Different roles for RNA silencing and RNA processing components in virus recovery and virus-induced gene silencing in plants

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Abstract

A major antiviral mechanism in plants is mediated by RNA silencing, which relies on the cleavage of viral dsRNA into virus-derived small interfering RNAs (vsiRNAs) by DICER-like enzymes. Members of the Argonaute (AGO) family of endonucleases then use these vsiRNA as guides to target viral RNA. This can result in a phenomenon known as recovery, whereby the plant silences viral gene expression and recovers from viral symptoms. Endogenous mRNAs can also be targeted by vsiRNAs in a phenomenon known as virus-induced gene silencing (VIGS). Although related to other RNA silencing mechanisms, it has not been established if recovery and VIGS are mediated by the same molecular mechanisms. We used tobacco rattle virus (TRV) carrying a fragment of the phytoene desaturase (PDS) gene (TRV–PDS) or expressing green fluorescent protein (TRV–GFP) as readouts for VIGS and recovery, respectively, in Arabidopsis ago mutants. Our results demonstrated roles for AGO2 and AGO4 in susceptibility to TRV, whereas VIGS of endogenous genes appeared to be largely mediated by AGO1. However, recovery appeared to be mediated by different components, as all the aforementioned mutants were able to recover from TRV–GFP inoculation. TRV RNAs from recovered plants associated less with ribosomes, suggesting that recovery involves translational repression of viral transcripts. Translationally repressed RNAs often accumulate in RNA processing bodies (PBs), where they are eventually processed by decapping enzymes. Consistent with this, we found that viral recovery induced increased PB formation and that a decapping mutant (DCP2) showed increased VIGS and virus RNA accumulation, indicating an important role for PBs in eliminating viral RNA.

Key words: Argonaute, VIGS, RNA silencing, Arabidopsis, tobacco rattle virus, DCP2, decapping.

Introduction

Plants have developed diverse mechanisms to defend themselves against viral infections, including RNA silencing. RNA silencing is a sequence-specific RNA mechanism that regulates the expression of endogenous genes as well as exogenous genetic elements, including viruses, transgenes, and transposable elements (Incarbone and Dunoyer, 2013; Bologna and Voinnet, 2014). As a defence against viruses in plants, RNA silencing relies on the recognition of viral dsRNA by DICER-like (DCL) enzymes, which cleave the dsRNA into virus-derived small interfering RNAs (vsiRNA) of 21–24 nt. These vsiRNAs in turn bind to RNA silencing complexes, which contain Argonaute (AGO) proteins that use the
vsiRNAs as guides to target ssRNAs (Mallory and Vaucheret, 2010). The targeting of viral RNAs is thought to largely involve RNA cleavage, although RNA silencing mechanisms can target endogenous genes by translational repression (Aukerman and Sakai, 2003; Brodersen et al., 2008; Lanet et al., 2009; Carbonell et al., 2012). Given the strong pressure exerted by RNA silencing, plant viruses have evolved viral suppressors of RNA silencing (VSRs), which interfere with the RNA silencing machinery at multiple steps (Pumplin and Voinnet, 2013).

The Arabidopsis genome encodes 10 AGO proteins and four DCL proteins, which play roles in multiple RNA silencing-related phenomena. DCL1 is reported mainly to process endogenous microRNAs (miRNAs) (Bologna and Voinnet, 2014) that target cellular transcripts and does not appear to produce significant amounts of vsiRNAs upon infection with cucumber mosaic virus (CMV) or turnip crinkle virus (TCV) (Vaucheret et al., 2004; Deleris et al., 2006). Both DCL2 and DCL4 play roles in antiviral defences against positive-sense ssRNA viruses, whereas DCL3 is thought to play a minor role in defence against RNA viruses (Deleris et al., 2006; Qu et al., 2008; Garcia-Ruiz et al., 2010). A number of AGO proteins show direct antiviral activity, and multiple VSRs have been shown to target AGO proteins (Pumplin and Voinnet, 2013; Schuck et al., 2013). Genetic analyses have reported a role for AGO1 in resistance to CMV and TCV (Azevedo et al., 2010; Morel et al., 2002), while AGO2 has been shown to play a role in resistance to CMV and TCV, as well as to potato virus X, tomato bushy stunt virus and turnip mosaic virus (Harvey et al., 2011; Jaubert et al., 2011; Scholthof et al., 2011; Wang et al., 2011; Carbonell et al., 2012; Zhang et al., 2012).

The phenomenon of recovery is typified by systemic virus infection with associated symptoms, followed by a decrease and disappearance of symptoms in young leaves (MacDiarmid, 2005). Recovered plants are subsequently resistant to further inoculations by the same virus, despite the continued presence of viral RNA in some cases (Jovel et al., 2007). Recovery can also provide resistance against sequence-related viruses, a phenomenon known as cross-protection (Ratcliff et al., 1999; Folimonova, 2013). Recovery occurs in a number of plant–virus interactions, including tobacco rattle virus (TRV) (Ratcliff et al., 1997–1999). Although traditionally defined by symptomology, in the latter case, the term recovery is used to describe a situation wherein TRV expressing green fluorescent protein (TRV–GFP) accumulates in Nicotiana benthamiana with few symptoms except green fluorescence, followed by a loss of fluorescence and a dramatic decrease in virus abundance (Ratcliff et al., 1999). Recovery is thought to be a consequence of RNA silencing, as it can induce sequence-specific gene silencing (Ratcliff et al., 1999; Jovel et al., 2007) and mutations in a viral VSR can result in viruses that undergo recovery and induce cross-protection (Lin et al., 2007). Likewise, the resistance of plants to VSR-defective viruses resembles recovery, and this has been shown to be dependent on RNA silencing components (Deleris et al., 2006; Azevedo et al., 2010; Garcia-Ruiz et al., 2010; Carbonell et al., 2012). Furthermore, the NbAgo1 protein has been reported as being required for recovery against tomato ringspot virus (ToRSV) in N. benthamiana (Ghoshal and Sanfacon, 2014).

