

## Molecular cloning of *BnZEP* and its expression in petals of different colors in *Brassica napus* L.

Jun JIANG\*, Lushen TIAN\*, Shixing GUO, Qingqing YU, Dezhi ZENG, Yingze NIU\*\*  
Rapeseed Research Center, Sichuan Agricultural University, Wenjiang, Sichuan, P.R. China

Received: 03.03.2014 • Accepted: 28.09.2014 • Published Online: 12.06.2015 • Printed: 30.06.2015

**Abstract:** Carotenoids are a class of pigment elements present in photosynthetic organisms. The transcripts of specific carotenoid biosynthetic genes are probably important for the formation of different colors of flower petals. Zeaxanthin epoxidase (*ZEP*) enzyme activity is important for the high content of carotenoids in petals. In the present study, the zeaxanthin epoxidase gene in *Brassica napus* L. (*BnZEP*) was cloned and bioinformatically analyzed. The expression levels of the *BnZEP* gene in different plant organs and petals of different colors were investigated and compared in *B. napus*. The results showed that the full-length cDNA sequence of *BnZEP* was 2228 bp, containing an open reading frame of 2010 bp and encoding a putative protein of 669 amino acids, with a molecular weight of 73.8 kDa and an isoelectric point (pI) of 6.16. The full-length genomic DNA sequence of *BnZEP* was 3268 bp, containing 16 exons and 15 introns, the same as in *Arabidopsis thaliana* (*AtZEP*). The predicted amino acid sequence of *BnZEP* revealed a close phylogenetic relationship among the cruciferous species, including *Arabidopsis thaliana*. The expression levels of *BnZEP* were remarkably different in different organs and in petals of different colors. Obviously higher expression levels of *BnZEP* were found in flower petals than in leaves and buds. Yellow petals had markedly higher expression levels of *BnZEP* than white petals. The results showed that the expression level of *BnZEP* was positively correlated with yellow petal color in *B. napus* L. However, the highest expression level of *BnZEP* was observed in the pale yellow flower petals of the F<sub>1</sub> hybrid (HW243 × ZHONGYOU 821). This implied that the expression level of *BnZEP* was not completely related to the degree of yellow petal color in *B. napus* L. Based on the roles of *ZEP* enzyme in the xanthophyll cycle, it was suggested that the yellow flower color in *Brassica napus* L. was probably associated with the accumulation of antheraxanthin and violaxanthin in the petals. However, further studies are necessary to identify the specific key carotenoid(s) for the yellow color in the petals of *Brassica napus* L.

**Key words:** *Brassica napus* L., flower color, gene cloning, real-time quantitative PCR, zeaxanthin epoxidase

### 1. Introduction

Carotenoids, a class of pigment elements present in all photosynthetic organisms, act as light sensors in photosynthesis in the form of pigment-protein complexes. They also prevent plants from photooxidation induced by strong light and peroxidation of lipids, and they are favorable for stabilization of the thylakoid membrane structure (Lu and Li, 2008). In horticultural crops, carotenoids play major roles in the coloration of fruits, roots, or tubers and contribute to nutritional qualities as well. Moreover, carotenoids are associated with flower colors, which may change with the relative proportions of individual carotenoids. For example, the main carotenoid in red lily flower petals is capsanthin, whereas a combination of violaxanthin, lutein, antheraxanthin, and  $\beta$ -carotene was observed in pink lily flowers (Yamagishi et al., 2010). The ratio

of transcript abundance of specific carotenoid biosynthetic genes is probably important for the determination of flower color. This has been shown in the changes in flower color in *Eschscholzia californica*, which was found to be a reflection of zeaxanthin accumulation depending on the downregulation of the zeaxanthin epoxidase (*ZEP*) gene (Wolters et al., 2010). The *ZEP* enzyme is widely present in photosynthetic chloroplast-containing tissues of plant and also in the nonphotosynthetic chromoplast-containing tissues such as fruits and flower petals (Ralley et al., 2004). It was seen that, in the developing endosperm of maize, carotenoid accumulation increased with the decrease in *ZEP* transcript abundance (Vallabhaneni and Wurtzel, 2009). In potato, reduced *ZEP* expression level was found responsible for the development of yellow-fleshed tuber (Wolters et al., 2010; McCord et al., 2012).

\* These authors made equal contributions to the paper.

\*\* Correspondence: niuyz01@126.com

In *Brassica napus* L., the color of flowers is generally yellow, while other different flower colors are also present, including pale yellow, orange, milky white, and white. Flower colors in rapeseed can be useful indicator characters in breeding and genetic studies. We developed a group of pure white-flowered rapeseed lines (*Brassica napus* L.) through crosses between common *Brassica napus* lines and white-flowered resynthesized *Brassica napus* lines. Different intermediate petal-color lines were also obtained. However, the specific pigments and genes responsible for the flower colors in *B. napus* L. are not understood. In the present study, we cloned the full-length cDNA and genomic DNA sequences of the *ZEP* gene in *Brassica napus* L. (designated as *BnZEP*) using the rapid amplification of cDNA ends (RACE) method. The sequence of *BnZEP* was bioinformatically analyzed and its expression in different organs and petals of different colors was investigated using the real-time quantitative PCR method. This study aimed for insight into the relationship between *BnZEP* expression levels and petal color in *Brassica napus* L.

