Isolation and Expression Analysis of Two DOPA Dioxygenases in *Phytolacca americana*

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Z. Naturforsch. **64c**, 564–573 (2009); received February 17/March 19, 2009

Betacyanins and anthocyanins, two main red flower pigments, never occur together in the same plant. Although the anthocyanin biosynthetic pathway has been well analyzed, the biosynthetic genes and the regulatory mechanism of the betacyanin biosynthesis are still obscure. We cloned two cDNAs of DOPA dioxygenase from *Phytolacca americana*, *PaDOD1* and *PaDOD2*, that may be involved in the betalain biosynthesis. The deduced amino acid sequence of *PaDOD1* and *PaDOD2* showed approximately 80% homology to each other. The promoter regions of *PaDOD1* and *PaDOD2* were isolated by inverse PCR and analyzed using PLACE database. Some putative MYB, bHLH, and environmental stress-responsive transcription factor binding sites were detected in the *PaDOD1* and *PaDOD2* promoter regions. Expression patterns of *PaDOD1* and *PaDOD2* in suspension cultures of *P. americana* were investigated by semiquantitative RT-PCR. The transcripts of *PaDOD*s were found in both betacyanin-producing red cells and non-betacyanin-producing white cells, suggesting that not only the expression of *DOD*, but also the supplementation of DOPA might be a regulatory step for the betalain biosynthesis in *P. americana*.

Key words: Betalain, DOPA Dioxygenase, *Phytolacca americana*

Introduction

The red colour in flowers is mainly produced by two types of pigments; betacyanins and anthocyanins. These two red pigments are stored in vacuoles, and serve important functions in plant reproduction through recruiting pollinators and seed dispersers (Grotewold, 2006). Though anthocyanins are broadly distributed among plants, betacyanins have replaced the anthocyanins in the Caryophyllales, excluding the families Caryophyllaceae and Molluginaceae (Strack *et al.*, 2003).

In physiological investigations on the betalain biosynthesis, the effects of various stimuli such as hormones (Biddington and Thomas, 1973; Piattelli, 1976; Sakuta *et al.*, 1991; Hirano *et al.*, 1992, 1996), nutrition (Sakuta *et al.*, 1986, 1987a, b; Sakuta and Komamine, 1987), and light (Giudici De Nicola *et al.*, 1973, 1974) on betacyanin accumulation have been analyzed. However, the detailed signaling pathways and biosynthetic genes of betacyanin biosynthesis are still poorly understood. Thus, further studies on betacyanin biosynthetic enzymes are required to clarify the regulatory mechanism of the betacyanin biosynthesis.

Betacyanin, a red-violet pigment, and betaxanthin, a yellow pigment, are members of the betalains, whose basic structure is betalamic acid. The extradiolic 4,5-cleavage of DOPA catalyzed by 4,5-DOPA dioxygenase (DOD) is required for the formation of betalamic acid (Fig. 1). A plant *DOD* gene encoding the DOD was first isolated from *Portulaca grandiflora* (*PgDOD*; Christinet *et al.*, 2004). The function of *PgDOD* in the betalain biosynthetic pathway was shown *in vivo* by genetic complementation in white petals of *P. grandiflora*, in which the set of genes for colour formation are missing (Christinet *et al.*, 2004). It is also reported that some non-betalain-producing plants possess *DOD* genes although the functions of these homologues are yet elusive (Christinet *et al.*, 2004). To assess the contribution of *DOD* to the betalain biosynthesis, we cloned two cDNAs of *DOD* from *Phytolacca americana*, *PaDOD1* and *PaDOD2*, and analyzed the promoter regions of *PaDOD1* and *PaDOD2*. The expression profiles of *PaDOD1* and *PaDOD2* in *P. americana* suspension cultures were investigated to examine the correlation between betacyanin accumulation and the expression levels of these *PaDOD* genes.

