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The cell walls of green algae: a journey through evolution and diversity

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The green algae represent a large group of morphologically diverse photosynthetic eukaryotes that occupy virtually every photic habitat on the planet. The extracellular coverings of green algae including cell walls are also diverse. A recent surge of research in green algal cell walls fueled by new emerging technologies has revealed new and critical insight concerning these coverings. For example, the late divergent taxa of the Charophycean green algae possess cell walls containing assemblages of polymers with notable similarity to the cellulose, pectins, hemicelluloses, arabinogalactan proteins (AGPs), extensin, and lignin present in embryophyte walls. Ulvophycean seaweeds have cell wall components whose most abundant fibrillar constituents may change from cellulose to β -mannans to β -xylans and during different life cycle phases. Likewise, these algae produce complex sulfated polysaccharides, AGPs, and extensin. Chlorophycean green algae produce a wide array of walls ranging from cellulose–pectin complexes to ones made of hydroxyproline-rich glycoproteins. Larger and more detailed surveys of the green algal taxa including incorporation of emerging genomic and transcriptomic data are required in order to more fully resolve evolutionary trends within the green algae and in relationship with higher plants as well as potential applications of wall components in the food and pharmaceutical industries.

Keywords: cell walls, scales, green algae, sulfated polysaccharides, glycoproteins

INTRODUCTION

The emergence of green algae (Chlorophyta and Streptophyta, Viridiplantae; *sensu* Leliaert et al., 2012) onto land roughly 470 million years ago represents one of the most important events in the history of life on the planet. Their successful colonization of land and subsequent evolution into modern land plants significantly altered the atmosphere, changed terrestrial substrates and paved the way for the evolution of other biota. Today, humans ultimately depend on the evolutionary “offshoots” of green algae (i.e., embryophytes or “land plants”) for food, textiles, building material, pharmaceuticals, and fuels. Yet these events and applications represent only parts of a much larger story of green algae. Contemporary green algae are ubiquitous. They are important members of the ocean’s phytoplankton, common and sometimes nuisance seaweeds of coastal marine habitats, peculiar symbionts of lichens and flatworms, and inhabitants of just about any freshwater ecosystem ranging from ponds, rivers, lakes, wetlands, and snow banks. In the 1.5 billion years since they first appeared (Lewis and McCourt, 2004; Becker and Marin, 2009; Finet et al., 2010; Leliaert et al., 2011; Wodnick et al., 2011), green algae have successfully adapted to virtually all photic zones of the planet.

Similar to their land plant offspring, the vast majority of extant green algae today are covered by a very large assortment of types of extracellular matrix (ECM). These external coverings are products of complex biosynthetic machineries that often make use of the bulk of the alga’s photosynthetically fixed carbon. The ECM is

integral to growth and development, affords the alga physical protection and defense against microbial attack, is involved in cell–cell and cell–substrate adhesion and in some cases, is involved in sexual reproduction. Some green algae are covered by multiple layers of intricately sculpted scales while others have crystalline glycoprotein coverings or thick multilaminar fibrillar cell walls. A few taxa though have cell walls with remarkable structural and biochemical similarity to cell walls found in land plants (Sørensen et al., 2010, 2011). How did these diverse extracellular coverings arise and what are the evolutionary links between them? Many analytical approaches and technologies are now being used to study green algal ECMs and are providing new and critical insight into structure, chemistry, and evolution of these coverings (Table 1). Nevertheless, we are only in an infancy stage in our understanding of the green algal extracellular coverings. In this review, we describe some of these recent discoveries and comment on future directions for study of the cell walls of green algae.

THE CURRENT STATE OF STUDY OF GREEN ALGAL CELL WALLS AND EXTRACELLULAR COVERINGS

While the green algae display a large and diverse array of ECM-coverings, only a few taxa have been studied in detail. It is widely accepted that taxa of the Ulvophyceae and the Charophycean green algae (CGA) possess fibrillar cell walls (Popper et al., 2011) consisting of various polysaccharide and proteoglycan constituents while other taxa, especially those of the Prasinophyceae,

Table 1 | Major methodologies used today in the study of green algal coverings.

