SCIENTIFIC REPORTS

Brassica juncea *CLAVATA1* *BjMc1*

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and , showed the phenotype of multilocular siliques due to the disturbance of stem cell growth balance[17,18]. e mutant showed multiloculus, and was found to be required for the normal accumulation of various miRNAs, indicating that miRNAs might be involved in the regulation of silique trait[19]. In addition, a recent study showed that another receptor kinase signaling pathway involving ( ) regulated the stem cell growth, and the mutant exhibited a similar silique trait with mutants[20]. In tomato, the mutation of the homologues of both and resulted in the increased number of fruit locules. Moreover, the mutation of the homologous genes in signaling pathway in maize[21,22] and rice[23], such as , and , could also the increase the seed number per in orescence, which was similar to multilocular trait in rapeseed.

As a member of signaling pathway that regulates expression, more than 10 alleles of with multiloculus have been discovered in , and the mutants exhibited weak, intermediate and strong multilocular phenotypes[12,24,25]. e gene encoded a putative receptor kinase[26], and mutation at di erent sites in the gene sequence could lead to di erent degrees of multilocular phenotype. Moreover, all the mutants with intermediate and strong multilocular phenotypes were dominant negative, and the mutant of homologous gene in tomato was also expected to be dominant-negative[16,27]. Similarly, most of the mutants with multiloculus discovered in showed variable valve numbers[10,11]. However, it has been unknown whether the molecular mechanism controlling multiloculus is similar between and or the dominant-negative character is associated with the instability of multilocular silique trait.

In previous study, we found that the trilocular silique always had three locules, and the trilocular plants had signi cantly higher yield per plant than the bilocular plants[2]. e trilocular trait of . was controlled by two independent recessive nuclear genes, and . e and gene were isolated from the same plants and mapped by molecular markers[28,29]. In present study, we cloned the bilocular gene and trilocular respectively. A Copia-LTR retrotransposable element 1 (RTE1) inserted in the coding region of was identi ed in trilocular plants, which interrupted the transcription of the target gene. We also found that two amino acid sites had undergone positive selection in the ancestor of genes, and purifying selection was the dominant force a er divergence of dicots and monocots from their common ancestor in the evolutionary process of genes, indicating that they were conserved in modern land plants.

BjMc1 gene was previously mapped to a genomic region between marker EC14MC14 and SC20, which could delimit an interval of 2.7 cM[28]. Compared with the bilocular siliques in NILs of gene, the trilocular siliques displayed shorter, wider and atter (Supplementary Fig. 1a,b). But the in orescence meristem and oral meristem did not show di erences between bilocular and trilocular plants in BC_6F_1 generation (Supplementary Fig. 2a,b). To identify the gene locus, we further screened a BAC library of purple-leaf mustard with the primer C1-1 (Supplementary Table 1). Two positive clones, 26P20 and 83D02, were identi ed, and four sca olds (designated as sca old 1, 2, 3 and 4) (Supplementary Data 1) were obtained by sequencing 83D02. Two sequence-characterized ampli ed region markers SC40 and SC151 (Supplementary Table 1) were identi ed according to sca old 2, and one SSR marker SR52 was identi ed based on sca old 4. Subsequently, polymorphic markers SC40, SC151 and SR52 were used to search for the recombinants identi able between SC13 and SC20. Among the 242 recombinants discovered from the NILs population which consisted of 9,300 individuals, 0, 4 and 8 recombination events were detected for SC40, SC151 and SR52 respectively. Finally, the candidate region of was found to be restricted between SC151 and EC14MC14 at a 1.14-cM region. Twenty- ve open reading frames (ORFs) of sca old 2 in which both the co-segregative SC40 and nearest marker SC151 located were predicted according to: (http://linux1.so berry.com/berry.phtml?topic=f-genesh&gr(m)19.ind opic]TJ1

