Gene Expression and Stress Response Mediated by the Epigenetic Regulation of a Transposable Element Small RNA

Andrea D. McCue¹, Saiavageethi Nuthikattu¹, Sarah H. Reeder¹, R. Keith Slotkin¹,²*

¹Department of Molecular Genetics, The Ohio State University, Columbus, Ohio, United States of America, ²The Center for RNA Biology, The Ohio State University, Columbus, Ohio, United States of America

Abstract

The epigenetic activity of transposable elements (TEs) can influence the regulation of genes; though, this regulation is confined to the genes, promoters, and enhancers that neighbor the TE. This local cis regulation of genes therefore limits the influence of the TE’s epigenetic regulation on the genome. TE activity is suppressed by small RNAs, which also inhibit viruses and regulate the expression of genes. The production of TE heterochromatin-associated endogenous small interfering RNAs (siRNAs) in the reference plant Arabidopsis thaliana is mechanistically distinct from gene-regulating small RNAs, such as microRNAs or trans-acting siRNAs (tasiRNAs). Previous research identified a TE small RNA that potentially regulates the UBP1b mRNA, which encodes an RNA–binding protein involved in stress granule formation. We demonstrate that this siRNA, siRNA854, is under the same trans-generational epigenetic control as the Athila family LTR retrotransposons from which it is produced. The epigenetic activation of Athila elements results in a shift in small RNA processing pathways, and new 21–22 nucleotide versions of Athila siRNAs are produced by protein components normally not responsible for processing TE siRNAs. This processing results in siRNA854’s incorporation into ARGONAUTE1 protein complexes in a similar fashion to gene-regulating tasiRNAs. We have used reporter transgenes to demonstrate that the UBP1b 3’ untranslated region directly responds to the epigenetic status of Athila TEs and the accumulation of siRNA854. The regulation of the UBP1b 3’ untranslated region occurs both on the post-transcriptional and translational levels when Athila TEs are epigenetically activated, and this regulation results in the phenocopy of the ubp1b mutant stress-sensitive phenotype. This demonstrates that a TE’s epigenetic activity can modulate the host organism’s stress response. In addition, the ability of this TE siRNA to regulate a gene’s expression in trans blurs the lines between TE and gene-regulating small RNAs.

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* E-mail: Slotkin.2@osu.edu

Introduction

Transposable elements (TEs) are mobile fragments of DNA that can accumulate and occupy large fractions of a genome, including over 45% of the human genome [1]. When active, TEs have the potential to create mutations by inserting into genes or generating breaks in DNA. To suppress the mutagenic potential of TEs, the eukaryotic genome has evolved defense mechanisms to inhibit TE proliferation, which are distinct from the developmental regulation of genes [2]. TEs are targeted for epigenetic repression mediated by the overlapping signals of cytosine DNA methylation, repressive histone tail modifications, and remodeling of chromatin into transcriptionally recalcitrant condensed heterochromatin (reviewed in [3]). Gene regulation can be influenced by the epigenetic regulation of TEs; however, this only occurs due to the proximity of a preexisting or newly transposed TE to a gene. This regulation of genes by neighboring TEs in cis can be due to multiple mechanisms, including interruption of a regulatory element, or by local spreading of repressive chromatin modifications such as DNA or histone methylation, resulting in position-effect variegation and potentially the formation of heritable epialleles [4–5].

TEs are major producers of small RNAs that act to maintain the TE in an epigenetically silenced state. In plants, and perhaps in animals, heterochromatin modifications are targeted by the activity of small RNAs. For example, in the mouse TE-derived piwi-interacting RNAs (piRNAs) guide DNA methylation to TEs [6]. In the reference plant Arabidopsis thaliana, the cycle of RNA-directed DNA methylation (RdDM) is initiated by the plant-specific RNA Polymerase IV (PolIV), which produces a non-protein coding transcript that is converted into double stranded RNA (dRNA) by the activity of RNA-dependent RNA Polymerase 2 (RDR2) [reviewed in [7]]. Dicer-like 3 (DCL3) cleaves this TE dRNA into small interfering RNAs (siRNAs) of 24 nucleotides (nt) in length, which are incorporated into either Argonaute 4 (AGO4), AGO6, or potentially AGO9 [8]. These siRNA-loaded Argonaute proteins act to maintain the heterochromatic state of TEs by targeting them for DNA and histone tail methylation.

Athila LTR retrotransposons are the largest family of TEs in Arabidopsis, occupying over 2.7% of the genome [9]. Athila elements are transcriptionally silenced, and silencing is dependent on symmetrical CG DNA methylation. When DNA methylation is removed, either in a DNMT1-homolog maintenance of DNA
The portion of the genome that does not encode for genes is often overlooked as a source of cellular regulatory information. Here, we demonstrate that regulatory information controlling expression and protein production from a gene called UBP1b is coming from a distant non-gene transposable element (TE). TEs are fragments of DNA that, unlike genes, are capable of duplicating themselves from one location in the genome to another, and occupy nearly half of the human genome. TEs are often referred to as “junk DNA,” as the study of cellular regulation and function is focused on genes. The regulation of TEs is distinct from genes, as a process termed epigenetic silencing heritably represses TE expression and activity. We have demonstrated that the epigenetic status (active versus silenced) of the Athila TE family regulates the UBP1b gene through the activity of a TE small RNA. The function of the UBP1b gene is to respond to and regulate cellular stress, and the epigenetic regulatory status of the Athila TE therefore modulates this stress response. This demonstrates that the epigenetic regulation of TEs can be a source of gene regulatory information, influencing a basic cellular function such as the stress response.

Author Summary

The portion of the genome that does not encode for genes is often overlooked as a source of cellular regulatory information. Here, we demonstrate that regulatory information controlling expression and protein production from a gene called UBP1b is coming from a distant non-gene transposable element (TE). TEs are fragments of DNA that, unlike genes, are capable of duplicating themselves from one location in the genome to another, and occupy nearly half of the human genome. TEs are often referred to as “junk DNA,” as the study of cellular regulation and function is focused on genes. The regulation of TEs is distinct from genes, as a process termed epigenetic silencing heritably represses TE expression and activity. We have demonstrated that the epigenetic status (active versus silenced) of the Athila TE family regulates the UBP1b gene through the activity of a TE small RNA. The function of the UBP1b gene is to respond to and regulate cellular stress, and the epigenetic regulatory status of the Athila TE therefore modulates this stress response. This demonstrates that the epigenetic regulation of TEs can be a source of gene regulatory information, influencing a basic cellular function such as the stress response.
produced from this region of the refutes previous data that characterized a specific microRNA is one member of a larger region of siRNA production. Our data leaf tissue, siRNA854 accumulates in *met1* compared to the extremely low levels in wt Col inflorescence and *Athila6* combined (Table 1). However, when epigenetic repression of *ddm1* and combined with the results of deep sequencing of small RNAs entire region is under the same epigenetic regulation as siRNA854, demonstrate that the production of 21–22 nt siRNAs from this siRNAs at levels comparable to those of siRNA854. These results reads per million than in pollen of wt Col plants, albeit to a lower level of 21 nt siRNA854 per million than in *met1* or *ddm1* mutants.

In the plant body, retrotransposons such as *Athila6* are tightly epigenetically suppressed by heritable symmetric DNA methylation and RdDM. In each case of 21 nt siRNA854 accumulation (*met1*, *ddm1* and pollen) loss of TE epigenetic silencing is known to occur [11–12,34–35]. To determine if the *Athila6* retrotransposon is specifically activated in these mutants and pollen, we performed real-time quantitative RT-PCR (qRT-PCR) and found that in *met1* and *ddm1* mutants and wt pollen, *Athila6* transcription accumulation is significantly increased compared to wt Col whole seedlings, leaf and inflorescence tissue (Figure 1A). We used qRT-PCR to measure *Athila6* expression (Figure 1), as well as a separate *Athila6* primer set specific to the microRNA stem-loop structure previously proposed (Figure S1) [29]. Both primer sets provided similar data, showing that neither the proposed stem-loop nor flanking *Athila6* region transcripts accumulate in wt Col seedlings, leaves or inflorescences, while both regions are expressed in *ddm1* and *met1* mutants. In addition, *Athila6* transcripts accumulate in wt Col pollen (Figure 1A). Compared relatively, pollen has considerably less *Athila6* transcript accumulation than either *ddm1* or *met1* mutants, perhaps due to the fact that pollen TE reactivation only occurs in the pollen vegetative nucleus, one of three nuclei expressing mRNA in mature pollen [12].

