Molecular Breeding (2024) 44:68
https://doi.org/10.1007/s11032-024-01503-7

Molecular Breeding

BnaC09.tfl1 controls determinate inflorescence trait in *Brassica napus*

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Received: 20 February 2024 / Accepted: 15 September 2024

Abstract

Determinate inflorescence is indeed a pivotal agricultural characteristic in crops, notably impacting the architecture modification of *Brassica napus* (AACC, 2n = 38). Previous study identified a crucial gene *Bnsdt2* that encodes the transcription factor *BnaC09.TFL1* (*Terminal Flower 1*). Here by two alleles were cloned and sequenced from indeterminate 2982 and determinate 4769, respectively, we found that *BnaC09.TFL1* harbors two T/C and G/C non-synonymous mutations in exon 1, and contains sixty-six differences in a 1.9 Kb promoter sequence. Subsequently, *BnaC09.TFL1* was introduced into *B. napus* 571 line by genetic complementation and overexpression, transgenic plants 571^{CTO} lines and 571^{TC}lines were all restored to the indeterminate inflorescence. Interestingly, after *BnaC09.TFL1* was knocked out in 'Westar', transgenic plants $Westar^{Tcr}$ lines were mutated to determinate inflorescences. Additionally, a NIL-4769 line was constructed to evaluate the effect of *BnaC09.TFL1* on agronomic traits of *Brassica napus*, the results demonstrated that *BnaC09.tfl1* reduced the plant height and increased the branch number and branch thousand grain weight of *Brassica napus.* Finally, we performed RT-qPCR, GUS staining and subcellular localization experiments to analyze the expression pattern of *BnaC09.TFL1*, the results showed that the expression of *BnaC09.TFL1* at shoot apex of NIL-4769 was higher than that of 4769, GUS activity was detected at apical of *Arabidopsis thaliana* and ***BnC09.TFL1-GFP*** was detected in cell membrane, nucleus and cytoplasm. Our findings provide a firm molecular foundation for the study of rapeseed's molecular mechanism of determinate inflorescence formation, as well as theoretical guidance for the application of determinate inflorescence in rapeseed breeding.

Keywords *Brassica napus* · Indeterminate and determinate inflorescence · Agronomic traits · Transgenesis · Gene knock-out

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Extended author information available on the last page of the article

Published online: 30 September 2024

Introduction

Rapeseed is one of the most vital oil crops in the world. In addition to being used as food oil, rapeseed meal is rich in high protein and can be used as feed. It is an important source of feed protein in China. Moreover, rapeseed can be used as vegetables, nectar, flower, green manure, environmental improvement and other multi-functional purposes (Wang et al. 2019). According to ecotype, it can be divided into winter rapeseed, semi-winter rapeseed, and spring rapeseed, in Europe, China, and Canada and Australia, respectively (Qian et al. 2006). Spring rapeseed does not require vernalization to complete its growth period and is planted and harvested in the same year. However, semi-winter rapeseed and winter rapeseed require a certain period of low temperature induction to complete vernalization and are planted in one year and harvested in the next (Quijada et al. 2006).The main spring rapeseed-producing areas in China are Qinghai, Xinjiang, Inner Mongolia, Gansu, and other regions with low temperatures and short frost-free periods (Du et al. 2018). According to morphological characteristics, rapeseeds can be classified into three groups, *Brassica napus* from Europe, *Brassica rapa* (AA, 2n = 20) from China and India, and *Brassica juncea* (AABB, 2n = 36) (Wang et al. 2006). *Brassica napus* is mainly cultivated in Qinghai Province's spring rapeseed area because of its excellent agronomic traits, such as high yield, stress resistance, disease resistance, and insect resistance (Lu et al. 2019). However, high plant height, easy lodging, and poor uniformity are main factors that limit rapeseed breeding development (Sun et al. 2016).

The determinate inflorescence trait of *Brassica napus* was found during a microspore culture in 2014 (Li et al. 2018). After several years of research, it was found that, compared with indeterminate inflorescence of *B.napus* under the same genetic background, *Brassica napus*' determinate inflorescence has early termination of flowering and maturity, which shortens the length of the main inflorescence, reduces plant height, enhances lodging resistance, and increases branch number (Li et al. 2021a).

During the flowering period of higher plants, flowers are arranged in a certain way on the floral axis, which is called the plant inflorescence (McKim 2019). An inflorescence indicates that plants have entered the reproductive growth stage (Liu et al. 2021). Plant inflorescences are subdivided into indeterminate and determinate inflorescences, the type of inflorescence is determined by the shoot apical meristem (SAM) (Miao et al. 2022). During plant reproductive growth, the SAM differentiates into the inflorescence meristem (IM) and flower meristems (FMs). If the FMs can maintain differentiation activity and produce flowers, the plant's inflorescence is indeterminate, otherwise, it is determinate (Liu 2020).

Generally, the differentiation activity of the IM and FM is controlled by multiple genes, such as inflorescence meristem attribute genes, flower meristem characteristic genes, and flower organ formation genes (Zhang 2002; Weigel and Nilsson. 1995; Weigel and Meyerowitz 1993). At present, the mechanism and signaling pathway of determinate inflorescence formation in plants have been reported. Shannon (1991) mutated Columbia wild-type *Arabidopsis thaliana*

using ethyl methanesulfonate (EMS) to obtain a mutant with a determinate inflorescence. After study, a new *TFL1* locus that controls inflorescence traits was obtained, belonging to the phosphatidyl ethanolamine binding protein (PEBP) family, which is involved in the regulation of inflorescence structure and flowering time of *Arabidopsis*. (Alvarez et al. 1992; Schultz et al. 1991; Desmond et al. 1997). During the vegetative growth of *Arabidopsis thaliana*, *TFL1* expression inhibits flowering time. (Oliver et al. 1998). Although it is involved in transcriptional regulation in higher plants, the gene is a small mobile protein that does not have a DNA domain and functions by binding to other transcription factors (Zhu et al. 2020). In the *Arabidopsis thaliana* flowering regulation pathway, *TFL1* inhibits the expression of the flowering pathway integration factor *LFY* (*LEAFY*) by interacting with zinc finger transcription factor *FD* (*FLOWERING LOCUS D*), thereby repressing the expression of floral meristem characteristic genes *AP1* (*APETALLA1*) and *CAL* (*CAULIFLOWER*) (Hanano 2011). At the same time, *LFY* and *AP1*/*CAL* also suppress the expression of *TFL1* in the flower meristem, the expression balance between them determines the plants' flowering time (Ratcliffe et al. 1999).

In *Brassica napus*, according to the current reports, there are five *BnaTFL1* copies, which are located at A02, A10, C02, C03, C09 sites, respectively. The results showed that *TFL1* homologous gene located on chromosome C03 had the function of delaying the flowering of *Brassica napus*. When the genes located on A02, A10, C02, C03 and C09 were mutated at the same time, the transgenic offspring of *Brassica napus* displayed altered plant architecture, which showed the plant height, branch initiation height, branch number, sillique number, number of seeds per sillique and silliques number of main inflorescence were significantly reduced (Sriboon S et al. 2020). In the latest research report, except for these five *TFL1* homologous gene copies, there is also a copy of *BnaA03.TFL1* in *Brassica napus. BnaC02.TFL1* could not delay the flowering and the other five paralogs are repressors of flowering time in *Brassica napus.* Meanwhile*, BnTFL1* paralogous genes also affect plant architeture but not seed yield (Wang et al. 2023). Therefore, the *TFL1* homologous gene of *B.napus* has the function of regulating plant architecture and flowering time.

