Short communication

Inhibition of *in vivo* Slicer activity of Argonaute protein 1 by the viral 2b protein independent of its dsRNA-binding functionLEI FENG^{1,2,†}, CHENG-GUO DUAN^{1,†,‡} AND HUI-SHAN GUO^{1,*}¹State Key Laboratory of Plant Genomics and National Center for Plant Gene Research, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China²University of Chinese Academy of Sciences, Beijing 100049, China**SUMMARY**

The 2b protein of *Cucumber mosaic virus* (CMV) has several unique properties, such as targeting to the nucleolus and interaction with both Argonautes (AGOs) and short and long double-stranded RNA (dsRNA). We have recently uncoupled the domain requirements for dsRNA binding and nucleolar targeting from the physical interactions with AGO proteins, and have found that the direct 2b–AGO interaction is sufficient to inhibit the *in vitro* AGO1 Slicer function independent of the other biochemical properties of 2b. Because the AGO binding activity of 2b is not required for its suppressor function *in vivo*, this raises the question of whether *in vivo* 2b–AGO interaction is possible to inhibit the *in vivo* AGO Slicer function. In this study, by taking advantage of a technology for the production of artificial *trans*-acting small interfering RNA (tasiRNA), a process uniquely associated with AGO1-mediated *in vivo* Slicer activity, we demonstrated that the expression of the 2b protein *in planta* interfered with the production of tasiRNA. Through further detailed analysis with deletion mutants of 2b proteins, we found that the inhibition of *in vivo* AGO1 Slicer function required the nucleolar localization signal (NoLS), in addition to the AGO-binding domain, of the 2b protein. Our finding demonstrates that *in vivo* 2b–AGO1 interaction is sufficient to inhibit AGO1 Slicer function independent of the dsRNA-binding activity of the 2b protein.

INTRODUCTION

RNA silencing is a conserved pathway that results in the blockage of gene expression in eukaryotic organisms. In plants, post-transcriptional gene silencing (PTGS) occurs in the cytoplasm and utilizes small RNAs, including small interfering RNAs and microRNAs (siRNAs and miRNAs), which specifically recognize and base pair with targeted RNA sequences (Baulcombe, 2004,

2005; Voinnet, 2008). Small RNAs are processed from long double-stranded RNAs (dsRNAs) or hairpin precursor RNAs by RNase III-type enzymes called Dicer-like ribonucleases (DCL) proteins (e.g. DCL1 and DCL2–4 which generate miRNA and siRNA, respectively). Small RNAs bind to the Argonaute (AGO)-containing RNA-induced silencing complex (RISC) which cleaves targeted RNA. AGO proteins are often named Slicer proteins because they cleave target transcripts at the duplex formed with the guide siRNAs or miRNAs (Baumberger and Baulcombe, 2005; Tolia and Joshua-Tor, 2007; Vaucheret, 2008). In plants, RNA silencing involves an amplification process that requires RNA-directed RNA polymerases (RDRs) for secondary siRNA production for persistent silencing (Baulcombe, 2007). The biogenesis of *trans*-acting siRNAs (tasiRNAs), one type of plant endogenous siRNA, represents a case of an RDR-dependent secondary siRNA-producing pathway. For example, tasiRNAs formed from primary *TAS1* transcript are initially targeted and sliced by the AGO1–miR173 complex at a 5' proximal site. The cleaved RNA products are then transcribed by RDR6, leading to dsRNAs that are sequentially processed by DCL4 to yield phased tasiRNAs (Gascioli *et al.*, 2005; Montgomery *et al.*, 2008a, b).

