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Research Article

Biotin attachment domain-containing proteins mediate hydroxy fatty acid-dependent inhibition of acetyl CoA carboxylase

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Abstract

Hundreds of naturally occurring specialized fatty acids (FAs) have potential as desirable chemical feedstocks if they could be produced at large scale by crop plants; however, transgenic expression of their biosynthetic genes has generally been accompanied by dramatic reductions in oil yield. For example, expression of castor (*Ricinus communis*) FA hydroxylase (FAH) in the *Arabidopsis thaliana* FA elongation mutant *fae1* resulted in a 50% reduction of FA synthesis rate that was attributed to inhibition of acetyl-CoA carboxylase (ACCase) by an undefined mechanism. Here, we tested the hypothesis that the ricinoleic acid-dependent decrease in ACCase activity is mediated by biotin attachment domain-containing (BADC) proteins. BADCs are inactive homologs of biotin carboxy carrier protein that lack a biotin cofactor and can inhibit ACCase. Arabidopsis contains three BADC genes. To reduce expression levels of *BADC1* and *BADC3* in *fae1*/FAH plants, a homozygous *badc1,3/fae1*/FAH line was created. The rate of FA synthesis in *badc1,3/fae1*/FAH seeds doubled relative to *fae1*/FAH, restoring it to *fae1* levels, increasing both native FA and HFA accumulation. Total FA per seed, seed oil content, and seed yield per plant all increased in *badc1,3/fae1*/FAH, to 5.8 µg, 37%, and 162 mg, respectively, relative to 4.9 µg, 33%, and 126 mg, respectively, for *fae1*/FAH. Transcript levels of FA synthesis-related genes, including those encoding ACCase subunits, did not significantly differ between *badc1,3/fae1*/FAH and *fae1*/FAH. These results demonstrate that BADC1 and BADC3 mediate ricinoleic acid-dependent inhibition of FA synthesis. We propose that BADC-mediated FAS inhibition as a general mechanism that limits FA accumulation in specialized FA-accumulating seeds.

Disrupting two genes encoding biotin attachment domain containing proteins relieves hydroxy fatty acid-dependent inhibition of acetyl CoA carboxylase, thereby increasing hydroxy fatty acid yield.

Introduction

A longstanding crop improvement goal has been to exploit our knowledge of specialized fatty acid (FA) synthesis from

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plants and microbes by reconstructing their synthetic pathways in crop plants (Napier, 2007). If successful, this would allow the production of chiral FA feedstocks in an inexpensive and scalable manner. However, a major barrier to progress in this area was the discovery that seed oil yields are significantly decreased upon the accumulation of specialized FAs (Cahoon et al., 2007; Haslam et al., 2013; Vanhercke et al., 2013; Bates et al., 2014). Perhaps the most intensively studied example of this comes from attempts to increase the accumulation of hydroxy fatty acid (HFA) in seed oils, of which much of the work has been performed in the model system Arabidopsis (Lu et al., 2006).

HFAs contain one or more hydroxy group(s) on a FA backbone, which confers beneficial properties such as higher viscosity and chemical reactivity. The hydroxyl group of HFAs makes them useful chemical feedstocks for the production of a wide range of industrial products, including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives for coatings and paints (Kim et al., 2000). Moreover, HFAs could be used as intermediates in the production of biodegradable plastics, cyclic lactones, and pharmaceuticals (Wang et al., 2012). Industrial use of HFAs is limited by their availability from natural sources such as the beans of castor (*Ricinus communis*). Isolation of the oleate hydroxylase fatty acid hydroxylase (FAH) from castor over two decades ago raised the possibility of ricinoleic acid production in high-yielding oil crops (van de Loo et al., 1995). However, in contrast to castor that accumulates approximately 90% of its FA as ricinoleic acid, transgenic Arabidopsis *fatty acid elongation1* (*fae1*) mutant expressing castor FAH, i.e., *fae1*/FAH, (a line designated CL37) accumulated only 17% HFA in its total seed oil (Lu et al., 2006). The seeds of *fae1*/FAH also displayed many physiological deficits including reduced oil content and seed weight, low seed yield per plant compared with its parental *fae1* line, and delayed seed germination (Adhikari et al., 2016).

Investigation of the reduced oil content of *fae1*/FAH revealed its FA synthesis rate was reduced compared to the parental *fae1* line (Bates et al., 2014). Whereas the molecular basis for this reduction in FA synthesis has not been reported, several attempts at overcoming it have proved at least partially successful, such as overexpressing a master transcriptional regulator of FA synthesis WRINKLED1 (Adhikari et al., 2016) or a lipid droplet associated factor SEIPIN1 to increase lipid droplet size (Lunn et al., 2018). Development defects of HFA-accumulating seeds are partially mitigated upon the expression of several castor acyltransferases (Lunn et al., 2018). Stacking the expression of several castor acyltransferases, including GPAT9, LPAT2, and PDAT1A, along with the castor hydroxylase *fae1*/FAH seeds produced abundant tri-HFA TAG, restored seed oil content, and partially restored seedling establishment (Lunn et al., 2019). The expression of phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT), encoded by the *REDUCED OLEATE DESATURATION1* (*ROD1*) gene (Lu et al., 2009) and that channels about 40% of the flux of polyunsaturated fatty from PC into DAG for TAG synthesis, was found to potentiate efficient accumulation of HFA in Arabidopsis (Hu et al., 2012).