0939–5075/2009/0700–0564 $ 06.00

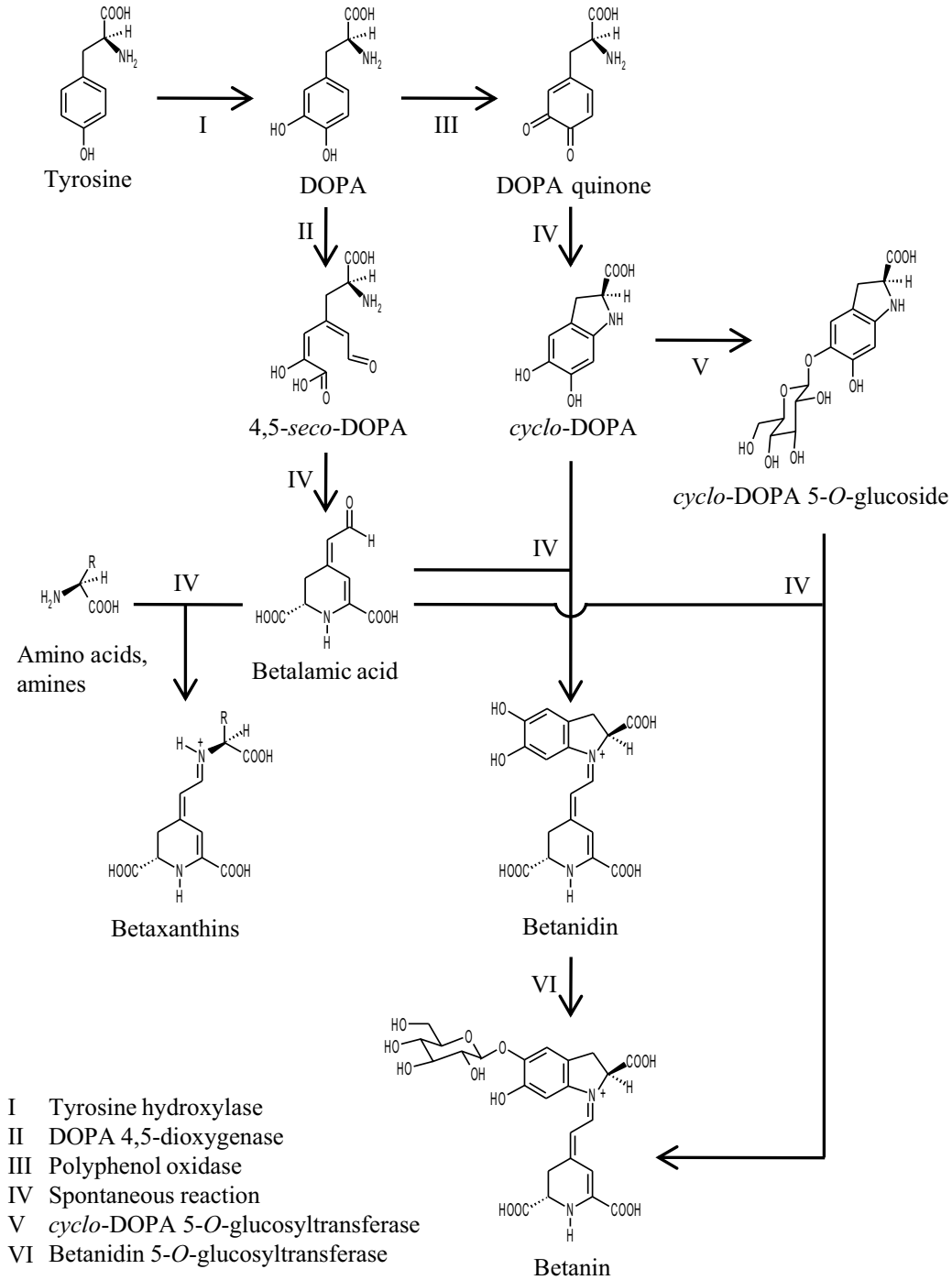

Fig. 1. Biosynthetic pathway of betalain.

Material and Methods

Plant materials

The suspension cultures were prepared from calli initiated from stem explants of *Phytolacca americana* (Sakuta *et al.*, 1986). The cells were maintained in Murashige and Skoog (1962) medium containing 3% (w/v) sucrose and 5 μM 2,4-dichlorophenoxyacetic acid.

Measurement of betacyanin content

The betacyanin content was estimated by measuring the absorbance at 530 nm after the extraction of 100 mg frozen cells in 1 ml of 80% methanol (Sakuta *et al.*, 1986).

