Increased seed number per silique in *Brassica juncea* by deleting cis-regulatory region affecting BjCLV1 expression in carpel margin meristem

Gang Wang, Xiangxiang Zhang, Wei Huang, Ping Xu, Zewen Lv, Lun Zhao, Jing Wen, Bin Yi, Chaozhi Ma, Jinxing Tu, Tingdong Fu and Jinxiong Shen*

National Key Laboratory of Crop Genetic Improvement/National Engineering Research Center of Rapeseed, Huazhong Agricultural University, Wuhan, China

Summary

Mustard yield per plant is severely restricted by the seed number per silique. The seed number per silique in the *Brassica juncea* trilocular mutant J163-4 is significantly greater than that in normal bilocular plants. However, how the trilocular silique of J163-4 is formed remains unclear. Here, we studied the gene structure and function of *mc2* in *B. juncea* and *Arabidopsis* using comparative morphology and molecular genetic experiments. We found that *mc2* is a CLV1 ortholog, *BjA7.CLV1*. The deletion of cis-regulatory region in *mc2* promoter, which affects *mc2* expression in carpel margin meristem (CMM), led to trilocular silique formation. The *BjCLV1* sequence with its complete promoter containing the cis-regulatory region can restore the *Bjclv1* and *clv1* mutant phenotypes in *B. juncea* and *Arabidopsis*, respectively. Additionally, this cis-regulatory region had a collinear segment in the promoter of *CLV1* homologous gene in most Brassicaceae species. Our results are consistent with the report that *BJCLV1* represents a conserved pleiotropic role in shoot meristem and CMM development, which contains a cis-regulatory sequence specifically expressed BJCLV1 in CMM in its promoter, and this cis-regulatory region is conserved in Brassicaceae species. These results offer a reliable approach for fine-tuning the traits of seed yield in Brassicaceae crops.

Introduction

*Brassica juncea* (*B. juncea*, AAB, 2n=36), one of the three most commonly planted rapeseeds worldwide, is widely used in the genetic improvement of *Brassica napus* as a donor resource. Many natural plant variants with multilocular siliques have been found for *B. juncea*. Its yield per plant is significantly higher than that of bilocular mustard, which has siliques with two locules, under the same genetic background, mainly due to the increased seed number per silique (Katsyar et al., 1998; Lv et al., 2012; Zhao et al., 2003).

The silique of *B. juncea*, derived from the gynoecium, typically comprises two fused carpels separated by two repla connected to a false septum, which divides the silique into two locules (bilocular) with two rows of seeds growing at the junction between the septum and replum. However, multilocular siliques comprise more than two fused carpels, and different false septa divide the silique into multiple locules, each containing more than one row of seeds. Genetic studies have indicated that the multilocular trait of the MVS7, MVS8 and MVS24 lines (Choudhary and Solanki, 2007), as well as the ‘duoshi’ cultivar (Xiao et al., 2013), is controlled by two independently inherited recessive nuclear genes in *B. juncea*. *Arabidopsis* gynoecium comprises two congenitally fused carpels that form a hollow tube (Smyth et al., 1990). In early gynoecium development, a tissue with meristematic identities forms along the margins where the carpels fuse, which is called carpel margin meristem (CMM) (Long et al., 1996; Reyes-Olalde et al., 2013). As development proceeds, the CMM gives rise to medial tissues and organs, including the placenta, ovules, septum and transmitting tract (Bowman et al., 1999).

Shoot meristems, including shoot apical meristem (SAM), inflorescence meristem (IM) and floral meristem (FM), are maintained by pluripotent stem cells that are controlled by classical CLAVATA (CLV)-WUSCHEL (WUS) feedback signalling established in *Arabidopsis* (Brand et al., 2000; Schoof et al., 2000) conserved in crops, such as maize, rice, tomato and rapeseed (Somsich et al., 2016). This pathway relies on communication between peptide ligands, a series of receptors, and transcription factors (TFs) expressed in different zones and coordinate stem cell proliferation with differentiation. The cores of this complex network are WUS, a mobile homeodomain TF expressed in the organizing centre (OC) that can move to the central zone (CZ) to promote stem cell fate (Yadav et al., 2011), especially by repressing differentiation (Mayer et al., 1998) and CLV3 (Fletcher et al., 1999), a small peptide ligand whose expression is induced by WUS in the CZ but can repress WUS expression when perceived by leucine-rich repeat receptor-like kinases (LRR-RLKs), such as CLV1 (Clark et al., 1997), and a leucine-rich repeat receptor-like protein (LRR-RLP) CLV2 (Jeong et al., 1999). Over the past 30 years, mutations in *CLV1* in *Arabidopsis* and *CLV1* orthologs in crops, such as the *CLV1* orthologs in rice (Suzaki et al., 2004), in maize (Bommert et al., 2005), in tomato (Xu et al., 2015) and in *B. juncea* (Chen et al., 2018; Xiao et al., 2018), often created great interest, which contains enlarged shoot meristems, flattened stems and increased floral and fruit...
organ number. However, despite mutations in CLV1 and CLV1 orthologs involved in shoot meristem activity that affect the formation of these gynoecial structures, little is known about their direct roles in CMM development.

J163-4, whose siliques exhibit a trilocular trait, is a landrace of *B. juncea*. Genetic studies have shown that the trilocular trait of J163-4 is controlled by a pair of recessive nuclear genes, *mc1* and *mc2* (Lv et al., 2012), and mapping studies have delimited *mc2* to 68 kb in the Scaffold 000019 physical map of *A7* in *Brassica rapa* (Wang et al., 2016). In this study, we cloned another trilocular gene of J163-4, *mc2*. By studying the gene structure and function of *mc2* in *B. juncea* and *Arabidopsis* using comparative morphological and molecular genetic experiments, we report that an 914-bp deletion in *mc2* promoter, which contains a cis-regulatory sequence, specifically expressed *BjCLV1* in CMM, and this cis-regulatory region is conserved in Brassicaceae species. These findings offer a new and reliable approach for fine-tuning the traits of seed yield in Brassicaceae crops.

**Results**

*mc2* mutation affects CMM size with increased yield

To specify the timing and phenotypic effects of *mc2* locus on chromosome A7 in *B. juncea*, near-isogenic lines BC1-F2, homozygous for wild-type and mutant alleles, were used for comparative developmental studies. The most prominent phenotype of the *mc2* plants was in the development of siliques, which at maturity were abnormally flattened and wider than normal (Figure 1a–d), with four carpels and three loculi separated by an ‘il’-type false septum (Figure 1f,h). Notably, *mc2* siliques can bear an average of 25 seeds (Figure 1f,h), versus 17 seeds for normal siliques (Figure 1e,g), without a reduction in the 1000-seed weight (Table 1), although they are shorter in length (Table 1), indicating that *mc2* plants have great potential for the future genetic improvement of yield traits in mustard. The architecture of the *mc2* plants was relatively normal (Table 1) compared to that of siliques.

