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Physaria fendleri FAD3-1 overexpression increases α-linolenic acid content in camelina seeds

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Abstract

Camelina (*Camelina sativa*) is an oil crop with a short growing period, resistance to drought and cold, low fertilizer requirements, and can be transformed using floral dipping. Camelina seeds have a high content of polyunsaturated fatty acids, especially -linolenic acid (ALA) at 32–38%. ALA is an omega-3 fatty acid (FA) that is a substrate for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the human body. In this study, ALA content was further enhanced by seed-specific expression of *Physaria fendleri* (*Pf*) *FAD3-1* in camelina. When *PfFAD3*-1 was introduced into camelina using the seed-specific glycinin promoter, ALA content increased by 48% in T2 seeds and 50% in T3 seeds. In addition, the weight and size of seeds increased. The expression of FA metabolism related genes in *GlyP:PfFAD3-1* transgenic camelina was different than that in the wild type, in which the expression of *Camelina sativa* (*Cs*) *FAD2* decreased and that of *CsFAD3* increased. In summary, we developed a high omega-3 FA containing camelina with up to 50% ALA content by introducing *PfFAD3-1*. This line can be used for genetic engineering to obtain EPA and DHA from seeds.

Introduction

Plant oils are composed of triacylglycerols (TAG), which comprise fatty acids (FAs) that form ester bonds with the glycerol backbone. Plant oils can be used in various ways, such as for food and industrial purposes, depending on their FA composition[1]. Generally, plant oils are mainly composed of five types of FAs—saturated FAs including palmitic acid (PA; 16:0) and stearic acid (SA; 18:0), and unsaturated FAs including oleic acid (OA; 18:1), linoleic acid (LA; 18:2), and α-linolenic acid (ALA; 18:3)[2].

The aim of this study was to enhance the production of omega-3 FAs. The methyl side of FAs is called omega. If there is a double bond at the third carbon of the methyl group, it is called an omega-3 FA, and if it has a double bond at the sixth carbon, it is called an omega-6 FA[3,4]. Omega-3 FAs include ALA, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA)[5]. ALA is an essential FA because it is not synthesized in humans[5]. ALA is found in nut and seed oils, while EPA and DHA are mainly found in fish[4,6]. In the human body, ALA is slightly converted into EPA and DHA. The conversion ratio of ALA to EPA is approximately 8%, but the ratio of ALA to DHA is much lower[7,8]. An adequate ratio of omega-3 to omega-6 FAs (1:1 to 1:4) should be maintained in the human body, depending on the disease condition, by consuming foods rich in omega-3 FAs[9]. However, it is concerning that omega-3 FAs, such as EPA and DHA, are abundant in fish but they contain high levels of mercury. In addition, vegetarians do not eat fish[6]. Therefore, plant-derived omega-3 FAs are a good alternative to fish-derived omega-3 FAs.

FATTY ACID DESATURASE 3 (FAD3) was identified to synthesize ALA in *Arabidopsis thaliana*. FAD3 synthesizes the ALA by desaturation of 18:2, located at *sn-2* of phosphatidylcholine (PC) in the endoplasmic reticulum (ER) membrane[10,11]. Functional studies of the *FAD3* gene have been conducted in various plants including Arabidopsis[12–17]. Recently, *Physaria fendleri FAD3-1* (*PfFAD3-1*) and *PfFAD3-2*

have been expressed in the Arabidopsis *fad3-2* mutant. Among the two *FAD3* genes, only *PfFAD3-1* restored 18:3 content to 29.9% when expressed in the *fad3-2* mutant, which has only 1.6% ALA[12]. The 18:3 content was increased when *PfFAD3-1* was transformed into soybean. The 18:3 content of cultivar 'Kwangankong' was 7.5%, which increased to 52.4% when *PfFAD3-1* was expressed under the β-conglycinin promoter and to 33.3% when expressed under the phaseolin promoter[18].

Camelina (*Camelina sativa*) is emerging as a tool to perform research related to biofuels and other biotechnological applications in oil crops[19–21]. Camelina is an allohexaploid (2n = 6x = 40) plant belonging to the Brassicaceae[22]. Camelina is grown mainly in Europe and North America. It has a short life cycle (85–100 days), low fertilizer requirements, and is resistant to cold and drought[23]. In addition, it can be easily transformed using the floral dipping method[24]. Polyunsaturated fatty acids (PUFAs) constitute approximately 50% of the FA content in camelina seeds; in particular, the 18:3 FA content is 32–38%. The contents of 18:1 FA and 20:1 FA are 12–18% and 12–17%, respectively [23].

A brief description of FA biosynthesis and metabolism in seeds of plants of the Brassicacea, such as camelina, is as follows. FA biosynthesis is initiated by the conversion of acetyl-coenzyme A (CoA) to malonyl-CoA in the plastid by acetyl-CoA carboxylase (ACCase)[25]. Then, malonyl-CoA is converted to malonyl-ACP by malonyl-CoA: acyl carrier protein (ACP) transacylase [26]. Malonyl-ACP is converted to 4:0-ACP by the fatty acid synthase (FAS) enzyme and β-ketoacyl-acyl carrier protein synthase (KAS) III, and it is synthesized as 16:0-ACP by KAS I and FAS enzymes[27]. Thereafter, it is synthesized as 18:0-ACP by FAS and KASII. Stearoyl-ACP desaturase (SAD) generates 18:1-ACP from 18:0-ACP[27]. Fatty acyl-ACP thioesterase B (FATB) specifically cleaves 16:0-ACP and 18:0-ACP, which are saturated FAs, while 18:1-ACP is specifically cleaved by FATA. The three free FAs produced by FATA and FATB are exported from the plastid to the cytosol[28]. Free FAs, 16:0, 18:0, and 18:1, combine with CoA in the cytosol to form an acyl-CoA pool. 18:1-CoA is synthesized as 20:1-CoA and 22:1-CoA in the cytosol by fatty acid elongase1 (FAE1)[29]. The desaturation of 18:1 to 18:2 and 18:3 involves the sequential reaction of FAD2 and FAD3, which target 18:1 at the *sn-2* position of PC. Three copies of *FAD2* were identified in camelina, and when their expression was suppressed using antisense RNA, the 18:2 content decreased[30]. When *CsFAD3* was overexpressed in Arabidopsis, the 18:3 content was enhanced[31]. The 18:1, 18:2, and 18:3 FAs in PC are released into the acyl-CoA pool through the reverse reaction of lysophosphatidylcholine acyltransferase (LPCAT)[32]. Various types of FAs in the acyl-CoA pool are transferred to glycerol to form TAG using glycerol-3-phosphate acyltransferase (GPAT), lysophospholipid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT)[25]. In this study, *PfFAD3-1* was introduced into camelina to increase the ALA content in seeds (Fig. 1).

