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Gurvan Michel, Thierry Tonon, Delphine Scornet, J. Mark Cock, Bernard Kloareg. Central and storage carbon metabolism of the brown alga *Ectocarpus siliculosus*: insights into the origin and evolution of storage carbohydrates in Eukaryotes. *New Phytologist*, 2010, 188 (1), pp.67-81. 10.1111/j.1469-8137.2010.03345.x . hal-01806417

HAL Id: hal-01806417

<https://hal.science/hal-01806417>

Submitted on 16 Nov 2018

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The carbohydrate metabolism of the brown alga *Ectocarpus siliculosus*.

Part I: Central and storage carbon metabolism. Insights into the origin and evolution of storage carbohydrates in Eukaryotes.

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Summary: 196 words

Total word count: 6365 words

Introduction: 715 words

Materials and Methods: 252 words

Results: 2371 words

Discussion: 3027 words

Tables: 0

Figures: 6

Supporting Information Tables : 3

Summary

- Brown algae exhibit a unique carbon storage metabolism. The photoassimilate D-fructose 6-phosphate is not used to produce sucrose but is converted into D-mannitol. These seaweeds also store carbon as β -1,3-glucan (laminarin), thus markedly departing from most living organisms, which use α -1,4-glucans (glycogen or starch).
- We identify the enzymes involved in carbon storage in the genome of the brown alga *Ectocarpus siliculosus* and trace their evolutionary origins. *Ectocarpus* possesses a complete set of enzymes for synthesis of mannitol, laminarin and trehalose, while the pathways for sucrose, starch and glycogen are completely absent.
- The synthesis of β -1,3-glucans appears to be a very ancient eukaryotic pathway. Brown algae inherited the trehalose pathway from the red algal progenitor of phaeoplasts, while the mannitol pathway was acquired by lateral gene transfer from Actinobacteria. The starch metabolism of the red algal endosymbiont was entirely lost in the ancestor of Stramenopiles, as was glycogen metabolism from the ancestral host.
- In the light of these novel findings we question the validity of the “Chromalveolate hypothesis”. We also propose that β -1,3-glucans were the archetypal carbon storage compound in Eukaryotes. In most extant lineages they were supplanted by glycogen following mitochondrial endosymbiosis.

Keywords: brown algae, Chromalveolate, Eukaryotic evolution, beta-1,3-glucan, glycogen, starch, mannitol, trehalose

1 Introduction

2 Brown algae (Phaeophyceae) are photosynthetic, multicellular organisms that dominate
 3 rocky coastal environments. These macroalgae are members of the Stramenopiles, a
 4 eukaryotic phylum which also includes diatoms, Oomycetes and various protists (Fig. 1)
 5 (Baldauf, 2008). Stramenopiles are characterized by the occurrence in their life history of
 6 cells with two unequal flagella and the monophyly of this group has been confirmed by
 7 molecular phylogenies (Ben Ali *et al.*, 2001). Stramenopile plastids arose via a secondary
 8 endosymbiotic event, in which a unicellular red alga was engulfed by an ancestral protist
 9 (Reyes-Prieto *et al.*, 2007). Other eukaryotic lineages also possess secondary plastids derived
 10 from red algae, particularly the Alveolates, Haptophytes and Cryptophytes (Fig. 1). Cavalier-
 11 Smith proposed that a single secondary endosymbiosis with a red alga gave rise to the plastid
 12 ancestor of all these eukaryotic groups (the Chromalveolate hypothesis) and, therefore, that
 13 the host lineages form a monophyletic super-group designated as Chromalveolates (Cavalier-
 14 Smith, 1999). The Chromalveolate hypothesis has been intensely debated in the last decade
 15 and is still a highly contentious issue (Bodyl *et al.*, 2009; Keeling, 2009; Lane & Archibald,
 16 2009).

17 This complex evolutionary history of brown algae is reflected by the uniqueness of their
 18 carbohydrate metabolism. With respect to the outflow of the Calvin cycle the photoassimilate
 19 D-fructose-6-phosphate (F6P) is not used by brown algae to produce sucrose as in higher
 20 plants, but it is mainly converted into D-mannitol. This alcohol sugar is localized in the
 21 cytosol and is by far the most abundant, organic soluble compound in brown algae (Gravot *et*
 22 *al.*, 2010, this issue). Mannitol is synthesized in two steps: F6P is reduced by mannitol-1-
 23 phosphate 5-dehydrogenase (M1PDH) into mannitol-1P, which is then converted into
 24 mannitol by mannitol-1-phosphatase (M1Pase). These enzymatic activities have been
 25 measured in several brown algae (Ikawa *et al.*, 1972), but the corresponding genes have not

1 been described yet. Mannitol is thought to be recycled by mannitol-2-dehydrogenase (M2DH)
 2 and hexokinase (Iwamoto & Shiraiwa, 2005).

3 Brown algae and other Stramenopiles also possess unique carbon storage polysaccharides.
 4 Most living organisms store carbon as linear or branched α -1,4-glucans: glycogen in
 5 Opisthokonta (animals and fungi) and in most bacteria, or starch in the diazotrophic
 6 cyanobacteria and Archaeplastida (i.e. red algae, green algae and plants, Deschamps *et al.*,
 7 2008). In contrast, the storage polysaccharide of brown algae is laminarin, a vacuolar β -1,3-
 8 glucan with occasional β -1,6-linked branches (Percival & Ross, 1951). This polysaccharide is
 9 polydisperse, consisting of a minor G-series with polymers containing only glucose residues,
 10 and a more abundant M-series with glucans terminated with a 1-linked D-mannitol residue
 11 (Read *et al.*, 1996). Diatoms and oomycetes also produce vacuolar β -1,3-glucans, known as
 12 chrysolaminarin (Beattie *et al.*, 1961) and mycolaminarin (Wang & Bartnicki-Garcia, 1974),
 13 respectively. These storage polysaccharides have a branched structure similar to that of
 14 laminarin, but they do not contain mannitol residues. In the sieve elements of Laminariales,
 15 plates are made of microfibrillar β -1,3-glucans deposits, reminiscent of plant callose (Parker
 16 & Huber, 1965). Self-assembling linear β -1,3-glucans also occur as structural components in
 17 the cell wall of Oomycetes (Bartnicki-Garcia, 1968).

18 Experiments with radioactive carbon demonstrated that laminarin and mannitol are
 19 interchangeable storage compounds in brown algae, as are sucrose and starch in higher plants
 20 (Yamaguchi *et al.*, 1966). In kelps mannitol can be remobilized and translocated via the sieve
 21 tubes from mature tissues to supply the rapidly-growing parts of the alga with carbon
 22 (Schmitz & Lobban, 1976; Lobban & Harrison, 1994).

23 The molecular bases of carbohydrate metabolism in brown algae are essentially
 24 uncharacterized. Until recently, expressed sequence tag (EST) libraries were the only
 25 molecular data available for inferences on central and storage carbon metabolisms in brown

1 algae. Analysis of a cDNA library produced from sporophytes of *Laminaria digitata* retrieved
2 six partial open reading frames corresponding to genes potentially involved in central carbon
3 metabolism (Moulin *et al.*, 1999). The complete genome sequence of the brown alga
4 *Ectocarpus siliculosus*, from the order Ectocarpales (Charrier *et al.*, 2008), is now available
5 (Cock *et al.*, 2010), providing, for the first time, a comprehensive view of brown algal
6 carbohydrate metabolism.

7 Here we decipher the metabolic routes for central sugar metabolism and carbon storage in
8 brown algae and reconstruct the phylogenetic relationships of key enzymes in these metabolic
9 pathways. This analysis provides novel insights into the origin and evolution of carbohydrates
10 in Eukaryotes and the results raise doubts with regard to the Chromalveolate hypothesis.

