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RESEARCH ARTICLE

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The peculiar distribution of class I and class II aldolases in diatoms and in red algae

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Abstract Diatom plastids probably evolved by secondary endocytobiosis from a red alga that was taken up by a eukaryotic host cell. Apparently, this process increased the complexity of the intracellular distribution of metabolic enzymes. We identified genes encoding fructose-bisphosphate aldolases (FBA) in two centric (*Odontella sinensis, Thalassiosira pseudonana*) and one pennate (*Phaeodactylum tricornutum*) diatoms and found that four different aldolases are present in both groups: two plastid targeted class II enzymes (FBAC1 and FBAC2), one cytosolic class II (FBA3) and one cytosolic class I (FBA4) enzyme. The pennate *Phaeodactylum* possesses an additional plastidic class I enzyme (FBAC5). We verified the classification of the different aldolases in the diatoms by enzymatic characterization of isolated plastids and whole cell extracts. Interestingly, our results imply that in plastids of centric and pennate diatoms mainly either class I or class II aldolases are active. We also identified genes for both class I and class II aldolases in red algal EST databases, thus presenting a fascinating example of the reutilization and recompartmentalization of different aldolase isoenzymes during secondary endocytobiosis but as well demonstrating the limited use of metabolic enzymes as markers for the interpretation of phylogenetic histories in algae.

Keywords Aldolase · Diatom · Intron · Plastid · Targeting

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Introduction

The eukaryotic cell is strongly compartmentalized. Hence, metabolic reactions that occur in different compartments often utilize individual isoenzymes. Prominent isoenzymes in plants are found to be involved in primary reactions in the cytosol as well as in the chloroplasts (Martin and Herrmann 1998). One of these isoenzymes is the fructose-1,6-bisphosphate aldolase (FBA, EC 4.12.1.13). This enzyme catalyzes the reversible aldol condensation of dihydroxyacetone-3-phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) to fructose-1,6-bisphosphate (FBP), an essential reaction in glycolysis, the Calvin cycle (reductive pentose phosphate pathway), and gluconeogenesis (Martin et al. 2000). Two different types of aldolases named class I and class II enzymes are known from prokaryotic as well as from eukaryotic organisms (Marsh and Lebherz 1992). The two classes do not share any significant sequence similarity and are characterized by different enzymatic mechanisms. Class I enzymes form a Schiff-base with the substrate via condensation of the ε-amino group of an active-site lysine residue with the carbonyl group of the substrate. Class II enzymes require bivalent cations, usually Zn^{2+}, as co-factors and can be inhibited by EDTA. Class I enzymes are homotetramers, whereas class II enzymes are homodimers. Structural analyses (Blom and Sygusch 1997), sequence alignments (Pelzer-Reith et al. 1993) and enzymatic characterization (Flechner et al. 1999) of both enzyme types clearly demonstrate that class I and class II enzymes are phylogenetically unrelated to each other; thus, they evolved convergently. Class II enzymes are further separated into two families, type "A" and "B", most probably due

to an early duplication within eubacteria (Henze et al. 1998).

The phylogenetic distribution of class I and II FBA enzymes is very complex. Class I enzymes are found within plastids and the cytosol of plants (Martin et al. 1996), green algae (Schnarrenberger et al. 1994; Pelzer-Reith et al. 1995) and red algae (Gross et al. 1999) as well as in the cytosol of metazoa. Fungi rely solely on class II enzymes (Mutoh and Hayashi 1994) and archaea on class I enzymes (Siebers et al. 2001), while in eubacteria both types can be found (Witke and Götz 1993). So far, *Euglena gracilis* is exceptional among eukaryotes as it contains a plastidic class I aldolase while a class II enzyme is located in the cytosol (Plaumann et al. 1997). On the other hand, the glaucophyte *Cyanophora paradoxa* has a plastidic class II enzyme operating the Calvin cycle (Gross et al. 1994). In cyanobacteria, which represent the prokaryotic ancestors of plastids (Rodríguez-Ezpeleta et al. 2005), both classes can be found in the respective genome database (http://www.kazusa.or.jp/cyanobase/); it is unknown, however, which of both is used primarily. Hitherto, red algae were thought to lack class II enzymes at all (Gross et al. 1999).

Diatoms (Bacillariophytes) are particularly interesting due to their importance for the global carbon cycles in the oceans (Treguer et al. 1995; Smetacek 1999) as well as to their evolution by secondary endocytobiosis. While plastids of glaucophytes, red algae, green algae and vascular plants are probably directly derived from a primary endocytobiosis event, in which a prokaryotic cyanobacterium was taken up by a eukaryotic host cell (for an actual discussion about a mono- or polyphyletic origin of plastids, see Palmer 2003), diatoms and other heterokont algae are supposed to have evolved in an even more complex fashion. In a secondary endocytobiosis event, a eukaryotic alga, probably an ancestor of modern red algae, was taken up by a eukaryotic host cell and subsequently transformed into a plastid (Delwiche and Palmer 1997; McFadden 2001; Kroth 2002). Thus, in heterokonts, two eukaryotic cells including their genomes and enzyme equipments have been recombined, creating a chimeric cell with a highly increased complexity. While the photosynthetic chloroplast was retained, most other organelles and cytosolic structures of the endosymbiont vanished during this process. Most of the nuclear genes of the endosymbiont were probably redundant to the host genome and therefore also were lost, while especially—but not solely—nuclear genes encoding plastid proteins were moved intracellularly into the nucleus of the host cell (Martin and Herrmann 1998).

Early experiments on aldolase activity in diatoms indicated different types of aldolases being dominant in pennate and centric diatoms, respectively (Antia 1967). In order to investigate the distribution of isoenzymes in diatoms, we cloned and sequenced cDNAs encoding plastidic aldolases from the diatoms *Odontella sinensis* and *Phaeodactylum tricornutum* and analyzed enzymatic activities in plastidic and cellular extracts. In a previous work, we showed that diatom plastid aldolase genes might have been substituted by genes donated by lateral gene transfer (Kilian and Kroth 2004). Similarly, Patron et al. (2004) recently discussed the common origin of chromaveolates on the basis of the presence of class II aldolases. However, the occurrence of genes for class II aldolases in the red algae *Chondrus crispus* and *Porphyra yezoensis* and in diatoms more likely points to an ancestral ubiquitous presence of class II and class I isoenzymes in algae derived by primary endocytobiosis. According to our data, class I and maybe class II aldolases of both endosymbiont and host have been utilized and recompartimentalized during secondary endocytobiosis, while some isoenzymes possibly were lost in various genera. Here we report an actual survey of class I and class II aldolases in diatoms and present a functional characterization of the different isoenzymes.

