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SHORT COMMUNICATION

Is chloroplastic class IIA aldolase a marine enzyme?

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Expressed sequence tag analyses revealed that two marine Chlorophyceae green algae, *Chlamydomonas* sp. W80 and *Chlamydomonas* sp. HS5, contain genes coding for chloroplastic class IIA aldolase (fructose-1, 6-bisphosphate aldolase: FBA). These genes show robust monophyly with those of the marine Prasinophyceae algae genera *Micromonas*, *Ostreococcus* and *Bathycoccus*, indicating that the acquisition of this gene through horizontal gene transfer by an ancestor of the green algal lineage occurred prior to the divergence of the core chlorophytes (Chlorophyceae and Trebouxiophyceae) and the prasinophytes. The absence of this gene in some freshwater chlorophytes, such as *Chlamydomonas reinhardtii*, *Volvox carteri, Chlorella vulgaris, Chlorella variabilis* and *Coccomyxa subellipsoidea*, can therefore be explained by the loss of this gene somewhere in the evolutionary process. Our survey on the distribution of this gene in genomic and transcriptome databases suggests that this gene occurs almost exclusively in marine algae, with a few exceptions, and as such, we propose that chloroplastic class IIA FBA is a marine environment-adapted enzyme. This hypothesis was also experimentally tested using *Chlamydomonas* W80, for which we found that the transcript levels of this gene to be significantly lower under low-salt (that is, simulated terrestrial) conditions. Expression analyses of transcriptome data for two algae, *Prymnesium parvum* and *Emiliania huxleyi*, taken from the Sequence Read Archive database also indicated that the expression of this gene under terrestrial conditions (low NaCl and low sulfate) is significantly downregulated. Thus, these experimental and transcriptome data provide support for our hypothesis.
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Fructose-1, 6-bisphosphate aldolase (FBA) catalyzes the reversible condensation of dihydroxyacetone-3-phosphate and glyceraldehyde-3-phosphate to fructose-1, 6-bisphosphate, an essential reaction in both the glycolytic and gluconeogenesis pathways, and the Calvin–Benson cycle. There are two classes of FBAs (class I and II), but they exhibit no similarity in amino acid sequence and differ in their catalytic mechanisms, and are generally considered to be an archetypical example of convergent functional evolution (Marsh and Lebherz, 1992). The distribution of chloroplastic FBAs in photosynthetic organisms is quite complex owing to the combination of endosymbiotic gene transfers and horizontal gene transfers (HGTs) that have occurred (Qiu *et al*., 2013), as well as gene transfers between nuclei and chloroplasts (Kleine *et al*., 2009), and thus provide a wealth of information about the evolutionary history of photosynthetic organisms, including cyanobacteria, eukaryotic algae and land plants.

Chloroplastic FBAs have been one of the strongest pillars of evidence supporting the 'chromalveolate hypothesis' first proposed by Cavalier-Smith (Cavalier-Smith, 1999; Keeling, 2009). This hypothesis proposes that the common ancestor of chromist (stramenopiles, haptohytes and cryptophytes) and alveolates (for example, dinoflagellates, ciliates and apicomplexans) acquired plastids via a single endosymbiotic event with a red alga. There are two types of chloroplastic class II FBAs, one the cyanobacterial type class IIB and the other the bacterial type class IIA. The commonly distributed nuclear-coded chloroplastic class IIA *FBA* genes among the chromalveolates are believed to have been acquired through HGT from bacteria, strongly suggesting the presence of a common ancestor of extant chromalveolate lineages, given that it is very unlikely that all chromalveolate lineages acquired class IIA *FBA* genes independently by HGT with bacteria (Patron *et al*., 2004). More recent multigene

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phylogenetic data indicate that stramenopiles and alveolates are more closely related to the Rhizaria than to a monophyletic group of haptophyta and cryptophyta (HC group; Baurain *et al*., 2010; Burki *et al*., 2012), however, suggesting that revision of the original chromalveolate hypothesis is warranted, but class IIA FBA as evidence for the chromalveolate hypothesis has not yet been challenged.

