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Characterization of the *BnA10.tfl1* Gene Controls Determinate Inflorescence Trait in *Brassica napus* L.

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Abstract: Determinate inflorescences have a significant effect on the genetic improvement of rapeseed, so understanding the molecular function underlying the inflorescence trait may be beneficial to oilseed breeding. A previous study found candidate gene *BnTFL1* (*Terminal Flower 1*) for control of the inflorescence trait on *Brassica napus* chromosome A10 (16,627–16,847 kb). However, little is known about the function of the *BnTFL1* gene in *B. napus*. In this study, we firstly studied the formation of the shoot apical meristem and gene expression in indeterminate and determinate inflorescences; the results showed that the inflorescence architecture and *BnA10.TFL1* expression showed significant differences in the shoot apex at the budding stage. Then, two alleles (named *BnA10.TFL1* gene from indeterminate and *BnA10.tfl1* gene from determinate) were cloned and sequence-analyzed; the results suggest that the open reading frame of the alleles comprises 537 bp, encodes 178 amino acids containing a conserved phosphatidylethanolamine-binding protein (PEBP) domain, and shares high similarity with *Arabidopsis thaliana* TFL1. To analyze the function of *BnA10.TFL1*, the *BnA10.TFL1* gene was introduced into the determinate *A. thaliana tfl1* mutant and *B. napus* 571 line by complementation experiment. The determinate traits were restored to indeterminate, and expression of *BnA10.TFL1* was increased in the indeterminate shoot apex. These results reveal that *BnA10.tfl1* is a gene controlling the determinate inflorescence trait. Moreover, the *BnA10.TFL1* protein was localized to the nucleus, cytoplasm, and plasma membrane. Collectively, the results of this study help us to understand the molecular mechanism of determinate inflorescences and will provide a reliable research basis for the application of determinate inflorescences in *B. napus*.

Keywords: *Brassica napus*; *Terminal Flower 1*; indeterminate; determinate; transformation; quantitative real-time PCR

1. Introduction

Rapeseed is a very important oil crop around the world. *Brassica napus* has the largest planting area among the three rapeseed cultivar species (*B. napus*, AACC, $2n = 38$; *Brassica juncea*, AABB, $2n = 36$; *Brassica rapa*, AA, $2n = 20$) in China [1,2]. The wild type of *B. napus* has an indeterminate

inflorescence. In production, the indeterminate growth habits have shown some defects, such as higher plants, easier lodging, long growth periods, and inconsistent ripening periods. With the realization of crop mechanization, overcoming the deficiency of indeterminate growth habits is a problem that breeders must face. However, it provides an opportunity for breeders to realize a transformation from indeterminate inflorescences to determinate inflorescences in plants. Current studies have shown that the determinate inflorescence mutant in *B. napus* has the characteristics of reduced plant height, lodging resistance, early growth period, and no negative impact on yield, which can complement the shortcomings of the indeterminate inflorescence growth habits in *B. napus* [3]. Therefore, it is essential to research the molecular mechanism of determinate inflorescences for the genetic improvement of *B. napus*.

At present, there are many reports on determinate inflorescence traits in plants [4–7]. The determinate inflorescence mutant *tfl1* gene was screened from wild-type *Arabidopsis thaliana* using treatment ethylmethane sulfonate (EMS). The *tfl1* mutant shoot apex produces a terminal flower, preventing further shoot apical meristem (SAM) differentiation, and determinate growth of *tfl1* can reduce plant height and early flowering. In contrast, the inflorescence SAM of wild-type *A. thaliana*, which has indeterminate growth, retains its differentiation capacity and continues to generate new buds; thus, it increases the plant height and growth period [8,9]. In leguminous crops, determinate inflorescence plants, controlled by *Vrdet1* (*TFL1* homologous), had earlier ripening pods than indeterminate plants [10]. *B. juncea* commercial varieties showed that determinate inflorescence plants had superior traits (plant height, flowering time, maturity consistency, harvest index, 1000-grain weight, etc.) when compared to indeterminate inflorescence plants [6]. In *B. napus*, Li et al. [3] assessed indeterminate and determinate plants from the BC3 population; the results showed that the plant height, maturation period, and number of branches in determinate inflorescence plants were more advantageous than those in the indeterminate plants. Thus, further study of the determinate trait gene has important guiding significance for crop breeding.

