Generation of Camelina Mid-Oleic Acid Seed Oil by Identification and Stacking of Fatty Acid Biosynthetic Mutants

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ABSTRACT

Modifying oilseeds to obtain a desired fatty acid composition is often necessary to enable use as feedstocks for specific applications such as food processing, biofuels, or biolubricants. A mutant population of camelina (*Camelina sativa*), an emerging specialty oilseed crop, was screened by high-throughput gas chromatography for lines with altered seed oil fatty acid composition. By leveraging knowledge of fatty acid synthesis in *Arabidopsis thaliana*, mutations in orthologs of *FATTY ACID ELONGASE1 (FAE1), FATTY ACID DESATURASE2 (FAD2), FATTY ACID DESATURASE3 (FAD3),* and *β-KETO-ACYL-ACP SYNTHASE II (KASII; FAB1)* were identified. The mutations altered conserved amino acid residues in the encoded proteins. The ability of the mutations in *FAE1*, *FAD2* and *FAD3* to affect enzyme function was demonstrated by comparing *in vivo* activities of wild-type and mutant alleles in yeast. In addition, expression of wild-type cDNA in camelina complemented fatty acid phenotypes of these mutants. As camelina has a hexaploid genome, the effect of a mutation in one of the three homeologs for each gene resulted in no or less severe growth phenotypes compared to similar mutations in Arabidopsis. Mid-oleic oils with nearly 40% oleic acid and reduced very long-chain (≤C20) fatty acid content were obtained by crossing to obtain a *fae1c/fad2a/fae1a/fad3a* quadruple mutant. Little effect on total seed oil content was observed in the stacked mutant line. The resulting mid-oleic acid oil had improved oxidative stability due to reductions in polyunsaturated fatty acid content, increasing its utility for biofuels and other applications.

Keywords: *Camelina sativa*, genetic improvement, vegetable oil, biofuels

1. INTRODUCTION

The primary component of vegetable oil derived from different oilseed crops is triacylglycerol (TAG), molecules with three fatty acids esterified to a glycerol backbone. Vegetable oil has mostly been produced for human consumption, but over the last few decades, an increasing amount of this valuable agricultural commodity has been used as a source of biodiesel, or for other industrial applications (Durrett et al., 2008; Msanne et al., 2020). This increased demand has led to the identification of new TAG sources, particularly for non-food applications. Camelina (*Camelina sativa*), a member of the Brassicaceae family, has emerged as one such suitable industrial oil seed crop. Camelina seed oil content ranges from 32-41% of seed weight, with the fatty acid profile being dominated by the polyunsaturated fatty acids (PUFAs) 18:2 and 18:3 (Bansal and Durrett, 2016; Iskandarov et al., 2014). Importantly, camelina can be grown without displacing other crops. In this regard, it is suited for cultivation on land not typically used for food crop production due to its productivity with limited rainfall and minimal soil fertility inputs. Under drought conditions, camelina achieves higher seed yields compared to other Brassicaceae oil seed crops (Enjalbert et al., 2013). Further, camelina has a relatively short growing season (85–100 days), and possesses winter and spring varieties, making this crop very attractive for integrating into existing agricultural practices as a relay crop. Camelina can be grown as a rotation crop during fallow years with wheat and other dryland cereals, without affecting the yield of these crops, thus making roughly 5-7 million acres of fallow land available in the U.S. each year, allowing the production of 750,000,000 to 1 billion gallons of camelina oil per year (Shonnard et al., 2010). Camelina oil has also been used as a feedstock for jet fuel production through conversion methods such as hydroprocessing, and life cycle analyses show

61 that production and use of jet fuel from camelina results in 75% lower greenhouse gas emissions
62 relative to petroleum-derived fuel (Shonnard et al., 2010).

63 In addition to its positive agronomic and sustainability traits, camelina also benefits from
64 the availability of a variety of genetic and biotechnology tools with which to further improve
65 different traits (Bansal and Durrett, 2016; Iskandarov et al., 2014). These include abundant
66 genomic and transcriptomic resources (Abdullah et al., 2016; Kagale et al., 2014; Kagale et al.,
67 2016; Mudalkar et al., 2014; Nguyen et al., 2013) and a facile *Agrobacterium* floral-infiltration
68 transformation system (Lu and Kang, 2008) that allows for the overexpression of both
69 endogenous and exogenous genes, as well as targeting gene expression through methods such as
70 CRISPR and RNA-interference. Further, camelina's close relationship with *Arabidopsis thaliana*
71 (Kagale et al., 2014) facilitates the straightforward transfer of the wealth of knowledge of the
72 model species into this oilseed crop. Camelina's hexaploid genome also offers advantages for
73 successful mutational breeding, allowing knockout of one or two of the three homeologous genes
74 that may be essential or compromise agronomic performance when disrupted in diploid oilseeds
75 such as pennycress (Hutcheon et al., 2010; Kagale et al., 2014; Sedbrook et al., 2014).

76 Despite its numerous positive attributes as a biofuel feedstock, the need remains to
77 improve camelina's agronomic and seed quality traits. In particular, changes in fatty acid
78 composition will greatly improve the utility of camelina oil, which is currently prone to
79 oxidation due to its high content of polyunsaturated fatty acids, making it less suitable for certain
80 applications. For example, biodiesel derived from camelina possesses a lower oil stability index
81 (OSI) compared to biodiesel from other feedstocks (Soriano and Narani, 2012). Additionally,
82 saturated fatty acids and very long-chain fatty acids (VLCFA) with chain-lengths ≥C20 are also

targets for enhancement of biofuel functionality to address deficiencies in pour-point and other qualities (Durrett et al., 2008).

Mutational breeding and biotechnological approaches can be applied to address deficiencies in camelina seed oil quality. The key target genes for these efforts include genes for fatty acid desaturases that control polyunsaturated fatty acid production (Figure 1), most notably genes for FATTY ACID DESATURASE2 (FAD2) that forms linoleic acid (18:2Δ9,12) by Δ12 desaturation of oleic acid linked to phosphatidylcholine (PC) and FATTY ACID DESATURASE3 (FAD3) for α-linolenic acid (18:3Δ9,12,15) production by subsequent Δ15 desaturation of linoleic acid bound to PC (Arondel et al., 1992; Okuley et al., 1994; Yadav et al., 1993). In addition, carbon chain extension of oleic acid to the VLCFAs eicosenoic acid (20:1Δ11) and docosenoic (or erucic) acid (22:1Δ13) is initiated by the *FATTY ACID ELONGASE1* (*FAE1*)-encoded β-ketoacyl-CoA synthase, and mutation of *FAE1* can provide further increases in seed TAG oleic acid content {Haslam, 2013 #902;James, 1995 #77}. Furthermore, the relative amounts of the saturated fatty acids palmitic acid (16:0) and stearic acid (18:0) are regulated by β-ketoacyl-acyl carrier protein (ACP) synthase II (KASII) in plastid-localized fatty acid biosynthesis encoded by the *FATTY ACID BIOSYNTHESIS1* (*FAB1*) gene. This enzyme initiates the two-carbon elongation of 16:0-ACP for formation of 18:0-ACP (Carlsson et al., 2002; Wu et al., 1994).

To improve the oil properties of camelina, we employed a forward genetics approach and screened a mutant population for lines with altered fatty acid composition by high through-put gas chromatography (GC). By using knowledge about fatty acid synthesis in Arabidopsis, we were able to rapidly identify the causative mutations in specific homeologs of genes for fatty acid biosynthetic enzymes. We crossed different combinations of the mutations to obtain mutant

stacks with further alteration of seed oil fatty acid composition, including a mid-oleic acid phenotype for enhanced oil oxidative stability for biofuel and biolubricant applications.

2. MATERIALS AND METHODS

2.1. Plant growth and characterization

Camelina sativa (cultivar Ames 1043) seed lines were planted into moist soil media (soil, perlite, vermiculture, and fertilizer) and grown at 21°C under a 16h day/8h night light cycle in greenhouses or growth chambers. Mature seeds were harvested, dried thoroughly, and stored at room temperature until further analyzed. Lines were crossed by emasculating green flower buds of the pollen recipient and then applying pollen from anthers removed from the donor plant.