In addition to the regulation of development, RNA silencing also functions as an antiviral mechanism in plants and invertebrates. To invade their host effectively, viruses have evolved a vast array of proteins, called viral suppressors of RNA silencing (VSR), which target different stages of the silencing process (Agius *et al.*, 2012; Ding and Voinnet, 2007). More than 35 VSR families have been identified so far. The sequestration of siRNAs by siRNA-binding suppressors is a common way to inhibit RISC assembly (Csorba *et al.*, 2009; Ding and Voinnet, 2007). Other VSRs inhibit RNA silencing through protein–protein interaction. Poliovirus P0 protein has been suggested to target the PAZ domain of AGO1 to direct its degradation (Baumberger *et al.*, 2007; Pazhouhandeh *et al.*, 2006). *Turnip crinkle virus* P38 and *Sweet potato mild mottle virus* P1 proteins have been suggested to bind AGO1 through the WG/GW motifs, and inhibit both existing and *de novo*-formed AGO1-containing RISCs (Azevedo *et al.*, 2010; Giner *et al.*, 2010). The 111-residue 2b protein encoded by *Cucumber mosaic virus* (CMV) is unique among the known plant and animal VSRs because it interacts directly with both siRNA and AGO proteins and is targeted to the nucleolus (Gonzalez *et al.*, 2010; Goto *et al.*,

*Correspondence: Email: guohs@im.ac.cn

†These authors contributed equally to this work.

‡Present address: Department of Horticulture & Landscape Architecture, Purdue University, West Lafayette, IN 47906, USA.

2007; Hamera *et al.*, 2012; Lucy *et al.*, 2000; Zhang *et al.*, 2006). Recently, we have identified regions of the 2b protein that determine these biochemical properties and their contributions to suppression activity (Duan *et al.*, 2012). We identified a functional nucleolar localization signal (NoLS), which includes two closely spaced nuclear localization signals (NLS1 and NLS2), located within the 61-amino-acid N-terminal dsRNA-binding domain (dsRBD) (Fig. 1A), which exhibits high affinity for short (siRNAs) and long dsRNA. Physical interaction of 2b with AGOs requires an

essential 33-residue region C terminal to dsRBD (Fig. 1A), and such an interaction is sufficient to inhibit *in vitro* AGO1 Slicer activity independent of its dsRNA-binding activities (Duan *et al.*, 2012). However, this AGO binding or suppression of AGO Slicer activity *in vitro* is dispensable for the suppression of RNA silencing by the 2b protein (Duan *et al.*, 2012). Taking into account that all assays of 2b protein inhibition of AGO Slicer activities were examined *in vitro* (Duan *et al.*, 2012; Hamera *et al.*, 2012; Zhang *et al.*, 2006), this raises the question of whether or not *in vivo* 2b-AGO

Fig. 1 Inhibition of *trans*-acting small interfering RNA (tasiRNA) production by 2b and its derivative mutants. (A) Amino acid sequence and functional domains of 2b protein. Two arginine-rich putative nuclear localization signals (NLSs) (blue letters), double-stranded RNA (dsRNA)-binding domain (dsRBD) and essential Argonaute (AGO)-binding domain are labelled. The C-terminal deletion mutant, 2b(1-61), and two N-terminal deletion mutants, 2b(38-111) and 2b(13-111), and three NLS substitution mutants (blue letters to red letters), 2bN1m, 2bN2m and 2bN1,2m, are shown. (B, C) Effects of 2b, NLSs and deletion mutants on tasiRNA synthesis. 2b, 2b mutant construct (as indicated on top of gels), 35S-P19 and control vector (V) were co-infiltrated with 35S-MiR173 and 35S-TAS1C constructs into *Nicotiana benthamiana* (B) or RDR6i (C) leaves. The expression of 2b, the 2b mutants, TAS1C mRNA, tasiRNAs and miR173 was detected as described in Fig. 2B.

interaction affects AGO Slicer function, as AGO binding is not required for the suppression activity of 2b *in vivo*. To address this question, in this study, we analysed, with a series of deletion mutants of 2b proteins using a technology associated with AGO1-mediated slicing *in planta*, and provided evidence that the *in vivo* 2b-AGO1 interaction endows the 2b protein with the ability to inhibit AGO1 Slicer function independent of its dsRNA-binding activity.

