

Article

Multiplex CRISPR/Cas9 Mutagenesis of *BrVRN1* Delays Flowering Time in Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Abstract: The *VERNALIZATION1* (*VRN1*) gene is a crucial transcriptional repressor involved in triggering the transition to flowering in response to prolonged cold. To develop Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) plants with delayed flowering time, we designed a multiplex CRISPR/Cas9 platform that allows the co-expression of four sgRNAs targeting different regions of the endogenous *BrVRN1* gene delivered via a single binary vector built using the Golden Gate cloning system. DNA sequencing analysis revealed site-directed mutations at two target sites: gRNA1 and gRNA2. T₁ mutant plants with a 1-bp insertion in *BrVRN1* exhibited late flowering after the vernalization. Additionally, we identified ‘transgene-free’ *BrVRN1* mutant plants without any transgenic elements from the GE₁ (gene-editing 1) and GE₂ generations. All GE₂ mutant plants contained successful edits in two out of three *BrVRN1* orthologs and displayed delayed flowering time. In GE₂ mutant plants, the floral repressor gene *FLC1* was expressed during vernalization; but the floral integrator gene *FT* was not expressed after vernalization. Taken together, our data indicate that the *BrVRN1* genes act as negative regulators of *FLC1* expression during vernalization in Chinese cabbage, raising the possibility that the ‘transgene-free’ mutants of *BrVRN1* developed in this study may serve as useful genetic resources for crop improvement with respect to flowering time regulation.

Keywords: *Brassica rapa*; *BrVRN1*; CRISPR/Cas9; late flowering; transgene-free; vernalization



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1. Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is an important vegetable crop that contributes to human health as a source of beneficial dietary fiber, vitamins, and minerals [1]. Because it is responsive to seed vernalization, Chinese cabbage is prone to premature bolting, which affects crop yield and quality. This is particularly true in spring varieties that are often exposed to extended periods of cold during winter [2–4].

A properly timed transition from vegetative to reproductive development is the most important step in the life cycle of a flowering plant. Flowering time plasticity has evolved to integrate plants’ perceptions of both seasonal and developmental changes to maximize reproductive success in various environments. Flowering time has also emerged as a key trait affecting yield in commercial agronomic and horticultural crops because certain biotic and abiotic stresses can be avoided altogether by inducing or delaying the transition to flowering. Multiple pathways, such as photoperiod sensation, vernalization, gibberellin signaling, and responses to autonomous signals, are involved in the timing of floral transition during specific seasons, and altered flowering time phenotypes have been generated in multiple cultivated species through targeted genetic modification [5–9]. Vernalization, the induction of flowering by prolonged cold, ensures that plants flower in favorable spring conditions, and is usually required for flowering in Brassicaceae. Because

Chinese cabbage can sense low temperatures during seed germination, it sometimes undergoes early flowering (bolting) due to low temperatures in the fall [10,11]. Premature bolting leads to a decline in commercial quality and a loss of market value, so a high bolting resistance is preferred when breeding cultivars of this species [10].

To facilitate these breeding efforts, extensive genetic analysis in *Arabidopsis thaliana* has identified several genes, including *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), that play vital roles in the flowering process. *FRI* encodes a plant-specific nuclear protein that promotes high expression of *FLC* [12]. *FLC* encodes a MADS-box transcription factor that prevents flowering by suppressing the expression of the floral integrator genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) [13–16]. The *FLC* expression level negatively correlates with flowering time after vernalization, and is suppressed by exposure to a long period of cold temperatures [17]. The duration of vernalization, therefore, represses *FLC* expression in order to promote flowering in vernalization-responsive late-flowering plants. Thus, *FLC* is thought to be a major determinant of the vernalization response. In *Arabidopsis*, *FLC* expression is decreased through a vernalization mechanism involving *VERNALIZATION INSENSITIVE 3* (*VIN3*), and this repression is epigenetically maintained by a Polycomb-like chromatin regulation involving *VERNALIZATION 2* (*VRN2*), a Su(z)12 homolog, *VERNALIZATION 1* (*VRN1*), and *LIKE-HETEROCHROMATIN PROTEIN 1* [18–23].

VRN1 encodes a DNA-binding protein containing two plant-specific B3 domains that is required for stable repression of the major target of the vernalization pathway, the floral repressor *FLC*, during prolonged cold treatment [23,24]. In *Arabidopsis*, expression levels of *AtVRN1* and *AtVRN2* are not affected by vernalization [22,23]. However, *vrn1–2* mutant plants grown in extended short-day (ESD) conditions showed reduced *FT* but wild-type *FLC* expression, suggesting that *VRN1* regulates *FLC* and *FT* [23]. In Chinese cabbage, the expression of *BrVRN1* genes is altered by cold treatment, according to patterns that vary among the three paralogous genes: two *BrVRN1* genes are downregulated during cold treatment, and one is upregulated. It is therefore possible that the suppression of *BrFLC* expression is dependent on the upregulation of *BrVRN1* by vernalization in *B. rapa* [25].

Despite genetic analysis of floral transition regulation in *Arabidopsis* and the identification of floral regulator genes in Chinese cabbage, there have been few reports on the mechanism of floral transition in Chinese cabbage specifically [26,27]. Recently, several studies have set out to directly control flowering time via CRISPR/Cas9-mediated genome editing [28,29]. In this study, we used the CRISPR/Cas9 system for targeted mutagenesis of *BrVRN1* homologs (*BrVRN1s*) to introduce the late-flowering trait into *B. rapa* [25]. CRISPR/Cas9-mediated multi-target mutagenesis of *BrVRN1s* gave new insights into flowering time regulation and demonstrated that genome editing can be used to improve agricultural traits in *B. rapa*.

2. Material and Methods

2.1. sgRNA Target Site Design and Vector Construction

sgRNAs were designed using the web tool Cas-Designer (<http://www.rgenome.net/cas-designer/>, accessed on 8 July 2019) [30], which displayed all optional sgRNA sequences (20 bp) immediately followed by a 5'-NGG protospacer-adjacent motif (PAM) sequence in the forward or reverse strand. Sequences of the Chinese cabbage *VRN1* genes (*BrVRN1a*, Bra022375, *BrVRN1b*, Bra037544, and *BrVRN1c*, Bra001729) were compared to find a region with matching sequences (Figures 1a and S1 and Table S1). Then, four sgRNAs were designed in the corresponding region to target the *BrVRN1a* (Bra022375) gene [25]. To construct a plasmid vector carrying four sgRNAs and the Cas9 cassette, we designed a multiplex CRISPR/Cas9 platform that allows the co-expression of four sgRNA modules targeting different sites of *BrVRN1* in one binary vector (pAGM4723) using Golden Gate assembly (Figure 1b) [31]. The sequence of Cas9 was assembled downstream of the *Cauliflower mosaic virus* (CaMV) 2× 35S promoter along with four custom sgRNAs driven by four *Arabidopsis* U6-26 promoters. The hygromycin-resistance gene (*hpt*) driven by

a *nos* promoter was used as a selectable marker. The final vector was introduced into *Agrobacterium tumefaciens* GV3101 and then used for the transformation of Chinese cabbage.

