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.
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 desaturase that converts oleic acid [18:1(Δ9)] to linoleic acid [18:2(Δ9,Δ12)]. 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 soybean 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 Penetration

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 casettes, the FAD2-1A intron splicing signals had been removed. The soy 7Sa′ 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 EPS 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 XbaI, 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 Mr (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).
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.

Homoyzogous 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 early in development.


to CTRL early in develop-

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.
Inverted Repeat Configurations

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 BamHI, SalI, and NsiI, 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 3′ UTR probe after XbaI 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 BamHI, SpeI, and SacI, 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 transform lines an additional single band is observed for each restriction enzyme digest. For each transformant, BamHI, SpeI, and SacI 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](image)

**Figure 3.** Genomic DNA gel-blot analysis of transgenic lines. Genomic DNA was digested with BamHI, SpeI, and SacI 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 points) is usually possible, since very little inverted repeat sequence is amplified (triangles indicate primer point). 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 7Sa’ 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 7Sa’ 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-
suppressed transfectants. 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 7Sa’ 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, 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 FAD2-1A 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, 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, 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.
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...
The number 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 7S" 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 (Bhushan and Keler, 1995; GenBank accession no. L17308) supplied the polyadenylation signals for each cassette. Each cassette was integrated into a standard binary vector that used Agrobacterium tumefaciens EPS130 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 Keller et al. (2001). 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 variety developed by Asgrow Seed Company and is a midmaturity group III variety with very high yield potential. Transformants were selected and variety developed by Asgrow Seed Company and is a midmaturity group III variety with very high yield potential.
reaction proceeded at 50°C for 5 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 deoxynucleotide triphosphates, 40 mM 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-32P]dCTP-labeled cotton H63 UTR DNA fragment, and then washed and exposed to film as described above.

Low-M, RNA Gel-Blot Analysis

Low-M, RNA was isolated from ground soybean seeds using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s protocol for small RNA-enriched preparation. RNA concentrations were measured using a Qubit fluorimeter (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 1200 µl × 1 mm in a Stratagene 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’-TTTGAGATTGAAGGGAGCTCTA-3’. Hybridization was performed with 100 ng of probe in Perfect-Hyb 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).

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