ARGONAUTE 2 AND ANTIVIRAL SILENCING IN PLANTS

A Thesis

by

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MASTER OF SCIENCE

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ABSTRACT

RNA silencing is a mechanism used by eukaryotes to defend them self against viruses. When encountering an external or foreign double-stranded RNA (dsRNA), plants activate their machinery to assemble an RNA induced gene silencing complex (RISC) in which Argonautes (AGOs) play an important role. Once activated plant use RISC to survey for those sequences identical to the original dsRNA and cleave these into small fragments.

This study provides evidence that RNA silencing against *Tomato bushy stunt virus* (TBSV) can depend on plant age in *Nicotiana benthamiana* (Nb) by using TBSV mutants that do not express P19 (TGdp19 or 157). I show that NbAGO2 plays role in older plants during viral silencing by measuring mRNA and comparing treatments. These results indicated that NbAGO2 is up-regulated after TBSV inoculation and that 6 week old plants have a better ability to silence TBSV compared to younger plants.

By using transgenic plants expressing a dsRNA NbAGO2 hairpin for down-regulation of NbAGO2, my studies provide evidence that NbAGO2 is required for viral silencing by conferring several layers of protection. This was demonstrated by infecting NbAGO2-hairpin transgenic plants with different TBSV variants, different Tombusvirus members and non-Tombusvirus. The results showed that reduced levels of NbAGO2 enhanced viral infection in general, these infections compromised plant integrity, TBSV not expressing the coat protein yielded severe systemic infections not otherwise observed, and TBSV not expressing P19 (TGdp19 and TBSV-157) caused more severe

infections compared to controls.

Additionally, my studies validate the use of two well-known viruses that express suppressors to avoid the effects of silencing by NbAGO2 or other components. This was shown for *Tobacco mosaic virus* (TMV) and TBSV that were used as viral vectors to express and co-express green fluorescent protein (GFP) and red fluorescent protein (RFP) in *N. benthamiana* and tomato. The results demonstrated that the vectors accumulate in the same plant and leaves, and even in the same cells, providing a tool for fast expression of potentially biomedically or otherwise valuable oligomeric proteins.

DEDICATION

To my family.

ACKNOWLEDGEMENTS

I would first like to thank my advisor Dr. Herman Scholthof for believing in me, by giving me the opportunity to join his laboratory and also because the door of his office was always open whenever I ran into trouble or had a question about my research or writing. He consistently allowed this work to be my own work, but steered me in the right the direction whenever he thought I needed it.

To my committee members, Dr. Karen-Beth Scholthof for her support during my studies, for always taking care of my research projects and me and to Dr. Hongmin Qin for her guidance and support throughout the course of this research.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

RNA SILENCING HISTORY

RNA silencing is a post-transcriptional modification of gene expression or post-transcriptional gene silencing (PTGS) in eukaryotes (Hannon, 2002; Voinnet, 2001). It is a mechanism that plants use to defend against viruses (Wang et al., 2012), to protect the genome from mutation and to regulate gene expression.

Unbeknown to the authors, the first example and indication of silencing was reported in 1928 during a host range evaluation for *Tobacco* (or tomato) *ring spot virus* susceptibility. This study described that infected tobacco plants recovered two weeks after inoculation and new plant growth appeared normal. They observed that new attempts to re-infect recovered plants failed to produce symptoms (Wingard, 1928). Many decades later, another case was reported whereby the chalcone synthase gene was overexpressed in Petunia hybrids; the desired phenotype, flowers with violet color was suppressed unexpectedly, resulting in white petals pigments (Napoli et al., 1990; van der Krol et al., 1990). Subsequently a study in *Caenorhabditis elegans*, using the same principle from the petunia experiments, aimed to overexpress a nonessential myofilament protein into the nematode, which resulted instead in silencing of the gene that led to muscle structural defects and impaired motility in the worm (Fire et al., 1998).

In plants, RNA silencing has been categorized in three pathways: cytoplasmic short interfering RNA (siRNA) guided silencing, endogenous mRNA silencing by micro-RNAs (miRNAs), and DNA methylation and suppression of transcription (Baulcombe, 2004; Chan et al., 2004). These three pathways are associated with Argonautes (Diederichs and Haber, 2007; Garcia-Ruiz et al., 2015)

The siRNAs are the result from available double-stranded RNA (dsRNA) that is captured by one of the RNAseIII like Dicer family proteins. This Dicer cleaves dsRNA into small fragments of 20-25 nucleotides that form the siRNA duplex (Castel and Martienssen, 2013; Colmenares et al., 2007). Dicer contains a multidomain protein with RNA helicase-like, double-stranded RNA binding domains and the RNaseIII regions (Bernstein et al., 2001). MicroRNAs (miRNAs) are involved in posttranscriptional gene regulation. They are small endogenous noncoding RNA segments. Mature miRNAs are formed when a Dicer cleaves a pre-miRNA hairpin (Ketting et al., 2001), whereby the resulting product enters the RNA silencing pathway (Petersen et al., 2006) where Argonautes can regulated the expression of genes (Diederichs and Haber, 2007). Finally, Cytosine DNA methylation has the ability to silence unwelcome DNA such as DNA viruses (Chan et al., 2004) to repress their gene transcription.

PLANT VIRAL SILENCING

Typically, antiviral RNA silencing starts when the plant encounters dsRNA from viral replication, which activates a pathway to detect these dsRNA (Alvarado and Scholthof, 2009) and redirects it to a Dicer-like protein (DCL) that initiates the cleavage

of dsRNA into 21 to 26 nucleotides fragments (Baulcombe, 2004). The association between DCL and dsRNA binding proteins (DRB) that creates these siRNAs (Forstemann et al., 2005; Hiraguri et al., 2005) is followed by unwinding whereby one of the siRNA strands is used to program the RNA induced silencing complex (RISC). Before siRNA enters RISC, a methylation process occurs by methyltransferase HUA enhancer 1 (HEN1) (Chen et al., 2002).

Once methylated viral siRNAs are incorporated into RISC, this programmed complex surveys for complementary mRNA or viral RNA, which then is subjected to a degradation process. During this process Argonautes (AGOs) play important roles (Ciomperlik et al., 2011; Omarov et al., 2007); other gene products such as RNA dependent RNA polymerase (RDR), silencing defective 3(SDE3), suppressor of gene silencing 3 (SGS3) (Vaistij et al., 2002), and DCL-DRB also contribute to regulate invasive RNA degradation by RISC (Alvarado and Scholthof, 2009).

The above-sketched pathway can seriously hinder virus infection and may lead to recovery of the plants (Scholthof, 2006). To counteract the effect of antiviral silencing many viruses encode proteins that interfere with specific steps resulting in suppression. There are many and diverse suppressors identified for different viruses (Alvarado and Scholthof, 2009) that help the virus to respond to plant silencing and making viral infections more virulent.

PROJECT DESCRIPTION, RESEARCH HYPOTHESIS AND OBJECTIVES

The focus of my research was to understand and to provide further evidence of the relationship that Argonautes have in antiviral silencing against different viruses and determine specifically how two different viruses can coexist by combatting antiviral silencing in the same plant. To achieve this I have outlined the following hypothesis with their objectives.

Hypothesis 1

Nicotiana benthamiana Argonaute 2 (NbAGO2) antiviral silencing activity against *Tomato bushy stunt virus* (TBSV) is dependent on plant age.

To test this hypothesis I addressed the following objectives:

- Determine *NbAGO2* transcript accumulation in 3-week old plants upon infection with different GFP-expressing TBSV constructs that either do or do not express a suppressor of gene silencing. This is determined by agroinfiltration of TG (31) or TGdp19 (33).
- Determine *NbAGO2* mRNA accumulation at two different plant ages upon infection with TBSV not expressing its P19 suppressor of RNA silencing (TBSV-157) and by following gene expression using quantitative real time PCR.

Hypothesis 2

Transgenic *Nicotiana benthamiana* plants in which NbAGO2 is down regulated via dsRNA hairpin technology are more susceptible to virus infection.

To pursue this hypothesis my objectives were:

- Validate that in plants positive for *NbAGO2*-hairpin expression, AGO2 expression is down regulated using reverse transcription PCR.
- Determine susceptibility to different TBSV variants.

Hypothesis 3

NbAGO2 may possess antiviral silencing against viruses other than TBSV. To test this hypothesis I designed the following experiments:

- Test transgenic *NbAGO2*-hairpin plants against different viruses and observe symptom progression.
- Determine virus accumulation by western blot analysis of infected plants.

Hypothesis 4

Viruses can be used as gene expression vectors, but the expression is often limited to a single protein, and this expression is often hampered by RNA silencing. Since TBSV expresses the P19 suppressors I postulated that TBSV and TMV virus vectors can be used to express different foreign proteins in several plant species and in the same cell.

To test this hypothesis I proposed the following objectives:

- Express GFP in three different plant species with TBSV-GFP and TMV-GFP vectors.
- Determine virus accumulation by western blot assay of agroinfiltrated plants.

- Determine if TBSV-GFP and TMV-RFP are co-expressed in the same cells.

CHAPTER II

ARGONAUTE 2, TBSV AND PLANT AGE

INTRODUCTION

Argonautes (AGOs) are highly conserved and ubiquitously expressed in all eukaryotes, bacteria and archaea. A crystal structure of an Argonaute from *Pyrococcus furious* revealed that it could be separated into distinct domains: amino-terminal, middle, PIWI (contains piwi protein and it is similar to ribonuclease H) and PAZ (Piwi Argonaute Zwille) domains (Hammond et al., 2000; Song et al., 2004). The presence of the PAZ domain allows for direct interaction with small RNAs in RISC (Song et al., 2003).

The presence and expression of AGOs has been demonstrated in many plants, for instance in *Arabidopsis thaliana* (Vaucheret, 2008), *Oryza sativa* (Kapoor et al., 2008), *Nicotiana benthamina* (Alvarado and Scholthof, 2011; Jones et al., 2006; Scholthof et al., 2011) and *Solanun lycopersicum* (Bai et al., 2012). In *Arabidopsis*, AGO1 (Zhang et al., 2006), AGO7 (Qu et al., 2008) and recently AGO2 and AGO5 have been characterized by their antiviral activity (Brosseau and Moffett, 2015; Jaubert et al., 2011). In *N. benthamiana* several AGOs are known to be expressed: AGO1 (Jones et al., 2006), AGO2, AGO3, AGO5, AGO7 and AGOX (Odokonyero, 2013); of these six AGOs only AGO1 (Ghoshal and Sanfaçon, 2014; Karran and Sanfaçon, 2014) and AGO2 (Scholthof et al., 2011) are shown to contribute to antiviral silencing. For our

studies we rely heavy on TBSV, and in sections below I will describe the virus in more detail.

Tomato bushy stunt virus (TBSV) is the type member of the genus *Tombusvirus* in the *Tombusviridae* that infects a wide variety of plant species (Martelli et al., 1988). This virus contains a positive-sense single-stranded RNA (ssRNA) genome (Figure 2.1) that encodes five different proteins (Yamamura and Scholthof, 2005): P33 and P92 genes encode replicase proteins. P41 represents the coat protein (CP) that is translated from subgenomic RNA1 (sgRNA1); the cell-to-cell movement protein P22, and P19 are expressed from sgRNA2. P19 is a strong silencing suppressor that forms homodimers to sequester siRNAs and prevent these from associating with RISC (Scholthof, 2006; Vargason et al., 2003). The TBSV replication process starts with the synthesis of the minus-strand RNA from the plus-strand template. After this initiation process the minus-strand is the template to produce progeny viral genome as well as the sgRNAs (King, 2012).

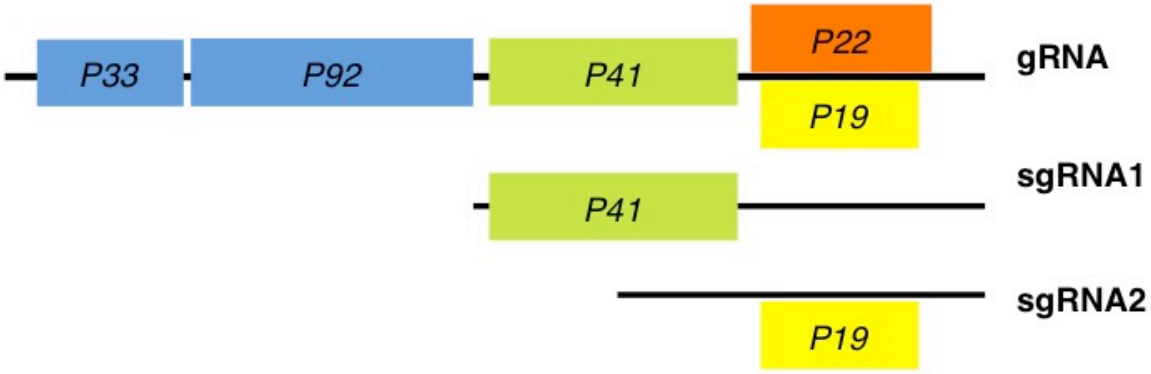

Figure 2.1. TBSV genome and its subgenomic (sg) RNA1 and sgRNA2. The P33 and P92 genes encode replicase proteins, P41 is used to translate the coat protein from sgRNA1, and P22 and P19 are translated from sgRNA2 to produce the cell-to-cell movement protein and the suppressor of silencing, respectively.

TBSV is a soil-borne pathogen and the primary infection occurs in roots (Martelli et al., 1988). Under laboratory conditions infection is often established via leaf inoculations (Yamamura and Scholthof, 2005). By using this technique it has been shown that CP is not strictly required for viral systemic infection in *N. benthamiana* (Qu and Morris, 2002; Scholthof et al., 1993) and that there is a difference for virus protein requirements depending on the host (Turina et al., 2003). Other studies provide evidence that it is important whether virus infections start in roots or leaves (Andika et al., 2015; Manabayeva et al., 2013), and that plant age difference has effect in viral infection as shown for *Arabidopsis thaliana* (Jackel et al., 2015)

My study is based on the following TBSV constructs (Figure 2.2): TG (also referred as 31) where the capsid protein is replaced by GFP, and TGdp19 (also referred

to as 33) also expresses GFP but lacks the ability to express the P19 silencing suppressor. These two vectors were used via agroinfiltration (Shamekova et al., 2014) while TBSV-157, that also lacks P19, is used as a template for synthesizing RNA transcripts for inoculation (Scholthof et al., 1995) (Figure 2.2).

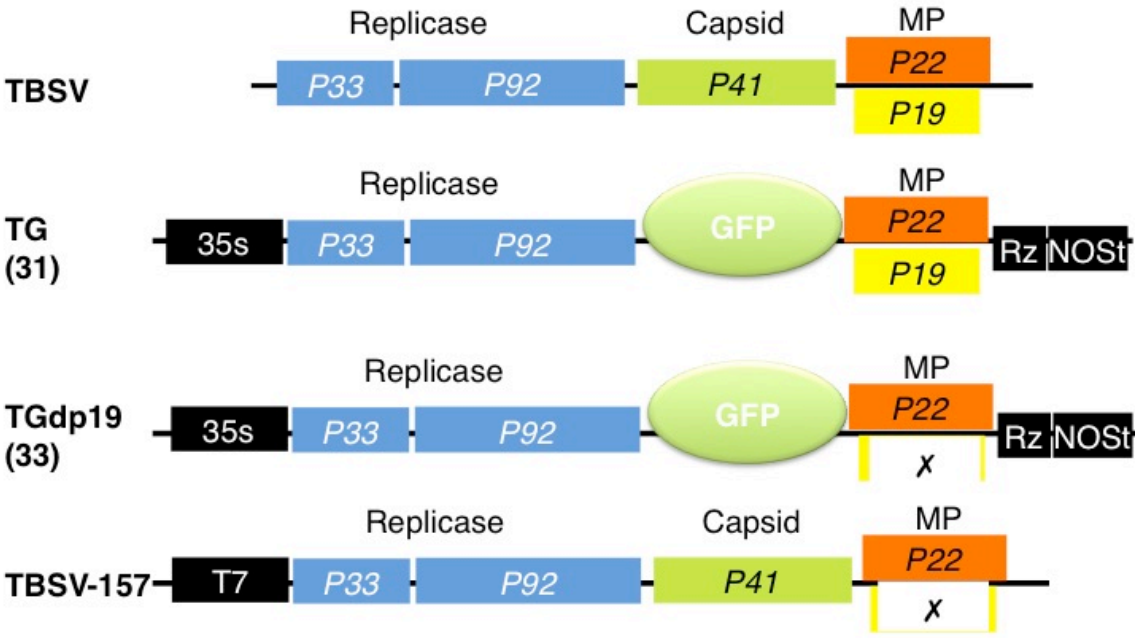

Figure 2.2. TBSV constructs. In TG or 31, GFP substitutes *P41*, TGdp19 has the same GFP-substitution of *P41* but lacks the ability to express a P19 protein. These two vectors are for use via agroinfiltration, whereas TBSV-157, which also lacks P19 expression, is used by rub-inoculation of RNA transcripts.

