Analysis of *Porphyra* Membrane Transporters Demonstrates Gene Transfer among Photosynthetic Eukaryotes and Numerous Sodium-Coupled Transport Systems[1][C][W][OA]

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Membrane transporters play a central role in many cellular processes that rely on the movement of ions and organic molecules between the environment and the cell, and between cellular compartments. Transporters have been well characterized in plants and green algae, but little is known about transporters or their evolutionary histories in the red algae. Here we examined 482 expressed sequence tag contigs that encode putative membrane transporters in the economically important red seaweed *Porphyra* (Bangiophyceae, Rhodophyta). These contigs are part of a comprehensive transcriptome dataset from *Porphyra umbilicalis* and *Porphyra purpurea*. Using phylogenomics, we identified 30 trees that support the expected monophyly of red and green algae/plants (i.e. the Plantae hypothesis) and 19 expressed sequence tag contigs that show evidence of endosymbiotic/horizontal gene transfer involving stramenopiles. The majority (77%) of analyzed contigs encode transporters with unresolved phylogenies, demonstrating the difficulty in resolving the evolutionary history of genes. We observed molecular features of many sodium-coupled transport systems in marine algae, and the potential for coregulation of *Porphyra* transporter genes that are associated with fatty acid biosynthesis and intracellular lipid trafficking. Although both the tissue-specific and subcellular locations of the encoded proteins require further investigation, our study provides red algal gene candidates associated with transport functions and novel insights into the biology and evolution of these transporters.

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Many vital cellular processes (e.g. osmoregulation, removal or compartmentalization of toxic compounds, and acquisition of nutrients) are dependent on electrochemical gradients of ions such as H^+, K^+, Na^+, and Cl^-, membrane potential, and/or a pH gradient (Harold, 1986; Raven and Brownlee, 2001; Bisson et al., 2006). Membrane transporters, usually heterogeneous in structure, are integral membrane proteins (that span the lipid bilayers) responsible for moving ions and biomolecules (both organic and inorganic) across the plasma membrane, and between the cytoplasm and organelles (e.g. plastids, mitochondria, vacuoles, endoplasmic reticulum). Primary (active) transporters such as proton pumps transfer ions as part of a chemical reaction, establishing gradients of electrochemical potential that are required for ion/solute transmission by secondary transporters through facilitated diffusion (Harold, 1986). Secondary transporters are essential for the

uptake of specific nutrients such as amino acids, sugars, and various forms of nitrogen, phosphorus, and sulfur.

Previous electrophysiological and radioactive tracer studies of algal membrane physiology (Raven and Brownlee, 2001) have revealed families of transporter proteins that are differentially regulated. The biochemistry of these proteins and the gene families that encode them have been examined in green algae and plants (Ward et al., 2009), but much less is known about the function and evolutionary histories of these transporters in Rhodophyta (red algae; for exception, see Barbier et al., 2005). The red algae comprise a major eukaryotic lineage that is implicated in secondary plastid endosymbiosis. This process led to the establishment of rhodophyte-derived plastids in other groups of algae, such as the stramenopiles (e.g. diatoms, brown algae) and alveolates (e.g. dinoflagellates; McFadden, 2001; Sanchez-Puerta and Delwiche, 2008; Keeling, 2009). Other instances of tertiary endosymbiosis of red algal-derived plastids occurred in some dinoflagellates (Ishida and Green, 2002; Yoon et al., 2005). As a result of gene transfer associated with secondary endosymbiosis, red algal genes were moved to the nucleus of these lineages, often to support plastid functions.

The position of the red algae in the tree of life and the extent of horizontal gene transfer (HGT) between red algae and other eukaryotes have often been controversial topics (Burki et al., 2007; Lane and Archibald, 2008; Nozaki et al., 2009; Stiller et al., 2009; Baurain et al., 2010; Parfrey et al., 2010). However, in a recent phylogenomic analysis of novel genes derived from two mesophilic red algae, *Porphyridium cruentum* (Porphyridiophyceae) and *Calliarthron tuberculosum* (Florideophyceae), Chan et al. (2011b) found support both for the Plantae hypothesis, i.e. the grouping of Rhodophyta, Viridiplantae, and Glaucophyta (Rodríguez-Ezpeleta et al., 2005), and reticulate (nonlineal) evolution involving sharing of red algal genes with various eukaryotic and prokaryotic phyla through endosymbiotic gene transfer (EGT) or HGT. A more recent analysis that included complete genome data from the glaucophyte *Cyanophora paradoxa* also provides robust support for Plantae monophyly (Price et al., 2012). Nevertheless, in another study of membrane transporters in diatoms, these genes were shown to have both red and green algal origins, suggesting that EGT/HGT has led to the extensive spread of Plantae genes among microbial eukaryotes (Chan et al., 2011a).

Here we examine two species of *Porphyra* in the red algal class Bangiophyceae. This class has a fossil history extending back 1.2 billion years (Butterfield, 2000), whereas *Porphyra*-like fossils have been found in rocks dated at approximately 425 million years old (Campbell, 1980; for review, see Blouin et al., 2011). Bangiophyceae is a sister to the Florideophyceae, which includes economically important phycocolloid-producing species. *Porphyra* (commonly known as laver), which has an agaran cell wall polysaccharide (i.e. porphyran; Correc et al., 2011), is a potentially useful target for understanding the evolution of phycocolloid-producing seaweeds or to unravel other aspects of red algal biology such as the formation of filaments linked by distinctive pit plugs (Graham et al., 2008; Blouin et al., 2011). Therefore, elucidating the evolutionary history of transporter proteins in *Porphyra* will advance our understanding of membrane transport systems in red algae, and more broadly among photosynthetic eukaryotes. Here, using a comprehensive transcriptome dataset derived from *Porphyra umbilicalis* and *Porphyra purpurea* (68,104 contigs assembled from approximately 4.3 million ESTs) we identified putative membrane transporters using a combination of computational and manual annotation. An automated phylogenomic approach was used to assess the evolutionary origins of the transporters. These data were also used to infer both shared and unique physiological features associated with specific transport processes in red algae relative to other algae and vascular plants.

