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ORIGINAL ARTICLE

Generation of early-flowering Chinese cabbage (*Brassica rapa* spp. *pekinensis*) through CRISPR/Cas9-mediated genome editing

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Abstract

The CRISPR system enables us to induce precisely targeted mutations in a plant genome. The widely used CRISPR system is composed of a Cas9 protein derived from *Streptococcus pyogenes* (SpCas9) and a target site-specific guide RNA. In this study, we successfully generated the early-flowering Chinese cabbage (*Brassica rapa* spp. *pekinensis*), which is one of the most important vegetables in the world. To generate early-flowering *B. rapa* without requiring vernalization, we designed seven guide RNAs which target *B. rapa* homologous genes to the *Arabidopsis thaliana FLOWERING LOCUS C* (*FLC*). We first examined the indel mutation efficacy of the designed guide RNAs in protoplasts isolated from young leaves of Kenshin (an inbred line of *B. rapa*). After selecting four guide RNAs, genome-edited plants were established by delivering the plant binary vectors harboring SpCas9 along with respective guide RNAs into *B. rapa* hypocotyl explants. In the T_0 generation, we found *BraFLC2* and *BraFLC3* double knockout lines with the indel efficiency of 97.7% and 100%, respectively. The simultaneous mutations of both *BraFLC2* and *BraFLC3* were inherited in T_1 generations with 100% of indel efficiency. The edited lines obtained showed an early-flowering phenotype that did not depend on vernalization. This study provides a practical gene-editing protocol for Chinese cabbage and verifies the function of its multi-copy *BraFLC* genes.

Keywords BraFLC · *Brassica rapa* · CRISPR · Heritable mutation · Protoplast · Vernalization

Introduction

The efficacy of the CRISPR system can be examined by generating gene-edited animals and plants. Hundreds of guide RNAs are easily designed for *Streptococcus pyogenes* Cas9 protein (SpCas9) even when only one gene is knocked out, but gene-editing efficacy differs depending on the loci of guide RNA-binding sites (Doench et al. 2014; Hinz et al. 2015; Isaac et al. 2016; Wilson et al. 2018). For plant genome editing, highly efficient guide RNAs should be selected, because the whole plant transformation process may take from several months to a year. Recently, a deep-learning algorism has been developed to predict the gene-editing efficacy of guide RNAs for Cas12a (Cpf1) system (Kim et al. 2018). However, it is still hard to predict the efficacy of guide RNAs in silico for SpCas9, which is extensively used for plant genome editing. Therefore, it would be better to validate the gene-editing efficacy of guide RNAs in interested plants, before plant transformation and regeneration occur.

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Chinese cabbage (*Brassica rapa* spp. *pekinensis*), an important vegetable crop, has been cultivated for many centuries. Late-flowering cultivars have been selected to increase crop productivity as in other leafy vegetables (Jung and Müller 2009; Blümel et al. 2015). In addition, many late-flowering *B. rapa* require cold treatment for several weeks to induce flowering and ultimately to harvest seeds (Osborn et al. 1997; Schranz et al. 2002). However, the late-flowering trait has several disadvantages for researchers and breeders who want to do forward and reverse genetics with *B. rapa*. Therefore, an early-flowering *B. rapa* that does not require vernalization is highly desirable as it allows scientist to analyze gene function for plant development, physiology, and ecology in Chinese cabbage (Williams and Hill 1986).

The molecular basis of vernalization-induce flowering has been well studied in the model plant, *Arabidopsis thaliana* (Blümel et al. 2015). Long-term cold treatment reduces the level of *FLC* transcripts, which accelerates the flowering of *Arabidopsis* (Michaels and Amasino 1999). Natural *Arabidopsis* mutants in the *FLC* gene show early-flowering phenotypes that do not require vernalization. Therefore, we hypothesized that mutations in *B. rapa* homologous genes to *Arabidopsis* FLC were supposed to generate an early flowering *B. rapa* (Kim et al. 2007). There are four *B. rapa FLC* genes (*BraFLC1*, *BraFLC2*, *BraFLC3*, and *BraFLC5*) with high sequence similarity to the *Arabidopsis FLC* gene (Schranz et al. 2002b; Kim et al. 2007). However, little is known about which genes are important in vernalization or whether there is functional redundancy among the multiple *BraFLC* genes (Kim et al. 2007). Hence, the purpose of this study was to introduce the targeted mutation into *FLC* genes using the CRISPR system and to generate an early flowering in Chinese cabbage without requiring vernalization.

Materials and methods

Plant materials and growth conditions

Chinese cabbage inbred line, Kenshin (*Brassica rapa* L. ssp. *pekinensis*) (Lee et al. 2013) was used in this study. The mature seeds were sterilized in 2% active chloride for 10 min, and rinsed eight times with sterilized distilled water. The seeds were placed in glass bottles (height 15 cm, diameter 7 cm) containing half-strength MS medium (Murashige and Skoog 1962, Duchefa Biochemie, Netherlands) containing vitamins, 30 g/L sucrose (Duchefa Biochemie) and 8 g/L phytoagar (Duchefa Biochemie). Glass bottles with seeds were maintained under long day (16 h:8 h, light:dark) conditions in a growth room at 25 ± 1 °C for 3 days, and seed-containing bottles were transferred to a growth room under dark conditions for 7 days.

