An Intron Sense Suppression Construct Targeting Soybean *FAD2-1* Requires a Double-Stranded RNA-Producing Inverted Repeat T-DNA Insert[OA]

Andrew Mroczka, Peter D. Roberts, JoAnne J. Fillatti, B. Elizabeth Wiggins, Tim Ulmasov, and Toni Voelker*

Monsanto Company, Calgene Campus, Davis, California 95616 (A.M., P.D.R., J.J.F., T.V.); Monsanto Company, Chesterfield Campus, Chesterfield, Missouri 63017 (B.E.W.); and Monsanto Company, Creve Coeur Campus, Creve Coeur, Missouri 63167 (T.U.)

We demonstrate that the transformation of soybean (*Glycine max*) with sense suppression constructs using intron sequences from the fatty acid oleyl Δ12 desaturase gene *FAD2-1A* leads to efficient and specific reduction of *FAD2-1* transcripts in developing seeds, increased oleic acid, and decreased polyunsaturated fatty acids. The related *FAD2-2* transcripts are only marginally affected. Despite screening a large number of independent transformants, no single-copy efficacious transformants could be found. Invariably, all the least complex transgenic loci have two T-DNA copies in an inverted repeat configuration, centered at the right borders. We show that this T-DNA configuration produces an inverted repeat transcript and that small interfering RNAs accumulate against the target sequence.

Posttranscriptional gene silencing (PTGS) is a widely used gene suppression approach that selectively silences genes in plants and animals (Watson et al., 2005). PTGS works through sequence-specific degradation of mRNA through endonucleolytic cleavage followed by exonuclease digestion (Van Eldik et al., 1998) and is considered to have evolved in plants for protection against pathogenic RNAs (Marathe et al., 2000). PTGS was discovered in 1990, when transgenic overexpression of an endogenous petunia (*Petunia hybrida*) chalcone synthase gene resulted in unexpected reduction of chalcone synthase in many transformants (Napoli et al., 1990). Since both the transgenic and endogenous chalcone synthase transcripts were reduced in these transformants, the phenomenon was originally termed "cosuppression." It is now called "sense suppression" because only a sense copy of the gene sequence is supplied by the suppression cassette (Nakamura et al., 2006). Subsequent studies demonstrated efficacious sense suppression of a variety of genes in many plant species (Baulcombe, 1996). In the years following the discovery of sense suppression, diverse mechanisms were proposed, attributing its efficacy to transgene insert copy number or to transgene transcript dosage (Dehio and Schell, 1994; Jorgensen, 1995; Jorgensen et al., 1996; Que and Jorgensen, 1998; Lechtenberg et al., 2003). Other studies demonstrated that sense suppression was not correlated to the level of transgene expression (Van Blokland et al., 1994; Stam et al., 1997). In general, sense suppression was found to have low penetrance, occurring typically only in about 15% of transformants (Hamilton et al., 1998), and of those, many with low efficacy (Que et al., 1997). Transgene loci causing sense suppression are generally complex, containing multiple intact and partial copies of the T-DNA, often with two copies in an inverted repeat orientation (Metzlaff et al., 1997; Stam et al., 1997). While many of these studies described the genomic structure of the inverted repeat and some characterized transcripts in the suppressed lines, no satisfactory biochemical mechanism for sense suppression emerged.

However, following the discovery of RNA interference (RNAi; Fire et al., 1998) and the subsequent rapid elucidation of the PTGS pathway (for review, see Voinnet, 2008), it was suggested that the low penetrance and efficacy of sense suppression constructs is due to the required chance assembly of two T-DNAs occurring in planta to produce an inverted repeat transcript (Chuang and Meyerowitz, 2000), making sense suppression basically an indirect and inefficient method of inducing RNAi. Indeed, efficacious, single-copy sense suppression transformants are rarely described in the literature (Stoutjesdijk et al., 2002). In addition, the production of small interfering RNAs (siRNAs) has been observed in sense suppression transformants (Hamilton and Baulcombe, 1999; Di Serio et al., 2001; Han and Grierson, 2002), which adds support for this mechanism. However, to our knowledge, the postulated inverted repeat suppression transcripts were never directly demonstrated.

* Corresponding author; e-mail toni.voelker@monsanto.com.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Toni Voelker (toni.voelker@monsanto.com).

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.110.154351

The nutritional value and utility of vegetable oils are affected by their fatty acid composition. Soybean (*Glycine max*) oil is highly polyunsaturated (more than 65%) and is not well suited for applications requiring high oil stability, such as commercial frying. To make soybean oil suitable for such applications, the polyunsaturates can be reduced via chemical hydrogenation, and this partially hydrogenated soybean oil was used for many food products in the United States (DiRienzo et al., 2006). However, the hydrogenation process leads to the production of trans-fatty acids, which are known to contribute to cardiovascular risk by elevating low-density lipoprotein ("bad cholesterol") and reducing high-density lipoprotein ("good cholesterol"; Allison et al., 1995; Hu et al., 1997). These negative health implications have led to the current phase-out of hydrogenation as a means to improve the oxidative stability of soybean oil (Moss, 2006). Alternatively, less polyunsaturated soybean oil can be produced naturally through alteration of soy fatty acid biosynthesis in developing seeds. A crucial determinant of polyunsaturation is the enzyme FAD2, an endoplasmic reticulum-localized fatty acid Δ12 desaturase that converts oleic acid [$18{:}1^{(\Delta 9)}$] to linoleic acid [$18{:}2^{(\Delta 9,\Delta 12)}$]. Simply reducing FAD2 activity during seed fill reduces the rate at which oleic acid is converted to linoleic acid, leaving more oleic acid available to be stored in the oil.

Suppression of *FAD2*, the subsequent reduction of polyunsaturates from 65% in normal soybean to 5%, and a concomitant elevation of 18:1 from 20% to about 85%, have previously been achieved in soybean using a *FAD2* cDNA sense suppression construct (Knowlton, 1999). More recently, a *FAD2* intron sequence used in a sense suppression construct led to transformants with a more moderate reduction of polyunsaturates, to about 20% (Fillatti, 2009). Here, we describe the large-scale production of soy transformants using sequence from the 420-bp single intron of the soy *FAD2-1A* gene driven by a seed promoter. We analyzed the timing and extent of the impact of transgene expression during seed development on 18:1 levels and the endogenous *FAD2-1A* transcript as well as the transcripts of the three related *FAD2* gene family members. We also investigated the structure of the transgenic loci, the nature of the transcript produced by such loci, and the induction of transgene-specific siRNAs.

