are distributed across four different chromosomes (A02, A06, A09, and A10) [6–8]. Six *B FLS* homologs, *B FLS1*, *B FLS2*, *B FLS3.1*, *B FLS3.2*, *B FLS3.3*, and *B FLS4.1*, were suggested to be syntenic orthologs of four *A FLS* genes, *A FLS1*, *A FLS2*, *A FLS3*, and *A FLS4*, respectively, but those corresponding to *A FLS5* and *A FLS6* are absent from the *B.* genome [7]. An additional *FLS* gene (named *B FLS4.2* in this study) was recently assigned to belong to the FLS4 group, although its syntenic relationship has not been investigated [5]. Therefore, a total of seven *B FLS* homologous genes have been assigned. Among them, *B FLS1* (Bra009358), a syntenic ortholog of *A FLS1*, was predicted to encode a functional FLS enzyme, since *B FLS1* expression was predominantly higher among other homologs but was not correlated with expression of *B DFR* genes or anthocyanin accumulation in purple Chinese cabbage [9]. However, the functional differences between the seven *B FLS* homologs have not been revealed, and the key *FLS* gene mainly responsible for flavonol biosynthesis in *B.* has yet to be determined.

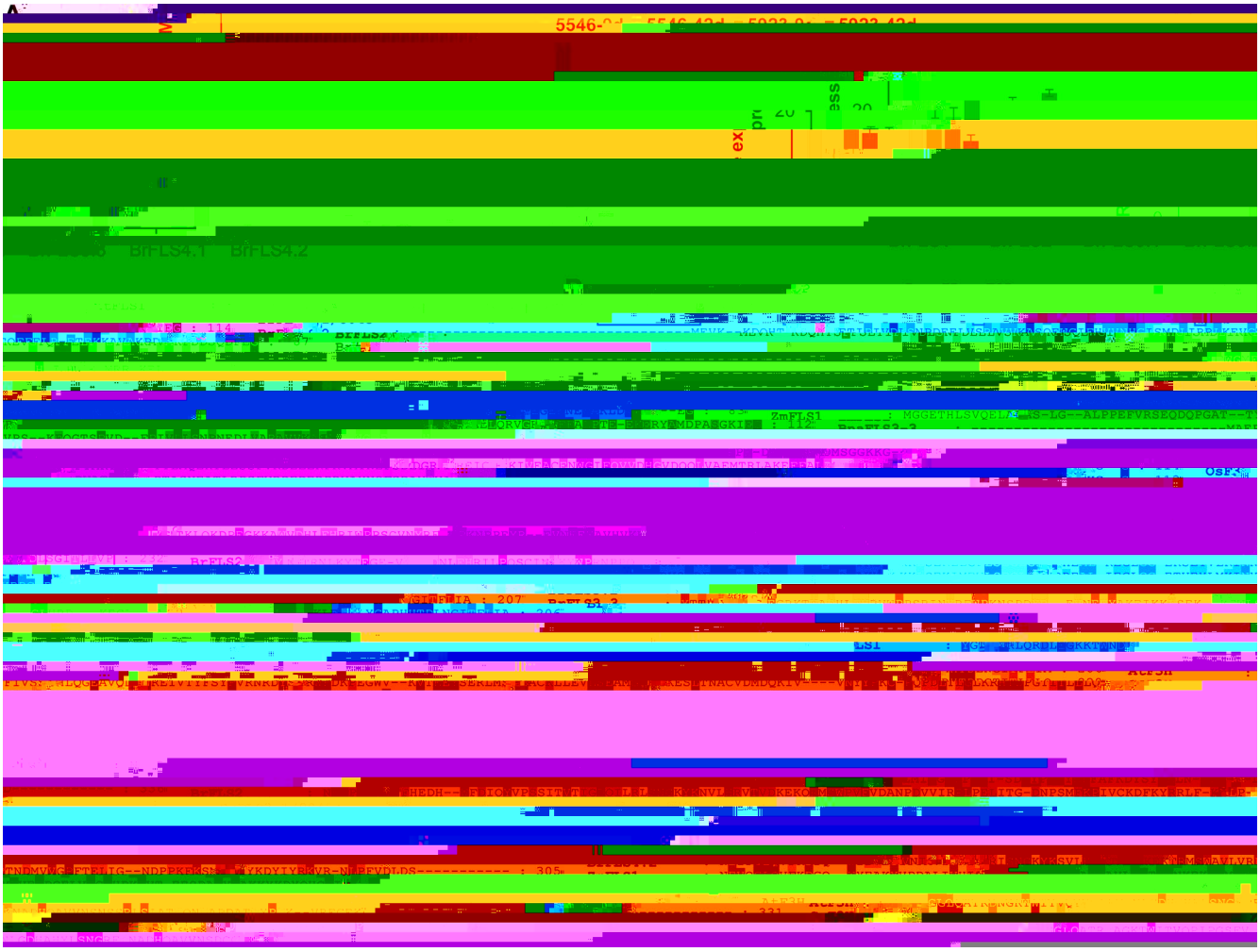
Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) technology-mediated genome editing (GE) has been used to edit DNA sequences in numerous species due to its simplicity, high efficiency, versatility, and capacity for multiplexing [10]. Since 2015, >45 applications of CRISPR/Cas-mediated GE have been reported in Brassicaceae crops. Most were conducted in *B* or *B.* to improve commercially important agronomic traits or nutritional values such as seed oil, carotenoids, or glucosinolate contents. Most of these studies used CRISPR/CRISPR-associated protein 9 (Cas9) to generate insertions/deletions (InDels) in single or multiple genes [11]. Only a few studies have reported modifying genes using CRISPR/Cas9 in *B.* . These studies focused on genes involved in flowering time [12], leaf color transition [13], methylation of pectin [14], self-incompatibility [15], and circadian rhythms [16]. However, considering that the end goal of CRISPR/-Cas9 GE is to obtain a transgene-free homozygous plant harboring a precise modification of a specific target gene, and the altered sequence and the resulting traits should be stably inherited, there is still a need for efficiency improvement and diversification of objectives in the CRISPR/Cas9 application for *B.* .

