Knockout of two *BnaMAX1* homologs by CRISPR/Cas9-targeted mutagenesis improves plant architecture and increases yield in rapeseed (*Brassica napus* L.)

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Summary

Plant height and branch number are essential components of rapeseed plant architecture and are directly correlated with its yield. Presently, improvement of plant architecture is a major challenge in rapeseed breeding. In this study, we first verified that the two rapeseed *BnaMAX1* genes had redundant functions resembling those of Arabidopsis *MAX1*, which regulates plant height and axillary bud outgrowth. Therefore, we designed two sgRNAs to edit these *BnaMAX1* homologs using the CRISPR/Cas9 system. The T_0 plants were edited very efficiently (56.30%–67.38%) at the *BnaMAX1* target sites resulting in homozygous, heterozygous, bi-allelic and chimeric mutations. Transmission tests revealed that the mutations were passed on to the T_1 and T_2 progeny. We also obtained transgene-free lines created by the CRISPR/Cas9 editing, and no mutations were detected in potential off-target sites. Notably, simultaneous knockout of all four *BnaMAX1* alleles resulted in semi-dwarf and increased branching phenotypes with more siliques, contributing to increased yield per plant relative to wild type. Therefore, these semi-dwarf and increased branching characteristics have the potential to help construct a rapeseed ideotype. Significantly, the editing resources obtained in our study provide desirable germplasm for further breeding of high yield in rapeseed.

Introduction

Plant architecture depends greatly on the fates of the axillary meristems, and their regulation by hormonal and environmental factors (Schmitz and Theres, 2005; Sussex and Kerk, 2001). Rapeseed (*Brassica napus* L., AACC, $2n = 38$) is a crucial source of edible oil and biofuel, and its branch number is a major factor affecting its architecture and yield. Plant breeders have been seeking the rapeseed 'ideotype' as it directly affects crop yield.

In the vegetative stage, axillary meristems initiate in leaf axils to form axillary buds, which subsequently remain dormant or continue to grow to form branches (Bennett and Leyser, 2006). Levels of auxin and cytokinin hormones have been thought to be the main factors that regulate bud outgrowth for many decades. Auxin synthesized in the primary shoot apex and then transported to the basal regions (basipetally) indirectly inhibits branching, which is termed apical dominance (Ljung *et al.*, 2001; Thimann and Skoog, 1933). In contrast, cytokinin is produced in the root and transported up to the axillary buds promoting their growth (Horvath *et al.*, 2003). Auxin probably partially reduces cytokinin export from roots and synthesis at the node, thus inhibiting bud growth (Chatfield *et al.*, 2000; Nordstrom *et al.*, 2004). Unexpectedly, a study revealed that auxin formed at the apex does not enter the buds, suggesting that another signal is involved in inhibition of bud growth (Morris, 1977).

Studies of several branching mutants, including *ramosus* (*rms*) in pea, *decreased apical dominance* (*dad*) in petunia, *more axillary growth* (*max*) in Arabidopsis and *dwarf mutants* (*d mutants*) in rice,

revealed that strigolactones (SLs) played a negative role in bud outgrowth and that this function was highly conserved in both monocots and dicots (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). The *MAX3/RMS5/DAD3/D17* and *MAX4/RMS1/DAD1/D10* genes encode carotenoid cleavage dioxygenases 7 (CCD7) and CCD8, respectively, and *MAX1* encodes a cytochrome P450 monooxygenase (CYP711A1) that acts downstream of *MAX3* and *MAX4*. All of these enzymes are involved in SL biosynthesis (Alder *et al.*, 2012; Booker *et al.*, 2005; Lazar and Goodman, 2006). *MAX1* converts carlactone to carlactonoic acid, and *max1* mutants display reduced stature with increased branching and rounder rosette leaves. In contrast, overexpression of *MAX1* represses bud outgrowth at the stem base (Abe *et al.*, 2014; Booker *et al.*, 2005; Lazar and Goodman, 2006). The *MAX2/RMS4/D3* and *DAD2/D14* genes encode an F-box protein and α/β-hydrolase, respectively, likely participating in SL signal transduction (Arite *et al.*, 2009; Stirnberg *et al.*, 2007). Recently, the identification of the SL receptor *SMAX1-LIKE/D53* gene in Arabidopsis and rice established an SL biosynthesis–signalling pathway in these plants (Jiang *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015; Zhou *et al.*, 2013). However, an SL biosynthesis–signalling pathway has not been identified in rapeseed.

Rapeseed is a relatively recent allopolyploid originating from the hybridization of *B. rapa* ($2n = 20$, AA) with *B. oleracea* ($2n = 18$, CC), and most orthologous genes are duplicated and functionally redundant (Chalhoub *et al.*, 2014). Consequently, characterization of a single gene is complicated, so simultaneous mutation of all members of a multi-copy gene family is preferable for analysis of

gene function. Recently, the CRISPR/CRISPR-associated 9 (CRISPR/Cas9) gene editing system which can effectively and precisely edit target sites in genomes of all organisms was created (Cong *et al.*, 2013; Gaj *et al.*, 2013; Mali *et al.*, 2013). This system has been successfully used in many plant species (Li *et al.*, 2013; Shan *et al.*, 2013). Moreover, CRISPR/Cas9-mediated gene editing has been performed in wheat, potato and cotton, showing that CRISPR/Cas9 can be used to edit multi-gene families in polyploid crops (Andersson *et al.*, 2017; Wang *et al.*, 2014, 2018). To date, several studies have reported using CRISPR/Cas9 for targeted mutagenesis in rapeseed to improve shattering resistance, seed production, plant architecture and disease resistance, respectively (Braatz *et al.*, 2017; Li *et al.*, 2018; Sun *et al.*, 2018; Yang *et al.*, 2017; Zhai *et al.*, 2019).

In this study, we designed sgRNAs to edit the two rapeseed homologs (*BnaMAX1*) of the Arabidopsis *MAX1* gene using the CRISPR/Cas9 system. We obtained plants that contained edited versions of both *BnaMAX1* genes and were free of T-DNA insertions in a spring-type rapeseed variety. This is the first report of creating semi-dwarf and increased branching germplasm resources in rapeseed using CRISPR/Cas9 technology and provides a useful germplasm for improving rapeseed plant architecture and yield.

Results and discussion

The *19-22* mutant is Arabidopsis *max1*

To investigate the molecular mechanisms controlling plant architecture in rapeseed, we first screened for branching mutants in an Arabidopsis T-DNA library. We obtained a line (Salk_036990, which we designated *19-22*) that displayed dwarfism with increased branching and rounder rosette leaves (Figure 1a–c). Using Tail-PCR amplification, we identified one putative insertion site, and linkage analysis showed that this insertion was not responsible for the phenotype of *19-22* (Figure S1). Further, an F_2 segregant from a cross with Col was used to show via genetic experiments that *19-22* was inherited as a single nuclear recessive mutation (Figure S2a; Table S1). MutMap showed that a 20-bp deletion in *At2g26170* (*MAX1*) was probably related to the dwarfism and increased branching phenotypes (Table S2). Linkage and sequence analyses confirmed that this deletion was present in *19-22* and the F_2 plants expressing the recessive phenotype (Figure S2b). The 20-bp deletion caused a frameshift and premature stop in the first exon of *MAX1*. This represented a novel allele compared with previous studies, and the phenotype of *19-22* resembled that of the Arabidopsis *max1-3* mutant (Figure 1; Lazar and Goodman, 2006).