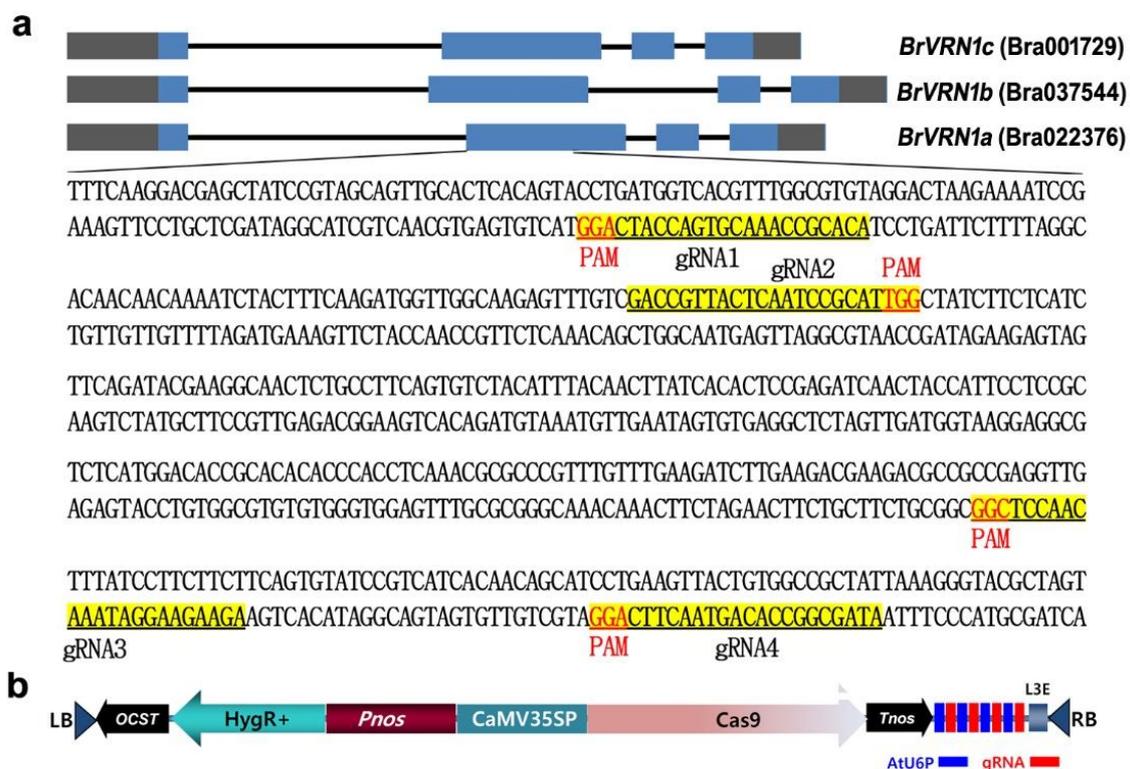


Figure 1. Multiplex gene editing construction of *BrVRN1* family gene. (a) Gene structure of *BrVRN1a* with target sites of CRISPR/Cas9 designed in the second exon. Blue stripe, exon; black line, intron; grey stripe, UTR (untranslated regions). The underlined nucleotides indicate the target sites (named gRNA1 to gRNA4). Nucleotides in red represent PAM sequences. PAM, protospacer adjacent motif. (b) Structure of CRISPR/Cas9 platform with *BrVRN1a* four sgRNAs vector based on pAGM4723. AtU6P, *Arabidopsis* U6 promoter; *Pnos*, nopaline synthase promoter; *Tnos*, nopaline synthase terminator; LB, left border; RB, right border; HygR+, hygromycin-resistance gene; OCST, octopine synthase terminator; L3E, end linker 3; CaMV35P, 35S promoter.

2.2. Generation of Transgenic Chinese Cabbage Plants

The late-flowering Chinese cabbage cultivar ‘inbred line DA001’ was used for transformation according to previously published methods with minor modifications [32,33]. Surface-sterilized seeds were placed on Murashige and Skoog (MS) medium (MS basal medium, 3% sucrose, and 0.8% Phytagar) and germinated in a culture room under long-day conditions (16 h light/8 h dark) at 25 °C for 7 days. Hypocotyls were cut into 0.5 cm segments and placed on pre-culture medium (MS basal medium, 1 mg/L NAA, 3 mg/L BA, 2 mg/L AgNO₃, 3% sucrose, and 0.8% Phytagar) for 2 days. The pre-cultured hypocotyls were inoculated with *Agrobacterium* suspension (OD₆₀₀ = 0.3) in MS liquid medium for 15–20 min with gentle shaking. The inoculated hypocotyls were blotted with sterilized filter paper to remove excess bacterial suspension and co-cultivated on co-cultivation medium (MS basal medium, 1 mg/L NAA, 3 mg/L BA, 2 mg/L AgNO₃, 100 μM acetosyringone, 3% sucrose, and 0.8% Phytagar) for 3 days in darkness at 25%. Hypocotyls were then washed with sterilized water containing 100 mg/L carbenicillin and 250 mg/L cefotaxime and transferred to selective medium (MS basal medium, 1 mg/L NAA, 3 mg/L BA, 2 mg/L AgNO₃, 3% sucrose, 100 mg/L carbenicillin, 250 mg/L cefotaxime, and 0.8% Phytagar) supplemented with 10 mg/L hygromycin as a selective agent. Callus that formed on hypocotyls was sub-cultured on fresh selective medium every 2–3 weeks. Healthy and elongated shoots were excised and transferred to a phytohormone-free MS medium to induce rooting. The transformed plantlets were grown in a greenhouse after acclimatization.

2.3. Confirmation of the Mutations of Transgenic Plants

To confirm the stable integration of transgenes in Chinese cabbage, the dual-genomic DNA-PCR was performed. Genomic DNA PCR was carried out using a pair of *hpt*-specific primers (forward, 5'-AGCCTGAACTCACCGCGACGTCTGTGCGAGAAGTTTC-3'; reverse, 5'-TCTACACAGCCATCGGTCCAGACGGCCGCGCTTCTG-3'; 961 bp PCR product) and two pairs of Cas9-specific primers (SP1-forward, 5'-TCCTCCCGAAAAGGAACAGCGAC-3'; SP1-reverse, 5'-CATTTCGTTTCCGGCCGTTTTCAAG-3'; 311 bp PCR product, SP2-forward, 5'-CAACTGGTTCAGACTTACAATCAGC-3'; SP2-reverse, 5'-TTCCAAGATGGGCTTAATAAATTTG-3'; 1031 bp PCR product). The PCR reactions were performed using 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 40 s at 72 °C, followed by a final extension at 72 °C for 10 min. The resulting PCR products were analyzed using electrophoresis and ethidium bromide staining.

