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ORIGINAL PAPER

Altering *Arabidopsis* Oilseed Composition by a Combined Antisense-Hairpin RNAi Gene Suppression Approach

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Abstract Antisense (AS) and hairpin (HP) RNA interference (RNAi) targeted gene suppression technologies have been used to modify seed oil composition. Larger numbers of AS transgenics have to be screened to achieve a targeted level of suppression compared to RNAi. We hypothesized combining AS with RNAi might result in enhanced gene suppression compared to either method individually. AS and HP-RNAi were combined as hairpin antisense (HPAS) constructs containing ~125 bp sense and antisense portions of an untranslated region of the target gene separated by an intron containing an antisense copy of a portion of the target coding region. The Δ12-desaturase FAD2, the ω3-desaturase FAD3 and β-ketoacyl-ACP synthase (KAS) II were targeted in *Arabidopsis* to evaluate changes in oil composition with AS, HP and HPAS constructs driven by the phaseolin promoter. Modest but statistically significant enhancements in oilseed phenotypes were observed with HPAS relative to AS and HP-RNAi. Phenotypes for HPAS suppression of FAD2 and FAD3 were indistinguishable from their strongest mutant alleles. Our data suggest that HPAS may be useful for: (1) achieving levels of suppression comparable to those of gene knockouts in a tissue specific manner. (2) Maximizing suppression of suboptimal RNAi constructs and (3) minimizing the screening of transgenics to achieve desired oilseed composition.

Keywords Gene suppression · RNA interference · Hairpin antisense

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Abbreviations

FAD2	Oleate desaturase
FAD3	Linoleate desaturase
KASII	β-Ketoacyl-ACP synthase
Oleic acid	18:1
Stearic acid	18:0
SCD	Stearoyl-CoA-desaturase

Introduction

Changes in the expression of various fatty-acid metabolizing enzymes can result in dramatic changes in seed oil composition [1, 2]. Seeds are remarkably plastic in that changes in oil composition [3] which rarely result in deleterious changes in the quantity of stored oil, or physiological properties such as germination [4]. Naturally occurring mutations in genes encoding fatty-acid metabolizing enzymes provide a resource for novel oilseed phenotypes, but the effects of such changes are evident in all tissues and organs of the plant [5]. In contrast, transgenic gene suppression approaches in conjunction with seed specific promoters has been used as a way to restrict the effects of targeted gene suppression to oil accumulating tissues within seeds [6].

Several approaches, including antisense [7] co-suppression [8] and RNA interference (RNAi) [9] have been applied to the modulation of gene expression in seed tissues. Antisense technology blocks the flow of information from DNA *via* RNA to protein by introducing an RNA strand complementary to the sequence of the target mRNA. Duplex formation may impair mRNA maturation and/or translation, or alternatively, lead to rapid mRNA degradation [10]. Simons [11] discovered the regulation of gene

expression by antisense RNA as a natural phenomenon occurring in bacteria. Natural antisense RNAs are found in a variety of eukaryotes. The expression of antisense- and sense- (co-suppression) RNA constructs in animal and plant systems has been successfully used to downregulate specific targeted genes [12, 13]. These methods typically generate a range of expression levels of the target in individual transgenics, thus providing useful tools to establish the influence of various levels of expression of the target gene construct on phenotype [2]. However, since most transformants exhibit only moderate gene suppression other techniques have been developed such as that employing a hairpin of the target mRNA that result in stronger average decreases in the expression of the targeted gene [14, 15].

Both antisense and hairpin RNAi methodologies are capable of eliminating detectable expression of the targeted genes. However, the practical consequence of the higher average suppression observed for hairpin RNAi compared to antisense is that fewer numbers of transgenic plants must be screened in order to identify individuals that exhibit a particular level of suppression to achieve a particular oilseed phenotype [12].

Because the detailed mechanisms of antisense[16] and hairpin RNAi [17] can differ [18], we hypothesized that employing both pathways simultaneously might result in stronger inhibition, which would allow the identification of desired levels of gene suppression by screening fewer transformants.

In this paper, we compare several methods of gene suppression with respect to the fatty-acid composition of resulting transgenic seed. We describe a method in which we combined antisense (AS) and hairpin (HP) RNAi into a single compact integrated construct (HPAS) under the control of the seed-specific phaseolin promoter. We targeted AS, HP, and HPAS against three fatty-acid metabolizing enzymes, viz., the oleate desaturase FAD2; the linoleate desaturase, FAD3; and, the β-ketoacyl-ACP synthase, KASII. The mean values for HPAS gene silencing showed statistically significant improvements in average gene suppression compared to either AS or HP alone; and the mean HPAS phenotypes resulting from targeting *Fad2* and *Fad3* were statistically indistinguishable from the strongest mutant alleles reported to date.

