Putative *Arabidopsis* THO/TREX mRNA export complex is involved in transgene and endogenous siRNA biosynthesis

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RNA silencing in plants and some animals has a non–cell-autonomous effect due to an RNA signal that moves between cells or organs. To identify unique factors involved in this process, we analyzed a group of *Arabidopsis* mutants with defective spread of RNA silencing from a transgene expressed specifically in the phloem. These mutants accumulated reduced amounts of small interfering (si)RNA from the transgene locus and from endogenous loci *TAS1*, *TAS2*, and an inverted repeat locus *IR71*. The defect in *TAS1* and *TAS2* siRNA biogenesis is in the processing of a long siRNA precursor. We mapped the mutations to a gene encoding the *Arabidopsis* homolog of a protein, TEX1, which is involved in intracellular transport of RNA in animals. TEX1 is a component of the THO/TREX complex, and we show that the *Arabidopsis* TEX1 interacts with other predicted components of a THO/TREX complex. Correspondingly, we found at least two other components of the *Arabidopsis* THO core complex that are involved in RNA silencing. To reconcile the effect of these mutations on transgene and endogenous gene siRNA, we propose a mechanism in which THO/TREX processes or transports a long RNA molecule so that it can be a template for secondary siRNA production.

RNA silencing | *trans*-acting siRNA | miRNA | inverted repeat DNA

In plants, *Caenorhabditis elegans*, and *Drosophila*, there is an effect of RNA silencing that spreads between cells or for long distances between organs (1–4). This spreading process operates in a nucleotide sequence-specific manner, indicating that the signal molecule includes one or more of the RNA species in RNA silencing pathways. Mobile silencing RNAs could be the 21- to 24-nt small (s)RNAs that are the specificity determinant of silencing (5–7). Alternatively, they could be the longer sRNA precursors that might exist in either a single or double-stranded (ds) form.

Genetic analyses are consistent with the signal, including an sRNA, that would have been processed from a long dsRNA precursor by a Dicer nuclease (8). However, these genetic analyses do not differentiate between effects that are required in the cells that generate the signal and those operating in the cells receiving the signal. Consequently, they do not rule out that the mobile molecule is the long dsRNA or one of the other intermediates in the pathway.

Various proteins that had been previously associated with silencing effects on DNA and chromatin have also been implicated in the spread of silencing between cells (6, 9, 10) and in long distance movement across a graft union (5, 7). There is no definitive information about the role of these proteins in the cell-to-cell movement mechanism, although in the grafting system they are required in cells that receive the signal (5, 7) and may be involved in an amplification process.

A further potential complexity of the spreading process derives from the existence of primary and secondary phases of sRNA biogenesis (11–14). The primary phase involves cleavage of a sRNA precursor by the Dicer nuclease (DCL in plants). The secondary phase is initiated following the interaction of this primary sRNA with a long, single-stranded sRNA target. The primary sRNA in this process is associated with an Argonaute nuclease, although the target RNA may not be cleaved (11, 15, 16). The secondary phase involves a cellular RNA-dependent RNA polymerase (RDR) that converts the target RNA into a double-stranded form. This dsRNA is then processed by Dicer into the secondary sRNAs (14, 17, 18). The production of secondary sRNA could be at least part of the amplification of RNA silencing.

The best characterized secondary sRNAs in plants are the *trans*-acting small interfering RNAs (tasiRNAs) (17–22). The primary sRNAs in tasiRNA pathways are micro (mi)RNAs. In some instances the tasiRNAs initiate further rounds of secondary siRNA production, and there is a cascade of secondary siRNA production that would enhance the amplification effect (12, 13, 23). Two recent reports provide evidence implicating some tasiRNAs with mobile silencing (24, 25).

In this paper we describe further analysis of *Arabidopsis* plants in which a silencer transgene was expressed under the control of a phloem-specific promoter. The wild-type plants expressing the transgene exhibited silencing around the veins that was reduced in the mutant plants that also exhibited a reduction in endogenous tasiRNAs (8, 9). This observation provided an additional link between spreading and tasiRNAs. Here we show that the mutant phenotype in these plants was due to an impaired processing of precursors for both the transgene RNA and tasiRNA, and we map the mutations to a homolog of *TEX1*. TEX1 proteins are components of THO/TREX complexes implicated in transport of mRNA precursors (26–29). We provide evidence that at least two other components of the THO complex influence tasiRNA production, and show that RNAs from an endogenous inverted repeat locus—*IR71*—are also affected by mutations in the *Arabidopsis* TEX1 homolog. These findings point to the existence of a variant RNA-silencing pathway that involves a THO/TREX complex.

