



Mechanistic insights from plant heteromannan synthesis in yeast

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Heteromannan (HM) is one of the most ancient cell wall polymers in the plant kingdom, consisting of β -(1–4)-linked backbones of glucose (Glc) and mannose (Man) units. Despite the widespread distribution of HM polysaccharides, their biosynthesis remains mechanistically unclear. HM is elongated by glycosyltransferases (GTs) from the cellulose synthase-like A (CSLA) family. MANNAN-SYNTHESIS RELATED (MSR) putative GTs have also been implicated in (gluco)mannan synthesis, but their roles have been difficult to decipher *in planta* and *in vitro*. To further characterize the products of the HM synthases and accessory proteins, we chose a synthetic biology approach to synthesize plant HM in yeast. The expression of a CSLA protein in *Pichia pastoris* led to the abundant production of plant HM: up to 30% of glycans in the yeast cell wall. Based on sequential chemical and enzymatic extractions, followed by detailed structural analyses, the newly produced HM polymers were unbranched and could be larger than 270 kDa. Using CSLAs from different species, we programmed yeast cells to produce an HM backbone composed exclusively of Man or also incorporating Glc. We demonstrate that specific MSR cofactors were indispensable for mannan synthase activity of a coffee CSLA or modulated a functional CSLA enzyme to produce glucomannan instead of mannan. Therefore, this powerful platform yields functional insight into the molecular machinery required for HM biosynthesis in plants.

plant cell wall | polysaccharide | glycosyltransferase | mannan | yeast

Plants thrive in a wide range of aqueous and terrestrial environments, as their cells are shaped and reinforced by a carbohydrate-rich extracellular matrix. Plant cell wall polysaccharides make up the bulk of plant biomass, and thus represent the most abundant source of renewable material on the planet (1). One class of wall polysaccharides, the hemicelluloses, are a heterogeneous group of polysaccharides consisting of β -(1–4)-linked backbones of glucose (Glc), mannose (Man), or xylose units (2). In addition to strengthening the wall, hemicelluloses facilitate cell expansion, store carbohydrates, and supply signal molecules in certain tissues (2). Heteromannan (HM) is regarded as the most ancient type of hemicellulose, and is found throughout the plant kingdom, including in red algae (3), early land plants (mosses, lycophytes), gymnosperms, and angiosperms (4). Along with these important biological functions, HM is important for human nutrition, and may play a role in the treatment of lifestyle diseases such as type 2 diabetes (5). In addition, HM polysaccharides are widespread ingredients used to stabilize foods and cosmetics, coat pharmaceutical drugs for the controlled release of active agents, and even waterproof dynamite sticks for the mining industry (6). The global demand for HM has increased substantially in recent years because of its utility as a fluid thickener in hydraulic fracturing for oil and gas (7). Despite the widespread distribution and important applications of HM, the roles of genetic factors that determine the polysaccharide's fine structure remain to be characterized.

Changes in HM monosaccharide composition, length, or substitution modulate the polymer's physical properties and determine, for instance, whether a polysaccharide forms a crystalline aggregate or is water-soluble. Two distinct HM backbones are found in plants (2): linear mannan consists almost exclusively

(>90%) of Man subunits, whereas glucomannan also includes Glc residues. Mannan accumulates primarily in palm seeds, whereas glucomannan is more widespread and is, for example, highly enriched in konjac corms (8). The solubility of HM polysaccharides is enhanced by the substitution of Man units in (gluco)mannan with galactose side chains. For example, coffee seeds accumulate a galactosyl-substituted mannan (9), whereas galactoglucomannan is the most abundant hemicellulose in softwood (10).

Similar to other plant hemicelluloses, HM is synthesized by Golgi-localized glycosyltransferases (GTs), and the resulting polysaccharides are secreted to the plant cell wall via exocytosis. GTs from the Cellulose Synthase-Like A (CSLA) family have been demonstrated to use GDP-Man, and sometimes GDP-Glc, to elongate the HM backbone *in vitro* (11–14), but the polymers made by different CSLA isoforms have not been characterized in detail. In addition, MANNAN-SYNTHESIS RELATED (MSR) putative GTs are associated with (gluco)mannan synthesis (15, 16), but their roles have been difficult to decipher *in planta* and *in vitro*. *Arabidopsis thaliana* (*At*) MSRs have been hypothesized to synthesize a primer to initiate HM synthesis, glycosylate CSLA enzymes to enhance their activity, or promote CSLA stability or activity via glycosylation-independent interactions (15). Because these hypotheses have not been tested, the machinery that synthesizes different HM types remains unclear.