Materials and methods

Identification and bioinformatic analyses of carbohydrate-related proteins

The proteins involved in carbohydrate metabolism encoded by the *E. siliculosus* genome were identified by homology with biochemically characterized proteins selected in the CAZY (<http://www.cazy.org/>, Cantarel *et al.*, 2009) and UniProt databases. For each identified *Ectocarpus* protein, evidence of conserved protein modules was queried using the Pfam database (Bateman *et al.*, 2004). The presence of additional, orphan modules was detected by BlastP searches against the UniProt database. Signal peptides and transmembrane helices were predicted using HECTAR (Gschloessl *et al.*, 2008) and TMHMM (Krogh *et al.*, 2001), respectively. Numerous proteins involved in carbohydrate metabolism belong to families encompassing several enzymatic activities and/or substrate specificities. To clarify their function, these proteins were further analyzed by a phylogenetic approach. For each different activity of a poly-specific family, a set of experimentally characterized proteins was selected in the UniProt database. These representative proteins were aligned with their homologues from *E. siliculosus* using MAFFT with the iterative refinement method and the scoring matrix Blosum62 (Kato *et al.*, 2002). Phylogenetic trees were derived from this refined alignment using the Maximum Likelihood method with the program PhyML (Guindon & Gascuel, 2003). The reliability of the trees was always tested by bootstrap analysis using 100 re-samplings of the dataset. The trees were displayed with MEGA (Kumar *et al.*, 2004). The functional annotation of *Ectocarpus* proteins was based on the proximity to specific characterized proteins in the phylogenetic trees. Genomic comparisons were performed using the genomic BLAST server at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

1 Results

2 From carbon fixation to carbohydrate biosynthesis: an overview of the carbohydrate 3 active enzymes in *Ectocarpus siliculosus*

4 As in most photosynthetic organisms, Rubisco is responsible for carbon fixation in brown
5 algae, releasing two molecules of glycerate-3-phosphate (Assali *et al.*, 1991). The enzymes
6 converting this triose-phosphate into F6P are well conserved in *E. siliculosus* (Supporting
7 Information Table S1). Based on bioinformatic predictions of signal peptides (Gschloessl *et*
8 *al.*, 2008), the corresponding isoenzymes mainly differ in their localization (cytosol,
9 mitochondrial or plastid matrices). Interestingly, the gene Esi0187_0027 encodes a modular
10 protein encompassing a triose-phosphate isomerase (TPI) fused to a glyceraldehyde-3-
11 phosphate dehydrogenase (GAPDH). Homologues of this translational fusion are only found
12 in diatoms (Armbrust *et al.*, 2004; Bowler *et al.*, 2008) and Oomycetes (Tyler *et al.*, 2006;
13 Haas *et al.*, 2009), suggesting that this bifunctional enzyme is characteristic of Stramenopiles.
14 The TPI/GAPDH fusion-protein from the diatom *Phaeodactylum tricornutum* has been shown
15 to be active and is localized in mitochondria (Liaud *et al.*, 2000). The enzymes that equilibrate
16 the hexose-phosphate pool (F6P, D-glucose-6P and D-glucose-1P) are also highly conserved,
17 with two isoforms each of glucose-6 isomerase (GPI, Esi0060_0128 and Esi0266_0033) and
18 of phosphoglucomutase (PGM, Esi0002_0317 and Esi0430_0005). Moreover, Esi0430_0005
19 displays an additional UDP-glucose-pyrophosphorylase (UGP) module at the N-terminus.
20 Again, homologues of this UGP/PGM fusion-protein are only found in the genomes of
21 Oomycetes and of the diatom *P. tricornutum*. In *Thalassiosira pseudonana*, these activities
22 are however encoded by two distinct genes (Armbrust *et al.*, 2004). The bifunctional enzymes
23 TPI/GAPDH and UGP/PGM catalyze consecutive reactions, suggesting that these fusion
24 events may have increased the efficiency of the metabolic steps leading to the activated form
25 of glucose (UDP-glucose) in most Stramenopiles.

1 Synthesis of polysaccharides and glycoconjugates is catalyzed by glycosyltransferases
 2 (GT), which use activated sugar donors, or by transglycosylases. The cleavage of glycosidic
 3 linkages is performed by glycoside hydrolases (GH) or by polysaccharide lyases (PL).
 4 Carbohydrate esterases (CE) remove methyl or acetyl groups from substituted
 5 polysaccharides. Collectively these enzymes are termed Carbohydrate Active enZymes
 6 (CAZYmes). Based on sequence similarities (Henrissat, 1991), CAZYmes have been
 7 classified into more than 200 protein families (<http://www.cazy.org/>, Cantarel *et al.*, 2009).
 8 The genome of *Ectocarpus* encodes 41 GH and 88 GT, but surprisingly lacks genes
 9 homologous to known PL or CE (Supporting Information Table S2). This seaweed possesses
 10 a slightly greater number of GH/GT genes than the marine green microalgae *Micromonas* and
 11 *Ostreococcus* (Worden *et al.*, 2009), but at least six times less than terrestrial plants.
 12 *Arabidopsis thaliana*, for example, has 730 GH/GT genes (Henrissat *et al.*, 2001). However,
 13 looking at the number of CAZY families, the difference between brown algae and land plants
 14 is less pronounced: *Ectocarpus* has members of 18 GH and 32 GT families, while
 15 *Arabidopsis* contains members of 34 GH and 40 GT families. The impressive number of
 16 CAZymes in plants is mainly explained by the presence of large multigenic families. For
 17 instance, *Arabidopsis* has 67 polygalacturonases (GH28), which participate in pectin
 18 recycling, and 121 GT1 that are mainly involved in secondary metabolite biosynthesis
 19 (Henrissat *et al.*, 2001). In contrast, *Ectocarpus* features less functional redundancy, with
 20 fewer genes in each CAZY family. Interestingly, this seaweed contains some families which
 21 are absent from plants but are conserved with other phyla, such as bacteria (GH88, Δ -4,5
 22 unsaturated β -glucuronyl hydrolase), fungi (GT15, biosynthesis of cell wall mannoproteins),
 23 animals and fungi (GH30, β -glucosidase; GT23, GT49 and GT54, N-glycosylation) and
 24 Amoebozoa (GT60 and GT74, O-glycosylation of Skp1 subunits of E3 ubiquitin-protein
 25 ligase).

1 Mannitol

2 Three *Ectocarpus* proteins (Esi0017_0062, Esi0020_0181 and Esi0080_0017) share
 3 significant sequence identity (~30%) with the M1PDH from the Apicomplexa *Eimeria*
 4 *tenella*. The only known M1Pase gene was also cloned from this parasite (Liberator *et al.*,
 5 1998), but no homolog has been found in *Ectocarpus*. Recycling of mannitol is probably
 6 performed by Esi0135_0010, which is similar to bacterial and fungal M2DH. However, no
 7 ortholog of eukaryotic hexokinases was found in *E. siliculosus*. Instead, this seaweed
 8 possesses a gene, Esi0139_0025, which is closely related to cyanobacterial fructokinases.
 9 Thus, brown algae apparently are an exception to the trend that broad specificity hexokinases
 10 are typical of multicellular eukaryotes, whereas sugar-specific kinases are distinctive of
 11 bacteria and unicellular eukaryotes (Cardenas *et al.*, 1998).

