Targeted mutagenesis of BnTT8 homologs controls yellow seed coat development for effective oil production in Brassica napus L.

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Abstract

Yellow seed is a desirable trait with great potential for improving seed quality in Brassica crops. Unfortunately, no natural or induced yellow seed germplasms have been found in Brassica napus, an important oil crop, which likely reflects its genome complexity and the difficulty of the simultaneous random mutagenesis of multiple gene copies with functional redundancy. Here, we demonstrate the first application of CRISPR/Cas9 for creating yellow-seeded mutants in rapeseed. The targeted mutations of the BnTT8 gene were stably transmitted to successive generations, and a range of homozygous mutants with loss-of-function alleles of the target genes were obtained for phenotyping. The yellow-seeded phenotype could be recovered only in targeted mutants of both BnTT8 functional copies, indicating that the redundant roles of BnA09.TT8 and BnC09.TT8B are vital for seed colour. The BnTT8 double mutants produced seeds with elevated seed oil and protein content and altered fatty acid (FA) composition without any serious defects in the yield-related traits, making it a valuable resource for rapeseed breeding programmes. Chemical staining and histological analysis showed that the targeted mutations of BnTT8 completely blocked the proanthocyanidin (PA)-specific deposition in the seed coat. Further, transcriptomic profiling revealed that the targeted mutations of BnTT8 resulted in the broad suppression of phenylpropanoid/flavonoid biosynthesis genes, which indicated a much more complex molecular mechanism underlying seed colour formation in rapeseed than in Arabidopsis and other Brassica species. In addition, gene expression analysis revealed the possible mechanism through which BnTT8 altered the oil content and fatty acid composition in seeds.

Introduction

Rapeseed (Brassica napus L., AACC, 2n = 38) is the third-largest oilseed crop worldwide after soya bean and oil palm, accounting for approximately 16% of the entire global vegetable oil production (Hu et al., 2017; Woodfield et al., 2017). It provides not only edible oils for human diets and high-quality animal feed proteins but also raw materials for industrial processes, such as biodiesel production. Achieving high oil yields, better oil and meal quality has always been the major breeding goals in rapeseed production. At present, most commercial rapeseed cultivars have brown to black seed colour. Previous studies showed that yellow-seeded B. napus has a thinner seed coat, a reduced percentage of pigment and hull, and a greater content of oil and protein than the black-seeded type (Marles and Gruber, 2004; Meng et al., 1998; Tang et al., 1997). With these superior characteristics, yellow seed is widely accepted as a good-quality trait and is a focus of rapeseed research globally (Hong et al., 2017; Jiang et al., 2019; Lian et al., 2017; Meng et al., 1998; Qu et al., 2013, 2016; Simbaya et al., 1995; Tang et al., 1997; Wen et al., 2012).

As in Arabidopsis, the formation of seed colour is due to the deposition of the oxidized form of a flavonoid, proanthocyanidins (PAs, the so-called condensed tannins), within the endothelial layer of the inner integument of the seed coat in Brassica species (Lepinec et al., 2006; Marles and Gruber, 2004). PA is synthesized through the common phenylpropanoid pathway into the flavonoid pathway to form anthocyanidin, and then PA is formed using anthocyanidin as a precursor (Debeaujon et al., 2003). During seed maturation, colourless PA precursors, such as epicatechin, polymerize and oxidize to form dark brown PAs, altering the colour of the seed to dark brown or black (Lian et al., 2017; Pourcel et al., 2005). In Arabidopsis, the flavonoid pathway has been well characterized at the molecular level mainly by utilizing transient testa (tt) and tannin-deficient seed (tds) mutants, which influence flavonoid accumulation and modify seed coat pigmentation (Albert et al., 2014; Lepinec et al., 2006; Shirley et al., 1995; Xu et al., 2014). They correspond to two groups of structural proteins, the so-called early and late biosynthetic genes (EBGs and LBGs, respectively), transporters and regulatory factors. The EBG proteins catalyse the early biosynthetic steps and include chalcone synthase (CHS/TT4), chalcone isomerase (CHI/TT5), flavanone 3-hydroxylase (F3H/TT6) and flavonoid 3’-hydroxylase (F3’H/TT7), resulting in the formation of dihydrolavonols (the common precursors of flavonoids). The LBG proteins catalyse the late steps of the pathway and include the downstream enzymes dihydrolavonol-4-reductase (DFR/TT3), leucoanthocyanidin dioxygenase/anthocyanidin...
synthase (LDOX/ANS/TT18) and BANYULS/anthocyanidin reductase (BAN/ANR), in addition to the transporters glutathione S-transferase 26 (GST26/GSTF12/TT19), auto-inhibited H-‘ATPase isoform 10 (AHA10/TT13) and MATE transporter (TT12), as well as a laccase gene, laccase 15 (LAC15/TT10), resulting in the production of anthocyanin/PAs (Lepiniec et al., 2006; Xu et al., 2014). This expression regulation of the genes involved in the flavonoid biosynthetic pathway is mainly controlled by different sets of transcription factors (TFs) in a tissue-specific manner (Lepiniec et al., 2006; Xu et al., 2014, 2015). In A. thaliana, a ternary complex (known as the MBW complex) comprising three TF regulators, namely ATM123/TT2 (R2R3-MYB), TT8 (basic helix-loop-helix, bHLH) and TTG1 (WD40 protein), plays a key role in activating PA-specific genes in seed coat development (Xu et al., 2014, 2015).

Accumulating evidence demonstrates that TT8 is a central component of the well-conserved complex that controls flavonoid accumulation in various crops (Escaray et al., 2017; Li et al., 2016; Li et al., 2019; Lim et al., 2017; Nemesio-González et al., 2013). Taking into account the close phylogenetic relationship between Arabidopsis and Brassica, TT8 gene homologs could play comparable roles in Brassica species. Indeed, the yellow-seeded trait of B. rapa var. yellow sarson is due to the loss of function of the TT8 gene caused by an insertion of a transposable element in its intron (Li et al., 2012a, 2012b). Similarly, natural mutations in two homologous TT8 genes, BjuA.TT8 and BjuB.TT8, control the yellow-seeded trait in allotetraploid B. juncea (Padmaja et al., 2014). In B. napus, studies have shown that most of the genes involved in the flavonoid biosynthetic pathway, including the homologs of TT8, are down-regulated in yellow seed compared to black seed, indicating that they are evolutionarily conserved in rapeseed (Hong et al., 2017; Jiang et al., 2019; Qu et al., 2013). However, the functions of these genes have yet to be verified. The BnTT8 gene is therefore regarded as appropriate for manipulation in the breeding of yellow-seeded varieties of rapeseed.

Brassica napus is an allotetraploid species that was formed by a recent hybridization of two diploid ancestors, B. rapa (AA) and B. oleracea (CC) (Chalhoub et al., 2014). Although both of its diploid ancestors possess yellow seed genotypes with stable phenotypes and qualitative inheritance, B. napus naturally lacks yellow-seeded mutants (Hong et al., 2017; Jiang et al., 2019; Lian et al., 2017). It emerges mainly in rapeseed that is amphidiploid nature, having a minimum of two similar copies of most of its genes. Spontaneous and induced random mutagenesis usually induces single mutants of these copies, which in most cases do not have the preferred effect in rapeseed. Yellow-seeded rapeseed has been exclusively developed through interspecific hybridization of Brassica species (B. rapa, B. juncea, B. carinata, and B. oleracea) or intergeneric hybridization with relevant genera (Rashid et al., 1994; Meng et al., 1998; Rahman et al., 2001; Wang et al., 2005; Li et al., 2009; Li et al., 2012a, 2012b; Wen et al., 2012). However, this strategy is time-consuming and inefficient, as the introduced yellow seed trait exhibits extreme variation in seed colour stability, which is recognized as a major obstacle in yellow-seeded rapeseed breeding (Liu et al., 2005). Extensive studies in resynthesized rapeseed resources have also revealed the complexity of yellow seed trait introgression from related species, which made the yellow-seeded feature in B. napus more difficult to study than Arabidopsis (Yu, 2013). Therefore, the utilization of new technologies that can simultaneously modify numerous copies of the gene becomes imperative to create new genetic variation for seed coat colour in polyploid rapeseed.