The SAM signaling pathways and inflorescence architecture have also been studied in *A. thaliana* [11,12]. The inflorescence architecture gene *TFL1* interacts with *Flowering Locus D* (*FD*) to affect the expression levels of *LEAFY* (*LFY*). *TFL1* also regulates the expression of downstream genes *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*). Intriguingly, *LFY* and *AP1* may affect normal expression of the *TFL1* gene in the SAM; thus, indeterminate inflorescences may continuously produce flowers [13,14]. These genes (*LFY*, *AP1*, and *CAL*) share major functions in floral meristem identity [15,16]. Meanwhile, studies have consistently indicated that *AP1*, a MADS-box transcription factor, is also a floral organ identity gene that acts in the development of floral sepals and petals; among the other floral organ identity genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) may control the development of petals and stamens [17–19]. In cucumber, *CsTFL1* interacts with *CsNOT2a* to inhibit determinate inflorescences and terminal flower formation, but low expression of *CsTFL1* caused non-interaction with *CsNOT2a* to produce determinate growth [20]. In *Phaseolus vulgaris*, *PvTFL1y* restored normal indeterminate growth, similar to that of wild-type *A. thaliana*, when it was introduced into the *tfl1* mutant, which has determinate inflorescences [21]. Therefore, the *tfl1* gene is essential for the maintenance and control of determinate plant growth.

Recent research has reported the determinate inflorescence trait in *B. napus*, and genetic studies showed that a related candidate gene, *BnTFL1* on chromosome A10, is homologous to the *A. thaliana* *TFL1* [3]. However, little is known about the function of *TFL1* in *B. napus*. In the present study, we mainly identified the function of *BnA10.TFL1* from indeterminate inflorescence plants by the complementation test. The full-length sequence containing the *BnA10.TFL1* gene was introduced into the determinate *A. thaliana* *tfl1* mutant and the determinate *B. napus* 571 line. The results indicated that the *BnA10.tfl1* gene may control the determinate inflorescence trait in *B. napus*.

2. Materials and Methods

2.1. Plant Materials

In this study, two *B. napus* lines, the indeterminate inflorescence line 2014 and the determinate inflorescence line 4769, were used as materials. Using 2014 as the donor parent and 4769 as the recurrent parent, near-isogenic lines (NIL) were constructed after three consecutive selective backcrosses and selfing for two generations. These offspring were then divided into homozygous groups for indeterminate and determinate NIL. These plants were grown in an experimental field at Qinghai University, Xining, China. All required routine field management was carried out.

An early maturing *B. napus* line with determinate inflorescences, named 571 (carrying the 4769 allele at the *BnA10.tfl1* locus), was used as the transformation recipient. We also purchased the *A. thaliana* CS3091 mutant (*tfl1-2*, determinate inflorescences) from the *Arabidopsis* Biological Resource Center (ABRC). Transgenic plants were grown in growth chambers (10,000 Lux) under 16 h light at 24 °C and 8 h dark at 16 °C. The total DNA of the plants was isolated using the CTAB (cetyltrimethylammonium bromide) method [22,23].

2.2. Paraffin Sectioning and Scanning Electron Microscopy Analysis

Previous studies have found that the determinate phenotype was caused by the development and differentiation of the shoot apical meristem (SAM) [3–5,14]. To further understand the phenotypic differences of apical SAM, we observed the inflorescence SAM in homozygous indeterminate and determinate plants at different stage. Paraffin sectioning of the plant SAMs was performed as previously described [24]. The samples were treated with 50% FAA (Formalin-acetic acid-alcohol), then safranin-dyed, rinsed, dehydrated, cleared, infiltrated, embedded, sliced, sealed, and examined with a Nikon microscope (Nikon, Tokyo, Japan) [25]. Scanning electron microscopy (SEM) was performed following the previously described methods [26]: the SAMs were transferred to 2.5% glutaraldehyde and then rinsed, fixed again, and dehydrated. After drying and spraying with gold powder, they were imaged using a JSM-6610LV scanning electron microscope (JEOL Ltd., Tokyo, Japan).