Results

Mutants with Reduced *PDS* Silencing Spreading Affect Transgene and Endogenous siRNA. To identify components involved in the spread of RNA silencing we have used a genetic screen (9) based on

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Data deposition: All proteomics data can be viewed at http://www.ringtail.tsl.ac.uk/hosted-data/ (see Yelina2010) and http://www.ebi.ac.uk/pride/, accession no. 10068.

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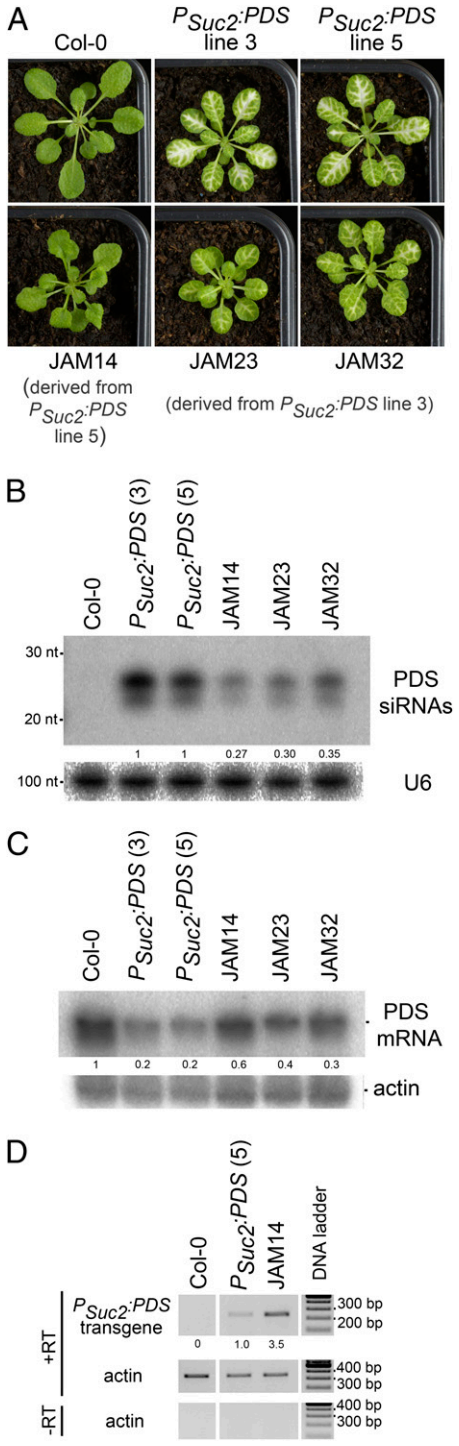

Fig. 1. The phenotypes of P_{Suc2}:*PDS* and mutant lines. (*A*) Photographs of 3-wk-old Col-0, two P_{Suc2}:*PDS* lines (3 and 5) expressing the P_{Suc2}:*PDS* transgene, and three EMS mutant lines: JAM14, 23, and 32. (*B* and *C*) Northern blot hybridizations showing the levels of *PDS* siRNAs (*B*) and *PDS* mRNA (*C*) in wild-type and mutant lines. Blots of total RNA from rosette leaves were hybridized with radiolabeled DNA probes complementary to *PDS* siRNAs (*B*) and *PDS* mRNA (*C*). Blots were subsequently reprobed for loading with a radiolabeled DNA oligonucleotide probe complementary to U6 RNA (*B*) and a radiolabeled DNA probe complementary to actin (*C*). Numbers under blots indicate the intensity of hybridization signal relative to (*B*) P_{Suc2}:*PDS* (lines 3 and 5) after normalization to U6 and (*C*) Col-0 after normalization to actin. (*D*) Total RNA isolated from the indicated lines was subjected to semiquantitative RT-PCR analysis to detect P_{Suc2}:*PDS* transgene expression and actin mRNA, as a control. Parallel reactions were carried out with (+RT) or without (–RT) the addition of reverse transcriptase. Numbers under the agarose gel indicate relative abundance of PCR products after normalization to actin.

Arabidopsis thaliana lines with a *PHYTOENE DESATURASE* (*PDS*) inverted repeat construct expressed from the phloem-specific *SUC2* promoter P_{Suc2}:*PDS* (Jawohl:AtSuc2:PDS) (9). The promoter in these transgenic lines directs dsRNA production in the phloem companion cells to initiate the silencing of *PDS*. Spread of an RNA silencing signal to the adjacent cells causes the more extensive silencing of *PDS* that is manifested as photobleaching around the veins of the wild-type plants (P_{Suc2}:*PDS* lines 3 and 5; Fig. 1*A*) (9). In this paper we focus on three ethyl methanesulfonate (EMS) mutants that belong to one complementation group (JAM lines for Jawohl:AtSuc2:PDS mutants) named JAM14 (derived from P_{Suc2}:*PDS* line 5), JAM23, and JAM32 (derived from P_{Suc2}:*PDS* line 3), in which there was reduced *PDS* photobleaching (Fig. 1*A*).