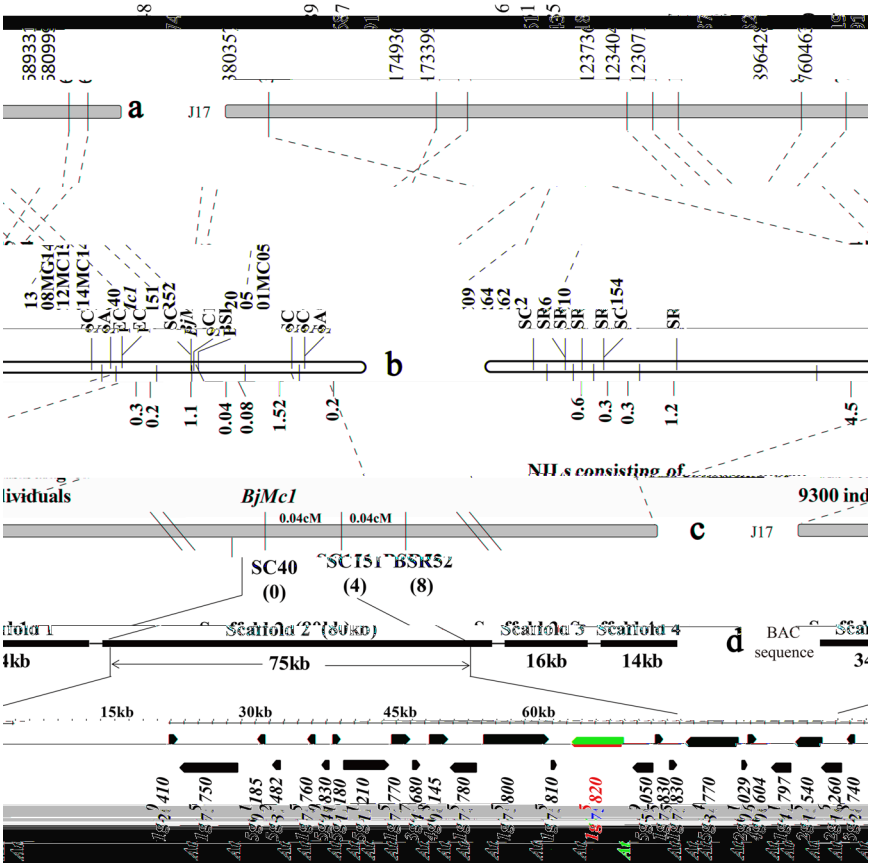

Fig e 1. Map-based cloning of . e number below the marker indicates the number of recombinants between individual markers and locus. e pentagons represent the predicted genes in the 75-kb target region on chromosome J17 of . . e candidate gene of is indicated by red color. (**a**) e physical location of molecular markers of on J17 chromosome. (**b**) e genetic linkage map of . (**c**) Genetic distance of the three markers identi ed in this research. (**d**) the sca olds of BAC clone 83D02.

lines with : (TGP1-4), 11 transgenic lines with :: (TGP5-15), and the bilocular and trilocular plants of BC_5F_1 generation were detected by qPCR using the primer DL2. As expected, compared with the bilocular plants of BC_5F_1 generation, the :: -transgenic plants showed much higher expression level of , while the : -transgenic plants exhibited similar or lower expression level (Supplementary Fig. 3a).

In addition, the constructs of :: and : were transformed respectively into multilocular mutant which showed four valves per silique (Fig. 4c). A total of 56 : transgenetic plants and 32 :: -transgenetic plants were obtained in T0 generation. 33 out of the 56 : transgenetic plants showed chimeric phenotype, while 17 out of the 32 :: -transgenetic plants showed completely bilocular phenotype (Fig. 3b,c). Further analysis of phenotypes and genotypes con rmed that the transgenic events were cosegregated with the bilocular trait in T1 progeny.

Bjmc1 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed to analyze the transcription levels of and of early in o-rescences of bilocular and trilocular plants in BC_5F_1 generation. C1-2 and M1-1(Supplementary Table 1) were designed according to di erent parts of the gene sequence (Fig. 2a). Both C1-2 and M1-1 could identify the transcription of in bilocular plants, while only C1-2 could identify the transcription of in trilocular plants, indicating that the truncation of transcription has occurred in gene. Although the transcription of and could be detected by C1-2, the expression was down-regulated in trilocular plants (Supplementary Fig. 1i).

