Cloning and characterization of zeaxanthin epoxidase (*BnZEP*) gene in *Brassica napus* L.

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1. Introduction

Carotenoids are a class of pigment elements present in all photosynthetic organisms. In forms of pigment-protein complexes, they act as light sensors in photosynthesis. They also prevent the photo-oxidation induced by too strong light intensities, the peroxidation of lipids, and stabilize the thylakoid membrane structure. In horticultural crops, carotenoids play major roles in the coloration of fruit, root, or tuber and also contribute to the nutritional qualities. In fact, some of these micronutrients are precursors of vitamin A, an essential component for human and animal diets. Carotenoids may also play important roles in the prevention from some chronic diseases (such as cancers), due probably to their antioxidant properties. Xanthophyll is an important component in the biosynthesis of carotenoids and may be involved in the adaptation of plants and green alga to high intensity light. Xanthophyll is also a precursor for the synthesis of abscisic acid (ABA), an important hormone in all kinds of higher plants, as well as in some mosses, green algae, fungi and bacteria. In higher plants, ABA 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. Zeaxanthin epoxidase (ZEP) is a key enzyme in the biosynthesis of xanthophyll and abscisic acid (ABA). In this study, the full length cDNA and genomic DNA sequences of the zeaxanthin epoxidase gene (*BnZEP*) from *Brassica napus* L. were cloned by the method of rapid amplification of cDNA ends (RACE) and their sequences were analyzed.

2. Materials and methods

Plant material and primers Plant tissues for the total RNA and total DNA extractions were obtained from a rapeseed cultivar "ZHONGYOU 821" (*Brassica napus* L) grown on the experimental farm of Sichuan Agricultural University, Ya'an, Sichuan, China. The primers used in present study are listed in Table1. The special primers used in the 3' RACE and 5' RACE and the primers to amplify the full length cDNA and DNA were designed with the Primer Premier 5.0(http://www.PremierBiosoft.com).

Isolation of total RNA and total DNA Total RNA was isolated from petal, stem, leaf and bud of *Brassica napus* using the TRNzol Reagent (Tiangen, Beijing, China). The plant tissues were ground on ice with at least 1 ml TRNzol Reagent in mortars. Extraction and purification of the total RNA was carried out following the manufacturer's instructions, including a treatment with DNase (TaKaRa, Dalian, China). The total RNA was finally dissolved in 50 μl DEPC-treated H_2O and stored at -80℃ for use. The total genomic DNA was extracted from young leaves and purified as described by Jia Li's method.

Synthesis of cDNA cDNA fragments were amplified by Reverse Transcription (RT) PCR using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Dalian, China), with 2 μg of total RNA and the GeneRacer™ oligo(dT) primer (table 1). The reaction was performed in accordance with the manufacturer's instructions and the product was stored at -20℃ for use.

Table 1 Sequences of the primers used in this study

Primer	Sequence(5'-3')
GeneRacer™ oligo dT primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG$(T)_{24}$
Z1(forward primer of conserved sequence)	GAGAGGGACAATACAGAGGACCTathcarathca
Z2(reverse primer of conserved sequence)	AACCTGGGACAAGGAGGAtgyatggcnat
Z3(GeneRacer™ 3′ primer)	GCTGTCAACGATACGCTACGTAACG
Z4(3' RACE gene specific primer)	AGGTAACGGTGGTGCTTGAGAATGG
Z5(GeneRacer™ 3′ nested primer)	CGCTACGTAACGGCATGACAGTG
Z6(GeneRacer™ 5′ primer)	CGACTGGAGCACGAGGACACTGA
Z7(5' RACE gene specific primer)	TCCTCCTCTGTGGCATTCAGTAAGTC
Z8(GeneRacer™ 5′ nested primer)	GGACACTGACATGGACTGAAGGAGTA
ZEPF(forward primer of the full length)	ATGGGCTCAACTCCCTTCTGCTAC
ZEPR(reverse primer of the full length)	TCAAGCAGCCTGAAGCAATTTACCG

