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It's time to go glyco in cell wall bioengineering

Cătălin Voiniciuc

Abstract

Tailoring the structure of cellulose, hemicellulose or pectin in plant cell walls can modulate growth, disease resistance, biomass yield and other important agronomic traits. Recent advances in the biosynthesis of microfibrils and matrix polysaccharides force us to re-examine old assumptions about the assembly and functions of cell wall components. The engineering of living or hybrid materials in microorganisms could be adapted to plant biopolymers or to inspire the development of new plant-based composites. High-throughput cellular factories and synthetic biology toolkits could unveil the biological roles and biotechnological potential of the large, unexplored space of carbohydrate-active enzymes. Increasing automation and enhanced carbohydrate detection methods are unlocking new routes to design plant glycans for a sustainable bioeconomy.

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Plant polysaccharides, Glycosyltransferases, Glycosyl hydrolases, Synthetic biology, Biomaterials, Biotic stress, Cell wall biochemistry.

Abbreviations

GH, glycosyl hydrolase; GT, glycosyltransferase; CBM, carbohydrate binding modules.

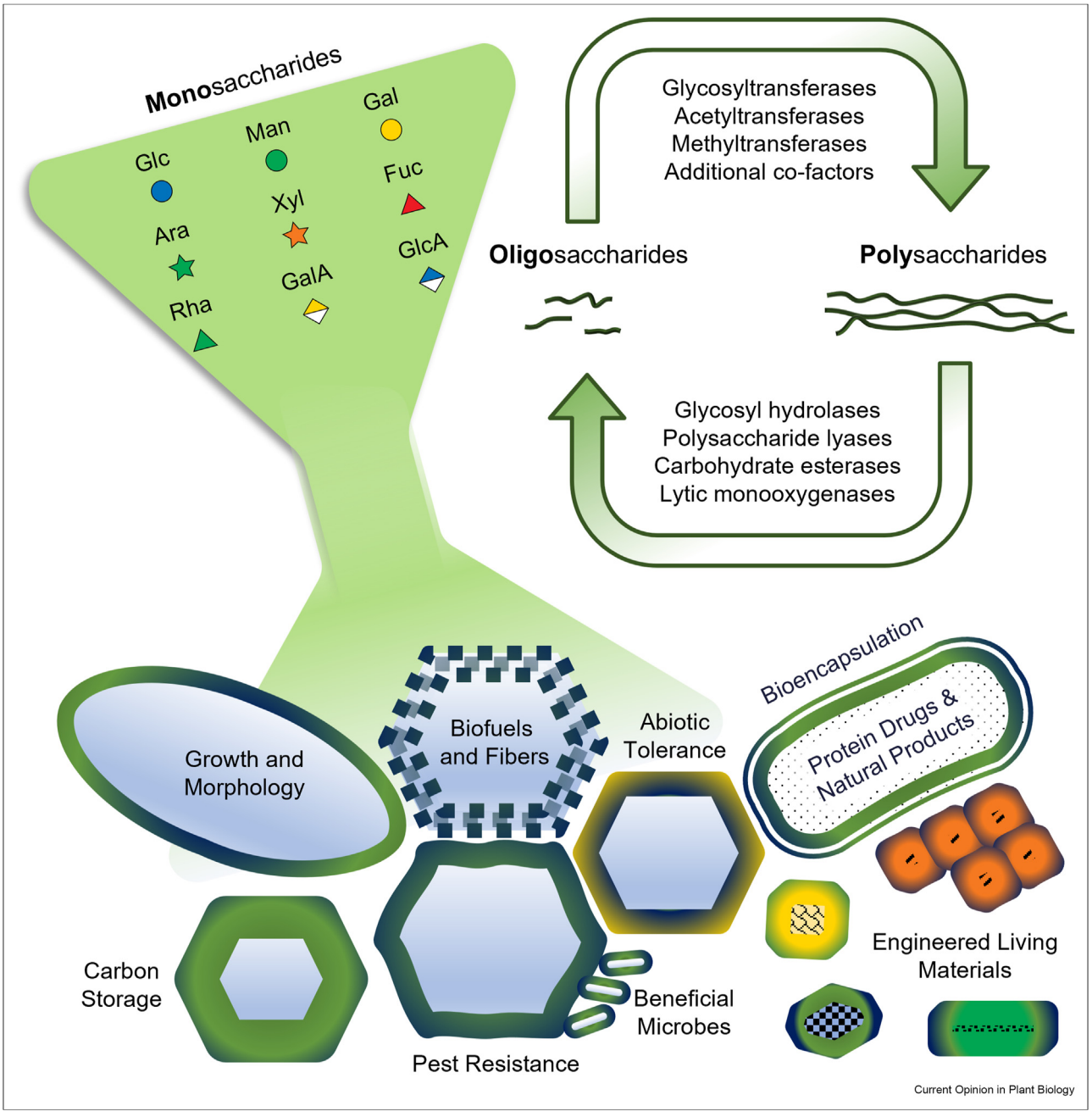
Introduction

Complex glycans (from the Greek 'glyko' for 'sweet') govern many aspects of biology: from controlling the growth of plant cells to post-translationally modifying proteins in eukaryotes. Polysaccharides are the most abundant components of the plant cell wall, a versatile exoskeleton that shapes development, morphology, and environmental responses. In addition to cellulose, the plant extracellular matrix can contain multiple hemicelluloses (β-1,4-linked xylans, mannans, and xyloglucans, plus mixed-linkage glucans containing both β-1,4 and β-1,3-linkages) and pectin. The biosynthesis and functions of cellulose, hemicelluloses and pectin have been recently reviewed [1], but these biopolymers still have untapped potential. Cell wall polysaccharides represent the main sink for carbon captured from the atmospheric CO_2 via