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Induction and Suppression of Antiviral RNA Silencing by Tomato Spotted Wilt Virus

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INDUCTION AND SUPPRESSION OF ANTIVIRAL RNA SILENCING BY *TOMATO SPOTTED WILT VIRUS*

by

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INDUCTION AND SUPPRESSION OF ANTIVIRAL RNA SILENCING BY *TOMATO SPOTTED WILT VIRUS*

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Tomato spotted wilt virus (TSWV) is an emerging pathogen with wide host range and one of the most important viruses of plants. Information regarding processing of negative single stranded RNA viruses such as TSWV in the RNA silencing pathway remains limited. In nature TSWV is only transmitted by thrips as vectors and since infection occurs in both thrips and plants, an experimental system to transmit using thrips and the detection of TSWV were established. In order to understand the processing of TSWV in the RNA silencing pathway, *Arabidopsis thaliana* as a model plant was used in the genetic analysis against TSWV. Core components of the RNA silencing machinery tested were Dicer-like proteins(DCLs), RNA dependent RNA Polymerases (RDRs), and Argonaute proteins (AGOs). Results suggest DCL4 and DCL2 proteins are involved in the recognition and processing of TSWV. RDR1 is needed for the amplification of siRNAs derived from TSWV. AGOs protein levels remain unaffected after TSWV

inoculation compared to mock inoculated plants suggesting that degradation by TSWV does not occur.

It is well known that plant viruses encode suppressors of RNA silencing to facilitate plant infection and TSWV encodes the Non-Structural-small protein (NSs) whose mechanism of suppression has not been elucidated. NSs protein from TSWV was cloned from viral RNA and tagged with a 6HIS-3xFLAG tag at the C terminus for detection (NSs-HF). In transient assays activity of NSs as a suppressor of RNA silencing was corroborated by preventing silencing of green fluorescent protein (GFP) at three days post infiltration (dpi). Infectious clones of TSWV are not available and to determine the mechanism of suppression, NSs-HF was introduced in a previously described inactive model virus *Turnip mosaic virus* (TuMV-GFP-AS9) which has an inactive suppressor a does not infect wild type plants. The rescue of pathogenicity of this virus was observed after GFP detection under UV light and the presence of the NSs protein by western blotting. These experiments establish a foundation to study TSWV within the RNA silencing pathway and are the basis for future experiments to understand the mechanism of suppression of RNA silencing by TSWV.

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LITERATURE REVIEW

INTRODUCTION

World's population is around 7.3 billion and is projected to reach 9.7 billion by 2050 (United Nations, 2015). To feed that population with today's technology seems almost impossible without compromising the environment. Changes are needed in the production systems focusing in sustainability for future generations and the planet. In order to improve today's technology advances in sciences are needed to elucidate important pathways and missing information.

Available arable land, erosion and water including high cost of supplies are abiotic factors affecting agricultural production. Biotic factors as damage by insects, weeds and diseases can also present a threat to agricultural production. Combination of biotic factors and abiotic factors such as climate change also affect the roles of vectors and compromise the immune responses of plants to plant pathogens (Bevan et al., 2017)

Plant pathogens and the diseases they cause are the origin of significant losses in valuable food crops throughout the world. Diseases account for at least 10% of crop loss globally and are partially responsible for the suffering of 800 million people who lack adequate food sources (De Wolf and Isard, 2007).

Viruses are among the most agriculturally important and biologically intriguing plant pathogens. Serious economic losses are associated to viral diseases by reducing yield and quality of agricultural products. Viruses are relatively simple genetic entities but still little is known about the mechanisms

they use to hijack and sequester host functions in order to favor their own replication (Kang et al., 2005).

DISEASES CAUSED BY TOMATO SPOTTED WILT VIRUS

The spotted wilt disease of tomato is the representative disease caused by *Tomato spotted wilt virus* (TSWV) and its transmission by western flower thrips *Frankliniella occidentallis* (Sherwood et al., 2009). TSWV and its transmission by thrips insects was initially associated and described in Australia around 1919 (Adkins, 2000; Jones, 2004; Scholthof et al., 2011).

TSWV has a global distribution and infects more than 1000 plant species in more than 70 families including both crops and ornamentals (Margaria et al., 2014). TSWV unique interaction with thrips is of exceptional importance since infection occurs in both thrips and plants (Gilbertson et al., 2015). Losses associated with this disease exceeded tens of millions dollars and for the US only in a 10 year period losses have ascended to 1.4 billion dollars (Rotenberg et al., 2015).

Symptoms of the disease vary with the host. The most common symptoms are yellowing in leaves, stunting of plants, reduced quality of fruits and sometimes necrotic/chlorotic rings in leaves, stems and fruits (Scholthof et al., 2011; Oliver and Whitfield, 2016). Some ornamental plants and some weeds are asymptomatic or present weak symptoms. Special attention should be given to alternative hosts of TSWV also known as “green bridges”, where TSWV and thrip species may overwinter for the next season (Jones, 2004).

TSWV is not transmitted through seeds or mechanical contact in field conditions (Scholthof et al., 2011). The only mode of transmission is through insect vectors (thrips) (Sherwood et al., 2009). For experimental purposes, and due to lack of infectious clones TSWV can be transmitted through mechanical inoculations (Mandal et al., 2008).

Management strategies of the disease are focused mainly in the vector and avoiding introduction of TSWV to new fields. Control of thrips is particularly difficult because of their small size and preference for protected places as petals. In addition, extreme polyphagy, rapid development of resistance to insecticides, fast generation of multiple populations and long distance movement when winged adults are present also complicate management (Gilbertson et al., 2015)

Crop rotations are not effective in the management of this disease because TSWV has also wide host range and thrips are polyphagous. Resistance cultivars are effective but available effective resistance may not be present for some crops or quickly overcome by natural variation of TSWV (Jones, 2004).

TOMATO SPOTTED WILT VIRUS GENOME ORGANIZATION AND REPLICATION

TSWV belongs to the genus *Tospovirus* in the family *Bunyaviridae* commonly known to infect vertebrates and invertebrates (Hedil and Kormelink, 2016). All *Bunyaviridae* species feature genomes composed of three RNA

segments encoded in a negative sense orientation and sometimes in an ambisense orientation (Kormelink et al., 2011). The genus *Tospovirus* is unique among the family because infects both thrips and plants (Oliver and Whitfield, 2016). After a century of TSWV's first detection and 30 years of intensive molecular research, TSWV is among the top 10 most economical, destructive and scientifically important plant viruses (Scholthof et al., 2011).

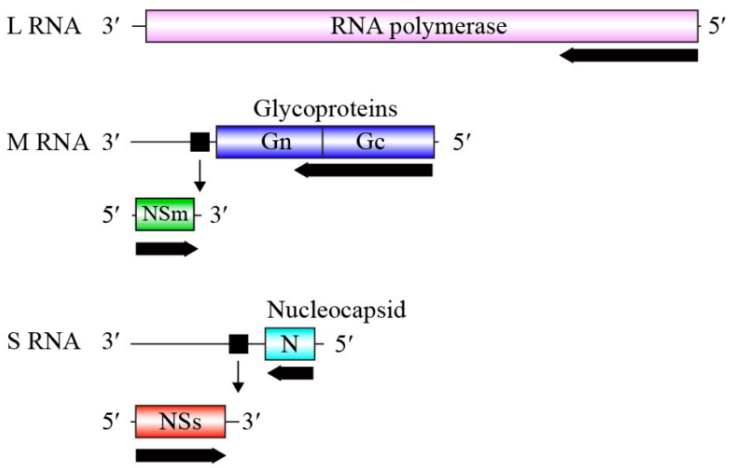

Figure 1.1 TSWV genome organization and replication strategies. Arrows show orientation of genes and black squares hairpin-like structures as terminators of transcription (Ocampo Ocampo et al., 2016) with permission.

The genome of TSWV consists of three single stranded RNA segments denoted L, M and S according to their size L (~8.9 Kb), M (~4.8 Kb), S (~2.9 Kb) respectively (Turina et al., 2016). The L segment encodes a RNA dependent RNA polymerase (L) in a negative sense orientation in the viral or genomic RNA (Figure 1.1 Segment L). The M segment encodes two proteins in an ambisense orientation, the GN-GC glycoprotein precursor encoded in a negative orientation

while the NSm movement protein is encoded in a positive sense orientation (Figure 1.1 Segment M). The S segment also encodes two proteins in an ambisense orientation, the nucleocapsid protein (N) in negative sense orientation and the NSs protein in a positive orientation (Figure 1.1 Segment S) (Kormelink et al., 2011; Oliver and Whitfield, 2016; Turina et al., 2016).

TSWV virions are spherical and measure from 80 nm to 120 nm (Adkins, 2000). Compared to other plant viruses TSWV virions have a phospholipid membrane embedded with glycoproteins forming projections (Kormelink et al., 2011). The phospholipid membrane is host derived and protects the RNA genome. In addition viral genomic RNA segments interact with nucleocapsid proteins (N) and RNA dependent RNA polymerases (RdRp also known as L proteins) to form ribonucleoproteins that act as templates of replication (Oliver and Whitfield, 2016). The genomic RNA segments have complementary terminal sequences that allow the ssRNA segments to adopt semicircular or panhandle structures (Adkins, 2000).

Replication of TSWV in plants starts with transcription of the genes encoded in a negative sense orientation by the RdRp to produce mRNAs. Transcription of ambisense segments is terminated after the first ORF in a conservative zone where a hairpin like structure (Figure 1.1 black squares) may release the RdRp (Kormelink et al., 2011; Jackson and Li, 2016; Turina et al., 2016). After initial rounds of transcription and production of subgenomic RNAs that serve as mRNAs for translation of the negative encoded ORF, the RdRp

switches to a replicative mode yielding complete positive single stranded antigenomic RNA segments. ORFs located in the new antigenomic segments in a negative orientation can be used as templates for the transcription of the remaining proteins NSm and NSs (Gielen et al., 1991; Jackson and Li, 2016).

TSWV TRANSMISSION BY WESTERN FLOWER THRIPS

Nearly all species in the genus *Tospoviruses* are known to be transmitted by thrips insects in the order *Thysanoptera* in a persistent propagative manner (Hedil and Kormelink, 2016). Of the thousands of recognized thrips species only about a dozen transmit *Tospoviruses* (Gilbertson et al., 2015). TSWV is transmitted by *Frankliniella bispinosa, Frankliniella cephalica, Frankliniella gemina, Frankliniella fusca, Frankliniella intonsa, Frankliniella occidentalis, Frankliniella schultzei, Thrips setosus and Thrips tabaci* (Oliver and Whitfield, 2016). Some vectors have wide host range and global distribution (Gilbertson et al., 2015).

Early literature reported onions thrips (*Thrips tabaci)* as the most important vector of TSWV. Recently TSWV is predominantly transmitted by western flower thrips (*Frankliniella occidentalis)*. Interestingly, today's TSWV is no longer able to efficiently infect and by transmitted by onions thrips (*Thrips tabaci)* (Jones, 2004; Oliver and Whitfield, 2016).

Even though *Tospoviruses* have specific interactions with thrips and many thrips are able to transmit more than one species, TSWV has become the most important *Tospovirus* species (Gilbertson et al., 2015; Turina et al., 2016).

Frankliniella occidentalis has become the most important and efficient *Tospovirus* vector and also an emerging threat to food security because of its role as a vector of many plant viruses (Gilbertson et al., 2015; Oliver and Whitfield, 2016). *Frankliniella occidentalis* has played a key role in the global emergence of the genus *Tospovirus* and specifically TSWV (Gilbertson et al., 2015).

TSWV transmission cycle begins with acquisition of TSWV by thrips and culminates with dispersal and inoculation of plants by adult thrips. In order to become competent vectors virus acquisition must occur in the first and second instar of the thrips' life cycle.

When TSWV is ingested by larval stages, enters and replicates in the midgut ephitelial cells and spreads in the gut tissues. TSWV disseminates in the vector and must reach the principal salivary glands (PSGs) for virus inoculation to a new plant host to occur (Montero-Astua et al., 2016; Oliver and Whitfield, 2016). In *Frankliniella occidentalis*, the gut is connected to the PSGs by the tubular salivary glands, and this tissue may serve as a conduit for virus dissemination in the vector (Oliver and Whitfield, 2016).

TSWV is not transovarially transmitted but can go through molting stages and still replicate transtadially (Whitfield et al., 2005; Oliver and Whitfield, 2016).

DEFENSE RESPONSES AGAINST PATHOGENS IN PLANTS

Plants have evolved a variety of immune systems conferred by host-encoded disease resistant genes to defend from pathogens including viruses (Pumplin and Voinnet, 2013). Mechanistically, plant defense mechanisms against viruses can be divided into two classes: protein-based and RNA silencing-based responses.

PROTEIN-BASED RESPONSES

These systems include pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Wang et al., 2012; Pumplin and Voinnet, 2013). Effective resistance against TSWV is a desirable trait as the introduction to commercial cultivar crops. However, sources of resistance against TSWV are limiting. The gene cluster Sw-5 from *Solanun peruviamun* is a promising source (Spassova et al., 2001). Non-adaptive effective resistance is found in the Sw-5b protein from the gene cluster Sw-5. Resistance from Sw-5b protein is monogenetic; only one protein triggers cell death after detection of TSWV NSm movement protein (Hoang et al., 2013). Even though there are more resistance genes found in other species, to date the molecular details of their resistance have not been fully described. In addition selection pressure and mixed infections generate new reassortants that are able to break resistance by changing as few as 2 nucleotides in the TSWV genome as in the case of the resistance provided by Sw-5b protein (Turina et al., 2016).

ANTIVIRAL RNA SILENCING AGAINST TSWV

RNA silencing is used to regulate invasive RNA via sequence-specific degradation (Alvarado and Scholthof, 2009). RNA silencing models explaining viral degradation of plant viruses are based on positive single stranded RNA viruses such as *Potyviruses* but genome organization and properties are different compared to negative single stranded RNA viruses. Processing of plant negative RNA viruses such as TSWV and its interactions within the RNA silencing pathway remain unknown (Jackson and Li, 2016). Some models proposed (Hedil and Kormelink, 2016) may be applicable to *Tospoviruses* in general but their demonstration to specific species has not been demonstrated.