Methodology	Technical aspects	Data obtained/status	Reference
Biochemical	Chemical and enzymatic fractionation; methylation analysis-GC/MS; NMR; electrophoresis	Monosaccharide composition, glycosidic linkage composition, conformational studies, molecular weights of various cell wall polysaccharides	Popper et al. (2011), Popper and Fry (2003), Estevez et al. (2009)
Carbohydrate microarrays	Sequential extraction of polysaccharides; immobilization onto nitrocellulose, mAb probing	Early divergent CGA walls differ from late divergent taxa walls; late divergent taxa possess HGA, RG-I, MLG, various hemicelluloses, AGPs, extensins	Sørensen et al. (2010, 2011), Moller et al. (2007)
Immunocytochemistry	Immunofluorescence and immunogold labeling of live cells and sections of fixed cells	Wall polymer mAbs may be used in live cell studies; <i>Coleochaete</i> walls possess lignin-like epitopes	Domozych et al. (2009, 2011), Eder and Lutz-Meindl (2010), Sørensen et al. (2011)
FTIR microspectroscopy	IR spectral arrays obtained from microscopically imaged covering	Analysis of presence and distribution of polymers in the cell wall of Ulvophyceae	Estevez et al. (2009), Fernández et al. (2011a), Carpita et al. (2001)
Molecular	Transcriptome and genome acquisition; annotation of genes	Genomes sequenced in <i>Volvox carteri</i> , <i>Chlamydomonas reinhardtii</i> , <i>Micromonas sp. RCC299</i> , <i>Ostreococcus tauri</i> , and <i>Ostreococcus lucimarinus</i> (see http://bioinformatics.psb.ugent.be/plaza/) and <i>Chlorella variabilis</i> NC64; several transcriptomes analyzed	Blanc et al. (2010), Timme and Delwiche (2010), Vannerum et al. (2011), Timme et al. (2012)

mAb, monoclonal antibody; RG-I, rhamnogalacturonan-I; MLG, mixed linkage glucans; AGP, arabinogalactan protein; FTIR, Fourier transform infrared; CGA, Charophycean green algae.

Chlorodendrophyceae, and some taxa of the Chlorophyceae, produce coverings that are structurally and biochemically unique. Presently, extant green algae are classified into six distinct clades (Delwiche and Timme, 2011; Leliaert et al., 2011, 2012). The following represent brief synopses of the current state of knowledge concerning the ECM of these groups (see also **Table 2**).

THE PRASINOPHYCEAE MATRIX: SUBTLE TO THE SPECTACULAR!

The Prasinophyceae or prasinophytes represent a group of motile and non-motile unicells that are presently classified in four clades (Leliaert et al., 2012) and are most commonly found in marine habitats. In photic zones of oceans, picoplanktonic prasinophytes (perhaps the smallest extant eukaryotes; 0.8 μm cell size), like *Ostreococcus* and *Micromonas*, exist in very large numbers. Previous microscopy-based research has shown that taxa like these are either covered with scales or do not have any discernable matrix at all (i.e., they are naked; Piganeau et al., 2011). However, recent analysis of the *Ostreococcus* genome plus immunocytochemical investigations in our laboratories together raise questions as to the nakedness of this picoalga. Other prasinophytes are significantly larger and covered with layers of thousands of distinctly shaped scales coating both the cell and flagellar membrane surfaces (Moestrup and Walne, 1979). Biochemical analyses have shown that these scales are comprised primarily of neutral and acidic sugars including 2-keto sugars such as 3-deoxy-lyxo-2-heptulosaric acid (DHA; Becker et al., 1991, 1994). All scales of prasinophytes are believed to be processed in the Golgi apparatus, packaged in secretory vesicles and secreted to the cell surface near the flagellar apparatus or to vacuole-like scale reservoirs before release to the cell surface.