To further characterize the products of CSLA enzymes and the roles of accessory proteins such as MSR, we genetically engineered yeast to synthesize and secrete plant HM to their

Significance

Heteromannans are ancient plant cell wall polysaccharides, ubiquitous throughout the plant kingdom. In plants, these hemicellulosic polymers strengthen the cell wall and facilitate energy storage, and are involved in cell expansion and developmental signaling. Heteromannans are used extensively by humans, as healthy dietary fibers and as thickening agents in food, cosmetic, pharmaceutical, and mining industries. The fine structure of heteromannan is important for the polysaccharide's biophysical properties, and thus its function and application. However, the biosynthesis of different heteromannan structures remains mechanistically unclear. We demonstrate that plant mannan and glucomannan can be synthesized heterologously in the yeast *Pichia pastoris*, and the polysaccharide's structure can be modulated by specific plant cofactors, allowing for the synthesis of tailored mannan structures.

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walls. *Pichia pastoris* (hereafter *Pichia*) was selected as a promising host because it only contains trace amounts of 4-linked Man in its wall (17), and active CSLA proteins have been expressed in this host for in vitro assays (8, 18). Related Cellulose Synthase-Like C enzymes that elongate the backbone of xyloglucan, another type of hemicellulose, were also functional in *Pichia* (17). We now show that CSLA expression in *Pichia* led to the abundant production of plant HM. Depending on the CSLA isoform used, yeast cells synthesized linear HM polymers built primarily of Man, or also incorporating Glc. Using this platform, we demonstrate that MSR proteins are important cofactors for the elongation and the composition of plant mannan and glucomannan.

Results

Engineering *Pichia* to Produce Plant HM in Its Cell Wall. We engineered *Pichia* cells to produce plant HM to characterize the polysaccharides produced by CSLA enzymes and the roles of MSR proteins. As a proof of concept, we stably integrated the *Amorphophallus konjac* (*Ak*) *CSLA3* coding sequence in the *Pichia* genome under the control of a methanol-inducible promoter. This gene was selected because *Pichia* microsomes expressing recombinant AkCSLA3 have glucomannan synthase activity in radiometric assays (8). *Pichia* cells containing the *AkCSLA3* transgene were grown for biomass production before inducing recombinant protein expression. *Pichia* cell wall alcohol-insoluble residue (AIR) was isolated and subjected to glycosidic linkage analysis. The wall AIR of an empty vector control *Pichia* strain was rich in 2-Man and 3-Glc linkages, derived from native yeast α -mannoproteins and β -1,3-glucans, respectively, but only contained trace amounts of 4-Man (Fig. 1*A* and *SI Appendix, Fig. S1A*). The expression of AkCSLA3 led to the abundant production of 4-Man, characteristic of plant mannan, and also showed an increased 4-Glc content (Fig. 1*A* and *SI Appendix, Fig. S1A*). The abundance of 4-Man was elevated up to 30% of total carbohydrates in the AkCSLA3 cell wall AIR (Fig. 1*A*). To confirm the stability of HM production in yeast cells, an AkCSLA3 colony examined in Fig. 1*A* was reanalyzed alongside five additional AkCSLA3 colonies (*SI Appendix, Fig. S1B*), isolated from an independent transformation. Indeed,

4-Man was the dominant wall glycosidic linkage for all AkCSLA3 transformants (*SI Appendix, Fig. S1B*). In contrast to its effects on wall AIR, AkCSLA3 did not alter the composition of ethanol-precipitable glycans secreted in the culture media (*SI Appendix, Fig. S1C*).

