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Mutating *BnEOD1s* via CRISPR-Cas9 increases the seed size and weight in *Brassica napus*

Jianwei Gu · Jiayin Chen · Chenqi Zhao · Dengfeng Hong

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Abstract Seed weight, which is highly correlated to seed size, is a critical agronomic trait that determines the yield of *Brassica napus*. However, there have been limited researches on the genes involved in regulating seed size. In *Arabidopsis thaliana*, *ENHANCER OF DA1* (*EOD1*), an E3 ubiquitin ligase gene, has been identified as a significant negative regulator in controlling organ size, but the function of its homologs in rapeseed remains unknown. Only two homologous of *EOD1*, *BnaEOD1.A04 and BnaEOD1.C04*, have been found in *B. napus* and were mutated using the CRISPR-Cas9 system. Three T-DNA-free lines, T_2-157-1-C8, T_2-390-2-B8, and T_2-397-2-E2, were identified from the homozygous T_2 mutant lines. The BnaEOD1.A04 showed a similar type of editing in these mutants, whereas the BnaEOD1.C04 in T_2-397-2-E2 was only missing 26 amino acids, and the translation was not prematurely terminated, which was different from the other two mutants. In parallel, mutation of *BnaEOD1s* resulted in a noteworthy increase in both seed size and seed weight in the three editing lines. Additionally, there was a significant decline in the number of seeds per silique (SPS) and silique length (SL) in T_2-157-1-C8 and T_2-390-2-B8, but T_2-397-2-E2 did not show any significant changes in the SPS and SL, possibly due to distinct types of editing in the three lines. The above results indicate the conserved function of *EOD1* homologs and provides promising germplasm for breeding novel high-yield rapeseed varieties by improving seed size and thousand-seed weight.

Keywords *Brassica napus* · Seed weight · CRISPR/Cas9 · Ubiquitin ligase · *BnEOD1s*

Abbreviations

B. napus	*Brassica napus*
WT	wild-type
TSW	thousand-seed weight
T-DNA	transfer DNA
SL	silique length
SPS	number of seeds per silique
ZNF	RING type zinc finger
CDS	coding sequence
MW	molecular weight
PI	isoelectric point
Nucl	nuclear
NP	number of plants used for phenotype analysis
NS	no significant difference

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J. Gu · J. Chen · C. Zhao · D. Hong (✉)
National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan 430070, China
e-mail: dfhong@mail.hzau.edu.cn

J. Gu
College of Life Science and Technology, Hubei Engineering University, Xiaogan 432000, China

Introduction

Rapeseed is widely regarded as one of the most important oil crop worldwide and serves as a vital source of vegetable oil for the human consumption (Wang 2018). To meet the increasing world demand for rapeseed oil, enhancing production has always been a fundamental objective for rapeseed breeding. Seed size and seed weight are highly positively correlated, and augmenting yield per plant through large seeds is a crucial strategy to boost crop productivity (Li et al. 2015). The regulation of seed size has been extensively studied in model plants such as *Arabidopsis* and rice, in which the ubiquitin–protease pathway is thought to have a crucial role in regulating seed size (Li et al. 2019).

Ubiquitin is a highly conserved small globular protein consisting of 76 amino acids. Protein ubiquitination is an important post-translational modification that impacts protein stability, subcellular localization, molecular interaction, and activity (Li and Li 2014; Li et al. 2019). The ubiquitin-mediated signaling pathway performs a function in various aspects of plant life, particularly in the regulation of seed and organ size (Shu and Yang 2017). The ubiquitin receptor protein DA1 negatively regulates seed size by inhibiting seed epidermal cell division in *Arabidopsis* and may functionally conserved in *B. napus* (Li et al. 2008; Wang et al. 2017). Three genes—*DA2*, *ENHANCER OF DA1* (*EOD1*, also known as *BIG BROTHER*), and *Grain Width 2* (*GW2*)—all encode a RING type E3 ubiquitin ligase and interact with *DA1* in *Arabidopsis* (Xia et al. 2013). The leaves of *eod1* mutant plants exhibited greater area than the WT at early developmental stages, and the lifespan of the mutant leaves was prolonged at later developmental stages. Notably, the phenotypic differences in the *da1-1_eod1-2* double mutants were even more striking, indicating that the *EOD1* negatively regulates seed and organ size (Vanhaeren et al. 2017). The double mutant *da1-1_da2-1* produces petals and seeds that are larger than those of the single mutants and WT, while the *eod1_da2* double mutants showed an additive effect on organ size, indicating the independent functions of *DA2* and E*OD1* in the regulation of organ size (Li et al. 2008; Xia et al. 2013; Dong et al. 2017; Vanhaeren et al. 2017). *EOD1* can ubiquitinated *DA1* and its closely related family members *DAR1* at multiple sites to activate *DA1* and *DAR1*, which disassembles various growth regulators in the plant and regulates seed and organ size (Dong et al. 2017). Meanwhile, *DA1* can also destabilize *EOD1*, indicating that these two genes may act as key nodes in the feedback regulation of the E3 ubiquitin ligase pathway in *Arabidopsis* (Li et al. 2019). The downregulation of *BnDA1* has been shown to significantly increase seed weight and organ size in *B. napus* (Wang et al. 2017). Although the role of the ubiquitin pathway in the regulation of seed and organ size has been extensively studied in model plants, there are still many unknowns in *B. napus*.

