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cDNA cloning and expression analysis of wheat (*Triticum aestivum* L.) phytoene and ζ-carotene desaturase genes

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Abstract Carotene desaturation, an essential step in the carotenoid biosynthesis pathway, is catalyzed by two carotene desaturases, phytoene desaturase (PDS) and ζ-carotene desaturase (ζ-carotene desaturase, ZDS). Full-length cDNAs designated *TaPDS* and *TaZDS* were cloned from wheat (*Triticum aestivum* cv. Chinese Spring) respectively, using the rapid amplification of cDNA ends (RACE) approach. The cDNA of *TaPDS* sequence was 2076 bp long, containing a 1731 bp open reading frame (ORF) which deduced protein having 576 amino acid residues with predicted molecular mass of 64.3 kDa and having a putative transit sequence for plastid targeting in the N-terminal region. While the cDNA sequence of *TaZDS* was 2150 bp long, contained an ORF sequence of 1707 bp, and encoded a putative protein of 568 amino acid residues with an estimated molecular mass of 62.5 kDa. Phylogenetic analysis demonstrated that TaPDS and TaZDS showed high homology with other PDSs and ZDSs in higher plant species, respectively. Moreover, sequences analysis also showed a high degree of conservation among plant PDSs and ZDSs. The deduced TaPDS and TaZDS protein both have the dinucleotide binding domain and conserved regions characteristic of other carotene desaturases. Analysis of the expression pattern of wheat *TaPDS* and *TaZDS* in different tissues revealed that the transcripts levels were higher in leaves and flowers petals, followed by in inflorescences, and were nearly absent in the roots and seeds. Southern analysis of genomic DNA indicated that the wheat *TaPDS* and *TaZDS* probably belong to a low-copy-number gene family.

Keywords Carotenoid biosynthesis · Phytoene desaturase · *Triticum aestivum* · ζ-Carotene desaturase

Abbreviations

ABA	Abscisic acid
CRTI	Bacterial carotene desaturase
DAP	Days after pollination
GGPP	Geranylgeranyl pyrophosphate
PDS	Phytoene desaturase
PSY	Phytoene synthase
QTL	Quantitative trait loci
ZDS	Zeta(ζ)-carotene desaturase

Introduction

Carotenoids are the second most abundant pigment in nature, and the carotenoid family consist of more than 750 members [1], which are yellow, red and orange pigments derived from isoprenoids. Most carotenoids are C_{40} polyenes produced by certain bacteria and fungi, and by all plants and cyanobacteria. In plants, carotenoids play a crucial role in light-harvesting and served as photo-protective compounds by quenching triplet chlorophyll and singlet oxygen derived from excess light energy; and as precursors to the hormone, abscisic acid (ABA) [2]. In human and animal diets, they also play an important protective role as antioxidants. In addition, β-carotene is a precursor of essential vitamin A [3].

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The main metabolic pathway of carotenoids is clear and may be common for most of the carotenogenic species [4]. The early steps of the carotenoid metabolic pathway, which consist of the biosynthesis of phytoene from terpenoid precursors by phytoene synthase as well as the desaturation of phytoene into lycopene followed by two cyclization reactions of lycopene to form β-carotene, have been extensively studied, and the corresponding genes have been isolated from yeast, bacteria, algae, and plants [5, 6]. The condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) to produce phytoene is catalyzed by phytoene synthase, which is encoded by *PSY* gene in plants and by *crtB* gene in bacteria. Plants and cyanobacteria employ two different and very non-homologous flavoenzymes, named phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) each mediating two steps, to carry out all four desaturation reactions necessary to convert the colorless phytoene into the red colored lycopene, whereas this is achieved in bacteria by one gene product, the bacterial carotene desaturase (CRTI).

Since Misawa cloned the carotenoid biosynthesis gene cluster from *Erwinia uredovora* for the first time [7], many works have been done on the identification of the carotenoid biosynthetic genes in plants and other organisms. Besides the assembly of carotenogenic genes into new pathways and molecular incubation of novel metabolic pathways, a large pool of available carotenogenic genes is also required to produce a mass of high value carotenoids in many fruits, vegetables and crop plants to improve the nutritional value [8]. However, very little is known about the global regulatory mechanisms underlying carotenoid metabolism and the genetic elements that regulate the expression of carotenoid biosynthetic genes.

Wheat is one of the major crops in the world and as such is a primary target for improvement of agronomic characteristics by genetic engineering. Carotenoids are the main components of flour yellow pigment, which determines the seed and products colour. This is a negative trait for the bread-making industry because of the consumer preference for white bread. On the contrary, it is a very valuable trait for the pasta industry because yellow macaroni are preferred by the market. The results of molecular biology and genetic studies showed that wheat endosperm yellow colour is highly heritable and under polygenic control [9, 10]. Genes involved in the carotenoid biosynthesis pathway are good candidates to explain some of the differences in endosperm colour among cultivars. Quantitative trait loci (QTL) for endosperm yellow colour accounting for a large proportion of the genetic variation have been mapped on chromosome arms 7AL and 7BL [11, 12]. The phytoene synthase (PSY) gene associated with the differences in carotenoids content of wheat grain, has been isolated from durum wheat [13] and hexaploid wheat [14]. Smaller QTLs were also detected on chromosomes 2A, 3A, 4A, 4B, 5A, and 5B [9, 10, 15]. However, very few genes responsible for the differences in carotenoid accumulation in the wheat grain have been identified.

Here, the isolation of cDNA sequences corresponding to phytoene desaturase (PDS) gene and ζ-carotene desaturase (ZDS) gene of wheat (*Triticum aestivum* L.) are reported. In addition, to understand the molecular phylogeny of PDS and ZDS, phylogenetic trees have been constructed using the neighbor-joining method. In the present paper the *PDS* and *ZDS* genes were first isolated and chosen to illuminate the possibly specific carotenoid metabolic pathway in wheat (*T. aestivum*). Cloning and analysis of the *PDS* and *ZDS* genes would lay a foundation to study the mechanisms for the β-carotene biosynthetic pathway and high accumulation in common wheat.

