

Artificial trans-Acting siRNAs Confer Consistent and Effective Gene Silencing

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Manipulating gene expression is critical to exploring gene function and a useful tool for altering commercial traits. Techniques such as hairpin-based RNA interference, virus-induced gene silencing, and artificial microRNAs take advantage of endogenous posttranscriptional gene silencing pathways to block translation of designated transcripts. Here we present a novel gene silencing method utilizing artificial trans-acting small interfering RNAs in *Arabidopsis* (*Arabidopsis thaliana*). Replacing the endogenous small interfering RNAs encoded in the *TAS1c* gene with sequences from the *FAD2* gene silenced *FAD2* activity to levels comparable to the *fad2-1* null allele in nearly all transgenic events. Interestingly, exchanging the endogenous miR173 target sequence in *TAS1c* with an miR167 target sequence led to variable, inefficient silencing of *FAD2*, suggesting a specific requirement for the miR173 trigger for production of small interfering RNAs from the *TAS1c* locus.

Loss-of-function mutations are the most basic tool of genetic analysis: The biological activity of a gene is inferred from the mutant phenotype. In traditional genetic analysis, a plant with a phenotype of interest is identified from a mutagenized population, and the gene responsible for the phenotype is identified by genetic mapping or by means of a T-DNA or transposon tag (Lukowitz et al., 2000). A complementary approach to investigating gene function, often called functional genomics, is to identify genes of interest based on their sequence and/or expression pattern, and then to use a transgenic approach to inactivate the gene to obtain a mutant phenotype. One strategy for functional genomics is to produce massive sequenced-indexed libraries of plants with genes randomly inactivated by T-DNA insertion (Sessions et al., 2002; Alonso et al., 2003). Although this approach has been of great value to the scientific community, T-DNA insertion sites are biased toward 5' and 3' untranslated region (UTR) sequences, and T-DNA insertions in a large proportion of genes, especially small genes, have never been recovered (Sessions et al., 2002; Alonso et al., 2003). In addition, indexed T-DNA insertion libraries are not available for many plant species used for basic research or for most agronomically important crop species. Thus, strategies to efficiently silence specific genes are essential.

Many different strategies have been used for gene silencing (for review, see Ossowski et al., 2008). Initial efforts to overexpress genes resulted in cosuppression (Napoli et al., 1990), and overexpression of antisense

transcripts was an early method to silence genes, albeit at a low frequency (Baulcombe, 1996). Later studies showed that expression of an inverted repeat of a gene that formed a hairpinRNA was effective at silencing endogenous genes (Waterhouse et al., 1998; Chuang and Meyerowitz, 2000). Subsequently, this method was refined through the use of an intron-spliced hairpin construct that gave more consistent silencing (Smith et al., 2000; Stoutjesdijk et al., 2002). A large-scale evaluation of inverted repeat-based gene silencing found that the success of this method varies greatly from gene to gene (McGinnis et al., 2005). More recently, artificial microRNAs (amiRNAs) have been designed to target endogenous genes, and have also proven to be effective (Alvarez et al., 2006; Schwab et al., 2006).

All of these strategies rely on the genetic machinery of an endogenous phenomenon called posttranscriptional gene silencing (PTGS). PTGS was first recognized as a plant immune response to viral infection (Hamilton and Baulcombe, 1999). In this process, viral RNAs are substrates for an RNA-dependent RNA polymerase that synthesizes a complementary RNA strand. This double-stranded RNA is cut into 21- to 24-bp small interfering RNAs (siRNAs) by an endonuclease called Dicer (Bernstein et al., 2001). One strand of the siRNA is loaded into the RNA-induced silencing complex (RISC). Guided by the associated siRNA, RISC recognizes and destroys complementary target viral RNA via the "slicer" Argonaute protein, the primary enzyme of the RISC complex (Song et al., 2004; Baumberger and Baulcombe, 2005). Another mechanism for gene silencing in plants is mediated by 21- to 24-bp microRNAs (miRNAs). miRNA genes are transcribed to form pre-miRNAs, which fold back on themselves to form stem-loop structures. The functional miRNA is processed from the pre-miRNA by the enzyme Dicer, which cuts the stem of the pre-miRNA to produce a double-stranded miRNA, only one strand of which is active (Park et al., 2002; Reinhart

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et al., 2002). In plants, miRNAs target endogenous mRNAs, usually in their coding regions, but sometimes in the 5' or the 3' UTR (Bartel, 2004; Wu and Poethig, 2006). Typically, an miRNA that is bound to its target mRNA marks it for cleavage by the RISC complex, though there are several cases in which miRNAs have been shown to cause translational inhibition in plants, as they frequently do in animals (Aukerman and Sakai, 2003; Chen, 2004; Arteaga-Vázquez et al., 2006; Gandikota et al., 2007).

Generation of a third class of PTGS-associated small RNAs, called trans-acting siRNAs (ta-siRNAs), involves both siRNAs and miRNAs. ta-siRNAs differ from conventional siRNAs in that they target genes in trans (Peragine et al., 2004; Vazquez et al., 2004). ta-siRNA genes produce nonprotein coding transcripts that are themselves targeted by an miRNA. One of the miRNA cleavage products is converted to double-stranded RNA by RDR6, at which point it can be cleaved into siRNAs by Dicer-like protein DCL4. Upon incorporation into RISC, some of these siRNAs have the ability to target other genes in trans (Allen et al., 2005; Yoshikawa et al., 2005). Thus, all three pathways converge with incorporation of a small RNA into the RISC complex, which then enables cleavage of the target RNA.

In this study, we describe our efforts to extend previous silencing strategies by engineering the *TAS1c* (trans-acting siRNA1c) locus to silence the *FAD2* gene in *Arabidopsis* (*Arabidopsis thaliana*). Processing of the *TAS1c* RNA is initiated by miR173-mediated cleavage, revealing six phased siRNAs downstream of the miR173 target site (Allen et al., 2005). We engineered the *TAS1c* locus to silence the *FAD2* gene by replacing a single native siRNA with an siRNA targeting *FAD2* (siFAD2), by replacing five native siRNAs with siFAD2, or by substituting these five native siRNAs with a 210-bp fragment of the *FAD2* gene. All three of these strategies resulted in silencing of the *FAD2* gene activity similar to levels seen in the *fad2-1* null mutant. Furthermore, almost all transgenic events showed a high degree of silencing, and the silencing effect was inherited over four generations. Interestingly, replacement of the miR173 trigger in *TAS1c* with miR167 did not give effective silencing, suggesting that miR173 may play a specific role in the biology of *TAS1c*.