2.2. Creation of the mutant population

A mutant population of camelina accession Ames 1043 was produced following methods described for Arabidopsis (Till et al. 2003). Treatment with 30 mM ethyl methanesulfonate (EMS) was for 17 h at room temperature using mild agitation. The M_1 seed was rinsed five times in water and ~7,000 were sowed in an open field. Seed was harvested from each individual M_1 plant and a single M_2 plant was grown per sib family. DNA of each M_2 plant was collected and subjected to TILLING (Targeted Induced Local Lesions IN Genomes) by sequencing to verify mutation density (Tsai et al. 2011). The M_3 seed was harvested individually from each M_2 plant. One thousand families (M_3 seed bags) were selected at random from the whole population and used for the experiments described here. According to the high mutation density observed (higher than 1/50kb), we expected that 1,000 families would carry at least a null mutation for the average gene and several missense mutations (Comai and Henikoff, 2006).

2.3. Screening of the mutant population

Fatty acid methyl esters (FAMEs) were prepared by transesterification with trimethylsulphonium hydroxide (TMSH; (Butte, 1983)). Single transgenic seeds were directly crushed in 50 µL of TMSH in glass GC vials. Heptane (450 µL) was added to each vial. After room temperature incubation with agitation for 30 min, FAMEs were quantified on an Agilent Technologies 7890A gas chromatograph fitted with a flame ionization detector and a 30 m length×0.25mm inner diameter HP-INNOWax column (Agilent, CA, USA) using helium as the carrier gas. The oven temperature was set to start at 90°C with a 1 min hold and then an increase of 30°C/min until it reached 235°C, for 5 min (Kim et al., 2015).

2.3. Fatty acid quantification of camelina seed

FAMEs were generated from ~10mg of dry camelina seeds using a previously established acid catalyzed method, with triheptadecanoin (Nu-Check Prep, MN) added as an internal standard (Miquel and Browse, 1992; Kim et al., 2015). The organic phase was transferred to autosampler vials and FAMEs quantified using gas chromatography as described above.

2.4. Identification and isolation of mutant alleles

Genomic DNA was extracted from young leaf tissue using the DNeasy Plant Mini kit (QIAGEN Sciences, MD, USA). Gene candidates were amplified with PCR using Phusion High-Fidelity DNA polymerase (Fisher Scientific) as per the manufacturer's protocol and primer pairs specific for all three gene homeologs (Supplemental Table 1). PCR products were ligated into pCRBlunt or pGEM-T Easy vectors (Invitrogen, CA, USA) and transformed into DH5α chemical competent *Escherichia coli* cells. Plasmid DNA was isolated from individual colonies and sequenced to identify mutant alleles. Because *FAE1A, FAE1C* and *FAD2A* lack introns, these cloned genomic sequences were also used for subsequent expression experiments. To isolate cDNA for *FAD3A*, which does contain introns, RNA was extracted from developing wild type

and CS2864 line seeds using a cetyltrimethylammonium bromide (CTAB) method (Bekesiova et al., 1999). Briefly, 1.4mls of RNA extraction buffer, which also included 0.5g/L spermidine, was added to the ground tissue and incubated for 30 min at 65°C with occasional mixing. 8M LiCl was used to precipitate the RNA. 45 0µl Buffer RLT from the RNeasy Plant Mini kit (QIAGEN Sciences, MD, USA) was added to the RNA and steps 4-11 of the kit were followed. 1µg RNA was treated with DNase and cDNA was synthesized using the RevertAid RT Reverse Transcription kit (Thermofisher Scientific, MA, USA). *FAD3A* homeologs were then amplified, cloned into pCRBlunt and then identified by sequencing.

2.5. Yeast expression

The open reading frames for wild type and mutant alleles of *FAE1A, FAE1C* and *FAD2A* were ligated into the *Eco*RI site of the yeast expression vector pYES2 (Invitrogen, CA, USA) under the control of the inducible *GAL1* promoter. Wild type *FAD3A* or the mutant *fad3a* allele were ligated into the *Bam*HI/*Xho*I sites of the yeast expression vector pESC-URA under control of the *GAL1* promoter; wild type *FAD2A* was cloned downstream of the *GAL10* promoter to enable expression of both genes. The pYES2 and pESC-URA plasmids were transformed into *Saccharomyces cerevisiae* yeast strain BY4741 using the Frozen-EZ Yeast Transformation II Kit (Zymo Research, CA, USA) and selected on minimal agar plates lacking uracil. Single colonies were inoculated into synthetic complete minimal medium lacking uracil (SC-Ura) which contained 2% raffinose as the exclusive carbon source and grown at 28 °C for 48 hours with shaking at 250 rpm. Cells were harvested, washed with sterile water and diluted to 0.2 OD_{600} in SC-Ura induction medium containing 2% galactose. After a further 48 h growth at 28 °C for the *FAE1* vectors and 96 hour growth at 20 °C for the *FAD2* and *FAD3* vectors, the yeast cells were pelleted and washed three times with water to remove media or other metabolites, cells were

dried in a freeze dryer and stored at -80°C until lipids were extracted and analyzed. Fatty acid methyl esters (FAMEs) were generated by resuspending dried yeast cells in 1.5ml of 2.5% sulfuric acid (H_2SO_4, v/v) in methanol and heated for 30 min at 90 °C in 13 x 100 mm glass-screw-capped tubes. Following cooling, 1.5ml of water and 2ml of heptane were added to the tubes and mixed. The organic phase was transferred to autosampler vials and analyzed on an Agilent Technologies 7890A gas chromatograph as for the camelina FAMEs (see Sec. 2.3; Kim et al., 2015)).

2.6. Camelina mutant complementation

pCRBlunt clones containing cDNAs for the *FAE1A*, *FAE1C, FAD2A* or *FAD3A* wild type alleles were amplified by PCR, digested with *Eco*RI/*Xho*I, and ligated under control of the CaMV35S promoter into the corresponding sites in the binary plant vector pBin35SRed, a variant of the previously described pBinGlyRed3 (Nguyen et al., 2015; Nguyen et al., 2013) with the glycinin-1 promoter replaced with a CaMV35 promoter. This vector also contains the DsRed (*Discosoma* coral) gene under control of the cassava mosaic virus 35S promoter as the seed selection marker. The resulting vectors were transformed into *Agrobacterium tumefaciens* strain C58CI. Transgenic camelina lines were generated using an *Agrobacterium* mediated vacuum method and DsRed-positive seeds were identified using a green LED flashlight with a red camera filter lens (Lu and Kang, 2008). FAMEs were prepared by transesterification with TMSH and quantified on an Agilent Technologies 7890A gas chromatograph as described above (Sec 2.3).

2.7. Genotyping using CAPS/dCAPS

CAPS (Cleaved Amplified Polymorphic Sequences) (Konieczny and Ausubel, 1993) or dCAPS (Derived Cleaved Amplified Polymorphic Sequences) (Neff et al., 1998) markers were designed to distinguish the different mutant alleles from the wild type genes. The dCAPS assays were

198 designed using the dCAPS Finder 2.0 software (Neff et al., 2002). Primer sequences and
199 restriction enzymes used for each assay are listed in Supplemental Table S2.

200 **2.8. Oxidative Stability Index calculations**

201 Oxidative stability index (OSI) was calculated based on bis-allylic position equivalences (BAPE)
202 that have been shown to correlate well with experimental values (Knothe and Dunn, 2003).
203 BAPE for different fatty acid compositions were calculated based on the 18:2 and 18:3 content
204 (Knothe, 2002).

