Published in final edited form as:

Science. 2008 September 12; 321(5895): 1490–1492. doi:10.1126/science.1163728.

Degradation of microRNAs by a family of exoribonucleases in *Arabidopsis*

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Abstract

microRNAs (miRNAs) play crucial roles in numerous developmental and metabolic processes in plants and animals. The steady-state levels of miRNAs need to be properly controlled to ensure normal development. While the framework of miRNA biogenesis is established, factors involved in miRNA degradation remain unknown. Here, we show that a family of exoribonucleases encoded by the *SMALL RNA DEGRADING NUCLEASE (SDN)* genes degrades mature miRNAs in *Arabidopsis*. SDN1 acts specifically on single-stranded miRNAs *in vitro*, and is sensitive to the 2'-*O*-methyl modification on the 3' terminal ribose of miRNAs. Simultaneous knockdown of three *SDN* genes *in vivo* results in elevated miRNA levels and pleiotropic developmental defects. Therefore, we have uncovered the enzymes that degrade miRNAs and demonstrated that miRNA turnover is crucial for plant development.

Plant miRNAs carry a 2'-*O*-methyl group that protects them from a 3'-to-5' exonucleolytic activity and a uridylation activity that adds an oligoU tail to the 3' ends of miRNAs (1,2). Maintaining proper steady-state levels of miRNAs is crucial for plant development (3–7). The steady-state levels of miRNAs are presumably determined by the opposing activities of miRNA biogenesis and degradation. A conserved exonuclease from *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, Eri-1, specifically degrades siRNA/siRNA* duplexes with 2 nt 3' overhangs *in vitro* and reduces RNAi efficiency *in vivo* (8,9). Exonucleases that degrade single-stranded small RNAs have yet to be identified.

To identify enzymes that degrade single-stranded miRNAs or siRNAs, we took a candidate gene approach. We presume that enzymes involved in miRNA metabolism evolved from enzymes that process structural and/or catalytic RNAs, a view supported by the fact that a number of known players in small RNA metabolism also function in the processing of rRNAs (10–13). We sought for *Arabidopsis* homologs of a class of exoribonucleases in yeast, Rex1p to Rex4p, which participate in 3'-end processing of rRNAs and tRNAs (14,15). BLAST searches using the four Rex proteins identified 15 *Arabidopsis* proteins containing an exonuclease domain (Fig S1). Notably, At3g15140, which belongs to a clade of six proteins (Fig S1), was the most similar to Eri-1 among the 15 proteins. Since we seek enzymes that degrade single-stranded small RNAs, we excluded proteins in this clade from our analysis.

From the remaining Rex homologs, we randomly chose At3g50100 from the five-member clade and At3g15080 from the outliers (Fig S1), expressed them as GST fusion proteins in *E. coli* (Fig S2), and tested their activities on miRNAs *in vitro*. A 5'-end labeled single-stranded RNA oligonucleotide corresponding to miR167 in sequence (but lacking a 2'-*O*-methyl group)

was incubated with GST-At3g15080, GST-At3g50100, or GST. While GST-At3g15080 or GST did not exhibit any activity on miR167, GST-At3g50100 degraded the full-length miR167 generating a product of approximately 8–9 nt (Fig 1A; the size of the final product was estimated from Fig 2D). GST-At3g50100 also acted on miR173 and 2'-*O*-methylated miR173 and generated products of roughly 8–9 nt (Fig 1A). We refer to At3g50100 as SMALL RNA DEGRADING NUCLEASE1 (SDN1) hereafter.