The near-isogenic lines were further compared in order to determine when *mc2* first affected meristem development. We found that *mc2* mainly affects gynoecium development but has no apparent effect on other tissues and organ development during the embryonic, vegetative seedling and early flowering stage (Figure S1). To further explore the potential effects of *mc2* on the early stages of gynoecium development, the early development of *mc2* gynoecium was examined using scanning electron microscopy (SEM). At stage 4 of flower development, the *mc2* flowers exhibited a normal phenotype (Figure 1i,n). However, when carpel primordia were initiated in the centre of FM, the difference between *mc2* and normal gynoecia could be seen at stage 5. At this stage, a tissue with meristematic identities called the CMM forms along the margins where the carpels fuse in the medial region of *mc2* gynoecia, which began to swell compared to normal gynoecia (Figure 1j,o). When the gynoecium began to develop as a raised ridge around a central cleft at stage 6, every enlarged CMM of *mc2* gynoecium developed into two CMMs (Figure 1p). The normal gynoecium comprises two congenitally fused carpels and two CMMs (Figure 1k), which arise as a cylinder-like structure because they are joined at the margins in the medial region (Figure 1l,m). In contrast, the *mc2* gynoecium comprised four acquired carpels and four CMMs, which arise as square-like structures (Figure 1a). This abnormal gynoecium was more obviously distinct than that of the normal gynoecium at stage 8 (Figure 1r). Compared to those of gynoecia, the stature and number of other floral organs of the *mc2* flowers were relatively standard (Figure 1n–r).

To further investigate the potential effects of additional CMMs on gynoecium development in later stages, the development of internal tissues of *mc2* gynoecium was analysed by cross section. As CMM development proceeds, the septum is initiated during stage 8 when the inner medial surfaces form ridges (Figure 1s,v) and forms properly during stage 9 when the leading edges of each medial ridge meet and fuse (Figure 1w). At stage 12, normal CMMs gave rise to an ‘i’-type false septum and two rows of ovules (Figure 1u); however, the *mc2* CMMs gave rise to an ‘il’-type false septum and more than two rows of ovules (Figure 1x). These phenotypes were consistent with the observation that mature *mc2* siliques are composed of four carpels and three loci (Figure 1f,h).

To summarize, *mc2* plants were defective in regulating CMM size in early gynoecium phases and showed no apparent abnormalities in other meristems. The *mc2* siliques produced extra carpels, and seeds resulted from enlarged CMMs. Therefore, *mc2* plants can be defined as weak mutants.

**Mc2** encodes CLV1, and a 914-bp deletion region exists in the *mc2* promoter

The *Mc2* gene was previously positioned in a region between markers ZX17 and BACs96, which has perfect collinearity with a 68-kb physical region between 946 and 1014 kb in Scaffold 000019 physical map of *A7* in *B. rapa* (Wang et al., 2016). Subsequently, the full-length sequencing of two positive clones of a purple-leaf mustard BAC library, 002-O-21 and 009-M-2, screened by the flanking markers linked to *Mc2*, was completed, and three contigs (designated as contig 1, 2, and 3) were obtained. Using BLAST, the physical positions of the markers ZX17 and BACs96 in contig 1 were found to be 89.5 and 54.6 kb, respectively (Figure 2a), covering a physical interval of 34.9 kb. When the whole genome sequencing of *B. juncea* was completed (Yang et al., 2016), markers ZX17 and BACs96 were aligned at the physical positions of 32 935 and 32 900 kb on Chr.A07, respectively (Figure 2a), indicating that the previous localization results of *Mc2* were reliable. Notably, this genomic region contains only six annotated open reading frames, including a homolog of *Arabidopsis thaliana* CLAVATA1 (*CLV1*, At1g75820), *BjuA029486* (Figure 2b; Table S1). Given that clv1 mutants could increase flower organ numbers, especially carpels (Clark et al., 1999), it is reasonable to speculate that *BjA7-CLV1* is a candidate gene for *Mc2*.

To confirm the candidate gene for *Mc2* loci, a series of gene-specific primers for amplifying *BjA7-CLV1* in bilocular and trilocular parents were designed based on the contig 1 sequence. Compared to the sequencing results, the CDS (3043 bp) and 3′-UTR (1248 bp) regions of *BjA7-CLV1* showed no difference in the bilocular and trilocular parents, but a 914-bp deletion was identified in the regulatory region –2865 bp upstream of *BjA7-CLV1* in J163-4 (Figure 2c). These results show that *BjA7-CLV1* is a reliable candidate gene for *Mc2*.

BLAST searching BRAD database revealed that *CLV1* is highly conserved in brassicaceous species and contains at least one homologous copy (Figure 2d). *Brassica* allotetraploids, such as *B. juncea* and *B. napus*, have two CLV1 homologs (Figure 2d).
Phylogenetic analysis showed that *Brassica* CLV1 genes were assigned to subclades following their localization to the A, B or C subgenomes (Figure 2d). *BjA7* CLV1 (Mc2) has high homology with *BraCLV1* derived from *B. rapa*, whereas *BjB3* CLV1 (Mc1) has high homology with *BniCLV1* derived from *B. nigra* (Figure 2d).

To further understand whether the 914-bp deletion sequence of the *mc2* promoter was similarly conserved in the promoter region of these homologous genes, BLAST analysis using the 914-bp deletion sequence as a query was performed in the BRAD database. Conservatory analysis showed that the 914-bp deletion sequence of the *mc2* promoter had a relatively high level of conservation, especially in *Brassica* species, with 602-bp and 282-bp sequences conserved within *Mc1* and *AtCLV1*, respectively (Figure 2d).

Trilocular siliques are formed owing to the 914-bp regulatory sequence deletion in the promoter

Real-time quantitative PCR analysis revealed no significant difference in the expression of *Mc2* transcripts in the seedling-stage young leaves and inflorescences of *pMc2::Mc2* and *pmc2::Mc2* transgenic plants (Figure 3b); however, there was a significant difference in the ovary (Figure 3b). Similarly, *Mc1* transcripts in *pMc1::Mc1* and *pmc1::Mc1* transgenic plants only showed significant differences in expression in the ovary (Figure 3a). Cross-sectional analysis results showed that *p3SS::Mc1* (Figure 3c), *pmC1::Mc1* (Figure 3d), *p3SS::Mc2* (Figure 3f) and *pMc2::Mc2* (Figure 3g) transgenic plants could restore the bilocular silique phenotype composed of two carpels and an ‘I’-typed septum (Figure 3c), while *pmc2::Mc2*...
### Table 1 Descriptive statistics of the traits

| Genotype            | Number of primary branches | Plant height (cm) | Number of siliques per plant | Thousand seed weight (g) | Seed yield per plant (g) | Silique length (cm) | Silique body length (cm) | Seed yield per silique (g) | Plant height (cm) |
|---------------------|-----------------------------|-------------------|-----------------------------|--------------------------|-------------------------|-----------------------|--------------------------|--------------------------|-------------------|
| J248-2 A-Bi: Mc1Mc1Mc2Mc2 | 7 ± 0.55                   | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |
| J248-2 B-BC6F2       | 7 ± 0.55                   | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |
| J248-2 A-BC7F2       | 7 ± 0.55                   | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |
| J163-4 B-Bi: Mc1Mc1Mc2Mc2 | 7 ± 0.55                  | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |
| J163-4 B-Tri: Mc1Mc1Mc2Mc2 | 7 ± 0.55                 | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |
| J163-4 A-Bi: Mc1Mc1Mc2Mc2 | 7 ± 0.55                 | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |
| J163-4 A-Tri: Mc1Mc1Mc2Mc2 | 7 ± 0.55                 | 358 ± 18.6        | 516 ± 0.62                  | 15.8 ± 0.81              | 3.15 ± 0.21*            | 31.5 ± 0.21*          | 30.5 ± 0.21*             | 22.07 ± 0.21*            |

(*) Significant at 1% level

Data and errors bars represent mean ± SD.