In dicotyledonous plants, heteromeric acetyl-CoA carboxylase (ACCase) catalyzes the first committed step of de novo FA biosynthesis. This enzyme complex consists of four catalytic subunits: biotin carboxylase (BC), carboxyltransferase (CT)-α, CT-β, and biotin carboxyl carrier protein (BCCP; Salie et al., 2016). The two BCCP isoforms (BCCP1 and BCCP2) of Arabidopsis ACCase can interact with Biotin/lipoyl attachment domain-containing (BADC) proteins (Feria Bourrellier et al., 2010). BADCs are BCCP homologs that contain a biotin attachment motif, but that critically lack a biotinylation site. BADC proteins can act as negative regulators of ACCase due to their lack of the biotin adduct required for carboxylation (Salie et al., 2016), and a role for them in ACCase assembly was recently proposed. These proteins have been reported to significantly inhibit ACCase activity in both *Escherichia coli* and Arabidopsis (Salie et al., 2016), and it was recently proposed that they can sense pH changes (Ye et al., 2020). An additional role for BADCs in ACCase assembly has also been proposed (Shivaiah et al., 2020).

Three BADC genes have been identified in Arabidopsis; the single Arabidopsis knock-out mutants *badc1*, *badc2*, and *badc3* do not exhibit significant changes in oil content relative to wild-type plants (Keereetaweep et al., 2018), whereas the *badc1badc3* (*badc1,3*) double mutant shows an increased FA synthesis rate and a remarkable 25% increase in seed oil content (Keereetaweep et al., 2018).

In this context, *badc1,3/fae1*/FAH homozygous plant was generated in this study by crossing the *badc1,3* double mutant with CL37, an Arabidopsis *fae1* line expressing FAH (Lu et al., 2006). Downregulation of BADC1 and BADC3 in *fae1*/FAH doubled the rate of FA synthesis in developing seeds, restoring it to *fae1* levels, and increased both native FA and HFA accumulation.

Results

Generation of *badc1,3/fae1/FAH* plants

To test the hypothesis that HFA-induced inhibition of FA synthesis results from BADC-dependent inhibition of ACCase, we crossed the *badc1,3* double mutant with CL37, a single-insertion homozygous FAH transgenic line in a homozygous mutant FA elongase1 (*fae1*) background (Kunst et al., 1992), the seeds of which are reported to contain 17% HFA (Lu et al., 2006). The level of 18:1, the FAH substrate, is only 13% of TFA in wild type Columbia. Therefore, *fae1*, which contains much higher levels (33%) of 18:1 in its seed oil, was used. Seeds resulting from this cross were germinated and genetically screened to identify heterozygous *badc1,3/fae1*/FAH plants. F2 seeds from the heterozygous *badc1,3/fae1*/FAH plants were planted to screen for homozygous plants which were used for the following studies. The *fae1* mutant (Kunst et al., 1992) and the *badc1* and *badc3* T-DNA insertion lines (Bohannon and Kleiman, 1978; Bolle et al., 2013) were all in the Arabidopsis Columbia-0 background.

To screen for *fae1* homozygous individuals, we first needed to determine the genetic lesion underlying the *fae1* mutant. To do this, we amplified the *fae1* open reading frame from CL37 and sequenced it. We identified a mutation encoding a premature termination at 1,395 bp (TGG1393TGA) in the *fae1* mutant allele (Supplemental Figure S1). We next designed primers to introduce a HinfI restriction site in the PCR amplification product of the *fae1* allele around the mutation site. Subsequent restriction digestion with HinfI of a 240-bp PCR fragment produced two fragments of 200 bp and 40 bp in the *fae1* mutant, and only a single 240-bp fragment in the wide type. Whereas the 40-bp fragment was weakly detectable on our gel system, the *fae1* mutant displayed the 200-bp fragment which could be distinguished from the wild type fragment that is characterized by the larger 240-bp band (Figure 1).

The genotypes of *badc1* or *badc3* were determined using gene-specific primer pairs in combination with a T-DNA specific primer. After screening more than 500 plants, five *badc1,3/fae1* homozygous plants carrying the *FAH* gene were identified. GC/MS analysis of 20 individual seeds for HFA accumulation from each of the five *badc1,3/fae1* homozygous lines was used to identify FAH-expressing homozygous lines characterized by the accumulation of HFA in all 20 seeds (Supplemental Figure S2). Finally, we identified two *badc1,3/fae1*/FAH homozygous individuals.