205

206 **3. RESULTS AND DISCUSSION**

207 **3.1. Isolation of camelina mutants with altered fatty acid composition**

208 We analyzed M_3 seeds from a *Camelina sativa* (cultivar Ames 1043) mutant population created
209 by EMS treatment for altered fatty acid composition using gas chromatography (GC). Among the
210 1,000 lines analyzed, we identified four different phenotypic classes of interest: 1) more 18:1 and
211 less 20:1 than wild type, suggesting possible mutations in *FAE1*, 2) more 18:1 and less 18:2 than
212 wild type, suggesting possible mutations in *FAD2*, 3) more 18:2 and less 18:3 than wild type,
213 suggesting possible mutations in *FAD3*, and 4) more 16:0 and less 18:0 than wild type,
214 suggesting possible mutations in *FAB1* encoding KASII. One TILLING line from each of the
215 four mutant classes was chosen for further analysis. Specific lines were selected based on the
216 magnitude of their fatty acid compositional phenotype, as well as whether they most closely
217 resembled the growth of wild type plants when grown together in the greenhouse (Supplemental
218 Figure S1). M_2 seeds from the four chosen lines were planted and allowed to self-pollinate. We
219 analyzed the fatty acid content and composition of the resulting M_3 seeds to confirm the
220 heritability of the phenotype observed in the M_2 seed (Figure 2A). Seeds from line CS2901 had
221 9.9% C20:1, lower than the 12.8% found in wild type, but 14.5% 18:1, approximately double the

levels present in wild type (9.2%). In addition, line CS2901 had a significant reduction in 22:1 (1.7%) compared to the 3.9% found in wild type. Seeds from line CS2362 had decreased amounts of 18:2 (17.4%) compared to wild type (21.5%), with an increase of 18:1 from 9.2% to 16.4% in the mutant. Seeds from line CS2864 had decreased amounts of 20:1 (8%) compared to wild type (12.8%) and increased amounts of 18:1 (15%) compared to wild type (9.2%). This mutant also had a significant decrease of 18:3 (18.4%) compared to wild type (33.7%) and a significant increase in the amount of 18:2 (37.9%) compared to wild type (21.5%). In addition, this line had a significant reduction in 22:1 (1%) compared to wild type (3.9%). Seeds from line CS1996 contained 9.8% 16:0 compared to 7.2% in wild type. Differences in total fatty acid content were observed in seeds of some of the lines, particularly for CS2864 which was ~80% that of wild type (Figure 2B).

3.2. Identification of *FAE1*, *FAD2*, *FAD3* and *FAB1* mutations

Based on its reduced levels of 20:1, we hypothesized that CS2901 contained a mutation in one of the *FAE1* homeologs encoding part of the enzyme complex that catalyzes VLCFA synthesis by elongation of 18:1 to 20:1. Arabidopsis *fae1* mutants synthesize very little VLCFA and have increased amounts of 18:1 (James Jr, 1990; Lemieux et al., 1990). Further, the simultaneous mutagenesis of all three camelina *FAE1* homeologs using CRISPR-based genome editing results in 20:1 levels of less than 1% with concomitant increases in 18:1 (Ozseyhan et al., 2018). Cloning and sequencing of the three camelina *FAE1* homeologs revealed that CS2901 had wild type alleles of *FAE1A* (*Csa11g007400*) and *FAE1B* (*Csa10g007610*), but *FAE1C* (*Csa12g009060*) contains a C625T mutation (Supplemental Figure S2B) which replaces an arginine residue with a stop codon (Figures 3A and S2C). This nonsense mutation results in a

244 truncated protein lacking the region highly conserved among condensing enzymes in VLCFA
245 biosynthesis (Moon et al., 2001).

246 Due to its higher 18:1 levels, we suspected that CS2362 had a mutation similar to that
247 affecting the activity of FAD2, the Δ12-desaturase that catalyzes the synthesis of 18:2 from 18:1
248 in Arabidopsis (Okuley et al., 1994). Similar to CS2362, Arabidopsis *fad2* mutants possess
249 increased levels of 18:1 and lower levels of 18:2 and 18:3 (Lemieux et al., 1990; Okuley et al.,
250 1994). Likewise, genome edited camelina lines with targeted mutations in multiple *FAD2*
251 homeologs show higher levels of 18:1 and reduced PUFA content (Jiang et al., 2017; Morineau
252 et al., 2017). Sequencing of all three camelina *FAD2* homeologs demonstrated that CS2362
253 possessed wild type alleles of *FAD2B* (*Csa15g016000*) and *FAD2C* (*Csa19g016350*), but
254 *FAD2A* (*Csa01g013220*) contained a G449A nucleotide change (Supplemental Figure S3A),
255 which results in the changed of a conserved glycine residue to glutamate (Figure 3B). This
256 G150E mutation lies close to the second of three conserved histidine boxes that are critical to the
257 function of the enzyme (Tocher, *et al*., 1998; Figures 3B and S3B) and is predicted by
258 PROVEAN (Protein Variation Effect Analyzer) to be deleterious (Choi et al., 2012).

259 The reduced levels of 18:3 in Arabidopsis *fad3* mutants are caused by mutations in the
260 Δ15-desaturase (Arondel et al., 1992; Yadav et al., 1993). Line CS2864 was therefore suspected
261 of having a mutation in one the *FAD3* homeologs, based on its lower levels of 18:3. CS2864
262 possessed wild type alleles of *FAD3C* (*Csa05g033930*) and *FAD3B* (*Csa07g013360*), but
263 *FAD3A* (*Csa16g014970*) contained a G301A mutation (Supplemental Figure S4A), resulting in a
264 G101S substitution. This mutation affects a conserved residue present in the second
265 transmembrane domain of *FAD3* and is located adjacent to one of the histidine boxes important
266 for enzyme function ((Rodríguez-Rodríguez et al., 2016); Figures 3C and S4B). The decreased

levels of C20:1 in seeds of CS2864 suggested that this line might also have a mutation in one of the *FAE1* homeologs. Sequencing demonstrated that *FAE1A* in CS2864 contained a C422T nucleotide change (Supplemental Figure S2A) with the consequent replacement of a conserved proline residue with leucine (Figures 3C and S2C). *FAE1B* and *FAE1C* did not possess any mutations in this line. The affected proline is conserved and leucine substitution is predicted by PROVEAN to be deleterious.

Similar to Arabidopsis *fab1* mutants, the increased C16:0 content of CS1996 suggested that this line contained a mutation affecting the activity of KASII (Carlsson et al., 2002; Wu et al., 1994). Indeed, sequencing demonstrated a C1425T mutation in the fifth exon of *FAB1C* (Csa09g079550) resulting in a P269L substitution (Supplemental Figure S5). The other *FAB1* homeologs Csa16g038860 (*FAB1A*) and Csa07g046400 (*FAB1B*) contained no mutations. The mutated proline residue of *fab1c* is highly conserved in orthologs from diverse species, including *fabF* from *E. coli* (Figure 3D), suggesting an essential function for this particular residue. Further, the P269L substitution is predicted by PROVEAN to be deleterious.

The partial changes in fatty acid composition in these camelina mutants compared to orthologous mutations in Arabidopsis reflect the fact that the three camelina subgenomes are highly related and undifferentiated (Kagale et al., 2014). Previous work has demonstrated that all three *FAE1* and *FAD2* homeologs are expressed in developing seeds (Hutcheon et al., 2010). Consistent with this, and similar to our results, camelina lines with mutations in individual *FAE1* or *FAD2* homeologs only possessed a partial phenotype whereas lines with mutations in multiple homeologs presented a stronger phenotype (Kang et al., 2011; Morineau et al., 2017; Ozseyhan et al., 2018).