To examine siRNA854 accumulation in more detail, we performed small RNA Northern blots and found in wt Col and *ddm1* and *met1* in pollens, a 24 nt version containing the siRNA854 sequence accumulates, while 21–22 nt versions of this sequence accumulate only in *ddm1* and *met1* (Figure 1B), as well as in pollen (Table 1). We then probed this Northern blot with a 300 bp siRNA854-flanking region of *Athila6* (*Athila6* 3’ probe) and found that this region also produces other 24 and 21–22 nt siRNAs at levels comparable to those of siRNA854. These results demonstrate that the production of 21–22 nt siRNAs from this entire region is under the same epigenetic regulation as siRNA854, and combined with the results of deep sequencing of small RNAs from *ddm1*, *met1* and pollen [12–13], demonstrates that siRNA854 is one member of a larger region of siRNA production. Our data refutes previous data that characterized a specific microRNA produced from this region of the *Athila6* retrotransposon [29].

The phenotype of *ddm1* mutant plants becomes more severe in progressive generations. Second generation homozygotes for the recessive *ddm1-2* allele (*ddm1 F2*) display little to no morphological phenotype, while after propagation as a homozygote for four additional generations (*ddm1 F6*), leaf size and infertility phenotypes are severe [36]. Figure 1C shows that increasing transcript accumulation of the *Athila6* retrotransposon is associated with the progression of *ddm1* from the F2 to F6 generation. To determine if the different transcript levels of *Athila6* directly correlate with the accumulation of 21–22 nt siRNA854 and flanking 21–22 nt *Athila6* 3’ siRNAs, we examined siRNA854 accumulation by Northern blot in *ddm1* F2 and F6 individuals. F6 *ddm1* individuals produce increased levels of siRNA854 and other *Athila6* 3’ siRNAs compared to F2 generation *ddm1* individuals (Figure 1D). These data, together with the transcript accumulation and siRNA accumulation in *met1* and pollen (Figure 1A and 1B, Table 1), suggests that the epigenetic activation and level of *Athila6* steady-state transcripts directly and positively correlates with the accumulation level of *Athila6* 21–22 nt siRNAs, including siRNA854.

**siRNA854 biogenesis is atypical for a retrotransposon siRNA and requires RDR6, DCL2, DCL4, and AGO1**

To determine the biogenesis mechanism responsible for producing the 21–22 nt versions of *Athila* siRNAs and siRNA854, we first screened four tissues of wt Col and *ddm1* mutant plants and determined that *Athila6* 21–22 nt siRNAs are not detected in wt Col seedlings, roots, leaves or inflorescences (Figure 2A). *Athila6* 21–22 nt siRNAs are most easily detectable in *ddm1* inflorescence tissue, while leaf and seedling tissues have lower relative levels, and the siRNAs are undetectable in roots (Figure 2A). In wt Col, 24 nt *Athila6* siRNAs weakly accumulate in the root and inflorescence (Figure 2A), and these inflorescence 24 nt siRNAs are dependent on the *PolIV* component *NRPD1A, RDR2*, and the small RNA-modifying protein *HEN1* (Figure 2B). *HEN1* is responsible for the accumulation of both microRNAs and siRNAs [37], while the requirement of *NRPD1A* and *RDR2* demonstrates that, like other known 24 nt TE siRNAs, *Athila6* 24 nt siRNAs are generated by the RdDM pathway which is responsible for maintaining epigenetically silenced regions of the genome [21,38]. The 24 nt siRNA854 accumulation in wt Col inflorescences is not dependent on *RDR6*, the *PolV* component *NRDE1*, or the microRNA processing *DCL1* (Figure 2B). Contrary to previously published results, these data demonstrate that there is no siRNA854 version in wt Col inflorescences that is dependent on the microRNA machinery.

To determine the small RNA biogenesis pathway responsible for producing 21–22 nt siRNA854 and *Athila6* siRNAs when *Athila* is epigenetically activated, we generated 12 double mutant combinations with *ddm1*, using mutants for genes with known

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**Table 1. Frequency of 21 nt siRNA854 in SBS small RNA libraries.**

| siRNA854 | genome matched reads | siRNA854 N 1M | Reference |
|----------|----------------------|---------------|-----------|
| Col      | 0                    | 2,516,337     | 0         | [13]      |
| *met1*   | 290                  | 1,506,711     | 192       | [13]      |
| Col      | 1                    | 3,200,398     | 1         | [12]      |
| *ddm1*   | 268                  | 3,568,226     | 75        | [12]      |
| Col      | 0                    | 926,951       | 0         | [16]      |
| Col pollen | 14                   | 551,394       | 25        | [12]      |

*A* Does not include tRNA, rRNA, snRNA, snoRNA reads.

*Number of siRNA854 reads normalized per 1 million.

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roles in the various Arabidopsis small RNA biogenesis pathways, such as different AGO, DCL and RDR genes. Double mutant inflorescences were used to assay the accumulation of siRNA854 (Figure 2C). As some of these double mutants are in Col x Landsberg erecta (Ler) mixed genetic backgrounds, as a control we confirmed that Ler ddm1 mutants also accumulate Athila6 siRNAs, while wt Ler does not. We found that the RdDM pathway responsible for producing Athila6 24 nt siRNAs involving NRPD1A and RDR2 does not generate the 21 nt or 22 nt siRNA854. This represents a change in siRNA biogenesis pathways for Athila6 siRNAs, as their epigenetic reactivation results in a new set of proteins responsible for the 21–22 nt siRNA production. We determined that RDR6 function is required for both 21 nt and 22 nt siRNA854 accumulation, as in ddm1 ror6 double mutants neither of these siRNAs accumulate (Figure 2C), while RDR6 is not responsible for the 24 nt version of these siRNAs (Figure 2B). RDR6’s involvement suggests that an Athila6 transcript is copied into dsRNA, which is required for 21–22 nt siRNA production. We also found that in ddm1 dcl4 double mutants, the 21 nt siRNA854 is absent, while increased levels of the 22 nt and 24 nt versions are detected. There are well-described hierarchical relationships among DCL2, DCL3, and DCL4. When DCL4 is not present to generate 21 nt siRNAs, DCL2 primarily substitutes for this function and generates 22 nt siRNAs, while DCL3 substitutes for DCL4 to a lesser degree and produces 24 nt siRNAs [39]. Conversely, ddm1 lsd2 double mutants lose the 22 nt version of Athila6 siRNAs, including siRNA854 (Figure 2D), demonstrating that the 21 nt and 22 nt versions of siRNA854 that accumulate in ddm1 mutants are generated by the activity of DCL4 and DCL2, respectively. The production of 21 nt or 22 nt siRNA854 in either ddm1 lsd2 or ddm1 dcl4 double mutants suggests that the processing by DCL4 and DCL2 proteins occurs after RDR6 converts the Athila6 transcript into dsRNA. In addition, the proteins responsible for the biogenesis of the 24 nt version of siRNA854 and,
separately, for the 21–22 nt version are identical to those responsible for generating the corresponding sizes of the flanking Athila6 siRNAs, further indicating that siRNA854 is not solely or specifically cleaved from this region.

To determine which Argonaute protein(s) are responsible for siRNA854 accumulation, we tested ddm1 double mutants with ago1, ago4, ago5, ago6, and ago10. ddm1 double mutants with ago1, ago5, and ago6 did not result in loss of siRNA854, and ago10 double mutants displayed only reduced accumulation (Figure 2C). In the ddm1;ago1 double mutant both the 21 nt and 22 nt versions of siRNA854 fail to accumulate (Figure 2E), demonstrating that AGO1 is essential for 21–22 nt siRNA854 accumulation. The requirement of RDR6, DCL2, DCL4, and AGO1 suggests that either the known VIGS pathway of post-transcriptional degradation of...
viral RNAs, or the tasiRNA pathway is responsible for Athila6 21–22 nt siRNA biogenesis.