Consistent with the qRT-PCR analyses, GUS activity was detected in most tissues and organs investigated when controlled by the full-length promoters (Figure 4c,f). These results suggest that Mc1 and Mc2 play vital roles in various tissues and organs of B. juncea and that the 914-bp deletion region of the Mc1 promoter and its collinearity segments in the Mc1 promoter contain cis-regulatory elements that are crucial for Mc2 and Mc1 expression in CMM.
Figure 2  Molecular characterization of the Mc2 gene. (a) Fine mapping of the Mc2 gene to a 35-kb region between the markers ZX17 and BACsr96 of chromosome A7. The numbers in parentheses indicate the number of recombinants of corresponding markers. The grey physical map below represents the corresponding purple-leaf mustard Brassica juncea BAC clone 002-O-09 and 009-M-2 region homologous to the region in which Mc2 resides. (b) The refined 35-kb region at the Mc2 locus contained six annotated genes, and the red arrow represents the candidate gene Mc2. (c) Gene structure of the candidate gene and polymorphisms between the two parents. Dark grey columns represent exons, and light grey columns represent the promoter and 3'-flanking region. The red dashed line represents a 914-bp deletion in mc2. The two red arrowheads indicate two single gRNA target sites for CRISPR/Cas9. (d) Phylogenetic tree and promoter conservatory analysis of Mc2 and homologous genes in the Brassicaceae family. The neighbour-joining phylogenetic tree was constructed using MEGA X. The numbers at the nodes represent percentage bootstrap values based on 1000 replicates. The lengths of the branches refer to the nucleotide variation rates. The red box represents the core region that may contain cis-regulatory elements specifically expressed in the carpel margin meristem (CMM).
Figure 3 Complementation test and overexpression of Mc1 and Mc2 in Brassica juncea. (a, b) Expression of Mc1 (a) and Mc2 (b) in different tissues of T1 transgenic lines, as revealed by quantitative RT-PCR. Error bars indicate SD of three replicates. (c–n) Cross sections to analyse the development of internal tissues of the gynoecium. (c, d) Stage 12 section of p35S::Mc1. (e, f) Stage 12 section of pMc1::Mc1. (g, h) Stage 12 and 10 sections of pmc1::Mc1, respectively. (i, j) Stage 12 section of p35S::Mc2. (k, l) Stage 12 and 11 sections of pMc2::Mc2, respectively. (m, n) Stage 10 and 8 sections of pmc2::Mc2, respectively. (o–z) T1 Phenotypes of plant complementation test and overexpression. (o, u) Plant and siliques of p35S::Mc1. (p, v) Plant and siliques of pMc1::Mc1. (q, w) Plant and siliques of pmc1::Mc1. (r, x) Plant and siliques of p35S::Mc2. (s, y) Plant and siliques of pMc2::Mc2. (t, z) Plant and siliques of pmc2::Mc2. M, medial region; Ca, carpel; Se, septum; O, ovule; *, carpel margin meristem (CMM). Scale bars = 100 μm for (c–n), 1 cm for (o–t) and 0.5 cm for (u–z). Significant differences by Student’s t-test are shown as NS, not significant at \( P \geq 0.05 \); ***, \( P < 0.001 \).
Figure 4  Expression pattern of Mc1 and Mc2. (a) Expression of Mc1 in various tissues from B-Bi and B-Tri detected using qRT-PCR. (b) Expression of Mc2 in various tissues from A-Bi and A-Tri detected using qRT-PCR. Error bars in (a) and (b) represent SD from three biological replicates. Significant differences by Student’s t-test are shown as NS, not significant at $P \geq 0.05$; ***$P < 0.001$. (c-f) GUS staining showing Mc1 and Mc2 expression in the carpel margin meristem (CMM) of pMc1::GUS (c) and pMc2::GUS (e) Arabidopsis, but no expression in the CMM of pmc1::GUS (d) and pmc2::GUS (f) Arabidopsis. Scale bars = 2.5 mm, 1 mm, 100 μm, 100 μm and 2 mm for (c) and (e) (from left to right) and 2.5 mm, 1 mm, 100 μm, 100 μm and 2 mm for (d) and (f) (from left to right).
–3512- to –3409-bp region of the Mc2 promoter for expression of Mc2 in CMM

To further identify the regulatory region for CMM-specific expression in the 914-bp deletion sequence, we studied the GUS reporter gene driven by a series of progressive 5’-deletion Mc2 promoters in Col. The results of staining in flowers revealed an intense GUS activity in the CMM of gynoecium from three transgenic plants [GUS2-1 (–3631bp), GUS2-2 (–3562bp) and GUS2-3 (–3512bp)] (Figure 5a–c), while slight GUS staining was also detected in the CMM of GUS2-4 (–3468bp) (Figure 5d) gynoecium. However, GUS staining was not detected in the CMM of gynoecium of the six other constructs (Figure 5e–j).

Additionally, Mc2 gene expression was driven by different deletions of its native promoter (Figure 5f) in which cvl1-1 mutant was studied. The phenotypic results showed that the Mc2G-1, Mc2G-2 and Mc2G-3 transgenic plants exhibited a similar stature phenotype and siliques architecture to Ler (Figure 5a–c), and the average proportion of bilocular siliques per plant exceeded 98% (Figure 5k; Table S2), indicating that these plants resulted in a bilocular phenotype. Mc2G-4 (Figure 5d) transgenic plants included part of tricarpellate siliques, the average proportion of which was less than 20% (Figure 5k; Table S2), indicating that these plants only partially restored a bilocular phenotype. In contrast, the other six transgenic plants (Mc2G-5, Mc2G-6, Mc2G-7, Mc2G-8, Mc2G-9 and Mc2G-10) (Figure 5e–j) exhibited a similar stature phenotype and siliques architecture to pmc2::Mc2, and the average proportion of bilocular siliques per plant accounted for <40% (Figure 5k; Table S2), indicating that these plants could not restore a bilocular phenotype. Mc2 expression was also analyzed using qRT-PCR in the ovaries of these transgenic plants. Consistent with the complementation phenotype analyses, the higher the average proportion of bilocular siliques in these transgenic plants, the higher the Mc2 transcription level in the ovary (Figure 5k), suggesting that Mc2 expression in CMM is required for normal stem cell homeostasis.

In summary, when the Mc2 promoter was truncated to –3511-bp from 5’–3’, it did not affect Mc2 expression in the CMM and would not increase the number of carpels in the siliques. When it was truncated to –3467-bp, Mc2 expression in CMM decreased, increasing the number of carpels in some siliques. When truncated to –3409-bp, Mc2 expression in CMM was undetectable, increasing the number of carpels in more siliques and trilocular plant formation. Therefore, the –3512- to –3409-bp region of Mc2 promoter was necessary for Mc2 expression in CMM.

Phenotypes of null mc2 alleles created by CRISPR-Cas9

As the 914-bp deletion of Mc2 promoter in trilocular plants does not affect its expression in most tissues, to gain insight into its loss-of-function effects on IM and FM development, we generated several null alleles of Mc2 gene using clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) genome editing, with two single guide sequences, S1 and S2; S1 was located in the upstream 599-bp promoter region, and S2 was located in the first exon 2077 bp of the coding region (Figure 2c). We obtained four lines with one- to seven-bp deletions in the protein-coding region that likely disrupted Mc2 functionality (Figure 6a) from more than a hundred primary transformants. In all cases, the CR-mc2 lines yielded fascinated inflorescences, flowers and siliques resembling the phenotype observed in cvl1-4, which is the strongest reported allele of cvl1 (Clark et al., 1993). The four loss-of-function alleles had the same phenotypes, and we chose line CR-mc2-1, which has a 4-bp deletion (nucleotide 2072–2075), for further experiments. Expression analysis using qRT-PCR revealed that Mc2 was not expressed in CR-mc2-1 (Figure 6b).

