Molecular tools for the identification and localization of algal cell wall components

Raimundo, Sandra Cristina Carneiro

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Molecular tools for the identification and localization of algal cell wall components

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Sandra Cristina Carneiro Raimundo

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SUPERVISOR
Dr. Zoë A. Popper
Botany and Plant Science
Ryan Institute for Environment, Marine and Energy Research
School of Natural Sciences
National University of Ireland Galway
Galway
Ireland

EXAMINERS
Dr. Maria Tuohy
Molecular Glycobiotechnology Group
Department of Biochemistry
School of Natural Sciences
National University of Ireland Galway
Galway
Ireland

Professor Azeddine Driouich
Laboratoire Glycobiologie et Matrice Extracellulaire
Normandie Université
Institute for Research and Innovation in Biomedicine, Végétal, Agronomie, Sol, et Innovation
76821 Mont-Saint-Aignan
France

CHAIR
Professor Peter Dockery
Anatomy
School of Medicine
National University of Ireland Galway
Galway
Ireland
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ABSTRACT

Cell wall glycan-directed monoclonal antibodies (mAbs) are sensitive tools for the detection of wall components in vitro (glycome profiling) and in situ (immunolocalization). Although extensively applied to land plant and green microalgal research, their application to seaweeds is rare and mAbs generated against commercially valuable red- (carrageenans) and brown- (sulfated fucans and alginates) seaweed polysaccharides were never made available to the scientific community. Although brown seaweeds are only distantly related to land plants and the other algal groups, the glycome profile of Fucus vesiculosus indicated that several mAbs, belonging to a group that recognizes arabinogalactans (RG-I/AG), bound to extracts and gave distinct in situ labelling patterns. This led to the conclusion that epitopes from structurally different galactans are present and have distinct spatio-temporal distributions within seaweed tissues. The LAMP mAb, generated against laminaran, recognizes a (1,3)-β-glucan epitope. While it is commonly used for the immunolocalization of callose in plants it has only rarely been applied to brown seaweeds. However, distinct in situ labelling patterns in F. vesiculosus and Laminaria digitata indicate that LAMP is a valuable probe for brown seaweed research. The LM7 mAb recognizes an epitope present in homogalacturonan, localized in land plants to the corners of the intercellular spaces between adherent and separated cell walls. In situ labelling of F. vesiculosus and L. digitata highlight this mAb as an excellent marker for alginates. These results show that some land plant cell wall glycan epitopes are present in brown seaweeds, providing new insights into cell wall evolution. Additionally identification of existing mAbs that can recognize epitopes present in seaweed cell walls has diversified and added valuable additional tools for algal research.

The Vreeland mAbs, generated against seaweed polysaccharides, were tested to see if they remained viable after nearly 40 years storage. Some of the mAb supernatants indeed proved viable and can be used to produce hybridoma lines, creating antibodies for seaweed research. The green microalga Penium margaritaceum, used as a unicellular model organism for primary cell walls, was shown to be highly sensitive to plant hormones that had severe repercussions for growth and morphology. However, more pertinently, using mAbs, many of the impacts were seen to specifically involve the cell wall. These studies pave the way for future research regarding the restructuring of specific cell wall components, and wall architecture, by hormones as well as investigation of the mode of action of inhibitors.
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<th>Definition</th>
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<tbody>
<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>AG-3</td>
<td>Arabinogalactan-3</td>
</tr>
<tr>
<td>AGP</td>
<td>Arabinogalactan protein</td>
</tr>
<tr>
<td>AIR</td>
<td>Alcohol insoluble residue</td>
</tr>
<tr>
<td>Ara</td>
<td>Arabinose</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCRC</td>
<td>Complex Carbohydrate Research Center</td>
</tr>
<tr>
<td>CGA</td>
<td>Charophyceae green algae</td>
</tr>
<tr>
<td>CHPAA</td>
<td>3-chloro-4-hydroxyphenylacetic acid</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>COS</td>
<td>Chitosan oligosaccharides</td>
</tr>
<tr>
<td>DIC-LM</td>
<td>Differential interference contrast light microscopy</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular polymeric substance</td>
</tr>
<tr>
<td>FCSPs</td>
<td>Fucose-containing sulphated polysaccharides</td>
</tr>
<tr>
<td>Fuc</td>
<td>Fucose</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberellic acid</td>
</tr>
<tr>
<td>Gal</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalA</td>
<td>Galacturonic acid</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Gas chromatography/mass spectrometry</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GulA</td>
<td>Guluronic acid</td>
</tr>
<tr>
<td>HG</td>
<td>Homogalacturonan</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole-3-butyric acid</td>
</tr>
<tr>
<td>KPBS</td>
<td>Potassium phosphate buffered saline</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>ManA</td>
<td>Mannuronic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MBS</td>
<td>3-maleimidobenzoic acid N-hydroxy succinimide ester</td>
</tr>
<tr>
<td>MLG</td>
<td>Mixed-linkage glucan</td>
</tr>
<tr>
<td>MSB</td>
<td>Microfilament stabilizing buffer</td>
</tr>
<tr>
<td>MYA</td>
<td>Million years ago</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PME</td>
<td>Pectin methylesterase</td>
</tr>
<tr>
<td>Rha</td>
<td>Rhamnose</td>
</tr>
<tr>
<td>RG-I</td>
<td>Rhamnogalacturonan I</td>
</tr>
<tr>
<td>RG-I/AG</td>
<td>Rhamnogalacturonan I/Arabinogalactan</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TMS</td>
<td>Per-O-trimethylsilyl</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>VPSEM</td>
<td>Variable pressure scanning electron microscopy</td>
</tr>
<tr>
<td>WHM</td>
<td>Woods Hole medium</td>
</tr>
<tr>
<td>Xyl</td>
<td>Xylose</td>
</tr>
<tr>
<td>1-NOA</td>
<td>1-Naphthoxyacetic acid</td>
</tr>
<tr>
<td>2-NOA</td>
<td>2-Naphthoxyacetic acid</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>6-BAP</td>
<td>6-Benzylaminopurine</td>
</tr>
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</table>
PUBLICATIONS AND COMMUNICATIONS

BY THE AUTHOR

PUBLICATIONS

Raimundo SC, Pattathil S, Eberhard S, Hahn MG, Popper ZA. β-1,3-Glucans are structural components of brown seaweed (Phaeophyceae) cell walls. Submitted to Protoplasma.


ORAL PRESENTATIONS

Raimundo SC and Popper ZA. Immunolocalization of cell wall carbohydrate epitopes in the brown seaweed Fucus vesiculosus (Phaeophyceae). Botany and Plant Sciences Internal Research Seminar Series (NUI Galway), March 2014;


Raimundo SC. Composition and localization of algal cell wall components. THREESIS — Open your mind... and get to the point! (NUI Galway), December 2012;

Raimundo SC and Popper ZA. Composition and localization of algal cell wall components II. Botany and Plant Sciences Internal Research Seminar Series (NUI Galway), October 2012;

Raimundo SC and Popper ZA. Composition and localization of algal cell wall components I. Botany and Plant Sciences Internal Research Seminar Series (NUI Galway), April 2012.
Poster Presentations

**Raimundo, SC**, O’Callaghan, C, Popper, ZA. Algal cell walls: evolution and diversity. 6th European Phycological Congress (London, UK), August 2015;

**Raimundo S**, Hahn, MG, Popper, ZA. Application of vascular plant cell wall carbohydrate-directed monoclonal antibodies as molecular markers for the study of brown seaweed (Phaeophyceae) cell walls. Plant Cell Walls Gordon Research Conference (Bentley University, Waltham, MA, USA), July 2015;

Tinaz B, Sun L, Lietz AP, Patten M, Keehn P, Ritter E, **Raimundo SC**, Popper ZA, Domozych D. Dissecting plant cell wall development one polymeric domain at a time using the unicellular charophyte, *Penium margaritaceum*. Plant Cell Walls Gordon Research Conference (Bentley University, Waltham, MA, USA), July 2015;

**Raimundo S** and Popper, ZA. Arabinogalactan proteins in seaweeds: taxonomic and seasonal variation. XIII Cell Wall Meeting (Nantes, France), July 2013;


Leroux O, Pielach A, **Raimundo SC** and Popper ZA. Plant and algal cell walls: structure and function. Ryan Institute Launch and Symposium (NUI Galway), July 2012;

1

**General Introduction**
1.1. Evolution and diversity of land plants and algae

The phylogenetic eukaryotic tree of life has been gradually assembled over the last couple of decades, starting from the most commonly cultivated and well characterized organisms (mainly land plants, animals and fungi). It has gradually been complemented with genetic information, which has revolutionized previous ideas about eukaryotic evolution and resolved many of the relationships amongst the major eukaryotic groups (Baldauf, 2003, 2008). One of the primary goals of every phylogenetic tree, the oldest point in the tree that matches the theoretical last common ancestor, or root, has proven to be a true headache for scientists; in the case of eukaryotes it is believed to be ancient and complex (Baldauf, 2003, 2008). Evolution has resulted in a vast diversity of organisms but has also yielded in many problems related to nomenclature and associated issues regarding classification whereby some organisms do not appear to clearly fit into any groups, while others can fit in more than one group (Patterson, 1999). Figure 1.1 shows a simplified phylogenetic tree for the major groups of eukaryotes. The work presented in this thesis is based on the research performed with species from the Archaeplastida and Stramenopile lineages, where freshwater and marine algae are included; therefore this introduction is focused on these lineages.

![Phylogenetic Tree](image)

**Figure 1.1.** A consensus phylogeny of the major Eukaryotic groups based on molecular phylogenetic and ultrastructural data (modified from Baldauf, 2003).
The green lineage in the eukaryotic tree of life (Viridiplantae, Latin for “green plants”) is one of the major groups of photosynthetic eukaryote organisms, and includes the green algae (Chlorophyta and Streptophyta) and land plants (Embryophyta) (Figure 1.2) (Becker and Marin, 2009; Leliaert et al., 2012). Scientists agree that photosynthesis was acquired by eukaryotes through primary endosymbiosis; the entrapment of a cyanobacterium that later evolved to became a plastid (De Clerck et al., 2012). Within the molecular timeline of algal evolution, it is believed that such an event occurred after the split of the plantae lineage (algae and land plants) from the opisthokonts, around 1558 million years ago (MYA); the split between red and green algae occurred around 1500 MYA, and the red algal secondary endosymbiosis that is the origin for the plastid in e.g. brown algae occurred around 1300 MYA (Yoon et al., 2004).

Figure 1.2. Overview of the phylogeny of the green lineage (modified from Leliaert et al. 2012 and Timme et al. 2012).

The time course of evolution from a green algal ancestor to the diversification of land plants is noted as a period of major innovation in natural history, where a particular event dating from 480–360 MYA marked one of the most remarkable developments in the history of life on Earth: the emergence of green algae to the land environment (Kenrick and Crane, 1997). The complexity and diversity that land plants have developed are innovations that
have allowed them to dominate terrestrial ecosystems, initiating the development of the entire terrestrial environment (Karol et al., 2001).

Understanding the evolutionary processes and the phylogenetic relationships between the four major lineages of land plants; (1) bryophytes (mosses), (2) lycopodiophytes (lycopods or clubmosses), (3) pteridophytes (ferns), and (4), angiosperms (flowering plants) is a controversial matter, which is starting to become more clear through ultrastructural, biochemical and molecular data (Leliaert et al., 2012; Qiu et al., 2006). Through evolution, photosynthetic organisms adapted to specific environmental conditions, and eventually developed some form of specialized external or internal material in direct contact with the plasma membrane that could protect them against stressful biotic and abiotic factors, a cell wall. Evidence shows that the origin of the machinery responsible for the cell wall synthesis of land plants, of their closest algal relatives, and of some of the oldest (red and brown algae) algal lineages dates back to ancient primary endosymbiotic and horizontal gene transfer events (Michel et al., 2010b; Niklas, 2004). This shared ancestry between algae and land plants reflects itself in similarities in their wall polymer composition (Popper and Tuohy, 2010; Popper et al., 2011), but in order to fully understand the early evolution of plant cell walls, it is necessary to go further down in the evolutionary tree, and study the living algal groups considered to be most similar to land plants.

Within the Streptophyta lineage, the Charophyceae green algae (CGA) are the closest extant group of green algae related to land plants, which makes their study important for unravelling cell wall evolution (Domozych et al., 2012; Eder et al., 2008; Eder and Lütz-Meindl, 2010; Popper and Fry, 2003; Popper and Tuohy, 2010; Popper et al., 2011; Sørensen et al., 2010; Sørensen et al., 2011; Timme et al., 2012). Charales, Coleochaetales, Zygnematales, Klebsormidiales, Chlorokybales and Mesostigmatales are the six orders included within the CGA, and the transition to land is believed to have been driven by the progressive occupation of dry environments by freshwater algae (Becker and Marin, 2009; Lewis and McCourt, 2004). There is a lot of debate regarding which of the CGA groups are the closest living ancestors of the embryophytes; often the literature fails to address this question (Turmel et al., 2002); some studies place the Charales as the closest living relatives of land plants (Karol et al., 2001; Turmel et al., 2006), while other reports present the Zygnematales as the closest group (Timme et al., 2012; Wodniok et al., 2011). Nonetheless, the Charales, Coleochaetales and Zygnematales are the three most advanced CGA orders, and share cell walls that are comparable to the primary walls of land plants, e.g. detection of lignin-like polymers in a Coleochaete alga, led to the conclusion that the ability to synthesize
many of the wall components typical of land plants evolved during divergence within CGA (Sørensen et al., 2011). The CGA have been, therefore, considered to be model organisms for the study of evolutionary traits related to land plants and to cell walls (Sørensen et al., 2012).

Red algae (phylum Rhodophyta) are a distinct and ancient eukaryotic algal lineage (Yoon et al., 2004). The oldest taxonomically resolved eukaryotic fossil is 1.2 billion years old and is believed to belong to an extant red algal species (De Clerck et al., 2012). This phylum represents the most species-rich group of marine macroalgae, with around 7000 described species (Guiry, 2012), divided into seven classes (Yoon et al., 2006). Red seaweeds are important elements in many marine ecosystems, including rocky intertidal shores and coral reefs (Graham and Wilcox, 2000; Lobban and Harrison, 1994). Some of the most common species found on the Irish coast such as Porphyra sp. and Chondrus crispus are economically important as food and as sources of gelling agents. Red algal plastid and nuclear genomes contributed, via secondary endosymbiosis, to several eukaryotic lineages, including the brown algae (Yoon et al., 2004), hence the genes of the red algal ancestors are widely spread amongst eukaryotes. The recent sequencing of the genome of the red alga, Chondrus crispus, has contributed new knowledge regarding the evolutionary origins of the Archaeplastida, including proof of polyphyly of cellulose synthesis within this lineage (Collén et al., 2013).

Stramenopiles (formally, Heterokontophyta) form an independent eukaryotic lineage (Figure 1.1) that is neither related to the other algal groups, nor related to land plants, and where Phaeophyceae, commonly known as brown seaweeds, are included (Patterson, 1999). This class comprises approximately 1500 species of multicellular photosynthetic organisms (Norton et al., 1996). With a global distribution, in almost exclusively marine environments (Cock et al., 2010), brown seaweeds commonly dominate intertidal and subtidal areas of rocky shores in the northern hemisphere, and can form ecologically important three-dimensional structures for associated species (Bold and Wynne, 1978; Graham and Wilcox, 2000; Lobban and Harrison, 1994; Schiel and Foster, 2006). Although less complex than most vascular plants, brown algae have the highest morphological complexity of the major macroalgal groups (Charrier et al., 2012). Some ecologically important species are very common on the Irish coast, and include mid-littoral species such as Fucus vesiculosus and Ascophyllum nodosum (Fucales), as well as Laminaria digitata (Laminariales), that inhabits the lower intertidal zones (Bold and Wynne, 1978). The intertidal environment can be an extremely challenging place to live, where abiotic factors such as light, turbulence, nutrients, temperature and salinity vary frequently (Schiel and Foster, 2006). It is not, therefore
surprising, that during the course of evolution, seaweeds acquired different characteristics from other algae and from land plants, that allowed them to thrive in such demanding environments.

Highly heterogeneous, Phaeophyceae include some of the largest organisms known, e.g. the giant kelp *Macrocystis pyrifera*, and the smallest, such as the microscopic, filamentous *Ectocarpus siliculosus* (Charrier et al., 2012). This small species has recently emerged as a model organism for brown algal research. Its sequenced genome provided not only new insights for the independent evolution of brown algae (Cock *et al*., 2010), but also unprecedented information about the evolutionary processes that led to multicellularity and cellular differentiation and the uniqueness of the polysaccharides used by algae as storage sugars and cell wall components (Coelho *et al*., 2012). Such studies give evidence that the ability to produce some cell wall compounds shared with land plants and red algae, such as cellulose, were inherited via endosymbiosis of the ancestral red alga, while the unique ability to produce alginates was acquired through horizontal gene transfer from an Actinobacterium; the biosynthetic pathway for sulfated fucans is ancestral and conserved with the animal lineage (Baurain *et al*., 2010; Michel *et al*., 2010b; Stiller *et al*., 2009). However, in the case of cellulose, the sequencing of *Chondrus crispus*, indicated that cellulose biosynthesis in brown algae has an ancient, but distinct origin from the other lineages (Collén *et al*., 2013).

1.2. **Comparison between land plant and algal cell walls**

Algal cell walls are quite unique and different from land plant cell walls, nonetheless they share some polymers (Popper, 2008; Raimundo *et al*., 2015). Marine algal cell walls have higher percentages of matrix polysaccharides in relation to fibrillar ones, e.g. cellulose microfibrils (Naylor and Russel-Wells, 1934). They also contain high levels of sulfated polysaccharides, as well as higher quantities of acidic over neutral polysaccharides (Percival, 1979). The presence of sulfated polysaccharides in seaweeds, sulfated fucans and galactans in brown and red seaweeds, respectively, is evolutionarily correlated with the adaptation to life in the presence of high salinity environmental conditions, which was retained by marine green algae during plant evolution (Aquino *et al*., 2011; Usov, 1998). On the other hand, the acquisition of compounds such as the hemicellulose xyloglucan may be part of the adaptation process that resulted in the colonization of land (Popper and Fry, 2003), although evidence suggests that the most developed CGA taxa may have a different form of xyloglucan (Sørensen *et al*., 2010; 2011). Land plants can also generate a secondary wall layers in which
microfibrils are laid down after the cell has lost the ability to expand, and are part of specialized tissues such as xylem vessel elements, tracheids, or cork; they may also be impregnated with lignin (Fry, 2010). To date, secondary walls are unknown in other lineages, although the existence of lignin in a coralline red alga has been reported (Martone et al., 2009).

1.3. Plant cell walls

Studies on the plant cell wall are vast and complex research fields. Many books could be mentioned, even more scientific reviews could be cited. Cell wall models have been proposed, changed, rearranged, and are not yet fully resolved. Although this thesis is primarily focused on brown seaweeds and, to a smaller extent, on red and green algae, it is noteworthy to briefly discuss land plant cell walls, to give a deeper understanding regarding the differences between land plants and algal cell walls which is the focus of chapter 2.

The primary cell walls of land plants are composed of carbohydrates that bind and link in complex ways to dynamically modulate its architectural design and function during development, as well as in response to the environment (Tan et al., 2013; Wang et al., 2015b; Xue et al., 2013). A commonly cited model for plant cell walls, which is based on onion cell walls, is shown in Figure 1.3. The major polysaccharides in land plant walls are cellulose, pectins and hemicelluloses. Briefly, cellulose (a linear β-1,4-linked glucan) microfibrils form a network tethered by cross-links with hemicelluloses, which are non-cellulosic polysaccharides that have the ability to form hydrogen bonds with cellulose, and include xyloglucans, xylans, arabinoxylans, mannans and mixed-linkage (1,3),(1,4)-β-D-glucans (MLG) (Albersheim et al., 2011; Fry, 2010). In the primary walls of many taxonomic groups such as gymnosperms, dicots and non-graminaceous monocots, these structural polymers are embedded in a matrix of structurally different pectins that include homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II (see Chapter 2 for details), and lower amounts of proteins and glycoproteins (Ridley et al., 2001).

The proteins and glycoproteins are well studied but nonetheless quite mysterious, due to their structural and functional complexity. Nevertheless, hydroxyproline-rich glycoproteins, such as arabinogalactan proteins (AGPs) are reported to be involved in the communication between cell walls and cytoplasm (Driouich and Baskin, 2008), as well as in a diversity of biological functions (Seifert and Roberts, 2007). These include implications in cell expansion (Lamport et al., 2006), signaling (Lamport and Vármai, 2012) and
development (Majewska-Sawka and Nothnagel, 2000). The biology of roots and pollen tubes are also affected by AGPs (Nguema-Ona et al., 2012), including root cell-microbe interactions (Nguema-Ona et al., 2013) and male-female attraction during sexual reproduction (Coimbra et al., 2007; 2009; 2010).

A secondary wall develops in woody and vascular tissues when microfibrils are deposited after a cell has lost the ability to expand (Fry, 2010). They contain typically more hemicelluloses than pectin and are reinforced by the hydrophobic phenylpropanoid lignin (Boerjan et al., 2003), a non-polysaccharide polymer that is deposited in specialized cells such as tracheids, vessels, fibers of xylem and phloem and sclerenchyma (Novo-Uzal et al., 2012).

Plant cell walls are tailored by the combination of these components, in different quantities and structure according to the requirements imposed by biotic and abiotic factors, reproductive and developmental demands, or combinations thereof (Sørensen et al., 2011). The cell wall polysaccharides are not synthesized within the wall itself. The synthesis, dynamics and architecture of the extracellular cell wall depends on the intracellular trafficking of the wall components in addition to the wall-modifying and-synthesizing machinery to and from the plasma membrane. In land plants, cellulose and callose are synthesized on the apoplastic side of the plasma membrane by cellulose synthase complexes (Cosgrove, 2005). By contrast, non-cellulosic polysaccharides, such as hemicelluloses and pectins, are assembled within the Golgi apparatus. These compounds are secreted into the apoplast by fusion of Golgi-derived vesicles with the plasma membrane and linked to newly synthesized cellulose microfibrils in the wall (Driouich et al., 1993; Driouich et al., 2012). Although information regarding cell wall components deposition in algae is scarce, the Golgi apparatus also appeared to be involved in the synthesis of polysaccharides in algal species (Keidan et al., 2009). Brown algal wall components such as cellulose microfibrils are produced and deposited by cellulose synthase complexes (Peng and Jaffe, 1976). Whilst cellulose synthase terminal complexes form rosettes in land plants, in brown algae these complexes form single rows, resulting in flat ribbon-like shaped microfibrils (Tamura et al., 1996). In algae alginates, sulfated fucans and phlorotannins are also synthesized in the Golgi apparatus, further transported to the plasma membrane and secreted into the wall (Schoenwaelder and Wiencke, 2000). Cell wall growth and expansion in brown algae is driven by turgor pressure as in land plants, but the exact mechanisms responsible for these events remain to be elucidated (Michel et al., 2010b).
Figure 1.3. General models for the cell walls of A. land plants (modified from McCann and Roberts. 1991) and of B. brown algae from the order Fucales (modified from Deniaud-Bouët et al. 2014).
1.4. Algal cell walls

1.4.1. Seaweed cell walls

The precise polysaccharide composition, linkages between cell wall polymers and their organization within the wall is a dynamic process, and depends on the species, thallus part, tissue, developmental stage, season and/or habitat (Kloareg and Quatrano, 1988; Kropf et al., 1988; Lahaye et al., 1994; Lechat et al., 2000; Mabeau and Kloareg, 1987; Stengel et al., 2011). Brown algal cell walls are composed mainly of polysaccharides and lower amounts of phenols, proteins and halide compounds, such as iodide (Deniaud-Bouët et al., 2014; Nitschke et al., 2011). Cellulose microfibrils provide a crystalline phase, while alginites and sulfated fucans form the amorphous, or matrix phase, that makes up the main portion of the wall (Kloareg and Quatrano, 1988). The three-dimensional organization of the polymers within the cell wall is not fully resolved, but researchers have begun to uncover the architectural features of brown algal cell walls (Deniaud-Bouët et al., 2014; Ropartz et al., 2015; Tesson and Charrier, 2014). Figure 1.3 shows the most recent model proposed for the cell wall structure of Fucales order within the brown algae (Deniaud-Bouët et al. 2014). According to this model, although the relationships are unclear, the sulfated fucans are suggested to interlock the cellulose microfibrils and play an important structural role in the wall by cross-linking the other polysaccharides, while phenols and iodide are likely to be associated with alginites and proteins in the wall. This network is likely to be embedded within alginites, which have a critical role in regulating the rigidity of the wall. Red seaweeds contain low amounts of cellulose in their cell walls (up to 12%), and also contain other structural polysaccharides such as β-1,4-mannans and β-1,3-linked or β-1,4-linked xylans (Domozych, 2011). Similarly to brown seaweeds, they contain high amounts of matrix polymers, and sulfated polysaccharides account for up to 70% of the dry matter of some species which in the red algae are sulfated galactans, including agars and carrageenans, found in diverse forms of different seaweeds (Rinaudo, 2007). However, the structural features that each polymers has in the cell wall architecture of red algae is not yet resolved.

Laminaran is a glucan composed of linear β-1,3-linked chains with occasional β-1,6-linked branches (Beattie et al., 1961; Read et al., 1996) (a diagram of the chemical structure can be found in Figure 1.4). Brown algae have a unique carbon storage metabolism. Instead of producing sucrose, laminaran is their main storage glucan (Michel et al., 2010a). Although reported to be located in the cytoplasm and not considered to be a cell wall component (Mian
and Percival, 1973; Quatrano and Stevens, 1976), this polysaccharide is worth mentioning, as Chapter 3 is focused on the presence of β-1,3-linked glucans in seaweed cell walls.

Phycocolloid is a common term used to describe high molecular weight polysaccharides composed of polymers of sugar residues extracted from freshwater and marine algae (Cardozo et al., 2007). Seaweed phycocolloids are well known for their gelling abilities, which are based on the associations between the polymers resulting from the glycosidic linkage angles, and the ability to form intra- and inter-molecular physical linkages, which are affected by negative charges from carboxylic and/or sulfate groups. In fact, the type of structure formed by polysaccharides depends more on the glycosidic linkage geometry rather than on the nature of the building blocks. Linkages found in cellulose, mannan, chitin are parallel and almost co-linear with the sugar residues, therefore they form flat ribbons able to pack into microfibrils; on the other hand, linkages found in alginates (and pectins) allow cavities that can accommodate and stabilize ions, forming ordered structures such as buckled ribbons; linkages found in agar and carrageenans are not parallel or diagonally, rather they form a regular twist, resulting in a helix usually stabilized by co-axial packing (Lahaye, 2001).

1.4.1.1. Alginates

Alginates are a family of naturally occurring anionic polymers produced by marine brown algae. Some prokaryotes such as Azotobacter and Pseudomonas also synthesize alginates, with different compositions (Gorin and Spencer, 1966), but commercially available alginate is typically extracted from the brown seaweeds of the genera Laminaria, Macrocystis, Ascophyllum, Fucus and Pelvetia (Guiseley, 1989; Lee and Mooney, 2012; Radmer, 1996).

Alginates are linear copolymers of the uronic acids β-1,4-D-mannuronic acid, or M blocks, and its C₅ epimer, α-1,4-L-guluronic acid, or G blocks (a diagram of their chemical structures can be found in Figure 1.4), which are present in different ratios depending on the species, seaweed part and age, and habitat (Kim and Park, 1985; Kloareg and Quatrano, 1988; Percival, 1979; Smidsrød et al., 1973) and dictate the gelling characteristics and functional properties of the alginates. Both mannuronic and guluronic acid can be arranged in homopolymeric regions of guluronic (GGGG...) and mannuronic residues (MMMM...) together with mixed sequences, in the same molecule (Haug, 1964). Two antiparallel guluronic chains can engage in a complex structure, in which the carboxyl group of two
guluronic acid residues form a negatively charged pocket that can accommodate a calcium cation, building a stable cross-link between the chains. This characteristic of the G blocks leads to the formation of sequences of ionic bridges which are known as ‘egg-box’ structures (Figure 1.4), increasing the mechanical abilities of the polymer to form a gel (Haug, 1964; Morris et al., 1978; Smidsrød et al., 1973; Vreeland and Laetsch, 1990).

The epimerization of the M into G blocks is mediated by the enzyme mannuronan C5-epimerase (Haug et al., 1974; Nyvall et al., 2003), and it is proposed that alginates and mannuronan C5-epimerase determine some properties of the wall such as porosity and rigidity, analogous to pectins and pectin methylesterases in land plants, respectively (Deniaud-Bouët et al., 2014). Homogalacturonan is the most abundant pectic polysaccharide (~65%) (Mohnen, 2008). It is a linear polymer of (1→4)-α-galacturonic acid that can be partially methyl esterified (O’Neill et al., 1990) and, similarly to alginates, can adopt different conformations. Two antiparallel unesterified homogalacturonan chains can adopt the egg-box structures, in which the carboxyl groups of two galacturonic acid residues accommodate a calcium cation, building the cross-link between the chains, which in mechanical terms leads to gel formation (Liners et al., 1992; Willats et al., 2001). Pectins are a major polymer of land plant cell walls and are extremely complex and valuable as an industrial plant-based food component, due to their gelling properties (Vincken et al., 2003; Willats et al., 2006). Similarly, alginates have equivalent importance in nutritional and pharmaceutical industries, associated with their viscousifying abilities (Cardozo et al., 2007; Stengel et al., 2011).

1.4.1.2. Fucoidans

Fucoidans, also known as fucoidin, fucan, fucosan or sulfated fucans, are a family of naturally occurring sulfated polysaccharides produced by brown marine algae that contain significant amounts of α-L-fucose and sulfate ester groups (Ale et al., 2011; Li et al., 2008; Rioux et al., 2007). Although with different configurations, some marine invertebrates such as sea urchins and sea cucumbers also produce sulfated fucans (Pomin and Mourao, 2008; Usov and Bilan, 2009). This complex and not fully characterized heterogenous family has been collectively named fucose-containing sulfated polysaccharides, and in brown seaweed cell walls has an important role cross-linking cellulose microfibrils (Deniaud-Bouët et al., 2014). They are of high interest to research related to adaptation to saline environmental conditions (Aquino et al., 2011). Furthermore, an increasing number of research papers
regarding these polysaccharides is being published because of their potential biological activities in humans, such as anticoagulant, antivirus, antitumor, anti-inflammatory, antiviral and antioxidant (Ale et al., 2011; Li et al., 2008; Usov and Bilan, 2009).

Figure 1.4. Chemical structures of the main polysaccharides found in brown algae, simple sulfated fucoidans, laminaran, and alginites with the guluronic (G) and mannuronic (M) building blocks, and a schematic representation of the typical egg-box structure that is formed by the cross-linking of G blocks with Ca$^{2+}$ ions (Figure prepared by S. Raimundo using ChemDraw Prime 15.0 software).

From a structural standpoint, different polysaccharides can be found within fucoidans, their structure is highly complex, and the amounts of fucose/sulfate/other monosaccharide residues present varies among different species of brown seaweeds (Ale et al., 2011; Li et al., 2008; Usov and Bilan, 2009). Attempts to systematize the fine structure of fucoidans have not yet been successful, because only a few examples of regularity have been found; linkages, branching, sulfate position, other sugar residues are variable and thus the relationship between structure and biological activity is not clearly defined (Ale et al., 2011). Nonetheless, fucoidans generally contain a linear backbone of 1,3-α-L-fucose or alternating
1,3-α-L-fucose and 1,4-α-L-fucose, sometimes with branches of 1,2-α-L-fucose, with sulfate groups at C-2 or/and C-3, C-4 of the fucose (Figure 1.4) (Bilan et al., 2006; Chandía and Matsuhiro, 2008; Chizhov et al., 1999; Cumashi et al., 2007; Kloareg et al., 1986; Pereira et al., 1999). Some fucoidans may only contain fucose residues and sulfate groups (“fucan”), while others have additional monosaccharide residues such as D-xylose, D-mannose, glucuronic acid, and fewer sulfate groups (Rabanal et al., 2014). As fucoids vary between species, different residues are also detected; species of Laminaria have been reported to have high amounts of D-galactose (“galactofucan” or “fucogalactan”), although their structural complexity is still elusive (Usov and Bilan, 2009). Fucus vesiculosus and Ascophyllum nodosum fucoidan is described to be more simple, with a backbone of α-1,3-L-fucose residues (Patankar et al., 1993) while other reports describe a backbone of alternating α-1,3- and α-1,4-L-fucose residues with sulfation in positions C-2 and C-2,3 (Chevolot et al., 1999; Chevolot et al., 2001; Usov and Bilan, 2009).

1.4.1.3. Red seaweed galactans

Red seaweed galactans have great industrial importance as they are used widely in food industry because they form strong thermoreversible gels at low concentrations in aqueous solutions. These are also sulfated polysaccharides and are classified as agarans and carrageenans according to their stereochemistry; galactans with 4-linked α-galactose residues of the L-series are named agarans and those of the D-series are named carrageenans (Knutsen et al., 1994).

Carrageenans are the main components of the cell walls of many red marine algae, where they are organized as highly ordered molecules, both associated to the cellulose microfibrils and in the microfibril-less extracellular matrix (Gordon-Mills and McCandless, 1977; Gordon-Mills et al., 1978). Natural carrageenans are versatile mixtures of different sulphated galactans, and their proportions differ from species to species, as well as geographically (Tuvikene et al., 2006). They are classified according to their structural characteristics, including sulfation patterns and the presence or absence of anhydrogalactose on the D-residues. There are at least 15 different carrageenan structures known (Lahaye, 2001), with the most commercially valuable types being κ, τ and λ, depending on their sulfation levels. There are other types such as μ- and ν-, that are the precursors for κ- and τ-
carrageenans respectively (Zablackis et al., 1988). These polysaccharides are composed of linear chains of alternating α-1,3-D-galactose and β-1,4-D-galactose, modified by the presence of 3,6-anhydro bridges in the α-linked galactose residues and by the presence of sulfate, methyl or pyruvate groups (Figure 1.5) (Kloareg and Quatrano, 1988). κ-Carrageenan is extracted commercially from the red algae *Kappaphycus alvarezii* or *Eucheuma alvarezii*, and has alternating β-1,3-D-galactose residues which are sulfated at C-4; sulfation can be up to 20% (w/w) (Cardozo et al., 2007; Jiao et al., 2011; Zablackis et al., 1988). τ-Carrageenan is more homogeneous and flexible than κ-carrageenan; it can be extracted from *Eucheuma spinosum*, and its chemical structure has an additional sulfate group at C-2 on the 4 linked 3,6-anhydro-α-D-galactose residue (Funami et al., 2007). λ-Carrageenan can be extracted from species within the Gigartina and Chondrus (Zhou et al., 2006), lacks the anhydrogalactose, and contains three sulfate groups, with sulfation levels of up to 40% (w/w) (Cardozo et al., 2007).

Figure 1.5. Chemical structures of the main polysaccharides found in red algae, κ-, τ-, λ-carrageenans, and the primary structure of agar (Figure prepared by S. Raimundo using ChemDraw Prime 15.0 software).
The gel-forming ability of carrageenans is associated with a conformational transition that goes from random coils in solution to helices which aggregate on cooling and/or upon specific interactions with certain cations in the solution, such as potassium or calcium. The presence of the 3,6-anhydro-α-D-galactose residues is also necessary for gel formation (Lahaye, 2001; Usov, 1998). Low sulfate and high anhydrogalactose are associated with a high gel strength. Both κ- and τ-carrageenans contain anhydrogalactose and are known as gel-forming polymers; κ-carrageenan gelation occurs optimally with potassium ions and forms a stiff gel, whereas τ-carrageenan gelation occurs with calcium and magnesium ions, forming soft elastic gels; λ-carrageenan has no anhydrogalactose which results in aqueous solutions of very high viscosity, not forming gels (Vreeland et al., 1992b; Zablackis et al., 1991).

AGARS

Agar is a generic name for red seaweed galactans containing agarobiose, repeating units of α-(1-4)-3,6-anhydro-D-galactose and β-(1-3)-D-galactose residues, with up to 6% (w/w) of sulfated esterification groups (Figure 1.5) (Cardozo et al., 2007). It is primarily extracted from Gellidium, Pterocladia, Gelidiella and Gracilaria genera, and its specific physicochemical characteristics vary with environmental parameters, season, growth and developmental stage (Cardozo et al., 2007; Lee, 2008; Radmer, 1996). Agar, agarocolloids, agaroids, agaran, and agarose, are terms that reflect the structural complexity and variability in the gelling properties of these galactans, as well as the difficulty of resolving both practical and chemical definitions (Lahaye, 2001). The chemistry and physico-chemical properties of agar are very complex, and similarly to alginites and carrageenans, depend on the chemical structure (Lahaye and Rochas, 1991). The purity of agar dictates its commercial application, which can range from food and industrial products for low purified agar, to gel substrates for biology culture media and pharmaceutics for medium quality agar, to molecular biology applications for highly purified agar, also known as agarose (Cardozo et al., 2007; Radmer, 1996). The commercial applications of carrageenans and agar are discussed below (see section 1.4.1.4). There are still significant knowledge gaps regarding the secondary and tertiary (gel) structures of carrageenans and agars and physical and rheological information is missing, which is required in order to understand the conformations of the different polysaccharides that can explain their mechanisms of aggregation (Lahaye, 2001).
1.4.1.4. Commercial relevance and applications

More than 7.5–8 million tons of wet seaweeds are used by industry annually, either from naturally growing or cultivated seaweeds (McHugh, 2003). Many compounds produced by seaweeds are appreciated in industry for their bioactivity, including pigments, lipids, proteins, phenolic compounds and polysaccharides (Stengel et al., 2011). Although there is much to say about all of them, this section will focus on the polysaccharides. Seaweeds are rarely consumed as a primary food source, instead they are harvested for their unique cell wall polysaccharides that have desirable organoleptic and functional properties. The ability these phycocolloids have to provide texture in aqueous solutions is the feature exploited by industry (specifically food, pharmaceutical and cosmetic sectors), for gelling, thickening, emulsifying and stabilizing properties (Lahaye, 2001; Pulz and Gross, 2004; Stengel et al., 2011). Furthermore, seaweed cell wall polymer extracts are also broadly studied for their bioactivity with potential benefits for human health (Smit, 2004).

Probably the most widely recognized and studied bioactivity of marine sulfated polysaccharides is the heparin-like anticoagulant and antithrombotic activity reported for fucoidans (Boisson-Vidal et al., 1991; Kusaykin et al., 2008; Pereira et al., 1999). Fucoidans have chemical and functional similarities with heparin, which is known to stimulate the production of hepatocyte growth factor. Therefore, fucoidans have been studied for their application in biomedical sciences, related to tissue and organ regeneration derived from injuries and diseases (Li et al., 2008). Fucoidans are also associated with the ability to inhibit the replication of enveloped viruses such as HSV, HIV and dengue, to stimulate immune responses or control immune cell activity and to lower negative effects associated with inflammation. They may have antioxidant benefits, reduce blood lipid levels, and inhibit tumor cell proliferation (Ale et al., 2011; Berteau and Mulloy, 2003; Boisson-Vidal et al., 1991; Guangling et al., 2012; Jiao et al., 2011; Li et al., 2008; Rabanal et al., 2014). In plant research, fucan oligosaccharides have been reported to induce defense responses in cell cultures against the tobacco mosaic virus (Klarzynski et al., 2003).