While generating the ddm1 double mutant plants, we encountered an unusual pattern of inheritance of Athila6 siRNAs, which stems from the atypical genetic inheritance of ddm1 mutants. For example, both the ddm1 mutant phenotype and Athila6 expression become more severe over increasing generations (Figure 1C) [36]. In addition, ddm1/+ heterozygous plants produced by crossing a plant homozygous for the ddm1-2 recessive allele to wt Col inherit epigenetically decondensed and transcriptionally uncontrolled chromatin from the ddm1 parent, which is not fully restored in the ddm1/+ heterozygote [40–42]. This mutant chromatin in a ddm1/+ heterozygous individual continues to express TEIs [42]. In Figure 2F we demonstrate that a ddm1/+ heterozygote produced from a ddm1 homozygous parent (Col x ddm1 in Figure 2F) still produces 21–22 nt siRNA854 and Athila6 3’ siRNAs, although to a considerably lower level than the ddm1 homozygote. This is in contrast to a ddm1/+ heterozygote that was not the progeny of a homozygous parent, but has been backcrossed to wt Col for six generations while being maintained as a heterozygote. In this ddm1/+ heterozygote (ddm1/+ in segregating family, Figure 2F) the amount of mutant chromatin inherited from the ddm1 homozygous parent has been diluted away by segregation in each generation of crossing to wt Col, demonstrating that there is an effect of the parent’s genotype on the production of Athila6 siRNAs in ddm1/+ heterozygous plants. The requirement of AGO1 for the production of 21–22 nt siRNA854 in ddm1/+ heterozygotes was demonstrated using an F2 family segregating for ago1 and ddm1, which was produced from a ddm1 homozygous P1 individual (Figure 2E). In ago1 mutants that are ddm1/+ heterozygotes (ago1/ddm1+), neither 21 nor 22 nt versions of siRNA854 accumulate, while they do in the corresponding ago1+/+ddm1/+ double heterozygote siblings. These data demonstrate that AGO1 is necessary for siRNA854 accumulation in ddm1 mutants and in the progeny of ddm1 homozygotes.

siRNA854 accumulation represses reporter gene transcripts with the UBP1b 3’UTR

The 21 nt version of siRNA854 was previously predicted to target the 3’UTR of the UBP1b gene in four locations using modified criteria that allows for non-canonical or ‘wobble’ G-U base pairing [29]. G-U base pairing has been demonstrated to be tolerated in microRNA target sites, even within the critical first 7 base pairs (bp) or ‘seed’ pairing region [43]. However, the targeting of the 3’UTR by small RNAs was not previously shown directly, and complementarity of siRNA854 to the UBP1b 3’UTR relies heavily on non-canonical base pairing and lacks a strong 7 bp seed-pairing region (shown in Figure S2). To directly test if the 21 nt siRNA854 sequence has the ability to target the UBP1b 3’UTR, we took advantage of the fact that wt Col inflorescences do not accumulate 21–22 nt siRNA854 (Table 1, Figure 1, Figure 2). To directly examine the role of the siRNA854 sequence, we constructed plants constitutively expressing a GUS reporter gene with the GUS mRNA fused to the UBP1b 3’UTR and transformed these plants with artificial microRNA (amiRNA) constructs expressing the siRNA854 sequence, or an unrelated sequence as a control, from the Arabidopsis microRNA319a stem-loop transcript [44]. Quantitative assays to detect GUS activity, as well as qualitative plant staining, demonstrate that the plants with the control amiRNA have high levels of GUS activity, while plants expressing the siRNA854 sequence from a microRNA stem-loop display significantly lower levels of GUS activity (Figure 3A). These data demonstrate that although the alignment of siRNA854 to the UBP1b 3’UTR lacks a strong seed pairing region and relies on G-U base pairing, the 21 and/or 22 nt siRNA854 sequence can target the UBP1b 3’UTR resulting in decreased reporter protein accumulation.

The developmental expression profiles of UBP1b and the six Athila6 elements on the Affymetrix ATH1 gene expression microarray are negatively correlated, with UBP1b expressed highly in all wt tissues except mature pollen, specifically where Athila6 activation occurs (Figure S3A). To determine if the increased levels of endogenous 21–22 nt siRNA854 observed when Athila6 is epigenetically activated can regulate the UBP1b 3’UTR, we transformed both wt Col and ddm1 plants with either the GUS-UBP1b 3’UTR transgene from Figure 3A, or a control transgene without the UBP1b 3’UTR. We assayed GUS activity in plants homozygous for the transgenes and found that in wt Col, the presence of the UBP1b 3’UTR did not affect GUS activity (Figure 3B). In contrast, when this same analysis was previously published, the same GUS-UBP1b 3’UTR transgene in a wt Col plant resulted in little to no GUS protein production in leaves and inflorescences [29]. Our data, which demonstrate no inhibition of the UBP1b 3’UTR in wt Col leaves and inflorescences, are supported by the fact that there is no 21/22 nt siRNA854 detected in leaves or inflorescence by either Northern blot (Figure 1, Figure 2A) or small RNA deep sequencing (1 read in a combined 6.6 million) (Table 1).

In ddm1 mutants, the GUS-UBP1b 3’UTR and GUS control (no 3’UTR) transgenes both display reduced GUS activity (Figure 3B). It remains enigmatic why the constitutively expressed GUS transgene without a 3’UTR has reduced expression in ddm1 compared to wt Col. However, the presence of the UBP1b 3’UTR resulted in a significant reduction of GUS activity compared to the no 3’UTR control in ddm1 (Figure 3B). To make sure that position effects of these transgene insertions were not the cause of this differential regulation, we crossed a wt Col plant with the UBP1b 3’UTR transgene that displayed high GUS activity to a ddm1 homozygote, as the resulting heterozygote will have siRNA854 accumulation (Figure 2F). The GUS activity in this ddm1 heterozygote is significantly reduced compared to both the wt Col homozygous GUS transgene parent and to wt Col plants heterozygous for the same GUS transgene (Figure 3C). Therefore, the regulation of the UBP1b 3’UTR is sensitive to the levels of siRNA854, with either the production of this sequence as an amiRNA, or accumulation of this sequence as an siRNA in ddm1 resulting in repression of GUS activity. We determined that all of the transgenes in either wt Col or ddm1 from Figure 3B have GUS transcripts that accumulate to similar levels (Figure 3D), indicating that the regulation of these transgene transcripts is not due to post-transcriptional degradation of the GUS RNA, but is likely rather due to the inhibition of translation of these mRNAs.

In addition to the increased levels of siRNA854 in ddm1 mutants, siRNA854 also accumulates in wt Col pollen (Table 1). To determine if endogenous siRNA854 in pollen is able to regulate the UBP1b 3’UTR, we performed similar transgene reporter assays as above in wt Col pollen. We used a pollen vegetative cell promoter to drive GFP and added one of three different 3’UTRs to these reporter transgenes. GFP fluorescence was quantitatively measured by subtracting the fluorescence of segregating pollen grains that did not inherit the transgene from the fluorescence of pollen grains that did inherit the transgene. With no 3’UTR, transgene protein accumulates, and a moderate level of fluorescence is observed (Figure 4). When the wt UBP1b 3’UTR is added to this transgene, significantly less fluorescence is observed, likely due to the accumulation of siRNA854 in wt Col pollen. To test if the binding of siRNA854 was specifically responsible for this regulation, we generated a version of the UBP1b 3’UTR that lacks
Figure 3. Accumulation of 21–22 nt siRNA854 negatively regulates transgene transcripts with the **UBP1b** 3' UTR. (A) Plants homozygous for a transgene constitutively expressing the GUS reporter protein fused to the **UBP1b** 3’ UTR (35S:GUS-**UBP1b** 3’ UTR) were transformed with an artificial microRNA (amiRNA) with the siRNA854 sequence (35S:amiRNA-854), or a second control sequence that does not target **UBP1b** (35S:amiRNA-control).
amiRNA-control). GUS activity was monitored using a quantitative assay (left) and inflorescence staining (right). Plants expressing the siRNA854 sequence as an artificial microRNA show significantly reduced GUS levels. (B) Col and ddmi plants homozygous for a constitutively expressed GUS transgene (35S::GUS) or the same reporter transgene with the UBP1b 3’ UTR from part A. As in A, GUS activity was monitored using a quantitative assay (left) and plant staining (right). Wt Col plants show no differential regulation between the two transgene variations, while in the ddmi mutant background the GUS activity of the UBP1b 3’ UTR transgene is significantly less than the control 35S::GUS transgene. (C) A 35S::GUS-UBP1b 3’UTR transgene in the wt Col background was crossed to a ddmi homozygote, and the GUS activity was measured in the F1 plant. GUS activity of the same hemizygous transgene in the wt Col background is shown as a control. (D) RT-PCR of the same transgenic individuals from part B. The GUS activity differences observed in part B are not reflected in transgene transcript levels, demonstrating that this regulation is not due to post-transcriptional mRNA degradation. In A, B and C, the box plot whiskers represent the minimum and maximum of the dataset, the top and bottom of the box are the 75th and 25th percentile (respectively), the middle line is the median, and + is the mean. The number of individuals assayed (n) is shown in or near the box.
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all four of the siRNA854 predicted target sites, resulting in a shorter 3’UTR (shown in Figure S2). This deleted 3’UTR transgene (DEL transgene) resulted in significantly increased fluorescence compared to the wt UBP1b 3’UTR (Figure 4). We also produced a UBP1b 3’UTR variation of the same length as the wt UBP1b 3’UTR, in which each of the perfectly complementary base pairs in all four of the siRNA854 predicted target sites have been switched to bases that do not show complementarity (or G-U pairing) with siRNA854 (Figure S2). Pollen grains with the base-modified 3’UTR (MOD transgene) on the GFP transcript display significantly increased fluorescence compared to the wt UBP1b 3’UTR (Figure 4), demonstrating that these bases are necessary for the regulation of the UBP1b 3’UTR. Pollen from both the MOD and DEL 3’UTR transgenes display fluorescence levels even higher than the control lacking a 3’UTR, likely due to the ability of the UBP1b 3’UTR, when not targeted by small RNAs, to stabilize transcripts or promote their translation. Lastly, we transformed the GFP transgene with and without the wt UBP1b 3’UTR into vrd6 mutants. We observed that the expression of Lat52-GFP (no 3’UTR) in vrd6 mutant pollen is higher than that of the same transgene in wt Col pollen (Figure 4). This difference is likely due to the role of RDR6 in post-transcriptional silencing of transgenes [45]. We speculate that the wt Col Lat52-GFP transgene is subject to a certain low amount of post-transcriptional regulation mediated by RDR6. When this transgene is present in vrd6 mutant plants, this post-transcriptional regulation does not occur, resulting in higher expression of the transgene compared to wt Col. Interestingly, we did not observe a reduction in pollen fluorescence for the Lat52-GFP-UBP1b 3’UTR transgene in vrd6 compared to the no-3’UTR control transgene in vrd6 (Figure 4), demonstrating that RDR6 is necessary for the targeting of the UBP1b 3’UTR in pollen. The combined data from Figure 3 and Figure 4 demonstrate that the RDR6-dependent accumulation of siRNA854 and base pairing with the UBP1b 3’UTR target sites are required for the inhibitory regulation of UBP1b 3’UTR reporter genes.

