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ORIGINAL PAPER

The effect of BnTT8 on accumulation of seed storage reserves and tolerance to abiotic stresses during Arabidopsis seedling establishment

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Abstract Previous studies have revealed that a basic helix–loop–helix domain transcription factor AtTT8 is not only involved in biosynthesis of anthocyanins and proanthocyanidins, but also regulates seed development and oil biosynthesis in Arabidopsis. However, the functions of BnTT8 in accumulation of seed storage reserves and seedling establishment remain largely unclear. Here, we obtained the *BnTT8* coding domain sequence from the *Brassica napus* cultivar QINYOU Seven, and analyzed its conserved protein domains. A subcellular localization experiment in tobacco leaves indicated that BnTT8 functioned as a transcription factor. Expression analysis indicated that *TT8* was widely expressed in various tissues, and was also predominantly present in developing seeds of QINYOU Seven. Our results strongly demonstrated that introduction of *BnTT8* into the *tt8-4* mutant could fully complement many phenotypes of *tt8-4* seeds, such as yellow seed coat, higher fatty acid content, and lower storage protein content. We also found that ectopic expression of *BnTT8* successfully corrected the sensitivity of the *tt8-4* mutant to salinity and glucose stresses during seed germination and seedling establishment. Moreover, altered expression levels of many stress-responsive genes in *tt8-4* seedlings were rescued to wild-type levels by ectopic expression of *BnTT8*. These results should improve understanding of the entire function of BnTT8 and provide promising targets for genetic manipulations of *B. napus* to improve oil quantity in seeds.

Keywords BnTT8 · *Brassica napus* · Seed germination · Seedling establishment · Stress-responsive genes

Shuanghui Qi and Kaige Liu they have contributed equally to this work.

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Introduction

Canola (*Brassica napus* L., AACC, 2n = 38), a major oil-producing crop, is an important source of vegetative oil. Canola oil modified through breeding practices has high nutritional value with high levels of unsaturated C18 fatty acids (FAs; >60%), traces of erucic acid (C22:1; <1%), and low concentrations of glucosinolates (Dupont et al. 1989; Prakash et al. 2011). Moreover, it is a suitable source for biofuels and raw industrial materials (Thelen and Ohlrogge 2002). In addition, canola meal as the byproduct after oil refining of canola seeds contains abundant protein and fiber, and is suitable for use as animal feed. In canola seeds, the storage reserves are mainly oils stored as triacylglycerols, proteins, and carbohydrates, which not only play essential roles in maintaining this species by joining two distinct sporophytic generations, but also provide energy for seed germination and seedling establishment (Sullivan and Deng 2003). Canola seeds include embryo, endosperm, and seed coat, with most of the seed reserves stored in the embryo. Demand for vegetable oil and proteins has greatly increased in recent years, and the increase of oil and protein contents is thus a major challenge for genetic improvement of oilseed crops. Therefore, understanding the functions of key canola genes on seed development and accumulation

of seed storage reserves is important in terms of social and economic significance.

For angiosperms, including Brassicaceae species like *Arabidopsis thaliana* and *B. napus*, seed development starts with a double fertilization event that produces a diploid zygote and triploid endosperm. Seed development consists of two major stages: embryonic morphogenesis and maturation (Baud and Lepiniec 2009). After double fertilization, the embryo enters the embryonic morphogenesis stage, during which the endosperm generally occupies most of the total seed volume (Baud et al. 2002). During development, the endosperm supplies nutrition for the growing embryo and is almost degraded and reduced to a single cell layer surrounding the embryo, which acquires the basic architecture of the plant (Jenik et al. 2007; Millar et al. 2015). The seed maturation stage is characterized by the accumulation of seed storage reserves and acquisition of desiccation tolerance (Baud et al. 2002; Fait et al. 2006). The seed coat plays important roles in releasing nutrients and transmitting signals from the maternal tissues to the embryo and the endosperm during seed development and in protecting the embryo and the endosperm against detrimental events outside, and is necessary for proper seed development (Weber et al. 1996; Debeaujon and Koornneef 2000; Millar et al. 2015).

Seed coat color is determined by the type of pigment accumulated in seed coat cells. It is related to important agronomic traits of seeds such as seed dormancy, longevity, and seed oil and protein contents. In canola, yellow seed is a desirable quality trait. The genes controlling seed coat color mainly correspond to enzymes and regulatory factors involved in flavonoid biosynthesis (Li et al. 2012). Previous studies demonstrated that some of these are also involved in accumulation of seed storage reserves in canola. TRANSPARENT TESTA 2 (TT2) was significantly associated with seed coat color and linoleic acid and total FA contents in canola (Zhou et al. 2016). BnTT16 plays multiple roles in regulating the development of inflorescences, flowers, siliques, seed embryo development, and FA biosynthesis in canola (Deng et al. 2012). Ectopic expression analysis of *BnaC.GLABRA2.b* from the C genome of *B. napus* in Arabidopsis indicated that it can negatively regulate oil accumulation in seeds (Chai et al. 2010). The functions and mechanisms of genes controlling seed coat color in canola and underlying seed storage reserves remain largely unknown and require more investigation.

Previous studies revealed that *AtTT8*, which encodes a basic helix–loop–helix domain transcription factor, is involved in the biosynthesis of anthocyanins and proanthocyanidins (Nesi et al. 2000; Baudry et al. 2006). More recently, we found that it could target *LEC1*, *LEC2*, and *FUS3* to regulate seed development and/or oil biosynthesis in Arabidopsis (Chen et al. 2014b). *TT8* from *B. rapa* could rescue the yellow seed phenotype of the Arabidopsis *tt8* mutant by regulating flavonoid accumulation in the seed coat (Li et al. 2012). In the present study, we showed that ectopic expression of *BnTT8* in the Arabidopsis *tt8-4* mutant restored the yellow seed coat, and altered seed oil and storage protein contents during seed development. In addition, we provided evidence that *BnTT8* exhibited similar functions to *AtTT8* on seed germination and seedling establishment under abiotic stresses.