Chloroplastic class IIA *FBA* genes found among various chromalveolates were once thought to be absent in Archaeplastida (land plants, green algae, red algae and glaucophytes), but in 2012 class IIA *FBA* genes were reported to occur in the prasinophytes green algae genera *Micromonas*, *Ostreococcus* and *Bathycoccus* (Allen *et al*., 2012). The discovery of chloroplastic class IIA *FBA* genes in these algae might support the 'cryptic endosymbiosis' hypothesis proposed by Moustafa *et al*., 2009, who, while analyzing the genomic data of the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, found that these diatoms have more 'green genes', which are primarily associated with prasinophytes, than 'red genes' of red algal origin. On the basis of these findings, the researchers hypothesized that these 'green genes' were the 'footprint' of an ancient endosymbiotic relationship between a cryptic prasinophyte-like green alga and a common ancestor of the chromalveolates. The chloroplastic class IIA *FBA* genes of chromalveolates may therefore have been acquired from a prasinophyte-like green alga through this cryptic endosymbiosis.

Here we report the presence of chloroplastic class IIA *FBA* genes in two species of Chlorophyceae marine green algae, *Chlamydomonas* sp. W80 (*C.* W80) and *Chlamydomonas* sp. HS5 (*C.* HS5). These genes show robust monophyly with those of marine Prasinophyceae algae, indicating that the acquisition of the chloroplastic class IIA *FBA* gene by an ancestor of the green algae lineage through HGT occurred prior to the divergence of the core chlorophytes (Chlorophyceae and Trebouxiophyceae) and the prasinophytes.

Samples of *C.* W80 and *C.* HS5 were collected from the shorelines of Wakayama Bay (Wakayama, Japan) and Sumaura Park (Hyogo, Japan), respectively, and maintained as described in Tanaka *et al*., 2011. Expressed sequence tag analyses of *C.* W80 and *C.* HS5 complementary DNA libraries were undertaken. In total, 960 (174 clusters and 786 singlets) unigenes were found in *C.* W80 and 883 (157 clusters and 726 singlets) unigenes were found in *C.* HS5 (see experimental procedures in the Supplementary Information). Analyses of homology demonstrated that the chloroplastic class IIA *FBA* genes occur in both *C.* W80 (seven expressed sequence tag clones) and *C.* HS5 (two expressed sequence tag clones); the accession numbers of these expressed sequence tag clones and full-length DNA sequences can be found in the Supplementary Information. The presence of chloroplast-targeting signal peptide sequences was examined using ChloroP (Emanuelsson *et al*., 1999), the scores for which (0.500 for *C.* W80 and 0.498 for *C.* HS5) predicted the presence of chloroplast-targeting signal peptide in both *C.* W80 and *C.* HS5 FBA sequences (chloroplast-targeting signal peptide values for the chloroplastic class II FBAs of the prasinophytes green alga *Micromonas*, *Ostreococcus* and *Bathycoccus* range between 0.458 and 0.569). The absence of homologs of the chloroplastic class IIA *FBA* genes of *C.* W80 and *C.* HS5 in some core chlorophytes, such as *Chlamydomonas reinhardtii*, *Volvox carteri, Chlorella vulgaris, Chlorella variabilis* and *Coccomyxa subellipsoidea*, was further confirmed through a BLASTN search of the genomic data of these algae using the class IIA FBA sequences of *C.* W80 and *C.* HS5 as the queries (data not shown). Figure 1a shows the phylogenetic tree of the chloroplastic class IIA FBAs from Chlorophyceae and Prasinophyceae green algae, and various chromalveolates. The chloroplastic class IIA FBAs of Chlorophyceae and Prasinophyceae green algae exhibited robust monophyletic relationships (bootstrap value: 100%).