Mechanisms related to virus recovery are thought to be involved in virus-induced gene silencing (VIGS), a technique used to downregulate a host gene through the use of a recombinant virus carrying a fragment of the host gene of interest. Upon infection, vsiRNAs are produced from the host gene fragment, which can in turn target the gene for degradation (Ratcliff et al., 2001; Burch-Smith et al., 2004). A small number of RNA silencing mutants have been tested, but only mutants in HEN1 and DCL2/DCL4 have been reported to be dramatically affected in VIGS, suggesting either redundancy or distinct mechanisms in VIGS as compared with other RNA silencing pathways (Deleris et al., 2006; Dunoyer et al., 2007). Thus, although VIGS and virus recovery are related to other RNA silencing mechanisms, the genetic requirements for these phenomena have not been studied extensively. In particular, it is unclear if they employ the same AGO proteins that mediate other RNA silencing phenomena.

Likewise, little is known about the fate of viral RNAs upon recovery, and we have investigated the possibility that this RNA silencing-related phenomenon might involve translational repression. RNA processing bodies (PBs) are cytoplasmic foci where translationally repressed mRNAs accumulate and are eventually processed by decapping enzymes and exoribonucleases (Parker and Sheth, 2007). In animals and yeast, mRNAs subjected to translational repression accumulate in PBs, and increases in translation repression can result in an increase in the number of PBs in the cell (Franks and Lykke-Andersen, 2008; Balagopal and Parker, 2009). In plants, the major enzymatic components of PBs are well conserved (Weber et al., 2008; Maldonado-Bonilla, 2014). Several PB component mutants have enhanced RNA silencing phenotypes, which are thought to be due to an accumulation of ‘aberrant’ RNAs that subsequently become substrates for the RNA silencing machinery (Souret et al., 2004; Gy et al., 2007; Thran et al., 2012).

Here, we used TRV to investigate the role of individual AGO proteins in VIGS and recovery using Arabidopsis mutants. Although ago2-1 and ago4-2 mutants were initially more susceptible to TRV, none of the 10 single ago mutant lines was compromised in virus recovery, whereas the ago1-27 mutant was compromised for VIGS, suggesting that VIGS and recovery are mediated by different components of the RNA silencing machinery. At the same time, we found that TRV RNAs in recovered plants showed reduced association with ribosomes, suggesting translational repression of viral RNAs. Consistent with this, plants expressing a PB marker showed increased numbers of PBs after recovery from TRV but not upon infection with TCV, from which Arabidopsis does not recover. These results indicated a connection between virus recovery, VIGS, and translational repression.

Materials and methods

Plants and viruses

Arabidopsis thaliana wild type (Col-0 and Ler) and the following previously described mutant lines were used: ago1-27 (Morel et al., 2002), ago2-1, ago3-2, ago6-3, ago8-1, and ago10-2 (Takeda et al., 2008), ago5, ago7, and ago9 (Katiyar-Agarwal et al., 2007), ago4-1 (Zilberman et al., 2003), ago4-2 (Agorio and Vera, 2007), triple DICER mutant dcl2-1dcl3-1dcl4-2 (Deleris et al., 2006), ago12,
Fig. 1A

KCl, 25 mM tridecyl ether, 5 mM phosphate buffer, pH 7.5, 1% deoxycholic acid sodium salt, 1% polyoxyethylene glycol (Igepal CA630), 1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20), 1% detergent mix [1% (w/v) polyoxyethylene(23) lauryl ether (Brij-35), 1% (v/v) Triton X-100, 1% (v/v) octylphenyl-polyethylene glycol (Igepal CA630), 1% (v/v) polyoxyethylene sorbitan monolaurate (Tween 20), 1% deoxycholic acid sodium salt, 1% polyoxyethylene (10) tridecyl ether, 5 mM dithiothreitol, 1 mM protease inhibitors, 50 μg ml⁻¹ of cycloheximide, 50 μg ml⁻¹ of chloramphenicol]. Aliquots from the input and from the pellet fractions were subsequently isolated with an equal volume of 8 M guanidine HCl and 3 vols of ethanol, and quantified. For polysome profiles, RNA pellets were overlaid on sucrose gradients [4.5 ml linear sucrose gradient of 15–60% sucrose (w/v), supplemented with 10 mM Tris/HCl, pH 7.5, 140 mM KCl, 1.5 mM MgCl₂, 100 μg ml⁻¹ of chloramphenicol and 100 μg ml⁻¹ of cycloheximide] and ultracentrifuged at 237 000 g for 2 h at 4 °C. Seventeen fractions (300 μl per fraction) were collected and an equal volume of 8 M guanidine/HCl and 3 vols of ethanol were added to each fraction to precipitate the RNA.

