How endogenous plant cell-wall degradation mechanisms can help achieve higher efficiency in saccharification of biomass

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Abstract

Cell-wall recalcitrance to hydrolysis still represents one of the major bottlenecks for second-generation bioethanol production. This occurs despite the development of pre-treatments, the prospect of new enzymes, and the production of transgenic plants with less-recalcitrant cell walls. Recalcitrance, which is the intrinsic resistance to breakdown imposed by polymer assembly, is the result of inherent limitations in its three domains. These consist of: (i) porosity, associated with a pectin matrix impairing trafficking through the wall; (ii) the glycomic code, which refers to the fine-structural emergent complexity of cell-wall polymers that are unique to cells, tissues, and species; and (iii) cellulose crystallinity, which refers to the organization in micro- and/or macrofibrils. One way to circumvent recalcitrance could be by following cell-wall hydrolysis strategies underlying plant endogenous mechanisms that are optimized to precisely modify cell walls in planta. Thus, the cell-wall degradation that occurs during fruit ripening, abscission, storage cell-wall mobilization, and aerenchyma formation are reviewed in order to highlight how plants deal with recalcitrance and which are the routes to couple prospective enzymes and cocktail designs with cell-wall features. The manipulation of key enzyme levels in planta can help achieving biologically pre-treated walls (i.e. less recalcitrant) before plants are harvested for bioethanol production. This may be helpful in decreasing the costs associated with producing bioethanol from biomass.

Keywords: Abscission, aerenchyma, bioenergy, cell wall, cell-wall polysaccharide, fruit ripening, pre-treatment, recalcitrance storage mobilization.

Introduction

The current increase in the world’s demand for energy and the need to reduce the dependence on fossil fuel, as well as the establishment of alternative fuels as a cost-effective production, has induced an increase in scientific efforts in plant biology. Although 23.5 million of gallons of first-generation (1G) bioethanol produced from sucrose and starch were generated in 2013 (Renewable Fuels Association, 2014, http://www.ethanolrfa.org/), the fuel industry can no longer rely solely on 1G technologies to supply the future demands. As a result, second-generation (2G) bioethanol is an alternative to overcome this challenge. Production of 2G bioethanol is based on plant biomass, which is considered the most abundant and highly wasted organic raw material available (Pauly and Keegstra, 2008). Grasses such as sugarcane, sorghum, switchgrass, maize, and Miscanthus have an appealing biotechnological potential to be used as feedstocks for 2G bioethanol production (Kim and Dale, 2004; Carroll and Somerville, 2009). However, none of them fulfils all the needs for an ideal biofuel crop (De Souza et al., 2014).

Abbreviations: 1G, first generation; 2G, second generation; AX, arabinoxylan; GH, glycosyl hydrolase; HG, homogalacturonan; MLG, mixed-linkage (1→3),[1→4]-β-d-glucan; XG, xyloglucan; XTH, xyloglucan transglycosylase/hydrolase.

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In order to convert plant biomass into fermentable sugars for bioethanol production, it is necessary to access the polysaccharides (e.g. cellulose, hemicelluloses, and pectins) that form plant cell walls, which means circumventing cell-wall recalcitrance. In industry, the breakdown of these polymers is usually accomplished by chemical biomass pre-treatment followed by hydrolysis with glycosyl hydrolase (GH) cocktails. Although considerable improvement in microbial fermentation and GH efficiencies have been made (Gilbert, 2014) and a few companies around the world are already producing 2G bioethanol, cell-wall recalcitrance to hydrolysis remains a major barrier. This is so because 2G bioethanol has not yet achieved full economic sustainability to complement the 1G technology and compete with other fuels (Lynd et al., 2008). Furthermore, the large amount of enzyme required for polysaccharide hydrolysis corresponds to 9% of the total cost for biomass conversion (Aden et al., 2002), which can render 2G-bioethanol unfeasible in a larger scale (Banerjee et al., 2010). In this sense, any scientific information that can help decrease cell-wall recalcitrance and, consequently, the amount of enzyme used by the industry will probably have an important impact on bioethanol production as a renewable and economically sustainable biofuel.