Recently, the determinate inflorescence mutant of *Brassica napus* was derived from the microspore culture process, genetic analysis showed that the determinate inflorescence trait was controlled by two pairs of recessive genes (*Bnsdt1* and *Bnsdt2*) (Li et al. 2018; Li et al. 2021). Previous studies mapped *Bnsdt1* to the 68 Kb position of the *B. napus* A10 chromosome and predicted that the candidate gene is *Bntfl1*. At the same time, two mutation sites, F→L and L→F were detected at the forty-sixth and forty-seventh amino acid positions in amino acid sequence, respectively. Further functional verification of the *Bnsdt1* (*BnaA10.tfl1*) gene proved that the *Bnsdt1* gene is one of the recessive genes responsible for determinate inflorescence trait in *B.napus* (Jia et al. 2019). The *Bnsdt2* gene was mapped to a 122.9 Kb interval between 68,586.2 kb and 68,709.1 on *B. napus* chromosome C09. A homologous gene, *BnaC09.TFL1*, controlled inflorescence traits in the interval, and the gene's open reading frame (ORF) region was cloned, two existing non-synonymous mutations identical to *BnaC09.TFL1* were found. In the present study, the 1.9 Kb sequence upstream of ATG of the *BnaC09.TFL1* gene was cloned. Next, we

identified the function of *BnaC09.TFL1* via overexpression, a complementary test, and a gene knock-out experiment. The overexpression vector, genetic complementary vector, and gene knock-out vector were transformed into the determinate early-maturing 571 line (determinate inflorescence) of *Brassica napus*. Meanwhile, the NIL-4769 line with a 4769 background was constructed, and the agronomic traits of near-isogenic lines were investigated. The expression pattern of *BnaC09.tfl1* was completed by RT-qPCR, GUS staining and subcellular localization experiments. The results were as follows: firstly, *BnaC09.tfl1* controls determinate inflorescence trait in *B. napus.* Secondly, *BnaC09.tfl1* affects some agronomic traits of *Brassica napus*. Thirdly, *BnaC09.tfl1* was expressed at the shoot apex of *Arabidopsis*, and the *BnaC09.tfl1* protein are concentrated in the cell membrane, nucleus, and cytoplasm.

Materials and methods

Plant materials

The determinate inflorescence material 4769, the indeterminate inflorescence material 2982, and the determinate inflorescence early flowering line 571 (carrying two alleles of 4769, *BnaA10.tfl1* and *BnaC09.tfl1*) of *Brassica napus* used in this study were all sourced from the Qinghai Branch of the China Oil Crop Improvement Center. Columbia wild-type *Arabidopsis thaliana* was purchased from ABRC (http://abrc.osu.edu/). NIL-4769 was built via backcrossed with the 4769 material, The backcross generation was the fifth generation (BC5F1), the combined preparation method is (2982 × 4769) × 4769. The background of transgenic lines were derived from the 571 line, and gene knock-out T-cr mutants, the background of *BnaC09.TFL1* gene-edited plants, were generated in the 'Westar' background.

Gene cloning and comparative sequencing

The homologous gene of candidate gene *BnaC09.TFL1* (*AT5G03840*) in the reference genome of *B. napus* Zhongshuang 11 (https://yanglab.hzau.edu.cn/bntir) is *BnaC09G0608000ZS* according to its homologous gene sequence information, special primers, ORF-sdt2F / ORF-sdt2R (Table S1), were designed, and PCR amplification was performed using indeterminate inflorescence 2982 and determinate inflorescence 4769 as templates. The amplified fragment was recovered using a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (code No. 9762), and the recovered product was connected to the cloning vector PMD-18 T (using a TaKaRa pMD™18-T Vector Cloning Kit, code No. 6011), and then transferred into *E. coli* DH5α Competent Cells (TaKaRa code No. 9057). Monoclonal positive bacteria were detected using universal primers M13-47 / M13-48, and positive clones were sent to be sequenced. Finally, we obtained the open reading frame sequence of gene *BnaC09.TFL1*/*BnaC09.tfl1*. Next, the two gene sequences were compared using DNA sequence alignment software (http://multalin.toulouse.inra.fr/multalin/multalin.html), and the different positions were identified between *BnaC09.TFL1*

and *BnaC09.tfl1*. To identify the sequence difference of *BnaC09.TFL1*/*BnaC09.tfl1* in the 5' UTR (untranslated region) upstream of ATG and the 3' UTR downstream of the stop codon, specific primers (5 ' sdt2-4F / 5 ' sdt2-3R and 3 ' sdt2-5F / 3 ' sdt2-2R, respectively) were designed (Table S2). Total RNA was extracted from NIL-4769 and 4769 using a Tiangen RNA kit (code No. DP432). Then, the total RNA was reverse transcribed using a Biosharp reverse transcription kit (code No. BL699A). The above operations were carried out according to the operators' instructions. *TFL1* cDNA-specific primers (Table S3) were designed to amplify the cDNA sequences of *BnaC09.TFL1* and *BnaC09.tfl1*, In order to distinguish different copies of CDS in *B.napus*, NIL-4769 was used as RNA extraction template in the cDNA amplification. Sequence alignment was performed using the online website MAFFT (https://www.genome.jp/tools-bin/mafft) to determine the number of introns and exons and the sequence differences in the genes.

Vector for genetic transformation

An approximately 3.78 Kb DNA fragment from *BnaC09.TFL1* containing a 1946 bp upstream sequence of start codon ATG, 1066 bp open reading frame sequence of *BnaC09.TFL1*, and 768 bp downstream sequence of stop codon TAA was cloned into the expression vector pCAMBIA2300 (restriction sites *Eco*R I and *Pst* I were selected) to generate the pCAMBIA2300-pro*BnaC09.TFL1*:*BnaC09.TFL1* genetic complementation construct. The coding region was amplified using *TFL1* cDNA-specific primers (restriction sites *Nco* I and *Spe* I were selected) and inserted into a modified pCAMBIA1302-overexpression vector to produce pCAMBIA1302-35S:*BnaC09.TFL1* constructs. For the *BnaC09.TFL1* gene knock-out vector, two small-guide RNA sequences from the *BnaC09.TFL1* coding region were inserted into a zmp1CRISPR/Cas9 vector. Homologous recombination primer and plasmid detection primer information is presented in Table S4.