**RNA extraction and northern blotting**

Total RNA was extracted from pools of six to eight plants using Trizol reagent as per the manufacturer’s instructions (Invitrogen). Total RNA (15–20 μg) was separated on a 1.2% (w/v) formaldehyde/agarose gel by electrophoresis and then transferred to a positively charged nylon membrane (Roche) and cross-linked with UV light (Spectrolinker XL-1000 UV Crosslinker; Spectrolink). Prehybridization, hybridization (DIG Easy Hyb; Roche) and washing (DIG Wash and Block Buffer Set; Roche) were performed following the manufacturer’s instructions. DNA probes labelled with α[32P]dATP (PCR DIG Probe Synthesis kit; Roche) corresponding to the coat protein of TR V RNA2 or the GFP insert were PCR amplified using primer pairs CP-F (5′-AT GGGAGATATGACAGAATCCAT-3′) and CP-R 5′-CTAGG GATAGGAGATCGGACCTC-3′) or GFP-F (5′-GTCAGTG GAGAGGTCAGGTT-3′) and GFP-R (5′-GTCGTCATGGT GAACGCTTCCAT-3′), and were used to detect TR V RNA2–PDS or TR V RNA2–GFP, respectively. Signals were revealed by CDP-Star ready-to-use substrate (Roche). Signal intensities were quantified using ImageJ software.

**SDS-PAGE and western blotting**

The entire aboveground portion of individual Arabidopsis plants was ground in liquid nitrogen, and 50 μg of the ground powder was mixed with 50 μl 1.5× sample buffer [18 mM Tris/HCl, pH 6.8, 7.5% glycerol, 0.6% SDS, 0.3 mg ml⁻¹ of bromophenol blue, 5% β-mercaptoethanol (v/v)] and incubated at 95 °C for 5 min. Protein samples from total leaf tissue extracts were separated by SDS-PAGE on 10.5% resolving gels and transferred to a polyvinylidene difluoride membrane (BioRad), followed by incubations with hors eradish peroxidase-conjugated GFP antiserum (Rockland Immunochemicals). The proteins were revealed by western blotting luminol reagent (Santa Cruz Biotechnology).

**Polysome RNA isolation**

Polysome extraction assays were performed as described previously (Mustroph et al., 2009), with some minor modifications. Briefly, tissues from the infiltrated leaf areas were ground to a fine powder in liquid nitrogen, resuspended in polysome extraction buffer (0.2 M Tris/HCl, pH 9.0, 0.2 M KCl, 25 mM EGTA, 35 mM MgCl₂, 1% detergent mix [1% (w/v) polyoxyethylene(23) lauryl ether (Brij-35), 1% (v/v) Triton X-100, 1% (v/v) octylphenyl-polyethylene glycol (Igepal CA630), 1% (v/v) polyoxyethylene sorbitan monolaurate 20], 1% deoxycholic acid sodium salt, 1% polyoxyethylene (10) tridecyl ether, 5 mM dithiothreitol, 1× protease inhibitors, 50 μg ml⁻¹ of cycloheximide, 50 μg ml⁻¹ of chloramphenicol) and centrifuged at 16 000 g for 15 min at 4 °C. Extracts were overlaid on a 1.6 M sucrose cushion solution (0.4 M Tris/HCl, pH 9.0, 0.2 M KCl, 5 mM EGTA, 35 mM MgCl₂, 1.7 M sucrose, 5 mM dithiothreitol, 50 μg ml⁻¹ of cycloheximide, 50 μg ml⁻¹ of chloramphenicol) and clarified by centrifugation at 16 000 g for 15 min at 4 °C. Extracts were overlaid on a 1.6 M sucrose cushion solution (0.4 M Tris/HCl, pH 9.0, 0.2 M KCl, 5 mM EGTA, 35 mM MgCl₂, 1.7 M sucrose, 5 mM dithiothreitol, 50 μg ml⁻¹ of cycloheximide, 50 μg ml⁻¹ of chloramphenicol) and ultracentrifuged at 116 000 g for 18 h at 4 °C. The RNA pellets were resuspended in resuspension buffer (0.2 M Tris/HCl, pH 9.0, 0.2 M KCl, 25 mM EGTA, 35 mM MgCl₂, 5 mM dithiothreitol, 50 μg ml⁻¹ of cycloheximide, 50 μg ml⁻¹ of chloramphenicol). Aliquots from the input and from the pellet fractions were subsequently isolated with an equal volume of 8 M guanidine HCl and 3 vols of ethanol, and quantified. For polysome profiles, RNA pellets were overlaid on sucrose gradients [4.5 ml linear sucrose gradient of 15–60% sucrose (w/v), supplemented with 10 mM Tris/HCl, pH 7.5, 140 mM KCl, 1.5 mM MgCl₂, 100 μg ml⁻¹ of chloramphenicol and 100 μg ml⁻¹ of cycloheximide] and ultracentrifuged at 237 000 g for 2 h at 4 °C. Seventeen fractions (300 μl per fraction) were collected and an equal volume of 8 M guanidine/HCl and 3 vols of ethanol were added to each fraction to precipitate the RNA.

**Microscopy and quantitation**

Confocal laser-scanning microscopy was performed on a FV300 imaging system (Olympus). Yellow fluorescent protein (YFP) was excited using a 488 nm argon laser and emission was detected using a 510–530 band-pass filter. Granules were counted from images of equal areas (244×244 μm) from each treatment using Cell-Profiler software (Jones et al., 2008). In this study, each image represents a Z-stack of 0.5 μm. Representative images are shown from experiments that were repeated at least three times.