The rapeseed genome contains two genes homologous to Arabidopsis *MAX1*

A BLASTP search identified two close homologs of *MAX1* in the rapeseed genome (*BnaA03g22900D* and *BnaC03g26960D*). However, *BnaA03g22900D* only has a partial coding sequence (CDS) in the 'Darmor-bzh' database, and therefore, we obtained the full-length CDS sequence (*BnA03 g0116320.1*) from the 'ZS11' database (Sun *et al.*, 2017). We named these *MAX1* homologs, *BnaA03.MAX1* and *BnaC03.MAX1*. *BnaA03.MAX1* shared 88.9% identity and 93.8% similarity with *MAX1* at the amino acid level, and *BnaC03.MAX1* shared 88.5% and 93.2% similarity with *MAX1* at these levels (Figure 2a). These results suggested that the *BnaMAX1* homologs might have similar functions as *MAX1*. In addition, the two *BnaMAX1* homologs

shared 98.5% identity and 99.4% similarity in their amino acid sequences (Figure 2a, b). Genomic-synteny analysis showed that the two *BnaMAX1s* originated from ancestors *B. rapa* and *B. oleracea*, respectively (Chalhoub *et al.*, 2014). Therefore, the two *BnaMAX1s* probably have redundant functions in regulating axillary bud outgrowth.

The SLs are thought to be synthesized in roots and transported to shoots through the xylem to inhibit bud outgrowth (Beveridge, 2006; Kohlen *et al.*, 2011). Our qRT-PCR analyses showed that the *BnaMAX1* transcripts were more abundant in axillary buds, and both were more highly expressed in roots, as was reported for Arabidopsis *MAX1* (Figure 2c, Figure S3a), suggesting that both have roles in SL biosynthesis.

BnaMAX1s have similar functions in regulating plant architecture as Arabidopsis *MAX1*

To further confirm the functions of *BnaMAX1s* in SL signalling, we constructed vectors expressing *BnaA03.MAX1*, *BnaC03.-MAX1* and *MAX1* controlled by the 35S promoter and transformed them into the Arabidopsis *19-22* mutant. All transgenic lines that expressed the three genes exhibited normal Col-0 leaf phenotypes at the seedling stage (Figure 3a, Figure S3b). Similarly, adult transgenic plants also showed normal plant architecture, including increased plant height (PH) and decreased branch number (BN) relative to the *19-22* mutant (Figure 3b). Previous studies reported that *MAX1* encodes a member of the CYP450 family, CYP711A1, which is a carlactone oxidase that catalyses the last step of SL biosynthesis (Booker *et al.*, 2005). Therefore, the two homologs of *BnaMAX1* could have a similar function in SL biosynthesis and repressing the vegetative growth of axillary buds in rapeseed.

Sequence identification for editing *BnaMAX1* homologs by CRISPR/Cas9 in rapeseed

In the light of the functional analysis of the two *BnaMAX1s* in Arabidopsis, we realized that we needed to simultaneously knock out both homologous genes in order to produce the expected phenotypes in rapeseed. We chose the BGK01 vector (Biogle, China) to generate the *Cas9-BnaMAX1* construct, which uses the AtU6-26 promoter to drive sgRNA expression (Figure 4a). We next designed two sgRNAs to target the first exons of the *BnaMAX1s* that each had the same target site sequences in their homologous region (Figure 4b). In addition, we analysed the likely off-target sites in the rapeseed genome using CRISPR-P (http://crispr.hzau.edu.cn/CRISPR/). The results showed that the two sgRNA sequences had several putative off-target sites in the rapeseed genome, but all had more than three mismatches compared with the original target sites (Figure S4). This indicated that the two target sites were good choices for specific mutagenesis without off-target effects.

The first target sequence was CCGTTCTTGCCAAGCAC-TATGGC (reversed, GCCATAGTGCTTGGCAAGAACGG), with a G/C SNP located 6-bp upstream of the CGG PAM motif in *BnaA03/C03.MAX1*, which was used to distinguish the homologous copies by sequencing transgenic plants (Figure 4b). After *Agrobacterium tumefaciens*-mediated transformation of variety '862' (a spring-type rapeseed) hypocotyls, we obtained 4735 calli and 141 positive transgenic plants (141 out of 150 plants) displaying hygromycin (Hyg) resistance. Most transgenic plants had different changes in the sequences of the target sites in the four *BnaMAX1* alleles (Figures 4c; S5). In order to distinguish the four *BnaMAX1* alleles, we named them a_1, a_2, c_1 and c_2,
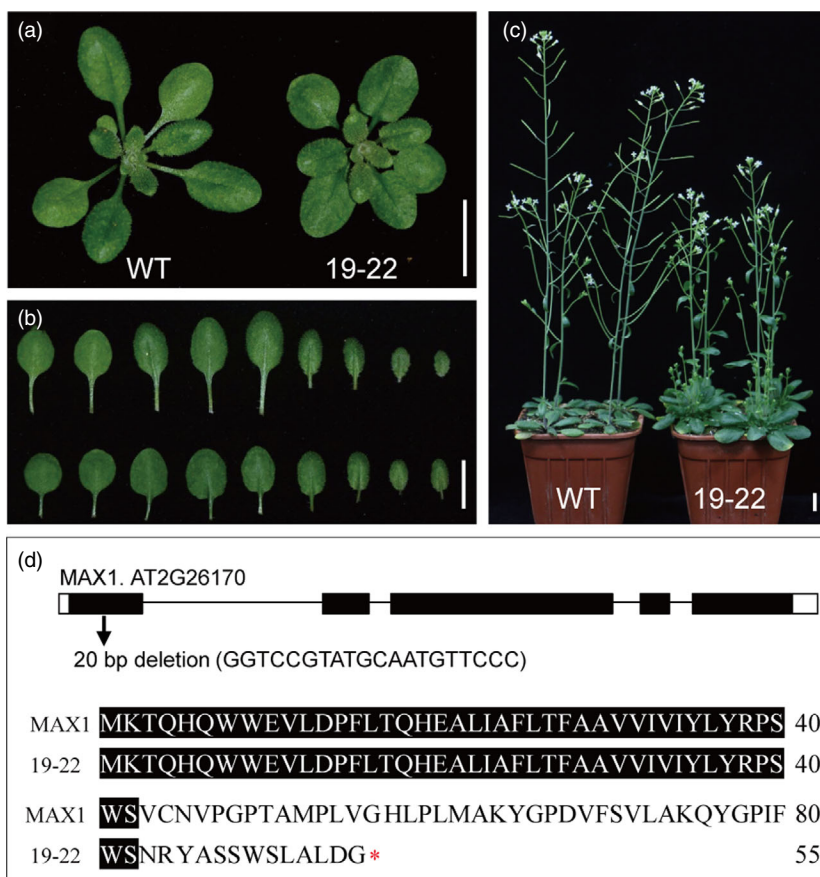
Figure 1 Phenotypes of Arabidopsis wild-type (WT) and *19-22* mutant, and alignment of MAX1 protein with its mutations. (a–b) Comparison of WT and *19-22* mutant vegetative rosettes. The *19-22* shows rounder leaves. Top is WT and bottom is *19-22* in (b). (c) *19-22* mutant showing dwarfism with increased branching phenotype at the flowering stage. (d) Structures of *MAX1* and a 20-bp nucleotide deletion in the first exon that caused a frameshift and premature stop in 19-22. Lines indicate introns, black boxes indicate exons, and white boxes indicate 5′- or 3′-UTR. Bars = 1 cm.

respectively. We observed that all four *BnaMAX1* alleles simultaneously had mutations in 29 T_0 lines (Table S4). 9.22%–12.06% of all the loci examined were bi-allelic mutations, 17.02%–18.44% of the loci were homozygous mutations, and 24.82%–29.79% and 7.80%–9.93% of the loci were heterozygous and chimeric mutations in the A/C genomes, respectively (Figure 4d; Table S4). Notably, in single nucleotide insertion events, 98.78%–99.07% of the loci were insertions of a 'T' (Figure 4e), while 57.89%–58.97% of deletions were less than 3 bp (Figure 4f).