Unlike mc2 allele, the CR-mc2-1 allele exhibited enlarged inflorescences (Figure 6c,d). The SEM results showed that in the CR-mc2-1 flowers, the carpel primordium arose as a ring of organs around an enlarged and still-proliferating central dome at stage 5 (Figure 6e), at which time the FM terminated and carpel primordia arose at the centre of FM in wild-type flowers. In the later stages, this enlarged dome still appeared at the centre of gynoecium (Figure 6f–i). At stage 12, the cross-sectional observations showed a large mass of meristem tissue inside the gynoecium, which could generate an additional gynoecium (Figure 6j). This inner gynoecium can take up most of the space of the ovary, which caused the ovule and false septum to develop abnormalities in the CR-mc2 lines (Figure 6j). During the flowering period, CR-mc2-1 flowers showed an increase in the number of petals, stamens and carpels (Figure 6j,k,m, S2), and the gynoecium enlarged with the stigma cracked (Figure 6j,k).

Hence, our results revealed that null mc2 alleles affected IM and FM development; the larger FM not only developed into a flower with additional organs in each whorl but also continued to proliferate and result in a large mass of undifferentiated cells in the centre of the flower instead of terminating like the wild-type FM. Thus, in both the shoot and FM, null mc2 alleles affected the balance between cell proliferation and differentiation.

Mc2 affecting the expression of genes involved in CMM development

As Mc2 encodes an LRR-RLK, suggesting that it might perceive CLV3 or a related CLE peptide. In some plants, exogenous application of synthetic CLE peptides in vitro induced the consumption of meristems in the shoots and roots. CLE26 could be expressed similarly to Mc2 in CMM (Jun et al., 2010); therefore, we treated seedlings with peptides (CLV3 and CLE26) and measured the inhibition of root and epicotyl growth. Peptide treatment results showed that trilocular seedlings were the same as bilocular seedlings. Roots and epicotyls in seedlings treated with a control, scrambled CLV3 peptide (sCLV3) grew normally but were inhibited in the presence of CLV3 and CLE26 (Figure 7a, d). Further observation found that this inhibition was due to the restricted development of SAM (Figure 7b,e) and root apical meristem (RAM) (Figure 7c,f), indicating that Mc2 functions in the SAM and RAM of trilocular seedlings, similar to that of bilocular seedlings. Furthermore, confocal microscopy revealed that the fluorescence expression of Mc2-GFP and Mc2-1-GFP showed plasma membrane localization in Nicotiana benthamiana epidermal cells (Figure S7), which is consistent with its proposed role as a membrane receptor (Stahl et al., 2013).

CLV1 is a major player in regulating the size of shoot apical, inflorescence and flower meristems. To better understand the molecular mechanisms in which BjCLV1 regulates CMM development, we monitored the expression of genes involved in CLE peptide signalling genes, homeodomain TFs and CUP-SHAPED COTYLEDON (CUC) genes in ovaries from stage 8 flowers. The expression of the homolog of WUS (BjA-WUS, BjBA001259, BjB-WUS, BjB033714) and a likely functional homolog of CLV3 (BjCLV3, BjB026230) was hardly detectable in the trilocular mutants and bilocular plants (Figure 7g), suggesting that BjWUS
Figure 5  Promoter deletion analysis of Mc2 in Arabidopsis. (a–j) The expression of complementary phenotype and analysis of GUS in gynoecium, containing different sections of Mc2 promoter. The number indicates the relative distance from the 5' site of the section to Mc2 gene translation initiator. (k) Quantification of carpel number (data are presented as average percentage of siliques per carpel number category; plant number per genotype = 20, silique number per plant >50) of indicated genotypes (T1) and expression of Mc1 or Mc2 in the ovary from indicated genotypes detected using qRT-PCR. Error bars represent SD from three biological replicates. Significant differences by Student’s t-test are shown as NS, not significant at $P \geq 0.05$; **$P < 0.05$; ***$P < 0.001$. Scale bars = 5 mm, 2 mm, and 100 μm for (a–j) (from left to right).
Figure 6 Phenotypes of the loss-of-function CRISPR/Cas9-mc2 (CR-mc2) lines. (a) Loss-of-function CR-mc2 alleles identified by cloning and sequencing PCR products from the Mc2-targeted region from four T0 plants. Blue dashed lines indicate InDel mutations, and black bold and underlined letters indicate protospacer-adjacent motif (PAM) sequences. (b) Expression of Mc2 in different tissues of two loss-of-function CR-mc2 alleles and A-Bi, as revealed by qRT-PCR. Error bars indicate the SD of three replicates. (c, d) Inflorescence of the wild-type (WT) (c; A-Bi) and CR-mc2-1 (d). (e–i) Scanning electron microscopy images of CR-mc2-1 flowers at stages 5 (e), 6 (f) and 7 (g), and a gynoecium (i) from (h). (j) Cross sections for analysing the development of internal tissues of the gynoecium in the indicated alleles. (k) CR-mc2-1 flower; the box is the top view of the gynoecium, the stigma. (l) CR-mc2-1, CR-mc2-3 and WT (A-Bi) gynoecium. (m) CR-mc2-1 floral organs, including sepal, petal and stamen. Scale bars = 5 mm for (c) and (d), 200 μm for (j) and 1 mm for (k) and (l).
Figure 7  CLE peptide assays and pathway analysis. (a, d) Seven-day-old Brassica juncea seedlings were grown on agar media with or without the individual peptides; epicotyl and root length measurements are shown in (d). (b, e) Effect of different peptides on the shoot apical meristems (SAMs) of A-Tri (b); SAM diameter was quantified (e). (c, f) Effect of different peptides on the root apical meristems (RAMs) of A-Tri (c); RAM diameter was quantified (f). (g) Expression of Arabidopsis homologous genes involved in meristem maintenance and expressed in CMM in the ovary of the near-isogenic lines. The expression levels were determined using qRT-PCR and normalized to Actin2. The values are presented as the mean ± SD (n = 4 biological replicates). (h) Proposed CMM maintenance via a feedback pathway containing the core receptor Mc2 in Brassica juncea. M, medial region; L, lateral region; CMM, carpel margin meristem; TF, transcription factor; the pentagram represents the core region, which may contain cis-regulatory specifically expressed in CMM. n = 20 for each genotype in (d), (e) and (f). Data in (d), (e) and (f) are presented as mean ± SD. Significant differences by Student’s t-test are shown as NS = not significant at P ≥ 0.05; **P < 0.05; ***P < 0.001. Scale bars = 1 cm for (a), 50 μm for (b) and 100 μm for (c).
and BjCLV3 may not be the main factors in establishing and maintaining CMM. Notably, one additional CLE gene (BjCLE26, BjA027560) was up-regulated in the trilocular mutants (Figure 7g). The expression of the homolog of SHOOT MERISTEM-LESS (STM) (BjA-STM, BjA033384; BjB-STM, BjB028275) was increased in the trilocular mutants compared to that in the bilocular plants (Figure 7g), suggesting that BjSTM may control CMM size in the same pathway as Mc2. Furthermore, the expression of BjCUC1 and BjCUC2, involved in shoot meristem initiation and required for formation and stable positioning of the CMMs in Arabidopsis (Kamuchi et al., 2014), was significantly affected in the trilocular mutants. In the trilocular mutants, an increase expression of both genes was detected (Figure 7g), suggesting that BjCUC1 and BjCUC2 could also be involved in CMM formation during B. juncea gynoecium development.