**Results**

**Recovery from TR V in Arabidopsis RNA silencing mutants**

TRV is widely used as a VIGS vector in a variety of plant species (MacFarlane, 2010) and is one of the only VIGS vectors based on an RNA virus to function in Arabidopsis (Deng et al., 2013). We have previously used TRV to silence AGO-encoding genes in N. benthamiana (Bhattacharjee et al., 2009; Scholthof et al., 2011). The latter experiments may seem paradoxical if AGO proteins are required for VIGS, and so we investigated which AGO proteins are required for viral recovery and VIGS by TRV in Arabidopsis. In certain cases, virus recovery is characterized by a continued presence of viral RNA but a dramatic decrease in viral protein translation (Jovel et al., 2007; Ghoshal and Sanfaçon, 2014). TRV engineered to express GFP (TRV–GFP) (MacFarlane and Popovich, 2000) has previously been used as a read out for recovery in N. benthamiana (Ratecliff et al., 1999). In TRV–GFP-infected Arabidopsis Col-0 plants, GFP fluorescence was observed on the inoculated leaves 2–3 dpi post-inoculation (Fig. 1A). At 5 dpi, GFP fluorescence appeared on the systemic leaves above the inoculated leaf (Fig. 1A). However, at 8 dpi, GFP fluorescence was no longer apparent on either the infected or the newly emerging leaves (Fig. 1A). Likewise, western blotting showed reduced levels of GFP protein over time, indicating that the plant had recovered from TRV–GFP infection (Fig. 1B). Northern blotting indicated a peak of viral RNA accumulation at 5 dpi with decreased viral RNA levels thereafter (Fig. 1C).

To explore the role of RNA silencing components in TRV recovery, we first infected a triple DICER (TD) mutant line (dcl2/dcl3/dcl4; MacFarlane, 1999). In TRV–GFP-infected N. benthamiana (Ratecliff et al., 1999). In TRV–GFP-infected Arabidopsis Col-0 plants, GFP fluorescence was observed on the inoculated leaves 2–3 d post-inoculation (dpi) (Fig. 1A). At 5 dpi, GFP fluorescence appeared on the systemic leaves above the inoculated leaf (Fig. 1A). However, at 8 dpi, GFP fluorescence was no longer apparent on either the infected or the newly emerging leaves (Fig. 1A). Likewise, western blotting showed reduced levels of GFP protein over time, indicating that the plant had recovered from TRV–GFP infection (Fig. 1B). Northern blotting indicated a peak of viral RNA accumulation at 5 dpi with decreased viral RNA levels thereafter (Fig. 1C).
Fig. 1. TRV-GFP as a model for virus recovery in Arabidopsis. (A) Several leaves of wild-type (Col-0; upper panels) and triple DICER mutant (TD; lower panels) Arabidopsis were infected with TRV–GFP and representative leaves are indicated with white arrows. GFP fluorescence (representative fluorescent areas indicated by green arrows) was photographed under UV illumination at 3, 5, 8, and 11 dpi. (B) Protein extracts from non-infected (NI) or TRV–GFP-infected Col-0 (upper panel) or TD mutant plants (lower panel), sampled at the indicated time points, were subjected to anti-GFP western blotting. Ponceau staining is shown to demonstrate equal loading. Each lane corresponds to a pool of five to six inoculated plants. (C) Total RNA was extracted
levels of viral RNA accumulation, accumulated GFP protein for a longer time period relative to wild-type plants, and showed viral symptoms in the form of stunted growth and leaf curling (Fig. 1D). Nonetheless, TD plants still recovered to a certain extent in that GFP fluorescence still disappeared at 11 dpi, and GFP protein and viral RNA decreased over time, albeit a later time point than in the wild type (Fig. 1A–C). Thus, although the TD mutant could eventually downregulate TRV–GFP expression, the virus appeared to accumulate sufficiently (or long enough) to induce symptoms. We next infected wild-type and AGO mutant lines. These included the hypomorphic ago1-27 allele, as ago1 null mutants are lethal (Morel et al., 2002), as well as T-DNA insertional null alleles for the AGO2 to AGO10 genes. Upon infection with TRV–GFP, GFP was detectable visually and by western blotting at 3–5 dpi in all genotypes (Fig. 2). Nonetheless, all 10 ago mutant lines still underwent recovery from TRV–GFP in that GFP fluorescence subsided 8–11 dpi (Table 1, Supplementary Fig. S1 at JXB online).

The fact that all mutant lines tested showed a loss of GFP fluorescence could be caused by properties inherent to the replication/expression strategies of TRV rather than by RNA silencing. To test whether TRV had the intrinsic ability to undergo sustained GFP expression, we co-infected wild-type plants with TRV–GFP and wild-type TCV, which encodes a strong VSR, P38 (Qu et al., 2003). Plants infected with both TRV–GFP and TCV showed dramatically increased GFP fluorescence after 8 d, compared with TRV–GFP alone (Fig. 1E). GFP fluorescence was observable up to 17 d, at which point the infected plants died (data not shown). Although we cannot rule out the possibility that TCV causes this effect via other mechanisms, the simplest interpretation of this result is that TRV can undergo sustained expression in Arabidopsis if RNA silencing mechanisms are fully inhibited by a more virulent virus.

AGO2 and AGO4 mutants show increased TRV susceptibility but still recover

Upon infection with TRV–GFP, we observed increased GFP intensity on both the inoculated and systemic leaves of ago2-1 and ago4-2 mutant plants compared with Col-0 (Fig. S2A, Supplementary Fig. 1A). This was also confirmed by anti-GFP western blotting (Fig. 2B) and northern blotting (Supplementary Fig. S2 at JXB online). The role of AGO2 in virus defence is well established, but to confirm the role of AGO4 in TRV susceptibility, we tested an additional ago4 mutant allele. Upon infection of the ago4-1 mutant, present in the Landsberg (Ler) background, and wild-type Ler plants, we observed the same result as with the ago4-2 allele in the Col-0 background (Supplementary Fig. S2). Despite these initial increased GFP levels, however, both ago2 and ago4 mutant plants still recovered, eventually losing GFP expression (Table 1, Fig. 2A, Supplementary Fig. S1).