Currently, it has been demonstrated that:

- TSWV NSs protein is a silencing suppressor by preventing GFP silencing and by restoring pathogenicity to two suppressor deficient viruses (Takeda et al., 2002; Ocampo Ocampo et al., 2016). NSs silencing suppressor has also been described preventing local and systemic silencing of GFP (Hedil et al., 2015).

- TSWV genome is processed into small interfering RNAs derived from all three genomic RNAs by detection of small interfering RNAs derived from TSWV in RNA sequencing analysis. The use of suppressor deficient viruses also led to an increase accumulation of 21 nucleotide small interfering RNAs (Margaria et al., 2015).

The mechanisms of induction and suppression of RNA silencing of TSWV remain to be determined.

ENDOGENOUS RNA SILENCING IN PLANTS

RNA silencing is an adaptive eukaryote mechanism used to regulate gene expression (Alvarado and Scholthof, 2009). In plants RNA silencing regulates a high number of genes with functions in developmental processes, including flowering and developmental timing, control of cell proliferation, meristem identity and patterning (Allen et al., 2005).

RNA silencing in plants depends on the production of small RNAs (Baulcombe, 2004). Plant small RNAs range from 21 to 24 nucleotides in length and can be divided in two main classes: micro RNAS (miRNAs) that regulate endogenous genes (Alvarado and Scholthof, 2009; Budak and Akpinar, 2015) and small interfering (siRNAs) involved in the silencing of transposons, repetitive DNA and viruses (Ruiz-Ferrer and Voinnet, 2009).

In *Arabidopsis thaliana* biogenesis of miRNAs occurs in the nucleus from transcription of genes from different loci in the genome by a cellular polymerase II (Bologna and Voinnet, 2014). After transcription miRNAs precursors form hairpin-like structures that are recognized and cleaved by a RNase III endonuclease protein called Dicer-like protein (DCL). There are four DCLs in the *Arabidopsis* genome and specific functions have been assigned for every member (Bologna and Voinnet, 2014). In the miRNA pathway DCL1 protein

cleaves and produces small RNA duplexes measuring 21 nucleotides in length showing 5’ phosphate groups and two nucleotides overhanging 3’ ends (Ding and Voinnet, 2007; Bologna and Voinnet, 2014). Small RNA duplexes are stabilized through 2′ -O-methylation by a HUA ENHANCER 1 (HEN1) protein to prevent degradation by endonucleases.

One strand of the RNA duplex (guide strand) is loaded into an Argonaute (AGO) family member of the 1O AGOs proteins found in *Arabidopsis* (Bologna and Voinnet, 2014). Argonaute proteins are the main effectors of RNA silencing across kingdoms and their protein complex known as RNA Induced Silencing Complex (RISC) (Bologna and Voinnet, 2014). Activated RISCs target complementary mRNA transcripts to the loaded small RNA and prevent translation through binding by blocking the ribosomes or through slicer activity by slicing mRNAs (Baulcombe, 2004; Carbonell et al., 2012).

RNA silencing core components also go through self-regulation via cellular miRNAs. Specific miRNAs target mRNA of DCLs and AGOs proteins. DCL1 is regulated by miR162. AGO1 is regulated by miR168 and AGO2 levels are regulated by miR403 (Bologna and Voinnet, 2014)

Biogenesis of siRNAs is complex and depending on the origin siRNAs can be subdivided in more groups based in their biogenesis and their targets. siRNAs are derived from dsRNA precursors and cleavage occurs by DCL2 to DCL4. DCLs generate dsRNA duplexes measuring 22 to 24 nucleotides in length that can be loaded into AGOs and direct cleavage of mRNAs of transgenes,

transposons and repetitive regions as in the miRNA silencing pathway. Trans-acting small interfering RNAs (tasiRNAs) are generated when *TAS* transcripts are targeted by miRNAs, after cleavage of *TAS* transcripts siRNAs are processed to produce tasiRNAs that are loaded in the AGOs to direct cleavage of several new mRNA targets (Borges and Martienssen, 2015).

A secondary amplification of siRNAs can occur via a cellular RNA dependent RNA polymerase (RdRp) that produces complementary sequences or double stranded RNA (dsRNA) to the remaining products of the mRNA transcripts and single stranded small RNAs. dsRNA is recognized by DCL proteins and incorporated in the RNA silencing pathway (Bologna and Voinnet, 2014). Plants can protect themselves from viral infections through RNA silencing using virus derived siRNAs (Ding and Voinnet, 2007). RNA viruses often resemble dsRNA structures in their genomes or replication intermediates generate dsRNA that is recognized by DCL2 and DCL4. Generation of virus derived small interfering RNAs (vsiRNAs) occurs in the cytoplasm where vsiRNAs are incorporated in the RISC and direct targeting of RNA viruses restricting replication and infection of viruses (Garcia-Ruiz et al., 2010). DCL2 and DCL4 have hierarchical roles and DCL4 appears to preferentially be expressed over DCL2. If DCL4 is not present DCL2 replaces DCL4 generating vsiRNAs 22 nucleotides in length compared to normal 24 nucleotides from DCL4 (Garcia-Ruiz et al., 2010; Bologna and Voinnet, 2014).

MOLECULAR TECHNIQUES FOR DETECTION OF TSWV

There are several techniques for virus detection and the use of them will depend on the nature of the virus and what needs to be detected. Detection of viral proteins is based in antibodies that binds to specific proteins such as nucleocapsid proteins. However, its effectiveness can be limited by nonspecific binding and consequent background signals (Wu et al., 2002)

TSWV is a single stranded RNA virus that can be detected using Northern Blotting. Northern Blotting is a molecular technique that can detect specific sequences in RNA preparations by using blotting and hybridization techniques that were originally developed for DNA (Southern Blotting)(Brown et al., 2004).

Hybridization of probes to complementary sequences and detection of probes previously labelled with radioactive isotope ^{32}P is the basis of this molecular technique and still a common and sensitive technique (Hloch et al., 2001). The main disadvantages of Northern blotting techniques using radioactive probes are the possible health hazards, inconvenience during handling and the short half-life of radioactive probes. Nevertheless, there are alternatives to radiation probes such as the digoxigenin-dUTP probes (DIG) (Roche, Basel, Switzerland) where detection occurs using specific antibodies that detect digoxygenin within the probes. Digoxygenin is incorporated in the new synthesized DNA strands during a normal PCR (Eisel et al., 2008).

CHAPTER 2:

ESTABLISHMENT OF AN EXPERIMENTAL SYSTEM TO REPLICATE TSWV IN ARABIDOPSIS

INTRODUCTION

A TSWV infectious clone is not available due to limitations in today's cloning strategies to amplify a full-length clone of the L segment (Jackson and Li, 2016). Recent advances in molecular techniques may overcome that limitation (Gibson et al., 2009; Kosuri and Church, 2014). Therefore, to work with this virus complete virions must be used. Mechanical inoculations and protocols to transmit TSWV are well established (Mandal et al., 2008); however, if repeated mechanical inoculations are used with the same inoculum mutations and loss of proteins involved during the thrips' infection cycle are common to appear (Spassova et al., 2001). In order to avoid mutations that may interfere with the normal infection and replication cycles of TSWV, a thrips transmission and maintenance protocol has been established (Ocampo Ocampo et al., 2016). *Emilia fosbergii* is used to maintain TSWV active because it is known to have less effects on virions (Spassova et al., 2001). Besides, continuous natural transmissions using living thrips as vectors is also a good strategy to avoid generation of mutant virions that may affect TSWV infection.

In this chapter, the establishment of a protocol to transmit TSWV using thrips as vectors, storage of infected tissue, propagation in *N. benthamiana*, inoculation of *Arabidopsis thaliana* as a model host for TSWV replication, detection of TSWV genomic RNA segments and total small RNAs derived from TSWV are described.

MATERIALS AND METHODS

TSWV maintenance in *Emilia fosbergii*

The Hawaii isolate of TSWV was transmitted by thrips (*Frankliniella occidentalis*) to *Emilia fosbergii* plants for maintenance. Adult thrips were collected from flowers of healthy *Arabidopsis* plants growth in the Plant Pathology Greenhouse (University of Nebraska-Lincoln East Campus) and fed on green beans inside pots in lab conditions (8 hours of white light, to mate and produce offspring). Leaves of *Emilia fosbergii* plants with TSWV infection were introduced inside pots to feed thrips in order to acquire TSWV. Pupal and adult stages were then moved to chambers containing healthy *Emilia fosbergii* plants in order to infect healthy plants. Plants were monitored to observe development of systemic TSWV infection. Infected leaves with systemic symptoms were collected and frozen or used for mechanical inoculation of *Nicotiana benthamiana* plants.

TSWV amplification in *Nicotiana benthamiana*

Before inoculation of *Arabidopsis thaliana*, TSWV inoculum was propagated in *N. benthamiana* plants. Using a mortar and pestle, inoculum was prepared by grinding 1 g of systemically infected *Emilia fosbergii* fresh leaves in 10 mL of TSWV inoculation buffer (final concentration 0.1 M potassium phosphate pH 7.0, 10 mM sodium sulfite, 10 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride PMSF) in the cold room at 4° C. The extract was

centrifuged at 4000 rpm for 10 min at 4° C, and the supernatant transferred to a new tube and kept on ice all the time.

Leaves were dusted with carborundum and rub-inoculated with 50 µL of inoculum per leaf. Plants were inoculated when they were 25-30 days old and kept in a growth chamber at 22°C under a 16:8 h light:dark cycle. Systemically infected *N. benthamiana* leaves were collected at 15 days post inoculation (dpi), respectively and these leaves were used as inoculum for mechanical inoculation of *Arabidopsis thaliana* plants.

Mechanical inoculation of *Arabidopsis thaliana*

Four *Arabidopsis* leaves per plant were dusted with carborundum and rub-inoculated with 10 µL of inoculum per leaf. Plants were inoculated when they were 23 days old. Control plants were mock-inoculated with only buffer solution. Plants were kept in a growth chamber at 22° C under a 16:8 h light:dark cycle. Inflorescences from systemic infected *Arabidopsis* plants were collected at 15 dpi, frozen in liquid nitrogen and stored at -80° C until protein and RNA extractions.

TSWV virion purification

TSWV virions were purified from systemically infected *N. benthamiana* plants as described in (Ocampo Ocampo et al., 2016). Total and virion RNA was extracted and analyzed in agarose gels as described in (Ocampo Ocampo et al., 2016).

Protein extraction

Samples from systemically infected *Arabidopsis thaliana* plants were ground for 3 minutes in glycine grinding buffer (final concentration 0.1 M glycine-NaOH pH 9.0, 0.1 M NaCl, 10mM EDTA, 2% sodium dodecyl sulfate SDS and 1% sodium lauroylsarcosine) using zirconia beads and a BeadBeater (Biospect Products, Bartlesville, OK USA). After grinding samples were centrifuged at 14,000 rpm for 3 min at 4° C. An aliquot of 100 µl supernatant was transferred to an equal amount of 2X Protein Dissociation Buffer (final concentration 0.0625 M Tris pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 10% 2-mercaptoethanol and saturated bromophenol blue) and boiled for three minutes at 100ºC. Protein samples were stored at -80ºC until further use. The remaining supernatant was kept on ice and used for RNA extraction.

Western blotting

5 µl of total protein extraction were loaded on TGX Stain-Free™ FastCast™ 12% Acrylamide gels or Mini-PROTEAN® TGX Stain-Free™ gels and proteins separated by electrophoresis at 150 V for 60 min using a Mini-Protean® Tetra Cell (Bio-Rad, Hercules, CA) in 1X Tris-Glycine/SDS buffer (final concentration 0.05 M Tris, 0.38 M Glycine and 0.01 M sodium dodecyl sulfate). Protein transfer to an Amersham protran 0.45 µm nitrocellulose protein membrane (GE Healthcare, Little Chalfont, UK) was done using a Mini Trans-Blot® Electrophoretic Transfer Cell in western transfer buffer (final concentration 25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). After being transferred,

blots were stained with Ponceau S solution (Sigma, St. Louis, MO, USA) to detect the Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein which was used as a loading control. Ponceau S solution was de-stained with potassium buffered saline solution 1X PBS (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4) containing 0.1% v/v Tween 20 (Thermo Fisher Scientific, Waltham, MA) to obtain 1X PBS-T. For blocking blots were incubated for thirty minutes at room temperature in 5% milk (Research Products International Corp, Prospect, Illinois) diluted in 1X PBS-T.

Nucleocapsid protein was detected using a primary antibody (Anti-TSWV-N) from rabbit (dilution was 1:64,000) as described in (Ocampo Ocampo et al., 2016). An incubation time with gently shaking of a least 1 hour at room temperature or overnight incubation at 4° C were used. A secondary antibody from goat anti-rabbit immunoglobulin G was used to detect the first antibody (1:10,000; NA934-1; GE Healthcare, Little Chalfont, UK). Chemiluminescence was detected with Clarity Western ECL Substrate and a ChemiDoc® MP Imaging system (Bio-Rad, Hercules, CA, USA).

RNA extraction

After protein extraction samples were kept on ice all the time to prevent RNA degradation and total RNA was extracted using TRIzol® (Invitrogen, Carlsbad, CA, USA) as described in (Ocampo Ocampo et al., 2016). Remaining aliquots were mixed with 800 µl of TRIzol® reagent and vigorously mixed for 10 seconds using a vortex, following centrifugation at 8,300 g for three minutes at 4°

C to remove debris. The supernatant was carefully transferred to a new tube containing 500 µl of chloroform and vigorously vortexed to remove TRIzol® and centrifuged at 14,000 rpm for 10 min at 4° C to separate the phases. The supernatant was again transferred to a new tube containing 500 µl of clean chloroform to remove remaining TRIzol®. For RNA precipitation, the supernatant was transferred to a new tube containing 1 mL of ice-cold isopropanol followed by fifteen minutes of incubation time at room temperature. Centrifugation at 14,000 rpm for 10 minutes at 4° C was done to precipitate the RNA in a pallet in the bottom of the tube. RNA pallets were washed with 70% ethanol and centrifuged to remove ethanol which was done carefully to avoid loss of RNA. RNA pallets were completely dried at room temperature for one hour or until complete removal of ethanol was observed. Once RNA was completely dried resuspension in 50 µl of 0.1 X TE in DEPC water was done for one hour with samples on ice.

