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

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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
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<tr>
<td>Asc, ascorbate</td>
<td>A vitamin required for various metabolic processes.</td>
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<td>Ax, anteraxanthin</td>
<td>A xanthophyll pigment.</td>
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<td>DDE, diadinoxanthin de-epoxidase</td>
<td>An enzyme that catalyzes the conversion of diadinoxanthin to violaxanthin.</td>
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<td>Pt, Phaeodactylum tricornutum</td>
<td>A genus of diatoms.</td>
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<td>VDE, violaxanthin de-epoxidase</td>
<td>An enzyme that catalyzes the conversion of violaxanthin to zeaxanthin.</td>
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INTRODUCTION

All photosynthetic organisms have developed protective 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-epoxidized to zeaxanthin via anteraxanthin 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, 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* 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.

METHODS

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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**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<sub>680</sub> 0.3–0.4 was used at the start of a 250 ml batch culture. The cultures were grown at 15ºC 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<sub>600</sub> 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 37ºC for 30 min.

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

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

<table>
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<tr>
<th>Name of genes</th>
<th>Primer name</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>VDE</td>
<td>VDEPhtriRev</td>
<td>5'-CCCAGTACCCTATATTGCGAGGAGGGTTCTC-3'</td>
</tr>
<tr>
<td></td>
<td>VDEPhtriFor</td>
<td>5'-GACCATATGAACTTCCGTGGTTACCG-3'</td>
</tr>
<tr>
<td>VDL1</td>
<td>VDL1PhtriRev</td>
<td>5'-CCCAGTCCCTAGCATGTCTGAGGTTATCTC-3'</td>
</tr>
<tr>
<td></td>
<td>VDL1PhtriFor</td>
<td>5'-GACCATATGCGGATTTGCGGTGGTGT-3'</td>
</tr>
<tr>
<td>VDL2</td>
<td>VDL2PhtriRev</td>
<td>5'-CCCAGTCAATGCTCTGACATTCTCG-3'</td>
</tr>
<tr>
<td></td>
<td>VDL2PhtriFor</td>
<td>5'-GACCATATGAACTTCCGTGGTTACCG-3'</td>
</tr>
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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 is 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 gene-expression of de-epoxidases. PCR products were purified using the Ez-10 Spin column DNA Gel extraction Kit (Lab Empire).

**Violaaxanthin de-epoxidation activity.** E. coli cells were collected, centrifuged 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 (pH 5.1) 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-epoxidation 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).
Expression of three diadinoxanthin de-epoxidase genes

Vol. 60

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 pet15b 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 PtVDE and PtVDL2 of Ph. tricornutum CCAP 1055/1 strain show differences in two positions. The amino acids of PtVDE of Ph. tricornutum UTEX 645 identified as differing from PtVDE 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 PtVDL2), 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 PtVDL2 (dark bands), whereas in BL21 and in Origami b lower expression was observed for PtVDE and PtVDL2, 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 violaxanthin 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 inducted 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.

REFERENCES


