Differential effects of viral silencing suppressors on siRNA and miRNA loading support the existence of two distinct cellular pools of ARGONAUTE1

Gregory Schott^{1,2}, Arturo Mari-Ordonez^{1,2},
Christophe Himber¹, Abdelmalek Alioua¹,
Olivier Voinnet^{1,2,*} and Patrice Dunoyer^{1,*}

¹IBMP-CNRS, Strasbourg Cedex, France and ²Department of Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

Plant viruses encode RNA silencing suppressors (VSRs) to counteract the antiviral RNA silencing response. Based on *in-vitro* studies, several VSRs were proposed to suppress silencing through direct binding of short-interfering RNAs (siRNAs). Because their expression also frequently hinders endogenous miRNA-mediated regulation and stabilizes labile miRNA* strands, VSRs have been assumed to prevent both siRNA and miRNA loading into their common effector protein, AGO1, through sequestration of small RNA (sRNA) duplexes *in vivo*. These assumptions, however, have not been formally tested experimentally. Here, we present a systematic *in planta* analysis comparing the effects of four distinct VSRs in *Arabidopsis*. While all of the VSRs tested compromised loading of siRNAs into AGO1, only P19 was found to concurrently prevent miRNA loading, consistent with a VSR strategy primarily based on sRNA sequestration. By contrast, we provide multiple lines of evidence that the action of the other VSRs tested is unlikely to entail siRNA sequestration, indicating that *in-vitro* binding assays and *in-vivo* miRNA* stabilization are not reliable indicator of VSR action. The contrasted effects of VSRs on siRNA versus miRNA loading into AGO1 also imply the existence of two distinct pools of cellular AGO1 that are specifically loaded by each class of sRNAs. These findings have important implications for our current understanding of RNA silencing and of its suppression in plants.

The EMBO Journal (2012) 31, 2553–2565. doi:10.1038/emboj.2012.92; Published online 24 April 2012

Subject Categories: RNA; plant biology

Keywords: AGO1; miRNA; RNA silencing; siRNA; viral silencing suppressor

Introduction

RNA silencing is an ancient eukaryotic process involved in sequence-specific control of gene expression. It plays essential role in many biological processes such as genome defence against mobile DNA elements or regulation of factors involved in plant and animal development. Although it operates through multiple pathways, RNA silencing relies on a set of core processes triggered by double-stranded RNA (dsRNA). dsRNA is processed by RNaseIII-like enzymes in the DICER family to generate 21–24 nt-long small RNA (sRNA) duplexes. The model plant *Arabidopsis thaliana* encodes four DICER paralogues with specialized functions (Baulcombe, 2004). Dicer-like (DCL)-1 mainly contributes to the production of micro (mi)RNAs from non-coding, imperfect stem-loop precursor RNAs (Voinnet, 2009), whereas populations of 21, 22 and 24 nt-long short-interfering (si)RNAs are synthesized from long, perfectly or near-perfectly base-paired dsRNAs through the action of DCL4, DCL2 and DCL3, respectively (Brodersen and Voinnet, 2006; Vazquez, 2006; Chapman and Carrington, 2007). Upon processing, sRNAs are incorporated into an RNA-induced silencing complex (RISC) containing 1 of the 10 Argonaute (AGO) proteins that affect RNA silencing in *Arabidopsis*. Most miRNAs load into AGO1-containing RISC to guide post-transcriptional gene silencing (PTGS) of complementary or partially complementary mRNA by inhibiting their stability and/or translation (Voinnet, 2009). miRNA targets include transcription factor mRNAs required for plant development, as well as transcripts encoding proteins involved in various metabolic and hormonal pathways. DCL4-dependent 21 nt-long siRNAs guide AGO1-dependent PTGS of endogenous transcripts including those involved in leaf polarization via the production of *trans*-acting (tasi) RNAs (Vaucheret, 2005).

Besides its essential roles in development, RNA silencing constitutes the primary plant immune system against viruses. First, vsRNAs are invariably detected during infection, diagnostic of the activation of antiviral RNA silencing (Hamilton and Baulcombe, 1999). Second, viral siRNA accumulation correlates with lower levels and reduced spread of the invading virus, as illustrated by the hypersusceptibility of mutant *Arabidopsis* affected in silencing pathways (Morel *et al.*, 2002; Blevins *et al.*, 2006; Deleris *et al.*, 2006; Qu *et al.*, 2008). Third, viruses have evolved a vast array of proteins, called viral suppressors of RNA silencing (VSR), whose expression is often prerequisite for them to multiply and invade their host systemically (Deleris *et al.*, 2006; Garcia-Ruiz *et al.*, 2010; Wang *et al.*, 2011). Antiviral RNA silencing is triggered by dsRNA replication intermediates or intra-molecular fold-back structures within viral genomes, which are processed by DCL4, or its surrogate DCL2, to produce viral-derived sRNAs (vsRNAs; Blevins *et al.*,

*Corresponding authors. O Voinnet, Department of Biology, Swiss Federal Institute of Technology Zurich, Universitatstrasse 2, 8092 Zurich, Switzerland. Tel.: +41 44 633 93 60; Fax: +41 44 632 10 81; E-mail: voinneto@ethz.ch or P Dunoyer, IBMP-CNRS, 12, rue du Général Zimmer, 67084 Strasbourg Cedex, France. Tel.: +33 3 88 41 72 60; Fax: +33 3 88 61 44 42; E-mail: patrice.dunoyer@ibmp-cnrs.unistra.fr

Received: 6 January 2012; accepted: 9 March 2012; published online 24 April 2012

2006; Deleris *et al*, 2006). Subsequently, vsRNAs are recruited by one or several endogenous AGO proteins to direct PTGS of viral RNA as part of antiviral RISCs. Among the AGOs implicated in antiviral defence, AGO1 and AGO2 have emerged as major effectors (Morel *et al*, 2002; Zhang *et al*, 2006; Qu *et al*, 2008; Takeda *et al*, 2008; Harvey *et al*, 2011; Wang *et al*, 2011).

VSRs target many stages of the antiviral silencing pathway, including the processing (i.e., DCL level) and effector (i.e., AGO level) steps (reviewed in Ding and Voinnet, 2007). The targeting of AGO1 is exemplified, for instance, with the 2b protein of *Cucumber mosaic virus* (CMV), which inhibits RISC activity via physical interaction with the PAZ domain of AGO1 (Zhang *et al*, 2006). The *Beet western yellows virus* (BWYV) P0 protein was suggested to act as an F-box protein targeting AGO proteins for ubiquitination and subsequent degradation, thereby preventing RISC assembly (Baumberger *et al*, 2007; Bortolamiol *et al*, 2007; Csorba *et al*, 2010). *Turnip crinkle virus* (TCV) P38 was recently shown to bind directly and specifically AGO1 through mimicry of host-encoded glycine/tryptophane (GW)-containing proteins normally required for RISC assembly/function in diverse organisms. Binding of P38 to AGO1 was suggested to inhibit loading of AGO1 with vsRNA and miRNA. In particular, miR162-mediated regulation of DCL1 is suppressed by P38 in a GW motif-dependent manner, leading to a dramatic increase in DCL1 accumulation, which in turn promotes downregulation of DCL3 and DCL4 (Azevedo *et al*, 2010). In addition to AGO1 suppression, sequestration of siRNA is another common VSR strategy to inhibit RNA silencing (Lakatos *et al*, 2006; Merai *et al*, 2006). The most compelling example of this mode of action was illustrated with the crystallization of the tombusvirus P19 protein in direct association with an siRNA duplex (Vargason *et al*, 2003; Ye *et al*, 2003). P19 acts as a head-to-tail homodimer that specifically sizes 21 bp siRNA duplexes, acting as a 'molecular calliper'. Incidentally, 21 bp siRNAs are the products of the main antiviral Dicer, DCL4, and point mutations that prevent P19 binding to these siRNAs abolish its silencing suppression activity.