Materials and methods

Plant material

Wheat seeds of the cultivar Chinese Spring (*T. aestivum*) were germinated and grown in soil at 18–20°C day and 14–15°C night temperatures under 16 h photoperiod provided by a mercury vapour lamp in a growth room. The green young leaves were collected after 12–15 days and immediately frozen in liquid nitrogen. The total RNA was extracted from the green leaves with Plant (leaves) RNA Mini Kits (Watson, Shanghai, China) under the manufacturer's instructions, and stored at −80°C until used.

Cloning of wheat PDS cDNA

A wheat EST clone (BG909132) showed 89% nucleotide identity and 91% deduced amino acid identity (97% similarity) to the rice *PDS* cDNA (AF049356), and showed 87% nucleotide identity and 91% deduced amino acid identity (97% similarity) to the maize *PDS* cDNA (L39266), indicating that the EST encoded a putative wheat PDS. Two primers, PDS-F1 and PDS-R1, were designed based on the wheat EST sequence (see Table 1). The RT–PCR reaction was carried out using RNA isolated from leaf of wheat Chinese Spring as template for the synthesis of cDNA. One μg total RNA was used as template for cDNA first strand synthesis (M-MLV Reverse Transcriptase, Promega, America), then, 1 μl of cDNA product (ca. 0.2–0.8 ng), was utilized in a 25 μl PCR reaction [20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 0.04 U μl^{-1} *Taq* DNA polymerase (Takara, Japan)]. The

Table 1 Primers designed on the phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) genes sequences used for the isolation and expression analysis of the two genes

Primer name	Sequence (5′ → 3′)	Source	Amplified region	Product size (bp)
PDS-F1	GCACGAGGGCTGGTCTATCAA	BG909132	Partial EST	680
PDS-R1	GGACCTCACCACCCAAAGACTGAA	BG909132	Partial EST	
PDS-GSP5-1	CCAACACATCTCTTGCCTCA	BG909132	5′ RACE	577
PDS-GSP5-2	CAGCGTCTGCCAGGTATTTTGC	BG909132	5′ RACE	537
PDS-GSP3-1	GCAGTGCATTCTGATTGCTCTA	BG909132	3′ RACE	1,089
PDS-GSP3-2	GCATTCTTGGATGGTAATCC	BG909132	3′ RACE	1,020
PDS5	GCTGTTGGTTGAATAAGGTTGAC	FJ517553	*TaPDS* clone	1,864
PDS3	CAATTCACTGGTGGACTCTAC	FJ517553	*TaPDS* clone	
PDS-F2	GAAGGAACACTCCATGATATTTGC	FJ517553	Probe fragment	417
PDS-R2	CATAGCCTTTCAGGAGGATTAC	FJ517553	Probe fragment	
ZDS-F1	CAGCAATCTTTTCCGCCTCA	BG909132	Partial EST	524
ZDS-R1	TACCACCCCTGTCTGTTATG	BG909132	Partial EST	
ZDS-GSP5-1	TGAGGCGGAAAAGATTGCTG	BG909132	5′ RACE	609
ZDS-GSP5-2	CATAACAGACAATGGTGGGG	BG909132	5′ RACE	528
ZDS-GSP3-1	CAGCAATCTTTTCCGCCTCA	BG909132	3′ RACE	1,346
ZDS-GSP3-2	GGGCTCACCTGATGTTTACTTA	BG909132	3′ RACE	1,232
ZDS5	TGGCTCCGTCCCTCACTGCT	FJ169496	*TaZDS* clone	1,802
ZDS3	GTTTGGCATTCTGGCTTTGGTG	FJ169496	*TaZDS* clone	
ZDS-F2	GCCCTAAGCCCAGTTGTTC	FJ169496	Probe fragment	503
ZDS-R2	TCCCATTCCCTCCATTCCGA	FJ169496	Probe fragment	
Actin5	CTTGTATGCCAGCGGTCGAACA	AB181991	RT–PCR	250
Actin3	CTCATAATCAAGGGCCACGTA	AB181991	RT–PCR	

PCR conditions were as follows: One cycle at 94°C for 5 min; 35 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 1 min; and one cycle at 72°C for 10 min. The expected amplification product of about 680 bp was cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced.

The rapid amplification of cDNA ends (RACE) approach was used to clone the 5′ and 3′ ends of the wheat *PDS* cDNA ends. All the reactions were performed with the SMART^{TM} RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) following the "User's Manual". The specific primers (see Table 1) were designed based on the known partial sequence from RT–PCR. Then, the specific cDNA from 3′ and 5′ RACE were cloned into pMD18-T vector and sequenced, respectively.

The overlapping regions with the wheat *PDS* clone were confirmed. A putative full-length *PDS* cDNA clone was obtained by PCR using a pair of specific primers located at the extreme 5′-end (PDS5, 54 nucleotides upstream from the translation initiation codon) and 3′-end (PDS3, 79 nucleotides downstream from the translational stop codon). The expected product of about 1800 bp was cloned into the pMD18-T vector and sequenced. More than ten clones have been sequenced for each of the four PCR products described above.

Cloning of wheat ZDS cDNA

The similar method was used for cloning of *ZDS* gene from the same wheat variety. A wheat EST clone (BG909132) showed 90% nucleotide identity and 94% deduced amino acid identity (97% similarity) to the rice *ZDS* cDNA (NM_001065680) and showed 90% nucleotide identity and 95% deduced amino acid identity (98% similarity) to the *Sorghum bicolor ZDS* cDNA (AY714266), indicating that the EST encoded a putative wheat ZDS. For RT–PCR, the primers ZDS-F1 and ZDS-R1 were designed based on the EST sequence. The components and conditions of RT–PCR reaction were performed as previously described. The expected amplification product of about 524 bp was cloned into the pMD18-T vector and sequenced. The 5′ and 3′ ends of the wheat *ZDS* cDNA sequence was also amplified by using the RACE method. The overlapped region with the wheat *ZDS* clone was confirmed, and a putative full-length *ZDS* cDNA clone was obtained by RT–PCR, using a pair of specific primers at the extreme 5′-end (ZDS5, 47 nucleotides upstream from the translation initiation codon) and 3′-end (ZDS3, 95 nucleotides downstream from the translational stop codon). An 1802 bp product was cloned into the pMD18-T vector and sequenced. More than ten clones

have been sequenced for each of the four PCR products described above.