3.3. Functional expression of wild type and mutant alleles in yeast cells

290 The wild type and mutated alleles of *FAE1A*, *FAE1C*, *FAD2A* and *FAD3A*
291 were expressed in yeast to determine if the mutations had an effect on the activity of the
292 enzymes and therefore were responsible for the altered fatty acid composition observed in the
293 mutant lines. In the control yeast cells transformed with the empty pYES2 vector, no VLCFAs
294 were produced (Figure 4E). However, yeast cells expressing wild type *FAE1A* were able to
295 elongate 18:0 into arachidic (20:0) and behenic (22:0) acids, as well as 18:1 into 20:1 and C22:1,
296 consistent with the activity of FAE1 (Figure 4A). However, when the *fae1a* mutant allele from
297 CS2864 was expressed, we did not detect any VLCFA (Figure 4A). Similarly, the expression of
298 the wild type *FAE1C* allele resulted in 20:1 synthesis, but not with the expression of the *fae1c*
299 mutant allele from CS2901 (Figure 4B). These results suggest these two camelina mutant *fae1*
300 alleles encode non-functional enzymes.
301 Normally no polyunsaturated acids are produced by *S. cerevisiae* (Figure 4E, F).
302 However, cells expressing wild type *FAD2A* synthesized hexadecadienoic acid (16:2) and 18:2
303 from the action of the Δ12-desaturase on palmitoleic acid (16:1) and 18:1, respectively. In
304 contrast, no 16:2 or 18:2 was detected with the expression of the *fad2a* mutant allele from
305 CS2362 (Figure 4C) suggesting the encoded desaturase lacks activity.
306 In the yeast cells expressing wild type copies of both *FAD2A* and *FAD3A*, there is
307 production of 16:2 and 18:2 from the activity of the FAD2A Δ12 desaturase and then conversion
308 of 18:2 to 18:3 due to the activity of FAD3A. However, when the mutant *fad3a* allele from
309 CS2864 is expressed in combination with *FAD2A*, there is a major reduction of 18:3, suggesting
310 this mutation substantially affects the activity of the encoded enzyme (Figure 4D).
311 **3.4. The *FAB1* mutant allele segregates with increased 16:0 content**

KASII functions in a Type II fatty acid synthase in plant cells, complicating its expression in yeast, which uses a Type I fatty acid synthase. To confirm that the *fab1c* allele in CS1996 is responsible for the high 16:0 phenotype of the mutant seed, we backcrossed the mutant line to the Ames 1043 wild type background. When we genotyped 18 F_2 plants, 14 possessed the C1425T mutation and 4 were wild type (Figure 5A). As the *fab1c* dCAPs marker (Supplemental Table S2) is unable to distinguish the three highly identical camelina *FAB1* homeologs, we could not differentiate plants that were homozygous for the mutation from those that were heterozygous. Quantification of 16:0 levels in the F_3 seed harvested from these plants revealed that the F_2 plants with a wild type genotype possessed lower levels of 16:0, comparable with those of control wild type seeds (Figure 5B). Seed from six of the F_2 plants containing the C1425T *fab1c* mutation had levels of 16:0 similar to that of CS1996 plants. Eight F_2 plants produced seed containing levels of 16:0 intermediate between the CS1996 and wild type controls. We hypothesized that the high 16:0 plants were homozygous for the *fab1c* mutation, with the intermediate 16:0 plants being heterozygous. We therefore genotyped F_3 progeny of selected plants to discover whether they were homozygous or heterozygous for the mutation. From two plants producing high levels of 16:0, all derived F_3 plants contained the C1425T mutation (Supplemental Figure S6). The result demonstrates that that the F_2 parents are homozygous for the mutation, consistent with the high 16:0 phenotype. F_3 plants derived from the seed containing intermediate amounts of 16:0 segregated 3:1 for progeny possessing the mutation versus those that only possess wild type homeologs of *FAB1*. Therefore, these F_2 plants were heterozygous for the mutation, explaining the intermediate C16:0 levels.

3.5. Complementation of altered fatty acid composition in Camelina TILLING lines

334 To determine if the altered fatty acid composition in mutant lines could be complemented
335 or restored to wild-type amounts, we cloned cDNAs for wild type alleles of *FAE1A*, *FAE1C*,
336 *FAD2A* or *FAD3A* under the control of the seed-specific glycinin-1 promoter. The fluorescent
337 protein DsRed was used as the selectable marker (Lu and Kang, 2008). Each mutant line was
338 transformed with the pertinent binary construct by vacuum infiltration of *Agrobacterium*
339 *tumefaciens*. Five T_1 red seeds were then selected for fatty acid composition analysis by GC, as
340 well as seeds from the mutant line transformed with the empty binary vector and from wild type
341 plants grown at the same time. The mutant line CS2864, containing the *fae1a* allele, had around
342 10% VLCFA (20:1+22:1), lower than wild type levels of 12%. In contrast, seeds of four of the
343 five T_1 lines contained levels higher than the mutant and two of them levels above wild type, up
344 to 16% (Figure 6A). Similarly, expression of a wild type copy of *FAE1C* in CS2901 increased
345 the levels of VLCFA from 11% up to 18% in the five T_1 lines (Figure 6B). The PUFA
346 (18:2+18:3) levels of CS2362, which contains a mutation in *FAD2A*, are 44% compared to wild
347 type levels of 52%. All five T_1 lines expressing *FAD2A* contained levels higher than the mutant,
348 with four of them having levels above wild type, up to 62% (Figure 6C). Likewise, the reduced
349 18:3 content of seeds from CS2864, containing the *fad3a* mutant, was complemented in four of
350 the five T_1 lines expressing *FAD3A*, with one line containing 18:3 levels up to 49% (Figure 6D).
351 These results further confirm that the mutant alleles are responsible for the changes in seed fatty
352 acid composition seen in the camelina mutants.

353 **3.6. Combining mutations to create mid-oleic camelina lines**

354 An oil with increased oleic and decreased PUFA content is more desirable as a biodiesel
355 feedstock because of the improved oxidative stability and ignition quality conferred by these
356 changes in fatty acid composition (Durrett et al., 2008). Likewise, a decrease in VLCFAs allows

357 better cold flow temperature properties (Lin and Lee, 2017). An improved fatty acid composition
358 such as this can be achieved by downregulating FAE1, FAD2 and FAD3 activity. We therefore
359 crossed the *fae1c* mutant, CS2901, with the *fad2a* mutant, CS2362. F_3 plants homozygous for
360 both mutations were identified by genotyping (Supplemental Figure S7) and the fatty acid
361 phenotype confirmed in the seed (Figure 7A). The *fae1c/fad2a* mutant cross has decreased levels
362 of 20:1,18:2 and 18:3 due to the two combined mutations, thereby increasing the amount of oleic
363 acid from 9% in wild type to 34% in the new line (Figure 7A). The reduced PUFA content in the
364 *fae1c/fad2a* line results in an increased oxidative stability index (OSI), as calculated according to
365 the bis-allylic position equivalences (BAPE) of the fatty acid composition (Knothe and Dunn,
366 2003). Here, the OSI for oil from *fae1c/fad2a* resulted in an OSI of 0.90, much greater than that
367 for wild type camelina oil, which is slightly negative (Figure 7B).
368 To further alter seed fatty acid composition, we crossed the *fae1c/fad2a* line with
369 CS2864, which contains mutations in *FAE1A* and *FAD3A*. Plants homozygous for all four
370 mutations were identified in the F_5 generation by genotyping (Supplemental Figure S7).
371 Consistent with previous work showing the additive effect of the expression of different
372 camelina homeologs, the amount of 20:1 was reduced even further with the combination of the
373 *fae1a* allele with *fae1c* (Figure 7A). Levels of 18:3 were reduced even further with the addition
374 of the *fad3a* mutant alleles, raising the amount of oleic acid to 43%. Consequently, the calculated
375 OSI for the quadruple mutant combination increased to 1.28 (Figure 7B). The *fae1c/fad2a* line
376 was slightly smaller than wild-type plants when grown in the greenhouse, but the quadruple
377 mutant appeared phenotypically normal (Supplemental Figure S1). Both lines containing mutant
378 combinations demonstrated slight reductions in total seed fatty acid content (Figure 7C).
379 Decreases in seed oil content have been observed in canola (*Brassica napus*) lines producing

very high levels of oleic acid (Bai et al., 2019). However, as these effects were only noted with 18:1 levels above 70%, it is unlikely that the changes in fatty acid composition in the mutant combinations are directly responsible for the reduced fatty acid content. Instead, these reductions and the minor changes in appearance may be the result of other EMS induced mutations in the genome not related to the ones in this study. Future work backcrossing these lines to eliminate such mutations will help clarify this question.