2.3. Gene Cloning and Sequencing

Sequence of the gene was obtained in the BRAD (<http://brassicadb.org/brad/>) based on the homologous to *TFL1* from *A. thaliana* (AT5G03840). Specific primers (TFL1-orf-F/TFL1-orf-R) were designed based on this sequence (Table S1). The gDNA and cDNA of *BnA10.TFL1/BnA10.tfl1* were isolated from indeterminate and determinate plants, and the sequences were cloned using PMD18-T vector and *E. coli* DH5a methods. The six positive clones which were verified with M13-specific primers were sequenced by Sangon Biotech (Shanghai, China). To obtain proteins homologous to BnA10.TFL1 from other species, the BnA10.TFL1 amino acid sequence was deduced and downloaded using GeneDoc (<http://www.nrbsc.org/gfx/genedoc/>) and NCBI (<https://blast.ncbi.nlm.nih.gov/>) BlastP. The phylogenetic tree was analyzed using the neighbor-joining method and 1000 bootstrap replicates in MEGA 7.0 (<https://www.megasoftware.net/>). The homologous proteins in NCBI were obtained, including BrTFL1 (XP_009122666.1) in *B. rapa*, BcTFL1 (ATQ37959.1) in *B. carinata*, BjTFL1 (ATQ37953.1) in *B. juncea*, BoTFL1 (BAA33420.1) in *B. oleracea*, AtTFL1 (AAM27947.1) in *A. thaliana*, RsTFL1 (XP_018474870.1) in *Raphanus sativus*, ThTFL1 (XP_010543489.1) in *Tarenaya hassleriana*, CcTFL1 (XP_006430226.1) in *Citrus clementina*, VcTFL1 (ART91289.1) in *Vaccinium corymbosum*, RiTFL1 (AXF50634.1) in *Rubus idaeus*, GmTFL1 (ACJ61501.1) in *Glycine max*, LaTFL1 (XP_019444254.1) in *Lupinus angustifolius*, DITFL1 (AGT41971.1) in *Dimocarpus longan*, FaTFL1 (AMR34693.1) in *Fragaria × ananassa*, VuTFL1 (AIA10351.1) in *Vigna unguiculata*, HbTFL1 (XP_021639026.1) in *Hevea brasiliensis*, and GsTFL1 (KHN18765.1) in *Glycine soja*.

2.4. Plasmid Construction

We explored the function of *BnA10.TFL1* via complementation tests. The primers were designed with NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) using the *BnaA10g26300D* (*TFL1* homologous) sequences from the BRAD. The enzyme restriction sites *EcoRI* and *PstI* and 15 bp sequences from both sides of the pCambia2300 vector were added to the ends of the primers (Table S1). The fragment of genomic DNA that included the upstream, full-length gene, and downstream sequences was amplified using Phusion Hot Start High Fidelity DNA polymerase (NEB, Ipswich, MA, USA) with recombinant primers (CP525-F/CP525-R, Table S1) from the indeterminate inflorescence line 2014. The complementation plasmid *pBnA10.TFL1:BnA10.TFL1* was produced using a sequenced genomic fragment and the pCambia2300 vector that had been digested with *EcoRI* and *PstI* using a One Step Cloning Kit (Vazyme, Nanjing, China), as previously described [24]. The confirmed recombinant plasmid was transformed into GV3101 (*Agrobacterium tumefaciens*) and stored at -80°C after adding sterilized glycerin.

2.5. Plant Transformation

For transformation of *A. thaliana*, we used the floral dip method described previously [27]. The fused construct was introduced into the *A. thaliana* *tfl1-2* mutant. For transformation of *B. napus*, the complementation plasmid, *pBnA10.TFL1:BnA10.TFL1*, was transformed into the determinate inflorescence 571 through the *Agrobacterium-tumefaciens*-mediated hypocotyl method [28]. The positive transgenic plants were confirmed and identified by antibiotic selection and PCR (Table S1).