RESULTS

Silencing with Artificial trans-Acting siRNAs

To determine if ta-siRNA loci could be engineered to silence genes of interest, we modified the *TAS1c* sequence to produce siRNAs targeting the *FAD2* gene. *FAD2* encodes an endoplasmic reticulum-localized $\Delta 12$ desaturase required for converting the monounsaturated oleic acid (18:1) to the polyunsaturated linoleic acid (18:2), itself the precursor to linoleic acid

(18:3; Okuley et al., 1994). *FAD2* is a classic reporter for silencing assays because it is a single-copy, nonessential gene in *Arabidopsis* with an easily assayed, quantifiable phenotype (Miquel and Browse, 1992). Using *FAD2* as a silencing reporter allowed direct comparison between current and previous methods as well (Stoutjesdijk et al., 2002).

We engineered a truncated *TAS1c* cDNA to silence *FAD2* by replacing the sequence encoding the five native *TAS1c* siRNAs with a sequence encoding five identical siRNAs targeting base pairs 98 to 118 of the *FAD2* coding sequence (CDS; *5XsiFAD2*; Fig. 1A). As a control, the miR173 binding site of *5XsiFAD2* was mutated to disrupt base pairing at base pairs 4, 10, and 11 of miR173, to produce *mut-5XsiFAD2*. Loss of the functional miR173 binding site in *mut-5XsiFAD2* should prevent production of phased *FAD2* siRNAs.

FAD2 expression is directly proportional to the amount of oleic to linoleic desaturase activity in a tissue (Okuley et al., 1994). Accordingly, silencing was quantified by expressing the degree of unsaturation of 18:1 to 18:2 and 18:3 fatty acids as oleic desaturation proportion (ODP; see "Materials and Methods"). Wild-type plants had an ODP of approximately 0.7 in seed, meaning that 70% of 18:1 fatty acids in seed are converted to 18:2 and 18:3 fatty acids (Fig. 1B). All 12 transgenic events we obtained for the *5XsiFAD2* construct showed strong silencing of the *FAD2* gene. Ten of the 12 families had average ODP values between 0.1 and 0.2, while the remaining two families had ODP values of approximately 0.25 and 0.3 (Fig. 1B). T2 seed from each family showed the same degree of silencing whether they were homozygous or hemizygous for the *5XsiFAD2* transgene. The *fad2-1* null allele had an ODP value of about 0.1 (Okuley et al., 1994; Stoutjesdijk et al., 2002), and thus the majority of our transgenic lines showed complete or almost complete silencing of the *FAD2* gene, with the remaining two lines showing strong silencing. Plants harboring the *mut-5XsiFAD2* construct had ODP values indistinguishable from the wild type (Fig. 1B), indicating that *FAD2* silencing initiated by *5XsiFAD2* occurs through an miR173-triggered mechanism. We propagated three lines for several generations more and found that silencing was maintained in the T5 generation (Fig. 1B).

To directly assay *FAD2* gene expression in these lines, we determined the levels of *FAD2* mRNA and siFAD2 by northern analysis of RNA from leaf tissue (Fig. 1D), and determined leaf fatty acid levels in the plants that we used for northern analysis (Fig. 1C). Using the same transgenic lines subjected to fatty acid analysis, we detected markedly decreased *FAD2* mRNA levels in *5XsiFAD2* lines compared to wild-type plants. This decrease is consistent with the large decrease in ODP seen in these lines. Transgenic lines harboring the *mut-5XsiFAD2* transgene showed no decrease in *FAD2* mRNA levels. In small RNA northrens, siFAD2 siRNAs were observed in *5XsiFAD2* plants, yet they were absent from wild-type and *mut-5XsiFAD2* plants (Fig. 1D). In addition, 5' RACE