Discussion

The tissues and organs of Arabidopsis gynoecium medial region, including the replum, placenta, septum, ovules and transmitting tract, are produced by CMM (Bowman et al., 1999); therefore, it is important to study the development mechanism of CMM to increase the Brassicaceae plant seed yield. In this study, we determined that trilocular silique formation was caused by CMM enlargement. There was a significant difference in CMM size between the bilocular and trilocular plants but no significant difference in the sizes of SAM, IM and FM. However, the SAM, IM and FM of multilocular plants were larger than those of bilocal plants in B. rapa ml4 (Fan et al., 2014) and B. juncea ‘duoshi’ (Chen et al., 2018; Xiao et al., 2018). Thus, multilocular phenotype formation was concurrent with enlarged SAMs, which could lead to an enlarged FM and, further allowing the initiation of more floral organ primordia, with extra gynoecium inside the silique as the FM failed to terminate. This showed that J163-4 was a natural weak mutant, which it will be more convenient to use, and the mechanism underlying trilocular silique formation differs from that of other multilocular plants.

In Arabidopsis, CLV1 encodes a fully functional LRR-RLK, which plays a key role in shoot meristem maintenance (Clark et al., 1997; Stone et al., 1998). The B. juncea genome encodes two CLV1 orthologs, BjA7.CLV1 and BjB3.CLV1. In this study, we cloned another trilocular gene of J163-4, mc2 and found a CLV1 ortholog, BjA7.CLV1. Unlike mc1, whose transcription is interrupted by the insertion of the BjB3.CLV1 coding region (Xu et al., 2017), there was no mutation in the coding region of mc2; however, there was a 914-bp deletion fragment in the promoter regulatory region, which did not affect the normal expression of Mc2 in trilocular plants, except in the CMM. Therefore, when bilocular and trilocular seedlings were treated with CLV3 peptides, BjCLV1 located in SAM and RAM could receive CLV3 peptide signals normally, thus inhibiting stem cell division, and seedlings showed stunted root and epicotyl growth (Figure 7a–f). However, both Bjln1 and Bjln2 in B. juncea ‘duoshi’ are homologous genes of CLV1. Bjln1, BjA7.clv1, is caused by a change in amino acids at positions 28 and 63 because of five SNPs in LRR domain of the coding region (Xiao et al., 2018), and Bjln2 is caused by the insertion of a 4961-bp fragment in the coding region that interferes with the normal BjB3.CLV1 transcription (Chen et al., 2018). This could explain why J163-4 and ‘duoshi’ have different phenotypes (Xiao et al., 2013; Xu et al., 2014), even though both have mutations in the BjCLV1 gene. Gynoecium cross sections have shown that a ‘+’-shaped false septum divides the ovary into four locules in ‘duoshi’ (Xiao et al., 2013; Zhao, 2014), while trilocular siliques of J163-4 were divided into three locules by a ‘Ⅲ’-shaped false septum in this study. Furthermore, null mc2 alleles developed more disorganized and fascinated siliques with low seed yield due to mutations in the coding region, leading to FM overproliferation. This suggests that compared to mutations in CDS of BjCLV1 that alter protein structure, the cis-regulatory variant which affect the expression of BjCLV1 in CMM only cause phenotypic change in siliques. Additionally, ml4 (tet-o) of ‘Yellow Sarson’ and sb (‘Sandglass’) in B. rapa are homologous genes of BjCLV3, and both are single-nucleotide mutations of CLE motif in the coding region and lead to amino acids changes (Fan et al., 2014; Yadava et al., 2014; Yang et al., 2021). Therefore, the J163-4 phenotype differs from that of other multilocular plants because of the different forms of mutation.

Conservatory analysis showed that the 914-bp deletion sequence of the mc2 promoter had a relatively high level of conservation in Brassicaceae species (Figure 2d). The BjCLV1 genome sequence with its complete promoter could rescue the Bjclv1 and clv1 mutant phenotype in B. juncea and Arabidopsis, respectively, while without the conserved deletion region of its promoter failed to rescue the mutant phenotype (Figure 3, S4). Similarly, promoters only including the 2691-, 2692-, 1945- or 2237-bp upstream regulatory sequence from TSS (Figure 2d), which the conserved deletion region, are not included, in the complementation constructs also partially rescue the mutant phenotype (Chen et al., 2018; Clark et al., 1997; Xiao et al., 2018; Xu et al., 2017). In contrast, GUS activity was detected in the CMM of gynoecium when 3.4-kb upstream regulatory sequence (Figure 2d) containing the conserved deletion region was fused to the GUS reporter gene in transgenic Arabidopsis (Durbak and Tax, 2011). In addition, Mc1 and Mc2 mRNA were expressed in a broad range of B. juncea (Figure 4). This suggests that the collinearity segments of the 914-bp deletion sequence in the CLV1 homologous promoter may contain cis-regulatory elements that affect their expression in CMM, and that Mc1 and Mc2 are a pair of functionally redundant genes that play vital roles in various tissues and organs of B. juncea. The B. napus genome also contains two CLV1 homologous genes. Mutation of only one homologous gene will not lead to phenotypic change; a multilocular plant can only be obtained by knocking out two homologous genes simultaneously (Yang et al., 2018). The CLV1 mRNA in Arabidopsis also constitutively expressed in diverse tissues (Clark et al., 1997; Dievart et al., 2003; Trotochaud et al., 1999). This conserved none-tissue-specific expression pattern suggested CLV1 and CLV1 orthology are a conserved pleiotropy.

Most functional studies of the CLV1 gene have been performed in Arabidopsis, and genetic studies of CLV1 homologs in other plants have shown conservation of meristem function (Somssich et al., 2016). Currently, it is known that uncovered pleiotropic roles of fruit-yield-related genes by generating CRISPR/Cas9 genome-edited promoter alleles in tomato and maize demonstrated that specific cis-regulatory regions control this pleiotropy (Hendelman et al., 2021; Liu et al., 2021; Rodríguez-Leal et al., 2017; Swinnen et al., 2016; Wang et al., 2021). This suggests that targeting the conserved cis-regulatory sequences of specially expressed CLV1 ortholog in CMM mutations could produce multilocular siliques with higher yields in Brassicaceae crops. From the phylogenetic tree, CLV1 is highly conserved with at least one homolog in all Brassicaceae species (Figure 2d). This conserved evolutionary manner suggested a conserved receptor
function of BjCLV1 in B. juncea multiple meristems. Carpel development involves two key TFs, STM and AGAMOUS (AG) MAD5 box genes (Scufield et al., 2007), which are activated in the centre of FM by the floral regular LEAFY (FY) and the stem cell-promoting factor WUS in the early stages of flower development (Lenhard et al., 2001; Lohmann et al., 2001). After stage 6, AG directly represses WUS expression by binding to WUS locus and recruiting Polycomb Group (PcG) that methylates histone H3 Lys-27 at WUS, causing stem cell maintenance to terminate and thus permitting carpel development (Lenhard et al., 2001; Liu et al., 2011; Lohmann et al., 2001). When the carpel primordia occupied the flower centre, no WUS or CLV3 expression was detected (Lenhard et al., 2001; Mayer et al., 1998). Similarly, the expression of the homolog of WUS and CLV3 was not detected in the mc2 and bilocular ovaries (Figure 7g). The homeodomain TF STM is a key regulator of CMM development. STM is required for the formation and maintenance of SAM and is expressed in the CMM at the early stages of gynoecium development (Long et al., 1996). Additionally, CUC1 and CUC2 are also required for the formation and stable positioning of CMMs, and between CUC and STM presence a positive feedback loop in CMM (Kamiuchı et al., 2014; Spinelli et al., 2011). Furthermore, CLE26 can be expressed similarly to Mc2 in CMM (Jun et al., 2010). In this study, the expression of the homolog of CLE26, STM and CUC genes in the ovary was higher in mc2 mutant than in bilocular plants, which may be because the expression of BjSTM in the OC of CMM, where it promotes stem cell division. BjCLV1 is activated by peptide BjCLE26, which regulates BjSTM in a negative feedback loop, to restrict CMM stemness (Figure 7h). Therefore, loss of BjCLV1 function in CMM leads to an expansion of the CMM, which leads to an increase in the number of CMMs and ultimately to trilocular siliques. However, evidence of direct interaction between Mc2 and BjCLE26 and the TFs regulating Mc2 expression in CMM are missing in this study; therefore, this will be the focus of our future studies. This did not affect our use of the excellent allele mc2. In breeding studies, we can utilize interspecific hybridization combined with molecular marker-assisted selection to transfer mc2 into the widely planted B. napus; this is expected to breed more seeds per silique of high yield varieties of B. napus without affecting other traits.