Gene bioinformatics analysis

The *BnaC09.TFL1* protein sequence was obtained via DNAMAN (version 9), and the protein sequence was submitted to the online website Prot Prarm (https://web.expasy.org/protparam/) to predict the chemical structure, amino acid composition, molecular weight, isoelectric point, and instability coefficient of the *BnaC09.TFL1*/*BnaC09.tfl1* protein. ProtScale (https://web.expasy.org/protscale/) was used to predict the hydrophilicity and hydrophobicity of the *BnaC09.TFL1*/*BnaC09.tfl1* protein. Interpro online website (https://www.ebi.ac.uk/interpro/search/sequence/) was used to predict the protein structure and clarify the function of protein. The *BnaC09.TFL1*/*BnaC09.tfl1* protein sequence was submitted to the NCBI website (https://www.ncbi.nlm.nih.gov/) for blastp analysis, protein sequences similar to those of other species were downloaded, phylogenetic analysis was performed using MEGA7 software, and the neighbor-joining (NJ) method was used to set the bootstrap parameter to repeat 1000 times.

Investigation of agronomic traits of near-isogenic lines

To evaluate the effect of *BnaC09.tfl1 gene* on yield-related traits of *B.napus*, we developed NIL-4769 using a 4769 background. The NIL-4769 and 4769 were planted in three different environments: 2022 Xining (36°62' N, 101°77′E), 2023 Xining, and 2023 Huzhu (36°50' N, 101°57′ E) in summer. A random block experiment was carried out using the NIL-4769 and 4769 constructed in this study. Five rows and three replicates were adopted in each environment, rows were 1.5 m long and 0.3 m wide. At the mature stage, 6 individual plants were randomly selected from each plot and the plot yield was measured. Agronomic characteristics such as plant height, primary branch number, thousand grain weight of the primary branch, secondary branch number, thousand grain weight of the secondary branch, length of the main inflorescence, thousand grain weight of the main inflorescence, seeds number per silique, effective silique number per plant, thousand grain weight per plant, and yield per plant were investigated, and data analysis was performed using WPS office 2023 and Raincloud-shiny (https://gabrifc.shinyapps.io/raincloudplots/).

Investigation of the agronomic traits of the T2 transgenic lines

Evaluation of the agronomic traits of the T2 transgenic lines were facilitated using T2 transgenic plants (overexpression plants and gene knock-out plants) and controls (the 571 line and 'Westar') planted in flowerpots (70 cm tall, 35 cm wide). Each flowerpot contained 50% transgenic plants and 50% controls, flowerpots were placed in a special artificial climate chamber for transgenic culture (Light for 16 h (22 degrees Celsius for 8 h, 18 degrees Celsius for 8 h), darkness for 8 h (4 degrees Celsius for 4 h, 10 degrees Celsius for 4 h). After the seeds matured, six plants from each transgenic line and the control were randomly selected and tested for agronomic traits, data analysis was performed using WPS office 2023 and Raincloud-shiny.

Quantitative real-time PCR (RT-qPCR) and RNA-Seq

Shoot apexes from *B.napus* line NIL-4769, at the budding stage, in the same time, the top of indeterminate inflorescence and the top of determinate inflorescence from 9 plants were used to analyze gene expression. RT-qPCR designed three biological replicates and two technical replicates with 2×SYBR Green qPCR Mix (AH0101-B, SPARKJadeon) on Light Cycler® 480 system. The total volume of PCR reaction was 10 μL, each primer was 0.2 μL, cDNA template was 3 μL, and the cycle conditions were as follows, denaturation at 94 ℃ for 3 min, amplified by 40 cycles of 94 ℃ for 20 s, 58 ℃ for 20 s, and 72 ℃ for 30 s. Relative expression values were calculated by normalizing against *BnActing 7* with the $2^{-\Delta\Delta CT}$ method. The primers used for gene expression analysis are listed in Table S5.

The shoot apexes from 6 plants ('Westar' and 'Westar^{Tcr1}') at budding stage in the same time were used an RNA-Seq experiment. Plants were grown in artificial climate culture room under 16 h light at 22 ℃ and dark 8 h (3 h at 10 ℃ and 5 h at 18 ℃). Three biological replicates and two technical replicates for *BnaC09.TFL1* and

BnaC09.tfl1 genes were sequenced by Mi-cro Science Alliance (Shenzhen, China). The 'ZS11' was used as reference genome (http://118.195.251.60/public/reference-genome-sequences-and-gene-annotation/B_napus_cv_ZS11/FAFU/), differentially expressed genes (DEGs) with p-value ≤ 0.05, $|\log2\ \text{FoldChange}| \geq 1$ were listed Table S6. Functional annotation of DEGs used *Arabidopsis* website of TAIR and Gene Ontology (GO).

Gus staining experiment

To analyze the expression pattern of *BnaC09.TFL1*, the 1.9-Kb sequence upstream of the initiation codon ATG was amplified and inserted into the pBWA (V) BII-GUS vector to generate pBWA (V) BII-$P_{BnaC09.TFL1}$:GUS constructs (Restriction sites *Bsa* I were selected). The correct sequenced plasmid was transformed into *Agrobacterium* (GV3101) and transferred into *Arabidopsis* wild-type Col-0. The T2 generation positive transgenic homozygous lines were screened and stained with GUS staining kit (SL7160-50 mL, Coolaber). After stained, they were observed and photographed under a stereo microscope with a CCD camera. The plasmid detection primer information are shown in Table S7.

Subcellular localization assay

To detect the specific location of *BnaC09.TFL1* expression in cells, full-length cDNA (without a stop codon) of *BnaC09.TFL1* from 2982 was amplified and introduced into a pBWA(V)HS-35S:GLosgfp vector. After vector purification, its were transferred into *Arabidopsis* protoplasts. Fluorescence signals captured using a confocal laser-scanning microscope, cDNA amplification primers, and plasmid detection primer information are available in Table S8.

Results

Comparative sequencing of BnaC09.tfl1

To identify gene variations in DNA sequences, *BnaC09.TFL1* and *BnaC09.tfl1* were isolated from 2982 and 4769 (Fig. 1a, b), respectively, followed by comparative sequencing. The indeterminate inflorescence gene's open reading frame was 1066 bp, and the determinate inflorescence gene's open reading frame was 1072 bp, these two genes contain four exons and three introns, and the cDNA sequences were 537 bp. sixty-six differences in the 1.9 Kb sequence were located upstream of ATG (Fig. S1), and we also detected six SNPs (+36 G > A, +136 T > C, +141 G > C, +177 A > C, +372 T > G, and +450 C > T) in the coding region (Fig. 1c). The two protein sequences of genes were obtained using DNAMAN software, protein sequence comparison revealed that both genes encode 178 amino acids. Two non-synonymous mutations were evident at the forty-sixth (F → L) and forty-seventh (L → F) in the amino acid sequence of the determinate inflorescence gene