RESULTS

FAD2-1A Intron Sense Suppression Constructs Produce Elevated 18:1 Phenotype with Low Penetrance

Our aim was to use sense suppression to generate soybean lines with 65% to 75% 18:1 in the oil. We chose sequences from the single 420-bp intron of the soy *FAD2-1A* gene as a suppression trigger. We reasoned that suppression could be dependent on the length of the suppression trigger; therefore, we built three different transformation vectors, one containing the full-length (420 bp) intron, one with the 5′-most 301-bp fragment, and one with the 5′-most 221-bp fragment of the *FAD2-1A* intron. In all cassettes, the *FAD2-1A* intron splicing signals had been removed. The soy 7Sα′ seed promoter was used for expression, and the cotton (*Gossypium hirsutum*) *H6* 3′ untranslated region (UTR) supplied the polyadenylation signals. The suppression cassettes were introduced into one T-DNA segment of a so-called 2-T plant transformation vector, with a second T-DNA segment containing the *Agrobacterium tumefaciens* EPSP synthase CP4 gene for glyphosate resistance as a plant-selectable marker. Our goal was to produce single-copy, marker-free transformants. The T-DNAs were transferred into the soy genome via *Agrobacterium* transformation, and 1,265 independent glyphosate-tolerant transformants were obtained. In a first screen at early vegetative stage, we measured the copy number of the transgene in the transformants by Invader, and transformants containing zero or more than two *FAD2* T-DNA copies were eliminated, which left 353 transformants. After seed set, fatty acid composition was measured. About 82% of the transformants harboring one or two copies of the transgene had only seeds with unaltered oil phenotype, indicating no significant *FAD2* suppression. Only 18% of the transformants had seeds with 18:1 greater than 35%. Overall, the screens removed approximately 95% of the transformants; only 63 transformants remained. Interestingly, while we used different lengths of the *FAD2-1A* intron for the suppression triggers of the three vectors, 18:1 levels from the efficacious transformants obtained from all three vectors were very similar, usually in the 60% to 75% range.

Multiple seeds of each of the remaining 63 transformants were planted, and plants that were homozygous for a single copy of the suppression insert, again measured by Invader, were selected. We also eliminated transformants where the CP4 selectable marker gene cosegregated with the *FAD2* transgene locus, to obtain marker-free transgenic lines. The Invader suppression cassette copy-number estimates were confirmed by genomic DNA gel-blot analysis using *Xba*I, which cuts the suppression T-DNA only once in the *FAD2-1A* suppression trigger, and probed with the transgenic *H6* 3′ UTR. For a single-copy insert, this analysis is expected to detect only one fragment, with a transformant-specific M_r (data not shown).

After this multistage selection process, only five apparently single-copy, marker-free transformants, MOC-1, MOC-2, MOC-3, MOC-4, and MOC-5, remained. All homozygous lines derived from these transformants had seeds with 18:1 levels elevated to 65% to 70% and a corresponding reduction of 18:2 (Fig. 1). Palmitic acid (16:0), linolenic acid (18:3), and stearic acid (18:0) were also slightly reduced, as observed previously when 18:1 was increased by suppressing *FAD2-1* activity using the *FAD2-1A* cDNA as a suppression trigger (Knowlton, 1999).

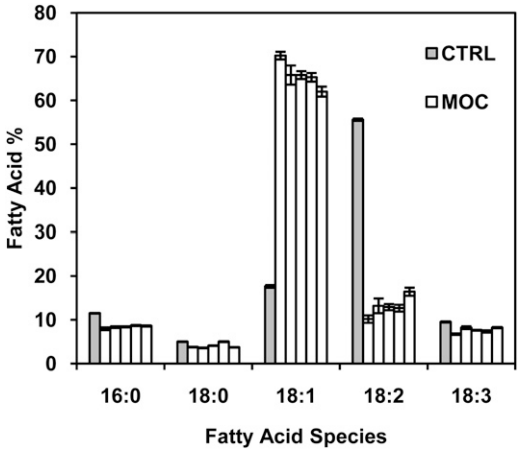

Figure 1. Impact of *FAD2* intron suppression on fatty acid composition of mature soybean seeds. For each line, 24 mature seeds were individually analyzed by GC-FAME. The values are averages of fatty acid mass percentage. Bars from left to right: CTRL, MOC-1, MOC-2, MOC-3, MOC-4, and MOC-5.

FAD2-1 Transcripts Are Greatly Reduced, But *FAD2-2* Transcripts Are Only Slightly Affected

We sought to determine why *FAD2-1A* intron sense suppression yielded lower 18:1 levels (60%–70%) than suppression using the *FAD2-1A* cDNA, which yielded 85% 18:1 (Knowlton, 1999). The soy genome contains four transcribed *FAD2* genes, in two subfamilies, *FAD2-1* and *FAD2-2*, each with two genes, denoted *A* and *B*. The *FAD2-1* genes are expressed in the seeds, while the *FAD2-2* genes are expressed in both seeds and vegetative tissues, but at lower levels (Heppard et al., 1996). *FAD2-1A* and *FAD2-1B* are highly related, with translated sequences of the same length and 95% sequence identity. *FAD2-2* genes are more distantly related to *FAD2-1A*, with translated sequences sharing only 72% sequence identity and completely diverged intron and 3′ UTR sequences (Schlueter et al., 2007). To determine the extent of suppression in the gene family, we measured transcript levels individually for each of the *FAD2* genes during seed development.

Homozygous lines from transformants MOC-1 and MOC-2 and two nontransgenic control lines were grown under identical conditions in the same greenhouse. Developing seeds were harvested at several developmental stages. The 18:1 content at all seed stages of nontransgenic control lines (CTRL) and *FAD2*-suppressed lines (MOC-1 and MOC-2) are shown in Figure 2A. In CTRL, 18:1 accounts for about 10% of total fatty acids in early seed stages and increases gradually to about 20% in mature seeds. For MOC-1 and MOC-2, 18:1 levels are equal to CTRL in stages 0 and 1, then increase sharply beginning at stage 2, consistent with the timing of the 7Sα' promoter that drives the gene suppression cassette (Walling et al., 1986). Oleic acid continues to increase through the period of heaviest oil deposition before gradually leveling off during desiccation (stages 7 and 8), reaching about 70% in the mature seeds.

To measure the effect of the MOC loci on each of the four endogenous *FAD2* transcripts, we designed gene-specific primer-probe sets targeting exon regions for real-time reverse transcription (RT)-PCR. Results for the two *FAD2-1* transcripts are shown in Figure 2, B and C. In CTRL, the levels of the endogenous *FAD2-1A* and *FAD2-1B* transcripts gradually increase in sync during seed fill, then sharply drop nearly 1,000-fold during seed desiccation, reaching their lowest levels in the mature seeds. *FAD2-1A* and *FAD2-1B* transcripts in MOC-1 and MOC-2 are equal to CTRL early in devel-