photosynthesis, and they can be broken down into various oligosaccharides and monosaccharides (Figure 1). While cell walls provide a beneficial barrier against (a)biotic stresses, their limited porosity and recalcitrance to degradation present obstacles for some biotechnological applications, such as the conversion of lignocellulosic biomass into bioenergy [2]. Increasing cell wall hexose sugar content can boost saccharification, as evidenced by the stacking of multiple genes to synthesize pectic galactan in *Arabidopsis thaliana* [3]. However, a variety of purpose-built cell walls (Figure 1) are needed to modulate agronomic traits and for advanced bioproducts. In this review, I highlight recent advances, biological questions, and emerging opportunities to build designer plant glycans with tailored functions or new-to-nature properties.

Recent advances in plant cell wall biology

An updated model on how extensible cell walls are built was proposed in 2022 [4], with interactions among cellulose microfibrils being most important for growth mechanics. In contrast to cellulose, coarse-grained molecular dynamics simulations found that matrix polysaccharides (xyloglucan and pectin) did not have a major impact on the biomechanical properties of epidermal cell walls [5]. Consistent with this model, a quintuple cellulose synthase-like mutant with no detectable xyloglucan displayed relatively normal growth [6]. However, a new publication shows a role for β-galactoglucomannan in elongating tissues lacking xyloglucan [7] since growth was further reduced by disrupting the biosynthesis of both polymers. The deposition of cellulose microfibrils in certain cell walls can also be influenced by the intracellular biosynthesis of pectin [8] and certain hemicelluloses [9]. In addition, the movement of cellulose synthases at the plasma membrane is coordinated by recently discovered cytosolic microtubule-related proteins in Arabidopsis [10], including the IQ67-domain (IQD) family [11] that was previously associated with cell wall biomass properties in *Populus* trees [12]. These findings signal the need to re-examine old assumptions and address gaps in our