siRNA854 represses endogenous UBP1b in pollen

We aimed to determine if siRNA854 has a regulatory effect on the endogenous UBP1b gene or transcript. To aid our characterization of UBP1b we isolated two mutant uphl alleles, which are in the Ws background. UBP1b insertion alleles result in a lack of polyadenylated transcripts, although un-spliced and non-polyadenylated transcripts are still produced (Figure S4).

First, we wondered if the sequence similarity between the 21, 22 or 24 nt versions of siRNA854 was directing DNA methylation to the UBP1b 3’UTR through the RdDM pathway, as a possible mechanism of epipalele production. We have determined that the DNA methylation status of the UBP1b 3’UTR is not altered in ddmi inflorescences relative to wt Col (Figure S3A). Next, we utilized microarray data and RT-PCR to analyze UBP1b transcript levels, and we found they accumulate to the highest levels in inflorescence tissue, intermediate levels in seedling and leaf tissues, and either to extremely low levels or not at all in wt Col pollen (Figure S3). We measured UBP1b transcript accumulation in ddmi1 mutants at two developmental time points: inflorescence buds and mature pollen. In inflorescence tissue, the transcript accumulation of UBP1b is not significantly altered in ddmi1 F2 or ddmi1 F6 inflorescences (Figure 5A). Therefore, qRT-PCR expression analysis and DNA methylation analysis both demonstrate that in inflorescence tissue there is no transcriptional or post-transcriptional effect of siRNA854 on UBP1b transcript accumulation. We continued to assay UBP1b in inflorescence tissue of wt Col and ddmi supposing that the regulation may be on the translational level, as was observed for the inflorescences of the GUS-UBP1b 3’UTR transgene in Figure 3. We assayed two known microRNA-induced alterations to transcripts associated with translational regulation (reviewed in [46]). We determined that in ddmi inflorescence tissue the polyA tail length of UBP1b is unaffected, and un-capped transcript does not accumulate (Figure S5B and S5C). Without the availability of a specific antibody to assay endogenous UBP1b protein accumulation, we can provide no direct evidence that endogenous UBP1b transcripts are regulated by the elevated siRNA854 levels that accumulate in ddmi inflorescences.

In contrast to inflorescence tissue, the transcript accumulation of UBP1b in pollen is regulated at the post-transcriptional level by siRNA854. In wt Col pollen, the UBP1b transcript does not accumulate (Figure S3). To determine if this is a consequence of the increased levels of siRNA854 in pollen, or if the UBP1b promoter is simply not active in mature pollen, we performed qRT-PCR in vrd6 mutant plants as well as from plants heterozygous for ago1. Plants homozygous for the recessive ago1-11 allele do not produce pollen, so we used an ago1-11/+ heterozygote that produces pollen segregating 1:1 wt and mutant for ago1. In both vrd6 pollen and ago1 segregating pollen there is a significant increase in UBP1b transcript accumulation, with vrd6 having a >16-fold increase (Figure 5A). This demonstrates that the UBP1b promoter is active in pollen, but the transcripts are subject to post-transcriptional degradation. Attempts at identifying siRNA854-induced cleavage sites in the UBP1b 3’UTR from inflorescence and pollen were inconclusive (data not shown), likely due to the high rate of non-small RNA-induced processing and degradation of the endogenous UBP1b 3’UTR detected in whole genome degradome analysis [47–48].

To determine if the UBP1b 3’UTR is specifically responsible for the differential UBP1b accumulation in inflorescence and pollen, we generated two transgenes with the native UBP1b promoter and coding region, with and without the 3’UTR. This transgene also has a 3’ FLAG epitope tag to distinguish it from the endogenous UBP1b. We found that the presence of the UBP1b 3’UTR in inflorescence tissue increases the transcript accumulation levels >11-fold, presumably by stabilizing this transcript (Figure 5B). In wt Col pollen the UBP1b promoter is active, and without the 3’UTR this transcript accumulates to levels 4-fold less compared
to inflorescence tissue. However, in contrast to inflorescence tissue, the addition of the UBP1b 3′UTR resulted in >73-fold reduced transcript accumulation in wt pollen. Together, these data demonstrate that in wt Col pollen the presence of the UBP1b 3′UTR causes a decrease in UBP1b transcript accumulation.

The mature pollen grain contains two sperm cells with highly condensed chromatin imbedded into the larger vegetative cell, which displays a chromatin-decondensed vegetative nucleus. Communication between the vegetative cell and imbedded sperm cells has been previously hypothesized to occur (reviewed in [49]). To determine in which cell post-transcriptional silencing in the pollen grain is taking place, we aimed to decipher where in the mature pollen grain the repression of the UBP1b 3′UTR is occurring. Since we demonstrated that both RDR6 and AGO1 are necessary for the repression of the endogenous UBP1b transcript in the mature pollen grain (Figure 5A), we examined the localization of the RDR6 and AGO1 proteins by fusing them to GFP and expressing them from their native promoters. We found that both of these proteins localize to the nucleus and cytoplasm of the pollen vegetative cell and are not detectable in sperm cells (Figure S6), in agreement with mined microarray data from purified sperm cells [50]. The pollen vegetative cell is also the location of epigenetic TE reactivation [12], suggesting that the activation of Athila6, cleavage into siRNAs and potentially the repression of the UBP1b 3′UTR are all occurring in this cell.

