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Seminars in Cell and Developmental Biology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Seminars in Cell and Developmental Biology

journal homepage: www.elsevier.com/locate/semcdb

Review

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ARTICLE INFO

Keywords:
Extracellular matrix
Brown algae
Alginates
Fucans
Glycosyltransferases
Sulfotransferases

ABSTRACT

In brown algae, the extracellular matrix (ECM) and its constitutive polymers play crucial roles in specialized functions, including algal growth and development. In this review we offer an integrative view of ECM construction in brown algae. We briefly report the chemical composition of its main constituents, and how these are interlinked in a structural model. We examine the ECM assembly at the tissue and cell level, with consideration on its structure in *vivo* and on the putative subcellular sites for the synthesis of its main constituents. We further discuss the biosynthetic pathways of two major polysaccharides, alginates and sulfated fucans, and the progress made beyond the candidate genes with the biochemical validation of encoded proteins. Key enzymes involved in the elongation of the glycan chains are still unknown and predictions have been made at the gene level. Here, we offer a re-examination of some glycosyltransferases and sulfotransferases from published genomes. Overall, our analysis suggests novel investigations to be performed at both the cellular and biochemical levels. First, to depict the location of polysaccharide structures in tissues. Secondly, to identify putative actors in the ECM synthesis to be functionally studied in the future.

1. Introduction

Over the course of eukaryote evolution, a limited number of lineages managed to elaborate complex multicellular organisms [1–3], which are namely the animals, fungi, land plants, green, red and brown algae. In all cases, multicellularity acquisition was associated with the development of an extracellular matrix (ECM) [1,2]. ECMs have been intensely studied in several eukaryotic lineages including animals, fungi and terrestrial plants (see [4–6] for reviews) but are less characterized in macroalgae, including brown seaweeds [2]. While strong compositional variations of these ECMs are evident on a phylogenetic basis, many common functions are found [1,3]. Being at the outer surface of the cell, ECMs are in position to modulate or mediate a variety of events critical to the development and functions of a multicellular organism. In brown algae, the ECM or cell wall, promotes cell adhesion [7,8], regulates cell expansion [9,10] and morphogenesis [11,12], influences polar axis fixation and cell fate [13,14], provides a support to osmotic adjustment [10,15,16], and protects the cell from pathogens [17,18]. Additionally, the ECM in brown algae is an important resource for hydrocolloids, raw material, feed and food. Some of its components have potential as bioactives of high value [19]. These functions and uses depend on the specific composition of the ECM. The major components of brown algal ECMs are polysaccharides. While knowledge on their chemical diversity expands, the understanding of the in situ ECM assembly is still at its infancy. In land plants and animals, much effort has gone into understanding the mechanisms of glycosylation and glycan synthesis at the subcellular level [3]. These investigations have been performed from cell surfaces to within the ER and the Golgi apparatus, sometimes down to distinct Golgi compartments (see [3,5,20,21] for reviews). In brown algae, the topographic locations of the biosynthetic actors within a cell have not been precisely established. Additionally, and in contrast to nucleic acids and proteins, polysaccharides are not template-encoded and their production likely depends on the sequential action of a complex web of metabolic and trafficking steps [22,23], making

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https://doi.org/10.1016/j.semcdb.2022.03.005
Received 15 November 2021; Received in revised form 3 February 2022; Accepted 4 March 2022

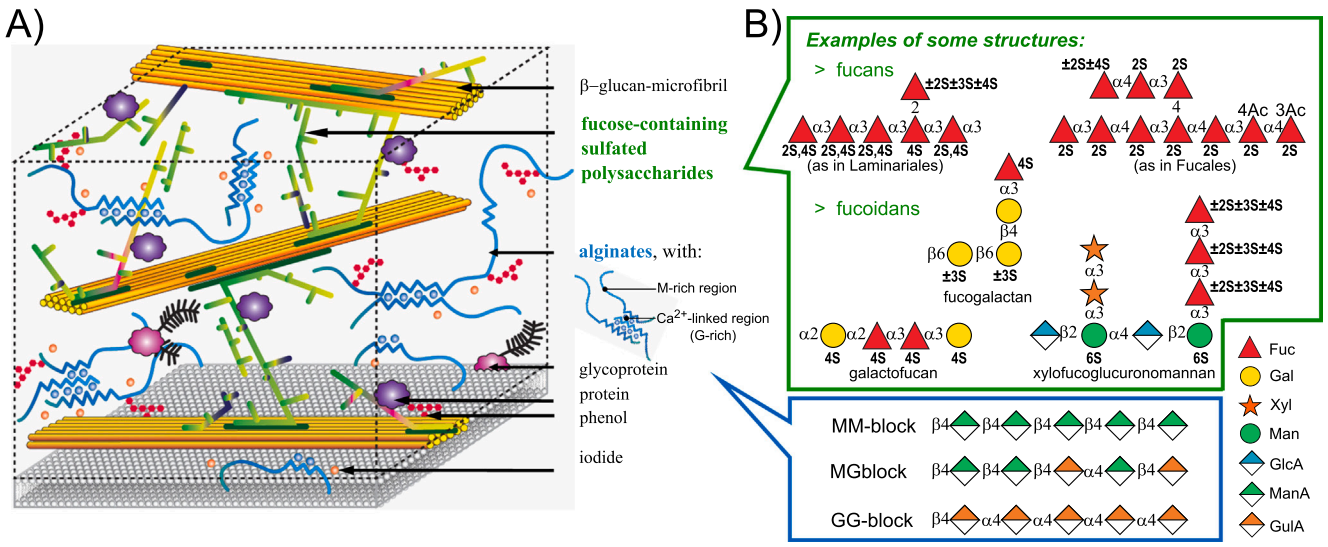

Fig. 1. Schematic representation of the ECM model in brown algae along with key structures of FCSPs and alginates, A) ECM model for brown algae from the order Fucales. Alginates and FCSPs are the main constituents. FCSPs interlock a β-glucan scaffold that includes cellulose and possible mixed linkage glucans, which have been reported to be insoluble in brown algal ECMs [87]. Most phenols are cross-linked to alginates. Proteins and halide compounds also are present. Glycoproteins related to arabinogalactan-proteins have been reported in brown algae [9], yet they still await a detailed chemical characterisation in these models. B) Examples of typical alginates and FCSP motifs are shown. Updated from Deniaud-Bouët et al. (2014).

carbohydrate structures impossible to predict on the basis of genetic information only. The establishment of the first genomic sequence for brown algae (*Ectocarpus* sp. Ec32 [24], also reffered as Ectocarpus species 7 [25]), and its in-depth molecular analysis [26], allowed to formalize hypothesis on the activation and incorporation of sugar residues into polysaccharide backbones (i.e. alginates and fucans *sensus stricto*). Yet, the function of only few enzymes has been experimentally validated so far, and none of them deals with the elongation of glycan chains, leaving this area of investigation still unexplored.

In this review we offer an integrative view of results obtained in relation to ECM construction in brown algae, beginning with a summary on its composition and architecture. We provide a comprehensive survey of the literature regarding ECM deposition *in planta* and of the possible subcellular sites for the enzymatic synthesis of polysaccharides. We further address the candidate genes involved in the synthesis of ECM polysaccharides, and discuss the functionally characterized proteins. Finally, we provide a re-examination of some glycosyltransferases and sulfotransferases in published genomes.

2. ECM architecture and composition in brown algae

Knowledge about the macromolecular organization of the ECM and its detailed chemistry is still limited in brown algae. However, there is increasing interest in studying brown algal biomass in details, and this might lead to a better understanding of ECM compositions in the near future. Polysaccharides are the main constituents, the majority of which being the alginates and the fucose-containing sulfated polysaccharides (FCSPs). These components have been integrated in an ECM model for Fucales species in which two polysaccharide-based networks are reported, the first being composed of FCSPs interlocking a β-glucan scaffold, embedded in the second made up of alginates and cross-linking phlorotannins [19,27,28] (Fig. 1 A). Proteins [29], glycoproteins [9], halide compounds and other ions [30], are additional components of brown algal ECMs (Fig. 1 A).