To investigate the transcription level of and in detail, the full-length complementary DNAs (cDNAs) were identi ed by RACE technology. e cDNA of was 3,155 bp in length, which was composed of two exons and consists of a 71 bp 5′ untranslated region, a 2,964 bp ORF and a 136 bp 3′ untranslated region. e cDNA of , with a full-length of 2,583 bp, comprised a 71 bp 5′ untranslated region which contained the same sequence with the cDNA of , a 2,349 bp ORF and a 159 bp 3′ untranslated region (Fig. 2b and Supplementary Data 4). encoded a putative protein of 987 amino acids and contained two putative transmembrane domains, a putative extracellular domain consisting of 6 complete Leu-rich repeats (LRRs) and a putative intracellular domain containing all of the conserved residues found in serine/threonine protein kinase.

e LRR region was anked by a pair of conservatively spaced cysteines (Fig. 2a,b). Sequence analysis indicated that the putative protein consisting of 782 amino acids contained the extracellular domain of , and further analysis showed that protein was derived from the truncation of gene at Gly[782] by Copia-LTR RTE1 (Fig. 2b and Supplementary Fig. 3c), proving that the serine/threonine protein kinase domain of gene was required for the gene to control the development of siliques in . .

The dominant negative effect of *Bjmc1* In back-cross populations, we observed that some bilocular individuals ( ) showed several triloculus-like siliques which showed trilocular shape but with two locules and only a few siliques had three locules (Fig. 4a,b). e silique trait of 219 bilocular plants was investigated in BC_8F_1 generation, and 92 individuals were found to have triloculus-like siliques.

A previous report has demonstrated that all intermediate and strong alleles in are dominant negative[27]. Similar to the homozygous mutant of homologous gene, the heterozygous plants also exhibited weak fasciation in tomato[16]. Moreover, the , a Leu-rich repeat receptor-like Ser/ r kinase, regulated organ shape and in orescence architecture, and a truncated protein that lacks the cytoplasmic kinase domain confers dominant-negative e ects[30]. In our research, was shown to be able to encode a truncated protein with a similar structure with protein, indicating the possibility of dominant negative e ect. To verify whether protein functions in the development process of carpel, the construct :: was transformed into - mutant. A total of 62 transgenic plants were obtained and 25 of them showed siliques with ve valves (Fig. 4c). e phenotype analysis of T_1 progeny showed that the transgenic events could be cosegregated with the phenotype of ve valves. e over-expression of could aggravate the multilocular phenotype of . ese results further supported the inference that the truncated protein showed the dominant negative character in controlling carpel development.