In this article, we discuss details of how the composition and assembly of wall domains can affect recalcitrance and saccharification, highlighting the key cell-wall aspects contributing to its insubtractibility to GHs. We report the main drawbacks of pre-treatment and molecular biology approaches as a result of lack of knowledge on how the selective and progressive removal of polysaccharides targeted by one reagent or enzyme activity can influence the overall cell-wall structure. Next, we discuss possible key features of endogenous plant mechanisms of cell-wall degradation that could be used as a guide to better complement and/or help direct the ongoing strategies to improve saccharification of biomass. Thus, the cell-wall hydrolysis underlying mobilization of cell-wall storage polysaccharides, abscission, fruit ripening, and aerenchyma formation are highlighted as possible ways to optimize the design of enzyme hydrolysis processing of plant biomass in the industry. In addition, the focus on how plant cells degrade their own walls can better direct the selection of candidate genes to be employed in the production of transgenic plants. This could provide a less-recalcitrant biomass input to the industry in order to contribute to reducing the costs of bioenergy production.

Recalcitrance: a whole-cell-wall problem

One of the key steps in comprehending cell-wall recalcitrance is the understanding of cell-wall composition and architecture. Cell walls are composed of cellulose, hemicelluloses, pectins, lignin, and proteins (Carpita and McCann, 2000) (Fig. 1). Commaulinid monocots, such as the grasses, present type II cell walls in which the cellulose network is associated with arabinoxylans (AXs), xylglucans (XGs), and mixed-linkage (1→3),(1→4)-β-d-glucans (MLGs). These polysaccharides are all embedded in a matrix containing branched AXs and pectins composed of homogalacturonan (HG), rhamnogalacturonans and arabinogalactans (Carpita, 1996; Carpita and McCann, 2000). In this type of wall, there are also hydroxycinnamic acids, and p-coumaric and ferulic acids, which may form dimers interconnecting AX, XG, and pectin molecules. Cell-wall proteins such as extensins may also interfere with recalcitrance. However, no literature has been found reporting experiments with extensin for biofuel purposes. All of these cell-wall components are arranged as an entangled structure that confers important cell-wall properties such as porosity, mechanical strength, cell shape, and signalling, to name but a few (Fig. 1). Cell walls are therefore extremely complex composites that are precisely assembled and constantly modified in plant cells.

Besides cellulose, hemicelluloses, pectins, and proteins, lignin is also a relevant cell-wall component. It consists of a complex polymer of aromatic alcohols and has been traditionally addressed as one of the main contributors to cell-wall recalcitrance (Simmons et al., 2010). However, recent studies have shown that recalcitrance does not rely on lignin alone but also on polysaccharides (Ray et al., 2012; Torres et al., 2013; Marriot et al., 2014). For instance, in switchgrass, xylan is indicated as a relevant recalcitrance factor, since the removal of this polysaccharide resulted in almost 100% glucose yield after enzyme action (De Martini et al., 2013). Similar results have been produced for Miscanthus biomass, in which the hemicellulose content was found to be the most relevant factor correlated to digestibility (Xu et al., 2012). For maize, Penning et al. (2014) revealed that quantitative trait loci for lignin abundance do not overlap with those related to saccharification, but rather are associated with whole-cell-wall metabolism, vascularization, and fibre formation. Remarkably, qualitative (i.e. polysaccharide–polysaccharide interactions and polysaccharide fine structure) rather than quantitative effects seem to play a role in recalcitrance/hydrolysis in Miscanthus sinensis (De Souza et al., 2015, this issue). In this sense, the interaction between polymers within the cell wall is also closely connected to its inherent resistance to hydrolysis. The assembly and range of possible cross-links between polymers defines the cell-wall architecture, divided into three domains (cellulose, hemicellulose, and pectins; Fig. 1a), which imposes several constraints on degradability.

The first barrier to hydrolysis is the selective cell-wall accessibility to GHs. This porosity feature is determined mainly by the pectin domain (Baron-Epel et al., 1988) and the number and size of the enzymes that can penetrate the biomass are probably selected by the pore sizes of the wall matrix (Buckeridge et al., 2015).

Another critical aspect for recalcitrance is the polymer–polymer interaction, which is closely associated with the hemicellulose domain. As these polysaccharides prevent access to cellulose, knowledge about their fine structure as well as their interactions with other polymers is mandatory. Differences in fine structure can be related to sugar (i.e. mono- or oligosaccharides) or non-sugar (i.e. acetylation, methylation, or feruloylation) branching decorations in the main chains of polysaccharides. These differences affect the interactions between polymers, which in turn are thought to display a
complex structure that appears to determine the mode of action of GHs.

Every fine structure of each different polysaccharide in the wall of a given cell or tissue is encoded by the biosynthetic system so that a given polysaccharide can, for instance, be ‘blocked’ or ‘open’ to hydrolytic enzyme action. In other words, the supramolecular structure formed by polysaccharides seems to derive a glycomic code, which is characterized by information properties that emerge from the assembly of cell-wall polymers (Buckeridge and De Souza, 2014; Barbieri, 2015). In this sense, an understanding of the glycomic code is one of the keys to open the way for efficient hydrolysis.