Chinese cabbage transformation

After removing cotyledons and root parts, dark-grown hypocotyls were cut into 0.7–1.0 cm segments, and then kept under long day (16 h:8 h, light:dark) conditions in a growth room at 25 ± 1 °C for 3 days. We used our plant binary vector system (pHAtC) (Kim et al. 2016) harboring 35S::SpCas9 and U6::guide RNA for *Agrobacterium*-mediated transformation. The following compositions of YEP medium (all from Duchefa Biochemie) were used for the growth of *Agrobacterium* (LBA4404); 50 mg/L streptomycin, 50 mg/L spectinomycin, 50 mg/L rifampicin in 50 mL of YEP medium. Cell cultures were grown overnight at 250 rpm on a rotary shaker at 28 °C until the culture reached the final concentration of $OD_{600nm} = 0.6$. Before transformation, *Agrobacterium* was washed three times with 30 mL of YEP medium. The following conditions were used for centrifugation: 22 °C, 5000 rpm for 15 min.

Agrobacterium was finally resuspended with 25 mL of YEP medium and poured into a Petri dish. The chopped hypocotyl explants were added to the Petri dish containing *Agrobacterium* and gently shaken for 10 min. The following composition of co-culture medium was used to incubate explants for 2 days under dark conditions at 25 ± 1 °C; 4.4 g/L of MS including vitamins (Duchefa Biochemie), 30 g/L of sucrose, 1 mg/L of NAA, 4 mg/L of BA, 4 mg/L of $AgNO_3$, 10 mg/L of acetosyringone, and 7.5 g/L of agar, pH 5.8. After 2 days, explants were washed with washing medium (4.4 g/L of MS including vitamins, 30 g/L of sucrose, 1 mg/L of NAA, 4 mg/L of BA, 4 mg/L of $AgNO_3$, 250 mg/L of cefotaxime and 10 mg/L of acetosyringone, pH 5.8) for at least 5 times. Washed explants were again placed on sterilized filter paper and dried for a minute to reduce media contamination and transferred on callus induction media (4.4 g/L of MS including vitamins, 30 g/L of sucrose, 1 mg/L of NAA, 4 mg/L of BA, 4 mg/L of $AgNO_3$, 250 mg/L of cefotaxime, 10 mg/L of acetosyringone, 10 mg/L of hygromycin and 8 g/L of agar, pH 5.8). The cultures were subcultured once per 1–2 weeks until the explants exhibited a leaf-like organ. The regenerated shoot parts were transferred into maturation medium (4.4 g/L of MS including vitamins, 30 g/L of sucrose, 250 mg/L of cefotaxime, 10 mg/L of hygromycin and 8 g/L of agar, pH 5.8). The cultures were maintained on the same maturation medium every 2 weeks until several leaves were regenerated. When multiple shoots were regenerated, this shoot part was transferred to rooting media (2.2 g/L of MS including vitamins, 30 g/L of sucrose and 10 g/L of agar, pH 5.8).

In vitro guide RNA synthesis

IVT primers (F: T7 promoter + 'G' + 18-20 bp of target-binding sites + universal, R: universal + guide RNA scaffold,

see Supplemental Table 1) were designed for in vitro guide RNA synthesis. Polymerase chain reaction (PCR) was performed to amplify a template DNA for guide RNA synthesis in total 50 µL of reaction mixture containing 1 µL of 100 µM forward primer, 1 µL of 100 µM reverse primer, 10 µL of 5X buffer, 1 µL of 10 mmol dNTPs, and 0.5 µL Phusion™ DNA polymerase (Thermo Scientific, USA). The following PCR conditions were used to amplify template DNA: 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 54 °C for 20 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR product was analyzed using 1.5% agarose gel, and PCR purification was done using Expin™ PCR SV kit (Gene All, Korea).

After PCR purification, guide RNAs were transcribed in vitro using T7 RNA polymerase (New England Biolabs, USA) with the following conditions: in total 100 µL of reaction mixture containing 1.5 µg of PCR products, 4 µL of 100 mM ATP, 4 µL of 100 mM UTP, 4 µL of 100 mM CTP, 4 µL of 100 mM GTP, 28 µL of $MgCl_2$ (50 mM), 10 µL of 10X buffer, 10 µL of 100 mM DTT, 2.5 µL of RNase inhibitor, and 10 µL of T7 RNA polymerase. This mixture was incubated overnight at 37 °C to maximize RNA transcription efficiency. After overnight synthesis, 10 µL of DNase I (New England Biolabs) was added to the mixture and kept at 37 °C for 30 min to remove template DNA in the mixture. The synthesized RNA concentration was quantified by nano-drop (Thermo Scientific, USA).

Protoplast isolation and transfection

Protoplast isolation was performed using 7-day-old seedlings as previously described (Woo et al. 2015; Kim et al. 2016, 2017). The ribonucleoprotein (RNP) premixture with 30 µg of SpCas9 protein and 80 µg of sgRNA was prepared in a Falcon tube (14 mL) and stabilized for 30 min at room temperature. Approximately, 2×10^5 protoplast cells were mixed with RNP premixture and suspended slowly with 300 µL of MMG solution (0.4 mM of mannitol, 15 mM of $MgCl_2$ and 4 mM of MES, pH 5.7) followed by the addition of the equal volume of PEG solution (40% of PEG 4000, 0.2 M of mannitol and 0.1 M of $CaCl_2$). In addition, the mixture was then incubated at 22 °C for 24 h. After incubation, protoplasts were washed at least three times with an equal volume of W5 solution (154 mM of NaCl, 125 mM of $CaCl_2$, 5 mM of KCl, 5 mM of glucose and 2 mM of MES).

