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Creating yellow seed *Camelina sativa* with enhanced oil accumulation by CRISPR-mediated disruption of *Transparent Testa 8*

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Summary

Camelina (*Camelina sativa* L.), a hexaploid member of the Brassicaceae family, is an emerging oilseed crop being developed to meet the increasing demand for plant oils as biofuel feedstocks. In other Brassicas, high oil content can be associated with a yellow seed phenotype, which is unknown for camelina. We sought to create yellow seed camelina using CRISPR/Cas9 technology to disrupt its Transparent Testa 8 (TT8) transcription factor genes and to evaluate the resulting seed phenotype. We identified three *TT8* genes, one in each of the three camelina subgenomes, and obtained independent *CsTT8* lines containing frameshift edits. Disruption of *TT8* caused seed coat colour to change from brown to yellow reflecting their reduced flavonoid accumulation of up to 44%, and the loss of a well-organized seed coat mucilage layer. Transcriptomic analysis of *CsTT8*-edited seeds revealed significantly increased expression of the lipid-related transcription factors *LEC1*, *LEC2*, *FUS3*, and *WRI1* and their downstream fatty acid synthesis-related targets. These changes caused metabolic remodelling with increased fatty acid synthesis rates and corresponding increases in total fatty acid (TFA) accumulation from 32.4% to as high as 38.0% of seed weight, and TAG yield by more than 21% without significant changes in starch or protein levels compared to parental line. These data highlight the effectiveness of CRISPR in creating novel enhanced-oil germplasm in camelina. The resulting lines may directly contribute to future net-zero carbon energy production or be combined with other traits to produce desired lipid-derived bioproducts at high yields.

Introduction

Plant oils (triacylglycerol or TAG) are increasingly used for biofuel feedstocks because they have a high energy density and they are compatible with the current energy infrastructure (Wang *et al*., 2022). *Camelina sativa*, an allohexaploid oil crop in the Brassicaceae family, has garnered attention for its relatively high oil yield, short generation time, stress resistance, and low resource requirements (Yuan and Li, 2020). Increasing seed oil content is one of the primary targets for improving camelina productivity (Marisol Berti *et al*., 2016).

In Brassicaceae, dark brown seed coats result from the accumulation of an oxidized flavonoid known as proanthocyanidin within the endothelium layer of the inner integument of the seed coat (Lepiniec *et al*., 2006). Flavonoid accumulation has been extensively studied in the model plant *Arabidopsis*, revealing two distinct groups of activities. The first group consists of early biosynthetic genes (EBGs), including chalcone synthase, chalcone isomerase, flavanone-3-hydroxylase, and flavanone-3′-hydroxylase. The second group comprises late biosynthetic genes (LBGs), such as dihydroflavonol reductase (DFR), leucocyanidin dioxygenase, and anthocyanidin reductase (ANR or BAN) (Hartmann *et al*., 2020). In addition to these enzymes, various regulatory proteins play pivotal roles in flavanol cell biology, including TT1, TT2, TT8, TT16, TTG1, TTG2, TT12, TT19, and AHA10, which are likely involved in the compartmentation of flavonoids (Lepiniec *et al*., 2006). It has been established that the regulation of gene expression, particularly that of LBGs such as DFR and BAN, is tightly coordinated by a ternary complex of TT2 (R2R3-MYB), TT8 (basic helix–loop–helix, bHLH), and the WD40 regulatory protein encoded by TTG1, referred to as the MYB-bHLH-WD40 (MBW) complex which ultimately regulates the biosynthesis of proanthocyanidins (Baudry *et al*., 2004; Hichri *et al*., 2011). The MBW complex activates proanthocyanidin biosynthesis in developing seeds, influencing seed colour (Hartmann *et al*., 2020). The *TT8* gene, known as *Transparent Testa 8*, is a transcriptional repressor that plays a pivotal role in metabolism, specifically in the regulation of proanthocyanin biosynthesis (Baudry *et al*., 2004). Mutations or variations in *TT8* led to alterations in anthocyanin production,

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affecting the coloration of plant tissues (Chen *et al.*, 2014; Padmaja *et al.*, 2014). Moreover, TT8 (bHLH42) plays negative roles in regulating seed fatty acid biosynthesis by repressing LEC1, LEC2, and FUS3 (Chen *et al.*, 2014). Knocking out *TT8* not only changes seed coat colour but also increases oil content in Arabidopsis (Chen *et al.*, 2014) and *Brassica napus* (Li *et al.*, 2023; Zhai *et al.*, 2020).

Genome editing, particularly by CRISPR/Cas9 technology, has become a key method for understanding and manipulating gene function (Doudna and Charpentier, 2014). This technology has been successfully applied to camelina, where knocking out the Fatty Acid Desaturase 2 (*FAD2*) genes increased oleic acid content while reducing the levels of long-chain polyunsaturated fatty acids (Han *et al.*, 2022; Jiang *et al.*, 2017; Lee *et al.*, 2021; Morineau *et al.*, 2017). Deactivating Fatty Acid Elongase1 (FAE1) resulted in reduced production of very long-chain fatty acids and increased levels of oleic acid or α-linolenic acid (Ozseyhan *et al.*, 2018). Editing seed storage protein CRUCIFERIN C (CsCRUC) did not affect the total seed protein content but altered the abundance of cruciferin isoforms and other seed storage proteins (Lyzenga *et al.*, 2019). In another example, the multiplex editing of genes in camelina enabled the creation of a stable early-flowering trait (Bellec *et al.*, 2022).

Yellow seed is a highly desirable trait in Brassica oilseed crops (Marles and Gruber, 2004; Meng *et al.*, 1998; Rahman and McVetty, 2011; Tang *et al.*, 1997) because it is often associated with higher oil content compared to dark-seed varieties. In addition, yellow seeds typically have lower pigment content and reduced hull mass. Because the oil-rich interior is a higher proportion of the overall seed mass, oil extraction from yellow seed varieties is more cost-effective. The reduced pigment also improves oil quality (Meng *et al.*, 1998). Camelina has no naturally occurring yellow seed variants and it is an open question as to whether their oil contents would be higher in *TT8*-disrupted lines. To explore this knowledge gap, we identified three *CsTT8* isoforms in *C. sativa* and used CRISPR/Cas9 technology to create null mutants. In the C*stt8* mutants, flavonoid accumulation was reduced by 44%. Carbon allocation was redirected toward enhanced synthesis of fatty acids which accumulated to as high as 38% of dry weight (DW) without changes in starch and protein contents. Disrupting all three *TT8* alleles successfully created yellow seed camelina with increased TAG yield of more than 21%.

Results

Identification of *TT8* homologues in *Camelina sativa*

Using the sequence of *AtTT8* to BLAST against the camelina genome of *C. sativa* (Kagale *et al.*, 2014), we identified three *TT8* isoforms designated as *CsTT8-2* (*Csa02g028180.1*), *CsTT8-8* (*Csa08g037600.2*) and *CsTT8-13* (*Csa13g044750.1*) located on chromosomes 2, 8 and 13, respectively. *CsTT8-2* and *CsTT8-8* each contain six exons, while *CsTT8-13* has an extra exon (Figure 1a). Like *AtTT8*, the predicted amino acid sequences of all three *CsTT8*s contain a series of conserved domains: the N-terminal MYB interaction region (MIR), the bHLH domain in the C-terminal region (Figure 1a). The CsTT8-2 protein shares 92.46% and 91.87% identity to CsTT8-8 and CsTT8-13, respectively. CsTT8-8 and CsTT8-13 protein are 97.84% identical at the amino acid level. The high identity shared by all three CsTT8 genes suggests that they encode transcription factors with the same or similar functions. Phylogenetic analysis shows that all three *CsTT8* copies cluster in a clade with *AtTT8* (Figure S1). In addition, assessment of gene synteny relationships with the GenomicusPlantv49.01 online resource (https://doi.org/10.1093/nar/gkx1003) showed that *CsTT8-2*, *CsTT8-8* and *CsTT8-13* are syntenic orthologs to *AtTT8*. Taken together this suggests similar, or equivalent functions of these three *TT8* genes in camelina.