To verify *BrVRN1* mutations in gene-edited plants, genomic DNA was isolated from transgenic leaf samples using the CTAB method, and then PCR, Sanger sequencing, and deep sequencing were performed. To confirm *BrVRN1* mutations in T₀ and T₁ generation plants, as well as in gene-edited plants without transgenes (GE₁ plants, sequences adjacent to the four sgRNA target positions were amplified with primer sets (target1 and target2, 5'-AGGACGAGCTATCCGTAGCA-3', 5'-GGCAGAGTTGCCTTCGTATC-3'; target3 and target4, 5'-TACCATTCTCCGCTCTCAT-3', 5'-TAGGAGTTGGTGTGCGCTCT-3'). PCR products were subjected to deep sequencing performed at ToolGen Inc. (ToolGen, Seoul, Korea) to detect mutations [34]. Also, to verify the mutations in the three *BrVRN1* genes in the GE₁ and GE₂ plants, Sanger sequencing was performed using three gene-specific amplicons generated using intron-specific primer sets (*BrVRN1a*, 5'-TATACAATGTTCCAGGAATAGTAATGGAC-3', 5'-ACCAATCATCACAATCAATCCAAG AATG-3'; *BrVRN1b*, 5'-GAACCATTGGATTGTGATTTAAATTA-3', 5'-AATTTTCGGTT CAGTTCAGCCGGTTTGG-3'; *BrVRN1c*, 5'-CCTAAAGGTTACAAGCA0GTCTCATTAT-3', 5'-TCACTACTAAACAATAGTCTGAAGAATC-3'). PCR amplicons from GE₁ and GE₂ plants were cloned into the pGEM-T vector (Promega, Madison, WI, USA), and 100 plasmids were Sanger-sequenced. For verification of on- and off-target editing events, as well as homozygosity, in the GE₂ generation, SNP and insertion/deletion (indel) analyses were performed using next-generation sequencing (NGS) at SEEDERS Inc. (SEEDERS, Daejeon, Korea). Raw sequencing reads of individual paralogs were used as queries, which were then compared to the provided non-edited reference genome sequence (Table S2) [35–40].

2.4. RNA Extraction and Real-Time Reverse Transcription Quantitative PCR (qPCR) Analysis

To analyze the expression of floral-related genes in transgenic plants, qPCR was performed using primer sets specific to flowering genes (*BrFLC1*, 5'-GTGCGCTGCTTGAACA T-3', 5'-CCTGACCAGGCTGGAGAAA-3'; *BrFT*, 5'-ACTTTGGTTATGGTGGATCCAGATG T-3', and 5'-GGAAGGCCGAGATTGTATAGGGAA-3') [41,42]. Samples were collected from the leaf tissue of GE₂ plants at different growth stages. Total RNA was extracted from 100 mg of leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with DNase I (New England Biolabs, Ipswich, MA, USA) to remove genomic DNA. First-strand cDNA was synthesized from 2 µg of total RNA using MMLV reverse transcriptase (RNaseH-free; Toyobo, Osaka, Japan) according to the manufacturer's instructions. The cDNA was resuspended in nuclease-free water (Promega, Madison, WI, USA) and used for qPCR analysis with a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). *BrActin* was used as an internal control for normalizing mRNA levels [41,42]. Three biological replicates were analyzed for each qRT-PCR assay, and Student's *t*-test was used for statistical analyses.

2.5. Flowering Time Analysis

Transgenic Chinese cabbage plants with 4–5 leaves were transferred to a 4 °C growth chamber (for vernalization) under long-day conditions (16 h light/8 h dark) for 40–60 days. After vernalization, plants were transplanted into 25 cm diameter pots and transferred to

a greenhouse to observe flowering time phenotypes in March and September [32]. The flowering time of GE₁ and GE₂ plants was recorded as the number of days until the appearance of a bolt with a floral bud. Progeny seeds were obtained using bud-pollination.

3. Results

3.1. Identification and CRISPR-Cas9 Mutagenesis of *BrVRN1* Genes

To identify orthologs of the *AtVRN1* gene in Chinese cabbage, we used an *AtVRN1* (At3g18990) sequence for a BLAST query in the *B. rapa* database (BRAD, <http://brassicadb.org>, accessed on 15 March 2019), which revealed three paralogs (Table S1), which we refer to as *BrVRN1a*, *BrVRN1b*, and *BrVRN1c*. The sequence similarity between the paralogs ranged between 89.1% and 90.7%. A previous study showed that under cold treatment, *BrVRN1a* (Bra022376) is upregulated, while its paralogs *BrVRN1b* (Bra037544) and *BrVRN1c* (Bra001729) are downregulated [25,41]. We expected that knocking out any one paralog in Chinese cabbage would give comparable phenotypic results and therefore focused on *BrVRN1a*. We selected four target sites (gRNA1-gRNA4) in the second exon of *BrVRN1a* (Figures 1a and S1 and Table S3) and transformed the corresponding multiplex plasmid vector carrying four sgRNAs and a Cas9 cassette into Chinese cabbage (*B. rapa* L. ssp. *pekinensis*) via *Agrobacterium*-mediated transformation (Figure 1b).

Next, we performed hypocotyl transformation to introduce the CRISPR/Cas9 construct into the late-flowering Chinese cabbage cultivar ‘inbred line DA001’. The efficiency of callus formation from hypocotyls infected with *Agrobacterium* on selection medium supplemented with hygromycin was 36% (data not shown), and we obtained nineteen independent T₀ transformants (Table S4). Before transferring the transgenic plants to the soil, we used genomic DNA extracted from T₀ leaf tissue to examine the CRISPR/Cas9-induced mutations at the target sites using deep-sequencing analysis (data not shown). We identified mutations in 10 independent T₀ lines regenerated into whole plants, demonstrating the efficacy of the selected sgRNAs in Chinese cabbage. Deep-sequencing of these lines revealed an overlay of more than two different sequences (Figure S2), suggesting that these were chimeric plants containing different mutations and that some mutations might have occurred somatically. Among these 10 mutants, we were able to harvest seeds from three lines (#1, #2, and #7), which we planted under vernalization conditions in Jeonju, Korea, and used for further studies.