B. napus (AACC, $2n = 38$) is a typical allotetraploid with a complex genome structure, for which only limited genes related to seed size regulation are currently available for use. Previous studies have demonstrated the significant role of *EOD1/BB* in the regulation of organ size in *Arabidopsis*, but its functionality in *B. napus* requires further investigation. In this study, we identified the homologous gene of *EOD1* in *B. napus* using gene sequence BLAST and verified the functions of *BnaEOD1s* in *Bnaeod1s* mutant plants that generated using the CRISPR-Cas9 system. In addition, we screened for homozygous mutant lines that was stably inherited and without T-DNA insertion, which will provide promising germplasm for the studying the genetic basis of seed size variation in *B. napus* and the improvement of yield.

Materials and method

Materials

The spring *B. napus* Xiaoyun was used for genetic transformation in this study. *B. napus* plants were cultivated in the growth chamber at 22°C, light 22 h/dark 2 h, humidity 60–70%, the light source was LED (red, green; blue, 6:2:2), and the light intensity was maintained at approximately 500 μmol/m^2/s.

Bioinformatics analyses of *EOD1* homologous genes in the *B. napus* genome

Previously reported *EOD1* gene sequences of *Arabidopsis* were used for BLAST in BnGDXY (http://localhost/BnGDXY/index.html). The expression profile of all copies of the *BnaEOD1*s can be analyzed in

BnIR database (http://yanglab.hzau.edu.cn/BnTIR/). CDS and amino acid information were collected from BnGDXY. The molecular weight and theoretical pI were analyzed using ProParam (https://web.expasy.org/cgi-bin/protparam/protparam) (Wilkins et al. 1999). WOLF PSORT (http://www.genscript.com/wolf-psort.html) and ProtComp v. 9.0 (http://linux1.softberry.com/) were employed to predict the subcellular localization of EOD1 proteins in rapeseed (Horton et al. 2007; Tong et al. 2020).

Phylogenetic and sequence feature analysis of *EOD1* homologous genes

The full length of EOD1 homologous proteins was collected from NCBI and BnGDXY. The phylogenetic tree of EOD1 homologous proteins was constructed using MEGA 11.0 software (https://www.megasoftware.net/) with the neighbor-joining (NJ) method, and the bootstrap test was performed with 1000 iterations (Li et al. 2021).

The exon–intron organizations of EOD1 homologous genes were presented by GSDS2.0 (http://gsds.cbi.pku.edu.cn/) based on the aligning results of the corresponding genomic DNA sequences. The MEME (http://meme-suite.org/tools/meme) was utilized for identifying the conserved motifs in EOD1 homologous protein. The motif distribution type was set as zero or one occurrence per sequence. The number of motifs was set as 20, and the motif width was between 6 and 50 amino acids (Li et al. 2021).

Vector construction, gene transformation, mutation identification, and phenotypic screening of transgenic plants

Firstly, two single guide RNA (sgRNA) that targeted the CDS sequence of all *BnEOD1*s was designed through CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/) (Table S1) (Liu et al. 2017). PCR was performed using primers XX-DT1-BsF, XX-DT1-F0, XX-DT2-R0, and XX-DT2-BsR (Table S1) to construct a DNA fragment containing two sgRNAs (Xing et al. 2014). Subsequently, the DNA fragment was connected to the pSKE401 skeleton vector using Golden Gate reactions to obtain the gene editing vector *BnaEOD1-A4/C4-pKSE401* as previously described (Xing et al. 2014). The resultant gene editing vector was introduced into Xiaoyun via *Agrobacterium*-mediated genetic transformation as previously described by Dai et al. (2020).

To identify the mutant plants, genomic DNA was isolated from the leaves of both transgenic and non-transgenic *B. napus* plants using the CTAB protocol with minor modification (Doyle 1987). Subsequently, positive transgenic plants were screened using the positive detection primers 505F/505R and U626-IDF/U629-IDR. Only a plant with positive PCR results from both primer pairs was designated as a positive transgenic plant. The identification of gene editing for target gene was then conducted in positive transgenic plants through Sanger and Hi-TOM analyses using primers provided in Table S1 as described by Liu et al. (2019).

The homozygous T_2 generation lines were planted in both a greenhouse and the field for phenotype investigation. In the greenhouse, three T_2 generation edited lines were planted with 9 plants per line, and 6 WT plants were used as control. The field experiment was conducted at Huazhong Agricultural University, Wuhan, China, where five edited lines were planted in a three-row plot with 8–10 plants per row and 24 cm spacing between lines. Fifteen plants per line were used for the measurement of agronomic traits, including seedling growth status, organ size, plant architecture, SL, SPS, TSW, and seed germination. SL refers to the average length of 10 siliques on the main stem, which was measured and counted for each plant. SPS refers to the average seed number of 10 siliques on the main stem, which was also measured and counted for each plant. TSW was determined by collecting all seeds of each plant and removing impurities and then weighing the seeds on a SC-G seed detector (Hangzhou Wanshen detection technology Co., Ltd.). Curating the raw data, analyzing phenotypic data using a *t*-test, and creating charts were all performed using WPS Office.