RNA concentration was measured with a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) and normalized to 1 mg/µl using 0.1 X TE. Samples containing 1 mg RNA were mixed with 5 µl of RNA loading dye (final concentration 95% formamide, 0.025% SDS, 0.025% Bromophenol blue, 0.025% xylene cyanol and 0.5 mM EDTA) and run for 40 minutes at 85 volts on a 1% non-denaturing agarose gel previously stained with ethidium bromide (1 µl at 10 mg/µl per every 10 mL of agarose) to visualize quality of total RNA.

DIG-labelled oligos and cDNA probes

TSWV genomic RNAs: L, M, and S were detected in high molecular RNA gels with DIG random labelled dsDNA PCR products amplified from DNA plasmids harboring cDNA regions of TSWV genomic segments using DIG DNA labeling kit (11175033910 Roche, Basel, Switzerland) and following manufacturer's instructions.

Corresponding cDNA regions in the dsDNA probes: L segment from nucleotide 1 to nucleotide 1508, M segment from nucleotide 1 to nucleotide 1477 including the Gn/Gc ORF and S segment from nucleotide 1 to nucleotide 1491 including the NSs ORF (Figure 2.1 A). A dsDNA DIG random labelled 18S ribosomal probe was used as loading control in high molecular gels and detected as described in (Garcia-Ruiz et al., 2015).

Endogenous miR166, miR168, miR390, miR403, siRNA02, siRNA255 and nuclear RNA U6 (Garcia-Ruiz et al., 2010; Garcia-Ruiz et al., 2015) were detected in small RNA gels using DIG labeled oligonucleotides probes. TSWV virus derived small RNAs were detected using the random labelled DIG probes used for the detection of TSWV RNA segments.

High molecular weight Northern blotting

Samples were run in a 1% agarose, 2.7% formaldehyde high molecular RNA gel at 90 V for one hour at room temperature to separate TSWV genomic segments. RNA was overnight transferred to a positively charged nylon membrane at room temperature (Roche, Basel, Switzerland) by capillarity using

a 10X SSC (1.5 M NaCl and 0.15 M Trisodium Citrate) salt gradient and paper towels. Membrane was auto-crosslinked in two positions using a UV Stratalinker® 1800 (Stratagene, La Jolla, CA, USA) to immobilize RNA to the membrane.

Pre-hybridization of blots was done using 10 mL of PerfectHyb™ Plus Hybridization Buffer (Thermo Fisher Scientific, Waltham, MA) for one hour at 38° C. For hybridization, probes for L, M, and S segments were added in equal amounts to the blots containing 10 mL of PerfectHyb™ Plus Hybridization Buffer solution and 16 hours or overnight incubation at 38° C were required. For surplus probe removal two washes at 42° C with 50 mL of high salt solution (100 mL 20X SSC, 20 mL 10% SDS and 880 mL Water) and a final wash with 50 mL low salt solution (50 mL 20X SSC, 10 mL 10% SDS and 940 mL Water) were required. To remove SDS from blots two washes in 3X SSC were done at room temperature. Special care was taken to prevent dried of the blots at any time according to manufacturer's instructions.

For DIG detection blocking of the blots for at least 30 minutes was done using 15 mL of 1X DIG blocking solution (11 175 033 910; Roche). After blocking 0.75 µl of Anti-DIG antibody were added to the blots and one hour of incubation at room temperature were needed. For DIG detection blots were equilibrated in DIG detection Buffer (0.1M Tris-HCl, 0.1M NaCl, pH 9.5) for five minutes. Chemiluminescence was detected using substrate CDP-Star (Roche, Basel,

Switzerland) and a ChemiDoc® MP Imaging system (Bio-Rad, Hercules, CA, USA).

Small RNA Northern blotting

A protocol (Llave et al., 2002) was modified to detect small RNAs, 17% PAGE urea gels (12.6 g of Urea, 1.5 mL of 10X TBE buffer, 2 mL of water and 17 mL of 30% of acrylamide:bisacrylamide 37.5: 1) were made using glass plates previously treated with RNase*Zap*® RNase Decontamination Solution (Thermo Fisher Scientific, Waltham, MA), washed with 10% sodium dodecyl sulfate (SDS) and rinsed with 70% alcohol.

Gels were equilibrated on an Owl™ P9DS Dual-Gel Vertical Electrophoresis System (Owl Separation Systems Inc. Marietta, OH, USA) for at least one hour in 0.5 X TBE running buffer. 15 µl of RNA samples normalized 1 µg/1 µl were mixed with 15 µl of RNA loading dye, denatured for five minutes at 95° C and run for three hours and fifteen minutes.

After separation of the RNA samples gels were stained with 8 µl (10 mg/µl) of ethidium bromide and a picture was taken under UV light to visualize RNA quality and separation of the bands. Transfer of the gels to a positive charged nylon membrane was done using a Bio-Rad Semi-dry transfer unit (Bio-Rad, Hercules, CA) following manufacturer's instructions. Membranes were twice auto-cross linked using a UV Stratalinker® 1800 (Stratagene, La Jolla, CA, USA) and stored in sheets of filter paper until hybridization protocols.

Membranes were cut in two parts, bottom parts for detection of small RNAs and upper parts for detection of U6 nuclear RNA that was used as a loading control. In parallel upper blots were pre-hybridized in glass cylinders with 10 mL of NorthernMax® Prehybridization/Hybridization Buffer (Thermo Fisher Scientific, Waltham, MA) at 38° C. Hybridization was done by addition of 4 µl of U6 oligo labelled DIG probe and blots were processed in parallel with the bottom parts of the membranes.

Pre-hybridization of bottom blots was done in glass cylinders and 10 mL of PerfectHyb™ Plus Hybridization Buffer (Thermo Fisher Scientific, Waltham, MA) for thirty minutes at 38° C in a Autoblot® Micro Hybridization Oven (Bellco Glass Inc, Vineland, NJ, USA). Hybridization was followed by addition of 10 µl of corresponding oligo DIG labelled probe or 15 µl of random labelled probes (a mixture of 5 µl per TSVW segment) to the blots and overnight incubation for 16 hours at 38° C. Washes to remove exceeding probes were repeated at 42° C as described in High Molecular RNA gels. Dig detection of 21-24 nucleotides small RNAs and nuclear U6 RNA were also followed as described in the High Molecular Weight RNA gels.

RESULTS

TSWV segments are detected by northern blotting from total and nucleocapsids RNA extractions.

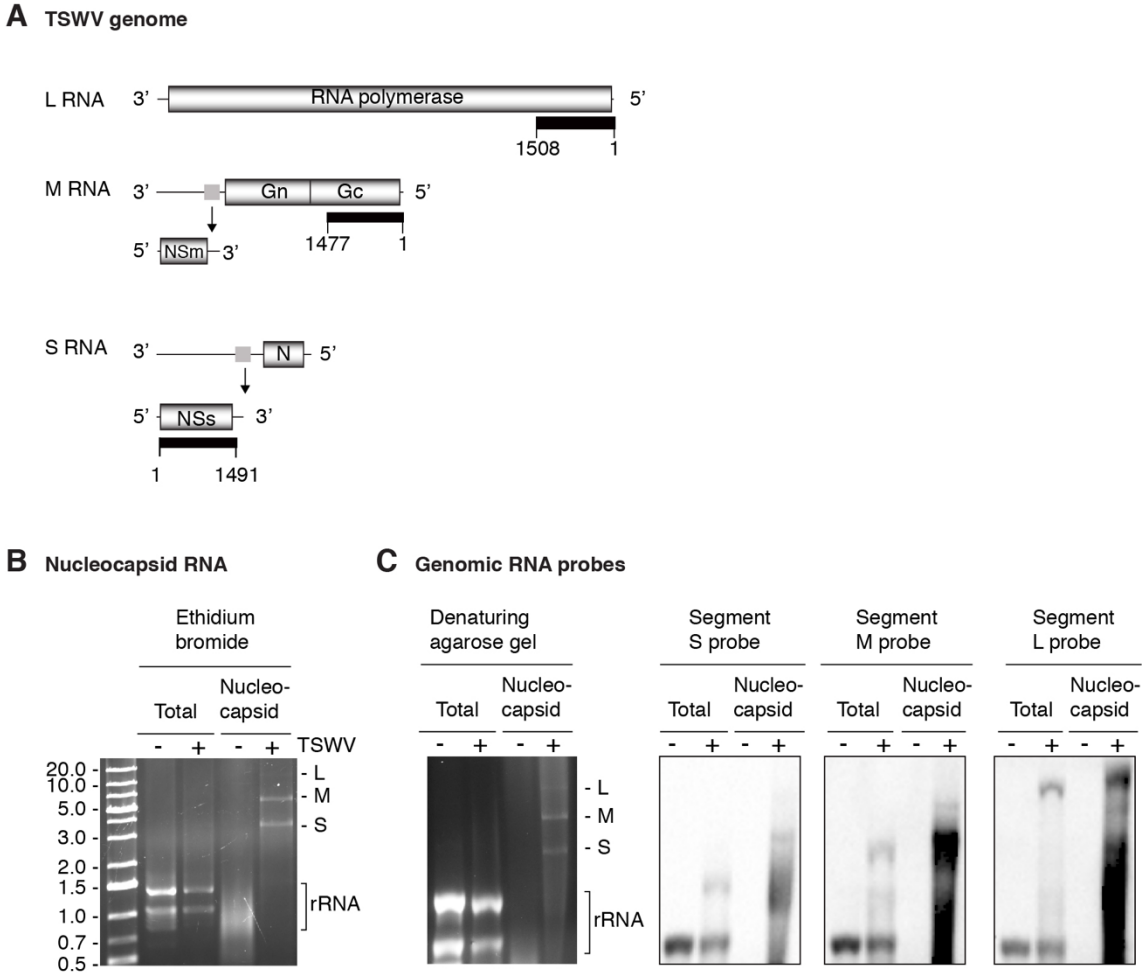

Figure 2.1 TSWV RNA detection in *N. benthamiana* using genomic RNA-specific probes. (A) L RNA (8.8 Kb), M RNA (4.8 Kb), S RNA (2.9 Kb) Black lines represent the length of the random labelled DIG probe used for detection of RNA segments. (B) Representative blot showing accumulation of TSWV genomic RNA in total RNA samples and in nucleocapsid preparations. 18S rRNA was used as a loading control. (C) TSWV genomic RNAs were detected with DIG-labelled probes made by random priming from cDNA corresponding to parts of genomic RNA L, M, or S. Triplicate gels were run and segment specific probes used in equal amounts.

Based in cDNA obtained from RT-PCR from TSWV nucleocapsid RNA extractions, regions in black represent the length of the dsDNA probe used to detect TSWV genomic RNA (Figure 2.1 A).

In a non-denaturing agarose gel stained with ethidium bromide, TSWV L, M and S segments could not be observed in samples from total RNA extractions (Figure 2.1 B). Nonetheless, TSWV segments were detected in samples where RNA extractions were made from nucleocapsids isolations. According to their size they corresponded to TSWV segments and were clearly visible in a normal ethidium bromide gel.

TSWV segments were consecutively detected by Northern blotting using dsDNA probes to target the sequences from cDNA regions in black (Figure 2.1 A). In duplicative blots detection of each segment using only one probe for each segment detected the corresponding segment in total RNAs extractions and RNA extractions from nucleocapsids (Figure 2.1 C).

In samples where RNA extractions were made from nucleocapsids multiple segments were detected using only one probe as in the case of the segment S probe that was able to detect the M segment. Segment M probe detected the S segment and the L segment. Finally segment L probe detected not only segment L but M and S (Figure 2.1 C).

TSWV induces symptoms and accumulates in *Arabidopsis thaliana* wild type and selected mutants.

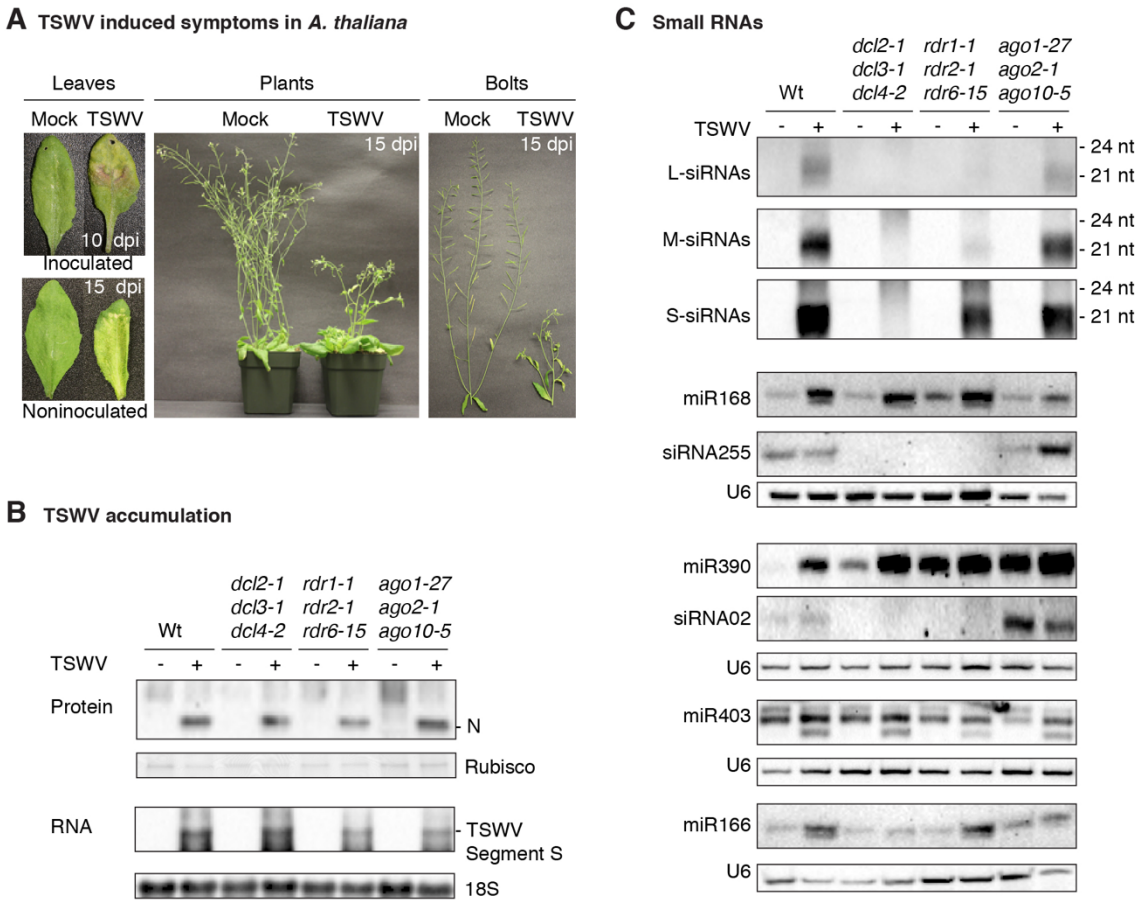

Figure 2.2 TSWV is routed through antiviral RNA silencing in *Arabidopsis thaliana*. Selected Arabidopsis mutants lacking core components of antiviral RNA silencing were mechanically inoculated with TSWV. Pictures were taken and samples were collected at 15 days post inoculation. (A) Local and systemic symptoms of TSWV infection. (B) Representative immunoblots showing accumulation of TSWV nucleocapsid protein in inflorescence. The large unit of Rubisco was used as a loading control. (C) Representative blots showing accumulation of endogenous and TSWV-derived siRNAs. U6 was used as a loading control. TSWV-derived siRNAs were detected with DIG-labeled random probes. Endogenous small RNAs were detected with DIG-labeled oligonucleotide probes.