Consistent with the mutant phenotype, the levels of the 21-nt and 24-nt *PDS* siRNAs produced from the inverted-repeat transcript P_{Suc2}:*PDS* were lower, and the levels of *PDS* mRNA were higher, than in wild-type plants expressing the P_{Suc2}:*PDS* transgene (Fig. 1 *B* and *C*). The suppression of silencing in these mutants is not due to reduced transcription of the P_{Suc2}:*PDS* transgene: in the JAM 14 line in which the loss of the *PDS* silencing was stronger than with JAM23 or 32 (Fig. 1*A*), the transgene transcript detected by RT-PCR was 3.5-fold more abundant than in the corresponding wild-type line (Fig. 1*D*). It is likely, therefore, that the mutants affect the processing of the P_{Suc2}:*PDS* transgene RNA into siRNA.

In addition to the *PDS* silencing phenotype, the JAM14, JAM23, and JAM32 mutants have reduced levels of endogenous tasiRNAs (9). The levels of tasiRNAs derived from *TAS1a-c* and *TAS2* loci were markedly reduced (9) (Fig. 2*A*), as in *dcl4-2* and *rdr6-15*. However, the levels of *TAS3* tasiRNAs (Fig. 2*A*), together with NRPD1a-dependent 24-nt siRNAs and miRNAs (e.g., miR159 and miR167), were not reduced or showed minimal reduction (9).

To find out which stage of tasiRNA biogenesis was blocked in JAM14 (and JAM 23 and 32) mutants, we analyzed the precursor RNAs (pre-RNA) of *TAS1* and *TAS2*. In the *TAS1* and *TAS2* tasiRNA pathway, *TAS1* and *TAS2* pre-RNAs are cleaved by Argonaute nuclease AGO1, and the cleavage is guided by miR173 (19, 22). We analyzed intermediate RNAs that are formed by Argonaute-mediated cleavage of the pre-RNA. In the tasiRNA biogenesis pathway, the cleavage product on the 3′ side of the miR173 target side is converted into a dsRNA form by an RDR. The 5′ cleavage product does not participate in tasiRNA biogenesis, but it is an indicator of the miR173 cleavage process (Fig. 2*B*).

Figure 2*C* illustrates RNA gel blot analysis showing that *TAS2* pre-RNA was more abundant in the mutants JAM14 and *attex1-4* than in wild-type plants (*attex1-4* mutation is allelic to the mutation in JAM14, 23, and 32 and is described in more detail below). However, there was no corresponding increase in the *TAS2* (5′) cleavage product (22). The level of the *TAS1* long RNA precursor also increased (Fig. S1 *A–C*). In both instances the increase was approximately 2-fold, as estimated by quantitative PCR (Fig. S1*C*). A *dcl1-7* mutant in which tasiRNA biogenesis is suppressed due to the reduced levels of miRNAs, including miR173, also showed increased *TAS1* and *2* pre-RNA and low levels of the 5′ cleavage products (Fig. 2*C* and Fig. S1*B*). By contrast, an *rdr6-15* mutant in which tasiRNA biogenesis is blocked at a later step in the pathway had wild-type levels of the pre-RNAs and higher-than-wild-type levels of the cleavage products, as reported (22) (Fig. 2*C* and Fig. S1*B*). It is, therefore, likely that the JAM14 and *attex1-4* mutants are blocked in tasiRNA biogenesis at or before the step in which there is miR173-directed cleavage of the *TAS* pre-RNA and in advance of the step involving RDR6.

It is unlikely that the increased level of the *TAS1* and *2* pre-RNA is due to a defect in miR173 biogenesis because the JAM14, 23, and 32 mutants and wild-type plants have similar levels of

