

Role of the cell wall in cell shape acquisition

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Umeå

Doctoral thesis

Swedish University of Agricultural Sciences

Umeå 2018

Acta Universitatis agriculturae Sueciae

2018:10

Cover: Yin and yang of leaf epidermal pavement cells
(photo: Mateusz Majda)

ISSN 1652-6880

ISBN (print version) 978-91-7760-160-9

ISBN (electronic version) 978-91-7760-161-6

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Print: Arkitektkopia, Umeå 2018

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Abstract

The growth and development of an organism depend on the coordinated expansion and shape acquisition of individual cells. The epidermis, primarily controls morphogenesis as well as acts as an essential component at the interface with the environment. In plants, the cell wall, a polysaccharide network located outside the plasma membrane, ensures tight junctions between cells and determines the expansion rate and direction of each neighbouring cell, thereby determining cell shape and tissue morphology. Interestingly, plant cells are characterized by a great diversity of shapes, which vary from simple isodiametric forms to more complex structures such as in the puzzle-shaped pavement cells (PCs), displaying alternating lobes and necks, which are observed in the leaf epidermis.

In our studies, we investigated the role of wall composition and mechanical properties in cell shape acquisition. We found that in *Arabidopsis thaliana*, cell wall integrity is essential for proper PC shape formation and that the mechanical properties of the cell wall between two mature PCs are heterogeneous. Further detailed examinations revealed the existence of a stiffness gradient across the curved cell wall at the lobes. We then showed that locally softer regions display an increased accumulation of specific pectic components such as galactans and arabinans, demonstrating their role in the regulation of wall mechanical properties. Furthermore, the appearance of these local heterogeneities precedes the cell morphological changes, indicating that the wall modifications are needed to initiate the lobing process. The cell wall composition was also studied in another species, *Cinnamomum camphora* (camphor tree), revealing a polarization of some cell wall components in PCs, and, uniquely, the presence of wall lignification in both epidermal and mesophyll cells. We also demonstrated that PC division pattern and development are correlated with an auxin gradient generated by directional transport, making a direct link with what is known on auxin stimulated acid growth and transcriptional response of genes controlling cell wall biosynthesis and remodelling.

Altogether, our results support a major role for plant cell walls in cell shape acquisition. Our data reveal a striking dynamicity of PC cell walls, displaying the polarly distributed mechano-chemical properties required for lobing, which change according to the cell developmental stage. Furthermore, our work tightly links the master growth regulator auxin to the regulation of cell shape via a complex and dynamic control of cell wall remodelling.

Keywords: cell walls, mechanics, polarity, heterogeneity, pectins, galactans

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Dedication

To Nicola

the scientist must keep his eyes open to see what others do not see.
Zygmunt Hejnowicz

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I **Majda M.**, Grones P., Sintorn I-M., Vain T., Milani P., Krupinski P., Zagórska-Marek B., Viotti C., Jönsson H., Mellerowicz E.J., Hamant O., Robert S.* (2017). Mechanochemical Polarization of Contiguous Cell Walls Shapes Plant Pavement Cells. *Developmental Cell*, 43 (3), pp. 290–304.
- II Grones P., **Majda M.**, Robert S.* Specific auxin distribution regulates lobe formation in pavement cells (Manuscript).
- III **Majda M.**, Robert S.* The role of auxin in cell expansion (Review Manuscript).
- IV **Majda M.***, Robert S., Zagórska-Marek B., Mellerowicz E.J. Occurrence of secondary walls in leaf epidermal and mesophyll cells of camphor tree (Manuscript).

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Additional publications and manuscripts from the author, which are not part of the thesis:

- Dejonghe W., Kuenen S., Mylle E., Vasileva M., Keech O., Viotti C., Swerts J., Fendrych M., Ortiz-Morea F.A., Mishev K., Delang S., Scholl S., Zarza X., Heilmann M., Kourelis J., Kasprowicz J., Nguyen L.S.L., Drozdzecki A., Van Houtte I., Szatmári A-M., **Majda M.**, Baisa G., Bednarek S.Y., Robert S., Audenaert D., Testerink C., Munnik T., Van Damme D., Heilmann I., Schumacher K., Winne J., Friml J., Verstrecken P., Russinova E.* (2016) Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification. *Nature Communications*, 7, Article number: 11710.
- **Majda M.**, Grones P., Robert S.* Dissecting the role of anticlinal and periclinal walls in the growth of pavement cells (Manuscript).
- Doyle S.M.§, Rigal A.§, Grones P., Karady M., **Majda M.**, Barange D.K., Pěnčík A., Karampelias M., Zwiewka M., Almquist F., Novák O., Ljung K., Robert S.* The tryptophan precursor anthranilic acid plays a role in *Arabidopsis thaliana* root gravitropism via regulation of PIN protein polarity (§ joint first authors) (Manuscript).

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The contribution of Mateusz Majda to the papers included in this thesis was as follows:

- I. Experimental work, planning and data analysis, writing the manuscript.
- II. Experimental work.
- III. Data analysis and writing the manuscript.
- IV. Experimental work, planning and data analysis, writing the manuscript.

Abbreviations

GALS	β-1,4-GALACTAN SYNTHASE
ABCB	ATP-BINDING CASSETTE SUBFAMILY B
AF	actin filament
AFM	atomic force microscopy
AGP	ARABINOGALACTAN PROTEIN
ARP2/3	ACTIN RELATED PROTEIN 2/3
AUX/LAX	AUXIN RESISTANT/LIKE-AUX
BOT1	BOTERO1
CA-ROP2	CONSTITUTIVELY ACTIVE-ROP2
CBM1	CARBOHYDRATE BINDING MODULE FAMILY 1
CCRC M1	Complex Carbohydrate Research Center Monoclonal Antibodies M1
CD	Cytochalasin D
CDC42	CELL DIVISION CONTROL PROTEIN 42 HOMOLOG
CDK	CYCLIN-DEPENDENT KINASE
CESA	CELLULOSE SYNTHASE
CLASP	CLIP-ASSOCIATED PROTEIN
CMF	cellulose microfibrils
CSC	CESA protein complex
DER1	DEFORMED ROOT HAIRS1
<i>E. coli</i>	<i>Escherichia coli</i>
Ea	elastic modulus
EM	electron microscopy
eP	Euclidean point
EXP	EXPANSIN

EXT	EXTENSIN
F-actin	filamentous actin
FEM	fine element modeling
FRA2	FRAGILE FIBER2
<i>gal10-1</i>	B-GALACTOSIDASE deficient
GalAT	GALACTURONOSYLTRANSFERASE
GALS	GALACTAN SYNTHASE
GAX	glucuronoarabinoxylan
GRP	GLYCINE-RICH PROTEIN
HG	homogalacturonan
JIM	John Innes Monoclonal Antibody
KOR1	ENDO-(1,4)- β -D-GLUCANASE KORRIGAN1
<i>kor1-1</i>	ENDO-1,4- β -D-GLUCANASE deficient
MAP	MICROTUBULE-ASSOCIATED PROTEIN
MOR1	MICROTUBULE ORGANIZER 1
MT	microtubule
<i>mur1-2</i>	GDP-D-MANNOSE-4,6-DEHYDRATASE deficient
<i>mur3-1</i>	GALACTOSYLTRANSFERASE deficient
<i>mur4-1</i>	ARABINOTRANSFERASE deficient
NAA	naphthalene-1-acetic acid
NADPH	NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSHPATE
NPA	1-N-naphthylphthalamic acid
Pa	pascal
PAE	PECTIN ACETYL-ESTERASE
PC	pavement cell
Per	PEROXIDASE
PG	POLYGALACTURONASE
PGI	POLYGALACTURONASE INHIBITING PROTEIN
picloram	4-amino-3,5,6-trichloropicolinic acid
PIN	PIN-FORMED
<i>pin</i>	PIN-FORMED deficient
PL	PECTATE LYASE
PME	PECTIN METHYLESTERASE
PMEI	PECTIN METHYLESTERASE INIHIBITOR
<i>pom1-2</i>	CELLULOSE SYNTHASE-INTERACTIVE PROTEIN deficient

POM2/CSII	CELLULOSE SYNTHASE INTERACTING 1
PRP	PROLINE-RICH PROTEIN
<i>qua1-1</i>	GLYCOSYLTRANSFERASE deficient
<i>qua2-1</i>	GLYCOSYLTRANSFERASE deficient
RGI	rhamnogalacturonan I
RGII	rhamnogalacturonan II
RIC4	ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN4
ROI	region of interest
ROP	RHO OF PLANTS
ROS	reactive oxygen species
<i>rsh</i>	ROOT-, SHOOT-, HYPOCOTYL-DEFECTIVE deficient
SLGC	stomatal lineage ground cell
TEM	transmission electron microscope
XEH	XYLOGLUCAN ENDOHYDROLASE
XET	XYLOGLUCAN ENDOTRANSGUCOSYLASE
XGA	xylogalacturonan
XTH	XYLOGLUCAN ENDOTRANSGUCOSYLASE/HYDROLASE
XXT	XYLOGLUCAN XYLOSYLTRANSFERASES
XyG	xyloglucan
YUC	YUCCA
β -GAL	β -GALACTOSIDASE

1 Introduction

All living organisms from unicellular prokaryotes to multicellular eukaryotes are characterized by a great variety of cell shapes. The cell contours can vary from simple spheres in bacteria to very complex and specialized shapes in animal cells such as dendritic neurons. The common feature of all cells is the presence of the plasma membrane, which determines the cell borders. In animals, outside of the plasma membrane the extracellular matrix formed by extracellular components is present, while bacteria, fungi and plant cells are surrounded by the wall (Kost & Chua, 2002), a rigid structure composed mainly of various polysaccharides. Cell shape acquisition differs among different organisms. In animals, the cell form is driven by the intracellular fibrillar structure known as the cytoskeleton, and the extracellular matrix (Mattila & Lappalainen, 2008; Fletcher & Mullins, 2010), giving rise to different forms such as highly elongated muscle cells or small and flat biconcave blood cells (Klinken, 2002; Thakar *et al.*, 2009). In walled cells, the shape is mainly coordinated by the wall, the inside turgor pressure and the cytoskeleton (Peters *et al.*, 2000). Cell wall is important, because if the wall is removed from these cells, the protoplast acquires a spherical shape (Baluška *et al.*, 2003). The shapes of bacterial cells vary from simple spheres in *Staphylococcus* to spirals in *Spirillum*, while in fungi, comprising unicellular and multicellular organisms, their reproductive structures (spores) can develop diverse shapes such as round with spikes in *Laccaria*. In the case of plants, cells can vary from isodiametric meristematic cells to complex multi-lobed pavement cells (PCs) (Mathur, 2005). The shape of the plant cell, its acquisition and its maintenance, display common features with other kingdoms, however outstanding plant-specific features have been observed, highlighting their unique nature.

1.1 Plant cell shape

In contrast to freely moving animal cells, plant cells are tightly connected to each other within a tissue (Traas & Sassi, 2014). For this reason, plant cells can undergo i) symplastic growth, which is defined as the simultaneous expansion of neighboring cells, mutually adjusting growth to each other without shifting the walls (e.g. epidermal cells); ii) intrusive growth in which one cell elongates, breaking existing contacts between two cells (e.g. pollen tubes and vascular fibers); or iii) protrusive growth, defined as the less restricted growth of a cell exposed to the environment (e.g. root hairs and trichomes) (Priestley, 1930; Green, 1962; Erickson, 1986; Guerriero *et al.*, 2014). Most plant cells are initially isodiametric before entering the differentiation stage, which often results in size and shape changes (**Figure 1** and **Table 1**). Cell differentiation generates different anisotropic forms that display asymmetry either along one (elongated and tip growth) or multiple axes (multifocal growth) (Mathur, 2004; Baskin, 2005).

Anisotropy along the apical-basal axis leads to cell elongation and occurs, for example, in the epidermal cells of the hypocotyl (Gendreau *et al.*, 1997). Because epidermal cells are less restricted than other tissues, some of the cells can differentiate into specific shapes such as root hairs in roots or trichomes in leaves (Guimil & Dunand, 2007; Kasili *et al.*, 2011). Root hairs grow by a local swelling at the basal end of the cell, which then extends via tip-growth (Guimil & Dunand, 2007). This tip-growth is initiated in a small part of the cell, which progressively extrudes into a single cell outgrowth (Bannigan & Baskin, 2005; Baskin, 2005). Another example of tip-growth is that which occurs to form the pollen tube that, from an initially spherical pollen grain, forms a local protrusion (Cheung, 1996; Smith & Oppenheimer, 2005). Similar to root hairs, leaf trichome initiation starts through a single axis of growth that is perpendicular to the organ surface. At later stages, this outgrowth develops three or four branches through which multiple axis polarity is established *de novo* (Szymanski *et al.*, 1999; Mathur, 2004; Smith & Oppenheimer, 2005). At the end of their development, trichomes are composed of a stalk and several branches. This type of growth is defined as being multifocal because it leads to the formation of more complex contours generated by outgrowth within different cell sub-domains (Mathur, 2004; Panteris & Galatis, 2005).

Multifocal growth has been described in the algae *Micrasterias sp.* (Meindl, 1993) and *Vaucheria sp.* (Blatt & Briggs, 1980). In higher plants, multifocal growth occurs in aerenchyma tissue in the monocot *Juncus sp.* (Peters *et al.*, 2000), astrosclereids (branched, lignified cells) (Evert 2006), lobed spongy parenchyma cells (Panteris & Galatis, 2005), branching trichomes and PCs in

flowering plants including *Arabidopsis thaliana* (Bannigan & Baskin, 2005; Smith & Oppenheimer, 2005; Zhang *et al.*, 2011). Lobed spongy parenchyma cells are initially well-connected but when the leaf expands, they form intercellular spaces between neighboring cells with local cell wall junctions (Galatis, 1988; Panteris & Galatis, 2005).