At the same time, ago1-27 plants consistently showed less GFP, and viral RNA accumulation and systemic movement of TRV–GFP proceeded more slowly in this mutant (Fig. 2A, Supplementary Figs S1 and S3 at JXB online). We also inoculated a double mutant, possessing the ago1-27 allele and an ago2 mutant allele (ago1ago2), with TRV–GFP. In this mutant, we observed increased GFP, visually and by western blotting, similar to that seen in the ago2 single mutant (Fig. 2 and Supplementary Fig. S1). However, virus infection progressed more slowly in this mutant, as seen in ago1-27 plants, with the virus moving into systemic leaves at 6–8 dpi, compared with 5 dpi for wild-type plants (Supplementary Fig. S1).

TRV VIGS in Arabidopsis RNA silencing mutants

Virus recovery differs from VIGS in that the former targets viral RNAs, whereas the latter is due to the targeting of endogenous mRNAs by vsiRNAs. To determine if these mechanisms might use different components, we infected the same mutant lines with TRV carrying an insert from the Arabidopsis PDS gene (TRV–PDS) (Liu et al., 2002). Consistent with the results observed with TRV–GFP, ago2 and ago4 mutant plants showed increased levels of TRV–PDS viral RNAs, although this was not accompanied by an altered VIGS phenotype (Fig. 3, Table 1). Indeed, with the exception of ago1-27, all single ago mutant lines showed similar VIGS phenotypes, as assessed visually by the intensity and extent of photobleaching (Table 1, Supplementary Fig. S3). VIGS in ago1-27 and in several double and triple mutants with the ago1-27 allele (ago12, ago1ago5ago10, ago1ago7, ago1ago5) (Supplementary Fig. S4A at JXB online) was compromised, with greatly reduced photobleaching, which was restricted to areas around primary veins (Table 3, Supplementary Fig. S4A and Table 1). This decrease in VIGS efficiency was similar to but not as severe as in the TD mutant (Fig. 3A), in which VIGS has previously been reported to be severely compromised (Deleris et al., 2006). Surprisingly, whereas TRV–GFP showed decreased accumulation of viral RNA in the ago1-27 mutant (Supplementary Fig. S3), we consistently observed an increase in TRV–PDS RNA accumulation in this mutant compared with the wild type (Fig. 3B). At the same time, viral TRV–PDS RNA levels were even higher in the ago12 mutant (Supplementary Fig. S4B). These observations suggested independent functions of AGO1 and AGO2 in defence against TRV–PDS. They also suggested differences in the mechanisms that inhibit viral gene expression (recovery) versus those that regulate viral RNA accumulation and the targeting of endogenous genes for silencing.

VIGS intensity of dcl2/dcl3/dcl4 plants is temperature dependent

Previous studies have shown that environmental conditions such as elevated temperature can enhance silencing efficiency against

from plants infected with TRV–GFP-infected Col-0 (left panel) or from TD mutant plants (right panel) at the time points indicated and subjected to northern blotting with an anti-GFP probe. TRV RNA2 genomic (g) and subgenomic (sg) are indicated. Ethidium bromide (Etbr)-stained RNA (prior to transfer) is shown as a loading control. (D) Symptoms in non-infected (mock) and TRV–GFP-infected Arabidopsis. Photographs were taken 3 weeks after infection. (E) Wild-type Arabidopsis was co-infected with TRV–GFP and TCV. GFP fluorescence was photographed under UV illumination at 8 dpi. All experiments were repeated three to five times, and representative results are shown.
RNA viruses, often resulting in decreased symptom development and/or enhanced recovery (Szittya et al., 2003; Qu et al., 2005; Velázquez et al., 2010; Zhang et al., 2012; Ghoshal and Sanfacon, 2014). As the TD and ago1-27 mutants showed reduced VIGS intensity in the experiments outlined above (at 19 °C), we repeated TR V–PDS infections at 19 and 26 °C. As shown in Fig. 4, growing infected plants at 26 °C resulted in significantly increased photobleaching in TD mutant plants compared with plants grown at 19 °C. The ago1-27 mutant showed a slight increase in VIGS efficiency but not to the same extent as the TD mutant at 26 °C (Fig. 4). These results further indicated that AGO1 is a limiting factor in silencing endogenous genes by VIGS and suggest that the DICER protein remaining in the TD mutant, DCL1, can function in VIGS under certain circumstances.

**TRV recovery involves translational repression and PB formation**

Plants that have recently recovered from TRV–GFP infection contain significant levels of viral RNA (Fig. 1C), begging the question of why these RNAs do not produce greater amounts of GFP protein. A recent report suggested that recovery involves the repression of translation of viral proteins (Ghoshal and Sanfacon, 2014). To test this possibility in our experimental system, we analysed the association of TRV–GFP transcripts with actively translating ribosomes. We performed polysome profiling by sucrose density-gradient fractionation on plants infected with TRV–GFP showing green fluorescence (5 dpi) and recovered plants (8 dpi). As seen in Fig. 5A, total polysome profiles did not differ among uninfected, fluorescing, and recovered plants, indicating that recovery is not associated with a global repression of translation. Polysome profiling protocols for plant ribosomes included a ribosome pelleting step in which extracts were passed through a 1.6 M sucrose cushion to pellet all ribosomes prior to separation on a sucrose gradient (Mustroph et al., 2009). Initial attempts at polysome profiling of TRV RNAs, however, were hampered by low amounts of RNA in the ribosome pellets from recovered plant samples. As shown in Fig. 5B, northern blotting showed a mild reduction in total RNA in recovered versus non-recovered plants. However, much less viral RNA was present in the ribosome pellets of recovered plants. In the northern blot shown in Fig. 5B, which is a representative result from three biological repetitions, we observed that in non-recovered plants the ratio of signal in total versus ribosome pellet RNAs was 1.3:1, whereas in recovered plants the ratio was 3.2:1. Thus, when compared with total viral RNA, TRV–GFP RNAs were reduced more...
than 2-fold in the ribosomal pellet in recovered plants compared with non-recovered plants. These results indicated that, after recovery, a significant proportion of TRV–GFP RNAs do not associate with ribosomes, suggesting that their translation may be inhibited.