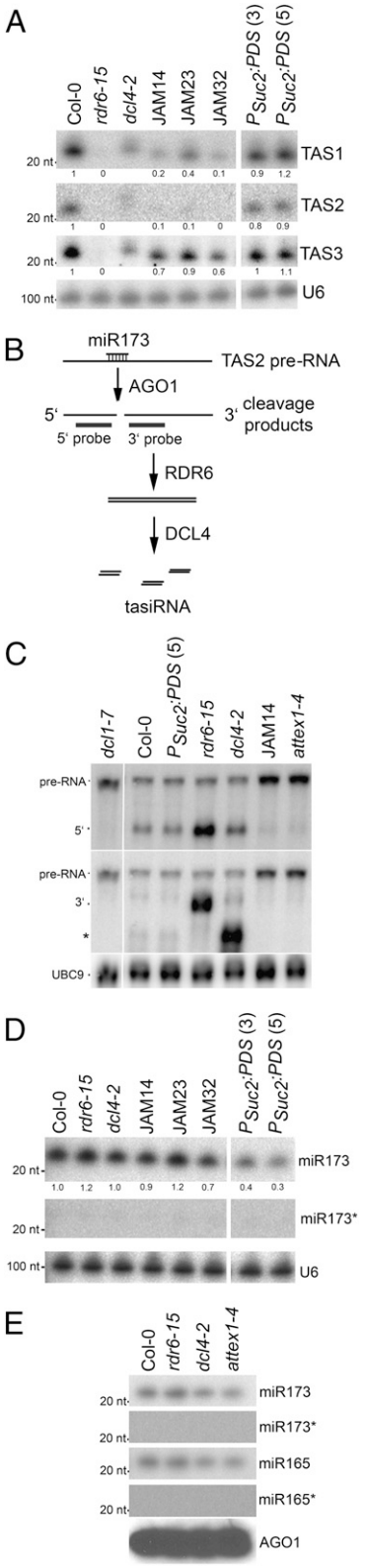

Fig. 2. Effect of mutations in *AtTEX1* on siRNAs derived from tasiRNA-producing loci and on *TAS2* pre-RNA. (*A*) Northern blot hybridization of total RNA from rosette leaves of the indicated wild-type and mutant lines. Blots were hybridized with radiolabeled DNA oligonucleotide probes complementary to siR255 (*TAS1* locus), siR1511 (*TAS2* locus), and a radiolabeled RNA transcript probe complementary to siRNAs in *TAS3* locus, and subsequently reprobed with a radiolabeled DNA oligonucleotide complementary to U6 RNA (as a loading control). Numbers under blots indicate the intensity of hybridization signal relative to Col-0 after normalization to U6 (siRNAs accumulating in the *dcl4-2* background are 22 nt long; therefore, quantification has not been done for this mutant). (*B*) Schematic representation of *TAS* pathway. Black bars indicate regions used for hybridization probes. (*C*) Northern blots of total RNA from the indicated *Arabidopsis* lines hybridized with radiolabeled DNA probes complementary to the upstream (5′) and downstream (3′) parts of the cleaved *TAS2* pre-RNA (*Top* and *Middle*, respectively). Hybridization with ubiquitin-conjugating enzyme 9 probe (*Bottom*) is shown as a loading control. Mobility shift in the *dcl1-7* background is most likely due to impurities in the sample. An unknown processing product is indicated with an asterisk. (*D*) Total RNA from the indicated wild-type and mutant lines was hybridized with radiolabeled DNA oligonucleotide probes complementary to miR173 and miR173*. U6 hybridization is shown as a loading control. Numbers under the blot indicate the intensity of hybridization signal relative to Col-0 after normalization to U6. (*E*) AGO1 was immunoprecipitated, and associated sRNAs were detected by hybridizing with radiolabeled DNA oligonucleotides as indicated. The presence of AGO1 was detected by immunoblot (*Bottom*).

miR173 and miR173* (Fig. 2*D*). We can also rule out that miR173 failed to associate with AGO1, because we could coimmunoprecipitate AGO1 and miR173 in both wild-type plants and in a mutant *attex1-4* (Fig. 2*E*). Therefore, the effect of the mutations in JAM14, 23, and 32 on the processing of *TAS1* and *TAS2* pre-RNAs does not affect miR173.

siRNA Production from an Endogenous Inverted-Repeat (IR) Locus Is Affected in the Mutant Lines. The *Arabidopsis* genome contains several IR loci that give rise to siRNAs (30), and we reasoned that the mutant protein in the JAM14, 23, and 32 lines could have an effect on the abundance of siRNAs derived from these loci, as at the *PDS* transgene locus. We tested three such loci that generate siRNAs (30) and found that one of them, *IR71*, accumulates less siRNA in the *attex1-4* mutant than in the wild type (Fig. 3*A*). The siRNAs from the two other tested loci (described in ref. 30), which we refer to as *IR13489* and *IR07679*, were at wild-type levels or had a minimal reduction in *attex1-4* (Fig. 3 *B* and *C*).

An RNA Transport Protein Involved in siRNA Biogenesis. The mutations in JAM 14, JAM 23, and JAM 32 lines mapped to the *At5g56130* gene. JAM 14 and JAM 32 have splice-site mutations, and JAM 23 has a missense mutation in this gene, as shown in Fig. S1*D*. Transformants of the mutant lines with a transgene extending 1,999 bp upstream and 521 bp downstream of the predicted initiation and termination codons regained the photobleaching phenotype characteristic of the P_{Suc2}:*PDS* transgene (Fig. S2*A*). These plants also had wild-type levels of siRNAs corresponding to *TAS2* endogenous locus (Fig. S2*B*). An *Arabidopsis* line with a T-DNA insertion in *At5g56130*, SALK_100012, also exhibited the loss or strong reduction of the *TAS1* and *TAS2*, but a minimal or no reduction of *TAS3*-derived tasiRNAs (Fig. S2*C*).

