Cytoplasmic Arabidopsis AGO7 accumulates in membrane-associated siRNA bodies and is required for ta-siRNA biogenesis

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Formation of trans-acting small interfering RNAs (ta-siRNAs) from the *TAS3* precursor is triggered by the AGO7/miR390 complex, which primes *TAS3* for conversion into double-stranded RNA by the RNA-dependent RNA polymerase RDR6 and SGS3. These ta-siRNAs control several aspects of plant development. The mechanism routing AGO7-cleaved *TAS3* precursor to RDR6/SGS3 and its subcellular organization are unknown. We show that AGO7 accumulates together with SGS3 and RDR6 in cytoplasmic siRNA bodies that are distinct from P-bodies. siRNA bodies colocalize with a membrane-associated viral protein and become positive for stress-granule markers upon stress-induced translational repression, this suggests that siRNA bodies are membrane-associated sites of accumulation of mRNA stalled during translation. AGO7 congregates with miR390 and SGS3 in membranes and its targeting to the nucleus prevents its accumulation in siRNA bodies and ta-siRNA formation. AGO7 is therefore required in the cytoplasm and membranous siRNA bodies for *TAS3* processing, revealing a hitherto unknown role for membrane-associated ribonucleoparticles in ta-siRNA biogenesis and AGO action in plants.

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Introduction

Trans-acting small interfering RNAs (ta-siRNAs) are plant-specific endogenous small regulatory RNAs that are produced from non-coding *TAS* genes and guide the cleavage of specific mRNA targets. ta-siRNA biogenesis requires an initial micro RNA (miRNA)-mediated cut and the conversion of one of the two cleavage products into a double-stranded RNA (dsRNA) by

the cellular RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and the SGS3 RNA-binding protein (Peragine *et al.*, 2004; Vazquez *et al.*, 2004; Allen *et al.*, 2005). The resulting dsRNA is then processed into 21 nt ta-siRNAs by DICER-LIKE 4 (DCL4) (Dunoyer *et al.*, 2005; Gascioli *et al.*, 2005; Hiraguri *et al.*, 2005; Xie *et al.*, 2005; Yoshikawa *et al.*, 2005). ta-siRNAs have the ability to act non-cell autonomously (Chitwood *et al.*, 2009; Schwab *et al.*, 2009) and regulate the abundance of a diverse set of genes (Allen *et al.*, 2005; Williams *et al.*, 2005; Yoshikawa *et al.*, 2005; Fahlgren *et al.*, 2006; Hunter *et al.*, 2006; Rajagopalan *et al.*, 2006; Howell *et al.*, 2007). An essential and yet enigmatic feature of ta-siRNA biogenesis is the specific routing of the miRNA-cleaved *TAS* precursors to RDR, different from most miRNA-guided mRNA cleavage products, which enter the 5' → 3' or 3' → 5' RNA degradation pathways because they lack either a 5' Cap or a 3' polyA tail.

ta-siRNAs produced from the *TAS3* precursors target mRNA coding for the *AUXIN RESPONSE FACTOR 3* (*ARF3*) and *ARF4*, which encode transcription factors mediating the effects of the phytohormone auxin (Williams *et al.*, 2005; Fahlgren *et al.*, 2006; Hunter *et al.*, 2006). By way of this regulation, the *TAS3* pathway controls the developmental timing of the transition between juvenile and adult leaves, contributes to the specification of leaves ad/abaxial polarity and controls the growth of lateral roots (Adenot *et al.*, 2006; Fahlgren *et al.*, 2006; Garcia *et al.*, 2006; Hunter *et al.*, 2006; Marin *et al.*, 2010; Yoon *et al.*, 2010). This pathway is one of the most ancient small RNA pathway and is conserved both mechanistically and functionally across all land plants (Axtell *et al.*, 2006; Talmor-Neiman *et al.*, 2006; Nogueira *et al.*, 2007, 2009; Douglas *et al.*, 2010). The *TAS3* precursor is transcribed by RNA polymerase II as a long primary RNA and recognized by a complex of miR390 and AGO7 (Allen *et al.*, 2005; Axtell *et al.*, 2006; Montgomery *et al.*, 2008). The *TAS3* mRNA contains two sites complementary to miR390. Whereas the 3' site is competent for cleavage (Allen *et al.*, 2005; Axtell *et al.*, 2006), the second 5' site exhibits mismatches at positions 9–11, which prevent RNA cleavage (Axtell *et al.*, 2006). Although miR390-mediated cleavage at the 3' site does not exclusively rely on AGO7, ta-siRNA biogenesis requires AGO7 at the 5' site (Montgomery *et al.*, 2008; Cuperus *et al.*, 2010a). This suggests that the miR390/AGO7 complex directs *TAS3* precursors to the siRNA pathway.