In conclusion, the isolation and functional characterization of CLV1 ortholog genes in B. juncea were reported. A novel 914-bp deletion fragment, which containing cis-regulatory elements was specifically expressed in CMM, in BjA7.CLV1 promoter is essential for controlling CMM size and the number of locules and seeds per silique. Moreover, this cis-regulatory region is highly conserved in the promoter of CLV1 ortholog genes in Brassicaceae, suggesting that this region can be edited to fine-tune CMM development without altering protein structure. These findings provide a new method for improving the seed yield-related traits in Brassicaceae crops.

Experimental procedures

Plant materials and growth conditions

Homozygous bilocular lines (A-Bi, genotype mc1mc1Mc2Mc2) and homozygous trilocular lines (A-Tri, genotype mc1mc1mc2mc2) from BC2F2 populations, which was constructed by using a trilocular line J163-4 and a bilocular line J268-1, were used for phenotypic and expression analyses of Mc2. Homozygous bilocular lines (B-Bi, genotype Mc1Mc1mc2mc2) and homozygous trilocular lines (B-Tri, genotype mc1mc1mc2mc2) from BC2F2 plants, which was constructed by using a trilocular line J163-4 and a bilocular line J248-2, were used for gene cloning and expression analysis of Mc1. Brassica juncea plants were grown at the experimental farm at Huazhong Agricultural University, Wuhan, China, in autumn. Transgenic B. juncea plants were grown in an isolated experimental station. Arabidopsis plants (Col-0 ecotype, Ler ecotype and clv1-1 mutant) were grown in a plant growth chamber at 20–22°C and 70% humidity under a photoperiod of 16/8 h of light/dark.

Gene cloning and sequence analysis

The genomic DNA of Mc2 (9118-bp, including 4827-bp upstream regulatory sequence from the translation start site (TSS) and 3043-bp coding region) and mc2 (8204-bp, including 3913-bp upstream regulatory sequence from TSS and 3043-bp coding region) were amplified with specific primers (Table S3) and sequenced. To identify the Mc2 homologous genes in Brassicaceae, BLAST analysis using the coding sequence (CDS) as a query was performed in BRAD database (http://brassicadb.org/brad/). The most similar sequence was selected from each species. Sequence alignment and clustering of these homologs were performed with MEGA-X (http://www.megasoftware.net) using the neighbour-joining method with 1000 bootstrap replications. To determine whether 914-bp deletion sequence of mc2 promoter has collinearity segments in Mc2 homologous genes promoter region of Brassicaceae, BLAST analysis using 914-bp deletion sequence as a query was performed in BRAD database.

Plasmid construction and transformation

Four plasmids were constructed for complementation assay. Genomic DNA fragments of Mc2 and mc2, including 3818-bp and 2864-bp upstream regulatory sequence from TSS, respectively, were amplified, respectively, cut with EcoR I/Kpn I and cloned into pCAMBIA2300, generating pMc2::Mc2 and pmc2::Mc2 constructs. Genomic DNA fragments of Mc1, including 4581-bp and 2499-bp upstream regulatory sequence from TSS, respectively, were amplified, respectively, cut with Kpn I/Pst I and cloned into pCAMBIA2300, generating pMc1::Mc1 and pmc1::Mc1 constructs. To overexpress Mc1 and Mc2, four promoter-reporter plasmids were constructed. The 4581-bp and 2499-bp upstream regulatory sequence from TSS of Mc1 was amplified by PCR from B-Bi, respectively, the 3818-bp upstream regulatory sequence from TSS of Mc2 was amplified by PCR from B-A, and 2864-bp upstream regulatory sequence from TSS of mc2 was amplified by PCR from A-Tri and then fused to the gene for β-glucuronidase (GUS) reporter in the modified binary vector pCAMBIA2300 at Hind III/Bam HI sites, respectively, generating pMc1::GUS, pmc1::GUS, pMc2::GUS and pmc2::GUS constructs. To identify the CMM-specific promoter region, ten promoter-reporter plasmids and ten complementary assay plasmids were constructed. The 3631-bp, 3562-bp, 3512-bp, 3468-bp, 3410-bp, 3353-bp, 3324-bp, 3308-bp, 3236-bp and 3064-bp upstream regulatory sequence from TSS of Mc2 were amplified by PCR from A-Bi and then fused to the gene for GUS reporter in the modified binary vector pCAMBIA2300 at Hind III/Bam HI sites, respectively, generating GUS2-1, GUS2-2, GUS2-3, GUS2-4, GUS2-5, GUS2-6, GUS2-7, GUS2-8, GUS2-9 and GUS2-10 constructs. And a series of genomic DNA fragments with 5′-deletion in the promoter of Mc2, including 3631-bp, 3562-bp,
3512-bp, 3468-bp, 3410-bp, 3353-bp, 3324-bp, 3318-bp, 3236-bp and 3064-bp upstream regulatory sequence from T5, respectively, were amplified by PCR from A-Bi, cut with KpnI/PstI and cloned into pCAMBIA2300, respectively, generating Mc2G-1, Mc2G-2, Mc2G-3, Mc2G-4, Mc2G-5, Mc2G-6, Mc2G-7, Mc2G-8, Mc2G-9 and Mc2G-10 constructs. Two knockout constructs, targeting Mc2, were produced. Two sequence-specific sgRNAs were designed using the web-based tool CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2), one targeting the promoter region (S1) and one targeting the first exon (S2) for Mc2. Following the previously described method (Ma et al., 2015), two sgRNA cassettes driven by the promoters of ATU3d and ATU3b, respectively, were cloned into pYLCRISPR/Cas9Pubi-H and pYLCRISPR/Cas9P35S-H, in which Cas9p is driven by maize ubiquitin promoter (Ptubi) and cauliflower mosaic virus 35S promoter (P35S), generating pMHMc2 and pDHMc2 constructs. The primers used for above plasmid construction were listed in Table S3. Finally, the constructed plasmids were introduced into B. juncea by Agrobacterium-mediated transformation using the hypocotyl infection method (Xu et al., 2017) or into Arabidopsis by Agrobacterium-mediated transformation using the floral dip method (Clough and Bent, 1998). The receptor plants used were listed in Table S4.

To investigate the subcellular localization of Mc2, CDS without the termination codon and CDS without the kinase domain were amplified from A-Bi using primers (Table S3). The amplified cDNA fragments were independently cloned into the pMD83 vector at KpnI/BamHI site, to generate a C-terminal fusion with GFP under control of CaMV 35S promoter, generating Mc2-GFP and Mc2-1-GFP constructs.