Preparation of RNA

Total RNA was extracted from 2 g frozen cells with extraction buffer (0.1 M Tris-HCl, pH 9.0, 0.1 M NaCl, 10 mM EDTA, 0.5% SDS, 14 mM 2-mercaptoethanol), and was deproteinized by phenol/chloroform extraction. The samples were precipitated with LiCl (Ozeki *et al.*, 1990).

Single-stranded cDNA synthesis

The single-stranded cDNA was synthesized from 2.5 μg total RNA using the Prime Script reverse transcriptase (Takara) with 5 μM oligo-dT primer. The synthesized cDNA was used as the template for the series of PCR reactions described below.

Cloning and reverse transcription-PCR (RT-PCR) of PaDODs

The single-stranded cDNA was synthesized from total RNA of 7-day-old *P. americana* suspension cultures. The primers used in PCRs are described in Table I. The RACE (rapid amplification of cDNA ends) products were cloned into pT7Blue vector (Novagen) and sequenced. To clone DOPA dioxy-

Table I. Oligonucleotide primers and uses for PCR.

Name	Sequence	Description
GSP-T15	5′-CAACAACGCACAGAATCTAGC$(T)_{15}$-3′	3′-RACE reverse transcription for *PaDOD1* 3′-RACE reverse transcription for *PaDOD2*
GSP	5′-CAACAACGCACAGAATCTAGC-3′	3′-RACE 1st PCR for *PaDOD1* 3′-RACE for *PaDOD2*
GSP-T3	5′-CAACAACGCACAGAATCTAGCTTT-3′	3′-RACE 2nd PCR for *PaDOD1*
TdT	5′-GGCCACGCGTCGACTAGTAC$(G)_{15}$-3′	5′-RACE for *PaDOD2*
PaDOD-f1	5′-T(C/T)(A/T)CTGCTCA(C/T)TGGGA(A/G)AC(C/T)G-3′	RACE for *PaDOD1* fragment
PaDOD-r1	5′-GCAGCCCA(A/G/C)GGAGCAAC-3′	RACE for *PaDOD1*fragment RACE for *PaDOD2* fragment
PaDOD1-f1	5′-ACACTCCCCATGCTGTTGG-3′	3′-RACE 1st PCR for *PaDOD1* 3′-RACE 2nd PCR for *PaDOD1*
PaDOD1-f2	5′-GGGCTACGCCTGCTACAAGT-3′	5′-RACE 1st PCR for *PaDOD1*
PaDOD1-f3	5′-CATGGGCCTTGTCGTGGTCA-3′	5′-RACE 2nd PCR for *PaDOD1*
PaDOD1-f4	5′-CCTTCGAACGACACTCCC-3′	Semiquantitative RT-PCR for *PaDOD1*
PaDOD1-r1	5′-CGAACCGCCATGGCT-3′	5′-RACE reverse transcription for *PaDOD1*
PaDOD1-r2	5′-CCCGTGTCGCCAAATCTGGA-3′	5′-RACE 1st PCR for *PaDOD1*
PaDOD1-r3	5′-GAGCTGGTACATGGGAGCAG-3′	5′-RACE 2nd PCR for *PaDOD1*
PaDOD1-r4	5′-GAATTTCGCAGTGGTGAACT-3′	Semiquantitative RT-PCR for *PaDOD1*
PaDOD2-f1	5′-TCCTCCTTGTTCGAG-3′	RACE for *PaDOD2* fragment
PaDOD2-f2	5′-GGCAACACCCCTGGTTCATT-3′	3′-RACE for *PaDOD2* Semiquantitative RT-PCR for *PaDOD2*
PaDOD2-r1	5′-TGCGGTTAACACTTCT-3′	5′-RACE for *PaDOD2*
PaDOD2-r2	5′-TGCTTGTACATTGTCGG-3′	Semiquantitative RT-PCR for *PaDOD2*
PaDODcis-f1	5′-TTCTGTGCACGGGGG-3′	1st inverse PCR (*Bam*HI) for *PaDOD1* promoter 1st inverse PCR (*Mlu*I) for *PaDOD1* promoter 1st inverse PCR (*Bam*HI) for *PaDOD2* promoter

Table I continued.