To investigate whether the products of AkCSLA3 were deposited in the yeast cell wall, *Pichia* spheroplasts were immunolabeled with LM21 and LM22, two HM-specific monoclonal antibodies (19) that cannot cross the plasma membrane. The AkCSLA3 cells were labeled by LM21 or, to a lesser extent, LM22 antibodies (*SI Appendix, Fig. S2 A–C*). Although LM21 epitopes were only detected in AkCSLA3-expressing cells, native yeast β -glucans were stained in a similar fashion by the calcofluor dye in both the AkCSLA3 and empty vector *Pichia* strains (*SI Appendix, Fig. S2 D–G*). Confocal microscopy confirmed that the LM21 signals detected in the AkCSLA3-expressing cells colocalized with the yeast wall counterstained with calcofluor (Fig. 1*B*). The subcellular localization of the AkCSLA3 enzyme was investigated using a superfolder green fluorescent protein (sfGFP) N-terminal tag (20). Although the free sfGFP tag was distributed throughout the cytosol (*SI Appendix, Fig. S2H*), the sfGFP-AkCSLA3 fusion proteins were localized in small punctae (*SI Appendix, Fig. S2I*), interior to the plasma membrane stained by FM4-64 (Fig. 1*C*). The sfGFP-AkCSLA3 protein was functional, but AkCSLA3 yielded more HM sugars (*SI Appendix, Fig. S2J*), so only untagged proteins were expressed in further experiments. Therefore, as observed in plants, *Pichia* cells expressed CSLA recombinant proteins in intracellular compartments, but deposited their HM products in the extracellular matrix.

Product Specificity of CSLA Enzymes from Different Species. The products of AkCSLA3 and the *At* CSLA2 protein were compared to investigate whether different CSLA isoforms yield distinct HM structures. When expressed in *Pichia*, the two enzymes produced equally high amounts of 4-Man in the wall AIR, but only AkCSLA3 significantly increased the content of 4-Glc relative to the empty vector control (Fig. 2*A*). This suggests that the AkCSLA3 enzyme produces glucomannan, but AtCSLA2 synthesizes a relatively pure mannan in yeast. We enriched the novel HM polysaccharides by washing the cell wall AIR with hot 1 M sodium hydroxide (*SI Appendix, Fig. S3A*), which removes yeast α -mannoproteins (21). The resulting alkaline-insoluble (AKI) material was then treated with Zymolyase, which digests yeast β -1,3-glucans proteins (21), to yield an enriched HM (EM) fraction (*SI Appendix, Fig. S3A*). These sequential extractions removed most of the native yeast polymers, and thus enriched the CSLA product (*SI Appendix, Fig. S3B*). The EM polysaccharides were enzymatically characterized using purified β -mannanase and cellulase recombinant enzymes, which cleave β -1,4-Man and β -1,4-Glc bonds, respectively. Relative to the empty vector and undigested samples, the AkCSLA3 protein produced HM polymers that could be digested by β -mannanase and, to a lesser extent, cellulase treatment (Fig. 2*B*). In contrast, the AtCSLA2 product was only digested by β -mannanase (Fig. 2*B*), indicating a lack of β -1,4-Glc bonds. To further investigate their structure, EM polysaccharides were subjected to proton NMR ($^1\text{H-NMR}$) spectroscopy (Fig. 3*C*), alongside standards for plant HM polymers and yeast glucans (*SI Appendix, Table S2*). No carbohydrate peaks were observed in the $^1\text{H-NMR}$ spectrum of the empty vector control (Fig. 3*C*). The $^1\text{H-NMR}$ spectrum of the EM polymers made by AkCSLA3 contained the anomeric peaks characteristic of β -1,4-glucan and β -1,4-mannan (Fig. 3*C*), as well as other aliphatic signals also found in konjac glucomannan (*SI Appendix, Table S2*). In contrast, the AtCSLA2 product lacked the β -1,4-glucan anomeric peak (Fig. 3*C*), but had signals with the identical chemical shifts as pure mannan extracted from ivory nut (*SI Appendix, Table S2*). Therefore, *Pichia* cells expressing AkCSLA3 and AtCSLA2 produced glucomannan and mannan, respectively.