Materials and methods

Plant material and growth condition

Arabidopsis wild-type (WT) Columbia (Col-0) and the *AtTT8*-deficient Arabidopsis mutant *tt8-4* (Chen et al. 2014b) were used in this study. Arabidopsis plants were grown in a growth chamber under long-day (16/8 h of light/dark) conditions at 22 °C. The light intensity was 160 µmol m^{-2} s^{-1}, as detected at the middle of plants.

The canola cultivar QINYOU Seven was grown in the Agricultural Farm of North Campus, Northwest A&F University, China. All canola plant materials (roots, stems, leaves, flowers, and developing seeds) were collected from the farm-grown plants, immediately frozen in liquid nitrogen, and then stored at −80 °C until use.

Gene cloning and plasmid construction

According to the full-length coding domain sequence (CDS) of *TT8* from *B. napus*, which are *BnaA.TT8* (Accession, ADV03941) and *BnaC.TT8* (Accession, ADV03942), primers BnTT8_P1 (5′-ATGGATGAATTAAGTAT-3′) and BnTT8_P2 (5′-GAGTTTATTATTATATATGATTTG ATGG-3′) were used to amplify the *BnTT8* gene from total RNA isolated from QINYOU Seven developing seeds at 15 days after pollination (DAP). The PCR products were cloned into the pMD19-T vector (TaKaRa Bio, Dalian, China), and then eight randomly selected single colonies were sequenced by Sangon Biotechnology (Shanghai, China).

To construct *35S:BnTT8*, the *BnTT8* cDNA was amplified with primers BnTT8_P3_XhoI (5′-ATTCTCGAG ATGGATGAATTAAGTAT-3′) and BnTT8_P4_ HindIII (5′-ATTAAGCTTGAGTTTATTATTATATATGAT TTGATGG-3′). The PCR products were digested by XhoI and HindIII and then cloned into pGreen-35S to obtain an in-frame fusion of *BnTT8* under control of the 35S promoter. Similarly, *BnTT8* cDNA was also amplified with primers BnTT8_P5_XmaI (5′-TATTCCCGG GATGGATGAATTAAGTAT-3′) and BnTT8- _P6_SpeI (5′-GGACTAGTGAGTTTATTATTATATATGATTTGA

TGG-3′) and then cloned into pGreen-35S–eGFP (where GFP is green fluorescent protein) to obtain an in-frame fusion of BnTT8–eGFP under control of the 35S promoter (*35S:BnTT8–eGFP*).

Subcellular localization of BnTT8–eGFP fusion protein

The *35S:BnTT8–eGFP* construct was transiently expressed in *Nicotiana benthamiana* leaves as previously reported (Yang et al. 2000). Images were collected on a confocal laser scanning microscope (Zeiss LSM 700, Germany) within 72 h following agroinfiltration.

Generation of Arabidopsis transgenic plants

The construct *35S:BnTT8* was transformed into *Agrobacterium tumefaciens* strain GV3101, which was then used for the transformation of Arabidopsis *tt8-4* plants via floral dip (Clough and Bent 1998). The transgenic plants were selected on soil using Basta® until T_3 transgenic plants were generated.

Morphological observations of mature seeds

The seeds were harvested from siliques at the basal part of a major inflorescence. The mature seeds from different lines were randomly selected and photographed using an Olympus SZ 61 stereomicroscope.

Determination of seed FAs and storage proteins

Seed FAs were detected as reported by Chen et al. (2012a). In brief, total FAs were converted to FA methyl esters in methanol solution containing 1 M hydrochloric acid for 2 h at 80 °C. FAs in seeds were subsequently determined using a gas chromatograph (GC-2014; Shimadzu). Storage proteins in seeds were measured as described by Chen et al. (2012b). Briefly, in the presence of Polyclar, seeds were homogenized in the extraction buffer: 50 mM HEPES, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 10% (v/v) ethylene glycol (pH 7.5). After centrifugation (18,000×*g* for 10 min at 4 °C), the supernatant was utilized for storage protein measurements. Gammaglobulin (Bio-Rad) was used for calibration.

Analysis of gene expression by reverse transcription PCR and real-time quantitative PCR

The RNA extraction was conducted using a TaKaRa MiniBest Plant RNA Extraction Kit (TaKaRa Bio) following the manufacturer's instructions. The RNA samples were treated with RNase-free DNAse I supplied by the kit to remove any trace of genomic DNA. The first-strand cDNA was synthesized in a 20-μL reaction solution containing approximately 2.0 μg of total RNA, using PrimerScript™ reverse transcriptase (TaKaRa Bio) and oligo (dT) 12–18 as a primer. *AtACTIN7* (At5g09810) and *BnACTIN7* were regarded as the internal control. The real-time quantitative PCR (qPCR) and the calculation of relative gene expression levels were performed as described in detail by Chen et al. (2014a). Primers used for reverse transcription PCR (RT-PCR) and qPCR are listed in Supplementary Table S1.

Analysis of seed germination rate and seedling establishment under stressed conditions

The Arabidopsis seeds used for the germination assays were collected at the same time from plants grown under the same growth conditions as previously described, allowed to mature for 6 weeks at room temperature, and stored at −20 °C. The seeds were pre-chilled at 4 °C for 5 d and then surface sterilized twice with 75% (v/v) ethanol for 30 s. Then, seeds were washed five times with ddH_2O and subsequently sown onto solid Murashige and Skoog (MS) stock medium containing 3% (w/v) glucose (Glc) and 100 mM sodium chloride (NaCl). The seed germination rate (defined as radicle emergence) was scored daily (Cernac et al. 2006). Arabidopsis seedlings at 17 days after sowing (DAS) were photographed and collected for RNA analysis.

Statistical analysis

Completely randomized block designs with at least three biological replicates were used in the study. Data were classified with Win-Excel and analyzed via ANOVA using the SPSS statistical package (version 8.0). Seed size, seed weight, and seed oil and protein contents for the WT and *tt8-4* mutant were compared using Student's *t*-test. Tukey's highly significant difference test and Student's *t*-test were used to analyze gene expression and seedling fresh and dry weights under abiotic stresses. P-values ≤ 0.05 were considered statistically significant.