In recent years, sequence-specific nucleases (SSNs) have been demonstrated to be an amazing tool for the improvement of crops via site-specific genome editing, and CRISPR/Cas9 is considered the most simple and efficient SSN. The CRISPR/Cas9 system has been effectively utilized in rapeseed to produce the targeted null mutations required to improve agronomic traits (Braatz et al., 2017; Hu et al., 2018; Li et al., 2018; Yang et al., 2017; Yang et al., 2018; Zhai et al., 2019; Zheng et al., 2019). Hence, we utilized the CRISPR/Cas9 system to generate efficient knockouts of BnTT8 homeologs in the flavonoid biosynthesis pathway with stable transformation in rapeseed. In the T1 and T2 generations, mutant plants containing the desired gene modification but not the transferred DNA were obtained by segregation. This is the first report on the creation of yellow-seeded mutants in rapeseed using CRISPR/Cas9 technology and provides valuable germplasm resources for further breeding of yellow-seeded varieties of rapeseed. The current study also used transcriptomic analysis and metabolite profiling of mutant plants from BnTT8 to investigate the molecular mechanism that regulates seed colour.

**Results**

**Molecular cloning and characterization of TT8 homologs in B. napus**

Previous studies revealed that TT8 gene function is essential for seed coat colour and is highly conserved in Brassica species. Loss-of-function TT8 mutants showed yellow-seeded traits in B. rapa and B. juncea (Li et al., 2012a, 2012b; Padmaja et al., 2014). Thus, TT8 is one of the ideal candidates for creating yellow-seeded germplasm resources in rapeseed. B. napus contains three TT8 copies, one on chromosome A09 (BnaA09g22810D, designated as BnaA09.TT8) and two tandem duplicates on chromosome C09 (BnaC09g24860D/BnaC09g24870D, designated BnaC09.TT8a/b, respectively). To check for putative mutations in the target genes, we confirmed their genomic DNA sequences in the B. napus pure line J9707. In every one of the three cases, the sequenced ORFs matched the length of the high-confidence gene models in the Darmor-bzh reference genome assembly (Chalhoub et al., 2014). As for Arabidopsis TT8, the predicted amino acid sequences of BnaA09.TT8/BnaC09.TT8b both contain several conserved domains: the N-terminal MYP interaction region (MIR), the WD/AD and the bHLH domain in the C-terminal region (Figs. S1 and S2). However, the predicted BnaC09.TT8a lacked the MIR and WD/AD domain and part of the bHLH domain, which are essential for TT8 function (Figs. S1 and S2). Thus, the sequence analysis suggested that BnaA09.TT8 and BnaC09.TT8b encode functional bHLH proteins and that BnaC09.TT8a is most likely a pseudogene. For this reason, BnaC09.TT8a was not used in further experiments.

BnaA09.TT8 and BnaC09.TT8b were 96.85% identical at the nucleotide level and share 97.89% amino acid identity, suggesting that these genes encode enzymes with similar functions. According to the sequence alignment of the two copies of BnaTT8 gene, polymorphisms distinguished the origins of these gene copies (Fig. S3).

Phylogenetic investigation showed that all BnTT8 copies were clustered together with AtTT8 and its homologs from different plant species in a well-supported clade, all of which are involved in flavonoid biosynthesis (Fig. S4). It also revealed that BnaA09.TT8 and BnaC09.TT8a/b were closely related to their homologs in
B. rapa and B. oleracea, respectively (Fig. S4), which is in line with their origination from two diploid progenitors. Similarly, B. oleracea contained two tandem duplicates, that is, BoTT8a (Bo9g086910) and BoTT8b (Bo9g086920), with one truncated copy of BoTT8a (Figs. S1 and S2).

Expression analysis of the BnTT8 gene

The expression pattern of BnTT8 copies in J9707 was initially examined using quantitative real-time PCR (qRT-PCR) with RNA from different plant tissues (Fig. 1a). No transcript was identified for the BnC09.TT8a, but various amounts of transcript were detected for BnA09.TT8 and BnC09.TT8b in all tissues, with the highest expression in seeds. Evidently, BnA09.TT8 had a significantly higher expression level than BnC09.TT8b in these tissues. This result suggested that BnTT8 principally plays a role in seed development.

To confirm and further characterize the expression of the BnA09.TT8 and BnC09.TT8b copies, their expression was assessed during various stages of seed coat development: 7, 14, 21, 28, 35 and 42 days after flowering (DAF). The expression of both copies exhibited a steady increase from 7 DAF to a peak value at 21 DAF and then decreased at later stages (Fig. 1b). Overall, BnA09.TT8 had a significantly higher expression level than BnC09.TT8b during seed formation. Analysis of mRNA accumulation patterns for BnTT8 copies based on recent public RNA-seq data in brown/black-seeded rapeseed lines showed their expression profiles in the developing seeds with different genetic backgrounds (Fig. 1c–e). In all cases, the BnC09.TT8a transcripts were undetectable at all developing stages, whereas the expression of BnA09.TT8 and BnC09.TT8b gradually increased during the early stage of seed development and peaked at 23 DAF (Fig. 1d and e) or 35 DAF (Fig. 1c). The expression level of BnA09.TT8 was higher than that of BnC09.TT8b during later seed development, especially at and after the peaking stages (Fig. 1c–e). Thus, we further confirmed that the B. napus genome contains two functional AtTT8 homologs, BnA09.TT8 and BnC09.TT8b.

Creation of CRISPR/Cas9-targeted mutations in BnTT8

To generate Cas9-induced knockout mutations in the functional copies of BnTT8, four sgRNAs were designed using the CRISPR-P program (Lei et al., 2014; Fig. 2a). Three of these sgRNAs, that is, sgRNA1 (S1) through sgRNA3 (S3), were designed to target the MIR domain, and the targeting sequence of sgRNA4 (S4) was within the WD/AD domain; this was expected to induce mutations in the functional domain of the gene and thus inactivate the BnTT8 protein (Figs 2a and S3). The sgRNAs matched well with BnA09.TT8 and BnC09.TT8b (Fig. 2a). A CRISPR/Cas9 construct containing these four sgRNAs with Cas9 driven by the P35S promoter (Fig. 2b) was produced based on the CRISPR/Cas9 multiplex genome-editing vector as previously described by Yang et al. (2018). The resulting construct was transformed into J9707 using Agrobacterium-mediated transformation, and 333 T0-positive transgenic plants were generated. A total of 48 targeted mutants were identified by Sanger DNA sequencing of the PCR products of the target sites, with five plants showing a visible knockout phenotype (i.e., yellow seed; Table 1).