Based on the P19 precedent, additional VSRs were subsequently suggested to suppress RNA silencing through sRNA binding. These VSRs include *Beet yellows virus* P21, Potyviral HcPro, *Peanut clump virus* (PCV) P15, *Barley stripe mosaic virus* γ b, *Rice hoja blanca tenuivirus* NS3, *Tobacco mosaic virus* P122, TCV P38 (Lakatos *et al*, 2006; Merai *et al*, 2006; Csorba *et al*, 2007; Hemmes *et al*, 2007). In most cases, however, these results must be interpreted with caution as they are generally obtained using *in-vitro* binding assays or via transient heterologous expression systems. Moreover, binding is frequently observed under non-physiological amounts of VSR, and functional correlation between siRNA binding and silencing suppression activity is lacking, in most cases, owing to the unavailability of adequate loss-of-function point-mutant VSR alleles. This was recently illustrated by the use of CMV 2b mutant derivatives that allowed the authors to discriminate the respective contribution of siRNA binding and AGO1 slicer inhibition with respect to 2b-mediated silencing suppression activity *in vivo* (Duan *et al*, 2012). Finally, RNA binding is often non-specific. For instance, the TCV P38 protein was shown to bind both long and short dsRNA (Merai *et al*, 2006), yet disruption

of two GW residues is sufficient to abolish P38 VSR activity by preventing P38 association with AGO1 (Azevedo *et al*, 2010). It is in fact plausible that vsRNA binding often reflect additional, silencing-unrelated functions of VSRs, which require their close association with viral nucleic acids. For instance, TCV P38 and TMV P122 suppress silencing, but they are also required for encapsidation and replication of the viral RNA, respectively. Consequently, whether dsRNA binding is a genuine feature of silencing suppression remains, in most cases, unaddressed.

Based on the robust data obtained with P19 (Vargason *et al*, 2003; Chapman *et al*, 2004; Dunoyer *et al*, 2004), dsRNA binding by VSRs is generally thought to quantitatively impair vs/siRNA incorporation into antiviral RISCs, but, here again, *in-vivo* experimental evidence is often lacking to support this general inference. Inhibition of si/vsRNA loading into AGO1 is commonly used to extrapolate how VSR might hinder miRNA-mediated mRNA regulation, which is frequently observed in transgenic VSR-expressing plants (Chapman *et al*, 2004; Dunoyer *et al*, 2004; Zhang *et al*, 2006). While this is indeed clearly the case of P19, which binds miRNA duplexes and thereby stabilizes the otherwise labile miRNA* strand (Chapman *et al*, 2004; Dunoyer *et al*, 2004; Csorba *et al*, 2007), it remains uncertain if VSR commonly sequester both siRNAs and miRNAs away from AGO1 and if this forms the basis for their miRNA-inhibitory effects.

The present study was aimed at answering the above questions by analysing siRNA and miRNA loading into AGO1 *in vivo*, using transgenic Arabidopsis lines expressing four distinct VSRs: the TCV-encoded P38, the P15 protein of PCV, Hc-Pro of *Turnip mosaic virus* (TuMV), and the tombus-viral P19 protein evoked above. The analysis revealed that the four VSRs indeed quantitatively prevent *in-vivo* siRNA loading into AGO1. Furthermore, inhibition of siRNA loading in the case of P38 did not involve siRNA sequestration, as previously suggested by *in-vitro* analyses, but relies largely, if not exclusively, on its direct and specific interaction with AGO1. Interestingly, among the four VSR tested, only P19 was found to efficiently prevent miRNA loading into AGO1, even if all VSRs were found to inhibit miRNA-mediated target regulation and to stabilize miRNA* strands *in vivo*. Collectively, these and other observations suggest the existence of at least two distinct pools of cellular AGO1. One pool seems preferentially loaded with siRNAs, while the other seems preferentially loaded with miRNAs, with each pool being differentially affected by VSRs. These results unravel further layers of complexity in the plant RNA silencing pathway and its perturbation by biotic stress.

Results

Effect of transgenic P38 expression on endogenous and exogenous sRNA accumulation

The TCV-encoded P38 protein was recently demonstrated to bind directly and specifically AGO1 via two GW motifs (Azevedo *et al*, 2010). The resulting sequestering of AGO1 was proposed to prevent its loading with vsRNA, or to impair the action of AGO1-loaded vsRNA by competing with an as yet unidentified GW-motif protein required for RISC function. Moreover, during TCV infection, binding of P38 to AGO1 was also associated with a strong increase in DCL1 accumulation, which, in turn, promotes a decrease in both DCL3 and DCL4

Figure 1 Effect of P38 expression on SUC:SUL plants. **(A)** Phenotypes of wild-type (SUC:SUL) or P38-expressing (SSxP38#1 and #2) SUC:SUL plants. **(B)** Northern analysis of SUL siRNA (@SUL), miRNA (@173, @390, @159) and trans-acting siRNA (@255, @TAS3) accumulation in wild-type or P38-expressing plants. SUC:SUL plants carrying the double *dcl3/dcl4* (SSxdlc3/4) mutations are used as a control for DCL2-dependent 22 nt-long siRNA accumulation. Ethidium bromide staining of ribosomal RNAs (rRNAs) is used as loading control of the gel. Figure source data can be found with the Supplementary data.

levels through undefined mechanisms. Increased DCL1 levels were suggested to result from of a deficit in miR162-loading or activity into AGO1, a process whereby miR162 normally controls DCL1 mRNA levels (Xie *et al*, 2003; Azevedo *et al*, 2010). However, as TCV infection also causes a dramatic and general decrease in miRNA accumulation (Azevedo *et al*, 2010), this issue could not be formally addressed. Moreover, recombinant TCV carrying point mutations in the P38 GW motifs is only able to infect hypomorphic *ago1* or *dcl2/dcl4* double-mutant Arabidopsis, precluding a direct comparative analysis of vsRNA loading into AGO1 in presence of WT versus mutated P38. In order to explore the effects of P38 on siRNA and miRNA loading into AGO1, we introduced a 35S-P38 transgene into the SUC:SUL Arabidopsis system. In this system, an inverted repeat (IR) construct driven by the phloem companion cell (CC)-specific SUC2 promoter directs silencing of the *SULPHUR* (*SUL*) endogenous mRNA (Dunoyer *et al*, 2005). Processing of the phloem-specific *SUL* dsRNA generates 21 nt- and 24 nt-long siRNAs and causes non-cell autonomous silencing manifested by a chlorotic phenotype expanding 10–15 cells beyond the vasculature (Figure 1A; Dunoyer *et al*, 2005). Of the two siRNAs species, only the DCL4-dependent 21 nt-long siRNAs are required for non-cell autonomous *SUL* silencing (Dunoyer *et al*, 2007, 2010b).

Although fertile, several P38-expressing lines displayed elongated and downward-curved rosette leaves with moderate serration (Figure 1A). These morphological characteristics are reminiscent of tasiRNA or weak miRNA mutants, and are typical of VSR-expressing lines (Kasschau *et al*, 2003; Chapman *et al*, 2004; Dunoyer *et al*, 2004; Zhang *et al*, 2006). Therefore, we first investigated if these P38-induced phenotypic defects correlated with changes in the accumulation of various endogenous sRNAs. Northern analysis indicated that miRNA steady-state levels in the

35S-P38 plants were comparable to those of non-transgenic plants, with the notable exception of miR390, for which increased accumulation was observed in the P38 plants (Figure 1B). miR390 is almost exclusively associated with AGO7 and mediates the initial cut of non-coding TAS3 precursor transcripts. By contrast, TAS1 and TAS2 precursors are cleaved by miR173, associated with AGO1 (Allen *et al*, 2005; Montgomery *et al*, 2008a,b; Felippes and Weigel, 2009). Interestingly, 21 nt-long TAS3 tasiRNAs were below detection levels in P38-transgenic plants and were replaced by a DCL2-dependent 22 nt siRNA species, whereas TAS1 siRNA255 accumulation was strongly reduced (Figure 1B). P38 expression also completely abolished the appearance of the *SUL* silencing phenotype (Figure 1A). However, this suppression was not accounted for by an inhibition of DCL4-mediated processing of the *SUL* dsRNA, as northern analysis revealed that 35S-P38 plants accumulated similar amounts of 21 nt *SUL* siRNAs to those of control plants (Figure 1B). Therefore, transgenic P38 expression does not affect DCL4-dependent IR-derived dsRNA processing while it strongly (TAS1) or completely (TAS3) impairs DCL4-dependent TAS-derived dsRNA processing or TAS siRNA stability.

