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SI GENOME TO PHENOME

Cell wall traits that influence plant development, immunity, and bioconversion†

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†Dedicated to the memory of Peter Albersheim

SUMMARY

The architecture of the plant cell wall is highly dynamic, being substantially re-modeled during growth and development. Cell walls determine the size and shape of cells and contribute to the functional specialization of tissues and organs. Beyond the physiological dynamics, the wall structure undergoes changes upon biotic or abiotic stresses. In this review several cell wall traits, mainly related to pectin, one of the major matrix components, will be discussed in relation to plant development, immunity and industrial bioconversion of biomass, especially for energy production. Plant cell walls are a source of oligosaccharide fragments with a signaling function for both development and immunity. Sensing cell wall damage, sometimes through the perception of released damage-associated molecular patterns (DAMPs), is crucial for some developmental and immunity responses. Methodological advances that are expected to deepen our knowledge of cell wall (CW) biology will also be presented.

Keywords: plant cell wall, pectin, growth, egg-boxes, cell wall damage, cell wall DAMPs.

INTRODUCTION

The plant cell wall (CW) contains a dynamic and complex array of components, the function of which goes far beyond mechanical and structural support. Many components of the CW are known to mediate cell–cell adhesion and communication, providing the spatial context for many signaling events that govern cell functions, differentiation, developmental patterning, and growth. The structural polysaccharides of the CW determine, on one side, the shape and size the cells, tissues and organs and, on the other side, provide the mechanical strength to resist to both external stresses and internal turgor pressure. Moreover, they are the main component of the plant biomass that is considered an important source of biofuels and other industrial products in the green chemistry era. In addition to their structural function, CW polysaccharides are also a repository of molecules acting as signals in intercellular communication and as elicitors of immunity during microbial attacks (Bacete *et al.*, 2018; Cosgrove, 2018; Voiniciuc *et al.*, 2018).

The role of cellulose, the preponderant CW polysaccharide, and the role of the non-saccharide CW component lignin in development, defense and bioconversion have been well covered by several recent reviews (Chen *et al.*, 2018; Liu *et al.*, 2018; Meents *et al.*, 2018). The classical model of the CW architecture depicts cellulose microfibrils as interconnected at their surface with hemicelluloses in turn embedded in a matrix of pectin, which forms a sort of glue that provides flexibility or stiffness to the CW. NMR studies, however, have revealed that pectin–cellulose interactions are much more abundant than those foreseen according to the classical models of primary wall architecture (Cosgrove, 2018). Moreover, unique, stable covalent interactions between the two components have been suggested (Broxterman and Schols, 2018). Here we discuss several biologically relevant CW traits, focusing on pectin, given the growing evidence that this component plays a critical role in development (Bou Daher *et al.*, 2018), immunity (Benedetti *et al.*, 2015, 2018), and bioconversion

(Lionetti *et al.*, 2010; Biswal *et al.*, 2018). Pectin is a very complex entity containing rhamnogalacturonan I (RGI), homogalacturonan (HG), xylogalacturonan (XGA), and rhamnogalacturonan II (RGII), which exhibit structures that are variable among the different species and the different tissues and organs within a species. The structure of these polysaccharides is also variable upon changing the environmental conditions. While the single components of pectin have been characterized chemically and structurally, a comprehensive view of the architecture of pectin in the context of the other CW components and its influence on plant biology is still a challenging task.

Bullet point summary of the main points covered in the review

- Dissection of the cell wall (CW) structure and composition and parallel progress in identifying the function of genes has elucidated the role of several CW components in plant development and immunity.
- The machinery that monitors and maintains cell wall integrity (CWI) initiates adaptive cellular responses to damage. Cell wall damage (CWD) is likely to induce the production of endogenous elicitors that promote pattern-triggered immunity (PTI) and dampen growth processes (growth-defense trade-off).
- The impact of specific CW components like pectin on biomass processing points to potential targets for genetic improvement of crops for industrial purposes.
- Identification of CW-related genes has been accelerated by advanced analytical technologies for detailed cell wall structure analysis.

Bullet point summary of the open questions

- The number of genes involved in CW synthesis, remodeling and turnover in Arabidopsis is over 2000, suggesting that the CW has a structural complexity still to be explored. What are all these genes doing in relation to development and immunity, and what is their importance for the possible utilization of biomass?
- Subtle and cryptic CW variations affecting plant phenotype can be detected and analyzed only by advanced analytical tools. Will we be able to analyze and understand the organization of CW components in a given group of cells or in a single cell?
- The knowledge of genes involved in CW biosynthesis and turnover is still limited. Will genome-editing technology allow for rapid progress in identifying these functions?
- The structural complexity of the CW could serve as a reservoir of latent signal molecules involved in development and defense. How many CW-derived elicitors exist and what role do they play in immunity and development?
- What is relationship between the maintenance of CWI, development and immunity?

DEVELOPMENT

Plant cells are immobile and maintain a fixed position during growth and development, while they undergo cell division, selective cell expansion and differentiation concomitantly with changes in cell wall biosynthesis and re-modeling. The local complexity and diversity of the CW determines the cell size and shape and, ultimately, the remarkably rich plant morphology. A comprehensive review has been recently published on this topic (Tucker *et al.*, 2018).

