

## Supplementary materials

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

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Bra022376 : ATGCCACGCCCTTCTTCCACAAGTTGATTTCTCATCCACTATCCAAGAAAACGCCCTGAGAGTTCAGATAAGTTTGTGAGTAATTTC : 90
Bra037544 : .....C.....T.....A... : 90
Bra001729 : .....G..T..A.....GA... : 90

                                gRNA1
Bra022376 : AAGGACGAGCTATCCGTAGCAGTTGCACTCACAGTACCTCATGGTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACAACAACA---AA : 177
Bra037544 : ....GG.....G..T.....T.....A...G.....T..G...T...A----- : 177
Bra001729 : .....G..T.....C.....T.....G...G..T.....ACA.G : 180

                                gRNA2
Bra022376 : ATCTACTTTCAGATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGATACGAAGGCAACTCT : 267
Bra037544 : ....GG.....T.....C...T.G..T..T..... : 267
Bra001729 : ..T.GG.....T.....C.....T..C...T.G...T..... : 270

                                gRNA3
Bra022376 : CTCAAACGCGCCCGTTTGTGTTGAAGATCTTGAAGACGAGACGCCCGGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA : 441
Bra037544 : T.....T..G.....C..C...T...T..TT-----C.....G-----T... : 429
Bra001729 : T.....TC.....C.....T...T..T-----AC.C.GC.CA.AA.CG- : 425

                                gRNA4
Bra022376 : CAGCATCCTGAAGTTACTGTGGC-CGCTATTAAGGGTACGCTAGTCCAGCTATCCAGAGCTTCTTCGCTGGACCACCTGTAAAGCTGA : 530
Bra037544 : .....GAC.....A.....A.....C.TA...T.....A..... : 515
Bra001729 : -----GAC.....T...C.....A.....C..... : 467

Bra022376 : AGAGGCGACACCAACTC-----CTAAAGTTACTAAGAAGAGAGGGAGGAAGAAGAACGCTGT---TCCTGAGGAAGTAACTCATC : 611
Bra037544 : ....A.A.....C-----A.....C...A.....A---.....A...T.... : 596
Bra001729 : .....A.....A.AACCT.A.....C...A.....T...ATCA..... : 557

Bra022376 : TGCTCCGAGGGATGATGACCCGGAGAGCCGTTCAAAGTTCTACGAGAGTGCTTCTGCGAGAAGAGAACGGTGACTGCAGAGAGAGAGA : 701
Bra037544 : ....AC.A...C...A...A.....T.....A.....C.....G..A.... : 686
Bra001729 : ..A...A.....A.....A.....A.....A...T..... : 647

Bra022376 : GAGAGCCATCAATGCAGCTAAACGTTTCGAGCCAACGAACCCCTTCTTCAGAGTTGTTCTCCGACCATCTTATCTATACAGAGGCTGCAT : 791
Bra037544 : .....C.....A.....A.....C.....T..... : 776
Bra001729 : ..G..TG.T.....C.....T.....A.....GA...G.....T..... : 737

Bra022376 : CATGTATCTGCCTTCTGGATTGCTGAGAGTACTTAAAGTGGGATCTCGGGATTCATCAAGGTCCAGCTCGGGGAGAAACAGTGGCCAGT : 881
Bra037544 : .....G..A..G.....T...C..... : 866
Bra001729 : .....T.....T.....A.....A.....T... : 827

Bra022376 : GAGATGCCTCTACAAAGCAGGGAGAGCCAAGTTCAGCCAGGATGGTACGAGTTCACCTCTGGAGAACACCTAGGAGAGAGGTGACGCTCTG : 971
Bra037544 : ..G.....T.....T.....G.....T..... : 956
Bra001729 : .....A.....T.....T...T...T.....G...T.....T..... : 917