The FCSPs designation is used as a collective term to group both fucans and fucoidans. Fucans are highly sulfated polysaccharides with a backbone structure based on sulfated L-fucose residues, to which additional branches of various natures can be observed [19] (Fig. 1B). As a chemotaxonomical trait, it has been postulated that fucans from the Fucales are mostly harboring an alternative structure of α-(1−3) and α-(1−4)-linked fucose residues, while other orders, including the Laminariales, tend to have a α-(1−3)-linked structure [31], albeit exceptions to this statement exist [27]. Fucoidans encompass a set of heterogeneous polymers, harboring diverse backbones, which are based on neutral sugars and/or uronic acid residues (Fig. 1B). In addition to fucose, other monosaccharides are reported such as galactose, mannose, xylose, rhamnose and glucuronic acid [19,31]. Recently, Ponce et al. (2020) have performed an extensive examination of the literature regarding the compositional analyses of FCSPs. More than 100 species were examined [31]. As a matter of fact, it is very difficult to find a common trait in the structures so far analyzed. Across the Phaeophyceae, no obvious relationship between the fucoidan composition and the taxonomic classification can be drawn. If present, this relationship is likely overridden by other factors which are known to dramatically change the biomass and its polysaccharide composition, such as the geographic location and the season of harvest of the seaweed [31]. Additionally, the structures reported are characterized after extraction, pre-treatment and biochemical analyses that differ from one lab to another, making comparisons even harder. At this point, it can be assumed that the description of existing FCSP structures is still incomplete.

Alginates are linear polymers made solely of two 1,4-linked epimers: β-D-mannuronic acid (M) and α-L-guluronic acid (G). No regular repeating structure can be found throughout the entire polysaccharide chain. However, monomers can be arranged in blocks of G, M and MG of varying length and distribution (Fig. 1B). Each block has different conformational preferences and, hence, different physico-chemical properties [32]. In particular, the G-blocks regions form “egg-box” junctions with calcium, which contribute to gel stiffening. While most seaweed species have an M/G ratio of 1.2–1.6 in alginate [33], variations of the M/G ratio have been frequently reported between tissues [32,33]. This statement is of biological significance as a low or a high M/G ratio will impart the alga with mechanical strength or flexibility, respectively. Bacteria of the *Azotobacter* and *Pseudomonas* genera are also known to synthesize alginates, albeit in an acetylated form. Additionally, and in contrast to bacteria, alginates from brown algae all contain a broad distribution of G-blocks [34].

The ECM composition varies among species, across cell types within a specific alga, in response to abiotic or biotic factors [31,35–39], and during responses of many aspects of cell development and properties. A schematic model of the ECM architecture has been proposed [27]

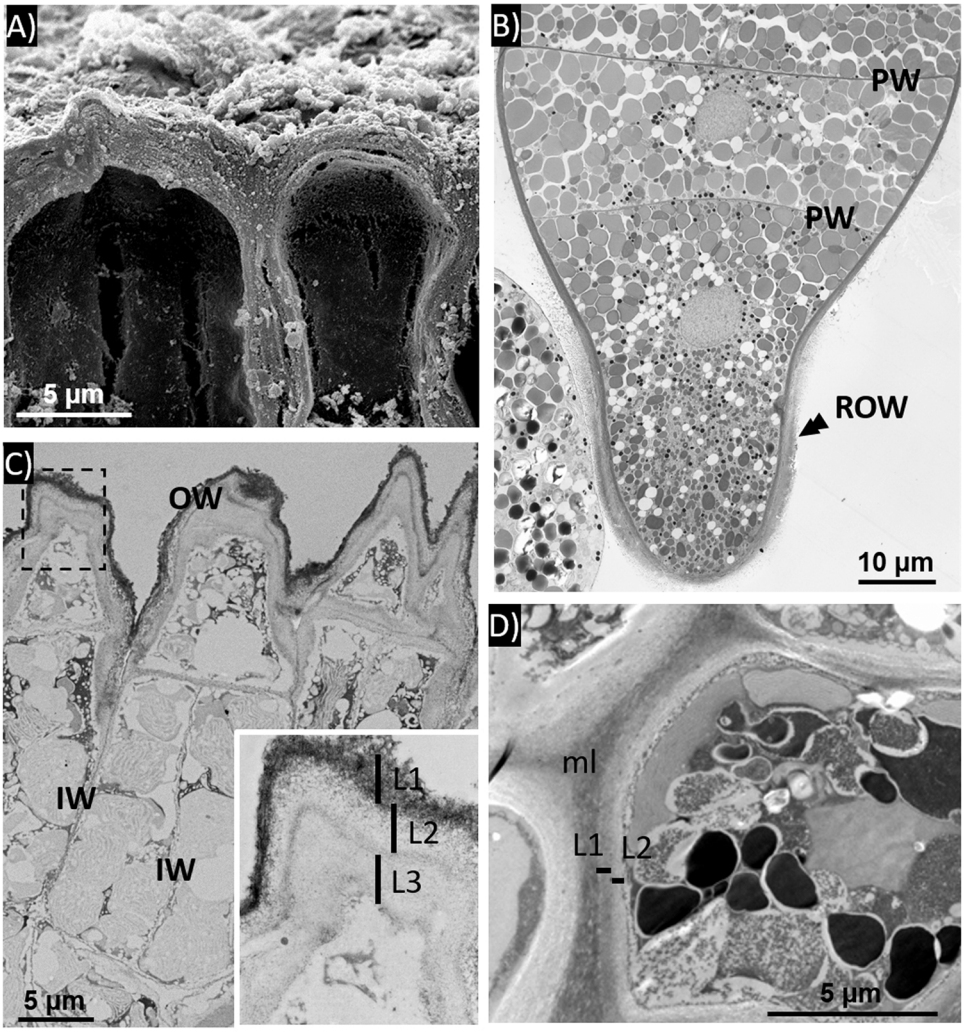

Fig. 2. The ECM frequently follows a layered-ultrastructure in brown algae, A) SEM micrograph of thick-walled meristoderm cells of *A. nodosum*, fouled with epibionts. Up to six ECM layers have been reported prior to epidermal shedding [49]. B) TEM micrograph of a *S. babingtonii* embryo at 24 h after fertilization. The layered structure of the rhizoid outer wall (ROW) is shown and contrasts with the thin and monolayered periclinal wall (PW). C) TEM micrograph of *D. dichotoma* indicating epidermal tissues. Cells in the epidermis are highly polarized with thick outer walls (OW) and thin inner walls (IW). The inset shows a higher magnification of the OW in which distinct layers numbered L1 to L3 are visible. D) TEM micrograph of *S. latissima* showing a medullar cell with ECMs of equal thickness. Distinct layers numbered L1 and L2 are visible. ml, middle lamellae. Figure developed from micrographs provided by Halat et al. (2020), Yonamine et al. (2021) and personal collections.

(Fig. 1 A). However, such a model is generic, applied to some analyzed Fucales only. While it helps to raise our understanding of 'the brown algal ECM', it cannot document the entire ECM diversity in brown algae. Similarly, the current model does not address the specificity of a multilayered wall, which is the most reported case in brown algal tissues (see below).

3. ECM generation and deposition in planta

3.1. ECMs are frequently multilayered in brown algae

In land plants, when they have ceased growth, some cells elaborate a multilayered secondary wall within the primary wall. This formation is associated with cell differentiation and the layers harbor different polysaccharide compositions. A thick secondary wall as defined in plants, e.g. associated with a specific cell type and in non-expanding cells, has not been reported in brown algae. The relationship between the cell type, the cell wall architecture and its composition is still poorly understood in these organisms. Yet, many ultrastructural observations indicate the occurrence of two, three and up to six ECM layers (Fig. 2A). Some ultrastructural differences can be evident regarding the ECM thickness. In *Dictyota dichotoma* [40], *Pelvetia canaliculata* [41], and *Fucus virsoides* [42], and supposedly in all brown algae with complex tissues, epidermal cells are highly polarized with thick outer walls and thin inner walls (Fig. 2B, C). In contrast medullary cells have ECMs of more or less equal thickness. In complex tissues, amorphous materials are found between the ECMs of neighboring cells. No clear definition exists in the literature for this region, referred to as 'middle lamellae' in this review (Fig. 2D). The middle lamellae is extensive in the medulla but rather scarce in cortical or epidermal regions.