Material and methods

Cultivation of diatoms

Odontella sinensis (Grev. Grunow) is an isolate from the North Sea and was cultivated axenically in artificial seawater (Tropic Marin, Wartenberg, Germany) as described (Pancic and Strotmann 1990). The cells were grown at 16°C in 10-l flasks at a light density of 15 μE $m^{-2}s^{-1}$ in a 14:10 h light:dark cycle. Cells were harvested through a 50-μm gauze and washed with 3.5% NaCl solution prior to preparation. *P. tricornutum* (Bohlin, University of Texas Culture Collection, strain 646) was grown at 22°C with continuous illumination at 35 μE $m^{-2}s^{-1}$. The media consisted of Provasoli's enriched seawater (Starr and Zeikus 1993) using "Tropic Marin" artificial seawater at 50% concentration, compared to natural seawater. Solid media contained 1.2% Bacto Agar (Diffco).

Isolation of genomic DNA

Cells were pelleted as described above and one volume of 2 × CTAB-buffer was added (100 mM Tris/HCl pH 8, 1.5 M NaCl, 20 mM EDTA, 2% cetyltrimethylammonium bromide (CTAB), supplemented with 0.2% β-mercaptoethanol prior to use). The mixture was incubated for 1 h at 65°C under gentle shaking, supplemented with one volume chloroform:isoamyl alcohol and incubated for one additional hour under gentle shaking at room temperature. After centrifugation (15 min, 3,000 g, room temperature) the liquid phase was mixed with the same volume of isopropanol and the DNA precipitated by incubation at −20°C overnight. The DNA was pelleted by centrifugation (4°C, 30 min, 10,000 g) and subsequently washed with ethanol (70%). After gentle air drying the DNA was resuspended in TE-buffer and stored at 4°C.

Genetic transformation of *Phaeodactylum tricornutum*

Transformation was performed according to the protocol described in Zaslavskaia et al. (2000). In contrast to the described procedure, Tungsten particles with an average size of 0.7 μm (M10, BioRad, Hercules) and a helium pressure of 1350 psi were employed. Cells were selected on plates containing 75 μg/ml Zeocin (Invitrogen) under continuous light for 2–3 weeks. Transformants were replated on Zeocin plates and were checked for GFP fluorescence. Fluorescence microscopy of transformed diatoms was performed with an Olympus BX51 fluorescence microscope equipped with a Nikon DMX-1200 camera using the filter sets HQ480/20 for GFP-, and U-MWSG2 for chlorophyll-fluorescence (Olympus, Hamburg).

Cloning of the *Fba* genes from diatoms

For the identification of *Fba* genes from the diatom *O. sinensis*, a cDNA library was utilized as described in Pancic and Strotmann (1993). Random sequencing of clones resulted in one fragment displaying homology to *Fba* sequences. This cDNA was completely sequenced and PCR primers were designed according to the 5́- and 3́-end of the *Fba* gene. With these primers the respective genomic region were amplified from genomic DNA from *O. sinensis* using the "High Fidelity Expand" PCR Kit (Roche) according to standard protocols. The PCR product was purified by gel elution and directly cloned into the pGEM-T vector (Promega) for further sequencing. For cloning of the *Fba* genes from *Phaeodactylum*, EST sequences from a mass-sequencing program were scanned and positive sequences were used for isolation of the complete genes by RACE-PCR. Total RNA from *Phaeodactylum* was reverse transcribed and a poly-G tail was added at the 5-ends using a terminal transferase according to the protocol of the supplier (MBI Fermentas), but incubation occurred in the presence of 1 mM dGTP for 30 min. The modified cDNA was used as template for PCR employing a Poly-C-primer (15-mer) and a gene-specific primer.

Isolation of diatom plastids

Odontella cells were harvested on a 50-μm nylon gauze. Preparation of intact plastids was performed according to Wittpoth et al. (1998). After isolation the plastids were washed thoroughly. Morphological integrity was routinely checked by measuring CO_2-dependent oxygen evolution of the plastids in an oxygen electrode (Hansatech, Norfolk) under illumination as described before (Wittpoth et al. 1998) and after addition of $NaHCO_3$. To obtain stromal extracts, plastids were ruptured by osmotic shock for 30 s in a small volume of 50 mM Tricine, pH7, containing 1 mM of the protease inhibitor phenyl methylsulfonyl fluoride (PMSF). The plastids were additionally sheared by pipetting up and down in a narrow tip. After the plastids were completely ruptured, the thylakoids were sedimented at 15,000 *g* for 30 min and the supernatant containing the stromal extract was stored on ice for further enzymatic analysis.

Enzymatic assays

Protein concentrations of the stromal extracts were determined by Bradford assay (BioRad, Hercules) according to protocols of the manufacturer. Enzyme activity was measured in an optimized coupled test based on the protocol by Gross et al. (1999). The standard reaction assay contained 100 mM Tricine, pH 7.5; 10 mM $MgCl_2$; 1 U/ml TIM; 1 U/ml glycerole-3-phosphate dehydrogenase; NADH (0.2 mM); stromal extract (varying volumes). The decreasing concentration of NADH was monitored spectrophotometrically at $\lambda = 366$ nm. The reaction was started at 25°C by the addition of 2 mM fructose-1,6-bisphosphate. If necessary, stromal extracts were preincubated in the presence of bivalent ions (10 mM either $MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$) or 10 mM EDTA on ice for 1 h.

Phylogenetic analyses

For the calculation of phylogenetic trees, FBA amino acid sequences (without presequences) were aligned using the clustal W program, followed by a first calculation of trees by the Neighbor-Joining method (pairwise deletion) in MEGA 3.0 (Kumar et al. 2004). For a more thorough analysis, maximum likelihood calculations were performed using the web-based program PHYML with the dayhoff PAM matrix as model (Guindon and Gascuel 2003; http://atgc.lirmm.fr/phyml/). Bootstrap values were determined with a replicate number of 100.

Results

Identification and cloning of diatom *Fba* genes

Screening of a cDNA library from *O. sinensis* led to the identification of an open reading frame with similarities to class II fructose-1,6-bisphatases (*OsFbaC1*). By screening an EST database from *P. tricornutum* and subsequent combination of individual cDNAs, we additionally obtained *Fba* sequences designated *PtFbaC1*, *PtFbaC2* and *PtFba3* (Fig. 1). The respective *PtFbaC3* sequence was not complete; therefore, we isolated the respective full-length clone by applying the RACE technique.

