Tissue-Specific Silencing of Arabidopsis SU(VAR)3-9 HOMOLOG8 by miR171a*1[W][OA]

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MicroRNAs (miRNAs) are produced from double-stranded precursors, from which a short duplex is excised. The strand of the duplex that remains more abundant is usually the active form, the miRNA, while steady-state levels of the other strand, the miRNA*, are generally lower. The executive engines of miRNA-directed gene silencing are RNA-induced silencing complexes (RISCs). During RISC maturation, the miRNA/miRNA* duplex associates with the catalytic subunit, an ARGONAUTE (AGO) protein. Subsequently, the guide strand, which directs gene silencing, is retained, while the passenger strand is degraded. Under certain circumstances, the miRNA*s can be retained as guide strands. miR170 and miR171 are prototypical miRNAs in Arabidopsis (Arabidopsis thaliana) with well-defined targets. We found that the corresponding star molecules, the sequence-identical miR170* and miR171a*, have several features of active miRNAs, such as sequence conservation and AGO1 association. We confirmed that active AGO1-miR171a* complexes are common in Arabidopsis and that they trigger silencing of SU(VAR)3-9 HOMOLOG8, a new miR171a* target that was acquired very recently in the Arabidopsis lineage. Our study demonstrates that each miR171a strand can be loaded onto RISC with separate regulatory outcomes.

Primary microRNA (miRNA) transcript in plants are first processed into miRNA precursors and then into miRNA duplexes, which are subsequently sorted and loaded into one of the ARGONAUTE (AGO) proteins. It has been proposed that the more thermodynamically unstable strand is retained during loading, while the other is removed and degraded. This sorting and degradation mechanism causes the mismatched steady-state levels of the two duplex strands (Voinnet, 2009). Generally, the more abundant strand is equated with the miRNA, also called the guide strand, while the rarer one is known as the passenger strand or miRNA*. Through sequence complementarity, the guide strand lets the RISC interact with a target mRNA, which is either cleaved or translationally inhibited (Voinnet, 2009). The miRNA* strands were originally thought to be mere by-products of the miRNA biogenesis pathway. Recent reports, however, have indicated that miRNA*s can be active in posttranscriptional gene silencing in Drosophila melanogaster, humans, and plants. The two strands of a miRNA/miRNA* duplex can even be sorted into different AGOs and thus are likely to have distinct functions (Okamura et al., 2008; Czech et al., 2009; Jazdzewski et al., 2009; Jay et al., 2011; Zhang et al., 2011). Large-scale sequencing has revealed not only that some miRNA*s are quite abundant but also that there are mRNA degradation products consistent with miRNA* guided cleavage (Devers et al., 2011). In agreement with the biological activity of miRNA*s, many are enriched in AGO1 complexes (Manavella et al., 2012).

Here, we identify miR170* and miR171a*, which have identical sequences, as active miRNA*s. This small RNA is one of the most abundant miRNA*s in Arabidopsis (Arabidopsis thaliana) and highly enriched in AGO1 complexes. It silences the expression of SU(VAR)3-9 HOMOLOG8 (SUVH8; also known as SET DOMAIN GROUP21), a member of the large SET domain protein family (Baumbusch et al., 2001). A biological function has not yet been assigned to SUVH8, which belongs to the SUVH1 subgroup (Naumann et al., 2005), although SUVH1 weakly affects monomethyl and dimethyl H3K9 methylation in pericentromeric heterochromatin (Naumann et al., 2005; Ebbs and Bender, 2006; Fischer et al., 2006). Here, we show that miR171a*-mediated silencing of SUVH8 in specific tissues is important for normal plant development. Two other Arabidopsis SUVH genes, SUVH5 and SUVH6, which belong to the SUVH5 subgroup, are targeted by an evolutionarily young miRNA, miR778 (Pant et al., 2009; Vergouil et al.,...
2012). Thus, at least three of 10 Arabidopsis SUVH genes are miRNA regulated.