3.2. Generation of ‘Transgene-Free’ Mutant Lines

We expected that chimeric T₀ plants would give rise to complex segregation patterns in the T₁ generation and that homozygous mutants could only be selected from later generations. We tested thirty-seven T₁ plants (19 from line #1, 13 from line #2, and 5 from line #7) for the presence of transgenes. Also, to obtain *BrVRN1a* mutants without any transgenic elements of the sgRNAs/Cas9 vector, we screened ‘transgene-free’ plants through a dual-PCR strategy that used two sets of primers flanking the *Cas9* and *hph* (hygromycin B phosphotransferase) genes (Figure S3). This showed that two (#2-2 and #2-8) of the 13 #2 T₁ mutants were ‘transgene-free’, and their 20 offspring plants were all ‘transgene-free’ *BrVRN1* mutants (Figure S4). We then conducted deep-sequencing of two selected ‘transgene-free’ GE₁ (gene-editing 1) lines, which confirmed *BrVRN1* gene editing based on the proportion of indels. The 1-bp insertion type was most frequently identified in the *BrVRN1*-gRNA1 and *BrVRN1*-gRNA2 target regions but was not found in the *BrVRN1*-gRNA3 and *BrVRN1*-gRNA4 target regions (Figure S5).

Next, we sequenced the PCR products from the genomic DNA of each mutant to search for mutations within the targeted regions in three paralogs (*BrVRN1a-c*). Cloning the amplicons containing the target regions into plasmids and subsequent sequencing confirming the chimeric nature of the T₁ plants. Interestingly, five (#2-1, -2, -3, -8, and -13) mutant plants showed three types of mutations at two target sites in two genes (*BrVRN1a, b*) (Figure 2). Two types of mutations were found at target sites *BrVRN1a*-gRNA1 and -gRNA2 (A-insertion and C-insertion), and the 1-bp insertion type was most frequently

identified. A type of mutation was also found at target site *BrVRN1b*-gRNA2 (A-insertion), and the 1-bp insertion type was most frequently identified. However, no mutations at the target sites *BrVRN1c*-gRNA1 and *BrVRN1c*-gRNA2 were generated in T₁ plants (Figure 2). All types of frameshift mutations induced by CRISPR/Cas9 at target sites of *BrVRN1a* and *BrVRN1b* generated premature translation termination codons (Figure 3). Unfortunately, no mutations at target sites gRNA3 and gRNA4 in *BrVRN1a* were identified in T₁ plants. This suggests that the mutations at these two target sites were not inherited from the T₀ generation to the T₁ generation.

<i>BrVRN1a</i> (Bra022376-target)	
Wt	CAGTACCTGAT–GGTCACGTTTGGCGTGTAGGACT
#2-1	CAGTACCTGAT A GGTCACGTTTGGCGTGTAGGACT
#2-2	CAGTACCTGAT A GGTCACGTTTGGCGTGTAGGACT
#2-3	CAGTACCTGAT A GGTCACGTTTGGCGTGTAGGACT
#2-8	CAGTACCTGAT A GGTCACGTTTGGCGTGTAGGACT
#2-13	CAGTACCTGAT A GGTCACGTTTGGCGTGTAGGACT
Wt	TTTGTCGACCGTACTCAATCCG–CATTGGCTATC
#2-1	TTTGTCGACCGTACTCAATCCG CC ATTGGCTATC
#2-2	TTTGTCGACCGTACTCAATCCG CC ATTGGCTATC
#2-3	TTTGTCGACCGTACTCAATCCG CC ATTGGCTATC
#2-8	TTTGTCGACCGTACTCAATCCG CC ATTGGCTATC
#2-13	TTTGTCGACCGTACTCAATCCG CC ATTGGCTATC
<i>BrVRN1b</i> (Bra037544)	
Wt	TTGTTGACCGTACTCAATCCG–CATTGGTTACC
#2-1	TTGTTGACCGTACTCAATCCG A CATTGGTTACC
#2-2	TTGTTGACCGTACTCAATCCG A CATTGGTTACC
#2-3	TTGTTGACCGTACTCAATCCG A CATTGGTTACC
#2-8	TTGTTGACCGTACTCAATCCG A CATTGGTTACC
#2-13	TTGTTGACCGTACTCAATCCG A CATTGGTTACC

Figure 2. Target gene mutations by the multiplex CRISPR/Cas9 system in the T₁ generation. The mutated DNA sequences of target regions are provided. Gene mutations are in red. Wt, untransformed wild-type plants; lanes #2-2 to -8, selected GE1 lines, respectively.

3.3. Stable Inheritance of Induced Mutations and Phenotypes of the Mutants

To validate whether CRISPR/Cas9-mediated mutagenesis of *BrVRN1s* was linked to a late-bolting phenotype, we compared the flowering times of the three T₁ mutant lines (#1, #2, and #7) with that of wild-type plants under vernalization conditions. All three mutant lines had delayed flowering time (Figure S6 and Table 1). After a 60-day vernalization period, the average time to bolting after the end of the vernalization period was approximately nine days in wild-type plants, but 19–20 days in the CRISPR/Cas9-mutagenized #1 and #2 T₁ plants. In addition, after 40 days of vernalization, wild-type plants bolted 15–16 days after the end of vernalization, while the T₁ mutant #7 plants were delayed by 8–11 days, bolting 23–27 days after the end of vernalization (Table 1). Thus, bolting time was delayed in all mutants regardless of cold treatment season and duration

(Table 1), suggesting that the targeted gene-edits of *BrVRN1s* are sufficient to induce late-bolting phenotypes in Chinese cabbage. In order to use the mutants showing delayed bolting as breeding material, we attempted to select mutants in which the transgenes had been removed and the mutations were homogeneous. We then analyzed GE₂ generations of the two GE₁ lines that had the Cas9 gene removed (Figure S3) and selected two ‘transgene-free’ mutants (#2-2 and #2-8) for phenotypic analysis. We self-crossed the #2-2 and #2-8 GE₁ plants to obtain GE₂ seeds and analyzed 10 plants (20 total) from each line for their genotypes and bolting characteristics.