To acquire stable lines with targeted mutations, eight independent T0 editing lines of BnTT8 were self-pollinated to produce T1 and T2 progeny. The targeted mutations of progeny from these T0 lines were verified by high-throughput tracking of mutations (Hi-TOM) sequencing analysis of the target sites. A total of

Figure 1

Expression pattern of BnTT8 in rapeseed. Relative gene expression of BnTT8 in various tissues of J9707 (a) and different stages of seed development in J9707 (b) were determined by qRT-PCR; values are the means ± SE of three biological replicates. (c-e) mRNA accumulation patterns for BnTT8 were calculated by fragments per kilobase of transcript per million mapped reads (FPKM) based on public RNA-seq data, including the seed coat transcriptomes of a brown-seeded NIL (c; Hong et al., 2017), and the seed transcriptomes of two black-seeded rapeseed inbred lines, 1L99 (d) and 1L363 (e), respectively (Shahid et al., 2019).
eighteen T2 lines with homozygous mutations in BnTT8 were detected, including two BnA09.TT8 single mutants, four BnC09.TT8b single mutants and twelve BnTT8 double mutants (Table 1; Fig. 2c). All of these detected homozygous mutations at the target sites within BnTT8 were predicted to cause frame shifts and result in non-functional proteins, with the exception of T2 line TT8-281-5-5, which had a 6-nucleotide-long deletion resulting in two amino acid (Val31 and Gly32) deletions in the conserved MIR domain of BnC09.TT8b (Table 1; Fig. 2c; Fig. S5). As expected, all of those double mutants could produce yellow seeds, while the single mutants showed a comparable phenotype to that of the WT (Table 1; Fig. 3a). Thus, both copies of the BnTT8 gene function redundantly in yellow seed development.

We performed PCR assays of the T1 and T2 plants to explore the potential for targeted changes without integrating foreign DNA into the rapeseed genome. A variety of BnTT8 homozygous T-DNA-free mutants were identified (Table 1).

**Targeted mutations in BnTT8 lead to defective PA accumulation in the inner seed coat**

Dissecting the seed coat and embryo of WT and BnTT8 double-mutant seeds clearly shows that the seed colour difference is mainly determined by the seed coat, not by the embryo (Fig. 3b). The brown to dark colour of mature seeds in Brassica species is due to the PA oxidations during seed desiccation that cause the accumulation of colourless compounds in the seed coat (Lepiniec et al., 2006; Marles and Gruber, 2004). The dissected seed coats of WT, single (TT8-291-6-4, TT8-291-9-7, TT8-384-14-9, TT8-270-11-3) and double (TT8-96-3-2, TT8-148-5-7) homozygous mutants of BnTT8 were used for the vanillin and DMACA staining test to determine the dynamic variation of PA accumulation during seed development. Red coloration with vanillin and blue coloration with DMACA started at 21 DAF in the black seed coats of both WT and single mutants, and the colour gradually became darker during development (Fig. 3c and d). The double-mutant seeds at any stage were not obviously stained (Fig. 3c and d).

Microscopy of Safranin O and Fast Green-stained transverse sections showed that the accumulated PAs were deposited in the endothelial cells of the WT and single-mutant seed coats (indicated by arrowheads in Fig. 4a). However, the red-stained PA was completely absent in the double-mutant seed coat (Fig. 4a). Additionally, the seed coat thickness had the most drastic reduction in the double mutant, by 27.0% lower relative to WT seeds; the BnA09.TT8 single mutant showed a significant decrease (9.8%) in the thickness of the seed coat with respect to WT plants, whereas this trait in the BnC09.TT8b single mutant was similar to the WT trait (Fig. 4b).

Together, these findings indicate that the disruption of BnTT8 affected seed development and hindered the PA deposition in the inner layer of the seed coat, which is consistent with previously described tt8 mutant seed phenotypes in Arabidopsis and other Brassica species (Li et al., 2012a,2012b; Nesi et al., 2000; Padmaja et al., 2014). The findings also suggest that the most important stage of seed coat colour formation occurs at 21 DAF in B. napus.

**Figure 2** CRISPR/Cas9-induced null mutants of BnTT8 in B. napus. (a) The BnTT8 gene model includes seven exons (box) separated by six introns (represented by the solid line). The positions of the N-terminal MYB interaction region (MIR) domain, the WD/AD domain and the bHLH domain in the C-terminal region are marked in the model. The vertical line in the gene model indicates the target site, and the arrow indicates the sgRNA direction. The target sequences are shown with the PAM underlined. (b) The CRISPR/Cas9 construct houses the following: a hygromycin resistance cassette consisting of the hygromycin phosphotransferase coding sequence driven by the cauliflower mosaic virus 35S promoter; a Cas9 expression cassette comprising the sequence encoding Cas9 driven by P35S; and four sgRNAs (S1-S4) driven by the U3d, U3b, U6-1 and U6-29 promoters from Arabidopsis. (c) Sequences at the sgRNA target sites of BnTT8 homozygous mutants in T2 generation. The protospacer adjacent motif (PAM) is underlined, and nucleotide indels are marked in red, with details labelled at right, ‘a’ and ‘c’ represent the mutated alleles of the target gene on BnA09.TT8 and BnC09.TT8b, respectively. ‘aaCC’, ‘AACC’ and ‘aaccc’ represent homozygous mutations of the target gene in BnA09.TT8, BnC09.TT8b and both copies, respectively.
| Plant ID   | Generation | Positive | S1        | S2        | S3        | S4        | S1        | S2        | S3        | S4        | Seed colour |
|------------|------------|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|--------------|
| TT8-96     | T0         | Y        | Hetero    | Homo (+1bp) | wt        | Hetero    | Homo (+1bp) | /         | wt        | Hetero    | Yellow       |
| TT8-96-2   | T1         | Y        | Homo (+1bp) | Homo (+1bp) | wt        | Hetero    | Homo (+1bp) | /         | wt        | Hetero    | Yellow       |
| TT8-96-3   | T1         | N        | Homo (+1bp) | Homo (+1bp) | wt        | wt        | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-96-3-2 | T2         | N        | Homo (+1bp) | Homo (+1bp) | wt        | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-96-3-3 | T2         | N        | Homo (+1bp) | Homo (+1bp) | wt        | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-148    | T0         | Y        | Hetero    | Homo (+1bp) | wt        | Hetero    | Homo (+1bp) | /         | wt        | Hetero    | Yellow       |
| TT8-148-5  | T1         | Y        | Homo (+31bp, -9bp) | Homo (+1bp) | wt        | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-148-9  | T1         | Y        | Homo (+31bp, -9bp) | Homo (+1bp) | wt        | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-148-9-2| T2         | Y        | Homo (+31bp, -9bp) | Homo (+1bp) | wt        | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-145    | T0         | Y        | Homo (+1bp) | Homo (+1bp) | wt        | Hetero    | Homo (+1bp) | /         | wt        | Hetero    | Yellow       |
| TT8-145-3  | T1         | Y        | wt        | Homo (+1bp) | wt        | Hetero    | Homo (+1bp) | /         | wt        | Hetero    | Yellow       |
| TT8-145-13 | T2         | Y        | wt        | Homo (+1bp) | /         | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-281    | T0         | Y        | Homo (+1bp) | /         | /         | Hetero    | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-281-5  | T1         | Y        | Homo (+1bp) | wt        | wt        | wt        | Homo (+1bp) | /         | wt        | /         | Yellow       |
| TT8-281-5-5| T2         | Y        | Homo (+1bp) | wt        | wt        | wt        | Homo (+1bp) | /         | wt        | /         | Yellow       |
| TT8-299    | T0         | Y        | Homo (+1bp) | /         | /         | Hetero    | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-299-9  | T1         | Y        | Homo (+1bp) | wt        | wt        | wt        | Homo (+1bp) | /         | wt        | /         | Yellow       |
| TT8-299-9-10| T2        | N        | Homo (+1bp) | /         | /         | Hetero    | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-299-12 | T1         | Y        | Homo (+1bp) | Homo (+1bp) | /         | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-299-12-2| T2        | N        | Homo (+1bp) | /         | /         | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-299-12-4| T2        | Y        | Homo (+1bp) | Homo (+1bp) | /         | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-291    | T0         | Y        | wt        | Homo (+1bp) | /         | wt        | Homo (+1bp) | /         | wt        | /         | Yellow       |
| TT8-291-6  | T1         | Y        | wt        | Homo (+1bp) | wt        | wt        | Homo (+1bp) | /         | wt        | /         | Yellow       |
| TT8-291-6-4| T2         | Y        | wt        | Homo (+1bp) | wt        | wt        | Homo (+1bp) | /         | wt        | /         | Yellow       |
| TT8-291-9  | T1         | N        | wt        | Hetero    | wt        | wt        | Hetero    | Hetero    | Hetero    | Yellow       |
| TT8-291-9-7| T2         | N        | wt        | Hetero    | wt        | wt        | Hetero    | Hetero    | Hetero    | Yellow       |
| TT8-384    | T0         | Y        | wt        | Hetero    | /         | wt        | Hetero    | Hetero    | Hetero    | Yellow       |
| TT8-384-4  | T1         | Y        | wt        | Homo (+1bp) | wt        | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-384-4-10| T2        | Y        | wt        | Homo (+1bp) | wt        | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-384-13 | T2         | Y        | wt        | Homo (+1bp) | /         | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-384-14 | T1         | Y        | wt        | wt        | wt        | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-384-14-9| T2        | Y        | wt        | wt        | wt        | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-384-14-10| T2       | Y        | wt        | wt        | wt        | Homo (+1bp) | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-270    | T0         | Y        | wt        | wt        | wt        | wt        | wt        | Biallelic | /         | /         | Yellow       |
| TT8-270-1  | T0         | Y        | wt        | wt        | wt        | Hetero    | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-270-1-9| T2         | N        | Homo (+1bp, +2bp) | Homo (+1bp) | wt        | /         | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-270-11 | T1         | N        | wt        | wt        | wt        | Hetero    | Homo (+1bp) | /         | /         | /         | Yellow       |
| TT8-270-11-3| T2       | N        | wt        | wt        | wt        | Hetero    | Homo (+1bp) | /         | /         | /         | Yellow       |