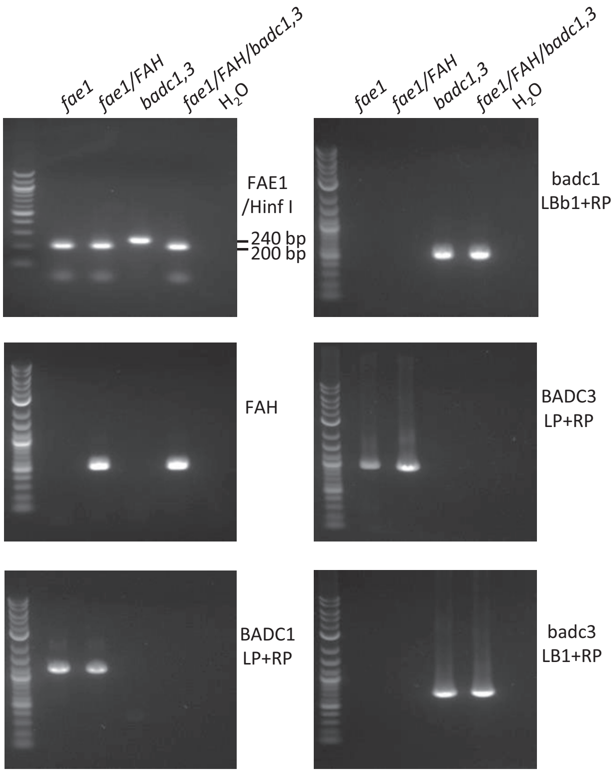

Figure 1 Genotyping of *badc1,3/fae1*/FAH. Individual plants were genotyped to be *fae1* homozygous via HinfI digestion of a *FAE1* PCR fragment; *badc1* or *badc3* homozygous plants were verified using PCR with the indicated gene-specific primer pairs and combinations with T-DNA-specific primer LBb1.

Knocking out *BADC1* and *BADC3* did not change FAH transcription

To assess whether *badc1,3/fae1*/FAH plants were null mutants for *BADC1* (AT3G56130) and *BADC3* (AT3G15690), we harvested developing seeds from siliques 11–13 d after flowering (DAF), and for comparison from *fae1*, *fae1*/FAH, and *badc1,3* seeds grown in parallel. Reverse transcription-quantitative PCR (RT-qPCR) analysis of total RNA extracted from developing seeds confirmed that both *BADC1* and *BADC3* transcription were dramatically decreased in *badc1,3/fae1*/FAH and the *badc1,3* double mutant (Figure 2A and B). To evaluate whether knocking out *BADC1,3* affects FAH expression, we also quantified FAH transcription. As shown in Figure 2C, FAH transcription showed no significant change between *badc1,3/fae1*/FAH and *fae1*/FAH seeds, showing that knocking out *BADC1* and *BADC3* genes did not significantly affect FAH expression (Figure 2C).

Disruption of *BADC1* and *BADC3* did not significantly alter transcript levels of other FA synthesis genes

To investigate whether disrupting *BADC1* and *BADC3* expression affects the transcription of FA synthetic genes, the expression levels of several genes involved in the FA biosynthetic pathway were quantified by RT-qPCR. Using relative expression (REST)-specific analysis (Pfaffl et al., 2002) designed for comparing qPCR data, no significant changes in transcript abundance were observed for ACCase subunit-encoding genes, including those encoding BCCP1 (AT5G16390), BCCP2 (AT5G15530), ACCASE BIOTIN CARBOXYLASE (BC, AT5G35360), α-CT (AT2G38040), and β-CT (ATCG00500), and the two key enzymes in FA synthesis 3-KETOACYL ACP SYNTHASE I (KASI; AT5G46290) and KASIII (AT1G62640; Maeo et al., 2009; To et al., 2012; Supplemental Figure S3). WRI1 was previously shown to regulate a number of FA synthesis genes (Maeo et al., 2009) and all three *BADC* genes (Liu et al., 2019). Analysis of *WRI1* from the same materials showed no significant changes in *WRI1* transcript levels (Supplemental Figure S3). The observation that the levels of transcripts corresponding to these genes were not significantly different from controls suggests that the alleviation of FA synthesis inhibition is not the result of increased transcription of other FA synthesis genes.

Badc1,3/fae1/FAH plants exhibited increased FA content and seed yield

FA content in seeds was quantified to determine if *badc1,3* alleviated the feedback inhibition of FA synthesis in seeds with HFA production. The *fae1* seeds contain 6.00 ± 0.07 μg of total FA, and overexpression of FAH in *fae1* significantly reduced FA to 4.94 ± 0.10 μg per seed. After introduction of *badc1,3*, the FA content of the seeds significantly increases by 16.8% to 5.77 ± 0.04 μg per seed (Figure 3A). Correspondingly, seeds of *fae1* plants yielded 34.3 ± 0.4% oil content, expression of FAH significantly decreased the oil content to 32.7 ± 0.7% and the introduction of *badc1,3*