Genomic DNA extraction and in vitro cleavage assay

The genomic DNA of Chinese cabbage was extracted with DNeasy Plant Mini Kit (Qiagen, USA) and used as a template for amplifying DNA fragments containing guide RNA-binding sites. The PCR reaction was performed with the following parameters: 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 65 °C for 15 s, 72 °C for 15 s, and a final extension at 72 °C for 5 min. The primers used for amplifying guide RNA-binding products (*BraFLC1*-F, TCTCTGCCCTATACATGTTCCA; *BraFLC1*-R, ACATGTGTTTTACCCACTCCT; *BraFLC2*-F, TCTCCGGCGAGAGTTGAAAC; *BraFLC2*-R, ACCGAGAAATCCACATGCGA; *BraFLC3*-F, CCTTGTGTCGAGAGCCTCAA; *BraFLC3*-R, CCAAAATGCCCTAATCTCGACA; *BraFLC5*-F, ACCTCTCGGAGACAGAAGCT; *BraFLC5*-R, GCTCATCACAACATTGTTCTTCCT) were designed by GENEIOUS program (https://www.geneious.com). An in vitro cleavage assay was performed in total 20 µL of reaction mixture containing 240 ng of DNA template, 2 µg of the SpCas9 protein (Toolgen, Korea), 1.5 µg of guide RNA and 2 µL of 10X NEB 3.1 buffer (NEB). The mixture was incubated at 37 °C for 1 h, and 4 µL of 20 mg RNase A (NEB) was added and incubated at 37 °C for 30 min. Finally, 6 µL of 5X STOP solution (1.2% of SDS, 12.5 mmol of EDTA) was added. DNA fragments were purified by Expin™ PCR SV kit (Gene All) and analyzed by 1.5% agarose gel electrophoresis.

Next-generation sequencing (NGS) analysis

Targeting regions of SpCas9 and guide RNA complex were amplified by three rounds of PCR. First, the genomic region containing guide RNA-binding sites was amplified with the size of approximately 1 kb. First-time PCR products were diluted at 1:100 or 1:1000 and used as a template for second-time PCR. Illumina adaptors and bar-code sequences were added to second- and third-time PCR products. Final PCR products were quantified by nano-drop (Thermo Scientific, USA) and purified with Expin™ PCR SV kit (Gene All). High-throughput sequencing was performed using Illumina MiSeq. Indel frequencies and mutation patterns were analyzed by CAS-Analyzer (Park et al. 2017).

Results

Protein alignment of putative FLC homologous genes in Chinese cabbage

The full lengths of four putative *FLC* homologous genes in Chinese cabbage (Kim et al. 2007; Schranz et al. 2002) were isolated by BLAST search against the genome sequence of cv. Kenshin cultivar using the *Arabidopsis* FLC (At5g10140) protein sequence (Fig. 1). Gene names are based on the sequence similarity between *B. rapa* and *Brassica napus* FLCs. As previously reported (Schranz et al. 2002), we did not find the homologous gene of *B. napus FLC4* in *B. rapa*. The protein sequence of BraFLC1, BraFLC2, and BraFLC3 showed high similarity to the protein sequence of *Arabidopsis* FLC (BraFLC1, Identifies = 81%, Positives = 87%,

Gaps = 5%; BraFLC2, Identifies = 84%, Positives = 91%, Gaps = 2%; BraFLC3, Identifies = 83%, Positives = 90%, Gaps = 1%). BraFLC5 had 58% identical amino acid sequence (Positives = 62%, Gaps = 27%) and no 50 amino acids in the middle region when compared with *Arabidopsis* FLC. Since the BraFLC proteins showed high protein similarity to *Arabidopsis* FLC, we expected functional redundancy among BraFLC proteins (Kim et al. 2007). Therefore, we decided to generate single- or double-knockout mutants of *BraFLC* genes simultaneously via conserved guide RNAs.

Testing guide RNA efficiency in protoplasts isolated from Chinese cabbage

Among various CRISPR systems (Hsu et al. 2014; Sander and Joung 2014; Sternberg and Doudna 2015; Puchta 2017), engineered Cas9 and guide RNA originated from *S. pyogenes* (Jinek et al. 2012) has been widely used for plant genome editing. To introduce insertion and deletion (indel) mutations in the Chinese cabbage *FLC* genes, we designed a total of seven guide RNAs (Fig. 2a): guide RNA1 (gRNA1) targets the first exon of the *BraFLC1* and *BraFLC2* genes; both gRNA2 and 3 target the second exon of the *BraFLC1* and *BraFLC2* genes; gRNA4 targets the first exon of *BraFLC2* and *BraFLC3* genes; gRNA5 targets the fifth exon of *BraFLC2* and *BraFLC5* genes; and both gRNA6 and gRNA7 target the *BraFLC5* gene. We first performed the in vitro cleavage assay to examine the activity of the pre-assembled ribonucleoprotein (RNP) complex, consisting of in vitro synthesized guide RNAs and recombinant SpCas9 proteins purified from *E. coli*. This CRISPR RNP complex cleaved PCR products containing the guide RNA target site (Fig. 2b).