Previous experiments in the laboratory revealed two findings that attracted my attention and interest. First, NbAGO2 seemed to have an antiviral silencing role

((Scholthof et al., 2011) and see also Chapter III), and secondly consistent observations were made that silencing against TGdP19 (Figure 2.3) is more effective in older plants compared to younger plants TBSV (Alvarado, unpublished data). Therefore, I was interested in determining if these two observations were related and aimed to measure mRNA of Argonautes (including NbAGO2) expression in *N. benthamiana* at different ages. For this, I formulated the following hypothesis and its objectives to investigate silencing at different ages in *N. benthamiana* in more detail.

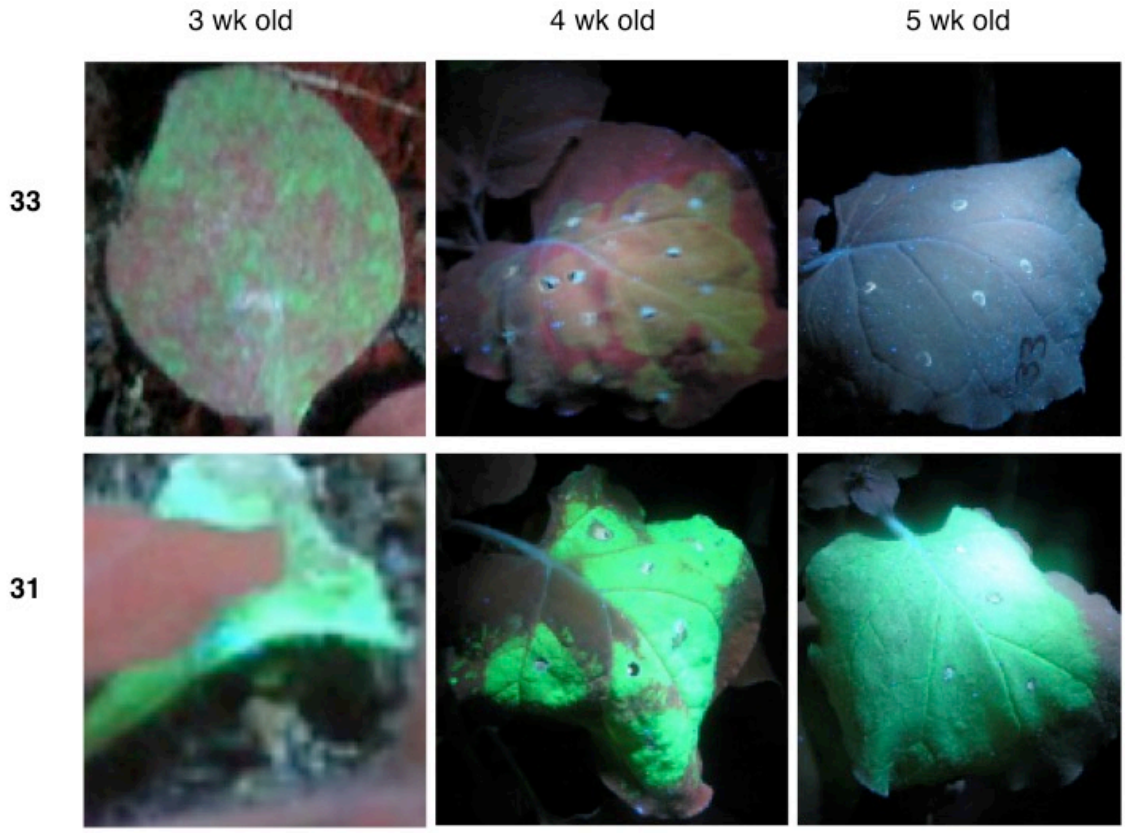

Figure 2.3. Plant silencing against TBSV not expressing P19 (TGdP19 (33)) in 5 week old *N. benthamiana*. TG (31), expressing P19 (second panel), is not silenced by the plant (Alvarado, unpublished data).

Hypothesis 1

Nicotiana benthamiana Argonaute 2 (NbAGO2) antiviral silencing activity against *Tomato bushy stunt virus* (TBSV) is dependent on plant age.

To test this hypothesis I addressed the following objectives:

- Determine *NbAGO2* transcript accumulation in 3-week old plants upon infection with different GFP-expressing TBSV constructs that either do or do not express a

suppressor of gene silencing. This is determined by agroinfiltration of TG (31) or TGdp19 (33).

- Determine *NbAGO2* mRNA accumulation at two different plant ages upon infection with TBSV not expressing its P19 suppressor of RNA silencing (TBSV-157) and by following gene expression using quantitative real time PCR.

RESULTS

NbAGO2 is expressed upon TBSV inoculation

Agroinfiltration in 3-week old *N. benthamiana* was performed at the abaxial side, and after infiltration all plants were kept under the same conditions until harvest time. To establish that effects were due to virus infection and not to the *Agrobacterium* infection upon agroinfiltration, it was required to use a negative agroinfiltrated control. This is represented by "00", which corresponds to a *Tobacco rattle virus* (TRV) vector that only expresses one of the two genomic RNAs and is not infectious by itself. The relevance of using *Agrobacterium* only expressing "GFP" was to exclude possible effects on NbAGOs induction due to GFP expression, because GFP is expressed in TG and TGdP19. TG and TGdp19 were used to compare the effect of P19-suppressor presence and absence, respectively. From each infiltrated plant, 50 mg of tissue was collected from infiltrated leaves and a non-infiltrated leaf, to extract RNA for qRT-PCR analysis.

Absolute quantities obtained from agroinfiltrated treatments were normalized against actin gene expression and expression in control plants. The results show that compared to other treatments NbAGO2 expression is highly up-regulated in TG (31)

inoculated leaves (Figure 2.4), and noticeably upregulated in TGdP19 (33) inoculated leaves. However, effects were also observed in control treatments, for instance in GFP-agroinoculated leaves. This experiment was reproduced for three times showing the same trend. Plotted data in Figure 2.4 represent two technical replicates in one biological replication.

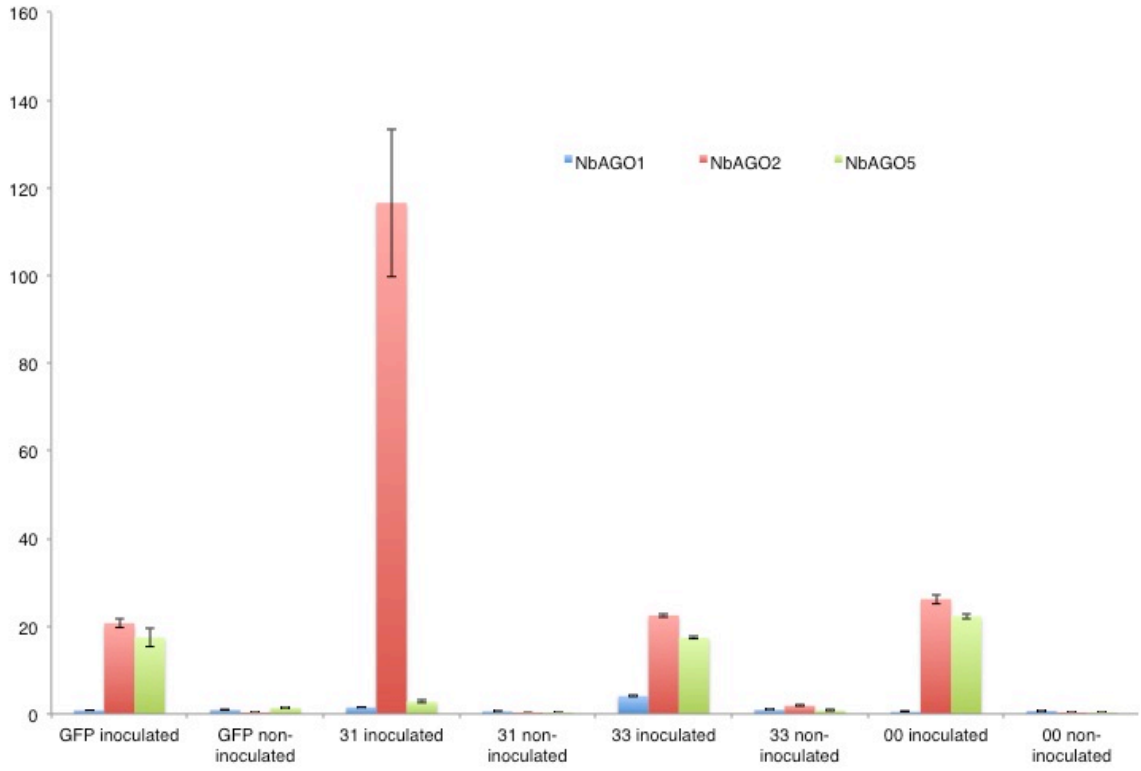

Figure 2.4. *N. benthamiana* Argonaute2, 1 and 5 as measured by fold change from normalized data of RT-PCR products in agroinfiltrated leaves and upper non-inoculated leaves. Transcript levels of actin (housekeeping gene) was used to normalize RT-PCR. Gene expression values are from one independent biological replicate that contains three mixed plants; each biological replicate had two technical replicates and values are relative to mock.

To reduce the contributing effect of *Agrobacterium* on AGO induction, a new experiment was designed, which only contain treatments with viral RNA, plus controls. In this case the experiment consisted of 3 plants at 3 and 6 weeks old at the time of inoculation treatment. I rub-inoculated RNA for each treatment into leaves, then collected plant tissue from these leaves and mixed the three samples (one from each plant) to make one biological replicate. Each biological replicate had two time points, one at 3 dpi and the second at 6 dpi. Results plotted in Figure 2.5 represent one biological replicate with three technical replications; the same experiments were repeated several times on plants with similar age and resulting up-regulation of NbAGO2 showed same tendencies.

In this experiment NbAGO2 levels were highly induced after TBSV treatment in younger and older plants compared to that of NbAGO1 (Figure 2.5). During the experiment, plants treated at 3 weeks old were more vulnerable in comparison with plants at 6 weeks old, often resulting in necrosis in inoculated leaves at 6 days post inoculation. Therefore, no collections were made at 6 dpi on 3 week old plants. Another piece of information obtained from this experiment was that after infection of 6 weeks old plants with TBSV-157, NbAGO2 levels were elevated when compared to TBSV wild-type treatment. In conclusion, these experiments showed that compared to other NbAGOs, NbAGO2 is particularly sensitive to TBSV infection.

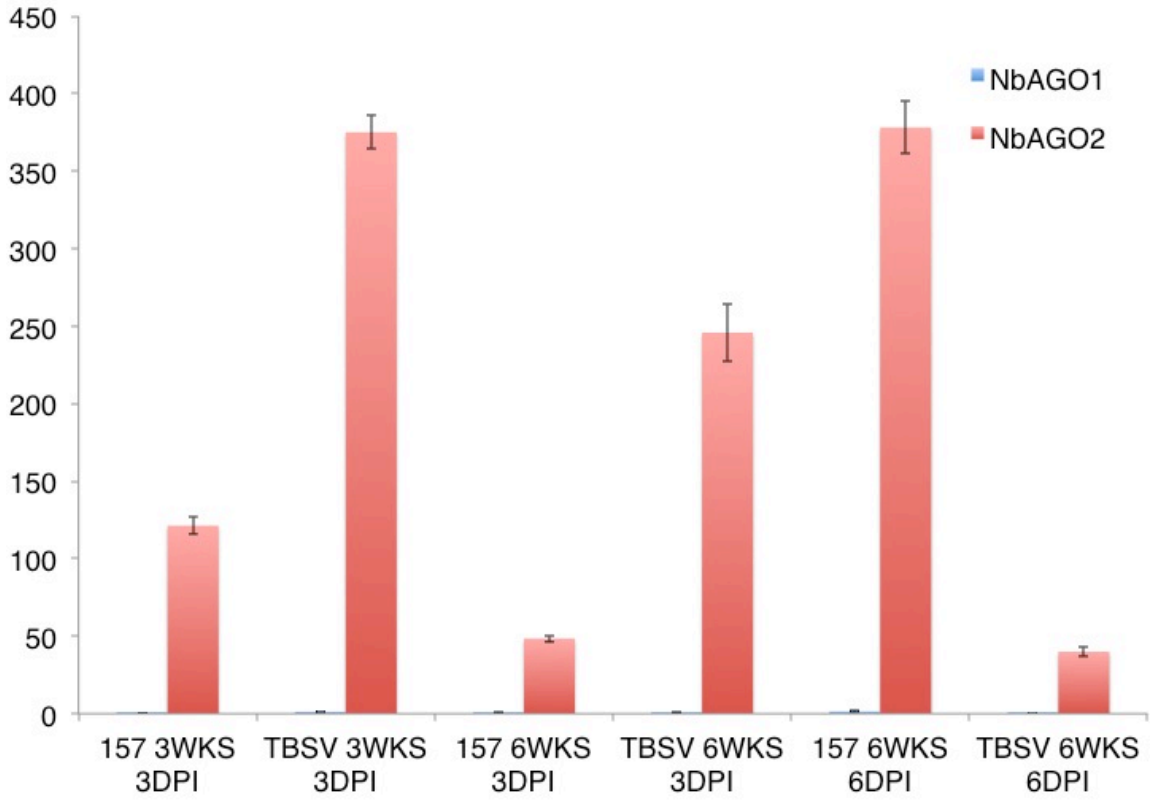

Figure 2.5. NbAGO1 and NbAGO2 fold change of RNA accumulation after TBSV wild type and TBSV-157 inoculation of 3 week or 6 week old plants. Transcript levels of actin (housekeeping gene) were used to normalize RT-PCR. Gene expression values are from an independent biological replicate that contains three mixed plants; each biological replicate had three technical replicates and values are relative to mock.

DISCUSSION

The use of *Agrobacterium* to launch TBSV constructs (TG and TGdP19, also known as 31 and 33) was the basis for our observation that anti-TBSV silencing was more effective in young compared to older plants (Fig. 2.3) (Alvarado, et al., in preparation). In this case TGdp19 that does not express the P19 viral suppressor protein,

gives rise to GFP expression in younger plants, but under the same experimental conditions and with the only variant of plant age, older plants were able to silence TGdP19 virus and GFP was not detected. Based on this observation I proposed that NbAGO2 expression is activated in TBSV inoculated leaves, especially in younger 'resistant' 3-week-old plants to explain the prevention or restriction of the viral infection.

Normal plant symptoms observed upon wild type TBSV infection in *N. benthamiana* include severe necrosis while TBSV-157 induces mostly mosaic symptoms, leaf distortion and stunting growth, which are apparent when plants are inoculated at a young age (Scholthof et al., 1995), as we also these observed in our 3 weeks old plants (Figure 2.6). The qRT-PCR results indicated that both upon agroinfiltration and RNA transcript inoculations, NbAGO2 was preferentially induced upon infection of leaves with different TBSV constructs. This is in agreement with the hypothesis that NbAGO2 is playing an important role in plants for silencing against viral accumulation.

Figure 2.6. Symptoms on *N. benthamiana* 6 days after inoculation of 3 week old plants with TBSV (left), TBSV-157 (center) or Mock (right).

After infection, it appeared that the up-regulation of NbAGO2 expression is not only present in older plants but especially in younger plants. Interestingly, however, 6-week old plants effectively silence the inoculated virus (TBSV-157) showing that the comparatively reduced levels of NbAGO2 are sufficient for silencing in older plants, as is an agreement with our previous results (Odokonyero, 2013; Odokonyero et al., 2015; Scholthof et al., 2011). In the present study we additionally find that NbAGO2 is highly up-regulated when P19 is not expressed (TBSV-157) which suggest that NbAGO2 plays a vital role, especially in the absence of a viral suppressor, to ultimately clear the virus infection. However, in young plants this up-regulation is either too late or still insufficient to induce silencing, suggesting that in young plants a vital component in addition to NbAGO2 is unavailable, resulting in unhindered virus accumulation and expression. Also, it is possible that some of my findings are also influenced by how fast a virus can replicate, and that may be more effective in younger plants compared to older

plants and due to this relative enhanced accumulation, the levels of NbAGO2 are insufficient to provide protection against the virus in these younger tissues.

Additionally, two studies reported that genes such as AGO1 and AGO5 are involved in antiviral silencing in Arabidopsis (Brosseau and Moffett, 2015; Vaucheret et al., 2004). However even though NbAGO1 was shown not to be involved against TBSV in *N. benthamiana* (Scholthof et al., 2011), a more recent observation suggests an ancillary role for an NbAGO5-like component (Odokonyero, unpublished). We also evaluated NbAGO1 and NbAGO5 induction and found that NbAGO1 did not respond noticeably to TBSV infection, but the modest response of NbAGO5 suggests it may have an auxiliary role for silencing TBSV.