RNAs subjected to translational repression often accumulate in PBs prior to degradation (Maldonado-Bonilla, 2014). The decapping protein DCP1 is a well-established marker for PBs in Arabidopsis (Xu et al., 2006; Weber et al., 2008; Xu and Chua, 2009). We investigated the role of PBs in virus recovery using transgenic Arabidopsis plants expressing DCP1 fused to YFP (YFP–DCP1) from the DCP1 promoter (Merret et al., 2013). Plants were infected with TRV (with no insert) or with TCV (from which Arabidopsis does not recover) on d 0, followed by infection of a second batch of plants of the same age 4 d later. PBs were then visualized 8 d later as, based on the results found with TRV–GFP (Fig. 1 and Supplementary Fig. S1), the first batch of infected plants would have recovered from TRV, whereas the second batch would not. PBs, as indicated by foci of YFP–DCP1, were observed by confocal microscopy in inoculated leaves and the number of PBs in a fixed area (244 × 244 μm) was counted. Uninfected plants showed very few PBs and we observed no significant difference in PB number in plants infected with TRV for 4 d, or in plants infected with TCV for 4 or 8 d, as compared with uninfected plants (Fig. 6). However, we observed a dramatic increase in the average number of PBs in TRV recovered plants (8 d infected), suggesting that viral RNAs in recovered

<table>
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<th>Mutants</th>
<th>GFP intensityab</th>
<th>PDS VIGS intensityb</th>
<th>TRV–GFP recovery</th>
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<td>+</td>
<td>8 dpi</td>
</tr>
<tr>
<td>ago1/7</td>
<td>+</td>
<td>+</td>
<td>8 dpi</td>
</tr>
<tr>
<td>ago1/2</td>
<td>+++</td>
<td>+</td>
<td>11 dpi</td>
</tr>
<tr>
<td>ago1/2/7</td>
<td>+++</td>
<td>+</td>
<td>11 dpi</td>
</tr>
<tr>
<td>ago1/2/10</td>
<td>+++</td>
<td>+</td>
<td>11 dpi</td>
</tr>
</tbody>
</table>

a GFP intensity is based on visual assessment at 5 dpi of at least 30 plants of a given genotype using Col-0 as a standard (++). Examples of varying degrees of fluorescence (+, ago1–27; ++++, ago2–1; ++++, TD) are shown in Figs 1 and 2.

b VIGS intensity is based on visual assessment of the relative extent (portion of entire plant) of photobleaching at 13 dpi of at least 30 plants of a given genotype using Col-0 as a standard (++). Examples of varying degrees of VIGS intensity (+/−, TD; +, ago1–27; ++++, its1) are shown in Figs 3 and 4.

c At 19 °C only.
plants contribute to an increase in PB activity, likely due to their translational repression (Fig. 5).

To investigate further the role of PB components in virus recovery/VIGS, we infected the Arabidopsis increased transgene silencing (itsl) mutant (Thran et al., 2012) with TRV–PDS and TRV–GFP. Most mutants in genes encoding components of the decapping complex have severely or lethal phenotypes (Maldonado-Bonilla, 2014). However, the itsl phenotype is caused by a hypomorphic mutation in DECAPPING 2 (DCP2), which encodes for the catalytic subunit of the decapping complex found in PBs (Xu et al., 2006). We observed an increase in the time of onset, intensity, and the extent of TRV–PDS VIGS in the itsl mutant (Fig. 7A, left). In contrast, the timing and extent of infection by TRV–GFP, as well as recovery, was not noticeably different from the wild type except for a slight increase in GFP protein accumulation (Fig. 7B, C). However, the itsl mutant consistently accumulated much higher levels of TRV–GFP and TRV–PDS RNA compared with Col-0 (Fig. 7C, Supplementary Fig. S6 at JXB online).

Discussion

In the first systematic study, to the best of our knowledge, of the involvement of AGO proteins in VIGS and recovery, we found that these phenomena were surprisingly robust, in contrast to other RNA silencing mechanisms. Although the TD, ago2, and ago4 mutants showed delayed recovery (Table 1), this was probably due to increased initial levels of virus and GFP. Thus, either recovery involves non-RNA silencing mechanisms, or the involvement of DCL and AGO proteins in recovery is highly redundant. We do not rule out the former possibility, but the fact that co-infection with TCV allowed TRV to escape recovery, presumably by providing a strong VSR in trans, suggests that recovery is mediated by RNA silencing-related mechanisms. The TCV VSR P38 interferes with AGO activity and has been reported to co-immunoprecipitate with AGO1 but not AGO4 or AGO7 (Azevedo et al., 2010). Our data suggest that, in addition to AGO1, P38 must also inhibit whichever other AGO proteins mediate recovery. The identification of these other AGO proteins will require further study; however, this redundancy is perhaps not surprising. For example, of the AGO proteins tested, AGO1, AGO2, and AGO5 but not AGO4 and AGO7 bind vsiRNAs derived from viruses (TCV and CMV) with VSRs that target AGOs (Zhang et al., 2006; Takeda et al., 2008; Azevedo et al., 2010; Hamera et al., 2012). At the same time, AGO1, AGO2, AGO3, AGO4, AGO5, and AGO9 are able to bind to small interfering RNAs (siRNAs) derived from a viroid, which does not encode a VSR (Minoa et al., 2014). These results suggest that, in the absence of VSR, most AGO proteins are able to bind siRNAs. Thus, given the relatively weak activity of the TRV VSR (Martin-Hernandez and Baulcombe, 2008), it is possible that multiple unrelated AGO proteins target TRV. Likewise, although DCL2 and DCL4 are thought to be the most important DICER proteins for defence against viruses, TD mutants infected with TRV do nonetheless produce small amounts of TRV-derived siRNAs (Deleris et al., 2006). Thus, although the latter DICER proteins may normally mediate antiviral defence, our results indicate that DCL1 does have some capacity to mediate recovery. The fact that VIGS in the TD mutant is temperature dependent is consistent with the temperature-dependent property of RNA silencing, which has been well documented in previous studies (Szittya et al., 2003; Qu et al., 2005; Tuttle et al., 2008; Zhang et al., 2012).