We then verified the genome-editing efficiency of the guide RNAs in vivo in Chinese cabbage protoplasts. Each CRISPR RNP complex was delivered into the protoplasts by PEG transfection, and 2 days later, the target regions were amplified by PCR. The indel frequency and mutation patterns of each guide RNA were analyzed by targeted deep sequencing (Fig. 2c). To consider the naturally occurring mutations and sequencing errors, the same guide RNA-targeted region was amplified and sequenced in the protoplasts harboring only the SpCas9 protein (control protoplasts). As expected, there were almost no mutations (less than 0.1% mutation) in the control protoplasts. Each RNP complex induced indel mutations at various efficacies ranging from 3 to 39%: 7.4% for *BraFLC1* and

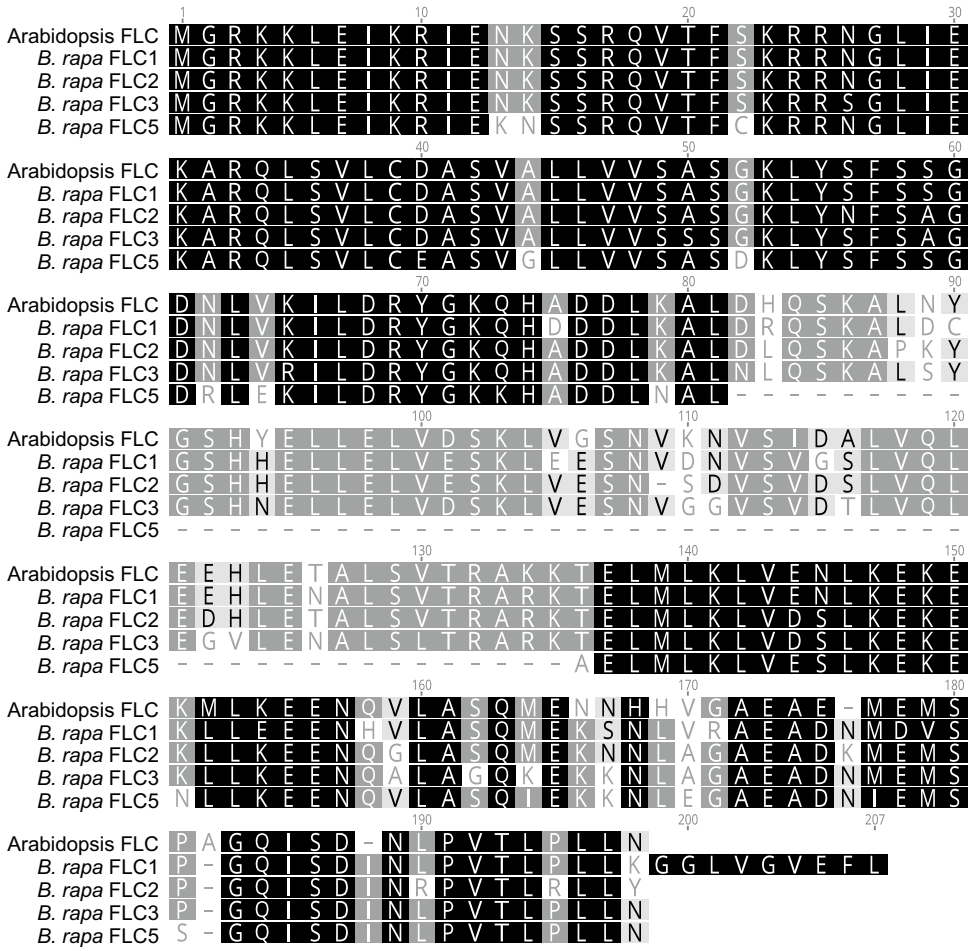

Fig. 1 Protein alignment of putative *Arabidopsis* FLC orthologs in *Brassica rapa*. Full-length amino acid sequences were aligned using the Geneious software V (https://www.geneious.com). The accession number of *Arabidopsis thaliana* FLC is At5g10140. FLC, flowering locus C