METHODS

For the first objective, I infiltrated *Agrobacterium* containing TG, TGdp19, 00 (an empty viral vector) and GFP (virus-free binary vector that contains GFP) constructs in 3-week old *N. benthamiana* plants. Using quantitative PCR, expression of *NbAGO1*, *NbAGO2* and *NbAGO5* was analyzed. To approach this, plant RNA was extracted in 1.5 ml tubes containing RNA extraction buffer (78.57 mM Tris, 39.28 mM LiCl, 2 mM EDTA, 0.4265% SDS, 78.57 mM sodium acetate, 43.65% phenol, 8.72% chloroform and 4.5 mM β-mercaptoethanol). Then, after centrifugation at 13,000 rpm for 6 min, I collected the 700 ul supernatant. Subsequently, 700 ul phenol:chloroform:isoamyl alcohol (25:24:1) was added followed by centrifugation of the samples for 6 min at 13,000 rpm. The obtained supernatant was transferred to a new tube, mixed with 650 ul

chloroform and centrifuged for 6 min at 13000 rpm. Then 600 ul of supernatant was collected into a new tube, mixed with 200 ul 8 mM LiCl and incubated overnight at -20°C. After incubation, the sample was spun down at 14,000 rpm for 20 min at 4°C, the RNA pellet was washed with 70% ethanol, centrifuged for 10 min to remove the ethanol and he pellet was resuspended in DEPC treated dH_2O. The RNA was treated with DNase I to remove possible DNA contamination. This RNA was used to synthetize cDNA via oligo-dT (18) by using a kit from Invitrogen Technologies. The acquired cDNA was used for qPCR detection of AGOs with primers reported previously (Odokonyero, 2013).

For the second experiment I used 3 and 6 weeks old plants. Plants were rub-inoculated with TBSV-157 (Δp19) or wild-type TBSV transcripts. Plant tissue was collected for RNA extraction at 3 and 6 days after inoculation, cDNA was synthesized using oligo-dT (18) primers and PCR was carried out using primers described previously (Odokonyero, 2013). To monitor amplification, the accumulation of actin was used as control. qRT-PCR was performed by using SYBR GREEN (BioLabs Technology) and the protocol suggested by the manufacturer with primers sets reported before (Odokonyero, 2013). The data was analyzed by comparing relative expression of genes toward their control, which generated the fold induction for each treatment.

CHAPTER III

SILENCING OF ARGONAUTE 2 AND EFFECTS ON VIRUSES INFECTION*

INTRODUCTION

Antiviral RNA silencing is a host RNA-mediated defense mechanism that specifically recognizes and degrades single-stranded viral RNA (Baulcombe, 2004). During replication of RNA viruses, double-stranded (ds) or highly structured single-stranded (ss) RNA accumulates and that triggers the host silencing cascade. DICER-LIKE proteins assisted by dsRNA binding proteins cleave these RNAs into short interfering RNAs (siRNAs) of 21–24 nucleotides. Upon their methylation (Yang et al., 2006) siRNAs are recognized by and programmed into an RNA induced silencing complex (RISC) which targets and specifically cleaves cognate mRNA (Alvarado and Scholthof, 2009). The proposed model for RNA silencing in eukaryotes suggests that members of the ARGONAUTE protein (AGO) family form key catalytic units of RISC, which target RNAs for cleavage or translational repression (Baulcombe, 2004).

In the dicotyledenous plant model *Arabidopsis thaliana*, the functions and developmental regulatory capabilities of its 10 known AGOs have been fairly well characterized (Mallory and Vaucheret, 2010; Morel et al., 2002; Vaucheret, 2008) 2008 and Mallory and Vaucheret, 2010). For example, while AGO4, −6, and −9 carry

* Reprinted from Virology, Vol 486, Denis Odokonyero, Maria R. Mendoza (*shared first outhorship*), Veria Y. Alvarado, Jiantao Zhang, Xiaofeng Wang, Herman B. Scholthof. Transgenic down-regulation of ARGONAUTE2 expression in *Nicotiana benthamiana* interferes with several layers of antiviral defenses, 10p, Copyright 2015, with permission from Elsevier.

out transcriptional RNA silencing involving 24-nucleotide small RNAs (Havecker et al., 2010; Zheng et al., 2007; Zilberman et al., 2003); AGOs 1 and −7 are known to be programmed with 21- to 22-nucleotide small RNAs such as miRNAs, ta-siRNAs, or exogenously derived siRNAs, such as those from viruses and transgenes (Baumberger and Baulcombe, 2005; Montgomery et al., 2008; Qi et al., 2005). AGOs 1 and −10 are also required for translational control of other miRNA targets and autoregulation (Brodersen et al., 2008; Mallory et al., 2009). AGO1 is a critical developmental regulator, and *ago1* mutants display multiple phenotypes, most notably tubular shaped leaves that resemble the tentacles of an argonaute squid (hence the name-argonaute) (Bohmert et al., 1998). Up-regulation of *AGO1* mRNA has been observed to be a general response to virus infection (Csorba et al., 2007; Havelda et al., 2008; Zhang et al., 2006) probably as an innate defense mechanism. Accordingly, *ago1* mutants exhibit extreme susceptibility to virus infections (Morel et al., 2002). This and other work (Harvey et al., 2011; Jaubert et al., 2011; Qu et al., 2008; Wang et al., 2011) and as reviewed (Alvarado and Scholthof, 2009; Ding and Voinnet, 2007) strongly suggest that even though in Arabidopsis AGO2 and AGO7 may contribute to antiviral silencing, AGO1 is key to establishing an antiviral response, presumably because once programmed with siRNAs it forms the slicer component of RISC to specifically target the homologous viral RNA for degradation.

The functional analysis of AGOs in Arabidopsis was possible in part through the availability of shared genetic resources, including specific gene knock-outs. However, for studying plant–virus interactions Arabidopsis has its limitations because of the

relatively few viruses that infect this plant. Instead over many decades plant virologists have preferred to use *Nicotiana benthamiana* as the platform to study plant–virus interactions. However, even though the genome sequence (Bombarely et al., 2012) and transcriptome (Nakasugi et al., 2013) of *N. benthamiana* have recently been characterized there is not yet a library available with gene specific knock-outs. Instead gene knock-down studies on *N. benthamiana* are now routinely performed using *Tobacco rattle virus* (TRV) vectors to induce virus-induced gene silencing (VIGS) of specific host mRNAs (Burch-Smith et al., 2004). Even though the knock-down that is achieved yields incomplete loss-of-function (Orzaez et al., 2006; Pflieger et al., 2008), this may occasionally be advantageous, and importantly, sufficient to observe causal effects.

Despite the numerous advantages of the use of the VIGS approach, skeptics often point to possible limitations. For instance, an often-expressed concern is that VIGS necessitates the infection of a host with a virus (*e.g.*, TRV) that may perturb numerous host functions that in turn may mask or interfere with the manifestation of expected silencing phenotypes. This may become especially problematic when the (TRV)-infected plants are challenged with another virus to study its performance in a background where specific mRNAs are targeted by TRV-mediated VIGS. Even when including "empty-vector" TRV controls, this can lead to unexpected synergistic or antagonistic interactions that can influence observations and conclusions. Also, with VIGS experiments there is the potential influence of variation in experimental conditions, and plant-to-plant variation. When studying the antiviral silencing response there is also a paradoxical

situation that one depends on an active VIGS to inactivate silencing components that are necessary for VIGS.

Using TRV-mediated VIGS to reduce expression of individual AGOs in *N. benthamiana*, we recently reported that instead of AGO1 (as in Arabidopsis), the antiviral response in *N. benthamiana* against *Tomato bushy stunt virus* (TBSV) is controlled by an AGO2 (Scholthof et al., 2011) analog (NbAGO2). Partly because of the aforementioned reasons relating to possible issues with VIGS, but importantly also to: (i) address the question whether NbAGO2 is specifically used against viral RNA or also for silencing of endogenous (ds)RNA; (ii) to create a stable platform of plants with the inheritable *NbAGO2*-silenced trait; and, (iii) to permit studies on the involvement of NbAGO2 in developmental processes and antiviral defense, we aimed in the present study to transgenically silence *NbAGO2* in *N. benthamiana* without resorting to VIGS. For this purpose a dsRNA-hairpin approach was employed to effectively trigger transient or transgenic gene silencing of *NbAGO2* in *N. benthamiana.*

According with the topics mentioned above I established two different hypotheses and accompanying objectives that address NbAGO2 and antiviral activity.

Hypothesis 2

Transgenic *Nicotiana benthamiana* plants in which NbAGO2 is down regulated via dsRNA hairpin technology are more susceptible to virus infection.

To pursue this hypothesis my objectives were to:

- Validate that in plants positive for *NbAGO2*-hairpin expression, Argonaute 2 expression is down regulated using reverse transcription PCR.
- Determine susceptibility to different TBSV variants.

Hypothesis 3

NbAGO2 may possess antiviral silencing against viruses other than TBSV. To test this hypothesis I designed the following experiments:

- Test transgenic *NbAGO2*-hairpin plants against different viruses and observe symptom progression.
- Determine virus accumulation by western blot analysis of inoculated plants.

RESULTS

Biological properties of transgenic NbAGO2hp-expressing plants

Based on growth under kanamycin selection, putative transformants were selected and transferred to tissue culture containers (Magenta, Sigma-Aldrich). A leaf portion was used for DNA extraction that was then subjected to PCR screening for the presence of the *NbAGO2hp* containing T-DNA expression cassette (data not shown). Also, using primers designed to selectively amplify the endogenous *N. benthamiana AGO2* transcripts, we confirmed that the levels of *NbAGO2* had been reduced in at least 4 of the 6 selected putative *NbAGO2*-silenced transgenic plant lines, whereas the control plants expressing only the vector did not show the same reduction in transcript levels (Figure 3.1; and data not shown). Even though several independent transformant lines

(T0 and progeny) exhibiting the most substantial reduction on *NbAGO2* mRNA levels were used at various stages through the study, the results were similar as those presented in the following sections for two separate transgenic lines referred to as A21A and A24C. Because of the similar results obtained with independent transformants, the effects noted in this study are not line- or insertion-site specific, but are due to the silencing of *NbAGO2* caused by expression of *NbAGO2hp*. Semi-qRT PCR analyses of the T1, T2 and T3 generations showed that the *NbAGO2hp* constructs were maintained and expressed in subsequent generations resulting in a reduced level of *NbAGO2* expression, as shown for A21A-T1 in Figure 3.1. The expression level of other selected *NbAGO*s was not noticeably affected (Figure 3.1), confirming that the *NbAGOhp*-mediated silencing is specifically targeted to *NbAGO2* mRNA. Transformants were transferred to soil and plants grew normally and were brought to flower and seed. This indicated that reduction of NbAGO2 had no effect on normal developmental processes. Curiously, for reasons that are not clear, seeds of the transgenic plants failed to germinate on agar plates in the presence of kanamycin selection at 50 mg/L, therefore, seeds were placed directly in the soil and prior to experiments described in the following sections, plants were screened with PCR to verify their transgenic status. Plants were also periodically screened with RT-PCR or by TGdP19 inoculation (see section below), to verify the maintenance of decline in *NbAGO2* expression.

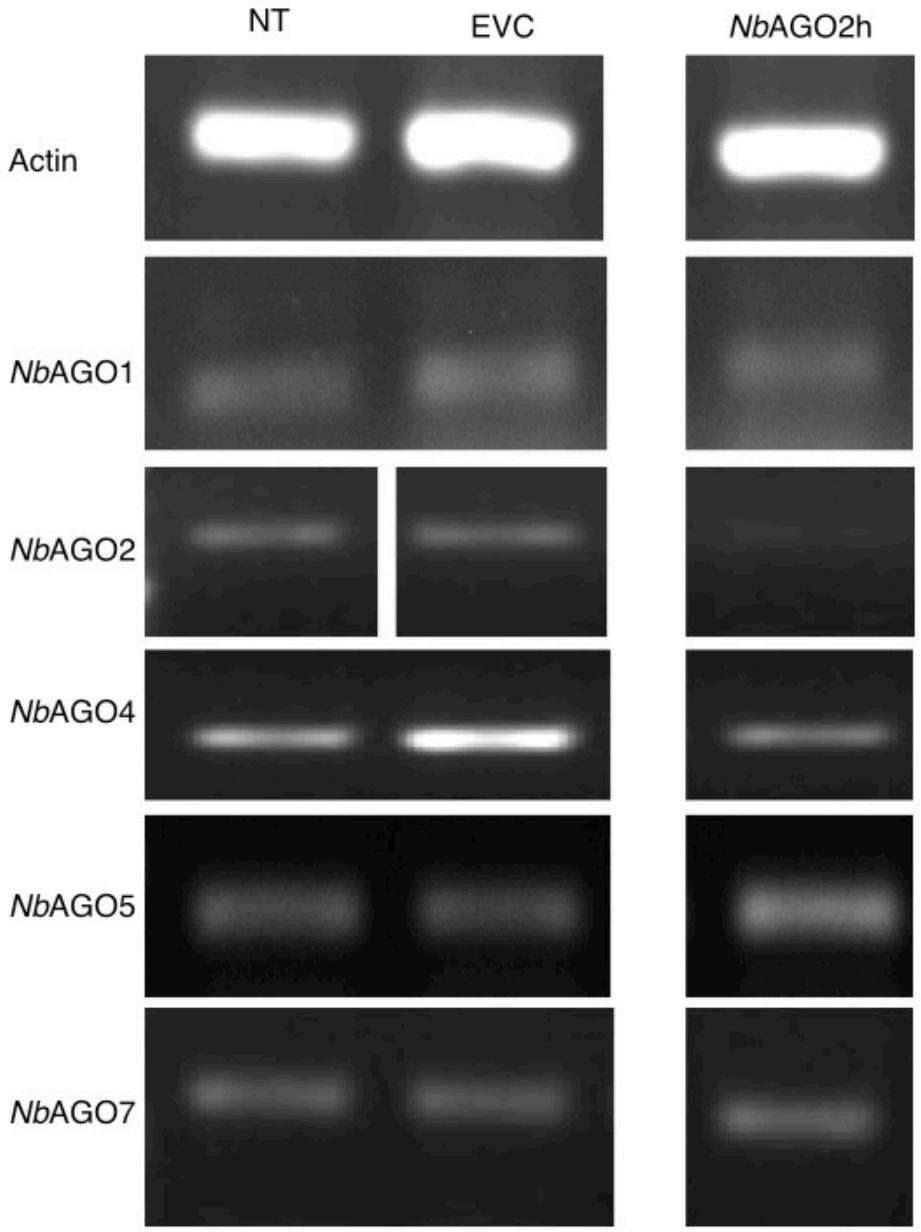

Figure 3.1. Hairpin-mediated down-regulation of *NbAGO*2 in transgenic *N. benthamiana*. RT-PCR was used to monitor mRNA accumulation in 4-week old non-transgenic (NT) plants, transgenic empty vector control (EVC) T1 plants, and *NbAGO2hp* transgenic T1 plants. As a control RT-PCR for actin was run for 26 cycles together with *NbAGO1*, *NbAGO*2 and *NbAGO4*; *NbAGO5* and *NbAGO7* were run for 35 cycles. Except for *NbAGO2* NT and EVC, PCR bands in each row are from the same gel with intervening lanes removed.

When seeds were germinated and plants grown under normal growth-chamber conditions, the next generation plants (T1, T2, etc.) developed normally (Figure 3.2), just as was observed for the T0 plants described above. However, as a result of serendipitous changes in growing conditions we observed that under sub-optimal conditions the transgenic plantlets were seriously stalled in developing beyond the 3–4th leaf stage (Supplementary Figure 3.1). During the course of study it was noted that these phenotypic effects correlated with the presence of the transgene as verified by PCR. At this point it is not known which external stimulus is responsible for this effect but it does indicate that under stress conditions, NbAGO2 is required at its normal levels early during development.