RESULTS AND DISCUSSION

Identification of Membrane Transporters in the *Porphyra* Transcriptome

Of 68,104 nuclear-encoded contigs that were assembled from *Porphyra* ESTs (20,704 in *P. umbilicalis* and 47,400 in *P. purpurea*), we identified 482 predicted proteins that encode putative membrane transporters (see Supplemental Materials and Methods S1 and Supplemental Table S1 for more details). More putative transporters (314 EST contigs) were recovered from *P. purpurea* than from *P. umbilicalis* (168 EST contigs). As expected with most transcriptome studies, our EST data include partial gene fragments (e.g. incomplete, expressed transcripts) that may comprise a protein subunit or domain. Therefore, determination of the total number of membrane transporter genes in *P. umbilicalis* and *P. purpurea* awaits analysis of complete genome data (Blouin et al., 2011). However, the

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greater number found in *P. purpurea* likely reflects sampling of the entire life history of *P. purpurea* for this project, i.e. both diploid sporophyte filaments (conchocelis) and haploid gametophytic blade. Only blades of *P. umbilicalis* were sampled because northwestern Atlantic populations of *P. umbilicalis* appear to recycle blades directly by asexual spores without a conchocelis phase (Blouin et al., 2007). This list of *Porphyra* EST contigs that encode putative membrane transporters was derived from sequence similarity searches (BLASTX, *E* value $\leq 10^{-10}$) against the manually curated protein database at Swiss-Prot. The 482 contigs represent approximately 5.6% of the total number of contigs (8,638) in *Porphyra* that have significant hits in Swiss-Prot. At the more-lenient *E*-value thresholds of $\leq 10^{-5}$ and $\leq 10^{-3}$, the number of putative transporters rises to 589 and 670 (out of 11,331 and 13,013 contigs), respectively. Therefore, we recovered at least 482 contigs (approximately 500–600) in our dataset that encode putative membrane transporter proteins or protein fragments.

In this study, we restricted our analysis to the 482 EST contigs as defined by the most stringent BLASTX threshold. Based on the annotated function and sequence similarity of the Swiss-Prot top hit in the Transporter Classification Database (TCDB; Saier et al., 2009), we manually grouped 455 of the contigs into 57 distinct transporter families. The distribution of transporter families, each with at least five protein (EST contig) members, is shown in Figure 1 (see Supplemental Table S2 for complete list). The remaining 27, although annotated as general secretory and transport proteins, were not classified due to lack of significant matches to TCDB. The 57 families encompass diverse transport functions related to carriers of organic and inorganic molecules, as well as metals and metal ions.

As shown in Figure 1 (full membrane transporter family names are shown in the figure legend), the most common putative transporters in *Porphyra* (based on the count of EST contigs) are members of the large, multisubunit, and abundant ATP-binding cassette (ABC) superfamily (97 contigs), the H^+ or Na^+ translocating F-type, V-type, and A-type superfamily (F-ATPase; 54 contigs), the mitochondrial carrier family (MC; 53 contigs), followed by the drug/metabolite transporter superfamily (DMT; 26 contigs), the P-type ATPase superfamily (P-ATPase; 23 contigs), and the major facilitator superfamily (MFS; 19 contigs). The ABC transporter family consists of proteins that contain one or more ABC domains and derive energy from ATP hydrolysis to transport hydrophilic molecules across membranes. In plants and animals, ABC transporters are detoxifying agents that purge cells of drugs and xenobiotics (Gottesman and Pastan, 1993). These transporters are also involved in developmental processes in plants (Schulz and Kolukisaoglu, 2006) and the transport of bile acids into vacuoles (Hörtensteiner et al., 1993). Within the ABC transporters in *Porphyra*, we found many that are related to multidrug resistance, bile salt pumps, and to the transport of lipids into the plastid (see also below).

In addition to various V-type proton pumps (H^+-ATPase) in the F-type, V-type, and A-type superfamily, we identified a wide variety of *Porphyra* transporters in families that are associated with metal ions, including the monovalent cation:proton antiporter families (both

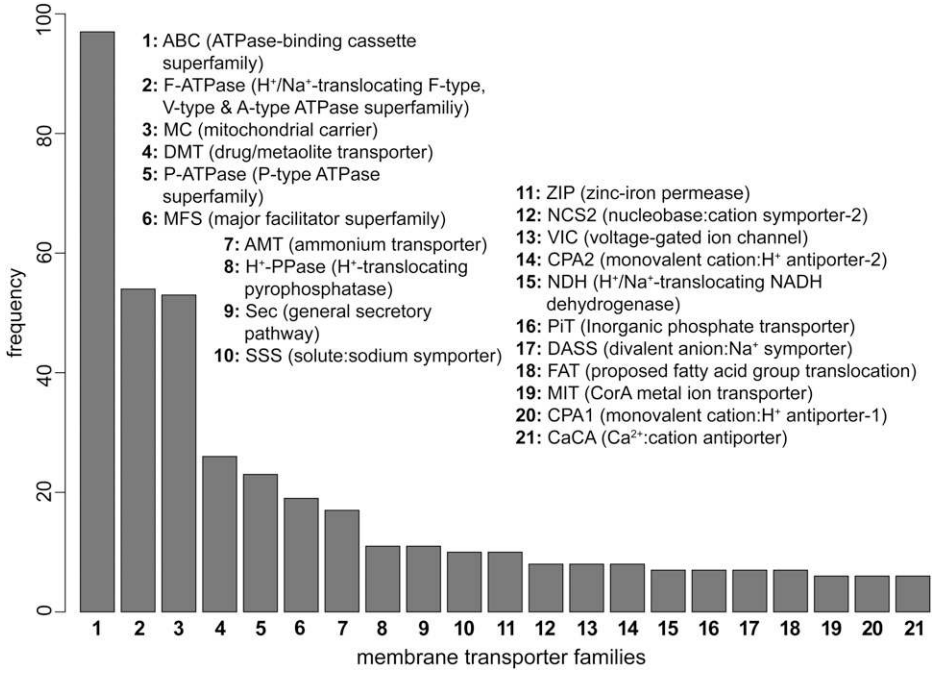

Figure 1. Distribution of *Porphyra* EST contigs that encode putative membrane transporter functions based on the classification in TCDB. Only 21 families, each with at least five contigs, are shown. The complete list of EST contigs with their corresponding number of transporters is shown in Supplemental Table S2.

CATION:PROTON ANTIPORTER1 [CPA1] and CPA2, i.e. Na^+, K^+, and H^+ antiporters; 14 contigs), the zinc-iron permease family (ZIP; 10 contigs), the voltage-gated ion channel family (VIC; eight contigs), the corA metal ion transporter family (MIT; i.e. the Mg^{2+} transporters; six contigs), and the Ca^{2+}:cation antiporter (CaCA) family (six contigs). In marine algae, transport of Na^+ and K^+ along with Cl^- is central to the maintenance of osmotic balance between cellular compartments. Ca^{2+} transport plays a role in many signaling processes. Many other metal cations, and particularly divalent metal ions (e.g. Mg^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+}), are essential cofactors associated with several metalloproteins (Hanikenne et al., 2005) that are required for numerous metabolic processes in the cell including chlorophyll and isoprenoid biosynthesis, primary metabolism, and photosynthetic CO_2 fixation. The photosynthetic machinery represents a very large sink for Fe^{2+}, Cu^{2+}, and Mn^{2+}, which are integral to photosynthetic reaction center and electron transport proteins. In addition to the MFS family that includes most nitrate and sugar transporters, we also identified transporters among the *Porphyra* EST contigs that are associated with diverse organic and inorganic compounds (see Fig. 1).