Another important aspect of the cell walls related to recalcitrance and architecture (the final expression of the glycomic code) is the way cellulose forms crystals, resulting in microfibrils that vary along the plant body and tissues (Driemeier et al., 2012). In grasses, these microfibrils can form bundles, possibly tethered by hemicelluloses (i.e. xylans and/or XGs), generating macrofibrils (Ding and Himmel, 2006; Kozlova et al., 2014; Langan et al., 2014; Buckeridge et al., 2015) (Fig. 1B).

Surpassing the recalcitrance issue has proven to be more challenging than previously foreseen. Solving this problem relies on the early production of GHs with lower diameter associated with knowledge related to the emergent properties of polymer–polymer interactions (e.g. the glycomic code). In this sense, the first set of enzymes (those allowed to cross the cell-wall pores) would open the access to further ones aimed at attacking key hemicelluloses in order to open accessibility to amorphous cellulose stretches. Following these steps is crucial for gaining control on how to precisely attack polysaccharides, making cell walls as a whole more amenable to hydrolysis (Buckeridge and De Souza, 2014; Ong et al., 2014). Furthermore, this would help to design rational enzyme cocktails able to facilitate the complete disassembly of the cell wall (Buckeridge and De Souza, 2014). As will be discussed in the following sections, the strategies currently employed to hydrolyse the cell wall often do not take into account all the features responsible for its recalcitrance.

### Current approaches to increase cell-wall digestibility

Currently, cell-wall recalcitrance is overwhelmed with the use of biomass pre-treatments that are responsible for removing lignin and/or decreasing cellulose crystallinity. However, those procedures can interfere with macro- and microfibril associations, reducing the access to cellulose and also removing pectins and some soluble hemicelluloses from cell walls. For instance, Alonso-Simón et al. (2010) demonstrated that the use of liquid hot-water pre-treatment removes xylans and AX from wheat straw. Similarly, Li et al. (2014) showed loss of xylans, MLGs, XGs, and pectins in corn stover and goldenrod (Solidago canadensis) when they were pre-treated with alkaline hydrogen peroxide. Thus, considering the percentage of hemicelluloses in cell walls of the main biomass feedstocks (Pauly and Keegstra, 2008), pre-treatments might represent a loss of polysaccharide of about 12–33% that could potentially be used to produce 2G bioethanol.
In this sense, different approaches other than chemical pre-treatments have been attempted to improve biomass saccharification, such as the production of genetically modified plants in order to synthesize less-recalcitrant cell walls in planta. Although several efforts have aimed at decreasing lignin (Jung et al., 2012; Vanholme et al., 2012; Van Acker et al., 2013) or overexpressing different microbial and plant GHs in planta (Brunecky et al., 2011; Mahadevan et al., 2011; Bartley et al., 2013; Sumiyoshi et al., 2013), those strategies had limitations. This is mainly due to undesirable traits in plants harbouring mutations in lignin biosynthesis, such as reduced biomass yields (Simmons et al., 2010; Fu et al. 2011a, b), low germination frequency, lower seed viability, decreased height, and high pollen sterility (Goujon et al., 2003; Mandal and Datta, 2014), besides the increased sensitivity to pathogens (Boerjan et al., 2003). Moreover, some authors suggest that GH overexpression in planta increased the accessibility to polysaccharides other than the one targeted by the heterologous enzyme (Kaida et al., 2009; Lionetti et al., 2010; Mahadevan et al., 2011; Biswal et al., 2014). For instance, decreased cellulose crystallinity was observed after xyloglucanase expression in poplar (Kaida et al., 2009). On the other hand, monosaccharide proportions other than glucose were kept constant when Thermotoga maritima cellulase was overexpressed in tobacco (Mahadevan et al., 2011), emphasizing the need for a more complex set of enzymes to successfully attack a broader set of polysaccharides. Furthermore, cellulose hydrolysis in poplar xylem cells overexpressing xyloglucanase was higher than in xylem overexpressing xylanase or cellulase (Kaida et al., 2009).

In a similar fashion, changes in pectin content and/or its substitution pattern led to increased saccharification. In poplar, tobacco, wheat, and Arabidopsis, the overexpression of plant pectinases such as endopolygalacturonase, pectate lyase, or pectin methylesterase led to increased release of simple sugars. It is noteworthy that not only pectins but tate lyase, or pectin methylesterase led to increased release of plant pectinases such as endopolygalacturonase, pec

the impact of the step of pre-treatment in industrial processes in the future.

