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ORIGINAL RESEARCH

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).

Highlights

- The balance between FAE1 abundance and its substrate availability is crucial not only to the yield of VLCFAs but also to the types of VLCFAs that accumulate.

Fatty acid modification and plant oil biosynthesis involve a complex metabolic network with multiple subcellular compartments, parallel pathways, and cycles (Bates, 2016). De novo synthesized fatty acids are either modified, as in elongation to VLCFAs in the acyl-CoA pool, or esterified to phospholipids such as phosphatidylcholine (PC). Fatty acid desaturases in seeds, primarily FAD2 and FAD3, usually act on fatty acids esterified to PC (Ohlrogge & Browse, 1995), whereas the KCSs use cytosolic acyl-CoAs as substrates. Most de novo C18 acyl-CoAs are first incorporated into PC, where the oleoyl moiety undergoes desaturation; and acyl moieties can be exchanged between the acyl-CoA pool and PC pools via Lands cycle or accumulated in TAG via DAG (Bates et al., 2009, 2012; Karki et al., 2019; Lands, 1965; Wang et al., 2012). Therefore, relative substrate populations in the acyl-CoA pool are affected by other kinds of fatty acid modifications.

Generally, fatty acid accumulation efficiency is determined by substrate abundance and enzyme kinetics. How can PC-based desaturation and acyl editing affect substrate availability for biosynthesis of VLCFAs? In the Arabidopsis *fad2* mutant, the 18:1 proportions largely increased and the proportions of VLCFAs reportedly increased (Lemieux et al., 1990). Similarly, this phenotype was observed in a *fad2* mutant of *Brassica napus* (Bai et al., 2019). The relative content of VLCFAs increased in the *lpcat1lpcat2* double mutant, in which the Land cycle was impaired, and 18:1 increased. In contrast, VLCFA content decreased when *LPCAT*s were overexpressed (Wang et al., 2012). These findings suggest that PC desaturation and acyl editing may affect substrate abundance for FAE1 and thus, the final elongation product. On the other hand, VLCFAs were increased when Arabidopsis *FAE1* was overexpressed in seeds, although a VLCFA decrease in some lines was speculated to be due to co-suppression (James et al., 1995). Therefore, we hypothesized that increasing both substrate abundance (indirectly) and the expression level of *FAE1* might further improve the accumulation of VLCFAs in Arabidopsis seeds.

When we tested this hypothesis, unexpected results were observed: there was no improvement in VLCFA proportions found in lines with increased 18:1, and a very strong co-suppression was observed in 90% of sense *FAE1* transgenic lines. This strong co-suppression was effectively released in the *rdr6-11* (an RNA-dependent RNA Polymerase 6 mutant [Dalmay et al., 2000]) mutant host and the resulting *FAE1* overexpression produced a wide range of seed VLCFAs (other than the naturally preferred product 20:1), including 22:1, 20:0, 20:2, 20:3, 22:0, 22:2, 22:3, and ≥24C fatty acids. This suggests that FAE1 acts

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 under abundant FAE1. 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

