ACYL-ACYL CARRIER PROTEIN DESATURASE2 and 3 Are Responsible for Making Omega-7 Fatty Acids in the Arabidopsis Aleurone[1][OPEN]

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Omega-7 monounsaturated fatty acids (*ω*-7s) are specifically enriched in the aleurone of Arabidopsis (*Arabidopsis thaliana*) seeds. We found significant natural variation in seed *ω*-7 content and used a Multiparent Advanced Generation Inter-Cross population to fine-map a major quantitative trait loci to a region containing *ACYL-ACYL CARRIER PROTEIN DESATURASE1* (*AAD1*) and *AAD3*. We found that *AAD3* expression is localized to the aleurone where mutants show an approximately 50% reduction in *ω*-7 content. By contrast, *AAD1* is localized to the embryo where mutants show a small reduction in *ω*-9 content. Enzymatic analysis has previously shown that AAD family members possess both stearoyl- and palmitoyl-ACP Δ^9 desaturase activity, including the predominant isoform SUPPRESSOR OF SALICYLIC ACID INSENSITIVE2. However, *aad3 ssi2* aleurone contained the same amount of *ω*-7s as *aad3*. Within the AAD family, AAD3 shares the highest degree of sequence similarity with AAD2 and AAD4. Mutant analysis showed that AAD2 also contributes to *ω*-7 production in the aleurone, and *aad3 aad2* exhibits an approximately 85% reduction in *ω*-7s. Mutant analysis also showed that *FATTY ACID ELONGASE1* is required for the production of very long chain *ω*-7s in the aleurone. Together, these data provide genetic evidence that the *ω*-7 pathway proceeds via Δ^9 desaturation of palmitoyl-ACP followed by elongation of the product. Interestingly, significant variation was also identified in the *ω*-7 content of *Brassica napus* aleurone, with the highest level detected being approximately 47% of total fatty acids.

Oils containing omega-7 monounsaturated fatty acids (*ω*-7s; with double-bond 7-carbon atoms from the methyl end of the acyl chain) are generally considered to be of very low abundance in plant seeds, with just a few rare exceptions (Nguyen et al., 2015). In the seeds of species from the Brassicaceae such as Arabidopsis (*Arabidopsis thaliana*) and rapeseed (*Brassica napus*), *ω*-7 fatty acids account for <4 mol% of total fatty acids. However, analysis of separate tissues has revealed that *ω*-7 fatty acids are not uniformly distributed within the seed and are in fact highly concentrated in the endosperm (aleurone), which is reduced to a single cell layer surrounding the embryo in the mature seed (Penfield et al., 2004; Li et al., 2006). In the aleurone (plus seed coat), the *ω*-7 fatty acids constitute approximately 15 mol% in Arabidopsis and approximately 35 mol% in rapeseed, with the major species being cis-vaccenic acid ($18{:}1\Delta^{11}$) and paullinic acid ($20{:}1\Delta^{13}$; Li et al., 2006). These levels of *ω*-7 fatty acids are similar to those found in macadamia nuts (*Macadamia sp*.) or in the seeds of sea buckthorn (*Hippophae rhamnoides*; Nguyen et al., 2015). The biosynthetic pathway responsible for *ω*-7 fatty acid production in the aleurone of Arabidopsis seed is unknown, and no physiological role has been ascribed to this class of fatty acids despite their striking abundance in this tissue.

In the seeds of other species, *ω*-7 fatty acids are believed to be produced by Δ^9 desaturation of palmitic acid (16:0) to produce palmitoleic acid ($16{:}1\Delta^9$), which can then be elongated (Cahoon et al., 1997, 1998). In plants, two structurally unrelated classes of Δ^9 desaturase have been identified that use different substrates. The first class of Δ^9 desaturases are the acyl-acyl carrier protein (ACP) desaturases (*AAD*), which are soluble enzymes situated in the plastid stroma (Shanklin and

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Somerville, 1991). The archetype for this class is the stearoyl-ACP Δ^9 desaturase that catalyzes the formation of oleic acid (18:1Δ^9) from stearic acid (18:0) in plants and is therefore the primary route by which unsaturated fatty acids are generated. This enzyme also exhibits low levels of palmitoyl-ACP Δ^9 desaturase activity (McKeon and Stumpf, 1982), and variant forms that display a distinct substrate specificity for 16:0-ACP, rather than 18:0-ACP, have been isolated from several plant species that accumulate high levels of ω-7 fatty acids (Cahoon et al., 1997, 1998). It is therefore most likely that *AAD* genes are responsible for ω-7 fatty acid production in Arabidopsis. The *AAD* gene family in Arabidopsis encodes seven proteins of which SUPPRESSOR OF SALICYLIC ACID INSENSITIVE2 (SSI2/FAB2) is the predominant isoform. The *ssi2* mutant accumulates 18:0 at the expense of 18:1Δ^9 in both seed and vegetative tissues, indicating a reduction in Δ^9 desaturation (Lightner et al., 1994). SSI2, AAD1, AAD3, AAD4, and AAD5 have all been shown to desaturate 18:0-ACP at the Δ^9 position, although the specific activity of SSI2 for 18:0-ACP was found to be >40-fold greater (Kachroo et al., 2007). Of the five AAD proteins tested, SSI2 and AAD3 were also found to have the highest specific activity on 16:0-ACP (Kachroo et al., 2007). Kachroo et al. (2007) were unable to recover homozygous T-DNA mutants in *AAD2*, *AAD3*, and *AAD5*, implying that they may be lethal. However, *aad1* and *aad4* mutants exhibited an approximately 5-fold increase in 16:1 in stems, and *aad1* also showed an approximately 2-fold increase in the ω-7 fatty acid 18:1Δ^{11} in flowers (Kachroo et al., 2007).