Results

FA composition analysis of seeds from the T1 to T3 generations of GlyP:PfFAD3-1 Camelina transgenic plants

To enhance ALA production in seeds, *PfFAD3-1* was overexpressed in camelina. The pBinGlyRed3 vector was used for seed-specific expression[33]. This vector contained the glycinin promoter, which is a seed-specific promoter, and DsRED, a red fluorescent protein, as a selection marker. *PfFAD3-1* cDNA was cloned into this vector (Fig. 2), which was named *GlyP:PfFAD3-1*. The *GlyP:PfFAD3-1* vector was transformed into the camelina cv. Suneson using the floral dipping method[24]. Seeds were harvested from the transformed T0 plants and only fluorescent seeds were selected using a green flash under a red filter. Thus, 22 T1 plants were selected. After germination of these 22 T1 lines and wild type (WT), they were grown in the same environmental conditions in the culture chamber to obtain the next generation. To observe the changes in FA content upon the introduction of *GlyP:PfFAD3-1* into camelina, the FA composition of the T2 seeds of 22 plants was analyzed and compared with that of the WT (Fig. 3, Table S1). The transgenic plants have been indicated as *GlyP:PfFAD3-1* # number in the following description. In most transgenic plants, the 18:3 content was increased compared to that in WT and led to a decrease in 18:2 content, which is the substrate of FAD3. On average, the 18:3 content in the WT was 41.4% and that in the transformant was 45.5%, showing an increase of approximately 4%. Furthermore, on average, 18:2 content in the WT was 15.8% and that in the transformant was 8.7%, showing a reduction of approximately 7%. The average 18:1 content was 12% in the WT but increased to an average of 16.8% in the transformant. The contents of other FAs did not significantly differ between WT and transgenic plants (Fig. 3).

Because the T1 generation was not a homozygous line, the *GlyP:PfFAD3-1* #6, #19, and #22 lines, which showed the highest 18:3 content of 48.3%, 48.4%, and 48.5%, respectively, among the 22 transformants, were selected and progressed to the T2 generation. After selecting the fluorescent red seeds from the T2 generation and sowing them in the soil, T3 seeds were harvested from each plant. The FA composition was analyzed using gas chromatography (GC) (Table S2). The average 18:3 content in the WT was 35.7%, whereas that in the *GlyP:PfFAD3-1* #6, #19, and #22 lines was 46.1%, 48.7%, and 49.8%, respectively. In the WT, the average 18:2 content was 16.3%, whereas that in the *GlyP:PfFAD3-1* #6, #19, and #22 lines was 4.1%, 2.9%, and 3.4%, respectively, showing a decrease of more than 12%.

*GlyP:PfFAD3-*1 #6–9, #19 − 5, and #22 − 1 were selected for further analysis as they had the highest 18:3 contents (Table S2). To determine the FA composition and oil content of the T3 generation lines of *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1, the FA analysis was performed five times. On average, the 18:3 content was 48%, 49.4%, and 48.8%, respectively, which was significantly higher than that of the WT (35.7%) (Fig. 4a). The content of saturated FAs 16:0 and 18:0 in the WT was 7.1% and 3.2%, respectively, and that in the *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1 lines was 7.4 ~ 7.6% and 3.5 ~ 4.0%, respectively, showing no significant difference from the WT (Fig. 4a). The 18:2 content in the WT was 16.3%, whereas that in the *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1 was 2.6 ~ 3.4% (Fig. 4a). Furthermore, 20:1 and 22:1 contents in the WT were 12.5% and 2.1%, respectively, and that in the *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1 were 13.0 ~ 13.3% and 1.6 ~ 1.7% respectively, showing no significant difference compared to the WT. In contrast, 18:1 content in the WT was 18.9%, which was

increased slightly to 19.8% in the *GlyP:PfFAD3-1* # 6–9 lines and decreased in *GlyP:PfFAD3-1* #19 − 5 (17.4%) and 22 − 1 (18.2%) lines (Fig. 4a, Table S3).

The total oil content of the seeds was measured as the total fatty acid methyl ester (FAME). The FAME content of WT was 272.5 µg/seed, and that of *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1 lines was 251.8, 263.8, and 268.9 µg/seed, respectively (Fig. 4b). In the *GlyP:PfFAD3-1* #19 − 5 and #22 − 1 lines, the total FAME (µg/seed) content was similar to that of the WT; however, the total FAME in the *GlyP:PfFAD3-1* #6–9 line was slightly lower than that of the WT (Fig. 4b).

In summary, the results of FA analysis up to the T3 generation revealed that the seed-specific expression of *PfFAD3-1* enhanced 18:3 content in camelina seeds and decreased the content of its substrate, 18:2 FA. Moreover, the effect of *PfFAD3-1* was confirmed to be maintained until the T3 generation.