Here, comparative analyses of gene expression and enzyme activities of the *B FLS* homologs characterized the *B FLS* family genes and indicated that *B FLS1* is the only candidate for the major active *FLS* gene in *B.* . The CRISPR/Cas9 targeting *B FLS1* was introduced to a commercial inbred line of green Chinese cabbage to generate *B FLS1*-knockout (1) plants, and we obtained transgene-free homozygous 1 lines in the T_1 generation. Flavonol glycosides, the major class of flavonoids in the background variety, dramatically decreased, and instead dihydroflavonol glycosides accumulated in the 1 lines. We showed an effective way of metabolic engineering to develop new varieties of Chinese cabbage with modified flavonoid profiles using CRISPR/Cas9 GE system. Furthermore, our study provides insights into understanding the phenylpropanoid and flavonoid biosynthetic pathway in *B.* .

Results

BrFLS

We estimated the expression levels of each *B FLS* homolog in the leaves of two 9- and 42-day-old green Chinese cabbage varieties, 5546 and 5923, using transcriptome deep sequencing (RNA-



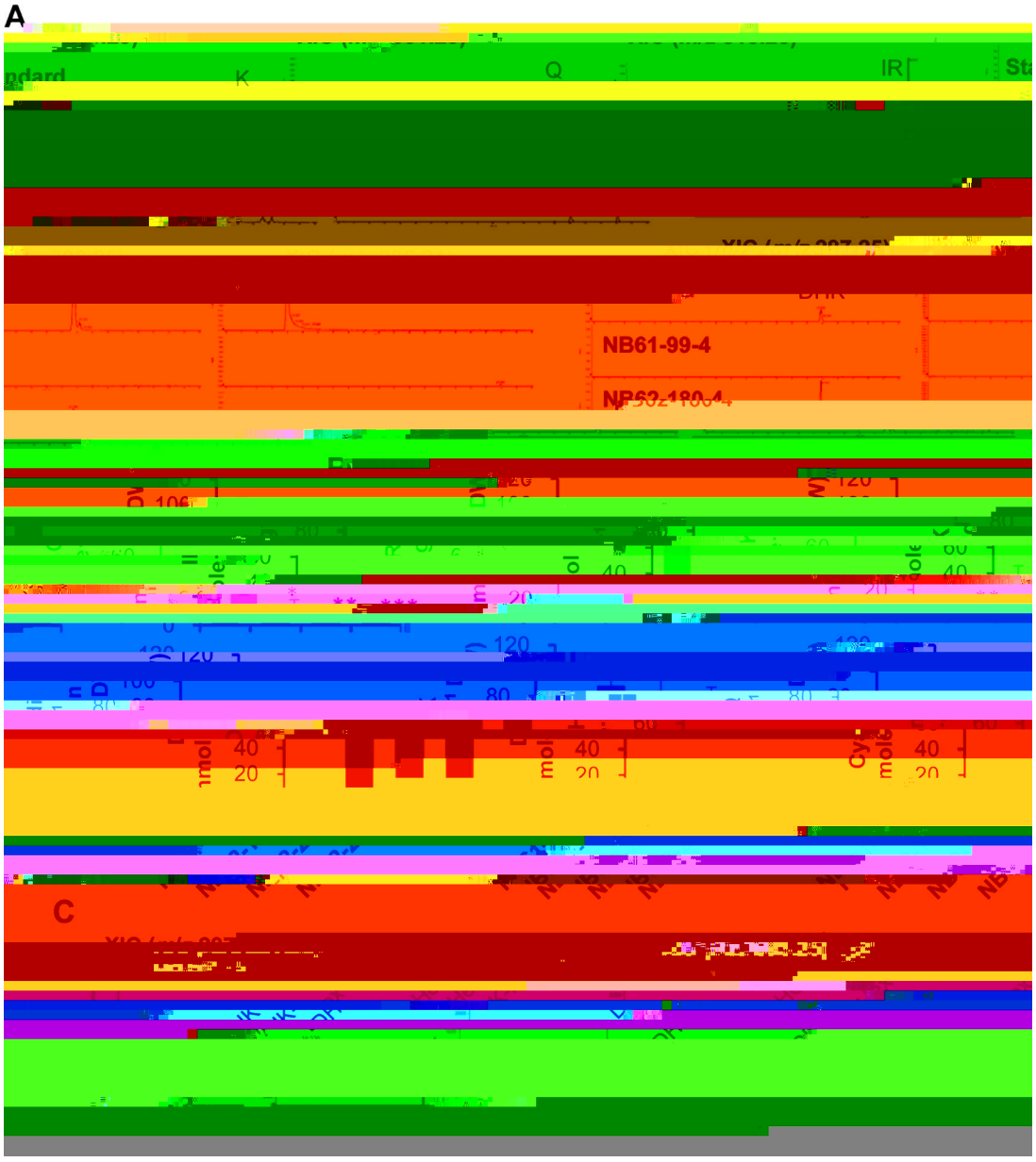