The multilayered ECM organization was only investigated in an handful of early studies, focusing on zygotes [14,43] and thallus cells [42,44] in the Fucales, meristematic cells of *D. dichotoma* [40], the meristoderm of *Ascophyllum nodosum* [45] and apical cells of *Sphacelaria rigidula* [46]. The distinction of the layers was made by observation of a shift in the distribution of fibers and amorphous material, yet these studies failed to give a consensus view about architectural patterns of the multilayered ECM in brown algae. Initial observations were made using conventional chemical fixation of the tissues. These methods possess inherent limitations as compared to rapid freezing/freeze substitution and electron tomography techniques, which instead offer excellent preservation of morphological details. The extension of these methods in brown algae allowed for a better understanding of the spatial arrangement of the ECM layers, but only rare species have been analyzed so far. This includes *Ectocarpus* sp. Ec32 sporophytes, in which the lateral ECM of thallus cells contains two to three layers [47]. Electron-dense fibrils in the innermost layer run parallel to the plasma membrane, while the outermost layer consists of non-fibrillar amorphous material, and the central layer is made of randomly arranged fibrils. This situation of the denser fibers being located in the innermost layer and the amorphous materials at the outermost layer is reminiscent in *Silvetia babingtonii* zygotes [48] (Fig. 3). Rapid freezing/freeze

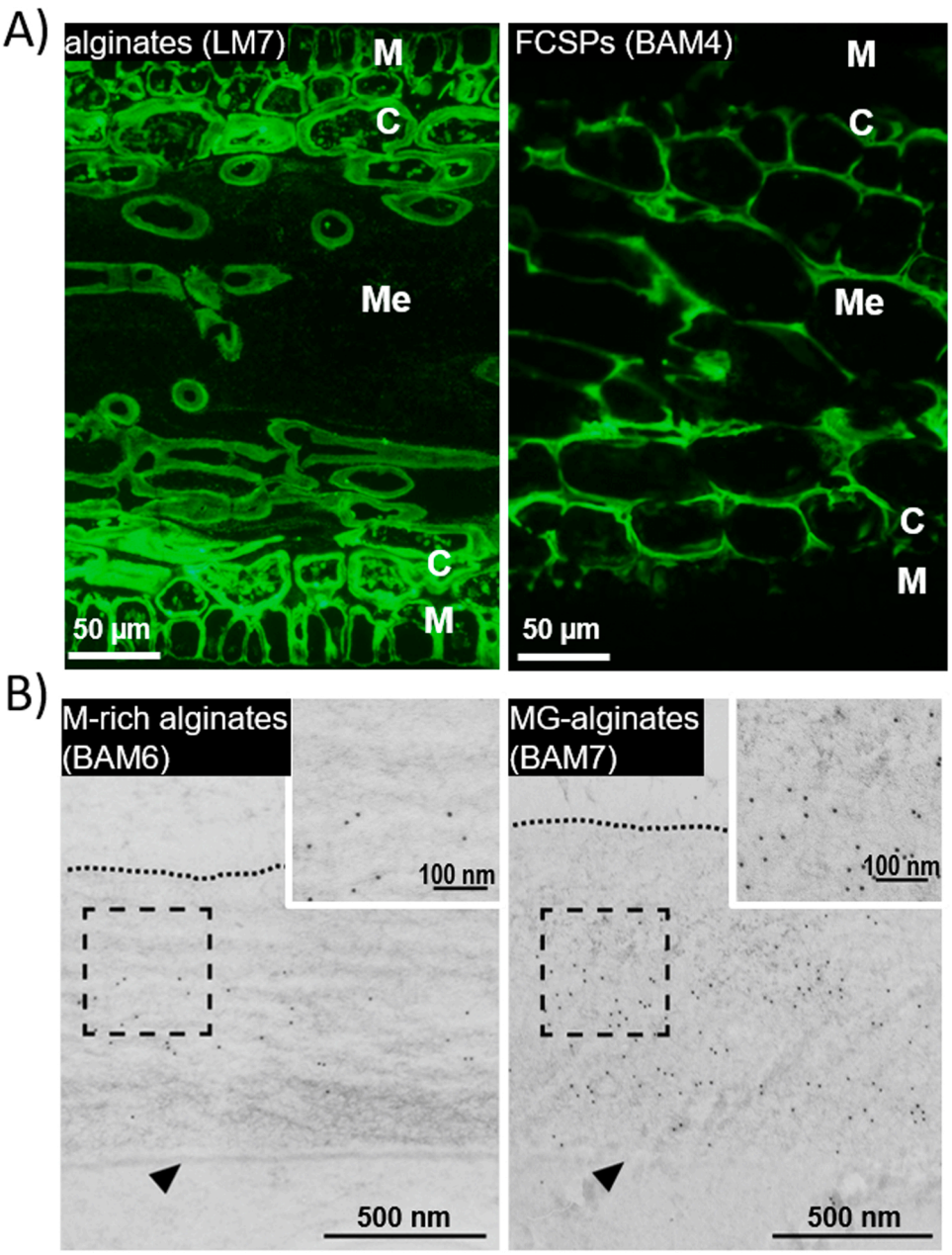

Fig. 3. Examples of immunolabelling of alginates and FCSPs at the tissue and cell levels, A) Immunofluorescence analysis of ECMs in transverse sections of developing apices of *Fucus vesiculosus*. Moving in from the surface there are a meristoderm cell layer (M), cortical cell layers (C), and the medulla cells (Me) in the center. Probes used are the anti-pectin monoclonal antibody LM7, cross-reacting and detecting alginates in this case [16], and the FCSP/sulfated fucans monoclonal antibody BAM4. The two probes show contrasting labeling. LM7 binds to all of the ECMs although with different intensities, while the BAM4-labelling is observed in all ECMs except within the cell layers present at the tissue surface. At the cell level, distinct ECM layers seem to be differently labelled by the two probes, although this would deserve investigations at higher resolutions. Figure developed from micrographs provided by Torode et al. (2015). B) Immunogold localization of anti-alginate epitopes in the ECM of the thallus cell of a *S. babingtonii* embryo at 24 h after fertilization. Probes used are targeting M-rich alginates (BAM6) and MG-alginates (BAM7). BAM6 epitopes have a more restricted occurrence as compared to BAM7 epitopes. Figure developed from micrographs provided by Yonamine et al. (2021).

substitution has also been applied in *A. nodosum* during surface shedding. The amorphous materials are sandwiched between two layers of fibrillary structures, where they mark the site of the future rupture zone [49]. More generally, previous reports often indicated dense fibers in the innermost layers of ECMs.

3.2. ECM composition at the cell and tissue levels

Surprisingly the literature reports only a few attempts to specifically localize ECM polysaccharides in brown algae. At the tissue level, the use of metachromatic stains has suggested that FCSPs are the main components of the surface walls and in the medulla, notably within the middle lamellae [32,42]. However these techniques are not sensitive and specific enough to offer a conclusive view of polysaccharide distribution. Immunohistochemical approaches, based on the use of specific antibodies, are more appropriate for this purpose. In 1993, Green et al. indicated that the FCSP composition in the aforementioned locations is more complex and that the occurrence of FCSP structures varies between thallus parts [50] (Fig. 3 A). This statement was further validated in *Fucus* species, where distinct FCSPs epitopes show contrasting distribution patterns between tissues [16]. By contrast, alginates seem to be present in all tissues (Fig. 3A), although their abundance differs with the cell type and the organ. In vegetative tissues of *Fucus distichus*, alginates were detected in abundance in the medulla, with lower occurrences in cortical and epidermal ECMs [51].

At the cell level, studies attempting to relate the multilayered architecture of the ECM to different polysaccharide compositions are also scarce. Immunolabelling procedures in cells of *S. rigidula*, *Scytosiphon lomentaria* [52], *Ectocarpus* sp. Ec32 [47] and in *S. babingtonii* zygotes [53] indicate that alginates tend to be distributed more evenly toward the inner layers of the ECM. It was further concluded that the fibrils observed by TEM in the layers in the vicinity of the plasma membrane are composed of alginate chains. Those alginate fibers, with an average width of 4 nm, are likely shaped by the assembly of multiple alginate chains [47]. The use of the anti-alginate BAM antibodies in *S. babingtonii* zygotes showed that the M-rich epitopes have a more restricted

