Deep Sequencing from *hen1* Mutants to Identify Small RNA 3' Modifications

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microRNAs (miRNAs) function via targeting of messenger RNAs, suppressing protein levels, and playing important roles in biological processes of plants and animals. The pathway for miRNA biogenesis is well established, but less is known about miRNA turnover, largely because of difficulties in capturing miRNAs during the process of decay, in which they are both rare and ephemeral. The HEN1 protein methylates the 3' terminus of small RNAs (sRNAs), protecting them from poly-uridylation and degradation. Recent progress using deep sequencing to study sRNAs in *hen1* reveals the potential for *hen1* mutants to serve as a platform for studies of miRNA turnover, with the sequencing data providing unprecedented precision and detail in the characterization of 3' modifications.

In plants, microRNAs (miRNAs) (Reinhart et al. 2002; Jones-Rhoades et al. 2006) and small interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999; Chapman and Carrington 2007) are the two major classes of sRNAs. microRNAs in plants function in a wide variety of roles principally in development but have demonstrated roles in modulation of transcriptional responses to abiotic and biotic stress, and to nutrient deficiencies (Chen 2005; Sunkar et al. 2007; Rubio-Somoza et al. 2009; Jeong et al. 2011). siRNAs in plants are predominantly but not exclusively composed of heterochromatic siRNAs that function in genome defense via the maintenance of heterochromatin, presumably important for the suppression of transposon activity (Volpe and Martienssen 2011).

miRNAs are processed from long single-stranded transcripts that originate from distinct genomic loci and are transcribed by RNA polymerase II (Pol II) like a typical messenger RNA (mRNA). These miRNA precursor transcripts fold into imperfectly paired, hairpin-like structures and are subsequently processed via a series of nuclear-localized events (in plants, because some processing events in animals occur in the cytoplasm) that lead to the generation of a single, mature sRNA of typically 21 or 22 nucleotides (Bartel 2004; Voinnet 2009). In an early processing step, an sRNA “duplex” comprised of both the miRNA and miRNA-star is cleaved from the precursor hairpin by the DICER-LIKE1 (DCL1) protein. The miRNA strand, also known as the “guide” molecule eventually becomes associated with (or “loaded” into) an ARGONAUTE (AGO) protein to form an active RNA-induced silencing complex (RISC). The miRNA-star strand is usually nonfunctional (hence, it is also known as the “passenger” strand), and it is preferentially decayed leading to a substantially lower abundance than the miRNA. In contrast, siRNAs are often not per se encoded in the genome but are derived from precursor RNA molecules that have been made double

stranded by RNA-dependent RNA polymerases (RDRs). These double-stranded RNA (dsRNA) precursors are processed by DCL proteins into multiple distinct siRNA classes that represent both strands of the original dsRNA (Baulcombe 2004; Chapman and Carrington 2007). Plant siRNAs come in several forms; for example, heterochromatic siRNAs are produced from RNA polymerase IV-dependent precursors generated from a wide variety of repetitive sequences (Wierzbicki et al. 2008), whereas *trans*-acting or phased siRNAs are produced via Pol II from long, noncoding mRNAs or even from protein-coding transcripts (Montgomery et al. 2008; Cuperus et al. 2010; Zhai et al. 2011). The *Arabidopsis* genome includes four *DCL* genes that are responsible for generating distinct classes of sRNAs and of different sizes, ranging from 21 to 24 nucleotides. Mutant analysis has revealed some functional redundancies among the *DCL* genes (Deleris et al. 2006; Henderson et al. 2006). Despite the diversification of sRNA types and biogenesis pathway proteins, current data suggest that all plant sRNAs require 3' methylation performed by a protein known as HEN1. This protein also has a conserved function in the maturation of specific subclasses (namely, the Piwi-interacting RNAs [piRNAs]) of animal sRNAs. Deep sequencing of sRNAs in mutants of *hen1* can describe the types of 3' modifications to which sRNAs are subjected in the absence of a protective methyl group. In this chapter, we discuss the use of these deep sequencing data, types of modifications that are observed, and insights that may be made from these analyses when performed on *hen1* mutants.

