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Brief Communication

Creating ultra-high linolenic acid camelina by co-expressing *AtFAD2sm* with synonymous mutations and *BnFAD3* in the *fae1* mutant

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Alpha-linolenic acid (α-linolenic acid, ALA, 18:3) is an ω-3 polyunsaturated fatty acid (PUFA), which along with linoleic acid (LA, 18:2, ω6) is essential for human nutrition that must be obtained through dietary sources due to the absence of Δ12/Δ15 fatty acid desaturases in mammals. Vegetable oils rich in 18:3, such as flaxseed oil, are prone to oxidation, leading to issues like a short shelf-life. The modern oilseed industry addressed this issue by developing high-oleic/low-18:3 vegetable oils to improve their oxidative stability. However, this contributed to the severe imbalance of these essential fatty acids and the very high dietary ω6/ω3 ratio that promotes the pathogenesis of many diseases, including cardiovascular disease, cancer, and inflammatory and autoimmune diseases. Therefore, it is desirable to develop high-18:3 oil crops to improve human health. Camelina (*Camelina sativa* L. Crantz), a flexible and low-input oilseed crop, contains a high content of 18:3 (31%–40%) in seeds, which represents a potential source (Berti *et al*., 2016).

The 18:3 biosynthesis pathway initiates with oleic acid (OA, 18:1), which is sequentially converted to 18:2 on phosphatidylcholine via Δ12-desaturase (FAD2) and further desaturated by Δ15-desaturase (FAD3) to form 18:3 (Arondel *et al*., 1992; Okuley *et al*., 1994). In *Brassica napus*, co-expressing *FAD2* and *FAD3*—or their fusion proteins via a codon-optimized LP4-2A hybrid linker peptide—successfully increased the 18:3 content up to 53% and 37%, respectively, from 8.6% to 9.6% in wild-type (Liu *et al*., 2024; Xue *et al*., 2023). In camelina, heterologous overexpression of *FAD3* from *Physaria fendleri* also elevated the 18:3 content to 50% (Park *et al*., 2023). Notably, *FAD3* overexpression effectively diverted 18:2 towards 18:3 biosynthesis, while 18:1 remained unchanged. Therefore, it was suggested that co-overexpression of *FAD2* in *FAD3*-overexpressing lines could enhance metabolic flux from 18:1 to 18:2 and further increase the 18:3 content (Park *et al*., 2023). In this study, we aimed to test whether co-expressing *FAD2* and *FAD3* can further increase 18:3 in camelina. Previously, we demonstrated that overexpression of Arabidopsis *FAD2* in camelina and Arabidopsis triggered severe co-suppression (Du *et al*., 2019), but this effect was effectively circumvented by synonymous mutations in Arabidopsis *FAD2* (*FAD2sm*) and PUFAs were increased (Chen *et al*., 2024). In camelina seeds, 18:1 can be elongated using fatty acid elongases (FAE1) and resulted in the accumulation of approximately 20% very-long-chain fatty acids (VLCFAs). The targeted knockout of three *FAE1s* via gene editing blocks VLCFA biosynthesis, thereby redirecting metabolic flux of 18:1 towards PUFA accumulation, resulting in a significant increase of 18:3 to 50% from 39% in wild-type, and the gene-edited *fae1* mutants showed no phenotypic differences from wild-type plants in both seed physiology and overall growth (Ozseyhan *et al*., 2018). Based on these successes, we hypothesized that the level of 18:3 can be further improved in camelina by co-expressing *AtFAD2sm* with *B. napus FAD3* (*BnFAD3*) in the *fae1* mutant (Figure 1a,b).

Initially, we designed the constructs containing a seed-specific Phaseolin promoter-driven Pha::*AtFAD2sm* with *DsRed* selection marker (Chen *et al*., 2024) and a pTF101-*BnFAD3* vector (*Bar* selection marker). First, the efficiency of Pha::*AtFAD2sm* alone was tested in wild-type camelina. Compared with the wild-type, a 4.2% decrease of 18:1, a 5.3% increase of 18:2 and a 1.8% increase of 18:3 in transgenic T_1 seeds suggested that AtFAD2sm catalysed more 18:1 into 18:2, pushing the formation of 18:3 (Figure S1 and Table S1). Then, *AtFAD2sm* and *BnFAD3* were co-transformed into Arabidopsis wild-type Col-0 (Figure 1b). Dual-vector transgenic lines, designated as 'OE^{DV}' to distinguish them from the following overexpression lines of the tandem vector, were selected via DsRed fluorescence followed by Basta resistance. Gas chromatography (GC) analysis of two T_3-generation lines (OE^{DV} 1-1, OE^{DV} 2-3) revealed that OE^{DV} 1-1 increased the 18:3 content of seeds from 17.3% (wild-type) to 21.9% (Figure 1c and Table S2), a 26.6% increase. We then applied this dual-gene co-transformation strategy to camelina wild-type. Two dual-transgenic lines (OE^{DV} 12, OE^{DV} 13) co-expressing *AtFAD2sm* and *BnFAD3* were identified. Subsequently, three homozygous transgenic lines of each OE^{DV} line were obtained. The best-performing OE^{DV} 13-1 line elevated the 18:3 content from 31.1% to 54.3% (Figure 1d and Table S3), a nearly 75% increase. These results clearly indicated the efficacy of overexpressing the two fatty acid desaturase genes in Arabidopsis and camelina for enhancing 18:3 accumulation.