The second target sequence was CCACTTGCCCATGCTGGC-TAAGT (reversed, ACTTAGCCAGCATGGGCAAGTGG). We changed the first A to G in this sgRNA sequence to suit the AtU6-26 promoter. A G/C SNP was located 31-bp downstream of the TGG PAM motif in *BnaA03/C03.MAX1* to help identify gene-edited progeny (Figure 4b). We transformed this construct into hypocotyls of variety '862' and obtained 3382 calli and 119 positive transgenic plants (119 out of 123 plants) showing Hyg resistance. After sequencing the PCR amplicons of the two *BnaMAX1* genomic DNAs, we found bi-allelic mutations in both AC genomes in 7 lines (Figure 4d; Table S4). Similarly, 28.57%–41.18% (34-49/119) of all loci examined were single heterozygous alleles in the A/C genomes, while the homozygous mutation rate was much lower than that of the sgRNA-1 edited sites, ranging from 3.36% to 5.04% (Figure 4d; Table S4). Notably, in single nucleotide insertion events, 62.30%–74.24% of the loci were G insertions, whereas T insertions occurred in 19.70%–34.43% of the loci (Figure 4e). As expected, most mutations were adjacent to the PAM sequence in the plants edited with our

two sgRNAs. However, three T_0 lines contained large deletions (85–165 bp) that lost the entire sgRNA target sites (Figure S5; Table S4). Finally, most of these indels resulted in frameshifts, premature stop codons or amino acid deletions in the first or third exons of the targeted *BnaMAX1s*.

Previous studies in rapeseed using the CRISPR/Cas9 system selected 20-nt sequences 5′ to the PAM as target sequences (Braatz *et al.*, 2017; Yang *et al.*, 2017). In our study, we selected 19-nt targeting sequences and achieved high editing efficiencies. This shows that 19 nt target sequences are also suitable for CRISPR/Cas9 editing in rapeseed, that is the target sequence may be loosened to 18–20 nt (Shan *et al.*, 2013). The sgRNAs targeting-specific sequences were transcribed by U3 or U6 promoters that initiated with A or G, respectively (Cong *et al.*, 2013; Shan *et al.*, 2013). It was reported that an additional A or G at the 5′ end of sgRNAs that started with a T or C also resulted in genome editing in rice (Ma *et al.*, 2015; Xie and Yang, 2013). In our sgRNA-2 sequence, the first nucleotide A was an initiation site for U3 promoter transcription, and in subsequent constructs, we changed this A to G to suit the U6 promoter. Intriguingly, the positive transgenic plants expressing sgRNA-2 also showed high editing levels (56.30%–63.87%, Figure 4e), showing that replacing the transcription start nucleotide to suit the relative promoter functioned in a rapeseed CRISPR/Cas9 system.

1-bp insertions are typical CRISPR/Cas9 mutations and were found at high rates in both sgRNA-1 and sgRNA-2 lines (17.65%–21.05%; Figure 4f). Previous studies showed that single insertions were observed in the 3–4 bp adjacent to the PAM (Cong *et al.*, 2013; Lawrenson *et al.*, 2015). Gs were inserted 4 bp
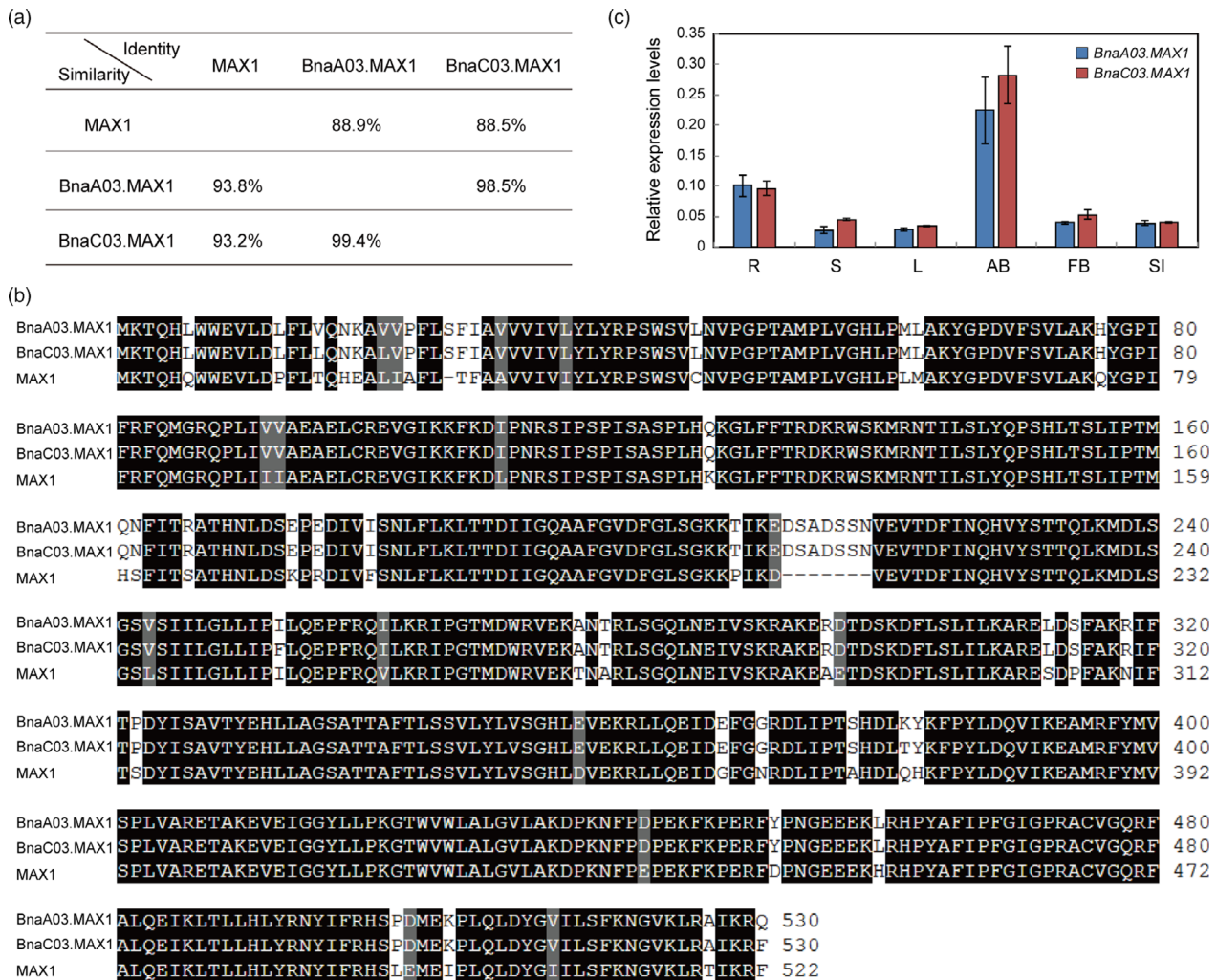
(a)

Identity / Similarity	MAX1	BnaA03.MAX1	BnaC03.MAX1
MAX1		88.9%	88.5%
BnaA03.MAX1	93.8%		98.5%
BnaC03.MAX1	93.2%	99.4%	

(c)

(b)

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BnaA03.MAX1 MKTQHLWWEVLDLFLVQNKAVVPFLSFIAVVVIVLYLYRPSWSVLNVPGPTAMPLVGHLPMLAKYGPDVFSVLAKHYGPI  80
BnaC03.MAX1 MKTQHLWWEVLDLFLLQNKALVPFLSFIAVVVIVLYLYRPSWSVLNVPGPTAMPLVGHLPMLAKYGPDVFSVLAKHYGPI  80
MAX1        MKTQHQWWEVLDPFLTQHEALIAFL-TFAAVVIVIYLYRPSWSVCNVPGPTAMPLVGHLPLMAKYGPDVFSVLAKQYGPI  79

BnaA03.MAX1 FRFQMGRQPLIVVAEAELCREVGIKKFKDIPNRSIPSPISASPLHQKGLFFTRDKRWSKMRNTILSLYQPSHLTSLIPTM 160
BnaC03.MAX1 FRFQMGRQPLIVVAEAELCREVGIKKFKDIPNRSIPSPISASPLHXKGLFFTRDKRWSKMRNTILSLYQPSHLTSLIPTM 160
MAX1        FRFQMGRQPLIIIAEAELCREVGIKKFKDLPNRSIPSPISASPLHKKGLFFTRDKRWSKMRNTILSLYQPSHLTSLIPTM 159