Discovery of HEN1 and Its Conserved Function in Many Species

The *HEN1* (*HUA ENHANCER1*) gene was first identified in a screen for *Arabidopsis* floral development mu-

tants (Chen et al. 2002). The phenotype was identified as an enhancer of a floral-organ phenotype in the *hual-1 hua2-1* double mutant; when the Chen laboratory segregated out the *hen1* mutant from the *hua* mutant genes, the single-gene mutant *hen1* was observed to have a pleiotropic phenotype, including a short stature, delayed flowering, reduced fertility, and multiple inflorescence aberrations (Chen et al. 2002). These phenotypes were later revealed to be attributable to widespread reductions in sRNAs and their activities. In subsequent work by the Chen laboratory, the HEN1 protein was shown to function as a methyltransferase; the methyltransferase activity is specific to sRNAs, adding a 2'-O-methyl group on the 3'-terminal nucleotide of template sRNAs (Yu et al. 2005; Yang et al. 2006). In more recent work, the crystal structure of HEN1 suggests that it can specifically recognize the sRNA duplex as its template via multiple RNA-binding domains and a methyltransferase domain (Huang et al. 2009). Thus, HEN1 activity is highly specific, but a loss of *HEN1* can have a widespread impact on sRNAs, particularly in plants in which all known small RNA pathways converge on the methylation activity of HEN1.

miRNAs examined by RNA gel blot analysis show unusual length heterogeneity in a *hen1* mutant background (Li et al. 2005). Comparisons performed by cloning and sequencing individual miRNAs in wild-type and *hen1-1* backgrounds showed the addition of predominantly uridine (U) nucleotides at the 3' end of some miRNAs in a *hen1-1* mutant (Li et al. 2005). This U "tailing" is presumably a result of deprotection caused by a lack of 3' methylation owing to the loss of HEN1 activity. RNA gel blot analysis of miRNAs has shown that miRNAs in plant *hen1* mutants display a "laddering" of length, reflecting the 3' tails, whereas sizes shorter than the wild-type length are also observed, suggesting that 3' truncation occurs as well (Fig. 1A) (Li et al. 2005). As we describe below, our analyses of deep sequencing data have confirmed that both 3' truncation and 3' tailing are common for most miRNAs in plant *hen1* mutants. An additional and likely related observation was that the abundance of most miRNAs is considerably reduced in the *hen1* mutants, with no corresponding decrease in the rate of miRNA biogenesis, implying that in *hen1*, miRNAs have increased rates of degradation (Li et al. 2005; Yu et al. 2005). At that point in time, it was unclear whether U tailing is a cause or a consequence of miRNA degradation. The 3' tailing of miRNAs is easily detected by today's "next-generation" sequencing, and thus, sRNAs in plant *hen1* mutant are good candidates for molecular characterization using modern techniques.

The *hen1-1* allele in the *Arabidopsis* Landsberg *erecta* (*Ler*) ecotype came out of an enhancer screen of the *hual-1/hua2-1* background (Chen et al. 2002). It was the first *hen1* mutant to be identified in any species and therefore contributed the name by which even animal *HEN1* genes are now known. In *Arabidopsis*, a variety of alleles have since been characterized via both forward- and reverse-genetics approaches. These include the weaker *hen1-2* allele, also from *Ler*, and the identical

point mutation in the Columbia (Col) background isolated from an independent genetic screen and known as *hen1-8* (Yu et al. 2010); curiously, the same allele in different backgrounds produces different phenotypes, with a more severe developmental impact (on fertility, for example) in the Col background as well as greater molecular effects such as a reduction in miRNA levels (Yu et al. 2010). In rice, two mutant alleles in the ortholog *WAVY LEAF1* (*WAF1*) of *Arabidopsis* *HEN1* have been described; the rice *waf1-1* and *waf1-2* mutants each bear a single-base substitution, leading to a premature stop codon in the second exon and a nonfunctional splicing site of the fourth intron, respectively (Abe et al. 2010). Whereas *HEN1* is also single copy in rice, there are numerous phenotypic differences in the rice *hen1* ("waf") mutants, compared with *Arabidopsis*, most of which reflect a more severe impact on normal biological functions. For example, rice *hen1* mutants show seedling lethality and phenotypes reminiscent of *trans*-acting siRNA (tasiRNA)-defective mutants in rice (Abe et al. 2010). Because mutants in the rice ortholog were identified only recently, work on the plant *HEN1* gene has been primarily performed in *Arabidopsis*; however, the characterization of *wavy leaf1* (*waf1*) mutants represents an opportunity to expand our understanding of HEN1 function in monocots.

