Genome-wide identification of genes involved in carbon fixation in *Saccharina japonica* and responses of putative C₄-related genes to bicarbonate concentration and light intensity

Zhanru Shao, Wenli Wang, Pengyan Zhang, Jianting Yao, Fahe Wang, Delin Duan

**ARTICLE INFO**

**Keywords:** Carbon fixation, Environmental influences, Enzymatic activity, mRNA abundance, Saccharina japonica

**ABSTRACT**

Brown algae play a dominant role in the primary productivity of coastal ecosystems and may have an efficient carbon fixation. In this work, 56 genes involved in inorganic carbon fixation were identified from the *Saccharina japonica* genome. Sequence structure analysis of these genes showed the existence of corresponding function domains and active amino acid sites highly conserved with other stramenopile species. The predicted subcellular localizations showed that Calvin cycle-related enzymes predominantly reside in the plastid and that putative C₄-related enzymes are mainly distributed in the mitochondrion. We determined the transcriptional profiles and enzymatic activities of these C₄-related enzymes in response to the KHCO₃ concentrations and light intensities. Pyruvate orthophosphate dikinase (PPDK) presented the greatest response to low HCO₃⁻ concentrations and high light intensity. Phosphoenolpyruvate carboxykinase (PEPCK) was up-regulated at low HCO₃⁻ concentrations to compensate for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and might be the crucial decarboxylase in this kelp. We propose that *S. japonica* might possess a PPDK- and PEPCK-dependent C₄-like pathway that enables its rapid growth in natural coastal environments.

1. Introduction

Seaweeds are important marine photoautotrophs and play an important role in global primary production (Nellemann et al., 2009). Marine macroalgae mainly inhabit littoral zones and account for approximately 50% of the carbon fixation (∼210–240 Tg C y⁻¹) in coastal ecosystems (Duarte et al., 2005), with important economic value as sources of ‘3rd generation biomass’ (Xu et al., 2014).

Approximately 90% of dissolved inorganic carbon in seawater environments (pH 8.1–8.4) exists as bicarbonate ions (HCO₃⁻) and the remainder as carbonate ions (CO₃²⁻) and dissolved CO₂ (Skirrow, 1975; Donley et al., 2009). Macroalgae assimilate inorganic carbon via three mechanisms: free diffusion of CO₂ across cell membranes, conversion of extracellular HCO₃⁻ into CO₂ by a secreted carbonic anhydrase, and active transport of HCO₃⁻ (Gao and McKinley, 1994; Raven, 1997; Larsson and Axelsson, 1999; Riebesell et al., 2000). Due to the low diffusion rate of CO₂ in seawater, most macroalgae directly absorb HCO₃⁻ rather than CO₂ (Hurd et al., 2014). Seaweeds possess carbon-concentrating mechanisms (CCMs), and these CCMs are increasingly important because levels of HCO₃⁻ have been rising in seawater during the past few decades (Millero et al., 2002; Raven and Beardall, 2014). Studies of CCMs are much more limited in marine macroalgae compared with phytoplankton (Bowes, 2011; Lachmann et al., 2016; Xu et al., 2017).

Giant brown seaweeds predominantly inhabit cold and temperate seawater with dim light and limited dissolved inorganic carbon (DIC) concentrations. Many brown algae form kelp forests in deep waters and may have a net annual primary productivity of up to 3 kg C m⁻² (Abdullah and Fredriksen, 2004; Hurd et al., 2014). This productivity may be attributed to co-function of the Calvin cycle and C₄ pathways,
which has been reported in Fucales and Laminariales (Küppers and
Kremer, 1978; Kremer, 1981). For example, 14C isotopic tracing ana-
lysis in Fucus and Laminaria showed that 14C accumulates in C4 acids (Ji
et al., 1980; Bidwell and Mclachlan, 1985). Cabello-Pasini and Alberte
(2001) detected the C4-related enzymatic activities in Laminaria setch-
ellii and reported different carboxylation pathways in its thallus. Al-
though genome and transcriptome data are available (Cock et al., 2010;
Ye et al., 2015), whether an inducible C4-like metabolism exists in
brown algae remains unclear (Gravot et al., 2010; Bi and Zhou, 2016).