Plant cells grow by turgor-driven expansion that depends on a continuous and irreversible process of extension or 'creep', in which cellulose microfibrils and matrix polysaccharides slide within the wall. Beyond the cellulose microfibrils, which are known to confer tensile resistance and mechanical anisotropy to the wall, matrix polysaccharides like pectin and hemicelluloses also influence the different stages of growth and development. Pectin is likely to be involved in CW loosening and re-modeling (Hamant and Haswell, 2017). Specifically, α-expansins and bacterial expansive-like proteins with unknown enzymatic function that are involved in wall loosening at low pH, carry a pectin-binding region besides the cellulose-binding region (Georgelis *et al.*, 2012; Wang *et al.*, 2013; Nardi *et al.*, 2015). Disruption by site-directed mutagenesis of the pectin-binding domain increases wall creep, suggesting that the interaction of expansin with pectin is important to control the extent of creeping. This and other evidence led to a revised concept of wall extensibility that is postulated to be controlled at limited sites ('biomechanical hotspots') subjected to stress relaxation by the action of expansins and other wall-loosening proteins (Cosgrove, 2016, 2018). Expansin loss-of-function mutants are impaired in CW loosening and the resulting impairment in cell elongation and volume hampers cell differentiation. Volume changes of cells depend on the concomitant activation of several expansins and the AHA1 and AHA2 proton pumps in response to cytokinin, providing a link between cytokinin-dependent CW acidification, re-modeling, and differentiation (Pacifici *et al.*, 2018).

Pectins display methyl-ester side chains that are removed by pectin methylesterases (PMEs) as a necessary prerequisite for the action of pectinases like pectate lyases and polygalacturonases (PGs). PME isoforms operating in a block-wise fashion generate stretches of negatively charged galacturonic acid residues that are cross-linked by calcium and form the 'egg box' structure, a rigid gel that strengthens the cell-to-cell adhesion. Conversely, de-methylesterified stretches may be more susceptible to degradation by

pectinases, favoring CW loosening. Because of this dual role, the action of PME could either increase or decrease CW stiffness and, in either case, is predicted to affect cell growth (Lionetti *et al.*, 2010; Senechal *et al.*, 2014). Methylation and de-methylation are tightly regulated and rely not only on the localized expression of specific PME isoforms and the local presence of calcium ions (Bascom *et al.*, 2018) but also on the localized expression of PME inhibitors (PMEI) (Di Matteo *et al.*, 2005). Overexpression of PMEI causes changes in growth behavior, through the activation of the BR signaling pathway that in turn enhances transcription of CW-modifying genes, including PME genes (Wolf, 2017). Moreover, the cell type/tissue-specific expression of PMEI5 leads to widely diverse defective phenotypes. These results indicate that the pectin status may influence cell/tissue-specific functions and point to a potential influence of PMEI expression and lifetime on plant growth during distinct developmental stages (Li, 2018).

The growth of pollen tubes and root hairs is particularly amenable to study CW dynamics and the complex interplay between synthesis and re-modeling of the CW polysaccharides (Mravec *et al.*, 2017a; Cameron and Geitmann, 2018; Tucker *et al.*, 2018). Control of pectin synthesis, de-methylesterification and assembly is essential to prevent bursting of the growing cells caused by a turgor pressure not properly balanced by the CW tensile strength, with consequent perturbation of the cell wall integrity (CWI). Several plasma membrane receptor kinases have been shown to be essential for maintaining CWI and allowing growth. Most of these belong to the family of malectin-like receptor kinases (RLKs), also known as the *Catharanthus roseus* RLK-like proteins (*Cr*RLK1Ls), which in Arabidopsis comprises 17 members (Franck *et al.*, 2018). Within this family, *ERULUS* (ERU) is an important component of the fertilization pathway and regulates pollen tube targeting *in vivo* (Schoenaers *et al.*, 2017). ERU also regulates the dynamics of pectin methylation/de-methylation during the growth of the root hair tip by functionally interacting with another CrRLK1L member, FERONIA (FER) (Schoenaers *et al.*, 2018; Kwon *et al.*, 2018). FER is required for growth of root hairs as well as of trichomes, which both lose their integrity upon or shortly after emergence in the loss-of-function *fer* mutants (Franck *et al.*, 2018). The ectodomain of FER is capable of binding pectin *in vitro* (Feng *et al.*, 2018; Verger and Hamant, 2018). In a submitted paper, FER has been proposed to sense the presence of de-methylated pectin and directly activate the ROP6 GTPase signaling pathway. In this paper, both FER loss-of-function mutations and defects in the pectin biosynthesis and de-methylation have been described to cause changes in the pavement cell shape of leaf epidermis and ROP6 GTPase signaling (Lin *et al.*, 2018). FER also binds the peptides RAPID ALKALINIZATION FACTOR (RALF) 1 (Haruta *et al.*, 2014) and RALF23 (Stegmann *et al.*, 2017). The interaction between RALF1 and FER regulates stomatal aperture (Yu *et al.*, 2018). RALFs are cysteine-rich peptides of 80–120 amino acids derived from proteolytic cleavage of secreted preproproteins that appear to play important roles in growth, development, and immunity (Campbell and Turner, 2017). How the interaction with pectin and RALFs is integrated in FER-mediated signaling is still unclear.

Four other CrRLK1L members, i.e. Buddha's Paper Seal I (BUPS1), BUPS2, ANXUR1 (ANX1) and ANX2, control pollen growth by binding RALF4 and RALF19 (Ge *et al.*, 2017). The receptor–ligand interactions between ANXs/BUPSs and their RALF ligands is interfered with the female-derived ligand RALF34, which induces pollen tube bursting and is the ligand of another CrRLKL member, THESEUS1 (THE1) (Gonneau *et al.*, 2018). THE1 is known to be essential for response to CW damage (CWD) triggered by perturbation of cellulose synthesis (see below). In addition, pollen growth and integrity requires the LEUCINE-RICH REPEAT EXTENSIN (LRX) proteins LRX8, LRX9, and LRX11 (Wang *et al.*, 2018) that, like ANXs/BUPSs, also bind RALF4 and RALF19 (Mecchia *et al.*, 2017; Li and Yang, 2018; Sede *et al.*, 2018). The deposition pattern of pectin and callose is impaired in the tubes of the triple mutants (Mecchia *et al.*, 2017; Wang *et al.*, 2018). The extensin domain of LRX is required for its CW localization (Fabrice *et al.*, 2018) and, in general, extensins (EXTs) are thought to mediate cell wall stiffening (Lamport *et al.*, 2011). Among the 59 Arabidopsis EXTs, EXT3 has been shown to be a self-assembling molecule that forms scaffolds and binds de-methylated homogalacturonan through acid–base interaction and serves as a template for pectin assembly (Cannon *et al.*, 2008; Marzol *et al.*, 2018).