The above results prompted us to investigate if transgenic P38 affects the accumulation of DCL4 itself, as observed during TCV infection, when P38 is produced at comparatively much higher levels than in transgenic plants (Azevedo *et al*, 2010). Western blot analysis revealed that P38-transgenic plants accumulate DCL4 to levels similar to those of non-transgenic plants (Figure 2A). Likewise, the levels of DRB4, a dsRNA-binding protein required for optimal DCL4-mediated processing of both IRs and TAS precursors (Adenot *et al*, 2006; Dunoyer *et al*, 2007; Nakazawa *et al*, 2007), were unchanged (Figure 2A). Therefore, the contrasted effect of TCV P38 on tasiRNA and *SUL* siRNA levels, both of which are DCL4 dependent, cannot be explained by a destabilization of

Figure 2 Effect of P38 on silencing factors or miRNA targets accumulation. (A) Accumulation of endogenous AGO1, DCL3, DCL4, DRB4 and the miRNA targets DCL1 and CIP4 was assessed by protein blot analysis of wild-type (SS) or P38-expressing (P38) *SUC:SUL* plants. Arrows indicate the specific band as opposed to crossreacting bands detected by the antibodies used. Equal loading was verified by Coomassie staining of the membrane after western blotting. (B) Quantitative real-time PCR of the miRNA or *trans*-acting siRNA targets accumulation in the same plants as described in (A). The small RNA that targets each of these endogenous mRNA is indicated under brackets. mRNA levels were normalized to that of *Actin2* (At3g18780) and then to the WT plants which were arbitrarily set to 1. Error bars represent standard deviation from two independent experiments involving triplicate PCRs each.

dsRNA-processing factors. The strong accumulation of 22 nt-long TAS3 siRNAs also excludes an effect of P38 on upstream factors such as RDR6 or SGS3, both of which are required for the conversion of cleaved TAS precursors into long-dsRNA, but dispensable for IR-derived siRNA processing (Peragine *et al*, 2004; Vazquez *et al*, 2004; Allen *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005; Dunoyer *et al*, 2007). The strong accumulation of 22 nt-long TAS3 siRNAs also excludes an inhibition, by P38, of miR390 loading into AGO7, consistent with the absence of affinity of P38 for this effector (Azevedo *et al*, 2010). Finally, the observation that P38 inhibits neither DCL4-dependent IR-derived siRNA accumulation nor DCL2-dependent TAS3-derived tasiRNA accumulation, argues against the previous proposal that long dsRNA binding underlies the VSR activity of P38 (Merai *et al*, 2006).

Transgenic P38 inhibits silencing of endogenous targets of siRNAs and miRNAs

Given that developmental anomalies in VSR-transgenic Arabidopsis have been associated with aberrant regulation of miRNA- or tasiRNA-targeted mRNA expression (Kasschau *et al*, 2003; Chapman *et al*, 2004; Dunoyer *et al*, 2004; Zhang *et al*, 2006; Jay *et al*, 2011), we compared the expression levels of several of these targets in control and P38-transgenic

lines. Figure 2B shows that in P38-expressing plants, accumulation of *At5g18040* (target of *TAS1* siRNA255), *ARF3* and *ARF4* (targets of *TAS3* tasiRNA) was upregulated. Although trivially explained in the case of *TAS1* targets (based on the low level of *TAS1* tasiRNA255 accumulation), upregulation of *TAS3* targets was interesting because 22 nt-long *TAS3* siRNAs accumulated to much higher level in 35S-P38-transgenic plants than the 21-nt-long *TAS3* siRNAs in control plants (Figure 1B). Since effective tasiRNAs are phased in 21 nt increments, DCL2-mediated processing of the *TAS3* dsRNA in P38-expressing plants might result in the formation of offset siRNAs with suboptimal complementarity with target sequences. Alternatively, the 22-nt *TAS3* siRNAs may not be properly loaded into AGO1 because their offset may cause a change in their 5'-terminal nucleotide, which influences loading into AGOs, including AGO1 (Mi *et al*, 2008; Montgomery *et al*, 2008a). A last, non-mutually exclusive possibility is that P38 might interfere with 22 nt siRNA loading into AGO1, either through siRNA-binding properties, as observed *in vitro* (Merai *et al*, 2006), or through direct interaction with AGO1, as observed *in vivo* (Azevedo *et al*, 2010).

miRNA targets such as *CUC2* (miR164), *MYB65* (miR159), *HAP2B* (miR169), *SCL6-III* (miR171), *ARF10* (miR160), *ARF17* (miR160) and *ARF8* (miR167) were also found upregulated in

Figure 3 Effect of P38 on small RNA loading into AGO1. Immunoprecipitation experiments were conducted in wild-type or P38-expressing SUC:*SUL* plants using an AGO1-specific antibody. Total RNA extracted from the respective IPs was subjected to northern analysis using the indicated probes by sequential rounds of probing and stripping the same membrane. The presence of AGO1 in each IP was confirmed by protein blot analysis (data not shown). rRNA: loading control. Figure source data can be found with the Supplementary data.

35S-P38 plants as opposed to WT plants, as assessed by Q-RT-PCR analyses (Figure 2B). Moreover, western blot analysis of DCL1 and CIP4, respectively, targeted by miR162 and miR834, revealed their increased accumulation in the P38-expressing plants (Figure 2A). As shown in Figures 1B, 2 and 3, and in sharp contrast to what is observed during authentic TCV infections (Azevedo *et al*, 2010), this enhanced accumulation of miRNA targets was not due to decreased accumulation of the corresponding miRNA, suggesting that transgenically expressed P38 inhibits AGO1 activity. This inhibition can be either due to the sequestration of miRNA/miRNA* duplexes by P38, or to the interaction of P38 with AGO1 resulting in impaired loading of the miRNA guide strand into RISC, or impaired RISC activity.

P38 prevents loading of siRNAs into AGO1 without affecting loading of miRNAs

SUL silencing requires the specific loading of 21 nt *SUL* siRNAs into AGO1 (Dunoyer *et al*, 2007). As P38 suppresses *SUL* silencing and inhibits miRNA-loaded AGO1 target regulation without affecting miRNA or *SUL* siRNA accumulation, these observations prompted us to test if P38 compromised AGO1 loading with either sRNA species. We first confirmed that P38 expression affected neither AGO1 stability (Figure 2A) nor the amount of immunoprecipitated AGO1 (data not shown). As shown previously (Dunoyer *et al*, 2007), analysis of the sRNA fraction of AGO1 immunoprecipitates isolated from SUC-*SUL* plants showed that, out of the 21-nt and 24-nt *SUL* siRNA, only the former is loaded

into AGO1. AGO1 immunoprecipitates from 35S-P38-transgenic plants were, by contrast, devoid of 21 nt *SUL* siRNAs, suggesting that this pool of siRNAs is sequestered away from AGO1 (Figure 3), likely accounting for *SUL*-silencing suppression in P38-expressing plants. Of note, similar immunoprecipitation experiments conducted in TCV-infected plants showed a more modest effect of P38 on *SUL*-derived siRNA loading into AGO1 (Azevedo *et al*, 2010). This difference likely reflects dissimilar pattern and timing of P38 accumulation when expressed from a transgene or from the cognate virus. Indeed, in transgenic plants, P38 is expressed from a ubiquitous promoter in many tissues of the plants and at very early developmental stages. By contrast, not all cells are infected during TCV infection, and in those cells, P38 accumulation has to build up from viral replication, which presumably allows a significant proportion of *SUL* siRNAs to incorporate into AGO1 before the onset of P38-mediated inhibition of siRNA loading. This likely explains the more modest effect of P38 observed in infected tissues. Moreover, as suggested by Azevedo *et al* (2010), P38 may only be able to prevent siRNA incorporation into non-loaded RISC. Therefore, only siRNAs produced concomitantly with P38 expression will be prevented from incorporation into AGO1.

Interestingly, in P38-transgenic plants, TAS1 and TAS3 siRNAs were also below detection levels in AGO1 immunoprecipitates. As evoked above, this result can be explained, in the case of TAS3 siRNAs, by a change in the identity of their 5'-terminal nucleotide. However, this cannot be the case for TAS1 tasiRNA255, given the strong disproportion of their

levels in total RNA fraction versus AGO1 immunoprecipitates, suggesting that P38 also prevents loading of TAS-derived siRNA into AGO1.