Results

Sequence analysis of the BnTT8 protein

The *BnTT8* CDS isolated from developing seeds of *B. napus* cultivar QINYOU Seven is designated *BnTT8* in this study. We used the *BnTT8* CDS to BLAST with the *B. napus* nucleotide database in NCBI (National Center for Biotechnology Information), and found that *BnTT8-like* located on the A8 genome shared the highest identity in nucleotide

sequence with *BnTT8* CDS, except for *BnaA.TT8* and *BnaC.TT8*. The TT8 from canola and Arabidopsis shared high nucleotide and protein sequence similarities (Fig. 1, Supplementary Figure S1). All four canola TT8 proteins had 521 amino acids, and BnTT8 shared highest identity in amino acid sequence with BnaC.TT8 (99.62%), compared with BnaA.TT8 (98.85%) and BnTT8-like (98.85%) (Fig. 1). Consistently, *BnTT8* shared highest nucleotide

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AtTT8          MDESSIIPAEKVAGAEKKELQGLLKTAVQSVDWTYSVFWQFCPQQRVLVWGNGYYNGAIK
BnaA.TT8       MDELSIIPLWKVIGAEKEEIQGLLKAVVQSVGWTYSVFWQLCPQRRKLMWSSGYYNGAIK
BnTT8-like     MDELSIIPLWKVIGAEKEEIQGLLKAVVQSVGWTYSVFWQLCPQRRKLMWSSGYYNGAIK
BnaC.TT8       MDELSIIPLWKVIGAEKEEIQGLLKAVVQSVGWTYSVFWQLCPQRRKLLWSSGNYNGAIK
QINYOU.BnTT8   MDELSIIPLWKVIGAEKEEIQGLLKAVVQSVGWTYSVFWQLCPQRRKLMWSSGNYNGAIK
                  *   ** *       *     **    *         *     * * ** *

AtTT8          TRKTTQPAEVTAEEAALERSQQLRELYETLLAGESTSEARACTALSPEDLTETEWFYLMC
BnaA.TT8       TRKTTQPAEVTAEEAASERSQQLMELYETLFAGESSMEARACTALSPEDLTDPEWFYVLC
BnTT8-like     TRKTTQPAEVTAEEAASERSQQLMELYETLFAGESSMEARACTALSPEDLTDPEWFYVLC
BnaC.TT8       TRKTTQPAEVTAEEAASERSQQLMELYETLFAGESSMEARACTALSPEDLTDPEWFYVLC
QINYOU.BnTT8   TRKTTQPAEVTAEEAASERSQQLMELYETLFAGESSMEARACTALSPEDLTDPEWFYVLC
                               *      *      *    **              **    **

AtTT8          VSFSFPPPSGMPGKAYARRKHVWLSGANEVDSKTFSRAILAKSAKIQTVVCIPMLDGVVE
BnaA.TT8       FTYSFEPPSGMPGKAYARRKHIWLSGANEVDNKIFSRAISAKSAKIQTVVCIPVLDGVLE
BnTT8-like     FTYSFEPPSGMPGKAYARRKHIWLSGANEVDNKIFSRAISAKSAKIQTVVCIPVLDGVLE
BnaC.TT8       FTYSFEPPSGMPGKAYARRKHIWLSGANEVDNKIFSRAISAKSAKIQTVVCIPVLDGVLE
QINYOU.BnTT8   FTYSFEPPSGMPGKAYARRKHIWLSGANEVDNKIFSRAISAKSAKIQTVVCIPVLDGVLE
               *** *                *         *       *             *    *

AtTT8          LGTTKKVREDVEFVELTKSFFYDHCKTNPKPALSEHSTYEVHEEAEDEEEVEEEMTMSEE
BnaA.TT8       LGTTNKVKESEEFVEHIKSFFHNHPKSNTKPTLSEHFINEEHEEDEEEVE-EEEMTMSEE
BnTT8-like     LGTTNKVKESEEFVEHIKSFFHNHPKSNTKPTLSEHFINEEHEEDEEEVE-EEEMTMSEE
BnaC.TT8       LGTTNKVKESEEFVDHIKSFFHNYPKSNTKPTLSEHFINEEREEDEDEVE-EEEMTMSEE
QINYOU.BnTT8   LGTTNKVKESEEFVDHIKSFFHNYPKSNTKPTLSEHFINEEREEDEDEVE-EEEMTMSEE
                   * * *    **    **** * *  *    *** **  * * * *

AtTT8          MRLGSPDDEDVSNQNLHSDLHIESTHTLDTHMDMMNLMEEGGNYSQTVTTLLMSHPTSLL
BnaA.TT8       IRLGSPDDDDVSNQNLLSDFHIEATNSLDTHMDMMNLMEEGGNYSQTVSTLLMSQPTSLL
BnTT8-like     IRLGSPDDDDVSNQNLLSDFHIEATNSLDTHMDMMNLMEEGGNYSQTVSTLLMSQPTSLL
BnaC.TT8       IRLGSPDDDDVSNQNLLSDFHIEATNSLDTHMDMMNLMEEGGNYSQTVSTLLMSQPTSLL
QINYOU.BnTT8   IRLGSPDDDDVSNQNLLSDFHIEATNSLDTHMDMMNLMEEGGNYSQTVSTLLMSQPTSLL
               *      *      * *  * **                        *    *

AtTT8          SDSVSTSSYIQSSFATWRVENGKEHQQVKTA-----PSSQWVLKQMIFRVPFLHDNTKDK
BnaA.TT8       SDSVSTSSYVQSSFVSWRVENVKEHQQYQRVEKAAWSSSQWMLKHIILKVPFLHDNTKNK
BnTT8-like     SDSVSTSSYVQSSFVSWRVENVKEHQQYQRVEKAAWSSSQWMLKHIILKVPFLHDNTKNK
BnaC.TT8       SDSVSTSSYVQSSFISWRVENVKEHQQYQRVEKAASSSSQWMLKHIILKVPFLHDNTKNK
QINYOU.BnTT8   SDSVSTSSYVQSSFISWRVENVKEHQQYQRVEKAAWSSSQWMLKHIILKVPFLHDNTKNK
                        **    **    *      **********    *  ** **         *

