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An antiviral RISC isolated from *Tobacco rattle virus*-infected plants

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ABSTRACT

The RNAi model predicts that during antiviral defense a RNA-induced silencing complex (RISC) is programmed with viral short-interfering RNAs (siRNAs) to target the cognate viral RNA for degradation. We show that infection of *Nicotiana benthamiana* with *Tobacco rattle virus* (TRV) activates an antiviral nuclease that specifically cleaves TRV RNA *in vitro*. In agreement with known RISC properties, the nuclease activity was inhibited by NaCl and EDTA and stimulated by divalent metal cations; a novel property was its preferential targeting of elongated RNA molecules. Intriguingly, the specificity of the TRV RISC could be reprogrammed by exogenous addition of RNA (containing siRNAs) from plants infected with an unrelated virus, resulting in a newly acquired ability of RISC to target this heterologous genome *in vitro*. Evidently the virus-specific nuclease complex from *N. benthamiana* represents a genuine RISC that functions as a readily employable and reprogrammable antiviral defense unit.

Introduction

RNA interference (RNAi) or silencing is a highly conserved molecular mechanism known to regulate gene expression and/or combat invasive nucleic acids across species including plants (Baulcombe, 2004), fungi (Romano and Macino, 1992), insects (Hammond et al., 2001) and mammals (Liu et al., 2004). As RNAi pathways were initially studied most intensely in *Drosophila melanogaster* and mammalian cells, accordingly the original RNAi models were based on those systems. Recently, these have been substantially augmented by (reverse) genetic studies using *Arabidopsis* (Li and Ding, 2006; Voinnet, 2005).

In the current RNAi model, the process is triggered by the presence of double-stranded RNA (dsRNA) structures; for instance, those that accumulate in the cell via viral infection or upon artificial introduction (Filipowicz, 2005). A Dicer protein cleaves these dsRNAs into smaller segments, giving rise to short duplex RNAs such as microRNAs (miRNAs) or short-interfering RNAs (siRNAs), with one strand of these used to program a multi-protein RNA-induced silencing complex (RISC). The programmed RISC then targets single-stranded (ss) RNAs complementary to the incorporated RNA for cleavage or translational repression, resulting in post-transcriptional silencing of specific genes.

RISC is postulated to be a high-molecular weight complex composed of at least one protein from the Argonaute (Ago) family, and possibly one Dicer family protein (MacRae et al., 2008; Song and Joshua-Tor, 2006; Tomari et al., 2007). Ago proteins represent the catalytic effector unit(s) of RISC, and as such, are signature proteins of this pathway (Baumberger and Baulcombe, 2005; Hammond et al., 2001). Two protein domains, Piwi–Argonaute–Zwille (PAZ) and Piwi, are found associated with this family in addition to N-terminal and middle domains (Song et al., 2004; Song and Joshua-Tor, 2006). The Ago Piwi domain is thought to have an RNase H-type fold (Liu et al., 2004) containing a Mg^{2+} ion to catalyze the cleavage of target RNA (Schwarz et al., 2004).

As there are several Ago proteins contributing to different modes of RNAi, the specific role that many individual Ago proteins fulfill is an active area of investigation (Meister et al., 2004; Tolia and Joshua-Tor, 2007). This is especially relevant for plant–virus interactions (Alvarado and Scholthof, 2009), and thus far, antiviral roles have been suggested for Ago1, Ago7, and Ago4 (Baumberger and Baulcombe, 2005; Baumberger et al., 2007; Bhattacharjee et al., 2009; Bortolamiol et al., 2007; Csorba et al., 2010; Qu et al., 2008). Once RISC is activated, the incorporated siRNA ensures sequence-specific binding to a cognate target ssRNA, followed by Ago-mediated cleavage of the target RNA in a manner similar to that of RNase H, 10-nt in from the 5′-end of the bound siRNA (Ameres et al., 2007).