To assess if P38 also compromised loading of AGO1 with DCL2-dependent siRNAs, we exploited endogenous IRs recently identified in the Arabidopsis genome (Kasschau *et al*, 2007; Lindow *et al*, 2007). One representative of these endogenous IRs, IR71, is processed mainly into 22 nt- and 24 nt-long siRNAs, with the former being efficiently loaded into AGO1 (Dunoyer *et al*, 2010a). However, in the presence of P38, these DCL2-dependent 22 nt-long siRNA were also below detection levels in AGO1 immunoprecipitates (Supplementary Figure S1). Thus, P38 prevents the loading of both DCL4- and DCL2-dependent siRNAs into AGO1. Surprisingly, however, miRNA loading into AGO1 remained unaffected by P38, or was even slightly elevated compared with control SUC:*SUL* plants (Figure 3). Interestingly, accumulation of several miR* strands, such as miR160*, was strongly upregulated in total RNA fraction isolated from P38-transgenic plants (Figure 3, data not shown). However, no signal for the miRNA* strand was observed in AGO1 immunoprecipitates, whereas the corresponding miRNA guide strands were loaded as efficiently as in control SUC:*SUL* plants. Thus, P38 does not prevent unwinding of the miRNA/miRNA* duplexes or the subsequent loading of miRNA guide strands into AGO1. Furthermore, the fact that TCV P38 does not block loading of mature miRNA into AGO1, yet inhibits AGO1 activity on miRNA targets, suggests that P38 interacts and interferes with preloaded AGO1 miRISC.

Collectively, these results allow us to draw two important conclusions. First, they unravel strikingly contrasted effects of P38 on siRNA- and miRNA-loaded AGO1, which are most easily reconciled by the existence of at least two distinct AGO1 pools, of which one is specifically loaded with siRNAs, and the other specifically loaded with miRNAs; the results further suggest that the ensuing siRNA- and miRNA-loaded RISCs are targeted differently by P38. Second, the results strongly argue against a prevalent contribution of sRNA binding to the VSR action of P38, a point further addressed in the following section.

CC-specific expression of P38 does not affect *SUL* silencing in the neighbouring cells

In plants and nematodes, RNA silencing spreads beyond its sites of initiation, owing to the movement of signal molecules. We recently established that exogenous and endogenous siRNAs act as mobile silencing signal between plant cells (Dunoyer *et al*, 2010a,b). siRNAs are unlikely to move from cell-to-cell bound to AGO1 because we demonstrated that the action of AGO1 is strictly cell autonomous. If P38-mediated silencing suppression *in vivo* involved its direct binding to sRNAs, then we reasoned that phloem CC-specific expression of P38 into SUC:*SUL* plants should compromise the *SUL*-silencing phenotype. On the other hand, if silencing suppression by P38 *in vivo* relied on its ability to prevent siRNA loading into AGO1, owing to the AGO1-binding property of the P38 GW motifs (Azevedo *et al*, 2010), then specific expression of P38 within CCs should have no impact on *SUL* silencing and its movement into neighbouring cells. In order to investigate this aspect, an SUC-P38 transgene was introduced into the SUC:*SUL* plants.

In most of the transformants generated (83%, $n = 64$), *SUL*-silencing movement remained unaltered, even in high P38-expressing lines (Figure 4A). Moreover, *in-situ* hybridization revealed that the P38 mRNA was specifically expressed within the CCs of SUC-P38-transgenic plants displaying an unaltered *SUL*-silencing phenotype. By contrast, it was ectopically expressed in those transformants exhibiting reduced *SUL* silencing (Figure 4B). Furthermore, analysis of the sRNA fraction of AGO1 immunoprecipitates revealed that, in SUC-P38 plants, the amount of 21 nt-*SUL* siRNAs was similar or only slightly reduced compared with the amount isolated from AGO1 immunoprecipitates in control SUC:*SUL* plants (Figure 4C). These observations were in sharp contrast with previous results obtained with SUC-driven expression of the P19 and P21 VSRs, both of which sequester 21 nt-long siRNAs *in vivo* and alleviate the *SUL*-silencing phenotype (Vargason *et al*, 2003; Ye *et al*, 2003; Dunoyer *et al*, 2010b). Collectively, the results imply that P38 does not bind directly the mobile *SUL* siRNA fraction that accounts for the *SUL*-silencing phenotype (Dunoyer *et al*, 2010b). Taking into further account the results of the previous sections of this manuscript, these observations rule out a significant contribution of sRNA binding to the VSR activity of P38 *in planta*. To note, in plants exhibiting CC-specific P38 expression, no P38 hybridization signal was observed in the AGO1 immunoprecipitates (Figure 4C). This lack of detection does not result from a weak P38-AGO1 interaction as it was shown before that this complex is unaltered by treatments of up to 800 mM KCl (Azevedo *et al*, 2010) and is therefore highly stable. More likely, this reflects the relative discrete number of cells in which P38 is expressed, resulting in a strong dilution of the P38-AGO1 complex in the immunoprecipitation extract as those experiments were performed on whole leaf samples.

Distinct effects of P15, P19, Hc-Pro and P38 on siRNA and miRNA loading into AGO1

To assess if the differential effects of P38 on siRNA versus miRNA loading into AGO1 were a general property of VSRs, we employed transgenic plants expressing PCV P15, Tomato bushy stunt virus (TBSV) P19 and TuMV P1-HcPro. We also included a fourth suppressor called P15ΔN6, a derivative of PCV P15 where the C-terminal peroxisomal targeting signal (PTS1) has been removed. This C-terminal PTS1 has been shown to be required for peroxisomal localization of P15 but dispensable for its silencing suppression activity (Dunoyer *et al*, 2002). Together with the TCV P38, these silencing suppressors were introduced into 35S-CHS-RNAi-transgenic plants, in which an IR against the Chalcone synthase (CHS) endogenous gene is expressed (Wesley *et al*, 2001; Dunoyer *et al*, 2004; see Materials and methods). The rationale for using a ubiquitous, highly expressed RNAi trigger was to rule out the possibility that selective P38-mediated block of siRNAs loading into AGO1 was a CC-specific effect or was artificially facilitated by the low amounts of siRNAs accumulating in the SUC:*SUL* experimental system. Indeed, we previously showed that siRNA production is >50 times higher from the strong, ubiquitous 35S promoter than from the phloem-specific SUC promoter (Dunoyer *et al*, 2007).

Northern analysis revealed that all VSR mRNAs accumulated at their expected size (Figure 5B, upper panel). Moreover, CHS silencing was efficiently suppressed in all

Figure 4 Companion cell-specific expression of P38 does not suppress cell-to-cell SUL-silencing movement. (A) Leaf phenotypes of companion cell-specific (#1 and #2) or ectopic (#5) expression of P38 in SUC:SUL transgenics. (B) *In-situ* hybridization of P38 mRNA in transversal sections of leaves depicted in (A). (C) Immunoprecipitation experiments were conducted using an AGO1-specific antibody in control plants (SS), blend of transgenic SUC:P38 T0 or selected T1 line (#1). The presence of AGO1 in each IP was confirmed by protein blot analysis (upper panel). Total RNA extracted from the respective IPs was subjected to northern analysis using the indicated probes.

Figure 5 Differential effect of several VSRs on siRNA and miRNA loading into AGO1. (A) Protein blot analysis of AGO1 accumulation in wild-type or transgenics CHS-RNAi plants expressing various VSRs (namely, TBSV P19, TuMV Hc-Pro, PCV P15 or P15ΔN6 and TCV P38). (B) RNA gel blot analysis of VSRs (upper panel) and Chalcone synthase expression (lower panel) in transgenics CHS-RNAi plants. (C) RNA gel blot analysis of CHS siRNA (@CHS) or miRNA accumulations in total RNA and AGO1 immunoprecipitated fractions from lines characterized in (A) and (B). (D) Immunoprecipitation experiments were conducted in VSR-expressing lines to assess the loading of endogenous inverted repeat-derived siRNA (@IR71) in AGO1. Figure source data can be found with the Supplementary data.