## 2. Materials and methods

### 2.1. Plant materials

Rapeseed cultivar ZHONGYOU 821 was used for cloning and characterization of the *BnZEP* gene in *Brassica napus* L. Three rapeseed materials with different flower colors, ZHONGYOU 821 (yellow flowers), HW243 (white flowers), and the F<sub>1</sub> hybrid of HW243 × ZHONGYOU 821 (pale yellow flowers), were used to observe the expression levels of the *BnZEP* gene in petals of different colors and in different plant organs, including the leaf, stem, bud, petal in bud (younger unopened petals), and fully open petal. The plant materials were grown on the experimental farm of Sichuan Agricultural University, Yaan, Sichuan Province, China. Plant organ tissues were taken at the full-blossom stage, immediately frozen in liquid nitrogen, and then stored at -80 °C for use.

### 2.2. Isolation of total RNA and genomic DNA and synthesis of cDNA

Total RNA was isolated from different organs separately using the RNAPrep Pure Plant Kit (Tiangen, China). Total genomic DNA was extracted from young leaves of ZHONGYOU 821 and purified as described by Li et al. (1994). Reverse-transcription PCR was performed to amplify cDNA using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, China) and GeneRacer oligo (dT) primers with 2 µg of total RNA as templates according to the manufacturer's instructions (TaKaRa). The cDNA products were stored at -20 °C for further use.

### 2.3. Cloning and sequencing of the conserved sequence of *BnZEP*

The conserved sequence of the *BnZEP* gene was amplified using the degenerate primer pair 5'-GAGAGGGACAATACAGAGGACCTatthcarathca-3' and 5'-AACCTGGGACAAGGAGGAtgyatgcnat-3', designed according to Henikoff and Henikoff (1994). PCR was performed with a reaction volume of 50 µL containing 1.25 U of PrimeSTAR HS DNA Polymerase (TaKaRa) and 1 µL of cDNA in a PTC1000 PCR Thermal Cycler (Bio-Rad, USA). The amplification conditions were 30 cycles of 98 °C for 10 s and 68 °C for 1 min.

The amplified conserved fragment of *BnZEP* was cloned into the pMD19-T Vector (TaKaRa), and transformed into *Escherichia coli* DH5α competent cells (Tiangen). The DNA inserts in the recombinant clones were amplified using the above-mentioned degenerate primers and sequenced in both directions by Invitrogen Life Technologies Co. Ltd. (China).

### 2.4. The 3' and 5' rapid amplification of cDNA ends (3' RACE and 5' RACE)

The 3' RACE and the 5' RACE were performed using the GeneRacer Kit and the GeneRacer oligo (dT) primer 5'-GCTGTCAACGATACGCTACGTAACGGCATGACA GTG(T)<sub>24</sub>-3' (Invitrogen, USA). The 3' RACE was carried out using PrimeSTAR HS DNA polymerase and the primer pair 5'-AGGTAACGGTGGTGCTTGAGAATGG-3' and 5'-GCTGTCAACGATACGCTACGTAACG-3'. Nested PCR was carried out using similar conditions with 1 µL of 3' RACE product as a template and the primer pair 5'-AGGTAACGGTGGTGCTTGAGAATGG-3' and 5'-CGCTACGTAACGGCATGACAGTG-3'. The 5' RACE was performed accordingly using the primer pair 5'-CGACTGGAGCACGAGGACACTGA-3' and 5'-TCCTCCTCTGTGGCATTTCAGTAAGTC-3'. Nested PCR was carried out using 1 µL of 5' RACE product as a template and the primer pair 5'-GGACACTGACATGGACTGAAGGAGTA-3' and 5'-TCCTCCTCTGTGGCATTTCAGTAAGTC-3'. The products of 3' and 5' nested PCRs were recovered, purified, and cloned into the pMD19-T Vector. The recombinant clones were amplified using the sequencing primer M13 and the aforementioned gene-specific primers, and were then sequenced as described above. The full-length cDNA sequence of *BnZEP* was obtained by electronic assembly of the 3' RACE, the 5' RACE, and the conserved sequence of *BnZEP* using the software DNASTAR. The open reading frame (ORF) was predicted with the software ORFfinder (<http://www.ncbi.nlm.gov/gorf/gorf.html>) (Li et al., 2002). The full lengths of cDNA and genomic DNA sequences were confirmed using the primer pair 5'-ATGGGCTCAACTCCCTTCTGCTAC-3' (*ZEPF*) and 5'-TCAAGCAGCCTGAAGCAATTTACCG-3' (*ZEPR*), with 1 µL cDNA or genomic DNA as the template.

## 2.5. Analysis of nucleotide and amino acid sequences

Nucleotide sequences of the full-length cDNA and genomic DNA of *BnZEP* were analyzed using the ABI PRISM 3730 sequencer (Applied Biosystems, USA). The theoretical molecular weight and isoelectric point (pI) of the predicted protein were calculated using the ExPASy Proteomics tool (<http://www.expasy.ch/tools/>). The homologous amino acid sequences were searched by BLAST P from the nonredundant protein sequence (nr) database of the NCBI (<http://www.ncbi.nlm.nih.gov>) (Shen et al., 2002). The numbers of exons and introns in the full-length genomic DNA sequence of *BnZEP* were analyzed using the Spidey tool (<http://www.ncbi.nlm.nih.gov>). A phylogenetic tree was constructed using MEGA version 3 by the neighbor-joining method (Kumar et al., 2004).

## 2.6. Reference gene selection and quantitative real-time PCR

Four candidate reference genes belonging to different functional classes (18S, GAPDH, TIP41, and UBC21) were selected from the commonly used reference genes in the literature in order to avoid coregulation. Primers used for the target and reference gene amplifications are described in Table 1 and were synthesized by Invitrogen Life Technologies Co. Ltd.