BrVRN1a (Bra022376-target)

	M P R P F F H K L I F S S T I Q E K R L R V P D K F V	
1	ATGCCACGCCCTTTCATCCACAAGTGTATTTCTCATCCACTATCCAAGAAAACCGCTGAGAGTCCAGATAAGTTTGT	80
1	TACGGTGC GGGAAGAAGGTGTTCAACTAAAAGAGTAGGTGATAGGTTCTTTTTCGGGACTCTCAAGGCTATTCAAACA	80
	S N F K D E L S V A V A L T V P D R S R L A C R T K	
81	GAGTAAATTCAGGACGAGCTATCCGTAGCAGTTGCACACTCACAGTACCTGATAGGTCAGCTTTGGGGTGAAGGACTAAGA	160
81	CTCATTAAAGTTCCTGCTCGATAGGCATCGTCAAAGTGTGTCATGGACTATCCAGTGC AAACCCGACATCCTGATTCT	160
	K I R Q Q Q N L L S R W L A R V C R P L L N P P L A I	
161	AAATCCGACAACAACAAAATCTACTTTCAAGATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCCATTGGCTATC	240
161	TTTAGGCTGTGTGTTTAGATGAAAGTTCACCAACCGTTCCTCAAACAGCTGGCAATGAGTTAGCGGTAMCCGATAG	240
	F S S S D T K A T L P S V S T F T T Y H T P R S T T I	
241	TTCTCATCTCAGATACGAAGGCAACTTCGCCTCAGTGTCTACATTTACAACCTATCACACTCCGAGATCAACTACCAT	320
241	AAGAGTAGAAGTCTATGCTCCGTGAGACGGAAGTCACAGATGTAATGTTGAATAGTGTGAGGCTCTAGTTGATGGTA	320
	P P L S W T P H T P T S N A P V C L K I L K T K T P	
321	TCCTCCGCTCTATGGACACCCGACACACCCACCTCAAACGCGCCGTTGTTTGAAGATCTTGAAGAGAGAGCGCCGC	400
321	AGGAGGCGAGAGTACCTGTGGCGTGTGGGTGGAGTTTTCGCGGGCAAACAACCTCTAGAACTCTGCTCTCGGGCG	400
	P R L F I L L L Q C I R H H N S I L K L L W P L L K G	
401	CGAGGTGTTTATCCCTTCTTTCAGTGTATCCGTATCACAACAGCATCCTGAAGTACTGTGGCCGCTATTAAAGGGT	480
401	GCTCCAACAATAGGAAGAAGTACATAGGCAGTAGTGTGTCGTTAGGACTTC AATGACACCGGGGATAATTTCCCA	480
	T L V Q L S R A S S L D H L L K L K R R H Q L L K L L	
481	ACGCTAGTCCAGCTATCCAGAGCTTCTTCGCTGGACCACCTGTTAAAGCTGAAGAGGCGACCAACTCCTAAAGTTACT	560
481	TGCATCAGGTCGATAGGTCGGAAGAAGGACCTGGTGGACAATTCGACTTCTCGCTGTGGTTGAGGATTCAATGA	560
	R R E G G R R R T L F L R K *	
561	AAGAAGAGAGGGAGGAGAGAAAGAACCGCTTCCTGAGGAGTAACTCATCTGCTCCGAGGGATGATGACCCGGAGAG	640
561	TTTCTCTCCCTCCTTCTTCTTTCGACAAAGGACTCCTTCATTTGAGTAGACGAGGCTCCTACTACTGGCCTCTC	640

BrVRN1b (Bra037544)

	M P R P F F H K L I F S S T I Q E K R L R V P D K F V	
1	ATGCCACGCCCTTTCATCCACAAGTGTATTTCTCATCCACTATCCAAGAAAACCGCTGAGAGTCCAGATAAGTTTGT	80
1	TACGGTGC GGGAAGAAGGTGTTCAACTAAAAGAGTAGGTGATAGGTTCTTTTTCGAGACTCTCAAGGCTATTCAAACA	80
	S K F K D E L S V A V A L T V P D G H V W R V G L R	
81	GAGTAAATTCAGGACGAGCTATCCGTAGCAGTTGCACACTCACAGTACCTGATGGTCAATGTTGGCGTGTAGGACTAAGGA	160
81	CTCATTAAAGTTCCTGCTCGATAGCCAAAGTCAACGTGAGTGTATGGACTACCAGTACAACCCGACATCCTGATTCT	160
	K A D N N K I W F Q D G W Q E F V D R Y S I R H W L P	
161	AAGCTGACAACAACAAAATCTGGTTCAAGATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGACATTGGCTACCT	240
161	TTGACTGTTGTGTTTAGACCAAAGTTCACCAACCGTTCCTCAAACAACCTGGCAATGAGTTAGGCTGTAAACCGATGGA	240
	F D F *	
241	TTTGATTTTTAGATACGAAGGCAACTTCGCCTCAGGCTCTACATTTACAACCTTATCACACTCCGAGATCAACTACCATT	320
241	AAACTAAAAATCTATGCTCCGTTGAGACGGAAGTCCGAGATGTAATGTTGAATAGTGTGAGGCTCTAGTTGATGGTAA	320

Figure 3. Nucleotide and deduced amino acid sequences of insertion variants of *BrVRN1* homologs in #2 GE₁ mutant lines. Nucleotide residues are numbered starting with the methionine initiation codon (ATG). The deduced amino acid sequences are presented by a single letter code above the nucleotide sequence. The original amino acid sequences and changed codes are colored in green and red, respectively. The stop codon is denoted by an asterisk.

Table 1. Phenotypes of flowering time in Chinese cabbage GE1 (T₁) mutant plants after vernalization. GE1 mutant plants were exposed to low temperature (4 °C) for 40 or 60 days.

Vernalization Period	Target Gene	GE1 Line	Days to Visible Flowering Bud in Plants
12 June–12 October 2020 (60 days)	No gene targeted	Wt-1~10	9 (9 ± 0) ^a
	<i>BrVRN1</i> (multi-target)	#1-1~19	19-20 (19.4 ± 0.116 **)
	<i>BrVRN1</i> (multi-target)	#2-1~13	19-20 (19.2 ± 0.104 **)
28 September–10 November 2020 (40 days)	No gene targeted	Wt-1~5	15-16 (15.6 ± 0.245)
	<i>BrVRN1</i> (multi-target)	#7-1~5	23-27 (25.4 ± 0.748 **)

^a (parentheses): The range of values obtained by mean ± SE with 95% confidence limits (**; *p*-value < 0.001, Student's *t*-test).

To determine whether the *BrVRN1* mutants could transmit the induced mutations and phenotypes to their progenies, we grew the GE₂ progeny of the GE₁ #2-2 and #2-8 lines under vernalization conditions. We examined Sanger sequencing results of three gene-specific amplicons from the 20 GE₂ plants and found that targeted mutagenesis of *BrVRN1s* induced by CRISPR/Cas9 was stably inherited and maintained consistent mutation types from the GE₁ generation to the GE₂ generation (Figure S7). The average time to bolting after 40-day vernalization was approximately 16 days in wild-type plants, but ranged from 37 to 59 days in the #2-2 and #2-8 GE₂ plants, meaning that bolting was delayed by 21–43 days (Table 2). These results confirmed that the *BrVRN1* mutant GE₂ plants showed a delayed flowering phenotype under vernalization conditions, suggesting that the *BrVRN1a* and *BrVRN1b* knockout carried the controlled late-flowering trait in Chinese cabbage (Figure 4).

