

**ISOLATION AND CHARACTERIZATION OF
AN ANTI-VIRAL RISC IN PLANTS**

A Senior Scholars Thesis

by

JESSICA CIOMPERLIK

Submitted to the Office of Undergraduate Research of
Texas A&M University
In partial fulfillment of the requirements for the designation of

UNDERGRADUATE RESEARCH SCHOLAR

April 2006

Major: Biology

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Approved by:

Research Advisor:
Associate Dean for Undergraduate Research:

Herman B. Scholthof
Robert C. Webb

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ABSTRACT

Isolation and Characterization of an Anti-viral RISC in Plants (April 2006)

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As a defense against viral infection, plants are only very recently thought to use an RNA-induced silencing complex (RISC) as part of the RNA interference (RNAi) pathway to target and cleave viral RNA. To counteract this, some viruses have evolved proteins to inhibit RISC-mediated activity, thus ensuring their continued virulence. This research focused on the elucidation and analysis of anti-viral RISC in plants, to gain an understanding of how both the plant defense and countering viral mechanisms operate at the molecular level. A virus-host model system was used to examine this; *Nicotiana benthamiana* plants were infected with *Tomato bushy stunt virus* (TBSV) and time was allowed for RISC to target and cleave the viral RNA. If the TBSV defense protein, called P19, was present, the virus was not degraded by RISC and infection culminated in the death of the plant. When the plants were instead inoculated with a mutant of the virus, in which P19 was not expressed, RISC cleared the viral RNA and the plant survived the infection. This agrees with the model that RISC can (be programmed to) act as an anti-TBSV complex. To examine

the composition of the anti-TBSV RISC, plant proteins from both scenarios (with the presence or absence of P19) were subjected to various biochemical analyses and purification techniques. Proteins most closely associated with RISC activity *in vitro* (degradation of viral RNA in the test tube) were purified toward determining their identity. Future experiments are planned to examine if the antiviral RISC proteins are unique to TBSV infected plants or whether they are also associated with other virus infections.

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TABLE OF CONTENTS

	Page
ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES.....	vii
INTRODUCTION.....	1
PROBLEM STATEMENT.....	13
METHODS AND MATERIALS.....	14
Inoculation and analysis in plants.....	14
Protein analysis.....	14
SDS-PAGE detection of proteins in fractions and western blot analysis.....	15
Detection of siRNAs.....	16
RISC activity in vitro.....	16
RESULTS AND DISCUSSION.....	18
Isolation of RISC.....	18
Protein composition of RISC.....	21
Future projects.....	28
CONCLUSION.....	29
REFERENCES.....	30
CONTACT INFORMATION.....	33

LIST OF FIGURES

Figure		Page
1	Model of the RNAi pathway	2
2	Genomic organization of TBSV	10
3	Identification of RISC activity	20
4	Blocking of RISC activity by P19.....	20
5	Analysis of RISC	23
6	Western blot for detection of TBSV capsid protein after anion exchange chromatography	25
7	Assay of RISC activity in presence of catalytic metals.....	26
8	Identification of RISC proteins.....	27

INTRODUCTION

RNA interference (RNAi) is a species non-specific method of post-transcriptional gene silencing, initiated by double-stranded RNA (dsRNA) present in the cell (Fig. 1). In RNAi, target RNAs are degraded in a sequence-specific manner in many organisms. Plants, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammalian and human cells are all shown to use the RNAi pathway for regulation of gene expression.

Although the mechanism was not known at that time, the existence of an RNAi pathway in plants can be traced to a 1928 study with *Tobacco ringspot virus* showing that an infected tobacco plant lost symptoms in upper leaves, and became resistant to secondary infection (Baulcombe, 2004). The cause of this observation remained unexplainable until much later.

Depending on the organism of study, several terms were used to describe gene silencing, but it is now known that they are all related to RNAi. For example, co-suppression was a term used to explain the silencing of homologous genes in plants, as evidenced by the silencing of transgenes and their related endogenous counterparts (Que and Jorgensen, 1998). In 1998, a gene silencing pathway was also described using *C. elegans*. In an attempt to show that anti-sense and sense RNA could both be used to silence genes, a team lead by Fire, Mello and others tested a blend of the two (Fire et al., 1998). They found that this was ten times more effective as a trigger than either sense or anti-sense RNA alone (Hannon, 2002). They also observed that the gene silencing was a

This thesis follows the style and format of *Virology*.