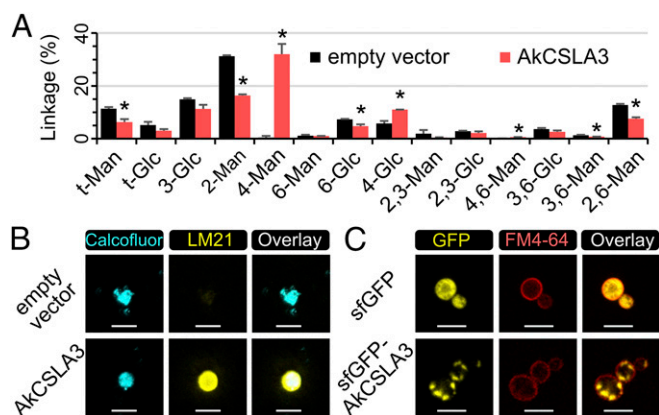


Fig. 1. Yeast cells expressing AkCSLA3 accumulate plant HM in their walls. (A) Glycosidic linkage analysis of *Pichia* wall AIR. Values represent molar percentage of total carbohydrates detected. Data show mean + SD of two independent transformants for each strain. Asterisks indicate significant changes (two-tailed *t* test, $P < 0.05$) between the strains. (B) Yeast wall polysaccharides with stained with calcofluor, a general β -glucan dye, and immunolabeled with the HM-binding LM21 antibody. (C) Subcellular localization of AkCSLA3 enzymes in *Pichia* cells. The plasma membrane was stained with the FM4-64 dye. The signal intensity of all channels for the fusion protein sfGFP-AkCSLA3 sample was increased postacquisition, relative to sfGFP control. (Scale bars, 5 μm .)

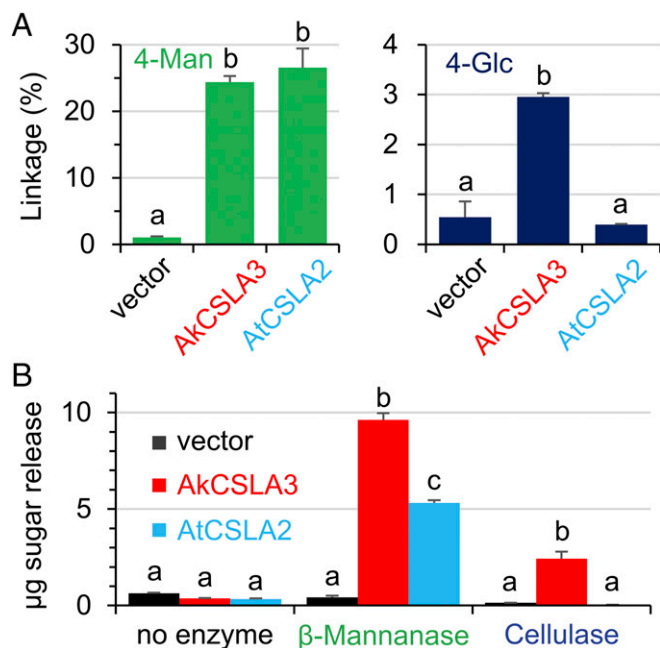


Fig. 2. Products after AkCSLA3 and AtCSLA2 expression. (A) Content of 4-Man and 4-Glc glycosidic linkages in the *Pichia* wall AIR. Values represent percentage of total carbohydrate area detected and show mean + SD of two biological replicates per *Pichia* strain. Vector, empty vector control. (B) Hexose sugars enzymatically released from enriched HM polysaccharides. Data show mean + SD of three technical replicates. For each linkage or digestion, different letters indicate significant changes based on one-way ANOVA with post hoc Tukey HSD Test ($P < 0.01$).

The AtMSR1 Protein Is an Optional Cofactor for Glucomannan Synthesis.

We designed an in vitro multimer assembly strategy to efficiently integrate two or more transgenes in the *Pichia* genome (SI Appendix, Fig. S4 A and B). We domesticated (removed) the *PmeI* site in the methanol-inducible promoter of the *pPICZ B* vector to create a novel *pPICZ X* vector that was used to clone additional transcriptional units, which can be subsequently fused (SI Appendix, Fig. S4B). The *Pichia* wall AIR composition was not significantly altered by the expression of *AtMSR1* alone (SI Appendix, Fig. S4 C and E), indicating that this gene is not sufficient for HM production. Interestingly, no *AtMSR1* homolog was identified in *Ak* transcriptome datasets (8, 22). We thus evaluated the function of *AtMSR1* in combination with the AkCSLA3 enzyme. Based on monosaccharide and glycosidic linkage analyses, coexpression of *AtMSR1* with AkCSLA3 increased the amount of glucomannan in the *Pichia* EM fraction by at least 70% relative to AkCSLA3 alone (Fig. 3 A and B). The elevated content of EM polysaccharides in the two-gene construct (Fig. 3A) did not simply result from changes in *CSLA* gene expression (SI Appendix, Fig. S4E), nor from changes in HM solubility, as the addition of *AtMSR1* also boosted the ability of AkCSLA3 to incorporate 4-Man and 4-Glc into cell wall AIR (SI Appendix, Fig. S4C). It is noteworthy that *AtMSR1* only boosted the content of glucomannan produced by konjac AkCSLA3 without significantly altering its Man:Glc ratio (Fig. 3 and SI Appendix, Fig. S4C).