Plant CWs are a source of oligosaccharide fragments with a signaling function for both development and immunity. For example, the hydrolysis of de-methylated HG by PGs releases oligogalacturonides (OGs), which activate immunity (see later) but also inhibit stem growth and root formation by antagonizing auxin (Branca *et al.*, 1988; Bellincampi *et al.*, 1993; Savatin *et al.*, 2011). The molecular mechanism underlying the effects of OGs on auxin responses is not yet elucidated. The presence of endogenous OGs *in planta* was recently demonstrated (Pontiggia *et al.*, 2015). In Arabidopsis, hundreds of pectic enzymes are regulated during development and can potentially release OGs (Senechal *et al.*, 2014). Under physiological conditions microlesions occurring in developmental processes like cell expansion, lateral root formation, abscission, tissue maturation, and pollen–stigma interactions may be perceived through the release of low amounts of OGs. Larger ruptures, which generally occur during pathogenic events and mechanical injury, are likely to cause the accumulation of larger amounts of OGs that are sensed as danger signals (damage-associated molecular patterns or DAMPs). In order to study the effect of OGs generated *in*

muro, Arabidopsis plants have been engineered to conditionally express a fusion between a fungal PG and a plant PGIP named 'OG machine'. High levels of OGs produced by the OG machine led to the formation of salicylic acid, inhibition of growth, and eventually death of the plant, reflecting the role of these signal molecules in the growth-defense trade-off (Benedetti *et al.*, 2015).

A novel enzymatic mechanism has been identified that is likely to control the homeostasis of OGs and prevents the deleterious effects of their hyper-accumulation. It relies on H_2O_2-generating FAD-dependent oxidases (OGOXs) that belong to the large subfamily of berberine bridge enzyme-like (BBE-like) proteins and inactivate OGs (Benedetti *et al.*, 2018). Enzymes such as the OG oxidase, by acting on the local micro-environment where ruptures of the CW polysaccharides are caused during growth and development, also locally produce hydrogen peroxide that may be useful to rapidly repair the broken polymers by cross-linking. Conversely, the localized production of hydrogen peroxide at the sites of such ruptures during physiological re-modeling may function as a positional signal for those tissues and cells that are engaged in developmental or growth responses. The activities of other BBE-like members may be directed to the oxidation of other CW-derived signaling molecules, such as cellulose fragments, which also act as DAMPs. Interestingly, the single berberine bridge enzyme-like homolog identified in *Physcomitrella patens* was shown to have cellobiose oxidase activity, probably relevant during the degradation of cellulose for energy production (Toplak *et al.*, 2018).

Xyloglucan oligosaccharides have been shown to cause CW loosening by acting as acceptors of the cleaved xyloglucan chain catalyzed by xylosyltransferases (XTH) (Kaku *et al.*, 2004). An α-xylosidase TGR1/XYL1 has been identified that could finely tune the levels of xyloglucan oligosaccharides and their action on the mechanical properties of the CW. The expression of the *TGR1/XYL1* gene is high in growing tissues such as the root tip and the upper hypocotyl, but relatively low in the elongating cells in the embryo of the germinating seed (Shigeyama *et al.*, 2016).

Oligosaccharides derived from galactoglucomannan also act as auxin antagonists and affect elongation, morphogenesis and xylogenesis, influence the root growth in the presence of indole-3-acetic acid (IAA) and decrease the accumulation of flavonoids that act as modulators of auxin transport (Kucerova *et al.*, 2016).

IMMUNITY

The activation of defenses upon recognition of exogenous pathogen-derived (pathogen-associated molecular patterns, PAMPs) or endogenous danger signals referred to as DAMPs is a key function of the plant innate immunity. Sensing damage is a crucial trait for survival of all living organisms. Both mechanical damage and infections often disrupt the homeostatic cellular processes and this is sensed as danger by the organisms. The recognition of 'damaged self' may occur independently of an invading pathogen and, therefore, the activated response may be not specific against a given threat (Heil and Land, 2014). DAMPs may originate from the lesion of the cell structures upon injuries and developmental ruptures and do not act only in defense against infection but are also important in pathogen-independent processes such as tissue damages and repair (De Lorenzo *et al.*, 2018).

A well known class of DAMPs is represented by oligosaccharides released from the CW. The structural integrity of CW is strictly monitored, is continuously perturbed upon CW re-modeling during growth, development, and formation of organs and is dramatically altered by a mechanical damage or an infectious event (Ferrari *et al.*, 2013; Bellincampi *et al.*, 2014; Hamann, 2015). The breakage products of homogalacturonan (HG), i.e. oligogalacturonides (OGs), are perceived as DAMPs both for defense against microbes and local signal for repairing mechanical injuries (Benedetti *et al.*, 2015; Gramegna *et al.*, 2016). During infection, OGs are released through the action of microbial pectin-degrading enzymes while, upon wounding and mechanical damage, they are likely to be produced by plant-derived endogenous enzymes (Savatin *et al.*, 2014b). The immune responses are strongly activated by OGs comprised from 10 to 15 residues, while shorter oligomers (2–6 residue long) exhibit lower activity (Davidsson *et al.*, 2017). The activity is maximal when OGs are engaged in calcium-mediated intermolecular ionic bonds, which confer to these molecules the conformational state called 'egg boxes' (Cabrera *et al.*, 2008). The degree of methylesterification and/or acetylation of HG that varies in different organs during plant development, may influence the nature of OGs released during pathogen infection or upon wounding and, consequently, their biological activity. As stated before, while a moderate accumulation of OGs triggers a proper and balanced immune response, an excess of OGs may provoke hyperimmunity, which severely affects growth and eventually leads to cell death (Benedetti *et al.*, 2015).