Saccharina japonica is one of the most important cultivated brown
seaweed species. Carbohydrates may account for as much as 60% of its
dry weight, and we presume that kelp may possess relatively efficient
inorganic carbon fixation pathways considering that it fixes DIC to
build complex carbon-based molecules. Wang et al. (2011) previously
proposed the possible existence of a C4-like pathway in S. japonica by
comparing ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)
activity in different generations of the organism. An annotation of C4-
related genes from the S. japonica transcriptome database indicated the
possible existence of a C4-like pathway (Wang et al., 2013). Chi et al.
(2014) analyzed the phylogenetic relationships of C4-related genes from
19 Phaeophyceae species. However, the above evidence is not sufficient
to show that C4 photosynthesis functions in S. japonica. It is required to
show evidence for expression of these enzymes in an intracellular lo-
cation compatible with C4 photosynthesis and their response to dif-
f erent carbon, light or temperature conditions (Gowik and Westhoff,
2011; Xu et al., 2012; Raven and Giordano, 2017). In this study, we
screened genes encoding enzymes in the Calvin cycle and C4 pathways
through a genome-wide survey in S. japonica and predicted their sub-
cellular localization. We investigated the responses of putative C4-re-
lated enzymes to bicarbonate concentrations and light intensity. Our
results provided explanations for the physiological factors under-
pinning the rapid growth and strong environmental adaptability of this
kelp.

2. Materials and methods

2.1. Algal sample treatment

Juvenile sporophytes of S. japonica (ca. 50 cm in length and ca. 5 cm
in width) were collected from cultivated rafts in April 2017 in
Rongcheng, Shandong, China. Robust samples were selected and rinsed
with filtered seawater samples and preincubated in sterilized sea-
water enriched with 11.76 mM NaNO3 and 7.35 μM KH2PO4 at 15 °C in
darkness overnight. To explore the effects of bicarbonate availability,
the individual sporophytes were similarly exposed to solutions with
different concentrations of bicarbonate (0 and 0.1 M KHCO3 added into
sterilized seawater) for 2 h. To explore the influence of light intensity,
the individual sporophytes were exposed to different irradiances (0 and
100 μmol m−2 s−1) for 6 h.

2.2. RNA extraction and cDNA synthesis

Total RNA extraction was performed using an RNaseasy Plant Mini Kit
following the manufacturer’s instructions (Qiagen, Germany). RNA was
quantified with a Nanodrop 2000 Spectrophotometer (Thermo
Scientific, USA), and RNA quality was verified with 1% agarose gel
electrophoresis. First-strand cDNA was synthesized using a PrimeScript
II cDNA Synthesis Kit (Takara, Japan) and stored at −20 °C for the
subsequent gene cloning and quantitative real-time PCR (qRT-PCR)
analysis.

2.3. Sequence analysis of carbon fixation-related genes

We screened C3 and C4 pathway-related unigenes from our S. ja-
ponica genome libraries in the NCBI database (Accession: MEH0Q00000000). The amino acid sequences from these genes were then
aligned with the NCBI database using the BLASTP algorithm. The
online software SMART was used to screen the protein modular archi-
tecture (PFAM domains), signal peptides and transmembrane helices
(Letunic et al., 2015; Letunic and Bork, 2017). HECTAR v1.3 and Tar-
getP jointly predicted possible localizations to the plastid, mitochon-
drion and cytoplasm etc. (Emmanuelsson et al., 2007; Gschloessl et al.,
2008). C-terminal retention signals (KDEL, DDEL or DEEL) were
manually identified in endoplasmic reticulum (ER) proteins. Enzymes
without recognizable targeting sequences were regarded as cytosolic or
targeted to other cellular compartments.