AtMSR1 Enables AtCSLA2 to Produce Glucomannan Instead of Mannan.

Interestingly, the *AtMSR1* cofactor significantly altered the product of *AtCSLA2*, which is required for glucomannan elongation in *Arabidopsis* (23–25). Glycosidic linkage analysis of wall AIR as well as EM polymers indicated that *AtCSLA2* alone produced mannan, but the *AtCSLA2* + *AtMSR1* *Pichia* strain synthesized glucomannan (Fig. 3B and SI Appendix, Fig. S4C and

Table S1). To confirm this structural change, EM polymers were examined with $^1\text{H-NMR}$. The presence of *AtMSR1* enabled the *AtCSLA2* enzyme to produce both β -1,4-glucosyl- and β -1,4-mannosyl residues instead of pure mannan (Fig. 3C). On the basis of the $^1\text{H-NMR}$ anomeric signals, the EM polymers contained a 4-Glc to 4-Man ratio of 1:3.2 (Fig. 3C), consistent with the 1:3.5–4.5 ratio in the glycosidic linkage analysis (Fig. 3B). We performed size-exclusion chromatography to confirm that these glycosyl units are part of the same polymer (i.e., glucomannan), and to investigate its size. EM polymers produced by AkCSLA3 or *AtCSLA2*, with or without *AtMSR1*, could only be solubilized by overnight incubation in 10% (wt/vol) sodium hydroxide. Polysaccharides solubilized in this manner were heterodisperse, ranging in size from below 5 kDa to above 270 kDa (Fig. 4A). EM

