Concerted increases of FAE1 expression level and substrate availability improve and singularize the production of very-long-chain fatty acids in Arabidopsis seeds

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Abstract

Our initial goal was to evaluate the contributions of high 18:1 phosphatidylcholine and the expression level of FAE1 to the accumulation of very-long-chain fatty acids (VLCFAs), which have wide applications as industrial feedstocks. Unexpectedly, VLCFAs were not improved by increasing the proportions of 18:1 in fad2-1 mutant, FAD2 artificial miRNA, and FAD2 co-suppression lines. Expressing Arabidopsis FAE1 resulted in co-suppression in 90% of transgenic lines, which was effectively released when it was expressed in the rdr6-11 mutant host. When FAE1 could be highly expressed, apart from its naturally preferred product, 20:1, other saturated and polyunsaturated VLCFAs also accumulated in seeds. We postulated that overabundant FAE1 might cause the diversified VLCFA profile. When FAE1 was highly expressed, knocking down FAD2 increased the content of 20:1, suggesting that the 18:1 availability in the acyl-CoA pool increased from the high 18:1-PC via acyl editing. Concurrent decreases of side products like 22:1 and 20:0 in these lines suggest that increasing availability of the preferred substrate could suppress the side elongation reactions and reverse the effect of VLCFA product diversification due to overabundant FAE1. Re-analysis of FAD2 knockdown lines indicated that increasing 18:1 led to a decrease of 22:1, which also supports the above hypothesis. These results demonstrate that 18:1 substrate could be increased by a downregulation of FAD2 and that a balance between the levels of enzyme and substrate may be crucial for engineering-specific VLCFA products.

KEYWORDS

Arabidopsis, balance in abundance of FAE1 enzyme and its preferred substrate, co-suppression, FAE1, side product, substrate availability, VLCFAs

1 | INTRODUCTION

Very-long-chain fatty acids (VLCFAs) are those of ≥20 carbons in length. Plant VLCFAs, as precursors of cuticular waxes, sphingolipids, and vegetable oils (Bach & Faure, 2010; Kunst & Samuels, 2003; Yeats & Rose, 2013), provide protective barriers to the environment (impermeability to water and ions), lipid signaling molecules, and energy-storage compounds in...
seeds, respectively (Franke et al., 2012; Kunst & Samuels, 2009; Leonard et al., 2004; Pollard et al., 2008; Raffaele et al., 2009; Vanhercke et al., 2013). Plant oils high in VLCFAs, such as erucic and nervonic acids, have many industrial applications, for example, as feedstocks in the manufacture of bioplastics, biopolymers, enhanced oil recovery surfactants, and biojet fuels (Marillia et al., 2014; Piazza & Foglia, 2001). A full understanding of the biosynthesis and accumulation of VLCFAs in seeds is critical for improving their yield through bioengineering.

Arabidopsis contains about 20% VLCFAs in its seed oil and therefore it is a useful model for investigating the biosynthesis and accumulation of VLCFAs in seed triacylglycerols (TAGs). The biochemical processes of fatty acid biosynthesis in the plastid and TAG assembly in the endoplasmic reticulum (ER) have been extensively reviewed (Li-Beisson et al., 2010; Ohlrogge & Jaworski, 1997). Unlike the elongation of acyl-ACP up to 18 carbons by the fatty acid synthase (FAS) complex in the plastid, VLCFAs are synthesized from acyl-CoA substrates by the ER-based fatty acid elongase (FAE) complex, which consists of four enzymes: 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HACD), and enoyl-CoA reductase (ECR). Under the synergistic action of these four enzymes, two carbons from malonyl-CoA are transferred to the 18-carbon-CoA (Joubès et al., 2008). The resulting 20-carbon chain can be further elongated to C22 and C24 fatty acids (Haslam & Kunst, 2013; Kunst & Samuels, 2003) by subsequent elongase cycles. Then the very-long-chain acyl-CoAs are assembled into TAGs. The first condensation reaction in the FAE complex, catalyzed by the 3-ketoacyl-CoA synthase (KCS), is a rate-limiting step for the accumulation of VLCFAs in Arabidopsis seeds (Lemieux et al., 1990; Millar & Kunst, 1997). On the basis of a large number of studies, it is generally believed that the specificities of KCS enzymes determine the VLCFA products (Guo et al., 2009; James et al., 1995; Mietkiewska et al., 2004; Millar & Kunst, 1997; Taylor et al., 2009).