To determine whether SDN1 is an endonuclease cleaving the RNAs between nt 8 and 9 from their 5' ends, or a 3'-to-5' exonuclease that cannot process RNAs of 8 nt or shorter, we labeled miR173 with ³²pCp at the 3' end and incubated miR173-³²pCp with GST-SDN1. miR173-³²pCp was resistant to GST-SDN1 and phosphatase treatment of miR173-³²pCp to remove the 3' phosphate rendered the miRNA susceptible to GST-SDN1 (Fig 1B). Furthermore, a product of 15 nt, which would be expected if SDN1 were an endonuclease cleaving between nt 8 and 9 from the 5' end, was not observed on phosphatase-treated miR173-³²pCp (Fig 1B). These data indicated that SDN1 is a 3'-to-5' exonuclease.

GST-SDN1 did not have any effect on a single-stranded DNA oligonucleotide (Fig 2B) and is therefore a ribonuclease. Unlike Eri-1 (9), GST-SDN1 failed to degrade miR173 in a miR173/miR173* duplex (Fig 2B, Fig S3). To look at SDN1 substrate size, synthetic RNA oligonucleotides of 17, 18, 20, 21 (miR167), 22 (miR173), 23, 24, and 27 nt (Table S2) were incubated with GST-SDN1 separately. SDN1 degraded all tested RNA oligonucleotides and yielded an end product of approximately 8–9 nt regardless of the length of the substrates (Fig 2A). However, SDN1 cannot act on longer RNAs. pre-miR167 or a 300 nt RNA from the protein-coding *APETALA1* (*API*) gene was not detectably degraded by GST-SDN1 (Fig 2C). Therefore, SDN1 acts specifically on single-stranded small RNAs in a sequence-independent manner.

The 2'-*O*-methyl group present in all plant small RNAs (1,2) deters the activities of SDN1. When miR173 or 2'-*O*-methyl miR173 was incubated with varying concentrations of GST-SDN1, a degradation intermediate of ~ 20 nt was present in reactions on 2'-*O*-methyl miR173 under lower enzyme concentrations but was barely detectable in reactions on miR173 (Fig 2E, top panel). In a time course using a low enzyme concentration (Fig 2E, bottom panel), the rate of degradation of miR173 was faster than that of 2'-*O*-methyl miR173, as judged by the time of appearance of the final product. The 20 nt intermediate was much more prominent and lingered longer in the 2'-*O*-methyl miR173 reaction (Fig 2E, bottom panel).

SDN1 is a multiple turnover enzyme. In the reactions in Fig 2A and E, the great majority of the substrates (4pmoles) was degraded by GST-SDN1 (278 fmoles) in 60 min. Therefore, one molecule of enzyme degrades 14 molecules of small RNA.

miRNAs are uridylated on their 3' ends when not methylated (1). miR173 with 2 or 5 additional Us on the 3' end was not degraded as efficiently as miR173 by GST-SDN1 (Fig 2D), as judged by the delayed appearance of the final product and delayed disappearance of the full-length substrates or shorter intermediates. This suggests that uridylation of miRNAs in the absence of methylation could have a protective role against exonucleolytic degradation.

To determine whether SDN1 limits miRNA accumulation *in vivo*, we identified a homozygous T-DNA insertion mutant, *sdn1-1* (Fig S4). This mutant is unlikely a null allele (Fig S5A) and it shows no obvious developmental defects or much difference in the abundance of seven tested miRNAs from that of wild type (Fig 3). The lack of miRNA defects in *sdn1-1* could be due to redundancy with the other four members of the clade, At3g50090, At5g05540 (*SDN2*), At5g67240 (*SDN3*), and At5g25800 (Fig S1). We obtained T-DNA insertion alleles in the three genes most closely related to *SDN1* (Fig S4). The abundance of seven tested miRNAs was largely unaffected in all four single mutants (*sdn1-1*, *sdn2-1*, likely a reduction-of-function

allele (Fig S5), *sdn3-1*, a reduction-of-function or null allele (Fig S5), and the T-DNA allele in At3g50090, a possible pseudo gene) (Fig 3). Three of the seven tested miRNAs, miR159, miR167, and miR173, and siR1003, an endogenous siRNA, accumulated to 1.5–1.8 times of the wild-type levels in the *sdn1-1 sdn2-1* double mutant (Fig 3).