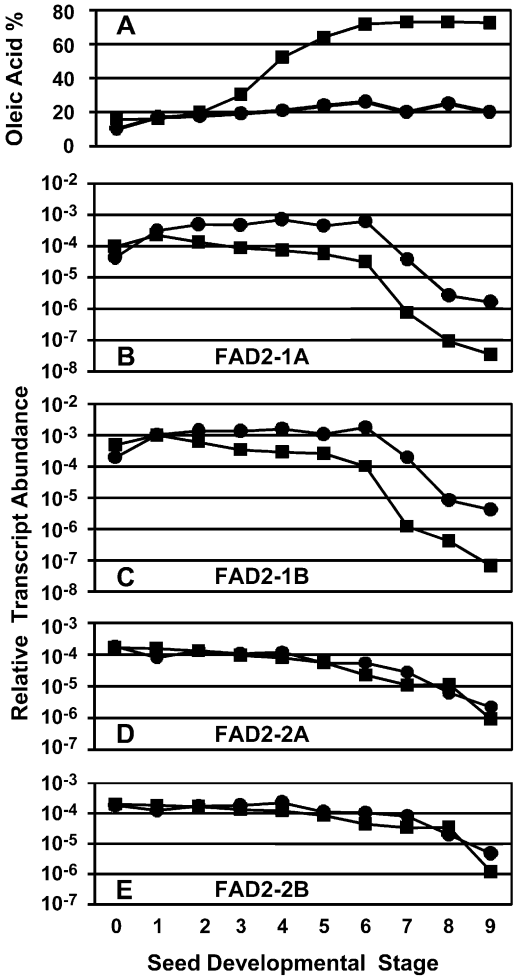

Figure 2. Impact of *FAD2* intron suppression during seed development on 18:1 content and *FAD2* transcripts. Developing seeds from greenhouse-grown plants were sorted into 10 stages as defined in "Materials and Methods," from 10 mg fresh weight (stage 0) to maximum weight (290 mg) green seeds (stage 6), from the onset and midpoint of seed desiccation (stages 7 and 8, respectively), to fully mature dry seeds (stage 9). A, Oleic acid composition via GC-FAME. B to E, Total RNA from all stages was isolated and subjected to gene-specific quantitative RT-PCR, with the respective specificities indicated. All data are averages of two lines. Circles represent CTRL plants, and squares represent averaged homozygous suppressed lines MOC-1 and MOC-2.

opment, but from stage 2 on they show a strong reduction, which becomes more pronounced during seed development, in correlation with the observed timing of 18:1 elevation. This differential is maintained during desiccation and is up to 2 orders of magnitude lower in the mature dry seed. Overall, the extent of the suppression is nearly identical for both transcripts. This is not surprising, since the single introns of *FAD2-1A* and *FAD2-1B* are 420 and 406 bp long, respectively, and share 79% sequence identity overall. Their sequences differ by nine short deletions and insertions but contain some extended stretches of complete identity. In addition, the translated sequences of the mature mRNAs are 95% identical.

Figure 2, D and E, shows the transcript analysis of the *FAD2-2* genes. In the CTRL, both transcripts are at their highest levels early in seed development and gradually diminish, reaching their lowest levels in the mature seeds, about 100-fold reduced relative to stage 0. When comparing the respective seed developmental stages, there is on average a 1.4-fold reduction in transcript abundance between MOC-1 and MOC-2 and the CTRL throughout seed development, indicating that *FAD2-2* transcript sequences are sufficiently distant from the *FAD2-1A* intron trigger to be not strongly affected.

In summary, the *FAD2-1A* intron suppression cassette driven by the 7Sα' promoter reduced both endogenous *FAD2-1* transcripts beginning in stage 2 of seed development, with transcript suppression increasing during the seed-fill phase. Only a minor impact on *FAD2-2* transcript levels was observed. Accumulation of 18:1 is synchronized with the reduction of *FAD2-1* transcripts, which demonstrates that the accumulation of 18:2 in soybean seeds relies primarily on FAD2-1; FAD2-2 appears to play only a moderate role. The fact that the *FAD2-1A* intron suppression trigger only marginally affects *FAD2-2* transcripts could be the reason for lower 18:1 levels as compared with the *FAD2-1A* cDNA sense suppression (Knowlton, 1999), which likely affects the transcripts of all four genes significantly.

All MOC Transformants Contain Two T-DNA Copies in Inverted Repeat Configurations

As described above, five CP4 marker-free transformants remained after selecting for elevated 18:1 content and single-copy insertions, as predicted by Invader and single restriction enzyme digest assays. Each of these five transformants was then subjected to a more rigorous genomic DNA gel-blot analysis to more precisely assess the integrity of the respective T-DNA insertions. Genomic DNA was digested with *Bam*HI, *Sal*I, and *Not*I, all with a single site on the suppression T-DNA, close to the left border (Fig. 4, full-length map). After digestion of genomic DNA with any of these enzymes, the *FAD2-1A* intron probe is expected to detect a single fragment of unique size for each single-copy transgene locus. Indeed, all enzymes produced a single band for each transformant. Strikingly, however, M_r values of the detected fragments for all transformants and enzymes were similar and approximately twice that of the suppression T-DNA (data not shown). Together with the results obtained with the *H6* 3′ UTR probe after *Xba*I digestion, these results suggested two-copy, inverted repeat T-DNA insertions in each of our presumed single-copy lines, all converged at the right borders.

To further test this assumption, we digested genomic DNA of the MOC transformants and CTRL with *Bam*HI, *Spe*I, and *Sac*I, enzymes that cut progressively closer to the right border of the T-DNA (Fig. 4, full length). Inverted T-DNA repeat loci with the right borders at the center and the left borders at the flanks are predicted to yield a pattern of progressively smaller DNA fragments with each of the three enzymes above when probed with *FAD2-1A* intron. The result of this analysis is shown in Figure 3. The *FAD2-1A* intron probe detects the endogenous soy *FAD2-1* introns in CTRL and the transformants, as expected. In addition to the common signal, in each of the transgenic lines an additional single band is observed for each restriction enzyme digest. For each transformant, *Bam*HI, *Spe*I, and *Sac*I produce progressively shorter fragments, reconfirming the predicted right border/right border inverted repeat inserts without exception. Besides this general trend, a recurring pattern of minor fragment length variation is observed with each enzyme. Most of this pattern can be explained by the length differences of the *FAD2* intron suppression sequences used in the three constructs from which the transformants were generated. Additional transformant-to-transformant variation is most likely due to small insertions and deletions at the right borders, which might have occurred during integration of the T-DNAs into the soy genome.