Expression analysis of the *CsTT8* genes

The tissue specific expression patterns of *CsTT8* isoforms were investigated in camelina variety Suneson using quantitative real-time PCR (RT-qPCR) (Figure 1b). Various amounts of transcript were detected for *CsTT8-2*, *CsTT8-8* and *CsTT8-13* in leaf, root, stem and seeds of 12, days afer flowering (DAF) 24 DAF and mature seeds. All three *TT8* genes exhibit high expression levels in developing seeds with the highest levels observed at 12 DAF. Their transcription in roots is much lower than in seeds, but is higher than that in leaves and stems, where it is barely detectable (Figure 1b). These results suggest that *CsTT8*s plays a role in seed development. Overall, *CsTT8-8* had a significantly higher expression level (by 1.5 to 3-fold) compared to *CsTT8-2 and CsTT8-13* during seed development (Figure 1b). Further analysis of *CsTT8* genes in developing seeds by RNAseq revealed strong expression for all three *TT8* genes (Figure 1c). High expression levels were detected at early stages that peaked at 10–12 DAF, and subsequently decreased from 14–22 DAF. Again, *CsTT8-8* demonstrated the highest expression at the peak stages, followed by *CsTT8-2*, while CsTT8-13 exhibited the lowest expression level (Figure 1c). These results are consistent with those obtained from RT-qPCR, although the 24 DAF seeds showed higher expression by RT-PCR, possibly due to different growth conditions.

Creation of CRISPR/Cas9-targeted mutations in *CsTT8s*

To generate Cas9-induced knockout mutations in all three *CsTT8* genes, two sets of sgRNAs (S1 and S2) were designed using the CRISPR-P algorithm (Lei *et al.*, 2014). Both sets are in the first exon of *TT8*, with S1 close to the start codon, and S2 targeting the MIR domain (Figure 1a). To facilitate the screening of transgenic lines and achieve transgene-free *CsTT8* mutagenized camelina lines, a *DsRed* expression cassette was inserted into the pHEE401E vector (Wang *et al.*, 2015; Xie *et al.*, 2015). We obtained and planted 33 transgenic seeds with DsRed fluorescence, of which 14 lines were genotyped by DNA sequencing. In the first generation (T_1) (Table 1), lines 13, 22, 26 and 27 showed DNA sequence changes in all three *CsTT8* isoforms, and lines 13 and 22 showed one homozygous target site changed for only a single isoform. Lines 24, 25, 31, 32, and 33 showed mutations in at least one target site in one or two of the three isoforms. But lines 23, 28, 29, 30 and 34 remained unedited in all three isoforms. We then planted dark and red seeds from lines 13, 22, 26, and 27 and genotyped the progeny (T_2) plants (Table 1). Lines 26–6, 26–7, and 27–9 contained homozygous changes in all three isoforms (Figure 2a). Mutations in all three genes resulted in frameshifts that truncate TT8 within the first 65 amino acid resulting in loss of function except for tt8-2 gene in line 26–7, in which deletion of 48 bp of DNA resulted in deletion of 16 amino acid (Figure S2a) resulting in loss of function. Moreover, no changes were observed in the predicted off-target sites (Figure S2). Thus, we obtained three homozygous lines containing edits in all three isoforms of *CsTT8* for subsequent studies. Lines 22–1, 22–2, 27–8, 27–10, and 27–11 had at least one homozygous mutation.

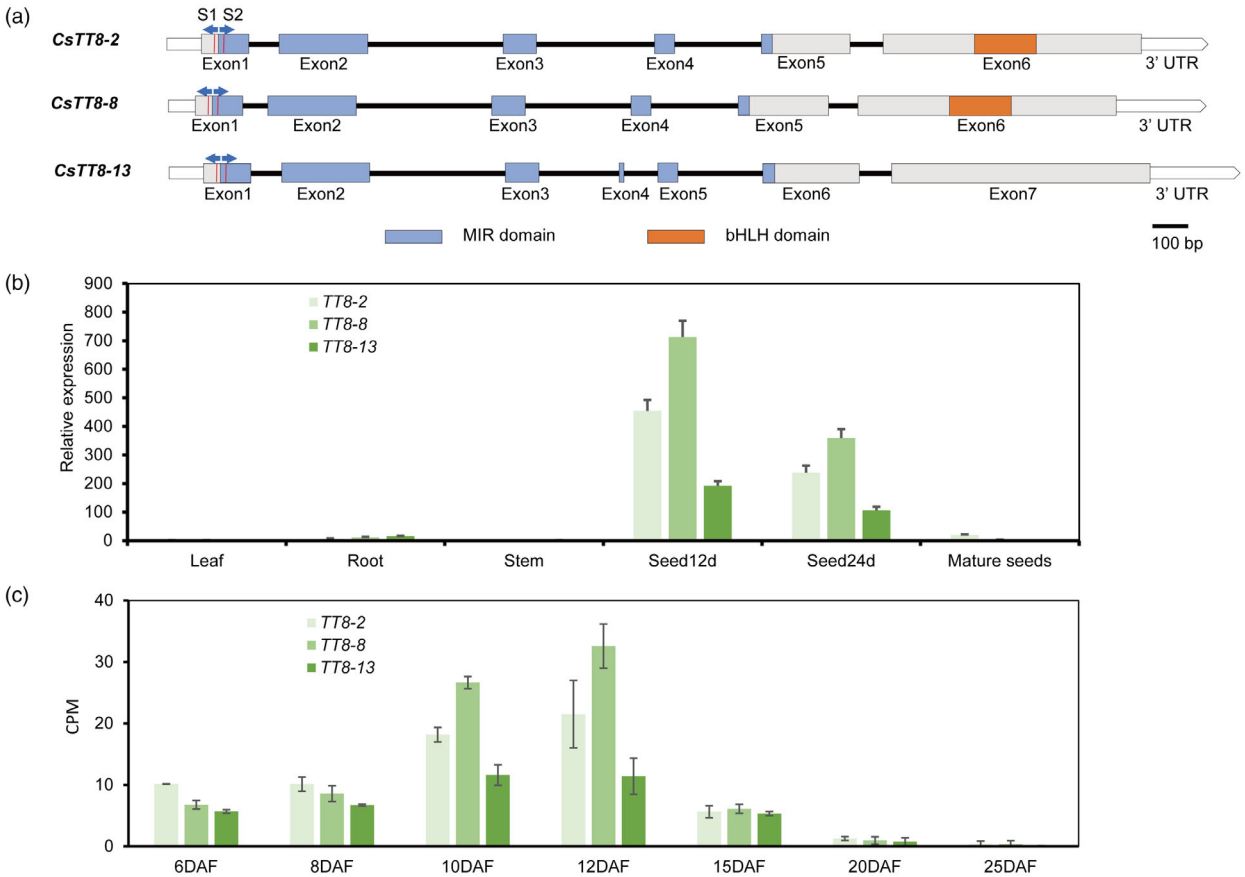

Figure 1 Identification of three TT8 genes in camelina. (a) Two CsTT8 isoforms comprise six exons (box) separated by five introns (represented by the solid line) and one contains 7 exons with six introns. The N-terminal MYB interaction region (MIR) domain and the bHLH domain in the C terminal region are coloured blue and orange, respectively. The vertical dashed lines in the gene model indicate the CRISPR target sites, and the arrows indicate the sgRNA direction. The target sequences are shown with the PAM in red. (b) Expression pattern of three isoforms of *CsTT8* in camelina. Relative gene expression of *CsTT8* in leaf, root, stem, seeds of 12 DAF, 24 DAF and mature seeds of Suneson were determined by qRT-PCR; values are the means ± SE of three biological replicates. (c) Expression of three CsTT8 homologous genes in camelina during seed development. Data (normalized counts per million) are derived from RNAseq experiments.