Name	Sequence	Description
PaDODcis-f2	5′-ACCCATGCGCAGCTCGTGCG-3′	1st inverse PCR (*Bam*HI) for *PaDOD1* promoter 2nd inverse PCR (*Mlu*I) for *PaDOD1* promoter 2nd inverse PCR (*Dra*I) for *PaDOD1* promoter 1st inverse PCR (*Dra*I) for *PaDOD1* promoter 2nd inverse PCR (*Bam*HI) for *PaDOD2* promoter
PaDODcis-f3	5′-ATGGATGTGAAAGATATG-3′	1st inverse PCR (*Nco*I) for *PaDOD1* promoter 2nd inverse PCR (*Nco*I) for *PaDOD1* promoter
PaDODcis-f4	5′-TGTACGTGCGTCTCCTCG-3′	Inverse PCR (*Eco*RV) for *PaDOD1* promoter
PaDODcis-f5	5′-GGGCTACGCCTGCTACAAG-3′	1st inverse PCR (*Eco*RI) for *PaDOD2* promoter
PaDODcis-f6	5′-CATGGGCCTTGTCGTGGTCA-3′	2nd inverse PCR (*Eco*RI) for *PaDOD2* promoter
PaDODcis-r1	5′-AGAGAGACCGACCTGGTACATGG-3′	1st inverse PCR (*Bam*HI) for *PaDOD1* promoter 2nd inverse PCR (*Bam*HI) for *PaDOD1* promoter 2nd inverse PCR (*Mlu*I) for *PaDOD1* promoter 2nd inverse PCR (*Nco*I) for *PaDOD1* promoter 1st inverse PCR (*Bam*HI) for *PaDOD2* promoter 2nd inverse PCR (*Bam*HI) for *PaDOD2* promoter
PaDODcis-r2	5′-CATTGCTGAGCATTAGCTGC-3′	1st inverse PCR (*Mlu*I) for *PaDOD1* promoter
PaDODcis-r3	5′-ACTCTAGGGAACTGAGAATCC-3′	1st inverse PCR (*Nco*I) for *PaDOD1* promoter
PaDODcis-r4	5′-TGTTGTCTCCATCTTATGGAG-3′	2nd inverse PCR (*Nco*I) for *PaDOD1* promoter
PaDODcis-r5	5′-TCGTTCAAGAATCCAAAGACTCG-3′	2nd inverse PCR (*Dra*I) for *PaDOD1* promoter
PaDODcis-r6	5′-AAATGTTCATGGGCGGGGG-3′	Inverse PCR (*Eco*RV) for *PaDOD1* promoter
PaDODcis-r7	5′-AGCTGGTACATGGGAGCAG-3′	1st inverse PCR (*Eco*RI) for *PaDOD2* promoter 2nd inverse PCR (*Eco*RI) for *PaDOD2* promoter

genase (*DOD*) orthologues, the degenerate primers were designed from the most conserved region of known *DOD* sequences from Caryophyllales (Christinet *et al.*, 2004). For 3'-RACE of *PaDOD1* cDNA, single-stranded DNA was synthesized, and then the product was used as the template for PCR. For the 5'-full RACE of *PaDOD1*, cDNA was synthesized using the 5'-Full RACE Core Set (Takara) according to the manufacturer's instructions, and then PCR was performed.

The *PaDOD2* fragment was amplified partially using *PaDOD1*'s sequence. The 3'-RACE of *PaDOD2* was carried out using the same conditions as for 3'-RACE of *PaDOD1*. For the 5'-RACE of *PaDOD2*, a single-stranded cDNA was used as the template for the terminal deoxynucleotide

transferase (TdT) tailing reaction, and then the product was used as the template for PCR.

The expression level of *PaDOD1* and *PaDOD2* was estimated by the semiquantitative RT-PCR reaction. Aliquots of total RNA from suspension cultures of *P. americana* were subjected to RT-PCR analysis using Prime Script reverse transcriptase (Takara). PCR was performed for 28 to 31 cycles. After PCR, 5 μl of PCR products were separated by 1% TAE agarose gel electrophoresis.

DNA sequencing

The nucleotide sequences were determined using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems).