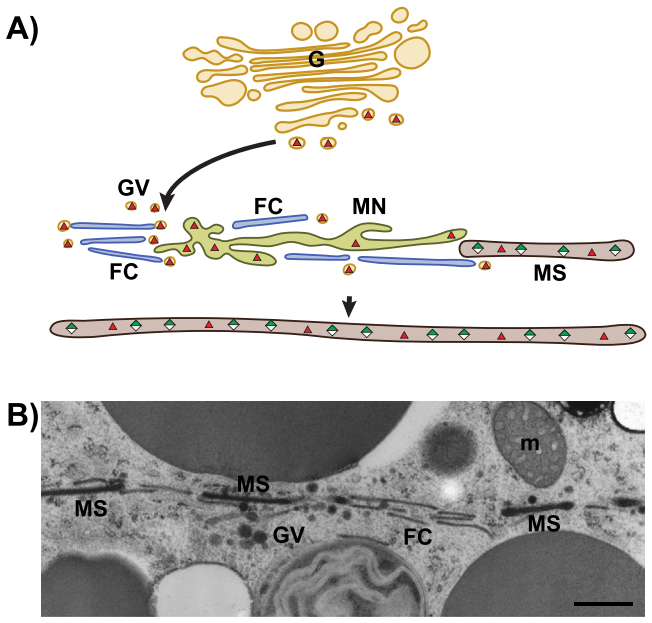

Fig. 4. Centrifugal outgrowth of a partition membrane during cytokinesis in brown algae, A) Diagram showing the formation of a continuous cell partition membrane during cytokinesis. The Golgi vesicles (GVs) derived from the Golgi body and deliver FCSPs in the vicinity of flat cisternae (FC). These structures combine to shape a membranous network (MN). Disappearance of the gaps between MNs occurs when several MNs fuse to grow into membranous sacs (MS). Alginates are accumulating at this stage. The red triangles and the white/green diamonds show FCSPs and alginates, respectively. Updated from Nagasato et al. (2010). B) TEM micrograph of the formation of the cell partition membrane during the second division in a *S. babingtonii* zygote. GVs and FCs have accumulated to shape MSs. The MSs appear in patches at the future cytokinetic plane and will fused to complete the partition membrane. m, mitochondria. Figure developed from micrographs provided by Yonamine et al. (2021).

distribution pattern as compared to MG-motifs (Fig. 3B). This indicates a rapid conversion of some M residues into G residues after alginate incorporation; a conclusion also reached in *Fucus serratus* zygotes by light microscopy [48,53]. Cellulose fibers were also observed in the *Ectocarpus* cells, albeit with a clear anisotropy and in a very small quantity [47]. So far, immunolabelling of distinct FSCP epitopes has not been thoroughly investigated at the ECM level.

3.3. ECM assembly and expansion

Depending on the species observed, two patterns of cytokinesis have been reported in brown algae, with a completion either through the furrowing of the plasma membrane, or centrifugally by outgrowth of a partition membrane from within the cytoplasm [54]. In both cases flat cisternae are necessary for the completion of the new septum and Golgi vesicles play an important role in this process [55]. Flat cisternae are structures observed in the vicinity of the endoplasmic reticulum from which they likely derived [54,55] (Fig. 4A). During the partition process, both flat cisternae and Golgi vesicles accumulate in several places around the future cytokinetic plane and shape elongated membranous sacs which further fuse to achieve the division (Fig. 4B). During cytokinesis, immunolabelling studies have indicated that the Golgi vesicles deliver FCSPs to the future site of division. Polyclonal anti-alginates antibodies, likely targeting MG-alginates, indicate that the polysaccharide only starts to be detected at a later stage on the newly formed membrane [55]. So far cellulose has not been detected within this new cross wall and its content is therefore estimated to be low.

Once the new cell has been shaped, in the vast majority of algal cells, growth and the deposition of new ECM material supposedly occur uniformly along the entire expanding wall. Yet, this statement still needs to be fully explored. The situation has been better described in some cells featuring tip growth, notably in the zygote of various *Fucus* species. This model system will not be detailed here as it has been largely described in dedicated reviews [56]. One can just briefly notice the essential role of the ECM as a source of position-dependent information required for cell polarization in those zygotes. Cellulose and alginates are the first polysaccharides to be deposited uniformly into the ECM after fertilization. FCSPs are deposited at a later stage, during the establishment of polarity axis and starting specifically at the emerging, rhizoid tip [14,53]. In *E. subulatus* fluorescent imaging also indicates the incorporation of highly sulfated FCSPs at the dome of apical cells [10].

3.4. Subcellular compartmentalization of ECM synthesis

In *Pelvetia canaliculata*, autoradiography using $^{35}SO_4^{2-}$ has shown that sulfated materials, which probably include FCSPs, are synthesized by all cell types [41] (Fig. 5 A). In *D. dichotoma*, Evans et al. (1973) reported that some of the vegetative cells are specialized for secretion. In distinct species from the Laminariales order (*Laminaria digitata, Laminaria hyperborea, Saccharina latissima*), this activity was shown to be confined to highly specialized secretory cells, which discharge the sulfated materials into mucilage canals [41,57] (Fig. 5B). There was no evidence of involvement of any other cell type in the production of these sulfated materials. This highly specific location for the sulfation is puzzling, as the deposition of FSCPs would be expected to be rather uniform at least between some cell layers. Work on these substances at the cellular level is very limited and would therefore deserve further investigation.

In all instances the process of sulfation appears to occur in the Golgi-rich perinuclear region. Ultrastructural MET studies based on the use of $^{35}SO_4^{2-}$ indicated the location of silver grains within the Golgi bodies in zygotes of *F. serratus* [43]. Similar observations were made in zygotes of *F. distichus* [8], *P. canaliculata* [41] and in the secretory cells of Laminariales [58] (Fig. 5 C, 5D). It is believed that FCSPs are first polymerized into neutral polysaccharides prior to their sulfation by sulfotransferases. This point, together with the aforementioned observations, suggest that the carbohydrate sulfotransferases are located in the Golgi complex. Consequently, these observations indicate that the synthesis of the FCSPs is localized within the Golgi apparatus. This suggestion was validated using specific antibodies in *S. babingtonii* zygotes and which locate FCSP antigens in the Golgi [55] (Fig. 6 A). This result is in agreement with the reported characterization of a galactosyltransferase activity from an isolated Golgi fraction from *F. serratus*, and which transfers UDP-galactose to endogenous acceptors, fucose and fucoidans [59]. Early ultrastructural TEM studies also indicate the common occurrence of granular vesicles in close vicinity of the membrane, and that the material delivered by those vesicles was seen as continuous to identical materials forming the inner ECM [40], supposedly alginates. Latter on Nagasato et al. (2010) reported by immunolabelling observations that alginates are not detected in Golgi bodies, but in specific osmiophilic vesicles closely associated to the plasma membrane (Fig 6 B).

4. The synthesis of ECM polysaccharides in brown algae

4.1. Predicted metabolic pathways

The alginate pathway was first explored at the biochemical level in *Fucus gardneri* by Lin et al. (1966), with the identification of key enzymatic activities [60]. It was only with the first release of a brown algal genome, 44 years later, that a more comprehensive view was obtained at the molecular level [26]. Since then, additional functional annotations were made, as for instance enzymes involved in the degradation of alginates (see below). Fig. 7 includes those predictions along with the characterized proteins. Michel et al. (2010) were also the first to provide