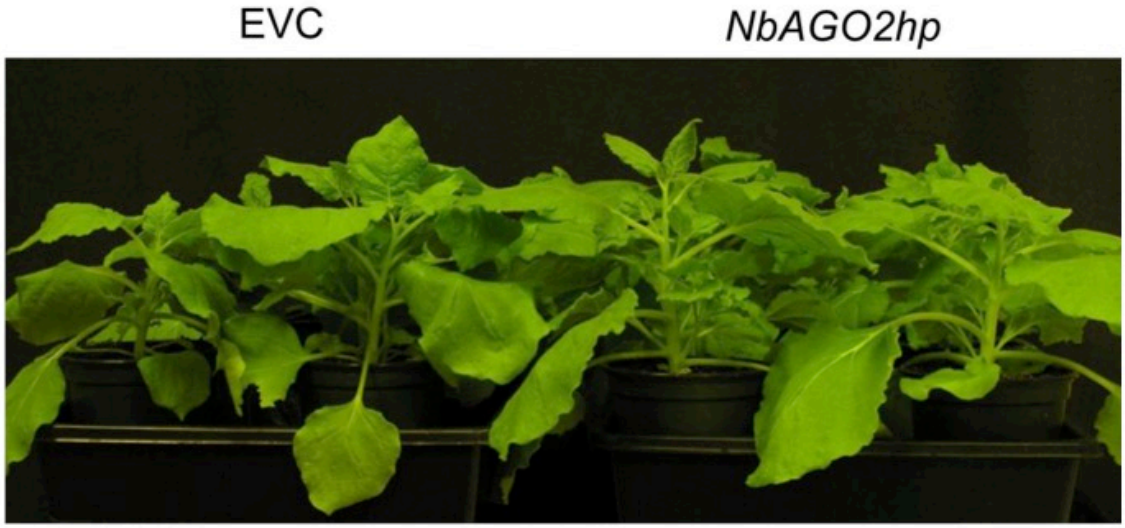

Figure 3.2. Phenotype of *NbAGO2hp*-transgenic *N. benthamiana* plants. The two plants on the left-half of the picture are empty vector control (EVC), and the two on the right-half are *NbAGO2hp*-transgenic. Five week old plants were grown at 25–26 °C, 60% humidity and 12 h light at 114 mm/m^2/s

Silencing against TBSV-GFP not expressing both CP and P19 is substantially impeded in NbAGO2hp-transgenic plants

Agroinfiltration assays with the CP-substitution TBSV-GFP (TG) and TGdP19 constructs on control plants (non-transgenic and empty-vector control plants) revealed that these plants mounted an effective antiviral response since little or no GFP accumulated (Figure 3.3) in the absence of the P19 suppressor. This was similar to previous results, just as in the presence of P19 (TG) no silencing occurred (Shamekova et al., 2014). However, in leaves of *NbAGO2hp*-transgenic plants, infections with TGdP19 resulted in an abundant accumulation of GFP almost resembling that of levels obtained with TG based on fluorescence (Figure 3.3A) and western blot assays (Figure

3.3B). This loss of silencing against TBSV phenotype was maintained into the T2 generation (Supplementary Figure 3.2). In the context of what is known for this system (Scholthof et al., 2011; Shamekova et al., 2014) this supports the conclusion that the antiviral response against TBSV not expressing both P19 and CP is seriously impeded in these transgenic plants.

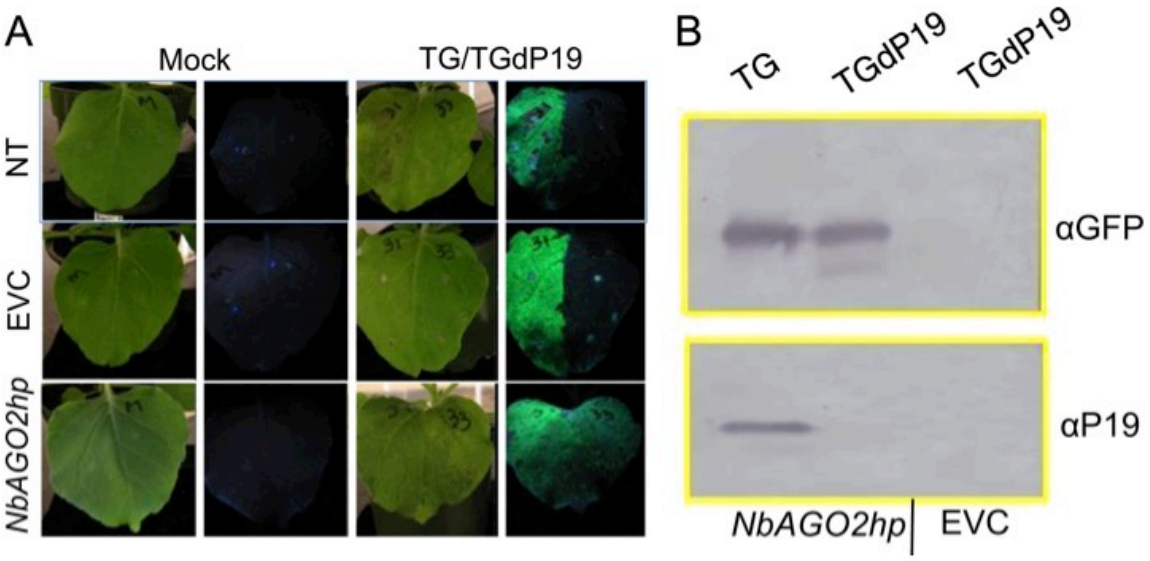

Figure 3.3. Silencing activity against TBSV-GFP. (A) Half leaves of 4-week old *N. benthamiana* plants were infiltrated with mock (left panel), or TG (31 on image) and TGdP19 (33 on image) (right panel), and inspected at 7 dpi. The leaves on the left of each panel are shown under ambient light and on the right under UV light. NT, non-transgenic; EVC, empty vector control; *NbAGO2hp*, hairpin transgenic. (B) Western blot showing GFP and P19 expression in 4 week old *N. benthamiana* plants infiltrated with TG and TGdP19, sampled at 8 dpi. Samples are from transgenic *NbAGO2hp* or EVC transgenic plants. Primary antiserum was for GFP (αGFP) or P19 (αP19).

Augmented systemic infections and symptoms by individual TBSV CP or P19 mutants, and other Tombusviruses

Infections of control *N. benthamiana* plants with a TBSV variant expressing its native CP but not expressing P19 (TdP19), yielded systemic infections with expected (Scholthof, 2006) mild mosaic symptoms. In contrast, TdP19 infections of *NbAGO2hp* transgenic plants resulted in the onset of severe symptoms such as extreme stunting, chlorosis and eventual necrosis (Figure 3.4), similar to what was observed when this same TdP19 was used to infect *N. benthamiana* in which *NbAGO2* expression was reduced by TRV-mediated VIGS (Scholthof et al., 2011). Clearly, reduction of NbAGO2 expression not only prevents silencing in inoculated leaves but is permissive for systemic infections and symptoms, somewhat resembling those elicited by infections in presence of P19, albeit much delayed.

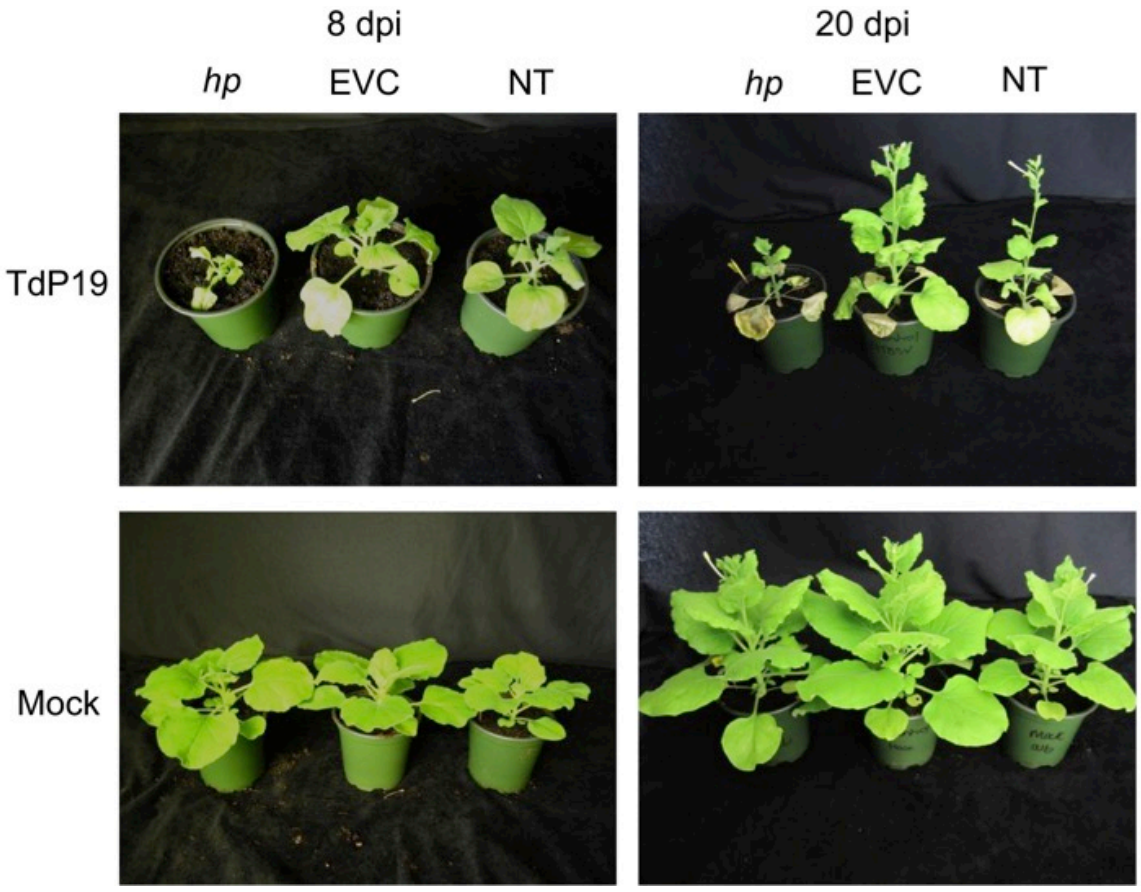

Figure 3.4. Symptoms on *N. benthamiana* upon rub-inoculation with TdP19 transcripts. Plants at 8 dpi (left) or 20 dpi (right). *NbAGO2hp* is indicated by *hp*; EVC, empty vector control; NT, non-transgenic.

In the P19-expressing TG construct the CP gene was replaced with GFP (Shamekova et al., 2014), and normally, infections of *N. benthamiana* with TBSV constructs in which CP is substituted are seriously impeded in the establishment of systemic infections and symptoms (Desvoyes and Scholthof, 2002; Everett et al., 2010; Scholthof et al., 1993; Shamekova et al., 2014). The same was observed in the present study upon infection of control plants with TG (Figure 3.5A). Conversely, infections of

NbAGO2hp transgenic plants with TG resulted in systemic infection accompanied by severe symptoms (Figure 3.5A), eventually culminating in a lethal necrosis. Evidently, the reduction in NbAGO2 also affects systemic infections, even in the presence of P19, suggesting that normally CP protects the viral RNA during systemic invasion but this protection is not needed when *NbAGO2* expression is compromised.

To test whether *NbAGO2* down-regulation still affected infections even when P19 and CP are both expressed, the *NbAGO2hp* transgenic plants were inoculated with wild-type TBSV. This resulted in accelerated plant death, compared to symptom progression in control plants (Figure 3.5B). We also tested other viruses in the *Tombusvirus* genus of the *Tombusviridae,* including *Cymbidium ringspot virus* (CymRSV), *Carnation Italian ringspot virus* (CIRSV), and *Cucumber necrosis virus* (CNV). In all instances systemic infections on *NbAGO2hp* transgenic plants progressed more rapidly than in control plants and the virus-dependent symptoms were more pronounced (Supplementary Figure 3.3), including accelerated onset of apical necrosis for CNV. Evidently, even when the virus is able to suppress silencing (using P19) and protect its genome during systemic transport (using CP) the effect of down-regulating NbAGO2 remains noticeable, indicative that the *NbAGO2hp* transgenic plants exhibit a general enhanced susceptibility to *Tombusvirus* infection.

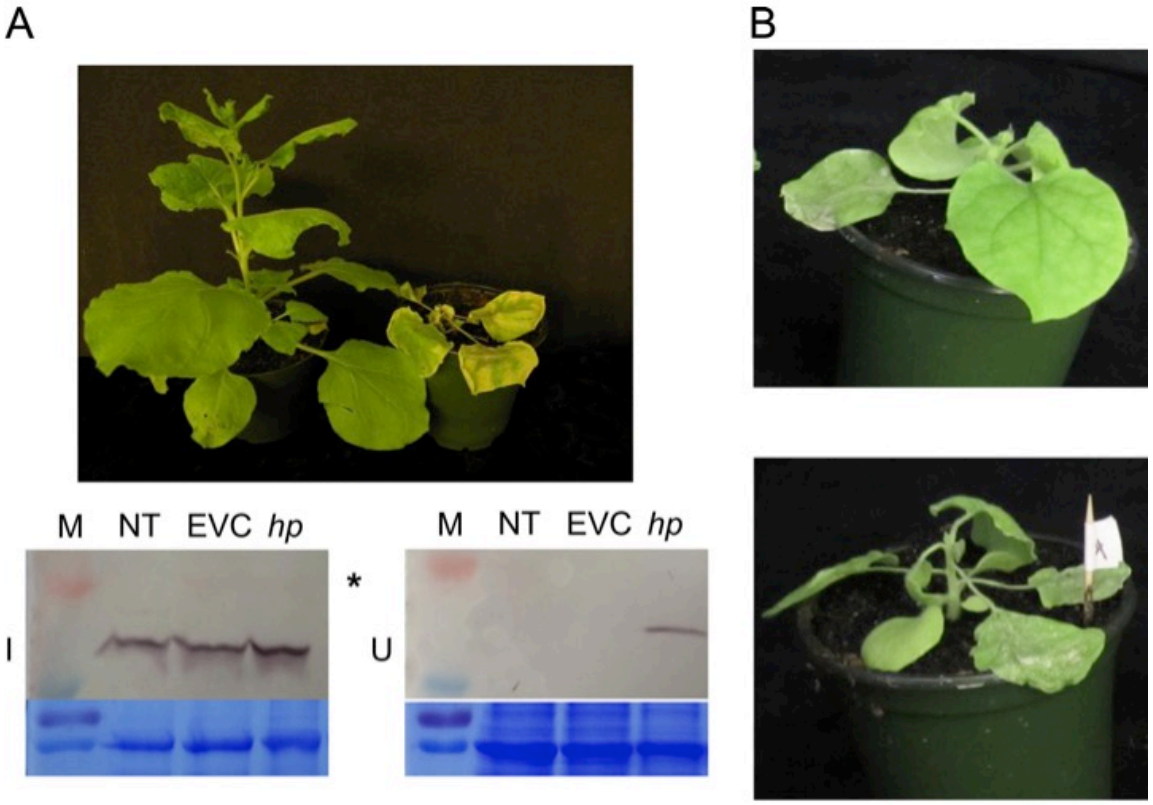

Figure 3.5. Symptoms on *NbAGO2hp*-transgenic plants upon inoculation of TBSV in absence or presence of coat protein. (A) Plants photographed 3 weeks after agroinfiltration with TG on non-transgenic (left) or *NbAGO2hp*-transgenic plants (right). The bottom panels show westerns using αP19 for extracts from non-transgenic (NT), EVC, and *NbAGO2hp*-transgenic plants (*hp*), taken from inoculated leaves (I) at 8 dpi, or upper leaves (U) at 13 dpi, on plants inoculated at the 5-week stage. The 25 kDa size marker (M) is indicated (*) and the bottom panels compare loading by staining with Coomassie Brilliant Blue. (B) Plants at 9 dpi with wild-type TBSV transcripts on non-transgenic (top) or *NbAGO2hp*-transgenic plants (bottom). Plants were 4 weeks old upon inoculation. The systemic necrosis is more advanced for *NbAGO2hp* plants compared to control plants where apical necrosis is just developing. Under the conditions used here (ambient light and temperature in laboratory), at 12 dpi *NbAGO2hp* died while the non-transgenic plants remained alive.

NbAGO2 and non-Tombusviruses

To test the effect of NbAGO2 on susceptibility of *N. benthamiana* to viruses other than TBSV, we initially experimented with TRBO-G a robust GFP-expressing *Tobacco mosaic virus* (TMV) variant that does not express CP (Lindbo, 2007) and thus only accumulates in inoculated leaves. We also experimented with a *Foxtail mosaic virus* (FoMV; *Potexvirus*) based vector (Liu and Kearney, 2010a) and a *Sunn-hemp mosaic virus* (SHMV; *Tobamovirus*) vector (Liu and Kearney, 2010b) that express GFP and do not accumulate in absence of an exogenously supplied suppressor, indicating that these viral RNAs are very susceptible to silencing. GFP expression by TRBO-G may have been somewhat elevated in plants in which *NbAGO2* was targeted by TRV-mediated VIGS, compared to controls (Odokonyero, 2013). However, this was not apparent upon inoculation of the *NbAGO2hp* transgenic plants, and likewise the inability of the FoMV and SHMV vectors to establish an infection was not restored upon TRV-mediated VIGS of *NbAGO2* (Odokonyero, 2013), or in *NbAGO2hp* transgenic plants (data not shown).

The above results suggested that NbAGO2 played no clearly discernable role in establishing an infection with the tested TMV, FoMV, and SHMV constructs in inoculated leaves. However, the results with TBSV had taught us that not all noticeable effects may be evident in the inoculated leaves, but rather the effect may surface at the level of systemic infection and symptom development. For this purpose, we infected *NbAGO2hp* transgenic plants with JL24, which is similar to TRBO-G with the difference that it expresses CP which promotes systemic infection (Lindbo, 2007). The results in

Figure 3.6 show systemic GFP expression in transgenic and control plants, indicating the establishment of systemic infections. However, when compared to controls it is evident that JL24 infections in *NbAGO2hp* transgenic plants exhibited much more severe symptoms to include excessive stunting, wilting of leaves, and ultimately plant death (Figure 3.6). Also, time-course studies using western blot analysis to better quantify GFP expression showed that systemic infections with JL24 were reproducibly accelerated by ~24 h in *NbAGO2hp* plants (Figure 3.7).