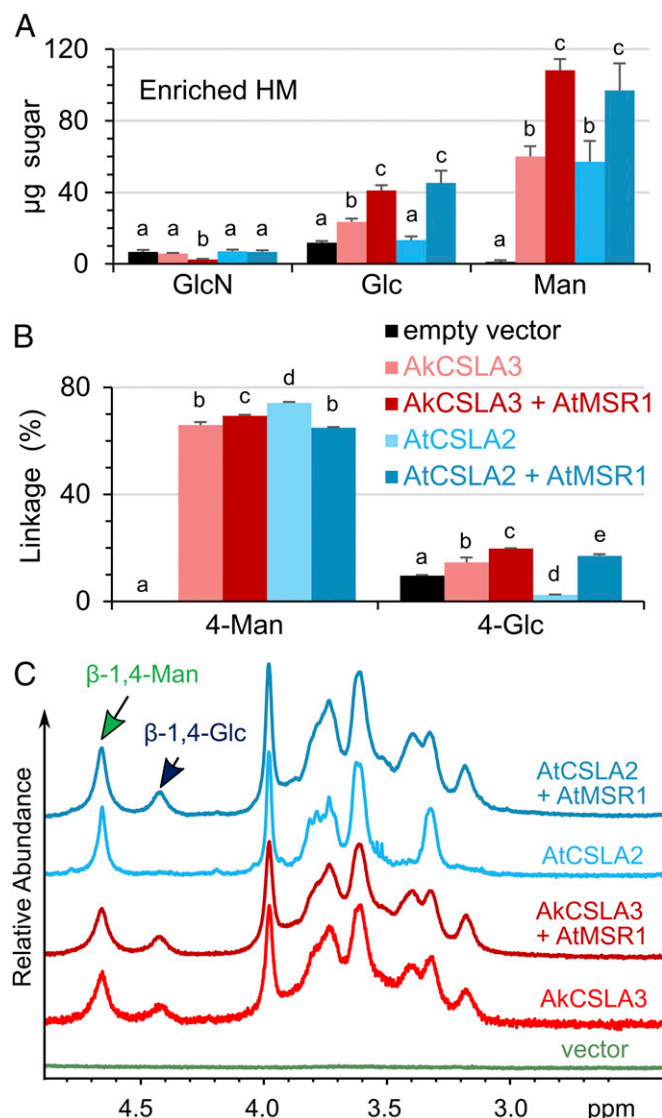


Fig. 3. *AtMSR1* is a cofactor for glucomannan synthesis. (A) Absolute composition of monosaccharides in EM polymers, enriched from *Pichia* wall AIR. (B) Relative content of 4-Man and 4-Glc linkages in the material analyzed in A. Values represent molar percentage of total carbohydrates detected. Data show mean + SD of at least three technical replicates. For each sugar or linkage, different letters denote significant changes based on one-way ANOVA with post hoc Tukey HSD Test ($P < 0.05$ for A; $P < 0.01$ for B). (C) $^1\text{H-NMR}$ analysis of EM polymers. Arrows indicate diagnostic anomeric peaks.

polysaccharides from three *Pichia* strains (AkCSLA3, AkCSLA3 + AtMSR1, and AtCSLA2 + AtMSR1) were cleaved into fragments smaller than 5 kDa by β -mannanase digestion and contained around 20% Glc (Fig. 4B). In contrast, AtCSLA2 alone produced EM polysaccharides that were proportionally smaller (Fig. 4A) and contained more than 95% Man (Fig. 4B). Only 34% of AtCSLA2 solubilized products were above 50 kDa compared with around 50% of carbohydrates for the three glucomannan-producing strains. In summary, AtMSR1 modulated glucomannan production by AtCSLA2, which increased the content (Fig. 3A) and relative size (Fig. 4A) of EM polymers isolated from *Pichia* walls.

Influence of MSR1 Proteins on Other Mannan Synthases. We also investigated how AtMSR1 modulates the activity of other mannan synthases such as AtCSLA7, which belongs to a phylogenetic clade distinct from AtCSLA2 (12) and only elongates mannan in vitro (13). Indeed, *Pichia* expressing AtCSLA7 incorporated significantly more 4-Man, but not 4-Glc, linkages into AKI polysaccharides (SI Appendix, Fig. S4D). In contrast to the AtCSLA2 + AtMSR1 combination (Fig. 3), the coexpression of the *AtMSR1* gene with *AtCSLA7* (SI Appendix, Fig. S4F) decreased 4-Man production in *Pichia* relative to the single gene controls, without altering the content of 4-Glc in the AKI fraction (SI Appendix, Fig. S4D).

The *Coffea canephora* (Cc) MANS1 mannan synthase represents a prime candidate for the production of mannan in coffee seeds (26), but was inactive when expressed in *Pichia* microsomal membrane fractions and assayed in vitro (27). Unlike AtCSLA2 and AkCSLA3, expression of CcMANS1 alone did not produce any significant amounts of β -1,4-mannan in *Pichia* walls (Fig. 5).