RESULTS AND DISCUSSION

To evaluate the *in vivo* suppression activity of the 2b protein, we took advantage of a technology for the production of artificial tasiRNAs in *Nicotiana benthamiana*. The processes of generation of tasiRNAs include miR173-guided AGO1-dependent cleavage of *TAS1C*-derived transcripts, which are then synthesized to dsRNA by RDR6 and subsequently diced by DCL4 (Fig. 2A) (Montgomery *et al.*, 2008b; Ying *et al.*, 2010). In this assay, 35S-MIR173, which produces active miR173, and 35S-TAS1C were co-infiltrated into *N. benthamiana* leaves, so that *TAS1C* transcripts from 35S-TAS1C were targets of AGO1-dependent cleavage by miR173. As expected, the production of tasiRNAs was detected in the leaves co-infiltrated with 35S-TAS1C and 35S-MIR173 (Fig. 2B, lane 3), but not in the leaves infiltrated solely with 35S-TAS1C (Fig. 2B,

lane 2). Also, as expected, the production of tasiRNAs was undetectable following the co-infiltration of 35S-MIR173 and 35S-TAS1C in RDR6i plants (Fig. 2B, lane 5), in which Nb-RDR6 was silenced (Schwach *et al.*, 2005; Ying *et al.*, 2010). RDR6 silencing in RDR6i plants stabilized the 5' cleavage products of *TAS1C* transcripts by miR173, which were detected as a discrete band (Fig. 2B, lane 5), rather than as a smear in wild-type plants (Fig. 2B, lane 3). 5' Rapid amplification of cDNA ends (RACE) products from the co-infiltration in RDR6i plants were cloned and sequenced, confirming that miR173-mediated cleavage of the *TAS1C* transcripts was at the complementary region of the miR173/*TAS1C* pairing area (Fig. 2C).

We then examined whether the expression of the 2b protein would interfere with the production of tasiRNA in this system. On co-infiltration of 35S-2b/35S-TAS1C/35S-MIR173, we found that the expression of 2b inhibited the production of tasiRNAs and resulted in an increased accumulation of *TAS1C* transcript in the infiltrated leaves (Fig. 2B, lane 4). These results indicated that 2b suppressed the production of tasiRNAs in the infiltrated leaves. Notably, the 5' cleavage products of *TAS1C* transcripts were undetectable in the leaves of RDR6i plants that were co-infiltrated with 35S-2b (Fig. 2B, lane 6). To determine whether or not the failure to detect the 5' cleavage products of *TAS1C* transcripts was caused by the expression of 2b, which reversed the silencing of *RDR6*

Fig. 2 Detection of inhibitory effects of 2b on Argonaute protein 1 (AGO1)-mediated cleavage of miR173-targeted *TAS1C* transcripts and production of RNA-directed RNA polymerase 6 (RDR6)-mediated *trans*-acting small interfering RNA (tasiRNA) *in vivo*. (A) A sketch map of miR173-AGO1-mediated cleavage and RDR6-dependent tasiRNA synthesis. DCL4, Dicer-like ribonuclease 4; RISC, RNA-induced silencing complex. (B) 2b protein-inhibited production of tasiRNA. 35S-2b and control vector (V) were co-infiltrated with 35S-MIR173 and 35S-TAS1C constructs into wild-type *Nicotiana benthamiana* (Nb) and RDR6i leaves. The expression of 2b, *TAS1C* mRNA and 5' cleavage products was detected using 2b- and *TAS1C* 5'-specific DNA probes (labelled in A). miR173 and tasiRNA accumulation in infiltrated leaves was detected with ³²P-labelled specific DNA oligonucleotide probes. Methylene blue-stained rRNA is shown as the mRNA loading control. (C) Analysis of miR173-mediated cleavage of the *TAS1C* transcript. The arrows indicate the cleavage sites of the *TAS1C* RNA detected by 5' rapid amplification of cDNA ends (5' RACE). The proportion of cloned 5' RACE products corresponding to cleavage at a site is shown above the arrows. (D) 2b protein did not reverse double-stranded RNA (dsRNA)-induced *RDR6* silencing. RNA gel analysis of samples collected from Nb and RDR6i plants infiltrated with vector (V) or 35S-2b. ³²P-labelled *RDR6* DNA probes were used. The silencing trigger (*RDR6* RNAi transcript) detected in leaves infiltrated with 35S-2b is indicated.