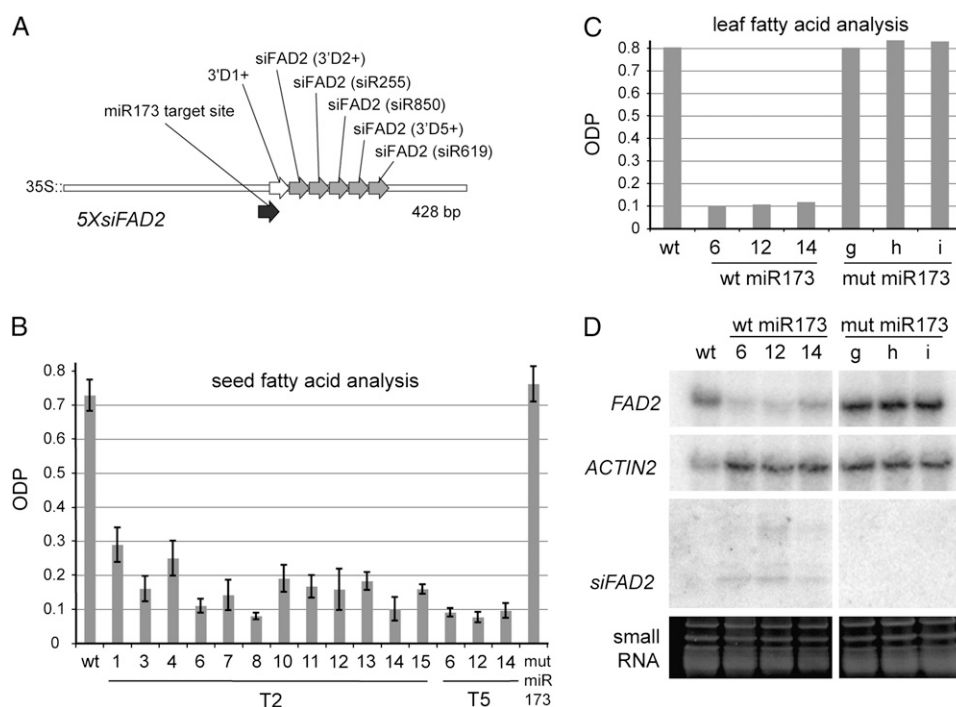


Figure 1. Silencing of *FAD2* with *5XsiFAD2*. **A**, Diagrammatic representation of the *5XsiFAD2* construct. This construct was engineered by replacing the sequence corresponding to five native siRNAs from the *TAS1c* cDNA at positions 3'D2+ to siR619 with five copies of a 21-bp DNA sequence (shown in gray) complementary to base pairs 98 to 118 of the *FAD2* CDS. The name of the native siRNA replaced at each position is shown in parentheses. The miR173 target site is shown in black, and the 3'D1+ siRNA is shown in white. In planta expression of the *5XsiFAD2* construct is driven by the CaMV35S promoter. **B**, Seed fatty acid analysis of T2 and T5 families harboring the *5XsiFAD2* construct. Fatty acid analysis is expressed as ODP. ODP values (with *SD*) represent the average of at least five individual seeds. Wild type and *mut-5XsiFAD2*, a version of the *5XsiFAD2* construct with a mutated miR173 binding site, are shown as controls. **C**, Leaf fatty acid analysis of wild type, three T2 transgenic lines harboring the *5XsiFAD2* construct (lines 6, 12, and 14), and three T2 transgenic lines (g, h, and i) harboring *mut-5XsiFAD2*. **D**, Northern analysis of levels of *FAD2* transcripts and siFAD2 siRNAs. RNA samples were extracted from the transgenic plants subjected to fatty acid analysis (shown in C). *ACTIN2* is shown as a loading control for *FAD2* transcript levels; a picture of the small RNA gel used to separate small RNAs for blotting is shown as a loading control for the siFAD2 hybridization.

revealed cleavage at the miR173 site of *5XsiFAD2* (data not shown).

A Single Copy of siFAD2 Is Sufficient for Silencing

Next we sought to determine if a single copy of siFAD2 could effectively silence *FAD2*. We replaced the sequence encoding the native siRNA at the 3'D2+ position within the full-length *TAS1c* cDNA with a sequence encoding siFAD2, producing the construct *1XsiFAD2* (Fig. 2A). As a control we engineered *mut-1XsiFAD2*, identical to *1XsiFAD2* except that the miR173 binding site was mutated to prevent miR173-induced cleavage.

When transformed into plants, *1XsiFAD2* decreased fatty acid ODP from about 0.75 in wild-type seed to an average of 0.18 in 10 independent T1 lines (Fig. 2B). All 10 independent transgenic lines showed approximately the same degree of *FAD2* sense silencing: The *SD* in ODP score was only about 0.03. The silencing was heritable, as the majority of the T2 families examined

showed ODP scores similar to those of T1 lines. Thus, engineering *TAS1c* with a single siFAD2 decreased ODP nearly as much as what was achieved with five copies of siFAD2. This level of silencing persisted in almost all lines in the T2 generation (Fig. 2B).

Silencing of the *FAD2* Gene with the *fragFAD2* Construct

The experiments described above demonstrated that the *TAS1c* locus can be successfully modified to silence the *FAD2* gene by substituting DNA encoding a deliberately designed *FAD2* siRNA for a native *TAS1c* siRNA. However, it may not always be straightforward to design siRNAs targeting a gene of interest, as the characteristics of successful siRNAs are not completely known. For this reason, we determined if inserting a larger fragment of the *FAD2* gene into *TAS1c* would effectively produce siRNAs. A 210-bp sense fragment of the *FAD2* gene was inserted into the full-length *TAS1c* cDNA in place of the segment encoding the phased siRNAs 3'D2+ to 3'D6+ to produce the *fragFAD2*

construct (Fig. 3A). As a control for miR173-dependent production of phased siRNAs, the miR173 target site was mutated to produce construct *mut-fragFAD2*.

As shown in Figure 3B, T1 transgenic plants containing the *fragFAD2* construct had an average ODP of less than 0.1, similar to levels seen in the *5XsiFAD2* transgenic lines and in the *fad2-1* mutant (Miquel and Browse, 1992). This level of silencing was maintained in six out of seven T2 transgenic lines. We checked ODP levels for three lines in the T3 generation and found that silencing was maintained. Transgenic lines containing the *mut-fragFAD2* construct had ODP levels similar to the wild type, indicating that miR173-induced cleavage of *fragFAD2* was required for siRNA production.

To verify gene silencing, levels of *FAD2* mRNA and *FAD2* siRNAs were determined in *fragFAD2* and *mut-fragFAD2* transgenic plants (Fig. 3C). As expected, *FAD2* mRNA levels were markedly decreased in *fragFAD2* transgenic plants and were similar to the wild type in *mut-fragFAD2* transgenic plants. In addition, *FAD2* siRNAs were detected in *fragFAD2* plants, but not in wild-type or *mut-fragFAD2* transgenic plants. Thus, the substitution of native *TAS1c* siRNAs with a large fragment of the *FAD2* gene leads to efficient and stable silencing of this target gene.

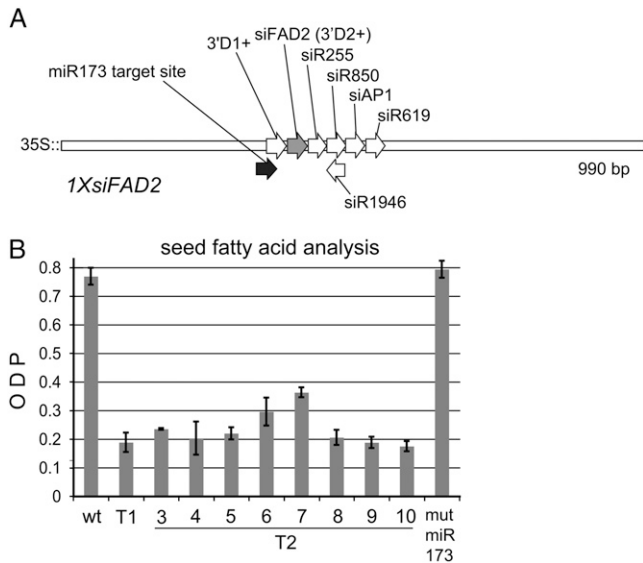


Figure 2. Silencing of *FAD2* with *1XsiFAD2*. A, Diagrammatic representation of the *1XsiFAD2* construct. Sequence coding for the 3'D2+ siRNA was replaced with base pairs 98 to 118 of the *FAD2* CDS coding for the 21-bp siFAD2 sequence (shown in gray). The miR173 target site is shown in black, and sequences encoding other siRNAs are shown in white. In planta expression of the *1XsiFAD2* construct is driven by the CaMV35S promoter. B, Fatty acid analysis of wild type; T1 and T2 transgenic seed harboring the *1XsiFAD2* construct; and T2 transgenic control seed harboring the *mut-1XsiFAD2* construct, a version of the *1XsiFAD2* construct in which the miR173 site has been mutated. Data for T1 seed are the average of 10 individual seeds, and data for T2 seed are the average of four individual seeds each from independent families. Fatty acid profiles are expressed as ODP. The SD is shown at the top of each bar.

