Molecular cloning, **in vitro** expression and enzyme activity analysis of violaxanthin de-epoxidase from *Oryza sativa* L.

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**Abstract** The violaxanthin de-epoxidase gene was cloned from rice (*Oryza sativa* subsp. *japonica*). The full length of the cDNA is 1887 bp, encoding a 446-amino acids protein with the transit peptide of 98 amino acids. The bacterial expression vector pET-Rvde was constructed and the expression quantity of the exogenous protein increased with the induction time by 0.4 mmol/L IPTG. Its molecular weight was similar with that of the native VDE. Western blotting indicated that the expressed protein has immunological reaction with the VDE polyclonal antibody. The absorbance spectrum together with xanthophyll pigments quantification by HPLC demonstrated that the expressed VDE has its enzyme activity, which can de-epoxidate violaxanthin into antheraxanthin and zeaxanthin **in vitro**.

**Keywords:** *Oryza sativa* L., violaxanthin de-epoxidase, clone, expression, enzyme activity.

When plants absorb more light than what can be used for photosynthesis, the excessive energy can cause photo-inhibition and even photo-oxidation of the photosynthetic apparatus. But plants have developed multiple photoprotective mechanisms to cope with excess light [1]. Xanthophyll cycle-dependent heat dissipation is widely used for photosynthesis, the excessive energy can cause photodamage [4, 5]. Therefore, the xanthophyll cycle plays an important role against photooxidation in plants. Rice is one of the major crops in the world. During the past few years, studies have focused on the physiological role of xanthophyll cycle. But there is no report involved in the molecular aspect of violaxanthin de-epoxidase in rice. In the present study we described the molecular cloning and sequence analysis of *RVDE* gene from rice (the cDNA sequence has been deposited in the GenBank database under accession number AF411133). The enzyme activity of the expressed protein was also examined **in vitro**.

1 **Materials and methods**

(i) **Materials.** Rice (*Oryza sativa* subsp. *japonica*) cv. Zhonghua 8 seedlings were grown in the greenhouse. *E. coli* strains DH5α, BL21(DE3) and plasmid pET-3d were stored in our laboratory. Plasmid pGEM-T Easy was purchased from Promega and Trizol was from Gibco. RACE kits were purchased from Takara and Clontech. VDE polyclonal antibody was kindly provided by Prof. Hans-Erik Åkerlund of Lund University.

(ii) **Plant total DNA and RNA extraction.** Rice total DNA was extracted according to the method described in ref. [6]. Total RNA was isolated using Trizol as the manufacturer described.

(iii) **Full-length cDNA cloning and sequence analysis.** Two degenerate primers p5 (5′-GACGA(GA)TTCAA(C(T)GAGTGTGC-3′)') and p2 (5′-TTAACCCCA-(AG)TA(GT)CCATCCCA-3′) were designed to conduct RT-PCR using total RNA as the template. The reaction system was as follows: 50°C reverse transcription for 30 min; 94°C for 2 min; then 30 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 1 min; and final extension at 72°C for 8 min. Primers for 3′ RACE and 5′ RACE were designed according to the first sequenced fragment. After synthesis of the first strand cDNA, p5 was used along with pM4 (5′-GTGTTCACCTGACGC-3′) in the first round of 3′ RACE PCR. A nested 5′ primer p9 (5′-AGCGAACTTGACATGGAGAA-3′) and pM4 were used in the secondary round of 3′ RACE PCR. The 5′ end sequence of the cDNA was completed by using Smart RACE cDNA Amplification kit with primer p16 (5′-CCACTTGGCGTTGAATCGCCAT-3′) and 5′ Universal Primer Mixture according to the manufacturer’s instruction. The basic PCR system was 94°C, 3 min; then 35 cycles of 94°C, 30 s, 55°C, 40 s and 72°C, 2 min; and a final extension at 72°C for 8 min. Open reading frame searching was performed on NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Amino acids were analyzed using DNASTar and homology analysis was performed using AlignX.

(iv) **VDE genomic DNA cloning.** Primers p18 (5′-CTGGATCCGCAACTAGAAGCTACCT-3′') and p19 (5′-TCGTCCATGGCGCAAGACCGAGA-3′) were used to amplify encoding region of *RVDE* using total DNA as the
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The PCR products were ligated into pGEM-T Easy vector and transferred to E. coli strain DH5α, and then sequenced.

Vector construction and expression in E. coli.
The cDNA sequence encoding RVDE mature protein was amplified by RT-PCR using oligonucleotides p17 (5’-GTCCATGGTTGATGCTCTCAAGACA-3’) and p18. The PCR product was introduced into the expression vector pET-3d through the NcoI and BamHI sites and named pET-Rvde, which was transferred in E. coli strain BL21(DE3). The bacterial expression method was described by Sun et al.[7] with some modifications. The bacteria E. coli with pET-Rvde and pET-3d were inoculated in LB medium and grown with shaking at 37°C for 3 h. Proteins were induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.4 mmol/L final concentration) for 2, 4 and 6 h and used for SDS-PAGE analysis.

Western blotting was performed using the method described by Eskling et al.[8].

Violaxanthin isolation. Pigments in 100% acetone extracts from spinach were separated on thin-layer plates (acetone : hexane = 1 : 1). Violaxanthin, the second band from the start position was scraped off and extracted with methanol.

VDE enzyme activity assay. 40 mL bacterial culture was collected after 4 h induction with IPTG. The bacterial pellet was resuspended in 2 mL of 10 mmol/L Tris-HCl (pH 7.4) and 1 mmol/L EDTA, and lysed using an ultrasonic cell disrupter on ice bath. The extract was centrifuged at 12000×g for 10 min and 100 μL of the supernatant were used for in vitro enzyme activity assay as described[8]. The absorption spectra at 502 and 540 nm were detected on UVIKON-943 Double Beam UV/Visible Spectrophotometer every minute. For pigment quantification, the reactions were stopped at 0, 3, 6, 10, 15 and 20 min, respectively, with addition of solid Tris. The pigments were extracted three times with diethyl ether and solubilized in methanol. Then the extracts were filtered through a 0.45-μm membrane filter and used for HPLC analysis[9].

2 Results

Cloning of the violaxanthin de-epoxidase gene. A couple of degenerate primers were designed according to the conservative regions of VDE cDNAs from the other species. A 450-bp product was amplified by RT-PCR using total RNA as the template. Two fragments of 1.1-kb and 0.9-kb were also obtained in the 3′ RACE and 5′RACE, respectively. Sequencing of the PCR products reveals that the full-length cDNA of RVDE is 1887 bp. The first initiation codon starts at nucleotides 175—177 and the stop codon is located at nucleotides 1513—1515. Thus the cDNA has a 1341 bp open reading frame encoding a 446-amino acid protein. It is predicted to be a water-soluble protein (fig. 1). The mature protein is deduced to have 348 amino acids with a calculated molecular weight of 39.9 ku and a calculated isoelectric point of 4.71. Sequence alignments between cDNA and genomic DNA indicated that the RVDE gene contains four introns: 105, 327, 81 and 69 bp, respectively.

VDE expression in E. coli. The expression vector pET-Rvde containing RVDE gene was transferred into E. coli BL21(DE3) and the protein was induced by addition of 0.4 mmol/L IPTG. SDS-PAGE result showed that a distinct band about 43 ku, similar to that of the native protein from spinach[10], appeared as compared with pET-3d as control. The quantity of the protein increased with the induction time by IPTG. It accounted for 25% of the total proteins after induction for 4 h (fig. 2).

It was further confirmed by Western blotting probed with the VDE polyclonal antibody. The result demon-