Results

Molecular characterization of *EOD1* homologous genes in *B. napus*

A BLASTN searching was conducted to identify homologous genes of *EOD1* in *B. napus* by using the DNA sequence of *AtEOD1* (*AT3G63530*). It reveals that the rapeseed genome harbors two homologous genes for

EOD1: *BnaEOD1.A04* (*BnaA04G0000200XY*) and *BnaEOD1.C04* (*BnaC04G0257100XY*). The CDS sequence for *BnaEOD1.A04* and *BnaEOD1.C04* is 729bp and 723bp, respectively, and both copies comprise seven exons and six introns, consistent with the *AtEOD1* (Table 1; Fig. 1A). The predicted encoded products of *BnaEOD1.A04* and *BnaEOD1.C04* comprise 242 and 240 amino acids, respectively, and both possess a ZNF domain (Table 1; Fig. 1B–D). Upon comparing the amino acid sequences, it was found that AtEOD1 shared a similarity of 85.9% with BnaEOD1.A04 and 83.7% with BnaEOD1.C04. Additionally, there was a high similarity of 95.1% between BnaEOD1.A04 and BnaEOD1.C04 (Fig. 1B and C).

To comprehend the phylogenetic connections among EOD1 proteins in rapeseed and other species, we constructed an unrooted phylogenetic tree comprising members of EOD1 family. As shown in Fig. 2, two copies of BnaEOD1s are found to be closely related with EOD 1 in *B. rapa* and *B. oleracea*, respectively.

Implement genetic editing for two *BnaEOD1s* using CRISPR-Cas9

The expression profile analysis of *BnaEOD1s* revealed that the expression levels of the two copies of *BnaEOD1s* were relatively high in the main stem, flower, and the early stages of silique and seed development (Figure S1). This suggests that *BnaEOD1s* may primarily regulate the developmental processes of seed in *B. napus* and there is evident functional redundancy between the two copies of *BnaEOD1s* (Figure S1). In order to validate the role of *BnaEOD1s* in the regulating seed size in *B. napus*, we constructed CRISPR-Cas9 target editing for these genes with dual targets (Fig. 3A and B), and then the gene editing vector was introduced into Xiaoyun to generate mutant plants.

A total of 118 T_0 positive plants was obtained, generating 59 editing plants with various types of editing at the targeted site of *BnaEOD1.A04* and *BnaEOD1.C04*. Of these, 58 plants underwent simultaneous editing at two copies, mainly involving three editing types: single-base insertion, single-base deletion, and multiple-base deletion (Table S2). T_0-149 and T_0-293 had the longest base deletions, with both having a 77bp base deletion in the copy on chromosome C4, and their DNA fragments between sgRNA1 and sgRNA2 were all deleted (Table S2). Editing events mostly observed 3–4 bases upstream of the PAM (Fig. 4). Hi-TOM sequencing analysis of target sequences from the T_0 edited plants of *BnaEOD1s* revealed up to six different editing types in the T_0-226 plant, which had simultaneous editing events with single-base insertion, single-base deletion, two-base deletion, three-base deletion, four-base deletion, and five-base deletion at the sgRNA2 position of *BnaEOD1.A04* (Table S2).

Subsequently, we conducted in-depth analysis on the editing situation of 36 randomly selected editing lines (Table 2); the results revealed that the probabilities of editing by the two sgRNAs were 75.00% and 97.22% for *BnaEOD1.A04*. Additionally, the probability of base insertion and base deletion was 72.22% and 11.11% for sgRNA1 and 38.89% and 63.89% for sgRNA2. Similarly, we observed that the probability of editing by the two sgRNAs was 83.33% and 100.00% for *BnaEOD1.C04*. Furthermore, the probability of base insertion and base deletion was 66.67% and 16.67% for sgRNA1 and 47.22% and 80.56% for sgRNA2.

The results demonstrate that base editing in the T_0 editing plants could be efficiently inherited to the next generation, and homozygous mutants with different types of editing were isolated (Fig. 4). The T_1 offspring of T_0-157, T_0-315, T_0-390, and T_0-397 lines were used for selecting homozygous edited lines. The descendants of T_0-157 presented two types of single-base insertion at the sgRNA1

Table 1 Physicochemical properties of the *EOD1* homologous in the *B. napus*

Gene name	Gene ID	CDS (bp)	Amino acids	MW (Da)	PI	Subcellular localization
BnEOD1.A04	*BnaA04G0000200XY*	729	242	27354.21	4.79	Nucl
BnEOD1.C04	*BnaC04G0257100XY*	723	240	27215.99	4.8	Nucl
AtEOD1	*AT3G63530*	747	248	27982.6	4.47	Nucl

