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Molecular tools to explore the biology of diatoms

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INTRODUCTION

Diatoms have fascinated botanists since they were first discovered by light microscopy and mistakenly addressed as unicellular animals due to their brownish colour and their capability to move on substrates. Diatoms are single celled, sometimes colonial organisms, almost all of them are photoautotrophic and can be found in nearly any aquatic and even in some terrestrial habitats. Diatoms also play a significant ecological role. About half of the global annual net primary production in the oceans is due to phytoplankton which is dominated by diatoms (Falkowski et al. 1998). Diatoms thus produce and represent the main input into the marine food web. It is not yet fully understood how diatoms filled aquatic niches so successfully, even though they have the capability to grow at a wide range of light intensities (Falkowski et al. 2004) and apparently are able to perform a kind of C4 photosynthesis or carbon concentrating mechanisms (Reinfelder et al. 2004).

Another peculiar aspect of diatoms is their evolution. Various analyses indicate that the plastids of all the different algae we know today may be traced back to a single endosymbiosis event ("primary endosymbiosis" or more correctly "primary endocytobiosis"), a process in which a cyanobacterium was taken up by a eukaryotic host cell and subsequently transformed into a plastid (Delwiche & Palmer 1997). An apparently inevitable consequence of this "domestication" is the transfer of most of the endosymbiont´s genes into the nucleus of the host cell. As we know today, several algal groups - including the diatoms - are supposed to have evolved in an even more complex way by secondary endocytobiosis, a process that successfully happened at least twice during evolution (Cavalier-Smith 2003). Here, eukaryotic algae, themselves possessing plastids, were taken up by host cells and were transformed into plastids. Due to redundancy, nearly all of the cytoplasmic structures of the endosymbiotic algae have vanished - usually including their nuclear genome - while the plastids have been preserved. Probably because of their ability to perform photosynthesis they must have been highly attractive for the host cell. Diatom plastids are also termed 'complex' plastids, because in comparison to land plant plastids they possess two additional surrounding membranes probably originating from the phagotrophic membrane of the host and the plasma membrane of the endosymbiont (Delwiche & Palmer 1997). In diatoms the outermost plastid membrane contains bound ribosomes and is connected to the cytosolic endoplasmic reticulum (ER), the plastids are thus essentially located in a sac of ER (Gibbs 1981). Although some genetic tools to explore diatom biology like genomes and genetic transformation are available, several methods are still lacking, which might improve the usability of these organisms for research. Here the actual status of these methods in diatoms is described.

GENOME STRUCTURES

The recent sequencing projects exploring the genomes of the centric diatom *Thalassiosira pseudonana* Hasle & Heimdal (Armbrust et al. 2004) and the pennate *Phaeodactylum tricornutum* Bohlin (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html) (Fig. 1) indicated a rather small genome size of about 31 and 26 MBp, respectively, but supported the expected

genetic complexity of diatoms indicating that only half of the genes encode proteins similar to red algae, green algae and plants, but also about 7 % of genes for “animal”-like proteins that may been derived from the secondary host cell (Armbrust et al. 2004). The nuclear genome of *T. pseudonana* contains 24 chromosomes, while *P. tricornutum* possesses 33 different chromosomes.