Isolation of promoter sequences

The promoter regions of *PaDOD1* and *PaDOD2* were isolated by the inverse PCR (IPCR) method. The primers used in PCRs are described in Table I. The genomic DNA was extracted from the suspension culture of *P. americana* with extraction buffer [0.3 M NaCl, 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% (w/v) SDS, 10 mM 2-mercaptoethanol, 5 M urea, 5% (v/v) phenol], then with phenol/chloroform. The genomic DNA (1 μg) was digested with 50 U of restriction enzymes (*Bam*HI, *Mlu*I, *Nco*I, *Dra*I and *Eco*RV for *PaDOD1*, *Eco*RI and *Bam*HI for *PaDOD2*) in each 500 μl of the total reaction volume. The digested DNA was circularized in the presence of T4 DNA ligase (Fermentas, Maryland, USA) in a 500 μl total reaction volume and used as the template for PCR after ethanol precipitation. The first PCRs for the promoter region of *PaDOD1* were performed for 33 cycles (94 °C for 30 s, 58–62 °C for 1 min, 72 °C for 8 min), the second PCRs were also carried out for 33 cycles (94 °C for 30 s, 60–64 °C for 1 min, 72 °C for 8 min). The promoter region of *PaDOD2* was amplified under the conditions used for amplification of the 5'-upstream region of *PaDOD1*.

The fragments of the IPCR product were directly sequenced and determined. Transcription factor binding sites were determined using the PLACE signal scan search program (http://www.dna.affrc.go.jp/PLACE/signalscan.html; Higo *et al.*, 1999).

The nucleotide sequence data reported in this article can be found in the GenBank data libraries under accession numbers: AB451869 (*PaDOD1*), AB451870 (*PaDOD2*), AB451871 (5'-upstream region of *PaDOD1*), and AB451872 (5'-upstream region of *PaDOD2*).

Results and Discussion

Isolation of PaDOD1 and PaDOD2

To elucidate the origin and regulation system of betalain biosynthesis, we attempted to clone cDNA of *DOD* from *P. americana*, and isolated two full-length cDNAs of *PaDOD* (*PaDOD1* and *PaDOD2*).

Using single-stranded cDNA prepared from the total RNA of *P. americana* suspension cultures as template, the full-length cDNAs of *PaDOD1* and *PaDOD2* were isolated by RACE. The fragment of *PaDOD* was amplified using degenerate primers designed for the reported *DOD*s from Caryophyllales (Christinet *et al.*, 2004), and the full-length cDNAs of *PaDOD1* and *PaDOD2* were determined by 3'- and 5'-RACE. The *PaDOD1* and *PaDOD2* cDNAs contained 798-bp and 924-bp open reading frames encoding proteins that corresponded to sizes of 29.8 kDa and 34.6 kDa, respectively. The deduced amino acid sequences of *PaDOD1* and *PaDOD2* showed approximately 80% homology with each other (Fig. 2), and *PaDOD1* and *PaDOD2* share 68% and 65% identities, respectively, with *PgDOD,* a 4,5-DOPA dioxygenase involved in betalain biosynthesis (Christinet *et al.*, 2004). These results indicate that *PaDOD1* and *PaDOD2* may be homologues of *PgDOD*.

Cloning and structural analysis of PaDOD1 and PaDOD2 promoters

To analyze the promoter regions of *PaDOD1* and *PaDOD2* genes, the 5'-upstream regions of these *PaDOD*s were isolated from *P. americana* genomic DNA using the IPCR method. The fragments amplified by IPCR were sequenced and the approximately 630-bp and 1000-bp upstream regions from the transcription start site of *PaDOD1* and *PaDOD2*, respectively, were determined. The promoter regions of *PaDOD1* and *PaDOD2* were analyzed using the PLACE database (http://www.dna.affrc.go.jp/PLACE/signalscan.html; Higo *et al.*, 1999).