Table 1. *Variety of plant cell shapes*

Growth		examples
isotropic	diffusive	meristematic cells mesophyll cells
	elongated	most epidermal cells (root, hypocotyl) palisade parenchyma cells cortex and endodermis in root phloem vascular fibers
anisotropic	tip growth	pollen tubes root hairs
	multifocal	pavement cells (PCs) spongy parenchyma cells trichomes

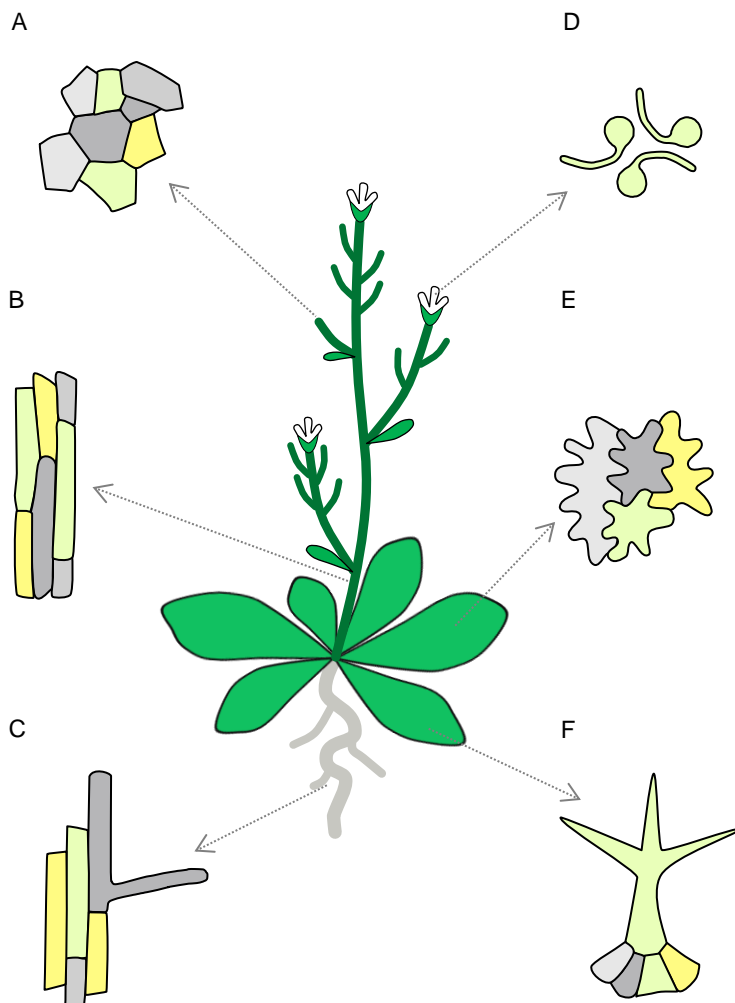


Figure 1. Illustration of different cell shapes in *Arabidopsis thaliana*. Isodiametric meristematic cells (a), elongated stem cells (b), tip growing root hairs (c), pollen tubes (d), epidermal pavement cells (e) and trichomes (f).

This study particularly focused on leaf epidermal PCs. Leaf epidermis is a heterogeneous tissue as it is composed of different organ-specific cells, such as PCs, guard cells or stomata, trichomes and sometimes secretory cells (Evert 2006). Expansion of the leaf in its early stage of development takes place at the basal part of the leaf, in which cells actively divide and then later expand (Dale, 1988). Additionally, meristematic cells are present across the entire leaf surface and they follow a stereotypical division pattern (Robinson *et al.*, 2011). These cells divide to produce new stomata and PCs (Robinson *et al.*, 2011) in

order to enable gas exchange and increase the leaf surface, respectively (Dale, 1988).

PCs are initially isodiametric and develop interdigitation during their growth, acquiring a jigsaw-puzzle structure (**Figure 2**), as observed in most flowering plant species (Panteris *et al.*, 1993a; Panteris & Galatis, 2005). In this way, PCs form alternative patterns of lobes and necks, while the growth of one cell lobe inevitably leads to an indentation (neck) in the neighboring adjacent cell (Deeks & Hussey, 2003; Bannigan & Baskin, 2005; Panteris & Galatis, 2005). As a result of this growth, many outgrowths around the cells are created. Although the reason for this peculiar shape remains mysterious, it has been suggested that the lobed shape of PCs may have a role in increasing the contact area between cells to reinforce their cell-cell contact.

Overall, a great diversity in plant cell shape has been widely observed. The question of how such diverse shapes are achieved and what purposes they serve is still a major subject of research and debate.

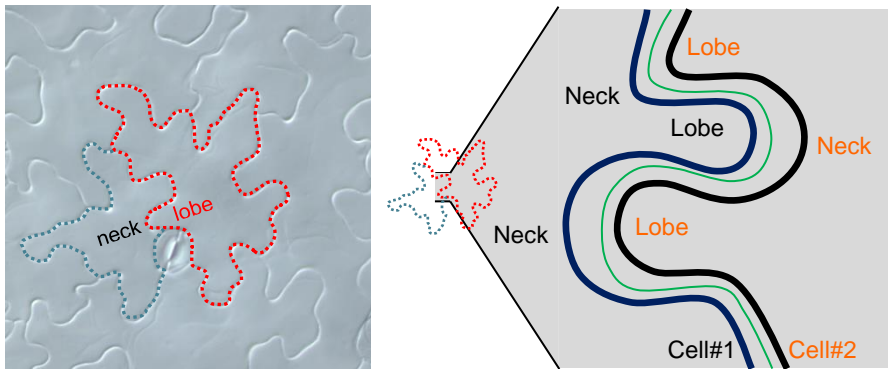


Figure 2. Epidermal pavement cells (on the left) and drawing illustrating anticlinal pavement cell walls (on the right)

1.2 Cell shape acquisition at the subcellular scale

Different cell shapes are acquired due to temporal changes within the cell and polarity establishment on the subcellular level (Harold, 1990; Drubin & Nelson, 1996; Fowler & Quatrano, 1997; Huang & Ingber, 1999). Polarity occurs as spatial differences within the cell, such as the presence of growing and non-growing zones, which regulate cell extension (Baluška *et al.*, 2003). Cell extension is caused by local cell growth, which is associated with the accumulation of specific cell components, and this kind of growth is observed, for instance, in tip-growth. Polar extension can also be mediated via non-

growing zones, which restrict (or inhibit) growth in specific cell zones while other places are free to grow, as occurs in elongating cells.

In most organisms, cell polarity is established and maintained not only by the orientation of the cytoskeleton, but also by the subcellular localization of regulatory molecules, which accumulate in specific cell zones (Li & Gundersen, 2008). Nonetheless, structural differences among cells from different kingdoms underlie diverse mechanisms of polarity establishment. For instance, in animal cells, the cytoskeleton is the primary cause of cell polarity establishment, while in bacteria, fungi and plants, the shape is defined mainly by the cell wall and turgor pressure, with the cytoskeleton playing an important but indirect role by controlling the deposition of different cell wall components (Peters *et al.*, 2000). In eukaryotes, the cytoskeleton consists of microtubules (MTs) and actin filaments (AFs), and additionally of intermediate filaments in animals. In prokaryotes, a cytoskeleton is also present and consists of proteins homologous to eukaryotic MT and AF proteins (Pogliano, 2008).

MTs are composed of tubulin proteins that are heterodimerized to form protofilaments that are attached to each other and enclosed within a load-bearing cylinder with a diameter around 20nm. MTs are very dynamic structures within cells, because they continuously assemble and disassemble their subunits, contributing to cell growth anisotropy (Desai & Mitchison, 1997). This dynamic remodeling is controlled by MICROTUBULE-ASSOCIATED PROTEINs (MAPs). For instance, in human cells MTs are stabilized by MAP4 (Permana *et al.*, 2005), while spacing of the MTs is controlled by MAP1 (Chen *et al.*, 1992). In animals, MTs control the movement of cilia and flagella in addition to controlling the shape of different cells such as the axon part of neurons (Desai & Mitchison, 1997).

AFs are comprised of actin monomers built up in thin and flexible filaments resembling a double helix with a diameter around 7 nm. AFs play a role in vesicular transport and accumulation of materials to build the cell. Like MTs, AFs are very dynamic and can be easily assembled and disassembled, contributing to local growth and cell movement (Hall, 1998). AFs can be found close to the cell surface and are able to give a specific shape and structure to the cell. The dynamics of AFs and their function are modulated by various associated proteins, such as ACTIN RELATED PROTEINs 2/3 (ARP2/3), which facilitate the remodeling of AFs required for adjusting cell movement or shape (Mullins *et al.*, 1998). Disassembly of AFs is mediated by cofilin, while filaments are assembled by profilin (Didry *et al.*, 1998). Examples of AF-enriched growth can be found in animal cells such as dendrites in neurons, and this kind of growth contributes to the motility of microvilli or lamellipodia. This growth is also present in fungal budding yeast (*Saccharomyces*

cerevisiae), algae such as *Micrasterias sp.*, and cells in higher plants such as pollen tubes (Belanger & Quatrano, 2000; Hepler *et al.*, 2001; Baluška *et al.*, 2003).

The existence of the cytoskeleton was already proposed in the 19th century, but the question of how this intracellular structure controls cell shape acquisition only started to be unveiled around 30 years ago. It began with the discovery that the signaling G proteins of RHO GTPases function as a “bridge” between signal perception and cellular response, regulating various subcellular processes such as dynamics of the cytoskeleton and vesicle trafficking (Chant, 1996, 1999; Van Aelst & D’Souza-Schorey, 1997; Hall, 1998; Vernoud *et al.*, 2003; Gu *et al.*, 2004; Bannigan & Baskin, 2005). RHO proteins were shown to be involved in local actin accumulation in neurons (Hall, 1998). In yeast, RHO analog CELL DIVISION CONTROL PROTEIN 42 HOMOLOG (CDC42) was found to be specifically located at the tip of budding yeast where AFs were accumulated (Chant, 1996, 1999; Hall, 1998). In plants, RHO analogs called RHO OF PLANTS (ROP) play similar functions to those described in animals and yeast. In the growing pollen tube, ROP1 proteins are concentrated at the tip, marking the place where the AFs will accumulate (Fu *et al.*, 2001). These lines of evidence showed the importance of ROP proteins for local cell growth.

The above-mentioned examples refer to the polarity established on the basis of growing zones within the cell. Another way to form polarity is based on the non-growing domains established beforehand and is typical for the rod-shaped bacterium *Escherichia coli* (*E. coli*). These non-growing domains are enriched with actin-like proteins, which are not found in spherical-shaped bacteria, indicating that these proteins determine the polarization of the *E. coli*. Moreover, when the cell grows, newly synthesized proteins are added to the growing membrane, but not to the non-growing limiting membrane. This process is thought to be the cause of the non-spherical shape in bacteria (Nanninga, 1998; Hoppert & Mayer, 1999; Jones *et al.*, 2001; Baluška *et al.*, 2003). Baluška *et al.*, 2003 suggested that a similar mechanism is also present in elongating plant cells. In the expanding zones, MTs are present, while the non-growing zones lack MTs and display accumulation of dense AFs. In contrary to AF-enriched growing tips, the local accumulation of AF and AF-like proteins in non-growing zones suggests that mechanisms mediating polarization of prokaryotic rod-shaped bacteria and the polarized shape of plant cells might be conserved. However, plant cell dynamics are certainly more complex and will be discussed in the following chapter.

1.2.1 Role of the cytoskeleton in plant cell shape acquisition

In plants, cell wall is the main factor determining why each cell acquires a characteristic shape. Nevertheless, the cytoskeleton controls cell wall deposition and thus influences the process of shape acquisition (Bringmann *et al.*, 2012). MTs are highly dynamic polymers and their (re)organization and local accumulation precede cell morphological changes (Desai & Mitchison, 1997). For instance, during anisotropic growth, MT orientation occurs preferentially along one axis, which generates reinforced places within the cell, resulting in cell expansion perpendicular to the orientation of the MTs (Bichet *et al.*, 2001). An illustrative example is represented by the *Arabidopsis* gene *BOTERO1* (*BOT1*)/*FRAGILE FIBER2* (*FRA2*), which encodes for the kinesin subunit that severs MTs. Mutants of this gene display short and swollen hypocotyl cells, caused by a defect in MT reorganization, which results in a reduced anisotropic growth (Bichet *et al.*, 2001). Moreover, the mutants display reduced cell length (Burk *et al.*, 2001) and aberrant cell differentiation in the root (Webb *et al.*, 2002). Other examples are the MAP CLIP-ASSOCIATED PROTEIN (CLASP) (Ambrose *et al.*, 2007; Kirik *et al.*, 2007) and MICROTUBULE ORGANIZER 1 (MOR1) (Whittington *et al.*, 2001). These proteins have been described as regulators of MT dynamics, stabilization, organization/orientation, polymerization and disassembly. The *clasp* mutants display fewer cells in the root and defects in hypocotyl elongation, with shorter and radially swollen cells. The mutants also have smaller, less-undulated PCs and less-branched trichomes (Ambrose *et al.*, 2007; Kirik *et al.*, 2007). The *MOR1* deficient mutant *mor1-1* is characterized by short, deformed and detached hypocotyl epidermal cells and curly root hairs (Whittington *et al.*, 2001). All *mor1* mutants display cell elongation defects reflected in smaller leaves and overall shorter plants, coupled with altered cell shape. These results indicate that MTs play an important role in the maintenance of cell polarity.

AFs are the second group of cytoskeletal elements critical for plant cell shape acquisition, because they accumulate in actively growing cell zones and guide directional transport of Golgi vesicles containing materials for local cell expansion. In plants, AFs are accumulated locally at the tips of root hairs, pollen tubes, and trichomes (Szymanski *et al.*, 1999; Hepler *et al.*, 2001; Mathur & Hülskamp, 2002; Deeks & Hussey, 2003; Smith, 2003; Wasteneys & Galway, 2003; Bannigan & Baskin, 2005; Smith & Oppenheimer, 2005; Guimil & Dunand, 2007). The *deformed root hairs1* (*der1*) mutant for the gene encoding ACTIN2 displays altered root hair development, including changes in the site of emergence and the overall outgrowth (Ringli *et al.*, 2002;

Vaškebová *et al.*, 2017), indicating that ACTIN2 plays an important role in root hair tip growth.