The ago2 and ago4 mutants showed an initial increase in susceptibility to TRV, as indicated by increased protein and RNA levels (Figs 2B and 3B). The antiviral role of AGO2 has been well established in previous studies (Harvey et al., 2011; Jaubert et al., 2011; Scholthof et al., 2011; Wang et al., 2011) and appears to function through direct slicing of viral RNAs (Carbonell et al., 2012). However, increased susceptibility of the ago4 mutants to a virus is novel. The role of AGO4 in TRV infection is less clear, as AGO4 is best characterized for its role in RNA-directed DNA methylation and transcriptional gene silencing of transposons and repeats (Zilberman et al., 2004; Li et al., 2006; Li et al., 2008; Ye et al., 2012; Bologna and Voinnet, 2014). This activity may play a general role in plant defences, as ago4 mutants are also more susceptible to the
bacterium *Pseudomonas syringae* (Agorio and Vera, 2007). Nonetheless, the *ago2* and *ago4* mutants still undergo recovery, suggesting a temporal aspect to antiviral defences and that susceptibility (i.e. the ability of the virus to accumulate initially in plant cells) and recovery may be carried out by different molecular mechanisms. Thus, AGO2 and AGO4 may play important roles in restricting viruses early in infection, whereas other AGO proteins (or other mechanisms) may later mediate recovery. This idea is consistent with DCL proteins having differential effects in local and systemic infection, in that DCL4 alone is required for defence against turnip mosaic virus in inoculated leaves, whereas both DCL4 and DCL2 are required later in infection, in systemic tissues (Garcia-Ruiz et al., 2010). The *ago1-27* mutant showed decreased susceptibility to TRV–GFP, as indicated by reduced GFP intensity (Fig. 2, Supplementary Fig. S1) and reduced viral RNAs level in TRV–GFP-infected plants (Supplementary Fig. S3). This phenotype contrasts with the increased susceptibility of *ago1* mutants to CMV and VSR-defective TCV (Vaucheret et al., 2004; Deleris et al., 2006) and with the requirement for *NbAgo1* for recovery against ToRSV (Ghoshal and Sanfacon, 2014). On the other hand, it is consistent with a lack of requirement for *NbAgo1* for recovery from a VSR-deficient tomato bushy stunt virus infection (Scholthof et al., 2011), a lack of increased susceptibility to potato virus X in *ago1-27* plants (Jaubert et al., 2011), and a slower infection of *NbAgo1*-silenced plants by ToRSV (Ghoshal and Sanfacon, 2014). AGO1 regulates the expression of *AGO2*, which is targeted by a miRNA, and consequently AGO2 protein levels increase in *ago1* mutants (Harvey et al., 2011). The *ago1/ago2* double mutant allows GFP expression from TRV similar to the *ago2* mutant (Fig. 2) and greater accumulation of TRV–PDS RNA (Supplementary Fig. S4), suggesting that *ago1-27* phenotypes may be due to increased AGO2. However, TRV–GFP still moves more slowly in *ago1/ago2* plants, indicating that this mutant is still somewhat more resistant to TRV. This observation is consistent with previous reports showing that silencing or mutation of *AGO1* can result in constitutive expression of defence-related genes and enhanced resistance gene responses (Yi and Richards, 2007; Bhattacharjee et al., 2009; Li et al., 2010 2012; Shivaprasad et al., 2012).

To date, only mutations in *DCL2*, *DCL4*, and *HEN1* have been reported to have dramatic effects on VIGS (Deleris et al., 2006; Dunoyer et al., 2007). In contrast to recovery, we were able to identify one AGO protein, AGO1, that appeared to be a major effector of VIGS (Fig. 3). The *ago1-27* mutant showed some residual VIGS, although this may be due to the fact that this allele encodes a hypomorphic allele (Morel et al., 2002). A role for AGO1 in VIGS is in agreement with the fact that AGO1 appears to be the major effector of miRNA-mediated control of endogenous gene expression (Bologna and Voinnet, 2014). This result underlines an important difference between recovery and VIGS in that the former targets viral RNAs, whereas the latter targets cellular transcripts. Our results suggest that, although both AGO1 and AGO2 can bind vsiRNAs (Takeda et al., 2008), they somehow distinguish between different types of target RNA. Thus, even though virus infection results in massive production of vsiRNAs, AGO2 (and possibly others) appear to use these guide RNAs only to target viral RNAs, whereas AGO1 can be loaded with vsiRNAs but largely targets cellular mRNA, at least in the case of TRV. Our speculation is...
supported by a recent report indicating the existence of two distinct pools of cellular AGO1 that are specifically loaded by siRNAs and miRNAs, respectively (Schott et al., 2012).