Amplification of the conserved sequence of BnZEP A pair of degenerate primers Z1 and Z2 (see table 1) were designed as described by Henikoff S and Henikoff J G's method. The PCR was conducted with 1 μl cDNA in 50 μl reaction volume by a PTC1000 PCR Thermal Cycler (Bio-RAD, USA) using the PrimeSTAR® HS DNA Polymerase (TaKaRa, Dalian, China). The reaction was programmed for 30 cycles at 98℃ for 10 sec, 68℃ for 1 min. The amplified product was separated on a 2% agarose gel and stained with Glod-View (a substitute of ethidium bromide). The band with the expected size was excised out and the amplified DNA fragment was recovered and purified using the AxyPrep DNA Gel Extraction Kit (AxyGEN, USA).

Cloning and sequencing of the conserved sequence The recovered conserved sequence of *BnZEP* gene was cloned into the pMD®19-T Vector (TaKaRa, Dalian, China), and then transformed into *E. coli* DH5α competent cells (Tiagen, Beijing, China). The DNA insert in the recombinant clones was amplified by PCR with the designed degenerate primers used above, and then were sequenced in both directions by the Invitrogen Life Technologies Co. LTD (Shanghai, China).

Rapid amplification of cDNA ends (3' RACE and 5' RACE) The 3' RACE and 5' RACE amplifications were performed using the GeneRacer™ Kit (Invitrogen, USA) and the GeneRacer™ oligo(dT) primer (table 1). The 3' RACE was conducted with the primer pair Z3 and Z4 (table 1) using the PrimeSTAR® HS DNA Polymerase. A nested PCR was carried out using similar conditions with 1 μl of the 3' RACE product as template, together with the primer pair Z4 and Z5. The 5' RACE was accordingly performed using the primer pair Z6 and Z7. A nested PCR was conducted with 1 μl of the 5' RACE product as template, and the primer pair Z7 and Z8 using the same PCR program. The 3' and 5' nested PCR products were recovered and purified, then cloned into the pMD®19-T Vector. The recombinant clones were identified by PCR amplification with the sequencing primer (M13) and the gene specific primer pair Z4-Z5 or Z7-Z8. They were then sequenced as described above. The full length cDNA sequence of *BnZEP* was obtained by an electronic assembly of the 3' RACE, the 5' RACE and the conserved sequence using the DNAStar software. The open read frame (ORF) was predicted using the ORFFinder program (http://www.ncbi.nlm.gov/gorf/gorf.html). The full length cDNA and DNA sequences were confirmed using the primer pair ZEPF and ZEPR (table 1) with 1 μl genomic DNA or cDNA from the previous experiments as templates.

Sequence analysis The nucleotide sequences of the cDNA and DNA fragments of *BnZEP* gene were determined by an ABI-PRISM 3730 sequencer (USA). Homologous amino acid sequences were searched by BlastP from the non-redundant protein sequences (nr) database on the NCBI (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed using the MEGA version 3.1 by the Neighbor-Joining method.

3. Results and Discussion

The full length cDNA and genomic DNA Sequences of BnZEP

A 582 base pair (bp) fragment was obtained with the degenerate primers Z1 and Z2 (Figure 1, lane 2). A fragment of 1480 bp (Figure 1, lane 3) was obtained by the 3' RACE, and a fragment of 755 bp (Figure 1, lane 4) by the 5' RACE. The full length cDNA sequence of *BnZEP* gene obtained by the electronic assembly of the 3' RACE, the 5' RACE, and the degenerate primer amplified sequences was 2226 bp (GenBank accession number, GU361616). The full length cDNA sequence of *BnZEP* obtained from the cDNA sample by the primer pair ZEPF and ZEPR was same in length (Figure 1, lane 5). The full length genomic DNA sequence of *BnZEP* (GenBank accession number, GU561839)was 3268 bp (Figure 1, lane 6).