AtTEX1 Is Part of a Putative Arabidopsis THO/TREX Complex. The protein encoded by *At5g56130* is a WD-40 repeat family protein. It does not have any close homologs in *Arabidopsis*, but there are similar proteins in other plants, yeasts, *Drosophila melanogaster*, *C. elegans,* mouse, and humans as shown in Table S1. The protein encoded by these genes in animals is the TEX1 subunit of the THO/TREX complex that is involved in mRNA export (26, 27), and we refer to this gene in *A. thaliana* as *AtTEX1*. The serine residue that is mutated into phenylalanine (S86F) in JAM23 is in a 6-aa motif that is conserved in *A. thaliana*, *D. melanogaster*, mouse, and human (Fig. S2*D*). We conclude therefore that AtTEX1 is a protein involved in biogenesis of tasiRNA or siRNA from transgenic and some of the endogenous inverted repeat transcripts. In the following text we refer to the mutant alleles as JAM14 – *attex1-1*, JAM23 – *attex1-2*, JAM32 – *attex1-3*, Col-0 T-DNA insertion line in *At5g56130* – *attex1-4*.

The TEX1 protein in animals and yeast is part of a THO/TREX complex (26–28) in which core components include Tho2, Hpr1,

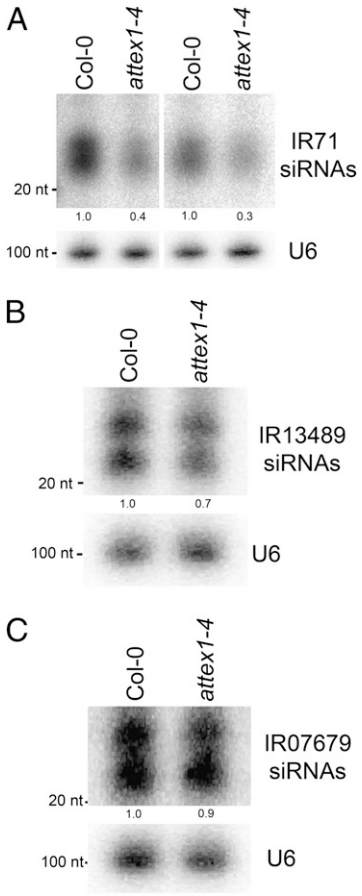

Fig. 3. The effect of *attex1-4* on siRNAs derived from endogenous inverted repeat (IR) loci. Total RNA was subjected to hybridization with radiolabeled RNA transcript probes to detect siRNA arising from *Arabidopsis IR71* (*A*), *IR13489* (*B*), and *IR07679* (*C*) loci in Col-0 and *attex1-4* plants (two biological replicates shown in *A*). Blots were reprobed for U6 as a loading control. Numbers under the blot indicate the intensity of hybridization signal relative to Col-0 after normalization to U6.

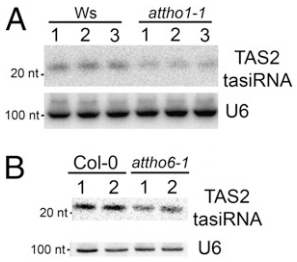

Fig. 4. Mutations in *AtTHO1* and *AtTHO6* lead to a reduction in *TAS2* siRNAs. Total RNA from fully expanded mature leaves of three Ws and three *attho1-1* mutant plants (*A*) and two Col-0 and two *attho6-1* mutant plants (*B*) was hybridized to a radiolabeled DNA oligonucleotide probe complementary to siR1511 (*TAS2*) and reprobed for U6 as loading control.

THOC5-THOC7 in *Drosophila* (27), and fSAPs 79, 35, and 24 in humans (28). TEX1 is not part of the core; it is part of an associated TREX complex together with a helicase homolog UAP56 and REF1/Aly (27, 28). A similar THO/TREX complex is likely to exist in *Arabidopsis* because the genome encodes homologs of all five components of the core THO complex (Table S2) and an Aly protein component of TREX.

To confirm the THO/TREX complex in *Arabidopsis*, we assembled an HA epitope-tagged *AtTEX1* construct and expressed it from its endogenous promoter in JAM23 and JAM32 lines. The transformants contain a protein of the expected size of 35.4 kDa detected with an anti-HA antibody (Fig. S3*C*). In addition, they have wild-type levels of *TAS2*-derived tasiRNAs and *PDS* photobleaching as in the wild-type progenitors of JAM23 and JAM32 (Fig. S3 *A* and *B*).

