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Addressing various compartments of the diatom model organism *Phaeodactylum tricornutum* via sub-cellular marker proteins

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ABSTRACT

The diatom *Phaeodactylum tricornutum* is a prominent organism to study protein import mechanisms into complex plastids surrounded by four membranes. In this regard, various molecular techniques were adapted and established for this organism, so that nowadays a broad range of questions in a variety of research disciplines can be addressed. At least in the case of sub-cellular localization studies, the different compartments of this complex structured organism have to be addressed by marker molecules. Here we present an arsenal of subcellular marker proteins, which provide defined localizations even on the sub-compartmental level and imply new and fascinating topics for further research.

1. Introduction

In the last decades, tremendous knowledge of cellular processes has been generated by studying model systems. However, in eukaryotes, studying cell biology is still restricted to a couple of model organisms only, which belong, in most cases, either to the Ophistokonta or to the Archaeplastida. Other domains frequently show unique physiologies and might be, therefore, terrain for new cell biological discoveries. The chromalveolates [8,9], a group with enormous ecological impact, are expected to be one of these. Most members of this group show a complex cellular morphology reflecting their evolutionary origin by merging two eukaryotic cells. In extant chromalveolate taxa, one of the once free-living partners is represented by non-plastid compartments (host cell). Whereas in the engulfed cell, the symbiont, initially a phototrophic red alga, is presently reflected by a complex organized plastid [18,56]. The pleiotropy of the cellular structures of the plastid is explained by co-evolution of two cellular organisms, which led to elimination and reduction of many compartments from the symbiont. Thereby, in numerous members so-called complex plastids evolved, which are surrounded by three (peridinin-containing dinoflagellates) or, in most cases, four membranes (cryptophytes, heterokonts, haptophytes, chromerids and apicomplexa) [19,20]. Thus, chromalveolates show, with respect to their complex plastids, a peculiar membrane arrangement, but other structures such as the cell wall or vacuoles are assumed to be specific as well, although they can be very different even within this group. The apicomplexan parasites are important members of the chromalveolates [18] and, therefore, not only in focus of basic research, but also a major target of medical research worldwide. Especially their complex plastid (the apicoplast) is a promising target for the development of drugs dealing with malaria and toxoplasmosis [35].

It is no surprise that the first sequenced chromalveolates were apicomplexan parasites [15,32]. However, shortly after these milestones, the genomes of different phototrophic chromalveolates were deciphered as well. One of those was the nuclear genome of the diatom *Phaeodactylum tricornutum* [5], for which a transformation protocol was already available [2]. Over the last years, additional molecular tools for genetic engineering of this diatom were developed. These included manipulation of gene expression via gene silencing [13], the development of a protocol for TALEN (transcription activator-like effector nucleases) [51], a plastid transformation protocol [52], the establishing of episomes delivered by bacterial conjugation [30] and quite recently the CRISPR/Cas9 system could be adopted to *P. tricornutum* as well [38].

Since diatoms are phototrophic organisms, which can be easily cultivated, *P. tricornutum* was additionally noticed as a potential valuable bioreactor to synthesize commercially interesting products. And indeed the initial design of strains synthesizing pharmaceuticals and bioplastic turned out to be successful [25–27].

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For detailed cell biological analyses, knowledge of ultrastructure is very helpful, if not mandatory. An electron micrograph of *P. tricornutum* is shown in Fig. 1. The most dominant organelle is the complex plastid, which is surrounded by four membranes [49].

Diatom cells possess ER cisternae, which are expected to deliver material to the Golgi apparatus (beside other functions). This compartment is also well preserved by the methods used here (Fig. 1). Another dominant structure is a vacuolar-like compartment. In close proximity to the complex plastid is the mitochondrion (Fig. 1; see also: [4,43]).

Another powerful basic cell biological technique is in vivo localization of proteins on the subcellular level. For that, the protein of interest or only its targeting signal is fused to a fluorescent marker protein (e.g. eGFP) and expressed as fusion protein in the appropriate model system. Fortunately, this is possible in diatoms as well and eGFP can be targeted to different cellular destinations (e.g. [3,31]), and several inducible promoters and terminators are established for protein expression [2,23,41,42]. However, in many cases localization studies in diatoms were restricted to visualize the compartments of the complex plastid, the ER, the cytoplasm or the cell wall by this approach up to now (for recent examples see: [34,37]). In order to extend the repertoire of addressable subcellular destinations in diatoms and to better understand their complex compartmentalization and targeting mechanisms, further compartment-specific marker proteins or targeting signals need to be established.

Here we show subcellular localizations of several not yet investigated proteins in the diatom *P. tricornutum*. We present a variety of marker proteins localized in the different membranes of the complex plastid and its non-membrane structures. Further here investigated marker proteins specify putative ER sub-compartments and the nucleus, mitochondrion, the Golgi apparatus and the retromer, the cytoplasmic membrane, peroxisomes, as well as a vacuolar structure of the diatom *P. tricornutum*. Thus, our results extend knowledge on subcellular fluorescence patterns of compartment-specific marker proteins and membranes significantly and provide the basis for co-localization studies with proteins of unknown subcellular localization.

