

# Molecular cloning of two ortholog genes of *Arabidopsis thaliana* *TTG1* from oilseed rape (*Brassica napus* L.)

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## Abstract

Using RACE method, 2 members (*BnTTG1-1* and *BnTTG1-2*) of *Brassica napus* *TRANSPARENT TESTA GLABRA 1* (*BnTTG1*) gene family encoding WD40 regulatory proteins of proanthocyanidin biosynthesis were isolated. The genomic sequence and the full-length cDNA of *BnTTG1-1* are 1511 bp and 1355 bp, while 1555 bp and 1303 bp for *BnTTG1-2*, respectively. They both have an intron, 156 bp for *BnTTG1-1* and 252 bp for *BnTTG1-2*. BLASTn indicated that their coding regions show the highest homologies to intrafamily *AtTTG1* and *Matthiola incana* *TTG1* and wide homologies to sequences encoding WD40 proteins from distant plant species. Deduced *BnTTG1-1* and *BnTTG1-2* proteins both are of 337 aa, and SUPERFAMILY alignment revealed that they both belong to the WD40 family. NCBI blastp and pair-wise alignments indicated that *BnTTG1-1* and *BnTTG1-2* show a wide range of similarity degrees to a diverse group of WD40 proteins from various plants, with the highest homologies to intrafamily *AtTTG1*. Notably, NCBI blastp also found that *BnTTG1-1* and *BnTTG1-2* share moderate to low similarities to WD40 proteins from other kingdoms. NCBI Conserved Domain (CD) search detected two conserved domains, WD40 and COG2319, dominating the central party of *BnTTG1-1* and *BnTTG1-2*. They both are conserved domains of WD40 proteins. RT-PCR detection results indicated that *BnTTG1-1* and *BnTTG1-2* showed some differences in tissue specificity. Transcripts of *BnTTG1-2* can be detected in all the 11 organs/tissues. The transcription of *BnTTG1-1* is more tissue-specific. This research will help to reveal the molecular mechanism of seed color formation of rapeseed and lay the basis for transgenic creation of stably inherited novel yellow-seeded stocks through seed-coat-specifically suppression of *BnTTG1* expression.

**Key words:** *Brassica napus* L., Flavonoid, Gene cloning; WD40, *TRANSPARENT TESTA GLABRA1* (*TTG1*)

## Introduction

More and more evaluations have revealed many differences of quality traits between brown-/black-seeded and yellow-seeded rapeseed stocks. It is one of the most important breeding goals to select yellow-seeded oilseed rape cultivars with stably inherited yellow seed trait and good agronomic traits.

Recent genetic studies on the flavonoid biosynthetic pathway show that transcription factors are efficient new molecular tools for plant metabolic engineering to increase or decrease the production of valuable compounds (Gantet & Memelink, 2002). The transcriptional regulators for anthocyanin/proanthocyanidin biosynthesis include members of proteins containing an R2R3-MYB domain, a bHLH (basic helix-loop-helix) domain and conserved WD40 repeat (WDR). Spatial and temporal expression of the structural genes encoding the enzymes for anthocyanin/proanthocyanidin biosynthesis is thought to be determined by combinations of the R2R3-MYB domain, bHLH and WD40 repeat (Morita et al., 2006). Among the phenylpropanoid mutants in *Arabidopsis* a group representing 20 loci shows a reduction or complete absence of pigmentation in the testa. These *tt* mutants have distinct yellow or pale brown seeds in contrast to the wild-type dark brown seeds. Mutation at the *TTG1* locus has a large range of pleiotropic effects. It is known that *ttg1* mutant has a glabrous phenotype with no leaf or stem hairs (trichomes). No purple anthocyanin pigments are present in the seed coat leading to the yellow cotyledons being visible through the transparent testa. Mucilage is absent from *ttg1* mutant seeds and the seeds show no secretion of mucilage. Seeds of *ttg1* mutant plants do not require drying and cold treatments to germinate (Bharti & Khurana, 2003; Galway et al., 1994). *TTG1* control the expression of downstream structural genes like *DFR/TT3* (Shirley et al., 1995), *BAN/ANR* and *LDOX/TT18* (Pelletier et al., 1997). Manipulation of the phenotypic characteristics of plants may be achieved by altering the *TTG1* gene by up or down regulating its expression. Up-regulation or suppression of *TTG1* expression may used to alter pigmentation patterns of seeds, flowers, fruits and leaves for agronomical purposes.

## Material and Methods

### *Plant materials and nucleic acids isolation*

Typical black-seeded *B. napus* line 5B was bred and grown by Chongqing Rapeseed Engineering Research Center. A CTAB method described by Jaakola was used to isolate total RNA of each sample while total genomic DNA was extracted from fresh leaves using a CTAB method.