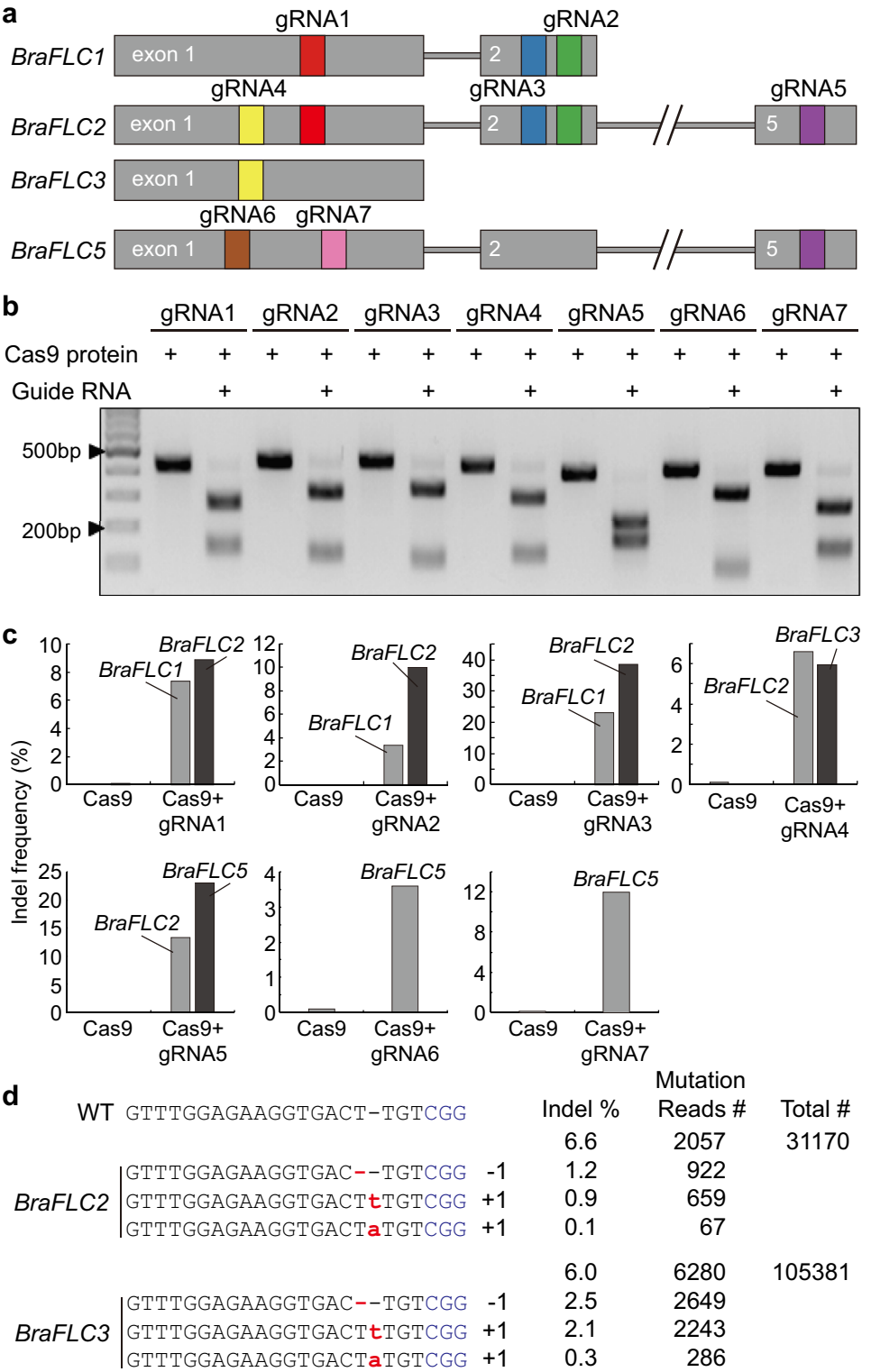

Fig. 2 In vivo activity of guide RNAs targeting *BraFLC*s. **a** Seven guide RNAs were designed to target one or two *BraFLC* genes. **b** An in vitro cleavage assay was performed to check the activity of the SpCas9 protein and synthesized guide RNA complex. **c** Indel (insertion and deletion) ratio (%) in *B. rapa* protoplasts induced by the SpCas9 and guide RNA complex. **d** Indel mutation patterns and ratio induced by the SpCas9 and gRNA4 complex. The mutation ratio (indel %) was calculated by dividing the number of reads containing indels at the target site (Read #) by the number of total sequencing reads (Total #). WT, wild-type sequence of the gRNA4-binding site; blue, the protospacer adjacent motif sequence; red, inserted or deleted sequence; + 1 and − 1 indicates one-base insertion and deletion, respectively, in the guide RNA-binding sequence

8.9% for *BraFLC2* in gRNA1-transfected protoplasts; 3.4% for *BraFLC1* and 10.0% for *BraFLC2* in gRNA2-transfected protoplasts; 23.0% for *BraFLC1* and 38.5% for *BraFLC2* in gRNA3-transfected protoplasts; 6.6% for *BraFLC2* and 6.0% for *BraFLC3* in gRNA4-transfected protoplasts; 13.3% for *BraFLC2* and 23.1% for *BraFLC5* in gRNA5-transfected protoplasts; 3.6% for *BraFLC5* in gRNA6-transfected protoplasts; and 12.0% for *BraFLC5* in gRNA7-transfected protoplasts (Fig. 2c). Analysis of the mutation pattern revealed that a single-nucleotide insertion

or deletion mainly occurred in the 3 bp upstream region of a PAM sequence (Fig. 2d).

Generation of *BraFLC* knockout mutants

To generate the knockout plants of *BraFLC* genes, we selected four guide RNAs (gRNA3, gRNA4, gRNA5, and gRNA7), all of which showed high efficiency in the protoplast assay, and cloned each guide RNA into a plant binary vector (pHAtC) (Kim et al. 2016) harboring SpCas9. We next delivered the binary vectors into *B. rapa* hypocotyl explants via *Agrobacterium*-mediated transformation. *B. rapa* seeds were germinated in a high-height glass bottle to get seedlings with a long hypocotyl (Fig. 3a). After cutting the hypocotyl into small pieces, the explants were inoculated with the binary vector-containing *Agrobacterium* (Fig. 3b), and antibiotic-resistant calli were selected on callus-inducing medium (Fig. 3c). Then, we regenerated shoot and root from the selected calli (Fig. 3d–f). The proportion of calli showing hygromycin resistance was about 1.3–5.6%. Six T_0 regenerated plants were obtained from each gRNA2- and gRNA4-transformed calli, and a single T_0 plant was obtained from each gRNA5- and gRNA7-transformed calli (Fig. 3g).