FAE1 (KCS18), which is involved in the synthesis of VLCFAs, was first identified in Arabidopsis seeds (James et al., 1995). Subsequently, FAE1 homologs were isolated and characterized from other plants rich in seed VLCFAs (e.g., Mietkiewska et al., 2004; Taylor et al., 2009). KCSs from different plant sources show preferences for substrates of different carbon chain lengths (Cahoon et al., 2000; Guo et al., 2009; Han et al., 2001; Lassner et al., 1996; Mietkiewska et al., 2004; Nguyen et al., 2013; Rossak et al., 2001; Taylor et al., 2009) and the expression or inhibition of different KCS genes in seeds also increase or decrease the length or types of VLCFAs that accumulate (Millar & Kunst, 1997; Taylor et al., 2009). AtFAE1 (hereafter FAE1) gene function has been extensively studied: for example, substrate preference tested by in vitro enzymatic activity; transformation into yeast and analysis of the fatty acid profiles (Blacklock & Jaworski, 2002, 2006; Trenkamp et al., 2004). FAE1 has a high substrate specificity for 16C and 18C fatty acids, especially 18:1-CoA, and FAE1 is only highly expressed in seeds (James et al., 1995; Kunst et al., 1992).
on a wide range of substrates in planta. We propose that this new pattern of diverse VLCFA products might be due to an imbalance in abundance between FAE1 and its substrate, that is, that an overabundance of FAE1 produces the diverse VLCFAs. Although acyl-CoA esters in plant tissues can be determined (e.g., Larson & Graham, 2001), actual acyl-CoA substrates of ER-based FAE1 cannot be accurately quantified as acyl-CoAs are present in different organelles and various acyl-CoAs are unevenly distributed in ER lumen. Alternatively, FAD2 was further knocked down in FAE1 overexpression lines to test how high 18:1-PC might affect VLCFA production. A large increase in proportions of 20:1 and an obvious decrease in 22:1 in the amiFAD2 FAE1 lines suggested that the high 18:1-PC may increase the preferred substrate availability to overabundant FAE1, which considerably weakens the accumulation of VLCFA side products. Re-examination of 22:1 proportions in FAD2 knockdown and co-suppression lines also supported this hypothesis.

2 RESULTS

2.1 The accumulation of VLCFAs cannot be improved in seeds with high 18:1 content

As discussed above, the high 18:1 on PC might re-enter the acyl-CoA pool and increase 18:1-CoA via Lands cycle. To investigate and confirm the effect of high 18:1-PC on VLCFA accumulation, the fad2-1 mutant, and wild type were first characterized. In seeds of wild-type Col-0, the relative proportions of 18:1 and total VLCFAs were about 15% and 20%–22%, respectively. Compared with wild type, in the fad2-1 mutant, the proportion of 18:1 accumulated to more than 60%. However, the levels of VLCFAs were not increased, and both of 20:1 and 22:1 were even slightly decreased (Figure 1a).

This result was quite different from that previously reported for two fad2 mutants, JB9 (fad2-1) and JB12 (fad2-2) (Lemieux et al., 1990). Careful analysis suggested the main difference was the VLCFA proportions in seeds of wild types. There are different

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**FIGURE 1** Changes in relative proportions of 20:1 + 22:1 and 18:1 in fad2-1 null mutant and FAD2 knockdown lines. (a) The fatty acid composition in Col-0 and fad2-1 mutant; (b) Relative content of 20:1 + 22:1 and 18:1 in T2 seeds of Pha::amiFAD2/Col-0 lines. Pha::amiFAD2/Col-0 refers an artificial miRNA targeting FAD2, driven by the phaseolin promoter, expressed in the wild-type Col-0. The T2 lines were sorted in the order of 20:1 + 22:1 content; (c) Relative content of 20:1 + 22:1 and its ratio to 18:1 in T2 seeds of Pha::FAD2/Col-0 lines. Pha::FAD2/Col-0 refers to FAD2, driven by the phaseolin promoter, expressed in the wild-type Col-0. Relative content of 20:1 + 22:1 was indicated by the light blue columns and red dots represent the ratio of 20:1 + 22:1/18:1 of each line. Error bars represent SD of three replicates.
sub-ecotypes of wild-type Columbia and the Col-0 in the current study might be different from the Col used in the earlier Lemieux et al. (1990) study. In the latter report, the WT exhibited an atypically low proportion of 20:1 at 16.7% compared with the average value of around 20% for Col-0 prevalent in most of the literature.

Accordingly, for the present study, we deemed it to be more accurate to evaluate the effect of FAD2 suppression by using a same genetic background. Therefore, artificial miRNA was used to knock down the expression of FAD2 in our Col-0 wild type to further test the effect of high 18:1-PC on the accumulation of VLCFAs. Eight T2 lines with red fluorescence were used to determine the fatty acid composition of seeds. The relative proportions of 18:1 were around 60% in all eight lines, which suggested that FAD2 was strongly knocked down. Similar to the phenotype of the fad2-1 mutant, VLCFA proportions also remained similar to wild type in the eight T2 lines possessing such high 18:1 levels (Figure 1b). In our previous study, a strong co-suppression was observed when FAD2 was overexpressed (Du et al., 2019). Accordingly, content of VLCFAs were investigated in those FAD2 co-suppression lines and also compared with their background Col-0. Similar to the result in the fad2-1 background, no increase (slight decrease) of VLCFAs was found in these co-suppression lines showing increased 18:1, which was indicated by their low ratios of (20:1 + 22:1)/18:1 proportions (Figure 1c); 20:1 proportions were 17.1%–19.2% in co-suppression lines versus 20.8% in wild type. Taken together, these results supported two interpretations: (a) the preferred substrate abundance of FAE1 might be not effectively improved via acyl editing and (b) the proportion of substrate available to FAE1 may not be a limiting factor for improving VLCFAs in Arabidopsis seeds.