Brown seaweeds are commercially harvested primarily as food and as raw materials for the extraction of alginates. The main species used as food sources belong to the genera Laminaria and Undaria (recognized in food products as kombu and wakame, respectively), as well as Hizikia; they are best known in Asian countries including Japan, China and Korea (McHugh, 2003). The key species harvested as sources of alginate belong to the genera Laminaria, Macrocystis, Ascophyllum, and Undaria, and are valuable in both the Northern
and Southern Hemispheres. Alginates commercial importance arises from their three major properties: the ability to 1) dissolve in water and to thicken solutions, 2) form gels of calcium alginates, 3) form films of sodium or calcium alginates and fibers of calcium alginates (McHugh, 2003). The thickening property of alginates is used e.g. in sauces and in syrups and toppings for ice cream, they also improve texture and stabilize milk products, and reduce the formation of ice crystals during freezing (Draget, 2000). Alginates (and their derivatives) are highly biocompatible and are consequently used in biomedical sciences and pharmaceutical research, where their conventional roles include serving as thickening, gel forming, and stabilizing agents. Alginate hydrogels are further used for immobilization or isolation of bioactive compounds by encapsulation, in controlled-release drug products and in efficient gene and drug delivery systems (Grassi et al., 2009; Lee and Mooney, 2012; Zhao et al., 2012). Recent approaches in biofuel research have been reported, involving the use of brown macroalgae as feedstocks for bioethanol production (Enquist-Newman et al., 2014; Wargacki et al., 2012).

Red seaweeds are commercially harvested primarily as food and also as sources of carrageenans and agars. The main species used as food sources belong to the genus Porphyra: its sheet-like thallus being used for the production of the nori sheets in the Japanese sushi dishes that have become very popular in western countries. Palmaria palmata, or dulse is also popular in coastal communities in countries including Ireland, Iceland, and Canada (McHugh, 2003; Radmer, 1996). The key species harvested for agars belong to the genera Gelidium, Pterocladia, Acanthopeltis, Ahnfeltia, and Gracilaria. Many countries globally harvest them, including Spain, Portugal, Mexico, Chile, Indonesia, and many others, while Chondrus crispus, Gigartina sp. and Eucheuma sp. are valued in European countries as sources of carrageenans (McHugh, 2003). Carrageenans have been reported to stimulate defense responses against pathogens in plants (Vera et al., 2011), but their main application is in the industrial preparation of dairy desserts like puddings, milk-based products such as chocolate, ice cream or evaporated milk, due to their ability to form gels in milk at lower concentrations compared to other gelling agents. Carrageenans are also valued in the preparation of cake glazes, water dessert gels, jellies, jams, dressings, meat products, pet food, etc. (Cardozo et al., 2007; Saha and Bhattacharya, 2010).

Agar is used in industry according to its quality/purity; 90% of the extracted agars are used in the food industry (McHugh, 2003). Lower quality agar is commercially used in food products such as frozen food, canned meat, fish, poultry products, bakery goods such as icings, meringues, dessert gels, candies, and fruit juices. Other industrial applications include
for paper sizing/coating, adhesives, textile printing/dyes, castings and impressions (Cardozo et al., 2007; Saha and Bhattacharya, 2010). Medium quality agar is used as the gel substrate in biological culture media; bacto-agar or bacteriological agar is widely used in research for cell culture media production (McHugh, 2003). Agars are also applied in the medical/pharmaceutical fields as bulking agents for laxatives, suppositories, capsules, tablets and anticoagulants. The highest quality agar type, or agarose, is used in molecule separation techniques in molecular biology research: electrophoresis, immunodiffusion and gel chromatography (Cardozo et al., 2007). Despite the relatively low level of commercial exploitation for agar outside of the hydrocolloid industry, it is increasingly employed in medicinal and pharmaceutical research, for example, as a therapy against cancer cells since it can induce apoptosis in these cells in vitro (Chen et al., 2004).

1.4.2. Charophycean green algal cell walls

CGA are the extant algal group closest to land plants (Figure 1.2). It is not surprising that they share some characteristics with land plants, including specific features of their cell walls. Similarly to land plants, most green algal cells are covered by different types of extracellular matrix that provide a physical protective barrier against grazers, parasites, toxins, abiotic stressors such as temperature or osmotic stress. Cell walls also enable nutrients to be sequestered, enhance cell-cell and cell-substrate adhesion, and are involved in sexual reproduction (Domozych et al., 2005; 2012). Several studies support that in addition to cellulose, many CGA contain polymers common to embryophyte walls (Domozych et al., 2007a; Eder et al., 2008; Popper and Fry, 2003). Homogalacturonan (HG) is an abundant polysaccharide in the walls of the unicellular desmid Penium margaritaceum and in the Charales species Chara corallina, and, similarly to the HG present in land plant cell walls, it can form complexes with calcium (Domozych et al., 2007a; Proseus and Boyer, 2006). Different degrees of methylesterification in the HG and spatial distribution of epitopes in P. margaritaceum and Netrium digitus were shown to be part of specific developmental events, as is reported for embryophytes (Domozych et al., 2007a; Eder and Lütz-Meindl, 2010; Popper and Fry, 2003). AGPs have also been detected in the cell walls of several CGA species and their implication in development and adhesion has been documented (Domozych et al., 2007b; Eder et al., 2008). Some evidence for the presence of hemicelluloses such as xyloglucan and MLG in Micrasterias denticulata exist (Eder et al., 2008) and, even though xyloglucan oligosaccharides have not been detected after enzyme treatments, putative
xyloglucan and MLG-related enzymes have been reported in several members of the CGA (Franková and Fry, 2011; Van Sandt et al., 2007). The ability to produce the β-D-1,3-glucan, callose, in Klebsormidium and Zygnema in response to desiccation tolerance has recently been reported (Herburger and Holzinger, 2015). The combination of immunocytochemistry and comprehensive glycan microarray analysis of the cell walls of several species included in the more recently diverged members of the CGA, including the Charales, Coleochaetales and Zygnematales, allowed the detection of cellulose, mannan, pectic polymers, xylan/arabinoxylan, MLG and AGPs (Domozych et al., 2010; Sørensen et al., 2011). The presence of xyloglucan is somewhat controversial, because although epitopes have been detected in some tissues, unequivocal evidence for the presence of the polymer has not yet been found, indicating the possibility that a different form of xyloglucan may have evolved before the transition to land habitats (Sørensen et al., 2010; 2011).

CGA have remarkably similar biochemical and structural features to embryophytes. This is very important in order to have a better understanding of cell wall evolution, as these characteristics imply that some features of land plant cell walls evolved before colonization of terrestrial habitats by land plants, rather than having evolved as a result of the natural selection intrinsic to that transition (Domozych et al., 2010; Sørensen et al., 2010; 2011). This sets CGA as interesting model organisms to study the composition and dynamics not only of cell walls, but also to address other biological questions. Additionally they are complex and have large cell sizes, and are easy to grow in controlled laboratory conditions. The possibilities for using them in different research fields is unlimited, including to study cellular morphogenesis, effects of environmental stressors, cytoplasmic streaming, plant cell development (Domozych, 2012; Sørensen et al., 2012), and biophysical aspects of membrane potential, classically studied in the multicellular Chara and Nitella (Hope, 1961; Kitasato, 1968; MacRobbie, 1962).
1.5. *Penium margaritaceum* as a model organism

*Penium margaritaceum* is a unicellular freshwater microalga member of the CGA order Zygnematales that has been having a great impact in the cell wall research. There are several physiological and practical characteristics that make this microalga an exceptional model organism. Very conveniently, this species can be easily cultured and grows either in a controlled laboratory with artificial light, or just close to a window with natural light; it produces a primary wall (described in section 1.4.2) that contains many of the polysaccharides found in land plant cell walls. This makes it complex enough to allow elaborate comparative studies, but simple enough to allow these studies to be conducted in a single cell. Additionally, its robust physiology allows it to be easily handled in cytological studies (Domozych *et al.*, 2005; 2012).

*P. margaritaceum* has a simple cylindrical shape with two semicells surrounding a central isthmus region (see Chapter 6 for details regarding the cell characteristics). Along with cellulose microfibrils, this alga secrets HG in a methylesterified form, that is further demethylesterified and complexed with Ca$^{2+}$, similar to HGs in land plant cell walls, resulting in a distinct lattice that covers the cell surface (Domozych and Lambiasse, 2009; Domozych *et al.*, 2014). These events can be visualized in a live timeframe because one of the biggest advantages of this alga is the fact that it is robust enough to allow the live-labelling procedure with monoclonal antibodies (mAbs) specific for cell wall glycan epitopes (Domozych *et al.*, 2014). Labelled cells can be cultured for a period of time and co-labelling with different mAbs can be further performed in order to monitor specific events of polymer deposition and wall development (Domozych and Lambiasse, 2009). Another advantage is the possibility to perform rapid high throughput screening of wall polymer deposition when cells are grown in the presence of specific compounds such as enzymes, inhibitors, or hormones (Domozych *et al.*, 2011). Single cells can also be selected from the culture medium and subjected to specific treatments or treated cells can be grown in normal media in order to monitor cell recovery (Chapter 6). While its genome is in the process of being sequenced, recent reports of high-throughput Agrobacterium *tumefaciens*-mediated stable transformation of *P. margaritaceum* has opened the possibility for functional genomics, exponentially increasing its potential applications (Sørensen *et al.*, 2014). Semicell expansion and morphogenesis in *P. margaritaceum* is typical of polar growth known as tip-growth (Domozych and Lambiasse, 2009), a specialized type of anisotropic expansion in which the focal point of cell growth is at
an apex or tip. Tip growth is typically observed in specialized cells such as pollen tubes and root hairs in land plants (Hepler et al., 2001). *P. margaritaceum* is a valuable model organism for understanding cell expansion and its inherent cellular mechanisms, in a single cell (Rydhall et al., 2015). Because of the abundance of extracellular matrix (ECM), formed by a multilayered cell wall with a network of pore-like openings, and an external layer of extracellular polymeric substances (EPS) (also known as mucilage) (Domozych et al., 2007a; 2007b), this species is also a good model for biofilm-related studies (Domozych et al., 2005).

1.6. Monoclonal antibodies as tools for the study of cell walls

Chemical and enzymatic extractions are essential tools for providing detailed biochemical information about the monosaccharide residues present in a polymer, as well as the linkages between them, and how they associate within the wall structure (Deniaud-Bouët et al., 2014; Popper and Fry, 2003; 2004). However, most of the characterized carbohydrates are extracted from whole plants or parts of a plant, and therefore represent a mixture of cells and tissue types (Vreeland and Kloareg, 2000). Any information relating to the localization of cell wall components at cellular and sub-cellular levels is impossible to acquire solely through biochemical analysis of extracts (Avci et al., 2012; Knox, 1997). Plant research has seen significant advances since the development of tools that enable imaging of specific compounds to be performed through the complementation of pure chemical compositional and structural characterization with *in situ* techniques, such as immunolabelling (Knox, 1997). Immunocytochemistry is a major break-through in cell wall research, because it allows detailed localization of specific components in cell walls and provides important information regarding their structure, organization and dynamics at the cellular level (Domozych, 2012; Lee et al., 2011; Willats et al., 2000). Immunological approaches apply the use of monoclonal antibodies (mAbs) that recognize specific epitopes within cell wall polymers and are used extensively for plant cell wall/biomass characterization in two different and complementary approaches: glycome profiling/microarrays and immunohistochemistry (Moller et al., 2007; 2008; 2012; Pattathil et al., 2012). Although there are some limitations, for example regarding the recognition of a carbohydrate epitope that might not be available in a purified form by a specific antibody (O’Rourke et al., 2015), immunocytochemistry has a significant value for plant research.
MAbs are very sensitive, because they detect antigens even if they are present in a tissue in very low levels. They allow the detection of antigens within specific organelles, and also the visualization of epitopes at the ultrastructural level using electron microscopy techniques. Specificity is another advantage of mAbs, which can be generated towards differences in polymers as small as anomic configurations of monosaccharide residues, the linkages between them, or their degree of esterification (McCandless et al., 1980). At the same time, such specificity is also a limitation of this technique, because it relies on the purity of the antigen isolated to produce the specific antiserum and some cell wall components may be modified during some extraction procedures, for example acetyl groups are typically lost during chemical extraction (McCandless et al., 1980; Vreeland and Laetsch, 1985). The specificity of the antigen-antibody interactions is also dependent on the incubation conditions, particularly when probing polymers with ionic charges, such as alginates, as the carbohydrate conformation and ability to cross-react with other polymers depends on the pH, the presence of ions, temperature and type of buffer used (Larsen et al., 1985; Vreeland et al., 1984).

1.6.1 Monoclonal antibody production

Antibody production is a complex, multistep procedure, and its success depends on several factors such as the appropriate antigen preparation, characterization, and the immunization protocol (Figure 1.6 shows a summarized scheme of mAb production) (Vreeland and Laetsch, 1985). Outlined below is a brief explanation of the procedure adopted by the Complex Carbohydrate Research Center (www.ccrc.uga.edu) to generate the toolkit of plant cell wall glycan-directed mAbs (Pattathil et al., 2010). The first step in mAb production involves isolation of the polysaccharide from the cell wall (although in some cases entire cell walls have been used to inoculate the mouse). The polysaccharide is then conjugated to a protein such as Bovine Serum Albumin (BSA), that will act as a immunopotentiator (or “Super Carrier”), as carbohydrates do not generally stimulate a strong immune response (Smith and Ginsberg, 1980). This neoglycoconjugate is also mixed with Freund’s adjuvant prior to immunizing the rodents (mice or rats) to further stimulate their immune system.
Mice or rats are immunized a second time with the glycoconjugate, and after monitoring with immunoassay for the presence of the antibodies of interest, the serum with the lymphocytes is isolated from the animal and fused with myeloma cells. The cell lines that secret the mAbs are called hybridomas, and can be proliferated indefinitely in culture (McCandless et al., 1980; Vreeland and Laetsch, 1985). A portion of the hybridoma culture is cryopreserved; stable hybridoma cell lines are used to continuously produce the mAbs. To characterize the epitopes recognized by the mAbs the polysaccharides they recognize must be fragmented and the binding ability toward specific fragments assayed using competitive ELISAs. This is an essential step to enhance the value of mAbs as tools, but it is a very time consuming and complex process and less than half of the commercially available mAbs generated against land plant cell wall glycans have been fully characterized with respect to their epitope recognition.
1.6.2. Plant cell wall-glycan directed mAbs

Currently over 200 mAbs, largely generated against epitopes present in vascular plant cell wall glycans are available and widely used by the scientific (predominantly cell wall) community. These mAbs recognize epitopes present in all of the major classes of plant cell wall polysaccharides, including hemicelluloses (xyloglucans, xylans, mannans), pectins (homogalacturonan, rhamnogalacturonan-I), and arabinogalactans (AGPs, extensin) (Moller et al., 2008; Pattathil et al., 2010). Different series are available, mainly according to where they were generated. The Complex Carbohydrate Research Center Monoclonal Antibodies (CCRC-M series), John Innes Monoclonal Antibodies (JIM series) and MAC series are available from http://cell.ccrc.uga.edu/~carbosource/CSS_home.html; Leeds Monoclonal Antibodies (LM series) are available from http://www.plantprobes.co.uk/. A smaller number of very important mAbs, including those that recognize MLG and callose, are available from Biosupplies (http://www.biosupplies.com.au/).
1.6.2.1. New emerging tools

A new class of non-immunological molecular probes directed toward homogalacturonan and chitosan (a product of chitin deacetylation) have recently been developed for the study of plant cell walls (Mravec et al., 2014), derived from the natural affinity between the positively charged amino groups on chitosan oligosaccharides (COS) and the negatively charged carboxyl groups on oligogalacturonate fragments of HG that typically form egg-box structures (Cabrera et al., 2010). The production of highly sensitive reciprocal oligosaccharide-based probes for chitosan and for demethylesterified homogalacturonan, COS, have some advantages over mAbs: 1) the experimental protocol involves a simple and fast one-step labelling; 2) the probe is smaller than mAbs allowing excellent tissue penetration and high resolution; 3) the direct conjugation to a variety of tags facilitate dual labelling and time course experiments; 4) COS probes were generated in order to be used at a pH compatible with plant growth conditions (~pH 5.7), while mAbs are generated close to mammalian physiological pH 7. Consequently, localization studies can be performed more effectively in live tissues (Mravec et al., 2014).

1.6.3. Seaweed cell wall-glycan directed mAbs

Dr. Valerie Vreeland (Department of Botany, University of California, CA, USA) generated a large number of mAbs toward the most commercially important cell wall polysaccharides derived from red and brown seaweeds. The first reports of the generation of immunological tools for seaweeds date back to 4 decades ago, where polyclonal serum specific for alginates (Vreeland, 1970, 1972, 1974) and sulfated fucans (Vreeland, 1975; Vreeland and Chapman, 1978) was described for brown seaweed. These antibodies allowed the first suppositions regarding variation in the different alginate and fucan epitopes particularly with respect to their occurrence, proportion, and amount within the different taxonomic groups (Vreeland and Chapman, 1977), and also within the different stages of embryo development (Vreeland et al., 1981). Hybridization probes of fluorescein-conjugated polyuronate oligosaccharides were also reported (Vreeland and Laetsch, 1989). Following these initial studies, mAbs were generated against brown seaweed cell wall carbohydrates (Green et al., 1993; Jones et al., 1988; Vreeland et al., 1982; 1984). In the case of red algae, hybridization probes of fluorescein-conjugated carrageenan oligosaccharides probes were also generated (Zablockis et al., 1988, 1991). Anti-carrageenan mAbs were further developed with specificity to κ-, τ- and λ-carrageenans (Vreeland et al., 1988, 1992a). Fewer reports
exist on the agarose wall portion, nonetheless, hybridization probes were also produced from agarose oligosaccharides (Vreeland et al., 1995), although no further application has been reported. The mAbs collection generated by Vreeland et al. and by Green et al. was never made available to the scientific community or commercialized, their epitopes were not fully characterized, and their potential was not fully explored; the remaining collection was donated to Professor Michael G. Hahn (Complex Carbohydrate Research Center (CCRC), University of Georgia, GA, USA) where they have been stored in liquid nitrogen or frozen at -20°C. Details of the mAb collection is presented in Chapter 5, Table 5.1, and includes supernatants and/or hybridoma lines.

These is a clear gap between the availability of immunological tools for plant and algal cell walls. Although there is a distinct historical repertoire regarding the production of seaweed glycan-directed mAbs, these probes remain unavailable. Nonetheless, seaweed research is producing new knowledge that will lead to a better understanding of polysaccharides in the wall architecture as well as the cellular (and physiological) properties of commercially valuable (marine) algae. New mAbs have recently been generated against brown seaweed sulfated fucoidans (Torode et al., 2015), which will likely increase research directed toward seaweed cell wall components and interactions.

1.7. Plant hormones

Plant hormones, also known as phytohormones or plant growth regulators, are a group of structurally unrelated small molecules that regulate every stage of a plant’s life cycle and have a primary role in the regulation of plant growth, development and defense (Wang and Irving, 2011). Ten structurally different types of plant hormones have been recognized (Santner and Estelle, 2009; Wang et al., 2015a). The classic hormones, identified during the first half of the twentieth century are auxins, abscisic acid, cytokinins, gibberellins, and ethylene. Other hormones have been identified more recently and include brassinosteroids, jasmonates, salicylic acid, nitric oxide and strigolactones (Davies, 1995; Santner and Estelle, 2009).

Auxins are a class of hormones that have an essential role in almost every aspect of plant growth and development, by affecting cell expansion and division (Kepinski and Leyser, 2005). Their regulation, transport and response involve complex and redundant processes that allow a complete system of auxin utilization that has multiple functions in plant development (Woodward and Bartel, 2005). Abscisic acid regulates the expression of
many genes in plants and is a stress hormone, with important roles in responses to environmental stress, mainly drought, by triggering stomata closure and reducing water loss (Fujii et al., 2009; Schroeder et al., 2001). Cytokinins influence many aspects of plant growth and development, such as cell division, chloroplast development, leaf expansion and seed germination, regulation of leaf senescence, root and shoot growth and branching (Hwang et al., 2012; Schaller et al., 2011). Gibberellins are also involved in many aspects of plant development and are implicated in all stages of the plant life cycle, such as flower induction and development, sex determination, pollen tube growth, seed and fruit development, and stem and leaf elongation (Bethke and Jones, 1998; Murase et al., 2008; Phillips, 1998; Singh et al., 2002). Ethylene is a gaseous hormone that regulates processes such as fruit maturation, stimulates germination, senescence, abscission, inhibits elongation, initiates adventitious roots and programs cell death; it is also involved in responses to biotic and abiotic stresses (Qiao et al., 2012; Wang and Irving, 2011). Brassinosteroids are a group of plant steroid hormones found in pollen, leaves, shoots, flowers, stems and seeds, but not for roots. They regulate a wide range of developmental and physiological processes, such as elongation, division, photomorphogenesis, seed germination, reproductive development, and senescence (Wang and Irving, 2011; Wang et al., 2012). Jasmonate, or jasmonic acid, is found in the highest levels in areas of active division such as stem apex, young leaves, immature fruits, and root tips, and its implications are numerous. It can inhibit germination in non-dormant seeds and stimulate germination of dormant seeds, inhibit root growth, is crucial for plant fertility and reproduction, and is involved in plant defense against wounding, herbivores and certain pathogens (Sheard et al., 2010; Wang and Irving, 2011). Salicylic acid is a phenol hormone that regulates immunity and induces systemic acquired resistance in the presence of pathogens (Fu et al., 2012). Strigolactones inhibit shoot branching and modulate seed germination and seedling photomorphogenesis (Zhou et al., 2013). Nitric oxide is involved in reproductive processes, control of development and in the regulation of physiological responses such as stomatal closure (Wilson et al., 2008).
1.7.1. Plant hormones in algae

The evolutionary time frame at which hormones became involved in the complex biological functions they are known to control in plants is controversial and not fully determined (Tarakhovskaya et al., 2007).

A very recent study presented by Ju et al. (2015) brought key insights with the potential to transform our understanding of plant evolution and the ways in which plants react to environmental stress. This study shows that at least some algal lineages use the same molecular machinery as land plants to respond to a hormone. Five species of the late-divergent CGA were studied using a combination of advanced bioinformatics and experimental approaches, and the ethylene signaling pathway was documented in all of them. The genes and cellular machinery responsible for the ethylene pathway were found to have remained almost unchanged across species for more than 450 MYA, prior to land colonization, when CGA and land plants started diverging from their shared aquatic ancestor. This study also concludes that, although ethylene is involved in a variety of responses in plants, and can have different responses according to environmental conditions, the molecular machinery behind ethylene sensing is exactly the same in all plants, and nearly identical in the CGA (Ju et al., 2015).

Comparative genomics and phylogenetic analysis have provided significant evidence into the origin and evolutionary mechanisms of plant hormone signaling pathways. Auxin and cytokinin pathways probably have an early evolutionary origin in charophytes, while gibberellin signaling evolved after the divergence of bryophytes from land plants (Lau et al., 2009; Wang et al., 2015a). Auxins are known to be present in algal lineages and to affect algal development (Lau et al., 2009; Tarakhovskaya et al., 2007). Auxins, particularly indole-3-acetic acid (IAA) have been detected in the late divergent CGA Chara corallina, and evidence of polar auxin transport has been reported for this species (Boot et al., 2012), although the proteins responsible for the auxin transport were not identified. Microalgae do not seem to have the major components necessary for auxin signaling found in land plants, but their responses to auxins indicates the possibility that they may have an alternative auxin signaling pathway (Lau et al., 2009). IAA has been found in many red and brown seaweed species (Cooke et al., 2002; Stirk et al., 2004). The presence of IAA, which has a function related to cell-cell positional information, in Ectocarpus siliculosus suggested the induction of a signaling pathway different from the one known in land plants (Le Bail et al., 2010). Information on the presence of other hormones in microalgae is scarce, with a few reports on
the presence of cytokinins in *Chara* (Zhang *et al*., 1989), and in members of the Chlorophyta microalgae (Ördög *et al*., 2004). In seaweeds, though, cytokinins are reported to exist in many red and brown species, while there are only a few data indicating the presence of endogenous compounds with gibberellin activity in brown algae (Stirk *et al*., 2004; Tarakhovskaya *et al*., 2007). Some seaweed species, such as *Ascophyllum nodosum*, are applied in agricultural crops as biostimulants because their extracts can elicit a wide range of responses in plants including root and shoot growth, improvement of nutrient uptake and flowering and fruit yield (Khan *et al*., 2009). One such biostimulant, Kelpak®, is extracted from the kelp *Ecklonia maxima* and some studies have shown that this seaweed concentrate contains auxins, cytokinins, abscisic acid, gibberellins and brassinosteroids, although in low concentrations. A novel phlorotannin named eckol has also been detected and found to act as a plant growth stimulant (Rengasamy *et al*., 2015; Stirk *et al*., 2014). Abscisic acid has been found in many classes of seaweeds and they synthesize ethylene, although confirmation requires further research; reports on brassinosteroids, jasmonates, salicylic acid and strigolactones are rather scarce (Stirk and Van Staden, 2014).

### 1.7.2. Interactions between plant hormones and cell walls

Hormones are involved in every aspect of the vegetative and reproductive stages of the plant life cycle, therefore is not surprising that some will have effects on cell walls. Auxins are known to affect the rigidity (elasticity) of plant tissues (Cleland, 1958, 1971; Tagawa and James, 1957), which is in turn, related to growth (Braybrook *et al*., 2012; Kierzkowski *et al*., 2012; Peaucelle *et al*., 2011; 2012). Pectins are cell wall polymers known to be responsible for growth through changes in their chemistry, which in turn will affect the rigidity of the wall (Peaucelle *et al*., 2011), and are well reported in algal growth research (Domozych *et al*., 2014; Peaucelle *et al*., 2012). Auxins have a direct influence on *Arabidopsis thaliana* growth, acting through the activation of the demethylsterification of the homogalacturonan, causing the reduction in cell wall rigidity, meaning that the demethylsterification of pectin alone is enough to induce local tissue growth in meristems (Braybrook and Peaucelle, 2013). Auxin-induced wall loosening is also associated with xyloglucan hydrolysis and transglycosylation (Hoson *et al*., 1991), and the expression of wall-modifying xyloglucan endotransglycosylase genes is reported to be regulated by auxins and brassinosteroids (Xu *et al*., 1995). Auxins are also reported to interfere with the cytoskeleton machinery, which has consequences for polar growth and the necessary cell wall deposition that is controlled by the actin bundles that form the cytoskeleton (Kropf *et al*.,
This is a delicate process, where the cell wall synthesis must be accurately processed at the growing area, with a precise and coordinated relationship between the cell’s endomembrane system and the cytoskeleton network (Heath and Geitmann, 2000). Auxins have an influence in this mechanism, causing the de-bundling of the actin filaments into finer strands in plant coleoptile growth (Holweg et al., 2004; Waller et al., 2002). However, the auxin/actin relationship is bidirectional. Auxins that exhibit polar transport (such as IAA, but not other auxins, such as 2,4-D) can restore actin configuration in cells that have de-bundled actin filaments (Nick et al., 2009).

Steroid hormones (brassinosteroids) are known to stimulate pollen tube growth (Ylstra et al., 1995) and other plant growth regulators such as auxins, cytokinins and gibberellins are reported to be important players in pollen tube (Singh et al., 2002; Wu et al., 2008b) and root hair growth (Lee and Cho, 2013; Overvoorde et al., 2010; Pitts et al., 1998), although their physiological role has been well established only for auxins. For example, IAA stimulates the synthesis of pectin in pollen tubes and increases the activity of pectin methylesterases which results in cell wall extension (Wu et al., 2008a; Wu et al., 2008b).
1.8. Research Objectives

This thesis describes research focused on algal cell walls using immunocytochemical approaches. Different algal taxa were studied: 1) brown seaweeds *Fucus vesiculosus* (Chapters 2, 3, 4, 5) and *Laminaria digitata* (Chapters 3, 4, 5), 2) red seaweeds *Chondrus crispus* and *Porphyra* sp. (Chapter 5), and 3) green microalga *Penium margaritaceum* (Chapter 6).

There are ~200 glycan-directed mAbs currently available that have been used worldwide as valuable probes in plant cell wall research. These mAbs have enabled the elucidation of many cell wall details; for example, regarding their diversity at the cellular and subcellular level, as well as aspects of their metabolism and physiology. In contrast, such tools are lacking for algal cell wall research, hence there is a significant knowledge gap related to many of their compositional and structural characteristics, even though some of their wall components have high commercial value.

The starting hypothesis was based on the principle that algae, especially brown seaweeds, although evolutionarily only distantly related to land plants and to the other algal groups (Charrier *et al.*, 2012), have some cell wall polysaccharides that contain epitopes similar to those existing in land plants, and can, thus, be recognized by the land plant glycan-directed mAbs currently available. These tools, widely used in land plant research, may hypothetically have the potential to become valuable tools for algal cell wall research.

In collaboration with Professor Michael G. Hahn, at the CCRC, University of Georgia, GA, USA, during two research visits, a collection of over 155 mAbs was tested against different seaweeds. The localization of specific wall components was investigated in brown seaweeds using a multifaceted approach that included Glycome Profiling of cell wall extracts, *in situ* immunolocalization, and compositional analyses (Chapters 2, 3, 4). Those mAbs that gave positive enzyme-linked immunosorbent assay (ELISA) signals were further tested for immunolocalization on different vegetative and reproductive thallus parts in order to identify and understand labeling patterns.

A wider aim within this project related to the identification and description of mAbs that can be used as valuable molecular probes for brown seaweed cell wall research (Chapter 2). After the ELISA experiments, some mAbs were highlighted due to their surprising ability to recognize specific thallus parts and tissues in brown seaweeds (Chapters 3 and 4). One of those mAbs, known as LAMP (LAMP2H12H7; Biosupplies 400-2), was generated against the brown seaweed storage polysaccharide laminaran, but has not often been applied in algal
research. It recognizes a (1→3)-β-glucan epitope and has primarily been used in the immunolocalization of callose in vascular plant cell walls. It was, therefore, hypothesized that the epitope recognized by this mAb is broadly distributed throughout the brown seaweed thallus parts and might be a valuable probe not only for in situ localization in seaweed tissues but also in studies related to specialized cell walls such as sieve plates (Chapter 3).

Another mAb that showed promising results was LM7, generated against lime pectin, that recognizes an epitope of unesterified galacturonic acid residues within methylesterified HG. With a very discrete epitope distribution in land plant cell walls, LM7 mAb has never been reported to be used for immunolocalization in seaweeds. However, from the unique distribution of the labelling patterns observed after immunolabelling different seaweed thallus parts, supported by reports on the cross-reactivity of this mAb with pectic polysaccharides (Vreeland and Laetsch, 1985), and possibly alginates (Torode et al., 2015), it was hypothesized that LM7 mAb is a unique and as yet unreported probe for the identification and localization of the commercially valuable brown seaweed alginate polysaccharides (Chapter 4).

Dr. Valerie Vreeland (Department of Botany, University of California, CA, USA) generated a large number of seaweed cell wall-directed mAbs several decades ago (in the 1970’s and 1980’s). Although they have never been generally available to the scientific community they were transferred from the Lawrence Berkeley Laboratories to the care of Professor Michael G. Hahn at the CCRC several years ago. These unique probes were generated against the most valuable red and brown seaweed polysaccharides, carrageenans and alginates, respectively. These mAbs do not exist anywhere else, and neither their viability nor the epitopes which they recognize was certain. Therefore, the main objective in Chapter 5, was to test the available mAb supernatants against a variety of cell wall extracts obtained, using a range of extraction procedures, from different brown and red seaweed species to verify if any of the mAbs are able to react with any of the wall extracts and are, therefore, still usable. The major objective of this work was to make any of the viable mAbs available for the scientific community in the future, in order to stimulate the use of mAbs and immunolocalization toward the study of seaweeds. For example the ability to identify specific epitopes in situ opens up new pathways to investigate their taxonomic distribution, interactions, metabolism and the impact of seasons and environmental stress.

Chapter 6 of this thesis started from evidence relating to the existence of hormones and the genetic machinery for plant hormone signaling pathways in algae (Ju et al., 2015; Wang et al., 2015a). Hormones interplay with many cell wall acting enzymes to mediate
changes in expansive cell growth in land plants; auxins, for example, have been found to impact pectin HG demethylesterification (Braybrook and Peaucelle, 2013; Peaucelle et al., 2012). It is of interest to investigate the similarities of hormone interactions in the cell wall that may exist between plants and algae; this is particularly important for model organisms. The late divergent CGA *P. margaritaceum* is emerging as a model for cells walls and has a defined and reported HG machinery associated with its cell wall (Domozych et al., 2007a). It was hypothesized that this alga is sensitive and has the ability to respond to different plant hormone and hormone inhibitor treatments, and the effects will be visible in the cell morphology and cell wall microarchitecture. In collaboration with Professor David Domozych (Skidmore College, NY, USA), the ability of hormones, and their various inhibitors, to induce changes in *P. margaritaceum* cell walls was assessed with immunolocalization using mAbs directed against pectin epitopes and a range of microscopy techniques.

The research hypotheses investigated within this thesis and the chapters in which the results are presented are summarized in the list below:

1. Some land plant glycan-directed mAbs have the ability to recognize brown seaweed wall epitopes. Based on the distribution of different epitopes in plant cell walls it was suggest that algal cell walls are likely to be similarly dynamic with epitopes having different localizations across the algal thallus parts (Chapter 2);
2. The epitope recognized by the LAMP mAb is broadly distributed throughout brown algal tissues. LAMP is a useful probe for brown algal cell wall studies (Chapter 3);
3. LM7 mAb is a useful probe for the identification and localization of alginates (Chapter 4);
4. Some of the seaweed cell wall-glycan mAbs generated by Dr. Valerie Vreeland are still viable and have the potential to become available for the scientific community (Chapter 5);
5. Plant hormones can affect the cell wall morphology and wall architecture of *P. margaritaceum* (Chapter 6).
IMMUNOLOCALIZATION OF CELL WALL CARBOHYDRATE EPITOPES IN SEAWEEDS: PRESENCE OF LAND PLANT EPITOPES IN FUCUS VESICULOSUS L. (PHAEOPHYCEAE)
2.1. Introduction

Marine brown macroalgae (Phaeophyceae, Heterokontophyta), or brown seaweeds, form an independent eukaryotic lineage consisting of approximately 1500–2000 species of diversified multicellular photosynthetic organisms with a global distribution (Cock et al., 2010). Some species, such as Laminaria digitata, Ascophyllum nodosum and Fucus vesiculosus, can form ecologically important extensive beds in intertidal and subtidal zones of rocky shores in the northern hemisphere (Bold and Wynne, 1978; Graham and Wilcox, 2000; Lobban and Harrison, 1994). Other species may be epiphytic such as Sargassum muticum which is invasive in Europe and North America (Global Invasive Species Database, 2005). Phaeophyceae includes the most morphologically complex macroalgal species, although they are still considered less complex than vascular plants (Charrier et al., 2012). The Phaeophyceae is a highly heterogeneous class, comprising the largest known marine alga, Macrocystis pyrifera, as well as many diminutive species such as the sequenced model, Ectocarpus siliculosus (Cock et al., 2010; Coelho et al., 2012). Algal polysaccharides, such as agars and carrageenans from red seaweeds, and alginates from brown seaweeds (primarily sourced commercially from L. digitata, A. nodosum, F. vesiculosus) are the most important economically valuable compounds extracted from algae. These phycocolloids have important rheological gelling, emulsifying and stabilizing properties, with significant applications within food, pharmaceutical, cosmetic (Cardozo et al., 2007; Pulz and Gross, 2004; Stengel et al., 2011), and biofuel industries (Enquist-Newman et al., 2014; Wargacki et al., 2012).

Brown algae evolved independently from green- (Chlorophyta) and red- (Rhodophyta) algae, and land plants; and so did their cell walls (Baldauf, 2008; Niklas, 2004). Although they are photosynthetic organisms and share complex, dynamic, carbohydrate-rich cell walls with the other groups (Charrier et al., 2012; Popper et al., 2011), adaptive diversification has led to a vast diversity of cell wall polymers (Popper, 2008); consequently, brown algae are only evolutionarily distantly related to the other groups (Yoon et al., 2004).

The cell walls of brown marine algae primarily consist of a crystalline phase composed of cellulose, and an amorphous or matrix phase made up of alginates and fucans (Kloareg and Quatrano, 1988). Cellulose, a cell wall component common to land plants and algae, occurs at much lower quantities in algae (Naylor and Russel-Wells, 1934). Marine algal cell walls are unique, not only because they have higher amounts of matrix
polysaccharides in relation to the fibrillar polymers, but also due to the dominance of acidic over neutral polysaccharides and the presence of sulfated polysaccharides (Percival, 1979). Alginites and sulfated fucans are major components of brown seaweed cell walls and are constituents of the fibrillar wall and of the intercellular space matrix respectively (Kloareg, 1984; Mabeau and Kloareg, 1987). Alginites are linear acidic polysaccharides composed of β-1,4-D-mannuronic acid (ManA) and its C₅ epimer, α-1,4-L-guluronic acid, (GulA) at different ratios depending on the species and on environmental conditions (Kim and Park, 1985; Kloareg and Quatrano, 1988; Percival, 1979). They have high commercial value derived from their nutritional and pharmaceutical importance, which is associated with their viscosifying properties (Cardozo et al., 2007; Stengel et al., 2011). Fucoidan is a heterogeneous family of fucose-containing sulfated polysaccharides (FCSPs) that is thought to play a key role in wall architecture by cross-linking cellulose microfibrils (Deniaud-Bouët et al., 2014). Increased research interest on FCSPs, triggered by their bioactive potential such as anticoagulant, antivirus, antitumor, anti-inflammatory, antiviral and antioxidant activities, has revealed an enormous structural complexity (Ale et al., 2011; Li et al., 2008; Usov and Bilan, 2009). Different polysaccharide structures are found within the fucoidans. High proportions of L-fucose (Fuc) and/or D-galactose (Gal) (“galactofucan” or “fucogalactan”) are often found, with sulfate groups mainly positioned on C-2, C-3 and/or C-4. Other FCSPs may contain only L-Fuc and sulfate (“fucan”), while others contain different monosaccharide residues such as D-xylose (Xyl), D-mannose (Man), uronic acids, and less sulfate (Rabanal et al., 2014). The structural backbone of fucoidans varies between species. Fucus vesiculosus fucoidan has been reported as consisting of α-1,3-L-fucopyranose residues (Patankar et al., 1993), but recent reports also describe a backbone of alternating α-1,3- and α-1,4-L-fucopyranose residues (Chevolot et al., 2001; Usov and Bilan, 2009).