We also did a TBLASTN search of the Transcriptome Shotgun Assembly (TSA) database of the National Center for Biotechnology Information (NCBI) using the deduced amino acid sequence of *C.* W80 class IIA FBA as the query, finding that this gene occurs in *Chlamydomonas moewusii*, *Chlamydomonas acidophila*, *Tetraselmis subcordiformis* (chlorophytes, Chlorodendrophyceae), and *Caulerpa taxifolia* (chlorophytes, Ulvophyceae; for their Transcriptome Shotgun Assembly accession numbers, Supplementary Table S2). Figure1b shows the phylogenetic tree based on the 18S *rRNA* gene sequences of various green algae and the distribution of chloroplastic class IIA *FBA* genes among them. The discovery of chloroplastic class IIA FBAs in the two Chlorophyceae marine algae, *C.* W80 and *C.* HS5, along with their monophyletic relationship with Prasinophyceae green algae, clearly indicates that the acquisition of this gene by an ancestor of the green algal lineage through HGT —most likely from eubacteria—occurred prior to the divergence of Chlorophyceae and Prasinophyceae (the position circled with asterisk mark in Figure 1b). In addition, the absence of this gene in the genome of many freshwater core chlorophytes (Chlorophyceae and Trebouxiophyceae), such as *C. reinhardtii*, *V. carteri, C. vulgaris, C. variabilis* and *C. subellipsoidea*, as shown in Figure 1b, can therefore be explained by the loss of this gene during the evolutionary process. *C. moewusii* isolated from soil and *C. acidophila* isolated from acidic lakes are the exceptions of non-marine green algae that contain chloroplastic class IIA FBA; the marine-like habitat (that is, acidic lakes with high salt concentrations) of *C. acidophila* may account for the presence of this gene in this species.

Given the absence of chloroplastic class IIA *FBA* genes in many freshwater chlorophytes, as described above, we propose that the chloroplastic class IIA FBA enzyme is a marine environment-adapted enzyme and is generally unsuitable for terrestrial

habitats, and was lost in many non-marine algae as a result of selection pressures during the evolutionary transition to freshwater environments.

We also observed that all 24 of the chromalveolate algae known to have chloroplastic class IIA *FBA* genes are marine algae (Supplementary Table S1).

