Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation

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Virus infections induce the expression of *ARGONAUTE1* (*AGO1*) mRNA and in parallel enhance the accumulation of miR168 (regulator of *AGO1* mRNA). Here, we show that in virus-infected plants the enhanced expression of *AGO1* mRNA is not accompanied by increased *AGO1* protein accumulation. We also show that the induction of *AGO1* mRNA level is a part of the host defence reaction, whereas the induction of miR168, which overlaps spatially with virus-occupied sectors, is mediated mainly by the Tombusvirus p19 RNA-silencing suppressor. The absence of p19 results in the elimination of miR168 induction and accompanied with the enhanced accumulation of *AGO1* protein. In transient expression study, p19 mediates the induction of miR168 and the down-regulation of endogenous *AGO1* level. P19 is not able to efficiently bind miR168 in virus-infected plants, indicating that this activity is uncoupled from the small RNA-binding capacity of p19. Our results imply that plant viruses can inhibit the translational capacity of *AGO1* mRNA by modulating the endogenous miR168 level to alleviate the anti-viral function of *AGO1* protein.

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Introduction

During virus infection, siRNAs are generated from viral double-stranded RNA products and secondary RNA structures by the activity of RNase-III ribonuclease Dicer-like (DCL) proteins (Molnar *et al*, 2005; Ding and Voinnet, 2007). Virus-specific siRNAs are then incorporated into the RNA-induced-silencing complex (RISC) determining its specificity and bringing about the degradation of complementary viral RNAs (Pantaleo *et al*, 2007). RNA-silencing suppressors of viruses can inhibit this mechanism at different steps by

binding to viral siRNAs, double-stranded RNAs or directly interacting with *AGO1* (Merai *et al*, 2006; Burgyán, 2008; Mlotshwa *et al*, 2008). Therefore, elimination of RNA-silencing suppressors from the virus-infection process can result in decreased virus accumulation and the development of the recovery phenotype (Qiu *et al*, 2002; Qu and Morris, 2002; Szittyá *et al*, 2002).

Plant miRNAs represent another RNA-silencing pathway and are indispensable for the control of wide variety of biological functions, including development, hormone responses, feedback mechanisms and biotic and abiotic stresses (Voinnet, 2009). MiRNAs are generated by sequential processing of genome-coded long single-stranded RNA precursor molecules possessing specific secondary structures by DCL1 and other factors. DCL1 processing liberates the miRNA/miRNA* duplex and the selected strand (the miRNA) is exported to the cytoplasm, whereas the miRNA* strand is usually degraded. In contrast, the generation of virus-specific siRNAs is mediated mainly by DCL4 and also by DCL2 (Ding and Voinnet, 2007). Small RNAs generated by the activity of DCL enzymes are incorporated into ARGONAUTE (*AGO*) proteins, which are the central components of the RISC complex. *AGO1*, the most important *AGO* protein in the miRNA pathway, is responsible for the cleavage or translational inhibition of target RNAs determined by the loaded miRNA (Mallory and Bouche, 2008; Mallory *et al*, 2008). As a feedback mechanism, *AGO1* homeostasis itself is controlled by coordinated action of miR168 (Rhoades *et al*, 2002; Vaucheret *et al*, 2004) and *AGO1*-derived siRNAs (Mallory and Vaucheret, 2009) on *AGO1* mRNA. Moreover, additional components of the miRNA-mediated feedback regulation of *AGO1* have been also described involving the *AGO1*-mediated post-transcriptional stabilization of miR168 and the co-regulated expression of *AGO1* and *MIR168* genes (Vaucheret *et al*, 2006). These results show the existence of a complex refined feedback regulatory loop, which balances *AGO1* and miR168 accumulation.

Analyses of affinity-purified *AGO1* revealed that it is preferably associated with miRNAs and mediates target mRNA cleavage (Baumberger and Baulcombe, 2005; Qi *et al*, 2005; Mi *et al*, 2008). However, *AGO1* also recruits virus-specific siRNAs and is involved in RNA-silencing-mediated defence mechanisms (Zhang *et al*, 2006). It was also shown that virus-specific siRNAs of Cymbidium ringspot virus (CYMRV) and miRNAs co-fractionate with large protein complexes, which contain *AGO1* likely corresponding to RISC complexes (Pantaleo *et al*, 2007; Csorba *et al*, 2010). Moreover, it was shown that cucumber mosaic virus (CMV) 2b RNA-silencing suppressor directly interacts with siRNA-loaded *AGO1* inhibiting its slicing activity (Zhang *et al*, 2006). P0 RNA-silencing suppressor of a Polerovirus mediates the targeted degradation of *AGO1* (Pazhouhandeh *et al*, 2006; Baumberger *et al*, 2007; Bortolamiol *et al*, 2007; Csorba *et al*, 2010). In line with these observations, it was found that *ago1* hypomorphic

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mutant was more susceptible to CMV infection (Morel *et al*, 2002). This mutant showed also increased susceptibility to coat protein (CP) deletion mutant turnip crinkle virus (TCV), which is compromised also in RNA-silencing suppressor function (Qu *et al*, 2008). Plant virus infections are often associated with changes in endogenous miRNA levels (Bazzini *et al*, 2007; Csorba *et al*, 2007). RNA-silencing suppressors of plant viruses have been shown to be responsible for altering endogenous miRNA levels inducing changes also in target mRNA accumulations (Kasschau *et al*, 2003; Dunoyer *et al*, 2004; Zhang *et al*, 2006). Moreover, several recent works described the enhanced expression of miR168 and AGO1 mRNA in virus-infected plants (Zhang *et al*, 2006; Csorba *et al*, 2007; Havelda *et al*, 2008).

As AGO1 is one of the central components of RNA-silencing-mediated host defence, we investigated the regulation of AGO1 and miR168 expressions and their function during virus infections.

Here, we show the specific induction of miR168 accumulation in CymRSV-infected plants, which spatially overlaps with virus-occupied sectors. The enhanced accumulation of miR168 was also characteristic for all the other plant-virus interactions investigated in this study, indicating its importance in virus-infection process. Moreover, we show that in virus-infected plants the elevated level of miR168 is associated with the induced AGO1 mRNA expression but reduced AGO1 protein accumulation, indicating that miR168-mediated translational repression mechanism might be responsible for the control of AGO1 level. We also show that p19 RNA-silencing suppressor of Tombusviruses is responsible for the specific induction of miR168 and this activity is independent from the RNA-binding capacity of p19. On the basis of

our results, we propose a new layer in the combat between plants and viruses in which the virus-induced expression of miR168 controls the accumulation of AGO1 limiting the effect of host-mounted RNA-silencing defence.