The first-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (Perfect Real Time) with 2 µg of total RNA in a total reaction volume of 50 µL following the manufacturer's recommendations (TaKaRa). Quantitative real-time PCR was performed in a BIO-RAD CFX96 (Bio-Rad) system with 20 µL of mixture containing 10 ng of cDNA, 10 µL of SsoFast EvaGreen Supermix (Bio-Rad), and 0.1 µM of each primer. To calculate the relative expression levels of the genes, serial dilutions (0.2–125 ng) were prepared to produce standard curves for each gene. PCR was performed in triplicate in 96-well optical reaction plates with a program comprising an initial heating step of 98 °C for 2 min, followed by 40 cycles of 98 °C for 2 s and 55 °C for 10 s. The fluorescence was measured at the end of the 55 °C extension step. The amplification specificity of each primer pair was confirmed by analysis of the melting

curves of the final PCR products in the temperature range of 65–95 °C where the fluorescence was acquired after each step of 0.5 °C increment. The fluorescence threshold value and the gene expression levels were calculated using CFX96 system software. Relative expression levels of reference genes were determined according to Vandesompele et al. (2002). The relative expression levels of the target gene were normalized to the geometric means of the most stable reference genes by CFX96 system software. All samples were run in duplicate and mean values were used for further calculations.

Serial dilution curves of cDNA were produced to calculate the amplification efficiency for all primers. A graph of threshold cycle versus  $\log_{10}$  picograms of diluted sample series was produced. The slope of the curve was used to determine the amplification (PCR) efficiency according to Pfaffl (2001): efficiency =  $10^{(-1/\text{slope})}$ . The values of amplification efficiency are given in Table 1. The gene expression stability and the selected most suitable reference genes were evaluated with CFX96 system software.

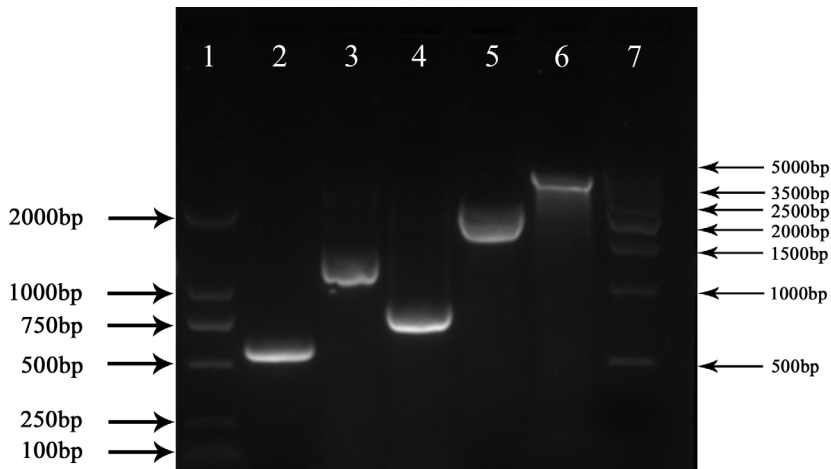
## 3. Results

### 3.1. Full-length sequences of cDNA and genomic DNA of *BnZEP*

A 582-bp fragment was obtained with the degenerate primers (Figure 1, lane 2). The sequence of this fragment revealed an ORF of 194 amino acids. Alignment of the deduced amino acid sequence showed a high degree of identity to the central coding regions of the known *ZEP* sequences in other plant species. The cloned conserved sequence of *BnZEP* was further elongated in both directions by the 3' and 5' RACEs. A fragment of 1480 bp (Figure 1, lane 3) was obtained by 3' RACE and a fragment of 755 bp (Figure 1, lane 4) was obtained by 5' RACE. The BLAST of the deduced amino acid sequences revealed good correspondence to the expected gene. Comparison of the sequences obtained by 3' and 5' RACEs and the central amplified part with the degenerate primers indicated that the overlapping regions matched perfectly. It was indicated that the sequences from the 3' and 5' RACEs represented the missing parts of the gene.

**Table 1.** Primer sequences of target genes and candidate reference genes for normalization.

Gene symbol	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length	PCR efficiency (%)	Tm (°C)
qzep	ACCTGTGACTCGGGTGATTAG	AAGCACCACCGTTACCTTATC	133	101.8	80
18S	AACCAAACATCTCACGACAC	GCAAGACCGAAACTCAAAG	193	100.4	83.5
GAPDH	CAACATTGTTTCCAACGCTAG	ACGGTGGTCATAAGTCCCTC	102	99.8	80.5
TIP41	AGAGTCATGCCAAGTTCATGGTT	CCTCATAAGCACACCATCAACTCTAA	69	101.6	76.5
UBC21	CCTCTGCAGCCTCCTCAAGT	CATATCTCCCCTGTCTTGAATGC	77	101.9	77.5



**Figure 1.** Electrophoresis of the RACE and the full-length PCR products on 2% agarose gel. Lane 1: DL2000 marker; lane 2: fragment cDNA; lane 3: 3' RACE product; lane 4: 5' RACE product; lane 5: the full-length cDNA of *BnZEP*; lane 6: the full-length genomic DNA of *BnZEP*; lane 7: 500-bp DNA ladder marker.