4. CONCLUSIONS

Improving the oil quality of camelina in a targeted manner is necessary for its use in different applications. For food processing purposes, oil with increased levels of saturated fatty acids, such as 16:0, improve the shelf life of vegetable oils and can act as replacements for hydrogenated vegetable oils (Kok el al., 1999; Shen et al., 1997). Similarly, the increased oxidative stability of high-oleic oils makes them valuable not only for cooking and frying, but also as biofuels and bio-based lubricants. We screened a camelina EMS mutant population and identified lines with altered seed fatty acid composition. By taking advantage of camelina's close similarity to Arabidopsis and information about orthologous mutant phenotypes in that model species (Kagale et al., 2014; Kagale et al., 2016; Nguyen et al., 2013), we were able to rapidly identify the mutant genes. The combination of different mutations resulted in different camelina lines producing oil with increased 16:0 content, or with reduced PUFA and VLCFA content. As the latter oil is predicted to have increased oxidative stability, this example serves to illustrate the utility of the mutant populations to improve camelina oil quality through a genetic approach. We acknowledge that the mutants and mutant stacks from our studies will require additional back-crossing to remove secondary mutations from EMS mutagenesis as well as possible crossing to

403 elite high-yielding germplasm or germplasm better adapted to specific geographic regions. These
404 efforts will be facilitated by the CAPS/dCAPS markers developed in this study for each mutant
405 allele. While similar results have been achieved by our groups and others using CRISPR/Cas9
406 genome editing (Jiang et al., 2017; Morineau et al., 2017; Ozseyhan et al., 2018) or RNAi
407 suppression (Nguyen et al., 2013), potential limitations on the large scale cultivation of lines
408 obtained through these approaches exist due to concerns about intellectual property freedom to
409 operate status with CRISPR-Cas9 gene-editing and the high cost of de-regulation of transgenic
410 germplasm derived from RNAi . Furthermore, while US regulatory agencies have long indicated
411 that plants generated using genome editing technologies but lacking transgenic DNA will not be
412 regulated by the agency (Wolt et al., 2016), the recent decision from the European Union to
413 classify plant lines altered using these new technologies as genetically modified, has limited the
414 commercial development of such modified crops (Wight, 2018). In contrast, the use of EMS-
415 generated mutant lines should be free of such IP and regulatory barriers to more quickly advance
416 the traits described here for commercial application.
417

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6. FIGURE LEGENDS

Figure 1. Fatty acid synthesis and desaturation pathways in plants. A simplified overview of fatty acid synthesis and desaturation pathways in developing seeds, with a focus on enzymes relevant to this study. ACP, acyl carrier protein; CoA, Coenzyme A; ER, endoplasmic reticulum; FAS, fatty acid synthase complex; FAE1, FATTY ACID ELONGASE1; FAD, FATTY ACID DESATURASE; KASII, β-ketoacyl-ACP synthase II; VLCFA, very long chain fatty acid; PC, phosphatidylcholine; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α-linolenic acid.

Figure 2. Altered fatty acid composition of camelina mutant lines. Fatty acid composition (A) and content (B) of M_4 seed from camelina mutant lines. Fatty acid composition is expressed as the weight percentage (wt%) of each fatty acid relative to the total weight of fatty acids. Fatty acid content is expressed as the wt% of total fatty acids relative to the weight of the seed sample. Data represents the mean ± standard deviation of at least three biological replicates. *, $P < 0.05$, ** $P < 0.01$ (Student's *t* test).

Figure 3. Mutations in fatty acid synthesis genes result in changes to conserved amino acids. Amino acid alignments of regions of the FAE1C (A), FAD2A (B), FAD3A and FAE1A (C), and KASII (D) homeologs from *Camelina sativa* with orthologs from *Arabidopsis thaliana, Oryza sativa, Sorghum bicolor, Chlamydomonas reinhardtii, Escherichia coli* and

448 *Staphylococcus aureus*. Black triangles indicate the mutations present in the alleles in the
449 different mutant lines; asterisks denote nonsense mutations. Conserved amino acids are shaded
450 black and similar amino acids are shaded gray. Red boxed regions represent conserved histidine
451 boxes present in fatty acid desaturases. GenBank accession numbers for the proteins used in the
452 alignments are located in Supplemental Table 2.
453
454 **Figure 4. Camelina mutant alleles are non-functional when expressed in yeast.**
455 Gas chromatograms showing the fatty acid composition of total lipids extracted from yeast
456 transformed with wild type or mutant alleles of *FAE1A* (A), *FAE1C* (B), *FAD2A* (C) or *FAD3A*
457 (D). For A, B and C, pYES2 was used as the empty vector (EV) controls (E). For D, the empty
458 vector used was pESC-URA (F) and *FAD2A* was co-expressed with *FAD3A* or *fad3a*.
459
460 **Figure 5. The *fab1c* allele segregates with high palmitate content.**
461 A) dCAPS genotyping to detect the *fab1c* allele in a segregating F_2 population resulting from the
462 backcrossing of CS1996. Black triangles indicate the absence of the *fab1c* allele. B) Palmitate
463 (16:0) content of the F_3 seed derived from the genotyped plants in (A), expressed as the weight
464 percentage (wt%) of 16:0 relative to the total weight of fatty acids. Dark purple bars represent
465 the CS1996 parent line, light purple bars indicated F_2 plants possessing a *fab1c* allele, purple-
466 grey striped bars represent F_2 plants with a wild-type genotype for *FAB1*, and grey bars represent
467 wild-type (Ames 1043) plants. Arrows indicate which lines were further genotyped in the F_3
468 generation to confirm homozygosity or heterozygosity.
469
470 **Figure 6. Complementation of fatty acid biosynthesis gene mutants**

Fatty acid composition of seeds from *Camelina sativa* wild type, CS2864 (*fad3a*/*fae1a*) and CS2864 transformed with pBinGlyRed3/*FAE1A* (A), CS2901 (*fae1c*) with pBinGlyRed3/*FAE1C* (B), CS2362 (*fad2a*) with pBinGlyRed3/*FAD2A* (C) and CS2864 with pBinGlyRed3 (D). The results shown are 20:1+22:1 composition (as weight percent of total fatty acids) of wild type, CS2864 parent and five independent T_1 seeds (A), 20:1+22:1 composition of wild type, CS2901 parent and five independent T_1 seeds (B), 18:2+18:3 composition of wild type, CS2362 parent and five independent T_1 seeds (C), and 18:3 composition of wild type, CS2864 parent and five independent T_1 seeds (D).

Figure 7. Combining mutations results in oil with increased oxidative stability. .

Fatty acid composition (A), oxidative stability index (B) and fatty acid content (C) of seeds from camelina lines created by crossing mutant plants to combine different loss of function alleles. Fatty acid composition is expressed as the weight percentage (wt%) of each fatty acid relative to the total weight of fatty acids. Fatty acid content is expressed as the wt% of total fatty acids relative to the weight of the seed sample. Data represents the mean ± standard deviation of at least three biological replicates. *, $P < 0.05$, ** $P < 0.01$ (Student's *t* test).