Table 2. Phenotypes of flowering time in Chinese cabbage GE2 mutant plants after vernalization. ‘Transgene-free’ GE2 plants were exposed to low temperature (4 °C) for 40 days.

Vernalization Period	Target Gene	GE2 Line	Days to Visible Flowering Bud in Plants
21 January–2 March 2021 (40 days)	No gene targeted	Wt-1~10	16 (16 ± 0) ^a
	<i>BrVRN1</i> (multi-target, transgene-free)	#2-2-1~10	37–59 (52.7 ± 2.395 **)
	<i>BrVRN1</i> (multi-target, transgene-free)	#2-8-1~10	38–56 (48.3 ± 2.408 **)

^a (parentheses): The range of values obtained by mean ± SD with 95% confidence limits (**; *p*-value < 0.001, Student's *t*-test).

**Figure 4.** Flowering-time phenotype in ‘transgene-free’ *BrVRN1* GE2 mutant lines. Pictures were taken 31 days at 25 °C after a 40-day exposure to 4 °C. Wt, untransformed wild-type plants; #2-2 and #2-8, selected ‘transgene-free’ GE2 mutant lines, respectively.

3.4. Expression Patterns of Floral Regulators in GE₂ Mutant Plants

Because the *BrVRN1* GE₂ mutants displayed late-flowering phenotypes, we considered the possibility that *BrVRN1*s may regulate the expression of other floral genes, such as *FLC1* and *FT* [23]. Therefore, we analyzed the expression patterns of *BrFLC1* and *BrFT* in the leaves of vernalized and unvernallized wild-type and ‘transgene-free’ GE₂ mutant plants with different flowering times using qPCR (Figure 5). Unvernallized wild-type Chinese cabbage showed high *BrFLC1* expression, whereas *BrFT* expression was undetectable, probably because of the high level of *BrFLC1* expression. *BrFLC1* expression was reduced in the wild-type plant during a 40-day cold treatment. *BrFT* expression was undetectable before vernalization, but began to increase towards the end of vernalization; in general, the *BrFT* expression level was inversely proportional to that of *BrFLC* [32]. In GE₂ mutant plants, the expression of *BrFLC1* decreased more slowly than in wild-type plants and remained detectable after vernalization. In addition, *FT* expression was never observed in the mutants regardless of vernalization status. However, *BrVRN1a* expression in mutants at the RNA level was similar to that of the wild type, occurring only after vernalization (Figure S8). These results revealed that the altered patterns of *BrFLC1* and *BrFT* expression induced by *BrVRN1* mutations could result in the late flowering time phenotypes of mutant plants. These data indicate that the *BrVRN1*s are important determinants of vernalization-responsive flowering time through their regulation of *BrFLC1* and *BrFT* expression in Chinese cabbage.

3.5. Analysis of Potential Off-Target Changes

To confirm the specificity of *BrVRN1* gene editing via CRISPR/Cas9 in Chinese cabbage, and to avoid affecting the phenotypic statistics by including off-target mutations when identifying homologous mutants, we analyzed the potential off-target effects based on SNP and indel proportions in the 10 GE₂ mutant plants (five plants each of the #2-2 and #2-8 lines) using NGS (Tables S3 and S5). All of the examined potential off-target sites possessed mismatches of only 1–3 bp compared with the on-target guide sequences. We did not observe any mutations in the potential off-target sites we examined in GE₂ plants (Table 3). Also, the mutation types (*BrVRN1a*; A-insertion and C-insertion, *BrVRN1b*; A-insertion) in GE₁ mutants (#2-2 and #2-8) were inherited without any transgenic elements in the GE₂ generation (Tables 4 and 5). In this experiment, we identified homozygous double mutations (*BrVRN1a* and *BrVRN1b*), but no heterozygous mutations in the 10 GE₂ mutant (Table 5).

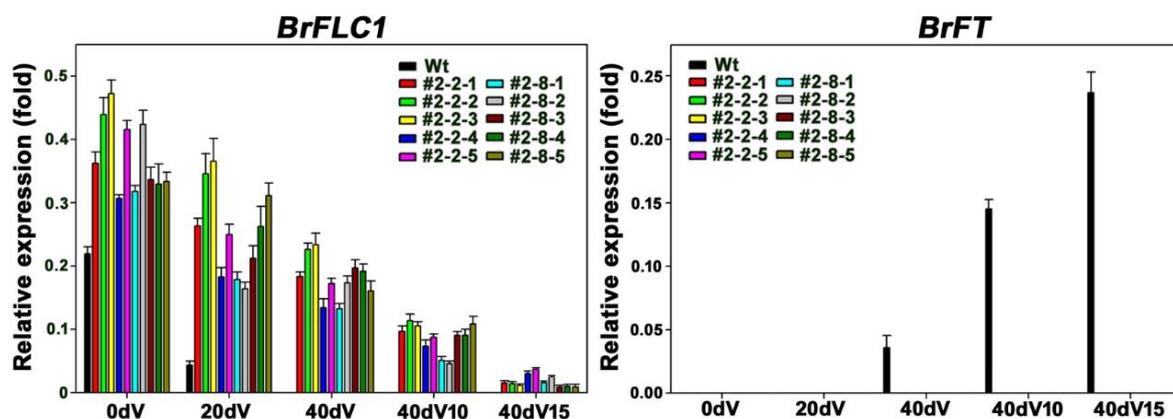


Figure 5. Expression patterns of floral regulator genes in ‘transgene-free’ *BrVRN1* mutant GE₂ lines. *BrFLC*, *B. rapa* FLOWERING LOCUS C; *FT*, FLOWERING LOCUS T. 0dV, 20 day-old plants before cold treatment (4 °C); 20dV, 20-day exposure to 4 °C; 40dV, 40-day exposure to 4 °C; 40dV10 and 40dV15, 10 days and 15 days at 25 °C after a 40-day exposure to 4 °C, respectively. Wt, untransformed wild-type plants; lanes #2-2-1 to #2-8-10, selected ‘transgene-free’ GE₂ mutant lines, respectively.

Table 3. Effect of chromatin structure on Cas9 activity at off-target sites of the ‘transgene-free’ GE2 mutant plants.