mRNA in RDR6i plants, the accumulation of *RDR6* mRNA was examined in 35S-2b infiltrated leaves. Silencing of *RDR6* mRNA in RDR6i plants was not reversed by the expression of 2b, although the accumulation of the silencing trigger (*RDR6i* transcript) was detected in the 35S-2b infiltrated leaves (Fig. 2D, lane 4). These results were consistent with a previous report that CMV 2b did not reverse silencing in silenced tissue (Brigneti *et al.*, 1998).

The processes for the synthesis of tasiRNAs, for example AGO1-mediated slicing of the *TAS1C* transcripts and RDR6-mediated synthesis of dsRNAs, might be affected by the 2b protein. To separate the two inhibition processes, we first used 35S-2b(1-61), which carries a deletion mutant of 2b that retains the N-terminal 61 amino acids of dsRBD, but lacks the AGO-binding domain (Duan *et al.*, 2012), to examine the possibility of whether the 2b dsRNA-binding activity was sufficient for the inhibition of the production of tasiRNAs in this system. We co-infiltrated 35S-*TAS1C*/35S-MIR173 with 35S-2b(1-61). Co-infiltration with empty vector or with the tombusviral suppressor P19, which can bind perfectly matched double-stranded 21-nucleotide siRNA duplex, but not imperfectly matched miRNA/miRNA* duplexes or long dsRNA (Duan *et al.*, 2012), was used in parallel assays. A slight effect on tasiRNA production was observed in the co-infiltration with 35S-P19, relative to co-infiltration with the vector control (Fig. 3, lanes 1 and 3); however, the 5' cleavage products of *TAS1C* transcripts were detected in the leaves of RDR6i plants co-infiltrated with 35S-P19 (Fig. 3, lane 5). This result indicated that P19 did not inhibit the process of miR173-AGO1-mediated cleavage of *TAS1C* transcripts, a result consistent with the observation that P19 is incapable of binding to single-stranded

miRNA or miRNA/miRNA* duplexes (Duan *et al.*, 2012). When co-infiltrated with 35S-2b(1-61), inhibition of tasiRNA production was observed, which was similar to the co-infiltration of 35S-2b in the infiltrated leaves of wild-type *N. benthamiana* (Fig. 3, lanes 4 and 2). However, the 5' cleavage products of *TAS1C* transcripts were detected in the leaves of RDR6i plants co-infiltrated with 35S-2b(1-61), but not with 35S-2b (Fig. 3, lanes 5–7). This result indicated that 2b(1-61) did not inhibit the process of miR173-AGO1-mediated cleavage of *TAS1C* transcripts, but could bind the dsRNA derived from the cleaved products to suppress the production of tasiRNAs. This is consistent with the observation that 2b(1-61) is unable to physically interact with AGO proteins in the absence of the AGO-binding domain (Duan *et al.*, 2012). Taking into account that no 5' cleavage products of *TAS1C* transcripts were detected by the expression of the full-length 2b protein in RDR6i plants (Fig. 2, lane 6 and Fig. 3 lane 6), our results indicated that 2b suppressed the production of tasiRNAs in the infiltrated leaves, including substantial inhibition of the AGO1-mediated slicing of the *TAS1C* transcripts, in addition to its ability to bind to long dsRNA.