Because *AtTEX1* is expressed in the flowers as well as leaves (www.arabidopsis.org/), we bound the HA-AtTEX1 from flower extracts of two transgenic lines to an HA-affinity matrix, eluted the bound protein with an HA peptide, and analyzed the eluted proteins by mass spectrometry. As negative controls, we performed the same procedure with plants not expressing an HA-fused protein (JAM23 or JAM32). This immunoaffinity purification was carried out from nuclear extracts as well as from whole-cell extracts because THO/TREX in animals is a nuclear complex (28). The analysis was carried out in triplicate for each nuclear extract and once for total extracts. The detected peptides include those corresponding to AtTHO1 or AtHPR1 (*At5g09860*), AtTHO2 (*At1g24706*), AtTHO3 or AtTEX1 (*At5g56130*), AtTHO5 (*At5g42920*, *At1g45233*), AtTHO6 (*At2g19430*), and AtTHO7 (*At5g16790*, *At3g02950*) in the fractions that were specifically bound to HA-affinity matrix (Table 1 and Datasets S1 and S2). It is therefore likely that *Arabidopsis* contains a THO/TREX complex. We did not find either Aly proteins or a helicase that could be a homolog of UAP56 in our immunoprecipitation experiments. The absence of these proteins could be because, as in humans (28), the TREX complex is not stable under high-salt conditions used in our experiments.

In a complementary genetic approach, we tested insertional mutants of *Arabidopsis* genes encoding homologs of the AtTHO1 (AtHPR1, At5g09860) and AtTHO6 (At2g19430) THO/TREX components. We reasoned that, if a THO/TREX complex is involved in siRNA biogenesis, these mutants would phenocopy the *attex1* alleles described previously. The analysis focused on these genes because they are present as single copies in the *Arabidopsis* genome and the outcome may not be complicated by genetic redundancy. These lines had markedly reduced levels of the *AtTHO1* and *AtTHO6* mRNA (Fig. S3 *D* and *F*) and also had a reduction in *TAS2* tasiRNAs (Fig. 4 *A* and *B*).

We obtained T-DNA insertion mutant lines for another THO/TREX gene *AtTHO2*. However, the homozygous insertion mu-

Table 1. Summary of mass-spectral sequencing analysis of peptides coimmunoprecipitated with HA-AtTEX1

THO component	Predicted *A.thaliana* homolog	Molecular weight according to TAIR	Mascot score	Unique peptides	Times detected (of four biological replicates)
THO1 (HPR1)	At5g09860	68335.0	259	5	3
THO2	At1g24706	202719.0	708	20	4
THO3 (AtTEX1)	At5g56130	35375.0	246	9	4
THO5	At5g42920	92578.0	163	7	4
	At1g45233	51813.0	251	6	2
THO6	At2g19430	40073.0	69	1	1
THO7	At5g16790	26985.0	69	2	1
	At3g02950	27461.0	174	5	3

tant in *AtTHO2* could not be recovered, and we infer that it has a lethal phenotype. We did not analyze T-DNA insertion lines that target functionally redundant members of multigene families of the putative THO/TREX components. Nevertheless, the finding that AtTEX1, AtTHO1, and AtTHO6 have an effect on *TAS2* tasiRNA (Figs. 2 and 4) is good evidence that a complex including the THO core is required for a subset of siRNA biogenesis pathways in plants.

Discussion

AtTEX1 is not a general requirement for sRNA biogenesis because miRNAs and most cellular siRNAs, including those that are dependent on Pol IV, are not affected by mutation of the *AtTEX1* locus (9). However, the finding that *TAS1* and *TAS2* tasiRNAs, *IR71* siRNAs, and P_{Suc2}*:PDS* transgene siRNAs are affected by *attex1* mutations (Figs. 1–3) indicates that there is an siRNA biogenesis pathway that does require this protein. This pathway may also involve AtTHO1 and AtTHO6 as part of THO/TREX because mutations in these genes also affected TAS2 siRNAs (Fig. 4 *A* and *B*). However, the TAS RNA phenotype was less pronounced with *AtTHO1* and *AtTHO6* mutants than with loss of *AtTEX1* function (Figs. 1–3). This quantitative difference implies that AtTEX1 may affect TAS RNA independently of AtTHO1 and AtTHO6, and that there are variants of the THO/TREX complex that are required for siRNA biogenesis.

By analogy with the role of the THO/TREX complex in RNA transport in animals and yeasts (26–28), and based on our analysis of tasiRNA precursors in *attex1* mutants (Fig. 2 and Fig. S1), we consider that the THO/TREX complex in siRNA biogenesis is likely to affect transport of an siRNA precursor from its site of synthesis to a subcellular location where processing of siRNA takes place. Unfortunately, we do not yet have information about the site of this location. Both miRNAs and AGO proteins are located in both nuclear and cytoplasmic compartments (31, 32) so that, although THO/TREX complexes mediate RNA transport to the cytoplasm, we cannot rule out that they also transport the siRNA precursor to a nuclear location for processing into siRNAs. However, our data are consistent with a recent report that suggests a model where tasiRNA pre-RNA is cleaved by AGO in P-bodies in the cytoplasm, then transported to another cytoplasmic compartment where it is converted to dsRNA by RDR6, followed by DCL4 cleavage in the nucleus (33).