**Mechanisms of cell-wall degradation by plants**

Several plant endogenous mechanisms of cell-wall disassembly share common features (Rose and Bennett, 1999; Roberts et al., 2002; Grandis et al., 2014). The step-by-step procedure undertaken by plants to modify their own cell walls commonly starts with cell expansion and separation, in which pectins are the main targets for hydrolysis. Some natural cell-wall degradation events seem to rely on cell targeting, from which the carbohydrate breakdown activation spreads through the surrounding cells (McManus et al., 1998; Grandis et al., 2014). Downstream signalling activates hydrolysis of cell components through programmed cell death (Bouranis et al., 2007). Afterwards, first hemicelluloses and later on cellulose are degraded (Roberts et al., 2002; Grandis et al., 2014). These common steps required for cell-wall degradation in a broad spectrum of plant endogenous processes can be seen in a modular perspective. Thus, each module (i.e. cell separation, cell expansion, cell targeting, programmed cell death, hemicellulose hydrolysis, and cellulose hydrolysis) is a relatively conserved sequence of events taking place in fruit ripening, aerenchyma formation, leaf abscission, mobilization of cell-wall storage polysaccharides, and so on. On this basis, a modularity theory of hydrolysis has been proposed by Grandis et al. (2014) as a guide for a more successful strategy to achieve efficient hydrolysis in planta. In other words, plants could be induced to modify their cell walls by means of activation of key players from one or more modules so that cell walls could be swollen before harvesting. Thus, the biomass taken to industry could be more accessible to pre-treatment and hydrolysis by GHs.

During endogenous cell-wall hydrolysis, the plant GHs are produced and secreted to the wall in a temporal sequence compatible with the architectural feature of the cell wall of that specific cell and tissue (see Fig. 6 in De Souza et al., 2013). In this way, expansins and pectinases should be recruited for the cell separation and expansion modules, whereas a whole set of xyloglucanases and cellulases would be used afterwards in the process in order to loosen the walls.

Considering key modifications and/or degradation of cell-wall polysaccharides found in these natural processes, in the following sections we will discuss how they can drive improvements in the technologies for 2G bioethanol production. Next, we will highlight how the modifications on each polysaccharide present in the cell walls of the main bioenergy feedstocks could lead to further changes potentially enhancing biomass hydrolysis.

**Pectins: targets to change cell adhesion and porosity**

Pectins are polysaccharides related to plant growth, development, and morphogenesis. They are also relevant in plant defence, interaction with ions, cell adhesion and cell
expansion, and cell-wall porosity (Baron-Epel et al., 1988; Ridley et al., 2001). The latter is specifically relevant to bioenergy, since the average pore size of wall matrices is likely to limit the traffic of GHs (Buckeridge et al., 2015). Thus, in order to reduce the limitation of enzyme action in intact cell walls caused by the presence of the pectin domain, pectinases should be present in enzymatic cocktails (Buckeridge et al., 2015) as part of the strategy to modify plants by engineering (Grandis et al., 2014).

In nature, the attack on pectins seems to be an early step in distinct endogenous cell-wall degradation events. This reinforces the idea that it is necessary first to hydrolyse those polysaccharides in order to access other cell-wall polysaccharides. In natural processes such as lyssigenous aerenchyma formation, which leads to the opening of gas spaces after cell death and cell-wall degradation (Evans, 2003), the first event seems to be cell separation and cell expansion (Yamauchi et al., 2013; Grandis et al., 2014). It is noteworthy that changes in the pattern of pectin esterification are observed in the middle lamella during the early stages of aerenchyma development (Gunawardena et al., 2001). These changes are possibly related to the increase in polygalacturonase and pectin lyase expression during aerenchyma formation (Rajhi et al., 2011).

Pectin hydrolysis also occurs during fruit softening (Brummell, 2006). As with aerenchyma formation, the first event observed during fruit ripening seems to be the attack on HG present in the middle lamella (Gunawardena et al., 2001). Similarly, in kiwi fruits, the reduction in the molecular weight of polysaccharides present in soluble fractions strongly suggests HG depolymerization by endopolygalacturonase in the middle lamella (Redgwell et al., 1992). Similar results have been obtained for papaya fruits, where an increase in the release of pectin-derived oligosaccharides was observed concomitant with an increase in polygalacturonase activity during fruit ripening (Saúndo-Barajas et al., 2009).