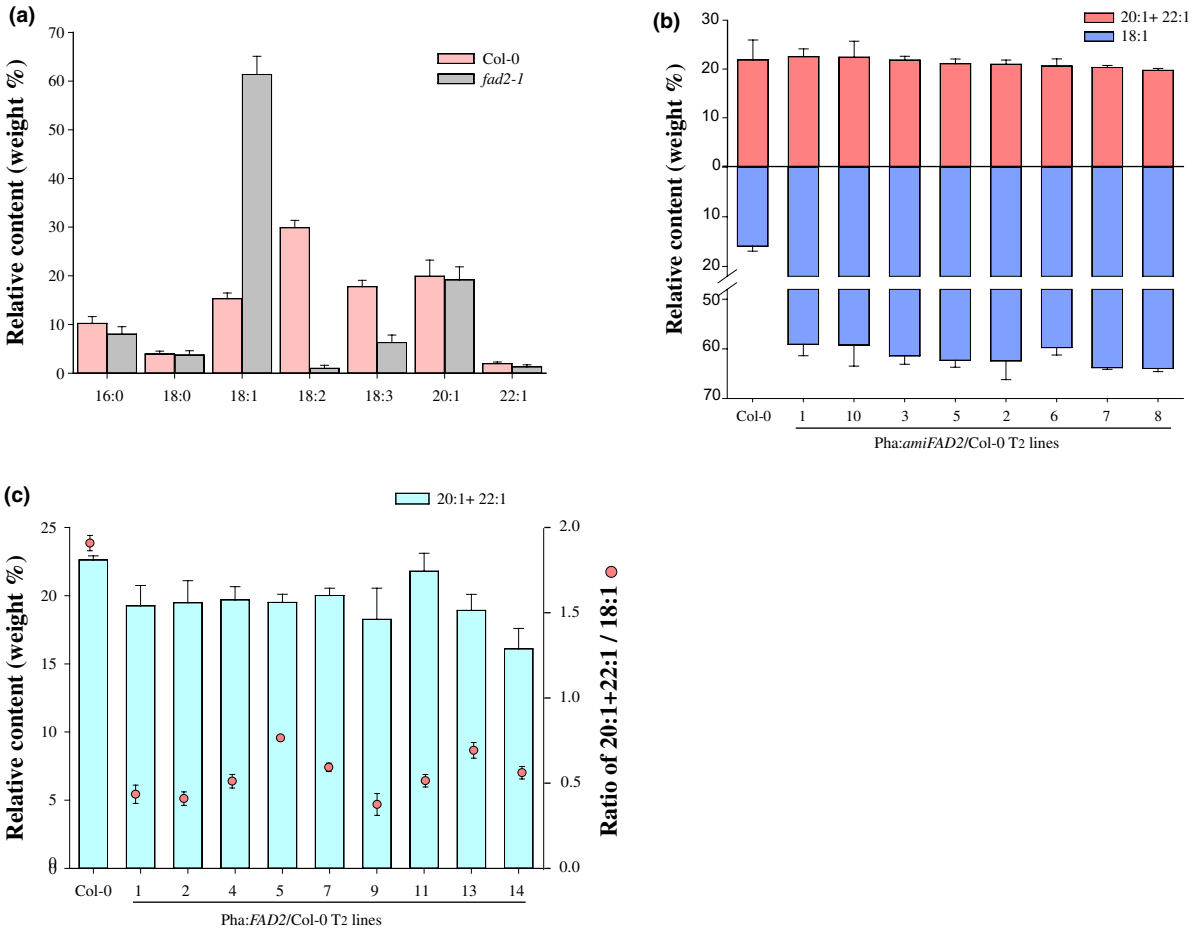

FIGURE 1 Changes in relative proportions of 20:1 + 22:1 and 18:1 in *fad2-1* null mutant and *FAD2* knockdown lines. (a) The fatty acid composition in Col-0 and *fad2-1* mutant; (b) Relative content of 20:1 + 22:1 and 18:1 in T_2 seeds of Pha::ami*FAD2*/Col-0 lines. Pha::ami*FAD2*/Col-0 refers an artificial miRNA targeting *FAD2*, driven by the phaseolin promoter, expressed in the wild-type Col-0. The T_2 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 T_2 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 T_2 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 T_2 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 T_1-transformed seeds were identified using red fluorescence. Because the fatty acid composition of a single Arabidopsis seed can be accurately determined by the SAST method (Ma et al., 2020), 39 T_1 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 T_1 seeds showed an increase of the ratio of (20:1 + 22:1)/18:1, whereas other T_1 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 *fae1-1* mutant. T_1 seeds were pooled, and their fatty acid composition was analyzed. Results showed that VLCFAs, including 20:1 and 22:1, accumulated in T_1 seeds, compared with undetectable VLCFAs in the *fae1-1* mutant, suggesting that the Pha::*FAE1* complemented the null *fae1* mutant and the vector was properly assembled. However, it was worth noting that the relative content of VLCFAs averaged 7.5% in these T_1 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 T_1 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 T_1 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 T_3 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 primers 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.