CHLORODENDROPHYCEAE: WALLS FROM A FUSION

Taxa of the small Chlorodendrophyceae group of green algae consist of motile or non-motile and sometimes stalked unicells (*Tetraselmis* and *Scherffelia*) that are covered by a single cell wall or layers of cell walls. Furthermore, the wall or theca consists of regular repeating subunits and unlike the cell walls of other green algae, this wall is believed to be a product of fused scales. The subunits of the theca are scale-like and are processed in the Golgi apparatus like those of scaly prasinophytes. The acid sugars, 2-keto-3-deoxy-D-manno-octulosonic acid, 5-O-methyl 2-keto-3-deoxy-D-manno-octulosonic acid, and DHA comprise 60% of the sugars present in the theca (Becker et al., 1991).

TREBOUXIOPHYCEAE: WALLS OF UNUSUAL POLYMERS

The Trebouxiophyceae consists of an assemblage of primarily freshwater and terrestrial forms that exhibit diverse phenotypes ranging from unicells to colonies to filaments as well as representing most of the photobiont green algae of lichens (e.g., *Trebouxia*). Some are considered highly attractive candidate genera for use in algal biofuel production (e.g., *Chlorella*; Rodrigues and da Silva Bon, 2011). Most members of this group possess cell walls but surprisingly little is known about their biosynthesis, composition, or architecture. In *Chlorella*, the wall contains cellulose and in some species, the wall is coated by a highly resistant outer stratum consisting of “algaenan,” an aliphatic polymer containing long polymethylene chains that are decorated with amide and *N*-alkyl substituted pyrroles (Rodrigues and da Silva Bon, 2011). In *Trebouxia* isolated from lichens and grown separately from its fungal partner, β-galactofuranan has been demonstrated, a polysaccharide previously found in fungi but not known from green algae (Cordeiro et al., 2006).

Table 2 | Summary of the composition of extracellular coverings in green algae.

Taxon	Covering type	Biochemical composition	Reference
<i>Prasinophyceae</i>	“Scales,” coatings	2-Keto sugars (e.g., DHA), mannans, glycoproteins	Becker et al. (1991, 1994), Moestrup and Walne (1979)
<i>Chlorodendrophyceae</i>	Wall of fused scales	2-Keto sugars (e.g., DHA), proteins	Becker et al. (1991)
<i>Trebouxiophyceae</i>	Cell walls	Cellulose, algaenan, β -galactofuranan	Rodrigues and da Silva Bon (2011), Cordeiro et al. (2006)
<i>Chlorophyceae</i>	Crystalline glycoprotein walls; fibrillar cell walls	Hyp-rich glycoproteins, cellulose pectins, AGP, extensin	Voigt et al. (2001, 2007), Kirk (1998), Estevez et al. (2008)
<i>Ulvophyceae</i>	Cell walls	Cellulose, β -mannans, β -xylans, sulfated (sometimes pyruvylated) polysaccharides or sulfated rhamnogalacturonans, AGP, extensin	Ciancia et al. (2012), Estevez et al. (2009), Percival (1979), Lahaye and Robic (2007)
<i>Charophyceae-early divergent clades</i>	Scales, cell walls	2-Keto sugars, cellulose, homogalacturonans, 1,3 β -glucans, AGP	Sørensen et al. (2011), Domozych et al. (1991)
<i>Charophyceae-late divergent clades</i>	Cell walls	Cellulose, homogalacturonans, RG-I xyloglucans, mannans, xylans, mixed linkage glucans, 1,3 β -glucans, AGP, extensin, lignin	Sørensen et al. (2011, 2012), Popper and Tuohy (2010)

For further detailed information, key references are provided. AGP, arabinogalactan proteins; Hyp, hydroxyproline.