The composition of algal cell walls differs depending on factors such as species, thallus part, developmental and life-cycle stage, season and habitat (Guibet et al., 2008; Kloareg and Quatrano, 1988; Kropf et al., 1988; Lahaye et al., 1994; Lechat et al., 2000; Mabeau and Kloareg, 1987), further complicating their full characterization. Additionally, many algal cell walls and their components have been characterized after extraction, which allows detailed biochemical characterization. However, information regarding localization of wall components, particularly at the cellular level is lost. In plants, significant advances have been made by complementation of biochemical characterization of structure and composition of cell wall components performed on extracts with in situ techniques, such as
immunolocalization (Knox, 1997). Currently 200+ monoclonal antibodies (mAbs), largely generated against epitopes present in vascular plant cell wall glycans (Pattathil et al. 2010), are available and extensively used by the scientific community as tools for detailed imaging of specific cell wall components in terms of their structure, organization and dynamics at the cellular level (Domozych, 2012; Lee et al., 2011). MAbs can also be used as a screening tool for larger scale characterization of specific cell wall and biomass extracts (Moller et al., 2007; Moller et al., 2008; Moller et al., 2012; Pattathil et al., 2012). Although the majority of mAbs currently available were generated against epitopes present in vascular plant cell walls, some have been demonstrated to bind to wall components present in freshwater- (Domozych et al., 2007; Domozych and Lambiasse, 2009; Domozych et al., 2011; Estevez et al., 2008) and marine- green algae (Estevez et al., 2009; Fernández et al., 2010). Despite the fact that mAbs have been generated against epitopes present in brown (Jones et al., 1988; Torode et al., 2015; Vreeland, 1970, 1972) and in red seaweeds (Vreeland et al., 1992) and their localization patterns have been studied (Green et al., 1993; Vreeland et al., 1984), information regarding the in situ distribution of cell wall components in seaweeds remains scarce.

_Fucus vesiculosus_ (Phaeophyceae) is a common species in the intertidal zone of rocky coastlines in the temperate northern hemisphere (Bold and Wynne, 1978). The dichotomously branched thallus (Fig. 2.1a) consists of a discoid holdfast which attaches the seaweed to a substratum, a narrow stipe (Fig. 2.1b) and a flattened blade (Fig. 2.1c) with a central midrib and, often, air bladders (Fig. 2.1d) (Graham and Wilcox, 2000; Lee, 2008). Specialized reproductive organs of this dioecious species, called receptacles, are located at the periphery of the algal thallus (Fig. 2.1e). Dispersed over the surface of the receptacles are the small openings of the conceptacles (Fig. 2.1e). Several oogonia are formed in each female conceptacle; each oogonium contains eight haploid eggs and is enclosed by three cell wall layers, (1) an exochite, (2) a thick mesochite surrounding the entire oogonia, and (3) an endochite enclosing each egg (Farmer and Williams, 1898). By contrast, the antheridia inside the male conceptacles produce 64 haploid biflagellate sperm cells (Bold and Wynne, 1978), each of which is enclosed by two cell walls, an outer and an inner wall (McCully, 1968). Desiccation during low tide can trigger the burst of the exochite; resubmersion leads to the swelling of the endochite, and subsequent mesochite rupture releases the eggs into the seawater. Similarly antheridia are discharged into the seawater following rupture of the outer wall. Their inner wall remains intact and is later dissolved releasing the sperm cells (Bold and Wynne, 1978). To effect fertilization, after release into the water, the male gametes are
attracted by molecules released by the female eggs that act like pheromones (Kochert, 1978; Müller and Gassmann, 1985). Fertilization also involves compounds derived from the sperm that recognize sulfated glycosidic compounds on the egg plasma membrane (Wright et al., 1995a; Wright et al., 1995b). Therefore, for this species the cell wall is intrinsically important for successful reproduction.

Although the Archaeplastida and Heterokontophyta form independent lineages, it has been speculated that, through horizontal gene transfer (Baurain et al., 2010; Michel et al., 2010; Stiller et al., 2009), at least some of their wall components have similarities (Popper and Tuohy, 2010). Therefore, plant cell wall component-directed mAbs may have the ability to bind epitopes present in algal cell walls allowing their application as important markers to investigate the distribution, localization and metabolism of (marine) algal cell wall components of significant physiological and commercial value (Vreeland et al., 1987). However, no reports of the application of mAbs generated against plant cell wall components toward detailed localization of epitopes present in the cell walls of members of the Phaeophyceae currently exist. In the present study, a multifaceted approach, including Glycome Profiling of cell wall extracts and in situ immunolocalization, was used to investigate the cell wall composition and localization of specific wall components in *Fucus vesiculosus*. Immunolocalizations were performed on different thallus parts in order to identify and understand labeling patterns and, since the cell wall is known to play an essential role in the reproduction of this species, a detailed examination of the reproductive structures was carried out.
Figure 2.1. Morphology of *Fucus vesiculosus* (a–e), and histology of the main tissues found in the respective thallus part in cross-sections stained with toluidine blue (f–i): (a) The thallus is differentiated into a holdfast (not shown), stipe (b), a dichotomously branched blade with a midrib (c), and air bladders (d). The reproductive structures, conceptacles, are formed inside the receptacles (e). The vegetative tissues of the stipe (f), blade (g) and air bladders (h) are mainly differentiated into meristoderm, cortex and medulla. The receptacles of this species produce several conceptacles (i), where the male and female gametes are produced, depending on the specimen. The cross-section shown is a male receptacle. *ab*, air bladder; *bl*, blade; *cp*, conceptacle; *cor*, cortex; *med*, medulla; *mer*, meristoderm; *mid*, midrib; *rp*, receptacle; *st*, stipe. Scale bars: *a* = 3 cm, *b–e* = 500 µm, *f–i* = 200 µm (Figure prepared by S. Raimundo).
2.2. Materials and methods

2.2.1. Algal material

Male and female specimens of the dioecious brown macroalga *Fucus vesiculosus* L. (Phaeophyceae, Heterokontophyta) were collected at Spiddal, Co. Galway (53°14.4’N, 9°18.7’W), on the west coast of Ireland in February 2012, washed with tap water and cleaned of visible epiphytes and grazers.

2.2.2. Cell wall extraction

Algal cell walls were extracted using methods similar to those previously described for vascular plant cell walls (Pattathil *et al.*, 2012), but with a somewhat different set of extractive solvents. Alcohol insoluble residue (AIR) was prepared from seaweed biomass. Briefly, algal material was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was resuspended in 80% (v/v) ethanol, shaken overnight at room temperature, and centrifuged at 3,000 × g for 15 min. The supernatant was discarded and the pellet washed with 80% (v/v) ethanol followed by final washing steps using 100% ethanol and acetone. AIR was air-dried for 24 h at room temperature. Sequential extractions of the AIR were carried out with selective solvents as outlined below. For each extraction the seaweed AIR was incubated with constant mechanical stirring (200 rpm) at 70°C for 24 h. After incubation, the suspension was centrifuged at 3,000 × g for 10 min, and the supernatant decanted from the pellet and stored at 4°C. Seaweed AIR was extracted with water. The insoluble residue was then extracted with 2% (w/v) calcium chloride (CaCl₂), followed by 0.01 M hydrochloric acid (HCl), pH 2, and then with 3% (w/v) sodium carbonate (Na₂CO₃) (Rioux *et al.*, 2007). The alginites, present in the Na₂CO₃ supernatant, were precipitated with acetone, resuspended in deionized water and dialyzed. Further extractions were performed on the Na₂CO₃-extracted residue, first with 1 M potassium hydroxide (KOH) containing 1% (w/v) sodium borohydride (NaBH₄), followed by 4 M KOH containing 1% (w/v) NaBH₄, after which the pellet was washed with deionized water and stored at 4°C. The 1 M KOH and 4 M KOH extracts were neutralized prior to dialysis. To neutralize the extracts, they were placed in upright and uncapped tubes in ice, 2 drops of 2-octanol were added, and glacial acetic acid slowly added to adjust the pH to 7. All extracts were dialyzed (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA,
USA) against three changes of deionized water in 4°C for a total of 48 h, freeze-dried, and weighed. The extracts were stored at room temperature in a desiccator containing a drying agent (silica) prior to further analysis.

2.2.3. Monosaccharide composition

Monosaccharide composition analysis of the extracts was carried out by the Complex Carbohydrate Research Center Analytical Services, University of Georgia and performed by combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis (Merkle and Poppe, 1994; York et al., 1986).

2.2.4. Enzyme-Linked Immunosorbent Assay

ELISAs were carried out as described by (Pattathil et al., 2010). Aliquots (50 µl) of F. vesiculosus extracts (10 µg/ml in deionized water) were added to each well of 96-well plates (Costar 3598, Corning Inc., NY, USA) and dried overnight at 37°C. Deionized water was added to the negative control wells. Tamarind xyloglucan (Megazyme, Bray, Ireland) probed with CCRC-M104 was used as a positive control. Plates were blocked with 200 µl of 1% (w/v) nonfat dry milk (instant nonfat dry milk, Publix supermarket, USA) in Tris-buffered saline (2 mM Tris-Base, 8 mM Tris-HCl, pH 7.6, containing 100 mM sodium chloride) for 1 h. The next steps of aspiration and washing employed an ELx405 VRS Microplate Washer (BioTek Instruments Inc., Winooski, VT, USA); incubations were at room temperature. After aspirating the blocking solution, 50 µl of undiluted hybridoma supernatant of the mAbs was added (LAMP and BG-1 were diluted 1:70 in 0.1 M Tris-buffered saline) to the wells and incubated for 1 h. Antibody solutions were aspirated and wells were washed three times with 300 µl of 0.1% (w/v) nonfat dry milk in Tris-buffered saline (wash buffer). Horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rat IgG secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) were diluted 1:5,000 in wash buffer and 50 µl added to the wells and incubated for 1 h. Antibody solutions were aspirated and the wells were washed with 300 µl of wash buffer (five times). 3,3′,5,5′-Tetramethylbenzidine peroxidase substrate (TMB peroxidase substrate kit SK-4400, Vector Laboratories, Inc., Burlingame, CA, USA) was prepared according to manufacturer’s instructions, and 50 µl was added to each well and incubated for 30 min. The reaction was stopped by adding 50 µl of 1 N sulfuric acid (H2SO4) and the optical density was
immediately read as the difference between $A_{450}$ and $A_{655}$ with a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the Microplate Manager Version 5.2 Build 103 software. The reading for the corresponding negative control well was also subtracted from each sample well. ELISA results were presented as a heat map using R-Console software (Team, 2011).

2.2.5. Light microscopy

Algal material was fixed and embedded as previously described (Coimbra et al., 2007) with minor modifications. Small pieces of the stipe, blade and receptacles (2–5 mm) were cut with a razor blade and immediately placed in an Eppendorf tube containing freshly prepared fixative solution [2% (v/v) paraformaldehyde; 0.25% (v/v) glutaraldehyde; 0.025 M PIPES buffer; 0.001% (v/v) Tween 80; pH 7.2]. The material was fixed for 2 h at room temperature followed by overnight incubation at 4°C. During the fixation time, a high content of mucilage is released. Therefore the samples must be shaken often, so the fixative can reach every cell within the tissue. The material that did not sink was discarded, and the remaining material was washed with 0.025 M PIPES buffer, pH 7.2, for 10 min, followed by a second wash for 20 min. Samples were then dehydrated in a graded ethanol series [25% (v/v), 35% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), and 3 times with 100%] with 15 min for each grade and transferred to glass tubes. Infiltration was performed by a sequential immersion in different proportions of 100% ethanol and LR White Resin (Medium grade, Agar Scientific, Cambridge, UK) over 6 days (ethanol/LR White proportion per day was 3:1; 2:1; 1:1; 1:2; 1:3; 0:1). After the final resin change, samples were placed in gelatin capsules (Electron Microscopy Sciences, Hatfield, PA, USA) with fresh LR White, hermetically sealed and placed in an oven at 60°C for 2 days.

2.2.6. Monoclonal antibodies

MAbs generated against diverse plant cell wall glycans were obtained as hybridoma cell culture supernatants from stocks at the Complex Carbohydrate Research Center (CCRC). The CCRC, PN, JIM and MAC series are available from CarboSource (www.carbosource.net). The antigens used to generate the collection of mAbs, and correspondent preparation methodology was previously described (Pattathil et al., 2010). LAMP and BG-1 antibodies are available from Biosupplies (Parkville, Victoria, Australia,
A list of the antibodies used, including links to the online database, WallMabDB (www.wallmabdb.net), is provided in Table 2.1.

Table 2.1. List of the plant cell wall glycan-directed mAbs used for Glycome Profiling (Fig. 2.2). The antibodies were grouped according to a hierarchical clustering of the predominant polysaccharides they recognize, based on ELISA data generated from a comprehensive screening of all mAbs against a series of plant polysaccharide preparations (Pattathil et al., 2010). These antibodies are listed in the WallMabDB plant cell wall monoclonal antibody database (www.wallmabdb.net) that provides detailed information about each mAb, including immunogen, antibody isotype, epitope structure (when known), supplier information, and related literature citations.

<table>
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<th>Glycan group recognized</th>
<th>mAb names</th>
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<tr>
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</tr>
<tr>
<td>JIM131</td>
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<tr>
<td>CCRC-M22</td>
<td></td>
</tr>
<tr>
<td>JIM132</td>
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<tr>
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</tr>
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<td>CCRC-M8</td>
<td></td>
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<tr>
<td>CCRC-M93</td>
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</table>
| Arabinogalactan-2 | JIM94  
|                  | JIM11  
|                  | MAC204 
|                  | JIM20  
| Arabinogalactan-3| JIM14  
|                  | JIM19  
|                  | JIM12  
|                  | CCRC-M133  
|                  | CCRC-M107 
| Arabinogalactan-4| JIM4   
|                  | CCRC-M31 
|                  | JIM17  
|                  | CCRC-M26 
|                  | JIM15  
|                  | JIM8   
|                  | CCRC-M85 
|                  | CCRC-M81 
|                  | MAC266 
|                  | PN 16.4B4 
| Unidentified     | MAC207 
|                  | JIM133 
|                  | JIM13  
|                  | CCRC-M92 
|                  | CCRC-M91 
|                  | CCRC-M78 
|                  | MAC265 
|                  | CCRC-M97 |
2.2.7. Immunohistochemistry

Immunolabelling was performed as described by (Avci et al., 2012). In brief, semi-thin sections (250 nm) were cut with a Leica EM UC6 ultramicrotome (Leica Mikrosysteme GmbH, Vienna, Austria), at room temperature, in a humid chamber. Sections were mounted on glass slides (Fisherbrand Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA) and dried at 50°C. To provide reference for anatomical information, sections were routinely stained with toluidine blue; a drop of 1% (w/v) toluidine blue in 1% (w/v) sodium borate was placed on the sections for 20 s (McCully et al., 1980), washed with H2O and the sections were then dried. For immunolabelling, sections were blocked with 3% (w/v) nonfat dry milk (instant nonfat dry milk, Publix) in potassium phosphate buffered saline (KPBS) (0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl) for 30 min, and then incubated with undiluted hybridoma supernatant of the mAbs (with the exceptions that the LAMP and BG-1 mAbs concentrations were according to manufacturer’s instructions). After 60 min, the sections were washed with KPBS, 3 × 5 min, and incubated with goat anti-mouse IgG or goat anti-rat IgG conjugated with AlexaFluor 488 (Invitrogen Molecular Probes, Eugene, OR, USA) secondary antibody (1:100 in KPBS) for 90 min, after which the sections were washed with KPBS, 2 × 5 min. After a final wash with distilled water (5 min), the sections were mounted with Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA, USA) and covered with a coverslip. Labeling was visualized with a Nikon Eclipse 80i (Nikon Instruments, Melville, NY, USA) microscope equipped with epifluorescence optics and Nikon B-2E/C filter. Images were captured with a Nikon DS-Ri1 camera (Nikon Instruments, Melville, NY, USA) using NIS-Elements Basic Research software, and assembled with Adobe Photoshop Elements 11.0 (Adobe Systems, San Jose, CA, USA) software.
2.3. Results

2.3.1. Cell wall monosaccharide composition

The monosaccharide compositions of each extract obtained from whole *F. vesiculosus* are shown in Table 2.2. The main sugar residues present in the *F. vesiculosus* extracts were Fuc and the uronic acids, ManA and GulA. ManA and GulA are indicative of the presence of alginates (Haug, 1964) and were present at the highest relative quantities in the H$_2$O (67.0%), CaCl$_2$ (72.1%) and Na$_2$CO$_3$ (95.2%) extracts. The HCl extract has the highest relative quantity of Fuc (52.1%), the main monosaccharide present in fucoidan (Conchie and Percival, 1950). The 1 M KOH and 4 M KOH extracts were composed of Man, Xyl, Gal and glucose (Glc). This is consistent with previous reports of these monosaccharides from fucoidan preparations from other brown seaweeds (Ale et al., 2011; Li et al., 2008). However, the absence of fucose in these extracts indicates that the polysaccharides present are unlikely to be part of fucoidans. Carbohydrates in the final insoluble residue corresponded entirely to Glc, most likely derived from cellulose (Naylor and Russel-Wells, 1934).

2.3.2. Glycome Profiling

Results of Glycome Profiling performed on different extracts (H$_2$O, CaCl$_2$, HCl, Na$_2$CO$_3$, 1 M KOH and 4 M KOH) of *F. vesiculosus* cell walls using a selection of 155 plant glycan-directed mAbs are presented as a heat map (Fig. 2.2). Very little if any antibody binding was observed to most of the extracts. The only exceptions were the antibodies of the rhamnogalacturonan I/arabinogalactan (RG-I/AG) clade, some of which bound strongly to the H$_2$O and CaCl$_2$ extracts. The highest signals were observed with CCRC-M22 and JIM131 for the H$_2$O extract and with CCRC-M13, CCRC-M22, CCRC-M77, CCRC-M79, CCRC-M80, CCRC-M121, CCRC-M122, CCRC-M123, CCRC-M134, JIM1 and JIM131 for the CaCl$_2$ extract.
Table 2.2 Monosaccharide composition analysis of each extract obtained from entire *F. vesiculosus* thallus. Carb: carbohydrate, Res: residue.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Crude seaweed Mol %&lt;sup&gt;1&lt;/sup&gt;</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;O Mol %&lt;sup&gt;1&lt;/sup&gt;</th>
<th>CaCl&lt;sub&gt;2&lt;/sub&gt; Mol %&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HCl Mol%&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Na&lt;sub&gt;2&lt;/sub&gt;CO&lt;sub&gt;3&lt;/sub&gt; Mol%&lt;sup&gt;1&lt;/sup&gt;</th>
<th>1 M KOH Mol%&lt;sup&gt;1&lt;/sup&gt;</th>
<th>4 M KOH Mol%&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Res Mol%&lt;sup&gt;1&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Res</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ara</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Rha</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Fuc</td>
<td>2.6</td>
<td>21.9</td>
<td>17.5</td>
<td>52.1</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Xyl</td>
<td>2.8</td>
<td>3.1</td>
<td>2.8</td>
<td>0.6</td>
<td>1.0</td>
<td>7.0</td>
<td>23.1</td>
<td>-</td>
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<tr>
<td>GlcA</td>
<td>-</td>
<td>0.7</td>
<td>0.7</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GalA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Man</td>
<td>-</td>
<td>2.1</td>
<td>1.8</td>
<td>0.4</td>
<td>1.0</td>
<td>7.5</td>
<td>52.6</td>
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<tr>
<td>Gal</td>
<td>2.1</td>
<td>3.3</td>
<td>3.2</td>
<td>0.5</td>
<td>0.5</td>
<td>1.8</td>
<td>7.1</td>
<td>-</td>
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<tr>
<td>Glc</td>
<td>90.8</td>
<td>2.0</td>
<td>1.9</td>
<td>-</td>
<td>0.1</td>
<td>83.7</td>
<td>17.1</td>
<td>100.0</td>
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<tr>
<td>ManA</td>
<td>-</td>
<td>67.0</td>
<td>72.1</td>
<td>46.4</td>
<td>95.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GulA</td>
<td>-</td>
<td>67.0</td>
<td>72.1</td>
<td>46.4</td>
<td>95.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carb [%]</td>
<td>2.0</td>
<td>42.0</td>
<td>71.0</td>
<td>90.0</td>
<td>27.0</td>
<td>3.0</td>
<td>0.4</td>
<td>4.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are expressed as mole percent of total carbohydrate. The total percentage may not add to exactly 100 % due to rounding. The values presented result from the analysis of one replicate.

Ara, arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; GlcA, glucuronic acid; GalA, galacturonic acid; Man, mannose; Gal, galactose; Glc, glucose; ManA, Mannuronic acid; GulA, guluronic acid.
Figure 2.2. Glycome profiling of sequential cell wall extracts prepared from whole thallus of *F. vesiculosus*. The data, which are presented as a heat map, result from ELISA screening of the extracts using 155 plant cell wall glycan-directed monoclonal antibodies (Pattathil et al., 2010). The panel on the right details the antibodies used, colour-coded as groups based on a hierarchical clustering of the cell wall glycans recognized by each antibody group (Table 2.1). The ELISA signal strength is displayed by a black-yellow scale, with black indicating no binding and yellow illustrating strongest binding. Labels at the bottom indicate the reagents used in each extraction step.
2.3.3. Immunolocalization

A broad immunolocalization study was performed on LR White-embedded sections of different thallus parts of *F. vesiculosus* using representative mAbs from each clade shown in the panel at the right of the glycome profiles (Fig. 2.2). The results of the immunolocalization studies are largely in accordance with the ELISA results, with only one mAb clade, RG-I/AG, showing significant binding to sections (Table 2.3). An interesting exception was observed with the LAMP mAb from the β-glucan clade, which strongly labelled the cell walls of all thallus parts *in situ* (Fig. 2.3c, Fig. 2.4c, Fig. 2.5c–d, Fig. 2.6c–d), even though it gave only a weak signal in the H<sub>2</sub>O extract on the glycome profiles (Fig. 2.2). The LAMP mAb was therefore used as a positive control in all subsequent immunolocalization experiments. A second exception was CCRC-M26, from the Arabinogalactan-3 (AG-3) clade, which showed modest binding to the 1 M KOH extract in the glycome profiles, and bound to some cell walls of some *F. vesiculosus* tissues (Table 2.3).

Table 2.3 Summary of immunolocalization patterns observed in *F. vesiculosus* for RG-I/AG and AG-3 mAb clades.

<table>
<thead>
<tr>
<th>Thallus part</th>
<th>Group 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Group 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Group 3&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Group 4&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Stipe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Male receptacle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cells, stronger binding to antheridia</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Antheridia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Antheridia inner cell wall</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Female receptacle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oogonia and paraphyses</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oogonia mesochite</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> mAbs: CCRC-M79, CCRC-M80, CCRC-M122, CCRC-M126, CCRC-M134.

<sup>b</sup> mAbs: JIM131, CCRC-M22, CCRC-M24, CCRC-M25, CCRC-M121, CCRC-M123, CCRC-M128.


Note: Labeling with mAbs CCRC-M13, CCRC-M15, CCRC-M44, CCRC-M125 was not sufficiently strong and/or defined to be grouped and JIM1, JIM16, JIM132 did not label the sections.
Figure 2.3. Immunolabelling of cross sections of *F. vesiculosus* stipe with the mAbs LAMP (c) and CCRC-M134 (d, e, f) and stained with toluidine blue (a). a Morphology of a stipe section. *mer* meristoderm, *cor* cortex, *med* medulla. b Negative control where no primary mAb was used, showing that autofluorescence is not present at significant levels. c LAMP labeling of all cell walls. d CCRC-M134 labeling of the meristoderm, cortex and medulla cells. e and f CCRC-M134 labeling of the medulla (e) and meristoderm and cortex (f) visualized at higher magnification. *Scale bars*: a = 100 µm, b–f = 50 µm.

The RG-I/AG clade of mAbs exhibited similar labeling patterns across the different organs/tissues examined. For example, the extracellular matrix was never labeled in any thallus part, and all of these mAbs bound very strongly to the male antheridia walls. Nonetheless, some distinctive labelling patterns were observed for different antibodies within the RG-I/AG clade. Each of the 31 mAbs belonging to this clade was used for immunolabelling, and they could be separated into three groups based on the binding patterns observed in the different thallus parts (Table 2.3). Group 1 includes CCRC-M79, CCRC-M80, CCRC-M122, CCRC-M126 and CCRC-M134; Group 2 includes JIM131, CCRC-M22, CCRC-M24, CCRC-M25, CCRC-M121, CCRC-M123 and CCRC-M128; Group 3 includes CCRC-M7, CCRC-M8, CCRC-M9, CCRC-M12, CCRC-M21, CCRC-M32, CCRC-M33, CCRC-M41, CCRC-M42, CCRC-M60, CCRC-M77 and CCRC-M112. A fourth labeling pattern, exhibited by CCRC-M26 from the AG-3 clade was also observed (Table 2.3). The
RG-I/AG mAbs CCRC-M13, CCRC-M15, CCRC-M44 and CCRC-M125 were not included in any group because, although they bound to the antheridia in similar patterns as the mAbs listed above, their labeling was not strong and/or defined; JIM1, JIM16 and JIM132 did not show any labeling in the tissues examined.

![Image](image.png)

**Figure 2.4.** Immunolabelling of cross sections of *F. vesiculosus* blade with the mAbs LAMP (c) and CCRC-M134 (d, e, f) and stained with toluidine blue (a). a Morphology of a blade section, *mer* meristoderm, *cor* cortex, *med* medulla. b Negative control where no primary mAb was used, indicating lack of autofluorescence. c LAMP labeling of all cell walls. d CCRC-M134 labelling of the meristoderm, cortex and medulla cells. e and f CCRC-M134 labelling of the medulla (e) and (f) meristoderm and cortex visualized at higher magnification. *Scale bars:* a = 100 µm, b–f = 50 µm.

The Group 1 set of RG-I/AG mAbs was the only one that showed generalized labeling of the cells in every thallus part (Table 2.3). These mAbs (CCRC-M79, CCRC-M80, CCRC-M122, CCRC-M126 and CCRC-M134) labeled the walls of all vegetative tissues, meristoderm, cortex, medulla and paraphyses in the stipe (Fig. 2.3d, e, f), blade (Fig. 2.4d, e, f) and in both male and female receptacles (Fig. 2.5f, j, n; Fig. 2.6f, j, n). Despite this general pattern, the medulla cells in the midrib of the blade labeled in distinctive patterns; some cells were entirely labeled, while others exhibited strong labeling in only one part of their cell walls (Fig. 2.4e). In the conceptacles, both antheridia (Fig. 2.5j) and oogonia (Fig. 2.6j) were
strongly labeled. This was the only group of mAbs that labeled the medullary cells (Fig. 2.5n, Fig. 2.6n). The Group 2, 3 and 4 mAbs did not label these cells (Fig. 2.5o, p; Fig. 2.6 o, p).

The Group 2 set of RG-I/AG mAbs (JIM131, CCRC-M22, CCRC-M24, CCRC-M25, CCRC-M121, CCRC-M123 and CCRC-M128) labeled the male and female conceptacles (Table 2.3), but no other cell or tissue. These mAbs specifically labeled the antheridia in the male conceptacle (Fig. 2.5g, k), and the oogonia and the paraphyses in the female conceptacle (Fig. 2.6g, k).

The Group 3 set of RG-I/AG mAbs (CCRC-M7, CCRC-M8, CCRC-M9, CCRC-M12, CCRC-M21, CCRC-M32, CCRC-M33, CCRC-M41, CCRC-M42, CCRC-M60, CCRC-M77 and CCRC-M112) showed the same pattern in the male conceptacle as the Group 2 mAbs; only the antheridia were labeled. However, in contrast to the Group 2 mAbs, none of the Group 3 mAbs labelled the female conceptacle (Table 2.3).

Only two mAbs outside of the RGI/AG clade had the ability to bind to epitopes present in *F. vesiculosus*. These are the LAMP mAb, which was used as a control because of its ability to bind to all *F. vesiculosus* cell walls, and CCRC-M26. CCRC-M26 is the only mAb from the panel of 155 mAbs that was not from the RG-I/AG clade and that showed binding to *F. vesiculosus* cell walls. CCRC-M26 belongs to the AG-3 clade and only showed labeling within the male and female reproductive tissues (Table 2.3, Group 4), where it bound exclusively to the antheridia inner cell wall (Fig. 2.5h, l), and the female oogonia mesochite (Fig. 2.6h, l).
Figure 2.5. Immunolabelling of cross sections of the male receptacle of *F. vesiculosus* with the mAbs LAMP (c, d), CCRC-M134 (f, j, n), CCRC-M7 (g, k, o), CCRC-M26 (h, l, p) and stained with toluidine blue (a, e, i, m). a Male receptacle, where the conceptacles (cp) containing the antheridia (ant) are formed, the ostiole (ost) is the opening to the exterior. Meristoderm (mer), cortex (cor) and medulla (med) are also indicated. b Negative control where no primary mAb was used, showing the absence of autofluorescence. c Conceptacle area labelled with LAMP, which labels all cell walls. d Medulla area labelled with LAMP. e Part of a male conceptacle stained with toluidine blue inside of which the sterile paraphyses (par) and the antheridia (ant) containing the sperm cells (sp) can be observed. f CCRC-M134 labels all of the receptacle cells and bound more strongly to the antheridia. g CCRC-M7 showed specific binding to the antheridia. h CCRC-M26 shows a distinctive pattern, labeling only the inner cell wall of the antheridia. i The male conceptacle stained with toluidine blue, showing the paraphyses (par) and the antheridia (ant) outer (out) and inner (inn) cell walls, and the sperm cells (sp). j CCRC-M134 labels the conceptacle cells, including the antheridia (ant) and the paraphyses (par). k Binding of CCRC-M7 to the antheridia. l Binding of CCRC-M26 to the antheridia inner cell wall (inn). m Medulla stained with toluidine blue. n CCRC-M134 labels the medulla cells (mc). o CCRC-M7 does not label the medulla. p CCRC-M26 does not label any cells in the medulla. Scale bars: a = 100 µm, b–p = 50 µm.
**Figure 2.6.** Immunolabelling of cross sections of the female receptacle of *F. vesiculosus* with the mAbs LAMP (c, d), CCRC-M134 (f, j, n), CCRC-M22 (g, k, o), CCRC-M26 (h, l, p) and stained with toluidine blue (a, e, i, m). a A female receptacle showing the conceptacles (cp) containing the oogonia (oog), the ostiole (ost) is the opening to the exterior. Meristoderm (mer), cortex (cor) and medulla (med) are also indicated. b Negative control where no primary mAb was used, showing the absence of autofluorescence. c Conceptacle area labelled with LAMP, which labels all cell walls. d Medulla area labelled with LAMP. e Part of a female conceptacle stained with toluidine blue inside of which the sterile paraphyses (par) and the oogonia (oog) can be observed. f CCRC-M134 shows a general labeling pattern in all the cells of the receptacle. g CCRC-22 shows specific binding to the oogonia and the paraphyses. h CCRC-M26 shows specific labeling of the oogonia cell walls. i Oogonium stained with toluidine blue, showing the paraphyses (par) and the three cell walls of the oogonium, exochite (exo), mesochite (mes) and endochite (end). Some of the nuclei (nuc) of the 8 eggs that each oogonium contains are also visible. j CCRC-M134 labels the oogonium (oog) and the paraphyses (par). k Binding of CCRC-M22 to the oogonium endochite (end). l Binding of CCRC-M26 to the oogonium mesochite (mes). m Medulla stained with toluidine blue. n CCRC-M134 labeling of the medulla cells (mc). o CCRC-M7 does not label the medulla. p CCRC-M26 does not label any cells in the medulla. Scale bars: a = 100 µm, b–p = 50 µm.
2.4. Discussion

The data presented in this chapter shows, for the first time, that some plant cell wall glycan-directed mAbs are able to bind to epitopes present in *F. vesiculosus* both *in vitro* (Glycome Profiling of cell wall extracts) and *in situ* (immunolocalization), indicating the presence of glycan epitopes in the walls of brown algae that previously have been identified in vascular plant cell walls. Glycome Profiling of *F. vesiculosus* extracts showed that mAbs from the RG-I/AG clade gave the strongest signals. Additionally, immunolabelling of LR-White embedded sections using the mAbs from this mAb clade demonstrated the presence of these pectic arabinogalactan epitopes *in situ* in the alga. Of particular interest is the observed intra-thallus variation in labelling patterns, which allowed the clustering of RG-I/AG mAbs into three groups (Table 2.3, Group 1–3). An additional arabinogalactan-directed mAb from clade AG-3 was placed into a fourth group (Table 2.3, Group 4), because of its unique specificity to the oogonia and antheridia cell walls.

2.4.1. Glycome Profiling

The strongest signals for the RG-I/AG clade mAbs were observed in the H$_2$O and CaCl$_2$ extracts and were higher for the CaCl$_2$ extract than for the H$_2$O extract. Alginites and fucoidans, the major constituents of the cell walls of brown seaweeds (Li *et al.*, 2008; Mabeau and Kloareg, 1987), are known to be extractable with CaCl$_2$ and HCl, respectively. Na$_2$CO$_3$ is also known to extract alginites (García-Ríos *et al.*, 2012; Rioux *et al.*, 2007) and the corresponding extract gave the highest percentages of uronic acids and lowest of Gal, but no ELISA signals. On the other hand, the 4 M KOH extract contained the highest percentage of Gal (7.1%), but did not react with the RG-I/AG mAbs; so the highest percentage of AG did not correlate with the ELISA results. The epitopes recognized by the RG-I/AG clade mAbs were probably extracted with the first two solvents, as indicated by the positive ELISA signals in those extracts. KOH is used in plant cell wall extractions, primarily to extract the hemicellulosic polysaccharides, xylans and xyloglucans (Carpita, 1984; Fry, 2000; Thornber and Northcote, 1962; Zablackis *et al.*, 1995). The 1 M KOH and 4 M KOH extracts contained a mixture of monosaccharides (Xyl, Man, Glc, Gal) known to be present in fucoidans (Ale *et al.*, 2011; Li *et al.*, 2008) of some brown seaweed species. Nonetheless, *F. vesiculosus* fucoidans are reported to have only Fuc and sulfate groups, therefore the residues found in the KOH extract are not likely to be from fucoidans. Rather, they may be part of the
hemicelluloses, which have been hypothesized to exist in brown seaweeds, acting as intermediates between cellulose microfibrils and FCSPs (Deniaud-Bouët et al., 2014). Analysis of the monosaccharide composition of the extracts (Table 2.2) confirms that the H$_2$O and CaCl$_2$ extracts are mainly composed of alginates, with high percentages of uronic acids and low percentages of other non-cellulosic neutral sugars, such as Xyl, Man and Gal. It is therefore likely that the epitopes recognized by the RG-I/AG mAbs are minor components of these algal wall extracts.

2.4.2. β-Glucan epitope

LAMP, a mAb that was generated against laminaran extracted from Laminaria digitata, recognizes a (1→3)-β-glucan epitope of five consecutive Glc residues typically found in callose and laminaran, but does not bind to cellulose or to (1→3),(1→4)-β-D-glucan (mixed linkage glucan) (Meikle et al., 1991; Meikle et al., 1994). LAMP was shown here to be a good positive control for the immunolocalization studies in F. vesiculosus because all cell walls of the alga were labeled with this mAb, regardless of the tissue or thallus part. Furthermore, none of the other mAbs used for in situ labelling in this study showed a similar ubiquitous labeling pattern. (1→3)-β-Glucans are present in brown seaweeds primarily as laminaran, their main storage glucan. However, laminaran has not been considered to be an algal cell wall component previously, but was reported instead to be located in the cytoplasm (Mian and Percival, 1973; Quatrano and Stevens, 1976). Nonetheless, immunolabelling of F. vesiculosus tissues with LAMP gave strong binding that was localized to the cell walls and no labeling of the cell interior was observed. Regardless of the precise nature of the polymer/s recognized, the labeling results show that the β-glucan epitope recognized by the LAMP mAb, [βGlc-(1,3)-βGlc-(1,3)-βGlc-(1,3)-βGlc-(1,3)-Glc], is present in the cell walls of this seaweed. Several land plant cell wall polysaccharides, including xyloglucan and (galacto)mannans (Buckeridge, 2010) serve as both cell wall structural components and storage polysaccharides. However, whether this is the case for the β-glucan epitope recognized by LAMP in F. vesiculosus, remains unclear.

2.4.3. Pectin-associated epitopes

The two groups of antibodies that bind to F. vesiculosus cell walls are the RG-I/AG clade that is comprised of mAbs that are directed against arabinogalactan epitopes frequently present in side chains of rhamnogalacturonan I (RG-I), and the AG-3 clade that is comprised
of mAbs that are directed to arabinogalactan epitopes occurring in other molecular contexts [e.g., arabinogalactan proteins (AGPs)] (Pattathil et al., 2010). Known to be present in seed mucilage and in primary cell walls of plants (Caffall and Mohnen, 2009), RG-I is one of the major components of pectins and consists of a backbone of a repeated [-α-D-GalA-1,2-α-L-Rha-1-4-]n disaccharide (Mohnen, 2008), with side chains of arabinans, galactans, or arabinogalactans (Lau et al., 1985). Despite the fact that mAbs in the RG-I/AG clade recognize similar compounds, the epitopes recognized by most of them have not yet been characterized in detail. One exception is CCRC-M7, which binds to an epitope consisting of a trimer or larger of β-(1,6)-Gal (Puhlmann et al., 1994; Steffan et al., 1995). Monosaccharide composition of *F. vesiculosus* walls and wall extracts showed only the presence of low amounts of Gal and no arabinose (Ara) (Table 2.2), so it is unlikely that classic higher plant arabinogalactans are present in the walls of this brown alga. Nonetheless, CCRC-M7 and 11 other mAbs from the RG-I/AG clade exhibited similar labelling patterns on *F. vesiculosus* tissues (Table 2.3, Group 3). In particular, labelling with these mAbs was observed in the antheridia inner and outer cell walls in the conceptacle tissue, showing that the β-galactan epitope recognized by CCRC-M7 is specific to these structures (Figure 5g, k, o). This is the first report of immunolocalization in brown seaweeds with plant cell wall-generated mAbs. However, this mAb was previously reported to label the primary cell wall of suffultory cells (that form the oogonial structures) in the green alga *Oedogonium* (Estevez et al., 2008). These results suggest that polymers containing the β-galactan epitope may have properties important for reproduction.

Interestingly, other mAbs from the RG-I/AG clade showed different labeling patterns in the *F. vesiculosus* tissues examined in this study. Some labeled all the cell walls of the different tissues, regardless of the thallus part, as was observed for the stipe (Fig. 2.3d), blade (Fig. 2.4d) and male– (Fig. 2.5f, j, n) and female– (Fig. 2.6f, j, n) conceptacles. Other mAbs bound only to the antheridia walls (Fig. 2.5g, k), and some additionally bound to the oogonia and paraphyses (Fig. 2.6g, k). The most striking labeling pattern observed, however, was that all the RG-I/AG mAbs bound very strongly to both the antheridia inner and outer cell walls. Monosaccharide composition analysis did not detect Ara in any of the algal wall extracts (Table 2.2), which precludes the possibility that these mAbs bind to arabinogalactan-related epitopes, suggesting instead that *F. vesiculosus* cell walls contain epitopes that are probably part of galactan-rich compounds. High sensitivity is one of the advantages of immunolocalization techniques; an epitope can be detected even if it only represents a small proportion of the total polysaccharide composition. In this case, labelling signals could result
from galactan epitopes associated to fucoidans. Some brown seaweed fucoidans are reported
to have associated galactans (Usov and Bilan, 2009), although they have not been described
for *F. vesiculosus* (Chevolot et al., 2001; Patankar et al., 1993). Nonetheless, Gal was
detected at low percentages in all of the wall extracts, which suggests that the Gal detected in
*F. vesiculosus* could be associated with galactan structures that are recognized by the RG-I/AG clade of mAbs.