Editing *CsTT8* produced yellow seed coat

We expected that successful mutagenesis of the *CsTT8* by CRISPR could result in lighter colour in camelina seeds, as disruption of *AtTT8* in *Arabidopsis* resulted in reduced seed pigmentation (Nesi *et al.*, 2000). The seeds of T_1 lines 13, 22, 26, and 27 appeared yellow, whereas seeds of other lines remained brown (Figure S3). In lines 21, 30, 31, 32, and 34, seed coat colour appeared light brown or tan. A reddish-brown colour was observed in the seeds of lines 24 and 33. The seed coat colour changed from brown to pale yellow in the T2 lines 26–7, 26–8, and 27–9 (Figure 2b), correlated with their homozygous CRISPR-induced *CsTT8* mutations (Figure 2a). Interestingly, no discernible alterations in growth or development were observed in these camelina plants, in which all three *CsTT8* genes had been disrupted but were free of the Cas9 and DsRed marker transgenes (Figure S4a).

Effect of *CsTT8* mutations on flavonoid content in camelina seeds

Seeds were sectioned and examined under a dissection microscope. A brown coloration was evident in the wild type (WT) camelina seed coat endothelium layer. However, in all three TT8 modification lines containing homozygous disruptions of all three *TT8* genes, this colour changed to a light-yellow hue, as illustrated in Figure 3a. In Brassicaceae plants, the dark seed coat is attributed to the accumulation of an oxidized form of a flavonoid known as proanthocyanidin within the endothelium layer of the inner integument of the seed coat (Lepiniec *et al.*, 2006). To investigate the relationship between *CsTT8* and the production of flavonoids in camelina seeds, we extracted and quantified their flavonoid contents. The flavonoid content decreased markedly in all three *CsTT8* mutant lines albeit with minor variation at 35.5% in line 26–6, 43.9% in line 26–7, and 44.1% in line 27–9, respectively (Figure 3b). These findings are consistent with the key role of *CsTT8* in the synthesis or regulation of the flavonoids responsible for brown pigmentation in the seed coats of Brassicaceae plants.

Editing *TT8* in camelina changed the seed coat structure

Under the confocal microscope, Differential Interference Contrast (DIC) imaging of wild type seeds showed a well-organized outermost layer of mucilage. In contrast, in all three *Cstt8* mutant lines, the mucilage layer was either partially reduced or completely absent as shown in Figure 4a. To gain further insights

Table 1 Genotyping of CRISPR-CsTT8 transgenic T1 and T2 plants

		Genotype at targets of *CsTT8-2*		Genotype at targets of *CsTT8-8*		Genotype at targets of *CsTT8-13*		
Plant ID	Generation	S1	S2	S1	S2	S1	S2	Seed colour
13	T1	Homo (−3 bp)	Homo (−4 bp)	Hetero	Hetero	Hetero	Hetero	Yellow
22	T1	Hetero	Hetero	WT	Homo (−4 bp)	Hetero	Hetero	Yellow
22–1	T2	Homo (−17 bp)	Homo (−18 bp)	WT	Homo (−4 bp)	Hetero	Hetero	Yellow
22–2	T2	Homo (−17 bp)	Homo (−18 bp)	WT	Homo	Hetero	Hetero	Yellow
22–3	T2	Hetero	Hetero	WT	Homo (−4 bp)	Homo (−15 bp)	Homo (−18 bp)	Yellow
24	T1	WT	WT	WT	Hetero	WT	Hetero	Brown
25	T1	WT	Hetero	WT	WT	Hetero	Hetero	Brown
26	T1	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Yellow
26–4	T2	Hetero	Hetero	Homo (−17 bp)	Homo (−18 bp)	Hetero	Hetero	Yellow
26–5	T2	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Yellow
26–6	T2	Homo (+1 bp)	Homo (−4 bp)	WT	Homo (−4 bp)	Homo	(−49 bp)	Yellow
26–7	T2	Homo	(−48 bp)	WT	Homo (−4 bp)	WT	Homo (−1 bp)	Yellow
27	T1	WT	Hetero	Hetero	Hetero	Hetero	Hetero	Yellow
27–10	T2	WT	Hetero	WT	Homo (−5 bp)	WT	Homo (−4 bp)	Yellow
27–11	T2	WT	Homo (+1 bp)	Hetero	Hetero	Hetero	Hetero	Yellow
27–8	T2	WT	Hetero	Hetero	Hetero	Homo (−1 bp)	Homo (−5 bp)	Yellow
27–9	T2	WT	Homo (+1 bp)	WT	Homo (−5 bp)	WT	Homo (−5 bp)	Yellow
31	T1	Hetero	Hetero	WT	WT	WT	WT	Brown
32	T1	Hetero	Hetero	WT	WT	WT	WT	Tan
33	T1	WT	Hetero	Hetero	Hetero	Hetero	WT	Brown

into changes in the mucilage layer, we stained seed cross-sections with toluidine blue O, a basic thiazine metachromatic dye (Sridharan and Shankar, 2012) that stains polysaccharides purple and nucleic acid blue. The wild type seeds consistently exhibited a clearly stained mucilage layer that was notably absent in the seeds of the *TT8*-modified lines, as exemplified in Figure 4b. Loss of this layer in the seeds of *TT8* mutagenized lines is a visible indicator of the disruption of *TT8* genes on seed coat morphology. We extracted and quantified their mucilage contents. The mucilage content decreased from 2.1% to as low as 0.4%, i.e., an approximately 80% decrease (Figure 4c).

While changes in the levels of mucilage has been reported to influence seed germination (Arsovski *et al.*, 2010), the *CsTT8* edited lines exhibited robust germination on soil (Figure S4b). Consistent results were observed when 10-month-old seeds were germinated on wet filter paper at a similar rate to that of WT (Figure S4c). We also observed longer radicles after 20 h of imbibition in the *TT8* edited lines compared to those in the WT, suggesting accelerated germination.

Editing *TT8* in camelina increased seed oil content

To investigate the potential impact of editing the *CsTT8* genes on other seed metabolites, we performed a comprehensive analysis focusing first on TFA and TAG content. As shown in Figure 5, seed TFA contents increased in all three edited lines. In WT camelina controls, the TFA content was 32.4% of dry weight (DW). However, disruption of *CsTT8* resulted in large increases in TFA content to 37.5%, 38.0%, and 36.1% in the three distinct *CsTT8* mutant lines, respectively. The TAG content also reached levels as high as 34.6% from 28.5% in WT. This concurrent rise of both TFA and TAG in gene edited seeds demonstrates a substantial influence of *CsTT8* on seed lipid metabolism.

We next analysed fatty acid composition by GC–MS and observed a distinct shift in the fatty acid profile. Notably, the editing of *CsTT8* led to a significant increase in 18:1 and 18:2 fatty acids, along with a corresponding decrease in 18:3 fatty acids.