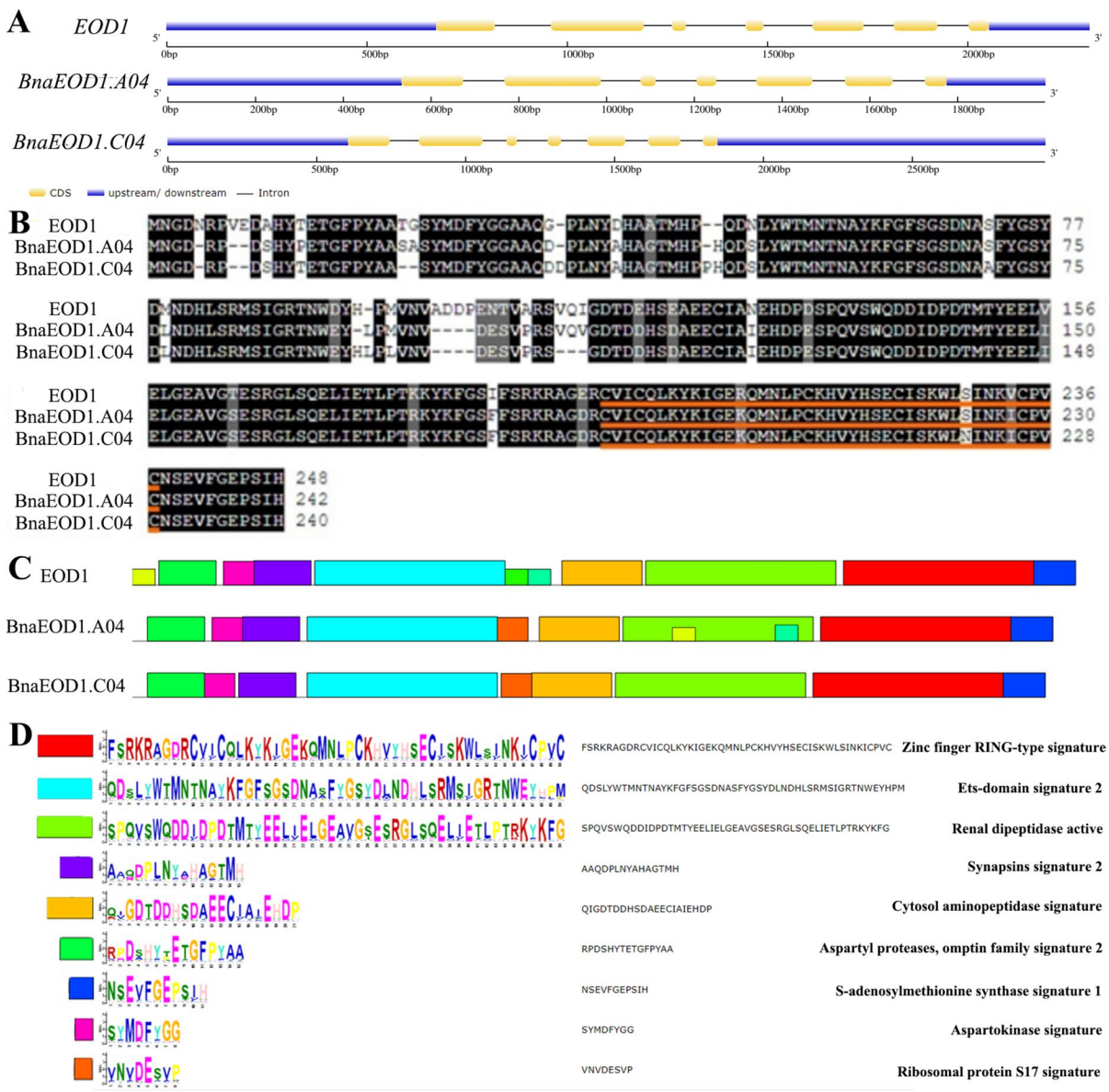


Fig. 1 Gene structures and conserved domain analysis of EOD1s. **A** Exon–intron structures of *AtEOD1* and *BnEOD1s*. **B** Amino acid sequence alignment and conserved domain analysis. **C** Motif locations. **D** Amino acid sequences of the motif conserved across rapeseed EOD1 proteins. The bigger the font size, the more likely the amino acid is at that location across all EOD1 proteins

position of *BnaEOD1.C04*, one with the insertion of adenine (A) and the other with thymine (T). Similarly, the offspring of T_0-315 had two types of single-base insertion at the sgRNA1 position of *BnaEOD1.C04*, with insertion of cytosine (C) and thymine (T), respectively. They also presented two types of multi-base deletion at the sgRNA2 target site, with a deletion of 2 bases and 12 bases, respectively. The descendants of T_0-390 presented two types of multi-base deletion at the sgRNA2 target site of *BnaEOD1.C04*, with deletion of 2 bases and 15 bases, respectively. The offspring of T_0-397 isolated two types of single-base insertion with one guanine (G) and one adenine (A) at the sgRNA1