The full-length cDNA sequence of *BnZEP*, assembled electronically with the 3' and 5' RACE sequences and the degenerate primer amplified central fragment, was 2228 bp (GenBank accession number GU361616). The same length of sequence was obtained from the cDNA sample of *BnZEP* using the primer pair ZEPF and ZEPR (Figure 1, lane 5). The full-length genomic DNA sequence of *BnZEP* (GenBank accession number GU561839) was 3268 bp (Figure 1, lane 6).

### 3.2. Analyses of cDNA and genomic DNA sequences of *BnZEP*

The full-length cDNA sequence of *BnZEP* showed an ORF of 2010 bp, encoding a protein of 669 amino acids with a calculated molecular weight of 73.8 kDa and a pI of 6.16. This cDNA sequence contained a 5' untranslated region (UTR) of 57 bp and a 3' UTR of 161 bp.

The full-length genomic DNA sequence of *BnZEP* was analyzed using the NCBI Spidey tool online (<http://www.ncbi.nlm.nih.gov/spidey/>). This sequence contained 16 exons and 15 introns (Figure 2A). The exon and intron

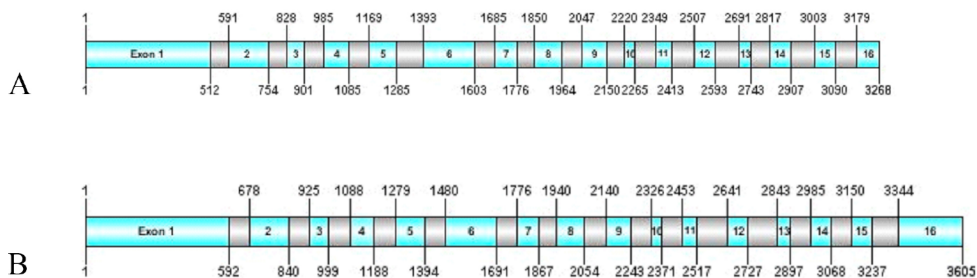
numbers were the same as in *Arabidopsis thaliana* (*AtZEP*), whereas their sizes were different (Figure 2B).

The nucleotide and amino acid sequences of *BnZEP* were compared among 12 different plant species using the BLAST software online (<http://www.ncbi.nlm.nih.gov/>). High homologies were observed in the available *ZEP*s from the GenBank database (Figure 3). The sequences of nucleotides and amino acids of *BnZEP* were 96% and 99% homologous with *Brassica rapa* (FJ606830), 89% and 93% homologous with *Thellungiella halophila* (AY842302), and 87% and 90% homologous with *Arabidopsis thaliana* (AF281655), respectively.

A phylogenetic tree was constructed using MEGA 4 software by the neighbor-joining method (Figure 4). It was indicated that the amino acid sequence of *BnZEP* was highly similar in the different cruciferous species, including *Arabidopsis thaliana* (Figure 4).

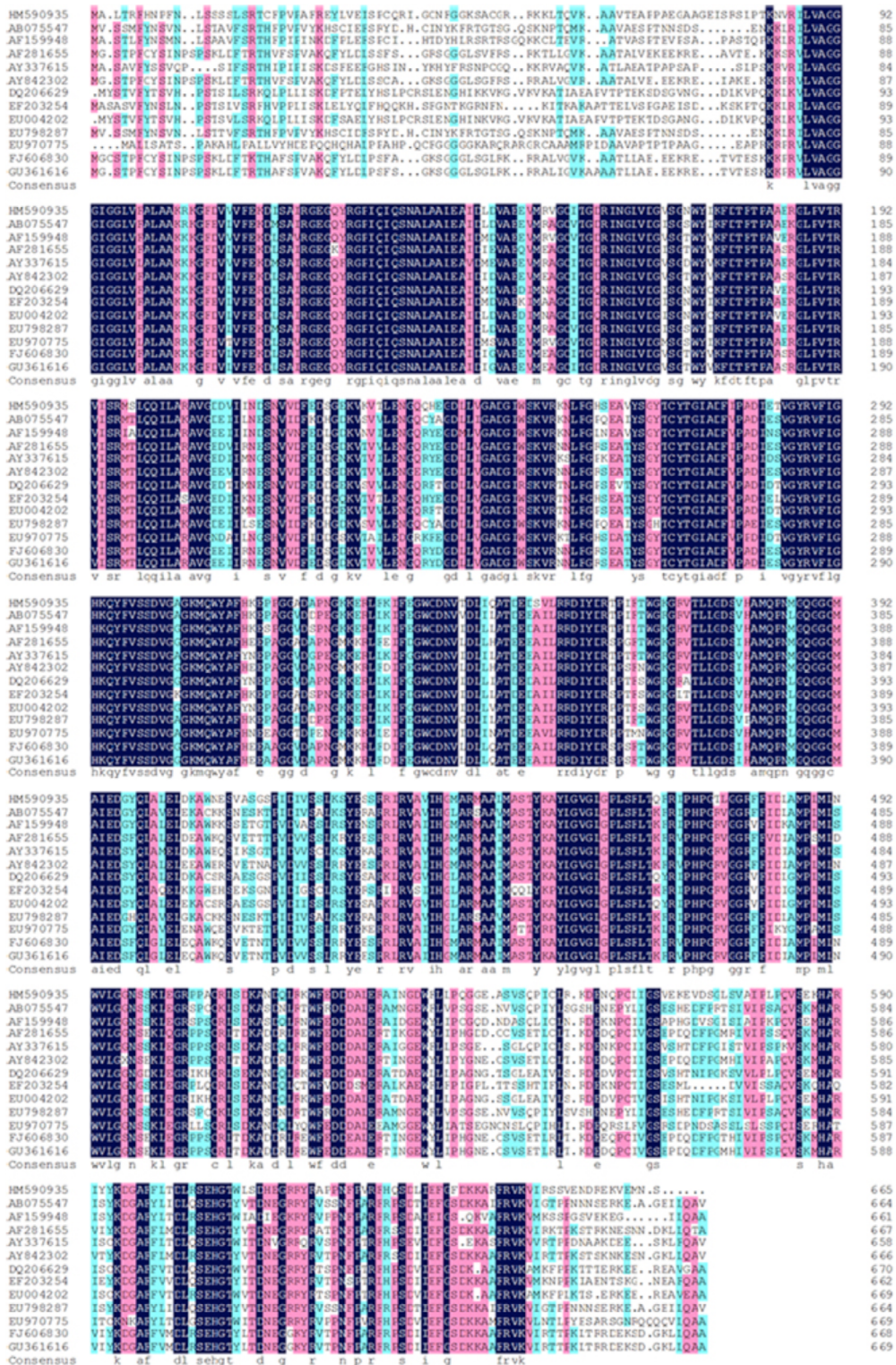
### 3.3. Analysis of amino acid sequence of *BnZEP*