To test whether diverting 18:1 flux from the elongation pathway would further increase 18:3 levels, we transformed the *fae1* mutants in both Arabidopsis and camelina. We constructed a tandem dual-expression cassette vector with a DsRed marker

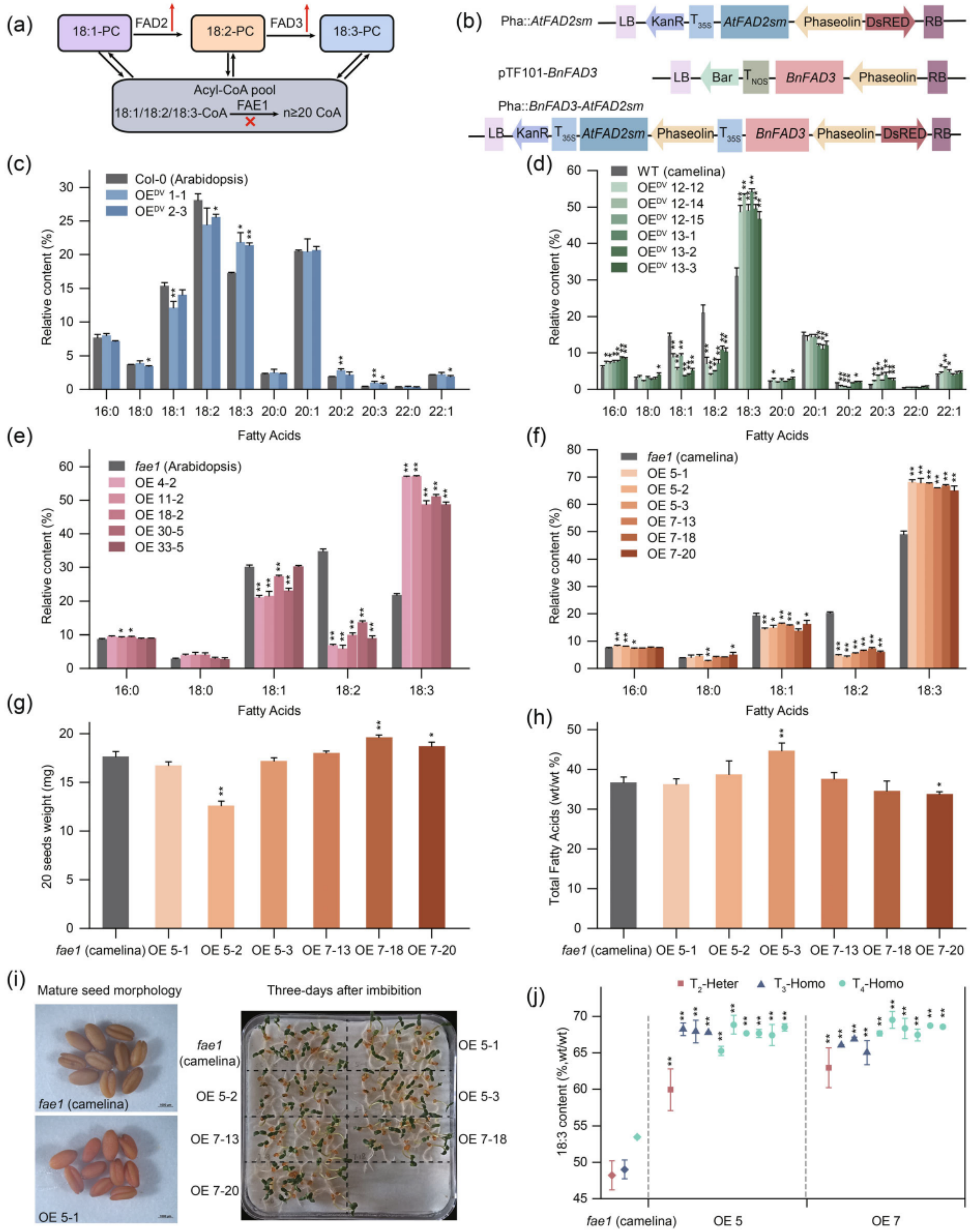

Figure 1 Co-expression analysis of *AtFAD2sm* and *BnFAD3* in wild-type and *fae1* mutant backgrounds of Arabidopsis and camelina. (a) Schematic diagram of fatty acid desaturation/elongation pathway and polyunsaturated fatty acid (PUFA) enhancement strategy. (b) Vector construction diagram, where Pha::*AtFAD2sm* was derived from Chen *et al*. (2024). (c, d) Fatty acids content of transgenic lines in wild-type backgrounds of Arabidopsis (c) and camelina (d). (e, f) Fatty acid content of transgenic lines in *fae1* mutant backgrounds of Arabidopsis (e) and camelina (f). (g–j) Seed weight (g), oil content (h), which is indicated by the relative fatty acid content (wt/wt%), mature seed morphology and seed germination at 3 days post-imbibition (i) and generational stability (j) of transgenic lines in camelina *fae1* mutant background. Heter and Homo refer to heterozygous and homozygous seeds, respectively. 'OE^{DV}' denotes the dual-transgenic lines harbouring two vectors (Pha::*AtFAD2sm* and pTF101-*BnFAD3*), while 'OE' refers to the single transgenic lines containing the tandem vector (Pha::*BnFAD3-AtFAD2sm*). Data in panels (c–h, j) are presented as mean ± standard deviation (SD) (n = 3 biological replicates). Asterisks indicate statistically significant differences compared with respective controls by Student's t-test (*$P < 0.05$, **$P < 0.01$).

(Pha::*BnFAD3*-Pha::*AtFAD2sm*), and 'OE' refers to the single transgenic lines containing the tandem vector (Figure 1b). Transgenic homozygous lines in the Arabidopsis *fae1* mutant (*kcs18-1*) background were first obtained (Figure 1e). The best transgenic line, OE 11-2, showed an increase in the 18:3 content from 21.8% to 57.2% (Figure 1e and Table S4), a 162% increase. We also generated two co-expressing lines in the camelina *fae1* gene-edited mutant (OE 5 and OE 7). Three homozygous transgenic lines from each OE line were analysed. The best-performing line, OE 5-1, elevated the 18:3 content from 49.0% to 68.3% (Figure 1f and Table S5), a 39.4% increase.

All the Arabidopsis and camelina transgenic lines were confirmed to co-overexpress *AtFAD2sm* and *BnFAD3* via RT-qPCR. The expression of their endogenous *FAD2* and *FAD3* fluctuated around background levels, suggesting no co-suppression occurred (Figure S2 and Table S6). Our results thus show that simultaneously enhancing fatty acid desaturation and eliminating the elongation pathways can effectively increase 18:3 accumulation in seeds. The increase of 18:3 was mainly at the expense of 18:2, suggesting that FAD3 exerts a major pulling force particularly when the elongation was eliminated (Figure 1e,f). Unlike the unchanged 18:1 content in *FAD3* transgenic camelina lines (Park *et al*., 2023), the increase of 18:3 in our lines was concurrent with the decreases of both 18:2 and 18:1 (Figure 1d), suggesting that *AtFAD2sm*, in addition to *BnFAD3*, enhances 18:3 production. Camelina and Arabidopsis are closely related within the Brassicaceae family, and their seeds contain similar fatty acid profiles. Differential transgenic effects on the 18:3 increase levels in wild-type and *fae1* backgrounds of these two species may reflect camelina's polyploidy, which possesses about three homeologs of the Arabidopsis orthologs. FAD3 activities and other possible factors, such as triacylglycerol assembly pathways, may also differ between Arabidopsis and camelina and require further investigation.

To assess the inheritance stability and growth effects of ultra-high 18:3 in camelina, we examined T_3 and T_4 homozygous lines co-expressing *AtFAD2sm* and *BnFAD3* in the *fae1* mutant. Seed weight remained unaffected except for a minor reduction in OE 5-2, potentially attributable to variation in seed size among individual plants (Figure 1g and Table S7). Oil content was unaffected except in OE 7-20 (Figure 1h), which was also observed in Arabidopsis/camelina WT high 18:3 lines (Figure S3). Most transgenic seeds (including OE 5-1, highest 18:3 content) exhibited normal morphology and similar sizes to their *fae1* background (Figure 1i and Table S7). All transgenic lines and controls showed 100% germination 3 days after imbibition (Figure 1i and Figure S4). High 18:3 levels (65.1%–69.5%) were stably inherited across T_3 to T_4 generations, with lower contents in T_2 likely reflecting dosage effects (Figure 1j and Tables S5, S8 and S9).

In summary, enhancing the fatty acid desaturases by co-expressing *AtFAD2sm* and *BnFAD3* and eliminating the competing 18:1 elongation pathway by the FAE1 substantially increased the 18:3 contents in Arabidopsis and camelina. The ultra-high 18:3 can be stably transmitted to the T_4 generation. The lines developed normal seed weight and oil content, and germination was not negatively affected. The ultra-high 18:3 camelina lines may provide valuable resources to produce such healthy oils.

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Conflict of interest

The authors have declared no conflict of interest.

Author contributions

NL and MZ designed the research. NL and MZ wrote the manuscript. NL, XL, YC, HW and YZ performed the experiments and analysed the data. CD and ZM supervised the experiments. NL, CD, CL and MZ revised the manuscript. All authors read and approved the final manuscript. This work is respectfully dedicated to the memory of Dr. John Ohlrogge (Michigan State University).

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Materials and methods.
Table S1–S9 Supplemental tables.
Figure S1–S4 Supplemental figures.