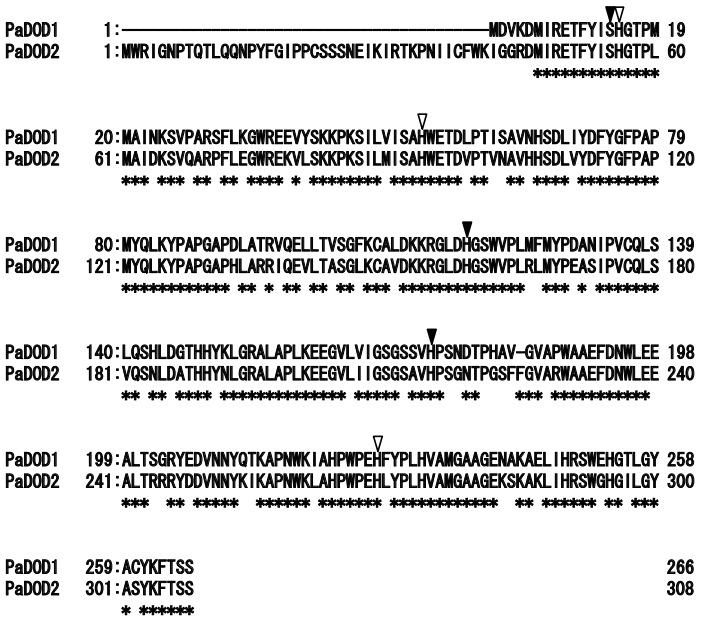

Fig. 2. The alignment of deduced amino acid sequences of *PaDOD1* and *PaDOD2*. Identical amino acid residues are marked with asterisks. The amino acid residues that are considered to contribute to the binding of aromatic substrates are marked with black arrows (Christinet *et al*., 2004). The putative binding sites for Fe^{2+} are marked with white arrows (Christinet *et al*., 2004).

Several putative TATA boxes were found in the *PaDOD1* and *PaDOD2* promoter regions (Fig. 3). Some putative MYB homologue binding sites (CCWACC, Grotewold *et al*., 1994; TAACTG, Urao *et al*., 1993; AACGG, Planchais *et al*., 2002; TAACAAA, Gubler *et al*., 1995; and WAACCA, Abe *et al*., 2003) were identified in *PaDOD1* and *PaDOD2* promoters (Fig. 3). Some putative recognition sites for the basic helix-loop-helix (bHLH) transcription factor, which activate abscisic acid (ABA) signaling, were also found in both *PaDOD1* and *PaDOD2* promoter regions (CANNTG, Abe *et al*., 2003). In addition, one putative binding site of the bZIP transcription factor involved in ABA response, ACACNNG (Kim *et al*., 1997), was detected in the *PaDOD1* promoter region, and five putative WRKY transcription factor recognition sites involved in pathogen defense (TGAC, Eulgem *et al*., 1999) were found in the *PaDOD2* promoter region. Moreover, some putative binding sites for environmental stress-responsive transcription factors (ACGTG, Simpson *et al*., 2003; NGATT, Sakai *et al*., 2000; CTCTT, Stougaard *et al*., 1990; GAAAAA, Park *et al*., 2004; TATTAG, Fusada *et al*., 2005; GTAC, Quinn *et al*., 2000; ACTTTA, Baumann *et al*., 1999; CATGCA, Ezcurra *et al*., 1999; and ACTCAT, Satoh *et al*., 2002) were also identified in *PaDOD1* and *PaDOD2* promoter regions (Fig. 3). The betalain biosynthesis is expressed in specific tissues and is affected by various environmental stresses such as light and hormones, as well as anthocyanin biosynthetic genes (Giudici De Nicola *et al*., 1973, 1974; Sakuta *et al*., 1991; Chalker-Scott, 1999; Irani and Grotewold, 2005; Grotewold, 2006). In addition, MYB, bHLH, and WD40 repeat transcription factors regulate the tissue-specific anthocyanin biosynthesis in various higher plants (*Vitis vinifera*, Kobayashi *et al*., 2002; Deluc *et al*., 2008; *Petunia hybrida*, Quattrocchio *et al*., 2006; and *Zea mays*, Hernandez *et al*., 2004). In *PaDOD1* and *PaDOD2* promoter regions, some putative MYB, bHLH, and environmental stress-responsive transcription factor binding sites were identified (Fig. 3). Therefore, it is possible that these transcription factors might regulate the *PaDOD*s and betacyanin biosynthesis in *P. americana*.

