

Cloning and characterization of phytoene desaturase (*BnPDS*) gene in the different flower color lines in *Brassica napus* L.

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1. Introduction

Carotenoids, a group of membrane-bound lipid soluble yellow, red, and orange pigments, including carotene and xanthophyll, are a class of C₄₀ hydrocarbon compounds formed through the condensation of isoprenoids. They play important roles in a large number of physiological processes in plants. Carotenoids act as accessory pigments in photosynthesis, forming the basic structural units of photosynthetic antennae. They also serve as photo-protection agents by quenching singlet oxygen that might otherwise damage chlorophyll. Moreover, carotenoids serve as precursors in the biosynthesis of vitamin A and abscisic acid (ABA). Many clinical studies have demonstrated that carotenoids function as free radical quenchers in many physiological pathways. Carotenoids biosynthesis takes place in the plastid, but all known enzymes in the pathway are nuclear-encoded and post-translationally imported into the organelle. The first committed step of carotenoids synthesis, the condensation of GGPP to phytoene, is mediated by the soluble enzyme phytoene synthase (PSY). The subsequent steps in the pathway, leading to the colored carotenoids, are carried out by the membrane-localized enzymes, some of which may function in forms of heterogeneous complexes. In the first step, phytoene is converted to colored carotene by a two-step desaturation reaction by phytoene desaturase (PDS). The phytoene desaturation is a rate-limiting step in the synthesis of carotenoids in cyanobacteria. However, similar situation has not yet been directly demonstrated in higher plants. The *PDS* gene was first cloned from cyanobacterium. Homologous *PDS* genes were then cloned from soybean, pepper, tomato, arabidopsis and maize. These homologous proteins share about 60% identity and 75% similarity in amino acid sequences to that from cyanobacterium. In this study, we cloned the full length cDNA and genomic DNA sequences of the phytoene desaturase gene (*BnPDS*) from *Brassica napus* using the Rapid Amplification of cDNA Ends (RACE) and their sequences were analyzed.

2. Materials and methods

Plant materials and primers Petals were collected from a yellow-flower cultivar (ZHONGYOU 821) and a white-flower line (HW243) of rapeseed (*Brassica napus* L.) grown on the experimental farm of Sichuan Agricultural University, Ya'an, Sichuan, China at the full blossom stage. The petals were frozen immediately in liquid nitrogen and stored at -80°C for use. The primers used in this study are listed in Table 1. The special primers used in the 3' RACE and 5' RACE and the primer to amplify the full length cDNA and DNA were all designed with the Primer Premier 5.0 (<http://www.PremierBiosoft.com>).

Table 1 Sequences of the primers used in this study

Primer	Sequence(5'-3')
GeneRacer™ oligo dT primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T) ₂₄
P1(forward primer of conserved sequence)	TGGAAGGATGAGGATGGAGAygtggtaygara
P2(reverse primer of conserved sequence)	AGCATAGATCGGTTAGGATCGTAGtayctytrca
P3(GeneRacer™ 3' primer)	GCTGTCAACGATACGCTACGTAACG
P4(3' RACE gene specific primer)	GCTATGGTCGGAGGTCAGGCTTAT
P5(GeneRacer™ 3' nested primer)	CGCTACGTAACGGCATGACAGTG
P6(GeneRacer™ 5' primer)	CGACTGGAGCACGAGGACACTGA
P7(5' RACE gene specific primer)	GCAGCGATTTTCGTCAGGGAAGAG
P8(GeneRacer™ 5' nested primer)	GGACACTGACATGGACTGAAGGAGTA
P9(forward primer of the full length)	GGCGTTATGGTTGTGTTTGGGAATG
P10(reverse primer of the full length)	GGCTTCCTGCCTCATGTTGATAGAGT

Isolation of total RNA and total DNA Petals were ground on ice with at least 1 ml TRNzol Reagent (Tiangen, Beijing, China). Total RNA was extracted using the TRNzol Reagent with a treatment of DNase (TaKaRa, Dalian, China) and purified following the manufacturer's instructions. The total RNA was finally dissolved in 50 µl DEPC-treated H₂O and stored at -80°C for use. The total genomic DNA was extracted from young leaves and purified as described by Jia Li's method.

