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Expression of three diadinoxanthin de-epoxidase genes of *Phaeodacylum tricornutum* in *Escherichia coli Origami b* and BL21 strain*

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In the diadinoxanthin cycle the epoxy group is removed from diadinoxanthin and diatoxanthin is created. This conversion takes place e.g. in diatoms with the involvement of the enzyme diadinoxanthin de-epoxidase. In one of the diatom species, *Phaeodactylum tricornutum* (CCAP 1055/1 strain with genome sequenced) three de-epoxidase genes (*PtVDE, PtVDL1, PtVDL2*) have been identified, but only one of them (*PtVDE*) corresponds to violaxanthin de-epoxidase, an enzyme which is commonly found in higher plants. In these studies, the expression of two de-epoxidase genes of another *Phaeodactylum tricornutum* strain (UTEX 646), which is commonly used in diatom studies, were obtained in *Origami b* and BL21 *E. coli* strains. The molecular masses of the mature proteins are about 49 kDa and 60 kDa, respectively, for VDE and VDL2. Both enzymes are active with violaxanthin as a substrate.

Key words: diadinoxanthin cycle, marine diatoms, violaxanthin, de-epoxidation

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INTRODUCTION

All photosynthetic organisms have developed photoprotective mechanisms with carotenoids playing a fundamental role in the dissipation of excess light energy. Photoprotection is connected with the de-epoxidased forms of xanthophyll pigments, which are formed by the enzymatic removal of epoxy groups under high light conditions. These reactions occur in those processes commonly known as xanthophyll cycles (Jahns *et al*., 2009). Two of the most common among them are the violaxanthin and diadinoxanthin cycles. In the violaxanthin cycle, violaxanthin is de-epoxidased to zeaxanthin via antheraxanthin by the enzyme called violaxanthin de-epoxidase (VDE), whereas in the diadinoxanthin cycle the epoxy group is removed from diadinoxanthin and diatoxanthin is created. Such conversion takes place e.g. in diatoms and is catalyzed by the enzyme diadinoxanthin de-epoxidase. In the past, in diatoms only one de-epoxidase enzyme, known as DDE, was postulated (Goss & Jacob, 2010). Nowadays three genes of this enzyme have been identified in one of the diatoms, the *Phaeodactylum tricornutum* CCAP 1055/1 strain with genome sequenced (Siaut *et al*., 2007). However, only one of them is similar to *VDE* of the violaxanthin cycle. This gene is marked as *PtVDE*. The product of this gene is called VDE, and may be involved in the conventional xantophyll cycle. Two others genes of *Phaeodactylum tricornutum* de-epoxidase are referred to as VDE-like de-epoxidases (designated as *PtVDL1* and *PtVDL2*, respectively), and are thought to be more specialized in the chromist-specific diadinoxanthin cycle (Coesel *et al*., 2008).

The purpose of our research was to obtain *Pt*VDE, *Pt*VDL1 and *Pt*VDL2 genes of *Phaeodactylum tricornutum* (UTEX 646 strain), and their expression with the goal to obtain active enzymes with polyhistidine tag.

METHODS

***Phaeodactylum tricornutum* growth conditions**. The *Ph. tricornutum UTEX 645* strain was obtained from the Institute of Botany at Leipzig University. The culture was grown in an f/2 medium (Guillard & Ryther, 1962; Guillard, 1975) made with 1.6% sea salt (Tropic Marin), supplemented with f/2 vitamins (filter sterilized and added after autoclaving). Approximately 70 ml of inoculum with optical density OD_{600} 0.3–0.4 was used at the start of a 250 ml batch culture. The cultures were grown at 15°C under white light at approximately 40 μmol $m^{-2}s^{-1}$ in a 10/14 h photoperiod. The cultures were shaken several times a week during the light phase to keep cells in suspension and maintain an optimal exchange of gas and nutrients.

RNA purification and reverse transcription. Total RNA was prepared from a 5 day old *Ph. tricornutum* batch culture, with OD_{600} 0.2–0.3, using GeneJET Plant RNA Purification kit (Thermo SCIENTIFIC) according to manufacturer's instructions. Genomic DNA was removed from RNA by the incubation of 1 μg RNA with DNaseI, RNase-free (Thermo SCIENTIFIC) at 37°C for 30 min.

First-strand cDNA was synthesized using 1 μg total RNA, M-Mul V Reverse transcriptase, Thermo Scientific

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Abbreviations: Asc, ascorbate; Ax, anteraxanthin; DDE, diadinoxanthin de-epoxidase, MGDG, monogalactosyldiacylglycerol; PCR, polimerase chain reaction; *Pt, Phaeodactylum tricornutum; VDE,* violaxanthin de-epoxidase gene; *VDL1,* precursor of violaxanthin de-epoxidase like protein 1 gene; *VDL2,* precursor of violaxanthin de-epoxidase like protein 2 gene; VDE, violaxanthin de-epoxidase; VDL1, precursor of violaxanthin de-epoxidase like protein 1; VDL2, precursor of violaxanthin de-epoxidase like protein 2; Vx, violaxanthin; Zx, zeaxanthin

Table 1. List of primers used for PCR amplification of the de-epoxidase genes (BamHI and NdeI site underlined).