The acyl-CoA desaturase-like (*ADS*) gene family is the second class of Δ^9 desaturases that have been identified in plants (Chen and Thelen, 2013; Smith et al., 2013). The Arabidopsis genome encodes a group of nine ADS proteins with homology to the Δ^9 acyl-lipid desaturases of cyanobacteria and the Δ^9 acyl-CoA desaturases of yeast (*Saccharomyces cerevisiae*) and mammals (Fukuchi-Mizutani et al., 1998; Heilmann et al., 2004a). The most studied of the *ADS* genes is *ADS3* (*FATTY ACID DESATURASE5*) that encodes a plastidic palmitoyl-monogalactosyldiacylglycerol Δ^7 desaturase (Browse et al., 1985; Kunst et al., 1989; Heilmann et al., 2004a). The *fad5* mutant is deficient in 16:3$\Delta^{7,10,13}$, which is normally a major constituent of thylakoid membrane lipids (Browse et al., 1985). More recent work has shown that *ADS2* is involved in membrane lipid remodeling at low temperatures (Chen and Thelen, 2013). ADS2 can desaturate 16:0 at the Δ^9 position when expressed in yeast (Heilmann et al., 2004b), but has also subsequently been shown to produce ω-7 very long chain fatty acids (VLCFAs; Smith et al., 2013). Other members of the ADS family have yet to be ascribed physiological roles, but some are also capable of producing ω-7 VLCFA (Heilmann et al., 2004a, 2004b; Smith et al., 2013).

It has been shown previously that there is significant genetic variation in seed fatty acid composition among different Arabidopsis accessions (Millar and Kunst, 1999; O'Neill et al., 2003). This has allowed several groups to exploit recombinant inbred (RI) populations to identify quantitative trait loci (QTL) controlling the abundance of major fatty acid species (Hobbs et al., 2004; Sanyal and Randal Linder, 2012; O'Neill et al., 2012). However, because ω-7 fatty acids are of low abundance in whole seeds and are also not straightforward to separate from their more common ω-9 isomers using gas chromatography, they do not feature in any of these studies. Here we used the powerful Multiparent Advanced Generation Inter-Cross (MAGIC) RI population created by Kover et al. (2009) to screen for QTL controlling ω-7 fatty acid content in Arabidopsis and identify AAD3 as the major Δ^9 desaturase responsible for the synthesis of these fatty acid species in the aleurone.

RESULTS

Aleurone ω-7 Fatty Acid Content Varies between the MAGIC Parental Accessions

To investigate whether ω-7 fatty acid content varies between different ecotypes of Arabidopsis, we performed fatty acid analysis on dissected aleurones (plus seed coat) and embryos of 19 different accessions. Kover et al. (2009) previously selected these accessions to construct the Arabidopsis MAGIC RI population, because they represent a wide range of genotypic and phenotypic diversity. In all 19 accessions, 18:1Δ^{11} and 20:1Δ^{13} were the major ω-7 fatty acid species we detected, and they were concentrated in the aleurone, as has been shown previously for ecotype Col-0 (Penfield et al., 2004; Li et al., 2006). However, there was also significant variation ($P < 0.05$) in endosperm total ω-7 fatty acid content across the accessions, ranging from 13% to 27% (Fig. 1A). In contrast, embryo total

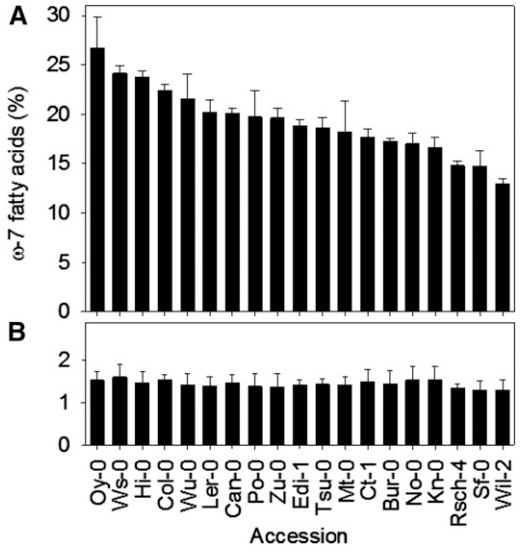


Figure 1. Variation in ω-7 fatty acid content of seed tissues among the 19 parental accessions of the Arabidopsis MAGIC population. Total ω-7 fatty acid content as a percentage of all fatty acids in aleurone plus seed coat (A) and embryo (B). Values are the mean ± SE of measurements on seed from four separate plants of each genotype.

ω-7 fatty acid content did not vary significantly ($P > 0.05$) and was consistently around 1.5%. Linear regression analysis also showed that the level of ω-7 fatty acids in whole seeds is positively correlated with that in the endosperm plus seed coat ($r^2 = 0.97$).

A Major QTL Determines the ω-7 fatty Acid Content in the MAGIC Population

Given that the ω-7 fatty acid content of whole seeds from the MAGIC population founder accessions (Kover et al., 2009) could be used as a proxy to monitor variation in the aleurone, we decided to perform a QTL analysis using the RI population. We grew approximately 450 MAGIC RI lines as three replicates in a random block design. We harvested seeds and determined their fatty acid composition by gas chromatography. Across the complete RI population, total ω-7 fatty acid content ranged from 0.8% to 4.3% in whole seeds, and the estimated broad sense heritability was 0.86. We analyzed the data using the multiple-QTL modeling method described by Kover et al. (2009). Three QTL were detected with genome-wide *P* values of < 0.05 (Supplemental Table S1). The most significant QTL had a $\log P_{max}$ value (-log10 ANOVA *P* value) of 20.0 and was located on Chromosome 5 at approximately 5.2 Mb (Fig. 2). The 90% confidence interval for QTL5, given the $\log P_{max}$ value, is estimated to be approximately 0.8 Mb (Kover et al., 2009). The other two minor QTL were located on chromosomes 1 and 3 at approximately 0.8 and 5.0 Mb, respectively. The logP values for QTL1 and QTL3 are 4.8 and 3.7, respectively (Supplemental Table S1).