Reverse-transcription PCR and qRTPCR analysis
Total RNA was extracted from various plant tissues using RNeasy® Plant Mini Kit (Qiagen, Chadstone Center, VIC, Australia) supplemented with RNase-free DNase I set to remove contaminating DNA according to the manufacturer’s instructions. First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo, http://www.thermofisher.com/cn/zh/home.html). cDNA was amplified on a CFX96TM Real-time PCR Detection System (Bio-Red, http://bio-rad.com/). Actin2 gene was used as the internal controls for B. juncea and Arabidopsis. Quantitative RT-PCR measurements were obtained using relative quantitative $^{−ΔΔC_{T}}$ method (Livak and Schmittgen, 2001). Data were expressed as the mean of three biological replicates ± SD. The primer sequences were listed in Table S3.

In vitro peptide assay
cSLV3 (PPTRGLSHHPVD, scrambled peptide), CLV3 (RTVPSGPDPLHH) and CLE26 (RKVPGRPGDPHHN) peptides with >95% purity were synthesized by friendsBio Science & Technology (Wuhan) Co., Ltd, Wuhan, China. The gel culture assay as described by Fan et al., (2014), B. juncea seeds were germinated on half MS gel medium overnight after surface-sterilized. Synchronized germinating seeds were selected and transferred to half MS gel medium containing sCLV3 (5 μM) or CLV3, or CLE26 peptides in square plates placed vertically in a growth chamber. After 7 days, root and epicotyl length were measured, and RAM measurements were obtained using Image J (https://imagej.net/). The RAM diameter was defined by measuring the width of the meristem where the first primordia were visible on each side.

Phenotype characterization, histochemical analysis and microscopy
Brassica juncea and Arabidopsis phenotype were photographed with a Nikon digital camera (D750). Brassica juncea gynoecium and Arabidopsis flower images were taken using an Olympus dissection microscope with an Olympus digital camera. Cross sections were performed as described by Fan et al., (2014). Photographs were captured with a Leica DM750 microscope with a Leica digital camera. buds at different developmental stages were collected from A-Bi and A-Tri inflorescence and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7). The fixed samples were dehydrated with a graded ethanol series, dried using a critical point dryer (Leica, https://www.leica-microsys tems.com), sputter-coated with gold (Nanotech SEMPrep II sputter coater) and images were taken under a JSM-6390 scanning electron microscope.

To measure the stem meristem size, the shoot apices of the embryos were excised under a dissecting microscope, cleared in Hoyer’s solution as described by Fan et al., (2014), analysed by differential interference contrast microscopy and measured with Image J. The GUS staining was conducted by incubating various tissues or organs of transgenic plants in a solution containing 0.5 mg/mL X-Gluc at 37 °C overnight followed by washing three times with 70% ethanol (Jefferson et al., 1987). The stained tissues or organs were photographed under a stereomicroscope (Olympus SZX16, https://www.olympus-ims.com/).

Trait measurements
The number of primary branches per plant, number of siliqua per plant and plant height at physiological maturity were recorded in 15 competitive plants per genotype. After complete maturity, siliqua length, siliqua body length and number of seeds per siliqua were recorded in 30 competitive siliques per plant, and 1000-seed weight and seed yield per plant were recorded. The mean values of each genotype were subjected to statistical analysis.

Subcellular localization
Subcellular localization analysis was performed as described previously (Duan et al., 2020). Briefly, A. tumefaciens (GV3101) cells containing the desired constructs were co-infiltrated into four- to five-week-old N. benthamiana leaves using an infiltration buffer (10 mM MES, pH 5.7, 10 mM MgCl2 and 150 μM acetoxy-syringone) to an OD600 = 0.2. The infiltrated plants were left for 2 days. Samples of the infiltrated leaves were collected from the infected area and visualized using a confocal laser scanning microscope (Leica SP8). The excitation wavelengths for GFP and RFP were 488 and 543 nm, respectively.

Acknowledgements
The authors are grateful to Dr. Cilla Luo in New Zealand for critically reading the manuscript. This work was financially supported by the National Natural Science Foundation of China (NSFC, grant No. 31571698), the National Key Research and Development Program of China (grant No. 2016YFD0101300).
and the Program for Modern Agricultural Industrial Technology System of China (grant No. CARS-12).

Conflict of interest
The authors declare no conflict of interest.

Author contributions
JS and GW designed the experiments. GW performed most of the experiments and wrote the manuscript. XZ and PX constructed the mapping population. WH and ZL collected the phenotypic data. LZ, JW, BY, CM, JT and TF supervised this study. JS conceived and supervised the research and writing.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