2.2 Overexpression of FAE1 leads to decreases of VLCFAs in more than 90% of transgenic lines

To further explore the effect of expression level of FAE1 on VLCFA accumulation in Arabidopsis seeds, the FAE1 (At4G34520) was cloned into an expression vector under the control of a seed-specific phaseolin promoter, which avoids the abnormal growth of vegetative tissues in constitutive expression lines (Reina-Pinto et al., 2009). The Pha::FAE1 was transferred into Arabidopsis wild-type Col-0 and T1 transformed seeds were identified using red fluorescence. Because the fatty acid composition of a single Arabidopsis wild-type Col-0 and T1 transformed seeds were accurately determined by the SAST method (Ma et al., 2020), 39 T1 seeds were phenotyped by GC analysis and the untransformed wild-type Col-0 single seeds were used as a control. The ratio of (20:1 + 22:1)/18:1 proportions was used to indicate the efficiency of substrate conversion to VLCFAs in these lines. Unexpectedly, only 3 out of 39 T1 seeds showed an increase of the ratio of (20:1 + 22:1)/18:1, whereas other T1 seeds showed the opposite change in the ratio (Figure 2a). The combined proportions of these two VLCFAs decreased to 2%–5% in nearly half of the Pha::FAE1 lines, compared with 24.2% in wild type.

To preclude the possibility of the effect being due to an unsuitable vector choice, the same Pha::FAE1 vector was transformed into the fad2-1 mutant. T1 seeds were pooled, and their fatty acid composition was analyzed. Results showed that VLCFAs, including 20:1 and 22:1, accumulated in T1 seeds, compared with undetectable VLCFAs in the fad2-1 mutant, suggesting that the Pha::FAE1 complemented the null fad2-1 mutant and the vector was properly assembled. However, it was worth noting that the relative content of VLCFAs averaged 7.5% in these T1 seeds, which was much lower than that in wild type (Figure 2b).

To further explore the effect of high 18:1-PC on this phenotype in overexpressing FAE1 lines (see Figure 2a), the same Pha::FAE1 vector was transformed into the fad2-1 mutant, in which the proportion of 18:1 was about fourfold that of wild type. Similarly, the transgenic T1 seeds were pooled and analyzed. As shown in Figure 2c, a significant decrease of 20:1, from 20% in the fad2-1 mutant to less than 4%, was found in transgenic lines, whereas the proportion of 18:1 in T1 positive seeds was further increased by about 20%. This result suggested that high 18:1-PC was not a reason for decreased VLCFA content when sense FAE1 was overexpressed in Arabidopsis seeds.

2.3 Overexpression of FAE1 in Arabidopsis seeds triggers an exceptionally strong co-suppression

Our results above also implied that co-suppression might be occurring when FAE1 is overexpressed in seeds of both wild type and the fad2-1 mutant. To test this hypothesis, fatty acid composition and gene expression were further investigated in T2 homozygous lines. Results showed that proportions of VLCFAs (20:1 and 22:1) only obviously increased in one out of ten lines (line 6–1), and it decreased or remained at a similar level to that found in wild type in the other nine lines. For example, as shown in Figure 3a, the proportion of VLCFAs in line 8–4 and line 1–6 decreased by 90% compared with wild type, whereas the corresponding proportions of 18:1 increased almost twofold over that of wild type. To test gene expression levels by q-PCR in six representative lines and wild-type Col-0, primers in the 5′ UTR were designed for endogenous FAE1 expression (Endo FAE1) and primes within the coding region were created to gauge expression of both endogenous and exogenous FAE1s (total FAE1). Results showed that the expression levels of both were decreased in five lines (1–6, 3–4, 4–1, 10–5, and 11–3), which exhibited higher 18:1 and lower 20:1 (Figure 3b). However, in line 6–1 with an increased VLCFA content, the expression of endogenous FAE1 remained unchanged, whereas the expression of total FAE1 increased by more than threefold compared with Col-0 (Figure 3b). Thus, the accumulation of VLCFAs was closely correlated to the total FAE1 expression level. Collectively, these results suggested that transgenic sense FAE1 triggered strong co-suppression during seed development, leading to a decrease in the expression level of FAE1 with a consequent decrease in VLCFA accumulation.
2.4 | FAE1 co-suppression is released in the rdr6 mutant and high expression of FAE1 leads to an accumulation of different types of VLCFAs

RDR6 is a key factor mediating posttranscriptional gene silencing, including transgenic co-suppression (Dalmay et al., 2000; Harmoko et al., 2013). To further identify whether the decrease of FAE1 expression levels was a classical type of co-suppression, the same Pha::FAE1 was expressed in rdr6-11 mutant seeds. Transgenic Pha::FAE1/rdr6-11 T1 seeds were first phenotyped, and the ratio (20:1 + 22:1)/18:1 proportion was also used to indicate the relative changes of VLCFAs and their substrate (Figure 1a). Contrary to the changes in transgenic seeds of Pha::FAE1/Col-0 lines, only one out of 17 single Pha::FAE1/rdr6-11 T1 seeds showed a decreased ratio of (20:1 + 22:1)/18:1 compared with the rdr6 host, whereas all other lines showed a relative increase in proportions of VLCFAs. The release of the co-suppression phenotype in the rdr6-11 mutant background suggested that RDR6 mediated the FAE1 co-suppression in Arabidopsis seeds.