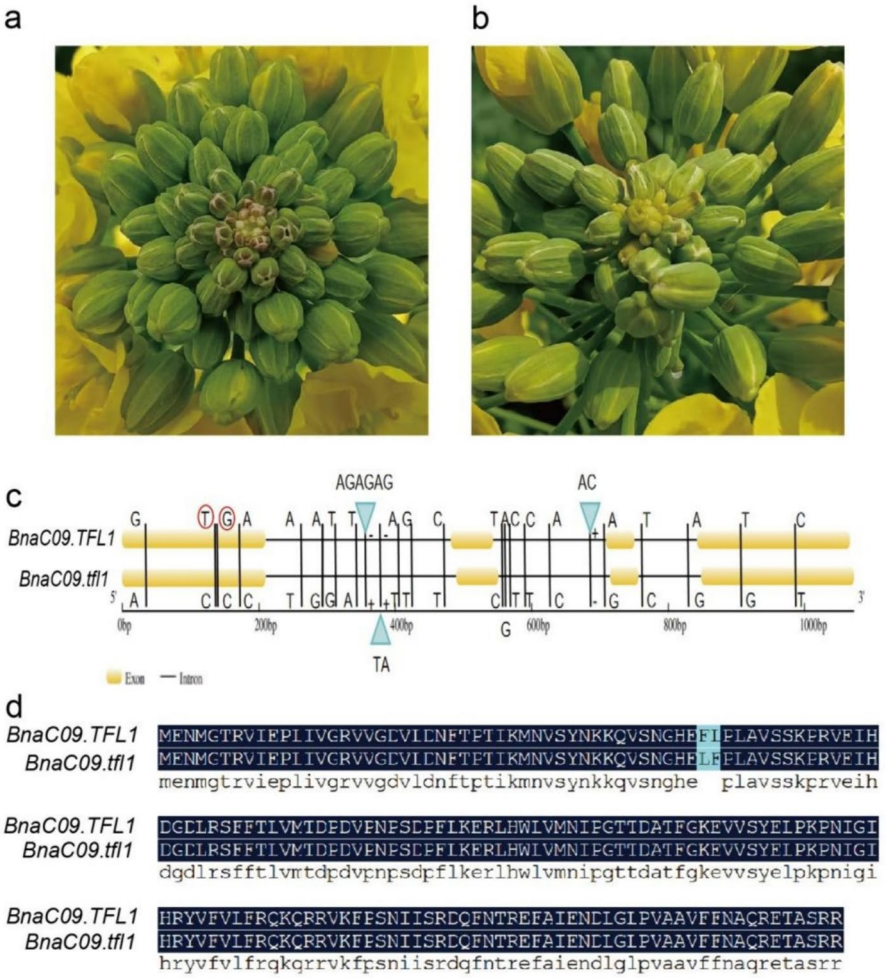

Fig. 1 Top phenotype and gene sequence analysis. **a** Indeterminate inflorescence. **b** Determinate inflorescence phenotypes of *Brassica napus*. **c** Two gene structure diagrams, red circles indicate two SNPs with nonsynonymous mutations. **d** Amino acid sequence comparison between *BnaC09.TFL1* and *BnaC09.tfl1*

compared with the indeterminate inflorescence gene (Fig. 1d). Therefore, two SNPs (+136T > C and +141G > C) mutations resulted in variations in the protein sequence.

Gene bioinformatics analysis

We used the Expasy ProtParam online website to predict the physical and chemical properties of the *BnaC09.TFL1* protein. Results indicated that its molecular formula is $C_{918}H_{1446}N_{258}O_{259}S_5$, it contains 2886 atoms, and it has 20 negatively charged residues and 24 positively charged residues, thus, the protein is positively charged and

is an alkaline protein. Results also indicated that its molecular weight is 20,401.45, its theoretical isoelectric point is 9.51, and its instability coefficient is 49.66, therefore, it is an unstable protein. We used Expasy Protscale to forecast the gene amino acid sequence's hydrophilicity and hydrophobicity, results indicated that *BnaC09.TFL1* has a maximum hydrophobic value of 2.267 at 162 points and a maximum hydrophilic value of -2.744 at 174 positions. The *BnaC09.TFL1* protein's N-terminus and C-terminus are hydrophilic; therefore, the protein is soluble (Fig. 2a).

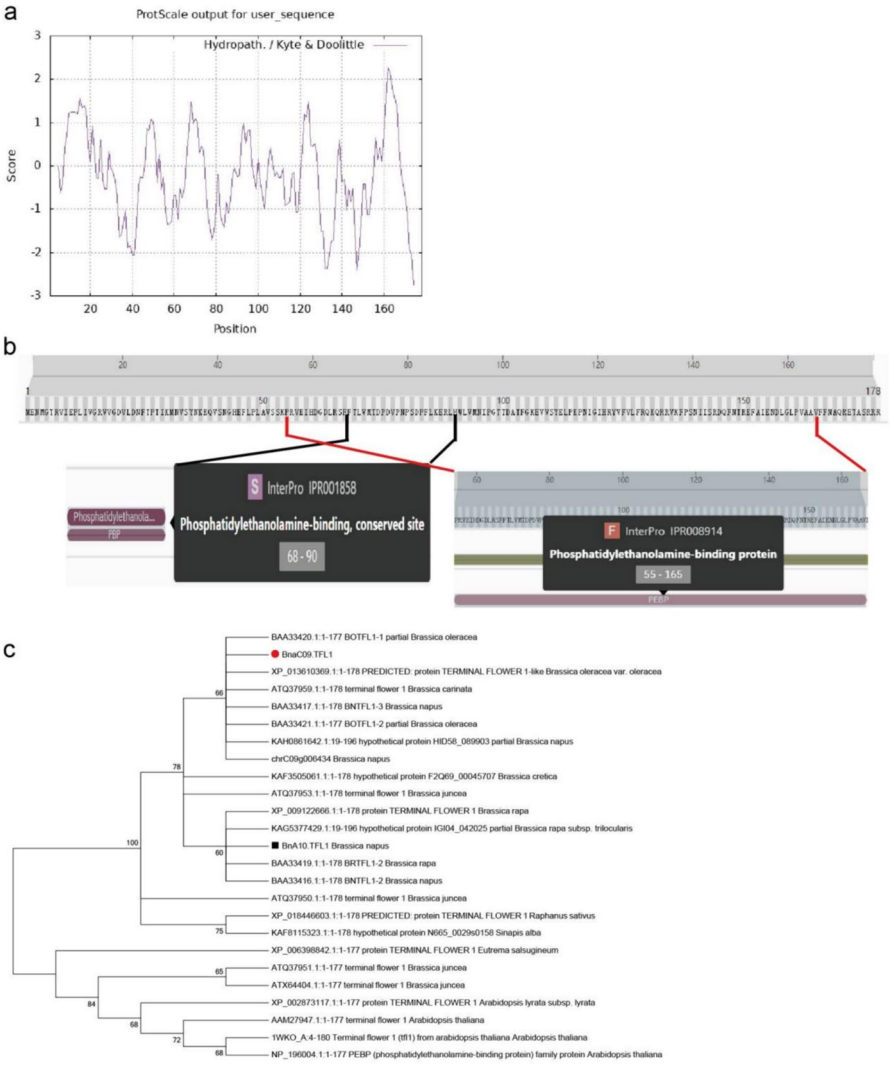

Fig. 2 Bioinformatics analysis of *BnaC09.TFL1*. **a** Analysis of hydrophilicity and hydrophobicity of the *BnaC09.TFL1* protein. the vertical axis represents the physical positions of amino acids, and the horizontal axis represents the hydrophilic/hydrophobic levels of amino acids. **b** Structure prediction of the protein encoded by the *BnaC09.TFL1* gene. **c** Phylogenic tree analysis of the *BnaC09.TFL1* protein