Synthesis of cDNA cDNA was synthesized by Reverse Transcription PCR (RT-PCR) with 2 µg of total RNA using the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Dalian, China) and the GeneRacer™ oligo(dT) primer. The reaction was performed according to the manufacturer's instructions. The product was stored at -20°C.

Cloning and sequencing of the conserved sequence of BnPDS A pair of degenerate primers (P1 and P2, Table 1) were designed as described by Henikoff S and Henikoff J G's method. The PCR reaction was conducted with 1 µl of cDNA synthesized with the total RNA from the white flower line 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°C for 10 sec, 68°C for 1 min. The amplified products were 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 DNA fragment was recovered and purified using the AxyPrep DNA Gel Extraction Kit (AxyGEN, USA). The recovered fragment was cloned into the pMD®19-T Vector (TaKaRa, Dalian, China) and transformed into the *E. coli* DH5α competent cells (Tiagen, Beijing, China). The DNA insert in the recombinant clones was amplified by PCR using the degenerate primers described above and the sequencing primers (M13), and then sequenced in both directions by the Invitrogen Life Technologies, Co. LTD (Shanghai, China).

Rapid amplification of cDNA ends (3' RACE and 5' RACE) 3' RACE and 5' RACE were performed with the conserved sequence amplified from the white-flower line(HW243), using the GeneRacer™ Kit (Invitrogen, USA). The 3' region was amplified using the GeneRacer™ 3' primer (P3) and the 3' RACE gene specific primer (P4), together with the PrimeSTAR® HS DNA Polymerase. The PCR program used an initial denaturation at 98°C for 10 sec, followed by 30 cycles of 98°C for 10 sec, 55°C for 5 sec and 72°C for 10 sec. A nested PCR was performed using similar conditions with 1 µl of the 3' RACE product as template, together with the primer pair P4 and P5. Accordingly, the 5' region was amplified using the primer pairs P6 and P7. The 5' nested PCR was performed with 1µl of the 5' RACE product as template, together with the primer pair P7 and P8. The products from the 3' and 5' nested PCR's were recovered and purified, and then cloned into the pMD®19-T Vectors. The recombinant clones were identified by PCR with the sequencing primer (M13) and the gene specific primer pairs P4-P5 or P7-P8, and then sequenced as described above. The full length cDNA sequence was obtained by

electronic assembly of the 3' RACE, the 5' RACE sequences and the degenerate primer amplified conserved sequence, using the DNAMAN 6.0 software. The open read frame (ORF) was predicted by the ORFFinder program (<http://www.ncbi.nlm.gov/gorf/gorf.html>). The full length cDNA and DNA sequences were confirmed using the primer pair P9 and P10 with 1 µl cDNA or genomic DNA as template. The PCR program used an initial denaturation at 98°C for 10 sec, followed by 30 cycles at 98°C for 10 sec, 55°C for 5 sec and 72°C for 4 min, and with a final extension at 72°C for 10 min.

Sequence analysis The nucleotide sequences of the cDNA and DNA fragments 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 software MEGA version 3.1 by the Neighbor-Joining method.

3. Results and Discussion

Isolation of full length cDNA and DNA of *BnPDS* from *Brassica napus*

A 208 base pair (bp) fragment of *BnPDS* gene was obtained with the degenerate primers P1 and P2 from the white-flower line of rapeseed (*B. napus* L.) (Figure 1, lane 2). The sequences obtained by the 3' RACE and the 5' RACE were 1246 bp (Figure 1, lane 3) and 1527 bp (Figure 1, lane 4), respectively. The full length cDNA of *BnPDS* gene obtained by electronic assembly of the 3' RACE, 5' RACE, and the conserved sequence amplified with the degenerate primers from the white-flower line was 1935 bp (GenBank accession number, HM989806), with an ORF of 1695 bp (Figure 1, lane 5). The ORF in the *BnPDS* gene from the yellow-flower line was 1698 bp (GenBank accession number, HM989808). The full length DNA sequence of *BnPDS* gene from the white-flower line was 3046 bp (Figure 1, lane 6) (GenBank accession number, HM989807), and that from the yellow-flower line was 3701 bp (Figure 1, lane 7) (GenBank accession number, HM989809).