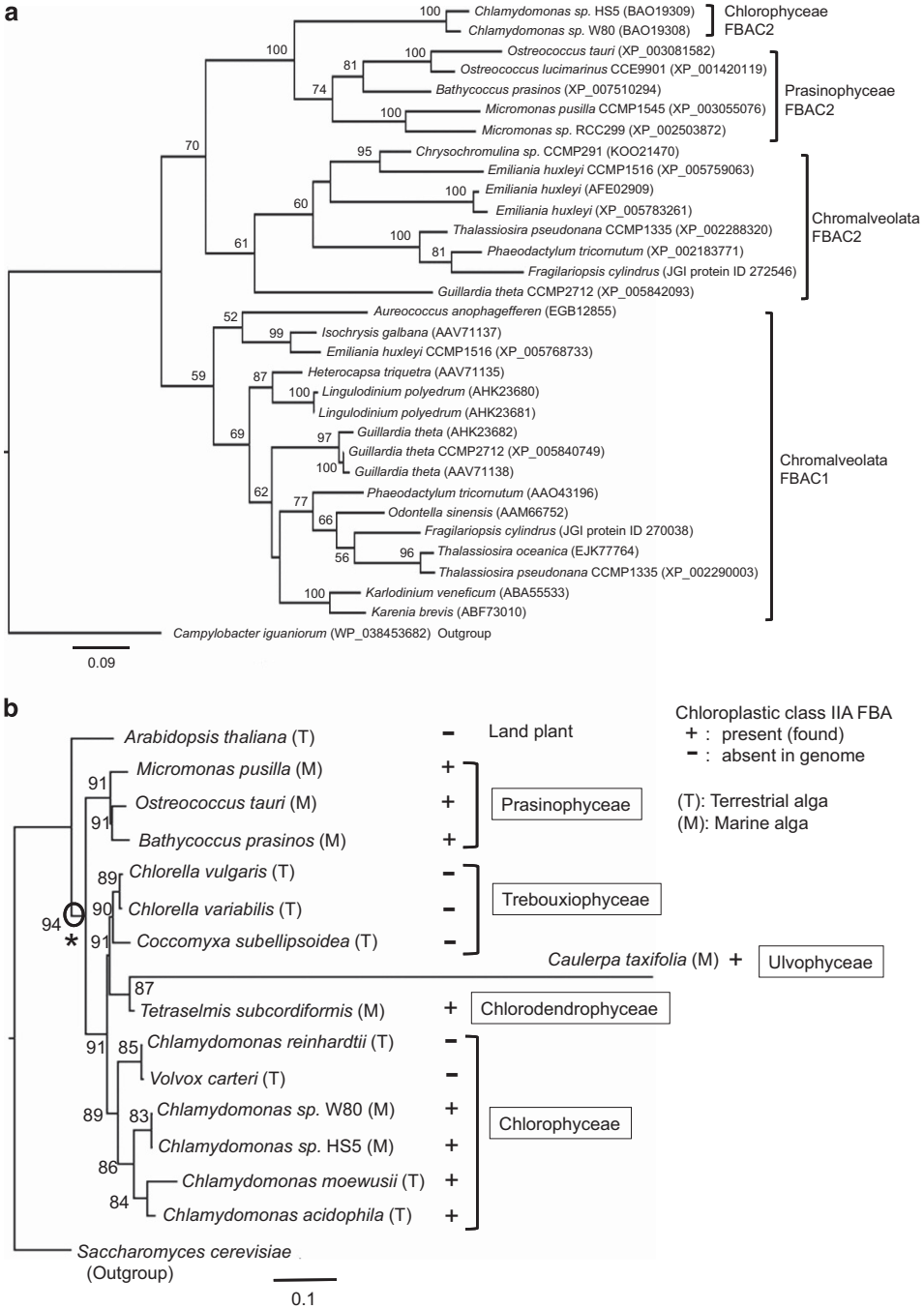

Figure 1 (**a**) Phylogeny of chloroplastic class IIA FBAs among various algae, and (**b**) phylogeny of prasinophytic and chlorophytic algae based on 18S ribosomal RNA (rRNA), and presence/absence of chloroplastic class IIA *FBA* genes. (**a**) Maximum-likelihood tree of 31 amino acid sequences (317 positions) of chloroplastic class IIA FBA (9 Stramenopiles, 4 Cryptophyta, 6 Haptophyta, 5 Dinoflagellata, 5 Prasinophyceae, and 2 Chlorophyceae; see Supplementary Table S1 for more details) rooted by using *Campylobacter iguaniorum* class IIA FBA (WP_038453682) as the outgroup. Bootstrap supports were calculated from the analyses of 100 replicates; values greater than 50% are shown. The chloroplastic class IIA FBAs are categorized as FbaC1 (plastidic-pyrenoid) and FbaC2 (plastidic) types (Allen *et al*., 2012). (**b**) Maximum-likelihood tree of 18S *rRNA* gene sequences (3 Prasinophyceae, 3 Trebouxiophyceae, 6 Chlorophyceae, 1 Ulvophyceae, and 1 Chlorodendrophyceae green algae, and 1 land plant, 1213 positions) rooted by using *Saccharomyces cerevisiae* (AF548094) as the outgroup. Bootstrap supports were calculated from the analyses of 100 replicates; values greater than 50% are shown. The point of divergence of Chlorophyceae and Prasinophyceae is circled and marked with an asterisk. The presence/absence of chloroplastic class IIA *FBA* genes in the genome of each alga are represented by + (present), and − (absent) symbols. For accession numbers are listed in the Experimental Procedures in the Supplementary Information.

We also did TBLASTN search of the Transcriptome Shotgun Assembly database using chloroplastic class IIA *FBA* genes of Stramenopiles (diatom), Cryptophyta, Haptophyta and Dinoflagellata as the queries, and found an additional 17 chromalveolates with this gene (Supplementary Table S2). Of these 17 chromalveolates, 16 are marine strains and only one (*Nitzschia* sp. ChengR-2013, Transcriptome Shotgun Assembly accession number: GAKA01004743) is a freshwater strain. It should be noted, however, that the genomic and transcriptome data of chromalveolates within the databases we examined are extremely skewed toward marine strains, most likely owing to their ecological, environmental and industrial importance, and further investigation is needed to understand the correct distribution of class IIA FBA genes in freshwater chromalveolates. The distribution of chloroplastic class IIA *FBA* genes among various algae (47 marine and 3 terrestrial species, and including Chromalveolata and Archaeplastida) is shown in Supplementary Table S3.

