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Extracellular matrix of the charophycean green algae

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The Extracellular Matrix of the Charophycean Green Algae

By

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This dissertation, "The Extracellular Matrix of the Charophycean Green Algae," is hereby approved in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the field of Biological Sciences.

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Date _____

This work is dedicated in loving memory of Josephine Getty and Madison Kiemle, who always supported me in all my endeavors; also to my wonderful parents, Dee and Fred, and my sister, Kate, who have sustained my education and always encouraged me to pursue my love of science.

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"If it looks like a duck, and quacks like a duck, we have at least to consider the possibility that we have a small aquatic bird of the family Anatidae on our hands."
Douglas Adams, *(1952 - 2001)*

Abstract

A comprehensive knowledge of cell wall structure and function throughout the plant kingdom is essential to understanding cell wall evolution. The fundamental understanding of the charophycean green algal cell wall is broadening. The similarities and differences that exist between land plant and algal cell walls provide opportunities to understand plant evolution. A variety of polymers previously associated with higher plants were discovered in the charophycean green algae (CGA), including homogalacturonans, cross-linking glycans, arabinogalactan protein, β-glucans, and cellulose. The cellulose content of CGA cell walls ranged from 6% to 43%, with the higher valuescomparable to that found in the primary cell wall of land plants (20-30%). (1,3)β-glucans were found in the unicellular *Chlorokybus atmophyticus*, *Penium margaritaceum*, and *Cosmarium turpini*, the unbranched filamentous *Klebsormidium flaccidum*, and the multicellular *Chara corallina*.

The discovery of homogalacturonan in *Penium margaritaceum* represents the first confirmation of land plant-type pectins in desmids and the second rigorous characterization of a pectin polymer from the charophycean algae. Homogalacturonan was also indicated from the basal species *Chlorokybus atmophyticus* and *Klebsormidium flaccidum*. There is evidence of branched pectins in *Cosmarium turpini and* linkage analysis suggests the presence of type I rhamnogalacturonan (RGI). Cross-linking β-glucans are associated with cellulose microfibrils during land plant cell growth, and were found in the cell wall of CGA. The evidence of mixed-linkage glucan (MLG) in the

charophytes is both suprising and significant given that MLG was once thought to be specific to some grasses. The organization and structure of *Cosmarium turpini* and *Chara corallina* MLG was found to be similar to that of *Equisetum* spp., whereas the basal species of the CGA, *Chlorokybus atmophyticus* and *Klebsormidium flaccidum*, have unique organization of alternating of 3- and 4-linkages. The significance of this result on the evolution of the MLG synthetic pathway has yet to be determined.

The extracellular matrix (ECM) of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* spp. exhibits significant biochemical diversity, ranging from distinct "land plant" polymers to polysaccharides unique to these algae. The neutral sugar composition of *Chlorokybus atmophyticus* hot water extract and *Spirogyra* extracellular polymeric substance (EPS), combined with antibody labeling results, revealed the distinct possibility of an arabinogalactan protein in these organisms. Polysaccharide analysis of Zygnematales (desmid) EPS, indicated a probable range of different EPS backbones and substitution patterns upon the core portions of the molecules. Desmid EPS is predominately composed of a complex matrix of branched, uronic acid containing polysaccharides with ester sulfate substitutions and, as such, has an almost infinite capacity for various hydrogen bonding, hydrophobic interaction and ionic cross-bridging motifs, which characterize their unique function in biofilms.

My observations support the hypothesis that members of the CGA represent the phylogenetic line that gave rise to vascular plants and that the primary cell wall of vascular plants many have evolved directly from structures typical of the cell wall of filamentous green algae found in the charophycean green algae.

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List of Abbreviations

AG	arabinogalactan
AGP	arabinogalactan protein
AIR	alcohol insoluble residue
Ara	arabinose
BS-I	*Bandeiraea simplicifolia* agglutinin
CDTA	trans-1,2-diaminocyclohexane N,N,N',N',-tetracetic acid
CGA	charophycean green algae
Ch	*Chlorokybus*
CLSM	confocal laser scanning microscopy
Co	*Cosmarium*
CoMPP	comprehensive microarray polymer profiling
ConA	concanavalin agglutinin
CW	cell wall
DIC	differential interference contract microscopy
DMSO	dimethylsulfoxide
DNS	3,5-dinitrosalicylic acid
ECM	extracellular matrix
EPS	extracellular polymer substances
FITC	fluorescein isothiocyanate
Fuc	fucose
Gal	galactose

GalA	galacturonic acid
GCMS	gas chromatography-mass spectrometry
GlcA	glucuronic acid
HG	homogalacturonan
HPA	*Helix pomatia* agglutinin
HPAEC-PAD	high-performance anion exchange chromatography with pulsed amperometric detection
HPLC	high-pressure liquid chromotography
HRGP	hydroxyproline-rich glycoprotein
HW	hot water soluble fraction
K	*Klebsormidium*
KOH	potassium hydroxide
LcH	*Lens culinaris* agglutinin
LN_2	liquid nitrogen
Man	mannose
MKIR	methonalic possium hydroxide insoluble residue
MLG	mixed linkage glucan
N	*Netrium*
NMR	nuclear magnetic resonance
OLIMP	oligosaccharide mass profiling
P	*Penium*
PAD	pulsed amperometric detector
Pl	*Pleurotaenium*
PSA	*Pisum sativum* agglutinim

RGI	rhamnogalacturonan I
RGII	rhamnogalacturonan II
Rha	rhamnose
Rib	ribose
SEM	scanning electron microscopy
T	*Tetmemorus*
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TRITC	tetramethylrhodamine-5-(and 6)-isothiocyanate
UEA	*Ulex europaeus* agglutinin
VPSEM	variable pressure scanning electron microscopy
WGA	wheat germ agglutinin
WHM	woods hole media
XyG	xyloglucan
Xyl	xylose

Chapter 1: Algal Extracellular Matrix to Land Plant Cell Walls

Charophycean Green Algae

One of the most important developments in the evolution of life in terrestrial environments occurred 470-450 million years ago in the Ordovician period (Sanderson et al. 2004; Gray et al 1982). The plant lineage (includes glaucophytes, rhodophytes, chlorophytes, charophytes, and embryophytes; Figure 1.1) purportedly originated from a common ancestral group. This eukaryotic ancestor had an endosymbiosis with a photosynthetic cyanobacterium (Gray et al. 1982; Bowmen et al. 2007). During this time, descendants of green algae (Streptophytes) colonized terrestrial habitats (Graham 1993; Kenrick and Crane 1997; Bateman et al. 1998). Streptophytes are a monophyletic group comprised of the charophycean green algae (CGA) and the land plants. This group represents one of the main lineages of multicellular eukaryotes (Figure 1.1). A classification of the charophyceae was developed by Mattox and Stewart (1984). Mattox and Stewart based their phylogenetic tree on cell division, reproduction, and flagellar apparatus ultrastructure, to define this group of green algae. Recent molecular and phylogenetic studies support this hypothesis that land plants indeed have a charophytic ancestry (Delwiche et al. 1989; Manhart and Palmer 1990; Melkonian et al. 1995; Chapman et al. 1998; Karol et al. 2001; Petersen et al. 2006; Lemieux et al. 2007; Turmel et al. 2007) though the position of the orders inside the CGA is still undetermined. There

are two main theories on the order of the charophytes lineage, based on a different number of characteristics and different number of species. The first phylogenetic study shows Charales as the sister clade to the land plants, Zygnematales, and Coleochaetales, this is based on molecular analysis with a high number of characters examined and a low number of species (Turmel et al. 2007). The second theory, which is also the classical ultrastructural theory, states that the Charales are the closest group to the land plants (Karol et al. 2001). Their phylogenic position within the green plants means the CGA have a special significance for our understanding of the early origins of cell walls.

The CGA include approximately 65 genera and a few thousand species with morphotypes ranging from unicells to complex, branching forms (Kenrich and Crane 1997; Karol et al. 2001; McCourt et al. 2004). Six groups are presently recognized in the CGA: Mesostigmales, Chlorokybales, Klebsormidiales, Zygnematales, Charales and Coleochaetales (Figure 1.1).

Mesostigmales. The Mesotigmales has been only recently incorporated into the charophytes as the basal extant order (Lewis and McCourt 2004, Melkonian 1989) it was once considered part of the Prasinophyceae (Bold and Wynne 1985). This placement in the CGA is based on the multilayered structure of the flagellum (Rogers et al. 1981; Melkonian 1989), also recent molecular analysis have confirmed a close phylogenetic relationship (Melkonian et al. 1995). There is only a single genus of this group, *Mesostigma viride*, which is a freshwater scaly biflagellate. The cells are without a well-defined wall but possess a unique extracellular matrix (ECM) with evidence of a mucilage envelope with three layers. Each layer contains a novel non-cellulosic scale

type (Domozych et al. 1991). Asexual reproduction occurs by division into daughter cells (Graham and Wilcox 2000).

Chlorokybales. The Chlorokybales are located taxonomically at the base of the charophytes as a sister clade to the Mesostigmales and contain only one extant genus, *Chlorokybus atmophyticus* (Figure 1.1; Rogers et al. 1980; Turmel et al. 2006, 2007; Karol et al. 2001). *Chlorokybus atmophyticus* is a rare terrestrial or freshwater green algae (Rogers et al. 1980). The thallus has a simple sarcinoid form, with cells arranged in packets of 2-8 cells held together by a thick mucilaginous layer of carbohydrate and protein. Each vegetative cell is surrounded by a cell wall (CW). During cell development, new CW material is only produced at the septum (Lockhorst et. al. 1988). This process is similar to that observed in more advanced CGA taxa. It is regarded as the simplest charophycean green algae to have a non-motile vegetative stage (Graham et al. 1991; Rogers et al. 1980). *Chlorokybus atmophyticus* is considered a rare freshwater or terrestrial alga as it has only been isolated from two terrestrial habitats (Rogers et al. 1980).

Klebsormidiales. The Klebsormidiales is also classified as a basal taxon of the CGA (Figure 1.1). This order is represented by several genera including the type genus, *Klebsormidium*, which displays a morphotype of single, unbranched filaments and is found in soils and freshwater habitats. The cells produce only a single zoospore, which lacks an eyespot and possess two laterally emergent flagella with a multi-layer flagellar root. Cell division occurs by the development of a constricting furrow from the periphery with an open mitotic spindle and an internuclear vacuole that separates daughter nuclei

(Floyd et al. 1972, Cook 2004). Early biochemical studies suggested that the *Klebsormidium* CW possessed polysaccharides that are similar to other CGA forms (Hotchkiss et al. 1989, Domozych et al. 1980).

Zygnematales. The Zygnematales are the largest and most diverse group of the CGA with widespread global distribution in fresh and brackish water. There are over 4,000 species classified as members of Zygnematales (Gerrath 2003). The members of this order may be unicellular, colonial, or filamentous. The Zygnematales are uninucleate and characterized by elaborate chloroplasts with prominent pyrenoids, the frequent presence of a copious mucilage sheath surrounding the cells and filaments, the absence of flagellate gametes, and reproduction involving the union of usually amoeboid gametes. All are haplobiontic and haploid with zygotic meiosis. During early development and cell expansion, the cell is often covered by a primary CW which is ultimately displaced by a thick secondary CW (Lutz-Meindl and Brosch-Salomon 2000; Hogetsu 1992). The CW proper is surrounded by an extensive thick sheath that is typically carbohydrate rich (Kiemle et al. 2007; Domozych et al. 1993; Domozych et. al. 2005).

Charales. The Charales grow submerged in fresh standing water, upon muddy or sandy bottoms and can form extensive subaquatic blooms. This group is composed of six extant genera of nonvascular hydrophytes that occur worldwide and contain the greatest complexity within the CGA. The branched thallus is differentiated into a regular succession of nodes and internodes. The shoot possesses a single meristematic cell which, upon multiple cell division cycles, yields the complex nodal zone and elongated

internodal cells. Cell division in the Charales occurs at the nodes where branches are then formed sequentially in a radial arrangement. Sexual reproduction is oogamous, with a one-celled antheridia united in uniserate filaments which are surrounded by a spherical envelope composed of packets of cells (Bold and Wyne 1985; Graham and Wilcox 2000).

Coleochaetales. This clade is comprised of two genera, *Coleochaete* and *Chaetosphaeridium* with the form of disc-shaped epiphytes. The Coleochaetales are a key taxon in the elucidation of the evolution of embryophytes, based on evidence of zygote retention on the female gametophyte, putative placental transfer of nutrients to the zygote, and the presence of sheathed hairs (Lewis and McCourt 2004, Graham and Wilcox 2000, Graham 1993). Cell division is accomplished by a cell-plate phragmoplast system with circumferential or radial division, depending on the pattern in the peripheral meristomatic cells. In *Coleochaete orbicularis* and *C. scutata*, T-shaped cell divisions occur in peripheral meristematic cells to yield parenchymatous thalli. Cytokenisis occurs in *C. orbicularis* through the development of a cell plate associated with a phragmoplast, whereas in *C. scutata*, furrowing takes place. In addition, these species produce plasmodesmata in their cross walls which is considered a major evolutionary development.

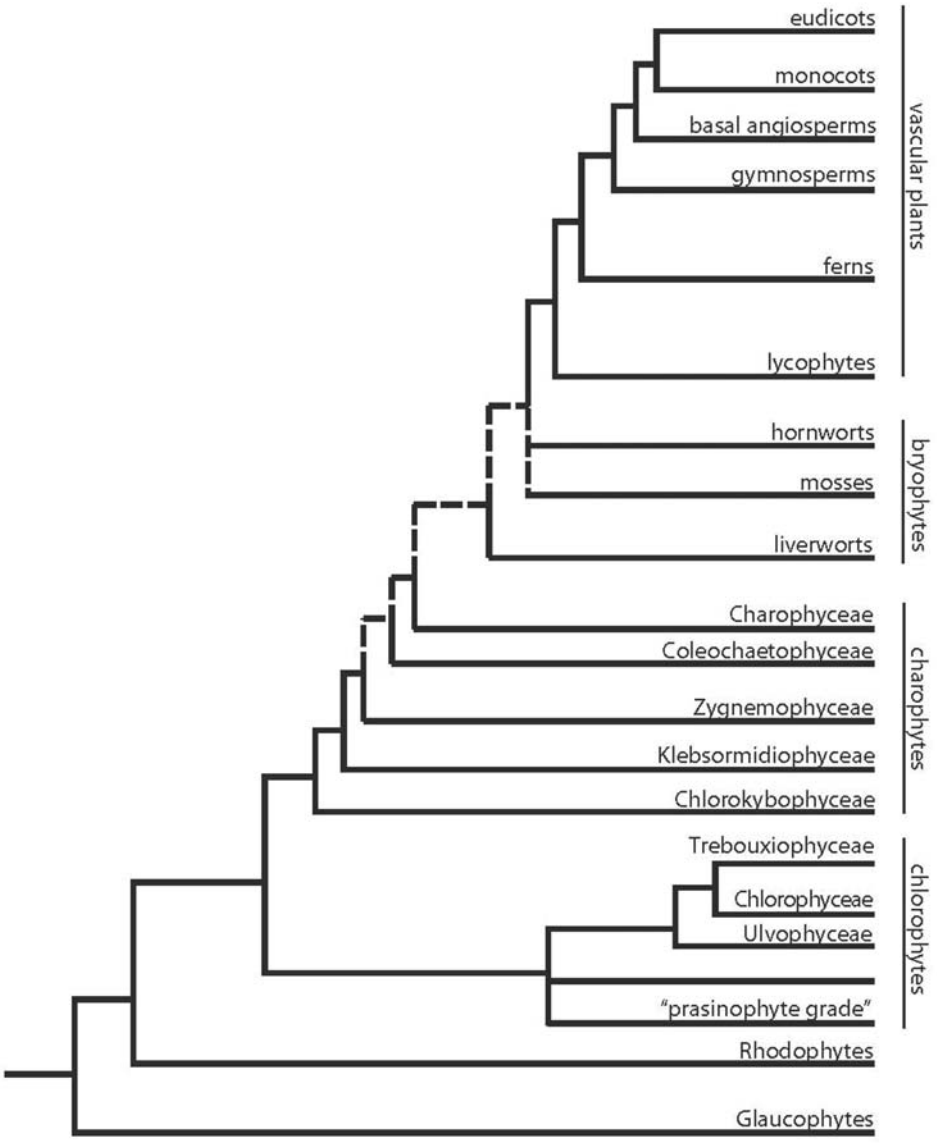


Figure 1.1: Relationships among the CW lineages: glaucophytes (freshwater algae), rhodophytes (red algae), and the green plants (chlorophytes, charophytes, and land plants). Adapted from Bowman et al. 2007.

Formation and Diversification of Cell Walls

The formation and diversification of CWs has a role of fundamental importance in the evolution of land plants and algae (Mattox and Stewart 1984; Stewart and Mattox 1978; Swain 1974). The CWs of early plant-like forms may have provided rigid structures for protections and osmotic control, enabling the formation of multi-cellular structures (Bold and Wynne 1985). While there is considerable variability in the fine structure of the CW throughout the embryophytes, the CW fundamentally consists of an intricate network of gel-like pectins and proteins, in which an ordered array of cellulose microfibrils with cross-linking glycans are embedded (O'Neill and York 2003; Reiter 1998; Cosgrove 1997; Fry 2000; Willats et al. 2001; Matsunaga et al. 2004). The polysaccharides and proteins of the CW are involved in such essential functions as cell-cell communication, cell expansion, signal transduction, adhesion and physical/chemical defense.

Recent biochemical and molecular dissections of land plant CW components along with their synthetic pathways have greatly enhanced our understanding of land plant development and physiology. The process of CW formation includes glycan synthesis, ordered deposition, and degradation, which may involve over 1000 genes (Somerville et al. 2004). Unfortunately, few of the regulatory genes involved in the coordinated process of CW formation and that modulate changes in existing CW structure have been identified (Scheible and Pauly 2004). The information gleaned from previous studies has been limited, due to the circuitous linkage between the glycan product and the CW-related genes.

Evolution of Plant Cell Walls through Cell Wall Polymers.

Knowledge regarding the structure of CW of land plants has increased considerably since about 1970 (Carpita and McCann 2000). Although most of the current research on land plant CWs is focused on *Arabidopsis thaliana* and other angiosperms, recently more focus has been given to the CWs of lower plants including algae. The transition from an aquatic environment to a terrestrial environment was once thought to have triggered physical and structural changes in the cell-wall structures (Graham 1991; Karol et al. 2001; Kenrick and Crane 1997), leading to predictions of a CW structure in CGA quite different from that of land plants. Discovery of polymers of terrestrial plants occurring in algae in aquatic environments leads to questions about the vitality of this opinion. These findings are highly significant for understanding CW evolution, since many of the features found in land plant CWs appear to have evolved prior to the transition from aquatic to terrestrial environments, instead of evolution due to selection pressure during the transition, therefore being a pre-adaption to the transition to land.

The CWs of all plants including the CGA share several common features including a mechanical framework of cellulose microfibrils and a matrix phase that creates cross-linking among the microfibrils (McCann and Roberts 1991; Carpita and Gibeaut 1993; O'Neill and York 2003; Somerville et al. 2004; Sarkar et al 2009). Very few comprehensive studies of the CWs of CGA have been undertaken. In surveys of selected polymers (Popper and Fry 2003, 2004) and comparative analyses of previous literature (Niklas 2004), several differences in CW chemistry and function have been the basis for significant speculation. Detailed chemical profiling of CW macromolecules in

charophytes and lower green plants will be needed in order to accurately characterize evolutionary trends in CW structure and function.

Cellulose

The principal component of plant cell walls is cellulose, a fibrous polymer consisting of linear chains of β-(1,4)-linked glucose molecules (Figure 1.2). These ribbon-like glucan chains crystallize to form microfibrils that impart the characteristic flexible strength of higher plant cell walls (Fry 2000; Carpita and McCann 2000). Cellulose represents the main structural component of the CW and is tightly associated with cross-linking glycans. Cellulose is abundant throughout the phylogeny of life. Many prokaryotic organisms make cellulose including gram-positive anerobic bacteria (*Sarcina*) and purple bacteria (*Acetobacter*, *Rhizobium*, *Alcaligenes*, and *Agrobacterium*). Cellulose is also present in a variety of algal phylogenies including chlorophytes (*Oocystic apiculata*, *Valonia*, and *Boergensenia*), pheophytes (*Pelvetia*), chrysophytes (*Vaucheria* and *Pleurochrysis*), and rhodophytes (*Erthrocladia*). Other eukaryotic organisms that make cellulose include the protists (*Dictoyostelium discoideum*), fungi (*Saprolegnia*, *Allomyces*, *Achlya*) and in the tunicates (*Metandrocarpa* and *Hyalocynthia*) which are classified as animals (*Reviewed in* Brown et al. 1996).

The configuration of cellulose synthesizing terminal complexes (TCs) in the plasma membrane is related to the cross-sectional profile of resultant cellulosic microfibrils. TCs are composed in part of cellulose synthase catalytic subunits (CesAs), which not only catalyze the polymerization of glucan chains, but also play a role in TC assembly. There is a correlation between the structure of the cellulose microfibrils and

the organization of the TC arrays in the plasma membrane, which synthesize the microfibrils (Tsekos 1999; Roberts and Roberts 2007). Microfibrils are deposited in highly organized layers to form the structural backbone of the cell wall. (Tsekos 1999; Okuda and Brown 1992; Gidding and Staehelin 1991; Hotchkiss and Brown 1987; Kiermayer and Sleytr 1979). The first visual confirmation of the terminal complex was in 1975 by Brown and Montezinos who described a linear synthesizing complex associated with the terminus of cellulose microfibril in the bacteria. The TC of the CGA *Mougeotia* sp (Hotchkiss et al. 1989) and *Mesotaenium caldariorum* (Roberts et al. 2002) contain a plant-like rosette structure. The *CesA* genes sequences reveal important evolutionary relationships between CESA sequences, terminal complex configuration and microfibril structure (Roberts et al. 2002, Roberts and Roberts 2007, Tsekos1999). The *CesA* genes from the alga *Mesotaenium caldariorum* have rosette terminal complexes which are similar to seed plant *CesA*s with deduced amino acid sequence identities of up to 59%. (Roberts et al. 2002).

Cellulose

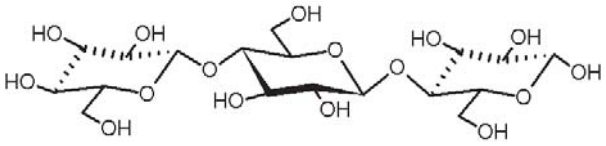


Figure 1.2 Diagrams of polymer structure of cellulose. Polymers were drawn with ChemSketch

Cross-linking glycans

Cross-linking glycans were originally called hemicelluloses, a term proposed in 1892 by Schulze to designate polysaccharides that were extractable by aqueous alkaline solution from land plants (Fry et al. 2000). Up to 50% of the biomass of land plant CWs consists of cross-linking glycans; this group includes a diverse array of polymers including xylans, mannans, mixed linkage β-glucan, and xyloglucans. Cross-linking β-glucans are associated with cellulose microfibrils during cell growth (Buckeridge et al. 2004). A gene superfamily called *CSL* (cellulose synthase-like) has been proposed to synthesize the hemicellulose backbone. It is purported that members of the *CSLA* family function in the biosynthesis of mannan, galactomannan, and glucomannan (Dhugga et al. 2004; Liepman et al. 2005, 2007) whereas *CSLC* family members may synthesize the glucan backbone of xyloglucans (Cocuron et al. 2007), and *CSLF* members in β-glucans (mixed-linkage glucans) (Burton et al. 2006). In a recent survey examining the land plants and algae genomes, six chlorophyte algae were found to have single-copy *CSL* gene, similar to the *CSLA* and *CSLC* genes in land plants (Yin et al. 2009). This may indicate that these *CSL*s may have arisen in an ancient origin. Therefore, the diversification of the *CSL* is important to the evolution of the hemicelluloses.

Xyloglucans (XyG), contains backbone identical to cellulose with a linear polymer of β-(1→4)-linked D-glucan with numerous side branches (70-80%) at the O-6 position composed primarily of xylose, galactose, arabinose, and fucose (Figure 1.3; Carpita and McCann 2000; Fry 2000). Important in cell expansion, xyloglucans constitute about 20% of the primary CWs of dicots and much less (1-5%) in the primary

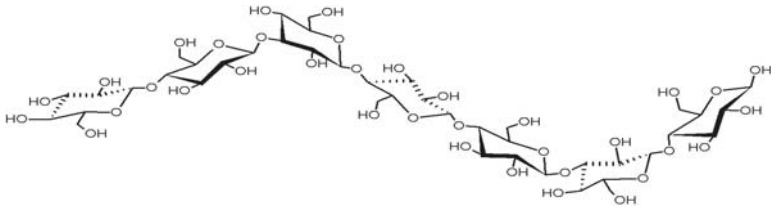
walls of the grasses. Popper and Fry (2003) reported that *Klebsoridum flaccidium*, *Coleochaete scuata*, and *Chara corallina* showed no significant amounts of xyloglucans, though there is conflicting antibody labeling where Comparative Microarray Polymer Profiling (CoMPP) analysis indicates *Klebsormidium* spp. and *Spirogyra* spp. labels with CCRC-M1 for α-Fuc-(1,2)-β-Gal epitopes (fucosylated xyloglucans/rhamnogalacturan I) (Willats CoMPP unpublished). The CW of *Nitella* spp. may contain xyloglucan as indicated by the 1M KOH fraction which contains fucose (10 %), xylose (22%), galactose (10%) and glucose (46%) (Morrison et al. 1993). Xyloglucan endotransglycosylase/hydrolases that have been postulated to have critical function in CW modifications allowing cell expansion have recently been reported highly conserved during the evolution of the vascular plants (Van Sandt et al. 2006) and have been found in *Coleochaete sculata* (Fry et al. 2008b).

Mixed-linkage glucans (MLG) are a cross-linking polysaccharide homopolymer with an alternating (1,3) (1,4) β-glucan backbone that is found in the primary CW of growing cells (Figure 1.3). It is integrated with cellulose and other noncellulosic polysaccharides (Carpita 1984). The cellulose microfibrils are tethered by MLG polymers, which strengthen the CW (Buckeridge et al. 2004). MLG are found in large amounts in some grasses such as *Hordeum vulgare* (barley), *Avena sativa* (Oat), and *Secale cereale*. Recent studies have also shown that they are present in *Equisetum* species (Fry et al. 2008b; Sorensen et al. 2008), MLG were originally presumed to only be present in the Poales. Now, MLG-like polymers have been reported in the lichen *Cetraria islandica* (Olafsdottir and Ingolfsdottir 2001), the fungus *Aspergillus fumigates*

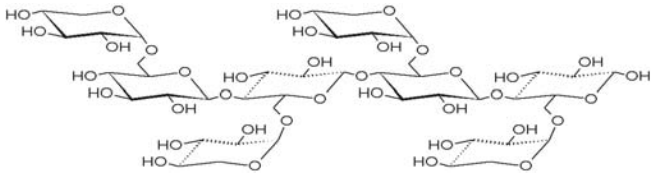
CWs (Fontaine et al. 2000), and possibly in the dinoflagellate *Peridinium westii* (Nevo and Sharon 1969). Popper and Fry (2003) found a MLG-related polymer in lichenase digests of the chlorophyte, *Ulva lactata.* MLG was not detected in material derived from the charophytes *Chara corallina* and *Coleochaete scutata* (Popper and Fry 2003, 2004). COMPP analysis indicates the presents of MLG in *Micraceterias* sp. and *Pleurotaenium* sp. (Willats COMPP data unpublished). MLG synthesis in the grasses is reported to be mediated by a function *CSLF* and *CSLH* proteins (Burton et al. 2006, Doblin et al. 2009).

Xylan. Another hemicellulosic polymer is xylan; an often branched polymer with a β (1, 4)-D xylan backbone and side chains including arabinose and/or glucuronic acid. (Figure 1.3). Xylans are only about 5% of the primary CW in the Magnoliophales and 20% in Poales (Fry 2000). The xylans from different organisms have high variation in composition and structure. *Porphyra umbilicalis*, a member of the rhodophytes, contains a xylan with both 1,3- and 1,4-xylosyl linkages (Turvey and Williams 1970), which serves as the structural component in the CW much like cellulose. In the CGA, there is preliminary evidence of xylans from antibody labeling with LM11 in *Penium margaritaceum*, *Netrium* sp. *Cosmarium* sp, *Spirogyra* sp. *Klebsormidium* sp. *Chlorokybus* sp. and *Chara* sp. (Willats COMPP data unpublished). Also Hotchkiss et al. (1989) found 4-linked xylan and 2, 4-linked xylan in the CWs of *Mougetia* sp, which may indicate a xylan witharabinose and glucuronic acid containing side chains or substitutions. Arabinoxylan is one of the most abundant hemicellulosic polymers in the secondary CW of higher plant vascular tissue (Carpita and McCann 2000).

Mixed-Linkage Glucans



Xyloglucan



Xylan

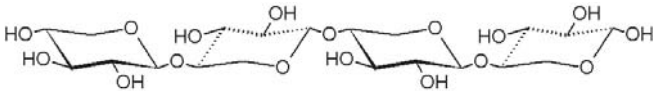


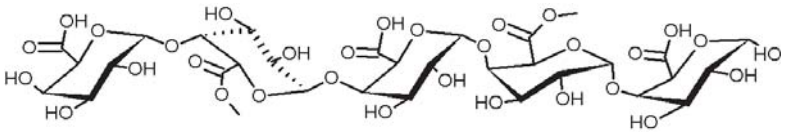
Figure 1.3 Diagrams of polymer structure of cross-linking glycans examined during this project. Polymers were drawn with ChemSketch

Pectin

Pectins are found throughout the plant primary CW; first isolated and described by Henri Braconnot in 1825. Pectins are covalently linked to form a network throughout the primary CW matrix and middle lamellae (Willats et al. 2001). They represent a complex mixture of heterogeneous, branched polymers with two primary backbones, homogalacturonan (HG) and rhamnogalacturonan I (RG-I). Two types of structurally modified HG include xylogalacturonan (XGA) and rhamnogalacturonan II (RG-II). The hydrated, 3-D matrix created by pectins in the CW contributes to the control of stretching of the cellulose microfibril network and accommodates cell expansion driven by turgor pressure (Verhertbruggen and Knox 2007; Seymour and Knox 2002; Janeau et al. 1998). The functions of pectin polymers include providing surfaces which regulate both the pH

and ionic status of the CW, control of porosity of the cell surface, cell adhesion or signaling molecules in defense (Seymour and Knox 2002; Mohnen 2008).

Homogalacturonan (HG)



Rhamnogalacturonan I (RGI)

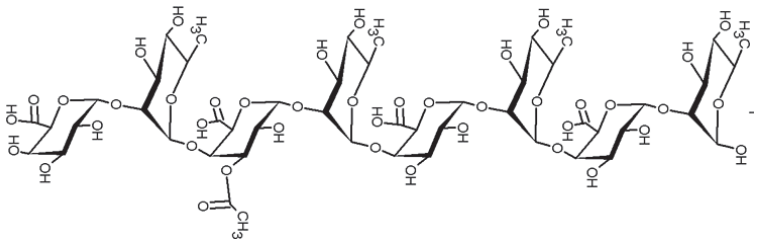


Figure 1.4 Diagrams of polymer structure of pectins examined during this project. Polymers were drawn with ChemSketch

Homogalacturonan (HG) has been demonstrated to play an important role in control of cell adhesion, division, expansion and polarity (*reviewed in* Capodicasa et al. 2004). HG is a linear homopolymer of (1→4)-α-linked D-galacturonic acid (GalA) and is thought to contain about 100-200 galacturononsyl residues (Figure 1.4; Zhan et al. 1998; Thibault and Ralet 2001). Up to 60% of the pectin in the dicot primary CW is HG (O'Neill et al. 2003). Cherno et al. (1976) found *Chara aculeolata* to contain GalA in the pectin fraction (78.8%) along with Glc, Ara, and Xyl. The IR spectra were similar to that of pectins of land plants, with uniform MW that contained 98% D-GalA and 1% OCH_3. In *Chara corallina,* Popper and Fry (2003) found that the alcohol insoluble fraction was

rich with GalA with glucuronic acid (GlcA), Man, and 3-O-Me-Rha. Large amounts uronic acids (GlcA and GalA), indicative of pectin-like molecules, were found in *Chara corallina* and *Coleochaete* sp. (Popper and Fry 2003). In the CWs of the Streptophytes GalA and GlcA were found in the chlorophye *Ulva lactuca*, and in charophytes such as *Penium margaritaceum*, *Chara corallina*, *Chara aculeolata*, *Coleochaete scuata*, and *Closterium acerosum* (Popper and Fry 2004; Baylson et al. 2001; Cherno et al. 1976; Domozych et al 2007a). Baylson et al. (2001) found a pectin-like polymer in *Closterium acerosum* that was rich in galacturonic acid.

In higher plants, HG is synthesized as a methylated neutral polymer. The O-methyl group is converted by methyl esterase proteins in the wall. At neutral pH, the GalA group is ionized allowing the formation of salt bridges and the chelation of small metals such as calcium, providing rigidity to the matrix. At low pH, the acids become protonated and are no longer able to form bridging bonds (Mohnen et al 2008). An antibody binding to unesterified HG epitopes (JIM5) labeled the outer layer of the primary CW of *Micracterias denticulata* and JIM7 (antibody binding to HG epitopes with a high degree of methyl esterification) labeled the primary CW (Lutz-Meindl and Brosch-Salomom 2000).

Rhamnogalacturonan I (RGI) represents a large diverse family of polymers with a backbone comprising (1→2)-α-L-Rha-(1→4)- α -D-GalA disaccharide repeat units (Figure 1.4). Neutral sugar side chains are attached to the O-4 of approximately half of the Rha residues (ex. arabinans, galactans, type I arabinogalactans). RGI has been shown to bind specifically to cellulose (Zykwinska et al. 2005) and the dependence of physical

properties of the CW on RGI side chains (Jones et al. 2005 and Ulvskov et al. 2005) may indicate an important structural role of this pectic polymer in the CW. The only report of possible RGI in the CGA was in *Mougeotia* sp. (UTEX #758). Hotchkiss et al. (1989) found a 3-linked galactan, 4-Ara, 2,4-Ara, and 3-O-methyl-6-deoxyhexoses in the hot water soluble fraction indicating the possible existence of RGI.

Rhamnogalacturonan II (RGII) is a very complex modified HG with at least 12 different glycosyl residues, including some rare substituents, such as apiose, aceric acid, 2-O-methyl Fuc, 2-O-methyl Xyl, 3-deoxy-D-manno-2-octulosonic acid (KDO), and 3-deoxy-D-lyxo-2-heptulosaric acid (DHA) (Carpita and McCann 2000). This structure is highly conserved throughout the land plants including the pteridophytes, lycophytes, and flowering plants (Carpita and McCann 2000) but it is not widely found in the bryophtes, suggesting that RG-II appeared early in land plant CW evolution (Matsunaga et al. 2004). Ryden et al. (2003) demonstrated the importance of RG-II structure, which can form borate ester cross-links (Matoh et al. 1996) and xyloglucans crosslinking in wall strength. 3-*O*-methylrhamnose has been detected in the CWs of charophytes (*Klebsormidium flaccidum* and *Coleochaete scuata*) bryophytes and homosporous lycopodiophytes (Popper and Fry 2003), in the pteridophyte *Osmunda* (Akiyama et al. 1988) and as a component of an acidic polysaccharide in the green alga *Chlorella* (Ogawa et al. 1997)

Beta- (1,3) glucans

Beta- (1,3) glucans are widely distributed among different lineages, such as the red algae, green algae, and embryophytes and in the chromalveolates, some alveolates, and in some chromistans and are not considered pectins, or crosslinking glycans (Bacic et

al 2009). The simplest (1→3)-β-glucans are linear, unbranched chains as found in callose, curdlan, paramylon and pachyman. The structure of callose is a linear (1→3)-β-glucan (Figure 1.5), found in the CWs of yeast, some filamentous fungi and some bacteria cells (Stone and Clark 1992; Kauss 1996). In higher plants, callose typically produced during specific developmental events such plasmodesmata development, cell plate formation and wound responses. Callose deposits are formed during normal growth and in response to wounding and are widespread in CWs of higher plants (Stone and Clarke 1992). (1,3)-β-glucan synthesis believed to occur at the plasma membrane is catalyzed by C-2 members of the glucan synthase-like (*GSL*) gene family (Brownfield et al. 2007), though callose synthesis also occurs in some cases of cellulose synthesis disruption (Delmer 1999)

In land plants and multicellular chlorophycean and charophycean green algae, (1, 3)-β-glucan appears during cytokinesis (Scherp et al. 2001). Scherp et al. (2001) discovered the presence of (1,3)-β-glucan by antibody labeling of wound-induced callose in several charophyte species including *Chlorokybus atmophyticus*, *Mesostigma viride*, *Klebsormidium flaccidum*, *Spirogyra* sp., *Coleochaete scutata*, *Coleochaete nitellarum*, and *Chara vulgaris*. In addition to wound-induced callose formation, *Klebsormidium flaccidum* and *Spirogyra* sp. show an indication of callose in the plane of cell division and cell plate during cytokinesis. In *Coeochaete scutata*, *Coleochaete nitellarum*, and *Chara vulgaris* juvenile CW contain callose but the polymer is no longer detectable once cytokinesis is complete. This also occurs in the meristematic cells of of the bryophytes *Riella*, *Marchantia*, and *Funaria* (Scherp et al. 2001). Preliminary evidence indicates of

callose was detected with antibody labeling in *Pleurotaenium* sp., *Micraceterias* sp., *Cosmarium* sp., *Klebsormidium* sp. and *Chlorokybus* sp (unpublished Willats COMPP data).

β-(1,3)-glucan

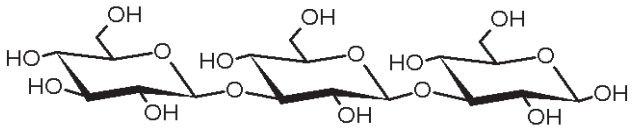


Figure 1.5 Diagrams of polymer structure of β-(1,3)-glucan. Polymers were drawn with ChemSketch

Cell Wall Proteins.

The CW also contains a diverse array of proteins, one major group are hydroxyproline-rich glycoproteins (HRGPs) which encompass the well-studied arabinogalactan proteins (AGPs) and extensions (Seifert and Roberts 2007). HRGPs are highly glycosylated and are derived from several large multigene families. AGP and extension are found throughout all land plant groups, CGAs and other non-charophyte green algae (e.g. from chlamydomonad flagellates). Their functions are both diverse and numerous in plants and include CW expansion, embryogenesis, adhesion, defense, reproduction (e.g. pollen and style development and function) and programmed cell death (Showalter 2001, 1993; Cassab 1998; Tan et al. 2004; Bucher et al. 2002). There are two types of arabinogalactan(AG) structures, type I AGs are associated only with pectins and

are composed of (1→4)β-D-galactan chains with mostly t-Ara units at the O-3 of the Gal units. Type II AGs constitute a broad group of short (1→3) and (1→6) β-D-galactans chains connected to each other by (1→3, 1→6)-linked branch point residues. The backbone of Type II AGs (AGP) is linked to arabinose with smaller amounts glucuronic acid, galacuronic acid, manose, and rhamnose. The size varies from 30-150 sugar residues although 90-99% of the molecular mass of the AGP may be carbohydrateand are associated with AGPs. AGPs have been shown to have a role in apical cell expansion in protonema of *Physcomitrella* (Lee et al. 2005). Recent evidences suggest that AGP in the CGA functions in adhesion and in cell development. Lutz-Meindl and Brosch-Salomon (2000) showed that primary CW at all stages of a non-growing semi-cell of *Micracterias denticulata* labeled with the monoclonal antibody JIM 1 (for AGP epitopes). *Cosmarium* sp., *Pleurotanium* sp., *Spirogyra* sp., and *Netrium* sp. all labels with LM1, the monoclonal antibody for extension eptitopes (Willats COMPP unpublished).