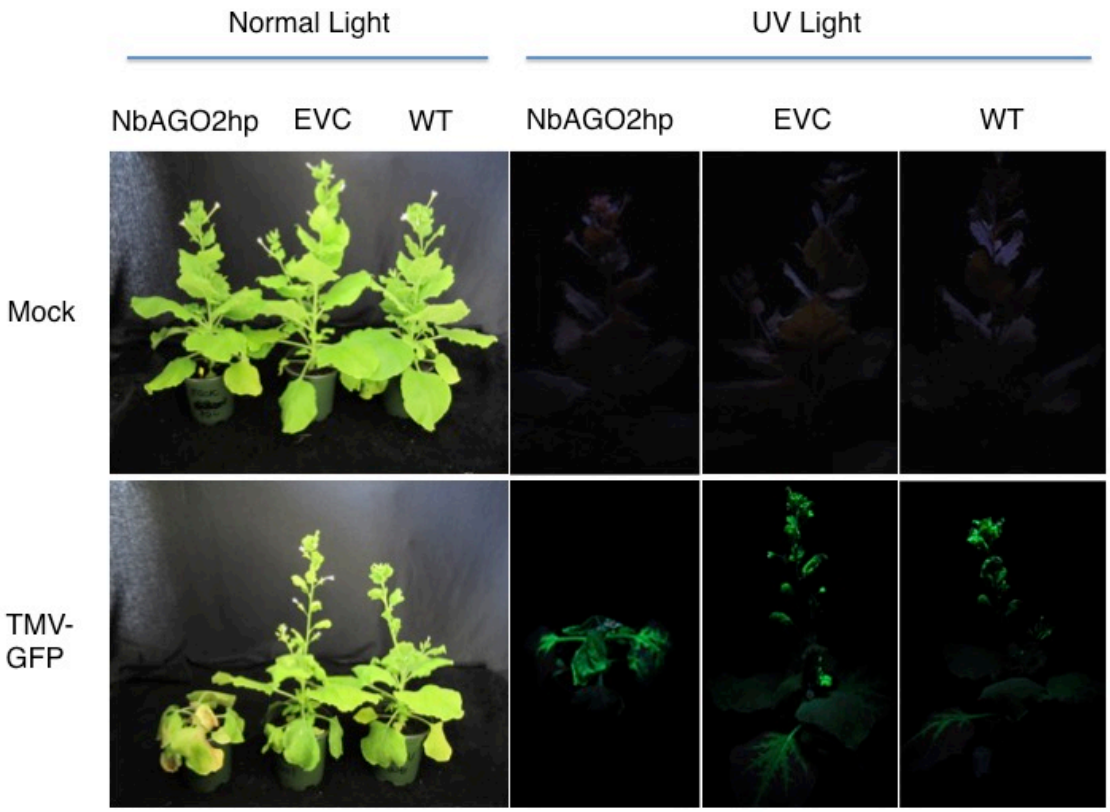

Figure 3.6. Infection of plants with TMV-GFP. Infiltration with JL24 (TMV-GFP) was performed on 5 week old plants and images were taken 3.5 weeks later either under normal light or under UV illumination. *NbAGO2hp*, hairpin transgenic; EVC, empty vector control; NT, non-transgenic.

When a *Potato virus X* expressing GFP (PVX-GFP) construct was agroinfiltrated on transgenic and control plants, we detected systemic GFP expression at 10–14 days accompanied by very mild symptoms (Supplementary Figure 3.4). However, compared to control plants, systemic infections (*i.e*., the appearance of green fluorescence in upper leaves) were noticeably and consistently accelerated by ~24 h in the *NbAGO2hp* transgenic plants (Supplementary Figure 3.4) and symptoms progressed to necrotic spots. This is in agreement with a reported defensive role for AGO2 against PVX in Arabidopsis (Jaubert et al., 2011). Also, at later stages, mature *NbAGO2hp* transgenic plants infected with PVX-GFP exhibited leaf malformations like those normally associated with reduced NbAGO1 accumulation (Jones et al., 2006; Scholthof et al., 2011).

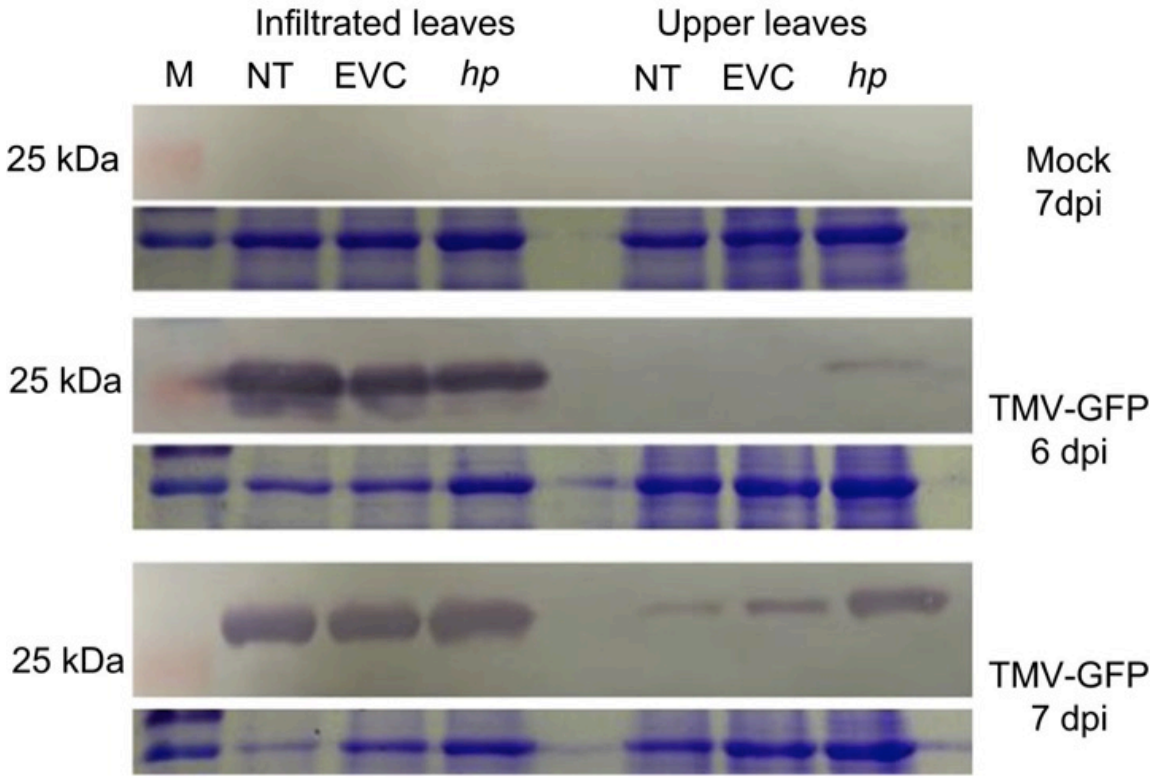

Figure 3.7. Western blot for GFP detection upon infection of *N. benthamiana* plants with TMV-GFP. In each of the three panels GFP is shown in upper half while the lower half shows the loading upon staining of the gels with Coomassie brilliant blue. Mock represent plants infiltrated with infiltration buffer alone and protein samples for this treatment were collected at 7 days post-infiltration. pJL24 (TMV-GFP) infiltrated samples were collected at 6 (middle) and 7 (lower) days post-infiltration. NT, non-transgenic; EVC, empty vector control transgenic plants; *hp*, *NbAGO2hp* transgenic plants. The position of the 25 kDa molecular size marker (M) is indicated.

Even though, as mentioned earlier, no defense-negating effects were measurable in *NbAGOhp* transgenic plant leaves inoculated with the SHMV-GFP *Tobamovirus* construct, effects were noted at the systemic infection level for the JL24 derivative of TMV, the type *Tobamovirus* species (Figure 3.6). This raised the possibility that a similar scenario could hold for FoMV (*Potexvirus*) for which no effect was seen in leaves inoculated with the FoMV-GFP vector (Odokonyero, 2013), and that possible

effects for FoMV should be investigated at the systemic level. For this purpose, transgenic and control plants, were inoculated with wild-type FoMV. However, no obvious differential effects were noted regarding the accumulation in inoculated infected leaves (Supplementary Figure 3.5) while measurements of effects on the ineffective systemic invasion were inconclusive. Therefore, NbAGO2-associated effects noted for PVX are not measurably recapitulated for its *Potexvirus* relative FoMV.

DISCUSSION

Transient silencing of NbAGO2

Transient assays with the *NbAGO2hp* or control infiltrated *N. benthamiana* leaves showed that upon agroinfiltration of these same leaves with TGdP19, GFP expression was evident only in *NbAGO2hp* treated leaves. This indicates the persistence of the effects in the host without significantly interfering with normal physiological processes. These observations not only further confirm the antiviral defense role of NbAGO2 against TBSV as previously reported (Scholthof et al., 2011), but also prove the effectiveness of hairpin RNA as a tool for silencing of *N. benthamiana AGO* genes. In fact, the efficiency levels are comparable to the commonly used TRV-virus induced gene silencing systems. Therefore, both techniques yield very comparable results and may remain attractive options if gene knock-out strategies, that are becoming increasingly applied for *N. benthamiana,* for instance by implementation of the CRISPR/Cas9 system (Li et al., 2014; Nekrasov et al., 2013), are not desired.

The results also suggest that a systemic 'hairpin-associated signal' had spread to adjacent leaves not infiltrated with the hairpin construct. RNA silencing has been shown to be non-cell-autonomous, with the capability of being induced locally and then spread to distant sites throughout the plant (Boerjan et al., 1994; Palauqui et al., 1996; Palauqui et al., 1997; Vaucheret et al., 1997) and propagated by means of some 'mobile signal' (Palauqui and Vaucheret, 1998).

Development of NbAGOhp transgenic plants

The results in Figure 3.1 and Figure 3.3 show that the transgenic dsRNA approach effectively and specifically down-regulated *NbAGO2* mRNA expression in transgenic *N. benthamiana* indicating that NbAGO2 is either not, or only at low levels, involved in transgenic dsRNA-mediated silencing. This supports our earlier observations using VIGS that NbAGO2 was neither involved in the ability of TRV to induce silencing nor in transient dsRNA-mediated silencing (Scholthof et al., 2011).

In earlier experiments no phenotypic effects were noticed when *NbAGO2* expression was down-regulated in *N. benthamiana* plants using TRV-based VIGS that was initiated on ~3 week old plants (Scholthof et al., 2011). Likewise, agroinfiltration with a *NbAGO2hp*-expressing construct in the transient assays did not lead to any noticeable phenotype either. Similarly, when *NbAGO2hp* transgenic plants are grown under normal conditions they are, at the macroscopic level, phenotypically indistinguishable from control plants. Furthermore, the transgenic plants flowered and set seed normally, and for the different generations these seeds were viable and

germinated at rates comparable to the wild-type and EVC-transformed plants. These findings suggest that under our conditions *NbAGO2* reduction did not cause irregularities in plant development. Alternatively, if in the future it turns out that *NbAGO2* is required for regulation of plant growth and development, it may be needed only in the trace amounts that are still produced upon its hairpin-mediated down-regulation.

When growth conditions were sub-optimal the development of the *NbAGO2hp* transgenic plants was compromised at early stages. This may be related to the observation that in comparison to the controls, we experienced some recalcitrance in the efficacy of regenerating *NbAGO2hp*-transgenic explants from callus. These phenomenological observations need further experimental scrutiny but they allude to a notion that NbAGO2 may have a supportive role early during development of *N. benthamiana* that surfaces under stress conditions, while at later stages it is no longer needed, or only at low levels, and can then be recruited into the plant's antiviral defense arsenal.

Antiviral effects against P19 and/or CP inactivated viruses versus wild-type versions

Upon agroinfiltration of the TGdP19 construct onto successive generations of *NbAGO2*-down-regulated transgenic plants (*e.g.*, Figure 3.3), we observed the accumulation of GFP in leaf tissue confirming the trans-generational persistence of *NbAGO2* gene silencing that protects the otherwise silencing-susceptible TGdP19. Clearly, when the virus is no longer protected from silencing by the P19 suppressor, it is

susceptible to the NbAGO2-mediated defense. But, this defense can be disarmed by reducing NbAGO2 accumulation.

In contrast to controls plants, *NbAGO2hp* transgenic plants infected with TdP19 (expressing CP, but not P19) exhibited severe and often lethal systemic symptoms, that are normally associated with P19 expression. This suggests that part of the severe symptoms associated with TBSV on *N. benthamiana* are attributable to interference with antiviral silencing to protect the virus, that can either be achieved naturally by P19-mediated suppression (Scholthof, 2006) or experimentally by down-regulation of *NbAGO2*.

Infections of *NbAGO2hp* transgenic plants with TG (expressing P19, but not CP) resulted in severe systemic symptoms (Figure 3.4) eventually leading to a lethal necrosis. Such severe systemic invasions are normally not seen for TG (Shamekova et al., 2014) or other CP-defective TBSV mutants in *N. benthamiana* (Desvoyes and Scholthof, 2002; Qiu et al., 2002; Qiu and Scholthof, 2001; Scholthof et al., 1993). These results suggest that the reduction of NbAGO2 in *NbAGO2hp* plants affects systemic infections of TBSV without the CP. It is plausible that under normal conditions the CP protects the virus against NbAGO2-associated antiviral defenses during systemic invasion, but when *NbAGO2* expression is compromised this protection by CP is less important, resulting in a severe systemic infection even in absence of CP.

Even though wild-type TBSV infections are accelerated in *NbAGO2hp* transgenic plants, the effect is much less extensive than as discussed above for the P19 and CP mutants, which suggest that other biological studies with wild-type versus

mutant viruses could also easily lead to different interpretations. For instance, it is known that various proteins of tombusviruses can cause a HR upon infection but even without a (functionally active) elicitor of the necrotic response the defense in the form of lesions or ringspots can remain operative (Angel et al., 2011; Angel and Schoelz, 2013; Chu et al., 2000; Chu et al., 1999). Specifically, when comparing results of defense responses on resistant *N. tabacum* (a necrotic local lesion host for TBSV, with P19 as elicitor) using TGdP19 not expressing CP (Sansregret et al., 2013) but reported as retracted (Sansregret et al., 2015) versus TdP19 expressing CP (Scholthof et al., 1995) important differences surface. The authors of the former study are reporting a finding that there is an extreme resistance (ER) response when using TGdP19, but in the much earlier study with TdP19 this ER was not noted since lesions still formed. Also, the two systems yield different interpretations about the possible involvement of siRNA binding for the resistance response (Hsieh et al., 2009; Sansregret et al., 2013) except that the second reference is retracted (Sansregret et al., 2015). These findings strongly suggest that the lack of CP influences the outcome of the studies, and it seems to seriously hinder TBSV invasion of *N. tabacum*, which apparently manifests itself as ER.

Our findings also show that while NbAGO2 is clearly involved in anti-TBSV defense, its antiviral activity may be more widespread than previously thought. GFP-expressing *Tobamovirus* and *Potexvirus* variants that are very sensitive to silencing, are not able to infect *NbAGO2hp* transgenic plants. However, the wild-type based *Tobamovirus* TMV-GFP did exhibit an enhanced ability to infect *NbAGO2h* transgenic plants while this was not evident for the *Potexvirus* FoMV. Yet, NbAGO2 down-

regulated plants show enhanced susceptibility to systemic infection with PVX-GFP, and also for several tombusviruses, and the same trend was observed upon infections with *Brome mosaic virus* (not shown). Collectively, the results show that depending on the virus and whether the virus construct expresses a suppressor and/or a coat protein, the contribution of NbAGO2 to the antiviral response may surface in inoculated leaves, during systemic transport, or in the onset and severity of systemic symptoms.

Roles of different NbAGOs in N. benthamiana

Several studies have shown that in Arabidopsis AGO1 plays a vital antiviral defense role with different levels of contribution by AGO2 and AGO7 (Alvarado and Scholthof, 2011; Carbonell et al., 2012; Harvey et al., 2011; Jaubert et al., 2011; Qu et al., 2008; Wang et al., 2011; Zhang et al., 2012) and more recently shown for AGO5 (Brosseau and Moffett, 2015). Much less is known regarding the antiviral activities of AGOs in *N. benthamiana,* but it was established that NbAGO4 has an effect on translation during a viral resistance response (Bhattacharjee et al., 2009). Furthermore, although NbAGO1 did not contribute to silencing of TBSV (Scholthof et al., 2011), it does have a novel translation-mediated defensive effect against *Tomato ringspot virus* infection in *N. benthamiana* (Ghoshal and Sanfaçon, 2014; Karran and Sanfaçon, 2014). The present study with transient and transgenic expression of *NbAGO2* hairpin RNA, firmly confirms that NbAGO2 is necessary for antiviral silencing against TBSV. Considering that Arabidopsis AGO2 has 'slicing' activity (Carbonell et al., 2012), it is possible that NbAGO2 represents a catalytic component of the enzymatically active

RISC that was previously isolated from TBSV (P19 mutant) infected plants (Omarov et al., 2007). Collectively, the reports thus far seem to support the premise that depending on the circumstances, several AGOs in plants can be deployed to combat virus infections.