Arabidopsis wall-associated kinase 1 (WAK1) acts as a receptor for OGs (Brutus *et al.*, 2010) and plays a role in immunity and in local response to wounding, along with its interactors glycine-rich protein-3 (GRP-3; extracellular) and kinase associated protein phosphatase (KAPP; cytoplasmic) (Gramegna *et al.*, 2016). Analysis of several mutants suggests a complexity of the OG perception that is unprecedented in plant immunity (Gravino *et al.*, 2017) and resembles the complexity of the perception systems of hyaluronan fragments in vertebrates (Cyphert *et al.*, 2015; Frevert *et al.*, 2018).

The transcriptional profiles of Arabidopsis seedlings treated with OGs and flagellin, one of best characterized PAMPs, are almost identical at the early stages of the

response (Denoux *et al.*, 2008). Typical early signaling events involved in both OG- and flagellin-triggered defenses include the plasma membrane depolarization and alkalization of extracellular pH, the activation of calcium fluxes and specific calcium-dependent protein kinases (CDPKs), the production of ROS and nitric oxide and the activation of specific MAP kinase cascades (Galletti *et al.*, 2011; Savatin *et al.*, 2014a; Gravino *et al.*, 2015). In Arabidopsis, MPK6 has a crucial role in resistance against *Botrytis cinerea* induced by OGs and flagellin (Ferrari *et al.*, 2007), while the mitogen-activated triple kinases ANPs act in the cascades activated by OGs and elf18, another well characterized PAMP (Savatin *et al.*, 2014a). Moreover, simultaneous loss of the Arabidopsis CPK5, CPK6, and CPK11, important for flagellin-mediated responses, also affects responses induced by OGs (Gravino *et al.*, 2015).

Additional CW polysaccharides have been described to act as DAMPs in plants. For example, cellulose-derived oligomers including cellobiose induce several defense responses and may play a role in CWI surveillance. However, unlike OGs, cellobiose does not elicit a detectable ROS production and callose deposition (Aziz *et al.*, 2007; Souza *et al.*, 2017). Also cellotriose has been recently shown to induce a moderate defense-like response, including the production of ROS, changes in membrane potential and expression of genes involved in defense and regulation of growth and root development (Johnson *et al.*, 2018; Oelmuller, 2018). The presence of CW fragments functioning as DAMPs is an open chapter in plant biology. Circumstantial evidence of the existence of CW fragments other than OGs and cellulose-derived fragments that may work as DAMPs has been recently reported (Bacete *et al.*, 2017, 2018; Gallego-Giraldo *et al.*, 2018). Also the involvement of several orphan receptors in phenomena such as CWD suggests that other molecules derived from CW fragmentation may possibly work as DAMPs (see below).

Cell wall DAMPs may condition the tissue for faster activation of defense responses upon infection, conferring a long-term protection against microbes. This phenomenon termed 'priming' is considered a form of non-specific memory that does not rely on activated defense responses but makes an organism capable of responding faster and more efficiently to a secondary infection (Ramirez-Prado *et al.*, 2018; Tugizimana *et al.*, 2018). Treatments with OGs, for example, cause the accumulation of the endogenous antimicrobial compound camalexin in Arabidopsis plants inoculated with *B. cinerea* (Gravino *et al.*, 2015), while the expression of genes involved in camalexin biosynthesis is induced only transiently (Ferrari *et al.*, 2013).

An overlooked aspect of the DAMP biology is the presence of these molecules during the physiological re-modeling and turnover of the cellular structures. Molecules having physiological function may behave as DAMPs when their levels increase. In this respect, it would be interesting to see whether the receptors for DAMPs acting under damage conditions differ from those acting under physiological conditions. It is plausible to hypothesize that, under physiological conditions, certain DAMPs that are present at low levels, activate DAMP receptors with high affinity, whereas, under tissue damage conditions, DAMPs are perceived by receptors with lower affinity. OGs and other possible CW DAMPs are located in key positions to act as indicators of CWI, both in adverse conditions and during normal growth (see later).

DAMPs may also influence grafting compatibilities (Melnyk and Meyerowitz, 2015). Upon grafting, wound healing and formation of vascular connections occurs between two plants and is usually restricted to closely related species. Interfamily grafting is often incompatible and whether this is due to the non-specific release of DAMPs or some other mechanisms is currently unknown.

CELL WALL DAMAGE

The involvement of the CW in development, immunity and interaction with the environment imposes a strict control of its structure, dynamics, and homeostasis. Mechanisms for perception and response to perturbations of CWI have been widely studied in fungi (Sanz *et al.*, 2017) and analogous mechanisms have been proposed to occur in plants (Hamann, 2015; Voxeur and Hofte, 2016). Evidence supports the existence of a system of plant CWI maintenance in the primary cell walls but not in the secondary walls, likely to be because the program leading to secondary CW thickening is part of a wider committed program that culminates with cell death (Faria-Blanc *et al.*, 2018).