Besides plants, HEN1 orthologs have also been described and studied in metazoans, in which HEN1 methylates different subsets of sRNAs, primarily including piRNAs but excluding miRNAs (see Table 1 for a summary). piRNAs are a class of animal sRNAs that are abundant in germline cells; they are longer and distinct from miRNAs and target transposons to effect silencing (Juliano et al. 2011). Unlike animal miRNAs that are unmethylated (as a reminder, plant miRNAs are methylated), piRNAs are methylated, a modification performed by HEN1. Thus, the mouse *HEN1* homolog (*mHEN1*) is expressed specifically in the testes, in which it functions to methylate piRNAs (Kirino and Mourelatos 2007a,b; Ohara et al. 2007). HEN1 substrates in *Drosophila* are determined by AGO binding: AGO1-bound miRNAs remain unmethylated, but AGO2-bound siRNAs and Piwi-bound piRNAs are methylated (Horwich et al. 2007; Iwasaki et al. 2009). This suggests that animal HEN1 acts on AGO-bound single-stranded sRNAs (ssRNAs), whereas the plant HEN1 acts on sRNA duplexes before they are incorporated into AGO proteins. The HEN1 homolog in zebrafish has also been studied; as in *Drosophila* and mouse, the zebrafish HEN1 methylates piRNAs in germline cells and is required for oocyte development, and deep sequencing of sRNAs in the zebrafish *hen1* mutant revealed addition of U tailing on the unmethylated piRNAs (Kamminga et al. 2010). Although miRNAs in animals mostly remain unmethylated, they are fully methylated in plants as a crucial step for stabilization and protection from enzymatic activities such as the 3' truncation and uridylation mentioned above. miRNAs in *Arabidopsis* *hen1* mutants, without the protection of 3' methylation, are often uridylated and in some cases truncated.

Figure 1. Data handling for truncation and tailing analysis. (A) miRNAs may be 3' modified in several ways in plant *hen1* mutants. The blot on the left shows the laddering effect observed for miR167 in an *Arabidopsis hen1-1* mutant versus a *Landsberg erecta* (*Ler*) control; reprinted from Li et al. (2005). At top right, in wild-type (WT) plants, miRNAs are 3' methylated by HEN1, but may be decayed by the 3' to 5' exonuclease SDN1/2. In the lower box at right, in a *hen1* mutant, miRNAs may be 3' tailed by HESO1, 3' truncated by an as-yet-unknown protein (perhaps one of the SDN family of nucleases) or may have some combination of both 3' truncation and 3' tailing. (B) Schema for analysis of sRNA truncation and tailing from deep sequencing data, as described in the main text. For sRNA reads that are not genome matched, one nucleotide is chopped off from the 3' end, in successive rounds until the remaining 5' sequence perfectly maps to genome. Thus, any nongenome-matched sRNA read could be split into two parts: the longest 5' genome-matched component (5GMC) and a 3' "tail." With all reads processed into the format of the 5GMC plus tail, the 5GMC of each read is aligned to annotated miRNAs in the miRNA database (miRBase) for the genome of origin. This then determines the extent of "tailing" (additional of nonconventional 3' nucleotides) and "truncation" (shortening of the miRNA from the 3' end), as diagrammed in A. Each miRNA in an organism could have a different, distinguishable pattern of 3' modifications.