We also examined our hypothesis experimentally using cells of *C.* W80. We expected that the expression patterns of the extant chloroplastic class IIA *FBA* genes would reflect their 'marine environment-adapted' characteristics, and thus respond to low-salt (simulated terrestrial) conditions. *C.* W80 has both chloroplastic class I and IIA *FBA* genes, and the changes in the transcript levels of these genes in response to low-salt conditions were examined by quantitative reverse transcription-PCR. In addition to these two types of chloroplastic *FBA* genes, the transcript levels of chloroplastic *GAPDH*, ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit (*RbcS*), and sedoheptulose-1, 7-bisphosphatase (*SBPase*) genes were also examined as reference genes. The results are shown in Figure 2a; the transcript levels of class I FBA, class IIA FBA, RbcS, and SBPase are presented as relative values to that of chloroplastic GAPDH. The NaCl concentration of seawater is ~0.5 M; when cells of *C.* W80 were cultured at NaCl concentrations lower than that of seawater, a significant decrease in class IIA FBA (at 0.05 M) and a significant increase (at 0.075 and 0.05 M) in class I FBA transcript levels were observed, whereas there were no significant changes in the transcript levels of RbcS and SBPase. Terrestrial environments differ from marine environment in that they typically have low salt concentrations, and thus the downregulation of class IIA FBA and upregulation of class I FBA transcript levels under low-salt conditions would support our hypothesis that class IIA FBA is an enzyme specially adapted to marine environments.

The difference in the transcript levels of chloroplastic class I *FBA* and IIA *FBA* genes between the cells cultured under marine and terrestrial conditions were also compared using the transcriptome data of a euryhaline haptophyta alga, *Prymnesium parvum*, obtained from the Sequence Read Archive (SRA) database. We compared transcriptome data for *P. parvum* cultured under conditions of 30 p.s.u. (practical salinity unit; seawater level) and 5 p.s.u (Figure 2b). The accession numbers of the SRA database are SRX319965 for 30 p.s.u. and SRX319966 for 5 p.s.u. conditions, respectively. We found that under 5 p.s.u. conditions, the transcript level of class I FBA increased significantly ($P<0.001$), whereas that of class IIA decreased significantly ($P<0.001$, results that are consistent with our reverse transcription-PCR data for *C.* W80.

In addition to the high NaCl concentration, the relatively high concentration of sulfate (SO_4^{2-}) is another unique characteristic of the marine environment. Sulfate concentrations in seawater and different types of terrestrial freshwaters range between 25–28 mM and 10–50 μM, respectively. Bochenek *et al.* (2013) examined the response of the marine haptophyta *Emiliania huxleyi* CCMP1516 to sulfate limitation using a system biology approach. We used their SRA data (accession numbers: ERS654078 for 25 mM sulfate and ERS654075 for 5 mM sulfate conditions, respectively) in the NCBI database to analyze the responses of chloroplastic class I and class IIA FBA expression in *E. huxleyi* to low-sulfate conditions (5 mM), finding that the expression of two class I genes increased significantly under such conditions, whereas the expression of three class IIA genes decreased significantly (Figure 2c).

The responses of chloroplastic class I *FBA* and class IIA *FBA* gene expression in *C.* W80, *P. parvum*, and *E. huxleyi* exposed to terrestrial conditions (that is, low-NaCl and low-sulfate conditions) indicate that the shift from marine environment to terrestrial environment conditions is recognized by the cells, which respond by downregulating the expression of class IIA *FBA* genes and upregulating the expression of class I *FBA* genes. Although the decline in the transcript levels of class IIA FBA under terrestrial culture conditions observed in the present study are relatively small with respect to biological significance in daily metabolic activities, even a subtle change in enzyme activity might have enough selective force to drive the eventual loss of its gene over the time-scale on which evolutionary change occurs.