To further confirm this, fatty acid composition and gene expression levels of FAE1 were determined in homozygous lines. As shown in Figure 1b, VLCFAs were increased to varying degrees in 15 of 16 T1 lines, and the 20:1 + 22:1 content was largely increased by 20%-60% in most lines compared with the rdr6-11 background, whereas the relative content of 18:1 was decreased in most lines. Thereafter, six representative FAE1/rdr6-11 co-suppression-released lines as well as the host rdr6-11 mutant were used for detection of endogenous FAE1 and total FAE1 expression levels in developing seeds. In the six FAE1 transformants, the expression levels of endogenous FAE1 were similar to that in the rdr6-11 mutant alone, whereas the expression levels of total FAE1 increased up to 10-fold. In addition, the expression level of total FAE1 showed a positive correlation with the relative contents of VLCFAs in these lines (Figure 1b,c). These results further demonstrated that co-suppression of FAE1 can be released in the rdr6-11 mutant and therefore FAE1 can be effectively overexpressed, which largely increases the accumulation of VLCFAs. These results suggest that the expression level of FAE1 is a limiting factor for improving VLCFAs in Arabidopsis seeds.

In the lines where co-suppression was released in the rdr6-11 mutant host, it was found that not only monounsaturated VLCFAs but also other types of VLCFAs, including saturated and polyunsaturated fatty acids, were significantly increased. Three lines with this phenotype were selected for a detailed analysis. In general, both 16C and 18C fatty acids decreased, and 20C fatty acids increased

![Figure 2](image_url)

FIGURE 2  Changes of relative proportions of VLCFAs in Pha::FAE1 transgenic lines within different backgrounds. (a) Ratio of (20:1 + 22:1)/18:1 proportions in Pha::FAE1/Col-0 single T1 seeds. Pha::FAE1/Col-0 refers the FAE1, driven by the phaseolin promoter, expressed in wild-type Col-0. The red dot and dashed line stand for the average ratio in untransformed seeds of Col-0. (b) Fatty acid composition in T1 transgenic seeds expressing FAE1 in the fae1-1 mutant and (c) in the fad2-1 mutant. The transformed T1 seeds were selected by red fluorescence from transformed plants. Five T1 transgenic seeds of (b) and (c) were pooled for fatty acid analysis. Error bars represent SD of three replicates.
polyunsaturated VLCFA in native seeds, we hypothesized that both substrates. Considering the natural paucity of these saturated and FAE1 can exhibit elongation activity with other kinds of fatty acid substrate preference and the ratio of substrate availability to FAE1 abundance determine the accumulation of final VLCFA products. We deduced that in FAE1/rd6 lines, the preferred substrate, 18:1-CoA, might not be prevalent enough to fully engage/bind to the overly abundant FAE1; the overabundant FAE1 might elongate other available acyl-CoA substrates despite their lower binding capacity, causing the diversified VLCFA product profile. Considering the two possibilities incited in the above FAD2 knockout/down experiments, this hypothesis might be tested such that one of two possibilities might be excluded. This would be done by knocking down FAD2 when FAE1 is highly expressed. Two expression vectors, the Phaselin::FAE1 vector with the DsRed marker (cited above) and a Phaselin::amiFAD2 vector without DsRed, were co-transferred into rd6-11 mutants and seed fatty acid composition was phenotyped. Phaselin::FAE1 T1 positive seeds were screened by the DsRed fluorescent marker, whereas successful transformation with Phaselin::amiFAD2 was identified by the large decrease in the proportion of 18:2 (Figure S1a). Eight out of 24 T1 seeds were identified as double-transgenic lines. The average fatty acid composition of Phaselin::FAE1 single-transgenic seeds (Ave FAE1) as well as the highest increase of FAE1 single-transformed line (Highest FAE1) and the untransformed rd6-11 background (rd6-11) were compared with the changes in double-transformation lines (Ave of DE). The sum of 20:1 + 22:1 was used to indicate the increase of elongation product of 18:1 (Figure S1b), whereas 20:1 and 22:1 were used to evaluate the preferred reaction and side reaction, respectively (Figure 6a,b). The proportion of 20:1 increased in the amiFAD2 FAE1/rd6-11 double-transgenic lines (Figure 6a), suggesting that the high 18:1-PC under the fad2 mutation effectively increased the 18:1-CoA availability to FAE1 via the Lands cycle.

Figure 3 Changes of relative proportions of VLCFAs and expression level of FAE1 in seeds of Pha::FAE1/Col-0 lines. (a) Relative proportions of 20:1 + 22:1 and 18:1 in seeds of Pha::FAE1/Col-0 T3 homozygous lines. Pha::FAE1/Col-0 refers the FAE1, driven by the phaseolin promoter, expressed in wild-type Col-0. The T3 lines were sorted in the order of 20:1 + 22:1 content. Error bars represent SD of three replicates. (b) Relative expression levels of total FAE1 and endogenous FAE1 (Endo FAE1) in 12–14 DAF developing seeds of Pha::FAE1/Col-0 T3 lines. Endo FAE1 is detected by primers in 5' UTR of FAE1, while total FAE1 was determined by primers within coding region. EF1 and UBQ10 were used as double internal standards. Error bars represent SD of three replicates.
Notably, although 20:1 increased, the average proportion of 22:1 still decreased to 4.1% (Ave of DE) compared with 6.8% the FAE1/rdr6-11 single-transgenic lines (Ave of FAE, Figure 6a). Knocking down FAD2 by artificial miRNA allowed us to propose that the proportion of the preferred FAE1 substrate, 18:1, was effectively increased. As a result, compared with the FAE1/rdr6-11 single-transgenic lines, the proportion of 22:1 decreased and was negatively correlated with the increased proportion of 18:1. The lowest proportion of 22:1 in double-transgenic line T3 was even lower than that found in the rdr6-11 mutant host (Figure 6b). These results suggest that a further indirect increase in availability of the preferred FAE1 substrate can weaken the accumulation of the more diverse VLCFA side products. Additionally, the average proportion of 20:1 increased to 30% in amiFAD2 FAE1/rdr6-11 double-transgenic lines compared with 24% in the FAE1/rdr6-11 single-transgenic lines (Figure 6a), suggesting that simultaneous increases of substrate availability and FAE1 enzyme levels is an effective way to further produce a target VLCFA (in this case, 20:1).