Table 1: Genotypic analysis of BnTT8 mutants and their transmission to T1 and T2 generations
Effects of *BnTT8* targeted mutants on seed oil and protein contents, fatty acid (FA) composition and yield-related traits

To characterize the effect of the *BnTT8* targeted mutations on oil and protein contents and FA composition, all double homozygous T0 and T2 lines with diverse frame-shift targeted mutations (Table 1) were chosen for subsequent phenotypic characterization. The oil content from WT seeds was 45.34%–47.32% dry weight (Table 2). However, the oil contents of the double-mutant seeds were approximately 51.80% in T0 plants and 48.01% in T2 plants, increased by 9.47% and 5.89% relative to WT seeds, respectively (Table 2). The protein contents of the double-mutant seeds were also simultaneously significantly increased by 16.95% in T0 plants and 16.00% in T2 plants relative to WT seeds, respectively (Table 2), which is thought to improve the nutritional quality of the oilseed. Examination of FA profiles in T0 and T1 plants showed a consistent alteration in the FA composition in *BnTT8* mutants, including increases in palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3) and decreases in stearic acid (C18:0) and oleic acid (C18:1) relative to WT seeds (Table 2).

To comprehensively characterize the *BnTT8* mutant phenotypes, single (TT8-291-9-7, TT8-270-11-3, TT8-384-14-9, TT8-384-14-10) and double (TT8-299-12-2, TT8-270-1-9, TT8-384-13-3, TT8-96-3-2, TT8-281-5-5) homozygous mutant T3 lines without T-DNA were grown in the field following a randomized block design with three replicates. The oil content in the single-mutant seeds of *BnTT8* was similar to that in the WT control ranging from 44.94% to 45.50% (Table 3). However, the oil contents of all double-mutant lines, which ranged from 46.69% to 48.97%, were significantly increased (by 5.90% on average) relative to WT seeds (Table 3). The protein content of the single-mutant seeds was similar to that of WT, whereas this trait in all double-mutant lines was higher than that in the WT control with two of these lines showing significant differences (Table 3). The overall alteration in FA composition in double mutants showed a similar trend to the T0 and T1 generations, with some variations among different lines (Table 3). We also evaluated the yield-related traits of these T3 mutant lines. Although different degrees of changes in these yield-related traits were observed among different lines, the seed yields of these mutants were similar to those of the WT (Table S2). Thus, the simultaneous targeted mutation of *BnA09.TT8* and *BnC09.TT8b* conferred a high oil yield potential with modified FA composition and improved the nutritional quality. Therefore, the *BnTT8* double mutants generated in this study could serve as excellent starting materials for rapeseed breeding.

Off-target activity of CRISPR/Cas9 in T0 transgenic *B. napus* plants

To ascertain whether off-targeting occurred in the present study, we searched the *B. napus* genome for putative off-target sites with high homology to the three sgRNAs that detected on-target mutations according to the CRISPR-P program (Lei et al., 2014). These potential off-target sites are listed in Table S3. There were 8, 9 and 9 putative off-target sites for S1, S3 and S4, respectively (Table S3).

High-throughput sequencing of the PCR products of these 26 potential sites from 30 T0 gene-edited plants exhibited no mutations (Table S3), indicating that the off-target effect is
negligible when the sgRNA specificity is well considered according to the genome sequence. Thus, the CRISPR/Cas9 system has a high specificity for targeted mutagenesis in *B. napus*.

**BnTT8 regulates the expression of the phenylpropanoid and flavonoid biosynthesis genes**

As the investigation above showed that the PAs accumulated more accumulated after 21 DAF in the seed coat of *WT* (Fig. 3c and d), we collected the developing seed coats at 14 DAF and 35 DAF for comparison of the expression profiles between the BnTT8 double mutants (TT8-299-12-2, TT8-96-3-2, TT8-270-1-9) and the WT to investigate the transcriptional changes underlying the seed coat colour phenotypes (Table S4). A total of 52 962 genes were expressed in the developing seed coats at the two stages and were included in the subsequent analysis. The Pearson correlation coefficient between any two of the three biological replicates at each stage was high (*R* = 0.91–0.98) in both WT and mutant, indicating that the sequencing data utilized in the present study were highly reliable (Fig. S6).

Comparison of transcript abundances in these developing seed coats uncovered 1298 differentially expressed genes (DEGs) between each double mutant and its corresponding *WT*, among which 145 DEGs were shared in common by the two stages (Table S5; Fig. S7). The number of DEGs at stage 14 DAF (963 DEGs) was almost twofold of that at stage 35 DAF (480 DEGs), indicating extensive changes in gene expression preceding observable changes in seed coat colour (Fig. S7). Generally, 732 genes exhibited up-regulation, and 711 genes exhibited down-regulation in the mutant seed coat, which might be associated with seed colour development (Fig. S8). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis for these identified DEGs showed that the phenylpropanoid and flavonoid metabolic processes were significantly enriched among down-regulated DEGs in mutants relative to WT, especially at 35 DAF, an important stage during the formation of seed coat colour (Figs. S9–S11; Tables S6–S8).