Target	Target Sequence	Mismatch	Number of Found Targets	Number of SNP Positions	Number of In/Del Positions
gRNA1	ACACGCCAAACGTGACCATCNGG	1	0	0	0
		2	0	0	0
		3	1	0	0
gRNA2	GACCGTTACTCAATCCGCATNGG	1	0	0	0
		2	0	0	0
		3	0	0	0
gRNA3	AGAAGAAGGATAAACAACCTNGG	1	0	0	0
		2	0	0	0
		3	6	1	0
gRNA4	ATAGCGGCCACAGTAACTCNGG	1	0	0	0
		2	0	0	0
		3	0	0	0
total			7	1	0

Excluded the on-target sites searched of three *BrVRN1* genes. Nucleotides in red represent PAM sequence.

Table 4. Statistics of alignment to *BrVRN1a*-4gRNAs vector.

Line	No. of Total Trimmed Reads * ¹	No. of Mapped Reads * ²	Mapped Region * ³ (%)
WT	143,832,424	24 (0.00%)	374 (2.81%)
#2-2-1	110,834,798	1 (0.00%)	0 (0.00%)
#2-2-2	144,407,538	13 (0.00%)	0 (0.00%)
#2-2-3	129,575,654	6 (0.00%)	0 (0.00%)
#2-2-4	160,433,726	8 (0.00%)	0 (0.00%)
#2-2-5	120,246,026	4 (0.00%)	0 (0.00%)
#2-8-1	130,463,146	4 (0.00%)	0 (0.00%)
#2-8-2	153,956,546	7 (0.00%)	0 (0.00%)
#2-8-3	151,410,950	2 (0.00%)	0 (0.00%)
#2-8-4	160,952,626	6 (0.00%)	0 (0.00%)
#2-8-5	126,426,790	12 (0.00%)	0 (0.00%)

*¹ No. of Total trimmed reads: Total number of clean reads used for read alignment through pre-processing. *² No. of Mapped reads: When clean reads are aligned, the number of reads mapped to the vector sequence. *³ Mapped region: Compared to the vector 13,301 bp sequence, the base pair of the region covered by read alignment.

Table 5. Summary of zygosity and genotype of *BrVRN1* mutations in the ‘transgene-free’ GE2 mutant plants.

Line	Target	Type of Mutation	<i>BrVRN1a</i>			<i>BrVRN1b</i>			<i>BrVRN1c</i>			
			Reads	Read Rate (%)	Zygosity	Reads	Read Rate (%)	Zygosity	Reads	Read Rate (%)	Zygosity	
	gRNA1	Wt	10	91.15	Homozygous	115	0	Wt	171	0	Wt	
		+A	103			-			-			-
		Total	113			115			171			
#2-2-1-5	gRNA2	Wt	11	90.90	Homozygous	4	95.87	Homozygous	165	0	Wt	
		+C/+A	110			93			-			-
		Total	121			97			165			
	gRNA3	Wt	147	0	Wt	-	-	-	-	-	-	
		SNP/indel	-			-			-			-
		Total	147			-			-			
	gRNA4	Wt	124	0	Wt	-	-	-	-	-	-	
		SNP/indel	-			-			-			-
		Total	124			-			-			
	gRNA1	Wt	5	95.83	Homozygous	108	0	Wt	171	0	Wt	
		+A	115			-			-			-
		Total	120			108			171			
#2-8-1-5	gRNA2	Wt	11	92.46	Homozygous	8	93.80	Homozygous	155	0	Wt	
		+C/+A	135			121			-			-
		Total	146			129			155			
	gRNA3	Wt	151	0	Wt	-	-	-	-	-	-	
		SNP/indel	-			-			-			-
		Total	151			-			-			
	gRNA4	Wt	149	0	Wt	-	-	-	-	-	-	
		SNP/indel	-			-			-			-
		Total	149			-			-			

* Read rate indicates the percentage of the number of mutations in the total mapped read count. Plus symbol denotes insertion. Homologous: read rate $\geq 90\%$ and heterozygous: $40\% \leq$ read rate $< 90\%$.

4. Discussion

The transition from vegetative to reproductive growth is a key developmental switch in the life cycle of flowering plants. Flowering is regulated by a complex regulatory network that modulates flowering time in response to various environmental stimuli and developmental statuses [32,43]. Chinese cabbage is a typical vernalization-sensitive plant. Genetic analyses have identified several genes, including *VERNALIZATION1* (*VRN1*), involved in the control of flowering time in Chinese cabbage during vernalization [23,25,41,44].

VRN1 encodes a DNA binding protein containing two plant-specific B3 domains that is required for the increase in H3K27me3 (trimethylation of lysine 27 in histone H3) epigenetic marks that causes transcriptional repression of *FLC* during prolonged cold treatment [23,26]. In previous studies, expression levels of *AtVRN1* and *AtVRN2* were not affected by vernalization in Arabidopsis [22,23]. In contrast to Arabidopsis, in Chinese cabbage one of the three *BrVRN1* paralogs (Bra022376) was upregulated by cold treatment, whereas the other two (Bra037544 and Bra001729) were downregulated [25,41]. These results suggest that the flowering pathway in Chinese cabbage is somehow different and more complex than that of Arabidopsis [25,41]. Despite the importance of *VRN1* genes in controlling vernalization-responsive flowering time, phenotypic effects of the *BrVRN1* genes in Chinese cabbage have not been fully investigated.

The CRISPR/Cas9 system has been reliable means to perform functional studies on multi-gene clusters of unknown function and is becoming one of the most effective tools for creating desirable phenotypes in crop plants. In this study, we assessed the molecular variation in the flowering time of Chinese cabbage via CRISPR/Cas9-mediated mutagenesis of *BrVRN1* genes. Among the three *BrVRN1* genes, we chose four target sites in the second exon of *BrVRN1a* (Figures 1 and S1 and Table S1) [25,41]. This multiplex CRISPR/Cas9 system-generated target gene mutations with a highly diverse pattern of indel mutations in the T₀ generation. CRISPR/Cas9 editing using constitutively strong promoters such as CaMV 35S may generate unwanted somatic mutations in various tissues and growth periods. The various mutations induced through CRISPR/Cas9 in the T₀ generations may then be unstably inherited, and it is, therefore, difficult to maintain consistent mutation types between the T₀ and T₁ generations. These undesired and unstable mutations in Chinese cabbage can be segregated out of the genome via self-pollination [45]. Notably, not all T₀ plants were capable of generating seeds, with only three out of ten edited lines (#1, #2, #7 lines) forming seeds (Figure S2 and Table S4). By deep sequencing of T₁ plants from the #2 line without transgenes, we verified the presence of A and C base insertions at the gRNA1 and gRNA2 target sites (Figures 2 and S5), confirming that the CRISPR/Cas9-induced mutations were passed down from the T₀ to the T₁ generation. In addition, targeted mutations in two *BrVRN1* orthologs (*BrVRN1a* and *BrVRN1b*) in the T₁ heterozygous mutant lines were stably transmitted to the GE₂ generation (Figures 2 and S7 and Table 4). These mutations, caused by base insertions in the coding regions, were expected to inactivate the *BrVRN1a* and *BrVRN1b* proteins by causing frameshifts (Figure 3). In contrast, no mutations at target sites *BrVRN1a* gRNA3 or gRNA4 or *BrVRN1c* gRNA1 or gRNA2 were identified in the T₁ and GE₂ mutant plants (Figures 2 and S5 and Table 5). This suggests that the gene targeting efficiency of sgRNAs may be affected by their relative position in the expression cassette and the chromosome structure of targeted genes, as has also been observed in other plant species [31,46]. Taken together, our results demonstrate the feasibility of introducing transmissible mutations induced by CRISPR/Cas9 through the propagation of stable transgenic plants, which could be widely used for reverse genetics in Chinese cabbage.