RESULTS AND DISCUSSION
Loading of Highly Abundant and Conserved miR171* into AGO1
Since small RNAs that are not incorporated into AGO complexes are preferentially degraded, we reasoned that biologically active miRNA*s should be identifiable based on their steady-state levels. Based on the results of deep sequencing of small RNAs extracted from rosette leaves of Arabidopsis, we calculated the ratio between miRNAs and their corresponding miRNA*s (Fig. 1A; Supplemental Fig. S1A). Several of the more abundant miRNA*s have an adenine at their 5’ end, a feature that may favor loading of these miRNA*s into AGO2, as is the case for miR393* (Zhang et al., 2011). Among the miRNAs/miRNA*s with preferential accumulation of the miRNA*

Figure 1. miR171a* properties. A, Ratios between miRNAs and miRNA*s in small RNA libraries. Arrows indicate miRNA*s enriched in AGO1 coimmunoprecipitated material (Manavella et al., 2012). The color code indicates the 5’ nucleotide of miRNA* for those pairs with a ratio below 2. For pairs with a ratio above 5, see Supplemental Figure S1A. B, Alignment of SUVH8 and miR171a*. C, Phylogenetic tree of MIR171 precursors from land plants. The displayed clade includes homologs closely related to Arabidopsis MIR171A; a complete tree is shown in Supplemental Figure S1A. D, Relative entropy of miR171a* sequences, calculated as Shannon’s entropy corrected for the background.
We found several miRNA*s to be enriched in material that coimmunoprecipitated with AGO1 material (Fig. 1A; Manavella et al., 2012). Similar to canonical miRNAs, these miRNA*s often have a 5' uracil (Fig. 1; Supplemental Fig. S1B). Since AGO1-associated miRNA*s are likely to engage in conventional silencing, we decided to focus on three examples of this group, miR170*/miR171a*, miR846*, and miR840*.

Using established algorithms (Ossowski et al., 2008), we could identify a high-probability target for miR170*/miR171a*, SUVH8. Despite five mismatches between miR171a* and SUVH8, the hybridization energy (-36.5 kcal mol⁻¹) and mismatch positions are in agreement with boundary conditions for miRNA targeting (Schwab et al., 2005; Fig. 1B). A phylogenetic tree of MIR171 precursors together with a comparison of miR171a-complementary sequences revealed conservation of miRNA* sequences in a clade that includes the Arabidopsis MIR171A precursor (Fig. 1, C and D). Such conservation can be a consequence of selection for miRNA duplex pairing, although this does not necessarily require maintenance of the exact DNA sequence, as long as a minimum of pairing is present throughout the miRNA/miRNA* duplex. The analysis of the miR171a outgroups indicated that only the miR171a-like precursors have highly conserved star sequences.

![Figure 2](image1.png)

**Figure 2.** Conservation of the miR171a*-SUVH8 interaction. A, Phylogenetic tree of land plants with MIR171 precursors. Asterisks mark species with at least one sequence identical to miR171a* from the Arabidopsis reference genome. B, Alignment of SUVH8 and miR171a* in different dicots. Numbers in parentheses indicate mismatches between miR171a* and its target. C, miR171a* polymorphisms in Arabidopsis accessions.

![Figure 3](image2.png)

**Figure 3.** Association of miR171a* with AGO1. A, RNA blot after coimmunoprecipitation of small RNAs with HA-AGO1, HA-AGO2, and HA-AGO7 in stable transgenic Arabidopsis plants. U6 was used as a control. B, Enrichment of miR171a and miR171a* in coimmunoprecipitated (IP) material with different AGOs.
miR171a* sequences identical to the ones in Arabidopsis are found in many dicots but not in monocots (Fig. 2A; Supplemental Table S1). Strikingly, the miR171a* target site in SUVH8 apparently evolved recently in the Arabidopsis lineage, since it is not shared with the closest relative, Arabidopsis lyrata, or with other dicots (Fig. 2B). To date this event, we inspected the target site in 80 fully resequenced accessions of Arabidopsis (Cao et al., 2011). The miR171a* appears indeed to be derived, since the reference sequence is shared with only 10 other accessions (Fig. 2C). At position 15 of the target site, 70 out of 80 accessions have the ancestral variant found in A. lyrata and Brassica rapa, an adenine, which is predicted to interfere with miR171a* targeting (Fig. 2B). The derived allele encodes a nonconservative Asn-to-Gly substitution. Although only found in a minority of accessions, the fact that the miR171a*-targetable version of SUVH8 occurs in several accessions suggests that it is not deleterious and perhaps even adaptive. These properties of miR171a* encouraged us to further investigate its possible biological function. It has recently been shown that miRNAs and the corresponding miRNA*s can take different routes and be loaded into different subset of AGO proteins (Zhang et al., 2011). We first corroborated that miR171a* is associated with AGO1, but not with AGO2 or AGO7, by coimmunoprecipitation and subsequent RNA blots (Fig. 3A). To substantiate this observation and to expand it to other AGOs, we analyzed available deep sequencing data of AGO-associated small RNAs (Mi et al., 2008; Montgomery et al., 2008; Cuperus et al., 2010; Wang et al., 2011; Zhu et al., 2011). This analysis confirmed that miR171a*, like miR171a, is preferentially loaded into AGO1. In addition, miR171a* is associated with AGO10 (Fig. 3B). Because of lack of information, we could not draw any conclusion about the association with AGO3, AGO6, or AGO9. In contrast to some other miRNA/miRNA* pairs (Zhang et al., 2011), miR171a and miR171a* are both loaded into AGO1, suggesting that AGO1’s preference for retaining one or the other strand could be tissue or environment dependent.