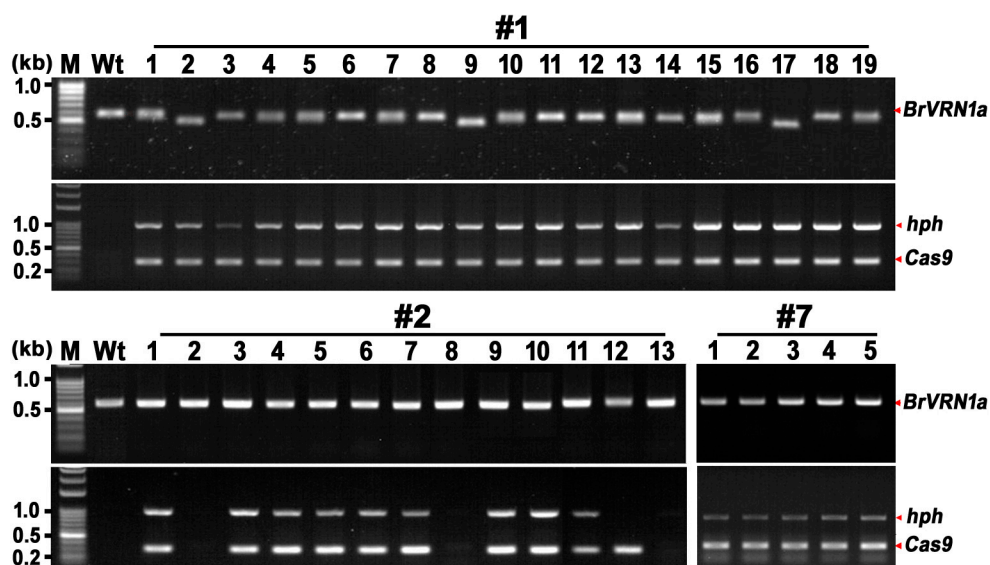
Bra022376 : CGTGTTCCGAGCTCCTCAGAACAGAGACTTCGTCCTGAAAGTAAACGGCCTATCGAGTCAAC---TCAATCTAG----- : 1041
Bra037544 : .....G.....T..C...G.....T..T.....GAG.ACG...GA----- : 1029
Bra001729 : .....T.....G..G.....T.....G.....GAG.ATG...GA----- : 990

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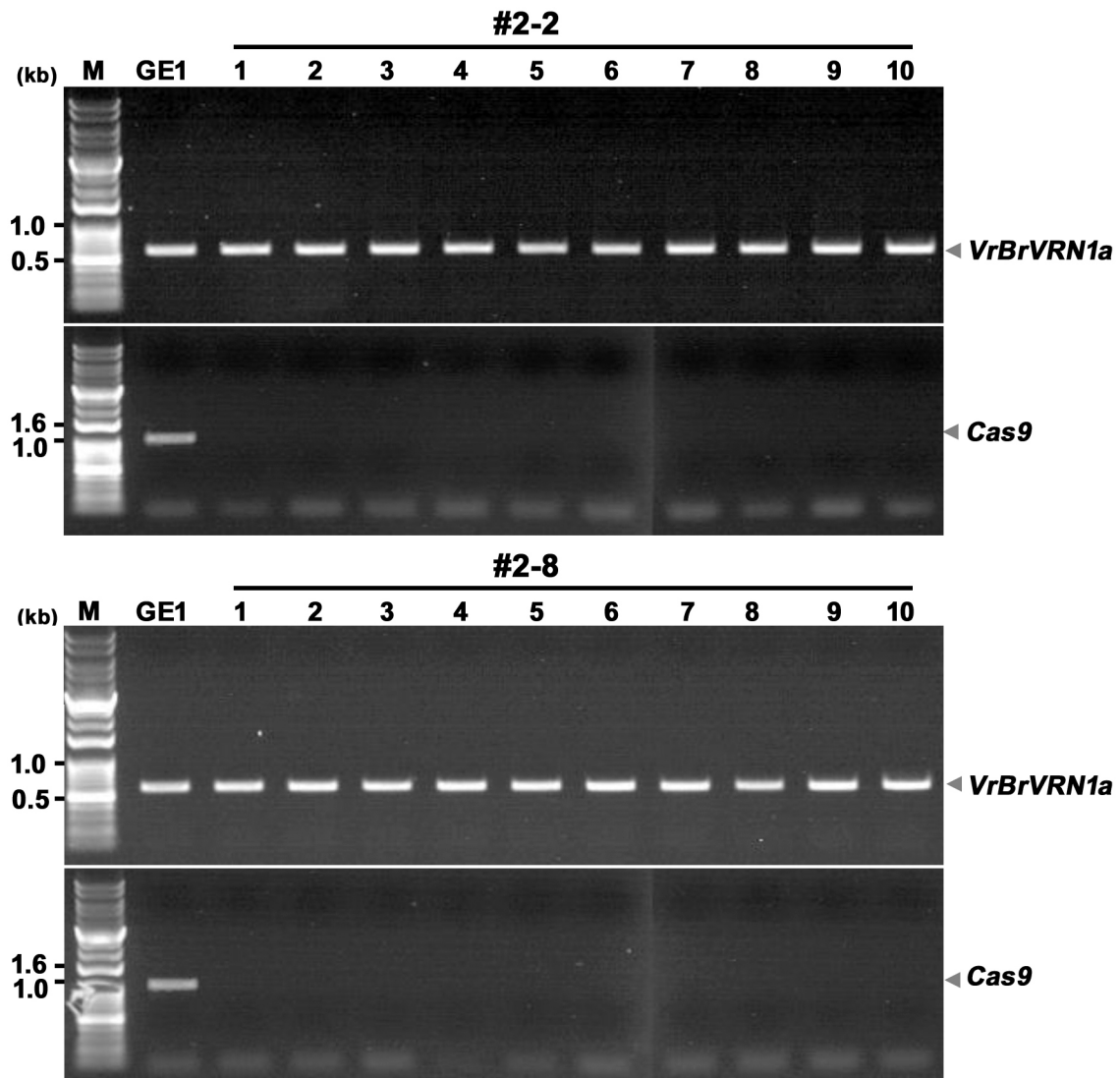
**Figure S1.** Comparison of the nucleotide sequences of three *BrVRN1* genes with target sites of CRISPR/Cas9. The gene accession numbers for these sequences are as follows: Bra022376 (*BrVRN1a*), Bra037544 (*BrVRN1b*), and Bra001729 (*BrVRN1c*). Dots indicate identical sequences. Dashes represent gaps introduced to maximize the alignment.