The amino acid sequence of *BnZEP* was analyzed with SMART software online (<http://www.ncbi.nlm.nih.gov/>).

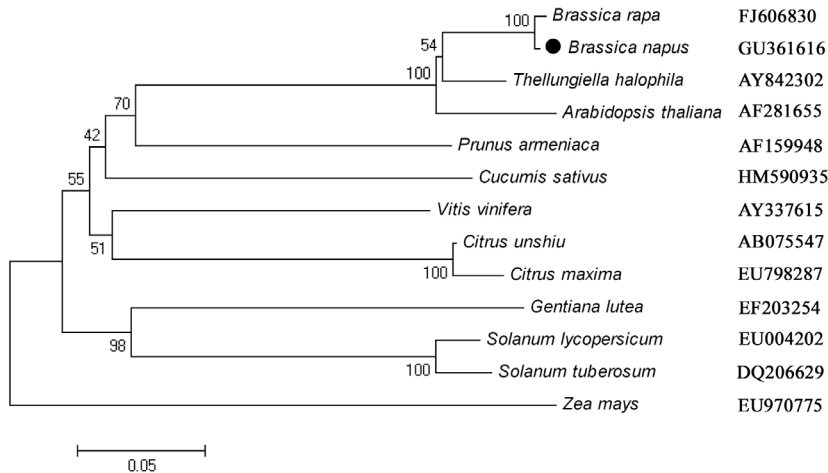


**Figure 2.** Schematic representation of the genomic sequence of the *BnZEP* gene (A) and the *AtZEP* gene (B). The numbers of exons and introns are same but their sizes are different.





**Figure 3.** Alignment of the deduced amino acid sequences determined for ZEP from *Brassica napus* (GU361616), *Brassica rapa* (FJ606830), *Thellungiella halophila* (AY842302), *Arabidopsis thaliana* (AF281655), *Prunus armeniaca* (AF159948), *Vitis vinifera* (AY337615), *Citrus unshiu* (AB075547), *Cucumis sativus* (HM590935), *Citrus maxima* (EU798287), *Solanum lycopersicum* (EU004202), *Gentiana lutea* (EF203254), *Solanum tuberosum* (DQ206629), and *Zea mays* (EU970775). The amino acid residues marked in dark blue indicate a 100% sequence identity.



**Figure 4.** Phylogenetic analyses of *ZEP*. Distances along the horizontal axes are proportional to sequence difference. GenBank accession numbers are shown after the species names.

The deduced amino acid sequence revealed a large number of domains. Two FAD\_binding\_3 domains spanned 216 amino acids (from amino acid 82 to 297) and 79 amino acids (from amino acid 356 to 434), respectively. A conserved forkhead (FHA) domain was identified in the C-terminal of the predicted amino acid sequence, which spanned 56 amino acid residues (amino acid 559 to 614). Two less complex regions with 15 and 16 amino acids were identified in the *BnZEP* amino acid sequence (Figure 5A). Alignments of the sequences also showed a homologous region of the FHA domain comprising 57 residues (Figure 5B).

The predicted protein of *BnZEP* was analyzed using TMHMM-2.0 online (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>). It was revealed that the *BnZEP* enzyme is a transmembrane protein with an N-terminal chloroplast transit peptide (Destefano-Beltrán et al., 2006). The *BnZEP* protein is localized in the stromal side of the thylakoids (Bouvier et al., 1996) (Figure 6).

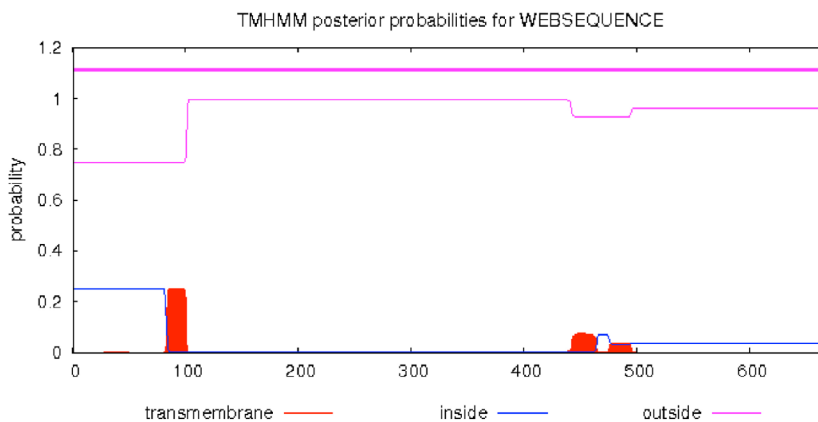
### 3.4. Real-time quantitative PCR of *BnZEP* in different organs and petals of different colors

Real-time quantitative PCRs were performed with total RNA isolated from leaf, stem, bud, petal in bud, and fully opened petal of the white-flowered plant HW243 and the petals of different colors from 3 different materials of *Brassica napus* L. The expression levels of *BnZEP* gene were compared among different organs and different flower petals.