The finding that the inhibition of tasiRNA production by the 2b protein included the prevention of miR173-guided AGO1-mediated cleavage of *TAS1C* transcripts allowed us to examine whether 2b was capable of inhibiting *in vivo* AGO1 Slicer activity in the absence of the long dsRNA-binding function. We used five 2b mutants that lacked long dsRNA-binding activity, but retained the ability to physically interact with AGOs and inhibit AGO1 Slicer activity *in vitro* (Duan *et al.*, 2012) for the co-infiltration assay. These 2b mutants included: 2b(13-111), in which the N-terminal 13 amino acids were deleted, but the intact NoLS was retained; 2b(38-111), in which the N-terminal 38 amino acids, including the intact NoLS, were deleted (Fig. 1A); and 2bN1m, 2bN2m and 2bN1,2m, in which NLS1, NLS2 and both NLSs in the NoLS region were mutated, respectively (Fig. 1A). In a previous study, we noted that the accumulation levels of these 2b mutants were lower than that of the wild-type 2b in the infiltration assay; hence, co-infiltration with P19 was used to restore their accumulation (Duan *et al.*, 2012). The above co-infiltration assays showed that P19 had little effect on miR173-guided AGO1-mediated cleavage of its precursor *TAS1C* transcripts (Fig. 3, lane 5). Therefore, co-infiltration with P19 was used to restore the accumulation of these 2b mutants in this assay (Fig. 1B). The inhibition activity of mutant 2b(13-111), which retains the intact NoLS (Fig. 1A), was very effective compared with the wild-type 2b (Fig. 1B, lanes 3 and 2). Partial inhibition activity was observed in leaves co-infiltrated with 35S-2bN2m, which contains NLS1 (Fig. 1B, lane 6), whereas little effect on tasiRNA production was observed in leaves co-infiltrated with 35S-2b(38-111), 35S-2bN1m or 35S-2bN1,2m (Fig. 1B, lanes 4, 5 and 7). These results indicated that not all of the 2b mutants were able to inhibit AGO1 Slicer activity *in vivo*, although they all retained the essential AGO-binding domain and

Fig. 3 Inhibition of *trans*-acting small interfering RNA (tasiRNA) production by 2b, 2b(1-61) and P19. 35S-2b, 35S-2b(1-61), 35S-P19 and control vector (V) were co-infiltrated with 35S-MIR173 and 35S-*TAS1C* constructs into *Nicotiana benthamiana* (Nb) or RDR6i leaves. The expression of 2b and 2b(1-61) RNA (2b/mut), *TAS1C* mRNA and 5' cleavage product was detected as described in Fig. 2B.

were capable of suppressing AGO1 Slicer function *in vitro* (Duan *et al.*, 2012).

To further confirm that efficient inhibition of tasiRNA production by 2b(13-111) resulted from the inhibition of AGO1-mediated cleavage of *TAS1C* transcripts, we co-infiltrated 35S-TAS1C/35S-MIR173/35S-2b(13-111) with 35S-P19 in RDR6i plants. Co-infiltration with 35S-2b and 35S-2b(1-61) in the presence of 35S-P19 was also carried out in parallel assays. Although the 5' cleavage products of *TAS1C* transcripts were detected in RDR6i leaves co-infiltrated with 35S-2b(1-61) and/or 35S-P19 (Fig. 1C, lanes 3 and 2), no 5' cleavage products were detected with the expression of 2b and 2b(13-111) in RDR6i plants (Fig. 1C, lanes 4 and 5). Our results clearly indicated that suppression of the production of tasiRNAs by 2b(13-111) resulted from the inhibition of AGO1-mediated slicing of the *TAS1C* transcripts, rather than from the action of binding to long dsRNA, as observed with 2b(1-61).