Seed phenotype of T3 generation GlyP:PfFAD3-**1 camelina**

Seed weight and size were measured to determine whether overexpression of *PfFAD3-1* affects camelina seeds. The width, length, and size of 50 seeds per line were measured in three repetitions (Fig. 5a). There was no significant difference in seed weight between WT (44.6 mg) and the *GlyP:PfFAD3-1* #6–9 (44 mg), #19 − 5 (45.3 mg), and #22 − 1 lines (45.8 mg) (Fig. 5b). Seed width and length of all three transgenic lines were significantly increased compared to those of the WT (Fig. 5c,d). On average, the seed width of the WT was 0.83 mm, whereas that of *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1 lines was 0.85, 0.88, and 0.87 mm, respectively (Fig. 5c). Seed length of the WT was 1.62 mm and that of *GlyP:PfFAD3-1* #6–9, #19 − 5, and #22 − 1 lines was 1.73, 1.74, and 1.66 mm, respectively (Fig. 5d). Seed size was calculated by multiplying the width and length. Thus, the seed size was also increased in the transgenic lines compared to that in the WT (Fig. 5e).

Analysis of FA composition and FA synthesis-related gene expression by seed development stage in GlyP:PfFAD3-1 **T4 camelina transgenic plants**

To investigate whether the introduction of *PfFAD3-1* affects the expression of endogenous FA synthesis genes in camelina, seven genes related to FA metabolism were selected—*KASIII, SAD, FATA*, and *FATB*, which are involved in FA biosynthesis; *FAD2* and *FAD3*, which are FA desaturase genes; and *FAE1*, which is an elongate gene (Fig. 6). For the *GlyP:PfFAD3-1* #6-9-3, #19-5-2, and #22-1-3 lines of the T4 generation, the development of seeds was divided into three stages (S1, S2, S3), and the FA composition and expression of FA biosynthesis genes in the seeds were analyzed (Fig. 6a,b).

In both WT and the three *GlyP:PfFAD3-*1 lines, 16:0 FA and 18:0 FA continued to decrease from S1 to S3, showing no significant difference. The 18:1 content decreased from S1 to S2 and increased in the S3 stage, and the three transgenic plants showed a higher content than the WT plants at all stages. The opposite trend was observed between the 18:2 and 18:3 contents—18:2 content decreased from S1 to S3, whereas 18:3 content increased in both the WT and transformants. The decrease and increase in the 18:2 and 18:3 contents, respectively, in the transformants gradually became different from those in the WT as

the seed development progressed. The contents of 20:1 and 22:1 showed a tendency to increase from the S1 to S2 stages and then slightly decrease at the S3 stage. However, there was no significant difference between the WT and transformants for both 20:1 FA and 22:1 FA (Fig. 6a).

The expression of FA biosynthesis genes in camelina was analyzed in the WT and *GlyP:PfFAD3-1* plants at each stage of seed development (Fig. 6b). The expression of *PfFAD3-1* was not detected in the developing seeds of WT; however, *PfFAD3-1* was highly expressed in the developing seeds of the three transgenic lines. *CsFAD3*, which has the same function as *PfFAD3-1*, showed different expression patterns between the WT and transgenic plants. In the S2 and S3 stages, the transgenic plants showed slightly higher upregulation of *CsFAD3* than the WT plants. The expression patterns of *CsSAD*, *CsFATB*, and *CsFAD2* showed the same tendencies in the WT and transformants. However, the expression levels of *CsSAD* and *CsFAD2* were slightly lower in transgenic plants than those in WT plants. During the S2 stage, *CsFATB* expression was elevated in the *GlyP:PfFAD3-1 #6-9-3* line compared to that in the WT; however, it showed a similar or a decreasing trend in the *GlyP:PfFAD3-1* #19-5-2 and #22-1-3 lines compared to that in the WT. *CsKASII, CsFATA*, and *CsFAE1* showed different expression patterns between WT and transgenic plants (Fig. 6b). In summary, 18:3 content increased and 18:2 content decreased in the developing seeds of transgenic plants compared to those of the WT plants. This was due to the introduction of *PfFAD3-1*, and the expression of exogenous *PfFAD3-1* induced the upregulation of *CsFAD3* genes and downregulation of *CsFAD2* genes.

Discussion

In this study, *PfFAD3-1*, which converts 18:2 FA to 18:3 FA, was transformed into camelina (Fig. 1). Consequently, the ALA (18:3) content was improved by up to 48% in the T2 seeds and up to 50% in the T3 seeds of the transgenic camelina plants (Fig. 3, 4). In a previous study, when *PfFAD3-1* was transformed into soybean under the control of the seed-specific promoter, β-conglycinin, the ALA content increased up to 52% in T1 seeds and up to 42% in T2 seeds[18]. In soybean, *PfFAD3-1* expression increased ALA content under the control of the phaseolin promoter and 35S promoter; however, the increase in ALA content was the greatest under the control of β-conglycinin[18]. *PfFAD3-1* expression in soybean did not result in significantly different oil content from that of the WT; however, it increased both seed size and weight[18]. Similarly, in this study, the seed oil content of *GlyP:PfFAD3-1* #6–9 line was slightly lower than that of WT, whereas that of *GlyP:PfFAD3-1* #19 − 5 and #22 − 1 lines was similar to that of the WT (Fig. 4b). Seed weight did not differ between WT and the three transgenic lines. However, seed width, length, and size increased compared to those of the WT (Fig. 5b-e). This showed a seed phenotype similar to that observed when *PfFAD3-1* was expressed in soybean[18]. Hence, the seed-specific expression of *PfFAD3-1* in camelina did not significantly affect seed weight but increased seed size. Because seed size increased without a significant effect on seed oil content, further research is needed to determine whether ALA content affects seed size.