The degree of actin polymerization is controlled via the ARP2/3 complex, which regulates the local accumulation of filamentous actin (F-actin) present in locally growing cells, contributing to cell shape acquisition. Mutations that impair any of the components within the ARP2/3 complex cause formation of dense AF bundles and defective AF organization (Blanchoin *et al.*, 2000; Volkmann *et al.*, 2001; Carlier *et al.*, 2003; Deeks & Hussey, 2003; Bannigan & Baskin, 2005; Mathur, 2005; Panteris & Galatis, 2005; Szymanski, 2005; Guimil & Dunand, 2007). For example, mutants for the genes *WURM* and *DISTORTED1*, the paralogs of *ARP2/3*, display cell shape defects such as deformed trichomes, non-lobed and small PCs, short hypocotyl epidermal cells with defective cell adhesion, and curving epidermal root hairs (Mathur *et al.*, 2003). *CROOKED* is another subunit of the *ARP2/3* complex, and *crooked* mutants also display shape defects such as curling and deformed trichomes, smaller and randomly dividing hypocotyl cells, detached hypocotyl epidermal cells, isodiametric and small PCs, and curling root hairs (Mathur, 2003). *BRICK1* is one of the elements within the Scar/*WAVE* complex, which activates *ARP2/3*. The *brick1* mutants display alterations in actin polymerization similar to those observed in *arp2-3* mutants, resulting in unbranched and deformed trichomes and misshaped PCs with less indentations than wild type (Djakovic, 2006). PCs of *brick1* mutants in *Zea mays* (*brk1*, *brk2*, and *brk3*) do not even form lobes (Frank & Smith, 2002; Frank, 2003). Mutants defective in the *SPIKE1* gene, encoding a guanine nucleotide exchange factor which activates ROPs, display altered cytoskeleton reorganization and form unbranched trichomes and almost isodiametric PCs with gaps between these two types of cells (Qiu *et al.*, 2002; Ren *et al.*, 2016).

The importance of the cytoskeleton for cell shape acquisition and directional growth has been demonstrated using pharmacological approaches to perturb cytoskeleton integrity. Colchicine is a drug that disrupts MT organization, and its application leads to isodiametric cell shape (Armour *et al.*, 2015). Similarly, the use of Cytochalasin D (CD) to disrupt AFs results in the formation of PCs with reduced interdigitation (Armour *et al.*, 2015). However, lobing does not disappear completely, as in the case of application of drugs perturbing MTs (Panteris & Galatis, 2005). Application of CD or latrunculin B leads to actin bundle disruption at the tips of directionally growing cells and thus inhibits root hair and pollen tube elongation (Baluška *et al.*, 2001).

1.2.2 Mechanism of pavement cell interdigitation

PCs display peculiar jigsaw-like shapes characterized by an alternating pattern of lobes and necks (**Figure 2**). The relationship between neighboring cells can be thought of as resembling the ancient Chinese philosophic concept of the Yin and Yang, in which two forces oppose each other but at the same time are interdependent and could not exist in the absence of one or another. This complex relationship between neighboring PCs and the factors and molecular mechanisms that give rise to this distinctive shape have intrigued researchers for many years; the prominent mathematical biologist D'Arcy Wentworth Thompson noted over a century ago, "the more coarsely sinuous outlines of the epithelium in many plants is another story, and not so easily accounted for" (Thompson, 1917; Carter *et al.*, 2017).

The shape of sinuous PCs has been proposed to be the consequence of uneven cell wall thickness (Panteris *et al.*, 1993b). The curved wall zones are thicker and locally reinforce the wall, while the straight zones are thinner and are thought to be extensible under turgor pressure (Panteris *et al.*, 1993b, 1994). This theory was further supported by the analysis of the cell wall composition (Sotiriou1 *et al* 2017).

Another suggested explanation for the shape of PCs is that the cytoskeleton contributes to the shape acquisition. The role of the cytoskeleton in the lobing of mesophyll cells was implicated by the application of drugs perturbing MTs and AFs, which lead to lobe-less cells (Wernicke and Yung 1992; Smith, 2003). The shaping of PCs was thought to be MT-dependent, as the MT-deficient mutant *fra2* displays a PC interdigitation defect and the cells remain isodiametric (Burk *et al.*, 2001). Additionally, AFs have been shown to be accumulated in the places where the lobes form, marking the sites where the future lobes will appear (Frank & Smith, 2002; Fu *et al.*, 2002; Frank, 2003). The contribution of both cytoskeletal elements, AFs and MTs, to the shaping of PCs was demonstrated by the finding that AFs and MTs localize in the cell lobes and neck zones, respectively (Fu *et al.*, 2005). Furthermore, it was suggested that not only the local accumulation but also the local polymerization of AFs seems to be important for the lobing process (Higgs & Pollard, 2001; Eden *et al.*, 2002; Deeks & Hussey, 2003, 2005). The mechanism of lobing of PCs has been speculated to be analogous to tip-growth (Smith, 2003). However, in contrast to freely growing pollen tubes or root hairs, PCs are tightly connected by their anticlinal walls. The local growth of one cell (lobing) inevitably leads to the indentation of the neighboring cell, which requires a simultaneous (symplastic) growth of neighboring cells.

At the molecular level, the localization of AFs and MTs is driven by two different ROP proteins (**Figure 3**), which display an alternating pattern along the lobes and necks: in the growing cell regions (lobes), ROP2, through ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN4 (RIC4), activates the local accumulation of AFs, promoting a local growth resulting in lobe formation; in the neck zones where growth is inhibited, ROP6 activates RIC1, which prompts MT array formation, leading to a local growth inhibition, resulting in indentation formation (Fu *et al.*, 2005, 2009). Simultaneously, ROP2, by mediating the inactivation of the RIC1-ROP6 effector, leads to the inhibition of cortical MT formation at the lobes, while local accumulation of MTs leads to suppression of RIC4-ROP2 (Gu *et al.*, 2004; Bannigan & Baskin, 2005; Fu *et al.*, 2005, 2009; Pietra & Grebe, 2010; Xu *et al.*, 2010).

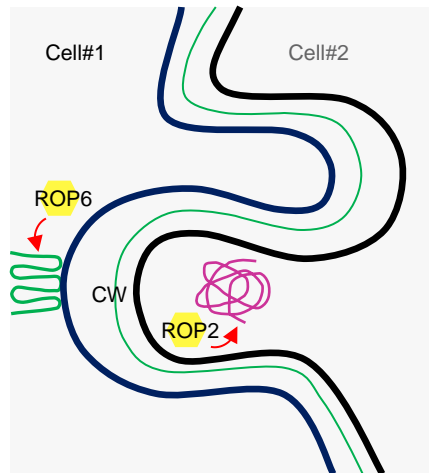


Figure 3. Drawing illustrating contact sides between two neighbouring pavement cells (Cell#1 and Cell#2). ROP6 and MTs (in green) are localized in the neck while ROP2 and AFs (purple) are localized in the lobe.

The plant hormone auxin has been proposed to play a role in PC shape acquisition (Xu *et al.*, 2010, 2014). It has been shown that the application of the synthetic auxin naphthalene-1-acetic acid (NAA) at low concentration increases the lobing of PCs (Xu *et al.*, 2010; Grones *et al.*, 2015), while the application of the auxin efflux inhibitor 1-N-naphthylphthalamic acid (NPA) reduces the number of lobes (Xu *et al.*, 2010). Moreover, the auxin biosynthesis deficient quadruple mutant *yucca* (*yuc4 yuc6 yuc1 yuc2*) displays a reduced lobe number (Cheng *et al.*, 2006; Xu *et al.*, 2010) and this effect can be rescued by application of exogenous auxin (Xu *et al.*, 2010).

Furthermore, auxin has been shown to control the polar distribution of the PIN-FORMED (PIN) auxin efflux carriers (Paciorek *et al.*, 2005). It has been suggested that in PCs, PIN proteins that are localized in the lobes may promote a directional auxin flow (Xu *et al.*, 2010; Nagawa *et al.*, 2012). Taken together, a correlation between auxin, ROP2 and PINs has been proposed as follows: auxin activates the ROP2 pathway and ROP2 signaling simultaneously stimulates auxin efflux by regulating distribution of PIN proteins into the lobes, leading to an increase in the extracellular auxin level. This elevated auxin concentration activates the ROP6 pathway in the neighboring cell, which promotes the formation of the neck (Xu *et al.*, 2010). It is accepted that auxin participates in the regulation of directional cell growth by activating ROP signaling pathways, and that ROPs are necessary for auxin-mediated cell shape regulation.

Initially, PIN1 was proposed as the player in the auxin-ROPs-PINs model (Xu *et al.*, 2010). However, Belteton *et al.*, 2017 showed that *PIN1* was not expressed in PCs. Moreover, analysis of PIN1-GFP showed that PIN1 was only localized at the leaf base and over the veins (Le *et al.*, 2014). These results imply that PIN1 most probably is not involved in the shape acquisition of PCs. Considering high PIN redundancy, it might be that other PINs such as PIN3, PIN4, and PIN7, rather than PIN1, are involved in lobe formation.

Although the role of the cytoskeleton in the lobing of PCs and overall cell shape acquisition is well defined, the contributions of other cellular components remain elusive. Nonetheless, it is known that cell shape acquisition can be mediated by the cell wall.

1.3 Plant cell wall

Plant cell wall consists of cellulose microfibrils (CMFs), which are embedded in a matrix consisting of different polysaccharides, structural proteins and glycoproteins, as well as lignins. Matrix polysaccharides include hemicelluloses, which reinforce the wall, and highly hydrated pectins (Carpita & Gibeau, 1993; Cosgrove, 2005). However, cell walls are characterized by a great diversity of composites, which are not only species-specific, but also vary with the cell type, at different wall domains or along the plant's development. This heterogeneity is known to be spatially and temporarily controlled (Freshour *et al.*, 1996; Refrégier *et al.*, 2004; Derbyshire *et al.*, 2007a; Burton *et al.*, 2010; Wolf *et al.*, 2012; Majda *et al.*, 2017; Phyo *et al.*, 2017). For instance, the amount and distribution of specific cell wall composites depend on the cell developmental stage and differ between meristematic and mature

cells. Young cells display porous walls, through which water, nutrients and hormones can easily enter the cells. In contrast, mature cell walls are thicker, multi-lamellate, and sometimes even impregnated by phenolic compounds such as lignins, making them impermeable to water (Burton *et al.*, 2010). The walls formed in growing cells are called the primary walls, and are divided into type I and type II according to the presence and amount of different matrix polysaccharides (Carpita & Gibeaut, 1993). The wall layers deposited in some specific non-growing cell types such as xylem vessels or fibers are called the secondary walls. These walls are thick and multi-lamellate and they increase the cell wall strength. In cells having secondary wall layers, the cell walls become impregnated with lignins, which further dehydrate the wall and provide additional mechanical strength (Ralph *et al.*, 2004; Cosgrove, 2005; Burton *et al.*, 2010; Wolf *et al.*, 2012). Recent method developments of *in situ* approaches have allowed the study of cell wall heterogeneities within a single cell wall (Majda *et al.*, 2017), highlighting their potential in the regulation of cell shape.

1.3.1 Cell wall composition

Despite the high variability of wall composition, the main elements are always present (**Table 2**). CMFs are the largest cell wall polymers, forming crystals with approximate diameter of 3–5 nm (Cosgrove, 2005). Cellulose varies in the degree of its crystallinity, however its basic chemical structure is the same among different walls (Burton *et al.*, 2010). Each CMF is built of (1,4)- β -D-glucan chains in parallel arrays (Doblin, 2002; Somerville, 2006). CMFs are stiff load-bearing wall components, displaying a high resistance to tensional stress (Cosgrove, 2005; Burton *et al.*, 2010; Wolf *et al.*, 2012). Their orientation defines the stiffness pattern within the wall, causing anisotropy and controlling growth direction (Baskin, 2005; Chen *et al.*, 2010; Wolf *et al.*, 2012). Cellulose deposition determines cell shape, and accordingly, cellulose deficient mutants display cell elongation defects (Fagard, 2000; Robert *et al.*, 2004). CMFs are cross-linked, forming a honey comb-like structure and can be linked with non-cellulosic polysaccharides such as hemicelluloses and pectins (Keegstra *et al.*, 1973; Gibson, 2012).

Non-cellulosic matrix polysaccharides are very complex. The structure and amount of matrix polysaccharides vary among cell walls across the plant kingdom. Primary cell walls of type I, present in dicotyledons and non-commelinid monocotyledons (alismatid and lilioid), are characterized by high amount of xyloglucans (XyGs) and pectins. In contrast, primary cell walls of type II, found in commelinid monocots such as rice (*Oryza sativa*), display

increased amounts of glucuronoarabinoxylans (GAX) and (1,3;1,4)- β -D-glucans, together with decreased amounts of pectins and XyGs (Carpita & Gibeaut, 1993; Carpita, 1996; Yokoyama & Nishitani, 2004). Interestingly, non-cellulosic polysaccharides have been shown to be involved in growth regulation and signaling (Burton *et al.*, 2010; Wolf *et al.*, 2012).