2.4. Determination of cellular contents of chlorophyll a

The chlorophyll a content was measured for subsequent determi-
nation of enzyme activity. ~0.1 g fresh tissue was ground with liquid
nitrogen and then transferred to a 2 mL centrifuge tube with 80% (V/V)
acetic. The tube was centrifuged at 4000 rpm for 15 min after in-
cubation in darkness for 5 min. The supernatant was then transferred to
a volumetric flask, and 80% acetone was added to a total volume of
10 mL. Absorbance was measured at 630, 647 and 664 nm with a mi-
croplate spectrophotometer (Eon, BioTek, USA). The concentration of chlorophyll a (chl a) was calculated using the following formula (Guo
et al., 2015):

\[
\text{chl a (mg/l)} = 11.85A_{664} - 1.54A_{647} - 0.08A_{630}
\]
2.5. Transcriptional profiles of putative C\textsubscript{4}-related genes

Using Primer Premier 5 software, we designed the specific primers for the Rubisco large subunit gene, and genes involved in possible C\textsubscript{4} pathways: MDH, ME, PEPC, PEPCK and PPDK. The transcriptional levels of each gene were normalized against those of \(\beta\)-actin (Accession: FJ375360.1). qRT-PCR detection was performed with a SYBR Premix Ex Taq™ II kit (Takara, Japan) using a TP800 Thermal Cycler Dice™ (Takara, Japan). A total volume of 25μL of a mixture containing 12.5μL of Premix, 1μL of each primer (10μM), 2μL of diluted cDNA and 8.5μL of RNase-free water was prepared for each reaction. Each sample was amplified using a standard PCR protocol consisting of 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s. Triplicate technical replicates were performed independently, and relative quantitative values were calculated by the \(2^{-\Delta\Delta Ct}\) method (Schmittgen et al., 2000).

2.6. Detection of the endogenous activities of putative C\textsubscript{4}-related enzymes

Total crude protein was extracted from approximately 0.2 g of kelp sample (fresh weight, FW) in 1 mL of ice-cold Bicine-KOH (1 M, pH 9.0) solution containing 5 mM DTT and protease inhibitors (modified from Osorio et al., 2014). After incubation in an ice bath for 20 min, the homogenate was centrifuged at 12,000 rpm for 5 min. The supernatant was transferred to new tubes for the measurement of PEPCK and PEPC. The carboxylation and decarboxylation activities of PEPCK were measured in HEPES buffer (100 mM, pH 7.0) and Tris-acetate buffer (65 mM, pH 7.4), respectively (Walker et al., 2002). PEPC activity was determined by placing 20 μL of extract in 1 mL of EPPS-NaOH buffer (100 mM, pH 8.0) according to the method described by Cousins et al. (2007), and Chlorophytum comosum served as a positive control.

For the MDH, ME, and pyruvate orthophosphate dikinase (PPDK) activity measurements, approximately 0.1 g (FW) of sample was ground and homogenized in prechilled 100 mM Tris-HCl (pH 8.3) extraction solution containing 5 mM EDTA, 2 mM NAD\textsuperscript{+} and 2 mM OAA. The conversion of malate to pyruvate by ME was assayd in 50 mM Tris-HCl (pH 7.8) buffer with 50 mM l-malate and 1 mM NAD\textsuperscript{+} (Freschia et al., 2010). PPDK activity was detected using an assay mixture containing 50 mM Tris-HCl (pH 8.3), 6 mM MgSO\textsubscript{4}, 10 mM DTT, 1 mM PEP, 1 mM trisodium phosphate, 0.15 mM NADH, 1 mM AMP and 2 U/mL lactic dehydrogenase according to the method of Xu et al. (2012).

The total protein was quantified with an Easy Protein Quantitative Kit (TransGen, Beijing, China) according to the Bradford method (Bradford, 1976). A 200 μL mixture containing 100 μL of enzyme extracts was used for each assay. The activity was initiated by adding different substrates and was measured in a continuous assay at 26°C. The OD\textsubscript{340} absorption value was detected at 10 min using a PowerWave HT microplate spectrophotometer. A mixture without enzyme extracts was used for each assay as a negative control.

