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ORIGINAL PAPER

# Molecular characterization and primary functional analysis of *PeVDE*, a violaxanthin de-epoxidase gene from bamboo (*Phyllostachys edulis*)

**Zhimin Gao · Qing Liu · Bo Zheng · Ying Chen**

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**Abstract**
***Key message*** ***PeVDE*** **was expressed primarily in bamboo leaves, which was up-regulated under high light. The protein encoded by** ***PeVDE*** **had enzyme activity of catalyzing violaxanthin (V) to zeaxanthin (Z) through antheraxanthin (A) as assay shown in vitro.**
*Abstract* Violaxanthin de-epoxidase (VDE), a key enzyme of xanthophyll cycle, catalyzes conversion from violaxanthin (V) to zeaxanthin (Z) through antheraxanthin (A) to protect photosynthesis apparatus. A cDNA, *PeVDE*, encoding a VDE was isolated from bamboo (*Phyllostachys edulis*) by RT-PCR and RACE methods. *PeVDE* is 1,723 bp and contains an ORF encoding 451 amino acids, with a transit peptide of 103 amino acids. The mature protein is deduced to have 348 amino acids with a calculated molecular weight of 39.6 kDa and a theoretic isoelectric point of 4.5. Semi-quantitative RT-PCR assay indicated that the highest expression level of *PeVDE* was in leaf, which agreed with the accumulation pattern of PeVDE protein. Real time PCR results showed that *PeVDE* was up-regulated and reached the highest level after the treatment (1,200 μmo1 $m^{-2}$ $s^{-1}$) for 2 h, then decreased and kept at the level similar to that of 0.5 h after treatment for 8 h. To investigate the function of PeVDE, mature protein was heterologously expressed in *Escherichia coli* and the enzymatic activity assay was carried out using V as substrate. The pigments that formed in the reaction mixture were extracted and analyzed by HPLC method. Besides V, A and Z were detected in the reaction mixture, which indicated that the recombinant protein exhibited enzymatic activity of catalyzing V into Z through A. This study indicates that PeVDE functions through regulating the components of xanthophyll cycle, which might be one of the critical factors that contribute to the growth of bamboo under naturally varying light conditions.

**Keywords** Bamboo · Violaxanthin de-epoxidase · Western blotting · Enzymatic assay in vitro · Real time PCR

**Abbreviations**

| | |
|---|---|
| cDNA | Complementary DNA |
| DTT | Dithiothreitol |
| EDTA | Ethylene diamine tetraacetic acid |
| HPLC | High performance liquid chromatography |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| kDa | Kilodaltons |
| MGDG | Monogalactosyldiacylglycerol |
| NPQ | Non-photochemical fluorescence quenching |
| ORF | Open reading frame |
| PBS | Phosphate-buffered saline |
| PSII | Photosystem II |
| RACE | Rapid amplification of cDNA ends |
| RT-PCR | Reverse transcription-polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| VDE | Violaxanthin de-epoxidase |
| ZE | Zeaxanthin epoxidase |

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Z. Gao (✉) · Q. Liu · B. Zheng · Y. Chen
Key Laboratory on the Science and Technology of Bamboo and Rattan, International Center for Bamboo and Rattan, Beijing 100102, People's Republic of China
e-mail: gaozhimin@icbr.ac.cn

## Introduction

It is known for plants to survive under naturally varying light conditions. Plants utilize the process of photosynthesis to convert light energy to chemistry energy. However, photosynthetic apparatus can be damaged when plants absorb excessive light (Niyogi 1999). Therefore, plants need to modulate light-harvesting efficiency to avoid high light radiation damages. In the long-term evolution process, higher plants have developed multiple effective mechanisms to minimize the risk of photodamage. An important protective mechanism that plants use to deal with excessive radiant energy to dissipate energy non-radiatively is known as xanthophyll cycle, i.e. in the conversion of the di-epoxide xanthophyll violaxanthin into the epoxide-free zeaxanthin, catalyzed by the enzyme violaxanthin de-epoxidase (VDE) (Jahns et al. 2009). Under high light, the energy input exceeds its photosynthetic capacity which leads to over acidification of the thylakoid lumen, and the drop of pH in lumen activates VDE which shifts the xanthophyll balance from violaxanthin (V) toward zeaxanthin (Z) through antheraxanthin (A) (Hager 1969; Rockholm and Yamamoto 1996). Experimental evidence supports the view that Z and A can transfer excess energy from chlorophyll and releases as heat by thermal dissipation and efficiently scavenges reactive oxygen species, thus protecting the photosynthetic apparatus from photodamage (Eskling et al. 1997; Müller et al. 2001). Z enhances the photoprotection capacity and it has been shown to increase the quenching of excited chlorophyll states as well as the scavenging of reactive oxygen species eventually formed (Li et al. 2009; Rockholm and Yamamoto 1996). Whereas, Z is converted back to V through A by zeaxanthin epoxidase (ZE) in the stromal side of the thylakoid membrane under low light (Arnoux et al. 2009), which can increase light-harvesting efficiency by avoiding unnecessary quenching of excitation energy. The xanthophyll cycle is a mechanism found in many different photosynthetic eukaryotes (Coesel et al. 2008), in which the VDE characteristics and activation are well studied.