The possible metabolic basis of the marine-adapted nature of the class IIA FBA enzyme might be because of the difference in the bivalent ion requirements between class I and II FBAs. Class II FBAs require divalent metal ions, such as Mg^{2+}, Mn^{2+} and Zn^{2+}, for proper functioning, whereas class I FBAs do not. The concentration of Mg^{2+} in seawater is about 50 mM but typically ranges between 0.1 and 0.5 mM in terrestrial freshwaters; in other words, divalent metal ions are far more available in marine environments than in freshwater environments. The Mg^{2+} concentration in soils, usually ranging between 1 and 10 mM, is generally higher than in rivers and lakes, which may help to explain the presence of class IIA *FBA* genes in *C. moewusii*, a terrestrial alga native to soil.

Chromalveolate algae have important roles in marine ecosystems, as well as having important

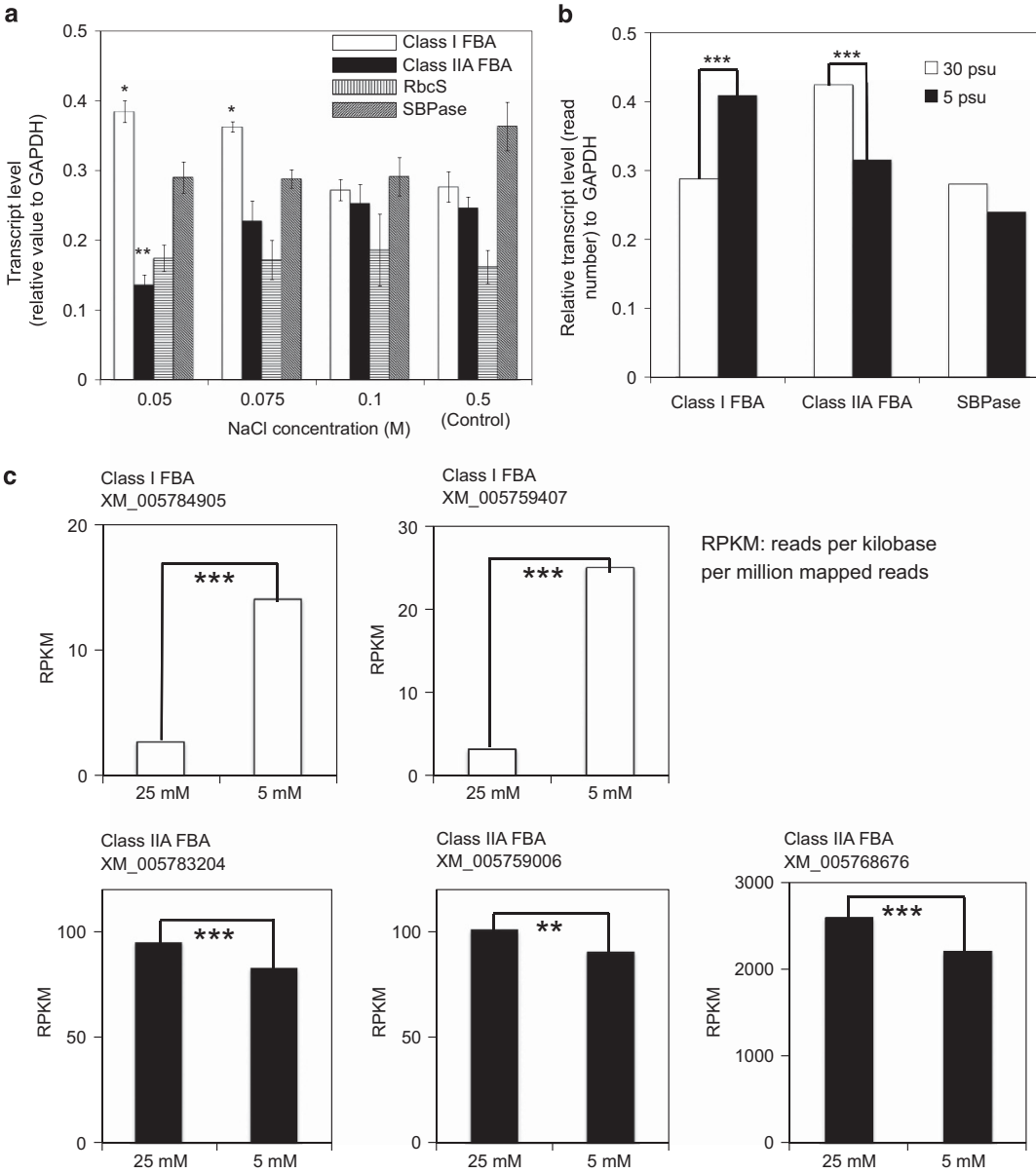

Figure 2 (**a**) Changes in the transcript levels of the chloroplastic class IIA *FBA* gene in response to low-salt conditions, in comparison with chloroplastic class I *FBA*, *RbcS* and *SBPase* genes in *C.* W80 measured by quantitative RT-PCR. (**b**) Changes in the transcript levels of chloroplastic class I and IIA *FBA* genes in *Prymnesium parvum* in response to low-salt conditions; estimates are based on SRA transcriptome data. (**c**) Changes in the transcript levels of chloroplastic class I and IIA *FBA* genes in *Emiliania huxleyi* CCMP1516 in response to low-sulfate conditions; estimates are based on SRA transcriptome data. (**a**) The cells of *C.* W80 were cultured in medium with NaCl concentrations of 0.05, 0.075, 0.1 and 0.5 M (NaCl concentration in seawater is ~0.5 M) for 72 h, and the transcript levels of chloroplastic class IIA *FBA*, chloroplastic class I *FBA*, *GAPDH*, *RbcS* and *SBPase* genes were examined by quantitative RT-PCR. The transcript levels of class IIA FBA, class I FBA, RbcS and SBPase are shown as relative values to that of chloroplastic GAPDH. Data are means+s.d. ($n=3$). Statistical significance was determined using the Student's *t*-test (two-tailed, unpaired). Asterisks indicate results that were significantly different ($*P<0.05$, $**P<0.01$) from the control (NaCl 0.5 M). (**b**) The transcriptome data of *Prymnesium parvum* cultured in medium with seawater level NaCl (30 p.s.u.: practical salinity unit) and low NaCl (5 