Plant responses to cell wall damage (CWD) have been mainly studied upon interference with the deposition of cell wall polysaccharides or using mutants of genes necessary for cell wall biogenesis and re-modeling (Hamann, 2015; Voxeur and Hofte, 2016; Wolf, 2017) or upon plant transformation with CW degrading enzymes (Capodicasa *et al.*, 2004; Ferrari *et al.*, 2008; Reem *et al.*, 2018). Typical readouts of the CWD response are a compensatory production of CW components like callose and lignin, the accumulation of jasmonic acid, salicylic acid, and ethylene as well as the expression of genes like *PDF1.2*, a marker of pattern-triggered immunity (PTI) and *TOUCH4,* a marker of mechanical stimulation (Hamant and Haswell, 2017; Engelsdorf *et al.*, 2018). Some phenotypes related to growth, development, and immunity described in several mutants or in some plants transformed with CW-modifying enzymes may be a consequence of the alterations of CWI sensing and signaling (Wolf, 2017). Notably, all the responses to CWD are turgor-sensitive, while the responses of PTI are not. Both mechano-perception and osmo-perception are required for induction of responses to CWD but not for PTI. CWD requires elements shown to be involved in sensing mechanical (i.e. the putatively

stretch-activated Ca^{2+} channel MCA1) and hypo-osmotic stress (i.e. channels *MSL2* and *MLS3*; *MCA1*) (Engelsdorf *et al.*, 2018). The NPK1-related protein kinases ANP1, ANP2, and ANP3 belonging to the MAP kinase kinase kinase superfamily also appear to play a role in CWI maintenance (Gigli-Bisceglia *et al.*, 2018).

Receptor-like kinases and proteins capable of binding cell wall polysaccharides are obvious candidates as sentinels of CWI. Wall-associated kinases (WAKs) have been proposed to perform this function. WAK1 binds OGs and homogalacturonan (Decreux *et al.*, 2006; Kohorn, 2016) and is a receptor of OGs (Brutus *et al.*, 2010). OGs have been proposed as components of the CWI system, because they are indicators of the CWD caused by pathogens or mechanical injury (Ryan *et al.*, 1981; Roberts, 1992; Ferrari *et al.*, 2013; Savatin *et al.*, 2014b). In addition, WAK1 has been shown to increase the local response to wounding when overexpressed (Gramegna *et al.*, 2016). However, recent experiments show that OGs do not significantly induce JA, a hallmark of the CWD response and that typical responses to OGs are not turgor-sensitive, suggesting that OGs alone are not the only players in the CWD responses (Gigli-Bisceglia *et al.*, 2018). It remains to be elucidated whether signaling by exogenous OGs is equivalent to signaling by *in muro* release of OGs, which necessarily involves a concomitant rupture in the wall and the release of OGs. A synergistic action of two simultaneous wall-related events is also possible and an additional contribution of other wall-related DAMPs such as cellulose fragments may occur. Treatment with pectinase, but not with cellulase, induces turgor-sensitive accumulation of JA and SA, while a combined treatment with the two enzymes elicits a turgor-sensitive response higher than that induced by pectinase alone (Engelsdorf *et al.*, 2018). This effect may be due to increased accessibility of cellulose microfibrils to cellulase upon pectinase action and/or to a synergistic signaling by two different released fragments from cellulose and pectin.

The receptor kinase THE1 is required for the CWD-dependent growth repression and responses, but not for PTI (Gigli-Bisceglia *et al.*, 2018). This implies that the receptor is somehow devoted to monitoring cell wall status and the mere damage is not *per se* responsible for all the complex alterations caused by the CWD. The leucine-rich repeat (LRR) RLKs, MIK2 and FEI2, also appear to be involved in sensing the CWD, by acting downstream of THE1 (Van der Does *et al.*, 2017; Engelsdorf *et al.*, 2018). Instead, no major role in CWD response emerges for other RLKs such as WAK2, several LLR RLKs (FEI1, BAK1, BAK1-LIKE 1, PEPR1, PEPR2, BIK1), the LRR receptor-like protein RLP44 or FER (Engelsdorf *et al.*, 2018).

It has to be noted, however, that the *fer* mutant shows constitutive expression of some typical CWD responses (Gonneau *et al.*, 2018). Recently, FER has been shown to maintain CWI in root cells under salt stress, which causes softening of the wall (Feng *et al.*, 2018). In the presence of high salt concentrations, root cells in *fer* mutants explode instead of recovering growth. A similar defect is observed in the *mur1* mutant that has altered pectin cross-links. In both *fer* and *mur1* mutants, growth is recovered after treatment with calcium or borate, which both facilitate pectin cross-linking, supporting the hypothesis that FER senses the status of pectin (Feng *et al.*, 2018; Verger and Hamant, 2018). Interestingly, FER shares with WAK1 the characteristics of being tightly bound to the cell wall and both proteins can be extracted only by boiling tissue in the presence of high concentrations of SDS and a reducing agent (Lin *et al.*, 2018). Taken together, these observations suggest that the action of FER, rather than during the initial CWD alert, is crucial in the recovery phase, probably allowing a proper reorganization of the architecture of the pectic polysaccharides.

BIOCONVERSION

The past decade has witnessed an explosion of basic and applied research in the field of CW biology. One reason is that plant-derived biofuels are an interesting and renewable alternative to fossil fuels. However, first generation biofuels, mostly ethanol from sugarcane and corn starch and oils from food crops, have met increasing opposition due to competition with food production and land use. Instead, the second generation biofuels derived from lignocellulosic biomasses, either from agricultural wastes or dedicated energy crops, represent a sound alternative. Conversely, concerns regarding the effect of bioenergy crops on climate, biodiversity, nitrogen loss, and water use can be addressed with appropriate crops and management choices (Robertson *et al.*, 2017). Furthermore, recent technical advances have contributed to an industrial-scale production of cellulosic bioethanol, although the economic feasibility of some industrial projects is not yet optimal (Ko and Lee, 2018). One of the limits to the large-scale production of second generation biofuels is the recalcitrance of the CW to degradation into simple sugars ('saccharification'). Recalcitrance increases the costs and energy requirements of the conversion processes and reduces the recovery of the desired products (McCann and Carpita, 2015). Pretreatments are often required to overcome recalcitrance and make cellulose fibers prone to conversion into fermentable sugars. Additionally, efficient microbes must be found that are capable of utilizing the wide range of sugars obtained from the complex CW matrix including uronic acids and pentoses and cope with the inhibitory compounds released during the saccharification process. For these reasons, understanding how the CW composition and architecture affects the different physical, chemical, and enzymatic processes of

saccharification as well as the downstream utilization by fermentative microorganisms is a priority to ensure a viable production of cellulosic biofuels.