<b>gRNA1</b>			
<b>Wt</b>	TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGGTAGGACTAAGAAAAATCCGACA	Target sequence	
	<u>Most frequent sequences</u>	Type	Reads
<b>#1</b>	TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGGTAGGACTAAGAAAAATCCGACA	Wild-type	412113
<b>#2</b>	TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGGTAGGACTAAGAAAAATCCGACA TAGCAGTTGCACTCACAGTACCTGATAGGTCACGTTTGGCGGTAGGACTAAGAAAAATCCGACA TAGCAGTTGCACTCACAGTACCTGAT----ACGTTTGGCGGTAGGACTAAGAAAAATCCGACA	Wild-type 1 bp insertion(Out of frame) 4 bp deletion(Out of frame)	325 177752 366916
<b>#7</b>	TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGGTAGGACTAAGAAAAATCCGACA TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGGTAGGACTAAGAAAAATCCGACA	Wild-type 1 bp insertion(Out of frame)	681881 1229
<b>gRNA2</b>			
<b>Wt</b>	ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGATTGGCTATCTTCTCATCTTCAGAT	Target sequence	
	<u>Most frequent sequences</u>	Type	Reads
<b>#1</b>	ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGACATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCC-CATTGGCTATCTTCTCATCTTCAGAT	Wild-type 1 bp insertion(Out of frame) 1 bp deletion(Out of frame)	1468 144083 165072
<b>#2</b>	ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGACATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGACATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGTCATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCC-CATTGGCTATCTTCTCATCTTCAGAT	Wild-type 1 bp insertion(Out of frame) 1 bp insertion(Out of frame) 1 bp insertion(Out of frame) 1 bp deletion(Out of frame)	114957 208846 187989 42573 6108
<b>#7</b>	ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGTCATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGACATTGGCTATCTTCTCATCTTCAGAT ATGGTTGGCAAGAGTTTGTGCACCGTTACTCAATCCGACATTGGCTATCTTCTCATCTTCAGAT	Wild-type 1 bp insertion(Out of frame) 1 bp insertion(Out of frame) 1 bp insertion(Out of frame)	297577 270036 4450 2380
<b>gRNA3</b>			
<b>Wt</b>	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Target sequence	
	<u>Most frequent sequences</u>	Type	Reads
<b>#1</b>	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA -----TTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGG-TGTTTATCCTTCTTTCAGTGTATCCGTCATCACA -----TTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGG-TGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type 6 bp deletion(In frame) 1 bp deletion(Out of frame) 6 bp deletion(In frame) 1 bp deletion(Out of frame)	1,370 263,973 143,821 1,513 1,115
<b>#2</b>	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGTTT----- TCTTGAAGACGAAGACGCCGCCGAG--TGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAG-GTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGG-TGTTTATCCTTCTTTCAGTGTATCCGTCATCACA -----TTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAG--GTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGG---TTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGTTT----- TCTTGAAGACGAAGACGCCGCCGAGGATTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type 15 bp deletion(In frame) 2 bp deletion(Out of frame) 1 bp deletion(Out of frame) 1 bp deletion(Out of frame) 6 bp deletion(In frame) 1 bp insertion(Out of frame) 3 bp deletion(In frame) 4 bp deletion(Out of frame) 15 bp deletion(In frame) 1 bp insertion(Out of frame)	375,915 110,060 26,672 13,226 6,978 5,066 3,707 1,236 1,062 1,053 561
<b>#7</b>	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGA-----ACA TCTTGAAGACGAAGACGCCGCCG-----ATCCTTCTTTCAGTGTATCCGTCATCACA TCTTGAAGACGAAGACGCCGCCGAGG-TGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type 22 bp deletion(Out of frame) 9 bp deletion(In frame) 1 bp deletion(Out of frame)	18,202 290,381 128,997 12,103
<b>gRNA4</b>			
<b>Wt</b>	ATCCGTCATCACAACAGCATCTGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	Target sequence	
	<u>Most frequent sequences</u>	Type	Leads
<b>#1</b>	ATCCGTCATCACAACAGCATCTGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	Wild-type	226,803
<b>#2</b>	ATCCGTCATCACAACAGCATCTGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG -----GTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	Wild-type 6 bp deletion(In frame)	320,744 132,344
<b>#7</b>	ATCCGTCATCACAACAGCATCTGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG A-----CGCTATTAAAGGGTACGCTAGTCCAG	Wild-type 17 bp deletion(Out of frame)	239,818 12,039