Figure 4. Catalytic activity of AGO1-loaded miR171a*. A, Detection by 5′ RACE of miR171a*-triggered cleavage products of SUVH8 mRNA. Images of gels from the first and second rounds of RACE-PCR are shown; black arrowheads indicate purified bands. The fraction of clones with the expected 5′ end among all sequenced products is indicated above the SUVH8 target site on the bottom. MW, Molecular weight standard. B, SUVH8 protein accumulation. Normalized band intensities, determined with ImageJ software, are shown on top. C, SUVH8 mRNA accumulation.

miR171* Mediates SUVH8 Silencing Affecting Normal Plant Development

Because SUVH8 is expressed in only a few tissues and at low levels (Baumbusch et al., 2001), cleavage products caused by RISCmiR171* action were expected (Supplemental Fig. S2). miR171a* sequences identical to the ones in Arabidopsis are found in many dicots but not in monocots (Fig. 2A; Supplemental Table S1).

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Figure 5. Developmental defects in plants with altered miR171a*/SUVH8 balance. A, Rosettes of 15-d-old (top) and 25-d-old (bottom) plants. Bars = 1.5 cm. B, Alignment of miR171a* and SUVH8. The introduced synonymous mutations in the miR171a*–resistant version of SUVH8 occurs in several accessions suggests that it is not deleterious and perhaps even adaptive.

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miR171* Mediates SUVH8 Silencing Affecting Normal Plant Development

Because SUVH8 is expressed in only a few tissues and at low levels (Baumbusch et al., 2001), cleavage products caused by RISCmiR171* action were expected.
to be rare. An activity sensor indicated that miR171a* was expressed in pistils (see Fig. 6 below). Using poly (A') RNA from pistils and 5' RACE, we detected SUVH8 mRNA degradation products consistent with miR171a*-mediated slicing (Fig. 4A). In agreement with the repression of SUVH8 by miR171a*, both mRNA and protein levels increased when miR171a* action was suppressed with an artificial target mimic (Franco-Zorrilla et al., 2007) and decreased when the MIR171A precursor was overexpressed (Fig. 4, B and C).

Since these observations supported the restriction of SUVH8 function by miR171a*, we wanted to know whether altering the interaction between the two led to phenotypic changes. To answer this question, we analyzed plants expressing a miRNA-resistant form of SUVH8 mRNA, rSUVH8, either from a constitutive promoter or from the SUVH8 promoter (Fig. 5A). The miRNA-resistant variant was generated by introducing synonymous mutations into the miR171a*-complementary motif in the SUVH8 coding sequence (Palatnik et al., 2003; Chen, 2004; Fig. 5B). We compared these with plants that overexpressed the MIR171A precursor (Song et al., 2010), with plants that expressed either miR171a* or a miR171a target mimic (Todesco et al., 2010), and with plants that had an extra copy of the unmodified SUVH8 gene.

Plants specifically altered in the SUVH8-miR171a* balance (Pro35S:MIM171A*, Pro35S:rSUVH8, and ProSUVH8:rSUVH8) presented various developmental defects (Fig. 5A). These were most evident in plants expressing the miR171a*-resistant form of SUVH8 and

Figure 6. miR171a and miR171a* expression and activity domains. Different stages and tissues stained for activity of GUS reporters are shown. The miRNA target sites in the reporters are indicated on the left, and promoters driving reporters are shown on the right. Note the increased activity when reporters are assayed in the presence of ubiquitously expressed target mimics, Pro35S:MIM171A or Pro35S:MIM171A*. Individuals with and without the target mimics are from the same reporter lines. Insets show closeups of root tips.