Since a functional AGO1 protein is required for the accumulation of siRNA854 (Figure 2E), and UBP1b transcript levels increase in segregating mutant ago1 pollen (Figure 5A), we aimed to determine if siRNA854 is incorporated into AGO1 protein complexes. We performed an immunoprecipitation (IP) of AGO1 complexes using a commercially available AGO1 antibody and purified the incorporated small RNA. To verify the specificity of the AGO1 antibody, we demonstrated that this antibody does not detect any other proteins by first performing a Western blot on protein extracts from Col, Ler, and ago1-11 inflorescences. We found that the AGO1 antibody yields no cross-reactive bands (Figure S7A). Additionally, we used Western blot analysis to confirm the success of the IP by both detecting the presence of AGO1 protein in the IP Input extract and AGO1 IP samples, and observing the absence of AGO1 protein in the no antibody IP control (Figure S7B). After the IP, we purified the AGO1-bound small RNAs and used qRT-PCR to assay levels of siRNA854, a known AGO1-incorporated microRNA (miR161), a known AGO1-incorporated tasiRNA (TAS3a-D8), and two 24 nt heterochromatic siRNAs not present in AGO1 complexes (siRNA02 and siRNA1003) [51–52]. We found no AGO1-IP enrichment of siRNA854 or the control siRNAs, siRNA02 and siRNA1003, in the wt Col plant body, while we did detect enrichment of the control microRNA miR161 and control tasiRNA TAS3a-D8 (Figure 5C). In contrast, in abin1 F2 and F6 plants we found
Figure 5. Tissue-specific regulation of UBP1b by siRNA854. (A) qRT-PCR of UBP1b in inflorescence tissue and mature pollen. In inflorescence tissue, UBP1b transcript accumulation is not affected in ddm1 mutants. In wt Col pollen, UBP1b expression significantly increases in rdr6 mutants and in a pool of pollen that is segregating 1:1 ago1 mutant pollen (seg ago1). (B) qRT-PCR of FLAG-tagged UBP1b transgenes with and without the UBP1b 3'UTR and under control of their native promoters. In inflorescence tissue, the addition of the UBP1b 3’UTR results in increased transcript levels. In pollen, the UBP1b promoter is active, and addition of the UBP1b 3’UTR results in significantly decreased levels of mRNA. (C) qRT-PCR of small RNAs from AGO1-IP biological replicates demonstrating that in the plant body of ddm1 mutants siRNA854 is enriched in AGO1 protein complexes, while it is not in wt Col. Relative enrichment values over 1.0 indicate AGO1-enrichment, whereas relative enrichment values under 1.0 indicate no enrichment. Relative enrichment was calculated based on amplification of the input sample for each IP. MiR161 and TAS3a-D8 are shown as positive controls while siRNA02 and siRNA1003 are 24 nt siRNA negative controls not bound by AGO1. qRT-PCR melting curves for siRNA854 amplification products.
enrichment of siRNA854 in AGO1. Analysis of the melting curves generated from the products of the qRT-PCR demonstrate that the background levels of siRNA854 from wt Col plants are the larger 24 nt version (which have higher melting temperatures) compared to the AGO1-enriched 21–22 nt siRNA854 from ddm1 plants (lower melting temperatures) (Figure 5C). The level of enrichment of siRNA854 in AGO1 complexes in ddm1 mutants is not as high as miR161, but is more similar to the level of enrichment of the tasRNA TAS3a-D8 in wt Col (Figure 5C), likely due to the fact that both siRNA854 and TAS3a-D8 are single siRNAs from transcripts that generate multiple siRNAs through the activity of RDR6 and DCL4. In addition, we detected no difference in enrichment levels between ddm1 F2 and ddm1 F6 plants. However, since there are higher levels of siRNA854 in the ddm1 F6 plants (Figure 1D) and input (non-IP) sample that was used for normalization, there are likely more AGO1 protein complexes interacting with siRNA854 in F6 ddm1 plants compared to the F2 generation. These data demonstrate that only when epigenetically activated, the Athila6-generated 21–22 nt siRNA854 is incorporated into AGO1, and this complex is responsible for the regulation of the UBP1b transcript.

Altered stress-regulation in ddm1 mutants

TIA-1 has a known role in the sensing and response to cellular stress, and mutant cells unable to form stress granules display increased sensitivity to stress [53–55]. We have experimentally determined that the germination and growth of ubp1b mutant plants are also sensitive to both ionic (+NaCl) and osmotic (+mannitol) stress conditions compared to its corresponding wt backgroundWs, particularly when grown on 100 mM NaCl or 300 mM mannnitol (Figure 6A). In addition, wtWs itself is more sensitive to these stresses than wt Col, as at higher NaCl or mannnitol concentrations, wt Col survives but wtWs does not. Since ddm1 plants have increased levels of siRNA854 (Table 1, Figure 1), and siRNA854 can target the UBP1b 3’ UTR (Figure 3, Figure 4), we tested ddm1 seedlings to determine if they display a similar stress sensitivity as ubp1b mutant plants. Plants that have been homozygous ddm1 for six generations (ddm1 F6) are significantly more sensitive than the corresponding wt Col for both ionic and osmotic stress, while ddm1 F2 is only sensitive to ionic stress (Figure 6A). Similar to ubp1b mutants, ddm1 mutant plants are sensitive to ionic and osmotic stress conditions. These data, combined with our demonstrated regulation of UBP1b levels by siRNA854 [Figure 5], suggest that the ddm1 stress sensitivity acts directly through epigenetic activation of Athila6 and production of siRNA854, which results in the post-transcriptional and/or translational repression of UBP1b.

The mammalian homolog of UBP1b is TIA-1, an RNA binding protein localized to the nucleus that moves into the cytoplasm and aggregates into stress granules upon induction of stresses such as treatment with arsenite, glucose deprivation, and viral infection [56–58]. To visualize the sub-cellular localization of the UBP1b protein in response to cellular stress in Arabidopsis, as well as to determine the influence of ddm1 on this process, we expressed an siRNA854-resistant version of UBP1b (without its native 3’ UTR) fused to GFP, under constitutive expression. In wt Col seedling roots this protein is localized to the nucleus, with distinct bright peri-nuclear foci observed [Figure 6B]. However, when transformed into ddm1 plants, this same transgene displayed cytoplasmic fluorescence [Figure 6B]. We aimed to induce stress in both the wt Col and ddm1 UBP1b-GFP lines; however, the ionic and osmotic conditions from Figure 6A inhibited growth in the ddm1 plants. Therefore, we experimentally determined that germination and growth in the dark (etiolation) would generate a UBP1b protein stress response, without killing the plant. We germinated and grew the wt Col UBP1b-GFP plants under etiolation conditions and observed a shift in the sub-cellular localization of UBP1b-GFP, as fluorescence accumulated around the periphery of the nucleus and in the cytoplasm (Figure 6B). This change in sub-cellular distribution of UBP1b-GFP under a condition of stress (etiolation) is analogous to the movement of TIA-1 out of the nucleus under stress conditions [59]. In etiolated ddm1 plants, the UBP1b-GFP fluorescence pattern is the same as in non-stressed ddm1 plants (data not shown). We quantified these fluorescence patterns by measuring the amount of nuclear vs. cytoplasmic fluorescence and found a statistically significant difference between the localization of UBP1b-GFP in unstressed wt Col compared to unstressed ddm1 (Figure 6C). The unstressed ddm1 fluorescence pattern resembles the stressed wt Col roots (Figure 6B and 6C). Additionally, in a very small number of ddm1 cells (roughly 1/1000), we observe a second accumulation pattern that displays distinct cytoplasmic foci reminiscent of mammalian stress granules (red arrows, Figure 6B), as well as fluorescence at the periphery of the nucleus. Together, these data suggest that the translocation of the UBP1b protein in ddm1 mutant cells is a response to an intracellular stress as a result of the ddm1 mutation, perhaps due to altered gene expression in ddm1 mutants, or due to the loss of DNA methylation, loss of repressive histone modifications, and activation of TEs [4,60].

Discussion

The epigenetic control of Athila6 and siRNA854

siRNA854 is a gene-regulating endogenous siRNA that is produced from the Athila6 family of LTR retrotransposons, and its accumulation is strictly dependent upon the transcriptional epigenetic activation of the Athila6 element. Athila elements, as well as nearly all other types of TEs in wt Col Arabidopsis, are normally transcriptionally silenced and are associated with DNA methylation and 24 nt siRNAs involved in maintaining the transcriptionally silenced state [7,13]. The 21–22 nt versions of siRNA854 are only produced upon Athila6 transcriptional activation, such as in ddm1 and met1 mutants, or in the vegetative cell of wt pollen. Like Athila6 activity itself, siRNA854 production displays two unusual epigenetic trans-generational inheritance patterns. First, siRNA854 is produced in a ddm1/+ heterozygote that was generated from a ddm1 homozygote, and the pathway of this accumulation remains dependent on AGO1 in the ddm1/+ heterozygote. Second, there is a positive correlation between the increasing levels of Athila6 mRNA and accumulation of Athila6 21–22 nt siRNAs (including siRNA854) as ddm1 mutants are propagated from the F2 to the F6 generation. The progressively increasing transcript levels in ddm1 F2 to F6 generations could be due to increased rates of transcription, perhaps due to progressive loss of heterochromatin control, such as Athila DNA methylation, each generation. The positive correlation in Athila mRNA and siRNA levels suggests that the Athila6 transcript is the limiting factor in siRNA854 production, and that at least some Athila6 mRNA transcripts can accumulate although the 3’ region of