Four candidate reference genes were tested using CFX96 system software to rank their expression stability values (M). The most stable reference gene produces the lowest M value, while the least stable one produces the highest M value. Results showed that the most stable candidate reference genes were *TIP41/UBC21* (M = 0.26) in the different organs and *GAPDH/TIP41* (M = 0.18) in the different petals. They were used as the internal control genes for the expression level analyses of *BnZEP*. The expression levels of *BnZEP* were normalized to



**Figure 5.** (A) The domain analyses of predicted amino acid sequence. (B) Highly conserved residues sites of the FHA domain in the proteins of *Brassica napus* (GU361616), *Brassica rapa* (FJ606830), *Thellungiella halophila* (AY842302), *Arabidopsis thaliana* (AF281655), and the FHA domain sequence. Red frames represent the invariant residues.



**Figure 6.** The transmembrane protein prediction of the *BnZEP* amino acid sequence.

the reference genes using CFX96 system software and compared among different organs and different petals of different colors.

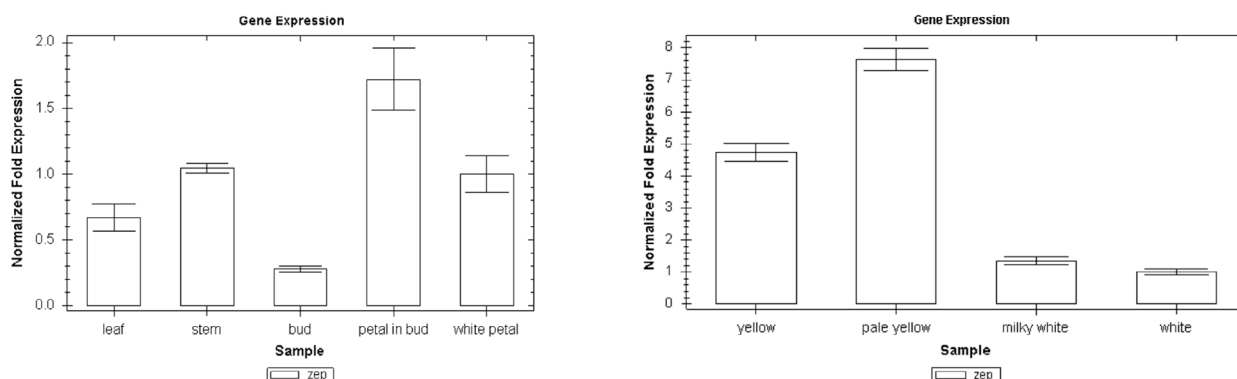
Higher expression levels of *BnZEP* were observed in the petals, while the highest transcript abundance was observed in the younger unopened petals (petals in bud) with a milky-white color (Figure 7A). Compared with the fully opened white petals, the expression level of *BnZEP* was 1.72 times higher in the younger unopened petals, but it was only about 0.28 and 0.67 times that value, respectively, in the bud and leaf (Table 2).

The expression levels of *BnZEP* were also markedly different in the different types of flower petals with different colors. Higher expression levels were observed in the yellow types of petals, including yellow and pale yellow petals. The highest expression level was observed in the fully open pale yellow petals of the F1 hybrid of HW243 × ZHONGYOU 821. The white and milky-white petals

showed lower expression levels of *BnZEP* (Figure 7B). In comparison with the white flower petals, the expression levels of *BnZEP* were 7.65 times higher in pale yellow petals, 4.74 times higher in yellow petals, and 1.35 times higher in milky-white petals (Table 3).

#### 4. Discussion

*ZEP* is a key enzyme responsible for the accumulation of antheraxanthin and violaxanthin (Zhu et al., 2003). It is also the first important enzyme in the abscisic acid (ABA) biosynthesis pathway (Rock and Zeevart, 1991; Marin et al., 1996; Audran et al., 1998; Seo and Koshiba, 2002) and in the xanthophyll cycle. ABA is an important hormone in all kinds of higher plants, which has been implicated in the control of a wide range of essential physiological processes, including seed development, seed germination, and plant tolerance to different stresses (Zeevaert et al., 1988). Xanthophyll is an important



**Figure 7.** (A) The expression levels of the *BnZEP* gene in different organs of the white-flowered line HW243 (*Brassica napus* L.): petal in bud (milky-white petal), white petal (fully opened flower). *TIP41* and *UBC21* ( $M = 0.26$ ) were used as the reference genes in the different tissues. (B) The expression levels of the *BnZEP* gene in petals of different flower colors in *Brassica napus* L.: yellow flower (petal of ZHONGYOU 821), white flower (petal of HW243), pale yellow flower (fully opened flower of HW243 × ZHONGYOU 821), and milky-white flower (unopened petal in bud of HW243). *GAPDH* and *TIP41* ( $M = 0.18$ ) were used as the reference genes in different colors of petals.