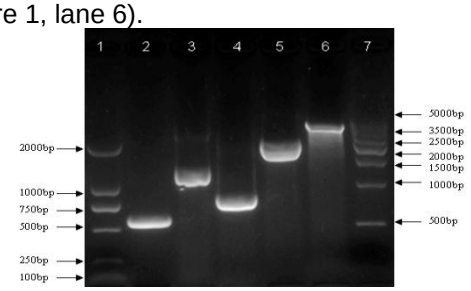

Figure 1 Electrophoresis of the RACE and the full length PCR products on 2% agarose gel.
Lane 1: DL2000 marker; lane 2: fragment cDNA; lane 3: 3' RACE product; lane 4: 5' RACE product; lane 5: the full length cDNA of *BnZEP*; lane 6: the full length genomic DNA of *BnZEP*; Lane 7: 500bp DNA ladder marker.

Sequence Analysis of the BnZEP

The full length cDNA sequence and the deduced amino acid sequence (below the nucleotide sequence) of the *BnZEP* gene were shown in Figure 2. The full length cDNA sequence was 2228 bp, with an ORF of 2010 bp, encoding a 669 amino acid polypeptide. The predicted molecular weight of the polypeptide was 73.8 k Daltons, with an isoelectric point (pI) 6.5. Analyses indicated that the cDNA sequence of *BnZEP* gene was composed of a 57 bp 5'-end UTR and a 161 bp 3'-end UTR. The full length DNA sequence of *BnZEP* gene from the genomic DNA sample contained 16 exons and 15 introns. From a protein-protein BLAST and a multiple alignment analysis it was shown that the deduced amino acid sequence of *BnZEP* had a high homology with those from other plant species (Figure 3). The amino acid sequence of *BnZEP* was 98% homologous with *Brassica rapa* (FJ606830), 92% with *Thellungiella halophila* (AY842302), 89% with *Arabidopsis thaliana* (AF281655), 71% with *Prunus armeniaca* (AF159948), 69% with *Vitis vinifera* (AY337615) and *Citrus unshiu* (AB075547), 68% with Cucmis sativus (HM590935) and *Citrus maxima* (EU798287) , 67% with *Solanum lycopersicum* (EU004202), 66% with *Gentiana lutea* (EF203254), 65% with *Solanum tuberosm* (DQ206629), and 63% with *Zea mays* (EU970775). Further studies are underway to understand the functions of the cloned *BnZEP* gene.