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from the AGO1-IP demonstrate that the non-enriched siRNA854 in wt Col is the larger 24 nt version (higher melting temperature), while the AGO1-enriched siRNA854 in ddm1 is the 21–22 nt version (lower melting temperature).
**TE Epigenetic Regulation Controls Gene Expression**

**A**

![Graph showing survival rates under different conditions](image)

**B**

![Images of cellular structures under different conditions](image)

**C**

![Box plot showing nuclear GFP fluorescence](image)
Figure 6. UBP1b protein localization and stress sensitivity of ddm1. (A) Survival of plants grown under conditions of ionic and osmotic stress. Seeds were spotted on plates containing no stressor, increasing levels of NaCl or increasing levels of mannitol. After 11 days the number of surviving plants was counted. Wt plants of the Ws background are more sensitive to the stressors tested than wt Col. Compared to the corresponding wt Ws mutant plants and wt pollen are not

**siRNA854 biogenesis**

Most *Arabidopsis* TEs lose siRNA production when epigenetically activated. There is an unknown factor that causes some TEs, such as *Athila*, to produce siRNAs even when transcriptionally active. In contrast to some TE families such as *ATGP1*, which simply retains 24 nt siRNAs when activated, *Athila* is one of only very few element families identified to date that produces 21–22 nt siRNAs when epigenetically activated and expressed [12,15–16]. The production of 21–22 nt siRNAs represents a shift in small RNA biogenesis pathways from PolIV-dependent 24 nt siRNAs processed by the RdDM pathway, to a post-transcriptional silencing pathway that presumably uses PolII-derivates transcripts and involves DCL2, DCL4, RDR6 and AGO1, with at least siRNA854 eventually incorporated into AGO1. These DCL, RDR and AGO proteins act both in the tasiRNA and VIGS pathways, and *Athila* processing shows hallmarks of both. For example, the VIGS pathway likely acts on *Athila6* transcripts, as *Athila* has evolved from an LTR retrovirus, and, due to the conservation of the ***envelope*** protein coding domain, the Athila4 subfamily has even been classified as an *Arabidopsis* endogenous retrovirus [61]. *Athila* siRNAs may have been produced via the VIGS pathway, however, siRNA854’s ability to regulate *UBP1b* in trans is functionally more similar to the tasiRNA pathway. Therefore, we defer classifying *Athila6* 21–22 nt siRNAs as either tasiRNAs or VIGS siRNAs.

The classification of *Athila6* 21–22 nt siRNAs as either tasiRNAs or VIGS siRNAs perhaps can be resolved once the initiation of their production is understood. We currently have three models for how the initiation of *Athila6* 21–22 nt siRNAs may occur. First, the secondary structure of the Athila6 transcript, particularly in the region of siRNA production, may fold back into hairpin-like structures, producing a substrate for DCL4 cleavage. Second, overlapping sense and antisense Athila6 transcripts may result in the formation of a dsRNA trigger, as *Athila* elements accumulate in nested arrays of elements near the centromere that favor the production of read-through transcripts [reviewed in [62]]. A pathway of natural antisense transcript siRNA (nat-siRNA) production exists in *Arabidopsis*, however, PolIV and RDR2 are required for this pathway [63–64], and we have experimentally determined that these proteins are not required for *Athila6* 21–22 nt siRNA accumulation. A third proposed mechanism of 21–22 nt Athila6 siRNA initiation may be similar to tasiRNA initiation and the initiation of islands of 21–22 nt siRNA accumulation in maize and rice. MicroRNA[s] may initiate the cleavage of an Athila6 transcript, causing the production of RDR6- and DCL4-dependent siRNAs. In rice, the production of 21 nt phased siRNAs is dependent first on microRNA cleavage, and then on OsDCL4 for production, and these siRNAs preferentially accumulate in male reproductive organs [65]. One microRNA that shows potential seed regions complementarity to *Athila6* is the 22 nt microRNA845b; however, *Athila* siRNAs are produced on either side of the predicted *Athila6* cleavage site, and our experiments to date provide no evidence that microRNA845b is required for *Athila6* 21–22 nt siRNA biogenesis (data not shown). In addition to acting downstream of siRNA854 production, it is currently unknown if AGO1 acts upstream of DCL4 and RDR6 to initiate *Athila6* transcript cleavage, but it is likely that a 21 nt siRNA initiates the RDR6-dependent amplification of *Athila6* siRNAs [66]. If initiated by a tasiRNA-like mechanism, tasiRNA-like phasing should be detected in the *Athila6* siRNA production. We have not detected any such phasing of *Athila6* siRNAs (data not shown), but this analysis is complicated by the 12 nearly identical *Athila6* elements that carry siRNA854, and dozens more *Athila* elements that are cleaved into siRNAs at the same time. If each element that produces 21–22 nt siRNAs is correctly phased, but not in the same register as each other, our analysis would detect no phasing. Therefore, although we have identified AGO1, RDR6 and DCL4 as necessary for the accumulation of siRNA854, the trigger for *Athila6* siRNA initiation remains to be elucidated.

**The regulation of UBP1b by siRNA854**

We used the *UBP1b* 3′UTR in reporter assays to demonstrate that whenever we observe the accumulation of the 21–22 nt siRNA854 sequence (in *ddm1* mutants, wt pollen, or expressed as an amiRNA), we observe decreased reporter protein accumulation. Expression of the siRNA854 sequence as an amiRNA in the vegetative tissue of wt plants demonstrates that the potentially complicating secondary effects occurring from loss of heterochromatin control in *ddm1* mutant plants and wt pollen are not responsible for repression of the *UBP1b* 3′UTR. Both the siRNA854-amiRNA and endogenous siRNA854 are able to inhibit protein production from reporter transcripts bearing the *UBP1b* 3′UTR, and in pollen this regulation is dependent on the siRNA854 target sites in the 3′UTR, as well as on RDR6.

We have also demonstrated that the endogenous *UBP1b* transcript is regulated by siRNA854. In inflorescence tissue, this regulation is likely on the translational level, and this result is supported by the translational regulation of the GUS-UBP1b 3′UTR transcript in inflorescences. In contrast, in mature pollen we detect post-transcriptional regulation of the endogenous *UBP1b* transcript by siRNA854. This pollen post-transcriptional regulation of the endogenous *UBP1b* transcript is under the control of RDR6 and AGO1, suggesting that the accumulation of siRNA854 is necessary for this regulation. The basis of the switch from translational control in inflorescence tissue to post-transcriptional...
control in pollen remains puzzling. One possibility is that the four predicted target sites for siRNA854 in the UBP1b 3’UTR are not equally available to base pair in inflorescence tissue compared to pollen. Therefore, in pollen the interaction of siRNA854 with one target site may cause transcript cleavage, while in inflorescence tissue the interaction with a different target site may lead to translational inhibition. Lastly, the observation of 21–22 nt siRNA854 still present in ddm1 heterozygotes produced from ddm1 homozygous parents suggests that there may be a trans-generational epigenetic component to the regulation of UBP1b, as UBP1b may continue to respond to Athila activity even when the plant is no longer homozygous for ddm1. This trans-generational regulation was observed with the GUS-UBP1b 3’UTR transgene in an individual heterozygous for the recessive ddm1-2 allele, due to the inheritance of mutant chromatin from the parental plant.

**Athila and stress**

Under the stress condition of etiolation, the UBP1b-GFP protein traffics from the nucleus and accumulates in the cytoplasm. In unstressed ddm1 mutants, this siRNA854-resistant form of UBP1b is also located in the cytoplasm, suggesting that some aspect of the ddm1 mutation triggers this stress-sensing change in protein location, independent of siRNA854. It is currently unknown which characteristic(s) of the ddm1 mutation triggers this response, as ddm1 mutants display aberrant control of genic epialleles, global TE activation, TE mobilization, and general chromatin decondensation [4,35,60,67]. Conversely, ddm1 mutant seedlings show a phenotype similarly sensitive to ionic and osmotic stress as upb1b mutants. Several studies have shown that TEs are reactivated during stress conditions [68–69]; however, in this case TEs are regulating the stress-responsive pathway. Taken together, these data suggest that an antagonism exists between the UBP1b-induced stress response, which is activated in ddm1 mutants, and Athila6, which inhibits this response by targeting UBP1b through siRNA854. This antagonism may also exist in animal cells, as some DNA viruses generate microRNAs that specifically target the UBP1b homologue TIA-1 mRNA [70], while other RNA viruses specifically target stress granule proteins for proteolysis [71], presumably for the same reason that Athila targets UBP1b. Since TIA-1 is known to repress the activity of some animal viruses and retrotransposons through the formation of stress granules [72–73], we speculate that the same is true for Athila. Therefore, we envision a three-layered host repression of Athila activity. First, transcriptional regulation dependent on DNA methylation epigenetically silences Athila. Second, when transcriptionally active, Athila mRNA accumulation is inhibited by the post-transcriptional regulation mediated by the tasiRNA/VIGS siRNA pathway components DCL2, DCL4, RDR6 and AGO1. Third, we speculate that Athila transcripts may be translationally inhibited due to their sequestration in stress granules, targeted by the UBP1b protein. Transcripts in stress granules are not degraded, but are not translated due to their separation from active ribosome complexes (reviewed in [34]). Akin to a virus encoding a suppressor of gene silencing [20], Athila may encode siRNA854 to inhibit UBP1b protein formation and interfere with the function of this translational-level repression.