2.6. RNA Extraction and Quantitative Real-time PCR

Total RNA was isolated from the plants using a TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China), while cDNA synthesis was performed using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). The primers for qRT-PCR were designed with Primer-BLAST in NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). For the relative quantification of transcriptional levels, Actin7 was used as the reference gene (Table S1), as recommended by ICG (http://icg.big.ac.cn/index.php/Brassica_napus) [29]. qRT-PCR was performed on a LightCycler® 480 Instrument II (Roche, Basel, Switzerland) in a total volume of 20 μL containing 2 μL gene-specific primers (10 ng/ μL), 8 μL cDNA (12.5 ng/ μL), and 10 μL SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Dalian, China). qRT-PCR was performed in three biological replicates and three technical replicates. The data were processed using the $2^{-\Delta\Delta\text{CT}}$ method [30].

2.7. Subcellular Localization of the BnA10.TFL1 Protein

To analyze the subcellular localization of the BnA10.TFL1 protein, primers (without the stop codon “TAA”) relating to subcellular localization were designed with Primer5 using the cDNA sequence. Then, the enzyme restriction sites *XbaI* and *BamHI* and the homologous sequences from the pCambia3301-GFP vector were added to the ends of the primer. The cDNA of *BnA10.TFL1* was amplified using Phusion Hot Start High Fidelity DNA polymerase (NEB, Ipswich, MA, USA) with recombinant primers (XWB-3301-XbaIF/XWB-3301-BamHIR, Table S1) from the indeterminate inflorescence line 2014. The confirmed sequence was inserted into the modified pCambia3301-GFP vector (containing the GFP gene) that had been digested by *XbaI* and *BamHI* using a One Step Cloning Kit (Vazyme, Nanjing, China). The fusion plasmid BnA10.TFL1:GFP, transformed into GV3101 (*Agrobacterium tumefaciens*), was introduced into the leaf epidermal cells of *Nicotiana benthamiana* by injection methods, as previously described [31]. The GFP signals were obtained using a confocal laser scanning microscope (Leica, Wetzlar, Germany).

3. Results

3.1. Observations of the SAM Apex in *B. napus*

To find the developmental stage at which SAM architecture variation originates in the determinate trait, we observed the formation of the SAM in indeterminate and determinate plants by scanning electron microscopy and paraffin sectioning techniques (Figure 1). The SAM architecture showed no differences between the indeterminate (Figure 1a,b,g,h) and determinate (Figure 1d,e,j,k) plants during both the vegetative meristem (two leaves) and early inflorescence meristem stages (four leaves). At the budding stage (eight leaves), we observed that SAM of indeterminate inflorescence (Figure 1c,i) was similar to that of two and four leaves stages. However SAM of the determinate inflorescence (Figure 1f,l) showed morphology similar to the floral organ. These findings can help us to provide a reference for the observation and sampling of determinate inflorescences in *B. napus*.

Figure 1. Scanning electron microscopy and paraffin section images of indeterminate (a–c,g–i) and determinate (d–f,j–l) shoot apical meristems (SAMs). (a,d,g,j) SAMs of indeterminate and determinate inflorescences at the vegetative growth stage (two leaves). (b,e,h,k) SAMs of indeterminate and determinate inflorescences at the early inflorescence meristem stage (four leaves). (c,f,i,l) SAMs of indeterminate and determinate inflorescences during the budding period (eight leaves).

3.2. Expression of *BnA10.TFL1* in Different Tissues of *B. napus*

The expression levels of *BnA10.TFL1* were examined using qRT-PCR in different tissues between the indeterminate and determinate NIL, including the shoot apex at the budding stage, roots, stems, leaves, and flowers (Figure 2). The expression levels of *BnA10.TFL1* in the shoot apex were higher than those in the other tissues. However, *BnA10.TFL1* was significantly up-regulated in the shoot apex of the indeterminate NIL compared with the determinate NIL at the budding stage (Figure 2, ** $p < 0.01$). The results indicated that *BnA10.TFL1* may be specifically expressed in the shoot apex. This is similar to the expression of the *TFL1* gene in *A. thaliana*. Thus, the expression levels of *BnA10.TFL1* may cause changes in the determinate inflorescence trait.