Red Algal Membrane Transporters Have Diverse Evolutionary Origins

For each of these EST contig-encoded proteins, we searched for homologs (BLASTP, E value $\leq 10^{-10}$) within an in-house, taxonomically broadly sampled protein sequence database that comprises approximately 15 million sequences from a number of public repositories (as of January 14, 2011; see "Materials and Methods" and Supplemental Table S3), including the recently published red algal EST data from the unicellular red alga *P. cruentum* and predicted proteins from partial genome data from the coralline red alga *C. tuberculosum* (Chan et al., 2011b). Of the 482 EST contig-encoded membrane transporters in *Porphyra*, 14 (2.9%) have hits only to other red algae in the database, suggesting these proteins could be specific to red algae (see Supplemental Table S1). The remaining 468 (97.1%) have homologs in diverse taxa, with nine showing exclusive gene sharing between rhodophytes and one other taxon (i.e. absence of putative homologs in other taxa in the database). In addition, 404 (83.8%) of these 482 proteins in *Porphyra* have significant hits to one or more other red algal genera (i.e. *Eucheuma*, *Gracilaria*, *Cyanidioschyzon*, *Porphyridium*, and/or *Calliarthron*), substantiating their red algal provenance.

To ensure the capture of useful phylogenetic signal across taxa, we refined the protein sequence alignments to minimize the contribution of phylogenetically noninformative sites (Talavera and Castresana, 2007), excluded short alignments (<75 amino acid positions), and removed protein sets with less than four sequences from the subsequent analysis (this removed 22 trees; see "Materials and Methods"). We reconstructed the phylogeny for each of the remaining 460 protein sets (of the initial 482) using a maximum likelihood approach (Stamatakis, 2006), and inspected these phylogenies for a strongly supported monophyletic relationship between red algae and other phyla. Adopting a similar approach as in Chan et al. (2011b), we restricted our analysis to 438 phylogenies that contained greater than or equal to three distinct phyla and ≥20 terminal taxa to minimize potential artifacts caused by inadequate taxon sampling or stochastic sequence variation that could mislead phylogenetic inference. Supplemental Table S1 shows the 482 *Porphyra* transporters and the results of the phylogenomic analysis.

Of the 438 trees, 102 (23.2%) show a moderately well-supported monophyletic relationship (bootstrap ≥70%) between *Porphyra* and one other lineage (five of which show two such monophyletic groups in the tree). At the more stringent bootstrap threshold value ≥90%, this number becomes 54 (see Supplemental Table S1 for details). Given the limited number of algal genes in the current database (compared to prokaryotes, metazoans, and fungi), monophyletic clades inferred at the bootstrap threshold ≥70% provide reasonably well-supported evidence for phylogenetic affinity. This approach of assessing the phylogenetic output with increasing levels of stringency combined with a careful initial screening of the phylogenetic trees has been rigorously tested in previous studies (Chan et al., 2011a; Price et al., 2012). Similar to the analysis shown in Chan et al. (2011b) of gene sharing involving the Plantae lineages (red with green and/or glaucophyte algae), we included phylogenies with strongly supported monophyletic clades that contain foreign taxa (<10% of the total lineages within the clade; i.e. instances of nonexclusive gene sharing). This is an expected outcome of secondary EGT involving red or green algae. The various phyla with a history of gene sharing with the red algae as inferred from phylogenomic and BLAST analyses are shown in Figure 2 (i.e. 108 contigs inferred from phylogenetic analysis plus nine contigs for which no tree was generated due to less than four hits in the BLAST analysis [117 total]). The most common gene-sharing partners for the red algae are other members of the Plantae supergroup (Viridiplantae and/or Glaucophyta [30]), the stramenopiles (19) that contain a red-algal-derived plastid and are well represented in our genome database (219,962 sequences), as well as bacteria that are not Cyanobacteria (13). In addition, we recovered evidence of red algal transporters that show a strong affinity to the Opisthokonta (13 contigs with Fungi, 10 with Choanoflagellida, and nine with Metazoa). The finding of a close association with choanoflagellates is not surprising given their phagotrophic lifestyle, i.e. putative HGT from red algal prey to the choanoflagellate nuclear genome (Sun et al., 2010). Nevertheless, the observed association of red algae with Fungi and Metazoa (especially for cases of exclusive gene sharing) may be explained by the imbalanced taxon sampling in the current database, in

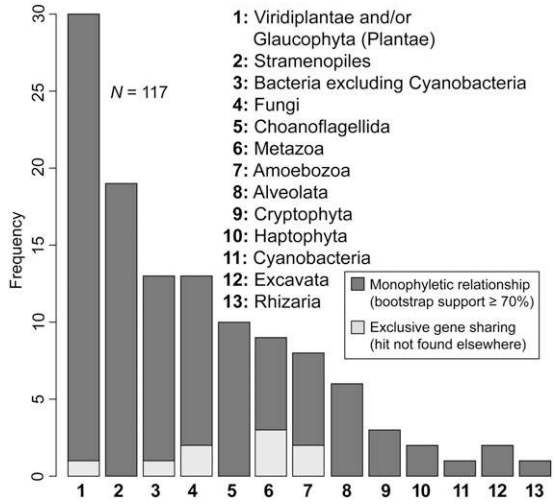

Figure 2. Phylogenetic distribution of phyla that are implicated in membrane transporter gene sharing with red algae. The yellow bars show the extent of exclusive gene sharing between the two phyla (instances where the gene was not recovered in taxa other than the two phyla) and the blue bars represent the number of phylogenetic trees that show strongly supported (bootstrap ≥70%) monophyletic relationship between the two phyla.

which the opisthokonts constitute approximately 20% of the sequences (3 million sequences), combined with the limited genome data from other taxa such as microbial eukaryotes.

An example of a protein phylogeny that is consistent with Plantae monophyly is shown in Figure 3A. This tree includes members of the MFS in which a bacterium-derived gene appears to be uniquely shared by Rhodophyta and Viridiplantae (bootstrap 100%). An alternative explanation for this tree is an ancient origin of the transporter gene in the eukaryote common ancestor followed by loss in all non-Plantae lineages in our database. The relatively straightforward interpretation of the tree in Figure 3A does not, however, hold up for the majority of the phylogenies. Most of these (76.7%, 336 of 438) do not show a highly resolved monophyletic relationship involving red algae. Rather, they suggest a convoluted (and likely reticulate) gene evolutionary history that is difficult to interpret. This is in agreement with recent phylogenomic studies that often show an unresolved position of rhodophyte genes within the eukaryote tree of life (Burki et al., 2007; Lane and Archibald, 2008; Nozaki et al., 2009; Baurain et al., 2010; Chan et al., 2011b).

Nitrate and Ammonium Transports

We found that all four phylogenies of high-affinity nitrate transporters (two from each species of *P. umbilicalis* and *P. purpurea*) within the MFS family support a rhodophyte-stramenopile association. The strongly supported (bootstrap 92%) monophyly between red algae and stramenopiles in the nitrate transporter phylogeny (Fig. 3B) suggests a gene-sharing history between these lineages, likely as a consequence of secondary endosymbiosis. In addition, we also observe a strong association (bootstrap 99%) between the Viridiplantae and haptophytes, suggesting an independent history of gene sharing among these taxa. Nitrate is a common nitrogen source for plant and algal cells.