Sequencing and phylogenic analysis

The resulting wheat *PDS* and *ZDS* cDNA sequences were deposited in GenBank with accession numbers of FJ517553 and FJ169496, respectively. Sequences analysis and alignments were done with the DNAStar software package (Lasergene). The amino acid sequences were subjected to TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) for transmembrane analysis, SignalP 3.0 server [16] (http://www.cbs.dtu.dk/services/SignalP/) for the prediction of protein signal sequence, and CholoP 1.1 network-based method [17] (http://www.cbs.dtu.dk/services/ChloroP/) for the prediction of chloroplast transit peptides and their cleavage sites in proteins. For construction of a phylogenic tree, ClustalX 2.0 [18] and TREECON 2.0 [19] computer programs were used.

Wheat RNA extractions and semi-quantitative RT–PCR

The semi-quantitative RT–PCR analysis was used to examine the expression pattern of the wheat PDS and ZDS genes in different tissues. To prepare total RNA samples from different wheat organs, i.e., roots (R) of 2-week-old seedlings, leaves (L) of 3-week-old plants, stems (S), inflorescences (I), flower petals (F), immature seeds (IS) at 14 DAP (days after pollination), and mature seeds (MS), the Plant RNA Mini Kits (Watson, Shanghai, China) was used following the manufacturer's instructions. To eliminate genomic DNA contamination, total RNA samples were treated with RNase-free DNase I (Takara, Japan) according to the manufacturer's instructions.

Two pairs of primers were designed for amplification of wheat *PDS* and *ZDS* genes based on the obtained sequences: PDS-F2 and PDS-R2 for *PDS* gene, and ZDS-F2 and ZDS-R2 for *ZDS* gene. In all of the semi-quantitative RT–PCR experiments conducted in this work, the amplification of wheat *actin* gene transcripts was used to normalize the cDNA contents of various reverse transcription mixtures before PCR, and to monitor the kinetics of thermo amplification during PCR. A varying number of thermo cycles (35, 40, 45 and 50) were used in order to check the linearity of the amplifications. RT–PCRs were performed in a total volume of 50 μl with the Access RT–PCR System (Promega, USA), containing 1× AMV/*Tfl* reaction buffer, 0.2 mM of each dNTP, 1 μM of each primer, 1 mM $MgCl_2$, 0.1 U μl^{-1} of AMV reverse transcriptase, 0.1 U μl^{-1} of *Tfl* DNA polymerase and 2 μg total RNA for each sample. Amplification was carried out according to the following temperature profile: 45°C for 45 min for first-strand cDNA synthesis, 94°C for 2 min for denaturation, then 40 cycles at 94°C for 30 s, 57°C for 30 s, 68°C for 1 min, and a final extension of 68°C for 7 min.

The relative amounts of each PCR product were readily quantified by direct scanning with a densitometer of ethidium-stained 1.5% TAE-agarose gels with a Molecular Imager Gel Doc 2000 System (Bio-Rad Laboratories Inc., California, USA) equipped with the Quantity One 1-D analysis software. To normalize for equal amounts of total RNA and efficiency of cDNA synthesis from various tissue samples, the intensities of the band were normalized with the average intensity of the *actin* product across the samples investigated. An arbitrary value of 100% was assigned to the *actin* product level of each sample. The reproducibility of the transcriptional patterns revealed by semi-quantitative PCR was tested by at least three independent assays, and each percentage value is the mean of the three independent samples of the same experiment.

Wheat genomic DNA extractions and Southern blotting

Total genomic DNA was isolated from leaf material of 3-week-old seedlings using a cetyltrimethylammonium bromide (CTAB) extraction method [20]. About 15 μg genomic DNA was digested with restriction enzyme *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII and *Pst*I, separately, and then separated in a 0.6% (w/v) agarose gels. For wheat PDS, *Bam*HI and *Dra*I cut once in the sequence corresponding to the probe, and *Eco*RI and *Hin*dIII are non-cutters. For wheat ZDS, *Eco*RI and *Hin*dIII cut once in the sequence corresponding to probe, and *Bam*HI and *Pst*I are non-cutters. After being electrophoresed, DNA was transferred to nylon membrane by capillary blotting. The same fragments of semi-quantitative RT–PCR products above were used as probes to estimate the number of copies of wheat *PDS* and *ZDS* gene in wheat genome by hybridization to hexaploid wheat (cv. Chinese Spring). Then, the membrane was labeled with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Southern hybridization was carried out overnight after half an hour of pre-hybridization at 42°C. After hybridization the membranes were washed twice for 5 min each with 2× SSC, 0.1% SDS at 42°C for 5 min, and further washing twice for 15 min each with 0.5× SSC, 0.1% SDS at 68°C under constant agitation, and finally exposed to X-ray films at 37°C for several hours.

Results

Isolation and characterization of *TaPDS* cDNA

A cDNA fragment was isolated by RT-PCR with gene-specific primers (PDS-F1 and PDS-R1), which were designed based on the EST clone (BG908924). This cDNA