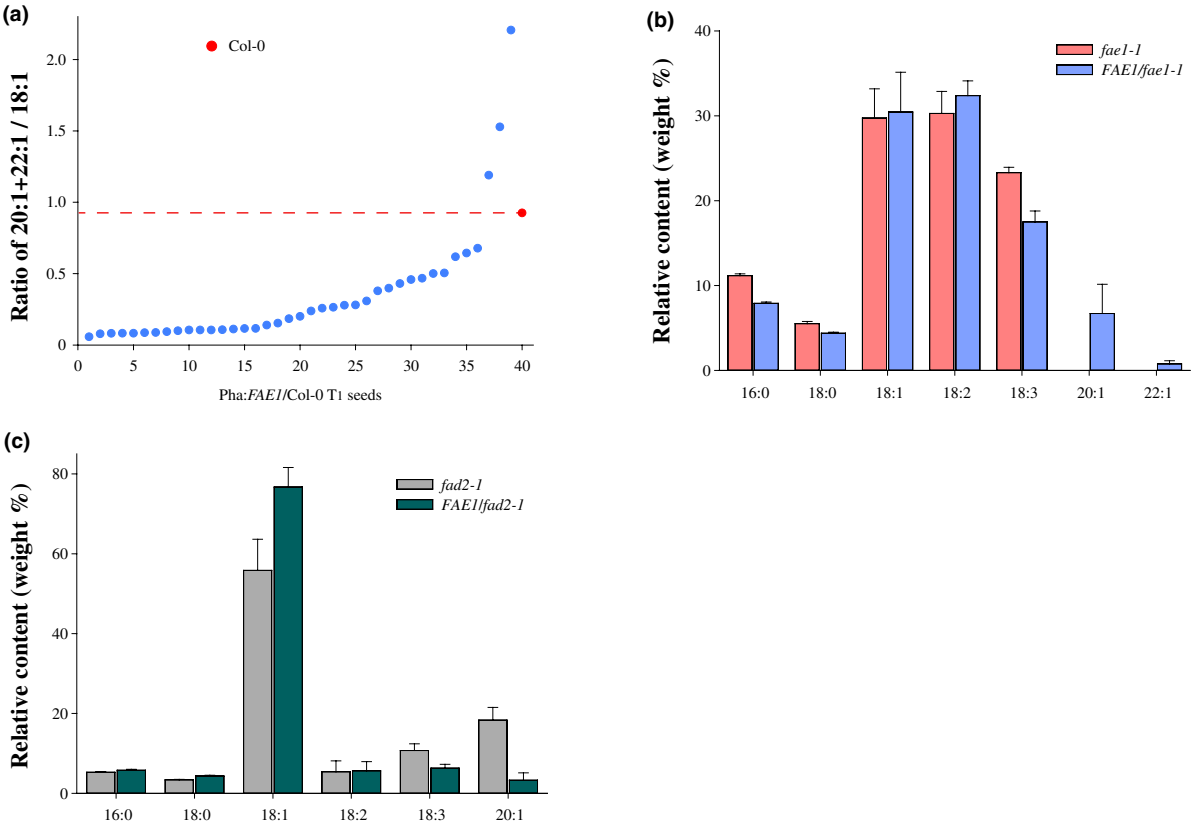

FIGURE 2 Changes of relative proportions of VLCFAs in Pha::*FAE1* transgenic lines within different backgrounds. (a) Ratio of (20:1 + 22:1)/18:1 proportions in Pha::*FAE1*/Col-0 single T_1 seeds. Pha::*FAE1*/Col-0 refers the *FAE1*, driven by the phaseolin promoter, expressed in wild-type Col-0. The red dot and dashed line stand for the average ratio in untransformed seeds of Col-0. (b) Fatty acid composition in T_1 transgenic seeds expressing *FAE1* in the *fae1-1* mutant and (c) in the *fad2-1* mutant. The transformed T_1 seeds were selected by red fluorescence from transformed plants. Five T_1 transgenic seeds of (b) and (c) were pooled for fatty acid analysis. Error bars represent *SD* of three replicates

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* T_1 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 4a). Contrary to the changes in transgenic seeds of Pha::*FAE1*/Col-0 lines, only one out of 17 single Pha::*FAE1*/*rdr6-11* T_1 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 4b, VLCFAs were increased to varying degrees in 15 of 16 T_3 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 4b,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