Conclusions

A comprehensive knowledge of CW structure and function throughout the plant kingdom is essential to understanding CW evolution. The overall goal of this project was to examine the composition of the charophycean cell wall and associated extracellular polymers; this goal includes identifying the polymers of the CGA ECM, determining the organization and the details of the biochemistry and characterizing the CW synthesis machinery. Different species from along the whole lineage of the CGA from the basal

spcies to the more advanced morphoforms were examined (Table 1.1). A variety of methods were utilized to examine the CGA ECM, including cytochemical screening with monoclonal antibodies, lectins, analytical methods (including linkage, neutral sugar, colorimetric, gravimetric and NMR analyses), and enzyme susceptibility to examine the extracellular matrices . The similarities and differences that exist between plant and algal cell walls need to be further examined, and through an understanding of the charophyte extracellular matrices, an understanding of the evolution of the land plant cell wall and the unique characteristics that allow for life in aquatic habitats will be achieved.

Table 1.1. This study incorporates different species from along the whole lineage of the CGA, from the basal species through the more advanced morphoforms. Five main species were examined (starred *)

Order of the CGA	Species Examined
Charales	*Chara corallina**
Coleochaetales	
Zygnematales	*Penium margaritaceum**
Zygnematales	*Penium cylindris*
Zygnematales	*Penium spirostriolatum*
Zygnematales	*Cosmarium turpini**
Zygnematales	*Cosmarium sp. 1*
Zygnematales	*Cosmarium sp. 2*
Zygnematales	*Micraceterias radiata*
Zygnematales	*Pleurotaenium trabecula*
Zygnematales	*Tetmemorus brebissonii*
Zygnematales	*Netrium digitus*
Zygnematales	*Netrium oblongum*
Zygnematales	*Netrium interruptum*
Zygnematales	*Spirogyra sp.*
Klebsormidiales	*Klebsormidium flaccidum**
Chlorokybales	*Chlorokybus atmophyticus**
Mesostigmales	--

Objectives

Objectives of this work

To my knowledge, no previous study has attempted a comprehensive approach to understanding the complex fundamentals of cell wall composition of the CGA. My goal was to understand the composition and structure of the extracellular matrix of charophycean green algae such as *Penium margaritaceum*, *Cosmarium turpini*, *Chara corallina*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum*.

Specific objectives included:

1) isolation and characterization of the cellulosic and hemicellulosic fractions of *Chara corallina, Penium margaritaceum*, *Cosmarium turpini*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum*;

2) isolation and characterization of the pectin fractions of *Penium margaritaceum*, *Cosmarium turpini*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum*;

3) isolation and characterization of the physiological role of arabinogalactan protein in the extracellular matrix of *Chlorokybus atmophyticus* and similarly to understand the unique algal polymer in *Klebsormidium flaccidum* that is known to be arabinogalactan protein-like;

4) determining the relationship between the above structural and distribution information to the evolution of the land plants, and;

5) isolation and characterization of the extracellular polymeric substances (EPS) of a subgroup of the CGA, the desmids (including *Penium cylindris*, *Penium spirostriolatum*, *Cosmarium sp. 1*, *Cosmarium sp. 2*, *Pleurotaenium trabecula*, *Tetmemorus brebissonii*, *Netrium digitus*, *Netrium oblongum*, *Netrium interruptum* and *Netrium interruptum 2509*) and. additionally, to understand how the composition of the EPS varies in a given individual as changes occur in its environment.

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Chapter 2: Implications for polymer evolution of beta-glucans in the charophycean green algae

Abstract

The aim of this work was to obtain further insight into the presence, distributions and amounts of simple β-glucans (cellulose and (1,3)-β-glucans) in the CGA and implications for evolution of land plants by examining the sister clades in the CGA. Cells walls of the charophycean green alage were investigated by means of biochemical, cytochemical, and gravimetric analyses. The cellulose content from 6% to 43% (w/w), the higher range comparable to the primary cell wall of land plants (20-30%). When the Updegraff residues were examined, the cell walls were clean shells and free of cytoplasmic contaminants. Glycosyl substitution analysis of the insoluble residues revealed the majority of residues to be 4-linked glucosyl. (1,3)-β-glucans were found localized in the hemicellulosic portion of fractionated cell walls with abundance. The cells walls of CGA were digested with endo-(1→3) β-glucanase which hydrolyses (1,3)-β-glucans. The oligosaccharides produced were analyzed by oligosaccharide mass profiling, extending the application of oligosaccharide mass profiling (OLIMP) to another polymer.

Keywords: charophycean green algae, cellulose, callose, β-(1, 3) glucan, charophytes, evolution, cell walls, β-glucan, endo-(1→3) β-glucanase.

Introduction

Beta-glucans are widely distributed among different plant lineages. They are found in the red algae, green algae, and embryophytes and in the chromalveolates, some alveolates, and in some chromistans (Bacic et al 2009). The roles they play are as diverse as the polymers they form; types of β-glucans include: crystalline (1,4)-β-glucan (cellulose), linear (1,3) β-glucan, branch-on-branch (1,3;1,6)- β-glucan, linear (1,3;1,4)-β-glucan, cyclic (1,3;1,6)- β-glucan, and branched (1,3;1,2)- β-glucan, just to include the major products. The molecular mass, solubility, viscosity, and function are similarily diverse.

The most abundant CW polymer is cellulose, an unbranched polymer joined by β-(1,4) glucosyl linkages (Fry 2000; Buchanan 2000), which constitutes 20-30% (w/w) of the primary CW of land plants and up to 90% of the secondary CW. It is also tightly associated with cross-linking glycans. The configuration of cellulose synthesizing terminal complexes (TCs) in the plasma membrane is related to the cross-sectional profile of the resultant cellulosic microfibrils (Tsekos 1999). The first visual confirmation of the terminal complex was in 1975 by Brown and Montezinos who described a linear terminal synthesizing complex associated with the terminus of cellulose microfibrils in the bacteria. Terminal complex structures in algae range from rosettes (plant-like) to linear forms (bacterium-like). In cellulose-walled algae and land plants, cellulose microfibrils are synthesized at the plasma membrane by arrays of membrane particles (Kiermayer and Sleytr 1979; Hotchkiss and Brown 1987; Gidding and Staehelin 1991; Okuda and Brown

1992). The TCs of the CGA examined so far appear to be a plant-like rosette structure in *Mougeotia* sp (Hotchkiss et al. 1989) and *Mesotaenium caldariorum* (Roberts et al. 2002). The cellulose synthase (*CesA*) gene sequences reveal important evolutionary relationships between CESA sequences, terminal complex (TC) configuration and microfibril structure (Roberts et al. 2002; Roberts and Roberts 2007; Tsekos1999). The *CesA* genes from the algae *Mesotaenium caldariorum*, a member of the order Zygnematales, which has rosette terminal complexes, are remarkably similar to seed plant *CesA*s, with deduced amino acid sequence identities of up to 59%. (Roberts et al. 2002).

The simplest form of (1, 3)-β-glucans are linear, unbranched chains as found in callose, curdlan, paramylon and pachyman. The structure of callose is a linear (1, 3)-β-glucan, and is found in the CWs of yeast, some filamentous fungi and some bacteria cells (Stone and Clark 1992; Kauss 1996). The (1, 3)-β-glucans have a variety of conformation and physical properties, curdlan is a linear unbranched (1, 3)-β -glucan which may be 12,000 glucose units in length and isfound as a capsular polysaccharide of Gram-negative bacteria and Gram-positive species. Paramylon is a high molecular mass, insoluble, linear (1, 3) -β -glucan occurring naturally in a highly crystalline form, occurs in membrane-bound granules in the cytoplasm of euglenid protozoans. Pachyman is insoluble linear (1, 3) -β -glucan isolated from the sclerotia of basidiomycete fungus *Poria cocus*. Callose is a linear (1, 3) -β -glucan typically produced during specific developmental events such plasmodesmata development, cell plate formation and wound responses (*reviewed in* Bacic et al. 2009). Deposits of callose are widespread in the CWs

of higher plants and are formed during normal growth and also in response to wounding (Stone and Clarke 1992).

(1, 3)-β-glucan synthesis is believed to be catalyzed by C-2 members of the glucan synthase-like (GSL) gene family (Brownfield et al. 2007) and biosynthesis is widely believed to occur at the plasma membrane. In land plants and multicellular chlorophycean and charophycean green algae (1, 3)-β-glucan appears during cytokinesis (Scherp et al. 2001). The genes encoding (1, 3)-β-glucan synthesis are in two distinct glycosyl transferase (GT) families. The first, GT2, is found in gram-negative bacteria and is an ancient gene family. It occurs throughout the eubacteria, the form in the eubacterium of (1, 3) β-glucan is the linear curdlan, cyclic, and with 2-linked side chains. The other GT family, GT48, is found in angiosperms, fungi and yeast.

The charophycean green algae (or charophytes) (Streptophyta, Viridiplantae) are a diverse group of green algae which include about 65 genera and a few thousand species. Six groups are presently recognized in the CGA: Mesostigmales, Chlorokybales, Klebsormidiales, Zygnematales, Charales and Coleochaetales. These algae are a small group of freshwater algae from which the embryophytes evolved (McCourt et al. 2000, 2004; Gontcharov et al. 2003, 2004; Mattox and Stewart 1984; Graham et al. 1991). CGA exhibit characteristics that distinguish them as the ancestral lineage to land plants (Mattox and Stewart, 1984). For example, CGA possess CWs whose inclusive polymers bear significant resemblance to that of land plant CWs. CWs are composed of a relatively small number of basic building blocks and biochemical analyses have revealed that all plant CWs share several common components, including the cellulose microfibrils that

form the mechanical framework of the CW and a matrix phase that forms cross-linking among the microfibrils (McCann and Roberts 1991; Carpita and Gibeaut 1993; O'Neill and York 2003; Somerville et al 2004; Sarkar et al 2009).

The aim of this work was to obtain further insight into the presence, distributions and amounts of simple β-glucans (cellulose and (1, 3)-β-glucans) in the evolution of land plants by examining representatives of the CGA.

Methods

Organism and growth conditions. The organisms examined in this study are diverse and from throughout the CGA (Streptophyta) line including the orders Chlorokybales (*Chlorokybus atmophyticus)*, Klebsormidiales (*Klebsormidium flaccidum)*, Zygnematales (*Cosmarium turpini*, *Penium margaritaceum*, *Pleurotaenium trabecula*, *Micrasterias* sp., and *Netrium digitus*), and Charales (*Chara corallina). Chara corallina, Cosmarium 2turpini, Pleurotaenium trabecula, Micrasterias sp, Netrium digitus* and *Penium margaritaceum* were collected from a freshwater wetland in Porter Corners, NY (USA) and subsequently cultured in greenhouse facility of Skidmore College. *Chlorokybus atmophyticus* (LB2591) and *Klebsormidium flaccidum* (LB321) were obtained from the University of Texas Culture Collection (Austin, TX, USA). Both cultures were made axenic by treatment in a commercial anti-biotic/anti-mycotic cocktail (Sigma Chemical, St. Louis, MO, USA) using the manufacturer's instructions. In brief, cells were treated for 24 h in the antibiotic solution, washed repeatedly in sterile Woods Hole Medium (WHM) (Nichols 1973) and subsequently cultured in the same media. After 5 days growth, aliquots of cells and extracts were removed and plated on a bacterial

assay agar (Sigma) and viewed microscopically in order to determine if cultures were axenic (Domozych et al. 2005).

Defatting of the cell walls. The *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* cells were ground under liquid nitrogen (LN_2) and extracted with methanolic KOH (MeKOH) (Aronson and Lin 1978). Briefly, ground cells were treated with 5% KOH in 80% MEOH (KOH-methanol) at 98°C for 15 minutes, centrifuged and the pellet washed sequentially with hot KOH-methanol, dH_2O, 1N acetic acid, and dH_2O several times. This procedure was repeated until the pellet was white and then the pellet was dialyzed against dH_2O for 48 hours and then lyophilized; this fraction was the methanolic KOH insoluble residue (MKIR). For *Cosmarium turpini*, *Penium margaritaceum*, *Pleurotaenium trabecula*, *Micrasterias* sp., *Netrium digitus* and *Chara corallina,* cells were isolated at described by Fry (2000). Briefly, the cells were ground under LN_2, placed in (CCC) acetone at 21°C for 16h. Then the pellet was extracted with boiling 80% ethanol for 30 minutes and washed with 80% ethanol and then 95% ethanol, dialyzed against dH_2O for 48 hours and then freeze-dried; the resulting pellet was termed alcohol insoluble residues (AIR).

Cellulose content. Cellulose content was determined by the gravimetric method of Updegraff (1969). The defatted samples were ground in a mortar and pestle to a fine powder, lyophilized and weighed. The pellet was boiled with intermittent stirring in acetic-nitric acid-water (8:1:2, v:v:v) for 1hr, washed with dH_2O until neutral, lyophilised and re-weighed, yielding percent cellulose.

Fractionation of cell walls. Defatted CWs were fractionated using the methods of Fry (2000). MKIR/AIR were successively extracted with the following process: (i) dH_2O at 95°C for 1h followed by centrifugation at 700 x g for 10 min, and supernatant collected (repeated 3x) (HW-soluble fraction); (ii) 50mM CDTA (21°C , pH 7.5) for 6h, centrifuged as above, washed with dH_2O (3x) and supernatants collected, and dialyzed against 0.5M imidazole for 12h (CDTA soluble fraction); (iii) 50mM Na_2CO_3 containing 20mM $NaBH_4$ (1°C) for 16h, centrifuged as above, washed with dH_2O (3x), and supernatants collected (Na_2CO_3 soluble fraction); (iv) 1M KOH containing 20mM $NaBH_4$ for 2h at 1°C, centrifuged as above (repeated 2x), washed with dH_2O (3x), supernatants collected (1M KOH soluble fraction); (v) 4M KOH containing 20mM $NaBH_4$ for 2h at 1°C, centrifuged as above (repeated 2x), washed with dH_2O (3x), supernatants collected (4M KOH soluble fraction). The remaining CW remnant was washed with 0.5M imidazole (1x) and dH_2O (4x) and lyophilized. The supernatants from each stage were neutralized with acetic acid where appropriate, dialyzed against dH_2O exhaustively at 4°C and lyophilized.

Neutral sugar analysis. Neutral sugar analysis was performed by the methods described in Wustman et al. (1997). Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°C for 3 h, treated with 1 M NH_4OH for 2 h, reduced with $NaBH_4$ and acetylated as in Harris (1984) and Wustman et al. (1997). Gas chromatography/mass spectrometry (GC/MS) was performed using a SP-2330 column (30m, 0.25 mm i.d., isothermal at 230°C, injector 240°C) on a Finnigan-MAT Magnum mass spectrometer (San Jose, CA) operated in the electron-impact mode and alditol acetates were identified

by their mass spectra and relative retention times to a *myo*-inositol internal standard. Quantification was based on response factors of standard monosaccharides that had been subjected to the same procedure.

Linkage analysis. Fractions containing uronosyl residues were treated with Dowex 50W-X12 (H+ form) prior to per-O-methylation (Waeghe et al., 1983) and reduced with 1M lithium triethylborodeuteride in tetrahydrofuran for 1.5 h at 22°C following per-O-methylation (York et al., 1985). Per *O*-methylation of the polymers was carried out with butyllithium in DMSO and iodomethane (Stevenson and Furneaux 1991). Sep Pac C-18 reverse phase chromatography was used to purify the methylated polymers (Waeghe et al. 1983). Methylated polymers were hydrolyzed with 2M TFA at 121^{o}C for 1.5 hrs and reduced with $NaBD_4$ in ethanolic ammonia. The per-*O*-methylated alditols were acetylated as in Harris et al. (1984) and separated on a SP-2330 column (30 m, 0.25 mm i.d., 150-245°C at 4^{o}C/min, held at 245°C for 20 min, injector 240°C). Mass spectra were obtained by GC/MS (Finnegan-MAT) operated in the electron-impact mode and used to detect column effluent. Linkage patterns were assigned based on previously determined diagnostic mass ion fragments and relative retention times (Jansson et al. 1976, Waeghe et al. 1983, Wustman et al. 1997, 1998) and compared to a library created by injection of standards of incompletely methylated alditol acetate derivates of each sugar. Darvill et al. (1980) glycosyl linkage/ substitution nomenclature was used. Quantification of per-*O* methylated alditol acetates was based on effective carbon response factors, as described in Sweet et al. (1975).

Enzymatic digestion. Pretreatment prior to enzymatic digestions was done by

swelling MKIR (*Klebsormidium flaccidum* and *Chlorokybus atmophyticus*) or AIR (*Cosmarium turpini*, *Penium margaritaceum*, *Pleurotaenium trabecula*, *Micrasterias* sp., *Netrium digitus* and *Chara corallina*) insoluble material with 1M KOH containing 20mM $NaBH_4$ for 16 hours at 4°C. The swollen material was then neutralized with concentrated acetic acid and EtOH was added to a final concentration of 75% (Sorensen et al. 2008). Following centrifugation at 500 x g for 10 minutes, the precipitate was washed with 80% ethanol (2x), then dH_2O (2x), and finally with sodium phosphate buffer (pH 6.5) (3x). Approximately 10mg swelled MKIR / AIR walls were treated with ten units of endo-1, 3-ß-D-Glucanase (*Trichoderma* sp.) EC 3.2.1.39 from Megazyme (Cat # E-LAMSE) in phosphate buffer (0.2M) pH 6.5 and incubated at 60°C for 16 hours. Ethanol was added to stop the reaction and precipitate polymers, samples were chilled to 4°C for 1 hour, centrifuged at 500g for 10 minutes, and the supernatant dried under filtered air. A portion of the supernatant was isolated for linkage analysis and a portion was used for OLIMP. Parallel treatments were performed on the standard linear (1, 3)-β-glucan Pachyman, which is derived from the sclerotia of the basidiomycete fungus *Poria cocus* (Warsi and Whelan 1957, Saito et al. 1968).

Oligosaccharide characterization. Oligosaccharide Mass Profiling (OLIMP) was performed as described in Lerouxel et al. (2002), Obel et al. (2006) and Leboeuf et al. (2008). The oligosaccharides were dissolved in ddH_2O and desalted to remove buffer salts (Bio-Rex MSZ 501(D) resin Bio-Rad) (Obel et al. 2006). The oligosaccharide solution was spotted onto a MALDI-TOF sample plate containing vacuum-dried 2, 5-dihydroxybenzoic acid (10 mg per well) and crystallized under vacuum. Samples were

analyzed on a Voyager DE-Pro MALDI-TOF-MS instrument (Applied Biosystems) in positive reflectron mode with an acceleration voltage of 20 kV and an extraction delay time of 350 ns. The peak intensities were used to calculate the relative abundance of each ion peak and to perform pair-wise comparisons (Pauly et al. 2009).

Monoclonal antibody labeling. The CW fractions were analyzed by immuno-dot assay (using the methods of Willats et al. 2001). Briefly, a 5-fold serial dilution (in dH_2O) was prepared from the fractions and applied to nitrocellulose as 1 µl aliquots. The membranes were air-dried for approximately 16h at 21^oC. The membranes were treated 5%M/PBS for 1h, then 1.5h in primary antibody solution (BS-400-2 BioSupplies (Melbourne, Australia)). The antibody BS-400-2 was diluted 1/10 in 5% M/PBS. The nitrocellulose membranes were washed in PBS extensively prior to the incubation for 1.5h in secondary antibody solution. The secondary antibody was an anti-mouse horseradish peroxidase conjugate (Sigma), diluted 1/1000 in 5% M/PBS and washed again extensively in dH_2O and PBS. Development was in the substrate solution (25ml dH_2O, 5ml methanol containing 10mg/ml 4-chloro-l-napthol, 30ul 6% (w/w) H_2O_2).

The monoclonal antibody BS-400-2 for (1, 3) β-D-glucan was obtained from BioSupplies (Melbourne, Australia). Cells were prepared as in Domozych et al.(2005, 2007a). Cell pellets were fixed at RT in 1% paraformaldehyde in WHM for 20 min, washed 3X with WHM and 3X with phosphate-buffered saline (PBS; pH 7.2) and subsequently blocked for 30 min in 2% BSA in PBST (PBS plus 0.1% Tween-20). The cells were washed 3X with PBST and incubated for 2h at RT in PBST. Cells were gently mixed throughout the treatment period. The cells were washed three times in PBST,

incubated in block solution for 30 min and washed again 3X in PBST. The cells were incubated in a 1/100 dilution (in PBST) of anti-mouse antibody conjugated with TRITC (Sigma Chemical) for 2 h at RT in the dark. The cells were then washed and used for subsequent microscopic observation. The controls for this study included eliminating the primary antibody incubation. CW were analyzed using standard fluorescence microcopy, an Olympus BX-60 light microscope (LM) was used for general and fluorescence microscopy and images were captured using an Olympus DP70 camera.

Results

Cellulose content. The Updegraff method was used to determine the percent cellulose in the following species: *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, *Netrium oblongum*, *Micrasterias* sp., *Pleurotaenium trabecula*, *Penium margaritaceum*, *Cosmarium turpini*, and *Chara corallina* (Figure 2.1). The cellulose content ranged from 6% to 43% in *Cosmarium turpini* (Figure 2.1). When the Updegraff residues were examined, the CW "ghosts" were clean shells and free of cytoplasmic contaminants (Figure 2.2a-e). The *Chara corallina* Updegraff residue (Figure 2.2a) appeared as a sheets or ribbons whereas *Micrasterias* sp. residue (Figure 2.2b) resembled the flattened semicells with protruding star projections of the native alga. *Pleurotaenium trabecula* and *Netrium digitus* CWs were intact, whereas the CW of *Chlorokybus atmophyticus* waswere distorted and shrunken.

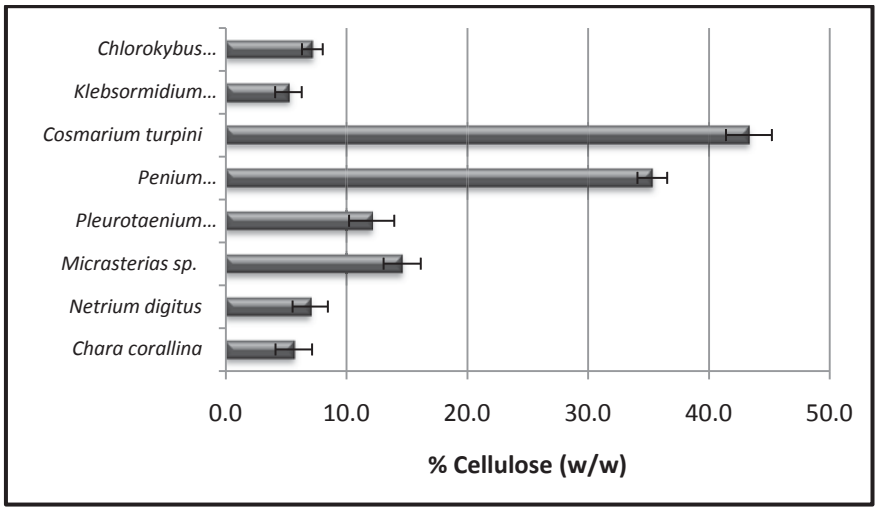

Figure 2.1: Cellulose content determined by the gravimetric Updegraff methods for the CWs of the CGA and presented as weight percent. The Updegraff procedure was performed on the defatted walls: MKIR (*Klebsormidium flaccidum* and *Chlorokybus atmophyticus*) and AIR (*Chara corallina, Netrium digitus, Penium margaritaceum, Pleurotaenium trabecula, Micrasterias* sp. and *Cosmarium turpini*)

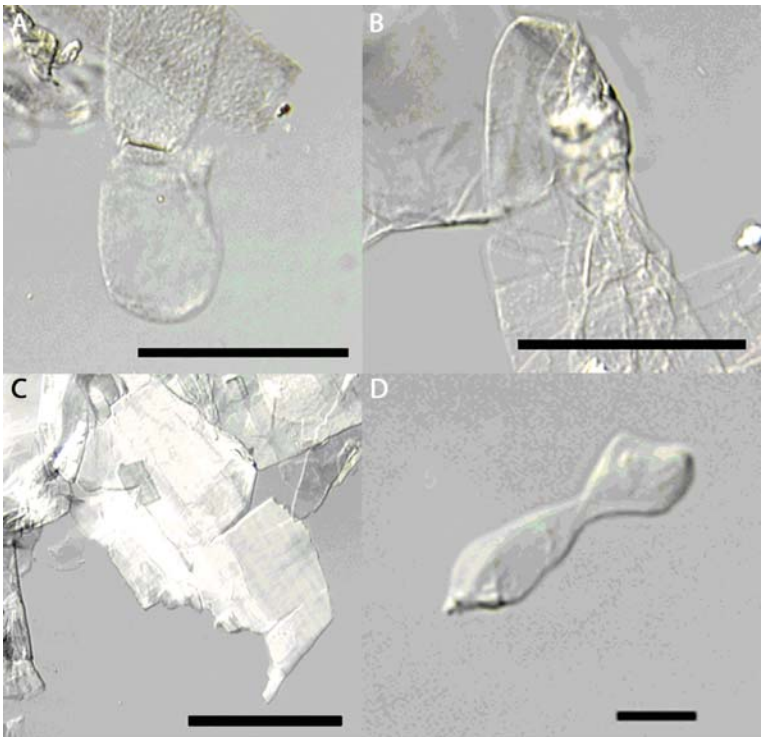

Figure 2.2: DIC images of Updegraff residues A) *Pleurotaenium* sp the scale bar =100um B) *Netrium digitus* the scale bar =100um. C) *Chara corallina* the scale bar =100um D) *Chlorokybus atmophyticus* the scale bar =10um

Chemical composition of the insoluble residue. The chemical composition of insoluble residue (IR) of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, *Penium margaritaceum*, *Cosmarium turpini*, and *Chara corallina* contained glucose as the major sugar. Glycosyl substitution analysis of the insoluble residues revealed the majority of residues to be 4-linked glucosyl (Table 2.1). When considering the ratio of 4-linked glucosyl residues to terminal residues, *Chara corallina* contained the lowest amount of terminal residues with a ratio of 100:1.8, followed by *K. flaccidum* (100:2.8), *Cosmarium turpini* (100:4.0), *and Chlorokybus atmophyticus* (100:5.9) and, with the largest amount of terminal residues, *P. margaritaceum* with 100:21.7. Other common residues in all of the CGA insoluble residues examined include: terminal-glucosyl and terminal-xylsoyl residues.

Penium margaritaceum insoluble residue contained 74.4% glucose with 4-linked, terminal, 4, 6-linked, and 2, 3-linked residues (Table 2.1). The other neutral sugar present in the insoluble residue was galactose with 23%, the major glycosyl residue being 6-linked also consisting of 3-linked, 4, 6-linked, 4-linked, 3, 6-linked, 2, 6-linked and terminal galactosyl residues. *Cosmarium turpini* IR also consisted of a significant amount of galactose (19%) in the form of 4, 6-linked galactosyl residues. The main neutral sugar component of the *C. turpini* IR fraction was glucose (73%), the major glucosyl residue was 4-linked, in addition terminal, 3-linked, and 3, 6-linked residues are present.

In *Chara corallina* the major neutral sugar present in the IR fraction was glucose (66%), with the majority 4-linked, 4, 6-linked, 2, 3-linked and terminal residues (Table

2.1). The other neutral sugars present include mannose and xylose. *Chlorokybus atmophyticus* IR fractions contained 98% glucose with 4-linked, 3-linked, 3,6-linked, and terminal glucosyl residues The insoluble residue of *Klebsormidium flaccidum* also contained mostly glucose with 4-linked, 2,3-linked, and terminal residues (Table 2.1)

Table 2.1 Glycosyl substitution patterns of post alkali extraction insoluble residue. Glycosyl linkages reported as % mole detected.

		C atmophyticus Insol. Residue	*K flaccidium* Insol. Residue	*P margaritaceum* Insol. Residue	*C turpini* Insol. Residue	*C coralina* Insol. Residue
Glc	4-	35.2	47.6	19.0	70.8	75.4
	t-	2.0	1.3		2.8	1.4
	3-	2.3			0.4	
	2,3-		2.6	1.7		2.9
	3,6-	2.1			0.9	
	4,6-			2.7		9.0
	2,4,6-			0.9		
Gal						
	t-			0.5		
	3-		2.5	4.5		
	4-			1.7		
	6-			9.5		2.9
	2,3-					
	2,4-	1.1	1.1	9.9	1.4	
	2,6-			0.5		
	3,6-		0.5	3.9	1.7	
	4,6-			0.6		
	3,4,6-		2.3	0.4	9.0	1.7
Man						
	4-	1.6				
	6-	0.6	0.4		1.6	
	2,3-	0.4	0.7		2.6	
Xyl						
	t_p-	0.1	0.7	0.3	1.6	
	3_p-			1.3		
	4_p-	0.1	1.5		0.7	
	$2,4_p$-			0.4	1.0	
	$2,3,4_p$-	40.9	19.4			
Ara						
	t_f-	0.5	1.8			
	2_f-	0.3				
	4_p-	0.4				
	$3,4_p$-		4.2			
	$2,3,4_p$-	11.0	3.5		1.1	
Fuc						
	t-			6.5		
	3-			4.4		
	3,4-					1.8
	2,3,4-				2.8	5.0
Rha						
	t-	0.6		9.2		
	2-			5.7		
	4-			4.1		
	2,3-			0.7		
	2,4-			11.4		
	2,3,4-	0.6	9.7		1.5	

Characterization of (1, 3) β-glucan in the cell wall. Beta (1, 3) glucan was widespread in the CGA including: *Chara corallina*, *Cosmarium turpini*, *Penium margaritaceum*, *Netrium digitus*, *Klebsormidium flaccidum*, and *Chlorokybus atmophyticus*. To determine presence of (1, 3) β-glucan in the CGA, oligosaccharides produced by endo-1, 3-β-Glucanase (*Trichoderma* sp.) digestion were analyzed with MALDI-TOF MS. The products indicated the presence of (1,3)-β-glucans in the cell walls. As a control, a parallel analysis was also performed on the (1, 3)-β-glucan pachyman by enzyme digestion (Table 2.2). Pachyman is a linear β-(1, 3)-d-glucan isolated from *Poria cocos* mycelia). The results indicate that most of the CGA species have a (1, 3) β-glucan similar to that of pachyman with most of the oligosaccharides being cellolaminarose. *Chlorokybus atmophyticus* is the only species in this examination that contained more trisaccharides and tetrasacharides than cellobiose. When the CW remnants from the *endo*-1, 3-ß-D-glucanase (*Bacillus* sp.EC 3.2.1.73) treatment were examined (Figure 2.3), the residual shells were cleaned and appeared as whole CW. The enzyme did not completely degrade the whole CW.

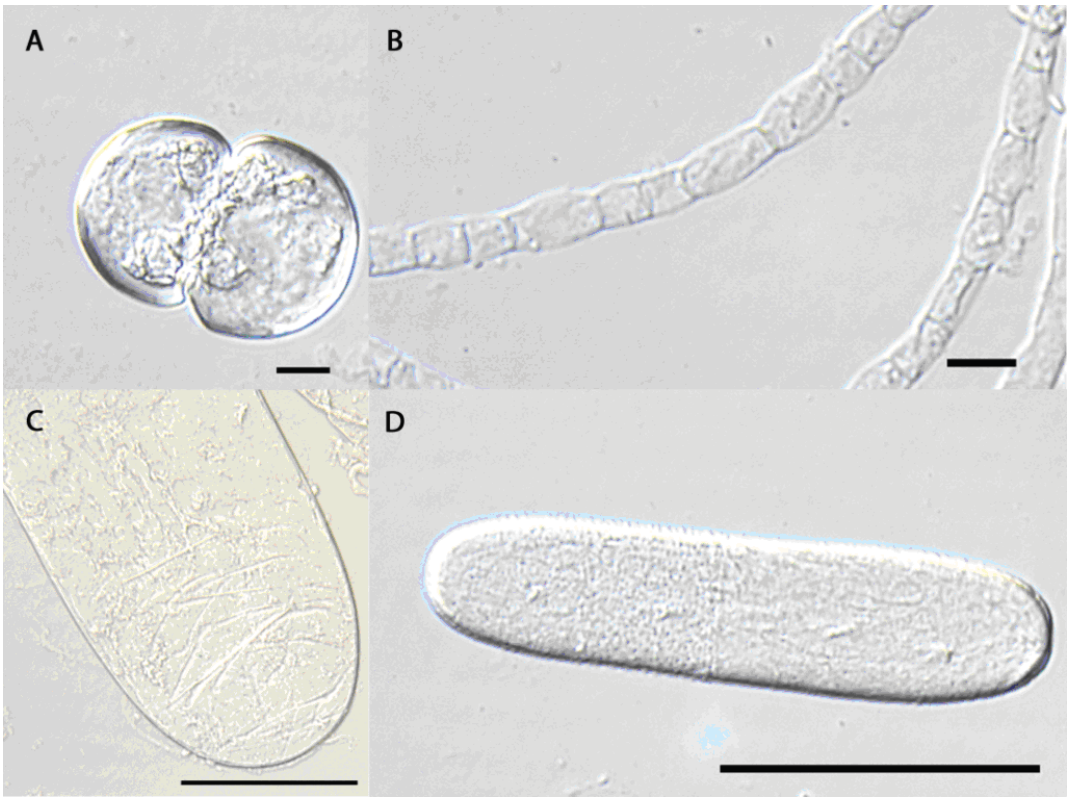

Figure 2.3: Wall remnant post enzyme degradation with *endo*-1,3-ß-D-glucanase (*Bacillus* sp.)EC 3.2.1.73 products (Megazyme) A) *Cosmarium turpini* scale bar=10um B) *Klebsormidium flaccidum* scale bar=10um C) *Netrium digitus* scale bar=10um, D) *Penium margaritaceum*. Scale bar=100um

Table 2.2: Percent of oligosaccharides released from MALDI-TOF MS analysis of *endo*-1,3-ß-D-glucanase (*Bacillus* sp.)EC 3.2.1.73 products. OLIMP of MLG oligosaccharides released from MKIR (*Klebsormidium flaccidum* and *Chlorokybus atmophyticus*) and AIR (*Chara corallina, Netrium digitus, Penium margaritaceum,* and *Cosmarium turpini*) preparations digested with *endo*-1, 3-ß-D-glucanase.

Oligosaccharide units	*Ch. atmophyticus*	*K. flaccidum*	*P. margaritaceum*	*Co. turpini*	*N. digitus*	*C. corallina*
G3G	11.7	67.2	51.2	48.3	33.3	40.3
G3G3G	38.6	13.3	12.8	37.2	10.4	18.1
G3G3G3G	40.4	5.9	12.4	9.6	7.9	11.9
G3G3G3G3G	2.8	3.0	9.2	0.7	6.1	9.8
G3G3G3G3G3G	4.4	2.6	7.4	1.2	10.7	7.5
G3G3G3G3G3G3G	1.5	1.8	3.9	1.5	7.9	5.3
G3G3G3G3G3G3G3G3G	0.4	2.3	3.1	0.8	9.3	4.1
G3G3G3G3G3G3G3G3G3G	0.3	3.8	0.0	0.5	14.4	2.9

(1, 3) β-glucan localization in the cell wall. In *Chlorokybus atmophyticus* and *Klebsormidium flaccidum*, cytochemical labeling revealed the presence of (1, 3) β-glucans once the pectin fractions had been removed (Figure 2.4a-e). The HW and CDTA insoluble residues of *Chlorokybus atmophyticus* were not labeled with the anti-(1,3)- β-glucan (BS-400-2) although the post-Na_2CO_3, 1M KOH, and 4M KOH treatment residues labeled with anti-(1,3)- β-glucan. The insoluble residue after Na_2CO_3 extraction of *Chlorokybus atmophyticus* were patchy and weakly labeled (Figure 2.4a). However, the post 1M KOH treatment residue is smoothly labeled with clear bright colorations. *Klebsormidium flaccidum* post-CDTA, post-Na_2CO_3, and post-1M KOH insoluble residues labeled strongly with the antibody for (1,3)-β-glucan (Figure 2.4c-e) though not

smoothly for any of the insoluble fractions the terminal end of *K. flaccidum* appear to label clearer than the middle sections.

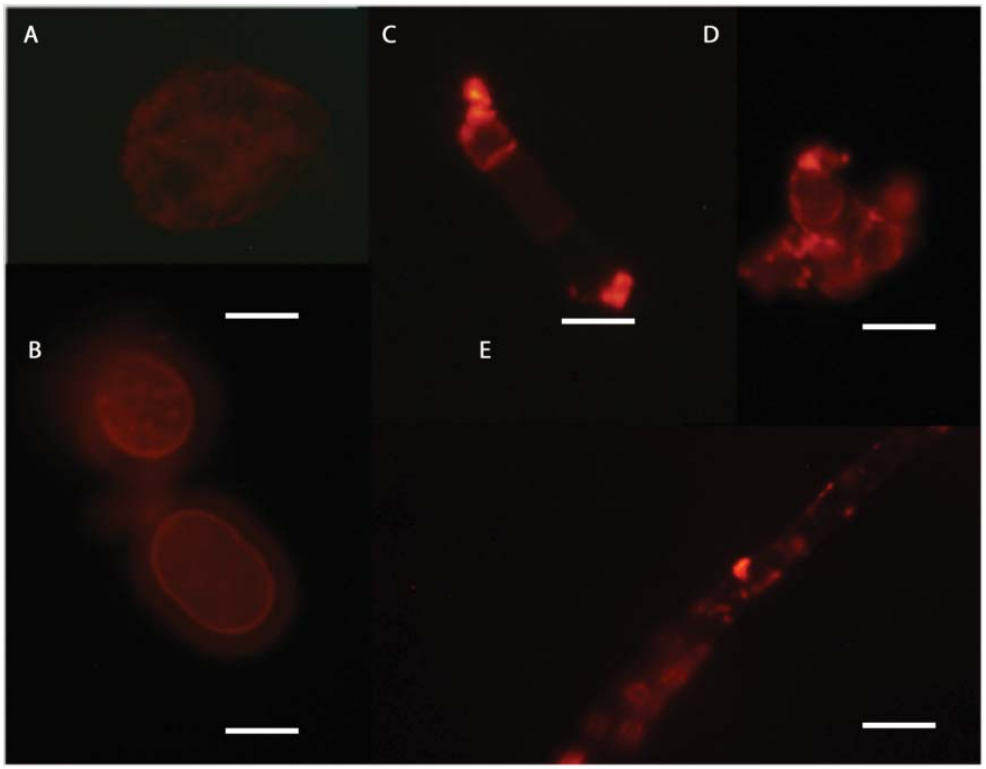

Figure 2.4: Cytochemical labeling for anti-(1, 3) β -glucans antibody (BS-400-2 Biosupplies Australia). A) Post Na_2CO_3 insoluble residue of *Chlorokybus atmophyticus* Scale bar = 15µm, B) Post 1MKOH insoluble residue of *C. atmophyticus*. Scale bar = 15µm C) Post CDTA insoluble residue of *Klebsormidium flaccidum* Scale bar = 10µm D) Post Na_2CO_3 insoluble residue of *K. flaccidum*. Scale bar = 10µm E) Post 1MKOH insoluble residue of *K. flaccidum.* Scale bar = 10µm

Discussion

Our results demonstrate the presence of cellulose and (1, 3) β-glucan in the CW of a variety of CGA, ranging from unicellular algae (*Chlorokybus atmophyticus*, *Penium margaritaceum*, and *Cosmarium turpini*), unbranched filaments (*Klebsormidium flaccidum*), and multicellular algae (*Chara corallina*). Calcofluor labeling revealed the presence of ß-glycans in *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* (data not shown).

