CRISPR/Cas9-Targeted Mutagenesis of BnaFAE1 Genes Confers Low-Erucic Acid in Brassica napus

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INTRODUCTION

Rapeseed (Brassica napus) is an important oilseed crop widely planted in the world, providing substantial edible oil and other nutrients for mankind. The composition of fatty acids affects the edible and processing quality of vegetable oils, among which erucic acid (EA) is potentially to cause health problems. Therefore, low erucic acid (LEA) has always been a breeding trait of B. napus. Fatty acid elongase 1 (FAE1) plays a decisive role in the synthesis of EA. There are two functional homologous copies of FAE1 on the A08 and C03 chromosomes in B. napus. In this study, we used CRISPR/Cas9 technology to create targeted mutations on these two homologous copies of BnaFAE1 in three B. napus germplasms with high EA (>30%) and high oil (>50%). Our results show that the EA content was significantly reduced by more than 10 percentage points in the mutant of BnaC03.FAE1 (c03), while the double mutation of BnaA08.FAE1 and BnaC03.FAE1 (a08c03) resulted in nearly zero EA in three BnaFAE1-edited germplasms, and the oleic acid content was increased in different degrees. In addition, knockout of BnaA08.FAE1 or/and BnaC03.FAE1 mildly decreased seed oil content, but had no significant effect on other agronomic traits. In general, we successfully created low EA germplasms of B. napus, which provides a feasible way for future low EA breeding.

Keywords: Brassica napus, CRISPR/Cas9, FAE1, erucic acid, seed oil content
genes, and finally obtained BnaFAE1 with reduced EAC. The EAC of BnaA08.FAE1
B. napus evaluate the impact of BnaFAE1 in breeding because many high SOC germplasms contain high
with high seed oil content (SOC), but they cannot be well utilized
chromosome A08 and C03 play major roles in the synthesis of EA
could significantly reduce VLCFAs from 22 to
Camelina sativa
in good agreement with the theoretical ratios under regulation
variety displayed intermediate EAC between those of its parents,
Erucic acid (EA, cis-D13 C22:1 fatty acid, hereafter abbreviated as C22:1) is found in
many vegetable oils. It has been publicly recognized that
Rapeseed is one of the most important oil crops and produces
13% of edible oil globally (Tang et al., 2021). Erucic acid (EA, cis-D13 C22:1 fatty acid, hereafter abbreviated as C22:1) is found in
many vegetable oils. It has been publicly recognized that
in the history of rapeseed genetic improvement, low
lowering of the EAC of rapeseed oil containing high EA for edible oil (Knutsen et al., 2016). In the history of rapeseed genetic improvement, low
erucic acid (LEA) revolution made great contributions to the
popularization of rapeseed in 1960s. In 1960s, a natural LEA mutant was identified in a feed rapeseed “Liho” (Stefansson et al., 1961). F1 seeds from cross between this mutant and a high EA
parents variety displayed intermediate EAC between those of its parents,
suggesting the genetic regulators of EAC act in an additive
manner. The segregation ratios of EACs in F2 and F3 seeds were
in good agreement with the theoretical ratios under regulation
two genes (Harvey and Downey, 1964). These two genes were
identified as BnaA08.FAE1 in rapeseed and two BnaFAE1 genes on
of chromosome A08 and C03 play major roles in the synthesis of EA
(Gupta et al., 2004; Qu et al., 2017).
At present, breeders own multiple Brassica napus germplasms
with high seed oil content (SOC), but they cannot be well utilized
in breeding because many high SOC germplasms contain high
EAC. In order to improve the EAC of three germplasms and evaluate the impact of BnaFAE1 on the agronomic traits of B. napus, we used gene editing technology to knock out the BnaFAE1 genes, and finally obtained BnaFAE1 knockout mutants with reduced EAC. The EAC of BnaA08.FAE1 and BnaC03.FAE1
double mutants were almost reduced to zero, while the content of C18:1 was greatly increased to more than 66%. This study provides new LEA germplasm resources for the breeding of B. napus.

MATERIALS AND METHODS

Plant Materials
The germplasms used in this study were three high SOC and high
EA B. napus inbred lines WH3411, WH3417, and GY284, which were obtained from the National Engineering Research Center of Rapeseed, Wuhan, China.