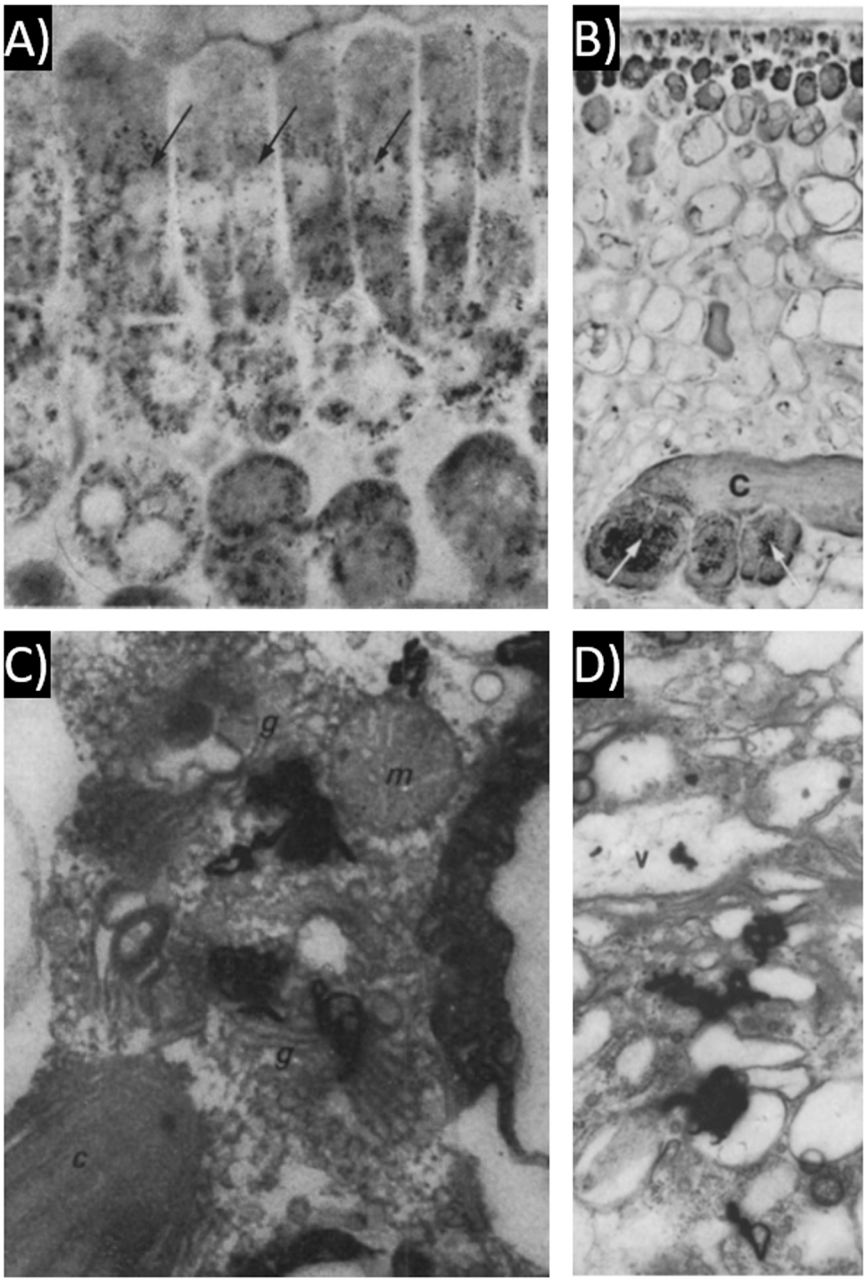

Fig. 5. Autoradiograph detection of $^{35}SO_4^{2-}$ in brown algal cells, The detection is shown at the tissue level (A, B) and at the cell level (C, D) for Fucales species (A, C) and Laminariales species (B, D). A) Light-microscope autoradiograph in an apex of *P. canaliculata* incubated 2 h in ^{35}S followed by 4 h chase. Silver grains are seen around the nuclei (arrowed) and basal regions of epidermal cells and in outer cortical cells. B) Light-microscope autoradiograph in the thallus of *L. hyperborea* incubated 3 h in ^{35}S followed by 26 h chase. The subsidiary secretory canal (white arrows) is more heavily labelled than the secretory cells surrounding it. The main canal (C) is not labelled. C) Electron-microscope autoradiograph of a 22 h-old zygote pulsed 15 min with ^{35}S. The silver grains are associated with the Golgi bodies (g). A mitochondrion (m) and part of a chloroplast (c) also are visible. D) Electron-microscope autoradiograph of a secretory cell showing the association of silver grains with one Golgi body and its associated secretory vesicles (v).
(A) Reproduced with permission from Evans et al. (1973).
(B) Reproduced with permission from Evans et al. (1973).
(C) Reproduced with permission from Callow et al. (1978).
(D) Reproduced with permission from Evans and Callow (1974).

a comprehensive view of the main steps leading to the synthesis of sulfated fucans (Fig. 7). The FCSP populations being highly heterogeneous, it is anticipated that more genes are recruited for their synthesis than just those involved in the synthesis of fucan *sensus stricto*. This would include some epimerases for the synthesis of additional GDP-precursors (e.g. GDP-galactose) and a variety of glycosyltransferases beyond the proper fucosyltransferases. Yet, those predictions are still the best available so far and are described in more details below.

4.2. A recent boost in functionally validated genes

All enzymes biochemically characterized so far are involved either in the synthesis of sugar precursors or in the remodeling of alginates. They are essentially coming from *Saccharina japonica* as a source, with some exceptions made from *Ectocarpus* sp. Ec32.

a. *Characterized enzymes involved in the synthesis of sugar-precursors*

GDP-mannose is an essential activated sugar used in the biosynthetic pathway of alginates, but also in the production of fucans through its conversion to GDP-fucose [26]. The three enzymatic steps which leads to the production of GDP-mannose from fructose-6 phosphate, are essential actors in building up major cell wall polysaccharides in brown algae (Fig. 7). The mannose-6-phosphate isomerase (MPI) catalyzes the production of mannose-6-phosphate from fructose-6-phosphate. In *S. japonica*, three full-length MPI genes have been isolated and the function of one SjaMPI4 protein was validated [61]. This enzyme has an additional mannose-1-phosphate guyanylyltransferase (MPG) activity (Fig. 7). This situation is reminiscent to bacterial MPI homologues which possess a dual MPI/MPG activity and would explain why genuine MPG genes were not identified in brown algal genomic and transcriptomic data [26,61].

The bifunctional phosphomannomutase (PMM)/

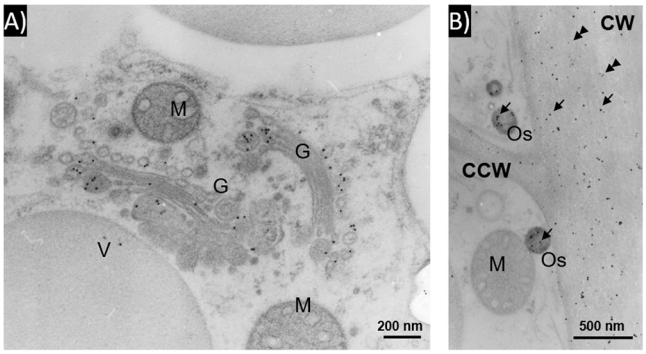

Fig. 6. Immunogold localization of FSCPs and alginates at the subcellular level in *S. babingtonii* zygotes, A) FCSPs localize in the Golgi vesicles. B) Double labeling using 10 nm-gold particles for the anti-FSCP antibody (double arrowheads) and 15 nm-gold particles for the anti-alginate antibody (arrows). Alginates, but not FCSPs, localize in osmiophilic vesicles. CCW cross cell wall, CW cell wall, G Golgi body, M mitochondrion, Os osmiophilic vesicle, V vacuole. Figure developed from micrographs provided by Yonamine et al. (2021).

phosphoglucomutase (PGM) enzymes are fundamental to primary metabolism across all the kingdoms of life [62]. In brown algae, besides providing precursors for the synthesis of alginates and fucans (*de novo* pathway) (Fig. 7), they are possibly involved in the generation of other glycosylated components. Two distinct studies report the characterization of PMM activity in *S. japonica*. The corresponding enzyme was named SjPMM/SjPGM by Zang et al. (2018) [63]. While there is not an obvious transparency on the sequence used by Chi et al. (2018), it likely derives from the same gene and the protein was named SjaPMM [61]. In both cases biochemical characterizations were made on the recombinant and purified forms of the protein. Similar to most PMM/PGMs from other species, this enzyme catalyzes the reversible interconversion of glucose-6-phosphate and mannose-6-phosphate, with the corresponding bisphosphorylated sugar as an intermediate and cofactor. This SjPMM/SjPGM enzyme harbors a preference to the glucose phosphosugar as a substrate [61,63] and as such might represent a key branch point in carbohydrate metabolism in *S. japonica*. As reported by Zhang et al. (2018), the corresponding gene was 95% similar to a previously unannotated gene sequence of *Ectocarpus sp.* Ec32, yet differing from another PMM/PGM candidate gene predicted by Michel et al. (2010). Therefore, additional PMM/PGM enzymes with better affinities toward mannose phosphosugars may lay uncharacterized in brown algae.

Once GDP-mannose has been synthesized, its oxidation to GDP-mannuronic acid will serve the elongation of an alginate polymer as a form of polymannuronate. The oxidation of GDP-mannose is catalyzed by GDP-mannose dehydrogenase (GMD), generally seen as a rate-limiting enzyme in the alginate biosynthetic pathway [64]. Such enzyme belongs to the superfamily of GDP-mannose/UDP-glucose dehydrogenases (GMD/UGDs) which are ubiquitously present in all