Algae are known to take up urea and ammonium from the environment and assimilate nitrogen from these compounds (Kakinuma et al., 2008). Ammonium is taken up preferentially by many algae including *Porphyra* (Kim et al., 2007) when compared to NO_3^-, which reflects the reduced energy requirement for assimilation of the former. Although NO_3^- is usually more abundant than NH_4^+, NO_3^- can be reduced to NO_2^- and subsequently to NH_4^+ by nitrate and nitrite reductases. The assimilation of NH_4^+ is critical to the synthesis of other nitrogen-containing compounds such as amino acids, and nucleic acids (Vitousek et al., 1997; Galloway et al., 2008). The number of putative NH_4^+ transporters (ammonium transporter family) described in the completely sequenced genomes of other algae varies from two to nine (Armbrust et al., 2004; Matsuzaki et al., 2004; Merchant et al., 2007; Bowler et al., 2008; Cock et al., 2010). Here we found 17 distinct *Porphyra* EST contigs that putatively encode NH_4^+ transporters (10 in *P. purpurea*, seven in *P. umbilicalis*), suggesting that genes with functions related to NH_4^+ transport are highly expressed and important for cell survival in red seaweeds. The evolutionary histories for many of these transporters are unclear in red algae based on our approach. Six (out of 10) *P. purpurea* contigs have homologs in *P. umbilicalis*, and likewise five (out of seven) *P. umbilicalis* contigs have homologs in *P. purpurea*. Therefore, we observed five to 10 distinct EST contigs encoding NH_4^+ transporters in each of the *Porphyra* species. The exact number of genes encoding NH_4^+ transporters in *Porphyra* remains to be verified with complete genome data, because we cannot dismiss the possibility of alternative splicing, inclusion of gene fragments, bias in gene expression, and assembly errors in the current transcriptome data, which could lead to over- or underestimation in the number of genes. The ability to assimilate NH_4^+ allows seaweeds such as *Porphyra* to utilize NH_4^+ excreted by farmed fish, which in addition to NO_3^- utilization contributes both a food crop and bioremediation function when *Porphyra* is used in integrated aquaculture (Kim et al., 2007).

Red Algae Possess a Small Number of Known Aquaporins

The water channel proteins or aquaporins are critical for osmotic regulation and glycerol transport in vascular plants (Maurel, 1997), which in turn can mediate CO_2 permeability and photosynthetic activity (Uehlein et al., 2003). Whereas aquaporins are ubiquitous in vascular plants (Maurel and Chrispeels, 2001) and have been characterized in humans and other

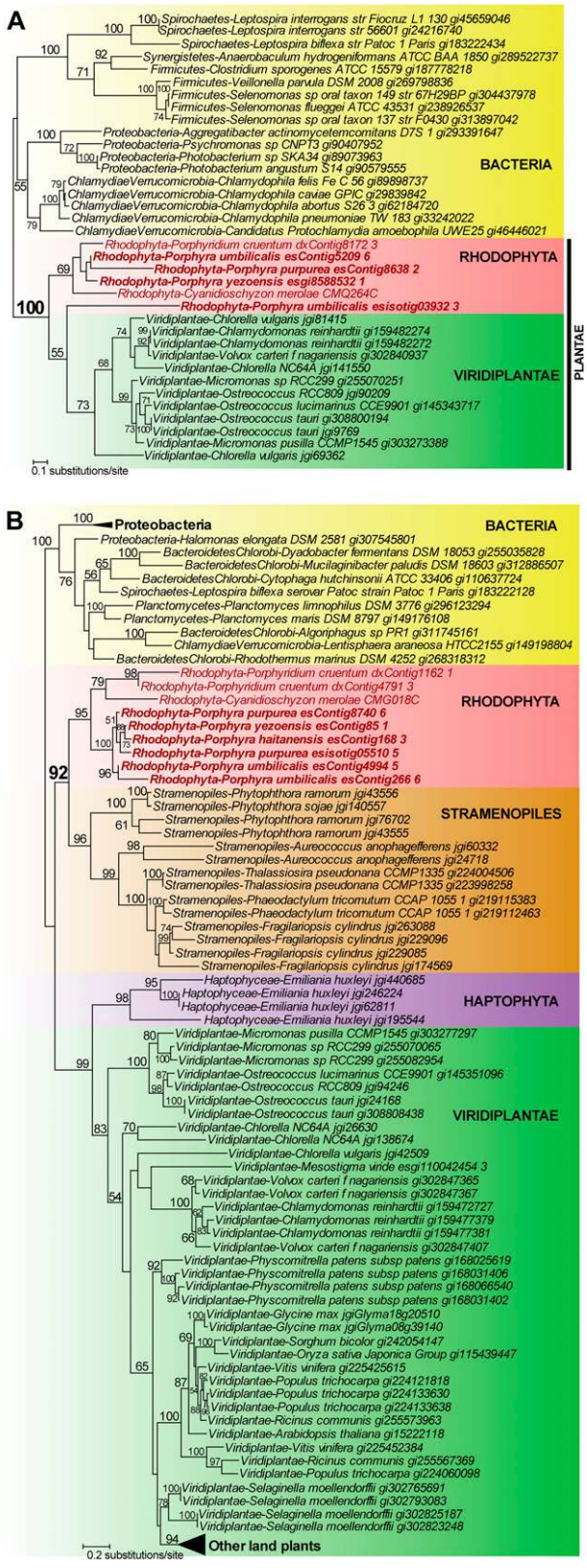

Figure 3. Phylogenies of membrane transporters in *Porphyra*. Phylogenies are shown for MFS protein (A), in which a strongly supported monophyly (bootstrap 100%) of red and green algae (the Plantae supergroup) is observed, and high-affinity nitrate transporter with a strongly supported monophyly (bootstrap 92%) of red algae and stramenopiles (B), suggesting a history of gene sharing between these two phyla. RAxML bootstrap values (>50%) are shown at each internal node. *Porphyra* species are highlighted in boldface. The unit of branch length is number of substitutions per site. [See online article for color version of this figure.]

animals (Murata et al., 2000; King et al., 2004), much less is known about the specific functions of these channel proteins and their evolutionary history in algae (for exception, see Anderberg et al., 2011). We found three EST contigs that putatively encode aquaporins in *P. purpurea* (none in *P. umbilicalis*) in our dataset. In comparison, a single aquaporin has been reported in green algae and is involved in glycerol uptake. This gene is highly divergent when compared to known aquaporin proteins (Anderca et al., 2004). Two of the three *P. purpurea* aquaporins show strong support for monophyly with green algae (i.e. likely Plantae monophyly) and with stramenopiles (i.e. a putative EGT history), whereas the other shows an inconclusive evolutionary history, suggesting these genes could have arisen from different sources or at different times in evolution.