Although high levels of endopolygalacturonase mRNA have proven not to be essential for tomato fruit softening (Kramer et al., 1992), depolymerization of linear pectins was impaired when this gene was downregulated by antisense RNA (Smith et al., 1990). The role of this enzyme in fruit softening was assessed further in papaya and apple fruits (Atkinson et al., 2012; Fabi et al., 2014), leading to a decreased molecular mass of pectins. This confirmed the profound changes exerted by this enzyme in the cell-wall structure.

During tomato fruit ripening, pectin becomes gradually de-esterified due the action of pectin methyl esterases. The latter enzymes are required to open access for polygalacturonases and consequently degrade the main chain of methyl-esterified pectin (Micheli, 2001). Together, these two enzymes are known to affect middle lamella integrity, controlling cell adhesion. Also, pectin dimethyl esterification favours other enzymes besides polygalacturonases by modifying cell-wall porosity (Baron-Epel et al., 1988; Ehwald et al., 1992; Rondeau-Mouro et al., 2008). In terms of bioenergy, it has already been demonstrated that the reduction of dimethyl-esterified HG in Arabidopsis, wheat, and tobacco increased saccharification efficiency by 35–40%. This suggests that cellulose and hemicelluloses are more easily digested when methyl-esterified HGs are reduced in the wall, possibly facilitating the access of enzymes to the cell-wall network (Lionetti et al., 2010).

Other endogenous cell-wall degradation events also require polygalacturonase and pectin methylesterase activities. Microscopic and spectroscopic approaches showed low levels of esterified pectin associated with high levels of polygalacturonase activity in the abscission zone of cells of oil palm (Elaeis guineensis) (Henderson et al., 2001). In Brassica napus, a gene related to polygalacturonase seems to be expressed in the abscission zone from the base of the anther filaments, petals, and sepals (González-Carranza et al., 2002). Once abscission starts, cell swelling is evident in the abscission zone where endo-β-glucanase and polygalacturonase mRNAs accumulate or have their activity increased (David et al., 1999; Roberts et al., 2002). As a result, middle lamella dissolution and abscission have been observed. Thus, the synergistic effect of pectin-degradation enzymes and cellulases is noticeable (González-Carranza et al., 2007).

Additionally, fruit softening is significantly reduced by impaired β-galactosidase activity during the early steps in the ripening process (González-Carranza et al., 2007), highlighting the relevance of this enzyme to cell-wall degradation. This GH is part of an array of enzymes required for pectin debranching. It is believed that removal of galactan substitutions from pectins will also be mandatory in other fruits (Ali et al., 1995; Carrington and Pressey, 1996; Ali et al., 2004; Gulzar and Amin, 2012; Kivi and Sartipnia, 2014), as well as for cotyledon cell-wall degradation (Buckeridge and Reid, 1994; Buckeridge et al., 2005), allowing subsequent cell-wall changes to occur (Brummell and Harpster, 2001).

Arabinofuranosidases are thought to act synergistically with β-galactosidases on pectins (Hirano et al., 1994), with transcripts for both enzymes showing a similar pattern (Sozzi et al., 2002). Arabinofuranosidases catalyse the removal of α-L-arabinoyl branches from arabinogalactans and AXs (Yoshioka et al., 1995). The upregulation of the corresponding gene is associated with peach fruit softening (Hayama et al., 2006), and an increased loss of arabinose residues has been demonstrated to contribute to the ripening of several fruits (Yamaki et al., 1979; Peña and Carpita, 2004; Rosli et al., 2004; Tateishi et al., 2005).

Therefore, the presence of β-galactosidases as well as arabinofuranosidases, endopolygalacturonases, and pectin methyl esterases in plant tissues seems to be relevant for the early steps of cell-wall degradation. The use of such a set of enzymes for bioenergy purposes could favour not only 2G processes but 1G ones as well. By softening sugarcane stem with these enzymes, the cells would become easier to break, which could significantly reduce the energy necessary to extract sucrose.

Expansins

The process of cell expansion is an important step in natural systems that could be explored for improvement of bioethanol.
production. During cell expansion, the polysaccharides that coat cellulose microfibrils should be rearranged (Cosgrove, 2000). This rearrangement involves expansins, which are proteins that provoke cell-wall relaxation, affecting the cellulose-XG network (McQueen-Mason and Cosgrove, 1995; Rose and Bennett, 1999). Expansins lack hydrolytic activity and are thought to ease the action of GHs on cellulose by destabilizing hydrogen bonds and promoting slippage of XGs on the surface of cellulose microfibrils (Sampedro and Harpster, 2001). For more details on the synergistic activity of microbial GHs and plant expansins, see Baker et al. (2000) and Arantes and Saddler (2010).