The results above lead us to re-examine the proportions of 22:1 in FAD2 knockdown lines, in which 18:1 substrate availability might be increased and FAE1 remained at a normal expression level. Results showed that the proportions of 22:1 decreased to different degrees in these FAD2 knockdown lines (Figure 6c,d). The proportions of 18:1 and 22:1 in both the microRNA FAD2 knockdown lines and the FAD2 co-suppression lines showed a strong negative correlation with R² = 0.924 and 0.898, respectively (Figure 5a,b). These results further suggested that high 18:1-PC might increase the availability of the preferred FAE1 substrate, 18:1-CoA, via Lands cycle/acyl editing, which, in turn, could reduce the accumulation of side product VLCFAs like erucic acid.

Additionally, considering that 20:0 is also a side product of FAE1 and its substate 18:0 abundance is not largely affected in amiFAD2 lines, the accumulation level of 20:0 was investigated in the FAE1/rdr6-11 single-transgenic lines and amiFAD2 FAE1/rdr6-11 double-transgenic lines. Results showed that average proportion of 20:0 increased up to 4.3% in FAE1/rdr6-11 T3 lines compared with 3.5% in rdr6 host (Figure 5a). Similarly, it was nearly doubled in FAE1/rdr6-11 T3 lines (Figure 5a). However, average proportion of 20:0 decreased to 2.3% in amiFAD2 FAE1/rdr6-11 double-transgenic lines (Figure S3a). Moreover, in amiFAD2/Col lines, the proportions of 20:0 obviously decreased to
a range of 0.75%–1.26% compared with the 1.82% 20:0 in Col wild type (Figure S3b). These results further demonstrated that a proposed increase of the proportion of the preferred substrate weakened the accumulation of the side product *in planta*.

### 3 | DISCUSSION

Although co-suppression was postulated in a previous study of FAE1 when function of FAE1 was investigated (Millar & Kunst, 1997), no further evidence has been collected. In this study, a strong co-suppression of FAE1 was characterized in Arabidopsis seeds (Figures 2 and 3). Although co-suppression was first reported in plants three decades ago (van der Krol et al., 1990; Napoli et al., 1990), and co-suppression lines are frequently found in transgenic plants, only a few cases of strong co-suppression have been investigated in plants (e.g. Zhang et al., 2015). The strong co-suppression of FAE1 impedes the improvement of VLCFA, and it was released in the *rdr6-11* mutant host (Figure 4). The high expression of FAE1 in *rdr6-11* made it feasible to evaluate the contribution of FAE1 abundance to the accumulation of VLCFAs. This led to identification of diverse substrates and products of FAE1 *in planta*, allowing us to explore the effect of high 18:1 PC and the effect of the balance between substrate availability and FAE1 enzyme abundance on VLCFA accumulation.

#### 3.1 | The alteration of acyl-CoA pools via manipulating acyl desaturation on PC and the limiting factors in the accumulation of VLCFAs

Increasing substrate content and improving the amount or activity of an enzyme often result in an accumulation of the end product, although it may be affected by other factors, such as storage or usage of product and interactions with other metabolic steps/pathways. In this study, alteration of substrate availability via manipulating the desaturation of acyl moieties on PC and FAE1 gene expression level were evaluated for their effects on the accumulation of VLCFAs. As mentioned above, the 18:1-CoA in the acyl-CoA pool (but not 18:1 esterified to PC or PE) is a direct substrate of FAE1 (Haslam & Kunst, 2013). It is known that 18:1-CoA content is positively correlated to the content of 18:1 on PC, through the Lands cycle (Wang et al., 2012). Therefore, it is inferred that the relative content of 18:1-CoA is increased in the acyl-CoA pool when the proportion of 18:1 on PC is upregulated. On the other hand, most de novo fatty acids are incorporated into PC and most DAG used for TAG synthesis comes from PC (Bates et al., 2009, 2012; Karki et al. 2019). It remains unclear how much the high 18:1-PC impacts VLCFA accumulation via acyl editing. Although an apparent increase of VLCFAs was reported in Arabidopsis *fad2* mutants compared with the wild type (Lemieux et al., 1990), we noticed that, in that early study, the proportions of VLCFAs in the wild type were atypically low. In this study, relative contents of 18:1 increased from around 15% in wild type up to 60% in *fad2-1* mutant, *fad2* miRNA knockdown lines (Figure 1a, b) and *fad2* co-suppression lines (Figure 2 in Du et al., 2019), yet no obvious increases of VLCFAs were observed in any of these lines with different genetic backgrounds (Figure 1c). In agreement with our results, 20:1 content did not show obvious increases when 18:1 content was increased from 15% in wild type to 23% in a *fad3* (α-linoleate desaturase) (BL1) mutant (Lemieux et al., 1990). Similarly, VLCFAs, including 20:1 and 22:1, slightly decreased in the rod1 (DH4) mutant in which the content of 18:1 was doubled (Lemieux et al., 1990; Lu et al., 2009).