The processing location in *TAS1* and *TAS2* tasiRNA biogenesis is likely to be a site at which the siRNA precursor interacts with a miR173/AGO complex. This interaction would then initiate conversion of the cleaved precursor into a double-stranded RNA by an RDR, and the resulting dsRNA would be cleaved by Dicer into siRNA (Fig. 5). In the absence of AtTEX1 (or of miR173), the miR173 interaction would not occur and the precursor would be stabilized so that its accumulation would be at a higher level than in wild-type plants (Fig. 2 and Fig. S1).

Our model to explain the involvement of AtTEX1 in *IR71* and P_{Suc2}*:PDS* siRNAs invokes two types of transcript from the corresponding genomic loci (Fig. 5), and it is based on the idea that, like the tasiRNA pathway, these inverted repeat genes generate primary and secondary sRNAs. An inverted repeat transcript would be processed into primary sRNAs by Dicer without the involvement of AtTEX1 or RDR proteins. In addition, according to the model, there would also be shorter, single-stranded transcripts that would not extend through both arms of the inverted repeat. These single-stranded transcripts would be the functional equivalent of the pre-*TAS1* and -*TAS2* RNAs, and they would interact by base pairing with the primary sRNAs. By analogy with the tasiRNA pathway, we envision that transport of the single-stranded RNAs to the site of this interaction would be dependent on AtTEX1. The interaction would then initiate secondary siRNA production through a process that would be RDR dependent.

The model is attractive because it accounts for the involvement of an RDR in sRNA production from loci that would generate an inverted transcript. It would be RDR2 in the production of the siRNAs from P_{Suc2}*:PDS* rather than RDR6 of the tasiRNA pathways (9, 17, 20) and at IR71 it could be either or both of RDR2 and RDR1 (Table S3). The model could also explain how multiple DNA-dependent RNA polymerases could be involved in production of these sRNAs. At P_{Suc2}*:PDS*, Pol II would produce the precursor of the primary sRNAs from the P_{Suc2} promoter, and Pol IV would produce the precursor of the secondary sRNAs (9). Production of IR71 sRNA is influenced by Pol IV (30, 34) but, as there is a residual sRNA accumulation in a Pol IV mutant (*nrpd1a*) (Table S3), it is likely that other polymerases are also involved.

The evidence for the involvement of Pol IV is from the finding that most siRNAs from P_{Suc2}*:PDS* locus are dependent on NRPD1a—the largest subunit of Pol IV (9). It is similarly likely that the 24-nt siRNAs from *IR71* are dependent on Pol IV. Evidence that a second polymerase is involved is indirect. With P_{Suc2}*:PDS*, it is based on the silencing effect being specific to the veins where the P_{Suc2} promoter should be expressed under Pol II. At the *IR71* locus, it is from the finding that a subset of the siRNAs from this locus—corresponding to one of the two possible strands—is produced independently of Pol IV (30). Pol II is assumed to be involved by default, although formally we cannot rule out either Pol I or III.

This two-phase silencing pathway with a THO/TREX complex involved in the secondary phase would be important in the spreading of silencing because it has amplification properties. As the silencing signal moves away from its initial source, the am-