To verify the proposed inverted repeat structure, we attempted to obtain a junction sequence. Amplifying a

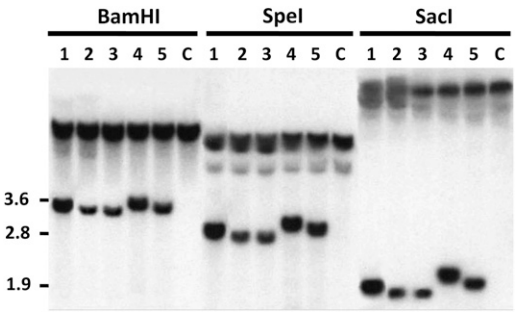

Figure 3. Genomic DNA gel-blot analysis of transgenic lines. Genomic DNA was digested with *Bam*HI, *Spe*I, and *Sac*I as indicated. After agarose gel electrophoresis and transfer, the genomic DNA was hybridized to the 420-bp *FAD2-1A* intron probe. M_r markers are indicated to the left. From left to right, MOC-1, MOC-2, MOC-3, MOC-4, MOC-5, and CTRL are indicated by numbers and C, respectively. The signals shared between CTRL and the transgenic lines represent the endogenous soy *FAD2* sequence. Each of the transgenic lines also shows one extra band corresponding to the transgene insert.

perfect inverted repeat from genomic DNA template is generally not possible. However, if an inverted repeat is imperfect due to a truncation of one of the arms, a primer can be placed close to the terminus of the truncated arm and amplification through the fusion point is usually possible, since very little inverted repeat sequence is amplified (triangles indicate primer positions in Fig. 4). Since the relatively lower M_r values observed from the above DNA gel-blot analysis for MOC-1 suggested the presence of small deletions in the T-DNA for that line, we used PCR to scan for such a configuration using several primer combinations. In genomic DNA from MOC-1, a successful reaction amplified a 381-bp DNA. The sequence of this product confirmed the predicted inverted repeat structure at the right borders, with one of the arms completely intact, while the other arm is truncated. The right border sequence of this copy is missing as well as the 3′ terminal 220 bases of the *H6* 3′ UTR from the suppression cassette. To further verify the proposed inverted repeat locus, we determined approximately 1.4 kb of the flanking soybean genomic DNA sequence on either side of the transgene insert by inverse PCR. Each of the flanking genomic sequences was found adjacent to the left border of one of the T-DNAs (data not shown), providing additional evidence for their inverted repeat orientation (for a map of this insertion locus, see Fig. 4).

It is interesting that the Invader assay could not detect the two-copy nature of the MOC-1 to MOC-5 inverted repeat loci, even though this method is usually very reliable, especially when applied to segregating R1 plants. The sequence targeted by the Invader probe is positioned at the 5′-most region of the *H6* 3′ UTR, as shown in Figure 4. Despite the 220-bp deletion of the 3′ end of *H6* in one copy of the T-DNA in MOC-1, the sequence targeted by the *H6* Invader probe still remained intact. We can only speculate that the proximity of the two probe target locations in an inverted repeat structure reduces the efficiency of the reaction, such that the signal produced is more similar to that of a single-copy transformant.

In summary, after generating hundreds of independent transformants, we were unable to obtain a single-copy sense-suppressed transformant. Even the putative single-copy transformants, as determined by Invader, actually contained two copies of the suppression T-DNA, all in a right border/right border inverted repeat configuration.

An Inverted Repeat Suppression Transcript Is Detected in the Developing Seeds of MOC-1

The genomic analysis invariably detected right border/right border inverted repeats of the *FAD2-1A* suppression T-DNA in our lowest copy number transformants. This suggested that such a configuration is possibly essential for triggering suppression, but it does not supply direct evidence for a particular mechanism of action. We hypothesized that in these right border/right border inverted repeats, the suppression transcripts, driven from one or both 7S*α*′ promoters, might continue beyond the end of the respective T-DNA. Such an extension would transcribe *FAD2-1A* intron antisense sequence, resulting in *FAD2-1A* double-stranded RNA (dsRNA) upon folding back on itself. This dsRNA would then trigger RNAi.

To obtain evidence for this hypothesis, we attempted to detect a transcript in MOC-1 containing sequence from both T-DNAs on one molecule. We used the same PCR primers described above for amplifying across the internal junction of the inverted repeat in genomic DNA, but with cDNA template reverse transcribed from seed total RNA. The gel-separated RT-PCR products were blotted and probed with an *H6* 3′ UTR probe to confirm their sequence identity, as shown in Figure 5. As expected, CTRL seeds yielded no detectable product. The PCR amplified a single fragment from MOC-1 cDNA, with the predicted M_r for a template reverse transcribed from the hypothesized inverted repeat transcript. The product was most abundant at stage 3, greatly reduced at stage 6, and barely detectable at stage 9, consistent with the timing of the 7S*α*′ promoter driving the suppression cassette. Reactions lacking reverse transcriptase for MOC-1 did not yield a product.

This result is evidence for the hypothesized inverted repeat transcript in the developing seeds of sense-

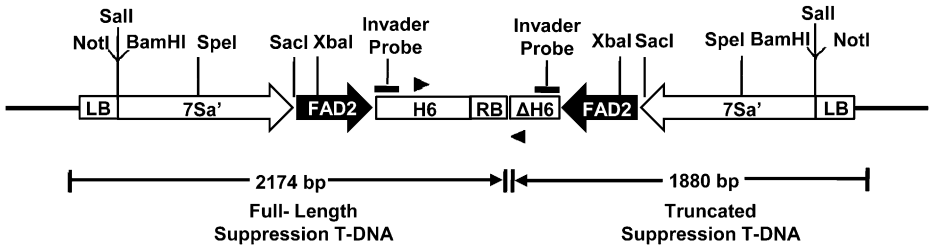

Figure 4. Map for the transgenic locus for MOC-1. The locus consists of two copies of the *FAD2-1A* intron suppression cassette in an inverted repeat orientation. The left copy is complete, and the right copy has a deletion of the 3′ terminal 220 bp of the *H6* 3′ UTR as well as the entire right border (RB) sequence. The *H6* 3′ UTR sequence targeted by Invader assay for copy number estimation is indicated. The black triangles indicate the locations and orientations of the inverted repeat primers. The map is not to scale. LB, Left border.

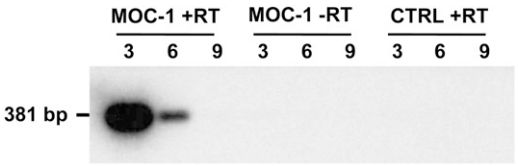

Figure 5. Detection of inverted repeat transcripts in the seeds of MOC-1. Total RNA was isolated from developing and mature seeds, treated with DNase, and used as template for RT, followed by 20 cycles of PCR. Products were transferred to a membrane and probed with DNA sequence from the cotton *H6* 3′ UTR. The nontransgenic CTRL line shows no signal, and a signal is present in MOC-1. The signal was absent when the RT step for MOC-1 was omitted (−RT), demonstrating the absence of genomic DNA contamination.

suppressed transformants. Likely, truncations in the 3′ UTRs of the inverted repeats diminished or abolished transcriptional termination. The ensuing read-through into the inverted copy then created dsRNA, triggering the gene suppression.

FAD2-1 Transcripts Are Degraded in MOC-1 Developing Seeds, and *FAD2-1*-Specific siRNAs Accumulate

We have demonstrated that the *FAD2-1A* intron-suppressed line MOC-1 produces inverted repeat transgenic transcripts in developing soybean. Because of this similarity to conventional RNAi transcripts, we wanted to compare the impact of the MOC-1 locus on *FAD2-1A* intron-containing transcripts with the impact of a conventional RNAi construct. For this comparison, we chose the transformant INT-1.The RNAi plant transformation vector used to generate INT-1 contained the identical splicing-disabled 420-bp soy *FAD2-1A* intron in an inverted repeat configuration, driven by the 7Sα′ promoter, and seed 18:1 levels were the same as in MOC-1, indicating a similar extent of suppression (T. Voelker, personal communication).