Results

Induction of miR168 is ubiquitous in plant-virus interactions

Using the CymRSV—*Nicotiana benthamiana*—system, we investigated the induction of miR168 relative to other miRNAs. Small RNA northern blot analyses of RNA samples derived from symptomatic systemically infected leaves at 4 days post-inoculation (dpi) revealed that CymRSV infections induced elevated accumulation of miR168 compared with the mock-infected plants (Figure 1A). The induction of miR168 was not a part of a general response at the level of miRNA expressions to the virus infection as the level of other miRNAs (miR159, miR171 and miR319) remained unchanged. We experienced similar results using *N. benthamiana* test plants infected with crucifer-infecting tobacco mosaic virus (crTMV), potato virus X (PVX) and tobacco etch virus (TEV) (Figure 1B). In all cases, we observed markedly increased accumulation of miR168 in virus-infected plants, whereas no or moderate changes occurred in the level of miR159 in the same samples. TEV infection was an exception as miR159 also showed increased accumulation as described previously (Bazzini *et al*, 2007). Next, we tested several other plant-virus interactions for the accumulation of miR168 (Figure 1C). In *Arabidopsis thaliana*, both TCV and ribgrass mosaic virus (RMV) infections induced miR168 expression. Sunn-hemp mosaic virus (SHMV) infection on

Figure 1 Induction of miR168 is ubiquitous in plant-virus interactions. Total RNAs were prepared from systemically infected leaves of different host plants or mock-inoculated tissues and used for small RNA northern blot analyses using LNA probes for miRNAs as indicated. (A) *N. benthamiana* infected with CymRSV. (B) *N. benthamiana* infected with distinct unrelated viruses. (C) Investigation of various host-virus interactions. Relative gel loadings and viral accumulations are indicated by ethidium bromide staining of ribosomal RNAs (rRNA) and viral genomic RNAs (v).

Medicago truncatula and TMV U1 and PVX infections on *Solanum lycopersicum* were also accompanied with drastic induction of miR168. The induction of miR168 was not a general unspecific answer to these infections as miR159 level did not change significantly or in line with previous data showed only moderate increases in Tobamovirus (RMV and SHMV)-infected plants (Csorba *et al*, 2007) in the investigated time points.

These results show that infection of the investigated viruses is always associated with the enhanced accumulation of miR168 irrespective of their various ability to interfere with the expression of other endogenous miRNAs. The general feature of miR168 over-accumulation implies its important function in the virus-infection processes and/or host defence.

Induction of miR168 spatially overlaps with virus accumulation and is linked to enhanced accumulation of pre-miR168a-loop intermediate

As virus infections have strong effect on miR168 accumulation, we wanted to know how the accumulation of miR168 is

spatially regulated in virus-infected plant. We performed *in situ* analyses on consecutive sections of young developing symptomatic leaf of CymRSV-infected *N. benthamiana* to detect the relative accumulation of viral RNA and miR168 (Figure 2A). We revealed that virus accumulation and the increased miR168 expression spatially overlapped. We did not detect any alteration in miR159 accumulation level at the virus-occupied regions showing that the enhanced miR168 level is a specific response to virus infections. This finding indicates that enhanced miR168 accumulation is directly activated by the viral-derived products and does not represent a general whole plant response.

There are several possibilities, which can account for the increased accumulation of miR168 in virus-infected plants. Mechanisms such as the increased stability of miR168, transcriptional activation of the *MIR168* precursor and/or the more efficient processing of the precursor RNAs can be responsible for enhanced miR168 accumulation. As recent data described the transcriptional activation of the promoter of *MIR164a* precursor in virus-infected plants (Bazzini *et al*, 2009), we also tested the accumulation of *MIR168a* precursor

Figure 2 Analyses of miR168 expression in virus-infected plants. (A) Spatial analysis of the accumulation of virus-derived RNAs (CymRSV), miR168 and miR159 on consecutive sections (12 µm) of systemically infected *N. benthamiana* leaves. Cross-sections show the basal part of a young developing apical leaf. Section represented in the upper panel was hybridized with RNA probe detecting viral RNAs (CymRSV). The dark colour indicates the accumulation of viral RNAs showing the virus-occupied sector. Arrowheads point to the front of virus infection. Consecutive section hybridized with LNA probe detecting miR168 displays very similar expression pattern. The enhanced miR168 accumulation spatially overlaps with the virus-occupied zone. Arrowheads point to the border of increased miR168 accumulation. The next section was hybridized with miR159 detecting LNA probe and no altered miR159 expression was detected on the tissue section. Chicken-specific miR449 was used as negative control to detect the possible technical background signals. Bar shows 100 µm. (B) *A. thaliana* total RNA samples originated from mixed flowers and virus and mock-inoculated leaves were separated on polyacrylamide gel and used for northern blot analyses using RNA probe complementary to *MIR168a* precursor (upper panel). The membrane was stripped and used for subsequent hybridizations with LNA probes-specific mature miR168 and miR171 or DNA probe detecting U6 snRNA (loading control). Relative gel loadings are also indicated by ethidium bromide staining of ribosomal RNAs (rRNA).

RNA. The *MIR168* precursors of *N. benthamiana* are not known; therefore, we investigated the accumulation *MIR168a* precursor in virus-infected and -non-infected *A. thaliana* RNA samples by northern blot analyses (Figure 2B). We detected the same *MIR168a* precursor processing products, the 104nt stem-loop and 64nt-loop intermediates, which have been described in *MIR168a*-overexpressing transgenic plants (Vaucheret *et al*, 2006). We showed that in virus-infected leaves the loop intermediates accumulated to a much higher level than in mock-infected leaves. Surprisingly, the stem-loop intermediate did not show enhanced accumulation in virus-infected leaves. RNA sample originating from the flowers shows higher stem-loop intermediate accumulation level; however, this was not accompanied by proportionally higher accumulation levels of the loop intermediate and mature miR168 in comparison with virus-infected samples (Figure 2B). For example, in TCV-infected sample, less loop intermediate is present compared with flower sample; however, it is associated with higher level of mature miRNA accumulation. These findings suggest that in virus-infected plants the processing of the stem-loop intermediate to loop intermediate and mature miRNA is more efficient than in wild-type flowers in normal conditions. Another possibility is that the RNA-binding activity of RNA-silencing suppressors can interfere with the stability of miR168 precursor intermediates and mature miR168. However, in this case, we would expect a more profound effect on other miRNAs. Indeed, we also showed that p19 was not able to bind efficiently to miR168 (see Figure 6). Alternatively, it is also possible that accumulation of miR168 is controlled by an RNA-silencing-mediated mechanism, which is inhibited by the RNA-silencing suppressor bringing about the increased accumulation of miR168.

In the *in situ* experiments, we showed that in CymRSV-infected plants the miR168 induction shows spatial overlap with viral accumulation, indicating the direct effect of viral-derived products on miR168 accumulation. Moreover, we detected high-level accumulation of processed *MIR168a* precursor intermediate, suggesting that the activation of the expression of *MIR168a* precursor and its increased processing rate can be mainly responsible for the enhanced accumulation of miR168 in virus-infected plants.

Increased miR168 accumulation is accompanied by AGO1 mRNA induction

Several recent publications described that in addition to the increased miR168 accumulation, its target *AGO1* mRNA also showed induced expression in virus-infected plants (Zhang *et al*, 2006; Csorba *et al*, 2007; Havelda *et al*, 2008). First, we investigated the relative expression of *AGO1* mRNA and miR168 in CymRSV-infected *N. benthamiana* plants at 4 and 5 dpi using a probe specific for *AGO1* mRNA downstream from the miR168 recognition site. The parallel increase of miR168 and *AGO1* mRNA was not followed by the marked accumulation of potential 3' cleavage products detected in both mock- and virus-inoculated samples (Figure 3A). Quantitative RT-PCR analyses of mock-, CymRSV- and Cym19Stop-inoculated plants also revealed that the majority of virus-infection-induced *AGO1* mRNA is present in intact form and no significant over-accumulation of 3' cleavage product is detectable (Supplementary Figure S1). These observations suggested that virus-infection-induced miR168 accumulation does not, or only with very low efficiency, mediate the cleavage of *AGO1* mRNA. This finding was surprising as previous studies showed that *AGO1* mRNA level is regulated through miR168-mediated cleavage (Vaucheret *et al*, 2004; Mallory and Vaucheret, 2009).