**TE regulation of genes**

AGO1 is known to mediate gene regulation via siRNAs in the tasiRNA pathway [74–75]. We have demonstrated that an siRNA which is not part of one of the four known tasiRNA producing loci (TASI-4), but rather part of an epigenetically regulated TE, is able to act on genic transcripts in trans in a similar fashion to a tasiRNA. We think the key aspect of this regulation is the incorporation of siRNA854 into AGO1. AGO1 is the main Argonaute protein responsible for gene regulation in Arabidopsis (reviewed in [76]). This protein is likely unable to distinguish between an siRNA generated from a transcriptionally reactivated TE and one generated from a tasiRNA precursor transcript, at least in the case of siRNA854. Sequencing from AGO1 immunoprecipitations has demonstrated a higher than expected level of siRNAs [52,77], providing evidence that AGO1 is likely regulating both genic transcripts using microRNAs, as well as viral, TE or other repeat transcripts via siRNAs and post-transcriptional silencing. Further investigation is required to understand if and how AGO1 protein complexes determine which siRNAs should target genic mRNAs in trans and which should not. Therefore, the possibility currently exists that siRNA854 does not act alone, and the genome-wide regulation of many transcripts is altered by TE or viral siRNAs loaded into AGO1. It is an intriguing possibility, since both TE epigenetic activation and viral infection lead to a series of still unexplained changes in gene regulation and phenotype. In fact, one longstanding enigmatic viral symptom of the Cucumber mosaic virus was recently found to be caused by a viral satellite siRNA targeting a host gene in trans [20]. In animals, many viruses encode microRNAs that target cellular genes to generate a favorable cellular environment [78].

In order to gain this same advantage, plant TEs may carry sequences that do not require a microRNA stem-loop structure, but utilize a different mechanism by co-opting the tasiRNA/VIGS siRNA biogenesis machinery to regulate a diverse set of cellular transcripts.

**Materials and Methods**

**Plant material**

The mutant alleles used in this study are listed in Table S1. All mutants are in the Col background except ago1 (Ler), ago10 (Ler), hnn-1 (Ler), ubp1b (FLAG_298B04)(Ws), upb1b (FLAG_071F09)(Ws), and ddm1 Ler. Plants were grown under standard long-day growth chamber conditions. Etiolated and stress-test plants were stratified for 3 days at 4°C and grown for 11 days on 1/2X MS media+Gamborg’s vitamins with supplemented sucrose in 16 hours of light per day, with the exception of etiolated seedlings, which were grown without light. For the stress-test analysis, the number of plants surviving after 11 days was counted. Fifty seedlings of each genotype for each condition were grown, and the analysis was replicated three or more times.

**qRT–PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen) or the RNasy Plant Kit (Qiagen). Total RNA was DNaseI treated and reverse transcribed using an oligo-dT primer and SuperScript III Reverse Transcriptase (Invitrogen). qRT-PCR was performed with iQ SYBR Green Supermix (BioRad Laboratories) using 3 technical replicates each of 3 or more biological replicates. qRT-PCR primers are shown in Table S1. qPCR reactions were annealed at 63°C unless otherwise noted. Since most standard qRT-PCR control genes are not highly expressed in pollen, the relative expression values for all experiments were calculated based on the expression of the experimentally validated control gene Atg60200. qPCR was performed on a CFX96 thermocycler and the results analyzed on the CFX Manager Software package (BioRad Laboratories). Relative expression was calculated using the ‘delta-delta method’ formula 2^−(ΔΔCt) sample−ACt control, where 2 represents perfect PCR efficiency. Statistical significance was calculated using unpaired T-tests.

**Small RNA Northern blots**

Total RNA was extracted using TRIzol reagent (Invitrogen), and small RNA was enriched by polyethylene glycol precipitation.
The quantity of small RNA loaded in each lane ranged from 16–60 μg between blots, though the same amount of RNA was loaded per lane on each blot for comparison between samples. We accounted for the equal loading and sizes of small RNAs by re-probing our Northern blots with a known 21 nt microRNA (miR161) and/or a known 24 nt siRNA (siRNA02). In addition, our small RNA Northern blot analysis is supported by independent small RNA deep sequencing data [33]. Gel electrophoresis, blotting and cross-linking were performed as in Pall et al. [79]. Probes for siRNA854, miR161, and siRNA02 were generated by 5’ labeling DNA oligonucleotides with P32-ATP, whereas the probe for Athila 3’ was generated by randomly degrading a P32-UTP labeled in vitro transcribed RNA as in [80]. Sequences of DNA oligonucleotides and primers for generating the in vitro transcription template are listed in Table S1.

Transgene construction and analysis

The 35SamiRNA-siRNA854 transgene was generated by cloning the sequence 5’GATGGAGGATGAGGAGGAG into the microRNA319a stem-loop transcript as in [44]. This transcript was sub-cloned into the 35S promoter binary plasmid pB2GW7. The wt version of the UBPIb 3’UTR was amplified from the wt Col genome, and the 35S:GUS-UBPIb 3’UTR transgene was produced as in [29]. GUS staining was performed as in [81]. For GUS protein activity quantification, protein was quantified using the DC assay (BioRad Laboratories), and 1 μg was used to assay the cleavage of MUG into fluorescent 4-MU as in [82–83]. Fluorescence was measured in 96-well format with a Tecan-SpectraFluor Plus microplate reader, and the specific activity of GUS in each sample was calculated as nmol of 4-MU formed per hour per mg of protein (nmol 4-MU/h/mg). RT-PCR of these lines was performed with oligo-dT primed cDNA for 28 cycles of PCR using primers listed in Table S1.

The modified and deleted versions of the UBPIb 3’UTR were synthesized by IDT. The Lat52 promoter-driven GFP-UBPIb 3’UTR transgene was constructed by cloning the either the wt UBPIb 3’UTR, Modified UBPIb 3’UTR or Deleted 3’UTR version into the SacI site at the end of the mGFP coding sequence of the binary plasmid pMD107, and then by adding the Lat52 promoter to the KpnI site upstream of mGFP in these clones. GFP fluorescence quantification was performed on a Nikon C2 confocal microscope with the NIS-Elements software suite (Nikon Corporation). GFP quantification was performed with the same microscope settings (exposure time, laser power) on the same day. Subtraction of the fluorescence of pollen grains that did not inherit the GFP transgene from the same hemizygous plant negated the background pollen auto-fluorescence.

The FLAG-UBPIb transgene was constructed by adding the FLAG epitope sequence to the 5’ end of the UBPIb CDS as in [51]. This FLAG-UBPIb fragment was amplified and cloned into pENTR/D-TOPO (Invitrogen). The UBPIb promoter and 5’UTR were inserted 5’ to the FLAG tag, and the UBPIb 3’UTR was inserted 3’ of the UBPIb coding region by In-Fusion Recombination (Clontech). Subsequent constructs were recombined into pBGW by Gateway LR Recombination (Invitrogen). Specific qRT-PCR primer sets detecting the FLAG-UBPIb transgene are shown in Table S1. Attempts at identifying the FLAG-UBPIb protein using a FLAG-epitope antibody were repeatedly unsuccessful.

The 35S:UBPIb-GFP transgene was generated by cloning the UBPIb coding region into the binary plasmid pK7FWG2. Seedlings were grown on 1/2X MS media for 11 days before their roots were analyzed by confocal microscopy. The ratio of nuclear to cytosolic fluorescence was calculated by using the NIS-Elements software (Nikon Corporation) by manually defining the average fluorescence touching an analysis line transecting the nucleus and cytosol of an individual cell. The ratio of 25 cells were examined per condition.