### *Amplification of full-length cDNAs and corresponding genomic sequences of the 2 BnTTG1 members*

Mixture of total RNA from reproductive organ samples of line 5B was used as template to generate first-strand total cDNA using Rapid Amplification of cDNA Ends (RACE) in terms of the user manual (GeneRacer kit, Invitrogen, USA).

After obtaining both the 5' and the 3' cDNA ends, FTTG15 was paired with RTTG17 and RTTG11 respectively to carry out amplifications of full-length cDNAs and corresponding genomic sequences of 2 unique genes. Alignment, open reading frame translation, and parameter calculation of nucleotide and protein sequences, were performed on Vector NTI Advance 9.0. Sequence BLAST analyses were done on NCBI website (<http://www.ncbi.nlm.nih.gov/>). Protein structure predictions were carried out on website (<http://www.expasy.org>).

#### *Southern blot detection of paralogous TTG1 genes in B. napus*

Fifty µg of 5B total genomic DNA for each enzyme was fully digested with *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Xba*I respectively. Southern blot hybridization (DIG Easy Hyb, Roche) was performed at 48.6°C for 16 h using DIG-dUTP labeled *BnTTG1-1* fragment as probe (PCR DIG Probe Synthesis Kit). Stringent washing and immunological detection (DIG Wash and Block Buffer Set, DIG Nucleic Acid Detection Kit, Roche) were performed in terms of kit instructions.

#### *RT-PCR detection of transcription levels of the 2 BnTTG1 members in various organs of B. napus*

Semi-quantitative reverse transcription-PCR (RT-PCR) was adopted to detect the transcription levels of the 2 *BnTTG1* members in 11 organs of line 5B. Five µg of each total RNA sample were reverse-transcribed using primer Oligo(dT)<sub>20</sub> (SuperScript III First-Strand Synthesis SuperMix, Invitrogen). The quantity uniformity of each cDNA sample was monitored by a 24-cycle PCR checking using internal control primers FATACT2 and RATACT2 to amplify a 542-bp fragment.

## Results

#### *Molecular characterization of nucleotide sequences of BnTTG1-1 and BnTTG1-2*

The genomic sequence and the full-length cDNA of *BnTTG1-1* are 1511 bp and 1355 bp in length respectively. When they were pair-wise aligned, a 156-bp intron was detected. The genomic sequence and the full-length cDNA of *BnTTG1-2* are 1555 bp and 1303 bp in length respectively. When they were pair-wise aligned, a 252-bp intron was detected. BLASTn indicated that the coding regions of *BnTTG1-1* and *BnTTG1-2* show the highest homologies to intrafamily *AtTTG1* (GenBank accession numbers of [AK227211.1](#) etc.) and *Matthiola incana TTG1* ([AJ586861.1](#) etc.) and wide homologies to sequences encoding WD40 proteins from distant plant species.

#### *Conservation and structural features of the deduced BnTTG1-1 and BnTTG1-2 proteins*

The ORFs of *BnTTG1-1* and *BnTTG1-2* both encode polypeptides of 337 amino acid residues. *BnTTG1-1* possesses a calculated molecular weight of 37.28 kDa and an isoelectric point (pI) value of 4.66, while *BnTTG1-2* is 37.26 kDa with a pI value of 4.66. The results of SUPERFAMILY alignment (Madera et al., 2004) revealed that *BnTTG1-1* and *BnTTG1-2* both belong to the WD40 family. NCBI blastp and pair-wise alignments indicated that *BnTTG1-1* and *BnTTG1-2* show a wide range of similarity degrees to a diverse group of WD40 proteins from various plants, with the highest homologies to intrafamily *AtTTG1*. Notably, NCBI blastp also found that *BnTTG1-1* and *BnTTG1-2* share moderate to low similarities to WD40 proteins from other kingdoms. For example, *BnTTG1-1/BnTTG1-1* shows 49.0%/48.7% identities and 63.9%/63.9% positives to Cg14614-prov protein from *Xenopus laevis* (AAH44040.1). NCBI Conserved Domain (CD) search detected two conserved domains, WD40 and COG2319, dominating the central party of *BnTTG1-1* and *BnTTG1-2*. They both are conserved domains of WD40 proteins.

#### *Southern blot detection of TTG1 homologues in the genome of B. napus*

Using *B. napus* genomic DNA to perform Southern gel blot analysis under moderate stringency conditions, *Dra*I, *Eco*RV, *Hind*III and *Xba*I digestions all resulted in two unambiguous bands and no weak band was observed (Fig. 1). Because all the 4 enzymes have no cutting site in *BnTTG1* and *BnTTG2*, it is suggested that *BnTTG1* family most possibly contains two gene members that are reported here.

