

perceiving effectors on the plasma membrane or in the cytoplasm, R proteins also localize in the nucleus at some stage during pathogen invasion, indicating that these proteins activate signaling pathways linked to genetic reprogramming. The barley Mla10 protein, for example, interacts with the transcriptional repressor WRKY and the transcriptional activator MYB6 (Chang et al., 2013; Shen et al., 2007). Potato Rx needs a balanced distribution in both nucleus and cytoplasm for full functionality, although recognition of the Potato virus X Avr coat protein (CP) and resistance signaling seem to take place in the cytoplasm (Slootweg et al., 2010; Tameling et al., 2010). Eventually, nuclear localization of R proteins does seem vital for resistance activation. On the other hand, Arabidopsis RPS5 and RPM1 are only present on the plasma membrane, likely reflecting the location of their guardees and cognate effectors from *Pseudomonas syringae* (Chung et al., 2011; Dowen et al., 2009; Gao et al., 2011; Liu et al., 2011; Shao et al., 2003).

Effectors are assumed to be sensed via direct or indirect interaction with their cognate R proteins in plants. In most cases studied, a direct interaction has not been observed which has led to the design of the most commonly accepted "Guard" and "Decoy" models (Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008). The first one states that R proteins (guard) survey other host proteins (guardees) that are modified by effectors for pathogen virulence. The perception of these modifications activates the R protein and results in disease resistance. The second model also considers the presence of host intermediates, but questions their relevance for pathogen virulence. In other words, these host intermediates just evolve as "decoys" of effectors and do not have other cellular functions.

The *Sw-5b* gene codes for a CC-NB-LRR protein that confers resistance against *Tomato spotted wilt virus* (TSWV) and several other related important tospovirus species (Genus *Tospovirus*, Family *Bunyaviridae*) (Brommonschenkel et al., 2000; Hallwass et al., 2014; Spassova et al., 2001). This gene originates from *Solanum peruvianum* L., a wild tomato species, and has been introgressed in different cultivars of commercial tomato, *Solanum lycopersicum* L. (Ferraz et al., 2003; Giordano et al., 2000; Langella et al., 2004). The cell-to-cell movement protein ($NS_M$) activates the Sw-5b-mediated resistance (Chapter 2). Four *Sw-5b* paralogs (*Sw-5a*, *Sw-5c*, *Sw-5d*, and *Sw-5e*) have been reported in tomatoes crossed with *S. peruvianum* (Spassova et al., 2001), but only the paralog *Sw-5b* is able to halt tospovirus infections (Chapter 4). Tomatoes not crossed with *S. peruvianum* and susceptible to TSWV, harbor highly conserved *Sw-5* orthologs (Chapter 4). Dissection of the Sw-5b protein and of two other Sw-5 homologs has indicated that the NB domain is required for signal transduction leading to cell death, while the CC region seems to suppress this process. On the other hand, the LRR domain is essential for effector recognition (Chapter 4), but whether this involves direct or indirect recognition of $NS_M$ remains to be investigated.

Here we have determined the localization of the Sw-5b protein and derivatives that contain only one or two of its three main domains, using Green Fluorescent Protein (GFP) fusions, in the absence or presence of $NS_M$ from a resistance-inducing (BR-01) TSWV isolate. In addition, pilot experiments were performed to investigate the interaction between LRR, NB-ARC and $NS_M$ using Bimolecular Fluorescence Complementation assays (BiFC).

## RESULTS

### The Sw-5b protein localizes in both nucleus and cytoplasm

Due to the absence of a specific antibody towards Sw-5b, its localization and those of the individual domains CC, NB-ARC, and LRR and for the truncated versions CC-NB-ARC and NB-ARC-LRR (Figure 1A) were studied by fusion to GFP to their N-terminus. Constructs were made and cloned into binary vectors and via agroinfiltration expressed in *N. benthamiana* leaves. Optimal fluorescence images were captured at 4 days post agroinfiltration (dpa). The results showed that GFP-Sw-5b localized in both nucleus and cytoplasm (Figure 1B), and this was similarly observed for the individual domains and GFP-CC-NB-ARC (Figure 1C). GFP-NB-ARC-LRR was only observed in the cytoplasm and seemed absent from the nucleus. Co-expression of all aforementioned constructs in the presence of $NS_M$ resulted in an apparent decrease in nuclear localization for NB-ARC and LRR (data not shown).

### $NS_M$ and LRR domain do not seem to directly interact *in vivo*

Since the LRR domain is required for effector recognition (Chapter 4), its direct interaction with $NS_M$ was tested. To this end, binary vector BiFC constructs were made of LRR and $NS_M$ fused to either the N- or C-terminal halves of YFP and, after agroinfiltration into *N. benthamiana* leaves, checked for fluorescence recovery, indicative for protein interaction. As a result, no fluorescence was observed when both LRR and $NS_M$ were present (Figure 2A). The $NS_M$ protein was used as positive control, earlier shown to form homo-dimers and localizing to the cell periphery (Leastro et al., 2015). These results suggest that LRR and $NS_M$ do not directly interact.

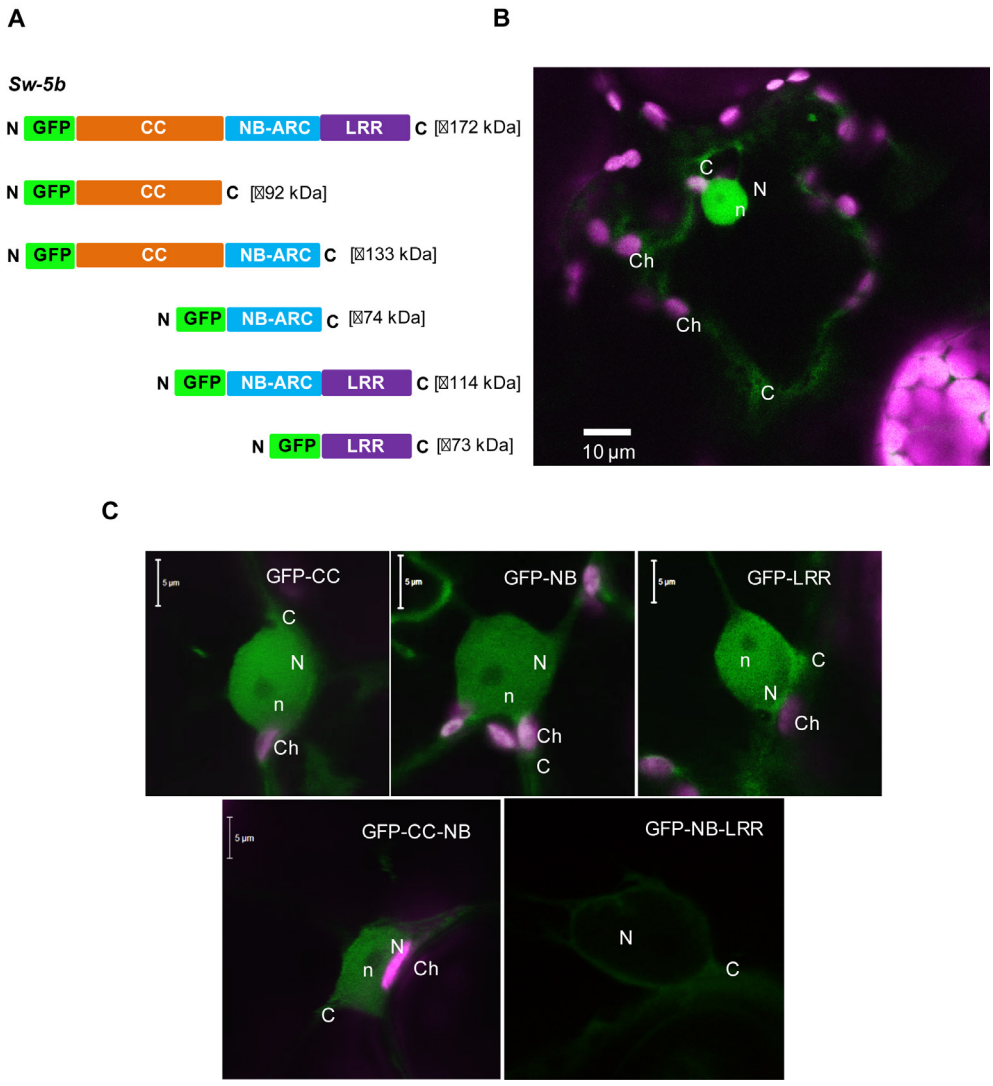


**Figure 1. Localization of full length and truncated Sw-5b proteins fused to GFP in *N. benthamiana* leaves.** (A) Schematic illustration of the full length and truncated Sw-5b proteins. (B) Transient expression of the full-length Sw-5b protein. (C) Expression of truncated Sw-5b proteins. Confocal images were captured at 4dpa. C cytoplasm; N nucleus; n nucleolus; and Ch chloroplast.

**$NS_M$ and NB-ARC seem to directly interact *in vivo***

A possible direct interaction was also tested between NB-ARC and $NS_M$, especially since an apparent decreasing of nuclear localization was observed when GFP-NB-ARC was co-expressed with non-tagged $NS_M$. Surprisingly, upon co-expression of NB-ARC and $NS_M$ fused to split-YFP, fluorescence recovery was observed for three out of four combinations as visualized in the Figure 2B. The nucleocapsid protein of Salmonid alphavirus (SAV) was used as negative control in combination with NB-ARC constructs and, since it formed dimers, as a positive control as well (Figure 2B). To strength the possibility of a direct interaction between NB-ARC and $NS_M$, BIFC constructs harboring the NB-ARC-LRR region were also co-infiltrated with split YFP-tagged $NS_M$. The same combinations, considering the position (N- or C-terminal) of the split-YFP halves, ended up in fluorescence recovery (Figure 2C). Nevertheless, only two combinations (nYFP-NB-ARC-LRR and cYFP-$NS_{M\text{-}BR01}$; nYFP-NB-ARC-LRR and $NS_{M\text{-}BR01}$-cYFP) resulted in $NS_M$-dependent HR triggering upon expression in *N. benthamiana* leaves, indicating that the other split-YFP fusions might have hampered the functionality of NB-ARC-LRR in recognizing $NS_M$ as Avr. The co-expression of NB-ARC-LRR with $NS_M$, included as positive control, triggered HR as earlier demonstrated (Chapter 4).

**The NB-ARC domain forms dimers and interacts with the LRR domain**

In analogy to the BiFC approach to test for a direct interaction between individual Sw-5b subdomains and $NS_M$, NB-ARC and LRR were analyzed for possible interactions. Pilot experiments revealed that co-expression of nYFP-NB-ARC and cYFP-NB-ARC resulted in fluorescence recovery, indicating the formation of dimers. The same outcome was not observed after co-expression of a pair of LRR proteins fused to split-YFP (Figure 3). Furthermore, fluorescence was observed after co-expression of nYFP-NB-ARC and cYFP-LRR, pointing towards a direct interaction between these two domains as well (Figure 3). No fluorescence in this experiment was seen in the nucleus in contrast with the single subdomains fused to GFP but in agreement with the earlier found cytoplasmic location of GFP-NB-ARC-LRR (Figure 1).

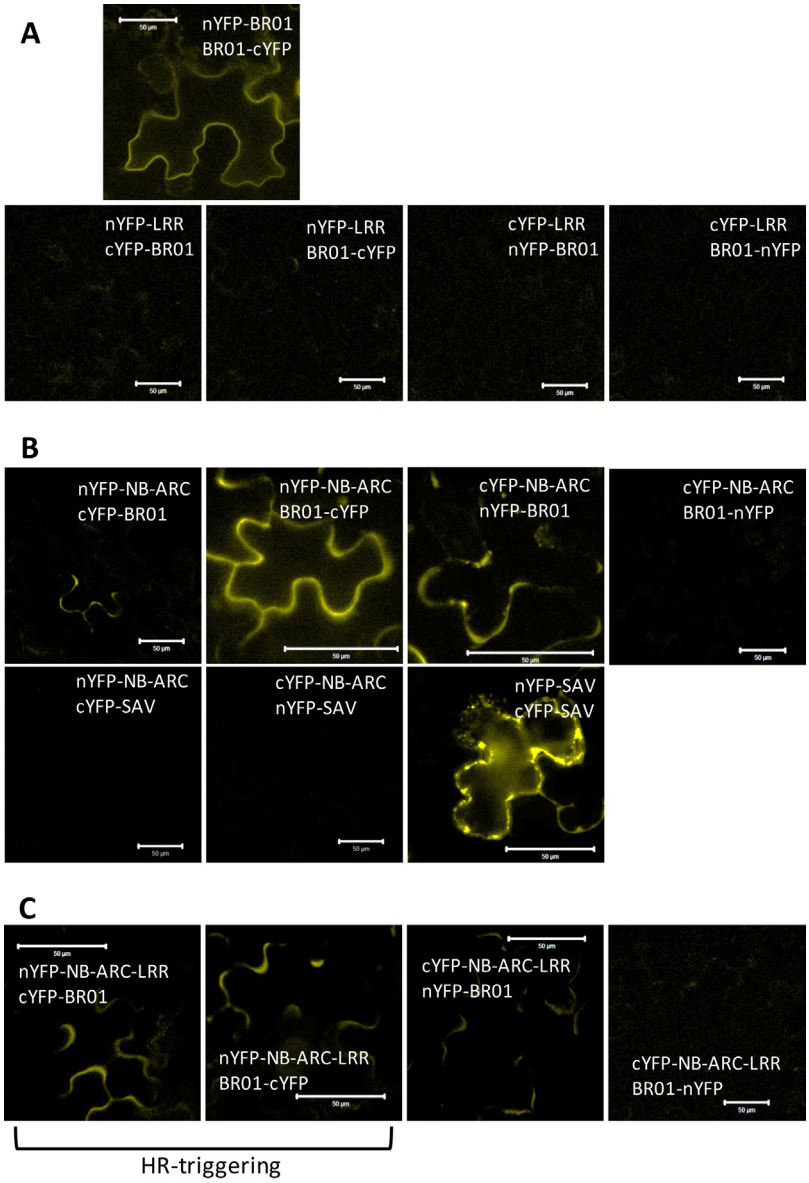


**Figure 2. BIFC assay in *N. benthamiana* leaves between Sw-5b subdomains and $NS_M$ fused to split-YFP.** (A) Transient expression of LRR and $NS_M$ (from the resistance-inducing TSWV BR-01 isolate) fused to the N- or C-terminal halves of YFP. The $NS_M$ protein, which forms dimers, was used as positive control for YFP fluorescence recovery (Leastro et al., 2015). (B) Transient expression of NB-ARC and $NS_M$ fused to the N- or C-terminal halves of YFP. The capsid protein of Salmonid alphavirus (SAV) in combination with NB-ARC was used as negative control. The capsid protein of SAV was also used as positive control for YFP fluorescence recovery since it forms dimers. (C) Transient expression of NB-ARC-LRR and $NS_M$ fused to the N- or C-terminal halves of YFP. The first two combinations resulted in HR-triggering of infiltrated leaves from 2/3 dpa. Confocal images were captured at 3 dpa. C cytoplasm; N nucleus; n nucleolus; and Ch chloroplast.

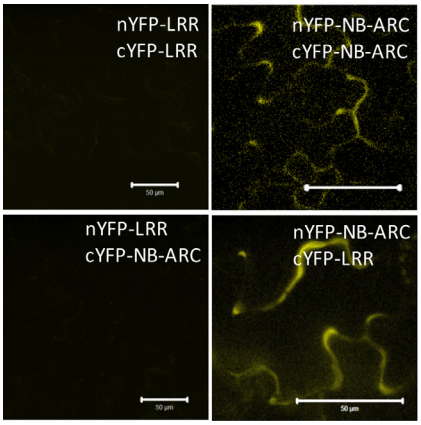


**Figure 3. BIFC assay in *N. benthamiana* leaves between NB-ARC and LRR fused to split-YFP.** Transient expression of NB-ARC and LRR fused to the N- or C-terminal halves of YFP. The $NS_M$ protein was used as positive control for YFP fluorescence recovery (not shown). C cytoplasm; N nucleus; n nucleolus; and Ch chloroplast.