To further interrogate the gene family, we introduced an artificial miRNA (amiRNA) (16) that targets the exonuclease region in four of the five genes in the clade (Fig S4) into *sdn1-1*. In the T1 population, plants with various pleiotropic developmental defects were observed (Fig 4A-F, Table S3). Notably, type I plants (Fig 4B-D), which were most severely affected, had small and often serrated leaves. Some plants had pin-like protrusions emanating from the abaxial side of the rosette leaves (Fig 4C). Similar protrusions have been found in leaves of plants carrying an antisense *AGO1* cDNA or those undergoing sense *AGO1* cDNA-mediated co-suppression (17).

Levels of amiRNA-targeted *SDN1*, *SDN2*, and *SDN3* transcripts were severely reduced in one individual line and moderately reduced in another line (Fig 4H,I). miR167 accumulated to 2–4 times that of the wild-type level in the two amiRNA lines (Fig 4G). Consistent with the presence of pin-like structures in the first individual line, a strong reduction in *AGO1* mRNA levels was found (Fig 4I). To analyze the amiRNA lines more extensively, we pooled T1 plants according to the severity of the developmental phenotypes. Type I plants (lanes 1, 2, and 4 in Fig 4J,K) had the highest levels of the amiRNA (Fig 4J), greatly reduced levels of *SDN1* and *SDN2* transcripts, and a slight reduction in *SDN3* transcript levels (Fig 4K,S6). In these lines, miR167, miR159, and siR1003 levels were to 2–3 times that of wild type, and miR172 levels, which were not elevated in the *sdn1-1 sdn2-1* mutant (Fig 3), were up to 3 fold of the wild type level (Fig 4J). The remaining miRNAs, except for miR164, all showed some elevation in abundance in some of the type I plants. Type II and type III plants (lanes 3 and 5, respectively, in Fig 4J,K) had moderate levels of the amiRNA (Fig 4J), a moderate to severe reduction in *SDN1* and *SDN2* transcript levels (Fig 4K,S6), and a moderate or no elevation in the abundance of endogenous small RNAs (Fig 4J).

We did not observe any 3' extended forms of the 5S or 5.8S rRNAs, which readily accumulate in the yeast *rex* mutants (15) and in the *C. elegans eri-1* mutant (13), respectively, in any of the *sdn* single mutants, the *sdn1-1 sdn2-1* double mutant, or the amiRNA lines (Fig S7). This, together with the inability of SDN1 to digest small RNA duplexes, pre-miRNAs or longer RNAs *in vitro*, suggests that single-stranded small RNAs are the most likely *in vivo* substrates of SDN1. However, a role of these genes in the metabolism of other classes of RNAs cannot be excluded.

In conclusion, we have identified a family of exonucleases that degrades single-stranded small RNAs *in vitro* and that limits the accumulation of small RNAs *in vivo*. SDN1 and the only other known small RNA exonuclease, Eri-1, have distinct substrate specificities. The pleiotropic developmental phenotypes associated with reduction-of-function of the *SDN* gene family indicates that small RNA turnover is crucial for developmental patterning in plants. This family of genes is universally present in eukaryotes and it is likely that the animal homologs of *SDN1* perform similar functions in small RNA metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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18. We thank Liu Bi for technical assistance and Theresa Dinh, Lijuan Ji, Bin Yu, and Binglian Zheng for helpful discussions and careful reading of the manuscript. This work was supported by grants from NSF (MCB-0718029) and NIH (GM61146) to X.C.

Figure 1.