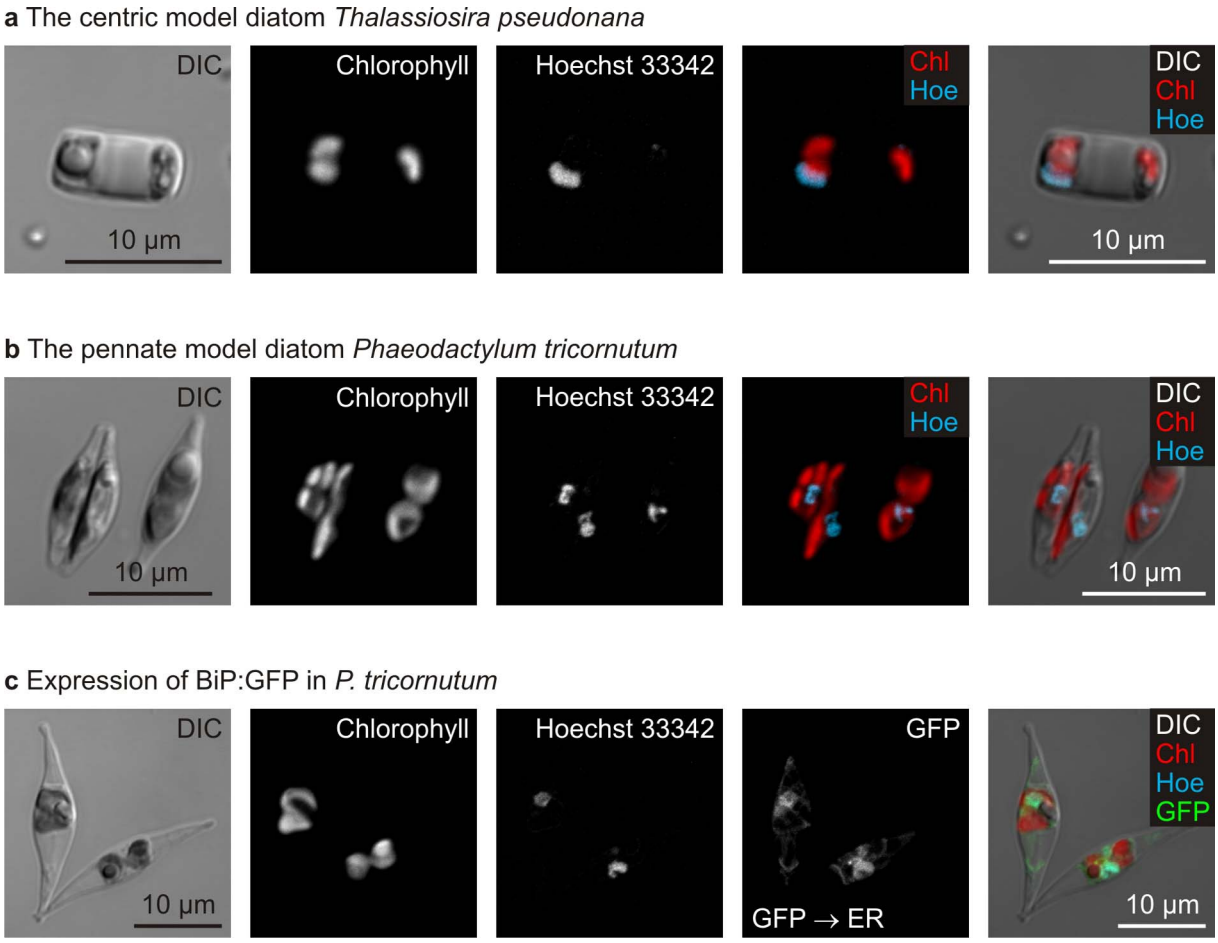

Fig. 1. *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, actual model organisms for centric and pennate diatoms **a**: *T. pseudonana*, DNA stained with Hoechst 33342 dye (bisbenzimide, Behring Diagnostics, La Jolla, USA), maximum intensity projection obtained from 10 optical slices of a 3 µm z-stack; **b**: *P. tricornutum*, DNA staining as in **a**, maximum intensity projection obtained from 8 optical slices of a 2.4 µm z-stack; **c**: *In vivo* expression of green fluorescent protein (GFP) in a genetically transformed *P. tricornutum* cell line, the presequence of an endoplasmic reticulum (ER) localised chaperone (BiP) was fused to the GFP gene (see Apt et al. 2002, Kilian et al. 2005), DNA staining as in **a**, maximum intensity projection obtained from 5 optical slices of a 1.5 µm z-stack; Nomarski’s differential interference contrast (DIC), Chlorophyll autofluorescence (Chl), Hoechst 33342 (Hoe) fluorescence, GFP fluorescence and merged images showing the respective channels in the indicated colours are shown from left to right. (Images taken by Ansgar Gruber, University of Konstanz).

The chloroplast genomes of *P. tricornutum* and the centric diatoms *T. pseudonana* and *Odontella sinensis* (Grev.) Grunow also have been completely sequenced. All three genomes are rather compact (117, 128, 118 KBp, respectively) (Oudot-Le Secq et al. 2007, Kowallik et al. 1995) with small intergenic regions and no introns. They are similar in gene content with 127–130 protein-coding genes, and genes for 27 tRNAs, three ribosomal RNAs and two small RNAs (tRNA and signal recognition particle RNA). The gene order is not strictly conserved, but there are a number of conserved gene clusters showing remnants of red algal origin. The mitochondrial genome of T. pseudonana is a small circular chromosome of 43 KBp containing about 42 protein coding genes and 22 tRNAs (Armbrust et al. 2004). No indications were found that diatoms might possess any form of nucleomorph, reduced nuclei from the secondary endosymbionts, which are found in cryptophytes within the periplastidic space (Maier et al. 2000).