VDE belongs to a multigenic protein family called lipocalins, whose members are characterized by a conserved structural organization with an eight-strand β-barrel and often bind hydrophobic molecules (Hieber et al. 2002). The VDE lipocalin domain (VDEcd) structure has been resolved from crystals grown at acidic and neutral pH (Arnoux et al. 2009). The activation of VDE is associated with a pH-dependent conformational change (Kawano and Kuwabara 2000) and the protein association to the thylakoids membrane, where its substrate violaxanthin is found (Hager and Holocher 1994; Morosinotto et al. 2002).

Bamboo is a monocotyledonous plant belonging to the *Bambusoideae* subfamily of *Poaceae* family. As one of the most important non-timber forest products among the world's plant and forest resources, bamboo is widely distributed in the tropical and subtropical areas (Jiang 2002). Bamboos demonstrate a fundamental association between physiology and characteristic of the extraordinary rate of shoot elongation, some of them can produce upright culms more than 30 m tall in one growing season. Photosynthesis is one of the necessary factors which supply carbon-hydrates for the rapid expansion of cells. The main heat source for bamboo growth is from solar energy (sun light), which is essential for photosynthesis but potentially results in over-excitation and photo-oxidative damage to the photosynthetic reaction center if the intensity exceeds its photosynthetic capacity. Bamboo is fast growing even under fluctuating light conditions, the function of light harvesting might be important for the fast-growing character of bamboo. However, the study on spectroscopic features, capacity of forming homotrimers and structural stabilities of different bamboo isoforms (Lhcb 1–3) showed that they possess similar characteristics as those in other higher plants in spite of small differences (Jiang et al. 2012). It means that bamboo may possess unique light regulation mechanism though it is unknown. As a key enzyme in xanthophylls cycle, VDE might play an essential role in bamboo photoprotection response to light stress. However, there is no information about VDE in bamboo.

In this paper, the full-length cDNA of *PeVDE* gene was isolated using RT-PCR and RACE methods from bamboo (*Phyllostachys edulis*). To understand the function of *PeVDE*, we analyzed the expression of *PeVDE* in bamboo and characterized the biochemical identity of the protein encoded by the gene in vitro. Our results show that the transcript pattern of *PeVDE* in different tissues was similar to that of protein accumulation and it was up-regulated under high light. The enzymatic activity assay confirmed that PeVDE functioned in catalyzing V into Z through A.

## Materials and methods

### Plant materials and strains

The seeds of bamboo (*Phyllostachys edulis*), collected from Guilin in Guangxi Province of China in 2009, were sterilized and germinated on moistened tissues and then transplanted into vermiculite. Plants were grown under long-day conditions (16 h light/8 h dark) at 25 °C with the light intensity of 200 μmol $m^{-2}$ $s^{-1}$. 1-year-old seedlings were selected until the fifth leaf of the plant's aerial parts was emerged; the second leaf, sheath, stem and root were collected, respectively, and stored at −80 °C for further experiment.

*Escherichia coli* strain, DH5α was used as the recipients for routine cloning experiments, and Rosetta-gamin B (*DE3*) was used for gene expression in vitro.

### Isolation of full-length cDNA and the genomic DNA containing encoding region

Total RNA was isolated from leaves of *P. edulis* with Trizol according to the protocol recommended by the manufacturer (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from 500 ng of RNA using the Promega cDNA synthesis system. The 3′ cDNA and 5′ cDNA were synthesized using the SMART™ RACE kit (Clontech, Mountain View, CA, USA). Degenerate primers for the conserved peptide motifs (PeVDE-F: 5′-CC(A/T)GA(T/C)GA(A/G)AC(T/C/G)GA(A/G)TG(C/T)CA-3′; PeVDE-R: 5′-C(C/A)(A/G)CC(A/T)CCATA(T/G)CCATCCCA-3′) were designed based on the sequences of the conservative domain of VDE from monocots. Gradient PCR amplification was performed with PeVDE-F and PeVDE-R to optimize the annealing temperature.

Specific primers were designed according to the sequence obtained using the procedure described above. The primers used for 5′ rapid amplification of cDNA ends (RACE) were PeVDE5-1 (5′-GAGTATTGCGGGTTGTGAGGGGTCCTGC-3′) and PeVDE5-2 (5′-AGGGACTGGGAACTCGCCGACATCAGAC-3′); those used for 3′ RACE were PeVDE3-1 (5′-TCTGATGTCGGCGAGTTCCCAGTCCCTG-3′) and PeVDE3-2 (5′-TGACATGGAGAATCCGCACCCCCGACAG-3′). Touch down PCR was performed with PeVDE5-1, PeVDE3-1 and a universal primer mix (UPM) as supplied with the SMART™ RACE cDNA amplification kit, and then the PCR amplicons were used as template for a subsequent nested PCR using primer pairs of PeVDE3-2 or PeVDE5-2 with the NUP primer supplied in kit. The PCR fragments were cloned into pGEM-T easy vectors (Promega, Madison, WI, USA) using standard protocol and sequenced using an ABI 3730 sequencer (Applied Biosystems, Bedford, MA, USA). The full-length cDNA was obtained by the combination of conserved sequence with the 5′ and 3′ end sequences. On the basis of this assembled sequence, the sequence of the open reading frame (ORF) was obtained from leaves' cDNA using the Pyrobest DNA polymerase (Takara Biotechnology Co., Ltd, Dalian, China) with primers of $PeVDE_0F$ (5′-ATGATGTCGCGGCAGTGCG-3′) and $PeVDE_0R$ (5′-CTACCTTAGCTTCCTTATTGGCAGGGA-3′).