In the general phenylpropanoid pathway, the expression of genes encoding three classes of enzymes, namely PAL, C4H and 4CL, was suppressed to different extents at least in the 35 DAF mutant seed coat, including four copies of BnPAL1, six copies of BnPAL2, five copies of BnC4H, two copies of Bn4CL3 and a copy of Bn4CL1 (Fig. 5; Table S8).

In flavonoid biosynthesis, all the structural genes showed notable down-regulation at both stages in most cases, including five copies of BnCHS, six copies of BnCHI, three copies of BnF3H and BnDFR, two copies of BnCHI, a copy of BnF3H, and four copies of BnLODX and BnBAN genes (Fig. 5; Table S8). Similarly, three key transporter genes that act downstream of the structural genes were also repressed in the mutant seed coat, including four copies of BnGST26 and two copies of BnTT12 and BnHAH10 (Fig. 5; Table S8). A homolog of LAC1S, encoding a laccase-like oxidase involved in the formation of flavonoid end products, was down-regulated more than sixfold in the mutants compared with WT (Fig. 5; Table S8). The expression of several key regulatory genes controlling PA accumulation in the seed coat was also changed at different stages in the mutants (Fig. 5; Table S8). As the targeted mutated gene, BnA09.TT8 was down-regulated by almost fivefold at 14 DAF and 330-fold at 35 DAF, and BnCO9.TT8b was only down-regulated at 35 DAF (Fig. 5; Table S8), which indicates that BnTT8 controls its own transcription in the seed coat similar to that in *Arabidopsis* (Baudry et al., 2006). Again, no transcript was detected for BnCO9.TT8a, which further confirmed it as a non-functional copy. The down-regulated DEGs also included two copies of BnMYBL2 and a copy of BnTT2 (Fig. 5; Table S8). Conversely, a copy of BnMYB5 as a key member of the MBW ternary complexes showed significant up-regulation only at 14 DAF. For flavone biosynthesis, one out of 16 copies of BnFOMT was markedly down-regulated at both stages (Table S10).

To validate the RNA-seq data, subsets of 29 DEGs in developing seed coats were chosen for qRT-PCR analysis. These genes included 16 DEGs involved in phenylpropanoid and flavonoid biosynthesis and 13 randomly chosen DEGs. Linear regression analysis showed very high correlation coefficients (*R* = 0.803–0.900; Fig. S12) between the transcript levels assayed by the two analytic systems, further confirming the reliability of the RNA-seq data.

Together, these results agree with the mutation phenotypes of BnTT8 and further demonstrate that BnTT8 is a key regulator during flavonoid biosynthesis in the seed coat of *B. napus*.

**Targeted mutations in BnTT8 change flavonoid composition in the seeds**

To assess the impact of targeted mutation of BnTT8 on the flavonoid metabolic pathway, metabolite profiling of the double-mutant (TT8-299-12-2, TT8-96-3-2, TT8-270-1-9) and its WT control seeds was analysed using an LC-ESI-MS/MS system. Approximately half of the identified flavonoid metabolites showed a significant difference between the double-mutant and WT seeds (Tables S9, S10). All of these differential metabolites were lower in mutant seeds than in WT seeds (Table S10). Epicatechin, the PA precursor, showed the most drastic reduction in the mutants, by more than 230-fold lower relative to WT seeds (Table S10; Fig. 6). Targeted mutation of BnTT8 also blocked the accumulation of other flavonoid compounds, including flavones, anthocyanidins and naringenin (Table S10; Fig. 6). Interestingly, the flavone compounds were obviously enriched in the differential metabolite profiling (Table S10; Fig. 6). These results are in line with the phenotypes and the transcriptomic analysis of the mutant seeds and further demonstrate that BnTT8 plays an important role in the regulatory network controlling flavonoid accumulation during seed development in *B. napus*.

**The BnTT8 targeted mutation results in altered expression of genes involved in FA biosynthesis during seed development**

Since targeted mutation of BnTT8 resulted in significantly increased oil content and alteration of FA composition, we speculated that BnTT8 participates in the regulation of genes involved in the FA biosynthesis pathway during seed development. To test this hypothesis, the expression of several critical TFs controlling seed development and FA accumulation, such as LEAFY COTYLEDON1 (LEC1), LEC2 and FUSCA3 (FUS3), and key enzymes involved in the FA biosynthesis pathway, including FATTY ACID ELONGASE 1 (FAE1), FATTY ACID DESATURASE 2 (FAD2) and FAD3, was then compared between BnTT8 double mutants and the corresponding WT during seed development. Relative to WT, the expression of FUS3, FAD2 and LEC1 was significantly up-regulated in the BnTT8 mutant at 14 DAF and/or 28 DAF seeds (Fig. 7). There were no significant changes in the expression of LEC2, FAD3 and FAE1 when compared to WT and BnTT8 mutant (Fig. 7). The expression changes in these genes agreed well with the phenotypic variations of oil content and FA composition between WT and the BnTT8 mutants. Thus, these
findings reveal significant roles for BnTT8 in controlling FA biosynthesis and accumulation.

Discussion

The CRISPR/Cas9-targeted mutations in BnTT8 is a promising strategy for yellow-seeded rapeseed breeding

Yellow seed is a desirable trait for Brassica oilseed crop breeding due to its better quality than the black-seeded variety (Marles and Gruber, 2004; Meng et al., 1998; Tang et al., 1997). Unfortunately, no natural yellow-seeded germplasm has been found in rapeseed, which is recognized as a major obstacle in yellow-seeded rapeseed breeding (Liu et al., 2005). Thus, the constant creation and use of novel genetic variants are important to the improvement of this trait. To this end, an effective approach is needed to produce targeted mutations in these well-conserved tt homologs in B. napus. The newly developed CRISPR/Cas9 technology provides a powerful approach to create novel allelic variation. Thus far, it has been successfully utilized to modify several important agronomic traits in rapeseed, such as multilocular silique, plant height and architecture, and pod shatter resistance, by generating specific gene knockouts (Yang et al., 2017; Yang et al., 2018; Zhai et al., 2019; Zheng et al., 2019).

However, no example of the yellow-seeded improvement in rapeseed via genome editing has been published yet. In this study, we show the successful utilization of CRISPR/Cas9 for targeted mutations of the BnTT8 gene in rapeseed with high efficiency. A visible yellow-seeded knockout phenotype can only be recovered after targeted mutations in both functional copies of the BnTT8 gene in rapeseed (Table 1; Fig. 3a), which further supports the idea that the role of the TT8 gene is essential for seed coat colour and is highly conserved in Brassica species. Chemical staining of seed coats further confirmed that the specific PA deposition in the seed coat was blocked in the double mutants but not in either of the single mutants (Fig. 3c and d), indicating that the two gene copies have redundant functions in seed colour formation. However, differences between the two BnTT8 copies were observed, with only the BnA09.TT8 single mutant resulting in significantly reduced seed coat thickness, whereas the BnC09.TT8b single mutant had no significant effects (Fig. 4b). Thus, the two functional copies of the BnTT8 gene have partially redundant functions in seed coat development, with BnA09.TT8 making a greater contribution than BnC09.TT8b. These observations are in accord with the higher expression level of BnA09.TT8 than BnC09.TT8b during seed development.