Next, we analyzed the indel mutation frequencies in T_0 plants. Unfortunately, we found no mutation on the target sites of gRNA3-, gRNA5-, and gRNA7-transformed T_0 plants (data not shown). However, all gRNA4-transformed lines contained 50% or 100% mutations on the target sites (Fig. 4a). The SpCas9 and gRNA4 complex induced one-base insertion or deletion mutation at the target sites of *BraFLC2* and *BraFLC3* genes as observed in the protoplasts (Fig. 2d). For instance, *Braflc2flc3-3* lines mainly had one-base (T) deletion (87%) at the cleavage site on *BraFLC2* and one-base (T) deletion (49.8%) and one-base (T) insertion (41.6%) on *BraFLC3*. *Braflc2flc3*-4 lines mainly had one-base (T) deletion (46.7%) at the cleavage site on *BraFLC2*, and one-base (T) deletion (49.8%) and one-base (A) insertion (40.6%) on *BraFLC3*. The *Braflc2flc3*-9 line mainly had one-base (T) deletion (48.1%) and one-base (T) insertion (38.2%) on *BraFLC2*, and interestingly showed a large deletion of 23 bases (66.9%) and one-base (T) insertion on *BraFLC3* (Fig. 4b).

The *B. rapa* cv. Kenshin we used in this study requires long-term cold treatment (vernalization) to produce a flower. Whereas a wild-type *B. rapa* produced only leaves and no flower without vernalization, double-knockout mutants in

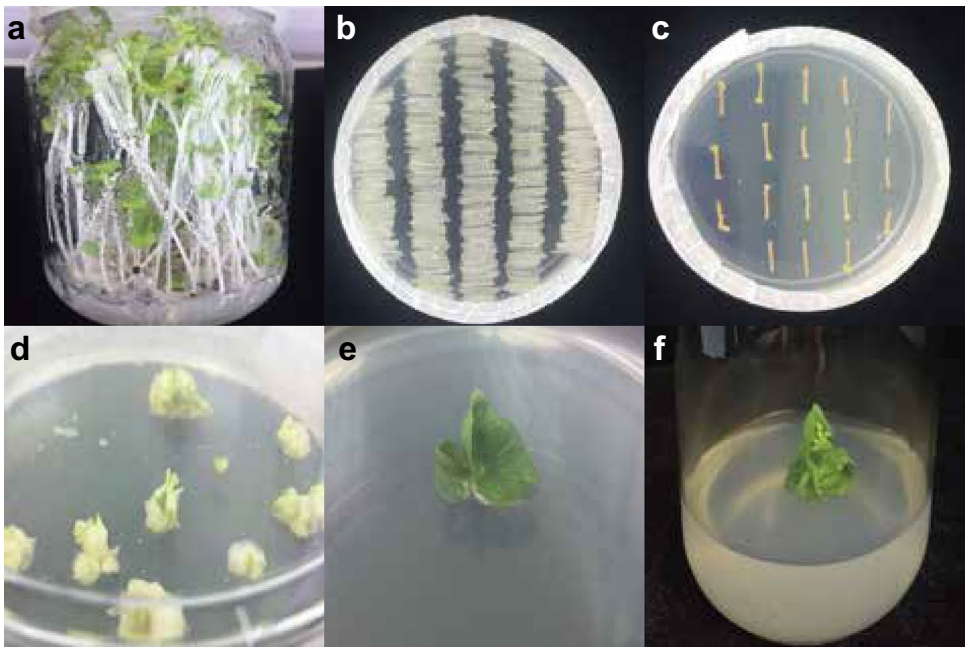

Fig. 3 Transformation and regeneration of gene-edited *Brassica rapa*. **a**–**f** Regeneration procedure of *B. rapa* after delivering the plant binary vector harboring the SpCas9 and guide RNA by *Agrobacterium*-mediated transformation. **g** The number of explants used for *Agrobacterium*-mediated transformation and the number of T_0-regenerated plants

g

	Inoculated explants	Hygromycin resistant calli	T0 plants
gRNA3	560	18 (3.2%)	6 (1.07%)
gRNA4	720	40 (5.6%)	6 (0.83%)
gRNA5	830	11 (1.3%)	1 (0.12%)
gRNA7	330	7 (2.1%)	1 (0.30%)