2. Materials and methods

2.1. Bioinformatic analysis

Screening of the *P. tricornutum* genome database (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html) was done with protein sequences of known proteins from *A. thaliana*, *Saccharomyces cerevisiae* or *Thalassiosira pseudonana* in BLAST searches using default settings [1].

Annotations of predicted gene models were checked by expressed sequence tags (EST) data retrieved from the NCBI database using tblastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Putative N-terminal targeting sequences were predicted using the CBS prediction server tools TargetP (http://www.cbs.dtu.dk/services/TargetP/) SignalP (3.0 server) (http://www.cbs.dtu.dk/services/SignalP-3.0/), or (SignalP 4.1 server) (http://www.cbs.dtu.dk/services/SignalP/) using default settings. To compare the predictions of transmembrane helices in proteins, four different prediction algorithms were used: TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), TOPCONS (http://topcons.cbr.su.se/), ΔG predictor (http://dgpred.cbr.su.se/index.php?p=fullscan) and SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html).

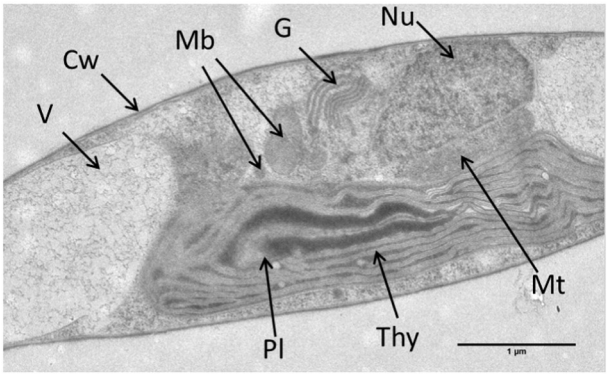

Fig. 1. Electron micrograph of a longitudinal section through a *P. tricornutum* wild type cell. Scale bar represents 1 µm. **Cw**, cell wall; **G**, Golgi; **Mb**, microbodies; **Mt**, mitochondrium; **Nu**, nucleus; **Pl**, plastid; **Thy**, thylakoids; **V**, vacuole.

2.2. Organism and culture conditions

Phaeodactylum tricornutum (Bohlin, UTEX646) cells were cultured in Erlenmeyer flasks containing f/2 medium constantly shaken at 225 rpm or on f/2 agar plates under 24 h light condition (8000–11,000 Lux) at 22 °C. For selection of transformed clones, zeozin™ (InvivoGen) was added in a concentration of 75 µg/ml. The clones were cultured for about 3–4 weeks until they were transferred to new plates. Strain maintenance was carried out by continuous cultivation on selective plates for what cells were transferred to new plates every four weeks.

2.3. Plasmid construction and transfection of P. tricornutum

P. tricornutum genomic DNA and total RNA were isolated using standard procedures [47]. Synthesis of cDNA was done by use of Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Amplification of sequences was performed using PHUSION High Fidelity DNA polymerase (Finnzymes) and specific oligonucleotides (Sigma) introducing restriction sites, in standard polymerase chain reactions. Products were subcloned into pJET1.2 (MBI Fermentas) and verified by sequencing. Sequences were fused upstream to eGFP or mRFP coding sequences and cloned into pPha-NR vector (GenBank: JN180663) or pPha-dual-2xNR vectors (GenBank: JN180664). For transfection of *P. tricornutum*, cells from an exponential culture were collected. The biolistic transfection was carried out as outlined in [2] using tungsten M10 particles and 1350 psi rupture disks together with the Biolistic PDS - 1000/He Particle Delivery System from Biorad [26].

2.4. In vivo localization

Induction of protein expression in the pPha-NR vector was performed for 24 h to 48 h depending on different constructs by transferring cells from ammonium containing media (1.5 mM NH_4^+) to nitrate-containing media (0.9 mM NO_3^-). For each construct all clones (up to 50) were analyzed to avoid effects on the localization resulting from the genomic integration site of the plasmid. For staining of the mitochondria and plasma membrane or integrated vesicles, cells were harvested and washed once in 1× phosphate buffered saline (PBS, pH 7.5), then incubated for 30 min in 0.5 mM MitoTracker Orange CMTMRos or for 25 min in 0.08 mM FM®4–64 in the dark, respectively. After staining, cells were washed twice with 1× PBS and then fixed with 4% paraformaldehyde–0.0075% glutaraldehyde in 1× PBS buffer for 20 min. After fixation, cells were resuspended in 30 µl PBS for microscopy. The cells from the staining of FM®4–64 dye were not fixed and directly resuspended in 30 µl PBS for microscopy.

Subsequently, cells were analyzed with a confocal laser scanning microscope Leica TCS SP2 using a HCX PL APO 40×/1.25 to 0.75 oil CS objective. Fluorescence of eGFP and plastid autofluorescence was excited with an argon laser at 488 nm and detected with two photomultiplier tubes at bandwidths of 500 to 520 nm and 625 to 720 nm for eGFP and plastid autofluorescence, respectively. Fluorescence of mRFP, MitoTracker® Orange CMTMRos (Molecular Probes) and FM®4–64 (Molecular Probes) was excited with a HeNe 1.2 mW laser at 543 nm and detected with a photomultiplier tube at a bandwidth of 580–