The visible yellow-seeded knockout phenotype was first present in five independent T0 transgenic plants and presented the fastest possible scenario in targeted mutations to a polyploid crop. The targeted mutations were stably transmitted to consecutive generations, and a collection of homozygous mutants with loss-of-function alleles of the target genes was acquired for phenotyping (Fig. 2c; Table 1). Consistent with previous studies, the seed oil contents of the double mutants steadily increased by 9.47%, 5.89% and 5.80% relative to the corresponding WT seeds from generations T0 to T3, respectively (Tables 2, 3). Among the selected double mutants, TT8-96-3-2 showed relatively lower oil content (Table 3). Comparison of the mutated locations in these double mutants revealed that TT8-96-3-2 is the only one carrying mutations at the last target (S4) in the BnTT8 gene (Figs 2c; S5), which may represent a weaker allele mutant and
contribute to its weaker phenotypic effect. Since a 1% increase in the seed oil content of rapeseed is equivalent to a 2.3%–2.5% increase in seed yield (Wang, 2004), the yellow-seeded mutants produced in the present study might offer excellent starting germplasms for promoting high oil production breeding in rapeseed. The protein contents of the double-mutant seeds were also consistently enhanced across generations (Tables 2 and 3), which were statistically significant in T0, T2 and two of five T3 generations (Tables 2 and 3). The simultaneous increases in both oil and protein contents in the BnTT8 double mutant were consistent with the advantages of yellow-seeded B. napus over its black-seeded type reported previously (Marles and Gruber, 2004; Meng et al., 1998; Tang et al., 1997). In contrast, the tt8 mutation in Arabidopsis markedly increased oil content and lowered the protein accumulation of mature seeds (Chen et al., 2014). Thus, BnTT8 has functionally diverged from its orthologues in Arabidopsis. The double mutants also produced seeds with consistently altered FA composition across generations (Tables 2, 3). The simultaneous increases in both oil and protein contents in the BnTT8 double mutant were consistent with the advantages of yellow-seeded B. napus over its black-seeded type reported previously (Marles and Gruber, 2004; Meng et al., 1998; Tang et al., 1997). In contrast, the tt8 mutation in Arabidopsis markedly increased oil content and lowered the protein accumulation of mature seeds (Chen et al., 2014). Thus, BnTT8 has functionally diverged from its orthologues in Arabidopsis. The double mutants also produced seeds with consistently altered FA composition across generations (Tables 2, 3). Assessing the yield-related traits revealed that these mutants did not cause any severe defects (Table S2), which was consistent with previous reports that most yellow-seeded materials appear normal morphologically (Yu 2013).

We assayed all potential off-target loci, and none of them displayed evidence of a CRISPR/Cas9 system-induced mutation, indicating that the off-target effect is negligible for well-designed specific sgRNAs (Table S3). In addition, a variety of transgene-free B. napus plants with homozygous mutations in the target gene were obtained through genetic segregation (Table 1), which would provide valuable resources for rapeseed breeding programmes.

BnTT8 is critical for the specific accumulation of PAs in the inner seed coat

In Brassicaceae, PAs are the prominent pigments that accumulate especially in the inner integument of the seed coat, where they confer on seeds a dark colour by their oxidation during maturations (Lepinec et al., 2006; Marles and Gruber, 2004). Based on our vanillin and DMACA assays, it was clearly shown that PA accumulation intensely started at 21 DAF in the black-seeded WT but not in the yellow-seeded mutants of BnTT8 (Fig. 3c and d). Recently, Hong et al. (2017) also observed the same phenomenon by chemical staining of developmental seeds in rapeseed, indicating the 21 DAF is an essential stage for seed coat coloration in rapeseed. Consistent with this finding, the expression level of BnTT8 showed a gradual increase during the early seed developmental stage and peaked at 23 DAF or 35 DAF (Fig. 1), which suggested a positive connection between the expression levels of the BnTT8 gene and seed coat coloration in rapeseed. Further histological analysis showed that PA accumulation was deposited specifically in the inner integument of the

Figure 4

Mutations in the BnTT8 gene affected seed development and blocked the PA deposition in the seed coat. (a) Microscopy of Safranin O and Fast Green-stained transverse sections from 28 DAF and 42 DAF seeds of WT and BnTT8 mutants. ‘aaCC’, ‘AAcc’ and ‘aacc’ represent homozygous mutations of the target gene in BnA09.TT8, BnC09.TT8b and both copies, respectively. Arrowheads indicate the red-stained PA deposited in the endothelial cell of seed coats; ep, epidermis; p, palisade layer; en, endothelial cell layer. Bars, 100 µm. (b) Microscopic observation of the thickness of mature seed coat. Bars, 50 µm. Data are presented as means ± SE (n ≥ 15); Student’s t-test was used for statistical analysis between the mutant and its WT (**, P < 0.01).
Table 2 Seed oil and protein contents, and FA composition of BnTT8 double mutant (aacc) and WT in T0 and T2 lines

| Genotype | Generation | Oil content (%) | Protein content (%) | C16:0 (%) | C18:0 (%) | C18:1 (%) | C18:2 (%) | C18:3 (%) | C20:1 (%) |
|----------|------------|-----------------|--------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| WT       | T0         | 47.32 ± 0.58    | 15.40 ± 0.28       | 3.92 ± 0.04| 2.57 ± 0.13| 72.22 ± 0.66| 13.84 ± 0.25| 6.85 ± 0.36| 0.60 ± 0.10|
| aacc     | T0         | 51.80 ± 0.74**  | 18.01 ± 1.13**     | 4.17 ± 0.08**| 1.65 ± 0.21**| 64.45 ± 0.74**| 19.69 ± 0.38**| 9.26 ± 0.80**| 0.77 ± 0.03**|
| WT       | T2         | 45.34 ± 1.12    | 18.94 ± 2.65       | 3.95 ± 0.24| 2.83 ± 0.65| 68.13 ± 4.23| 16.78 ± 3.28| 7.46 ± 1.47| 0.86 ± 0.11|
| aacc     | T2         | 48.01 ± 1.08**  | 21.97 ± 1.64**     | 4.41 ± 0.43**| 2.17 ± 0.47**| 63.84 ± 2.63**| 19.84 ± 2.32**| 8.92 ± 0.90**| 0.83 ± 0.10|

The data represent the mean ± SD; Student’s t-test was used for statistical analysis between the mutant and its corresponding WT (*, P ≤ 0.05; **, P ≤ 0.01).