## DISCUSSION

Activation of NB-LRR proteins usually requires nuclear localization that is associated with transcriptional reprogramming. Upon presence of effectors, some NB-LRR signature proteins have been observed to accumulate in the nucleus and interact with transcription factors. Although a nuclear localization seems crucial for a resistance response, the *Arabidopsis* RPM1 protein, has only been observed to localize in the plasma membrane, likely reflecting the location of its Avr and "guardee" proteins (Gao et al., 2011). In the current study the functional GFP-fused Sw-5b protein distributed in both nucleus and cytoplasm in the absence of its Avr, the TSWV cell-to-cell movement protein $NS_M$. From the various Sw-5b truncations or individual domains tested, the GFP-NB-ARC-LRR localized exclusively in the cytoplasm, while all other fusion proteins showed a nucleocytoplasmic distribution similar to the full length Sw-5b protein. These data seem to suggest an important role for the CC domain in nuclear translocation. The fusion proteins GFP-NB-ARC and GFP-LRR, containing single domains, also were seen in the nucleus, but their localization might be due to passive diffusion into the nucleus. For humans cells, even proteins larger than 60 kDa can diffuse though the nuclear pore complex (Wang and Brattain, 2007).

The CC domain corresponds to 41% of the full-length Sw-5b protein. This large CC domain is due to an extended N-terminus, which has also been observed for other NB-LRR proteins from Solanaceae plants (Lukasik-Shreepaathy et al., 2012). Intriguingly, a Sw-5b derivates lacking this entire CC domain still triggered $NS_M$-dependent HR (Chapter 4).

Together with the observation that NB-ARC-LRR -lacking the CC domain- localizes to the cytoplasm only, the signal transduction for effector-dependent HR seems to occur in the cytoplasm. Earlier studies, however, already indicated that HR and pathogen resistance are two process that can be uncoupled for certain R proteins (Coll et al., 2011). Whether HR is part of the intrinsic disease resistance response of Sw-5b remains to be determined. For other R proteins, the CC domain has been seen indispensable for correct functioning. Dimers of CC from barley MLA10 are necessary and sufficient for interaction with WRKY transcription factors and CC is also sufficient for cell death triggering (Maekawa et al., 2011). Although speculative, it might well be possible that the CC domain of Sw-5b directs the nuclear targeting to trigger transcriptional reprogramming leading to the actual TSWV resistance response. In conclusion, it remains to be investigated if the CC domain from Sw-5b is necessary for the signal transduction that leads to disease resistance against tospoviruses.

Since the Sw-5b LRR domain specifies effector recognition, this domain has been tested for direct interaction with $NS_M$ from BR-01, a resistance-inducing TSWV isolate. However, our first pilot experiments using BiFC ended up negative, indicating that a direct interaction between these two proteins seems to be absent. Since the experiment was only performed with split-YFP fusions at the N-terminus of LRR, which might have caused a failure due to steric hindrance, experiments need to be repeated using fusions at its C-terminus as well. Although the LRR domain seems to specify effector recognition for most NB-LRR proteins, the CC domain of rice Pik binds directly to its cognate Avr from the ascomycete fungus *Magnaporthe oryzae* (Kanzaki et al., 2012). This physical interaction determines the specific recognition of the pathogen leading to HR triggering. Since the cellular localization of the Sw-5b NB-ARC domain apparently changed upon co-expression with non-tagged $NS_M$, these two proteins were also tested by BiFC. Interestingly, YFP fluorescence recovery was observed, which strengthen the earlier indication for a putative direct interaction between NB-ARC and $NS_M$. A similar outcome was also observed between co-expression of NB-ARC-LRR and $NS_M$ fused to split-YFP, suggesting that a direct interaction may indeed occur between $NS_M$ and Sw-5b NB-ARC rather than that LRR binds directly to $NS_M$. Besides this unexpected interaction, the LRR domain specifies Avr recognition for Sw-5b since NB-ARC has to be covalently bound to LRR for $NS_M$-dependent HR (Chapter 4). Altogether, this leads to the hypothesis that binding between $NS_M$ from a resistance-inducing tospovirus isolate and NB-ARC leads to a conformational change sensed by LRR, which in turn, controls the inactive/active states of NB-ARC. More experiments, however, need to be performed to test this model.

BiFC has also been used to follow the interaction dynamics between NB-ARC and LRR domains in cells. Surprisingly, NB-ARC seemed to form dimers as previously observed for the CC domain of the barley MLA protein. CC-dependent dimerization of MLA is required for cell death triggering (Maekawa et al., 2011). Although NB-ARC and CC are completely

different domains, further studies will be needed to reveal if cell death triggering depends on NB-ARC dimerization for Sw-5b. The NB-ARC domain also seems to interact with LRR, which was expected based on the abovementioned activation model and on models of other NB-LRR proteins. Physical interaction and *trans*-complementation, for example, are seen for CC-NB-ARC and LRR from tomato Mi-1.2 (van Ooijen et al., 2008) and from potato Rx, while a wrong pairing of NB-ARC and LRR ends up in auto-HR (Rairdan and Moffett, 2006). Although a generic model still seems too speculative with the limited data, in most cases NB-ARC and LRR interactions occur and play a role in the activation of NB-LRR proteins. The cellular localization data presented here have provided a first glimpse on the signaling pathway behind Sw-5b-mediated resistance, i.e. on the cellular compartment in which effector-dependent HR takes place and the activation of the Sw-5b protein by $NS_M$.

## MATERIALS AND METHODS

### Plant material

*Nicotiana benthamiana* plants were kept under glasshouse conditions (24ºC, 16h light/8h dark a day).

### Molecular cloning

The pK7WGF2-based constructs were made as described in Chapter 4. The pDONR207 harboring $NS_M$ and *Sw-5b*-based genes (Chapter 2 and 4), the capsid gene from SAV (kindly supplied by Mia Hikke, Wageningen University) and pENTR-GUS (supplied by Invitrogen) were recombined into pSITE-BiFC-C1 vectors (Martin et al., 2009) by LR Clonase (Invitrogen). The molecular cloning procedures followed the manufacturer's protocol and methods described by (Green et al., 2012).

### Agroinfiltration

Agroinfiltrations were performed following the protocol described by Bucher et al. (Bucher et al., 2003). *A. tumefaciens* COR308 harboring single pK7WGF2- or pSITE-BiFC-C1-based constructs were grown overnight at 28 °C in LB3 medium with 2 µg/ml tetracycline and 250 µg/ml spectinomycin. A volume of 600 µl LB3 inoculum was added into 3 ml induction medium and, once again, grown overnight. After harvesting and resuspending the

bacteria in MS-MES buffer, *N. benthamiana* leaves were infiltrated with single or combinations of suspensions with a final $OD_{600}$ of 1.0 for pK7-based constructs (single) and 0.5 for each pSITE construct (co-infiltration). Pictures were taken 4 days post agroinfiltration (dpa) for GFP-tagged proteins and 3 dpa for BiFC experiments.

**Confocal microscopy**

Images of epidermal cells from *N. benthamiana* leaves were obtained using a Zeiss LSM 510-META 18 confocal laser scanning microscope with an x40 1.3-numerical aperture oil-corrected objective. GPF imaging was obtained by 488-nm excitation from an argon laser and emission was detected through a 505- to 570-nm filter. For YFP, images were obtained by 514-nm excitation from an argon laser and emission was detected through a 518- to 572-nm filter. Chlorophyll emission was detected through a 650-nm filter. The parameters for image acquisition were kept the same for all constructs.

# Chapter 7

# General Discussion

Athos Silva de Oliveira

Despite the continuous use of the *Sw-5*-resistance gene cluster in breeding of tomato cultivars, not much was known about the molecular and cellular mechanisms leading to resistance against *Tomato spotted wilt virus* (TSWV) and other tospoviruses. This thesis, aimed to investigate the functionality of different Sw-5 homologs, focusing on the mode of activation and the recognition of tospoviruses. The instability of the Sw-5 proteins in triggering cell death under determined conditions and lack of molecular tools such as a TSWV reverse genetic system have made the exploration of biological questions more difficult and limited the execution of some experiments. Nevertheless, several interesting results have been obtained.

**_Sw-5b_ seems to be the only _Sw-5_ paralog that triggers disease resistance against tospoviruses**

Besides TSWV, *Sw-5b*-tranformed *N. benthamiana* plants also showed resistance to isolates of the tospovirus species *Alstroemeria necrotic streak virus* (ANSV), *Groundnut ringspot virus* (GRSV), *Tomato chlorotic spot virus* (TCSV), *Chrysantemum stem necrosis virus* (CSNV) and *Impatiens necrotic spot virus* (INSV), revealing that the Sw-5b protein is sufficient for broad-spectrum resistance (Chapter 2, Figure 1). These tospoviruses have host-ranges encompassing species of the family Solanaceae (including tomatoes), confirming that they have had a close evolutionary relationship with these host plants that also have an American origin. The current *S. lycopersicum* (not bred to *S. peruvianum*) either lost the ability to perceive American tospoviruses or did not suffer selection pressure as much as *S. peruvianum,* which harbors *Sw-5a* and *Sw-5b*, a typical example of gene duplication that has not been observed for the ortholog *Sw-5a*$^{S}$ from *S. lycopersicum* so far. Tospoviruses that have evolved in non-solanaceous host plants are unlikely to be perceived by *Sw-5b.* This indeed appeared to be the case for the virus studied in Chapter 4, Bean necrotic mosaic virus (BeNMV) which was found naturally infecting common beans (family Fabaceae).

Considering the presence of at least five *Sw-5* paralogs in *S. Peruvianum* (Spassova et al., 2001), the other Sw-5 proteins (other than Sw-5b) were hypothesized to possibly play part in the recognition of this large number of tospovirus species. Whether any of these other paralogs indeed triggers resistance to tospoviruses remains to be investigated but, based on the studies presented mainly in Chapter 5, they seem not to do so. Nevertheless, it cannot be ruled out that these other *Sw-5* paralogs encode functional proteins triggering resistance against other pathogens. The Potato Rx and Gpa2 paralogs, for example, share 88% identity and provide resistance against *Potato virus X* (PVX) and the nematode *Globodera pallida*, respectively (van der Vossen et al., 2000).

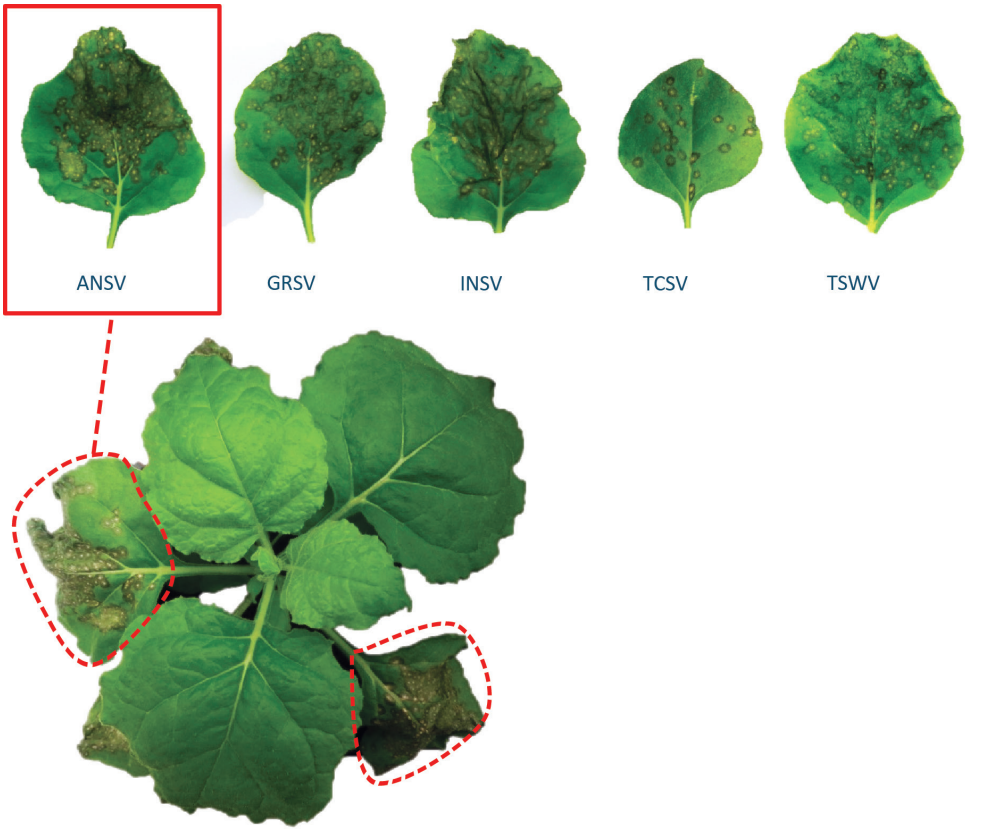


**Figure 1. *Sw-5b*-transformed *N. benthamiana* challenged with different tospovirus species.** Plants were mechanically inoculated with tospovirus-infected suspensions as described in Chapter 4. Only inoculated leaves show necrotic spots. Pictures were taken 12 days post mechanical inoculation.

### Sw-5b senses a broad-spectrum of tospoviruses via their movement proteins

Previous studies have suggested the $NS_M$ protein as the avirulence determinant (Avr) of the Sw-5b-mediated resistance. First, viruses with reassorted genome fragments, that contained the M RNA from a resistance-breaking TSWV isolate, in the context of S and L RNAs from a resistance-inducing TSWV isolate, overcame the *Sw-5* resistance (Hoffmann et al., 2001). This indicated that either the viral glycoproteins $G_N$ and $G_C$ or the movement protein $NS_M$ would be the Avr for the triggering of *Sw-5*-resistance. Later it was found that nucleotide changes in resistance-breaking TSWV isolates led to shared amino acid replacements in the $NS_M$ protein. These mutations were likely the consequence of the *Sw-5* selection pressure and pointed towards $NS_M$ as the putative Avr (Lopez et al., 2011). In this

thesis, it was shown that the $NS_M$ protein from resistance-inducing tospovirus isolates triggers HR in both Sw-5-resistant tomato isolines and in *Sw-5b*-transformed *N. benthamiana* (Chapter 2). Transient co-expression of Sw-5b with the $NS_M$ proteins from TSWV and INSV resulted in HR, in contrasts to co-expression with the homologous $NS_M$ of BeNMV (Chapters 4-5).

Besides the $NS_M$ protein of tospoviruses, there are two other cases in which a viral movement protein turned out to be a Avr determinant, namely the tobamovirus 30 kD protein sensed by the tomato Tm-2 protein and the hordeivirus TGB1 protein sensed by the false brome Brs1 protein (Lee et al., 2012; Weber et al., 1993). In most cases though, capsid proteins have been reported as viral Avr recognized by cloned NB-LRR genes (de Ronde et al., 2014a). Bearing in mind the definition of a viral effector, it remains unknown how $NS_M$ interferes in the innate immunity of plants. So far $NS_M$ has only been shown to induce tubule formation and associate with RNPs to facilitate their transport through modified plasmodesmata to enter other cells (Kormelink et al., 1994; Soellick et al., 2000; Storms et al., 1995; Storms et al., 1998). However, non-functional $NS_M$ protein is still recognized by Sw-5b, based on observations that truncated $NS_M$ proteins lacking motifs associated to cell-to-cell and systemic movements still induce Sw-5b-dependent HR (Chapter 3).