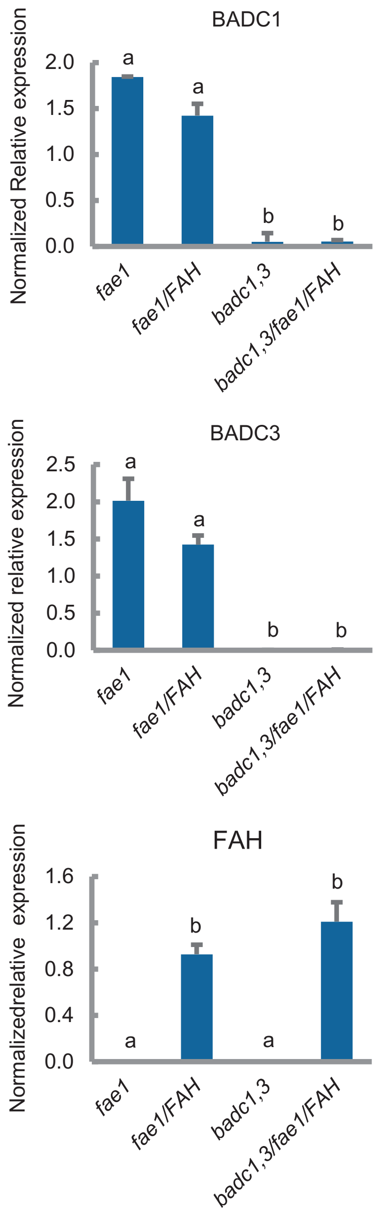

Figure 2 Analysis of *BADC1,3* and *FAH* gene expression in developing seeds. Transcript levels of *BADC1,3* were analyzed by RT-qPCR in 11–13-DAF developing seeds of *fae1*, *fae1*/FAH, *badc1,3/fae1*/FAH, and *badc1,3*, $n = 3$ biological replicates, and error bars represent SD. The relative expression levels are reported relative to the expression of the UBQ10 (At4g05320) transcript. Columns with different letters are significantly different ($P < 0.05$) computed by the relative expression (REST) software algorithm using three biological replicates (Pfaffl et al., 2002).

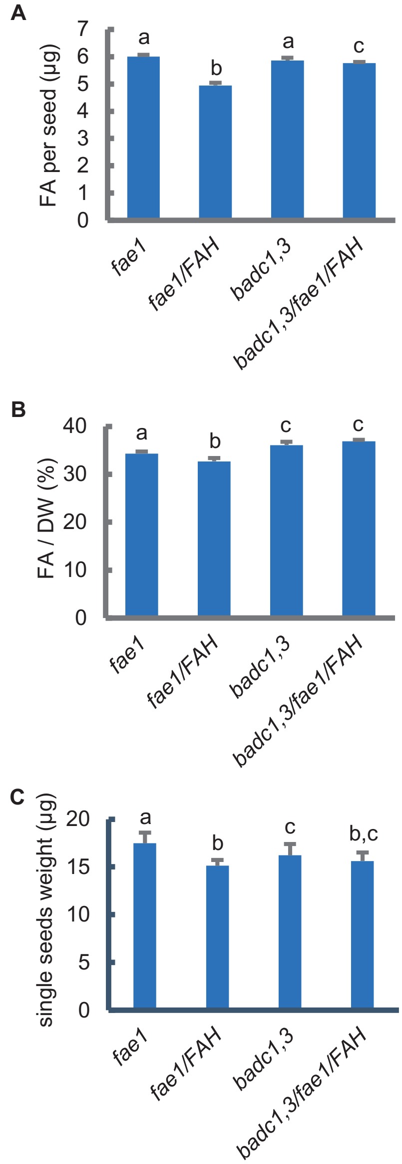

Figure 3 Seed weight and FA content in seeds. A, FA per seed. FA was determined by 5 pooled sets of 100 seeds each. B, Oil content in seeds as a proportion of dry seed weight. Seed oil content, represented by total acyl lipids, was quantified by GC of FA methyl esters. C, Mean weight of transgenic seeds determined by five pooled sets of 100 seeds each. Error bars represent SD. Columns with different letters are significantly different (Student's *t* test; $P < 0.05$; five biological replicates).

increased the oil content to 36.9 ± 0.3% (Figure 3B). The lower oil content in *fae1*/FAH has been reported to reduce seed weight (Adhikari et al., 2016). Indeed, expression of FAH in *fae1* seeds decreased average seed weight from 17.5 ± 1.1 µg to 15.1 ± 0.7 µg (Figure 3C), but the introduction of *badc1,3* did not significantly increase seed weight (15.6 ± 1.0 µg per seed). The small significant differences in FA content and seed yield reported herein can be attributed to differences in *BADC* and *FAE* gene expression, since both of the T-DNA lines (Bolle et al., 2013) and the *fae1* (Kunst et al., 1992) line were created in the Arabidopsis Columbia-0 (Arabidopsis Genome I, 2000) background.

Both HFA and unmodified FA increased in *badc1,3/fae1/FAH*

The *badc1,3* double mutant increased total FA in *badc1,3/fae1*/FAH seeds. To determine whether the increase of FA was specific for either unmodified FAs or HFAs, FAMEs from the respective seed backgrounds were analyzed. HFA in

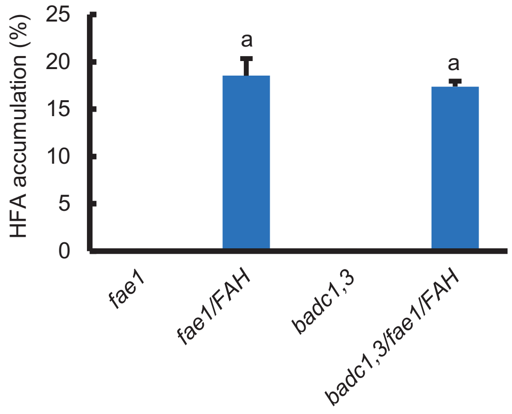

Figure 4 Hydroxy FA content in seeds. HFA is expressed as a weight percentage of the total seed FA. Values represent means ± SD (n = 3 pooled sets of 100 seeds). Student's t test analysis found no significant difference between *fae1*/FAH and *badc1,3/fae1*/FAH ($P > 0.05$).