BnaA03.MAX1 QNFITRATHNLDSEPEDIVISNLFLKLTTDIIGQAAFGVDFGLSGKKTIKEDSADSSNVEVTDFINQHVYSTTQLKMDLS 240
BnaC03.MAX1 QNFITRATHNLDSEPEDIVISNLFLKLTTDIIGQAAFGVDFGLSGKKTIKEDSADSSNVEVTDFINQHVYSTTQLKMDLS 240
MAX1        HSFITSATHNLDSKPRDIVFSNLFLKLTTDIIGQAAFGVDFGLSGKKPIKD-------VEVTDFINQHVYSTTQLKMDLS 232

BnaA03.MAX1 GSVSIILGLLIPILQEPFRQILKRIPGTMDWRVEKANTRLSGQLNEIVSKRAKERDTDSKDFLSLILKARELDSFAKRIF 320
BnaC03.MAX1 GSVSIILGLLIPFLQEPFRQILKRIPGTMDWRVEKANTRLSGQLNEIVSKRAKERDTDSKDFLSLILKARELDSFAKRIF 320
MAX1        GSLSIILGLLIPILQEPFRQVLKRIPGTMDWRVEKTNARLSGQLNEIVSKRAKEAETDSKDFLSLILKARESDPFAKNIF 312

BnaA03.MAX1 TPDYISAVTYEHLLAGSATTAFTLSSVLYLVSGHLEVEKRLLQEIDEFGGRDLIPTSHDLKYKFPYLDQVIKEAMRFYMV 400
BnaC03.MAX1 TPDYISAVTYEHLLAGSATTAFTLSSVLYLVSGHLEVEKRLLQEIDEFGGRDLIPTSHDLTYKFPYLDQVIKEAMRFYMV 400
MAX1        TSDYISAVTYEHLLAGSATTAFTLSSVLYLVSGHLDVEKRLLQEIDGFGNRDLIPTAHDLQHKFPYLDQVIKEAMRFYMV 392

BnaA03.MAX1 SPLVARETAKEVEIGGYLLPKGTWVWLALGVLAKDPKNFPDPEKFKPERFYPNGEEEKLRHPYAFIPFGIGPRACVGQRF 480
BnaC03.MAX1 SPLVARETAKEVEIGGYLLPKGTWVWLALGVLAKDPKNFPDPEKFKPERFYPNGEEEKLRHPYAFIPFGIGPRACVGQRF 480
MAX1        SPLVARETAKEVEIGGYLLPKGTWVWLALGVLAKDPKNFPEPEKFKPERFDPNGEEEKHRHPYAFIPFGIGPRACVGQRF 472

BnaA03.MAX1 ALQEIKLTLLHLYRNYIFRHSPDMEKPLQLDYGVILSFKNGVKLRAIKRQ 530
BnaC03.MAX1 ALQEIKLTLLHLYRNYIFRHSPDMEKPLQLDYGVILSFKNGVKLRAIKRF 530
MAX1        ALQEIKLTLLHLYRNYIFRHSLEMEIPLQLDYGIILSFKNGVKLRTIKRF 522
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Figure 2 The rapeseed genome contains two *BnaMAX1* homologs. (a) Amino acid identities and similarities of MAX1 proteins in Arabidopsis and rapeseed. (b) Alignment of MAX1 and BnaMAX1s amino acid sequences. Black shading indicates identical or similar residues, and numbers indicate amino acid positions. (c) qRT-PCR analysis of the expression of *BnaMAX1*s in diverse tissues. Error bars $\pm$ standard deviations ($n = 3$). R, root; S, stem; L, leaf; AB, axillary bud; FB, flower bud; SI, silique.

adjacent to the PAM in sgRNA-2 lines, while Ts were inserted 6 bp adjacent to the PAM in sgRNA-1 lines, showing that insertions occurred 3–6 bp upstream of the PAM. Previous studies reported that most insertions were As or Ts (Ma *et al.*, 2015; Zhang *et al.*, 2014), whereas most insertions in our sgRNA-2 plants were Gs. Thus, the base inserted by this type of mutation might be random. In addition, we found that 1-bp deletions occurred at the lowest rates at both sgRNA target sites, in contrast to previous reports. A possible explanation for these findings is that they were caused by microhomology-mediated repair (Sanchez-Leon *et al.*, 2018; Yang *et al.*, 2017). Homozygous mutations occurred at a high rate in the two targeted sites (3.36%–18.44%) in T$_0$ rapeseed plants and occurred simultaneously in both A and C homologs with the same mutation type. It is likely that one chromosome was edited by the Cas9/sgRNA complex, and another homologous chromosome or sub-homologous chromosome was cleaved by the Cas9/sgRNA complex and repaired using the originally mutated allele as template (Ma *et al.*, 2015). Therefore, both non-homologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms might have occurred simultaneously in our edited plants.

Isolation of edited rapeseed *BnaMAX1* lines that eliminated the T-DNA

To assess the inheritance of the CRISPR/Cas9-induced mutations and obtain stably inherited lines containing mutations in both *BnaMAX1* genes that also eliminated the T-DNA, we self-pollinated four T$_0$ lines (S1-5, S1-8, S1-11 and S2-5, which had diverse types of mutations). Real-time PCR using a Hyg probe in S1-8 T$_1$ segregants showed that the T-DNA insertion site was not linked with the plant phenotypes (Table S3). We then screened five independent S1-8 T$_1$ lines (S1-8-8/12/23/36/39) and collected each of their T$_2$ progenies. A quarter of S1-8-23 or S1-8-39 T$_2$ plants lacking the hygromycin resistance gene, and eight T$_2$ progeny of S1-8-8 were homozygous plants contained T-DNA that corresponded to their T$_1$ parents (Table 1). Notably, in S1-8 T$_1$ segregants, if one *BnaMAX1* allele was normal the plant resembled wild type (Figure S6; Table S3), whereas simultaneous knockout of all four *BnaMAX1* alleles resulted in semi-dwarf and increased branching phenotypes in several T$_0$ plants (e.g. S1-11/-12/-59 and S2-5/-41/-47), indicating that the two *BnaMAX1* homologs had functional redundancy.
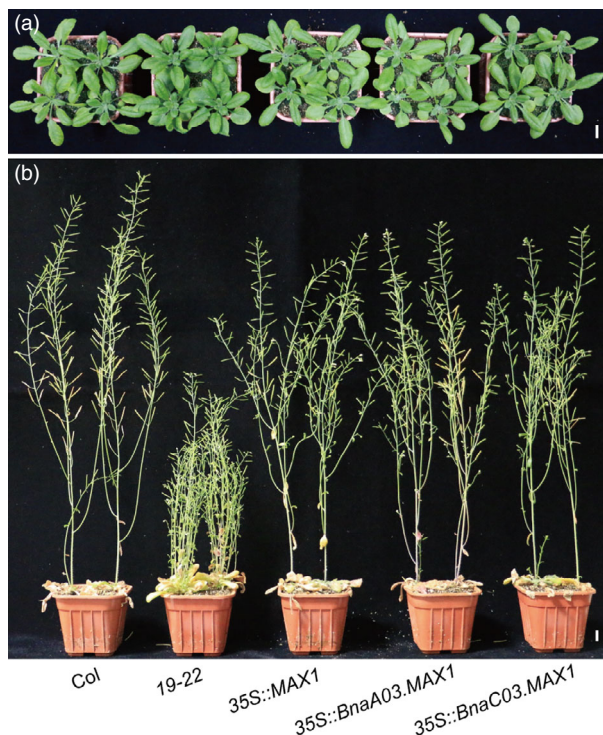
Figure 3 Morphological comparison of wild-type (WT), *19-22* and transgenic Arabidopsis plants overexpressing *MAX1* or *BnaMAX1*s in *19-22* mutant. (a–b) WT, *19-22* and transgenic plants at vegetative (a) and mature (b) stages. Bars = 1 cm.