Calcium Signaling

We identified two classes of ion channels in *Porphyra* that are likely to be involved in Ca^{2+} signaling in response to environmental stimuli (i.e. three contigs encoding the ionotropic Glu receptor [GLR], and one contig encoding the inositol triphosphate receptor [IP_3R]). The evolutionary histories of these transporters in *Porphyra* are inconclusive based on available data (although one contig encoding GLR potentially has a bacterial origin; see Supplemental Table S1), suggesting reticulate evolution of the encoding genes. IP_3Rs mediate the release of Ca^{2+} from intracellular storage in animal cells (Berridge, 2009), but despite extensive research efforts, there is no molecular evidence for the occurrence of an IP_3R in land plants (Krinke et al., 2007). IP_3R-like genes have been identified in *Chlamydomonas* and *Ectocarpus*, and our identification of an EST contig encoding IP_3R in the red alga (specifically in conchocelis tissue of *P. purpurea*; see Supplemental Table S1) suggests that this class of ion channel could have a widespread distribution in algae despite its apparent absence in some species as observed in whole-genome studies (Wheeler and Brownlee, 2008; Verret et al., 2010). Although the presence of IP_3R in *Porphyra* remains to be validated as genome data become available, a recent study (Li et al., 2009) provides biochemical evidence that IP_3Rs may be required to establish cell polarity in germinating archeospores in *Porphyra yezoensis*.

Eukaryotic cells use Ca^{2+}-ATPases and Ca^{2+}/cation exchangers to mediate Ca^{2+} efflux from the cytosol, maintaining very low concentrations of cytosolic Ca^{2+}

(approximately 100 nM). There are two major groups of Ca^{2+}-ATPases, the P_{2A}-ATPases (endoplasmic reticulum type Ca^{2+}-ATPases) and the P_{2B}-ATPases (autoinhibited Ca^{2+}-ATPases), both of which are found in animal and plant cells (Baxter et al., 2003). Analysis of the *Porphyra* ESTs indicates both types are present in red algae (six endoplasmic reticulum type Ca^{2+}-ATPases and two autoinhibited Ca^{2+}-ATPases in *P. purpurea*). The Ca^{2+}/cation exchangers (CaCA) generally have a lower affinity for Ca^{2+} than Ca^{2+}-ATPases but exhibit a higher transport capacity. In animal cells, an inward Na^+ gradient is used to drive Ca^{2+} efflux via the K^+-independent (NCX) and K^+-dependent (NCKX) Na^+/Ca^{2+} exchangers (Cai and Lytton, 2004). In contrast, Na^+/Ca^{2+} exchangers are absent from land plants, which use Ca^{2+}/H^+ exchangers (CAX) to establish a proton gradient that drives Ca^{2+} efflux from the cytosol into an acidic vacuole. CAX proteins appear to be absent from animals (Cai and Lytton, 2004). The *Porphyra* ESTs contain six sequences encoding members of the CaCA family. Based on phylogenetic analyses, four of these sequences fall into the SLC24 class of NCKX transporters, with the others belonging to the CAX class of Ca^{2+}/H^+ transporters.

Lipid Transport

The transport of fatty acids from the chloroplasts where they are synthesized to the endoplasmic reticulum where they are incorporated into phosphoglycerolipids represents an essential physiological feature in plants. Fatty acids are further modified, either bound to phosphatidylcholine or to CoA, resulting in the synthesis of eicosapentaenoic acid (20:5). Eicosapentaenoic acid is also incorporated at high concentrations into the plastid galactolipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol. This distribution of lipid species involves extensive trafficking of lipids from the endoplasmic reticulum to the plastid. In vascular plants this process is mediated by a transport complex associated with the inner chloroplast membrane and is composed of three proteins designated TGD1, TGD2, and TGD3 (Benning, 2009), that are named after the diagnostic lipid trigalactosyldiacylglycerol that accumulates in Arabidopsis (*Arabidopsis thaliana*) mutants defective for these transport proteins. These three components resemble a bacterial-type ABC transporter with TGD1 as the transmembrane permease, TGD2 as the substrate-binding subunit, and TGD3 as the subunit involved in ATP hydrolysis (which powers the uptake of the substrate against a concentration gradient). The components of the transporter have been predicted for most Gram-negative bacteria including Cyanobacteria (ancestor of plastids), and are organized in operons (Lu et al., 2007). In vascular plants and green algae, all TGD orthologs are encoded in the nucleus (Xu et al., 2003, 2008; Awai et al., 2006; Lu et al., 2007).

We identified the ESTs encoding the putative TGD3 subunit in both *P. umbilicalis* (one contig) and *P. purpurea* (one contig). The TGD1 and TGD2 proteins are encoded on the *P. purpurea* plastid genome (Reith and Munholland, 1995); sequences encoding these proteins were absent from our *Porphyra* transcriptome dataset. The TGD3 gene in *P. purpurea* shows a strong phylogenetic association with Cyanobacteria and Viridiplantae (Supplemental Table S1). In addition to the TGD complex, some proteins involved in fatty acid biosynthesis are also encoded on the plastid genome, which differs from what has been observed in vascular plants (Li-Beisson et al., 2010) and green algae (Merchant et al., 2007). To confirm the identity and localization of the two plastidic sequences encoding TGD1 and TGD2 in *P. purpurea*, we used a PCR approach to amplify intergenic fragments flanking both genes. Primers were designed based on the available sequence information of *P. purpurea* strain Avonport (Reith and Munholland, 1995). Interestingly, TGD2 is located in the same operon as *accA* (see Supplemental Fig. S1). The *accA* gene encodes the α-subunit of the acetyl-CoA carboxylase, an enzyme required for fatty acid synthesis (Fig. 4). Whereas the TGD1, 2, and 3 orthologs in bacteria such as *Escherichia coli*, *Pseudomonas putida*, and *Xyllella fastidiosa* are located in the same operon, TGD1 is separated from the other genes in cyanobacteria (not shown; see Lu et al., 2007 for details). In vascular plants and green algae, all three proteins are nuclear encoded. The proximity of TGD2 and a fatty acid synthase gene suggests coregulation of fatty acid synthesis and intracellular lipid trafficking in red algae. Lipid import into the chloroplast in Arabidopsis involves another enzyme named TGD4. No homolog for this gene was identified in EST sequences or the plastid genome of *Porphyra*. Interestingly, no candidate gene for any of the four mentioned TGD genes was found in secondary plastids of the stramenopiles (e.g. *Ectocarpus*, *Phaeodactylum*, and *Thalassiosira*).