![Carbon fixation pathway in photosynthetic organisms](https://example.com/carbon-fixation.png)

**Fig. 1.** Carbon fixation pathway in *Saccharina japonica* generated by KEGG (Accession: PRJNA148185). The red rectangle represents genes annotated from the *S. japonica* genome database (Accession: MEHQ00000000). The green cross indicates the absence of the enzyme. The numbers in rectangles are the EC numbers of the enzymes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
3. Results

3.1. Annotation and sequence analysis of carbon fixation related genes in S. japonica

In total, 56 genes involved in the carbon fixation pathways were screened. The schematic domain architecture of these enzymes is shown in Fig. S1 and Fig. S2, which indicates the presence of signal peptides, transmembrane helices and conserved motifs. Except for the sedoheptulokinase, fructose-6-phosphate phosphoketolase and phosphoketolase genes, all the other enzymes involved in the Calvin cycle, C4-Dicarboxylic acid cycle and CAM pathway were found according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Fig. 1).

The annotated genes were verified by the BLASTX algorithm, and 40 of these 56 sequences (71%) contained complete open reading frames (ORFs) (Table S1). With 5’ and 3’ RACE-PCR, we cloned the full-length CDS of 10 putative C4-related genes and submitted the nucleic acid sequences to GenBank (Table 1). Approximately 93% of the genes were highly similar (average 80% identities) to sequences of genes from brown algae, including S. japonica (Accession: ACJ38542.1), and this represents a typical characteristic for C4-specific PEPC genes (Fig. 2b). The predicted PEPC sequence contains a series of active sites, e.g., OAA-binding sites (R-Y-R), ATD-binding sites (H-R), and Mg2+-binding sites (R-I-T) (Fig. 2c). S. japonica possesses two ME sequences that both have five highly conserved regions (I-V) (Fig. 2d). Three adjacent residues (GNQ) in NADP-ME are responsible for binding NADP in S. japonica and E. siliculosus, relative to SN1 in NAD-ME from S. japonica and the CNN in malic enzyme in T. pseudonana (Fig. 2d).

3.2. Subcellular targeting prediction of C4-related enzymes

The predicted intracellular targeting of S. japonica carbon fixation enzymes is summarized in Table S3. Except for phosphoribulokinase (PRK), we find that other Calvin cycle enzymes contain at least one entry to be localized in the chloroplast using HECTAR prediction. The presence of phenylalanine at cleavage sites provides further evidence for the localization of these enzymes in the plastid (Table S3). On the other hand, the putative C4-related enzymes PEPC, AAT6, MDH2, PEPPCK, NAD-ME and ALT are predicted to localize to the mitochondrion. Similar to the Calvin cycle enzymes, the chloroplast-localized pyruvate kinase (PK1) and PPDK also contain phenylalanine at the cleavage sites. Based on these predictions of subcellular localization, we deduced the compartmentation of the carbon fixation pathways in S. japonica (Fig. 3). In the chloroplast, pyruvate is catalyzed by PPDK to yield PEP. Three entries annotated as PEP/Phosphate translocator were listed in Table S1, which all contain 7-10 transmembrane helices for their location in membranes. Therefore, plastid PEP is transported to the mitochondrion through PEPP translocators and then carboxylated via PEPC to form OAA. OAA is either decarboxylated by PEPPCK or catalyzed by MDH2 and NAD-ME to release CO2 (Fig. 3). We presumed that the enriched CO2 could diffuse to the plastid and initiate the Calvin cycle from the production of glycerate-3-phosphate (3-PG) to the regeneration of RuBP (Fig. 3).

Furthermore, we compared the types, numbers and localizations of the annotated C4-related genes in S. japonica with those of their orthologues in E. siliculosus, Phaeodactylum tricornutum and T. pseudonana (Table 2). The putative C4-related genes are present in greater abundance in E. siliculosus and S. japonica than in diatoms, especially for ME and MDH isozymes. ME and PEPPCK were identified as mitochondrion-targeted proteins in the four stramenopiles species, indicating that they may produce CO2 in mitochondria through a C4-like pathway. PEPC and MDH are also predicted to be located in mitochondria, suggesting that the production of OAA and malate and their subsequent decarboxylation both occur in mitochondria. In addition, ALT is predicted to catalyze the interconversion of pyruvate and alanine in mitochondria, thereby regulating the ME activity and indirectly compensating the pyruvate in the chloroplast.