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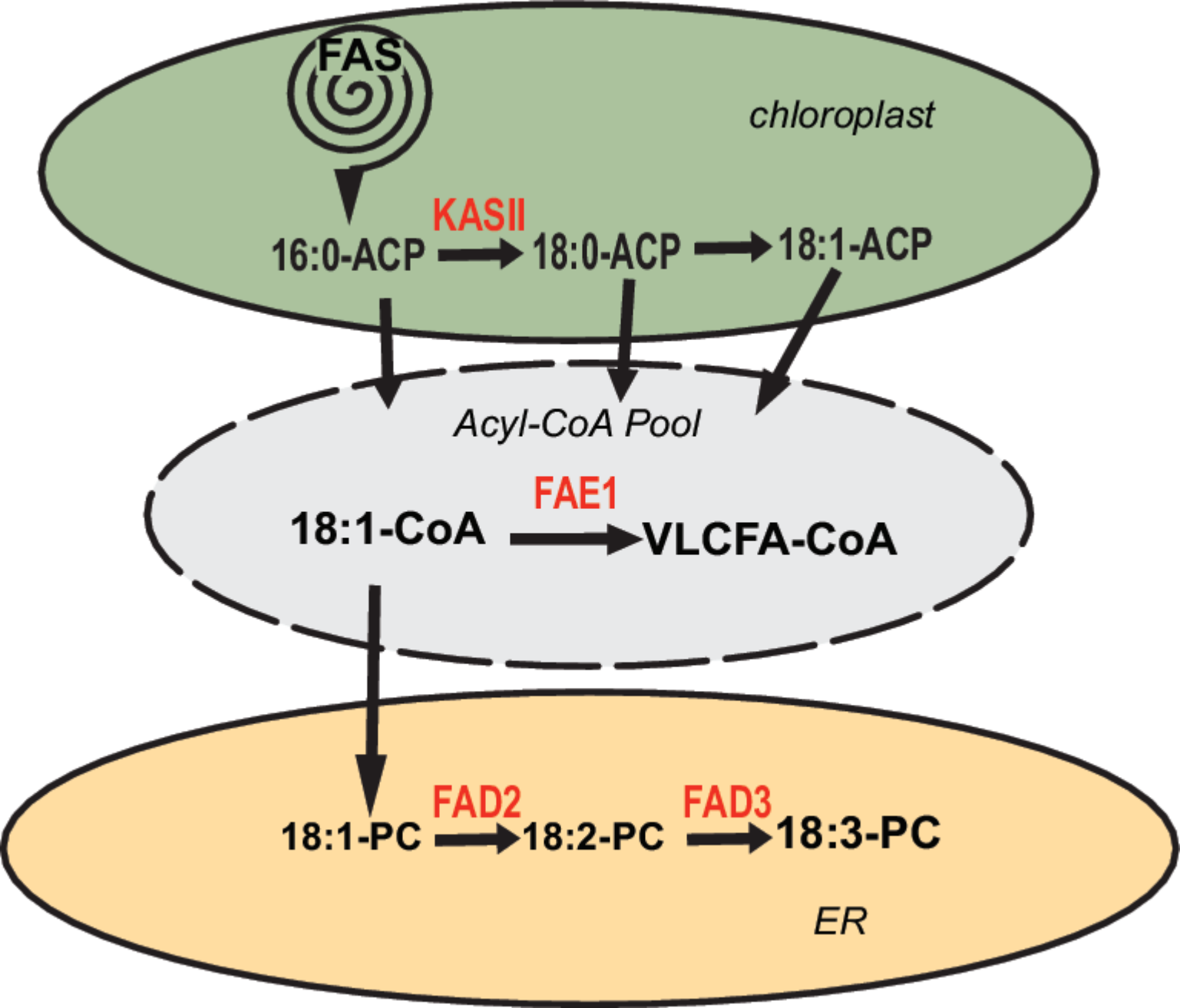
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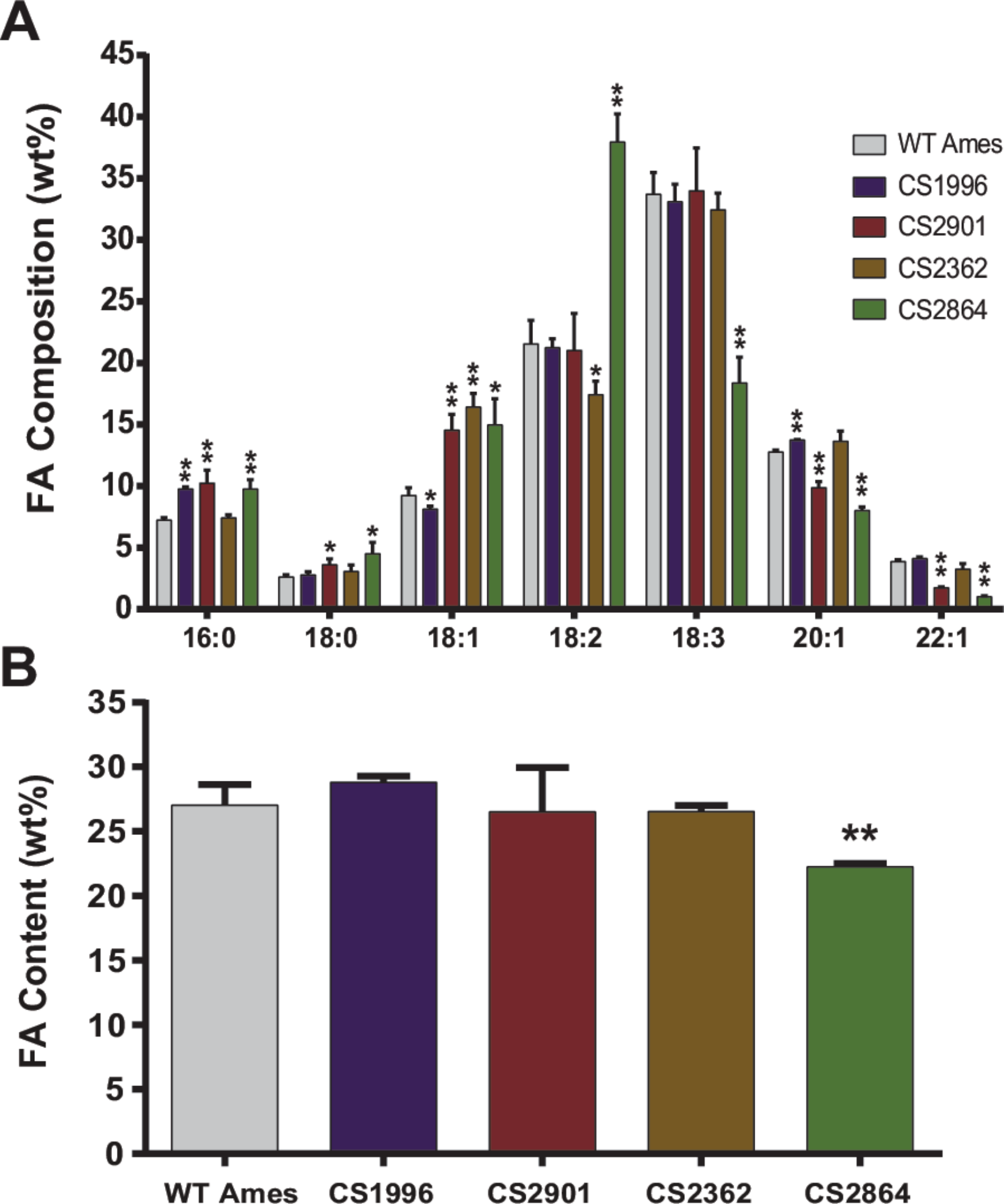
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CS2864
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18:0
18:1
18:2
18:3
20:1
22:1
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FA Content (wt%)
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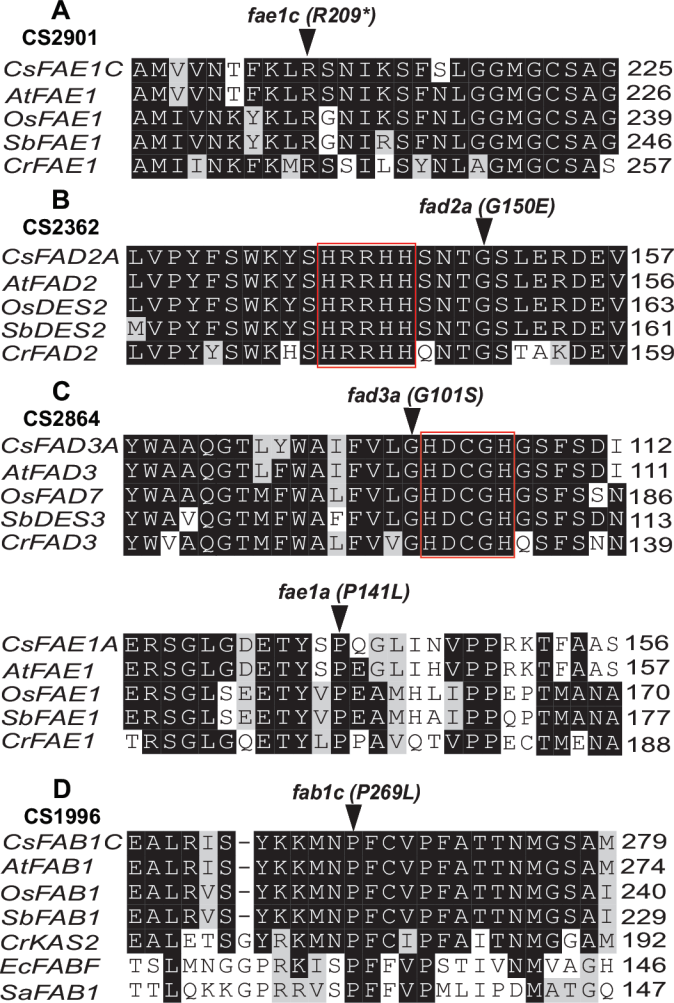
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SbFAE1 AMIVNKYKLRGNIRSFNLGGMGCSAG 246
CrFAE1 AMIINKFKMRSSILSYNLAGMGCSAS 257
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CS2362
fad2a (G150E)