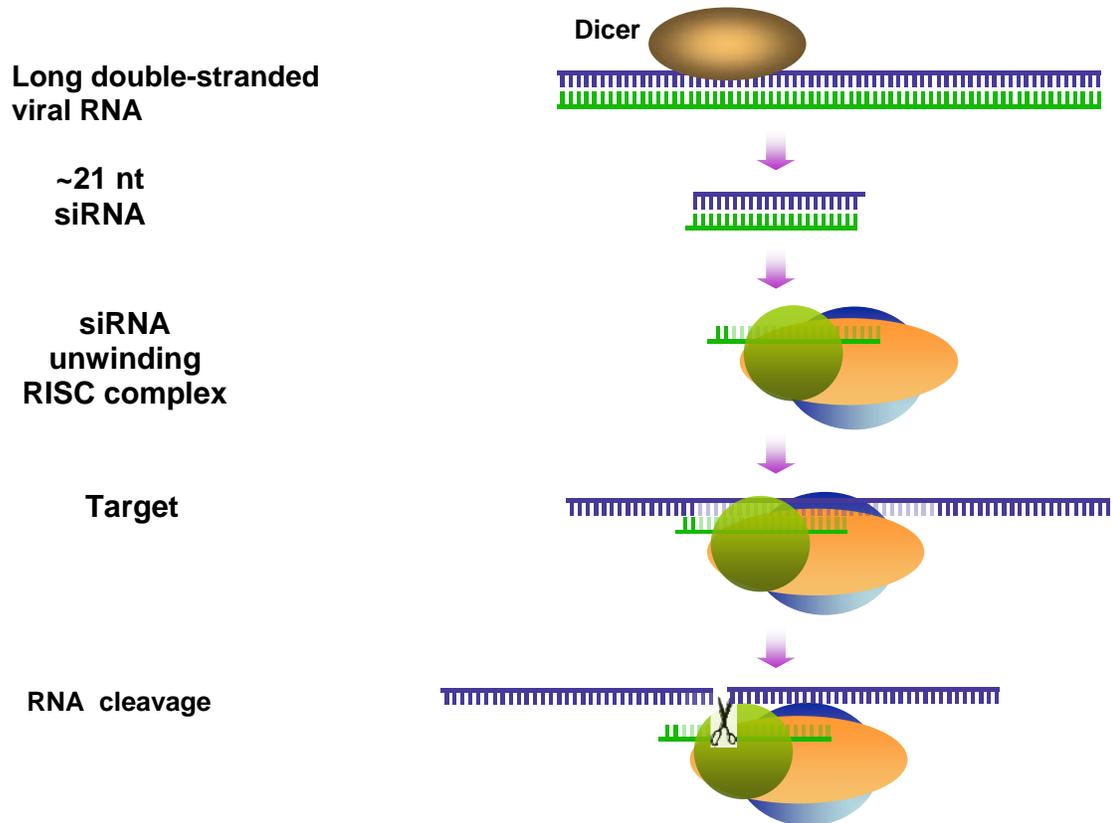


Figure 1. Model of the RNAi pathway. dsRNA in the cell is recognized by a host nuclease Dicer, which cleaves it into 21 nt duplexes. These duplexes are loaded into an RNA-induced silencing complex (RISC), and the passenger strand (RNA strand, shown in blue) dissociates. The active RISC then binds in a sequence-specific manner to single stranded RNA and cleaves it, rendering it unviable.

systemic effect; the entire organism was affected by introduction of dsRNA in a cell by either artificial introduction, by feeding the organism bacteria expressing dsRNA, or feeding it dsRNA directly (Timmons et al., 2001). This gene silencing protection extends to the next generation of *C. elegans* from those initially exposed (Fire et al., 1998).

RNAi is currently a very popular topic due to the seeming potential for gene regulation (Fig.1). As mentioned above, the gene suppression pathway has been observed in *Drosophila*, humans (Gregory et al., 2005) and other mammals, fungi (quelling in *Neurospora crassa*) (Cogoni, 2001), and *Arabidopsis thaliana* (Hannon, 2002). The cellular trigger of this pathway depends on the origin of the RNA duplex, and both microRNAs (miRNAs) and small interfering RNAs (siRNAs) can be involved. The miRNAs originate from hairpin structures produced in the nucleus by Drosha, an RNaseIII enzyme in conjunction with the proteins Pasha in *Drosophila* or DGCR8 in mammals (Valencia-Sanchez et al., 2006) from transcripts that form stem-loop structures. These transcripts can be assembled through inverted repeat RNAs resulting in double-stranded regions. The miRNAs then enter the common RNAi pathway at this step. Small interfering RNAs (siRNAs) are made from dsRNA produced in the cell. Alternately, these can be synthesized artificially and introduced into the cell. This research will focus on RNAi as initiated by the siRNA pathway (Fig. 1), and the current knowledge will be described in some detail below.

DsRNA (Fig. 1), either transcribed directly from DNA or by RNA synthesis from complementary strands, can accumulate in the cell due to viral infection or by artificial introduction (Filipowicz, 2005). The dsRNA is then cleaved into smaller segments by a

protein from the Dicer family (Fig. 1). Highly conserved in many species (Hannon, 2002), Dicer proteins are RNase III-class endonucleases that usually contain an N-terminus RNA-helicase domain, two RNaseIII domains, a dsRNA binding domain, a PAZ domain (to be covered in greater detail later), and another domain whose function remains unknown. Non-typical Dicers also have been described in *A. thaliana* that do not contain PAZ domains, instead having some sort of adaptor molecule (Carmell and Hannon, 2004). As seen in models of Dicer generated by crystallography, the two RNase III domains interact to form a dimer, and the center of this acts as two independent catalytic sites. Each of these sites contains an Mg^{2+} ion surrounded by 4 conserved acidic amino acid residues. These centers each have been thought to cleave one strand of the RNA duplex (Fig. 1) (Filipowicz et al., 2005). It has been suggested that Dicer preferentially binds the ends of its substrates, but that the nature of this end may not make a difference, and this preference may apply primarily to miRNAs (Carmell and Hannon, 2004). Dicer generates non-sequence specific siRNAs. Depending on the type of Dicer present, the size of the siRNA differs; *A. thaliana* generates two distinct sizes of siRNAs due to the presence of two different Dicers, and this trend is seen in studies of other species. For the most part, Dicer recognizes dsRNA at specific sites and cleaves it into duplex siRNAs, of 21 to 25 nucleotides with two single nucleotide overhangs (Hannon, 2002; Baulcombe, 2004; Filipowicz et al., 2005; Tomari and Zamore, 2005).

Once the siRNA has been generated, it dissociates from Dicer; the siRNA is then re-loaded onto the RNA induced silencing complex (RISC) in a strand-specific manner

(Leuschner, et al., 2006). As to which strand RISC prefers to bind, it has been suggested that 'guide' siRNAs retained when the duplex is unwound are anti-sense to the mRNA to be cleaved (Khvorova et al., 2003). Those anti-sense siRNAs seem to be most efficient with the least stably paired 5' end (Gong and Ferrell, 2004), and require a free 5' phosphate (Khvorova et al., 2003).

This results in the programming of RISC with single strands of the siRNAs to continue the RNAi pathway (Fig. 1). This complex has been shown to be composed of several proteins through isolation of high molecular weight complexes from *Drosophila* and human extracts, though the exact molecular structure and loading mechanism remains unidentified (Gregory et al., 2005). It is postulated that RISC consists of a protein from the aforementioned Dicer family, proteins from the Argonaut family, and a protein or proteins that contribute to the loading process. Because *Drosophila* and human RNAi pathways have been studied the most intensely, therefore those are the models available. There are similarities between the two pathways but the processes are not identical; it follows that the RISC for plants would share some similar elements but may also have specific properties.