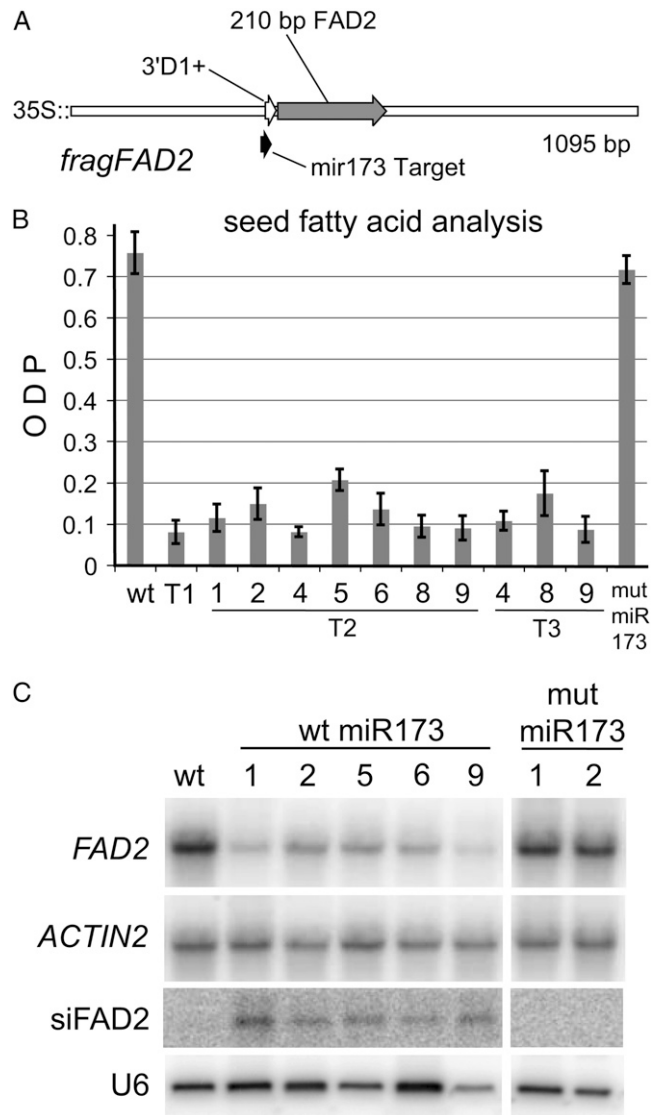


Figure 3. Silencing of *FAD2* with *fragFAD2*. A, Diagrammatic representation of the *fragFAD2* construct. This construct was engineered by replacing the DNA sequence coding for the native siRNAs from the 3'D2+ to the 3'D6+ positions of the *TAS1c* cDNA with a 210-bp fragment corresponding to base pairs 1 to 210 of the *FAD2* CDS. The miR173 target site is shown in black, and the siRNA at the 3'D1+ position is shown in white. In planta expression of the *fragFAD2* construct is driven by the CaMV35S promoter. B, Fatty acid analysis of wild type; T1, T2, and T3 transgenic seed harboring the *fragFAD2* construct; and T2 seed harboring the *mut-fragFAD2* construct, a version of the *fragFAD2* construct with the miR173 binding site mutated. Data for T1 seed are the average of 10 seeds, and data for T2 and T3 seed are the average of four seeds each from independent families. Fatty acid profiles are expressed as ODP. The SD is shown at the top of each bar. C, Northern analysis of levels of *FAD2* transcripts and siFAD2 siRNAs of T2 plants. RNA samples were extracted from T2 transgenic plants grown from the same families subjected to fatty acid analysis (shown in B). *ACTIN2* is shown as a loading control for *FAD2* transcript levels; U6 is shown as a loading control for the siFAD2 hybridization.

An Alternate miRNA Trigger Leads to Inefficient Silencing

Whether each *TAS* locus requires its native miRNA trigger for proper function remains an open question. To address this, we substituted the endogenous miR173 target site in the *5XsiFAD2* construct with the target site for miR167, a highly expressed miRNA in leaves (data not shown) to produce *miR167-5XsiFAD2* (Fig. 4A). As a control to show that any siRNAs produced from this construct required miR167 for cleavage, we also made *mut-miR167-5XsiFAD2*, a construct with a mutated miR167 binding site.

When transformed into plants, *miR167-5XsiFAD2* T1 seed had approximately half the ODP of the wild type, although there was considerable variation in the degree of silencing among T1 individuals, as shown by the large SD for the average ODP value of T1 seed (Fig. 4B). ODP levels climbed to approximately 75% of wild type in most T2 families. No difference was observed in ODP levels between *mut-miR167-5XsiFAD2* control plants and the wild type. Such moderate and variable silencing was also noted in leaf tissue (Fig. 4C).

Modified 5' RACE showed a reduced frequency of cleavage at the miR167 site of *miR167-5XsiFAD2* mRNA in leaf tissue. Out of 30 sequenced cleavage products, only two were cleaved at the miR167 target site (Fig. 4A). The remaining 28 5' RACE products showed cleavage throughout the *miR167-5XsiFAD2* mRNA. For comparison, we performed 5' RACE on an endogenous target of miR167, *ARF8*. We detected robust miR167 cleavage events in *ARF8* (data not shown), results similar to those published previously (Yang et al., 2006). The inefficiency of miR167 in triggering ta-siRNA production suggests that miR173 is specially adapted to participating in ta-siRNA biogenesis.