It is generally hypothesized that during virus infection of eukaryotic hosts, components of the RNAi pathway similar to those outlined above generate virus-derived siRNAs to program an antiviral RISC. Recently, a suppressor-defective mutant of *Tomato bushy stunt virus* (TBSV) (Omarov et al., 2006; Scholthof, 2006; Yamamura and Scholthof, 2005) was used to isolate one such RISC from extracts of infected *Nicotiana benthamiana* plants. Biochemical analysis revealed a discrete RISC-like effector that was associated with TBSV-derived siRNAs and specifically targeted the viral RNA for degradation during *in vitro* reactions (Omarov et al., 2007). This provided direct evidence

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that the anti-TBSV RISC was cleaving the target RNA rather than causing translational repression. Using a somewhat different approach, these findings were independently corroborated for a related tombusvirus (Pantaleo et al., 2007).

It is reasonable to hypothesize that the above processes are not unique to tombusviruses, as specifically illustrated by other virus gene vectors used to trigger virus-induced gene silencing (VIGS) (Batten et al., 2003; Burch-Smith et al., 2004; Ratcliff et al., 2001). Furthermore, many animal and plant viruses encode silencing suppressors (Li and Ding, 2006; Omarov and Scholthof, in press; Scholthof, 2007; Voinnet, 2005), also suggesting they encounter host enforced RNA silencing defenses during infection. Nevertheless, the demonstration of detectable and *in vitro* active antiviral RISC thus far remains unique to the tombusvirus–host system.

To examine the validity of a model predicting the existence of a conserved antiviral silencing pathway in plants and to determine if it corresponds to those based on other systems like *Drosophila*, it is necessary to biochemically analyze the defense response of plants towards different viruses. We hypothesized that an antiviral RISC can be isolated from plants infected with a virus not related to tombusviruses, and analysis of its biochemical characteristics would help to determine if the same or different RISCs target RNAs from different virus species. For this we chose a tobravirus *Tobacco rattle virus* (TRV) variant, which is commonly used as an effective VIGS vector in a wide range of plants (Burch-Smith et al., 2006; Ratcliff et al., 2001).

The results show that column chromatography of extracts from *N. benthamiana* plants infected with TRV yielded fractions exhibiting ribonuclease activity specific for TRV RNA. This activity was inhibited in the presence of NaCl and EDTA, and stimulated by addition of divalent metal cations. Moreover, ribonuclease activity exhibited a preference for longer RNA segments as a cleavage substrate. Interestingly, the specificity of the nuclease activity could be reprogrammed *in vitro* with the addition of total RNA extract from TBSV-infected plants. The evidence strongly supports the view that induction of an antiviral RISC is a common molecular defense pathway and that the composition of this effector is conserved while its specificity is strictly governed by the siRNA content.

Results

Ribonuclease activity associated with siRNAs and Piwi proteins in extracts of TRV-infected plants

To establish TRV infections in *N. benthamiana,* plants were infiltrated with *Agrobacterium* cultures containing T-DNA plasmids that express TRV RNA1 as well as the RNA2 vector with a segment of the *phytoene desaturase* (*pds*) gene (Burch-Smith et al., 2004). Following infiltration of *N. benthamiana*, silencing of *pds* expression is observed as a whitening of tissue (Fig. 1). This type of silencing is virus replication-dependent; agroinfiltrations of the RNA2 construct in absence of RNA1 does not elicit the silencing response (data not shown).

The earliest experiments for this project involved the harvest of tissue at 8 or 2 weeks post-infiltration. Following column chromatography, many fractions exhibited extensive non-specific *in vitro* degradation of any RNA transcripts (data not shown). In an attempt to avoid or minimize non-specific degradation, tissue was harvested from infected *N. benthamiana* plants at the onset of *pds* silencing-induced bleaching, 5 days post-infiltration. Tissue extract was clarified by centrifugation and separated on a 40-ml hydroxyapatite chromatography column. The resultant fractions were individually tested for ribonuclease activity that was still found to be non-specific, as both TRV and TBSV RNAs were degraded (data not shown). These observations suggested that if RISC activity was present, there was insufficient separation from contaminating nucleases, and that additional purification steps were required.