An elegant scheme used to illustrate potential RISC loading in *Drosophila* diagrams the involvement of a holoRISC, comprised of proteins called VIG, TSN, dFMR1, and Ago2/Dcr-1 in conjunction with Dcr-2/R2D2. R2D2 is thought to interact with the 5' phosphorylated end, leaving Dcr-2 to bind to the other. This interaction is then thought to force apart the dsRNA, expelling the passenger strand for degradation. The holoRISC then becomes involved, associating the single stranded siRNA with the

Ago2 proteins (Tomari and Zamore, 2005). Similar mechanisms to load RISC have been seen in other systems; humans use the protein TRBP also present in the miRNA processing pathway to load RISC (Chendrimada et al., 2005) and *C. elegans* has been shown to use RDE-4 to help load the RDE-1 (Ago) protein (Gregory et al., 2005). In order to load the siRNA, it has been shown that the passenger strand must be cleavable at a site 10 nt from the 5' end – if this cannot occur, the RISC activity is inhibited due to ineffective removal of the passenger strand (Leuschner et al., 2006). The loading of RISC and subsequent unwinding of the dsRNA has shown to be facilitated by the presence of phosphates, though the process itself does not require ATP as initially suggested (Hannon, 2002). Recent studies propose that the docking of the RISC loading duplex with the Argonaute protein initiates a conformational change that causes the siRNA duplex to unwind, and the dissociated passenger strand to be released (Tomari and Zamore, 2005). However, other studies conflict with this notion, and propose instead that the passenger strand is released in the same manner that later viral mRNA would be, cleaving in such a way that 9 and 12 nt strands would be created, as anticipated by difference in thermodynamic profiles detected from this (Leuschner et al., 2006). In essence, this process largely remains unknown.

The Argonaute family is a group of proteins that are present in varying numbers in many species, said to be the largest related group of proteins implicated in the silencing of dsRNA (Meister et al., 2004). At last count, there are at least 5 Argonaute proteins in *Drosophila*, 10 present in *Arabidopsis* (Baumberger and Baulcombe, 2005), 8 in humans, 20 in *C. elegans*, etc., and many of these are closely related in function but

do not overlap (Meister et al., 2004). This group can be divided into two subfamilies by the domains present. The PIWI domain, found in humans, for example, has been shown to be involved in loading the RISC complex, by acting in protein-protein interaction between Argonaute and Dicer (Meister et al., 2004). It is also thought that this domain contains the Mg^{2+} that catalyzes the cleavage of the target RNA (Tomari and Zamore, 2005) in a fold similar to that of RNase H (Liu et al, 2004) . The other subfamily contains the PAZ domain, for Piwi-Argonaute-Zwille (Baulcombe, 2004). (This domain is found in Dicer, too.) This recognizes the two nt overhangs on the duplex siRNA (Meister et al., 2004), and is a highly conserved 130 amino acid sequence (Carmell and Hannon, 2004). The functions of the different proteins in the family are varied as well. Studies have recently determined that Ago-1 is associated with the miRNA pathway, while Ago-2 is able to cleave target RNAs (Baumberger and Baulcombe, 2005), and Ago-4 is involved in chromatin modification (Meister et al., 2004). The roles of the other Ago proteins are still being investigated. In addition to roles in the RNAi pathway, Argonaute proteins are found in many cellular processes (Meister et al., 2004). For example, Ago-2 seems to be crucial for *Drosophila* embryo development, though it is currently unknown what function the protein plays in this process (Baumberger and Baulcombe, 2005).

Once RISC is loaded, the incorporated siRNA allows for sequence specific binding to a target mRNA (Fig. 1). Next, cleavage of the target RNA occurs, earning this the name 'Slicer' (Baumberger and Baulcombe, 2005). This endonuclease activity is similar to that of RNase H, and produces 5'-phosphate and 3'-OH termini (Filipowicz,

2005). The binding energy for this association with the target RNA comes from the 5' half of the siRNA in the RISC complex (Doench and Sharp, 2004). Again, the site of cleavage is at the 10 nt site from the 5' end; data shows that mutations in this area drastically reduce RISC activity. The cleavage, once more, is ATP-independent (Hannon, 2002). The 'Slicer' itself has been isolated in *A. thaliana* fractions of about 150 kDa, possibly indicating the presence of only the Argonaute protein and its corresponding siRNA (Baumberger and Baulcombe, 2005). When this data is reconciled with the model set forth for RNAi based on evidence from *Drosophila* (Tomari and Zamore, 2005), it suggests that while RISC may contain multiple proteins, only the Argonaute protein is necessary for the cleavage reaction. It is also worth noting that the exact proteins that constitute an active RISC might vary between species.

Upon cleavage, the mRNA is rendered unviable, effectively silencing it. It is theorized that RISC has 'multiple turnover of the target' (Khvorova et al., 2003); it acts in such a manner that once the target RNA has been cleaved, RISC dissociates from the product and goes on to further react with other mRNA sites (Khvorova et al., 2003; Gregory et al., 2005). It is suggested that the flexible region (9-14 nt from the 5' end) on the guide siRNA assists in this (Khvorova et al., 2003).

Alternately, siRNAs and miRNAs have been shown as triggers for gene silencing by methylation, like that seen in RITS (RNA-induced initiation of transcriptional gene silencing), but that is not further considered here.

A RISC loaded with virus-derived siRNAs would allow for systemic protection of the organism by being a mobile element. An active RNAi pathway in plants would

produce a mobile signal, likely dsRNA or siRNAs due to size issues (Hannon, 2002) that spreads through the plants, giving systemic virus protection to the host (Lakatos et al., 2004). Recent studies have shown evidence of RISC activity in *N. benthamiana* (Omarov et al, unpublished). According to these studies, viruses have evolved mechanisms to overcome the RNAi pathway by encoding a suppressor (Scholthof, 2005). The step in the pathway where the suppressor operates is only known for a few examples; one of these is discussed below.

Tomato bushy stunt virus (TBSV) has been shown to infect over 120 species of plants in 20 separate families, though the host range remains limited in nature. The few economically important crops it can impact are primarily vegetable crops. It belongs to the family Tombusviridae, which also includes *Cucumber necrosis virus*, *Carnation mottle virus*, and *Panicum mosaic virus*. Individuals in this family can usually infect either dicots or monocots, are very stable, and are soil-borne (Agrios, 2005).

Symptoms caused by TBSV vary by host. They usually include stunting with a profusion of lateral stems from the base, lending a bushy appearance, as well as the general crinkling and drooping of leaves. The leaves bear chronic chlorotic and necrotic lesions, varying from mild to severe mosaic, which can potentially result in the eventual death of the plant, depending on the host species.