Table 2. *The main groups of cell wall polysaccharides and proteins*

Characteristics	Component	Building domains
Microfibrils	Cellulose	Crystalline
		Non-crystalline
	Hemicelluloses	Xyloglucan (XyG)
		Xylan
		Mannan
		Homogalacturonan (HG)
Pectins	Rhamnogalacturonan I (RG I)	
	Rhamnogalacturonan II (RG II)	
	Xylogalacturonan (XGA)	
Matrix	Structural proteins, non-enzymatic proteins and proteoglycans	Extensins (EXTs)
		Expansins (EXPs)
		Arabinogalactan proteins (AGPs)
		Glycine-rich proteins (GRPs)
		Proline-rich proteins (PRPs)
		Cysteine-rich thionins
		Histidine-tryptophan-rich proteins

Hemicelluloses interact with cellulose and lignin to regulate the strengthening of the walls. Hemicelluloses are characterized by β -(1 \rightarrow 4)-linked backbones and branches consisting of more specific sugar residues (**Table 3**). The main types are XyGs, xylans (including glucuronoxylan, arabinoxylan and GAX), mannans (including galactomannan (Edwards *et al.*, 1992), glucomannan (Goubet *et al.*, 2009) and galactoglucomannan (Schroder *et al.*, 2001)), and β -(1 \rightarrow 3,1 \rightarrow 4) linked glucans, present mostly in type II primary walls of some of the monocotyledons (Poales) and few other groups (Scheller & Ulvskov, 2010). XyGs are composed of a cellulose-like (1,4)- β -D-glucan backbone, with xylose at about 70% of the glycosyl residues, further connected with galactose and fucose (Cosgrove, 2005; Burton *et al.*, 2010; Scheller & Ulvskov, 2010). XyGs are abundant in young, actively growing primary cell walls of dicotyledons, and are involved in cell elongation (Hayashi, 1989; Takeda *et al.*, 2002; Cavalier *et al.*, 2008; Eckardt, 2008). The

degree of XyG fucosylation seems to be important for formation of root hairs, which display an increase in non-fucosylated XyGs (Cavalier *et al.*, 2008). Xylans are characterized by a common (1,4)- β -D-xylose backbone, which can be decorated with glucuronosyl residues (glucuronoxylan in secondary cell walls of dicotyledons and GAX in type II primary walls of grasses and related species) or arabinose residues (arabinoxylan and GAX in type II primary walls) (Scheller & Ulvskov, 2010; Wolf *et al.*, 2012). Mannans including homomannans and galactomannans are characterized by β -(1 \rightarrow 4)-linked mannose units in their backbone, whereas glucomannans also have β -(1 \rightarrow 4)-glucose in their backbone. Mannans have been found in all cell walls and are abundant in early land plants such as mosses and lycophytes (Moller *et al.*, 2007). Mannans are fundamental for plant development, as demonstrated by the embryo lethality of an *Arabidopsis* GLUCOMANNAN SYNTHASE-deficient mutant (Goubet *et al.*, 2003; Scheller & Ulvskov, 2010)

Table 3. Diversity of plant hemicelluloses

Polysaccharide	Monomers*	Occurrence	References
Xyloglucan (XyG)	<u>D-Glucose</u>	Primary walls in most land plants, less abundant in type 2 primary walls	Popper & Fry, 2003; Moller <i>et al.</i> , 2007; Popper, 2008; Scheller & Ulvskov, 2010; Sorensen <i>et al.</i> , 2010
	<u>D-Xylose</u>		
	<u>D-Galactose</u>		
	<u>L-Fucose</u>		
Homoxylan	<u>D-Xylose</u>	Red and green algae, guar	Scheller & Ulvskov, 2010
Glucuronoxylan	<u>D-Xylose</u>	Secondary cell walls of dicots	Scheller & Ulvskov, 2010
	<u>D-Glucuronic acid</u>		
Arabinoxylan	<u>D-Xylose</u>	Cereal grains	Bochicchio & Reicher, 2003; Scheller & Ulvskov, 2010
	<u>L-Arabinose</u>		
Glucuronoarabinoxylan (GAX)	<u>D-Xylose</u>	Abundant in type 2 primary walls and in cereal grains	Harris <i>et al.</i> , 1997; Carnachan & Harris, 2000; Scheller & Ulvskov, 2010
	<u>D-Glucuronic acid</u>		
	<u>L-Arabinose</u>		
Homomannan	<u>D-Mannose</u>	Abundant in early land plants including mosses and lycophytes	Moller <i>et al.</i> , 2007; Scheller & Ulvskov, 2010
Galactomannan	<u>D-Mannose</u>	Storage cell wall polysaccharides in leguminous seeds	Edwards et al. 1999
	<u>D-Galactose</u>		
Glucomannan	<u>D-Mannose</u>	Mosses, ferns, secondary walls of gymnosperms and angiosperms, and primary walls of monocots and dicots	Goubet <i>et al.</i> , 2003, 2009; Scheller & Ulvskov, 2010
	<u>D-Glucose</u>		
Galactoglucomannan	<u>D-Mannose</u>	Gymnosperm secondary walls	Schroder <i>et al.</i> , 2001; Scheller & Ulvskov, 2010
	<u>D-Glucose</u>		
	<u>D-Galactose</u>		
(1,3;1,4)-β-D-glucan	<u>D-Glucose</u>	Type 2 primary walls of monocot grasses (Poales), and primary cell walls in horsetails, liverworts, Charophytes, and red algae	Smith & Harris, 1999; Popper & Fry, 2003; Fry <i>et al.</i> , 2008; Sørensen <i>et al.</i> , 2008; Scheller & Ulvskov, 2010

* the component (s) of the main backbone is (are) underlined

Pectins determine wall porosity and thickness as they form hydrated gels and lead to wall swelling. Pectins push CMFs apart and facilitate their sliding during cell growth and they stabilize microfibrils in non-growing regions (Burton *et al.*, 2010; Cosgrove, 2017). Pectins also control cell adhesion as the main composite of the middle lamella, which glues cell walls together (Ridley *et al.*, 2001; Willats *et al.*, 2001b; Iwai *et al.*, 2002; Verger *et al.*, 2016). Pectins are involved in tip growth in pollen tubes (Rojas *et al.*, 2011; Nezhad *et al.*, 2014) and in local growth in the green algae *Chara* and *Micrasterias* (Eder & Lütz-Meindl, 2008; Boyer, 2016). Interestingly, study of the cell wall composition in different developmental zones along the *Arabidopsis* stem has revealed differences in the pectic composition. The younger parts of the stem contain pectins with higher hydration, esterification and branching than the older parts (Phyo *et al.*, 2017). Recent studies have revealed that pectins, especially galactans and arabinans, locally soften the cell walls, leading to wall bending and the formation of lobes in PCs (Majda *et al.*, 2017). Pectins are the most complex and heterogeneous polysaccharides, consisting of four distinctive domains most likely covalently linked to each other: homogalacturonan (HG), rhamnogalacturonan I (RGI), xylogalacturonan (XGA) and rhamnogalacturonan II (RGII) (**Table 4**) (Willats *et al.*, 2001a; Vincken, 2003; Caffall & Mohnen, 2009; Round *et al.*, 2010). HGs are the earliest form of pectins, having been found in charophycean and *Micrasterias* green algae (Eder & Lütz-Meindl, 2008; Domozych *et al.*, 2009; Sorensen *et al.*, 2010). HGs consist of a main chain formed by galacturonic acid residues, which are modified by methylesterification, influencing their properties, such as hydration. RGIs are composed of galacturonic acid and rhamnose with some side chains of galactose, arabinose or arabinogalactans (Ridley *et al.*, 2001; Willats *et al.*, 2001b; Vincken, 2003). RGIIs are very complex and are composed of different sugar residues, which bind to borate esters (Willats *et al.*, 2001a; Vincken, 2003; Matsunaga *et al.*, 2004; Cosgrove, 2005). XGAs are composed of a D-galacturonic acid chain, substituted with D-xylose. XGA has also been proposed to be a side chain of RGIs (Vincken, 2003; Zandleven *et al.*, 2007).

Table 4. *Diversity of pectins in plant cell walls*

Polysaccharide	Monomer*	Occurance	References
Homogalacturonan (HG)	<u>D-Galacturonic acid</u>	charophycean green algae, abundant in type 1 primary walls in land plants	Domozych <i>et al.</i> , 2007, 2009; Eder & Lütz-Meindl, 2008; Wolf <i>et al.</i> , 2012
Rhamnogalacturonan I (RG I) (including arabinan, galactan, arabionogalactans)	<u>D-Galacturonic acid</u> <u>L-Rhamnose</u> D-Galactose L-Arabinose	Type 1 primary cell walls and mucilage of higher plants	Yapo, 2011
Rhamnogalacturonan II (RG II)	<u>D-Galacturonic acid</u> L-Rhamnose D-Galactose L-Galactose L-Arabinose L-Fucose D-Xylose D-Glucuronic acid Hydroxycinnamic acid L-Aceric acid D-Apiose D-Dha Keto-deoxyoctulosonic acid	Mainly in type 1 primary walls of vascular plants	Popper, 2008; Sorensen <i>et al.</i> , 2010
Xylogalacturonan (XGA)	<u>D-Galacturonic acid</u> D-Xylose	Peas, soybeans, watermelons, apples, pears, onions, potatoes, pine pollen, and cotton	Zandleven <i>et al.</i> , 2007

*The component(s) of the main backbone is (are) underlined

Cell wall structural proteins represent around 10% of the cell wall content (Cassab, 1998; Wolf *et al.*, 2012). They undertake many important functions such as a contribution to cell wall strength, and the regulation of cell wall assembly, expansion, hydration and permeability. The most abundant structural cell wall proteins are EXTENSINS (EXTs), ARABINOGALACTAN PROTEINS (AGPs), GLYCINE-RICH PROTEINS (GRPs) and PROLINE-RICH PROTEINS (PRPs) (Carpita, 1996). To a lesser extent, other structural protein can also be found such as CYSTEINE-RICH THIONINS, and HISTIDINE-TRYPTOPHAN-RICH PROTEINS (Cassab and Varner *et al.*, 1988). In addition to structural proteins, cell walls contain many active enzymes and EXPANSINS (EXP).

Among the structural cell wall proteins, the well-characterized EXTs are non-enzymatic hydroxyproline-rich glycoproteins, which form a crosslinked network in primary walls (Lampert, 1963). EXTs consist of two repetitive amphiphilic motifs. EXTs are essential for cell wall assembly, and cell plate and wall formation (Lampert, 1963; Showalter, 1993; Kieliszewski & Lampert, 1994; Lampert *et al.*, 2011). The *Arabidopsis root-, shoot-, hypocotyl-defective (rsh)* mutant deficient in EXT3 is embryo lethal (Cannon *et al.*, 2008; Wolf *et al.*, 2012) showing the importance of these proteins in plant development.

Other important proteins found in the primary cell wall are EXPs. These proteins are nonenzymatic, pH dependent, wall-loosening proteins, which promote cell wall enlargement and overall cell growth (McQueen-Mason *et al.*, 1992; Cosgrove, 2000). Moreover, EXPs induce loosening of the walls during the emergence of root hairs (Cho & Cosgrove, 2002) and pollen tube growth, and are important for fruit softening, abscission (Cosgrove, 2000), and leaf shape development (Cho & Cosgrove, 2000; Pien *et al.*, 2001).

AGPs are present in primary and secondary walls of higher plants, being an abundant component of arabic gum in *Acacia senegal*, and also occur in lower plants such as liverworts. AGPs were found to create a physical barrier to the environment in wounded plants (Kreuger & Van Hoist, 1996; Cassab, 1998). They are fundamental for cell wall growth and development, as exogenous AGPs added to cell cultures alter cell fate (Kreuger & Van Hoist, 1993). AGPs are also involved in control of leaf and branch development in bryophytes through the suppression of cell division and growth (Cassab, 1998).

In summary, plant cell wall consists of complex and highly heterogeneous polysaccharides. This heterogeneity results from distinct biosynthetic pathways and continuous post-synthetic modifications.

1.3.2 Cell wall biosynthesis and modification

The enzymes, structural proteins and matrix polysaccharides involved in cell wall establishment are sorted through the endomembrane system before reaching the cell wall. Hemicelluloses and pectins are synthesized in the Golgi apparatus, before being secreted along AFs, ultimately reaching the cell surface via exocytosis (Toyooka *et al.*, 2009; Rose & Lee, 2010; Zhu *et al.*, 2015; Kim & Brandizzi, 2016). The synthesis of these wall polysaccharides in the Golgi requires two groups of glycosyl transferases: the polysaccharide synthases, which catalyze the polymerization of monomers, and glycosyl transferases, which add glycosyl residues (or short oligosaccharide chains) to the polymer chain (Burton *et al.*, 2010). Pushed by turgor pressure, these polysaccharides

have the ability to diffuse through the cell wall matrix (Proseus & Boyer, 2005; Cosgrove, 2017).

In the cell wall, post-synthetic modifications further alter the polysaccharides' chemical and physical properties (Burton *et al.*, 2010). HGs are subjected to methylesterification, lysis or hydrolysis. For instance, HGs can be de-methyl-esterified by PECTIN METHYL-ESTERASEs (PMEs), de-acetylated by PECTIN ACETYL-ESTERASEs (PAEs), or depolymerized by POLYGALACTURONASEs (PGs) and PECTATE LYASEs (PLs) (Hocq *et al.*, 2017). This PME-mediated cell wall modification is important for many developmental processes such as, initiation of organ primordia (Peaucelle *et al.*, 2011), hypocotyl development (Derbyshire *et al.*, 2007b; Pelletier *et al.*, 2010; Peaucelle *et al.*, 2015), resistance to wall degradation (Willats *et al.*, 2001b; Wolf *et al.*, 2009), and cell to cell adhesion (Wen *et al.*, 1999; Krupková *et al.*, 2007; Mouille *et al.*, 2007; Durand *et al.*, 2009; Verger *et al.*, 2016). At the cellular level, in pollen tubes PMEs locally methylesterify pectic HGs, influencing wall extensibility and pollen tube growth. Along the pollen tube, two zones can be defined: the neck with accumulation of low methylesterified HGs (being softer) and the tip with highly methylesterified HGs (being stiffer) (Bosch & Hepler, 2005; Bosch *et al.*, 2005; Jiang *et al.*, 2005; Parre & Geitmann, 2005; Bove *et al.*, 2008; Röckel *et al.*, 2008; Fayant *et al.*, 2010). XyGs are transglycosylated by XYLOGLUCAN ENDOTRANSGLUCOSYLASE (XET) or hydrolyzed by XYLOGLUCAN ENDOHYDROLASE (XEH), jointly known as XYLOGLUCAN ENDOTRANSGUCOSYLASE/HYDROLASEs (XTHs), or by ENDO-(1,4)- β -D-GLUCANASEs (Nishitani & Tominaga, 1992; Antosiewicz *et al.*, 1997; Steele *et al.*, 2001; Cosgrove, 2005; Shipp *et al.*, 2008; Caffall & Mohnen, 2009; Scheller & Ulvskov, 2010). All of these property processes indicate that cell wall matrix polysaccharides are very dynamic components, being subjected to various modifications over cell development.

In contrast to matrix polysaccharides, cellulose is synthesized at the plasma membrane by CELLULOSE SYNTHASE (CESA), which is assembled in large, rosette-shaped multimeric CESA protein complexes (CSCs) containing the ENDO-(1,4)- β -D-GLUCANASE KORRIGAN1 (KOR1) (Doblin, 2002; Somerville, 2006). CSCs move along AFs to reach the plasma membrane. Then, the cortical MTs (cMTs) that lie beneath the membrane act like rails along which the CSCs move, synthesizing glucan chains as they do so, which then aggregate to form microfibrils. In this way, the cMTs regulate the positioning of CESAs at the plasma membrane, as well as their velocity and density (Wasteneys & Galway, 2003; Wasteneys, 2004; Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Chen *et al.*, 2010; Wolf *et al.*, 2012). As a result, the

positioning of the cMTs reflects the arrangement of the CMFs (Paredes *et al.*, 2006). CELLULOSE SYNTHASE INTERACTING 1 (POM2/CS11) connects cMTs with CESAs and is required for the movement of the CESAs along the cMTs (Bringmann *et al.*, 2012). Via a pharmaceutical approach, using taxol (a MT-stabilizing drug) and oryzalin (a MT-depolymerizing chemical), cMTs have been shown to influence CSC mobility, but not their presence at the plasma membrane (Lloyd 2011).