3' Modifications of Plant sRNAs: HEN1 and Its Sidekicks

The Chen laboratory and collaborators have published biochemical data demonstrating the specificity of HEN1 activity. Their initial work in this area showed the methyltransferase activity by which HEN1 transfers a methyl group to the 2' hydroxyl of the 3'-terminal nucleotide of a sRNA (Yu et al. 2005; Yang et al. 2006). They showed that both strands of either an miRNA/miRNA* duplex or siRNA/siRNA* duplex are methylated *in vitro*, and HEN1 has a preference for 21–24-nucleotide RNA duplexes with a 2-nucleotide overhang typical of DICER cleavage (Yang et al. 2006). HEN1 in plants possesses double-stranded RNA-binding domains (dsRBDs), domains missing in animal orthologs, and structural data

showed that plant HEN1 uses the dsRBDs to recognize ~16 bps of its duplexed sRNA substrate, measuring the length of the substrate but transferring the methyl group to the duplex in a nonsequence-specific manner (Huang et al. 2009). The transfer of the methyl group to the sRNA duplex occurs via a novel mechanism dependent on Mg^{2+} , coordinated between four invariant residues in the active site of the methyltransferase domain and the 2' and 3' hydroxyls on the 3'-terminal nucleotide of the template (Huang et al. 2009). Analogous biochemical work on the mouse HEN1 protein has shown that its activity is specific for single-stranded piRNAs (Kirino and Mourelatos 2007a), consistent with the lack of a dsRBD in the animal orthologs identified to date. Therefore, HEN1 proteins function to add methyl groups to the

Table 1. HEN1 homologs in various species

Name	Organism	Mutant alleles	Substrates	sRNAs sequenced?	Reference(s)
HEN1	<i>Arabidopsis</i>	<i>hen1-1, hen1-2, hen1-4, hen1-8</i>	miRNAs and siRNAs	Yes	Li et al. 2005; Zhao et al. 2012
WAF	Rice	<i>waf-1, waf-2</i>	miRNAs and siRNAs	No	Abe et al. 2010
mHEN1	Mouse	N/A	piRNAs	No	Kirino and Mourelatos 2007a,b
HEN1	Zebrafish	<i>hen1</i>	piRNAs	Yes	Kamminga et al. 2010
homolog					
DmHEN1	<i>Drosophila</i>	<i>hen1^{f00810}</i>	siRNAs and piRNAs	Yes	Horwich et al. 2007; Ameres et al. 2010; Ghildiyal et al. 2010
Hen1p	<i>Tetrahymena</i>	Δ <i>HEN1</i>	scnRNA	No	Kurth and Mochizuki 2009

3' end of small RNAs, with no sequence specificity for the substrate yet shown other than a preference for either ssRNA (in animals) or dsRNA (in plants).

Other than HEN1, several proteins have been identified for their role in modifying the 3' end of plant sRNAs. These proteins, functioning as “sidekicks” to HEN1 in 3' modifications, use unmethylated sRNAs as substrates, presumably those either missed by HEN1 or demethylated via natural processes. Work from the Chen laboratory in *Arabidopsis* has identified a family of SDN (small RNA degrading nuclease) proteins that degrade mature miRNAs (Ramachandran and Chen 2008). Whereas it is still unclear which proteins are responsible for the 3' truncation that occurs at a higher rate in *hen1* mutants (Fig. 1A), SDN proteins are candidates for this activity. However, in vitro analysis of the SDNs suggests that they degrade miRNAs 3' to 5', leaving a small fragment of 8 or 9 nucleotides in length, and this is quite distinct from the typical 1- or 2-nucleotide shorter size observed for truncated miRNAs in RNA gel blots from *hen1* mutants (Li et al. 2005; Yu et al. 2005). More recent work from Chen and Yu laboratories identified a nucleotidyl transferase *HEN1 SUPPRESSOR1* (*HESO1*) that is responsible for the addition of the uridine-rich tail to the end of the miRNAs, which are not protected by methylation (Ren et al. 2012; Zhao et al. 2012). Other genes in the same family, which include poly(U) polymerases, were previously shown in animals to modify and regulate the stability of the pre-miRNA (cleaved hairpin) of *let-7* (Lehrbach et al. 2009). In the green alga *Chlamydomonas*, it was shown that uridylation added by nucleotidyltransferase MUT68 can promote the degradation of miRNAs and siRNAs (Ibrahim et al. 2010) and in *C. elegans*, CDE-1 uridylates 22G-RNAs in the germline to promote their degradation and thus prevent over accumulation (van Wolfswinkel et al. 2009). The methylation of sRNAs by HEN1 blocks both 3' truncation and 3' tailing, suggesting that either or both of these 3' modifications may be important marks for RNA destabilization.