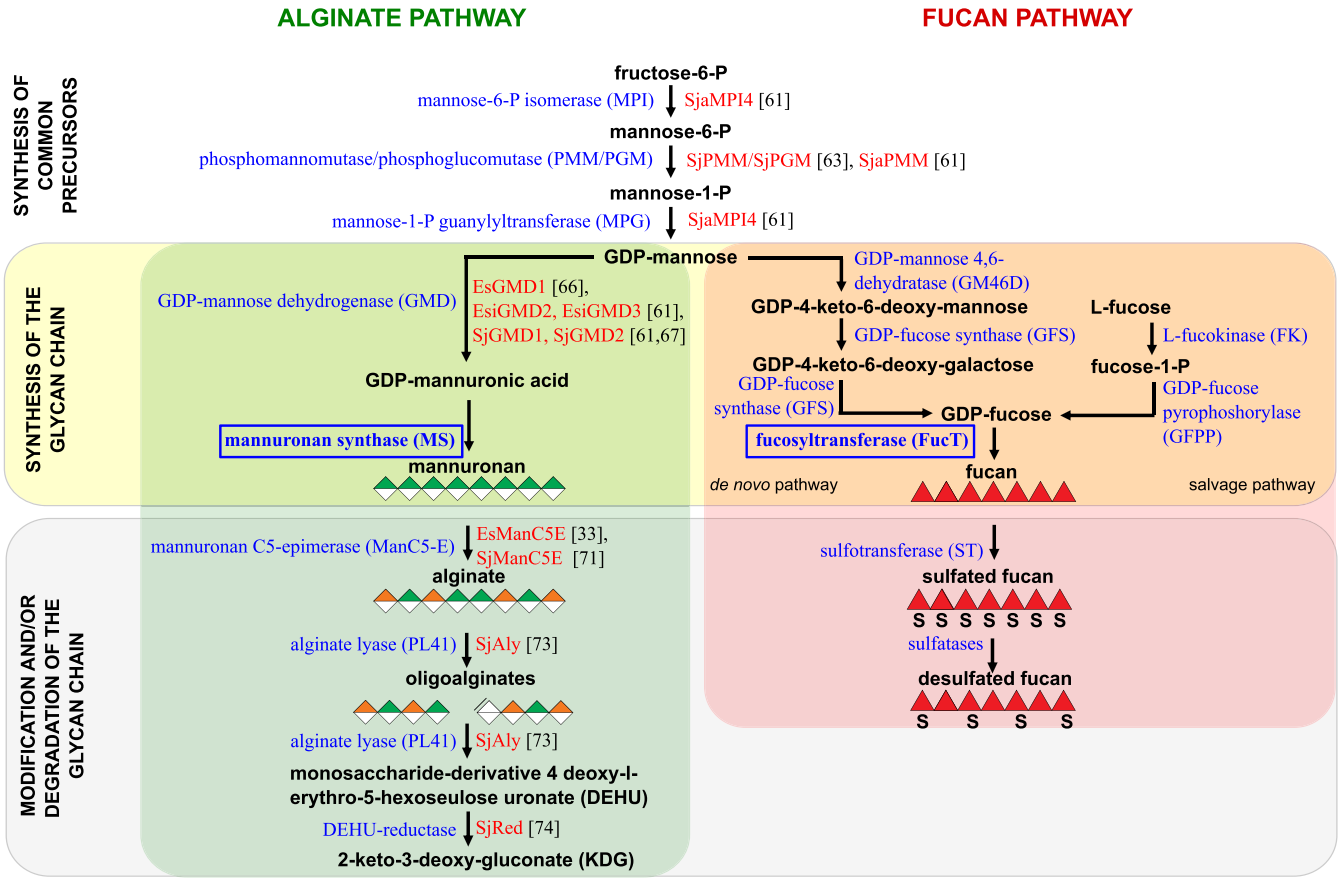

Fig. 7. Schematic representation of the metabolic pathways for alginates and sulfated fucans *sensus stricto,* The different enzymatic activities (in blue) are indicated between each product (in black). The enzymes for which a biochemical validation has been obtained are indicated (in red) with their short names and corresponding references. Key enzymes (e.g. glycosyltransferases) involved in the elongation of the glycan chains are still uncharacterized and are indicated in a blue frame. Note that the catalytic steps involved in the synthesis or early precursors are common to both pathways (upper part). Some reactions are related to the synthesis of the polymeric chains (middle part), whereas others refer to the remodeling and/or degradation of the elongated chains (lower part). The schematic representation of the pathways has been updated from Michel et al. (2010) with the addition of the newly characterized activities, notably the alginate lyase [73] and DEHU reductase [74].

organisms [65]. In bacteria this superfamily includes enzymes that oxidize different sugars such as UDP-glucose, GDP-mannose, UDP-N-acetylglucosamine, UDP-N-acetyl-D-mannosaminuronic acid, and UDP-galactose [66]. Outside brown algae, all known eukaryotic members of this superfamily convert only UDP-glucose (e.g. UGDs) [66]. GMD/UGDs are found in small multi-copy genes in brown algae, with a total of 3 and 4 identified genes in *S. japonica* and *Ectocarpus* sp. Ec32, respectively [61]. Initial studies were reporting the isolation of one GMD gene from *Ectocarpus* sp. Ec32 (EsGMD1) [66] and two GMD genes from *S. japonica* (SjGMD1, SjGMD2) [67] and the corresponding recombinant proteins were produced, purified and biochemically validated. Chi et al. (2018) reported the characterization of all the 3 predicted GMD/UGDs from *S. japonica*, in addition to 3 others from *Ectocarpus* sp. Ec32. While a comparison with the previous work from Zhang et al. (2016) was not made, the characterized SjaGMD1 and SjaGMD2 likely correspond to the previously published enzymes from *S. japonica.* The catalytic activity of two other GMD proteins from *Ectocarpus* was confirmed (EsiGMD2, EsiGMD3). Two additional enzymes were characterized and referred as UGDs (SjaUGD, EsiUGD) [61], however the substrate preference by these enzymes was deduced from their clustering in a UGD clade in phylogenetic studies [61] and their use of UDP-glucose as a substrate has not been biochemically established. Overall these brown algal enzymes have the ability to produce the alginate precursor, GDP-mannuronic acid and are the first eukaryotic GMDs characterized so far.

By contrast to the alginate pathway, all the candidate genes involved in the synthesis of fucan precursors after the GDP-mannose generation still await functional validations. GDP-fucose was predicted to be synthesized through either a *de novo* pathway or a salvage pathway (Fig. 7). Some candidate genes were found in a gene cluster in *Cladosiphon okamuranus* [68], along with a sulfotransferase and a hydrolase, which shared a synteny in *Ectocarpus* sp. Ec32 and *Nemacystus decipiens* [69]. These enzymes offer ideal targets for future work dealing with the biochemical characterization of activities within the fucan pathway.

b. *Characterized enzymes involved in the remodeling of alginates*

Once the mannuronic acid residues have been assembled in an alginate chain, the final step of synthesis or remodeling is the epimerization of some M residues into G residues by mannuronan C5-epimerases (ManC5-E) (Fig. 7). Most of the biochemical knowledge about ManC5-Es originates from the characterization of bacterial enzymes [70]. Two ManC5-Es from brown algae have been expressed in their recombinant forms, purified and characterized [33,71]. However heterologous expression of these algal enzymes, a challenging task, is still a bottleneck which impairs the thorough characterization of the epimerization patterns generated. The brown algal ManC5-Es are putatively located into the walls [72] were they are believed to modify their substrate *in muro*. There is a large number of ManC5-E genes in brown algae, which expression presumably yields polymers with a variety of either random or blockwise distribution of G residues, and thus alginates of distinct rheological properties. These modifications would be regulated in response to physiological requirements. However this is a one-way process, and there is no evidence of modification of the G residues once in ECMs. This suggests the existence of other mechanisms to modulate the alginate and ECM properties further. While the interactions of additional actors may be one option to modify the hydrogel network (i.e. regulation of apoplastic Ca^{2+} concentration, phlorotannins cross-linking, etc.), Inoue et al. (2019, 2021) have demonstrated the occurrence of an alginate-degrading pathway in brown algae, with the biochemical characterization of key enzymes described below [73,74].

Alginate lyases are common in alginate-assimilating organisms and several of those enzymes have been thoroughly characterized in alga-associated bacteria such as *Zobellia galactanivoran* [75] and other Flavobacteria. Like glycoside hydrolases, glycosyltransferases and others carbohydrate-acting enzymes, polysaccharide lyases (PL) have been classified based on amino-acid sequence similarities in the CAZy database (www.cazy.org) [76]. Most bacterial alginate lyases described so far belong to families PL5, PL6, PL7, PL15, PL17 and PL18. Additionally, alginate lyase activities were demonstrated in families PL8 and PL14 in eukaryotes [77,78]. Several new PL families with alginate lyase activities were discovered during the last two years, namely PL31, PL32, PL36 and PL39 in bacteria [79,80], and PL41 in brown algae [73]. Indeed, in 2019, Inoue et al. characterized the first PL41-alginate lyase from *S. japonica*, thereof bringing the first evidence of such a lyase in an alginate-producing eukaryotic organism. This SjAly enzyme preferentially uses alginates enriched in M-blocks and can cleave the polymer endolytically into unsaturated tetra-, tri-, disaccharides, and down to the monosaccharide-derivative 4-deoxy-L-erythro-5-hexoseulose uronate (DEHU) (Fig. 7). Homologous genes have been subsequently reported in *E. subulatus* [81] and are likely to be present in other brown algae. The authors later characterized an active DEHU-reductase from the same alga, which reduces DEHU into 2-keto-3-deoxy gluconate (KDG) [74]. DEHU cannot be directly used as a unit structure for alginate synthesis, therefore its further degradation allows its usage as an energy source. In alginate-degrading organisms KDG is further cleaved into pyruvate and either glyceraldehyde-3-phosphate or glyceraldehyde as the final products. Such an enzymatic activity needs to be assessed in brown algae, yet its presence is very likely.