References
Bommert, P., Lunde, C., Nardmann, J., Vollbrecht, E., Running, M., Jackson, D., Hake, S. et al. (2005) thick tassel dwarf1 encodes a putative maize ortholog of the Arabidopsis CLAVATA1 leucine-rich repeat receptor-like kinase. Development, 132, 1235–1245.
Bowman, J.L., Baum, S.F., Eshed, Y., Putterill, J. and Alvarez, J. (1999) Molecular genetics of gynoecium development in Arabidopsis. Curr. Top. Dev. Biol., 45, 155–205.
Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M. and Simon, R. (2000) Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science, 289, 617–619.
Chen, C., Xiao, L., Li, X. and Du, D. (2018) Comparative mapping combined with map-based cloning of the Brassica juncea genome reveals a candidate gene for multicellular rapeseed. Front. Plant Sci., 9, 1744.
Choudhary, B.R. and Solanki, Z.S. (2007) Inheritance of silique locule number and seed coat colour in Brassica juncea. Plant Breeding, 126, 104–106.
Clark, S.E., Running, M.P. and Meyerowitz, E.M. (1993) CLAVATA1, a regulator of meristem and flower development in Arabidopsis. Development, 119, 397–418.
Clark, S.E., Williams, R.W. and Meyerowitz, E.M. (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell, 89, 575–585.
Clough, S.J. and Bentm, A.F. (1998) Floral dip: a simplified method for CLAVATA3 gene expression and overexpression activity in Arabidopsis. Plant Physiol., 154, 1721–1736.
Kamiuchi, Y., Yamamoto, K., Furutani, M., Tasaka, M. and Aida, M. (2014) The CUC1 and CUC2 genes promote carpel margin meristem formation during Arabidopsis gynoecium development. Front. Plant Sci., 5, 165.
Katiyar, R.K., Chamola, R. and Chopra, V.L. (1998) Tetralocular mustard, Brassica juncea: new promising variability through interspecific hybridization. Plant Breeding, 117, 398–399.
Lenhard, M., Bohnert, A., Jurgens, G. and Lax, T. (2001) Termination of stem cell maintenance in Arabidopsis floral meristems by interactions between WUSCHEL and AGAMOUS. Cell, 105, 805–814.
Liu, L., Gallagher, J., Arevalo, E.D., Chen, R., Skopolitis, T., Wu, Q., Bartlett, M. et al. (2021) Enhancing grain-yield-related traits by CRISPR-Cas9 promoter editing of maize CLE genes. Nat. Plants, 7, 287–294.
Liu, X., Kim, Y.J., Muller, R., Yumul, R.E., Liu, C., Pan, Y., Cao, X. et al. (2011) AGAMOUS terminates floral stem cell maintenance in Arabidopsis by directly repressing WUSCHEL through recruitment of Polycomb Group proteins. Plant Cell, 23, 3654–3670.
Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25, 402–408.
Lohmann, J.U., Hong, R.L., Hobe, M., Busch, M.A., Parcy, F., Simon, R. and Wiegel, D. (2001) A molecular link between stem cell regulation and floral patterning in Arabidopsis. Cell, 105, 793–803.
Long, J.A., Moan, E.J., Medford, J.J. and Barton, M.K. (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. Nature, 379, 66–69.
Lv, Z., Xu, P., Zhang, X., Wen, J., Yi, B., Ma, C., Tu, J. et al. (2012) Primary study on anatomic and genetic analyses of multi-loculus in Brassica juncea. Chinese J. Oil Crop Sci., 34, 461–466.
Ma, X., Zhang, Q., Zhu, Q., Liu, W., Chen, Y., Qiu, R., Wang, B. et al. (2015) A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Mol. Plant, 8, 1274–1284.
Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. Cell, 95, 805–815.
Reyes-Olalde, J.I., Junigaye-Noyo, M.V., Chavez-Montes, R.A., Marsch-Martinez, N. and de Folter, S. (2013) Inside the gynoecium: at the carpel margin. Plant Cell, 25, 644–655.
Rodriguez-Leal, D., Lemmon, Z.H., Man, J., Bartlett, M.F. and Lipman, Z.B. (2017) Engineering quantitative trait variation for crop improvement by genome editing. Cell, 171, 470–480.
Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F.X., Jurgens, G. and Laux, T. (2000) The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell, 100, 635–644.
Scotﬁeld, S., Dewitt, W. and Murray, J.A. (2007) The KNOX gene SHOOT MERISTEMLESS is required for the development of reproductive meristematic tissues in Arabidopsis. Plant J., 50, 767–781.
Smyth, D.R., Bowman, J.L. and Meyerowitz, E.M. (1990) Early flower development in Arabidopsis. Plant Cell, 2, 755–767.
Somssich, M., Je, B.I., Simon, R. and Jackson, D. (2016) CLAVATA-WUSCHEL signaling in the shoot meristem. Development, 143, 3238–3248.
Spinelli, S.V., Martin, A.P., Viola, I.L., Gonzalez, D.H. and Palatnik, J.F. (2011) A mechanistic link between STM and CUC1 during Arabidopsis development. Plant Physiol., 156, 1894–1904.
Stahl, Y., Grabowski, S., Bleckmann, A., Kühnemuth, R., Weidtkamp-Peters, S., Pinto, K., Kirschner, G. et al. (2013) Moderation of Arabidopsis root stenness by CLAVATA1 and ARABIDOPSIS CRINKLY4 receptor kinase complexes. Curr. Biol., 23, 362–371.
Stone, J.M., Trotochaud, A.E., Walker, J.C. and Clark, S.E. (1998) Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. Plant Physiol., 117, 1217–1225.
Suzaki, T., Sato, M., Ashikari, M., Miyoshi, M., Nagato, Y. and Hirano, H.Y. (2016) Lessons from domestication: targeting cis-regulatory elements for crop improvement. Trends Plant Sci., 21, 506–515.
Trostchand, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. Plant Cell, 11, 393–406.
Wang, G., Zhang, X., Xu, P., Lv, Z., Wen, J., Yi, B., Ma, C. et al. (2016) Fine mapping of polycyclic gene (Bjmc2) in Brassica juncea L. Acta Agronomica Sinica, 42, 1735–1742.
Wang, X., Aguirre, L., Rodríguez-Leal, D., Hendelman, A., Benoît, M. and Lippman, Z.B. (2021) Dissecting cis-regulatory control of quantitative trait variation in a plant stem cell circuit. Nat. Plants, 7, 419–427.
Xiao, L., Li, X., Liu, F., Zhao, Z., Xu, L., Chen, C., Wang, Y. et al. (2018) Mutations in the CDS and promoter of BjA07.CLV1 cause a multilocular trait in Brassica juncea. Sci. Rep., 8, 5339.
Xiao, L., Zhao, H., Zhao, Z., Du, D., Xu, L., Yao, Y., Zhao, Z. et al. (2013) Genetic and physical fine mapping of a multilocular gene Bjh1 in Brassica juncea to a 208-kb region. Mol. Breeding, 32, 373–383.
Xu, C., Liberatore, K.L., MacAlister, C.A., Huang, Z., Chu, Y.-H., Jiang, K.-E., Brooks, C. et al. (2015) A cascade of arabinosyltransferases controls shoot meristem size in tomato. Nat. Genet., 47, 784–792.
Xu, P., Cao, S., Hu, K., Wang, X., Huang, W., Wang, G., Lv, Z. et al. (2017) Trilocular phenotype in Brassica juncea L. resulted from interruption of CLAVATA1 gene homologue (BjMc1) transcription. Sci. Rep., 7, 4398.
Yadav, R.K., Perales, M., Gruel, J., Girke, T., Jonsson, H. and Reddy, G.V. (2011) WUSCHEL protein movement mediates stem cell homeostasis in the Arabidopsis shoot apex. Genes Dev., 25, 2025–2030.
Yadava, S.K., Pantosh, K., Panjabi-Massad, P., Gupta, V., Chandra, A., Sodhi, Y.S., Pradhan, A.K. et al. (2014) Tetralocular ovary and high silique width in yellow sarson lines of Brassica rapa (subspecies trilocularis) are due to a mutation in Bra034340 gene, a homologue of CLAVATA3 in Arabidopsis. Theor. Appl. Genet., 127, 2359–2369.
Yang, J., Liu, D., Wang, X., Ji, C., Cheng, F., Liu, B., Hu, Z. et al. (2016) The genome sequence of allopolyploid Brassica juncea and analysis of differential homoeolog gene expression influencing selection. Nat. Genet., 48, 1225–1232.
Yi, B., Li, H., Hu, L., Fan, C. and Zhou, Y. (2021) Genetic analysis and molecular characterization of multilocular trait in the srb mutant of Brassica rapa L. Acta Agronomica Sinica, 47, 385–393.
Yi, B., Zhu, K., Li, H., Han, S., Meng, Q., Khan, S.U., Fan, C. et al. (2018) Precise editing of CLAVATA genes in Brassica napus L. regulates multilocular silique development. Plant Biotechnol. J., 16, 1322–1335.
Zhou, H. (2014) Fine mapping of multilocular gene Bjh1 in Brassica juncea L. MS thesis, Qinghai University, Wuhan, China.
Zhuo, H., Du, D., Liu, Q., Li, X., Yu, Q. and Fu, Z. (2003) Performance in main characteristics of multilocular Brassica juncea. Acta Agric. Boreali-occident. Sinica, 12, 62–64.

Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Shoot apical meristems and the floral phenotype of bilocular and trilocular plants in A-Bi and A-Tri lines.
Figure S2 Average numbers of Brassica juncea floral organs in indicated genotypes.
Figure S3 Transgenic plants (T1) obtained in the overexpression and complementation experiment and quantification of carpel number in indicated genotypes of Brassica juncea (T1).
Figure S4 Complementation test of Mc1 and Mc2 in Arabidopsis.
Figure S5 Activity differences in the normal and mutant promoter of Mc1 and Mc2 gene during reproductive growth.
Figure S6 Schematic representation of the constructs that expressed Mc2, driven by ten truncated promoters.
Figure S7 Subcellular localization of Mc2.

Table S1 Gene annotations in the Mc2 candidate region.
Table S2 Quantification of carpel number in the indicated genotypes of Arabidopsis.
Table S3 Primers used in this study.
Table S4 The receptor plants used in the constructed plasmids transformation.