Sequence Alignment and Gene Expression Analysis
Amino acid sequences in this research were found from the Tair1 and B. napus transcriptome information resource (BnTIR)2 (Liu et al., 2021). Amino acid sequence alignment was performed by MEGA7 and gene expression data of FAE1s in B. napus were obtained from BnTIR (Liu et al., 2021).

Construction of CRISPR/Cas9 Vector
To generate BnaFAE1 mutants, two sgRNAs simultaneously targeting at BnaA08.FAE1 and BnaC03.FAE1 were designed by CRISPR-P3 (Lei et al., 2014) and putative off-target sites were manually eliminated. U6-26 and U6-29 promoters from Arabidopsis were employed to separately drive these two sgRNA cassettes, which were fused in T-DNA region of pKSE410 vector carrying a Kanamycin selection marker (Xing et al., 2014). Primers used in the construction of the CRISPR/Cas9 vector were listed in Supplementary Table 1.

Agrobacterium-Mediated Transformation of Brassica napus
Agrobacterium tumefaciens (GV3101 strain) cells were transfected with the BnaFAE1-CRISPR-Cas9 recombinant plasmid by electroporation method. A. tumefaciens-mediated hypocotyl transformation in B. napus were conducted as previously described (Dai et al., 2020).

Identification of BnaFAE1 Mutants
T0 plants were obtained by kanamycin screening (25 mg/L), and the Cas9 protein was identified by primer pairs Cas9F/R. Then the positive plants with Cas9 were selected to amplify BnaA08.FAE1 and BnaC03.FAE1, respectively, and the amplified fragments were sequenced and analyzed to identify edited T0 mutants. To obtain homozygous mutants, the T0 mutants were self-crossed for T1 and T2 generations and confirmed by sequencing. Primers used in the identification were listed in Supplementary Table 1.

1https://www.arabidopsis.org/
2http://yanglab.hzau.edu.cn/BnTIR
3http://crispr.hzau.edu.cn/CRISPR/
Field Experiments and Investigation of Agronomic Traits

T0 and T1 mutant plants and WT plants were grown in a greenhouse (16/8 h of light/dark at 22°C) in 2018 and 2019, respectively. The confirmed homozygous T2 mutant lines without Cas9 were grown in the winter-type growing season (2020–2021) in the experimental farm of Huazhong Agricultural University, Wuhan, China. The field experiment followed a randomized complete block with three replications. Each line was planted in one row with 8–10 plants, with a distance of 21 cm between plants within each row and 30 cm between rows. The field management was performed in line with standard breeding practice. Yield-related traits including plant height, branch height, branch number, silique length, number of siliques per plant, 1000-seed weight, and yield per plant were measured as described previously (Cai et al., 2016).

Analysis of Seed Quality-Related Traits

Mature seeds were harvested and dried for the measurement of seed quality-related traits, including fatty acid composition and SOC. Fatty acids were extracted using the gas chromatograph (GC) fatty acid methyl ester method as described previously (Lu et al., 2016). A total of nine fatty acid species were measured with an Agilent 6890 GC. SOC is scanned by near infrared spectroscopy using 2000–3000 seeds per scan (Gan et al., 2003).

![FIGURE 1](image1.png)

**FIGURE 1** | Fatty acid composition and oil content of three germplasms (WH3411, WH3417, GY284). (A) Fatty acids were extracted from mature seeds and analyzed using the gas chromatograph method. Values are means ± SD (n = 3–5). (B) Seed oil content is determined by near infrared spectroscopy. Values are means ± SD (n = 12–20).

![FIGURE 2](image2.png)

**FIGURE 2** | BnaFAE1 gene analysis and mutant generation. (A) Illustration of desaturation and elongation of fatty acids. Red cross indicates mutation of FAE1 genes to block the synthesis of EA. (B) Expression pattern of BnaFAE1s in different tissues. (C) Location of CRISPR/Cas9 sgRNA-1 and sgRNA-2 targeting BnaFAE1 genes and sequencing identification of T2 homozygous mutants. PAM is indicated in green. Red * * means deletions. Red font indicates nucleotide insertions and substitutions.
RESULTS

Selection and Identification of Three High Erucic Acid and High Seed Oil Content Brassica napus Seeds

Three natural B. napus germplasms WH3411, WH3417, and GY284 were selected and their fatty acid composition characters were measured. Fatty acids were determined by GC analysis, and the results show that EA of these three germplasms were between 31.05 and 34.95 mol% (Figure 1A). SOC was measured by near infrared spectroscopy, and the SOC of three germplasms ranged from 51.28 to 53.08% (Figure 1B). The results show that WH3411, WH3417, and GY284 have high EAC and high SOC.