Figure 7. Activity domains of miR171a* and SUVH8. Blue marks tissues where only the MIR171A promoter is active, red where SUVH8 is expressed but not silenced, and green where SUVH8 is silenced. Yellow indicates tissues where we observed SUVH8 silencing but not MIR171A promoter activity.
Activity Domains of miR171a and miR171a*

To determine whether the activity domains of miR171a and miR171a* strands are spatially regulated, we analyzed a set of GUS reporter constructs containing miR171a and miR171a* target sites expressed under the control of the MIR171A or SUVH8 promoter (Supplemental Fig. S3). Artificial target mimics were used to confirm the functionality of the reporters (Fig. 6).

Although expressed from the same promoters, the activity patterns of the two reporters differed. The only tissue in which the miR171a reporter escaped silencing was the shoot apical meristem. In contrast, the miR171a* reporter escaped silencing not only in the shoot apical meristem but also at the top of anther filaments, at the base of young leaves, and in stomata (Fig. 6). These observations suggested that efficient incorporation of miR171a* into the AGO1 effector complex is tissue dependent. As expected, miR171a* silencing of the reporter under the control of the SUVH8 promoter was only apparent where activity of the MIR171A* and SUVH8 promoters overlapped (Fig. 6). The miR171a* reporter was also silenced in cotyledon veins and at the top of the style, despite these tissues lacking detectable MIR171A promoter activity (Fig. 6). This may reflect that the MIR171a promoter fragment used does not reproduce the endogenous expression pattern, or it may be due to miR171a* mobility. It is also possible that silencing is caused by miR170*, which has the same sequence as miR171a*, although we were not able to ascertain where the MIR170 promoter is active (Supplemental Fig. S4). Figure 7 summarizes the miR171a* and SUVH8 activity domains deduced from our experiments, although we cannot exclude that the endogenous targets are silenced or expressed in additional tissues.

CONCLUSION

During processing of their precursor, both the miRNA and its complementary miRNA* are produced in equimolar ratio. Subsequently, biased incorporation into and retention by AGO proteins changes the steady-state ratio of miRNA and miRNA*. That miR171a* is frequently detected in small RNA libraries suggests that this species is often retained in the effector complex and, therefore, protected from degradation. That miR171a* can silence a reporter in almost all tissues where the precursor is likely to be produced indicates that miR171a* is often loaded into RISC. The differences in silencing activities between miR171a and miR171a* reporters suggest that loading of the two strands is under tissue-specific control. Alternative scenarios, such as tissue-specific modifiers, strand-specific decoys, differential processing of transcribed primary miRNA, or even restricted accessibility to the miRNA/miRNA* target site due to tissue-specific folding of the target mRNA, could also explain the observations but would not be any less interesting than regulated loading. Our results define the tissue-specific action of both miR171a strands, but the exact underlying mechanisms await discovery.

MATERIALS AND METHODS

Computational Analyses

MIR171 precursor sequences were obtained from miRBase (Griffiths-Jones et al., 2006) and aligned with ClustalW2 using the EMBL-EBI framework (Larkin et al., 2007; Goujon et al., 2010). A phylogenetic tree was reconstructed using SplitsTree4 (Huson and Bryant, 2006). miR171* sequences were extracted as the region complementary to the canonical miR171 offset by two nucleotides. Nucleotide frequencies and relative entropy were visualized using the WebLogo 3 Web tool (Crooks et al., 2004). A phylogenetic tree of land plant species was obtained using the iTOL Web tool (Letunic and Bork, 2011).

Data sets for small RNAs from ecotype Columbia (Col-0) wild-type leaves and from AGO-immunoprecipitated material have been published (Mi et al., 2008; Montgomery et al., 2008; Cuperus et al., 2010; Wang et al., 2011; Zhu et al., 2011; Manavella et al., 2012). miR171a* targets were predicted using WMD3 (http://wmd3.weigelworld.org; Ossowski et al., 2008).

Plant Material

Arabidopsis (Arabidopsis thaliana) plants, accession Col-0, were grown on soil at 23°C in long days (16 h of light/8 h of dark) in growth rooms with 65% humidity under a 2:1 mixture of cool-white and warm fluorescent light. Alternatively, plants were grown on Murashige and Skoog medium plates with 0.4% agar in growth chambers (Percival Scientific). Plants expressing hemagglutinin-tagged (HA)-AGO1 in agei-25, HA-AGO2 in Col-0, and HA-AGO7 in zip1 have been described (Montgomery et al., 2008; Cuperus et al., 2010). Plants expressing a miR171a target mimic have been reported (Todesco et al., 2010). Transgenic seedlings were selected with 50 mg mL⁻¹ kanamycin on plates or 0.1% ammonium glucofusinate (BASTA) on soil.