Genomic DNA was extracted from leaves of *P. edulis* by the CTAB method (Murray and Thompson 1980). PCR amplification was carried out with genomic DNA using primers of $PeVDE_0F$ and $PeVDE_0R$. Nucleotide sequences of the PCR products and cloned cDNAs were determined by sequencing.

### Sequence analysis

Sequence analysis was carried out with DNASTAR software package. The full-length cDNA sequence was subjected to a similarity search against the NCBI database (http://www.ncbi.nlm.nih.gov) using the BLASTX algorithm with default parameters. A neighbor joining (NJ) tree was constructed using the MEGA4.0 software package and the CLUSTAL algorithm in conjunction with the amino acid sequences of known VDEs (Tamura et al. 2007). Known motifs within the sequence were identified by comparison with a database of such motifs using the web-based MOTIF SCAN tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan) (Sigrist et al. 2010). By comparing the mRNA sequence and genomic DNA sequence of *PeVDE*, gene structure was determined. The *cis*-regulatory element was also analyzed for introns using publicly available database: database of Plant Cis-acting Regulatory DNA Elements (PLACE, http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al. 1999; Prestridge 1991).

### Expression of the recombinant protein

The fragment encoding mature protein of PeVDE was re-amplified by PCR to introduce *Nco*I (forward) and *Xho*I (reverse) sites. The fragment with *Nco*I and *Xho*I sites was cloned into pET-32b vector. The primers used to generate fragment with *Nco*I and *Xho*I sites were PeVDE-F1 (5′-CATGCCATGGCCACCGATGCTCTCAAGAC-3′) and PeVDE-R1 (5′-CCGCTCGAGCCTTAGCTTCCTTATTGGC-3′) based on the full-length cDNA of *PeVDE*. The *Nco*I and *Xho*I recognition sequences in primers were underlined.

After sequencing of the fragment on both strands, the recombinant plasmid carrying pET-32b-*PeVDE* was transformed into competent *E. coli* strain Rosetta-gamin B (*DE3*) cells for protein expression. *DE3* cells harboring pET-32b-*PeVDE* or an empty vector (pET-32b) were cultured at 37 °C, in lysogeny broth (LB) liquid medium containing 100 μg $ml^{-1}$ of ampicillin until an $OD_{600}$ of approximately 0.6 was attained. The medium was then supplemented with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were cultured at 20, 30 and 37 °C, respectively, for an additional 4 h to select the optimum temperature for recombinant protein.

The recombinant proteins were purified with His Bind Purification Kit as described by the manufacturer (Cat. No. 70239-3 Novagen, Darmstadt, Germany). The recombinant

protein was analyzed using SDS-PAGE (5 % stacking gel and 12 % separating gel) according to the Molecular Clone procedure (Sambrook et al. 1989).

### Qualitative enzyme activity assay of the recombinant protein

After 4 h induction with IPTG, 50 mL bacterial culture was collected and centrifuged. The bacterial pellet was resuspended in 2 mL of 10 mM Tris–HCl (pH 7.4) and 1 mM EDTA, and lysed using an ultrasonic cell disrupter on ice bath. The extract was centrifuged at 12,000*g* for 10 min and the protein concentration in the supernatant was determined by a dye-binding method (Bradford 1976) using bovine serum albumin as the protein standard. The reaction of in vitro enzyme activity assay was performed at room temperature (25 °C) in total volume of 3.0 ml containing 100 μL protein, 100 μl violaxanthin (0.1 mM) (Sigma-Aldrich Co.), 100 μl monogalactosyldiacylglycerol (MGDG) in methanol (0.27 mM), 1.5 ml sodium citrate buffer (0.2 M, pH 5.1), 1.17 ml Mill-Q water and 30 μl sodium ascorbate (3.0 M) to start the reaction (Eskling and Åkerlund 1998). The reaction was stopped with addition of solid Tris for 15 min. The pigments in the mixture of reaction were extracted for three times with diethylether and solubilized in methanol. Then the extracts were filtered through a 0.45-μm membrane filter and analyzed by HPLC. Hypersil GOLD column (250 × 4.6 mm, 5 μm) produced by Thermo Scientific was used in this separation. The pigments were eluted using 100 % Solvent A (acetonitrile:water = 88:12) for the first 25 min followed by a 5 min linear gradient to 40 % solvent A and 60 % solvent B (ethyl acetate) which continued isocratically until the end of the 20 min separation. The column was allowed to elute with a 2 min linear gradient to 100 % Solvent A which continued to re-equilibrate for 8 min prior to the next injection. The column temperature was 25 °C and the flow rate was 1 ml $min^{-1}$. The pigments were detected by their absorbance at 445 nm. As a control, an equal volume of enzyme was replaced by the protein expressed in *E. coli* harboring pET-32b. The concentration of violaxanthin in the extract was quantified by the peak area of HPLC with the calibration curve which was constructed by plotting peak area versus concentrations of violaxanthin (Sigma-Aldrich Co.), and the activity of the recombinant PeVDE was calculated through the concentration change of violaxanthin.

### Antibody preparation and Western blotting analysis

The polyclonal antibody was prepared by immunizing rabbit with purified PeVDE protein, the serum was collected from the blood of rabbit's ears for Western blotting analysis which was carried out using the polyclonal antibody of GAPDH as loading control (Gao et al. 2012). The crude proteins from different tissues of *P. edulis* seedlings were separately extracted using a modified protocol (Berüter and Feusi 1997) and analyzed by SDS-PAGE. 15 μg of the extracted protein was loaded on each lane and electro-transferred to a nitrocellulose membrane in the transfer medium. Western blotting was carried out using the ECL kit (Thermo Scientific, Waltham, MA, USA). The signals of Western blotting were exposed with X-ray film and scanned; the integrated density value (IDV) of each band was captured using an Alpha-Image 2200 analysis system (Alpha Innotech Corporation) (Gao et al. 2012). The relative accumulated level of PeVDE was quantified using the IDV ratio of PeVDE/GAPDH.