12 M1PDH genes have been identified in cDNA libraries from the brown algae *L. digitata*
 13 (Roeder *et al.*, 2005) and *Sargassum binderi* (Wong *et al.*, 2007), but are absent from the
 14 genome of diatoms and Oomycetes. Homologues of M1PDH have been found in only a few
 15 organisms so far, namely Gram positive bacteria, some fungi and two species of *Micromonas*
 16 (Worden *et al.*, 2009). The presence of M1PDH in *Micromonas* is surprising, since this gene
 17 is absent from the genomes of two other green algae, namely *Ostreococcus* (Derelle *et al.*,
 18 2006) and *Chlamydomonas* (Merchant *et al.*, 2007), from the red alga *Cyanidioschyzon*
 19 *merolae* (Matsuzaki *et al.*, 2004) and from terrestrial plants. However, mannitol has been
 20 reported in some other Prasinophytes (Kremer, 1980) and M1PDH activity was detected in
 21 the green alga *Platymonas subcordiformis* (Richter & Kirst, 1987). Phylogenetic analyses
 22 suggest that bacterial M1PDH genes were acquired independently by fungi and brown algae
 23 (Fig. 2a). In contrast, the M1PDH sequences of *E. tenella* and of the *Micromonas* species are
 24 nested within the brown algal clade. The *Micromonas* M1PDH genes (MICPUN_62892 and
 25 MICPUC_48208) both encode fusion proteins that include a haloacid dehalogenase-like

domain (HAD). Interestingly the HAD superfamily contains various phosphatases, including sucrose-phosphate and trehalose-phosphate phosphatases (Ridder & Dijkstra, 1999). The two *Micromonas* species possess a second HAD-like enzyme existing as a standalone protein (MICPUC_47598 and MICPUN_101665). These M1PDH/HAD proteins are reminiscent of the fusion sucrose-phosphate synthase / sucrose-phosphate phosphatases (SPS/SPP) and trehalose-phosphate synthase / trehalose-phosphate phosphatases (TPS/TPP), suggesting that the HAD modules provide the missing M1Pase activity. This assumption is further strengthened by the identification in *Ectocarpus* of two standalone proteins (Esi0080_0016 and Esi0100_0020) highly similar to the *Micromonas* putative M1Pases (63% sequence identity with MICPUC_47598). In addition the putative M1Pase Esi0080_0016 is located next to the M1PDH gene Esi0080_0017. This would be a rare example of functional clustering in *Ectocarpus* (Cock et al., 2010). A conserved putative M1Pase was also found in the EST library of the brown alga *Fucus serratus* (Pearson *et al.*, 2009), while additional significant homologues occur in some bacteria and fungi only. Comparison with crystal structures of HAD-like phosphatases confirm that the catalytic machinery (Ridder & Dijkstra, 1999) is strictly conserved in the putative M1Pases (data not shown). Phylogenetic analyses indicate that brown algal putative M1Pases are also basal to the *Micromonas* standalone enzymes, while putative M1Pases fused to M1PDH form a sister group (Fig. 2b).

Non-reducing disaccharides

Sucrose and trehalose are the most commonly occurring non-reducing disaccharides. Their metabolic pathways are biochemically similar, but involve enzymes which are not homologous (Paul *et al.*, 2008). Phylogenetic analyses of sucrose-related proteins indicate that plant sucrose metabolism was acquired from the cyanobacterial progenitor of chloroplasts (Salerno & Curatti, 2003). Sucrose metabolism is completely absent in *Ectocarpus*, as deduced from the lack of genes encoding SPS (family GT4), sucrose synthase (GT4), SPP and

invertases (families GH32 and GH100). These enzymes are also absent in diatoms and Oomycetes, with the exception of the GH32 family of invertases present in *Phytophthora* species. However, these latter enzymes are related to fungal orthologues, suggesting that *Phytophthora* uses invertases to feed on plant sucrose. In contrast, *Ectocarpus* possesses a complete trehalose pathway. This sugar is synthesized by a family of six bifunctional enzymes, including a TPS (family GT20) fused to a TPP, while the recycling of trehalose is assured by a single trehalase (family GH37).

TPS are widespread in bacteria, suggesting a prokaryotic origin for these glycosyltransferases. Bacterial TPS exists in two forms, either as standalone protein or as a fusion with TPP. Both types of TPS exist in fungi and they form two distinct clades in phylogenetic trees (data not shown). In contrast, TPS are only present as fusion-proteins in *Dictyostelium discoideum* (Amoebozoa), insects, red algae, green algae, plants, Alveolates and Stramenopiles. Phylogenetic analyses of the TPS and TPP domains of these fusion-proteins result in similar topologies, with similar clades observed for both domains (Fig. 3), even though the TPS domains are more divergent and score weaker bootstrap values. Therefore the TPS and TPP domains have co-evolved, indicating that all these eukaryotic phyla have acquired TPS directly as fusion-proteins. Bacterial and archaeal TPS domains constitute a well-supported clade, which was chosen as the outgroup (Fig. 3a). The TPS from insects emerge as an isolated cluster, while the fungal bifunctional TPS are distantly related to the TPS domain of *D. discoideum*. The TPS from Archaeplastida, Alveolates and Stramenopiles form three distinct clades, which group together. The red alga *C. merolae* possesses three TPS isoforms (CMI293C, CMO053C and CMP219C). Green algal and plant TPS cluster together into two well-supported clades, which are sister groups of CMI293C and CMO053C, respectively. However, CMP219C-like TPS genes are absent from the green lineages (Fig. 3a), indicating either gene loss in the ancestor of green algae and plants or a

specific gene duplication in red algae. Red algal TPS are basal to the TPS from Stramenopiles and Alveolates, indicating that these eukaryotic phyla acquired TPS from ancestral red algal endosymbiont(s). *Ectocarpus* possesses the three types of red algal TPS and has even evolved a complex CMI293-like TPS family. Oomycetes conserved the CMP219C- and CMO053C-like TPS but they lost the CMI293C-like TPS. In contrast, diatoms and Apicomplexa have conserved only this latter type of TPS (Fig. 3a).

Laminarin and other β -1,3-glucans

The biosynthetic pathway of laminarin is essentially unknown, but we identified several genes which are likely to be involved in this metabolism in the *Ectocarpus* genome. This seaweed contains two cytosolic isoforms of UDP-glucose pyrophosphorylase (UGP, Esi0144_0004 and Esi0430_0005), supporting the assumption that UDP-glucose is the activated sugar needed for the production of laminarin. Beta-1,3-glucan synthases fall into two different GT families: the GT2, a polyspecific family which includes bacterial β -1,3-glucan synthases, and the GT48, which only contains eukaryotic β -1,3-glucan synthases (Cantarel *et al.*, 2009). *Ectocarpus* harbors eleven GT2, none of which are related to bacterial β -1,3-glucan synthases (Michel *et al.*, 2010, this issue). In contrast, the three members of the GT48 family (Esi0033_0138, Esi0193_0029 and Esi0338_0032) display significant similarities with plant callose synthases (~35% sequence identity). The phylogenetic tree of the GT48 family (Fig. 4) is congruent with the currently accepted phylogeny of the Eukaryotes (Fig. 1), with the β -1,3-glucan synthases from plants, fungi, Apicomplexa and Stramenopiles consistently emerging as distinct clades. The β -1,3-glucan synthases of Stramenopiles are further divided into three groups. Clade A, which includes Esi0338_0032, is common to brown algae, diatoms and Oomycetes suggesting that these glycosyltransferases are responsible for the polymerization of the backbones of laminarin, chrysolaminarin and mycolaminarin, respectively. The β -1,3-glucan synthases of clade B are only found in

Phytophthora and, therefore, they are likely to be involved in the biosynthesis of the Oomycete cell wall β -1,3-glucans. Clade C is unique to brown algae, but the exact role of Esi0033_0138 and Esi0193_0029 is unclear. These β -1,3-glucan synthases could specifically catalyze the production of laminarin M-series, which is a distinctive feature of brown algae (Read *et al.*, 1996). Alternatively, they might be involved in callose biosynthesis, this molecule having been found in the sieve plates of Laminariales (Parker & Huber, 1965). In addition, *Ectocarpus* possesses two proteins (Esi0100_0034 and Esi0243_0020) which are homologous to KRE6, a GH16 family transglycosylase involved in β -1,6-branching of cell wall β -1,3-glucans in yeasts (Montijn *et al.*, 1999). Therefore, these two proteins represent good candidates for the synthesis of β -1,6-linked branches of laminarin. This hypothesis is strengthened by the conservation of KRE6-like proteins in diatoms and Oomycetes. Remarkably, the KRE6-like protein PITG_03335 from *P. infestans* (Haas *et al.*, 2009) is fused to a GT48 family β -1,3-glucan synthase. This GT48 module belongs to the clade A (Fig. 4), the very subgroup that we suggest to be responsible for laminarin polymerization.