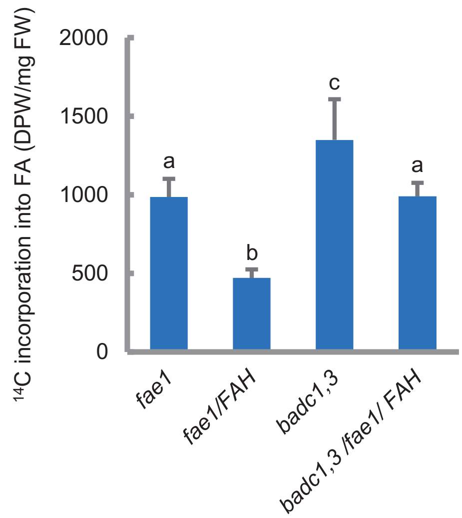

Figure 5 [^{14}C]Acetate incorporation assay in developing seeds. [^{14}C]Acetate incorporation into total lipids showed ACCase activity in 11–13-DAF developing seeds of *fae1*, *badc1,3/fae1*/FAH, and *badc1,3*. Specified different letters indicate significant differences ($P < 0.05$) as determined by Student's t test. Values are presented as means ± SD of three biological replicates.

fae1/FAH and *badc1,3/fae1*/FAH were 18.6 ± 1.8% and 17.4 ± 0.6% of the total FAs, respectively (Figure 4), showing that *badc1,3* did not significantly change the HFA percentage in mature seeds (Student's t test, $P > 0.05$), rather, the increases are in both HFAs and native FAs.

FA synthesis rate is restored in *badc1,3/fae1/FAH*

It was previously reported that the production of HFA in *fae1* seeds expressing FAH was associated with a reduced rate of de novo FA synthesis that resulted in the observed decrease in oil content compared with the *fae1* parental line (Bates et al., 2014). The introduction of *badc1,3* in the *fae1*/FAH line restored the FA content, suggesting that it had alleviated the previously observed inhibition of FA synthesis reported in non-HFA producing lines (Salie et al., 2016; Keereetaweep et al., 2018). To test this hypothesis, mid-phase developing seeds 11–13 DAF were collected and their FA synthesis rates were determined by measuring the rate of [^{14}C]acetate incorporation into FAs by total lipid extraction and scintillation counting. We first validated the assay by showing linear incorporation of [^{14}C]acetate between 20 and 100 min using *badc1,3* seeds (Supplemental Figure S4) and chose 60-min incubations for subsequent experiments. As shown in Figure 5, compared to *fae1*, the *badc1,3* double mutant showed a 36.8% increase in FA synthesis rate, whereas expression of FAH in *fae1* decreased FA synthesis rate by 52.2% with respect to that of *fae1*. When FAH was expressed in *badc1,3/fae1*, the FA synthesis rate was fully restored to that of parental *fae1* seeds.

Seed germination and development

Overexpression of FAH in *fae1* has been reported to decrease seed germination (Adhikari et al., 2016; Lunn et al., 2018; Lunn et al., 2018). To test if the restored FA content in *badc1,3* can mitigate the germination defects, seeds of *badc1,3/fae1*/FAH were tested for germination and seedling establishment relative to the *fae1*/FAH, *badc1,3* parental lines, and *fae1*. Emergence of the radicle was used as a germination marker, and the appearance of roots and green cotyledons was used as a marker for establishment. Germination of *fae1*/FAH lines was reduced to 88% compared with 99% for *fae1* (Figure 6A). The germination rate of *badc1,3/fae1*/FAH was even lower than *fae1*/FAH at 76%. *badc1,3* showed a germination rate of 95%, i.e., similar to that of *fae1*. The seedling establishment rates of *fae1* and *badc1,3* were the same as their germination rates (Figure 6B). 90% of geminated *fae1*/FAH seedlings continued to establishment, whereas 99% of germinated *badc1,3/fae1*/FAH seeds continued to establishment, resulting in similar establishment rates with respect to all seeds for these two genotypes. Comparison of seedling establishment rates at 7 and 10 d showed that combining *badc1,3* with *fae1*/FAH had the effect of reducing germination while increasing seedling establishment (Supplemental Figure S5). Whereas the growth rate of *badc1,3/fae1*/FAH was higher than that of *fae1*/FAH, no visible differences were observed at maturity with respect to plant height and leaf size. However, *fae1* plants produced 158 mg of seeds per plant, which decreased to 126 mg in *fae1*/FAH, whereas the introduction of *badc1,3* in the *fae1*/FAH lines more than compensated, increasing seed yield per plant to 162 mg (Figure 6C). In summary, combining *badc1,3* with *fae1*/FAH improved seedling establishment and restored seed yield.