Experimental Procedures

Arabidopsis Growth and Transformation

Arabidopsis plants were grown in soil under continuous exposure to 300 microEinsteins of light (1 microEinstein = 1 mole of photons) in E7/2 controlled environment growth chambers (Conviron). Approximately 16 plants per construct were subject to transformation according to Clough and Bent's method [19] using *Agrobacterium tumefaciens* strain GV3101. We identified individual T_1 seeds carrying the transgenes by the red fluorescence emitted [20] upon illumination with green light from an X5 LED flashlight (Inova) in conjunction with a 25A red camera filter [6]. Seed-specific expression was achieved by placing all constructs under the control of the phaseolin seed-storage protein promoter [21, 22].

Plasmid Construction

The general arrangements of DNA elements for antisense (AS), hairpin (HP) and hairpin antisense (HPAS) constructs, detailed below, are shown in Fig. 1.

Fad2-AS

A portion consisting of the ORF of *Fad2* was amplified with primers FAD2-5′XhoI (CCCTCGAGATGGGTGCA GGTGGAAGAAT) and FAD2-3′PacI (CCTTAATTAAT CATAACTTATTGTTGTACCA), and used to replace the corresponding region of the *Fad2*-HP cassette at the corresponding PacI-XhoI restriction sites to yield a *Fad2* ORF in the antisense direction (Fig. 2).

Fad2-HP

A 118 bp fragment of the *Fad2* 3′UTR was amplified from *Arabidopsis* genomic DNA in both sense (using primers Fad2-UTR5′-PstI CCCTGCAGAAACGGATGATGGTGA AGAAATT and Fad2-UTR3′-SacI/XhoI GGGAGCTCCT CGAGCAGCCAAAATGTCATAACAC) and antisense orientations (using primers Fad2-UTR5′-NheI CCGCTAG CGGATGATGGTGAAGAAATT and Fad2-UTR3′-PacI CCTTAATTAAGCAGCCAAAATGTCATAACAC) and used to replace the 5′UTR sense and antisense portions of KasII in plasmid pGEMT-Easy-HTM3. The resulting *Fad2* hairpin sequence was excised at PacI/XhoI from that plasmid and inserted into pDs-Red-PHAS as a PacI/XhoI fragment to produce pPHAS-fad2-*HP* ([6] and Fig. 2).

Fad2-HPAS

A 1,152 bp fragment of the *Fad2* gene was amplified with primers FAD2-5′SphI (CGCATGCATGGGTGCAGGTGG AAGAAT) and FAD2-3′SpeI (CCACTAGTTCATAACTT ATTGTTGTACCA), and the fragment was used to replace the corresponding SpeI-SphI segment of the *Fad2* intron i.e., in the antisense direction (Fig. 2).