Arabidopsis At3g50100 (SDN1) possesses 3'-to-5' exonuclease activity on miRNAs. A, enzymatic activity assays on single-stranded miRNAs *in vitro*. RNA oligonucleotides were 5'-end labeled, incubated with buffer alone (1), purified GST (2), or purified GST-At3g50100 (3), and resolved on a denaturing polyacrylamide gel. miR173-me, a miR173 oligonucleotide containing a 2'-*O*-methyl group on the 3' terminal ribose. B, enzymatic activity of GST-At3g50100 (GST-SDN1) on miR173 labeled at the 3' end with ³²pCp. miR173-³²pCp was treated (+) or not (-) with phosphatase before incubation with GST-SDN1. The arrow indicates the position of the expected 15 nt product if SDN1 were to cleave the RNA between nt 8 and 9 from the 5' end. The radioactivity at the bottom corresponds to the position of free nucleotides.

Figure 2.

Substrate specificity of SDN1. A, RNA oligonucleotides ranging from 17 to 27 nt in length were 5'-end labeled and incubated with GST-SDN1. S, substrates alone; E, substrates + GST-SDN1. B, a 5'-end labeled, single-stranded DNA oligonucleotide (ssDNA) and a miR173/miR173* duplex labeled at the 5' end of miR173 (dsRNA) were each incubated with buffer (S), GST (G), or GST-SDN1 (E). C, an *in vitro* transcribed and 5'-end labeled pre-miR167 was incubated with buffer (S), GST (G), or GST-SDN1 (E). An *in vitro* transcribed and 5'-end labeled AP1 RNA of approximately 300 nt was incubated with GST-SDN1 for 0, 30, and 60 min. D, miR173 or miR173 with 2 or 5 additional Us at the 3' ends was each incubated with GST-SDN1 for 0, 10, or 30 min. E, effects of the 2'-O-methyl group on GST-SDN1 activity.

Top panel: 5'-end labeled miR173 or 2'-*O*-methyl miR173 was incubated with increasing amounts of GST-SDN1 (numbers 0–5 represent 0 ng/μl, 0.33 ng/μl, 0.67 ng/μl, 1.33 ng/μl, 2.0 ng/μl, and 2.67 ng/μl of GST-SDN1, respectively). Bottom panel: miR173 or 2'-*O*-methyl miR173 was incubated with 0.67 ng/μl GST-SDN1 for the time specified periods (min). The arrow indicates a ~20 nt intermediate. Note that under low enzyme concentration, another intermediate of 9–10 nt was also present (in D and E). The bottom band corresponds to the final, 8–9 nt product.

Figure 3.

Northern blot to detect the steady-state levels of seven miRNAs and an siRNA in mutants of *SDN1* and related genes. The U6 blots serve as a loading control. The numbers below the blots indicate the relative abundance of the small RNAs in the different genotypes. 1, wild type; 2, *sdn1-1*; 3, the mutant in At3g50090; 4, *sdn2-1*; 5, *sdn3-1*; 6, *sdn1-1 sdn2-1*.

Figure 4.

Effects of an amiRNA that targets *SDN1* and three related genes. A, an *sdn1-1* plant. B-F, amiRNA lines (in *sdn1-1*) with developmental defects of varying severity. B-D, type I plants. Arrowhead in C indicates a pin-like protrusion. E, a type II plant that has small, mildly serrated leaves. F, an early-flowering type III plant. G, accumulation of miR167 in wild type (Col), *sdn1-1*, and two individual type I amiRNA lines. The numbers below the gel images indicate the relative abundance of the miRNA. H-I, levels of *SDN* and *AGO1* mRNAs as determined by RT-PCR (H) and realtime PCR (I) in the four genotypes in G. J, northern blotting to detect the amiRNA and endogenous small RNAs in pooled amiRNA lines. The numbers below the gel images indicate the relative abundance of the small RNAs. K, RT-PCR to detect *SDN*

transcripts in wild type (Col), *sdn1-1*, and six pools of amiRNA lines. The “-RT” controls did not yield any products and were not shown. The three bands for *SDN2* probably represent alternative transcripts since all three were missing in the “-RT” control.