METHODS

Agroinfiltrations and virus inoculations

Plasmids carrying TG and TGdP19 (Shamekova et al., 2014)(Shamekova et al., 2013), PVX-GFP (Peart et al., 2002) and JL24 (Lindbo, 2007) were also cultured in *Agrobacterium* GV3101 and agroinfiltrated (OD_{600}: 0.5) onto *N. benthamiana,* as above. In the case of wtTBSV, TBSVdp19 (Qiu and Scholthof, 2001), and the other tombusviruses *in vitro* transcription reactions were conducted on *Sma*I digested plasmids with T7 RNA polymerase (Fermentas Life Sciences). Transcripts were mixed in RNA inoculation buffer (0.05 M Potassium phosphate monobasic, 50 mM Glycine pH 9.0, 1% bentonite, and 1% Celite) and inoculated onto *N. benthamiana* plants, as described previously (Scholthof et al., 1993). FoMV-infected plant sap (from laboratory virus collection repository, (Mandadi et al., 2014)) was used as the source of inoculum in the present experiments.

Imaging of GFP fluorescence was performed as previously described (Everett et al., 2010).

Protein extraction and western blot assays

For each treatment 50 mg of plant tissue was collected, ground in liquid nitrogen and 300 µL of 5× Laemmli sodium dodecyl sulfate (SDS) protein extraction buffer was added. The samples were denatured in boiling water for 5 min and 25 µL loaded onto a 10% polyacrylamide-SDS gel and initially run at 60 V for 1 h and then 2.5 h at 110 V. The proteins were transferred to a nitrocellulose membrane (Biorad, CA) in transfer buffer (25 mM Tris, 192 mM glycine) at 260 mAmp for 90 min. Then, the membrane was blocked in 5% non-fat milk in a Tris-buffered saline solution (TBS) (0.2 M NaCl, 50 mM Tris, pH 7.4) for 1 h and incubated with the primary antibody (anti-GFP (B-2) mouse monoclonal (Santa Cruz Biothechnology) at 1:10,000 dilution, or rabbit P19 antibodies and anti-FoMVH93 (laboratory stock) at 1:3000 dilution) for 1 h. After incubation, three washes for 5 min each were performed. The secondary antibodies were anti-mouse or anti-rabbit alkaline phosphatase (Sigma) at 1:10,000 dilution. These were incubated for another hour, then 4 washes were performed with TBS and 1 wash with TBST (0.05% Tween) for 5 min. Finally, each membrane was incubated for colorimetric detection by using 1× alkaline phosphatase buffer (100 mM Tris, pH 9.5, 1 M $NaCl_2$, and 0.5 M $MgCl_2$ 6(H_2O)), 33 µL of NBT (250 mg/ml) and 66 µL of BCIP (100 mg/ml).

RNA and DNA extractions, and (RT)-PCR conditions

For total RNA extraction, 0.1 g of plant tissue was collected and ground using a mortar and pestle in 750 µl extraction buffer (80 mM Tris pH 8.2, 40 mM LiCl, 1.96 mM EDTA, 0.44% SDS, 78.57 mM NaAc pH 4.0, 0.44 M phenol, 87 mM

chloroform and 0.44 mM β-mercaptoethanol) and centrifuged at 12,000 rpm for 6 min at room temperature. The supernatant was collected and mixed in a 1:1 ratio with a phenol:chloroform:isoamyl-alcohol (25:24:1) mixture. The mixture was further centrifuged for 6 min and the supernatant was collected, mixed with chloroform at 1:1 ratio and centrifuged at 12,000 rpm for 6 min. The supernatant was collected, mixed with 1/3 volume of 8 M LiCl and precipitated overnight at −20 °C. The RNA was pelleted by centrifugation at 4 °C at 13,000 rpm for 20 min, and the pellet washed in 70% ethanol and resuspended in 30 µl DEPC-treated water. The obtained RNA was treated with Turbo DNA-free kit (Applied Biosystems) prior to cDNA synthesis using oligo-d(T) primers and M-MLV reverse transcriptase (Invitrogen).

To extract plant genomic DNA, approximately 200 mg of leaf tissue was thoroughly macerated in 750 µL of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol) using a mortar and pestle. Then, 35 µL of a 20% SDS solution was added and incubated in a 65 °C heat block for 5 min followed by addition of 130 µl of potassium acetate, gentle mixing and incubation on ice for 5 min. The resulting debris was then pelleted by centrifuging at 15,000*g* for 10 min at room temperature. The supernatant was extracted and 750 µL of isopropyl alcohol followed by 75 µL of 3 M NaAc pH 5.2, were added, the tubes gently inverted to mix and at incubated in a −20 °C for at least 1 h. DNA was then pelleted by centrifugation at 15,000*g*, the supernatant was discarded and 70% ethanol was used to wash the resulting pellet. Excess ethanol was evaporated in a spin-vacuum centrifuge for approximately 30 min. The DNA was then resuspended in 30 µL of TE containing

20 µg/mL RNase. The mixture was incubated at 37 °C for 15 min and then centrifuged at 15,000*g* for 5 min and diluted to a final volume of 60 µL for immediate use or storage at −20 °C.

PCR was carried out to verify the presence of the *NbAGO2hp* construct as well as transcript levels of *NbAGO2*. Primary transformants were screened by regular DNA extraction followed by conventional PCR and first and second generation transgenic plants (T1 and T2) were screened with Terra PCR Direct Polymerase kit (Takara Biotech) using primers for native *NbAGO2* sequence 5′-GAGCACTTGGCTGAACATGA-3′ and vector sequences 5′-CTTGTAGTTTTATTAACTTCT-3′. Primers for amplification of cDNA to measure relative transcript levels are presented in Table 1.

Table 1. Primers sequence used for quantification of RT-products

Gene	Forward	Reverse
Actin	5'-GCTTCAGTGAGTAGTACAGGGTGTTC-3'	5'-ATGGCAGACGGTGAGGATATTCA-3'
AGO1	5'-CATACCCAGTGGCCTTGTCT-3'	5'-ATTCGATTGCCAAACTCC-3'
AGO2	5-CTAGCCAACAAGGGACAAGAT-3'	5'-TAGGCAACAAGGTCAGCATAG-3'
AGO4	5'-AAGGGCGTGTTCTGCCTGCC-3'	5'-GCCTGCATGGGCACACAGGT-3'
AGO5	5'-GCCACCTGCCTATTACGCCCA-3'	5'-TCCGAAACCCACCATACAGTTGC-3'
AGO7	5'-CGGCCGGGATGTCAAAGGTGT-3'	5'-CGCTCGGGGAGTTTTAGAAGC

CHAPTER IV

VIRUSES AS PROTEIN EXPRESSION TOOLS

INTRODUCTION

Expression of foreign proteins in plants is normally achieved via transformation with *Agrobacterium tumefaciens* or by biolistics, to generate stable transgenic plants within several months by incorporating foreign DNA into the plant chromosome (Scholthof et al., 2002). Another technique that recently has been optimized is to use virus systems to express proteins in plants (Lindbo, 2007). This has the advantage of fast expression and high yields within a few days after infection, and consequently larger protein quantities can be isolated from plant host (Gleba et al., 2007; Gleba et al., 2004; Porta and Lomonossoff, 2002; Scholthof et al., 2002) compared with normal expression approaches.

Several plant viruses have been designed to serve as vectors for expression of foreign proteins and the most promising to use are ssRNA viruses, such as TMV, PVX, CMV (*Cucumber mosaic virus*), and CPMV (*Cowpea mosaic virus*) (Hefferon, 2014). One disadvantage of virus-mediated protein expression is that due to size constraints most viruses only support the expression of one (often small) protein (Gleba et al., 2004), while many bioactive proteins are often oligomers of different proteins. To overcome this limitation requires the use of two non-competitive plant viral vectors to achieve expression of more than one protein. This has been reported by co-expressing two polypeptides using two vectors, TMV and PVX (Giritch et al., 2006).

In the laboratory there is an ongoing interest to design and use new TBSV-based expression vectors. As mentioned before, TBSV is an ssRNA virus with an icosahedral structure that can be transmitted by mechanical inoculation, and it is dispersed by seed and soil; this virus does not have a biological vector for transmission (Yamamura and Scholthof, 2005). Previous results have shown that TBSV-GFP constructs successfully infect *N. benthamiana*, cowpea, tomato and lettuce (Shamekova et al., 2014), which provides evidence for a wide host range for transient expression of proteins. Another promising vector is based on TMV (Lindbo, 2007). TMV rapidly infects plants, accumulating to high titers. TMV has no known biological vector and it is very easily transmitted by rub-inoculation (Scholthof, 2004). TMV expresses four different proteins, the 126-kDa protein and 183-kDa (expressed by a read-through amber stop codon) replicase proteins, a 30-kDa movement protein and the 17-kDa capsid protein (Scholthof, 2004). A most promising TMV virus vector TRBO, has the coat protein replaced with GFP to yield high levels of protein expression (Lindbo, 2007) (Figure 4.1). The question I posed was whether TBSV and TMV virus vectors could express foreign proteins at high levels in different plant species and in the same cells even upon the induction of silencing because that would be suppressed by P19.

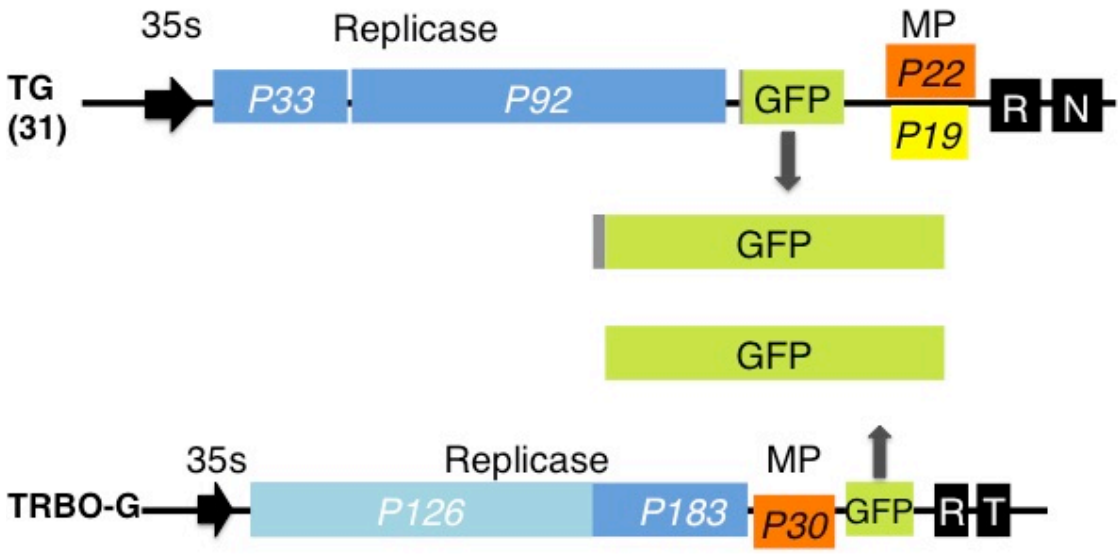

Figure 4.1. TG (31) and TRBO viral vectors expressing GFP. GFP from TG contains 17 extra amino acids extra that correspond to capsid protein. The dark box labeled 'R' at the 3' end represents cleaving ribozymes, 'N' refers to nopaline synthase terminator and 'T' refers to the CaMV poly (A) signal.

It is not known if TBSV and TMV can co-infect plants, even thought these viruses have been studied for almost 100 years. This is an interesting biological question in itself but also in the context of using virus vectors. For my research, I plan to test if TG (p31) and TRBO can infect several host species and test whether these two constructs can coexist for expression of different proteins in the same cell without competition.

Hypothesis 4

Viruses can be used as gene expression vectors, but the expression is often limited to a single protein, and this expression is often hampered by RNA silencing.

Since TBSV expresses the P19 suppressors I postulated that TBSV and TMV virus vectors can be used to express different foreign proteins in several plant species and in the same cell.

To test this hypothesis I proposed the following objectives:

- Express GFP in three different plant species with TBSV-GFP and TMV-GFP vectors.
- Determine virus accumulation by western blot assay of agroinfiltrated plants.
- Determine if TBSV-GFP and TMV-RFP are co-expressed in the same cells.

RESULTS

Agroinfiltration in different hosts

N. benthamiana is a common experimental plant to study virus-mediated expression of proteins. However, other host platforms are desirable, for instance in vegetables to express nutritionally beneficial proteins. Previous work in the laboratory had pointed towards tomato, lettuce and cowpea as good expression platforms for TBSV (Seaberg et al., 2012). However in those experiments the virus was inoculated as RNA, which has much lower efficiency than the agroinfiltration technique used in my studies. Therefore, I agroinfiltrated *N. benthamiana*, tomato, lettuce and cowpea with TG (also known as 31), and the results (Figure 4.2) showed that these plants support TG-mediated GFP expression. Even though TRBO-GFP was able to infect *N. benthamiana* and tomato, it did not yield observable levels of GFP flourescence in lettuce and cowpea

(Figure 4.2). The co-infections and therefore these GFP images did not yet reveal whether both vectors were co-infecting and/or co-expressing.

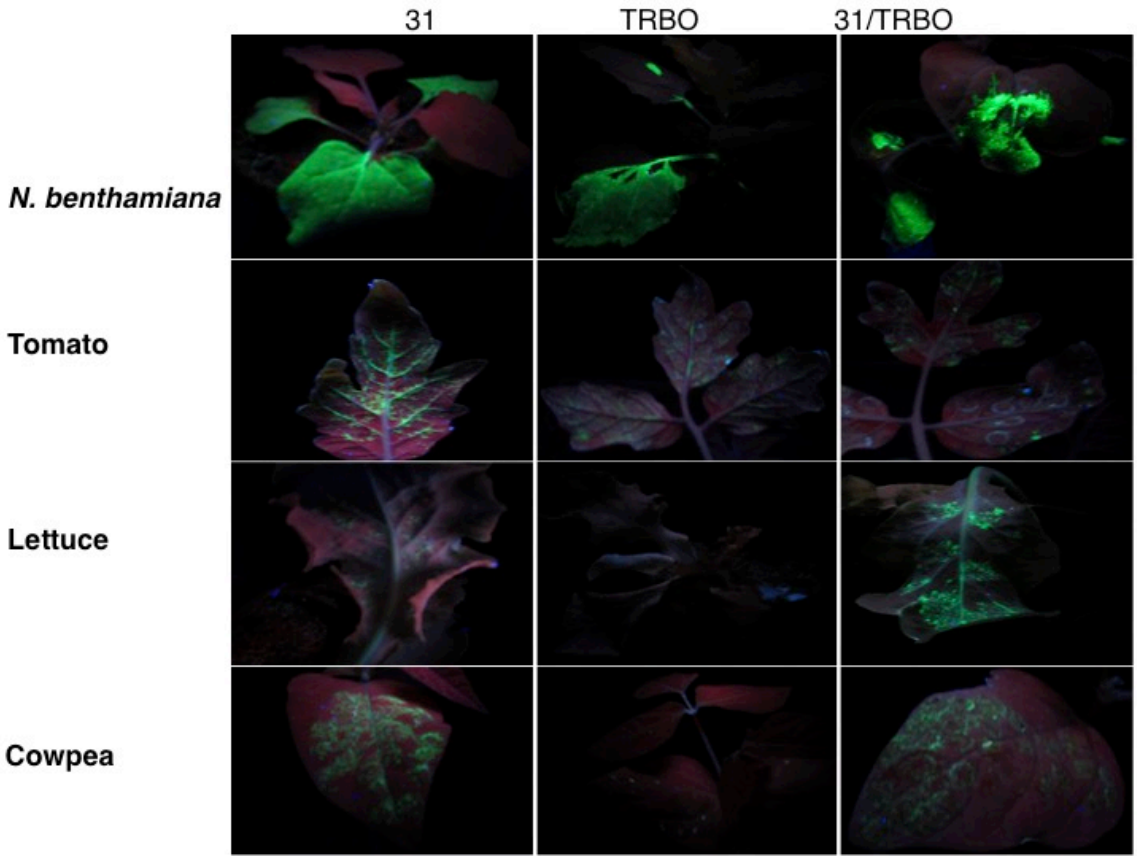

Figure 4.2. GFP expression upon *Agrobacterium* mediated infiltration of viral vectors in 4 week old *N. benthamiana*, 3 week old tomato, 2 week old lettuce and 1 week old cowpea and observation GFP expression was visualized with UV illumination at 3 days post infiltration.

Western blot analysis was performed to detect GFP (Figure 4.3) and the results indicated that tomato is a host for TRBO and that a co-infection of 31 and TRBO

permits protein expression (two faint bands, see below for details). In this particular experiment the GFP expression in tomato did not show GFP signals for TG but it was observed in other experiments (data not shown). In the case of lettuce and cowpea, the TG vector expresses GFP but not TRBO-GFP.