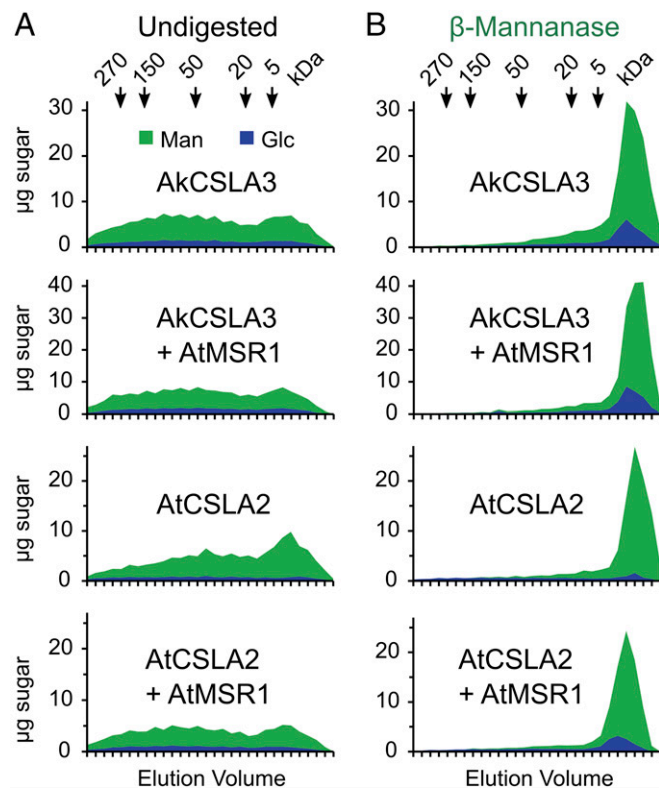


Fig. 4. Size-exclusion chromatography of enriched HM. (A) EM polysaccharides were solubilized in 10% NaOH overnight, neutralized, and analyzed by size-exclusion chromatography. Arrows indicate the elution of Dextran standards of known sizes. Data show the Glc and Man content (stacked). (B) An equal amount of the EM material used in A was digested with β -mannanase before SEC analysis.

Also, coexpression of the *Arabidopsis* AtMSR1 with the coffee CcMANS1 did not produce HM polysaccharides that could be detected via β -mannanase digestion (Fig. 5A) or glycosidic linkage analysis of the AKI fraction (Fig. 5B). However, coexpression of CcMANS1 with CcMSR1, the cofactor found natively in coffee seeds (26), produced β -1,4-mannan in *Pichia* cells (Fig. 5A) without increasing the 4-Glc content of AKI polymers (Fig. 5B). Therefore, AtMSR1 enhanced glucomannan synthesis by AtCSLA2 and AkCSLA3, but did not enhance the CcMANS1 and AtCSLA7 mannan synthases. Unlike an auxiliary role of AtMSR1 in glucomannan production by AtCSLA2, we demonstrated that the CcMSR1 protein is an essential cofactor for the synthesis of mannan by the coffee CcMANS1 enzyme.

Functional Characterization of AtMSR1 Conserved Motifs. The AtMSR1 protein has an N-terminal transmembrane (TM) domain and a large PF10250 motif (SI Appendix, Fig. S5A), which defines a large family of putative GTs in land plants, and has structural similarity to the catalytic domain of protein *O*-fucosyltransferases (PoFUT1) from the animal kingdom (15). Multiple protein alignment pointed to three residues that are conserved in the PF10250 domain of the human (*Homo sapiens*) HsPoFUT1 and MSR-related proteins from *Arabidopsis* (SI Appendix, Fig. S5B). For example, the AtMSR1 R265 and D355 amino acids correspond to key residues of the HsPoFUT1 sugar-binding motif (SI Appendix, Fig. S5B). There, the R240 residue is indispensable for HsPoFUT1 catalytic activity, and D340 is within hydrogen-bonding distance of the guanine ring on GDP-fucose (28). Moreover, disulfide bridges contribute to the formation of the PoFUT1 sugar-binding site (29), with the C274 amino acid of AtMSR1 putatively involved in this process (SI Appendix, Fig. S5 A and B). To functionally characterize these motifs, we evaluated how AtMSR1 variants without the TM domain ($\Delta 28$) or with missense mutations in three conserved amino acids (R265A, C274A, and D355A) influence HM production by AtCSLA2. Relative to the coexpression of AtCSLA2 with wild-type AtMSR1, the AtMSR1 $\Delta 28$ and AtMSR1^{C274A} mutant variants decreased glucomannan production (SI Appendix, Fig. S5C). The coexpression of AtCSLA2 with mutated AtMSR1 proteins yielded 4-Glc levels similar to the sfGFP control and significantly reduced the 4-Man content relative to the AtCSLA2 + AtMSR1 strain (SI Appendix, Fig. S5D). Two mutations in AtMSR1 sugar-binding residues impaired glucomannan synthesis by AtCSLA2 to different degrees (SI Appendix, Fig. S5 C and D), and were thus characterized in more detail. Coexpression of AtCSLA2 + AtMSR1^{R265A} not only failed to produce glucomannan but also reduced 4-Man production by more than 50% compared with the AtCSLA2 enzyme alone (Fig. 5). Interestingly, the AtCSLA2 + AtMSR1^{D355A} strain produced HM containing both 4-Man and 4-Glc, albeit at significantly lower levels than the wild-type multimeric construct (Fig. 5). Although less severe than the AtMSR1^{R265A} variant, the addition of AtMSR1^{D355A} still reduced 4-Man accumulation compared with AtCSLA2 expressed alone (Fig. 5B). In summary, the AtMSR1 TM domain and sugar-binding motif were important modulators of glucomannan synthesis, and mutations in these regions reduced β -1,4-mannan production by AtCSLA2.