Figure 1

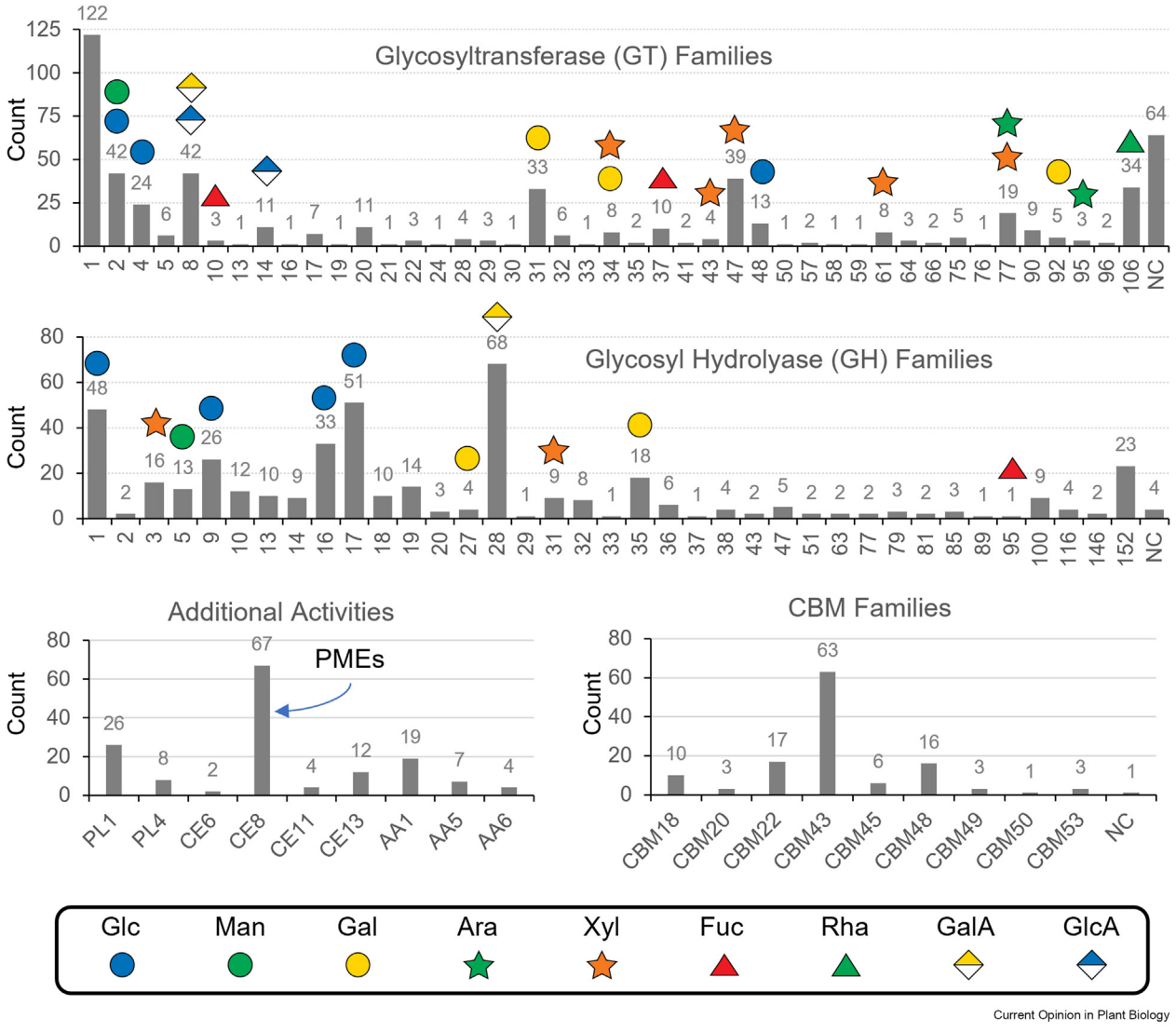


Conceptual model for tailor-made plant polysaccharides. Glycosyl residues (via activated nucleotide sugars; not shown) can be incorporated into cell wall polysaccharides by a large suite of glycosyltransferases and additional proteins. A variety of hydrolytic enzymes can work in synchrony to degrade carbohydrate polymers into oligosaccharides and even monosaccharides. Genetic engineering efforts could funnel glycans into extracellular structures that modulate agronomic traits (e.g. plant stature and biomass yields), carbon sequestration, (a) biotic resistance, and the production of various bio-products. The diversity of cell wall structures displayed symbolizes that purpose-built cell walls are needed for each application, such as bioencapsulated drugs and programmable biomaterials. Abbreviations for the most common glycosyl residues in plant cell walls: Glc, Glucose; Man, Mannose; Gal, Galactose; Ara, Arabinose; Xyl, Xylose; Fuc, Fucose; Rha, Rhamnose, GalA, Galacturonic Acid; GlcA, Glucuronic Acid.

knowledge of cell wall assembly, such as the roles of xyloglucans and mannans [4,6,7,13], and the supporting players for cellulose deposition [11].