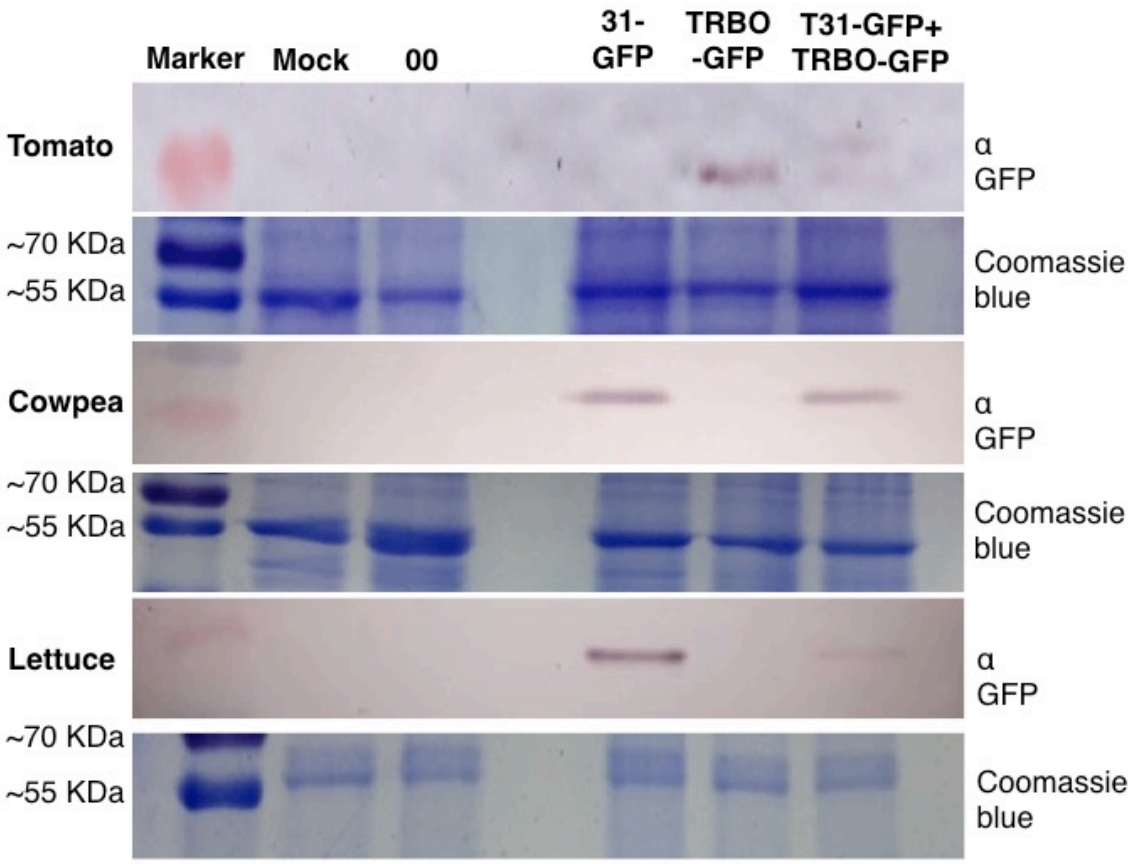

Figure 4.3 Western blot for GFP in tomato, cowpea and lettuce. TG-GFP (31) samples were collected at 3 dpi while Mock, 00, TRBO-GFP and TRBO-GFP+T31-GFP were collected at 7 dpi. Primary GFP antibodies were used (αGFP), and Coomassie staining was performed to provide loading comparisons.

Co-expression by vectors

As depicted in Figure 4.1 the GFP protein expressed from TG is expected to be somewhat larger than the expressed by TRBO-GFP due to the addition of 17aa N-terminal from TBSV coat protein. This provided a convenient tool to measure if upon co-infection both vectors indeed expressed GFP. The western blot analysis did show that the GFP expressed from the TG vector has indeed a slightly larger molecular mass compared to GFP from TRBO and both are detected when a co-infiltration is performed in tomato (Figure 4.3) and *N. benthamiana* (Figure 4.4). These data demonstrate that TG (31) infiltrated together with TRBO-GFP can coexist in the same leaves of *N. benthamiana* and tomato.

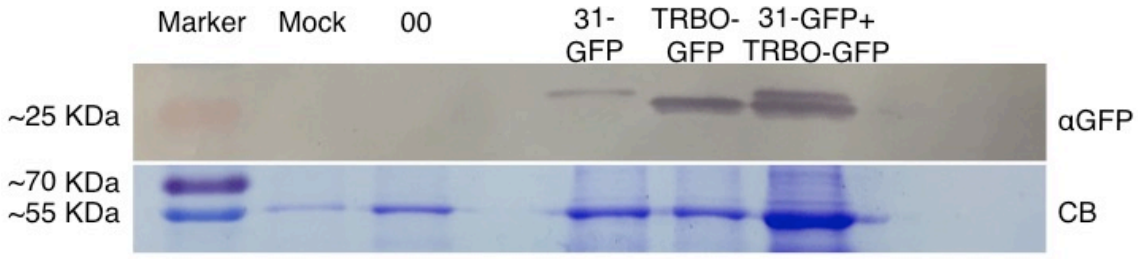

Figure 4.4. GFP accumulation in *N. benthamiana* plants after agroinfiltration with cultures containing a viral vector. Plant samples were collected for 31/TG at 3 days post infiltration (dpi) and for TRBO and combination of vectors at 7 dpi. The upper panel is a western blot for GFP detection using anti-GFP antiserum (αGFP), the lower panel the corresponding Coomassie blue (CB) stained gel for loading comparison.

Once it was routine to detect co-expression of GFP from the two constructs, I evaluated how many days protein expression was maintained. For this, agroinfiltrated plants were monitored every day under UV light and 50 mg of tissue was collected from each plant for protein analysis. The resulting western blot shows that the TG vector starts expressing detectable amounts of GFP starting at day 3, and protein intensity increase up to 7 days. After this time infiltrated *N. benthamiana* leaves started to wilt and were no longer conducive for protein assays because of P19. In the case of TRBO, this vector start accumulating GFP was observed at 3 days post infiltration, and reaching the highest level at 4 dpi, which was maintained until the end of the time course study (Figure 4.5).

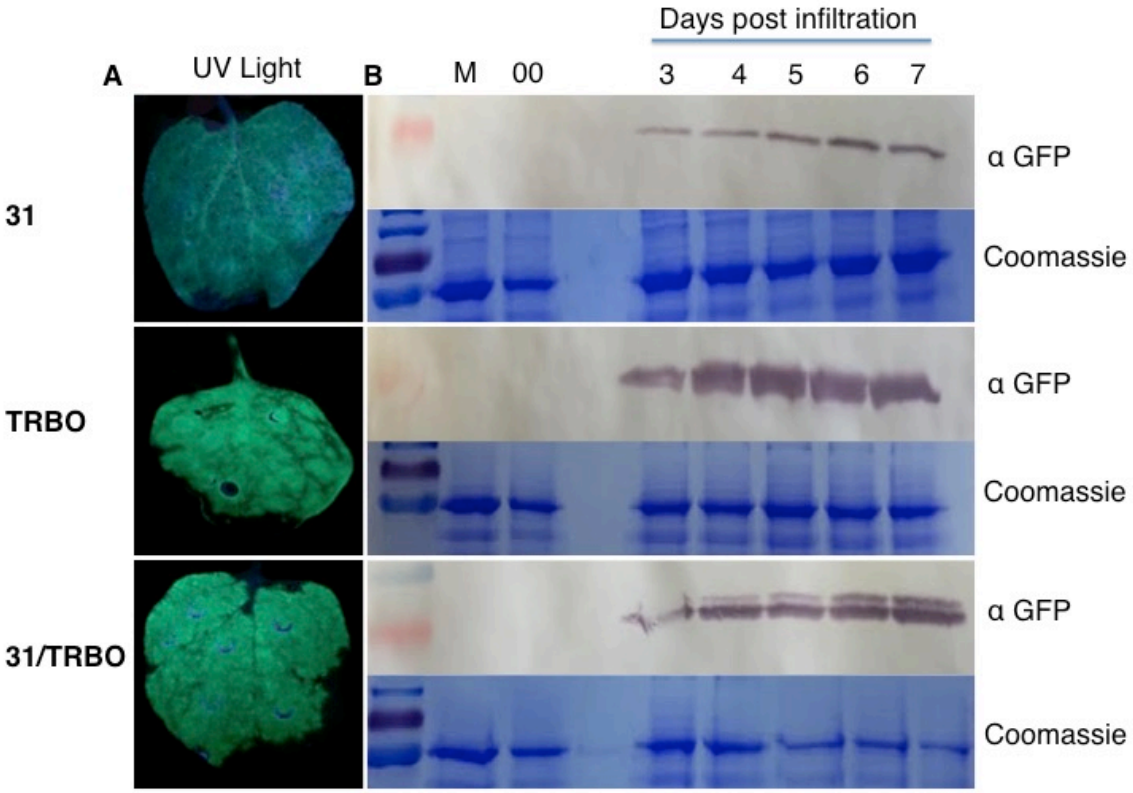

Figure 4.5. GFP analysis in *N. benthamiana* from western blotting experiments and ultraviolet light examination. (A) GFP analysis at 3 dpi in *N. benthamiana* infiltrated with TG (31) and TRBO, individually or co-infiltrated (B) GFP protein analysis in *N. benthamiana* infiltrated as for A. GFP expression for TG (31), TRBO and TG (31+ TRBO (31/TRBO) was monitored each day at 3 to 7 days post infiltration (dpi) with mock (M) and 00 collected at 7 dpi. The upper panels are western blow assays for GFP detection, the lower panels show the corresponding Coomassie blue stained gel for loading comparison.

The above experiments showed that TG and TRBO were co-expressing GFP in the same leaves. However, these analyses could not address whether both vectors were present and expressing in the same cells. Therefore, it was necessary to take another approach by having one of the two vectors express RFP instead of GFP. For this purpose, TRBO-GFP was digested to remove the GFP and replaced with RFP. The resulting TRBO-RFP was infiltrated in *N. benthamiana* leaves individually or in combination with TG. Fluorescence microscopy (Figure 4.6) showed that TG-mediated GFP expression and TRBO mediated RFP expression were readily apparent in single epidermal cells. This was inferred from the orange fluorescence, as would be expected by overlaying green and red fluorescence. Therefore, collectively the results showed that TG and TRBO vectors can co-express in the same plant, the same leaves, and in the same cells.

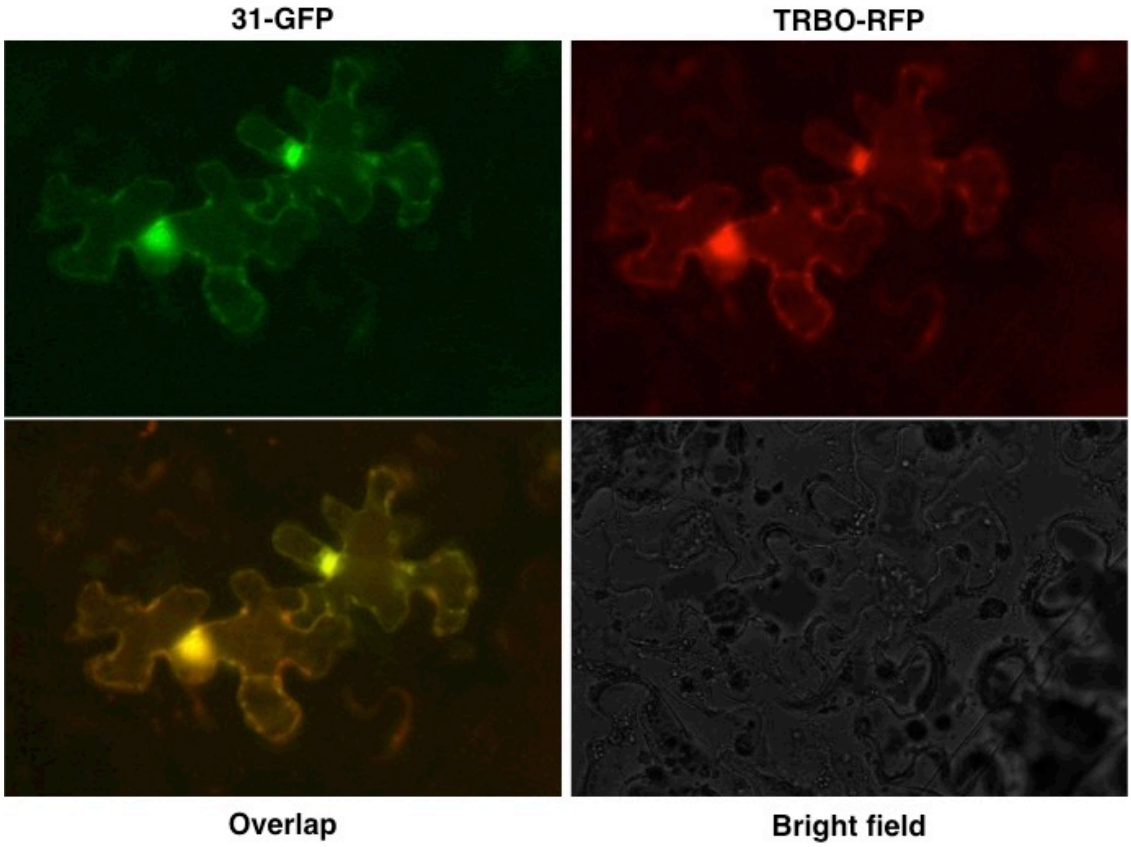

Figure 4.6. 31/TG and TRBO co-agroinfiltration on 4 week old *N. benthamiana*. Plant epidermal cells were observed 10 days after infiltration. Fluorescence occurs throughout the cytoplasm and the bright spots probably reflect accumulation of fluorescent components in the cytoplasm surrounding the nucleus.

DISCUSSION

The use of Agrobacterium to express foreign proteins has been widely reported. The utility of TG expressing GFP in *N. benthamiana*, tomato, lettuce and cowpea has just recently been described (Shamekova et al., 2014). TRBO has been reported as a powerful vector with the capacity to infect several plants (Lindbo, 2007). In the present study, I verified the utility of these infection vectors and compared their expression in

the aforementioned hosts: as individual and as co-expressing vectors. I conclude that TG and TRBO are capable of infecting and expressing high levels of proteins in *N. benthamiana* and tomato. However, only the TG vector is available to infect and express proteins in cowpea and lettuce. Visual comparisons indicate that TRBO expression is greater than observed for TG but the differential host susceptibility suggests that the added utility of the TG system is that it infects a wider range of plant species.

It was also shown that TG and TRBO co-express their respective foreign proteins in the same leaves in *N. benthamiana* and tomato. Imaging assays performed under UV-light, western blot analyses, and fluorescent microscopy verified that co-expression occurs for these two vectors. Only a few papers have reported that two different backbone virus vectors can co-exist in the same host (Gleba et al., 2007; Gleba et al., 2004). Here, the novelty is that TRBO, a TMV vector with high levels of protein expression, can be combined with TG, a TBSV-based vector reported with similar capability of expressing foreign proteins. Because the two viral vectors can co-exist at the cellular level this opens up possibilities to co-express peptides and proteins in vegetables for nutrition and for biomedical proposes such as generation of substrates for vaccines, and other bioactive oligomers.

Another interesting feature is that both TBSV and TMV have been extensively studied the past century (Hull, 2014), but no information is available on co-infections in nature or under experimental conditions. In my experiments the two were forced together by agroinfiltration and this demonstrated that at the molecular levels there is no hindrance for both viruses to accumulate in the same plants, leaves, and cells. A question

thus far unanswered is whether in nature some unknown property is prohibiting their co-existence, or that it readily occurs but simply has not been reported.

METHODS

To prepare infiltration cultures of the viral vector constructs, Agrobacterium GV3101 containing a viral construct was grown at 28°C overnight in LB media containing 50 mg/L kanamycin. Then, a subculture was prepared to grow bacteria cultures in LB media with 50 mg/L kanamycin, 20 μM acetocyringone and 10 mM MES. The day of infiltration bacteria were collected by centrifugation at 4000 rpm for 20 min, the supernatant was discarded and bacteria pellets were resuspended at 0.5 600 OD in infiltration media (10 mM $MgCl_2$, 10 mM MES pH 5.6 and 200 μM acetosyringone). After cultures were suspended in infiltration media, they were incubated for 4-6 hours in the dark at room temperature. Leaves of plants were infiltrated at the abaxial side using 1 ml syringe.

N. benthamiana was infiltrated at 3-4 weeks, and based on preliminary tests, it was determined that cowpea required 1-week old plants, Grand Rapids lettuce 2 week old plants, and tomato 3-week old plants. Plants were grown in a growth chamber with 60% humidity, 22°C for 16 h under light, 20°C for 8h under dark. The treatments for these experiments were: Mock (infiltration buffer only), TRV-00 (which contains an empty TRV vector), TG and TRBO-GFP constructs. After infiltration, plants were monitored at 3-5 days and 50 mg of plant samples were collected for protein expression

analysis. GFP imaging and western blot analyses were performed as described in Chapter III.