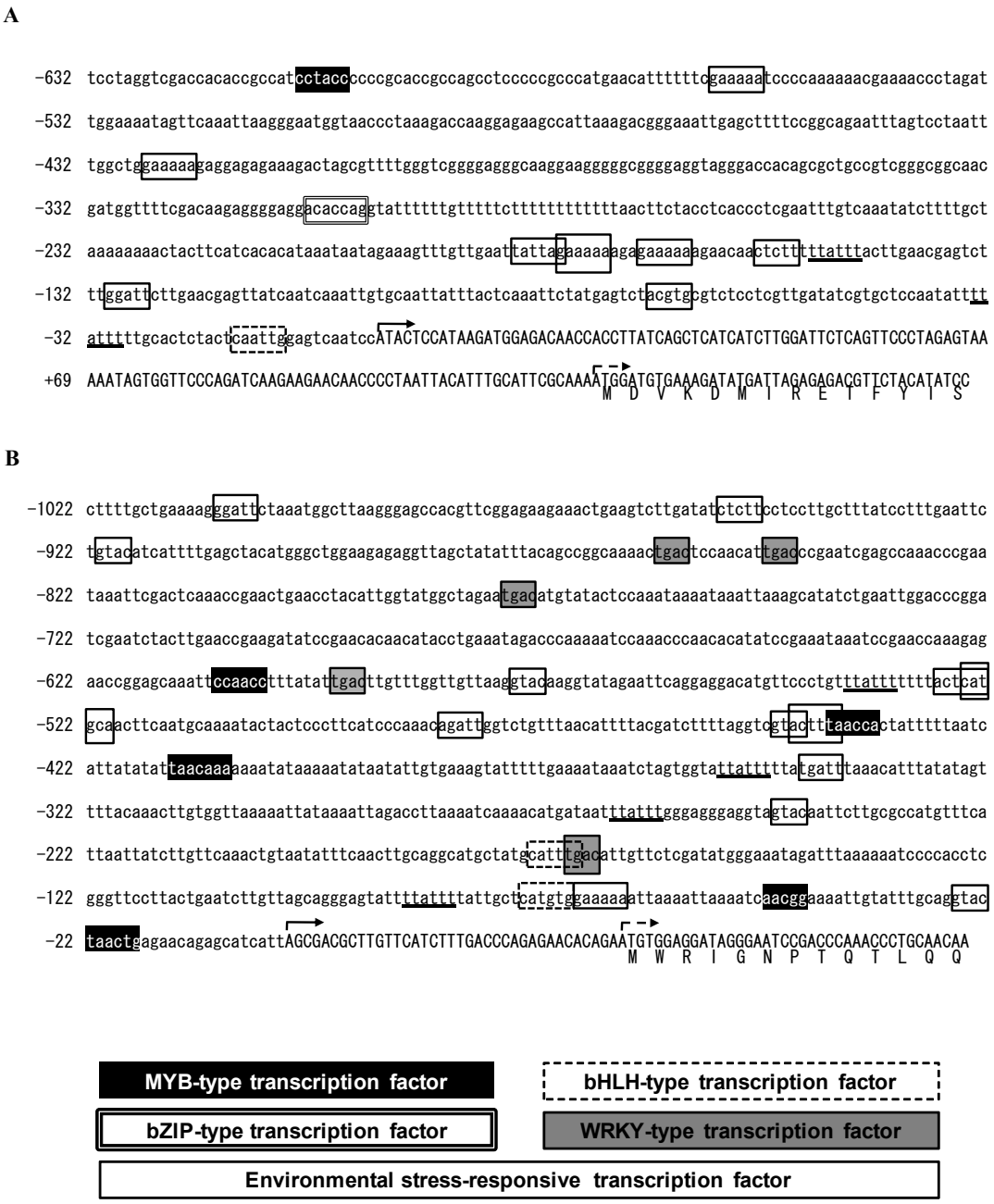

Fig. 3. Sequences of promoters of (A) *PaDOD1* and (B) *PaDOD2* genes. Upper case letters indicate the transcription region. Lower case letters indicate the 5'-upstream region. The putative transcription start sites are shown by solid arrows. The putative translation initiation sites are indicated by dashed arrows. The numbering starts from the transcription start site. The putative TATA boxes are underlined. The sequences in boxes indicate binding sites for transcription factors.