Similar results were obtained in RMV-, CMV- and TCV-infected *A. thaliana* in a time course analyses (Figure 3B). It was found that miR168, miR168* and *AGO1* mRNA expressions were always increased in parallel with the advance of virus-infection processes. Previously, it has been described that miR168* also showed enhanced accumulation in virus-infected plants (Zhang *et al*, 2006; Csorba *et al*, 2007). We also showed that miR168* accumulated to high levels in RMV, CMV and TCV infections (Figure 3B). We found that high-level miR168* accumulation is a part of the normal miR168a precursor processing, as presence of miR168 in uninfected tissues is also accompanied with high-level miR168* accumulation (Supplementary Figure S2). The expression level of other miRNAs (miR159 and miR171) showed no or only moderate changes. Slightly increased expression of miR159 was detected in RMV- and CMV-in-

Figure 3 Induction of *AGO1* mRNA level is associated with increased miR168 accumulation. (A) Northern blot analyses of CymRSV-infected *N. benthamiana* plants for *AGO1* mRNA and miRNA expressions. *AGO1-1* mRNA was detected with a DNA probe specific for region downstream from the miR168 recognition site. LNA probe was used for miR168 detection. The accumulation of the potential 3' cleavage products are indicated. (B) Northern blot analyses of virus-infected *A. thaliana* plants for *AGO1* mRNA and miRNA expressions. *AGO1* mRNA was detected with a DNA probe specific for region downstream from the miR168 recognition site. LNA probes were used for miRNA detections as indicated.

fect samples. This simultaneous increase of *AGO1* mRNA and miR168 levels can be explained by the previous observation that *AGO1* and miR168 possess a co-expressional regulation mechanism (Vaucheret *et al*, 2006). We were not able to detect the increased accumulation miR168-mediated *AGO1* mRNA cleavage products even in a sample in which the parallel accumulation of miR168 and *AGO1* mRNA reached an extremely high level (RMV infection 15 dpi.). Moreover, we did not detect the appearance of potential cleavage products in *xrn-4* mutant plants, which have been described to be affected in degradation of mRNAs and selected miRNA targets (Gazzani *et al*, 2004; Souret *et al*, 2004) (Supplementary Figure S3).

Although it cannot be excluded that cleavage products are undetectable because of their rapid degradation, these results suggest that in virus-infected plants the increased expression of *AGO1* mRNA and miR168 is not accompanied with drastically enhanced accumulation of miR168-mediated *AGO1* mRNA cleavage products.

***AGO1* accumulation is repressed in virus-infected plants**

As several recent reports described miRNA-mediated translational control in plants (Aukerman and Sakai, 2003; Chen, 2004; Brodersen *et al*, 2008; Dugas and Bartel, 2008; Beauclair *et al*, 2010), we investigated the accumulation of *AGO1* protein relative to the expression of miR168 and *AGO1* mRNA in virus-infected plants. For this purpose, we infected *N. benthamiana* plants with CymRSV and investigated the accumulation of *AGO1* in systemically infected leaves at 7 dpi using an antibody raised against endogenous *N. benthamiana* AGO1-1. We found that in virus-infected plants the *AGO1* level was maintained close to the normal level in spite of the strongly increased expression of *AGO1* mRNA (Figure 4A). This phenomenon was associated with the presence of drastically increased miR168 level. Similar results were observed when FLAG-*AGO1* transgenic *A. thaliana* plants (Baumberger and Baulcombe, 2005) were infected with crTMV and TCV. RNA and protein samples from the symptomatic systemically infected leaves showed that enhanced level of *AGO1* mRNA produced less *AGO1* protein than in mock-inoculated control plants (Figure 4B). In contrast to CymRSV-infected *N. benthamiana* plants the down-regulation of *AGO1* protein was more profound in *A. thaliana* plants especially in the case of crTMV. In the investigated samples, we detected again the high-level accumulation of miR168 compared with mock-inoculated sample as well.

These results showed that in spite of the induction of *AGO1* mRNA in virus-infected plants the level of *AGO1* protein remained unchanged or showed down-regulation implying a translational control mechanism on *AGO1* mRNA mediated by miR168.

Elimination of p19 RNA-silencing suppressors from virus-infection process results in loss of miR168 induction and in parallel-enhanced *AGO1* accumulation

RNA-silencing suppressors of viruses are important symptom determinants and can interfere with the accumulation and activity of endogenous miRNAs (Kasschau *et al*, 2003; Dunoyer *et al*, 2004; Zhang *et al*, 2006). We used the previously described CymRSV *N. benthamiana* system to test the function of an RNA-silencing suppressor in the control of miR168 and *AGO1* accumulation. CymRSV-en-

Figure 4 Reduced accumulation of *AGO1* protein in virus-infected plants. Systemically infected leaves of *A. thaliana* and *N. benthamiana* plants were homogenized and divided into RNA and protein extractions. The corresponding samples were used for detecting *AGO1* mRNA, *AGO1* protein and miR168 expressions. (A) Top: northern blot analyses of CymRSV-infected GFP transgenic *N. benthamiana* plants for *AGO1* mRNA accumulation. The membrane was striped and used for hybridization with DNA probe specific for COX mRNA (loading control). Middle: western blot of total protein extract for *AGO1* accumulation using AGO1-1 (*N. benthamiana*) antibodies. Equal loading was verified by detecting GFP accumulation using GFP-specific antibody and Ponceau staining of the membrane after western blotting. Bottom: small RNA northern blot analyses using miR168-specific LNA probe. (B) Top: northern blot analyses of virus-infected FLAG-*AGO1* transgenic *A. thaliana* plants for *AGO1* mRNA accumulation. The membrane was striped and used for hybridization with DNA probe specific for GAPDH mRNA (loading control). Middle: western blot of total protein extract for *AGO1* accumulation using FLAG antibodies. Equal loading was verified by detecting actin accumulation using actin-specific antibody and Ponceau staining of the membrane after western blotting. Bottom: small RNA northern blot analyses using miR168-specific LNA probe.