Using InterPro software to predict protein function, we found that a conserved site of the PEBP family existed in the fifty-eighth to ninetieth amino acid sequence, and the fifity-fifth to one hundred sixty-fifth amino acid sequence belongs to the domain of the PEBP gene family. Therefore, the protein belongs to the PEBP gene family and has the function of PEBP. (Fig. 2b), the function of this gene annotated for vegetative to reproductive phase transition of meristem and negative regulation of flower development in Go terms, therefore the protein function encoded by *BnaC09.TFL1* is closely related to inflorescence formation. The *BnaC09.TFL1* protein sequence was submitted to the NCBI database for BlastP analysis, and other plant protein sequences with high similarity to the *BnaC09.TFL1* protein sequence were selected for phylogenetic analysis (see the amino acid sequences of other species in Text S1). Results showed that the protein was most closely related to the *TFL1* proteins of *Brassica oleracea*, *Brassica oleracea* var. capitata, and *Brassica juncea*, which are all C subgenomes. The relationship to *Brassica rapa* and *Brassica juncea* was close; similarity with the *TFL1* protein of the model plant *Arabidopsis thaliana* was greater than 70% (Fig. 2c). Therefore, the homologous genes of *BnaC09.TLF1* protein sequences were highly conserved during evolution.

BnaC09.tfl1 controls determinate inflorescence trait in *Brassica napus*

BnaC09.tfl1 encodes a plant-usual transcription of PEBP family and plays an important role in the establishment of the plant type. To validate that *Brassica napus*' determinate inflorescence is controlled by *BnaC09.tfl1*, a panel of genetic transformation experiments was carried out. As *Brassica napus* is an allotetraploid crop, homologous interference was encountered when cloning the upstream sequence of the *BnaC09.tfl1* initiation codon ATG, and the gene promoter could not be amplified in time. Over-expression experiments were introduced to verify the gene's function. The constructed pCAMBIA1302-35S:*BnaC09.TFL1*-cdna vector was transferred into *Brassica napus* 571 line with determinate inflorescence traits, we obtained constitutive *BnaC09.TFL1*-overexpression of transgenic lines (571^{CTO1} and 571^{CTO2}). The 571^{CTO} lines were restored to an indeterminate inflorescence and promoted *BnaC09.TFL1* (Fig. 3a, b). When ZS11 V10 genome assembly was completed, the sequence differences of *BnaC09.tfl1* in the *Brassica napus* cv. ZS11 FAFU and *Brassica oleracea* (JZS. v2) reference genomes were analyzed, we found a pair of specific primers that can amplify the promoter sequence approximately 1.9 Kb upstream of ATG. To produce allelic promoter complementary lines (571^{TC1} and 571^{TC2} lines), we introduced *BnaC09.TFL1*'s promoter and open reading frame into *B. napus* 571. The 571^{TC1} and 571^{TC2} complementary transgenic lines also were restored to indeterminate inflorescence, and the *BnaC09.TFL1* expression level was higher than that of *B. napus* 571 (Fig. 3c, d). Therefore, *BnaC09.tfl1* controls the determinate inflorescence in *Brassica napus*.

Additionally, two gene knock-out lines (Tcr1, Tcr2) based on the CRISPR/Cas 9 system were generated (background in 'Westar'). The $Westar^{Tcr1\sim Tcr2}$ lines were edited at the target site, and T0 generation phenotypes were mutated into determinate

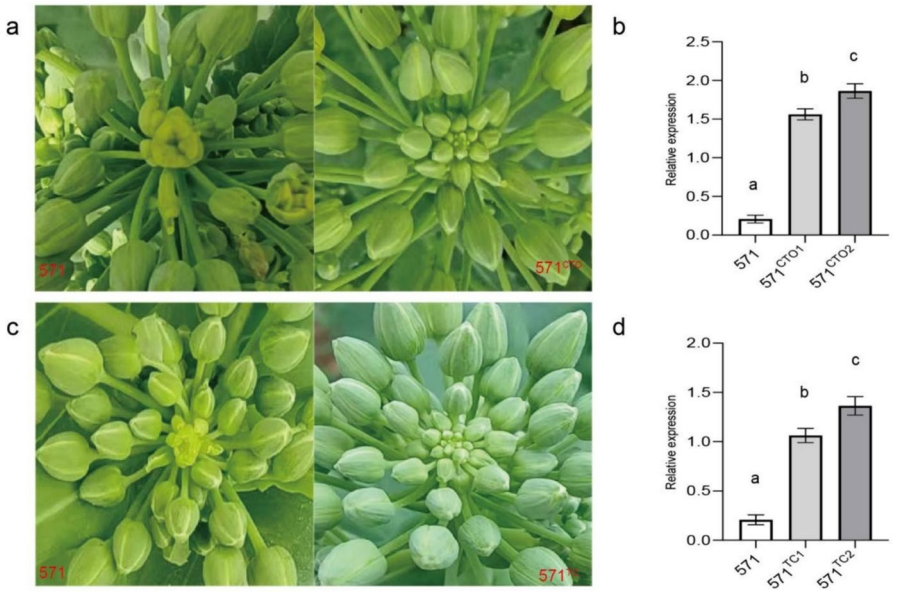

Fig. 3 Phenotype of 571, 571^{CTO}, and 571^{TC} lines and *BnaC09.TFL1* expression at the inflorescence apex in *Brassica napus* transgenic plants. **a** Left, the 571 line has a determinate inflorescence; right, 571^{CTO} lines have an indeterminate inflorescence. **b** *BnaC09.TFL1* expression level in *BnaC09.TFL1*-overexpressing transgenic plants. **c** Left, the 571 line has a determinate inflorescence; right, the 571^{TC} line has an indeterminate inflorescence. **d** *BnaC09.TFL1* expression levels in the complementary detection of transgenic plants

inflorescences (Fig. 4a, b, c). These results also indicated that *BnaC09.tfl1* acts as an important regulator of determinate inflorescence in *Brassica napus*.

Expression patterns of *BnaC09.TFL1*

We used RT-qPCR to detect the expression difference of *BnaC09.TFL1* between shoot apexes of NIL-4769 and 4769, the results indicted that *BnaC09.TFL1* was significantly higher expressed in the top of NIL-4769 than the top of 4769 (Fig. 5a), the *BnaC09.TFL1* expression level was consistent with previous research (Jia YP et al. 2019, Sriboon et al 2020), further proved that the *BnaC09.TFL1* is involved in the regulation of the apical phenotype of *Brassica napus*.

Further to understand the expression pattern and tissue specificity of *BnaC09.TFL1* in plants. We inserted the 1.9 kb promoter sequence of *BnaC09.TFL1* gene upstream of the initiation codon ATG into the pBWA (V) BII vector to construct the pBWA (V) BII-BBGUS promoter analysis vector, transformed into wild-type *Arabidopsis thaliana* (Columbia). The T2 homozygous transgenic positive lines were stained, and the empty vector pBWA (V) BII-GUS was introduced into *Arabidopsis* as a control. During flowering, GUS staining was deep at the top of *Arabidopsis thaliana.* (Fig. 5b).