Priming of other *TAS* precursors by a 22-nt miRNA associated with AGO1, but not by a 21-nt miRNA associated with AGO1, was recently shown to be instrumental for the routing of 3' cleavage products to RDR6 (Chen *et al.*, 2010; Cuperus *et al.*, 2010b). However, another mechanism must be at play for *TAS3* because miR390 is 21 nt long and routes a 5' cleavage product to RDR6. As the miR390–AGO7 complex is specific to *TAS3* processing, a careful analysis of its role might uncover an essential step in ta-siRNA biogenesis and further clarify the relationships between miRNA and siRNA pathways.

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In plant and animal cells, certain AGO proteins have been shown to accumulate in cytoplasmic foci called P-bodies, sites at which numerous RNA decay enzymes are concentrated (Liu *et al*, 2005; Pillai *et al*, 2005; Sen and Blau, 2005; Zhang *et al*, 2006; Pomeranz *et al*, 2010). In most eukaryotes, a second set of ribonucleoprotein granules exists, the so-called stress granules. They represent cytoplasmic aggregates of non-translated mRNPs and have been suggested to serve as sorting sites, where mRNAs are targeted for storage, reinitiation or degradation by transfer to P-bodies (Anderson and Kedersha, 2009; Erickson and Lykke-Andersen, 2011). Their formation is triggered by the massive disassembly of polyosomes as it occurs under stress conditions such as heat, oxidative or UV stress. Whereas P-bodies contain components of the mRNA decay machinery, stress granules contain components of the translation initiation machinery.

Very little is known about the subcellular compartments involved in ta-siRNA biogenesis. Whereas miRNAs are generated in the nucleus and exported into the cytoplasm, the key determinants of siRNA biogenesis, RDR6 and SGS3, accumulate in the cytoplasm (Glick *et al*, 2008; Elmayan *et al*, 2009; Kumakura *et al*, 2009). This raises the question of whether the miR390-AGO7-mediated priming of the *TAS3* precursor occurs in the nucleus or in the cytoplasm.

Here, we show that a functional GFP-tagged version of AGO7 accumulates together with SGS3 and RDR6 in cytoplasmic foci. These foci, herein referred to as siRNA bodies, are distinct from P-bodies. Upon stress-induced translational repression, siRNA bodies become positive for stress-granules markers, suggesting that these bodies may accumulate stalled mRNAs. We also show that AGO7 colocalizes with the membrane-associated protein VP6 of the Tobacco Etch Virus (TEV), suggesting that AGO7 might interact with membranes. Furthermore, AGO7, together with miR390 and SGS3 associates tightly with the microsomal fraction, suggesting a link between AGO function and membrane-associated ribonucleoproteins. By modifying the subcellular localization of AGO7, we demonstrate that accumulation of AGO7 in the cytoplasm and the siRNA bodies is necessary for *TAS3* processing.

Results

AGO7 localizes in cytoplasmic foci

To determine the subcellular localization of AGO7, we generated an N-terminal translational fusion with GFP and transiently expressed the fusion protein from the constitutive p35S promoter in tobacco leaves and Arabidopsis protoplasts. In both systems, GFP-AGO7 signal was detected diffusely in the cytoplasm and in discrete foci (Figure 1A; Supplementary Figure S1, arrowheads). In tobacco cells, these foci were highly mobile and never observed in the nucleus. We also determined the localization of the GFP-AGO7 fusion after transformation of the *ago7-1* mutant. Expression of the p35S:GFP-AGO7 construct complemented the typical *zippy* phenotype caused by loss of AGO7 function (narrow leaves; Hunter *et al*, 2003), indicating the functionality of the GFP-AGO7 fusion protein. However, because the T-DNA of the *ago7-1* mutant causes partial transcriptional silencing of p35S-driven transgenes (Daxinger *et al*, 2008), the p35S:GFP-AGO7 construct was partially silenced, and the restoration of *TAS3*-derived ta-siRNA synthesis was below

that of wild-type plants (Figure 1B and C). Moreover, we could not directly detect the fluorescence emitted by GFP-AGO7, even if a protein of the expected molecular weight was detected by western blot using an anti-GFP antibody (Figure 1D). We thus revealed GFP-AGO7 localization by immunofluorescence using an anti-GFP antibody on root meristem cells. We observed GFP-AGO7 signal in discrete cytoplasmic foci (Figure 1E and F); in the same conditions, no signal was detected in non-transgenic plants. These foci were of similar aspect as the ones observed in tobacco and Arabidopsis protoplast cells.

To rule out the possibility that the foci observed for the GFP-AGO7 fusion expressed under the control of the p35S promoter are due to mislocalization induced by overexpression of the protein, we examined the subcellular localization of AGO7 fused to another tag (HA), and expressed at physiological levels from the native *AGO7* promoter. The *pAGO7:HA-AGO7* construct, herein abbreviated HA-AGO7, reverted both macroscopically and molecularly the *ago7* (*zip-1*) mutant phenotype (Montgomery *et al*, 2008). In these plants, immunolocalization of AGO7 using an anti-HA antibody revealed accumulation of AGO7 in cytoplasmic granules comparable to the ones detected with the *p35S:GFP-AGO7* (Figure 1G). These results indicate that AGO7 accumulates in cytoplasmic foci and that these foci very likely represent the physiological localization of AGO7 in plant cells.