coded p19 protein has been previously characterized as an RNA-silencing suppressor (Silhavy *et al*, 2002) and also as an important symptom determinant (Scholthof *et al*, 1995; Burgyan *et al*, 2000). CymRSV infection induces the necrosis of the systemically infected *N. benthamiana* leaves, which later culminates in the death of the plant (Burgyan *et al*, 2000). In contrast, Cym19Stop (p19 defective mutant virus)-infected plants show non-necrotic symptoms on the first systemically infected leaves and the plants grow further exhibiting the development of RNA-silencing-associated recovery phenotype and drastically reduced levels of virus accumulation (Silhavy *et al*, 2002). RNA and protein samples

were taken from *N. benthamiana* plants infected with either CymRSV or Cym19Stop at 21°C. Northern blot analyses using an *AGO1* mRNA-specific probe revealed that in both CymRSV- and Cym19Stop-infected plants the level of *AGO1* mRNA significantly increased compared with the mock-inoculated plants (Figure 5A). In line with previous experiments, we found that in CymRSV-infected plants the *AGO1* level did not display changes in spite of the strong *AGO1* mRNA induction. In contrast, in Cym19Stop-infected plants the comparable level *AGO1* mRNA induction was followed by enhanced accumulation of AGO1, indicating that p19 has a central function in the control of AGO1 accumulation. Next, we investigated the miR168 level in the samples. We found that in striking contrast to CymRSV infection, in which miR168 accumulated to extremely high levels, in Cym19Stop-infected plants the miR168 (21 nucleotide in length) level showed no changes. Only the moderate increase of a higher-molecular weight-type miR168 was detected whose origin and biological competence is unknown (Figure 5A). According to a recent study, this higher-molecular weight miR168 species can be the product of *MIR168b* precursor (Vaucheret, 2009). However, according to the western blot data the moderate increase of this miR168 species cannot efficiently interfere

with the translation of AGO1 (Figure 5). The level of miR159 and miR171 remained unaffected in these samples. At 21°C, Cym19Stop accumulation is severely inhibited (compare viral accumulations in Figure 5A), which could account for the loss of increased miR168 expression. To exclude this possibility, we carried out similar experiments at 15°C. It has been described previously that at 15°C the activity of siRNA-mediated RNA silencing is inhibited and the Cym19Stop-infected plants do not show the recovery phenotype as inefficient RNA silencing allows the accumulation of the mutant virus to the wild-type level (Szittyá *et al*, 2003). Our experiments at 15°C showed that the wild-type level accumulation of Cym19Stop did not induce the enhanced accumulation of normal size miR168 (Figure 5B). Moreover, similar to the previous results, the absence of miR168 increase in Cym19Stop-infected plants was associated with the massive accumulation of AGO1 compared with wild-type virus infection (Figure 5B). These findings also show that the inhibited activity of RNA silencing at low temperature is not the outcome of the lack of proper AGO1 amount, suggesting that other molecular step(s) are affected. The level of control miRNAs remained unchanged also in this experiment.

These data show that the induction of AGO1 mRNA accumulation in virus-infected tissue is a part of a host defence reaction, whereas enhanced accumulation of miR168 is primarily mediated by viral-encoded p19 RNA-silencing suppressor. Moreover, the finding that the absence of miR168 induction in Cym19Stop-infected plants is tightly correlated with increased accumulation of AGO1, supporting the function of miR168 in the translational control of AGO1 mRNA.

P19 does not bind miR168 efficiently

P19-silencing suppressor inhibits the intermediate step of RNA silencing through binding to siRNAs (Lakatos *et al*, 2006). To test whether the small RNA-binding ability of p19 is interfering with miR168 accumulation and/or activity we used gel filtration assays (Lakatos *et al*, 2006) (Figure 6). We found that in mock-inoculated plants the majority of miR168 and miR168* is eluted in the fractions corresponding to the size of protein-free siRNAs (fractions 33–39) (Figure 6A). We also identified an miR168 containing high-molecular mass protein complex, which co-fractionate with AGO1 proteins, suggesting that these are AGO1-bound miR168 (670 kDa; fractions 4–9). In contrast, the majority of miR159 is present in AGO1 containing fractions and no significant amount of free miR159 is detected in mock-inoculated samples. These findings confirm a previous observation, suggesting that the loading of miR168 onto AGO1 is inefficient (Vaucheret *et al*, 2006). In CymRSV-infected plants the fractions accommodating p19-bound viral siRNAs (fractions 24–28) contained only small amount of miR168 (fraction 27) and most of the miR168 and miR168* were detected in the protein-free fractions (fractions 33–39) (Figure 6B). Analyses of samples derived from Cym19Stop-infected plants revealed that the unbound miR168, miR168* and viral siRNAs co-fractionate (fractions 33–39) (Figure 6C).

The outcome of the gel filtration assays shows that miR168 excess in CymRSV-infected plant is present in unbound form allowing their incorporation into AGO proteins showing that small RNA-binding ability of p19 is not able to efficiently inhibit the loading of miR168 onto protein complexes.

Figure 5 P19 RNA-silencing suppressors of CymRSV is responsible for miR168 induction. (A) Systemically infected leaves of CymRSV-, Cym19Stop- and mock-inoculated *N. benthamiana* plants were homogenized and divided into RNA and protein extractions. The corresponding samples were used for detecting *AGO1* mRNA, AGO1 and miRNA expressions. Top: northern blot analyses of virus-infected and mock-inoculated plants for *AGO1* mRNA at 21°C. Middle: western blot of total protein extract for AGO1 accumulation using AGO1-1 (*N. benthamiana*) antibodies. Bottom: small RNA northern blot analyses using miR168-, miR159- and miR171-specific LNA probes. (B) The same experiments at 15°C. CymRSV-infected plants were harvested at 20 dpi, whereas Cym19Stop-infected plants at 25 dpi to allow the mutant virus to accumulate at same level as the wild-type virus.

Figure 6 Small RNA-binding ability of p19 does not interfere with miR168 activity. Gel filtration of crude extracts prepared from systemically infected leaves (7 dpi.). Mock-inoculated (A), CymRSV- (B) and Cym19Stop- (C) infected *N. benthamiana* materials were used. Even number fractions were used for AGO1 western blot analyses, whereas odd number fractions were used for small RNA northern blotting for miR168 (LNA). Membranes were stripped and subsequently hybridized for miR168* (LNA) and miR159 (LNA) or virus-specific siRNAs (RNA probe). Hybridization of samples deriving from CymRSV-infected plants identifies fractions containing p19-bound siRNAs (B).

Moreover, these results also show that the enhanced accumulation of miR168 is not due to small RNA-binding and/or -stabilizing effects of p19.

Transient expression of p19 induces miR168 expression and is associated with the down-regulation of AGO1 accumulation

The clear association of miR168 induction with the presence of p19 RNA-silencing suppressor prompted us to investigate whether the transient expression of p19 brings about the enhanced accumulation of miR168. We found that the transient expression of p19 in *N. benthamiana* leaves by *Agrobacterium* infiltration induced the accumulation of miR168 compared with control infiltrations (empty vector and GFP) (Figure 7A). This was specific for miR168 as the level of other miRNAs such as miR171 and miR159 showed no or only moderate changes. Importantly, the induced miR168 accumulation was associated with the down-regulation of endogenous AGO1 protein compared with control samples. Quantitative RT-PCR analyses of RNA samples deriving from the infiltrated patches expressing p19 or empty vector showed that full-length intact AGO1 mRNA

level was slightly increased because of p19 expression (Figure 7A). This result indicates that the miR168 excess exerts its effect mainly by inhibiting the translation of AGO1 mRNA rather than inducing its efficient cleavage. We also tested the miR168 inducing capacity of another p19 encoded by carnation Italian ringspot virus (CIRV; Vargason *et al*, 2003). We found that CIRV p19 also induced the miR168 expression and the parallel down-regulation of AGO1 (Figure 7B).

To test whether transient p19 expression-mediated miR168 induction is directly responsible for the inhibition of AGO1 protein accumulation, we transiently expressed the *MIR168a* precursor (pre168) using *Agrobacterium* infiltration. We found that transient expression miR168a precursor produced high amount of miR168 and induced the down-regulation of AGO1 protein (Figure 7C). Moreover, we have constructed a target mimicry construct to inhibit the activity of miR168 (MIM168) as described previously (Franco-Zorrilla *et al*, 2007). Transient co-expression of pre168 and miR168 inhibitor (MIM168) resulted in the enhanced accumulation of AGO1 protein further confirming the direct involvement of miR168 in the control AGO1 protein accumulation (Figure 7D).

