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Research article

CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in *Brassica napus*☆

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ABSTRACT

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9-mediated genome editing system has been widely applied as a powerful tool for modifying preferable endogenous genes. This system is highly expected to be further applied for the breeding of various agronomically important plant species. Here we report the modification of a fatty acid desaturase 2 gene (*FAD2*), which encodes an enzyme that catalyzes the desaturation of oleic acid, in *Brassica napus* cv. Westar using the CRISPR/Cas9 system. Two guide RNAs were designed for *BnaA.FAD2.a* (*FAD2_Aa*). Of 22 regenerated shoots with *FAD2_Aa* editing vectors, three contained mutant alleles. Further analysis revealed that two of three mature plants (Aa1#13 and Aa2#2) contained the mutant alleles. The mutant *fad2_Aa* allele had a 4-bp deletion, which was inherited by backcross progenies (BC_1) in the Aa1#13 line. Furthermore, plants with the *fad2_Aa* allele without transgenes were selected from the BC_1 progenies and plants homozygous for *fad2_Aa* were then produced by self-crossing these BC_1 progenies (BC_1S_1). Fatty acid composition analysis of their seeds revealed a statistically significant increase in the content of oleic acid compared with that in wild-type seeds. These results showed that the application of the CRISPR/Cas9 system is useful to produce desirable mutant plants with an agronomically suitable phenotype by modifying the metabolic pathway in *B. napus*.

1. Introduction

Oilseed rape (*Brassica napus*) is an important oil crop with seeds that contain three major unsaturated fatty acids: oleic acid (C18:1); linoleic acid (C18:2); and linolenic acid (C18:3). The contents and relative abundance of these fatty acids are important in the nutritional and processing applications of oilseeds. Higher oleic acid content in *B. napus* seed oil is one of the breeding objective because it increases the thermal stability of the oil and is more suitable as an edible oil. Stearoyl-acyl carrier protein desaturase (SAD) catalyzes desaturation of steric acid (C18:0) to oleic acid (C18:1). Desaturation of oleic acid (C18:1) to linoleic acid (C18:2) and linoleic acid (C18:2) to linolenic acid (C18:3) are catalyzed by fatty acid desaturase 2 (FAD2) and 3 (FAD3), respectively. Genes encoding SAD, FAD2, and FAD3 are involved in synthesis of C18 fatty acids during seed development in *B. napus* (Knutzon et al., 1992; Yang et al., 2012). *B. napus* (AACC, 2n = 38) is an allotetraploid species that consists of an AA genome from *B. rapa* (2n = 18) and a CC genome from *B. oleracea* (2n = 10). Based on the genomic sequences of *B. rapa* and *B. oleracea*, Yang et al. (2012) reported that the *B. napus* genome contains four *FAD2* genes, originating from two genes of *B. rapa* (*BnaA.FAD2.a* and *BnaA.FAD2.b*) and the other two of *B. oleracea* (*BnaC.FAD2.a* and *BnaC.FAD2.b*). The *BnaA.FAD2.a* gene in the SW Hickory variety of *B. napus* contains a 4-bp insertion sequence in the coding region that is responsible for the high oleic acid content (Yang et al., 2012). In addition, the variety Cabriolet, which has high oleic acid content, has a frame shift mutation in the coding region of the *BnaA.FAD2.a* and *BnaA.FAD2.b* genes. By ethyl methanesulfonate treatment of the Cabriolet variety and screening of its M_2 population, several lines which show further increases in the content of oleic acid were obtained and responsible mutations were identified as *BnaC.FAD2.a* (Wells et al., 2014). These reports indicated oleic acid content is controlled by these *FAD2* loci.

Abbreviations: BC_1, first generation of backcross; BC_1S_1, self-pollinated progenies of BC_1; CAPSs, Cleaved amplified polymorphic sequences; CRISPR, Clustered regularly interspaced short palindromic repeats; FAME, Fatty acid methyl esters; GC-MS, Gas chromatography-mass spectrometry; OAI, Oleic acid index; PCR, Polymerase chain reaction; RNAi, RNA interference; TALEN, Transcription activator-like effector nuclease; WT, Wild-type

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Genetic engineering is an effective approach to increase the oleic acid content in *B. napus*. The endogenous *FAD2* gene in *B. napus* can be silenced using an RNA interference (RNAi) construct (Peng et al., 2010). Transgenic analysis has shown that the content of oleic acid increased, while that of polyunsaturated fatty acid decreased. Although the RNAi approach is useful to analyze gene function, the inheritance of T-DNA that carries an RNAi-inducing gene cassette is continuously required to confer the desired phenotype, which is unprefarable in the practical application of RNAi technologies in plant breeding strategies.