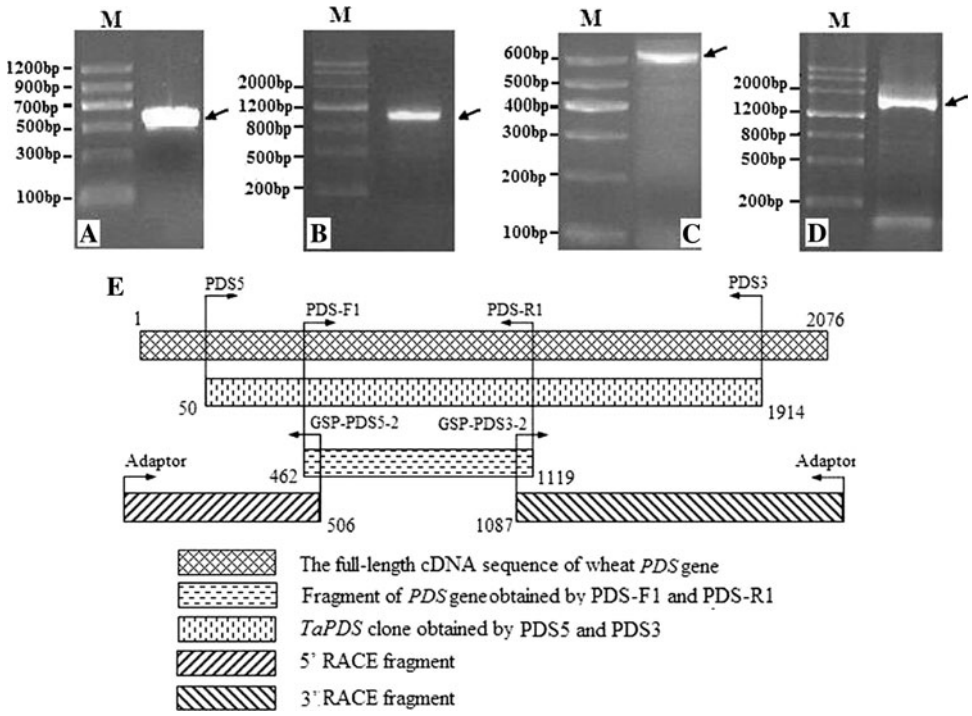

Fig. 1 Cloning of the full-length cDNA of phytoene desaturase (PDS) gene from wheat. **a** The fragment of PDS cDNA was amplified from wheat by a pair of primers (PDS-F1 and PDS-R1) designed according to high conserved regions of the EST clone (GenBank BG908924). **b** 3′-end of PDS cDNA isolated by 3′RACE. **c** 5′-end of PDS cDNA isolated by 5′RACE. **d** The cDNA fragment named *TaPDS* clone was obtained by RT–PCR with a pair of gene-specific primers (PDS5 and PDS3) designed according to the reconstructed full-length cDNA sequence. **e** Diagram of overlapping relationship of subclone fragments and positions of corresponding primers

fragment showed a 680-bp band in a 1% agarose gel (Fig. 1a). Various clones were sequenced and sequencing results showed the same nucleic acid sequence which was fully consistent with the EST clone. The full-length cDNA sequence of wheat was then cloned using RACE strategies based on obtained fragment. First strand cDNA derived from total RNA was used as template to amplify the 3′-end (Fig. 1b) and the 5′-end (Fig. 1c) of cDNA with the specific primers and universal primers. The wheat *PDS* gene was assembled according to overlapping sequences from the three fragments above (Fig. 1e). The reconstructed full-length cDNA sequence, 2076 bp long, obtained from 3′ and 5′RACE, was confirmed from the sequence of the *TaPDS* clone (1865 bp) (Fig. 1d), containing a 1731 bp CDS, 140-nucleotides of 5′-untranslated region (UTR), and 206-nucleotides of 3′-UTR (data not shown). The predicted protein displayed a sequence of 576 amino acid residues (unprocessed), with a calculated molecular mass of 64.3 kDa.

The BlastP (Standard protein–protein Basic Local Alignment Search Tool, NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) search results demonstrated that the cloned TaPDS showed at the protein level 86% identity and 91% similarity with *Zea mays* counterpart, 89 and 94% with the *Oryza sativa* counterpart, 81 and 89% with the *Arabidopsis thaliana* counterpart, and 79 and 87% with the *Narcissus pseudonarcissus* counterpart. These data demonstrated that the cloned TaPDS belongs to the PDS family. The resulting *TaPDS* sequence was deposited in GenBank with accession number of FJ517553.

Figure 2a shows the comparison of the amino acid sequence of available higher plant PDS proteins. Other PDS sequences used for comparison were plant-type, from rice (AF049356), maize (L39266), *Arabidopsis* (L16237), daffodil (X78815), soybean (M64704), pepper (X68058), and bacterial-type, from *Erwinia* (D90087). Based on nucleotide and amino acid primary structure homology, the TaPDS shows higher homology to the sequence of the plant-type rather the bacterial (CRTI)-type gene (nucleotide comparison not shown). The phylogenic trees (Fig. 2b) suggested that PDS genes could be largely classified into two groups, a monocotyledonous PDS group and a dicotyledonous PDS group. The TaPDS has been placed close to the rice and maize, and showed a lower homology with other dicotyledonous plants, such as pepper, soybean and *Arabidopsis*.

A putative transit sequence for plastid targeting was predicated to be the N-terminal residues of 1–61 by concordance with the predicated transit peptides of rice, maize, *Arabidopsis*, and daffodil, which are 87, 79, 75 and 56 residues, respectively [21–23]. A CholoP 1.1 method predicts a transit peptide cleavage site between residues 61R and 62A (Fig. 2a). The predicted molecular mass of the mature wheat PDS polypeptide found in plastids is about 57.7 kDa. The deduced amino acid sequence of wheat PDS revealed a typical pyridine dinucleotide binding motif generally associated with the binding of FAD, which was represented by GenBank Pfam00890, and was present in other plant PDS proteins.