The virion itself is icosahedral (T=3), about 32-33 nm in size and contains about 4800 nucleotides of positive-sense RNA (Fig. 2), which indicates that the genome is in the same sense as the mRNA produced (Yamamura and Scholthof, 2005). This RNA encodes for 5 open reading frames (ORFs). Two directly translate the proteins P33 and

P92. These are involved in replication. P92 is an RNA-dependent RNA polymerase (RdRP) that interacts with P33 to form the replicase complex. The next ORF for P41, the capsid protein, is translated from subgenomic RNA1 (sgRNA1). P22 is translated from sgRNA2, and encodes for a movement protein involved in the viral membrane associated cell-to-cell movement (Scholthof, 2005). It has been suggested that complexes composed of TBSV RNA and P22 are transported systemically through the plasmodesmata, possibly by altering the plasmodesmata size. The P19 ORF is nested within the larger P22 ORF and is also translated from sgRNA2. Both of these proteins have been hypothesized to contribute to the pathogenesis, perhaps by interaction with host proteins, like the transcriptional factor HFi22 that interacts with P22 (Desvoyes and Scholthof, 2002), and P19 interacts with Hin19, an RNA processing protein (Park et al., 2004).

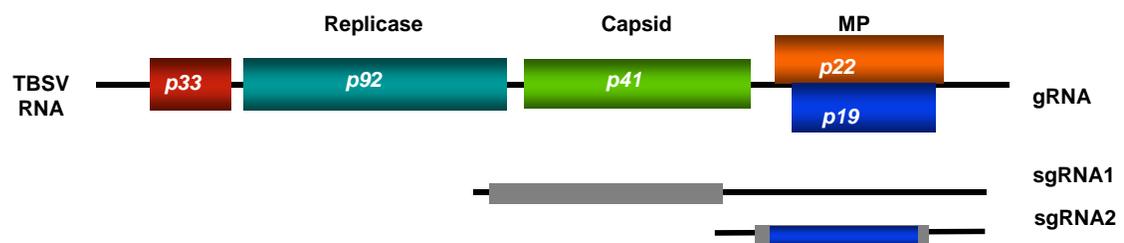


Figure 2. Genomic organization of TBSV. The virus' genomic RNA encodes for 5 ORFs. Replicase proteins P33 and P92 are translated from genomic RNA. Capsid protein P41 is translated from sgRNA1, and the proteins P22 and P19 are translated from sgRNA2. The P19 and P22 ORFs are overlapping, with P19 produced from an out-of-frame alternate start codon. In addition to movement protein functions, P19 has also been shown to be a silencing suppressor.

P19 is highly conserved by many Tombusviruses (Scholthof, 2006). P19, however, has been shown to be involved in several functions. It is responsible for systemic necrosis of the plant (*Nicotiana benthamiana*), and has been shown to induce a hypersensitive response in *N. tabacum*, but most importantly, it seems to inhibit the RNAi pathway in several studies (Lakatos et al., 2004; Omarov et al., 2006; Scholthof, 2006). This may be related to the observation that P19 is required in a host-specific manner for systemic movement (Turina et al., 2003). The activity of P19 for these functions requires that P19 be expressed at high levels within the cell (Scholthof, 2006). This corresponds with the start-codon preference shown by the ribosomes for P19 over P22, resulting in early and high levels of P19 expression (Yamamura and Scholthof, 2005).

Crystal structures show that P19, encoded by *Carnation Italian ringspot virus* (of the Tombusvirus genus) with a sequestered siRNA, forms a homodimer that interacts with the sugar-phosphates present on the duplex siRNA backbone, independent of the RNA bases themselves. A P19 monomer has one four-stranded beta sheet and 4 alpha helices, with the N-terminals overlapping in dimer form (Vargason et al., 2003). The beta4 strand and alpha5 helix (as well as possibly the beta3 strand) form a carboxy region that interacts with each of the nt overhangs on each side of the siRNA (Vargason et al., 2003) sequestering it and potentially preventing the siRNA's loading into a RISC complex. This arrangement agrees with the observation that the presence of P19 corresponds to RNA silencing suppression (Qiu et al., 2002; Vargason et al., 2003; Lakatos et al., 2004; Park et al., 2004; Tomari and Zamore, 2005; Yamamura and

Scholthof, 2005; Omarov et al., 2006; Scholthof, 2006). It has been shown that P19 mutants incapacitated for binding TBSV-derived siRNA during infection are no longer capable of suppressing RNAi (Omarov et al., 2006). This illustrates that key role of siRNAs for RNAi and RISC programming.

PROBLEM STATEMENT

This project utilizes a host/virus model interaction system using *N. benthamiana* and TBSV. The hypothesis is that in *N. benthamiana* plants, infected with the wild-type TBSV, the P19 silencing suppressor of RNAi accumulates and inhibits the loading of an active anti-viral RISC. This leads to a lethal TBSV infection – the plant is unable to clear the viral load and succumbs. In a plant infected with a mutant of TBSV engineered to not produce the P19 protein, the RNAi pathway is active, and the viral mRNA is cleaved by an active RISC leading to a recovery phenotype. This mRNA is rendered unviable, and the plant is able to clear the viral load and ‘recover’. This unique arrangement allows for the observance of both the plant defense RNAi as well as the viral counter, P19.

The existence of a virus activated RISC has not yet been demonstrated in previous work – no high molecular weight siRNA-protein complexes have been isolated from plants as they have in *Drosophila* cell extracts (Lakatos et al., 2004). If the hypothesis is correct, we should be able to isolate the RISC and potentially determine the identity of the proteins present.

MATERIALS AND METHODS

The model uses TBSV and a mutant of TBSV mutated with a premature P19 stop codon that prevents its expression. These constructs were generated in previous experiments (Hearne et al., 1990; Scholthof et al., 1995).

Inoculation and analysis of plants

Transcripts of full length cDNAs were prepared *in vitro* for both the wild type TBSV and the TBSV mutant not producing p19 (referred to as 157). As outlined in previous experiments (Omarov et al, 2006), the viral cDNA was inserted into a plasmid conferring resistance to Ampicillin to select *E. coli*, grown in a broth containing tryptone, yeast extract, salt, and dextrose (Sambrook et al, 1989). The plasmids were harvested using a Qiagen maxi-prep kit according to the manufacture's directions, and the plasmids present were linearized at the viral 3' terminus using the *SmaI* restriction enzyme. Transcripts were generated using a T7 RNA polymerase. The transcripts were mixed with a sodium phosphate (monobasic) buffer with 1% cellite. Each leaf was rub-inoculated with 20 microliters (μ l) of the transcript/buffer mixture. Plants were incubated overnight at 25°C, and then transferred to a 27°C humidity controlled growth chamber for 7 days.