Figure 7 Transient expression of p19 RNA-silencing suppressors and *MIR168a* precursor using *Agrobacterium* infiltration in *N. benthamiana* leaves. Endogenous AGO1 protein accumulation was detected by using AGO1-1 antibody. (A) P19 expressing (BINp19), empty (BIN) and GFP containing binary vectors were used for *Agrobacterium* infiltration. RNA samples extracted from infiltrated leaves were investigated for the accumulation of miR168, miR168*, miR159 and miR171 in small RNA northern blot analyses using LNA probes as indicated. Quantitative RT-PCR analyses of *AGO1* mRNA from RNA samples extracted from BIN or BINp19 expressing infiltrated patches. White column represents data generated by primer pair encompassing the miR168 target site detecting full-length mRNA. Black column shows the accumulation of the 3' part of mRNA using primer pair downstream from the miR168 target site detecting full-length mRNA and potential cleavage products. (B) Endogenous AGO1 protein accumulation in CIRV p19 expressing *Agrobacterium* infiltrated patches. (C) *Agrobacterium*-mediated transient expression of *MIR168a* precursor (pre168) and negative controls expressing artificial GFP miRNA precursor (amiRGFP) and an unspecific *A. thaliana* RNA (pre134). (D) Co-expression of pre168 and target mimicry constructs targeting miR168 (MIM168) or miR399 (MIMWT) by *Agrobacterium* infiltration.

These results revealed that transient expression of both CymRSV and CIRV p19 induces the accumulation of miR168 bringing about the inhibition of AGO1 protein accumulation possibly by imposing translational control on *AGO1* mRNA. Altogether, these data show that p19 is the important viral factor responsible for the observed control of AGO1 protein.

Down-regulation of AGO1 protein is inhibited in mutants impaired in miR168-mediated control or translational repression

Our results strongly suggested that virus-infection-induced miR168 accumulation is responsible for AGO1 protein down-regulation through the inhibition of the translational capacity of *AGO1* mRNA. Next, we investigated wild-type and

mutant *A. thaliana* plants disabled in the activity of miR168-mediated control (4mAGO1; Vaucheret *et al*, 2004, 2006) or compromised in the activity of ZWILLE/PINHEAD/AGO10 (zll-3; Moussian *et al*, 1998) and AGO1 (ago1-25; Morel *et al*, 2002). As CymRSV does not infect *A. thaliana* plants next, we used crTMV-infected plants, which show the drastic down-regulation of AGO1 protein (Figure 4B) in the following experiments assuming that miR168 induction in these plants also contributes for the control of *AGO1* mRNA.

To test whether virus-infection-associated repression of AGO1 protein accumulation is specific phenomenon, we investigated the accumulation copper chaperone for superoxide dismutase (CCS1), which have been previously shown to be under miR398-directed translational control

(Beauclair *et al*, 2010). We found that CCS1 protein level did not change in crTMV-infected wild-type *A. thaliana* plants, indicating that the observed down-regulation of AGO1 protein is not a part of general enhancement of translational repression in the infected plants (Figure 8A). The specificity of AGO1 protein down-regulation suggested that crTMV-infection-associated increase of miR168 level can be directly responsible for this phenomenon. We investigated the function of miR168 in this process by infecting 4mAGO1 mutant plants (Vaucheret *et al*, 2004, 2006) possessing *AGO1* gene, which contains four silent mutations in the miR168 target site rendering the mRNA to be resistant for miR168-mediated control. In these mutant plants the down-regulation of AGO1 protein was inhibited in spite of the enhanced accumulation of miR168 (Figure 8B). As 4mAGO1 mutant plants express both the mutant and the wild-type versions of *AGO1* mRNAs, we expected a balanced answer in this experiment depending on the efficiency of down-regulation of the wild-type mRNA and the induction level of the mutant mRNA. The gained results showed that miR168 is directly involved in the down-regulation of AGO1 as elimination of correct target site from the mRNA resulted in less effective inhibition of AGO1 accumulation. In contrast to 4mAGO1

plants, ago1-25 hypomorphic mutants (Morel *et al*, 2002) showed no efficient inhibition of AGO1 repression (Figure 8C) showing that this mutant is not affected in the translational control of *AGO1* mRNA.

Previously, it has been shown that ZWILLE/PINHEAD/AGO10 is able to exert translational regulation on *AGO1* mRNA (Mallory *et al*, 2009). Moreover, using *zll-15* (ecotype Ler), it was shown that ZWILLE/PINHEAD/AGO10 is responsible for the translational control mediated by some miRNAs (Brodersen *et al*, 2008). To test the function of ZWILLE/PINHEAD/AGO10 in the control of AGO1 accumulation, we used *zll-3* (ecotype Ler) for crTMV infection. Virus-infected wild-type plants (Ler ecotype), similarly to Col ecotype, also showed the induction of *AGO1* mRNA and miR168 and in parallel the inhibition of AGO1 protein accumulation (Figure 8D). In contrast, analyses of crTMV-infected *zll-3* plants revealed that down-regulation of AGO1 protein is inhibited in spite of the enhanced accumulation of miR168 (Figure 8E). VARICOSE mutant (*vcs-7*; Goeres *et al*, 2007) plants, which was previously revealed to be involved in miRNA-mediated translational repression (Brodersen *et al*, 2008) also showed the moderate inhibition of AGO1 repression (Supplementary Figure S4).

Figure 8 Accumulation of AGO1 protein in wild type and in mutant *A. thaliana* plants infected with crTMV. Infected *A. thaliana* plants at 12dpi were homogenized and divided into RNA and protein extractions. Top panels show northern blot analyses of *AGO1* mRNA accumulation. Middle panels show western blot analyses of total protein extract for AGO1 (using antibody detecting AGO1 protein) protein accumulation. Loading was revealed by detecting actin accumulation and Ponceau staining of the membrane. Bottom panels show the small RNA northern blot analyses using miR168-specific LNA probe. (A) Accumulation of AGO1 and CCS1 (bottom panel) in wild type (Col ecotype). Accumulation of AGO1 in 4mAGO1 (Col ecotype) (B), ago1-25 (Col ecotype) (C), wild type (Ler ecotype) (D) and *zll-3* (Ler ecotype) (E) *A. thaliana* plants.

Altogether, these data imply that crTMV-infection-induced miR168 excess is directly involved in the control of AGO1 protein accumulation. Moreover, the enhanced accumulation of AGO1 is connected to the disabled activity of ZWILLE/PINHEAD/AGO10 in virus-infected plants (*zll-3* in Ler ecotype), implicating the pivotal function of ZWILLE/PINHEAD/AGO10 in translational regulation of AGO1 mRNA by miR168.