Expression of PaDOD1 and PaDOD2 in P. americana

To examine the correlation between the expression profiles of the two *PaDOD* genes and betacyanin accumulations in red and white cells of *P. americana* suspension cultures, the expression levels of *PaDOD1* and *PaDOD2* were monitored by semiquantitative RT-PCR (Fig. 4A). The accumulation of betacyanin decreased during the first 3 days of culture, and an increase in the betacyanin content was observed thereafter. The betacyanin accumulation reached a maximum 9 days after transfer, following the degradation of betacyanin in old cells (Fig. 4B). High expression of *PaDOD1* was observed in 5-, 7-, and 10-day-old red cells, along with high betacyanin accumulation (Figs. 4A, B). In contrast, high levels of transcripts of *PaDOD2* were detected in 1-, 3-, and 5-day-old cultures of both white and red cells (Fig. 4B). These results demonstrate that expression of *PaDOD1* and *PaDOD2* was detected in white cells as well as in betacyanin-producing red cells. The correlation between *PaDOD* expression and betalain accumulation in *P. americana* was not clear. These results suggest that not only the expression of *DOD*, but also the supplementation of DOPA, the substrate of DOD, might be a regulatory step for betalain biosynthesis in *P. americana*. Some DODs from non-betalain-producing plants also show DOD activity *in vitro* (Tanaka *et al.*, 2008), supporting the hypothesis that lack of betalain in these plants is due to the absence of DOPA. DOPA is assumed to be synthesized by hydroxylation of tyrosine in betalain-producing Caryophyllales (Steiner *et al.*, 1996, 1999; Yamamoto *et al.*, 2001). However, the biological function and regulatory mechanisms of the DOPA metabolism in higher plants are still poorly understood. To reveal the contribution of *DOD* to the betalain biosynthesis and DOPA metabolism in the Caryophyllales, further investigations of *DOD* genes are desired. The functional analyses of *PaDOD1* and *PaDOD2* in *P. americana* are in progress.

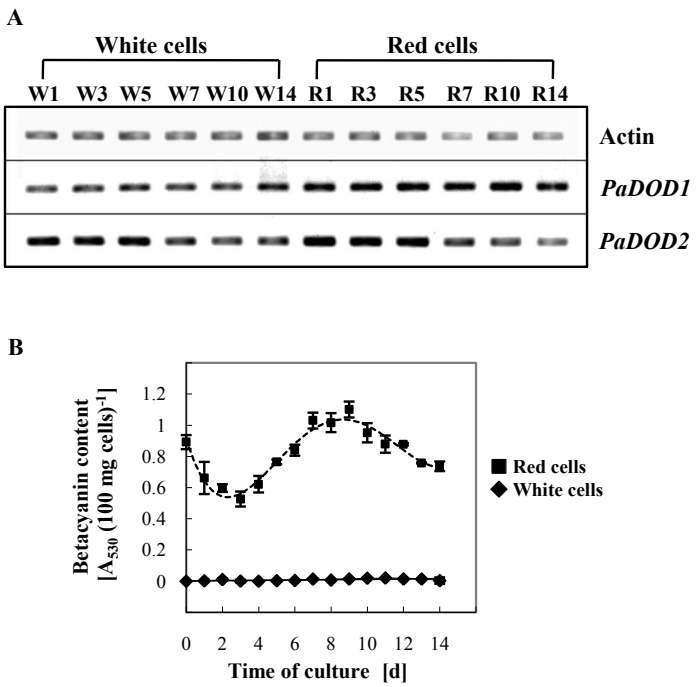

Fig. 4. Expression profiles of *PaDOD1* and *PaDOD2* in *P. americana* suspension cultures. (A) Expression level of *PaDOD1* and *PaDOD2* in suspension cultures. Total RNA extracted from white and red cells at days 1, 3, 5, 7, 10, 14 was used for the semiquantitative RT-PCR analysis with gene-specific primers, with actin as a loading control. (B) Betacyanin accumulation during culture (0–14 days) in *P. americana* suspension cultures. The betacyanin content was measured in white cells and red cells. The vertical lines indicate the SD (n = 3).

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