AtTT8          RLPREDLSHVVAERRRREKLNEKFITLRSMVPFVTKMDKVSILGDTIAYVNHLRKRVHEL
BnaA.TT8       RLPREELNHVVAERRRREKLNERFITLRSLVPFVTKMDKVSILGDTIEYVNHLSKRIHEL
BnTT8-like     RLPREELNHVVAERRRREKLNERFITLRSLVPFVTKMDKVSILGDTIEYVNHLSKRIHEL
BnaC.TT8       RLPREELNHVVAERRRREKLNERFITLRSLVPFVTKMDKVSILGDTIEYVNHLSKRIHEL
QINYOU.BnTT8   RLPREDLNHVVAERRRREKLNERFITLRSLVPFVTKMDKVSILGDTIEYVNHLSKRIHEL
                    * *              *      *                  *    *  *

AtTT8          ENTHHEQQHKRTRTCKRKTSEEVEVSIIENDVLLEMRCEYRDGLLLDILQVLHELGIETT
BnaA.TT8       ESTHHEPNQKRMRIGKGRTWEEVEVSIIESDVLLEMRCEYRDGLLLNILQVLKELGIETT
BnTT8-like     ESTHHEPNQKRMRIGKGRTWEEVEVSIIESDVLLEMRCEYRDGLLLNILQVLKELGIETT
BnaC.TT8       ESTHHEPNQKRMRIGKGRTWEEVEVSIIESDVLLEMRCEYRDGLLLNILQVLKELGIETT
QINYOU.BnTT8   ESTHHEPNQKRMRIGKGRTWEEVEVSIIESDVLLEMRCEYRDGLLLNILQVLKELGIETT
                *    ***  *  * ** *          *                *     *

AtTT8          AVHTSVNDHDFEAEIRAKVRGKKASIAEVKRAIHQVIIHDTNL
BnaA.TT8       AVHTALNDNHFEAEIRAKVRGKKPTIAEVKIAIHQ-IIYNNKL
BnTT8-like     AVHTALNDNHFEAEIRAKVRGKKPTIAEVKIAIHQ-IIYNNKL
BnaC.TT8       AVHTALNDHHFEAEIRAKVRGKKPTIAEVKIAIHQ-IIYNNKL
QINYOU.BnTT8   AVHTALNDNHFEAEIRAKVRGKKPTIAEVKIAIHQ-IIYNNKL
                   ** **          **    *    *  ****
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Fig. 1 Alignment of protein sequences of TT8 from canola and Arabidopsis. The alignments of AtTT8, BnaA.TT8 (ADV03941), BnaC.TT8 (ADV03942), BnTT8-like (NM_001315974.1), and BnTT8 were analyzed using the MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/). Their conserved regulatory domains were predicted by Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). *Asterisks* represent different amino acids. The two domains 20–201 and 366–417 indicated by *black lines* are the N-terminal regions of a family of MYB and MYC transcription factors and the helix–loop–helix DNA-binding regions, respectively

sequence identity with *BnaC.TT8* (99.23%), compared with *BnaA.TT8* (99.04%) and *BnTT8-like* (99.04%) (Supplementary Figure S1). These indicated that *BnTT8* should be located on the C genome of QINYOU Seven. Moreover, they were highly conserved in the N-terminal region of the family of MYB and MYC transcription factors and the helix–loop–helix DNA-binding region (Fig. 1).

Subcellular localization of BnTT8 protein

Using laser scanning confocal microscopy, the fluorescence signal was specifically localized in the nucleus of *N. benthamiana* leaves transformed using the fusion construct, *35S:BnTT8–eGFP* (Fig. 2a). This result supported the suggestion that BnTT8 functions as a transcription factor.

Expression pattern of *TT8* in the canola genome

At least two copies of *TT8* were present in the A and C genomes of QINYOU Seven, and may have had different expression patterns. However, the expression pattern of all *TT8* copies in QINYOU Seven would still help in better understanding the biological function of *TT8*. Therefore, the temporal and spatial mRNA distributions of all *TT8* copies were analyzed using qPCR in QINYOU Seven. *TT8* was widely expressed in various tissues (Fig. 2b), and was predominantly present in developing seeds (Fig. 2c). The *TT8* transcript level was higher in stems and flowers than in leaves and roots (Fig. 2b). During seed development, *TT8* expression progressively increased at 15 DAP to a maximum at 20 DAP, then remained stable, and slightly decreased at 35 DAP (Fig. 2c).

BnTT8 affects the accumulation of seed storage reserves in Arabidopsis

To explore the BnTT8 function, we transformed the construct *35S:BnTT8* into the Arabidopsis mutant *tt8-4*. At least 20 T_1 plants were obtained by Basta® selection. Four selected independent *tt8-4 35S:BnTT8* T3 homozygous transgenic lines were identified by PCR amplification of *BnTT8* with the specific primers 35S_P/BnTT8_R (Supplementary Figure S2; Table S1). Furthermore, heterologous expression of *BnTT8* was also detected in these lines by RT-PCR (Supplementary Figure S2).

Previous studies indicated that loss of function of *AtTT8* resulted in a yellow seed coat (Nesi et al. 2000) and higher oil and lower storage protein contents in Arabidopsis seeds (Chen et al. 2014b). Ectopic expression of *BnTT8* corrected the yellow seed coat phenotype of *tt8-4* (Fig. 3a). However, seed length and width (Fig. 3b) and seed weight (Fig. 3c) did not significantly differ among the WT, *tt8-4*, and *tt8-4 35S:BnTT8* plants. In addition, the higher FA and lower storage protein contents in *tt8-4* seeds could be fully rescued to WT levels by ectopic expression of *BnTT8* (Fig. 4). These results together indicated that BnTT8 had similar functions to AtTT8 in the accumulation of seed storage reserves in Arabidopsis.