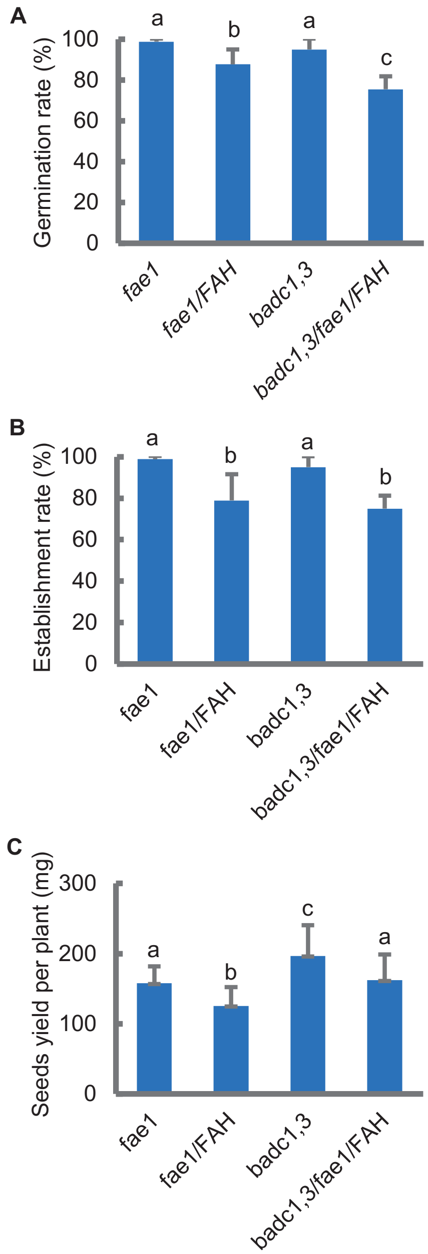

Figure 6 Seed germination and establishment. A total of 180 seeds in five equal replicates from each line were plated in half MS media containing 1% sucrose for 14 d. Germination was scored as seeds that produced a radicle, and seedlings that produced roots and green cotyledons were counted as being able to establish. The germination rates (A) and establishment rates (B) were calculated as the percentage of total seeds plated. Values are presented as means ± SD of five biological replicates. C, Seed yield per plant. $n = 10$, and error bars represent ±SE. Columns with different letters are significantly different ($P < 0.05$, $n = 5$) as determined by Student's *t* test.

Discussion

It was previously reported that the accumulation of HFA in Arabidopsis seeds resulted in feedback inhibition of FA synthesis (Bates et al., 2014), with ACCase activity reduced by ~50% relative to the parental *fae1* line. ACCase is the first committed step in FA synthesis and is often considered rate-limiting. It is therefore under tight genetic and biochemical regulation by a variety of mechanisms (Salie et al., 2016; Ye et al., 2020). In this study, we investigated the effects of null mutations in two negative regulatory subunits of ACCase, i.e., *badc1* and *badc3* in FAH-expressing Arabidopsis seeds with respect to FA synthesis, common FA, and HFA accumulation. The data demonstrate that eliminating BADC1 and BADC3 alleviates the HFA-dependent feedback inhibition of ACCase that results in a doubling FAS rate in *badc1,3/fae1*/FAH seeds restoring them to that of the parental *fae1* line. Seed FA content of *badc1,3/fae1*/FAH was also restored to that of the parental *fae1* line. No significant increases were observed for transcripts corresponding to key FA synthesis-related genes in *badc1,3/fae1*/FAH, which is consistent with the increases being attributed to relief of BADC1- and BADC3-dependent inhibition of ACCase. Thus, data presented here employing *badc1,3* null mutants demonstrate both the mechanism of HFA-dependent inhibition of ACCase and an approach to largely mitigating its effects by reducing or eliminating BADC isoforms 1 and 3. The increased seed oil content in *badc1,3/fae1*/FAH did not fully rescue seed weight relative to the parental *fae1* line, which is consistent with previous reports in which the *badc1,3* double mutant exhibited a small decrease in seed weight compare to that of wild-type seeds. This likely resulted from a buildup of nonesterified FA under conditions in which their supply exceeds cellular demand. Support for this view comes from studies showing that excess FAs can be associated with negative cellular consequences, including reductions in axillary bud growth in tobacco (Tso, 1964), microalgal growth (Bosma et al., 2008), cell elongation in Arabidopsis (Li et al., 2011), and cell death in Arabidopsis (Fan et al., 2013; Yang et al., 2015).

The work presented here is an extension of our previous studies that focused on understanding mechanisms underlying lipid homeostasis under conditions in which FA supply exceeds that of cellular demand. Using a *Brassica napus* cell suspension culture, we fed FA in the form of Tween esters and monitored reductions in the rate of FAS. Exposure of oleoyl-Tween for up to 2 d resulted in oleoyl-ACP-dependent reversible inhibition of ACCase (Andre et al., 2012), whereas prolonged exposure resulted in irreversible BADC-dependent inhibition (Keereetaweep et al., 2018). It is intriguing that BADC-dependent inhibition of ACCase activity can be elicited by chronic exposure to excess oleate, a common naturally occurring monounsaturated FA, and ricinoleic acid, a non-native FA. Evidence is accumulating that BADCs are conditional inhibitors of ACCase activity, i.e., that upon the accumulation of excess FA, biotin-lacking, and therefore inactive, BADC subunits replace active BCCP subunits in the BC/BCCP ACCase subcomplex (Salie et al., 2016; Keereetaweep et al., 2018; Liu et al., 2019). Based on in vitro studies in which a one-unit pH change caused small changes in the dissociation constants of BADCs and BCCP for BC, it has been proposed that this might contribute to in vivo changes in the inhibition of ACCase related to light- and dark-dependent pH changes (Ye et al., 2020). However, in vivo evidence to support this hypothesis is lacking, and the experiments were conducted under nonphysiological conditions. Thus, whether excess FA causes BCCP to dissociate from BC, allowing BADC to join the complex, or whether excess FA drives BADCs into the complex displacing BCCP subunits is an open question that requires additional investigation to resolve.