Similarly, we identified three class II aldolase genes (named *TpFbaC1*, *TpFbaC2* and *TpFbaC3* by putative homology to the respective genes in *P. tricornutum*)

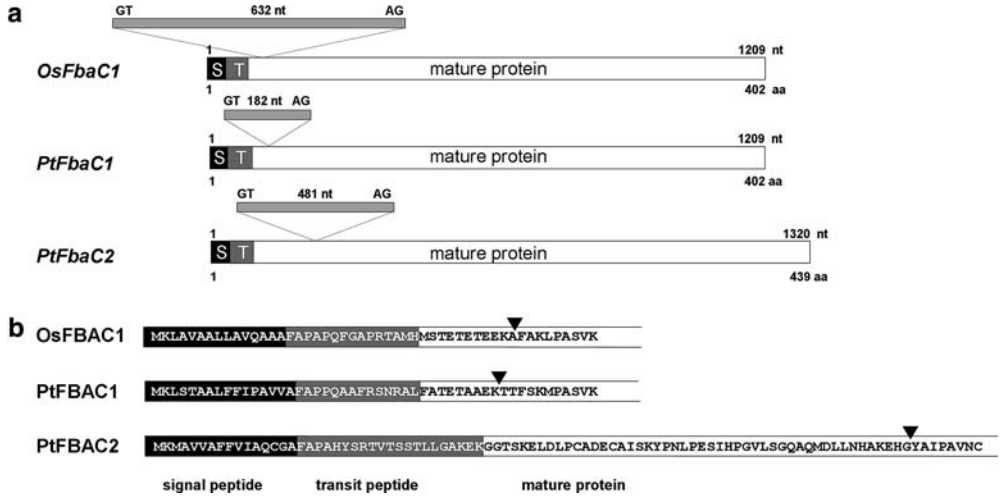

Fig. 1 Schematic presentation of the structure of aldolase genes cloned and analyzed in this work. **a** In-scale presentation of the structure of the aldolase genes showing the localization of the signal (*S*) and transit (*T*) peptide domains and the introns (*gray bar* above). Length of the introns in nucleotides (*nt*) and *GT–AG* borders are shown above the *bars*. The size of the genes and the deduced gene products is given in *nt* or amino acids (*aa*), respectively. **b** Amino acid sequences of the N-termini deduced from the *FbaC1* and *FbaC2* genes from *Odontella sinensis* (*Os*) and *Phaeodactylum tricornutum* (*Pt*). A *vertical line* indicates putative cleavage sites between signal and transit peptides and the mature proteins. *Filled arrows* indicate the position of introns within the respective DNA strands

within the genome of the centric diatom *Thalassiosira pseudonana* by searching the respective database (accessible at http://genome.jgi-psf.org/thaps1; Armbrust et al. 2004). We could not identify genes for class II aldolases in the complete genome sequence of the red alga *Cyanidioschyzon merolae* (accessible at http://merolae.biol.s.u-tokyo.ac.jp/; Matsuzaki et al. 2004), but could identify several overlapping ESTs encoding class II aldolases of *Chondrus crispus* (Genbank) and *Porphyra yezoensis* (Nikaido et al. 2000, accessible at http://www.kazusa.or.jp/).

Looking for class I aldolases in the genomes of the diatom *Thalassiosira* and the red alga *Cyanidioschyzon* and within the EST database of *Phaeodactylum* (Maheswari et al. 2005; accessible at avesthagen.sznbowler.com), we found a single gene for a class I aldolase in *Thalassiosira* (designated *TpFba4*), two in *Phaeodactylum* (*PtFba4* and *PtFbaC5*, meanwhile having been described by Montsant et al. 2005) and two in *Cyanidioschyzon* (accordingly named *CmFba4* and *CmFbaC4*; see Table 1).

Intron sequences

We compared the cDNA fragments of *OsFbaC1*, *PtFbaC1* and *PtFbaC2* with respective DNA fragments obtained by PCR amplification using genomic DNA as template. In all three DNA fragments, we found insertions in the proximal regions starting with "GT" and ending with "AG", which is characteristic for spliceosomal introns (Fig. 1; Tolstrup et al. 1997). Introns in these positions were frequently found in diatoms (Kilian and Kroth 2004). The intron sequences found in the *Fba* genes from *Odontella* and *Phaeodactylum* have lengths of 632 bp (*OsFbaC1*), 182 bp (*PtFbaC1*) and 481 bp (*PtFbaC2*). Structural analyses revealed that they apparently cannot form significant secondary structures. Possible branching points (Tolstrup et al. 1997) have been found in all three sequences and fit a possible consensus sequence deduced from other diatom introns: NNCT(G/C)A(T/C) (Kilian and Kroth 2004). We also observed typical accumulations of thymidine bases ("pyrimidine stretches") in the 3-regions of the introns.

Table 1 Localization of class I and class II aldolases in the red algae *Cyanidioschyzon merolae* (Cm) and *Porphyra yezoenis* (Py) and in the diatoms *Phaeodactylum tricornutum* (Pt) and *Thalassiosira pseudonana* (Tp)

	Cyanidioschyzon	*Porphyra*	*Thalassiosira*	*Phaeodactylum*
Cytosolic class I	CmFBA4	PyFBA4	TpFBA4	PtFBA4
Plastidic class I	CmFBAC5	?	–	PtFBAC5
Cytosolic class II	–	?	TpFBA3	PtFBA3
Plastidic class II	–	PyFBAC2[a]	TpFBAC1 TpFBAC2	PtFBAC1 PtFBAC2

A "–" indicates that no respective gene has been identified in the complete genome
[a]The *Porphyra* FBAC2 sequence does possess the complete N-terminus of the mature protein; however, as yet, it is unclear whether the short upstream regions might represent a plastidic targeting domain

All introns are inserted in phase 0 of the respective genes. The *FbaC1* introns displayed a much lower GC content than the respective coding regions (*Odontella*: 49% GC in the intron region versus 62% in the coding region; *Phaeodactylum*: 45% GC versus 63%), while in the *FbaC2* gene the GC content of the intron is similarly high (53% GC versus 52%). We were not able to demonstrate that these introns are in fact related. Alignments of the introns are possible without introducing larger gaps, but the individual sequence identity is less than 50%.

Analysis of the *Thalassiosira* genome revealed that the introns found in the respective *FbaC1* and *FbaC2* sequences are at similar positions. In addition to these introns, *TpFbaC2* contains two other introns which are in the region encoding the mature protein (data not shown).

The identical locations of one intron in *FbaC1* of *Phaeodactylum*, *Odontella* and *Thalassiosira* and also in *FbaC2* of *Phaeodactylum* and *Thalassiosira* strongly indicate that these genes evolved—probably by the duplication of an ancestral *FBA* gene—prior to and not after the separation of pennate and centric diatoms.

Functional characterization of the plastid-targeting domains

Comparison of the amino acid sequences of diatom class I and class II aldolases with cytosolic aldolases from other organisms revealed an N-terminal extension of about 30 amino acids in all FBAC1 and FBAC2 proteins and the *Phaeodactylum* ptFBAC5 protein. Such extensions are not present in FBA3 and FBA4 and can definitely be excluded at least in PtFBA3 since a stop codon is located immediately upstream of the putative start of translation of respective *Phaeodactylum* ESTs. Similarly, putative presequences upstream of the regions encoding the mature TpFBA3 and TpFBA4 proteins could not be identified in the respective genomic DNA sequences.