**Figure S2.** Genome-editing analysis of T<sub>0</sub> plants by deep-sequencing. Wt, untransformed wild-type plants; #1, #2, and #7, selected transgenic lines, respectively.



**Figure S3.** The confirmation of the presence of hygromycin resistant gene (*hph*) and *Cas9* transgenes using genomic PCR analysis in T<sub>1</sub> transgenic lines. Wt, untransformed wild-type plants; lane numbers, selected #1, #2, and #7 T<sub>1</sub> transgenic lines, respectively.



**Figure S4.** The confirmation of the presence of *Cas9* transgenes using genomic PCR analysis in ‘transgene-free’ *BrVRN1* mutant GE2 lines. Wt, untransformed wild-type plants; lanes 1 to 10, selected #2-2 and #2-8 GE2 lines, respectively.



<b>gRNA1</b>			
<b>Wt</b>	TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	Target sequence	
	<u>Most frequent sequences</u>	Type	Leads
#2-2	TAGCAGTTGCACTCACAGTACCTGATAGTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	Wild-type(mismatch)	10
	TAGCAGTTGCACTCACAGTACCTGATAGGTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	1 bp insertion(Out of frame)	720,101
	TAGCAGTTGCACTCACAGTACCTGATAGGTCACG-TTGGCGTGTAGGACTAAGAAAATCCGACA	1 bp ins/1bp del(Out of frame)	167
	TAGCAGTTGCACTCACAGTACCTGAT-GTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	1 bp deletion(Out of frame)	2
#2-8	TAGCAGTTGCACTCACAGTACCTGATGGTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	Wild-type(mismatch)	15
	TAGCAGTTGCACTCACAGTACCTGATAGGTCACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	1 bp insertion(Out of frame)	726,623
	TAGCAGTTGCACTCACAGTACCTGATAGGTCGCGTTTGGCGTGTAGGACTAAGAAAATCCGACA	1 bp insertion(Out of frame)	3,284
	TAGCAGTTGCACTCACAGTACCTGATAGGTCACG-TTGGCGTGTAGGACTAAGAAAATCCGACA	1 bp ins/1bp del(Out of frame)	132
	TAGCAGTTGCACTCACAGTACCTGAT---ACGTTTGGCGTGTAGGACTAAGAAAATCCGACA	4 bp deletion(Out of frame)	92
<b>gRNA2</b>			
<b>Wt</b>	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGAT	Target sequence	
	<u>Most frequent sequences</u>	Type	Leads
#2-2	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGAT	Wild-type	24
	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGAT	1 bp insertion(Out of frame)	596,480
	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCC-CATTGGCTATCTTCTCATCTTCAGAT	1 bp deletion(Out of frame)	2
#2-8	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGAT	Wild-type	2
	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGAT	2 bp deletion(Out of frame)	34