Discussion

Here, we describe a new aspect in the combat between the host plants and the invading viruses further confirming the function of AGO1 in the plant defence. It has been described previously that infection of plant viruses can interfere with the accumulation of endogenous miRNAs at various level (Bazzini *et al*, 2007; Csorba *et al*, 2007). In this study, we have focused on the investigation of miR168, which targets AGO1 mRNA encoding one of the most important central executor molecules of miRNA- and siRNA-mediated regulatory processes. In addition to the moderate changes of other miRNAs, all of the various virus-infected host plants investigated in this study showed the consequent and drastic increase of miR168. We revealed that miR168 induction, which is paralleled with enhanced AGO1 mRNA accumulation, is associated with the inhibition of AGO1 protein accumulation. These findings indicated that virus-mediated induction of miR168 can be a counter-defence action aiming the control of AGO1 protein the main component of RISC. This hypothesis is further supported by finding that enhanced miR168 accumulation spatially overlaps with virus-occupied regions indicating direct involvement of virus-derived products in this phenomenon. The spatial overlap between the virus-infected zones and induced miR168 can ensure that cells accommodating replicating viruses contain controlled amounts of AGO1 enabling efficient virus replication.

Using a mutant virus (Cym19Stop), disabled in RNA-silencing suppressor (p19) activity, we showed that the infected plant is able to induce the AGO1 mRNA expression as a defence reaction, which results in the enhanced accumulation of AGO1. Moreover, in striking contrast to wild-type virus Cym19Stop-infection-induced increase of AGO1 mRNA level was not accompanied with significant changes in miR168 accumulation, indicating that elimination of miR168 excess from the infection process brings about the loss of AGO1 down-regulation. As a consequence in Cym19Stop-infected plants the elevated level of AGO1 could accommodate higher amounts of free virus siRNAs facilitating the efficient cleavage of viral RNAs resulting in restricted levels of viral RNAs and in development of the recovery phenotype. In addition, our observation also shows that the previously described transcriptional co-regulation of miR168 and AGO1 mRNA (Vaucheret *et al*, 2006) was uncoupled in Cym19Stop-infected plants. These data indicate that AGO1 mRNA induction and subsequent accumulation of AGO1 is a component of the host defence response, whereas the induction of miR168 in effect is a counter-defence action of the invading virus mediated mainly by p19.

There are several possible mechanisms, which can be accounted for down-regulation of AGO1 level in virus-infected plants such as targeted degradation of AGO1 or miR168-mediated cleavage and/or translational inhibition of AGO1 mRNA. The first possibility is the targeted degradation

of AGO1 as described for P0 RNA-silencing suppressor of Poleroviruses (Pazhouhandeh *et al*, 2006; Baumberger *et al*, 2007; Bortolamiol *et al*, 2007). However, p19 also has been investigated in transient co-expression studies for the destabilization of AGO1 protein, but no AGO1 degradation was observed (Baumberger *et al*, 2007). Important to note here is that in p19 transient expression studies, we investigated the accumulation of endogenous AGO1 protein as the quantity of transient expression of AGO1 protein by *Agrobacterium* infiltration exceeds the regulatory capacity of endogenous miR168. To date, the miR168-mediated cleavage of AGO1 mRNA has been described as the main mechanism for controlling AGO1 homeostasis in healthy plants (Rhoades *et al*, 2002; Vaucheret *et al*, 2004). The accumulation of full-length AGO1 mRNAs and the lack of significantly higher accumulation of potential 3' cleavage products characteristic for miRNA-mediated cleavages in virus-infected plants argue against the function of miRNA-mediated efficient cleavage of AGO1 mRNAs. Although it cannot be excluded that miR168 cleavage products show very fast degradation evading the sensitivity of the various detection methods. However, the presence of high-level intact AGO1 mRNA and less AGO1 protein suggests that the translational control by miR168 excess is the most likely mechanism for the virus-infection-mediated inhibition of AGO1 accumulation. Recent findings also support the potential occurrence of miR168-associated translational inhibition of AGO1 mRNA in virus-infected plants. Growing evidences indicate that translational control can be one of the main mechanisms used by ARGONAUTE proteins. The miR168-mediated translational control of AGO1 mRNA is supported by recent findings in which AGO1 and mature miR168 were associated with active polysomes, suggesting their involvement in translational repression (Lanet *et al*, 2009). In addition, recent findings showed that NB-LRR proteins-mediated resistance can be based on AGO4 dependent translation inhibition of viral RNAs (Bhattacharjee *et al*, 2009). Moreover, AGO1 level can be controlled through negative regulation at the protein level through the activity of ZWILLE/PINHEAD/AGO10 (Mallory *et al*, 2009). CrTMV infection of mutant plants having AGO1 mRNA with mutated miR168 target site (Vaucheret *et al*, 2004, 2006) resulted in the inhibition of AGO1 down-regulation showing the activity of miR168-mediated control of AGO1 in *A. thaliana*. In contrast, ago1-25 mutant (Morel *et al*, 2002) showed no significant changes in the efficiency of the inhibition of AGO1 protein accumulation. Moreover, crTMV infection of *zll-3* (Ler ecotype) plants, disabled in the activity ZWILLE/PINHEAD/AGO10 (Moussian *et al*, 1998), also showed less effective AGO1 down-regulation in spite of the increased accumulation of miR168 and AGO1 mRNA. We used Ler ecotype mutant for studying the function of ZWILLE/PINHEAD/AGO10 in the regulation of AGO1 as this mutant, in contrast to Col ecotype *zwille/pinhead/ago10* mutant (Mallory *et al*, 2009), shows strong developmental defects, indicating the importance of AGO10 activity. On the basis of these data, we can hypothesize that similar factors are responsible for the control of AGO1 accumulation also in Col ecotype. Altogether, these findings support the hypothesis that in virus-infected plants the AGO1 protein accumulation can be, at least partly, under miR168-driven translational control. The coexistence of high-level miR168 and AGO1 mRNA indicates that, in contrast to the translational repres-

Figure 9 Schematic representation of the proposed model. CymRSV infection induces the host defence responses including the production of viral siRNAs and induction of AGO1 mRNA expression. In the case of wild-type virus infections the expressed RNA-silencing suppressors act through their specific functions (binding to viral siRNAs). However, in the same time the RNA-silencing suppressor has the ability to induce miR168 accumulation. This enhanced accumulation of miR168 is associated with the translational repression of its target mRNA encoding AGO1. As a result of the dual functions of p19 RNA-silencing suppressor the loading of viral siRNAs onto the AGO1 is inhibited in cells accommodating replicating virus enabling its efficient replication and the development of severe symptoms. Elimination of the silencing suppressor from the infection process (Cym19Stop infection) is associated with the lack of induced miR168 expression and consequently the translational repression on AGO1 mRNA is relieved producing increased amount of AGO1. The freely produced viral siRNAs are able to associate with the high amount of AGO1, resulting in inhibited virus accumulation and development of recovery phenotype.

sion, the cleavage activity of miR168 excess is inhibited. Recent evidences show that miRNA targets can be under dual, translational and slicing, regulation (Brodersen *et al*, 2008; Beauclair *et al*, 2010). One possible explanation for this phenomenon in Ler ecotype can be the different activities of AGO proteins. It is possible that virus-infection-induced miR168 is sorted mainly into ZWILLE/PINHEAD/AGO10, which exerts its activity at the level of translational repression, whereas miR168 incorporated into AGO1 mediates cleavage of AGO1 mRNA. However, the exact nature of factors responsible for determining the slicing or translational inhibitory activities of miR168 and the function of translational repression during the developmental processes remains to be investigated.