DISCUSSION

Here we have described a novel method of PTGS utilizing the *TAS1c* locus in Arabidopsis. Engineering *TAS1c* to produce single or multiple copies of an artificial trans-acting siRNA (ata-siRNA) targeting the *FAD2* gene, or with a 210-bp fragment of the *FAD2* gene, resulted in consistent and very efficient silencing of *FAD2*. Notably, we also showed that miR167 was not an effective trigger for silencing in a *TAS1c* context, suggesting that miR173 may be specifically required to cleave *TAS1c* and/or may have a more sophisticated role in initiating phased siRNA from the *TAS1c* transcript.

TAS1c-mediated silencing consistently reaches complete phenotypic penetration. Nearly all T1 seed for constructs encoding single or multiple ata-siRNAs targeting *FAD2* showed *FAD2* silencing comparable to that of the *fad2-1* null allele. In hairpinRNA-mediated silencing, a continuous spectrum of silencing was obtained in T1 plants with ODP scores ranging from similar to the *fad2-1* mutant to as high as 50% of the

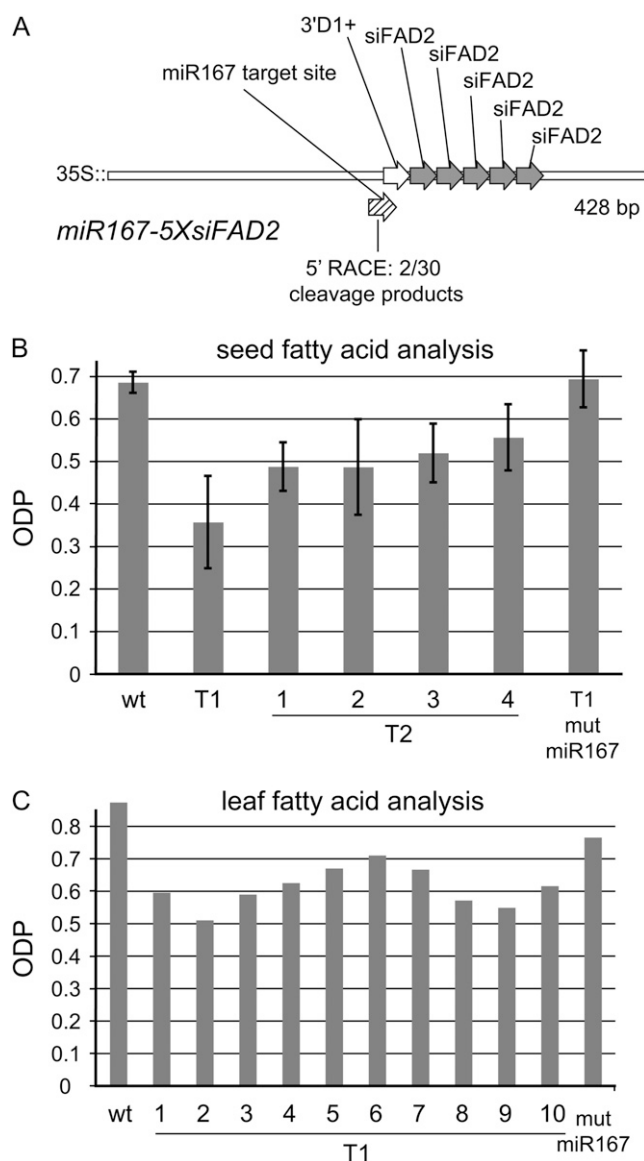


Figure 4. Inefficient silencing of *FAD2* by *miR167-5XsiFAD2*. A, Diagrammatic representation of the *miR167-5XsiFAD2* construct. This construct was engineered by replacing the miR173 target site in the *5XsiFAD2* construct with an miR167 target site. The miR167 target is shown with hatched bars, the 3'D1+ siRNA in white, and the siFAD2 siRNAs in gray. The number of cleavage events detected by 5' RACE at the miR167 target site in *miR167-5XsiFAD2* transgenic plants is shown. B, Fatty acid analysis of wild type; T1 and T2 transgenic seed harboring the *miR167-5XsiFAD2* construct; and T2 seed harboring the *mut-miR167-5XsiFAD2* construct, a version of the *miR167-5XsiFAD2* construct in which the miR167 target site has been mutated. Fatty acid profiles are expressed as ODP. Value for wild type is the average of 10 seeds; values for T1 and T2 are the average of four seeds each from independent families. C, Fatty acid analysis of a single leaf of wild-type and T1 plants harboring the *miR167-5XsiFAD2* construct and the *mut-miR167-5XsiFAD2* construct.

wild type (Stoutjesdijk et al., 2002). Considering that up to 50% of Arabidopsis genes may be difficult to silence using long hairpin RNA interference con-

Table 1. *Oligonucleotide primers used in this study*

*. These oligonucleotides have the attB primer tagged pair to clone the fragment of interest in the pDONR/Zeo vector (Invitrogen) and were used in the three constructs to amplify the 5' end and the 3' end.