Camelina seed oil can be used for various purposes such as baking, frying, and salad dressings[34]. The camelina developed in this study is beneficial for health because it contains up to 10% (mol %) or more omega-3 FA content compared to the WT. Camelina seeds contain eicosenoic acid (20:1) and erucic acid (22:1) at 12–17% and 2–3%, respectively[23]. The 20:1 FA and 22:1 FA were synthesized from 18:1 by FAE1[35]. Camelina was identified to have three copies of the *FAE1* genes[29]. When the three copies of *FAE1* were knocked out in camelina using CRISPR/Cas9 technology, 20:1 and 22:1 synthesis was blocked, thus increasing the 18:1 content and continuously increasing the ALA content by up to 50%[36]. In this study, *PfFAD3-1* expression increased the 18:3 content by up to 50% (Fig. 4). Therefore, knocking out *CsFAE1* genes using CRISPR/Cas9 in the *PfFAD3-1* overexpression line will further increase the ALA content by 50% or more. In addition, under *PfFAD3-1* expression, the 18:2 content was 2.6–3.4% on average, indicating that *PfFAD3-1* sufficiently synthesized 18:3 using 18:2 as a substrate. Therefore, if the *FAD2* gene is additionally overexpressed in the *PfFAD3-1* overexpression line, the flux from 18:1 to 18:2 would be increased; thus, PfFAD3-1 can use 18:2 to further enhance ALA content.

ALA exerts beneficial effects on the human body only when it is converted into EPA and DHA in the human body. However, the conversion efficiency is very low[8]. The development of crops that produce EPA and DHA from ALA by expressing desaturase and elongase involved in EPA and DHA synthesis has been progressed[37–39]. Recently, a transgenic camelina strain that produces 12% DHA in seeds was developed through multigene metabolic engineering[38]. In addition, the total omega-3 FA content was increased to 33% by crossing the plants whose ALA was increased to 47% by knocking out *csfae1* and the plants that had 27% omega-3 FA content including ETA, EPA, DPA, and DHA[40]. Camelina, with an ALA content of 50%, developed in this study can be used to produce omega-3 FAs.

Materials And Methods

Vector construction of PfFAD3-1 gene

cDNA was synthesized using mRNA from developing seeds of *Physaria fendleri* using a cDNA kit (Takara, Japan). *P. fendleri* seed WCL-LY1 (PI596362) was provide by the U.S National Plant Germplasm System (NPGS). The synthesized cDNA was used as a template, and PCR was performed using primers targeting the *PfFAD3-1* ORF (Table S4). The *PfFAD3-1* sequence was obtained from a previous study[12]. The PCR product was eluted using a PCR purification kit (Cosmogenetech, Korea) and cloned into the pGEMT-Easy vector (Promega, USA). Thereafter, we checked whether there was a PCR error using Sanger sequencing and proceeded to the next step. Because there is an *Eco*RI enzyme site on both sides of the pGEMT-Easy vector and a multi-cloning site, including the *Eco*RI enzyme site, in the pBinGlyRed3 vector, it was cut with *Eco*RI enzyme, and the PCR product was ligated into the pBinGlyRed3 vector.

Selection of PfFAD3-1 transformed camelina

Camelina sativa cv. Suneson was used for all experiments in this study. Camelina seeds were provided by Dr. Edgar Cahoon (University of Nebraska-Lincoln Lincoln, Nebraska, USA). Camelina seeds were

germinated on filter paper containing water in a culture chamber at 16°C under a 16 h light/8 h dark photoperiod. After a week, the camelina seeds from which the cotyledons emerged were transplanted into the soil. Eighteen camelina plants were grown in the soil, and transformation was performed when Camelina flowers bloomed. The *Agrobacterium* strain GV3101 was used. *Agrobacterium* cells (500 µl) and antibiotics were added to 500 ml LB medium and incubated overnight at 28°C until an OD value of 1 was obtained. This solution was centrifuged at 3000 × g for 10 min and resuspended in 500 ml solution containing 1% sucrose and 0.05% Silwet L-77. Camelina flowers were immersed into the solution for 1 min. After floral dipping, camelina was placed in a growth chamber for 24 h in the dark. This procedure was performed three times at intervals of 5 days. After harvesting the transformed plants, fluorescent seeds were selected using a green flashlight and a red filter. All procedures were conducted including the collection of plant material and experimental research in accordance with the institutional, national and international guidelines.

Analysis of PfFAD3-1 gene expression in camelina transformants

To perform RT-qPCR, RNA was extracted from the developing seeds 30, 40, and 50 days after flowering. RNA was extracted using the method described in the reference [41]. After RNA isolation, 2 µg cDNA was synthesized using a cDNA synthesis kit (Takara, Japan). RT-qPCR analysis was performed using the synthesized cDNA as a template and the SYBR Green Master Mix (TOYOBO, Japan) reagent in the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA). Camelina *ACTIN2* was used as an endogenous gene control, and primers capable of targeting all three copies of camelina were designed and used in this study (Table S4).

Fatty acid analysis

Seven camelina seeds were subjected to GC. Next, put 500 ml of toluene and 1 ml of 5% H_2SO_4 including pentadecanoic acid (15:0) standard (100 µg/ml) into seeds and allowed to react for 2 h in an 85 ℃ water bath. The mixture was shaken every 30 min. After the reaction was complete, 1 ml of 0.9% NaCl and 1 ml of hexane were added, mixed well, and centrifuged at 330 × g for 2 min. The supernatant was transferred to a 6 ml tube. The procedure of adding hexane and centrifuging was repeated thrice. A total of 3 ml of the supernatant was purged with nitrogen gas, dissolved in 200 µl hexane, and transferred to a GC vial. A DB-23 column (30 m × 0.25 mm, 0.25 µm film, Agilent, USA) was used, and analysis was performed using a GC-2030 instrument (Shimadzu, Japan). The GC oven temperature was increased from 190°C to 230°C at a rate of 5°C per min.

Analysis of camelina seed phenotype

With *Camelina sativa* cv. Suneson as a control, seed weight, length, width, and size were measured for the transgenic line. To determine the seed phenotype, 50 seeds from each line were analyzed in triplicates. The seed photographs were captured using an optical microscope (NIKON, Japan). The seed weight was measured using an electronic scale (OHAUS, USA). Seed length and width were measured using ImageJ, and seed size was calculated by multiplying the seed length and width.