AGO7 colocalizes with RDR6 and SGS3 in cytoplasmic siRNA bodies

To identify the nature of the cytoplasmic foci in which AGO7 accumulates, we performed colocalization experiments using protein markers of specific subcellular compartments in tobacco leaves. We first tested whether the AGO7 foci are P-bodies by co-expression of p35S:RFP-AGO7 with p35S:YFP-DCP1. As previously described (Xu *et al*, 2006; Iwasaki *et al*, 2007; Xu and Chua, 2009), the YFP-DCP1 signal was exclusively cytoplasmic and consisted of small discrete foci, yet none of these foci colocalized with RFP-AGO7 ($n = 24$ DCP1-positive bodies) (Figure 2A).

During ta-siRNA biogenesis, AGO7 acts in conjunction with RDR6 and SGS3, two proteins that accumulate in specific cytoplasmic bodies (Glick *et al*, 2008; Elmayan *et al*, 2009; Kumakura *et al*, 2009), hereafter called siRNA bodies. To test whether AGO7 colocalizes with RDR6 and/or SGS3, we expressed GFP-AGO7 together with RFP-SGS3 or RFP-RDR6. GFP-AGO7 foci colocalized with both RFP-SGS3 and RFP-RDR6 foci (~90% colocalization, $n = 50$ foci) (Figure 2B and C).

Plant viruses induce a significant reorganization of the endomembrane system and induce the formation of replication bodies that differ from P-bodies and partially overlap with membrane-linked hot spots for initiation of viral replication complex formation (Laliberté and Sanfaçon, 2010). The TEV 6 kDa protein (VP6) is a membrane-associated protein required for viral replication (Restrepo-Hartwig and Carrington, 1994) and formation of viral replication complexes from the endoplasmic reticulum (ER) (Schaad *et al*, 1997). In tobacco cells, VP6 accumulates in cytoplasmic foci marking an intermediary compartment between the ER and the cis-Golgi (Schaad *et al*, 1997; Lerich *et al*, 2011). Because AGO7, RDR6 and SGS3 have been implicated in plant defense

Figure 1 AGO7 accumulates in cytoplasmic foci. (A) Confocal section of a *Nicotiana tabacum* leaves expressing GFP-AGO7. Cytoplasmic AGO7 foci are indicated by arrowheads. The cytoplasm appears as a thin peripheral layer, while the vacuole (v) fills most of the cell volume. Inset: GFP-AGO7 is not detected in the nucleus (n). (B) Morphology of 3-week-old *ago7-1* or *p35S::GFP:AGO7/ago7-1* Arabidopsis plants. *ago7-1* plants display the typical zippy phenotype (narrow pointed leaves). (C) RNA gel blot analysis of 15 µg of total RNA from 7-day-old wild-type (WT), *ago7-1* mutant or four independent *p35S::GFP:AGO7/ago7-1* plants. The blot was probed with DNA complementary to ta-siARFs. U6 snRNA served as a loading control. (D) Western blot analysis of 7-day-old *p35S::GFP:AGO7/ago7-1* plants. The blot was probed with an anti-GFP. The arrowhead indicates the position of the GFP-AGO7 band (127 kDa). (E) Indirect immunofluorescence detection of GFP-AGO7 in epidermal cells of the root meristem in 7-day-old *p35S::GFP:AGO7/ago7-1* plants using an anti-GFP antibody. In meristem cells, the vacuole is not yet formed. The inset represents a non-transgenic plant processed and imaged in the same conditions. (F) Same as in (E) but counterstained with DAPI to mark nuclei (n). (G) Indirect immunofluorescence detection of HA-AGO7 in epidermal cells of the root meristem in 7-day-old *pAGO7::HA:AGO7/zip-1* plants using an anti-HA antibody. The lower left inset represents a non-transgenic plant processed and imaged in the same conditions, whereas the upper right one is a higher magnification view. Scale bars: (A, F and G inset) 5 µm; (E, G) 25 µm. Figure source data can be found in Supplementary data.

against virus (Mourrain *et al*, 2000; Qu *et al*, 2008), we investigated whether siRNAs bodies and VP6 colocalized. We expressed GFP-AGO7 together with VP6-CFP and observed that the AGO7 signal partially overlapped with VP6 (~60% colocalization, $n = 25$ foci) (Figure 2D). We then looked at the relationships between siRNA bodies, the ER and cis-Golgi. For this, we co-expressed in tobacco leaves GFP-AGO7 and either a resident ER marker (*p35S::ER-mCherry*; Nelson *et al*, 2007) or the cis-Golgi marker *p35S::MAN1-RFP* (Lerich *et al*, 2011). Although no overlap between GFP-AGO7 and the ER could be observed (Figure 2E), we noticed that MAN1 and AGO7 signals tend to be adjacent to one another (Figure 2F), reminiscent of the relative disposition of VP6 and MAN1 (Lerich *et al*, 2011). Taken together, these results indicate that AGO7, RDR6 and SGS3 accumulate in cytoplasmic siRNA bodies.