The degradation of laminarin is potentially catalyzed by ten endo-1,3- β -glucanases belonging to three different families (GH16: 4 genes; GH17: 1 gene; GH81: 5 genes), and by two exo-1,3-beta-glucanases (family GH5). These numerous laminarinases have homologues in bacteria (family GH16), fungi (families GH5 and GH81) and plants (family GH17), underlining the complexity of laminarin metabolism in brown algae. Laminarin oligosaccharides would be further hydrolyzed by β -glucosidases of the GH1 (Esi0061_0010, Esi0176_0045 and Esi0212_0019) or GH3 families (Esi0010_0226). The end-product, glucose, would be subsequently phosphorylated by a glucokinase (Esi0000_0270) before entering glycolysis.

***Ectocarpus siliculosus* has not retained any trace of the starch metabolism of its red algal endosymbiont.**

The uptake of a red alga by the heterotrophic ancestor of brown algae resulted in the acquisition of photosynthesis. In plants and red algae, the acquisition of photosynthetic capacity following the capture of a cyanobacteria was accompanied by the transformation of the glycogen metabolism of the ancestral host cell into a starch biosynthetic pathway (Ball & Morell, 2003; Deschamps *et al.*, 2008). In contrast to plants, red algae synthesize starch in their cytoplasm, and not in their plastids, and they use UDP-glucose as the activated sugar instead of ADP-glucose (Nyvall *et al.*, 1999; Viola *et al.*, 2001). The question then arises as to whether there is any remnant of this red algal starch metabolism in extant brown algae.

Starch and glycogen are synthesized and recycled by homologous enzymes. The α -1,4-glucan backbone is produced by starch synthase (GT5) or glycogen synthase (GT3 and GT5), while branching enzymes (GH13) are responsible for the creation of α -1,6 branches (Ball & Morell, 2003). Recycling is mainly performed by the combined action of glycogen/starch phosphorylases (GT35), debranching enzymes (GH13) and α -1,4-glucanotransferases (GH77). Additional hydrolytic enzymes are also involved in α -1,4-glucan catabolism: α - and β -amylases (GH13 and GH14) and α -glucosidases of the GH31 family (Ball & Morell, 2003). Starch degradation is specifically initiated by glucan water dikinases (GWDs) and phosphoglucan water dikinases (PWDs), which loosen the crystalline starch granules (Edner *et al.*, 2007). A systematic search for all these enzymes in *Ectocarpus* did not retrieve any corresponding gene. This seaweed does contain two isoforms of UGP, but these enzymes are not specific for glycogen/starch metabolism as they could also be involved in other biosynthetic pathways (cellulose, laminarin, glycosylation). The ADP-glucose pyrophosphorylase (AGP), which is responsible for ADP-glucose synthesis in plants, is also

- 1 absent from brown algae. Diatom and Oomycete genomes also completely lack starch- or
- 2 glycogen-related genes.

For Peer Review

Discussion

The central and storage carbohydrate metabolism in brown algae

Figure 5 shows the central and storage carbon metabolism of *E. siliculosus*, as reconstructed from the whole genome analyses reported above. In brown algae, this metabolism is profoundly different from what is known for fungi, animals and plants, a uniqueness which is further reinforced in the phylogenomic reconstruction of the metabolic routes for the biosynthesis of cell wall polysaccharides (Michel *et al.*, 2010). With the exception of GDP-mannose pyrophosphorylase (Michel *et al.*, 2010), the enzymes involved in the equilibration of the hexose-phosphate pool and in the generation of activated sugars are conserved with other eukaryotes. However, these key sugars are then used by specific enzymes to generate two unique storage compounds, mannitol and laminarin. Fructose-6-phosphate plays a central role in these pathways. As in the other photosynthetic lineages, this photoassimilate is the precursor of all activated sugars. In brown algae, however, F6P can be also directly transformed into a soluble storage compound (mannitol), without prior conversion into an activated sugar (Fig. 5). Two other activated sugars assume pivotal functions, (i) UDP-glucose (UDPG), as the starting point for the biogenesis of trehalose, laminarin and cellulose, and (ii) GDP-mannose, which is the direct precursor of alginates and the indirect precursor of sulfated fucans (Michel *et al.*, 2010).

This study highlights numerous candidate-genes for which further experimental analyses are needed to confirm their exact biochemical function. Particularly attractive targets are the putative M1Pases and the KRE6-like proteins, which are potentially responsible for the final steps of mannitol and laminarin biosynthesis, respectively. Our findings also open several novel questions as to the nature of the central and storage carbohydrate metabolism in brown algae. To the best of our knowledge, the presence of trehalose was never reported in brown

algae before this study, even in recent reviews (Elbein et al., 2003; Iturriaga et al., 2009). In plants, trehalose and trehalose-6-P (T6P) are in low abundance and difficult to assay. The role of trehalose in green plants is uncertain, but it may regulate starch breakdown (Paul *et al.*, 2008). T6P is considered to be a signal of glucose-6-P (G6P) and UDPG pool size and, hence, to acts as an effective indicator of sucrose. This signal metabolite is thus thought to coordinate carbon metabolism with plant development in response to carbon availability and stress (Paul *et al.*, 2008). By analogy with plants, and since F6P, G6P and UDPG are also interconnected to T6P in *Ectocarpus* (Fig. 5), trehalose and T6P might act as metabolic regulators in brown algae.

Another unresolved question is the biochemical route which connects mannitol and laminarin and the reason why the majority of the laminarin chains are terminated by a mannitol residue at their reducing end (Read *et al.*, 1996). An attractive working hypothesis is that the addition of a mannitol residue is probably catalyzed by specific glycosyltransferases, for instance the GT48 isoforms which are unique to brown algae (Fig. 4), or by a new, undiscovered family of glycosyltransferases. Considering the catalytic mechanism of glycosyltransferases (Lairson *et al.*, 2008), transfer of activated glucose onto mannitol is likely to be the first step in the biosynthesis of the laminarin M-chains. Indeed, the simplest way to form a covalent bond between mannitol and glucose residues would be for an as yet undescribed GT to use mannitol as an acceptor molecule while the donor sugar substrate would be UDPG. The newly formed glucose-mannitol disaccharide would be subsequently used as an acceptor molecule by a GT48 which would further elongate the laminarin chain.

Origin of central and storage carbohydrates in brown algae

Brown algae and the other Stramenopiles have a rich evolutionary history. This lineage arose from two independent endosymbiotic events, the symbiosis of a protoeukaryote with an alphaproteobacterium and the symbiosis between a protist and a red alga. The

1 alphaproteobacterial symbiont was progressively converted into mitochondria whereas the
 2 red-algal symbiont gave rise to plastids. As red algae had themselves originated from a
 3 primary endosymbiosis, between a protist and a cyanobacterium, the emergence of
 4 Stramenopiles is described as a secondary endosymbiosis, which involved two eukaryotic
 5 cells (Reyes-Prieto *et al.*, 2007). Based on molecular clock analyses calibrated by
 6 paleontological constraints, Stramenopiles are thought to have diverged from other major
 7 eukaryotic groups about 1 billion years ago and it is also thought that the secondary
 8 endosymbiosis happened shortly after the primary endosymbiosis (Douzery *et al.*, 2004).

9 A recent genomic study has further complicated this already complex picture by suggesting
 10 that a cryptic endosymbiosis with a green alga, related to extant Prasinophytes such as
 11 *Micromonas*, had occurred in Stramenopiles before the capture of the red algal secondary
 12 endosymbiont. This hypothesis is based on the presence, in Stramenopile genomes, of a high
 13 number of genes phylogenetically related to those of green microalgae (Moustafa *et al.*,
 14 2009). However, the only available whole-genome resource for the red algal lineage so far is
 15 the genome sequence of *C. merolae*, an unusual red microalga which lacks cell wall, lives in
 16 acidic hot water and has a reduced genome (Matsuzaki *et al.*, 2004). Therefore, the
 17 interpretation of Moustafa and coworkers is somewhat weakened from the lack of genomic
 18 data from archetypal red algae, which may also possess many of the proposed “green algal”
 19 genes (Dagan & Martin, 2009). In this intricate context we discuss below the possible origins
 20 of the various carbohydrates from brown algae.