Plastid-targeting presequences in diatoms apparently consist of two domains: a hydrophobic domain resembling a signal peptide for cotranslational ER import and a more hydrophilic domain with characteristics of transit peptide domains necessary for targeting proteins into plastids of green algae and higher plants (Kroth 2002). The N-terminal extensions deduced from the *FbaC1*, *FbaC2* and *FbaC5* genes show a structural similarity to a variety of other plastid-targeting domains from diatoms, especially in a conserved motif at the boundaries between signal and transit peptide (Kilian and Kroth 2005). Computer analyses [SignalP: http://www.cbs.dtu.dk/services/SignalP/ (Nielsen et al. 1999) and ChloroP: http://www.cbs.dtu.dk/services/ChloroP/ (Emanuelsson et al. 1999)] indicated a cleavage site for a signal peptidase between amino acids 15/16, 16/17 and 16/17 (OsFBAC1, PtFBAC1 and PtFBAC2, respectively), and similarly a transit peptide cleavage site between positions 30/31, 30/31 and 37/38 (Fig. 1). To verify that these presequences are in fact plastid-targeting domains, we genetically fused the complete presequences of OsFBAC1 and PtFBAC1 including a small portion (13 and 29 amino acids, respectively) of the putative mature proteins in frame to the GFP protein. The constructs were cloned into a diatom transformation vector and transformed into *Phaeodactylum* wild-type cells by particle bombardment as described previously (Zaslavskaia et al. 2000, 2001). GFP localization in transformants was analyzed by epifluorescence microscopy. Figure 2 shows that expressed GFP is clearly located inside the plastids; the red chlorophyll autofluorescence serves as a marker for the plastid boundaries. Analysis of the respective *Cyanidioschyzon* class I aldolases also revealed an N-terminal extension in CmFBAC5 which, according to ChloroP (Emanuelsson et al. 1999), is a chloroplast-targeting transit peptide. Unfortunately, the EST data for *Chondrus* and *Porphyra* aldolases were incomplete and lacked N-terminal regions.

Taken together, these results strongly suggest that the diatom FBAC1, FBAC2 and FBAC5 are directed into the respective chloroplasts, while the diatom FBA3 and FBA4 are cytosolic enzymes (Table 1).

Phylogenetic analysis of the *Fba* genes in diatoms and red algae

We have aligned the amino acid sequences of the putative mature domains of the diatom FBAC1, FBAC2, FBA3, FBA4 and FBAC5 aldolases with those from other organisms, clearly identifying FBAC1, FBAC2 and FBA3 as class II and FBA4 and FBAC5 as class I enzymes. Similarly, we identified partial genes encoding class II aldolases from the red algae *P. yezoenis*, *Gracilaria gracilis* and *C. crispus*, and class I aldolases from *C. merolae* and *Galdieria sulphuraria*. The results from maximum-likelihood analyses indicate complex evolutionary events in red algae and diatoms, including putative gene transfers and gene duplications.

Class I enzymes

We performed phylogenetic analysis with Class I aldolases from diatoms, red algae and other groups (Fig. 3). Interestingly, the diatom FBA4 sequences were grouped together with class I enzymes from the cyanobacterium *Synechocystis* PCC6803 and other eubacteria, indicating that cytosolic diatom FBA4 is a prokaryotic-type enzyme. Although the internal branches of this clade were only weakly supported by bootstrap analyses, it is obvious that FBA4 is only distantly related to the other class I enzymes that are clustered together with FBAC5. It is also important to note that we could not identify FBA4 homologs in other genomes of cyanobacteria published so far. In contrast, the plastidic PtFBAC5 aldolase apparently is more closely related to plastidic

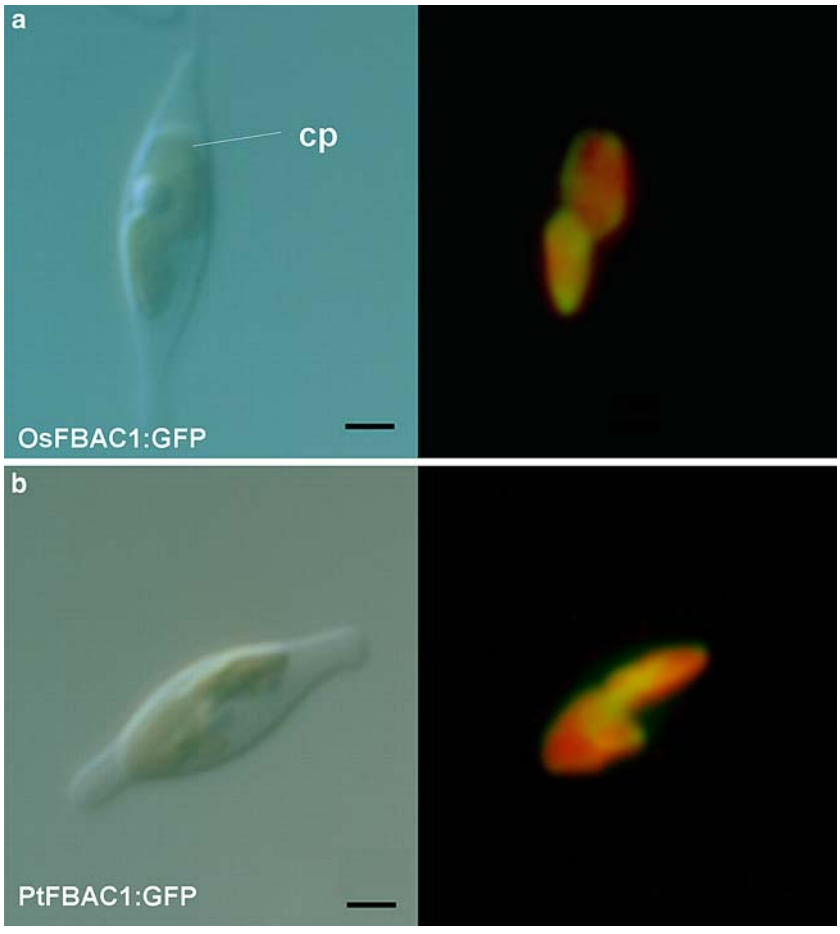

Fig. 2 In vivo targeting of FBAC1-presequence: GFP fusion protein from *Odontella sinensis* (**a**) and from *Phaeodactylum tricornutum* (**b**). *Left*: Light microscopical images of cells showing the pigmented plastids (*cp*). *Right*: Corresponding fluorescence image (*green*: *GFP* fluorescence; *red*: chlorophyll autofluorescence) obtained by UV excitation. The *scale bars* at the lower right corners represent 2 μm

red algal sequences, thus probably representing the original plastidic aldolase of the rhodophytic endosymbiont. Interestingly, no FBAC5 was found in the genome of *Thalassiosira*, indicating that the respective gene might have been lost in centric diatoms. We also found a class I aldolase closely related to PtFBAC5 by screening EST sequences of the dinoflagellate *Karenia brevis* (Fig. 3).