Protein analysis

Thirty to forty grams of the infected plants were harvested by cleaving the leaves and stem of the plant at the base, and grinding in a blender with 100 ml of a 200 mM

sodium phosphate buffer, pH 7.5. The plant extracts were respectively centrifuged at 4000 rpm for 30 minutes at 4°C, the supernatant was strained through cheese cloth, and centrifuged again at 10,000 rpm for 15 minutes at 4°C. The 50 ml of resulting soluble extract was then loaded onto a 40 ml anion exchange chromatography column, packed with Macro-Prep DEAE Support (Bio-Rad, Hercules, CA). The column was washed with about 150 ml of the sodium phosphate buffer, and the proteins were eluted off by increasing concentrations of sodium chloride (0.1-.09 M). Separate 2 ml fractions were collected and every two fractions combined to analyze for RISC activity (see below). Fractions displaying RISC activity were further concentrated and purified by filtering and fractionation on an S-200 HR gel-filtration chromatography column. These fractions were also eluted in 2 ml fractions, and combined in a similar fashion as above.

SDS-PAGE detection of proteins in fractions and western blot analysis

The fractions were first assayed on a ~ 1% agarose gel and stained with ethidium bromide for 10 minutes, then analyzed for nucleic acid content under UV light. Following this, the proteins were subjected to ~15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels or ~7.5% SDS-PAGE. Proteins were visualized using Coomassie Blue stain and later with Silver staining techniques to determine size of proteins present.

For western blot analysis, proteins were separated in ~7.5% polyacrylamide gels by SDS-PAGE, and then transferred to nitrocellulose membranes (Osmonics, Westborough, MA). These were stained with Ponceau S (Sigma, St. Louis, MO) to

confirm protein transfer, then the membranes were incubated with about 1:5000 dilution of viral protein specific antibodies. Secondary antibody of alkaline phosphatase-conjugated to goat anti-mouse or rabbit antiserum (Sigma, St. Louis, MO) serum at a dilution of 1:1000 was added, and the immuno-complexes were determined by a standard alkaline phosphate color precipitation reaction.

Detection of siRNAs

The presence of siRNAs was determined by treating 350 μ l of the collected fractions with 50 μ l 10% SDS at 65° C for 15 minutes to dissociate the protein complexes from the fractions. These samples were then purified with phenolchloroform, precipitated with 100% ethanol, and then analyzed by electrophoresis through a ~17% acrylamide gel in presence of 8 M urea, followed by electro-blotting onto a nylon membrane (Osmonics, Westborough, MA). SiRNAs specific to TBSV were identified on the membrane by hybridization with [32P]dCTP-labeled TBSV specific probes made for that purpose, using standard northern blot techniques (Sambrook et al, 1989; Omarov et al, 2006).

RISC activity *in vitro*

To determine RISC activity *in vitro*, 2 μ l of TBSV transcripts were added to 5 μ l of chromatography fractions. Mixtures were incubated at 37°C for 30 minutes, and the sample was loaded onto a ~1% agarose gel. After electrophoresis, the presence or

absence of transcripts was verified upon staining with ethidium bromide. The resulting gel was subjected to UV light to determine cleavage of the transcripts.

RESULTS AND DISCUSSION

Isolation of RISC

Plants were infected with the TBSV mutant not producing P19. For this TBSV mutant, the RISC should be active due to the lack of the silencing suppressing P19. These active RISCs would be able to cleave TBSV RNA because RISC complexes allow for systemic removal of specific viral RNA corresponding to the siRNA they contain. Our results showed indeed that *N. benthamiana* plant infected with the mutant recovered from the infection. This systemic protection agrees with prior results, where plants first infected with the non-P19 producing TBSV mutant do not allow for later infection by the wild type of that virus (Omarov et al., 2006). These results also correspond with RNAi pathway observation in other organisms (Fig. 1) (Timmons et al., 2001; Baulcombe, 2004).

The plant material was harvested and the proteins separated by charge and purified by eluting them from anion exchange chromatography column with an increasing sodium chloride gradient. These fractions were then analyzed for RISC activity by incubating small amounts of TBSV RNA transcripts with each of the eluted fractions. The result in Fig. 3 showed that the TBSV RNA was not cleaved in the majority of the fractions. However, in some fractions the transcripts were degraded. This degradation was most conspicuous and reproducible for fractions 5-8, resulting in the generation of a 'wash' of RNAs. Based on the use of standard molecular weight markers added during chromatography, we know that fractions 5-8 contain complexes of ~500 kDa. Thus, the results suggest that these fractions contain RISC, and that RISC is a high

molecular weight complex. These results have not been seen in any other host/virus model system. While previous results for other systems suggest that Ago-1 was the sole part of RISC (Baumberger and Baulcombe, 2005), our results correspond more with the model based on data from *Drosophila* lysates (Tomari and Zamore, 2005); an active RISC consists of a unit of proteins. Another possibility is that our methods isolate the RISC complex as a whole, instead of only the Argonaute protein. Additional analysis is currently being carried out to further separate co-eluting proteins and determine the activity of each (Omarov et al; unpublished data).

As one would expect from the model used in the hypothesis, for fractions collected from a plant infected with the wild-type TBSV, P19 would sequester the siRNAs (Fig. 1) as they are produced (Omarov et al., 2006). These siRNAs would not be loaded onto a RISC complex, and the RISC complex would be inactive. To test this, fractions were collected from plant material infected with the wild type TBSV. As before, aliquots of the fractions were tested for their ability to cleave transcripts. The results in Figure 4 show that when transcripts were added to fractions from a plant infected with the wild-type virus, the TBSV transcripts remained intact. The results support the hypothesis that the sequestration of siRNAs by P19 (Vargason et al., 2003; Havelda et al, 2005; Omarov et al., 2006) prevent the programming of an anti-TBSV RISC. In fact, residual nuclease activity for the P19 mutant in other fractions (Fig. 3) was also less prevalent for fractions collected from plants infected with the wild-type (Fig. 4). Thus, the presence of P19 gave enhanced protection against any type of RNA degradation.