To detect the inheritance of bi-allelic mutations, we sequenced S1-11 and S2-5 T_1 and T_2 plants. We found that the mutations identified in S1-11 T_2 plants matched those observed in the corresponding S1-11 T_0 and T_1 lines. We also identified homozygous or bi-allelic individuals which did not contain T-DNA in the T_2 progeny (Figure 5a; Table 1). Interestingly, a new mutation (+TT) was found in two T_2 plants (S2-5-14-6 and S2-5-14-16 of S2-5-14), even though it was not detected in S2-5 T_0 and T_1 lines. This showed that the sgRNA-2 site on chromosome A was further edited during the growth of the transgenic plants, even though it has one mismatch with the sgRNA (Figure 5b; Table 1).

As expected, the plants with homozygous or bi-allelic mutations resulting in premature stop codons in the two *BnaMAX1* genes displayed similar mutant phenotypes. In the S1-5 line, insertion of a T led to premature stop codons in *BnaA03.MAX1* on the A chromosome and an amino acid deletion in *BnaC03.MAX1* on the C chromosome. However, homozygous mutant plants displayed normal phenotypes similar to the wild type, suggesting that the single L_{73} amino acid deletion in *BnaC03.MAX1* did not affect its protein function (Figure S7). We also obtained non-transgenic plants with semi-dwarf and increased branching phenotypes in S1-8/S1-11/S2-5 segregating populations, suggesting that the Cas9-induced mutations were stably transmitted to T_2 rapeseed plants independently of the T-DNA construct, and the mutations in *BnaMAX1s* were heritable (Figure S8; Table 1).

Searching for putative off-target activities

To determine whether the increased branching phenotype resulted from mutations of off-target sites in the edited lines, we searched for off-target effects in our edited plants. We

therefore designed PCR primers to detect the possible off-target sites predicted by CRISPR-P (9 annotated genes for sgRNA-1 and only one for sgRNA-2). After sequencing all PCR products of the putative off-target genes, no mutations were found in S1-8 for sgRNA-1 as well as in S2-5 for sgRNA-2. We have also analysed 36 additional independent T_0 plants in both sgRNAs, and no off-target events were found (Figure S9). These results demonstrated that the two sgRNAs had a high specificity for targeting the *BnaMAX1* genes in rapeseed, and such high specificity ($\geq$3 mismatches in sgRNA) can avoid off-targeting (Tang *et al.*, 2018; Yang *et al.*, 2017). In mammals, a single nucleotide mismatch in the 5′ half of an sgRNA sequence showed a high ratio of off-site targeting, whereas up to five nucleotide mismatches also had low off-target scores (Doench *et al.*, 2014; Fu *et al.*, 2013; Hsu *et al.*, 2013). In *Brassica oleracea*, four-nucleotide mismatches in the sgRNA showed no off-target effects on the homologs of *BolC.GA4.a* (*BolC.GA4.b*), while two mismatches in sgRNA on *BolC.GA4.b* resulted in off-target events (Lawrenson *et al.*, 2015). These results suggest that sgRNA with two mismatches or less can simultaneously target multi-copy genes in plants.

Knockout of two *BnaMAX1* homologs can significantly decrease plant height and increase branch number, silique number and yield

We next chose T-DNA-free homozygous lines in S1-5 (S1-5-6-8), S1-8 (S1-8-39-1) and S1-11 (S1-11-7-3) for subsequent phenotypic characterization. Five weeks after we sowed seeds in the greenhouse, the WT and all three mutant lines entered the bolting stage. S1-8 and S1-11 displayed different phenotypes than WT and S1-5, with more buds at the stem base. Remarkably, all of the axillary buds were outgrowths in S1-8 and S1-11 plants, but these were partially repressed at the bases of leaf petioles of WT and S1-5 plants (Figure 6a–d). At the mature stage, S1-5 displayed similar architecture as WT, including PH, BN and yield-related factors. In contrast, the S1-8 and S1-11 lines displayed dramatic semi-dwarf phenotypes. Height was about 31.9%–36.5% shorter than WT (Figure 6e–i); the total primary branch numbers (axillary and base part) increased from 3 (WT) to 9 (Figure 6i); and the total silique numbers (SN) increased about 62.3%–71.8% relative to WT (Figure 6i). Finally, S1-8 and S1-11 had increased yields per plant of about 29.9%–31.3% compared with WT (Figure 6i). We also assessed the yield-related traits of the two T-DNA-free lines (S1-8 and S1-11) in the field. Both lines exhibited decreased PH and increased BN and SN as well as yield per plant compared with WT (Figure S10; Table S5).

The 'Green revolution gene' was successfully adopted in cereal crops in the late 1960s (Peng *et al.*, 1999; Evenson and Gollin, 2003). Presently, rice breeding has moved from 'breeding for dwarfs' and 'using hybrid heterosis' into 'super rice' to obtain greater yield. It was reported that selecting for reasonable PH is an effective method for breeding rapeseed for high yield (Fu and Zhou, 2013). Although dwarf types are adapted to mechanical harvesting, this is sometimes accompanied by decreased biomass (Salas Fernandez *et al.*, 2009). Decreasing PH and increasing BN would benefit the stabilization of biomass. However, the lack of special semi-dwarf germplasm resources and their biomass constraints has limited rapeseed breeding. So far, increased branching germplasms have not been identified in rapeseed. In a previous study, Cardoso *et al*. (2014) found that *Bala* (a rice *indica* cultivar) displayed more tillers than *Azucena* (a *japonica* cultivar) due to lack of two *MAX1* homologs in the *indica* genome, indicating that abolishing *MAX1* could benefit
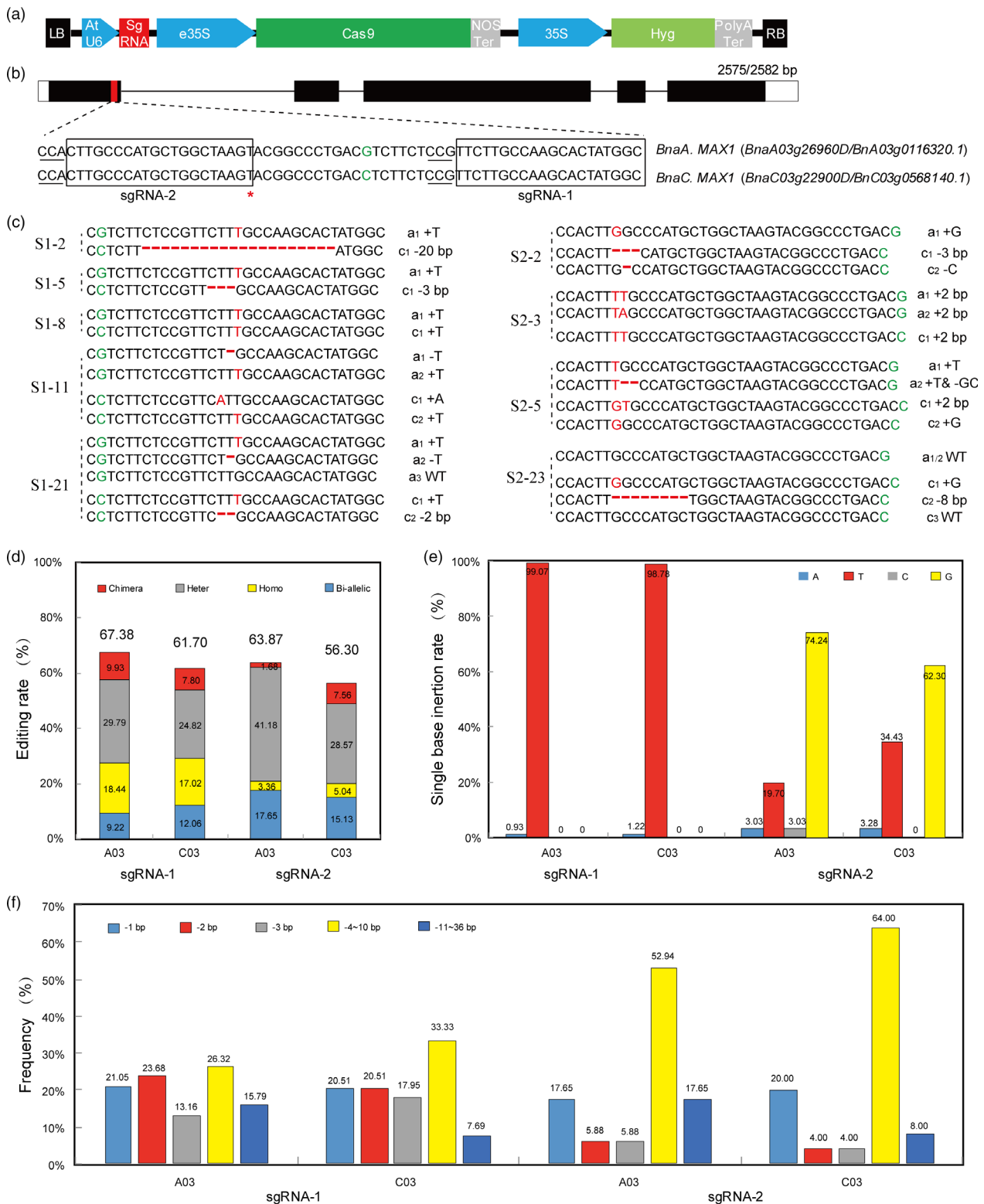
Figure 4 Characterization of editing of *BnaMAX1* genes using the CRISPR/Cas9 system in rapeseed. (a) Structures of the BGK01–CRISPR/Cas9 binary vectors. (b) CRISPR/Cas9 sgRNA-1 targets the first exon of *BnaMAX1*s. The green colours indicate the SNP upstream sgRNA-1 target. The protospacer adjacent motif (PAM) is underlined. The box indicates the target sequences. (c) Sequencing of the *BnaMAX1* sites targeted by two sgRNAs. Red colours and red hyphens indicate insertions and deletions, respectively. a_1, a_2, c_1 and c_2 indicate the four *BnaMAX1* alleles, respectively. (d) Editing efficiencies of the two sgRNAs in rapeseed T_0 plants. (e) The single nucleotide types and insertion rates in gene editing guided by sgRNAs. (f) Deletion frequencies at the two targeted sites.