Fig. 2 Phylogenetic connections of EOD1s. A total of 47 protein sequences was subjected to multiple sequence alignment in ClustalW prior to constructing a phylogenetic tree using the neighbor-joining method in MEGA 11. The aligned EOD1 proteins originating from *A. thaliana* and *B. napus* are marked by red triangles

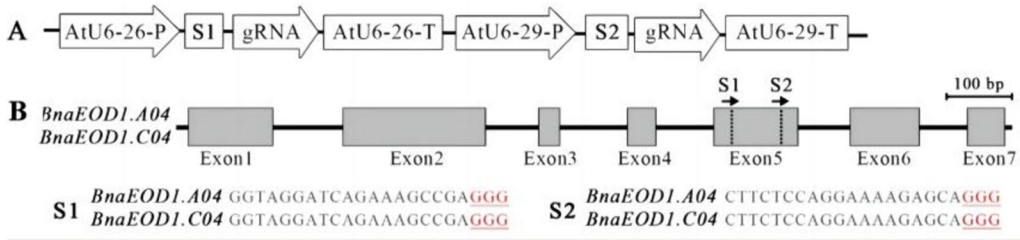


Fig. 3 Schematic diagram of CRISPR-Cas9 vector structure and target site sequence and location. **A** Structure of *pKSE401* vector. **B** sgRNA sequence and the target site in *BnaEOD1s*. S1 and S2 are 2 sgRNA sequences, with arrows indicating the direction and the red base for the PAM sequence

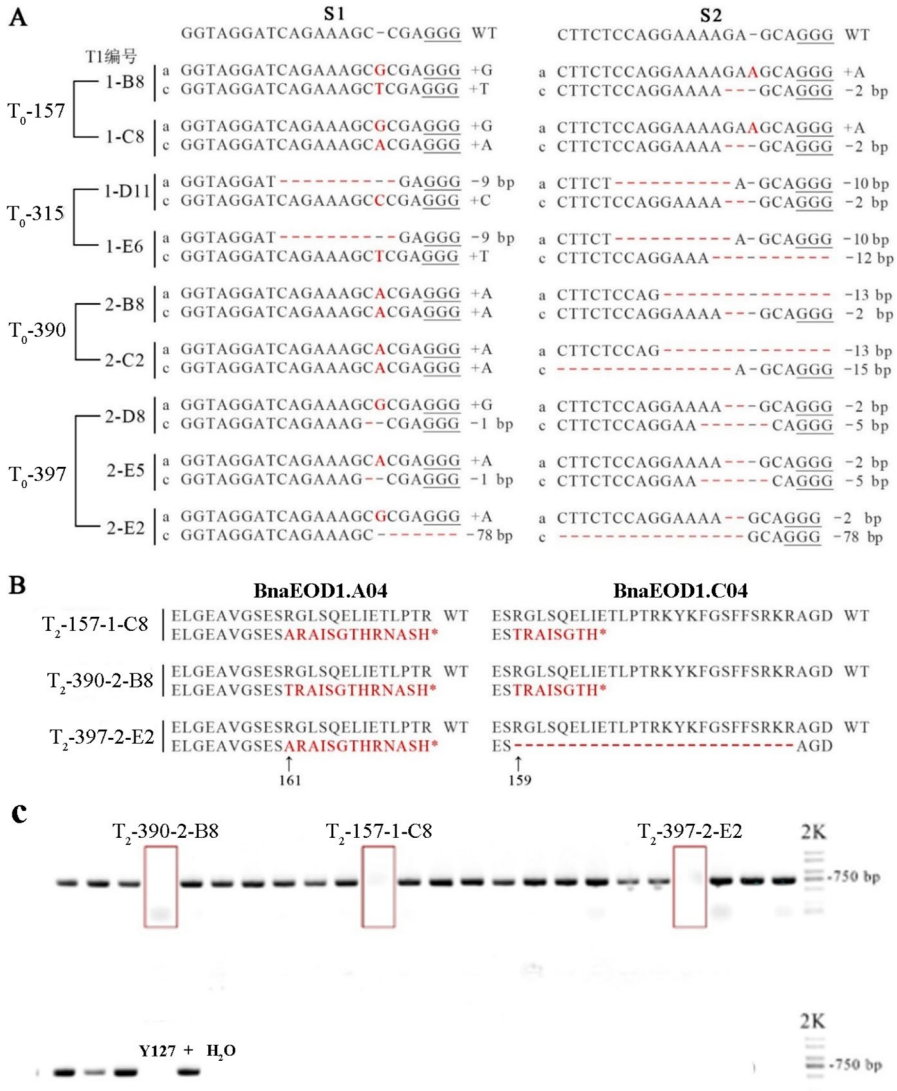


Fig. 4 Target editing and amino acid sequence analysis of homozygous editing plants for *BnaEOD1s*. **A** Analysis of target editing of T_1 edited plants of *BnaEOD1s*. **B** Amino acid sequence analysis of T_2 edited plants. S1 and S2 are sgRNA1 and sgRNA2, respectively; WT is wild-type; a means *BnaEOD1.A04* on A4 chromosome; c means *BnaEOD1.C04* on C4 chromosome. **C** Identification of T-DNA free lines. Y127 and H_2O, negative control for identification; +, positive control for identification