In natural systems such as fruit ripening, the overexpression of an expansin in ripening tomato resulted in softer fruits, which was correlated to extensive depolymerization of hemicelluloses and pectins (Brummell et al., 1999; Brummell and Harpster, 2001). The same can be suggested for aerenchyma formation, where an increase in expansin transcripts was correlated to higher expression of pectin- and XG-active enzymes acting on cell-wall loosening in maize roots (Rahji et al., 2011). Thus, the synergistic action between hemicellulose- and pectin-degrading enzymes and expansins seems to be crucial for coordinated wall disassembly.

In this sense, the optimization of enzymatic cocktails to 2G bioethanol is already considering the inclusion of expansins as an optimization that would reduce biomass recalcitrance to hydrolysis (Mohanram et al., 2013). As stated above for pectinases, expansins also have the potential to help IG processing for the same reasons. Cells with loosened walls may become easy to break and facilitate the extraction of sucrose from sugarcane.

**Hemicellulose removal: opening the doors towards cellulose**

Hemicelluloses are the main domain present in cell walls of grass bioenergy feedstocks. Thus, the degradation of these polysaccharides is crucial to achieve efficient cell-wall disassembly. In dicots, hemicelluloses such as XGs, mannans, glucomannans, and galactomannans are involved in exceptionally efficient hydrolytic systems (Meier and Reid, 1982; De Mason et al., 1983; Reid, 1985; Buckeridge et al., 2000; Buckeridge, 2010). In cotyledons and endosperms of several species, cell-wall polysaccharides occurring in large proportions are completely hydrolysed in order to provide free sugars to be used for respiration and growth (Buckeridge, 2010). Although glucomannans are found in small amounts in cell walls of bioenergy crops, they can be cross-linked with cellulose microfibrils (Carpita and Gibeaut, 1993), interfering with cellulose saccharification. The same could be inferred for galactomannans and mannans, which seem to be associated with increased biomass saccharification (De Souza et al., 2015, this issue). In this way, enzymes such as endo-β-mannanases and α-galactosidases could improve the potential of enzymatic cocktails. Natural mechanisms that involve mannann degradation are related mainly to seed germination. In lettuce, for example, β-mannannase enzyme activity starts in the radicle tip and endosperm cap (Toorop et al., 1998) and may be involved in cell-wall extensibility (Karssen et al., 1989). Some indication that these enzymes could improve cell-wall saccharification were given by Banerjee et al. (2010), who used a fungal mannanase and found an enhancement of hydrolysis of dried distillers' grains.

One of the main hemicelluloses in grasses is MLG (reviewed by Stone and Clarke, 1992). Due to its relatively simple structure, MLG requires only two enzymes (lichenase and β-glucosidase) to be completely hydrolysed (Fincher, 2009). Because of this, it has already been suggested that an increase in MLG content in feedstocks could increase glucose yields (McCann and Carpita, 2008).

Degradation of MLG was observed during grass and cereal seed germination (Buckeridge et al., 2004) and mobilization in leaves of barley (Roulin et al., 2002), as well as during aerenchyma formation in sugarcane roots (D. C. C. Leite, A. Grandis, E. Q. P. Tavares, S. Pattathil, U. Avici, A. P. De Souza, B. Mattei, M. J. Hahn, and M. S. Buckeider, unpublished results). Crops like sugarcane and Miscanthus offer great opportunities for improvement of MLG degradation, making cell-wall composition more adapted for use as a bioenergy feedstock.

AX is another important hemicellulose in grasses. In contrast to MLGs, the structure of AX requires a broader set of enzymes to be degraded. In endogenous plant cell-wall degradation, hydrolysis of AX has rarely been observed. Some enzymes capable of degrading AX have been identified in germinating cereals, comprising mainly α-arabinofuranosidase and xylanase (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977). Arabinofuranosidases act by removing arabinosyl side chains from AX, opening the way for degradation by xylanases (Preene and MacDougall, 1958). In fruit ripening, the presence of arabinofuranosidase mRNA and enzyme activity has been observed (Itai et al., 2003). However, no report of the presence of xylanases has been made to date, suggesting that the complete degradation of this hemicellulosic polysaccharide during fruit ripening might not occur.