Cellulose is not only an embryophyte-unique polymer; it is abundant throughout the tree of life, including prokaryotic organisms such as gram-positive anaerobic bacteria (*Sarcina*) and purple bacteria (*Acetobacter*, *Rhizobium*, *Alcaligenes*, *and Agrobacterium*). It also present in a variety of algal groups including chlorophytes (*Oocystic apiculata*, *Valonia*, and *Boergensenia*), phaeophytes (*Pelvetia*), chrysophytes (*Vaucheria* and *Pleurochrysis*), and rhodophytes (*Erthrocladia*). Cellulose is also in other eukaryotic organisms including the protists (*Dictoyostelium discoideum*), fungi (*Saprolegnia*, *Allomyces*, *and Achlya*) and in the tunicates (*Metandrocarpa* and *Hyalocynthia*) which are classified as animals (*Reviewed in* Brown et al. 1996).

The cellulosic structure of the CGA has homologies with vascular plant CWs. The CW of CGA (*Mougeotia* sp.) has been shown to contain cellulose and also terminal rosette complexes in the plasma membrane (Hotchkiss et al. 1989). The cellulose content in the CGA ranged from 5% to 43% cellulose (Figure 2.1). As a group, the desmids (Zygnematales) range in cellulose content from 7% to 43% with the saccoderm desmid *Netrium oblongum* containing the lower amount of 7%. The basal species of the CGA,

Chlorokybus atmophyticus and *Klebsormidium flaccidum,* both contain low amounts of cellulose in their CWs. *Mougeotia* sp. was reported to contain 13.4% cellulose in the CW (Hotchkiss et al. 1989). Compared to land plants, which usually range from 20-30% cellulose in the primary CW (Fry 2000), or unusually up to 50% (Siegel and Siegel 1973), CGA and algal cellulose content in general is very low. The cellulose content of the heterokontophytes, for example, ranges from 0.6-1.5% in *Pelvetia canaliculata* to as high as 4-10% in *Laminaria hyperborea* (Siegel and Siegel 1973). In the rhodophytes the cellulose content ranges from 1.0% (*Endocladia muricata*) - 9.0 % (*Gelidium cartilagineum*) with a mean of 5.2%. The mean of the CGA cellulose content is 16%, which is more than that found in both the heterokontophytes and rhodophytes (Siegel and Siegel 1973).

The presence of β-(1, 3)-glucan has been indicated in a multitude of charophyte species (Table 2.3). Like cellulose, (1,3) β-glucans are found throughout living organisms, such as rhodophytes, chlorophytes, embryophytes, chromalveolates, some alveolates (dinoflagellates), chrysophytes, heterokontophytes including diatoms, oomycetes, and some haptotophytes, and many fungi (*reviewed* in Harris and Stone 2009). They are also found in both gram-negative and gram-positive bacteria. The structure of (1, 3) β-glucan has evolved to accommodate a variety of functions and includes polymers such as callose and chrysolaminarin used as a storage polymer in diatoms and brown algae. In diatoms the chrysolaminarin is an intracellular storage polymer of β-1, 3-D-glucan which is a hot-water-extractable polymer (Allan et al. 1972; Myklestad 1978).

In CGA, 3-linked glucosyl residues mostly occur in the hemicellulosic fractions of the wall (see Chapter 3); in *Chlorokybus atmophyticus* the 1M KOH fraction contains 65 mole % 3-Glc (36% in the 1M KOH fraction pretreated for uronic acids) in a fraction that contains 87% glucose. The 4M KOH fraction also contains significant 3-Glc (14mole% in a fraction with 97% glucose). The 1M KOH fraction of *Klebsormidium flaccidum* is also enriched with 3-Glc with 86 % (19 % in the 1M KOH fraction pretreated for uronic acids); the 4M KOH fraction contains 14.5 % 3-Glc (9.3% in pretreated uronic acid fractions). *Cosmarium turpini* and *Chara corallina* contain lesser amounts of 3-linked glucosyl residues in the 1M KOH fractions with 10% in *Cosmarium turpini* and 7% in *Chara corallina* (Kiemle et. al. 2010; published in Chapter 3). The 3-linked glucosyl residues in the 1M KOH and 4M KOH contribute to at least two polymers including mixed linkage glucans, which are a cross-linking polysaccharide homopolymer with a (1→3), (1→4) β-glucan backbone. MLG is found in the growing cells of the primary CW, integrated with cellulose and other noncellulosic polysaccharide (Carpita 1984).In many embryophytes and charophytes, (1,3)-β-glucan is found as a polymer and is typically produced during specific developmental events such plasmodesmata development, cell plate formation and wound responses (callose) (Aidemark et al. 2009). In addition to the 3-linked glucosyl residues, the hemicellulosic fractions also contain a characteristic residue of callose with 3, 6-linked glucosyl residues. The 1M KOH fraction of 3, 6-linked glucosyl residues was found in *Chlorokybus atmophyticus* (12-14 mole %), in *Klebsormidium flaccidum* (3-6 mole %), and in *Chara corallina* (2 mole %) (Kiemle et. al. 2010; published in Chapter 3). In the

insoluble wall remnant (Table 2.1), there is evidence of 3-linked and 3, 6-linked glucosyl residues in *Chlorokybus atmophyticus* and *Penium margaritaceum*.

Callose is involved in pollen development, pollen tube growth, biotic stress response, abiotic stress response, sieve pore development and regulation, plasmodesmata regulation, cell plate formation, and functional megaspore selection (*Reviewed in* Chen and Kim 2009). The presence of the polysaccharide callose in the charophytes, lower land plants and also higher plants was first discovered as a natural component of non-damaged sieve tubes (Stone and Clarke 1992). Scherp et al. (2001) indicated the presence, by antibody labeling, of wound-induced callose in several charophyte species including *Chlorokybus atmophyticus*, *Mesostigma viride*, *Klebsormidium flaccidum*, *Spirogyra* sp, *Coleochaete scutata*, *Coleochaete nitellarum*, and *Chara vulgaris*. In addition to wound-induced callose, *Klebsormidium flaccidum* and *Spirogyra* spp. shows indication of callose formation in the plane of cell division and cell plate during cytokinesis. No callose was detected in the dividing cells of the CWs of *Coleochaete scutata*, *Coleochaete nitellarum* and *Chara vulgaris* were covered by callose with β-(1, 3)-glucan disappearing upon the completion of the cytokinesis, the same phenomenon that was observed in the meristematic cells of gametophytes of bryophytes (*Riella*, *Marchantia*, and *Funaria*) and the sporophytes of the water fern *Azola*.

In the insoluble wall remnant, in addition to the 4-linked glucosyl residues (Table 2.1), there is evidence for other land plant polymers. In *Penium margaritaceum* the insoluble residue contains possible linkages with xyloglucan (XyG). XyG is a linear polymer of (1, 4) β-glucans with branches (xylosyl (terminal and 2-linked), galactosyl

(terminal and 2-linked), and fucosyl (terminal)) at the 6- position of glucosyl residues. It is involved in CW expansion and binds to cellulose. It is present in the primary walls of dicots, gymnosperms, and noncommelinoid grasses (Carpita and McCann 2000).

In *P. margaritaceum* IR, the glucosyl residues were 4- and 4,6-linked with terminal galactosyl, xylosyl, and fucosyl residues, and 2-linked galactosyl and xylosyl residues were not present (Table 3.1). The possible presence of XyG was found in the 1M KOH fraction of *Chara corallina*, where the glucosyl residues 4- and 4,6- (42mole%) linked were abundant and, in addition, there weres both terminal and 2-linked xylosyl residues (Kiemle et al 2010; published in Chapter 3). 4, 6-linked glucosyl residues were also in the insoluble wall residue of *Chara corallina* (Table 2.1). Evidence for/against XyG in the Charales has been presented. Domozych et al. (2009) reported cytochemical labeling for XyG in all of the thallus parts of *Chara corallina*. However, the absence of XyG in the CGA was reported by Popper and Fry (2003) where XyG was found in all of the land plants.

Additionally, there was an abundance of 6-linked galactosyl residues in the IR of *C. atmophyticus*, *P. margaritaceum*, and *C. corallina*. *Penium margaritaceum* contains additional galactosyl linkages including 3-, 2,3-, 2,6- and 3,6-linked residues, which indicates the possibility of arabinogalactan protein (AGP) given that the protein core is decorated with branched glycans based on (1-3),(1-6)-ß-galactan backbones with terminal residues of Ara or GlcA (Showalter, 2001). 90-99% of the molecular mass of the AGP may be carbohydrate. Although they are very common throughout land-plant taxa, the specific functions of AGPs are poorly understood and may include roles in cell-cell

recognition, adhesion, cell development, gametogenesis and embryogenesis (Lee et al., 2005; Lamport et al., 2006; Johnson et. al., 2003; Showalter, 2001). Cytochemical labeling indicated AGP in the CWs of the desmid *Micrasterias* sp. (Eder et al. 2008) and *Pleurotaenium trabecula* (Domozych et al. 2007). The major hot water soluble neutral polysaccharide was identified as a 3-linked galactan in the charophyte *Mougetia* sp (Hotchkiss et al 1989).

The rhamnosyl residues in the insoluble wall remnant of *Penium margaritaceum* are confounding in that they are typical rhamnogalacturonan I (RGI) residues including terminal, 2-, and 2, 4-linked residues, which should be extracted in the pectin fractions with CDTA and Na_2CO_3. *P. margaritaceum* CWs shows evidence of true homogalacturonan, (1, 4) α-galacturonosyl residues (Domozych et al. 2007 published in Chapter 4). The major monomers in RGI are α-GalA, α-Rha, β-Gal, α-Ara, Fuc, and Xyl (Fry 2000). The CDTA fraction of *Penium margaritaceum* consists of GalA, Gal, Rha, Ara, Fuc, and Xyl (Domozych et al. 2007; published in Chapter 4). RGI in land plants consists of a backbone with a repeating disaccharide repeating unit of GalA-Rha, which forms a contorted rod that is more flexible than the pure HG (Marry et al. 2000). HG, RG-I and its side chains are conserved and both may be found in the same wall (Marry et al 2000, Carpita and Gibeaut 1993, Albersheim et al. 1994, Fry 2000, Lau et al. 1985). RG-I is thought to be glycosidically attached to HG domains (Willats et al 2001), which may explain the present of the rhamnosyl residues in *P. margaritaceum* CWs. Or this could be a uniquely algal polymer which localizes with cellulose, in the hemicellulose of

the green algae *Enteromorpha* sp. consists of rhamnose and xylose with minor amounts of glucose (Siegel and Siegel 1973).

Our observations support evidence which suggests that members of the Charophyceae represent the phylogenetic line that gave rise to vascular plants and that primary CW of vascular plants may have evolved directly from structures typical of the filamentous green algal CWs found in the Charophyceae. Previous workers have speculated that the transition from an aquatic environment to a terrestrial environment would have triggered physical and structural changes in the cell-wall structures (Graham 1996, Karol et al. 2001, Kenrick and Crane 1997), such that the CW structure in CGA is thought to be quite different from that of land plants. The CWs of CGA have been shown to contain both cellulose with terminal rosette complexes (Hotchkiss et al. 1989) and homogalacturonans (Domozych et al. 2007, Cherno et al. 1976), which indicates similarities in CW organization between primitive CGA and land plants. The presence of beta-glucans including cellulose and hemicellulosic 3-glucans (and possibly AGP) in a wide variety of CGA species provides support for the notion that the CWs of the CGA are highly conserved with those of land plants.

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Chapter 3: Hemicellulosic Polymers in the CGA: Unique mixed-linkage (1 → 3),(1 → 4)-β-D-glucan structure

Abstract

The fundamental understanding of the charophyte cell walls is broadening. Mixed linkage (1, 3-1, and 4) glucans (MLG), which until 2008 were purported to only be found in certain Poales, are also found in the cell walls of the charophytes. The work presented in this paper examines the MLG found in the hemicellulosic fractions of the CGA cell walls. The cells walls of CGA were digested with lichenase which hydrolyses MLG. The oligosaccharides produced were analyzed by oligosaccharide mass profiling (OLIMP). Lichenase released oligo-β-glucans from the walls of the CGA species, *Chlorokybus atmophyticus, Klebsormidium flaccidum*, *Cosmarium turpini* and *Chara corallina*. The digestion products of *Cosmarium turpini* and *Chara corallina* are similar to the digestion products of barley flour, with the major product being a tetrasaccharide (G4G4G3G-) followed by trisaccharide (G4G3G-). Whereas, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum* yielded the disaccharide laminaribiose (G3G-) as the abundant product. The hemicellulosic fractions were examined for additional higher plant hemicellulosic polymers. Preliminary evidence suggests the CGA walls may contain xylans and/or xyloglucans in the CGA. Land plants evolved from an ancestral

pool of freshwater green algae known as the charophycean green algae. CGA possess cell walls whose inclusive polymers bear significant resemblance to those of land plant cell walls.

Keywords: mixed linkage glucans, charophytes, evolution, cell wall, lichenase, β-glucans, hemicellulose, cross-linking glycans, charophycean green algae.

Introduction

Land plant cell walls (CWs) have been studied intensively and a comprehensive body of knowledge about CW composition, structure and biosynthesis has been assembled for several species (Rose 2003). The CW is a semi-crystalline aggregation of gelatinous and fibrillar polymers which form a cell covering that functions in defense, rigidity, signaling, adsorption, and turgor control. The CW provides support for the plant body structure, acts as a defensive barrier and also has roles in cell signaling, fate determination, and development (Carpita and McCann 2000). The structures of CWs are dynamic and involved in response to genetic and environmental cues.

The CW usually consists of cellulosic microfibrils tethered by cross-linking polysaccharides, which is the load-bearing network that is embedded in a pectin matrix with varying amounts and types of proteoglycans (eg. extensions and arabinogalactans proteins) (Carpita and McCann 2000; O'Neill and York 2003; Knox 2008). The cross-linking polysaccharides were originally called hemicelluloses, which a term was proposed in 1892 by Schulze to designate polysaccharides that were extractable by

aqueous alkaline solution (Fry 2000). Up to 50% of the biomass of the higher plant CW consists of cross-linking glycans this group includes a diverse array of polymers including xylans, mannans, mixed linkage β-glucan, and xyloglucans.

Cross-linking β-glucans are associated with cellulose microfibrils during plant cell growth (Buckeridge et al. 2004). Originally identified in the commeloid grasses, mixed-linkage glucan (MLG) is a cross-linking polysaccharide homopolymer with a (1, 3) (1, 4) β-glucan backbone, found in the primary wall growing cells. The polymer is hydrogen bonded with cellulose and other noncellulosic polysaccharides (Carpita 1984). The cellulose microfibrils are tethered by MLG polymers and strengthen the CW (Buckeridge et al. 2004). MLG are found in large amounts in some grasses such as *Hordeum vulgare* (barley), *Avena sativa* (Oat), and *Secale cereale*. Long believed to be restricted to the commeloid grass, recent studies have also shown that this polymer is present in *Equisetum* species suggesting a more ancient origin (Fry et al. 2008b; Sorensen et al. 2008).

Land plants evolved from an ancestral pool of freshwater green algae known as the charophycean green algae (CGA) or charophytes (McCourt et al. 2000, 2004; Gontcharov et al. 2003, 2004; Mattox and Stewart 1984; Graham et al. 1991). CGA possess CWs whose inclusive polymers bear significant resemblance to those of land plant CWs. The charophycean green algae (Streptophyta, Viridiplantae) are a diverse group of green algae (Mattox and Stewart 1984) containing, as classified at the ordinal level, six extant groups: Mesostigmales, Chlorokybales, Klebsormidiales, Zygnematales, Charales and Coleochaetales. **CGA are thought to be progenitors of land plants. The**

presence of homologous polymers in the CGA has implications in our understanding of the function of the CW. In this study we identify in the CGA a variety of hemicellulosic polymers previously only found in land plants utilizing a combination of monoclonal antibody screening, analytical methods, and enzyme susceptibility analysis coupled with oligosaccharide characterization.

Methods

Organism and growth conditions. The organisms examined in this study are diverse and within the CGA (Streptophyta) line including representatives of the Chlorokybales (*Chlorokybus atmophyticus)*, Klebsormidiales (*Klebsormidium flaccidum)*, Zygnematales (*Cosmarium turpini* and *Penium margaritaceum*), and Charales (*Chara corallina)*. *C. corallina, Co. turpini* and *P. margaritaceum* were collected from a freshwater wetland in Porter Corners, NY (USA) and was subsequently cultured in a greenhouse facility in Skidmore College. *Ch atmophyticus* (LB2591) and *K. flaccidum* (LB321) were obtained from the University of Texas Culture Collection (Austin, TX, USA). Both cultures were made axenic by treatment in a commercial anti-biotic/anti-mycotic cocktail (Sigma Chemical, St. Louis, MO, USA) using the manufacturer's instructions. In brief, cells were treated for 24 h in the antibiotic solution, washed repeatedly in sterile Woods Hole Medium (WHM) (Nichols 1973) and subsequently cultured. After 5 days growth, aliquots of cells and extracts were removed and plated on a bacterial assay agar (Sigma) and viewed microscopically in order to determine if cultures were axenic (Domozych et al. 2005).

Mechanical and chemical isolation of cell walls. Prior to analysis, the *Ch. atmophyticus* and *K. flaccidum* cells were ground under liquid nitrogen (LN_2) and extracted with methanolic potassium hydroxide (MeKOH) (Aronson and Lin 1978). The CWs were treated with 5% KOH in 80% MEOH and then placed in a hot water bath for 15 minutes at 98°C. The preparation was then centrifuged at 700 x g for 10 mins, the supernatant removed and the resultant pellet washed in the following sequence: hot MeKOH, with RT dH_2O, with 1N acetic acid, and, as a final rinse, with RT dH_2O. This procedure was repeated until the pellet was white. The pellet was then dialyzed against dH_2O for 48 hours and then freeze-dried; this is the methanolic KOH insoluble residue (MKIR). For *C. corallina*, *Co. turpini*, and *P. margaritaceum*, the defatted CW pellet was prepared according to Fry (2000). Briefly, the cells were ground under LN_2 and placed in acetone for 16h. Then the pellet was extracted with boiling 80% ethanol for 30 minutes and washed with RT 80% and then RT 95% ethanol. The pellet was then dialyzed against dH_2O for 48 hours and then freeze-dried; this is the alcohol insoluble residue (AIR).

Fractionation of cell walls. Fractionations of CW polymers from methanolic KOH-processed (MKIR) cell walls was accomplished using the methods of Fry (2001). The pellet was successively extracted with the following process: (i) dH_2O at 95°C for 1h followed by centrifugation at 700 x g for 10 min at RT, and supernatant collected (repeated 3x) (HW-soluble fraction); (ii) 50mM CDTA (21°C , pH 7.5) for 6h, centrifuged as above, washed with dH_2O (3x) and supernatants collected, and dialysed against 0.5M imidazole for 12h (CDTA soluble fraction); (iii) 50mM Na_2CO_3 containing

20mM $NaBH_4$ (1°C) for 16h, centrifuged as above, washed with dH_2O (3x), and supernatants collected (Na_2CO_3 soluble fraction); (iv) 1M KOH containing 20mM $NaBH_4$ to prevent "peeling" for 2h at 1°C, centrifuged as above (repeated 2x), washed with dH_2O (3x), supernatants collected (1M KOH soluble fraction); (v) 4M KOH containing 20mM $NaBH_4$ for 2h at 1°C, centrifuged as above (repeated 2x), washed with dH_2O (3x), supernatants collected (4M KOH soluble fraction). The remaining CW remnant was washed with 0.5M imidazole (1x) and dH_2O (4x) and lyophilized. The supernatants from each stage were neutralized with acetic acid where appropriate, dialysed against dH_2O exhaustively at 4°C and lyophilized.

Neutral sugar analysis. Neutral sugar analysis was performed by the methods described in Wustman et al. (1997). Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°C for 3 h, saponified with 1 M NH_4OH for 2 h, reduced with $NaBH_4$ and acetylated as in Harris (1984) and Wustman et al. (1997). Gas chromatography/mass spectrometry (GC/MS) was performed using a SP-2330 column (30m, 0.25 mm i.d., isothermal at 230°C, injector 240°C) on a Finnigan-MAT Magnum mass spectrometer (San Jose, CA) operated in the electron-impact mode and alditol acetates were identified by their mass spectra and relative retention times to a *myo*-inositol internal standard. Quantification was based on response factors of standard monosaccharides that had been subjected to the same procedure.

Linkage analysis. Linkage analysis was done as described in Wustman et al. (1997). Uronic acid containing polymers were protonated with Dowex 50W-X8 resin (Sigma Aldrich, St. Louis, MO, USA) and pre-reduced following methylation and before

hydrolysis. Per *O*-methylation of the polymers was carried out with 1.6 M butyllithium in DMSO and iodomethane (Stevenson and Furneaux 1991). Sep Pac C-18 reverse phase chromatography was used to purify the methylated polymers (Waeghe et al. 1983). Samples were hydrolyzed with 2M TFA at 121^{o}C for 1.5 hrs and reduced with $NaBD_4$. The per-*O*-methylated alditols were acetylated as in Harris et al. (1984) and separated on the GC/MS as described for monosaccharide analysis with column parameters 150^{o}C to 245^{o}C at 4^{o}C/min with an isothermal hold at 245^{o}C for 20 min. Linkage patterns were assigned based on previously determined diagnostic mass ion fragments and relative retention times (Jansson et al. 1976, Waeghe et al. 1983, Wustman et al. 1997, 1998) and compared to a library created by injection of standards of incompletely methylated alditol acetate derivates of each sugar. Quantification of per-*O* methylated alditol acetates were based on effective carbon response factors, as described in Sweet et al. (1975).

Monoclonal antibody labeling. The CW fractions were analyzed by immune-dot assay (using the methods of Willats et al. 2001). Briefly, a five-fold serial dilution (in dH_2O) were prepared from the fractions and applied to nitrocellulose as 1ul aliquots. The membranes ere air-dried for approximately 16h at 21^{o}C. The membranes were treated 5%M/PBS for 1h, then 1.5h in primary antibody solution (BS-400-2 BioSupplies (Melbourne, Australia)). The antibody BS-400-2 was diluted 1/10 in 5% M/PBS. The nitrocellulose membranes were washed in PBS extensively prior to the incubation for 1.5h in secondary antibody solution. The secondary antibody was anti-mouse horseradish peroxidase conjugate (Sigma), diluted 1/1000 in 5% M/PBS and washed again extensively in dH_2O and PBS. Development was in the substrate solution (25ml

dH_2O, 5ml methanol containing 10mg/ml 4-chloro-l-napthol, 30ul 6% (w/w) H_2O_2).

The monoclonal antibody BS-400-2 for (1, 3) β-D-glucan was obtained from BioSupplies (Melbourne, Australia). Cell pellets were fixed at RT in 1% paraformaldehyde in WHM for 20 min, washed 3X with WHM and 3X with phosphate-buffered saline (PBS; pH 7.2) and subsequently blocked for 30 min in 2% BSA in PBST (PBS plus 0.1% Tween-20). The cells were washed 3X with PBST and incubated for 2h at RT in PBST. Cells were gently mixed throughout the treatment period. The cells were washed three times in PBST, incubated in block solution for 30 min and washed again 3X in PBST. The cells were incubated in a 1/100 dilution (in PBST) of anti-mouse antibody conjugated with TRITC (Sigma Chemical) for 2 h at RT in the dark. The cells were then washed and used for subsequent microscopic observation. The controls for this study included eliminating the primary antibody incubation. CW were analyzed using standard fluorescence microcopy, an Olympus BX-60 light microscope (LM) was used for general and fluorescence microscopy and images were captured using an Olympus DP70 camera

Enzymatic Digestion. In order achieve adequate enzymatic digestion, MKIR (*K. flaccidum* and *Ch. atmophyticus*) or AIR (*C. corallina, P. margaritaceum* and *Co. turpini*) insoluble material, and barley flour control were swelled with 1M KOH containing 20mM $NaBH_4$ for 16 hours at 4^oC. The swollen material was then neutralized with concentrated acetic acid and EtOH was added to a final concentration of 75% (Sorensen et al. 2008). Following centrifugation at 700x g for 10 mins, the precipitate was washed with 80% ethanol (2x), then dH_2O (2x), and finally with sodium phosphate buffer (pH 6.5) (3x). As a control, a parallel analysis was also performed on MLG

derived from barley flour and by lichenase digestion. Ten units of lichenase (*endo*-1, 3(4)-ß-D-glucanase) (*Bacillus* sp.) EC 3.2.1.73 from Megazyme (Cat # E-LICHN) in phosphate buffer (0.2M) pH 6.5 was added to approximately 10 mg of swollen walls, swollen barley flour and barley glucan (Sigma # G6513) and incubated at 60^{o}C for 16 hours. Ethanol (3 additions) was added to precipitate polymers; samples were chilled to 4^{o}C for 1 hour, centrifuged at 500 x g for 10 minutes, and the supernatant dried under filtered air. A portion of the supernatant was isolated for linkage analysis and a portion was used for oligosaccharide characterization. Lichenase cleaves MLG at the 1-4 linkage after a (1, 3) linkage in the sequence G3G4G- (Meikle et al. 1994) therefore the digestion products will have (1, 3) linkage next to the reducing terminus (Fry et al. 2008a).

Reducing sugar assays. Reducing sugar from enzymatic digestions were measured using the 3, 5-dinitrosalicylic acid reagent assay (DNS) method (Miller 1959), with a glucose standard. Controls including heat killed enzyme, substrate only, and all values were adjusted to subtraction reducing sugar contribution from enzyme preparation.

Oligosaccharide characterization. Oligosaccharide Mass Profiling (OLIMP) was performed as described in Lerouxel et al. (2002), Obel et al. (2006) and Leboeuf et al. (2008). The oligosaccharides were dissolved in ddH_2O and desalted with ion exchange resin to remove buffer salts (Bio-Rex MSZ 501(D) resin Bio-Rad) (Obel et al. 2006). The oligosaccharide solution was spotted onto a MALDI-TOF sample plate containing vacuum-dried 2, 5-dihydroxybenzoic acid (10 mg per well) and crystallized under vacuum. Samples were analyzed on a Voyager DE-Pro MALDI-TOF-MS (Applied Biosystems) in positive reflectron mode with an acceleration voltage of 20 kV and an

extraction delay time of 350 ns. The peak intensities were used to calculate the relative abundance of each ion peak and to perform pair-wise comparisons.

Results

Mixed-linkage oligosaccharide composition. Digestion of *C. corallina*, *Co. turpini*, *K. flaccidum*, and *Ch. atmophyticus* defatted CWs with *Bacillus subtilis* lichenase (*endo*-1, 3(4)-β-glucanase) produced oligosaccharides, which revealed the presence of MLG by OLIMP and DNS reducing sugar assay. *P. margaritaceum* was digested with lichenase and there was no reducing sugar released indicating the absence of MLG in the CW (Figure 3.1).

OLIMP results indicated a distribution of the variable-sized blocks of (1, 4)-linked glucosyl residues up to the octasaccharide level (Table 3.1). Consistent with previous analysis, the most abundant hexose oligomers in barley MLG were oligosaccharides with degree of polymerization 3 and 4. The digestion products in barley flour (*Hordeum vulgare*) were mainly tetrasaccharides (G4G4G3G-) followed by trisaccharides (G4G3G) and pentasaccharides (Table 3.1). The digestion products of *Co. turpini* and *C. corallina* are similar to the digestion products of barley flour, with the major product being a tetrasaccharide (G4G4G3G-) followed by trisaccharide (G4G3G-). Whereas, in *Ch. atmophyticus*, and *K. flaccidium*, the disaccharide laminaribiose (G3G-) was the most abundant product (Table 3.1). When the major units for the typical Poaceae MLGs are compared; the ratio of trisaccharide to tetrasaccharide units varies between species: 0.3:1 in *C. corallina*, 0.6:1 in *Co. turpini*, 1.7:1 in *K. flaccidum*, and 1.2:1 in *Ch.*

atmophyticus. *K. flaccidum* and *Ch. atmophyticus* both have an unusual ratio because the MLG contains a large number of disaccharide units.

Linkage analysis of the oligosaccharides produced from the lichenase digestions of the CGA CWs (Figure 3.2), revealed terminal-, 3- and 4-Glc linkages with a molar ratio of the barley glucan and barley flour standards indicating similar ratios. (with 0.4:1:2 and 0.6:1:2). The CGA molar ratio varied from 1:1:4 in *C. corallina* to the 7:1:2 in *K. flaccidum* (Figure 3.2). *Co. turpini* and *Ch. atmophyticus* were both very similar to the standards with ratios of 0.4:1:3 and 0.6:1:2 respectively (Figure 3.2). Additional linkages were identified (Table 3.2). In the barley glucan and barley flour standards additional glucosyl residues including 2-, 2,3-, 2,4-, 3,6-linked residues, 2,3-linked galactosyl residues, and 3,6-mannosyl residues were detected. In the CGA species, most of the species had additional linkages with 6- and 3, 6-linked glucosyl residues and 6-linked galactosyl residues predominate. In addition, *C. corallina* contained 2, 4- and 4, 6-linked glucosyl residues and 3-linked xylosyl residues. Also found in *Ch. atmophyticus* were terminal- and 6-linked galactosyl residues. The oligosaccharides released in *K. flaccidum* digests included 2- and 2, 6-linked glucosyl residues, terminal-galactosyl residues, and 2-linked arabinosyl residues. *C. turpini* had the most additional oligosaccharides solubilized including 2-, and 4,6-linked glucosyl residues, 3-, 6-, 2,3-, 2,4-linked galactosyl residues, terminal arabinosyl linkage and an abundance of fucosyl residues including terminal, 3-, 4-, 3,4-linked residues (Table 3.2).

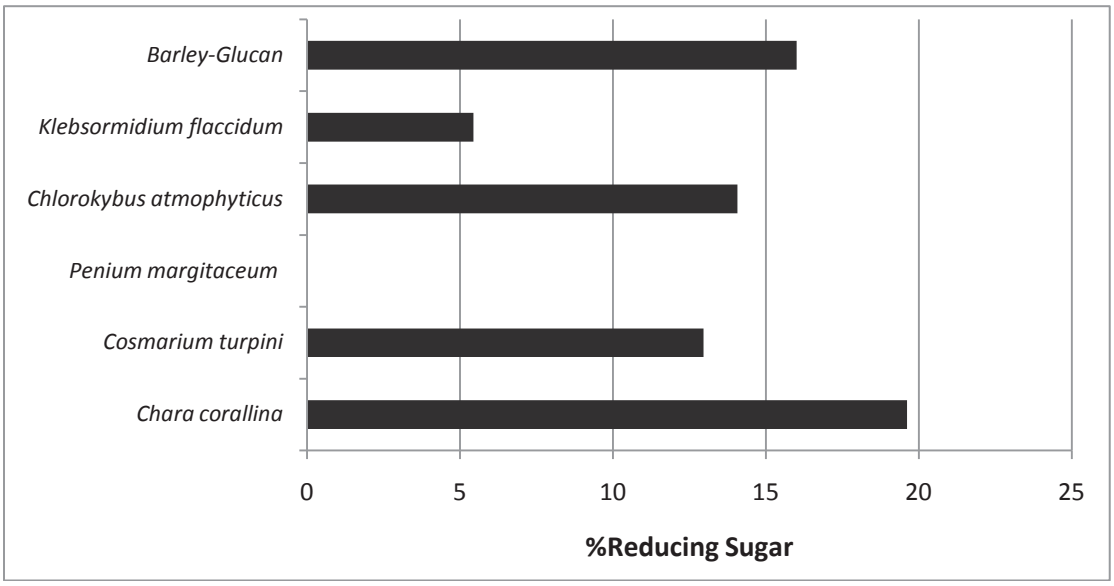


Figure 3.1: Reducing sugar assay of saccharide solubilized following (1, 3), (1, 4)-β-d-glucan-specific endoglucanase (lichenase) digestions of AIR from *Cosmarium turpini* and *Chara corallina* and methonolic KOH insoluble residue (MKIR) from *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* were utilized. Barley glucan was used as a positive control.

Table 3.1: Oligosaccharide detected by MALDI-TOF MS analysis digested of Lichenase (*endo*-1, 3(4)-ß-D-glucanase) (*Bacillus* sp.)EC 3.2.1.73 products expressed as percent. OLIMP of MLG oligosaccharides released from MKIR (*Klebsormidium flaccidum* and *Chlorokybus atmophyticus*) and AIR (*Chara corallina* and *Cosmarium turpini*) preparations digested with lichenase

Oligosaccharide units	*C. corallina*	*Co. turpini*	*K. flaccidum*	*Ch. atmophyticus*
G3G4G3G4G (DP 2)	7.8	5.9	69.1	74.0
G3G4G4G3G4G (DP 3)	17.0	26.9	16.4	10.2
G3G4G4G4G3G4G (DP 4)	61.9	45.0	9.9	8.2
G3G4G4G4G4G3G4G (DP 5)	3.2	5.7	3.1	4.7
G3G4G4G4G4G4G3G4G (DP 6)	10.1	7.4	0.8	2.6
G3G4G4G4G4G4G4G4G3G4G (DP 7)	0.0	7.4	0.7	0.4
G3G4G4G4G4G4G4G4G4G3G4G (DP 8)		1.6		

Table 3.2: Glycosyl substitution patterns of saccharide solubilized following lichenase digestion (*endo*-1, 3(4)-ß-D-glucanase) (*Bacillus* sp.)EC 3.2.1.73. Digestions of AIR from *Cosmarium turpini* and *Chara corallina* and MKIR of *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* were utilized. Barley glucan and barley flour were used as a positive control. Products expressed as % mole detected.

		Barley Glucan	Barley Flour	*Chara corallina*	*Cosmarium turpini*	*Chlorokybus atmophyticus*	*Klebsordium flaccidum*
Glc							
	t-	12.9	11.6	12.9	5.4	12.4	38.0
	3-	23.9	27.6	13.1	14.9	21.1	5.2
	4-	35.2	43.7	49.9	42.0	50.5	11.1
	2-	1.0	1.9	—	1.3	—	2.8
	6-	—	2.1	3.3	1.6	3.8	8.5
	2,3-	1.8	1.3	—	—	—	—
	2,4-	1.7	1.6	4.6	—	—	—
	2,6-	1.5	—	—	—	—	3.9
	3,6-	0.5	0.5	—	0.4	3.1	—
	4,6-	—	—	9.3	2.9	—	—
Gal							
	t-	—	4.1	—	—	3.1	1.8
	2-	2.5	—	—	—	—	—
	3-	—	—	—	2.2	—	—
	6-	—	—	—	1.5	5.9	—
	2,3-	15.6	2.6	—	2.7	—	—
	2,4-	—	—	—	1.9	—	—
Man							
	4-	0.1	—	—	—	—	—
	3,6-	1.2	1.9	—	—	—	—
Ara							
	t-	—	—	—	2.3	—	—
	2-	—	—	—	—	—	28.7
Xyl							
	t-	—	—	—	1.0	—	—
	3-	—	—	6.9	—	—	—
Rha							
	2-	0.8	0.4	—	2.0	—	—
Fuc							
	t-	0.3	—	—	0.9	—	—
	3-	—	—	—	10.3	—	—
	4-	—	—	—	1.2	—	—
	3,4-	—	—	—	4.3	—	—

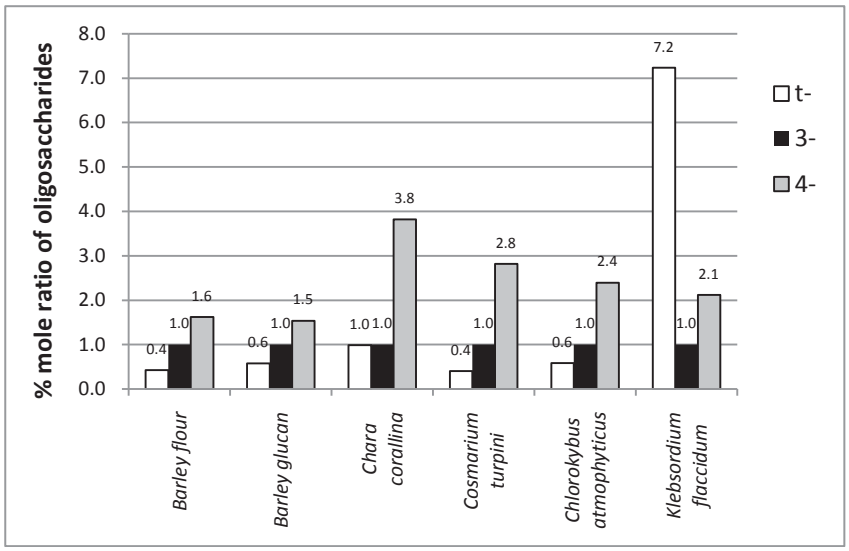


Figure 3.2: Molar ratio of lichenase solubilized oligosaccharide (terminal-, 3- and 4-Glc) Glycosyl linkage composition of the products produced by digestion of MKIR (*Chlorokybus atmophyticus* and *Klebsormidium flaccidum*) and AIR (*Chara corallina* and *Cosmarium turpini*) residues (Aliquots of the same product were used in Table 1). Ratios of terminal-, 3- and 4-Glc where the 3-Glc mean value was set to 1 and other mean values adjusted accordingly.

MLG localization. Dot blot analysis of fractions with the antibody BS 400-3, for mixed linkage glucans (Biosupply, Australia) labeled the 1M KOH and fractions of *Ch. atmophyticus* and *K. flaccidum* indicating the presence of MLG (dot blots not shown; Figure 3.3). The walls of *Ch. atmophyticus* and *K. flaccidum* were labeled with BS-400-3 (Meikle et al. 1994), though only following the extraction of the pectin layer with HW, CDTA, and Na_2CO_3 (Figure 3.3). There was masking of the polymer under of the mucilage layer and other polymers. The labeling in *Ch. atmophyticus* was continuous and followed the smooth outline of the cells (Figure 3.3a, b). The antibody labeling of *K.*

flaccidum cells appeared blotchy which may be related to the growth of this filamentous alga (Figure 3.3c).

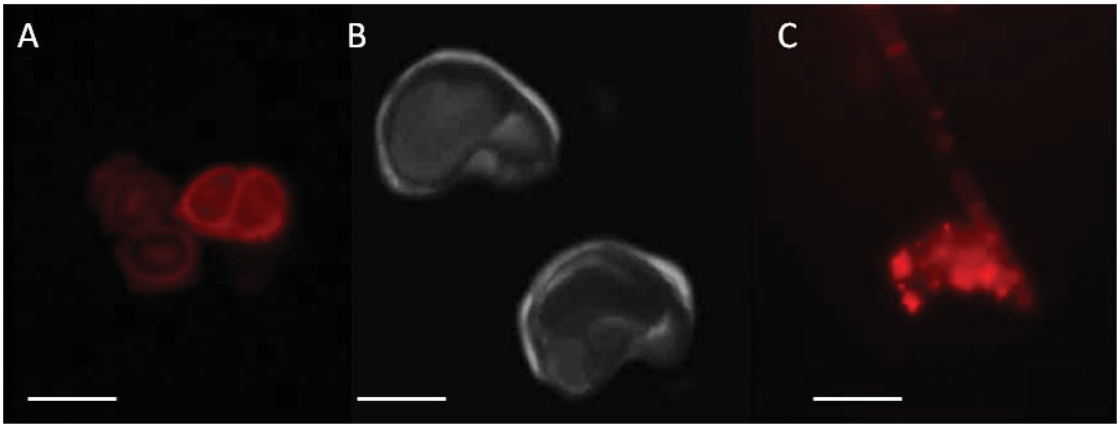


Figure 3.3: Cytochemical labeling with the anti-(1-3)(1-4)-ß-glucan antibody (BS 400-3, Meikle et al. 1994) *A) Chlorokybus atmophyticus* HW insoluble residue (red) Scale bar = 25µm B)Whole cell wall of *C. atmophyticus,* Scale bar = 10µm, C) *Klebsormidium flaccidum* HW insoluble residue Scale bar = 25µm .