Table 1 Genetic analysis of CRISPR/Cas9-induced mutations in *BnaMAX1s* and their transmission to the T_1 and T_2 generations.

T_0 plants line	T_1 plants				T_2 plants	
	No. of tested plants	Line	Chr. A03	Chr. C03	No. of homozygous plants	Plants without T-DNA
S1-5	6	S1-5-1	+ T/WT (He)	− CTT (Ho)	10/24	None
a_1 + T		S1-5-4	+ T (Ho)	− CTT (Ho)	8/8	None
a_2 WT		S1-5-6	+ T (Ho)	− CTT (Ho)	12/12	S1-5-6-8
c_1 − CTT						
c_2 WT						
S1-8	60	S1-8-8	+ T (Ho)	+ T (Ho)	8/8	None
a_1 + T		S1-8-12	+ T (Ho)	+ T (Ho)	10/10	None
a_2 WT		S1-8-23	WT	WT	0/23	S1-8-23-3/7/10/14/17/18
c_1 + T		S1-8-36	+ T/WT (He)	+ T (Ho)	0/2	None
c_2 WT		S1-8-39	+ T (Ho)	+ T (Ho)	11/11	S1-8-39-1/4/6/10
S1-11	47	S1-11-1	− T (Ho)	+ T (Ho)	20/20	S1-11-4/7/12/19
a_1 − T		S1-11-7	− T/+ T (Bi-a)	+ A (Ho)	10/22	All
a_2 + T		S1-11-13	− T/+ T (Bi-a)	+ T (Ho)	9/22	All
c_1 + A		S1-11-16	− T (Ho)	+ T (Ho)	22/22	All
c_2 + T						
S2-5	14	S2-5-2	GC − T (Ho)	+ G (Ho)	16/16	S2-5-2-6/10/15
a_1 + T		S2-5-6	+ T/GC − T (Bi-a)	+ GT/+ G (Bi-a)	4/16	S2-5-6-3/5
a_2 GC − T		S2-5-14	+ T (Ho)	+ GT/+ G (Bi-a)	6/16	S2-5-14-2/8
c_1 + GT						
c_2 + G						

He, heterozygous; Ho, homozygous; Bi-a, bio-allelic. '−' and '+' indicate the deletion and insertion in the sgRNAs target sites, respectively.
*Bold indicates the numbers of T_0 plants.

production. We analysed the resequencing data of 991 worldwide rapeseed germplasm accessions from Wu *et al.* (2019), and no insertion or deletion sites were found in each *BnaMAX1* exon on Chr. A03 or C03, indicating that natural mutations in both *BnaMAX1s* are difficult to obtain. In our study, we found that knockout of the two *BnaMAX1* homologs mediated by CRISPR/Cas9-targeted mutagenesis resulted in semi-dwarf and increased branching phenotypes and also increased SN and yield per plant. Significantly, the semi-dwarf habit can reduce the risk of lodging and facilitate mechanical harvesting, and more branching could maintain stable biomass and increase SN. In the future, it will be necessary to continue aggregating other traits (smaller branch angle, erect leaf, shattering resistance, etc.) in order to create an 'ideotype' that can be used for breeding high yield in rapeseed.

Experimental procedures

Plant materials

We used '862' (wild-type, a spring-type rapeseed variety) as the donor plants for transformation in our study. All wild-type and transgenic plants were grown in a greenhouse (16/8 h of light/dark at 20–23 °C). The Arabidopsis *19-22* mutant was obtained from TAIR (https://www.arabidopsis.org/, Salk_036990). All Col, *19-22* and transgenic plants were grown in a greenhouse (16/8 h of light/dark at 20 °C).

MutMap

One bulked DNA sample was prepared by mixing an equal ratio of DNA of 30 plants that displayed an increased branching phenotype from an F_2 population. The libraries for Illumina sequencing were constructed from Arabidopsis Col (wild type) and the bulked pool. The clean data (1.7 Gb) from Col were first reconstructed to determine the new reference genome, and then, the clean data (3.5 Gb) from bulked samples were BLASTed with the new reference genome to find mutation sites (Indel or SNP) using Coval and selected Indel or SNP index > 0.8 as candidate sites (Abe *et al.*, 2012).

qRT-PCR

Total RNA was extracted using the RNA Prep Pure Plant Kit (Tiangen), and cDNA was synthesized using Oligo (dT) 18 primer and the First cDNA transcriptase kit (Takara). qRT-PCR was performed using a Fast Start Universal Probe Master Mix (Roche) in an ABI 7500 Fast PCR system, and the rapeseed *TMA7* gene (*BnaC05g11560D*) was used as an endogenous control (Zhu *et al.*, 2017). Primers for qRT-PCR are listed in Table S6. Data from three biological replicates were analysed following the relative quantification method ($2^{-\Delta\Delta C_T}$).