More attractive genetic engineering tools have been recently developed to modify specific sites of targeted genes. Of these, TALEN (transcription activator-like effector nuclease) has been applied to knockout the *FAD2-1A* and *FAD2-1B* genes to modify the composition of soybean oil (Haun et al., 2014). In addition, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system is an innovative genome editing tool that can modify any region of the genome of any species with high precision and accuracy (Doudna and Charpentier, 2014; Endo and Toki, 2014). The CRISPR system, which can directly and effectively introduce mutations into the genomes of model plants, *Arabidopsis*, rice, and tobacco (Belhaj et al., 2013; Fauser et al., 2014; Feng et al., 2013), has been rapidly expanded to a wide range of major crops and vegetable species, such as maize, wheat, tomato, and cucumber (Scheben et al., 2017). Moreover, the T-DNA region of genome-edited mutant plants can be easily removed in successive generations by self-pollinating or crossing with wild-type (WT) in most plant species.

Here we report the application of the CRISPR/Cas9 system to *B. napus* to create a novel mutated allele *BnaA.FAD2.a* (abbreviated as *FAD2_Aa*), with premature stop codon or amino acid substitution to increase the oleic acid content. *Agrobacterium*-mediated transformation with the CRISPR/Cas9 vector having guide RNA designed for *FAD2_Aa* was conducted, and two transgenic plants with mutated *FAD2_Aa* alleles were obtained. Fatty acid composition was determined in the mutant progenies that did not carry the CRISPR/Cas9 transgene.

2. Material and methods

2.1. Vector construction and transformation

Two *FAD2_Aa*-targeted vectors were constructed according to the procedure described by Ito et al. (2015) using the target sequence cloning vector *pUC19_AtU6oligo* and the binary vector *pZD_AtU6gRNA_FFCas9_NPTII* constructed by Mikami et al. (2015). Two target sites (*Aa1* and *Aa2*) of the *FAD2_Aa* gene (Gene ID, LOC106452409) were manually selected. The specificity of the target sequences *Aa1* and *Aa2* was confirmed using CRISPRdirect (https://crispr.dbcls.jp) with the rapeseed (*B. napus*) genome reference 'Genoscope v4.1 (Aug, 2014)' in the site. Then, two complementary synthesized oligo DNAs (Sigma-Aldrich Corporation, St. Louis, MO, USA) of each two target sites of *FAD2_Aa*, *Aa1* (5′-attggttcctcttctgattgtcaa-3′; 5′-aaacttgacaatcagaagaggaac-3′) and *Aa2* (5′-attggaccatcgaggcaactcct-3′; 5′-aaacaggagttgcctcgatggtc-3′), were heated at 95 °C for 5 min and left at room temperature for 20 min to be annealed. Each annealed target sequence was cloned into the *Bbs*I restriction site between the *AtU6* promoter region and a *single guide RNA* (*sgRNA*) scaffold of the plasmid *pUC19_AtU6oligo*. These target sequence cloned vectors were digested at I-*Sce*I restriction sites; then, each *sgRNA* expression cassette region was extracted and ligated into the I-*Sce*I site between the right border and the *Cas9* expression cassette (*pZD_AtU6gRNA_FFCas9_NPTII*) and named *pFAD2Aa1* or *pFAD2Aa2*. Then, the plasmids *pFAD2Aa1* and *pFAD2Aa2* were transferred into *A. tumefaciens GV3101*. Rapeseed (*B. napus* cv. Westar) seedlings were grown at 23 °C under a 14 h:10 h light:dark cycle for *Agrobacterium*-mediated transformation, according to the procedure described by Kohno-Murase et al. (1994). Rapeseed containing *pFAD2Aa1* or *pFAD2Aa2* was selected by kanamycin and cultivated in a closed growth chamber at 23 °C under a 13 h:11 h light:dark cycle. Backcross progenies were grown on Jiffy-7 peat pellets (Jiffy Products International B.V., Moerdijk, The Netherlands) and then transferred to plastic pots with soil and grown under the same conditions as the transformants.