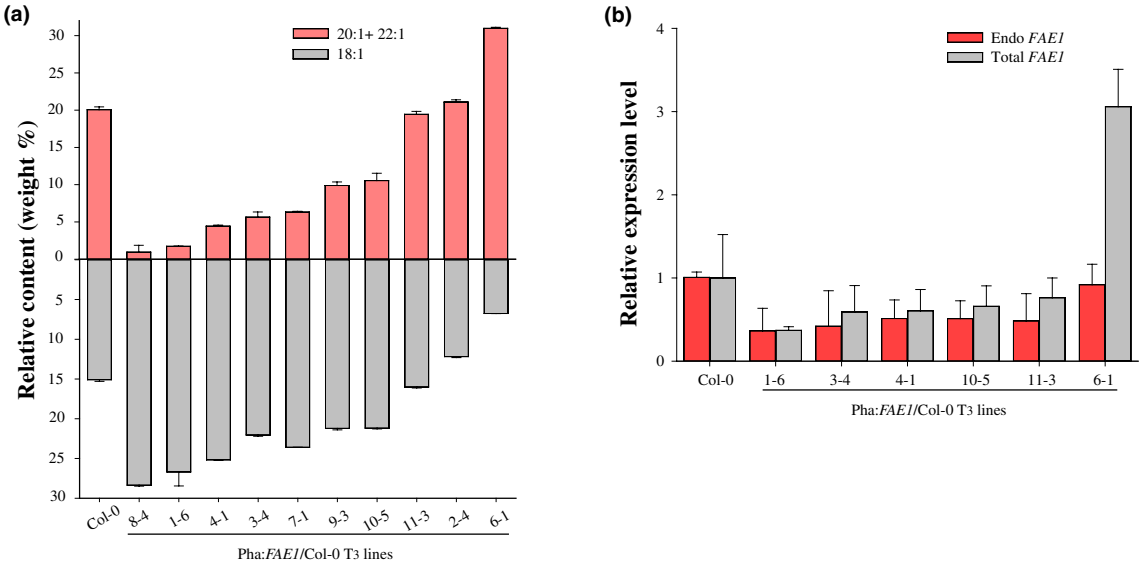

FIGURE 3 Changes of relative proportions of VLCFAs and expression level of *FAE1* in seeds of Pha::*FAE1*/Col-0 lines. (a) Relative proportions of 20:1 + 22:1 and 18:1 in seeds of Pha::*FAE1*/Col-0 T_3 homozygous lines. Pha::*FAE1*/Col-0 refers the *FAE1*, driven by the phaseolin promoter, expressed in wild-type Col-0. The T_3 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 T_3 lines. Endo *FAE1* is detected by primers in 5' UTR of *FAE1*, while total *FAE1* was determined by primers within coding region. *EF1α*and *UBQ10* were used as double internal standards. Error bars represent *SD* of three replicates

(Figure 5a). The relative content of 18:1 was decreased by about 45% compared with the *rdr6-11* line and its elongation product, 20:1, was increased by about 20% in two lines. Moreover, a large proportion of 20:1 could be further elongated to 22:1. The relative content of 22:1 increased up to sixfold. These results may suggest a fatty acid selectivity for monounsaturated fatty acids. Strikingly, fatty acids with ≥24 carbon length (not precisely determined) were also detected (Figure 5a,b). It is worth noting that the relative contents of 20:0 and 22:0 increased 80%–91% and 120%–162%, respectively, compared with the *rdr6-11* background, whereas both 16:0 and 18:0 decreased to varying degrees, suggesting that Arabidopsis FAE1 can elongate saturated fatty acid *in planta*. Noticeably, the relative contents of polyunsaturated fatty acids (20:2, 20:3, 22:2, and 22:3) were also significantly increased, whereas those of 18:2 and 18:3 correspondingly decreased. Collectively, diversified VLCFA products suggest that Arabidopsis FAE1 can use a wide range of substrates when it is highly expressed *in planta*.