Class II enzymes

Individual comparisons of the diatom class II aldolase sequences with enzymes from other organisms resulted in similarities between 34 and 60% identical amino acids for class II aldolases of the A-type from bacteria and fungi, while the similarity to B-type enzymes was significantly lower (15–20%). Sequence identity between the FBA sequences of the same type (e.g., FBAC1) within diatoms was rather high (68–86%), whereas the identity between the different isoforms (e.g., FBAC1 vs. FBAC2) was lower (47–59%). It is likely that FBAC1 and FBAC2 arose by a duplication probably before the divergence of the ancestors of diatoms. This view is supported by the close relationship of the diatom FBAC1 sequences to the respective aldolases in dinoflagellates (Fig. 4). Interestingly, the high divergence of the cytosolic FBA3 sequences compared to the other type A-aldolases and the closer relationship to fungi and oomycetes implies that FBA3 may represent the original cytosolic enzyme of the secondary host rather than originating from duplication of FbaC1/2 genes. Interestingly, no type A-aldolases have been identified yet in cyanobacteria. According to our analysis, the class II FBAC1/2 enzymes from diatoms, cryptophytes and dinoflagellates are most closely related to respective γ-proteobacterial enzymes and next to red algal class II enzymes. However, the relative position to the red algal class II enzymes was not clearly resolved in different phylogenetic analyses we performed. Thus, it remains unclear whether all FBAC1/2 sequences of diatoms/cryptophytes and dinoflagellates might have originated either by lateral gene transfer from a proteobacterium, from the plastid of the red algal endosymbiont or from the cytosolic FBA of the oomycete host cell.

Taken together, the phylogenetic data clearly indicate that diatoms as well as red algae possess both class I and class II aldolases. The different patterns of presence or absence of different aldolase classes in red algae and diatoms—i.e., the presence of (1) a class II aldolase in *Porphyra* but not in *Cyanidioschyzon* and (2) of a plastidic class I aldolase in *Phaeodactylum* but not in *Thalassiosira*—therefore illustrates that specific aldolase

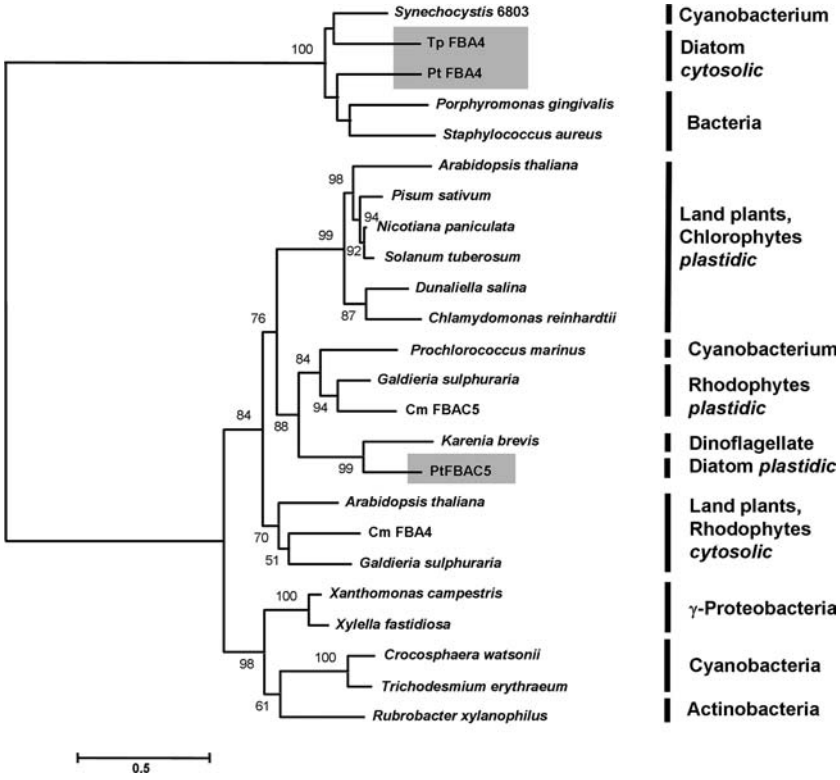

Fig. 3 Maximum likelihood phylogeny of class I aldolases from different organisms. *Synechocystis* sp PCC6803 (Genbank #NP_4417239), *Thalassiosira pseudonana* (according to Tp genome database), *Porphyromonas gingivalis* (NP_905858), *Staphylococcus aureus* (NP_370130), *Arabidopsis thaliana* (NP_974710), *Pisum sativum* (Q01517), *Nicotiana paniculata* (BAA77603), *Solanum tuberosum* (#T07418), *Dunaliella salina* (# AAM76969), Chlamydomonas reinhardtii (#CAA59590), Prochlorococcus marinus (YP_291383), *Karenia brevis* (EST #C005991), *Galdieria sulphuraria* (AAF27641), *Cyanidioschyzon merolae* (according to Cm genome database), *Arabidopsis thaliana* (NP_850759), *Galdieria sulphuraria* (AAF27640), *Xanthomonas campestris* (AAY48053), *Xylella fastidiosa* (ZP_00681287), *Crocosphaera watsonii* (ZP_00517428), *Trichodesmium erythraeum* (ZP_00675751), *Rubrobacter xylanophilus* (ZP_00600636). If known, the intracellular location is given. The diatom sequences from this work are boxed in *gray*. Bootstrap values for 100 calculations are given at the respective nodes if higher than 50

distribution in diatoms and modern red algae might be due to independent gene duplications, transfer processes and gene losses that are not directly related to secondary endocytobiosis.