4.3. The core proteins involved in glycan elongation and modification are still unknown

a. *Candidate genes for the biosynthesis of glucan chains*

As stated above, some functional assessment has been made on ECM enzymes in brown algae, however none of them is catalyzing the elongation or grafting of glucan chains, which is a mandatory step in generating the wall. The synthesis of glycans as stand-alone polysaccharides or glycoconjugates, is catalyzed by glycosyltransferases (GT). Among the available genomes of brown algae, only 3 had their CAZy contents analyzed, namely *Ectocarpus* sp. Ec32 [26], *E. subulatus* [81] and *S. japonica* [42,82,83]. The first census of this CAZyme content was detailed for *Ectocarpus* sp. Ec32 and indicated a content of 41 GH and 88 GT members, representing 18 GH and 32 GT families respectively [84]. Those analyses showed that brown algae have a peculiar distribution in GTs among eukaryotes, with for instance some families absent in land plants but shared with other taxonomic groups such as chlorophyte green algae and amoeba (GT60), sac fungi (GT49) and animals (GT23). As compared to other eukaryotes such as land plants, *Ectocarpus* sp. Ec32 harbors less functional redundancy with fewer genes in each of its CAZy families.

Ye et al. (2015) report a total number of GTs within the same range between *Ectocarpus* sp. Ec32 and *S. japonica*, except the slight expansion of some GT families in *S. japonica* (GT2, GT23, GT47, GT77) [83]. However, Liu et al. (2019) indicated that in *S. japonica* the gene number of GTs and GHs is twice more than the gene number in *Ectocarpus* sp. Ec32 [82]. While they speculate that this may relate to more complex tissues in kelps as compared to other species, more in-depth annotation of brown algal genomes from different taxonomic origins would be needed to assess this statement. Notably there is a discrepancy in the total number of GH and GT genes referenced by those authors, with for instance 82 GHs/131 GTs identified by Ye et al. (2015) versus 107 GHs/94 GTs identified by Liu et al. (2019) for *S. japonica*. This situation might lead to false statements regarding specific gene loss or expansion. While this is likely the consequence of different annotation tools applied to such 'exotic' genomes as compared to reference/model species, the accounting of domain duplicates within proteins and/or fragments might also have important impacts on the conclusions. Hence, to clarify this, we have undertaken an expert analysis of the CAZyme content of 4 published genomes of brown algae, namely *S. japonica*, *Ectocarpus* sp. Ec32, *N. decipiens* and *C. okamuranus.* This investigation was motivated by our expertise in maintaining the worldwide reference CAZy

Table 1
Gene number of GT families known to contain FucT activities in *Ectocarpus* sp. Ec32, *N. decipiens, C. okamuranus* and *S. japonica*.

CAZy database		Number of candidate genes in brown algae			
GT families	Known FucT activities in CAZy	*Ectocarpus* sp. Ec32	*N. decipiens*	*C. okamuranus*	*S. japonica*
GT37	α-1,2-FucTs	–	–	–	–
GT74	α − 1,2-FucTs	–	–	–	–
GT11	α − 1,2/3-FucTs	–	–	–	–
GT10	α − 1,3/4-FucTs	**1** [1]	**1** [1]	**1** [1]	**1** [2]
GT23	α − 1,6-FucTs	**2** [7]	**2** [5]	**6** [10]	**2** [9]
GT41	protein O-FucTs	**4** [10]	**7** [14]	**10** [15]	**2** [9]
GT65	protein O-FucTs	–	–	–	–
GT68	protein O-FucTs	–	–	–	–

The synthesis of homofucan backbones in brown algae involves fucosyltransferases which have not been characterized yet. It is still unknown to which GT families they belong to but some speculations can be made. In eukaryotes, all known FucTs are classified in a restricted number of CAZy GT families as shown. The generated glycosidic linkages identified from characterized enzymes (outside brown algae) are indicated. Our annotation retrieves full length proteins and fragments. Numbers in bold indicate the number of full-length proteins identified. The framed numbers indicate the number of the full-length proteins supplemented by the fragments identified. Note that distinct fragments can derive from the same protein. The hyphen sign ('-') indicates that our analysis retrieved no proteins or fragments in the corresponding genome.

classification that we initiated 30 years ago (www.cazy.org) [85,86]. In *S. japonica*, we observed a lower number of GHs than previously reported with 58 full-length genes, however, the number of predicted GTs was higher with 156 full-length genes. We additionally identified gene fragments not incorporated in this counting, with 30 and 37 fragments for GHs and GTs, respectively. Note that several of those fragments may possibly be part of the same gene. These quantities were in the same range for the 3 other genomes as highlighted for some GTs discussed hereafter, therefore not supporting the hypothesis of an extending GT content in Laminariales as compared to Ectocarpales.

Michel et al. (2010) were the first to speculate on the possible GT members involved in the synthesis of the alginate and fucan backbones. In alginate-producing bacteria, the synthesis of polymannuronate is catalysed by the Alg8 GT2 family member. While the *Ectocarpus* sp. Ec32 genome encodes 9 GT2s homologous to the cellulose synthases (CESA) and cellulose synthase-like (CSL) proteins, no significant homologues to Alg8 have been identified. Some of these GT2 members are likely involved in the synthesis of cellulose and mixed linkage glucans and possible candidate genes have been previously discussed [1,87]. Mannuronate synthases in brown algae might represent additional GT2 members, albeit this hypothesis is still highly speculative [26].

Sulfated fucans/fucoidans from brown algae being highly heterogeneous in term of sugar monomers and glycosidic bonding types, it is anticipated that their biological synthesis involves multiple GTs and accessory enzymes such as sulfotransferases (ST) or acetyltransferases. One current challenge is to integrate the genome-based evidences with our glycome-based evidence, which is much more heterogeneous. While the present review does not aim to gain an exhaustive view of all GTs involved in the FCSP synthesis in brown algae, one focus can be made on fucosylstransferases (FucTs) potentially involved in the synthesis of homofucan backbones. In the CAZy database, several FucTs have been identified and classified in the families GT37, GT74 (α-1,2-FucTs), GT11 (α-1,2/3-FucTs), GT10 (α-1,3/4-FucTs), GT23 (α-1,6-FucTs) and GT41, GT65, GT68 (protein O-FucTs) [76,88,89]. Michel et al. (2010) initially indicated putative FucTs, from families GT10, GT23 and GT65 in *Ectocarpus* sp. Ec32 as the best targets for fucan synthesis. However, no GT65 members were reported in neither *E. subulatus* nor *S. japonica* [81,83], excluding those genes as realistic actors in fucan synthesis. In addition, the GT11, GT37, GT68 and GT74 were not detected in brown algae so far, which excludes those members as well. By contrast, in our analysis, the GT23 family seems to be a robust candidate given its presence in several copies in the four genomes analysed (Table 1, Supplementary Table S1). Interestingly in *Fucus serratus*, some GT23 genes have been shown to be up-regulated during the first stages of embryogenesis, which correlates with the early deposition of ECM polysaccharides, notably fucans [90]. However, all characterized GT23 enzymes in other eukaryotes catalyze the incorporation of α-1,6-linked fucose, while a fucan synthase would be expected to incorporate α-1,3/4-linked fucose. Members of the GT10 family are known to have such a linkage specificity [88,89,91,92], although they are present in a very restricted gene number in the brown algal genomes analyzed (Table 1, Supplementary Table S1). One should note that the GT41 family seems to have been excluded from the initial genomic analyses, while some members are found in those genomes (Table 1, Supplementary Table S1). In other eukaryotic phyla, these enzymes are usually involved in protein glycosylation, therefore it is difficult to predict whether these FucTs are genuine fucan synthases. To conclude, this would leave the GT10 and GT23 families as the best candidates for the synthesis of fucan backbones in brown algae. Albeit, this does not exclude the evolution of distinct and specific GTs dedicated to the synthesis of this glycan in brown algae. In *Ectocarpus* sp. Ec32, more than 36% of the predicted proteins were novel, being *Ectocarpus* or brown algal specific, with no counterpart in other taxonomic groups [24,93]. This point, together with the fact that novel enzymatic activities have been recently discovered by biochemical-screening approaches (see above alginate lyase, DEHU-reductase), leaves opened the possibility of novel and unclassified GT families to be described in future.

b. *Candidate genes for the sulfate incorporation and depletion on carbohydrates*

The sulfation and desulfation of FCSPs in brown algae are predicted to be catalyzed by sulfotransferases (ST) and formylglycine-dependent sulfatases, respectively [26,94,95]. These enzymes are crucial in determining the position and the number of sulfate groups in FCSPs, and thus in catalyzing the last steps of FCSP biosynthesis.