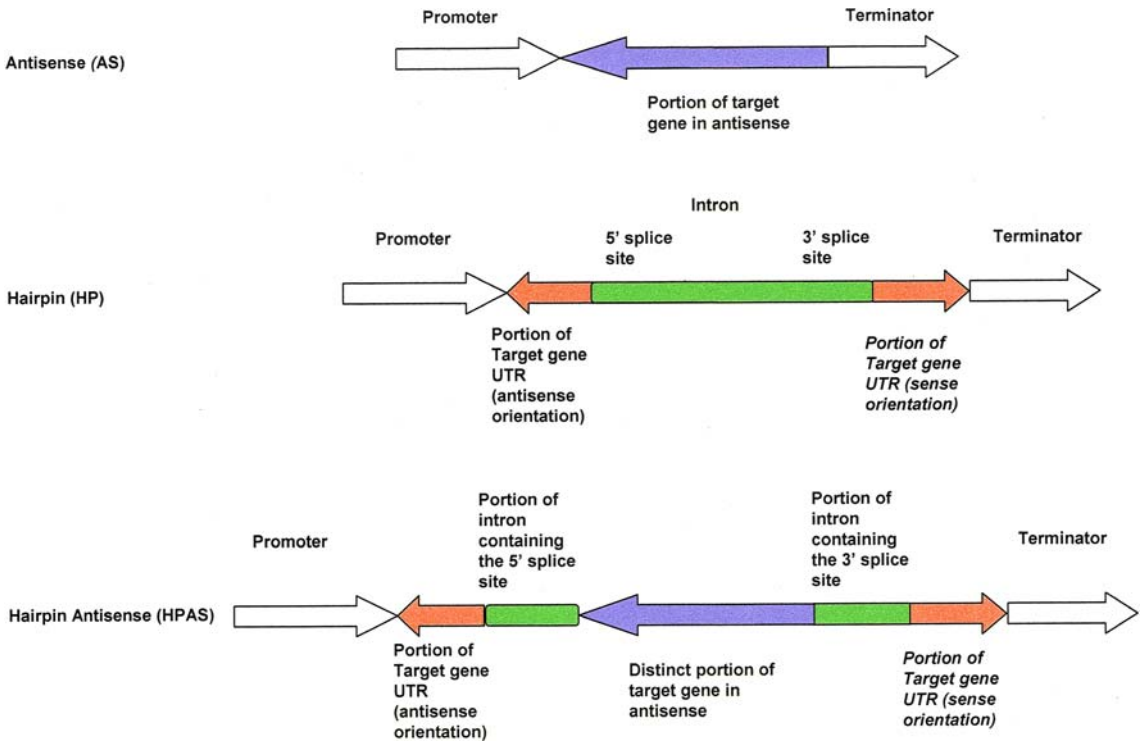

Fig. 1 Schematic diagram showing the relationship of DNA elements comprising antisense (*AS*), hairpin (*HP*) and hairpin antisense (*HPAS*) constructs

Fad2-HPAS(t)

A portion of the *Fad2* intron was replaced by *Fad2* coding sequence at the NheI-SpeI sites resulting in an antisense construct lacking the splice donor site (Fig. 2).

Fad3-AS

The 309 bp first exon of the *Fad3* gene was amplified from genomic *Arabidopsis* DNA using primers Fad3-anti-5′PacI (GGGTTAATTAACGTGGCCGAGAACAAAGATG) and Fad3-anti-3′XhoI (CCCTCGAGAGTTGTTGCTATGGAC CAACGC). The amplified fragment was used to replace the *Fad3*-HP cassette, employing the corresponding PacI and XhoI restriction sites (Fig. 2).

Fad3-HP

A 138 bp fragment of the *Arabidopsis Fad3* 3′UTR was amplified from genomic DNA in both sense (Fad3-UTR5-PstI CCCTGCAGAAACCCGGGGCTCTATTAGGAATA AACC and Fad3-UTR3-XhoI CCCTCGAGAACAAAACT TTACGCCTTGT) and antisense (Fad3-UTR5-PacI GGGT TAATTAACAAAACTTTACGCCTTGT and fad3-UTR3-NheI GGGCTAGCAGCTCTATTAGGAATAAACC) orientations. We replaced the 5′UTR sense and antisense portions of *Fab1* in *Fab1*-HP, respectively, with these fragments (Fig. 2).

Fad3-HPAS

The 309 bp first exon of the *Fad3* gene was amplified use primers Fad3-anti-5′BglII (GGAGATCTGGCGCGCCC GTGGCCGAGAACAAAGATG) and Fad3-anti-3′SpeI (GGGACTAGTGTTGTTGCTATGGACCAACGC), and the fragment was used to replace part of the *Fad2*-intron at the corresponding BglII-SpeI sites, resulting in an antisense *Fad3* fragment orientation (Fig. 2).

Fab1-AS

The 178 5′UTR of the *Fab1* gene was amplified using primers KasII-UTR5-NheI/XhoI (GGCTCGAGCTAGCC GCATCGAAGCTCTCTGCACGC) and KasII-UTR3-PacI (GGTTAATTAAGGCTTTGAGAAGAACCCAG), the fragment was used to replace the entire *Fab1* hairpin-intron in *Fab1*-HP with the use of the corresponding PacI-XhoI restriction sites (Fig. 2).

Fab1-HP

The construction of a *Fab1* hairpin RNAi was described earlier [6].