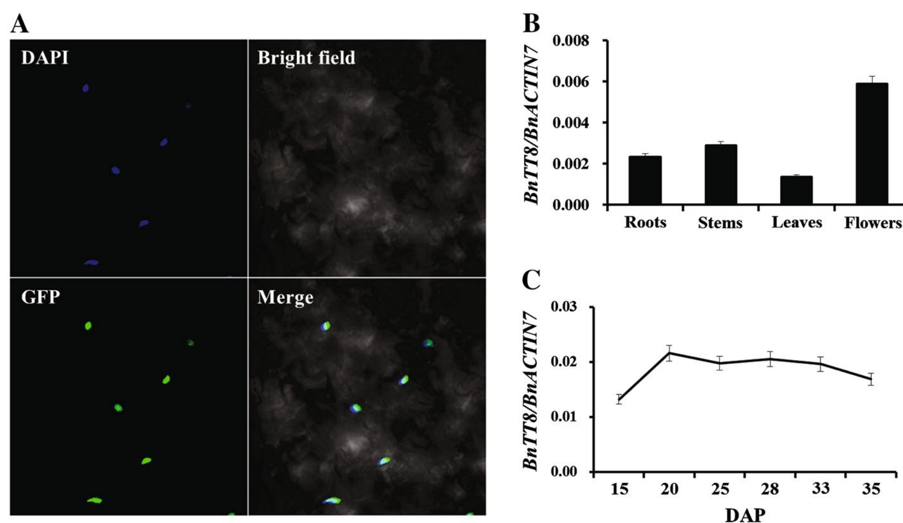

Fig. 2 Expression analysis of canola TT8. **a** Subcellular localization of BnTT8 protein fused with GFP (*35S:BnTT8–eGFP*) in *N. benthamiana* leaves. DAPI, fluorescence of 4′,6-diamino-2-phenylindole; Merge, merging of GFP, DAPI, and bright field images. qPCR analysis of canola *TT8* expression in various tissues (**b**) and developing seeds at different developmental stages (**c**) in cultivar QINYOU Seven. The qPCR result was normalized against *BnACTIN7* expression as an internal control. Values are means of three replicates carried out on cDNA dilutions obtained from three independent RNA extractions. *Error bars* denote standard deviations. DAP, days after pollination

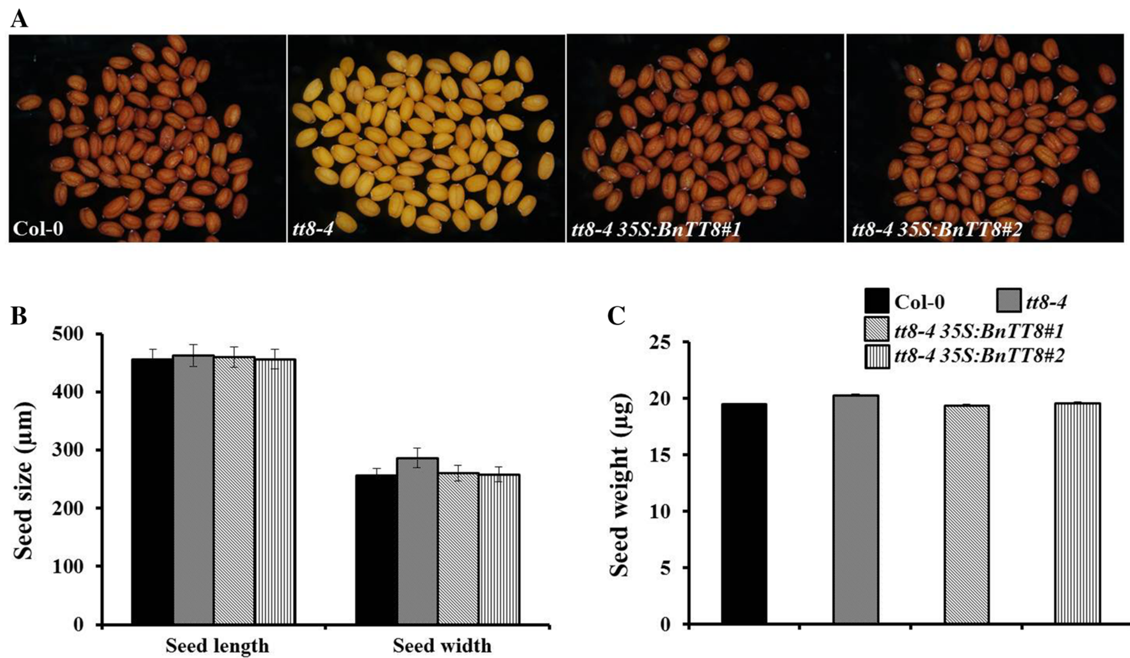

Fig. 3 Analysis of seed coat color, seed size, and seed weight among WT (Col-0), *tt8-4*, and *tt8-4 35S:BnTT8* plants. **a** Microscopic observations of randomly selected mature seeds. Comparison of seed size (**b**) and seed weight (**c**) of mature seeds. Values of seed size and weight are means of three biological replicates, and each of the three assays for each biological replicate contained 200 seeds from 12 individual plants grown in different pots arranged randomly within one of three blocks. There were no significant differences in seed size and weight between WT and the *tt8-4* mutant (Student's *t*-test, $P \leq 0.05$). *Error bars* denote standard deviations

BnTT8 affects tolerance to abiotic stresses during seedling establishment in Arabidopsis