### Semi-quantitative RT-PCR

For analysis of gene expression in different tissues, the cDNAs isolated from leaves, sheaths, stems and roots of the seedlings were used for semi-quantitative RT-PCR with F1 (5′-GCCACCGATGCTCTCAAGAC-3′) and R1 (5′-TCTCTGCAAGAGGAGGCTCAG-3′) primers designed based on *PeVDE* sequence. The final volume was 20 μL including 10 μL of 2 × GC Buffer I ($Mg^{2+}$ Plus), 3.2 μL of dNTPs (2.5 mM each of dATP, dTTP, dCTP and dGTP), 2 μL of F1 (5 μM), 2 μL of R1 (5 μM), 1 μL cDNA template, 1.6 μL Milli Q- water and 0.2 μL LA Taq DNA polymerase ($5U \cdot \mu l^{-1}$) (Takara Biotechnology Co., Ltd).

The PCR program involved in an initial denaturation period at 94 °C for 4 min, followed by 25 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min; then with a final extension period at 72 °C for 10 min. *Pe-actin* was used as a positive control in the same program (Gao et al. 2012).

### Real time PCR analysis

Primers were designed from non-conserved region of the isolated *PeVDE* using ABI Primer express 3.0 (F2: 5′-TGTCAAGGTTACCCGTTCCGAG-3′; R2: 5′-ACA-GCACAGCACCAC CATATCC-3′). The cDNA templates were synthesized using the total RNA isolated from leaves of bamboo seedlings treated with high light (1,200 μmo1 $m^{-2}$ $s^{-1}$) for 0, 0.5, 1, 2, 3, 4, 6, 8 and 10 h, respectively. PCR amplification and analysis were carried out using Light Cycler® 480 real time PCR System with LightCycler® 480 SYBR Green I Master kit (Roche Applied Science). The final volume was 20 μl containing 2 × SYBR Premix Ex Taq 10 μl, 0.4 μl of each primer (5 μM), 2 μl of cDNA and 7.2 μl of nuclease-free water. Amplification was performed according to the standard LightCycler 480 System procedure. The relative value of

the gene expression was done with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using the bamboo *Actin* gene (FJ601918) as a reference gene.

## Results

### Sequence isolation

Homologous genes of *VDE* showed high similarities among plant species which facilitate the design of primers for RT-PCR to clone *PeVDE*. As a result of the RT-PCR with PeVDE-F and PeVDE-R, a 500-bp long fragment was obtained with leaf cDNA as template. Subsequently, a 700-bp long fragment from the 5′-end region and a 1,000 bp long fragment from 3′-end region were obtained by 5′- and 3′-RACE with 5′ and 3′ cDNA templates from leaf, respectively. After analysis of the obtained sequences, a 1,690 bp full-length cDNA was identified, which contains a 1,356 bp ORF, a 74 bp 5′ untranslated region (UTR) and a 260 bp 3′ UTR (Fig. 1). Finally, the ORF was determined by end-to-end PCR. The ORF encoded a putative peptide of 451 amino acids, the theoretic isoelectric point (p*I*) and calculated molecular mass of PeVDE were 5.2 and 51.1 kDa, respectively. The GenBank accession number of *PeVDE* is JQ347804. There was another *PeVDE* homolog gene in bamboo by searching the whole genomic sequence (Peng et al. 2013), which indicated that there had two VDE isoforms in bamboo.

### Characteristics of PeVDE

Hydropathy plot of the deduced amino acid sequence of PeVDE was calculated as described (Kyte and Doolittle 1982) with a window of nine amino acids (S1). The deduced amino acid sequence of PeVDE consists of a mature protein (position 104–451) and a transit peptide of 103 amino acids. The transit peptide of PeVDE shares the bipartite feature with other lumen-located proteins according to the hydrophilicity plot. It contains a hydrophilic N-terminal stroma-targeting sequence followed by a short hydrophobic thylakoid-targeting and translocation sequence; the cleavage site also fits the consensus pattern of Ala-X-Ala ↓ of the thylakoid-localized proteins (Robinson et al. 1998). The mature protein of PeVDE is hydrophilic overall similar to other lumen-located proteins with 56.03 % of the total amino acid residues having polar side chains, which indicate it would be a water-soluble protein. The mature protein is deduced to have 348 amino acids with a calculated molecular weight of 39.6 kDa and a theoretic isoelectric point of 4.5.

Motif Scan analysis (Sigrist et al. 2010) showed that three interesting domains were identified in the mature protein of PeVDE including a cysteine-rich domain (position 7–50), a glutamic acid-rich domain (position 251–336) and a violaxanthin de-epoxidase domain (position 61–258). There are also one N-myristoylation site (position 108–113), one N-glycosylation site (position 133–136), four protein kinase C phosphorylation sites (position 68–70, 135–137, 151–153, 182–184) and five casein kinase II phosphorylation sites (position 75–78, 183–186, 261–264, 317–320, 332–335) (Fig. 1).