Among all tospovirus proteins, evolutionary analyses have indicated evidence of episodic diversifying selection only for $NS_M$ and RdRp in the branches leading to the "American" clades, which encompasses the tospoviruses experimentally proven to trigger Sw-5b resistance (Chapter 4). This suggests that the Sw-5b resistance may be one of the constraints leading to positive selection on $NS_M$. As described in Chapter 4 two tospovirus lineages circulate in the American continent. The first one contains tospovirus species such as TSWV that has a broad host-range, including many solanaceae species (e.g. tomato). So far the newly identified, second American clade contains two tospovirus species, BeNMV and Soybean vein necrosis-associated virus (SVNaV), which have been found from naturally infecting beans respectively soybeans (Family Fabaceae) and that may have a preference for leguminous hosts. Although they have been classified as "American" (or New World) tospoviruses, the co-expression of BeNMV $NS_M$ and Sw-5b did not result in HR triggering, indicating that this $NS_M$ protein is not recognized as Avr (Chapter 4). Altogether, these data suggest that BeNMV and SVNaV have experienced a different host adaptation history compared to TSWV. Since the BeNMV and SVNaV $NS_M$ proteins have suffered positive selection as well, it is likely that selection pressure comes from constraints other than Sw-5b. The absence of HR upon co-expression of BeNMV $NS_M$ and Sw-5b in *N. benthamiana*, however, does not mean that this virus can infect tomato cultivars. In light of this, attempts to infect a TSWV susceptible tomato cultivar with BeNMV by mechanical inoculation have failed.

So far only BeNMV and SVNaV have been found as members of their evolutionary lineage, but more viruses may be isolated in the future that belong to this lineage. This assumption comes from the number of tospovirus species grouped in other clades. An in-depth survey of leguminous plants may reveal more members of the BeNMV-SVNaV clade. Up to now, other BeNMV isolates have not been reported. The difficulty of transmission by mechanical inoculation and an apparent narrow host-range make BeNMV unique but currently inoffensive for crop production. On the other hand, SVNaV is already the most widespread soybean virus in the USA, and has drawn the attention of farmers and researchers (Zhou and Tzanetakis, 2013).

**Sw-5b triggers both $NS_M$-dependent and –independent HR**

Although HR and restriction of pathogen growth can be dissociated for tested *R* genes (Coll et al., 2011), HR has been a reliable phenotypic indication for activation of Sw-5b-mediated resistance. This is supported by observations made with transgenic lines of *N. benthamiana* that have been transformed with a copy of *Sw-5b* and respond with a clear HR after challenge with TSWV (Chapter 2). Previously, *N. tabacum* plants transformed with *Sw-5b* did not show HR after challenge with TSWV (Spassova et al., 2001). In those transformants, *Sw-5b* was under control of its own tomato promoter and terminator elements, while in *N. benthamiana* plants *Sw-5b* was under control of the strong Cauliflower mosaic virus-derived 35S promoter. Although it still needs to be proven, the cause for a strong HR response in transformed *N. benthamiana* is likely due to a difference in the regulation of its expression by different promoters. Expression levels obtained with the strong 35S promoter (as applied in the transgenic *N. benthamiana*) seems to be the limit for a tolerable *Sw-5b* accumulation since $NS_M$-dependent HR and disease resistance were still observed (Chapters 2-5). However, further elevation of these expressions levels led to auto-HR, as observed when *Sw-5b* was under control of two 35S promoters and co-expressed with RNA silencing suppressors (Chapter 5). Auto-HR occurred in the absence of $NS_M$ and thereby allowed the investigation of *Sw-5* mediated HR-triggering in a $NS_M$-independent manner while the first scenario (without RNA silencing suppressors) allowed a study on effector-recognition, i.e. $NS_M$-dependent HR-induction. During all these studies, the $NS_M$ protein from a resistance-breaking field isolate (GRAU) did not lead to HR triggering and therefore, was constantly used as an important negative control. Moreover, our results also indicated that certain *R* genes under control of a strong promoter may compromise the viability of transformed plants/cells. In this perspective tolerable expression levels of a given *R* gene have to be determined before the development of transgenic plant lines. The overexpression of a resistance gene can eventually lead to extreme resistance as well (Sato et al., 2014).

Even though Sw-5b and the paralog Sw-5a share around 95% amino acid identity, Sw-5a was only able to trigger auto-HR but not capable to recognize $NS_M$ (Chapter 5). This outcome explains the susceptible status of *Sw-5a*-transformed *N. tabacum* to TSWV (Spassova et al., 2001). These observations also support the idea that other paralogs from *S. peruvianum* (Sw-5c, Sw-5d, and Sw-5e) are likely incapable in sensing TSWV as well. Firstly, they are not only less similar to Sw-5b than Sw-5a but secondly, functional analysis of Sw-5b has indicated that a few amino acid mismatches observed among the Sw-5 proteins will disrupt effector recognition and HR triggering. In addition to Sw-5 paralogs from *S. peruvianum*, the closest ortholog of Sw-5a and Sw-5b from *S. lycopersicum*, Sw-$5a^S$, failed to recognize $NS_M$ as Avr and, surprisingly, also did not trigger auto-HR.

**NB-ARC domains from Sw-5 proteins are sufficient for the cellular signal transduction that leads to cell death**

Dissection of Sw-5b identified the role of CC, NB-ARC, and LRR domains in HR-triggering and effector recognition (Chapter 5). The Sw-5b NB-ARC domain alone was sufficient to trigger auto-HR but not $NS_M$-dependent HR, supporting a role in the signal transduction leading to cell death. Similar observations have been reported for potato Rx, which is one of the best studied R proteins. Structural modelling of Rx NB-ARC suggests that the ARC region can be subdivided into ARC1 and ARC2 (Rairdan and Moffett, 2006). The first subdomain plays a role in recruiting the LRR domain to bind the CC-NB-ARC domains, while ARC2 seems to be involved in controlling the active and inactive states of potato Rx. Wrong pairing of NB-ARC and LRR by domain-swap experiments with potato Rx and Gpa2 leads to auto-activation, indicating that LRR regulates NB-ARC transition from inactive to active states (Rairdan and Moffett, 2006). In our studies, auto-HR has been due to high protein accumulation, and CC rather than LRR seems to inhibit the active state of Sw-5b. However, auto-HR has been observed for Sw-5b by introduction of a D-to-V mutation at position 857, which is located between the NB-ARC and LRR domains (Postma et al., 2012). Thus, LRR also seems to control the active status of NB-ARC for Sw-5b. Similar to the full length proteins, the NB-ARC domain of Sw-5a was able to trigger HR, in contrast to the comparable domain of Sw-$5a^S$. The alignment of the NB-ARC domains of these three proteins revealed three major mismatches in the most conserved region, involving replacement of amino acids residues with divergent properties. Introduction of the mutation Q599R, one of these mismatches, led to a gain-of-function in Sw-$5a^S$ NB-ARC, as deduced from the ability to induce auto-HR upon overexpression. This modification is not located in or adjacent to the ADP/ATP binding site of NB-ARC, ruling out that the absence of HR is caused by the inability to bind ATP. Rather, this mutation seems to interfere with the activation of the downstream signal transduction leading to HR. Intriguingly, the introduction of the Q-to-R mutation into

the full length Sw-5a$^S$ did not lead to auto-HR, not even after removal of its CC domain that was likely to suppress auto-HR, as was seen for Sw-5b. In summary, this indicated that turning non-functional receptors into functional ones is quite tricky, even in situations of high identity/similarity between homologs.

The Sw-5b NB-ARC covalently bound to LRR triggered both auto-HR and $NS_M$-dependent HR, indicating that LRR perceives $NS_M$ as Avr. Nevertheless, the substitution of the LRR domain from Q599R Sw-5a$^S$ for the one from Sw-5b still did not trigger $NS_M$-dependent HR. On the other hand and interestingly, when the Q599R Sw-5a$^S$ CC domain was removed both auto- and $NS_M$-dependent HR were observed again. Thus, CC from Sw-5a$^S$ seems to interfere with the protein folding thereby preventing $NS_M$ recognition, indicating that a harmonic combination of the three domains is required for correct functioning. A similar chimera made from potato Rx (CC-NB-ARC) and GPa2 (LRR) ended up in auto-activation (Rairdan and Moffett, 2006). In the same study the C-terminal half of the LRR domain from Rx was proven to be required for recognition of its Avr, the PVX CP protein. A further dissection of Sw-5b may reveal which part of the LRR domain is essential for recognition of $NS_M$. Such information may contribute to the engineering of new NB-LRR receptors that are able to perceive tospovirus $NS_M$, using a scaffold of different R proteins appended with the sensing part from Sw-5b LRR.

One of the most unexpected outcomes was that the CC domain is not required for $NS_M$ recognition and HR activation. This domain covers about 41% of the Sw-5b protein sequence and seems to direct its nuclear localization (Chapter 6). Altogether, these results indicate that HR either plays part in the resistance mechanism as reinforcement, or alternatively, HR and disease resistance are uncoupled events also for Sw-5b. CC as a vestigial protein sequence for the Sw-5 homologs seems to be unlikely based on the activation model of other R proteins. So far the only function observed for CC is suppression of NB-ARC-triggered HR. Whether CC is indeed necessary for the signal transduction that leads to disease resistance remains to be investigated, but it would rather be expected based on our subcellular studies and the activation models of other R proteins. As discussed in chapter 5, the Sw-5 proteins present an extended CC domain compared to other R proteins. For tomato Mi-1.2, an extended N-terminus is also present and has been divided in three subdomains named Solanaceae Domain 1 (SD1), Solanaceae Domain 2 (SD2) and CC (Lukasik-Shreepaathy et al., 2012). SD1 suppresses HR triggering, while SD2 and CC work as positive regulators of HR-triggering. The removal of the extended CC domain from Mi-1.2 leads to HR inactivation, which differs from *Sw-5b* that still triggers both auto- and $NS_M$-dependent HR. The proteins encoded by the *Sw-5* homologs vary in secondary structure prediction of the CC domain. While Sw-5a, Sw-5b, Sw-5a$^S$, Sw-5d, and Sw-5e have coiled-coils distributed over the whole N-terminal part, the orthologous proteins Sw-5c and Sw-5f present such structures only in a small portion of the N terminal region (Figure 3). These differences may be reflected in their

activation models.

CC and TIR have been reported as the domains responsible for cell death signaling for some R proteins. The CC domain of the maize Rp1 protein, for example, has been shown sufficient for cell death triggering, while NB-ARC suppressed HR through direct interaction with the CC domain (Wang et al., 2015). The LRR in auto-active versions of maize Rp1 caused de-repression and, in that way, inhibited the HR suppression. In general, R proteins are activated leading to a similar downstream response but in which the subdomains not all seem to play an analogous role. In addition, it is not clear whether these differences are due to genuine mechanistic differences during protein activation or that experimental conditions, e.g. levels of expressions, tags and points of truncations, may have affected the results.

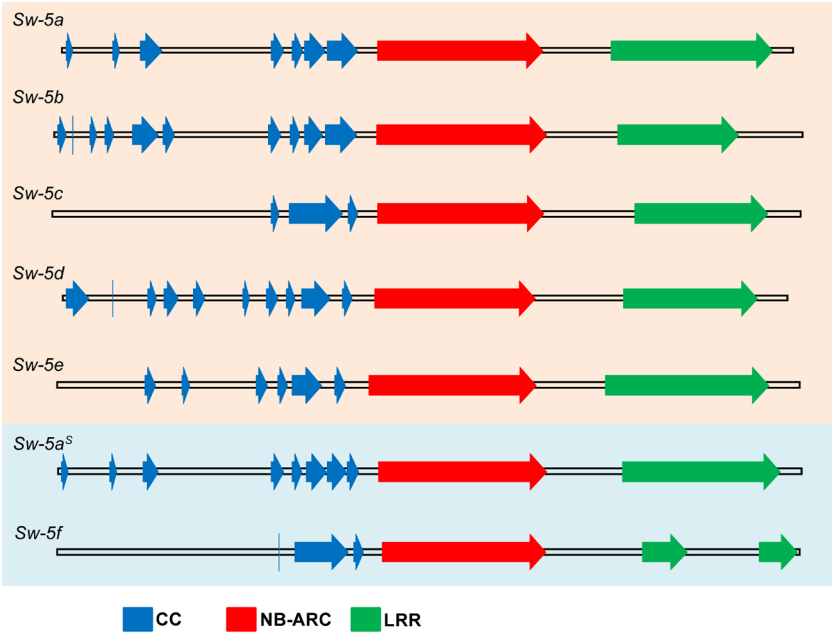


**Figure 2. Prediction of conserved domains of different *Sw-5* homologs.** The upper five homologs (pink background) correspond to protein sequences from *S. peruvianum*, the lower two (blue shading) from *S. lycopersicon* Heinz 1706. The bioinformatics tools used for predictions of domains are described in Chapter 5.

### Preliminary data suggest direct interaction between Sw-5b and $NS_M$

With the availability of the complete genome from the tomato cultivar Heinz 1706, more *Sw-5* orthologs have been mapped (Figure 3). In total there are three complete orthologs and a truncated *Sw-5* in Heinz 1706 including *Sw-5a*$^{S}$. All these orthologs seem to

be present in most *S. lycopersison* cultivars that have not been crossed with *S. peruvianum*. This gene cluster also includes the ortholog referred to as *Sw-5f*, which was first reported in the tomato cultivar Money Maker (susceptible to TSWV). The *Sw-5f*-encoded protein has been identified from a Yeast Two-Hybrid System screen as a protein interacting with the effector SPRYSEC-19 from the nematode *Globodera rostochiensis* (Rehman et al., 2009). This effector suppresses HR triggered by several CC-NB-LRR proteins including an auto-active Sw-5b. SPRYSEC-19 is a cyst nematode effector that is secreted with the saliva and allows the nematode to suppress cell death and resistance responses mediated by different R proteins in order to be able to feed and survive on host plants (Postma et al., 2012). Since SPRYSEC-19 directly interacts with the LRR domain of the Sw-5f protein, a possible direct interaction between Sw-5b and $NS_M$ has been speculated as well. Although the ability of Sw-5b to sense tospoviruses resides in its LRR domain, no direct interaction seemed to occur between LRR and $NS_M$ in BIFC assays (Chapter 6). Surprisingly, a direct interaction has been observed for NB-ARC and $NS_M$ using the same methodology. This outcome, however, does not rule out the participation of LRR in $NS_M$-recognition. More general, it could indicate that a direct and stable binding between the LRR and an Avr may only happen for activation of an R protein in the presence of NB-ARC.