In applying CRISPR/Cas9 gene editing, it is important to consider off-target sites, and the homology between sgRNAs and candidate off-target sites has been reported to be the main factor influencing off-target mutations [47,48]. To examine the specificity of CRISPR/Cas9 in Chinese cabbage and avoid confounding our phenotype statistics by including off-target sites, we investigated the off-target regions of four target sites of *BrVRN1* through NGS analysis. We observed no mutations at any of the off-target

sites (Table 3), most likely as a result of careful target selection. The risk of off-target edits by CRISPR/Cas9 in Chinese cabbage may also be low in general on account of the low frequency of somatic mutations in tissue-culture-based transformation [48]. In addition, undesired off-target mutations in plants could be eliminated by hybridization [45]. Therefore, we predicted that the complex mutation patterns of the *BrVRN1* in T₀ plants could be eliminated by hybridization (Figures S2 and 2).

Chinese cabbage possesses three *BrVRN1* genes, but it is not known whether all three are involved in controlling flowering time [25,41]. However, the *BrVRN1a* and *BrVRN1b* double-mutant plants did display a delayed flowering phenotype (Figure 4 and Table 2). Although our data do not indicate whether the *BrVRN1b* gene directly or indirectly influenced late-flowering phenotypes, they do suggest that the induced late-flowering phenotypes by the double-mutations of *BrVRN1* genes attests to the complexity underlying multiple signalling networks. Therefore, further experiments are required to define how much the *BrVRN1* genes contribute to the regulation of flowering time by vernalization in comparison with *BrFLC* genes. However, the frameshift mutations induced by CRISPR/Cas9 at two target sites of *BrVRN1* generated premature translation termination codons through amino acid substitution in the corresponding protein (Figure 3). This result suggests that the mutated genes were not translated as complete proteins containing two B3 domains, resulting in a loss of protein function. Therefore, their inability to suppress *FLC* expression in warm conditions following prolonged cold exposure resulted in a late-flowering phenotype [23,44]. In this study, the mutants for which *BrVRN1* gene editing was confirmed showed a delayed flowering time in each generation (from the T₀ to the T₂ generation), regardless of the season or the low-temperature treatment period (Tables 1 and 2). In addition, the bolting time of GE₂ mutant plants ranged from 37 to 59 days, whereas the GE₁ mutant plants required from 23 to 27 days, which may have been caused by converting heterozygous mutations in the targeted genes to homozygous using selfing-crossing (Table 5). Our results suggest that *BrVRN1* genes play an important role in the regulation of flowering time in Chinese cabbage by regulating the expression of the floral repressor *FLC* and floral integrator *FT* (Figure 5). In addition, these results suggest the possibility of developing breeding material with a delayed flowering time in Brassicaceae with a similar flowering pathway mechanism using *VRN1* gene knockouts.

Recently, genome-editing technology has provided a new approach for developing crops that can replace genetically modified organisms (GMOs), whose safety has been debated [45]. The CRISPR/Cas9 system can accurately generate site-directed mutations in specific genes. The exogenous transgenes (Cas9, sgRNA, promoters, and selectable markers) inserted into a plant genome by *Agrobacterium*-mediated transformation can be removed in later generations via genetic segregation through selfing-crossing or hybridization approaches. These 'transgene-free' mutants have been generated by CRISPR/Cas9 editing in many plant species, such as Arabidopsis [49,50], rice [51,52], wheat [53], tomato [54], and soya bean [45]. In the present study, we obtained 'transgene-free' targeted genome-modified Chinese cabbage that was homozygous for the null alleles of endogenous *BrVRN1a* and *BrVRN1b* without any transgenic elements in the GE₁ and GE₂ generations (Figures S3 and S4, Tables 4 and 5). Therefore, 'transgene-free' *BrVRN1* mutants can provide breeding materials for developing Brassica crops with delayed flowering time. Chinese cabbage is very sensitive to low temperatures, which limits its geographical range of cultivation, and our study may contribute to expanded regional introduction and domestication of this species.

5. Conclusions

In conclusion, this is the first report of the development of a Chinese cabbage strain with a delayed flowering time trait through *BrVRN1* gene editing using a multiplex CRISPR/Cas9 system. *BrVRN1*-specific gene editing notably delayed flowering times and induced the production of heritable mutations. Taken together, our data indicate that *BrVRN1* acts as a positive regulator of the induction of flowering in Chinese cabbage, thus

raising the possibility that this may serve as a useful genetic source for crop improvement with respect to local environments and climate change.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agriculture11121286/s1>, Figure S1. Comparison of the nucleotide sequences of three *BrVRN1* genes with target sites of CRISPR/Cas9. Figure S2. Genome-editing analysis of T₀ plants by deep-sequencing. Figure S3. The confirmation of the presence of hygromycin resistant gene (*hph*) and *Cas9* transgenes using genomic PCR analysis in T₁ transgenic lines. Figure S4. The confirmation of the presence of *Cas9* transgenes using genomic PCR analysis in ‘transgene-free’ *BrVRN1* mutant GE2 lines. Figure S5. Genome-editing analysis of T₀ lines by deep-sequencing. Figure S6. Flowering-time phenotype in *BrVRN1* mutant GE1 plants. Figure S7. Target gene mutations by the multiplex CRISPR/Cas9 system in the GE2 generation. Figure S8. Expression patterns of *BrVRN1a* in ‘transgene-free’ *BrVRN1* mutant GE2 lines. Table S1. Features of *BrVRN1* genes used in this study. Table S2. Summary of *Brassica rapa* L. (v3.0) reference genome. Table S3. Statistics of target four-gRNA position on the reference v3 genome. Table S4. CRISPR/Cas9-mediated targeted mutagenesis of *BrVRN1* in the T₀ generation. Table S5. Statistics of final trimmed whole genome resequencing data from ‘transgene-free’ *BrVRN1* mutant GE2 lines.

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