Symptoms after mechanical inoculation of TSWV in *Arabidopsis thaliana* plants were clearly visible after 10 days post inoculation. Systemic symptoms as yellowing, reduced size of bolts and necrosis were more evident at 15 days post inoculation (Figure 2.2 A)

TSWV accumulation in selective mutant plants as result of infection was demonstrated by the presence of nucleocapsid protein (Figure 2.2 B) and the detection of TSWV S segment by Northern blotting.

Production of small RNAs derived from TSWV (Figure 2.2 C) accumulated and were detected in samples with TSWV infection and not in mock inoculated plants. Endogenous small RNAs are clearly upregulated and downregulated when TSWV is present and even production of smaller miR403 siRNAs were detected (Figure 2.2 C).

DISCUSSION

TSWV segments in total RNA extractions could not be detected in a normal ethidium bromide gels due to degradation or available quantity. TSWV segments were only visualized in a normal ethidium bromide gel if samples were from nucleocapsids isolations; TSWV genomic RNA was enriched and protected during the nucleocapsid isolation which incremented RNA quantity and quality during the RNA extraction.

Nonetheless, the use of the DIG probes and Northern blotting detected TSWV RNA segments in both total and nucleocapsid samples which may be

attributed to the sensitivity of the technique but also indicating the presence of TSWV genomic RNA segments in total RNA extractions (Figure 2.1 C).

It is worthwhile noticing that the design of the probes corresponded to conservative and repetitive zones of TSWV RNA segments (Adkins, 2000). Segments were detected based in complementary with regions matching the cDNA regions of the dsDNA probe and since this region is conserved by the three TSWV segments, probes used were able to detect multiple segments. Detection of specific segments *i.e.* genomic, antigenomic cannot be differentiated with the probes generated in this study since probes are dsDNA products; matching both genomic and antigenomic TSWV RNA segments. Development of new probes will be helpful to resolve differences between genomic, antigenomic segments and even subgenomic RNAs.

TSWV infection in *Arabidopsis thaliana* induced typical symptoms of tomato spotted wilt disease such as yellowing, necrosis and reduced sized of bolts and flowers which were a sign of positive infection. TSWV is not transovarially transmitted (Oliver and Whitfield, 2016) and collection of adults harboring wild type TSWV or other *Tospoviruses* may include additional variation to the system. The use of offspring as vectors fed on green beans before they acquired the TSWV isolate was an important step to prevent variation by introduction of exogenous viruses.

A positive infection in selective mutant plants lacking core components of the RNA silencing pathway was required to determine the differences in small

RNA biogenesis of endogenous and virus derived upon TSWV infection and was corroborated by the accumulation of the nucleocapsid protein.

Small RNA analysis showed TSWV segments are differently processed in the RNA silencing pathway. Small RNAs derived from the L segment are less abundant than small RNAs from the M and S segments in the WT plant. Small RNAs from the S segment are the most abundant suggesting that this segment is transcribed at higher levels in order to trigger RNA silencing.

Small RNAs derived from any TSWV segment were not detected in the *dcl* triple mutant suggesting TSWV is processed by DCL2, DCL3 or DCL4 or a combination. Production of small RNAs from the S segment are also detected in the *rdr1-1, rdr2-1* and *rdr6-15* triple mutant which is interesting since RDR1, RDR2 and RDR3 produce and amplify secondary siRNA; these siRNAs could have been originated from another RDR protein or derived from the virus after recognition and processing by Dicer proteins. No clear role of the Argonaute proteins was found in the initial small RNA analysis after TSWV infection.

TSWV infection affected the biogenesis of several endogenous *Arabidopsis* miRNAs and tasiRNAs. Upregulation of miR168 involved in the negative feedback regulation of AGO1(Bologna and Voinnet, 2014) accumulates after TSWV infection. Production of smaller and bigger miR403 which regulate AGO2 (Bologna and Voinnet, 2014) are also found after TSWV infection, the role of this variants is not clear. miR390 involved in the targeting of TAS3 transcripts and guided by AGO7 (Cuperus et al., 2010) is also upregulated after TSWV

infection but this response may be triggered as a defense response from the plant in the form of auxin hormone responses (Baldrich and San Segundo, 2016).

small RNAs from tasiR255 and siRNA02 used as genetic controls did not accumulate in the mutants lacking core components of the RNA silencing pathway except in the *ago* triple mutant plants due to the involvement of AGO proteins other than AGO1, AGO2 and AGO10 in their processing and biogenesis (Garcia-Ruiz et al., 2010).

These results establish a foundation to investigate the mechanisms of induction and suppression of antiviral RNA silencing by *Tospoviruses*, using *Arabidopsis* and TSWV as model systems.

CHAPTER 3:

GENETIC ANALYSIS OF RNA SILENCING AGAINST TOMATO SPOTTED WILT VIRUS

INTRODUCTION

Arabidopsis thaliana encodes 4 Dicer proteins DCL1 to DCL4. DCL1 is the primary synthesizer of miRNAs, whereas DCL2, DCL3 and DCL4 process long double stranded RNA structures from different cellular origins into siRNAs measuring 22, 24 and 21 nucleotides in length respectively. (Bologna & Voinnet, 2014; Ding & Voinnet, 2007). The *Arabidopsis* genome encodes 10 AGOs with unique properties and specific interactions with small RNAs (Alvarado & Scholthof, 2009b). RNA Dependent RNA Polymerases (RDRs) are defined by conserved domains used for copying single stranded RNA and *Arabidopsis* encodes 6 RDR genes. RDR1, RDR2 and RDR6 share similarities in the C-terminal catalytic DLDGS motif. RDR3a, RDR3b and RDR3c share an atypical DFDGD motif and a their role has not yet been established (Bologna & Voinnet, 2014). Effector complexes RISC are assembled after loading one small RNA strand into one AGO protein. Viral replicates of RNA viruses often assemble dsRNA structures that can be recognized by DCL proteins and be processed in the natural occurring RNA silencing pathway (Ding & Voinnet, 2007).

The main objectives of these experiments are to analyze what are the effects of core components of the RNA silencing pathway in *Arabidopsis thaliana* when mutant plants lacking specific core components are challenged with TSWV infection and to determine how TSWV is processed in the same pathway.

MATERIALS AND METHODS

Arabidopsis genotypes

Mutant *Arabidopsis thaliana* plants were used as follows: *Arabidopsis col-0* wild type ecotype*, dcl1*, *dcl2, dcl3* and *dcl4* single mutants, *dcl2 dcl3, dcl-2 dcl4, dcl3 dcl4,* double mutants and *dcl2 dcl3 dcl4* triple mutants (Xie et al., 2004; Xie et al., 2005). Arabidopsis, *rdr1-1, rdr2-1, rdr-3a, rdr-3b, rdr-3c, rdr6-15* single mutants (Allen et al., 2005; Xie et al., 2005). Transgenic plants expressing catalytic mutant 3xHA-tagged-ago1-dah (*Col-0*), 3xHA-ago2-dad (*ago2-1*) and 3xHA-ago10-dah (*ago10-4*) (Garcia-Ruiz et al., 2015). Transgenic plants expressing GUS (*Col-0*) used as controls described in (Carbonell et al., 2012; Garcia-Ruiz et al., 2015).

Mechanical inoculation of *Arabidopsis* plants.

At 21 days after emergence *Arabidopsis* plants were mechanically inoculated with TSWV, as described in chapter II. Leaves were dusted with carborundum and rub-inoculated with 10 µL of inoculum per leaf. TSWV inoculum was prepared by grinding systemically infected *N. benthamiana* at 15 dpi in TSWV inoculation buffer. For each plant 4 leaves were inoculated and 18 to 24 *Arabidopsis* genotype plants in total. In parallel, equal number of *col-0* wild type plants were mock-inoculated with only TSWV inoculation buffer. Plants were grown at 22° C and long day (16 h light and 8h dark) in growth chambers. After 15 days post inoculation 10 clusters per systemically infected plant showing

TSWV infection symptoms were collected using 2 mL epitubes, frozen in liquid nitrogen and stored at -80° C degrees until protein and RNA extractions.

DNA plasmids

pCB302 plasmids expressing HA tagged Argonaute proteins **HA-AGO1, HA-AGO2, HA-AGO4, HA-AGO5, HA-AGO7 and HA-AGO10** and harboring the kanamycin and rifampicin resistance genes previously described (Carbonell et al., 2012; Garcia-Ruiz et al., 2015) were used during transient assays. **pMDC32-NSs-6HIS-3xFlag**. TSWV NSs protein was tagged at the C terminus with 6HIS and 3xFlag and expressed from the 35S promoter in pMDC32 vector. This clone was made using the NSs clone described in (Takeda et al., 2002). Using oligos NSs-ORF-F-947(caccATGTCTTCAAGTGTTTATGAGTC) where cacc TOPO® recognition site was included and NSs-HA-R 948 (CTAAGCGTAATCTGGAACATCGTATGGG) to produce pENTR-NSs-HA using TOPO® directional cloning kit (Invitrogen, Carlsbad, CA, USA). This plasmid was used as template for addition of 6HIS and 3xFlag epitope by PCR mutagenesis with oligos NSs-ORF-F-947 (caccATGTCTTCAAGTGTTTATGAGTC) and 6xHIS3xFLAG-NSs-714 (ggcggccgctctagaTCACTTGTCATCGTCATCCTTGTAGTCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCGTGATGGTGATGGTGATGTTTTGATCCTGAAGC) resulting in pENTR-NSs-6xHIS3xFLAG this plasmid was moved into pMDC32 by LR recombination using Gateway™ LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, Waltham, MA).

Agro-infiltrations

Glycerol stocks of *Agrobacterium tumefaciens* strain GV3101 containing **HA-AGOs** pCB302 plasmids were activated in plates containing solid Lysogenic broth (LB) with Rifampicin (0.1 mg/mL) and Kanamycin (0.1 mg/mL). After three days single colonies were used for initial 1 mL cultures.

100 ng of **pMDC32-NSs-6HIS-3xFlag** DNA plasmid were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, four single colonies were tested to determine silencing suppression activity (Chapter IV Transient RNA silencing suppression assays). Glycerol stocks were prepared using colonies with strong inhibition of GFP silencing and used for co-expression with AGOs proteins.

1.5 ml epitubes with 1 mL of liquid LB + Rifampicin (0.1 mg/mL) and Kanamycin (0.1 mg/mL) where used for initial activation of *Agrobacterium tumefaciens* stored in glycerol stocks for 32 hours at 28° C with shaking in dark conditions. Induction of *vir* genes was made by diluting *Agrobacterium* cells to optical density (OD_{600}) of 0.002 on LB *vir* induction solution (10 mM MES pH 5.2, 100 µM Acetosyringone 0.1 mg/mL Rifampicin and 0.1 mg/mL Kanamycin) and incubated for 16 hours at 28° C with shaking. Cell cultures where centrifuged for 10 minutes at 4° C and VIR induction media was discarded. Cells were re-suspended in equal volumes of infiltration solution (10 mM MgCl2, 10 mM MES, 150 µM of acetosyringone) and diluted to a final infiltration OD_{600} of 0.5 and kept in the dark for two hours. Corresponding AGOs + NSs treatments were made by

mixing diluted constructs to a final concentration of 0.5. *N. benthamiana* plants with six to eight leaves where used for infiltration and two upper leaves were used. After infiltration plants were kept at 25° C for 2 days and 0.15 g of infiltrated leaves were collected for protein extraction.

Western blotting

Protein extraction was done using protocol described in chapter II. 0.15 g of *N. benthamiana* leaf samples were collected at 2 days post infiltration (dpi). For gel electrophoresis 10 µl of total undiluted protein sample were separated using Mini-PROTEAN® TGX Stain-Free™ gels at 150 V for 60 minutes at room temperature and transferred onto nitrocellulose membranes.

Nucleocapsid protein was detected as described in chapter II. NSs protein was detected using 2 µl of Anti-Flag antibody (Sigma-Aldrich, St. Louis, MO). HA-AGOs (Carbonell et al., 2012; Garcia-Ruiz et al., 2015) were detected using 10 µl anti-HA antibody with peroxidase (Roche, Basel, Switzerland). Luminescence was measured with Clarity Western ECL Substrate and a ChemiDoc® MP Imaging system (Bio-Rad, Hercules, CA, USA).

RNA extraction

Total RNA used in small RNA analysis was extracted using TRIzol® and concentration was measured with a NanoDrop® as described in chapter II. Samples were normalized to 1 µg/µl and 1 µg of RNA was run on a 1% non-denaturing agarose gel to visualize ribosomal RNA quality.

Northern blotting for analysis of small RNAs.