Due to the desirability of creating an HFA-accumulating variant of a high-yielding crop, work to date has mostly focused on increasing the accumulation of HFA without deleterious effects on seed oil content. Previous studies have shown that negative HFA-dependent deficits including decreased seed oil and seed weight could be mitigated by the overexpression of several common FA accumulation factors. For example, overexpression of OLEOSIN1, a lipid droplet protection protein involved in TAG biosynthesis, with FAH was shown to enhance HFA accumulation (Lu et al., 2006). Likewise, overexpression of SEIPIN, a lipid droplet development factor that was previously reported to increase total seed oil (Cai et al., 2015), in HFA-accumulating seed increased both total oil and HFA content by more than 60%, likely by increasing LD size and creating a larger sink for TAG-accumulation (Lunn et al., 2018). Seed-specific expression of the WRINKLED1 transcription factor in *fae1*/FAH restored FA content (Adhikari et al., 2016). Other efforts have focused on the use of factors isolated from species that naturally accumulate modified FAs (mFAs), in which FA-metabolizing enzymes have evolved preference for mFAs. These studies were initially focused on enhancing the transfer of mFA from PC into TAG (Burgal et al., 2008; Kim et al., 2011; van Erp et al., 2011; Hu et al., 2012; Li et al., 2012). In another interesting example, the 18C ricinoleic acid is elongated to the corresponding 20C lesquerolic acid by a specialized *Physaria* elongase (Snapp et al., 2014). Lesquerolic acid alleviates feedback inhibition of FAS, which likely reflects decreased discrimination against lesquerolic relative to ricinoleic in its transfer from PC to TAG. Coexpression of multiple mFA-preferring enzymes, e.g., three castor acyltransferases: GPAT9, LPAT2, and PDAT1A in *fae1*/FAH seeds resulted in the production of abundant tri-HFA TAG and restored seed oil content relative to the parental *fae1* line (Lunn et al., 2019).

The reduced levels of seed oil accumulation reported for HFA-accumulating seed is a general phenomenon common to other mFAs, including epoxy (Li et al., 2012), conjugated (Cahoon et al., 2006), and cyclopropane (Yu et al., 2014) FA. The findings presented here demonstrating that knocking out *BADC1* and *BADC3* in FAH-producing Arabidopsis seeds restored the FA synthesis rate, total FA, seed yield may not be specific for HFA. Indeed, we speculate that reducing or eliminating *BADC1* and *BADC3* gene expression in other mFA-accumulating plants will have similar beneficial effects on mFA accumulation. Further, combining our BADC reduction strategy with the coexpression of other genes, or combinations of genes and/or factors described above, will likely increase mFA accumulation to levels equivalent to, or exceeding, those of their natural hosts.

Germination rates typically decline with increasing accumulation levels of mFA accumulation in non-native hosts, even in plants that accumulate normal levels of TAG such as that described herein and in previous studies (Lunn et al., 2019). This suggests that mFAs generally impair the mobilization of lipid reserves needed for energy production during the critical stages of germination (Lunn et al., 2019). Thus, cellular components that participate in the mobilization mFA-containing TAG, mFA transport, and β-oxidation represent additional targets for characterization and expression in non-native hosts to improve cellular energy supplies needed for germination to create robust mFA crops of the future.

Conclusions

We tested the hypothesis that HFA-dependent reduction in FA synthesis can be mediated by BADCs by the introgression of *badc1,3* into *fae1*/FAH. Consistent with the hypothesis, knocking out *BADC1* and *BADC3* expression increased FA synthesis rates in developing seeds by 2-fold, restoring the FA synthesis rate to that of the parental *fae1* line. This equally increased both normal FA and HFA accumulation in seeds. The total FA per seed, total oil content in seeds, and seed yield per plant all increased to an average of 5.8 μg, 37%, and 162 mg, respectively, compared to 4.9 μg, 33%, and 126 mg in *fae1*/FAH, respectively. FA synthesis-related genes including those encoding ACCase subunits, FA condensing enzymes, and transcription factors were not significantly increased upon knockout of *BADC1* and *BADC3*, which is consistent with the role of BADCs as inhibitors of FA synthesis. Knocking out *BADC1* and *BADC3* alleviated the inhibition of ACCase, providing a corresponding increase in the FA synthesis rate and improvement in seedling establishment. Combining the decreased expression of BADCs described herein along with the expression of other demonstrated mFA accumulating factors has the potential to realize the goal of creating crops with industrially relevant levels of HFA-accumulation. We speculate that this strategy may be applicable to increasing accumulation of many other mFAs in seed oils.

Materials and methods

Plant growth conditions

Arabidopsis *badc1,3* double mutant, CL37 (*fae1*/FAH), and *fae1* mutant lines were used in this study. Seeds were surface sterilized with 70% (v/v) ethanol, followed by 20% (v/v) bleach with 0.01% (v/v) Triton X-100, and washed three to 4 times with sterile water. Seeds were stratified for 2 d at 4°C in the dark and germinated on half-strength Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose at 23°C with a light/dark cycle of 18 h/6 h and photon flux density of 250 μmol m^{-2} s^{-1}. Plants were grown in walk-in growth chambers at 22°C with 16-h photoperiod and photon flux density of 70 µmol m^{-2} s^{-1}.