Figure 2. Expression of the *BnA10.TFL1* gene in different tissues of indeterminate and determinate near-isogenic lines (NIL) in *B. napus*. The error bars show the standard errors from three biological replicates. ** indicates significant differences at $p < 0.01$.

3.3. Cloning and Sequencing Analysis of the *BnA10.TFL1*/*BnA10.tfl1* Genes

A previous study reported that *BnTFL1*, being homologous to the *TFL1* gene of *A. thaliana*, is a candidate gene for the inflorescence trait in *B. napus* chromosome A10 [3]. This candidate gene was isolated from indeterminate and determinate plants, and the two alleles were named *BnA10.TFL1* and *BnA10.tfl1*. The sequences of the *BnA10.TFL1*/*BnA10.tfl1* gene were cloned from the indeterminate line 2014 and the determinate lines 4769/571 using the specific primer (TFL1-orf-F/TFL1-orf-R) by PCR amplification (Table S1). In 2014 (indeterminate) (Figure 3a), 1066 bp gDNA and 537 bp cDNA fragment sequences of *BnA10.TFL1* were found, and these included four exons and three introns by Blastn analysis. In 4769/571 (determinate) (Figure 3a), the sequences of *BnA10.tfl1* also had four exons and three introns; the gDNA and full-length cDNA of the gene comprised 1072 bp and 537 bp, respectively. Sequence analysis of the gDNA and cDNA of both *BnA10.TFL1* and *BnA10.tfl1* revealed that there are differences between 2014 (indeterminate) and 4769/571 (determinate) (Figure 3a): there are four single-nucleotide polymorphism (SNP) mutations in the exons and 18 SNP/Indel in the introns (15 SNP mutations, two insertions of 6 bp and 2 bp, and one instance of 2 bp deletion).

The amino acids were analyzed using GeneDoc and NCBI BlastP. The results indicated that the *BnA10.TFL1* and *BnA10.tfl1* alleles encode 178 amino acids; only two different amino acids were observed i.e., the phenylalanine (F) and leucine (L) in indeterminate 2014 becomes the leucine (L) and phenylalanine (F) in determinate 4769 (571) (Figure 3b). In addition, this protein contains a conserved phosphatidylethanolamine-binding protein (PEBP) domain and belongs to the PEBP family, and the amino acid sequence of *BnA10.TFL1* showed 85% identity to *A. thaliana* TFL1 (AT5G03840) (Figure 3b). The phylogenetic analysis showed that the *BnA10.TFL1* and TFL1 proteins from other plant species

can be divided into two separate clades; BnA10.TFL1 was grouped closely with AtTFL1 (AAM27947.1) from *A. thaliana* (Figure 3c).

Figure 3. Sequence analysis of *BnA10.TFL1*. (a) Gene structure and natural variations of the alleles from the indeterminate line 2014 and determinate lines 4769/571 of *BnA10.TFL1/BnA10.tfl1*. (b) Amino acid sequences of *BnA10.TFL1* and *BnA10.tfl1* from the indeterminate 2014 and determinate 4769/571 plants were aligned with those of other species, including *A. thaliana* AtTFL1 (AT5G03840), BnTFL1_Ref (BnaA10g26300D) from the *B. napus* reference genome, and BrTFL1 (Bra009508) from the *B. rapa* reference genome. The black line indicates the Phosphatidylethanolamine-binding protein (PEBP) domain. The red border indicates the position of amino acid difference between indeterminate 2014 and determinate 4769 (571). (c) Phylogenetic tree of TFL1 homologous proteins. These proteins are marked with their gene names and NCBI accession numbers. The black square indicates BnA10.TFL1, I and II show the two separate clades.