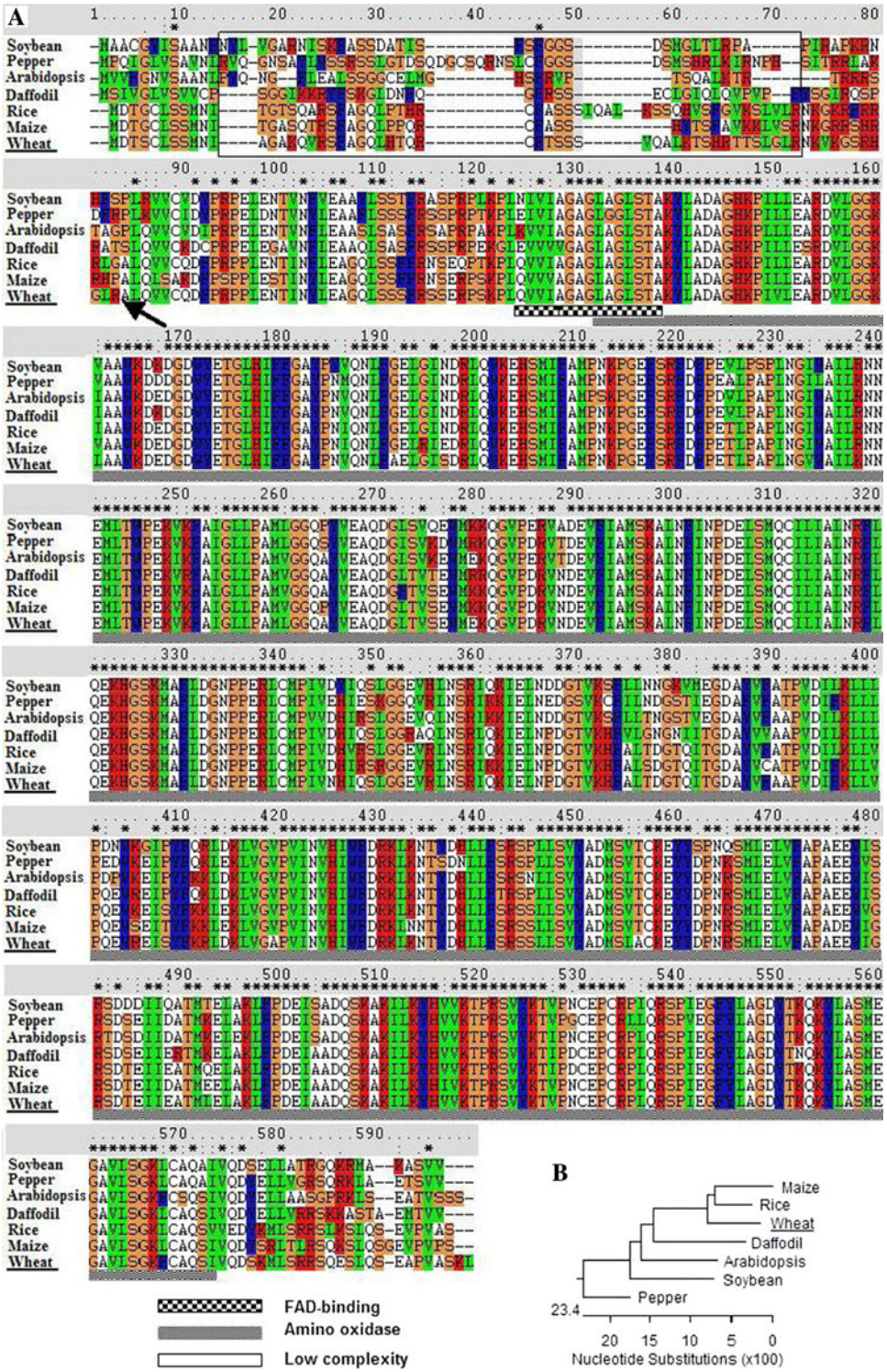

Fig. 2 Amino acid alignment (**a**) and polygenetic tree (**b**) of novel TaPDS with other selected phytoene desaturase. Selected conserved domains are as follows: Pfam00890, FAD binding domain; Pfam01593, amino oxidase, flavin containing amine oxidoreductase; COG1233, phytoene dehydrogenase and related proteins. The *asterisks* show the same amino acid residues. The *arrow* shows a likely chloroplast transit peptide cleavage site. The phenogram (Neighbour-Jointing) indicates that TaPDS shows higher homology to other PDS proteins; the length of branches corresponds to phenetic distance

Cloning and characterization of *TaZDS* cDNA

A cDNA fragment was isolated by RT–PCR with gene-specific primers (ZDS-F1 and ZDS-R1) designed on the basis of the sequence of the EST clone (BG909132) encoding a putative wheat ZDS. This cDNA fragment showed a 524 bp band in a 1.5% agarose gel (Fig. 3a). Sequencing results showed that it was fully consistent with the EST clone. The full-length cDNA sequence of wheat was cloned using RACE strategies based on the obtained 524-nucleotides fragment. First strand cDNA derived from total RNA was used as template to amplify the 3′-end