In previous studies, we found that the 2b-green fluorescent protein (GFP) fusion protein was localized in the nucleolus (Duan *et al.*, 2012). The removal of both NLSs in 2bN1,2m or the deletion of the N-terminal 38 amino acids, including both NLSs of 2b, in 2b(38-111) led to the cytoplasmic localization of the resulting 2b fusion protein. The removal of NLS1 in 2bN1m specifically excluded the protein from the nucleolus, whereas the removal of NLS2 in 2bN2m showed a weak nucleolar presence (Duan *et al.*, 2012). The three 2b mutants, 2b(38-111), 2bN1m and 2bN1,2m, which failed to target to the nucleolus (Duan *et al.*, 2012), also failed to inhibit AGO1-mediated slicing of the *TAS1C* transcripts (Fig. 1B), although they contained the essential AGO-binding domain (Fig. 1A); in contrast, the weak nucleolar localization of 2bN2m exhibited partial inhibitory activity of AGO1 slicing of the *TAS1C* transcript (Fig. 1B). These results were consistent with our previous observations that nucleolar targeting of 2b is required for co-localization of 2b and AGO proteins (Duan *et al.*, 2012). These findings indicated that, in addition to containing the AGO-binding domain, a functional NoLS is also required for the 2b protein to achieve the inhibition of *in vivo* AGO1 Slicer activity. Our previous results showed that 2b(13-111), which retains the intact NoLS, but lacks long dsRNA-binding activity, is co-localized with AGO proteins in the nucleus (Duan *et al.*, 2012). In this study, we showed that 2b(13-111) retained almost full inhibitory ability of *in vivo* AGO1 Slicer activity (Fig. 1B). Together, these results reveal that 2b mutants which retain the domains essential for AGO-binding activity and nucleolar targeting, without dsRNA-binding activity, are capable of inhibiting *in vivo* AGO1 Slicer activity.

The finding that 2b(1-61) effectively suppressed tasiRNA production is in agreement with our previous findings that 2b mutants, which retain the activity to bind dsRNAs, but without the AGO1-binding domain, exhibit efficient suppression of PTGS, and these results suggest that direct 2b-AGO interaction is not essential for 2b suppression of PTGS and RNA-directed DNA methylation (RdDM) (Duan *et al.*, 2012). With no requirement of binding to

AGOs for the silencing suppression activities of 2b *in vivo*, *in vivo* 2b-AGO interaction might not inhibit *in vivo* AGO1 Slicer function. In this study, we have provided evidence that the 2b protein is able to inhibit *in vivo* AGO1 Slicer activity in the absence of its dsRBD. These findings further support the idea that 2b may suppress PTGS and RdDM *in vivo* by the binding and sequestration of siRNA and the long dsRNAs precursor in a process that can be facilitated by its *in vivo* interactions with and inhibition of the Slicer function of AGO proteins. However, we cannot rule out the possibility that the *in vivo* 2b-AGO interaction may represent another layer of host anti-suppression of RNA silencing of the 2b protein, even though binding to the 2b protein may somewhat sacrifice the *in vivo* activities of AGOs.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

RDR6i transgenic *N. benthamiana* has been described previously (Schwach *et al.*, 2005). Transgenic and wild-type *N. benthamiana* plants were grown in a glasshouse at 25 °C with 16-h light/8-h dark cycles.

Plasmid constructs

pCAMBIA-MIR173 (35S-MIR173) and pCAMBIA-TAS1C (35S-TAS1C) were constructed by polymerase chain reaction (PCR) amplification of the Arabidopsis *MIR173* precursor sequence with the primers MIR173 5' (GGTCTAGAATAATTAGCAAGTAATAAGG; italic letters for *Xba*I) and MIR173 3' (CCGAGCTCATCTGTTATACAACCAATCC; italic letters for *Sac*I), and of the *TAS1C* sequence with the primers TAS1C 5' (GGTCTAGAAAACCTAAACCTAAACGGCTAAG; italic letters for *Xba*I) and TAS1C 3' (CCGAGCTCAACAAACAAATGCTGTTTCATC; italic letters for *Sac*I). The PCR fragments were cloned into pGEM-Teasy vector (Promega, Madison, WI, USA) to obtain pGEM-MIR173 and pGEM-TAS1C, respectively. The *Xba*I-*Sac*I fragments containing MIR173 or TAS1C were inserted into pCAMBIA1300-221 digested with *Xba*I and *Sac*I to obtain pCAMBIA-MIR173 and pCAMBIA-TAS1C, respectively.