All classes of plant cell wall polymers have been subjected to cell wall genetic engineering efforts in the last two decades, with lignin and cellulose (the dominant components of lignocellulosic biomass in trees and bioenergy grasses) representing the primary targets for gene overexpression, knockout or knock-down studies (historically via RNA interference, RNAi). While an overall reduction in lignin content compromises plant biomass yield [14], the incorporation for more cleavable linkages or high-value monomers in cell walls can lead to a lignin-first biorefinery that initially makes use of aromatic products [15]. In contrast to the relatively well-defined steps for lignin engineering, the biological roles and biotechnological potential of most plant glycosyltransferases that have been cloned [16] in Arabidopsis (Figure 2) remain unclear. There is also a

Figure 2



Carbohydrate-active enzymes and binding modules in *Arabidopsis thaliana*. All the Y-axes indicate the number of members, and the X-axes denote the protein family. Glycan symbols indicate carbohydrates associated with characterized cell wall-related members of a particular family. The data was extracted from the CAZy database (http://www.cazy.org/) and NC indicates non-classified members. Additional activities include Polysaccharide Lyases (PL), Carbohydrate Esterases (CE), and Auxiliary Activities (AA), such as lytic monooxygenases. Carbohydrate-binding modules (CBMs) are non-catalytic domains that confer binding specificities to carbohydrate-active enzymes. PMEs denote pectin methylesterases.

new appreciation of the complex coordination of many carbohydrate-active enzymes and their products that must occur at the subcellular level [10]. Recent advances in this area include biosynthetic insights on β-mannans [7,17,18], xylan-based nanocompartments [19], as well as the elongation of homogalacturonan and rhamnogalacturonan I pectic domains [20–22].

Another interesting discovery of the last two years is that many *A. thaliana* mutants with altered cell wall polysaccharide composition have enhanced immune responses to different types of pathogens [23]. Plant glycans could be applied or bioengineered to confer disease resistance while minimizing developmental defects. Apoplastic pectin can be modified by a variety of hydrolytic enzymes, including recently identified copper-dependent lytic polysaccharide monooxygenases in the oomycete *Phytophthora infestans* [24], a devastating pathogen of potato and tomato crops. Cell wall damage can also activate transcription factors that stimulate healing and regeneration [25]. Interestingly, grafting compatibility in several species is facilitated by putative β-1,4-glucanases [26], whose secretion has also been linked to host-parasite tissue adhesion [27]. Therefore, carbohydrate-active enzymes that bind and cleave different polysaccharides could be targeted to plant protection, to improve grafting efficiency, and/or to anchor microbes with desirable functions to plant tissues.

For the food industry, polysaccharides found in seed mucilage are in-demand as hydrocolloids with valuable rheological properties or as beneficial fibers for the treatment of lifestyle diseases such as type-II diabetes [28]. In Arabidopsis, the content of pectin and mucilage properties can now be precisely modified by expressing glycosyl hydrolases using seed coat-specific promoters [29]. Besides seeds, mucilaginous glycans are also found on the surface of other plant tissues such as maize aerial roots, where they are associated with the attraction or maintenance of nitrogen-fixing bacteria [30]. Therefore, tailoring the content and composition of secreted polysaccharides in specific tissues could help to engineering beneficial plant microbiomes for non-legume plants, while minimizing the unwanted attention of agricultural pests. For instance, galactosyl residues in flaxseed mucilage attract root-knot nematodes parasites that feed on host plants [31].