Expression pattern and subcellular localization of *BjMc1*

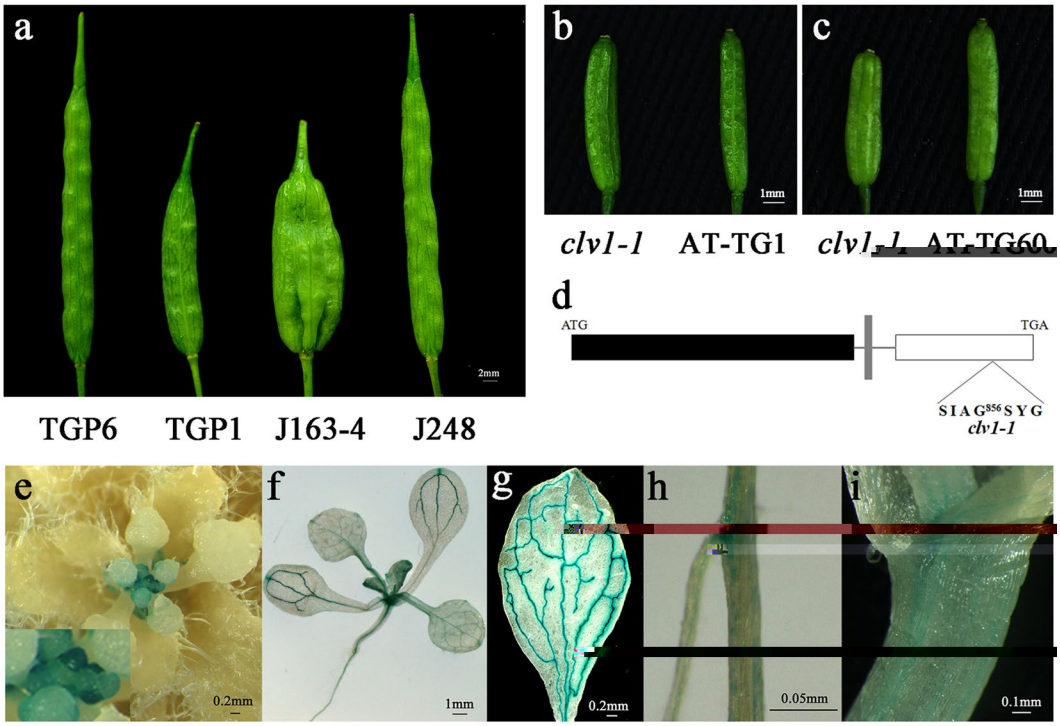

Fig e 3. Functional analysis and expression pattern of . (**a**) Silique phenotypes of :: - transgenetic T_0 line TGP6, : transgenetic T_0 line TGP1, multilocular parental J163-4 and bilocular parental J248. (**b**) Silique phenotypes of mutant and : - transgenetic T0 line AT-TG1. (**c**) Silique phenotypes of mutant and :: - transgenetic T0 line AT-TG60. (**d**) e mutation sites of . (**e**–**i**) Representative histochemical analysis of GUS expression in tissues under the control of the promoter in the transgenic T_2 plants. (**e**) Early in orescence with ower bud meristems. e solid arrow indicates the ower bud meristems on the early in orescence (**f**) Seedling 10 d a er germination. (**g**) Rosette leaf. (**h**) Root. (**i**) Stem.

To investigate the subcellular localization of , we fused the coding region containing putative transmembrane domains and LRR domains, a without serine/threonine protein kinase of with the coding region of an enhanced GFP driven by a double 35S promoter. is chimeric plasmid was transformed into . e result showed that the -GFP fusion protein was localized in the plasma membrane (Supplementary Fig. 1c–h), which was consistent with the previous study[31] and suggested that could retain some conserved functions of its homologs in other species.

Expression analysis of genes involved in early inflorescence. According to the expression pattern of gene, the homologous genes of singling pathway, including , , , , , , , and , and the homologous genes of ABC model of oral organ identity, including , , , and , were chosen to perform the qPCR analysis using the total RNA extracted from the early in orescence of bilocluar and trilocular plants in BC_5F_1 generation, and primers (Supplementary Table 1) were design according to these homologous gene sequences. e results showed that, in trilocular plants, the A class genes, and , were down-regulated signi cantly, the B class genes, and were up-regulated signi cantly, and the C class gene, , was also down-regulated signi cantly (Fig. 5). In trilocular plants, the signaling pathway genes, , , , , , and , were down-regulated signi cantly, and was up-regulated. However, as two key components of signaling pathway, and did not show signi cant variations between bilocular and trilocular plants in early in orescence (Fig. 5). ese results indicated that gene participated in signaling pathway, but it was not the key pathway to control the carpel development in . . e mutant of gene in trilocular plants resulted in the signi cantly expression variation of ABC class genes, indicating that involved in the pathway of ower bud formation to control the carpel development.

Mc1 Blast searching against the plant database revealed that genes were widespread in land plants and each sequenced land plant genome contained at least one gene encoding homologue. To explore the evolutionary process of in land plants, we characterized CDSs and proteins of genes from species representing the main lineages of land plants, including moss , lycophyte , Amborellaceae , 9 monocot species and 24 dicot species. e genes could be divided into three major branches on the phylogenetic tree (Supplementary Fig. 4), and was in the branch of the Cruciferae species. Structural analysis of genes

Fig e 4. Dominant negative phenotype. (**a**) Silique phenotype of the bilocular plant in BC_5F_1 generation. (**b**) Bilocular silique, trilocular silique and trilocular-like silique. (**c**) Silique phenotypes of Arabidopsis mutant and :: -transgenetic T0 line AT-TG100.