To provide a more comprehensive analysis, we analysed the protein and starch contents of the *TT8* mutant lines. As shown in Figure S6, the protein content in these lines remained largely unaffected. While there was some variation in starch content in the mutant lines, the means were not significantly different from those of WT (Figure S6). The lack of significant changes to protein and starch contents underscores the specificity of *TT8* with respect to lipid metabolism. The hundred-seed weight of 26–6 exhibited no significant change, whereas 26–7 and 27–9 showed a slight decrease (Figure S6c).

Editing *CsTT8* changed the expression levels of genes involved in FA biosynthesis in camelina seeds

Previous studies suggested that TT8 can negatively regulate seed FA biosynthesis by binding to the promoters of the *LEC1*, *LEC2* and *FUS3* transcription factors (Chen *et al.*, 2014). RT-qPCR analysis showed that editing of Cs*TT8* led to substantial increases in the expression levels of these genes in camelina seeds (Figure 6a). Expression of the transcription factor *WRI1* and several key genes associated with fatty acid synthesis, including α *carboxyltransferase* (*CT*), *biotin carboxyl carrier protein (BCCP)1*, *BCCP2*, *3-KETOACYL ACP SYNTHASE* (*KAS)I*, *KASII* displayed an increasing trend in their expression levels whereas *KASIII* showed a significant 2.6-fold increase in abundance. This implies that disruption of *TT8* genes has a positive regulatory effect on these downstream targets that contribute to increased fatty acid production within the seeds.

The disruption of *TT8* also resulted in subtle changes in the expression of fatty acid desaturase genes. Specifically, the *FAD2* transcript was elevated while the *FAD3* transcript displayed a small decrease in the *CsTT8* edited lines (Figure 6c). These

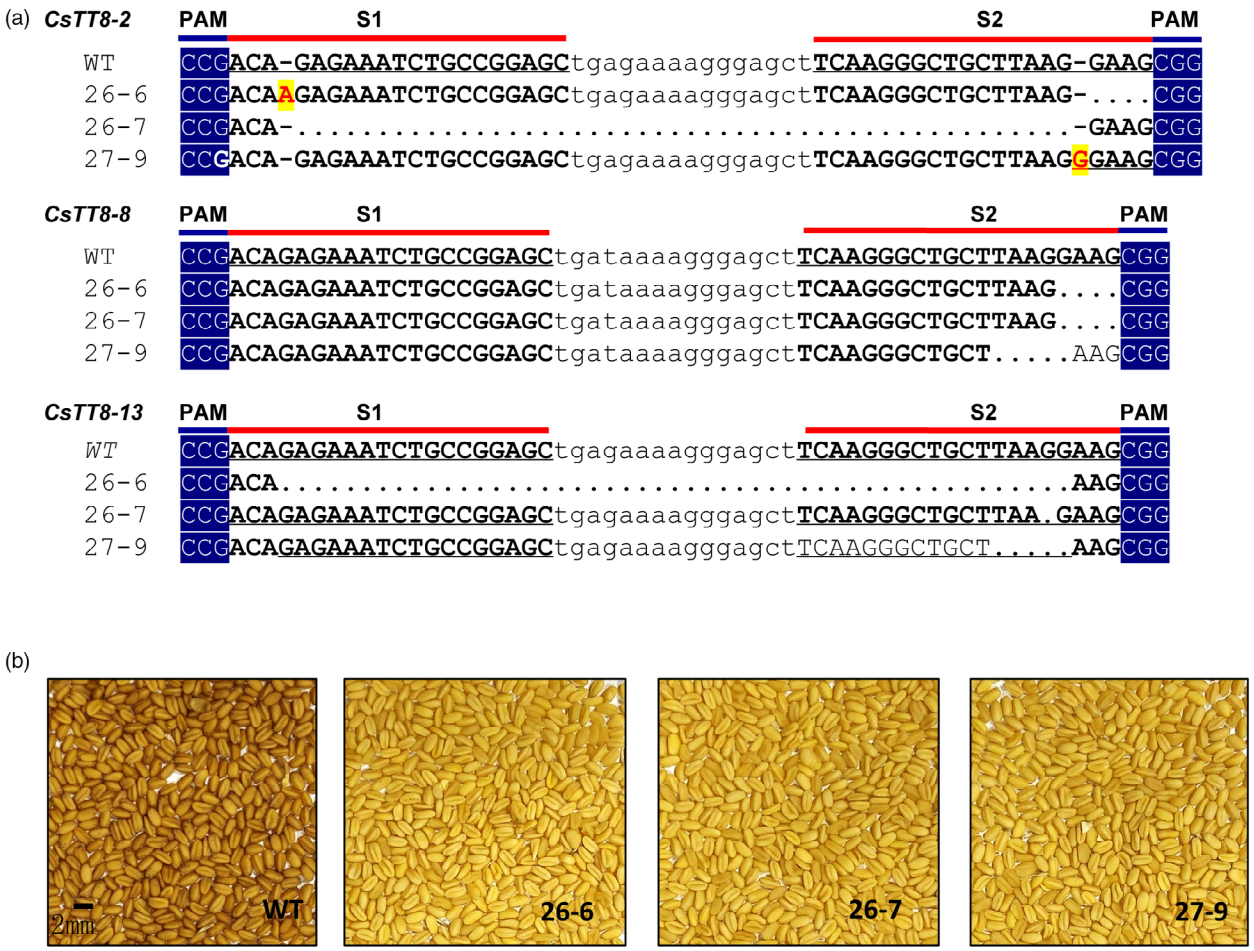

Figure 2 Editing of the *CsTT8* changed seed coat colour to light yellow. (a) Sequences at the sgRNA target sites of null CsTT8 homozygous mutants. The protospacer adjacent motif (PAM) is highlighted in blue, and target site S1 and S2 are labelled. Red letters refer to insertions. (b) Seed coat colour of WT Suneson and three null *TT8* modification lines, 26–6, 26–7 and 27–9 respectively.

changes may have contributed to alterations in the fatty acid composition.

Editing *CsTT8* increased FA biosynthesis rate in camelina seeds

To further elaborate the effect of CsTT8 on seed oil accumulation, developing seeds at 13-15 DAF were collected and their fatty acid synthesis rates were determined by measuring the rate of [^{14}C] acetate incorporation into FAs. As shown in Figure 6d, compared to WT, all three *CsTT8* triple mutants showed a significant increase in its fatty acid synthesis rate relative to the parental line consistent with the observed changes in FAS-related transcripts.

Discussion

CRISPR/Cas9-targeted mutations in *CsTT8* created yellow seed camelina

Within Brassica oil crops, yellow seeds are commonly associated with elevated oil content and reduced pigmentation and hull content (Xie *et al.*, 2020; Zhai *et al.*, 2020). In this study, we successfully created CRISPR camelina lines with mutated *CsTT8* genes that resulted in yellow seeds. Consistent with earlier reports for both Arabidopsis and *Brassica napus*, the camelina mutants exhibited enhanced fatty acid synthesis, leading to a significant 21.4% increase in seed TAG. Furthermore, a close examination of the seeds confirmed a reduction in flavonoid deposition in the seed coat of *Cstt8* mutants, as shown in Figure 3a.