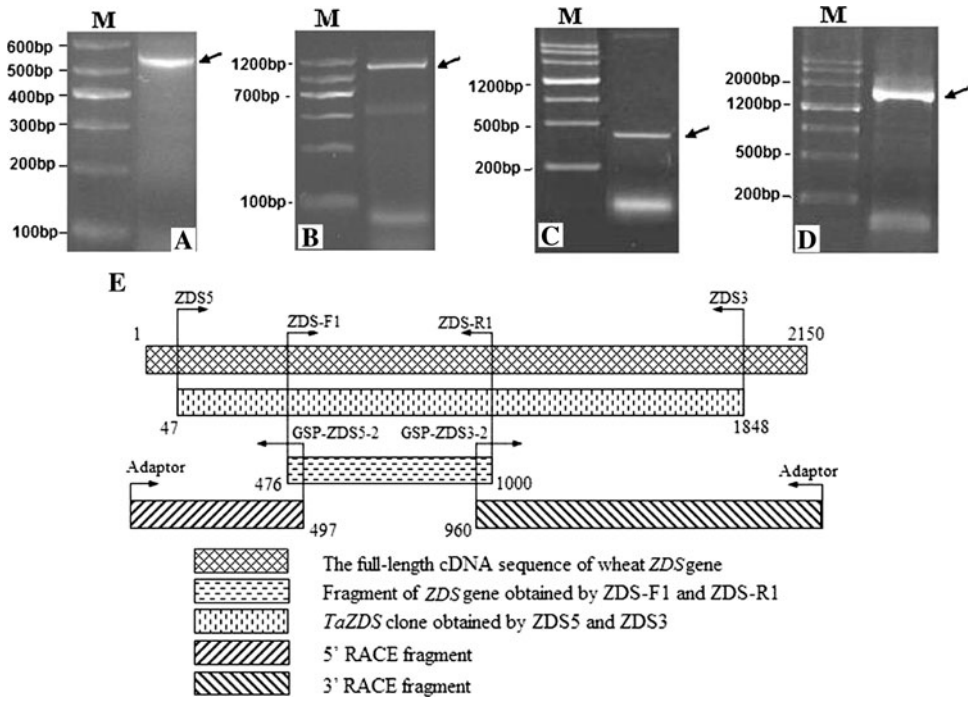

Fig. 3 Isolation of the full-length cDNA of ζ-carotene desaturase (ZDS) gene from wheat. **a** The fragment of *ZDS* cDNA amplified from wheat by a pair of primers (ZDS-F1 and ZDS-R1) designed according to high conserved regions of the EST clone (GenBank BG909132). **b** 3′-end of *ZDS* cDNA isolated by 3′RACE. **c** 5′-end of *ZDS* cDNA isolated by 5′RACE. **d** The fragment named *TaZDS* clone obtained by a pair of gene-specific primers (ZDS5 and ZDS3), which were designed according to the reconstructed full-length cDNA sequence (GenBank FJ169496). **e** Diagram of overlapping relationship of subclone fragments and positions of corresponding primers

(Fig. 3b) and the 5′-end (Fig. 3c) of cDNA with gene-specific primers and RACE universal primers. The wheat *ZDS* gene was assembled according to overlapping sequences from the three fragments above (Fig. 3e). The reconstructed full-length cDNA sequence obtained from 3′ and 5′ RACE was confirmed from the sequence of the clone *TaZDS* (1802 bp) (Fig. 3d). The full-length cDNA was 2150 bp long, and contained a 1707 bp CDS, 108-nucleotides of 5′-UTR, and 335-nucleotides of 3′-UTR (data not shown). The predicted protein displayed a sequence of 568 amino acid residues, with a calculated molecular mass of 62.5 kDa.

The BlastP search results demonstrated that the cloned TaZDS showed at the protein level 94% identity and 97% similarity with *Oryza sativa* counterpart, 93 and 97% with the *Sorghum bicolor* counterpart, 87 and 92% with the *Zea mays* counterpart, 80 and 91% with *Narcissus pseudonarcissus* counterpart, 79 and 89% with the *Helianthus annuus* counterpart. These data demonstrated that the cloned TaZDS belongs to the ZDS family. The resulting *TaZDS* sequence was deposited as GenBank accession number of FJ169496.

The deduced amino acid sequence of TaZDS protein was compared with other selected carotene desaturase amino acid sequence. The selected carotene desaturase genes were as follows: rice (NM_001065680), maize (AF047490), sorghum (AY714266), daffodil (AJ224683), sunflower (AJ438587), *Arabidopsis* (ATU38550), and tomato (DQ412572). Figure 4a shows the comparison of the amino acid sequence of available higher plant ZDS proteins. Based on amino acid and nucleotide primary structure homology, the TaZDS shows a higher homology to the sequences of monocotyledonous crop plants, i.e., rice, maize, and sorghum, rather than the dicotyledonous plants, i.e., daffodil, sunflower, tomato, and *Arabidopsis* (nucleotide comparison not shown). The phylogenic analysis (Fig. 4b) clearly demonstrated that the *TaZDS* is clustered to rice, maize and sorghum ZDS genes, while the ZDS for the monocotyledonous plant daffodil (*Narcissus pseudonarcissus*) is no more closely related to the grass carotenoid desaturase than the ZDS proteins from the selected dicotyledonous plants *Arabidopsis* and sunflower.

A putative transit sequence for plastid targeting was predicted to be the N-terminal residues of 1–32 by concordance with the predicated transit peptides of rice, maize, *Arabidopsis*, and daffodil, which are 42, 30, 34 and 42 residues, respectively [24–26]. The CholoP 1.1 method predicts a transit peptide cleavage site between residues 32R and 33C. The predicted molecular mass of the mature wheat ZDS polypeptide found in plastids is about 59.4 kDa. The deduced amino acid sequence of wheat ZDS revealed a typical pyridine dinucleotide binding domain (FAD-binding domain), which was represented by GenBank Pfam00890, and present in maize ZDS [24]. The similar enzymatic function of ZDS and PDS was also reflected in the sequence similarity of the mature protein, which showed an amino acid identity of about 32% and a similarity of 52%.

Expression analysis of wheat *TaPDS* and *TaZDS* genes

The organ-specific expressions of *TaPDS* and *TaZDS* were analyzed by semi-quantitative RT–PCR assays (Fig. 5),