Statistical analysis for data

Statistical analysis was performed using one way analysis of variance (ANOVA)and Tukey's multiple comparison test using the GraphPad Prism program to determine the significance of differences. Different letters indicate significant changes compared to WT plants in FA and seed phenotype analyses. Significant differences in the FA composition and gene expression patterns at different seed development stages are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Declarations

Author contributions

M-EP and HUK performed the experiments and analyzed the data. M-EP, HUK and H-AC wrote the manuscript. All the authors have read and approved the final manuscript.

Data availability

All data generated or analyzed in this study are included in this published article (and its supplementary information files).

Competing interests

The authors declare no competing interests.

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Figures

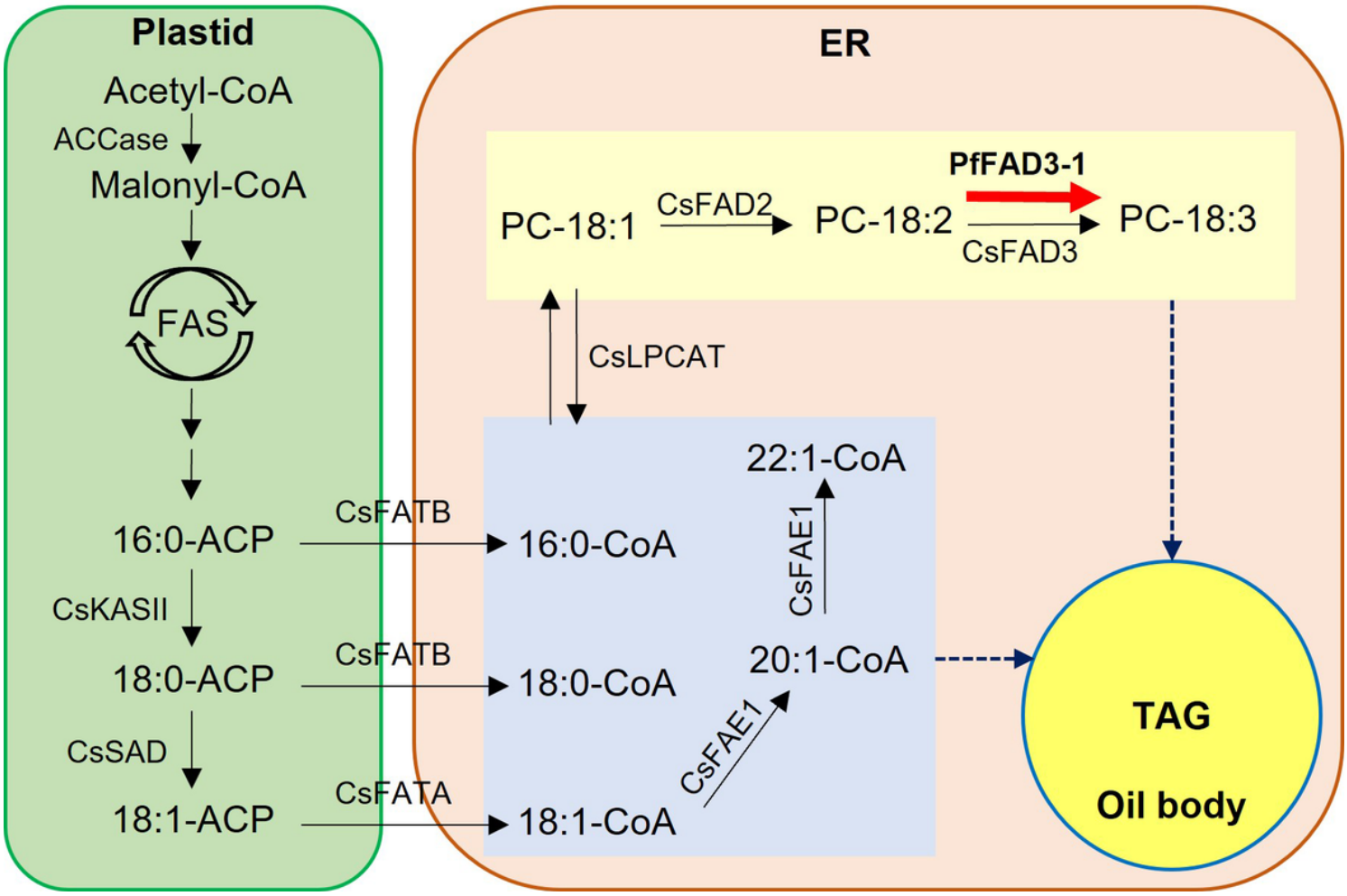


Figure 1

Schematic diagram of fatty acid synthesis pathway in *Camelina sativa* seeds.

Fatty acid synthesis occurs in plastids. Acetyl-CoA is converted to malonyl-CoA by ACCase, and malonyl-CoA is then elongated by FAS, KASIII, and KASII. KASII elongates palmitic acid (16:0) to form stearic acid (18:0). SAD catalyzes the desaturation of stearic acid to oleic acid (18:1). Fatty acids can be transported to plastids by FATA and FATB. The linoleic acid (18:2) and α-linolenic acid (18:3) are synthesized from oleic acid in the PC membrane of the ER by FAD2 and FAD3 enzymes. Additionally, oleic acid can be elongated to eicosenoic acid (20:1) and erucic acid (22:1) by FAE1. The black arrows indicate fatty acid flow, and the red arrow shows the pathway for enhancing α-linolenic acid by *PfFAD3-1* expression. The blue dotted arrow shows the process by which various fatty acids transfer into TAG by several enzymes. ACP, acyl carrier protein; CoA, coenzyme A; ER, Endoplasmic reticulum; FAD2, fatty acid desaturase 2; FAD3, fatty acid desaturase 3; FAE1, fatty acid elongase 1; FAS, fatty acid synthase; FATA, fatty acyl-ACP thioesterase A; FATB, fatty acyl-ACP thioesterase B; KASII, β-ketoacyl-acyl carrier protein synthase II; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; SAD, stearoyl-ACP desaturase; TAG, triacylglycerol.