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1       agaagaagcgcaccacaaagaaaactttgtatccttagcttgctcctctcatcgagATGGGCTCAACTCCCTTCTGC
1                                                          M  G  S  T  P  F  C
79      TACTCAATCAACCCATCTCCATCTAAGCTCGATTTCACCAGGACCCACGCGTTCAGCCCCGTCGCCAAACAGTTCTAC
8        Y  S  I  N  P  S  P  S  K  L  D  F  T  R  T  H  A  F  S  P  V  A  K  Q  F  Y
157     CTCGACTTACCGTCGTTCTCCGGCAAATCGGGAGGGGGATTATCGGGGTTAAGAAAGCGTCGAGCTTTAATCGGAGTA
34       L  D  L  P  S  F  S  G  K  S  G  G  G  L  S  G  L  R  K  R  R  A  L  I  G  V
235     AAGGCCGCGGCGGCGACTTTGTTGGCGGAAGAGGAGAAGCGGGAGACAGTAACCGAGAGTAAGAAGAAACCGAGAGTT
60       K  A  A  A  A  T  L  L  A  E  E  E  K  R  E  T  V  T  E  S  K  K  K  P  R  V
313     CTCGTGGCCGGAGGCGGAATCGGAGGTTTGGTGTTCGCTCTCGCGGCGAAGAAGAAAGGGTTCGATGTGTTGGTGTTC
86       L  V  A  G  G  G  I  G  G  L  V  F  A  L  A  A  K  K  K  G  F  D  V  L  V  F
391     GAGAAAGACTTAAGCGCCATAAGAGGAGAAGGACAGTACAGAGGTCCGATTCAGATACAGAGCAACGCCTTGGCTGCT
112      E  K  D  L  S  A  I  R  G  E  G  Q  Y  R  G  P  I  Q  I  Q  S  N  A  L  A  A
469     CTGGAAGCTATCGATATCGGTGTTGCTGAAGAAGTCATGGAGGCTGGATGTATCACCGGTGATCGGATTAACGGCCTC
138      L  E  A  I  D  I  G  V  A  E  E  V  M  E  A  G  C  I  T  G  D  R  I  N  G  L
547     GTCGACGGTGTCTCTGGCACTTGGTATGTCAAGTTTGATACTTTCACTCCTGCGGCGTCACGTGGACTACCTGTGACT
164      V  D  G  V  S  G  T  W  Y  V  K  F  D  T  F  T  P  A  A  S  R  G  L  P  V  T
625     CGGGTGATTAGCAGAATGACTCTGCAGCAGATCTTGGCACGTGCGGTTGGTGAAGAAATTATTAGAAACGAGAGTAAT
190      R  V  I  S  R  M  T  L  Q  Q  I  L  A  R  A  V  G  E  E  I  I  R  N  E  S  N
703     GTTGTTGATTTTGAAGATTCTGGAGATAAGGTAACGGTGGTGCTTGAGAATGGACAACGTTACGACGGTGATCTACTT
216      V  V  D  F  E  D  S  G  D  K  V  T  V  V  L  E  N  G  Q  R  Y  D  G  D  L  L
781     GTTGGTGCAGATGGCATATGGTCTAAAGTGAGAAACAACTTGTTTGGTCGGAGTGAAGCTACTTATTCAGGCTACACT
242      V  G  A  D  G  I  W  S  K  V  R  N  N  L  F  G  R  S  E  A  T  Y  S  G  Y  T
859     TGTTACACTGGTATTGCAGATTTTGTACCAGCTGATATCGAGTCTGTTGGCTACCGAGTTTTCTTGGGACACAAGCAA
268      C  Y  T  G  I  A  D  F  V  P  A  D  I  E  S  V  G  Y  R  V  F  L  G  H  K  Q
937     TACTTCGTTTCTTCTGATGTTGGTGGTGGAAAGATGCAATGGTATGCGTTCCATGAGGAAGCAGCTGGTGGGGTTGAT
294      Y  F  V  S  S  D  V  G  G  G  K  M  Q  W  Y  A  F  H  E  E  A  A  G  G  V  D
1015    GCTCCCAATGGTATGAAGAAAAGACTGTTTGATATATTTGAAGGTTGGTGCGACAATGTGCTGGATTTATTGCAAGCC
320      A  P  N  G  M  K  K  R  L  F  D  I  F  E  G  W  C  D  N  V  L  D  L  L  Q  A
1093    ACTGAGGAGGAAGCGATTCTGAGAAGAGATATATATGATAGAAGTCCTAGTTTCACTTGGGGTAAAGGACGTGTTACG
346      T  E  E  E  A  I  L  R  R  D  I  Y  D  R  S  P  S  F  T  W  G  K  G  R  V  T
1171    CTTCTCGGTGATTCTATTCATGCAATGCAGCCAAATATGGGTCAAGGTGGATGCATGGCCATTGAGGACAGTTTTCAA
372      L  L  G  D  S  I  H  A  M  Q  P  N  M  G  Q  G  G  C  M  A  I  E  D  S  F  Q
1249    CTAGGATTGGAGCTTGAACAAGCATGGAAACAGAGTGTTGAAACTAATACACCTGTTGATGTTGTTTCCTCTTTGAGA
398      L  G  L  E  L  E  Q  A  W  K  Q  S  V  E  T  N  T  P  V  D  V  V  S  S  L  R
1327    AGATACGAGGAATCTAGAAGACTAAGAGTTGCTATTATCCATGGAATGGCGAGAATGGCTGCAATAATGGCTTCCACT
424      R  Y  E  E  S  R  R  L  R  V  A  I  I  H  G  M  A  R  M  A  A  I  M  A  S  T
1405    TACAAAGCATACTTGGGTGTTGGGCTTGGTCCTCTCTCTTTCTTGACCAAGTTTAGAGTACCGCATCCGGGAAGAGTA
450      Y  K  A  Y  L  G  V  G  L  G  P  L  S  F  L  T  K  F  R  V  P  H  P  G  R  V
1483    GGTGGGAGATTCTTCATTGACATTGCTATGCCTTTGATGCTTAACTGGGTCCTTGGTGGTAACAGTGAGAAACTCGAA
476      G  G  R  F  F  I  D  I  A  M  P  L  M  L  N  W  V  L  G  G  N  S  E  K  L  E
1561    GGAAGGCCACCTAGTTGCAGACTCACTGACAAAGCTGATGACCGCCTTCGTGAGTGGTTTGAAGACGACGAAGCTCTT
502      G  R  P  P  S  C  R  L  T  D  K  A  D  D  R  L  R  E  W  F  E  D  D  E  A  L
1639    GAACGTACTATTAACGGAGAGTGGTATCTCATTCCACATGGCAACGAGTGTAGCGTTTCTGAAACATTACGTCTAACC
528      E  R  T  I  N  G  E  W  Y  L  I  P  H  G  N  E  C  S  V  S  E  T  L  R  L  T
1717    AAAGATGAGGATCAGCCTTGCATTGTCGGAAGTGAACCAGACCAAGATTTTCCTGGAATGCACATTGTGATCCCTTCC
554      K  D  E  D  Q  P  C  I  V  G  S  E  P  D  Q  D  F  P  G  M  H  I  V  I  P  S
1795    CCTCAGGTATCGAAGATGCATGCGCGTGTGATTTACAAAGATGGAGCTTTCTTCGTGATGGATCTTCGAAGCGAACAC
580      P  Q  V  S  K  M  H  A  R  V  I  Y  K  D  G  A  F  F  V  M  D  L  R  S  E  H
1873    GGGACCTATCTCACCGATAACGAAGGAGGAAAATACAGAGTGACACCAAACTTCCCGGCCCGGTTTAGACCGTCTGAT
606      G  T  Y  L  T  D  N  E  G  G  K  Y  R  V  T  P  N  F  P  A  R  F  R  P  S  D
1951    ATCATCGAGTTTGGTTCGGACAAGAAGGCGGCGTTTAGGGTCAAGGTGATCAGGACAACTCCCAAATTGACGAGAAGG
632      I  I  E  F  G  S  D  K  K  A  A  F  R  V  K  V  I  R  T  T  P  K  L  T  R  R
2029    GATGAGAAGAGTGACGGTAAATTGCTTCAGGCTGCTTGAtacttaagtaaaccgacggtgaatttaagaaaaaaaaga
658      D  E  K  S  D  G  K  L  L  Q  A  A  *
2107    tcagtttggcagataatgtgtagtaattgtcacaaaaaagaaaattaatatattacctgcattgcaatgtatatagtc
2185    tgaaacaaaggataatttatacagaacgttttctctttaaaaaa
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Figure 2 Nucleotide and deduced amino acid sequence of *BnZEP*. The nucleotides underlined show the positions of primers used in the experiment. The start codon ATG is indicated with bold and the stop codon TGA is indicated with bold and by an asterisk.