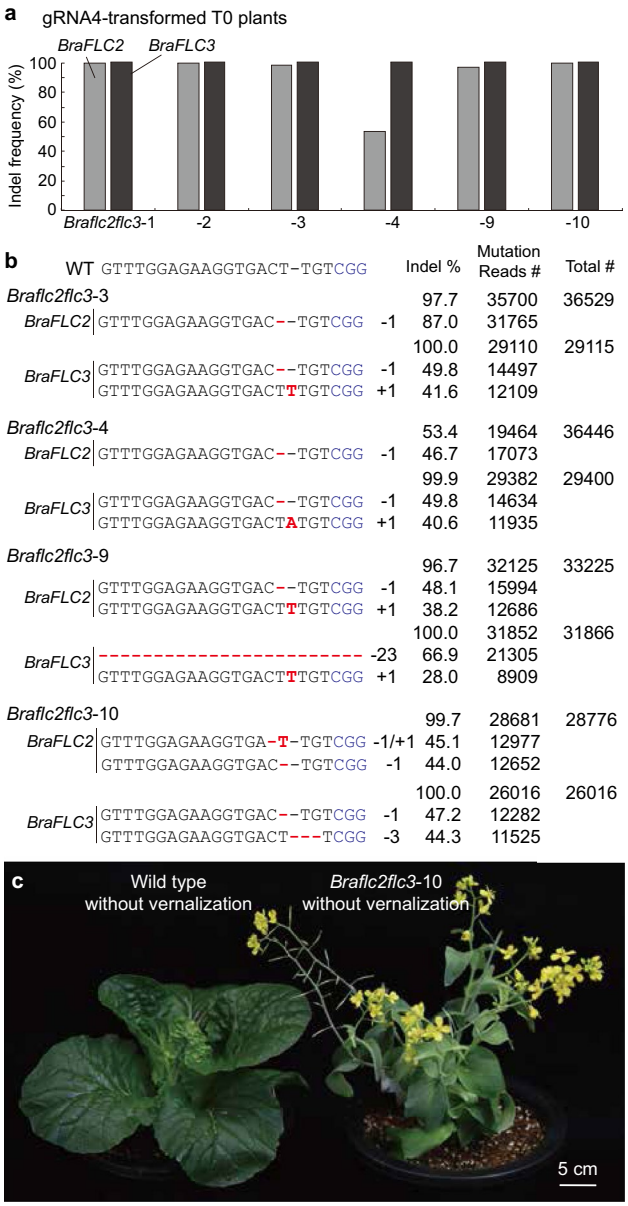

Fig. 4 Generation of T_0 *BraFLC2* and *BraFLC3* double-knockout plants. **a** The mutation ratios (indel frequency, %) in gRNA4-transformed T_0 plants. **b** Indel mutation patterns and ratios in gRNA4-transformed T_0 plants. The mutation ratio (indel %) was calculated by dividing the number of reads containing indel at the target site (Mutation Read #) by the number of total sequencing reads (Total #). WT, wild-type sequence of the gRNA4-binding site; blue, the protospacer adjacent motif sequence; red, inserted or deleted sequence; + 1 and − 1 indicates one-base insertion and deletion, respectively, in the guide RNA-binding sequence. **c** Early-flowering phenotype in *Braflc2flc3*-10 line without vernalization

BraFLC2 and *BraFLC3* genes produced flowers without vernalization (Fig. 4c).

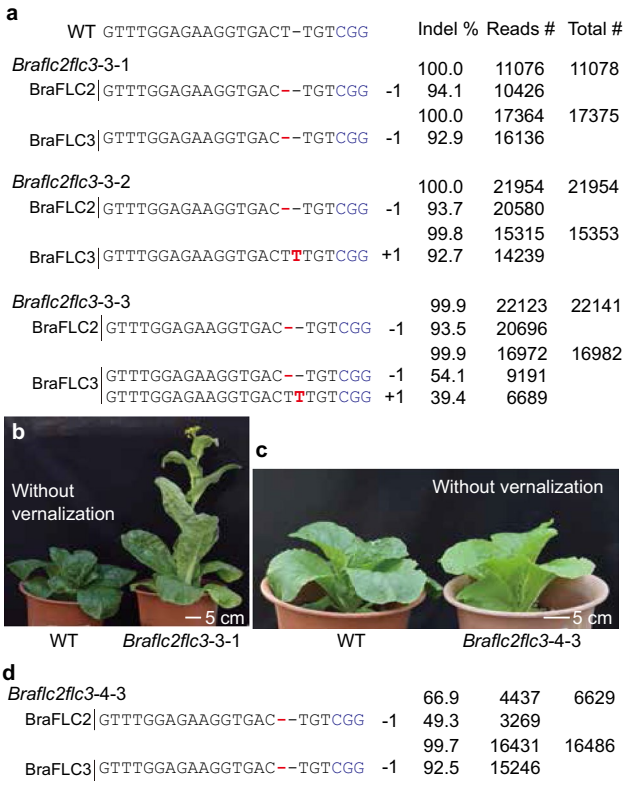

Fig. 5 Inheritability of CRISPR-induced mutations in *Brassica rapa*. **a** Indel mutation patterns and ratios in T_1 progenies of T_0 *Braflc2flc3*-3 plant. **b** Early-flowering phenotype in *Braflc2flc3*-1 line without vernalization. **c** Phenotypes of wild-type and T_1 *Braflc2flc3*-4-3 lines without requiring vernalization. The monoallelic wild-type allele of *BraFLC2* in biallelic *BraFLC3* mutation background is enough to suppress flowering of *B. rapa* without requiring vernalization. **d** Indel mutation patterns and ratios in T_1 progeny of T_0 *Braflc2flc3*-4 plant

Heritability of Cas9-induced mutations in *B. rapa*

To check the heritability of the mutations and the edited trait, we germinated T_1 seeds collected from all *Braflc2flc3* T_0 lines and performed the targeted sequencing. The progeny of *Braflc2flc3*-3 mutant follows the Mendelian segregation pattern: on the *BraFLC2* target site, all *Braflc2flc3*-3 progeny (T_1 plants) had one-base deletion (T), and on the *BraFLC3* target site, the *Braflc2flc3*-3-1 mutant showed 92.9% of one-base deletion (T), the *Braflc2flc3*-3-2 mutant showed 92.7% of one-base insertion (T), and the *Braflc2flc3*-3-3 showed 54.1% of one-base deletion (T) and 39.4% of one-base insertion (T) (Fig. 5a). Those T_1 double-knockout plants kept an early-flowering phenotype unlike wild-type plants (Fig. 5b).