3.4. *BnA10.TFL1* Rescues the *tfl1-2* Mutant Phenotype in *A. thaliana*

To confirm the function of *BnA10.TFL1* in *A. thaliana*, the full-length genomic DNA of *BnA10.TFL1* amplified from the indeterminate line 2014 was used to construct *pBnA10.TFL1:BnA10.TFL1* in the pCambia2300 vector. This vector, containing a 3816 bp genomic fragment incorporating the *BnA10.TFL1* gene (1860 bp upstream, 1066 bp coding region, and 890 bp downstream) (Figure S1) was transformed into the *A. thaliana tfl1-2* mutant (determinate inflorescences). The transgenic *A. thaliana* and *tfl1-2* mutants showed the expected results (Figure 4b–d). The transgenic lines (Figure 4c,d) had indeterminate inflorescence stem growth similar to that of wild-type *A. thaliana* (Figure 4a). The terminal flowers of *tfl1-2* mutants (Figure 4b) inhibited the differentiation of the SAM and caused the development of a determinate inflorescence. Nevertheless, the positive T1 *A. thaliana* plants grew many buds, showing an indeterminate phenotype (Figure 4c,d). These results showed that *BnA10.TFL1* has similar function to *TFL1*.

Figure 4. Architecture of terminal racemes in transgenic *A. thaliana*. (a) Wild-type *A. thaliana* (WT) shows an indeterminate phenotype. (b) The *tfl1-2* mutant has a determinate phenotype with terminal flowers. (c) T1 *A. thaliana* transgenic plants transformed with *pBnA10.TFL1:BnA10.TFL1-1T1* returned to an indeterminate phenotype. (d) T1 *A. thaliana* transgenic plants transformed with *pBnA10.TFL1:BnA10.TFL1-2T1* also became indeterminate.

3.5. *BnA10.TFL1* Rescues the Determinate Inflorescence Trait in *B. napus*

In *B. napus*, to determine whether the *BnA10.TFL1* controls the gene of the inflorescence trait, we transformed the complementation plasmid *pBnA10.TFL1:BnA10.TFL1* sequence into the hypocotyl of the determinate line 571 (Figure 5b) by the *Agrobacterium-tumefaciens*-mediated method. The transgenic plants containing the *pBnA10.TFL1:BnA10.TFL1* construct produced numerous buds (Figure 5c,d). These positive plants exhibited phenotypic reversions parallel to the indeterminate inflorescence architecture (Figure 5a). Then, the SAMs of the positive *B. napus* plants were examined at the budding stage to analyze the expression levels of the gene. The relative expression of *BnA10.TFL1* in the indeterminate line 2014 was significantly up-regulated compared with that in the determinate line 571 (Figure 5e). In addition, *BnA10.TFL1* showed significantly higher expression in the positive plants than in the determinate line 571 (Figure 5e). Thus, high expression of *BnA10.TFL1* was associated with the indeterminate inflorescence growth habit. In conclusion, *BnA10.tfl1* is a gene controlling determinate inflorescences in *B. napus*.

Figure 5. Architecture and *BnA10.TFL1* expression in the inflorescence apex of transgenic positive plants in *B. napus*. (a) The 2014 line is naturally indeterminate. (b) The 571 line has a determinate inflorescence and abnormal floral organs. (c,d) *B. napus* transgenic plants positive for *pBnA10.TFL1:BnA10.TFL1-1* and *pBnA10.TFL1:BnA10.TFL1-2* show indeterminate phenotypes. (e) *BnA10.TFL1* expression in transgenic positive plant inflorescence apices in *B. napus*. Actin7 was used as the reference gene; the error bars show the standard errors from three biological replicates. * indicates significant differences at $p < 0.05$. The arrows indicate the inflorescence apices.

3.6. Subcellular Localization of the *BnA10.TFL1* Protein

To confirm the subcellular localization of the *BnA10.TFL1* protein in *B. napus*, the fusion plasmid *BnA10.TFL1-GFP* was transiently expressed in the leaves of tobacco (*N. benthamiana*). The *BnA10.TFL1-GFP* protein signals were localized to the nucleus, cytoplasm, and plasma membrane (Figure 6). This provides a further basis for research into the molecular function of *BnA10.TFL1*.