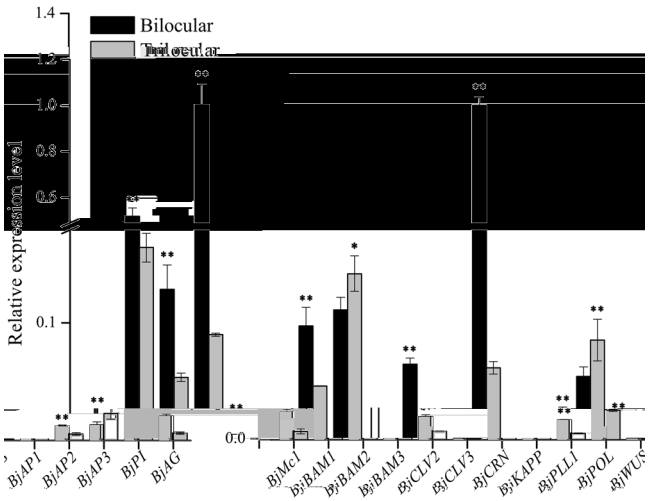

Fig e 5. e di erence of expression level of related genes between bilocular and trilocular plants during the ower bud di erentiation period in BC_5F_1 generation. *P < 0.05; **P 5

in land plants was performed by comparing the exon-intron organization (http://gsds.cbi.pku.edu.cn/). It was shown that the coding regions of all genes of land plants were interrupted by 1–2 introns except for , which contained 8 introns and 9 exons (Supplementary Fig. 5).

Mc1 In this research, we attempted to further reveal the evolutionary process of homologous genes in land plant species. e LRT of positive selection was applied using method and the codon substitution models[32–34], and all genes of sampled land plants were tested respectively. First, one-ratio model was used to determine whether there were variations in : ratio of the codon position for genes in land plants. Overall, the maximum likelihood estimates for : values were close to zero (Supplementary Table 3), suggesting that purifying selection was the predominant force in the evolution of in land plants. Second, the LRTs to compare the data t to models M1a vs M2a and M7 vs M8 were used to determine whether positive selection promoted the divergence of genes in land plants. No amino acid site was in uenced by positive selection during the evolution of genes in land plants. ese results indicated that the primary constraint for genes in land plants was purifying selection.

To assess whether the ancestors of gene had undergone a pattern of molecular evolution in the evolutionary process of land plants, branch-model of was performed. Six branches were selected from the phylogenetic tree (branch I, II, III, IV, V and VI) (Supplementary Fig. 4).We found that for branch I and II, the branch-model permitting a class of positively selected codons with : >1 had a signi cantly better t to the data than the branch-model in which this class of codons were restricted to : $=1$. However, this was not the case for branch III, IV, V and VI (Supplementary Table 4). e results indicated that the evolution process of gene might be in uenced by positive selection in its ancestors of branch I and II. Because LRTs suggested that positive selection acted on the ancestral species of gene, the method of Bayes empirical Bayes[34], namely branch-site-model of , was used to evaluate the positively selected sites and their posterior probabilities. A total of 32 codons were identi ed with a $>50\%$ posterior probability of : >1 along branch I. Of these codons, 2 amino acid sites had a $>95\%$ posterior probability of positive selection. Although 2 and 6 codons were identi ed with a $>50\%$ posterior probability of : >1 along branch III and branch IV respectively, no amino acid site had a posterior probability $\geq 95\%$ of positive selection (Supplementary Table 5 and Supplementary Fig. 6). e two positive sites of branch I were located in the rst cysteine domain and a key component of serine/threonine protein kinase domain respectively. When dicots and monocots were diverged from Moss, Lycophyte and Amborellaceae, the 139th amino acid tryptophan (W) located in the rst cysteine domain was mutated into phenylalanine (F), and the 899th amino acid cysteine (C) located in a component of serine/threonine protein kinase domain was mutated into valine (V) except for in , and (Supplementary Fig.

multiloculus had variable valve numbers in the siliques of the same plant[10, 11]. However, in this study, J163-4 planted in both central and northwestern China exhibited four valves stably in trilocular siliques. We transformed the gene into the allele in for over-expression, and proved that the truncated protein had a potential dominant negative e ect, which could have played a key role in maintaining the