In this paper, we showed that in CymRSV infection the p19 RNA-silencing suppressor has dual functions. P19 possesses the specific well-described suppressor activity attacking the RNA-silencing mechanism by binding to small RNAs (Burgan, 2008). However, we revealed that p19 has another function, the induction of miR168 bringing about the inhibition of AGO1 protein accumulation, which is one of the most important components of the antiviral RISC (Figure 9). Moreover, our data imply that virus-infection-associated miR168 excess acts through inhibition of the translational capacity of AGO1 mRNA. These data indicate that the efficient activity of a plant virus-encoded RNA-silencing suppressors may require multiple actions targeting the host defence at different levels at the same time.

Materials and methods

Plant materials and viruses

The following plants were used in the experiments, *A. thaliana* (Col) and (Ler), FLAG-AGO1 transgenic *A. thaliana* (Baumberger and Baulcombe, 2005), hypomorphic ago1–25 mutant (Morel *et al*, 2002), *N. benthamiana* (line 16c; Ruiz *et al*, 1998), *M. truncatula* (R-108) and *Lycopersicon esculentum* cv. Kecskeméti Jubileum Standard. Heterozygous individuals of the seedling lethal VARI-COSE mutant *vcs-7* (corresponds to SALK_032031) were identified by genotyping with oligonucleotides described previously (Goeres *et al*, 2007). *Arabidopsis zll-3* (ecotype Ler; Moussian *et al*, 1998) plants showed very strong phenotype; those individuals, which reached the size suitable for virus infection, were used in the subsequent experiments. Seeds originated from hemizygous siblings of 4mAGO1 (m7-1) (Vaucheret *et al*, 2004, 2006) were sowed and individuals showing spoon-shaped leaves characteristic for the mutant plants were selected for the subsequent experiments. The plants were used for infections using sap inoculation method or purified virions. See Supplementary data for details.

Plasmids and primers

Fragments of *A. thaliana* AGO1 and GAPDH, *N. benthamiana* AGO1-1 and COX1 were amplified from cDNA and cloned into pGEMT Easy vector. cDNAs of MIR168a precursor (pre168) and unrelated *A. thaliana* sequence (pre134) were cloned into pGEMT Easy vector. This vector served as a template for radioactive RNA probe detecting the MIR168a precursor RNA. The precursor RNAs were further cloned under the control of 35S promoter into pGreen0029 vector and transformed into AGL1 *Agrobacterium* strain and used for infiltration experiments (see Supplementary data).

A. thaliana IPS1 (MIMWT) was cloned and mutated into MIM168 (Franco-Zorrilla *et al*, 2007) and cloned into pGreen0029 under the control of 35S promoter and after transformation into *Agrobacterium* (AGL1) used for infiltration experiments. See Supplementary data for more details.

RNA isolation and northern blotting

The total RNA was extracted from virus-infected plants at different time points as indicated. Northern blot analyses of higher-molecular weight RNAs were performed by separating 8–10 µg RNA samples in formaldehyde permeated, 1.2% agarose gels, blotted to Nytran N membrane. Small RNA northern blot analysis was performed as described previously (Várallyay *et al*, 2008) using LNA-modified oligonucleotide probes. See Supplementary data for more details.

In situ hybridization and quantitative real-time PCR

In situ hybridization of paraffin-embedded tissue sections was carried out with digoxigenin-11-UTP-labelled RNA probes or 5' digoxigenin-labelled LNA probes (Exiqon) specific for virus RNAs or miRNAs, respectively, as described previously (Havelda *et al*, 2005; Valoczi *et al*, 2006). For quantitative RT-PCR analyses, we used two sets of primers for detecting AGO1 mRNA. AGOmiRNA primer pair was designed to encompass the miR168 recognition site, whereas AGO3' primer pair was specific to the 3' end of AGO1

mRNA downstream of miR168 recognition site. See Supplementary data for more details.

Agrobacterium tumefaciens infiltration

A. tumefaciens pBIN61 (C58C1) harbouring the appropriate plasmid (pBINp19 (CymRSV), pBINp19 (CIRV), pBIN (Empty), pBIN-GFP or pGreen0029 (AGL1) harbouring pre168, pre134, amirGFP, MIMWT and MIM168 was infiltrated in leaves of young *N. benthamiana* leaves (5 plants per construction) at optical density at 600 nm [OD₆₀₀] = 1.0 according to the method described previously (Vargason *et al*, 2003). The infiltrated leaves containing the individual constructs were pooled and used for northern and western blotting. See Supplementary data for more details.

Protein analysis

For protein analyses, 0.05–0.1 g plant material was collected (from virus-infected or agroinfiltrated leaves) and homogenized in an ice cold mortar in 400 µl of extraction buffer (0.1 M glycine-NaOH, pH 9.0, 100 mM NaCl, 10 mM EDTA, 2% sodium dodecyl sulphate and 1% sodium lauroylsarcosine). A total of 100 µl of 2 × Laemmli buffer was added to 100 µl of the homogenized sample and used for protein analysis. The remaining sample was complemented with 300 µl extraction buffer and further processed for RNA extraction. This method ensures the comparability of protein and RNA samples. Protein samples were treated as described previously (Csorba *et al*, 2007) and 20–30 µl of sample was resolved on 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, blotted to PVDF Transfer Membrane (Amersham Hybond-P) and subjected to western blot analysis. See Supplementary data for more details.

Gel filtration assay

The protein crude extracts were prepared at 7 dpi from systemically infected (CymRSV and Cym19Stop) and mock-inoculated leaves and size separated by a Superdex-200 gel filtration column as described previously (Lakatos *et al*, 2004; Havelda *et al*, 2005) with minor modifications. See Supplementary data for more details.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

- Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. *Plant Cell* **15**: 2730–2741
- Baumberger N, Baulcombe DC (2005) Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci USA* **102**: 11928–11933
- Baumberger N, Tsai CH, Lie M, Havecker E, Baulcombe DC (2007) The Ploverovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. *Curr Biol* **17**: 1609–1614
- Bazzini AA, Almasia NI, Manacorda CA, Mongelli VC, Conti G, Maroniche GA, Rodriguez MC, Distefano AJ, Hopp HE, del Vas M, Asurmendi S (2009) Virus infection elevates transcriptional activity of miR164a promoter in plants. *BMC Plant Biol* **9**: 152
- Bazzini AA, Hopp HE, Beachy RN, Asurmendi S (2007) Infection and coaccumulation of tobacco mosaic virus proteins alter microRNA levels, correlating with symptom and plant development. *Proc Natl Acad Sci USA* **104**: 12157–12162
- Beauchair L, Yu A, Bouche N (2010) MicroRNA-directed cleavage and translational repression of the copper chaperone for superoxide dismutase mRNA in Arabidopsis. *Plant J* **62**: 454–462
- Bhattacharjee S, Zamora A, Azhar MT, Sacco MA, Lambert LH, Moffett P (2009) Virus resistance induced by NB-LRR proteins involves Argonaute4-dependent translational control. *Plant J* **58**: 940–951
- Bortolamiol D, Pazhouhandeh M, Marrocco K, Genschik P, Ziegler-Graff V (2007) The Ploverovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. *Curr Biol* **17**: 1615–1621
- Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**: 1185–1190