2.5 | Knocking down *FAD2* under high expression of *FAE1* further increases the proportion of 20:1 but decreases the proportion of 22:1

The above results suggested that the expression level of *FAE1* is the limiting factor for improving VLCFAs and that Arabidopsis FAE1 can exhibit elongation activity with other kinds of fatty acid substrates. Considering the natural paucity of these saturated and polyunsaturated VLCFA in native seeds, we hypothesized that both substrate preference and the ratio of substrate availability to FAE1 abundance determine the accumulation of final VLCFA products. We deduced that in *FAE1/rdr6* 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 Phaseolin::*FAE1* vector with the DsRed marker (cited above) and a Phaseolin::*amiFAD2* vector without DsRed, were co-transferred into *rdr6-11* mutants and seed fatty acid composition was phenotyped. Phaseolin::*FAE1* T_1 positive seeds were screened by the DsRed fluorescent marker, whereas successful transformation with Phaseolin::*amiFAD2* was identified by the large decrease in the proportion of 18:2 (Figure S1a). Eight out of 24 T_1 seeds were identified as double-transgenic lines. The average fatty acid composition of Phaseolin::*FAE1* single-transgenic seeds (Ave FAE1) as well as the highest increase of *FAE1* single-transformed line (Highest FAE1) and the untransformed *rdr6-11* background (*rdr6-11*) 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/rdr6-11* 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.