21 **Trehalose was acquired from the red algal endosymbiont**

22 Sucrose is mainly limited to plants, green algae and cyanobacteria, while trehalose is found
 23 in a large range of bacteria and Eukaryotes (Salerno & Curatti, 2003). The widespread
 24 occurrence of trehalose led to the hypothesis that this non-reducing disaccharide is a more
 25 ancient metabolite than sucrose (Goddijn & van Dun, 1999). Sucrose metabolism in green

1 algae and land plants (Chloroplastida) originated from the cyanobiont (Salerno & Curatti,
 2 2003). Consequently, red algae probably acquired sucrose metabolism during primary
 3 endosymbiosis as well, even though these photosynthetic organisms are not currently known
 4 to produce sucrose (Kremer, 1980). Inspection of the genome of *C. merolae* (Matsuzaki *et al.*,
 5 2004) indicates that this red alga does not contain any sucrose-related gene. Similarly, genes
 6 involved in sucrose metabolism are completely absent from Stramenopiles genomes, as
 7 deduced from the lack of sucrose synthase, sucrose-phosphate synthase and of invertase.
 8 Therefore, it is very likely that the red alga that gave birth to the phaeoplasts of extant
 9 Stramenopiles (Reyes-Prieto *et al.*, 2007) had already lost the cyanobacterial sucrose-
 10 metabolism when the secondary endosymbiosis took place. Nevertheless, this hypothesis
 11 needs additional genomic data on red algae to be definitively confirmed.

12 In the case of trehalose, the question also arises as to the origin of this carbon storage
 13 disaccharide in brown algae; whether it was originated from the host or from the red algal
 14 endosymbiont. Trehalose synthesis requires two enzyme activities: trehalose-phosphate
 15 synthase (TPS) and trehalose-phosphate phosphatase (TPP). Here we show that the six
 16 TPS/TPP of *Ectocarpus* are closely related to their orthologs from *C. merolae* (Fig. 3).
 17 Therefore, it is likely that trehalose metabolism was transmitted into Stramenopiles by the
 18 endosymbiont and that the red algal genes supplanted preexisting host orthologs, if any
 19 existed. The assimilation of the red algal pathway by the host was probably facilitated by the
 20 fact that this pathway already consisted of a single bifunctional enzyme, with both TPP and
 21 TPS activities, in ancestral red algae (Fig. 5).

22 **Mannitol: evidence for a major horizontal gene transfer event from Actinobacteria**

23 Brown algae differ from other eukaryotes, including diatoms and Oomycetes, by their
 24 capacity to synthesize an additional carbon storage sugar, mannitol. The conversion of F6P
 25 into mannitol involves two enzymatic steps (Fig. 5), catalyzed by M1PDH and M1Pase.

These enzyme activities are indeed rather rare among living organisms and here we show that the M1PDH and putative M1Pase genes from *Ectocarpus* are closely related to those of Gram positive bacteria (Fig. 2). Based on this close phylogenetic relationship, we propose that, after the divergence of brown algae from diatoms and Oomycetes, these two enzymes were imported into brown algae by a horizontal gene transfer involving an ancestral Gram positive bacterium related to extant Actinobacteria. It is noteworthy that this bacterial phylum is common and diversified in the marine environment (Bull *et al.*, 2005). As reported in more detail in the second part of this study (Michel *et al.*, 2010), this HGT event extended beyond the acquisition of mannitol metabolism. The actinobacterium would also have contributed to the enzyme machinery that synthesizes alginate and hemicelluloses, two major cell wall polysaccharides which are absent from diatoms and Oomycetes. Therefore, this bacterial HGT was probably instrumental in the emergence of complex multicellularity in brown algae, by providing two essential elements in this evolutionary process: (i) extracellular matrix polysaccharides for the construction of multicellular tissues and organs, (ii) and mannitol for the long distance translocation of photoassimilates (Parker & Huber, 1965).

***Micromonas* acquired the capacity to synthesize mannitol from brown algae**

In the phylogenetic analyses of M1PDH and putative M1Pases, the Stramenopile genes cluster with their orthologues in *Micromonas* (Prasinophytes), suggesting that these microalgae feature a mannitol metabolism of brown algal origin. Beyond the robustness and the congruence of the phylogenetic trees (Fig. 2), several pieces of evidence support the direction of this potential HGT, from brown algae to these Prasinophytes: (i), M1PDH and putative M1Pases are standalone genes in *Ectocarpus* and the closest orthologs of brown algal M1Pases in *Micromonas* are also standalone proteins, whereas the M1Pases fused to M1PDH are more divergent (Fig. 2b). This suggests that a gene duplication specifically occurred in *Micromonas* followed by a gene-fusion event, between M1PDH and the putative M1Pase

domains; (ii), M1PDH and putative M1Pase genes are not conserved in other Prasinophyte genomes and are absent from red algae, Chlorophytes, mosses and higher plants. In contrast, orthologues of these genes are present in brown algal EST libraries (Roeder *et al.*, 2005; Wong *et al.*, 2007; Pearson *et al.*, 2009); and (iii), the other genes of actinobacterial origin in *Ectocarpus* are not present in Prasinophytes (Michel *et al.*, 2010), suggesting that there was not a direct HGT from Actinobacteria to Prasinophytes. Altogether these findings indicate that the mannitol-related genes in *Micromonas* were acquired relatively recently from a brown alga by these Prasinophytes.

Laminarin biosynthesis is ancestral in Stramenopiles

The β -1,3-glucan synthases from brown algae and other Stramenopiles belong to the GT48 family, a family that is conserved in most eukaryotic phyla, but which is absent from Bacteria and Archaea. The phylogenetic tree of the GT48 family is congruent with the currently accepted phylogeny of Eukaryotes (Fig. 4). This finding indicates that β -1,3-glucans were present in the last common ancestor of eukaryotes and that laminarin is an ancestral metabolite in Stramenopiles. The conservation between fungi (Unikonts) and Stramenopiles (Bikonts) of KRE6-like proteins (GH16) and some laminarinases (GH5 and GH81) is also consistent with the ancestral character of laminarin metabolism. It is likely that intracellular β -1,3-glucans were the ancestral forms of these carbohydrates and that they subsequently evolved to be secreted as extracellular polysaccharides as complex multicellularity emerged. Interestingly, such an evolution occurred independently in fungi and in plants, as well as within the Stramenopiles, with Oomycetes and Laminariales producing both storage and cell wall β -1,3-glucans (Parker & Huber, 1965; Bartnicki-Garcia, 1968).

1 **Insights into the origin and evolution of storage polysaccharides in Eukaryotes**

2 Biosynthesis and remobilization of carbon stores is a fundamental process in all living
3 cells. Extant Eukaryotes exhibit a striking diversity in the nature of their storage
4 polysaccharides, which probably reflects their complex evolutionary history. We discuss
5 below various evolutionary scenarios which would account for such a high diversity.

6 *The starch metabolism from the red algal endosymbiont was not retained in* 7 *Stramenopiles*

8 Starch metabolism is a complex process, involving a minimal set of ten different enzymes
9 in Rhodophyceae (Deschamps *et al.*, 2008). From the seminal work of Steven Ball and
10 colleagues on the origin of starch metabolism, it is now well recognized that this biosynthetic
11 pathway, characteristic of Archaeplastida, results from the merging of the glycogen
12 metabolism of the eukaryotic host cell with the starch metabolism of the cyanobacterial
13 endosymbiont (Ball & Morell, 2003; Deschamps *et al.*, 2008; Plancke *et al.*, 2008). In
14 particular, all the genes required for the complete pathway of glycogen metabolism in
15 heterotrophic Eukaryotes are maintained in red algae. The transition from glycogen to starch
16 metabolism was due to the transfer of a small number of cyanobacterial genes, namely
17 isoamylase (GH13), granule bound starch synthase I (GBSSI, GT5) and α -(1,4)-
18 glucanotransferase (GH77), to the host nuclear genome (Deschamps *et al.*, 2008). This fate
19 contrasts with the usual loss of storage polysaccharides by bacterial parasites and obligatory
20 symbionts (Henrissat *et al.*, 2002; Gil *et al.*, 2004). The success of the acquisition of starch
21 metabolism in the Archaeplastida following plastid primary endosymbiosis was probably due
22 to compatibility between biosynthetic pathways in the eukaryotic host cell and in its
23 cyanobiont. The limited number of transferred genes needed for this metabolic transformation
24 was also a favorable factor (Deschamps *et al.*, 2008).