All Arabidopsis transformations were performed using Agrobacterium strain GV3101 and standard laboratory techniques. Statistical significance was calculated using unpaired T-tests.

AGO1 immunoprecipitation and small RNA analysis

The AGO1 protein was immunoprecipitated as follows using a commercially available polyclonal AGO1 antibody (Agrisera AB) specific to the unique N-terminal peptide of AGO1, which has been demonstrated to lack cross-reactivity with various over-expressed AGO proteins [84]. Inflorescence tissue was ground with liquid nitrogen and homogenized in 2 ml extraction buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 5 mM DTT) containing 1 tablet/10 mL protease inhibitor cocktail (Roche) per gram of tissue. In a standard immunoprecipitation reaction, Arabidopsis protein extract was pre-cleared with incubation with 10 μl of goat anti-rabbit magnetic beads (NEB). Pre-cleared extracts were then incubated overnight with goat anti-rabbit magnetic beads pre-incubated with 1 μg α-AGO1. All washes were performed with extraction buffer. Immunoprecipitated, mock-immunoprecipitated and input sample RNA was isolated using TRIzol (Invitrogen). 125 ng of each RNA sample was subjected to polyA tailing, CDNA synthesis, and qRT-PCR according to the QuantiMir product specifications (System Biosciences Incorporated). The PCR was annealed at 61.5°C and performed on 2–3 biological replicate immunoprecipitations for each genotype tested, each one having 3 technical qPCR replicates. Each qRT-PCR IP Ct(φ) value was normalized to the amplification of its own input sample, using the ‘delta-delta method’ formula 2^[ΔCT-ΔCT Input], where 2 represents perfect PCR efficiency.

Supporting Information

Figure S1 Transcription analysis of the proposed microRNA854 stem loop structure. qRT-PCR using primers specific for the previously proposed microRNA854 stem loop structure [29]. Transcripts from this region of the Athila6 retrotransposon do not accumulate in vegetative tissues of wt Col. Similar to other regions of Athila6, transcript accumulation is activated in ddm1 and met1 mutants. (EPS)

Figure S2 Base pairing of siRNA854 to the wt, MOD and DEL versions of the UBPIb 3’UTR. The UBPIb 3’ UTR is shown from 5’ to 3’. The four sites of the UBPIb 3’ UTR targeted by siRNA854 predicted by Arteaga-Vázquez et al. are shown [27]. In the MODified 3’ UTR variant, all perfect complementary base pairing was replaced. In the DELetd 3’ UTR variant, the entire target sites have been removed, resulting in a shorter 3’ UTR. (EPS)

Figure S3 Expression analysis of UBPIb in wt Col. (A) Mined data from microarray experiments performed and normalized by Schmid et al. were accessed through AtGenExpress (http://jsp. weigelworld.org/expviz/expviz.jsp) [79]. GC-RNA normalized values for the accumulation of UBPIb and the six Athila6 elements on the ATH1 gene expression microarray are shown. (B) Expression level of the UBPIb mRNA in seedlings, rosette leaves, inflorescence and mature pollen as in part A are shown on a linear scale. (C) RT-PCR of UBPIb mRNA accumulation in wt Col tissues. Polyadenylated transcripts do not accumulate in pollen,
while they do accumulate in inflorescences, leaves and seedlings. RT-PCR was performed on 200 ng of total RNA, reverse transcribed with an oligo-dT primer or random hexamers using Superscript III Reverse Transcriptase (Invitrogen). PCR was performed for 20 cycles.

(EPS)

Figure S4 Analysis of ubp1b mutant plants. RT-PCR of ubp1b homozygous mutant plants. For both the FLAG298B04 and FLAG071F09 insertion alleles, UBP1b is still transcribed, but not spliced correctly, and the transcript is not polyadenylated. These insertions are in the Ws background. RT-PCR was performed on 200 ng of total RNA reverse transcribed with an oligo-dT primer or random hexamers using Superscript III Reverse Transcriptase (Invitrogen). PCR was performed for 28 cycles. (TIF)

Figure S5 Transcript-level regulation of UBP1b by siRNA854 is not observed in inflorescence tissue. (A) Bisulfite PCR analysis of the DNA methylation levels of the UBP1b 3’UTR in wt Col and ddm1. DNA was treated by the manufacturer’s instructions using the Epitext Bisulfite Kit (Qiagen), amplified using the primers in Table S1, TOPO-TA cloned into pCR4 (Invitrogen) and sequenced. Both the sense DNA strand (top) and antisense DNA strand (bottom) were interrogated separately. Each circle represents a cytosine in the DNA sequence, with the color of the circle corresponding to the sequence context of the cytosine (CG = red, CHG = blue, CXX = green, H = A,T,C). Closed circles represent methylated cytosines, while open circles represent unmethylated. The location of the cytosine corresponds to the map of the UBP1b last exon and 3’ UTR shown below for each DNA strand. The locations of the four predicted siRNA854 target sites on the UBP1b 3’UTR are shown as red lines on the maps. (B) 3’ RACE-PAT analysis of the polyA tail length of the UBP1b transcript shows no differences between wt Col and ddm1. Transcripts from the ubp1b mutant are not polyadenylated. This technique was performed as in [80] using primers shown in Table S1. (C) Modified 5’ RACE-PCR detecting full-length uncapped transcripts shows no difference in the level of UBP1b full-length uncapped transcripts between wt Col and ddm1. Uncapped UBP1b transcripts accumulate in the ubp1b mutant. A modified GeneRacer (Invitrogen) 5’ RACE protocol was performed using 5 μg TRIzol-isolated total RNA. RNA was ligated to a 5’ RNA adaptor by T4 RNA Ligase I and reverse transcribed with an oligo-dT primer and SuperScript III Reverse Transcriptase (Invitrogen). Uncapped transcripts were detected by two rounds of nested PCR using adaptor-specific and gene-specific primers, listed in Table S1. (EPS)

Figure S6 Pollen localization of RDR6 and AGO1. (A) Expression values from microarray data mined from Borges et al. of purified sperm cells and whole mature pollen [45]. UBP1b, RDR6 and AGO1 transcripts are not enriched in sperm cells. AGO5 is shown as a control of a known sperm-specific protein [81]. (B) Fluorescence microscopic images of mature pollen grains expressing GFP fused to the RDR6, AGO1 or AGO5 protein, each under control of their own native promoters. The transgenes were generated by cloning the promoters and open reading frames of the proteins (including introns) into the binary plasmid pMDC107. Transgenes were transformed into a line expressing RFP in the pollen vegetative nucleus (VN-RFP) [82]. Plants hemizygous for the transgene were used for analysis, and pollen grains that did not inherit the transgene are marked with an asterisk. pAGO5:AGO5-GFP is shown as a control for a protein that has known sperm cell localization [81]. In the images of pAGO1:AGO1-GFP and pRDR6::RDR6-GFP, dark shadows of the sperm cells in the vegetative cell cytoplasm can be seen. Scale bars are 20 microns. (C) Complementation of the nd6 mutant narrow leaf phenotype with the RDR6::RDR6-GFP transgene from part B. All plants are 14 days old. The pAGO1:AGO1-GFP transgene did not complement the ago1-11 seedling phenotype (data not shown). (TIF)

Figure S7 The AGO1 antibody is specific to the AGO1 protein. (A) Western blot of Col, Ler, and ago1-11 inflorescence tissue protein extract using the same AGO1 antibody as in Figure 5. The ago1-11 allele contains a single nucleotide polymorphism in a splice acceptor site that causes exon skipping and results in a weak allele that retains some AGO1 function [83]. Proteins sized 50–200 kDa were transferred to nylon, which was stained with Ponceau-S to show even loading and then blotted with α-AGO1. No cross-reaction bands were detected. The ago1-11 allele has decreased protein levels, but a small amount of AGO1 protein is still produced. (B) Western blot of Col inflorescence protein extract (Input), no antibody immunoprecipitation control (Mock IP) and AGO1 IP using the same AGO1 antibody as in Figure 5. The Input sample contains 1/15 of the total input protein compared to the Mock IP and AGO1 IP samples. The Western blot only detects AGO1 protein in Input and AGO1 IP samples, while no AGO1 protein is detected in the Mock IP. (EPS)

Table S1 PCR primers and mutant alleles used in this report. (XLS)

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Author Contributions

Conceived and designed the experiments: ADM SN RKS. Performed the experiments: ADM SN SHR. Analyzed the data: ADM SN RKS. Wrote the paper: ADM RKS.

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