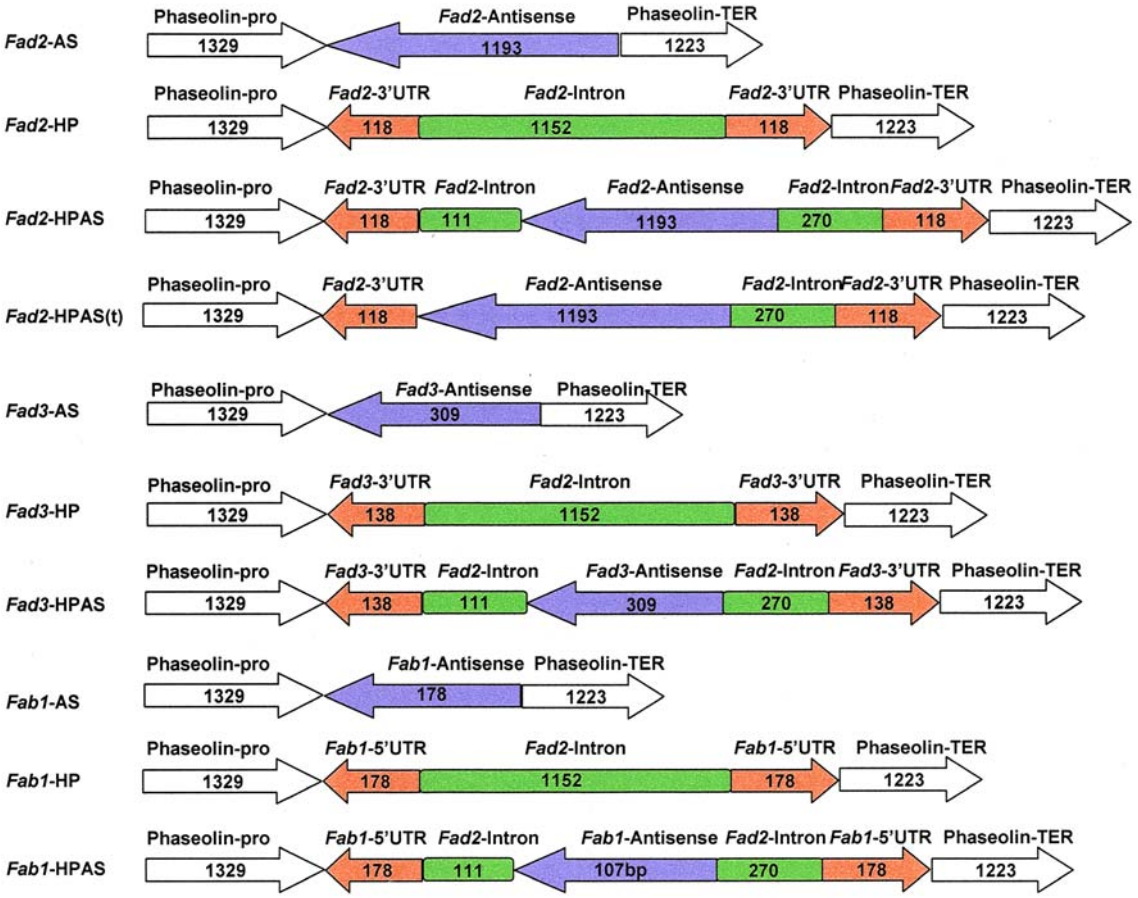

Fig. 2 Schematic diagram of the plasmid constructs. *Numbers* in base pairs are shown for individual elements. Functional elements are *color coded*. Note: elements are not drawn to scale

Fab1-HPAS

A 107 bp fragment of the first exon of the *Fab1* gene was amplified from genomic DNA using primers KasII-5′exon-BglII (GGGAGATCTGGCGCGCCGGCTATCTCCTCCA CCGTGA) and KasII-3′exon-SpeI (GGGACTAGTTCTTC CTTTTTATGCCATGG). We used this fragment to replace part of *Fad2*-Intron by restriction, *via* the SpeI and BglII restriction sites in pGEM-T-Easy-HTM3. The cassette containing the *Fab1* hairpin, intron and *Fab1* antisense replaced the HP fragment of *Fab1*-HP with the use of the corresponding BamHI and XhoI sites (Fig. 2).

Fatty-Acid Analysis

To analyze the fatty-acids of single seeds, we prepared fatty- acid methyl esters (FAMEs) by incubating the seeds with 0.2 M trimethylsulfonium hydroxide in methanol [23]. To similarly analyze bulk seeds, FAMEs were prepared by incubation them in 0.5 ml BCl_3 for 1 h at 80 °C, extracting them with 1 ml of hexane and then drying under N_2. FAMEs were analyzed either with an HP6890 gas chromatograph-flame ionization detector (Agilent Technologies) or an HP5890 gas chromatograph-mass spectrometer (Hewlett–Packard) fitted with 60-m × 250-μm SP-2340 capillary columns (Supelco). The oven temperature was raised during the analyses from 100 to 240 °C at a rate of 15 °C min^{-1} with a flow rate of 1.1 ml min^{-1}. Mass spectrometry was performed with an HP5973 mass selective detector (Hewlett–Packard). We determined the double-bond positions of monounsaturated FAMEs by dimethyl disulfide derivatization [24].