In contrast to Unikonts and Archaeplastida, the genomes of brown algae, diatoms and Oomycetes completely lack starch- or glycogen-related genes. It follows that the starch metabolism of the red algal secondary endosymbiont was not retained in Stramenopiles. Instead, their storage compounds consist of laminarin, a β -1,3-glucan. Here we demonstrate that the metabolism of β -1,3-glucans involves ancient eukaryotic enzymes. Therefore, we propose that the carbon storage machinery of the protistean ancestor of Stramenopiles was exclusively dedicated to the synthesis of β -1,3-glucans, a metabolism totally unrelated to the biosynthesis of α -1,4-glucans in its red algal endosymbiont. Based on the reasoning developed by Ball and coworkers for Archaeplastida, and as observed for obligatory symbionts (Henrissat *et al.*, 2002; Gil *et al.*, 2004), this metabolic incompatibility resulted in a rapid loss of the starch metabolism of the engulfed red alga.

Synapomorphies of Stramenopiles with respect to carbon storage metabolism do not support the Chromalveolate hypothesis

In comparison to the other major eukaryotic groups, Stramenopiles and Alveolates are rather closely related lineages (Baldauf, 2008). They are thought to constitute the kingdom of Chromoalveolates, distinct from the two other monophyletic super-groups, Unikonts and Archaeplastida (Fig. 1). In spite of their phylogenetic relatedness with Stramenopiles, Alveolates such as Dinoflagellates and Apicomplexa accumulate starch in their cytoplasm (Coppin *et al.*, 2005). As observed in the Archaeplastida, the starch pathway of Alveolates is a hybrid metabolism. The indirect debranching enzymes from Apicomplexa have homologues in animals and fungi, but not in Archaeplastida, indicating that the alveolate host cell contained its own pathway for glycogen synthesis (Coppin *et al.*, 2005). Phylogenetic analyses of other key enzymes for starch synthesis demonstrate that, following the secondary endosymbiosis, the ancestral host-cell glycogen pathway evolved into a genuine starch metabolism through the transfer of red algal starch-related genes (Coppin *et al.*, 2005).

Altogether, Stramenopiles and Alveolates have evolved completely different pathways for carbon storage: (i), the host cells of Stramenopiles and Alveolates differed in their ancestral carbon storage polysaccharide forms, β -1,3-glucan versus glycogen respectively; (ii), these metabolic divergences resulted in radically different fates for red algal endosymbionts, complete loss of the red algal starch pathway in Stramenopiles as opposed to transition from glycogen to red algal-like starch metabolism in Alveolates. It is very unlikely that two lineages with the same original genetic background, that is, genomes from the same ancestral host and rhodobiont cells would have evolved two so strikingly different carbon storage metabolisms. It follows that extant Stramenopiles and Alveolates arose from related, yet distinct eukaryotic host cells and from independent secondary endosymbiotic events. The occurrence of two independent, secondary endosymbiotic events contradicts the Chromalveolate hypothesis (Cavalier-Smith, 1999).

Origin of carbon storage polysaccharides in Eukaryotes

In conclusion, compared to the other eukaryotic phyla, Stramenopiles feature very distinctive traits in their storage carbon metabolism. Long-term carbon storage is based on laminarin, a soluble vacuolar β -1,3-glucan, whereas most other eukaryotes use polysaccharides based on α -1,4-glucan, either on a soluble (glycogen) or insoluble (starch) form. Interestingly, Euglenophytes (Excavata) and Haptophytes, two lineages independent from Stramenopiles (Fig. 1), also use β -1,3-glucans as carbon storage (Kiss *et al.*, 1988; Andersen, 2004).

The question then arises of the origin of these two classes of storage polysaccharides and whether one class predates the other. As shown in this study, the polymerization of β -1,3-glucans is catalyzed by GT48, a protein family conserved in most Eukaryotic groups, but absent in Prokaryotes. Therefore, we propose that β -1,3-glucans are the archetypal storage polysaccharide in Eukaryotes (Fig. 6). In contrast, most of the genes involved in eukaryotic

glycogen metabolism are shared with bacteria (Ball & Morell, 2003), suggesting that glycogen is of bacterial origin. It is difficult to trace back the bacterial group which could have transmitted this metabolism to Eukaryotes. The most parsimonious scenario is that Eukaryotes acquired glycogen metabolism from the alphaproteobacterial progenitor of mitochondria (Fig. 6). If this is true, two forms of carbon storage, β -1,3-glucan and glycogen, may have been transiently present after the mitochondrial endosymbiosis in all of the emerging eukaryote lineages. In most of them, glycogen then supplanted β -1,3 glucan as the form of carbon storage. However, the enzymes responsible for β -1,3-glucan synthesis were conserved in several eukaryotic groups (e.g. fungi, plants, Oomycetes, brown algae), where they evolved to produce extracellular β -1,3-glucans. In Archaeplastida and Alveolates, the ancestral glycogen metabolism of the host cell evolved into a starch metabolism, after the plastid primary and secondary endosymbioses, respectively (Coppin *et al.*, 2005; Deschamps *et al.*, 2008).

In the ancestors of extant Stramenopiles, the bacterial glycogen metabolism was lost (Fig. 6). As discussed above, we propose that this evolutionary route was probably completed before the secondary endosymbiosis took place, thus preventing the incorporation of the red algal starch metabolism into the host cell. Finally, in brown algae only, a significant HGT happened with an actinobacterium, leading to the *de novo* acquisition of an additional pathway for carbon storage, based on mannitol. Considering also that they have inherited an ancestral trehalose metabolism from their red algal endosymbiont, brown algae have evolved a very unique central and storage carbon metabolism. From a similar phylogenomic analysis of the enzymes involved in cell wall polysaccharides synthesis, we show that a variety of other major speciation events further contributed to the striking metabolic uniqueness of this lineage (Michel *et al.*, 2010).

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Figure legends

Fig. 1. Simplified phylogeny of the major groups of eukaryotes (adapted from Baldauf, 2008).

Fig. 2. Unrooted phylogenetic tree of the mannitol-1-phosphate 5-dehydrogenases (A) and of the putative mannitol-1-phosphatases (B). The phylogenetic trees were derived using the Maximum Likelihood (ML) approach with the program PhyML (Guindon & Gascuel, 2003). Numbers indicate the bootstrap values in the ML analysis. The full listing of the aligned proteins is reported in Supporting Information Table S3. The sequences marked by black diamonds correspond to the *Ectocarpus* proteins.

Fig. 3. Comparison of phylogenetic trees of modular trehalose-phosphate synthases (A) and of their appended trehalose-phosphate phosphatase modules (B). These phylogenetic trees were derived using the Maximum Likelihood (ML) approach with the program PhyML (Guindon & Gascuel, 2003). Each tree has been rooted using the bacterial orthologs. Numbers indicate the bootstrap values in the ML analysis. The full listing of the aligned proteins is reported in Supporting Information Table S3. The sequences marked by black diamonds and a square correspond to the proteins from the brown alga *Ectocarpus siliculosus* (◆) and the red alga *Cyanidioschyzon merolae* (■), respectively.

Fig. 4. Unrooted phylogenetic tree of the β -1,3-glucan synthases (family GT48). This phylogenetic tree was derived using the Maximum Likelihood (ML) approach with the program PhyML (Guindon & Gascuel, 2003). Numbers indicate the bootstrap values in the ML analysis. The full listing of the aligned proteins is reported in Supporting Information Table S3. The sequences marked by black diamonds correspond to the *Ectocarpus* proteins.