Cytoplasmic siRNA bodies form stress granules under heat-shock conditions

In plant cells under stress conditions, the pre-mRNA splicing factor UBP1 and the eukaryotic translation initiation factor 4E

(eIF4E) are markers of stress granules (Weber *et al*, 2008). Under normal growth conditions, UBP1 accumulates in the nucleus, whereas eIF4E is detected diffusely in the cytoplasm. When co-expressed with either SGS3 or AGO7 and observed under normal growth conditions, neither UBP1 nor eIF4E accumulated in the siRNA bodies (Figure 3A and B; Supplementary Figure S2). However, under conditions triggering the formation of stress granules (42°C for 5 min), siRNA bodies (as marked by SGS3 and AGO7) increased dramatically in number and showed perfect colocalization with UBP1 and eIF4E (Figure 3C and D; Supplementary Figure S2). This result shows that under stress conditions siRNA bodies become positive for markers of stress granules.

siRNA bodies associate with a membrane-containing fraction

To further characterize the intracellular distribution of siRNA bodies and their association with membranes, we performed subcellular fractionations. HA-AGO7 seedlings were homogenized, large cellular debris and intact cells were removed

Figure 2 AGO7 accumulates in membrane-linked cytoplasmic siRNA bodies. (A–F) Confocal sections of *Nicotiana tabacum* leaves expressing the indicated fluorescent fusion proteins. For each condition, the signals of the individual fluorophores are presented in the first two images whereas the signals are merged in the last image. The arrows indicate the AGO7/siRNA bodies. The dotted squares depict the location of the higher magnification insets. Scale bars: 5 μ m.

by a first centrifugation and the supernatant was further centrifuged in presence of detergent (0.5% Triton) to generate a nuclei pellet (N) and a post-nuclear supernatant (PNS). The partition of AGO7 in these two fractions was tested by western blot using a monoclonal anti-HA antibody. AGO7 accumulated exclusively in the PNS (Figure 4A). The PNS association was confirmed in the *p35S::GFP-AGO7/ago7-1* background (using an anti-GFP antibody) and in wild-type Arabidopsis plants using an anti-AGO7 antibody (Supplementary Figure S3). Therefore under physiological conditions, AGO7 accumulates in the cytoplasm.

In animal cells, select AGO proteins (human AGO2, *Drosophila* AGO1) associate with membranous compartments (Gibbings *et al*, 2009; Lee *et al*, 2009). To further investigate if AGO7 associates with membrane-containing compartments, we centrifuged homogenized HA-AGO7 seedlings to generate a low speed soluble fraction supernatant (LSS) and a pellet containing dense organelles (nuclei, plastids and mitochondria). The LSS was then centrifuged at 100 000 g, resulting in a microsomal pellet (P100) and a supernatant containing soluble proteins (S100). The bulk of AGO7 was retrieved in the microsomal pellet (P100; Figure 4B). In the same conditions, the soluble protein fructose 6 bisphosphatase remained mainly in the soluble fraction (S100; Figure 4B).

To analyse whether other markers of the siRNA bodies also accumulated in the microsomal fraction, we analysed the presence of SGS3 in the P100 fraction of wild-type Arabidopsis seedlings by western blot. Anti-SGS3 signal was detected in the P100 (Supplementary Figure S4), confirming that at physiological levels, AGO7 and SGS3 reside in subcellular fractions with similar properties.

The microsomal fraction consists of dense particles and membranous material (membranes and vesicles). To test if AGO7 is associated with a membranous compartment, we

treated the P100 fraction with various detergents (1% Triton X-100 and 1% deoxycholate) before recentrifugation for 30 min at 100 000 g. Whereas treatment of the microsomal fraction with the non-ionic detergent Triton X-100 resolubilized partially AGO7, the ionic detergent deoxycholate solubilized most of AGO7 (Figure 4C). This resolubilization of AGO7 upon detergent treatment further reinforces an association with membranes. We then tested whether AGO7 was located within vesicles. For this, the P100 fraction was treated, in absence of any detergent, with proteinase K and then recentrifuged for 30 min at 100 000 g. The AGO7 signal was lost upon proteinase K treatment, indicating that AGO7 did not localize in a compartment protected from the protease action such as the lumen of vesicles (Figure 4D).

To further characterize the nature of the membranous compartment associated with AGO7, we resolved the resolubilized P100 fraction over a continuous sucrose gradient. HA-AGO7 signal peaked in fractions of a density of 40–45% sucrose (w/v) (Figure 4E, fractions 7–8); the resident ER protein calnexin was detected in the same fractions (Figure 4E).