Complementary and CRISPR/Cas9 plasmid construction, transformation and positive transgenic-plant identification

We performed a BLASTP search of the MAX1 protein against the rapeseed reference genome (http://www.genoscope.cns.fr/brassicanapus/, Sun *et al.*, 2018) and identified two *MAX1* homologs (*BnaA03g22900D* and *BnaC03g26960D* or *BnA03g0116320.1* and *BnC03g0568140.1*). The full-length CDS of *MAX1* and the two *BnaMAX1*s were cloned into gateway 100 vectors controlled by the 35S promoter, and the three plasmids were transformed into the *Arabidopsis 19-22* mutant using the floral dip method mediated by *Agrobacterium tumefaciens* (GV3101). To select positive lines, 250 mg/L phosphinothricin was sprayed on Arabidopsis T_1 transgenic seedlings.
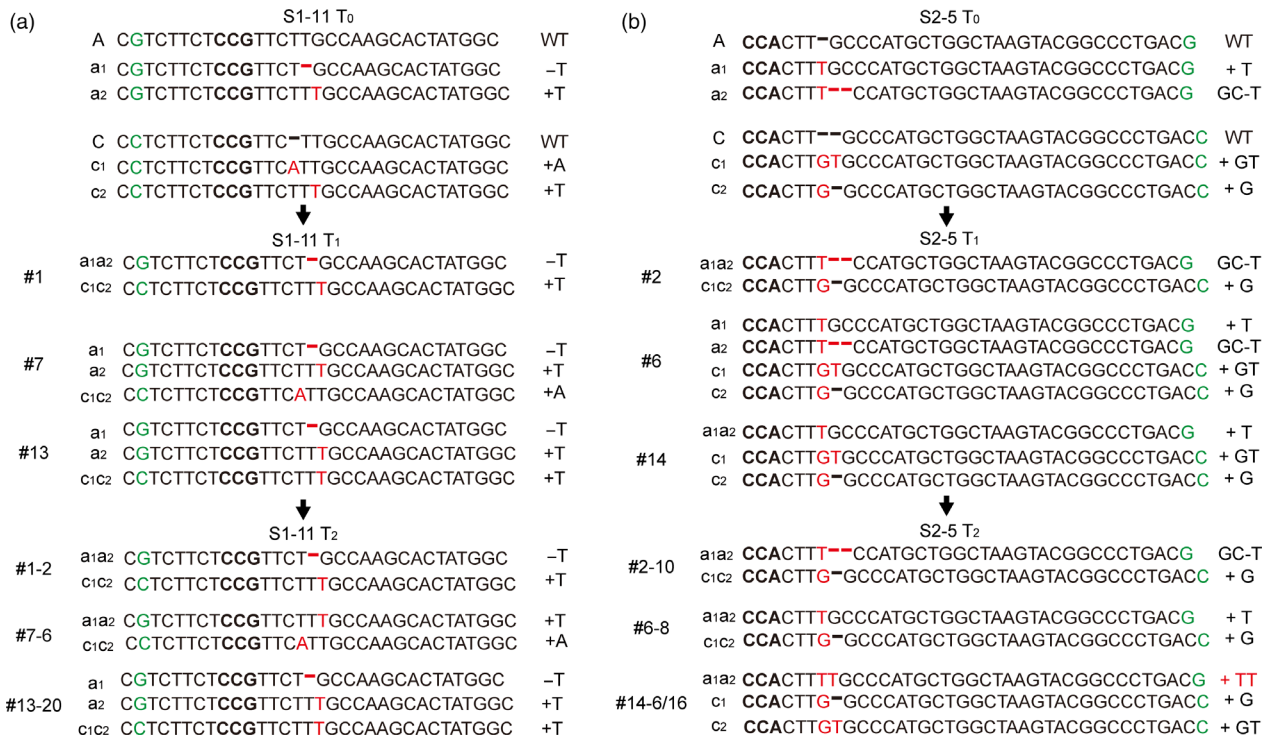
(a)
```
                    S1-11 T0
    A   CGTCTTCTCCGTTCTTGCCAAGCACTATGGC       WT
    a1  CGTCTTCTCCGTTC-GCCAAGCACTATGGC        –T
    a2  CGTCTTCTCCGTTCTTTGCCAAGCACTATGGC      +T

    C   CCTCTTCTCCGTTC-TTGCCAAGCACTATGGC      WT
    c1  CCTCTTCTCCGTTCATTGCCAAGCACTATGGC      +A
    c2  CCTCTTCTCCGTTCTTTGCCAAGCACTATGGC      +T
                        ↓
                    S1-11 T1
       a1a2  CGTCTTCTCCGTTCT-GCCAAGCACTATGGC    –T
 #1    c1c2  CCTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T

       a1    CGTCTTCTCCGTTCT-GCCAAGCACTATGGC    –T
 #7    a2    CGTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T
       c1c2  CCTCTTCTCCGTTCATTGCCAAGCACTATGGC   +A

       a1    CGTCTTCTCCGTTCT-GCCAAGCACTATGGC    –T
 #13   a2    CGTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T
       c1c2  CCTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T
                        ↓
                    S1-11 T2
 #1-2  a1a2  CGTCTTCTCCGTTCT-GCCAAGCACTATGGC    –T
       c1c2  CCTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T

 #7-6  a1a2  CGTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T
       c1c2  CCTCTTCTCCGTTCATTGCCAAGCACTATGGC   +A

       a1    CGTCTTCTCCGTTCT-GCCAAGCACTATGGC    –T
 #13-20 a2   CGTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T
       c1c2  CCTCTTCTCCGTTCTTTGCCAAGCACTATGGC   +T
```

(b)
```
                        S2-5 T0
   A   CCACTT-GCCCATGCTGGCTAAGTACGGCCCTGACG    WT
   a1  CCACTTTGCCCATGCTGGCTAAGTACGGCCCTGACG    + T
   a2  CCACTTT--CCATGCTGGCTAAGTACGGCCCTGACG    GC-T

   C   CCACTT--GCCCATGCTGGCTAAGTACGGCCCTGACC   WT
   c1  CCACTTGTGCCCATGCTGGCTAAGTACGGCCCTGACC   + GT
   c2  CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G
                           ↓
                        S2-5 T1
      a1a2  CCACTTT--CCATGCTGGCTAAGTACGGCCCTGACG   GC-T
 #2   c1c2  CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G

      a1    CCACTTTGCCCATGCTGGCTAAGTACGGCCCTGACG   + T
 #6   a2    CCACTTT--CCATGCTGGCTAAGTACGGCCCTGACG   GC-T
      c1    CCACTTGTGCCCATGCTGGCTAAGTACGGCCCTGACC   + GT
      c2    CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G

      a1a2  CCACTTTGCCCATGCTGGCTAAGTACGGCCCTGACG   + T
 #14  c1    CCACTTGTGCCCATGCTGGCTAAGTACGGCCCTGACC   + GT
      c2    CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G
                           ↓
                        S2-5 T2
 #2-10   a1a2  CCACTTT--CCATGCTGGCTAAGTACGGCCCTGACG   GC-T
         c1c2  CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G

 #6-8    a1a2  CCACTTTGCCCATGCTGGCTAAGTACGGCCCTGACG   + T
         c1c2  CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G

         a1a2  CCACTTTTGCCCATGCTGGCTAAGTACGGCCCTGACG   + TT
 #14-6/16 c1   CCACTTG-GCCCATGCTGGCTAAGTACGGCCCTGACC   + G
         c2    CCACTTGTGCCCATGCTGGCTAAGTACGGCCCTGACC   + GT
```

Figure 5 The target site mutations at the T_0, T_1 and T_2 generations in S1-11 and S2-5 lines. The green colours indicate the SNP between the two *BnaMAX1* genomes, and the bold fonts indicate the PAMs. The red fonts and hyphens indicate insertions and deletions, respectively.

We designed the two sgRNA target sequences using the online tool CRISPR-P1.0 (http://crispr.hzau.edu.cn/CRISPR/, Lei *et al.*, 2014). Two target sequences that each had high similarity with both *BnaMAX1* genes, and few putative off-target sites were selected as candidate sgRNAs. The sgRNA-1 target sequence initiated with G, and the sgRNA-2 initiated with A which we replaced with G. The two sgRNA sequences were cloned into BGK01 vector (Biogle, China; http://www.biogle.cn/) to complete the constructs and then transformed into 862 hypocotyls mediated by *Agrobacterium tumefaciens* (GV3101).