Figure 4. Investigation of changes in the flavonoid profile of . 1 plants using LC-ESI-QTOF-MS analysis. LC-ESI-QTOF-MS analysis of flavonoid aglycones extracted from the leaves of four T_2 individuals generated from the NB61–99 (WT) and . 1 (NB62–180, NB62–203, NB62–204) with negative ionization mode. (A) Representative XICs displaying mass spectra of flavonoid aglycones in the T_2 plants. XICs at ⊠⁄ 285.23, ⊠⁄ 287.25, ⊠⁄ 301.23, ⊠⁄ 303.25, ⊠⁄ 315.26, and ⊠⁄ 286.24 show deprotonated K, DHK, Q, DHQ, IR, and cyanidin Cya aglycones, respectively. (B) The average values of flavonoid content in the four individuals of each T_2 line were calculated based on the areas of corresponding standards. The mean values ± SD of four independent biological samples are shown. Significant differences were determined by Student's -tests. Asterisks indicate significant differences from the WT ($P < 0.05$, $P < 0.01$, $P < 0.001$). (C) Representative XICs of dihydroflavonol glycosides extracted from the . 1 T_2 plants using 70% methanol (Hex, hexoside; diHex, dihexoside).

. 1 heads. Their shape tended to be vertically elongated due to the increased mid-vein lengths compared to the controls (Fig. 6A and 6B). In terms of reproductive phenotypes, there were no significant differences in bolting, flowering, seed color, and seed weight between . 1 and the controls (Supplementary Data (.8Sn