Engineering plant-based living materials

Engineered living materials can be designed using synthetic biology [32], and plant cell walls could be the renewable fibers or hydrogels for many of these applications, including 3D printing. In a recent study inspired by the microbial community used to ferment kombucha, the cellulose produced by *Komagataeibacter rhaeticus* bacteria was coated with programmable functions via co-culture with genetically engineered *Saccharomyces cerevisiae* yeast strains secreting cellulose-binding recombinant proteins [33]. Since carbohydrate-binding modules (CBMs) are available for many plant polysaccharides [34], protein-encoded functions such as biosensors could also be added to plant carbohydrate polymers. Alternatively, functionalized groups could be incorporated during polysaccharide elongation as recently shown for the *in vitro* synthesis of xylan microparticles [35]. Plant photosynthesis has already been exploited to strengthen and heal 3D-printed structures [36], so cells capable of secreting polysaccharides could be added to produce hybrid materials. While some steps for plant matrix polysaccharide biosynthesis remain to be elucidated, the sheer diversity of carbohydrates found in nature indicates that a wealth of materials can be biomanufactured and likely repurposed. Synthetic biology can even benefit the production of existing plant fibers (e.g. neofunctionalized cotton) in conventional farming, or could be applied to create dwarf plant varieties or efficient cell lines that produce high-value glycans in indoor environments such as vertical farming (which could reduce the high water and pesticide use that are typically required in the field).

Since plant polysaccharides are already widely consumed and evolved to protect sensitive biological cargo, they could provide a low-cost solution for protein drug encapsulation [37]. Bioencapsulated drugs are inexpensive to manufacture in edible plants, and wild-type plant cell walls already protect recombinant proteins against degradation [37]. Furthermore, plant seeds or freeze-dried leaves can be stable at ambient conditions for more than a year, bypassing the typical need for cold storage and distribution. An immunoglobulin A-like antibody produced in plant seeds (Arabidopsis and soybean) or secreted from the yeast *Pichia pastoris* protected piglets from infection by enterotoxigenic *Escherichia coli* when mixed with food [38]. Tailoring the composition of polysaccharides could fine-tune the release of natural products or recombinant proteins from biological capsules for a broader range of delivery systems or scenarios. For example, different mutations or combinations thereof can dramatically alter how mucilaginous polysaccharides are released from Arabidopsis seeds and their architecture [9]. Heterologous expression systems such as *Pichia* yeast cells can not only provide mechanistic insights into the function of polysaccharide synthases and their co-factors [17], but could also represent biological factories for plant-like materials and programmable capsules.

Biological factories to build programmable walls

A major bottleneck in tailoring cell wall polysaccharides has been the limited pace and capacity to genetically engineer plants (Box 1). For *in vitro* studies, the chemical synthesis of oligosaccharides and glycan arrays can facilitate high-throughput screens of carbohydrate-

Box 1. Prominent questions for programming polysaccharides in cells.

- What organisms or new tools will accelerate plant cell wall bioengineering?
- What are the first strategies that will succeed to enzymatically diversify plant glycans?
- Which glycan changes will modulate (a)biotic stress responses without yield penalties?
- How can plant-inspired cell wall polysaccharides be assembled from the bottom up?
- What infrastructure and standards will facilitate an inclusive community of wall builders?
- How will laboratory findings scale to agricultural and manufacturing environments?

active enzymes [39]. Nevertheless, many plant glycosyltransferases (particularly those with multiple transmembrane spans) are challenging to purify in active forms and have low yields. These current limitations render *in vitro* activities of polysaccharide biosynthetic enzymes more suitable for biochemical studies rather than for biotechnological applications. Randomly mutagenized plant populations have been instrumental to identify cell wall related genes, but this approach can be difficult to scale even with the advent of mapping mutations by next-generation sequencing. Instead, novel plant systems and/or transformation pipelines are needed to enable parallel engineering of targeted genome modifications. The bryophytes *Physcomitrium patens* and *Marchantia polymorpha* can be cultivated in multi-well plates and are great models for cell biology [40]. They could also facilitate more rapid design, build, test and learn (DBTL) cycles for cell wall polysaccharide engineering in the future. For instance, *Marchantia* has a haploid-dominant life cycle, reduced genetic redundancy, and benefits from a modular toolkit for genetic manipulations [41].