Bioinformatics Analysis of sRNAs from *hen1* Mutants

Nearly all studies of 3' modifications made to miRNAs in *hen1* mutants were performed with RNA gel blots

(“northern”) that can only show the changes in miRNA length and relative abundance but cannot identify precisely any changes in miRNA sequence composition. In earlier work, cloning followed by Sanger sequencing of miR173 and miR167 in *hen1-1* revealed 3' truncation as well as 1–5 nucleotides of uridylation at the 3' end of miRNAs, consistent with observations on the blots (Fig. 1A) (Li et al. 2005). With the tremendous advances in high-throughput sequencing technologies, it is possible to systematically study sRNA populations in *hen1* mutants with unprecedented depth. We have been performing such studies, and although the results are both too extensive to report here and as-yet incomplete, the methodologies that we have devised may have utility for the analysis of mutants of *hen1* and the “sidekick” genes that are also involved in 3' sRNA modifications.

Our bioinformatics and visualization pipeline assists in the characterization of 3'-end modifications of miRNAs detected using deep sequencing data, typically from an Illumina sequencing instrument. The first step in our process is to identify nongenome-matched sRNA sequences, because most 3' “tailed” sRNAs will no longer match the genome (unless there is a chance identity between the tail and the adjacent nucleotides in the precursor) (Fig. 1B). For sRNA reads that were not genome matched, 1 nucleotide was chopped off from the 3' end in successive rounds until the remaining 5' sequence can be mapped to the *Arabidopsis* genome with no mismatches (Fig. 1B). Thus, any initially nongenome-matched sRNA read could be split into two parts: the longest 5' genome-matched component (5GMC) and a 3' “tail.” The tail is nonexistent for reads that matched the genome perfectly in the first round of analysis. For subsequent analyses, we focus on miRNAs because plant heterochromatic siRNAs are both too variable in sequence (owing to their typical origins from repetitive sequences) and too weakly abundant. With all reads processed into the format of 5GMC plus tail, we can also determine the degree of 3' truncation relative to annotated, mature miRNAs sequences by comparison of the 5GMC to all annotated miRNAs in miR-Base for the species of interest (Griffiths-Jones et al. 2008). This then determines the extent and composition of the tail (additional, noncanonical 3' nucleotides) and truncation (shortening of the miRNA from the 3' end). The sequence data provide the exact composition of the truncated miRNA and the added 3' tails.

We have also devised a graphic view that simultaneously displays the degree of both truncation and tailing for specific sRNAs (usually individual miRNAs) (Fig. 2). In this image, the wild-type or canonical miRNA length is assigned to the position in the lower right of a grid. Using the 5GMC and tailing lengths determined as described above, miRNA derivatives of identical length can be assigned to different positions in the grid based on the 5GMC value (x axis, indicating truncation) and the tail length (y axis). The size of the spot at each position in the grid corresponds to the proportion of total reads for a given sRNA that had that amount of truncation and tailing, regardless of the nucleotide composition of the tail. Any spots on the same diagonal share the same final length and would be indistinguishable on an RNA gel blot, but based on this plot, would have different 3' ends. We provide several examples in Figure 2. For example, *Arabidopsis* miR166 is significantly truncated and mildly tailed in the *hen1-8* mutant, with a small degree of truncation and tailing visible even in wild-type plants (Fig. 2, on left). In *Drosophila* and zebrafish, there is no obvious difference for miRNAs in a mutant *hen1* background, presumably because miRNAs are not methylated in animals; however, we were able to detect a small amount of 3' variation in wild-type animals (Fig. 2, middle and right). This could reflect variation in processing

or posttranscriptional 3' modifications resulting from processes similar to those that we have described in plants.