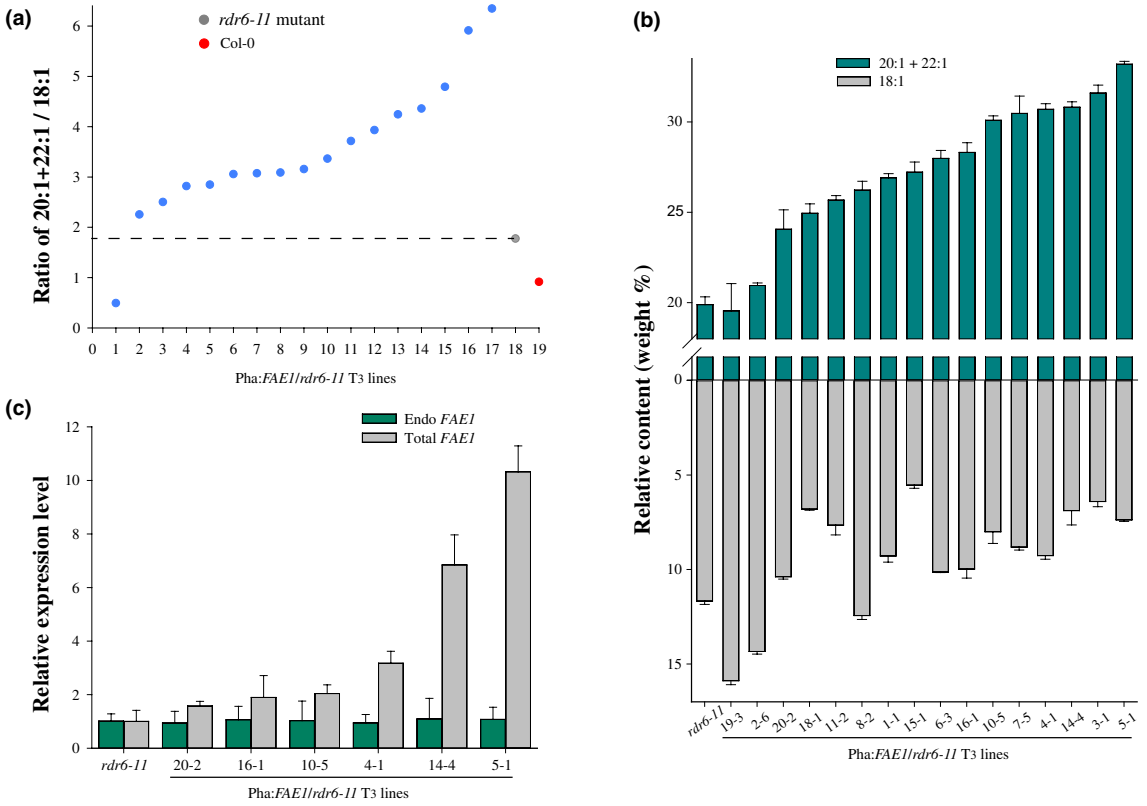

FIGURE 4 Changes of relative proportions of VLCFAs and expression level of *FAE1* in seeds of Pha::*FAE1* /*rdr6-11* lines. Pha::*FAE1/rdr6-11* refers the *FAE1*, driven by the phaseolin promoter, expressed in the *rdr6-11* mutant. (a) Ratio of (20:1 + 22:1)/18:1 proportions in T_1 single seeds expressing Pha::*FAE1* in the *rdr6-11*. The gray dot and dashed line represent the average ratios in seeds of the *rdr6-11* host mutant, and the red dot indicates the average ratio in seeds of Col-0. (b) Relative content of 20:1 + 22:1 and 18:1 in seeds of Pha::*FAE1/rdr6-11* T_3 homozygotes. The T_3 lines were sorted in the order of 20:1 + 22:1 content. Error bars represent *SD* of three replicates. (c) Relative expression levels of total *FAE1* and endogenous *FAE1* (Endo *FAE1*) in 12–14 DAF developing seeds of Pha::*FAE1/rdr6-11* homozygous lines. Endo *FAE1* is detected by primers in the 5' UTR of *FAE1*, whereas total *FAE1* was determined by primers within the coding region. *EF1α* 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 6b). 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 T_1-15 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 led 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 R^2=0.924 and 0.898, respectively (Figure S2a,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 ami*FAD2* 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* T_1 lines compared with 3.5% in *rdr6* host (Figure S3a). Similarly, it was nearly doubled in *FAE1/rdr6-11* T_3 lines (Figure 5a). However, average proportion of 20:0 decreased to 2.3% in ami*FAD2 FAE1/rdr6-11* double-transgenic lines (FigureS 3a). Moreover, in *amiFAD2*/Col lines, the proportions of 20:0 obviously decreased to

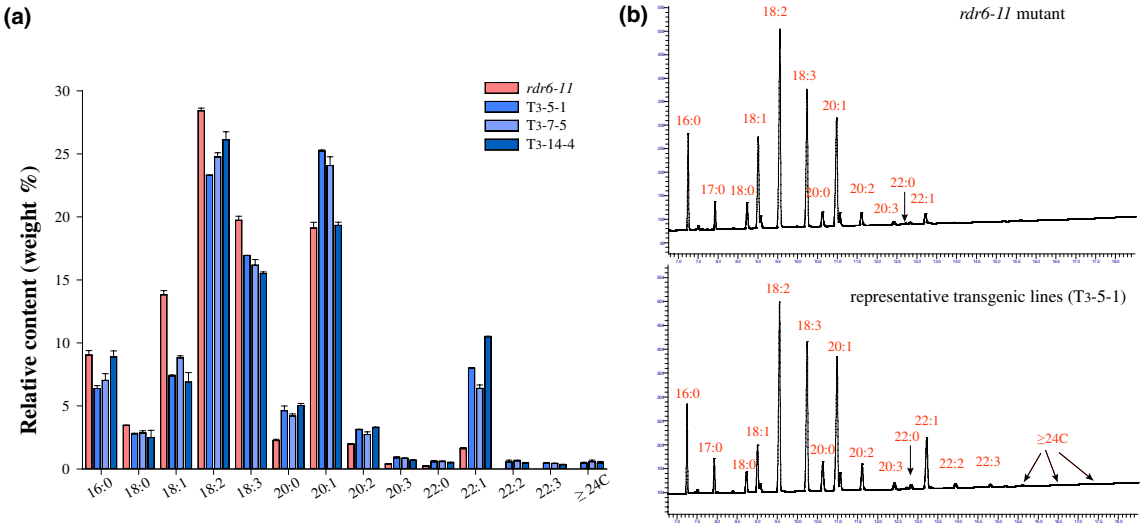

FIGURE 5 Fatty acid composition in seeds of Pha::*FAE1/rdr6-11* lines and a representative gas chromatograph. (a) Total fatty acid composition in seeds of three Pha::*FAE1/rdr6-11* T_3 homozygous lines. Pha::*FAE1/rdr6-11* refers *FAE1*, driven by the phaseolin promoter, expressed in the *rdr6-11* mutant. Error bars represent *SD* of three replicates. (b) Representative GC graph of fatty acid analysis of Pha::*FAE1/rdr6-11* line T_3-5-1 and the *rdr6-11* mutant