In the case of grasses, AX can be cross-linked to ferulic acids, so its degradation should involve feruloyl esterases. During aerenchyma formation in sugarcane roots, an increase in transcripts related to a precursor of a feruloyl esterase has been observed (E. Q. P. Tavares, A. P. De Souza, A. R. Piovezani, and M. S. Buckeider, unpublished results), which might be indicative that this enzyme is acting during aerenchyma development. However, although endoxylanase transcripts are also found, there is no evidence of a reduction in AX in the cell wall during this same process (D. C. C. Leite, A. Grandis, E. Q. P. Tavares, S. Pattathil, U. Avici, A. P. De Souza, B. Mattei, M. J. Hahn, and M. S. Buckeider, unpublished results). The AX present in cell walls of bioenergy crops such as sugarcane also contains decorations with acetyl groups that can interfere in enzymatic cleavage (De Souza et al., 2013). Thus, in this case, an acetylemesterase should be necessary.

Concerning XG, in storage tissues XG transglycosylase/hydrolase (XTH) has been demonstrated to play a key role in
XG mobilization (Fanutti et al., 1993). Decreasing levels of storage XG have been reported during seedling development in cotyledons of the tropical tree Hymenaea courbaril (Tinté et al., 2000), fruit ripening in avocado (O’Donoghue et al., 1994), and papaya fruit development (A. A. Cavalieri-Coreti, A. P. De Souza, and M. S. Buckeridge, unpublished results). In maize, increased expression of a gene coding for an XTH was observed during aerenchyma formation (Saab and Sachs, 1996; Rajhi et al., 2011). The XG depolymerization by endo-β-1,4-glucanases can be increased further by exoenzymes that will accomplish XG side-chain removal, making more sites available to the attack of endoenzymes (Rose and Bennett, 1999; Brummell and Harpster, 2001). In fact, Tinté et al. (2000) showed that the complete degradation of XG requires α-xyllosidases, β-galactosidases, and β-glucosidases to remove the XG side chains. As XG seems to be the hemicellulose that tethers cellulose, the mechanism by which it is hydrolysed in the plant cell can be further explored by plant scientists in order to reveal how to increase the access of enzymes to micro- and macrofibrils to cellulose (Buckeridge, 2010, Buckeridge et al., 2015).

Additionally, the presence of expansins (as discussed above) is critical in the process of detaching XG bound to the surface of cellulose micro/macrofibrils. This is due mainly to the reduced XG accessibility by XTH action when it is bound to cellulose (Pauly et al., 1999).

**Cellulose**

Most of the work on 2G bioethanol technologies regards cellulose degradation and cellulase activities as the main targets to solve the problem of recalcitrance. In this way, the comprehension of cellulose degradation in natural processes could help to understand how this polymer interacts with other cell-wall polysaccharides, improving knowledge on how to effectively access microfibrils.

Reports on cellulase activity are relatively common in descriptions of processes of cell-wall degradation in plants by fungi and bacteria. Usually, it is either a consequence of other polymer disassembly or overall modifications, but it rarely takes place as an isolated event. The detection of high cellulase activity in expanded cells during bean leaf abscission indicates that cell expansion must be coupled with cellulose hydrolysis. Both features are related to cell-wall loosening required for accomplishment of the abscission process (Ueda et al., 1996). In fact, cellulase action does occur in parallel with endopolygalacturanase activity in the abscission of orange calyx (Burns et al., 1998) and cotton leaves (Du et al., 2014).

As discussed previously, pectin modifications also seem to be relevant to cellulase action in lysigenous aerenchyma formation in maize roots (Gunawardena et al., 2001). In fact, for the cellulases to act on cellulose, an increase in porosity mediated by β-galactosidase and polygalacturonase is required for further disruption of hydrogen bridges between XG and cellulose by expansin (Rose and Bennett, 1999; Brummell, 2006). This raises an interesting interpretation: for cellulose to be targeted for enzymatic breakdown, cell metabolism recruits other enzymes in order to remove polysaccharides that are coating cellulose microfibrils. This reinforces the idea that a whole series of ‘accessory enzymes’ and enzymes other than cellulases should be used in enzymatic cocktails for 2G bioethanol technologies.

From a bioenergy perspective, in some cases cellulase activity can be detrimental to saccharification. For instance, it has been observed that the action of cellulase on amorphous sites of cellulose microfibrils increased cellulose crystallinity proportion in avocado ripening. Cellulose and other cell-wall polymer contents did not change and also XG associated with cellulose was kept constant (O’Donoghue et al., 1994). These findings suggest that the rearrangements of microfibrils may interfere with cellulase activity, increasing crystallinity and possibly increasing recalcitrance.