To enable the removal of CRISPR/Cas from lines with desired edits, we introduced a DsRed marker into the CRISPR/Cas vector. T_1 transgenic seeds exhibiting DsRed fluorescence were planted for genotyping, and yellow seeds edited for *CsTT8* genes but lacking the CRISPR/Cas9 were identified by visual screening. These targeted mutations were stably inherited in subsequent generations, resulting in the establishment of a pool of homozygous mutants harbouring loss-of-function alleles of the target genes for subsequent phenotyping (see Figure 2a and Table 1). The yellow seed phenotype initially appeared in four distinct T_1 transgenic plants, demonstrating that CRISPR-Cas9 editing is very efficient in the hexaploid camelina genome, as previously reported (Ozseyhan *et al.*, 2018).

Camelina sativa is a hexaploid with its three sub-genomes arising from two separate polyploidization events (Chaudhary *et al.*, 2020; Kagale *et al.*, 2014). We identified three *CsTT8* homeologs in camelina, with *CsTT8-8* and *CsTT8-13* exhibiting a closer relationship to each other than to *CsTT8-2* (Figure S1). Notably, all three isoforms are preferentially expressed during seed development, with *CsTT8-8* demonstrating a higher

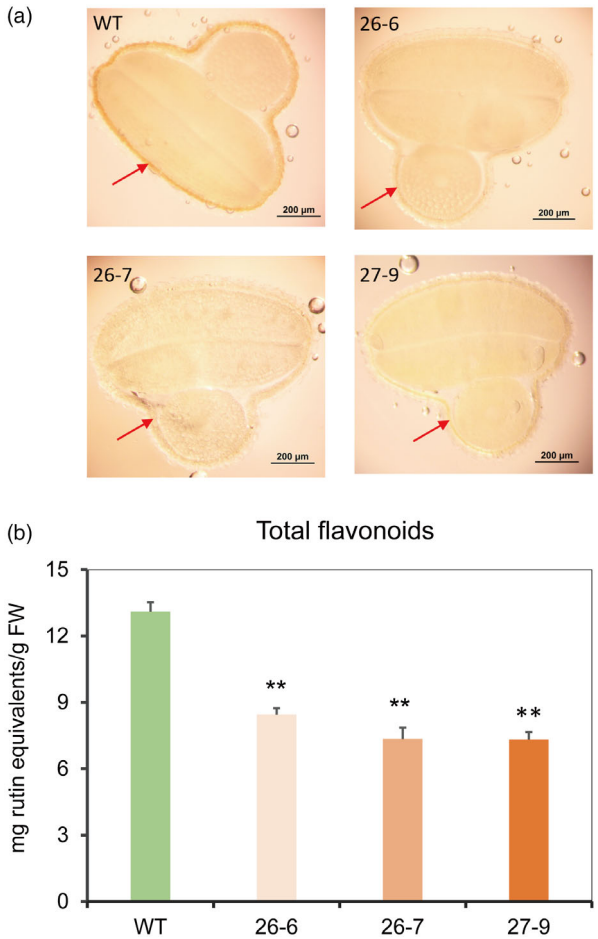

Figure 3 Effect of editing *CsTT8* on flavonoid content in camelina seeds. (a) Transverse section of camelina seeds. Arrowheads indicate the endothelial cells of seed coat, (b) Flavonoid content in camelina seeds. Total flavonoid was extracted from ground seeds and its content was measured and displayed as mg of rutin equivalents per g FW seeds. The values represent the mean ± standard deviation of three biological replicates. **Student *t*-test $P < 0.01$.

expression level compared to the other two isoforms (Figure 1b, c). This observation is consistent with a general increase of gene expression in the Cs-G3 subgenome compared to the other two sub-genomes that was attributed to the two-stage polyploidization pathway (Kagale *et al.*, 2014). Our results suggest that all three *CsTT8* genes play overlapping roles in controlling seed coat colour because mutagenesis of one or two isoforms did not result in a change of seed coat colour from brown to yellow. Indeed, the seed colour change was only observed when all three *CsTT8* genes were mutated (Table 1, Figure 2b, Figure S2). The low likelihood of loss of function mutations arising simultaneously in all three *CsTT8* genes provides a likely explanation as to why no naturally occurring yellow seeds in *Camelina sativa* populations have been identified to date.

CsTT8 mutation accelerated fatty acid synthesis by upregulating the expression of genes involved in FA biosynthesis

Disruption of *TT8* in camelina increased gene expression especially in key lipogenic transcription factor genes, including *LEC1*, *LEC2*, and *FUS3*. This result is consistent with previous reports in Arabidopsis and *B. napus* (Chen *et al.*, 2014). We also observed an upregulated expression of *WRI*, the master transcriptional activator of fatty acid synthesis, and correspondingly increased expression levels of its targets, *αCT*, *BCCP1*, *BCCP2*, *KASI*, *KASII*, and *KASIII* (Kuczynski *et al.*, 2022). The increased expression levels of factors in fatty acid biosynthesis is consistent with the enhanced fatty acid production observed in the *TT8*-edited camelina lines.

Interestingly, disruption of *TT8* also had an impact on the expression of genes encoding fatty acid desaturases. Specifically, the *CsTT8* edited lines had higher *FAD2* but decreased *FAD3* expression favouring the accumulation of 18:2 at the expense of 18:3 (Figure 5c). This differs from *TT8* modification in *B. napus* and Arabidopsis. *FAD2* was significantly upregulated in the *Bntt8* mutant, which led to increases in 16:0, 18:2, and 18:3, accompanied by decreases in 18:0 and 18:1 (Zhai *et al.*, 2020). In contrast, the *tt8* mutation in Arabidopsis resulted in substantial increases in the expression levels of both *FAD2* and *FAD3*, and the *tt8* mutant showed elevated levels of 18:1 and reduced 16:0, 18:2, and 18:3 (Chen *et al.*, 2014). Hence, CsTT8 appears to exhibit a distinct function compared to its orthologues in Arabidopsis and *Brassica napus*. These findings illustrate the complex regulatory networks involved in seed fatty acid biosynthesis, with TT8 emerging as a player that influences both the content and composition of fatty acids in oilseeds, thus providing the potential tool for enhancing oil production and tailoring its composition in camelina.

CsTT8s are involved in mucilage formation

Mucilage is a gelatinous substance composed of pectins, cellulose, hemicellulose and proteins that can be found in the seeds of many plants, including Arabidopsis (Haughn and Western, 2012). It is produced by specialized epidermal cells in the seed coat and serves various functions in seed dispersal and provides a protective barrier for the seed (Arsovski *et al.*, 2010). Inactivating all three *CsTT8* genes did not visibly change the internal structure of mucilage secretion cells (MSC), which retained their central volcano-shaped cellulosic structures called the columella, but it effectively minimized mucilage accumulation in the outermost layer of the seed coat, leading to a noticeable reduction in seed coat thickness (Figure 4). Thus, it can be concluded that CsTT8, a *bHLH* transcription factor, is a positive regulator of seed coat mucilage synthesis. In contrast to camelina, in Arabidopsis the mucilage biosynthetic pathway seems to be redundantly controlled by multiple *bHLH* transcription factors. In Arabidopsis, mutants of the TT8 locus alone do not display any defect in columellae development and mucilage production (Nesi *et al.*, 2000; Zhang *et al.*, 2003), reflecting a possible functional redundancy between TT8 and ENHANCER OF GLABRA3 (EGL3) because *egl3 tt8* double mutants have collapsed columellae with no releasable mucilage (Zhang *et al.*, 2003).