The differences in *in situ* labeling patterns observed within *F. vesiculosus* tissues for
the RG-I/AG mAbs suggests that the different groups of RG-I/AG mAbs recognize distinct
epitopes. These epitopes are not uniformly distributed within the seaweed tissues, with some
being present in all tissues and others observed only in reproductive tissues, suggesting the
occurrence of structurally different galactans in seaweed walls, possibly with different
functions.

The binding of the RG-I/AG antibodies to the algal cell walls indicates that glycan
epitopes present in land plants are also present in the walls of brown algae. None of the mAbs
previously generated against brown seaweeds including *Fucus* species (Jones et al. 1988;
Green et al. 1993; Vreeland 1970, 1972; Vreeland et al. 1984, 1992) and *Macrocystis
pyrifera* (Eardly et al., 1990) are currently widely available and many of the hybridoma cell
lines that produced these antibodies are no longer viable (Professor JR Green, University of
Birmingham, personal communication). Additionally, the epitopes recognized by these mAbs
were not clearly delimited in most cases. Therefore, although new seaweed cell wall-directed
mAbs have been developed recently (Torode et al., 2015), the potential to also use mAbs
generated against plant cell walls, including those whose epitopes have been at least partially
characterised, not only gives potential insight into cell wall evolution, but diversifies and
adds additional tools for cell wall investigations in brown seaweeds as well for studies of
various fundamental aspects of seaweed physiology.

2.4.4. AG-3 mAb

CCRC-M26 belongs to the AG-3 clade of mAbs thought to recognize AGPs epitopes
(Pattathil et al., 2010). This mAb specifically bound to *F. vesiculosus* antheridia inner cell
walls (Fig. 2.5h, l), which enclose 64 gametes, and to the oogonia mesochite (Fig. 2.6h, l),
which encloses 8 eggs, after their release from the conceptacles into seawater prior to
fertilization. AGPs belong to a family of hydroxyproline-rich glycoproteins and are present in
the cell walls of vascular plants (Pennell et al., 1989). They are implicated in a wide range of
physiological functions (Lamport and Várnai, 2012; Majewska-Sawka and Nothnagel, 2000; Seifert and Roberts, 2007) and in sexual reproduction (Coimbra et al., 2009; Coimbra et al., 2010) including in male-female communication during sexual reproduction (Coimbra et al., 2007; Pereira et al., 2014); the presence of AGP-like epitopes in the cell walls of the green macroalga Codium fragile was previously reported and was based on immunolocalization of epitopes in Codium fragile with the mAbs JIM11, JIM16, JIM20, LM1, and MAC206 (Estevez et al., 2009; Fernández et al., 2010). MAbs have previously been generated against Fucus serratus (Green et al., 1993; Jones et al., 1988) and were shown to recognize epitopes present on carbohydrate side chains of a specific glycoprotein present on the surface of sperm cells (Jones et al., 1990), as well as epitopes present within side chains that have fucose-containing polysaccharides on gametes and on vegetative tissues (Green et al., 1993).

Yariv phenylglycosides are a group of chemicals developed initially as antigens for the purification of sugar-binding proteins (Yariv et al., 1962; 1967), and extensively used for staining, detection, purification and quantification of AGPs (Lamport et al., 2006; 2013; Popper, 2011; Seifert and Roberts, 2007). However, the structures required for Yariv reactivity were not identified until 2013, when it was shown that Yariv specifically binds to β-1,3-galactan chains (longer than five residues), but the protein core is not required for Yariv reactivity (Kitazawa et al., 2013); this makes its application for the study of AGPs controversial. Instead, the entire collection of the AG-3 and AG-4 mAb clades were tested, which include AGP-directed mAbs, e.g. JIM8, JIM13 and MAC207 (Table 2.1), on F. vesiculosus sections, and no positive labelling was observed. Therefore, although F. vesiculosus extracts have the ability to bind to and precipitate with Yariv reagent in radial-gel diffusion assays (S. C. Raimundo and Z. A. Popper; unpublished data), one cannot conclude with certainty that AGPs are present in the F. vesiculosus cell walls to which CCRC-M26 bound for two reasons; 1) the epitope recognized by CCRC-M26 is not yet characterised, and 2) the glycan composition analysis did not indicate the presence of Ara in the extracts. Nonetheless, Glycome Profiling (Fig. 2.2) revealed that the 1 M KOH extract contained the epitope recognized by CCRC-M26 and contains some Gal as shown by monosaccharide composition analysis (Table 2.2). These results suggest that the epitope recognized by CCRC-M26 is part of a galactan different from the galactans recognized by the RG-I/AG mAbs. The localization specificity to the sperm inner wall and egg mesochite suggests that the epitope recognized by CCRC-M26 is part of a molecule that is involved in events preceding sexual reproduction in this species.
Regardless of the epitope recognized by the CCRC-M26 mAb, it's specificity towards an epitope present only in specific cell walls gives new information about *F. vesiculosus* sexual reproduction, particularly the male-female interactions. Specific molecules were detected and proven to be involved in the chemotaxis mediated by proteins produced by the sperm cells that recognize glycosydic compounds present on the oogonium cell wall, both discovered in monoecious (Müller and Gassmann, 1985) and in dioecious Fucales species (Wright *et al.*, 1995a; Wright *et al.*, 1995b). *F. vesiculosus*, the species investigated in this study is dioecious, and reproduction occurs following release of both female and male gametes into the seawater, when the oogonial mesochite and the antheridial inner wall are exposed. Therefore, this study presents a mAb with specificity to male and female gamete cell walls, which can be further applied as a suitable tool for studies of *F. vesiculosus* sexual reproduction.

In addition to the presence of AGP-like epitopes, Estevez *et al.* (2009) and Fernández *et al.* (2010) also reported immunolocalization of epitopes typically present in higher plant mannans in the green seaweed *Codium fragile*. However, green seaweeds share a common, although distant, evolutionary lineage with land plants (Yoon *et al.*, 2004), so co-occurrence of cell wall epitopes in land plants and green algae is perhaps somewhat less surprising than the finding of the shared occurrence of some cell wall localized epitopes in brown seaweeds and land plants. Brown seaweeds and land plants last shared a common ancestor that was concurrent with the origins of the Eukaryotic lineage ~3,500 MYA (Yoon *et al.*, 2004) and the cell wall components of brown seaweeds and land plants are generally considered to be quite different.

### 2.5 Acknowledgments

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3

APPLICATION OF THE LAMP MONOCLONAL ANTIBODY AS A MOLECULAR MARKER FOR THE STUDY OF BROWN SEAWEED (PHAEOPHYCEAE) CELL WALLS
3.1. Introduction

Phaeophyceae, commonly known as brown seaweeds, is an almost entirely marine class belonging to the Stramenopiles (phylum Heterokontophyta) lineage (Patterson, 1999). It includes approximately 1500 species of multicellular photosynthetic organisms (Norton et al., 1996) composed of cells surrounded by a complex carbohydrate-rich wall, features which they share with land plants and with green (Chlorophyta) and red (Rhodophyta) algae (Charrier et al., 2012; Popper et al., 2011). Phylogenetic studies have shown that brown algae are not related to these other lineages, which are collectively grouped as Archaeplastida, and therefore, their cell walls evolved independently (Baldauf, 2008; Cock et al., 2010; Michel et al., 2010a, b; Niklas, 2004; Yoon et al., 2004). However, the complete genome sequence of the filamentous brown alga, and model organism, Ectocarpus siliculosus (Cock et al., 2010) has provided unprecedented insight into the evolutionary processes that resulted not only in multicellularity and cellular differentiation, but also into the uniqueness of the cell wall composition of brown algae (Coelho et al., 2012). These studies show evidences of horizontal gene transfer, to brown algae from ancestral organisms, for example of the genes responsible for biosynthesis of some of their cell wall components (Baurain et al., 2010; Michel et al., 2010b; Stiller et al., 2009). Not surprisingly, although less complex than most vascular plants, brown algae have the highest morphological complexity of the major macroalgal groups (Charrier et al., 2012).

Brown algae are important organisms as they can form extensive beds in intertidal and subtidal zones of rocky shores in the northern hemisphere, playing a key role in coastal ecosystems (Bold and Wynne, 1978; Graham and Wilcox, 2000; Lobban and Harrison, 1994). Moreover, brown algal polymers have pronounced industrial importance as sources of: (1) bioactive compounds, extracted from several species (including Fucus vesiculosus and Laminaria digitata), with applications in food, pharmaceutical and cosmetic industries; (2) phycocolloids including alginates and sulfated polysaccharides (Stengel et al., 2011) important as gelling and viscosifying agents in the food industry and pharmaceutical applications for example alginates have been used in the preparation of efficient gene and drug delivery systems (Zhao et al., 2012); and (3) biofuel applications (Enquist-Newman et al., 2014; Wargacki et al., 2012).

Brown algal cell walls are composed mainly of polysaccharides and lower amounts of phenols, proteins and halide compounds such as iodide (Deniaud-Bouët et al., 2014). Full
characterization of algal cell walls is complicated because their composition can vary depending on different factors such as species, thallus part, developmental stage, season and habitat (Guibet et al., 2008; Kloareg and Quatrano, 1988; Kropf et al., 1988; Lahaye et al., 1994; Lechat et al., 2000; Mabeau and Kloareg, 1987). Nonetheless, much is already known about their composition, and the association between their constituent polymers is becoming clearer (Deniaud-Bouët et al., 2014; Ropartz et al., 2015; Tesson and Charrier, 2014). Cellulose microfibrils account for a smaller fraction of the cell wall than in land plants (Naylor and Russel-Wells, 1934), while alginates and sulfated fucans make up the main portion of the walls (Kloareg and Quatrano, 1988). They have a higher proportion of matrix polymers in relation to the fibrillar ones, sulfated polysaccharides are abundant, and acidic sugars dominate over neutral sugars, hence the uniqueness of brown algal cell walls (Percival, 1979). Alginates are linear acidic polysaccharides composed of β-1,4-D-mannuronic acid (ManA), or M blocks, and its C₅ epimer, α-1,4-L-guluronic acid, (GulA), or G blocks, which are present at different ratios depending on the species, seaweed part and age, and on environmental conditions (Kim and Park, 1985; Kloareg and Quatrano, 1988; Percival, 1979; Smidsrød et al., 1973). The ratios of these uronic acid residues determines the gelling characteristics of alginates; M block-rich alginate does not form gel in the presence of divalent cations, while G block-rich alginate forms the so called egg-box junctions with calcium, which increases the mechanical abilities of gel formation (Haug, 1964). Fucose-containing sulfated polysaccharides (FCSPs) is the term attributed to a complex and not yet fully characterized family of sulfated polysaccharides containing α-L-fucosyl residues, usually known as fucoidan (Ale et al., 2011; Rioux et al., 2007). Algal fucoidans are very complex, heterogeneous, and their structure is species-dependent (Li et al., 2008). They contain a linear backbone of (1→3)-α-L-Fucose (Fuc) or alternating (1→3)-α-L-Fuc and (1→4)-α-L-Fuc, sometimes with branches of (1→2)-α-L-Fuc, with sulfate groups at C-2 or/and C-3, C-4 of the Fuc (Bilan et al., 2006; Chandía and Matsushiro, 2008; Chizhov et al., 1999; Cumashi et al., 2007; Kloareg et al., 1986; Pereira et al., 1999). Residues of D-xylose (Xyl), D-galactose (Gal) and D-mannose (Man) are also present (Côté, 1959; Mabeau et al., 1990; Percival and Ross, 1950). While FCSPs were found to be associated with cellulose microfibrils, and are thought to play an important role in the wall architecture, cross-linking other polysaccharides, this network seems to be embedded within alginates (Deniaud-Bouët et al., 2014).
In situ techniques, such as immunolocalization, are a major breakthrough in cell wall research, because they allow the detailed localization of specific components present in cell walls and provide important information for the study of their structure, organization and dynamics at the cellular level (Domozycz, 2012; Lee et al., 2011; Willats et al., 2000). Biochemical information, which allows the study of detailed composition and structure of wall polymers, is complemented by in situ techniques as they provide information regarding cellular or subcellular localization of cell wall components, which biochemical analysis on extracts cannot provide (Avci et al., 2012; Knox, 1997). Monoclonal antibodies (mAbs) are directed to specific epitopes present in cell wall polymers, and are applied as screening tools for high-throughput characterization of specific cell wall and biomass extracts (Moller et al., 2007; Moller et al., 2008; Moller et al., 2012; Pattathil et al., 2012). Almost all the mAbs currently available were generated towards epitopes present in vascular plant cell walls, but some of them have been shown to bind to epitopes present in freshwater- (Domozycz et al., 2007; Domozycz and Lambiasse, 2009; Domozycz et al., 2011; Estevez et al., 2008) and marine- green algae (Estevez et al., 2009; Fernández et al., 2010). Although mAbs have been generated against epitopes present in brown- (Jones et al., 1988; Vreeland, 1970, 1972) and red- seaweed (Vreeland et al., 1992) cell wall polysaccharides, and localization studies were performed (Green et al., 1993; Vreeland et al., 1984), those mAbs are no longer available to the scientific community. Nonetheless, seaweed research is producing new knowledge that will lead to a better understanding of polysaccharides in the wall architecture as well as the cellular properties of commercially valuable (marine) algae. New mAbs have recently been generated against brown seaweed fucoidans, and different localization patterns associated with different epitope specificities were reported (Torode et al., 2015). Using an alternative approach, mAbs generated against plant cell wall polysaccharides were shown to bind to epitopes present in brown seaweed cell walls, and have thallus-specific localization patterns (Raimundo et al., 2015).

F. vesiculosus (Fucales) and L. digitata (Laminariales) are brown seaweeds common in the intertidal zone of rocky coastlines in the temperate northern hemisphere (Bold and Wynne, 1978). F. vesiculosus, commonly known has bladder wrack, is a mid-littoral species. It has a dichotomously branched thallus (Fig. 3.1A) with a discoid holdfast, a narrow stipe (Fig. 3.1B) and a flattened blade with a central midrib (Fig. 3.1C) and, often, air bladders (Fig. 3.1D). Specialized reproductive organs of this dioecious species, called receptacles, are located at the periphery of the algal thallus (Fig. 3.1E) (Graham and Wilcox, 2000; Lee, 2008; Lobban and Harrison, 1994).
Figure 3.1. Morphology of the brown seaweeds *F. vesiculosus* and *L. digitata*. A. General appearance of *F. vesiculosus*. The thallus is differentiated into holdfast (not shown), stipe (B) and blade (C) with midrib and air bladders (D). The reproductive structures, receptacles, (E) inside which the conceptacles are formed, are found at the thallus tips. F. General appearance of *L. digitata*. The sporophytic thallus is differentiated into holdfast, stipe (G) and blade, with an intercalary meristem located at the junction of the stipe and blade (H). Sori appear as dark patches scattered over the blade (I). *ab*, air bladder; *bl*, blade; *cp*, conceptacle; *hd*, holdfast; *mid*, midrib; *mt*, meristem; *rp*, receptacle; *so*, sori; *st*, stipe. Scale bars: 5 cm (Figure prepared by S. Raimundo).

Laminariales, commonly known as kelps, include the largest known seaweeds, such as *Macrocystis* that can grow up to 50 m long. Within this order, *L. digitata* is a key species,
and forms important extensive beds from the upper sublittoral fringe downwards (Lobban and Harrison, 1994). This species has been implicated in ecologically and environmentally important events, and is known to accumulate iodine from seawater in higher amounts than other seaweeds (Nitschke and Stengel, 2015), playing an important role on iodine emissions, as a mediator of coastal iodine fluctuations (Nitschke et al., 2013). L. digitata is characterized by a diploid sporophytic thallus (Fig. 3.1F) differentiated into a holdfast which anchors the alga to a hard substratum, a smooth and flexible stipe (Fig. 3.1G) and a long, large blade, with intercalary meristem located at the junction of the stipe and blade (Fig. 3.1H) (Graham and Wilcox, 2000; Lobban and Harrison, 1994). Unlike Fucus, which forms reproductive receptacles at the tips of the fronds, the reproductive organs of Laminaria are clustered in patches or sori, usually scattered over the blade (Fig. 3.1I). Sporangia are formed inside these, producing haploid spores that after released into the seawater, will settle and develop into dioecious gametophytes (Schiel and Foster, 2006).

LAminarin Pentaose, or LAMP (LAMP2H12H7; Biosupplies 400-2) is one of the 200+ mAbs, that bind to epitopes present in vascular plant cell wall glycans (Pattathil et al., 2010), which are commercially available and extensively used by the scientific community both for glycome profiling (Moller et al., 2007; Moller et al., 2012; Pattathil et al., 2012), and immunolocalization studies (Avci et al., 2012). Normally applied to plant cell wall studies, this mAb proved to bind strongly to different thallus parts of F. vesiculosus resin-embedded sections (Raimundo et al., 2015). Although this mAb was generated against laminaran extracted from Laminaria digitata, and its epitope is characterized and described (Meikle et al., 1991; Meikle et al., 1994), immunolabelling reports where LAMP mAb is used are almost non-existent for algae.

In this study the LAMP mAb was used to perform in situ immunolocalization in different thallus parts of the two brown seaweeds F. vesiculosus and L. digitata. This rarely used mAb is highlighted as a valuable molecular marker for the study of cell walls of brown seaweeds. Results indicate the presence of (1→3)-β-glucans in brown seaweed cell walls as part of a polymer which is not callose; this is supported by the fact that although the walls clearly label with LAMP they are not stained with the aniline blue fluorochrome that is widely used as a stain for callose. Finally, the presence of callose in the sieve plates of these seaweeds is also shown. Although this was assumed to be the case since the discovery of sieve plates in brown seaweeds (Smith, 1939; Yamanouchi, 1908), it was not yet proven.
3.2. Materials and methods

3.2.1. Algal material

Male and female specimens of the brown macroalga *Fucus vesiculosus* Linnaeus and sporophytes of *Laminaria digitata* (Hudson) J. V. Lamouroux (Phaeophyceae, Heterokontophyta) were collected at Finavarra, Co. Clare (53°09'25"N, 09°06'58"W), on the west coast of Ireland in July 2014, washed with tap water and cleaned of visible epiphytes and/or grazers.

3.2.2. Cell wall extraction

Algal cell walls were extracted based on previously described for vascular plant cell walls (Pattathil et al., 2012). The algal material was ground to a fine powder in liquid nitrogen and the powder was resuspended in 80% (v/v) ethanol, shaken overnight at room temperature, and centrifuged at 3,000 × g for 15 min. The supernatant was discarded and the pellet washed with 80% (v/v) ethanol followed by final washing steps using 100% ethanol and acetone. The final alcohol insoluble residue (AIR) was air-dried in a hood for 24 h and subjected to sequential extractions with increasingly harsh reagents as described below. All extractions were carried out in suspensions at 10 mg mL⁻¹ based on the starting weight of the AIR used, with constant mechanical stirring (200 rpm) at 70°C for 24 h. After each extraction the supernatant was collected by centrifugation at 3,000 × g for 10 min, and stored at 4°C. The AIR was extracted with 50 mM ammonium oxalate (pH 5), 50 mM sodium carbonate (Na₂CO₃) containing 0.5% (w/v) sodium borohydride (NaBH₄) (pH 10), 1 M potassium hydroxide (KOH) containing 1% (w/v) NaBH₄, 4 M KOH containing 1% (w/v) NaBH₄, 100 mM sodium chlorite, and the pelleted residue was treated once more with 4 M KOH containing 1% (w/v) NaBH₄ (KOHPC). The 1 M KOH, 4 M KOH and 4 M KOHPC fractions were neutralized using glacial acetic acid. All extracts were dialyzed (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against three changes of deionized water in 4°C for a total of 48 h, freeze-dried, and weighed. The extracts were stored at room temperature in a desiccator containing a drying agent (silica) prior to further analysis.
3.2.3. Total sugar estimation

Total sugar content of the cell wall extracts estimation was done using the phenol-sulfuric acid method in microplates (Dubois et al., 1956; Masuko et al., 2005). This procedure was performed in order to adjust the total carbohydrate content in all solubilized fractions to an equal amount, prior to ELISA assay. Briefly, the cell wall extracts were dissolved in deionized water at a concentration of 0.2 mg mL⁻¹. 5% (v/v) Phenol was added to each dissolved extract, followed by 18 M sulfuric acid for 20 min in a fume hood. Samples were transferred to 96-well plates (Costar 3598, Corning Inc., NY, USA). Optical density was read at A₄₉₀ nm with a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the Microplate Manager Version 5.2 Build 103 software. A standard curve prepared using D-glucose solutions with different concentrations (5 µg to 50 µg) was used to calculate the glucose equivalents of sugars in the extracts.

3.2.4. Enzyme-Linked Immunosorbent Assay

ELISAs were carried out as described previously (Pattathil et al., 2010). Briefly, 96-well plates (Costar 3598, Corning Inc., NY, USA) were used to load aliquots of diluted seaweed extracts (10 µg/ml in distilled water). Tamarind xyloglucan (Megazyme, Bray, Ireland) probed with CCRC-M104 mAb was used as a positive control, while distilled water was added to the negative control wells. After being left to dry overnight at 37ºC, the plates were incubated with 1% (w/v) nonfat dry milk (instant nonfat dry milk, Publix supermarket, USA) in Tris-buffered saline (2 mM Tris-Base, 8 mM Tris-HCl, pH 7.6 containing 100 mM sodium chloride) for 1 h. The aspiration and washing steps employed an ELx405 VRS Microplate Washer (BioTek Instruments Inc., Winooski, VT, USA); incubations were done at room temperature. After aspirating the blocking solution, 50 µl of undiluted hybridoma supernatant of the mAbs was added (LAMP and BG-1 were diluted 1:70 in 0.1 M Tris-buffered saline) to the wells and incubated for 1 h. After aspiration of the antibody solutions and a 3 times wash step with 0.1% (w/v) nonfat dry milk in Tris-buffered saline, the wells were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rat IgG secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:5,000 in wash buffer. Antibody solutions were aspirated and the wells were washed 5 times with wash buffer. 3,3',5,5'-Tetramethylbenzidine peroxidase substrate (TMB peroxidase substrate kit SK-4400, Vector Laboratories, Inc., Burlingame, CA, USA) was prepared based on the
manufacturer’s instructions, added to each well and incubated for 30 min. 1 N sulfuric acid (H₂SO₄) was added to stop the reaction and the optical density was immediately read as the difference between A₄₅₀ and A₆₅₅ with a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the Microplate Manager Version 5.2 Build 103 software. The reading for the corresponding negative control well was also subtracted from each sample well. ELISA results were presented as a heat map using R-Console software (Team, 2011).

3.2.5. Preparation of algal material for microscopy

Algal material was fixed and embedded as previously described (Coimbra et al., 2007) with minor modifications. Small pieces of the seaweed different thallus parts (2–5 mm) were cut and put in a fixative solution [2% (v/v) paraformaldehyde; 0.25% (v/v) glutaraldehyde; 0.025 M PIPES buffer; 0.001% (v/v) Tween 80; pH 7.2]. The material was fixed for 2 h at room temperature followed by an overnight incubation at 4°C. During the fixation time, a high content of mucilage is released. Therefore the samples must be shaken often, so the fixative can reach every cell within the tissue. Samples were washed with 0.025 M PIPES buffer, pH 7.2, for 10 min, followed by a second wash for 20 min. Dehydration was done in a graded ethanol series [25% (v/v), 35% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), and 3 times with 100%] with 15 min for each grade and infiltration was performed by a sequential immersion in different proportions of 100% ethanol and LR White Resin (Medium grade, Agar Scientific, Cambridge, UK) over 6 days (ethanol/LR White proportion per day was 3:1; 2:1; 1:1; 1:2; 1:3; 0:1). After the final resin change samples were placed either in gelatin capsules or polyethylene molds (Electron Microscopy Sciences, Hatfield, PA, USA) depending on the preferred sample orientation, and fresh LR White was added. The capsules were hermetically sealed while the molds were covered with Aclar embedding film (Electron Microscopy Sciences, Hatfield, PA, USA), and all the samples were placed in an oven at 60°C for 2 days.

3.2.6. Monoclonal antibodies

Monoclonal antibodies generated against different cell wall glycans were obtained as hybridoma cell culture supernatants from stocks at the Complex Carbohydrate Research Center (CCRC). The CCRC, PN, JIM and MAC series are available from CarboSource (www.carbosource.net). LAMP2H12H7 and BG-1 antibodies are available from Biosupplies
(Parkville, Victoria, Australia, www.biosupplies.com.au). A list of the antibodies used, including links to the online database, WallMabDB (www.wallmabdb.net), is provided in Table 2.3 (Chapter 2).

3.2.7. Immunohistochemistry

Immunolabelling was executed as previously reported (Avci et al., 2012). In brief, semi-thin sections (250 nm) were cut with a Leica EM UC6 ultramicrotome (Leica Mikrosysteme GmbH, Vienna, Austria), at room temperature, with a histo-diamond knife (DiATOME, Hatfield, PA, USA). Sections were mounted on glass slides (Fisherbrand Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA) and dried at 50ºC for 1 min. The sections used for future reference regarding anatomical information were stained with toluidine; a drop of 1% (w/v) toluidine blue in 1% (w/v) sodium borate was put on the sections for 20 s (McCully et al., 1980), washed with distilled water and dried. The sections used for immunolabelling were blocked with 3% (w/v) nonfat dry milk (instant nonfat dry milk, Publix) in potassium phosphate buffered saline (KPBS) (0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl) for 30 min, and then incubated for 60 min with LAMP2H12H7 mAb (Biosupplies, Parkville, Victoria, Australia) (concentration used directly from the manufacturer’s instructions). The sections were washed with KPBS, 3 × 5 min, and incubated with goat anti-mouse IgG conjugated with AlexaFluor 488 (Invitrogen Molecular Probes, Eugene, OR, USA) secondary antibody (1:100 in KPBS) for 90 min, after which the sections were washed with KPBS, 2 × 5 min. After a final wash with distilled water (5 min), the sections were mounted with Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA, USA) and covered with a coverslip.

3.2.8. Aniline blue staining

Aniline blue fluorochrome (Biosupplies, Parkville, Victoria, Australia) was prepared according to the manufacturer’s instructions. The stock solution of 0.1 mg mL⁻¹ of the fluorochrome in distilled water was diluted in KPBS (1:3) and semi-thin sections (250 nm) of the resin-embedded algal tissue were incubated for 30 min at 20ºC. After washing with distilled water, the sections were mounted with Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA, USA) and covered with a coverslip.
3.2.9. Enzymatic digestion

Sections on slides were incubated at 40°C with 5U of endo-1,3-β-d-glucanase (purified from Trichoderma sp., Megazyme, Bray, Ireland) in 50 mM acetate buffer (pH 4.5) for 60 min, washed with KPBS and then labelled with LAMP2H12H7 mAb, as described above.

3.2.10. Microscopy

Immunolabelling was observed with a Nikon Eclipse 80i (Nikon Instruments, Melville, NY, USA) microscope equipped with epifluorescence optics. Images were captured with a Nikon DS-Ri1 camera (Nikon Instruments, Melville, NY, USA) using NIS-Elements Basic Research software, and assembled with Adobe Photoshop Elements 11.0 (Adobe Systems, San Jose, CA, USA) software. The aniline blue and the post enzyme-digested immunolabelling imaging was performed with a Nikon Eclipse E400 (Nikon Instruments, Melville, NY, USA) microscope equipped with epifluorescence optics, the images were captured with an Olympus DP72 camera using CellSens software (Olympus Inc., Melville, NY, USA).
3.3. Results

The LAMP mAb was used in the immunolocalization performed on LR White-embedded sections of different thallus parts of *F. vesiculosus* and *L. digitata*. The Glycome Profiling matches the ELISA results previously described for *F. vesiculosus* (Raimundo et al., 2015), and again, no signals were observed for the LAMP mAb in any of the extracts (Fig. 3.2). Additionally, no signals were detected for *L. digitata* extracts. Nonetheless, the immunolocalization showed strong and specific labelling patterns in every tissue examined for both of the species studied. The only cells that did not label, in either of the species, were the meristoderm. The presence of callose in the sieve plates of the conductive cells was also confirmed with aniline blue staining (Fig. 3.9). The pretreatment with an endo-1,3-β-d-glucanase resulted in the digestion of the epitope recognized by the LAMP mAb, therefore confirming the presence of (1,3)-β-glucans in the seaweed cell walls (Fig. 3.10).
Figure 3.2 Glycome profiling of cell wall extracts from whole thallus of *F. vesiculosus* and *L. digitata*. The data is presented as a heat map, resulting from the ELISA screening using 155 plant cell wall glycan-directed monoclonal antibodies. The detailed list of the antibodies used is described on the right panel, colour-coded as groups based on a hierarchical clustering of the cell wall glycans recognized by each antibody group [Table 2.3, Chapter 2 (Pattathil et al., 2010)]. The ELISA signal strength is displayed by a black-yellow scale, with black indicating the absence of binding and yellow showing strongest binding. Labels at the bottom indicate the reagents used in each extraction step for each alga.
3.3.1. *Fucus vesiculosus*

The stipe sections of *F. vesiculosus* showed a generalized positive labelling throughout the medullary cell walls. The longitudinal sections show the elongated primary filament cells (Fig. 3.3B, F) that appear round in cross section (Fig. 3.3A, D). The LAMP mAb labelled all the cell walls of the primary filament cells which are very visible in cross section (Fig. 3.3E). In longitudinal sections the presence of cross-walls at the end of each contiguous medullary primary filament becomes visible; these are sieve elements (Fig. 3.3F), and the LAMP mAb bound particularly strongly to these walls (Fig. 3.3G). The labelling was also strong in the blade and air bladder tissues (Fig. 3.4 A, B). The blade tissues showed a generalized labelling in the cortex cell walls, but not in the meristoderm (Fig. 3.4E, F). The blade interior also showed labelling of the medulla cell walls, some labelling inside the primary filaments, and particularly strong labelling of the thick cell walls of the secondary hyphae (Fig. 3.4G, H). The dense medullary filament cell walls in the midrib region also labelled strongly (Fig. 3.4I, J), and the labelling was also observed in the air bladder cell walls (Fig. 3.4K, L). Both male (Fig. 3.5A) and female (Fig. 3.5B) receptacle tissues showed a general labelling throughout the cortex and medulla cell walls (Fig. 3.5E, F, G, H). However, as observed previously the meristoderm cell walls didn’t label (Fig. 3.5E, F). Generalized labelling was also observed for the conceptacles, where both male and female paraphyses cell walls showed positive labelling (Fig. 3.5J, L). However, while the three walls that surround each (female) oogonium, i.e. the exochite, mesochite and endochite, labelled (Fig. 3.5K, L), only the male antheridia inner cell walls label, while the outer walls did not (Fig. 3.5I, J). Aniline blue fluorochrome staining did not correlate with the immunolabelling patterns observed using the LAMP mAb; no aniline blue staining was observed in the algal tissues using the fluorochrome (Supplementary Figure 3.11).
**Figure 3.3** Sections of *F. vesiculosus* stipe immunolabelled with the LAMP mAb and stained with toluidine blue. **A.** Morphology of a stipe cross section stained with toluidine blue. **B.** Morphology of a stipe longitudinal section stained with toluidine blue. **C.** Negative control in which no primary mAb was used during the immunolabelling. **D.** Cross section of medulla cells stained with toluidine blue. **E.** LAMP labelling showed binding to the medulla cell walls. **F.** Medulla cells in longitudinal section stained with toluidine blue. The arrow points to a sieve plate. **G.** LAMP labelling showed binding to the medulla cell walls. The arrow points to the labelling of a sieve plate. *cor*, Cortex; *med*, medulla.

*Scale bars*: **A, B** = 100 µm, **C–G** = 25 µm.
Figure 3.4 Cross sections of *F. vesiculosus* blade and air bladder immunolabelled with the LAMP mAb and stained with toluidine blue. A. Morphology of a blade section stained with toluidine blue. B. Morphology of the air bladder stained with toluidine blue. C, D. Negative controls of the blade (C) and of the air bladder (D) where no primary mAb was used during the immunolabelling. E. Cortex region stained with toluidine blue. F. LAMP labelling showed binding to the cortex cell walls, but not to the meristoderm. G. Medulla region stained with toluidine blue. H. LAMP labelling showed binding to the medullary hyphae and filament cell walls. I. Medulla region of the midrib stained with toluidine blue. J. LAMP labelling showed binding to the midrib cell walls. K. Air bladder stained with toluidine blue. L. LAMP labelling showed binding to the air bladder cell walls. *cor*, Cortex; *fil*, primary filament; *hy*, secondary hypha; *med*, medulla; *mer*, meristoderm; *mid*, midrib. Scale bars: A, B = 100 µm, C–L = 25 µm.
Figure 3.5 Cross sections of *F. vesiculosus* receptacles immunolabelled with the LAMP mAb and stained with toluidine blue. A. Male receptacle stained with toluidine blue. B. Female receptacle stained with toluidine blue. C, D. Negative controls of the male (C) and female conceptacles (D) where no primary mAb was used during the immunolabelling. E. Cortex region stained with toluidine blue. F. LAMP labelling showed binding to the cortex cell walls, but not to the meristoderm. G. Medulla region stained with toluidine blue. H. LAMP labelling showed binding to the medulla cell walls. I. Part of the male conceptacle stained with toluidine blue. J. LAMP labelling showed binding to the male paraphyses and antheridia inner cell walls. K. Oogonium from a female conceptacle stained with toluidine blue. L. LAMP labelling showed binding to the female paraphyses and oogonia exochite, mesochite and endochite cell walls. *ant*, antheridium; *cor*, cortex; *cp*, conceptacle; *end*, endochite; *exo*, exochite; *inn*, inner wall; *med*, medulla; *mer*, meristoderm; *mes*, mesochite; *oog*, oogonium; *ost*, ostiole; *out*, outer wall; *par*, paraphyse; *sp*, sperm cells. Scale bars: A, B = 100 µm, C–L = 25 µm.
3.3.2. *Laminaria digitata*

Labelling was observed in the holdfast cells of *L. digitata* (Fig. 3.6A), namely the medullary cells (Fig. 3.6C), although the labelling was not contiguous but included some non-labelled areas (Fig. 3.6D). The stipe cells appear round in cross-section (Fig. 7A, D) and little labelling was observed in some thick cross-walls (Fig. 3.7E), the sieve plates. Longitudinal sections allowed distinction between the hyphae and the trumpet hyphae, also known as sieve cells (Fig. 3.7B, F). LAMP labelled specifically the thick cell walls in the trumpet hyphae sieve plates, which are the conducting elements in *L. digitata* (Fig. 3.7G). The blade and the stipe have a similar anatomy (Fig. 3.7B, 8A), although the hyphae are more disperse, with more extracellular mucilage between the cells, and they are not as parallel in the blade as they are in the stipe. Similarly to the stipe, positive labelling was detected in the thick walls of the trumpet hyphae sieve plates (Fig. 3.8E, F), but some labelling was also present inside the hyphae cells (Fig. 3.8F). In contrast, a different labelling pattern was observed on the fertile blade (Fig. 3.8B): the sorus cortex cell walls labelled intensely, but no labelling was observed in the sporangia (Fig. 3.8G, H).

Aniline blue labelling was distinct, intense, and clearly restricted to some of the cross-walls of the medullary hyphae (Fig. 3.9A, B). Autofluorescence, at the wavelength used to observe aniline blue, was visible for the negative control (Fig. 3.9C). However, it was clearly localized to the interior of the cortex cells whereas aniline blue staining was found to be specific to the sieve plates of the trumpet hyphae (Fig. 3.9E, G) typically present as conductive elements, in the medullary tissue (Fig. 3.9D, F).
Figure 3.6 Cross sections of *L. digitata* holdfast immunolabelled with the LAMP mAb and stained with toluidine blue. 

**A.** Morphology of a cross section stained with toluidine blue. 

**B.** Negative control in which no primary mAb was used during the immunolabelling. 

**C.** Medulla cells stained with toluidine blue. 

**D.** LAMP labelling showed binding to the medulla cell walls. *cor*, Cortex; *med*, medulla; *mer*, meristoderm. *Scale bars:* A = 100 µm, B–D = 50 µm.
Figure 3.7 Sections of *L. digitata* stipe immunolabelled with the LAMP mAb and stained with toluidine blue. **A.** Cross section of the medulla region stained with toluidine blue. **B.** Longitudinal section of the medulla region stained with toluidine blue. **C.** Negative control in which no primary mAb was used during the immunolabelling. **D.** Medulla cells in cross section, stained with toluidine blue. Some sieve plates are visible (arrows). **E.** LAMP labelling showed binding to the sieve plates (arrow). **F.** Medulla cells in a longitudinal section stained with toluidine blue. The arrows indicate the hyphae and the trumpet hyphae sieve plates. **G.** LAMP labelling showed specific binding to the trumpet hyphae sieve plates (arrows). A close up of a sieve plate is shown in the box. *hy*, Hypha; *sp*, sieve plate; *th*, trumpet hypha. *Scale bars*: **A, B** = 100 µm, **C–G** = 25 µm.
**Figure 3.8** Cross sections of *L. digitata* blade and fertile blade immunolabelled with the LAMP mAb and stained with toluidine blue. **A.** Morphology of the medulla region stained with toluidine blue. **B.** Morphology of the fertile blade stained with toluidine blue. **C, D.** Negative controls of the blade (C) and of the sorus (D) where no primary mAb was used during the immunolabelling. **E.** Medulla cells of the blade stained with toluidine blue, the arrows point to the sieve plates of the trumpet hyphae. **F.** LAMP labelling showed binding to the sieve plates, and inside the cells (arrows). A close up of the labelling i. of a sieve plate and ii. inside the hyphae cells. **G.** Sorus stained with toluidine blue. **H.** LAMP labelling showed binding to the sorus cortex cell walls, but not to the sporangia. A detail of the labelling is shown. *cor*, Cortex; *hyphae*, medulla; *sp*, sieve plate; *spr*, sporangia; *th*, trumpet hyphae. *Scale bars: A, B = 100 µm, C-H = 25 µm.*
3.3.3. Enzymatic digestion

The pretreatment of sections with endo-1,3-β-D-glucanase resulted in the enzymatic digestion of the epitope recognized by the LAMP mAb, in both species (Fig. 3.10). Labelling was observed in the positive controls, where no enzyme was used; localized to the cell walls in *F. vesiculosa* (Fig. 3.10B) and to the sieve plates in *L. digitata* (Fig. 3.10E). The labelling was abolished when the sections were pretreated with the enzyme, where only autofluorescence inside the cells was observed (Fig. 3.10C, F), similarly to the negative controls (Fig. 3.10A, D).
Figure 3.10 Sections of *F. vesiculosus* (A–C) and *L. digitata* (D–F) blades immunolabelled with the LAMP mAb (B, C, E, F) and after digestion with endo-1,3-β-d-glucanase (C, F). A. *F. vesiculosus* negative control where no primary mAb was used. B. *F. vesiculosus* positive control, where immunolabelling was performed without enzyme treatment; the labelling of the medullary cell walls is visible. C. No labelling was observed in *F. vesiculosus* when sections were preincubated with the enzyme. D. *L. digitata* negative control where no primary mAb was used. E. *L. digitata* positive control, where immunolabelling was performed without enzyme treatment; the labelling of the sieve plates of the trumpet hyphae is visible (arrows). F. No labelling was observed in *L. digitata* when sections were preincubated with the enzyme. The arrow points to an area where a sieve plate is located. Scale bars: 50 µm.
3.4. Discussion

This study describes for the first time the existence, and distribution of the (1→3)-β-glucan epitope throughout the cells of the brown seaweeds *F. vesiculosus* and *L. digitata*, and its intra-thallus distribution, using immunolocalization with the LAMP mAb, confirmed by enzymatic digestion with an endo-(1,3)-β-glucanase. This antibody proved to be an excellent marker for the cell walls in most of the thallus parts studied, showing it to be a valuable tool for *in situ* studies of brown seaweeds. Aniline blue fluorochrome was also used to confirm the presence of callose as part of the sieve plates composition. This fluorochrome also suggested the presence of a novel wall constituent in brown seaweeds because the majority of the cell walls which labelled with LAMP, indicating the presence of (1→3)-β-glucan, were not stained with aniline blue. A cell wall polymer that bears the (1→3)-β-glucan epitope amongst its structure was recognized with the LAMP antibody. This mAb has no cross-reactivity with (1→4)-β-glucan or (1→3, 1→4)-β-glucan (Meikle et al., 1991; Meikle et al., 1994) and no other potential cross-reactivities of this mAb have been reported.