Figure 6 Effect of P19, Hc-Pro, P15 and P38 expression on silencing factors or miRNA targets accumulation. (A) Quantitative real-time PCR of miRNA or *trans*-acting siRNA targets accumulation in wild-type or VSR-expressing transgenic plants. The small RNA that targets each of these endogenous mRNA is indicated under brackets. mRNA levels were normalized to that of *Actin2* (At3g18780) and then to the WT plants which were arbitrarily set to 1. Error bars represent standard deviation from two independent experiments involving triplicate PCRs each. (B) Accumulation of endogenous AGO1, DCL3, DCL4, DRB4 and the miRNA targets DCL1, AGO2 and CIP4 was assessed by protein blot analysis in the same plants as described in (A). Arrows indicate the specific band as opposed to crossreacting bands detected by the antibodies used. Control western blots showing the specificity of the various antibodies are available in Supplementary Figure S2. Equal loading was verified by Coomassie staining of the membrane after western blotting.

VSR-expressing plants compared with control plants, as revealed by high CHS transcript levels in VSR lines (Figure 5B, bottom panel). Similarly to what was previously shown for the SUC-SUL system, only the 21-nt-long CHS siRNA was found associated with AGO1 in control CHS-RNAi plants (Figure 5C). By contrast, while none of the four VSRs affected AGO1 steady-state levels (Figure 5A), the 21-nt CHS siRNA was below detection level in AGO1 immunoprecipitates from all VSR-expressing lines (Figure 5C). Conclusions for P15 or P15ΔN6 could not be drawn due to the strong destabilizing effects of these VSR alleles on total CHS siRNA accumulation. We thus looked at the accumulation of endogenous IR-derived sRNAs (e.g., IR71), which, we showed, follow processing and activity patterns undistinguishable from those of exogenous IRs such as SUC-SUL or 35S-CHS (Dunoyer *et al*, 2010a). Northern analysis of total RNA showed that P15 or P15ΔN6 expression has no effect on IR71-derived siRNA accumulation. However, IR71-derived siRNA levels were strongly reduced in AGO1 immunoprecipitates from the P15- and P15ΔN6-transgenic plants whereas they were readily detected in immunoprecipitates from control plants. Thus, like P19, Hc-Pro or P38, P15 and P15ΔN6 inhibit loading of IR-derived siRNAs into AGO1 (Figure 5D).

We then analysed the loading of conserved miRNAs, including miR160, miR164, miR162, miR168, miR156 and miR159, into AGO1. We found that miRNA loading was unchanged in all VSR-transgenic plants tested, except in P19-expressing plants, confirming previous observations (Chapman *et al*, 2004; Dunoyer *et al*, 2004). Thus, among all VSRs tested, only P19 prevents loading of both siRNAs and miRNAs into AGO1 (Figure 5C). Surprisingly, however, all

VSR tested stabilized miRNA* steady-state levels (Figure 5C) and efficiently inhibited miRNA-mediated target regulation, as previously observed (Chapman *et al*, 2004; Dunoyer *et al*, 2004). Hence, endogenous miRNA targets such as CUC2 (miR164), HAP2B (miR169), ARF10 (miR160), ARF17 (miR160) and SPL10 (miR156) accumulated to higher levels in VSR-transgenic plants than in control plants (Figure 6A). Moreover, western blot analysis of DCL1 (miR162), CIP4 (miR834) and AGO2 (miR403) also showed their increased accumulation, albeit to various extent, in all VSR-expressing lines (Figure 6B). Collectively, these results further substantiate the existence of at least two specific pools of AGO1, differentially affected by VSR in terms of sRNA loading. Furthermore, the results also show that miRNA* stabilization by VSR, a common effect of these proteins, is not necessarily linked to impaired miRNA loading into AGO1 as initially established with P19.

Discussion

Although the transgenic systems presented here may provide an oversimplified view of authentic viral infections, this study nonetheless brings forward novel insights into the mechanisms of VSR action and simultaneously delineates a series of up-front procedures and tools that will be useful to the community to readily discriminate the respective contribution of sRNA binding (as often established *in vitro*) as opposed to other biochemical properties, to VSR action *in planta*. The analysis also provides new information regarding a central component of PTGS, AGO1, further emphasizing the value of VSRs as molecular probes of endogenous RNA silencing pathways.

The relevance of *in-vitro* sRNA binding to VSR action *in planta*

Based on *in-vitro* studies, several VSRs were proposed to suppress silencing through direct binding of siRNA, as was originally established with P19 (Vargason *et al*, 2003; Ye *et al*, 2003; Lakatos *et al*, 2006; Merai *et al*, 2006); also by analogy to P19, those VSRs with *in-vitro* siRNA-binding properties were predicted to prevent siRNA loading into RISC *in vivo*. Both of these assumptions, however, were never formally tested experimentally. We have now designed experiments to examine these hypotheses *in planta* and showed that, indeed, all of the VSRs tested prevent siRNA loading into AGO1. P19, used as a reference, was confirmed to prevent loading of both siRNAs and miRNAs into AGO1, consistent with a silencing suppression strategy primarily based on sRNA sequestration. Results obtained with the additional VSRs tested in this study indicate, however, that inhibition of siRNA loading into AGO1 may not necessarily always entail *in-vivo* siRNA binding.

First, the TuMV Hc-Pro tested here, like its counterpart from *Potato virus Y*, displays no or only weak siRNA-binding properties *in vitro* (Merai *et al*, 2006), yet it strongly prevented siRNA loading into AGO1 *in vivo* (Figure 5). Second, at least three lines of evidence indicate that P38 inhibition of siRNA loading into AGO1 does not proceed through direct sequestration of sRNA *per se* but, rather via specific P38–AGO1 associations mediated by GW motifs, as proposed by Azevedo *et al* (2010). Hence, P38 binding to sRNA duplexes *in vivo*, as originally suggested by *in-vitro* studies (Merai *et al*, 2006), would predict that siRNAs would co-precipitate with AGO1 pull-downs from P38-transgenic plants, because P38 was shown to display remarkably high affinity to AGO1 *in planta* (Azevedo *et al*, 2010). However, siRNAs were below detection limit in AGO1 immunoprecipitates isolated from P38 transgenics (Figures 3 and 5). Perhaps more compellingly, the *SUL*-silencing phenotype was unchanged in *SUC-P38* background despite high P38 accumulation in CCs (Figure 4). siRNA sequestration by CC-specific P38 was, on the contrary, expected to result in reduced *SUL*-silencing phenotype, as was observed using CC-specific P19 or P21, two VSRs with demonstrated *in-vivo* siRNA-binding properties (Dunoyer *et al*, 2010b). A third, indirect evidence comes from analysis of miRNA and miRNA* strand accumulation in AGO1 immunoprecipitates from P38 transgenics. siRNA binding by P38 was indeed assumed to explain the stabilization of the otherwise labile miRNA* strand and the concomitant suppression of miRNA-mediated target regulation in transgenic 35S-P38 plants (Chapman *et al*, 2004; Merai *et al*, 2006). However, analysis of the AGO1 immunoprecipitated fraction revealed that loading of miRNA guide strands into AGO1 was not affected, despite miRNA* stabilization (Figures 3 and 5). Moreover, the miRNA* strand was not associated with AGO1 in the presence of P38, ruling out that the miRNA signal detected in AGO1 immunoprecipitates comes from miRNA/miRNA* duplexes bound by P38 and co-IPed with AGO1. We conclude, based on these collective results with P38, that sRNA binding *in vitro* cannot be used to infer any biological properties of VSR *in vivo*.

The contrasted effects of P38 on IR-derived and TAS-derived siRNAs

A second interesting observation pertains to the contrasted effects of P38 on two separate DCL4-dependent pathways.