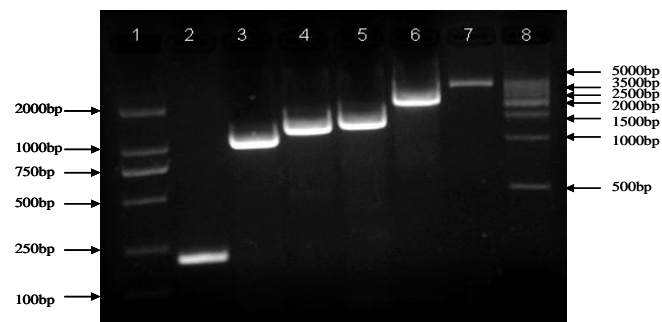


Figure 1 Electrophoresis of the RACE and the full length PCR products on 2% agarose gel.

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 *BnPDS* from the white-flower line; lane 6: the full length DNA of *BnPDS* from the white-flower line; lane 7: the full length DNA of *BnPDS* from the yellow-flower line; Lane 8: 500bp DNA ladder marker.

Sequence Analysis of the *BnPDS*

The deduced amino acid sequences of the *BnPDS* gene from the white-flower and the yellow -flower lines were shown in Figure 2. The full length cDNA sequence of *BnPDS* gene contained a 68 bp 5'-end UTR, a 172 bp 3'-end UTR and an ORF of 1695 bp, encoding a 564 amino acid polypeptide. The predicted molecular weight of the polypeptide was 62.9 k Daltons, with an isoelectric point (pI) of 7.2. The amino acid sequence alignments of the two different flower color lines varied at 5 positions, i.e. at the amino acid No.'s 43, 201, 398, 450 and 560. However, further studies were necessary to understand whether these differences in amino acid sequences led to the variation in flower color in *B. napus* L. From a protein-protein BLAST and a multiple alignment analysis it was indicated that the deduced amino acid sequence of *BnPDS* had a high homology with those from other plant species. The amino acid sequence of *BnPDS* was 98% homologous with *Brassica rapa* (FJ606826), 95% with *Brassica oleracea* (EF682130), 93% with *Arabidopsis thaliana* (L16237), 79% with *Oryza sativa* (AF049356), 78% with *Lycopersicon esculentum* (M88683) and 75% with *Zea mays* (L39266) (Figure 3).