3.4.1. *In situ* versus *in vitro* results

The *in situ* results show strong labelling patterns in all parts of the thalli of *F. vesiculosus* and *L. digitata*. However, the *in vitro* experiments performed on cell wall extracts that resulted in the Glycome Profiling did not correlate with the *in situ* labelling as no signal for the LAMP mAb was observed in the Glycome Profile for either species. These results are an example of how important it is not to neglect both *in vitro* (Glycome Profiling) and *in situ* (immunolocalization) analyses when immunological approaches are used. Glycome Profiling is a powerful tool, where increasingly harsh chemicals are used sequentially to extract specific cell wall compounds. During this process, epitopes that may have previously been masked by their close association with other polymers are accessible, once extracted, and able to be recognized by the mAbs used to perform the ELISAs. However, some epitope structures may also be modified by the chemicals used to perform the extractions, for example acetate groups may be removed; hence negative signals can appear in ELISA despite the fact that positive signals might be expected on the basis that the particular wall component is present *in vivo*. Immunolocalization, though, will show the presence of the intact epitope, unless it is masked by other wall components, or altered during the tissue fixation and embedding processes (Pattathil et al., 2012). In the light of the results presented
here, the epitope recognized by the LAMP mAb appears to either be chemically modified or lost during the extraction procedures, but was retained in the resin sections as observed by the strong labelling observed after the immunolabelling procedure. The Glycome Profiles of the microalga *Penium margaritaceum*, of the green seaweed *Ulva lactuca*, and of the red seaweeds *Chondrus crispus* and *Porphyra* sp. also did not show positive signals for any of the extracts; moreover, immunolabelling with the LAMP mAb was carried out on these green and red seaweeds but no labelling was observed for any of the tissues examined (data not shown).

3.4.2. LAMP epitope distribution — link to callose

LAMP2H12H7 mAb is reported as anti-(1,3)-β-glucan antibody, or simply LAMP. It was generated against commercially available laminaran and extracted from *Laminaria digitata*. It recognizes a (1→3)-β-glucan epitope composed of five consecutive glucose residues (βGlc- (1,3)-βGlc- (1,3)-βGlc- (1,3)-βGlc- Glc); it does not bind to cellulose or to (1→3),(1→4)-β-D-glucan (mixed linkage glucan) (Meikle et al., 1991; Meikle et al., 1994). This epitope is typically found in callose, a polymer characterized by a linear (1→3)-β-glucan with some (1→6)-branches (Aspinall and Kessler, 1957). Callose is present in specific cells in vascular plants including phloem elements, cell plates, plasmodesmata, root hair, and tracheids. Its biosynthesis can also be induced by wounding, pathogen infection, and stress (Kauss, 1996; Stone and Clarke, 1992). The LAMP mAb has been mostly used as a callose-specific antibody in land plants, for immunolocalization studies related to pollen tubes (Ferguson et al., 1998; Geitmann et al., 1995; Mollet et al., 2013), pathogen infected cells (Chowdhury et al., 2014) and tracheid cell walls (Altaner et al., 2010). Similarly to vascular plants, Laminariales also have a system of translocation of photoassimilates (Schmitz and Lobban, 1976); they have sieve elements that consist of cells with perforated terminal end walls, or sieve plates, arranged in long continuous longitudinal rows to form sieve tubes throughout the thallus, except in the holdfast (Sykes, 1908; Van Went et al., 1973). The ends of each sieve plate can be expanded so it resembles a trumpet, hence the name trumpet hyphae. Although some researchers considered the trumpet filaments as artifacts (Van Went and Tammes, 1973), they are usually mentioned in the literature (Graham and Wilcox, 2000; Lee, 2008) and the results show these structures clearly (Fig. 3.7B, F; Fig. 3.8A, E). Fucales similarly have sieve elements in the central medullary filaments with smaller pores, although they don’t form trumpet shaped hyphae (Moss, 1983). They are smaller and not so easily
detected, but were visible in the toluidine blue stained longitudinal sections (Fig. 3.3B, F). In vascular plants, callose is an important constituent of the walls around plasmodesmata (Radford et al., 1998); although not proved yet, the literature mentions the presence of callose in the sieve elements in brown seaweeds (Lee, 2008; Smith, 1939; Van Went et al., 1973), and the results show specific labelling of the sieve plates in both L. digitata (Fig. 3.7E, G; Fig. 3.8F) and F. vesiculosus (Fig. 3.3G).

To compare and/or complement the observations for the distribution of a (1→3)-β-glucan in seaweed cell walls sections were stained with the aniline blue fluorochrome. This fluorochrome is frequently used for the detection of callose (Evans et al., 1984; Stone et al., 1984). The commercially available aniline blue fluorochrome was used because it has high specificity for callose; many other available aniline blue dyes are heterogeneous, variable, and can bind to other compounds (McKay, 1962; Smith and McCully, 1978). Autofluorescence localized to the interior of L. digitata cortex cells was observed at the wavelength used to observe aniline blue staining (Fig. 3.9C); similarly autofluorescence was seen for F. vesiculosus particularly within the receptacle tissues (Supplemental Figure 3.11). So far, the compounds that contribute to this are not identified; nonetheless, the autofluorescence was not associated with the cell walls and did not interfere with the observations. The results were surprising because, with the exception of the sieve plates (Fig. 3.9), aniline blue did not stain any cell walls (Supplemental Figure 3.11). Such results can be explained as a matter of sensitivity, one of the biggest advantages of the use of mAbs. They detect and allow the localization of specific glycan epitopes that are present in extremely low percentages in the wall (Raimundo et al., 2015). On the other hand, a fluorochrome such as aniline blue will recognize a certain compound when it’s present in a certain amount enough to be recognized, which probably is the case of the sieve plates. Other possibilities include the fact that aniline blue binding may be influenced by the interaction of the glycan with the surrounding wall components; for instance, quenching of aniline blue fluorescence by the presence of phenolic compounds was reported (Smith and McCully, 1978). These compounds are known to be present in high levels (~2.5% of total dry weight) in brown seaweeds (Audibert et al., 2010), and are present in the cell walls (Deniaud-Bouët et al., 2014; Schoenwaelder, 2008), therefore, they may also contribute for the inability of the aniline blue to stain the cell walls.

Callose was recently reported to be part of the defense mechanisms of filamentous brown seaweeds with (1,3)-β-glucans deposited in pathogen-host contact areas, post-infection (Tsirigoti et al., 2015). The presence of callose in the sieve plates of the Laminariales is
reported (Schmitz and Srivastava, 1976; Smith, 1939; Yamanouchi, 1908), as well as β-(1,3)-glucans in *Fucus* embryo cell walls (Novotny and Forman, 1975), but not unambiguously proved. After the complete genome sequence of the brown alga *E. siliculosus* (Cock et al., 2010), bioinformatic and phylogenetic approaches allowed the identification of three homologous genes from a family that contains eukaryotic (1,3)-β-glucan synthases that have significant similarities (35% sequence identity) with plant callose synthases (Michel et al., 2010a). This shows that brown seaweeds may, in fact, be able to synthesize callose. In this paper we show the possibility of the existence of callose both in sieve plates of the conductive elements, as well as in other cell walls, although we cannot exclude the possibility that the epitope recognized by the LAMP mAb in the seaweeds may be part of another polysaccharide. The immunolabelling, together with the aniline blue staining and the enzyme digestion prove the presence of the (1,3)-β-glucan epitope; nonetheless, the presence of the epitope may not represent the specific polysaccharide, especially in the case of brown seaweed, that are evolutionarily distantly related to vascular plants, with different cell wall compositions.

3.4.3. LAMP epitope distribution — storage polysaccharides

Phototrophic eukaryotes use different molecular forms of glucans as storage polysaccharides and their intracellular localization varies depending on the lineage of the organism (Suzuki and Suzuki, 2013). Laminaran is a glucan that comprises β-1,3-linked chains with occasional β-1,6-linked branches (Beattie et al., 1961; Read et al., 1996). It is the main storage glucan in brown seaweeds, located in the cytoplasm, and not considered to be a cell wall component (Mian and Percival, 1973; Quatrano and Stevens, 1976). The use of the LAMP antibody outside vascular plants and associated callose labelling is very scarce. There are reports on the use of this mAb in brown seaweeds, namely the application of immunogold labelling to study the ultrastructure and development of the pyrenoids in the chloroplasts of four species (Tanaka et al., 2007), however, the authors did not present evidence for labelling of the cell walls of the species studied. The LAMP antibody has also been used to study storage glucans in chlorarachniophytes (McFadden et al., 1997), a group of small wall-less amoeboïd eukaryotes (Hibberd and Norris, 1984; Ludwig and Gibbs, 1989) that have enclosed a green algal endosymbiont, retaining it as a photosynthetic organelle (McFadden et al., 1994; McFadden et al., 1997). Immunolabelling showed that the storage polysaccharides produced by the endosymbiont, (1→3)-β-glucans, are stored within the vacuole of the host.
cell (McFadden et al., 1997), and nowhere else. In comparison with the existing reports, surprisingly, the results show only weak labelling inside the cells, which was specifically observed inside the medullary primary filaments in *F. vesiculosus* blade (Fig. 3.4H), and inside the medullary hyphae in *L. digitata* blade (Fig. 3.8F). No labelling could be seen in the cell interior of any of the other thallus parts. The results suggest that, although the LAMP mAb was generated towards laminaran, the (1→3)-β-glucan epitope it recognized is predominantly part of a cell wall polymer, rather than a storage sugar.

### 3.4.4. LAMP epitope distribution — cell walls

The results show for the first time the distribution of the (1→3)-β-glucan epitope in brown seaweeds, including the species that was used to generate the LAMP mAb. A broad distribution of the epitope throughout the cell walls of both species studied, with intra-thallus specificity was observed. Immunolocalization of *F. vesiculosus* showed a broad labelling of all the cortex and medulla cell walls, regardless of the thallus part. In this species, two types of filaments are present: the primary filaments, with a larger diameter, and the thicker secondary hyphae (McCully, 1965), that labelled more strongly, and are especially visible in the blade (Fig. 3.4F, H). These hyphae are formed through a process of secondary thickening (Pennington, 1937), where cortical and medullary cells give rise to long, thick-walled hyphae, more slender than the medullary filaments, with very narrow protoplasm, which grow into the mucilage of the medulla; these hyphae cells also stained more intensely than the filaments with toluidine blue (Fig. 3.4E, G). Their thick walls were intensely labelled, indicating that the (1→3)-β-glucan epitope is a major component of its cell walls. The fact that the epitope was present in all walls including in the male and female conceptacle cell walls, indicates that this epitope is part of an important structural wall polymer. The only exception was that the outer walls of the male antheridia that did not label. In contrast, *L. digitata* did not show any labelling of the stipe and blade cell walls, with the exception of the trumpet hyphae sieve plates; this indicates that the epitope recognized is part of a more specialized polymer, in the case of this species. The only exception was the fertile blade, where a strong labelling was observed in the cortex cells directly below the sporangia. It is difficult to interpret the physiological meaning of the changes observed in epitope distribution. However, they may be related to changes in cell function associated with the life cycle. Another possibility is that callose may play a role in contributing to the desiccation
tolerance of *F. vesiculosus*, the intertidal species, in a similar way to recently reported for the freshwater green alga *Klebsormidium flaccidium* (Herburger and Holzinger, 2015).

The LAMP mAb is very specific and therefore rarely mentioned in cell wall localization studies within vascular plants or other organisms, except within callose-related research, and some localization studies on yeast cell walls (Humbel *et al.*, 2001). Based on labelling results, the β-glucan that the LAMP mAb recognizes is part of the cell walls of these seaweeds, rather than a storage polysaccharide, and the labelling patterns observed are unique and widespread. This suggests that the β-glucans are part of a structural component in these brown seaweeds. The possibilities for the application of this mAb are, therefore, broadened to the algal field, as another tool for the localization of cell wall compounds in brown seaweeds.

3.5. Acknowledgments

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3.6. Supplementary Information

Figure 3.11. Cross sections of *F. vesiculosus* blade and female and male receptacles stained with toluidine blue and with aniline blue. No aniline blue staining was observed in *F. vesiculosus*, which co-localized with the LAMP labelling. Autofluorescence in the cells was associated to the cell interior rather than the cell walls. The compounds that cause the autofluorescence are not yet identified. *Scale bars*: 50 µm.
IMMUNOLOCALIZATION OF ALGINATES IN BROWN SEAWEEDS (PHAEOPHYCEAE) REVEALED BY THE MONOCLONAL ANTIBODY LM7
4.1. Introduction

Brown seaweeds (Phaeophyceae) form an almost entirely marine class within the Stramenopiles (phylum Heterokontophyta) independent lineage (Baldauf, 2008; Niklas, 2004; Patterson, 1999; Yoon et al., 2004). Many brown seaweed species play a central role in coastal ecosystem dynamics especially in extensive subtidal and intertidal zones of rocky shores of the northern hemisphere (Bold and Wynne, 1978; Graham and Wilcox, 2000; Lobban and Harrison, 1994). For example, *Fucus vesiculosus* (Fucales), commonly known as bladder wrack, is a mid-littoral species and *Laminaria digitata* (Laminariales), or kelp, inhabits the lower intertidal zones (Bold and Wynne, 1978). Both are very common inhabitants of the Irish coast. Apart from their ecological significance, they are also major sources of commercially important polysaccharides derived from their cell walls. Brown algae have structurally complex cell walls primarily composed of polysaccharides (Charrier et al., 2012; Popper et al., 2011) that are very different than the cell walls of land plants, green algae and red algae. Macroalgal polysaccharides, including alginates extracted from brown seaweeds, and agars and carrageenans extracted from red seaweeds, are the most economically important products extracted from algae. Also called phycocolloids, these polysaccharides are primarily exploited for their gelling, thickening and emulsifying properties which find many diverse and essential applications in the food, pharmaceutical and cosmetics industries (Cardozo et al., 2007; Pulz and Gross, 2004; Stengel et al., 2011). For example, alginates are used in biomedical sciences for pharmaceutical applications such as controlled-release drugs and efficient gene and drug delivery systems (Zhao et al., 2012). As important, they are being investigated for possible starting materials for biofuels (Enquist-Newman et al., 2014; Wargacki et al., 2012).

Brown seaweed cell walls are composed mostly of polysaccharides and lower percentages of phenols, proteins and halide compounds, such as iodine (Deniaud-Bouët et al., 2014). However, the elucidation of the precise composition, linkages between polymers and the deposition of polysaccharides in the wall, is a challenging task because differences have been consistently observed depending on the species, on the seaweed part, maturity, season or habitat (Guibet et al., 2008; Kloareg and Quatran, 1988; Kropf et al., 1988; Lahaye et al., 1994; Lechat et al., 2000; Mabeau and Kloareg, 1987; Stengel et al., 2011). New techniques, however, have shed light concerning the organization of brown algal cell walls (Deniaud-Bouët et al., 2014; Ropartz et al., 2015). The cell walls of brown algae are largely composed of alginates and sulfated fucoidans, (Kloareg and Quatran, 1988), with cellulose accounting
for a smaller portion (Naylor and Russel-Wells, 1934); the matrix and acidic sugars are more abundant than fibrillar and neutral sugars, respectively (Percival, 1979). They are basically the only Eukaryotes that have alginites, some of the biosynthetic pathways acquired through horizontal gene transfer from an Actinobacteria (Michel et al., 2010). Fucoidans are also unique to brown algae, except for some related biopolymers found in marine invertebrates (Usov and Bilan, 2009). From a structural standpoint, fucoidans are thought to have an important role in the wall architecture, cross-linking other polysaccharides, and were found to be connected to cellulose microfibrils; this network appears to be embedded within alginites (Deniaud-Bouët et al., 2014). Fucoidans are a very heterogeneous and highly complex family of sulfated polysaccharides named fucose-containing sulfated polysaccharides (FCSPs) that are investigated in a diversity of potential bioactive activities, such as anticoagulant, antiviral, antitumor, anti-inflammatory, antiviral and antioxidant (Ale et al., 2011; Li et al., 2008; Usov and Bilan, 2009), and also correlated to the adaptation to saline environmental conditions (Aquino et al., 2011). FCSPs have structural backbones that varies between species and may contain only L-fucose (Fuc) residues with sulfate groups (“fucan”) positioned on C-2, C-3 and/or C-4, or may have different monosaccharide residues such as D-xylose (Xyl), D-Galactose (Gal) and D-mannose (Man), uronic acids, and less sulfate (Rabanal et al., 2014). High proportions of D-gal (“galactofucan” or “fucogalactan”) were detected in some Laminaria species, although their structure is still elusive (Usov and Bilan, 2009). *Fucus vesiculosus* fucoidan backbone is described as α-1,3-L-fucopyranose residues (Patankar et al., 1993) and other reports describe a backbone of alternating α-1,3- and α-1,4- L-fucopyranose residues (Chevolot et al., 2001; Usov and Bilan, 2009). Alginates are made of linear blocks of the uronic acid residues β-1,4-D-mannuronic acid (M blocks), and the C₅ epimer, α-1,4-L-guluronic acid (G blocks). The ratios of G: M blocks differ depending on factors such as species, part of the alga, age, and environmental conditions (Kim and Park, 1985; Kloareg and Quatrano, 1988; Percival, 1979; Smidsrød et al., 1973) and dictate the gelling characteristics of the alginates. For example alginates rich in M blocks do not form gels in the presence of divalent cations, while G block-rich alginates form the so called egg-box structures in the presence of calcium, increasing the mechanical abilities of gel formation (Haug, 1964). It is important for industry that the differences in the occurrence of these traits and the basis for them is understood because although most brown seaweeds are possible sources of alginate their properties, and suitability for particular applications, differs from one species to another (McHugh, 1987).
Immunolocalization allows the detailed in situ localization of specific components present in cell walls and provides important information about structure, organization and dynamics within the cell (Domozych, 2012; Lee et al., 2011; Willats et al., 2000a). It complements the information derived from biochemical studies, which gives information regarding the composition and linkages of the polymers, but neglects cellular or subcellular localization of components (Avci et al., 2012; Knox, 1997). Immunolabelling approaches apply the use of monoclonal antibodies (mAbs) that recognize specific epitopes within cell wall polymers and have been used in high-throughput characterization of specific cell wall and biomass extracts (Moller et al., 2007; Moller et al., 2008; Moller et al., 2012; Pattathil et al., 2012). The mAbs that were initially generated against seaweed cell wall polysaccharides (Jones et al., 1988; Vreeland, 1970, 1972; Vreeland et al., 1992) are not available for the scientific community, but new mAbs have recently been generated against brown seaweed fucoidans (Torode et al., 2015). Nonetheless, plant wall-directed mAbs have been applied in freshwater- (Domozych et al., 2007; Domozych and Lambiasse, 2009; Domozych et al., 2011; Estevez et al., 2008) and marine- green algae (Estevez et al., 2009; Fernández et al., 2010). They were also shown to bind to epitopes present in brown seaweed cell walls (Raimundo et al., 2015).

LM7 is a mAb that recognizes non-blockwise unesterified galacturonic acid (GalA) residues in the linear homogalacturonan (HG) pectic polysaccharides present in vascular plant cell wall glycans (Clausen et al., 2003; Willats et al., 2001). It is commonly used for vascular plant cell wall research together with other mAbs to localize HG epitopes with different patterns of methylesterification (Christiaens et al., 2011; Jarvis et al., 2003). The use of the LM7 mAb in immunolocalization studies in algal research is very scarce, except for its application in green algae (Domozych et al., 2007), and the recent report where the reaction of this mAb to seaweed alginates is reported (Torode et al., 2015).

Recently, a new class of non-immunological molecular probes has been reported for the study of plant cell wall HG. These chitosan oligosaccharides COS488 are highly sensitive reciprocal oligosaccharide-based probes for chitosan and for demethylesterified HG that present some advantages in relation to the use of mAbs: 1) the experimental protocol involves a simple and fast one-step labelling; 2) smaller probe than compared to mAbs, which allow an excellent tissue penetration and high resolution; 3) the direct conjugation to a variety of tags facilitate dual labelling and time course experiment; 4) these probes were generated for a pH compatible with plant growth conditions (~5.7), while mAbs are
generated within the mammalian physiological pH 7, the probe can be used in live tissues (Mravec et al., 2014).

The LM7 mAb was used in this study to inform in situ immunogold labelling of different thallus parts of two representative brown seaweeds *Fucus vesiculosus* and *Laminaria digitata*. The importance of this mAb is highlighted and, in the light of the results combined with recent reports, the possibility of LM7 cross-reacting with alginates is discussed, and therefore the localization results pointing to the distribution of alginates, presenting LM7 as a valuable molecular marker for in situ studies of brown seaweed alginates. COS488 probe was also tested in some thallus parts (courtesy of Professor David Domozych) and its potential as a useful probe for the localization of uronic acids is discussed.
4.2. Materials and methods

4.2.1. Algal material

Specimens of the brown macroalga *Fucus vesiculosus* L. and sporophytes of *Laminaria digitata* (Hudson) J. V. Lamouroux (Phaeophyceae, Heterokontophyta) were collected at Finavarra, Co. Clare (53°09'25"N, 09°06'58"W), on the west coast of Ireland, washed with water and cleaned of visible epiphytes and/or grazers.

4.2.2. Preparation of algal material for light microscopy

Freshly collected algal material was prepared as described previously (see Chapters 2 and 3 for details). Briefly, small pieces (2–4 mm) of the seaweeds were fixed for 2 h at room temperature and overnight at 4°C. The samples were washed with 0.025 M PIPES buffer and dehydrated with a graded ethanol series. Infiltration was performed over 6 days with different proportions of 100% ethanol and LR White Resin (Medium grade, Agar Scientific, Cambridge, UK). Samples were placed either in gelatin capsules or polyethylene molds (Electron Microscopy Sciences, Hatfield, PA, USA). The capsules were hermetically sealed while the molds were covered with Aclar embedding film (Electron Microscopy Sciences, Hatfield, PA, USA), and all the samples were polymerized in an oven at 60°C for 2 days.

4.2.3. Preparation of algal material for electron microscopy

Freshly collected algal material was cut into small pieces (2–4 mm) and placed in a fixative solution of 1% (v/v) glutaraldehyde in filtered seawater for 60 min. After a 3 × wash with filtered seawater for 5 min each, samples were incubated for 2 h in a post-fixative solution of 1% (v/v) osmium tetroxide in filtered seawater. Samples were kept on ice during all the procedures. After a second washing step performed as described above, the samples were brought to room temperature. Dehydration, embedding and polymerization steps were performed in the same way as for the samples prepared for light microscopy.
4.2.4. Light microscopy — immunohistochemistry

Sections used for light microscopy were prepared as described in Chapters 2 and 3. Briefly, semi-thin sections (250 nm) were cut at room temperature, mounted on glass slides and dried at 50°C for 1 min. Sections were stained with toluidine for future anatomical reference; a drop of 1% (w/v) toluidine blue in 1% (w/v) sodium borate was put on the sections for 20 s (McCully et al., 1980), washed with distilled water and dried again. The sections used for immunolabelling were blocked with 3% (w/v) nonfat dry milk (instant nonfat dry milk, Publix) in potassium phosphate buffered saline (KPBS) (0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl) for 30 min, and then incubated for 60 min with LM7 mAb (Plant Probes, Leed, UK) diluted 1:10 in KPBS. The sections were washed with KPBS, 3 × 5 min, and incubated with goat anti-rat IgG conjugated with AlexaFluor 488 (Invitrogen Molecular Probes, Eugene, OR, USA) secondary antibody (1:100 in KPBS) for 90 min, after which the sections were washed with KPBS, 2 × 5 min. After a final wash with distilled water (5 min), the sections were mounted with Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA, USA) and covered with a coverslip.

4.2.5. COS⁴⁸⁸ labelling

Sections obtained as described above were labelled with a 1:1000 (v/v) dilution of COS probe coupled to Alexa Fluor 488 (COS⁴⁸⁸) (courtesy of Jozef Mravec, University of Copenhagen) in 0.025 M MES buffer, pH 5.8 for 90 min. The sections were then washed with MES buffer and observed with LM. This work was performed by Professor David Domozych (Skidmore College, Saratoga Springs, NY, USA).

4.2.6. Transmission electron microscopy — immunogold labelling

Sample preparation for transmission electron microscopy (TEM) was performed as described previously (Domozych, 2007) with some modifications. Thin sections (60–80 nm) of osmicated algal material were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Bannockburn, IL, USA), at room temperature, with a histo-diamond knife (DiATOME, Hatfield, PA, USA) and collected on formvar-coated (EMS, Hatfield, PA, USA) nickel grids. Sections were treated for 5 min with 5% (v/v) hydrogen peroxide, washed with dH₂O, treated for 20 min with 0.26% (w/v) ammonium chloride and re-washed with dH₂O. Sections were then blocked with 1% (w/v) instant nonfat milk (Carnation) in phosphate
buffer saline (PBS) for 30 min at room temperature. After washing with \(d\)H\(_2\)O, the sections were incubated overnight at 4ºC with a 1:10 dilution (in PBS) of the LM7 primary mAb. After a wash with \(d\)H\(_2\)O, sections were re-blocked for 30 min, washed with \(d\)H\(_2\)O, and incubated for 90 min at 37ºC with a 1:80 dilution (in PBS) EM goat anti-rat IgG secondary antibody conjugated with 15 nm gold particles (Ted Pella, Inc., Redding, CA, USA). Sections were washed with \(d\)H\(_2\)O and stained with conventional uranyl acetate/lead citrate: grids were incubated with 1\% (w/v) uranyl acetate for 15 min, washed with \(d\)H\(_2\)O, incubated with 1\% (w/v) lead citrate for 2–3 min and washed again with \(d\)H\(_2\)O.

4.2.7. Alginate lyase digestion

Sections on slides for light microscopy or nickel grids for TEM were incubated at room temperature in PBS for 30 min and then washed with \(d\)H\(_2\)O. After incubation at room temperature with alginate lyase (Megazyme; 5U, i.e., 5 \(\mu\)l/ml of the original stock) in PBS for 60 min, the sections were washed with \(d\)H\(_2\)O and then labelled with LM7 as previously described (see Chapters 2 and 3 for immunolocalization protocol for light microscopy). This work was kindly performed by Professor David Domozych (Skidmore College, Saratoga Springs, NY, USA).

4.2.8. Monoclonal antibody

LM7 mAb was purchased from Plant Probes (University of Leeds, Leeds, UK) (www.plantprobes.net/index.php).

4.2.9. Microscopy

Immunolabelling was observed with a Nikon Eclipse 80i (Nikon Instruments, Melville, NY, USA) microscope equipped with epifluorescence optics. Images were captured with a Nikon DS-Ri1 camera (Nikon Instruments, Melville, NY, USA) using NIS-Elements Basic Research software, and assembled with Adobe Photoshop Elements 11.0 (Adobe Systems, San Jose, CA, USA) software. TEM micrographs were taken with a Zeiss-Libra® 120 transmission electron microscope (Carl Zeiss SMT Inc., Thornwood, NY, USA). Images were captured with a Cantega G2 camera (Olympus Soft Imaging Solutions GmbH, Münster Germany), using iTEM 5.1 TEM Imaging Platform.
4.3. Results

The LM7 mAb was used to perform immunogold localization studies on LR White-embedded sections of different thallus parts of *L. digitata* and *F. vesiculosus*. The epitope this mAb recognizes was localized throughout the cell walls of the different vegetative tissues in both species. Interestingly, no labelling was observed in any of the reproductive structures. There was a broad distribution in the extracellular matrix of the medulla areas particularly in the blades of both species, where the tissues are known to accumulate high amounts of mucilage.

4.3.1. *Laminaria digitata*

Positive labelling was observed in the holdfast and stipe (Fig. 4.1A, 4.2A) medullary cell walls in resin-embedded cross sections of *L. digitata*. The presence of gold particles was detected in the transmission electron micrographs, throughout the walls of both thallus parts (Fig. 1B, 2B). However, some differences were observed, namely in the holdfast, where the labelling was located in the cell junctions (Fig. 4.1C, D) rather than in the fibrillar area, while the labelling in the stipe was located less in the cell junctions and more in the fibrillar area, and also in the cross-walls (Fig. 4.2C, D). Details of the plasmodesmata showed the labelling flanking these structures (Fig. 4.2E) but not within them. The blade tissues are known to have extensive extracellular matrixes surrounding the medullary cells that are easily observed with toluidine blue staining (Fig. 4.3A, G). LM7-bound immunogold particles were found to be spread throughout these areas (Fig. 4.3B), showing a general distribution both in areas with a smooth appearance and in areas where some microstructures were observed that closely resemble those previously described for alginate microstructures (Schuster et al., 2014) (Fig. 4.3C, D). The labelling of the fertile blade (Fig. 4.3E, G) showed the presence of gold particles in the extracellular matrix of the medulla area (Fig. 4.3H), while no labelling was detected in or between the sporangia structures of the sori (Fig. 4.3F).
Figure 4.1 Transmission electron micrographs of *L. digitata* holdfast cross sections immunogold labelled with LM7 antibody. **A.** Ultrastructure of the medulla cells. **B.** LM7 labelling showed binding to the medullary cell walls. **C.** LM7 labelled mostly the cell junction areas (arrows). **D.** Detail of the gold particles showing the labelling of the cell junctions. The arrows point to the area labelled. IS, intracellular space; MdCJ, medulla cell junction; PM, plasma membrane. Scale bars: **A** = 5 µm, **B** = 1 µm, **C, D** = 500 nm.
Figure 4.2 Transmission electron micrographs of *L. digitata* stipe cross sections immunogold labelled with LM7 antibody. **A.** Ultrastructure of the medulla cells. **B.** LM7 labelling showed binding to the medullary cell walls. **C.** LM7 labelled areas in the most fibrillar part of the cell walls (arrows). No labelling of the cell junctions was observed. **D.** Labelling was observed in the thinner walls between cells. **E.** Detail of a plasmodesmata area showing the labelling flanking the plasmodesmata. The arrows point to areas labelled. IS, intracellular space; MdCJ, medulla cell junction; Pd, plasmodesmata; PM, plasma membrane. *Scale bars: A = 5 \mu m, B = 1 \mu m, C–E = 500 nm.*
Figure 4.3 Cross sections of *L. digitata* blade and fertile blade stained with toluidine blue (A, E, G) and transmission electron micrographs immunogold labelled with LM7 antibody (B, C, D, F, H). A. Medulla area of the blade stained with toluidine blue. B. LM7 showed extensive labelling of the extracellular matrix of the medulla. C, D. Close up of the immunogold particles spread throughout the matrix area where some parts have a smooth texture (C) and others have a characteristic matrix texture (D). E. Sporangia in the fertile blade stained with toluidine blue. F. LM7 didn’t label neither the sporangia nor the spaces between them. G. Medulla area of the sorus, stained with toluidine blue. H. LM7 showed labelling of the extracellular matrix of the medulla of the fertile blade. The arrows point to areas labelled. ExtM, extracellular matrix; Spr, sporangia. *Scale bars:* A, E, G = 10 µm, B = 1 µm, C, D, F, H = 500 nm.
4.3.2. *Fucus vesiculosus*

The extracellular matrix of the blade of *F. vesiculosus* (Fig. 4.5A) showed generalized and extensive labelling throughout the medulla area with both fluorescence microscopy (Fig. 4.4A) and TEM (Fig. 4.5), similarly to that observed for *L. digitata* (Fig. 4.5B, C). This shows that the epitope recognized by LM7 is also common in the extracellular matrix of this species. However, in contrast with *L. digitata*, no labelling was observed in the stipe of *F. vesiculosus* (Fig. 4.5D, E). Positive labelling was observed in the receptacles, with extensive labelling of the extracellular matrix in the medulla area with both fluorescence (4.4B) and TEM immunogold labelling (Fig. 4.6A, B); gold particles were also detected in the cross-walls (Fig. 4.6D). Once again, plasmodesmata structures did not label (Fig. 4.6E).

Concerning the conceptacle structures, positive labelling was observed in the walls and extracellular matrix of the cells enclosing the conceptacles (Fig. 4.6C), but no labelling was detected inside the male and female conceptacles, and no labelling was observed either in the antheridia or in the oogonia (Fig. 4.6F, G). Immunofluorescence procedures were also performed in *F. vesiculosus*, and confirm the fluorescence patterns across the extracellular matrix in the tissues, consistent with the immunogold results.

![Figure 4.4](image)

**Figure 4.4** Cross sections of *F. vesiculosus* blade (A) and female receptacle (B) immunolabelled with the LM7 mAb. Labelling is extended to the mucilaginous extracellular matrix throughout the vegetative tissues of the blade (A) and the receptacles (B). C. Negative control. *Scale bars*: 25 µm.
Figure 4.5 Cross sections of *F. vesiculosus* blade and stipe stained with toluidine blue (A, D) and transmission electron micrographs immunogold labelled with LM7 antibody (B, C, E). A. Medulla area of the blade stained with toluidine blue. B. LM7 showed extensive labelling of the extracellular matrix of the medulla. C. Close up of the extracellular matrix, showing points of labelling (arrows). D. Medulla area of the stipe stained with toluidine blue. E. LM7 was not observed to label the stipe. ExtM, extracellular matrix; IS, intracellular space. Scale bars: A, D = 10 µm, B = 1 µm, C, E = 500 nm.
Figure 4.6 Transmission electron micrographs of *F. vesiculosus* receptacle cross-sections immunogold labelled with LM7 antibody. **A.** Ultrastructure of the female receptacle medulla area. **B.** LM7 labelling showed binding to the extracellular matrix of the medulla. **C.** LM7 labelled the walls of the cells surrounding the conceptacles. **D.** Close up of the LM7 labelling of the cross-walls. **E.** A plasmodesmata area detail, no LM7 labelling is observed. **F.** LM7 did not label inside the female conceptacle. **G.** LM7 did not label inside the male conceptacle. The arrows point to areas labelled. Ant, antheridium; Cpln, conceptacle interior; ExtM, extracellular matrix; IS, intracellular space; Oog, oogonium; Pd, plasmodesmata. *Scale bars:* **C, E** = 1 µm, **A, B, D, F, G** = 500 nm.
4.3.3. **COS\textsuperscript{488} labelling**

Labelling patterns observed with the chitosan oligosaccharide probe COS\textsuperscript{488} were the same as observed for LM7 (Fig. 4.7, courtesy of Professor David Domozych). The extracellular matrix showed a generalized labelling in both *F. vesiculosus* (Fig. 4.7A) and *L. digitata* (Fig. 4.7B) blades.

**Figure 4.7** Cross sections of *F. vesiculosus* and *L. digitata* blades labelled with the chitosan oligosaccharide probe COS488 (A-C), and TEM micrographs immunogold labelled with the LM7 mAb after digestion with alginate lyase (D, E). COS488 labelling is extended to the mucilaginous extracellular matrix of both *F. vesiculosus* (A) and *L. digitata* (B) blades. C. Negative control. No labelling was observed in either *F. vesiculosus* (D) or *L. digitata* (E) when sections were preincubated with alginate lyase. Images are courtesy of Professor David Domozych. *Scale bars*: A-C =50 µm, D-E = 1 µm.

4.3.4. **Alginate lyase treatment**

No labelling was observed after treatment with alginate lyase and subsequent labelling with LM7, either for *F. vesiculosus* (Fig. 4.7D) or *L. digitata* (Fig. 4.7E) by light microscopy or TEM (personal communication, Professor David Domozych).
4.4. Discussion

This study describes the distribution of the epitope recognized by the LM7 mAb throughout the tissues of the brown seaweeds *F. vesiculosus* and *L. digitata* LR-White embedded sections, and its intra-thallus distribution, using immunogold localization. This antibody proved to be an excellent probe, not only within the cell walls, but also for the extracellular matrix in the seaweed parts studied. The possibility of LM7 cross-linking to alginates is discussed, and therefore the localization results indicate the alginates distribution, presenting LM7 as a valuable tool for *in situ* studies of brown seaweed alginates.