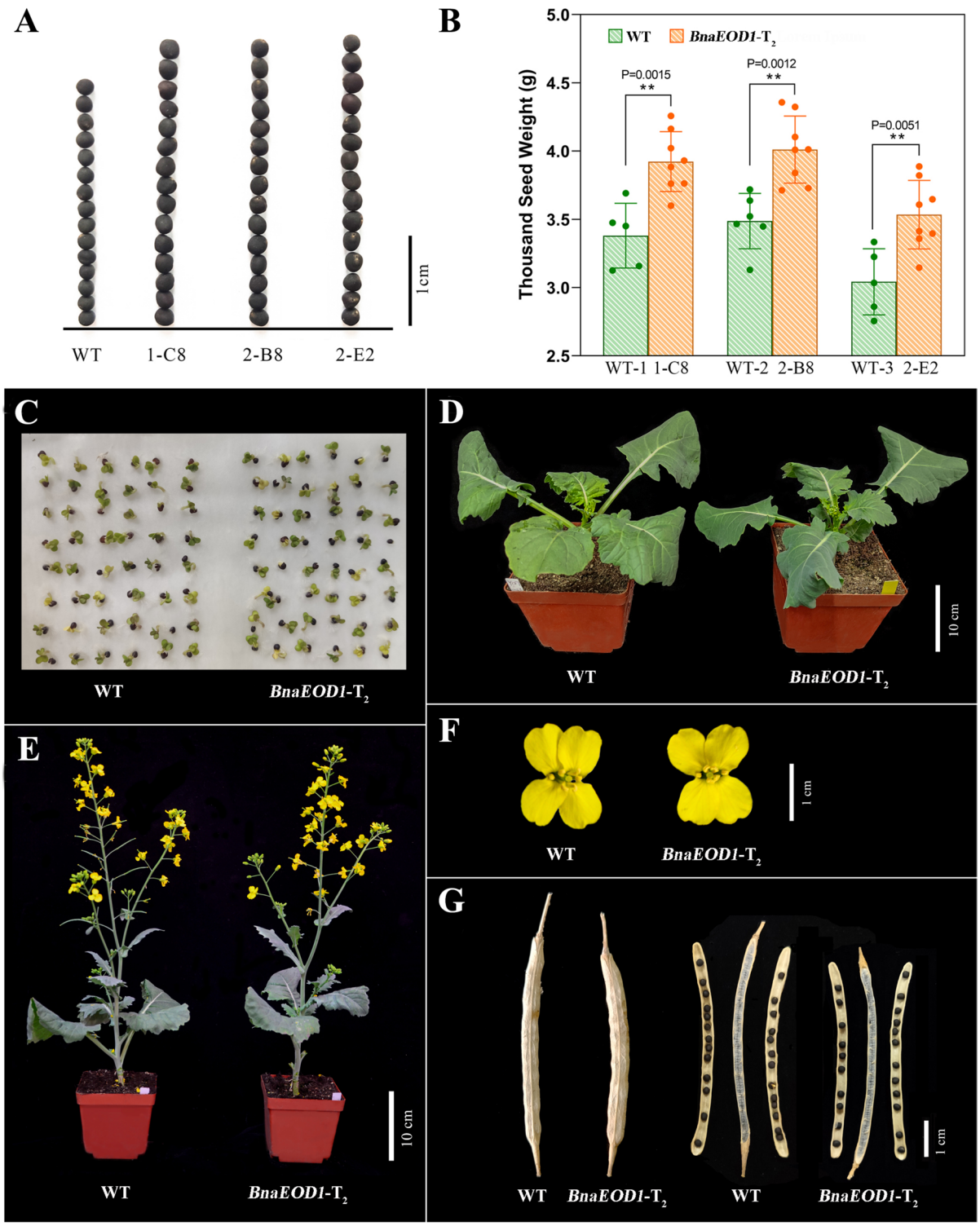
Table 2 Statistics of editing events of *BnaEOD1s* in T_0 edited plants

Gene	sgRNA	GC (%)	NP	Base insertion	Percentage	Base deletion	Percentage	WT	Editing rate
BnaEOD1.A04	S1	52.60%	36	26	72.22%	4	11.11%	9	75.00%
	S2	47.40%	36	14	38.89%	23	63.89%	1	97.22%
BnaEOD1.C04	S1	52.60%	36	24	66.67%	6	16.67%	6	83.33%
	S2	47.40%	36	17	47.22%	29	80.56%	0	100.00%

position of *BnaEOD1.A04*, while a new type of editing was isolated in the offspring of T_0-397, with a 78-base deletion between sgRNA1 and sgRNA2, possibly due to the CRISPR-Cas9 tool still works in the edited plants (Fig. 4A).