CHLOROPHYCEAE: GLYCOPROTEINS AND CELLULOSE

The Chlorophyceae are the largest group of green algae and exhibit great morphological diversity ranging from motile unicells to large filaments to blade-like thalli. The extracellular coverings of the Chlorophycean algae are also very diverse and consist of a distinct assortment of “cell walls.” In *Oedogonium*, the cell wall resembles those of higher plants in containing microfibrillar cellulose, homogalacturonans and rhamnogalacturonan-I, extensin, and arabinogalactan constituents (Estevez et al., 2008). Many of the polysaccharides that are common to embryophyte walls are thought to have evolved within the CGA, so these results were unexpected. Further biochemical study and a much wider screening of this and other Chlorophycean taxa will be required to ascertain the similarity of these polymers with those of the CGA and embryophytes. However, in the *Chlamydomonas*–*Volvox* assemblage (i.e., volvoclean flagellates), the cell wall does not contain cellulose but is made of crystalline glycoproteins, specifically one based upon aggregates of hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich glycoproteins (Imam et al., 1985; Adair et al., 1987; Kirk, 1998; Voigt et al., 2001, 2007). Extensins of plants are a group of cell wall glycoproteins that probably share at least some glycosylation motives and a common ancestry with the HRGPs of *Chlamydomonas*–*Volvox* assemblage. The glycosylation motives that govern extensin-type glycosylation comprise the SPPPP sequence (i.e., serine-proline-proline-proline-proline) usually occurring several times. The prolines are hydroxylated by prolyl hydroxylases prior to glycosylation. Showalter et al. (2010) used SPPSPPPP to define the class of extensins in their bioinformatic classification of HRGPs. The genetic encoding of the repetitive structures allows for substantial genetic drift without loss of function (Kieliszewski and Lampion, 1994), this being the reason why clear orthologies between individual vascular plant and chlorophyte extensins cannot be traced. The core arabinosylation machinery is clearly equivalent, however. Egelund et al. (2007) used the phylogenetic distances between family GT77

glycosyltransferases to deduce the function of the *Arabidopsis* GT77 clade A genes. This proposition that the genes encode extensin arabinosyltransferases, was later corroborated by extensin phenotypes of mutants knocked out in these genes (Velasquez et al., 2011). The prolyl hydroxylases described in that paper also have orthologs in the chlorophyte genomes and one from *Chlamydomonas* has been shown to be involved in cell wall biogenesis (Keskiäho et al., 2007) and the CAZy-database classifies *Chlamydomonas* gene BAF46284 to family GT75. The encoded protein is 68.6% identical to *Arabidopsis* At3g02230, one of the mutases that catalyzes the interconversion of UDP-Arap and UDP-Araf (Rautengarten et al., 2011), which we believe to be the donor substrate also of these arabinosyl transferases.

ULVOPHYCEAE: SULFATED POLYSACCHARIDES, FIBRILLAR POLYMERS, AND GLYCOPROTEINS

The Ulvophyceae area diverse assemblage of organisms including the best known marine seaweeds with siphonocladous, siphonous, and filamentous forms (Leliaert et al., 2012). Some of them have cellulose as a major fibrillar component of their cell walls. However, others possess (1 \rightarrow 4)- β -mannans or (1 \rightarrow 3)- β -xylans (Percival and McDowell, 1981; Painter, 1983; Yamagaki et al., 1997; Dunn et al., 2007; Estevez et al., 2009; Ciancia et al., 2012) and in some, the type of fibrillar polysaccharide is life cycle stage-dependent (Huizing and Rietema, 1975; Wutz and Zetsche, 1976; Huizing et al., 1979; Dunn et al., 2007).