	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCCGCATTGGCTATCTTCTCATCTTCAGAT	1 bp insertion(Out of frame)	613,710
	ATGGTTGGCAAGAGTTTGTGACCGTTACTCAATCC-CATTGGCTATCTTCTCATCTTCAGAT	1 bp deletion(Out of frame)	4
<b>gRNA3</b>			
<b>Wt</b>	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Target sequence	
	<u>Most frequent sequences</u>	Type	Leads
#2-2	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type	559,014
	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type(mismatch)	3,444
	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATC---CTTCTTTCAGTGTATCCGTCATCACA	3 bp deletion(In frame)	1,095
	TCTTGAAGACGAAGACGCCGCCGAGGTTG-TTATCCTTCTTTCAGTGTATCCGTCATCACA	1 bp deletion(Out of frame)	162
	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	3 bp insertion(In frame)	52
#2-8	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type	597,019
	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	Wild-type(mismatch)	3,600
	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATC---CTTCTTTCAGTGTATCCGTCATCACA	3 bp deletion(In frame)	1,211
	TCTTGAAGACGAAGACGCCGCCGAGGTTG-TTATCCTTCTTTCAGTGTATCCGTCATCACA	1 bp deletion(Out of frame)	101
	TCTTGAAGACGAAGACGCCGCCGAGGTTGTTTATCCTTCTTTCAGTGTATCCGTCATCACA	3 bp insertion(In frame)	49
<b>gRNA4</b>			
<b>Wt</b>	ATCCGTCATCACAACAGCATCTGGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	Target sequence	
	<u>Most frequent sequences</u>	Type	Leads
#2-2	ATCCGTCATCACAACAGCATCTGGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	Wild-type	423,517
	ATCCGTCATCACAACAGCATCTGGAAGTTACTGTGGCCGCTA-TAAAGGGTACGCTAGTCCAG	1 bp deletion(Out of frame)	14
#2-8	ATCCGTCATCACAACAGCATCTGGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	Wild-type	461,600
	ATCCGTCATCACAACAGCATCTGGAAGTTACTGTGGCC-CTATTAAAGGGTACGCTAGTCCAG	1 bp deletion(Out of frame)	15
	ATCCGTCATCACAACAGCATCTGGAAGTTACTGTGGCCGCTATTAAAGGGTACGCTAGTCCAG	1 bp insertion(Out of frame)	3

**Figure S5.** Genome-editing analysis of ‘transgene-free’ *BrVRN1* mutant GE1 lines by deep-sequencing. Wt, untransformed wild-type plants; Lanes #2-2 and #2-8, selected ‘transgene-free’ GE1 lines, respectively.

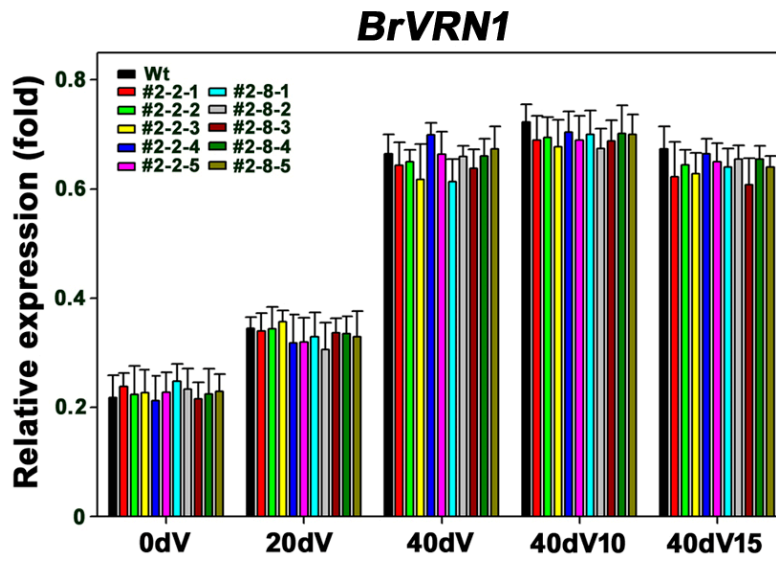


**Figure S6.** Flowering-time phenotype in *BrVRN1* mutant GE1 plants. Pictures were taken 20 days at 25°C after a 40-day exposure to 4°C. Wt, untransformed wild-type plants; GE1, gene-editing 1 mutant plants.