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

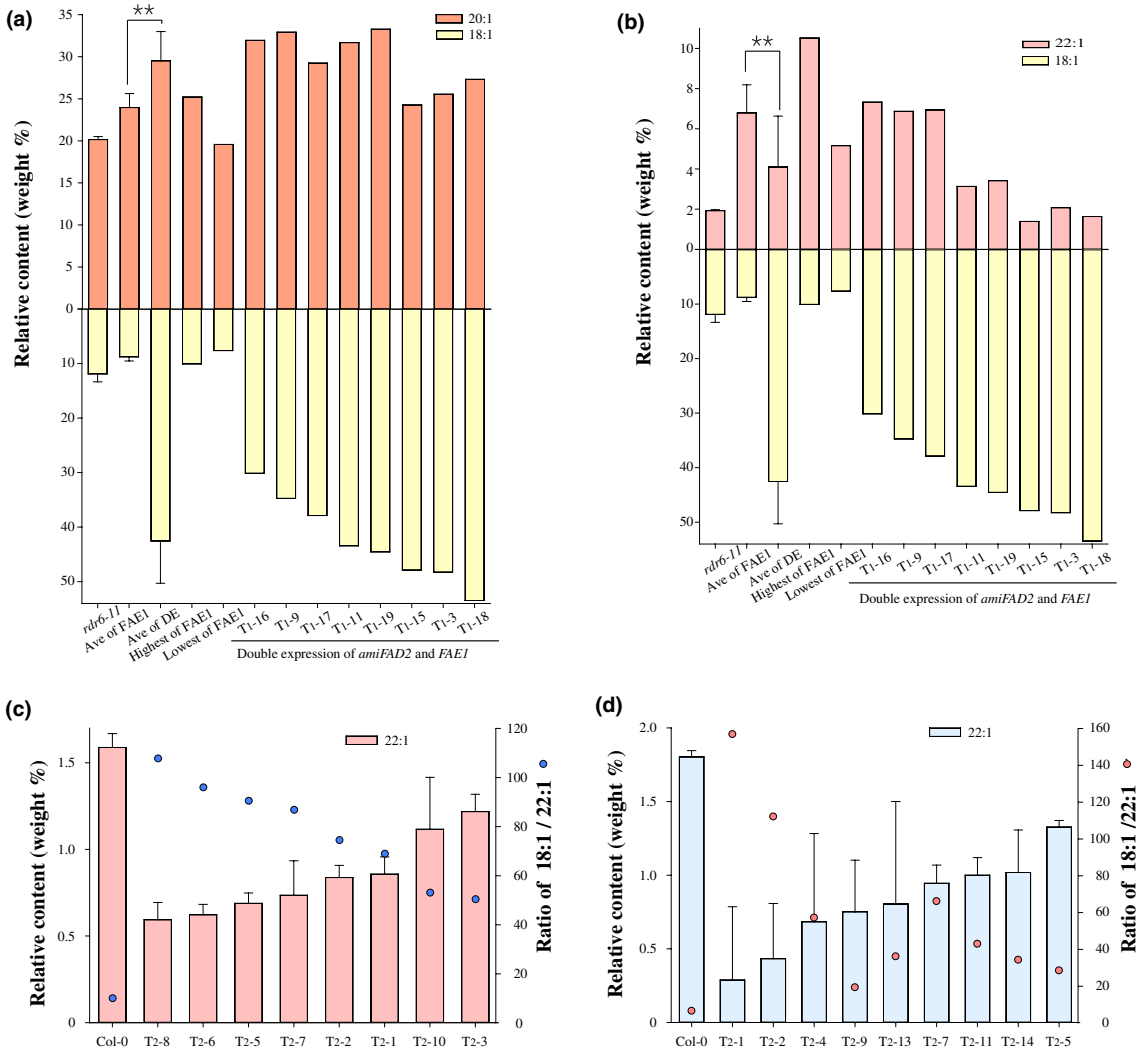

FIGURE 6 Changes of monounsaturated VLCFA proportions and 18:1 abundance in different transgenic seeds. (a) The relative content of 20:1 and 18:1 and (b) the relative content of 22:1and 18:1 in T_1 seeds of Pha::*FAE1* single-transgenic lines and Pha::*FAE1*+Pha::*amiFAD2* double-transgenic lines within 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 T_1 seeds; Ave of DE refers to the average value of all Pha::*FAE1*+ Pha::*amiFAD2* double-transgenic T_1 seeds; Highest of FAE1 refers to a line with the highest proportion of 20:1 or 22:1 in all Pha:*FAE1* single-transgenic T_1 seeds. Lowest of FAE1 refers to a line with the lowest proportion of 20:1 or 22:1 among all Pha::*FAE1* single-transgenic T_1 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 T_2 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 lines. (d) Relative content of 22:1 and ratios of 18:1 to 22:1 in T_2 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 T_2 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 43% 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 35S 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 metabolon.