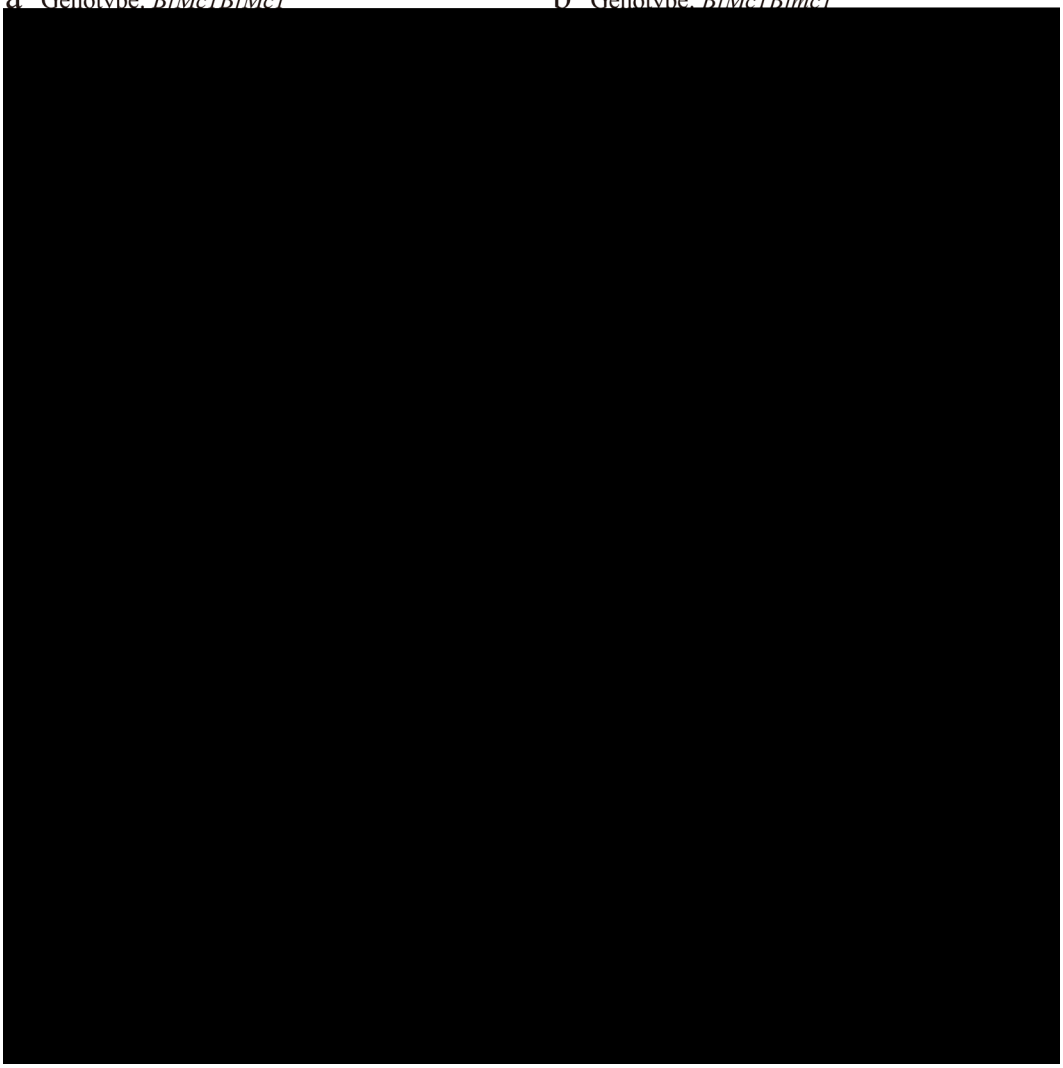

Fig e 6. Model of dominant negative receptor action in . . A putative model for the role of protein, ( ) protein and the ligand protein in regulating the carpel development. Bold arrows indicate that the protein plays a dominant role in regulating the development of carpel. (**a**) Scenarios for plants with genotype of . e ligand could bind to both the and protein, but protein was the dominant receptor kinase. (**b**) Scenarios for plants with genotype of . e ligand could bind to the , and protein, and the protein was the dominant receptor kinase. (**c**) Scenarios for plants with genotype of . e ligand could bind to the and protein, but only the protein which does not play the dominant role in regulating pathway has the normal function. (**d**) Scenarios for plants with genotype of and overexpression. More ligands are possessed by the , and little ligand could bind to the protein.