It was the mature protein of PeVDE which performs function in xanthophyll cycle, but its encoding sequence was separated by four introns (S2). The introns were subjected for *cis*-acting regulatory element analysis by searching the PLACE database. The result indicated that there were many light regulated motifs such as GT1CONSENSUS (GRWAAW), INRNTPSADB (YTCANTYY), IBOXCORE (GATAA) and GATABOX (GATA). The functions of these motifs under the varying light environment need to be further confirmed.

### Alignment analysis of PeVDE

PeVDE showed high identities with those of gramineous VDEs such as in *Triticum aestivum* (AAK38177), *Oryza sativa* (AAF97601) and *Zea mays* (NP_001147756), especially for the part of mature proteins. The most closely related sequence to PeVDE was that from *O. sativa*, with identity of 91.7 % (319/348 amino acids). However, the transit peptides of *P. edulis*, *T. aestivum*, *Z. mays* and *O. sativa* were 103, 106, 102 and 98 amino acids, respectively, which had more divergence in this region (Fig. 2).

Phylogenetic analysis showed that VDEs from monocotyledon such as *P. edulis*, *T. aestivum*, *O. sativa*, *Z. mays* and *Zingiber officinale* were clustered in the same group which indicated closed relationship among them, while those from *Vitis vinifera*, *Citrus sinensis*, *Spinacia oleracea*, *Coffea canephora*, *Arabidopsis thaliana*, *Glycine max*, *Chrysanthemum* × *morifolium*, *Medicago truncatula*, *Camellia sinensis*, *Cucumis sativus*, *Lactuca sativa*, *Solanum lycopersicum* and *Nicotiana tabacum* were clustered in another group belong to dicotyledon, which agrees with the morphological classification (S3).

### Purification and functional analysis of recombinant PeVDE

To confirm the isolated cDNA encoded a catalytically active VDE, the fragment encoding mature protein of PeVDE was subcloned into pET-32b vector, and functional analysis was carried out using the recombinant protein heterogeneously produced in *E. coli*. The optimal condition for protein expression was induced by