- Burgyan J (2008) Role of silencing suppressor proteins. *Methods Mol Biol* **451**: 69–79
- Burgyan J, Hornyik C, Szittya G, Silhavy D, Bisztray G (2000) The ORF1 products of tombusviruses play a crucial role in lethal necrosis of virus-infected plants. *J Virol* **74**: 10873–10881
- Chen X (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* **303**: 2022–2025
- Csorba T, Bovi A, Dalmay T, Burgyan J (2007) The p122 subunit of Tobacco Mosaic Virus replicase is a potent silencing suppressor and compromises both small interfering RNA- and microRNA-mediated pathways. *J Virol* **81**: 11768–11780
- Csorba T, Lozsa R, Hutvagner G, Burgyan J (2010) P1 protein of Tobacco Mosaic Virus prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. *Plant J* **62**: 463–472
- Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. *Cell* **130**: 413–426
- Dugas DV, Bartel B (2008) Sucrose induction of Arabidopsis miR398 represses two Cu/Zn superoxide dismutases. *Plant Mol Biol* **67**: 403–417
- Dunoyer P, Lecellier CH, Parizotto EA, Himber C, Voinnet O (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* **16**: 1235–1250
- Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, Garcia JA, Paz-Ares J (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat Genet* **39**: 1033–1037
- Gazzani S, Lawrenson T, Woodward C, Headon D, Sablowski R (2004) A link between mRNA turnover and RNA interference in Arabidopsis. *Science* **306**: 1046–1048
- Goeres DC, Van Norman JM, Zhang W, Fauver NA, Spencer ML, Sieburth LE (2007) Components of the Arabidopsis mRNA decapping complex are required for early seedling development. *Plant Cell* **19**: 1549–1564
- Havelda Z, Hornyik C, Valoczi A, Burgyan J (2005) Defective interfering RNA hinders the activity of a tombusvirus-encoded posttranscriptional gene silencing suppressor. *J Virol* **79**: 450–457
- Havelda Z, Várallyay E, Valoczi A, Burgyan J (2008) Plant virus infection-induced persistent host gene downregulation in systemically infected leaves. *Plant J* **55**: 278–288
- Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. *Dev Cell* **4**: 205–217
- Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, Dolja VV, Calvino LF, Lopez-Moya JJ, Burgyan J (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J* **25**: 2768–2780
- Lakatos L, Szittya G, Silhavy D, Burgyan J (2004) Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J* **23**: 876–884
- Lanet E, Delannoy E, Sormani R, Floris M, Brodersen P, Crete P, Voinnet O, Robaglia C (2009) Biochemical evidence for translational repression by Arabidopsis microRNAs. *Plant Cell* **21**: 1762–1768
- Mallory AC, Bouche N (2008) MicroRNA-directed regulation: to cleave or not to cleave. *Trends Plant Sci* **13**: 359–367
- Mallory AC, Elmayan T, Vaucheret H (2008) MicroRNA maturation and action—the expanding roles of ARGONAUTES. *Curr Opin Plant Biol* **11**: 560–566
- Mallory AC, Hinze A, Tucker MR, Bouche N, Gascioli V, Elmayan T, Lauressergues D, Jauvion V, Vaucheret H, Laux T (2009) Redundant and specific roles of the ARGONAUTE proteins AGO1 and ZLL in development and small RNA-directed gene silencing. *PLoS Genet* **5**: e1000646
- Mallory AC, Vaucheret H (2009) ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways. *EMBO Rep* **10**: 521–526
- Merei Z, Kerenyi Z, Kertesz S, Magna M, Lakatos L, Silhavy D (2006) Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J Virol* **80**: 5747–5756
- Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Wu L, Li S, Zhou H, Long C, Chen S, Hannon GJ, Qi Y (2008) Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. *Cell* **133**: 116–127
- Mlotshwa S, Pruss GJ, Vance V (2008) Small RNAs in viral infection and host defense. *Trends Plant Sci* **13**: 375–382
- Molnar A, Csorba T, Lakatos L, Várallyay E, Lacomme C, Burgyan J (2005) Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. *J Virol* **79**: 7812–7818
- Morel JB, Godon C, Mourrain P, Beclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H (2002) Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**: 629–639
- Moussian B, Schoof H, Haecker A, Jurgens G, Laux T (1998) Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during Arabidopsis embryogenesis. *EMBO J* **17**: 1799–1809
- Pantaleo V, Szittya G, Burgyan J (2007) Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. *J Virol* **81**: 3797–3806
- Pazhouhandeh M, Dieterle M, Marrocco K, Lechner E, Berry B, Brault V, Hemmer O, Kretsch T, Richards KE, Genschik P, Ziegler-Graff V (2006) F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. *Proc Natl Acad Sci USA* **103**: 1994–1999
- Qi Y, Denli AM, Hannon GJ (2005) Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421–428
- Qiu W, Park JW, Scholthof HB (2002) Tombusvirus P19-mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenicity. *Mol Plant Microbe Interact* **15**: 269–280
- Qu F, Morris TJ (2002) Efficient infection of Nicotiana benthamiana by Tomato bushy stunt virus is facilitated by the coat protein and maintained by p19 through suppression of gene silencing. *Mol Plant Microbe Interact* **15**: 193–202
- Qu F, Ye X, Morris TJ (2008) Arabidopsis DRB4, AGO1, AGO7, and RDR6 participate in a DCL4-initiated antiviral RNA silencing pathway negatively regulated by DCL1. *Proc Natl Acad Sci USA* **105**: 14732–14737
- Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP (2002) Prediction of plant microRNA targets. *Cell* **110**: 513–520
- Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell* **10**: 937–946
- Scholthof HB, Scholthof KB, Jackson AO (1995) Identification of tomato bushy stunt virus host-specific symptom determinants by expression of individual genes from a potato virus X vector. *Plant Cell* **7**: 1157–1172
- Silhavy D, Molnar A, Lucioli A, Szittya G, Hornyik C, Tavazza M, Burgyan J (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J* **21**: 3070–3080
- Souret FF, Kastenmayer JP, Green PJ (2004) AtXRN4 degrades mRNA in Arabidopsis and its substrates include selected miRNA targets. *Mol Cell* **15**: 173–183
- Szittya G, Molnar A, Silhavy D, Hornyik C, Burgyan J (2002) Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. *Plant Cell* **14**: 359–372
- Szittya G, Silhavy D, Molnar A, Havelda Z, Lovas A, Lakatos L, Banfalvi Z, Burgyan J (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO J* **22**: 633–640
- Valoczi A, Várallyay E, Kauppinen S, Burgyan J, Havelda Z (2006) Spatio-temporal accumulation of microRNAs is highly coordinated in developing plant tissues. *Plant J* **47**: 140–151
- Várallyay E, Burgyan J, Havelda Z (2008) MicroRNA detection by northern blotting using locked nucleic acid probe. *Nat Protoc* **2007.528**: 190–196
- Vargason JM, Szittya G, Burgyan J, Tanaka Hall TM (2003) Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**: 799–811
- Vaucheret H (2009) AGO1 homeostasis involves differential production of 21-nt and 22-nt miR168 species by MIR168a and MIR168b. *PLoS One* **4**: e6442
- Vaucheret H, Mallory AC, Bartel DP (2006) AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol Cell* **22**: 129–136
- Vaucheret H, Vazquez F, Crete P, Bartel DP (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev* **18**: 1187–1197
- Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell* **136**: 669–687
- Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ, Chua NH (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev* **20**: 3255–3268