<table>
<thead>
<tr>
<th>EC number</th>
<th>Enzyme name</th>
<th>Abbreviation</th>
<th>CDS (bp)</th>
<th>genome ID</th>
<th>Blastp accession/identities</th>
<th>Length (aa)</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 4.1.1.31</td>
<td>phosphoenolpyruvate carboxylase</td>
<td>PEPC</td>
<td>2856/2850</td>
<td>MRNA_029840</td>
<td>AIT70056.1/84%</td>
<td>949</td>
<td>AW66292.1</td>
</tr>
<tr>
<td>EC 2.6.1.1</td>
<td>aspartate aminotransferase</td>
<td>AAT1</td>
<td>999/1371</td>
<td>MRNA_001797</td>
<td>AIT70206.1/72%</td>
<td>456</td>
<td>AW66295.1</td>
</tr>
<tr>
<td>EC 2.6.1.1</td>
<td>malate dehydrogenase</td>
<td>MDH1</td>
<td>1293/1218</td>
<td>MRNA_002455</td>
<td>AIT70171.1/97%</td>
<td>349</td>
<td>AW66294.1</td>
</tr>
<tr>
<td>EC 2.6.1.1</td>
<td>pyruvate orthophosphate dikinase</td>
<td>PPDK</td>
<td>681/1734</td>
<td>MRNA_002455</td>
<td>AIT70171.1/97%</td>
<td>349</td>
<td>AW66294.1</td>
</tr>
<tr>
<td>EC 2.6.1.1</td>
<td>NAD-malic enzyme</td>
<td>NAD-ME</td>
<td>1749/2103</td>
<td>MRNA_001190</td>
<td>AIT70133.1/99%</td>
<td>561</td>
<td>AW66292.1</td>
</tr>
<tr>
<td>EC 2.6.1.1</td>
<td>NADP-malic enzyme</td>
<td>NADP-ME</td>
<td>1686/1686</td>
<td>MRNA_001334</td>
<td>AIT70147.1/94%</td>
<td>405</td>
<td>AW66292.1</td>
</tr>
<tr>
<td>EC 2.6.1.1</td>
<td>alanine transaminase</td>
<td>ALT</td>
<td>1506/1506</td>
<td>MRNA_001814</td>
<td>AIT69925.1/99%</td>
<td>501</td>
<td>AW66297.1</td>
</tr>
<tr>
<td>EC 2.7.9.1</td>
<td>pyruvate orthophosphate dikinase</td>
<td>PPDK</td>
<td>2970/2970</td>
<td>MRNA_019490</td>
<td>AIT69955.1/89%</td>
<td>989</td>
<td>AW66298.1</td>
</tr>
</tbody>
</table>

* The left number is the length of CDS from genome sequencing; the right number is the length of ORF from gene cloning in this study.

b The left ID is the NCBI accession number of entries showing the highest identities with our sequences; the right number is the percentage of identities.

c The accession number of our putative C4-related genes.

d The accession number of our putative C4-related genes.
3.3. Transcriptional profiles of C4-related genes in response to HCO$_3^-$ concentration and light intensity