HM989806	MVVFQNVSAANLFPYONGFLKAISSGGCDLMGHRSEKISTSEKLT	TRRRRSAGFLQVVCVDIPRPELENTVNFLEAASLSASFRSA	84
HM989808	MVVFQNVSAANLFPYONGFLKAISSGGCDLMGHRSEKISTSEKLT	TRRRRSAGFLQVVCVDIPRPELENTVNFLEAASLSASFRSA	85
Consensus	mvvfgnvsaanlpynqngflkaiissggcdlmghrsfkistsekt	trrrrsagplqvvcvdiprpeleentvnfleaaaslsasfrsa	
HM989806	PRFAKFLKVVVIAGAGLAGLSTAKYLADAGHKPILLLEARDVLGGKIAAWKDEDDGWYETGLHIFFGAYPNVONLFGELGINDRLOW		169
HM989808	PRFAKFLKVVVIAGAGLAGLSTAKYLADAGHKPILLLEARDVLGGKIAAWKDEDDGWYETGLHIFFGAYPNVONLFGELGINDRLOW		170
Consensus	ppfakplkvvviagaglaglstaakyladaghkpllleardvlggkiaawkdedgdwyetglhiffgaypvnvnlfgelgindrlqw		
HM989806	KEHSMIFAMPSKPGFESRFDFPDVLPAPLNG	WAILRNNEMLTWPEKIKFAIGLLPAMVGGQAYVEAQDGLSVEQWRRKQGVPDF	254
HM989808	KEHSMIFAMPSKPGFESRFDFPDVLPAPLNG	WAILRNNEMLTWPEKIKFAIGLLPAMVGGQAYVEAQDGLSVEQWRRKQGVPDF	255
Consensus	kehsmifampskpgefesrfdfpdpvlpaplng	wailrnnemltwpekikfaigllpamvggqayveaqdglsvewmrkqgvpdf	
HM989806	VTDEVFIAMSKALNFINPDELSMOCILIALNRFLQEKHGSKMAFLDGNPPERLPCMPIVEHIRSLGGEVRLNSRIRKIELEDDGTV		339
HM989808	VTDEVFIAMSKALNFINPDELSMOCILIALNRFLQEKHGSKMAFLDGNPPERLPCMPIVEHIRSLGGEVRLNSRIRKIELEDDGTV		340
Consensus	vtdevfiamskalfinpdelsmqciliialnrflqekhgskmafldgnpperlcmpivehirsllggevrlnsrirkieleddgtv		
HM989806	KSFLLTDGTTIQGDAYVFATPVDILKLLLPDSWKEIPYFKRLEKLVGVPVINVHIWFD	KLKNTYDHLFSLRNLSSVYADMSLT	424
HM989808	KSFLLTDGTTIQGDAYVFATPVDILKLLLPDSWKEIPYFKRLEKLVGVPVINVHIWFD	KLKNTYDHLFSLRNLSSVYADMSLT	425
Consensus	ksflltdgttioggdayvfatpvdilklllpdswkeipyfrkrlkvlvgvpvinvhiwfd	klkntydhllfsrnlssvyadmslt	
HM989806	CKEYYDPNRSMLLELVFAPAEWISR	DSDIIDATMKELERLFPDEIAADQSKAKILKYHVVKTPRSVYKTIPTDCEPCRFLORSPI	509
HM989808	CKEYYDPNRSMLLELVFAPAEWISR	DSDIIDATMKELERLFPDEIAADQSKAKILKYHVVKTPRSVYKTIPTDCEPCRFLORSPI	510
Consensus	ckeeydpnrsmlelvfapaeewisr	dsdiidatmkelerlfpdeiaadqskakilkvkvktprsvyktipdcepcrplqrspl	
HM989806	KGFYLAGDYTKQKYLASMEGAVLSGKFCQSOSILODYELLAASSGPKLSE	TLST	564
HM989808	KGFYLAGDYTKQKYLASMEGAVLSGKFCQSOSILODYELLAASSGPKLSE	TLST	565
Consensus	kgfyilagdytkqkylasmegevlsqkfcqsosilqdyellaassgpklse	tlst	

Figure 2 Amino acid alignment of *BnPDS* in the white-flower line (HM989806) and the yellow-flower line (HM989808)

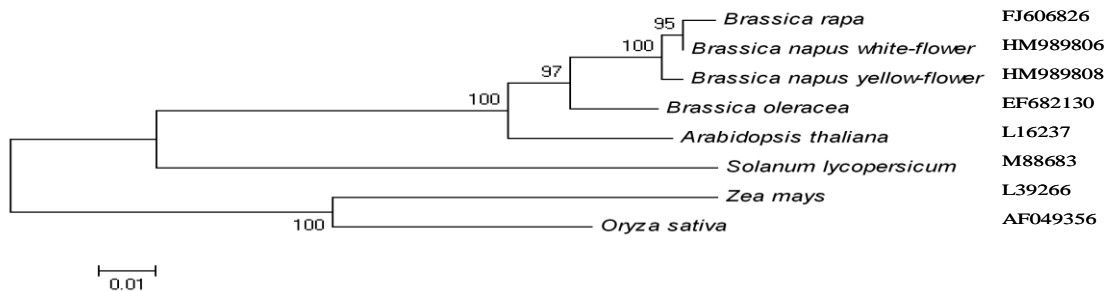


Figure 3 Phylogenetic relationship based on the amino acid sequence comparisons of *BnPDS*. GenBank accession numbers are shown after the species names.

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