#### *Transcription levels of BnTTG1-1 and BnTTG1-2 in various organs of B. napus*

RT-PCR detection results indicated that *BnTTG1-1* and *BnTTG1-2* showed some differences in tissue specificity. Transcripts of *BnTTG1-2* can be detected in all the 11 organs/tissues analyzed, with a nearly constitutive pattern. Organs like root, hypocotyl, leaf, seed of 20 d after flowering (DAF) and silique pericarp all showed extensive expression of *BnTTG1-2*, with the highest in the root. Only cotyledon was detected with distinctly low expression of this member. The transcription of *BnTTG1-1* is more tissue-specific. Its transcripts in the 3 stages of seed analyzed are obviously lower than in other 8 organs/tissues and those of *BnTTG1-2*. It is also obvious that as the seed develops, its expression also declines. In seed of 30 DAF, almost little transcription of *BnTTG1-1* was detected. In other 8 organs/tissues, *BnTTG1-1* shows the highest expression in the bud, followed by flower, stem and silique pericarp (Fig. 2).

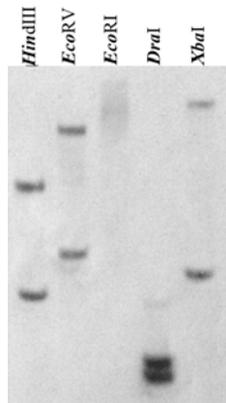


Fig. 1. Southern blot detection for members of *BnTTG1* gene family in the *B. napus* genome

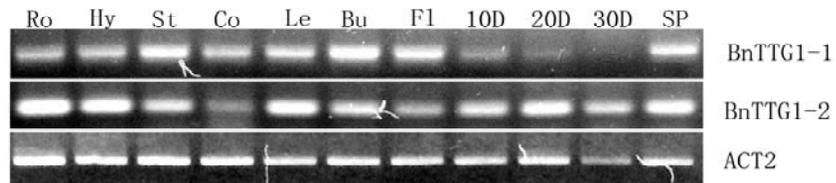


Fig. 2. RT-PCR detection of *BnTTG1* transcription levels in various organs of *B. napus*

## Discussion

Though *BnTTG1-1/BnTTG1-2* shares only 55.1%/56.2% identities on genomic scale and 76.8%/75.8% identities on mRNA scale to *AtTTG1* respectively, the deduced proteins have astonishingly high similarities to *AtTTG1* (92.7%/92.4% identities and 95.6%/96.6% positives). By analysis of the *BnTTG1-1* and *BnTTG1-2* amino acid sequences, we observed the presence of 3 predicted significant WD40 repeats which are remarkably well conserved among plants, yeast, nematodes and humans, suggesting the importance of these domains for their functions. *AtTTG1* was reported with 4 WD40 repeats, but only 3 WD40 repeats can be predicted as we sent it to InterProscan. In the region corresponding to the presumed third WD40 repeat of *AtTTG1*, *BnTTG1-1* and *BnTTG1-2* have only one residue of change (Y<sub>234</sub> to C<sub>230</sub>). Consistent with the pleiotropic mutant phenotype, Northern blot experiments showed that *AtTTG1* is expressed in most major plant organs (Walker et al., 1999). Transcripts of *AtTTG1* homologues from other species, namely *an11*, *PFWD* and *paci* are also present in both pigmented and unpigmented tissues. Expression patterns of the 2 *BnTTG1* genes cloned here are quite similar to that of *AtTTG1*, though certain divergence is found between these 2 members. OrthoMCL analyses (<http://orthomcl.cbil.upenn.edu>) revealed that *BnTTG1-1* and *BnTTG1-2* both are highest similar to *ath24718*, *ath24717* and *ath24716* (*At5g24520*, *AtTTG1*), with Scores of 613 and 612 bits and Expect values of  $e^{-175}$  and  $e^{-174}$  respectively. Other proteins show high scores are also WD-40 proteins involved in regulation of anthocyanin biosynthesis. Based on above analyses, it is concluded that, in viewing of homology, structural similarity, protein location and expression patterns, *BnTTG1-1* and *BnTTG1-2* are orthologous genes of *AtTTG1* and most probably have similar functions in planta as *AtTTG1*.

This experiment result will help to reveal the molecular mechanism of seed color formation of rapeseed and lay the basis for transgenic creation of stably inherited novel yellow-seeded stocks through seed-coat-specifically suppression of *BnTTG1* expression.

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