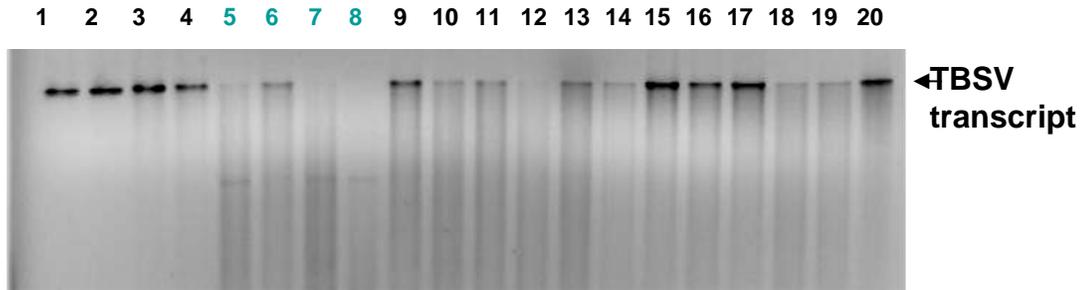


Figure 3. Identification of RISC activity. *In vitro* cleavage of exogenously added TBSV transcripts to gel-filtration S-200 chromatography fractions isolated from plants infected with a TBSV mutant, not expressing P19. Small amounts of TBSV RNA transcripts were added to aliquots of the fractions, incubated, loaded onto a ~1% agarose gel, subjected to electrophoresis, and stained with ethidium bromide. This resulted in the detection of RISC activity in fractions 5-8, as visualized by a wash of RNA smear in those fractions as seen above. The strong band in fractions 1-4 and later, piecemeal in fractions 9-20, is the un-degraded TBSV RNA transcript.

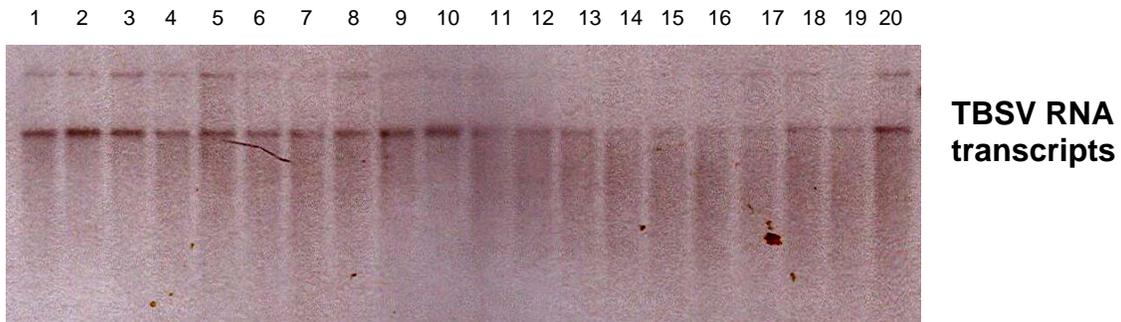


Figure 4. Blocking of RISC activity by P19. Assay for *in vitro* cleavage of TBSV transcripts added to gel S-200 chromatography fractions collected from plants infected with wild-type TBSV. Small amounts of TBSV RNA transcripts were added to aliquots of the fractions, incubated, loaded onto a ~1% agarose gel, subjected to electrophoresis, and stained with ethidium bromide. The band present in all fractions is unaltered TBSV RNA transcript. As predicted in the hypothesis, there is no RISC cleavage activity detected.

Protein composition of RISC

These preliminary results indicate the importance of siRNAs for this system. If the siRNAs are present, then those fractions should exhibit RISC activity. To test this, we set out to determine the protein and siRNA composition of fractions displaying RISC activity. For the presence of siRNAs, fractions from a plant infected with the TBSV mutant were treated with 10% SDS and the remaining proteins were separated on a ~8M urea acrylamide gel. These proteins were transferred to a nylon membrane. The result in Fig. 5a shows a preponderance of proteins in the active fractions. To test that TBSV-specific siRNAs were present, a TBSV specific probe was synthesized and then used in hybridization. The membrane was exposed to film, and the resulting image captured. The result in Fig. 5B shows the presence of TBSV siRNAs, particularly in fraction 20. This fraction is from just the ion exchange chromatography step, but corresponds to the gel-filtration fractions in which RISC activity is present for the *in vitro* assay (Fig. 3).

Along with TBSV specific RNA, the fractions were also incubated with transcripts made from other viruses (PMV and SPMV). There was no resulting degradation of the transcripts, indicating that the active RISCs are specific to only the virus used in the induction of the RNAi pathway. This agrees with the assumption that programming RISC with specific siRNAs is essential for RISC activity (Hannon, 2002; Baulcombe, 2004).

Collectively, the data strongly suggest that the fractions highlighted in Fig. 5 contain TBSV-specific RISC because they hold single stranded TBSV siRNAs and only cleave TBSV RNA. This narrowed our investigation to deciphering the protein

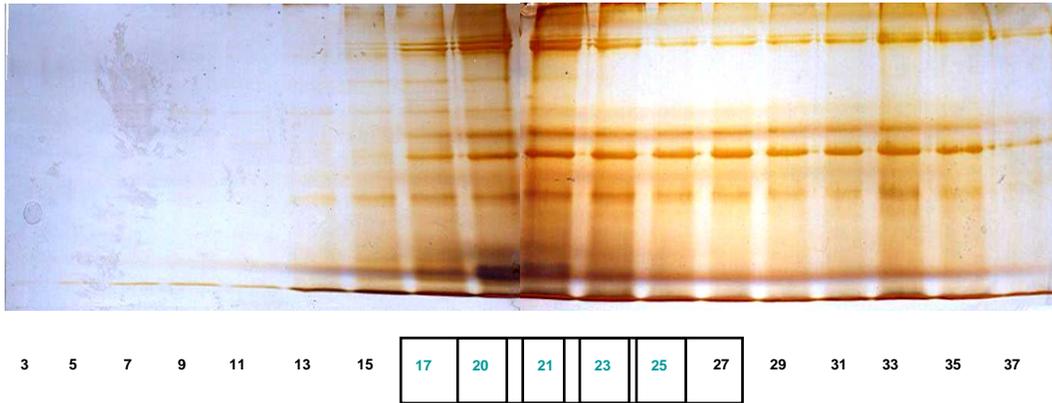
compositions of this novel antiviral RISC, and led me to perform additional gel-filtration after anion-exchange chromatography to analyze these fractions in more detail.