Thus, transgenic P38 did not significantly affect processing of DCL4-dependent IR-derived siRNA, whereas it strongly compromised DCL4-dependent TAS processing into 21 nt siRNAs (Figures 1, 3 and 5). An interpretation of this result is confounded, in the case of *TAS1*, by the fact that production of *TAS1* tasiRNAs relies on the initial cleavage of their precursor by miR173-loaded AGO1. Nonetheless, this is not the case for the initial cleavage of *TAS3*, triggered by AGO7, for which P38 displays no affinity *in vivo* (Azevedo *et al*, 2010). We also rule out differential effects of P38 on DCL4 or DRB4 steady-state levels, or on upstream factors, such as RDR6 or SGS3, required for TAS-derived, but not IR-derived 21 nt siRNA production (Figures 1 and 2). The differential effects of P38 on the two DCL4-dependent pathways are, in fact, reminiscent of the results of a forward genetic screen; this screen yielded *dcl4* point mutant alleles that uncoupled production of the two classes of DCL4-dependent 21 nt-long siRNAs (Dunoyer *et al*, 2005, 2007). More recently, a single substitution in a non-conserved Gly, between the PAZ and first RNaseIII domains of DCL4 was found to specifically shift *TAS3*- but not *TAS1*-processing towards 22 nt-long siRNA production (Cuperus *et al*, 2010). One possibility is that both the point mutant *dcl4* alleles and P38 impair the interaction or stability of an as-yet unidentified cellular partner of DCL4, required for tasiRNA- but not IR-derived siRNA production. Interestingly, similar conclusions could be drawn for the P19 and Hc-Pro VSRs (Supplementary Figure S3), suggesting that the elusive factor evoked above might be linked to the amplification step of RNA silencing, which is unique to tasiRNA synthesis and strongly required during antiviral silencing to keep pace with the high virus replication rates.

Stabilization of miRNA* strands and its significance for VSR action *in vivo*

Another puzzling result of the present analysis comes from the observed stabilization of the otherwise labile miRNA* strand by all VSRs. If this stabilization can be explained by the direct sequestration of miRNA/miRNA* duplexes in the case of P19, then this is probably not the case for the other VSRs tested, none of which compromised miRNA loading into AGO1. Moreover, these increased miRNA* steady-state levels cannot be explained by compromised miRNA duplex unwinding upon loading of the guide strand, because no miRNA* signal could be detected in AGO1 immunoprecipitates from VSR-transgenic plants (Figures 3 and 5). Interestingly, miRNA* stabilization was also previously observed in plants expressing the 2b protein from *CMV*, a VSR that inhibits RISC cleavage activity without preventing miRNA loading into AGO1 (Zhang *et al*, 2006). In *Drosophila*, siRNA guide strands direct AGO-catalysed cleavage of passenger strands, thereby facilitating their clearance (Matranga *et al*, 2005; Rand *et al*, 2005). It was thus suggested that, in plants, miRNA/miRNA* duplexes might be similarly loaded into AGO1 by a passenger-strand cleavage-assisted mechanism, such that 2b-mediated inhibition of AGO1 slicer activity would have also resulted in reduced turnover of miRNA*. However, this idea was recently challenged following the *in-vitro* reconstruction of cleavage-competent RISC from a plant cell-free system (Iki *et al*, 2010): while removal of an siRNA passenger strand indeed required AGO1 slicing activity in this system, it was

clearly dispensable for turnover of miRNA* strands (Iki *et al*, 2010).

What could, therefore, underpin the miRNA* stabilization observed in VSR-transgenic plants? One hypothesis is that as yet-undefined factors that degrade miRNA* strands are positively regulated by the miRNA pathway itself. VSR-mediated inhibition of the miRNA pathway would thus promote down-regulation of this hypothetical factor, directly or indirectly. In line with this hypothesis, *hyl1* and *ago1* mutants were recently shown to accumulate more miRNA* strands than wild-type control plants (Eamens *et al*, 2009). Alternatively, this hypothetical miRNA*-degrading factor may require a close association with the RISC complex in order to access and process efficiently the released miRNA* strand upon its unwinding; VSRs could potentially prevent this association. Independently of the underlying reasons, our results clearly show that stabilization of miRNA* strands cannot be reliably used as an indicator of sRNA sequestration by VSR *in vivo*.

Distinct cellular pools of AGO1 in Arabidopsis

siRNA and miRNA pathways in *Drosophila* proceed through clearly separate Dicer and AGO proteins: Dcr-2-AGO2 for the former and Dcr-1-AGO1 for the latter. siRNA loading into fly AGO2 is facilitated by the RISC-loading complex (RLC) containing Dicer-2 and its partner R2D2 (Liu *et al*, 2003; Pham *et al*, 2004; Tomari *et al*, 2004a). These proteins form a heterodimer that loads the strand with the less-stably paired 5' end into RISC (Tomari *et al*, 2004b). A similar mechanism has also been proposed for loading fly miRNAs into AGO1-RISC, but the factor(s) facilitating this process remain unknown (Okamura *et al*, 2004). Additional studies in *Drosophila* and *C. elegans* further indicate that, beside a facilitating role for the RLC, sRNA loading into specific AGO complexes is primarily determined by structural features of precursor duplexes, independently of biogenesis mechanisms (Forstemann *et al*, 2007; Steiner *et al*, 2007; Tomari *et al*, 2007). Hence, in flies, mismatches found in miRNA duplexes are necessary for their incorporation into AGO1, whereas perfectly matched siRNA duplexes bind AGO2. Moreover, including mismatches in an siRNA duplex is sufficient to promote its incorporation into AGO1.

The Arabidopsis siRNA and miRNA pathways are also initiated by distinct DCL proteins (DCL4 for the former; DCL1 for the latter) but, unlike in flies, both pathways converge through the common recruitment of the same, major effector: AGO1. Deep-sequencing analyses indicate that most Arabidopsis miRNAs follow asymmetry rules (Jones-Rhoades *et al*, 2006), strongly suggesting the existence of a plant RLC that might, therefore, account for both siRNA and miRNA loading into AGO1. Moreover, plant miRNAs and siRNAs share the same modes of action irrespectively of position, number and pairing degree with their targets (Brodersen *et al*, 2008). Consequently, both classes of sRNAs are usually assumed to load into the same AGO1 complex to carry out slicing and/or translational inhibition of target RNAs. The findings made in the present study suggest, however, that this simple view requires refinements in order to accommodate the existence of two distinct plant AGO1 pools, leading to a scheme not dissimilar, in essence, to the AGO1-AGO2 dichotomy found in flies.

Use of the SUC:SUL and CHS-RNAi transgenic systems have revealed that P38, P15 and HcPro, unlike P19, inhibit siRNA but not miRNA loading into AGO1 (Figures 3 and 5). The simplest explanation for this unexpected result is that at least two distinct AGO1 pools coexist in the cell, respectively, loaded with siRNAs and miRNAs. The existence of two distinct pools of miRNA- and siRNA-loaded AGO1 in Arabidopsis is fully consistent with recent observations that the miRNA pathway limits AGO1 availability during siRNA-mediated PTGS in transgenic Arabidopsis (Martinez de Alba *et al*, 2011). The authors proposed that during transgene PTGS initiation phase, transgene siRNAs and endogenous miRNA compete to bind AGO1, suggesting, indeed, that AGO1 might be partitioned into two distinct pools, each binding preferentially to one specific sRNA class. We further propose that these two pools might differ by their subcellular localization and/or specific AGO1-interacting cofactors to form molecularly distinct siRISCs and miRISCs. The first obvious candidates for such cofactors are the distinct DCLs that generate each class of mature sRNAs: specific DCL1-AGO1 or DCL4-AGO1 interactions may define the siRISC versus the miRISC in an RLC-like manner. According to this idea, licensing of a specific sRNA into a particular AGO1 complex might be directly coupled to its biogenesis. Accordingly, P38, P15 and HcPro inhibited AGO1 loading of miR822, which, unlike most plant miRNAs, is processed by DCL4 rather than by DCL1 (Rajagopalan *et al*, 2006; Supplementary Figure S2). Thus, unlike in animals, biogenesis mechanisms rather than structural features of their precursors might determine the 'loading fate' of sRNAs into specific AGO1 complexes. A second potential cofactor that may differentiate the siRISC from the miRISC is the recently identified ethylene-inducible transcription factor RAV2/EDF2 (Endres *et al*, 2010). Indeed, RAV2 interacts with TuMV Hc-Pro *in planta* is required for Hc-Pro- and P38-mediated block of primary siRNA activity, and is dispensable for Hc-Pro-mediated inhibition of miRNA activity (Endres *et al*, 2010). These attributes make RAV2 a good candidate as a specific cofactor of the proposed AGO1-siRISC. In this scenario, direct P38-AGO1 interactions may dissociate RAV2 from the siRISC while Hc-Pro could sequester RAV2 and thereby deplete it from the siRISC (Endres *et al*, 2010).