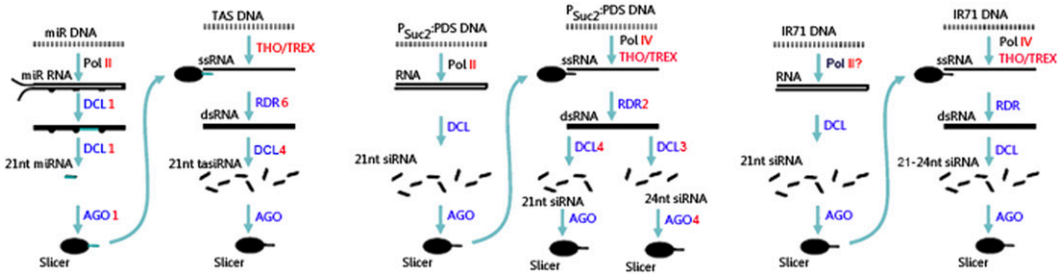

Fig. 5. Schematic representation of described and proposed pathways generating siRNAs from three different types of loci, *TAS*, P_{Suc2}*:PDS*, and *IR71*. All three pathways involve primary and secondary phases of sRNA biogenesis and the THO/TREX complex is proposed to act early in the secondary phase. In the tasiRNA pathway (*Left*), DCL1-mediated production of miRNA is the primary phase, and tasiRNAs production involving RDR6-mediated synthesis of the double-stranded (ds) tasi pre-RNA and DCL4-mediated cleavage of the dsRNA is the secondary phase. The resulting tasiRNAs are bound by AGO. In the *PDS* and *IR71* silencing pathways (*Center* and *Right*), two types of transcripts can be produced from the P_{Suc2}*:PDS* and *IR71* loci: full-length RNAs that form a hairpin, and shorter single-stranded (ss) ones that would not be able to fold into a hairpin structure. Dicing of sRNAs from the transgene hairpins is the primary phase of sRNA biogenesis. These sRNAs have a function similar to that of miRNAs in the tasiRNA pathway in that they prime synthesis of dsRNA on ss P_{Suc2}*:PDS* and *IR71* transgene transcripts. During the secondary phase, sRNA are diced from the dsRNA precursors. In the case of P_{Suc2}*:PDS*, two pathways—one involving DCL3 and AGO4 and the other involving DCL4—are proposed to give rise to *PDS* siRNAs (described in detail in ref. 9).

plification mechanism would compensate for dilution of the signal so that there could be a strong effect even at long distances from the source of the signal. The amplification phase could take place in cells that produce the signal so that more molecules are available to move. Alternatively, it could operate in cells that are distant from the source so that a few silencing RNAs could have a large effect in the cells that receive the signal. We have direct evidence for the link between this mobile silencing pathway and the P_{Suc2}:*PDS* transgene system, and our findings, together with those reported recently (35), are consistent with the intriguing possibility that tasiRNAs derived from *TAS1* and *TAS2* loci and endogenous siRNAs, such as those from *IR71*, may also spread in plants.

Our observation that THO/TREX-related mutations have an effect at *TAS1* and *TAS2* loci but not *TAS3* or at *IR71* and not other *IR* loci (Figs. 2 and 3) is consistent with findings that *Drosophila* THO/TREX influenced only <20% of all cellular mRNAs (27). Thus, in both animals and plants with coding or noncoding RNA, it seems that THO/TREX is selective. We do not know the molecular basis for this selectivity, or the structural clues THO/TREX employs to select particular RNA species. Similarly, we do not know whether THO/TREX in *Arabidopsis* is only committed to siRNA-producing RNAs. A whole-genome analysis of long and short RNAs in wild-type and *attex1* mutant *Arabidopsis* could provide answers to these questions.

Materials and Methods

Full details of experimental procedures are provided in *SI Materials and Methods*. In brief, P_{Suc2}:*PDS* lines 3 and 5, JAM14, JAM23, and JAM 32 were described previously (9). *attex1-4* (SALK_100012), *attho6-1* (SALK_051022), *attho1-1* (FLAG_204H05), and *attho2* alleles originated from the Salk Institute and INRA Versailles collections of T-DNA insertions (http://signal.salk.edu/cgi-bin/tdnaexpress; http://www-ijpb.versailles.inra.fr/en/sgap/equipes/variabilite/crg/index.htm; Table S2). For details of construct design for complementation analysis and for expression of N-terminal HA-epitope-tagged AtTEX1, as well as genetic mapping and genotyping *attex1*, *attho6-1*, *attho1*, and *attho2* mutant alleles, refer to *SI Materials and Methods*. For Northern blot hybridization, total RNA from aerial parts of 4- to 6-wk-old plants (only mature leaves for *attho1-1* and *attho6-1* analysis) was extracted using TRI Reagent (Sigma-Aldrich), separated on 1% formaldehyde (for high-molecular-weight RNA) or 15% PAGE/8 M urea gels (for low-molecular RNA), transferred to Hybond N+ (GE Healthcare) or Zeta Probe (Bio-Rad) membranes and subsequently hybridized with ^{32}P-radiolabeled DNA or RNA probes. For cDNA synthesis, reverse transcription reactions were carried out using SuperScript II or SuperScript III reverse transcriptase (Invitrogen) in the presence of random hexamers or oligo-dT. Oligonucleotides used in this study are listed in Table S4. For purification of AtTEX1 peptides, total or nuclear extracts from flowers were subjected to immunoprecipitation with HA-affinity matrix (Roche Applied Sciences), N-terminally HA-epitope-tagged AtTEX1 together with associated proteins was eluted from HA-affinity matrix by adding HA peptide (Roche Applied Sciences), precipitated, and analyzed as described in detail in *SI Materials and Methods*. In brief, LC-MS/MS analysis was performed using a LTQ Orbitrap Mass Spectrometer (Thermo Electron) and a nanoflow-HPLC system (Surveyor; Thermo Electron). Raw data were processed by using BioWorks 3.3 (Thermo Electron) and searched against the *Arabidopsis* genome supplemented with common contaminants (TAIR7 plus trypsin, keratins; sequences collated by Thermo Electron). Mascot results were combined in Scaffold (Proteome Software).

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