In summary, cell wall deposition and modification over cell development is controlled by the cytoskeleton. Additionally, the networks of different polysaccharides present in the wall interact with each other, which also heavily influences cell wall properties.

1.3.3 Interactions between cell wall components

Cell wall growth and maintenance are controlled by covalent and non-covalent interactions between the cell wall composites (**Table 5**) (Veysman & Cosgrove, 1998; Cosgrove, 2005). Covalent interactions involve atoms that share an electron pair (Langmuir, 1919) and occur, for example, during transglycosylation between XyGs and cellulosic substrates (Hrmova *et al.*, 2007). Non-covalent interactions, instead of sharing electrons, involve electromagnetic cooperations, for instance calcium ions and borate diester cross-links that together support cell wall components. The interactions between CMFs and non-cellulosic polysaccharides influence the physical properties of the cell wall (Cosgrove, 2005).

CMFs are composed of aggregated polymer chains with constrained configurations. Water molecules cannot access these chains inside the microfibrils, however, the chains on the CMF side surfaces display hydrophilic properties thanks to their free -OH groups. The top and bottom surfaces of the CMFs, on the other hand, are hydrophobic. The amount of hydrophilic and hydrophobic faces on the microfibrils determines the interactions between different microfibrils and other matrix components (Newman *et al.*, 2013; Cosgrove, 2014, 2017; Wang & Hong, 2016). CMFs are, at certain places, non-covalently connected to each other through hydrogen bonds present between the hydrophobic faces of the microfibrils, forming larger fibril complexes (Burton *et al.*, 2010; Zhang *et al.*, 2016; Cosgrove, 2017).

CMFs also form non-covalent crosslinks with XyGs on their hydrophobic face (Hanus & Mazeau, 2006; Whitney *et al.*, 2006; Hrmova *et al.*, 2007; Dick-Pérez *et al.*, 2011; Zhao *et al.*, 2014; Cosgrove, 2017; Zheng *et al.*, 2018). These XyG-cellulose connections reinforce and strengthen the cell wall (Hayashi, 1989; Talbott & Ray, 1992; Whitney *et al.*, 2006; Park & Cosgrove,

2015). XTHs mediate mechanical properties of the walls via controlling their strengthening/loosening (Fry *et al.*, 1992; Nishitani & Tominaga, 1992; Antosiewicz *et al.*, 1997; Thompson & Fry, 1997, 2001; Steele *et al.*, 2001; Rose *et al.*, 2002; Strohmeier *et al.*, 2004). Some XET isoforms catalyze the process of connecting XyGs to cellulose (Cosgrove, 2005; Vissenberg *et al.*, 2005), or link glucan chains of amorphous cellulose together (Shinohara *et al.*, 2017). The role of XyG in wall extension and cell growth has been studied using fungal endoglucanase treatment to hydrolyze XyG, which leads to a physical weakening and extension of the cell wall (Yuan, 2001; Cosgrove, 2005). A new insight into the cellulose-XyG interaction was brought by the recent study on XyG-deficient mutants *xyloglucan xylosyltransferases* (*xxt1, xxt2*) (Xiao *et al.*, 2016; Cosgrove, 2017). XyG-deficient mutants display more aligned and aggregated CMFs in comparison with the wild type, suggesting that XyGs promote spacing between the CMFs and influence microfibril lateral interactions. Moreover, *xxt1/xxt2* cell walls have been shown to stretch more easily than in the wild type under tensile stress conditions, being softer and weaker than the wild type wall. Consequently, dark-grown hypocotyls in the *xxt1xxt2* mutant grow more slowly, as its walls extend slowly (Xiao *et al.*, 2016; Cosgrove, 2017).

Besides cellulose-XyG interactions, CMFs also interact with pectins (Chanliaud & Gidley, 1999; Dick-Pérez *et al.*, 2011). In actively growing cells, pectins are constantly secreted into the existing network of wall polysaccharides, indicating that the cellulose-pectin ratio is constantly regulated, highlighting its importance in the cell wall growth process (Palme *et al.*, 2002; Yoneda *et al.*, 2010). CMFs interact with pectins through non-covalent bonds (Wang *et al.*, 2012, 2015), which stabilize the CMFs in non-growing places or induce the sliding of the CMFs in expanding cell walls and thus promote cell growth (Ridley *et al.*, 2001; Dick-Pérez *et al.*, 2011). In particular, arabinans and arabinogalactans cause swelling of the cell wall, influencing its extensibility and stiffness (Zykwinska *et al.*, 2005, 2007a; b).

Covalent interactions are also present within the different pectin domains (Ridley *et al.*, 2001; Taylor *et al.*, 2003; Burton *et al.*, 2010) and between pectin, xylan and AGP (Tan *et al.* 2013). Moreover, pectins are cross-linked via ion bonds involving calcium and borate (Cosgrove, 2005; Burton *et al.*, 2010). High pectin methyl-esterification decreases its capacity to crosslink via calcium ions, while de-methyl-esterification increases the negative charge of pectin, promoting its binding to calcium ions, leading to pectin gel formation and its interaction with positively charged EXTs (Virk & Cleland, 1990; Cabrera *et al.*, 2008, 2010; Valentin *et al.*, 2010; Hocq *et al.*, 2017). The removal of the methyl ester groups from HGs promotes the crosslink of

calcium ions, which increases HG viscosity (stickiness) and cell adhesion (Burton *et al.*, 2010). These interactions are essential for the scaffold formation of the new cell plate, pectin dehydration and cell wall compaction. Borate diester bonds are present between different RGII chains and are known to regulate cell wall porosity and thickness (Ridley *et al.*, 2001; Cosgrove, 2005). Additionally, other pectins such as arabinans and arabinogalactans interact with acidic pectins (Cosgrove, 2005; Zykwinska *et al.*, 2005; Dick-Pérez *et al.*, 2011; Wolf *et al.*, 2012).

Pectins have also been found to covalently bond to XyGs in cell walls. The pectin-XyG complex is formed by newly-made XyGs, just-deposited acidic pectin polysaccharides and several other mature wall polysaccharides (Keegstra *et al.*, 1973; Thompson & Fry, 2000; Cumming *et al.*, 2005; Park & Cosgrove, 2015). Half of newly synthesized XyGs are formed as a free (neutral) chain, while the other half interact with an anionic pectin primer, which leads to the formation of a pectin-XyG complex with a negative charge. These negatively charged pectin-XyG complexes are highly stable and left uncleaved for at least several days. The reason behind the stability of such complexes is thought to be a change from a string-like structure into a three-dimensional one, which aids the integration of the aforementioned complex into the wall. Yet, the function of the complex is still elusive (Popper & Fry, 2008).

In summary, recent studies have challenged the stereotypical model of the interactions within the wall with separated CMFs connected to XyGs, which make them resistant, and hydrated pectins between the microfibrils softening the wall (Carpita & Gibeaut, 1993). A new model has recently been proposed wherein cell wall biomechanical hotspots occur, consisting of merged CMFs with XyG in between, XyG linked with non-crystalline cellulose, as well as directly connected CMFs (Zhang *et al.*, 2016; Cosgrove, 2017).

Table 5. Overview of main covalent and non-covalent interactions within the primary type 1 cell wall.

Interactions	Cellulose	Xyloglucan	Pectins
Cellulose	non-covalent (hydrogen)	covalent / non-covalent (hydrogen)	non-covalent
Xyloglucan		covalent	covalent
Pectins			covalent / non-covalent (calcium ions/borate diester)

Interestingly, comparing the stiffness of the cell wall (10 MPa-10 GPa) to that of individual composites reveals their contribution to cell wall mechanical properties (**Table 6**): for instance the stiffness of CMFs (around 100 GPa) is at least ten times higher than the stiffness of the wall “mix”, while pectins (10-200 MPa), or hemicelluloses (around 40 MPa) are much softer than cellulose (Niklas 1992; Boudaoud, 2003; Keckes *et al.*, 2003; Zsivanovits *et al.*, 2004; Burgert, 2006; Mirabet *et al.*, 2011). In summary, cell wall is composed of different polysaccharides, whose amounts and interactions determine the wall properties and regulate growth.

Table 6. *Stiffness of different cell wall components (according to Niklas 1992; Boudaoud, 2003; Keckes et al., 2003; Zsivanovits et al., 2004; Burgert, 2006, reviewed in Mirabet et al., 2011)*

	Stiffness
Cell wall	10 MPa-10 GPa
Cellulose	~100 GPa
Hemicelluloses	~40 MPa
Pectins	10-200 MPa
Lignin	~2 GPa

1.4 Plant biomechanics

Plant cell vigor is influenced by the turgor pressure resulting from water accumulation in the cell. Turgor pressure is known as a driving force of plant cell growth and is applied by protoplasts to the surface of the wall, putting it under tension. This turgor pressure generates a strong force, reaching up to 2MPa. To reflect the magnitude of turgor pressure, we could compare it to the air pressure in a car tire (Beauzamy *et al.*, 2014, 2015), which is ‘only’ around 280-350 kPa.

The turgor pressure within every single cell creates a mechanical tension pattern within the tissue or organ and therefore the growth of a single cell is related to its neighboring cells. Remarkably, the tension can be tissue specific, which is the case for example in the epidermis, which is pushed by underlying tissues (Kutschera & Niklas, 2007). This kind of tension is also observed in other systems such as cardiac cells, which generate tensional forces either on vascular muscle cells, resulting in stretched arteries, or on epithelial cells (Leckband & de Rooij, 2014). Besides tension, other types of mechanical interactions exist such as shear and compression stresses. Shear stress acts in parallel to the cell surface, like for example in animal endothelial cells, stimulating blood flow. Compression stress is applied perpendicularly to the cell, inducing its compaction, such as in inner tissues of the leaf under the epidermis, or in root cortex cells during lateral root emergence in plants or in chondrocytes in animals, which are pushed by both body weight and muscle tension. Compression of slender materials could cause their buckling, which is defined as low energy-induced spontaneous changes of material geometry

(Green, 1999; Hejnowicz & Borowska-Wykreć, 2005). In plants, buckling can be observed in grass blades, and at the cellular level in the inner cell wall layer of etiolated hypocotyl cells (Hejnowicz & Borowska-Wykreć, 2005).

At the subcellular scale, turgor presses on cell walls. In plants, the cell wall is crucial for the dynamics of growth (Cosgrove, 2016), because during the adjustment of the cell shape, the cell wall extends and changes the contact area with the surrounding cells. Importantly, modifications of the balance in wall mechanical properties between wall loosening and wall synthesis determining the cell shape (Boudaoud, 2010; Hamant & Traas, 2010; Hejnowicz, 2011).

1.4.1 Growth as a physical process

Mechanics can be characterized as changes in an object shape under physical forces (Boudaoud, 2010; Mirabet *et al.*, 2011). Two kinds of mechanical property characteristics of such an object can be defined: the elasticity and the plasticity. An object is considered elastic when deformation is reversible. This can be observed for instance when a stretched elastic band is able to return to its original shape when the stretching force is removed. In contrast, plastic deformation appears as irreversible changes of the object (also called creeping). For example, a deformed paper clip is not able to return to its initial shape. Plastic deformations can be observed in the directional changes that occur during cell growth and can be measured by observing wall extension (Cosgrove, 1986, 2017; Boudaoud, 2010; Mirabet *et al.*, 2011).

Elastic properties of an object can be characterized by the analysis of its deformation through the use of physical formulae. An object's length (L_0) and cross-section area (S) need tension in order to deform. Tension is produced by the application of forces (F) at the object's ends. The measure of deformation of the object relative to its initial length is known as strain, and can be calculated by the formula $\varepsilon = \frac{L-L_0}{L_0}$. The tension applied is dependent on the cross-section area of the object. This relationship is known as stress (σ) and can be calculated by the formula $\sigma = \frac{F}{S}$. The resistance to elastic deformation or stiffness of an object is known as the elastic modulus (E) and is the relationship between stress and strain, $E = \frac{\sigma}{\varepsilon}$, which is measured in Pascals, $Pa = \frac{N}{m^2}$. The higher the elastic modulus, the stiffer the object. Such properties can be observed and measured in living plant cells, for example by transiently increasing or decreasing the water potential of the medium surrounding the plant tissue thus influencing the turgor pressure inside the cells. Artificial increase of turgor pressure will increase the force (F) applied to the cell wall and thus the tensile stress (σ). This can lead to reversible deformations (ε) of

the tissue depending on the physical properties of the cell walls (S and E) (Kierzkowski *et al.*, 2012)

Plasticity of an object can be analyzed by measuring the irreversible extensibility of the material, using the formula $\mu = \frac{\epsilon}{\sigma - Y}$, which is time dependent. Strain, over a specific time frame, is measured as $\epsilon = \frac{dL}{dt}$. Yield threshold (Y) defines the minimum stress (σ) necessary to extend the object. If the stress is smaller than the yield threshold (Y), the object will not extend but will go back to its initial length after the force is removed. On the other hand, in the presence of a larger stress, the object will deform and extend irreversibly. The extensibility is measured as $\frac{m^2}{Ns}$, which could suggest that the higher the extensibility, the softer the object. However, Cosgrove (2016) proposes that measuring a softer cell wall with atomic force microscopy (AFM) does not mean that the cell wall will be more extensible. The extensibility depends on the direction of the softness (elastic modulus).

The properties of the wall can be measured using AFM, which specifically measures the wall elasticity (Milani *et al.*, 2011; Majda *et al.*, 2017). AFM, as well as cellular force microscopy (CFM), can also be used to measure turgor pressure (Routier-Kierzkowska *et al.*, 2012; Beauzamy *et al.*, 2015). In both cases, cell deformations can be measured by quantifying the cell shape change over time with imaging tools like confocal microscopy (Kierzkowski *et al.*, 2012). Turgor driven cell wall deformation depends on the cell wall thickness (Beauzamy *et al.*, 2015), which can be defined using transmission electron microscopy (TEM), but also on the extensibility of the wall, which can be measured with an extensometer (Robinson *et al.*, 2017).

1.4.2 Plant cell growth

Growth of the cell can be defined by Lockhart's equation (Lockhart, 1965), which describes the irreversible increase of the volume and wall plasticity. Growth is characterized by three parameters: rate, anisotropy and direction. Growth rate is the modification of the cell size over time, compared to the initial size. Anisotropy is the unequal growth among the different axes of the cell. Growth direction is the preferred axis by which anisotropy occurs. The final organ shape is established by the cooperation between the cell division activity and the cell growth rate (Schmundt *et al.*, 1998; Rolland-Lagan *et al.*, 2003; Grandjean *et al.*, 2004; Reddy, 2004; Mirabet *et al.*, 2011). Plant morphogenesis is controlled by cell divisions and differential growth of cells, being a consequence of irreversible wall expansion (deformation).