First, in order to determine whether viral proteins are involved, the fractions were subjected to western blot analysis. These involve the use of antibodies generated against the separate viral proteins, and these antibodies are then detected chemically. Fractions seventeen through thirty-one (odds) were assayed, along with a molecular weight marker and a positive control (raw plant protein from a wild type TBSV infected plant) (Fig. 6). Darkened areas present on the membrane indicate a positive result.

The western blot shown in Fig. 6 exhibited a positive response for the TBSV capsid protein. It is unclear at this time whether this positive result is due to the sheer amount of capsid protein present in the samples, or if the viral capsid protein is somehow involved in the RNAi pathway. Potentially, the capsid protein might be involved in some sort of trigger for RNAi or other activity, but it remains more likely that because the capsid protein is present in such a high amount in the infected cell (Yamamura and Scholthof, 2005), it co-purifies with many fractions. These capsid proteins are not associated with any anti-viral RISC activity (Scholthof, 2006).

As expected, for fractions collected from a plant infected with the TBSV mutant, no P19 is present in fractions exhibiting RISC activity, as also shown previously (Omarov et al., 2006). For the corresponding fractions collected from plants infected with wild type TBSV and lacking RISC activity, P19 is present. This validates the hypothesis (Fig.1) (Omarov et al., 2006); in plants infected with the virus that produces P19, there is no RISC activity, and thus no *in vitro* nuclease activity in Fig. 4.

a:



b:

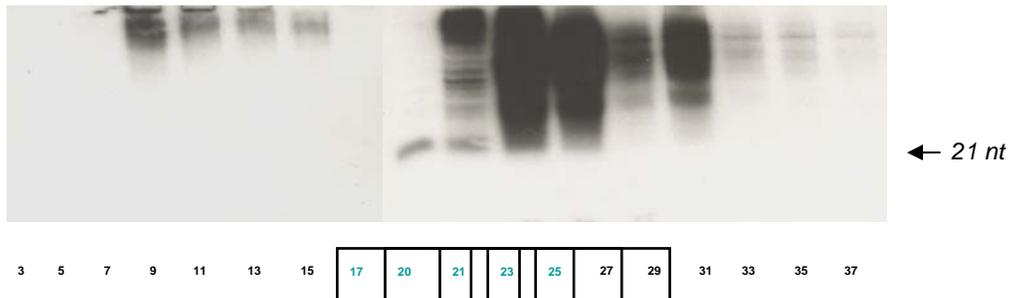


Figure 5. Analysis of RISC. (a.) Proteins detected in TBSV mutant-infected plants, as visualized by silver staining after anion exchange chromatography and gel filtration. The fractions highlighted are those corresponding to RISC activity in Figure 3. These will be the fractions focused on in later figures. (b.) Detection of siRNAs in fractions from figure 5a in a ~17% acrylamide gel in presence of 8M urea. The 21 nt band is clearly visible in fraction 20, indicating the presence of siRNAs in that fraction. This corresponds to RISC activity in Figure 3, and suggests the presence of loaded RISCs in these fractions.

The addition of Mg^{2+} and Mn^{2+} to the RISC active fractions increased the RISC activity with Mg^{2+} being more effective, whereas the addition of EDTA to fractions exhibiting activity prevent transcript cleavage *in vitro* (Fig. 7). These results correspond to observations in other systems with active RNAi (Filipowicz, 2005; Tomari and Zamore, 2005).

Once RISC activity was confidently and reproducibly verified, the fractions containing this activity were again purified (Fig. 8a, 8b) and further separated by gel filtration column chromatography. Again, RISC activity was verified, and those fractions concentrated (Fig. 8c). The ~7.5% acrylamide SDS-PAGE gels display the potential proteins that could be involved in RISC activity. Three protein bands (50 kDa, 70 kDa, and 200 kDa) were selected and gel purified. The thought is that these proteins could be related to those seen in other systems, and it seemed reasonable to speculate that the Argonaute and Dicer PAZ and PIWI domains would be conserved across species. However, there was also the possibility that the proteins could be novel. These proteins were sent for analysis on mass spectrometry (Yale University's Keck Center). At this time, several bands have been sequenced. The densest bands were chosen for this, with the expectation that since these proteins are present in the largest quantities, they could potentially be involved in the RNAi pathway. However, these have been shown to be the plant protein ribulose-1,5-bisphosphate carboxylase (Rubisco) and one of its precursors, and another of the bands is phosphoenolpyruvate carboxylase. As these proteins are involved in energy synthesis of the plant and would be present in high quantities, it is highly unlikely that they contribute to RISC, and only co-purify as they



Figure 6. Western blot for detection of TBSV capsid protein after anion exchange chromatography. The bands present indicate the presence of coat protein in fractions containing RISC activity, (+) is a positive control, and a standard, know molecular weight marker is shown in Lane M.