Enzymatic characterization of diatom aldolases

To analyze the abundance of class I and class II aldolases in the chloroplasts and the cytosol of diatoms, we measured aldolase activity in cell and stromal extracts, respectively, and investigated the amount of class I and class II aldolase activity by specific inhibition of class II aldolases by EDTA. So far, we could isolate intact chloroplasts only from the centric diatoms *O. sinensis* and *Coscinodiscus granii* (Wittpoth et al. 1998). We purified intact plastids from *Odontella* and subsequently broke them by osmotic shock as described in Materials and methods. The specific aldolase activity of stromal *Odontella* extract was rather low (0.4 mU mg^{-1} protein) compared to the activity of cellular extracts (26 mU mg^{-1} protein). One hour of incubation with 10 mM EDTA led to a complete inhibition of FBA activity in stromal extracts (Fig. 5a). This effect was reversible: subsequent addition of Mn^{2+}- or Mg^{2+}-ions in higher concentrations than the added amount of EDTA recovered aldolase activity. Comparative analyses of stromal extracts from isolated spinach plastids did not show significant effects of bivalent cations or EDTA on the aldolase activity (data not shown). This is in agreement with a previous work demonstrating that plastidic class I aldolases from land plant plastids do not depend on bivalent cations (Flechner et al. 1999).

Our data clearly show that class II aldolases are the dominant, if not the only FBAs in plastids of *O. sinensis* at standard growth conditions. Analyses of the aldolase activity in stromal extracts at various pH values revealed a narrow pH range for maximal activity. Maximum activity was observed at pH 7.5 while only 50% of the activity remained at pH 6.7 or 8.5, respectively (data not shown). A rather small pH tolerance is also typical for class II-enzymes (Rutter 1964). In control experiments,

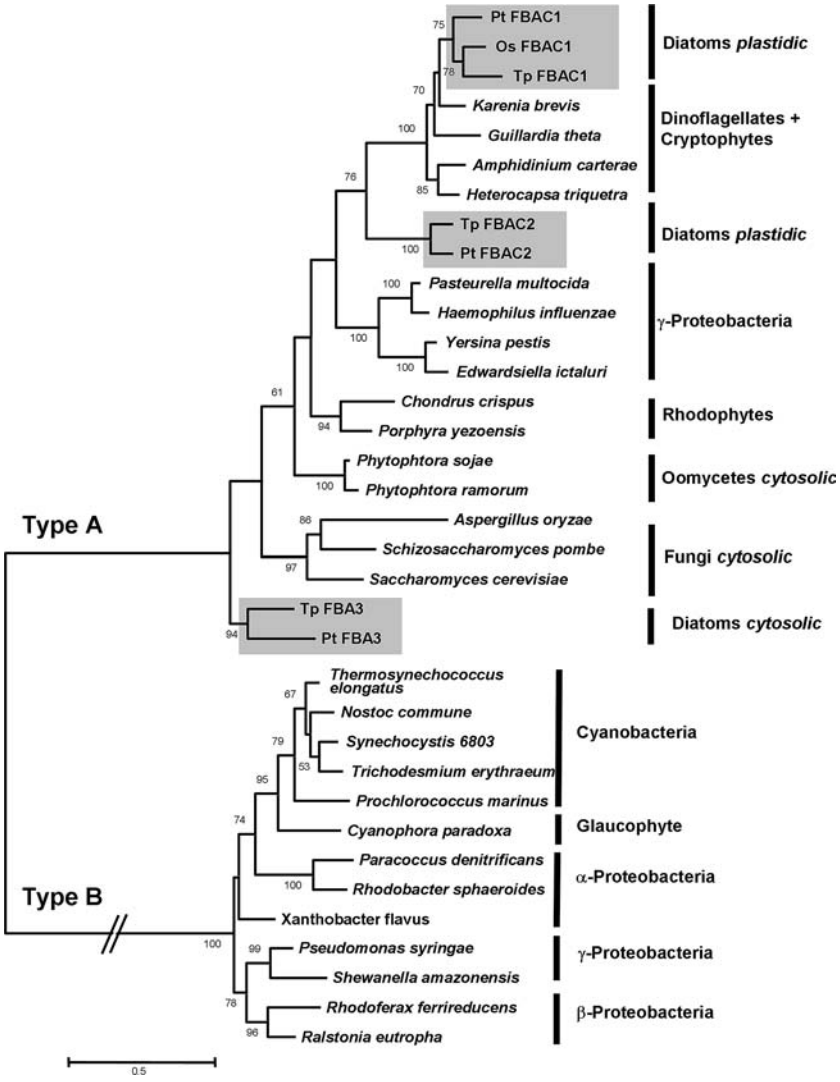

Fig. 4 Maximum likelihood phylogeny of class II aldolases from diatoms and other organisms. *Phaeodactylum tricornutum* (Genbank AAO43196), *Odontella sinensis* (AAM66752), *Thalassiosira pseudonana* (according to Tp genome database), *Karenia brevis* (ESTs C0064997, C0063474, C0061711, CV173752), *Guillardia theta* (AAV71138), *Amphidinium carterae* (Genbank ESTs CF065713, CF065864, CF065854, CF067842, CF0667809), *Phaeodactylum tricornutum* (AAO43262), *Pasteurella multocida* (NP-246800), *Haemophilus influenzae* (AAX87573), *Yersinia pestis* (NP_670606), *Edwardsiella ictaluri* (O52402), *Chondrus crispus* (ESTs C0649769, C0653005, C06549763, C0650873, C0650844, C0649362), *Porphyra yezoensis* (ESTs AU189958, AU186953, AU192994, AU189590, AU188334, AU189386), *Phytophtora sojae*/*ramorum* (according to the *Phytophtora sojae*/*ramorum* genome databases at JGI), Aspergillus oryzae (Q9HGY9), *Schizosaccharomyces pombe* (P36580), *Saccharomyces cerevisiae* (NP_012863), *Thermosynechococcus elongatus* (NP_681166), *Nostoc commune* (Q9XPD3), *Synechocystis* sp PCC 6803 (Q5564), *Trichodesmium erythraeum* (ZP_00671396), *Prochlorococcus marinus* (NP_892899), *Cyanophora paradoxa* (CAB46249), *Paracoccus denitrificans* (EAN67008), *Rhodobacter sphaeroides* (P29271), *Xanthobacter flavus* (Q56815), *Pseudomonas syringae* (AAZ33250), *Shewanella amazonensis* (EAN40289), *Rhodoferax ferrireducens* (EAO40944), *Ralstona eutropha* (YP_294783). If known, the intracellular location is given. The diatom sequences from this work are boxed in *gray*. Bootstrap values for 100 calculations are given at the respective nodes if higher than 50

stromal class I enzymes from spinach also shared a pH optimum at pH 7.5, but still revealed 70% of maximal activity at pH 10.0 (data not shown).

In contrast, in total cell extracts of *Thalassiosira* and *Odontella* aldolase activity was not completely inhibited by EDTA (Fig. 5b). These results suggest that a considerable amount of class I aldolase activity is present in both centric diatoms. As no class I aldolase activity remained in stromal extracts incubated with EDTA, class I aldolases thus are located only within the cytosol of *Odontella*. Interestingly, aldolase activity of *Phaeodactylum* cell extracts was not affected considerably when preincubated with EDTA, indicating the predominance of class I aldolases (data not shown).