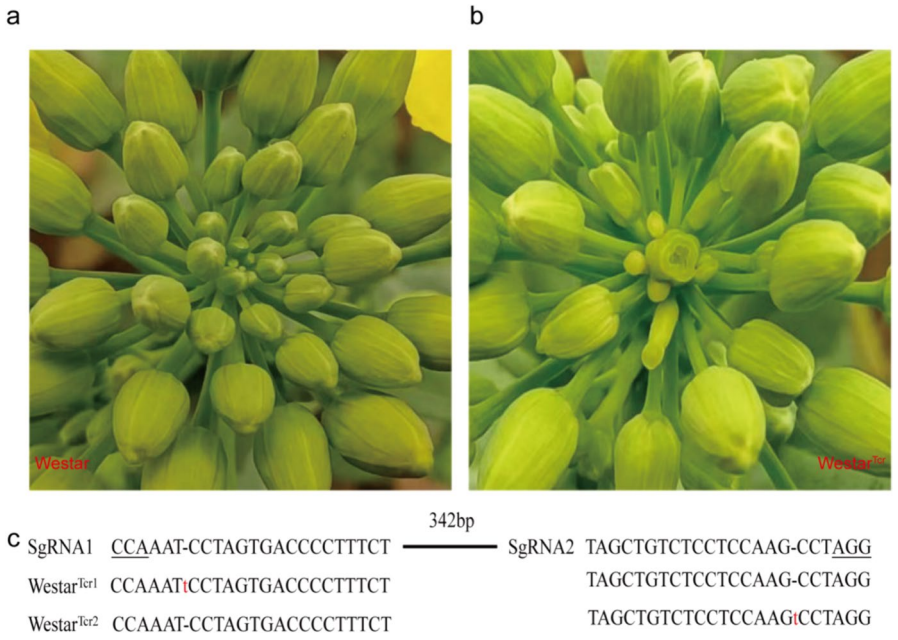

Fig. 4 Phenotypes of 'Westar', Westar^{Tcr}, and CRISPR/Cas9 knock-outs of *BnaC09.TFL1* in T1 generation transgenic plants. **a** 'Westar' has an indeterminate inflorescence. **b** Westar^{Tcr} lines have a determinate inflorescence. **c** T1 transgenic knock-out plants' editing sites

Finally, to Identify the expression position of *BnaC09.TFL1* in the cell, the CDS sequence of *BnaC09.TFL1* was fused with the GFP fluorescent protein on the pBWA (V) HS-gfp vector. The constructed vector was transferred into *Arabidopsis* protoplasts for transient expression, and then the location of ***BnaC09.TFL1-GFP*** green fluorescence in the cell was observed via confocal microscopy. The results indicated that ***BnaC09.TFL1-GFP*** emitted green fluorescence in the cell membrane, cytoplasm, and nucleus (**Fig**. 5c), which further indicated that *BnaC09.TFL1* was located in the same position as that of the *TFL1* protein in *Arabidopsis thaliana* and had similar functions as the *TFL1* protein. Therefore, the *BnaC09.TFL1* gene-encoded receptor enzymes were located in the cell membrane, cytoplasm, and nucleus and played an important role in cell signal transduction.

BnaC09.tfl1 decreases plant height but increasesTGW

After the NIL-4769 and 4769 lines were harvested, their agronomic traits were investigated. Compared with NIL-4769, the 4769 line showed a significant reduction in plant height and main inflorescence length in the three environments (Fig. 6a,b), but significantly increased for the primary branch TGW and secondary branch number and TGW in the three environments. The thousand grain weight of the primary inflorescence increased in the 2022 Xining and 2023 Huzhu environments

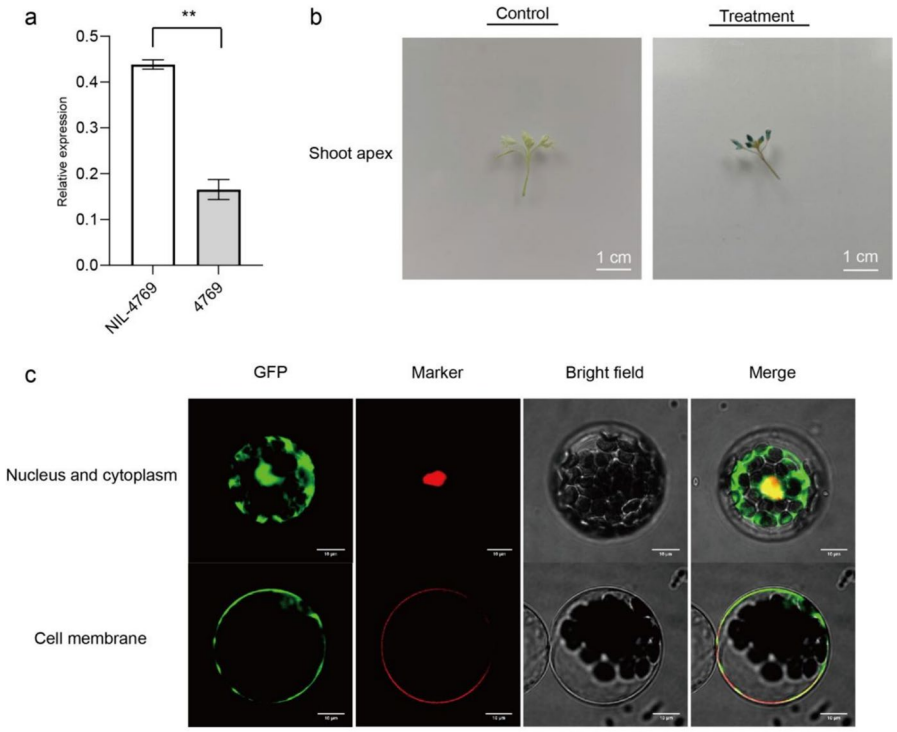

Fig. 5 Expression patterns of *BnaC09.TFL1*. **a** The expression difference of *BnaC09.TFL1* gene between NIL-4769 and 4769 lines. *, $P < 0.05$; **, $P < 0.01$. b GUS staining of *BnaC09.TFL1* promoter in *Arabidopsis*. c The green fluorescence position of the *BnaC09.TFL1*-GFP fusion protein detected in *Arabidopsis* protoplasts. Marker, MKate in the nucleus and cell membrane. Bright field, the same cell in a bright field. Merge, Overlay of light and dark (scale bar is 10 uM)

(Fig. 6c-f). We found no effect on the number of primary branches, effective siliques per plant, filled seeds number per silique, thousand grain weight per plant and yield per plant in the three environments; there was no difference in the plot yield between the NIL-4769 and 4769 lines (Fig. S2a-f).

Agronomic T2 generation, overexpression, and gene knock-out traits

To assess the effect of *BnaC09.TFL1* overexpression on agronomic traits, we tested 10 phenotypic data of 571^{CTO} lines of overexpressing transgenic T2 plants and found that plant height, the main inflorescence length, siliques on the main inflorescence and filled seed number per silique were significantly increased. The number of secondary branches, the main inflorescence of thousand grain weight, the yield per plant, and the effective siliques per plant and thousand grain weight were not affected (Table 1).