Name of genes	Primer name	Sequences
VDE	VDEPhtriRevBamH281111	5′-CCGGATCCTTATTGCTGGGAGGTTTCTC-3′
	VDEPhtriForNdel160511	5′-GAGCATATGAAGTTTCTCGGTGTTACCAG-3′
VDL1	VDL1PhtriRevBamH281111	5′-CCGGATCCTTAGCGTTTCGCCTTGTATTC-3′
	VDL1PhtriForNdel160511	5′-GAGCATATGCGATTCGCTTGGGTGGT-3′
VDL2	VDL2Phtri RevBamH281111	5′-CCGGATCCTTAGTTCTTGACATCTTCTG-3′
	VDL2Phtri For Ndel160511	5′-GAGCATATGAAGCGAGCCACGAGGAA-3′

RiboLock RNase Inhibitor and Random primer (Fermentas) by incubation for 10 min at 25ºC followed by 60 min at 37ºC. RNA and DNA concentration were determined spectrophotometrically at 260 nm (NanoDrop Litle Spectrophotometer, Thermo SCIENTIFIC).

PCR conditions. Primer pairs and cDNA were used as a template for the amplification of *PtVDE*, *PtVDL1* and *PtVDL2* (Pfu DNA Polymerase). The sense primer (Table 1) contained the initiation Met, N terminus codons of amino acid from the mature protein (*Ph. tricornutum* CCAP 1055/1 strain) and NdeI site (underlined). The antisense primers (Table 1) contained the stop codon, C terminus codons of amino acid from the mature protein and BamHI site (underlined). PCR was performed using the following thermal condition: initial denaturation 95ºC (3 min), denaturation 95ºC (30 s), annealing 56ºC (30 s), extension 72ºC (2 min) carried out in 35 cycles and to a final extension of 72ºC (15 min) (S1000 Thermal Cycler, BioRad).

PCR single products of the expected size were confirmed by 1% agarose gel electrophoresis visualized by ethidium bromide staining.

Construction of plasmids and recombinant expression of de-epoxidases. PCR products were purified using the Ez-10 Spin column DNA Gel extraction Kit (Lab Empire)

PtVDE, *PtVDL1* and *PtVDL2* were digested by NdeI (by night) and BamHI (2 h) at room temperature (Fermentas), and inserted to dephosphorylated (Shrimp Alcaline Phosphatase, Fermentas) digested by NdeI (by night) and BamHI (2 h) (Fermentas) — pET-15b vector (Novagen), using a ligation kit (T4 DNA Ligase Fermentas). Plasmids were named pET-15b/*PtVDE*, pET-15b/*PtVDL1* and pET-15b/*PtVDL2*, respectively.

The sequences of *PtVDE*, *PtVDL1* and *PtVDL2* inserted to pET-15b cloning region were compared with sequences of these genes of the *Ph. tricornutum* CCAP 1055/1 strain (Accession numbers (NCBI): XM_002178607, XM_002180599, XM_002180015, respectively) using BLAST and ExPASy. Plasmids were sequenced by Genomed (Poland). PET-15b/*PtVDE*, and pET-15b/*PtVDL2* plasmids (similar sequences to the respective *Ph. tricornutum* gene) were used to transform *E. coli Origami b* (DE3) (Novagen) and *BL21*(Novagen) cells. To 200 µl of competent cells 20 ng of respective pET-15b vector was added, mixed gently, and incubated for 30 min on ice. Transformation mixtures were transferred to a 42ºC water bath and incubated exactly 90 s followed by 2 min on ice. Transformed cells were added to pre-heated (37ºC) LB and incubated for 1 h at 37ºC. The bacteria from the transformation mix were spread on selective LB (Ampicillin 100) agar plates (modified method of Swords, 2003).

Electrophoresis and immunoblotting. Transformed *E. coli* cells after induction of protein overexpression by IPTG (0.5 mM) grown for 20 h at 22ºC were collected at 0 after 8 and 20 h and incubated with a urea lysis buffer (8M urea, 20 mM Tris, 9.0 pH) for 10 min at 100ºC. The presence of de-epoxidases was analyzed by gel electrophoresis (SDS-PAGE) stained with Coomassie Brillant Blue and transferred to PVDF membranes (pore size 0.45 µm, Immobilon-P) for western blotting. The membranes were then incubated for 16 h at 6ºC with a mouse anti-HisTag primary antibody (Sigma) followed by 1 h incubation with an alkaline phosphatase-conjugated anti-mouse secondary goat antibody (Sigma), and visualized by the BCIP/NBT liquid substrate (Sigma).