Interestingly, we found phenotypic segregation in the progeny of *Braflc2flc3*-4. Some progeny of *Braflc2flc3*-4 showed a similar flowering phenotype with a wild-type *B. rapa* (Fig. 5c) and some progeny showed the early-flowering phenotype that did not require vernalization. The T_1 mutant

plants with similar phenotypes to the wild type have the 49.3% indel frequency on *BraFLC2* and 92.5% indel frequency on *BraFLC3* (Fig. 5d), suggesting that the expression of one copy of *BraFLC2* gene is enough to suppress flowering.

Discussion

Homologous gene editing of *B. rapa* (Chinese cabbage)

In this study, we showed that the CRISPR system enables us to induce targeted mutation in *B. rapa FLC* genes and that the mutation pattern observed in parental lines is passed on to the next generation. To our knowledge, these *Braflc2flc3* double-knockout lines are the first genome-edited Chinese cabbage. Before generating gene-edited *B. rapa*, we tested the efficacy of designed guide RNA by delivering the SpCas9 and guide RNA complex into the protoplasts isolated from *B. rapa* leaves. Based on the gene-editing efficacy of each guide RNA, four guide RNAs (gRNA3, gRNA4, gRNA5, and gRNA7) with high efficiency were selected and delivered into *B. rapa* hypocotyl explants by *Agrobacterium*-mediated transformation. Although from one to six T_0 plants were regenerated from each guide RNA-transformed line, the targeted editing was identified only in regenerated plants containing gRNA4. The indel efficacy of gRNA3 in protoplasts was better than that of gRNA4, but we found no mutation in any of the six gRNA3-transformed plants. This phenomenon might be due to the difference of editing efficacy between leaf protoplasts and hypocotyl callus or the difference in the positions of T-DNA insertion affecting SpCas9 and guide RNA expression.

Vernalization and four *FLC* genes in *B. rapa*

The *Braflc2flc3* double-knockout lines we made in this study produced flowers without requiring long-term cold treatment. In addition, monoallelic wild-type *BrFLC2* in biallelic *BraFLC3* mutation background was enough to suppress the flowering of *B. rapa*, such as wild-type *B. rapa* (Fig. 5c). This result is consistent with the previous study in which overproducing a single *BraFLC* gene in *Arabidopsis* resulted in late flowering (Kim et al. 2007). Our results clearly show that there is the functional redundancy between *BraFLC2* and *BraFLC3* for *B. rapa*'s response to vernalization treatments. However, we know little about whether *BraFLC1* and *BraFLC5* play overlapping roles with *BraFLC2* and *BraFLC3* in the response to vernalization. This question remains to be tested.

Mutation pattern of tissue culture-based genome editing

Arabidopsis researchers have used a floral dipping method to deliver a plant binary vector harboring SpCas9 and guide RNA during floral development. SpCas9 proteins are normally expressed under strong promoters, such as 35S or ubiquitin. The 35S promoter-derived SpCas9 system works quite efficiently to induce targeted mutations in reproductive cells, but this system also produces many somatic mutations in leaves. To produce heritable mutations in plants, egg cell-specific promoters have been implemented to express the SpCas9 protein only in reproductive cells (Mao et al. 2015; Tsutsui and Higashiyama 2017).

However, the problem of non-inheritable mutations has not been well documented in attempts to edit plant genomes, except for that of *Arabidopsis*. We think that the differences in the transformation method are responsible for the inheritability of mutations. In most plants, tissue culture is required to deliver a plant binary vector into plant cells, and the single-transformed cell regenerates into a whole plant. If SpCas9 and guide RNA expression are fast and high enough to induce a mutation in this single cell, we can observe the simple mutation patterns in the whole regenerated plant, such as monoallelic (50% mutation) or biallelic (100% mutation) in T_0 plants (Xu et al. 2014; Kim et al. 2016; Wang et al. 2016; Sun et al. 2017; Zhang et al. 2017; Jiang et al. 2019).

Generation of a rapid cycling *B. rapa*

We generated a rapid cycling *B. rapa* by editing two functionally redundant *BraFLC* genes. The short life cycle of the edited Chinese cabbage is suitable for forward and reverse genetic study and allows us to examine the function of genes in *B. rapa* (Williams and Hill 1986). In many previous studies, to understand the function of *B. rapa* genes, the genes are typically over-expressed in *A. thaliana* and the altered phenotype is analyzed (Kim et al. 2007). However, the development of CRISPR technology has allowed us to develop a crop as a model plant; this in turn allows us to study the function of genes in the crop directly.

This study clearly shows how to examine the efficacy of guide RNAs in *B. rapa* protoplasts, how to regenerate gene-edited *B. rapa*, and how to calculate the mutation patterns and frequency in the transformed plant. By producing an early-flowering *B. rapa* that do not require vernalization, we have obtained materials that can be used to study how a gene functions in *B. rapa*. Shortly, CRISPR will be widely used to develop species-specific model systems for precisely targeted molecular and breeding studies.

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