Seed germination and establishment

Seeds of *fae1*, *fae1*/FAH, *badc1,3*/*fae1*/FAH, and *badc1,3* were sterilized with ethanol and bleach as described above. A total of 180 seeds in five replicates from each line were sown in plates with ½ MS media containing 1% (w/v) sucrose under the conditions described above for 14 d. Germination was scored as seeds that produced a radicle, and seedlings that produced roots and green cotyledons were counted as being able to establish (Adhikari et al., 2016).

Arabidopsis cross and screening of homozygous plants

The *badc1,3*/*fae1*/FAH was generated by crossing CL37 (*fae1*/FAH) with the *badc1,3* double mutant. Homozygous lines were identified by genotyping using PCR coupled with HinfI digestion of PCR products and GC/MS analysis of FA of individual seeds. The genotyping primers used for *BADC1* and *-3* are described previously (Keereetaweep et al., 2018). For genotyping *fae1*, the *FAE1* gene was amplified from CL37 with specific primers (gFAE-F0: catgagtttgagtatacacatgtcta and gFAE-R0: aaagaaatcatgtaaacctaaatagaaacgc) and purified for sequencing. According to the *fae1* gene sequence information, primers fae-LP: gtgatcgatgagctagagaagaac and fae-RP: caaggacta TTTGCCGATGCCTTGACA TTGCGTAGAGC**G**AC were designed to introduce a HinfI restriction site to the *fae1* mutant. PCR fragments were digested with HinfI and the *fae1* mutant allele produced two fragments of 200 bp and 40 bp.

RNA extraction and RT-qPCR

RNA from Arabidopsis seeds was extracted as previously described (Wu et al., 2002). RNA quality and concentration were determined by Nanodrop spectroscopy. The cDNA was prepared using SuperScript IV VILO Master Mix with ezDNase enzyme (Invitrogen) following manufacture's manual. SoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used in the reaction mix. RT-qPCR was carried out on the CFX96 Real-time PCR Detection System (Bio-Rad). Gene-specific primers used in the analysis for *BADC1* and *BADC3* are the same as previously described (Keereetaweep et al., 2018).

FAH-qF1, AATATAGCCATCGCCGCCACCATT and FAH-qR1: TGGCAAGCAAAGCGATCGTAAGGT for *FAH*.

The primers used for the *UBQ10* reference gene were UBQ10 qF, ACCATCACTTTGGAGGTGGA, and UBQ10 qR, GTCAATGGTGTCGGAGCTTT. Statistical analysis of RT-qPCR data was carried out with REST2009 (Pfaffl et al., 2002).

FA analyses

FA analyses were carried out as previously described (Broadwater et al., 2002). Lipids were extracted in methanol/chloroform/formic acid (20:10:1) from seeds and heptadecanoic acid (17:0) was added as an internal standard. Total seed lipids were converted into FA methyl esters (FAMEs) in 5% (v/v) H_2SO_4 in methanol at 90°C for 60 min and extracted with hexane. FAMEs from single seeds were prepared by incubating the seed with 30 µL 0.2 M trimethylsulfonium hydroxide in methanol (Butte et al., 1982). Lipid profiles and acyl group identification were analyzed on a Hewlett Packard 6890 gas chromatograph equipped with a 5,973 mass selective detector and Agilent DB-FATWAX UI capillary column (30 m × 0.25 µm × 0.25 µm). The injector was held at 225°C and the oven temperature was set at 170°C for 1 min and then increased to 250°C at 10°C/min, with a final hold at 250°C for 7 min. The FA percentage values were presented as a mean of at least three biological replicates.

[^{14}C]Acetate incorporation assay

[1-^{14}C]Acetic acid, sodium salt, was purchased from PerkinElmer. Developing seeds at 11–13 DAF were collected. Approximately 10 mg fresh developing seeds were labeled by incubating in 0.2 mCi of [^{14}C]acetate for 60 min at room temperature with constant shaking. Cells were subsequently rinsed 3 times with water. Total lipids were extracted with 500 mL of methanol:chloroform:formic acid (20:10:1, v/v). The organic phase was then extracted with 370 mL of 1 M KCl and 0.2 M H_3PO4 and suspended in 2 mL of Ultima Gold liquid scintillation cocktail (PerkinElmer). The incorporated radioactivity was measured in cpm with a scintillation counter (Packard BioScience).

Accession numbers

The following genes were used in this study: *BADC1* (AT3G56130), *BADC3* (AT3G15690), FAE1 (AT2G15090) and FAH (8267537).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. EMS mutation causes truncation of FAE1 in *fae1* mutant.

Supplemental Figure S2. GC/MS analysis of HFA accumulation in *badc1,3/fae1/FAH* seeds.

Supplemental Figure S3. Analysis of FA synthesis related gene expression in developing seeds.

Supplemental Figure S4. [^{14}C]acetate incorporation assay in developing seeds of *badc1,3*.

Supplemental Figure S5. Seed germination and establishment.

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Conflict of interest statement. The authors declare no conflict of interest.

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