Figure 6. The subcellular location of the BnA10.TFL1 protein in *Nicotiana benthamiana* epidermal cells. The ER-mCherry was used as a marker for ER-rb CD-960, FIB2-mCherry was used as a nuclear marker, 35S-GFP + ER-mCherry and 35S-GFP + FIB2-mCherry were used as control; the hollow white arrow refers to the nuclear, the solid white arrow refers to the filamentous substance in the cytoplasm, the solid blue arrow refers to plasma membrane; scale bar = 20 μ m.

4. Discussion

Plant growth habits (in particular, indeterminate or determinate inflorescences) have an important effect on the architecture and yield of plants such as oilseed [3,6,32], soybean [33,34], and tomato [35]. The development of the SAM determines the plant growth habit; the SAM is located at the top of the stem of the plant and is mainly composed of cells with unlimited growth capacity [36–38]. These SAM cells produce leaf buds, axillary buds, and flower buds that are closely related to the establishment of plant morphology and the economically important harvestable organs [39–41]. The determinate inflorescence trait was caused by development and differentiation of apex SAM [4,5,14]. In *B. napus*, the candidate gene for the inflorescence trait was identified [3]. In this study, the *BnA10.TFL1* was differentially more expressed in the inflorescence apex than in other tissues (Figure 2). Therefore, further research on the function of the *BnA10.TFL1* gene is necessary for oilseed breeding.

In this study, we observed the morphological characteristics of determinate and indeterminate inflorescences at different growth stages of the SAM in *B. napus*. The results showed that the SAMs of determinate inflorescences (Figure 1d,e,j,k) were structurally similar to those of indeterminate inflorescences (Figure 1a,b,g,h) at the vegetative meristem (two leaves) and early inflorescence meristem stages (four leaves). However, the SAM of determinate inflorescences (Figure 1f,i) showed differences compared to indeterminate (Figure 1c,i) during the budding period (eight leaves). The apex of the determinate SAM (Figure 1f,i) showed an organ (similar to the shape of a floral organ) which may cause determinate growth, in contrast to the SAM with unlimited differentiation capacity seen in the indeterminate plants (Figure 1c,i). In other reports, the determinate SAMs were observed to form a terminal flower in the *A. thaliana tfl1* mutant, halting SAM differentiation [4,8]. Meanwhile, the *centroradialis* mutant apex also developed a flower, terminating the normally indeterminate inflorescences in *Antirrhinum* [38]. In *Sesamum indicum* L., the shoot apex of determinate inflorescences showed a flower cluster [42]. Although the phenotypes of determinate inflorescences in these plants were different, they shared a common feature: the apex SAM continued to differentiate into new floral tissue, resulting in indeterminate plants becoming determinate.

Li et al. [3] reported that a candidate gene controlling plant growth habits, found on *B. napus* chromosome A10, was homologous to *A. thaliana TFL1*. In our research, the alleles *BnA10.TFL1* and *BnA10.tfl* were isolated from indeterminate and determinate plants, respectively, by PCR. The *BnA10.TFL1/BnA10.tfl* had a 537 bp ORF (open reading frame) which included four exons

and three introns. The amino acid sequences of the two alleles encoded a protein of 178 amino acids and contained a conserved PEBP domain (Figure 3a,b). The phylogenetic analysis showed that the BnA10.TFL1 and AtTFL1 proteins are highly homologous with 85% identities (Figure 3b,c). Cloned *TFL1* in other plants, such as in rice [43], soybean [44], apple [45], dogwood [46], and *Jatropha curcas* [47], showed similar results. These studies indicate that *BnA10.TFL1* may share a similar function with *TFL1*.