AAD3 and *AAD1* Colocate with QTL5 and *AAD3* Is Expressed in Only the Aleurone

By cross-referencing the location of the ω-7 fatty acid QTL mapping intervals with the position of the seven *AAD* and nine *ADS* Δ^9 desaturase family members present in Arabidopsis (Kachroo et al., 2007; Chen and Thelen 2013; Smith et al., 2013), we were able to identify *AAD1* (At5g16240) and *AAD3* (At5g16230) as candidate genes that are most likely to underlie the major QTL5. These two neighboring genes are situated within approximately 100 kb of the marker interval where QTL5 peaks (Fig. 2). It is noteworthy that *ADS3/FAD5* (At3g15850) and *ADS3.2* (At3g15870) also lie near QTL3 (Heilmann et al., 2004a). Recombinant AAD1 and AAD3 have both been reported to exhibit Δ^9 desaturase activity using 16:0-ACP as a substrate (Kachroo et al., 2007). However, in order for either *AAD1* or *AAD3* to play a role in seed ω-7 fatty acid production, they must be expressed in the aleurone during maturation (Penfield et al., 2004; Li et al., 2006). An initial investigation of the profile of *AAD1* and *AAD3* transcript abundance in different Arabidopsis tissues using public Ath1 gene chip microarray data (Winter et al., 2007) suggested that both genes are preferentially expressed in whole developing seeds during the phase of oil deposition. However, Le et al., (2010) have previously performed microarray experiments on microdissected tissues of developing Arabidopsis seeds. These data suggest that at the mature green stage *AAD3* is expressed specifically in the aleurone and seed coat tissues, while *AAD1* expression is restricted to the embryo (Le et al., 2010). We preformed quantitative PCR on RNA from dissected developing embryos and aleurone plus seed coat of ecotype Col-0, which confirmed these patterns of expression (Fig. 3).

Disruption of *AAD3* Reduces Aleurone ω-7 Fatty Acid Content

Kachroo et al. (2007) previously screened for T-DNA mutants in both *AAD1* and *AAD3* but were able to obtain only homozygous lines for *AAD1*. However, when we attempted to isolate mutants, we were successful in obtaining multiple independent homozygous Col-0 T-DNA lines for both *AAD1* and *AAD3* (Fig. 4). The fatty acid composition of dissected embryo and aleurone plus seed coat of *aad1* and *aad3* mutants was determined to investigate whether fatty acid content is altered (Fig. 4). The fatty acid profile of *aad3* aleurone plus seed coat was different from wild type in that the level of each of the ω-7 fatty acids ($16{:}1\Delta^9$, $18{:}1\Delta^{11}$, and $20{:}1\Delta^{13}$) was reduced by 40% or more (Fig. 4A). These data suggest that *AAD3* plays a major role in ω-7 fatty acids synthesis in the aleurone, and it is probable that

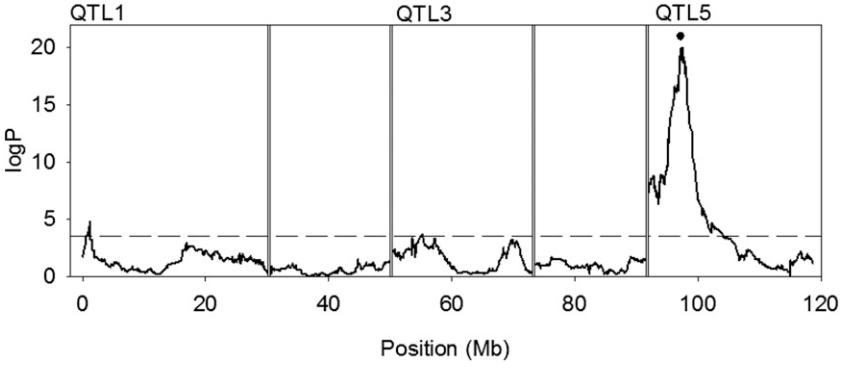


Figure 2. QTL analysis of total ω-7 fatty acid content of whole seeds from the Arabidopsis MAGIC population. Scan of logP value (-log10 ANOVA *P* value) against genomic position. Dotted line marks genome-wide significance threshold of 3 (Kover et al., 2009). The location of *AAD3* and *AAD1* is marked with a dot.

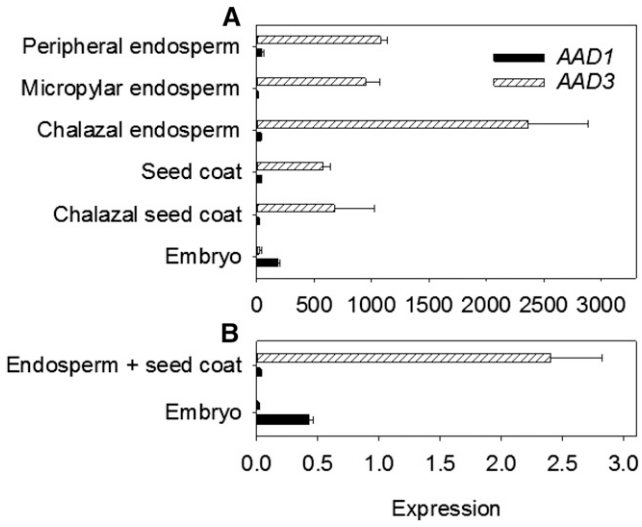


Figure 3. Patterns of expression of *AAD3* and *AAD1* in tissues of developing Arabidopsis seeds. A, Microarray data reproduced from the microdissection study of Le et al. (2010). B, Quantitative RT-PCR analysis. Values are the mean ± SE of measurements on seed from four separate plants.

these *ω*-7 fatty acids are created by Δ^9 desaturation of 16:0 followed by subsequent elongation. Kachroo et al. (2007) have previously shown that recombinant AAD3 and AAD1 can both desaturate 18:0-ACP but that AAD3 has an approximately 10-fold higher specific activity on 16:0-ACP than does AAD1. In *aad1* embryos, there was a small but significant ($P < 0.05$) increase in 18:0 and decrease in 18:1Δ^9 detected (Fig. 4B), suggesting that AAD1 plays a minor role in the Δ^9 desaturation of 18:0-ACP, together with SSI2 (Lightner et al., 1994).