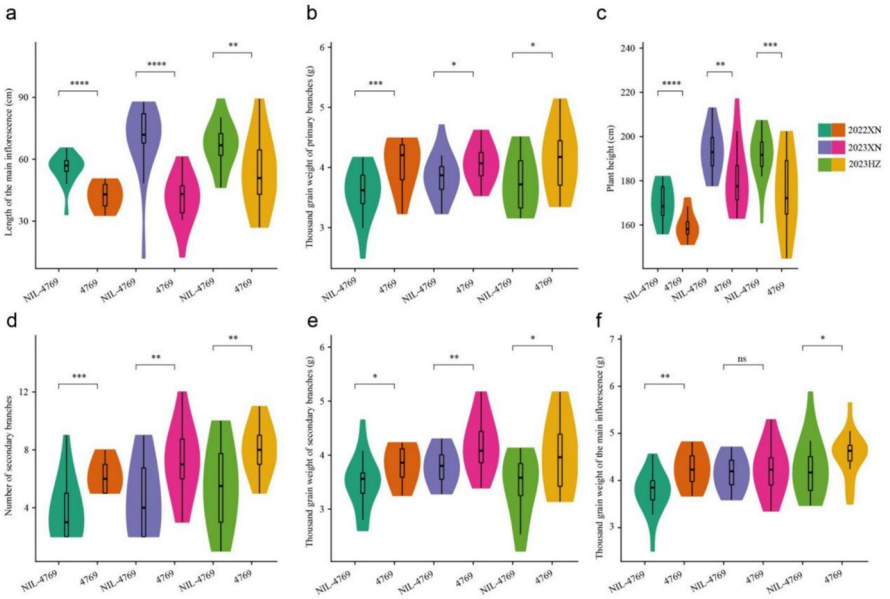

Fig. 6 Agronomic traits of the NIL-4769 and 4769 lines. **a** Y-axis, length of the main inflorescence. **b** Y-axis, thousand grain weight of primary branches. **c** Y-axis, plant height. **d** Y-axis, number of secondary branches. **e** Y-axis, thousand grain weight of secondary branches. **f** Y-axis, thousand grain weight of the main inflorescence. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$; ns, not significant

Table 1 Agronomic traits for T2 generation *BnaC09.TFL1* overexpression

Genotype	PH (cm)	NPB	NSB	LMI (cm)	SIMI	FSNS	TGW MI	YPP	ESP	TGWP
571^{CTO}	153.58	4.80	3.20	60.56	31.60	28.40	4.30	13.55	142.60	4.09
571	113.42	6.40	4.20	35.64	14.80	23.20	4.42	14.75	134.00	3.89
P	**	ns	ns	**	**	**	ns	ns	ns	ns

PH, plant height; NPB, number of primary branches; NSB, number of secondary branches; LMI, length of the main inflorescence; SIMI, siliques on the main inflorescence; FSNS, filled seeds number per silique; TGWMI, thousand-grain weight of the main inflorescence (g); YPP, yield per plant (g); ESP, effective siliques per plant; TGWP, thousand grain weight per plant (g); *, significant at $P<0.05$, **, significant at $P<0.01$, ns, not significant

BnaC09.TFL1 was knocked out in 'Westar' to obtain edited plants with a determinate inflorescence. To evaluate whether there were serious shortcomings in the agronomic traits of knocked-out plants, we investigated some T2 generation traits of edited plants. The results showed that the $Westar^{Tcr}$ lines' plant height and main inflorescence length decreased, and there were no significant differences in the number of effective siliques per plant, siliques on the main inflorescence, filled seeds per silique, thousand grain weight, and yield per plant (Table 2).

Table 2 Agronomic traits for T2 *BnaC09.TFL1* knock-out generation

Genotype	PH (cm)	ESP	LMI (cm)	SIMI	FSNS	TGWP	YPP
Westar	67.58	47.75	21.23	26.50	22.35	2.85	10.87
Westar^{Tcr}	48.36	48.29	12.26	25.43	21.80	2.68	10.54
P	**	ns	**	ns	ns	ns	ns

PH, plant height; ESP, effective siliques per plant; LMI, length of the main inflorescence; SIMI, siliques on the main inflorescence; FSNS, filled seeds number per silique; TGWP, thousand grain weight per plant (g); YPP, yield per plant (g); *, $P < 0.05$, **, significant at $P < 0.01$, ns, not significant

BnaC09.TFL1 regulates the expression of genes related to apical active in *Brassica napus*

According to the investigation of agronomic traits, while the gene was knocked out in 'Westar', the transgenic offspring showed the characteristics of reduced plant height, the resulted from the top of the knockout plant first formed a mutant floral organ and stopped the apical meristem differentiation. We wished to how *BnaC09.TFL1* regulate the activity of tip in *Brassica napus*, we performed the RNA-seq experiment in 'Westar' line and Westar^{Tcr} line, A total of 3015 transcripts were differentially expressed in apical tissues, of which 2259 genes were up-regulated and 756 genes were down-regulated (Fig. 7a, table S6), Go annotation and KEGG pathway analysis showed that the differentially expressed genes were mainly involved in the response to high light intensity and the pathway of glycerophospholipid metabolism (Fig. 7b, c, Table S9, Table S10). Previous studies have shown that the light intensity is closely related to the growth and development of plants (Fan et al 2013), Meanwhile, Glycerophospholipid metabolism plays a key role in plant growth and development, it's reported that *FT* interacts with phosphatidylglycerol to regulate the flowering process of plants (Susila et al 2021).

In conclusion, after *BnaC09.TFL1* mutation, the related pathway genes regulated the growth and development of *B.napus* were differentially expressed, which finally inhibited the apical activity of the limited inflorescence and caused the apical phenotype to alter.

Discussion

BnaC09.tfl1 from 4769 affects *Brassica napus* agronomic traits

The *TFL1* gene plays an important role in growth period of *Arabidopsis thaliana*, and its homologous gene *BnaC09.tfl1* participates in *Brassica napus*' inflorescence type regulation network (Cerise 2023). In our study, this gene affected some *B. napus* agronomic traits, such as decreased plant height, increased branch number, and increased branch thousand-grain weight. The decrease in plant height was due to the different characteristics of *Brassica napus*' indeterminate inflorescence formation and determinate inflorescence. The apical meristem (inflorescence and flower