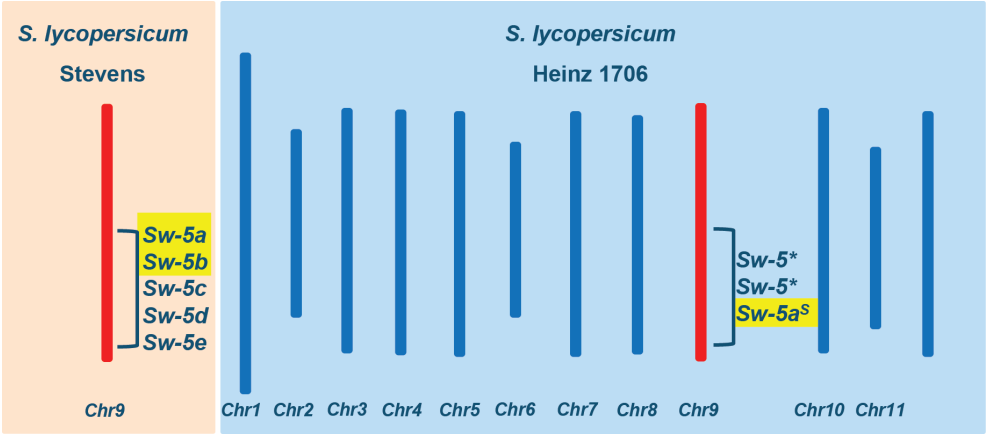


**Figure 3. Schematic illustration of the genome from tomato cultivar Heinz 1706 (shaded in blue).** Three full *Sw-5* genes have been mapped in chromosome 9 of the Heinz 1706 cultivar (using Basic Local Alignment Search Tool (BLAST)). *S. lycopersicum* Stevens, resistant to resistance-inducing TSWV isolates, has at least five *Sw-5* genes on chromosome 9, which originated from *S. peruvianum* (shaded in pink). The homologs analyzed in this thesis are highlighted in yellow.

**Final considerations**

So far the Sw-5b-mediated resistance is the only known efficient innate response against tospoviruses that has been able to overcome the first lines of defense in tomatoes (*S. lycopersicum* and *S. peruvianum*). Tospoviruses that enter a tomato cultivar lacking the *Sw-5b* gene are thought to primarily encounter the first line of, RNA interference (RNAi)-mediated, defense mechanism. They only achieve a successful infection if they are able to counter defend RNAi through RNA silencing suppressors. Since $NS_M$ is a non-structural protein, resistance-inducing tospoviruses are expected to replicate and transcribe their genome in infected cells of Sw-5b-resistant plants. Otherwise the $NS_M$ protein will not be available for interaction with the Sw-5b resistance protein. Therefore, after being injected by thrips, the RNPs of tospoviruses are thought to be released from the viral envelope and start genome transcription. In the presence of *Sw-5b*, disease resistance is activated upon $NS_M$ translation leading to cell death triggering. It remains uncertain whether cell death is the resistance mechanism itself or just a warrant to prevent virus movement after primary resistance responses.

From a practical point of view, the *Sw-5b* copy guarantees a broad-spectrum resistance against several distinct tospoviruses and therefore is one of the most interesting resistant genes for breeding of tomato. Correct management on the utilization of *Sw-5*-resistant tomato isolines via interchange with susceptible cultivars seems to safeguard durable resistance (Gordillo et al., 2008). This interchange of cultivars diminishes the selection pressure on the viruses, reducing the risk of appearance of large populations of resistance-breaking isolates.

Similar to the effect of the above mentioned Q599R mutation in the Sw-$5a^S$ NB-ARC domain, identification of key point mutations may enable the full activation of *Sw-$5a^S$* against tospoviruses in tomato cultivars using TALENs and CRISPR methodologies (Gaj et al., 2013). Plants with such small modifications may be more broadly accepted than classic transgenic plants. Moreover, the induction of random mutations by DNA shuffling techniques may end up in LRRs perceiving either a narrower or broader spectrum of tospoviruses. From a fundamental point of view, detailed information of the signaling pathway that leads to disease resistance is still very scarce and more work is needed to elucidate this pathway to identify the generic steps within the downstream signaling cascade common to all *R* genes. Using proteomics, candidate proteins may be identified that play a direct or indirect role in the perception of Avr determinants and downstream disease resistance activation. The findings presented in this thesis present an initial glimpse to this process for the Sw-5b-mediated resistance against tospoviruses.

# REFERENCES


Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 95:10306-10311.

Abascal, F., Zardoya, R., and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. Bioinformatics (Oxford, England) 21:2104-2105.

Abramovitch, R.B., Anderson, J.C., and Martin, G.B. (2006). Bacterial elicitation and evasion of plant innate immunity. Nature reviews. Molecular Cell Biology 7:601-611.

Ade, J., DeYoung, B.J., Golstein, C., and Innes, R.W. (2007). Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. Proceedings of the National Academy of Sciences of the United States of America 104:2531-2536.

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

Andolfo, G., Jupe, F., Witek, K., Etherington, G.J., Ercolano, M.R., and Jones, J.D. (2014). Defining the full tomato NB-LRR resistance gene repertoire using genomic and cDNA RenSeq. BMC Plant Biology 14:120.

Aramburu, J., Galipienso, L., Soler, S., and López, C. (2011). Characterization of Tomato spotted wilt virus isolates that overcome the Sw-5 resistance gene in tomato and fitness assays. Phytopathologia Mediterranea 49:342-351.

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

Asurmendi, S., Berg, R., Koo, J., and Beachy, R. (2004). Coat protein regulates formation of replication complexes during tobacco mosaic virus infection. Proceedings of the National Academy of Sciences of the United States of America 101:1415-1420.

Bahar, O., Pruitt, R., Luu, D.D., Schwessinger, B., Daudi, A., Liu, F., Ruan, R., Fontaine-Bodin, L., Koebnik, R., and Ronald, P. (2014). The Xanthomonas Ax21 protein is processed by the general secretory system and is secreted in association with outer membrane vesicles. PeerJ 2:e242.

Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., Xiao, W., Liu, Y., Chai, J., Takken, F.L., et al. (2012). Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. PLoS Pathogens 8:e1002752.

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

Beckers, G.J., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y., Zhang, S., and Conrath, U. (2009). Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in Arabidopsis thaliana. The Plant Cell 21:944-953.

Bendahmane, A., Kanyuka, K., and Baulcombe, D.C. (1999). The Rx gene from potato controls separate virus resistance and cell death responses. The Plant Cell Online 11:781-791.

Bendahmane, M., Szécsi, J., Chen, I., Berg, R.H., and Beachy, R.N. (2002). Characterization of mutant tobacco mosaic virus coat protein that interferes with virus cell-to-cell movement. Proceedings of the National Academy of Sciences 99:3645-3650.

Berger, P.H., Hunt, A.G., Domier, L.L., Hellmann, G.M., Stram, Y., Thornbury, D.W., and Pirone, T.P. (1989). Expression in transgenic plants of a viral gene product that mediates insect transmission of potyviruses. Proceedings of the National Academy of Sciences of the United States of America 86:8402-8406.

Bernoux, M., Ve, T., Williams, S., Warren, C., Hatters, D., Valkov, E., Zhang, X., Ellis, J.G., Kobe, B., and Dodds, P.N. (2011). Structural and functional analysis of a plant resistance protein TIR domain reveals interfaces for self-association, signaling, and autoregulation. Cell Host & Microbe 9:200-211.

Bertran, A., Oliveira, A., Nagata, T., and Resende, R. (2011). Molecular characterization of the RNA-dependent RNA polymerase from Groundnut ringspot virus (genus Tospovirus, family Bunyaviridae). Archives of Virology 156(8):1425-1429.

Bezerra, I.C., Resende, R.D., Pozzer, L., Nagata, T., Kormelink, R., and De Avila, A.C. (1999). Increase of tospoviral diversity in Brazil with the identification of two new tospovirus species, one from chrysanthemum and one from zucchini. Phytopathology 89:823-830.

Bielza, P. (2008). Insecticide resistance management strategies against the western flower thrips, Frankliniella occidentalis. Pest Management Science 64:1131-1138.

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

Boiteux, L.S. (1995). Allelic relationships between genes for resistance to tomato spotted wilt tospovirus in Capsicum chinense. TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik 90:146-149.

Boiteux, L.S.a.d.Á., A.C. (1994). Inheritance of a resistance specific to tomato spotted wilt tospovirus in Capsicum chinense 'PI 159236'. Euphytica 75:139-142.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology 60:379-406.

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

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

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

Chang, C., Yu, D., Jiao, J., Jing, S., Schulze-Lefert, P., and Shen, Q.H. (2013). Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling. The Plant Cell 25:1158-1173.

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

Chen, C.C., and Chiu, R.J. (1995). A tospovirus infecting peanut in Taiwan. International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops:57-67.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. Cell 124:803-814.

Cho, J., Custer, D., Brommonschenkel, S., and Tanksley, S. (1995). Conventional breeding: host-plant resistance and the use of molecular markers to develop resistance to tomato spot wilt virus in vegetables. Tospoviruses and Thrips of Floral and Vegetable Crops 431:367-378.

Chu, F.H., Chao, C.H., Peng, Y.C., Lin, S.S., Chen, C.C., and Yeh, S.D. (2001). Serological and molecular characterization of Peanut chlorotic fan-spot virus, a new species of the genus Tospovirus. Phytopathology 91:856-863.

Chung, E.H., da Cunha, L., Wu, A.J., Gao, Z., Cherkis, K., Afzal, A.J., Mackey, D., and Dangl, J.L. (2011). Specific threonine phosphorylation of a host target by two unrelated type III effectors activates a host innate immune receptor in plants. Cell Host & Microbe 9:125-136.

Ciuffo, M., Finetti-Sialer, M., Gallitelli, D., and Turina, M. (2005). First report in Italy of a resistance-breaking strain of Tomato spotted wilt virus infecting tomato cultivars carrying the Sw5 resistance gene. Plant Pathology 54:564-564.

Coll, N.S., Epple, P., and Dangl, J.L. (2011). Programmed cell death in the plant immune system. Cell Death and Differentiation 18:1247-1256.

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

Dangl, J.L., Horvath, D.M., and Staskawicz, B.J. (2013). Pivoting the plant immune system from dissection to deployment. Science (New York, N.Y.) 341:746-751.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature 411:826-833.

Day, B., Dahlbeck, D., Huang, J., Chisholm, S.T., Li, D., and Staskawicz, B.J. (2005). Molecular basis for the RIN4 negative regulation of RPS2 disease resistance. The Plant Cell 17:1292-1305.

de Avila, A.C., de Haan, P., Kormelink, R., Resende, R.e.O., Goldbach, R.W., and Peters, D. (1993). Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. The Journal of General Virology 74 ( Pt 2):153-159.

de Avila, A.C., Dehaan, P., Smeets, M.L.L., Resende, R.D., Kormelink, R., Kitajima, E.W., Goldbach, R.W., and Peters, D. (1993). Distinct levels of relationships between tospovirus isolates. Archives of Virology 128:211-227.

de Haan, P., Gielen, J.J.L., Prins, M., Wijkamp, I.G., Vanschepen, A., Peters, D., Vangrinsven, M., and Goldbach, R. (1992). Characterization of rna-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. Bio-Technology 10:1133-1137.

de Haan, P., Kormelink, R., Resende, R.D., Vanpoelwijk, F., Peters, D., and Goldbach, R. (1991). Tomato spotted wilt virus-l rna encodes a putative rna-polymerase. Journal of General Virology 72:2207-2216.

de Haan, P., Wagemakers, L., Peters, D., and Goldbach, R. (1990). The s-rna segment of tomato spotted wilt virus has an ambisense character. Journal of General Virology 71:1001-1007.

de Oliveira, A.S., Machado Bertran, A.G., Inoue-Nagata, A.K., Nagata, T., Kitajima, E.W., and Resende, R.O. (2011). An RNA-dependent RNA polymerase gene of a distinct Brazilian tospovirus. Virus Genes 43:385-389.

de Oliveira, A.S., Melo, F.L., Inoue-Nagata, A.K., Nagata, T., Kitajima, E.W., and Resende, R.O. (2012). Characterization of bean necrotic mosaic virus: a member of a novel evolutionary lineage within the Genus Tospovirus. PloS One 7:e38634.

de Ronde, D., Butterbach, P., and Kormelink, R. (2014a). Dominant resistance against plant viruses. Frontiers in Plant Science 5:307.

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

de Ronde, D., Pasquier, A., Ying, S., Butterbach, P., Lohuis, D., and Kormelink, R. (2014b). 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 15:185-195.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proceedings of the National Academy of Sciences 100:8024-8029.

Dianese, E.C., de Fonseca, M.E.N., Goldbach, R., Kormelink, R., Inoue-Nagata, A.K., Resende, R.O., and Boiteux, L.S. (2010). Development of a locus-specific, co-dominant SCAR marker for assisted-selection of the Sw-5 (Tospovirus resistance) gene cluster in a wide range of tomato accessions. Molecular Breeding 25:133-142.

Dianese, E.C., Fonseca, M.E.N., Inoue-Nagata, A.K., Resende, R.O., and Boiteux, L.S. (2011). Search in Solanum (section Lycopersicon) germplasm for sources of broad-spectrum resistance to four Tospovirus species. Euphytica 180:307-319.

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

Dowen, R.H., Engel, J.L., Shao, F., Ecker, J.R., and Dixon, J.E. (2009). A family of bacterial cysteine protease type III effectors utilizes acylation-dependent and -independent strategies to localize to plasma membranes. The Journal of Biological Chemistry 284:15867-15879.

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

Duijsings, D., Kormelink, R., and Goldbach, R. (2001). In vivo analysis of the TSWV cap-snatching mechanism: single base complementarity and primer length requirements. Embo J. 20:2545-2552.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annual Review of Phytopathology 42:185-209.

Edgar, R.C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. Bmc Bioinformatics 5:1-19.

Elliott, R., Pettersson, R., and Melin, L. (1996). Synthesis, Assembly, and Intracellular Transport of Bunyaviridae Membrane Proteins. In: The Bunyaviridae: Springer US. 159-188.

Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. Nature Protocols 2:953-971.

Erbs, G., and Newman, M.A. (2012). The role of lipopolysaccharide and peptidoglycan, two glycosylated bacterial microbe-associated molecular patterns (MAMPs), in plant innate immunity. Molecular Plant Pathology 13:95-104.

Ferraz, E., Resende, L.V., Lima, G.S.A., Silva, M.C.L., França, J.G.E.d., and Silva, D.J.d. (2003). Redenção: nova cultivar de tomate para a indústria resistente a geminivírus e tospovírus. Horticultura Brasileira 21:578-580.

Finetti Sialer, M., Lanave, C., Padula, M., Vovlas, C., and Gallitelli, D. (2002). Occurrence of two distinct Tomato spotted wilt virus subgroups in Southern Italy. Journal of Plant Pathology 84:145-152.

Finlay, K. (1953). Inheritance of spotted wilt resistance in the tomato II. Five genes controlling spotted wilt resistance in four tomato types. Australian Journal of Biological Sciences 6:153-163.

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

Fu, Z.Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. Annual Review of Plant Biology 64:839-863.

Gabriels, S.H., Vossen, J.H., Ekengren, S.K., van Ooijen, G., Abd-El-Haliem, A.M., van den Berg, G.C., Rainey, D.Y., Martin, G.B., Takken, F.L., de Wit, P.J., et al. (2007). An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins. The Plant journal : for cell and molecular biology 50:14-28.

Gaj, T., Gersbach, C.A., and Barbas, C.F., 3rd. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in Biotechnology 31:397-405.

Gao, Z., Chung, E.H., Eitas, T.K., and Dangl, J.L. (2011). Plant intracellular innate immune receptor Resistance to Pseudomonas syringae pv. maculicola 1 (RPM1) is activated at, and functions on, the plasma membrane. Proceedings of the National Academy of Sciences of the United States of America 108:7619-7624.

Garcia-Cano, E., Resende, R.O., Fernandez-Munoz, R., and Moriones, E. (2006). Synergistic interaction between Tomato chlorosis virus and Tomato spotted wilt virus results in breakdown of resistance in tomato. Phytopathology 96:1263-1269.

Giordano, L.D., de Avila, A.C., Charchar, J.M., Boiteux, L.S., and Ferraz, E. (2000). 'Viradoro': A tospovirus-resistant processing tomato cultivar adapted to tropical environments. Hortscience 35:1368-1370.

Gomez-Gomez, L., and Boller, T. (2002). Flagellin perception: a paradigm for innate immunity. Trends in plant science 7:251-256.