All data generated or analyzed during this study are included in this published article and its additional les.

Positive clones from the purple-leaf mustard ( ) bacterial arti cial chromosome (BAC) library were identi ed through a two-stage polymerase chain reaction (PCR) screening method with C1-1 primers designed from the sequence of which was located near the homologous sequence of molecular marker SC13 in A07 chromosome of . . e phenotypes were similar between J163-4 and mutant in (Crooijmans ., 2000). BAC DNA was sequenced as previously described by Yi .[40].

Bulked segregant analysis[41] was conducted to screen the molecular markers linked to locus. Simple sequence repeats (SSR) was designed according to the reference sequence retrieved from the positive BAC clone. e parents and bulks were subjected to SSR marker analysis. PCR was performed according to Xu .[28]. PCR products were then separated on 1% polyacrylamide denaturing

sequencing gel and shown by silver nitrate staining. Linkage analysis was performed using Joinmap4[42]. All genetic distances were expressed in centiMorgan (cM) using the Kosambi function[43].

cDNA preparation and 5′- and 3′- rapid-amplification of cDNA ends (RACE). Total RNA was extracted from various plant tissues using an RNA extraction kit (RNeasy Plant Mini Kit; QIAGEN). The first-strand cDNA was synthesized using 2 mg of RNA and 200 units of M-MLV reverse transcriptase (Promega Kit) in a volume of 25 ml. e 5′- and 3′- RACE reactions were performed using the SMARTer RACE Ampli cation Kit (Clontech) according to manufacturer's instructions.

e genomic fragments of the candidate gene were ampli ed from the biolocular plants from the NILs using high- delity PCR. A 6,613 bp genomic DNA fragment spanning from 2,692 bp upstream the translation start codon to 767 bp downstream the termination codon of using primer E48-2 (Supplementary Table 1) was ampli ed. e correct fragment con rmed by sequencing was then cloned into Pst I - kpn I site of pCAMBIA2300 vector to construct plasmid : . To prepare :: construction, we cloned a 3455 bp genomic DNA fragment spanning from start codon to 301 bp downstream the termination codon of using primer E46-1 (Supplementary Table 1) into Pst I - kpn I site of pCAMBIA2300, and double 35S promoter was cloned into Hind III - Pst I site of pCAMBIA2300. To prepare the Pro_{BjMc1}-GUS construct, the promoter region (from 1 to 2499 bp) using primer E58-1 (Supplementary Table 1) was ampli ed. A cassette containing GUS coding region followed by nopaline synthase polyadenylation signal from pBI101 vector was subcloned into the binary vector pCAMBIA 2300 with restriction enzymes Hind III and EcoR I to construct promoter-GUS fusions. e ampli ed fragments were subcloned into the modi ed binary vector pCAMBIA 2300 to yield the 2499 bp promoter-GUS construct. To prepare :: construct, a 3036 bp genomic DNA fragment spanning from the start codon to a 687 bp downstream DNA fragment using primer HY-4 (Supplementary Table 1) was ampli ed. e correct fragment con rmed by sequencing was cloned into Xba I - Sac I site of pMDC83 vector to construct plasmid :: . ese constructs were introduced into the host cells of GV3101. e : and :: were transformed into the trilocular line J163-4, whereas Pro_{BjMc1}-GUS construct was introduced into wild-type (ecotype) and :: was transformed into by oral dipping[44]. e T2 transgenic plants of Pro_{BjMc1}-GUS were grown for GUS staining.