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CS2864
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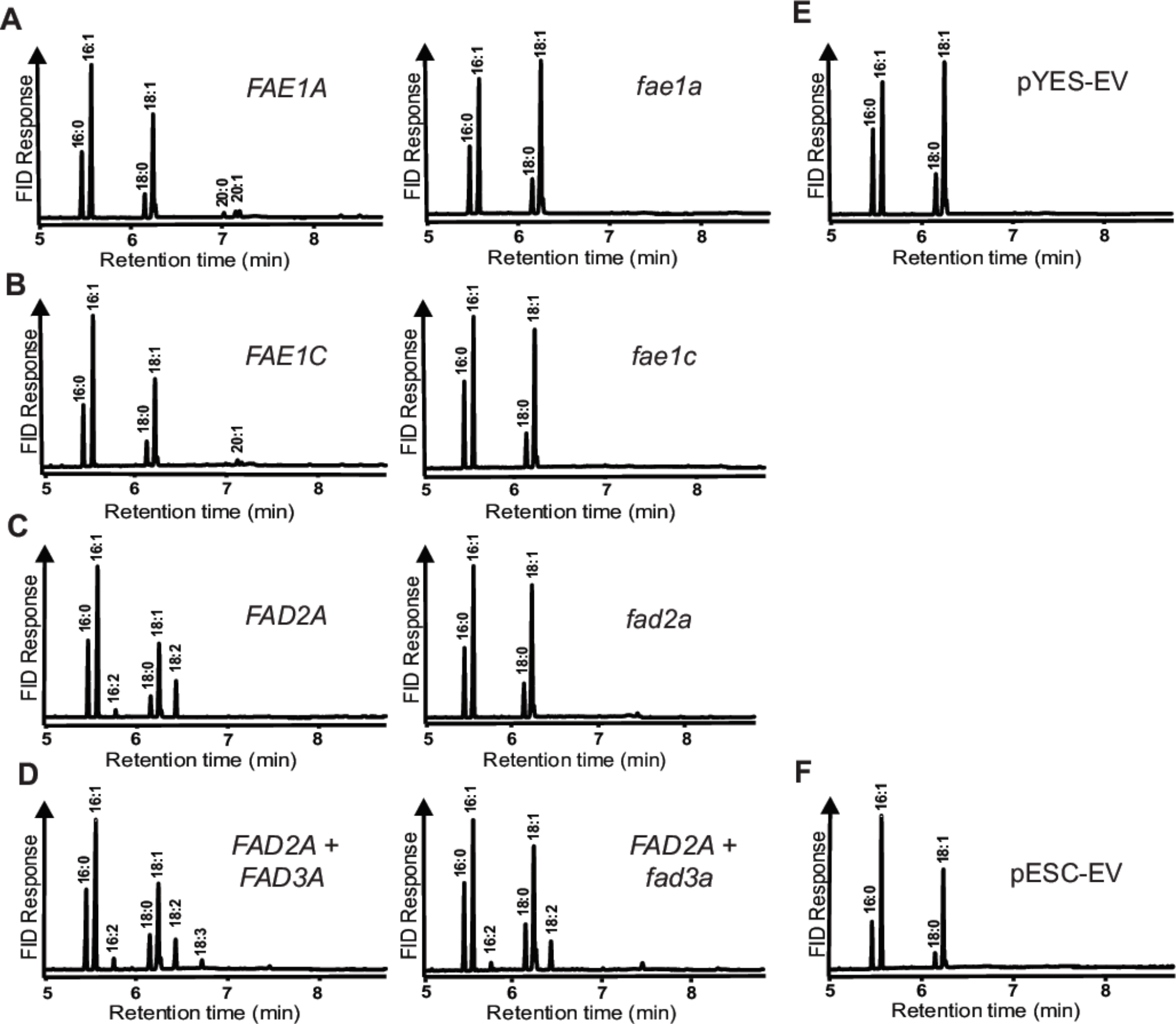
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FAD2A + fad3a
F
pESC-EV
FID Response
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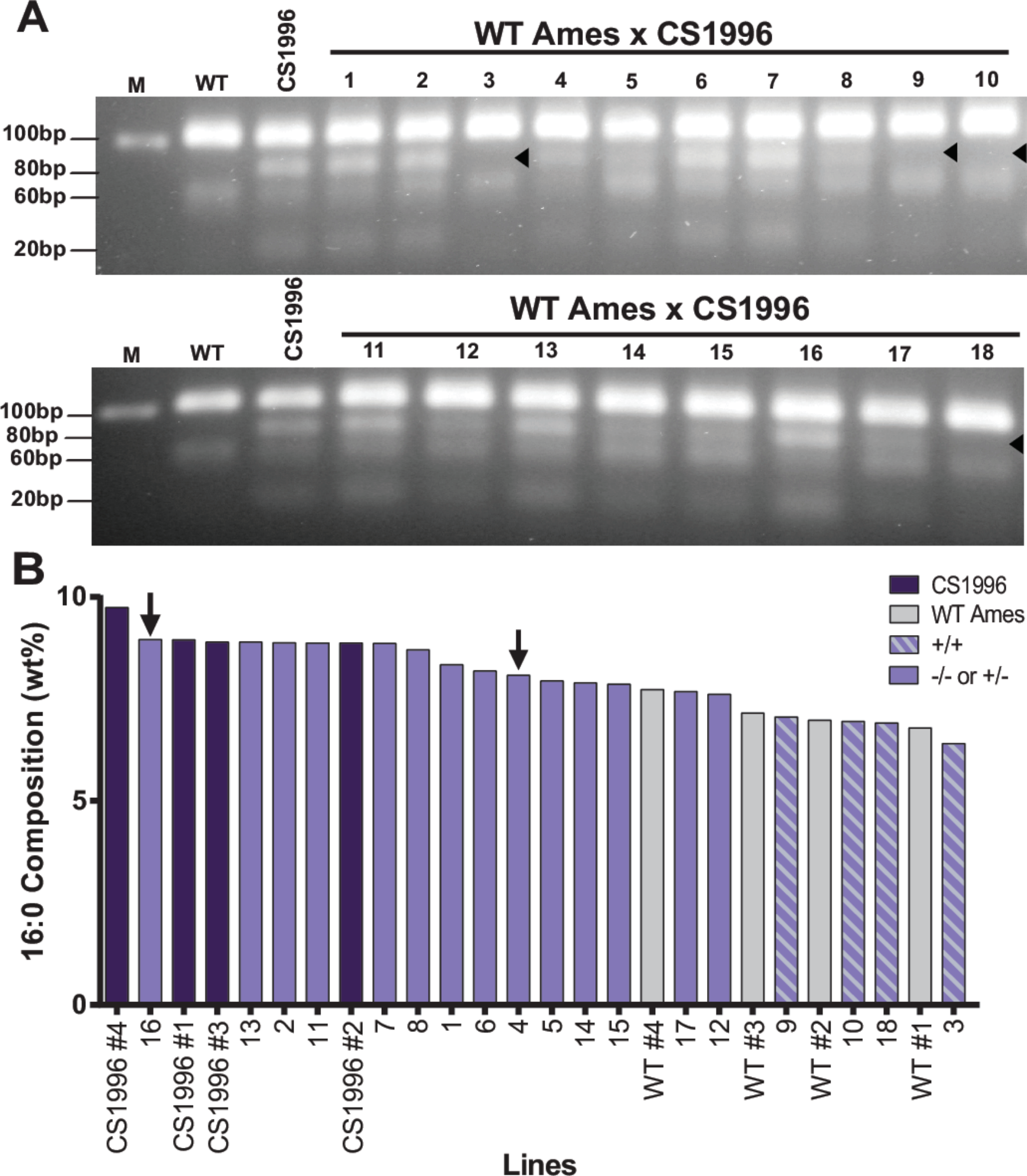
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CS1996
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WT Ames x CS1996
M
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CS1996
11
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100bp
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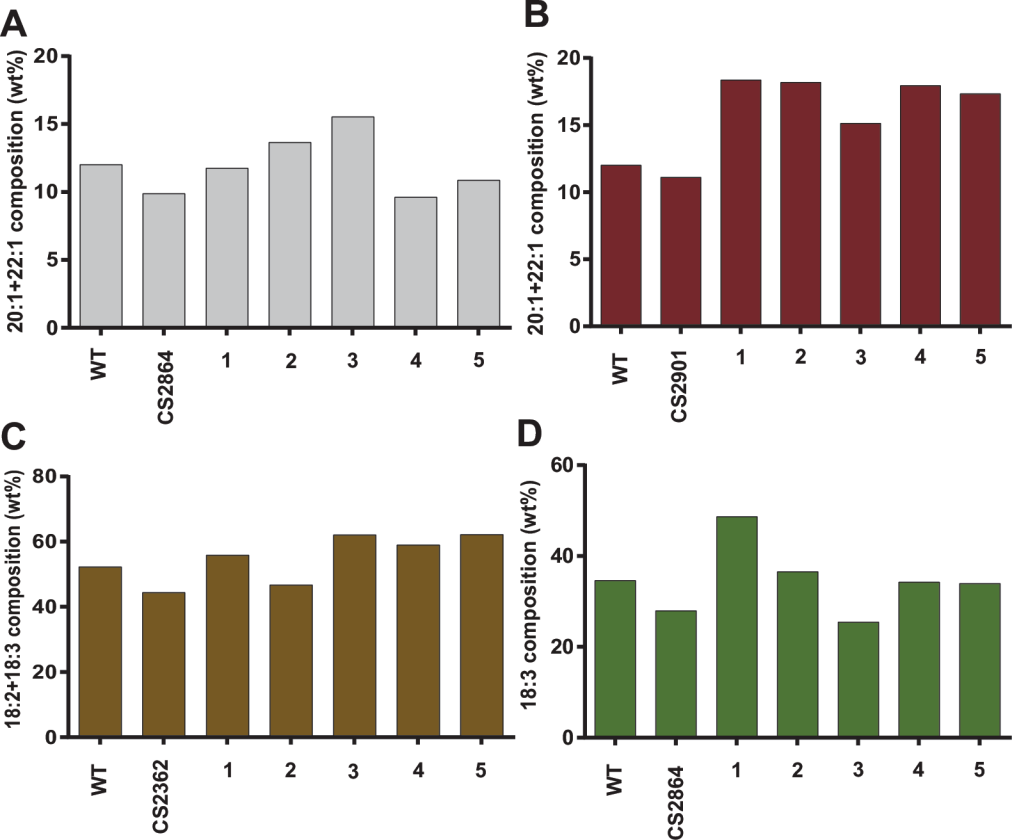
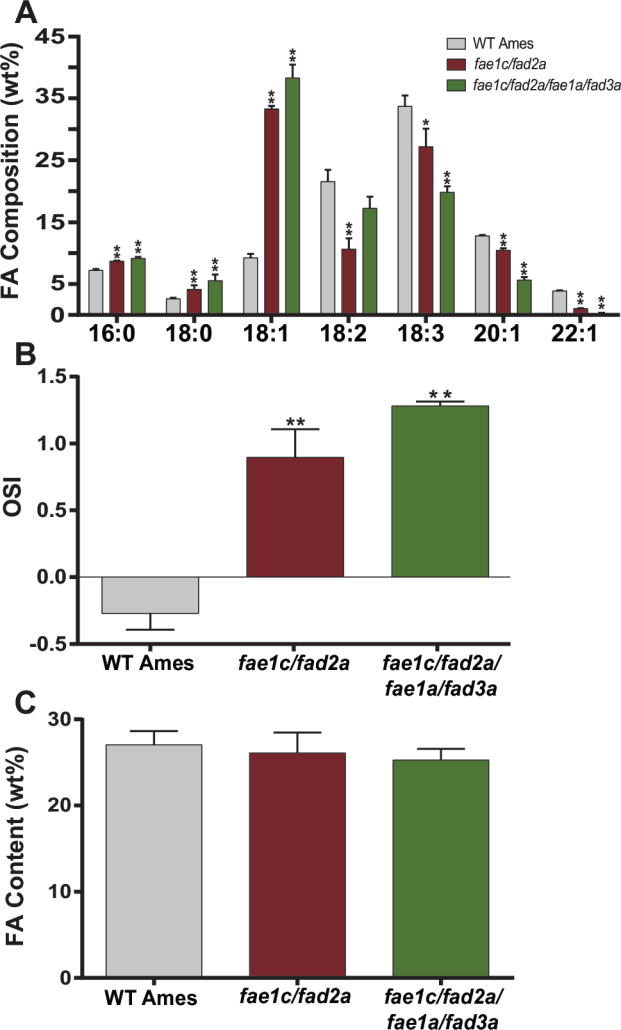