For rapeseed hypocotyl transformation, plump seeds were sterilized using 75% ethyl alcohol, 1.5% mercuric chloride and ddH_2O in turn. We placed them on filter paper to dry, then placed them on M_0 plates (2.2 g/L MS; 7.5 g/L agar; pH 6.0). After growth for about 1 week at 24 °C in the dark, the elongated hypocotyls were cut into 1 cm pieces in DM medium (4.4 g/L MS; 30 g/L sucrose; 0.1 mM AS; pH 6.0). These pieces were then soaked in resuspended *Agrobacterium* (harbouring the Cas9 plasmid) for about 15 min. Filter paper was then used to dry the hypocotyl pieces, which were placed on M_1 medium (2.2 g/L MS; 7.5 g/L agar; pH 6.0) and left for 2 days at 24 °C in the dark. The non-dehydrated hypocotyl pieces were selected on M_2 medium (4.4 g/L MS; 30 g/L sucrose; 18 g/L Mannitol; 1 mg/L 2,4-D; 0.3 mg/L KT; 7 g/L agar; 15 mg/L Hyg B; pH 6.0) for about 3 weeks at 24 °C under light. The putative transgenic pieces were placed on M_3 medium (4.4 g/L MS; 10 g/L glucose; 0.25 g/L xylose; 0.6 g/L MES; 10^{-4} mg/L IAA; 7 g/L agar; 300 mg/L TMT; 1 mg/L zeatin; 15 mg/L Hyg B; pH 6.0) for about 2 weeks at 24 °C under light, which was repeated at 2 week intervals. Finally, the regenerated shoots were allowed to take root in B5 culture medium.

The positive T_2 rapeseed plants were identified by PCR using the special Hyg TaqMan probe (Table S6). The T_0 seeds were identified after growing on MS medium containing 30 mg/L Hyg B in darkness for about 1 week, and seedlings with elongated hypocotyls were putative positive plants.

Mutant identification and off-target analysis

Genomic DNA of the T_0 and T_1 lines was isolated from the leaf samples by a CTAB extraction method. The specific primers that bracketed the CRISPR/Cas9 target sites were used to perform PCR amplification (Table S6), and the PCR products were directly sequenced or cloned into the pEASY-T vector (TransGen Biotech) and then sequenced using the Sanger method.

Putative off-target sites (9 annotated genes for sgRNA-1 and only one for sgRNA-2) were identified by CRISPR-P1.0 (http://crispr.hzau.edu.cn/CRISPR/) against the reference (*Brassica napus* v4.1). The specific primers that surrounded the putative off-target sites were used to perform PCR amplification (Table S6), and 37 independent T_0 plants in both sgRNAs were used for off-target analysis. The products were sequenced using the Sanger method.

Phenotypic observations

The wild-type and T_0 and T_1 transgenic lines were grown in a greenhouse. Whole plants were photographed at the bolting stage, especially at the branching part. At the mature stage, 15 each of wild-type, S1-5, S1-8 and S1-11 homozygous plants were selected to determine PH, BN and yield per plant, and then, ten plants of each were selected to determine SN per plant. For the field experiment, the wild-type and all T-DNA-free lines were grown in plots of 2 rows, with 10–11 plants in each row. Ten plants were selected from the centre of each row to measure
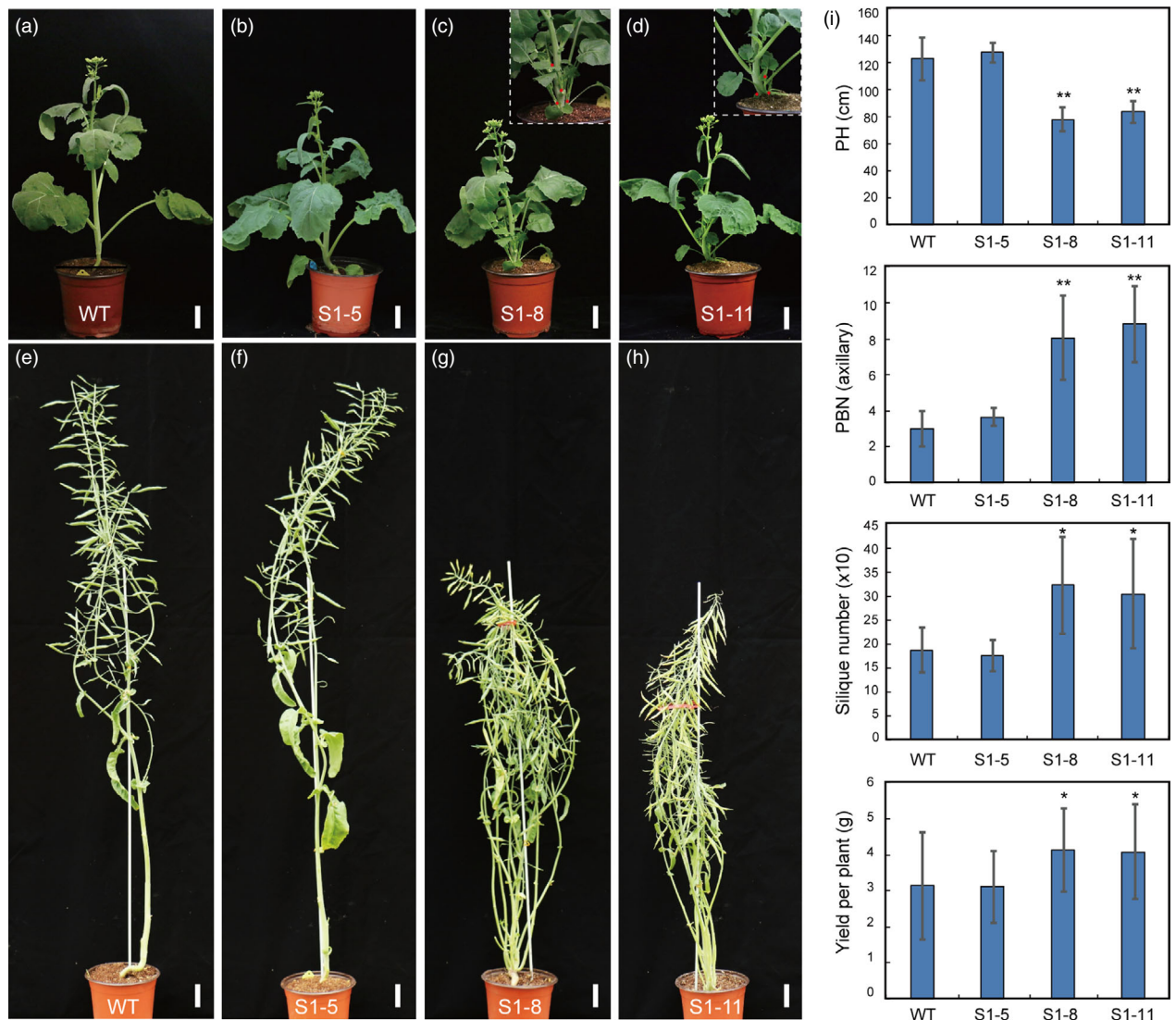
Figure 6 Morphological comparison of wild-type (WT) and *BnaMAX1* homozygous mutant plants at various developmental stages. (a–d) The S1-8-39-1 and S1-11-7-3 homozygous plants show more axillary buds than WT and S1-5-6-8 at the bolting stage. (e–h) The S1-8-39-1 and S1-11-7-3 homozygous plants show dwarfism and higher branch numbers than WT and S1-5-6-8 at the mature stage. (i) Statistical analysis of plant height (PH), branching number (BN), silique number (SN), and yield per plant in WT, S1-5-6-8, S1-8-39-1 and S1-11-7-3 homozygous plants in the greenhouse. Error bars ± standard deviation (n = 15 for PH, BN and yield; 10 for SN in WT, S1-5-6-8, S1-8-39-1 and S1-11-7-3). Student's *t*-test was used for statistical analysis (*$P \leq 0.05$; **$P \leq 0.01$). Bars = 5 cm.

the following four traits at maturity: PH, BN, SN and yield per plant.

Author contributions

M.Z. and W.H. designed the experiments; M.Z., L.Z., M.T., J.L. and H.Y. performed the experiments; H.L. and L.Z. analysed the genome and sequencing data; L.Z. and S.F. characterized the agronomic traits; M.Z. wrote the manuscript, and W.T., H.W. and W.H. revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no competing financial interest.

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Supporting information

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