pCAMBIA-SD2b (35S-2b) and its derivative mutant constructs, 35S-2b(1-61), 35S-2b(13-111), 35S-2b(38-111), 35S-2bN1m, 35S-2bN2m and 35S-2bN1,2m, and 35S-P19, used in *in vivo* Slicer activity assays, were constructed as described previously (Duan *et al.*, 2012).

Transient expression for *in vivo* inhibition of Slicer activity assays

Five-week-old *N. benthamiana* leaves were infiltrated with a culture of *Agrobacterium* EHA105 carrying the indicated DNA plasmid or empty vector pBI121-GUS to an optical density at 600 nm (OD₆₀₀) of 0.5 for infiltration. For the co-infiltration of 35S-TAS1C and 35S-MIR173, cultures of *Agrobacterium* carrying 35S-TAS1C and 35S-MIR173 were adjusted to OD = 0.5, and an equal volume was mixed and used. For *in vivo* inhibition of Slicer activity assays, 35S-MIR173 and 35S-TAS1C were co-infiltrated with empty vector, 35S-2b, 35S-2b(1-61), 35S-2b(13-111), 35S-2b(38-

111), 35S-SD2bN1m, 35S-SD2bN2m and 35S-SD2bN1,2m, and 35S-P19, into *N. benthamiana* (Nb) and Nb-RDR6i plants. The concentration of each culture was adjusted to OD₆₀₀ = 1.2, 1.2 and 0.6.

RNA extraction and RNA blot analysis

Plant total RNA used for RNA gel blot was extracted by the hot-phenol method as described previously (Fernandez *et al.*, 1997). For the detection of *TAS1C*, *2b* and *RDR6*, gene-specific DNA fragments were labelled with [α -³²P]-dCTP using the Rediprime II System. For small RNA blot, total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and small RNA was obtained via lithium chloride and ethanol precipitation from total RNA. For the detection of tasiRNAs, 30 µg of small RNA were separated on 17% polyacrylamide–8 M urea gels. The tasiRNA-specific probe, TTCTAAGTCCAACATAGCGTACCTGTCTC, and miR173-specific probe, GTGATTCTCTCTGCAAGCGAA, were labelled with [γ -³²P]-ATP (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) using polynucleotide kinase (NEB).

ACKNOWLEDGEMENTS

This work was supported by grants from the Natural Science Foundation of China (31030009 and 31123007) and the Ministry of Science and Technology of China (2011CB100703).