TFL1 has been reported to not only control inflorescence meristem identity but to also impact inflorescence architecture and the formation of the reproductive phase at the SAM [48,49]. The functions of *TFL1* homologous were studied in some plants. In *Vigna radiata*, Li and Ding et al. found that the *VrDet1* gene was transformed into the *A. thaliana tfl1* mutant, the progeny transgenic plants showed indeterminate traits similar to wild-type *A. thaliana*, and the expression level of *VrDet1* at the SAMs of indeterminate plants was higher than that in determinate plants [10]. In *Punica granatum* L., *PgTFL1* was transformed into the *tfl1-14* mutant, and the determinate trait restored growth to an indeterminate plant [50]. In this study, a full-length fragment of genomic DNA containing *BnA10.TFL1* was cloned from indeterminate plants and transformed into the determinate *tfl1-2* mutant (Figure 4b). The positive transgenic plants (Figure 4c,d) were restored to indeterminate inflorescences similar to wild-type *A. thaliana* (Figure 4a). The complementation plasmid *pBnA10.TFL1:BnA10.TFL1* was also introduced into the determinate *B. napus* line 571 (Figure 5b), and the results showed that the abnormal organ at the apex of the determinate SAM was absent, confirming the restoration of the indeterminate phenotype (Figure 5c,d). Additionally, the expression of *BnA10.TFL1* was also up-regulated in the indeterminate line 2014 and in the positive transgenic plants compared with in the determinate line 571 at the budding stage (Figure 5e). Thus, *BnA10.TFL1* may have similar functions to *A. thaliana TFL1*, which controls inflorescence architecture, as it restored determinate inflorescences in an *A. thaliana tfl1* mutant that has a terminal flower. Importantly, it also restored determinate inflorescences in the *B. napus* line 571 that showed a floral organ at the SAM. The low expression of *TFL1* gene (AT5G03840) in *A. thaliana* was caused by the single nucleotide substitution G to A at 3'-end of the second exon. In cucumber, what causes (mutation and deletion) to cause down-regulation of the *CsTFL1* is unclear [20]. In this study, whether the amino acid, promoter and others changed between indeterminate and determinate may cause binding to downstream genes and expression differences that remains unknown. This will be subject to further study. Previous studies showed that *TFL1* is expressed mainly in the SAM, and mobile *TFL1* may play a role in vesicle trafficking [48,51]. Hanano and Goto [52] found that the *TFL1* protein is localized to the nucleus and cytoplasm. However, *CmTFL1c* from *Chrysanthemum morifolium* also belongs to the nucleus and plasma membrane [16]. In this study, *BnA10.TFL1* protein was found to localize to the nucleus, cytoplasm, and plasma membrane (Figure 6). Therefore, our findings indicate that *BnA10.tfl1* acts an important gene controlling determinate inflorescences in *B. napus*.

5. Conclusions

In summary, the present study revealed mainly that the function of *BnA10.tfl1* controls determinate inflorescences in *B. napus*. First of all, we analyzed the morphological development of SAMs and gene expression by scanning electron microscopy, paraffin sectioning techniques, and qRT-PCR. The results showed that *BnA10.tfl1* may be specifically expressed in the shoot apex, and down-regulation of its expression was observed in determinate inflorescence plants. Phylogenetic analysis showed that the *BnA10.TFL1* protein has high similarity with *A. thaliana TFL1*. Further, when the *BnA10.TFL1* gene from an indeterminate inflorescence plant was transferred to determinate inflorescence *A. thaliana* and *B. napus*, the determinate traits were turned to indeterminate. These results indicate that *BnA10.tfl1* may control the determinate inflorescence trait. All these results taken together, this study may provide new insights into the molecular mechanism of and oilseed breeding for determinate inflorescences.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/9/11/722/s1>, Table S1: The primer sequences used in this study, Figure S1: The 3816 bp genomic fragment incorporating the

BnA10.TFL1 gene. The green background shows the 1860 bp of the gene upstream, the grey background shows the 1066 bp of the gene coding region, and the yellow background shows the 890 bp of the gene downstream.

Author Contributions: D.D. conceived and designed the research. Y.J., K.L., H.L. and L.Z. performed the experiments and analyzed the data. Y.J. and K.L. wrote and modified the article. All authors read and approved the manuscript.

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