This can for example be seen in an emerging primordium on the apical meristem, which displays distinct patterns of cell expansion, influencing

geometrical changes within the organ (Burian *et al.*, 2013). Changes in plant cell development can be tracked through live imaging (Rolland-Lagan *et al.*, 2003; Fernandez *et al.*, 2010; Robinson *et al.*, 2011) and imprinting methods (Dumais & Kwiatkowska, 2002; Kwiatkowska & Dumais, 2003). Additionally, recent developments of interdisciplinary approaches such as computational biology allow for automated quantification of plant morphogenesis (de Reuille *et al.* 2015). Modeling approaches such as finite element modeling (FEM) (Bidhendi and Geitmann 2017) allow the manipulation of different mechanical parameters *in silico* and the analysis of how they contribute to growth (Rolland-Lagan *et al.*, 2003; Bolduc *et al.*, 2006; Hamant *et al.*, 2008; Bidhendi & Geitmann, 2017). All together, these different approaches allow a comprehensive study and understanding of growth by: 1) integrating observed parameters from live imaging and 2) digitally reconstructing analyzed cell shapes into the virtual organ, which provides the opportunity to observe the changes over time during development (a 4D perspective). Moreover, modeling approaches allow: 3) the prediction of complex biological processes related to growth and the testing of hypotheses *in silico* that can be further investigated *in vivo* (Fernandez *et al.*, 2010; de Reuille *et al.*, 2015).

1.5 Epidermis controls plant growth

The epidermis is the outermost monolayer of plant tissues and forms the boundary between the plant and the external environment. It provides protection against external agents and controls the exchange of water, ions and nutrients with the environment (Javelle *et al.*, 2011). Therefore, epidermal cells need to be tightly connected to prevent any rupture. This connection is acquired thanks to their anticlinal walls, which sit perpendicularly to the leaf surface (Galletti *et al.*, 2016). The external periclinal cell walls (parallel to the leaf surface) are thicker than the anticlinal walls and are covered by a thick layer of cuticle and waxes (Kutschera & Niklas, 2007; Ingram & Nawrath, 2017).

An important role of epidermis in the regulation of organ growth was already proven at the end of the nineteenth century by the botanist Wilhelm Hofmeister, who performed two experiments that have since then become classical: 1) the dissection of the sunflower stem showed an outward recurvature of the segments, and 2) the removal of the epidermis from the stem caused a rapid and uncontrolled expansion of the pith (Kutschera & Niklas, 2007). These observations indicate that the epidermis is under tension and limits the growth of the tissues below, which are compressed within the organ.

Moreover, the epidermis is the most sensitive to the plant hormone auxin in comparison with other tissues. One hypothesis is that this is due to the proton pumps specifically located at or enriched in the epidermis (Kutschera *et al.*, 1987).

During organ growth, the tissues within press on the epidermis. This generates a tension, which is applied parallel to the cell surface, resulting in the expansion of the epidermal cells. However, the epidermis is thought to mechanically prevent an expansion of underlying tissues (Kutschera *et al.*, 1987; Savaldi-Goldstein & Chory, 2007; Szymanski, 2014). Taking once again the analogy of a tire, the epidermis can be compared to one that is filled with air and constantly under high pressure. The tire needs to be resistant to tension and at the same time be able to extend without breaking, which is why cells, within different tissues, need to adjust to each other.

In the leaf, the epidermis plays an important role in the regulation of the mesophyll growth rate and it controls the overall leaf size (Savaldi-Goldstein *et al.*, 2007; Procko *et al.*, 2016). The study of different chimeras has revealed that leaf size is controlled by the rate and amount of epidermal cell division. Moreover, it has been shown that epidermal cell divisions dictate the division activity of the mesophyll (Marcotrigiano, 2010). Interestingly, genetic studies have brought new insights into the role of the epidermis in plant growth. For example, the localized overexpression of CYCLIN-DEPENDENT KINASE (CDK) inhibitor genes in the epidermis inhibits cell division in the epidermal layer of the leaf, which is compensated by an increased epidermal cell volume. This defective epidermis was not able to influence the division of the tissues below (Savaldi-Goldstein & Chory, 2008).

In conclusion, the epidermis is a tissue of great importance in plants, not only because it provides the protection to the plant as the most external layer, but also because it controls the expansion of underlying tissues and contributes to the shaping of the organs.

2 Objectives

The objective of this study was to understand the role of the plant cell wall in cell shape acquisition. The following questions were addressed in this work:

- What is the role of the cell wall in PC shape acquisition?
(PAPER I)
- How does auxin regulate lobe formation in PCs?
(PAPER II)
- What is the relation between auxin, acidic growth and cell wall remodeling?
(PAPER III)
- What is the cause of cell wall thickening in epidermal and mesophyll cells?
(PAPER IV)

3 Results and Discussion

The work presented in this thesis focuses on how the plant cell wall contributes to cell shape acquisition. We addressed this question using not only the plant model species *Arabidopsis thaliana* but also camphor tree (*Cinnamomum camphora*), which is an anciently diverged dicot plant (Zeng *et al.*, 2014).

In this work, we showed that epidermal PC shape acquisition in *Arabidopsis* relies on cell wall composition and mechanical properties. Remarkably, these jigsaw-puzzle shaped epidermal cells display different cell wall mechano-chemical properties across the cell wall width and along the cell perimeter, matching with their sinuous outline. The appearance of these local heterogeneities in the cell wall precedes the wall bending and any cell morphological changes (**PAPER I**). Next, we reported that PC development follows a specific spiral division pattern, displaying an ontogenical sequence of cell differentiation. This process is regulated by the phytohormone auxin through the establishment of an auxin concentration gradient generated by directional transport (**PAPER II**). These findings are in accordance with what is found in the literature on the role of auxin in stimulating acid growth and activating the expression of genes controlling cell wall biosynthesis and remodeling (**reviewed in PAPER III**). Cell wall composition was also studied in camphor tree, which revealed a polarization of some cell wall components. Moreover, the epidermal cell walls and mesophyll spongy parenchyma cell walls display unique features in this species, such as lignified secondary cell wall deposition (**PAPER IV**).

3.1 Leaf epidermal pavement cells as a model to study cell shape acquisition

Leaf epidermal PCs are usually flattened and are surrounded by periclinal walls that are thick and parallel to the surface, and by anticlinal walls which are thin and perpendicular to the surface, connecting the cells to each other

(Szymanski, 2014). On the paradermal plane, PCs exhibit an interdigitated shape with an alternating pattern of lobes (bulges) and necks (indentations) (Fu *et al.*, 2005). The surrounding anticlinal cell walls display a sinuous contour (**Figure 4**) (Panteris & Galatis, 2005) alternating from curved to straight regions. Remarkably, the PC shape is dynamic during its development, transitioning from an isodiametric initial form to a lobed final shape (e.g. Panteris & Galatis, 2005). The shape of the PCs varies not only along leaf development, but also according to their position in the leaf. To systematically compare the shapes of the cells between different leaves, we measured the PCs located in the middle of the leaf.

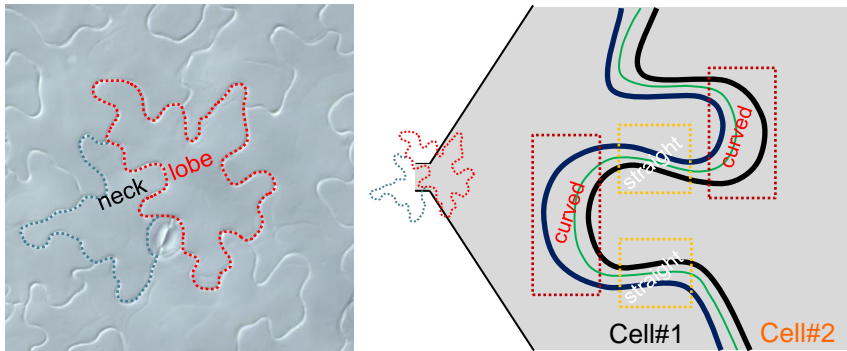


Figure 4. Epidermal pavement cells (on the left) and drawing illustrating curved and straight regions of anticlinal pavement cell walls (on the right).

The development of a novel approach to characterize the cell morphology (circularity) and cell wall curvature was established and performed in this study. The shape of the PCs can be characterized by measuring the widths of the necks (Fu *et al.*, 2002, 2009), widths of the lobes (Fu *et al.*, 2005), and number of lobes with an outgrowth longer than 1 μm (Xu *et al.*, 2010). However, these measurements seemed to be insufficient to reflect the complexity of PC shape. We therefore decided to characterize the PC shape by its circularity, which is defined as the ratio between the area and perimeter. Circularity oscillates from 0 to 1 with decreasing shape complexity (Armour *et al.*, 2015) and correlates with the lobing pattern of the PC: a cell with fewer lobes is more circular with circularity closer to 1, whereas a more complexly shaped cell with an increasing number of lobes has a circularity closer to 0. A high circularity therefore suggests a decrease in lobe number.

3.2 The native cell wall composition is important for pavement cell shape acquisition (PAPER I)

To investigate whether the cell wall composition is important for PC shape acquisition in *Arabidopsis*, we performed a confocal microscopic screen of a variety of cell wall deficient mutants. These mutants are affected in the biosynthesis and post-synthetic modifications of different cell wall polysaccharides - specifically, the main cell wall components, including cellulose, pectins and hemicelluloses. Our results showed that different cell wall mutants display a wide range of cell shape alterations. To investigate how these specific cell wall components, defective in these mutants, might influence the geometry of the PCs, we introduced three different measurement parameters: i) the cell area in the two-dimensional, paradermal plane, ii) the cell circularity, and iii) the lobe number.

We quantified these parameters in a semi-automated way using CellSeT, “a tool to segment confocal microscope images” (Pound *et al.*, 2012), which extracts the outlines of the cells in the vector scale. During this process, we were able to control the segmentation of every single cell analyzed, which allowed us to exclude stomata and the cells which were not entirely enclosed within the image (**PAPER I, Figure S1A**). Lobes were defined using “cytoskeletonisation” based on dendroid-like structures within a PC, while every end of this computer-generated “cytoskeleton” was treated as a lobe.

We investigated the PCs in the wild type and 16 different cell wall mutants (**Table 6**) (**PAPER I, Figure 1A**) and found that the PC population from each individual genotype is characterized by a great variance in cell size and shape (for the wild type see: **PAPER I, Figure 1B, C**), with cells varying from small and circular to big and interdigitated. Indeed, we noticed that the mean area of all PCs measured is different between the wild type and cell wall mutants (**PAPER I, Figure 1D**). For instance, *35::GALS-YFP* (β -1,4-galactan synthase mutant) (Liwanag *et al.*, 2012), *mur3-1* (GALACTOSYLTRANSFERASE deficient) (Reiter *et al.*, 1997), *mur4-1* (ARABINOTRANSFERASE deficient) (Reiter *et al.*, 1997), *pom1-2* (CESA-INTERACTIVE PROTEIN deficient) (Zhong, 2002), *xxt1/xxt2*, *xxt5*, *xxt1/xxt2/xxt5* (XXT defective mutants) (Cavalier *et al.*, 2008; Zabortina *et al.*, 2008) and *qual-1* (GLYCOSYLTRANSFERASE deficient) (Bouton, 2002) all have bigger cell areas in comparison with the wild type, implying that PCs in these lines might grow faster. By contrast, cell wall mutants *mur1-2* (GDP-D-MANNOSE-4,6-DEHYDRATASE deficient) (Bonin *et al.*, 1997) and *qua2-1* (GLYCOSYLTRANSFERASE deficient) (Bouton, 2002) display smaller cell areas. This variance in cell area among the mutants might mask any differences

in localized growth and cell shape defects. In an attempt to avoid any cell shape differences caused by growth defects in the mutants, we re-analysed cell size, and performed other analysis, selecting only the fully developed PCs (**PAPER I, Figure S1E**). After this analysis, we found that some lines including *mur3-1*, *xxt1xxt2*, *xxt1xxt2xxt5*, and *qua1-1* still display larger cell areas than the wild type, suggesting that some matrix polysaccharides, such as HGs, XyGs and galactosylated XyGs, may be involved in the regulation of overall PCs growth. To the contrary, *qua2-1* has smaller PCs than the wild type, indicating that HGs might not be involved in promoting cell growth.

We also found differences in cell circularity and lobe numbers in fully developed PCs among the wild type and different cell wall mutants (**PAPER I, Figure 1C, D**). It should be noted that these parameters measure specifically the differences in the localized but not global growth of PCs. Among the lines analyzed, *gal10-1* (β -GALACTOSIDASE deficient) (Sampedro *et al.*, 2012), *mur3-1*, *xxt5*, *xxt1/xxt2*, *xxt1/xxt2/5*, *kor1-1* (ENDO-1,4-BETA-D-GLUCANASE deficient) (Nicol *et al.*, 1998) and *qua1-1* display a higher circularity, which corresponds to a reduced lobe number. Interestingly, among the xyloglucan deficient mutants, an increase in circularity positively correlates with the number of mutated genes. In comparison with the wild type, *gal10-1*, *kor1-1* and *xxt5* display a decreased lobe number but no change in overall cell size, which suggests that specific cell wall enzymes such as β -GALACTOSIDASE, ENDO-1,4-BETA-D-GLUCANASE and XXT5 might be involved in local cell wall modifications that promote the lobing process. Interestingly, the mutant *mur1-2* displays an increased lobe number, while the cell circularity is not changed compared with the wild type, suggesting that this mutant might form shallow lobes. The opposite situation is observed in the *35::GALS-YFP* mutant where the cell circularity is decreased while the lobe number remains unchanged, suggesting the formation of wider lobes in this mutant. Moreover, *35::GALS-YFP* exhibits larger cell size, which may indicate that galactan is involved in the regulation of both overall cell expansion and localized cell growth. Altogether, our analysis of various cell wall deficient mutants revealed alterations in PC shape, indicating that native cell wall composition is important for PC shape acquisition, which requires both the synthesis and the remodeling of different cell wall components.

Table 6. An overview of different cell wall mutants used in PAPER I.