SSI2 Is Not Responsible for the Remaining *ω*-7 Fatty Acid Production in *aad3* Aleurone

SSI2 is the major 18:0-ACP Δ^9 desaturase in Arabidopsis (Lightner et al., 1994) and is expressed in both the embryo and aleurone (Le et al., 2010). Kachroo et al. (2007) have shown that the specific activity of recombinant SSI2 on 18:0-ACP is >40-fold greater than that of other AAD family members and that SSI2 also possesses a comparatively low level of activity on 16:0-ACP. To test whether SSI2 might be responsible for the remaining *ω*-7 fatty acid production in the aleurone of *aad3* mutants, we created a double mutant using an *ssi2* T-DNA mutant line in the same genetic background as *aad3* (Col-0). The fatty acid compositions of dissected aleurone plus seed coat of *aad3* and *aad3 ssi2* were compared (Fig. 5). In the *ssi2* mutant background, the aleurone contained much higher levels of 18:0 (and 20:0) than are present in wild type (Fig. 5). This finding is consistent with previous studies on whole *ssi2* seeds (Lightner et al., 1994). However, despite the effect on 18:0, the level of *ω*-7 fatty acids in *aad3 ssi2* aleurone plus seed coat was not significantly different ($P > 0.05$) from that in *aad3* (Fig. 5). These data suggest that SSI2 plays an important role in Δ^9 desaturation of 18:0 in the aleurone, but functions independently of AAD3 and does not contribute significantly to the production of *ω*-7 fatty acids.

AAD2 Accounts for Most of the Remaining *ω*-7 Fatty Acid Production in *aad3* Aleurone

Phylogenetic analysis of the AAD family shows that AAD3 is most closely related to AAD2 (At3g02610) and AAD4 (At3g02620), which are >90% identical at the amino acid level and are also encoded by neighboring genes (Kachroo et al., 2007). Kachroo et al. (2007) have previously demonstrated that recombinant AAD4 has a low specific activity on both 16:0 and 18:0-ACP substrates but did not test AAD2. Interestingly, microarray data suggest that *AAD2* and/or *AAD4* are also expressed almost exclusively in the aleurone at the mature green stage of seed development (Le et al., 2010). To test whether AAD2 or AAD4 might be responsible for the remaining *ω*-7 fatty acid production

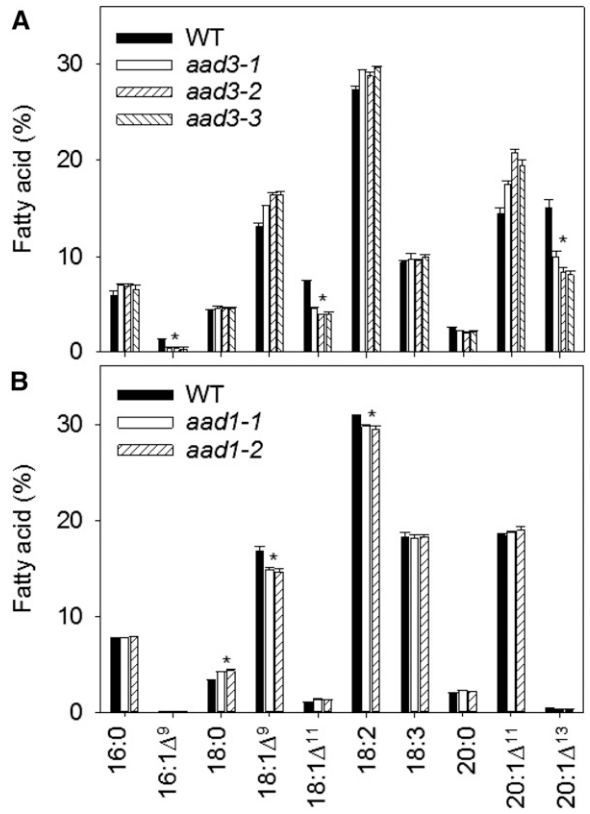


Figure 4. Fatty acid composition of *aad3* and *aad1* mutant seed tissues. A, Aleurone plus seed coat and (B) embryo. Values are the mean ± SE of measurements on seed from four separate plants of each genotype. The asterisk denotes *ω*-7 fatty acid values that are significantly different ($P < 0.05$) from wild type in A and all fatty acids that are significantly different ($P < 0.05$) from wild type in B.

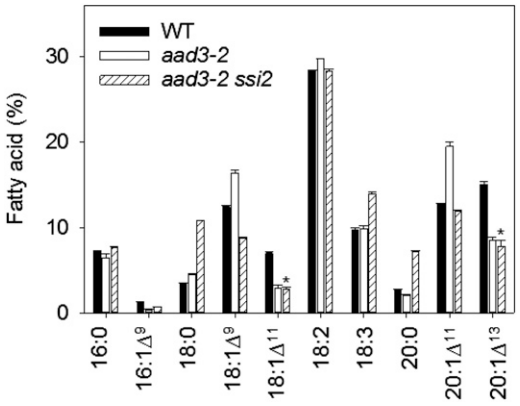


Figure 5. Fatty acid composition of aleurone plus seed coat from *aad3* and *aad3 ssi2* mutant seeds. Values are the mean ± SE of measurements on seed from four separate plants of each genotype. The asterisk denotes *ω*-7 fatty acid values in *aad3-2 ssi2* that are not significantly different ($P > 0.05$) from *aad3-2*.

that occurs in the aleurone of *aad3* mutants, we first isolated multiple homozygous T-DNA mutants in each gene. Analysis of the fatty acid composition of dissected aleurone plus seed coat showed that *aad2* mutants exhibit an approximately 30% reduction in *ω*-7 fatty acid levels, while the levels of *ω*-7 fatty acids in *aad4* mutants are not significantly ($P > 0.05$) changed (Fig. 6A). We then generated an *aad3 aad2* double mutant. Analysis of the fatty acid composition of dissected aleurone plus seed coat showed that the double mutant contains a significantly ($P < 0.05$) lower level of *ω*-7 fatty acids (approximately 3% in total) than either of the single mutants (Fig. 6B). AAD3 and AAD2 therefore account for the majority of *ω*-7 fatty acid production in the aleurone. The *aad3 aad2* seed do not differ in appearance to wild type and can germinate readily under standard laboratory conditions (Supplemental Table S2).