Knock-out of *BnaEOD1* by CRISPR-Cas9 significantly enhanced the seed size and TSW

To obtain stable inheritable mutant lines, homozygous mutant lines without T-DNA insertion were


A
WT
1-C8
2-B8
2-E2
1cm
B
WT
BnaEOD1-T2
Thousand Seed Weight (g)
5.0
4.5
4.0
3.5
3.0
2.5
P=0.0015
**
P=0.0012
**
P=0.0051
**
WT-1 1-C8
WT-2 2-B8
WT-3 2-E2
C
WT
BnaEOD1-T2
D
10 cm
WT
BnaEOD1-T2
E
10 cm
WT
BnaEOD1-T2
F
1 cm
WT
BnaEOD1-T2
G
1 cm
WT
BnaEOD1-T2
WT
BnaEOD1-T2

◂**Fig. 5** Phenotypic analysis of *BnEOD1* edited lines. **A** Seed size; **B** TSW; **C**–**G** represent results for germination of seeds, plant architectural at seedling, and the flowering stage, flowers, and silique. Bar represents 10 cm in **D** and **E**. Bar represent 1 cm in **A**, **F**, and **G**. 1-C8, 2-B8, and 2-E2 represents T_2-157-1-C8 and T_2-390-2-B8 and T_2-397-2-E2, respectively. WT-1, WT-2, and WT-3 represent the wild-type plants that were cultivated under the same environment as the mutant lines they compared, but these WT plants were planted in different pots

screened from 27 T_2 edited plants. Three T-DNA-free plants (T_2-157-1-C8, T_2-390-2-B8, and T_2-397-2-E2) with significant mutations in the *BnaEOD1.A04* and *BnaEOD1.C04* were successfully obtained from these homozygous T_2 mutants (Fig. 4B and C). Subsequently, the three homozygous edited lines were further examined for their phenotype. Notably, all three homozygous edited lines exhibited significantly larger seeds and an increased TSW compared with WT (Fig. 5A and B). Specifically, the TSW of three edited lines was approximately 15.98% ($P = 0.0015$), 14.90% ($P = 0.0012$), and 16.12% ($P = 0.0051$) higher than WT, respectively (Table 3; Fig. 5B).

Alongside the significant alterations in seed size and TSW, other agronomic traits closely related to *B. napus* development, including seed germination, cotyledons, true leaves, flowers, and plant architectural, were non-significant differences with WT (Fig. 5C–F). However, two edited lines (T_2-157-1-C8 and T_2-390-2-B8) exhibited notably shorter and wider silique, along with considerably lower SPS compared with WT (Table 3; Fig. 5G). Specifically, SL was reduced by 14.38% and 16.77%, and SPS was reduced 27.39% and 40.46%, respectively. In contrast, the edited line T_2-397-2-E2 did not show any significant change in SPS (Table 3). This disparity in outcomes may have resulted from distinct types of editing in the three lines, whereby the encoded product of *BnaEOD1.C04* in T_2-397-2-E2 missing only 26 amino acids and the translation was not prematurely terminated, possibly retaining partial gene function (Fig. 4B).

We also conducted a phenotype comparison of five homozygous T_3 editing lines and WT under field conditions. Consistent with previous results in greenhouse, all five edited lines displayed larger seeds and a noticeable increase in TSW while also showing shorter silique and fewer SPS (Table 4). In comparison to WT, the TSW of these edited lines experienced a significant uplift ranging from 0.72 g to 1.16 g (Table 4).

Discussion

Seed size shows a strong correlation with the TSW, which is a major determinant of crop yield. Extensive research has been conducted on the regulatory mechanisms of seed size in model plants like rice and *Arabidopsis*. *EOD1* is recognized as a key negative regulator in controlling organ size in *Arabidopsis thaliana*, while investigations into the function and regulatory mechanisms of this gene in rapeseed are lacking (Dong et al. 2017; Vanhaeren et al. 2017). In this study, we identified two *EOD1* homologous genes in *B. napus* through sequence homology BLAST, namely, *BnaEOD1.A04* (*BnaA04G0000200XY*) and *BnaEOD1.C04* (*BnaC04G0257100XY*). Evaluation of the gene and protein sequences proved that these homologous copies shared the same gene structure and exhibited significant sequence similarity with *AtEOD1* (Fig. 1). Moreover, the proteins encoded by these copies also displayed very similar physicochemical properties and subcellular localization (Table 1). Additionally, transcriptome data indicated highly similar expression profiles of the two homologous copies (Figure S1, Liu et al. 2021, http://yanglab.hzau.edu.cn/). These findings suggest that the function of BnEOD1s may be conserved with AtEOD1, without any evident sub-functionalization between the two copies.