We anticipate that most plant VSRs are programmed primarily to impact the siRNA-specific AGO1 pool through direct (e.g., P38) or indirect interactions. On this premise, we further suggest that the molecular effects of P19, which has been used over the years as a 'standard' in understanding VSR action, are, in fact, less stereotypical than anticipated. Indeed, the specific binding, by P19, of both miRNA and siRNA duplexes *in vivo* and its interference with the loading of AGO1 with miRNAs seems to be unique among the several VSRs tested in this study. In fact, primary targeting, by VSRs, of the siRNA-specific AGO1 pool would explain why developmental anomalies linked to miRNA dysfunction (Jay *et al*, 2011) are usually only apparent in transgenic plants that express VSRs to high levels, while their low-to-moderate expression is already sufficient to suppress RNAi. A similar phenomenon may also explain why successful transgenic expression of plant VSRs in *Drosophila* was not accompanied by developmental phenotypes regardless of expression levels (Berry *et al*, 2009). Presumably the plant VSRs were interfering primarily with the fly siRNA pathway

and the associated AGO2, leaving the AGO1-miRNA pathway unaffected.

Materials and methods

Plant material

The CHS-RNAi and corresponding P15, P19 and P1-HcPro-expressing plants (ecotype Col-0) have been previously described (Dunoyer *et al*, 2004). Binary vector constructs encoding P15ΔN6 and P38 were mobilized into *Agrobacterium* strain GV3101 and transformed into CHS-RNAi (Wesley *et al*, 2001) or into SUC:SUL (Dunoyer *et al*, 2005) *Arabidopsis* by the floral dip method, according to Bechtold and Pelletier (1998).

RNA analysis

Total RNA was extracted from *Arabidopsis* tissues with Tri-Reagent (Sigma, St Louis, MO) according to manufacturer's instructions. RNA gel blot analysis of high and low molecular weight RNA was on 10 and 30 µg of total RNA, respectively, and was as described previously (Dunoyer *et al*, 2004). Ethidium bromide staining of total RNA before transfer was used to confirm equal loading. Radiolabelled probes for detection of the SUL or CHS siRNAs were made by random priming reactions in the presence of α -³²P-dCTP (Amersham). The template used was a 400-bp-long (for SUL) and 256 bp-long (for CHS) PCR products amplified from *Arabidopsis* cDNA. DNA oligonucleotides complementary to miRNAs and tasiRNAs were end labelled with γ -³²P-ATP using T4 PNK (New England Biolabs, Beverly, MA).

Protein analysis

Total proteins were extracted from 4-week-old seedlings or from flower buds of *Arabidopsis* and were resolved on SDS-PAGE. After electroblotting onto Immobilon-P membrane (Millipore), protein gel blot analysis was carried out using the appropriate antiserum. The specificity of the various antibodies used in this study has been verified by western blot analysis on protein extracts from wild type and the corresponding T-DNA mutant plants and is presented in Supplementary Figure S4.

Immunoprecipitation

The peptide used to raise rabbit polyclonal antibodies against AGO1 was described previously (Qi *et al*, 2005). Antibodies were affinity purified before use. For immunoprecipitation, 1 g of 3- to 4-week-old seedlings or 0.2 g of flower buds was ground in liquid nitrogen, and homogenized in 3 ml/g of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP-40) containing 1 tablet/10 ml of protease inhibitor cocktail (Roche) for 1 h at 4°C. Cell debris was removed by centrifugation at 12 000 g at 4°C for 30 min. Extracts were precleared by incubation with Protein A-agarose (Roche) at 4°C for 1 h. Precleared extracts were then incubated with affinity-purified AGO1 antibodies and protein A-agarose overnight at 4°C. Immunoprecipitates were washed three times (20 min each) in extraction buffer. Aliquots of the immunoprecipitates and supernatant were collected before the first wash to assess by western blot analysis the efficiency of immunoprecipitation. For RNA analysis, immune complexes were subjected to Tri-Reagent extraction (Sigma).

Real-time RT-qPCR

In all, 2 µg of total RNA samples was reverse transcribed into polydT cDNAs using SuperScript III reverse transcriptase

(Invitrogen). The cDNA was quantified using SYBR[®] Green qPCR kit (EUROGENTECH) and gene-specific primers on a BIORAD iCycler apparatus according to manufacturer's recommendations. PCR was carried out in 96-well optical reaction plates heated to 95°C for 10 min to activate hot start Taq DNA polymerase, followed by 42 cycles of denaturation at 95°C for 15 s and annealing extension at 62°C for 30 s. miRNA and tasiRNA target quantifications were performed with specific primer pairs (primer sequences available upon request) surrounding the siRNA cleavage site and results were normalized with ACTIN2 (At3g18780) as previously described (Allen *et al*, 2005). For each cDNA synthesis, quantifications were made in triplicate. For each quantification, a melt curve was performed at the end of the amplification experiment by steps of 1°C from 53 to 95°C in order to ensure that quantification was not due to primers self-amplification. Error bars represent standard deviation from three PCRs performed each time in two independent experiments.

In-situ hybridization

RNA *in-situ* hybridizations were performed on transversal sections of SUC-SUL or SUC-P38/SUC-SUL young leaves. TCV P38 cDNA was cloned into the pGEM[®]-T Easy Vector (Promega). Antisense P38 Digoxigenin (DIG)-labelled riboprobe was *in vitro* synthesized using PCR-amplified fragments from pGEM-P38 plasmid with T7 and SP6 primers using T7 RNA polymerase (Promega) and DIG RNA Labelling Mix (Roche) according to manufacturer's instructions. Leaves were fixed in FAA (3.2% formaldehyde, 5% acetic acid, 50% ethanol) twice by vacuum infiltration for 30 min at room temperature and overnight incubation at 4°C prior to dehydration in ethanol and embedding in Paraplast Plus[®] (McCormick Scientific). Sections of 10 µm thickness were generated using a Leica microtome and mounted on Polysine[™] (Menzel-Glaser) slides. *In-situ* hybridization was performed as described by Jeff Long (<http://www.its.caltech.edu/~plantlab/protocols/insitu.htm>), with the following modifications: no SSC wash was performed prior to proteinase K treatment, triethanolamine and RNase treatments were omitted. Slides were hybridized with ~45 ng of DIG-labelled probe overnight at 52°C. Immunological detection of the DIG-labelled probes was performed using a DIG Nucleic Acid Detection Kit (Roche) according to manufacturer's instructions. Following detection, slides were rinsed in Tris-EDTA and mounted with Mounting Medium (Sigma) and a coverslip.

Supplementary data

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

Acknowledgements

This work was supported by research grants from Agence National pour la Recherche (ANR-08-JCJC-0063 and ANR-10-LABX-36) to GS and PD. We thank T Lagrange (DCL3) and A Simon (P38) for antibodies; R Wagner's team for plant care; and members of the Voinnet laboratory for critical reading of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Adenot X, Elmayan T, Lauressergues D, Boutet S, Bouche N, Gascioli V, Vaucheret H (2006) DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7. *Curr Biol* **16**: 927–932
- Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**: 207–221
- Azevedo J, Garcia D, Pontier D, Ohnesorge S, Yu A, Garcia S, Braun L, Bergdoll M, Hakimi MA, Lagrange T, Voinnet O (2010) Argonaute

- quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes Dev* **24**: 904–915
- Baulcombe D (2004) RNA silencing in plants. *Nature* **431**: 356–363
- 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
- Bechtold N, Pelletier G (1998) In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. *Methods Mol Biol* **82**: 259–266