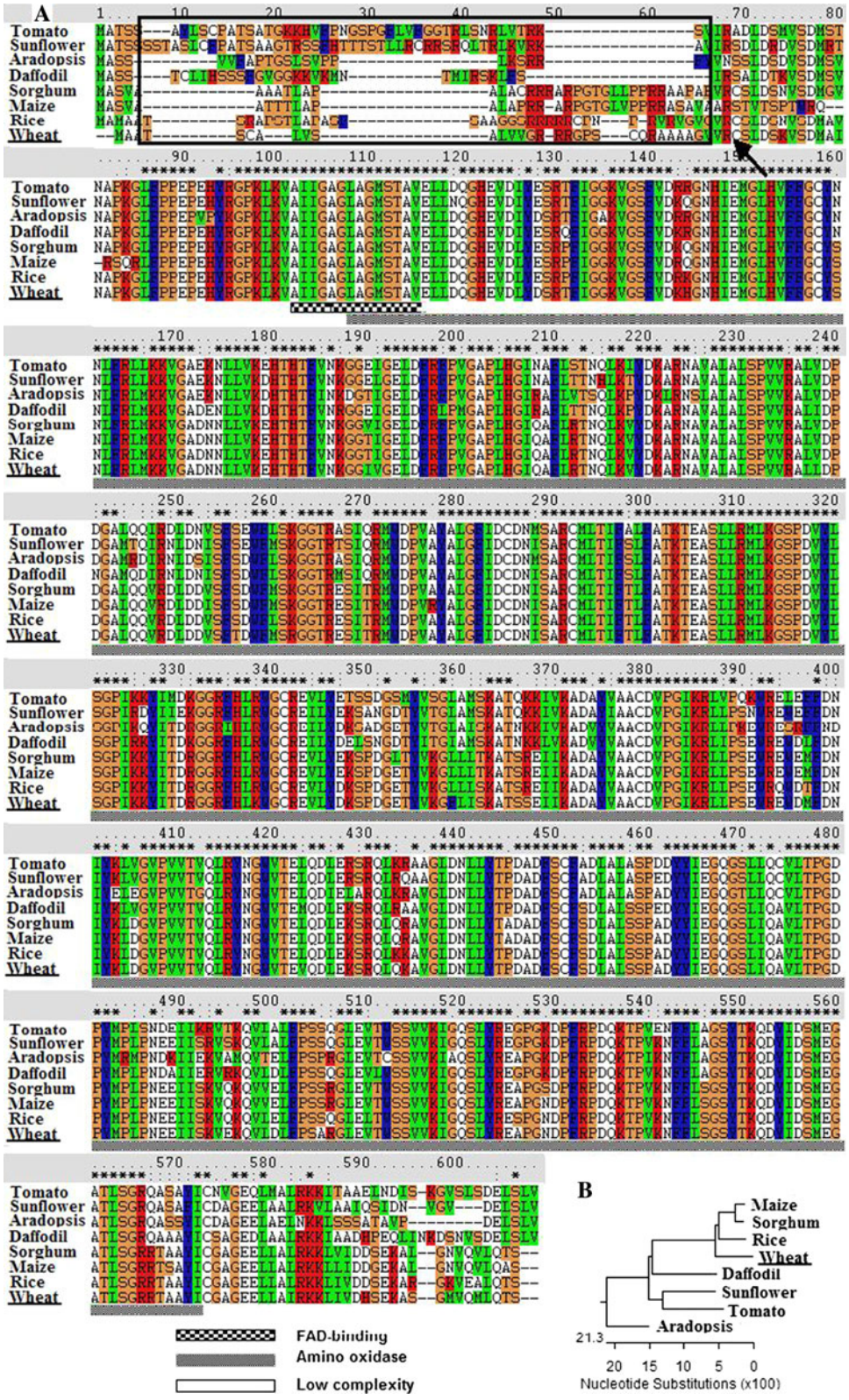

Fig. 4 Amino acid alignment (**a**) and phylogenic tree (**b**) of novel wheat ZDS with other selected ζ-carotene desaturases. Selected conserved domains are as follows: Pfam00890, FAD binding domain; Pfam01593, amino oxidase, flavin containing amine oxidoreductase; COG1233, phytoene dehydrogenase and related proteins. The *asterisks* show the same amino acid residues. The *arrow* shows a likely chloroplast transit peptide cleavage site. The phenogram (Neighbor-Jointing) indicates that wheat ZDS shows higher homology to other ZDS proteins; the length of branches corresponds to phenetic distance

which amplified a cDNA fragment of 417-bp product from *TaPDS* gene and a cDNA fragment of 503-bp product from *TaZDS* gene respectively. The results revealed that wheat *PDS* and *ZDS* transcripts showed similar expression patterns. Both were very highly expressed in leaves and flower petals, followed by stem and inflorescences which are

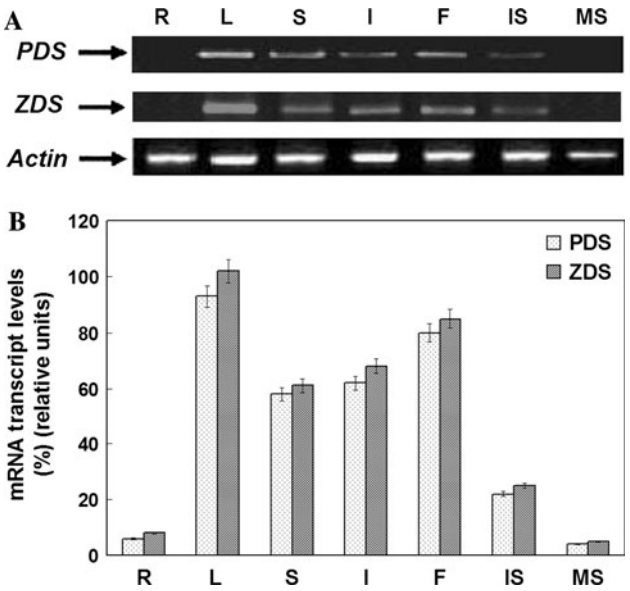

Fig. 5 Relative expression levels (%) of *PDS* and *ZDS* transcripts were estimated by RT–PCR assays. **a** The amplification products of *PDS*, *ZDS* and *actin* transcripts were obtained by RT–PCR using 2 μg of total RNA isolated from roots (R), leaves (L), stems (S), inflorescences (I), flower petals (F), immature seeds (IS) and mature seeds (MS) of plants. **b** The relative expression levels of the *PDS* and *ZDS* transcripts, expressed as a percentage with respect to the *actin* gene product level, were estimated by measuring the ethidium bromide fluorescence of the PCR products resolved by gel electrophoresis as in **a**

abundant in chloroplasts or chromoplasts. In contrast, transcript levels were comparatively lower in immature seeds, and nearly absent in the roots and mature seeds.

Genomic DNA isolation and Southern hybridization

The copy number of *TaPDS* and *TaZDS* genes in the hexaploid wheat genome was estimated by performing Southern blot hybridization. The genomic DNA of Chinese Spring leaf was digested with restriction enzyme *Bam*HI, *Dra*I, *Eco*RI, and *Hin*dIII, separately, and then was hybridized with a 417-bp probe synthesized by PCR amplification from wheat PDS ORF sequences. The result of hybridization revealed that the hybridization pattern of *PDS* was simple for several enzyme combinations, showing between one and four bands in hexaploid wheat (Fig. 6a). Otherwise, for estimating the copy number of the *TaZDS* gene, the genomic DNA was digested with restriction enzyme *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I, separately, and the probe was a 503-bp PCR product fragment. The hybridization result of *TaZDS* showed the simple hybridization pattern with 2–3 hybridizing bands per lane in Southern blots (Fig. 6b). Therefore, we assume that *TaPDS* and *TaZDS* genes are probably low-copy-number genes in the wheat genome.