Characterization of hemicellulosic fractions. Previous analyses have indicated the MLG in the Poaceae are homopolymers consisting only of glucose (Sorenson et al. 2008). Glucose was the prevalent monosaccharide in the alkaline fractions of *Co. turpini*, *Ch. atmophyticus*, and *K. flaccidum* (Figure 3.4), and in the 1M KOH fractions the percent of Glc ranged from 82% in *C. corallina* to 97% in *K. flaccidum*. Linkage analysis revealed the presence of terminal-linked, 3-linked and 4-linked glucosyl residues in the 1M KOH and 4M KOH fractions. In *Co. turpini* the ratio of the 3- to 4- linked glucosyl residues is 1: 7, whereas in *K. flaccidum* and *Ch. atmophyticus* the 3-linkage is more abundant; in *K. flaccidum* and *Ch. atmophyticus*, depending on the solubility of the

polymer, the ratio may be 29:2 for the 1M KOH samples or 4:1 or 2:1 for the "pretreated for uronic acids" 1M KOH fraction.

Relatively little is known about the structure and composition of the hemicellulose of the charophytes. To better understand the presence of MLG in the basal species (*Ch. atmophyticus* and *K. flaccidum)*, one of the desmids (*Co. turpini*), and a multicellular algae (*C. corallina*) comprehensive linkage analysis of the 1M KOH fractions were performed (Table 3.3). In addition to the terminal-, 3- and 4-linked glucosyl residues, *C. corallina* 1M KOH fraction contain significant amounts of 4, 6-linked glucosyl residues, this substitution was also present in the 4M KOH fraction of *Ch. atmophyticus* and in minor amounts in the 1M KOH fraction of *K. flaccidum.* There were also 3, 6-linked glucosyl residues found in all of the species examined but *Co. turpini*.

In addition to glucose in the hemicellulosic fraction: mannose, galactose, xylose, arabinose and fucose were found. The monosaccharide galactose ranged from 7% in *Ch. atmophyticus* to 1% in *K flaccidum* with significant amounts of 4-linked galactosyl residues present all of the species. *C. corallina* and *Co. turpini* also contained terminal-, and 2-galactosyl residues. There was only a minor amount of mannose (7.0%) in the 1M KOH fraction of *C. corallina* (Figure 3.4). Xylose ranged from 12% in *Ch. atmophyticus* to 0.5% in *K. flaccidum* with 4-xylosyl residues found. Terminal- and 2-linked xylsoyl residues were also present in the 4 MKOH fraction of *K. flaccidum* and in the 1M KOH fraction of *Co. turpini* and *C. corallina.* Minor amounts of arabinosyl branching occurred in the alkalini fractions of *K. flaccidum* (Table 3.3).

The CW fractions were screened with dot blot analysis for the epitopes from other land plant polymers. The 1M KOH fraction *of K. flaccidum* and *Co. turpini,* labeled with the monoclonal antibody LM11 for xylans /arabinoxylans epitopes (PlantProbes). The 1M KOH fraction of *C. corallina* and *Co. turpini* labeled with the monoclonal antibody LM15 for a xyloglucan epitope (PlantProbes) (Data not shown).

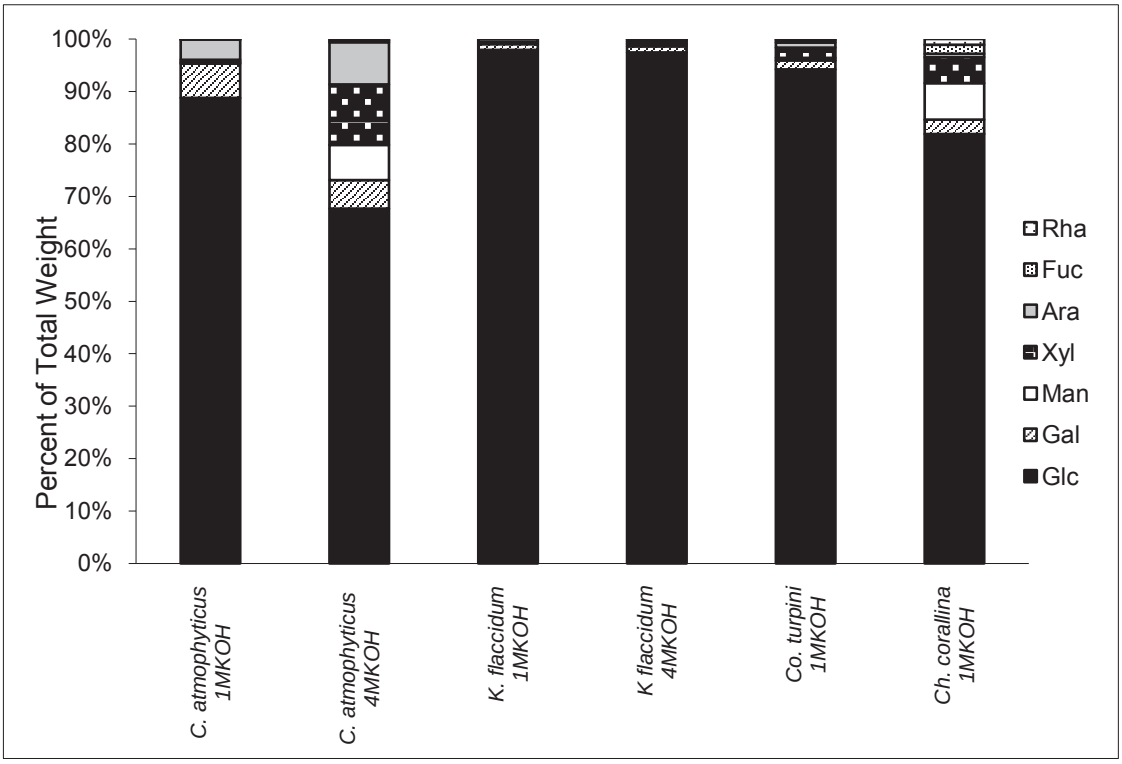


Figure 3.4: Monosaccharide profiles of hemicellulosic fractions expressed as percent detected neutral sugar.

Table 3.3: Glycosyl substitution patterns of CW fractions. Glycosyl linkages reported as % mole detected. uronic = Reduced prior to methylation

	Chlorokybus atmophyticus				*Klebsormidium flaccidum*				*Cosmarium turpini*	*Chara corallina*
	1MKOH (%mole)	*1MKOH (uronic) (%mole)*	4MKOH (%mole)	4MKOH (uronic) (%mole)	*1MKOH (%mole)*	*1MKOH (uronic) (%mole)*	4MKOH (%mole)	4MKOH (uronic) (%mole)	*1MKOH* (%mole)	*1MKOH (%mole)*
Glc										
t-	3.2	—	6.5	—	1.8	9.9	—	9.8	0.7	0.1
3-	**64.7**	35.5	14.0	—	**85.8**	19.0	14.5	9.3	9.8	7.1
4-	**17.0**	18.3	44.9	47.0	**3.0**	32.9	48.7	51.4	**77.3**	**28.4**
2,3-	2.9	3.9	—	—	5.2	1.5	—	1.3	—	—
2,6-	—	—	—	0.4	—	1.2	0.6	—	—	—
3,6-	12.2	14.2	—	—	2.8	6.4	2.7	3.7	—	1.9
4,6-	—	—	—	17.6	—	0.2	—	—	—	**42.2**
2,4,6-	—	—	—	—	—	—	—	—	—	1.4
Gal										
t-	—	—	—	—	—	—	—	—	1.1	—
2-	—	—	—	—	—	—	—	—	2.2	—
3-	—	—	—	—	—	7.0	—	—	0.7	—
4-	—	19.9	23.7	—	—	—	—	5.6	1.1	—
6-	—	—	—	5.4	—	—	—	—	1.0	8.2
2,3-	—	—	—	—	—	0.7	—	—	—	—
2,4-	—	—	6.5	—	—	—	1.6	—	—	—
2,6-	—	—	—	—	—	—	—	—	—	0.2
3,6-	—	—	—	—	—	—	0.6	—	—	—
2,4,6-	—	—	—	—	—	—	—	—	—	0.2
3,4,6-	—	—	4.3	—	—	—	4.2	—	0.1	—
Man										
2,4-	—	—	—	—	—	—	—	—	—	2.9
4,6-	—	—	—	—	—	—	—	—	—	1.0
Ara										
t-	—	—	—	—	—	2.3	—	—	—	—
2-	—	—	—	—	—	1.2	—	0.5	—	1.1
3-	—	—	—	—	—	4.5	—	—	—	—
2,3-	—	—	—	—	—	—	—	—	0.7	—
3,6-	—	—	—	—	—	—	—	—	tr	—
2,3,4-	—	—	—	—	1.4	—	—	—	—	—
Xyl										
t-	—	—	—	—	—	—	—	3.7	1.8	0.2
2-	—	—	—	—	—	—	—	2.8	2.5	1.9
4-	—	—	—	21.6	—	5.1	12.9	0.1	—	0.1
2,3,4-	—	—	—	—	—	—	10.9	—	—	—
Rha										
2-	—	—	—	—	—	—	2.3	—	—	—
4-	—	—	—	—	—	—	—	—	—	2.4
Fuc										
t-	—	—	—	—	—	—	—	—	0.1	0.5
3-	—	—	—	—	—	—	1.0	—	—	—
4-	—	—	—	—	—	—	—	—	0.9	—
GalA										
4-	—	3.5	—	5.0	—	—	—	11.8	—	—
GlcA										
3-	—	4.7	—	3.0	—	8.3	—	—	—	—

Discussion

MLGs were revealed to be present in a variety of charophyte species, including the early divergent species *Ch. atmophyticus* and *K. flaccidum*, and the advanced species, *Co. turpini* and *C. corallina.* In the charophyte species the ratio of trisaccharide units to tetrasaccharide units ranged from 1.24: 1 in *Ch. atmophyticus*, 1.65: 1 in *K. flaccidum*, 0.60: 1 in *Co. turpini* and 0.27: 1 in *C. corallina* (from Table 3.3). *C. corallina* and *Co. turpini* are different from the other species with more tetrasaccharide units than trisaccharide units, much like has been previously reported for *Equisetum* species (Sorenson 2008). Popper and Fry (2003) surveyed the bryophytes and charophytes using specific (1,3),(1,4)-β-glucan hydrolase digestion and reported the presence of (1,3),(1,4) β -glucan only in the liverwort *Lophocolea bidentata*, with the major oligosaccharides in the DP 2-6 range and yielding both glucose and arabinose on acid hydrolysis. Enzyme digests of barley endosperm, oat endosperm and maize coleoptiles are very similar, containing a majority of trisaccharide units (64-70 %) with fewer tetrasaccharide units (25-31 %). Digests of *Cetraria islandica*, a lichen, contain a majority of trisaccharide (86 %) with very little tetrasaccharide (3.5 %) units. The ratio of trisaccharide to tetrasaccharide units varies between species: from 3: 1 to 4.5: 1 in wheat, 2.9: 1 to 3.4: 1 in barley, 2.7: 1 in rye, and 1.8: 1 to 2.3: 1 in oats (*reviewed* Bacic et al. 2009).

In barley CWs the ratio of (1, 4) - and (1, 3)-linked glucose ranged from 2.2:1 to 2.6:1. The (1, 3)-β-D-glucosyl residues are believed to occur as single residues between (1, 4)-β-D-glucosyl residues. It is believed higher levels of oligosaccharide units are arranged at random (Staudete et al. 1983; Woodward et al. 1983a; Buliga et al. 1986). In

land plants, the cellulose synthase-like core domain of the MLG synthases complex is believed to make the tetrasaccharide units and the larger even-numbered (1→4)-glucose linked units (Urbanowicz et al.2004).

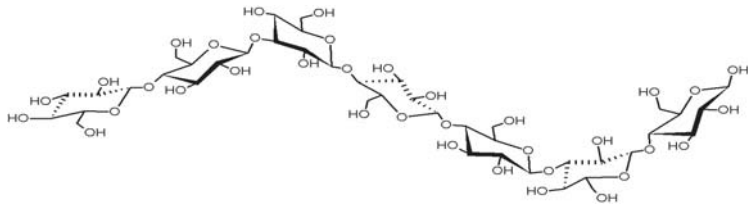
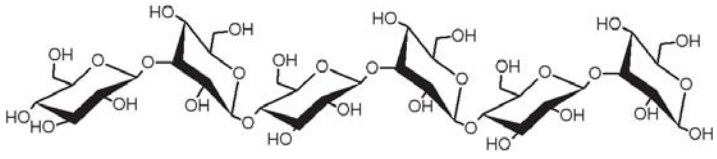
The (1, 3)-β-glucosyl residues are believed to be distributed randomly in a (1, 4) -β -glucan chain, which creates a disruption in the overall shape of the polymer, thus creating an irregular structure (Fincher 2009). Contiguous (1, 3)-linked glycosyl residue units have not been found (Lazaridou et al. 2004). Tetrasaccharide units and longer units may provide for alignment and a junction zone between other MLG polymer chains and other CW polymers (cellulose or arabinoxylans) (Carpita et al. 2001, Fincher and Stone 2004). Hrmova et al. (2007) and Mohand and Farkas (2006) suggest that XETs of barley may remodel the MLGs and/or the arabinoxylans. In *Tropaeolum majus,* Mohand and Farkas (2006) demonstrated heterotransglycosylating activity and Fry et al. (2008b) found a transglycosylase enzyme (MLG: xyloglucan endotrans glucosylase [MXE]) in *Equisetum* sp and *Coleochaete* sp that transfers finished molecules from MLG polymers to oligoxyloglucoside acceptors.

Equisetum sp. contains significant amounts of tetrasaccharide compared to trisaccharide; similar to what is found in *Co. turpini* and *C. corallina,*. The MLGs of lichens yield only the trisaccharide units under lichenase digestion (Stone and Clarke 1992). In *Equisetum sp*. the major product was the tetrasaccharide and the minor products were trimer glycosyl residues, which were sometimes exceeded in quantity by disaccharide laminaribiose units (Sorenson et al. 2008). *Co. turpini* and *C. corallina*

MLG seems to be similar to that found in *Equisetum* sp. but differs from that found in Poaceae and lichen (Table 3.1; Figure 3.2).

The MLG in the basal species (*K. flaccidum* and *Ch. atmophyticus*) of the charophytes was unique from that of *Equisetum*, Poaceae, and lichens. The pattern of 3- and 4-linkages revealed by OLIMP and the proportion of (1→3) to (1→4)-linked glucosyl residues from methylation analysis indicated that the MLG of *Ch. atmophyticus* and *K. flaccidum* appears to be unique (Table 3.1; Figure 3.2). The most prevalent oligosaccharides released upon treatment with lichenase of *K. flaccidum* and *Ch. atmophyticus*, are disaccharides. Disaccharide laminaribiose units, however, are not observed in barley endosperm, oat endosperm, maize coleoptiles and lichenin. In these species, linear runs of trisaccharide and tetrasaccharide units are joined by a single (1, 3) β-linkage (Staudte et al. 1983; Woodward et al. 1983). Contiguous (1, 3)-linked glycosyl bonds are not believed to occur and evidence for small quantities of the disaccharide units is a subject of debate (Roubroeks et al. 2000).

Table 3.4: Proposed possibility for the structure of the major MLG units in the CGA

Major MLG unit ("tetrasaccharide") in digests of *C. corallina* and *Co. turpini*.	
Major MLG unit ("disaccharide") in digests of *K. flaccidum* and *Ch. Atmophyticus*	

Lichenase solubilized other oligosaccharides in the CWs of the CGA. Popper and Fry (2003) reported (1, 3), (1, 4)-β-glucan hydrolysis of the liverwort *Lophocolea bidentata* produced arabinose, also *K. flaccidum* yielded arabinose (Table 3.2). The lichenase solubilized xylosyl residues from the whole CW of *C. corallina*. Arabinoxylans contains 3-linked xylosyl residues, it was found that in arabinoxylans 23−24% of the Xyl residues were 2-Xyl or 3-Xyl (McNeil et al., 1975; Bacic and Stone, 1981). Therefore, the xylosyl residues may have been in a separate polymer like arabinoxylan or it may have been attached to the MLG backbone. Additionally, deoxysugars were released from *Co. turpini* with an abundance of fucosyl residues, indicating that the fucose was released when the CWs were treated with lichenase. Fucose is not a major sugar in the 1M KOH fraction of *C. turpini*, which lends to the idea that it was hydrolased from another polymer.

The presence of MLG in the charophytes, including the basal species, indicates the origin and synthesis of these polymers may be a primitive adaption. MLG until very recently was thought to only occur in the Poales, though Fry et al. (2008b) found a MLG remodeling enzyme (MLG:xyloglucan endotrans glucosylase [MXE] in *Equisetum* and CGA *Coleochaete*). There is also the possibility of an alternate evolution of this polymer which begs the question, "What is the function of the MLG and how does it interact with cellulose?" In the Poales, MLGs are found in elongating cells to a maximum that coincides with the maximum rate of elongation (Kim et al. 2000), which is associated with high levels of GAX. There is no indication of GAX in the charophytes or in the MLGs previously identified in the four *Equisetum* species. These finding have

implications to the understanding of the functional relationship of the CW polymers, and the evolution of the plant CW.

In addition to the MLG polymer there is evidence for other "land plant" polymers in the hemicellulosic fractions of *Ch. atmophyticus*, *K. flaccidum*, *Co. turpini* and *C. corallina*. In the 1M KOH fraction of *C. corallina* there is preliminary evidence of xyloglucans (Table 3.3). Xyloglucans (XyG) are present in the primary walls of dicots, gymnosperms, and noncommelinoid grasses and are thought to bind to cellulose and be involved in CW expansion (Carpita and McCann 2000). They are composed of a linear polymer of (1,4)β-glucans with branches off the 6- position of glucosyl residues. The sides branches consist of xylosyl (terminal and 2-linked), galactosyl (terminal and 2-linked), and fucosyl (terminal) residues. In *C. corallina*, the glucosyl residues 4- and 4,6-(42%) linked were abundant. In addition, there were both terminal and 2-linked xylosyl residues. The typical galactosyl residues were present in the 1M KOH fraction. Other evidence for XyG in the Charales include Domozych et al. (2009) report of cytochemical labeling for xyloglucan in all of the thallus parts of *C. corallina*. However, the absence of XyG was reported by Popper and Fry (2003) where XyG were found in all of the land plants but absent from the CGA.

Another land plant polymer that may be present in the CGA is xylan. Xylans are hemicellulosic polymer in the primary and secondary CWs of dicots and grasses. The structure is a linear (1,4) β-xylosyl backbone with side branches of (1,2)-α-L-arabinosyl and (1,3) α -D-glucuronosyl. Xylose with the characteristic 4-linked residue were present in the 4M KOH fraction of *C. atmophyticus* and 4-linked xylosyl residues were also

present in the 1M KOH and 4M KOH fraction of *K. flaccidum* and in the 1M KOH fraction *C. corallina*. Willats CoMPP analysis (Willats CoMMP unpublished) indicated *Ch. atmophyticus*, *K. flaccidum*, *Co. turpini*, and *C. corallina* CWs labeled with the (1,4) β-xylan/arabinoxylan epitope (LM11). Hotchkiss et al (1989) indicated the presents of xylan in the hemicellulosic wall of *Mougetia* sp with evidence of a 4-xylosyl backbone and 2,4-xylosyl branch residues. Domozych et al. 2009 found labeling with LM11 in the CW of NaOH extracts for *C. corallina* thallus parts but no labeling with LM10, which suggests that the xylan in *C. corallina* is substituted. Xylan has been reported from the brown alga *Fucus* sp. also in water-soluble portion of *Rhodymenia* sp CW. The chlorophytes, *Bryopsis*, *Caulerpa*, *Halimeda*, *Udotea*, *Chlorodesius*, and *Pseudodichotomosiphon* contain linear chains of (1,3) β-xylosyl residues (*reviewed in* Siegel and Siegel 1973), which may are also be present in small amounts the insoluble residue of *Penium margaritaceum* (Table 1).

The CWs of CGA have been shown to contain cellulose with terminal rosette complexes (Hotchkiss et al. 1989), homogalacturonans (Domozych et al. 2007; Cherno et al. 1978) and now additional hemicellulose polymers including mixed linkage glucans. The hemicellulosic polymers were extracted using the traditional methods developed for land plant CWs, indicating similarities in CW organization between primitive CGA and land plants. Transition from an aquatic to a terrestrial environment is thought to have triggered physical and structural changes in the cell-wall structures (Graham 1996; Karol et al. 2001; Kenrick and Crane 1997); however, CGA CWs contain polymers that are

homologous to those of land plants, which calls into question as to how the functions of these CW polymers differ in aquatic and terrestrial environments.

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Chapter 4: Homologous Land Plant Pectins in the Charophycean Green Algae: *Penium margaritaceum*, *Cosmarium turpini*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum*

PORTIONS OF THIS CHAPTER HAVE BEEN PREVIOUSLY PUBLISHED.
DOMOZYCH DS, SERFIS A, KIEMLE SN, AND GRETZ MR. (2007) THE STRUCTURE AND BIOCHEMISTRY OF CHAROPHYCEAN CELL WALLS: I. PECTINS OF PENIUM MARGARITACEUM. PROTOPLASMA 230(1-2): 99-115.

Abstract

Pectin polymers create a hydrated, 3-D matrix that contributes to the control of stretching of the cellulose microfibril network within the CW. They also function in controlling porosity at the cell surface, providing charged surfaces which regulate both the pH and ionic status of the CW, promoting cell adhesion via their gel-like properties and serving as elicitors or signaling molecules in defense. Thisr report of homogalacturonan (HG) in *Penium margaritaceum* represents the first confirmation of land plant-type pectin in desmids and the second rigorous characterization of a pectin polymer from the charophycean algae. Evidence of HG in *Chlorokybus atmophyticus*, *Klebsormidium flaccidum* and the desmid *Cosmarium turpini* including glycosyl linkage analysis, neutral sugar analysis, and immunocytochemical labeling is also provided.

Typical (1→4)-linked galacturonosyl residues were present in the pectin fractions of *P. margaritaceum*, *C. turpini*, *K. flaccidum*, and *C. atmophyticus*. There was evidence of branching of the pectin backbone in all of the species but especially in *C. turpini* andlinkage analysis and other data suggests the presence of a possible RGI in *C. turpini*, *C. atmophyticus*, and *K. flaccidum*. Immunocytochemical labeling with monoclonal antibody JIM5 (recognizing largely un-esterified galacturonosyl epitopes) reveals labeling in all of the species examined. *Penium margaritaceum* also labels with monoclonal antibody JIM7, which recognizes mostly esterified galacturonosyl residues.

Keywords: charophycean green algae, charophytes, evolution, homogalacturonan, rhamnogalacturonan, pectin, cell wall.

Introduction

Pectins, first isolated and described by Henri Braconnot in 1825, are found throughout the plant primary cell wall (CW) and are one of its major components. The pectin structures in CWs have been described as block-like (Jarvis 1984) with smooth and hairy regions. The hydrated, 3-D matrix created by pectins in the CW contributes to the control of stretching of the cellulose microfibril network and accommodates cell expansion driven by turgor pressure (Verhertbruggen and Knox 2007, Seymour and Knox 2002, Janeau et al. 1998). The functions of pectin polymers include providing surfaces which regulate both the pH and ionic status of the CW, controlling the porosity of the cell

surface, aiding cell adhesion and signaling molecules in defense (Knox 2002, Mohnen 2008). In higher plants pectins are covalently linked to form a network throughout the primary CW matrix and middle lamellae (Willats et al. 2000). Pectins comprise a complex mixture of heterogeneous, branched polymers with two major types of primary backbone, homogalacturonan (HG) and rhamnogalacturonan I (RGI).

Smooth, unbranched pectin regions consist of homogalacturonans (McNeil et al. 1984). HG is involved in control of cell adhesion, division, expansion and polarity (Reviewed in Capodicasa et al. 2004) and HG is abundant and widespread throughout plant kingdom, constituting up to 60% of the pectin in the primary CW of higher plants (ONeill et al. 1990). HG is a linear homopolymer of (1→4)-alpha-linked D-galacturonic acid and is thought to contain about 100-200 residues (Zhan et al. 1998; Thibault et al. 1993). This polysaccharide often displays different degrees of methyl-esterification involving the C6 carboxyl group of galacturonic acid (GalA). The degree of methylation esterification varies within the HG polymer. Non-esterified chains are able to form calcium bridges creating a stiffened pectate gel (Goldberg et al. 1996). Pectin methyl esterases (PME) catalyze specific demethylation of methyl-esterified HG during deposition and modification of CWs. PMEs belong to large multigene families in all plant species examined to date (Pelloux et al. 2007). In the *Arabidopsis* genome there are 52 genes encoding PMEs. (Mohen 2008)

The hairy regions of pectin consist of RG-I and RG-II. RG-I contains alternating rhamnose and galacturonic acid residues in the backbone, specifically (1→ 2)-α-L-rhamnosyl residues-(1→4)-α-D-galacturonosyl residues disaccharide repeat units, with

neutral sugar side chains (e.g. arabinans, galactans, type I arabinogalactans) attached to O-4 of approximately half of the rhamnose residues. RG-I has been shown to bind specifically to cellulose (Zykwinska et al. 2005) and, given that the physical properties of the CW are dependent on RG-1 side chains (Jones et al 2003 and Ulvskov et al 2005), this may indicate a important structural role of this pectic polymer in the CW. Oomen et al (2002) found evidence to indicate that plants containing endoglycanses that fragment the RG backbone and side chains have both positive and also detrimental effects on growth and development.

Rhamnogalacturon II is a structurally modified HG containing the richest diversity of sugar and linkage structures known including apiose, aceric acid, 2-O-methyl fucose, 2-O-methyl xylose, 3-deoxy-D-manno-2-octulosonic acid (KDO), and 3-deoxy-D-lyxo-2-heptulosaric acid (DHA). Although it is present in exceedingly low amounts in the CW, its structure is very highly conserved among the flowering plants (Carpita and McCann 2000) (including the pteridophytes and lycophytes) suggesting that RGII appeared early in land plant CW evolution (Matsunaga et al. 2004). 3-*O*-methyl rhamnose has been detected in the CWs of charophytes, bryophytes and homosporous lycopodiophytes (Popper and Fry 2003).

Molecular analyses indicate that the embryophytes are derived from a common ancestor that would be classified with the charophytes. Analysis also indicates the bryophytes diverged early from the embryophyte line (Heisel et al. 1994; Waters et al. 1992). Very few comprehensive studies of the CWs of CGA have been undertaken. Detailed chemical profiling of CW macromolecules in charophytes and lower green

plants will be needed in order to accurately characterize evolutionary trends in CW structure and function.

Methods

Isolation and culture. *Cosmarium turpini*, and *Penium margaritaceum* were collected from a freshwater wetland in Porter Corners, NY (USA) and were subsequently cultured in the greenhouse facility of Skidmore College. *Chlorokybus atmophyticus* (LB2591) and *Klebsormidium flaccidum* (LB321) were obtained from the University of Texas Culture Collection (Austin, TX, USA). Both cultures were made axenic by treatment in a commercial anti-biotic/anti-mycotic cocktail (Sigma Chemical, St. Louis, MO, USA) using the manufacturer's instructions. In brief, cells were treated for 24 h in the antibiotic solution, washed repeatedly in sterile Woods Hole Medium (WHM) (Nichols 1973) and subsequently cultured (Domozych et al. 2007).

Defatting of the cell walls. The *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* cells were ground under liquid nitrogen (LN_2) and extracted with methanolic KOH (MeKOH) (Aronson and Lin 1978). Briefly, ground cells were treated with 5% KOH in 80% MEOH (KOH-methanol) at 98oC for 15 minutes, centrifuged and the pellet washed sequential with hot KOH-methanol, dH_2O, 1N acetic acid, and dH_2O several. This procedure was repeated until the pellet was white and then the pellet was then dialyzed against dH_2O for 48 hours and then lyophilized; this fraction was the methanolic KOH insoluble residue (MKIR). For all *Cosmarium turpini* and *Penium margaritaceum* cell were isolated at described by (Fry et al. 1985). Briefly, the cells were ground under

LN_2, place in 100% acetone at 21°C for 16h. Then the pellet was extracted with boiling 80% ethanol for 30 minutes and the washed with 80% and then 95% ethanol, dialyzed against dH_2O for 48 hours and then freeze-dried; resulting this was termed alcohol insoluble residues (AIR).

Fractionation of cell walls. Defatted CWs were fractionated using the methods of Fry (2001). MKIR/AIR were successively extracted with the following process: (i) dH_2O at 95°C for 1h followed by centrifugation at 700 x g for 10 min, and supernatant collected (repeated 3x) (HW-soluble fraction); (ii) 50mM CDTA (21°C , pH 7.5) for 6h, centrifuged as above, washed with dH_2O (3x) and supernatants collected, and dialyzed against 0.5M imidazole for 12h (CDTA soluble fraction); (iii) 50mM Na_2CO_3 containing 20mM $NaBH_4$ (1°C) for 16h, centrifuged as above, washed with dH_2O (3x), and supernatants collected (Na_2CO_3 soluble fraction); (iv) 1M KOH containing 20mM $NaBH_4$ for 2h at 1°C, centrifuged as above (repeated 2x), washed with dH_2O (3x), supernatants collected (1M KOH soluble fraction); (v) 4M KOH containing 20mM $NaBH_4$ for 2h at 1°C, centrifuged as above (repeated 2x), washed with dH_2O (3x), supernatants collected (4M KOH soluble fraction). The remaining CW remnant was washed with 0.5M imidazole (1x) and dH_2O (4x) and lyophilized. The supernatants from each stage were neutralized with acetic acid.

Compositional assays. Carbohydrate concentrations were determined using the phenol/sulfuric acid assay (Dubois et al. 1956) with glucose as the standard. Samples were suspended in water, phenol and H_2SO_4 were added, and absorbance was read at 490nm. Uronic acid was measured using the carbazole assay (Bitter and Muir 1962) with

a glucuronic acid standard. Samples were combined with the carbazole reagent ($Na_2B_4O_7$ and concentrated H_2SO_4) and the absorbance was read at 530nm.

Neutral sugar analysis. Neutral sugar analysis was performed by the methods described in Wustman et al. (1997). Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°C for 3 h, treated with 1 M NH_4OH for 2 h, reduced with $NaBH_4$ and acetylated as in Harris (1984) and Wustman et al. (1997). Gas chromatography/mass spectrometry (GC/MS) was performed using a SP-2330 column (30m, 0.25 mm i.d., isothermal at 230°C, injector 240°C) on a Finnigan-MAT Magnum mass spectrometer (San Jose, CA) operated in the electron-impact mode and alditol acetates were identified by their mass spectra and relative retention times to a *myo*-inositol internal standard. Quantification was based on response factors of standard monosaccharides that had been subjected to the same procedure.

Linkage analysis. Fractions containing uronosyl residues were treated with Dowex 50W-X12 (H+ form) prior to per-O-methylation (Waeghe et al., 1983) and reduced with 1 M lithium triethylborodeuteride in tetrahydrofuran for 1.5 h at 22°C prior to per-O-methylation (York et al. 1985). Per *O*-methylation of the polymers was carried out with butyllithium in DMSO and iodomethane (Stevenson and Furneaux 1991). Sep Pac C-18 reverse phase chromatography was used to purify the methylated polymers (Waeghe et al. 1983). Methylated polymers were hydrolyzed with 2M TFA at 121°C for 1.5 hrs and reduced with $NaBD_4$ in ethanolic ammonia. The per-*O*-methylated alditols were acetylated as in Harris et al. (1984) and separated on a SP-2330 column (30 m, 0.25 mm i.d., 150-245°C at 4°C/min, held at 245°C for 20 min, injector 240°C). Mass spectra

were obtained by GC/MS (Finnegan-MAT) operated in the electron-impact mode and used to detect column effluent. Linkage patterns were assigned based on previously determined diagnostic mass ion fragments and relative retention times (Jansson et al. 1976, Waeghe et al. 1983, Wustman et al. 1997, 1998) and compared to a library created by injection of standards of incompletely methylated alditol acetate derivates of each sugar. Darvill et al. (1980) glycosyl linkage/ substitution nomenclature was used. Quantification of per-*O* methylated alditol acetates were based on effective carbon response factors, as described in Sweet et al. (1975).

Analytical methods. Proton decoupled ^{13}C-NMR spectra were recorded with a Varian Unity INOVA 400 MHz NMR. Analyses were performed with a run time of 15.5 h at 45^{o}C, with a 90^{o} pulse width, 1 sec delay time, 0.64 sec acquisition time, and 8kHz Waltz for the decoupling of hydrogen. 30 mg of sample was dissolved in D_2O with an internal standard of DMSO (chemical shift 39.45 ppm). ^{13}C-NMR shift assignments were based on the work of Mukhiddinov et al. (2000) and Kramer et al. (2000).

Monoclonal antibody labeling. The CW fractions were analyzed by immune-dot assay using the methods of Willats et al. (2001). Briefly, five-fold serial dilutions (in dH_2O) were prepared from the fractions and applied to nitrocellulose as 1ul aliquots. The membranes were air-dried for approximately 16h at 21^{o}C. The membranes were treated with 5%M/PBS for 1h, then 1.5h in primary antibody solution. The monoclonal antibodies JIM5 or JIM7 were diluted 1/10 in 5% M/PBS. The nitrocellulose membranes were washed in PBS extensively prior to the incubation for 1.5h in secondary antibody solution. The secondary antibody was anti-rat horseradish peroxidase conjugate (Sigma),

diluted 1/1000 in 5% M/PBS and washed again extensively in dH_2O and PBS. Development was in the substrate solution (25ml dH_2O, 5ml methanol containing 10mg/ml 4-chloro-l-napthol, 30ul 6% (w/w) H_2O_2).

The monoclonal antibodies JIM5 and JIM7 were obtained from Plant Probes (Leeds, UK) and the protocol described by Domozych et al. (2007) was used. The controls for this study included eliminating the primary antibody incubation. Microscopic imaging was performed using an Olympus BX 61 light microscope equipped with the Fluoview 300 confocal laser system. CLSM Imaging was performed with either 20 x (Numerical aperture or NA= 0.5) or 60 x (NA= 1.4) objectives. An argon laser with 488 nm light was used for excitation for FITC visualization and a HeNe (G) laser with 543 nm light for TRITC visualization. The confocal aperture of 60 µ was used. Optical sectioning of 200 to 600 nm slices was performed and images of 800 by 600 pixels were captured as computer files. Reconstruction of slices into 3-D images and superimposition of FITC/TRITC- dual labeled cells employed Olympus Fluoview 300 software. CW were analyzed using standard fluorescence microcopy, an Olympus BX-60 light microscope (LM) was used for general and fluorescence microscopy and images were captured using an Olympus DP70 camera.

HPAEC-PAD System. *Cosmarium turpini* CDTA soluble fraction, *Chlorokybus atmophyticus* hot water soluble (HW) fraction and *Klebsormidium flaccidum* hot water soluble (HW) fraction were examined with the HPAEC-PAD with Dionex Bio-LC system which included a PAD 2 (gold working electrode) pulsed amperometric detector (E_1=0.15V, 480 ms; E_2=0.7 V, 120 ms; E_3 =-0.6V, 360ms), and a pressurized bottle post-

column delivery system that added 500mM potassium hydroxide prior to the detection. The Conditions for HPAEC-PAD were similar to those reported by Hotchkiss and Hicks (1990). This method allows for the separation of underivatized oligosaccharides up to DP 80 (Koizumi et al. 1989).

Results

Immunochemical localization. The CWs readily labeled with the monoclonal antibody JIM5 for the epitopes of unesterified galacturonosyl residues or flanking methyl-esterified residues (Clausen et al. 2003) (Figure 4.3). Dot blots with monoclonal antibody JIM5 labeled HW and CDTA fractions of both organisms. The tips of *K. flaccidum* labeled more intensely with JIM5 (Figure 4.3a, b). The cells walls of *C. atmophyticus* were labeled smoothly and fully by this antibody although labeled material may only be loosely associated with wall components (Figure 4.3e, d). The CW of *K. flaccidum* and *C. atmophyticus* did not label with the monoclonal antibody JIM7, which binds to the epitopes for methyl-esterified esterified galacturonosyl residues (Knox et al. 1990). *P. margaritaceum* labeling with monoclonal antibody JIM5 was interrupted by small, punctated, label-exclusion zones found over the CW surface and, in many cells, a narrow band in the isthmus zone (Figure 4.3c).

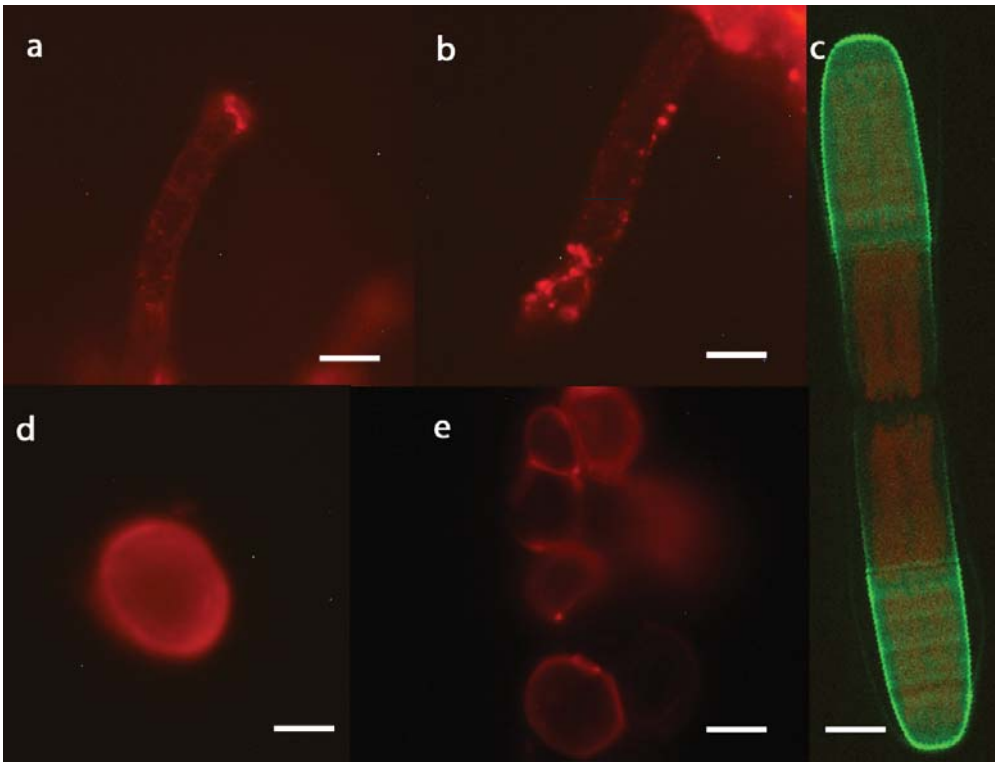

Figure 4.1: Cytochemical labeling with anti-homoglacturonan (HG) antibody JIM5. A and B) *Klebsormidium flaccidum;* the labeling (red) appears to be localilzated at the tip of the filaments, Scale bar = 10µm c) *Penium margaritaceum*, the anti-HG labeling in green and the autofluorescence of the chlorophyll (red) Scale bar = 10µm and D and E) *Chlorokybus atmophyticus* CW; labeling (red) appears to be at the periphery, maybe loosely associated wall components Scale bar = 20µm

Chemical composition. General pectin wall characteristics were examined for *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, *Cosmarium turpini* and *Penium margaritaceum*. The localization of the pectin polymers in the HW, the CDTA, and Na_2CO_3 fractions of the above species indicated that the HG found in the CGA is similar in structure and organization to that of higher plants (Figure 4.1, Table 4.1, and Figure 4.3). In *C. atmophyticus*, the following extracts were enriched in galacturonic acid at the

given percentage: HW at 49%, CDTA at 27% and Na_2CO_3 with much less at only 6% (Figure 4.1). The major linkage in *Ch. atmophyticus* in the HW fraction is 4-linked GalA with minor amounts of 2, 4-linked GalA. In the HW fraction of *K. flaccidum* the major glycosyl residue is 4-GalA with 53.6% saccharide detected. The major linkages of *Co. turpini* CDTA fractions are 4-GalA and 2,4-GalA with 15% of the saccharide content as GalA. 86.6% of saccharide detected in *Penium margaritaceum* CDTA extracts was GalA (Figure 4.1). Linkage analysis revealed a predominance of 4-linked GalA (4-GalA) (Table 4.1).