Taken together, these results indicate that AGO7 resides on the cytoplasmic side of subcellular compartments with membrane-like properties.

AGO7 and miR390 form an exclusive complex (Montgomery *et al*, 2008). To investigate where miR390 resides, we performed subcellular fractionation. We extracted small RNA from nuclear and post-nuclear fractions as well as from LSS, S100 and P100 fractions, and analysed by northern blot the presence of miR390 in these fractions. We could detect miR390 in both the nuclear and post-nuclear fractions (Figure 5A) and the P100 fraction (Figure 5B). In addition, miR390 partition in the post-nuclear and P100 fractions was not dependent on AGO7, as miR390 accumulated to a similar

Figure 3 siRNA bodies colocalize with stress granules after heat shock. (A–D) Confocal sections of *Nicotiana tabacum* leaves expressing the indicated fluorescent fusion proteins. For each condition, the green and red signals of the same plane are presented in two first images whereas the signals are merged in the last image. The arrows indicate the AGO7/siRNA bodies. The dotted squares depict the location of the higher magnification insets. Images in (C) and (D) were taken tangentially to the cytoplasm surface; n: nucleus. Scale bars: 5 μ m.

degree in *zip-1* mutant and wild-type backgrounds (Figure 5A; Supplementary Figure S5).

Nuclear localization impairs AGO7 function in ta-siRNA biogenesis

To test the functional relevance of AGO7 accumulation in cytoplasmic siRNA bodies, we engineered versions of AGO7 with a modified subcellular localization. For this, we added to AGO7 a nuclear localization signal (NLS: PKKKRKV) to force its localization to the nucleus. The NLS sequence was added at the N-terminal end of AGO7, a position where sequences such as trimerized HA tags or GFP can be added without compromising the function of the protein (Figure 6A). The effect of the NLS motif on AGO7 subcellular localization was tested by transiently expressing the NLS-AGO7 allele as a GFP fusion in tobacco leaves. Addition of the NLS motif led to

strong decrease in the cytoplasmic localization with no remaining signal in the siRNA bodies, and the concomitant appearance of NLS-AGO7 signal in the nucleus (Figure 6B).

We then tested the ability of this variant protein to functionally replace AGO7 in triggering formation of ta-siRNA when expressed at physiological levels. For this, we took advantage of a synthetic *TAS3* gene in which ta-siARFs were replaced by ta-siRNA targeting the *PDS* involved in β -carotenoid formation (Montgomery *et al*, 2008). In a wild-type background, the *syntasi-PDS* plants leaves are photobleached around the veins, whereas in absence of AGO7 function, in the *syntasi-PDS/zip-1* background, the leaves remain green (Montgomery *et al*, 2008). We expressed in *syntasi-PDS/zip-1* plants HA-tagged versions of AGO7 and NLS-HA-AGO7 from the native AGO7 promoter, and scored for bleached plants among the primary transformants

Figure 4 AGO7 copurifies with a membranous fraction. (A–E) Western blot analysis of 7-day-old *pAGO7:HA-AGO7/zip-1* plants. Unless indicated otherwise, the blots were probed with an anti-HA antibody (HA-AGO7: 113 kDa). (A) Analysis of 75 µg of protein from the total, nuclear (N) and post-nuclear (PNS) fractions. (B) Analysis of 75 µg of protein from the low speed supernatant (LSS), soluble fraction (S100) and microsomal fraction (P100). The blot was probed with anti-HA (upper panel) and an antibody against the cytosolic fructose-1,6-bisphosphatase (cFBPase, lower panel). (C) Resubilization of the microsomal fraction by treatment with detergents (DOC: deoxycholate). In all, 75 µg of protein was analysed. (D) Analysis of 75 µg of protein from the resuspended microsomal fraction treated (+) or not (–) by the proteinase K (Prot. K) before recentrifugation at 100 000 g. (E) Analysis of the different fractions after separation of the resuspended microsomal fraction on a continuous (20–60% w/v) sucrose gradient. The blot was probed with anti-HA (upper panel) and an antibody against calnexin (lower panel). Figure source data can be found in Supplementary data.

Figure 5 miR390 co-fractionates with AGO7. RNA gel blot analysis of 15 µg (A) or 30 µg (B) of RNA from 7-day-old plants. (A) Analysis of miR390 accumulation in the nuclear and post-nuclear fractions of wild-type and *zip-1* plants. (B) Analysis of miR390 accumulation in low speed supernatant (LSS), soluble fraction (S100) and microsomal fraction (P100) of wild-type plants. Ethidium bromide (EtBr) staining served as a loading control. Figure source data can be found in Supplementary data.