2.2. Genotyping of plants

Total DNA was extracted from the leaves of regenerated shoots or mature plants using the standard sodium dodecyl sulfate lysis buffer, and polymerase chain reaction (PCR) was performed using gene-specific primer pairs for *NPTII* (5′-tggagaggctattcggctat-3′/5′-aactcgtcaagaaggcgata-3′), *Cas9* (5′-gaacggtagaaagaggatgc-3′/5′-cgtagagtccagtgatagac-3′), *sgRNA* (5′-ggtgccactttttcaagttg-3′/5′-aggggccatagaaaagttgg-3′), *FAD2_Aa* (AaF2: 5′-tggacgacaccgtcggcctca -3′/AaR2: 5′-caatcccactcagacgagtca-3′), and *BnaC.FAD2.a* (gene ID LOC106452523), abbreviated as *FAD2_Ca* (5′-ctacggtctcttccgttacgcc-3′/5′-cgcgtgataatgcggcatcgtg-3′), using Ex Taq DNA polymerase or PrimeSTAR GXL DNA polymerase (Takara Bio, Inc., Shiga, Japan). PCR conditions were as follows. for *NPTII and FAD2_Ca*: 95 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, and a 3-min final extension at 72 °C; for *Cas9*: 95 °C for 1 min, followed by 30 cycles at 94 °C for 20 s, 64 °C for 20 s, and 68 °C for 20 s, and a 3-min final extension at 68 °C and for *FAD2_Aa*: 95 °C for 1 min, followed by 35 cycles at 94 °C for 20 s, 62 °C for 20 s, and 72 °C for 20 s, and a 3-min final extension at 68 °C; for *sgRNA*: 95 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a 3-min final extension at 72 °C. The PCR products of *FAD2_Aa* were purified using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) and digested with *Hinc*II (Takara Bio). To detect cleaved amplified polymorphic sequences (CAPSs), digested PCR products of *FAD2_Aa* were separated by electrophoresis with 1.5% agarose L03 (Takara Bio). For analyzing the *FAD2_Aa* target sequence, PCR products were amplified with a primer set AaF/AaR (5′-tggacgacaccgtcggcctca −3/5′-caatcccactcagacgagtca-3′) by following PCR conditions, 95 °C for 1 min, followed by 35 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 1 min, and a 3-min final extension at 72 °C. Then, PCR products were purified and cloned into the pGEM-T easy vector (Promega Corporation, Madison, WI, USA) and plasmid DNA was extracted using GENE PREP STAR PI-480 (Kurabo Industries, Ltd., Osaka, Japan), according to the manufacturer's instructions. Sequence reactions were performed with pGEM-T easy specific primers (5′-gttttcccagtcacgac-3′/5′-caggaaacagctatgac-3′) and analyzed using the 3500 series Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Sequence analysis were performed using Geneious 9.1.8 software (Biomatters Ltd., Auckland, New Zealand).

2.3. Production of backcross progenies

BC_1 progenies were produced by backcross between the transformant (Aa1#13) as a seed parent, and the wild type Westar as a pollen parent. BC_1S_1 progenies were produced by self-pollination of BC_1.

2.4. Analysis of fatty acid composition

For analyzing fatty acid composition, seeds were collected from plants (a parental cultivar Westar and six BC_1S_1 lines) grown under the same conditions. As the storage conditions of the seeds might affect oil composition, seeds were collected from plants grown under the same conditions and then stored in a freezer until they were analyzed. Total lipids were extracted from three replicates of 10 seeds using the Folch method (Folch et al., 1956) with some modifications. Briefly, the seed sample and 5.0 mm-diameter zirconium beads were placed in a 2-mL microtube (Eppendorf, Hamburg, Germany); the tube was then shaken in a mixer mill (Retsch MM400; Verder Scientific GmbH & Co. KG, Haan, Germany) for 2 min at a frequency of 30 Hz. Then, 1 mL of a chloroform:methanol mixture (2:1, v/v) was added to the tube, and the