The reduction in mucilage biosynthesis in our CsTT8 mutants might free up carbon resources for FA and TAG synthesis, and therefore could explain the increased oil contents (Figure 5) based on carbon re-allocation. The relationship between mucilage and oil content in seeds is complex and varies among plant species. For example, in flax, seed coat mucilage was positively correlated with seed oil content (Miart *et al.*, 2021). The transcriptional regulation associated with seed oil and fatty acid metabolism appears to occur in the seed coat during the mid-stage of seed development. (Arsovski *et al.*, 2010). However, an inverse relationship between mucilage and oil content has

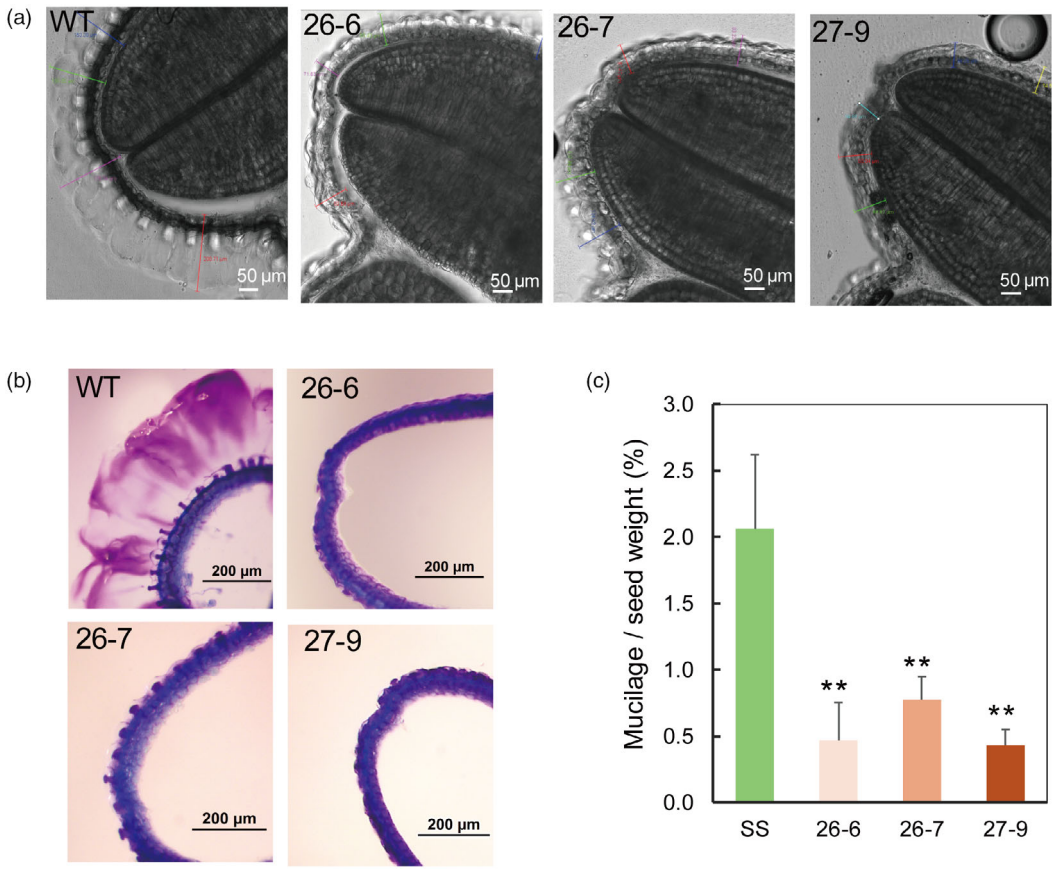

Figure 4 Effects of editing *CsTT8* on seed coat in camelina seeds. (a) Differential Interference Contrast (DIC) imaging of camelina seeds. (b) The seed coat of camelina. Camelina seeds were fixed and sectioned with a cryo microtome, and then stained with toluidine blue O (TBO) and observed under a dissection microscope. Images in a and b represent the dissection of 10–12 seeds from wild type and lines 26–6, 26–7 and 27–9. (c) Mucilage content in camelina seeds. Mucilage was extracted from seeds was quantified as a weight percent of dry seed weight. The values represent the mean ± standard deviation of four biological replicates. **Indicates a significant difference from wild type by student *t*-test $P < 0.01$.

been reported for other cases including legumes (Tookey and Jones, 1965), such as *Medicago orbicularis* (Tonnet and Snudden, 1974), and *M. truncatula* (Song *et al.*, 2017). These differences have been attributed to resource allocation in seeds of different species based on their reproductive and ecological strategies (Song *et al.*, 2017), that involve various transcription factors. For example, GL2 promotes the formation of non-glandular trichomes and regulates the production of mucilage in seed coat cells. It is also an inhibitor of oil content, impacting the balance between oil and mucilage production (Cheng *et al.*, 2021). AtMIF1 functions as a positive regulator, enhancing oil content by attenuating GL2 inhibition. Its interaction with MYB domain protein 5 (MYB5) disrupts normal transcriptional activation of the MBW complex. Consequently, the expression of GL2, a target of MBW, is reduced. Seeds of the AtMIF1-overexpressing plants no longer secrete mucilage normally, but the oil content is significantly enhanced (Cheng *et al.*, 2021). The MBW complex is implicated in seed coat mucilage and the biosynthesis of flavonoids in the endothelium (Zumajo-Cardona *et al.*, 2023). Downstream targets of TTG1/MYB/bHLH complexes include other transcription factors such as the Glabra2 (GL2) and Transparent Testa Glabra2 (TTG2) (Gonzalez *et al.*, 2009; Johnson *et al.*, 2002; Morohashi *et al.*, 2007; Rerie *et al.*, 1994). Both GL2 and TTG2 play positive roles in seed coat mucilage production. *CsTT8* editing may disrupt the formation of the camelina MBW complex leading to decreased transcription of GL2 and TTG2 (Figure S7) and consequently result in a reduction of mucilage. Investigating the mechanisms by which GL2 and TTG2 influence mucilage formation would be an interesting topic for further research. Studies also suggest that the *TT8* genes within this complex play a role in overcoming postzygotic hybridization barriers, specifically the triploid block (Baudry *et al.*, 2006; Nesi *et al.*, 2000).

TT8 mediates pleiotropic effects, but most significantly, editing of *CsTT8* in camelina results in a 44% decrease in flavonoid production. This reflects its ancestral role in the biosynthesis of flavonoids (Zumajo-Cardona *et al.*, 2023) involving in the final stages of this pathway that regulate the synthesis of anthocyanins and proanthocyanins (Baudry *et al.*, 2006; Nesi *et al.*, 2000). While mucilage has the potential to influence seed germination (Arsovski *et al.*, 2010), it is perhaps surprising, that the *CsTT8* edited lines exhibited robust germination on soil. The 10-month-old seeds of the *CsTT8*-edited lines also displayed a germination rate comparable to that of the WT, as depicted in Figure S4. The absence of mucilage in these edited lines could have facilitated more rapid water absorption by the seeds, thereby accelerating germination. Consistent with this is our observation that the edited seeds can more rapidly absorb water, thereby accelerating the germination process, resulting in longer radicles in the edited lines compared to those in the WT.