Statistical Analysis

Means and standard deviations were computed for fatty-acid methyl ester compositions for 10 randomly identified seeds per treatment. Means were compared with the use of students *t* test and results are reported for significance at the *p* values reported in the text.

Results and Discussion

To assess whether combining antisense and RNAi in a single contiguous construct (HPAS) would enhance changes in oilseed composition via gene suppression relative to those resulting from individual antisense and HP-RNAi approaches, we compared the fatty-acid phenotypes resulting from each of the three treatments. See Fig. 1 for a schematic illustration of the relative arrangement of DNA elements comprising AS, HP and HPAS constructs. The constructs were driven by the phaseolin seed- storage promoter, confining the effects to seed tissues which have been shown to tolerate substantial changes in the composition of stored lipids [4]. We chose to screen for transgenic seed using fluorescent proteins as described by Stuitje [20]. This method allows rapid assessment of the effects of the transgenic construct, but has the disadvantage that for lipid analysis the analysis is destructive which precludes the correlation of transcription with phenotype. However, this method is generally favoured because it gives a rapid indication of the effects of a construct in the hemizygous condition for a sample of the transgenics, the remainder are grown, allowed to self pollinate and produce segregating T2 seeds for subsequent analysis and further propagation.

For our initial target gene we chose the Δ12-desaturase-endcoding *Fad2* gene [25] because it is represented by a single copy in *Arabidopsis*, its product catalyzing the conversion of oleic- to linoleic- acid. *Fad2* was also chosen because it was previously used as a model for assessing the efficacy of several silencing approaches, such as antisense and co-suppression [8], RNAi [12] and ribosozyme [26]. To ascertain whether the observed effects were specific to *Fad2*, we also included the ω3-desaturase (*Fad3*) gene [27, 28]. For comparison, we analyzed the strongest mutant alleles of *Fad2* and *Fad3* characterized to date, i.e., *fad2-2* [29] and *fad3-2* [30]. Since we have an ongoing interest in modulating the levels of the β-ketoacyl ACP synthase (KAS) II, and recently reported its down-regulation by HP-RNAi [6], we also compared the effects of HPAS with those of HP-RNAi and antisense alone for KASII.

Fad2 Gene Suppression

AS suppression of Fad2 resulted in an increase of 18:1 fatty-acid from 15.2% in wt to 44.2% in seeds transformed with *Fad2*-AS. This level increased to 56.9% with *Fad2*-HP, and a further increase to a mean of 61.7% was observed in seeds transformed with *Fad2*-HPAS. All means were significantly different from all others ($P < 0.01$). The levels of di- and tri-unsaturated fatty-acids changed commensurately in these same transformants; the total of 18:2 + 18:3 being 43.3% in wt plants, which declined to 18.9% in the *Fad2*-AS seeds, and 9.4% in the *Fad2*-HP seeds; all means being significantly different ($P < 0.01$). The decline from 9.4% in the *Fad2*-HP line to 7.2% in the *Fad2*-HPAS line was significant ($P < 0.05$). In a control experiment designed to show that the HP is still operative when combined with AS, we engineered *Fad2*-HPAS(t), a truncated construct that lacks the 5′ intron border (Fig. 2) which should be either unable to excise the intron and therefore not be able to form the *Fad2* HP RNA, or should show reduced levels of intron excision [12]. The resulting fatty-acid phenotype was indistinguishable from that of the *Fad2*-AS (Fig. 3), suggesting that in this case excision of the intron did not occur. These data are consistent with our interpretation that both hairpin and antisense contribute to achieving the enhancing the levels of suppression seen for seeds containing the *Fad2*-HPAS construct.