B
CS1996
WT Ames
+/+
-/- or +/-
16:0 Composition (wt%)
10
5
0
CS1996 #4
16
CS1996 #1
CS1996 #3
13
2
11
CS1996 #2
7
8
1
6
4
5
14
15
WT #4
17
12
WT #3
9
WT #2
10
18
WT #1
3
Lines


A
20:1+22:1 composition (wt%)
0
5
10
15
20
WT
CS2864
1
2
3
4
5
B
20:1+22:1 composition (wt%)
0
5
10
15
20
WT
CS2901
1
2
3
4
5
C
18:2+18:3 composition (wt%)
0
20
40
60
80
WT
CS2362
1
2
3
4
5
D
18:3 composition (wt%)
0
20
40
60
WT
CS2864
1
2
3
4
5


A
FA Composition (wt%)
45
35
25
15
5
0
WT Ames
fae1c/fad2a
fae1c/fad2a/fae1a/fad3a
16:0
18:0
18:1
18:2
18:3
20:1
22:1
B
OSI
1.5
1.0
0.5
0.0
-0.5
WT Ames
fae1c/fad2a
fae1c/fad2a/
fae1a/fad3a
C
FA Content (wt%)
30
20
10
0
WT Ames
fae1c/fad2a
fae1c/fad2a/
fae1a/fad3a