*FAD2* was knocked down by amiRNA when FAE1 was overexpressed. The 18:1 in TAG dramatically increased in this case.
Changes of monounsaturated VLCFA proportions and 18:1 abundance in different transgenic seeds. (a) The relative content of 20:1 and 18:1 and (b) the relative content of 22:1 and 18:1 in T2 seeds of Pha::FAE1 single-transgenic lines and Pha::FAE1+Pha::amiFAD2 double-transgenic lines within the rdr6-11 background (18:1 proportion repeated from Figure 6a, for ease of comparison). Ave of FAE1 refers to the average value of all Pha::FAE1 single-transgenic T1 seeds; Ave of DE refers to the average value of all Pha::FAE1+Pha::amiFAD2 double-transgenic T1 seeds; Highest of FAE1 refers to a line with the highest proportion of 20:1 or 22:1 in all Pha::FAE1 single-transgenic T1 seeds. Lowest of FAE1 refers to a line with the lowest proportion of 20:1 or 22:1 among all Pha::FAE1 single-transgenic T1 seeds. The double-transgenic lines were sorted in the order of 18:1 proportion. (c) Relative proportions of 22:1 and ratios of 18:1/22:1 proportions in T2 seeds of Pha::amiFAD2/Col-0 lines. Pha::amiFAD2/Col-0 refers an artificial miRNA targeting FAD2, driven by phaseolin promoter, expressed in the wild-type Col-0. Relative content of 22:1 was indicated by pink columns and blue dots represent the ratio of 18:1/22:1 of each line. (d) Relative content of 22:1 and ratios of 18:1 to 22:1 in T2 seeds of Pha::FAD2/Col-0 lines. Pha::FAD2/Col-0 refers FAD2, driven by phaseolin promoter, expressed in the wild-type Col-0. The proportion of 22:1 is indicated by light blue columns and red dots represent the ratio of 18:1/22:1 proportions in each of the lines. The T2 lines were sorted in the order of 22:1 content in (c) and (d). Error bars represent SD of three replicates. Asterisk (*) indicates a significant difference: p < .01.

(Figure 6a), suggesting that the 18:1-CoA was effectively increased from high 18:1 PC, via the Lands cycle. Taken together, with the studies cited above, our findings also suggest that 18:1 availability may not pose a limitation factor for VLCFA accumulation in Arabidopsis seeds with normal FAE1 abundance. Furthermore, for reasons that are as yet, unclear, the situation in Arabidopsis is somewhat different from the cases observed in other oilseeds, wherein downregulating FAD2 led to a marked increase in erucic acid, as cited above.

At 7–13 days after flowering, the expression level of FAE1 in Arabidopsis thaliana seeds was increased, and the accumulation of VLCFAs closely paralleled the expression level of FAE1 (Rossak et al., 2001). When the expression level of the KCS (FAE1) gene was induced by abscisic acid in B. napus, VLCFA content was significantly increased in microspore-derived embryos (Qi et al., 1998). As mentioned above, expression level or activity of FAE1 is regarded as a limiting factor in VLCFA accumulation (Lemieux et al., 1990; Millar & Kunst, 1997). Decrease of FAE1 expression levels also led to a decrease of VLCFAs in co-suppression lines (Figure 3). However, due to the very strong co-suppression when FAE1 was overexpressed, we could not fully evaluate the effect of high FAE1 expression levels...
on VLCFA accumulation in a wild-type background. Therefore, the effect of an enzyme increase was tested by overexpressing FAE1 in the rdr6-11 background. Results showed that overexpressing FAE1 strongly increased VLCFA accumulation up to 60% compared with that in the rdr6-11 host (Figure 4a,b). Additionally, the accumulation of VLCFAs was positively correlated with the expression level of FAE1 (Figure 4b,c). These data suggest that the expression level of FAE1 is a limiting factor for improving VLCFA in Arabidopsis seeds and increasing expression of FAE1 is an effective way to enhance the accumulation of VLCFAs. Once the expression level of FAE1 is increased, a higher 18:1 content can further increase the accumulation of 20:1 (Figure 6a), suggesting that the combination of a high expression of FAE1 and an increase of substrate availability is an effective strategy for improving VLCFA accumulation. In this study, we obtained lines with up to 16% VLCFAs, 39.8% monounsaturated VLCFA and 33.3% 20:1, which, to our knowledge, are the highest proportions obtained in Arabidopsis seeds to date.

3.2 | Diversified substrates and products of FAE1 in planta

FAE1 homologs have different substrate preferences, which determine the length of the carbon chain of the final product fatty acid as well as its degree of unsaturation. The Arabidopsis FAE1 has a strong substrate specificity for 18:1-CoA (Blacklock & Jaworski, 2002; Kunst et al., 1992), while FAE1 homologs from Crambe abyssinica and Tropaeolum majus show preference for producing 22:1 (Mietkiewska et al., 2004, 2007). A 3D structural model suggested the differences between substrate pockets from different KCS proteins, which could possibly play a key role in the specificity of the products (Joubès et al., 2008). Yet another study shed light on the patterns of FAE1 gene evolution and discussed how sequence variations impact the production of erucic acid in plants (Sun et al., 2013). These topics require renewed attention, as many more KCS genes and their corresponding protein sequences from plants are now available for comparison and deep genetic and proteomic analyses.