Violaxanthin de-epoxidation activity. *E. coli* cells were collected, centrifugated and sonicated. The enzyme activity was measured with violaxanthin as a substrate (0.33 µM), in the presence of monogalactosyldiacylglycerol (MGDG) (9 µM) and 100 µl of sonicated cells in 0.1 M citric buffer (5.1 pH) were used as a source of the de-epoxidase. The reaction was initiated by the addition of ascorbate to a final concentration of 30 mM and this was carried out at room temperature. Samples were collected at 0, 5, 10 and 30 min of reaction. De-epoxydation was stopped by mixing 700 ml of assay mixture with 50 ml of 1 M KOH (Yamamoto, 1985). The level of xanthophyll pigments (violaxanthin as a substrate, antheraxanthin and zeaxanthin as products) was analyzed by reverse phase HPLC chromatography (Latowski *et al.*, 2002).

RESULTS AND DISCUSSION

The determination of the complete DNA sequence of *Ph. tricornutum* has provided a new opportunity for the gene expression analysis of diatoms, which are important component of marine phytoplankton, and which play an important role in global carbon cycling (diatoms fix more carbon then the most productive terrestrial ecosystem, the tropical rainforests) as well as in the regulation of the biogeochemical cycle of silicon in the ocean (Smetacek, 1985; Egge & Aksnes, 1992; Treguer *et al.*, 1995; Dugdale & Wilkerson, 1998; Geider *et al., 2001;* Yool & Tyrrell, 2003). The methodology for reverse ge-

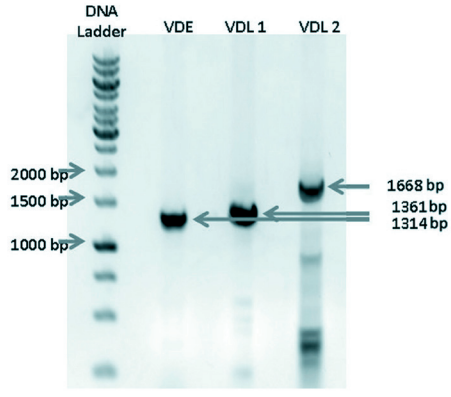

Figure 1. PCR amplification of *PtVDE, PtVDL1* and *PtVDL2*. cDNA used as a template was obtained by reverse-transcription of mRNA isolated from a 5 day old batch culture of *Ph. tricornutum* UTEX 645. The products were separated in 1% agarose gel and then visualized with ethidium bromide.

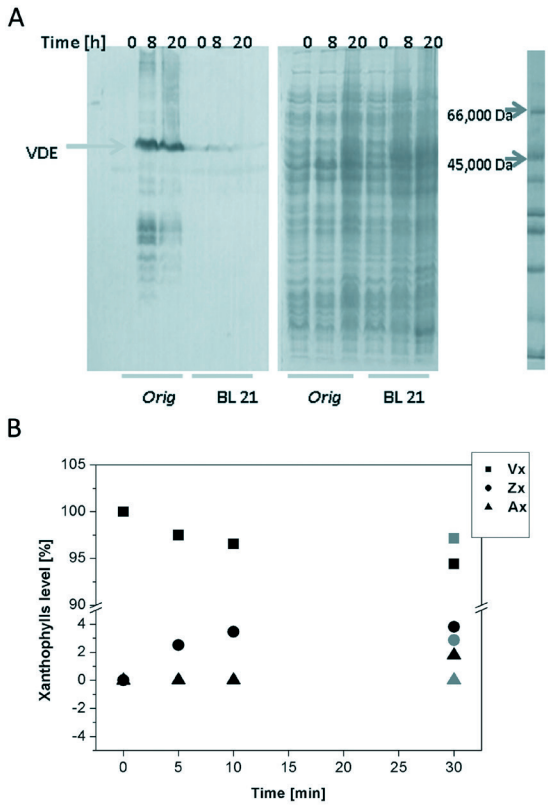

Figure 2. (A) SDS/PAGE electrophoresis gel (on the right) and western blot detection (on the left) of VDE after IPTG induction (0, 8 and 20 h at 22°C), in *Origami b* and BL21 cell extracts. (B) The kinetics of violaxanthin de-epoxidation (samples collected at 0, 5, 10, 30 min) catalyzed by overexpressed VDE present in bacteria cells extract: *Origami b* (gray) and BL21 (black). Vx — violaxanthin, Zx — zeaxanthin, Ax — anteraxanthin.