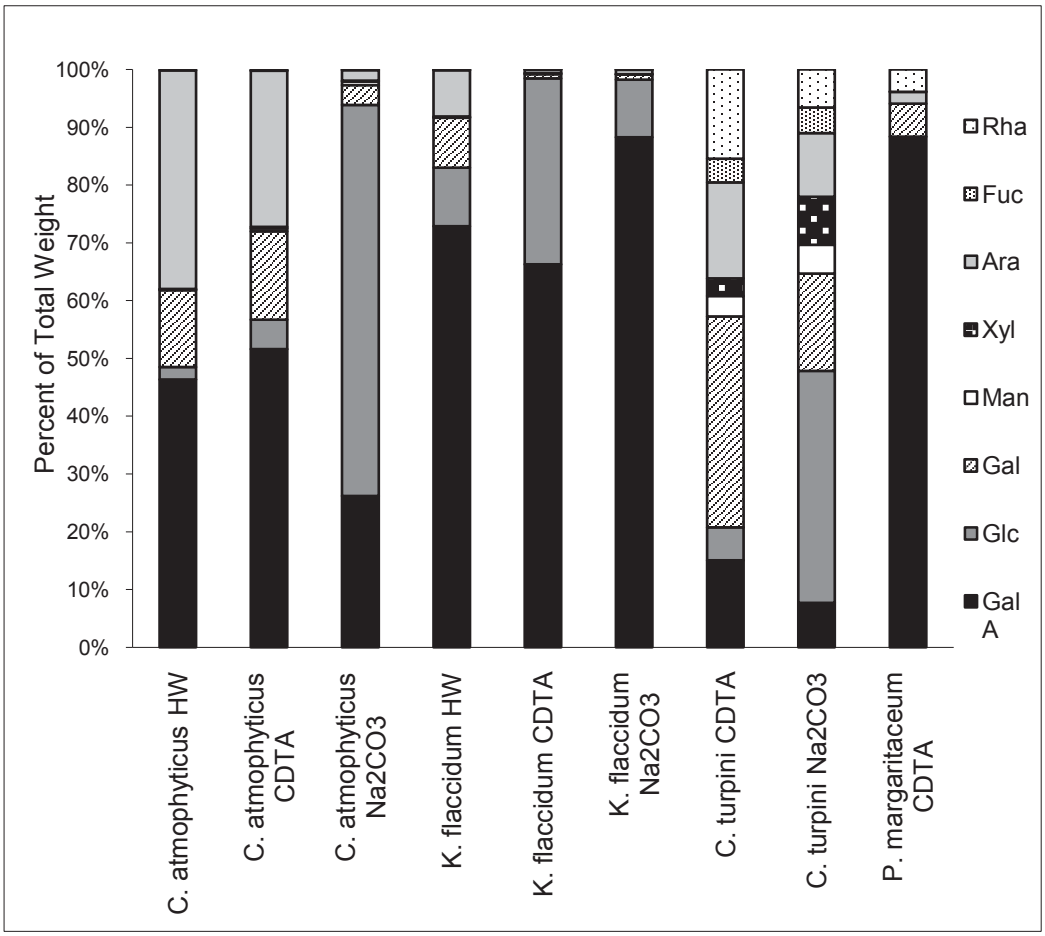

Figure 4.2: Monosaccharide profiles of pectin fractions expressed as percent detected neutral sugar.

Table 4.1 Glycosyl substitution patterns of pectin CW fractions. Glycosyl linkages reported as % mole detected. ur = Reduced prior to methylation

	Chlorokybus atmophyticus				*Klebsormidium flaccidum*		*Cosmarium turpini*	*Penium margaritaceum*
	HW (% mole)	HW (ur) (% mole)	CDTA (% mole)	Na_2CO_3 (% mole)	HW (% mole)	HW (ur) (% mole)	CDTA (ur) (% mole)	CDTA (ur) (% mole)
GalA								
t-	—	—	—	—	—	—	—	19.0
4-	—	43.0	—	—	—	18.8	**53.5**	68.0
2,4-	—	0.8	—	—	—	—	7.3	—
4,6-	—	—	—	—	—	—	—	5.0
GlcA								
3-	—	1.2	—	—	—	2.3	—	—
Glc								
t-	—	0.6	—	—	2.5	—	—	—
3-	—	0.9	—	—	—	0.6	—	—
4-	—	—	66.7	19.5	3.6	—	1.4	—
3,6-	—	—	—	—	0.9	—	—	—
2,4,6-	—	—	—	—	—	1.0	—	—
Gal								
t-	—	—	—	—	12.0	—	—	—
3-		0.7	3.9	2.6	1.6	2.9	—	—
4-	83.7	8.2	16.7	69.5	71.2	21.0	—	—
6-	—	—	—	—	—	—	3.7	8.0
2,3-	—	0.4	—	—	0.6	—	—	—
2,4-	—	0.2	—	—	—	2.0	—	—
2,6-	—	—	—	—	0.5	0.8	—	—
3,6-	—	1.1	—	0.8	—	1.7	—	—
4,6-	—	—	—	—	3.7	3.1	—	—
3,4,6-	—	—	—	—	0.2	—	7.9	—
Man								
2,3-	—	—	—	—	—	—	1.8	—
Ara								
t-	—	6.5	—	—	—	4.5	—	—
2-	5.5	20.2	12.7	6.2	2.4	18.7	—	—
4p-	—	3.2	—	—	—	9.1	—	—
2,4p-	—	11.6	—	—	—	6.6	—	—
2,3,4-	10.8	1.4	—	1.4	—	5.6	—	—
Fuc								
t-	—	—	—	—	0.7	—	—	—
3-	—	—	—	—	—	—	8.3	—
Rha								
2-	—	—	—	—	—	—	11.8	—
2,4-	—	—	—	—	—	—	3.2	—
2,3,4-	—	—	—	—	—	—	1.2	—

(ur) Prereduction prior to methylation.

* Fraction contain pectin by dot blots

Structural Analysis of HG. The due to the purity of the CDTA fraction of *Penium margaritaceum*, a thorough analysis was completed including ^{13}C-NMR (Figure 4.2). The ^{13}C-NMR spectrum confirmed the presence of HG in the CDTA fraction of *P. margaritaceum* with characteristic resonances for (1, 4)-D-GalA repeating units at 99.6, 63.4, 68.8, 78.6, 71.9 and 175.8 ppm, assigned to C1-C6, respectively, based on the work of Mukhiddinov et al. (2000) and Kramer et al (2000). Methyl esterification of the HG was indicated by shifts at 69.6 (C6) and 174.0 ppm (C5) as well as the characteristic methyl group shift at 55.2 ppm (Figure 4.2).

In addition to the galacturonosyl residues, the pectin fractions contain other neutral sugars. The *Penium margaritaceum* CDTA fraction also contained neutral sugars including galactose, rhamnose, arabinose, xylose, and fucose (Table 4.1). Gal represented 5.6% of the monosaccharide content with 6-Gal as the predominant galactosyl residue. Four other neutral sugars made up 7% of the total (Table 4.1). Unusual O-methylated sugars were detected, the most prevalent was 2-O-methyl-Fuc (2Me-Fuc) with lesser amounts of 2,3,4-tri-O-methyl-Ara (2,3,4Me-Ara), and 3-O-methyl-Xyl (3Me-Xyl). The presence of a small amount of 4,6-GalA indicated the potential for branching of the pectin polymer and several lower intensity resonances (96.7, 92.8, 77.9, 76.1, 73.5, 72.8, 72.3, 71.9, 71,4, 70.9, 70.2, 69.9, 69.5 and 68.8 ppm) were found in the NMR spectrum (Figure 4.2).

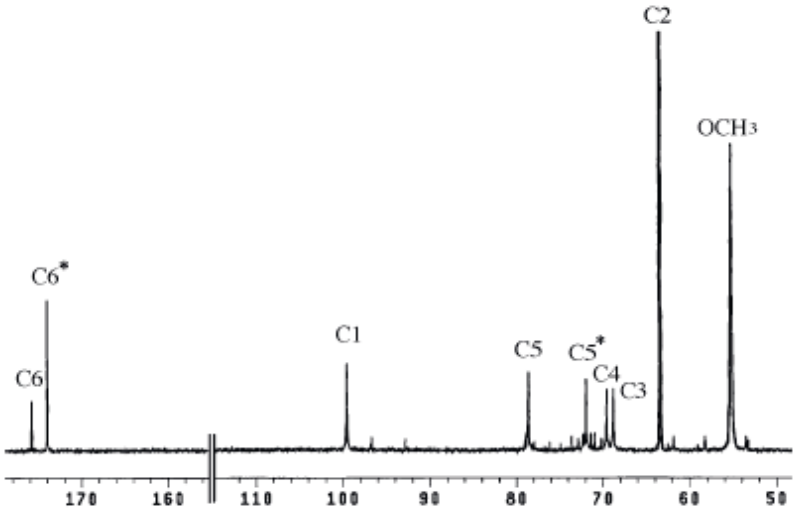

Figure 4.3: ^{13}C NMR spectrum of the CDTA-soluble fraction. Resonances characteristic of HG resonances (175.8, 99.6, 78.6, 71.9, 68.8, and 63.4 ppm) are labeled with the carbon equivalent. Methyl esterification is indicated by shifts in C-6* (174.0 ppm) and C-5* resonances (71.9 ppm). The lower intensity resonances which may indicate possible branching are 96.7, 92.8, 77.9, 76.1, 73.5, 72.8, 72.3, 71.9, 71.4, 70.9, 70.2, 69.9, 69.5, and 68.8 ppm

Evidence of other polymers in the cell walls of the CGA. In the *Cosmarium turpini* CDTA fraction, there are significant amounts of Gal (43%) with the predominant linkage being 6-Gal. In addition, Rha occurs with 2-, 2,4, and 2,3,4-linked residues; and Fuc with 3-linked residues. *Chlorokybus atmophyticus* HW fractions also contains significant neutral sugars including Ara (72%) as the major neutral sugar with terminal-, 2-, 4-, 2,4- and 2,3,4-linked arabinosyl residues, and Gal, which accounts for 23% of monosaccharide content, with 4-, 3-, 2,3-, 2,4- and 3,6-linked residues. The CDTA fraction also contains significant amounts of Ara and Gal, though the Na_2CO_3 extract is

enriched with Glc with 4-linked glucosyl residues. The neutral sugar present in *K. flaccidum* HW is Gal (with 4-Gal, terminal-, 4,6-, and 3-linked residues), Ara (with terminal, 2-, 4-, and 2,3,4-linked residues) and Glc (with terminal, 3-, and 4-linked residues).

The results of high-pH anion exchange chromatography with pulsed amperimetric detection (HPEAC-PAD) of *Cosmarium turpini* CDTA, *Klebsormidium flaccidum* HW, and *Chlorokybus atmophyticus* HW extract are shown in Figure 4.4 and give preliminary evidence for RGI in the three algal species. Peak B is an α-L-Rhap-(1→4)-α-D-GalA and peak A is an α-D-GalA oligosaccharide, based on retention times (Figure 4.4). For *C. turpini* HG, the three peaks in the location of an α-D-GalA oligosaccharide probably represents α-D-GalA oligosaccharides with different levels of esterification of. The *Cosmarium turpini* CDTA fraction contains almost equal amounts of rhamnose and galacturonic acid. This CDTA extract contains 2-, 2,4, and 2,3,4-linked rhamnosyl residues and 4- and 2,4-linked galacturonosyl residues. *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* contain minor amounts of rhamnose (0.5% and 0.4%), and no rhamnosyl residues were detected with methylation analysis (Table 4.2).

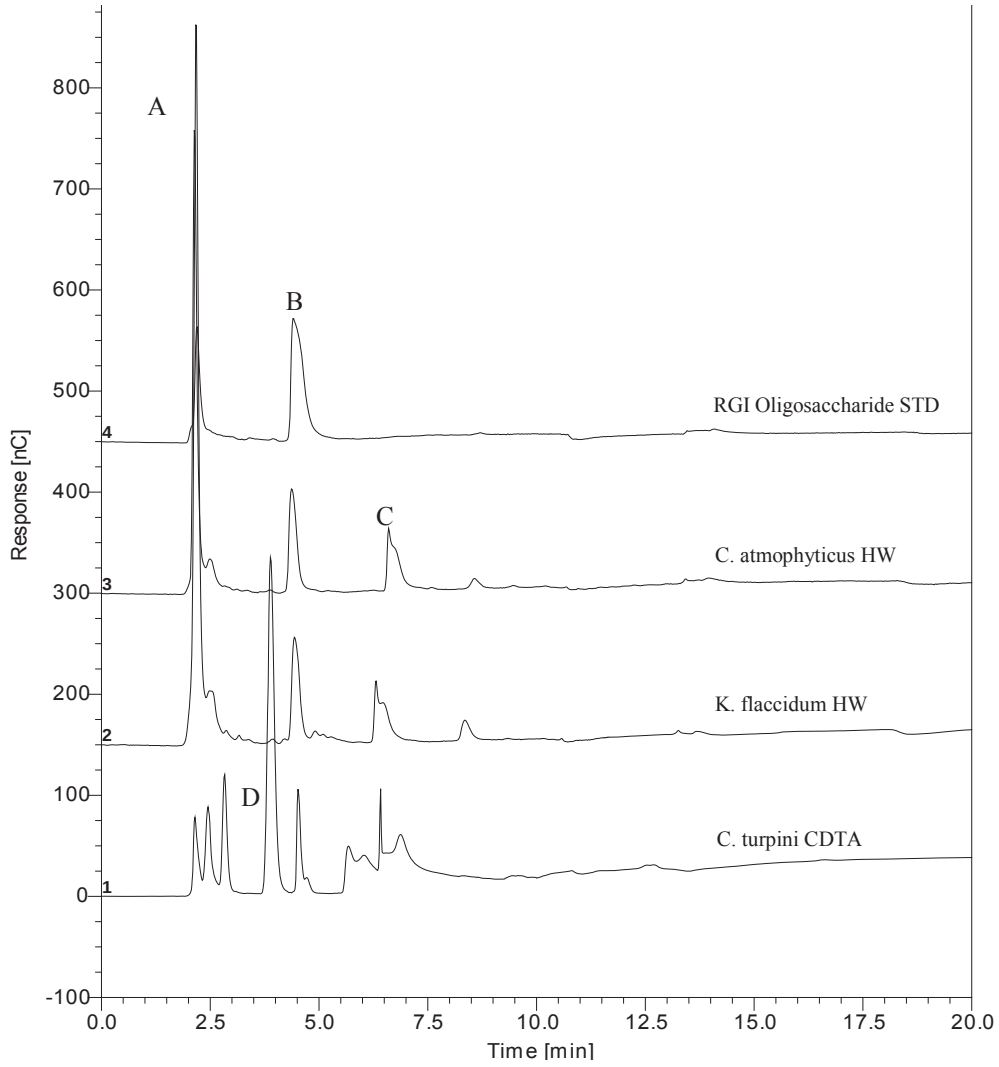

Figure 4.4: HPAEC analysis of rhamnogalacturonan oligosaccharide isolated from *Cosmarium turpini* CDTA, *Klebsormidium flaccidum* HW, and *Chlorokybus atmophyticus* HW extract. The rhamnogalacturonan oligosaccharides were confirmed based on retention times. Peak A is α-D-GalA oligosaccharide, peak B (at 4.5 min) is L-Rha-(1→4)- α -D-GalA, Peak C probably represents α -L-Rhap-(1→4)- α -D-GalA-(1→2)-L-Rhap. The large peak (Peak D) in the *Cosmarium turpini* CDTA is an esterified di-galacturonic acid.

Discussion

The pectin from *P. margaritaceum* appears to be a novel HG in the homogeneity of its base repeating unit. The CDTA extract was unique with 80% of polysaccharide a true homogalacturonan. The CDTA fraction of *Cosmarium turpini* also contained significant amounts of (1→4)-GalA, which also labeled with the antibody JIM5 indicating the presence of unesterified HG (Willats CoMMP data unpublished). Baylson et al. (2001) reported that *Closterium acerosum* showed that the CW contained a pectin-like polymer that was rich in galacturonic acid (60.1%). *Micrasterias denticulata* (Lütz-Meindl and Brosch-Salomon, 2000), and the mucilage of *Netrium digitus* (Eder and Lütz-Meindl 2009) were also shown to contain a pectin-like polymer under antibody labeling. The evidence of a true homogalacturonan in *P. margaritaceum* is the first chemical confirmation of HG in the Zygnematales. The charophyte alga, *Chara aculeolata*, contains an acid polysaccharide based on a fragment constructed of (1→4) α -D-galacturonic acid in the pyranose form. The pectin is characterized by a high homogeneity with a high amount of D-galacturonic acid (Cherno et al. 1978), similar to the HG found in *P. margaritaceum*.

There is evidence of HG in the basal species *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* as indicated by both biochemical and immunocytochemical testing. **This indicates the distinct possibility of plant-like HG in the basal CGA.** The HW extracts are rich in galacturonosyl residues, which account for 49% -54% of the polysaccharide. This is much higher than the low amounts of GalA found in another species of the CGA. Hotchkiss et al. (1989) found a low amount of uronic acid in

Mougeotia sp. CW with 4- and 2,4-arabinosyl linkages, suggesting that an arabinogalactan pectic carbohydrate may exist and indicating the possible presence of an RGII. Popper and Fry (2003) found that GalA was the major uronic acid in all the charophytes examined (*Klebsormidium flaccidum*, *Coleochaete scutata* and *Chara corallina*), a hornwort, thalloid and leafy liverworts and a basal moss. The concentration of GalA was higher in the charophycean green algae and bryophytes than in vascular plants. In the bryophyte family, antibody labeling with the monoclonal antibodies JIM5 and JIM7 indicated pectin in hornwort (*Megaceros gracilis*) and liverwort (*Rodula buccinifera*) (Kremer et al., 2004).

HG is an abundant and widespread domain of pectins. GalA residues in HG can be O-acetylated at C-3 and C-2; acetylated HG is abundant in sugar-beet roots and potato tubers (Ishii, 1997; Pauly and Scheller, 2000; Willats et al., 2001), there is no evidence of any acetylation of the C-3 and C-2 in *P. margaritaceum* HG. Apple pectins (Kikuchi et al., 1996), kidney bean cotyledon (Matsuura 1984), pea seed coats (Schols et al., 1995), watermelon fruit, and carrot cells consist of xylogalacturonan. The HG of *P. margaritaceum* contains xylose as a very small portion of the pectin (1.1%) A substitution of the C-4 with 20-80% rhamnose side chains are RG-I, this is not present in *P. margaritaceum* HG, those polymers consists of 3.8% rhamnose.

The preliminary evidence of the RG-I oligosaccharides in the pectin fractions of *C. turpini*, *C. atmophyticus*, and *K. flaccidum* would be the first report of this polymer in the CGA. The backbone of rhamnogalacturonan-I is composed of alternating 2-linked α-L-rhamnose and 4-linked α-D-galacturonic acid residues. The CDTA fraction of *C.*

turpini (Table 4.1) contains 15% GalA with both 4-linked galacturonsyl residues and 2,4-galacturonsyl residues; and 18% rhamnose with 2-linked rhamnosyl residues with 2,4-rhamnosyl residues. RGI carries side chains on the O4 position of rhamnose residues which are composed of primarily galactose and arabinose residues (Carpita and McCann 2000; Fry 2000) and *C. turpini* contains 43% galactose and 19.5% arabinose, which could be side chains of the RG-I polymer. In *Sphagnum* sp. walls, monosaccharide linkage analysis was performed and 4-GalAp, 2-Rhap, and 2,4-Rhap indicated the presence of pectin-like RG (Kremer et al., 2004). Meindl and Brosch-Salomon (2000) found that *Micraceterias denticulata* labels with the monoclonal JIM8 antibody (AGP and RGI epitope), which may indicate the presence of RG-I.

Chlorokybus atmophyticus and *Klebsormidium flaccidum* both contained the RG-I oligosaccharides, though linkage and monosaccharide analysis both indicate low amounts of rhamnose in the HW fractions. Less than 10% of the HG in *P. margaritaceum* contains side chains, as based on NMR and analytical chemistry. The presence of 4,6 GalA indicates a very small amount of branching such as would occur in RG-I. HG, RG-I, and its side chains are conserved and can be found simultaneously in the same wall (Marry et al 2000, Carpita and Gibeaut 1993, Albersheim et al. 1994, Fry 2000, Lau et al. 1985). RG-I is thought to be glycosidically attached to HG domains (Willats et al., 2001). The presence of possible RGI backbones in the group Zygnematales, Chlorokybales, and Klebsormidiales leads to the idea that possibly RGI is not an advanced character but a basic one.

The presence of homogalacturonan in CGA species is of considerable valuable in determining the type and nature of CW polymers that were fundamental in successful adaptation to terrestrial environments. This report of HG in *P. margaritaceum* represents the first confirmation of land plant-type pectin in desmids and the second rigorous characterization of a pectin polymer from the charophycean algae. The evidence of HG in *Chlorokybus atmophyticus*, *Klebsormidium flaccidum* and the desmid *Cosmarium turpini* leads to the idea that pectins in general and more specifically HG is a basic character in the CW of all of species in the plant lineage. They function of pectin in aquatic environments may include controlling porosity at the cell surface, providing charged surfaces which regulate both the pH and ionic status of the CW, promoting cell adhesion via their gel-like properties and serving as elicitors or signaling molecules in defense (Knox 2002; Mohnen 2008). The function as a hydrated 3-D matrix may have evolved in aquatic environments so the cells could avoid desiccation. The next step for investigating HG in the charophycean green algae is to look for the genes involved in pectin synthesis and maintenance including pectin biosynthetic transferases including GAUT1 (homogalacturonan alpha 1,4- galacturonosyl transferase) which produces HG and may also make the RGII backbone (*reviewed* in Mohnen 2008) and pectin methyl esterases (PME) which catalyze specific de-esterification of methyl esterified HG during deposition and modification of CW (Pelloux et al. 2007).

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Chapter 5: AGP-like polymer in *Chlorokybus atmophyticus* cell wall and *Spirogyra* sp EPS and an unique algal polymer in *Klebsormidium flaccidum*

Abstract

The extracellular matrix of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* sp. contains a great amount of biochemical diversity; it ranges from distinct land plant polymers to unique algal polymers. The majority of the neutral sugars in *C. atmophyticus* HW extract and *Spirogyra* EPS, along with the antibody labeling, reveal the distinct possibility of an arabinogalactan protein (AGP) present in the ECM of these organisms. AGPs represent a complex and diverse group of hydroxyproline-rich and highly glycosylated proteoglycans that are associated with the CW and plasma membrane of plant cells (Lee et al., 2005; Lamport et al., 2006; Johnson et. al., 2003; Showalter, 2001). The protein core is decorated with branched glycans based on (1-3),(1-6)-ß-galactan backbones with terminal residues of Ara or GlcA (Showalter, 2001). Although they are very common throughout land-plant taxa, the specific functions of AGPs are poorly understood and include roles in cell-cell recognition, adhesion, cell development, gametogenesis and embryogenesis. Glycosyl linkage analysis, neutral sugar analysis and immunocytochemical label suggest that *Chlorokybus atmophyticus*, *Klebsormidium*

flaccidum, and *Spirogyra* sp. have polymers similar to the carbohydrate domains decorating the AGPs of land plants.

Keywords: charophytes, evolution, arabinogalactan protein, extracellular polymetric substances, extracellular matrix, cell wall, charophycean green algae.

Introduction

The group of extant green algae most closely related to modern-day embryophytes are the Charophyceae (Streptophyta; Viridiplantae; McCourt et al. 2000, 2004, Turmel et al. 2007, Turmel and Lemieux 2006, Gontcharov et al. 2003, 2004, Mattox and Stewart 1984, Graham et al. 1991). The close phylogenic relationship of charophytes and land plants is based on ultrastructural, biochemical and molecular evidence derived from the study of modern forms (Graham 1993, McCourt 1995, Moestrup 1974, Pickett-Heaps and Marchant 1972). The molecular sequence evidence has established a close relationship of charophyceans to the land-plant ancestry (reviewed in McCourt et al 2000). In 2007, comparative analysis of chloroplast genomes sequences support the assertion that two sister basal taxa of the charophyte line are *Mesostigma* and *Chlorokybus* (Lemieux et al. 2007), with the Charales as a sister to the clade of Coleochaetales, Zygnematales and embryophytes (Turmel et al. 2007). Charophycean green algae include the orders of Mesostigmales, Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales, and Charales (Stewart and Mattox 1975, Mattox and Stewart 1977).

Chlorokybus atmophyticus has been placed taxonomically at the base of the charophytes with close affinity to *Mesostigma* (Turmel et al. 2006, 2007, Karol et al. 2001). *C. atmophyticus* is considered a rare freshwater or terrestrial alga. It has only been isolated from two terrestrial habitats (Rogers et al. 1981). Currently, it is the only extant genus present in Chlorokybales (Graham et al. 1991, Rogers et al. 1980). The thallus is a simple sarcinoid form, i.e., the cells are arranged in packets of 2-8 cells held together by a thick mucilaginous layer of carbohydrate and proteins. Each vegetative cell is surrounded by a cell wall (CW). During cell development, new CW material is only produced at the septum (Lockhorst et. al. 1988). This process is similar to that observed in more advanced CGA taxa. It is regarded as the simplest charophycean green algae having a nonmotile vegetative stage,

Klebsormidium is also classified as a basal taxon of the CGA and is the type genus of the order, Klebsormidiales. It is simple unbranched filamentous species found in soils and freshwater habitats. Cell division occurs by separation of the daughter nuclei during cross wall development with deep furrowing, an open mitotic spindle and an internuclear vacuole that separates daughter nuclei (Floyd et al. 1972, Cook 2004).

The Zygnematales represent the largest and most diverse group of the CGA. They are common inhabitants of freshwater habitats including pools, lakes, rivers and especially nutrient-poor mildly acidic streams and bogs. Many Zygnematalean taxa are considered model research systems for green plants and both singled-celled and filamentous species of this order have been used to investigate CW biosynthesis, cytoskeleton dynamics and other cellular and physiological features (Graham and Wilcox

2000; Bold and Wynne 1985). These algae are distinguished from other CGA most prominently by the fact that sexual reproduction occurs by conjugation, i.e., a process which involves nonflagellated gametes. During early development and cell expansion; the cell is often covered by a primary CW which is ultimately displaced by a thick secondary CW (Lutz-Meindl and Brosch-Salomon 2000, Hogetsu 1992). The CW proper is surrounded by an extensive thick sheath that is typically carbohydrate rich (Kiemle et al. 2007, Domozych et al. 1993, Domozych et. al. 2005). There are over 4,000 species classified as members of Zygnematales (Gerrath 2003). One of the most common forms is *Spirogyra*, an epiphytic filamentous form common in temperate pools, this genus consists of unbranched filaments with chloroplasts shaped like ribbons that spiral inside the cell. *Spirogyra* is very common (Bold and Wynne 1980). Sheath and Cole (1992) surveyed a wide variety of habitats and found it in tundra, temperate and rain forests and desert environments (already mentioned). In a North American continent-wide survey, McCourt et al. (1986) located it in at least 33% of 1000 locations sampled.

Evidence of land plant type polymers in the extracellular matrix of the charophycean green algae is growing. One of the most widespread polymers found from the angiosperms to the chlorophytes and has been found to function in various aspects of growth and development are arabinogalactan proteins (AGP). AGPs represent a diverse and complex group of hydroxylproline-rich and highly glycosylated proteoglycans that are associated with the CW and plasma membrane of plant cells (Lee et al., 2005; Lamport et al., 2006; Johnson et. al., 2003; Showalter, 2001). The protein core is decorated with branched glycans with (1-3),(1-6)-ß-galactan backbones with terminal

residues of Ara or GlcA (Showalter, 2001). There are two types of AG structures, type I AGs are associated only with pectins and are composed of (1→4)β-D-galactan chains with mostly t-Ara units at the O-3 of the Gal units. Type II AGs constitute a broad group of short (1→3) - and (1→6) β-D-galactans chains connected to each other by (1→3, 1→6)-linked branch point residues and are associated with specific proteins, called arabinogalactan proteins (AGPs).

Although they are very common throughout land plant taxa, the specific functions of AGPs are poorly understood and are believed to be involved in growth, development, fertilization, and apoptosis (Gao and Showalter 1999, Tan et al. 2004). A subclass of AGPs (fasciclins) is believed to be involved in cell adhesion and has been initially characterized in plants and green algae (Huber and Sumper 1994, Shpak et al. 2001, Johnson et al. 2003, Ito et al. 2005). The present investigation was initiated to elucidate the composition of the extracellular matrix of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* sp. and to compare the results with those reported for previously investigated members of the charophytes and the embryophytes.

Methods

Isolation and culture. *Chlorokybus atmophyticus* (LB2591) and *Klebsormidium flaccidum* (LB321) were obtained from the University of Texas Culture Collection (Austin, TX, USA). Both cultures were made axenic by treatment in a commercial anti-biotic/anti-mycotic cocktail (Sigma Chemical, St. Louis, MO, USA) using the manufacturer's instructions. In brief, cells were treated for 24 h in the antibiotic solution, washed repeatedly in sterile Woods Hole Medium (WHM) (Nichols 1973) and

subsequently cultured. After 5 days growth, aliquots of cells and extracts were removed and plated on a bacterial assay agar (Sigma) and viewed microscopically in order to determine if cultures were axenic. Axenic cultures were maintained in sterile WHM enriched to 5% with soil extract in 75 cm^2 tissue culture flasks (Fisher Scientific, Pittsburg, PA, USA). Cultures were kept in Bionette Growth Chamber (Fisher Scientific) set at 18^oC +/- 1^oC at a photoperiod of 14h light and 10 h dark with 35 W/m^2 of cool white fluorescent light. Subcultures were made every two weeks and cells from log-cultures (i.e., 3-6 weeks after sub culturing) were harvested for this study. Cells were collected by centrifugation at 2000 x g for 1.5 min in a clinical centrifuge (International Equipment Company, Needham Heights, MA, USA). The supernatants were discarded, the resultant pellets were resuspended in sterile WHM, shaken and centrifuged. This process was repeated three times.

Spirogyra EPS was isolated by the methods described in Domozych et al. (2005). Briefly, cells were collected by centrifugation at 3,000 x g for 6 min, 4^oC, in a Sorvall RC5C centrifuge equipped with a SS 34 rotor (Sorvall/Haraeus, Newtown, CT, USA). The supernatant with the EPS was collected and stored on ice. The cell pellet was resuspended in 20 ml of cold WHM, placed in a 100 ml bottle and vigorously hand-shaken for 1 min. The suspension was centrifuged as above, the supernatant was pooled with the first supernatant pool and the cell pellet was reshaken and centrifuged twice more. The pooled supernatants were placed in a 500 ml bottle, shaken vigorously for 2 min and recentrifuged. The supernatant was collected and LM observation of this EPS-containing supernatant revealed no cells or cell fragments. The supernatant pool was

dialyzed for 48 in 24 L of cold deionized water (dialysis tube cutoff= 3,500mw). The dialyzed supernatant was flash frozen in liquid nitrogen and freeze dried. This freeze dried material represented the EPS used for biochemical studies.

Defatting cell walls. Prior to analysis, the *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* cells were ground under liquid nitrogen (LN_2) and extracted with methanolic KOH (MeKOH) (Aronson and Lin 1978). For methanolic KOH, the CWs were treated with 5% KOH in 80% MEOH and then placed in a hot water bath for 15 minutes at 98^{o}C. The preparation was then centrifuged to remove the supernatant with the resultant pellet washed in the following sequence: hot KOH-methanol, water, 1N acetic acid, and, as a final rinse, water. This procedure was repeated until the pellet was white. The pellet was then dialyzed against dH_2O for 48 hours and then freeze-dried. This pellet was termed methanolic-KOH insoluble residue (MKIR). This produced the MeKOH supernatant termed later in the paper as colloidal EPS.

Fractionation of cell walls. Fractionations of CW polymers from defatted CWs were processed using the methods of Fry (2001). The pellet was successively extracted with the following process: (i) dH_2O at 95^{o}C for 1h followed by centrifugation at 700 x g for 10 min, and supernatant collection (repeated 3x) (HW-soluble fraction); (ii) 50mM CDTA (21^{o}C , pH 7.5) for 6h, centrifuged as in washed with dH_2O (3x) and supernatants collected, and dialysed against 0.5M imidazole for 12h (CDTA soluble fraction); (iii) 50mM Na_2CO_3 containing 20mM $NaBH_4$ (1^{o}C) for 16h, centrifuged as in washed with dH_2O (3x), and supernatants collected (Na_2CO_3 soluble fraction); (iv) 1M KOH containing 20mM $NaBH_4$ to prevent "peeling" for 2h at 1^{o}C, centrifuged as in (repeated

2x), washed with dH_2O (3x), supernatants collected (1M KOH soluble fraction); (v) 4M KOH containing 20mM $NaBH_4$ for 2h at 1°C, centrifuged as in (i) (repeated 2x), washed with dH_2O (3x), supernatants collected (4M KOH soluble fraction). The remaining CW remnant was washed with 0.5M imidazole (1x) and dH_2O (4x) and lyophilized. The supernatants from each stage were neutralized with acetic acid where appropriate, dialysed against dH_2O exhaustively at 4°C and lyophilized.

Compositional assays. Carbohydrate concentrations were determined using the phenol/sulfuric acid assay (Dubois et al. 1956) with Glc as the standard. Samples were suspended in water, phenol and H_2SO_4 were added, absorbances were read at 490nm. Uronic acid was measured using the carbazole assay (Bitter and Muir 1962) with a glucuronic acid standard. Samples were combined with the carbazole reagent ($Na_2B_4O_7$ and concentrated H_2SO_4) and the absorbances were read at 530nm. Ester sulfate was detected with methods of Craigie et al. (1984) with potassium sulfate as the standard. All glassware was acid washed (2% HCl). Samples were hydrolyzed with HCl (5:1 in 95% ethanol) for 2 hours at 100°C, diluted with H_2O, and an aliquot was combined with 0.5M HCl and $BaCl_2$ gelatin and the absorbances were read at 550nm. Protein content was measured with bovine serum albumin as the standard (Bradford 1976). Following a detergent solubilization, samples were combined with alkaline copper tartrate solution and Folin Reagent and the absorbances were read at 750nm.

Neutral sugar analysis. Neutral sugar analysis was performed by the methods described in Wustman et al. (1997). Samples were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121°C for 3 h, treated with 1 M NH_4OH for 2 h, reduced with $NaBH_4$ and

acetylated as in Harris (1984) and Wustman et al. (1997). Gas chromatography/mass spectrometry (GC/MS) was performed using a SP-2330 column (30m, 0.25 mm i.d., isothermal at 230^{o}C, injector 240^{o}C) on a Finnigan-MAT Magnum mass spectrometer (San Jose, CA) operated in the electron-impact mode and alditol acetates were identified by their mass spectra and relative retention times to a *myo*-inositol internal standard. Quantification was based on response factors of standard monosaccharides that had been subjected to the same procedure.

Linkage analysis. Fractions containing uronosyl residues were treated with Dowex 50W-X12 (H+ form) prior to per-O-methylation (Waeghe et al., 1983) and reduced with 1 M lithium triethylborodeuteride in tetrahydrofuran for 1.5 h at 22°C following per-O-methylation (York et al., 1985). Per *O*-methylation of the polymers was carried out with butyllithium in DMSO and iodomethane (Stevenson and Furneaux 1991). Sep Pac C-18 reverse phase chromatography was used to purify the methylated polymers (Waeghe et al. 1983). Methylated polymers were hydrolyzed with 2M TFA at 121^{o}C for 1.5 hrs and reduced with $NaBD_4$ in ethanolic ammonia. The per-*O*-methylated alditols were acetylated as in Harris et al. (1984) and separated on a SP-2330 column (30 m, 0.25 mm i.d., 150-245°C at 4^{o}C/min, held at 245°C for 20 min, injector 240°C). Mass spectra were obtained by GC/MS (Finnegan-MAT) operated in the electron-impact mode and used to detect column effluent. Linkage patterns were assigned based on previously determined diagnostic mass ion fragments and relative retention times (Jansson et al. 1976, Waeghe et al. 1983, Wustman et al. 1997, 1998) and compared to a library created by injection of standards of incompletely methylated alditol acetate derivates of each

sugar. Darvill et al. (1980) glycosyl linkage/ substitution nomenclature was used. Quantification of per-*O* methylated alditol acetates were based on effective carbon response factors, as described in Sweet et al. (1975).

Monoclonal antibody labeling. The monoclonal antibodies JIM13 and LM2 were obtained from Plant Probes (Leeds, UK) and the protocol described by Domozych et al. (2007) was used. The controls for this study included eliminating the primary antibody incubation. Microscopic imaging was performed using an Olympus BX 61 light microscope equipped with the Fluoview 300 confocal laser system. CLSM Imaging was performed with either 20 x (Numerical aperture or NA= 0.5) or 60 x (NA= 1.4) objectives. An argon laser with 488 nm light was used for excitation for FITC visualization and a HeNe (G) laser with 543 nm light for TRITC visualization. The confocal aperture of 60 μM was used. Optical sectioning of 200 to 600 nm slices was performed and images of 800 by 600 pixels were captured as computer files. Reconstruction of slices into 3-D images and superimposition of FITC/TRITC- dual labeled cells employed Olympus Fluoview 300 software. If the CW were analyzed using standard fluorescence microcopy an Olympus BX-60 light microscope (LM) the images were captured using an Olympus DP70 camera.

Results

Arabinogalactan protein (AGP) polymer characteristics were examined for the CWs of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and the extracellular polymeric substances of *Spirogyr*a EPS. Results from glycosyl linkage analysis, monosaccharide analysis, protein assay, and immunocytochemical analysis with

monoclonal antibodies against epitopes for AGP were utilized to compare the CWs and EPS of some of the CGA to the AGPs found in land plants.

Chlorokybus atmophyticus: Anti-AGP antibodies (JIM13, LM2) were used to determine the distribution of AGP in the walls and mucilage of *C. atmophyticus*. The cells were labeled with the antibodies JIM13, which recognizes AGP glycans (Knox et al. 1991), and LM2 which recognizes the glucuronic acid residues of extension/AGP (Smallwood et al. 1996), during live labeling (Figure 5.1a). This indicated the presence of AGP-like polymers on the surface of the cell. To determine whether the AGP-like polymer s were localized to the thick mucilage layer or in the CWproper, immunolabeling of MKIR were probed with monoclonal antibodies (JIM13 and LM2). The CWs also labeled with the AGP epitopes, indicating that the AGP is localized at the CW proper.