Figure 2

A vector map of *Physaria fendleri FAD3-1* (*PfFAD3-1*) for seed-specific expression.

T-DNA structure for *PfFAD3-1* expression in this study. CaMV P, cauliflower mosaic virus promoter; DsRed, red fluorescent protein; Gly P, glycinin promoter; Gly-T, glycinin terminator; LB, left border; RB, right border; Nos-T, nopaline synthase terminator.

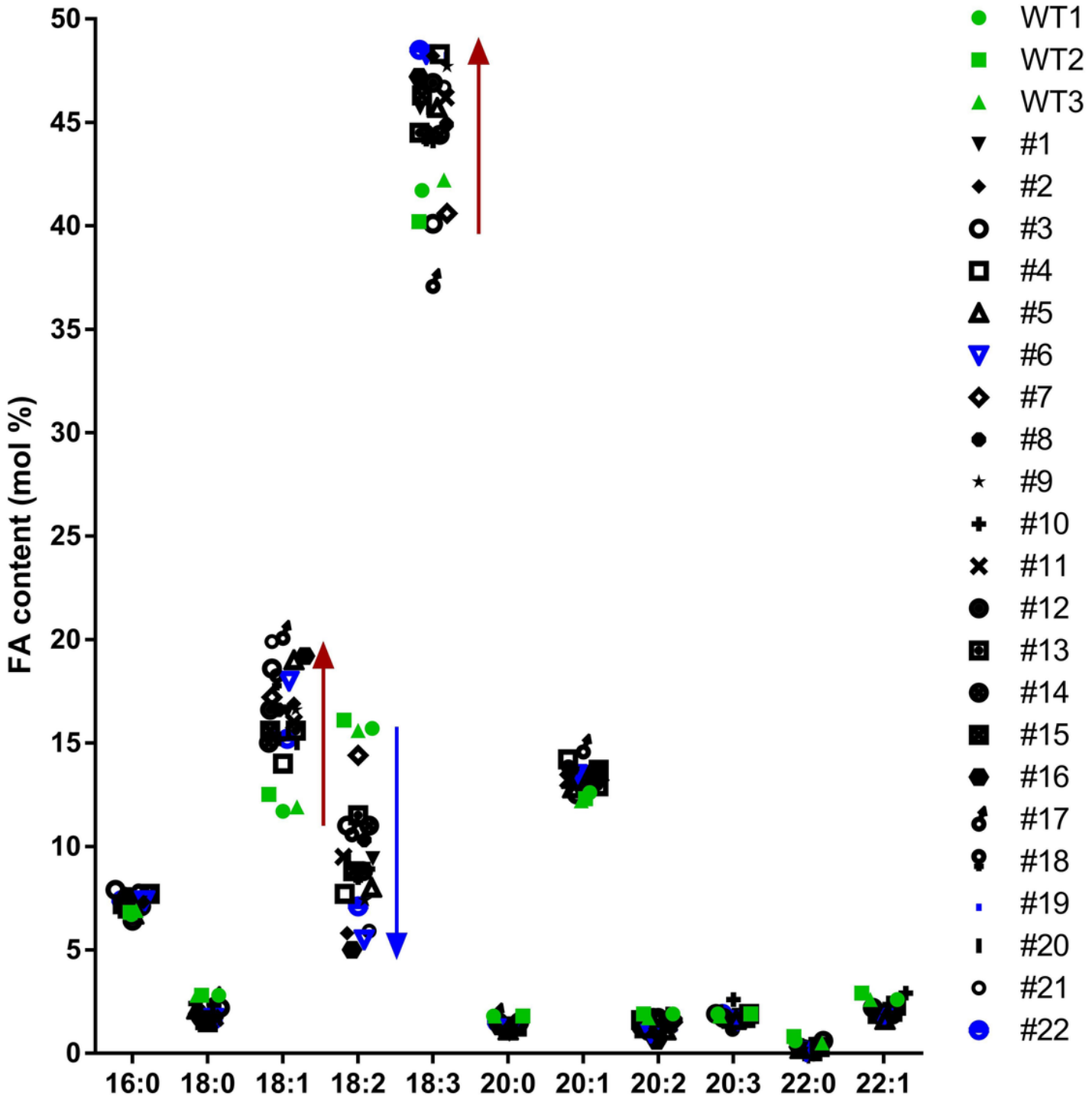


Figure 3

Fatty acid composition of *Camelina sativa* cv. Suneson and T1 transgenic lines expressing *PfFAD3-1* in T2 seeds.

Fatty acid methyl ester (FAME) was analyzed in the T2 generation seeds of the T1 transgenic plants and compared to the wild type. Green points indicate the wild type. The black points indicate the transgenic

plant, and *GlyP:PfFAD3-1* #6, #19, and #22 lines are marked in blue. *GlyP:PfFAD3-1* #6, #19, and #22 lines were selected for the T2 generation.