Surprisingly, in contrast to the decreased susceptibility to TRV–GFP (Fig. 2), we observed an increased TRV–PDS viral RNA level in ago1-27 and ago1 double and triple mutants plants compared with wild-type plants (Fig. 3B, Supplementary Fig. S4B). This result is similar in part to that seen with TCV; whereas TCV lacking its VSR (TCVΔCP) accumulated to higher levels in an ago1 mutant, TCV-ΔCP–GFP accumulated to lower levels (Qu et al., 2008). The reason for this difference is not yet clear, although it is possible that the GFP insert, due to some as-yet-undefined characteristic(s), makes the virus more susceptible to the increase in AGO2 caused by a lack of AGO1. Qu et al. (2008) suggested that this may be due to a lack of secondary structure in the GFP

Fig. 6. TRV recovery induces an increase in PB formation. Transgenic Arabidopsis plants expressing YFP–DCP1 were infected with TCV or TRV and monitored for YFP fluorescence at 4 and 8 d. (A) Confocal images of YFP fluorescence in epidermal cells of virus-infected or uninfected plants. A representative PB is indicated with a white arrow. YFP fluorescence was pseudocoloured using the software Cell-Profiler. (B) The number of YFP-expressing PBs (P-bodies) was quantified using Cell-Profiler software and represented graphically. See Supplementary Fig. S5 at JXB online for further details. (This figure is available in colour at JXB online.)
Fig. 7. VIGS and recovery in the Arabidopsis its1 mutant. (A) Col-0 and its1 mutant Arabidopsis plants were infected with TRV–PDS and photographed at the indicated number of dpi. (B) Col-0 and its1 mutant Arabidopsis plants were infected with TRV–GFP and photographed under UV illumination at the indicated number of dpi. (C) Total protein and total RNA was extracted from plants of the indicated genotypes that were either non-infected (NI) or infected with TRV–GFP at the time points indicated. Samples were subjected to anti-GFP western blotting (upper panels) and northern blotting with an anti-TRV CP probe (lower panels). Ponceau staining and ethidium bromide (Etbr)-stained RNA (prior to transfer) are shown as a loading controls.
activity, most likely mediated by AGO2, followed by translational repression mediated by additional AGO proteins.

A role for the decapping complex in viral defence was also seen with the hypomorphic DCP2 mutant istsl (Fig. 7). This mutation does not affect recovery from TRV–GFP infection. However, like ago1-27, istsl shows greatly increased accumulation of RNA from TRV–GFP (Fig. 7) and TRV–PDS (data not shown). Unlike ago1-27, however, istsl plants underwent VIGS to a much faster and greater extent. The latter phenotype is similar to the original increased transgene silencing phenotype (Thran et al., 2012). Plants with the istsl mutation have less uncapped RNA and transgenes have a greater tendency to undergo silencing and to give rise to siRNAs. This is thought to be due to a build-up of ‘aberrant’ RNAs that feed into an siRNA production/amplification mechanism based on RNA-dependent RNA polymerase 6, thus producing more siRNAs, which can in turn target the source gene. In contrast, however, whereas transgene RNAs decrease in istsl, TRV–GFP RNA increases. This suggests that the decapping/PB pathway may be an important mechanism for degrading viral RNA. Indeed, in contrast to the TD, ago2, and ago4 mutants, the greatly increased TRV–GFP RNA accumulation in istsl plants was not reflected in a similar increase in GFP protein (Figs 2 and 7). This suggests that much of the ‘excess’ viral RNA represents aberrant or non-translating RNA that would normally be degraded in PBs. Whether this degradation activity is linked to RNA silencing or represents an independent mechanism for clearing viral RNA remains to be determined. At the same time, the istsl mutant is, to our knowledge, the only mutant described with an increased efficiency of VIGS, and this mutant could be useful in functional genomics approaches in Arabidopsis.

In summary, we have shown that that virus susceptibility, recovery, and VIGS appear to be separable phenomena, with AGO2 and AGO4 playing important roles in the initial susceptibility to TRV, AGO1 playing an important role in VIGS, and as-yet-unidentified players mediating recovery. These results suggest the existence of distinct RNA-induced silencing complexes that target different RNA populations within the cell and over time, and provide a framework for further investigating their underlying molecular mechanisms. Furthermore, we showed that translational repression of viral RNA is likely to play an important role in virus recovery and that decapping/PB function plays an important role in clearing viral RNA from the cell.

Supplementary data

Supplementary data are available at JXB online.

Supplementary Fig. S1. TRV–GFP recovery in ago mutants.

Supplementary Fig. S2. AGO2 and AGO4 play antiviral roles against TRV–GFP.

Supplementary Fig. S3. Reduced TRV–GFP viral RNA in the ago1-27 mutant.

Supplementary Fig. S4. TRV phenotypes in ago1 and double and triple mutants.

Supplementary Fig. S5. Control images and PB numbers corresponding to Fig 6.

Supplementary Fig. S6. Increased TRV–PDS RNA levels in the istsl mutant.

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References


Folimonova SY. 2013. Developing an understanding of cross-protection by Citrus tristeza virus. Frontiers in Microbiology 4, 76.


interfering RNA biogenesis during turnip mosaic virus infection. Plant Cell 22, 481–496.


Sh-installation PV, Chen HM, Patel K, Bond DM, Santas BA, Baulcombe DC. 2012. A microRNA superfamily regulates nucleotide