These algae synthesize sulfated polysaccharides, an adaptation to the marine habitats that is also observed in many other marine organisms, including angiosperms and invertebrates (Aquino et al., 2011). These sulfated wall constituents may be classified into one of two groups as originally designated by Percival (1979): (1) uronic acid-rich polysaccharides also containing rhamnose, xylose, and sometimes galactose, and (2) uronic acid-limited polysaccharides consisting of major quantities of galactose, arabinose and, in some cases, xylose. The first group is represented by *Ulva*,

Monostroma, *Gayralia*, and *Acetabularia* (Ray, 2006; Chattopadhyay et al., 2007a; Dunn et al., 2007; Lahaye and Robic, 2007; Cassolato et al., 2008; Mao et al., 2008, 2009) and the second one includes *Caulerpa*, *Codium*, and *Bryopsis* (Bilan et al., 2007; Chattopadhyay et al., 2007b; Estevez et al., 2009; Ciancia et al., 2012).

Cell walls from the first group as represented by several *Ulva* species comprise two major polysaccharide components, soluble ulvans and cellulose, and two minor ones, an alkali-soluble linear xyloglucan and a glucuronan. Ulvan is the family of sulfated polysaccharides that consist of large quantities of glucuronic acid and rhamnose with the main repeating disaccharide being $\rightarrow 4$ - β -D-GlcAp-(1 \rightarrow 4)- α -L-Rhap-(1 \rightarrow). α -L-Iduronic acid or β -xylose can replace glucuronic acid to a certain extent with the latter sugar also found as a side chain linked to C-2 of some rhamnose residues. Sulfation appears mostly on C-3 of the rhamnose units and on C-2 of the glucuronic acid side chains. Distribution of these polymers in the cell walls of the bi-seriated thallus of *Ulva* was determined by cytochemical and physicochemical data (Lahaye and Robic, 2007). These polymers are structurally related to glycosaminoglycans from animal tissues, like chondroitin sulfate or heparin, but they have glucosamine units instead of rhamnose in their backbone, and the sulfation pattern is different.

The second group is represented by coenocytic *Codium* species, which produce a 4-linked β -D-mannan as fibrillar component. Three different sulfated polysaccharide structures were isolated from *C. vermilara* including: (1) highly ramified sulfated and pyruvylated β -(1 \rightarrow 3)-D-galactans, also present in other *Codium* species (Bilan et al., 2007; Ciancia et al., 2007; Farias et al., 2008; Ohta et al., 2009; Fernández et al., 2011a); (2) linear highly sulfated β -(1 \rightarrow 3)-L-arabinans, with the arabinose units in the pyranose form (P. V. Fernandez, J. M. Estevez, A. S. Cerezo, and M. Ciancia, unpublished results); and (3) linear β -(1 \rightarrow 4)-D-mannans partially sulfated mainly on C-2 of some of the mannose units (Fernández et al., 2011b). Also, HRGPs with characteristics similar to those of arabinogalactan proteins (AGPs) and extensins from vascular plants were detected in these cell walls (Estevez et al., 2009; Fernández et al., 2011a). Distribution of β -mannans and HRGP-like epitopes was shown to be found in two distinct cell wall layers, whereas sulfated polysaccharides were distributed in the middle area of the wall.

CHAROPHYCEAE: CELL WALLS AND THE ORIGIN OF LAND PLANTS

The Charophyceae or CGA are the extant group of green algae most closely related and ancestral to land plants. Over the past decade, a surge of research activity focusing on the walls of CGA taxa has revealed important insight into cell wall structure and evolution (Popper and Fry, 2003; Domozych et al., 2007, 2009; Eder et al., 2008; Eder and Lutz-Meindl, 2010; Popper and Tuohy, 2010; Sørensen et al., 2010, 2011; Popper et al., 2011). First, the cell walls of taxa of the early divergent CGA such as the Chlorokybales and Klebsormidiales, do not possess most of the polymers commonly found in late divergent CGA and land plants. The basal clade of CGA, the Mesostigmatales, does not even have a cell wall but rather produces layers of scales (Domozych et al., 1991). Second, the late divergent clades, the Charales, Coleochaetales,