CELL BIOLOGY – PROTEIN TRANSLOCATION INTO PLASTIDS

The establishment of a stable secondary endosymbiosis required the evolution of a protein import system to allow nucleus encoded and cytoplasmatically synthesized proteins to traverse the two additional membranes that surround diatom plastids. Diatom plastid preproteins possess presequences consisting of two domains: An N-terminal signal peptide sequence directs proteins across the endoplasmic reticulum (ER) membrane, which is continuous with the outermost plastid membrane in most heterokonts (Kroth 2002), followed by a second domain, resembling transit peptides for plastid import in land plants. Between these two domains a distinct signal peptide cleavage site and a very conserved motif were identified, both being essential for import into the plastids (Kilian & Kroth 2005, Gruber et al. 2007). However, the mechanism of transit of preproteins across all four membranes remains is still a matter of debate (Cavalier-Smith 2003, van Dooren et al. 2001). In land plants relatively similar transit peptides are used to target into plastids or mitochondria, making it sometimes difficult to predict the correct compartment. As diatom plastid presequences, however, differ significantly from those of land plants or green algae prediction of intracellular localization of proteins is much easier. The targeting properties can be investigated experimentally in *P. tricornutum* by expression of presequence: GFP fusion proteins (Fig. 1) (Gruber et al. 2007).

GENETIC TRANSFORMATION

Diatoms are almost unique among algae in being diplonts, and sexual reproduction is an obligate stage in the life cycle of most diatom species (Chepurnov et al. 2004). Most diatoms, however, often behave asexually in culture, because - depending on the species - sexual reproduction often only occurs at certain environmental conditions or when the cells reach a certain critical size (Edlund & Stoermer 1997). Genetic proofs for actual crosses of transgenic diatom lineages unfortunately have not yet been published, limiting the genetic methods available. One essential tool therefore is the transfer and integration of genetic material into the respective diatoms compartments containing DNA: the nucleus, the plastids or the mitochondria. Significant progress on gene transfer systems in eukaryotic algae has mainly been achieved in the last 20 years allowing the modification of algae either in order to obtain strains which produce certain compounds of commercial interest or to gain information about cellular, physiological or biochemical mechanisms by switching off, down-regulation or over-expression of existing or foreign genes, respectively (Bateman & Purton 2000, Kroth 2008).

NUCLEAR TRANSFORMATION

There are only few reports about nuclear transformation systems (Apt et al. 1996, Dunahay et al. 1995, Falciatore et al. 1999, Poulsen & Kröger 2005). Dunahay et al. (1995) were the first to report a genetic transformation system for the diatoms *Cyclotella cryptica* J.C.Lewin & Guillard and *Navicula saprophila* Lange-Bert. & Bonik. They used vectors containing the acetyl-CoA carboxylase from *Cyclotella* to drive expression of the neomycin phosphotransferase gene for selection. Soon thereafter reports appeared describing transformation protocols for *P. tricornutum* (Apt et al. 1996, Falciatore et al. 1999), *Cylindrotheca fusiformis* Reimann & J.C.Lewin (Fischer et al. 1999) and *T. pseudonana* (Poulsen et al. 2006) based on selection using the bacterial *sh ble* gene conferring resistance against phleomycin/Zeocin. A general purpose transformation vector was constructed for *P. tricornutum* possessing a resistance cassette and a multiple cloning site for the uptake of reporter genes both driven by endogenous Fcp promoters (Zaslavskaia et al. 2000). In all described protocols diatom cells are transformed by biolistic bombardment (Sanford et al. 1993). For this procedure DNA is precipitated on tungsten particles of defined sizes, and the particles are subsequently shot onto the diatoms which before have been spread in a monolayer on an agar plate (Kroth 2007). Other procedures that were successfully deployed in *Chlamydomonas* like shaking of the cells with glass beads (Kindle

1990) or silica carbide whiskers (Dunahay 1993) and electroporation (Shimogawara et al. 1998) have not yet been successful in diatoms.