Seven pairs of primers for putative C$_4$-related genes and one pair of primers for the large subunit of Rubisco (rbcL) were designed (Table S4) to detect transcriptional variations under various HCO$_3^-$ concentrations and light intensities. Fig. 4 shows the different expression levels of putative C$_4$ genes. The levels of PPDK transcript were increased by 13.9-fold at a low HCO$_3^-$ concentration (without addition of KHCO$_3$). NADP-ME, MDH1 and PEPCK transcription was 2.3-, 1.5- and 1.3-fold greater at 0M KHCO$_3$ than at 0.1M KHCO$_3$, respectively (Fig. 4a). On the contrary, the transcriptional levels of NAD-ME and PEPC were 2.3- and 4.5-fold lower at 0M KHCO$_3$ than at 1M KHCO$_3$, respectively (Fig. 4a). Similarly, the abundance of rbcL was hundred times higher than that of all C$_4$-related genes, and its expression levels were increased by 3.9-fold after HCO$_3^-$ enrichment (Fig. S3a). Juvenile sporophytes were incubated under darkness and light (100μmol m$^{-2}$ s$^{-1}$) for 6h and the mRNA levels of MDH1, MDH2, NAD-ME and PEPC were relatively greater in darkness than under light, and presented values that were 33.1-, 2.2-, 3.6- and 1.5-fold higher, respectively (Fig. 4b). In contrast, PPDK was highly transcribed when the sporophytes were subjected to light and exhibited 12.7-fold more transcripts under light than in darkness (Fig. 4b). The expression levels of rbcL were similarly increased by 2.1-fold after exposure to light (Fig. S3b).

3.4. Enzymatic activities in response to HCO$_3^-$ concentrations and light intensities

Fig. S4 shows that the chlorophyll a content is increased either by adding KHCO$_3$ to the culture or raising the light intensity. The initial activities of each C$_4$-related enzyme in response to light and KHCO$_3$ treatment were calculated by measuring the variation in OD340 relative to the kelp fresh weight, the Chl a weight and total protein content (Figs. 5 and S5). NAD-ME and PEPCK decarboxylation activities were the highest among all the C4-related enzymes under these bicarbonate and light treatments (Fig. 5). The addition of KHCO$_3$ barely changed the activities of MDH, ME and PEPCK but dramatically decreased the PPDK activity by 36.5-fold (Fig. 5a). Similarly, PPDK activity was most affected by light and presented 4.8-fold higher values under light irradiance than in darkness (Fig. 5b). NAD-ME activity was 1.3-fold higher under light, while PEPCK decarboxylation activity was 1.3-fold higher in darkness. NADH-MDH and NADP-ME activities were relatively low under both treatments. The carboxylation activities of PEPC, NADPH-MDH and PEPC were detected but only at negligible levels (data not shown).
Comparative profiles of transcription and enzymatic activities of each C4-related gene are summarized in Fig. 6. The relative transcriptional levels of most enzymes (PPDK, PEPCK and MDH) are consistent with their related activities. PPDK is the only protein whose mRNA and activity levels are up-regulated by light and the lower HCO$_3^-$ concentration. PEPCK and MDH are up-regulated in darkness and at the lower HCO$_3^-$ concentration. The NAD-ME decarboxylation activity decreased at the lower HCO$_3^-$ concentration but increased with light intensity.

**Fig. 3.** Proposed model of carbon fixation metabolism in *Saccharina japonica* based on annotations and the subcellular location of carbon fixation proteins. Enzyme abbreviations: AAT, aspartate aminotransferase; ALT, alanine transaminase; FBA: fructose-1,6-bisphosphate aldolase; FBP: fructose-1,6-bisphosphatase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; PEPCK, phosphoenolpyruvate carboxylase; PEPC, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PEPDK, pyruvate phosphate dikinase; PRK: phosphoribulokinase; RPE: ribulose-phosphate 3-epimerase; RPI: ribose-5-phosphate isomerase; Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase SBP: sedoheptulose-1,7-bisphosphatase; TAL: transaldolase; TKL: transketolase; TPI: triose phosphate isomerase.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th><em>P. tricornutum</em></th>
<th><em>T. pseudonana</em></th>
<th><em>E. siliculosus</em></th>
<th><em>S. japonica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviation</td>
<td>ID*</td>
<td>Localization</td>
<td>Protein ID*</td>
<td>Localization</td>
</tr>
<tr>
<td>PEPC</td>
<td>56026</td>
<td>ER/PPS</td>
<td>B8C1R7</td>
<td>CER/PPS</td>
</tr>
<tr>
<td>ME</td>
<td>20853</td>
<td>Mitochondria</td>
<td>B8BYW8</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>PEPCK</td>
<td>23074</td>
<td>Mitochondria</td>
<td>B8C274</td>
<td>Mito or CER</td>
</tr>
<tr>
<td>MDH</td>
<td>51297</td>
<td>Mitochondria</td>
<td>B5YNR1</td>
<td>—</td>
</tr>
<tr>
<td>PEPDK</td>
<td>21988</td>
<td>Plastid</td>
<td>B8C332</td>
<td>Cytosol</td>
</tr>
<tr>
<td>ALT</td>
<td>—</td>
<td>Cytosol</td>
<td>B8C017</td>
<td>Cytosol</td>
</tr>
</tbody>
</table>