REFERENCES

- Agius, C., Eamens, A.L., Millar, A.A., Watson, J.M. and Wang, M.B. (2012) RNA silencing and antiviral defense in plants. *Methods Mol. Biol.* **894**, 17–38.
- Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M.A., Lagrange, T. and 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.
- Baulcombe, D. (2005) RNA silencing. *Trends Biochem. Sci.* **30**, 290–293.
- Baulcombe, D.C. (2007) Molecular biology. Amplified silencing. *Science*, **315**, 199–200.
- Baumberger, N. and Baulcombe, D.C. (2005) Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc. Natl. Acad. Sci. USA*, **102**, 11 928–11 933.
- Baumberger, N., Tsai, C.H., Lie, M., Havecker, E. and Baulcombe, D.C. (2007) The Ploverovirus silencing suppressor P0 targets ARGONAUTE proteins for degradation. *Curr. Biol.* **17**, 1609–1614.
- Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W. and Baulcombe, D.C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J* **17**, 6739–6746.
- Csorba, T., Pantaleo, V. and Burgyn, J. (2009) RNA silencing: an antiviral mechanism. *Adv. Virus Res.* **75**, 35–71.
- Ding, S.W. and Voinnet, O. (2007) Antiviral immunity directed by small RNAs. *Cell*, **130**, 413–426.
- Duan, C.G., Fang, Y.Y., Zhou, B.J., Zhao, J.H., Hou, W.N., Zhu, H., Ding, S.W. and Guo, H.S. (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.
- Fernandez, A., Guo, H.S., Saenz, P., Simon-Buela, L., Gomez de Cedron, M. and Garcia, J.A. (1997) The motif V of plum pox potyvirus CI RNA helicase is involved in NTP hydrolysis and is essential for virus RNA replication. *Nucleic Acids Res.* **25**, 4474–4480.
- Gascioli, V., Mallory, A.C., Bartel, D.P. and Vaucheret, H. (2005) Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr. Biol.* **15**, 1494–1500.
- Giner, A., Lakatos, L., Garcia-Chapa, M., Lopez-Moya, J.J. and Burgyn, J. (2010) Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *Plos Pathog.* **6**, e1000996.
- Gonzalez, I., Martinez, L., Rakitina, D.V., Lewsey, M.G., Atencio, F.A., Llave, C., Kalinina, N.O., Carr, J.P., Palukaitis, P. and Canto, T. (2010) Cucumber mosaic virus 2b protein subcellular targets and interactions: their significance to RNA silencing suppressor activity. *Mol. Plant–Microbe Interact.* **23**, 294–303.
- Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T. and Masuta, C. (2007) Characterization of silencing suppressor 2b of cucumber mosaic virus based on examination of its small RNA-binding abilities. *Plant Cell Physiol.* **48**, 1050–1060.
- Hamera, S., Song, X., Su, L., Chen, X. and Fang, R. (2012) Cucumber Mosaic Virus suppressor 2b binds to AGO4-related small RNAs and impairs AGO4 activities. *Plant J.* **69**, 104–115.
- Lucy, A.P., Guo, H.S., Li, W.X. and Ding, S.W. (2000) Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J.* **19**, 1672–1680.
- Montgomery, T.A., Howell, M.D., Cuperus, J.T., Li, D., Hansen, J.E., Alexander, A.L., Chapman, E.J., Fahlgren, N., Allen, E. and Carrington, J. (2008a) Specificity of ARGONAUTE7–miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell*, **133**, 128–141.
- Montgomery, T.A., Yoo, S.J., Fahlgren, N., Gilbert, S.D., Howell, M.D., Sullivan, C.M., Alexander, A., Nguyen, G., Allen, E., Ahn, J.H. and Carrington, J.C. (2008b) AGO1–miR173 complex initiates phased siRNA formation in plants. *Proc. Natl. Acad. Sci. USA*, **105**, 20 055–20 062.
- Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K.E., Genschik, P. and 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.
- Schwach, F., Vaistij, F.E., Jones, L. and Baulcombe, D.C. (2005) An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol.* **138**, 1842–1852.
- Tolia, N.H. and Joshua-Tor, L. (2007) Slicer and the argonautes. *Nat. Chem. Biol.* **3**, 36–43.
- Vaucheret, H. (2008) Plant ARGONAUTES. *Trends Plant Sci.* **13**, 350–358.
- Voinnet, O. (2008) Post-transcriptional RNA silencing in plant–microbe interactions: a touch of robustness and versatility. *Curr. Opin. Plant Biol.* **11**, 464–470.
- Ying, X.B., Dong, L., Zhu, H., Duan, C.G., Du, Q.S., Lv, D.Q., Fang, Y.Y., Garcia, J.A., Fang, R.X. and Guo, H.S. (2010) RNA-dependent RNA polymerase 1 from *Nicotiana tabacum* suppresses RNA silencing and enhances viral infection in *Nicotiana benthamiana*. *Plant Cell*, **22**, 1358–1372.
- Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J. and Chua, N.H. (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* **20**, 3255–3268.