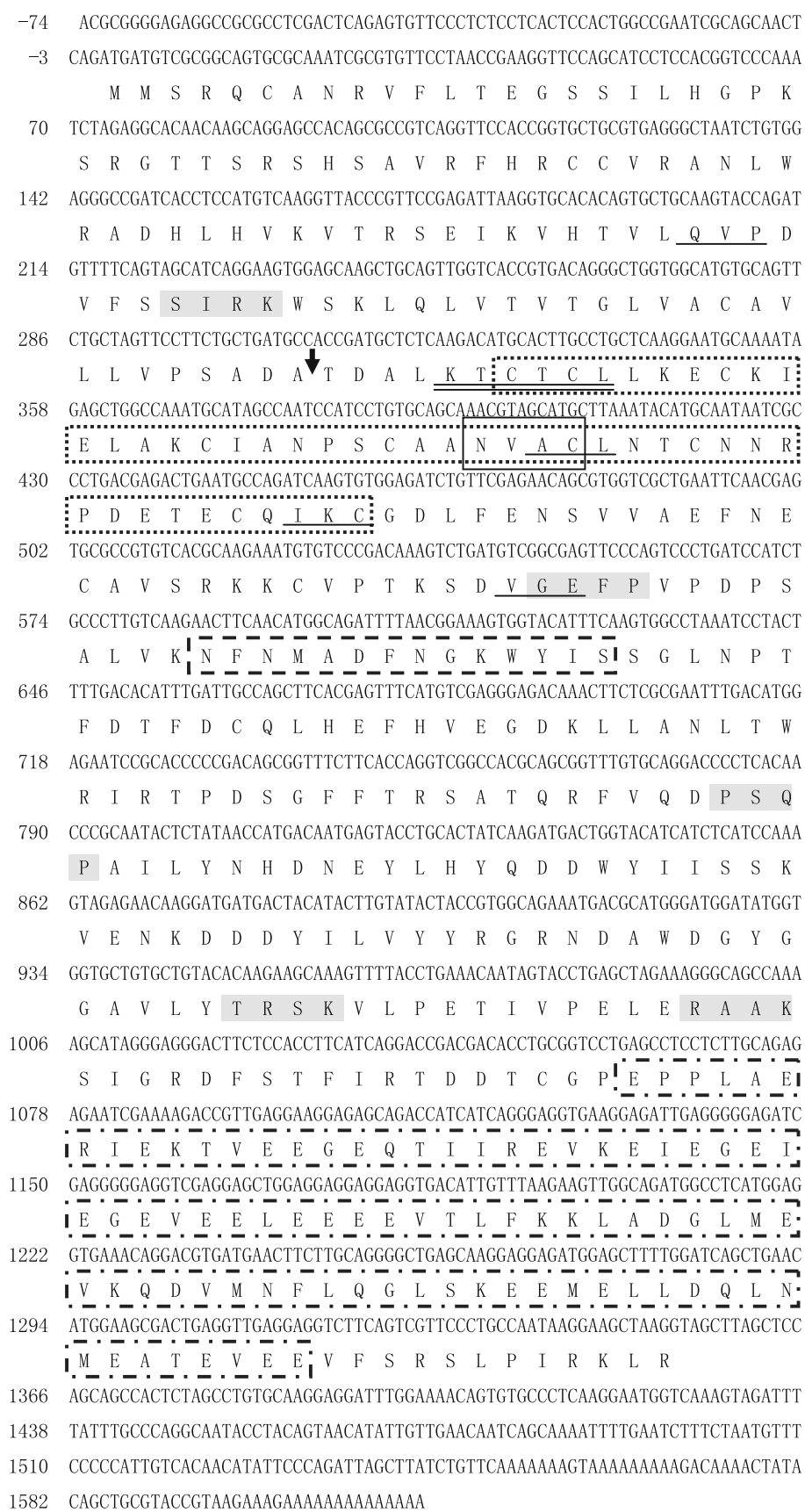


**Fig. 1** Nucleotide and deduced amino acid sequence for *PeVDE*. The *arrow* indicates the putative site of cleavage for the transit peptide. The *dot-boxed amino acids* are cysteine-rich domain; the *dash-boxed* amino acids are lipocalin signature; the *dot-dash-boxed amino acids* are glutamic acid-rich domain. *The amino acids underlined by double lines* is N-myristoylation site; *the boxed amino acids* is N-glycosylation site; *the amino acids underlined by a single line* are protein kinase C phosphorylation sites; the *shaded* indicates casein kinase II phosphorylation sites


**Fig. 2** Amino acid sequence comparison of violaxanthin de-epoxidase (VDE) proteins. The multiple alignments were performed with the DNAStar software. Identical amino acid residues are *shaded in black*. *Bars* indicate the region where gaps were inserted to maximize alignment. The cleavage site is marked with *arrow* and the proteins (with accession numbers) are as follows: *Phyllostachys edulis* (protein encoded by JQ347804), *Triticum aestivum* (AAK38177), *Oryza sativa* (AAF97601) and *Zea mays* (NP_001147756)

0.4 mM IPTG at 30 °C for 4 h. The recombinant $(His)_6$-tagged PeVDE protein was purified by a Ni-IDA column and eluted using imidazole buffer. The recombinant PeVDE protein was specifically eluted with 250 mM imidazole buffer and the protein molecular mass was about 65 kDa (Fig. 3), which agreed with the predicted mass of PeVDE (39.6 kDa) combined with tags of pET32b (25.4 kDa).

Compared with the chromatograms of the standard compound of V, A, and Z (S4), HPLC analysis showed that A and Z appeared consistent with sequential de-epoxidation and concomitant with the decrease in V (Fig. 4a), while there was only V in the control (Fig. 4b), which indicated that the recombinant protein could catalyze the two-step mono de-epoxidation reaction. The activity of recombinant PeVDE was 15.0 ± 2.2 nmol violaxanthin de-epoxidized $min^{-1}$ mg $protein^{-1}$, which was much lower than that of romaine lettuce (64.9 ± 5.4 nmol violaxanthin de-epoxidized $min^{-1}$ mg $protein^{-1}$) (Bugos and Yamamoto 1996).

## Expression patterns of *PeVDE*

Transcription and translation of *PeVDE* in different tissues of *P. edulis* seedling were detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively. The results demonstrated that the *PeVDE* expressed in all of the green tissues, with the highest expression in leaf, followed by sheath and the lowest in stem (Fig. 5).

The crude proteins from leaf, sheath, stem and root were further analyzed by Western blotting using anti-PeVDE antibody. The corresponding protein was detected in leaf, sheath and stem (Fig. 6), in which accumulation pattern was similar to its transcription. The highest protein accumulated level was in leaf, followed by sheath and stem, and no detectable in root. Chloroplasts were isolated from bamboo leaves (Asada et al. 1990; Endo et al. 1998), and Western blotting was performed with antibody of PeVDE. It was confirmed that the subcellular localization of PeVDE was in chloroplast (S5).

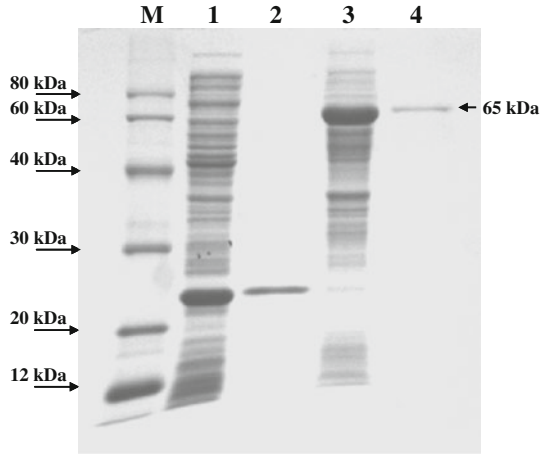


**Fig. 3** Recombinant protein of PeVDE detected by SDS-PAGE. 15 μg total proteins were loaded on each lane. *M* protein marker, *lane 1* The total protein of induced *E. coli* obtaining pET-32b, *lane 2* the purified protein of pET-32b, *lane 3* the total protein of induced *E. coli* obtaining pET-32b-PeVDE, *lane 4* the purified recombinant protein of PeVDE. The recombinant protein was around 65 kDa marked by *arrow*