Creation of BnaFAE1 Mutants by CRISPR/Cas9

In order to reduce EA in above three germplasms, CRISPR/Cas9 technology was employed to knock out BnaFAE1s (Figure 2A). There are four homologous copies of BnaFAE1 in B. napus and the expression data in different tissues showed that BnaA03.FAE1 and BnaC03.FAE1-2 were barely expressed in different tissues, while BnaA08.FAE1 and BnaC03.FAE1 were mainly expressed in the developing seeds, especially in the middle and late periods of seed development (Figure 2B). Based on the expression levels, BnaA08.FAE1 and BnaC03.FAE1 were selected to design target mutation sites. Both BnaA08.FAE1 and BnaC03.FAE1 were about 1500 bp in size and only consisted of one exon. We designed target sites at ~600 and 1300 bp, respectively. As a result, homozygous BnaC03.FAE1 mutations (c03) of WH3411, WH3417, and GY284, and homozygous BnaA08.FAE1 and BnaC03.FAE1 double mutations (a08c03) of WH3411 and WH3417 were identified by sequencing in T2 generation (Figure 2C and Supplementary Figure 1). All of them cause early termination of translation except a08c03WH3417 has one amino acid deletion and one amino acid mutations in the BnaA08.FAE1 (Supplementary Figure 2).

CRISPR/Cas9-Induced Mutations in BnaFAE1s Greatly Reduce Erucic Acid Content in Brassica napus Seed

We analyzed the fatty acids of mature seeds of T2 generation by GC method, and the results showed that the C22:1 of c03 and a08c03 was decreased from 34.9 to 19.3 and 0.07% when WH3411 was used as receptor. In addition, the composition of oleic acid (C18:1) in c03 and a08c03 was increased from 22.9 to 35.6 and
66.0%, respectively. Moreover, the composition of linoleic acid (C18:2) was increased to varied degrees (Figure 3A). In WH3417, the C22:1 of c03 and a08c03 was decreased from 31.0 to 18.8 and 0.03%, respectively. Meanwhile, C18:1 was increased from 25.0 to 32.9 and 66.2% in c03 and a08c03, respectively (Figure 3B).

Only homozygous a08c03 double mutant was obtained in GY284 background. The composition of C22:1 was reduced from 34.6 to 0.02%. C18:1 was increased from 22.8 to 67.3% and C18:2 was increased from 12.4 to 15.2% (Figure 3C). These results suggest that knocking out of BnaFAE1s can greatly reduce EAC and increase the content of oleic acid and linoleic acid in B. napus.

**Mutation of BnaFAE1 Results in Mild Decrease of Seed Oil Content**

To determine whether the mutation of BnaFAE1 affects the SOC, SOC of these mutant lines was analyzed by near infrared spectroscopy. The results indicate that the SOC of BnaC03.FAE1 mutant (c03) was not significantly altered in WH3411 and WH3417 background (Figures 4A, B). The SOC of BnaA08.FAE1 and BnaC03.FAE1 double mutants (a08c03) was significantly reduced from 51.28, 51.49, and 53.08% to 46.69, 49.96, and 50.17%, respectively, in WH3411, WH3417, and GY284 background (Figures 4A–C). The results indicate that knocking out of BnaA08.FAE1 and BnaC03.FAE1 simultaneously could slightly reduce seed oil accumulation in B. napus.

**Investigation of Agronomic Trait in Field**

To evaluate the impact of knockout of BnaFAE1s on the agronomic traits, mutant lines were sown in field under the natural environment. During the whole growth period, the mutants did not show obvious visible difference in growth. At mature stage, these mutants did not exhibit obvious morphological changes compared with WT (Figures 5A–C). Meantime, we investigated the agronomic traits including plant height, branch number, branch length, silique number, silique length, thousand seed weight, and yield. The results show that these agronomic traits were not significantly altered in these mutants (Figure 5D), indicating that knockout of BnaA08.FAE1 or/and BnaC03.FAE1 had no significant effect on plant growth and yield.