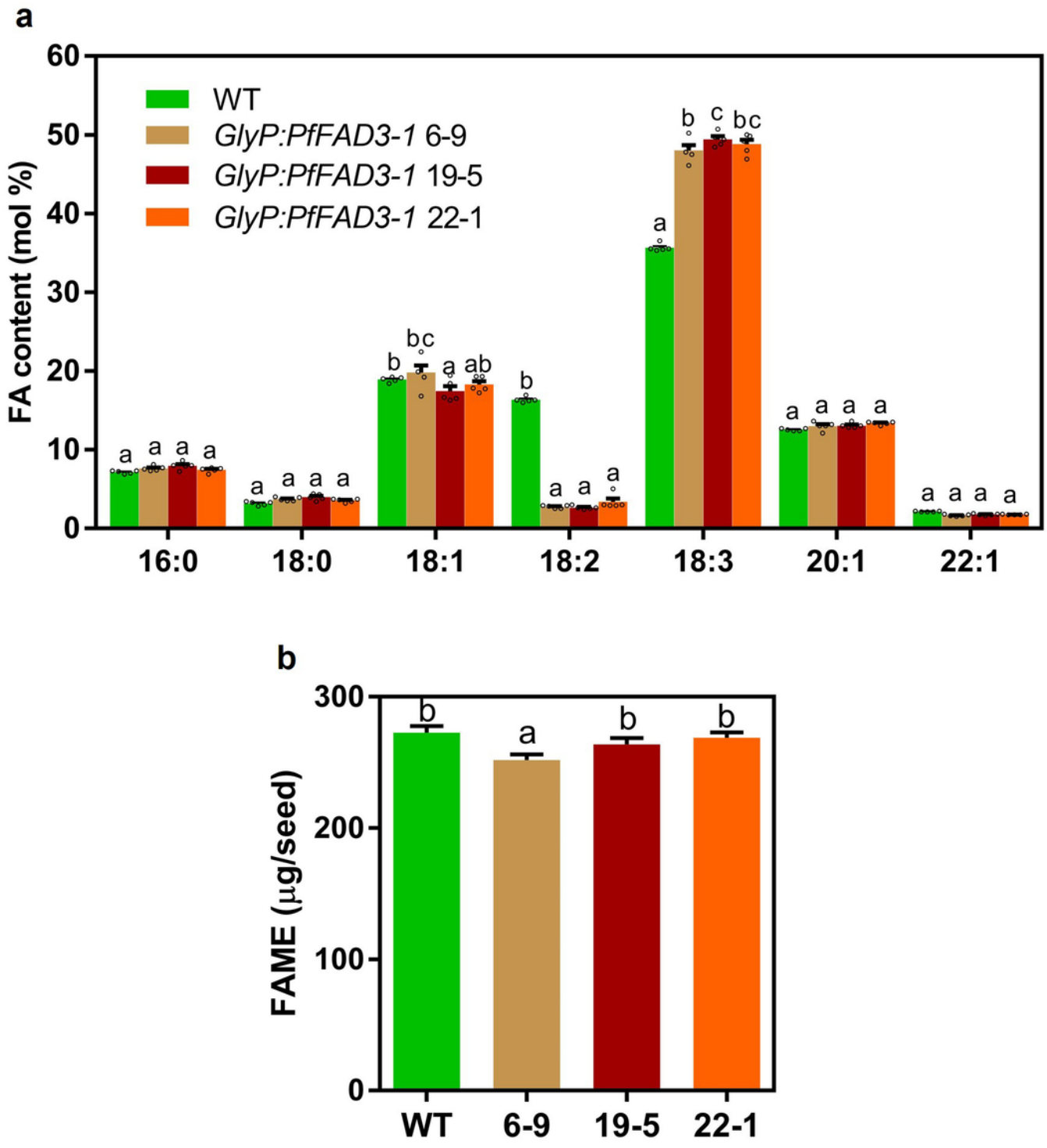


Figure 4

Fatty acid composition and oil content in T3 seeds of T2 generation transgenic lines.

a, Fatty acid composition of three transgenic plants (*GlyP:PfFAD3-1* #6-9, #19-5, #22-1) and the wild type. **b,** Total FAME (µg/seed) of transgenic and wild-typeplants. The results were measured in five replicates (n = 5). Error bars represent standard error of the mean (SEM). Statistical significance is indicated by different letters using one-way ANOVA and Tukey's multiple comparison test (*$p < 0.05$).