Ion Transport: Marine versus Freshwater Algae

Animal cells utilize a transmembrane Na^+ gradient established by the plasma membrane Na^+/K^+ ATPase to enable Na^+-coupled uptake of solutes such as sugars

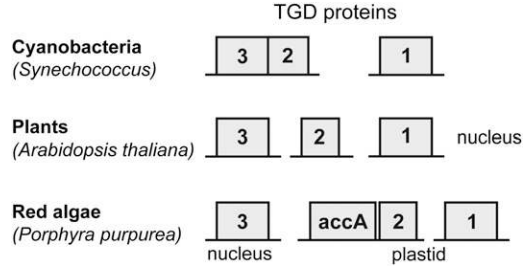

Figure 4. Structural organization of TGD genes in cyanobacteria, plants, and *Porphyra*. The numbers refer to open reading frames homologous to TGD1, 2, and 3 from Arabidopsis. In Arabidopsis all three genes are encoded in the nuclear genome, whereas TGD1 and 2 are encoded in the plastidial genome of *Porphyra*.

and amino acids across the plasma membrane. In contrast, land plants and fungi commonly use a H^+ gradient generated by a plasma membrane H^+-ATPase to energize secondary transport processes at the plasma membrane (Harold, 1986). H^+ symporters that function in the uptake of nutrients including hexose sugars and amino acids have been described in freshwater algae, including several species of the unicellular green alga *Chlorella* (Komor and Tanner, 1974; Tanner, 2000; Kato and Imamura, 2009), whereas a H^+-HCO_3^- symporter may be present in the streptophyte *Chara corallina* (Fisahn and Lucas, 1992). Marine algae exist in an alkaline environment (typically pH ≥ 7.8) where external Na^+ is abundant (450–500 mM), and electrophysiological and radioactive tracer studies demonstrate that they use Na^+-dependent acquisition of NO_3^-, PO_4^{3-}, Glc and amino acids, and silica (Hellebust, 1978; Bhattacharyya and Volcani, 1980; Raven and Smith, 1980; Boyd and Gradmann, 1999; Gimmler, 2000; Weiss et al., 2001) rather than H^+-energized transport at the plasma membrane. This contrast in H^+ versus Na^+ dependence of transport between freshwater and marine algae is not absolute. For example, Na^+ is required for PO_4^{3-} transport in the freshwater chlorophyte *Ankistrodesmus braunii* (Ullrich and Glaser, 1982). H^+ symport is typical of most Charales (Streptophyta), even those that can grow in brackish water, because the very-negative membrane potential of characean cells still allows H^+ symport to function at alkaline pH (Bisson et al., 2006; M.A. Bisson, personal communication to S.H.B.). However, there are several reports of Na^+-coupled transport systems operating at the plasma membrane of charophytes, including symport of urea (Walker et al., 1993), Cl^- (Sanders et al., 1985), and PO_4^{3-} (Reid et al., 2000), indicating that charophytes may utilize both Na^+- and H^+-energized transport. It is clear that the availability of Na^+ in the environment may have been a major influence on the evolution of plasma membrane transport mechanisms in different algae.

Our analysis of *Porphyra* membrane transporters provides evidence for the different usage of Na^+-coupled transporters between marine and freshwater algae. The *Porphyra* ESTs contain several NCKX transporters, suggesting that Na^+-coupled transport is important for Ca^{2+} homeostasis in this organism. NCKX transporters exchange one Ca^{2+} and one K^+ for four Na^+ ions; there is clearly a requirement for an inward Na^+ gradient to energize Ca^{2+} efflux. Interestingly, sequence analyses indicate that NCKX proteins are present in all marine algae with completely sequenced genomes (see "Materials and Methods" for data sources), including the prasinophytes (*Micromonas* and *Ostreococcus*), the pelagophyte *Aureococcus*, the three diatoms *Phaeodactylum*, *Thalassiosira*, and *Fragilariopsis*, and the brown seaweed *Ectocarpus*. However, these proteins are absent from the genomes of nonmarine algae, including *Cyanidioschyzon merolae*, *Chlorella*, *Volvox*, *Chlamydomonas*, and land plants. In contrast, the CAX transporters (which use a H^+ gradient to energize Ca^{2+} efflux from the cytosol) are found in all of the freshwater and marine algal genomes listed above, with the exception of *Aureococcus* and the prasinophytes. This finding suggests a fundamental difference in the mechanisms of Ca^{2+} homeostasis between marine and freshwater algae. Presumably, marine algae are able to utilize Na^+ as a counterion to drive Ca^{2+} efflux across the plasma membrane, but may also use a H^+ gradient to facilitate Ca^{2+} uptake into acidic intracellular compartments, as occurs in yeast (*Saccharomyces cerevisiae*) and land plants. In support of this hypothesis, the *Porphyra* ESTs contain numerous sequences exhibiting similarity to the P-type IIC ATPases that function as Na^+/K^+ pumps in animal and fungal cells to maintain the Na^+ gradient across the plasma membrane (see also Barrero-Gil et al., 2005). Plasma membrane Na^+-ATPases are likely to play a similar role in supporting Na^+-coupled transport in marine algae and a P-type IIC ATPase has been previously identified in a marine diatom (e.g. Gimmler, 2000), although they have not yet been experimentally characterized from algal cells.

Mechanisms for the transport of anions such as SO_4^{2-} may also be tailored to marine environments. We identified two EST contigs encoding proteins with the potential to be SO_4^{2-} permeases in *P. umbilicalis* based on annotation and homology with known divalent anion:Na^+ symporter (DASS)-type SO_4^{2-} transporters of *Chlamydomonas reinhardtii* (SLT1, SLT2, SLT3; Pootakham et al., 2010). Two analogous transporters were identified in *P. purpurea* (annotated as putative SO_4^{2-} deprivation response regulator). An alignment of the four *Porphyra* transporters is shown in Supplemental Figure S2. These transporters are in the DASS/SLC13 permease family (solute carrier 13), which contain eight to 13 transmembrane helices and are classified as Na^+-anion (such as SO_4^{2-}) transporters (Pajor, 2006). As shown in Supplemental Figure S3, these sequences have significant similarity to the SLT2 (and SLT1 and SLT3; not shown) sequences of *C. reinhardtii*, which encodes a low SO_4^{2-} deprivation-inducible permease associated with cytoplasmic membranes (Pootakham et al., 2010). Whereas the substrates for protein members of this family of permeases include SO_4^{2-}, some are more specific for other anions including dicarboxylates and arsenate. Currently, vascular plants have not been shown to contain the Na^+-SO_4^{2-} DASS transporters, although they may have members of the DASS-SLC13 family of transporters that are not associated with the transport of SO_4^{2-}, but appear to be involved in transporting organic acids; at least in one case such a transporter has been localized to the vacuolar membrane (Emmerlich et al., 2003).

In addition to having two SLC13 permease domains, the *Porphyra* DASS permeases have a TrkA-C domain in the central part of the protein. The function of the TrkA-C domain is not known, although it is thought to be located in the cytoplasm of the cell, to bind ligands, and potentially to function in controlling transporter activity (Barabote et al., 2006). The two SLC13 transporters identified in *P. umbilicalis* are very similar to those of *P. purpurea*, with one of the transporters from

each of these organisms having an insertion of 23 amino acids proximal to the TrkA-C domain relative to the other potential Na^+-SO_4^{2-} transporter. The DASS family symporters are present in both marine (*Ostreococcus* sp., *Micromonas*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana*) and freshwater algae (*C. reinhardtii* and *C. merolae*); similarity among these transporters from a number of marine algae is presented in Supplemental Figure S4, whereas a specific list of genes/cDNAs in various databases (see Supplemental List S1) that show significant homology to the SLT2 SO_4^{2-} permease of *C. reinhardtii* (Pootakham et al., 2010) is given in Supplemental File S1.