retrieved. About 50% of *pAGO7:HA-AGO7* primary transformants displayed a bleached phenotype (Figure 6C) whereas none could be retrieved for the *pAGO7:NLS-HA-AGO7* transformation (Figure 6C). This was not due to a change in protein stability, as we could detect the full-length protein by western blot (Supplementary Figure S6). This result suggested that accumulation of AGO7 in the nucleus impaired its function. To ascertain this result and to rule out that addition of the NLS inactivated AGO7 activity as a whole, we added a nuclear export signal (NES: ALPPLERLTL) to either HA-AGO7 or NLS-HA-AGO7. When expressed from the AGO7 promoter and transformed into *syntasi-PDS/zip-1* plants both the *NES-HA-AGO7* and (*NES + NLS*)-*HA-AGO7*

alleles restored the bleaching phenotype (Figure 6C). This suggested that accumulation of AGO7 in cytoplasmic siRNA bodies is crucial for its function and targeting to these bodies only occurs outside of the nucleus.

This result was further confirmed by expressing the HA-AGO7, NLS-HA-AGO7 and (NES + NLS)-HA-AGO7 from the AGO7 promoter in Arabidopsis *zip-1* mutants and scoring for reversion of the *zippy* phenotype both macroscopically (elongated leaves, precocious appearance of adult leaves) and molecularly (formation of ta-siRNAs). Similarly to the results obtained in the *syntasi-PDS/zip-1* line, NLS-HA-AGO7 failed to complement the *zip-1* phenotype, whereas HA-AGO7 and (NES + NLS)-HA-AGO7 did (Figure 6E). The *zip-1* mutant plants accumulate much reduced levels of ta-siARFs (Figure 6D). Accumulation of ta-siARFs was restored upon expression of NES-HA-AGO7 and (NES + NLS)-HA-AGO7 but not of NLS-HA-AGO7 (Figure 6D). However, the ta-siARFs levels were reduced for NES-HA-AGO7 and (NES + NLS)-HA-AGO7 compared with expression of HA-AGO7, indicating that although able to revert the *zip-1* phenotype, these alleles yield proteins less efficient than AGO7 (Figure 6D). Taken together, these results strongly argue that the presence of AGO7 in cytoplasmic siRNA bodies is essential for the formation of ta-siRNAs.

Discussion

In this paper, we show that in plant cells, cytoplasmic AGO7 accumulates in membrane-associated SGS3/RDR6-containing siRNA bodies. We establish the functional relevance of this localization by showing that nuclear relocalization of AGO7 impairs ta-siRNA biogenesis.

Figure 6 Nuclear localization impairs AGO7 function in ta-siRNA biogenesis. **(A)** Schematic representation of AGO7 and the NLS and NES alleles used. **(B)** Confocal sections of *Nicotiana tabacum* leaves expressing the indicated fluorescent fusion proteins. On the upper panels are views of the cytoplasm and on the lower panels of the nuclei (n). The arrowheads indicate the AGO7 siRNA bodies. Scale bars: 5 µm. **(C)** Morphology of 3-week-old *syntasi-PDS/zip-1* Arabidopsis plants expressing the indicated construct from the AGO7 promoter. Bleached plants harbour white sectors radiating from the veins. **(D)** RNA gel blot analysis of 30 µg of total RNA from *zip-1* inflorescence expressing the indicated construct from the AGO7 promoter. Each lane represents independent primary transformants (three for each allele). The blot was probed with DNA complementary to ta-siARFs. U6 snRNA served as a loading control and numbers indicate normalized intensities. **(E)** Morphology of 3-week-old wild-type and *zip-1* Arabidopsis plants expressing the indicated construct from the AGO7 promoter. Figure source data can be found in Supplementary data.

Our results establish that biogenesis of TAS3-derived ta-siRNA requires the addressing of AGO7 to a specialized cellular compartment in the cytoplasm. The presence in siRNA bodies of the key components of ta-siRNA formation and their co-purification with membranes suggest a model in which, upon accumulation of the ternary complex miR390/AGO7/TAS3 in siRNA bodies, the product of TAS3 miR390-mediated cleavage would be passed on to RDR6 and SGS3 for conversion into a dsRNA. In agreement with previous reports (Hiraguri *et al*, 2005; Kumakura *et al*, 2009; Hoffer *et al*, 2011), we could not detect DCL4 outside of the nucleus (data not shown), suggesting that further processing occurs outside of the siRNA bodies. Further work is required to test whether accumulation in the siRNA bodies occurs pre- or post-miRNA cleavage, and if this accumulation is cause or consequence of AGO7 recruitment onto the TAS3 precursor.