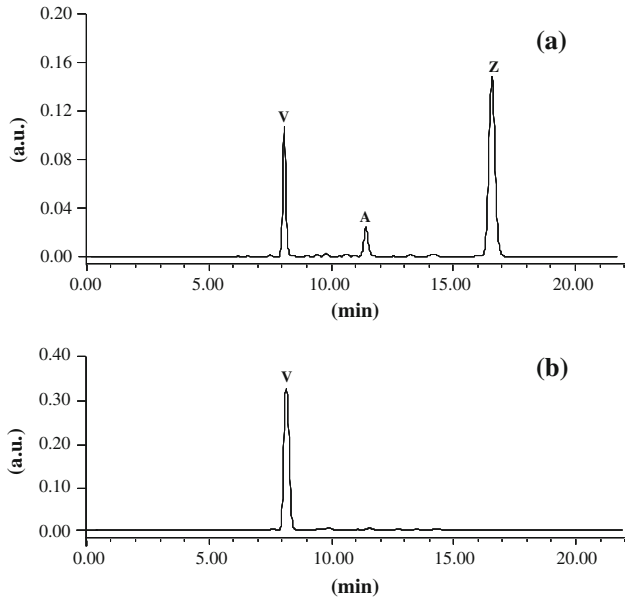


**Fig. 4** HPLC analysis of the products from the enzymatic reaction in vitro. **a** Reaction for 15 min, **b** control

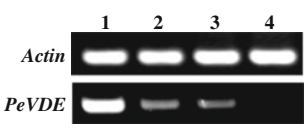


**Fig. 5** *PeVDE* transcriptional level was analyzed by RT-PCR in different tissues with actin as control. *Lane 1* leaf, *lane 2* sheath, *lane 3* stem, *lane 4* root. The amount of transcription of *PeVDE* and *Actin* was checked after 25 cycles by PCR

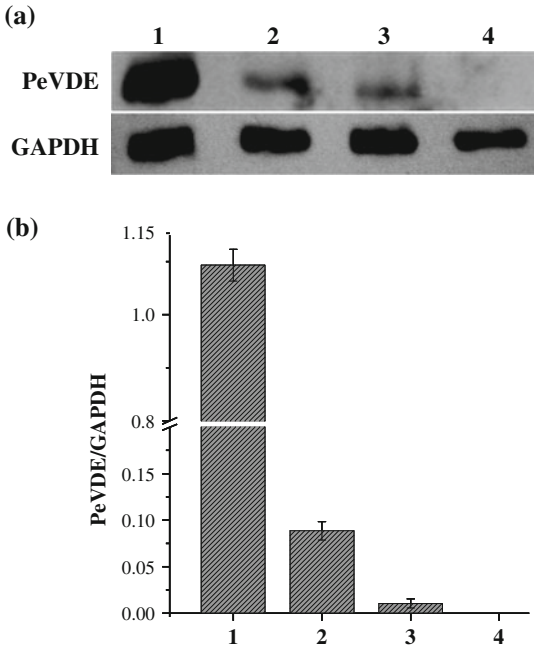


**Fig. 6** Western blotting analysis of PeVDE accumulation in different tissues (**a**) and the relative accumulated levels of PeVDE were normalized to GAPDH (**b**). *Lane 1* leaf, *lane 2* sheath, *lane 3* stem, *lane 4* root. 15 μg total proteins were loaded on each lane. Western blotting was repeated for three times, the average value was used as the experimental result

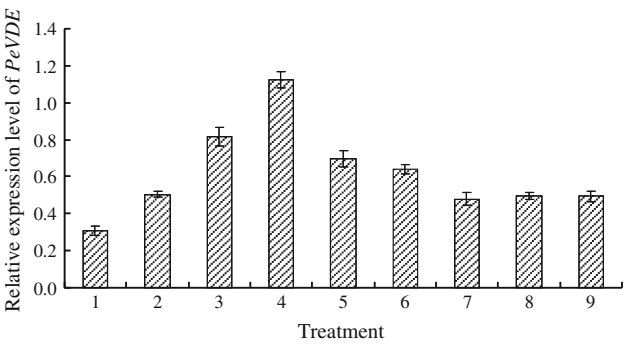


**Fig. 7** Relative expression level of *PeVDE* in leaves after treatment with high light (1,200 μmo1 $m^{-2}$ $s^{-1}$) at different time intervals. The biological replicate was made three times with high-light treatment, and three technical replicates were taken from each treatment. The real time PCR was repeated for three times, the average value was used as the experimental result, the *vertical bar* is indicated by STD. 1, 0 h; 2, 0.5 h; 3, 1 h; 4, 2 h; 5, 3 h; 6, 4 h; 7, 6 h; 8, 8 h; 9, 10 h

Real time PCR was used to detect the expression level of *PeVDE* in leaves of *P. edulis* seedlings after treatment with high light (1,200 μmo1 $m^{-2}$ $s^{-1}$). The results showed that *PeVDE* was up-regulated rapidly and reached to the highest level after treatment for 2 h, which indicated that the transcript of *PeVDE* was dramatically induced by high light. Though the expression level decreased in the following hours, there were highly significant differences ($P < 0.01$) when compared with that of 0 h, and it kept at

higher level similar to that of 0.5 h after treatment for 8 h (Fig. 7).

## Discussion

Higher plants have developed the ability to avoid or dissipate excess light energy to protect photosystem II (PSII) from photoinhibitory damage (Bugos and Yamamoto 1996). There are more than 1,000 bamboo species in the world (Jiang 2002), which have abundant biodiversity both in geographical distribution and ecotypes. Light stress is one of the adverse environmental factors that bamboos have to adapt to during lifetime. The de-epoxidation dependent energy dissipation through xanthophyll cycle is one of the key mechanisms for plants to acclimatize high-light environments over a wide dynamic range from sunflecks to long-term growth conditions (Demmig-Adams and Adams 1996). VDE is a crucial enzyme for the photoprotective xanthophyll cycle as it is responsible for the synthesis of zeaxanthin, a carotenoid with a seminal role in the strong light response (Li et al. 2009; Rockholm and Yamamoto 1996).