The effects of BnTT8 on stress responses, seed germination, and seedling establishment of WT, *tt8-4*, and *tt8-4 35S:BnTT8* plants were examined in MS medium containing 100 mM NaCl or 3% (w/v) Glc. These lines exhibited similar seed germination rates and seedling establishment (defined as the formation of true leaves and root elongation) on the medium without any stress treatment (Fig. 5). Under the stress of 100 mM NaCl, seed germination vigor was lower in the *tt8-4* mutant than the WT (Fig. 5a). The true leaves and roots developed poorly in the *tt8-4* mutant, suggesting that the loss of *AtTT8* function induced sensitivity to salinity stress (Fig. 5b). In the medium containing 3% (w/v) Glc, the rate and vigor of seed germination were significantly lower for the *tt8-4* mutant line than the WT. Almost all WT seeds germinated at 7 DAS; in contrast, only about 75% of *tt8-4* mutant seeds germinated (Fig. 5a). All WT plants successfully finished seedling establishment at 17 DAS, whereas most *tt8-4* plants failed to develop true leaves in the medium (Fig. 5b). Both fresh and dry weights of *tt8-4* seedlings were significantly lower compared with WT seedlings for each abiotic stress (Supplementary Figure S3). The phenotypes of seed germination and seedling establishment in the *tt8-4* mutant under the abiotic stresses could be fully rescued by introduction of *BnTT8* into the *tt8-4* mutant (Fig. 5; Supplementary Figure S3). This indicated that both AtTT8 and BnTT8 had similar functions in responses to abiotic stresses of high concentrations of NaCl and Glc.

To better understand how BnTT8 affects seedling establishment, we analyzed the transcript levels of some stress-responsive genes in WT, *tt8-4*, and *tt8-4 35S:BnTT8* seedlings at 17 DAS. Under control conditions, expressions of all detected stress-responsive genes were not significant, except for *AtABA1, AtNCED3*, and *AtRAB18* (Fig. 6). Under 100 mM NaCl and 3% Glc stresses, *AtABA1* expression was significantly reduced both in *tt8-4* mutant and WT seedlings, with its relative level uniformly higher in WT than in the *tt8-4* mutant. Expressions of *AtABA2* and *AtNCED3* in the *tt8-4* mutant were significantly decreased under high concentrations of Glc and NaCl, respectively. *AtABI2* was greatly induced in the WT and slightly induced in the mutant under both stresses. For 100 mM NaCl and 3% Glc stresses, *AtADH* was significantly induced both in the *tt8-4* mutant and WT plants, and its relative expression level was uniformly lower in the WT than the *tt8-4* mutant. In WT seedlings, the transcript level of *AtRD29A* was much higher under Glc stress than control conditions. The expression of *AtRAB18* was significantly elevated in both the *tt8-4* mutant and WT, and its relative level was uniformly much

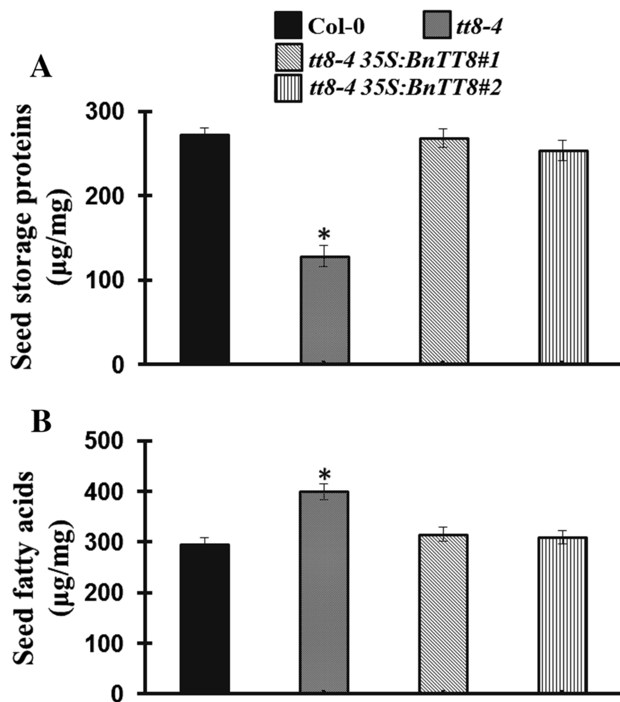

Fig. 4 Comparison of seed storage reserves including seed storage proteins (**a**) and seed fatty acids (**b**) among the WT (Col-0), *tt8-4*, and *tt8-4 35S:BnTT8* plants. Data presented are means of five biological replicates. *Error bars* denote standard deviations. *Asterisks* denote significant differences between the WT and *tt8-4* mutant (Student's *t*-test, $P \leq 0.05$)

higher in the WT than the *tt8-4* mutant. *AtKIN1* expression was significantly increased in the WT under both stresses. In addition, expressions of *AtERD15, AtRD22*, and *AtCBL1* in WT seedlings was significantly increased under Glc stress, whereas their expressions in the *tt8-4* mutant and WT seedlings did not significantly change under both stresses. *AtCBL9* expression was greatly reduced under the two stresses. Under 100 mM NaCl and 3% Glc stresses, all the measured stress-responsive genes exhibited similar expression patterns in the *tt8-4 35S:BnTT8* compared with WT seedlings (Fig. 6), indicating that ectopic expression of *BnTT8* could control expression of the genes involved in abiotic stresses, thus regulating the responses to abiotic stresses in Arabidopsis.

Discussion

Studies have shown that AtTT8, as a basic helix–loop–helix domain transcription factor, not only regulates the biosynthesis of anthocyanins and proanthocyanidins, but also controls seed development and accumulation of seed storage reserves in Arabidopsis (Nesi et al. 2000; Baudry et al. 2006; Chen et al. 2014b). The TT8 from *B. rapa* is involved in the accumulation of proanthocyanidins in the seed coat of Arabidopsis (Li et al. 2012). However, limited information is available about the role of BnTT8 protein in the accumulation of seed storage reserves and seedling establishment. In this study, BnTT8 obtained from the cultivar QINYOU Seven was present in the C genome (Fig. 1; Supplementary Figure S1). TT8 proteins from Arabidopsis and *B. napus* shared highly conserved MYB and MYC transcription factor and helix–loop–helix DNA-binding regions (Fig. 1). The subcellular localization experiment indicated that TT8 functioned as a transcription factor (Fig. 2a). Transcripts of all *TT8* copies were broadly present in different vegetative tissues (Fig. 2b), and highest in developing seeds (Fig. 2c) of cultivar QINYOU Seven, indicating that it might regulate many aspects of plant growth and development, especially seed development.