We probed total RNA from developing and mature seeds from CTRL, MOC-1, and INT-1 with a *FAD2-1A* intron DNA probe. Examining the RNA gel-blot results for CTRL, an approximately 1.6-kb band is observed in the developing seeds (Fig. 6A). This band has the appropriate M_r for the primary, unspliced, endogenous *FAD2-1* transcripts. The trend observed for *FAD2-1* transcripts in CTRL agrees with the quantitative real-time RT-PCR data obtained with the *FAD2-1* exon primer-probe set, with transcript levels rising between early and mid development and falling off sharply by seed maturity. For MOC-1 and INT-1, the full-length endogenous *FAD2-1* transcripts are much reduced or absent, but a large amount of lower M_r RNA is detected. We interpret this as intermediate breakdown products of both endogenous and transgenic transcripts. Overall, the patterns observed using the *FAD2-1A* intron probe in INT-1 and MOC-1 are very similar. We probed also with a *FAD2-1A* exon sequence (Fig. 6B). In the CTRL, there is a strong signal in stages 3 and 6, absent at stage 9 and in leaves, as expected for this gene (Heppard et al., 1996). In both MOC-1 and INT-1, only traces of signal are visible, indicating efficient breakdown of the exon part of the target transcripts as well, confirming the RT-PCR (Fig. 2).

Since the *FAD2-1A*-suppressed line MOC-1 produces inverted repeat transgenic transcripts (Fig. 5) and shows similar reduction of endogenous *FAD2-1* transcripts in the seeds as compared with a conventional RNAi transformant (Fig. 6), we sought to determine whether MOC-1 also accumulates *FAD2-1*-specific siRNAs in the seeds. To that end, we separated low-M_r RNA from developing and mature seeds from CTRL, MOC-1, and INT-1 and hybridized the resulting blot with a *FAD2-1A* intron RNA probe (Fig. 7). For CTRL, the RNA gel blot shows no siRNA accumulation for any seed stage. For MOC-1 and INT-1, 21-nucleotide *FAD2-1A* intron siRNAs are detected at every seed stage.

In summary, RNA gel-blot analysis shows that the MOC-1 locus causes degradation of target transcripts and gives rise to specific siRNAs in a manner very comparable to a classic double-stranded RNAi suppression locus. It is likely that in all of our "intron sense suppression" transformants, conventional RNAi is the mechanism of gene suppression.

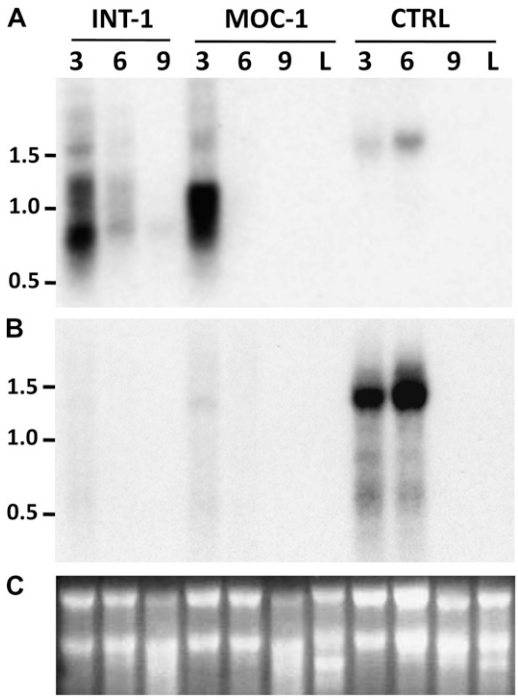

Figure 6. RNA gel-blot analysis of *FAD2* transcripts in developing and mature soy seeds. A and B, Total RNA was isolated from seed stages 3, 6, and 9 and leaves (L), separated by agarose gel electrophoresis, transferred to a membrane, and hybridized to the 420-bp *FAD2-1A* intron DNA probe (A) or a 316-bp *FAD2-1A* exon DNA probe (B). M_r markers are indicated at the left. C, The ethidium bromide fluorescence of the stained RNA.

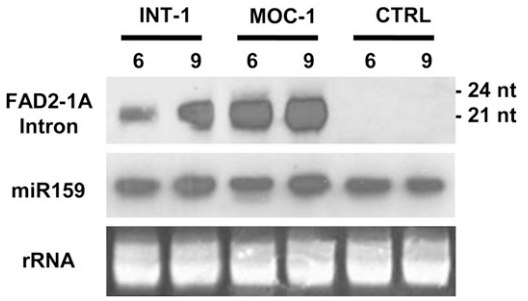

Figure 7. Small RNA analysis of developing and mature soy seeds. Total RNA was isolated as described, separated on a 17% acrylamide gel, and transferred to a nylon membrane. Top, the blot was hybridized with the 420-bp *FAD2-1A* intron RNA probe. The nontransgenic CTRL line shows no signal at any stage. MOC-1 shows the accumulation of *FAD2-1A* intron siRNAs at each seed stage. In comparison, a conventional RNAi transformant (INT-1), containing the same *FAD2-1A* intron suppression trigger sequence, also shows accumulation of *FAD2-1A* intron siRNAs. Middle and bottom, small RNA blot probed with a soy miR159 probe and ethidium bromide-stained ribosomal RNA to show equal loading. nt, Nucleotides.

DISCUSSION

Sense suppression of *FAD2* using full-length *FAD2-1A* cDNA increased oleic acid to 85% in seeds (Knowlton, 1999), and sense suppression using the *FAD2-1A* intron led to a more moderate oleic acid increase (Fillatti, 2009). Most of our efficacious current transformants produced oil with oleic acid between 60% and 70% as well. Interestingly, there was no clear correlation between the altered oleic acid levels and the size of the intron trigger fragments (420, 301, and 221 bp). Either suppression acts as an on/off switch or even shorter trigger fragments will be needed to further reduce the impact.

This lower level of oleic acid observed in the intron-suppressed transformants, when compared with the cDNA sense-suppressed transformants (Knowlton, 1999), likely indicates lower *FAD2* suppression. This may be due in part to the shorter length of the suppression trigger sequences used or to the nature of the intron. In contrast to the *FAD2*-translated sequences, where short stretches are completely conserved in all four *FAD2* genes, significant sequence identity to the *FAD2-1A* intron is found only in *FAD2-1B*. Introns of the *FAD2-2* genes have completely diverged (Schlueter et al., 2007). This is reflected in our gene-specific *FAD2* transcript analysis, which clearly shows that *FAD2-1A* and *FAD2-1B* transcripts are about equally reduced during seed development, but both *FAD2-2* transcripts are affected only slightly. Likely, the absence of transcript sequences targeted by the primary intron siRNAs makes *FAD2-2* transcripts only minor targets, even though fairly strong sequence identity exists in the exon regions between the subfamilies (65%). Secondary siRNAs (Chapman and Carrington, 2007) might not be produced in sufficient quantities to trigger effective *FAD2-2* transcript degradation.