FAE1 Is Responsible for the Elongation of C18 but Not C16 *ω*-7 Fatty Acids in the Aleurone

Our data suggest that AAD3 and AAD2 are the major Δ^9 desaturases responsible for *ω*-7 fatty acid production in the aleurone of Arabidopsis seeds. The depletion of all *ω*-7 fatty acid species in the aleurone of *aad3* and *aad3 aad2* suggests that the majority of the $18{:}1\Delta^{11}$ and $20{:}1\Delta^{13}$ is produced by elongation of $16{:}1\Delta^9$. In Arabidopsis seeds, the elongation of $18{:}1\Delta^9$ to produce the abundant VLCFA eicosenoic acid requires the microsomal 3-ketoacyl-CoA synthase FATTY ACID ELONGASE1 (James et al., 1995; Millar and Kunst, 1997). In contrast, the elongation of 16:0 to 18:0 is performed by plastidic *β*-ketoacyl-ACP synthase 2 (KASII/FAB1; Wu et al., 1994; Nguyen et al., 2010). To investigate the elongation of *ω*-7 fatty acids in the aleurone, we performed fatty acid analysis on the dissected aleurone plus seed coat of a *fae1* and a *fae1 fab1-1* mutant (James et al., 1995; Nguyen et al., 2010). Disruption of *FAE1* almost completely eliminated the production of $20{:}1\Delta^{13}$ but led to the accumulation of $18{:}1\Delta^{11}$ (Fig. 7). These data suggest that FAE1 is required for the elongation of $18{:}1\Delta^{11}$, as well as $18{:}1\Delta^9$, but that elongation of $16{:}1\Delta^9$ in the aleurone relies on an alternative mechanism that is highly active. In *fae1 fab1-1* aleurone more $16{:}1\Delta^9$ accumulated, but the abundance of $18{:}1\Delta^{11}$ also increased, suggesting that FAB1 is also unlikely to play a significant role in $16{:}1\Delta^9$ elongation.

Aleurone *ω*-7 Fatty Acid Content also Varies between Rapeseed Varieties

Li et al. (2006) reported that the aleurone of the spring high-erucic acid rapeseed variety 'Reston' contains approximately 35 mol% *ω*-7 fatty acids. Since we detected significant variation in *ω*-7 fatty acid content between Arabidopsis accessions (Fig. 1), we also carried out a preliminary screen of 10 rapeseed accessions from a fixed diversity set (Table I). This subset included representative spring and winter rapeseed varieties as well as forage rape, swede, and kale. Among the accessions were also high-erucic, high-oleic, and high-oleic

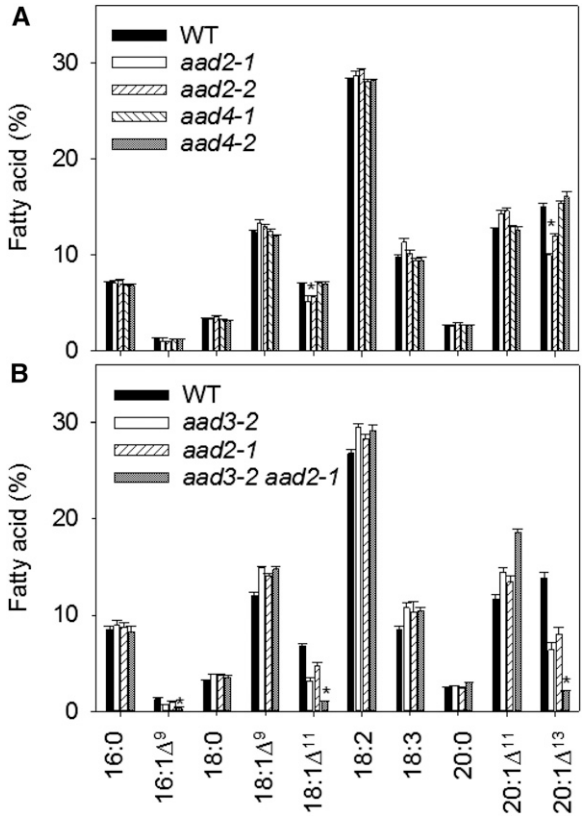


Figure 6. Fatty acid composition of aleurone plus seed coat from *aad2* and *aad4* seeds. A, Single mutants and B, *aad3 aad2* double mutant seeds. Values are the mean ± SE of measurements on seed from two to four separate plants of each genotype. The asterisk denotes *ω*-7 fatty acid values that are significantly different ($P < 0.05$) from wild type in A and single mutants in B.

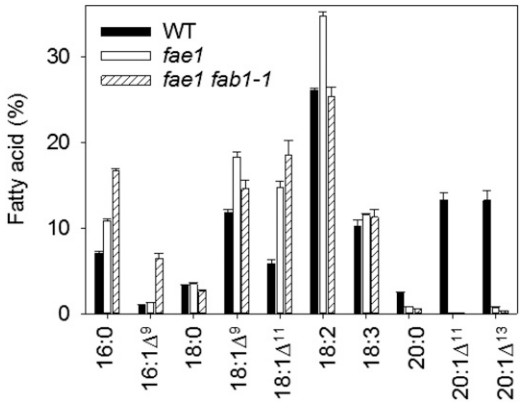


Figure 7. Fatty acid composition of aleurone plus seed coat from *fae1* and *fae1 fab1-1* mutant seeds. Values are the mean ± SE of measurements on seed from four separate plants of each genotype.

low-linolenic varieties. The total *ω*-7 fatty acid content of the aleurone varied between approximately 31% and approximately 47%. As in the Arabidopsis *fae*1 mutant background, accessions that were deficient in VLCFA synthesis did not produce VLC *ω*-7 fatty acids and instead accumulated 18:1Δ^{11}. However, unlike Arabidopsis, levels of 18:1Δ^{11} greatly surpass those of 18:1Δ^{9} in some accessions. The variety with the highest total *ω*-7 fatty acid content (Huguenot) contained approximately 43% 18:1Δ^{11} and only approximately 6% 18:1Δ^{9}.