p.s.u.) in the NCBI SRA database were used for the analysis (SRA accession numbers: SRX319965 for 30 p.s.u. and SRX319966 for 5 p.s.u., respectively). A BLASTN search of the SRA database using the mRNA sequences of class IIA *FBA* (GenBank accession number: DV099053), class I *FBA* (DV100842), *SBPase* (DQ508152) and *GAPDH* (KC899109) genes was carried out, and the number of hits with *E*-values lower than 10^{-10} were counted. Owing to the relatively low quality of the transcriptome data (36–64% of spots contain the read), the transcript levels of class IIA FBA, class I FBA, and SBPase (as a reference) are shown as relative values to chloroplastic GAPDH. Asterisks indicate significant differences ($***P<0.001$) estimated by a χ^2-test. (**c**) The transcriptome data of *Emiliania huxleyi* CCMP1516 cultured in medium with seawater level (25 mM) and low (5 mM) sulfate concentrations in the SRA database were used for the analysis (SRA accession numbers: ERS654078 for 25 mM sulfate and ERS654075 for 5 mM sulfate, respectively). BLASTN searches were done against these databases using the mRNA sequences of three class IIA FBAs (GenBank accession numbers: XM_005783204, XM_005759006 and XM_005768676) and two class I FBAs (XM_005784905 and XM_005759407), and the numbers of hits with *E*-values lower than 10^{-17} were counted as the read numbers of these genes. The transcript levels of class IIA *FBA* and class I *FBA* genes are shown in the form of RPKM (reads per kilobase per million mapped reads) values. Asterisks indicate significant differences ($**P<0.01$, $***P<0.001$) estimated using the χ^2-test.

commercial and industrial uses, whereas chlorophytic algae mainly inhabit terrestrial ecosystems (Lewis and McCourt, 2004). As a result, research interests and genetic data of the chromalveolate are skewed heavily toward marine strains, and those of chlorophytes are skewed heavily toward freshwater strains. Our hypothesis should, therefore, be examined further using freshwater chromalveolate algae in addition to marine chlorophytes, and thus experiments for the detection of chloroplastic class IIA *FBA* genes by degenerated PCR in various freshwater and marine algae, including both chromalveolates and chlorophytes, are currently in progress in our laboratory.

Conflict of Interest

The authors declare no conflict of interest.

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