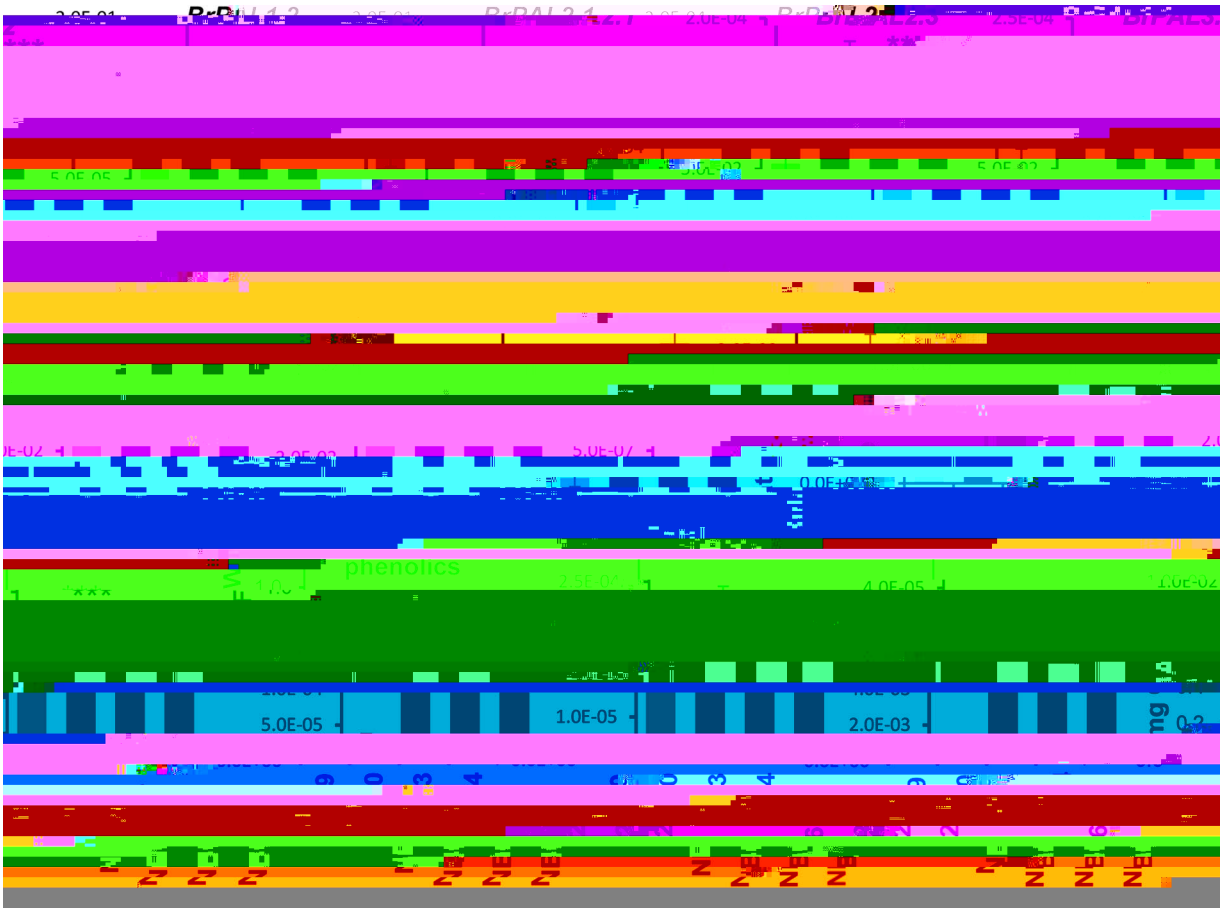


Figure 5. Analyses of changes in the expression of flavonoid biosynthetic genes and total phenolic contents in the . 1 T_2 plants. qPCR analysis was performed and total phenolic content was measured in the four T_2 individuals generated from the NB61–99 and . 1 (NB62–180, NB62–203, NB62–204). Expression values were normalized using *B. f f A . 7* (*B ACT7*) transcript. The total phenolic contents were calculated using gallic acid as a standard, and the mean values were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg GAE·g^{-1} FW). The mean values ± SD of four independent biological samples are shown. Significant differences were determined by Student's . -tests. Asterisks indicate significant differences from the WT ($^{\square}P < 0.05$, $^{\square\square}P < 0.01$, $^{\square\square\square}P < 0.001$).

nonfunctional *A FLS2*, a pseudogene encoding a protein lacking the key C-terminal [18], was inherited as a functional BrFLS2 exhibiting F3H activity *B. f f*. Furthermore, the BrFLS2 was inherited in

production is completely blocked [23

(sgRNA) sequences based on the genomic sequence of *B FLS1*. Among them, three sequences (sg1, 5′-AGGGATGGCTTCGGTGTG-GA-3′; sg2, 5′- CCGGCGATCACCACTTTCCG-3′; sg3, 5′-AACGTTCT-TCGAGCTTCCGT-3′) next to the protospacer adjacent motif (PAM) sequences (5′-NGG-3′) located within the first exon were selected, with expected out-of-frame scores of 68%, 60%, and 63%, respectively. DNA oligonucleotides of each sgRNA sequence were synthesized in the forward and reverse orientations and annealed by decreasing the temperature from 95°C to 25°C for 1.5 h. The resulting sgRNA DNA fragments were cloned into the binary vector pHAtC, according to a previously reported protocol [20]. The resulting CRISPR/Cas9 binary vector was introduced into Agrobacterium strain GV3101 by the freeze–thaw method.

The Chinese cabbage transformation method was described previously [16]. The newly formed T_0 plants were transferred to soil after acclimatization and grown in a glasshouse for 4–5 days, vernalized at 4°C for □45 days, and returned to the glasshouse. Genomic DNA of the cauline leaves from each T_0 plant was isolated and genotyped with PCR to detect the hygromycin resistance and *S Cf 9* genes. After flowering, bud pollination of T_0 plant was conducted to obtain T_1 seeds. Genotyping of T_1 lines was conducted by PCR, and individual plants lacking the hygromycin resistance and *S Cf 9* genes were selected and further analyzed by PCR and sequencing of the target site to verify editing of *B FLS1*. The primers used for genotyping are listed in Supplementary Table S1.

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Potential off-target edits of the sgRNAs were identified in the *B.* genome using the CRISPR-GE web tool (http://ski.scau.edu.cn) [41]. BLAST hits containing potential off-target sequences that had fewer than four mismatches and were located in the coding sequence were selected (Supplementary Table S2). The sequences of other *B FLS* genes corresponding to the target site were also considered as potential off-targets. PCR using specific primers (

Conflict of interest statement

No conflict of interest declared.

Supplementary Data

Supplementary data is available at *H* online.

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