Interestingly, even after analyzing hundreds of independently generated transformants, we were unable to find efficacious transgenic loci with only a single copy of the transgene. All five of the high-oleic acid transformants having the least complex loci contained two copies of the suppression T-DNA in an inverted repeat, invariably centered at the right borders. RT-PCR of total RNA extracted from developing seeds of MOC-1 detected a transcript spanning the inverted repeat region. This inverted repeat transcript likely also contains two copies of the suppression trigger in reverse orientation, which should allow dsRNA formation and trigger RNAi. This mechanism is supported also by the concomitant accumulation of *FAD2-1A* intron-specific 21-nucleotide siRNAs in developing seed tissues, in levels comparable to siRNAs from a transformant generated with a cognate RNAi vector with the same trigger sequences.

Only about 18% of our primary transformants containing one to two copies of the suppression cassette had a significant change in fatty acid composition. This is a much lower rate than we observe with RNAi vectors containing the *FAD2-1A* intron already preassembled in an inverted repeat, where the majority of transformants display the transgenic phenotype (T. Voelker, unpublished data). We believe that our analysis of MOC transformants can explain this low penetrance of sense suppression. If dsRNA is required to trigger significant gene suppression, only transformants with the chance in planta assembly, enabling the production of an inverted repeat transcript, will induce suppression. Since sense suppression of plant genes is generally connected with low penetrance and complex insertion loci (Hamilton et al., 1998), it is likely that the reason for this correlation is the requirement for in planta assembly or rearrangement of the T-DNA(s) to enable PTGS via dsRNA. Since such rearranged loci occur at a low frequency, penetrance is low. The idea that these in planta assembled inverted repeat transcripts could be the primary agent of sense suppression was proposed previously (Chuang and Meyerowitz, 2000). However, prior to our study, we could not find any published evidence for the presence of in planta assembled inverted repeat transcripts in sense suppression transformants.

We are unaware of other examples where intron sequences trigger gene suppression in plants. This might indicate that the processing of the *FAD2-1A* primary transcript is unusual, possibly frequently producing unspliced mRNA, which would lead to the efficient use of the intron as a gene suppression trigger. However, surveying several hundred *FAD2-1* ESTs from developing soybean seeds indicated efficient intron splicing (N. Wagner and T. Voelker, unpublished data). It is also possible that introns were not selected as gene suppression triggers, since PTGS is commonly believed to occur in the cytoplasm (Tang, 2005), where introns are not typically found. Finally, it is noteworthy that the *FAD2-1A* intron suppression displayed all the hallmarks of PTGS, suggesting a possible involvement

of the nucleus in this process. More evidence will be needed to confirm this hypothesis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Three different sense suppression cassettes were assembled for transformation into soybean (*Glycine max*). Each cassette consisted of the 7Sα′ seed promoter (Chen et al., 1986) driving one of three versions of the suppression trigger fragments: the full-length (420 bp) soy *FAD2-1A* intron (GenBank accession no. AJ271842), the 5′-most 301 bp of the intron, and the 5′-most 221 bp of the intron. All intron-splicing signals were excluded from the trigger fragments. The cotton (*Gossypium hirsutum*) *H6* 3′ UTR (John and Keller, 1995; GenBank accession no. L17308.1) supplied the polyadenylation signals for each cassette. Each cassette was integrated into a standard binary vector that used *Agrobacterium tumefaciens* EPSP synthase CP4 as the plant-selectable marker (Meyer, 2006). The resulting plant transformation vectors were named pM420, pM301, and pM221, respectively. Each vector was transformed into soybean variety A3525 according to the protocol of Martinell et al. (2002). A3525 is a nontransgenic conventional soybean variety developed by Asgrow Seed Company and is a midmaturity group III variety with very high yield potential. Transformants were selected and homozygous lines established using gene markers, fatty acid compositional analysis of individual seeds, and the Invader assay (designed to detect the *H6* 3′ UTR), which uses a Flap endonuclease and a universal fluorescence resonance energy transfer reporter system for semiquantitative sequence detection (Ryan et al., 1999). The homozygous lines investigated in more detail are here called MOC-1 (pM420), MOC-2 (pM221), MOC-3 (pM221), MOC-4 (pM420), and MOC-5 (pM301). These lines were compared with homozygous line INT-1, generated using a full-length *FAD2-1A* intron suppression trigger in a conventional RNAi cassette from construct pINT that contained the same promoter as the above lines (S. Ivashuta, A. Mroczka, P. Hoffer, J.M. Staub, X. Yang, Y. Zhang, N. Wagner, and T. Voelker, unpublished data). CTRL are plants from the nontransgenic soy variety A3525. Plants were grown in a greenhouse under 16-h photoperiods (1,200 μE average), with 31°C during the day and 20°C at night. Developing seeds were sorted into stages, defined as follows: stages (in mg per average seed) 0 (8), 1 (15), 2 (40), 3 (70), 4 (120), 5 (175), 6 (290), 7 (275), 8 (230), and 9 (130), where stage 6 is the largest green developing seed, stage 7 is yellowing, stage 8 is fully yellow, and stage 9 is mature.

Fatty Acid Analysis

Lyophilized soybean seeds were ground to a fine powder, and the lipids were extracted with heptane. The supernatant was transferred in a glass vial, and the heptane was evaporated with a flow of dry nitrogen gas at 80°C. An 8-mg aliquot of the extracted soybean oil was transesterified with sodium methoxide. Resultant fatty acid methyl esters (FAMEs) were separated by capillary gas chromatography (GC) and detected by a flame ionization detector. The column was a Supelcowax 10 with dimensions of 15 m × 0.25 mm × 0.25 μm film thickness (Sigma-Aldrich). An injection volume of 1 μL was used with a split ratio of 100:1. Peaks were identified based on their relative retention times compared with a FAME reference mixture. Results are reported as area percentage of total fatty acids.