The mean *Fad2*-HPAS level of 7.2% was not significantly different from the 18:2 + 18:3 level of 7.5% in the fad2-2 line (Fig. 3). Thus, for FAD2 the combination of hairpin and antisense gene silencing is higher than that seen for either method alone and the combination reduces *Fad2* expression to levels equivalent to those seen for the *fad2-2* mutant allele. While the mean levels of gene suppression for *Fad2*-HPAS do not show statistical differences from that of the *fad2* mutant background, we note that individual

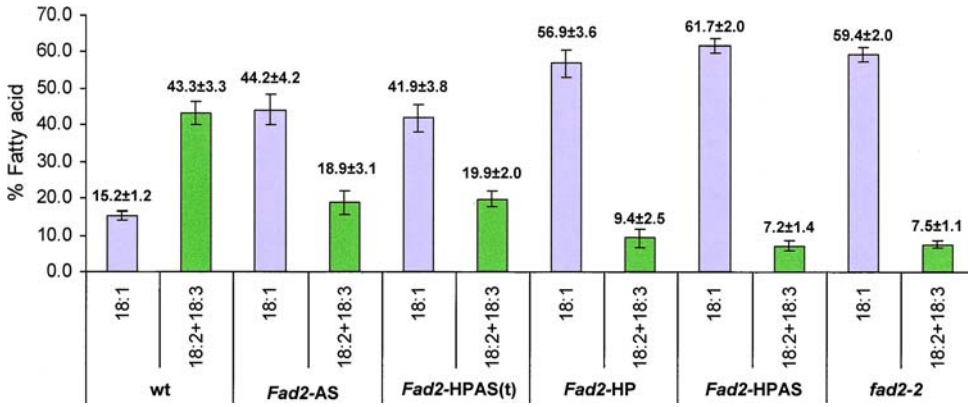

Fig. 3 Histogram of proportions of fatty-acid methyl esters derived from quantification of seed samples in which Fad2 was targeted for gene suppression. Samples of T1 seeds (*n*-10) from wild type (*wt*), *Fad2* antisense (*Fad2*-AS), *Fad2* hairpin (*Fad2*-HP), *Fad2* hairpin antisense (*Fad2*-HPAS), *Fad2* hairpin antisense in which the splice donor site is missing and the *fad2-2* mutant

strongly suppressed HP lines showed the same phenotype consistent with the results previously reported for *fad2* suppression by intron-spliced HP (iHP) reported by Green et al. [31].

Fad3 Gene Suppression

Our positive findings regarding *Fad2* gene silencing encouraged us to test our hypothesis on a second gene silencing target. We chose FAD3, the cytoplasmic linoleic acid desaturase, because like FAD2, its phenotype is sensitive to gene dosage effects [27] and the phenotypic effects of *Fad3* suppression can be reproducibly assessed using the methods developed for analyzing *Fad2* suppression.

Various *Fad3* constructs (Fig. 2) were transformed to wt plants. Seeds transformed with *Fad3*-AS increased in 18:2 + 18:1 from 39.2% in wt to 47.4%. Seeds transformed with *Fad3*-HP showed strong increases to 53.8%, while those transformed with *Fad3*-HPAS showed the largest increases over wt, yielding 57.5% 18:1 + 18:2. Compensatory changes were evident in 18:3, which fell from 17.1% in wt to 10.7, 4.5, and 3.0% in *Fad3*-AS, *Fad3*-HP, and *Fad3*-HPAS seeds, respectively (Fig. 4).

All of the treatments were significantly different from all others at the $P < 0.01$ level. The *Fad3*-HPAS line at 3.0% 18:3 was not significantly different from the strongest mutant *Fad3* allele, *fad3-2,* at 2.8%.

Fab1 Gene Suppression

We extended this study to include the condensing enzyme (KAS) II, encoded by the *Fab1* gene because we already had developed hairpin RNAi lines that increase the accumulation of palmitic acid [6]. We had previously used the *fab1-1 fae1* double mutant background, deficient in both plastidial (*fab1*)- [32]and cytoplasmic (*fae1*)- [33] 16 and 18 carbon elongation, as the background because it exhibits the highest seed 16:0 composition (~24%) described for a non-transgenic *Arabidopsis* genotype. We therefore used it again, introducing *Fab1*-AS and *Fab1*-HPAS constructs to *fab1-1 fae1 Arabidopsis* to compare their effects to those of the previously described *Fab1*-HP lines [6].