To understand the xanthophyll cycle in bamboo, the characterization and function of *PeVDE* was analyzed in this study. The full-length cDNA was 1,723 bp long, which was interrupted in the genomic sequence by four introns containing many *cis*-acting regulatory elements. Besides the light regulated motifs, the other most prominent motifs in introns were *cis*-acting elements for plant response to environmental stresses, including CBFHV (RYCGAC), CCAA TBOX1 (CCAAT), GT1GMSCAM4 (GAAAAA), MYB1 AT (WAACCA), MYB2AT (TAACTG), MYB2CONSENSUSAT (YAACKG), MYBCORE (CNGTTR), MYCCON SENSUSAT (CANNTG), PYRIMIDINEBOXOSRAMY 1A (CCTTTT), RAV1AAT (CAACA), WBOXATNPR1 (TTGAC) and WRKY71OS (TGAC), which indicated that the expression of *PeVDE* might be also regulated by transcription factors related to stress-tolerance.

Alignment of the PeVDE sequence with those from wheat, maize and rice showed high conservation in mature protein regions and more divergence in transit peptide regions. The transit peptide of PeVDE was bipartite and similar to other lumen-1ocated extrinsic proteins such as oxygen evo1ution complex protein (Clausmeyer et al. 1993) and plastocyanin (Vorst et al. 1988). The primary structure of the mature PeVDE protein consisted of three domains: cysteine-rich domain, lipocalin signature and highly charged domain featured by high composition of glutamic acid residues, which met the characteristics with members of lipocalin family (Bugos et al. 1998). The recombinant protein exhibited enzymatic activity of catalyzing V into Z through A, which confirmed it was specific for xanthophylls (Yamamoto and Higashi 1978). These results indicated that the isolated cDNA actually encoded a functional VDE with activity. The higher activity of VDE would result in the accumulation of Z in xanthophyll cycle on which thermal dissipation was depended in plant under high light. However, compared with orthologous VDEs in other species (Bugos and Yamamoto 1996; Lin et al. 2002), the activity of recombinant PeVDE was relatively lower. One reason for this might be explained by the amino acids difference of VDEs. Though PeVDE had high identities with VDEs from other plants, the difference in sequence would affect its structure which associated with enzyme activity. The VDE lipocalin domain structures illustrated the pH-dependent conformational changes associated with the protein activation, and the active VDE is a dimmer (Arnoux et al. 2009). Two residues (Asp-177 and Tyr-198) were suggested to participate in VDE catalytic mechanism besides the binding sites for violaxanthin and ascorbate (Saga et al. 2010). It was also confirmed that variants in four residues (D98, D117, H168 and D206) caused a reduction in enzymatic activity indicating a role in the activation of VDE while D86 mutants did not show any alteration (Fufezan et al. 2012). How about the enzymatic activity of PeVDE associated with the residues needs to be further studied by the combined approaches of characterizing its function in silico and site directed mutagenesis in vitro. The condition of enzyme reaction in vitro might be another reason for the lower activity. The activity tests in vitro were performed in excess of substrates which might mask alternations in substrate binding affinity (Saga et al. 2010). VDE also required a certain concentration of lipid and ascorbate for its activity besides the optimum pH. Therefore, the conditions need to be optimized for the measurement of PeVDE activity.

The analysis of *PeVDE* transcription in different tissues was in accordance with that of protein accumulation. *PeVDE* was expressed in green tissues with the highest level in leaves, but no signal was detected in root, which was similar to that of *AtVDE* in *Arabidopsis thaliana* (North et al. 2005). The subcellular localization of PeVDE was in chloroplast confirmed by Western blotting, which agreed that VDE was located in the thylakoid lumen of chloroplast (Hager and Holocher 1994). Real time PCR result suggested that *PeVDE* mRNA level increased gradually in leaves during the first 2 h treated with high light, which was consistent with the expression of *GVDE* in ginger (Huang et al. 2007). However, the *PeVDE* mRNA level decreased in the following hours instead of keeping the highest like that of 2 h, and after treatment for 8 h it reached a stable level similar to that of 0.5 h, which was about 1.3 time of that level of 0 h. This was different from that of *GVDE* (Huang et al. 2007) and *VDE* in Arabidopsis (Woitsch and Römer 2003) which kept an increasing

tendency during treatment for 8 h. The reason for the change of *PeVDE* transcript during high-light treatment still needs to be further analyzed.

The xanthophylls cycle is a conservative photo-protection mechanism for photosynthetic plants and algae, which protects the photosynthetic apparatus from excess light through converting V to A then Z by VDE, and the reverse reactions are catalyzed by ZE when light conditions become non-saturated (North et al. 2005). It is confirmed that over-expression of *LeVDE* increased the function of the xanthophyll cycle and alleviated photo-inhibition of PSII and PSI in tomato during high light and chilling stress with low irradiance (Han et al. 2010). The former study indicated that increasing VDE expression in Arabidopsis increased the de-epoxidation state of xanthophyll pigments, the rate of non-photochemical fluorescence quenching (NPQ) induction, and the level of NPQ achieved under subsaturating light (Chen and Gallie 2012). The increase in qN was accompanied with the Z-dependent thermal dissipation in rice plant under moderate and high light (Vaz and Sharma 2011). The relationship between qN and Z in bamboo for photoprotection is an interesting topic to be further studied.

This is the first study on bamboo *VDE*, which will open new opportunities for testing the mechanism of de-epoxidation dependent NPQ through xanthophyll cycle, and possibly improving photo-protection ability in bamboo through over-expression of *VDE*.

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