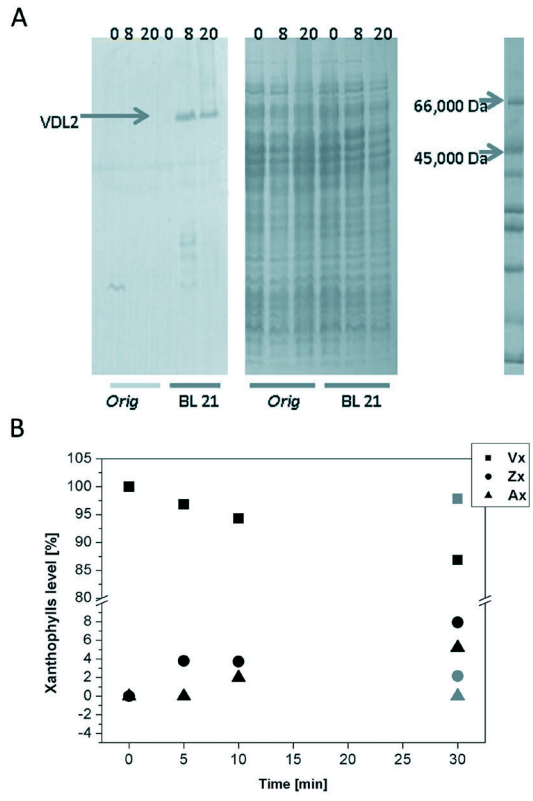

Figure 3. (A) SDS/PAGE electrophoresis gel (on the right) and western blot detection (on the left) of VDL2 after IPTG induction (0, 8 and 20 h at 22°C), in *Origami b* and BL21 cell extracts. (B) The kinetics of violaxanthin de-epoxidation (samples collected at 0, 5, 10, 30 min) catalyzed by overexpressed VDL2 present in bacteria cells extract: BL21 (black) and *Origami b* (gray). Vx — violaxanthin, Zx — zeaxanthin, Ax — anteraxanthin.

netics allows to the expression of cloned genes in heterologous systems in order to identify the function of the protein encoded (Siaut *et al.*, 2007). The typical methodology was applied to the expression of three genes of *Ph. tricornutum* de-epoxidase, and to test the enzymatic activity of the mature proteins.

During the first stage, three PCR products with bp values corresponding to *PtVDE*, *PtVDL1* and *PtVDL2* of *Ph. tricornutum* CCAP 1055/1 were obtained (Fig. 1)

PtVDE, PtVDL1 and *PtVDL2* were inserted to pet 15b vector cloning region and sequenced. The comparative analysis of both strains of *Ph. tricornutum* VDE-genes shows that *PtVDE* and *PtVDL2* had highly identical (99%) nucleotide sequences in diatom strains tested. Translation tool of the obtained *PtVDE* and *PtVDL2* DNA sequences in to protein sequences and their comparison with the protein sequences of *Pt*VDE and *Pt*-VDL2 of *Ph. tricornutum* CCAP 1055/1 strain show differences in two positions. The amino acids of *Pt*VDE of *Ph. tricornutum* UTEX 645 identified as differing from *Pt*-VDE of *Ph. tricornutum* CCAP 1055 /1 were localized in the N-terminal targeting sequence (Ser→Leu 13) and in the Glu-rich domain (Leu→Val 413). VDL2 of UTEX strain was different from VDL2 of CCAP in two sites located in the Cys-rich domain (Leu→Met 107) and in the lipocalin domain (Lys→Glu 378).

The de-epoxidase genes obtained (*PtVDE* and *Pt-VDL2*), showed a different expression level dependent on the time of induction and *E. coli* strains. The presence of VDE and VDL2 proteins after IPTG induction (8 and 20 h) were visualized on SDS/PAGE electrophoresis as a dark band up to the 45 kDa and close to the 65 kDa marker level (for VDE 49 kDa and VDL2 60 kDa, respectively). Western-blot analysis with His-tag antibodies confirmed proteins expression (Fig. 2A and 3A). The stable expression of the genes obtained were observed in *Origami b* for *PtVDE* and in BL21 for *Pt-VDL2* (dark bands), whereas in BL21 and in *Origami b* lower expression was observed for *PtVDE* and *Pt-VDL2*, respectively.

The activity of mature recombinant VDE and VDL2 after 20 h induction by IPTG was also observed. The results show that both enzymes expressed in *Origami b* and BL21 *E. coli* strain catalyzed violaxantin deepoxidation. The dynamics between conversion of Vx into Ax and Zx depended on the enzyme expression level. VDE inducted in *Origami b* strain converts about 4% Vx to Zx as the final product reaction whereas VDE inducted in BL21 only 3% after 30 min running of the reaction. Under the same experimental conditions VDL2 induced in BL21 converted 8% Vx, whereas VDL2 induced in *Origami b* strain converted 2% Vx (Fig. 2B and 3B) only.

These results show the effective expression of two of the three *Ph. tricornutum* active de-epoxidases.

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