Construct Name or Purpose	Primer Name	Sequence
<i>5XsiFAD2</i>	FAD2TAS 5' sense	GATCACAAGTTTGTACAAAAAGCAGGCTCATAGAAAGGTA CTTTTCGTTA- CTTCTTTGAGTATCGAGTAGAGCGTCGTCTATAGTTAGTTGAGATTGC- GTTTGTGAGAAGTTAGGTTCAATGTCCCGGTCCAATTTTCACCAAGCCA- TGTGTCAGTTTCGTTCCCTCCCGTCTCTTTGATTTCGTTGGGTTAC- GGATGTTTTCGAGATGAAACAGCATTGTTTTGTTG
	FAD2TAS 5' antisense	ATCACAACAAAAACAATGCTGTTTCATCTCGAAAAACATCCGTAACCCACGA- AATCAAAGAAGAGGACGGGAAGGAACGAACTGACACATGGCTGGT- GAAAATTGGACCGGGACATTGAACCTAATTCTGACAAACGCAATCTC- AACTAACTATAGACGACGCTCTACTCGATACTCAAAGAAGTAAACGA- AAGTACCTTCTATGAGCCTGCTTTTTGTACAAACTTGT
	FAD2TAS 3' sense	TGATTTTTCTTACAAGCGAATAGACCATTATTGCTTTCTTCAGATCTCCCA- TTGCTTTCTTCAGATCTCCCATTTGCTTTCTTCAGATCTCCCATTTGCTTTCT- TCAGATCTCCCATTTGCTTTCTTCAGATCTCCCAAAAAACAATGAATATTGTT- TTGAATGTGTTCAAGTAAATGAGATTTTCAAGTCGTCTAAAGAACAGTTG- CTAATACAGTTACTTAACCCAGCTTTCTTGTACAAAGTGGT
	FAD2TAS 3' antisense	AATTACCACCTTTGTACAAGAAAGCTGGGTTAAGTAACTGTATTAGCAACTG- TTCTTTAGACGACTTGAAAATCTCATTACTTGAACACATTCAAACAAT- ATTCATTGTTTTTGGGAGATCTGAAGAAAGCAATGGGAGATCTGAAGA- AAGCAATGGGAGATCTGAAGAAAGCAATGGGAGATCTGAAGAAACG- AATGGGAGATCTGAAGAAAGCAATAAATGGTCTATTCTGTTAGAG- AAAA
	FAD2TASmut 3' sense	TGATTTTTCTAAACAAGAGAATAGACCATTATTGCTTTCTTCAGATCTCCCA- TTGCTTTCTTCAGATCTCCCATTTGCTTTCTTCAGATCTCCCATTTGCTTTCT- CAGATCTCCCATTTGCTTTCTTCAGATCTCCCAAAAAACAATGAATATTGTT- TGAATGTGTTCAAGTAAATGAGATTTTCAAGTCGTCTAAAGAACAGTTG- CTAATACAGTTACTTAACCCAGCTTTCTTGTACAAAGTGGT
	FAD2TASmut 3' antisense	AATTACCACCTTTGTACAAGAAAGCTGGGTTAAGTAACTGTATTAGCAACTG- TTCTTTAGACGACTTGAAAATCTCATTACTTGAACACATTCAAACAAT- ATTCATTGTTTTTGGGAGATCTGAAGAAAGCAATGGGAGATCTGAAG- AAAGCAATGGGAGATCTGAAGAAAGCAATGGGAGATCTGAAGAAAG- CAATGGGAGATCTGAAGAAAGCAATAAATGGTCTATTCTGTTAGAG- AAAA
<i>1XsiFAD2</i>	TAS1c/FAD2-attB1*	GGGGACAAGTTTGTACAAAAAGCAGGCTAAACCTAAACCTAAACCGGCT- AAGCCCG
	TAS1c/FAD2-2R	GCATATCTGGAATATGTAGGATCATCTTCTTGATACAGCGATATGTTGAA- CTTAGAATACGCTATGTTGGACTTAGAATATTGCTTTCTTCAGATCTCCCA- AATGGTCTATTGCTTG
	TAS1c/FAD2-3F	CAAGCGAATAGACCATTGGGAGATCTGAAGAAAGCAATATTCTAAGTC- CAACATAGCGTATTCTAAGTTCAACATATCGCTGTATCAAGAAGATGAT- CCTACATATTCCAGGATATGC
	TAS1c/FAD2-attB-4*	GGGGACCACTTTGTACAAGAAAGCTGGGTATTTCACTTTACGATGTGG- TGTTT
<i>fragFAD2</i>	TAS1c/FAD2-frag-2R	GCATTTCCACCTGCACCCATAAATGGTCTATTGCTTGTAGAG
	TAS1c/FAD2-frag-3F	CAAGCGAATAGACCATTAAATGGGTGCAGGTGGAAGAATGCCGG
	TAS1c/FAD2-frag-4R	CAAAACAATATTCTGTTTTGGCGACGTAGTAGAAGCATGAGGC
	TAS1c/FAD2-frag-5F	CTCATGCTTCTACTACGTCGCCAAAACAATGAATATTGTTTGAATGTG
<i>miR167-5XsiFAD2</i>	miR167/TAS1c/5XsiFAD2-attB1	GGGGACAAGTTTGTACAAAAAGCAGGCTCATAGAAAGGTA CTTTTCGTTA- CTTCTTTGAGTATCGAGTAGAGCGTCGTCTATAGTTAGTTGAGATTGC- GTTTGTGAGAAGTTAGGTTCAATGTCCCGGTCCAATTTTCACCAAGCCA- TGTGTCAGTTTCGTTCCCTCCCGTCTCTTTGATTTCGTTGGGTTAC- GGATGTTTTCGAGATGAAACAGCATTGTTTTGTTG
	miR167/TAS1c/5XsiFAD2-2R	GAAAGCAATAAATGGTCTAACAAGCTGCCAGCCTGATCTAAAACAAAA- CAATGCTGTTTCATCTCGAAAAC
	miR167/TAS1c/5XsiFAD2-3F	GTTTTCGAGATGAAACAGCATTGTTTTGTTTGTAGATCAGGCTGGCAGCT- TGTTAGACCATTATTGCTTTT
	miR167/TAS1c/5XsiFAD2-attB4	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAGTAACTGTATTAGCA- ACTGTTT
To mutate miR173 target site	miR173 binding site mut 1for	GTGATTTTTCTAAACAAGAGAATAGACCATTG
	miR173 binding site mut 1rev	CCAAATGGTCTATTCTCTTGTTTAGAAAAATCAC

(Table continues on following page.)

Table 1. (Continued from previous page.)

Construct Name or Purpose	Primer Name	Sequence
<i>FAD2</i> northern hybridization	miR167 binding site mut 1for	TTAGATCAGGCAAACAGCATGTTAGACCATTTA
	miR167 binding site mut 1rev	TAAATGGTCTAACATGCTGTTTGCCTGATCTAA
	FAD2-3F	TAGGGGTGTTTCATCGTTATTA
<i>ACTIN2</i> northern control	FAD2-3R	AAGACCAACTGTGTCATCCA
	ACTIN2 F	AAGATGACTCAAATCATGTTTGAGAC
	ACTIN2 R	ACGACCTTAATCTTCATGCTGC
<i>1XsiFAD2</i> small RNA northern hybridization	siFAD2	TGGGAGATCTGAAGAAAGCAA
<i>fragFAD2</i> small RNA hybridization	FAD2 210-bp For	ATGGGTGCAGGTGGAAGAATGCCGG
Modified 5' RACE	FAD2 210-bp Rev	GGCGACGTAGTAGAAGCATGAGGC
	TAS1c-2rev	GACGACTTGAAAATCTCATT
	FAD-TAS 1rev	CAATATTCATTGTTTTGGGAGATCTGA
	ARF8 1Rev	GAGAGAGATGCCGAACGAATGGCATAT
	ARF8 1Nested	CCCACCACTGCCTTCTCCATGAT

structs (Small, 2007), alternative silencing methods such as engineered ta-siRNA genes represent a valuable resource for functional genomics. Full loss-of-function phenotypes such as those demonstrated here are useful for inferring gene function and for epistasis analysis. Manipulating construct promoters could also produce incomplete silencing or spatially and temporally restricted silencing that would be useful for studying essential genes or moderating gene activity.