Table 3 Seed oil and protein contents, and FA composition of WT, single and double homozygous mutant of BnTT8 without T-DNA in T3 generation

| Materials | Genotype | Oil content (%) | Protein content (%) | C16:0 (%) | C18:0 (%) | C18:1 (%) | C18:2 (%) | C18:3 (%) | C20:1 (%) |
|-----------|----------|-----------------|--------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| J9707     | WT       | 45.40 ± 0.36    | 19.06 ± 0.43       | 3.99 ± 0.13| 2.96 ± 0.41| 67.54 ± 2.16| 17.20 ± 1.43| 7.54 ± 0.62| 0.78 ± 0.05|
| T18-291-9-7| aCC    | 45.22 ± 1.04    | 18.22 ± 0.63       | 4.10 ± 0.15| 3.23 ± 0.27| 69.98 ± 0.48*| 14.39 ± 0.36**| 7.61 ± 0.09| 0.69 ± 0.02**|
| T18-270-11-3| AAcc | 44.57 ± 0.52    | 20.09 ± 0.60       | 4.25 ± 0.10**| 2.92 ± 0.07| 68.53 ± 0.52| 16.46 ± 0.48| 7.18 ± 0.14| 0.66 ± 0.01**|
| T18-384-14-9| AAcc | 45.19 ± 0.09    | 19.19 ± 0.07       | 4.43 ± 0.11**| 3.72 ± 0.07**| 68.39 ± 0.49| 16.19 ± 0.37| 6.59 ± 0.11*| 0.67 ± 0.01**|
| T18-384-14-10| AAcc | 44.94 ± 0.00    | 19.19 ± 0.08       | 4.04 ± 0.10| 2.57 ± 0.05| 68.87 ± 0.53| 16.83 ± 0.32| 6.88 ± 0.30| 0.81 ± 0.01|
| T18-299-12-2| aacc  | 47.92 ± 1.09**  | 20.15 ± 0.58*      | 4.02 ± 0.05| 2.22 ± 0.06**| 62.43 ± 0.31**| 21.34 ± 0.23**| 9.22 ± 0.10**| 0.77 ± 0.01|
| T18-270-1-9 | aacc   | 48.66 ± 0.74**  | 20.21 ± 0.24*      | 4.20 ± 0.01*| 2.53 ± 0.11| 63.41 ± 0.28**| 21.29 ± 0.27**| 7.80 ± 0.10| 0.77 ± 0.01|
| T18-384-13-3| aacc  | 48.97 ± 0.95**  | 19.43 ± 0.36       | 4.99 ± 0.13**| 2.63 ± 0.09| 62.77 ± 0.13**| 20.87 ± 0.29**| 8.02 ± 0.10| 0.71 ± 0.02*|
| T18-96-3-2  | aacc   | 46.69 ± 0.56*   | 19.38 ± 0.81       | 4.71 ± 0.02**| 2.86 ± 0.09| 61.48 ± 0.06**| 23.25 ± 0.16**| 7.01 ± 0.01| 0.68 ± 0.01**|
| T18-281-5-5  | aacc   | 48.16 ± 0.80**  | 19.12 ± 0.10       | 4.49 ± 0.12**| 2.80 ± 0.06| 63.69 ± 0.88**| 20.41 ± 0.56**| 7.87 ± 0.18| 0.73 ± 0.01|

The data represent the mean ± SD; Student’s t-test was used for statistical analysis between the mutant and its corresponding WT (*, P < 0.05; **, P < 0.01).
black seed coat (Fig. 4a). However, the disruption of BnTT8 completely blocked the PA deposition in the seed coat (Fig. 4a), suggesting a critical role for BnTT8 in the control of the specific accumulation of FAs in the seed coat.

Accumulating evidence indicates that PA biosynthesis in seeds is mainly controlled by the TT8-involved MBW ternary protein complexes (Xu et al., 2014). In Arabidopsis, TT8 is necessary for normal expression of LBGs by directly binding to their regulatory region but does not affect the expression of EBGs (Xu et al., 2014). Similar observations of the involvement of the TT8 gene in regulating the expression of LBGs have been reported in B. rapa and B. juncea (Li et al., 2012a, 2012b; Padmaja et al., 2014), which indicated that TT8 is a regulator of ‘late’ flavonoid metabolism (Nesi et al., 2000). In the present study, transcriptomic profiling revealed that the disruption of BnTT8 genes reduced not only the expression of LBGs but also that of EBGs, as well as 4CL, C4H and PAL, which participated in phenylalanine metabolism (Fig. 5; Table S8). Considering that only DFR, LODX, BAN, TT19 and AHA10 in the flavonoid biosynthesis pathways contain cis-regulatory motifs that can be directly targeted by TT8-involved complexes (Xu et al., 2014), the broad suppression of phenylpropanoid/flavonoid biosynthesis genes may be the outcome of unknown regulatory mechanisms in rapeseed. Recently, two transcriptomic studies also found that these down-regulated DEGs in yellow-seeded coats were enriched in phenylpropanoid and flavonoid biosynthesis in resynthesized yellow-seeded rape-seed as research materials (Hong et al., 2017; Jiang et al., 2019). Although the causal gene underlying the yellow-seeded trait is still not clear in their materials, the two functional copies of BnTT8 were significantly down-regulated in yellow seed compared with black seed in both cases (Hong et al., 2017; Jiang et al., 2019). It is reasonable to postulate that BnTT8 is involved in the down-regulation of these DEGs in phenylpropanoid and flavonoid biosynthesis pathways directly or indirectly. Together, these findings indicate that BnTT8 plays a key role in the regulation of PA accumulation in the seed coat, and the molecular mechanism underlying seed colour formation in rapeseed is much more complex than that in Arabidopsis and other Brassica species.

The mechanism by which BnTT8 alters oil content and FA composition in seeds

In Brassica oil crops, the increased oil content of yellow seeds is widely considered to be a consequence of thinner seed coats because most of the total oil of a seed is synthesized and stored in the embryo (Yu, 2013). Indeed, we observed a thinner seed coat because most of the total oil of a seed is synthesized and stored in the seed coat. Indeed, we observed a thinner seed coat because most of the total oil of a seed is synthesized and stored in the seed coat.

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Materials and methods

Plant materials

In this study, the B. napus pure line J9707 was used as the transformation receptor, and the seeds were obtained from the National Engineering Research Center of Rapseed, Wuhan, China. Flowers on the primary inflorescence were marked at anthesis, and the seeds at various developmental stages were collected for the following experiments of RNA-seq, microscopy and chemical staining.

Construction of the CRISPR/Cas9 vector and plant transformation

The binary pYLCRIPSR/Cas9 multiplex genome targeting vector system was utilized for gene editing in this investigation (Ma et al., 2015). The selection of sequence-specific sgRNAs in the target gene, CRISPR/Cas9 construct assembly and Agrobacterium tumefa-ciens-mediated hypocotyl transformation in B. napus were conducted as previously described (Yang et al., 2018). The oligos employed in constructing the sgRNA vectors are listed in Table S1. The resulting construct is described in detail in Fig. 2b.

Identification of transgenic plants and potential off-targets

The transgenic plants were screened by hygromycin selection (25 mg/L). Then, the presence of the T-DNA in the construct was assessed by PCR using the specific primer pairs BnTT8T2-F/TPB-R (Table S1).

The targeted mutations were determined in transgenic plants using the HI-TOM platform (Liu et al., 2019). Target-specific and barcoding PCR, that is two rounds of PCR, were performed to
Figure 5  Phenylpropanoid and flavonoid biosynthetic genes regulated by BnTT8 in rapeseed. The gene copy numbers from the genome and from the DEGs in the present study are listed in parentheses, respectively. Log2 fold changes (aac/wt) of DEGs at 14 DAF and 35 DAF are illustrated with green (down-regulation) or red (up-regulation) boxes ordered left to right. Names of genes are indicated in capital letters, and corresponding mutants in lower-case italics. EBGs and LBGs are indicated in red and blue, respectively. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase/anthocyanin synthase; ANR, anthocyanidin reductase; GST26, glutathione S-transferase 26; MATA, multidrug and toxic efflux transporter; AHA10, auto-inhibited H+ATPase isoform 10; LAC15, laccase 15; EGL3, enhancer of glabra 3; TT2/8, transparent testa 2, 8; TTG1, transparent testa glabra 1; EBGs, early biosynthetic genes; LBGs, late biosynthetic genes.
amplify the genomic region encompassing the specific targets of independent samples, and the resulting PCR products were mixed in equal amounts and purified for next-generation sequencing (the Illumina HiSeq platform at the Novogene Bioinformatics Institute, Beijing, China). The resulting sequencing data were then decoded by a corresponding online tool to track the mutations of the target sites (http://www.hi-tom.net/hi-tom/). The target-specific primer sets are listed in Table S1.