DISCUSSION

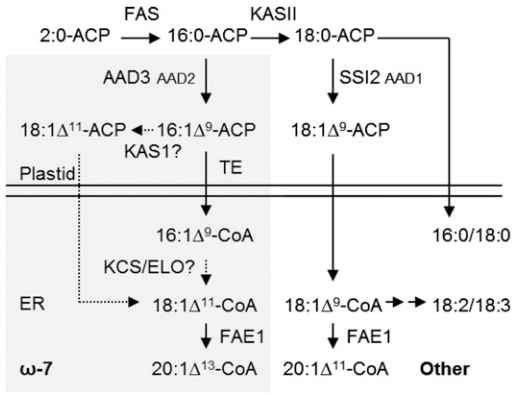
In this study, we found that significant natural variation exists in the *ω*-7 fatty acid content of Arabidopsis seed aleurone, and we used a MAGIC RI population, created by Kover et al. (2009), to map a major QTL responsible for this trait to an approximately 0.8-Mb region of chromosome 5 containing the acyl-ACP Δ^9 desaturases *AAD1* and *AAD3*. Gene expression analysis revealed that *AAD3* is restricted to the aleurone and seed coat during seed development, while *AAD1* is preferentially expressed in the embryo. Multiple homozygous T-DNA mutants were isolated in both genes. The *aad3* mutants showed an approximately 50% reduction in the quantity of *ω*-7 fatty acids present in the aleurone. AAD3 therefore plays a major role in *ω*-7 fatty acid production in these tissues. Furthermore, allelic variation within the MAGIC population at this locus could potentially explain the QTL. Kachroo et al. (2007) have previously shown that AAD3 has a relatively high specific activity on 16:0-ACP compared to other AAD family members. Given that *aad3* mutants are depleted in 16:1Δ^{9}, as well as the more abundant longer chain *ω*-7 fatty acids, it is probable that the pathway proceeds by Δ^9 desaturation of 16:0 followed by elongation (Fig. 8), as has been proposed from previous biochemical and molecular studies on other seed tissues that accumulate this class of fatty acids (Cahoon et al., 1997, 1998). It should be noted that AAD3 still retains activity on 18:0-ACP (Kachroo et al., 2007), and additional adaptations in the aleurone may serve to enhance *ω*-7 fatty acid production. For example, a reduction in KASII/FAB1 activity (Fig. 8) would likely increase the abundance of 16:0-ACP in the chloroplast and reduce that of 18:0-ACP, thereby favoring 16:0-ACP desaturation (Nguyen et al., 2010, 2015).

The major species of *ω*-7 fatty acids present in the Arabidopsis seed aleurone are 18:1Δ^{11} and 20:1Δ^{13} (Penfield et al., 2004; Li et al., 2006), which can be formed by elongation of 16:1Δ^{9}. We found that disruption of the seed-specific 3-ketoacyl-CoA synthase (KCS) gene *FAE1* (James et al., 1995; Millar and Kunst, 1997) blocked 20:1Δ^{13} production in the aleurone but led to an accumulation of 18:1Δ^{11}. 18:1Δ^{11} elongation therefore requires FAE1 but 16:1Δ^{9} elongation does not (Fig. 8). Nguyen et al. (2010) also found that when a 16:0-ACP Δ^9 desaturase was overexpressed in a *fae1* background, 18:1Δ^{11} accumulated in the seeds. The mechanism responsible for 16:1Δ^{9} elongation is not known (Fig. 8). Mutant analysis suggests that it does not rely on KASII (FAB1), since in a *fab1-1* background 18:1Δ^{11} still accumulates. However, it is conceivable that KASI elongates 16:1Δ^{9}-ACP (Mekhedov et al., 2000). Heilmann et al. (2004b) have also shown that overexpression of several ADS enzymes, which are located outside the plastid, can lead to 18:1Δ^{11} accumulation in Arabidopsis seeds (Heilmann et al., 2004b). 16:1Δ^{9}-CoA elongation could employ another member of the Arabidopsis *KCS* gene family that consists of

Table I. *Fatty acid composition of aleurone plus seed coat from 10 inbred rapeseed varieties*

The classes of *ω*-7 fatty acids are highlighted in bold. Varieties: HEAR, high-erucic acid; HO, high oleic acid, HOLL, high oleic acid low linolenic acid; SB, Siberische Boerenkool; S, spring; W, winter. Values are the mean ± SD of measurements on seed from three separate plants of each genotype.