**Table 2.** The expression values and C(t) values of *BnZEP* in different tissues of white-flowered cultivar HW243 (*Brassica napus* L.).

Sample	Expression	Mean C(t)
Leaf	0.66866 ± 0.10486	28.69 ± 0.21543
Stem	1.04435 ± 0.03855	25.44 ± 0.03846
Bud	0.27805 ± 0.02471	27.53 ± 0.01022
Petal in bud	1.72100 ± 0.23415	24.38 ± 0.16246
Fully opened petal*	1.00000 ± 0.13951	24.98 ± 0.19713

\*: Control.

**Table 3.** The expression values and C(t) values of *BnZEP* in petals of different colors in *Brassica napus* L.

Sample	Expression	Mean C(t)
Yellow petal	4.74107 ± 0.28757	23.05 ± 0.04411
Pale yellow petal	7.64687 ± 0.34270	22.16 ± 0.00863
Milky-white petal	1.35089 ± 0.11993	24.38 ± 0.10275
White petal*	1.00000 ± 0.08912	24.98 ± 0.12467

\*: Control.

component in the biosynthesis of carotenoids and may be involved in adaptation of plants and green algae to intensive light (Yamamoto, 1979; Demmig-Adams et al., 1992). Carotenoids may also play important roles in the prevention from some chronic diseases (such as cancers), due probably to their antioxidant properties (Krinsky, 1993).

In the present study, we cloned and analyzed the full-length cDNA and genomic DNA sequences of the *BnZEP* gene in *Brassica napus* L. using the RACE method. The full-length cDNA and genomic DNA sequences of the gene were 2228 bp and 3268 bp, respectively, with an ORF of 2010 bp. It encodes a putative protein of 669 amino acids, with a theoretical molecular weight of 73.8 kDa and a pI of 6.16.

The genomic DNA sequence of *BnZEP* contained the same numbers of exons and introns as in *Arabidopsis thaliana* (*AtZEP*), but their sizes were different. The nucleotide and amino acid sequences of *BnZEP* revealed high homology to those from other cruciferous species, including *Arabidopsis thaliana*. The sequence homology of *BnZEP* was closest to *Brassica rapa*.

Based on the amino acid sequence, it appears that the *BnZEP* enzyme belongs to the lipocalin protein family (Hieber et al., 2000; Grzyb et al., 2006), with a feature of FAD binding sites (Bouvier et al., 1996; Hieber et al., 2000).

It also has a C-terminal FHA domain normally found in the *ZEPs* of plants (Coesel et al., 2008).

The expression levels of *BnZEP* were markedly different in the different organs of the white-flowered plant. Relatively higher transcript levels were observed in the petals, especially in the younger unopened milky-white petals (petals in bud), than in leaf, stem, and bud (Figure 7A). This is consistent with the results observed in *Gentiana lutea* (Zhu et al., 2003; Yang et al., 2012), where the expression levels of the zeaxanthin epoxidase gene (*GlZEP*) were remarkably higher in the chromoplast-containing petals than in the chloroplast-containing younger petals and leaves. The higher levels of *BnZEP* expression in the petals may probably be due to the large amount of chromoplasts in the petals, especially in the unopened milky-white petals (Figure 7A). However, this was not in consistency with the result observed in Chinese cabbage (*Brassica rapa* L. subsp. *pekinensis*) by Tuan et al. (2012), where remarkably higher expression levels of zeaxanthin epoxidase gene (*BrZEP*) were found in leaves instead of flowers and stems. This implies that the expression patterns of the *ZEP* gene may be variable among cruciferous species. Further studies are necessary to clarify the difference in *ZEP* gene expression in *B. napus* and *B. rapa*.

The expression levels of *BnZEP* were also remarkably different in different kinds of flower petals with different



colors. The expression level of *BnZEP* was markedly higher in yellow petals (including pale yellow petals) than in white petals (Figure 7B). It was indicated that the expression level of *BnZEP* was positively correlated with yellow color in petals of *B. napus* L. However, the highest expression level of the *BnZEP* gene was observed in the pale yellow petals of the  $F_1$  hybrid (HW243 × ZHONGYOU 821) (Figure 7B). This was probably the result of heterosis between the 2 parents. It is also implied that the expression level of *BnZEP* gene was not completely correlated with the degree of yellow color in petals of *B. napus* L. There must be some other important gene(s) involved in yellow flower color of this species.

It has been observed that *ZEP* is an important enzyme in the xanthophyll cycle and a key enzyme responsible for the accumulation of antheraxanthin and violaxanthin (Zhu et al., 2003). Based on the results of present study,

the high expression levels of *BnZEP* may increase the accumulation of antheraxanthin and violaxanthin in the petals and be favorable for the yellow-colored flower formation in *Brassica napus* L. However, further studies are necessary to identify the key carotenoid(s) responsible for the yellow flower color in *B. napus* L.

### Acknowledgments

The authors are grateful to Dr Haojie Li and Dr Cheng Cui, Crop Research Institute, Sichuan Academy of Agricultural Sciences, and Dr Garry M Rosewarne, Senior Scientist of the International Maize and Wheat Improvement Center, CIMMYT, for writing assistance. This study was supported by the National High Technology Research and Development Program “863” of China (2011AA10A104), and the Crop Breeding Fund of Sichuan Province (2011NZ0098-5).