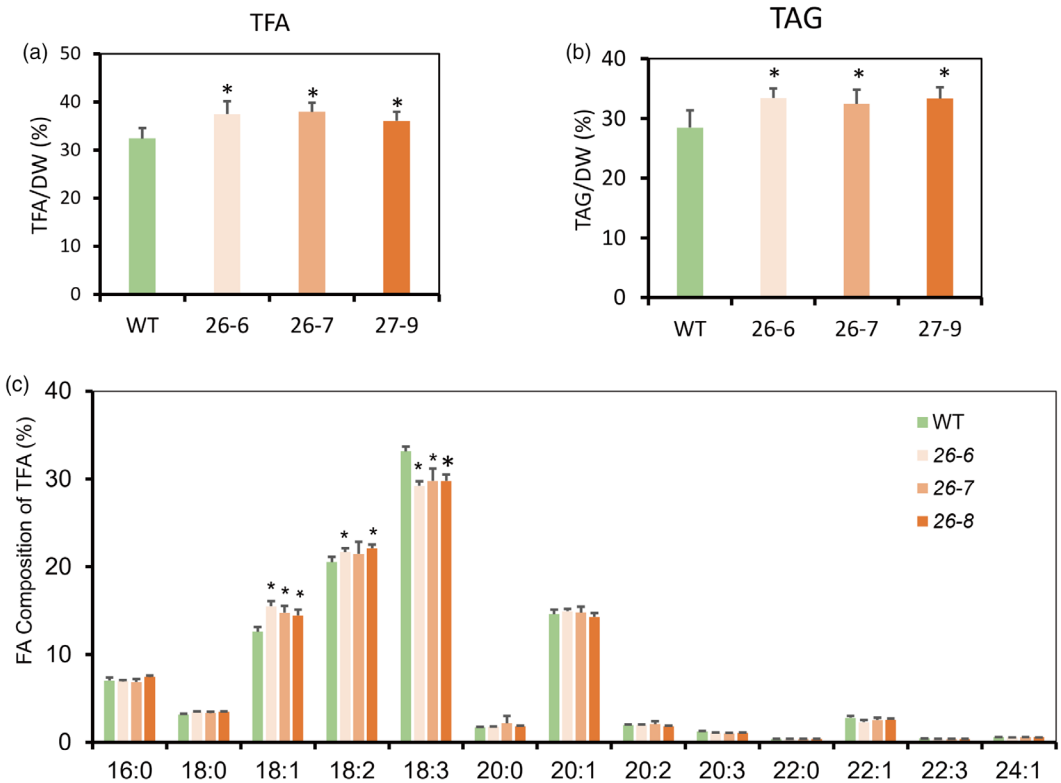

Figure 5 Editing of *CsTT8* changed fatty acid accumulation in camelina seeds. Seed total fatty acid (TFA) and TAG content was quantified by GC of fatty acid methyl esters. TFA (a) and TAG contents (b) in camelina seeds are presented as a proportion of dry seed weight. (c) Fatty acid composition in total fatty acid is expressed as a weight percentage of the total FA. Values represent means (±) standard deviation (n = 3). *Student t-test, $P < 0.05$ for differences between wild type and edited lines.

In summary, disruption of *CsTT8* via gene editing resulted in yellow seeds associated with reduced flavonoid accumulation and mucilage formation. Significantly, it caused substantial reprogramming of seed metabolism that led to increased TFA and TAG contents, along with changes in fatty acid composition. Our results demonstrate the potential of creating new germplasm in camelina by manipulating *TT8* to enhance lipid biosynthesis. Understanding the regulation of lipid metabolism by TT8 and other lipogenic factors may provide additional gene targets that can be manipulated to increase oil yields. The use of materials described herein with increased FA and TAG content, and others derived from them, have the potential to increase the yield of feedstocks for biofuels and bioproducts that can contribute toward a net-zero carbon bioeconomy.

Materials and methods

Plant material

Camelina sativa Suneson are grown in walk-in growth chambers at 22 °C with 16-h photoperiod and photon flux density of 70 μmol/m^2/s. Flowers on the primary inflorescence were marked at anthesis, and the seeds at 12 days after flowering (DAF), 24 DAF and mature seeds were collected for RT-qPCR and acetate incorporation experiment.

Construction of the CRISPR/Cas9 vector and camelina transformation

The DsRed expression cassette was amplified from phas-DsRed with its BsaI site removed by site-directed mutagenesis. The PCR fragments and *Eco*RI linearized pHEE401E (Wang *et al.*, 2015; Xie *et al.*, 2015) were used to assemble pHEE401E-DRM by Gibson assembly. Two sgRNA sites from CsTT8 coding region were selected to create pHEE401E-DRM-CsTT8 (Wang *et al.*, 2015; Xie *et al.*, 2015). All primers used are listed in Table S1.

The resulting constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into camelina via vacuum infiltration (Lu and Kang, 2008). Transgenic camelina seeds were screened for DsRed fluorescence as previously described (Pidkowich *et al.*, 2007) and planted.

Sanger sequencing analysis of target sites

Genomic DNA of *C. sativa* was extracted from young leaves with the extraction buffer (200 mM Tris–HCL, 0.5% SDS, and 25 mM EDTA, pH 8.0) and precipitated with two volumes of 100% ethanol. PCR was used to amplify the genomic regions encompassing the specific targets of the three *TT8* genes and the PCR products were sequenced by Stony Brook University. Primers used for vector construction and *TT8* gene amplification are listed in Table S1.

Microscopic examination of seed coat

Seeds of the WT and three *tt8* mutant lines were fixed in a FAA buffer (4% formalin, 5% glacial acetic acid, 50% ethanol, 41% water, V/V) within a vacuum for a duration of 25 min, as per our prior methodology (Cao *et al.*, 2023). After fixation, those seeds were embedded in Embedding Moulds filled with OCT compound (Tissue-Tek) and carefully submerged in a beaker containing

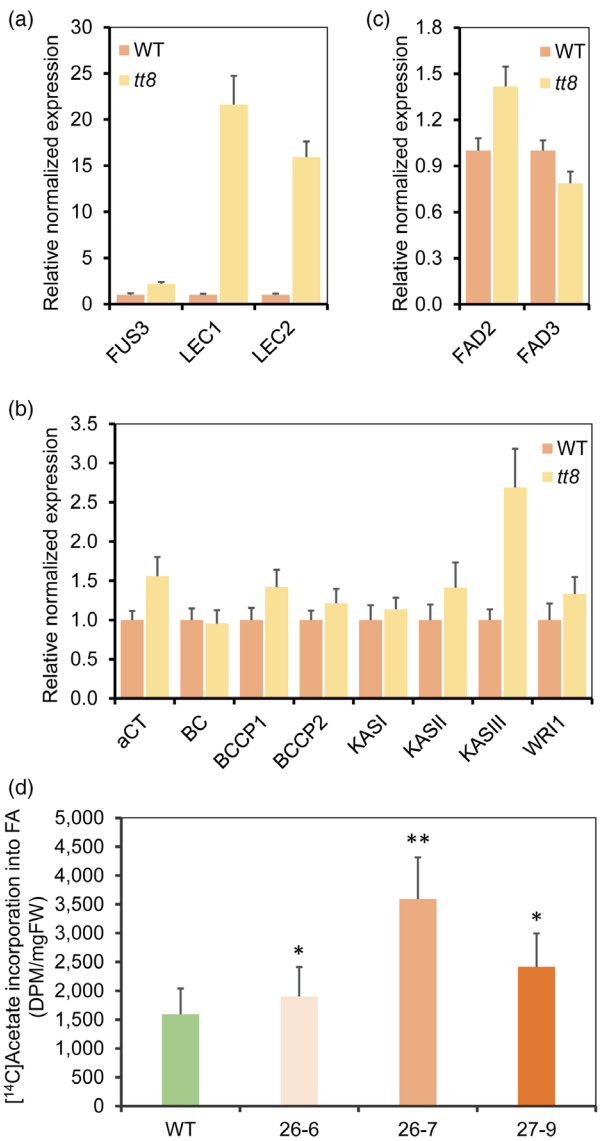

Figure 6 The *CsTT8* targeted mutation resulted in altered expression of genes involved in FA biosynthesis. Transcript levels of transcription factors (a), fatty acid synthesis (b) and fatty acid desaturation (c) genes were analysed by RT-qPCR in seeds, $n = 3$ biological replicates, and error bars represent SD. The relative expression levels are reported relative to the expression of the *Actin* transcript. (d) [^{14}C]Acetate incorporation assay in developing seeds. [^{14}C]Acetate incorporation into total lipids showed ACCase activity in 11–13-DAF developing seeds of WT, *TT8* editing lines. Stars indicate significant differences (*, $P < 0.05$, **, $p < 0.01$) as determined by Student's t test. Values are presented as means ± SD of four biological replicates.