Fig. 5. Schematic representation of the central and storage carbon metabolism of *Ectocarpus siliculosus* based on genome annotation data. Gene products contributing to

these pathways are indicated by the code XXXX_YYYY where XXXX indicates the superconting number and YYYY the gene number of the locus on this supercontig. The prefix Esi has been omitted to improve clarity. Mannitol metabolism: M1PDH, mannitol-1-phosphate 5-dehydrogenase; M1Pase, mannitol-1-phosphatase; M2DH, mannitol-2-dehydrogenase; FK, fructokinase. Laminarin synthesis: GPI, glucose-6-phosphate isomerase; PGM, phosphoglucomutase; UGP, UDP-glucose-pyrophosphorylase; GT48, β -1,3-glucan synthases (family GT48); KRE6-like proteins, putative 1,6- β -transglucosylases (family GH16). The question mark indicates the absence of an identified candidate for the reaction that links mannitol to β -1,3-glucan to produce the M-series of laminarin. The dotted arrows and boxes indicate biosynthetic pathways related to cell wall polysaccharides (cellulose, alginates and sulfated fucans).

Fig. 6. Schematic flowchart illustrating the evolution of carbon storage polysaccharides within major eukaryotic lineages. Endosymbiosis events are indicated by dotted lines. ME: Mitochondrial endosymbiosis; PE: plastid primary endosymbiosis; SE: plastid secondary endosymbiosis.

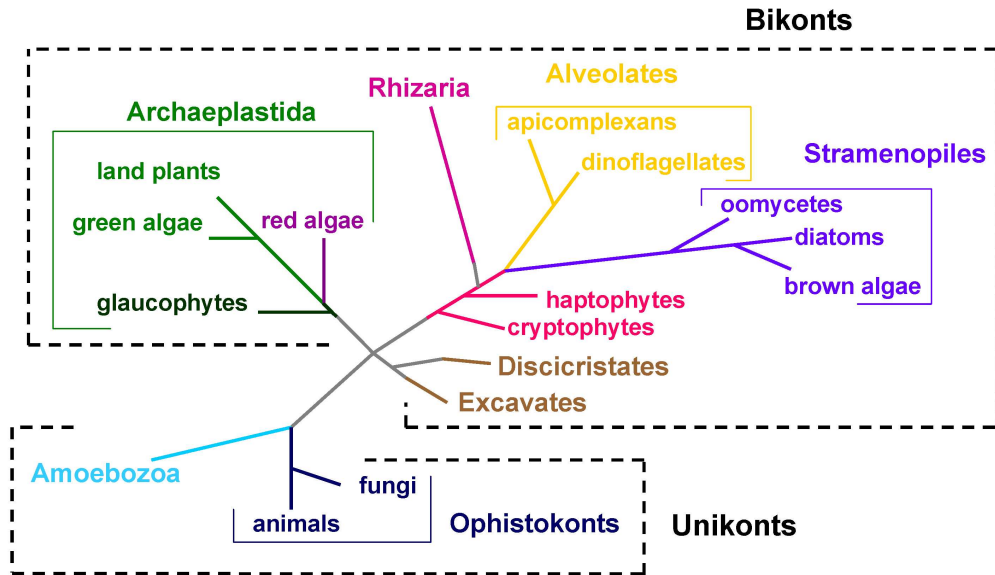


Figure 1
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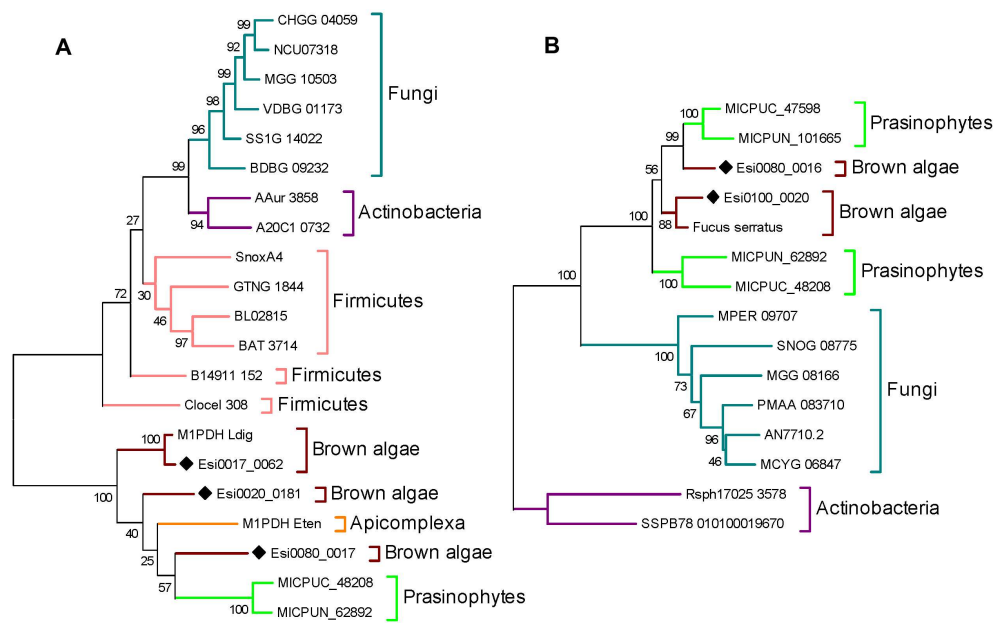


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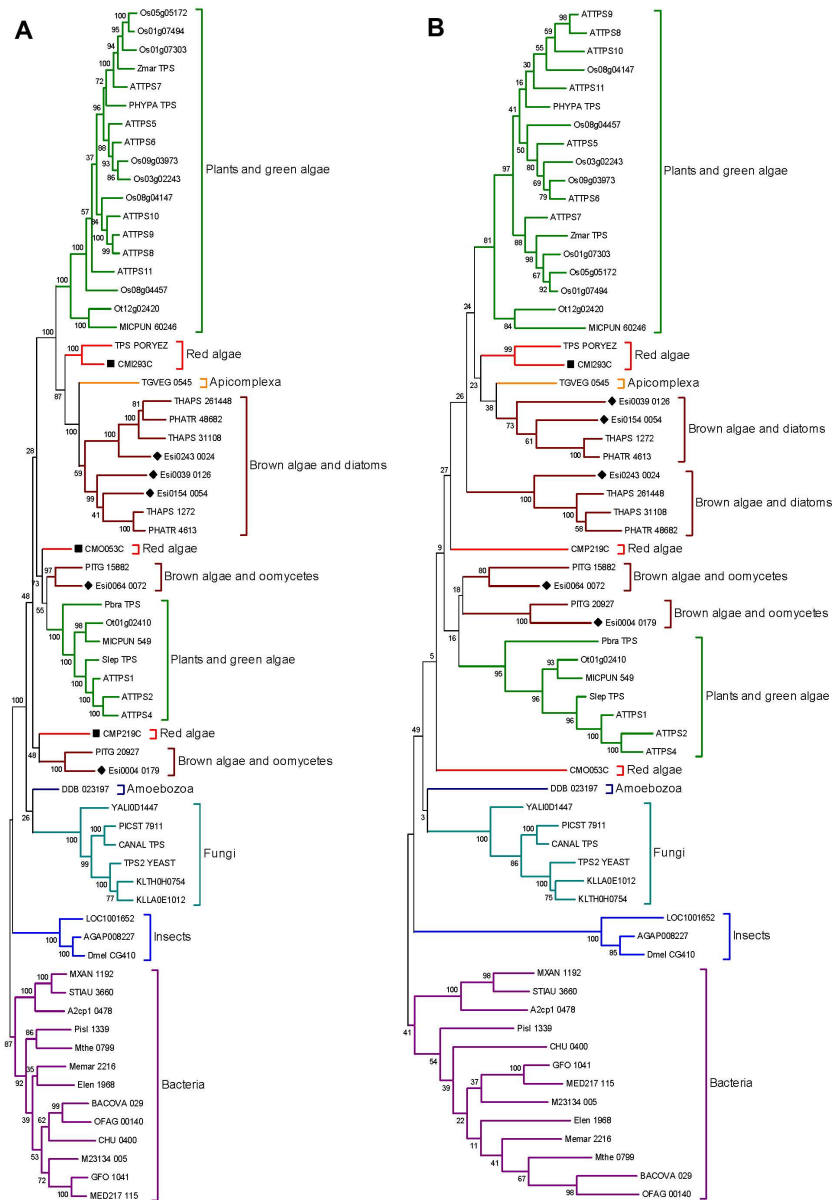