The 16:0 + 16:1 levels rose as follows: from 25.4% in *fab1-1 fae1* seeds to 32.8% in seeds transformed with *Fab1*-AS; and 46.6% in seeds containing *Fab1*-HP and 58.4% in seeds containing *Fab1*-HPAS (Fig. 5). Levels of 18:0, 18:1Δ9, 18:2 and 18:3 decreased commensurately: i.e., 66.5% for *Fab1*-AS, 53.4% for *Fab1*-HP and 41.6% for *Fab1*-HPAS seeds. The hemizygous T1 *Fab1*-HPAS lines were selfed and homozygous T2 individuals

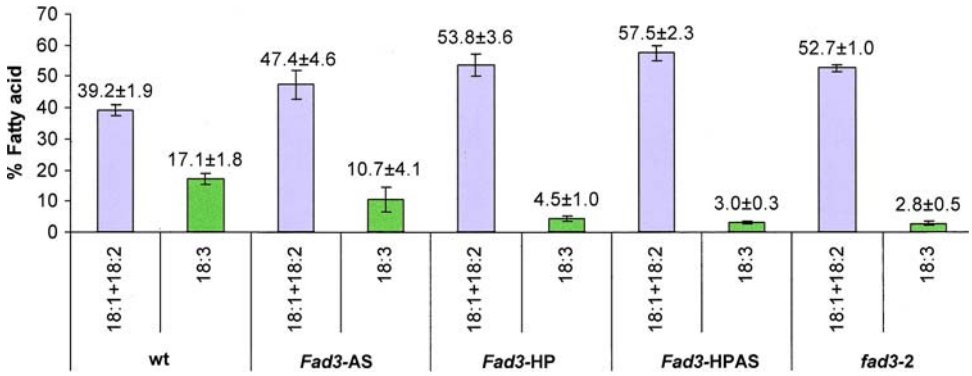

Fig. 4 Histogram of proportions of fatty-acid methyl esters derived from quantification of seed samples in which Fad3 was targeted for gene suppression. Samples of T1 seeds (n-10) from wild type (*wt*), *Fad3* antisense (*Fad3*-AS), *Fad3* hairpin (*Fad3*-HP), *Fad3* hairpin antisense (*Fad3*-HPAS), and *fad3-3* mutant

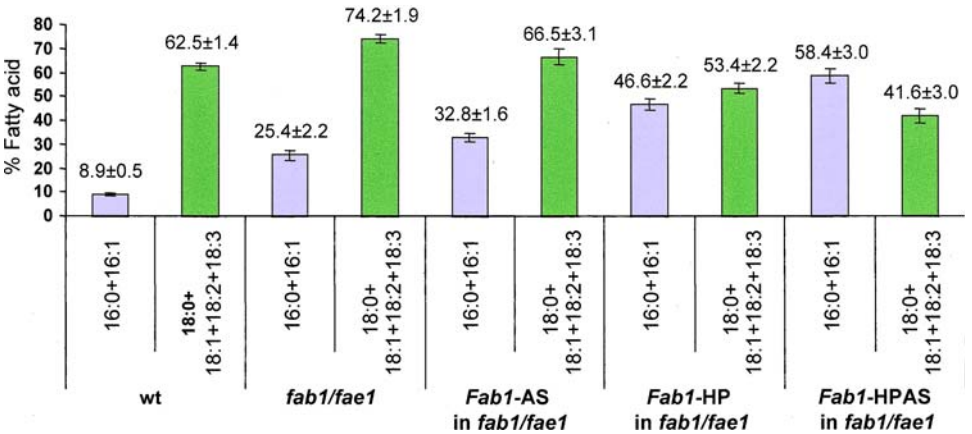

Fig. 5 Histogram of proportions of fatty-acid methyl esters derived from quantification of seed samples in which Fab1 was targeted for gene suppression. Samples of T1 seeds (*n*-10) from wild type (*wt*), *fab1/fae1*, *Fab1*-HP and *Fab1*-HPAS are shown