Plants and other eukaryotes have two other types of SO_4^{2-} transporters. One is a H^+-SO_4^{2-} symporter designated SULTR in plants (Takahashi et al., 2011), whereas the other is an ABC-type transporter (typical of bacteria) that is often composed of four subunits that include two integral membrane proteins that form a pore in the membrane, a peripheral membrane component involved in binding and hydrolyzing ATP, and a substrate-binding protein (Melis and Chen, 2005). There are homologs to H^+-SO_4^{2-} symporters of the SLC26 family in some of the marine algae based on current data (e.g. Supplemental File S2; *Micromonas* sp. RCC299 and *Pavlova lutherii*; there is a also a homolog in *P. tricornutum*, which is found in brackish waters), but no SLC26 transporters have been identified in some marine algae for which there is a full genome sequence (e.g. *T. pseudonana*, *Ostreococcus*). In addition, when we lowered the stringency of the BLAST analysis and queried with the SLC26 H^+-SO_4^{2-} symporters of *Chlamydomonas* (SULTR1, SULR2, SULTR3) and Arabidopsis (SULTR1;1, SULTR1;2, SULTR1;3, SULTR2;1, SULTR3;1, SULTR3;2, SULTR3;4, SULTR3;5, SULTR4;1, and SULTR4;2), we identified a short segment of putatively homologous sequence encoded on a contig of *P. purpurea*. The similarity of the various sequences from marine organisms and SULTR2 of *C. reinhardtii* is given in Supplemental Figure S5. Hence, whereas there are clearly some SLC26-type sequences in some marine algae, these sequences were not identified in at least two taxa that are represented by complete genome data, and it is unclear whether those sequences that were identified are involved in SO_4^{2-} transport. Based on these analyses, it seems likely that the DASS family of transporters may be important for Na^+-SO_4^{2-} symport in the oceans, but this needs to be demonstrated biochemically.

We have also examined the transporters associated with PO_4^{3-} uptake by *Porphyra*. The PTA and PTB transporters of *C. reinhardtii* have homology to the Pho84 and Pho89 transporters of yeast, respectively (Moseley et al., 2006). Previous work has shown that Pho84 is primarily an H^+-coupled transporter, whereas Pho89 is an Na^+-coupled transporter (Bun-Ya et al., 1991; Lenburg and O'Shea, 1996; Oshima, 1997; Pattison-Granberg and Persson, 2000; Zvyagilskaya et al., 2008). All of the putative PO_4^{3-} transporters that we identified in *Porphyra* were of the PTB type (Moseley et al., 2006), which are homologous to the Pho89 transporters of yeast and are represented by many contigs; no contigs from our *Porphyra* database had significant similarity to the PTA or the H^+-PO_4^3-type transporters (based on sequence similarity to *C. reinhardtii* or yeast sequences). Again, these findings likely reflect the fact that Na^+ is an abundant cation in marine environments, making it the preferred ion for symport, whereas both H^+ and Na^+ may more equally serve that function in soil and freshwater environments. Finally, subunits of an ABC-type transporter that is encoded on the *Porphyra* plastid genome (Reith and Munholland, 1993) is associated with the transport of SO_4^{2-} into plastids, which is similar to the plastid transporter identified in the green algae (Melis and Chen, 2005) and the cyanobacterial transporter (Laudenbach and Grossman, 1991).

CONCLUDING REMARKS

We identified 482 EST contigs that encode putative membrane transporters (or nonoverlapping transporter fragments) with diverse functions in the comprehensive transcriptome database from the red seaweed *Porphyra*. Of these only a small proportion (6.6%) show clear evidence of vertical inheritance, with even fewer (3.9%) implicated in an EGT/HGT history with stramenopiles. The majority of the transporter proteins show highly complex (or unresolved) evolutionary histories that defy a straightforward explanation, even using an enriched dataset of red algal genes for the phylogenetic analyses. The evolutionary history of these sequences may be unraveled using modern network approaches (Huson and Scornavacca, 2011; Schliep et al., 2011). Complicated red algal membrane transporter evolutionary histories reveal a number of interesting physiological features that are potentially specific to the rhodophytes or to marine algae in general (e.g. Barbier et al., 2005). This analysis supports earlier physiological research showing the importance of Na^+ in the marine environment as a counterion for the symport and antiport of a variety of anions such as SO_4^{2-} and PO_4^{3-}. The nuclear genome of *P. umbilicalis*, once sequenced to completion (Blouin et al., 2011), will enable comparative genomic approaches to further validate and elucidate the functions and subcellular locations of transporter proteins in *Porphyra*, and of the red algae, in general.

MATERIALS AND METHODS

Identification of Membrane Transporters in *Porphyra*

The *Porphyra* transcriptome was derived from RNAs isolated from blade tissues of *Porphyra umbilicalis* strain P.umb.1 (University of Texas at Austin Algal Culture Collection UTEX no. LB–2951), and from blade tissues and conchocelis filaments of *Porphyra purpurea*, grown under different conditions, including nutrient stress versus nutrient replete, different circadian times, high versus low light, and desiccation followed by rehydration (see Supplemental Table S4). Using 454 DNA pyrosequencing (Roche 454 life sciences), we generated a total of 4,676,380 raw EST reads from both *P. umbilicalis* (2,926,227) and *P. purpurea* (1,750,153). Initial filtering to remove bad-quality reads, ribosomal DNA, and bacterial sequences reduced the dataset to

4,259,550 sequences (2,849,102 and 1,410,448 from *P. umbilicalis* and *P. purpurea*, respectively; see Supplemental Table S4). Potential contaminants belonging to green algae and planctomycetes (Glöckner et al., 2003) were further removed from the dataset under a set of stringent criteria, particularly in the instance of *P. umbilicalis*, for which partial genome data are available from the ongoing genome sequencing project (Blouin et al., 2011). The ESTs were subsequently assembled into 68,104 distinct contigs (20,704 and 47,400 contigs for *P. umbilicalis* and *P. purpurea*, respectively) that are putatively encoded in the nucleus. See Supplemental Materials and Methods S1 for details in the generation of EST data, removal of potential contamination, and de novo transcriptome assembly.

We searched all EST contigs in the *Porphyra* transcriptome for those that putatively encode membrane transporters based on a keyword search across the top hit, for each of the contigs, in Swiss-Prot protein database (BLASTX, *E* value $\leq 10^{-10}$). We manually classified these contigs (482 in total) into transporter families as defined in the TCDB (http://www.tcdb.org/).