Gordillo, L.F., Stevens, M.R., Millard, M.A., and Geary, B. (2008). Screening two Lycopersicon peruvianum collections for resistance to Tomato spotted wilt virus. Plant Disease 92:694-704.

Green, M.R., Sambrook, J., and Sambrook, J. (2012). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Gubba, A., Gonsalves, C., Stevens, M.R., Tricoli, D.M., and Gonsalves, D. (2002). Combining transgenic and natural resistance to obtain broad resistance to tospovirus infection in tomato (Lycopersicon esculentum mill). Molecular Breeding 9:13-23.

Hallwass, M., de Oliveira, A.S., de Campos Dianese, E., Lohuis, D., Boiteux, L.S., Inoue-Nagata, A.K., Resende, R.O., and Kormelink, R. (2014). The Tomato spotted wilt virus cell-to-cell movement protein (NSM ) triggers a hypersensitive response in Sw-5-containing resistant tomato lines and in Nicotiana benthamiana transformed with the functional Sw-5b resistance gene copy. Molecular Plant Pathology 15:871-880.

Hassani-Mehraban, A., Botermans, M., Verhoeven, J.T.J., Meekes, E., Saaijer, J., Peters, D., Goldbach, R., and Kormelink, R. (2010). A distinct tospovirus causing necrotic streak on Alstroemeria sp in Colombia. Archives of Virology 155:423-428.

Hochreiter, S., Wagner, R., Fariselli, P., Molinini, D., Casadio, R., and Krogh, A. (2007). Prediction of Structurally-Determined Coiled-Coil Domains with Hidden Markov Models. In: Bioinformatics Research and Development: Springer Berlin Heidelberg. 292-302.

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

Inoue, T., and Sakurai, T. (2007). The phylogeny of thrips (Thysanoptera : Thripidae) based on partial sequences of cytochrome oxidase I, 28S ribosomal DNA and elongation factor-1 alpha and the association with vector competence of tospoviruses. Applied Entomology and Zoology 42:71-81.

Jahn, M., Paran, I., Hoffmann, K., Radwanski, E.R., Livingstone, K.D., Grube, R.C., Aftergoot, E., Lapidot, M., and Moyer, J. (2000). Genetic mapping of the Tsw locus for resistance to the Tospovirus Tomato spotted wilt virus in Capsicum spp. and its relationship to the Sw-5 gene for resistance to the same pathogen in tomato. Molecular plant-microbe interactions 13:673-682.

Jenkins, K.A., and Mansell, A. (2010). TIR-containing adaptors in Toll-like receptor signalling. Cytokine 49:237-244.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. Embo J. 19:4004-4014.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature 444:323-329.

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

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proceedings of the National Academy of Sciences of the United States of America 103:11086-11091.

Kanzaki, H., Yoshida, K., Saitoh, H., Fujisaki, K., Hirabuchi, A., Alaux, L., Fournier, E., Tharreau, D., and Terauchi, R. (2012). Arms race co-evolution of Magnaporthe oryzae AVR-Pik and rice Pik genes driven by their physical interactions. The Plant Journal : for cell and molecular biology 72:894-907.

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends in Plant Science 7:193-195.

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

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

Kim, C.-H., and Palukaitis, P. (1997). The plant defense response to cucumber mosaic virus in cowpea is elicited by the viral polymerase gene and affects virus accumulation in single cells. The EMBO Journal 16:4060-4068.

King, A.M., Adams, M.J., and Lefkowitz, E. (2011). Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses: Elsevier.

King, A.M.Q., Adams, M.J., Carstens, E.B., and Lefkowitz, E.J. (2012a). Family - Bunyaviridae. In: Virus Taxonomy San Diego: Elsevier. 725-741.

King, A.M.Q., Adams, M.J., Carstens, E.B., and Lefkowitz, E.J. (2012b). Ninth Report of the International Committee on Taxonomy of Viruses. United States of America: Elsevier.

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

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

Kormelink, R., Kitajima, E.W., Haan, P.D., Zuidema, D., Peters, D., and Goldbach, R. (1991). The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomao spotted wilt virus is associated with fibrous structures in infected plant cells. Virology 181:459-468.

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

Lanfermeijer, F.C., Dijkhuis, J., Sturre, M.J., de Haan, P., and Hille, J. (2003). Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-2(2) from Lycopersicon esculentum. Plant Molecular Biology 52:1037-1049.

Langella, R., Ercolano, M.R., Monti, L.M., Frusciante, L., and Barone, A. (2004). Molecular marker assisted transfer of resistance to TSWV in tomato elite lines. Journal of Horticultural Science & Biotechnology 79:806-810.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., et al. (2007). Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England) 23:2947-2948.

Latham, L., and Jones, R. (1998). Selection of resistance breaking strains of tomato spotted wilt tospovirus. Annals of Applied Biology 133:385-402.

Leastro, M.O., Pallas, V., Resende, R.O., and Sanchez-Navarro, J.A. (2015). The movement proteins (NSm) of distinct tospoviruses peripherally associate with cellular membranes and interact with homologous and heterologous NSm and nucleocapsid proteins. Virology 478:39-49.

Lee, M.Y., Yan, L., Gorter, F.A., Kim, B.Y., Cui, Y., Hu, Y., Yuan, C., Grindheim, J., Ganesan, U., Liu, Z., et al. (2012). Brachypodium distachyon line Bd3-1 resistance is elicited by the barley stripe mosaic virus triple gene block 1 movement protein. The Journal of General Virology 93:2729-2739.

Leipe, D.D., Koonin, E.V., and Aravind, L. (2004). STAND, a class of P-loop NTPases including animal and plant regulators of programmed cell death: multiple, complex domain architectures, unusual phyletic patterns, and evolution by horizontal gene transfer. Journal of Molecular Biology 343:1-28.

Leister, D. (2004). Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance gene. Trends in Genetics : TIG 20:116-122.

Les Erickson, F., Holzberg, S., Calderon-Urrea, A., Handley, V., Axtell, M., Corr, C., and Baker, B. (1999). The helicase domain of the TMV replicase proteins induces the N-mediated defence response in tobacco. The Plant Journal 18:67-75.

Letunic, I., Doerks, T., and Bork, P. (2012). SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Research 40:D302-305.

Li, F., Pignatta, D., Bendix, C., Brunkard, J.O., Cohn, M.M., Tung, J., Sun, H., Kumar, P., and Baker, B. (2012). MicroRNA regulation of plant innate immune receptors. Proceedings of the National Academy of Sciences 109:1790-1795.

Li, H.-W., Lucy, A.P., Guo, H.-S., Li, W.-X., Ji, L.-H., Wong, S.-M., and Ding, S.-W. (1999). Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. The EMBO journal 18:2683-2691.

Li, W., Lewandowski, D.J., Hilf, M.E., and Adkins, S. (2009). Identification of domains of the Tomato spotted wilt virus NSm protein involved in tubule formation, movement and symptomatology. Virology 390:110-121.

Liu, J., Elmore, J.M., Lin, Z.J., and Coaker, G. (2011). A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. Cell Host & Microbe 9:137-146.

Liu, Y., Burch-Smith, T., Schiff, M., Feng, S., and Dinesh-Kumar, S.P. (2004). Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. The Journal of Biological Chemistry 279:2101-2108.

Lopez, C., Aramburu, J., Galipienso, L., Soler, S., Nuez, F., and Rubio, L. (2011). Evolutionary analysis of tomato Sw-5 resistance-breaking isolates of Tomato spotted wilt virus. Journal of General Virology 92:210-215.

Lovato, F.A., Inoue-Nagata, A.K., Nagata, T., de Ávila, A.C., Pereira, L.A.R., and Resende, R.O. (2008). The N protein of Tomato spotted wilt virus (TSWV) is associated with the induction of programmed cell death (PCD) in Capsicum chinense plants, a hypersensitive host to TSWV infection. Virus Research 137:245-252.

Lovato, F.A., Nagata, T., de Oliveira Resende, R., de Avila, A.C., and Inoue-Nagata, A.K. (2004). Sequence analysis of the glycoproteins of Tomato chlorotic spot virus and Groundnut ringspot virus and comparison with other tospoviruses. Virus Genes 29:321-328.

Lukasik-Shreepaathy, E., Slootweg, E., Richter, H., Goverse, A., Cornelissen, B.J., and Takken, F.L. (2012). Dual regulatory roles of the extended N terminus for activation of the tomato MI-1.2 resistance protein. Molecular plant-microbe interactions 25:1045-1057.

Maekawa, T., Cheng, W., Spiridon, L.N., Toller, A., Lukasik, E., Saijo, Y., Liu, P., Shen, Q.H., Micluta, M.A., Somssich, I.E., et al. (2011). Coiled-coil domain-dependent homodimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death. Cell Host & Microbe 9:187-199.

Malcuit, I., Marano, M.R., Kavanagh, T.A., De Jong, W., Forsyth, A., and Baulcombe, D.C. (1999). The 25-kDa movement protein of PVX elicits Nb-mediated hypersensitive cell death in potato. Molecular Plant-Microbe Interactions 12:536-543.

Margaria, P., Ciuffo, M., Pacifico, D., and Turina, M. (2007). Evidence that the nonstructural protein of Tomato spotted wilt virus is the avirulence determinant in the interaction with resistant pepper carrying the Tsw gene. Molecular Plant-Microbe Interactions 20:547-558.

Martin, K., Kopperud, K., Chakrabarty, R., Banerjee, R., Brooks, R., and Goodin, M.M. (2009). Transient expression in Nicotiana benthamiana fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta. The Plant Journal : for cell and molecular biology 59:150-162.

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

Medeiros, R.B., Resende, R.D., and De Avila, A.C. (2004). The plant virus Tomato spotted wilt Tospovirus activates the immune system of its main insect vector, Frankliniella occidentalis. Journal of Virology 78:4976-4982.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. Nature reviews. Immunology 1:135-145.

Meyers, B.C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R.W. (2003). Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis. The Plant Cell 15:809-834.

Michelmore, R.W., and Meyers, B.C. (1998). Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Research 8:1113-1130.

Morel, J.-B., and Dangl, J.L. (1997). The hypersensitive response and the induction of cell death in plants. Cell Death and Differentiation 4:671-683.

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

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell 113:935-944.

Mound, L.A. (2002). The Thrips and Frankliniella genus groups: the phylogenetic significance of ctenidia. In: Thrips and Tospoviruses: Proceedings of the 7th International Symposium on Thysanoptera--R, M., and A, M.L., eds. Canberra: Australian National Insect Collection. 379-386.

Nakahara, S. (1997). Annotated list of the Frankliniella species of the world (Thysanoptera: Thripidae)*Contributions on entomology, international*: Associated Publishers.

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

Narusaka, M., Kubo, Y., Hatakeyama, K., Imamura, J., Ezura, H., Nanasato, Y., Tabei, Y., Takano, Y., Shirasu, K., and Narusaka, Y. (2013). Interfamily Transfer of Dual NB-LRR Genes Confers Resistance to Multiple Pathogens. PloS One 8:e55954.

Nürnberger, T., and Brunner, F. (2002). Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Current Opinion in Plant Biology 5:318-324.

Oh, J.-W. (1995). Open Reading Frames ofTurnip Crinkle Virus Involved in Satellite Symptom Expression and Incompatibility with Arabidopsis thaliana Ecotype Dijon. 8(6):979-987.

Pan, Q., Wendel, J., and Fluhr, R. (2000). Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. Journal of Molecular Evolution 50:203-213.

Panstruga, R., Parker, J.E., and Schulze-Lefert, P. (2009). SnapShot: Plant immune response pathways. Cell 136:978. e971-973.

Pappu, H., Jones, R., and Jain, R. (2009). Global status of tospovirus epidemics in diverse cropping systems: Successes achieved and challenges ahead. Virus Research 141:219-236.

Pawlowski, M., Bogdanowicz, A., and Bujnicki, J.M. (2013). QA-RecombineIt: a server for quality assessment and recombination of protein models. Nucleic Acids Research 41:W389-397.

Peng, J.C., Chen, T.C., Raja, J.A., Yang, C.F., Chien, W.C., Lin, C.H., Liu, F.L., Wu, H.W., and Yeh, S.D. (2014). Broad-spectrum transgenic resistance against distinct tospovirus species at the genus level. PloS One 9:e96073.

Peyret, H., and Lomonossoff, G.P. (2013). The pEAQ vector series: the easy and quick way to produce recombinant proteins in plants. Plant Molecular Biology 83:51-58.

Pond, S.L.K., Murrell, B., Fourment, M., Frost, S.D.W., Delport, W., and Scheffler, K. (2011). A Random Effects Branch-Site Model for Detecting Episodic Diversifying Selection. Molecular Biology and Evolution 28:3033-3043.

Postel, S., and Kemmerling, B. (2009). Plant systems for recognition of pathogen-associated molecular patterns. In: Seminars in cell & developmental biology: Elsevier. 1025-1031.

Postma, W.J., Slootweg, E.J., Rehman, S., Finkers-Tomczak, A., Tytgat, T.O.G., van Gelderen, K., Lozano-Torres, J.L., Roosien, J., Pomp, R., van Schaik, C., et al. (2012). The Effector SPRYSEC-19 of Globodera rostochiensis Suppresses CC-NB-LRR-Mediated Disease Resistance in Plants. Plant Physiology 160:944-954.

Price, D.L., Memmott, F.D., Scott, J.W., Olson, S.M., and Stevens, M.R. (2007). Identification of molecular markers linked to a new Tomato spotted wilt virus resistance source in tomato. Tomato Genetics Cooperative 57:35-36.

Prins, M., Kikkert, M., Ismayadi, C., deGraauw, W., deHaan, P., and Goldbach, R. (1997). Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NSM gene sequences. Plant Molecular Biology 33:235-243.

Qi, D., and Innes, R.W. (2013). Recent Advances in Plant NLR Structure, Function, Localization, and Signaling. Frontiers in Immunology 4:348.

Radhakrishnan, G.K., and Splitter, G.A. (2010). Biochemical and functional analysis of TIR domain containing protein from Brucella melitensis. Biochemical and Biophysical Research Communications 397:59-63.

Rairdan, G.J., Collier, S.M., Sacco, M.A., Baldwin, T.T., Boettrich, T., and Moffett, P. (2008). The coiled-coil and nucleotide binding domains of the Potato Rx disease resistance protein function in pathogen recognition and signaling. The Plant Cell 20:739-751.

Rairdan, G.J., and Moffett, P. (2006). Distinct domains in the ARC region of the potato resistance protein Rx mediate LRR binding and inhibition of activation. The Plant Cell 18:2082-2093.

Rehman, S., Postma, W., Tytgat, T., Prins, P., Qin, L., Overmars, H., Vossen, J., Spiridon, L.-N., Petrescu, A.-J., and Goverse, A. (2009). A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode Globodera rostochiensis interacts with a CC-NB-LRR protein from a susceptible tomato. Molecular Plant-Microbe Interactions 22:330-340.

Ribeiro, D., Foresti, O., Denecke, J., Wellink, J., Goldbach, R., and Kormelink, R.J.M. (2008). Tomato spotted wilt virus glycoproteins induce the formation of endoplasmic reticulum- and Golgi-derived pleomorphic membrane structures in plant cells. Journal of General Virology 89:1811-1818.

Riedl, S.J., Li, W., Chao, Y., Schwarzenbacher, R., and Shi, Y. (2005). Structure of the apoptotic protease-activating factor 1 bound to ADP. Nature 434:926-933.

Riley, D.G., Joseph, S.V., Srinivasan, R., and Diffie, S. (2011). Thrips vectors of tospoviruses. Journal of Integrated Pest Management 1.