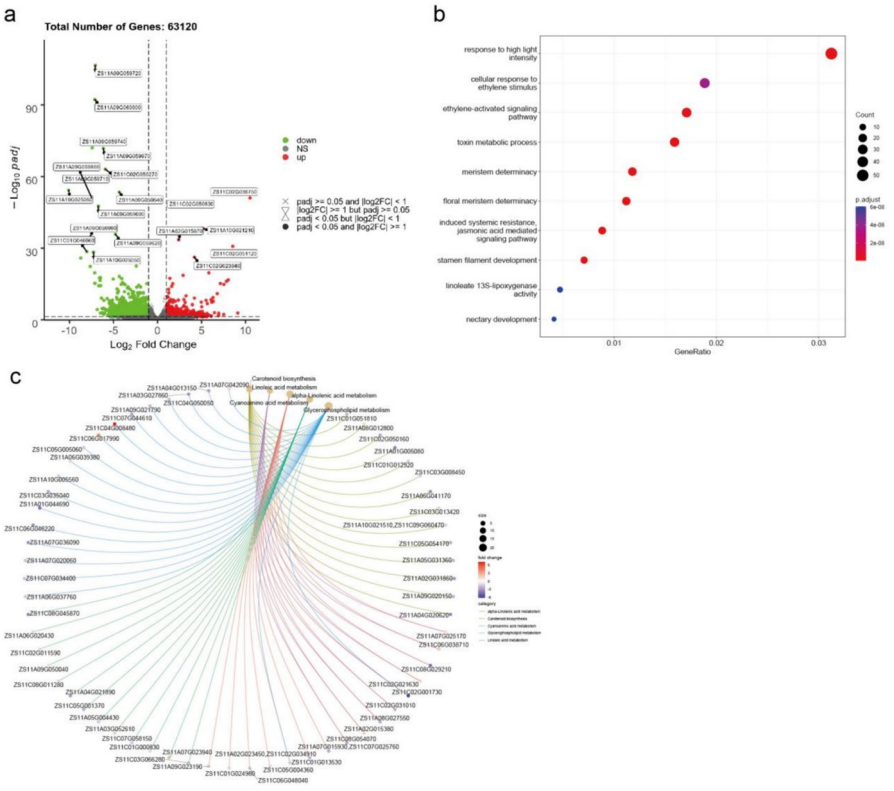

Fig. 7 Transcriptome analysis between the 'Westar' and *Bnac09.TFL1* homozygous mutant Westar^{Tcr1}. a Differentially expressed gene volcano map, display the first 20 genes. b Go annotation analysis, showing the top 10 processes. c KEGG pathway analysis, Showing the top 5 pathways

meristems) of *Brassica napus'* indeterminate inflorescence showed continuous inflorescence and flower meristem elongation under sufficient nutrient conditions, while the top of *Brassica napus*' determinate inflorescence was in the reproductive growth stage, which showed that the center of the top of the main inflorescence flowered first and that the meristem was not in the differentiation of inflorescence and bud stage. Therefore, the indeterminate inflorescence plant height was higher than that of the determinate inflorescence. Compared with *B. napus*' indeterminate inflorescence, its determinate inflorescence had a higher number of branches and thousand-grain weight (Shannon 1991; Wang 2023). According to the source–sink theory of plants, the plant height of *B. napus*' determinate inflorescence was lower, and nutrients used to elongate the inflorescence axis were allocated to other plant organs, such as branching organs and grains (Karnan et al 2023). Therefore, the number of branches and the thousand-grain weight of branches of *B. napus*' determinate inflorescence were higher than those of its indeterminate inflorescence.

BnaC09.tfl1 from 4769 plays a key role in plant inflorescence

Plant inflorescence plays an important role in plant reproduction and survival, different inflorescence structures affect plants' flowering time, flowering order, flower quantity, and nutrient distribution. The formation of a determinate inflorescence alters plants' morphological structure, for example, by reducing plant height or increasing plant lodging resistance. The earliest study of the plant inflorescence formation regulation mechanism was on *Antirrhinum majus*. Bradley (1996) isolated the *CEN* (*Centroradialis*) gene from wild-type *Antirrihum majus* L., which controls *A. majus*' indeterminate inflorescence trait, and analyzed its gene sequence. The *CEN* gene is similar to the PEBP in animals. Soon after, the homologous gene *FLO* of *CEN* was also found in *Antirrhinum majus*, and it was finally believed that these two genes jointly control the inflorescence traits of *Antirrhinum majus* (Coen et al. 1990). Then, three scientists (S. Shannon (1991), Alvarez (1992), and Schultz (1991) found that the *TFL1* gene in *Arabidopsis thaliana* has similar functions. The *TFL1* gene regulates *Arabidopsis thaliana*'s inflorescence. When the *TFL1* gene is mutated to the *tfl1* gene, the indeterminate inflorescence of *Arabidopsis thaliana* is transformed into a determinate inflorescence, and early flowering occurs. More interestingly, Bradley cloned and analyzed the *TFL1* gene and found that the gene sequence is highly similar to the *CEN* sequence and encodes the PEBP gene's family protein. Finally, it was determined that the *TFL1* gene and other floral meristem genes (such as *AP1*, *CAL*) jointly control *Arabidopsis thaliana*'s inflorescence traits (Hanano S, Goto K, 2011). With advances in genomics, *TFL1* homologous genes have been cloned from many crops, including the *GmTfl1* gene, which regulates soybean's (*Glycine max*) determinate inflorescence (Tian et al. 2010); the *Oscen1~2* gene, which delays the heading time and abnormal inflorescence structure of rice (Zhang et al. 2005); the *SiDt* gene, which maintains sesame's (*Sesamum indicum* L.) determinate inflorescence trait (Zhang et al. 2016); and the *AcTFL1* gene, which regulates the unlimited growth of the umbel inflorescence in onion (*Allium cepa* L.) (Dalvi et al. 2019). These genes have high sequence homology, a characteristic that can affect the structural characteristics of plant inflorescence.

In our study, Li (2018) isolated a determinate inflorescence gene, *BnaC09.tfl1*, in *B. napus* via map-based cloning. After analysis, it was found that this gene was highly homologous to the *AT5G03840* gene that controls the determinate inflorescence trait of *Arabidopsis thaliana*. We initially identified *BnaC09.tfl1* as the gene that controls the determinate inflorescence of *Brassica napus*. Then, a series of analyses and verifications were carried out. First, we performed a bioinformatics analysis of the *BnaC09.tfl1* gene to determine that it belongs to the PEBP family and has the general functions of that gene family, such as changing the plant's inflorescence structure and altering the number of branches. Through evolutionary analysis of the amino acid sequence encoded by *BnaC09.tfl1*, it was found that the gene was evolutionarily conserved. Therefore, it was theoretically speculated that *BnaC09.tfl1* controls the determinate inflorescence of *Brassica napus*. Second, we performed overexpression, complementary detection, and gene knock-out experiments, analyzed the gene's expression pattern and its expression position in cells, and finally verified that *BnaC09.tfl1* controls the determinate inflorescence of *Brassica napus*. These

results laid a solid theoretical foundation for the study of *Brassica napus*' determinate inflorescence regulatory mechanism.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11032-024-01503-7.

Acknowledgements The authors would like to thank Dr. Huiyan Zhao (Southwest University) and xin Li (Academy of Agricultural and Forestry Sciences, Qinghai University)for guidance of rapeseed transgenic system.

Author contribution Kaixiang Li and Dezhi Du conceived the project; Xutao Zhao performed the experiments; Lingxiong Zan and Niaofei He assisted in experiments; Xutao Zhao and Niaofei He analyzed experimental results and wrote the manuscript; Lingxiong Zan, Niaofei He, Haidong Liu, Xiaorong Xing and Guoyong Tang helped to revise the manuscript.

Funding This work was fnancially supported by the Qinghai Provincial High-level Scientist Responsibility System Project (2022-NK-170) and Earmarked Fund for China Agriculture Research System (CRAS-12).

Data Availability All data supporting the findings of this study are included in this paper and its supplementary files.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication All authors provide the consent for publication.

Competing interests The authors declare no competing interests.

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