The potential off-target sites were identified using CRISPR-P2.0 (http://crispr.hzau.edu.cn/CRISPR2/). The specific primers that surrounded the potential off-target sites (Table S1) were used to perform PCR amplification from mixed genomic DNA of 30 T0 editing plants. PCR amplification, DNA library construction, sequencing on the Illumina HiSeq 3000 system and data analysis were conducted in accordance with the approach previously described by Yang et al. (2018).

RNA extraction and qRT-PCR
Total RNA was prepared using the EasyPure Plant RNA Kit (TransGen Biotech, Beijing, China), and cDNA was synthesized using the Transcript RT Kit (TransGen Biotech). qPCR was carried out using the TransStart Top Green qPCR SuperMix Kit (TransGen Biotech) on a CFX384 Real-Time System (Bio-Rad). Relative quantification was performed using the comparative cycle threshold method, and the relative amount of PCR product that was amplified using the designed primer sets (Table S1) was normalized to the reference genes BnPP2A-1, BnACT7 and BnUBC10.

RNA-seq transcriptomic analysis
Seed coat tissues were sampled with three biological replicates at 14 and 35 DAF. At each stage, seed coats were hand-dissected from seeds gently on dry ice, immediately frozen in liquid nitrogen and stored at −80 °C until total RNA extraction.

RNA extraction, cDNA library construction, sequencing, quality control, and read mapping to the reference genome, identification of DEGs, and GO and KEGG pathway enrichment analysis of DEGs were performed essentially as described by Shahid et al. (2019). Fragments per kilobase of transcript per million mapped reads (FPKM) was calculated as a measure of the level of gene expression. Genes with a false discovery rate (FDR) ≤0.05 and an absolute value of log2 fold change ≥1 between mutant and wild type (WT) at each stage were defined as DEGs. The raw sequence data were deposited in the NCBI Sequence Read Archive (Accession No. MN399822).

Metabolite profiling
Flavonoids were extracted from mature seeds (100 mg dry weight) of the double mutants and WT with six biological replicates and were analysed by LC-ESI-MS/MS system at the National Key Laboratory for Crop Genetic Improvement (Huazhong Agricultural University, Wuhan, China). The sample extraction and flavonoid metabolic analysis were done essentially as previously described (Chen et al., 2013).

Light microscopy
Developing seeds at 28 DAF and 42 DAF were harvested for microscopic analysis. Tissue fixation, embedding, sectioning, and Safranin O and Fast Green staining were performed essentially as described by Li et al. (2012a). Images were obtained using a Nikon ECLIPSE 80i compound microscope.

Vanillin and DMACA staining
The vanillin and DMACA assays were both used for the specific detection of PA accumulation in the developing seed coat. The staining of dissected seed coats was done essentially as previously described (Hong et al., 2017).

Field experiments and phenotyping
The T0 and T1 transgenic and WT plants were grown in a greenhouse (16/8 h of light/dark at 22 °C) in 2016 and 2017, respectively. The selected homozygous mutant T2 lines without T-
DNA were grown in the winter-type oilseed rape growing season (2018–2019) on the experimental farm of Huazhong Agriculture University, Wuhan, China. The field experiment followed a randomized complete block design with three replications. Each line was planted in one row with 11–12 plants per row, with a distance of 21 cm between plants within each row and 30 cm between rows. The field management was performed in line with standard breeding practice.

Mature seeds were used for the measurement of seed quality traits, including the protein and oil contents, and FA composition. The FA profiles were determined using gas chromatography (GC) system with a Model 6890 GC analyser (Agilent Technologies, Inc., Wilmington, DE) according to the method previously described by Yang et al. (2012). The protein and oil contents were examined using a Foss NIRSystems 5000 near-infrared reflectance spectroscopy (NIRS) using the parameters described by Gan et al. (2003), and the content is expressed as a percentage of the total seed dry weight.

Yield-related traits, including plant height, branch height, branch number, silique length, number of seeds per silique, 1000-seed weight and yield/plant, were measured as previously described (Cai et al., 2016).

Figure 7 The BnTT8 targeted mutation resulted in altered expression of genes involved in FA biosynthesis during seed development. Relative gene expression in different stages of seeds of WT and the BnTT8 double mutant (aacc) was determined by qRT-PCR with normalization to BnUBC10. Values are the means ± SE of three biological replicates. Student’s t-test was used for statistical analysis between the mutant and its WT (*, P < 0.05).

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Conflict of interest
On behalf of all authors, the corresponding author states that there is no conflict of interest.

Author contributions
Conceived and designed the experiments: FC, ZY; Performed the experiments: ZY, CS, HL, XL, YY, MB, JY, ZC, SUK; Wrote the manuscript: FC, ZY, YK, AO, MHK; Bioinformatics analysis: YK, ZY.

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Supporting information

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

Figure S1 Organization of the predicted TT8 protein indicating the localization of the conserved domains in Arabidopsis and different Brassica species.

Figure S2 Alignment of TT8 homolog sequences identified from B. napus (BnaA09.TT8, BnC09.TT8a and BnC09.TT8b), B. rapa (BrTT8), B. oleracea (BoTT8a and BoTT8b), B. juncea (BjuA.TT8 and BjUB.TT8), and A. thaliana (AtTT8). The base differences are highlighted in grey boxes.

Figure S3 Sequence alignment of two functional BnTT8 gene copies in J9707.

Figure S4 Phylogenetic tree showing the sequence relationship among TT8 homologs identified from various plant species.

Figure S5 The predicted amino acid sequences of BnTT8 homozygous mutants in T2 generation.

Figure S6 Pearson correlation coefficient among counts of transcriptome data.

Figure S7 Venn diagrams summarizing the number of differentially expressed genes detected in 14 DAF and 35 DAF seed coats of BnTT8 double mutant (aacc) and WT.

Figure S8 Number of up- and down-DEGs between BnTT8 double mutant (aacc) and WT identified in developing seeds (14 DAF and 35 DAF).

Figure S9 Results of GO annotation of all up- and down-regulated genes.

Figure S10 Results of Top 20 GO annotation of all up- and down-regulated genes.

Figure S11 Results of KEGG pathway of all up- and down-regulated genes.

Figure S12 Validation of RNA-seq data by using qRT-PCR.

Table S1 Primers used in the present study.

Table S2 Yield related traits of WT, the single and double homozygous mutant of BnTT8 without T-DNA in T3 generation.

Table S3 Detection of potential off-target effect at each sgRNA target site.

Table S4 Statistics of RNA-seq reads and mapped reads.

Table S5 DEGs in BnTT8 double mutant (aacc) and WT seed coats at 14 DAF and 35 DAF.

Table S6 Enriched GO terms of DEGs related to the Phenylpropanoid and Flavonoid metabolites with significant difference between BnTT8 double mutant (aacc) and WT during seed development using Blast2GO.

Table S7 KEGG pathways of DEGs between BnTT8 double mutant (aacc) and WT during seed development.

Table S8 DEGs related to the Phenylpropanoid and Flavonoid metabolic processes.

Table S9 Flavonoid metabolites identified in the mature seeds of BnTT8 double mutant (aacc) and its WT control.

Table S10 Flavonoid metabolites with significant difference between BnTT8 double mutant (aacc) and its WT control.