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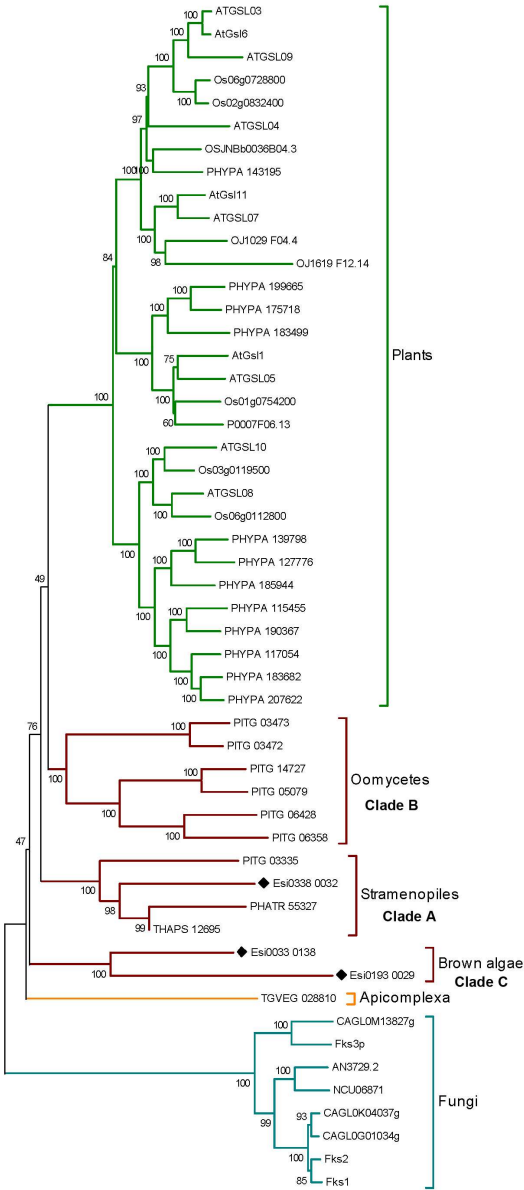


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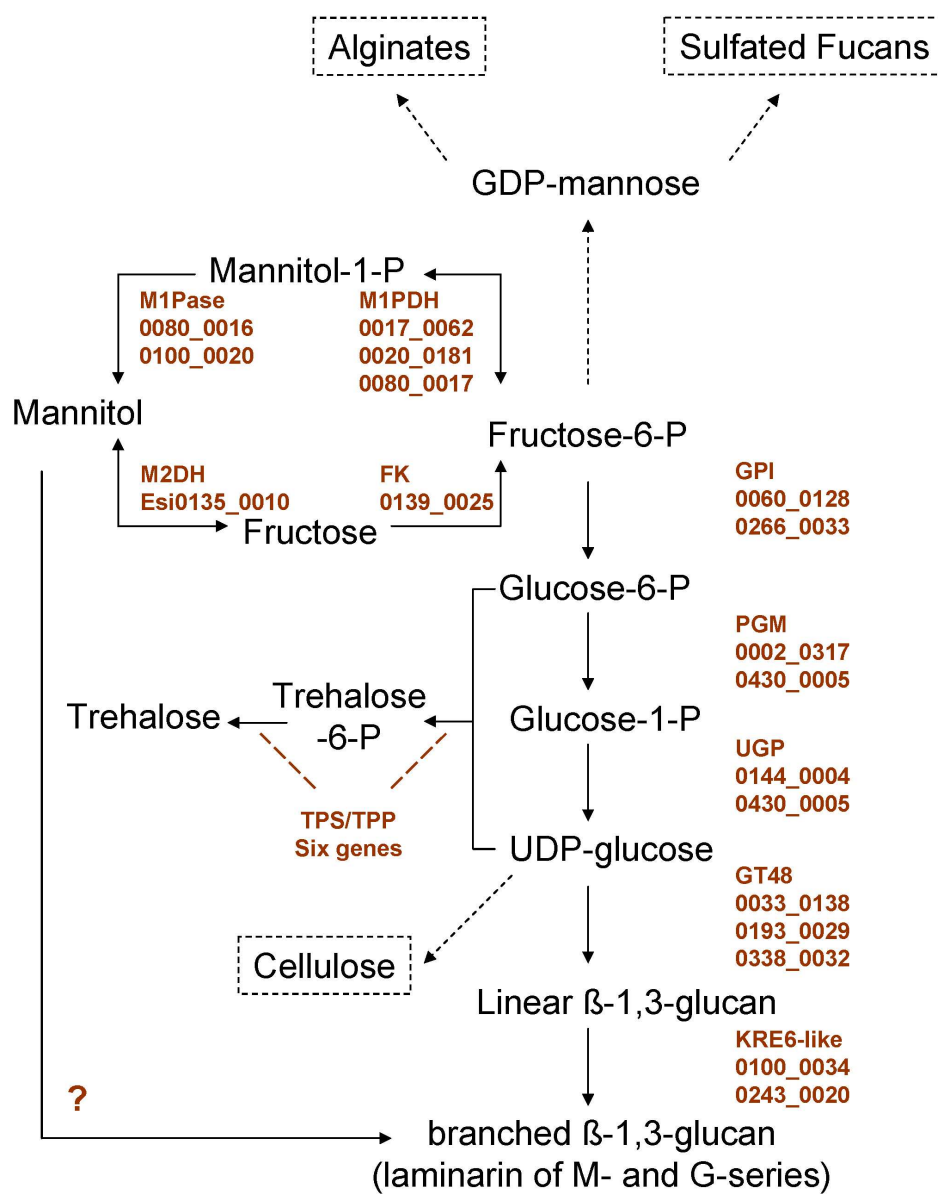


Figure 5
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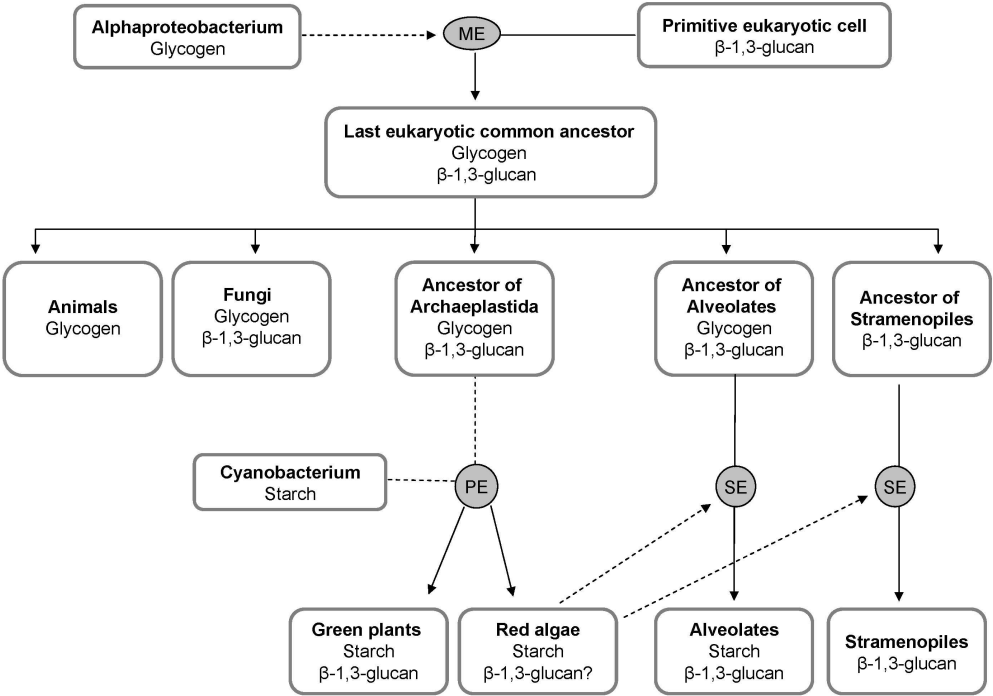


Figure 6
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