Variety	16:0	16:1Δ^{9}	18:0	18:1Δ^{9}	18:1Δ^{11}	18:2	18:3	20:0	20:1Δ^{11}	20:1Δ^{13}	22:1Δ^{13}	22:1Δ^{15}
Westar (SHO)	8.2 ± 0.1	**2.65 ± 0.1**	1.9 ± 0.2	17.7 ± 0.6	**31.9 ± 0.6**	29.8 ± 0.6	6.9 ± 0.3					
Bristol (WHO)	9.2 ± 0.6	**3.4 ± 0.4**	2.3 ± 0.2	13.5 ± 3.4	**33.5 ± 2.6**	29.2 ± 1.3	8.5 ± 0.8					
Sansibar (WHO)	9.6 ± 0.6	**3.6 ± 0.2**	2.2 ± 0.2	8.8 ± 2.7	**35.6 ± 1.4**	27.9 ± 3.3	7.3 ± 0.7					
E94197 (UHO)	8.9 ± 1.0	**3.6 ± 0.2**	2.3 ± 0.3	11.1 ± 0.9	**35.1 ± 1.9**	29.8 ± 0.6	6.9 ± 0.3					
Cabriolet (WHOLL)	7.9 ± 0.3	**4.3 ± 0.2**	2.5 ± 0.2	22.0 ± 1.2	**33.2 ± 0.9**	20.2 ± 0.4	8.4 ± 0.3					
Huguenot (Swede)	9.3 ± 0.5	**4.2 ± 0.2**	2.2 ± 0.3	6.5 ± 0.5	**43.4 ± 1.2**	24.5 ± 0.8	8.5 ± 0.4					
Kroko (SHEAR)	5.1 ± 0.9	**1.5 ± 0.2**	1.5 ± 0.1	5.8 ± 0.7	**8.0 ± 1.1**	20.5 ± 0.8	5.4 ± 0.3	0.9 ± 0.1	2.5 ± 0.3	**18.3 ± 0.2**	14.1 ± 0.9	**11.5 ± 1.0**
Major (WHEAR)	5.8 ± 1.3	**1.4 ± 0.3**	1.4 ± 0.1	6.5 ± 1.4	**5.9 ± 2.0**	20.5 ± 2.1	6.7 ± 0.5	0.8 ± 0.2	2.6 ± 0.5	**14.2 ± 0.3**	20.0 ± 4.3	**9.7 ± 2.3**
SB (Kale)	7.4 ± 0.2	**3.0 ± 0.2**	2.3 ± 0.2	6.9 ± 2.0	**16.1 ± 1.1**	22.2 ± 1.2	8.8 ± 0.2	0.5 ± 0.1	2.7 ± 0.3	**15.1 ± 0.7**	6.5 ± 0.9	**6.6 ± 1.2**
Dwarf Essex (Forage)	7.1 ± 0.6	**2.8 ± 0.9**	2.2 ± 0.2	4.8 ± 0.4	**4.2 ± 0.8**	19.0 ± 0.5	9.1 ± 0.2	0.8 ± 0.1	0.9 ± 0.1	**17.2 ± 0.3**	14.3 ± 1.8	**15.3 ± 2.0**



Fatty acids	ω-7	Other	Total in seed
Embryo	1-2%	98-99%	85-87%
Aleurone	13-27%	73-87%	13-15%

Figure 8. Cartoon depicting the pathways for fatty acid monounsaturation and elongation in the aleurone and the embryo of Arabidopsis seeds. The gray box highlights the *ω*-7 fatty acid species whose production in the aleurone depends on *AAD3* and *AAD2* expression in this tissue. Dotted arrows denote theoretical routes for elongation of 16:1Δ^9 to 18:1Δ^{11}. The table provides a summary of the range in fatty acid distribution between embryo and aleurone measured in the 19 parental accessions of the Arabidopsis MAGIC population. FAS, fatty acid synthase; ELO, elongation-defective; FAE1, FATTY ACID ELONGASE1; TE, acyl-ACP thioesterase.

21 members, or alternatively it might utilize a homolog of the yeast ELO (elongation-defective) family (Paul et al., 2006), of which there are four members in Arabidopsis.

The major 18:0-ACP Δ^9 desaturase in Arabidopsis is SSI2, which is expressed in all seed tissues (Lightner et al., 1994). Although the fatty acid composition of *aad1* aleurones is unchanged, the embryos exhibit a small mol% increase in 18:0 and decrease in 18:1Δ^9. These data suggest that AAD1 makes a minor contribution to 18:0-ACP Δ^9 desaturation (Fig. 8). Recombinant AAD1 is much less active on 18:0-ACP than SSI2, but it has been shown that this gene is able to complement the *ssi2* mutants when expressed under a strong promoter (Kachroo et al., 2007). SSI2 is also active on 16:0-ACP (Kachroo et al., 2007), and therefore it was possible that it could account for the residual *ω*-7 fatty acid production observed in the aleurone of *aad3* mutants. However, analysis of an *aad3 ssi2* double mutant suggests that this is not the case. Although the specific activity of SSI2 on 16:0-ACP is greater than that of AAD3, SSI2 is >40-fold more active on 18:0-ACP than other AAD family members (Kachroo et al., 2007). Therefore, the abundance of SSI2 need not be very high to account for the in vivo rate of 18:0-ACP Δ^9 desaturation.

Phylogenetic analysis of the AAD protein family in Arabidopsis has shown that AAD3 is most closely related to AAD2 and AAD4 (Kachroo et al., 2007). These two neighboring genes encode proteins with >90% amino acid sequence identity and are most likely to result from a recent duplication event. Analysis of multiple homozygous T-DNA mutants in both genes revealed that AAD2 also contributes to *ω*-7 fatty acid production in the aleurone. Furthermore, an *aad3 aad2* double mutant contains only approximately 3% *ω*-7 fatty acids. The molecular basis of AAD substrate specificity has previously been studied using the crystal structure of castor (*Ricinus communis*) 18:0-ACP Δ^9 desaturase as a guide (Cahoon et al., 1997, 1998). A polypeptide sequence alignment of the Arabidopsis AAD family (Kachroo et al., 2007) indicates that AAD3 and AAD2 contain several amino acid substitutions compared to the castor isoform (M147T, P212S, T214F) that lie near the bottom of the substrate-binding pocket (Cahoon et al., 1998) and might therefore be responsible for the altered chain length specificity reported by Kachroo et al. (2007). Microarray data suggest that *AAD2* (and possibly *AAD4*) is also expressed specifically in the aleurone, but the spatial pattern differs from that of *AAD3* (Le et al., 2010). Whereas *AAD3* expression is greatest in the chalazal region of the endosperm, *AAD2* is most strongly expressed in the peripheral endosperm (Le et al., 2010). The reason why *ω*-7 fatty acid production occurs in seeds is not clear, but the fact that AAD proteins with divergent substrate specificities have evolved implies that they likely provide a competitive advantage (Shanklin and Cahoon, 1998). In the case of Arabidopsis, the precise spatial control of expression within the seed aleurone also suggests that there may be a specific role in this tissue. What this role is remains to be determined. However, seeds from the *aad3 aad2* double mutant appear normal and can germinate readily in standard laboratory conditions.