Phylogenomic Analysis

For each of the 482 contigs in *Porphyra*, we identified homologous sequences (BLASTP, *E* value $\leq 10^{-10}$) within an in-house broadly sampled database consisting of all well-annotated proteins from the National Center for Biotechnology Information RefSeq release 45 (http://www.ncbi.nlm.nih.gov/RefSeq/; as of January 14, 2011), all predicted protein models available from the Joint Genome Institute (ftp://ftp.jgi-psf.org/pub/JGI_data/), and six-frame translated proteins from EST datasets of all publicly available algal and unicellular eukaryote sources, i.e. dbEST at GenBank (http://www.ncbi.nlm.nih.gov/dbEST/) and TBestDB (http://tbestdb.bcm.umontreal.ca/), as well as EST contigs from *Porphyridium cruentum* and predicted models from the *Calliarthron tuberculosum* partial genome (http://dbdata.rutgers.edu/data/plantae/), totaling 15,150,005 sequences (Supplemental Table S3). Most of the completely sequenced algal genome data are readily available from National Center for Biotechnology Information RefSeq (including the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*), and the Joint Genome Institute (including the prasinophytes [*Micromonas* and *Ostreococcus*], the green algae [*Chlorella* and *Volvox*], the pelagophyte *Aureococcus*, and the diatom *Fragilariopsis*). Data from the unicellular red alga *Cyanidioschyzon merolae* were obtained from http://merolae.biol.s.u-tokyo.ac.jp/. We applied two sampling criteria to ensure a reasonable representation of the diverse groupings in a protein set; i.e. less than or equal to five bacterial subgroups, no single species/strain/genome is represented greater than four times. Sequence alignments were generated using MUSCLE version 3.8.31 (Edgar, 2004), and noninformative sites within the alignments were removed using GBLOCKS version 0.91b (Talavera and Castresana, 2007), with the options b3 = 200, b4 = 2, and b5 = h. We used a strict set of criteria to ensure that the results obtained are phylogenetically meaningful: (1) each protein family (hence alignment) had greater than or equal to four members but were limited to ≤100 members, and (2) phylogenetically informative sites in each set of aligned protein sequences were ≥75 amino acid positions. Under these criteria, 22 protein sets were excluded. The phylogeny for each of the remaining 460 protein alignments was reconstructed using a maximum likelihood approach (Stamatakis, 2006) using Whelan and Goldman (2001) amino acid substitution model with a discrete γ distribution (Yang, 1994) and nonparametric bootstrap of 100 replicates. All sequence alignments and the resulting phylogenetic trees used in this study are available at http://dbdata.rutgers.edu/data/noriTransport and http://dblab.rutgers.edu/downloads/. For other sequence analyses, genome data of the brown seaweed *Ectocarpus siliculosus* was obtained from https://bioinformatics.psb.ugent.be/gdb/ectocarpus/.

Functional Annotation

Putative protein function encoded by each of the contigs was annotated using Blast2GO (Conesa et al., 2005), based on sequence similarity searches (BLASTX) against the manually curated Swiss-Prot protein database (*E* value $\leq 10^{-3}$).

Validation of TGD Gene Localization

To confirm the presence of TGD1 and TGD2 orthologs in the plastids of *P. purpurea* and *P. umbilicalis*, primers for both candidate genes were designed to target putative conserved regions, based on a sequence alignment of the published plastid genomes of *P. purpurea* strain Avonport (GenBank accession NC_000925) and *Porphyra yezoensis* (GenBank accession NC_007932). A fragment spanning both *accA* and *TGD2* (671 bp; primers PopuCp-F1: CTAGAGTAGTAGGTCTTTCTGATTG and PopuCp-R1: CGCATAGCATTTTAACACGCATTG), and an internal *TGD1* fragment (329 bp; primers PopuCp-TGD1-int-F: ACTCCTTTAGCCCCACCACTAC and PopuCp-TGD1-int-R: GCAACGATGGAGACCACAGAAC) were amplified from the isolated chloroplast DNA (template) using GoTaq Flexi DNA polymerase (Promega), under the following PCR conditions: initial denaturation (95°C, 2 min), 32 cycles of amplification (each cycle consists of 10 s at 95°C, 10 s at 48°C, and 30 s 72°C), and final elongation (72°C, 2 min). PCR products were cloned into the vector pGEM-T easy (Promega) and subjected to sequencing (two independent clones per organism). The *AccA-TGD2* spanning sequence and *TGD1* gene fragment for each of *P. purpurea* and *P. umbilicalis* generated from this study is available from GenBank (accessions JN817500–JN817503).

All EST sequence data from this article can be found in the GenBank Sequence Read Archive (project accession nos. SRP008761 and SRP008766).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignment of the *accA-TGD2*-spanning region of *P. purpurea* strain Avonport (NC_000925) with the corresponding sequences from *P. purpurea* and *P. umbilicalis* in this study.

Supplemental Figure S2. Alignment of the SLC13-DASS transporters identified in *P. umbilicalis* and in *P. purpurea*.

Supplemental Figure S3. Alignment of the *Porphyra* SLC13-DASS transporters (presented in Supplemental Fig. S2), with the SLT2 SO_4^{2-} transporter of *C. reinhardtii*.

Supplemental Figure S4. Alignment of the SLC13-DASS transporters from various marine algae.

Supplemental Figure S5. Identities of sequences in some marine algae to SULTR2 of *C. reinhardtii*.

Supplemental Table S1. The list of 482 EST contigs that putatively encode membrane transporters in *Porphyra*; shown for each contig is the size of the homologous protein set, top hit in the Swiss-Prot database, corresponding family classification, and the inference from the phylogenomic analysis.

Supplemental Table S2. The transporter families into which the *Porphyra* EST contigs are classified and their corresponding number of contigs.

Supplemental Table S3. The number of protein sequences in the database that is used for the phylogenomic analysis (based on phyla) in this study.

Supplemental Table S4. Experimental conditions for each of the *Porphyra* cDNA libraries used in this study, and the corresponding number of EST sequences generated.

Supplemental Materials and Methods S1. Algal culture and generation of EST data.

Supplemental File S1. Matches of the *C. reinhardtii* SLT1 sequence to proteins encoded by various algae, including many that live in marine environments.

Supplemental File S2. Matches of *C. reinhardtii* SULTR2 to proteins encoded by various algae, including many that live in marine environments.

Supplemental List S1. The genomes/cDNAs of algae that were probed for the DASS family symporters include *Alexandrium tamarense*, *Amphidinium carterae*, *Heterocapsa triquetra*, *Karenia brevis*, *Karlodinium micrum*, *Lingulodinium polyedrum* (dinoflagellates); *Goniomonas pacifica*, Guillardia θ, *Rhodomonas salina* (cryptophytes); *Emiliania huxleyi*, *Isochrysis galbana* CCMP1323, *Pavlova lutheri* (haptophytes); *P. tricornutum*, *T. pseudonana* (diatoms); *P. purpurea* NRv2, *P. umbilicalis* NRv2, *C. merolae*, *Galdieria sulphuraria*, Bangiophyceae CDS, Bangiophyceae EST, Florideophyceae CDS, Florideophyceae EST, Rhodophyta and others (red algae); *Glaucocystis nostochinearum*; *Cyanophora paradoxa* (glaucophytes) and some marine green algae including *Micromonas* spp.and *Ostreococcus* spp.

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