4.4.1. LM7 — Immunolocalization insights

LM7 is a mAb that binds to non-blockwise partially methyl-esterified HG (Willats *et al.*, 2001) that recognizes an epitope of four unesterified GalA residues with adjacent or flanking methyl-esterified GalA residues (Clausen *et al.*, 2003). HG is a linear polymer of (1→4)-α-GalA that is partially methyl esterified at the C-6 carboxyl and can also be O-acetylated at C-2 or C-3 (O’Neill *et al.*, 1990). Pectins have a prominent importance as industrial plant-based food product component, and HG is the most abundant pectic polysaccharide (~65%) (Mohnen, 2008), hence, several immunological probes towards pectic HG are currently available (Vandevenne *et al.*, 2012) that can be used to access different structural properties of these polysaccharides. Together with LM7, other mAbs, namely JIM5, JIM7 (Clausen *et al.*, 2003; Knox *et al.*, 1990), PAM1 (Willats *et al.*, 1999; Willats *et al.*, 2000b), 2F4 (Liners *et al.*, 1989; Liners *et al.*, 1992), LM18, LM19 and LM20 (Verhertbruggen *et al.*, 2009) that are currently available (PlantProbes; www.plantprobes.net), recognize portions of HG with different patterns of methyl esterification; JIM5 and JIM7, along with CCRC-M131, CCRC-M38 and JIM136 (Carbosource, www.carbosource.net), are part of the collection of mAbs currently used in high-throughput Glycome Profiling of cell wall extracts (Pattathil *et al.*, 2010). In this study, immunolabelling was also performed with JIM5, JIM7, CCRC-M131, CCRC-M38 and JIM136 mAbs in *F. vesiculosus* section, but no positive results were observed (data not shown). LM7 was the only mAb of the known anti-HG mAbs tested that showed positive labelling, although in a pattern never observed before. The LM7 pectic HG epitope is the only cell wall polysaccharide epitope to date that has been detected to be exclusively spatially regulated, with a consistent location within cell walls and intercellular matrices of land plant
tissues and species; with specific labelling patterns restricted to discrete regions at the corners of the intercellular spaces, the points between adherent and separated cell walls (Willats et al., 2001). Hence, LM7 is useful in the localization of different patterns and extents of methyl-esterification of HG that are key contributors to both matrix properties, responsible for intercellular adhesion and cell wall separation in vascular plants (Jarvis et al., 2003; Willats and Knox, 2003), also counteracting radial swelling stress induced, e.g. by heavy metals (Douchiche et al., 2010). Reports usually focus more on the other HG-directed mAbs, rather than on LM7, not only due to the spatial specificity of its epitope, but also because unlike its relatives, LM7 binding to plant tissues, including ferns, is often not apparent in vitro or in situ (Blake et al., 2008; Christiaens et al., 2011; Leroux et al., 2007; Leroux et al., 2015). A few studies on green algae, for example *Micrasterias*, indicate a lack of labelling (Eder and Lütz-Meindl, 2008) whereas LM7 has been reported to label specific epidermal areas in *Chara corallina* (Domozych et al., 2010), and in expanding polar regions in *Penium margaritaceum* (Domozych et al., 2007). Hence, the results show unique labelling patterns for LM7 because: 1) the labelling extended throughout the cell walls of the holdfast (Fig. 4.1) and stipe (Fig. 4.2), and rather than being restricted to the cell corners was found across the walls, including the thin cross-walls (Fig. 4.2.D), 2) the labelling was spread along the extracellular matrices of the medullary areas on the blade (Fig. 4.3, 4.5, 4.6), where the cells are spread across the tissue, and separated from each other by mucilage accumulation. The only walls LM7 did not label were the fertile tissues, sporangia in *L. digitata* (Fig. 4.3E, F), and the conceptacles in *F. vesiculosus* (Fig. 4.6F, G), although the vegetative tissue surrounding these structures was labelled (Fig. 4.3H, 4.6A).
4.4.2. COS\textsuperscript{488} labelling

Distinct labelling with the chitosan oligosaccharide probe COS\textsuperscript{488} was observed, that had a similar pattern than LM7. The COS probe was generated from a chitosan oligomer of 7 amino sugars, meant to label contiguous zones of negatively charges galacturonic acid sequences in the pectic HG (Mravec et al., 2014). This means that the probe will bind to any charged oligosaccharide in the wall with at least seven non-esterified uronic acids. The close similarity in size and structure of uronic acids that make up either the pectic HG in plants or the matrix alginites of brown seaweeds suggests that the probe should label both. It is, therefore, very likely that this probe recognizes sequences of alginites in the cell walls of these seaweeds. Therefore, this probe can also be applied not only in the localization of demethylesterified HG in and plants, but also of uronic acids in brown algae.

4.4.3. LM7 — A probe for alginites

The results presented in this study raise new questions related to exactly what compound LM7 is recognizes in brown seaweeds because Phaeophyceae cell walls are not known to have HG per se, nor do they contain the GalA residues, which are part of the epitope recognized by LM7. The labelling observed was strong and widespread throughout different thallus parts, which suggests the possibility of LM7 cross-reactivity to a common algal polymer. The cross-reactivity of mAbs generated against brown seaweed alginites with pectins was previously reported, and because both HG and alginites can form egg-box like-structures in the presence of calcium ions, the possibility that the reaction of an anti-alginate mAb with pectin may be caused by the specificity of the mAb towards the calcium salt rather than the chemistry of the uronic acid involved was suggested (Vreeland and Laetsch, 1985). A comparative analysis of mAbs binding to brown algal polysaccharides confirmed that LM7 binds to alginites, although which structural part of the polymer it recognizes remains elusive (Torode et al., 2015). The cross-reactivity of LM7 with alginites confirms that HG and alginites, which differ significantly in structural sugar residues and respective linkages, have at least one structural feature in common. Some of the TEM micrographs showed LM7-labelling in areas that resemble the typical microstructures observed in alginites (Schuster et al., 2014), these were particularly abundant in L. digitata (Fig. 4.3B, D). The same structure was also observed in F. vesiculosus receptacles, where mucilage is also abundant (data not shown). The tissue fixation, dehydration and embedding preparation steps may disrupt the fine alginate microstructure, but it still preserved the alginate structure that LM7 is able to
bind to. Moreover, the pretreatment of the sections with alginate lyase resulted in the lack of labelling (Fig. 4.7D, E). Alginate lyase is an enzyme that catalyzes the β-elimination cleavage of alginate (Wong et al., 2000). This evidence shows that, in fact, LM7 recognizes an epitope present within the alginate structure. Although it remains to be determined what specific structural feature of the seaweed polysaccharide LM7 is recognizing, the results, supported by the cross-reactivity described for this mAb, describe distribution patterns of alginates in brown seaweeds, abundant in the mucilaginous extracellular matrix. LM7 has a unique and not yet described labelling pattern, and can therefore be added to the already reported vascular plant cell wall glycan-directed mAbs that bind to brown seaweed cell walls (Raimundo et al., 2015), as a valuable probe for alginates.

4.5. Acknowledgements

I thank Professor David Domozych (Skidmore College, Saratoga Springs, NY, USA) for providing the facilities and equipment, including the TEM facilities, and for performing the enzymatic digestion, and the COS488 labelling. Thank you also to Dr. Jozef Mravec for providing the COS488 probe (University of Copenhagen, Denmark).
5

Vreeland antibodies:
Bringing the first monoclonal antibodies
generated against seaweeds back to science
5.1. Introduction

Phycocolloids is a term which refers to high molecular weight polysaccharides extracted from both freshwater and marine algae (Cardozo et al., 2007). The most important phycocolloids with commercial significance are alginates, extracted mainly from brown (class Phaeophyceae), and carrageenans and agars, extracted from red seaweeds (phylum Rhodophyta) (Vreeland et al., 1987). Their economic importance derives from their chemical properties such as high viscosity and excellent gelling, stabilizing and emulsifying properties in food, pharmaceutics, cosmetics (Cardozo et al., 2007; Smit, 2004; Stengel et al., 2011), and biofuel industries (Enquist-Newman et al., 2014).

Alginates, or alginic acid, primarily sourced commercially from Laminaria digitata, Ascophyllum nodosum, and Fucus vesiculosus (Lee and Mooney, 2012; Radmer, 1996; Stengel et al., 2011), are a family of linear polysaccharides composed of the uronic acids β-1,4-D-mannuronic acid (M blocks), and the C5 epimer, α-1,4-L-guluronic acid (G blocks) (a diagram of their chemical structures can be found in Figure 1.4 in the General Introduction) (Kim and Park, 1985; Kloareg and Quatrano, 1988; Percival, 1979). These sugar residues are arranged as homopolymers of M blocks, and G blocks together with mixed sequences, in the same molecule (Haug, 1964). The homopolymer blocks are stiff, while mixed blocks contain more flexible linkages. Divalent-cations, such as calcium, can bind two antiparallel G chains from different alginate molecules and form a sequence of ionic bridges which is known as an egg-box structure; this dimerization forms the basis for alginate gelation properties (Haug, 1964; Morris et al., 1978; Smidsrød et al., 1973). The epimerization of the M into G blocks is mediated by the enzyme mannuronan C5-epimerase (Haug et al., 1974; Nyvall et al., 2003), and it is proposed that alginates and mannuronan C5-epimerase determine some properties of the wall such as porosity and rigidity, analogous to pectins and pectin methylesterases in land plants, respectively (Deniaud-Bouët et al., 2014).

Carrageenans, primarily sourced commercially from Kappaphycus alvarezii and from Chondrus crispus (Cardozo et al., 2007; Lee, 2008; Radmer, 1996), are the major cell wall polysaccharides of many marine red algae, composed of linear chains of alternating α-1,3-D-galactose and β-1,4-D-galactose, modified by the occurrence of 3,6-anhydro bridges in the α-linked galactose residues and by substitution with ester sulfate, methyl or pyruvate groups (a diagram of their chemical structures can be found in Figure 1.5 in the General Introduction) (Kloareg and Quatrano, 1988). Natural carrageenans are mixtures with different sulfate levels, from which the most industrially important are κ-, τ- and λ-carrageenan, depending on
the sulfate level per disaccharide unit. The other two types known are µ- and ν-, and are the precursors for κ- and τ-carrageenans, respectively (Zablackis et al., 1988). κ-carrageenan has a 4-sulfate on the β-1,3-D-galactopyranosyl residue, with a sulfation percentage of up to 20% (w/w); τ-carrageenan has an additional sulfate at 2-sulfate on the 4 linked 3,6-anhydro-α-D-galactose residue; λ-carrageenan lacks the anhydrogalactose and contains three sulfate groups, with a sulfation percentage of up to 40% (w/w) (Cardozo et al., 2007; Kloareg and Quatrano, 1988; Zablackis et al., 1988). The ability of carrageenans to form a gel depends on the sulfate levels and of the presence of the anhydrogalactose; low sulfate and high anhydrogalactose are correlated with a high gel strength; both κ and τ contain anhydrogalactose and form gels; κ gelation occurs optimally with potassium ions and forms a stiff gel, whereas τ gelation occurs with calcium and magnesium ions, forming soft elastic gels; λ-carrageenan has no anhydrogalactose and do not form gels (Vreeland et al., 1992b; Zablackis et al., 1991).

Agar, primarily extracted commercially from Gracilaria and Gellidium species (Cardozo et al., 2007; Lee, 2008; Radmer, 1996), is a common name for certain red seaweed polysaccharides containing repeated agarobiose units of α-(1-4)-3,6-anhydro-L-galactose and β-(1-3)-D-galactose residues, with up to 6% (w/w) of sulfated esterification groups (a diagram of the chemical structure can be found in Figure 1.5 in the General Introduction) (Lee, 2008). Agar’s chemistry and physico-chemical properties are very complex, and similarly to alginates and carrageenans, depend on the chemical structure (Lahaye and Rochas, 1991). The purity of agar dictates its commercial application, which can range from food products (frozen food, bakery icing, dessert gels, candies, etc.) and industry (adhesives, textile printing/dyeing, etc.) for low purified agar; gel substrates for biology culture media and pharmaceutics (capsules, tablets, anticoagulants, etc.) for medium quality agar; or in molecular biology (electrophoresis, immunodiffusion, gel chromatography) for highly purified agar, also known as agarose (Cardozo et al., 2007; Radmer, 1996).

The advantages of the application of mAbs in seaweed research have been the main focus of the experimental approaches in this thesis (Chapter 2, 3, 4). Chemical and enzymatic extractions are essential tools, as they provide information about the monosaccharide residues present in a polymer, as well as the linkages between the residues, and the structural features of the polymers (Deniaud-Bouët et al., 2014; Popper and Fry, 2003; Popper and Fry, 2004). On the other hand, most of the characterized carbohydrates are extracted from entire plants, representative of a mixture of cell and tissue types, in addition to the possible presence of
intracellular polymers in some fractions (Vreeland and Kloareg, 2000). The application of immunological techniques, normally associated with medicine rather than with plant science, to algae is not new; labelled antibodies were a major breakthrough, with well known advantages, primarily: 1) sensitivity — antibodies can detect antigens even when they are present at very low levels, which allows detection of antigens within specific organelles and also the use other highly sensitive detection methods such as electron microscopy; and 2) specificity — antibodies can be generated in order to distinguish differences between polymers as small as anomeric configurations of monosaccharide residues in a polysaccharide (McCandless et al., 1980). Both monoclonal antibodies (mAbs) and hybridization probes are effective molecular markers to investigate algal carbohydrate synthesis, architecture and linkages between polymers, in order to understand cell wall structure, assembly and modifications; moreover, mAbs allow the comparison between the content of gelling sequences which has implications when choosing algal strains for higher content in gelling polymers (Vreeland et al., 1987).

Antibody production is very complex (Figure 1.6 in the General Introduction) and its success depends on several factors, including appropriate antigen preparation, characterization, and immunization protocols (Vreeland and Laetsch, 1985). Carbohydrates pose a specific problem when trying to generate mAbs as they are not naturally highly immunogenic, possibly because many form parts of a mammal’s food and natural environment. Carbohydrates may therefore be fused to proteins to help increase their recognition by the animal’s immune system (Smith and Ginsburg, 1980). Mice (predominantly albino female) or rats are immunized with the antigen of interest, the serum is isolated from the animal and the immunoglobulins are isolated from the antiserum to finally be tested with immunoassays. If the animal is producing a mAb of interest the lymphocytes are fused with myeloma cells. These hybrid cells, or hybridomas, secrete antibodies and can be proliferated indefinitely in culture. (McCandless et al., 1980; Vreeland and Laetsch, 1985).

The specificity of an antibody may be a limitation for the success of the immunological approach, as it relies on factors such as the purity of the antigen used to produce the specific antiserum (McCandless et al., 1980; Vreeland and Laetsch, 1985). Changes in specificity related to the antigen-antibody incubation conditions can also happen, especially with highly charged polymers such as alginates, as the carbohydrate conformation and cross reaction with other polymers can be influenced by pH, presence of ions, temperature, nature of the buffers used, therefore influencing the specificity of the antigen-
antibody interactions (Larsen et al., 1985; Vreeland et al., 1984). Even though mAbs identify specific epitopes, the antibody’s ability to recognize gelling sequences can be blocked by carbohydrate interactions; on the other hand, carbohydrate hybridization probes identify the gelling subunits of alginates and carrageenans even when they already formed gelled structures in the tissue (Vreeland et al., 1987), hence the usefulness of both techniques.

An extensive work with molecular probes for the study of the most important cell wall polysaccharides derived from brown and red seaweeds, alginates and carrageenans, respectively, was addressed by Dr. Valerie Vreeland (Department of Botany, University of California, CA, USA). The first reports of the production of alginate rabbit anti-serum date back to 4 decades ago, where the extracellular alginate distribution was described in *Fucus distichus* tissues (Vreeland, 1970, 1972), in different stages of life cycle, and from different brown algae species (Vreeland, 1974), although the polyclonal serum specificity was not directly correlated with alginate-block types. The production of antisera against sulfated fucans was also documented, and further study of its distribution was described for a number of seaweeds (Vreeland, 1975; Vreeland and Chapman, 1978), and provided the first conjectures regarding the different alginate and fucan antigen determinants, or epitopes, variation in proportion and amount with taxonomic groups (Vreeland and Chapman, 1977), and also within early developmental stages of embryos (Vreeland et al., 1981). Despite the new insights these first immunological approaches provided, they could not answer the questions that were rising related to the complexity of the patterns observed, therefore the need for the generation of mAbs that could be used to investigate individual epitopes separately was emerging (Vreeland et al., 1984). MAbs against brown seaweed cell wall carbohydrates were further developed (Vreeland et al., 1982; 1984), which brought new insights to the seaweed cell wall research, such as intracellular localization of the synthesis of cell wall epitopes in vegetative tissues, and wall modifications during early differentiation of *Fucus* zygotes (Vreeland and Laetsch, 1984, 1987). Studies on wall assembly of developing zygotes and of regenerating zygote protoplast cell walls using anti-fucan and anti-alginate mAbs (Boyen et al., 1988) led to relevant understanding about the complexity of zygote cell wall assembly, including alginase activity, alginate synthesis and structure during early development stages (Vreeland and Laetsch, 1989a, 1990), showing the intracellular synthesis of alginate gelling subunits as a time-, location- and gel type- dependent process (Vreeland et al., 1988a; Vreeland and Laetsch, 1988). Alginates were object of research in many studies, as its subunits identification were not possible due to the presence of other compounds with similar staining properties (Vreeland and Laetsch, 1988), thus preliminary work on alginate
molecular probes was undertaken (Vreeland and Laetsch, 1983, 1987; Vreeland et al., 1987; 1988). A further report on the preparation of fluorescein-conjugated polyuronate oligosaccharides and their application as specific probes, based on alginate G-block self-association, was applied to the localization of gelling subunits in cell walls (Vreeland and Laetsch, 1989b).

In the case of red algae, the first in situ probes for carrageenans were generated through the preparation of fluorescein-conjugated κ-carrageenan oligosaccharide hybridization probes based on the mutual interactions of the gelling κ-carrageenan in the presence of Ca²⁺, which provided first information about the gelling subunit distribution in the red alga *Eucheuma alvarezii* (= *Kappaphycus alvarezii*) (Zablackis et al., 1988). The gelling mechanisms of carrageenans, based on the differential labelling patterns of the cell walls and extracellular matrix of *K. alvarezii* were investigated using hybridization probes generated from κ- and τ-carrageenan oligosaccharides fluorescein-conjugated (Zablackis et al., 1991); hybridization probes were also applied in the study of τ-carrageenan secretion in *K. alvarezii* regenerating protoplasts (Zablackis et al., 1993). MAbs were later generated against carrageenans, with specificity to κ-, τ- and λ-carrageenans, which allowed the observation of the intracellular synthesis of epitopes related to the primary carrageenan subunits and their different extracellular localization (Vreeland et al., 1988b, 1992a). These molecular probes allowed further localization of specific carrageenans in red seaweeds with a number of procedures, such as tissue prints (Vreeland et al., 1992b), or immunogold-labelling, which showed the intracellular and Golgi-mediated biosynthesis of τ-carrageenan (Gretz et al., 1990; Gretz et al., 1991). Although fewer reports exist on the agarose wall portion, hybridization probes were also produced from agarose oligosaccharides (Vreeland et al., 1995), although no further application was ever reported.

The collection of MAbs generated by Dr. Vreeland and co-workers was unfortunately never made available to the scientific community or commercialized, so the potential of all these molecular tools was not fully exploited; ultimately the remaining collection was donated to Professor Michael G. Hahn (CCRC, University of Georgia, GA, USA). The entire collection known is presented on Table 5.1 and includes supernatants and/or hybridoma lines.

The main objective of this work was to test all the available MAb supernatants against a variety of cell wall extracts obtained from different brown and red species, and with different extraction protocols (summarized on Table 5.2), in the attempt to verify if any of the MAbs are able to react with any of the wall extracts and therefore viable. Extracts were
prepared from common brown seaweeds from the Irish West coast: 1) *Fucus*, although *Fucus distichus* was the specie chosen by Vreeland in her studies, *Fucus vesiculosus* was chosen mostly because it was the selected specie to all of the studies presented in the chapters of this thesis, therefore I preferred to obtain consistent data throughout the thesis; moreover, for extract test purposes, *F. vesiculosus* polysaccharides should have similar epitopes from other species within the same genera; and 2) the kelp *Laminaria digitata*, in order to increase the variety of extracts, and also because this species is a primary source of alginates, which were antigens used to produce many of the mAbs. The red seaweeds chosen for the extractions were *Chondrus crispus*, primarily because the antigens obtained to produce the red seaweed cell wall-directed mAbs were extracted from this species, so the probability to have positive results will be higher, and also because it is a representative species of one of the major red algal groups, the Florideophyceae class. *Porphyra sp.* was also chosen for extraction, in order to increase the variety of extracts to test, and as a representative of the other major red algal group, the Bangiophyceae class. The results obtained will elucidate if the mAbs are still viable or not, and if any of them can still be rescued, in order to proceed in a further attempt to create hybridoma lines that will allow the continuity of the mAb production and ultimately become available to the scientific community.
Table 5.1. Listing of the Dr. Vreeland mAbs supernatant inventory existing in the CCRC, the species used to extract the antigens, and references. The listing presents mAbs that are part of the supernatant inventory and published, mAbs that are part of the supernatant inventory but not published, and mAbs that are not part of the inventory, but were published.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Alga/species</th>
<th>Antigen</th>
<th>Origin of the antigen used for immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies part of the inventory that were published</td>
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<td></td>
<td></td>
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<tr>
<td>2-1.2</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-7.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-8.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-9.3</td>
<td><em>F. distichus</em></td>
<td>Alginates and fucans</td>
<td>Mixture of carbonate fucan, acid alginate, partially hydrolyzed acid alginate, carbonate alginate and partially hydrolyzed carbonate alginate (Vreeland et al., 1984)</td>
</tr>
<tr>
<td>2-10.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2-15.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2-18.2</td>
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<td></td>
<td></td>
</tr>
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<td>λ-carrageenan</td>
<td></td>
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<tr>
<td>5H12</td>
<td></td>
<td>κ- and τ-carrageenans</td>
<td>Short- and long-chain κ- and τ-carrageenans, and λ-carrageenan (purchased from Marine Colloids Division of FMC, Inc. Rockland, ME); carrageenan oligosaccharides: β-neocarrabiose, neocarrabiose 4-sulfate, neocarratetraose 4,4-disulfate and neocarratetraose 4-sulfate (purchased from Sigma, St. Louis, MO) (Vreeland et al., 1992a)</td>
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<tr>
<td>5H12-3C7</td>
<td></td>
<td>κ- and τ-carrageenans</td>
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<td>κ- and τ-carrageenans</td>
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<td>Agar</td>
<td>Not described (Vreeland and Laetsch, 1985)</td>
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<td>G blocks of alginates</td>
<td>Not described (Boyen et al., 1988)</td>
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<td>23-9F4</td>
<td>Brown seaweed</td>
<td>MG blocks of alginates</td>
<td>Not described (Vreeland and Laetsch, 1988)</td>
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<tr>
<td>Antibodies that were published that are not part of the inventory</td>
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<td>Alginates (Vreeland and Laetsch, 1985)</td>
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<td>Red seaweed</td>
<td>Agar (Vreeland and Laetsch, 1985)</td>
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<td></td>
</tr>
<tr>
<td><em>F. distichus</em></td>
<td>Fucans (Vreeland et al., 1984)</td>
<td></td>
<td></td>
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</tbody>
</table>
5.2. Materials and methods

5.2.1. Algal material

Specimens of the brown *Fucus vesiculosus* Linnaeus, *Laminaria digitata* (Hudson) J. V. Lamouroux (Phaeophyceae, Heterokontophyta) and red macroalgae *Chondrus crispus* Stackhouse and *Porphyra* sp. (Rhodophyta, Archaeplastida) were collected at Finavarra, Co. Clare (53°09′25″N, 09°06′58″W), on the west coast of Ireland in February 2012, cleaned of epiphytes and/or grazers, and rinsed with tap water.

5.2.2. Cell wall extractions

Algal material was prepared for further cell wall extraction according to methods previously described for vascular plant cell walls (Pattathil et al., 2012). The seaweeds were cut into small pieces and ground to a fine powder in liquid nitrogen using a mortar and pestle, and the powder was resuspended in 80% (v/v) ethanol, shaken overnight at room temperature, and centrifuged at 3,000 × g for 15 min. The supernatant was discarded and the pellet washed with 80% (v/v) ethanol followed by final washing steps using 100% ethanol and acetone. The final alcohol insoluble residue (AIR) was air-dried in a fume hood for 24 h and subjected to different sequential extractions for brown and red seaweeds, as described below. After drying, all the extracts were stored at room temperature in a desiccator containing a drying agent (silica) prior to further analysis. In order to simplify the results analysis and discussion, a code was attributed to each extract, according to species, extraction method, and extractor, as presented in Table 5.2.

5.2.3. Chemical sequential extractions 1 — Brown seaweeds

This method, described to chemically extract seaweed cell walls (Deniaud-Bouët et al., 2014), was used on *F. vesiculosus* and *L. digitata*. Instead of using AIR, ground and freeze-dried seaweed powder was used as the starting material for this method, in suspensions at 10 mg mL⁻¹ based on the starting weight of the algal powder, and with constant stirring (200 rpm). After each extraction the soluble materials were isolated by filtration with Miracloth (EMD Millipore), and the extracts were obtained after a purification step to remove alginate content from the solubilized material by precipitation with calcium.
chloride (with a final concentration of 4% (v/v)). The seaweeds were extracted with two serial incubations in deionized water containing 0.1% (w/v) of Na$_2$SO$_4$ as an antioxidant agent for 45 min at room temperature, 1 M NaCl containing 0.1% (w/v) of Na$_2$SO$_4$ for 2 h 30 min at room temperature, 4 M NaCl containing 0.1% (w/v) of Na$_2$SO$_4$ for 2 h 30 min at room temperature, deionized water containing 0.1% (w/v) of Na$_2$SO$_4$ for 2 h 30 min at 40ºC and 1 M urea for 5 h at room temperature. The extracts were stored at 4ºC until dialyzing (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against three changes of deionized water for a total of 48 h after which they were freeze-dried.

5.2.4. Enzymatic sequential extractions — Brown seaweeds

This method describes enzymatic extractions of seaweed cell walls (Deniaud-Bouët et al., 2014) and was used to make extracts from *F. vesiculosus* and *L. digitata*. All extractions were carried out in suspensions at 10 mg mL$^{-1}$ based on the starting 1 g of AIR used, in a water bath, using each enzyme in its corresponding buffer as the resuspending solution. The AIR was extracted with 1) 2.5 U of alginate lyase purified from *Sphingomonas* sp. (Megazyme, Bray, Ireland) in 100 mM Tris-HCl, pH 7.2 containing 1 mg mL$^{-1}$ of bovine serum albumin (BSA), for 70 h at room temperature; 2) 230 U of papain purified from *Papaya latex* (Sigma-Aldrich, St. Louis, MO, USA) in 100 mM sodium acetate, pH 6, 5 mM EDTA and 5 mM of L-cysteine for 24 h at 60ºC; 3) cellulase purified from *A. niger* (Megazyme, Bray, Ireland) diluted 1:13, pH 4.5, was added twice after 0 h and 24 h of incubation, respectively, in 50 ml of 0.2 % (w/v) sodium azide, for 40 h at 40ºC. The reactions were stopped by boiling, the soluble material was recovered by filtration through Miracloth and the extracts were stored at 4ºC until further dialyzes (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against three changes of deionized water for a total of 48 h and freeze-dried.

5.2.5. Chemical sequential extractions 2 — Brown seaweeds

This method, described to chemically extract seaweed cell walls (Vreeland et al., 1984), was used on *F. vesiculosus* and *L. digitata*. All extractions were carried out in suspensions at 10 mg mL$^{-1}$ based on the starting AIR used. A water bath was used to provide the correct temperatures for the incubations. After each extraction the soluble material was recovered by filtration through Miracloth and stored at 4ºC. The AIR was extracted with
three serial incubations in 0.2 M HCl for 1 h at 55°C. The first supernatant (Acid Extract) was neutralized with NaOH, precipitated by 4 volumes of 95 % (v/v) ethanol, washed with acetone and dried at 40°C; the second and third supernatants were discarded. After a further incubation with 3% (w/v) Na₂CO₃ for 1 h at 55°C, the residue was discarded and the supernatant was brought to pH 8.0 with HCl, filtered and precipitated by 2 volumes of 95 % (v/v) ethanol (carbonate). The carbonate-extracted material was dissolved in deionized water at 5 mg mL⁻¹, 5 M NaCl was added to a final concentration of 0.2 M NaCl, and pH was adjusted to 2.1 with HCl. The carbohydrate precipitate fraction was resuspended in deionized water and the pH adjusted to 7 with NaOH. The carbohydrates were then precipitated by 1 volume of 95 % (v/v) ethanol and dried at 40°C (acid insoluble fraction); the carbohydrate supernatant fraction was precipitated by 2 volumes of 95 % (v/v) ethanol and dried at 40°C (acid soluble fraction). Both acid soluble and insoluble fractions were further fractionated. After dissolving in deionized water at 5 mg mL⁻¹, 0.5 volumes of 2 % (w/v) CaCl₂ was slowly added to each fraction. The supernatants were dialyzed extensively against deionized water (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) for 48 h, filtered, brought to 0.05 M NaCl and precipitated with 3 volumes of 95 % (v/v) ethanol; the precipitates were dissolved in 0.33 M EDTA at pH 7.2 and dialyzed before precipitation by 1 volume of 95 % (v/v) ethanol.

5.2.6. Chemical sequential extractions 3 — Red seaweeds

This method, described to chemically extract red seaweed cell walls (Guangli et al., 2007), was used on C. crispus and Porphyra sp, with modifications. All extractions were carried out in suspensions at 10 mg mL⁻¹ based on the starting AIR used, in a water bath. After each extraction the supernatant was collected by centrifugation at 3,000 × g for 10 min. The AIR was extracted with two serial incubations in deionized water for 4 h at 25°C, the supernatant was freeze-dried and the residue was extracted with three serial incubations with deionized water for 1 h at 85°C. The residues were discarded and the supernatant was incubated with 1 volume of 0.25 M KCl for 1 h at room temperature. The residue was freeze-dried and the supernatant was precipitated by 4 volumes of 95% (v/v) ethanol, dissolved in 0.05 M (w/v) KCl and precipitated by 1.5 volumes of 95% (v/v) ethanol. The residue was freeze-dried and the supernatant was dialyzed (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against three changes of deionized water for a total of 48 h and precipitated with 4 volumes of 95 % (v/v) ethanol.
5.2.7. Chemical sequential extractions

This method, described to chemically extract plant cell walls (Pattathil et al., 2012), was used on *F. vesiculosus, L. digitata, C. crispus* and *Porphyra* sp. All extractions were carried out in suspensions at 10 mg mL\(^{-1}\) based on the starting weight of the AIR used, with constant mechanical stirring (200 rpm) at 70\(^\circ\)C for 24 h. After each extraction the supernatant was collected by centrifugation at 3,000 \(\times\) g for 10 min, and stored at 4\(^\circ\)C. The AIR was extracted with 50 mM ammonium oxalate (pH 5), 50 mM Na\(_2\)CO\(_3\) containing 0.5% (w/v) NaBH\(_4\) (pH 10), 1 M KOH containing 1% (w/v) NaBH\(_4\), 4 M KOH containing 1% (w/v) NaBH\(_4\), 100 mM sodium chlorite, and the pelleted residue was treated once more with 4 M KOH containing 1% (w/v) NaBH\(_4\) (KOHPC). The 1 M KOH, 4 M KOH and 4 M KOHPC fractions were neutralized with glacial acetic acid. All extracts were dialyzed (Spectra/Por 3 Dialysis Membrane 3,500 Da MWCO, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against three changes of deionized water in 4\(^\circ\)C for a total of 48 h and freeze-dried.

5.2.8. Enzyme-Linked Immunosorbent Assay

ELISAs were carried out as described previously (Pattathil et al., 2010). Aliquots of the commercial extracts and of the seaweed extracts were diluted in deionized water (10 \(\mu\)g/ml), put in a sonication bath for 15–20 min and loaded on 96-well plates (Costar 3598, Corning Inc., NY, USA) in duplicates (when tests were repeated, 8 replicates were loaded for each extract). Deionized water instead of extract was added to the negative control wells and Tamarind xyloglucan (Megazyme, Bray, Ireland) probed with CCRC-M104 was used as a positive control. After being left to dry overnight at 37\(^\circ\)C, the plates were incubated with 1% (w/v) nonfat dry milk (instant nonfat dry milk, Publix supermarket, USA) in Tris-buffered saline (2 mM Tris-Base, 8 mM Tris-HCl, pH 7.6 containing 100 mM sodium chloride) for 1 h. The aspiration and washing steps employed an ELx405 VRS Microplate Washer (BioTek Instruments Inc., Winooski, VT, USA); incubations were carried out at room temperature. After aspirating the blocking solution, 50 \(\mu\)l of undiluted hybridoma supernatant of the mAbs was added to the wells and incubated for 1 h. After aspiration of the antibody solutions and washing (\(\times\) 3) with 0.1% (w/v) nonfat dry milk in Tris-buffered saline, the wells were incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:5,000 in wash buffer. Antibody solutions were aspirated and the wells were washed 5 times with wash buffer. 3,3’5,5’-
Tetramethylbenzidine peroxidase substrate (TMB peroxidase substrate kit SK-4400, Vector Laboratories, Inc., Burlingame, CA, USA) was prepared according to manufacturer’s instructions, added to each well and incubated for 30 min. 1 N sulfuric acid (H$_2$SO$_4$) was added to stop the reaction and the optical density was immediately read as the difference between $A_{450}$ and $A_{655}$ with a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), using the Microplate Manager Version 5.2 Build 103 software. The reading for the corresponding negative control well was also subtracted from each sample well. ELISA results were presented as a heat map using R-Console software (Team, 2011).

5.2.9. Preparation of algal material for microscopy

*F. vesiculosus* and *C. crispus* blades were cut into small pieces and fixation and embedding procedures were conducted as previously described (Coimbra et al., 2007) with some modifications. Samples were put in a fixative solution (2% (v/v) paraformaldehyde; 0.25% (v/v) glutaraldehyde; 0.025 M PIPES buffer; 0.001% (v/v) Tween 80; pH 7.2) for 2 h at room temperature followed by an overnight incubation at 4°C. Samples were washed with 0.025 M PIPES buffer, pH 7.2, for 10 min, followed by a second wash for 20 min. Dehydration was carried out using a graded ethanol series (25% (v/v), 35% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v), and 3 times with 100%) with 15 min for each grade and infiltration was performed by sequential immersion in different proportions of 100% ethanol and LR White Resin (Medium grade, Agar Scientific, Cambridge, UK) over 6 days (ethanol/LR White proportion per day was 3:1; 2:1; 1:1; 1:2; 1:3; 0:1). After the final resin change samples were placed in gelatin capsules (Electron Microscopy Sciences, Hatfield, PA, USA) and fresh LR White was added. The capsules were hermetically sealed, and the samples were placed in an oven at 60°C for 2 days.
Table 5.2. Listing of the codes given to each algal extract, according to species, extraction method and chemical used. A code was also given to the commercially sourced extracts.

<table>
<thead>
<tr>
<th>Extraction steps</th>
<th>Species</th>
<th>Fucus vesiculosus</th>
<th>Laminaria digitata</th>
<th>Chondrus crispus</th>
<th>Porphyra sp.</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>dH₂O p.p. material</td>
<td>L1_Water.p</td>
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<td>dH₂O soluble material</td>
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<td>L1_Water</td>
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<tr>
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<td>dH₂O 40°C p.p. material</td>
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<td>-</td>
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<td></td>
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<td>F1_1MNaCl.p</td>
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<td></td>
<td></td>
<td>1 M NaCl soluble</td>
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<td></td>
<td>4 M NaCl soluble</td>
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<td></td>
<td></td>
<td>1 M urea soluble</td>
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<td>-</td>
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<td></td>
<td>Cellulase</td>
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<td>Vreeland et al. 1984</td>
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<tr>
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<td>-</td>
<td>C3_HWaterII</td>
<td>-</td>
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<td>Pattathil et al. 2010</td>
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<td>C4_Carbonat</td>
<td>P4_Carbonate</td>
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<td>L4_1MKOH</td>
<td>C4_1MKOH</td>
<td>P4_1MKOH</td>
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<td>C4_4MKOH</td>
<td>P4_4MKOH</td>
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<td>L4_Chlorite</td>
<td>C4_Chlorite</td>
<td>P4_Chlorite</td>
</tr>
<tr>
<td></td>
<td>4 M KOH post chlorite</td>
<td>F4_4MKOHhp</td>
<td>L4_4MKOHhp</td>
<td>C4_4MKOHhp</td>
<td>P4_4MKOHhp</td>
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<td>λ-carrageenan</td>
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<td>γ-carrageenanV</td>
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<td>C1138</td>
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<td></td>
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<td>22048</td>
<td>κ-carrageenan</td>
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<td></td>
<td></td>
<td>F2252</td>
<td>Fucose</td>
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</table>
5.2.10. Commercial seaweed extracts

The commercial seaweed polysaccharides tested, \( \lambda \)-carrageenan (Sigma 22049), \( \tau \)-carrageenan Type V (Sigma c4014), \( \tau \)-carrageenan Type II (Sigma C1138), \( \kappa \)-carrageenan (Sigma 22048) and fucose (Sigma F2252) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

5.2.11. Monoclonal antibodies

Monoclonal antibodies generated against different cell wall glycans were obtained as hybridoma cell culture supernatants from stocks at the Complex Carbohydrate Research Center (CCRC), kindly donated by Dr. Valerie Vreeland (Department of Botany, University of California, CA, USA). A list of the antibodies is provided in Table 5.1. CCRC-M104 used to probe the positive control is part of the CCRC hybridoma collection, available from CarboSource (www.carbosource.net).

5.2.12. Immunohistochemistry

Immunolabelling was executed as previously reported (Avci et al., 2012). In brief, semi-thin sections (250 nm) were cut with a Leica EM UC6 ultramicrotome (Leica Mikrosysteme GmbH, Vienna, Austria) with a histo-diamond knife (DiATOME, Hatfield, PA, USA). Sections were mounted on glass slides (Fisherbrand Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA) and dried at 50ºC for 1 min. The sections used for future reference regarding anatomical information were stained with toluidine blue O; a drop of 1% (w/v) toluidine blue in 1% (w/v) sodium borate was put on the sections for up to 20 s (McCully et al., 1980), after which the sections were washed with \( \mu \text{H}_2\text{O} \) and dried. The sections used for immunolabelling were blocked with 3% (w/v) nonfat dry milk (instant nonfat dry milk, Publix) in potassium phosphate buffered saline (KPBS) (0.01 M potassium phosphate, pH 7.1, containing 0.5 M NaCl) for 30 min, and then incubated for 60 min with undiluted hybridoma supernatant of the mAbs. The sections were washed with KPBS, 3 \( \times \) 5 min, and incubated with goat anti-mouse IgG conjugated with AlexaFluor 488 (Invitrogen Molecular Probes, Eugene, OR, USA) secondary antibody (1:100 in KPBS) for 90 min, after which the sections were washed with KPBS, 2 \( \times \) 5 min. After a final wash with distilled water (5 min), the sections were mounted with Citifluor antifade mounting medium AF1 (Electron Microscopy Sciences, Hatfield, PA, USA) and covered with a coverslip.
5.2.13. Microscopy

Labelling was observed with a Nikon Eclipse 80i (Nikon Instruments, Melville, NY, USA) microscope equipped with epifluorescence optics and Nikon B-2E/C filter. Images were captured with a Nikon DS-Ri1 camera (Nikon Instruments, Melville, NY, USA) using NIS-Elements Basic Research software, and assembled with Adobe Photoshop Elements 11.0 (Adobe Systems, San Jose, CA, USA) software.
5.3. Results

The collection of mAbs generated against seaweed cell wall glycans were tested for reactivity toward cell wall extracts obtained from the brown *F. vesiculosus* and *L. digitata*, and from the red seaweeds *C. crispus* and *Porphyra* sp. Different extraction methods were used in order to broaden the spectrum of possible positive signals. The methods described by Deniaud-Bouët et al. 2014, Vreeland et al. 1984 and Pattathil et al. 2010 were used to prepare extracts from the brown seaweeds, while the methods described by Guangli et al. 2007 and Pattathil et al. 2010 were applied to extract the red seaweeds. The ELISA results are presented as heat maps (Fig. 5.1, 5.2), and show that most of the mAbs did not give any positive signals, except for the 3G1.1C7.E4, 5H12.3C7, 26-10A5 and 26-10A5.4E66.C6 mAbs; the strongest signals observed are associated to the red seaweed extracts (Fig. 5.2). *In situ* immunolocalization performed on LR White-embedded sections of *F. vesiculosus* and *C. crispus* blades showed positive labelling with 3G1.1C7.E4, 26-10A5, 2-18.2 and 12-1.3 mAbs for *F. vesiculosus* (Fig. 3); 3G1.1C7.E4 and 26.10A5.4E66 were the only mAbs that showed positive labelling for *C. crispus* (Fig. 5.4). The codes assigned to the extracts mentioned in this section and the corresponding extraction step can be found in Table 5.2.