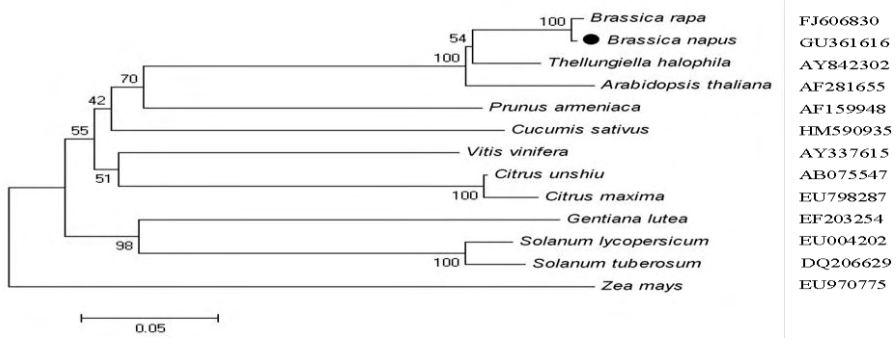

Figure 3 Phylogenetic relationship based on the amino acid sequence comparisons of *BnZEP*. GenBank accession numbers are shown after the species names.

Acknowledgments This work was financially supported by the China National High-tech "863" Program (2009AA101105) and the Chongqing City Natural Sience Fund Program (CSTC2009BA1088).