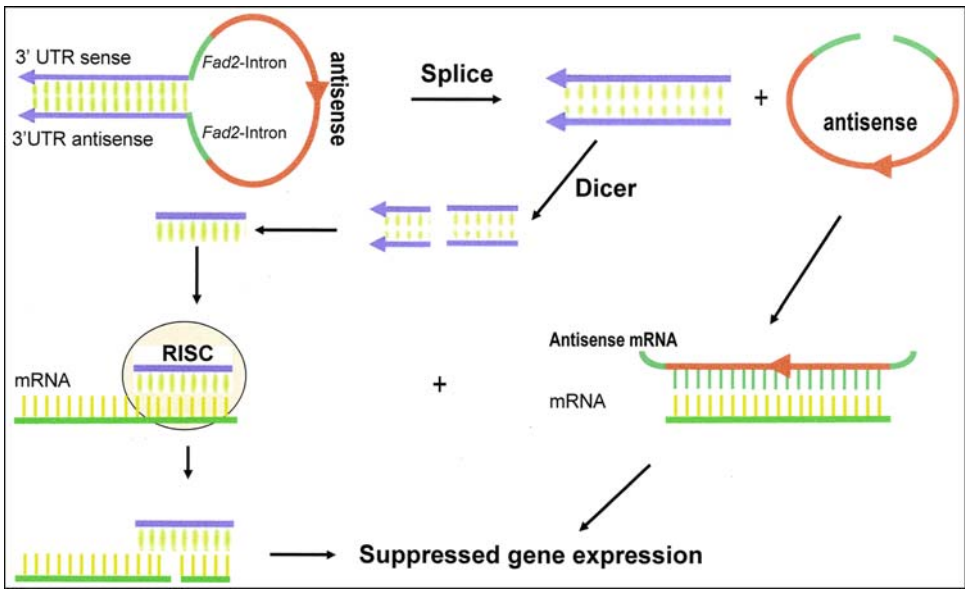

Fig. 6 A schematic representation of the possible roles of hairpin and antisense in suppressing a target gene

identified. Homozygous plants were propagated for several additional generations with stable inheritance of the *Fab1*-HPAS phenotype.

Hairpin Antisense Constructs Showed Stronger Average Gene Suppression than Either Hairpin or Antisense Constructs Alone

Smith [12] reported that HP-RNAi typically elicits stronger gene suppression than antisense or co-suppression constructs when an equivalent number of transgenic events are compared. We hypothesized that their effects should be additive because hairpin RNAi and antisense apparently are mediated *via* different mechanisms (see Fig. 6 for illustration), with the former being Dicer- and RISC-dependent [34], and the latter Dicer- and RISC-independent. Hence, we made our HPAS constructs to test this idea. For the three independent targets described herein, HPAS-RNAi exhibited significantly higher average levels of gene suppression than either method individually. This finding suggests that cleavage of an intron that contains an antisense portion of the target gene yields an antisense RNA capable of gene suppression. The design of our gene constructs affords a compact, efficient way of combining AS and HP-RNAi approaches to gene suppression. In the cases of *Fad2* and *Fad3*, the mean level of HPAS gene suppression was indistinguishable from that seen for their respective strongest natural alleles.

Potential Applications of HPAS Gene Suppression

The ability to strongly, rather than partly, suppress a target gene has a valuable role in probing the physiological function of specific genes. This is because many gene products occur in excess of their required levels, and accordingly, incomplete suppression fails to reveal a phenotype that is found only by investigating plants containing a gene knockout (KO). For example, 35-S-driven antisense of the 16:0-specific thioesterase *FatB* failed to reveal expected alterations in the level of leaf 16:0 [35], whereas significant changes in leaf fatty-acid composition was revealed when the FatB-KO was investigated [36].

There are efficient methods, such as tilling [37], for identifying the mutant alleles of target genes that can create plants exhibiting partial or complete suppression; however, because they are under the transcriptional control of their natural promoters, the effects of suppression are evident in all tissues for which the promoter is active. Thus, a desired change in oilseed phenotype may be associated with undesirable traits such as lower yield or susceptibility to pathogens. However, with HPAS, high levels of suppression can readily be attained in target tissues by judiciously choosing a tissue-specific promoter. This can be particularly useful when strong suppression in a tissue other that for which suppression is desired would generate a lethal phenotype. Alternatively, if deletion of the target gene encoding a metabolic enzyme in the tissue of interest yields a lethal phenotype, screening for transgenic HPAS-containing plants results in the identification of individuals suppressed to the maximum amount compatible with viability. In this case, analysis of resulting transgenics provides useful physiological information about the threshold amount of a metabolite required for survival [6]).

In summary, this study provides support for the hypothesis that a combination of antisense and HP-RNAi can increase the efficiency of gene suppression. The modest improvement in suppression of HPAS compared to AS and HP-RNAi suggest that HPAS may be useful for

maximizing suppression of suboptimal RNAi constructs, or achieving close to complete gene suppression in a tissue specific manner. The reason that the combined effects of HP and AS are modest likely is due to the efficacy of the HP alone, i.e., that there is little difference between the mean HP levels and the phenotype of a null mutant. However, the increased average suppression achieved by HPAS relative to HP-RNAi or AS employed separately may be of use in achieving desired oilseed phenotypes with the creation of fewer transgenic events. This may be particularly useful for modifying the seed composition of some monocots and species such as cotton for which obtaining high numbers of transformants remains costly and time consuming.

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