In one important aspect, ata-siRNAs are similar to amiRNAs: Both methods produce 21-bp single-stranded RNAs that target a specific sequence. These 21-bp species can be used to target one gene among a cluster of tandemly repeated genes, a specific allele, or a splice variant. This is a distinct advantage over intron-spliced hairpin constructs where much longer sequences are used, and thus more off-target silencing is possible.

It has been suggested that it might be possible to design polycistronic amiRNAs with multiple stem-loops that can encode distinct amiRNAs, in order to target multiple genes from the same construct (Ossowski et al., 2008). Targeting multiple genes with a single construct might be more easily achieved with ata-siRNAs. Instead of designing a precursor with multiple stem-loop structures, designing an ata-siRNA construct to silence multiple genes should be as simple as replacing two or more native sequences encoding siRNAs with artificial siRNAs targeting genes of interest. The main criterion that needs to be considered when designing an ata-siRNA construct is the characteristics of the encoded siRNA. Because ta-siRNAs are presumably derived from double-stranded RNA, not from an intramolecular fold as in miRNA, secondary structure considerations of the ata-siRNA precursor may not be critical.

Substituting miR167 for miR173 in *TAS1c* greatly reduces the ability of the *5XsiFAD2* construct to silence *FAD2* and suggests that an intriguing new

layer of ta-siRNA biogenesis remains to be explored. Even in leaves, where miR167 expression is high, the *miR167-5XsiFAD2* construct showed inefficient *FAD2* repression, precluding the possibility that weak silencing resulted from low miR167 expression. Instead, such poor silencing could indicate that *TAS1c* transcripts are not accessible to all miRNA. It is also possible that miR173 may have a specific role in promoting ta-siRNA biogenesis by recruiting an RNA-dependent RNA polymerase. In any case, once the hurdles to using miRNAs other than miR173 are overcome, one attractive possibility for silencing genes in a tissue-specific or temporally-specific manner would be to engineer *TAS* constructs triggered by miRNAs that are expressed in specific tissues or at specific times in development. The expression patterns of trigger miRNAs could be used to confer spatial or temporal specificity on *TAS*-induced silencing.

In addition to providing an excellent tool for gene silencing, the consistency and efficacy of silencing the *FAD2* gene with *TAS1c* could provide a valuable system for investigating the specificity of sequences and cofactors for the function of ta-siRNAs. We expect that refining the design of ata-siRNAs will go hand in hand with a deepening knowledge of the biology of ta-siRNAs.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All experiments were performed on the Columbia (Col-0) ecotype of *Arabidopsis* (*Arabidopsis thaliana*) plants. Plants were grown in long-day conditions (16 h of light at 22°C, 8 h of dark at 20°C).

Transgenic Constructs

The artificial *TAS1c* constructs targeting the *Arabidopsis FAD2* gene were made using a 21-bp or a 210-bp fragment of the 5' coding region of the gene. The 21-bp siFAD2 siRNA corresponds to base pairs 98 to 118 of the *FAD2* CDS. These 21-bp sequences were selected by scanning the CDS for target regions that would be recognized by a ta-siRNA of sequence

5'-(T/A)NNNNNNNNNNNNNNNNNN(C/G)NN-3' with GC content between 30% to 50%. Internal stability was analyzed by running the candidate 21mer through mFold (Zuker, 2003) to look for a secondary structure with a high deltaG (≥ 1) that had at least three unpaired nucleotides at the 5' and 3' ends.

5XsiFAD2 was made as follows: sense and antisense oligos for the 5' half and for the 3' half of the 484-nt piece were designed (FAD2TAS 5' sense, FAD2TAS 5' antisense, FAD2TAS 3' sense, FAD2TAS 3' antisense). The resulting DNA fragment contains the modified *TAS1c* fragment, flanked by attB sites and single-stranded overhangs (GATC and AATT). Oligos were annealed and ligated into pGEM7 (previously digested with *Bam*HI and *Eco*RI). The 5XsiFAD2 in pGEM7 was then recombined with pBC vector, using Gateway reactions. This placed the 5XsiFAD2 fragment downstream of the 35S promoter and upstream of the phaseolin terminator. *mut-5XsiFAD2* was made as above, except that oligos that contained mutations in the miR173 target site were used for the ligations (FAD2TASmut 3' sense, FAD2TASmut 3' antisense).

To build the 1XsiFAD2 construct, we used primers TAS1c/FAD2-attB1 and TAS1c/FAD2-2R to generate the 5' fragment by PCR, and primers TAS1c/FAD2-3F and TAS1c/FAD2-attB-4 to generate the 3' fragment by PCR. The TAS1c/FAD2-2R and TAS1c/FAD2-3F fragments overlapped in the region of *TAS1c* that produces phased siRNAs, and modifications to the siRNAs were introduced using these long primers. We also modified 3'D5+ to target the unrelated *AP1* gene. The 21-bp siAP1 sequence corresponds to base pairs 673 to 693 of the *AP1* CDS. Silencing of *AP1* was ineffective, likely due to the high intramolecular structure of the *AP1* siRNA. The 5' and 3' fragments were gel purified and used together as a template to amplify the whole fragment by PCR with primers TAS1c/FAD2-attB1 and TAS1c/FAD2-attB-4. In a similar manner, construct *miR167-5XsiFAD2* was generated using primers miR167/TAS1c/5XsiFAD2-attB1 and miR167/TAS1c/5XsiFAD2-2R to amplify the 5' fragment and miR167/TAS1c/5XsiFAD2-3F and miR167/TAS1c/5XsiFAD2-attB4 to amplify the 3' fragment. Both fragments were purified and used as a template to amplify the final product using primers miR167/TAS1c/5XsiFAD2-attB1 and miR167/TAS1c/5XsiFAD2-attB4.