Traditionally, the CW recalcitrance to saccharification has been associated to the negative effects of lignin and lignin–carbohydrate complexes. As described above, the plant CW has evolved the ability to resist to mechanical stresses as well and to the attack of enzymes secreted by pathogens and herbivores and lignification plays an important role in CW resistance. Plants with reduced cinnamate-4-hydroxylase, cinnamoyl CoA reductase, cinnamyl alcohol dehydrogenase and class III peroxidase (PRX) activities have reduced lignin deposition and increased saccharification (Chen and Dixon, 2007; Kavousi *et al.*, 2010). However, recalcitrance is dependent not only on lignin levels but also on the composition and architecture of the CW as a whole and differs greatly depending on the specific process for which the biomass is employed. Direct and reverse genetics supports an important role of matrix carbohydrates in determining recalcitrance. Screening of a mutagenized population of the model energy plant *Brachypodium distachyon*, coupled to CW characterization by spectroscopic, chromatographic, and immunostaining techniques, yielded several mutants with enhanced saccharification efficiency, normal lignin and crystalline cellulose content and alterations in the matrix carbohydrates. The mutation conferring the highest saccharification rate was mapped to a locus containing a gene involved in arabinoxylan substitution (Marriott *et al.*, 2014). Another matrix trait that affects the biomass recalcitrance is the content of de-esterified HG, which varies among Arabidopsis ecotypes and negatively correlates with cellulose degradability (Francocci *et al.*, 2013). Natural variation in CW composition can be employed to select genotypes of crops more amenable to bioconversion. High-throughput techniques of analysis of CW composition can greatly accelerate the rate of discovery of loci involved in the biosynthesis, modification, and assembly of CW that affect biomass conversion (Box 1). However, the genetic basis of biomass recalcitrance appears to be quite complicated, as exemplified by the finding of 86 different quantitative trait loci (QTLs) that affect the recalcitrance *Sorghum bicolor* (van der Weijde *et al.*, 2017). A recent analysis of *Brachypodium* inbred lines showed that a substantial phenotypic variation exists with respect to CW composition and biomass digestibility (Cass *et al.*, 2016).

To bypass genetic complexity, attempts to improve saccharification by directly engineering matrix carbohydrates have been made, sometimes with success (Lionetti *et al.*, 2010; Biswal *et al.*, 2018). Pectin is a major target for modifications that may improve biomass saccharification. A pioneering study showed that reduction of de-esterified HG by either expression of a fungal polygalacturonase or by overexpression of a PME inhibitor almost abolishes the need of pretreatments in Arabidopsis and wheat. Plants transformed with PMEI grow even better than the wild type plants (Lionetti *et al.*, 2010). This evidence highlighted the overlooked importance of pectin in the saccharification process and prompted further investigation to study different ways of modifying pectin. It is clear now that pectin composition strongly influences the accessibility of cellulose, and possibly of other CW components, to degradation and/or extraction. The overexpression of an endogenous pectate lyase in aspen increases solubility not only of pectin but also of hemicelluloses (Biswal *et al.*, 2014). Increased saccharification is obtained by downregulation of galacturonosyltransferase 4 (*GAUT4*), a gene involved in pectin biosynthesis in switchgrass and poplar (Biswal *et al.*, 2018). Similarly, downregulation by RNA silencing of aspen GAUT12, affecting xylan and pectin content, results in reduced recalcitrance and increased growth (Biswal *et al.*, 2015).

Several CW modifications increase biomass saccharification but compromise plant growth. Normally, plants manage to strike a balance between CW extensibility and rigidity, ensuring proper organ expansion and growth without compromising tissue rigidity and resistance to mechanical damage and to pest attack. As commented before, the stunted growth observed in plants with altered CW composition may not directly dependent on the initial CW defect but rather to responses that are activated by perception of the CWD. These responses strengthen the CW and therefore impair cell expansion, leading to reduced plant leaf area and biomass production. A clear example of this phenomenon comes from the analysis of Arabidopsis plants with reduced lignin levels in which reduced growth depends on the accumulation of salicylic acid (Gallego-Giraldo *et al.*, 2011). Changes in matrix polysaccharides trigger a CWD response that may affect biomass production. Arabidopsis plants transformed with fungal PGs have reduced content of de-esterified HG, increased saccharification and reduced biomass production (Ferrari *et al.*, 2008; Lionetti *et al.*, 2010; Francocci *et al.*, 2013). These plants show enhanced expression of a class III peroxidase gene (*AtPRX71*), strongly upregulated in response to CWD (Raggi *et al.*, 2015). AtPRX71 promotes ROS production and restricts the expansion of cells and organs under CWD conditions. Interestingly, *atprx71* loss-of-function mutations confer increased growth, compared with WT plants and partially complement the growth defects induced by CWD, including those of plants with altered HG (Raggi *et al.*, 2015). This gene and other genes activated by CWD and affecting CW properties might become interesting targets for genetic improvement of biomass crops.