Other studies have shown that Arabidopsis FAE1 can use additional substrates besides 18:1-CoA. Previous in vitro and in vivo elongase assays indicated that saturated, monounsaturated, and polyunsaturated fatty acyl-CoAs, including 16:0-, 18:0-; 20:0-; 16:1-, and 18:2-CoAs, could be elongated (Blacklock & Jaworski, 2002, 2006; Paul et al., 2006). The VLCFAs 20:2 and the 20:3 were produced when 18:2 and 18:3, respectively, were fed to a yeast strain in which Arabidopsis FAE1 was overexpressed (see table 2 in Trenkamp et al., 2004). Overexpression of Arabidopsis FAE1 in yeast resulted in accumulation of different VLCFAs, such as 20:0-24:0, 20:1, 22:0, 22:1, and 26:0 (Blacklock & Jaworski, 2006; Millar & Kunst, 1997; Trenkamp et al., 2004). The capacity for FAE1 to use diverse substrates was also observed in planta. Small amounts of 20:2, 22:2, 20:3, and 22:3 were detected in Arabidopsis leaves in which FAE1 was driven by 3SS promoter, and when FAE1 was driven by a seed-specific napin promoter in Arabidopsis, 18:2 and 18:3 were also decreased, whereas 20:2 and 20:3 were increased (Millar & Kunst, 1997); this indicated that FAE1 could extend these polyunsaturated fatty acids. The elongation of 18:0 decreased in the fae1 mutants (AC56 and AK57; Kunst et al., 1992), and the relative proportion of 20:0 decreased in the fae1 mutant (JB20) (table 4 in Lemieux et al., 1990), also suggesting that FAE1 can elongate a saturated fatty acid. In this study, the high expression of FAE1 combined with a full release of FAE1 co-suppression in the rdr6-11 mutant background allows one to evaluate the range of its substrate. Notably, the proportion of 22:1 increased up to sixfold, and the proportions of 20:0, 20:2, 20:3, and 22:0 were up to twofold higher in these FAE1 overexpression lines, compared with the rdr6-11 host. Correspondingly, their substrate fatty acids, such as 18:2 and 18:3, showed a large decrease (Figure 5). Taken together, our results demonstrate that FAE1 has so strong and wide capacity in planta to elongate 16 to 22 carbon fatty acids and thereby produce diversified VLCFAs ranging from saturated to polyunsaturated fatty acids. Our results also suggest that the KCS is a limiting factor and substrate preference determinant for fatty acid elongation in the four-enzyme complex; clearly when FAE1 expression is largely increased, the remaining part of the elongase complex can adapt to the new rate of flux and accept the new types of 3-keto acyl-CoA products coming from FAE1 and feeding into the metabolism.

3.3 | The balance between substrate availability and FAE1 abundance determines VLCFA products

The diversified elongation activities of Arabidopsis FAE1 raises the following questions: Although there are abundant 20:1, 18:2, and 18:3 substrates in wild-type seeds, why are there present, only trace amounts of 22:1, 20:2, and 20:3, respectively? If one accepts the hypothesis that depth of the "FAE pocket" alone determines product chain length (Joubès et al., 2008) and Arabidopsis FAE1 naturally produces 20:1, how can one explain the large amount of 22:1 (more than 10%) which accumulates when FAE1 is highly expressed (Figure 5)? We propose an alternative hypothesis: that the balance between the abundance of FAE1 and substrate availability, as well as substrate preference, determine the accumulation of the final VLCFA product(s). Studies on Arabidopsis FAE1 (KCS) specificity were conducted following functional expression in yeast (Blacklock & Jaworski, 2002). For the expressed Arabidopsis FAE1 when assayed in vitro, "20:1 product activity" was about eightfold of "22:1 product activity" (10.2 vs. 1.32 pmol/μg protein), indicating a substrate specificity directed heavily toward an 18:1-CoA substrate. The balance of substrate and enzyme abundance may be adaptive and has been created during the long evolution of Arabidopsis. This strong preference of FAE1 for 18:1-CoA clearly explains the high accumulation of 20:1 in native Arabidopsis seeds when 18:1-CoA and FAE1 abundance are balanced.