Finally, Li et al. (2006) reported that the aleurone of the spring high-erucic variety 'Reston' contains approximately 35 mol% *ω*-7 fatty acids. Given the relatively close phylogenetic relationship with Arabidopsis, it is probable that homologs of AAD3 are also responsible for *ω*-7 fatty acid production in rapeseed. To investigate whether significant variation exists in *ω*-7 fatty acids, we performed a preliminary screen of 10 rapeseed varieties. The highest level of *ω*-7 fatty acids we detected was approximately 47% in the Swede (Rutabaga) variety 'Huguenot.' There are a number of industrial applications for *ω*-7 fatty acids, ranging from polymer precursor production and "greener" biodiesel formulations to nutraceuticals (Nguyen et al., 2010, 2015). Tremendous progress has recently been made in genetically engineering Arabidopsis and *Camelina sativa* seeds to produce *ω*-7 fatty acids, and levels >60% have been achieved (Nguyen et al., 2010, 2015). However, it is interesting to consider that oilseed processors have repeatedly explored dehulling rapeseed before oil extraction as a means to improve the quality of the meal for livestock nutrition (Carré et al., 2015). It is possible that *ω*-7 fatty acids could be extracted from the hulls (which include the aleurone) as a coproduct of the process, thereby improving the overall economics of the crop. Only approximately 5% of the fatty acids in rapeseed are stored in the aleurone plus seed coat (Li et al., 2006), but it is possible to estimate that the current scale of cultivation (http://www.fas.usda.gov/commodities/oilseeds) would be sufficient to supply approximately 0.5 million MT of *ω*-7 fatty acids.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type (Col-0) Arabidopsis (*Arabidopsis thaliana*) seeds, the MAGIC population parental accessions and RI lines (N782242), and the T-DNA mutant lines *ssi2* (N536854), *aad1-1* (N666506), *aad2-1* (N664541), *aad2-2* (N672749), *aad3-1* (N679396), *aad3-2* (N637538), *aad3-3* (N686171), *aad4-1* (N658009), and *aad4-2* (N657947) were obtained from the European Arabidopsis Stock Centre (University of Nottingham, UK). The seeds were surface sterilized, plated on agar plates containing one-half strength Murashige and Skoog salts (Sigma-Aldrich), and imbibed in the dark for 4 d at 4°C. The plates were then placed in a growth chamber set to 16-h light (23°C)/8-h dark (18°C); photosynthetic photon flux density = 250 μmol m^{-2} s^{-1}; 70% relative humidity. After 5 d, seedlings were transplanted to moist Levingtons F2 compost in 7-cm^2 pots. Mutant lines were genotyped by performing PCR on genomic DNA with combinations of flanking gene-specific primers and T-DNA boarder primers as described on the SIGnAL T-DNA Web page (http://signal.salk.edu/tdnaprimers.2.html). The gene-specific primers used are listed in Supplemental Table S3, and the insertion sites are marked in Supplemental Figure S1. For the MAGIC parental accessions and RI lines, the seedlings were vernalized for 6 weeks at 4°C before being placed in a glasshouse with 16-h supplemental light. The plants were arranged into a one-way randomized design with one plant of each genotype per block and randomized within each block. When plants began to flower, a 60-cm stick was inserted in each pot and the pot was placed inside an upright, rectangular, transparent, perforated plastic bag (60 cm × 6 cm). The bag was sealed around the lower stem prior to seed shedding to ensure that the seeds from each plant were retained.

Seed Fatty Acid Analysis

Fatty acid composition was determined by gas chromatographic analysis of fatty acid methyl esters following direct transmethylation of seed tissues according to the method of Browse et al. (1985). The aleurone (plus seed coat) was separated from the embryo using the protocol described by Penfield et al., (2004). Briefly, the seeds were imbibed in water for 1 h and placed on a glass slide under a dissecting microscope. The seed coats were then ruptured individually using a needle and the embryos squeezed out with forceps.

QTL and Statistical Analyses

QTL mapping with the MAGIC population was performed as described by Kover et al. (2009) using the HAPPY R package available from http://mus.well.ox.ac.uk/magic/. The number of replicates (n) and the SE of the mean are shown for most measurements. One-way ANOVA was used to assess the differences between genotypes for measurements of fatty acids. Following significant ($P < 0.05$) F-test results, means of interest were compared using the appropriate LSD value at the 5% ($P = 0.05$) level of significance, on the corresponding degrees of freedom. The GenStat (2011, 14th edition, VSN International Ltd, Hemel Hempstead, UK) statistical system was used for these analyses.

Transcript Analysis

DNAse-treated total RNA was isolated from dissected embryos and aleurone plus seed coat of 'stage 6 to 9' developing Arabidopsis seeds (Winter et al., 2007) using the RNeasy kit from Qiagen Ltd with modifications described by Eastmond (2006). The synthesis of single-stranded cDNA was carried out using SuperScriptTM II RNase H- reverse transcriptase from Invitrogen Ltd. Quantitative real-time PCR was performed as described in Rajangam et al. (2013). The primer pairs used for real-time PCR were AAD1 (5′-AGATCTCGATGCCTGCTCAT-3′ plus 5′-TGGCAGTGTAGACACCAAGC-3′), AAD3 (5′-GGATCTGTTCAGGCGTTAGC-3′ plus 5′-GGCATTGT-GATCCGTTTCTT-3′), and Q18S (5′-TCCTAGTAAGCGCGAGTCATC-3′ plus 5′-CGAACACTTCACCGGATCAT-3′).

Accession Numbers

Sequence data for the genes described in this article can be found in the GenBank/EMBL data libraries using the Arabidopsis gene identifiers At2g43710 (*SSI2*), At5g16240 (*AAD1*), At3g02610 (*AAD2*), At5g16230 (*AAD3*), and At3g02620 (*AAD4*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Cartoon of T-DNA insertion sites in *AAD* family genes confirmed by PCR and sequencing.

Supplemental Table S1. Summary of data for *ω-7* fatty acid QTL derived from analysis of the MAGIC population using the 'HAPPY R' package.

Supplemental Table S2. Germination of *aad* mutants.

Supplemental Table S3. Primers used for genotyping.

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