Plant growth and photosynthetic efficiency are influenced by CW composition and changes in leaf area (Wormit *et al.*, 2012; Weraduwage *et al.*, 2015). For example,

Box 1: Tools and methods for cell wall studies

Tools and methods that have enhanced our knowledge of how cell wall composition and structure influence development, immunity and bioconversion in model and cultivated species

	Technique		References
Cell wall probes (CWPs)	*In situ* hybridization	CWPs have been useful in studying the dynamics of the cell wall components during growth and development as well as in response to stress. These comprise monoclonal antibodies, carbohydrate-binding modules, fluoro(chromo)phores, oligosaccharide conjugates and metabolic labelling reagents. A recently developed method, called comprehensive microarray polymer profiling (CoMPP) combines the high-throughput capacity of carbohydrate microarrays with the specificity of molecular probes	Fukui *et al.*, (2002); Fukui *et al.* (1988); Kitagawa *et al.* (1988); Kracun *et al.* (2017); Kuno *et al.* (2005); Rydahl *et al.* (2018); Wood *et al.* (2017)
	Click chemistry	Several microscopy techniques utilize specific probes to visualize the dynamics and mechanical properties of the CW. Innovative approaches based on 'click chemistry' utilize modified polysaccharides that can be metabolically incorporated into the CWs	Carella *et al.* (2018); Dumont *et al.* (2016); Mravec *et al.* (2017b)
Microscopy	Atomic force microscopy (AFM)	It allows to visualization of the surfaces of macromolecules and the spatial organization and orientation of the CW components. AFM-based images reveal at the nanometer scale the CW heterogeneity in growing tissues and the spatial distribution of soft and rigid matrix polymers. AFM has also been employed to study CWI during plant growth in mutants affected in cellulose synthase	Casdorff *et al.* (2017); Dufrene *et al.* (2017); Hu *et al.* (2018); Kirby *et al.* (1996); Round *et al.* (2001); Yakubov *et al.* (2016); Zhang *et al.* (2016)
	Scanning electron microscopy (SEM)	It has been useful to observe several CW components in different xylem structures	McCann *et al.* (1990); Sun *et al.* (2017)
	Confocal laser scanning microscopy (CLSM)	Allows visualization of tagged molecules in both fixed and living cells. Fluorescent dyes specific for cellulose, lignin and hemicelluloses are available as well as fluorescent pH indicators that allow assessment of the apoplastic pH. Fluorescent oligosaccharide-based probes have developed that bind to de-esterified homogalacturonan	Barbez *et al.* (2017); Choong *et al.* (2016); Komis *et al.* (2018); Lenartowska *et al.* (2001); Pien *et al.* (2001); Rydahl *et al.* (2018)
	Super-resolution microscopy	Improvement of CLSM and is especially useful to reveal lignin density and pattern distribution in the CW	Paes *et al.* (2018); Sahl *et al.* (2017); Vangindertael *et al.* (2018)
	Brillouin microscopy	Recently used to investigate how changes in cellular hydrostatic pressure modulate the mechanical properties of the CW	Elsayad *et al.* (2016)
Spectroscopy	Fourier-transform infrared (FT-IR) microspectroscopy Confocal Raman spectroscopy Small-angle neutron scattering (SANS) Solid-state NMR (SSNMR) spectroscopy	Spectroscopic techniques may reveal in a non-disruptive way the CW microstructures and may be used as a high-throughput method to characterize cryptic CW mutant phenotypes	Carpita and McCann, (2015); Gierlinger (2018); McCann *et al.* (1992) Phyo *et al.* (2017); Sene *et al.* (1994)
Genetics and genomics	QTL, GWAS, CRISPR/Cas	The genetic and genomics approaches have a tremendous potential to dissect complex phenotypes and identify genes controlling the structure, dynamics and function of the CW. Several CW traits have been analyzed by mapping quantitative trait loci (QTLs) in several species. Genome-wide association studies (GWAS) have identified novel loci/genes influencing morphology, metabolism and bioconversion.The genome-editing technology, i.e. the CRISPR/Cas system, is expected to provide new opportunities to gain insight into the genetic control of plant CW structure and function	Bourke *et al.* (2018); Farneti *et al.* (2017); Kaur *et al.* (2017); Longhi *et al.* (2013); Milano *et al.* (2018); Muchero *et al.* (2015); Paul and Qi (2016); van der Weijde *et al.* (2017); Whitehead *et al.* (2018)

(continued)

Box 1. (continued)

Tools and methods that have enhanced our knowledge of how cell wall composition and structure influence development, immunity and bioconversion in model and cultivated species

	Technique		References
	Cell/tissue and inducible promoters	Spatially and temporarily controlled expression of CW-modifying enzymes may elucidate their cell/tissue-specific functions and be useful to study their involvement in development, immunity and bioconversion. Examples include the expression of the PGIP–PG chimeric protein under the control of inducible promoters, the cell/tissue-specific expression of PMEI5 and the expression of a PG under a senescence-inducible promoter	Benedetti *et al.* (2015); Li (2018); Tomassetti *et al.* (2015)