We also analyzed the dependence of diatom aldolase activity in total extracts on bivalent cations (Fig. 5c). Preincubation of bivalent cations dramatically increased aldolase activity within the stromal *Odontella* extracts up to 30-fold when manganese cations were added, implying a loss of ions during the preparation of the cell

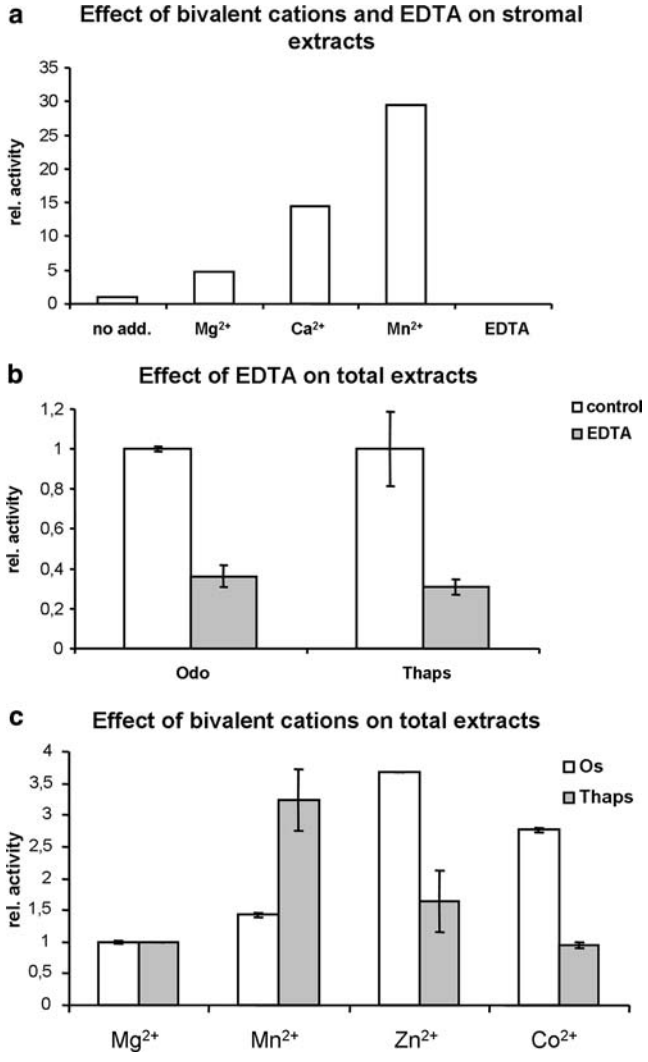

Fig. 5 **a** Relative activity of fructose-1,6-bisphosphate aldolase in stromal extracts of *Odontella sinensis* purified as described in Materials and methods. The stromal extracts were preincubated for 1 h without (set as 1) or with either 10 mM Mg^{2+}, Ca^{2+}, Mn^{2+}, and EDTA, respectively as indicated. **b** Enzymatic activity of fructose 1,6-bisphosphate aldolases in total cellular extracts of *O. sinensis* (*Odo*) and *Thalassiosira pseudonana* (*Thaps*). Standard deviation is given as *bars*. **c** Effect of different bivalent ions on the enzymatic activity of fructose-bisphosphate aldolase in cellular extracts from *Odontella* and *Thalassiosira*. Extracts were incubated for 1 h in 10 mM of ions as indicated. The activity in the presence of Mg ions was set as 1. Standard deviations are given as *bars*

extract. Incubation of *Thalassiosira* cell extracts with different bivalent cations revealed Zn^{2+} as the preferred ion. Preincubation of cellular extracts from *Phaeodactylum* with different bivalent cations had no effect on the aldolase activity (data not shown).

Discussion

Primary and secondary endocytobioses are regarded as the permanent establishment of prokaryotic and eukaryotic photoautotrophs, respectively, within heterotrophic eukaryotic host cells. The evolution of organelles from free-living organisms following endocytobiosis must have involved extensive transfer of genetic material from the endosymbiont to the nucleus of the host organism (Martin and Herrmann 1998). Although still controversial (Stiller 2003), substantial evidence suggests that all plastids can be traced back to a single primary endocytobiosis event (which led to the evolution of plastids in green algae and land plants, red algae and glaucophytes; see Delwiche and Palmer 1997; Moreira et al. 2000; Palmer 2003), whereas secondary endocytobioses probably occurred several times independently, resulting in even more algal lineages (Cavalier-Smith 2000; McFadden 2001; Palmer 2003). Secondary endocytobioses dramatically enhanced cellular complexity by combining two different eukaryotic organisms including their genomes and their cellular structures. In most secondary algae the nucleus of the eukaryotic endosymbiont disappeared during evolution; therefore, various genes of the endosymbiont must have been transferred to the nucleus of the secondary host before the nucleus of the endosymbiont vanished. For example, most of the nuclear genes for plastid proteins originally were donated by the prokaryotic endosymbiont during primary endocytobiosis, and then, during secondary endocytobiosis, had to be transferred again from the nucleus of the endosymbiont to the nucleus of the secondary host to prevent a loss of those genes and the respective gene products (Nisbet et al. 2005). This process also infers that the gene products of such relocated genes now had to be targeted from the new site of biosynthesis into the complex plastids. The mechanism of protein targeting into plastids surrounded by four membranes is still obscure, although individual transport steps have been identified (Bhaya and Grossman 1991; Lang et al. 1998; Waller et al. 2000; Apt et al. 2002). Diatom preproteins contain three signals important for targeting into the complex plastids: a hydrophobic signal peptide for cotranslational transport across ER membranes, a second domain resembling transit peptides of land plant plastids, and a conserved motif which is present between these two domains (Kilian and Kroth 2005). All three signals were also found in the plastidic FBA sequences analyzed in this work.

We identified cDNAs for genes encoding plastidic class I and II aldolases in the pennate diatom *P. tricornutum* as well as in the centric diatom *O. sinensis*. Together with the sequence information of the *Thalassiosira* genome and of the *Phaeodactylum* ESTs available in Genbank, we found four aldolases in centric diatoms: two plastid-targeted class II aldolases (FBAC1 and FBAC2), one cytosolic class II aldolase (FBA3) and one cytosolic class I enzyme (FBA4). Additionally, we were able to identify another class I enzyme in the pennate diatom *Phaeodactylum* (ptFBAC5) possessing an N-terminal extension typical for plastid-targeted proteins. Thus, in *Phaeodactylum*, class I aldolases might operate in the cytosol and in the plastids, whereas in *Thalassiosira* class I enzymes operate in the cytosol only (Table 1).