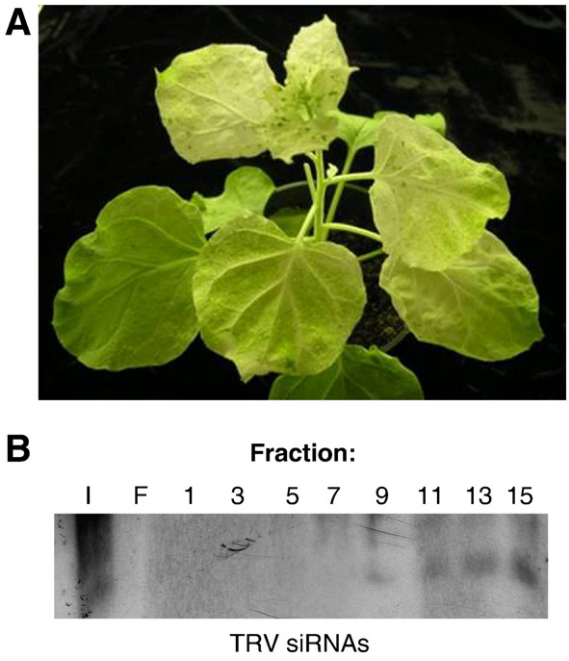

Fig. 1. TRV siRNAs in hydroxyapatite column chromatography fractions from TRV-*pds* infected *N. benthamiana* plants. (A) Image of TRV-*pds* infected plant at 14 days post-infiltration, with the upper leaves displaying bleaching due to *pds* silencing. (B) Infected plant tissue was collected 5 days post-infiltration and RNA was extracted from hydroxyapatite chromatography fractions, separated on a 17% acrylamide SDS-PAGE with 8 M urea, and blotted to a membrane for hybridization with a TRV RNA2 probe. Distinct TRV-derived short RNAs first eluted in fraction 9. I, column input; F, column flow-through.

To determine which fractions to select for further isolation, we tested each for biochemical features characteristic for RISC-associated ribonuclease. First, the presence of short RNAs (including siRNAs) was examined in fractions, followed by Northern hybridization with a TRV RNA2 probe (Fig. 1B). A positive signal for a distinct class of TRV-specific siRNAs was first evident in fraction 9, indicating that an anti-TRV RISC would be present in those fractions, based on our previous observations with TBSV (Omarov et al., 2007). Additionally, because a hallmark of RISC is the presence of one or more Ago proteins containing a conserved Piwi domain, we raised a Piwi-antiserum. When fractions were tested with Western blot assays, fractions 9–13 were enriched for multiple piwi-antiserum reactive proteins (Supplemental Fig. 1).

TRV RNA-specific ribonuclease activity

We realized that neither the ribonuclease test, the siRNA analyses, nor the piwi-Western blot assays by themselves provided sufficient evidence for RISC. However, since hydroxyapatite factions 9–15 (Fig. 1B) encompassed those positive for all three parameters: i) ribonuclease activity, ii) TRV-derived siRNAs, and iii) piwi-antibody reactive proteins, this strongly suggested the presence of RISC. Therefore, these fractions were combined for further separation. After Sephacryl S-200 HR gel column chromatography, fractions were again tested for ribonuclease activity by incubation with *in vitro* generated TRV or TBSV transcripts. The reactions were then subjected to standard agarose gel electrophoresis followed by Northern blot hybridization with probes for either TRV RNA1 (Fig. 2, top panel) or TBSV (Fig. 2, bottom panel) to examine effects on RNA integrity. The results in Fig. 2 reveal that gel filtration fractions 9–11 exhibit nuclease activity against TRV RNA1 transcripts (also observed for RNA2 transcripts, Fig. 3), while no comparable cleavage of TBSV RNA transcripts was evident. Some residual non-specific activity surfaced only at much lower RNA concentrations and upon prolonged reaction