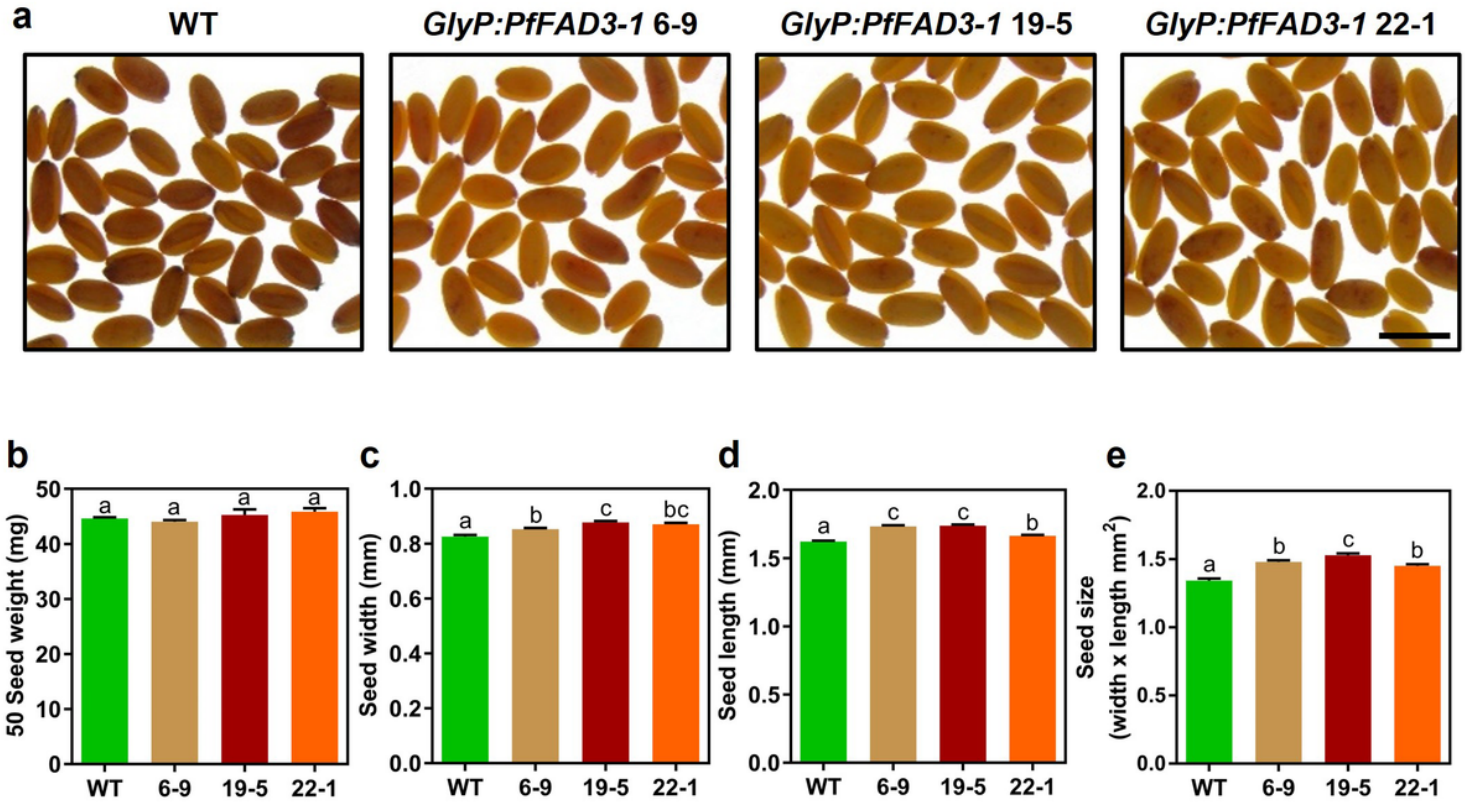


Figure 5

Seed phenotype of transgenic and wild type plants in the T3 generation.

a, Images of seeds of transgenic (*GlyP:PfFAD3-1* #6-9, #19-5, #22-1) and wild type plants. **b,** Seed weight. **c,**Seed width. **d,** Seed length. **e,** Seed size. The results were analyzed in triplicate (n = 30). Scale bar = 2 mm. Error bars represent SEM. Statistical significance is indicated by different letters using one-way ANOVA and Tukey's multiple comparison test (*$p < 0.05$).

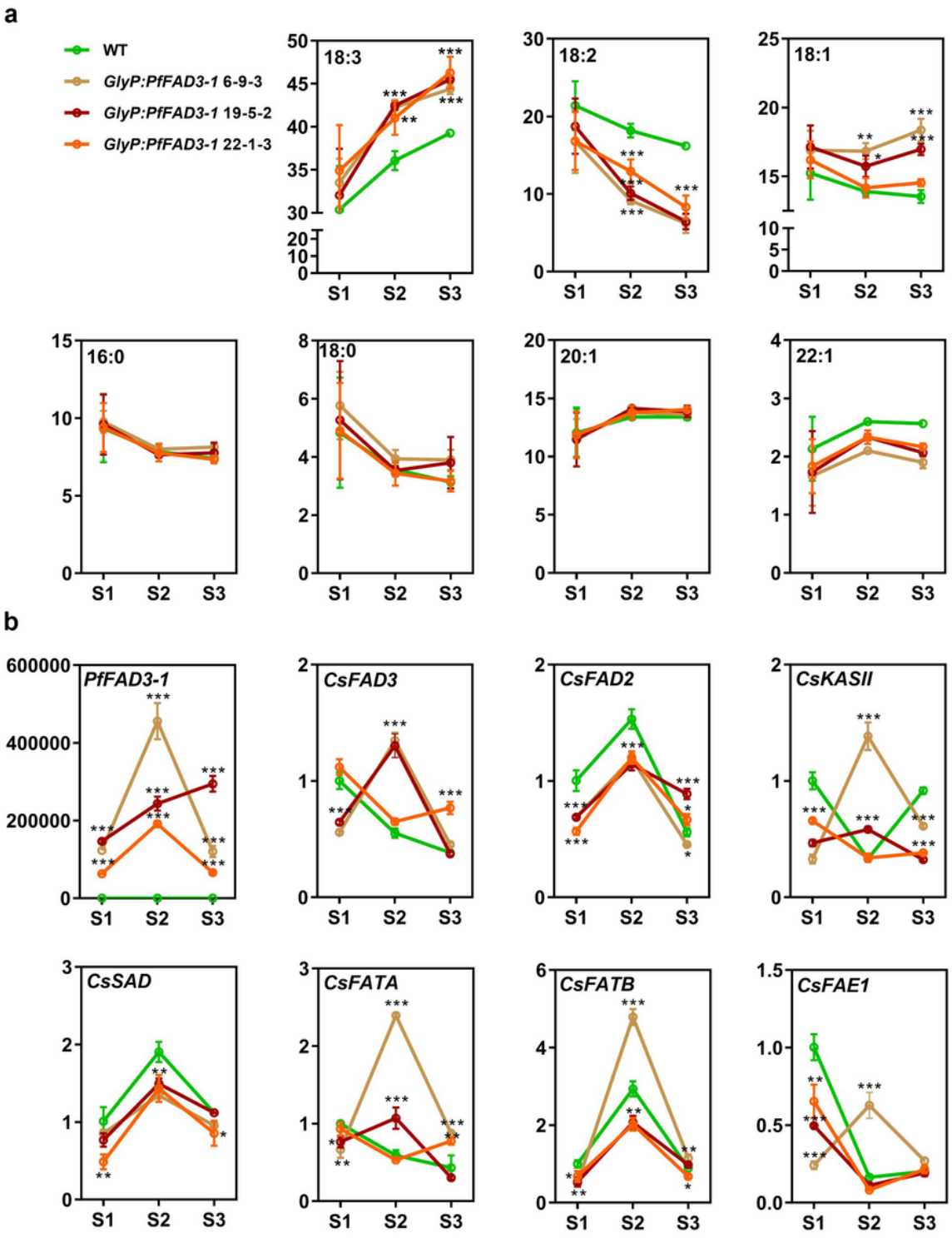


Figure 6

Fatty acid composition and gene expression in the stage of T4 generation developing silique

a, Fatty acid composition in transgenic lines and wild-type plants. **b,** Gene expression patterns in the developing stage of transgenic lines and the wild type. S1, S2, and S3 are developing seeds harvested 30, 40, and 50 d after flowering, respectively. Error bars represent SEM of the mean. Statistical analysis was

performed using one-way ANOVA with Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 22.11.08CamelinaHighALASupplementaryTableFinal1.xlsx