**References:** *P. tricornutum*: Kroth et al. (2008); *T. pseudonana*: Kustka et al. (2014); *E. siliculosus*: Gravot et al. (2010).

* Protein IDs for *P. tricornutum* were from the PlaR2 JGI database.
* Protein IDs for *T. pseudonana* were from the UniProt database.
* Protein IDs for *E. siliculosus* and *S. japonica* were from the NCBI database.
* Potential localization of C$_4$ enzymes from the results of Kustka et al. (2014).
* Potential localization of C$_4$ enzymes from the results of Tanaka et al. (2014).
The enzymatic activity. The two S. japonica carboxyl group, we presumed that this substitution would not affect its NAD-ME (Fig. 2d). Considering that NAD is bound by the oxygen of the is used less efficiently. However, glycine was replaced with a serine in a series of residues responsible for binding PEP in all the selected S. japonica PPDK, PEPC, ME and PEPCK sequences in (Fig. 2). We found that MEs are not as essential for CO₂ production as PEPCK. Although

### 4. Discussion

#### 4.1. Correlation between protein sequences and their putative functions

Highly conserved amino acids were identified in the predicted PPDK, PEPC, ME and PEPCK sequences in S. japonica (Fig. 2). We found a series of residues responsible for binding PEP in all the selected stramenopile PPDK sequences that matched those residues in the C₄-PPDK from Flaveria trinervia (Minges et al., 2017). This result indicated the potential activity of PPDK for PEP regeneration in stramenopile species. The two key residues (Glu491 and Arg496) responsible for tetramerization in S. japonica PEPC were believed to reduce its affinity for its substrate (PEP), which may explain the negligible PEPC activity detected in the kelp. Similar behavior has been reported in maize and diatoms (Wedding et al., 1994; Trimborn et al., 2009). We confirmed the presence of conserved substrate-binding, magnesium-chelating and coenzyme-binding residues in the S. japonica PEPCK sequence, and their presence strongly suggests that this mitochondrial PEPCK is an active enzyme catalyzing the decarboxylation reaction. Conserved region I exists in putative NAD-ME and NADP-ME sequences. Long et al. (1994) reported that region I utilizes both NAD⁻ and NADP⁺ and that NADP⁺ is used less efficiently. However, glycine was replaced with a serine in NAD-ME (Fig. 2d). Considering that NAD is bound by the oxygen of the carboxyl group, we presumed that this substitution would not affect its activity. The two S. japonica MEs are therefore likely to have decarboxylating activity and prefer NAD⁺ over NADP⁺. The enzymatic activity analysis showed that ME activity in the kelp was much higher with NAD⁺ than with NADP⁺ (Fig. 5), which might be explained by the preference of NAD-binding site.

![Fig. 5. Initial activity of different enzymes under various KHCO₃ concentrations and light irradiance. Enzymatic activities were calculated relative to kelp fresh weight, chlorophyll a weight and total protein content. a C₄-related enzymatic activity at different concentrations of KHCO₃. b C₄-related enzymatic activity at different light intensities. Error bars represent the SD of three replicates.](image)