Immunocytochemical dot blots were used to establish where the AGP epitopes where localized. The whole CW, HW and CDTA fraction labeled with LM2 and JIM13, therefore through chemical analysis was performed on the HW and CDTA extract. The prominent neutral sugars in the HW extract were arabinose (72%) and galactose (23%). The arabinosyl residues in the HW fraction were terminal-, 2-, 4- 2, 4-, and 2, 3, 4-linked residues, the galactosyl residues included 3-, 4-2, 3-. 2, 4- and 3, 6-linked residues (Table 5.1). The protein content was low (4%) and there was an abundance of uronic acid (49%). Other neutral sugars present included low amounts of glucose (4% and in the terminal and 3-linked form) and trace amounts of mannose, xylose, rhamnose. The acidic

sugars included GlcA and GalA, the glycosyl linkages of 3-GlcA, 4-GalA, and 2,4-GalA (Table 5.1).

The CDTA soluble extract was also enriched with arabinose (60%) with 2-linked arabinosyl and galactose (28%) with 3- and 4- linked galactosyl residues. The protein content was low (3%) and there was an abundant of uronic acid (27%). The colloidal EPS or methanolic KOH supernatant of *C. atmophyticus* is composed of 11% sugar, of which the majority is glucose followed by arabinose and galactose (Figure 5.2).

Klebsormidium flaccidum: Anti-AGP antibodies (JIM13, LM2) were used to determine the distribution of AGP-like polymers in the walls and mucilage of *K. flaccidum*. The *K. flaccidum* only labeled with the antibody LM2, which recognizes the glucuronic acid of extension/AGP but did not label with JIM13. The CWs labeled following the extraction with methanolic KOH and HW (Figure 5.1), which would remove the mucilaginous layer. The labeling of the post HW extracted CW was patchy with only light coverage. The labeling was not continuous but instead distinctively inconsistent (Figure 5.1d). The HW extract contains almost an equal distribution of glucose (37%), galactose (32%) and arabinose (30%). The glucosyl linkages were mostly 4-linked with terminal linked and lesser amounts of 3-, 3,6- and 2,4,6-residues (Table 5.1). There were many of galactosyl residues; the major linkages included 4-, 4,6-, 3- and terminal residues. The most prevalent substitutions of arabinose are 2-linked with 4-, 2, 4-. 2, 3, 4- and terminal residues. In the HW fraction there are significant amounts of uronic acids with 4-galacturonosyl and 3-glucuronosyl residues. Other neutral sugars present included trace amounts of mannose, xylose, fucose and rhamnose (Figure 5.2).

The colloidal EPS of *K. flaccidum* is composed of equal parts glucose and galactose with minor amounts of mannose, xylose, arabinose and rhamnose (Figure 5.2).

***Spirogyra* sp. EPS:** The carbohydrate content of *Spirogyra* sp EPS is galactose, fucose, and glucose (Figure 5.2). When the EPS is fractionated, the HW extract is enriched with galactose and fucose but with trace amounts of glucose. Arabinose, fucose and galactose are the major sugars in the EDTA extract of the EPS (Figure 5.2). The amount of fucose and galactose drop with sequential fractionation and the amount of arabinose increases. The uronic acid content of the EPS also decreases from 23% in the HW extract of the EPS to 0.1% in the EDTA extract. The protein content varies from 11% in the HW extract and only 1.8% in the EDTA fraction of *Spirogyra* sp. EPS. When linkage analysis is performed on the whole EPS the major linkages are 4-Fuc with terminal-Fuc, 4-Glc, 2, 3, 6-Gal with 2, 3- and 2, 6 Gal. In the unfractioned EPS there is an abundance of galactosyl residues with the major linkage at 2,3,6- with lesser amounts of 2,3-, 2,6-, 3,4,6-, 2-, 3- and terminal residues. Fucose is the most prominent neutral sugar with 44% the major linkages as 4-Fuc with terminal and 2,3-linked fucosyl residues. There was 11% glucose in the EPS with prominent amounts of 4-gluc with minor amounts of 2, 3-Glc. Xylose constitutes 9% of the EPS with terminal and 4-linked residues. There is only 3% arabinose with 2-linked residues (Table 5.1).

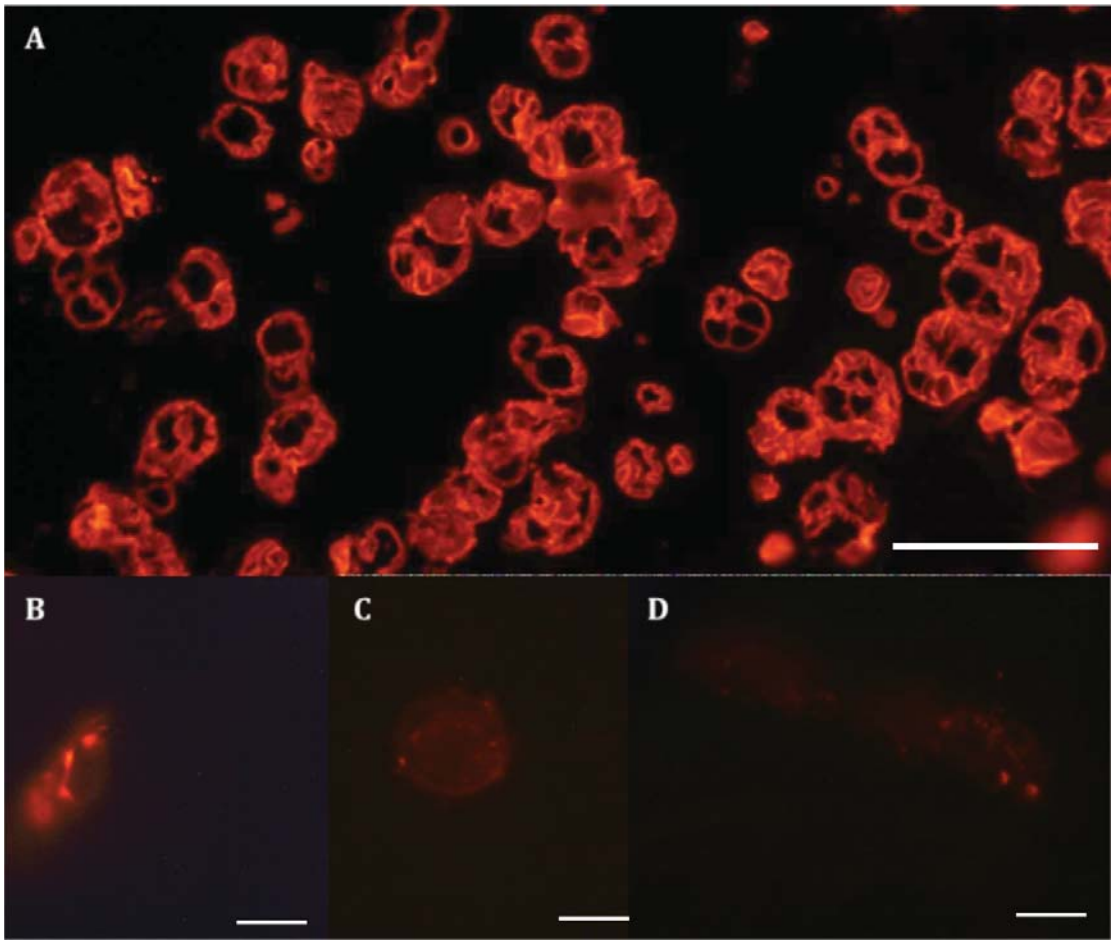

Figure 5.1: Cytochemical labeling for Arabinogalactan protein. A) *Chlorokybus atmophyticus* with anti-arabinogalactan protein antibody JIM13 Scale Bar=100µm , B) Wall methanolic KOH insoluble residue (MKIR) of *C. atmophyticus* labeled with anti-arabinogalactan protein antibody JIM13; Scale Bar=20µm C) Wall MKIR of *C. atmophyticus* labeled with anti-arabinogalactan protein antibody LM2; Scale Bar=20µm D) Wall residue -post HW insoluble residue of *K. flaccidum* labeled with anti-arabinogalactan protein antibody LM2; Scale Bar=20µm.

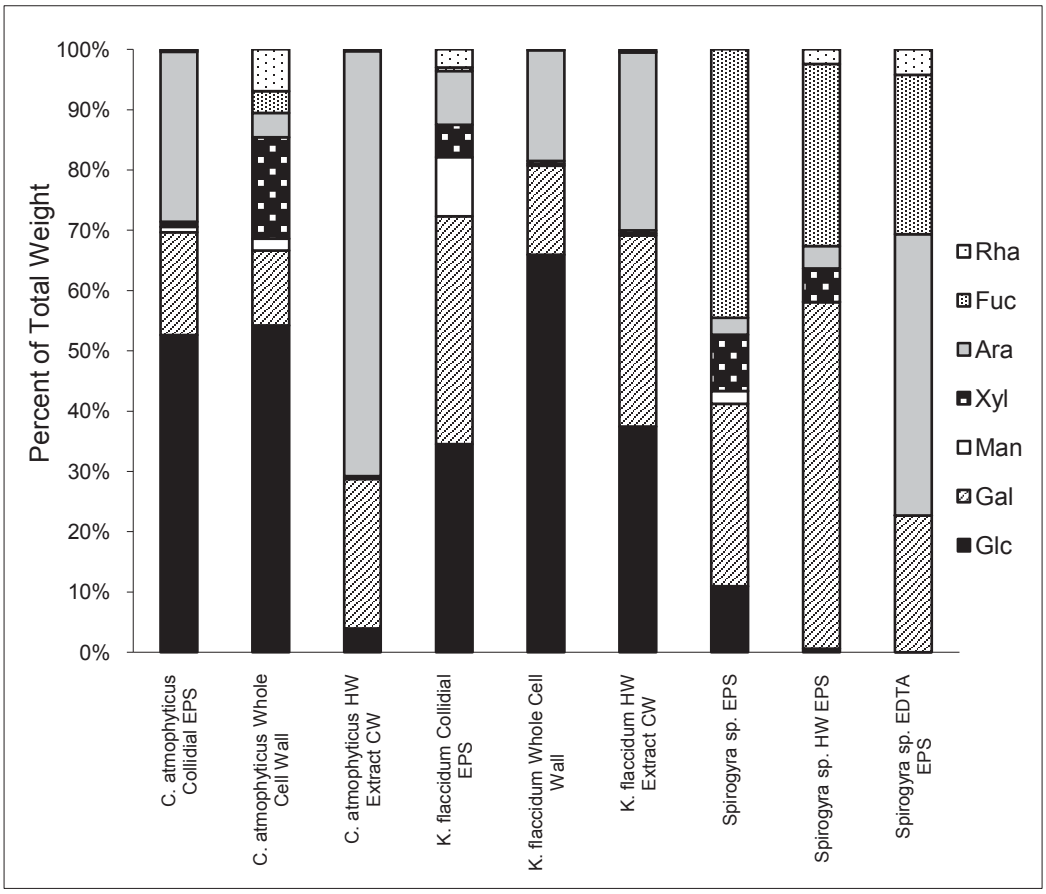

Figure 5.2: Percent neutral sugar detected in the different outer ECM carbohydrate fractions (colloidal EPS, whole CW, and HW soluble fraction of the CW of *Chlorokybus atmophyticus* and *Klebsormidium flaccidum*. Also the carbohydrate content of the different fractions of *Spirogyra* EPS (including unfractionated EPS, HW soluble EPS, and EDTA soluble EPS). Values are the mean, n=3

Table 5.1 Glycosyl substitution patterns of CW fractions. Glycosyl linkages reported as % mole detected. uronic = Reduced prior to methylation

	Chlorokybus atmophyticus		*Klebsormidium flaccidum*		*Spirogyra* spp.
	HW (%mole)	HW (uronic) (%mole)	HW (%mole)	HW (uronic) (%mole)	EPS (%mole)
Glc					
t-	—	0.6	2.5	—	—
3-	—	0.9	—	0.6	—
4-	—	—	3.6	—	4.9
2,3-	—	—	—	—	0.4
3,6-	—	—	0.9	—	—
2,4,6-	—	—	—	1.0	—
Gal					
t-	—	—	12.0	—	2.5
2-	—	—	—	—	1.6
3-	—	0.7	1.6	2.9	0.6
4-	83.7	8.2	71.2	21.0	—
2,3-	—	0.4	0.6	—	6.3
2,4-	—	0.2	—	2.0	—
2,6-	—	—	0.5	0.8	5.5
3,6-	—	1.1	—	1.7	—
4,6-	—	—	3.7	3.1	0.2
2,36-	—	—	—	—	18.3
3,4,6-	—	—	0.2	—	2.1
Man					
4,6-	—	—	—	—	0.4
Ara					
t-	—	6.5	—	4.5	—
2-	5.5	20.2	2.4	18.7	4.7
4p-	—	3.2	—	9.1	—
2,4p-	—	11.6	—	6.6	—
2,3,4-	10.8	1.4	—	5.6	—
Xyl					
t-	—	—	—	—	1.8
4-	—	—	—	—	2.9
Fuc					
t-	—	—	0.7	—	6.7
4-	—	—	—	—	36.9
2,3-	—	—	—	—	3.9
Rha					
2-	—	—	—	—	0.2
GalA					
t-	—	—	—	—	—
3-	—	1.2	—	2.3	—
4-	—	43.0	—	18.8	—
2,4-	—	0.8	—	—	—

Discussion

The extracellular matrix of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* sp. contain a great amount of biochemical diversity, ranging from distinct land plant polymers to unique algal polymers. The majority of the neutral sugars in *C. atmophyticus* HW extract and *Spirogyra* EPS, along with the antibody labeling, reveal the distinct possibility of an arabinogalactan protein (AGP) present in the ECM of these organisms. The rhizoids of *Spirogyra* sp labeled with the arabinogalactan protein antibody JIM16 (Sorenson et al. 2010) and the CW of *Chlorokybus atmophyticus* labeled with the arabinogalactan protein antibody JIM13 (Figure 5.1). The secretory vesicles and primary CWs of *Micrasterias denticulata* label with JIM8, JIM13, and JIM14 (Eder et al. 2008). Domozych et al. (2007) showed that the attachment centers of *Pleurotaenium trabecula* label with the monoclonal antibodies JIM13 and JIM15. *Klebsormidium flaccidum* labeled with LM2 but not JIM13 which leads to the question of the true plant-like AGP. LM2 antibody recognizes the glucuronic acid residues of extensin and labels AGP material only with a size between 100-180kDa and not the lower molecular mass polymers (Samaj et al. 2000). The primary CW surface of *Oedogonium bharuchae* labeled with the monoclonal antibody LM2 and the outer layer of the primary CW labeled with MAC-207 antibody (Pattathil et al. 2010).

Recent evidences suggests that AGPs in the CGA functions in adhesion and in cell development. Lutz-Meindl and Brosch-Salomon (2000) showed that *Micraceterias denticulata* labeled the primary CW at all stages of a non-growing semi-cell with JIM 1 (AGP). The AGP core protein of *Physcomitrella patens* was sequenced and the encoding

putative ESTs show 29% amino acid similarities to the sequences from *Arabidopsis*, *Brassica napus* and *Oryza sativa* (Lee et al. 2005). AGPs have been shown to have a role in apical cell expansion in protonema of *Physcomitrella patens* (Lee et al. 2005) , the tips of apical cells of *P. patens* labeled the monoclonal antibody LM6 for the 1,5-α-L-arabinan epitopes. Biochemical and bioinformatics analyses were used to identify seven *P. patens* ESTs encoding putative AGP core proteins from homology with *Arabidopsis thaliana*, *Brassica napus*, and *Oryza sativa* sequences and from peptide fragments isolated from βGlcYR-precipitated AGPs.

The protein proportion of the AGP-like polymers is low in all three species, ranging from 4-11%. The typical protein moiety of AGP is divided into two groups depending on their core protein. The classical AGPs are defined by the protein core which contains hydroxyproline, alanine, serine, threonine, and glycine. The nonclassical AGPs have a diversity of protein cores including hydroxyproline-poor AGPs and cysteine-rich AGPs (Showalter 2000).

The backbone of AGP is linked to arabinose with smaller amounts glucuronic acid, galacturonic acid, mannose, and rhamnose. The size varies from 30-150 sugar residues although 90-99% of the molecular mass of the AGP may be carbohydrate. There is a smaller amount of galactose than arabinose in the *Chlorokybus atmophyticus* HW soluble fraction and *Spirogyra* sp. EPS (Figure 5.2) contains 72% Ara, 23% Gal, with 4% protein and the CDTA soluble fraction contains 60% Ara, 28% Gal with 27% uronic acid with 3% protein. Linkage analysis reveals t- and 3-linked galactosyl residues and t, 2- and 2,4-linked arabinosyl residues. *K. flaccidum* HW extract contains 37% Glc,

32% Gal and 30% Ara. The major linkages include: t-, 3-, 4- and 3,6-glucosyl residues, t-, 3-, 4-, 3,6- and 4,6-galactosyl, and t-, 2-, 4-, and 2,4-arabinosyl residues. The linkage composition of precipitated AGPs from *P.* patens was rich in 3,6-Gal, 3-Gal, 5-Araf, t-Araf, t-Rha, and 4-GlcA (Lee et al. 2005), which is similar to the HW fraction of *K. flaccidum*. The polysaccharides portions of proteoglycans of *Micrasterias denticulata* were mostly xylose and galactose.

Fucose is the most abundant sugar present in *Spirogyra* EPS (Figure 5.2); it is also a polysaccharide that is copious in the EPS of other members of the Zygnematales. The substitution patterns of fucose vary drastically in the desmids with *Cosmarium* sp. (2,3,4-Fuc), *Pleurotaenium trabecula* and *Tetmemorus brebissonii* (3,4-Fuc) (Kiemle et al. 2007). The EPS of *Closterium* sp (Domozych et al. 1993), *Sp. panduriformis* (Paulsen and Viera 1994) and *Penium margaritaceum* contain high amounts of 4-linked fucosyl residues similar to the EPS of *Spirogyra* sp. The predominance of Fuc units in the EPS was significant because deoxy-sugars have been shown to have hydrophobic properties that may help maintain the gel-like matrix of the EPS sheaths (Wustman et al. 1997, 1998, Fattom and Shilo 1984, Hu et al. 2003, Hokputsa et al. 2003).

The AGP-like polymers of *Chlorokybus atmophyticus* and *Klebsormidium flaccidum* co-localized with the pectin fractions. Therefore some of the residues in the HW extracts are actually pectin polymers including homogalacturonan and possibly rhamnogalacturonan I including the 4-GalA and 2,4-GalA. (Kiemle et al. 2010, see chapter 4). In *P. patens AGP* there were evidences of high levels of 3-O-methylrhamnose in the terminal form. Popper and Fry (2003) found the presences of 3-O-methylrhamnose

in *Chara corallina*, *Coleochaete scuata*, and *Klebsormidium flaccidum*. It addition to the charophytes it was also found in bryophytes, pteridophytes and gymnosperms (Matsunaga et al. 2004, Popper and Fry 2003, 2004). Though because AGP sometimes co-localized with pectin polymers and 3-meRha is also founds as a side chain of RGII this brings up the question of the whether 3-O-methyl rhamnose is a pectin side chain that glycosylly linked to the AGP carbohydrate domain.

Biochemical and immunocytochemical evidence suggests that AGP-like polymers occur in all land plants and their charophyte ancestors, however there are probably functional differences, along with structural changes, in the protein and carbohydrate moieties. The carbohydrate moiety of AGPs may be instrumental in the evolution of the major groups of land plants and found all the down it the basal species of the group. The extracellular matrices of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* sp. contains a great amount of biochemical diversity, range from distinct land plant polymers to unique algal polymers. The majority of the neutral sugars in *C. atmophyticus* HW extract and *Spirogyra* EPS, along with the antibody labeling, reveal the distinct possibility of an arabinogalactan protein (AGP) present in the ECM of these organisms.

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Chapter 6: The extracellular polymeric substances of desmids (Conjugatophyceae, Streptophyta): chemistry, structural analyses and implications in wetland biofilms.

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Abstract

Desmids represent a group of advanced green algae that are commonly found in biofilm communities of freshwater wetlands. Desmids secrete significant amounts of extracellular polymeric substances (EPS) that form an extensive mucilaginous sheath external to the cell wall and function in adhesion, gliding-based movements and ultimate ensheathment within the biofilm complex. We have initiated biochemical and structural analyses of the EPS of desmids isolated from biofilms from the southeastern Adirondack region of New York including *Penium cylindris*, *Penium spirostriolatum*, *Cosmarium* sp. 1, *Cosmarium* sp. 2, *Pleurotaenium trabecula*, *Tetmemorus brebissonii*, *Netrium digitus*, *Netrium oblongum*, *Netrium interruptum*, and *Netrium interruptum* 2509. *Cosmarium* sp.

1 EPS appeared as a homogeneous sheath that encapsulated the cells, whereas *Pleurotaenium trabecula* EPS occurred as dispersed patches and *Netrium oblongum* EPS was reticulated and striated. Polysaccharides were the major component of the EPS (52-76%) and lectin labeling revealed differences in polymer organization between saccoderm (labeled with Con-A, WGA and HPA) and placoderm (labeled with BS-I, PSA, UEA, and HPA) desmids. Desmid EPS had significant uronic acid (3-29%) and protein (2-10%) content and the polysaccharides were sulfated to varying degrees. Xylose and Fucose were the predominate monosaccharides with the major glycosyl linkages t-Xylp, 3,4-Fuc, and t-Fuc. The unique EPS from *Netrium oblongum* was rich in galactose and uronic acid (29.3% w/w), extracellular polymers of *Penium spirostriolatum* were composed predominately of arabinose, and *Tetmemorus brebissonii* EPS was enriched in glucose and galactose. EPS of *Pleurotaenium trabecula* and *Tetmemorus brebissonii* was highly sulfated (10.2% and 14.7%, respectively). The EPSs from New York and UTEX strains of *Netrium interruptum* were unique from each other. Overall, desmid EPS exhibits a conserved motif with the predominant component an anionic polysaccharide. The presence of deoxy-sugar subunits indicates the potential for hydrophobic interaction where anionic components may play important role in ionic cross linking. The establishment of this baseline biochemistry provides the foundation for future dissection of core EPS molecules and associated functional groups and studies of EPS involvement in specific stages of biofilm development (e.g. adhesion).

Key Words: Biofilms, Carbohydrate chemistry, Conjugatophyceae, *Cosmarium*, Desmid, Extracellular polymeric substances, EPS, lectin labeling, *Netrium*, *Penium*, *Pleurotaenium*, Streptophyta, *Tetmemorus*, Wetlands

Introduction

In many oligotrophic or dystrophic freshwater ecosystems of the Northern Hemisphere, including diverse types of wetlands, desmids (Conjugatophyceae, Streptophyta) may display the greatest species diversity of any algal group. Desmids are part of a large taxon of green algae in the charophycean line of green algal evolution that are typically distinguished by conjugation-based sexual reproduction (Gontcharov et al. 2003, Stewart and Mattox 1984, Gerrath 1993, 2003). Two types of desmids have been traditionally recognized, saccoderm desmids, which have a one-piece CW and placoderm desmids, which have CWs in two or more pieces. Recent molecular studies have questioned this type of classification especially in light of the close phylogenetic affinity of saccoderm desmids to conjugating filamentous green algae (McCourt 1995, McCourt et al. 2000, Gontcharov et al. 2004). However, approximately 950 species of desmids have been reported to date in North America (Gerrath 2003). Wetland desmids are found in both benthic and epiphytic biofilms and often in significant numbers (Wantanabe et al. 2000, Woelkerling and Gough 1976a, b, Burkholder and Sheath 1984, Browder et al. 1994). Recently, in an ongoing survey of biofilms of shallow fens of the southeastern Adirondacks, we found that desmids constituted, on average, 25% of the total algal

population of biofilms formed on soft substrates (pers. obs.). Although desmids may be major components of freshwater biofilms and contribute significantly to community dynamics (e.g. photosynthesis/primary productivity/food chains, nitrate/phosphate/mineral biogeochemistry and substrate stabilization), we know very little about the specifics of their incorporation into aquatic biofilms or their biochemistry/physiology therein.

Biofilms are complex and cooperative consortia of surface-dwelling microorganisms. Biofilm dynamics are often centered around a distinct macromolecular matrix called the extracellular polymeric substance or EPS, which is secreted outside the CW. This complex of biopolymers typically ensheaths the biofilm community and provides far more stable conditions for resident microorganisms than that of the planktonic realm (Costerton 2004, 2000, Davey and O'Toole 2000, Watnick and Kolter 2000, Wimpenny 2000, Sutherland 2001a, b). The EPS constitutes a hydrated, 3-dimensional network of polymers and serves biofilm microbes in adhesion to substrates, physical protection from the overlying water column, motility mechanisms, protection against environmental stressors, various physiological and biochemical processes and as a conduit for interspecies interactions (Rittmann 2004, Wingender et al. 1999, Leppard 1995). The EPS may also serve as a C- (carbon) and N- (nitrogen) reserve, create reservoirs for adsorbed metals and organic materials, and form barriers to solute translocation, desiccation, biocides and UV light (Massieux et al. 2004). The EPS often constitutes the bulk of biomass of the biofilm and may be the major secreted component of the microbial residents (Decho 1990). The macromolecules that constitute the EPS of

biofilm microbes are diverse, complex, and in many cases, poorly understood. In prokaryotes, polysaccharides, proteins and lipids dominate EPS chemistry (Starkey et al. 2004, Sutherland 2001c). In most biofilm eukaryotes, the EPS typically contains polyanionic polysaccharides (i.e., containing sulfate esters or/and uronic acids) along with a small but important set of proteins (e.g. Hoagland et al. 1993). Yet, we are only in an infancy stage in our understanding of the specific biochemistry of eukaryotic EPS macromolecules, key substituent groups that define their function and the developmental mechanisms that regulate the secretion of these biopolymers.

In desmids, EPS is often produced in substantially large amounts and manifests itself in the form of gels, mucilages and slimes (Boney 1980, 1981, Domozych et al. 2005, Domozych et al. 1993, Linde et al. 2001, Paulsen and Vieira 1994). The EPS is processed through the Golgi Apparatus and associated vesicle network and is secreted through a network of channels or pores that are precisely positioned within the complex CW (Oertel et al. 2004, Domozych and Domozych 1993).

Two types of EPS have been identified in desmids to date: a) an encapsulating EPS that fully or partially engulfs the cells, and b) motility-associated EPS (Oertel et al. 2004). Motility-type EPS is secreted in specific locations on the cell surface. Upon release to the outside of the CW, hygroscopic swelling of the EPS is postulated to provide the force to move or "glide" the cell across the substrate surface (Domozych and Domozych 1993). Once secreted, desmid EPS has other purported functions including: 1) motility (Domozych et al. 2005, Domozych and Domozych 1993); 2) adhesion (Aubert et al. 1989); 3) phototactic behavior (Hader 1981, Hader and Wenderoth 1986); 4)

capture of scarce nutrients; 5) maintenance of organic phosphate metabolism (Spijkerman and Coesel 1996, 1997, 1998; Coesel 1994); 6) protection against desiccation; 7) protection against UV damage (Boney 1980, 1981; Gerrath 1993; Meindl and Lutz 1996); 9) low temperature adaption; 10) protection against grazers (Coesel 1997; Boney 1981; Porter 1993; Wilcox and Graham 2000); and 11) provides substrate for association with other microbes (Fisher and Wilcox 1996; Fisher et al. 1998a, b)

In an ongoing study of desmids and their extracellular matrices, we became interested in the role of desmid EPS in biofilm activities. We are delineating the different types of polysaccharides found in desmid EPS, their synthesis and secretion and ultimately, their role in such activities as adhesion, gliding and providing the structural framework for interspecies communication networks in wetland biofilms. In this paper, we report the results of a survey of the chemistry and organization of the complex polysaccharide-dominated EPS of a taxonomically-diverse assortment of desmid species isolated from biofilms of shallow wetlands of the southeastern Adirondack region of New York. This study serves as a framework for our general understanding of desmid EPS macromolecules and as the foundation for the dissection of specific polysaccharides and their role in biofilm dynamics.

Methods

Isolation and culture of desmids. Desmids were obtained from native biofilms growing on plexiglass substrates in a local beaver-engineered wetland in the southeastern Adirondack region of New York State (Porter Corners, New York USA). *Penium cylindris* (Ehren), *Penium spirostriolatum* (Barker), *Cosmarium* sp. 1, *Cosmarium* sp. 2,

Pleurotaenium trabecula (Ehren), *Tetmemorus brebissonii* (Menegh), *Netrium digitus* (Ehren), *Netrium oblongum* (DeBary), and *Netrium interruptum* (Breb) were isolated from biofilms as single cells using pulled glass pipettes. Clonal cultures of each taxon were successfully established (i.e., cells divided and grew), using Woods Hole Medium (WHM, Nichols 1973) at 20^{o}C +/- 1^{o}C in a 12:12-h light: dark cycle with 35 Wm^{-2} of cool white fluorescent light. WHM was chosen as the medium for our cultures because it yielded high growth rates and sustained all of the desmids used in this study. Aliquots of cells were treated with an antibacterial cocktail (A5955, Sigma Chemical, St. Louis, MO, USA) and then cultured in fresh WHM. *Netrium interruptum* (2509) was obtained from the UTEX culture collection (Austin, TX, USA) and treated as above. Axenicity of each clonal algal culture was subsequently confirmed by standard plating of algal cultures onto nutrient agar (Voight Global, Kansas City, MO, USA) and by microscopic observation of cells labeled with SYTO-9 (Molecular Probes, Eugene, OR, USA). Large cultures were maintained in 125 ml aliquots of WHM in 250 ml flasks and grown as above. In order to acquire large amounts of EPS, mass cultures of cells were grown in 50 ml aliquots in 125 ml plastic tissue culture flasks.

EPS was isolated by the methods described in Domozych et al. (2005). Briefly, cells were collected by centrifugation at 3,000 x g for 6 min, 4^{o}C, in a Sorvall RC5C centrifuge equipped with a SS 34 rotor (Sorvall/Haraeus, Newtown, CT, USA). The supernatant with the EPS was collected and stored on ice. The cell pellet was resuspended in 20 ml of cold WHM, placed in a 100 ml bottle and vigorously hand-shaken for 1 min. The suspension was centrifuged as above, the supernatant was pooled

with the first supernatant pool and the cell pellet was reshaken and centrifuged twice more. The pooled supernatants were placed in a 500 ml bottle, shaken vigorously for 2 min and recentrifuged. The supernatant was collected and LM observation of this EPS-containing supernatant revealed no cells or cell fragments. The supernatant pool was dialyzed for 48 in 24 L of cold deionized water (dialysis tube cutoff= 3,500mw). The dialyzed supernatant was flash frozen in liquid nitrogen and freeze dried. This freeze dried material represented the EPS used for biochemical studies.

Compositional assays. Carbohydrate concentrations were determined using the phenol/sulfuric acid assay (Dubois et al. 1956) with Glc as the standard. Samples were suspended in water, phenol and H_2SO_4 were added, and absorbances were read at 490nm. Uronic acid was measured using the carbazole assay (Bitter and Muir 1962) with a glucuronic acid standard. Samples were combined with the carbazole reagent ($Na_2B_4O_7$ and concentrated H_2SO_4) and the absorbances were read at 530nm. Ester sulfate was detected with methods of Craigie et al. (1984) with potassium sulfate as the standard. All glassware was acid washed (2% HCl). Samples were hydrolyzed with HCl (5:1 in 95% ethanol) for 2 hours at 100^{o}C, diluted with H_2O, and an aliquot was combined with 0.5M HCl and $BaCl_2$ gelatin and the absorbances were read at 550nm. Protein content was measured with bovine serum albumin as the standard (Bradford 1976). Following a detergent solubilization, samples were combined with alkaline copper tartrate solution and Folin Reagent and the absorbances were read at 750nm.

Monosaccharide analysis. Neutral sugar analysis was performed by the methods described in Wustman et al. (1997). Briefly, samples were hydrolyzed with 2 M

trifluoroacetic acid (TFA) at 121°C for 3 hrs, saponified with 1 M NH_4OH for 2 hrs, reduced with $NaBH_4$ and acetylated as in Harris et al. (1984) and Wustman et al. (1997). Gas chromatography/mass spectrometry (GC/MS) was performed using a SP-2330 column (30m, 0.25 mm i.d., isothermal at 230°C, injector 240°C) on a Finnigan-MAT Magnum mass spectrometer (San Jose, CA) operated in the electron-impact mode and alditol acetates were identified by their mass spectra and relative retention times to a *myo*-inositol internal standard. Quantification was based on response factors of standard monosaccharides that had been subjected to the same procedure.

Linkage analysis. Linkage analysis was done as described in Wustman et al. (1997). Uronic acid containing polymers were protonated with Dowex 50W-X8 resin (Sigma Aldrich, St. Louis, MO, USA) and pre-reduced following methylation and before hydrolysis. Per *O*-methylation of the polymers was carried out with 1.6 M butyllithium in DMSO and iodomethane (Stevenson and Furneaux 1991). Sep Pac C-18 reverse phase chromatography was used to purify the methylated polymers (Waeghe et al. 1983). Samples were hydrolyzed with 2M TFA at 121°C for 1.5 hrs and reduced with $NaBD_4$ in ethanolic ammonia. The per-*O*-methylated alditols were acetylated as in Harris et al. (1984) and separated on the GC/MS as described for monosaccharide analysis with column parameters 150°C to 245°C at 4°C/min with an isothermal hold at 245°C for 20 min. Linkage patterns were assigned based on previously determined diagnostic mass ion fragments and relative retention times (Jansson et al. 1976, Waeghe et al. 1983, Wustman et al. 1997, 1998) and comparison to a library created by injection of standards of incompletely methylated alditol acetate derivates of each sugar. Quantification of per-

O methylated alditol acetates were based on effective carbon response factors, as described in Sweet et al. (1975).

Light microscopy (LM), Confocal laser scanning microscopy (CLSM) and Lectin labeling. Prior to microscopy studies, EPS were removed the cell surface by repeated washing with WHM (3x, 5 min each) followed by centrifugation (800x g for 1 min). For live cell labeling, cells were collected by centrifugation (800 x g, 1 min) and extensively washed with growth medium (3x 5 min each). 50 µl drops of EPS-free cell suspension were placed on glass coverslips and incubated in a high humidity chamber for 12 hours under regular growth conditions. During this time the cells settled and adhered to the coverslip. Excess growth medium was aspirated off leaving a mass of adhered cells attached to the coverslip by EPS. For lectin labeling, the cells were treated with 35 µg/ml of lectin-FITC or –TRITC conjugate for 2 h at room temperature. All lectins were obtained from Sigma Chemical (St. Louis, MO, USA). After labeling, excess lectin solution was aspirated off; the cells were extensively washed with fresh growth medium and then viewed via CLSM.

For fixed cell preparations, cells were washed free of pre-existing EPS and processed as above. After 12 h, the drop of growth medium was aspirated off the adhered cells. The cells were fixed with 0.5% formaldehyde in growth medium for 30 min, the fixative aspirated off and adhered cells washed extensively with growth medium and then phosphate buffered saline with 0.001% Triton-X100, pH 7.2 (PBST). Cells were stained as above except that the lectin dilution was made in PBST and cells were washed with PBST and PBS before viewing. Control treatments for each lectin consisted of

incubation with the appropriate lectin-specific sugar at 1 mg/ml for 30 min prior to and during labeling (Bockelmann et al. 2002). Cells were viewed using a Olympus BX-61 LM equipped with a Fluoview 300 confocal laser system. A blue laser and appropriate filer set were used for FITC and a green laser and appropriate filter set was used for TRITC. Single X, Y scans were made as well as compilations of 10 scans at 300 nm ea.

Variable pressure scanning electron microscopy (VPSEM). 100 µl drops of washed cell suspension were placed on top of 7 mm diameter circles of Magna-R nylon filter (Sigma Chemical) and allowed to settle for 15 min, 1 h and overnight. The membrane substrates were quickly plunge frozen in liquid nitrogen (LN_2) and placed on a LN_2-cooled JEOL cryo-stub (Peabody, MA, USA). The cryostub was placed in a JEOL 6460-Variable Pressure Scanning Electron Microscope (VPSEM) and cells were visualized at 10 kV, 40 Pa and 60 nm spot size using the backscattered electron detector.

Results

EPS structure. The EPS produced by desmids in biofilms differs in composition and appearance with species, with a marked range of EPS morphology exhibited by the desmids in this study (Figures 6.1-6.6). *Cosmarium* sp. 1 (Figure 6.1) produced copious amounts of mucilage that entirely encompassed multiple cells and appeared homogeneous. The remarkable ornate CW substructure was revealed upon removal of the EPS (Figure 6.2) The EPS of *T. brebissonii* covered most of the CW surface but was most prominent at the termini of the semicell notches (Figures 6.3 and 6.5). The saccoderm desmid, *N. oblongum* (Figure 6.4) produced very large amounts of reticulated and striated strands, distinctly different from the other desmids. The mucilage matrix of

Pl. trabecula (Figure 6.6) appeared as heterogeneous patches around the CW.

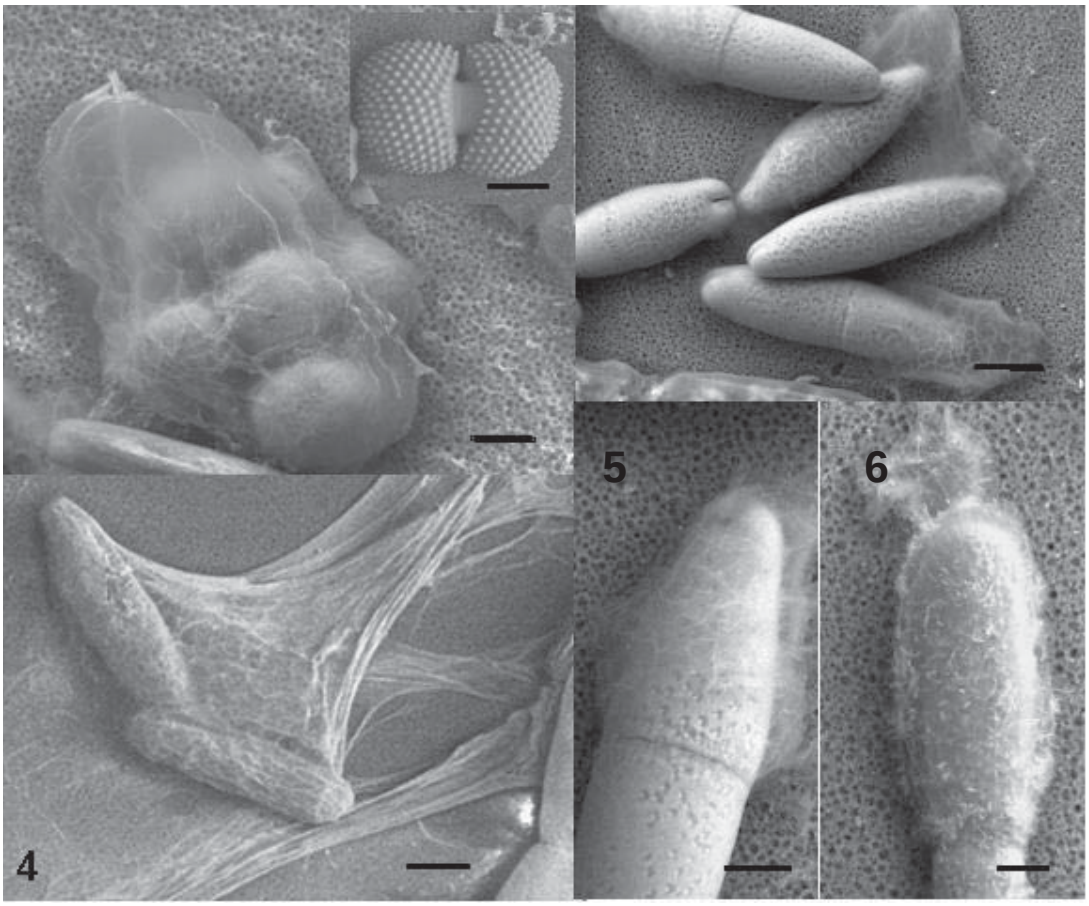

Figure 6.1-6.6: VPSEM images showing general features of various types of desmid EPS in biofilms. Fig.1 *Cosmarium sp.* 1 produced a thick EPS layer that ensheathed multiple cells (arrows) and masks the CW pores. (Scale bar = 10 μm). Fig. 2 Upon removal of EPS from *Cosmarium* sp 1, the CW pores are clearly visible. (Scale bar = 10 μm) Fig. 3 The EPS of *T. brebsonii* extruded from the notch at the cell apex. (Scale bar = 20μm). Fig. 4 *N. oblongum* produces prolific amounts of EPS which appear as fibrillar strands attaching the cells to the substrate. (Scale bar = 20μm). Fig. 5 Magnified view of the notch of *T. brebsonii* and mucilage. (Scale bar = 10μm). Fig. 6 *Pl. trabecula:* the EPS sheath (arrows) can be seen both surrounding the cell and on the CW surface. (Scale bar = 10μm).