Urea gels as described in chapter II were made to analyze small RNAs derived from the virus and cellular miRNAs derived from the plants. 15 µl of normalized to 1 µg/µl or 15 µg of total RNA were run in an Owl™ P9DS Dual-Gel Vertical Electrophoresis System. Transfer of gels to a positive charged nylon membrane was done using a Bio-Rad Semi-dry transfer unit. Blots were twice auto cross-linked using a UV Stratalinker® 1800. Blots were cut in half for small RNA analysis and detection of U6 nuclear RNA as a loading control. Blots were pre-hybridized and hybridized in glass cylinders in a Autoblot® Micro Hybridization Oven. DIG detection was made following protocol described in chapter II.

DIG-labelled oligos and cDNA probes

Random labelled DIG probes as described in chapter II were used for the detection of small RNAs derived from TSWV. 5 µl of dsDNA probe per segment were added in equal amounts (15 µl in total) for complete detection of small RNAs derived from the three segments.

Oligo labelled probes were made using kit 11 175 033 910 (Roche, Basel, Switzerland) following manufacturer's instructions for the detection of cellular miRNAs. In the *dcl* northerns: 10 µl of oligo DIG probes were used to detect miR390, miR255 used as genetic controls. In the *rdr* Northerns 10 µl of oligo DIG probes were used to detect miRNA390, miRNA255, and miRNA02 used as genetic controls. In the transgenic HA-AGOs northerns 10 µl of oligo DIG probes

were used to detect miR390, miR168 and miR403 used as genetic controls. 10 µl of oligo DIG labelled probe to detect U6 nuclear RNA used as loading control as described in (Garcia-Ruiz et al., 2010).

Blots were stripped to remove random labelled probes and re-hybridized with different oligo DIG labelled probe to measure cellular miRNAs using a stripping solution of (50% formamide, 0.1X SSC and 1% SDS). 50 mL of stripping solution were added to the blots in a hybridization glass cylinder and incubated to 80° C in a Autoblot® Micro Hybridization Oven for 2 hours. Stripping solution was discarded and new fresh 50 mL of stripping solution was added and incubated at 80° C overnight (16 hours). Two more washes steps were repeated two more times using the stripping solution. Two additional washes in 3X SSC were done to remove SDS and membranes were ready to be hybridized or stored in 3X SSC at 4° C.

RESULTS

TWSV-derived siRNAs are made by DCL4 and DCL2

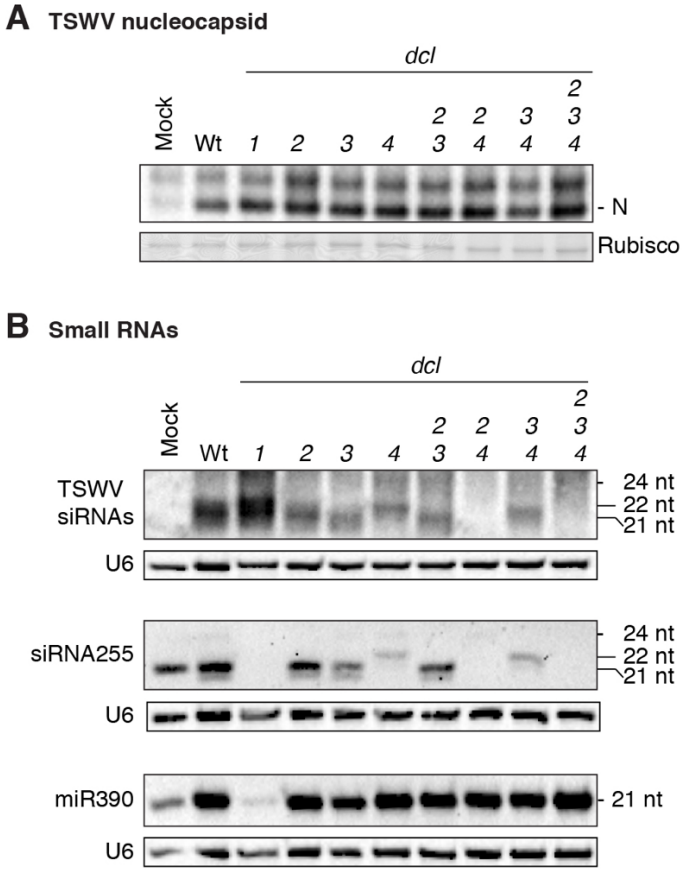

Figure 3.1 Accumulation of TSWV-derived siRNAs in Arabidopsis *dcl* mutants. Inflorescence samples were collected at 15 days post inoculation for total protein and RNA extraction. *dcl2-1 dcl3-1 dcl4-2* triple mutant plants were used as controls. TWSV N protein, genomic RNA and small RNAs were detected as described in chapter II. (A) TSWV nucleocapsid protein (N) accumulation. (B) TSWV genomic small RNA accumulation in selected *dcl* mutant plants and selective plant small RNA accumulation used genetic controls.

Detection of TSWV nucleocapsid protein (Figure 3.1 A) was an indicative of TSWV positive infection. Samples containing Nucleocapsid protein were used for Northern analysis (Figure 3.1 B).

Production of bigger 22 nucleotides small RNAs where detected in the *dcl4* single mutant (Figure 3.1 B). A complete reduction of small RNAs derived from TSWV were observed in the double mutant *dcl2* and *dcl4.* Dicer triple mutant was used as control and no production of small RNAs were observed.

siRNA255 did not accumulate in the *dcl1* single and *dcl2 dcl4* double mutant. When dcl4 was absent production of 22 nucleotides was observed suggesting this population was originated from 22 nucleotides producer *dcl2;* corroborating mutant plants were DCL mutants. Accumulation of miRNA390 was reduced in the *dcl1* mutant and an increment was observed after TSWV infection.

Amplification of antiviral silencing against TWSV is mediated by RDR1

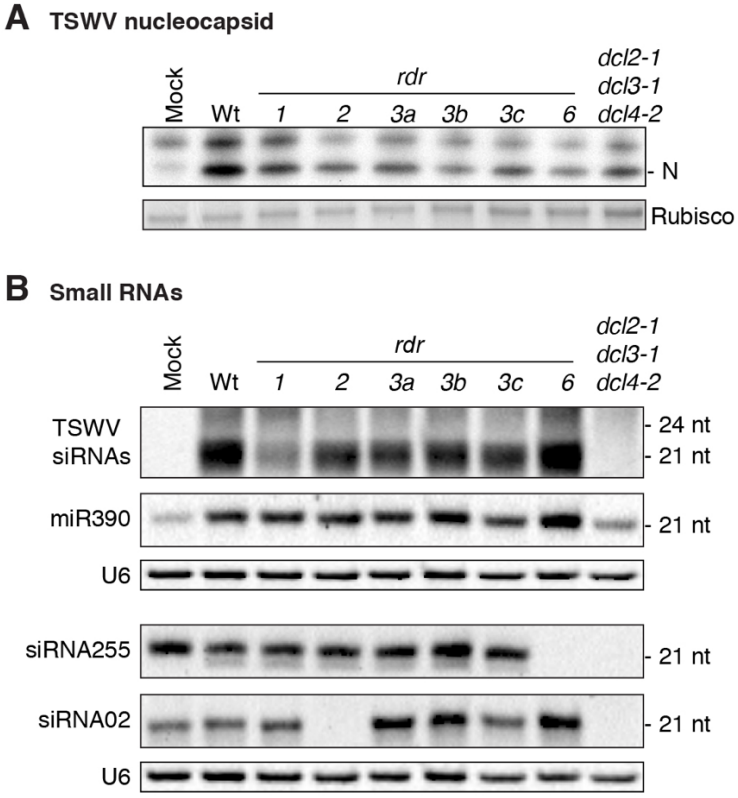

Figure 3.2 Accumulation of TSWV-derived siRNAs in single *rdr Arabidopsis* mutants. Inflorescence samples were collected at 15 days post inoculation for total protein and RNA extraction. *dcl2-1 dcl3-1 dcl4-2* triple mutant plants were used as controls. (A) Representative immunoblots showing accumulation of TSWV nucleocapsid protein (N) in inflorescence. The large unit of Rubisco was used as a loading control. (B) Representative blots showing accumulation of TSWV genomic RNA in inflorescence. Representative blots showing accumulation of TSWV-derived siRNAs and selected miRNAs. U6 was used as a loading control.

TSWV nucleocapsid protein accumulated and was detected in single mutant RDRs plants as indicative of TSWV infection (Figure 3.2 A).

A reduction in the levels of small RNAs derived from TSWV were only observed in the *rdr1* single mutant (Figure 3.2 B). Dicer triple mutant was used as control and no small RNAs derived from TSWV were detected.

siRNA225 and siRNA02 were used as genetic controls, siRNA225 did not accumulate in the *rdr6* and *dcl2-3-4* mutant. siRNA02 did not accumulate in the *rdr2* and *dcl2-3-4* mutant.

TSWV infection does not trigger degradation of AGO proteins

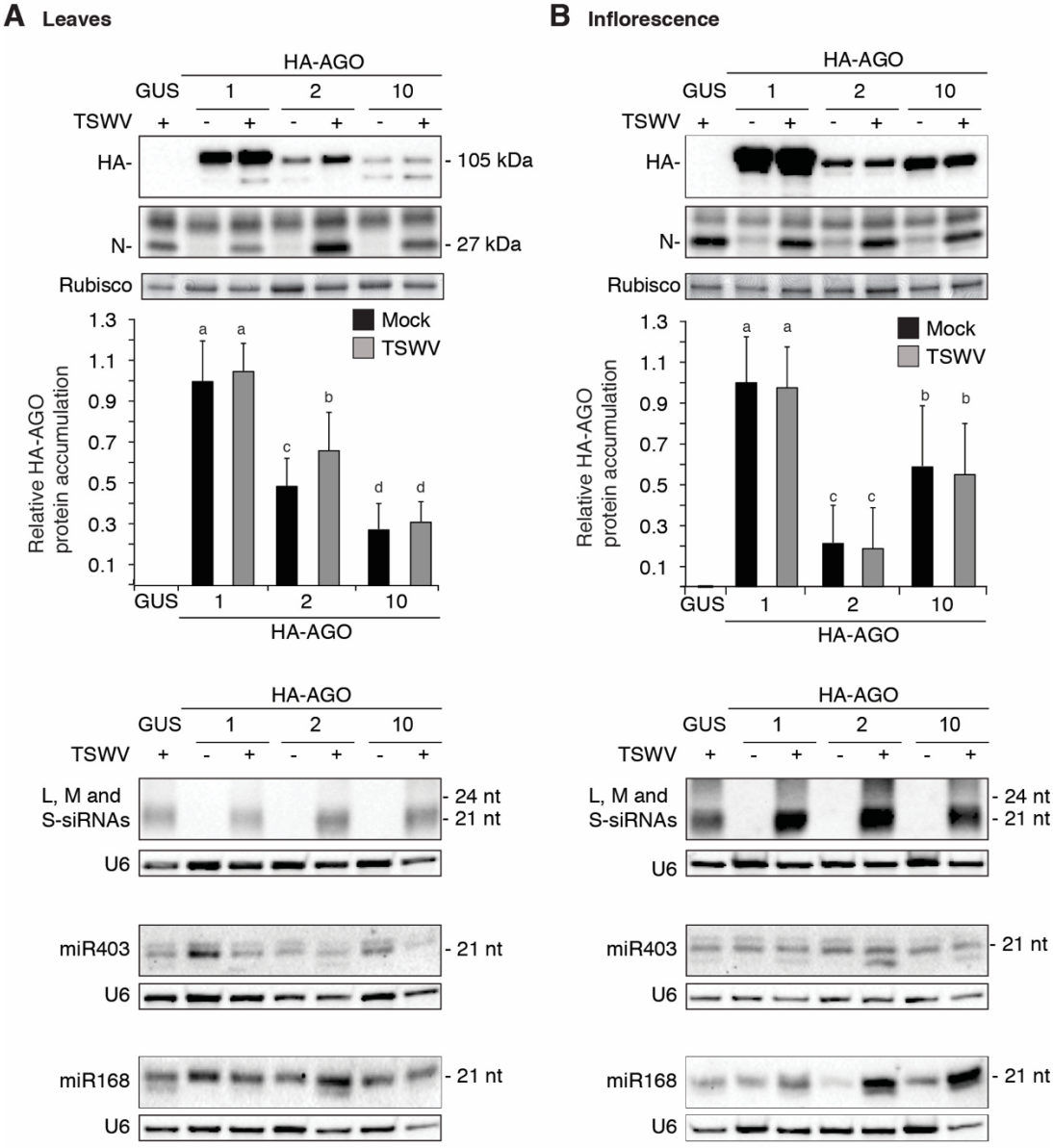

Figure 3.3 Accumulation of HA-AGO1, HA-AGO2 and HA-AGO10 in leaves and inflorescence of mock inoculated and TSWV-infected plants. (A) Representative immunoblots showing accumulation of HA-AGO1, HA-AGO2 and HA-AGO10 and TSWV nucleocapsid protein (N) in leaves. The large unit of Rubisco was used as a loading control. Representative blots showing accumulation of TSWV-derived siRNAs and selected miRNAs. U6 was used as a loading control. (B) Representative immunoblots showing accumulation of HA-AGO1, HA-AGO2 and HA-AGO10 and TSWV nucleocapsid protein (N) in inflorescence. The large unit of Rubisco was used as a loading control. Representative blots showing

accumulation of TSWV-derived siRNAs and selected miRNAs. U6 was used as a loading control.

Western blots showed HA-AGOs are not degraded after TSWV infection in samples from leaves infected with TSWV compared to mock inoculated leaves (Figure 3.3 A). The same pattern was also found in samples from inflorescences where no degradation of HA-AGO occurred compared to mock inoculated samples after TSVW infection (Figure 3.3 B).

Northerns detected siRNAs derived from TSWV in both leaves and inflorescences samples (Figure 3.3 A B). A reduction in the production of siRNAs derived from TSWV in leaves compared to inflorescences was found (Figure 3.3 A, B, L, M, S siRNAs gels) siRNAs derived from TSWV were also indicators of TSWV infection.