PLASTID TRANSFORMATION

As plastids contain genomes encoding various proteins involved in photosynthesis there have been successful efforts to establish a plastid transformation system for green algae and for land plants (Bateman & Purton 2000, Bogorad 2000). Because due to the low size of plastid genomes, integration or exchange of genes is mainly based on homologous recombination (Bateman & Purton 2000, Bogorad 2000). We have performed experiments to transform *P. tricornutum* with a modified plastidic psbA gene encoding the D1 protein of photosystem II. These modifications were supposed to alter the Q_B binding pocket of D1 reducing binding of the inhibitor DCMU. By screening on DCMU plates, we obtained several colonies that were resistant against high concentrations of DCMU (Materna et al., in preparation). Interestingly, the obtained cells all showed mutations within the Q_B binding pocket, but which not necessarily were identical to the fragment used for transformation, indicating that the obtained colonies were rather induced mutants instead of transformants. Control experiments performing bombardments with and without particles, with and without DNA, and with wild type or modified DNA fragments demonstrated that the mutations were only found when modified DNA fragments were shot into the cells and that these mutations were only in the area of the transformed gene, indicating that recombination processes might occur between wild type and modified genes, resulting in aberrant randomly mutated genes (Materna et al., in preparation). So far similar effects have only been described in bacteria (He et al. 2006), thus future work will have to show whether real targeted mutagenesis is feasible in diatoms.

NUCLEAR GENE SILENCING

In addition to expression of a foreign gene, another powerful benefit of molecular genetics is the possibility to knock-out or silence (knock-down) nuclear genes. One approach to achieve this goal can be the targeted disruption of a gene by insertion of a gene fragment or by replacement of the gene by a non-functional copy. Both approaches do not yet work in diatoms, probably because (i) vegetative diatom cells are diploid, thus if a gene should be knocked out there would still be another allele present, (ii) they require homologous recombination, a process by which the endogenous gene is replaced by a modified copy. Screening for such rare events requires transformation rates which are not yet available for diatoms. Another approach to silence a gene is by expression of antisense RNA or by RNAi resulting in a targeted destruction of the mRNA of the respective gene (van Dijk et al. 2005). In order to establish silencing in diatoms, we have utilized the diadinoxanthin de-epoxidase (DDE), an enzyme which is located in the thylakoids and which is involved in the protection of the cells against light stress by non-photochemical quenching (NPQ) (Lavaud, in press). This gene is ideal for a first approach, because even when the expression of the gene product is inhibited the cells should not be affected and should survive at low light conditions. We have designed different constructs that either contain DDE fragments of different lengths cloned in inverse orientation (antisense) or a complete gene together with an inverse gene separated by a spacer (RNAi) and expressed them in *P. tricornutum* (Maternal et al., in preparation). We found that both approaches usually resulted in a smaller amount of DDE transcripts and a lower NPQ indicating that the activity of the DDE is strongly reduced (data not shown). Interestingly we found that the phenotype of the RNAi transformants was stable over a period for more than a year, while the antisense constructs were stable for a few months only. This is the first promising example of gene silencing in diatoms, therefore further work will have to prove whether this method will allow the general knock-down of genes in diatoms. Especially in those cases where the gene products are essential for the cells, tight and inducible promoters will be needed to make sure that in the transformation, selection and recovery phases the RNAi constructs are not expressed.

DNA QUANTIFICATION

Ever since the existence of organellar genomes replicating and segregating independently from the nuclear chromosomes was reported, the apparently high ploidy levels of these genomes have been subject to controversy. It is still unclear whether changes in plastid genome copy numbers occur during development and aging of plastids, whether variable copy numbers of organellar genomes may regulate gene expression, and whether growth phase dependent changes in copy numbers may influence the evolution of organellar genomes. A crucial method to address these questions is the precise quantification of organellar genes or genomes as well as the visualization of segregating units within organelles. The need for accuracy and high throughput applicability to elucidate consequences of changing environmental conditions or the cell cycle on organellar ploidies further complicates the design of appropriate tools. We developed a protocol that allows the simultaneous determination of ploidy levels, i.e. genome copy numbers, in plastids and mitochondria. Relying on SYBR Green detection-based quantitative real-time PCR (qPCR) individual organellar genome copy numbers were quantified relative to the amount of nuclear genomes as a calibrator (Materna et al., in preparation). Analogous to the mathematical model for relative quantification presented by Pfaffl (2001) we derived a new algorithm, termed *GenQ*, that computes relative quantities of genes or genomes from raw data taking into account PCR efficiencies. We found that in *P. tricornutum* may contain more than 100 genome copies, while the mitochondrial network may harbour more than 600 genome copies, together representing more than 40 % of the cell's total DNA content. We demonstrated that organellar ploidies indeed may change due to culture conditions. Furthermore specific *in vivo* imaging of plastid nucleoids was achieved for the first time in an alga. Two different plastid phenotypes were identified containing either one or several nucleoids. The presented techniques support current research by providing access to the monitoring of organellar ploidies and spatial distributions of nucleoids.

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