- Berry B, Deddouche S, Kirschner D, Imler JL, Antoniewski C (2009) Viral suppressors of RNA silencing hinder exogenous and endogenous small RNA pathways in *Drosophila*. *PLoS One* **4**: e5866
- Blevins T, Rajeswaran R, Shivaprasad PV, Beknazariants D, Si-Ammour A, Park HS, Vazquez F, Robertson D, Meins Jr. F, Hohn T, Pooggin MM (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res* **34**: 6233–6246
- Bortolamiol D, Pazhouhandeh M, Marrocco K, Genschik P, Ziegler-Graff V (2007) The polerovirus 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
- Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. *Trends Genet* **22**: 268–280
- Chapman EJ, Carrington JC (2007) Specialization and evolution of endogenous small RNA pathways. *Nat Rev Genet* **8**: 884–896
- Chapman EJ, Prokhnovsky AI, Gopinath K, Dolja VV, Carrington JC (2004) Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev* **18**: 1179–1186
- 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) Polerovirus protein P0 prevents the assembly of small RNA-containing RISC complexes and leads to degradation of ARGONAUTE1. *Plant J* **62**: 463–472
- Cuperus JT, Montgomery TA, Fahlgren N, Burke RT, Townsend T, Sullivan CM, Carrington JC (2010) Identification of MIR390a precursor processing-defective mutants in Arabidopsis by direct genome sequencing. *Proc Natl Acad Sci USA* **107**: 466–471
- Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* **313**: 68–71
- Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. *Cell* **130**: 413–426
- Duan CG, Fang YY, Zhou BJ, Zhao JH, Hou WN, Zhu H, Ding SW, Guo HS (2012) Suppression of Arabidopsis ARGONAUTE1-mediated slicing, transgene-induced RNA silencing, and DNA methylation by distinct domains of the cucumber mosaic virus 2b protein. *Plant Cell* **24**: 259–274
- Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010a) An endogenous, systemic RNAi pathway in plants. *EMBO J* **29**: 1699–1712
- Dunoyer P, Himber C, Ruiz-Ferrer V, Alioua A, Voinnet O (2007) Intra- and intercellular RNA interference in Arabidopsis thaliana requires components of the microRNA and heterochromatic silencing pathways. *Nat Genet* **39**: 848–856
- Dunoyer P, Himber C, Voinnet O (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nat Genet* **37**: 1356–1360
- 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
- Dunoyer P, Pfeffer S, Fritsch C, Hemmer O, Voinnet O, Richards KE (2002) Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. *Plant J* **29**: 555–567
- Dunoyer P, Schott G, Himber C, Meyer D, Takeda A, Carrington JC, Voinnet O (2010b) Small RNA duplexes function as mobile silencing signals between plant cells. *Science* **328**: 912–916
- Eamens AL, Smith NA, Curtin SJ, Wang MB, Waterhouse PM (2009) The Arabidopsis thaliana double-stranded RNA binding protein DRB1 directs guide strand selection from microRNA duplexes. *RNA* **15**: 2219–2235
- Endres MW, Gregory BD, Gao Z, Foreman AW, Mlotshwa S, Ge X, Pruss GJ, Ecker JR, Bowman LH, Vance V (2010) Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing. *PLoS Pathog* **6**: e1000729
- Felippes FF, Weigel D (2009) Triggering the formation of tasiRNAs in Arabidopsis thaliana: the role of microRNA miR173. *EMBO Rep* **10**: 264–270
- Forstemann K, Horwich MD, Wee L, Tomari Y, Zamore PD (2007) Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell* **130**: 287–297
- Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, Bremel KJ, Carrington JC (2010) Arabidopsis RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during Turnip Mosaic Virus infection. *Plant Cell* **22**: 481–496
- Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**: 950–952
- Harvey JJ, Lewsey MG, Patel K, Westwood J, Heimstadt S, Carr JP, Baulcombe DC (2011) An antiviral defense role of AGO2 in plants. *PLoS One* **6**: e14639
- Hemmes H, Lakatos L, Goldbach R, Burgyan J, Prins M (2007) The NS3 protein of Rice hoja blanca tenuivirus suppresses RNA silencing in plant and insect hosts by efficiently binding both siRNAs and miRNAs. *RNA* **13**: 1079–1089
- Iki T, Yoshikawa M, Nishikiori M, Jaudal MC, Matsumoto-Yokoyama E, Mitsuhara I, Meshi T, Ishikawa M (2010) In vitro assembly of plant RNA-induced silencing complexes facilitated by molecular chaperone HSP90. *Mol Cell* **39**: 282–291
- Jay F, Wang Y, Yu A, Taconnat L, Pelletier S, Colot V, Renou JP, Voinnet O (2011) Misregulation of AUXIN RESPONSE FACTOR 8 underlies the developmental abnormalities caused by three distinct viral silencing suppressors in Arabidopsis. *PLoS Pathog* **7**: e1002035
- Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. *Annu Rev Plant Biol* **57**: 19–53
- Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA, Carrington JC (2007) Genome-wide profiling and analysis of Arabidopsis siRNAs. *PLoS Biol* **5**: e57
- 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
- Lindom M, Jacobsen A, Nygaard S, Mang Y, Krogh A (2007) Intragenomic matching reveals a huge potential for miRNA-mediated regulation in plants. *PLoS Comput Biol* **3**: e238
- Liu Q, Rand TA, Kalidas S, Du F, Kim HE, Smith DP, Wang X (2003) R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science* **301**: 1921–1925
- Martinez de Alba AE, Jauvion V, Mallory AC, Bouteiller N, Vaucheret H (2011) The miRNA pathway limits AGO1 availability during siRNA-mediated PTGS defense against exogenous RNA. *Nucleic Acids Res* **39**: 9339–9344
- Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**: 607–620
- Merai 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
- Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC (2008a) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* **133**: 128–141
- Montgomery TA, Yoo SJ, Fahlgren N, Gilbert SD, Howell MD, Sullivan CM, Alexander A, Nguyen G, Allen E, Ahn JH, Carrington JC (2008b) Inaugural article: AGO1-miR173 complex initiates phased siRNA formation in plants. *Proc Natl Acad Sci USA* **105**: 20055–20062

- Morel J-B, Gordon 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
- Nakazawa Y, Hiraguri A, Moriyama H, Fukuhara T (2007) The dsRNA-binding protein DRB4 interacts with the Dicer-like protein DCL4 in vivo and functions in the trans-acting siRNA pathway. *Plant Mol Biol* **63**: 777–785
- Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev* **18**: 1655–1666
- Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. *Genes Dev* **18**: 2368–2379
- Pham JW, Pellino JL, Lee YS, Carthew RW, Sontheimer EJ (2004) A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. *Cell* **117**: 83–94
- Qi Y, Denli AM, Hannon GJ (2005) Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell* **19**: 421–428
- Qu F, Ye X, Morris JT (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
- Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana. *Genes Dev* **20**: 3407–3425
- Rand TA, Petersen S, Du F, Wang X (2005) Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**: 621–629
- Steiner FA, Hoogstrate SW, Okihara KL, Thijssen KL, Ketting RF, Plasterk RH, Sijen T (2007) Structural features of small RNA precursors determine Argonaute loading in Caenorhabditis elegans. *Nat Struct Mol Biol* **14**: 927–933
- Takeda A, Iwasaki S, Watanabe T, Utsumi M, Watanabe Y (2008) The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. *Plant Cell Physiol* **49**: 493–500
- Tomari Y, Du T, Haley B, Schwarz DS, Bennett R, Cook HA, Koppetsch BS, Theurkauf WE, Zamore PD (2004a) RISC assembly defects in the Drosophila RNAi mutant armitage. *Cell* **116**: 831–841
- Tomari Y, Du T, Zamore PD (2007) Sorting of Drosophila small silencing RNAs. *Cell* **130**: 299–308
- Tomari Y, Matranga C, Haley B, Martinez N, Zamore PD (2004b) A protein sensor for siRNA asymmetry. *Science* **306**: 1377–1380
- Vargason JM, Szittyi G, Burgyan J, Tanaka Hall TM (2003) Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**: 799–811
- Vaucheret H (2005) MicroRNA-dependent trans-acting siRNA production. *Sci STKE* **2005**: pe43
- Vazquez F (2006) Arabidopsis endogenous small RNAs: highways and byways. *Trends Plant Sci* **11**: 460–468
- Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert JL, Bartel DP, Crete P (2004) Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Mol Cell* **16**: 69–79
- Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell* **136**: 669–687
- Wang XB, Jovel J, Udamporn P, Wang Y, Wu Q, Li WX, Gasciolli V, Vaucheret H, Ding SW (2011) The 21-nucleotide, but not 22-nucleotide, viral secondary small interfering RNAs direct potent antiviral defense by two cooperative argonautes in Arabidopsis thaliana. *Plant Cell* **23**: 1625–1638
- Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse P (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* **27**: 581–590
- Xie Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **102**: 12984–12989
- Xie Z, Kasschau KD, Carrington JC (2003) Negative feedback regulation of Dicer-Like1 in Arabidopsis by microRNA-guided mRNA degradation. *Curr Biol* **13**: 784–789
- Ye K, Malinina L, Patel DJ (2003) Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* **426**: 874–878
- Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev* **19**: 2164–2175
- 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