#2-2		<i>BrVRN1a</i> (Bra022376-target)		<i>BrVRN1b</i> (Bra037544)	
		PAM gRNA1	gRNA2 PAM	gRNA2 PAM	
Wt	CAGTACCTGAT-GGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCG-CATTGGGTATC	TTGTTGACCGTTACTCAATCCG-CATTGGTTACC		
1	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	1 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
2	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	2 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
3	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	3 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
4	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	4 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
5	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	5 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
6	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	6 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
7	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	7 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
8	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	8 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
9	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	9 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
10	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	10 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
#2-8		<i>BrVRN1a</i> (Bra022376-target)		<i>BrVRN1b</i> (Bra037544)	
		PAM gRNA1	gRNA2 PAM	gRNA2 PAM	
Wt	CAGTACCTGAT-GGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCG-CATTGGGTATC	Wt TTGTTGACCGTTACTCAATCCG-CATTGGTTACC		
1	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	1 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
2	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	2 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
3	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	3 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
4	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	4 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
5	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	5 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
6	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	6 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
7	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	7 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
8	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	8 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
9	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	9 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		
10	CAGTACCTGATAGGTACAGTTTGGCGTGTAGGACT	TTTGTGACCGTTACTCAATCCGCAATTGGGTATC	10 TTGTTGACCGTTACTCAATCCGACATTGGTTACC		

**Figure S7.** Target gene mutations by the multiplex CRISPR/Cas9 system in the GE2 generation. The mutated DNA sequences of target regions are provided. Gene mutations are in red. Wt, untransformed wild-type plants; lanes 1 to 10, selected #2-2 and #2-8 GE2 mutant lines, respectively.





**Figure S8.** Expression patterns of *BrVRN1a* in ‘transgene-free’ *BrVRN1* mutant GE2 lines. 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 S1.** Features of *BrVRN1* genes used in this study.

<i>Arabidopsis</i> gene	<i>B. rapa</i> Gene ID	<i>B. rapa</i> paralogs	Genomic sequence length (bp)	Exon/intron structure		Coding region (bp)	Protein size (aa)	Coding sequence identity with <i>AtVRN1</i> (%)	Amino acid Sequence identity with AtVRN1 (%)
				Exons	Introns				
At3g18990	Bra022376	<i>BrVRN1a</i>	2865	4	3	1041	346	87.1	90.9
	Bra037544	<i>BrVRN1b</i>	3110	4	3	1029	342	90.1	93.8
	Bra001729	<i>BrVRN1c</i>	2706	4	3	990	329	86.2	91.9

**Table S2.** Summary of *Brassica rapa* L. (v3.0) reference genome.

Chr. #	Chr. length (bp)	No. of Gene	Genelength (bp)	CDS length (bp)
SL4.0ch01	29,595,527	4,498	8,460,173	5,162,019
SL4.0ch02	31,442,979	4,586	8,509,646	4,957,257
SL4.0ch03	38,154,160	6,553	12,097,777	7,371,687
SL4.0ch04	21,928,416	3,258	5,872,957	3,543,990
SL4.0ch05	28,493,056	4,265	7,919,791	4,852,659
SL4.0ch06	29,167,992	4,499	8,726,395	5,354,649
SL4.0ch07	28,928,902	4,329	8,032,747	4,887,840
SL4.0ch08	22,981,702	3,578	6,621,748	3,960,462
SL4.0ch09	45,156,810	6,648	12,317,263	7,477,641
SL4.0ch10	20,725,698	3,381	6,477,353	3,948,051
Un-anchored*	56,564,952	655	962,088	551,304
Total	353,140,194	46,250	85,997,938	52,067,559

\* Un-anchored: Total length of 1,103 scaffolds.