Northerns to detect micro RNAs involved in the self-regulation of AGOs proteins showed miRNA403-AGO1 accumulates in leaves at similar levels in both TSWV and MOCK inoculated transgenic HA-AGO plants (Figure 3.3 A). In inflorescences of transgenic HA-AGO plants miRNA403 accumulates again at similar levels (Figure 3.3 B).

miRNA168-AGO2 in leaves of transgenic HA-AGO plants (Figure 3.3 A) was found at similar levels in both TSWV and mock inoculated samples however, an increment in miRNA168 was found in inflorescence samples of transgenic HA-AGO2, HA-AGO10 plants (Figure 3.3 B) after TSWV infection.

TSWV NSs does not degrade HA-AGOs proteins during transient assays

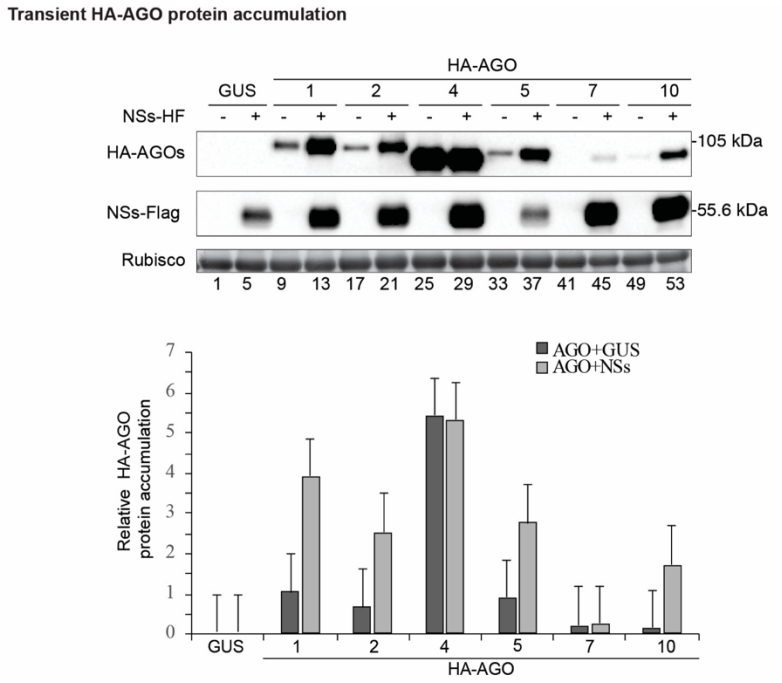

Figure 3.4 Transient accumulation of selected HA-tagged AGO proteins in the presence of NSs-HF. Two leaves of *Nicotiana benthamiana* plants were agro-infiltrated with *Agrobacterium tumefaciens* harboring selective HA-AGOs and NSs-HF alone or in combination at final infiltration 0.5 OD. Samples were collected from infiltrated leaves for total protein extraction at 2 dpi. Accumulation of HA-AGOs was measured relative to HA-AGO1.

During transient assays of NSs-6HIS-3xFlag in combination with HA-AGO1, HA-AGO2, HA-AGO4, HA-AGO5, HA-AGO7 and HA-AGO10, accumulation of HA-AGO proteins in the presence NSs-6HIS-3xFlag occurred and degradation of HA-AGOs proteins was not observed.

DISCUSSION

The effects of TSWV in *dcl* mutant plants show TSWV is recognized and processed by the generation of small RNAs in the WT *Arabidopsis* plant. The fact

that TSWV RNA segments form panhandle structures (Adkins, 2000) could represent a source of dsRNA as potential inducer of RNA silencing or replication intermediates reassembling dsRNA structures.

When DCL4 is not present DCL2 replace its functions and generation of bigger 22 nucleotides small RNAs compared to 21 nucleotides in length are produced (Bologna and Voinnet, 2014). This corresponded to what was observed and also it was corroborated when DCL2 and DCL4 were not present demonstrating their role in the recognition and processing of TSWV in the RNA silencing pathway. It seems TSWV can be recognized by the other DCLs at some extent but when absent these DCL do not have important roles, however, in absence of DCL2 and DCL4 they may replace their roles. TSWV in processed into virus-derived siRNAs by a mechanism that is dependent on DCL2 and/or DCL4. As expected siRNA255, did not accumulate in *dcl1* and *dcl4* mutant plants due to requirements of these proteins during the biogenesis of tasiRNAs(Garcia-Ruiz et al., 2010) and miR390 did not accumulate in *dcl1* mutant plants meaning that plants used for these experiments were in effect *dcl* mutants.

The role of RDRs in the amplification and generation of secondary small RNAs used in the RNA silencing pathway have been attributed to RDR1 and RDR6 (Garcia-Ruiz et al., 2010). Here we found amplification of TSWV-derived small RNAs occurred by RDR1 by comparing levels of TSWV-derived small RNAs in the *rdr1* mutant to the WT plant (Figure 3.2 A). No other significant result was attributed to other RDRs suggesting they may not be involved in the

secondary amplification of TSWV. Antiviral RNA silencing against TSWV was amplified by a mechanism that is dependent on RDR1.

In the AGO analysis after comparing the levels of HA-AGO protein in mock and TSWV infected transgenic plants no statistic difference was found, suggesting HA-AGO protein degradation is not occurring.

After Northern blots to measure cellular miRNAs used in the self-regulation of AGO1 and AGO2 some interesting results were observed in the TSWV infected samples. Accumulation of miRNA168 and miRNA403 involved in the negative regulation of AGO1 and AGO2 respectively (Bologna and Voinnet, 2014) hypothesize degradation of HA-AGO1 and HA-AGO2 but, these results do not correspond to HA-AGO protein levels found in the western blots (Figure 3.4). This finding also suggests TSWV may trigger negative regulation of AGOs by production of cellular miRNAs but at the same time NSs silencing suppressor may be sequestering total miRNAs (Schnettler et al., 2010) and as a result degradation is not occurring.

Antiviral RNA silencing against viruses is mediated by AGO1, AGO2, and/or AGO10 (Carbonell et al., 2012), the roles of suppressors and interactions with these core components of the RNA silencing pathway could be occurring at different steps or by not previously described mechanisms. This genetic analysis against TSWV is the foundation to understand how a negative RNA virus gets processed in the RNA silencing pathway.

CHAPTER 4

CHIMERIC TuMV EXPRESSING TSWV NSs SILENCING SUPPRESSOR

INTRODUCTION

In previous experiments the ability of TSWV NSs silencing suppressor to inhibit GFP silencing as the rescue of pathogenicity of two suppressor deficient viruses during transient assays have been previously described (Ocampo Ocampo et al., 2016). Co-expression of different proteins during transient assays is a potent tool to detect suppressors of RNA silencing using GFP as sensor. However, *Agrobacterium tumefaciens'* transgene expression may be silenced during normal plant defenses and mask the effects of the suppressors or proteins involved. One approach to reduce additional transgene silencing is to test the activity of suppressors expressed in infectious clones once its activity as suppressor of RNA silencing has been demonstrated. Infectious clones are useful tools to measure activity of viruses in plants using the *Agrobacterium* system. DNA plasmids harboring infectious clones can be modified through deletions, mutagenesis and even introduction of different proteins such as GFP sensors.

TuMV-GFP-AS9 (Garcia-Ruiz et al., 2010) chimeric infectious clone harboring an AS9 mutation is not able to infect both WT, *N. benthamiana* and *A. thaliana.* Introduction of TSWV NSs silencing suppressor will be useful to understand the mechanistic role of this protein in the rescue of pathogenicity of this mutant virus. The objectives of this study are to generate a chimeric virus harboring NSs suppressor and to test the pathogenicity compared to the WT virus.

MATERIALS AND METHODS

DNA plasmids

For inactivating the NSs protein point mutations involved the substitution of conserved amino acids by alanine and are described as the original amino acid, position number and followed by the alanine substitution.

pMDC32-NSs-S48A-R51A-6HIS3xFlag Inactivating mutations S48A and R51A in RNA binding motif 1 (de Ronde et al., 2013) were introduced by site directed mutagenesis by rolling circle amplification using oligos NSs_R_975 (ATACAGCTGGGTTTGAACTAGTGGAGAACC) and NSs_S48A_R51A_794 (gCaGAtTCAgctAGCAAAAGTAGCTTTGGC) using pENTR-NSs-6HIS-3xFlag as template, resulting plasmid pENTR-NSs-S48A-R51A-6HIS3xFlag was moved into pMDC32 by LR recombination. The pMDC32 vector harbors the left and right border for *Agrobacterium* transformation.

pMDC32-NSs-K182A-L413A-6HIS3xFlag. This construct harbors both the NSs-1 and NSs-2 inactivating mutations in the essential GKT motif (Zhai et al., 2014). The K182A mutation was introduced by PCR site directed mutagenesis using oligo NSs-1-R_977 (GCCTAAAGCTTGATTGTAGCACATCTCG) and NSs-1-F_976 (gctGTGAATGTTCTATCCCCTAACAG) and pENTR-NSs-6HIS-3xFlag plasmid as template, generating pENTR-NSs-K182A-6HIS-3xFlag. The L413A mutation was introduced by PCR site directed mutagenesis using oligos NSs-2-R_979 (GTAAGACATAGTTTGTGTGTTAGATGG) and NSs-2-F_978 (gctGACAGCATCCAAATCCC) using pENTR-NSs-K182A-6HIS-3xFlag as

template, generating pENTR-NSs-K182A-L413A-6HIS-3xFlag. The NSs-K182A-L413A-6HIS-3xFlag was moved into pMDC32 by LR recombination, generating pMDC32-NSs-K182A-L413A-6HIS3xFlag.

pPZP_ssGFP This plasmid was donated by Dr. Satyanarayana Tatineni and harbors a single stranded ORF of the soluble Green Fluorescent Protein from jellyfish *Aequorea victoria* expressed under the 35S promoter and between the left and right border. This plasmid also harbors the Spectinomycin and Rifampicin resistance gene (Qu et al., 2003)

pMDC32-p19 This clone was made by amplifying the ORF from the previously described pCB302-HA-p19 (Chapman et al., 2004) introduced in the cloning vector pENTR and moved by LR recombination to the pMDC32 vector (Gateway system).

pCB302-TuMV-GFP-AS9-NSs-HF Vector insert digestion protocol was followed using plasmid pCB302-TuMV-GFP-HGR as vector and insert from a cloning intermediate pENTR_NIb_NSsHF_CP(TuMV) harboring a partial Nib and CP region using restriction sites MluI and PvuI. pENTR_Nib-CP(TuMV) cloning intermediate was used to introduce the NSs ORF by stitching PCR. Using fragments amplified from three different plasmids: fragment A from plasmid pENTR_Nib-CP(TuMV) using oligos TuMV_Nib_ENTR_780 (caccGCGATGATTGAGTCGTGGGG) and Nib-NSs_R_1069 (tgCctggtgataaacacaagcctcagc), fragment B from plasmid pENTR_NSs-6HIS3XFlag (AT) using oligos Nib-NSs_F_1070

(TCTTCAAGTGTTTATGAGTCGATCATTCAGAC) and Flag-CP_R_1071 (CTTGTCATCGTCATCCTTGTAGTCG) and fragment C from plasmid pENTR_Nib-CP(TuMV) using oligos Flag-CP_F_1072 (gcaggtgaaacgcttgatgcagg) and pCB302_Ter_Rev_719 (ATCGCAAGACCGGCAACAGG) were used in a stitching PCR reaction to produce a fragment harboring a partial NIb fragment, NSs introduced with the addition of a NIb cleavage site CTGGTGATAGACACA at the N terminus and a partial CP, both partial NIb and CP harbor the MluI and PvuI site respectively. The full-length fragment was introduced into pENTR® cloning vector by TOPO® Cloning to produce pENTR_NIb_NSsHF_CP(TuMV) used as source of insert after digestion with 10 U of MluI and PvuI enzymes (New England Biolabs, Ipswich, MA) and ligated in the pCB302-TuMV-GFP-AS9 vector to produce final pCB302-TuMV-GFP-AS9-NSs-HF.

pCB302-TuMV-GFP-AS9-NSs-HF-N48A_R51A This plasmid was made in parallel and following the protocol used for the plasmid pCB302-TuMV-GFP-AS9-NSs-HF by replacing the source of insert within the MluI and PvuI site with plasmid pENTR_NIb_NSsHF_S48AR51A_CP(TuMV). To make this last plasmid fragment D was obtained from plasmid pENTR-NSs-6HIS3xFLAG_S48A_R51A(AT) with oligos Nib-NSs_F_1070 (TCTTCAAGTGTTTATGAGTCGATCATTCAGAC) and Flag-CP_R_1071 (CTTGTCATCGTCATCCTTGTAGTCG). Fragment B was replaced by fragment D in the stitching protocols to make pCB302-TuMV-GFP-AS9-NSs-HF-

N48A_R51. This plasmid harbors inactivating point mutations in the NSs ORF and was digested with 10 units of enzyme PvuI and MluI.

pCB302-TuMV-GFP-NSs-HF and pCB302-TuMV-GFP-NSs-HF-N48A_R51A

These plasmids were made using the same protocols described while making pCB302-TuMV-GFP-AS9-NSs-HF and pCB302-TuMV-GFP-AS9-NSs-HF-N48A_R51A. The only difference was the vector; instead of using pCB302-TuMV-GFP-AS9 with the AS9 mutation the TuMV-GFP WT plasmid whose HC-Pro does not harbor the AS9 mutation was used as vector.

Agro-infiltrations and transient RNA silencing suppression assays

Silencing suppression activity of TSWV NSs clones was determined using an established protocol (Johansen and Carrington, 2001). *Agrobacterium* strain GV3101 containing pMDC32 or pCB302 plasmids after electroporation were growth in 1 mL of Lysogenic broth (LB) containing Rifampicin (0.1 mg/mL) and Kanamycin (0.1 mg/mL) or Spectinomycin (0.1 mg/mL) and Rifampicin (0.1 mg/mL) for pPZP plasmids for 24 hours at 28° C with shaking for initial activation.

1 mL cultures were diluted to an optical density (OD_{600}) of 0.002 and growth in 10 mL of VIR infiltration solution (10 mM MES pH 5.2, 100 µM acetosyringone and corresponding antibiotic) for 16 hours at 28° C.