Abbreviations	Mutant name	Reference
<i>gal10-1</i>	β -galactosidase	(Sampedro <i>et al.</i> , 2012)
<i>gals1</i>	β -1,4-galactan synthase	(Liwanag <i>et al.</i> , 2012)
<i>GALS OX</i>	β -1,4-galactan synthase	(Liwanag <i>et al.</i> , 2012)
<i>gls8-2</i>	glucan synthase like 8	Chen <i>et al.</i> , 2009)
<i>mur1-2</i>	GDP-D-mannose-4,6-dehydratase	(Bonin <i>et al.</i> , 1997)
<i>mur2-1</i>	fucosyltransferase	(Reiter <i>et al.</i> , 1997)
<i>mur3-1</i>	galactosyltransferase	(Reiter <i>et al.</i> , 1997)
<i>mur4-1</i>	arabinotransferase	(Reiter <i>et al.</i> , 1997)
<i>pom1-2</i>	cellulose synthase-interactive protein	(Zhong <i>et al.</i> , 2002)
<i>prc1-1</i>	cellulose synthase 6	(Desnos <i>et al.</i> , 1996)
<i>qua2-1</i>	glycosyltransferase	(Bouton <i>et al.</i> , 2002)
<i>xxt5</i>	xyloglucan xylotransferase 5	(Zabotina <i>et al.</i> , 2008)
<i>xxt1/xxt2</i>	xyloglucan xylotransferase 1/2	(Cavalier <i>et al.</i> , 2008)
<i>xxt1/xxt2/xxt5</i>	xyloglucan xylotransferase 1/2/5	(Zabotina <i>et al.</i> , 2012)
<i>kor1-1</i>	endo-1,4-beta-D-glucanase	(Nicol <i>et al.</i> , 1998)
<i>qua1-1</i>	glycosyltransferase	(Bouton <i>et al.</i> , 2002)

3.3 Computational modeling shows that local inhomogeneity within anticlinal cell walls is necessary for the lobing of pavement cells (PAPER I)

To unveil how cell wall properties might influence the lobing process, we employed a computational modeling approach, named FEM, to study the dynamics of material geometry and complexity (Bidhendi & Geitmann, 2017). Plant cells are thought to be under compressive forces, which lead to the so-called buckling of the cell walls (Green, 1999; Shipman & Newell, 2004; Dumais, 2007), defined as the instability of sheets under compression (Hejnowicz & Borowska-Wykręt, 2005). However, PCs, as a composite of the epidermis, are subjected to tensional forces and the growth of epidermis is related to the stretching of the cell walls between individual cells (Sampathkumar *et al.*, 2014). We first tested the influence of tensional or compressional forces on the straight segments of cell wall-like materials with

either homogeneous or heterogeneous (softer and weaker materials alternating along and across the wall segment) properties by computational modeling (**PAPER I, Figure 2A-D**). Under compressive forces, homogeneous material buckles, while heterogeneous material bends, with the stronger segment being on the convex side. Under tensional forces, homogeneous material remains straight and does not bend, while heterogeneous material bends, with elastically softer material on the convex side. This result indicates that the direction of bending is different between tension and compression.

Next, we built a virtual PC, consisting of four anticlinal and initially straight wall segments, which were surrounded by other cells within an epidermis under tension (**PAPER I, Figure 2E-H**). We tested the effect of cell wall properties on the lobing of PCs under four different scenarios: i) walls were homogeneous, ii) walls displayed different properties along the perimeter (interchanging softer or stronger segments), iii) walls displayed different properties alternating along and across the walls, iv) different properties were present only across the walls. In summary, we observed that only the walls displaying mechanical properties under scenario iii) are able to lobe. In addition, we found that the size and number of the alternating heterogeneous wall segments influence the lobing of PCs. Furthermore, we observed that softer walls bend more easily than stiffer ones and cell walls are more likely to bend when the difference in the mechanical properties between the softer and harder segments becomes larger (**PAPER I, Figure 2I, J**). Our modeling results indicate that the lobing process depends not only on the mechanical heterogeneities of cell walls, but also on their size and density, plus the magnitude of the difference in overall wall stiffness.

3.4 Pavement cell walls display heterogeneous mechanical properties as shown by AFM analysis (PAPER I)

To validate the predictions of our model, we used AFM to characterize the mechanical properties of anticlinal cell walls. In order to access the anticlinal walls without any influence from leaf topography on our measurements, we prepared ultrathin, paradermal sections of the *Arabidopsis* leaf embedded in resin. We recorded high-resolution AFM images that present the mechanical properties expressed as apparent elastic modulus (E_a). From every AFM image we selected a region of interest (ROI) representing different cell wall regions. Within each ROI, different force curves were generated ($n > 100$), which were then processed in order to obtain stiffness values represented in pascals (Pa) (**PAPER I, Figure 3**).

We first investigated the mechanical properties of fully developed PCs in the wild type (**PAPER I, Figure 4**). We found that curved wall zones were stiffer (appx. 20%) than straight ones. This indicated that the alternating pattern of lobes and necks is correlated with a repetitive array of stiffer and softer wall zones. When the mechanical properties across the walls were examined, we also observed differences across both the curved and straight cell wall regions in the wild type. On average, the convex side was 10% softer than the concave side in the curved cell wall zones. To test whether the observed heterogeneities along and across the walls in the wild type are associated with the lobing process, we measured the mechanical properties of the straight cell walls of the cell polarity deficient, non-lobing *constitutively active-rop2* (*CA-rop2*) mutant. As expected, only homogenous walls, both along and across their perimeter, were observed in this mutant. To further test the observed association between wall heterogeneity and cell polarization using other another tissue, we preformed AFM analysis on the anticlinal cell walls of *Arabidopsis* root atrichoblasts that show no polarization. Again as expected, these walls displayed homogeneous mechanical properties (**PAPER I, Figure S3**). Overall, our AFM studies confirmed the prediction by the FEM modeling that PC walls display dual mechanical heterogeneity, which is present only in lobing cells.

3.5 Interdigitated pavement cells display a polar distribution of galactan and arabinan cell wall components (PAPER I)

Next, we wanted to know if different mechanical properties observed in sinuous anticlinal PC walls are due to local changes in polysaccharide distribution. To this end, we performed immunogold labeling of epitopes for different cell wall polysaccharides and detected them by high-resolution electron microscopy (EM). We used the carbohydrate binding module family 1 (CBM1) antibody to study the distribution of load-bearing cellulose microfibrils (crystalline cellulose), which are embedded in different matrix polysaccharides composed of pectins and hemicelluloses. Regarding pectins, we labeled the most common epitopes including acid and methylesterified HG using John Innes Monoclonal Antibody 5 (JIM5) and JIM7 antibodies, respectively, galactans using the Leeds Monoclonal Antibody 5 (LM5) antibody, and arabinans using the LM6 antibody. Among hemicelluloses, we targeted fucosylated and non-fucosylated xyloglucan using Complex Carbohydrate Research Center monoclonal antibody M1 (CCRC M1) and

CCRC M89 antibody, respectively. To precisely determine the positions of the gold particles, we developed a semi-automated algorithm to define the curved and straight cell wall zones within each EM image. By the same algorithm, we were able to define the densities of different cell wall epitopes within the curved and straight cell wall regions. We quantified the distributions of gold particles across the wall (polarity), between convex and concave sides within curved zones, and between two sides across the straight walls, in *Arabidopsis* PCs (**PAPER I, Figure S4**).

Our results indicated that different cell wall epitopes, especially galactan, were highly concentrated in the straight wall zones but less abundant in the curved cell wall regions in the wild type (**PAPER I, Figure 5 and S5**). This correlates with the results obtained by AFM, which showed that the straight cell wall zones are in general softer than the curved ones. Next, we investigated the gold particle distributions across the walls. We detected acidic HG and methylesterified HG highly concentrated in the proximity of middle lamella in both curved and straight cell wall regions. Interestingly, galactan and arabinan epitopes display a polar localization in the curved cell wall zones in the wild type. Galactan epitopes are accumulated close to the convex part of the curved cell wall zone. In the straight cell wall regions, galactan epitopes are more abundant in close proximity to both plasma membranes. Arabinan epitopes are concentrated closer to the convex and middle sides in the curved wall zones and are less abundant in the concave zone. In the straight zones, arabinan epitopes are more concentrated in the middle of the cell wall. Other cell wall epitopes are localized in the walls in a nonpolar way. As a control, we checked the distributions of the same epitopes in the straight cell walls of the *CA-rop2* mutant. Fucosylated xyloglucan and acid HG epitopes are enriched around the middle region of the cell wall. Galactan epitopes are located close to both plasma membranes, like in the straight cell walls of the wild type. Other cell wall epitopes are nonpolar in the straight cell walls in the *CA-rop2* mutant. In the wild type, an increased concentration of galactan epitopes in the straight cell wall regions, as well as the local accumulation of galactan and arabinan epitopes in the convex side of curved wall regions, is consistent with the presence of local cell wall softening. This result is in agreement with previous reports indicating that galactan and arabinan are elastic, water-retaining components (McCartney *et al.*, 2000; Ha *et al.*, 2005). Thus, our data implied that these components might locally soften the wall and mediate the lobing of the PCs. To test whether the specific polar distribution of galactan epitopes is also present in other plant species, we next analyzed galactan and arabinan epitope distributions in the anticlinal PC walls in camphor tree and observed

similar distributions of these epitopes to the ones found in *Arabidopsis* (**PAPER I, Figure S6**).

3.6 The heterogeneity of anticlinal cell walls in the pavement cell precedes the lobing process (**PAPER I**)

We demonstrated that sinuous PCs display local softening of the walls, corresponding with a restricted accumulation of galactan and arabinan epitopes in these zones. This indicates the importance of these epitopes in wall bending and overall lobe formation. However, our model predicts that the cell wall inhomogeneity must appear in the straight cell walls of isodiametric cells before the walls start to curve. To clarify this hypothesis, we performed AFM analysis on straight or early bending anticlinal walls of young *Arabidopsis* PCs. Young leaves are characterized by high division activity and their epidermal layer consists of constantly dividing meristemoid cells and cells in different developmental stages, from isodiametric to interdigitated (**PAPER I, Figure 6**). Our results showed that the straight cell walls of young PCs display different mechanical properties, being softer in the central zone of the walls and stiffer closer to the corners. Moreover, these walls display different mechanical properties across the walls, being softer at the future convex side and stiffer at the future concave side, which is consistent with heterogeneous mechanical properties detected across fully developed PC walls. Therefore, different mechanical properties detected in straight walls precede the lobing process, which validates the model presenting that only heterogeneous walls will lobe. In young epidermal PCs, the softer wall zones display an increased accumulation of specific cell wall epitopes such as galactan (**PAPER I, Figure 7**). Other matrix polysaccharides such as arabinan and acid and methylesterified HG are accumulated in the middle wall zone and are less present at the corners. Interestingly, we showed a spatial distribution difference of XyG epitopes according to their fucosylation status: fucosylated XyGs are abundant close to the corners, while non-fucosylated XyGs are more present in the central cell wall zone. In contrast to the wild type, straight cell walls in young PCs of the *CA-rop2* mutant display accumulation of different cell wall epitopes close to the cell corners, except for fucosylated XyGs that are present in the central zone of the cell wall. These results indicate that anticlinal PC walls display different mechano-chemical properties, which are present before lobe formation. Moreover, we demonstrated that wall mechanical properties and wall composition vary between different developmental stages, indicating a high dynamicity of the cell walls.

3.7 Dissecting first lobe formation in pavement cells (PAPER II)

In epidermis, asymmetrical divisions of the meristemoid mother cell lead to the formation of meristemoids and stomatal lineage ground cells (SLGCs). After three consecutive asymmetrical divisions, the meristemoid then undergoes asymmetrical division and forms two guard cells. We observed that the lobing process in SLGCs occurs in a highly coordinated way: small SLGCs always lobe into a larger, more mature neighbouring cell (for method **PAPER I, Figure 1** and **chapter 3.2**). We decided to use this unique system to better understand the process of lobe formation in epidermal PCs. We analysed different cell parameters in SLGCs, such as cell area and membrane length, as well as number of lobes in the neighbouring cells (**PAPER II, Figure S1**). Our quantifications indicated that the majority of non-lobed SLGCs were situated adjacent to neighbouring cells with a low number of lobes (3, 4 or 5 lobes) (**PAPER II, Figure 1**). We showed that the lobing process is not related independently to a specific cell area or to a specific length of the distance between the cell corners (Euclidean point (eP) distance). Moreover, only a simultaneous increase of both the eP distance and the cell area together promotes the formation of new lobes in SLGCs (**PAPER II, Figures 1 and S1**).

The plant hormone auxin is known to regulate the lobing process in PCs (Xu *et al.*, 2010; Grones *et al.*, 2015). To investigate the influence of auxin on lobe formation, we quantified the area and average lobe number of fully developed PCs after application of different auxin concentrations (**PAPER II, Figure 2**). We showed that different concentrations of the synthetic auxin NAA (1-Naphthaleneacetic acid) had various effects on the PCs: low auxin concentrations (5 and 20 nM) induced both local cell expansion (lobing of PCs) and overall cell growth, while high concentration (100 nM) did not influence the lobe number, but promoted the overall cell growth. We next quantified the cell geometry parameters of SLGCs after different NAA treatments (**PAPER II, Figure S2**). Low NAA concentrations caused a decrease in both cell area and eP distance in non-lobing SLGCs, while a high NAA concentration induced an increase in both cell area and eP distance in these cells. These results indicate that low auxin concentration can promote lobe formation while high auxin concentration suppresses the formation of lobes.

In *Arabidopsis* leaf epidermis, after three consecutive asymmetrical divisions of the meristemoid to produce PCs, guard cells are then formed through asymmetrical and symmetrical divisions of the meristemoid (Berger &

Altmann, 2000; Geisler, 2000). As a result, a newly formed stoma is surrounded by three cells displaying different sizes and stages of development in a spiral configuration, called an anisocytic stomatal complex (Metcalf & Chalk, 1950) (**PAPER II, Figure 2**). We analysed the distribution and signal strength of the auxin marker DR5 within the cells of anisocytic spirals. After the first asymmetric division of the meristemoid, we found that the DR5 expression level was similar in both newly formed cells. However, as the stomatal complex development progressed, the DR5 signal revealed an ascending auxin gradient within the spiral, with the weakest signal in the youngest SLGC. Interestingly, we found that once the first SLGC lobe has been formed, the occurrence of this ascending DR5 signal intensity pattern in the spiral significantly decreases, sometimes even reversing to reveal a descending auxin gradient (**PAPER II, Figure 2**). Our data imply that a local auxin minimum established in the centre of the spiral promotes lobe formation in the SLGC. Moreover, these results suggest that auxin levels in the SLGCs are not constant throughout the formation of lobes, but rather fluctuate according to the developmental stage.