Rinne, P.L., van den Boogaard, R., Mensink, M.G., Kopperud, C., Kormelink, R., Goldbach, R., and van der Schoot, C. (2005). Tobacco plants respond to the constitutive expression of the tospovirus movement protein NS(M) with a heat-reversible sealing of plasmodesmata that impairs development. The Plant Journal: for cell and molecular biology 43:688-707.

Román, A.C., González-Rico, F.J., Moltó, E., Hernando, H., Neto, A., Vicente-Garcia, C., Ballestar, E., Gómez-Skarmeta, J.L., Vavrova-Anderson, J., and White, R.J. (2011). Dioxin receptor and SLUG transcription factors regulate the insulator activity of B1 SINE retrotransposons via an RNA polymerase switch. Genome Research 21:422-432.

Roossinck, M.J. (2012). Plant virus metagenomics: biodiversity and ecology. Annual Review of Genetics 46:359-369.

Roossinck, M.J. (2013). Plant Virus Ecology. PLoS Pathogens 9:e1003304.

Roth, B.M., Pruss, G.J., and Vance, V.B. (2004). Plant viral suppressors of RNA silencing. Virus Research 102:97-108.

Saidi, M., and Warade, S. (2008). Tomato breeding for resistance to Tomato spotted wilt virus (TSWV): an overview of conventional and molecular approaches. Czech J. Genet. Plant Breed 44:83-92.

Sainsbury, F., Thuenemann, E.C., and Lomonossoff, G.P. (2009). pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnology Journal 7:682-693.

Sato, S., and Tabata, S., and Hirakawa, H., and Asamizu, E., and Shirasawa, K., and Isobe, S., and Kaneko, T., and Nakamura, Y., and Shibata, D., and Aoki, K., et al. (2012). The tomato genome sequence provides insights into fleshy fruit evolution. Nature 485:635-641.

Sato, Y., Ando, S., and Takahashi, H. (2014). Role of intron-mediated enhancement on accumulation of an Arabidopsis NB-LRR class R-protein that confers resistance to Cucumber mosaic virus. PloS One 9:e99041.

Satyanarayana, T., Gowda, S., Reddy, K.L., Mitchell, S.E., Dawson, W.O., and Reddy, D.V.R. (1998). Peanut yellow spot virus is a member of a new serogroup of Tospovirus genus based on small (S) RNA sequence and organization. Archives of Virology 143:353-364.

Scholthof, K.B., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., et al. (2011). Top 10 plant viruses in molecular plant pathology. Molecular Plant Pathology 12:938-954.

Seepiban, C., Gajanandana, O., Attathom, T., and Attathom, S. (2011). Tomato necrotic ringspot virus, a new tospovirus isolated in Thailand. Archives of Virology 156:263-274.

Sela, H., Spiridon, L.N., Ashkenazi, H., Bhullar, N.K., Brunner, S., Petrescu, A.-J., Fahima, T., Keller, B., and Jordan, T. (2014). Three-Dimensional Modeling and Diversity Analysis Reveals Distinct AVR Recognition Sites and Evolutionary Pathways in Wild and Domesticated Wheat Pm3 R Genes. Molecular Plant-Microbe Interactions 27:835-845.

Sela, H., Spiridon, L.N., Petrescu, A.-J., Akerman, M., Mandel-Gutfreund, Y., Nevo, E., Loutre, C., Keller, B., Schulman, A.H., and Fahima, T. (2012). Ancient diversity of splicing motifs and protein surfaces in the wild emmer wheat (Triticum dicoccoides) LR10 coiled coil (CC) and leucine-rich repeat (LRR) domains. Molecular Plant Pathology 13:276-287.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E., and Innes, R.W. (2003). Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science (New York, N.Y.) 301:1230-1233.

Shen, J., Araki, H., Chen, L., Chen, J.Q., and Tian, D. (2006). Unique evolutionary mechanism in R-genes under the presence/absence polymorphism in Arabidopsis thaliana. Genetics 172:1243-1250.

Shen, Q.H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I.E., and Schulze-Lefert, P. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science (New York, N.Y.) 315:1098-1103.

Shirasu, K. (2009). The HSP90-SGT1 chaperone complex for NLR immune sensors. Annual Review of Plant Biology

60:139-164.

Shivaprasad, P.V., Chen, H.M., Patel, K., Bond, D.M., Santos, B.A., and Baulcombe, D.C. (2012). A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. The Plant Cell 24:859-874.

Silva, M.S., Martins, C.R., Bezerra, I.C., Nagata, T., de Avila, A.C., and Resende, R.O. (2001). Sequence diversity of NS(M) movement protein of tospoviruses. Arch Virol 146:1267-1281.

Slootweg, E., Roosien, J., Spiridon, L.N., Petrescu, A.J., Tameling, W., Joosten, M., Pomp, R., van Schaik, C., Dees, R., Borst, J.W., et al. (2010). Nucleocytoplasmic distribution is required for activation of resistance by the potato NB-LRR receptor Rx1 and is balanced by its functional domains. The Plant Cell 22:4195-4215.

Slootweg, E.J., Spiridon, L.N., Roosien, J., Butterbach, P., Pomp, R., Westerhof, L., Wilbers, R., Bakker, E., Bakker, J., Petrescu, A.J., et al. (2013). Structural determinants at the interface of the ARC2 and leucine-rich repeat domains control the activation of the plant immune receptors Rx1 and Gpa2. Plant Physiol 162:1510-1528.

Snippe, M., Goldbach, R., and Kormelink, R. (2005). Tomato spotted wilt virus particle assembly and the prospects of fluorescence microscopy to study protein-protein interactions involved. Adv Virus Res 65:63-120.

Snippe, M., Smeenk, L., Goldbach, R., and Kormelink, R. (2007). The cytoplasmic domain of tomato spotted wilt virus Gn glycoprotein is required for Golgi localisation and interaction with Gc. Virology 363:272-279.

Soellick, T.-R., Uhrig, J.F., Bucher, G.L., Kellmann, J.-W., and Schreier, P.H. (2000). The movement protein NSm of tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the TSWV N protein, and identification of interacting plant proteins. Proceedings of the National Academy of Sciences 97:2373-2378.

Somssich, I.E., and Hahlbrock, K. (1998). Pathogen defence in plants—a paradigm of biological complexity. Trends in Plant Science 3:86-90.

Soosaar, J.L., Burch-Smith, T.M., and Dinesh-Kumar, S.P. (2005). Mechanisms of plant resistance to viruses. Nature reviews. Microbiology 3:789-798.

Spassova, M.I., Prins, T.W., Folkertsma, R.T., Klein-Lankhorst, R.M., Hille, J., Goldbach, R.W., and Prins, M. (2001). The tomato gene Sw5 is a member of the coiled coil, nucleotide binding, leucine-rich repeat class of plant resistance genes and confers resistance to TSWV in tobacco. Molecular Breeding 7:151-161.

Spoel, S.H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. Nature reviews. Immunology 12:89-100.

Staden, R. (1996). The Staden sequence analysis package. Molecular Biotechnology 5:233-241.

Stamatakis, A., Hoover, P., and Rougemont, J. (2008). A Rapid Bootstrap Algorithm for the RAxML Web Servers. Systematic Biology 57:758-771.

Stevens, M., Lamb, E., and Rhoads, D. (1995). Mapping the Sw-5 locus for tomato spotted wilt virus resistance in tomatoes using RAPD and RFLP analyses. Theoretical and Applied Genetics 90:451-456.

Stevens, M., Scott, S., and Gergerich, R. (1991). Inheritance of a gene for resistance to tomato spotted wilt virus (TSWV) from Lycopersicon peruvianum Mill. Euphytica 59:9-17.

Stevens, M., Scott, S., and Gergerich, R. (1994). Evaluation of seven Lycopersicon species for resistance to tomato spotted wilt virus (TSWV). Euphytica 80:79-84.

Storms, M.M., Kormelink, R., Peters, D., Van Lent, J.W., and Goldbach, R.W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology 214:485-493.

Storms, M.M.H., van der Schoot, C., Prins, M., Kormelink, R., van Lent, J.W.M., and Goldbach, R.W. (1998). A comparison of two methods of microinjection for assessing altered plasmodesmal gating in tissues expressing viral movement proteins. Plant Journal 13:131-140.

Sun, Q., Collins, N.C., Ayliffe, M., Smith, S.M., Drake, J., Pryor, T., and Hulbert, S.H. (2001). Recombination between paralogues at the Rp1 rust resistance locus in maize. Genetics 158:423-438.

Takahashi, H., Suzuki, M., Natsuaki, K., Shigyo, T., Hino, K., Teraoka, T., Hosokawa, D., and Ehara, Y. (2001). Mapping the virus and host genes involved in the resistance response in cucumber mosaic virus-Infected Arabidopsis thaliana. Plant & Cell Physiology 42:340-347.

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

Takken, F.L., and Goverse, A. (2012). How to build a pathogen detector: structural basis of NB-LRR function. Current Opinion in Plant Biology 15:375-384.

Tameling, W.I., Elzinga, S.D., Darmin, P.S., Vossen, J.H., Takken, F.L., Haring, M.A., and Cornelissen, B.J. (2002). The tomato R gene products I-2 and MI-1 are functional ATP binding proteins with ATPase activity. The Plant Cell 14:2929-2939.

Tameling, W.I., Nooijen, C., Ludwig, N., Boter, M., Slootweg, E., Goverse, A., Shirasu, K., and Joosten, M.H. (2010). RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. The Plant Cell 22:4176-4194.

Tang, X., Xie, M., Kim, Y.J., Zhou, J., Klessig, D.F., and Martin, G.B. (1999). Overexpression of Pto activates defense responses and confers broad resistance. The Plant Cell 11:15-29.

Thomma, B.P., Nurnberger, T., and Joosten, M.H. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. The Plant Cell 23:4-15.

Thompson, G., and Van Zijl, J. (1995). Control of tomato spotted wilt virus in tomatoes in South Africa. Tospoviruses and Thrips of Floral and Vegetable Crops 431:379-384.

Tordo, N., de Haan, P., Goldbach, R., and Poch, O. (1992). Evolution of negative-stranded RNA genomes. Semin. Virol. 3:341-357.

Ueda, H., Yamaguchi, Y., and Sano, H. (2006). Direct interaction between the tobacco mosaic virus helicase domain and the ATP-bound resistance protein, N factor during the hypersensitive response in tobacco plants. Plant Molecular Biology 61:31-45.

Ullman, D.E., German, T.L., Sherwood, J.L., Westcot, D.M., and Cantone, F.A. (1993). Tospovirus replication in insect vector cells - immunocytochemical evidence that the nonstructural protein encoded by the s-rna of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology 83:456-463.

Underwood, W. (2012). The plant cell wall: a dynamic barrier against pathogen invasion. Frontiers in Plant Science 3:6.

van der Biezen, E.A., and Jones, J.D. (1998). The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Current Biology : CB 8:R226-227.

van der Hoorn, R.A., and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. The Plant Cell 20:2009-2017.

van der Vossen, E.A., van der Voort, J.N., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D.C., Bakker, J., Stiekema, W.J., and Klein-Lankhorst, R.M. (2000). Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. The Plant Journal : for cell and molecular biology 23:567-576.

van Ooijen, G., Mayr, G., Albrecht, M., Cornelissen, B.J.C., and Takken, F.L.W. (2008) Transcomplementation, but not Physical Association of the CC-NB-ARC and LRR Domains of Tomato R Protein Mi-1.2 is Altered by Mutations in the ARC2 Subdomain. Molecular Plant 1:401-410.

Van Zijl, J., Bosch, S., and Coetzee, C. (1985). Breeding tomatoes for processing in South Africa. In: International Symposium on Fruit & Vegetables for Processing 194. 69-76.

Van poelwijk, F., Boye, K., Oosterling, R., Peters, D., and Goldbach, R. (1993). Detection of the l-protein of tomato spotted wilt virus. Virology 197:468-470.

Vogler, H., Kwon, M.O., Dang, V., Sambade, A., Fasler, M., Ashby, J., and Heinlein, M. (2008). Tobacco mosaic virus movement protein enhances the spread of RNA silencing. PLoS Pathogens 4:e1000038.

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathogens 2:e123.

Wang, G.-F., Ji, J., Ei-Kasmi, F., Dangl, J.L., Johal, G., and Balint-Kurti, P.J. (2015). Molecular and Functional Analyses of a Maize Autoactive NB-LRR Protein Identify Precise Structural Requirements for Activity. PLoS Pathogens 11:e1004674.

Wang, R., and Brattain, M.G. (2007). The maximal size of protein to diffuse through the nuclear pore is larger than 60kDa. FEBS letters 581:3164-3170.

Weber, H., Schultze, S., and Pfitzner, A. (1993). Two amino acid substitutions in the tomato mosaic virus 30-kilodalton movement protein confer the ability to overcome the Tm-2 (2) resistance gene in the tomato. Journal of Virology 67:6432-6438.

Wen, R.H., Khatabi, B., Ashfield, T., Saghai Maroof, M.A., and Hajimorad, M.R. (2013). The HC-Pro and P3 cistrons of an avirulent Soybean mosaic virus are recognized by different resistance genes at the complex Rsv1 locus. Molecular plant-microbe interactions 26:203-215.

Whitfield, A.E., Ullman, D.E., and German, T.L. (2005). Tospovirus-thrips interactions. Annual Review of Phytopathology 43:459-489.

Wijkamp, I., Vanlent, J., Kormelink, R., Goldbach, R., and Peters, D. (1993). Multiplication of tomato spotted wilt virus in its insect vector, frankliniella-occidentalis. Journal of General Virology 74:341-349.

Williams, L., Lambertini, P.L., Shohara, K., and Biderbost, E. (2001). Occurrence and geographical distribution of tospovirus species infecting tomato crops in Argentina. Plant Disease 85:1227-1229.

Xia, X., and Xie, Z. (2001). DAMBE: Software package for data analysis in molecular biology and evolution. Journal of Heredity 92:371-373.

Xia, X.H., Xie, Z., Salemi, M., Chen, L., and Wang, Y. (2003). An index of substitution saturation and its application. Molecular Phylogenetics and Evolution 26:1-7.

Xu, P., Chen, F., Mannas, J.P., Feldman, T., Sumner, L.W., and Roossinck, M.J. (2008). Virus infection improves drought tolerance. The New phytologist 180:911-921.

Yang, Z., and dos Reis, M. (2011). Statistical Properties of the Branch-Site Test of Positive Selection. Molecular Biology and Evolution 28:1217-1228.

Yoshikawa, N., Okada, K., Asamuma, K., Watanabe, K., Igarasi, A., Li, C., and Isogai, M. (2006). A movement protein and three capsid proteins are all necessary for the cell-to-cell movement of apple latent spherical cheravirus. Archives of Virology 151:837-848.

Zhang, R., Murat, F., Pont, C., Langin, T., and Salse, J. (2014). Paleo-evolutionary plasticity of plant disease resistance genes. BMC Genomics 15:187.

Zhang, Y., Dorey, S., Swiderski, M., and Jones, J.D. (2004). Expression of RPS4 in tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90. The Plant Journal : for cell and molecular biology 40:213-224.

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

Zhou, J., Kantartzi, S.K., Wen, R.H., Newman, M., Hajimorad, M.R., Rupe, J.C., and Tzanetakis, I.E. (2011). Molecular characterization of a new tospovirus infecting soybean. Virus Genes 43:289-295.

Zhou, J., and Tzanetakis, I.E. (2013). Epidemiology of soybean vein necrosis-associated virus. Phytopathology 103:966-971.

Zipfel, C. (2014). Plant pattern-recognition receptors. Trends Immunol 35:345-351.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125:749-760.