Components of Extracellular Matrix. The EPS from cultured desmids was primarily composed of polysaccharide as indicated by phenol-sulfuric positive material which ranged from 30% in *T. brebissonii* to 42% in *Pl. trabecula* (Figure 6.7). In the majority of desmids examined, the anionic nature of the EPS was in part conferred by significant amounts of uronic acid. Uronic acids accounted for 28% of *N. interruptum*, 30% of *N. oblongum*, and 24% of *Pl. trabecula* EPS dry weight. The uronic acid content varied widely among *Netrium* species (Table 6.1) and only 5% of the dry weight of *P. spirostriolatium* was uronic acid. Another important anionic group in all desmid EPS examined was ester sulfate, and *Pleurotaenium trabecula* and *Tetmemorus brebissonii* EPS were highly sulfated (10.2% and 14.7% of dry weight, respectively) (Figure 6.7, Table 6.1). Generally, protein was a minor component of desmid EPS, although *Pl. trabecula* EPS contained 10% (w/w) (Figure 6.7). Considering uronic acid and ester sulfate as components of the polysaccharide portion of the EPS, polysaccharides constitute 52 - 76% of EPS (w/w).

Eight neutral sugars; glucose (Glc), galactose (Gal), mannose (Man), xylose (Xyl), arabinose (Ara), ribose (Rib), fucose (Fuc) and rhamnose (Rha) were detected in desmid EPS. Although neutral sugar composition of the EPS varied considerably across the species examined, the predominate sugars in the majority of species were Xyl and Fuc (Table 6.1). The unique polysaccharides from *N. oblongum* EPS were rich in Gal, those of *P. spirostriolatum* were composed predominately of Ara and *T. brebissonii* EPS were enriched in Glc and Gal. The UTEX isolate of *N. interruptum* (2509) contained significant portions of Gal whereas the major monomer in the New York isolate was Xyl.

The EPS of both isolates of *N. interruptum* contained significant amounts of Fuc and Ara (Table 6.1).

Linkage analysis of the EPS revealed the presence of 3-Fuc and t-Xyl as common residues in all desmid EPS. Fuc was a major component and occurred as 2-, 3-, 4-, 2,3-, 2,4-, 3,4-, 2,3,4-, and t-linked/substituted fucosyl residues. The occurrence of multiply substituted residues (2,3-, 2,4-, 3,4- and 2,3,4-) indicated the potential for Fuc residues to be branch points and/or sulfated. The degree of Fuc substitution varied with the species, ranging from 13% in *N. oblongum* to 71% in *T. brebissonii*. Overall, linkage analysis provided evidence for species specific EPS chemistry.

Carbohydrate analysis revealed a significant disparity in EPS chemistry between placoderm and saccoderm desmids. In the placoderm desmid *Cosmarium* sp. 1, the major EPS monomer was Fuc (Table 6.1), with the major glycosyl linkages/substitution 2,3,4-Fuc (Table 6.2). The EPS of *Cosmarium* sp. 1 was highly branched/substituted with 61% of residues exhibiting multiple substitutions and 10% occurring as terminal residues. Fuc was also the major neutral sugar in *T. brebissonii*, although the predominate linkage/substitution was 3, 4-Fuc. The major saccharides present in *Pl. trabecula* were Fuc and Xyl, with predominance of 3-, and 3,4-linked fucosyl residues and 2- and t-linked xylosyl residues (Table 6.2). The polymers of *Pl. trabecula* EPS were 44% branched with 17% terminal residues, similar to *T. brebissonii* with 54% branched with 22% terminal residues. The EPS of saccoderm desmids examined was unique from those of placoderms, with Gal the major sugar of *N. oblongum* and analysis of *N. interruptum* revealing Xyl as the predominate neutral sugar (Table 6.1). Linkage analysis indicates

that monosaccharide residues of N. *oblongum* and *N. interruptum* were equally branched/substituted with 42% branching and 12-13% terminal linkages, which may indicate a similarity of organization. They share common linkages (ex. 6-Gal, 4-Glc, t-Xyl and 4_p (pyranose) /5_f (furanose)-Xyl) although the predominance of the linkages differed between species (Table 6.2).

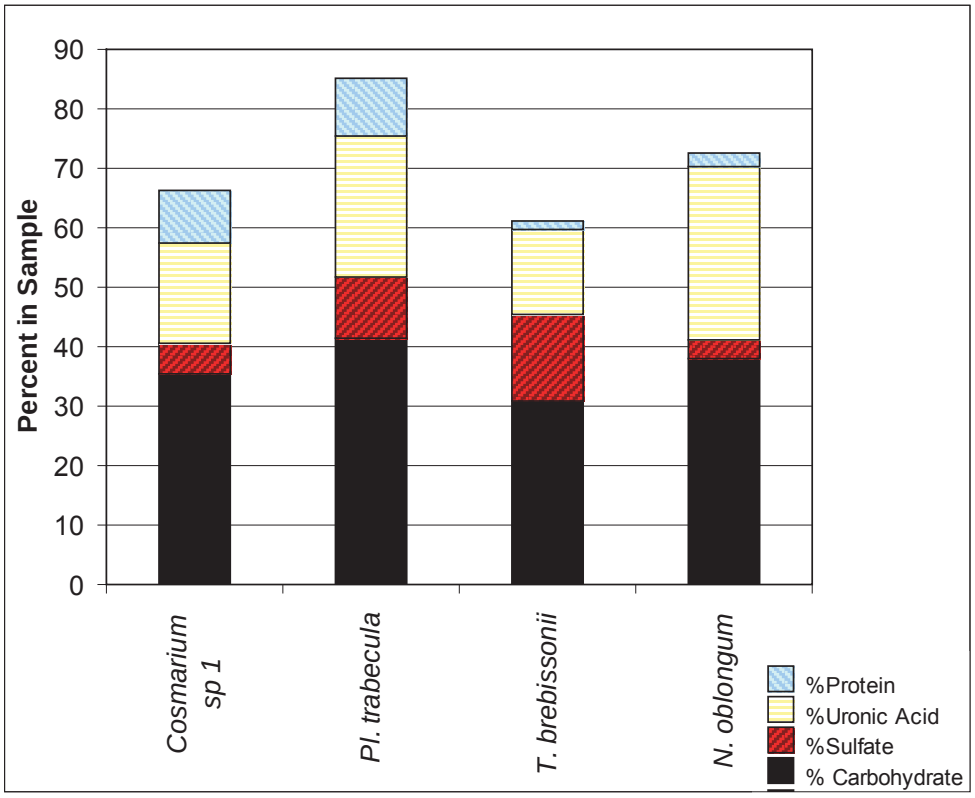

Figure 6.7: Total phenol-sulfuric carbohydrate, uronic acid, ester sulfate, and protein content of desmid EPS determined by colorimetric assay and presented as weight percent.

Table 6.1: Chemical composition of desmid EPS. Total phenol-sulfuric carbohydrate, uronic acid, ester sulfate, and protein content determined by colorimetric assay and presented as weight percent. Monosaccharide composition is expressed as percent detected neutral sugar with standard deviation. Number of replicates indicated in parentheses.

Desmids	% sugar detected								% Uronic Acid [A]	% Ester Sulfate [A]
	Glc	Gal	Man	Xyl	Ara	Rib	Fuc	Rha		
P. cylindrus	3.2 ± 1.2 (3)	6.6 ± 2.2 (3)	2.6 ± 0.90 (3)	30.7 ± 3.6(3)	11.3 ± 0.8 (3)	0.1 ± 0.09 (3)	41.1 ± 3.9 (3)	4.3 ± 0.4(3)	16.9	ND
P. spirostriolatum	5.4 ± 1.5 (3)	20.3 ± 3.7 (3)	2.2 ± 0.9 (3)	18.0 ± 2.9 (3)	40.4 ± 2.8 (3)	0.9 ± 0.3 (3)	10.8 ± 2.0 (3)	2.0 ± 0.8(3)	10.3	8.9
Cosmarium sp1	12.1 ± 3.7 (3)	16.4 ± 1.5 (3)	10.4 ± 4.2 (3)	10.3 ± 2.1 (3)	12.5 ± 4.9 (3)	2.7 ± 0.6 (3)	31.9 ± 4.9 (3)	3.7 ± 1.2 (3)	16.8	5.3
Cosmarium sp2	26.0 ± 2.5(3)	17.0 ± 0.9 (3)	9.8 ± 0.4 (3)	13.5 ± 1.1 (3)	10.0 ± 4.3 (3)	1.0 ± 0.08 (3)	16.4 ± 1.9 (3)	6.1 ± 0.6 (3)	ND	ND
Pl. trabecula	13.4 ± 1.3 (4)	16.9 ± 2.7 (4)	5.0 ± 0.4 (4)	25.9 ± 2.9 (4)	1.8 ± 0.08 (4)	5.7 ± 0.5 (4)	24.1 ± 1.6 (4)	7.2 ± 2.5(4)	23.8	10.2
T. brebissonii	19.4 ± 3.3 (4)	22.7 ± 2.2 (4)	6.8 ± 1.7 (4)	2.7 ± .9 (4)	5.6 ± 1.3 (4)	0.8 ± 0.2 (4)	36.7 ± 2.4(4)	5.2 ± 0.7(4)	14.3	14.7
N. digitus	7.6 ± 3.2 (5)	9.2 ± 1.3 (5)	3.6 ± 0.6 (5)	16.2 ± 2.9 (5)	9.1 ± 3.0 (5)	0.2 ± 0.1 (5)	45.0 ± 4.2(5)	9.1 ± 0.9 (5)	15.6	3.0
N. oblongum	6.6 ± 0.2 (4)	51.7 ± 1.3 (4)	2.7 ± .3 (4)	24.1 ± 0.4 (4)	7.1 ± 0.3 (4)	0.2 ± 0.07(4)	6.6 ± 0.8 (4)	0.9 ± 0.08(4)	29.3	3.0
N. interruptum [B]	4.3 ± 0.5 (3)	12.3 ± 1.9 (3)	2.8 ± 0.9 (3)	30.8 ± 3.5 (3)	18.8 ± 2.7 (3)	4.9 ± 2.4 (3)	21.7 ± 3.2 (3)	4.5 ± 1.3 (3)	27.7	2.8
N. interruptum (2509) [C]	4.2 ± 1.0 (3)	37.0 ± 2.9 (3)	4.4 ± 1.1 (3)	16.9 ± 3.5 (3)	12.1 ± 4.5 (3)	0.4 ± 0.5 (3)	21.7 ± 1.7 (3)	3.2 ± 1.9 (3)	ND	ND

[A] Uronic acid and sulfate content were determined by colorimetric assay and presented as weight percent.

[B] N. interruptum isolated from Adirondack wetlands NY

[C] N. interruptum from UTEX collections, number 2509

ND (not determined) - Not determined due to insufficient material

Table 6.2: Glycosyl unit and linkage/substitution site profiles of desmid EPS. Glycosyl linkage/substitution sites were determined by methylation analysis

	Cosmarium sp. 1	*Pl. trabecula*	*Pl. trabecula*[1]	*T. brebissonii*	*T. brebissonii*[1]	*N. oblongum*	*N. interruptum*
Ara							
2_f -			7.9				
2_p -			0.8			19.3	
$2,3_p$ -	1.5	3.7		8.1	4.7		
$2,3_f$ -			0.9				
$2,3,4_p/2,3,5_f$ -	14.0	6.6		2.8			
3_f -						2.5	
3_p -	4.9						4.3
$4_p/5_f$ -			5.1				
t_p -	2.3						
Fuc							
2 -	7.5	4.0		2.0	0.5		
2,3 -		3.0	2.5				
2,4 -	1.3			1.1	0.8	1.1	
2,3,4 -	20.0	6.5	1.8	7.3	10.1		9.7
3 -	2.7	9.8	12.7	1.7	1.3	2.3	4.8
3,4 -	2.9	13.5	19.7	14.0	18.6		13.5
4 -	5.7		3.5	3.7	2.0	2.6	3.8
t -	4.2	6.7	6.2	7.3	8.0	2.6	1.6
Gal							
2 -		0.8					
2,3 -					0.7		
2,6 -			1.8	0.4	0.5	2.7	
2,4,6 -		1.9					
2,3,4,6 -					3.5		
3 -		3.1	1.5	3.9		2.1	
3,4 -							3.5
3,6 -	3.8		0.3	1.5	0.6		
3,4,6 -	1.6			2.9	17.4		5.6
4 -			3.6	1.0			3.4
4,6 -					1.0		
6 -		1.9	3.5	1.9	2.1	4.6	1.9
t -					0.4		2.0
Glc							
2 -							7.9
2,3 -						17.9	2.1
2,6 -		1.2					
2,3,6 -	0.4				0.6		
3 -				1.6	1.5		
3,6 -		2.2					
4 -		2.9		5.2	1.9	9.3	7.7
6 -		0.5					
Man							
2,3 -			2.1				
2,4 -							0.6
4,6 -			1.1	0.8	3.5		
t -					1.4		
Rha							
2 -	0.7			2.5			4.6
2,4 -	0.3						
2,3,4 -	2.0						
4 -							1.4
Xyl							
2_f -		9.1					
$2,3,4_p/ 2,3,5_f$ -	13.5	8.1		6.4		20.2	5.7
3_p -	2.0			4.3	2.6		
$4_p/5_f$ -	5.1	5.2	3.9	8.2		3.5	6.5
t_p -	3.8	9.6	11.4	11.4	16.4	9.6	9.3
GlcA							
4 -			9.7				

[1] Prereductions prior to methylation

Polymer structure in biofilm. Lectin labeling revealed differences in polymer organization between saccoderm and placoderm desmids as summarized in Table 6.3. Lectin fluorescence was qualitatively judged as strong or absent or weak. The last designation referred to fluorescent signals that were visible but with only a fraction of the intensity observed with a strong signal. The EPS of the saccoderm desmids *N. oblongum* and *N. digitus* labeled with ConA and HPA and labeling appeared amorphous with distinct labeling patterns not observed (not shown). Their EPS also labeled with WGA though the signal was not strong. EPS of the placoderm desmid, *T. brebissonii* labeled weakly with BS-I exclusively in areas adjacent to the notch in the CW and labeled homogeneously with HPA (not shown). *Pl. trabecula* EPS gave a weak signal with WGA (Figure 6.8) and the mucilage can be seen surrounding the cell in patchy tufts. The prodigious amount of EPS produced by *Cosmarium* sp. 1 labeled with HPA (Figures 6.10 and 6.12) and UEA I (Figure 6.11), the EPS appeared homogeneous and encompassed the CW. LcH (*L. culinaris*), which is specific for α-D-Man and α-D-Glc, did not label any of the desmid EPS. For each of the lectins utilized, competitive controls resulted in no specific labeling patterns observed.

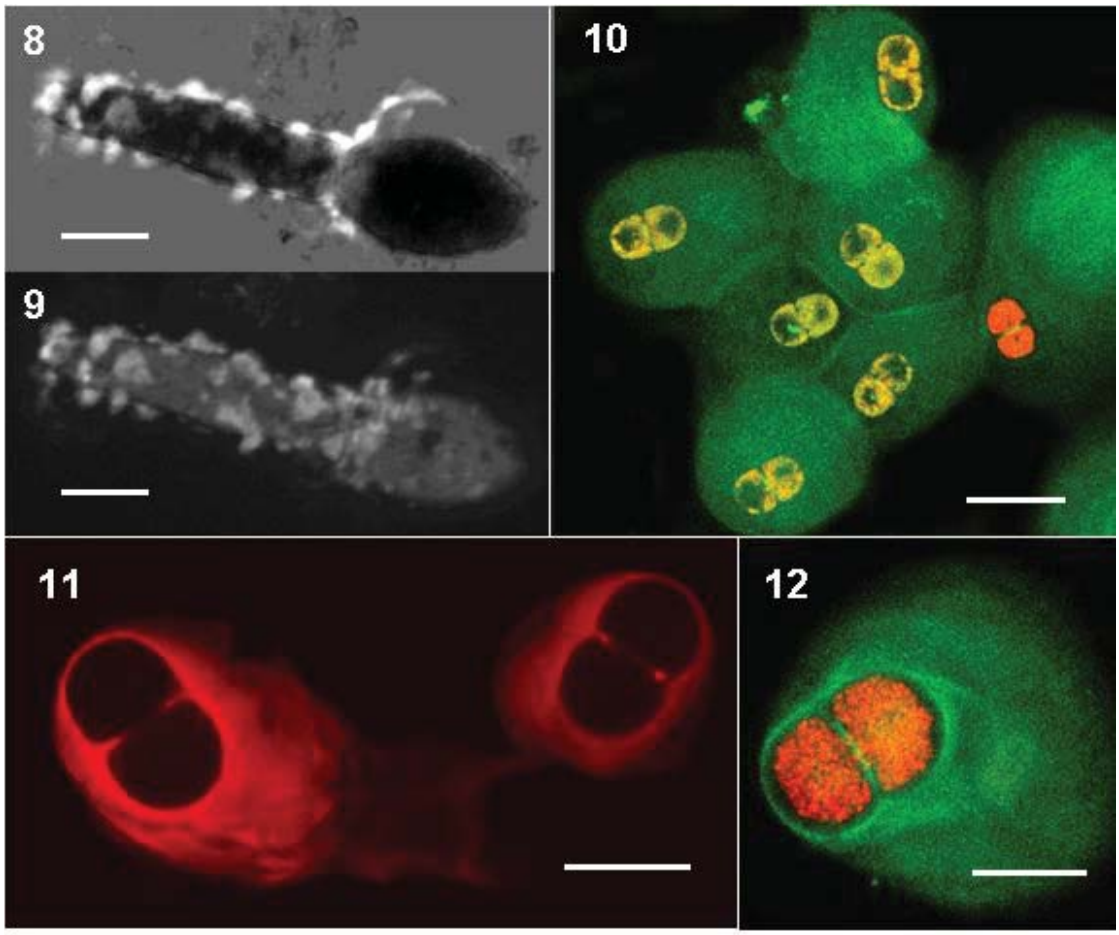

Figure 6.8-6.12: CLSM imaging of lectin labeled desmid biofilms. Fig. 8 *Pl. trabecula* labeled with WGA. The cell appears dark with wisps of EPS highlighted along the cell periphery. (Scale bar. = 20 μm). Fig. 9 *Pl. trabecula* labeled with WGA. The EPS distribution over the cell is heterogeneous. (Scale bar. = 20 μm). Figs 8 and 9 are different focal views of the same cell. Fig. 10 HPA labeling of *Cosmarium* sp. 1. The community forms capsule-like EPS sheaths, which engulf the cells and surrounding areas. The EPS appears green and the chloroplasts are yellowish and red. (Scale bar. = 50 μm). Fig. 11 Two cells of *Cosmarium* sp. 1 surrounded by EPS (in red) labeled with UEA. (Scale bar. = 20 μm). Fig. 12 Magnified view of *Cosmarium* sp. 1 EPS (green) labeled with HPA. The chloroplasts are visible as red. (Scale bar. = 20 μm).

Table 6.3 Lectin localization of desmid EPS in biofilms

Application	Specificity	*Cosmarium sp.*	*Pl. trabecula*	*T. brebsonii*	*N. digitus*	*N. oblongum*
Con A - FITC *(C. ensiformis)*	α-D-Man α-D-Glc	-	-	-	Strong	Strong
WGA - FITC *(Tr. vulgaris)*	N-acetyl-β-D-Glc D-acetyl-β-D-glucsamine oligomers	-	-	-	Weak	Weak
BS-I - FITC *(B. simplicifolia)*	α-D-Gal N-acetyl-α-D-Gal	-	-	Weak at notch	-	-
PSA -FITC *(P. sativum)*	α-D-Man	-	Weak	-	-	-
LcH -FITC *(L. culinaris)*	α-D-Man α-D-Glc	-	-	-	-	-
UEA I-FITC *(U. europaeus)*	(1→2)-α-L-Fuc	Strong	-	-	-	-
HPA -FITC *(H. pomatia)*	N-acetylgalactosamine	Strong	-	Strong	Strong	Strong

Discussion

Microscopic analyses revealed a diversity of EPS structure, ranging from reticulate to striated to amorphous (Figures 6.1- 6.6, Domozych et al. 2005, Freire-Nor et al. 1998, Paulsen and Vieira 1994, Domozych et al. 1993, Boney 1980). The prodigious amount of smooth sheath EPS produced from *Staurodesmus convergens* (Gouvea et al. 2002) and *Spondylosium panduriforme* (Paulsen and Vieira 1994; Freire-Nordi et al. 1998) completely engulfed the cell, as also shown for *Cosmarium* sp. 1 (Figure 6.1). In *Closterium* sp. (Domozych et al. 1993) and *T. brebissonii* (Figures 6.3 and 6.5) EPS is produced in apical regions. *Closterium* sp. has also been demonstrated to leave EPS motility trials in addition to producing engulfing amorphous EPS (Domozych et al. 1993). *Penium margaritaceum* produced prodigious amounts of EPS in the form of sheaths of mucilage that did not completely engulf the cells (Domozych et al. 2005),

which is similar to the type of EPS produced by *P. spirostriolatum* and *P. cylindrus* (images not shown). The EPS of *Cosmocladium saxonicum* (Surek and Sengbusch 1981) and *Mesotaenium chlamydosporum* (Boney 1980) was similar to *N. oblongum* in that it formed striated EPS which appeared to contain filaments, although, unlike *N. oblongum*, *Cosmocladium saxonicum* also produced amorphous EPS (Surek and Sengbusch 1981).

Biochemical analyses revealed that desmid EPS was unique at the species level and exhibited notable biochemical diversity among desmid taxa. Xyl and Fuc residues as well as significant acid groups (e.g. uronic acid or sulfate ester) were the most common components shared by desmid EPS. The differences in carbohydrate composition are wide ranging and include: 1) Xyl, Fuc and glucuronic acid (GlcA) residues as significant components of the EPS of *Closterium* sp. (Domozych et al.1993); 2) Fuc, GlcA, galacturonic acid (GalA), and Gal as the most common constituents of the EPS of *Staurastrum orbicular* (Giroldo et al. 2005), *Spondylosium panduriformis* (Paulsen and Vieria 1994), *Hyalotheca dissiliens* (Vieria and Paulsen 1994), and *Staurodesmus covergens* (Gouvea et al. 2002); 3) Rha and Man-rich constituents in *Micrasterias furcata* (Lombardi et al. 1998). Substitution/linkage patterns of each of the mucilages are unique as well and show a large degree of diversity between desmid species. For example, there were eight different linkages/substitution patterns for Fuc residues, which are the most abundant monomer in desmid EPS. In *Cosmarium* sp. 1 a highly substituted 2,3,4-linked Fuc residue predominated, whereas, in *Pl. trabecula* and *T. brebissonii*, fucose appeared as a 3,4-linked residue. *Pl. trabecula* also had a considerable amount of 3-linked fucosyl residues, similar to *St. orbiculare* EPS (Giroldo et al. 2005), whereas in EPS of *Sp.*

panduriformis (Paulsen and Vieira 1994) and *Closterium* sp. (Domozych et al. 1993), Fuc was mainly found in 4-linked residues. In *P. margaritaceum* EPS (Domozych et al. 2005) Fuc was found in equal portions of 3-linked, 4-linked, and 3,4- linked residues and in *H. dissiliens (*Vieria and Paulsen 1994*)*, Fuc substitution patterns were more complex, with Fuc residues representing the majority of the branched portion of the EPS. The predominance of Fuc units in desmid EPS was significant because deoxy-sugars have been shown to have hydrophobic properties that may help maintain the gel-like matrix of the EPS sheaths (Wustman et al. 1997, 1998, Fattom and Shilo 1984, Hu et al. 2003, Hokputsa et al. 2003). Fuc and Glc residues have been shown to be selectively removed during degradation of *Staurastrum orbiculare* EPS, as opposed to Rha and Ara containing portions of the polysaccharides which were resistant to bacterial action (Giroldo et al. 2005). Ara was a major neutral sugar in *Penium spirostriolatum*, *Netrium interruptum*, *Cosmarium* sp. 1, and P. *cylindrus* EPS. Ara has also been found to be a major component of an uronic acid and sulfate ester containing EPS of several green algae including *Chlamydomonas*, *Chlorosarcina* and *Gloeocystis* species (Lewin 1956). The EPS of *Ankistrodesmus densus*, a freshwater planktonic green alga, which consists of a large mucilaginous encompassing sheath, was shown to contain high amounts of Fuc and Man residues with 3-O-methylgalactose (Paulsen et al. 1998). *Sp. panduriforme* (Paulsen and Vieira 1994) EPS showed high amounts of Fuc, Gal, and GlcA units with 6% protein, which corresponds to the protein content of the desmid EPS described herein (2-10%) (Figure 6.7).

Lectin labeling of desmid EPS revealed marked differences between the saccoderm and placoderm desmids. Both saccoderm desmids, *N. oblongum* and *N. digitus*, labeled for α-D-Man and α-D-Glc (Con A), N-acetyl-β-D-Glc and D-acetyl-β-glucosamine oligomers (WGA), and N-acetylgalactosamine (HPA). The lectin labeling correlated with EPS chemistry, in that both *N. digitus* and *N. oblongum* contain Man and Glc, although they are not major components of the EPS. The EPS of the placoderm desmid *Cosmarium* sp. 1 labeled for (BS-I (directed 1→2)-α-L-Fuc (UEA), correlating with the neutral sugar profile and linkage analysis which demonstrated a predominance of 2-Fuc residues. The EPS of *T. brebissonii* labeled with against α-D-Gal and N-acetyl-α-D-Gal), though only weakly at the apical notch, which may indicate the possibility that the EPS structures may be modified post-excretion. Surek and Sengbusch (1981) found that the EPS of *Cosmocladium saxonicum* labeled weakly with ConA and not at all with UEA, although no chemical analysis of the EPS was performed.

Desmids share some chemical characteristics of their EPS with the other dominant eukaryotic EPS producer in freshwater biofilms, the diatoms. Both freshwater diatoms and desmids produce copious amounts of polysaccharide rich EPS which owe their anionic character to the presence of uronic acid and ester sulfate units. Comparing the relative abundance of monosaccharide building blocks of these polymers, desmid and diatom EPS are similar in the predominance of Xyl and Gal residues and the low amounts of Rha (Hoagland et al. 1993). The uronic acid component of *Pleurotaenium trabecula, Penium margaritaceum, Closterium* sp. and *Spondylosium panduriforme* was GlcA, which correlates with that reported from freshwater diatom EPS (Hoagland, 1993).

However, GlcA and GalA was been reported from the desmid *Staurodesmus convergens* (Gouvea et al. 2002). GlcA was identified in *Pl. trabecula* as 4-linked, in *P. margaritaceum* as 3,4-, 2,4-, and 4-linked (Domozych et al. 2005), in *Closterium* sp. as 4- and 3,4-linked (Domozych et al. 1993), and in *Sp. panduriforme* as t-, and 4-linked (Paulsen and Vieira 1994).

Although desmids have significant ester sulfate content (3-15%), freshwater diatom EPS is highly sulfated with up to 30% ester sulfate [Hoagland et al. 1993, Wustman et al. 1997]). As an exception, all *Netrium* species contain low amounts of sulfated polysaccharides (approx. 3%). It has been shown that ester sulfate substituent of bacterial polysaccharides are related to the adhesive properties of the EPS (Toida et al. 2003) and sulfate esters may play a role in desmid adhesion as well. As an example, *Pl. trabecula* EPS is highly sulfated and has unique characteristics which facilitate cell adhesion to substrates (Domozych et al. 2007). The anionic character of sulfate substitution and uronic acid units provides capacity for cationic cross-linking, leading to gel formation and/or high viscosity matrices as described for carrageenans and other sulfated galactans (Lahaye 2001). Anionic characteristics of desmid EPS may also play a role in resisting bacterial degradation, nutrient sequestering and ion exchange as has been proposed for diatom EPS (Hoagland et al. 1993). Polysaccharide biophysical character is dramatically altered when uronic acid residues and ester sulfate substituent are present, which has profound implications on inter-polymer relationships, three dimensional folding and inter- and intra-molecular associations within biofilms (Wustman et al. 1997, 1998, Fry 1988, Magaletti et al. 2004, Decho 2000).

Considering the complexity of polysaccharide substitution patterns found in this study (Table 6.2), desmid EPS is probably composed of multiple polymers that are highly branched and additional fractionation protocols must be applied in order to dissect details of the organization of residues within the polysaccharides. This information would allow determination of backbone structures of desmid EPS polysaccharides, which would provide a more useful starting point for interspecific comparisons. In diatoms, it has recently been established that, although the backbone polymers produced by each species remain constant, substituents on the backbone such as branching, sulfation, etc. are modified in response to environmental cues (Abdullahi, et al. 2006). Based on our current knowledge of desmid EPS, it is probable that different species of desmids will have different EPS backbones and substitution patterns upon the core portions of the molecules. Desmid EPS is predominately composed of a complex matrix of branched, uronic acid containing polysaccharides with ester sulfate substitution and, as such, have an almost infinite capacity for various hydrogen bonding, hydrophobic interaction and ionic cross-bridging motifs which characterize their unique function in biofilms.

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Chapter 7: Conclusions and Major Findings

The overall goal of this project was to examine the composition of the charophycean green algal (CGA) cell wall (CW) and associated extracellular polymers. Through an understanding of CGA extracellular matrices, we can develop an understanding of the evolution of the land plant CW and also the unique characteristics that allow for life in aquatic habitats. To demonstrate how the development of modern terrestrial plant form and function is dependent upon a comprehensive understanding of CW biochemistry and structure, we have used cytochemical screening with monoclonal antibodies, analytical methods (including linkage, neutral sugar, colormetric, gravimetric and NMR analyses), and enzyme susceptibility to examine the extracellular matrices of the CGA. The extracellular matrices (ECM) are structurally and biochemically diverse (Chapter 6) and also contain a number of characteristics found in the CWs of land plants, such as cross-linking glucans (Chapter 3), beta-glucans including (1, 3) β-glucans and cellulose, (Chapter 2), land plant pectin polymers (Chapter 4), and an arabinogalactan protein-like polymer (Chapter 5).

The hemicellulosic fractions of *Chara corallina, Penium margaritaceum*, *Cosmarium turpini*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum* were examined in an attempt to isolate and characterize the cross-linking glycans of the CGA. Mixed-linkage glucans (MLG) were determined to be present in the CW of *Chara corallina, Cosmarium turpini*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum*

(Chapter 3); this was confirmed with immunocytochemistry, biochemical methods and enzyme susceptibility with characterization of oligosaccharides. To examine the detailed structure of the MLG polymer, oligosaccharide mass profiling was utilized to examine the oligosaccharides solubilized following CW digestion with lichenase. The major product of enzyme digests of *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum* was laminaribiose with a degree of polymerization of two, which means that the MLG of the basal CGA consists of a majority of repeating (1→3), (1→4) linked glycosyl residues. Whereas, in *Chara corallina* and *Cosmarium turpini* the major product was a tetrasaccharide and the minor products were trimers which is similar to results for *Equisetum* sp. (Sorenson et al. 2008; Fry et al. 2008a,b). Until 2008, MLG was thought to be a polymer unique to the Poales but MLG has now been found in *Equisetum* sp. and in the CGA. The presence of MLG in the charophytes, including the basal species, indicates the origination and synthesis of these polymers may have occurred concurrently with the origination of homogalacturonans or cellulose. There is also the possibility of an alternate evolution of this polymer which begs the question, "What is the function of the MLG and how does it interact with cellulose?"

Other possible land plant polymers found in the hemicellulosic fractions of the CGA include xylan, which was suggested by: 1); *Chlorokybus atmophyticus* contains significant amounts of xylose in the 4M KOH fraction with 4-linked xylosyl residues; 2) *Klebsormidium flaccidum* contained a very small amount of xylose in the 1M KOH or 4M KOH fraction, and; 3) Comprehensive microarray polymer profiling (CoMPP) labeling with LM11 (specific for β(1,4)-xylan/arabinoxylan) indicated the presence of

substituted xylan (Willats CoMPP unpublished). There was no evidence of other cross-linking glycans in the CW of *C. atmophyticus* or *K. flaccidum*. CoMMP analysis also indicated the presence of fucosylated xyloglucans (Willats unpublished), however when oligosaccharide mass profiling was performed on the oligosaccharides solubilized by the xyloglucanase (Novo), the results showed a hexose ladder rather than a xyloglucan ladder, indicating that the backbone was cut apart but the side-chains of XyG were not present. The CWs of *Klebsormidium flaccidum*, *Cosmarium turpini*, *Spirogyra* sp., *Chara corallina*, and *Netrium digitus* were all examined with OLIMP for the presence of XyG but none was indicated. The solubilized products of *Chara corallina* did not produce a xyloglucan ladder. However, once again there was conflicting data; the glucosyl residues 4- and 4,6- (42%) linked were abundant, in addition there were both terminal and 2-linked xylosyl residues, and the typical galactosyl residues were not revealed in the 1M KOH fraction. At this point in time the debate is still raging on as to whether *Chara corallina* has xyloglucans as part of it's CW.

When examining the structure of the plant CW, the simple linear structures of the beta-glucans have to be considered. They are widely distributed among different plant lineages, and are found in the red and green algae, the chromalveolates, some alveolates, and in some chromistans (Bacic et al. 2009). One of the goals of this work was to obtain further insight into the presence, distribution and amount of simple β-glucans (cellulose and (1,3)-β-glucans) in the evolution of land plants by examining representative CGA species (Chapter 2). Cells walls of the charophycean green algae were investigated by means of biochemical, cytochemical, and gravimetric analyses. The cellulose content in

the CGA ranged from 6% to 43% with the higher range comparable to that in the primary CW of land plants (20-30%). When the Updegraff residues were examined, the CWs were clean shells and free of cytoplasmic contaminants. Glycosyl substitution analysis of the insoluble residues revealed the majority of residues to be 4-linked glucosyl. Preliminary work probing an EST database of *Chlorokybus atmophyticus* has revealed a putative CesA (Kiemle and Roberts, unpublished). CesA was not detected in the EST database of *Coleochaete* sp. however a putative *CSLD* has been found and there is some indication that *CSLD* may be involved in cellulose synthesis and may function as a co-ordinate gene.

(1,3)-β-glucans were found localized in the hemicellulosic fractions of CGA with abundance. The cells walls of CGA were digested with endo-(1,3) β-glucanase which hydrolyses (1,3)-β-glucans. The oligosaccharides produced were analyzed by oligosaccharide mass profiling which extends the application of OLIMP to another polymer. Endo-(1,3)- β -glucanase digested pachyman standard produced a ladder from disaccharide to octosaccharides, most of the oligosaccharides yield was disaccharides. The majority of CGA species yield a higher proportion of disaccharides that other oligosaccharide, with the exception of *Chlorokybus atmophyticus*. Enzyme digestion of *C. atmophyticus* produced trisaccharides and tetrasaccharides in larger numbers. The underlying reason for the disparity in the oligosaccharides profile is unkown at this time.

The pectin portion of Penium *margaritaceum*, *Cosmarium turpini*, *Chlorokybus atmophyticus*, and *Klebsormidium flaccidum*(Chapter 4) CW was analyzed. Anovel HG was found in the CW of *Penium margaritaceum, which* represents the first confirmation

of a land plant pectin in the Zygnematales. This was determined through a combination of immunocytochemistry, analytical methods and enzyme susceptibility with characterization of oligosaccharides. Homogalacturonan-like polymers were found to be present in the basal species of the CGA. The presence of homogalacturonans in CGA species may beconsiderably valuable in determining the type and nature of CW polymers that were key in a successful adaptation to terrestrial environments. The report of HG in *Penium margaritaceum* represents the first confirmation of land plant-type pectin in desmids and the second rigorous characterization of a pectin polymer from the charophycean algae. There was also evidence of HG in *Chlorokybus atmophyticus*, *Klebsormidium flaccidum* and the desmid *Cosmarium turpini* which leads to the idea that pectins in general (and more specifically HG) is a basic character in the CW of all of species in the plant lineage. The function of pectin in aquatic environments may include controlling porosity at the cell surface, providing charged surfaces which regulate both the pH and ionic status of the CW, promoting cell adhesion via their gel-like properties and serving as elicitors or signaling molecules in defense (Knox 1990, Mohnen 2008). The next step for investigating HG in the charophycean green algae will be to look for the genes involved in pectin synthesis and maintenance. There is preliminary evidence for the presence of possible RGI backbones in the Zygnamatales, Chlorokybales, and Klebsormidales, which would suggest that RGI is a basic rather than advanced characteristic.

The extracellular matrix of *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* sp is very biochemically diverse; it ranges from distinct land plant

polymers to unique algal polymers. The majority of the neutral sugars in *Chlorokybus atmophyticus* HW extract and *Spirogyra* EPS, along with the antibody labeling, reveal the distinct possibility of an arabinogalactan protein (AGP) present in the ECM of these organisms. Biochemical and immunocytochemical evidence suggests that AGP occurs in all land plants and their charophyte ancestors. However, there are probably functional differences along with structural changes in the protein and carbohydrate moieties. Glycosyl linkage analysis, neutral sugar analysis and immunocytochemical labeling suggest that *Chlorokybus atmophyticus*, *Klebsormidium flaccidum*, and *Spirogyra* sp have similar carbohydrate domains to that of the AGP of land plants. The carbohydrate moiety of AGPs may be instrumental in the evolution of the major groups of land plants and found all the way down to the basal species of the CGA.

Considering the complexity of polysaccharide substitution patterns found in this study (Chapter 6), Zygnematales extracellular polymeric substances (EPS) are probably composed of multiple polymers that are highly branched. Additional fractionation protocols must be applied in order to dissect the full details of the organization of residues within the polysaccharides. This information would allow determination of backbone structures of Zygnematales EPS polysaccharides, which would provide a more useful starting point for interspecific comparisons. Based on our current knowledge of the EPS, it is probable that different species will have different EPS backbones and substitution patterns upon the core portions of the molecules. Zygnematales EPS is predominately composed of a complex matrix of branched, uronic acid containing polysaccharides with ester sulfate substitution and, as such, have an almost infinite

capacity for various hydrogen bonding, hydrophobic interaction and ionic cross-bridging motifs which characterize their unique function in biofilms.