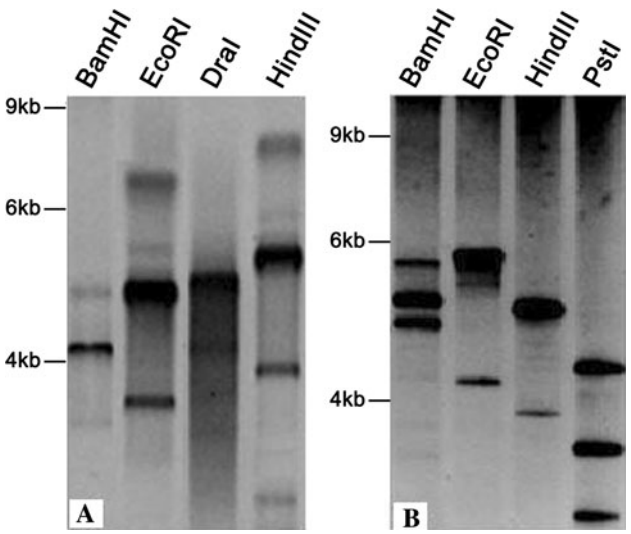

Fig. 6 Southern blot analysis of *TaPDS* (**a**) and *TaZDS* (**b**) in hexaploid wheat (cv. Chinese Spring). Genomic DNA used for *TaPDS* analysis was digested with the restriction enzymes *Bam*HI, *Dra*I, *Eco*RI and *Hin*dIII, and for *TaZDS* analysis with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I. The filters were probed with the RT–PCR product of *TaPDS* and *TaZDS* sequences

Discussion

A full-length cDNA sequence, which encoded a putative protein homologous to phytoene desaturase (PDS), has been cloned in wheat (*T. aestivum* L.) using the RACE method. The reconstructed cDNA sequence was 2076 bp in size, which contained a 1731 bp open reading frame (ORF) sequence. The predicted protein has a sequence of 576 amino acid residues with a molecular mass of 64.3 kDa (unprocessed). The putative wheat PDS amino acid sequences shows a high degree of homology with the PDS isolated from *Zea mays*, *Arabidopsis thaliana*, and *daffodil paracorolla* [21–23]. The phylogenetic tree (Fig. 2b) produced by alignment of monocot and dicot PDS amino acid sequences are consistent with current hypotheses of plant evolutionary relationships. However, unlike the nucleotide sequence, which is variable from 77 to 89% at the nucleotide level between monocots and dicots, the nuclear-encoded PDS proteins are highly conserved at the presumed mature protein region (data not show).

A full-length cDNA sequence encoding a putative ζ-carotene desaturase (ZDS), a key enzyme in carotenoid biosynthesis catalyzing the desaturation of ζ-carotene to produce neurosporene and lycopene, also has been cloned and characterized. The reconstructed full-length cDNA sequence was 2150 bp in size, which contained a 1707 bp ORF sequence. The predicted protein has a sequence of 568 amino acid residues with a molecular mass of 62.5 kDa (unprocessed). Sequence comparison suggests that the isolated TaZDS belongs to the ZDS family. Similar

to other enzymes in the carotenoid biosynthesis pathway, ZDS appears to be highly conserved in higher plants. Phylogenetic analysis demonstrated that the ZDS obtained from wheat was 97 and 91% homologous with that from *Oryza sativa* and *Zea Mays* respectively at amino acid level, and it also showed high homology with ZDS proteins from other higher plants.

Alignments at the protein level showed the sequence differences between monocotyledonous and dicotyledonous PDSs or ZDSs are mainly found at the N-terminus (Figs. 2a, 4a), due to the presence of a signal peptide responsible for the localization of these enzymes in chloroplasts and chromoplasts [6]. The deduced amino acid sequence of TaPDS and TaZDS both revealed the predicted chloroplast transit peptides, which were predicated to be 61 residues and 32 residues, respectively. The similar enzymatic function of TaZDS and TaPDS was also reflected in the sequence similarity of the mature protein, which showed that an amino acid identity of about 32% and a similarity of 52%.

The cloned cDNAs were used as tools to determine tissue-specific expression of these genes and to monitor the regulation of carotenogenic activity at the mRNA level in different tissues. The results demonstrated that the two genes showed a higher expression level in leaves and flower petals, followed by inflorescences and stem, which were abundant chloroplast or chromoplast. In contrast, transcript levels were lower in immature seed, and nearly absent in roots and mature seeds. The expression levels of *TaPDS* and *TaZDS* genes during wheat seeds development have also been detected here (data not show). No marked change in *TaPDS* transcript level was found in 5–25 DAP seeds and an imperceptible reduction in 30 DAP seeds was observed. However, this stage is late in endosperm development and well past the greatest period of carotenoid accumulation. This expression pattern was similar to the maize PDS in developing endosperms [21], and to the tomato *PDS* in developing tomato fruits [27]. The stable expression of *PDS* transcripts in developing endosperm may also serve as a useful internal experimental control for future studies of endosperm gene expression, including other genes involved in carotenogenesis.

Southern analysis of genomic DNA, carried out under varying stringency, indicated that *TaPDS* and *TaZDS* were probably low-copy-number genes, respectively, in hexaploid bread wheat genome. This result about *TaPDS* was in agreement with Travella et al., who reported that there were at least three copies in the hexaploid wheat genome, and mapped the wheat *PDS* gene on chromosomes 4A and 4D [28]. Otherwise, Cenci et al. also localized durum wheat (*Triticum durum* L.) bacterial artificial chromosome clones containing the *PDS* genes on chromosome 4A as well as 4B, and the ZDS genes on chromosome 2A and 2B [15]. The PDS and ZDS genes have been either confirmed as a single-copy gene in many other higher plants, such as *Arabidopsis*, maize and rice [21, 24, 29].

In summary, two cDNA sequences respectively encoding two carotene desaturase for the four-step desaturation reaction in carotenoids biosynthesis pathway, phytoene desaturase and ζ-carotene desaturase, have been cloned and characterized in hexaploid wheat (*T. aestivum* L.). The identification of the *TaPDS* and *TaZDS* genes provides useful materials for more comprehensive studies on the physiological function of carotenoids by genetic, biochemical and molecular approaches.

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