2-Methylbutane chilled by liquid nitrogen. Following this, transverse sections of the seeds with 80 μm thickness, were prepared using freezing-microtome (Leica CM 1950; Leica Biosystems Nussloch GmbH Inc., Heidelberger, Germany) at a temperature of −30 °C. To ascertain the seed coat thickness, Differential Interference Contrast (DIC) imaging was conducted using a Leica TCS SP5 laser scanning confocal microscope. For a more comprehensive understanding of the seed coat composition, images of seed sections were captured without staining or with 0.02% (w/v) Toluidine Blue O (TBO) staining, employing a Leica M125 light microscope.

Mucilage extraction and quantification

Mucilage was extracted from the seeds following Zhao *et al*. (2017). Briefly, 1 mL of water was added to 10 mg of seeds and incubated for 5 min with shaking at 100 rpm. The supernatants were then transferred to a new tube. Subsequently, the seeds were treated with ultrasonication for 20 s after adding 1 mL of water, and the resulting supernatants were combined with those from the previous step. The seeds were washed with 1 mL of water, and the supernatants were pooled. Finally, the pooled supernatants were subjected to freeze-drying and the dried mucilage samples were weighed.

Seed germination experiment

Camelina seeds were arranged on filter paper saturated with 4 mL of distilled water (ddH_2O) within a sterile petri dish. Subsequently, the petri dish was placed in a dark environment and maintained at room temperature. After a period of 20 h, the progression of seed germination was captured through photography. To assess seed germination in soil, it was moistened and firmly packed into pots. Subsequently, seeds were placed on the soil surface and the pots were transferred to an enclosed environment with 25% humidity. Seed germination was recorded at 3 days.

RNA extraction and qRT-PCR

RNAs from leaves, roots, stems and seeds of development stages of camelina plants were extracted according to Schultz *et al*. (1994). RNA quality and concentration was evaluated by gel electrophoresis and nanodrop spectroscopy. cDNAs were prepared using SuperScript IV VILO Master Mix (with ezDNase exzyme; Invitrogen). RT-qPCR was carried out on the CFX96 Real-time PCR Detection System (Bio-Rad) with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Gene-specific primers used in the analysis are listed in Table S1. *CsActin* was used as the internal control (Yu *et al*., 2019). Statistical analysis of RT-qPCR data was carried out with REST2009 (Pfaffl *et al*., 2002).

Fatty acid analyses

Fatty acid analyses were carried out as described (Broadwater *et al*., 2002). Lipids were extracted in methanol/chloroform (2:1) from seeds and heptadecanoic acid (17:0) was added as an internal standard. Total seed lipids were converted to their corresponding fatty acid methyl esters (FAMEs) in 3 M BCL_3 at 90 °C for 1 h and extracted with hexane. Lipid profiles and acyl group identification were analysed on a Hewlett Packard 6890 gas chromatograph equipped with a 5973 mass selective detector and an Agilent J&W DB 23 capillary column as previously reported (Yu *et al*., 2018). The FA percentage values were presented as a mean of at least three biological replicates.

[^{14}C]acetate incorporation assay

Developing seeds at 11–13 DAF were collected for [^{14}C]Acetate Incorporation Assay following our former protocol (Yu *et al*., 2021). Approximately 30 mg fresh developing seeds were labelled by incubating in 0.2 mCi of [^{14}C]acetate for 15 min. Cells were subsequently washed and total lipids were extracted and suspended in Ultima Gold liquid scintillation cocktail (PerkinElmer) for incorporated radioactivity measurement with a scintillation counter (Packard BioScience).

Accession numbers

CsTT8-2 (*Csa02g028180.1*,), *CsTT8-8* (*Csa08g037600.2*) and *CsTT8-13* (*Csa13g044750.1*).

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Conflict of interest

J.Sh. has a financial interest in AtTag Bio. Inc.

Author contributions

X.Y., J.Sh., Y.C. and Y.L. designed experiments. X.Y. performed experiments, analysed data and made figures and table with support from Y.C., Y.L., H.S., C.L., S.A. Y.C., Y.L. and X.Y. constructed the vectors and generated transgenic camelina plants. Jo.C, S.P. and X.Y. genotyped the CRISPR lines. H.S. and J.Sc. measured starch, protein and flavonoid content. X.Y. and J.C. analysed fatty acid. Y.L and X.Y. quantified genes expression and seed coat. S.A. and X.Y. tested fatty acid synthesis rate. X.Y. and J.Sh wrote the article with input from Y.L., Y.C., H.S., L.C. and J.Sc. All authors approved the article.

Data availability statement

All data generated or analysed during this study are included in this article and in its files. Source data are provided with this paper.

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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 Phylogenetic tree of the TT8 homologues from camelina and other plants. Protein sequences are from GenBank with the following accession numbers: AtTT8 (Q9FT81) in *Arabidopsis thaliana*; BnA09.TT8 (MN399821) and BnC09.TT8b (MN399822) in *Brassica napus*; BoTT8a (ADV03944), BoTT8b (ADP76654) in *Brassica oleracea*; BjuA.TT8 (AIN41653.1) in *Brassica juncea*; RsTT8 (ASF79354.1) from *Raphanus sativus* BrTT8 (XP_009113574) in *Brassica rapa*; LjTT8 (AB490778) in *Lotus japonicus*; RsTT8 (KY651179) in *Raphanus sativus*; LtTT8 (ARK19321.1) in *Lotus tenuis*; LcTT8 (KY196477) in *Lotus corniculatus*; AcbHLH42 (QAT77714) in *Actinidia chinensis*; MtTT8 (KM892777) in *Medicago truncatula*.
Figure S2 Editing of TT8 in camelina changed protein sequence but did not edit the predicted off target sites. (a) Protein sequences changes happened within the first 66 amino acid of tt8 mutants. The * represents stop codon, – represents deletion. (b) predicted off-target sites. (c) sequencing of predicted off-target sites.
Figure S3 Seed coat colour changes in some of the T1 transgenic lines.
Figure S4 Seed germination and plant growth of the mutated CsTT8 lines. (a) No growth differences were observed in the mutated CsTT8 lines. (b) Seed germination in soil. (c) Seed

germination on wet filter paper. The diameter of the petri dish is 9.8 cm.

Figure S5 Editing of TT8 in camelina change the FA composition in TAG. Fatty acid composition in TAG was expressed as a weight percentage of the total FA. Values represent means (±) standard deviation (n = 3). *Student t-test, $P < 0.05$.

Figure S6 Effect of editing TT8 on protein and starch content in camelina seeds. Protein content (a) and starch content (b) were expressed as a weight percentage of the total seed weight. Values represent means (±) standard deviation (n = 3). (c) Hundred seed weight. Values represent means (±) standard deviation (n = 4).

Figure S7 Effect of editing TT8 on the expression of GL2 and TTG2.

Table S1 Golden gate clone and qPCR primers.