RNA Isolation and Real-Time RT-PCR

Developing seeds were harvested and sorted into stages as defined above. Per line and stage, eight seeds were pooled and homogenized. Total RNA was isolated from 50 mg of ground tissue per sample using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Remaining DNA was removed via digestion with TURBO DNA-free DNase I (Ambion) according to the manufacturer's protocol. RNA concentrations were determined using a Qubit fluorometer (Invitrogen), and concentrations were equalized before analysis. To verify RNA integrity, 1 μg of total RNA from each sample was visualized on an ethidium bromide-stained 1.5% agarose gel, following electrophoresis. Real-time RT-PCR was performed with the Applied Biosystems 7900HT Fast Real-Time PCR system using the Applied Biosystems TaqMan One-Step RT-PCR Master Mix reagents. Endogenous soy *FAD2* transcripts were quantified with the following gene family member-specific primer-probe sets (probes labeled with 5′ FAM/3′ TAMRA): *FAD2-1A*, forward, 5′-ACTCATGTGGCTCACCATCTCTT-3′, probe, 5′-TCTACAATGCCACATTACCATGCAATGGA-3′, reverse, 5′-ATTGGCTTGATTGCATTGGTT-3′ (77-bp amplicon); *FAD2-1B*, forward, 5′-ACAAAGCCACCATTCACTGTTG-3′, probe, 5′-AACTCAAGAAAGCCATTCCACCGCACT-3′, reverse, 5′-GTGAGGAGGGAACGCTGAAAG-3′ (74-bp amplicon); *FAD2-2A*, forward, 5′-CCTTACTGGTGTTTGGGTCATTG-3′, probe, 5′-CATGAGTGTGGTCACCATGCATT-3′, reverse, 5′-ATCATCAAGCAGCTGGTAGTCACT-3′ (73-bp amplicon); *FAD2-2B*, forward, 5′-GGTCTCCTAGTCCCATACTTTTCATG-3′, probe, 5′-AAATACAGCCATCGCCGTCACCACTC-3′, reverse, 5′-CCGCTCAAGAGAACCAGTGTT-3′ (75-bp amplicon). The primer-probe set for the endogenous soy 18S rRNA transcripts was as follows: forward, 5′-CGTCCCTGCCCTTTGTACAC-3′, probe, 5′-CCGCCCGTCGCTCCTACCGAT-3′, reverse, 5′-CGAACACTTCACCGGATCATT-3′. Reactions, performed in duplicate, contained 20 ng of total RNA template, 800 nM each primer, 1.5 μM probe, 0.25 μL of MultiScribe reverse transcriptase with RNase Inhibitor Mix, 5 μL of TaqMan One-Step RT-PCR Master Mix reagents, and nuclease-free water to 10 μL total volume. Conditions for RT and amplification were 48°C for 30 min, then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Differences in loading between samples were accounted for by normalizing transcript abundance for each of the *FAD2* transcripts to that of endogenous 18S rRNA. No product was detectable after 40 cycles of amplification in *FAD2* control reactions with total RNA template but without reverse transcriptase, indicating the absence of genomic DNA.

Genomic DNA Gel-Blot Analysis

Genomic DNA was isolated from ground soybean leaves using a protocol similar to that of Webb and Knapp (1990). Ten-microgram samples of DNA were digested with restriction enzymes and separated on a 0.7% Tris-borate/EDTA (TBE)-agarose gel. Following electrophoresis, the gel was soaked in 0.25 M HCl for 10 min, then in 0.5 M NaOH and 1.5 M NaCl for 30 min, and blotted onto a positively charged nylon transfer membrane (Roche). The membrane was probed with a 420-bp [α-^{32}P]dCTP-labeled *FAD2-1A* intron DNA fragment. The hybridization was performed in 50% formamide, 6× sodium chloride/sodium phosphate/EDTA, 5% Denhardt's solution, 1% SDS, and 10 μg mL^{-1} denatured, sonicated salmon sperm DNA overnight at 42°C. The membrane was washed in 2× SSC, 0.1% SDS for 5 min at 42°C, then in 0.2× SSC, 0.1% SDS for 15 min at 55°C, and exposed to Biomax XAR film (Eastman Kodak).

Large RNA Gel-Blot Analysis

Soybean seeds were sorted into stages 3, 6, and 9 as described above, and total RNA was isolated from the seeds according to Jones et al. (1995). Remaining DNA was removed by digestion with TURBO DNA-free DNase I (Ambion) according to the manufacturer's protocol. RNA concentrations were measured using a Qubit fluorometer (Invitrogen). RNA samples were heated to 65°C for 5 min to denature before electrophoresis through a 1.5% agarose gel containing 1.5% formaldehyde and 1× MOPS (40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA, pH 7) in 1× MOPS running buffer. Following electrophoresis, the gel was blotted onto a positively charged nylon transfer membrane (Roche). The membrane was probed with a 420-bp [α-^{32}P]dCTP-labeled full-length *FAD2-1A* intron or a 316-bp *FAD2-1A* exon DNA fragment (forward, 5′-GGTCTAGCAAAGGAAACAACAATG-3′, reverse, 5′-TCACCCACACACCAGTGAGAAG-3′). The hybridization was performed in PerfectHyb hybridization buffer (Sigma) overnight at 68°C. The membrane was washed in 2× SSC, 0.1% SDS for 10 min at 42°C, then in 0.2× SSC, 0.1% SDS for 15 min at 60°C, and exposed to Biomax XAR film (Eastman Kodak).

RT-PCR Gel-Blot Analysis

Total RNA from soybean seeds was isolated from stages 3, 6, and 9, as described above. Five micrograms of RNA was denatured at 65°C for 5 min in the presence of 250 nM each primer and 1× manufacturer's annealing buffer from SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Primer sequences were 5′-CCTTGTTTTTCTCGTATTCTATGG-3′ and 5′-TAATTATGTTATTAAGAAACGTAG-3′. The manufacturers' 2× First-Strand Reaction Mix and SuperScript III/RNaseOUT Enzyme Mix were added, and RT

reaction proceeded at 50°C for 50 min, followed by 5 min of heat inactivation at 85°C. A 1/20th volume of reverse transcriptase product was used as DNA template in conventional PCR containing 200 μM deoxyribonucleotide triphosphates, 40 nM each primer, and 2.5 units of Taq DNA polymerase, with temperature conditions of 95°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min. Ten microliters of the resulting product from each reaction was separated on a 1.5% TBE-agarose gel. Following electrophoresis, the gel was transferred to a positively charged nylon membrane (Roche), probed with a 437-bp [α-^{32}P]dCTP-labeled cotton *H6* 3′ UTR DNA fragment, and then washed and exposed to film as described above.

Low-M_r RNA Gel-Blot Analysis

Low-M_r RNA was isolated from ground soybean seeds using the *mir*Vana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol for small RNA-enriched preparation. RNA concentrations were measured using a Qubit fluorometer (Invitrogen). Five micrograms of RNA from each sample was denatured at 95°C for 5 min before separation on a 17% PAGE gel containing 7 M urea in 0.5× TBE. Following electrophoresis, the gel was blotted onto a Nytran SuPerCharge membrane (Whatman-Schleicher & Schuell) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) according to the manufacturer's protocol. The resulting blot was cross-linked at 1,200 μJ × 100 in a Stratalinker 1800 (Stratagene). A 420-bp *FAD2-1A* intron RNA probe template was transcribed in vitro from a PCR product containing the T7 promoter, with T7 RNA polymerase, and labeled with digoxigenin using the DIG Northern Starter Kit (Roche) according to the manufacturer's protocol. The probe for Gm-miR159 detection was 5′-TTTGGATTGAAGGGAGCTCTA-3′ (http://microrna.sanger.ac.uk/sequences/index.shtml). Hybridization was performed with 100 ng of probe in PerfectHyb hybridization buffer (Sigma) at 38°C for 16 h. Detection was performed with the DIG Northern Starter Kit (Roche) according to the manufacturer's protocol, before exposure to Biomax XAR film (Eastman Kodak).