Zvereva, A.S., and Pooggin, M.M. (2012). Silencing and innate immunity in plant defense against viral and non-viral pathogens. Viruses 4:2578-2597.

# LIST OF ABBREVIATIONS

aa – amino acids
ADP - adenosine diphosphate
ANSV - Alstroemeria necrotic streak virus
ApaF-1 - apoptotic protease-activating factor-1
ATP - adenosine triphosphate
Avr – avirulence
BeNMV - Bean necrotic spot virus
BiFC - bimolecular fluorescence complementation
C - cysteine
CaCV - *Capsicum chlorosis virus*
CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CC – coiled coil
CCHFV - *Crimean Congo Hemorhagic Fever virus*
CCSV - Calla lily chlorotic virus
CNPq - Conselho Nacional de Desenvolvimento Científico e Tecnológico
CP – coat protein
CSNV - *Chrysanthemum stem necrosis virus*
*D. stramonium – Datura stramonium*
DCL - Dicer-like enzymes
DOT-ELISA - dot enzyme-linked immunosorbent assay
dpa - days post agroinfiltration
dpi - days post infiltration
dsRNA – double-stranded RNA
E – Glutamate
ETI - effector-triggered immunity
FAO - Food and Agriculture Organization of the United Nations
FAPDF - Fundação de Apoio à Pesquisa do Distrito Federal
Fig – figure
GBNV - *Groundnut bud necrosis virus*
GFP – Green fluorescent protein
GLU - 1,3-ß-D-glucan
GP – glycoprotein precursor
GRSV – *Groundnut ringspot virus*
HR - hypersensitive cell death response
INSV - *Impatiens necrotic spot virus*
IR – intergenic region
IYSV - *Iris yellow spot virus*

Ka - non-synonymous substitutions
Ks - synonymous substitutions
L protein – RNA-dependent RNA polymerase protein
L RNA – Large RNA
LRR - Leucine-Rich-Repeat
M RNA – Medium RNA
MAPK - mitogen-activated protein kinase
miRNA – microRNA
ML - maximum likelihood
mRNA – messenger RNA
MYSV - *Melon yellow spot virus*
N – asparagine
N protein – nucleocapsid protein
*N. benthamiana – Nicotiana benthamiana*
*N. tabacum – Nicotiana tabacum*
NB-ARC - Nucleotide-Binding adaptor shared by ApaF-1, Resistance proteins, and CED-4
NB-LRR - nucleotide-binding-leucine rich repeat
NLR proteins - Nucleotide-binding domain and leucine-rich repeat proteins
$NS_M$ – non-structural cell-to-cell movement protein
$NS_S$ - non-structural RNA silencing suppressor protein
nt – nucleotides
ORFs - open reading frames
*P. pubecens – Physalis pubecens*
*P. vulgaris - Phaseolus vulgaris*
PAMPs/MAMPs - Pathogen- or microbial-associated molecular patterns
PCD - programmed cell death
PCFSV - Peanut chlorotic fan-spot virus
Pd - plasmodesmata
PLA2 - phospholipase 2 (PLA2)
PolRSV - Poligonum ringspot virus
PR - pathogenesis-related
PRR - Pattern Recognition Receptors
PSMV - Physalis severe mottle virus
PTI - PAMP-triggered immunity
PVX - Potato virus X
PYSV - *Peanut yellow spot virus*
R - Resistance
RB – resistance-breaking
RdRp – RNA-dependent RNA polymerase

RI – resistance-inducing
RISC - RNA-induced silencing complex
RMSD - root mean squared deviation
RNPs – ribonucleoproteins
RSS - RNA silencing suppressors
RT-PCR - reverse transcription coupled with polymerase chain reaction
RVFV - *Rift Valley Fever virus*
S RNA – Small RNA
*S. lycopersicum – Solanum lycopersicum*
*S. peruvianum – Solanum peruvianum*
SAR - systemic acquired resistance
SAV - Salmonid alphavirus
SD - solanaceae domain
SD1 - Solanaceae Domain 1
SD2 - Solanaceae Domain 2
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA – small-interfering RNA
ssRNA – single-stranded RNA
SVNaV - Soybean vein necrosis associated virus
T - threonine
TCSV – *Tomato chlorotic spot virus*
TIR - Toll/interleukin-1 receptor domain
TNRV - Tomato necrotic ringspot virus
ToCV - *Tomato chlorosis virus*
TSWV - *Tomato spotted wilt virus*
TYRV - Tomato yellow ring virus
TZSV - Tomato zonate spot virus
UTR - untranslated region
WBNV - Watermelon bud necrosis virus
*WSMoV - Watermelon silver mottle virus*
Y - tyrosine
YFP – Yellow fluorescent protein
ZLCV - *Zucchini lethal chlorosis virus*

# SUMMARY

*Tomato spotted wilt virus* (TSWV) along with other tospovirus species (family *Bunyaviridae*) is responsible for substantial losses in crop production around the world. Tospoviruses are transmitted in a propagative and circulative manner by thrips vectors (Order Thysanoptera). As for other pathogens, disease management against tospoviruses pursues a holistic approach involving a combination of cultural, phytosanitary, chemical, and biological tactics, when suitable in addition to the use of resistant crops. Nevertheless, successful control has proven difficult given the broad host range of these viruses and their effective spread by thrips vectors. More importantly, increased reliance on the use of low-cost insecticides has exacerbated tospovirus spread by causing thrips resistance. To ease the financial and environmental constraints associated with insecticide abuse to control thrips, efforts have been increased to obtain genetically resistant cultivars as an integral component of disease management strategies.

So far there are two resistance sources available for commercial breeding of vegetables against TSWV. One of these sources is the *Sw-5* gene cluster, which has been found in *Solanum peruvianum* L., a wild species of tomato from Peru, and has been introgressed in *S. lycopersicum* L. cultivars (commercial tomatoes). After gene mapping, it has been reported that at least five paralogs compose the *Sw-5* gene cluster in *S. peruvianum*, which were named *Sw-5a* to *Sw-5e*. These paralogs encode NB-LRR receptors, a class of cytoplasmic proteins that activate disease resistance upon direct or indirect recognition of pathogens that have overcome the first lines of defense of the plant immune system. Transformation of tobacco plants with the paralogs *Sw-5a* and *Sw-5b,* revealed that only the latter triggers resistance against TSWV isolates. With the availability of the tomato genome, highly conserved orthologs have been mapped in *S. lycopersicum* as well. This thesis started off with a detailed description of the plant immune system and previous findings about the *Sw-5* gene cluster (Chapter 1). As part of this introduction, the problems caused by tospoviruses, to which *Sw-5b* locus confers resistance and the characteristics of these viruses have been described. With this knowledge as a starting point, this thesis focused on unraveling the key features of *Sw-5b*-mediated resistance against TSWV with special attention to the molecular and cellular events underlying the resistance mechanism. Functional analyses have been performed towards clarification of the genetic delimitations between functional and non-functional Sw-5 orthologs considering tospovirus recognition and resistance triggering.

Since earlier made *N. tabacum* transformants containing *Sw-5b* did not show a hypersensitive response (HR) upon challenging with TSWV, *N. benthamiana* have been transformed with *Sw-5b* aiming to obtain transgenic lines that would respond with a visual HR and thereby facilitate the identification of the avirulence determinant (Avr) from TSWV

(Chapter 2). While *N. tabacum* plants were transformed with *Sw-5b* gene under control of its own regulatory elements, the *N. benthamiana* plants were transformed with the *Sw-5b* gene under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Interestingly, the *Sw-5b*-transformed *N. benthamiana* plants presented a strong HR upon challenging with TSWV and four other tospoviruses: Alstroemeria necrotic streak virus (ANSV), *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV) and *Impatiens necrotic spot virus* (INSV) (Chapter 2 and 7). To identify the Avr, TSWV genes were cloned and expressed individually in leaves of *Sw-5b*-transformed *N. benthamiana* plants and Sw-5-resistant tomato isolines for HR monitoring. As a result, HR was triggered upon expression of the non-structural protein $NS_M$ from the resistance-inducing TSWV isolate BR-01, but not from the resistance-breaking TSWV isolate GRAU (Chapter 2). The research on $NS_M$ was continued in Chapter 3, in which truncated versions of this protein were transiently co-expressed with Sw-5b in wild type *N. benthamiana* leaves. These truncations lacked domains previously associated with tubule formation, cell-to-cell and systemic viral movement. Truncated $NS_M$ proteins lacking up to 50 amino acids (aa) from either the N- or C-terminus (full $NS_M$ is 301 aa in size) still triggered Sw-5b-mediated HR, suggesting that viral movement functions of $NS_M$ and its fate as Avr are independent from each other.

Chapter 4 described experiments to characterize a new tospovirus collected from bean plants showing necrotic mosaic symptoms during a field survey in São Paulo, Brazil (2006). Electron microscopy of symptomatic leaves revealed pleomorphic particles packed in vesicles. Due to its unusual natural host for a "Brazilian" tospovirus and being negative in ELISA for tospovirus species known to be circulating in Brazil, biological, serological, and molecular tests were performed to further characterize this putatively new tospovirus species. The virus appeared to have a narrow host-range, systemically infecting only three out of twenty test-plants of various species and presented unique serological properties when compared to other known tospovirus species. The genome sequencing of this tospovirus revealed a completely new species, which, together with Soybean vein necrosis-associated virus (SVNaV), represents a second evolutionary lineage of tospoviruses circulating in the American continent. This new tospovirus was tentatively named Bean necrotic mosaic virus (BeNMV). Since the *Sw-5b* protein recognizes at least five tospovirus species of "American" origin, the $NS_M$ protein of BeNMV was also tested for HR-triggering through co-expression with Sw-5b in *N. benthamiana* leaves. The outcome, however, indicated that the $NS_M$ protein of BeNMV is not a cognate Avr of the *Sw-5b* protein.

In Chapter 5 it is shown that the Sw-5b protein triggers both $NS_M$-dependent and -independent HR. The latter was achieved by co-expression of Sw-5b with RNA silencing suppressors (p19 and $NS_S$), which increased the Sw-5b cellular accumulation and lead to auto-HR. This observation allowed the screening of HR-triggering and $NS_M$-recognition as uncoupled events for other Sw-5 proteins as well. While Sw-5a could trigger auto-HR, it

lacked the ability to recognize $NS_M$ as Avr. On the other hand, the highest conserved ortholog of Sw-5a and Sw-5b from susceptible *S. lycopersicum*, named here as Sw-5a$^S$, lacked both auto-HR and $NS_M$-dependent HR. By co-expression of the individual Sw-5b domains CC, NB-ARC, LRR or combined versions (CC-NB-ARC and NB-ARC-LRR) with $NS_M$ and the silencing suppressor p19, the role of these domains in HR-triggering and $NS_M$-recognition was determined. While NB-ARC was sufficient for auto-HR, NB-ARC-LRR triggered both auto- and $NS_M$-dependent HR, indicating that LRR specifies the Avr recognition. The CC domain suppressed HR triggered by NB-ARC in *cis* and *trans*, pointing towards a regulatory function for CC. The overexpression of the NB-ARC domain from Sw-5a$^S$ did not result in HR, similar to the outcome with the full-length Sw-5a$^S$ protein. After alignment of the NB-ARC domain, three mismatches were found between Sw-5a, Sw-5b, and Sw-5a$^S$ which were reverted in the latter. When the glutamine at position 599 was converted into an arginine (Q599R), to mimic the situation in the Sw-5b protein at this position, the Sw-5a$^S$ NB-ARC domain became functional for auto-HR triggering. Modeling of this domain revealed that this mutation was outside of the ADP/ATP binding site, which is important for the switching between “on” and “off” states of NB-ARC domains. Finally, the fusion of the LRR domain of Sw-5b to the Q599R variant of the Sw-5a$^S$ NB-ARC domain resulted in both auto- and $NS_M$-dependent HR. The constructs encoding the various Sw-5b domains were used for subcellular studies in Chapter 6. To this end, all constructs encoded proteins were fused with Green Fluorescence Protein (GFP) at their N-terminus, to enable easy visualization by confocal microscopy in leaf tissue. Whereas the full-length Sw-5b protein, the individual domains CC, NB-ARC, and LRR and the combined CC-NB-ARC version showed a nucleocytoplasmic distribution, NB-ARC-LRR localized only in the cytoplasm, suggesting that CC signalizes nuclear import. The subdomains NB-ARC and LRR were also investigated for a possible direct interaction with $NS_M$ using Bimolecular Fluorescence Complementation (BiFC). While for LRR interaction with $NS_M$ was not observed, the NB-ARC domain seemed to directly bind to $NS_M$.

The specificities for $NS_M$ recognition have been discussed in Chapter 7 taking into consideration evolutionary aspects of tomatoes and tospoviruses. A model for Sw-5b activation is postulated throughout the text based on the dissection of this protein in HR-triggering and subcellular localization assays and on its putative direct interaction with $NS_M$.

# RESUMO

*Tomato spotted wilt virus* (TSWV) bem como outras espécies de tospovírus (Família *Bunyaviridae*) é responsável por perdas substanciais na produção de vegetais ao redor do mundo. Tospovírus são transmitidos de maneira circulativa e propagativa por tripes (Ordem Thysanoptera). Como para outros patógenos, ações contra tospovírus exigem um olhar holístico, envolvendo uma combinação de táticas culturais, fitosanitárias, químicas e biológicas, quando apropriadas, além do uso de cultivares resistentes. Entretanto, o controle de doenças causadas por tospovírus tem se mostrado difícil, dado o grande espectro de hospedeiros desses vírus e a grande eficiência dos tripes vetores em transmiti-los. Além disso, a dependência e o aumento no uso de inseticidas de baixo custo tem exacerbado a presença de tospovírus pela forte pressão de seleção sobre os tripes vetores que se tornam resistentes a diferentes inseticidas. Para solucionar de forma simples todas as questões financeiras e ambientais associadas ao uso abusivo de inseticidas para o controle de tripes, esforços têm aumentado para a obtenção de cultivares geneticamente resistentes como um componente integral das estratégias de controle de doenças.

Até o momento existem duas fontes de resistência disponíveis para o melhoramento genético de hortaliças à TSWV. Uma dessas fontes é o *cluster* genético *Sw-5*, o qual foi encontrado em *Solanum peruvianum* L., uma espécie de tomate selvagem do Peru, e tem sido introduzido em cultivares de *S. lycopersicum* L. (tomate comercial). Após mapeamento gênico, pelo menos cinco parálogos compõem o *cluster* genético *Sw-5* em *S. peruvianum*, os quais foram nomeados de *Sw-5a* a *Sw-5e*. Esses parálogos codificam receptores do tipo NB-LRR, uma classe de proteínas citoplasmática que ativam resistência após reconhecimento direto ou indireto de patógenos que tenham ultrapassado as primeiras barreiras de defesa do sistema imune vegetal. Transformação de plantas de tabaco com os parálogos *Sw-a* e *Sw-5b* revelou que somente o último ativa resistência contra isolados de TSWV. Com a disponibilidade do genoma do tomate, genes ortólogos também foram mapeados em *S. lycopersicum*. Esta tese inicia-se com uma descrição detalhada do sistema imune vegetal e descobertas anteriores sobre o *cluster* genético *Sw-5* (Capítulo 1). Como parte da introdução, os problemas causados por tospovírus e suas características são descritas. Tendo essas informações como ponto de partida, esta tese focou no entendimento de pontos-chaves da resistência mediada pela proteína Sw-5b contra tospovírus com uma atenção especial nos eventos moleculares e celulares envolvidos atrás desse mecanismo de resistência. Análises funcionais foram realizadas para esclarecer as delimitações genéticas entre os ortólogos funcionais e não funcionais do cluster *Sw-5* no reconhecimento de tospovírus e ativação de resistência.