One of the most interesting aspect of our findings is that the various types of aldolases in diatoms apparently did not all evolve simply by gene duplications within the diatoms but rather might have different origins. It is also striking that bacterial sequences can be found in all different main clades of class I as well as class II aldolases. This might be seen as an indication that some of the aldolase genes we find in algae and plants may have been transferred by lateral gene transfer from bacteria.

The plastidic FBAC5 aldolase of *Phaeodactylum* appears to be related to plastidic class I aldolases of red algae. Therefore, we suggest that this enzyme represents the original plastidic enzyme of the former rhodophytic endosymbiont which apparently was subsequently lost in centric diatoms. On the other hand, the cytosolic FBA4 enzyme shares highest similarity with bacterial enzymes and not with plastidic or cytosolic enzymes of red algae or land plants, indicating that it might have been acquired laterally from bacteria rather late in evolution. This is a more parsimonious assumption than a loss of this gene in red algae and in land plants (no genes encoding proteins closely related to FBA4 were found in the *Cyanidioschyzon* as well as in the *Arabidopsis* genome). However, this question might be solved when enough EST sequences or full genomes are available from algae like phaeophytes, cryptophytes and dinoflagellates.

Initially, the finding of class II aldolases in diatom plastids was surprising because diatom plastids evolved from red algae by endocytobiosis and *Galdieria*, the only red alga where FBAs were analyzed enzymatically so far, was described to possess class I aldolases only (Gross et al. 1999; Nikaido et al. 2000). This was consistent with the analysis of the genome of the red alga *C. merolae* which contains genes for cytosolic (CmFBA4) and plastidic (CmFBAC5) class I aldolases only. However, by analyzing EST databases, we found sequences encoding class II aldolases in *P. yezoensis, C. crispus* and *G. gracilis*, indicating that at least some rhodophytes do possess class II enzymes. Patron et al. (2004) interpreted the presence of class II aldolases in some red algae as an independent acquisition in the common ancestor of the floridophytes and bangiophytes after the divergence of the cyanidiales. However, in the light of the presence of class II aldolases in some rhodophytes (and their phylogenetical relationship to diatom class II enzymes), in cyanobacteria as well as in the glaucophyte *Cyanophora paradoxa*, it might be more reasonable to argue that both class II and class I enzymes were present in the common ancestor of red algae, whereas the class II enzyme may have been lost in the closely related cyanidiales *Galdieria* and *Cyanidioschyzon*. Due to the compactness of the *Cyanidioschyzon* genome, it is reasonable to assume that redundant aldolases genes have been eliminated. The presence of only class I but not class II aldolases might also be coincidental with the extreme acidic environment of these thermophilic algae, resulting in a preference for the more pH-tolerant metal independent class I aldolases. This together with the presence of class I and Class II enzymes in diatoms and in dinoflagellates does not exclude a shared origin of the proposed chromalveolata (Cavalier-Smith 2003), but demonstrates that the presence of class II aldolases in different members of the chromalveolata may not serve as an actual proof as proposed by Patron et al. (2004).

More obvious is our finding that FBAC1/C2 evolved by duplication of an ancestral *Fba* gene, which took place prior to the divergence of pennate and centric diatoms or even the heterokontophytes (Fig. 4). Unfortunately, the resolution of the phylogenetic trees in Patron et al. (2004) as well as in this work is not good enough to really resolve the ancestry of these class II aldolases. There are two likely scenarios: (1) The ancestor of the *FbaC1* and *FbaC2* genes was taken up by lateral gene transfer from a bacterium and was duplicated after secondary endosymbiosis. One example of such a lateral gene transfer in algae might be the presence of bacterial-type RubisCo in red and in chromophytic algae (Palmer 1993). Recent ultrastructural work on the close intracellular association of bacteria and diatoms (Schmid 2003) has stimulated the discussion about interactions between bacteria and eukaryotic algae in the early history of algal lineages, which might have promoted lateral gene transfer (Kowallik 2003). (2) A second possibility is the utilization of a gene from the secondary host cell. An ancestor of modern oomycetes is thought to have served as a host cell in secondary endocytobiosis that led to the evolution of the heterokont algae including the diatoms (Cavalier-Smith 2000). Unfortunately, there are no reports on aldolase activities in oomycetes, but genes encoding class II aldolases with high sequence similarity to the diatom FBAs are found in the genome of the oomycetes *Phytophtora sojae* and *Phytophtora ramorum*.

In an early report it was shown that in acetonic cell extracts from diatoms either mainly class I or class II enzyme activity can be found (Antia 1967). In this report aldolase activity in whole cell extracts of *Phaeodactylum* were not inhibited considerably by EDTA, indicating that these diatoms either do not contain class II enzymes at all or just in minor quantity. This result was surprising, since *Phaeodactylum* apparently expresses plastidic class II enzymes (FBAC1/2) just as the centric diatoms *Odontella* and *Thalassiosira* do. We therefore had a closer look at the cytosolic FBAs at the public *Thalassiosira* and *Phaeodactylum* EST databases and found that in *Phaeodactylum* the cytosolic class I enzyme (FBA4) is highly represented, whereas in *Thalassiosira* only one EST sequence for the class I enzyme is present. Additionally, *Phaeodactylum* possesses another plastid-targeted class I enzyme (ptFBAC5). In *Phaeodactylum*, ptFBAC5 might account for most of the plastidic aldolase activity, which presumably is maintained exclusively by class II enzymes in centric diatoms.

Aldolase activity in whole cell extracts of *O. sinensis* and *T. pseudonana* increased after preincubation with bivalent cations, indicating the presence of class II aldolases. EDTA treatment of the same cell extracts led

to a drastic but not complete decrease of aldolase activity, suggesting that in these diatoms both types of aldolases are active. In contrast, a complete inhibition of aldolase activity in stromal extracts of *Odontella* by EDTA treatment indicates that only class II type aldolases are active in the plastids of centric diatoms, which is consistent with the genome data of *Thalassiosira*. In summary, these results imply that cytosolic as well as plastidic aldolase activity in the pennate diatom *P. tricornutum* is mainly due to class I enzymes, whereas in centric diatoms class I aldolase activity may be restricted to the cytosol only while class II enzymes are present in the respective plastids exclusively.

It is yet unclear why the unicellular diatoms possess up to five different aldolases. All of the *Phaeodactylum* genes were obtained from cDNA, indicating that they are expressed and functional. Either FBAC1/C2/C5 and FBA3/4 in *Phaeodactylum* are simply redundant enzymes, or are expressed at different environmental conditions, or each of them might be involved in similar, but different enzymatic reactions. In eubacteria it has been shown that B-type class II aldolases may have other substrate specificities, e.g, for tagatose-1,6-bisphosphate or deoxyribose (see Plaumann et al. 1997). Future analyses of function and origin of metabolic enzymes in diatoms may help to unravel the complexity of processes involved in the establishment of secondary endocytobioses.

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