ACKNOWLEDGMENTS

We thank Greg Keithly, Jerri Koffman, and Dan Ovadya for growing plants, Tim Hickman for fatty acid analysis, Paul Hoffer for executing a northern blot, Nick Wagner and Paul Hoffer for helpful discussions, and Ken Gruys for support.

Received February 2, 2010; accepted April 25, 2010; published April 27, 2010.

LITERATURE CITED

Allison D, Denke M, Dietschy J, Emken E, Kris-Etherton P, Nicolosi R (1995) Trans fatty acids and coronary heart disease risk: report of the expert panel on trans fatty acids and coronary heart disease. Am J Clin Nutr **62:** 655S–708S

Baulcombe DC (1996) RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol Biol **32:** 79–88

Chapman EJ, Carrington JC (2007) Specialization and evolution of endogenous small RNA pathways. Nat Rev Genet **8:** 884–896

Chen ZL, Schuler MA, Beachy RN (1986) Functional analysis of regulatory elements in a plant embryo-specific gene. Proc Natl Acad Sci USA **83:** 8560–8564

Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **97:** 4985–4990

Dehio C, Schell J (1994) Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible transgene silencing. Proc Natl Acad Sci USA **91:** 5538–5542

DiRienzo MA, Astwood JD, Petersen BJ, Smith KM (2006) Effect of substitution of low linolenic acid soybean oil for hydrogenated soybean oil on fatty acid intake. Lipids **41:** 149–157

Di Serio F, Schöb H, Iglesias A, Tarina C, Bouldoires E, Meins F (2001) Sense- and antisense-mediated gene silencing in tobacco is inhibited by the same viral suppressors and is associated with accumulation of small RNAs. Proc Natl Acad Sci USA **98:** 6506–6510

Fillatti JJ, inventor. July 21, 2009. Nucleic acid sequences and methods of use for the production of plants with modified polyunsaturated fatty acids. US Patent Application No. 7563949

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:** 806–811

Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286:** 950–952

Hamilton AJ, Brown S, Yuanhai H, Ishizuka M, Lowe A, Solis AGA, Grierson D (1998) A transgene with repeated DNA causes high frequency, post-transcriptional suppression of ACC-oxidase gene expression in tomato. Plant J **15:** 737–746

Han HY, Grierson GD (2002) The influence of inverted repeats on the production of small antisense RNAs involved in gene silencing. Mol Genet Genomics **267:** 629–635

Heppard EP, Kinney AJ, Stecca KL, Miao GH (1996) Developmental and growth temperature regulation of two different microsomal omega-6 desaturase genes in soybeans. Plant Physiol **110:** 311–319

Hu FB, Stampfer MJ, Manson JE, Rimm E, Colditz GA, Rosner BA, Hennekens CH, Willett WC (1997) Dietary fat intake and the risk of coronary heart disease in women. N Engl J Med **337:** 1491–1499

John ME, Keller G (1995) Characterization of mRNA for a proline-rich protein of cotton fiber. Plant Physiol **108:** 669–676

Jones A, Davies HM, Voelker TA (1995) Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. Plant Cell **7:** 359–371

Jorgensen RA (1995) Cosuppression, flower color patterns, and metastable gene expression states. Science **268:** 686–691

Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol Biol **31:** 957–973

Knowlton S, inventor. November 1, 1999. Soybean oil having high oxidative stability. US Patent Application No. 5981781

Lechtenberg B, Schubert D, Forsbach A, Gils M, Schmidt R (2003) Neither inverted repeat T-DNA configurations nor arrangements of tandemly repeated transgenes are sufficient to trigger transgene silencing. Plant J **34:** 507–517

Marathe R, Anandalakshmi R, Smith TH, Pruss GJ, Vance VB (2000) RNA viruses as inducers, suppressors and targets of post-transcriptional gene silencing. Plant Mol Biol **43:** 295–306

Martinell BJ, Julson LS, Emler CA, Huang Y, McCabe DE, Williams EJ, inventors. May 7, 2002. Soybean Agrobacterium transformation method. US Patent Application No. 6384301

Metzlaff M, O'Dell M, Cluster PD, Flavell RB (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. Cell **88:** 845–854

Meyer JJ (2006) Petition for the determination of nonregulated status for Roundup RReady2Yield Soybean MON 89788. http://www.aphis.usda.gov/brs/aphisdocs/06_17801p.pdf (March 26, 2010)

Moss J (2006) Labeling of trans fatty acid content in food, regulations and limits: the FDA view. Atheroscler Suppl **7:** 57–59

Nakamura N, Fukuchi-Mizutani M, Miyazaki K, Suzuki K, Tanaka Y (2006) RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression. Plant Biotechnol **3:** 13–17

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell **2:** 279–289

Que Q, Jorgensen RA (1998) Homology-based control of gene expression patterns in transgenic petunia flowers. Dev Genet **22:** 100–109

Que Q, Wang HY, English JJ, Jorgensen RA (1997) The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. Plant Cell **9:** 1357–1368

Ryan D, Nuccie B, Arvan D (1999) Non-PCR-dependent detection of the factor V Leiden mutation from genomic DNA using a homogeneous invader microtiter plate assay. Mol Diagn **4:** 135–144

Schlueter JA, Vasylenko-Sanders IF, Deshpande S, Yi J, Siegfried M, Roe BA, Schlueter SD, Scheffler BE, Shoemaker RC (2007) The FAD2 gene family of soybean: insights into the structural and functional divergence of a paleopolyploid genome. Crop Sci **47:** S-14–S-26

Stam M, de Bruin R, Kenter S, van der Hoorn RAL, van Blokland R, Mol

JN, Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in petunia by inverted transgene repeats. Plant J **12:** 63–82

Stoutjesdijk PA, Singh SP, Liu Q, Hurlstone CJ, Waterhouse PA, Green AG (2002) hpRNA-mediated targeting of the Arabidopsis *FAD2* gene gives highly efficient and stable silencing. Plant Physiol **129:** 1723–1731

Tang G (2005) siRNA and miRNA: an insight into RISCs. Trends Biochem Sci **30:** 106–114

Van Blokland R, van der Geest N, Mol JNM, Kooter JM (1994) Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant J **6:** 861–877

Van Eldik G, Litiere K, Jacobs J, Van Montagu M, Cornelissen M (1998) Silencing of beta-1,3-glucanase genes in tobacco correlates with an increased abundance of RNA degradation intermediates. Nucleic Acids Res **26:** 5176–5181

Voinnet O (2008) Use, tolerance and avoidance of amplified RNA silencing by plants. Trends Plant Sci **13:** 317–328

Walling L, Drews GN, Goldberg RB (1986) Transcriptional and post-transcriptional regulation of soybean seed protein mRNA levels. Proc Natl Acad Sci USA **83:** 2123–2127

Watson JM, Fusaro AF, Wang M, Waterhouse PM (2005) RNA silencing platforms in plants. FEBS Lett **579:** 5982–5987

Webb D, Knapp S (1990) DNA extraction from a previously recalcitrant plant genus. Plant Mol Biol Rep **8:** 180–185