#### 4.2. Subcellular localization of carbon fixation pathways

Unlike the C₄ cycle, which depends on dimorphic chloroplasts and was identified in Suaedaodræa (Edwards and Voznesenskaya, 2011), the C₄-related enzymes in S. japonica may utilize the compartmentation of plastids and mitochondria to concentrate CO₂ (Table 2; Fig. 3). The chloroplast-targeting of PPDK is consistent with diatom PPDKs catalyzing the production of PEP (Kroth et al., 2008). In this study, transporters for PPDK substrate—pyruvate were not successfully annotated from Saccharina genome, which is also the same case for diatoms (Fig. 1 in Kroth et al., 2008). Bi and Zhou (2016) produced a schematic diagram for carbonic anhydrases and C₄ pathways and tentatively proposed that PEPC and PEPCK were localized in the cytosol. We annotated mitochondrion-localized PEPCK and PEPC in the S. japonica genome. The mitochondrion localization of PEPCK is consistent with its localization in diatoms but not most eukaryotes, which have both mitochondrial and cytosolic PEPCK isoforms (Yang et al., 2009; Tanaka et al., 2014). The lack of a cytosolic PEPCK indicates that CO₂ might not be produced in the cytoplasm. The single mitochondrial PEPC is different from the case in diatoms, which possess another ER/PPS-localized PEPC (Kustka et al., 2014; Tanaka et al., 2014). The mitochondrial MDH and NAD-ME complete the pathway from OAA to pyruvate. With regard to all these S. japonica enzymes localizing in mitochondria, we presume that the secondary endosymbiotic mitochondria have been undervalued in previous photosynthetic studies in stramenopiles.

#### 4.3. Influence of HCO₃⁻ and light intensity on putative C₄-related enzymes

PEPC and NAD-ME decarboxylation activity was the highest of all the C₄-related enzymes. PEPC is up-regulated at low HCO₃⁻ concentrations to supply carbon for Rubisco, but its transcription was less sensitive to light intensity. NAD-ME had a much higher activity than NADP-ME, but its insensitivity to HCO₃⁻ and light variations indicated that MEs are not as essential for CO₂ production as PEPC. Although
PPDK was not the most active among these enzymes, it presented the relatively high ability of carbon assimilation and fixation.

Kelp habitats typically experience low light intensity (less than 1500 lx, approximately 30 μmol m−2 s−1) (Huang and Yi, 1998). In our study, the transcriptional level and activity of PPDK were approximately 13- and 5-fold higher under illumination (100 μmol m−2 s−1) than in darkness, respectively. The increase in PPDK activity is comparable to the up-regulation of Rubisco carboxylase activity under light irradiance in our previous report (Shao et al., 2014). With regard to the co-increase of Rubisco and PEPCK activity, we presume that except for Rubisco, S. japonica relies on a PPDK-dependent pathway to maintain the dark reaction process and consequent metabolic pathways in the kelp under high light conditions.

Compared with the differentiation of MCs and BSCs in C4 higher plants, the lower brown alga S. japonica has a relatively simple structure, including cuticle, meristems, cortex and sieve cells, etc. (Fig. S6). Our integrated data describing the subcellular localization of putative C4-related enzymes and their response to HCO3− concentrations and light intensity provided clues that a C4 pathway may exist in the kelp in which CO2 is enriched in the mitochondria and then diffuses into the chloroplast for the Calvin cycle. Compared with the PPDK-independent mechanism in diatoms, PPDK in S. japonica responded intensively to low HCO3− concentrations and high light intensities. The subsequent up-regulation of PEPC and Rubisco might enable greater CO2 fixation, thereby allowing the kelp to grow more rapidly than if it merely relied on a C3 pathway.

Acknowledgements

This work was supported by the Shandong Key Research and Development Program (2018GY115023), Qingdao National Laboratory for Marine Science and Technology Project (No.2018ASJK03), Open Foundation of the State Key Laboratory of Bioactive Seaweed Substances, and CAS-Fujian STS Project (2017T3012). The authors are grateful to the suggestions and manuscript revision by Richard Dorrell from the Institut de Biologie de l’Ecole Normale Supérieure.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytoph.2019.01.032.

5. Compliance with ethical standards

Conflict of interest

The authors hereby declare no conflicts of interest.

Author contributions

ZS and DD conceived and designed research. ZS and PZ conducted experiments. WW and JY contributed the cultivation and the collection of kelp samples. WW and FW were involved in data analysis. ZS interpreted the data and wrote the manuscript. DD gave critical revision of the manuscript. All authors read and approved the manuscript.

References