We compared the phenotypes of seed coat color and seed storage reserves including FAs and storage proteins among the WT, *tt8-4*, and *tt8-4 35S:BnTT8* plants. Ectopic expression of *BnTT8* fully rescued many phenotypes of *tt8-4* seeds (Nesi et al. 2000; Chen et al. 2014b), such as yellow seed coat (Fig. 3a), higher seed FAs, and lower storage protein contents (Fig. 4). In canola developing seeds at 15 DAP, sucrose and other nutrients are delivered to the seed through the vascular tissue and then accumulate in the seed coat or endosperm. Large amounts of soluble sugar and nitrogen are present in the endosperm, accounting for about 80% of the total weight of seeds at 20 DAP (King et al. 1997; Lohaus and Moellers 2000). The seed embryo is fully developed and almost occupies the whole seed at 25 DAP, and then the seed storage reserves enter the rapid accumulation period (Dong et al. 2004; Xu et al. 2015). These, together with the observation of the highest and stable expression of all *TT8* copies in canola developing seeds during 15–33 DAP (Fig. 2c), suggested that BnTT8 is important in the regulatory network that suppresses the accumulation of seed storage reserves.

This study showed that the *tt8-4* mutant line was very sensitive to abiotic stresses caused by high concentrations of Glc and NaCl in the growth medium (Fig. 5; Supplementary Figure S3). The hormone abscisic acid (ABA) plays a major role in optimizing vegetative growth under stress conditions and, at the cellular level, ABA can promote tolerance to some abiotic stresses, including salinity, drought, and cold (Finkelstein et al. 2002). High concentrations of Glc inhibit seedling growth in an ABA-dependent manner, mediated by the osmotic effects of sugar and some aspects of sugar signaling (Gibson 2001; Finkelstein et al. 2002). The transcription levels of many stress-responsive genes differed between the WT and *tt8* seedlings under stressful environments (Fig. 6). Under Glc and NaCl stresses, expressions of all these genes were higher in the WT and *tt8-4 35S:BnTT8* seedlings than in the *tt8-4* mutant except

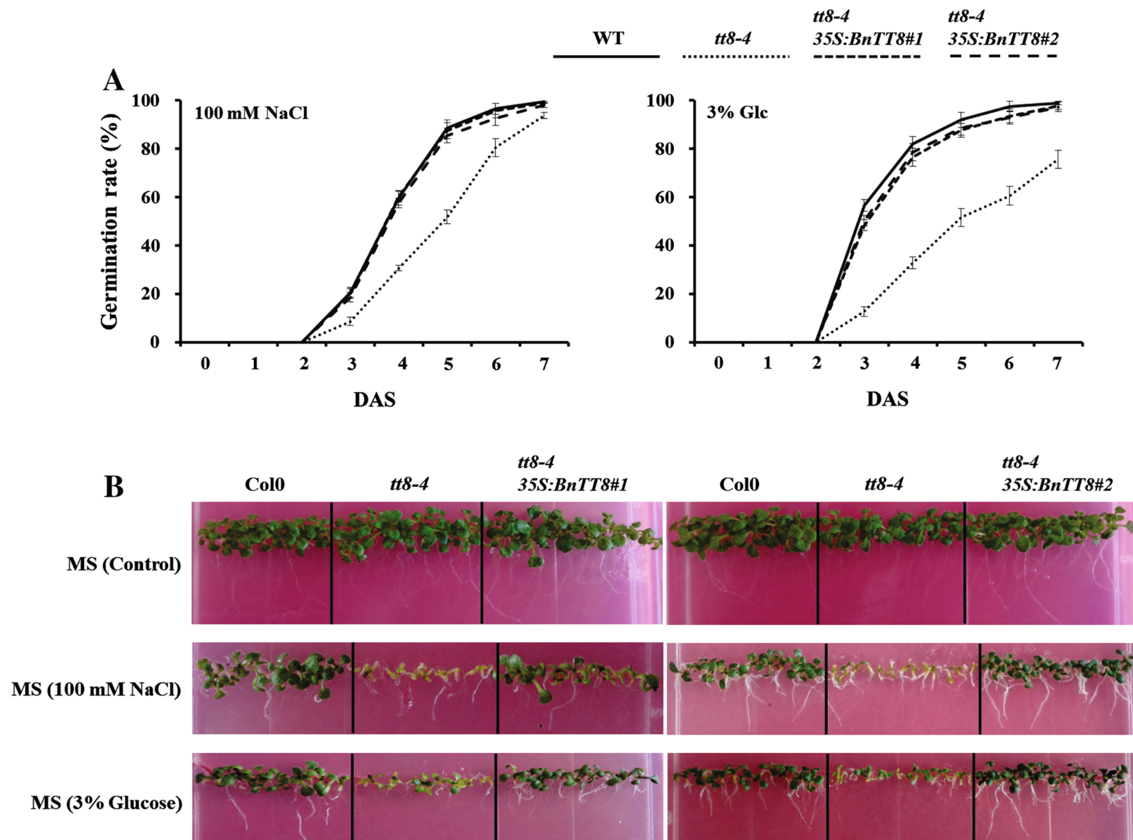

Fig. 5 Analysis of seed germination and seedling establishment of the WT (Col-0), *tt8-4*, and *tt8-4 35S:BnTT8* plants on MS agar medium containing 100 mM NaCl (*left*) or 3% (w/v) glucose (*right*). **a** Comparison of seed germination. Seed germination was scored daily after the radical tip had fully emerged from the seed coat. Data are the means from three independent experiments evaluating 200 seeds. *Error bars* denote standard deviations. **b** Comparison of seedling establishment. The pictures were taken at 17 DAS, showing the situations on the control medium (without stress, CK), MS agar medium containing 100 mM NaCl, and MS agar medium containing 3% (w/v) glucose