5.3.1. ELISAs — Reactivity of the Vreeland mAbs to brown seaweed extracts

The ELISA results for *F. vesiculosus* (Fig. 5.1, F1_Water.p to F4_4MKOHp) show that most of the mAbs did not react with the extracts. Low signals (~0.1–0.2) were observed for F1_HWater.p, F1_HWater and F1_Urea.p (Deniaud-Bouët et al. 2014 method) extracts with 26.10A5.4E66.C6 mAb; F2_S.Calcium.s (acid soluble calcium supernatant, Vreeland et al. 1984 method) with 5H12-3C7, and F4_Chlorite (Pattathil et al. 2010 method) with 3G1.1C7.E4 and 5H12-3C7 mAbs (Fig. 5.1).

*L. digitata* extracts showed positive signals for L1_Water, L1_Papain (Deniaud-Bouët et al. 2014 method), L4_Chlorite, L4_4MKOHp (Pattathil et al. 2010 method) with the 26-10A5 mAb; L4_Chlorite and L4_4MKOHp also reacted with 26.10A5.4E66.C6. Higher signals (~0.3) were observed with 5H12-3C7 also for the L1_Papain, L4_Chlorite and L4_4MKOHp extracts. The highest signals were detected for the 3G1.1C7.E4 mAb (~0.5) particularly for the L1_Papain, L4_1MKOH and L4_4MKOHp (Pattathil et al. 2010 method) extracts.
The data is presented as a heat map, resulting from the ELISA screening where seaweed cell wall glycan-directed monoclonal antibodies were tested. The detailed list of the antibodies used is described on the right panel, and the ELISA signal strength is displayed by a black-yellow scale, with black indicating the absence of binding and yellow showing strongest binding. The codes at the bottom correspond to the cell wall extracts, grouped by species, extraction method, and extractor. Table 5.2 shows the correspondence of each extract code to the respective species, extraction method and extraction step (Table 5.2). *F. vesiculosus* extracts according to 1) Deniaud-Bouët et al. 2014: F1_Water.p, F1_Water, F1_1MNaCl.p, F1_1MNaCl, F1_4MNaCl, F1_HWater.p, F1_HWater, F1_Urea.p, F1_Urea, F1_Alg.lyase, F1_Papain, F1_Cellulase; 2) Vreeland et al. 1984: F2_0.2MHC1, F2_S.Calcium.p, F2_S.Calcium.s, F2_I.Calcium.p; 3) Pattathil et al. 2010: F4_AmOxal, F4_Carbonate, F4_1MKOH, F4_4MKOH, F4_Chlorite, F4_4MKOHp. *L. digitata* extracts according to 1) Deniaud-Bouët et al. 2014: L1_Water.p, L1_Water, L1_1MNaCl.p, L1_1MNaCl, L1_4MNaCl, L1_HWater.p, L1_HWater, L1_Urea.p, L1_Urea, L1_Alg.lyase, L1_Papain, L1_Cellulase; 2) Vreeland et al. 1984: L2_0.2MHC1, L2_S.Calcium.p, L2_S.Calcium.s, L2_I.Calcium.p; 3) Pattathil et al. 2010: L4_AmOxal, L4_Carbonate, L4_1MKOH, L4_4MKOH, L4_Chlorite, L4_4MKOHp.
Figure 5.2. Profile of sequential cell wall extracts from whole thallus of *C. crispus* and *Porphyra* sp., and commercial extracts. The data is presented as a heat map, resulting from the ELISA screening where seaweed cell wall glycan-directed monoclonal antibodies were tested. The detailed list of the antibodies used is described on the right panel, and the ELISA signal strength is displayed by a black-yellow scale, with black indicating the absence of binding and yellow showing strongest binding. The codes at the bottom correspond to the cell wall extracts, grouped by species, extraction method, and extractor. Table 5.2 shows the correspondence of each extract code to the respective species, extraction method and extraction step (Table 5.2). *C. crispus* extracts according to 1) Guangli *et al.* 2007: C3_Water, C3_HWaterII, C3_HWaterII, C3_Residue; 2) Pattathil *et al.* 2010: C4_AmOxal, C4_Carbonate, C4_1MKOH, C4_4MKOH, C4_Chlorite, C4_4MKOHp. *Porphyra* sp. extracts according to: 1) Guangli *et al.* 2007: P3_Water, P3_HWater; 2) Pattathil *et al.* 2010: P4_AmOxal, P4_Carbonate, P4_1MKOH, P4_4MKOH, P4_Chlorite, P4_4MKOHp. Commercial extracts: λ-carrageenan, τ-carrageenanV, τ-carrageenanII, κ-carrageenan, Fucose.
5.3.2. ELISAs — Reactivity of the Vreeland mAbs to red seaweed extracts

All the extracts from *C. crispus* showed positive ELISA signals with the 3G1.1C7.E4, 5H12-3C7, 26-10A5 and 26.10A5.4E66.C6 mAbs. The highest signal (0.8) was observed for the C3_Residue (Guangli *et al*. 2007 method) extract with 3G1.1C7.E4; lower signals (~0.4-0.6) were observed for 5H12-3C7 and 26-10A5 mAbs, while a high signal (0.6) was observed for the C4_4MKOH (*Pattathil et al*. 2010 method).extract with 26.10A5.4E66.C6 mAb. Some positive signals were detected for *Porphyra* sp. with the same mAbs as observed for *C. crispus*; nonetheless, most of the extracts did not give significant signals. A high signal (0.8) for P4_Chlorite (*Pattathil et al*. 2010 method) extract was observed with 3G1.1C7.E4, and a lower signal (0.5) with the 26-10A5 mAb was observed for the same extract.

5.3.3. ELISAs — Commercial polysaccharides

The commercial polysaccharide extracts showed positive signals for the same mAbs as for the seaweeds; with the exception of fucose, which only gave a low signal (0.1) with the 26-10A5 mAb. The highest signals were observed for the commercial extracts derived from red seaweeds; λ-carrageenan extract (0.9) with 3G1.1C7.E4 mAb, for the τ-carrageenan type V and τ-carrageenan type II extracts (~0.6) with 26-10A5 mAb, and for the κ-carrageenan extract (0.6) with 26.10A5.4E66.C6 mAb.

5.3.4. Immunolocalization — *Fucus vesiculosus*

Similarly to the ELISA results, only a few of the seaweed cell wall glycan-directed mAbs showed positive immunolabelling to the resin-embedded cross sections of the seaweed blades. In *F. vesiculosus*, positive labelling was observed for the 3G1-1C7.E4 (Fig. 3D), 26-10A5 (Fig. 5.3E), 2-18.2 (Fig. 5.3F, G) and 12-1.3 (Fig. 5.3H, I). 3G1-1C7.E4, which also gave some positive signals with the ELISAs, was the only mAb that bound to mucilaginous areas of the extracellular matrix typically found in the medullary area (Fig. 5.3A) of the blade (Fig. 5.3D). Some labelling was observed in the cell walls of the medulla with the 26-10A5 mAb (Fig. 5.3E), but not in the extracellular matrix. 2-18.2 Mab did not show any ELISA signals, but showed positive in situ labelling (Fig. 5.3F), observed in the medullary cell walls, with a generalized punctuated pattern around the cells, very visible in the midrib part of the blade (Fig. 5.3G), where the cells are closer and the extracellular matrix is less pronounced.
A general labelling of all the blade cell walls was also observed with the 12-1.3 mAb (Fig. 5.3H, I), although it also did not produce signals using ELISA.

**Figure 5.3.** Cross sections of *F. vesiculosus* blade immunolabelled with the seaweed cell wall glycan-directed mAbs 3G1-1C7.E4 (D), 26-10A5 (E), 2-18.2 (F, G), 12-1.3 (H, I) and stained with toluidine blue (A, B). A. Morphology of the medulla area of a blade section stained with toluidine blue. B. Morphology of the medulla area of the midrib of a blade section stained with toluidine blue. C. Negative control of the blade where no primary mAb was used during the immunolabelling. D. 3G1-1C7.E4 labels the cell walls of the medulla, and shows some specificity in extracellular matrix areas. E. 26-10A5 labels the cell walls of the medulla area. F. 2-18.2 labels the cell walls of the medulla. G. 2-18.2 also labels the cell walls of the midrib cell walls, but with punctuation patterns. H. 12-1.3 labels the cell walls of the medulla. I. 12-1.3 also labels the cell walls of the midrib cell walls. *Scale bars*: 25 µm.
5.3.5. Immunolocalization — *Chondrus crispus*

From the collection of mAbs, only two of them showed positive *in situ* labelling to *C. crispus* resin-embedded cross sections, although with different labelling patterns. The thallus of this species consists of a simple sheet-like blade, which in cross section is quite similar to the blade of *F. vesiculosus*, with a layer of cortex cells and a medullary area very rich in mucilaginous extracellular matrix (Fig. 5.4A, B). 26.10A5.4E66 mAb, which revealed strong ELISA signals, showed a strong and generalized *in situ* labelling throughout the blade (Fig. 5.4D), localized in the extracellular matrix, around the medullary cells (Fig. 5.4E). 3G1-1C7.E4, which also showed ELISA signals for this species, presented a positive immunolocalization, although with a different pattern, localized along the blade (Fig. 5.4F); labelling was observed around the cortex cells, with some patches around the medullary cells, somewhat diffuse labelling was also observed in the extracellular matrix (Fig. 5.4G).
Figure 5.4. Cross sections of *C. crispus* blade immunolabelled with the seaweed cell wall glycan-directed mAbs 26.10A5.4E66 (D, E), 3G1-1C7.E4 (F, G) and stained with toluidine blue (A, B). A. General morphology of *C. crispus* blade stained with toluidine blue. B. Detail of the medulla area of the blade stained with toluidine blue. C. Negative control of the blade where no primary mAb was used during the immunolabelling. D. 26.10A5.4E66 shows a generalized and strong labelling throughout the blade. E. 26.10A5.4E66 labels the extracellular matrix of the blade. F. 3G1-1C7.E4 shows some labelling throughout the blade. G. 3G1-1C7.E4 labels the cell walls of the blade, and some areas of the extracellular matrix. Scale bars: A = 100 µm, B, C, E, G = 25 µm, D, F = 50 µm.
5.4. Discussion

Dr. Vreeland mAbs generated decades ago against seaweed cell wall-glycans were brought “out of the closet” and were tested with ELISA assays and immunolocalization procedures, in order to assess if some of them are still viable for future application. From the collection donated by Vreeland to the CCRC, most existed as supernatants, with no hybridoma lines in existence. If those mAbs give positive signals, they can be sent to the University of Georgia Monoclonal Antibody Facility (division of the UGA Bioexpression and Fermentation Facility), where the antibody supernatants will be used in the attempt to create viable hybridoma lines, from which more supernatant can be produced and eventually become available to the scientific community.

Unfortunately, most of the supernatants did not give positive ELISA signals, nor did they label resin-embedded sections. This leads us to conclude that those antibodies are no longer viable, and therefore impossible to clone. The different extraction protocols used in the experimental approach, combined with the different species used, were meant to generate a variety of extracts that could be used in the assays, to explore a range of possible epitopes that could be available for the mAbs to bind to, if these were still viable. Nonetheless, only 4 mAbs showed positive bindings to extracts (Fig. 5.1, 5.2). Those mAbs, namely 3G1.1C7.E4, 5H12-3C7, 26-10A5 and 26.10A5.4E66.C6, showed high ELISA signals, as well as positive in situ labelling, which is very promising, proving that these three lines are viable.

3G1-1C7.E4 mAb belongs to the 3G1 line series generated against C. crispus carrageenan antigen, and characterized to be specific to λ-carrageenan (Vreeland et al., 1992a). Although some in situ labelling was observed for F. vesiculosus (Fig. 5.3D), strong immunolocalization with this mAb was observed with C. crispus (Fig. 5.4F, G). Such circumstances have been previously reported (Vreeland and Laetsch, 1985), where a mAb was generated against a red algal antigen but cross reacted with some tissues of a brown seaweed, possibly showing the existence of fucan epitopes containing galactans (This was exploited in Chapter 2). Furthermore, the highest ELISA signals detected for 3G1-1C7.E4 (0.9) corresponded precisely to the commercial polysaccharide λ-carrageenan, which confirms the preference of this mAb for this type of carrageenan. This 3G1 line was already cloned, and hybridoma supernatant exists, so now the antibody can be prepared to be commercialized; this will be a valuable and unique probe, as the only mAb directed to the red algal non-gelling polysaccharide λ-carrageenan available to the scientific community.
5H12-3C7 mAb belongs to the 5H12 line series generated against *C. crispus* carrageenan antigen, and characterized as specific to κ- and λ-carrageenans (Vreeland et al., 1992a). Although no in situ labelling was observed for neither *F. vesiculosus* or *C. crispus*, the strongest ELISA signals were detected for the *C. crispus* C3_HWaterI (0.6), and for the commercial polysaccharide κ-carrageenan (0.6). C3_HWaterI extract has been characterized as κ-carrageenan (Guangli et al., 2007). The 5H12-3C7 line was already cloned, and hybridoma supernatant exists, so now the antibody can be prepared to be commercialized; this will be a valuable and unique probe, as this is the only mAb available at the moment with a specificity to the red algal polysaccharide κ-carrageenan.

26-10A5 and 26.10A5.4E66.C6 mAbs belong to the 10A5 line and were generated against *C. crispus* carrageenan antigen, and characterized to have specificity to both κ- and λ-carrageenans (Vreeland et al., 1992a). High ELISA signals were detected for 26-10A5 mAb with the λ-carrageenan commercial extracts (~0.7), and for 26.10A5.4E66.C6 mAb with the κ- and λ-carrageenan commercial extracts (0.6). This mAb showed the most striking labelling patterns, due to its strong and broad distribution throughout *C. crispus* extracellular matrix blade, which shows that the epitope recognized by this antibody localizes within the mucilage (Fig. 5.4D, E). Literature suggests that the 10A5 mAb might recognize an epitope that functions as a precursor to anhydrogalactose in gelling carrageenans (Vreeland et al., 1992a). The 10A5 line was already cloned, and hybridoma supernatant exists, so now the antibody can be prepared to be commercialized. Further studies need to be done in order to identify the epitope recognized the specificity of this mAb, nonetheless, this is another valuable mAb against red seaweed carrageenans that can soon become available for the scientific community. It is worth to mention that all the commercial extracts showed ELISA signals with the mAbs described above, except for the fucose extract, a result that was expected for these mAbs, because they are directed towards red algal carrageenans, and fucose is an important constituent of brown seaweed cell walls. Nonetheless, some signals were detected with brown seaweed extracts despite the fact that the mAbs were generated against red seaweeds. Although the reason for this is not determined, there is the possibility that the mAbs might also be recognizing an epitope present in some of the brown seaweed extracts as well. In order to confirm so, the mAbs will need to be purified, new hybridoma lines generated, and the epitopes recognized by them fully characterized.

The 12-1.3 mAb, for which no published information was found, only showed in situ labelling to *F. vesiculosus* blade, but no ELISA signals were detected. This is, unfortunately, not promising. However, it could be worth attempting to generate viable lines from the
existing supernatant, as the labelling patterns to the cell walls were very clean (Fig. 5.3H, I). The 2-18.2 mAb was published along with several other mAbs (Vreeland et al., 1984). It was generated against F. distichus using alginate and fucan extracts as antigens. The same extraction protocol was used in the experimental approach in the attempt to generate similar extracts (Fucus and Laminaria 0.2 M HCl, soluble calcium precipitate, soluble calcium supernatant and insoluble calcium precipitate extracts, Fig. 5.1) that the mAbs could recognize. However, no positive signals were detected by ELISA, with either of the extracts or the mAbs published by Vreeland et al. 1984. Despite the negative ELISA results, in situ labelling with the 2-18.2 mAb showed positive labelling to F. vesiculosus blade (Fig. 5.3F, G), therefore, it would be worth to attempt to start viable lines from the existing supernatant, as the labelling patterns observed corresponds to the previously documented labelling of the outer walls of the filament cells, and therefore, indicates that the mAb is still viable.

From the entire collection of mAbs generated years ago against seaweed glycan epitopes only a few are still viable. However, the viable mAbs are valuable tools that should be cloned for further generation of viable hybridoma lines, making their availability possible. These are important tools, that should be added to the existing toolkit of mAbs that can be used to study brown seaweed cell walls (Chapter 2, 3, 4), and importantly now also adding some other mAbs to study red seaweeds, and also commercially valuable carrageenan polysaccharides.

5.5. Acknowledgements

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Penium margaritaceum:
Designing a unicellular model organism-based toolbox for investigating the impacts of hormones on cell walls
6.1. Introduction

The green lineage (Viridiplantae, Latin for “green plants”) is one of the major groups of photosynthetic eukaryotic organisms, and comprises the green algae (Chlorophyta and Streptophyta) and land plants (Embryophyta) (Figure 2 in the General Introduction) (Becker and Marin, 2009; Leliaert et al., 2012). The emergence of green algae on to land dates back to 480–360 million years ago and marks one of the most remarkable events in the natural history of life on the planet (Kenrick and Crane, 1997). During these events, the cell walls of the green algae that were ancestral to the land plants evolved to have diverse chemistries associated with the different extant taxa some of which have distinct similarities with the cell walls of modern land plants (Niklas, 2004). Comparative analyses of early-divergent land plants show that the cell walls of bryophytes (mosses, liverworts and hornworts), lycopodiophytes (lycopods or clubmosses), pteridophytes (ferns), and angiosperms (flowering plants) share some notable similarities in their cell wall polymer constituents with the cell walls of some Charophyceae green algae (CGA, Streptophyta), i.e., the closest living ancestor of land plants (Domozych et al., 2012; Eder et al., 2008; Eder and Lütz-Meindl, 2010; Popper and Fry, 2003; Popper and Tuohy, 2010; Popper et al., 2011; Sørensen et al., 2010; Sørensen et al., 2011; Timme et al., 2012). More specifically, recent studies have shown that three late divergent CGA orders, Charales, Coleochaetales and Zygnematales, have cell walls constructed of many of the same polymers found in land plant walls. This has led to the supposition that the ability to synthesize many of the wall components typical in land plants evolved during divergence within CGA (Sørensen et al., 2011). It is now also believed that the evolutionary events that led ancestors of the late divergent CGA to occupy progressively drier environments resulted in the ultimate transition to land habitats (Becker and Marin, 2009; Lewis and McCourt, 2004; Turmel et al., 2013).

The primary cell wall of land plants is composed of carbohydrates that associate in a complex manner, to dynamically modulate its architectural design and function during development and in response to environmental conditions and/or stress (Tan et al., 2013; Wang et al., 2015b; Xue et al., 2013). Elucidating the structural and functional properties of specific components, especially at the resolution of individual cells in complex multicellular tissues, is a very difficult task. This is primarily a result of current technological limitations that do not allow the resolution of the polymeric domains of individual cells within the complexity of a tissue. CGA, as the closest extant ancestors of land plants, have many
characteristics that make them interesting as *in vitro* models of whole plants. They are relatively easy to culture and have been extensively used as models for many aspects of plant research. Partly because they have such large cells, the multicellular *Chara* sp. and *Nitella* sp. have been fundamental to investigations of ion fluxes, membrane potential, and biophysics (Hope, 1961; Kitasato, 1968; MacRobbie, 1962). Other members of the CGA offer unique opportunities for elucidating wall dynamics such as cellular morphogenesis, effects of environmental stressors, cytoplasmic streaming, cell cycle and plant cell development (Domozych, 2012). One CGA member, *Penium margaritaceum*, is a unicellular desmid (order Zygnematales) that has become a useful model organism for studying primary cell wall dynamics in plants. *P. margaritaceum* is a simple and convenient organism that is easy to grow in the laboratory and has a robust physiology that makes it easy to handle in experimental studies (Domozych *et al.*, 2005; Domozych, 2012). It produces only a primary cell wall, has well-defined secretory mechanisms during wall development and, most importantly, can be live-labelled with monoclonal antibodies (mAbs) specific for wall polymer epitopes of land plants (Domozych *et al.*, 2014b). Labelled cells can be cultured for a period of time and co-labelling with different mAbs can be further performed in order to monitor specific events of cell wall polymer deposition and wall development (Domozych and Lambiasse, 2009). The growth characteristics of *P. margaritaceum* also allow rapid high throughput screening of wall polymer deposition activities when cells are grown in the presence of specific compounds such as enzymes, inhibitors, or hormones (Domozych *et al.*, 2011). Added to the versatility of this species, recent reports of high-throughput Agrobacterium tumefaciens-mediated stable transformation of *P. margaritaceum* has opened the possibility for functional genomics using this species, exponentially increasing its potential applications (Sørensen *et al.*, 2014).

The majority of the CGA including *P. margaritaceum* are externally covered by different types of extracellular polymeric substances (EPS) that form the substrate for biofilms. EPS is a highly complex polysaccharide that is a product of a poorly understood, but highly complex, biosynthetic machinery. Once secreted, the EPS provides physical protection against grazers, parasites, toxins, abiotic stressors such as temperature or osmotic stress; they also aid the sequestering of nutrients, enhancing cell–cell and cell–substrate adhesion (Domozych *et al.*, 2005; Domozych *et al.*, 2012). In *P. margaritaceum*, the EPS is secreted through a network of pore-like openings in the cell wall and forms a gel-like mucilaginous covering (Domozych *et al.*, 2007a; Domozych *et al.*, 2007b).
P. margaritaceum has a cylindrical shape with two semicells surrounding a central isthmus region, and undergoes a bi-directional cell and cell wall expansion (Domozych et al., 2014b) that is focused at the isthmus zone prior to cell division. Here, cellulose is first deposited and serves as the structural foundation for the subsequent deposition of other polymers including pectins. Pectins are a family of extremely complex and abundant galacturonic acid-rich polysaccharides including homogalacturonan (HG), rhamnogalacturonan I and rhamnogalacturonan II, and xylogalacturonan, that are present in the cell walls and in the extracellular matrix of land plants (Mohnen, 2008). P. margaritaceum walls are rich in highly methylesterified HG that is secreted in a single band and is embedded in the cellulose domain. As this polymer is displaced outward toward both poles, it is demethylesterified, most likely by pectin methylesterase (PME) enzymes, and is subsequently complexed with Ca$^{2+}$. The cross-linking with Ca$^{2+}$ is critical to maintain the rigidity of the cell wall, and results in projections that cover the cell surface, forming a distinctive lattice (Domozych and Lambiasse, 2009; Domozych et al., 2014b). This mechanism can be monitored using live cell immunolocalization techniques and complemented with enzymatic removal of specific polymers. These studies have provided new insights into the evolutionary pathway which resulted in the formation of the adhesive middle lamella in multicellular land plants (Domozych et al., 2014b). Additionally, expansion and morphogenesis mechanisms in P. margaritaceum represent a type of “polar growth”. This is a type of anisotropic expansion where the focal point of cell growth is located at an apex or tip usually observed in only a few plant cells such as pollen tubes, or root hairs (Hepler et al., 2001). Here, polar growth occurs in one focal plane, the cell lengthens in relation to width, resulting in positive displacement as the growing cell tip extends from the preexisting cell. This growth involves precise and coordinated interaction between the endomembrane system and the cytoskeleton network, because cell wall synthesis must be accurate in fast growing polar tips (Heath and Geitmann, 2000).

Plant hormones have an essential role in the regulation of plant growth, development and defense. Ten structurally different types of plant hormones have been recognized (Santner and Estelle, 2009; Wang et al., 2015a), and briefly listed below:
- Auxins play a central role in almost every aspect of plant growth and development by affecting cell expansion and division (Kepinski and Leyser, 2005);
- Abscisic acid (ABA) is known as the stress hormone, as it plays an important role in responses to environmental stress, mainly drought, by triggering stomata closure (Fujii et al., 2009);
- Cytokinins influence many aspects of plant growth and development, such as cell division, chloroplast development, leaf senescence, root and shoot growth and branching (Schaller et al., 2011);
- Gibberellins regulate multiple aspects of plant development, including flower induction, sex determination, pollen tube growth, seed and fruit germination (Murase et al., 2008; Phillips, 1998; Singh et al., 2002);
- Ethylene is a gaseous hormone that regulates processes such as fruit ripening, senescence, abscission, programmed cell death, and responses to biotic and abiotic stresses (Qiao et al., 2012; Wang and Irving, 2011);
- Brassinosteroids is a steroid hormone that regulate a wide range of developmental and physiological processes, such as elongation, photomorphogenesis, seed germination, flowering, male fertility, and senescence (Wang et al., 2012);
- Jasmonate, or jasmonic acid, control plant defense against wounding, herbivores, certain pathogens, and are also crucial for plant fertility and reproduction (Sheard et al., 2010);
- Salicylic acid is a phenol hormone that regulates immunity and induces systemic acquired resistance in the presence of pathogens (Fu et al., 2012);
- Strigolactones inhibits shoot branching and modulates seed germination and seedling photomorphogenesis (Zhou et al., 2013);
- Nitric oxide is involved in reproductive processes, control of development and in the regulation of physiological responses such as stomatal closure (Wilson et al., 2008).

The evolutionary time frame at which hormones became involved in the complex biological functions they are known to control is poorly understood. The detection of auxins in algae and its influence on development suggests an early evolutionary origin (Lau et al., 2009). The genome sequencing and mass spectrometry of the CGA Klebsormidium flaccidum showed that this alga has the ability to produce several plant hormones, including auxin, ABA, cytokinin, jasmonic acid and salicylic acid, along with possible genes encoding plant hormone receptors (Hori et al., 2014). Comparative genomics and phylogenetic analyses between several land plant and charophyte species have provided new and exciting insights into the origin and evolutionary mechanisms of plant hormone signaling pathways. Ethylene signaling pathway appears to have been preserved during evolution and already existed in the
common aquatic ancestor between land plants and CGA, prior to the colonization of land (Ju et al., 2015). Furthermore, auxin and cytokinin signaling probably have their origins in the CGA, while gibberellin signaling evolved after the divergence of bryophytes from land plants (Wang et al., 2015a). The auxin indole-3-acetic acid (IAA) has been found in several algal lineages and has effects on thallus development (Lau et al., 2009; Tarakhovskaya et al., 2007). IAA has been identified in the late divergent CGA Chara corallina, and evidence of polar auxin transport has also been reported for this species although the proteins responsible for its transport have not yet been identified (Boot et al., 2012). The in silico analyses of auxin signaling components revealed that microalgae do not appear to have the same major components necessary for auxin signaling that are found in land plants. However, their responses to auxin application indicate that microalgae may have alternative auxin signaling pathways (Lau et al., 2009). No reports were found regarding the presence of other hormones in microalgae, with the exception of a few reports on the presence of cytokinins in C. corallina (Zhang et al., 1989) and in members of the Chlorophyta microalgae (Ördög et al., 2004).

This chapter will not be focused on seaweeds, but it is important to note that some plant growth regulators are present in seaweeds and have importance for their development. Even though they are only distantly related to land plants, and to the CGA (Popper et al., 2011), several plant growth regulators have been reported to be present in seaweeds (Stirk and Van Staden, 2014). The auxin, IAA, and cytokinins have been identified in large brown seaweeds such as kelps (Stirk et al., 2004). IAA has also been found in the filamentous brown alga Ectocarpus siliculosus, where it shows a function related to cell–cell positional information, inducing a signaling pathway different from the one known in land plants (Le Bail et al., 2010). The different hormones and their biosynthetic pathways in seaweeds compared to land plants (Stirk and Van Staden, 2014) may be explained by the early divergence of seaweeds from the common algal ancestor ~1600 million years ago (Yoon et al., 2004). Furthermore, ancestral genes have also evolved, potentially to recognize different hormonal compounds in seaweeds, which can explain the difference in the biosynthetic pathways in seaweeds compared to land plants (Stirk and Van Staden, 2014). Few reports on endogenous gibberellins exist for seaweeds, while ABA has been found in many classes; some evidence suggests that seaweeds synthesize ethylene, although further research needs to be undertaken to confirm this; reports on brassinosteroids, jasmonates, salicylic acid and strigolactones are quite scarce (Stirk and Van Staden, 2014).
In the present study, the late divergent CGA and model organism *P. margaritaceum* was subjected to an initial screening with plant growth regulators in order to test their sensitivity and response to plant hormones. The cell morphology and cell wall microarchitecture were accessed by immunolocalization using mAbs directed against pectin epitopes, and the results were documented using a range of microscopy techniques. *P. margaritaceum* was found to be highly sensitive to all of the hormones tested and the pectin microarchitecture was significantly affected. The relation between auxins and their involvement in pectin HG demethylesterification and consequently in cell expansion and growth has previously been reported in land plant genera (Braybrook and Peaucelle, 2013; Peaucelle *et al.*, 2012). Results are discussed in light of these facts and highlight some of the possible implications, as well as the potential to use hormones as a toolbox applied to this late divergent microalga, for future research.
6.2. Materials and methods

6.2.1. Algal material

Penium margaritaceum Brébisson (Skidmore College Algal Culture Collection, clone number Skd#8) was maintained in sterile liquid cultures of Woods Hole Medium supplemented with soil extract, pH 7.2 at 18 ± 1°C in a photoperiod of 14 h light/10 h dark cycle with 108 µmol photons m⁻² sec⁻¹ of cool white fluorescent light. The cells were subcultured every week and cells from log-phase culture (7 to 14 days old cultures) were used for the studies.

6.2.2. Culture medium

Woods Hole Medium (WHM) (Nichols, 1973) was prepared from stock solutions listed in Table 6.1, and supplemented with a soil-water supernatant extract (1:20). The soil extract was prepared from garden soil without chemicals added. Soil (± 10 g) was put in a 1L conical flask; the flask was filled to ¾ with deionized water, and after boiling for 5 min, the solution was kept on a hot plate slowly simmering (~75°C) for 4–6 h, after which it was left to cool overnight. After repeating the boiling and simmering steps the solution was filtered. The final solution was stored at 4°C.

6.2.3. Plant growth regulators

The following commercially available plant growth regulators, which include hormones and inhibitors, were used in the experiments: 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma D7299), indole-3-acetic acid (IAA) (Sigma I5148), indole-3-butyric acid (IBA) (Sigma I5386), 3-chloro-4-hydroxyphenylacetic acid (CHPAA) (Sigma 224529), 1-naphthoxyacetic acid (1-NOA) (Sigma 255416), 2-naphthoxyacetic acid (2-NOA) (Sigma N3019), naproxen (Sigma N8280), 6-benzylaminopurine (6-BAP) (Sigma B3408), gibberellic acid (GA) (Sigma G7645).
Table 6.1. Stock solutions used to prepare WHM (Nichols, 1973).

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Mol. Weight (g mol⁻¹)</th>
<th>Stock in 1L dH₂O (g)</th>
<th>Concentration of the stock (mM)</th>
<th>Per liter media (ml)</th>
<th>Concentration in media (µM)</th>
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<td>MgSO₄·7H₂O</td>
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<td>1000</td>
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<tr>
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<tr>
<td>Trizma-HCl</td>
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<td>250.0</td>
<td>2000</td>
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<tr>
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Note: All stock solutions were stored at 4°C.

6.2.4. Hormone treatment

Cells from log phase cultures were incubated in sterile microwell plates containing growth medium with different concentrations of growth regulators. An initial screening for cell wall or general morphological changes was used to select appropriate concentrations of plant growth regulators for further studies. The concentrations selected were those which caused visible morphological changes without causing death of the cells. The concentrations chosen were: 0.07 mM 2,4-D, 0.06 mM IAA, 0.15 mM IBA, 0.20 mM 1-NOA, 0.20 mM 2-NOA, 0.20 mM CHPAA, 0.33 mM naproxen, 0.04 mM 6-BAP and 0.01 mM GA.
6.2.5. Live-cell immunolabelling

Cells were labelled with the mAbs LM18 or JIM7, which recognize epitopes present in pectins, using the live labelling procedure previously described by Domozych et al., 2011. The incubation steps were performed at room temperature with constant gentle rotation. Cells were collected by centrifugation at 1000 × g for 1 min, supernatant was discarded, and the cells were washed 3 × with fresh WHM. The washing steps included resuspension of the cell pellet with fresh medium followed by 10 s vortexing before centrifugation. This washing process ensures that the EPS is removed from the cells (Domozych et al., 2005). The final washed cells were blocked with 1 % (w/v) nonfat milk (Carnation) in WHM for 30 min, washed 3 × with WHM, and then incubated with 1:10 diluted hybridoma supernatant of the primary mAb solution in WHM. After 90 min, the cells were washed 3x with WHM, blocked for 30 min as above described, and after 3 more washing steps, incubated with goat anti-rat IgG conjugated with tetramethylrhodamine (TRITC) (Life Technologies™ Molecular Probes®, Eugene, OR, USA) secondary antibody (1:50 in WHM) for 90 min in the dark, after which the cells were washed 3 × with WHM before microscopy visualization. The staining of the nucleus was performed by incubating LM18-labelled cells with 1 mg ml⁻¹ SYTO®9 green fluorescent nucleic acid stain (Life Technologies™ Molecular Probes®, Eugene, OR, USA) in WHM for 5 min, in the dark, with constant gentle rotation, and with a final 3 × washing step with WHM.

6.2.6. Rhodamine phalloidin labelling

Rhodamine phalloidin labelling was performed as described for P. margaritaceum (Ochs et al., 2014). The incubation steps were performed at room temperature with constant gentle rotation. The cells were collected by centrifugation at 1000 × g for 1 min, supernatant was discarded, and the cells were washed 3 × with fresh WHM. The final pelleted cells were resuspended in 1 ml of WHM and 1 µl of 0.1 M MBS (3-maleimidobenzoic acid N-hydroxy succinimide ester, Sigma-Aldrich, St. Louis, MO, USA) stock solution was added, to a final concentration of 100 µM, and incubated for 30 min. Cells were collected by centrifugation, washed once with WHM, and resuspended in a freshly prepared fixative solution containing 5% (v/v) formaldehyde in a microfilament stabilizing buffer (MSB) [50 mM PIPES, 5 mM MgCl₂, 25 mM KCl, 5 mM CaCl₂, and 5 mM EGTA pH 6.9] for 30 min in the dark. The cells were centrifuged and washed 3 × with the MSB for 15 min each wash. The final pelleted
cells were incubated with a solution of 6.6 µM rhodamine phalloidin (Life Technologies™ Molecular Probes®, Eugene, OR, USA) previously reconstituted according to the manufacturer, in MSB containing 0.5 % (v/v) Tween-20 detergent, in the dark for 90 min. The cells were collected by centrifugation, washed with 3 × MSB, and resuspended with 100 µl MSB before microscopic observation.

6.2.7. Single cell culture

Individual cells were isolated with a dissecting microscope and re-cultured individually in sterile microwell plates containing WHM with added plant growth regulators. Microscopic image capture of these cells was recorded daily. Recovery experiments were also performed, by selecting cells that were subjected to a particular hormone and showed significant morphological changes, and growing them in fresh WHM (free from exogenous growth regulators or inhibitors). Control cells grown in fresh WHM were also individually cultured.

6.2.8. Monoclonal antibodies

MAbs generated against low esterified HG LM18, and against high esterified HG JIM7, were obtained from Plant Probes, Leeds, UK (http://www.plantprobes.net).

6.2.9. Microscopy

For routine-, differential interference contrast light microscopy (DIC-LM), and for some immunolabelling- observations, cells were viewed with an Olympus BX60 (Olympus America Inc., Melville, NY, USA) and images were captured with an Olympus DP70 digital microscope camera using DP Controller 3.2.1.276 software. Single cell observations were performed using an Olympus IX70 Inverted Microscope (Olympus America Inc., Melville, NY, USA) and images were captured with an Olympus DP71 digital microscope camera using DP Controller 3.2.1.276 software. Confocal laser scanning microscopy (CLSM) was performed with an Olympus FluoView™ 300 Confocal Microscope using Fluoview 5.0 with O3D software. Variable pressure scanning electron microscopy (VPSEM) images were captured with a JEOL JSM-6480LV Scanning Electron Microscope (JEOL USA, Inc., Peabody, MA, USA) using SEM Control User Interface 7.05 software. Images were assembled with Adobe Photoshop Elements 11.0 (Adobe Systems, San Jose, CA, USA) software.
6.3. Results

6.3.1. General morphology

*P. margaritaceum* is a unicellular desmid with a cylindrical shape, and is composed of two equal semi-cells (Fig. 6.1A), each enclosing a large multi-lobed chloroplast that surrounds a central nucleus, visible when labelled with SYTO9 (Fig. 6.1D). This central zone is slightly narrowed and is named the isthmus. The isthmus is 17 μm wide while the length may vary from 150–250 nm depending on the stage of pre-division expansion. The cell wall forms an outer layer with lattice-like projections, clearly visible with VPSEM imaging (Fig. 6.1B). The cell wall of the isthmus contains a region of 1–2 μm that lacks the lattice projections and appears to be a smooth band (Fig. 6.1H). This area corresponds to a band that can be visualized with JIM7 immunolabelling (Fig. 6.1F). This mAb recognizes the epitope GalA1-4MeGalA1-4MeGalA1-4MeGalA1-4MeGalA1-4GalA (Clausen et al., 2003; Knox et al., 1990; Willats et al., 2000) in relatively high esterified HG, which co-localizes with the isthmus zone. In contrast, labelling with LM18, which recognizes the epitope α-GalA(1-4)-α-GalA(1-4)-α-GalA (Verhertbruggen et al., 2009) in relatively low esterified HG, shows the generalized labelling of the cell (Fig. 6.1C), except in the narrow band of the lattice-free isthmus zone (Fig. 6.1G). Rhodamine phalloidin allows visualization of the actin cytoskeleton and its detailed distribution has been reported for *P. margaritaceum* (Ochs et al., 2014). Arrays of actin filament bundles are aligned parallel to the long axis of the cell (Fig. 6.1E) and line the nuclear envelope (Fig. 6.1I).
Figure 6.1. Structural features of the cell and cell wall of *P. margaritaceum*. A. DIC-LM image of a typical cell. The two equivalent-sized semi-cells (SC) are found on either side of the isthmus zone (arrow). B. VPSEM-generated image of the cell that highlights the network of projections covering the cell wall surface. Two band-like regions that run perpendicular to the cell length can be observed: the isthmus (arrow) and a wall thickening (*). C. Live LM18-labeled cell observed with CLSM. The projections of the outer cell wall surface are highlighted; the dark band in the isthmus represents the zone where new cell wall material is added. D. SYTO9-labeled cell co-localized with LM18 and with the autofluorescent chloroplast (red) highlights the typical position of the nucleus (blue) in the isthmus zone (arrow). E. Rhodamine Phalloidin-labeled cell observed with CLSM shows the cortical actin network. Numerous actin filament bundles run parallel to the long axis of the cell. F. Live JIM7-labeled cell observed with CLSM merged with the chloroplast. A thin band of homogalacturonan secretion is found in the isthmus (arrow). G. Magnified LM18-labeled cell showing the isthmus zone (arrow) where no labeling is observed and showing the wall projections of the SC. H. Magnified VPSEM image of the isthmus (arrow) in the early stage of cell expansion. The isthmus zone possesses a narrow band devoid of the outer cell wall projections that are found on each SC. I. Magnified CLSM image of Rhodamine Phalloidin-labeled cell shows the actin filament bundle lining the nuclear envelop (arrow), and the bundles running along the cell cytoplasm. *Scale bars:* A–F, I = 20 µm, G, H = 10 µm.
6.3.2. Hormones

The initial screening for hormone sensitivity showed that *P. margaritaceum* morphology and cell wall microarchitecture were very responsive to the hormones and hormone inhibitors applied. Profound changes in cell morphology were observed with all the compounds tested, the cells lost their integrity and different ranges of shapes were observed. Generally, there was no clear differentiation between the morphological changes observed for individual hormones. Rather, all of the different hormone treatments appeared to induce a variety of different shapes.