Constructs and Plant Transformation

An artificial target mimic for miR171a* was designed as described (Todesco et al., 2010). For the expression from specific promoters, fragments of approximately 2,000 bp upstream of the translation start were used. Promoters and genomic fragments were combined by Gateway (Life Technologies) cloning into a modified pGREEN vector (pPK210) conferring resistance to BASTA (Hellens et al., 2000). For the HA-tagged version of SUVH8, pGWB415 was used (Nakagawa et al., 2007). Construct names are given in Supplemental Table S2. Plants were transformed using the flower dip method (Clough and Bent, 1998).

Expression Analysis and 5' RACE Assays

Total RNA was extracted from 12-d-old seedlings and 30-d-old inflorescences using TRIzol Reagent (Life Technologies). Complementary DNA was produced with the RevertAid First Strand complementary DNA Synthesis Kit (Fermentas) using 1 µg of total RNA pretreated with DNase I (Fermentas). qRT-PCR assays were performed as described (Manavella et al., 2006). Expression levels were normalized against β-TUBULIN2 (At5g22690). Biological
triplicates and technical duplicates were analyzed. Differences were considered significant at P < 0.01 (Student’s t test with Bonferroni’s correction). miRNA-guided 3’ cleavage products were detected using RNA ligase-mediated 5’ RACE (Llave et al., 2002). Poly(A+)-RNA was enriched using the FastTrack MAG mRNA Isolation Kit (Life Technologies). Oligonucleotide sequences are listed in Supplemental Table S3.

Small RNA Blots
RNA was extracted using TRizol reagent (Life Technologies). Total RNA (1–5 µg) was resolved on 17% polyacrylamide gels under denaturing conditions (7 M urea). RNA was transferred to HyBond-N* membranes (GE Healthcare) by semidry blotting, and membranes were hybridized with DNA oligonucleotide probes labeled with digoxigenin using the DIG Oligonucleotide 3’ End Labeling Kit, Second Generation (Roche). Oligonucleotide sequences are listed in Supplemental Table S3.

Protein Analysis
Proteins were extracted from 300 mg of ground tissue with 300 µL of extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% [v/v] glycerol, 1 mM dithiothreitol, 1 mM Pefablock, and one tablet of complete protease inhibitor cocktail [Roche]) and vortexing for 10 s. Tissue debris was removed by centrifugation at 12,000g at 4°C for 20 min. Twenty micrograms of crude protein extract per sample was resolved on an 8% polyacrylamide gel. Blotting, antibody incubation, and detection were performed as described (Brodersen et al., 2008). Anti-HA high-affinity monoclonal antibody was from Roche, and monoclonal anti-α-tubulin antibody for the loading control was from Sigma-Aldrich.

Immunoprecipitation
Leaves of 20-d-old plants were collected, and 1.5 g was ground in liquid nitrogen. The frozen powder was homogenized by vortexing in 4 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 5 mM MgCl2) at 4°C for 20 min. Cell debris was removed by centrifugation at 12,000g at 4°C for 30 min. Each sample was preincubated with 40 µL of Protein G Plus-Agarose (Santa Cruz Biotechnology) under rotation at 4°C for 30 min. Protein agarse was removed by centrifugation at 6,000g at 4°C for 1 min, and the supernatant was incubated with 40 µL of Monoclonal Anti-HA-Agarose (Sigma) overnight at 4°C under rotation. Immunoprecipitates were washed four times with lysis buffer before extracting RNA and proteins. RNA was isolated from the protein agarose by TRIzol extraction, and protein was precipitated with ice-cold acetone from the organic phase.

Histochemical Assays
Ten-day-old seedlings or inflorescences from 20-d-old plants were fixed in 90% (v/v) acetone. GUS activity was assayed as described (Blázquez et al., 1997). At least 10 independent T2 lines were analyzed for each reporter. Representative lines were crossed to target mimic lines. Seedlings of at least 15 independent T2 lines were analyzed for each construct.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_128034 (Arabidopsis thaliana), EF459118 (Arabidopsis MIR171a), and DQ606307 (Arabidopsis MIR170).

Supplemental Table S1. Sequence of ath-miR171* homologs.

Supplemental Table S2. Constructs information.

Supplemental Table S3. DNA oligonucleotide primers and probes.

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