**Conclusions and perspectives**

In this review, we have highlighted some of the main bottlenecks that need to be addressed in order to achieve better efficiency in the industrial processes for 2G bioethanol production. As the drawbacks of pre-treatments and enzymatic hydrolysis-limited efficiency are quite significant, we propose that the information about some natural cell-wall modification processes could be useful as guidelines to drive strategies for future research and technology development for bioenergy production.

Table 1 summarizes some features of the four main biological processes involving cell-wall modifications and how their key aspects could impact bioenergy production. In the four cases, the ability to hydrolyse cell walls in vivo could be manipulated to promote biological pre-treatment in order to provide less-recalcitrant biomass for hydrolysis in industry. This does not exclude the use of the pre-treatments already developed but rather would complement their application. The main goal is to decrease the energy input in this part of the process. Also, most of the enzymes that participate in these natural processes in plants could be characterized. In this sense, plant GHs would supplement the enzyme cocktails already in use in industry, in order to further optimize their performance.

If transgenic plants are to be largely used in bioenergy in the future, some important points should be considered. One of the major aspects is the cell adhesion involving changes in the pectins of the middle lamella. This can lead to cell separation and consequently increase the surface available to the GEs present in enzyme cocktails. As a result of some cell-wall modifications, recalcitrance could decrease due to changes in wall loosening in vivo.

Some challenges will have to be faced in order to achieve these goals from a practical viewpoint. In order to activate cell-wall modification modules in a given bioenergy crop, one will have to acquire control on how to activate that process at the right time. In this sense, plants holding inducible and/or tissue-specific promoters could be used to grow normally until the overexpression of the heterologous or endogenous enzyme is activated (Lionetti et al., 2010; Biswal et al., 2014). In addition, transcription factors controlling GH expression could be
Table 1. Biotechnological aspects related to bioenergy for possible uses of part of the four main biological processes in which cell-wall degradation takes place, in future programmes of synthetic biology

<table>
<thead>
<tr>
<th>Bioenergy aspects</th>
<th>Biological process</th>
<th>Fruit ripening</th>
<th>Aerenchyma formation</th>
<th>Abscission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Features to be used in synthetic biology</td>
<td>Increase one type of polysaccharide in the wall; regulation of timing of hydrolysis activation</td>
<td>Pectin hydrolysis; decrease cell adhesion; promote cell separation and cell expansion by changes in xyloglucan cellulose interactions; regulation of timing for process activation</td>
<td>Decrease cell adhesion; promote cell separation; modifications in pectins</td>
<td>Decrease cell adhesion; promote cell separation; regulation of timing of activation</td>
</tr>
<tr>
<td>CW domain attacked</td>
<td>Mainly the hemicellulose domain</td>
<td>Pectin, hemicellulose, and cellulose domains</td>
<td>Mainly the pectin domain</td>
<td>Pectin, hemicellulose and cellulose domains</td>
</tr>
<tr>
<td>Effect on pre-treatment</td>
<td>May make some pre-treatments easier due to decrease in recalcitrance</td>
<td>Loosening of walls and increase in substrate–enzymes contacts</td>
<td>Makes tissues loosen and increases effect</td>
<td>Little effect</td>
</tr>
<tr>
<td>Effect on saccharification</td>
<td>Increased facility for saccharification; avoidance of decrease of C5 impact</td>
<td>General increase in saccharification of walls as a whole</td>
<td>Limited increase in saccharification due to remaining recalcitrant walls</td>
<td>Limited increase in saccharification due to remaining recalcitrant walls</td>
</tr>
<tr>
<td>Impact on 1G</td>
<td>Little impact</td>
<td>Improvement of extraction of sucrose in sugarcane and sorghum</td>
<td>Limited effect as walls continue to be hard to break</td>
<td>Limited effect as walls continue to be hard to break</td>
</tr>
<tr>
<td>Main problems to be solved before application in synthetic biology</td>
<td>Gain control of polysaccharide biosynthesis mechanisms; find out which signalling mechanisms determine the partition of polysaccharides in the wall</td>
<td>Find what controls the timing of production of enzymes</td>
<td>Understand signalling so as to avoid total collapse of the wall</td>
<td>Understand how the system triggers only a specific group of cells instead of collapsing the whole tissue</td>
</tr>
</tbody>
</table>

relevant targets to understand how to activate cell-wall degradation processes.

The challenge is enormous and it will take some time before we can achieve a level of control that will be secure enough to be used in agriculture and profitable at the same time. However, some of the key basic knowledge is already available so that biotechnological strategies can now be devised. As a consequence, the efficiency of bioenergy production from plants can be even more improved in a scenario of intense demand for higher sustainability.

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