The identification and annotation of ST genes was made in 4 public genomes from brown algae, e.g. *Ectocarpus* sp. Ec32 [1,26,33], *S. japonica* [83,96], *N. decipiens* [69] and *C. okamuranus* [68]. A dedicated analysis by Lu et al. (2020) was recently completed on *S. japonica* and reports the expert identification of 44 ST genes in this species and 41 in *Ectocarpus* sp. Ec32. In contrast, only 24 and 6 STs were reported in *N. decipiens* and *C. okamuranus*, respectively. Such a difference in the ST gene numbers between the three Ectocarpales species is puzzling. In order to prevent any misinterpretation, we re-analysed the ST content on these 4 brown algal published genomes. We identified a rather similar number of full-length ST genes between these four genomes, i.e. 40, 59, 45 and 55 members in *Ectocarpus* sp. Ec32, *N. decipiens*, *C. okamuranus* and *S. japonica*, respectively.

Previous phylogenetic analyses have indicated that STs in brown algae are classified into distinct clades [1,26,96]. Michel et al. (2010) were pioneer in incorporating brown algal sequences in their analyses, later updated in Kloareg et al. (2021). They indicated the clustering of the brown algal sequences into 4 different clades and discussed their

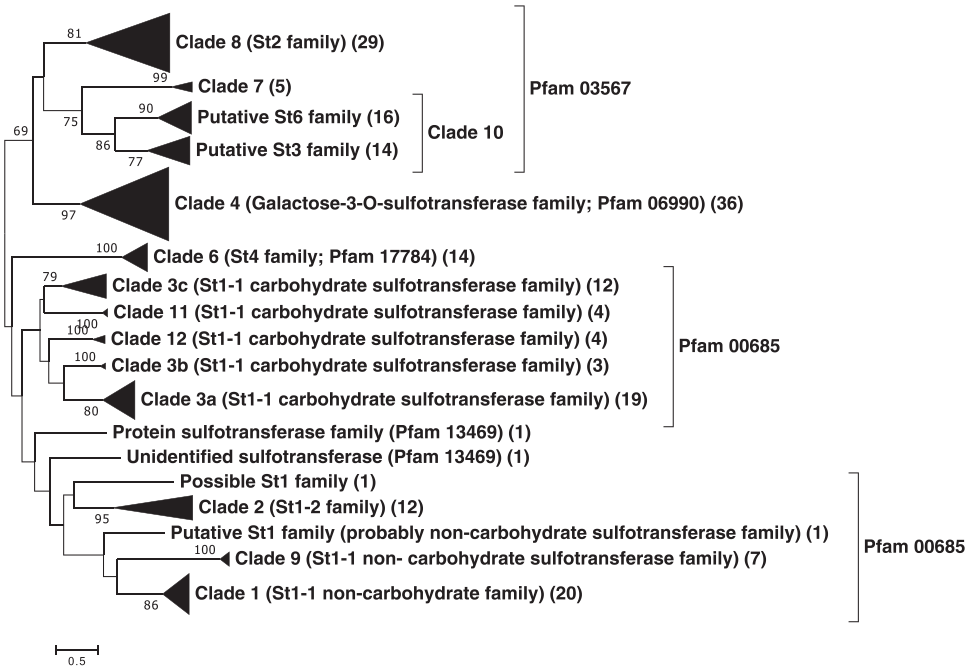

Fig. 8. Unrooted phylogenetic tree of sulfotransferases from *Ectocarpus* sp. Ec32, *N. decipiens*, *C. okamuranus* and *S. japonica*, The phylogenetic tree was constructed using the Maximum Likelihood approach. The reliability of the tree was tested by a bootstrap analysis using 1000 resamplings of the dataset. Only bootstrap values above 50% are shown. The Pfam domains and the classification in the different ST families are based from the Pfam 34.0 site and Uniprot database, respectively. We retained but extended the numbering followed by Kloareg et al. (2021). Note that clade 5 is not apparent as it did not retrieve algal sequences in the aforementioned analysis. Numbers in brackets refer to the number of sequences included in the collapsed clades. The detailed method and the uncompressed tree are shown in Supplementary Fig. S1.

relations to other eukaryotic STs. However only 15 brown algal sequences (all from *Ectocarpus* sp. Ec32) were incorporated, which only accounts for a subset of the whole ST content in these organisms. Later on, Lu et al. (2020) focused their phylogenetic analyses on brown algae only and likely identified additional clades (named clade D and clade E). But as stated above some sequences from *N. decipiens* and *C. okamuranus* were not incorporated. Thus, we have extended this analysis to the full set of ST genes we identified above (Fig. 8). A total of 198 sequences were retained in the analysis, the sequences with a low confidence or truncated, were removed. Our analysis indicates that the brown algal STs from these 4 genomes cluster into 15 distinct clades. As a matter of reference, we kept but extended the numbering followed by Kloareg et al. (2021) and in which clades 1–4 were previously discussed. Briefly, the STs from clade 1 likely are involved in the sulfation of phenolic components such as phlorotannins and/or flavonoids in brown algae [26]. Most other members form clades 2–4 are homologous to a variety of carbohydrate STs, including characterized enzymes involved in the synthesis of glycosphingolipids (galacosylceramide STs, clade 4), or glycosaminoglycans (carbohydrates STs, clade 2; heparan sulfate STs, clade 3) [26]. Populating the analysis causes clade 3 to subdivide further in additional clades (clades 3a, 3b, 3c which contain members from previous clade 3; and clades 11 and 12). These clades are well supported by the bootstrap values (Fig. 8., Supplementary Fig. S1), and they may enclose enzymes with distinct carbohydrate specificities. Additional clades are identified in the present analysis, notably clades 8 and 10 which both encompass a substantial number of ST members. While the fucose residues can be highly sulfated and at various positions in sulfated fucans, additional monosaccharides are known to be sulfated in FCSPs (i.e. galactose, mannose, Fig. 1) [97]. The number of ST members in brown algae, as their clustering in distinct clades, are likely to reflect this diversity in carbohydrate sulfation.

By contrast, the occurrence of sulfatases has not been extensively investigated in brown algae. Genome annotations retrieved 9 S1-sulfatases, all belonging to the S1_2 subfamily (http://abims.sb-roscoff.fr/sulfatlas/) [95] in *Ectocarpus* sp. Ec32 and in *E. subulatus* [26,81]. All these enzymes are related to GAG sulfatases from animals, which hydrolyze the 4-sulfate groups in GAGs. In brown algae, these enzymes probably hydrolyze 4-sulfate groups from FCSPs (Fig. 7). Sulfatases are anticipated to be ubiquitously present in brown algae.

5. Conclusions and perspectives

As in other eukaryotes, distinct functional roles for ECMs in brown algae are likely to be reflected by differences in their composition. The diversity of structures and ECM architectures are probably still underestimated in brown algae. Their heterogeneous chemistry is a limitation to their study. Our knowledge of the brown algal glycomes is only as complete as the methodology to define them. Progress has been made recently by the community as a whole to better define the isolated structures and their ECM context. However, despite the number of studies carried out on ECM polysaccharides, and the large quantities of alginate and FCSPs present in brown algae, little is known about their intracellular origin, subsequent maturation and transport, and of their specific locations at the cell and tissue levels. Similarly, we are still a long way from being able to infer the biochemical functions of many genes involved in the biosynthesis of glycans. Yet, the release of the first genomic sequences in brown algae has allowed progress on the matter, and many additional candidate genes will likely be examined in a near future. Connecting the morphological and molecular data will be an exciting subject to investigate in the long-term basis and the recent development of genetic transformation in brown algae [98] is opening up additional possibilities.

Funding

This work was supported by the French National Research Agency with regard to the BrownSugar project (grant agreement no. ANR-20-CE44–0011).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We sincerely thank Moira Galway (St. Francis Xavier University, Canada) for kindly providing a free-copyright micrograph of

A. nodosum. We are grateful to Sophie LePanse (MerImage facility, Station Biologique de Roscoff, France) for her help in providing TEM micrographs of brown algae. We also thank Bernard Kloareg for his critical reading of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.semcdb.2022.03.005.

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