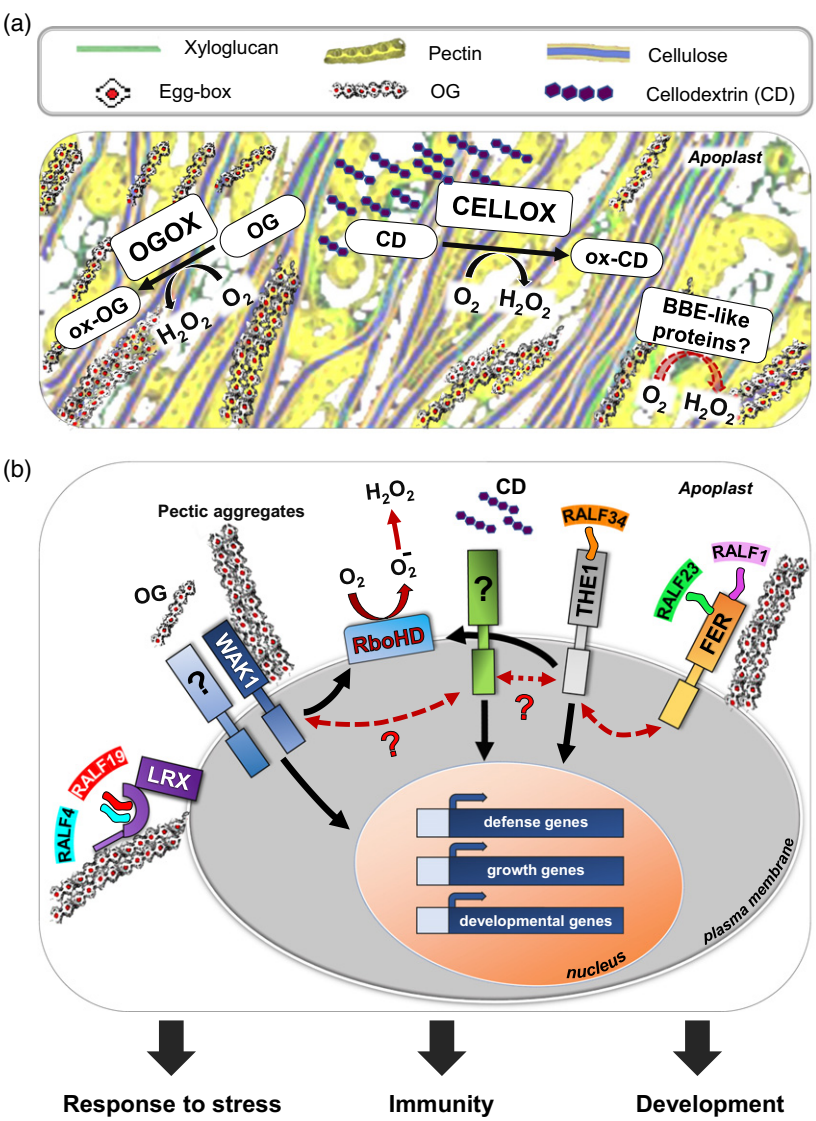

Figure 1. Overview of CW-mediated signaling and some of the main apoplastic or plasma membrane sensors involved. In (a) and (b), key elements that orchestrate cell wall surveillance and signaling are depicted. Solid lines between components represent known interactions, while dashed lines indicate hypothetical mechanisms or mechanisms requiring further confirmation.
(a) OGs and cellulose fragments (cellodextrins) are the only CW-derived DAMPs so far characterized. OGOXs are specific oxidases that dampen the signaling activity of OGs and belong to the BBE-like family. This family comprises enzymes for the oxidation of CDs and probably other cell wall-derived oligosaccharides. The background of the panel was adapted from Cosgrove (2018).
(b) Apoplastic and plasma membrane sensors/receptors that bind the egg box structure of demethylated pectin are WAK1, FER, and LXRs. WAK1 is also a receptor for OGs, the perception of which is likely to involve other receptors/sensor systems. FER and LRXs bind RALF peptides. THE1, a malectin-like receptor, also binds a RALF and is essential for response to cell wall damage (CWD). Activation of ROS production by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase respiratory burst oxidase homolog protein D (RBOHD) is a key event in the cell wall surveillance against microbes and signaling.

increased levels of pectin methylesterification in transgenic Arabidopsis led to increased growth and highly expanded leaves, whereas reduced esterification causes reduced growth and formation of thin and dense leaf mesophyll that limits CO_2 diffusion (Weraduwage *et al.*, 2015). The CW composition, therefore, can affect biomass production

by regulating not only cell expansion but also the relationship between photosynthesis and growth. As pectin affects biomass conversion in species as diverse as Arabidopsis, wheat, tobacco, and aspen (Lionetti *et al.*, 2010; Francocci *et al.*, 2013; Biswal *et al.*, 2014), it is conceivable to increase both photosynthetic efficiency and saccharification efficiency using crop varieties with altered pectin composition, without triggering a CWD response that might reduce biomass production. Novel crops with improved saccharification and increased biomass production may be selected in the future. This may depend, in part, on our ability to perform large-scale analysis of multiple parameters affecting CW characteristics and biomass degradability in targeted mutants (T-DNA insertions, TILLING lines, CRISPR/Cas9 lines) of genes potentially involved in CW biosynthesis and turnover and in genes involved in the adaptive responses to CWD (Box 1).

CONCLUSIONS

Sensing a breech in the wall is crucial for survival of plants and it is reasonable that this capacity was acquired even before developing the complex mechanism of immunity. It is now becoming clear that sensing the CWD is also important for regulation of growth and development (Figure 1). What is the link between CWD, development and immunity is a fascinating chapter of plant biology. The utilization of modern tools and methods to investigate the CW composition and structure will greatly contribute to our knowledge in the field (Box 1).

One of the ways by which plants sense the CWD is the perception of products released from the breach in the wall, i.e. the CW fragments. Only a few CW fragments with a biological activity as elicitors of immunity and/or regulators of growth and development are known at the moment but the amazing complexity of the plant CW polysaccharides, particularly pectin, prompts to search for others. CW fragments with elicitor activity were discovered in plants many years ago (Hahn *et al.*, 1981) and are now referred to as DAMPs, a term that was introduced much later (Land *et al.*, 2016). The involvement of DAMPs and CW fragments in physiological phenomena of tissue repairing and regulation of growth and development is an open field in plant biology (De Lorenzo *et al.*, 2018). The mechanism by which several CW fragments trigger defense but impair growth contributing to the so-called growth-defense tradeoff may be potentially exploited to enhancing plant immunity, while maintaining a normal growth phenotype. How microorganisms are capable of attacking the plant CW in order to fuel their growth is another promising research field that may give indication of how to better utilize the plant biomass to produce biofuel and other industrial products. Research on the structure and biology of the plant CW is expected to grow in the near future and the gained information will greatly contribute to improve both agricultural and industrial projects.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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