and Zygnematales, possess cell walls with notable similarity to those of land plants. Polysaccharides like cellulose, pectins (including homogalacturonans and rhamnogalacturonan-I), β -(1-3) glucans, and hemicellulosic polymers like xyloglucans, mannans, and xylans are found in the cell wall of these advanced CGA. Mixed linkage glucans or MLGs that were once thought to be found only in members of the grass family of angiosperms, were also found in Zygnematalean taxa, demonstrating a more widespread occurrence in green plants. Also surprising is the presence of lignin-like compounds in the cell walls of several species of the genus *Coleochaete*.

Arabinogalactan proteins and extensin have also been described in many CGA taxa. Extensins surprisingly are not detected in *Nitella* and *Chara* (Charales; Gotteli and Cleland, 1968). Arabinosyltransferases involved in extensin arabinosylation belong to the GT77 A- and C-clades sensu Petersen et al. (2011) and it is remarkable that the *Chara* transcriptome (courtesy of Gernot Glöckner, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Berlin) as well as that of *Nitella hyalina* (Timme et al., 2012) each have a putative member in family GT77 clade D, but none in clade A or C. The transcriptome of *Nitella*, but not that of *Chara* comprises putative prolyl hydroxylases allowing for the existence of AGPs even though extensins are missing. It thus appears that members of Charales have evolved and separated themselves significantly from other members of the CGA, notably the Zygnematales and Coleochaetales which feature cell walls that more closely resemble that of vascular plants and which are known to express enzymes involved in cell wall metabolism akin to that observed in angiosperms (e.g., Vannerum et al., 2011). These latter similarities suggest that late divergent taxa of the CGA (i.e., their ancestors 470 million years ago) may have possessed cell wall characteristics that pre-adapted them for successful emergence onto and life on land.

THE NEXT GOALS?

More detailed characterization of the various taxa will be required before we can fully understand the evolution of extracellular coverings of green plants as well as adaptations to ECM chemistry in response to life in marine, freshwater, and terrestrial habitats. Some specific questions and areas of focus for future study include:

- (1) Insights into the polysaccharide biosynthetic machinery of CGA are and will at the same time offer insights into the evolutionary events that accompanied adaptation to life on land. This will require full genomic sequencing as transcriptomic analysis is useful for demonstrating the functional expression of genes, but not for the absence. This endeavor offers many challenges as many relevant CGA genomes are estimated to be as big as if not bigger than that of *Arabidopsis* (Kapraun, 2007).
- (2) Detailed analysis of the roles of cell wall polymers in the CGA is critical. Although the CGA share many cell wall constituents with their embryophyte descendents, it is not clear if they are used in equivalent roles.
- (3) Sulfated polysaccharides from ulvophyte seaweeds have only recently been reexamined and have yet to be widely used in the hydrocolloid industry. Ulvan has been investigated as

potential dietary fiber for human diet (Lahaye and Robic, 2007) due to its medical properties and potentially profitable extraction yields from harvested specimens. In addition, ulvans have some interesting biological activities, including acting as antioxidants, modifying certain macrophage activities, and serving as potential anti-hyperlipidemic agent (Wijesekara et al., 2011). Sulfated polysaccharides from the Bryopsidales are not obtained in large yields (Ciancia et al., 2007, 2012), but have piqued interest as bioactive compounds with several potential pharmacological applications (Ohta et al., 2009; Ciancia et al., 2010; Costa et al., 2010; Lee et al., 2010). More detailed characterization of these molecules is needed in order to fully recognize their potential.

- (4) Comprehensive chemical and functional screening of the large but virtually unknown polysaccharide complexes secreted outside the cell walls of many Zygnematalean taxa (Domozych et al., 2005) will be important for understanding their physiology and importance to ecosystem dynamics.

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