Auxin homeostasis within plant tissues is achieved and maintained by auxin transporters. Therefore, to analyse whether auxin transporters could directly influence the lobing process, we analysed the geometry of PCs in a range of auxin transporter mutants defective in PIN proteins (auxin exporters), AUXIN RESISTANT (AUX)/LIKE-AUX (LAX) (AUX/LAX) proteins (auxin importers) and ATP-BINDING CASSETTE SUBFAMILY B (ABCB) proteins (auxin exporters) (**PAPER II, Figure 3**). Among the different *pin* mutants, *pin1-5*, *pin3/pin7*, *pin3/pin4*, *pin4/pin7* and *pin3/pin4/pin7* displayed reduced cell area and lobe number. Interestingly, the *pin3/pin4/pin7* triple mutant displayed an increase in the number of meristemoids. Additionally, the *aux1-21* mutant and *aux1/lax1/lax2* triple mutant also exhibited decreased cell area and lobe number. In contrast, *abcb1* and *abcb19* mutants showed an increase in cell area and an increase in the number of lobes. These results indicate that auxin transporters are important for lobe formation.

Next, we examined the localization of different fluorescently tagged auxin transporters which are expressed in epidermal PCs, such as PIN3, PIN7, AUX1, LAX1, ABCB1 and ABCB19 proteins, in the lobing SLGCs (**PAPER II, Figure 4**). We also performed plasmolysis experiments to distinguish upon which plasma membrane of two neighbouring cells these proteins were localized (**PAPER II, Figure S3**). Our results suggest auxin transport from the meristemoid toward the SLGC occurs before the first lobing event and is facilitated by PIN3, ABCB1 and ABCB19 proteins localized at the membrane of meristemoid and AUX1 protein localized at the basal membrane of the

SLGC. Once the first SLGC lobe is formed, we observed relocation of PIN3, PIN7, ABCB1, LAX1 proteins preferentially to the membranes of the SLGC and a relocation of the AUX1 protein, to become more equally distributed between the membranes of the SLGC and adjacent cells. This suggests an increase in auxin levels in the SLGC after lobe formation, via disruption of auxin flow out of the SLGC, which may suppress further lobe development.

In summary, our results suggest that lobing in young PCs is controlled via a complex and dynamic regulation of auxin gradients within spiral stomatal complexes via relocalization of auxin transporters.

3.8 Auxin controls cell expansion through the regulation of cell wall biosynthesis and remodeling (PAPER III)

The phytohormone auxin regulates many aspects of plant growth and development. Auxin activates the expression of genes controlling cell division, growth and differentiation (Nemhauser *et al.*, 2006). In Paper III, we reviewed the role of auxin in turgor driven cell growth and rapid cell wall expansion. We analysed publicly available gene expression data, especially that for which the synthetic auxin picloram (4-amino-3,5,6-trichloropicolinic acid) was used to induce hypocotyl cell elongation and cell wall expansion in *Arabidopsis* (Chapman *et al.*, 2012). We found that the expression of genes related to different cell wall composites, such as cellulose, hemicelluloses (xyloglucan, mannans), and xylan (the latter being present in secondary cell walls), are upregulated by picloram treatment. Interestingly, many classes of genes associated to pectin metabolism are differentially regulated by picloram treatment, such as *PME*, *PME INHIBITOR (PMEI)*, *PAE*, *PL*, *POLYGALACTURONASE INHIBITING PROTEIN (PGI)*, *GALS*, *GAL*, and *GALACTURONOSYLTRANSFERASE (GalAT)-LIKE*, *inter alia*. Among cell wall related structural proteins and enzymes, *AGP*, *EXP*, *EXP LIKE* and *PEROXIDASE (PER)* expressions are upregulated by picloram treatment. In summary, our analysis suggests that the auxin-induced expression of many cell wall-related genes may be related to regulation of cell elongation (PAPER III). Moreover, auxin is known to activate acid growth, inducing the loosening of the wall leading to cell growth and expansion (Rayle & Cleland, 1970; Hager *et al.*, 1971). In this process, auxin activates the expression of genes encoding proton pumps and potassium channels. Besides increasing their expression, auxin also stimulates the activity of these proton pumps, leading to acidification of the the apoplast and activation of potassium channels. The sunsequent accumulation of potassium in the vacuole induces water uptake and

enhances the vacuolar turgor forcing on the plasma membrane and walls (Hager *et al.*, 1971, 1991; Rayle & Cleland, 1980; Rück *et al.*, 1993; Frías *et al.*, 1996; Philippar *et al.*, 1999).

Due to the acidic pH, wall loosening EXP proteins and XET and CELLULASE enzymes are activated and cut the connections between CMFs and XyGs, inducing sliding of CMFs and wall loosening (McQueen-Mason & Cosgrove, 1994). PMEs mediate HG de-methyl-esterification, which in turn activates de-acetylation by PAEs and HG depolymerisation involving PGs and PLs (Hocq *et al.*, 2017). PMEs also activate the NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSHPATE (NADPH) OXIDASEs, which transport reactive oxygen species (ROS) into the wall, leading to the break-down of wall polymers (Bailey-Serres & Mittler, 2006; Wolf *et al.*, 2012; Francoz *et al.*, 2015; Tenhaken, 2015). The activities of these structural proteins and enzymes lead to loosening of the connections within the wall matrix polysaccharide network and increase porosity/hydration and swelling. Newly synthesized matrix polysaccharides are transported to the wall surface via vesicle trafficking. Then, driven by high turgor pressure, these non-cellulosic wall composites diffuse through the porous walls and finally integrate with other polysaccharides (Proseus & Boyer, 2006). Insertion of new polysaccharides allows the wall to extend and activates calcium channels to increase cytosolic calcium concentration, which inhibits the activity of the proton pumps and leads to wall alkalization (Nakagawa *et al.*, 2007; Monshausen *et al.*, 2009; Wolf *et al.*, 2012). In the resulting higher wall pH, the polysaccharides are again crosslinked tightly to each other or to different ions, which causes wall compaction and slows down the growth (Wolf *et al.*, 2012). In summary, auxin-regulated cell growth is mediated by many different proteins related to cell wall biosynthesis and modification, among which proteins related to pectin metabolism are strongly represented, indicating that pectins could play an important role in cell wall growth and dynamicity during cell development.

3.9 Unique secondary cell wall formation in leaf epidermal and mesophyll cells in camphor tree (PAPER IV)

Leaf epidermal PCs and mesophyll cells are surrounded by primary cell wall, with CMFs embedded in non-cellulosic components, such as HGs and XyGs, and a low amount of galactans and arabinogalactans. This is different from secondary cell wall, which is present in specific cell types such as xylem or sclerenchyma cells. Secondary cell wall layers display higher amounts of acetylated glucuronoxylan, galacto-glucuronomannan (Mellerowicz &

Gorshkova, 2012), and lignins. However, lignins can also occur in primary cell walls, as a response to different environmental stresses. In this work, we found that the PCs of camphor tree display extensively thickened walls and the spongy mesophyll cells develop local thickenings in areas of intercellular cell contacts (**PAPER IV, Figure 1**). In order to identify what causes these thicknesses, we performed ultrastructural studies using histochemistry, fluorescence, and immuno-gold labelling of different cell wall epitopes. Histological staining using phloroglucinol revealed lignification of epidermal cell walls, which was present not only in the inner periclinal walls, but also in the anticlinal walls (**PAPER IV, Figure 1**). Lignification was also detected in the spongy mesophyll cells, and was restricted to the intercellular contacts that correspond to the thickened regions of cell walls in these cells. Next, we performed high-resolution EM studies, which revealed that in such walls, several cell wall layers of different electron opacity could be distinguished, with the most electron-opaque layer (darkest) in the middle (**PAPER IV, Figure 1**). The darkest layer was continuous over the simple pit regions, where numerous plasmodesmata connecting the adjacent cells were present, whereas the more translucent layers (lighter) were absent in these regions. This wall ultrastructure strongly suggests that the lighter regions might be secondary wall layers. To test whether the thickened cell walls in epidermal and mesophyll cells have primary or secondary wall chemistry (Mellerowicz & Gorshkova, 2012), we performed immunogold labeling of different matrix components (**PAPER IV, Figure 1**). We detected the presence of unsubstituted and highly substituted xylan and arabinoxylan epitopes (LM11 antibody) in both the PC and spongy parenchyma cell walls, at the thickenings in the junctions between two neighboring cell walls, which is in agreement with the accumulation of lignins. These detected composites are known to be present in lignified secondary cell wall of xylan type, as found in S-layers in xylem and sclerenchyma tissues of dicotyledons. In particular, these epitopes are present in layers of xylem vessel elements, tracheids, xylem fibers, xylem parenchyma and phloem fibers (McCartney *et al.*, 2005; Donaldson & Knox, 2012; Kim & Daniel, 2012), and their presence has not previously been annotated in other cell types.

The secondary walls we observed in epidermal and mesophyll cells might be associated with mechanical reinforcements of camphor tree leaves. Secondary walls could help to maintain cell shape under low turgor pressure and thus may be part of a xeromorphic adaptation (Barros *et al.*, 2015) and a general strategy of the camphor tree to cope with drought and mechanical stresses. This discovery challenges the common view that epidermal and mesophyll cells only contain primary walls at maturity.

4 Conclusions and Future perspectives

In this work, we investigated the role of the cell wall in cell shape acquisition using epidermal pavement cells (PCs) as a model. These initially isodiametric cells acquire a fascinating jigsaw-puzzle shape, and their alternating lobes and necks imply a coordinated growth of neighbouring cells.

By devising a semi-automated method for quantifying PC shape geometry, we found that the acquisition of this peculiar lobed shape relies heavily on cell wall biosynthesis and modifications, regulated by the phytohormone auxin (**PAPERS I and II**). This effective analysis method could prove to be very useful for studying the complexity of cell shapes in other tissues.

We also employed novel and challenging *in situ* approaches to define local wall mechanical inhomogeneities at high-resolution (**PAPER I**). Remarkably, these data provided the first experimental evidences for the presence of distinct mechanical properties in the *Arabidopsis* PC wall at a micro scale, along the cell perimeter as well as across the wall curvature, which correlate with alternating distribution of lobes and necks. Thus, our work has improved the general understanding of cell wall mechanical functions and their regulation in plants in the context of cell shape acquisition regulation. It will be interesting future work to determine the roles of cell wall mechanical properties in regulating cell shape in other tissues.

Moreover, using high-resolution EM, we succeeded in defining cell wall ultrastructural composition in *Arabidopsis* PCs in relation to the characterized cell wall mechanical properties. In order to determine the accumulation and distribution of specific cell wall epitopes, we additionally developed a semi-automated method for quantifying the distribution of immuno-labeled cell wall epitopes. Interestingly, we uncovered polar distributions of galactan and arabinan epitopes within the local bending of the wall. We hypothesize that this distribution might influence the local mechanical wall properties, thus allowing controlled bending of the wall at specific sites (**PAPER I**). These findings represent a major step forward in the understanding of the link between cell

wall mechanical properties and composition *in planta*, and their contributions to cell shape acquisition. Additionally, application of this method in an anciently diverged dicot, the camphor tree, demonstrated that the differential pattern of galactan distribution in the PC wall is evolutionarily conserved among plant species, highlighting the importance of cell wall composition in regulating cell shape in the plant kingdom (**PAPER I**). Interestingly, we also showed that epidermal and spongy parenchyma mesophyll cell walls in camphor tree display the unique feature of lignified secondary cell wall deposition, which may play a role in mechanical reinforcement of the leaves to cope with mechanical and drought stresses (**PAPER IV**). Therefore, future studies in camphor tree could potentially shed more light on the importance of lignification in mechanical cell reinforcement.

Finally, to unravel the signalling mechanism upstream of the cell shape acquisition process, we questioned the potential function of the phytohormone auxin in PC lobe formation. We showed that the PC division pattern and shape acquisition are correlated with the establishment of a dynamic auxin concentration gradient, generated by directional transport, which alters according to PC developmental stages (**PAPER II**). This is consistent with the major role of auxin in plant development in general, and in particular its function in stimulating acid growth and activating the expression of genes controlling cell wall biosynthesis and remodelling (**PAPER III**).

Overall, our results show that cell wall native composition, as well as its synthesis and remodelling, are extremely dynamic and of major importance for complex shape acquisition in plants and these processes are regulated by precise gradients of the phytohormone auxin, established by complex, dynamic localization patterns of auxin transporter proteins.

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Acknowledgements

Foremost, I would like to thank my supervisor **Stéphanie Robert** for giving me the opportunity to join her research team and perform my doctoral studies. I am grateful for all the opportunities you gave me and for believing in me. I learnt a lot from you!

I would like to thank **Malcolm Bennett** for accepting to be my opponent and finding the time to come to Umeå. I would also like to thank **Arezki Boudaoud**, **Gregory Mouille**, **Hannele Tuominen**, and **Catherine Bellini** for accepting to be my committee members.

I thank my advisory group members **Rishikesh Bhalerao** and **Thomas Moritz** for your valuable comments and a special thanks to **Ewa J. Mellerowicz** for all your support and valuable advice.

I gratefully acknowledge **Olivier Hamant** for giving me the chance to be a part of his team during my research visits. Thanks for the opportunity to learn from you, and for your advice. A special thanks also to all the collaborators on our manuscripts for their valuable contributions.

I gratefully thank all the people who strongly supported me and made my defence possible, especially the following people. **Siamsa M. Doyle** for your great support and all the improvements of my writing and correcting of my thesis and all my papers. Also **Qian Ma**, **Peter Grones**, **Stéphane Verger**, **Sara Raggi**, and **Nicola Trozzi** for your proof reading of my thesis and all discussions and advice.

I would especially like to thank all my friends at Umeå Plant Science Centre, the team at Umeå Core Facility for Electron Microscopy and the team at the Department of Medical Biochemistry and Biophysics.

Finally, I would like to acknowledge my funding: Vetenskapsrådet (VR) for my research and Kempe Foundation for the travel grant funding my research visits at École Normale Supérieure de Lyon.