Viruses are known to hijack the early secretory system to trigger the formation of viral replication complexes (Laliberté and Sanfaçon, 2010). The colocalization observed between AGO7 and the membrane-linked VP6 protein suggests that siRNA bodies contain membranes. This is paralleled by the similar biochemical properties of AGO7 and VP6: retrieval from the microsomal fraction, resolubilization by detergent and light density on sucrose gradient (Schaad *et al*, 1997). In addition, SGS3 is also retrieved in the microsomal fraction (Supplementary Figure S4). The nature and mechanism of AGO7 association with membranes are unknown. Accumulation of AGO7 in the P100 fraction is unaffected in *sgs3* or *rdr6* mutants (Supplementary Figure S7), indicating that RDR6 and SGS3 are not required for AGO7 targeting. Several post-translational modifications of animal AGO proteins have been reported (for review, see Johnston and Hutvagner, 2011). Among those, prolyl-hydroxylation (Qi, *et al* 2008) might control accumulation of human AGO2 in multi-vesicular bodies (MVB). It may be that post-

translational modification of AGO7 and/or association with a yet unknown cofactor is required for membrane targeting.

Association of AGO proteins with the endomembrane system has been reported in animal cells Arabidopsis, MVB mature from the trans-Golgi network/early endosome whereas VP6 defines an intermediary compartment between ER and cis-Golgi (Gibbings and Voinnet, 2010; Lerich *et al*, 2011). In particular in *Drosophila* and mammalian cells, AGO proteins associate with MVB, and this contributes to AGO recycling (Gibbings *et al*, 2009; Lee *et al*, 2009). Although plant cells possess MVB, we consider it unlikely that the association of AGO7 with membranes reflects its localization in MVB. Indeed, in Arabidopsis, MVB mature from the trans-Golgi network/early endosome whereas VP6 defines an intermediary compartment between ER and cis-Golgi (Lerich *et al*, 2011).

The congregation of siRNA bodies, containing RDR6 and SGS3 both of which are essential for plant defense against virus, and VP6, a viral protein required for virus replication, suggest that siRNA bodies are a point of convergence between viral replication and the host defense mechanisms. In turn, endogenous small RNA pathways such as the miR390/TAS3 pathway, making use of the siRNA-production machinery, accumulate into the siRNA bodies.

Previous work has suggested that products of miRNA-mediated cleavage of TAS precursors transfer from P-bodies to the siRNA bodies for conversion into dsRNA (Kumakura *et al*, 2009). However, we never observed AGO7 in P-bodies marked with DCP1. This further reinforces the important differences between the mechanisms of ta-siRNA formation from TAS1/2 (dependent on 22 nt miRNA and AGO1) and from TAS3 (dependent on 21 nt miRNA and AGO7). The presence and still elusive role of the non-cleavable miR390 site in TAS3 (Montgomery *et al*, 2008) could be linked to the specific targeting of the miR390/AGO7/TAS3 complex to the siRNA bodies. It is possible that 22 nt miRNA-loaded AGO1 also accumulate in siRNA bodies.

A hallmark of mRNA targeted to processing by RDR6/SGS3 is their poor ability to be translated. This is either due to a very poor coding potential, as in the case of TAS3 (Ben Amor *et al*, 2009), or because, as a consequence of miRNA-mediated cleavage, these mRNAs lack the canonical marks (5' CAP and 3' polyA tail) required for efficient translation. Because siRNA bodies, unlike stress granules, are readily detected under normal growth conditions, this suggests that siRNA bodies could represent a microscopic aggregate of mRNAs stalled in translation. Upon stress-induced shut down of translation, siRNA bodies may serve as seeds for the formation of numerous stress granules. This is supported by the observation that upon massive shut down of translation induced by stress, siRNA bodies increase dramatically in number and accumulate canonical markers of mRNAs stalled at the translation initiation stage (as eIF4E, UBP1). The dynamic nature of siRNA bodies suggests that they are sites of mRNA triage, wherein mRNA could be sorted for degradation by P-bodies or enter the siRNA pathway.

Taken together, our results reveal a hitherto unknown role for specific cytoplasmic membrane-associated ribonucleoparticles in ta-siRNA biogenesis and AGO action in plants.

Materials and methods

Plant material and growth conditions

All *Arabidopsis thaliana* lines used are in the Columbia ecotype (Col-0) background. The following mutants were described previously: *ago7-1* (SALK_037458; Adenot *et al*, 2006), *zip-1* (CS24281; Hunter *et al*, 2003), and *tas3a-1* (GABI_621G08; Adenot *et al*, 2006). The *pAGO7:HA:AGO7/zip-1* and *p35S:TAS3aPDS-1/zip-1* lines were described in Montgomery *et al* (2008). For *in-vitro* conditions, plants were grown on sterile 0.5 × Murashige and Skoog (MS)/0.8% agar (1/2 MS agar) plates in a growth chamber under controlled conditions (150 mmol photon, 16 h light and 23°C temperature). For soil conditions, plants were grown in a growth room (150 mmol photon, 16 h light and 23°C temperature).