To determine co-expression, TRBO-RFP was constructed. For this, TRBO-GFP was digested with with *Pac*I and *Not*I to remove the GFP gene and this was substituted with the RFP sequence amplified with the same restriction enzyme sites at the termini, using standard PCR and cloning techniques. Agroinfiltration with this construct was performed at 0.3 600 OD together with TG (31). Infiltrated plants were visualized under the fluorescent microscope for GFP and RFP expression.

Microscopy was performed on Olympus BX51 microscope (Olympus America, Melville, NY, USA) and images were captured with Hamamatsu Orca-ER cooled CCD camera (Hamamatsu, Japan). For GFP visualization an Olympus U-MNIBA2 filter cube with excitation wavelengths from 470 to 480 nm, emission wavelengths from 510 to 550 nm, and a dichroic mirror at 505 nm. For RFP visualization an Olympus U-MNIBA2 filter cube was used with excitation wavelengths from 350 to 550 nm, emission wavelengths from 11 590 to 630, and a dichroic mirror at 570 nm. Images were acquired using Slidebook Version 5, which controlled a Prior shutter (Prior Scientific, Rockland, MA, USA).

CHAPTER V

CONCLUDING REMARKS

This study provides evidence regarding the role of NbAGO2 in antiviral silencing. In Chapter II, the results of measuring NbAGO2 accumulation upon TBSV inoculation by *Agrobacterium* indicated that NbAGO2 is highly regulated upon TG (31) inoculation. Likewise, plants rub-inoculated with viral transcripts showed that NbAGO2 was also highly induced compared to NbAGO1 in 3-week and 6-week old plants, demonstrating that NbAGO2 is sensitive to TBSV infections. Moreover, 6-week old plants have the ability to silence TBSV-157 (not expressing P19), which agrees with the notion that NbAGO2 is important for TBSV silencing. However, even though NbAGO2 is highly induced in 3-week old plants, this induction did not prevent virus infection. This suggests that NbAGO2 plays a role, probably with other host factors that we have not identified. Also, this study supports that NbAGO1 is not required for TBSV silencing and that the effect of TBSV on NbAGO5 accumulation would agree with the suggestion that an NbAGO5-like component contributes to viral silencing of TBSV. Ultimately, my hypothesis was partly supported in that NbAGO2 silencing against TBSV is dependent on plant age. I also confirmed that NbAGO2 is induced to stimulate TBSV silencing, especially when the virus does not express the viral suppressor P19 in older plants, which agrees with the observation that older plants are able to silence the virus better than younger plants. Why the younger plants are ineffective in antiviral silencing is not yet clear.

Chapter III supports my hypothesis that NbAGO2 is required for silencing by conducting experiments with transgenic NbAGO2-hairpin plants. Down-regulation of NbAGO2 in these plants was confirmed by measuring mRNA levels. Compared to non-transgenic plants, the transgenic hairpin plants were much more susceptible to infection with TBSV deficient in CP or P19 expression. This study also indicates a general role of NbAGO2 towards silencing of members in the tombusvirus genus, as inferred from the accelerated plant death in transgenic hairpin NbAGO2 plants compared to controls. Additionally, after testing viruses not related to TBSV, it can be concluded that NbAGO2 provides antiviral effects at several layers, because of enhanced symptoms after TMV (PJL24) infection and accelerated systemic infection of TMV (PJL24) and PVX-GFP. These results agree with the hypothesis that NbAGO2 possesses antiviral silencing against viruses other that TBSV. From this, I conclude that down-regulation of NbAGO2 in *N. benthamiana* increases the overall pathogenic effects of virus infection.

Chapter IV reports that two well-studied viruses, TBSV (TG) and TMV (TRBO), are excellent candidates to express and co-express high levels of proteins in the same host, the same leaf and at the cellular level. Co-expression can be achieved in *N. benthamiana* and tomato and high protein levels are detected after forcing TG (31) and TRBO to co-express proteins. Future experiments can determine if the expression levels will further benefit from NbAGO2 down regulation.

As a final conclusion, this study shows that *N. benthamiana* AGO2 is required for viral silencing in older plants and that viruses use suppressors to incapacitate plant RNA silencing. Thus, NbAGO2 provides protection against different viruses, an effect

that is abrogated when NbAGO2 is down regulated in the plant. Also, by overcoming silencing through the use of suppressors TBSV and TMV vectors can be engineered to co-express peptides and proteins that have potential for biotechnology applications.

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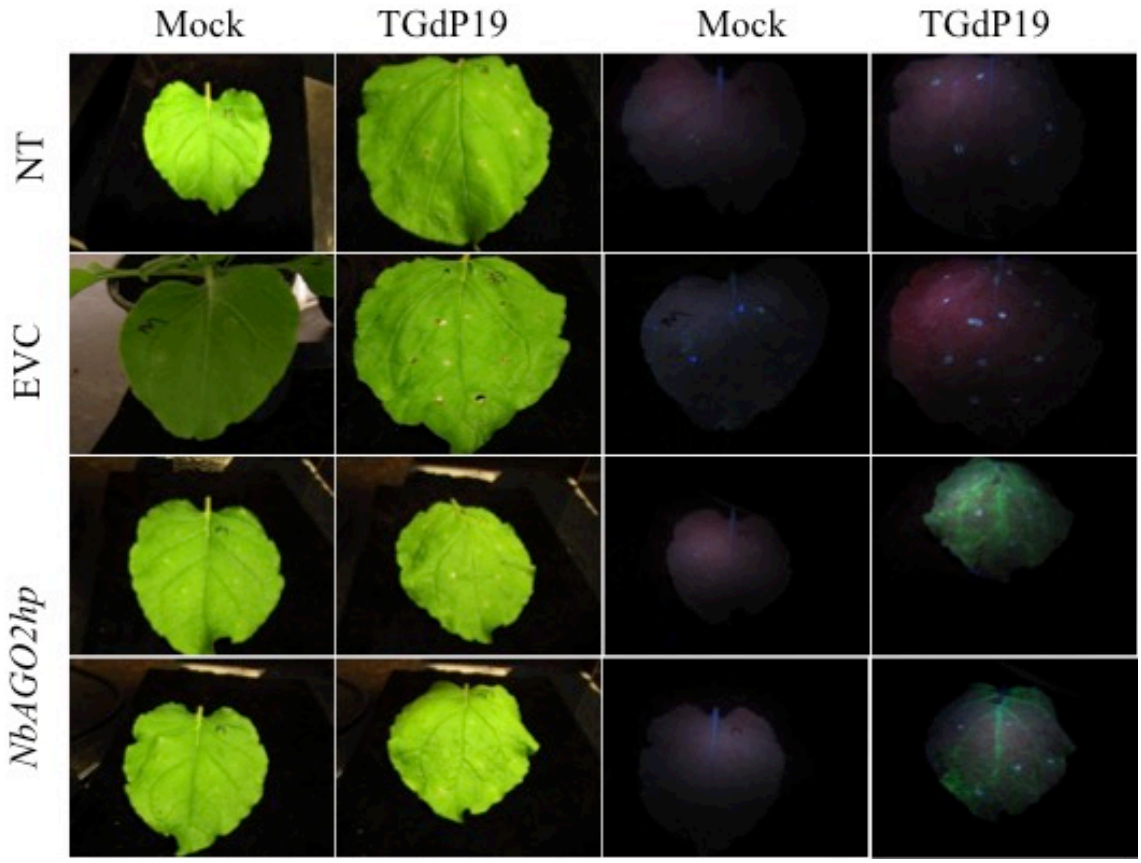
APPENDIX I

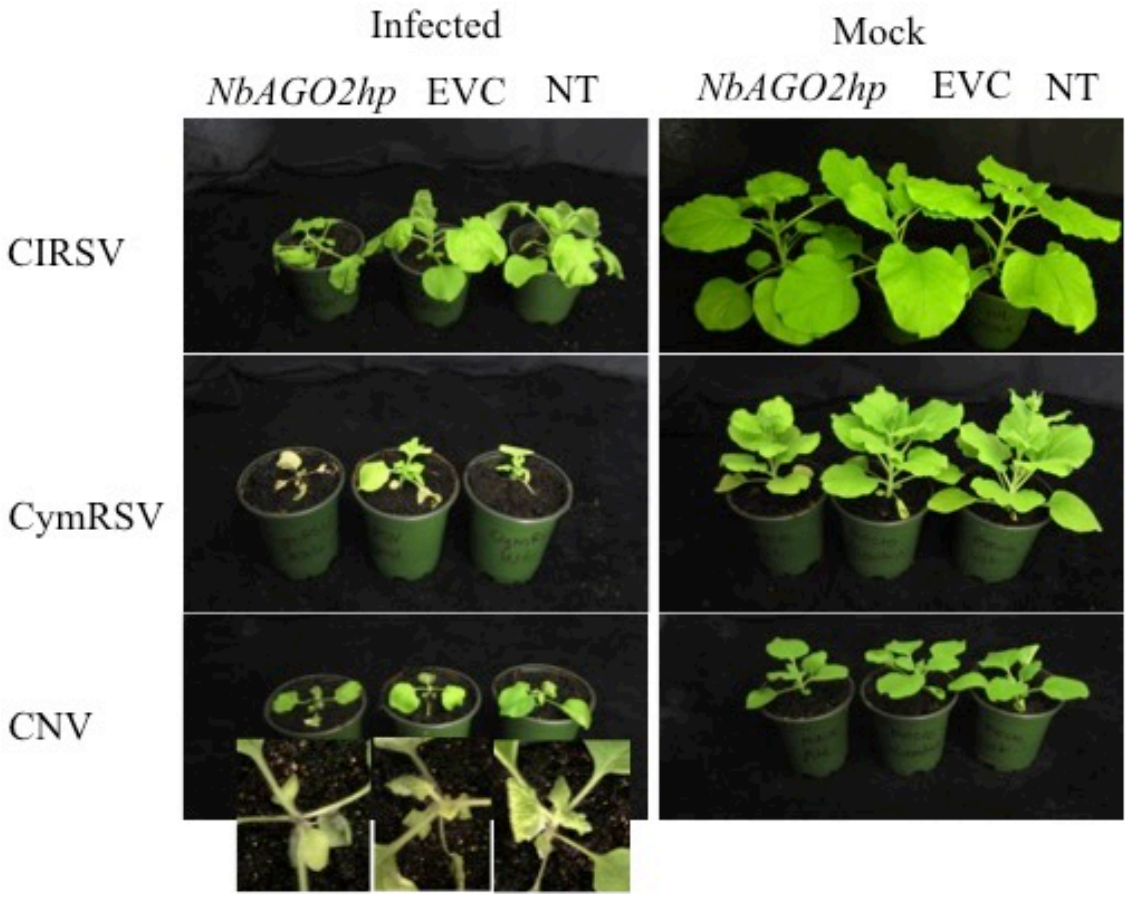
SUPPLEMENTARY FIGURES

Page

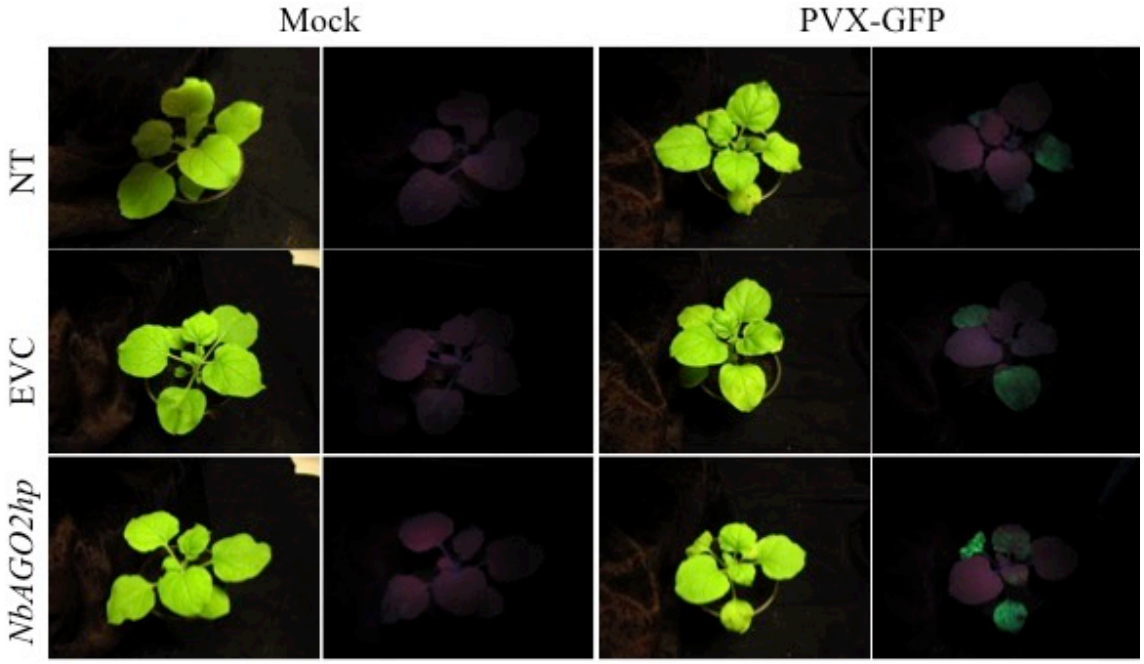
Supplementary Figure 3.1. Phenotypic effects at sub-optimal growth conditions. Five week old EVC or *NbAGO2hp* transgenic plants were grown in a laboratory space during the winter time at room temperature under ambient light.



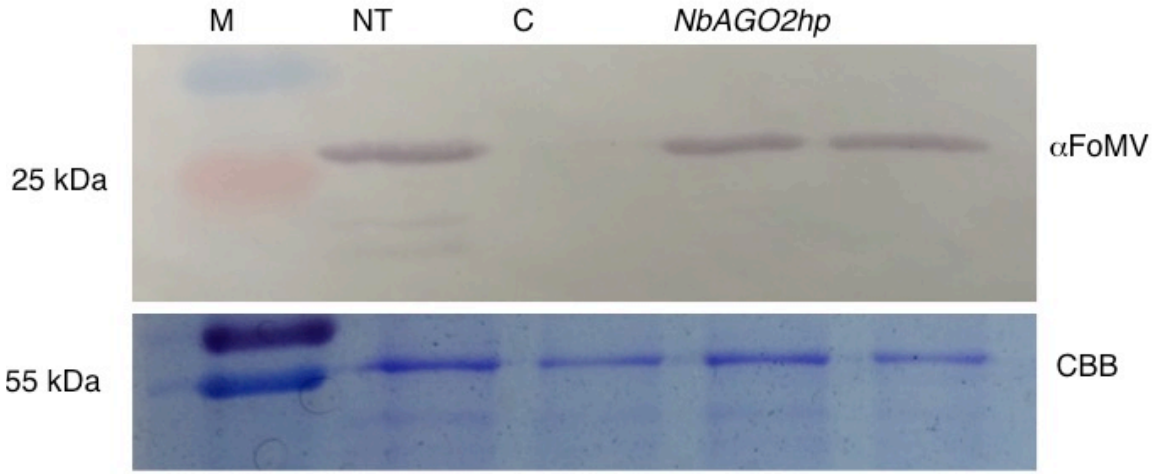
Supplementary Figure 3.2. Maintenance of *NbAGOhp*-associated antiviral effect in T2 generation. Seven week old leaves were either Mock infiltrated or infiltrated with TGdP19 and pictures were taken 10 days later. Leaves were imaged under normal light (left two vertical panels) or UV light (right two vertical panels). Top horizontal panel represents NT (non-transgenic); second horizontal panel is EVC (empty vector control); bottom two horizontal panels are from *NbAGO2hp* (hairpin transgenic).



Supplementary Figure 3.3. Symptoms on *N. benthamiana* upon infection with different tombusviruses. Three week old plants were inoculated and pictures taken 8, 11 or 16 dpi, respectively, upon infection with *Carnation Italian ringspot virus* (CIRSV), *Cymbidium ringspot virus* (CymRSV), or *Cucumber necrosis virus* (CNV) (including inserts showing apical necrosis for *NbAGO2hp* plants). *NbAGO2hp*, hairpin transgenic; EVC, empty vector control; NT, non-transgenic.



Supplementary Figure 3.4. Infection of *N. benthamiana* plants with PVX-GFP. Agroinfiltration was performed on 4 week old plants and images were taken 6 days post infiltration, under bright light (left) and UV light (right). NT, non-transgenic; EVC, empty vector control; *NbAGO2hp*, hairpin transgenic.



Supplementary Figure 3.5. Western blot analysis for *Foxtail mosaic virus* (FoMV). Virus inoculation was performed on 3 week old plants. Samples were collected at 19 days post rub-inoculation of *N. benthamiana*; non-transgenic (NT), or *NbAGO2hp*. Antiserum against FoMV (aFoMV) was used at 1:3000. C is an empty vector control plant that serendipitously did not get infected, showing that without FoMV infection there is no signal. The position of the 25 kDa molecular size marker (M) is indicated. CBB, Coomassie brilliant blue staining to verify loading.