The auxins tested, IAA, IBA and 2,4-D induced a variety of shapes that included notable elongation and swelling of the cells. DIC-LM imaging enabled screening of different shapes. The distinct forms observed included those with notable elongation (Figs. 2A, 3A, E, 4A), extreme swelling that caused the cells to lose their usual shape, and become almost rounded (Figs. 2B, C, 3D, 4C), “lemon-like” shaped cells (Figs. 2D, 3B, 4D), and cells where only parts of the cell were swollen (Fig. 6.2E, 6.3C, 6.4B). VPSEM enabled the surface of the cells to be visualized. The typical lattice projections of the elongated cells were noticeably affected as they looked faint compared to normal cells (Fig. 6.2F, 6.3F, H). In areas where the swelling is pronounced, it was observed that the HG layer was disrupted (Fig. 6.2F, 6.3G, 6.4E, G). This may have led to weak points in the cell wall, causing the cell to swell in those areas, sometimes in such an extreme way that they appeared to be close to bursting (Fig. 6.2G, 6.4F, G). Some cells were smaller than usual, but retained a typical lattice (Fig. 6.2I). However, slight disruption of the lattice, coinciding with curved features in the cells, was also observed (Fig. 6.3H, I); some cell wall constrictions in the isthmus zone were also sporadically observed (Fig. 6.2H). No JIM7 labelling was observed for the cells that presented the most dramatic morphotypes. Labelling corresponded to the isthmus zone in less pronounced shapes when the cells looked elongated, asymmetric, or with smaller swollen areas (Fig. 6.3J). Examination of the cell wall pectin using LM18 immunolabelling showed that the outer lattice was intact in the cells that did not have pronounced morphological changes (Fig. 6.2J, 6.3K). However, it was affected in cells that were notably elongated (Fig. 6.3M, 6.4H). Disruption of the outer HG-rich layer was often observed in expansion zones (Fig. 6.2K, 6.3L, 6.4H, I) and in dwarf cells (Fig. 6.2L); sheets of HG were also seen to slough off the inner wall layer (Fig. 6.4J).
**Figure 6.2.** Morphological changes observed after treatment with 0.07 mM 2,4-D. **A–E.** DIC images of treated cells. Note the elongation and pronounced swelling of some cells (**A, E**), while others are dwarf (**B**) and swollen (**C, D**). **F–I.** VPSEM imaging shows details of the cell wall surface. The disruption of the HG-rich outer wall layer lattice (**F**) is visible where swelling areas are pronounced (**i, ii**); the inner wall is exposed when the cell is extremely swollen (**G**). Cells that are constricted in the isthmus area have a thinner lattice (**H**), while dwarf cells (**I**) are not affected. **J–L.** LM18-labelling shows the pectin lattice. Note the disruption of the lattice in the isthmus zone (arrow) of curved (**K**) and dwarfed cells (**L**). **M–O.** Rhodamine phalloidin labelling shows the disorganization of the actin network of the cells that show severe elongation and swelling. *Scale bars: A–D, M–O = 25 µm, E, F, H, J–L = 50 µm, i, ii, G, I = 10 µm.*
Figure 6.3. Morphological changes observed after treatment with 0.06 mM IAA. A–E. DIC images of treated cells. Note the severe elongation (A) and pronounced swelling patterns observed (B–E). F–I. VPSEM imaging shows details of the cell wall surface. Elongated cells show some changes in the wall surface (F), swelling areas have a disruption of the HG-rich outer wall layer lattice (G), well visible in detail (i). Some cells start swell and curve at one tip (H); smaller cells have a disruption of the lattice in the isthmus zone (arrow) (I). J. Merged image of a JIM7-labeled cell with DIC. K–M. LM 18 labelling shows the pectin lattice. Note the disruption of the lattice in the isthmus zone (arrow) of smaller cells (L); elongated cells have gaps in the labelling (M). N–P. Rhodamine phalloidin labelling shows the disorganization of the actin network of the cells that lost shape integrity (N) and look swollen (O), although no significant changes occur in the network in cells only with constrictions of the wall (P). Scale bars: A, C–G = 50 µm, i = 10 µm, B, H–P = 20 µm.
Figure 6.4. Morphological changes observed after treatment with 0.15 mM IBA. A–D. DIC images of treated cells. Note the severe elongation (A) and pronounced swelling patterns observed (B–D). E–G. VPSEM imaging shows details of the cell wall surface. The swelling of the cells and disruption of the HG-rich outer wall layer lattice is visible (E) (arrow), leaving the smooth inner wall exposed (F, G) (arrow). H–J. LM18 labelling shows that the pectin lattice is affected in swollen areas (arrow) (H). Note the disruption of the HG layer in expansion zones (arrow) (I), and where swelling is extreme (arrow) (J). K–M. Rhodamine phalloidin labelling shows the disorganization of the actin network of the cells that lost shape integrity, especially when they are very swollen (L, M). Scale bars: A, E, F, H–J = 50 µm, B–D, G, K–M = 25 µm.
Rhodamine phalloidin showed that the actin cytoskeleton was also affected by the auxin treatment. Random distribution of microfilaments was associated with all of the *P. margaritaceum* cells with observed morphological changes, especially in the most swollen (Fig. 6.2M, N, O, 6.4L, M) cells. The cortical actin network was rearranged into a random network even in less dramatic cell shapes, such as curved or cells with little swollen areas (Fig. 6.3N, O, 6.4K). Few cells were able to retain the integrity of the actin bundles (Fig. 6.3P).

The auxin influx inhibitors NOA and CHPAA and the ABA inhibitor naproxen were found to induce severe changes in cell morphology. DIC imaging revealed that these chemicals induced a range of bizarre shapes ranging from extreme elongation (Fig. 6.5A, 6.6A, B, 6.7A), to other shapes (Fig. 6.5B, D, 6.6C, 6.7B, D), profound swelling (Fig. 6.5C, 6.7C, E), and dwarfism (Fig. 6.5E). VPSEM imaging showed that the outer HG layer was disrupted in expansion zones (Fig. 6.5F, G, 6.6D, E), and in extremely swollen cells (Fig. 6.7F), but dwarf cells seemed to have a normal lattice, although the isthmus zone was not well defined (Fig. 6.5H, 6.7G). SYTO9 labelling showed that the curved, or somewhat swollen, cells, only had one nucleus (Fig. 6.5I), indicating that the wall expansion and further swell occurred before mitosis. Two nuclei were observed for the most elongated and deformed cells showing that the cell went through one mitosis step, but no cytokinesis (Fig. 6.5L). Similarly to the auxin treated cells, JIM7 did not label the cells exhibiting the most dramatic morphotypes. Labelling was observed in the tip of an elongated and swollen cell (Fig. 6.5J, K), in contrast to the usual labelling in the central isthmus zone. The typical JIM7 labelling appeared to be misplaced in elongated cells (Fig. 6.7H), and some showed several scattered labelling bands (Fig. 6.6F). LM18 labelling showed that elongated cells *per se* displayed a typical labelling pattern (Fig. 6.5M), but disruption of the outer HG-rich layer in expansion zones was observed, as soon as there was swelling of the cell, so different labelling patterns were detected (Fig. 6.5N, O, 6.6G, H, 6.7I, J, K, L); visible sheets of HG slough off the inner wall layer (Fig. 6.5P). Rhodamine phalloidin showed that the actin cytoskeleton is also affected. With the exception of the dwarf cells, which seemed to have a relatively organized actin network (Fig. 6.5Q), the actin microfilaments were found randomly distributed in the cells (Fig. 6.5R, 6.7M, N, O).
Figure 6.5. Morphological changes observed after treatment with 0.20 mM NOA. A–E. DIC imaging shows extreme changes and severe elongation (A), combined with swelling (B–D); dwarf cells were also observed (E). F–H. VPSEM imaging shows details of the cell wall surface. Elongated cells show a less pronounced lattice (F) in the areas where the cell is stretched (i); swelled areas show the disruption of the HG-rich outer wall layer lattice (G) (arrow); while dwarf cells (H) are not affected. I, L. SYTO9-labeled cells co-localized with LM18 shows the presence of one nucleus in swollen cells (I), and two nucleus in the more elongated cells that underwent mitosis, but not cytokinesis (L). J. DIC
image and the correspondent JIM7-labeled cell (K) showed a thin labelling area in one edge of the cell (arrow). M–P. LM18 labelling shows the pectin lattice of an elongated cell (M), swollen areas do not label (N), rounded-shaped cells label but the typical punctuated lattice is missing (O). Severe disruption of the pectin lattice is observed outside the isthmus zone (P). Q–R. Rhodamine phalloidin labelling shows that dwarf cells apparently have a normal actin network (Q), while swollen cells that lost shape integrity have a disorganized actin network (R). Scale bars: A, E, G, H, i, J, K, O, Q, R = 20 µm, B–D, F, I, L–N, P = 50 µm.

Figure 6.6. Morphological changes observed after treatment with 0.20 mM CHPAA. A–C. DIC imaging shows severe elongation and deformation of the cells. D–E. VPSEM imaging shows details of the cell wall surface. Elongated cells have a less pronounced lattice (D, E, arrow); dwarf cells have a deformed shape and the lattice is disrupted (i). F. JIM7-labelling is found throughout the elongated areas of the cell (arrow). G–H. LM18-labelling shows that the pectin lattice is affected at the swollen isthmus zone (arrow) (G). Note the disruption of the HG layer in expansion zones (arrow) (H). Scale bars: A–D, E = 50 µm, i = 10 µm, F–H = 20 µm.
Figure 6.7. Morphological changes observed after treatment with 0.33 mM Naproxen. A–E. DIC imaging shows extreme changes in cell integrity. F, G. VPSEM imaging of rounded-shaped cells show the disruption of the HG-rich outer wall layer lattice (F), while dwarf cells (G) are not affected. H. DIC image merged with a JIM7-labeled cell shows a single labelling band (arrow). I–L. LM 18 labelling shows the pectin lattice. Note the irregular labelling at swollen areas (I, J), and at irregularly shaped cells (K, L). M–O. Rhodamine phalloidin of irregularly shaped cells shows a disorganized actin network. Scale bars: A, B, D, F, H–J = 50 µm, C, E, K–O = 25 µm, G = 10 µm.
Similarly to the characteristics described above, cytokinin, 6-BAP, and gibberellic acid (GA) treatment also resulted in pronounced loss of shape integrity. 6-BAP resulted in generally swollen features (Fig. 6.8A, B, C, D), and GA led to a mixture of elongation (Fig. 6.9A), pseudofilament formations (Fig. 6.9B), and swelling (Fig. 6.9C, D, E, F). The VPSEM showed disruption of the outer HG-rich layer in swollen and expanded cells, scattering of the lattice (Fig. 6.9H), and a smoother appearance of the surface of the inner wall layer in the swollen areas where the lattice disappeared (Fig. 6.8E, 6.9I). A difference observed in relation to the other hormones was that lattice was also disrupted in the dwarf-sized cells, despite their small size (Fig. 6.8F, 6.9G). The HG-rich wall layer of the swollen cells was only labelled irregularly with LM18 (Fig. 6.8G, H, 6.9J, L, M), and isthmus zone of the dwarf cells appeared not to be in its typical central position, although no disruption of the HG-rich layer was observed (Fig. 6.8I, 6.9K). Rhodamine phalloidin showed that, similarly to the other hormone treatments, the actin cytoskeleton was affected. With the exception of the dwarf cells, which had a relatively organized actin network (Fig. 6.8L), the actin microfilaments were found randomly distributed in the cells (Fig. 6.8J, 6.9N, P), being more pronounced in very swollen cells where the filament bundles seemed to distribute around the cells instead of parallel to the axis of the cell (Fig. 6.8K, 6.9O).
Figure 6.8. Morphological changes observed after treatment with 0.04 mM 6-BAP. A–D. DIC imaging show the different morphological changes observed in the cells. E–F. VPSEM imaging shows details of the cell wall surface. Elongated cells have a less pronounced outer layer lattice (E) in the areas where the cell is swollen (i); dwarf cells have a deformed shape and the lattice is disrupted (F). G–I. LM18-labeling of the pectin lattice. Note the irregular labeling of the swollen cells (G, H), while dwarf cells have a typical labeling pattern (I). J–L. Rhodamine phalloidin-labeling of irregularly shaped cells shows a disorganized actin network (J, K) while the actin network of dwarf cells does not appear to be affected (L). Scale bars: 20 µm.
Figure 6.9. Morphological changes observed after treatment with 0.01 mM GA. A–F. DIC imaging show the different morphological changes observed in the cells. Some cells are very elongated (A), others form pseudofilaments (B), while others show extreme swelling patterns (C–F). G–I. VPSEM imaging shows details of the cell wall surface. The disruption of the HG-rich outer wall layer lattice (G, H) is visible where swelling areas are pronounced (i, ii); elongated and deformed cells have a thinner lattice (I). J–M. LM18-labelling of the pectin lattice. Note the irregular labelling of curved and swollen cells (J, M); dwarf cells have a typical labelling pattern (K); only one semi-cell was labelled in some cases (L). N–P. Rhodamine phalloidin labelling shows the disorganization of the actin network of the cells that show severe swelling. Scale bars: A, B = 50 µm, C–P = 25 µm, i, ii = 10 µm.
6.3.3. Single cell culture

After 10 days treatment with the hormones, some of the individual cells exhibiting morphological changes were selected, cultured in fresh growth medium (lacking exogenous hormones or inhibitors), and the recovery of the cells recorded daily with bright field inverted microscopy. Figure 10 shows some examples of how the cells recovered over 6 days. Cells grown in unmodified WHM divide in a defined timeframe so that by day six one cell has divided to become a total of 16 daughter cells (Fig. 6.10A).

Hormone-treated cells, however, behave differently. The cell cycle of IAA-treated cells is affected. The cells divide slowly and the daughter cells were not able to keep the normal shape. By day 6 only 4 cells were observed, one died, and morphological features of the other daughter cells remained affected, looking curved in the isthmus zone (Fig. 6.10B). These cells were re-cultured in fresh WHM. After 13 days of recovery the daughter cells were continuing to divide but did not fully recover the typical *P. margaritaceum* shape. Other treated cells, e.g. with NOA, with swelling at the isthmus zone were able to divide. This was also found to be at a slower rate than non-treated cells. Furthermore, on reculturing in fresh WHM the tip of the daughter cells remained swollen. After 6 days, the cells divided again, and a total of 8 cells were observed. The daughter cells looked normal, while the initial mother cells kept the original swollen tip (Fig. 6.10C). Cells that suffered severe morphological changes, such as pronounced curved shape (Fig. 6.10D), or when the cells were swollen to a point where they looked almost rounded (Fig. 6.10F), lost the ability to divide, and died.

Cells with pronounced elongation and an irregular shape, however, showed a different recovery behavior. Severe elongation observed with naproxen-treated cells (Fig. 6.10E) elongated further and one of the tips divided into a second cell on day 3. On day 6 the elongated mother cell died and the daughter cell divided into two normally shaped cells. Severely elongated cells observed with gibberellin treatment (Fig. 6.10G) also divided from one of the tips of the semicells, and the daughter cell divided normally. The initial mother cells continued to elongate, and more daughter cells grew from the same tip, on day 6. In contrast to the naproxen-treated cells, the elongated mother cell did not die and retained the ability to divide.
In an attempt to observe the daily behavior following hormone treatment single cells were selected from a normal WHM culture and placed in multi-well plates in the presence of the hormones. It was difficult to follow changes though, because most of the cells died between days three and six. Nonetheless, a behavior common to all the different treatments was that after four days the rhythm of cell division was slowed. After 7 days, the first morphological changes were observed. Some cells died, while others looked normal, others began to undergo changes in morphology or failed to undergo cytokinesis, causing the irregular elongations. Several tiny cells were also observed, matching the results described above for the different hormones.
6.4. Discussion

Results presented in this chapter show that *P. margaritaceum* cell wall expansion and growth pattern is clearly disturbed by the application of exogenous hormones and hormone inhibitors. The range of morphotypes observed is an indication that the delicate machinery responsible for cell wall deposition, expansion, and division was severely disturbed by the treatments. The balance between the extensibility of the cell wall and the forces exerted on the wall by turgor pressure dictates plant cell growth. This balance can be controlled by changing either of the parameters but in most of the reported cases changes in growth have been found to result from modifications in cell wall extensibility (Cosgrove, 2005). Combining extensibility with strength seems to be a paradox. However, wall strength is determined by the number and strength of load-bearing compounds (such as cellulose microfibrils) and a wall relaxation mechanism that can break these bounds and transfer the load to new bonds would allow cell wall relaxation and cell expansion to occur, with or without changes in wall strength (Peaucelle et al., 2012). Endotransglycosylase enzymes such as xyloglucan endotransglucosylases (XETs) cut and ligate glycans together, mediating the integration of new polysaccharides into the existing network, also loosening the cell wall (Cosgrove, 2005; Van Sandt et al., 2007).

6.4.1. Impact of plant hormones on pectins

Auxins have been reported to affect the rigidity (elasticity) of plant tissues (Cleland, 1958, 1971; Tagawa and James, 1957); the relation between tissue rigidity and growth is also described (Braybrook et al., 2012; Kierzkowski et al., 2012; Peaucelle et al., 2011; Peaucelle et al., 2012). Studies focused on algal growth (Domozych et al., 2014b; Peaucelle et al., 2012) have shown that changes in pectins chemistry can influence cell growth by altering wall rigidity. Recent work on *Arabidopsis thaliana* showed that auxins have a direct influence on growth by triggering the demethylesterification of the pectic HG, which causes a reduction in cell wall rigidity; furthermore, the demethylesterification of pectin alone is enough to induce local tissue growth in meristems (Braybrook and Peaucelle, 2013). Steroid hormones are known to stimulate pollen tube growth (Ylstra et al., 1995) and plant growth regulators e.g. auxins, cytokinins and gibberellins are reported as important players in pollen tube- (Singh et al., 2002; Wu et al., 2008a) and in root hair- growth (Lee and Cho, 2013;
Overvoorde et al., 2010; Pitts et al., 1998), although their physiological role has been well established only for auxins. For example, IAA stimulates the synthesis of pectin in pollen tubes and increases PME activity, resulting in cell wall extension (Wu et al., 2008b). It is not therefore surprising that auxins had such a strong effect on the *P. margaritaceum* cell wall and growth patterns. The balance between the highly methylesterified HG that is secreted in the isthmus zone and the subsequent demethylesterification and complexing process with Ca\(^{2+}\), that results in the typical projections that cover the cell surface in a distinctive lattice, was clearly disturbed. The cross-linking with Ca\(^{2+}\) is essential to maintain the rigidity of the cell wall (Domozych et al., 2007a; Domozych and Lambiasse, 2009). A model for wall expansion suggested in *C. corallina* (a member of the CGA) suggests that newly deposited HG chelates Ca\(^{2+}\) from existing Ca\(^{2+}\)-HG complexes, preferentially from the load-bearing bonds that are distorted by the wall tension (Boyer, 2009). The loss of these cross-links cause the cell wall to relax resulting in irreversible wall expansion. New Ca\(^{2+}\) then cross-links the wall and the extension decelerates; new HG is deposited and the cycle repeats itself (Boyer, 2009; Proseus and Boyer, 2012).

*P. margaritaceum* has also been used as a model for pectin assembly in the cell wall (Domozych et al., 2014b). Studies on pollen-tube growth also indicate that turnover of the load-bearing Ca\(^{2+}\)-HG cross-links facilitates turgor-driven mechanical deformation of the wall. Furthermore, pectin production is controlled by its deposition and demethylesterification (Rojas et al., 2011). Observations show that hormones, including but not limited to auxins, interfere with cell expansion. The lattice was disrupted in cells which were stimulated by the hormone treatments to elongate significantly (Fig. 6.2F, 6.3G, 6.5F, 6.6E, 6.8E, 6.9I) and swelling (Fig. 6.2G, 6.4E, G, 6.5G, 6.7F, 6.8F, 6.9G, H); the areas of cell expansion lacked the lattice. These lattice-free areas showed deformations; potentially regions devoid of lattice are vulnerable to turgor pressure and therefore lose the ability to maintain their shape. As explained above, auxins trigger demethylesterification of HG, and cell wall expansion. Auxins, similarly to observed for the other hormones, may cause deregulation of HG deposition into the isthmus zone and demethylesterification of HG and/or cross-linking with Ca\(^{2+}\) was affected causing the cell wall to deform, extend and swell. For some elongated cells, the JIM7 labelling pattern was not restricted to the isthmus zone, but extended along the cell, indicating the presence of highly methylesterified HG (Fig. 6.6F). However, an important point to mention is that consistent morphological or labelling patterns that could be unequivocally assigned to specific treatments were not observed. This may have resulted from the fact that the cells within the cultures were not synchronized i.e. they were
not at the same stage of the cell cycle, and hormones may have caused different responses at different stages of the cell cycle. For example, a cell in the process of division and undergoing cell wall expansion to give rise to two daughter cells might be particularly susceptible to hormone-treatments, resulting in elongation and further swelling of the expanding zones (Fig. 6.2F, 6.3G, 6.5F, 6.6E, 6.8E, 6.9I); extreme cell elongation without swelling was also observed. Labelling of the nuclei showed that the most elongated cells seem to have gone through mitosis, because two nuclei could be observed with SYTO9 labelling, but instead of undergoing cytokinesis, the cells elongated and stayed connected to each other (Fig. 6.5L). This may have resulted from hormone treatment of cells in pre-mitotic stages. The hormone-treatment could weaken the isthmus zone, by impacting pectin deposition, and therefore result in the observed small cells with disrupted lattices, causing swelling, or angles, but not elongation (Fig. 6.2G, 6.3I, 6.4G, 6.6Di, 6.7F, G, 6.8F, 6.9G). Only one nucleus was observed for these cells (Fig. 6.5I), showing that the disruption of the cell wall occurred before mitosis. Some cells also seem to have responded to the hormone-treatments with an pronounced increase in the cell division rate; the cells divided before they could undergo expansive cell growth and reach the normal length, resulting in dwarfed cells (Fig. 6.5E, H, O, 6.7F, G, 6.8F, 6.9E, K).

6.4.2. Cytoskeleton network

Polar growth, and cell wall deposition are controlled by the cytoskeleton (Kropf et al., 1998). This process implies that cell wall synthesis must be accurately processed in the growing region, involving a precise and coordinated relationship between the endomembrane system and the cytoskeleton network (Heath and Geitmann, 2000). It would therefore be expected that any agent that interferes with this delicate process will affect the entire machinery responsible for the maintenance of this equilibrium. The relation between actin and auxins seems to be bidirectional and highly complex. Auxin-stimulated growth in rice coleoptiles has been shown to be accompanied by the de-bundling of the actin filaments into finer strands (Holweg et al., 2004; Waller et al., 2002). In contrast, auxins that are transported in a polar fashion (such as IAA) but not other auxins (such as 2,4-D) have been reported to restore actin configuration in cells that have de-bundled actin filaments (Nick et al., 2009). The mechanism of action of auxin influx inhibitors, such as 1-NOA, 2-NOA and CHPAA, is not clear although it is speculated that they may act through actin-mediated vesicle trafficking processes in eukaryotic cells (Lanková et al., 2010). As shown for tobacco
BY-2 cells, actin dynamics seems to be under both genomic and non-genomic control of auxin itself (Maisch and Nick, 2007; Nick et al., 2009). 1-NOA- and 2-NOA-application on tobacco BY-2 cells was seen to block the membrane dynamics, with subsequent detrimental effects on the polarity of cell division (Lanková et al., 2010). Interestingly, Dhonukshe et al., (2008) found that higher concentrations of auxin influx inhibitors can affect the vesicles that are transported towards the plasma membrane by a mechanism dependent on actin dynamics. The reported influences of different hormones on actin dynamics are evident in the results. In fact, all of the hormones tested seem to induce disorganization of the actin filament bundles which in P. margaritaceum normally run parallel to the long axis of the cell (Fig. 6.1E, I). Hormone treatment resulted in the random distribution of actin bundles which was particularly pronounced in the very swollen cells (Fig. 6.2N, O, 6.4M, 6.5R, 6.9P). In some cells the filament bundles appeared to be distributed in distinct bands around the cells (Fig. 6.2M, 6.8K, 6.9O). Microtubule network inhibitors disrupt cellulose deposition and pectin distribution in P. margaritaceum cell walls, causing thinning of the inner cellulosic wall layer, major disruption of the HG-rich outer wall lattice, and swelling at the central isthmus zone (Domozych et al., 2014a). These observations correlate with the results. Nevertheless, it is difficult to confirm whether the hormones directly induced changes in the cytoskeletal network, which lead to the HG-rich pectin disruption and cell morphology, or if the hormones affected pectin deposition in the central isthmus zone, leading to wall loosening and ultimately to the observed morphological changes, or both. In this case, the hormones would have influenced both the cell wall and the cytoskeletal network, and this associated with the different life cycle stages of the cells (because the cells were not synchronized), would explain the variety of morphological changes observed.
6.4.3. Single cell culture

The observations for the single cell cultures, isolated from hormone-treated cultures, showed that the cells that survive hormone treatment can still grow and divide (Fig. 6.10). The daughter cells retain part of the wall deformations that they inherit from the mother cell, but subsequent daughter cells show normal morphology and are able to complete the cell cycle normally. This indicates that the hormone treatments cause dramatic and permanent changes to cell morphology. However, they did not appear to have induced genetic mutations because the daughter cells appear normal (at least with respect to cell morphology and cell cycle). Exceptionally after 13-days recovery from IAA-treatment (Fig. 6.10B), although they continue to divide, the daughter cells do not fully recover the normal *P. margaritaceum* morphology. A possible genetic influence cannot be supposed; instead, IAA may have been stored within the cells and still affect the daughters in the subsequent generations. Eventually after each cell division it will become less concentrated to a point where the cells can cope with it and regain their usual morphology. The results presented in this chapter further highlight that *P. margaritaceum* is an excellent unicellular model. It has been used to study primary cell walls, which is extremely useful and adds new insights to evolutionary questions (Domozych and Lambiasse, 2009; Domozych *et al.*, 2011; Domozych *et al.*, 2014b), and a model to study cortical cytoskeletal network dynamics (Ochs *et al.*, 2014). This closely-related ancestor of land plants is presented as a model for the study of plant growth regulators in particular for investigation of responses to the stimuli and modes of action on the cell wall, cytoplasmic streaming, and cytoskeletal dynamics. The existence of endogenous hormones has not yet been unambiguously proven for *P. margaritaceum*. However, bioinformatics coupled to transcriptomics data applied to a number of species, including *P. margaritaceum*, have suggested that the genetic machinery necessary for the signaling pathway of auxins, cytokinins, strigolactones (Wang *et al.*, 2015a), and ethylene (Ju *et al.*, 2015) have older roots than the other hormones, and originated in the CGA lineage, prior to land colonization by plants, remaining almost unchanged across species.

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7

General Conclusions
The work presented in this thesis shows the results obtained from *in vitro* and *in situ* immunohistochemical approaches applied to algal cell walls. The main species chosen for investigation were the brown seaweeds *F. vesiculosus* and *L. digitata*. These species are very common on the Irish coastline and have a significant ecological and economical value, particularly as some of their cell wall components are extracted for their bioactivity, and are widely used as major sources of gellifying, emulsifying and stabilizing agents (alginites), as well in pharmaceutical industries (sulfated fucans) (Cardozo *et al.*, 2007; McHugh, 2003). Brown seaweeds (class Phaeophyceae) belong to the independent eukaryotic lineage Heterokontophyta (Cock *et al.*, 2010) which are only distantly related to land plants and to the red- and green-algal groups (Charrier *et al.*, 2012). Therefore during the course of evolution, adaptive diversification has led to their walls containing a diversity of cell wall components which differ significantly from those of the other algal groups (Popper, 2008; Popper *et al.*, 2011).

The use of algae, and more specifically the exploitation of a wider array of components, within a commercial context demands a deeper investigation of the factors that affect the production of such important compounds. Such factors, whether in natural or in cultivated conditions, need to take into account that bioactivity is extract-specific and varies within species (at tissue and cellular levels), with location, growth conditions, and season (Stengel *et al.*, 2011). Furthermore, the comparative lack of tools for the study of algal cell walls has resulted in a gap between what is known regarding the composition and structural features of algal cell walls compared to land plants. While a broad range of mAbs directed against almost all types of cell wall polysaccharides exists for land plants (Pattathil *et al.*, 2010), tools that allow detailed localization of specific compounds at a cellular level are almost non-existent for algal wall compounds. Exceptions are the range of mAbs (four in total) recently generated against seaweed fucans with different levels of sulfation (Torode *et al.*, 2015). Unfortunately, for several reasons including lack of viability of lines generated nearly 40 years ago, insufficient information regarding their epitope recognition, or because the cultures were no longer stored, most of the mAbs that were generated previously for seaweeds are not currently available (Green *et al.*, 1993; Vreeland *et al.*, 1988, 1992). Consequently, the perspective that brown algae have some cell wall polysaccharides that contain epitopes similar to those existing in land plants, and can, thus, be recognized by the land plant cell wall glycan-directed mAbs is tantalizing. If such a perspective is confirmed, a new range of already existing, and relatively well characterized, probes can be channeled for the study of seaweed cell walls.
The application of mAbs to the study of algal cell walls was further investigated in a more physiological context using the unicellular desmid and model organism *P. margaritaceum*, which was used in a pilot study on the effects of plant hormones on cell walls. This species belongs to the Zygnematales, a late divergent group within the CGA, and the most closely related living group to land plants (Sørensen *et al.*, 2011). This species has proven a good model system for the study of the primary cell wall (Domozych *et al.*, 2011); furthermore, recent reports on the existence of hormone pathways in members of the CGA provided new and exciting insights into the evolution of plant hormone signaling pathways (Ju *et al.*, 2015; Wang *et al.*, 2015). If *Penium* has the ability to respond to plant hormone treatments, it can potentially be used for a future hormone tool box.

**Presence of land plant cell wall epitopes in *F. vesiculosus***

The localization of specific cell wall components was investigated in the brown seaweed *F. vesiculosus* using a multitask approach that involved the use of a collection of 155 mAbs generated against plant cell wall epitopes. These mAbs were used to perform *in vitro* glycome profiling of cell wall extracts and *in situ* immunolocalization on different vegetative and reproductive thallus parts. The mAbs that belong to the RG-I/AG clade recognized and bound to epitopes present in different tissues, and they were divided into four groups, according to the immunolocalization patterns observed. This study revealed that some epitopes present in vascular plant cell walls are also present in brown seaweeds. Furthermore, the different *in situ* localization patterns of the mAbs suggested that they probably detect different epitopes that are present in structurally different galactans. Also, it seems likely that some of these epitopes play important roles in sexual reproduction, as they were restricted to the oogonia and antheridia cell walls. These results are a major breakthrough, because they show for the first time the presence of such epitopes in brown seaweed cell walls, providing new insights into cell wall evolution, and diversifying the currently available tools for the study of brown seaweed cell walls.
LAMP mAb is a valuable probe for brown seaweed cell walls

Immunolocalisation studies indicated that 3-linked glucans were present throughout the cell walls of *F. vesiculosus*, as detected by the β-glucan-directed LAMP mAb (Biosupplies 400-2). This mAb recognizes a (1→3)-β-glucan epitope, callose and although it was generated against a brown seaweed storage polysaccharide, laminaran (Meikle *et al*., 1991; Meikle *et al*., 1994) it has primarily been used to detect callose in plant cell walls. Callose is a polysaccharide present in specific cells such as pollen tubes (Geitmann *et al*., 1995), pathogen infected cells (Chowdhury *et al*., 2014) and tracheid cells (Altaner *et al*., 2010). The use of this mAb in algal research is almost inexistent.

*In situ* immunolocalization on the brown seaweeds *F. vesiculosus* and *L. digitata* with the LAMP mAb showed the presence and intra-thallus distribution of the (1→3)-β-glucan epitope throughout the algal tissues. The LAMP mAb also labelled the sieve plates of the conductive elements of *L. digitata*, which were previously reported to be composed of callose (Schmitz and Srivastava, 1976; Smith, 1939). This pattern was also observed with the fluorochrome aniline blue, commonly used to detect callose (Evans *et al*., 1984; Stone *et al*., 1984). These results show for the first time the presence of (1→3)-β-glucans in the cell walls of brown seaweeds, which might be associated to callose, or alternatively another polysaccharide that contains that epitope. LAMP mAb showed the presence of an epitope that was not known to be present in brown seaweed cell walls and identified another valuable probe not only for *in situ* localization in seaweed tissues but also in studies related to specialized cell walls, such as sieve plates.
LM7 mAb is a valuable probe for alginates

LM7 was a special mAb that deserved a chapter in this thesis, due to the potential importance raised by the in situ labelling patterns observed. LM7 was generated against lime pectin and recognizes an epitope of unesterified galacturonic acid residues with adjacent or flanking methyl-esterified residues present in homogalacturonan (Clausen et al., 2003; Willats et al., 2001). In vascular plants, this epitope is detected exclusively in very specific regions at the corners of the intercellular spaces between adherent and separated cell walls (Willats et al., 2001). Studies with LM7 in algae are scarce and to our knowledge, are non-existent for seaweeds.

The LM7 mAb labelled the cell walls and extracellular matrix of all the thallus parts of the brown seaweeds *F. vesiculosus* and *L. digitata*, especially within the blade mucilaginous medullary regions. This distinct labelling pattern was not observed for any other mAb previously used. Furthermore, treatment of sections with alginate lyase resulted in a lack of labelling. These results, supported by previous reports of 1) the cross-reactivity of previously generated alginate-directed mAbs with pectic polysaccharides (Larsen et al., 1985), 2) the similarity between pectic homogalacturonan egg-box structures with alginate M blocks gelling unit structure (Haug, 1964), and 3) recent reports on the cross-reactivity of LM7 with alginates (Torode et al., 2015) led to the conclusion that LM7 mAb is a unique and as yet unreported probe for the identification and localization of the brown seaweed (commercially) valuable polysaccharides alginates.
Dr. Valerie Vreeland and co-workers (Department of Botany, University of California, CA, USA) generated a large number of seaweed cell wall-directed mAbs several decades ago (in the 1970’s and 1980’s). These mAbs are highly valuable because they were directed against the most commercially important brown- and red- seaweed polysaccharides, alginates (Larsen et al., 1985; Vreeland et al., 1984) and carrageenans (Vreeland et al., 1992; Zablackis et al., 1991), respectively. Although these unique probes have never become available to the scientific community, they were eventually placed in the care of Professor Michael G. Hahn (CCRC, University of Georgia) several years ago. The potential to use them for cell wall studies requires that they are tested for the viability of the available mAb supernatants against extracts that may contain the epitopes that they recognize (the epitope recognition was never fully characterized). They were therefore tested against a variety of cell wall extracts obtained from different brown (Phaeophyceae) and red seaweed (Rhodophyta) species. The results showed that from the large collection of supernatants, only a few mAbs are still viable, which is unfortunate. Nonetheless, the ones that showed positive ELISA signals and/or positive labelling in sections, can now be cloned and viable hybridoma lines generated, so they can become available for the scientific community in the future. These probes will be added to the existing toolkit of mAbs that can be used to study seaweed cell walls, and help address the numerous gaps that currently exist related to the identification of seaweed cell wall polysaccharides, and the way they structurally organize within the wall architecture. This will push seaweed cell wall research further, opening new perspectives regarding evolutionary, physiological and ecological aspects. They will be valuable tools not only in the laboratory, but may also find application at the industrial scale, for fast and accurate identification of important commercialized polysaccharides, e.g. as part of quality control protocols.
**P. margaritaceum** as a model for designing a hormone toolbox

Plant hormones, or phytohormones, have vital roles in every aspect of plant development, reproduction, and defense against biotic and abiotic stress (Wang and Irving, 2011). Recent studies have shown that the evolutionary origin of plant hormone signaling pathways genetic mechanism is much older than previously thought, and are not restricted to land plants. The existence of endogenous hormones has not yet been unambiguously proven for *P. margaritaceum*. Nonetheless, the genetic machinery for auxins, cytokinins, strigolactones and ethylene signaling seems to have been preserved during evolution and to have already existed in the common ancestor of land plants and CGA, remaining almost unchanged across species (Ju et al., 2015; Wang et al., 2015). Hormones such as auxins have an important role in the mediation of expansive cell growth in land plants, for example, by affecting homogalacturonan demethylsterification (Braybrook and Peaucelle, 2013; Peaucelle et al., 2012). *P. margaritaceum* is a late divergent CGA and has a well-defined and studied HG machinery associated with its cell wall (Domozych et al., 2007). A primary screening showed that this species is very sensitive to hormone and hormone inhibitor treatments, and evident changes in the primary cell wall microarchitecture clearly showed that the treatments have a deep influence in the HG deposition and further methylesterification of the cell wall during cell growth and division, as well as in the cytoskeletal dynamics, by affecting the actin bundle filaments. This closely-related ancestor of land plants is presented as a model for the study of plant growth regulators in particular for investigation of responses to the stimuli and modes of action on the cell wall, cytoplasmic streaming, and cytoskeletal dynamics.

In conclusion, mAbs generated against land plant cell wall components have emerged as significant tools with the potential to develop algal cell wall research, complementing biochemical studies, and providing valuable information on the localization of specific compounds within the algal thallus part. However, it is important to note that some sample preparation practices, such as tissue fixation, could have influenced the results. For example, the fixation steps adopted in the work described here led to the loss of some mucilage. On one hand, this loss may have helped the mAbs in accessing the epitopes present and thus resulted in positive labelling in both light and electron microscopy. On the other hand, the loss of mucilage might have distorted the architectural structures, particularly for those seaweeds rich in mucilaginous polysaccharides. Therefore, future work should consider cryo-
based fixative techniques in order to allow a complete preservation of cell wall compounds and their architecture as they occur in situ. In addition, polysaccharide content of algae in temperate regions can vary with season (Stengel et al., 2011) with potential implications for their cell wall composition. Therefore, the time of the year should be considered when collecting specimens from nature, as their cell wall composition will likely vary.

The rehabilitation of the seaweed (commercially valuable) glycan-directed Vreeland mAbs will allow to deepen the current knowledge existing on seaweed cell wall composition and structure. At last, the use of plant hormones and inhibitors on a microalga can be used as a toolbox to comprehend not only plant cell wall modification phenomena, but also evolutionary mechanisms, using a unicellular organism. Algae, to be precise brown algae, have gained more interest since the past few years in the cell wall research areas. It is only natural that new insights are being brought to discussion within the scientific community, whilst exciting discoveries are showing how dynamic and complex algal cell walls are.
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