To build the *fragFAD2* construct, we first amplified three separate fragments. Fragment 1 was amplified with primers TAS1c/FAD2-attB1 and TAS1c/FAD2-frag-2R; fragment 2 was amplified with primers TAS1c/FAD2-frag-3F and TAS1c/FAD2-frag-4R; and fragment 3 was amplified with primers TAS1c/FAD2-frag-5F and TAS1c/FAD2-attB-4. Then fragment 1 and fragment 2 were used as templates to amplify fragment 4 using primers TAS1c/FAD2-attB1 and TAS1c/FAD2-frag-4R. The complete construct was then amplified by combining fragment 4 and fragment 3 using primers TAS1c/FAD2-attB1 and TAS1c/FAD2-attB-4. The 210-bp *FAD2* fragment corresponds to base pairs 1 to 210 of the *FAD2* CDS.

To create the constructs with mutated miRNA binding sites, we used constructs 5XsiFAD2, 1XsiFAD, and *fragFAD2* as templates for site-directed mutagenesis (Wang and Malcolm, 1999) using the following oligonucleotides: miR173 binding site mut 1for and miR173 binding site mut 1rev. To mutate the miR167 target site in the construct *TAS1c miR167-5XsiFAD2*, we used primers miR167 binding site mut1 and miR167 binding site mut1rev, as described previously (Wang and Malcolm, 1999).

The fragments of interest were amplified with attB-tagged primer pairs and cloned into the Gateway pDONR/Zeo (Invitrogen) vector to give rise to the entry clone. Sequences for all primers used in this study are listed in Table I. The pBC Yellow Gateway vector was used as a destination vector to generate the expression clone. pBC Yellow was generated from pBC (Aukerman and Sakai, 2003) by inserting a *PvuI-AlwNI* fragment generated by PCR containing the RD29A promoter driving Zs-Yellow YFP (CLONTECH Laboratories) expression into the *PvuI/DraIII* sites of pBC. This allowed transgenic seed to be selected based on YFP fluorescence. The *BAR* gene was eliminated from pBC Yellow by restriction digest and religation. Arabidopsis plants were transformed by pipetting *Agrobacterium tumefaciens* cell suspension directly on inflorescence buds (modified from Clough and Bent, 1998).

Fatty Acid Analysis

Lipid compositional analyses were conducted on mature seeds or rosette leaves, collected from wild-type and transgenic plants. Lipids were extracted from single seeds by homogenization with a pestle in an Eppendorf tube in MeOH:CHCl₃ (10 μ L; 2:1, v/v). After a 30-min incubation, 50 μ L of heptane was added. Samples were mixed thoroughly and spun at 10,000 rpm in a microfuge, and the organic layer was recovered and used for lipid compositional analyses as done by Damude et al. (2006). For leaf tissue, a single fully

expanded rosette leaf was ground, and then 1 mL of methanol/sulfuric acid derivatization solution (5% sulfuric acid in anhydrous methanol) was added. The resulting solution was heated at 95°C for 30 min, with vortexing every 10 min. The solution was then cooled to room temp, 1 M NaCl was added, and then 0.5 mL of heptane was added. Solution was then vortexed and centrifuged for 10 min. The resulting supernatant was then used for lipid compositional analysis as done by Damude et al. (2006).

Fatty acid profiles are expressed as ODP. Seed ODP is calculated as the percentage of 18:2 and 18:3 fatty acids out of the total amount of 18:1, 18:2, and 18:3 fatty acids, i.e. seed ODP = (%18:2 + %18:3)/(%18:1 + %18:2 + %18:3). Leaf ODP is calculated as the percentage of 18:2 fatty acids out of the total amount of 18:1 and 18:2 fatty acids, i.e. leaf ODP = (%18:2)/(%18:1 + %18:2). 18:3 fatty acids were not included in leaf ODP because the amount of 18:3 fatty acids in leaves is not dependent on FAD2 (Miquel and Browse, 1992). In leaf chloroplasts, an alternate pathway for 18:1 desaturation exists: The FADC enzyme desaturates 18:1 fatty acids to 18:2, and the FADD enzyme desaturates 18:2 fatty acids to 18:3 (Somerville and Browse, 1991).

Northern Analysis

Total RNA was isolated from aerial tissues of adult wild-type and transgenic plants using TRIZOL reagent (Sigma). RNA was separated on agarose gels, blotted onto a Hybond N⁺ membrane (Amersham), and probed with ³²P-labeled probes randomly primed with the RadPrime DNA labeling system (Invitrogen). Hybridization was carried out at 68°C using PerfectHyb Plus buffer (Sigma). Blots were washed once in 2 \times SSC and 0.1% SDS for 5 min at room temperature, twice in 0.5 \times SSC and 0.1% SDS for 20 min at 68°C, and once in 0.1 \times SSC, 0.1% SDS for 20 min at 68°C. The hybridization signal was detected with a Storm 860 (Molecular Dynamics).

Low-molecular-weight (LMW) RNA was purified from total RNA using the *mirVANA* miRNA isolation kit (Ambion). LMW RNA was separated on a 15% TBE-Urea Criterion gel (Bio-Rad) and transferred electrophoretically to Hybond N⁺ membrane (Amersham) using a TransBlot-SD apparatus (Bio-Rad). LMW blots were hybridized at 40°C using ULTRAhyb-oligo buffer (Ambion) with ³²P-end-labeled oligonucleotide probes. Probes were labeled with [γ -³²P]ATP using OptiKinase (USB). Blots were washed twice in 2 \times SSC and 0.5% SDS for 30 min at 38°C.

Validation of the miR167 Target Site in the miR167-5XsiFAD2 Construct and in the ARF8 Gene

Modified 5' RACE with the GeneRacer kit (Invitrogen) was adapted to validate the cleavage site determined by the miR167 target site. Primer TAS1c-2rev and nested primer FAD-TAS 1rev (*miR167-5XsiFAD2*) or ARF8 1Rev and ARF8 1Nested (*ARF8*) were used in PCRs, and the cleavage sites were revealed by sequence analyses of the PCR products.

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