Microbial hosts are even more amenable to biotechnology and can serve as a screening platform before engineering the most promising targets in model plants or crops [42]. Recombinant proteins and many eukaryotic enzymes can be rapidly produced in yeast such as *Pichia* cells. For example, chimeric cellulose synthase-like enzymes for mannan and xyloglucan synthesis can be modularly assembled using Golden Gate cloning and evaluated in yeast cells [18]. Several yeast species are already part of human diets as integral components of foods (fermented and baked goods) and live probiotics. As a biotechnological example, *Pichia* cells are used in the production of plant-based meat alternatives by companies such as Impossible Foods [43]. The baker's yeast *Saccharomyces cerevisae*, with even more genetic resources, was recently shown to engineer multi-step enzymatic pathways where cellulose synthase-like enzymes were unexpectedly found to be responsible for the transfer of glucuronic acid onto triterpenoid saponins [44,45], which represent potent sweeteners. The use of surrogate hosts, which could also include additional microbial organisms [42], for cell wall bioengineering will also increase the research opportunities available for undergraduate students from a range of programs including microbiology, engineering, and biomedical tracks. By lowering the technical barriers and costs required for students to start experimenting with cell wall bioengineering, we could enhance the diversity and inclusion of trainees in plant biology.

Conclusions

In the last eight years, the number of sequences indexed in the carbohydrate-active enzymes database (CAZy; http://www.cazy.org/) database has dramatically increased (e.g. by nearly 9-fold for CBMs) [46]. The CAZy sequence space that remains to be comprehensively explored is staggering even for model species such as Arabidopsis (Figure 2) and well-studied crops as such as rice [19]. Considering that the acetylation or methyl esterification patterns of matrix polysaccharides require the coordinated activities of additional proteins beyond CAZy families, tailoring glycan structures will greatly benefit from advanced genetic tools (e.g. high efficiency, multiplex genome editing), increased accessibility to lab automation and artificial intelligence. To support this, a global alliance of biofoundries was recently established to coordinate the activities of public infrastructures for the genetic reprograming of organisms [47]. Avenues to start automating molecular biology will facilitate large-scale experiments and biomanufacturing [48]. The decreasing cost of gene synthesis can also be leveraged to explore the sequence-to-function space of new carbohydrate-active enzymes, as recently demonstrated in bacteria [49]. Open access to AlphaFold protein structure predictions [50] (https://alphafold.ebi.ac.uk/), along with experimental data for polysaccharide synthases (e.g. for cellulose synthases [51,52]), will also help to prioritize natural or engineered enzymes for bench work. As an alternative to rational design, continuous directed evolution in yeast has started to be applied for the improvement of enzymes for plant applications [53]. The utility of this powerful technique could be expanded beyond primary metabolism by identifying yeast strains or cultivation conditions that couple growth to the use or synthesis of plant polysaccharides. With the development of powerful tools to simultaneously edit or activate multiple genes [54], the bioengineering of cell walls will become faster even in seed plants. An upcoming

bottleneck will likely be the analysis of polysaccharides in intact cell walls, for which relatively few non-invasive plant methods have emerged in the last two decades [34]. Fluorescent probes are available for many but not all cell wall glycans, so creating advanced molecular labels can help to visualize complex cell walls and their biosynthetic machinery [55]. Alternatively, existing labels such as 2-aminobenzamide (2-AB) can be applied in new contexts (e.g. flow cytometry) to empower new experimental approaches in microbial [56] or plant cells. Finally, the assembly of native plant cell walls can now be quantified using solid-state nuclear magnetic resonance (ssNMR; see recent review [57]), providing high-resolution insights into the molecular interactions and mobility of specific polysaccharide domains [58]. Therefore, this is the prime moment to "go glyco" and explore uncharted territories in the world of plant polysaccharides and their derivatives (Box 1).

Credit author statement

Cătălin Voiniciuc: Conceptualization, Writing, Visualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data for Figure 2 is from http://www.cazy.org/.

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