In the current study, if one takes into consideration the scenario wherein FAE1 was highly expressed in the Pha:FAE1 / rdr6-11 lines (up to 16-fold increase; Figure 4), it follows that the sixfold
increase in proportions of 22:1 (Figures 5 and 3 best T3 homozygous lines) might be due to a relative increase of FAE1/18:1-CoA available in the acyl-CoA pool; overabundant FAE1 can bind to 20:1-CoA. As deduced above, indirectly increasing 18:1 availability by knocking down FAD2 expression led to a low proportion of 22:1 (Figure 6b, Ave FAE1 vs. Ave DE), even though the proportion of its direct substrate, 20:1, was further improved (Figure 6a, Ave FAE1 vs. Ave DE). Although protein level of FAE1 could be examined by antibody, there is no accurate way to quantify the absolute abundance of acyl-CoAs in the ER pool and thereby arrive at a ratio of FAE1 to its substrate. Nonetheless, our results provide strong indirect evidence that the observed accumulation of more diversified VLCFAs in the FAE1/rdr6 lines may be due to an overabundance of FAE1 relative to the abundance of the preferred 18:1-CoA substrate in the acyl-CoA pool (i.e., with excess FAE1, other less-preferred acyl-CoAs can be extended). In the absence of overexpressed FAE1, the increased proportion of 18:1 in amiFAD2 and co-suppression lines also resulted in lower proportions of 22:1 (Figure 6c,d). Our hypothesis is also supported by the extraordinarily high proportion of 22:1 (15%) as well as a high proportion of 22:0 in the wrl1 mutant, in which total fatty acid biosynthesis largely decreased (meaning a lower absolute amount of 18:1-CoA) and wherein no feedback regulation on fatty acid elongation occurs (normal FAE1 abundance; table 2 in Focks & Benning, 1998). Additionally, in our study, another side product, 20:0, also showed a change similar to 22:1 when the proportion of preferred substrate was altered (Figure S3). Taken together, our results suggest that synergistically increasing substrate availability (deduced in this study) and enzyme abundance may be critical not only for the yield of product but also for a preferred product, if an enzyme can accept multiple substrates (see Figure 7).

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials and growth conditions

The Col-0, used as the wild type, the fae1-1 (N6245) and the fad2-1 (N8041) were obtained from the Nottingham Arabidopsis Stock Centre. The rdr6-11 mutant was kindly provided by Prof. Hongwei Guo (Southern University of Science and Technology, China). All the Arabidopsis seeds of wild type and mutants were sterilized with 75% alcohol (v/v) for one minute and rinsed with sterilized water three times, and then seeds were cultured on 1/2 MS medium containing 1.5% sucrose. Growth conditions for seedlings were described in a previous study (Zhang et al., 2016).

4.2 | Vector construction

To construct the Phaseolin::FAE1 (Pha::FAE1) vector, the full-length coding region of AtFAE1 (AT4G34520) was amplified from cDNA
of developing seeds of Col-0. The amplified fragment was cloned into pUC57 containing attL1 and attL2. Then the FAE1 was inserted into a binary vector under the control of the phaseolin promoter. To carry out a direct selection of transgenic seeds, a DsRed fluorescent marker was also cloned within expression vector via an LR reaction (Zhang et al., 2009).

For the Phaseolin::amiFAD2 (Pha::amiFAD2) vector, the the FAD2 target gene (At4G34520) was submitted to WMD3 (http://wmd3.weigelworld.org) a web service used for artificial miRNA design. Candidate 21nt mature amiRNA sequences were recommended that resemble natural miRNAs while minimizing possible off-target effects to other transcripts (Ossowski et al., 2008). A 21nt candidate (TATTGCATTATAATGCGGCAC) was introduced into a miRNA319a backbone by PCR and then cloned into a binary vector as described above.

4.3 Plant transformation

The expression vectors were transformed into Agrobacterium tumefaciens strain GV3101 and both wild-type and mutant plant hosts were transformed by the floral dip method (Clough & Bent, 1998). The transgenic seeds were identified by red fluorescence under green light, and double-transformed T1 seeds of Pha::FAE1 and Pha::amiFAD2 were selected by red fluorescence and the decrease of 18:2 content, respectively.

4.4 Fatty acid analysis

Mature T1 seeds were collected and methyl-esterified following the SAST method (Ma et al., 2020). Single seeds were treated with 200 µl methanol containing 5% (v/v) sulfuric acid, including 5 µl BHT plus 60 µl toluene and incubated at 85°C for 2 hr in a 1.5 ml GC vial. Then 200 µl 0.9% NaCl and 100 µl hexane are added. After vortexing and centrifuging, 60–80 µl of the upper hexane phase was transferred carefully to a GC sample insert and FAMES were measured by GC; the injection volume of GC was 4 µl. As for the T1 homozygous seeds, 10 seeds were used in same process described above, and the injection volume of GC was 2 µl. The GC analysis method was described in our previous study (Ma et al., 2020).

4.5 RNA extraction and gene expression analysis

For RNA extraction developing seeds at 12–14 DAF (days after flowering) were collected, and total RNA was extracted using Trizol™ reagent (Invitrogen). Reverse transcription was carried out using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) after RNA integrity was detected (Figure S4).

The primers endoFAE1-F and endoFAE1-R, located in the 5′ untranslated region, were used to analyze the endogenous FAE1 expression level. The total FAE1 expression level was analyzed using primers FAE1-F and totalFAE1-R, which were located within its coding region. Stability and validation of EF1α and UBQ10 internal standards were verified by following the previous method (Gutiérrez et al., 2008). The ChamQ Universal SYBR qPCR Master Mix (Vazyme) was used according to the manufacturer’s instructions and quantitative RT-PCR (q-PCR) was carried out using the ABI QuantStudio 7 Flex Real-Time PCR system with three biological replicates. The thermal treatment was 30 s at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 60°C, and 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C. The relative expression levels of endoFAE1 and totalFAE1 to double-reference genes were calculated by the 2−ΔΔCT method according to the MIQE guidelines (Bustin et al., 2009).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MZ and SM conceived the study. SM performed most of the experiments and CD performed the FAD2 co-suppression experiment. MZ, SM, and DCT drafted the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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