The CRISPR-Cas9 system has demonstrated high efficacy as a gene editing tool in *B. napus*, leading to the creation of numerous new rapeseed accessions (Zhai et al. 2020; Zhang et al. 2019; Wu et al. 2020; Khan et al. 2021). In order to investigate the function of Bna*EOD*1s in *B. napus*, we developed a double sgRNA editing vector using the *pSKE401* vector to simultaneously edit *BnaEOD1.A04* and *BnaEOD1.C04*. Assays of the editing events demonstrated that the vector successfully achieved simultaneous and efficient editing of two copies in *B. napus*, with single-copy editing being detected in only one T_0 generation line (T_0-110) (Table S2). Furthermore, the editing efficiency of sgRNA2 was found to be higher than that of sgRNA1 in all T_0 generation editing plants. The editing rates of sgRNA2 are against two homologous copies being 97% and 100%, while sgRNA1 targeted two homologous copies at 75% and 83%,

Table 3 Phenotype data of T_2 edited plants for *BnaEOD1s* in greenhouse

Lines	NP	TSW (g)	SPS	SL (mm)
WT-1	5	3.38 ± 0.21	25.90 ± 3.22	59.30 ± 4.43
T_2-157-1-C8	8	3.92 ± 0.21**	18.78 ± 2.75**	50.77 ± 2.24***
WT-2	6	3.49 ± 0.19	28.10 ± 1.32	65.52 ± 1.48
T_2-390-2-B8	8	4.01 ± 0.23**	16.73 ± 2.34****	54.53 ± 3.71***
WT-3	5	3.04 ± 0.22	22.27 ± 5.39	56.63 ± 3.97
T_2-397-2-E2	8	3.53 ± 0.23**	22.52 ± 2.01^{ns}	61.13 ± 2.50*

The statistical value is the mean ± *t*-test standard deviation; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.WT-1, WT-2, and WT-3 represent the wild-type plants that were grown under the same environment as the mutant lines they compared, but these WT plants were cultivated in different pot

Table 4 Phenotypic data of T_3 homozygous edited lines for *BnaEOD1s* under field

Lines	NP	TSW (g)	SPS	SL (mm)
WT	15	3.84±0.26	19.35±1.45	58.51±1.38
Bnaeod1-1	15	4.71±0.43****	16.58±2.71**	53.67±3.55****
Bnaeod1-2	15	5.00±0.48****	16.34±2.96**	55.36±2.99**
Bnaeod1-3	15	4.56±0.63***	14.16±3.21****	53.69±3.24****
Bnaeod1-4	15	**4.60±0.65*** **	14.86±2.57****	54.13±1.99****
Bnaeod1-5	15	**4.71±0.71*** **	13.09±2.28****	52.42±2.17****

The statistical value is the mean ± *t*-test standard deviation; ns, no significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. *Bnaeod1*-1, *Bnaeod1*-2, and Bnaeod1-3 are the offsprings of T_2-157-1-C8; *Bnaeod1*-4 and *Bnaeod1*-5 are the offsprings of T_2-390-2-B8

respectively. Additionally, the types of editing occurring at the two sgRNA target sites were significantly different. Base insertions were more frequent at the sgRNA1 target site, while base deletions are more frequent at the sgRNA2 target site. These results indicate that sgRNA1 and sgRNA2 exhibits distinct differences in editing efficiency and preference for editing types. However, it is still unclear whether these differences are related to the vector or the position of the sgRNA in the vector.

Through our comprehensive breeding platform, we have successfully obtained T_2 and T_3 generation lines with homozygous mutations in *BnaEOD1.A04* and *BnaEOD1.C04*. Phenotype analysis has demonstrated that the loss of function in *BnaEOD1*s leads to seed enlargement, indicating that *BnaEOD1*s play a negative role in regulating seed size in *B. napus*. Additionally, we have discovered that mutations in *BnaEOD1*s also have a significant impact on various important agronomic traits, including shorter silique and lower SPS. The phenotype of silique in the mutants varies depending on the types of editing, with lines edited to have premature termination of amino acid translation showing more dramatic phenotypic changes than lines edited for amino acid deletion (Fig. 4, Table 3). Previous research in *Arabidopsis* has shown that loss of function mutations in *EOD1* result in smaller organs like flowers and leaves (Li et al. 2008). However, our study in *B. napus* did not find any significant difference in the size of other organs. These results imply that *BnaEOD1*s might have distinct functional differentiation from *AtEOD1*, even though they still maintain their conserved function in regulating seed and silique size. Moreover, the function of *BnaEOD1*s seems to be dependent on the sequences beyond exon 5, and different types of mutations in this region distinctly affect the function of *BnaEOD1*s. Furthermore, we have managed to acquire T_2 and T_3 generation mutants with a diverse array of homozygous mutations in *BnaEOD1.A04* and *BnaEOD1.C04* within a mere year. This remarkable achievement was accomplished through the efficient utilization of both gene editing and comprehensive breeding platform. Ultimately, our success serves as a testament to the immense potential of combining these

innovative techniques and their ability to generate new germplasm for future breeding endeavors.

In conclusion, our study has demonstrated that *BnaEOD1*s play a vital role in regulating the growth and development of seed and silique. However, further investigation is required to elucidate the precise regulatory mechanisms of these genes in *B. napus*. Furthermore, we have successfully identified T-DNA-free homozygous edited mutants from the T_2 generation lines, which will serve as excellent material for future research on the genetic basis influencing seed size variation and enhancing yield in rapeseed.

Author contribution Material preparation, data collection, and analysis were performed by J.C. The first draft of the manuscript was written by J.G., and all authors commented on previous versions of the manuscript. D.H. participated in research design and manuscript proof. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate Not Applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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