The fundamental understanding of the charophycean green algae extracellular matrix is broadening our perspective on cell wall structure, function and evolution. A comprehensive knowledge of CW structure and function throughout the plant kingdom is essential to understanding CW evolution. The similarities and differences that exist between plant and algal CWs lead to further examination, with wall component patterns suggesting that existing diversity is likely to be a result of a variety of different evolutionary scenarios. A variety of higher plant polymers were discovered in the charophytes including homogalacturonans, cross-linking glycans, arabinogalactan protein, β-glucans, and cellulose. This supports evidence which suggests that members of the Charophyceae represent the phylogenetic line that gave rise to vascular plants and that primary CWs of vascular plants many have evolved directly from structures typical of the filamentous green algal CWs found in the Charophyceae.

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Table 7.1: Chemical characteristics of CGA CW

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Mesostigmales						
Mesostigma viride	1° Wall	*Scales do not contain GalA or GlcA (Becker et al 1991		* CesA were not detected in the EST database (Simon et al. 2006)		*Labeling for wound-induced callose (Scherp et al. 2001) *Basket scale consist of 24% carbohydrate and 5% protein and are complexed with calium phosphate (Domozych et al 1991)
Chlorokybales						
Chlorokybus atmophyticus	1° Wall	*HW fraction rich with GalA with 4-linked and 2,4-linked GalA. *CW labeled with JIM5 (Kiemle et al. 2010, Chap 4)	*MLG present in 1MKOH and 4MKOH fraction; CW digests with lichenase producing laminaribiose oligosaccharides. 1M / 4M KOH extracts rich in 4-, 3- and terrminal Glc; CW labeled with BS 400-3 for MLG (Kiemle et al. Chap 2) * Possible xylan with 4MKOH fraction rich in Xyl (12%) with 4-Xyl (Kiemle et al. Chap 2)	* CesA were detected in EST database (A. Roberts et al. unpublished) * CW is 7.2 % cellulose (Kiemle et al. 2010; Chap 3) * Insoluble residue contains a majority of 4-Glc with terminal-Glc, 3-Glc, 3,6-Glc, 4-Gal, 6-Gal, 2,3-Gal, t-Xyl, 4-Xyl, t-Ara, t-Rha (Kiemle et al. Chap 3)	*HW soluble fraction 72% Ara, 23% Gal, with 4% protein and the CDTA soluble fraction contains 60% Ara, 28% Gal with 27% uronic acid with 3% protein. Linkage analysis reveals t- and 3-linked galactosyl residues and t, 2- and 2,4-linked arabinosyl residues. (Kiemle et al. 2010; Chap 5) *CW labeled with JIM13 and LM2 for AGP (Kiemle et al. 2010; Chap 5)	* β-(1,3)-glucan present, CW digested with endo-(1,3)-glucanase producing characteristic oligosaccharides. *CW labeled with 3-linked Glc abundant throughout fractions including 1MKOH, 4MKOH, and insoluble remnant; CW labeled with BS 400-2 for (1,3) β-glucan (Kiemle et al.; Chap 3) *Labeling for wound-induced callose (Scherp et al. 2001)

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Chlorokybus sp.	1° Wall	*Labeled for JIM5 unesterified HGA (Willats CoMPP)	*CW labeled for LM11 for Xylans (Willats CoMPP)		* CW labeled for LM2 for AGP (Willats CoMPP)	*CW labeled for mannan and callose (Willats CoMPP)
Klebsormidales						
Klebsormidium flaccidium	1° Wall	*HW fraction rich with GalA with 4-linked and 2,4-linked GalA. *CW labed with JIM5 (Kiemle et al. 2010, Chap 4) ; *CW labeled LM6 for A(1-4)-Ara (Willats CoMMP) *Alcohol insoluble fraction: found 3-O-Me-Rha (+); No GalA or GlcA found (Popper and Fry 2004)	*MLG present in 1MKOH and 4MKOH fraction; CW digests with lichenase producing laminaribiose oligosaccharides. 1M / 4M KOH extracts rich in 4-, 3- and terrminal Glc; CW labeled with BS 400-3 for MLG (Kiemle et al. Chap 2) *No evidences of xyloglucan (Popper and Fry 2004; Kiemle et al. 2010; Chapter 2) *Possible xylan with 4MKOH fractin contains 4-Xyl (Kiemle et al. Chap 2) Labeled with LM11 for Xylan (Willats CoMMP)	* CesA were detected in EST database (A. Roberts et al. unpublished) *CW is 5.2 % cellulose (Kiemle et al. 2010; Chap 3) *Insoluble residue contains a majority of 4-Glc with t-Glc, 2,3-Glc, 3-Gal, 4, 6-Gal, t-Xyl, 4-Xyl, t-Ara, and 3,4-Ara (Kiemle et al. 2010; Chap 3)	*HW extract contains 37% Glc, 32% Gal and 30% Ara. The major linkages include: t-, 3-, 4- and 3,6-glucosyl residues, t-, 3-, 4-, 3,6- and 4,6-galactosyl, and t-, 2-, 4-, and 2,4-arabinosyl residues. *Labeled with LM2 but not JIM13 (Kiemle et al. 2010; Chap 5)	* β-(1,3)-glucan present, CW digested with endo-(1,3)-glucanase producing characteristic oligosaccharides. *CW labeled with 3-linked Glc abundant throughout fractions including 1MKOH, and 4MKOH; *CW labeled with BS 400-2 for (1,3) B-glucan (Kiemle et al.; Chap 3) *CW labeled for callose (Willats CoMPP) *Labeling for callose: wound-induced and in plane of cell division (Scherp et al. 2001)
Klebsormidium flaccidum #628	1° Wall	*Labeled with JIM5 for unesterified HGA, LM6 for A(1-4)-Ara (Willats CoMMP)	*Labeled with CCRC-M1 for fucosylated xyloglucan and with LM11 for Xylan (Willats CoMMP)		*CW labeled with LM2 for AGP (Willats CoMPP)	*CW labeled for mannan and callose (Willats CoMPP)

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Zygnematales						
Closterium acerosum	1,2 Walls	*CDTA fraction rich with GalA with GlcA, Glc, Rha, Gal, Fuc, Man, Ara, and Xyl. (Baylson et al 2001)		*Hexagonal arrays of rosettes (Hotchkiss and Brown 1989)		
Cosmarium turpini	1,2 Walls	*CDTA fraction rich with GalA with 4-linked and 2,4-linked GalA also significant amounts Rha with 2- and 2,4-linked Rha (Kiemle et al. 2010, Chap 4) *CW labeled with JIM5 (Kiemle et al. 2010, Chap 4)	*MLG present in 1MKOH and 4MKOH fraction; CW digests with lichenase producing oligosaccharides with tetrasaccharide and triaccharide oligos. 1M / 4M KOH extracts rich in 4-, 3- and terrminal Glc; (Kiemle et al. 2010 Chap 2)	*CW is 43% cellulose (Kiemle et al. 2010; Chap 3) * Insoluble residue contains a majority of 4-Glc with t-Glc, 3-Glc, 4, 6-Man, 2,3-Man, t-Xyl, 4-Xyl, 2,4-Xyl, t-Ara, and 3,4-Ara (Kiemle et al. 2010; Chap 3)		* β -(1,3)-glucan present, CW digested with endo-(1,3)-glucanase producing characteristic oligosaccharides. *3-linked Glc abundant throughout fractions including 1MKOH, and 4MKOH(Kiemle et al.; Chap 3)
Cosmarium sp.	1,2 Walls	*Labeled with JIM5 for unesterified HGA (Willats CoMMP)	*Labeled with LM11 for xylans, also labeled for mixed linkage glucans, and non-fucosylated xyloglucan with LM15 (Willats CoMMP)	*Hexagonal arrays of rosettes (Hotchkiss and Brown 1989)	*Labeled with LM1 extensin and LM2 for AGP (Willats CoMPP)	*CW labeled for callose (Willats CoMPP)
Mesotaenium caldariorum	1° Wall			*Rosette terminal complexes and both McCesA1 and McCesA2 are similar to seed plant CesAs in domain structure and intron position (Roberts et al. 2002)		

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Micraceterias denticulata	1,2 Walls	*CW labeled with JIM5 and JIM7, CW also labeled with JIM8 (AGP and RG1) Lutz-Meindl and Brosch-Salomom 2000	*MLG found in the 2^{o} wall but not primary CW (Eder et al. 2008), antibody labeling and HPEAC_PAD oligosaccharide results. Analytical results revealed that alkali-soluble polysaccharides in the secondary wall of consist mostly of (1,3, 1 ,4)-b-D-glucan. *Labeling indicated XyG in 1^{o} 2^{o} CW, CCRC-M1 (fucose/galactose side chain of XyG) did not recognize any structures. (Eder et al. 2008)	*CW is 14.6 % cellulose (Kiemle et al. 2010; Chap 2010)	*CW labeled with JIM1 for AGP (Lutz-Meindl and Brosch-Salomom 2000), and with JIM8, JIM13, and JIM14 for AGP (Eder et al. 2008) *No evidences of extension with antibody labeling (JIM11, JIM12, and JIM20) (Eder et al. 2008)	*No evidences of (1,3)-β glucans detected (Eder et al. 2008)
Micraceterias sp	1,2 Walls	*Labeled with JIM5 for unesterified HG with LM6 for A(1-5)-Ara (Willats CoMMP)	*Labeled for mixed link glucans (Willats CoMPP)			*CW labeled for mannan and callose (Willats CoMPP)
Mougeotia sp	1^{o} Wall	*Found 3-Gal, 4-Ara, 2,4 Ara, 3-O-Me-6-deoxyhexoses in HW soluble fraction (Hotchkiss et al. 1989	*4-linked xylan with 2,4-linked branch point with t-arabinosyl residues (Hotchkiss and Brown 1989)	* Rosette/globule terminal compleses - No developmental changes in microfibril orientation (Hotchkiss and Brown 1989)		*Methylated hexose (3-O-methyl-6-deoxy-hexoses) (Hotchkiss and Brown 1989)
Netrium digitus	1,2 Walls	Labeling with JIM5, JIM7, 2F4 indicate presences of HG in ECM (Eder and Lutz-Mendl 2009)		* CW is 7% cellulose (Kiemle et al. 2010; Chap 3)		* β-(1,3)-glucan present, CW digested with endo-(1,3)-glucanase producing characteristic oligosaccharides. (Kiemle et al.; Chap 3)

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Netrium sp #12	1,2 Walls	*Labled with JIM5 for unesterified HGA, with JIM7 for esterified, with LM5 for B(1-4)-Gal and with LM6 for A(1-5)-Ara HGA (Willats CoMPP)	*Labeled with LM11 for xylans, also labeled for mixed linkage glucans, and non-fucosylated xyloglucan with LM15 (Willats CoMMP)		*Labeled with LM1 extensin (Willats CoMPP)	
Netrium sp #48	1,2 Walls	*Labeled with JIM5 for unesterified HGA with LM5 for B(1-4)-Gal (Willats CoMMP)	*Labeled with LM11 for xylans and non-fucosylated xyloglucan with LM15 (Willats CoMMP)			
Penium margaritaceum	1° Wall	*Found (1,4) D-GalA, NMR found similar resonances, CDTA extract rich in GalA (86%) with Gal, Rha, Ara, Xyl, Fuc and 3-O-MeFuc: novel HGAs. Labeled with Jim5 and Jim7 (Domozych et al 2006)	*Labeled with LM11 for Xylan (Willats CoMMP)	*CW is 35% cellulose (Kiemle et al. 2010; Chap 3) *Insoluble residue contains a majority of 4-Glc with t-Glc, 2,3-Glc, 4,6-Glc, 6-Gal, 3-Gal, 4, 6-Gal, 3-Xyl, t-Fuc, 3-Fuc, t-Rha, 2-Rha, and 2,4-Rha (Kiemle et al. 2010; Chap 3)	*CW labeled with JIM1 for AGP (Domozych)	* β -(1,3)-glucan present, CW digested with endo-(1,3)-glucanase producing characteristic oligosaccharides. CW labeled with 3-linked Glc abundant throughout fractions including 1MKOH, 4MKOH, and insoluble remnant(Kiemle et al.; Chap 3)
Pleurotaenium trabecula				*CW is 12 % cellulose (Kiemle et al. 2010; Chap 3)	* Attachment centers label for AGP with JIM13 and JIM15 (Domozych et al. 2007) *Labeled with LM1 extensin (Willats CoMPP)	

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Pleurotaenium sp.		*Labeled with JIM5 for unesterified HGA with LM6 for A(1-5)-Ara (Willats CoMMP)	*Labeled for mixed link glucans (Willats CoMPP)			*CW labeled for callose (Willats CoMPP)
Spirogyra sp.	1,2 Walls	*Labeled with JIM5 for unesterified HGA (Willats CoMMP)	*Labeled with LM11 for xylans, CCRC-M1 for fucosylated xyloglucan, and non-fucosylated xyloglucan with LM15 (Willats CoMMP)	*Hexagonal arrays of rosettes (Hotchkiss and Brown 1989)	*Evidences for AGP in the EPS, rich in Gal with lesser amounts of Ara * EPS there is an abundance of Gal residues with the major linkage at 2,3,6- with lesser amounts of 2,3-, 2,6-, 3,4,6-, 2-, 3- and t- residues (Kiemle et al. 2010; Chap 5) *The rhizoid of labeled with the AGP antibody JIM16 (Sorenson et al. 2010)	*Labeling for callose: wound-induced and in plane of cell division, (Scherp et al. 2001) *Labeled with LM1 extensin (Willats CoMPP)
Tetmemorus sp		*Labeled with JIM5 for unesterified HGA (Willats CoMMP)	*Labeled for mixed link glucans and also labeled with LM11 for xylans (Willats CoMPP)			
Coleochaetales						
Coleochaete scuata	1,2 Walls	*Found abundant GalA with GlcA, 3-O-Me-Rha (Popper and Fry 2004	*MLG:xyloglucan endotransglucosylase found in CW (Fry et al. 2008b) *CW does not contain xyloglucan (Popper and Fry 2004)	*Putative CesA were detected in EST database (Roberts et al. unpublished)		*Labeling for callose: wound-induced and in plane of cell division, (Scherp et al. 2001)

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Charales						
Chara aculeolata	1,2 Walls	*Found (1,4) D-GalA, IR found similar spectra of pectins with uniform MW contained 89% D-GalA with 1% OCH3, Found GalA (79%), Glc (2%), Ara (1%), and Xyl (Cherno 1978)				
Chara corallina	1,2 Walls	*Found abundant GalA with GlcA, Man and 3-O-Me-Rha (Popper and Fry 2004)	*MLG present in 1MKOH and 4MKOH fraction; CW digests with lichenase producing oligosaccharides with tetrasaccharide and triaccharide oligos. 1MKOH extracts rich in 4-, 3- and terrminal Glc; (Kiemle et al. 2010 Chap 2) * Evidences for XyG with 4- and 4,6-Glc linked are abundant in addition there is both terminal and 2-linked Xyl. (Kiemle et al. 2010, Chap 2) *Labeling for xyloglucan in all of the thallus parts (Domozych et al. 2009) *Other reports found no evidences of XyG in CW (Popper and Fry 2004)	*CW is 5.6% cellulose (Kiemle et al. 2010; Chap 3) * Insoluble residue containing a majority of 4-Glc with t-Glc, 2,3-Glc, 3-Gal, 4, 6-Gal, t-Xyl, 4-Xyl, t-Ara, and 3,4-Ara (Kiemle et al. 2010; Chap 3)		* β-(1,3)-glucan present, CW digested with endo-(1,3)-glucanase producing characteristic oligosaccharides. CW labeled with 3-linked Glc abundant throughout fractions including 1MKOH (Kiemle et al.; Chap 3) *1MKOH fraction contains 2,4- and 4,6-linked Man (Kiemle et al. 2010; Chap 2) CW labeled for mannan (Willats CoMPP)

Taxon		Pectin	Hemicellulose	Cellulose	Structural Protein	Other CW Polymers
Chara sp.	1,2 Walls	*Labeled with JIM5 for unesterified HGA, with JIM7 for esterified, and with LM6 for A(1-5)-Ara HGA (Willats CoMPP)	*Labeled with LM11 for xylans, *LM15 for nonfucosylated xyloglucan, with CCRC-M1 for fucosylated xyloglucans (Willats CoMPP)			
Chara vulgaris	1,2 walls					*Labeling for callose: wound-induced and in plane of cell division, (Scherp et al. 2001)

Appendices

Procedures for Defatting and Cleaning Cell Walls

Alcohol Insoluble Residue protocol: (Fry 2000)

1. The cells are shaken in 70% ethanol at 4°C for 1 h and then the ethanol is removed (centrifuge cells or simply pour off ethanol for something like Chara.
2. Repeat step 1, 3 more times.
3. Transfer cells to glass beaker and add 100% acetone and shake at room temp for 5 min. Decant off acetone and repeat acetone shake 3-4 x or till the final pigmentation leaves the samples.
4. Resuspend cells in a little acetone and then pour all into into glass petri dish. Let the sample dry under a fume hood. Scrape off the AIR material and store at -20 till further use.

Methanolic KOH Extraction (*Chlorokybus* and *Klebsormidium*)

→ 5% KOH in 80% methanol: 25g KOH, 256 mL MeOH, and 64mL dH_2O
→ 0.5N acetic acid: 5.8mL of glacial acetic acid and 200mL dH_2O

1. Grind samples in LN_2 to fine powder
2. Add ~25mL of MeKOH to 50mL tube containing sample
3. Vortex thoroughly
4. Place in ~90°C water bath for 15 mins.
    - Watch that they do not explode!
5. Centrifuge (~5 mins on 2nd fastest setting)
6. Repeat steps (1 – 5) → 2 times (Total 3 times)
7. Add dH_2O, vortex, and centrifuge
8. Pour off supernatant and repeat step 6 (twice more)
9. Add .5N acetic acid (in refrig), vortex and centrifuge (1x)
10. Add dH_2O, vortex, and centrifuge (3x)

Cell Wall Fractionation

Hot Water Soluble Fraction:

1. Add about 20mL of dH_2O to MeKOH pellet,
2. Vortex thoroughly
3. Place in hot water bath (95^oC) for 1 hour
4. Remove allow to cool and centrifuge, collect supernatant
5. Repeat steps a-d → twice more
6. Remove small aliquot of pellet put in eppendorf tube and label: post HW pellet
7. Place supernatant in refrigerator and label: HW soluble fraction

CDTA Soluble Fraction:

→ 0.05M CDTA: 1.822g in 100mL of dH_2O
→ 0.5 M imidazole: 34.04g in 1000mL of dH_2O

1. Add ~20mL of 0.05M CDTA (on shelf) to HW pellet
2. Vortex thoroughly
3. Let sit at room temperature for 6 hours
4. Centrifuge and collect supernatant
5. Wash 3x with dH_2O and collect supernatant
6. Remove small aliquot of pellet put in eppendorf tube and label: post CDTA pellet
7. Combine washes and CDTA supernatant and place supernatant in refrigerator and label: CDTA soluble fraction

Na_2CO_3 Soluble Fraction:

→ 0.05M Na_2CO_3 / 20mM $NaBH_4$: 0.53g Na_2CO_3 and 75.6 mg $NaBH_4$ in 100mL of dH_2O

1. Add ~20mL of 0.05M Na_2CO_3 / 20mM $NaBH_4$ (on shelf) to CDTA pellet
2. Vortex thoroughly
3. Let sit in ice bath at 1^oC for 16 hours
4. Centrifuge and collect supernatant
5. Wash 3x with dH_2O and collect supernatant
6. Remove small aliquot of pellet put in eppendorf tube and label: post Na_2CO_3 pellet
7. Combine washes and supernatant and place in refrigerator and label: Na_2CO_3 soluble fraction

1 M KOH Soluble Fraction:

- → 1M KOH / 20mM $NaBH_4$: 5.6g KOH and 75.6 mg $NaBH_4$ in 100mL of dH_2O
- → Add ~20mL of 1M KOH / 20mM $NaBH_4$ (on shelf) to Na_2CO_3 pellet

1. Vortex thoroughly
2. Let sit in ice bath at 1^oC for 2 hours
3. Centrifuge and collect supernatant
4. Repeat steps a-d (1 more time)
5. Wash 3x with dH_2O and collect supernatant
6. Remove small aliquot of pellet put in eppendorf tube and label: post 1M KOH pellet
7. Combine washes and supernatant and place in refrigerator and label: 1M KOH soluble fraction

4 M KOH Soluble Fraction:

- → 4M KOH / 20mM $NaBH_4$: 22.4g KOH and 75.6 mg $NaBH_4$ in 100mL of dH_2O
- → Add ~20mL of 4M KOH / 20mM $NaBH_4$ (on shelf) to 1M KOH pellet

1. Vortex thoroughly
2. Let sit in ice bath at 1^oC for 2 hours
3. Centrifuge and collect supernatant
4. Repeat steps a-d (1 more time)
5. Wash 3x with dH_2O and collect supernatant
6. Remove small aliquot of pellet put in eppendorf tube and label: post 4M KOH pellet
7. Combine washes and supernatant and place in refrigerator and label: 4M KOH soluble fraction

Derivatization for Neutral Sugar Analysis (Monosaccharides)

TFA Hydrolysis

1. Unknowns - Add 100ul (50uL) of Inositol [Inos] (1ug/ul) to Reacti-vial and dry under filtered air or N_2. To the vial containing Inos, weigh in 0.5 to 4 mg of freeze-dried sample. Repeat for 4 replicates.
2. Standards - To each vial add 100ul of a 9 sugar standard containing 1ug/ul Glc, Man, Gal, Xyl, Ara, Rib, Fuc, Rha, Inos) and dry. Repeat for 4 replicates.
3. Add 200ul 2M TFA to each vial. Mix
4. Seal vials tightly and hydrolyze at 121°C for 3hrs in a hot block. Ensure that vials protrude from the block, there is airflow across the block (place in partly closed hood), and that samples are refluxing.
5. Cool vials to room temp. (after cool, tap vial to bring all solution to bottom of vial, ensure that no evaporation has taken place)
6. Dry under nitrogen on water bath (40°C)

Saponification of Uronics and makeup of borohydride

1. Add 200 ul of 1M ammonia. Let stand 1-2 h to saponify uronics and eliminate them from detection.
2. While the vials are standing, prepare a solution of $NaBH_4$ in dimethyl sulfoxide, DMSO, (fresh, anhydrous). [Add 0.5g $NaBH_4$ to 25 ml DMSO. Heat with stirring under hood @ 100°C for 10 min. Let cool to 20-22°C before use].

Reduction to Alditols

1. Add 500 ul of $NaBH_4$ in DMSO solution to each vial at room temperature. Seal vials with regular teflon-lined caps and mix well.
2. Incubate @ 40°C for 90 min. on water bath.
3. Cool to room temp.
4. Add 50 ul 18M (conc.) acetic acid.
5. Mix. Cool to room temp.

Acetylation

1. Add 100 ul 1-methylimidazole. Mix.
2. Add 1.0 ml acetic anhydride. Mix.
3. Incubate @ 20°C for 10 min.

4. Add 2.5 ml HPLC H_2O to quench reaction. Mix by inverting and mechanically agitating. Cool to room temp.

Phase extraction

1. Add 0.5 ml methylene chloride ($MeCl_2$). Mix well.
2. Let stand till phases separate completely.
3. Remove bottom layer ($MeCl_2$) and transfer to $MeCl_2$-rinsed (2x) Eppendorf tubes.
4. Check for floating water and remove using a glass pipet.
5. Dry under N_2 or filtered air @ 40°C on water bath. Store at -20 C until analysis.

GC/MS Analysis

* Inject 1.0 ul aliquot (from 5-10ul $MeCl_2$ added) onto GC column.
* Column: Supelco, Inc. SP2330 fused silica capillary, 30 meters
* Split injector head pressure: 6-9 psi
* Split vent: 68cc/min, approx. 1:100 split
* Column temp: 230°C isothermal
* Note: Retention times on 30 m for Inos (last off) are approx. 25 min. For shorter runs switch to 15m SP2330 and run temp program to optimize separation (15 min to Inos).

Quantification of Monosaccharide Analysis

Part I: Setting up Calibration

Ctrl D: enter file name

C open chromatogram (about 400 to 2000) place the curser over inos

Q → Build / Edit Internal Standard Calibration

- Inos
- 50 UG
- Internal yes,
- Fit cal (A) for average,
- F10, then space.

Then the rest of the sugars:

- 50 UG
- Internal No,
- Fit cal (A) for average,
- F10, then space.

Glc (–20 secs)
Gal (–20 secs)
Man (–20 secs)
Xyl (–20 secs)
Ara (–20 secs)
Rib (–20 secs)
Fuc (–10 secs)
Rha (–10 secs)

Part II:Calibration of Standards

Ctrl D: enter file name

Q , A

Turn off → Ctrl p (peak smoothing)
Turn on → Ctrl c (apply cali data)
Turn on → Ctrl s (single step)

F2

- Analyze all standards S1→ S4
- Check calibration reports

- F1, Shift + or –

Part III: Analyze Samples

Ctrl D: enter file name

Q , A

Turn off → Ctrl c (apply cali data)
Turn on → Ctrl s (single step)
Turn on → Ctrl p (peak smoothing)
F1 (integration)
Analyze all samples

Part IV: Reporting Quantification

* Set up Plot Spooler (P)
*
* Ctrl D: enter file name
  - Q , Q , F2 , Ctrl F2 , Alt S

* Back to main menu: C → open Chromatogram (400 to 2000)
  - Alt S

* Analyze all samples but remember to print standards

Linkage pattern analysis

Micro method for linkage/substution site analysis of oligosaccharides (<100ug)

Per-O-Methylation

1. Note: per-O-methylation should be done in glove box under nitrogen
2. Add 0.4 ml of dry DMSO, stir 2-4 h (or until solubilized). Do not heat or sonicate.
3. Add 0.2 ml Butyl lithium and stir for ~30 min (until sample turns faint yellow).
4. Cool sample to 0 C with frozen reacti-therm block. Add 0.2 ml of CH_3I. Let sample warm to room temp (~15 min), vortex briefly to remove crust from walls of vial.and stir for 1h.
5. Add 0.750 ml H_2O and mix.

Sep-Pak C-18 Clean up

1. Preflushed with 40 ml of 100% Ethanol
2. Preflush with 2 ml of 100 % acetonitrile (HPLC grade)
3. Preflush with 4 ml of water (HPLC grade)
4. Mix sample and load onto Sep-Pak C 18 cartridge (Push through slowly 1-2 drops per second)
5. Rinse reactivial with 500 ul of 1:1 (v/v) DMSO/ H2O (HPLC), and push through column.
6. Wash off contaminants with 2 ml H2O, repeat 3x (to dryness the last time) (Discard)

For Oligos of DP 2-10

7) Wash 4 x with 2 ml 3:17 (v/v) acetonitrile/H2O (both HPLC) (Discard)
8) Elute sample with 2 ml of acetonitrile into larger silanized tubes
9) Dry under filtered air at room temperature

For Oligos of DP >10

7) Wash 4 x with 2 ml 3:17 (v/v) acetonitrile/H2O (both HPLC) (Discard)
8) Wash 2x with 2 ml 1:4 (v/v) acetonitrile/H2O (to dryness the last time) (Discard)
9) Elute sample with 2 ml of 100 % acetonitrile and then 4 ml of 100 % EtOH into larger silanized tubes

10) Dry under N_2 at room temperature
11) Transfer to reactivial with 1 ml methylene chloride (Dichloromethane)
12) Rinse tube with 200 ul of MeCl2, transfer and Dry. Repeat 2x.

Hydrolysis

1. Add 200 ul of 2 M TFA (Aldrich, T6,2200)
2. Seal reactivials under N2
3. Heat at 121 C for 1 h
4. Let cool and add 10 ug of inositol (1/10 of sample weight)
5. Dry under N2 at room temperature
6. Add 200 ul methanol and dry under nitrogen

Reduction and Acetylation

1. Dissolve sample in 100 ul of 1 M NH_4OH.
2. Add 500 ul DMSO containing 10 mg of $NaBD_4$ and incubate for 90 min. at 40 C.
3. Add 100 ul glacial acidic acid and mix.
4. Add 100 ul anhydrous 1-methylinidazole and 500 ul of acetic anhydride and let incubate for 10 min at room temp.
5. Add 1.5 ml HPLC H_2O and cool to room temp.
6. Add 1 ml dichloromethane and shake and remove dichloromethane layer. Repeat 1x and combine extracts.
7. Wash 2x with 2ml of ddH_2O.
8. Dry samples under filtered air (a small amount of 2,2-dimethoxypropane can be added to speed up evaporation)

Methylation Analysis of Uronic Acid Oligosaccharides

Pretreatment with Dowex

1. Dissolve 5 mg (or less but > 2 mg) into 5 ml dH2O
2. Add 5 ml of clean Dowex (50 W, HCR-W2) and mix in 40 ml tube
3. Use Pasteur pipette to draw sample off
   Add 5 ml of dH2O to Dowex, dump in to syringe with 5 um filter, add to supernatant.
4. Freeze-dry the samples
5. Next steps are the same as mentioned in methylation protocols.

GCMS startup and Shut Down

Start-up:

1. Start computer
2. Turn on Helium (open silver knob)
3. Switch for GC (back left top – toggle switch)
4. Keep column cold – turn injector to 100 and transfer line to 240^{o} (Aux)
5. Turn up pressure (Injector must be hot before you turn up front column pressure)
6. Turn up split vent
7. Turn on box (heater) gas purifier
8. Turn on MS (white) master circuit breaker – turbo pump
9. Check for leaks
10. Turn injector up to 230^{o} (Column remains at 35^{o})
11. Wait about 1.5 hrs
12. Leak check again
13. Turn column temp to 140^{o} by program 10^{o}/min
14. Diagnostics

Shut-down:

1. Cool column to room temperature (30-35^{o}) (Open oven door and check temperature)
    a. On GCMS front computer panel press Build Modify, then Method 1, then type in 30 where it ask for initial temp then hit Activate. (The temperature should start going down)
2. From main screen of the computer press "Z" → Shut down procedure.
3. When injection temperature goes down to (about 200^{o}) turn pressure off the injector (Knob in the front of the GC turn pressure pass ZERO)
   Wait for turbo speed level (67%) don't wait once it happens (Do not have to wait for step 3)
4. Turn off heat zones (Automatic with program)
5. Turn off GC (Left back switch)
6. Turn off purifier (Left side switch)
7. Turn off tanks (Main silver valves turn clock-wise)
8. Turn off computer (Alt X)

Updegraff Procedure for Cellulose

1) Homogenize sample. (Freeze crack with Liquid N_2 and freeze-dry)
2) Weight sample, place in 12mL centrifuge tube.
3) Add 3.0 ml acetic nitric reagent; add 1.0ml mixing well, then add the remaining 2.0 ml and remixing
4) With a marble on top to reduce evaporation and create a refluxing action, place tubes in a boiling water bath for 30min. Maintain bath level at same level as liquid in the tubes.
5) Centrifuge 5min at high speed. Decant and discard supernatant.
6) Repeat #4 until pellet is neutral.
7) Freeze-dry

* Acetic / nitric reagent: mix 150mL of 80% acetic acid and 15 mL conc. nitric acid.

Updegraff, D. (1969) Semimicro Determination of Cellulose in Biological Materials. Anal Biochem. 32, 420-424.

Colormetric Assays

Phenol-Sulphuric

Remember! Phenol is toxic and a possible carcinogen. Use safe lab practices!

Standard Curve

1. Dissolve 1ml glucose stock (in freezer) in 99mL of dH_2O to make 100mL of solution.
2. Prepare 5% phenol solution by dissolving 5 grams of phenol in 100mL of dH_2O. Put solution in brown bottle. Work in the hood and expose the phenol to as little light as possible.
3. Dispense glucose solution into clean tubes with 4 replicates of each concentration and bring to 1mL as follows:

Glucose Standard Curve		
Concentration	µL Glucose	µL dH_2O
0	0	1000
10	100	900
20	200	800
40	400	600
60	600	400
80	800	200
100	1000	0

4. To 1mL of aqueous solution, add .5mL of 5% phenol solution. Vortex.
5. Jet into this mixture 2.5mL conc H_2SO_4 (5 pumps). This reaction is exothermic. The liquids will boil up. Take care. The head generated is necessary for the reaction.
6. Allow tubes to cool for 30 minutes.
7. Pour solution into cuvettes or small test tubes and measure the absorbance at 485 nm with spectrophotometer. Blank against distilled water blank. Measure between 30 minutes and 4 hours of reaction. The color is stable for this time.
8. Use Excel to plot the absorbance and concentration as a scatter plot. Find the r^2 value for the trend line, it should be above 99%. If not, do a new curve.

With samples

1. Measure .250 mg of sample and put into clean tubes. Use at least 3 replicates. Be sure to label and keep track of tubes throughout procedure to ensure accurate results.
2. Bring samples to 1mL by adding dH_20.

3. Proceed as stated above, steps 4-7.

Carbazole Assay (Total Uronic Acid % w/w)

1) Weigh out 0.277 g of sodium tetraborate
2) Add 55 ml of conc. H_2SO_4 (0.025M)
3) Pipete 5 ml of H_2SO_4 reagent in 10 test tubes with screw caps and freeze at -80 °C
4) Weigh out 1 g of carbazole
5) Add 100ml of absolute ethanol (stable for 12 weeks at 4 °C in the dark).
6) Weigh out 500 µg of standard and bring to volume, 5ml.
   Tube 1: 1ml H_2O
   Tube 2: 1ml Standard
   Tube 3: 800 µl standard + 200 µl H_2O
   Tube 4: 600 µl standard + 400 µl H_2O
   Tube 5: 400 µl standard + 600 µl H_2O
   Tube 6: 200 µl standard + 800 µl H_2O
7) Weigh out four 100 µg samples and bring each to volume, 1ml.
   Tube 7-8: 1ml sample
8) Add 1ml of standard or sample to each of the frozen testtubes with H_2SO_4 on ice.
9) Mix gently at first, then more vigorously (vortex)
10) Heat in boiling water bath for 12 min.
11) Remove and cool to room temp on ice.
12) Add 200 ul of carbazole reagent to each tube.
13) Mix by vortexing
14) Heat in boiling water bath for 15 min.
15) Remove and cool on ice to room temp.
16) Transfer to 2.5ml cuvettes
17) Read absorbance in Spec at 530 nm.

DNS reducing sugar Assay

1. In 200ml of warm distilled water add 8g of NaOH and dissolve.
2. Add 150g of sodium potassium tartrate and dissolve.
3. Add 5g of 3,5 dinitrosalicylic acid (DNS) and add heat to dissolve.
4. Adjust the volume to 500 ml with distilled water.
5. Add 100 ul of sample to 100ul of DNS reagent, mixed with a vortex and the tubes are placed in a water bath (100 C) for 5 min. and cooled.
6. Add 1ml of distilled water, mix with a vortex and read the OD at 540 nm.
7. Standard curve 0-500 ug/ml

Digestion for Oligosaccharide Mass Profiling

Digestion with Lichenase:

1. 10 mg whole cell was pre-swollen in 1ml 1M KOH supplemented with 10mg/ml NaBH4 overnight at 4^0C
2. Followed by neutralization with 180µL acetic acid and addition of 3 volumes 99% EtOH.
3. The precipitate was then collected by centrifugation and washed with 80% EtOH followed by buffer solution (0.2M disodium hydrogen phosphate buffer of pH 6.5). 0.5 ml 0.2M phosphate buffer of pH6.5 was then added to the precipitate.
4. Lichenase (100U) was added prior to incubation at 40^0C for 3 hours.
5. After incubation absolute EtOH (1.5 ml) was added and samples were chilled for 1 hour before collection of supernatants.
6. Supernatants were then dried under filtered air.
7. Barley flour was used as control.

Digestion with xylanase M2:

1. 10 mg whole cell was pre-swollen in 1ml 1M KOH supplemented with 10mg/ml NaBH4 overnight at 4^0C
2. Followed by neutralization with 180µL acetic acid and addition of 3 volumes 99% EtOH.
3. The precipitate was then collected by centrifugation and washed with 80% EtOH followed by buffer solution (50 mM sodium acetate of pH 4.5).
4. 1 ml 50 mM sodium acetate buffer of pH4.5 was then added to the precipitate Xylanse M2 (100U) was added prior to incubation at 50^0C. for 3 hours.
5. After incubation absolute EtOH (1.5 ml) was added and samples were chilled for 1 hour before collection of supernatants.
6. Supernatants were then dried under filtered air.
7. Beechwood xylan was used as control

Digestion with endo-1,3-β-D-glucanase:

1. 10 mg whole cell was pre-swollen in 1ml 1M KOH supplemented with 10mg/ml NaBH4 overnight at 4^0C
2. Followed by neutralization with 180µL acetic acid and addition of 3 volumes 99% EtOH.
3. The precipitate was then collected by centrifugation and washed with 80% EtOH followed by buffer solution (50 mM sodium acetate of pH4.5).
4. 1 ml 50 mM sodium acetate buffer of pH4.5 was then added to the precipitate . endo-1,3-β-D-glucanase was added prior to incubation at 50^0C for 3 hours.
5. After incubation absolute EtOH (1.5 ml) was added and samples were chilled for 1 hour before collection of supernatants.
6. Supernatants were then dried under filtered air.
7. Pachyman was used as control.

Preparation of MALDI –TOF –MS samples

1. Add 20µL of distilled water to each of the eppendorf tubes contain enzyme digested oligosaccharides and mix well.
2. Add 5-10 washed biorex MSZ501 resin beads (only the cation exchanging ones to each of the eppendorf tubes.
3. Transfer 10µL of the supernatant to a new tube and leave it in there for 10-15 minutes. Centrifuge.
4. Spot 2µL matrix (2,5-dihydroxy benzoic acid, 10mg/ml in water) onto target plate, dry under vaccum
5. Dilution series: in the 96 miro –well plate:
6. Dilution A (1/5): Take 2µL of desalted sample and ad 8µL of distilled water.
7. Dilution B (1/25): Take 2µL of dilution A and add 8µL of distilled water
8. Dilution C (1/25): Take 2µL of dilution B and add 8µL distilled water
9. Add 2µL of treated supernatant on to the dried DHB, spot sample for a maximum of 3 minutes and then wait 2 minutes before applying accum to the samples.
10. Spotted MALDI plate can be stored in dark at room temperatute for couple of days.

Parameters:

1. Collect mass fragments 300-2000.
2. Cal: xyloglucan 9 Jan 2007 14:17, Shimadzu Biotech Axima CFRplus 2.8.1.20080410:
3. Mode reflectron, Power:126, Blanked, P. Ext at 1400 (bin 115) % int. 185mV [Sum=18482 mV] Profiles 1-100 Smooth Av 2 – Baseline 6
4. In positive reflectron mode with an acceleration voltage of 20 kV and an extraction delay time of 350 ns.
    a. Lichenase fragments from 365-1175
    b. Xylanase fragments from 305-965

Oligosaccharide Masses for Maldi-TOF Results

XyG Ladder:

XXG 791
GXXG 953
XXXG 1085
XXLG 1247
XXLG +Ac 1289
XXFG 1393
XXFG +Ac 1435
XLFG 1555
XLFG + Ac 1597

Hexose Ladder:

H2 365
H3 527
H4 689
H5 851
H6 1013
H7 1175
H8 1337
H9 1499
H10 1515

Pentose Ladder:

P2 305
P3 437
P4 569
P5 701
P6 833
P7 965
P8 1097
P9 1229
P10 1361

Additions:

- Adding hexose into the middle: add 162
- Adding pentose into the middle: add 132
- Add Acetyl group: add 42

Xyloglucan Standard