CONCLUSIONS

The combination of these analysis and visualization tools will enable dissection of sRNA sequencing data to identify numerous attributes of the posttranscriptional modifications that occur to sRNAs. For example, it will be possible to compare the degree of truncation and tailing across miRNA families within a single organism to assess variability in 3' modifications; these may reflect sequence-specific effects or other factors that have yet to be described. As an example, it has recently been published that AGO10 in *Arabidopsis* specifically binds miR166 and promotes the degradation of this miRNA (Ji et al. 2011; Zhu et al. 2011), so it will be interesting to apply these tools to identify 3' modifications that may be promoted by AGO10. Given that *hen1* mutants are now available for rice, cross-species comparisons of 3' modifications for orthologous miRNAs may also identify conserved or divergent decay destinies for specific miRNAs. One advantage of sequencing-based approaches for studying posttranscriptional modifications to sRNAs is that it is an open-ended analysis that can test

Figure 2. Matrix for summarizing and visualizing miRNA 3' truncation and tailing from deep sequencing data. For each matrix, the x axis represents the length of the 5' genome-matched component (5GMC) of a particular miRNA related sequence; the y axis represents the length of the "tail" added to the 5GMC, mostly through uridylation. Annotated miRNA sequences are considered to have no truncation or tail and therefore are positioned at wild type, wild type (WT, WT) in the lower right corner of each matrix. In this figure, we show the results for a single miRNA (named at the top) from two libraries in each of three organisms, as labeled. The canonical miRNA size is indicated in the spot at the lower right of the top row of panels. (Lower row) For *Arabidopsis* and zebrafish, we analyzed a *hen1* mutant, whereas in *Drosophila*, we compared AGO1-IPed sRNAs with total sRNAs. Different colored spots in the top and bottom sets of panels represent different libraries. The data are interpreted as described in the main text. The sRNA sequencing data used in this analysis are described in previous studies (see Table 1 for summary). The GEO accession numbers for the libraries that we analyzed are GSE35479 (*Arabidopsis*), GSE33582 (zebrafish), and GSE18806 (*Drosophila*).

all known miRNAs in a single sequencing run. Furthermore, the data facilitate the analysis of other types of sRNAs, including heterochromatic siRNAs and *trans*-acting siRNAs, although alternative analysis approaches may be required to characterize the truncation and tails of such heterogeneous populations of sRNAs. We should also point out that there are a number of challenges or limitations to the approaches that we have described. These analyses depend on the accuracy of the miRNA annotation, and recent global analyses have shown that many miRBase-annotated miRNAs have siRNA-like qualities that may reflect older annotations that predate more strict, modern standards (Jeong et al. 2011). Biological factors can also confound the analyses, including (1) inconsistency in processing of a single precursor (optimal analyses require just one predominant miRNA species per precursor), (2) complex families with many loci that give rise to the same mature miRNAs, (3) weakly abundant miRNAs for which insufficient reads are available.

In conclusion, we believe that these are exciting times in which to study the posttranscriptional events that regulate sRNA function and stability. Numerous plant mutants have been described recently, led by the highly productive forward- and reverse-genetics approaches applied in the Chen laboratory that have identified a handful of genes critical to 3' modifications of sRNAs. The biochemistry of these genes remains to be worked out, but deep sequencing and genome-wide approaches comprise a powerful and sensitive readout for the activity of these genes. Future rounds of analyses will likely identify determinants that distinguish the varied activity of these proteins on different sRNAs, whereas cross-species and even cross-kingdom comparative analyses will provide insights into the biology of these processes.

ACKNOWLEDGMENTS

This material is based on work supported by the National Institute of Food and Agriculture, United States Department of Agriculture, under Agreement no. 2012-67013-19396. We also gratefully acknowledge ongoing collaborations with Xuemei Chen and her laboratory members (University of California–Riverside) and Bing Yang and his laboratory members (Iowa State University). Manoj Pillay was helpful with earlier implementations of the algorithms that we describe.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2012 77: 213-219 originally published online February 7, 2013

Access the most recent version at doi:[10.1101/sqb.2013.77.014779](https://doi.org/10.1101/sqb.2013.77.014779)

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