**Table S3.** Statistics of target four-gRNA position on the reference v3 genome.

<i>VRN</i> gene family (version 1.5)	Reference gene ID and position(version 3.0)					gRNA position in reference V3 genome			
	VRN gene family	Chr.	Start	End	Strand	Target	Start	End	Sequence
Bra022376 ( <i>BrVRN1a</i> -target)	BraA05g028310.3C (target)	A05	21,149,298	21,151,462	+	gRNA1	21,150,396	21,150,418	CCTGATGGTCACGTTTGGCGTGT
						gRNA2	21,150,480	21,150,502	GACCGTTACTCAATCCGCAT <b>TGG</b>
						gRNA3	21,150,667	21,150,689	CCGAGGTTGTTTATCCTTCTCT
						gRNA4	21,150,717	21,150,739	CCTGAAGTTACTGTGGCCGCTAT
Bra037544 ( <i>BrVRN1b</i> )	BraA01g033970.3C	A01	23,266,355	21,151,462	+	gRNA1	23,267,342	23,267,364	CCTGATGGTCATGTTTGGCGTGT
						gRNA2	23,267,426	23,267,448	GACCGTTACTCAATCCGCAT <b>TGG</b>
						gRNA3	-	-	-
						gRNA4	-	-	-
Bra001729 ( <i>BrVRN1c</i> )	BraA03g038610.3C	A03	19,118,034	19,120,080	-	gRNA1	19,119,053	19,119,075	ACACGCCAAACGTGACCATC <b>AGG</b>
						gRNA2	19,118,966	19,118,988	CCAATGCGGATGGAGTAACGGTC
						gRNA3	-	-	-
						gRNA4	-	-	-

\* Nucleotides in red represent PAM sequence



**Table S4.** CRISPR/Cas9-mediated targeted mutagenesis of *BrVRN1* in the T<sub>0</sub> generation.

Line (T0)	Mutagenesis	Harvested seed	Deep seq. result
#1	○	○	Figure S1
#2	○	○	Figure S1
#3	○	nh*	-
#4	X	-	-
#5	X	-	-
#6	X	-	-
#7	○	○	Figure S1
#8	X	-	-
#9	○	nh	-
#10	○	nh	-
#11	X	-	-
#12	X	-	-
#13	○	nh	-
#14	X	-	-
#15	X	-	-
#16	○	nh	-
#17	○	nh	-
#18	X	-	-
#19	○	nh	-
19 lines	10 lines	3 lines	

\*nh, not harvested seed.

**Table S5.** Statistics of final trimmed whole genome resequencing data from ‘transgene-free’ *BrVRNI* mutant GE2 lines.

Samples	No. of Reads	Avg. read length	Total read length (bp)	Trimmed/Raw (%) <sup>*1</sup>	Coverage <sup>*2</sup>
WT	71,916,212	132.85	9,553,709,532	62.97%	≈ 34.73X
	71,916,212	101.40	7,292,004,106	48.06%	
#2-2-1	55,417,399	104.64	5,798,673,684	60.27%	≈ 23.29X
	55,417,399	99.19	5,496,769,711	57.13%	
#2-2-2	72,203,769	133.25	9,621,293,493	66.29%	≈ 34.84X
	72,203,769	100.79	7,277,522,731	50.14%	
#2-2-3	64,787,827	132.47	8,582,441,617	63.86%	≈ 32.16X
	64,787,827	108.30	7,016,449,712	52.21%	
#2-2-4	80,216,863	118.41	9,498,436,349	66.15%	≈ 37.50X
	80,216,863	108.31	8,688,530,929	60.51%	
#2-2-5	60,123,013	133.18	8,006,934,424	68.77%	≈ 29.69X
	60,123,013	106.29	6,390,623,762	54.89%	
#2-8-1	65,231,573	120.25	7,844,113,865	66.95%	≈ 30.71X
	65,231,573	108.10	7,051,522,995	60.18%	
#2-8-2	76,978,273	119.91	9,230,265,110	66.87%	≈ 36.36X
	76,978,273	109.20	8,406,353,048	60.90%	
#2-8-3	75,705,475	118.58	8,977,072,374	65.87%	≈ 35.55X
	75,705,475	109.18	8,265,781,363	60.65%	
#2-8-4	80,476,313	117.68	9,470,709,283	66.31%	≈ 37.86X
	80,476,313	110.50	8,892,492,747	62.26%	
#2-8-5	63,213,395	130.88	8,273,659,352	71.54%	≈ 32.22X
	63,213,395	116.32	7,353,265,160	63.58%	
Total	1,532,540,224		176,988,625,347		

\*1) Trimmed/Raw(%): (Total length of trimmed reads / Total length of raw reads)\*100

\*2) Coverage: Total read length of each sample/estimated genome size (485Mb)