### References

- Audran C, Borel C, Frey A, Sotta B, Meyer C, Simonneau T, Marion-Poll A (1998). Expression studies of the zeaxanthin epoxidase gene in *Nicotiana plumbaginifolia*. *Plant Physiol* 118: 1021–1028.
- Bouvier F, d’Harlingue A, Huguency P, Marin E, Marion-Poll A, Camara B (1996). Xanthophyll biosynthesis. Cloning, expression, functional reconstitution, and regulation of beta-cyclohexenyl carotenoid epoxidase from pepper (*Capsicum annuum*). *J Biol Chem* 271: 28861–28867.
- Coesel S, Obornik M, Varela J, Falciatore A, Bowler C (2008). Evolutionary origins and functions of the carotenoid biosynthetic pathway in marine diatoms. *PLoS ONE* 3: e2896.
- Demmig-Adams B, Adams WW 3rd (1992). Photoprotection and other responses of plants to high light stress. *Annu Rev Plant Phys* 43: 599–626.
- Destefano-Beltrán L, Knauber D, Huckle L, Suttle JC (2006). Effects of postharvest storage and dormancy status on ABA content, metabolism, and expression of genes involved in ABA biosynthesis and metabolism in potato tuber tissues. *Plant Mol Biol* 61: 687–697.
- Grzyb J, Latowski D, Strzalka K (2006). Lipocalins—a family portrait. *J Plant Physiol* 163: 895–915.
- Henikoff S, Henikoff JG (1994). Protein family classification based on searching a database of blocks. *Genomics* 19: 97–107.
- Hieber AD, Bugos RC, Yamamoto HY (2000). Plant lipocalins: violaxanthin de-epoxidase and zeaxanthin epoxidase. *Biochim Biophys Acta* 1482: 84–91.
- Krinsky NI (1993). Actions of carotenoids in biological systems. *Annu Rev Nutr* 13: 561–587.
- Kumar S, Tamura K, Nei M (2004). MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5: 150–163.
- Li H, Wang MW, Wang GS, Chen RS, Ling LJ, Wang JH (2002). Rapid cloning and tissue expressive analyses of cDNA fragments in human gastric cancer. *Prog Biochem Biophys* 29: 604–609.
- Li J, Shen BZ, Han JX, Gan L (1994). A effective procedure for extracting total DNA in rape. *Journal of Huazhong Agricultural University* 13: 521–523.
- Lu S, Li L (2008). Carotenoid metabolism: biosynthesis, regulation, and beyond. *J Integr Plant Biol* 50: 778–785.
- Marin E, Nussaume L, Quesada A, Gonneau M, Sotta B, Huguency P, Frey A, Marion-Poll A (1996). Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *EMBO J* 15: 2331–2342.
- McCord P, Zhang LH, Brown C (2012). The incidence and effect on total tuber carotenoids of a recessive zeaxanthin epoxidase allele (*Zep1*) in yellow-fleshed potatoes. *Am J Pot Res* 89: 262–268.
- Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: 2002–2007.
- Ralley L, Enfissi EM, Misawa N, Schuch W, Bramley PM, Fraser PD (2004). Metabolic engineering of ketocatenoid formation in higher plants. *Plant J* 39: 477–486.
- Rock CD, Zeevart JA (1991). The aba mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *P Natl Acad Sci USA* 88: 7496–7499.
- Seo M, Koshiba T (2002). Complex regulation of ABA bio-synthesis in plants. *Trends Plant Sci* 7: 41–48.
- Shen B, Tian HS, Ma L, Li XL, Zhen SZ, Zhou DH, Zhu CL (2002). Cloning and sequence analysis of full length trypsin cDNA of *Culex pipiens pallens*. *Acta Bioch Bioph Sin* 34: 28–32.

- Tuan PA, Kim JK, Lee J, Park WT, Kwon DY, Kim YB, Kim HH, Kim HR, Park SU (2012). Analysis of carotenoid accumulation and expression of carotenoid biosynthesis genes in different organs of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*). *EXCLI Journal* 11:508-516.
- Vallabhaneni R, Wurtzel ET (2009). Timing and biosynthetic potential for carotenoid accumulation in genetically diverse germplasm of maize. *Plant Physiology* 150: 562–572.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paep A, Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3: research0034.
- Wolters AMA, Uitdewilligen JG, Kloosterman BA, Hutten RC, Visser RG, van Eck HJ (2010). Identification of alleles of carotenoid pathway genes important for zeaxanthin accumulation in potato tubers. *Plant Mol Biol* 73: 659–671.
- Yamagishi M, Kishimoto S, Nakayama M (2010). Carotenoid composition and changes in expression of carotenoid biosynthetic genes in tepals of Asiatic hybrid lily. *Plant Breeding* 129: 100–107.
- Yamamoto HY (1979). Biochemistry of the violaxanthin cycle. *Pure Appl Chem* 51: 639–648.
- Yang QJ, Yuan DW, Shi LX, Capell T, Bai C, Wen N, Lu XD, Sandmann G, Christou P, Zhu CF (2012). Functional characterization of the *Gentiana lutea* zeaxanthin epoxidase (GLZEP) promoter in transgenic tomato plants. *Transgenic Res* 21: 1043–1056.
- Zeevaart JAD, Creelman RA (1988). Metabolism and physiology of abscisic acid. *Annu Rev Plant Phys* 39: 439–473.
- Zhu C, Yamamura S, Nishihara M, Koiwa H, Sandmann G (2003). cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development. *Biochim Biophys Acta* 1625: 305–308.