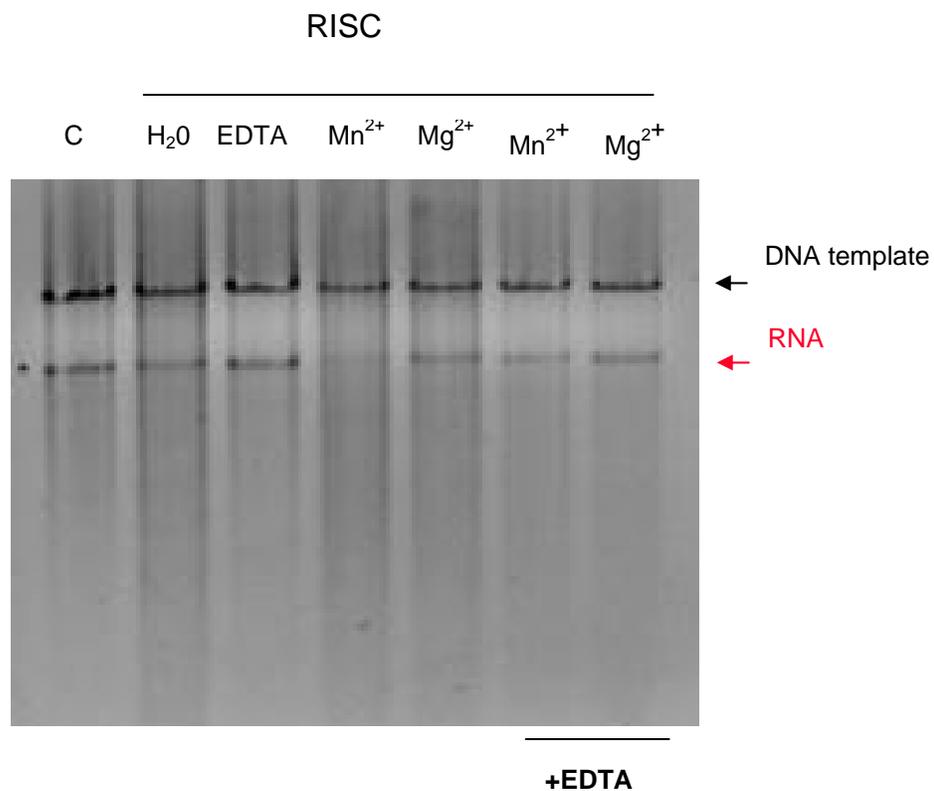


Figure 7. Assay of RISC activity in presence of catalytic metals. In the above gel, a control on the far left displays intact DNA template and RNA, followed by a mock with water and EDTA. The RNA is cleaved in the next lane when incubated with Mn²⁺ and the fraction displaying RISC activity, as it is in the following lane with the addition of Mg²⁺. When both the metals and EDTA are added, as they are in the two right-most lanes, there is protection of the RNA and no RISC activity. The DNA and RNA templates are labeled.

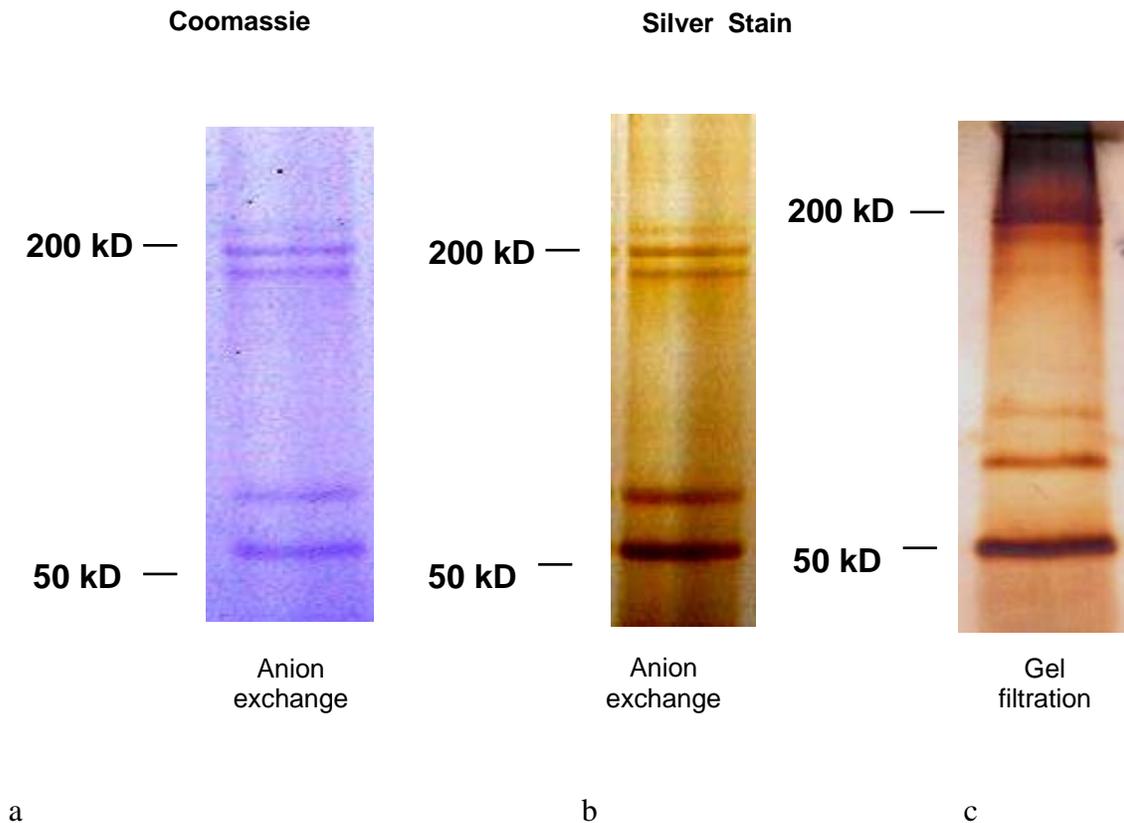


Figure 8. Identification of RISC proteins. (a.) RISC active fraction stained with Coomassie blue after anion-exchange chromatography; (b.) the same fraction as in a, stained with the more sensitive Silver stain. (c.) The protein after further concentration and purification following gel filtration, also silver stained. The position of selective size markers are indicated in kiloDaltons (kDa).

are also negatively charged molecules with a high molecular weight. At this time, the lab is currently investigating alternate methods of column chromatography to try and prevent this co-purification and make sure those proteins indeed are not needed for the RNAi pathway. Additional proteins also present in this fraction (Fig. 8c) that are currently undergoing mass spectrometry and sequencing analyses.

Regardless of the outcome, it seems that a high molecular weight anti-TBSV RISC has additional components compared to the Ago1 described previously in plants (Baumberger and Baulcombe, 2005). This data agrees with more complex models set forth for other systems (Tomari and Zamore, 2005).

Future projects

At this time, I am working to determine if a RISC similar to the one observed for TBSV is triggered by other viruses; currently utilizing *Tobacco rattle virus* for this purpose. So far, I have observed similar protein patterns in SDS-PAGE gels with coomassie and AgNO₃ staining, and am awaiting the results of siRNA analysis.

CONCLUSIONS

In agreement with my hypothesis, plants infected with a TBSV mutant defective for silencing suppressor (P19) expression display an active RNAi pathway. The RISC complex is loaded with a siRNA specific for the TBSV virus, enabling it to specifically cleave TBSV RNAs, as illustrated by results from *in vitro* experiments. Plants infected with the wild type TBSV, expressing the silencing suppressor, do not exhibit RISC activity in similar *in vitro* experiments.

As described in the results section, the RISC is a high molecular weight complex (about 600kDa). The degradation activity is most likely catalyzed by Mg^{2+} or Mn^{2+} , as anticipated.

Potential proteins that contribute to the RISC activity are undergoing sequencing for composition and identification.

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