Cell cultures were centrifuged at 6000 rpm for 10 min at 4 C and re-suspended in infiltration solution (10 mM MgCl2, 10 mM MES, 150 µM of Acetosyringone) and kept in the dark for 2 hours until infiltrations. Single stranded GFP was diluted and infiltrated to a final OD_{600} of 0.125, NSs clones to

a final OD_{600} of 0.5, TuMV constructs to a final OD_{600} of 0.05. GUS and p19 (Chapman 2005) were used as negative and positive controls, respectively and infiltrated to a final OD_{600} of 0.5. in 15 days old *N. benthamiana* plants with 5 to 6 leaves; two leaves were infiltrated by treatment. NSs infiltrated leaves were monitored for GFP expression, and pictures taken under UV light at 3 days post inoculation.

For TuMV constructs additional infiltration of 15 days old *A. thaliana* plants Col-0 WT and Dicer triple mutant plants were used to measure activity of different constructs. GFP of TuMV constructs was monitored at 5, 7, 10, 15 days post infiltration (dpi) and pictures taken at 7, 10 and 15 dpi.

Western blotting

Protein extraction was done using protocol described in chapter II. 0.15 g of *N. benthamiana* leaf samples were collected at 3 dpi for NSs clones, for TuMV constructs systemic infected leaves or 10 clusters of *Arabidopsis* samples were collected at 15 dpi.

For gel electrophoresis 10 µl of total undiluted protein sample were separated using Mini-PROTEAN® TGX Stain-Free™ gels at 150 V for 60 minutes and transferred onto nitrocellulose membranes.

GFP was detected using 5 µl of anti GFP antibody (Merck Millipore, Darmstadt, Germany). NSs protein was detected using 2 µl of Anti-Flag antibody (Sigma-Aldrich, St. Louis, MO). p19 protein (Chapman et al., 2004) was detected using 10 µl anti-HA antibody with peroxidase (Roche, Basel, Switzerland).

HSP70 used as loading control was detected using 3 µl of Primary Anti-HSP70 (Merck Millipore, Darmstadt, Germany) and 2 µl of secondary antibody from goat anti-rabbit immunoglobulin G used to detect the first antibody (1: 10,000; NA934-1; GE Healthcare, Little Chalfont, UK). TuMV CP was detected using 4 µl of Anti-CP from rabbit and 2 µl of secondary antibody from goat NA934-1. Luminescence was measured with Clarity Western ECL Substrate and a ChemiDoc® MP Imaging system (Bio-Rad, Hercules, CA, USA).

RESULTS

NSs inhibits GFP silencing at 3 dpi and point mutations inactivate this protein

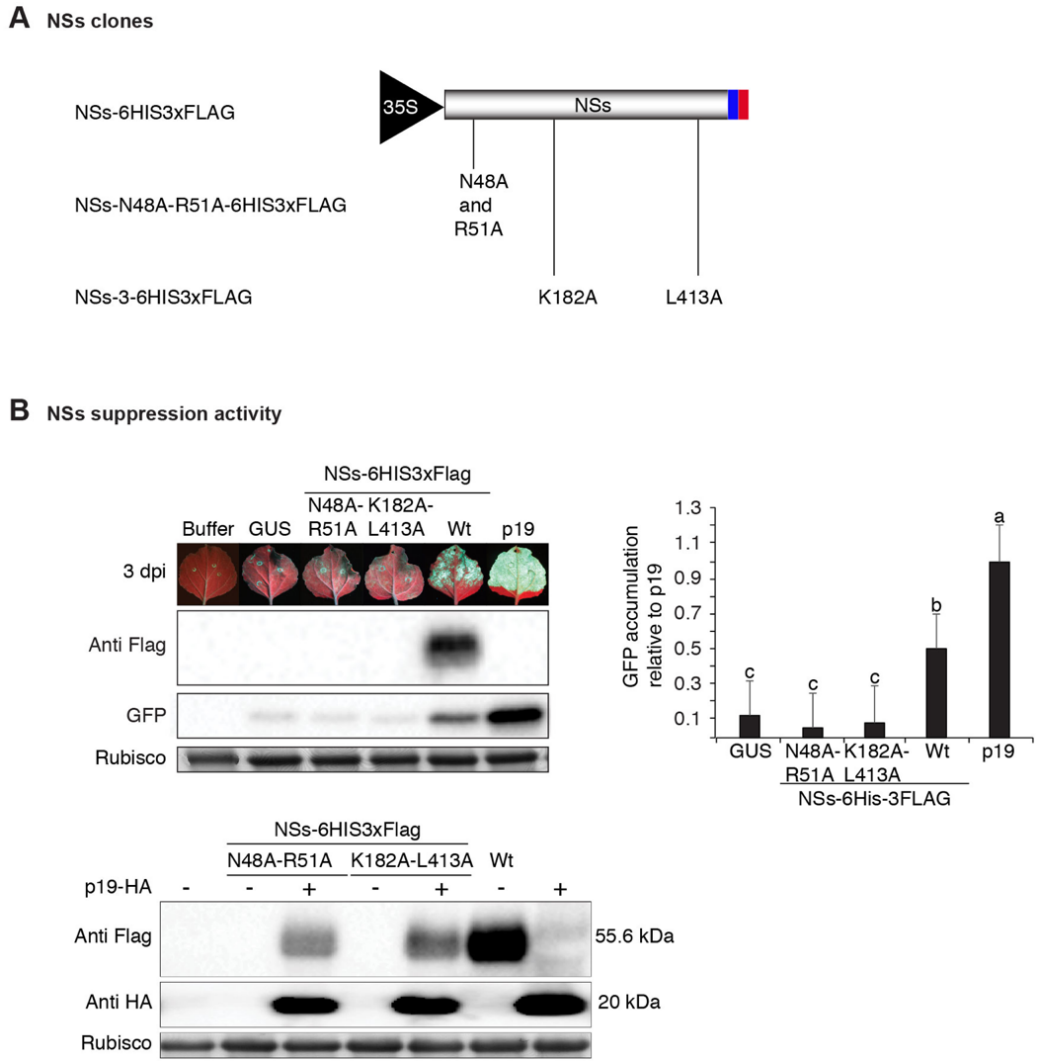

Figure 4.1 Suppression activity of NSs clones and GFP accumulation in *Nicotiana benthamiana* plants at 3 dpi. (A) NSs clones with relative positions of epitope His-Flag tag and point mutations used to inactivate the NSs protein. (B) Suppression activity of NSs clones in *N. benthamiana* plants at 3 dpi, the large unit of Rubisco protein was used as a loading control. NSs and GFP proteins were detected at 3 dpi using anti-Flag and anti-GFP antibodies respectively. Relative accumulation of GFP relative to p19, treatments with different letters are different Tukey HSD 0.05. Detection of NSs mutants using anti-Flag antibody when co-expressed with p19 at 3 dpi.

Previously described NSs clone (Takeda et al., 2002) was used as template for introduction of 6xHIS and 3xFLAG epitope tags at the C terminus (Figure 4.1 A). This clone was also used to introduce point mutations to inactivate the NSs protein based in conserved regions (Zhai et al., 2014).

NSs clones under the expression of the 35S promoter were used to determine suppression activity in co-expression with single stranded GFP. During transient RNA silencing suppression assays wild type NSs inhibited GFP silencing at 3 dpi. Mutant NSs clones pMDC32-NSs-S48A-R51A-6HIS3xFlag and pMDC32-NSs-K182A-L413A-6HIS3xFlag lost suppression activity and did not suppress GFP silencing at 3 dpi. GFP accumulation in the mutant NSs clones was different compared to wild type NSs. In addition, detection of mutant NSs clones using the FLAG antibody did not detect any proteins. To determine NSs protein was being produced in the mutant NSs clones and that reduction in the accumulation of GFP was not attributed to protein absence, co-expression of NSs mutants with p19 facilitated NSs detection but at lower levels compared to wild type NSs (Figure 4.1 B).

TuMV-AS9-GFP-NSs infects *Nicotiana benthamiana* and both WT and Dicer triple mutant *Arabidopsis thaliana*

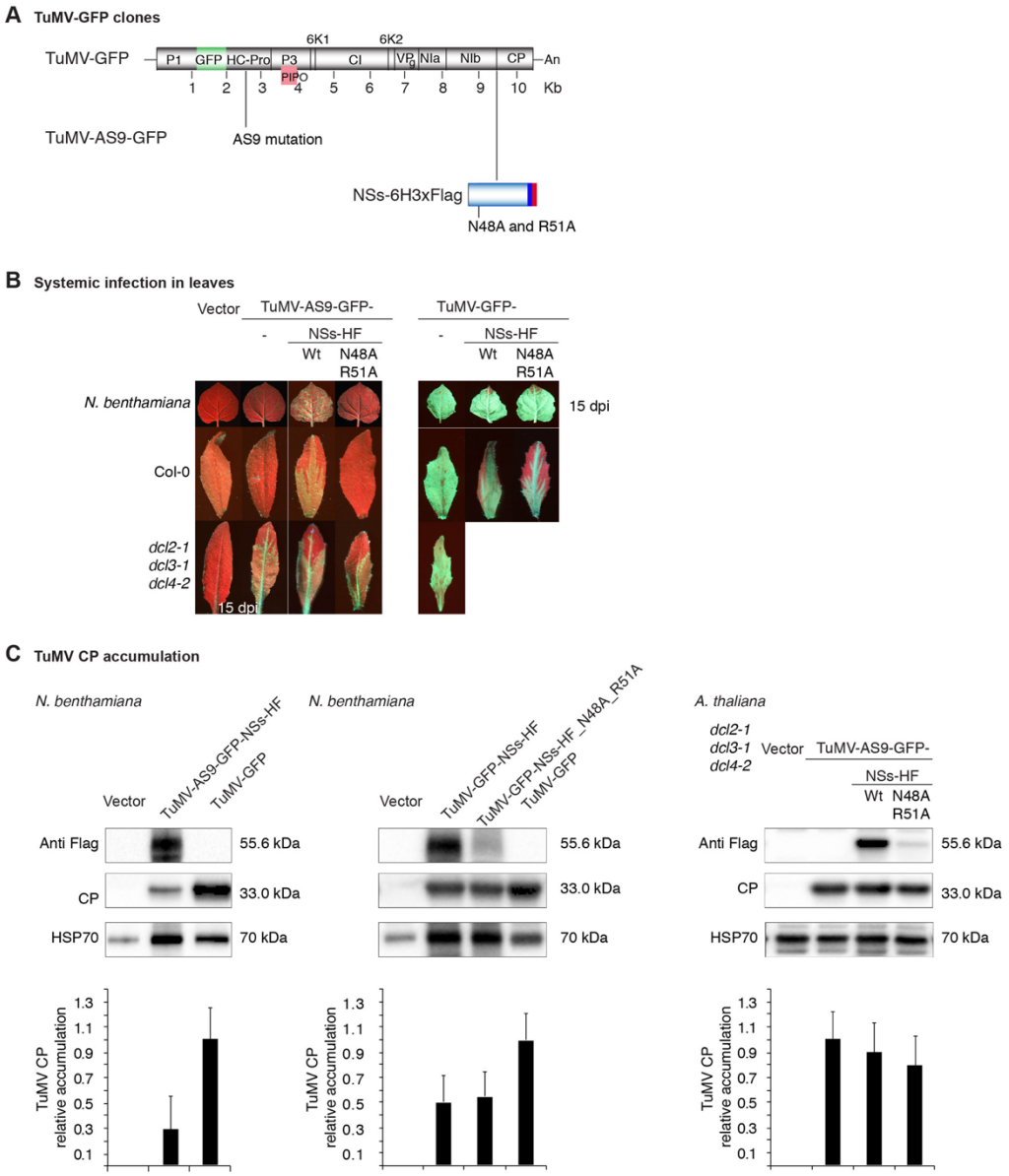

Figure 4.2 Stability and accumulation of TuMV-GFP-NSs clones expressing both WT and mutant NSs. (A) TuMV-GFP clones, relative position of AS9 mutation and position of NSs-6H3xFlag and S48A-R51A-6HIS3xFlag. (B) Systemic infection and GFP accumulation of TuMV-GFP clones in *N. benthamiana* and *A. thaliana* at 15 dpi. (C) NSs and CP protein accumulation from TuMV-AS9-GFP-NSs and TuMV-GFP-NSs at 15 dpi in *N. benthamiana* and NSs and CP accumulation of TuMV-AS9-GFP-NSs in *A. thaliana* dicer triple mutant. HSP70

was used as a loading control. TuMV CP accumulation was calculated relative to TuMV-GFP.

To determine NSs suppression activity when active in a virus, NSs ORF was introduced in the inactive infectious clone TuMV-GFP-AS9 (Garcia-Ruiz et al., 2010) which due to a AS9 mutation in the HC-Pro protein is not able to infect wild type plants. Both NSs-HF and NSs-HF_S48A_R51A variants were introduced between NIb and the CP after addition of a NIb cleavage site at the N terminus and removal of start and stop codon in order to process NSs as Potyviral protein. Relative position of TSWV NSs protein in the TuMV-GFP clone (Figure 4.2 A).

GFP accumulation from TuMV-GFP-AS9-NSs-HF was observed only in *A. thaliana* Col-0 ecotype and wild type *N. benthamiana*, no GFP was observed in controls TuMV-GFP-AS9 and TuMV-GFP-AS9-NSs-HF-NSs-K182A-L413A corroborating TuMV-GFP-AS9-NSs-HF construct was active and infective in wild type plants. *A. thaliana* Dicer triple mutant confirmed activity of controls, TuMV-GFP (WT) was used as positive control and brighter and full covering of GFP on leaves was observed in both *A. thaliana* Col-0 ecotype and wild type *N. benthamiana* (Figure 4.2 B).

NSs protein accumulation was detected by western blotting at 15 dpi using the anti-FLAG antibody in *N. benthamiana* (Figure 4.2 C). A reduction in the accumulation of NSs from the TuMV-GFP-AS9-NSs-HF-NSs-K182A-L413A clone was observed indicating NSs protein with point mutations was inactive and

tended to be degraded. A reduction of CP accumulation compared to TuMV-GFP (WT) was also observed which was also similar to GFP detection under UV light.