**DISCUSSION**

The synthetic pathway of EA involves a variety of enzymes, including 3-ketoacyl-CoA synthase (KCS), 3-ketoacyl-CoA reductase (KCR), 3-hydroxyacyl-CoA dehydratase (HCD), and trans-2,3-enoyl-CoA reductase (ECR) (Yu et al., 2011). Among these, the KCS encoded by FAE1 was the most critical one (Millar and Kunst, 1997). Therefore, finding or creating BnaFAE1 mutants has become an important way to cultivate LEA B. napus varieties. Until now, there are two main methods to acquire LEA mutants. One is to screen from natural or EMS mutagenic mutants (Harvey and Downey, 1964; Wang et al., 2008), and the other is to inhibit BnaFAE1 expression by RNAi (Js et al., 2017). In this study, new LEA mutants were created using CRISPR/Cas9-driven knockout of BnaFAE1 using three high SOC and high EAC germplasms, which broadens the breeding resources of B. napus with LEA.

**FIGURE 5 | Agronomic traits investigation. (A–C) Morphology of WH3411, WH3417, GY284, and their BnaFAE1 mutants. (D) Comparison of agronomic traits of WH3411, WH3417, GY284 with their FAE1 mutants. Values are means ± SD (n = 6–8).**
In addition, this study shows that CRISPR/Cas9 induced mutation of the BnaFAE1 genes significantly changed the fatty acid profiles in seeds, resulting in significantly decreased EA. The reduction of EA in the double mutant (a08c03) is much stronger than that in the single mutant (c03), which indicates that BnaA08.FAE1 and BnaC03.FAE1 have a dose effect on EA level, and BnaA08.FAE1 and BnaC03.FAE1 have certain functional redundancy. This is also consistent with previous results (Stefansson and Hougen, 1964). Previous studies have reported that the content of VLCFAs in the FAE1 mutants of Arabidopsis was greatly reduced, while the content of oleic acid was significantly increased (Leemieux et al., 1990). In this study, we also observed similar results, especially in the BnaA08.FAE1 and BnaC03.FAE1 double mutants (a08c03), and the oleic acid content significantly increased (over 66%), accompanying with the increase of linoleic acid (Figure 3). Taken together, our results demonstrate that knockout of the BnaFAE1s substantially improves the nutritional quality of B. napus seed oil.

Owing to the significance of high SOC and LEA in production, understanding of fatty acid metabolism and seed oil accumulation has obvious practical application value in oil crop breeding. Previous studies showed that BnaFAE1 was significantly associated with SOC (Li et al., 2014). Ecke et al. (1995) used the double haploid (DH) population to locate three SOC QTLs in the rape genome, and found that two of them were highly correlated with BnaA08.FAE1 and BnaC03.FAE1, and each additional high EA allele increased the SOC by 1 percentage point. Our results show that when BnaC03.FAE1 was knocked out, the SOC was not significantly decreased, and when both BnaA08.FAE1 and BnaC03.FAE1 were knocked out, the SOC was decreased by 1.53–4.59%. This is consistent with previous findings that inhibition of BnaFAE1 expression significantly reduces the SOC (Js et al., 2017). In order to make up this penalty on oil content, favorable genes/alleles such as DAGT may be introduced into the mutant (a08c03) to promote seed oil accumulation (Taylor et al., 2009). Both BnaA08.FAE1 and BnaC03.FAE1 are highly expressed in developing seeds while have low expression in other tissues. It is not surprising that knockout of BnaA08.FAE1 or/and BnaC03.FAE1 had no obvious effect on agronomic traits and plant architecture of B. napus. Above results suggest that it is feasible to breed LEA B. napus using high EA germplasms by direct genome editing of BnaA08.FAE1 and BnaC03.FAE1.

CONCLUSION

In brief, this is the first report using CRISPR/Cas9 to create LEA germplasms of B. napus by mutating BnaFAE1s in three germplasms with consistent results. The EAC was significantly reduced when BnaA08.FAE1 or/and BnaC03.FAE1 were mutated in different germplasms. The EA content was reduced to nearly zero when BnaA08.FAE1 and BnaC03.FAE1 were both knocked out. Our findings reveal that knockout of BnaA08.FAE1 or/and BnaC03.FAE1 had no remarkable effects on agronomic traits except mildly decreased SOC. Our work successfully generated new LEA germplasms for breeding LEA B. napus.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LG and ST designed this study. ZD, YL, SLi, and HL performed the experiments. YL and ST analyzed the data and wrote the manuscript. LG, ST, and SLi revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.848723/full#supplementary-material

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