Expression analysis. Total RNA was extracted using Trizol reagent (Invitrogen). e tissues of the early in orescence of trilocular plants and SAM, early in orescence, 1–4 mm ovary, 4–7 mm ovary, 7–10 mm ovary, silique peel, leaves, stem and root of bilocular plants in BC_5F_1 generation were selected, and 3 mg of total RNA from each tissue was treated with RNase-free DNase I to remove contaminated DNA respectively, then reverse transcribed into the rst-strand complementary DNA (cDNA) with M-MLV reverse transcriptase (Fermentas, Vilnius, Lithuania) using oligo $d(T)_{25}$ primer. e reverse-transcribed products from various tissues were used as templates for qPCR assay using the Bj3 primer (Supplementary Table 1) which could particularly amplify copy to examine the expression of gene. e qPCR was conducted according to Li .[4]. e measurements were obtained using the relative quanti cation method[45]. gene was used as the internal control for . [46]. All expression level data obtained by qPCR were based on three biological samples and three replicates for each sample.

e cDNA of and were detected from the reverse-transcribed products from early in orescence of trilocular and bilocular plants in BC_5F_1 generation using the semi-quantitative RT-PCR by C1-2 and M1-1. e gene was used as the control. Semi-quantitative RT-PCR was performed as following: 94 °C for 3 min; twenty- ve cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; and a nal 10-min elongation step.

Twelve independent T_2 transgenic lines of Pro_{BjMc1}-GUS were subjected to histochemical GUS assays. Seedlings of 10 d and various organs of the transgenic plants were incubated at 37 °C overnight in 5-bromo-4-chloro-3-indolyl-b-glucuronic acid solution and then cleaned in 75% (v/v) ethanol. e treated tissues were observed on an Olympus IX-70 Microscope equipped with Nomarski Optics[4].

BjMc1 e coding sequence without the termination codon (TAA) was ampli ed from the biolocular plants in BC_5F_1 generation by PCR using YXB4 primer (Supplementary Table 1). e ampli ed cDNA fragments were inserted downstream of the double 35S promoter through Xba I - BamH I site in frame with GFP in pMDC83 vector. is plasmid was transformed into . e roots of transgenic plants in T2 generation were incubated with 10 μM FM4-64 for at least 5 min before observation. e emission light was dispersed and recorded at 500–540 nm for GFP. Confocal images were taken with a Nikon Eclipse80i uorescence microscope equipped a water-immersed ×40 lens with an excitation wavelength of 488 nm and the following detection wavelengths: 500–540 nm for GFP and at >650 nm for FM4-64 (Nikon, Japan). All uorescence experiments were independently repeated at least three times.

Mc1 To identify homologous genes in Cruciferae, BLAST analysis using protein sequence of as a query was performed in Cruciferae (http://brassicadb.org/brad/, http://www.arabidopsis.org/, http://122.205.95.67/blast/blast.php).

To identify homologous genes in the land plants, the coding sequence (CDS) of was used as query to search the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), Ensembl Plants (http://plants.ensembl.org/index.html) and the Arabidopsis Information Resource database (http://www.arabidopsis.org/). e most highly similar sequence was selected from each species. e deduced nucleotide and protein sequences of land plant genes identi ed in this analysis were used for further analysis.

Using the MEGA5[47], Cruciferae amino acid sequences were aligned by ClastalW and land plant CDSs were aligned by ClastalW condons, and nally the FASTA formats were exported. e maximum-likelihood approach was used for the phylogenetic analysis of Cruciferae and all the land plants, respectively.

To test the selective pressure of genes during the long period of evolution in land plants, the value of : ratio (or) for genes was calculated with the program from Phylogenetic Analysis by Maximum Likelihood (PAML) v4.4[32]. In this study, three likelihood ratio tests (LRTs), M0, M1a vs M2a and M7 vs M8, were used to examine the selective pressure. M0 was used to calculate the average value of all codon sites, and the other two LRTs were used to detect the role of positive selection. For one LRT, the di erences of log likelihood of the two models were compared using chi-squared (χ^2) statistics. In our analyses, the degree of freedom was 1 for M1a/M2a and M7/M8 tests[33, 48].

e improved branch model and branch site model[49] were also used to detect the role of positive selection on the land plant genes. In these two models, six branches were selected from the phylogenetic tree (branch I, II, III, IV, V and VI); and when any one of the branches served as the foreground branch, the remaining branches were background branches (Supplementary Fig. 4). For the analysis of branch site model, we compared the null hypothesis (xed to 1) with the alternative hypothesis (free) to test whether positive selection a ected the evolution of land plant genes. e Bayes empirical Bayes procedure in [34] was used to calculate the posterior probability that each site in the foreground branch was subjected to positive selection.

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