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

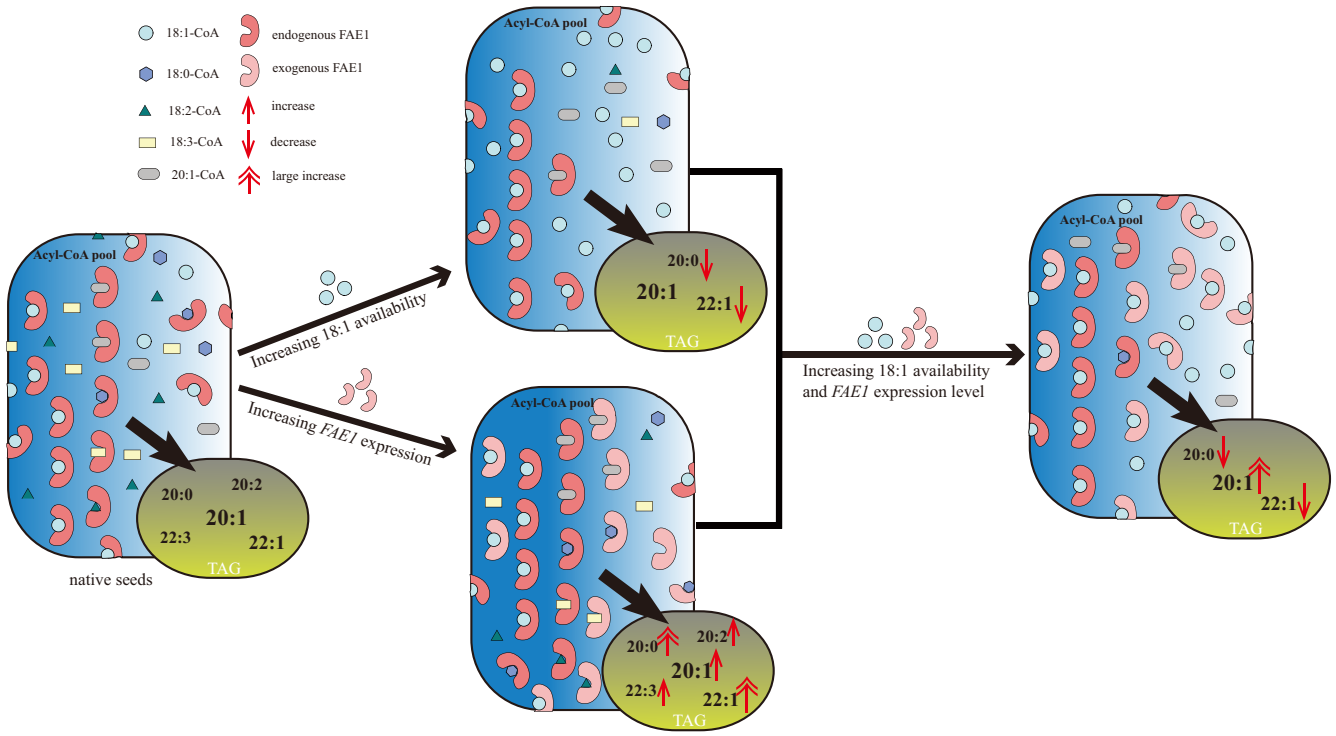

FIGURE 7 Schematic diagram of VLCFA products and the balance between FAE1 abundance and its preferred substrate 18:1-CoA. The abundance of FAE1 and substrates are indicated by their relative abundance in acyl-CoA pool in the ER lumen. Substrate preferences are depicted by the relative amounts of acyl-CoA species bound to FAE1 enzyme moieties

increase in proportions of 22:1 (Figures 5 and 3 best T_3 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 *wri1* 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 (TATTGCGTTATAATGCGGCAC) 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 T_1 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 T_1 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 T_3 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 (Gutierrez 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 endo*FAE1* and total*FAE1* to double-reference genes were calculated by the $2^{-\Delta\Delta CT}$ 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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