Protein samples from *A. thaliana* Dicer triple mutants showed TuMV-GFP-AS9-NSs-HF was active by detection of NSs protein at 15 dpi including TuMV-GFP-AS9-NSs-HF-NSs-K182A-L413A even though at lower levels. Similar accumulation levels of CP from TuMV-GFP-AS9-NSs-HF and controls were also detected.

To determine effects in TuMV-GFP-AS9-NSs-HF after addition of NSs ORF into TuMV-GFP-AS9 a new construct was made, TuMV-GFP-NSs-HF by introducing the NSs ORF in the TuMV-GFP (WT). This new construct showed to be different than TuMV-GFP-AS9-NSs-HF, systemic infection was slower in TuMV-GFP-NSs-HF but more similar to previous tested TuMV-GFP (WT) (Figure 4.2 B). Accumulation of NSs protein, wild type and mutant was similar to TuMV-GFP-AS9-NSs-HF. CP accumulation of TuMV-GFP-NSs-HF was also higher than TuMV-GFP-AS9-NSs-HF (Figure 4.2 C) but not higher than TuMV-GFP (WT) demonstrating there was an effect in the infection process by the addition of external sequences.

TuMV-GFP-AS9-NSs-HF infects *Nicotiana benthamiana* and *Arabidopsis thaliana* but symptoms are different than WT TuMV-GFP

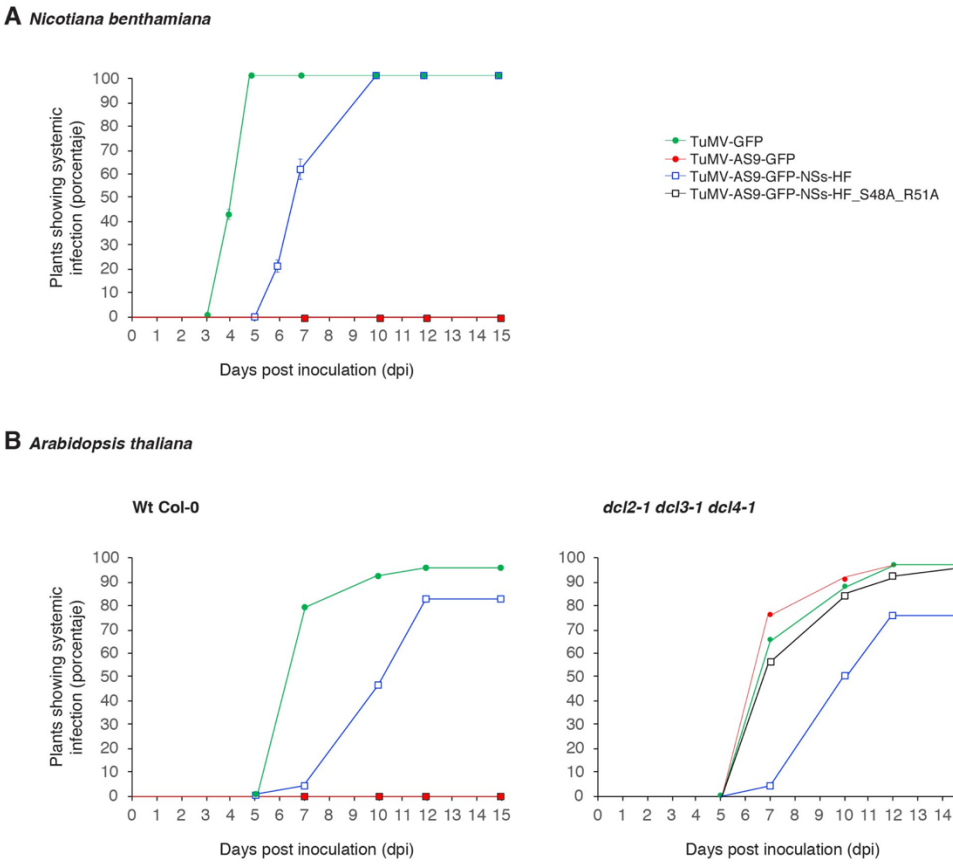

Figure 4.3 Systemic infection progression of TuMV-GFP-AS9-NSs constructs compared to TuMV-GFP. (A) Percentage of *N. benthamiana* plants showing systemic infection every day after post inoculation, 18 plants with 20 days old were Agro-infiltrated per treatment with a 0.05 final OD. (B) Percentage of *A. thaliana* WT and Dicer triple mutant plants showing systemic infection every day after post inoculation, 18 plants with 16 days old were Agro-infiltrated per treatment with a 0.05 final OD.

Agro-infiltrations of *Nicotiana benthamiana* and *Arabidopsis thaliana* plants showed chimeric TuMV-GFP-AS9-NSs-HF required more days to produce systemic infection compared to TuMV-GFP (Figure 4.3). While TuMV-GFP

required 4 to 5 days to produce systemic infection in *Nicotiana benthamiana* TuMV-GFP-AS9-NSs-HF required 7 to 10 days to show 100% of systemic infection (Figure 4.3 A).

The same pattern was found in both *Col-0* and Dicer triple mutant *Arabidopsis thaliana* plants (Figure 4.3 B). TuMV-GFP required only 6 to 10 days to reach systemic infection in *Col-0* compared to TuMV-GFP-AS9-NSs-HF that required 10 to 12 days. In the Dicer triple mutant ecotype TuMV-GFP-AS9-NSs-HF was also found to require additional days to systemically infect this ecotype compared to controls.

Systemic infection of TuMV-GFP-AS9-NSs-HF reassembles TSWV symptoms.

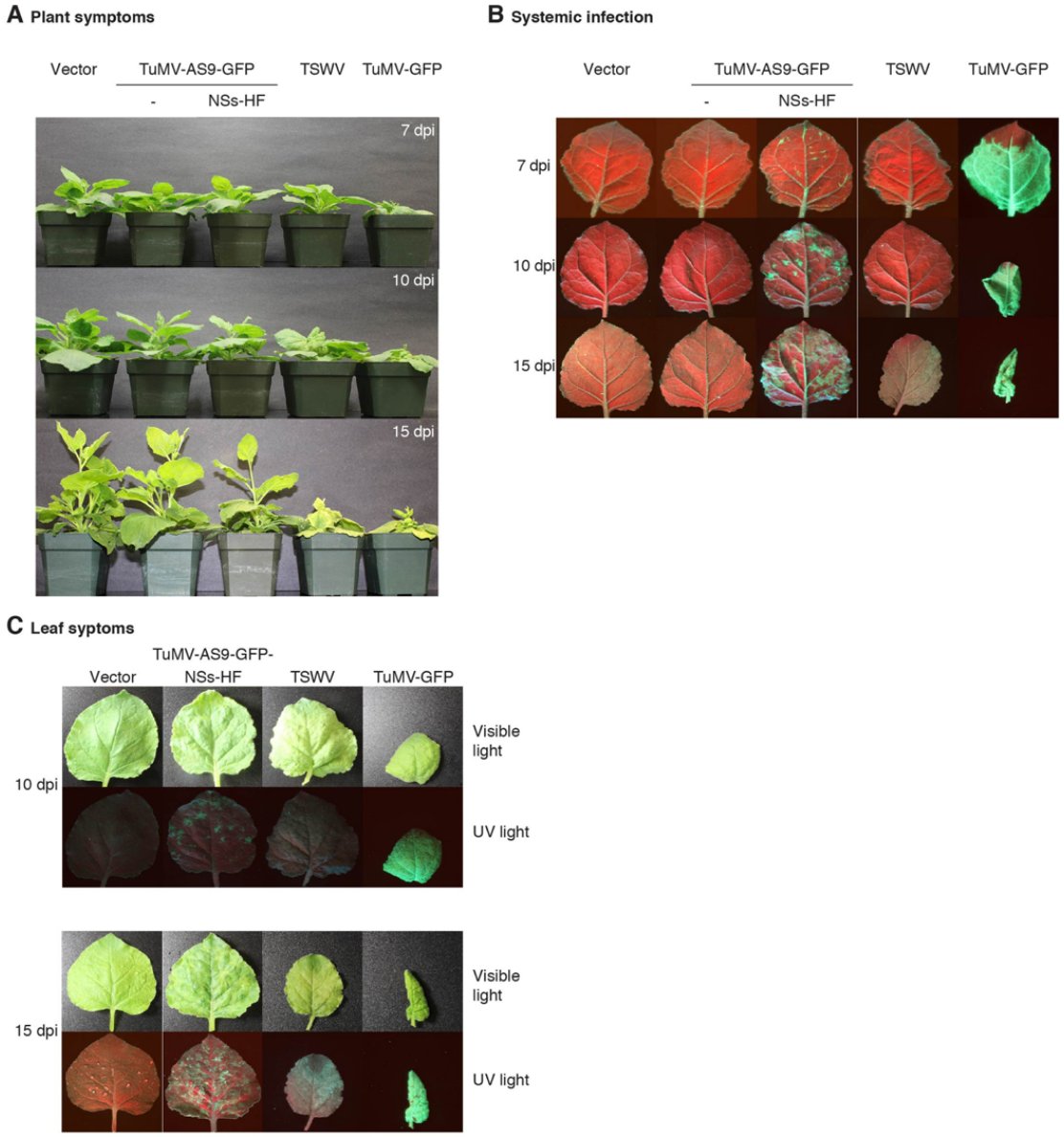

Figure 4.4 TuMV-GFP-AS9-NSs symptoms in *Nicotiana benthamiana* at 7, 10 and 15 dpi. (A) *N. benthamiana* plants were Agro-infiltrated at final infiltration 0.05 OD with TuMV- GFP-AS9 clones or infected with TSWV and plant symptoms pictures taken at 7, 10 and 15 days post inoculation under visible light. (B) Systemic infection of upper leaves under UV light at 7, 10 and 15 days post inoculation. (C) Leaf symptoms of TuMV-GFP-NSs clones under visible light and UV light at 15 days post inoculation.

After Agro-infiltrations pictures were taken at 7, 10 and 15 dpi and clear differences were observed between the TuMV-GFP-AS9-NSs-HF and controls (Figure 4.4 A) while TuMV-GFP affected growth of plants since day 7 and consequently killed plants at 15 dpi, TuMV-GFP-AS9-NSs-HF did not affect severely the growth of *N. benthamiana* at 15 dpi. Reduced sizes of plants were observed in the TSWV and TuMV-GFP WT infected plants compared to TuMV-GFP-AS9-NSs-HF where no reduction in sizes where evident at 15 dpi.

Systemic infection of *N. benthamiana* plants (Figure 4.4 B) with TuMV-GFP-AS9-NSs-HF showed GFP was scattered all over the leaf compared to TuMV-GFP where covering of the whole leaf was observed. In addition, no local infection by the presence of GFP in the infiltration zone of TuMV-GFP-AS9-NSs-HF was observed.

Visible and UV light pictures were taken at 10 and 15 dpi to detect distribution of GFP from TuMV-GFP-AS9-NSs-HF in leaves (Figure 4.4 C) yellow zones observed under visible light corresponded to GFP presence under UV light and these yellowing symptoms were similar when compared to TSWV infection.

DISCUSSION

Activity of NSs protein as a silencing suppressor has been previously described and NSs-6HIS-3xFlag clone inhibited GFP silencing at 3 dpi, (Figure 4.1 B) the fact that GFP protein accumulation is lower in the wild type NSs compared to p19 suggests this protein acts at a different step in the RNA

silencing pathway; p19 binds 21 nucleotides small RNAs (Chapman et al., 2004). Another important consideration is the quantity of NSs protein compared to p19, NSs differs in size by more than 30 kDa and this could affect protein movement, availability and even protein degradation. Important domains in the NSs protein may have additional or even new effects in the RNA silencing pathway and cannot be ruled out.

After introduction of point mutations to inactivate the NSs protein westerns showed NSs mutants where unstable and accumulation was not detected until co-expression of silencing suppressor p19. This highlights the importance of these amino acids in the protein as having important activity or involved in the folding of the NSs protein.

After suppression activity of NSs protein was corroborated introduction of this protein in the TuMV-GFP-AS9 clone was an important step to study the activity of this suppressor in the rescue of pathogenicity of a mutant virus. TuMV-GFP-AS9 in not able to infect *Nicotiana benthamiana* nor *Arabidopsis thaliana* but is able to infect the Dicer triple mutant ecotype. TuMV-GFP-AS9 was also used as a negative control in the infection of wild type *Nicotiana* and *Arabidopsis* plants and as positive control in the infection of Dicer triple mutants. Introduction of the NSs protein in the TuMV-GFP-AS9 rescues pathogenicity of this virus; making inactive TuMV-GFP-AS9 to be active again. Interestingly detection of the NSs protein by western blotting at the corresponding size suggests this protein is active and correctly processed inside the virus.

A reduction in the accumulation of TuMV CP suggested that addition of NSs protein has an effect in the pathogenicity of TuMV-GFP-AS9-NSs-HF; this was also shown when the percentage of plants showing systemic symptoms after Agro-infiltration where calculated, positioning TuMV-GFP-AS9-NSs-HF behind TuMV-GFP (WT). Introduction of NSs protein in the WT TuMV-GFP to compare effects showed there is an effect in the infection process due to presence of local infection compared to TuMV-GFP-AS9-NSs-HF where no local infection was observed (Figure 4.3 A 3 dpi). Nevertheless, differences in pathogenicity compared to TuMV-GFP (WT) could be attributed only to NSs silencing suppressor and the mechanistic roles this suppressor uses to interact with the RNA silencing pathway.

The protocol used to Agro-infiltrate *Arabidopsis* plants still needs to be reviewed and optimized due to some plants do not showing GFP in certain occasions; otherwise data generated by these plants may produce erroneous data in the appearance of local and systemic symptoms.

The presence of symptoms with high similitudes to TSWV infection in this system could attribute yellowing symptoms in natural host plants infected with TSWV to be caused only by presence of the NSs suppressor. These findings open interesting hypotheses to follow in current and future experiments involving the use of the chimeric TuMV-GFP-AS9-NSs-HF here described in order to understand the effects of this suppressor. The fact that the NSs protein is processed and cleaved after the introduction of NIb cleavage site in the region

used also opens the possibility to introduce different suppressors of different viruses.

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