for *AtADH* for both stresses, and *AtABA2* and *AtERD15* for NaCl stress (Fig. 6). Moreover, the introduction of *BnTT8* driven by the 35S promoter in the *tt8-4* mutant not only fully rescued impaired seed germination and seedling establishment of the *tt8-4* mutant under both salinity and Glc stresses (Fig. 5; Supplementary Figure S3), but also restored expression patterns of these stress-responsive genes in the *tt8-4* mutant to WT levels (Fig. 6). FAs or FA derivatives serve as essential dietary nutrients for human beings and animals, and also facilitate successful seed germination and seedling establishment (Sullivan and Deng 2003; Li et al. 2006; Mu et al. 2008). In addition, they act as essential components of cellular membranes and cellular signal or hormone molecules protecting the plant from environmental stresses (Ohlrogge and Jaworski 1997; Hong et al. 2008; Chen et al. 2012a, b). Flavonoids play a major role in plant responses to abiotic stresses (Winkel-Shirley 2002; Petrussa et al. 2013). It is possible that deficiency of flavonoids alters the expression patterns of stress-responsive genes during seedling establishment. However, the stress-sensitive phenotypes of the *tt8-4* mutant may also be caused by increased diffusion of stressful compounds through the *tt8-4* seed coat. Another possible explanation is that AtTT8 and BnTT8 function as important mediators in the regulatory network by directly regulating stress-responsive genes. The FA composition linolenic acid is the primary substrate for jasmonic acid biosynthesis in plants, and its content in *tt8-4* seeds was significantly increased (Supplementary Table S2; Chen et al. 2014b). A complex of bHLH factors (AtTT8, AtGL3, and AtEGL3) and MYB factors (AtMYB75 and AtGL1) was found to be required for jasmonic acid-dependent anthocyanin accumulation (Qi et al. 2011). Therefore, AtTT8 and BnTT8 probably combine these possibilities together to affect the tolerance to abiotic stresses during Arabidopsis seedling establishment.

In *B. napus*, yellow seeds have a significantly thinner seed coat than black seeds, and so have a lower hull proportion and higher proportions of oil and protein (Mahmood et al. 2006). The investigation and manipulation of transcription factors controlling seed coat color are of great

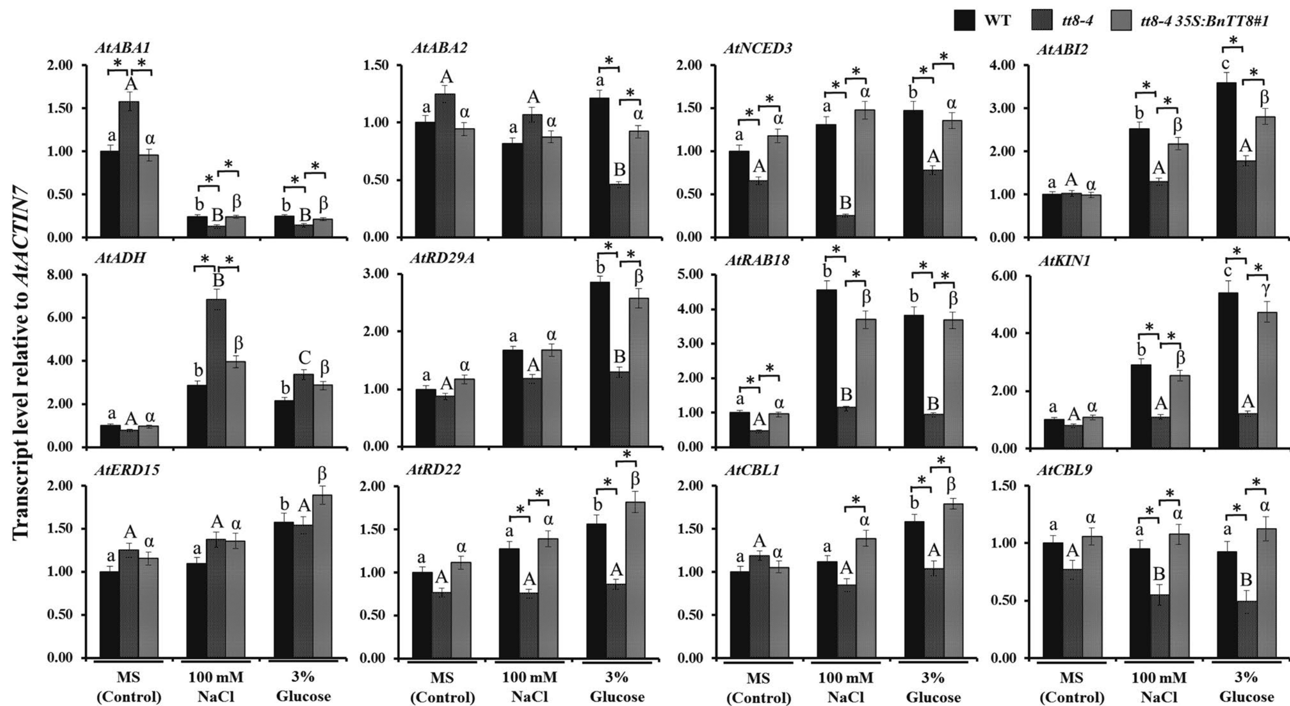

Fig. 6 Comparison of relative transcript levels of stress-responsive genes among the WT (Col-0), *tt8-4*, and *tt8-4 35S:BnTT8* plants under 100 mM NaCl or 3% (w/v) glucose stresses. Total RNA was isolated from whole seedlings at 17 DAS. The qPCR result was normalized against *AtACTIN7* expression as an internal control, and the transcript levels are relative to the WT on the MS medium, which was set to 1. Values are the means of two replicates carried out on cDNA dilutions obtained from two independent RNA extractions. *Different letters* within each treatment indicate significant differences at $P \leq 0.05$ (Tukey's honestly significant difference test); each kind of *letters* should only be compared with like letters. *Asterisks* denote significant differences between indicated samples (Student's *t*-test, $P \leq 0.05$). *Error bars* denote standard deviations

significance for agricultural production. In this regard, BnTT8 is a promising target to genetically manipulate *B. napus* to increase seed oil content. However, breeders should fully consider that the loss of BnTT8 might lead to a decreased ability of *B. napus* against abiotic stresses during seed germination and seedling establishment.

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