Subcellular fractionations

Nuclear/PNS fractionation was performed according to Park *et al* (2005). Briefly, plant material was ground and cell wall-disrupting buffer (10 mM potassium phosphate pH 7.0, 100 mM NaCl, 10 mM b-mercaptoethanol, 1 M hexylene glycerol) was added. The sample was filtered through Miracloth and centrifuged at 1500 g for 10 min at 4°C. After centrifugation, the supernatant was collected and recentrifuged at 13 000 g for 15 min at 4°C. The supernatant of this second centrifugation corresponds to the PNS. The first pellet was washed with nuclei preparation buffer (NPB) (10 mM potassium phosphate pH 7.0, 100 mM NaCl, 10 mM b-mercaptoethanol, 1 M hexylene glycerol, 10 mM MgCl₂, 0.5% Triton X-100) and centrifuged at 1500 g for 10 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was washed with NPB. Washing and centrifugation were repeated four to five times, and the final pellet was stored as the nuclear fraction.

Microsomal fractions were obtained as follow. Plant material was ground to fine powder under liquid nitrogen. In all, 2 × volume of homogenization buffer (Sorbitol 0.5 M, EDTA 10 mM, PVP 40 0.5%, Protease inhibitor cocktail) was added. The sample was filtered through Miracloth. The filter extract was centrifuged for 15 min at 8000 g at 4°C. The supernatant (low speed supernatant—LSS) was then centrifuged at 100 000 g for 30 min at 4°C. The supernatant corresponds to the S100 and the pellet to the P100. For resolubilization test, the P100 fraction was resuspended in 5 × volume of the same buffer supplemented with detergent, incubated for 30 min on ice and centrifuged at 100 000 g for 30 min at 4°C. The supernatant corresponds to the S100 and the pellet to the P100. The S100 fraction was precipitated (see below) before analysis.

Proteins extraction and immunoblot

Proteins were extracted as follow, 100 mg of plant material finely ground was mixed with 300 µl of extraction buffer (0.7 M Sucrose; 0.5 M

Tris-HCl pH 8; 5 mM EDTA; 0.1 M NaCl; 2% b-mercaptoethanol; protease inhibitor (Roche)). The same volume of phenol (pH 8) was added. The sample was mixed 20 min at room temperature and then centrifuged at 13 000 g for 5 min at 4°C. The phenolic phase was precipitated with five volumes of ammonium acetate (0.1 M) in absolute methanol at −20°C. The proteins were pelleted at 5000 g for 5 min at 4°C, washed with 80% acetone and finally resuspended in 300 µl of resuspension solution (3% SDS; 62.5 mM Tris-HCl pH 6.8; 10% glycerol). The samples were incubated at 65°C for 10 min, centrifuged at 13 000 g for 5 min and the supernatant was kept for quantification and analysis. Protein concentration was quantified using a detergent compatible BCA kit (Bio-Rad) and 75 µg of protein was loaded on gel.

The protein extracts were separated by SDS-PAGE (8%), and proteins were electroblotted onto nitrocellulose membranes (Amersham Hybond ECL). The membranes were incubated with primary antibodies and subsequent HRP-coupled secondary IgGs. Antigens were detected using chemiluminescence for HRP immunoblot (Amersham ECL Plus). The antibodies used were anti-HA (12CA5, 1/1000^e dilution; Roche); anti-GFP (sc-8334, 1/1000^e dilution; Santa Cruz); anti-SGS3 (sc-14068, 1/200^e dilution; Santa Cruz); anti-mouse HRP-coupled (NA931, 1/2500^e dilution; GE Healthcare), anti-rabbit HRP-coupled (NA934, 1/2500^e dilution; GE Healthcare), and anti-goat HRP-coupled (611620, 1/5000^e dilution; Invitrogen).

Whole-mount immunofluorescence

Whole-mount immunofluorescence was performed on 7-day-old roots according to Sauer *et al* (2006) with slight modifications. See Supplementary data for detailed protocol. The antibodies used were anti-HA (12CA5, 1/100^e dilution; Roche); anti-GFP (sc-8334, 1/100^e dilution; Santa Cruz); anti-mouse coupled to Alexa488 (A11017, 1/1000^e dilution; Invitrogen) and anti-rabbit coupled to Alexa488 (A11070, 1/1000^e dilution; Invitrogen).

Imaging

For confocal imaging, tobacco leaves were mounted in 15% glycerol and directly imaged on a Leica TCS SP5 (Leica Microsystems) with 488/543 nm excitation, 488/543 beamsplitter filter and 495–530 nm (green channel) and 550–700 nm (red channel) detection windows. All images were acquired with similar gain adjustments.

Arabidopsis plants were imaged after immunofluorescence on a Leica TCS SP5 (Leica Microsystems) with 488 nm excitation, 488 beamsplitter filter and 495–530 nm (green channel) detection window. For DAPI detection, a 364-nm UV laser (no beamsplitter filter set, detection window of 415–550 nm) was used.

Plasmid and cloning

See Supplementary data for details on the plasmids used and cloning procedures.

Supplementary data

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

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions: ABM generated materials and contributed to the colocalization studies. TE realized the biochemical analysis of SGS3. VJ performed all other experiments and helped writing the paper. AM, with the help of MC and HV, wrote the paper.

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