Já que transformantes de *N. tabacum* com o gene *Sw-5b* feitos anteriormente não apresentaram resposta hipersensitiva (HR) após inoculação com TSWV, plantas

de *N. benthamiana* foram transformadas com o gene *Sw-5b* buscando linhas transgênicas que poderiam responder com HR e assim facilitar a identificação do determinante de avirulência (Avr) de TSWV (Capítulo 2). Enquanto *N. tabacum* foi transformada com o gene *Sw-5b* sob controle de seus próprios elementos regulatórios, *N. benthamiana* foi transformada com o gene *Sw-5b* sob controle do promotor 35S do *Cauliflower mosaic virus* (CaMV). De forma interessante, as plantas transformadas de *N. benthamiana* apresentaram forte HR após inoculação de suas folhas com TSWV e outras quatro espécies de tospovírus: Alstroemeria necrotic streak virus (ANSV), *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV) and *Impatiens necrotic spot virus* (INSV) (Capítulos 2 e 7). Para identificação do Avr, os genes de TSWV foram clonados e expressos individualmente em folhas de *N. benthamiana* expressando Sw-5b e em isolinhas de tomate resistentes (contendo *Sw-5*) para monitoramento de HR. Como resultado, HR foi induzida após expressão da proteína não-estrutural $NS_M$ do isolado BR-01 de TSWV que induz resistência, mas não do isolado GRAU de TSWV que quebra resistência (Capítulo 2). Foco sobre a proteína $NS_M$ seguiu no capítulo 3. Versões truncadas dessa proteína foram transientemente co-expressas com Sw-5b em folhas de *N. benthamiana* (wild type). Tais versões truncadas excluíam domínios anteriormente associados com formação de túbulos, movimento viral célula-célula e sistêmico. Proteínas $NS_M$ truncadas faltando até 50 aminoácidos (aa) de ambos N e C terminais ($NS_M$ inteira contém 301 aa) ainda induziram HR, sugerindo que funções associadas ao movimento viral e comportamento como Avr são características independentes para $NS_M$.

No capítulo 4 experimentos foram realizados para caracterizar uma nova espécie de tospovírus observado em plantas de feijão que apresentavam sintomas de mosaico necrótico em São Paulo, Brasil (2006). Microscopia eletrônica de folhas sintomáticas revelou partículas pleiomórficas agrupadas em vesículas. Devido ao fato de feijão ser um hospedeiro não usual e pelo resultado negativo em testes de ELISA para outras espécies já conhecidas por circular no Brasil, testes biológicos, sorológicos e moleculares foram realizados para caracterização de uma provável nova espécie de tospovírus. Este vírus apresentou um estreito espectro de hospedeiros, infectando sistemicamente três de vintes espécies de plantas indicadoras e apresentou propriedades sorológicas únicas quando comparado com outras espécies de tospovírus encontradas no Brasil. O sequenciamento do genoma deste tospovírus revelou uma nova espécie que, junto com Soybean vein necrosis-associated virus (SVNaV), representa uma nova linhagem evolutiva de tospovírus circulando no continente americano. Este tospovírus foi tentativamente chamado Bean necrotic mosaic virus (BeNMV). Já que a proteína Sw-5b reconhece pelo menos cinco espécies de tospovírus de origem "americana", a proteína $NS_M$ do BeNMV também foi testada para monitoramento de HR através de sua co-expressão com Sw-5b em folhas de *N. benthamiana*. O resultado, entretanto, mostrou que a proteína $NS_M$ do BeNMV não é um Avr cognato da proteína Sw-5b.

No capítulo 5 é mostrado que a proteína Sw-5b induz HR dependentemente

e independentemente da presença de $NS_M$. A forma independente foi observada através da co-expressão da proteína Sw-5b com supressores de silenciamento gênico (p19 e $NS_S$), os quais aumentaram a acumulação celular da proteína Sw-5b, induzindo auto-HR. Esta observação permitiu o *screening* de indução de HR e reconhecimento de $NS_M$ como eventos independentes para as outras proteínas Sw-5. Enquanto Sw-5a induziu auto-HR, ela não foi capaz de reconhecer $NS_M$ como Avr. De forma diferente, o ortólogo mais conservado das proteínas Sw-5a e Sw-5b de *S. lycopersicum* susceptível a TSWV, nomeado Sw-$5a^S$, não induziu qualquer tipo de HR. Através da co-expressão dos domínios individuais de Sw-5b, CC, NB-ARC, LRR ou de versões combinadas (CC-NB-ARC e NB-ARC-LRR) com $NS_M$ e com o supressor de silenciamento gênico p19, o papel desses domínios na indução de HR e no reconhecimento de $NS_M$ foram determinados. Enquanto NB-ARC foi suficiente para induzir auto-HR, NB-ARC-LRR induziu tanto auto quanto HR dependente de $NS_M$, indicando que o domínio LRR especifica o reconhecimento do Avr. Já o domínio CC suprimiu HR induzida por NB-ARC em *cis* e *trans*, apontando para uma função regulatória de CC. A superexpressão do domínio NB-ARC de Sw-$5a^S$ não resultou em HR, similar ao resultado da proteína Sw-$5a^S$ completa. Após alinhamento dos domínios NB-ARC, três variações de aminoácidos (aa) foram encontradas entre as proteínas Sw-5a, Sw-5b e Sw-$5a^S$, os quais foram revertidos no último. Quando a glutamina da posição 599 foi convertida em uma arginina (Q599R), igual a proteína Sw-5b nesta posição, o domínio NB-ARC da proteína Sw-$5a^S$ tornou-se funcional para indução de HR. Modelagem deste domínio revelou que esta mutação encontra-se fora do domínio de ligação de ADP/ATP, o qual é importante para o *switching* entre os estados *on* e *off* dos domínios NB-ARC. Finalmente, a fusão do domínio LRR da proteína Sw-5b na variante Q599R do domínio NB-ARC de Sw-$5a^S$ resultou em auto-HR e HR dependente de $NS_M$. Os construtos codificando os domínios da proteína Sw-5b também foram usados para estudos subcelulares no capítulo 6. Para este propósito, todas as proteínas estavam fusionadas a *Green Fluorescence Protein* (GFP) na porção N-terminal para tornar possível a visualização por microscopia confocal em tecido folhear. Enquanto a proteína Sw-5b inteira, os domínios CC, NB-ARC e LRR e o combinado CC-NB-ARC apresentaram distribuição nucleocitoplasmática, NB-ARC-LRR localizou-se somente no citoplasma, sugerindo que CC sinaliza o importe nuclear. Os domínios NB-ARC e LRR também foram investigados para uma possível interação direta com $NS_M$ através da técnica *Bimolecular Fluorescence Complementation* (BiFC). Enquanto interação direta não foi observada entre LRR e $NS_M$, o domínio NB-ARC aparentou interagir com $NS_M$.

As especificidades para o reconhecimento de $NS_M$ como Avr são discutidas no capítulo 7, levando em consideração aspectos evolutivos de tomates e tospovírus. Um modelo da ativação da proteína Sw-5b é postulado no texto tendo como base a dissecção desta proteína e os ensaios de localização subcelular, além de sua putativa interação direta com $NS_M$.

## CO-TUTELLE OR DUAL-DEGREE AGREEMENT

This thesis has been a collaborative project between the Laboratory of Virology at Wageningen University, Wageningen, The Netherlands and the Laboratory of Virology (Department of Cell Biology) at University of Brasília, Brasília, Brazil.

By agreement of the two respective universities and signed by the two university boards this thesis by Athos Silva de Oliveira is considered a co-tutelle or dual-degree thesis in fulfillment of the requirements for a degree of doctor for both universities.

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Special acknowledgments to my friends and WUR paranymphs Giel and Corien. Thank you for accepting this task and for your friendships. You are a "social plus" at Virology. You are good people, so good that I feel like "The Joker" beside you (you know I like pop culture references). Thanks for the discussions and drinks we have shared together. Taking the chance, I should acknowledge other WUR Virology friends. Paulus, you made my first days in Wageningen easier, thanks for your friendship. It was nice to meet a "movie maniac" in the Netherlands ("Yeah, well, you know, that's just, like, your opinion, man."). Jelke, thanks for your friendship, beers we have shared and for listening to my non-sense stories and existential inquiries. Thank you to the ARBO girls Mia and Corinne, you have handled my complaining very well, getting impatient just once in a while. Thank you to the Chinese mates Qiushi and Han, my fellow countryman Leonardo, and Bob for good moments in and out of Virology. Kind regards to all PhD students who have passed by WUR Virology during my time there: Dryas, Amaya, Márcio, Stefan, Ali, Desrina, Fred and Stineke.

Ivo and Roel, thank you for your help and choosing me as your supervisor. The same is valid for Anna Fernanda in Brazil who helped me in the beginning of my PhD at UnB. Thank you to many master and bachelor students who have passed by WUR Virology, we have shared good moments. One, however, I have to mention. Wijnand, thanks for your friendship and, particularly, for taking me to the hospital when I fell off my bicycle and broke my arm with only six months left to the end of my contract at WUR (I know it was a shame and embarrassing for Dutch standards.).

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## ABOUT THE AUTHOR

Athos Silva de Oliveira was born on January the 14th of 1987 in Brasília, Brazil. He finished his bachelor in Biology in 2008 at the JK college of Biological Sciences, Brasília. In 2009 he started his master study in Molecular Biology at the University of Brasília (UnB) under supervision of Dr. Renato Resende. During this period, he started the experiments to characterize the novel Bean necrotic spot virus (BeNMV). In 2011 Athos got admitted to the PhD Program of Molecular Biology at UnB under supervision of Dr. Renato Resende. After two years working in Brasília, he moved to the Netherlands for a sandwich period at the Wageningen University (WUR) under supervision of Dr. Richard Kormelink. This sandwich period turned out to be a co-tutelle period since Athos got also admitted to the Graduate School of Experimental Plant Sciences (EPS) in the Netherlands. During his PhD, he studied the molecular and cellular aspects of the *Sw-5* gene cluster from tomato against tospoviruses.

**Education Statement of the Graduate School**

**Experimental Plant Sciences**

The Graduate School EXPERIMENTAL PLANT SCIENCES

**Issued to:** **Athos Silva de Oliveira**

**Date:** **2 December 2015**

**Group:** **Laboratory of Virology**

**University:** **Wageningen University & Research Centre**

| **1) Start-up phase** | *date* |
|---|---|
| ► **First presentation of your project** | |
| Unravelling the cellular signaling pathways of Sw-5-mediated dominant resistance against tospoviruses | Apr 04, 2011 |
| ► **Writing or rewriting a project proposal** | |
| Unravelling the cellular signaling pathways of Sw-5-mediated dominant resistance against tospoviruses | Apr 04, 2011 |
| ► **Writing a review or book chapter** | |
| ► **MSc courses** | |
| ► **Laboratory use of isotopes** | |
| *Subtotal Start-up Phase* | *7.5 credits** |

| **2) Scientific Exposure** | *date* |
|---|---|
| ► **EPS PhD Student Days** | |
| European Retreat for PhD Students in Plant Sciences, Amsterdam, Netherlands | Jul 01-04, 2014 |
| EPS Get2Gether | Jan 29-30, 2015 |
| ► **EPS Theme Symposia** | |
| EPS theme 2 symposium and Willie Commelin Scholten Day: “Interactions between plants and biotic agents” , University of Amsterdam | Feb 25, 2014 |
| EPS theme 2 symposium and Willie Commelin Scholten Day: “Interactions between plants and biotic agents”, Utrecht University | Feb 20, 2015 |
| ► **NWO Lunteren days and other National Platforms** | |
| NWO-ALW meeting Exp. Plant Sciences, Lunteren, NL | Apr 22-23, 2013 |
| NWO-ALW meeting Exp. Plant Sciences, Lunteren, NL | Apr 14-15, 2014 |
| NWO-ALW meeting Exp. Plant Sciences, Lunteren, NL | Apr 13-14, 2015 |
| ► **Seminars (series), workshops and symposia** | |
| DAVS symposium, Amsterdam | Mar 08, 2013 |
| Wageningen PhD Symposia | Dec 10, 2013 |
| DAVS symposium, Amsterdam | Mar 07, 2014 |
| ► **Seminar plus** | |
| ► **International symposia and congresses** | |
| XXIII Brasília congress of virology, Foz do Iguaçu, Paraná, Brazil. (4 days) | Sep 30 -Oct 03, 2012 |
| XVI International Congress on Molecular Plant-Microbe Interactions, Rhodes, Greece. (5 days) | Jul 06-10, 2014 |
| Xth International Symposium on Thysanoptera & Tospoviruses, San Francisco, USA. (5 days) | May 16-20, 2015 |

| | | |
|---|---|---|
| ► | **Presentations** | |
| | Oral: Bean necrotic mosaic virus: a new and distinct Brazilian tospovirus (Foz do Iguaçu, BR) | Oct 01, 2012 |
| | Oral: The Tomato spotted wilt virus cell-to-cell movement protein (NSM) triggers the Sw-5-mediated resistance (Lunteren, NL) | Apr 15, 2014 |
| | Poster: Effector-dependent and -independent hypersensitive cell death response triggered by Sw-5 resistance proteins (Rhodes, Greece) | Jul 09, 2014 |
| | Oral: The Sw-5 gene cluster: Unraveling the keys to resistance to Tomato spotted wilt virus (San Francisco, USA) | May 17, 2015 |
| ► | **IAB interview** | |
| | Meeting with a member of the Interanl Advisort Board (IAB) of EPS | Sep 29, 2014 |
| ► | **Excursions** | |
| | *Subtotal Scientific Exposure* | *14.0 credits** |

| **3) In-Depth Studies** | | *date* |
|---|---|---|
| ► | **EPS courses or other PhD courses** | |
| | Teacher Training on Molecular Biology (Treinamento Didático em Biologia Molecular) - UnB, Brazil | Mar 2011 |
| | Seminars on Molecular Biology (Seminários em Biologia Molecular) - UnB, Brazil | Mar 2011 |
| | Transmission Electron Microscopy on Biology (Microscopia Eletrônica de Transmissão em Biologia) - UnB, Brazil | Mar 2011 |
| | Purification and Characterization of Biologically-Active Peptides (Purificação e Caracterização de Peptídeos Biologicamente Ativos) - UnB, Brazil | Mar 2011 |
| | Seminars on Molecular Biology (Seminários em Biologia Molecular) - UnB, Brazil | Aug 2011 |
| | Fluorescence and Confocal Microscopies (Microscopia de Fluorescência e Confocal a Laser) - UnB, Brazil | Aug 2011 |
| | Seminars on Molecular Biology (Seminários em Biologia Molecular) - UnB, Brazil | Mar 2012 |
| | Seminars on Molecular Biology (Seminários em Biologia Molecular) - UnB, Brazil | Aug 2011 |
| | Spring School 'Host-Microbe Interactomics - WUR, Netherlands (3 days) | Jun 02-04, 2014 |
| ► | **Journal club** | |
| ► | **Individual research training** | |
| | *Subtotal In-Depth Studies* | *12.9 credits** |

| **4) Personal development** | | *date* |
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| ► | **Skill training courses** | |
| | The Essentials of Scientific Writing and Presenting (ESWP) | Nov 24, 2014 |
| | Writing grant proposals | Apr 07, 2015 |
| ► | **Organisation of PhD students day, course or conference** | |
| ► | **Membership of Board, Committee or PhD council** | |
| | *Subtotal Personal Development* | *3.2 credits** |

| | |
|---|---|
| **TOTAL NUMBER OF CREDIT POINTS*** | **37,6** |

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 credits

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

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