Discussion

Elucidating the mechanisms involved in the synthesis of cell wall polysaccharides remains a challenge in plant biology. Although the genomics era ushered in the identification of multiple genes involved in hemicellulose biosynthesis, many of them remain to be functionally characterized (30). We now demonstrate that yeast synthetic biology tools can be exploited to gain mechanistic insight into HM biosynthesis, which has been difficult to study *in planta* and *in vitro*. For example, as *Arabidopsis csla7* mutants are embryolethal (25), it has previously not been possible to investigate

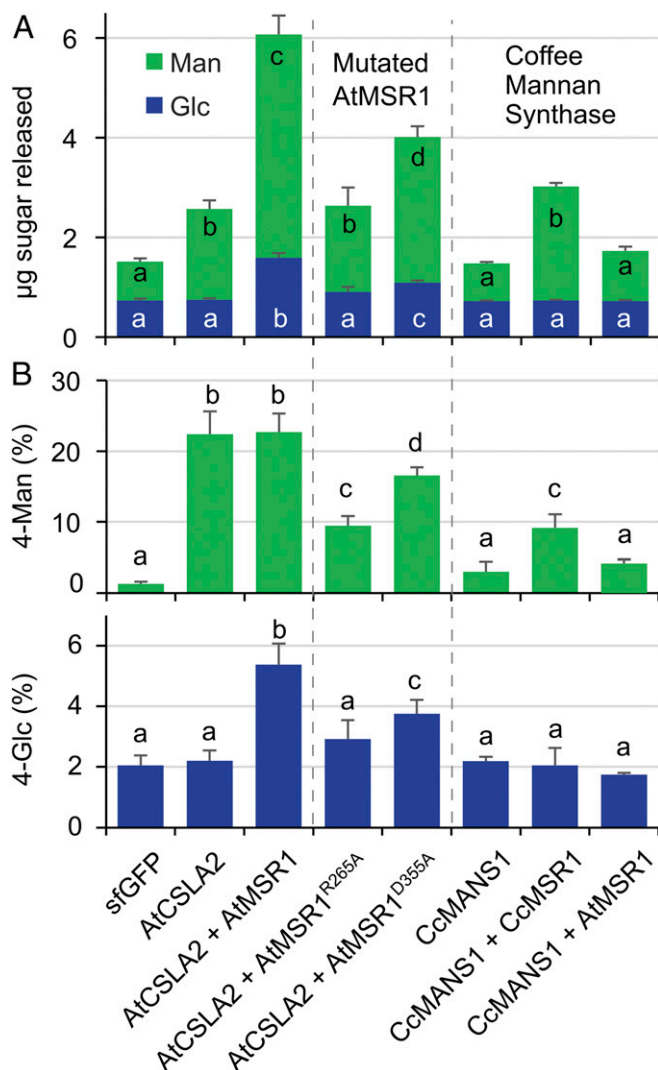


Fig. 5. Importance of MSR1 sugar-binding motif and protein specificity. (A) HM sugars released from AKI material digested with β -mannanase. Two point mutations (R265A) and (D355A) were introduced into AtMSR1. CcMANS1, coffee mannan synthase; CcMSR1, coffee MSR1. (B) Abundance of 4-Man and 4-Glc in AKI polymers. In all panels, data show mean + SD of three biological replicates. For each glycosyl residue or linkage, different letters denote significant changes based on one-way ANOVA with post hoc Tukey HSD Test ($P < 0.05$).

whether AtCSLA7 functions as a mannan synthase in vivo. In addition to CSLA enzymes, other putative GTs such as MSR proteins were implicated in HM elongation based on plant mutant chemotypes and in vitro assays using plant extracts (15). However, even in heterologous plant hosts, GT activity assays are subject to pleiotropic effects because of the presence of endogenous carbohydrate-related substrates and enzymes. For instance, expression of *Arabidopsis* Cellulose Synthase-Like D 5 (AtCSLD5) or coexpression of AtCSLD2 + AtCSLD3 enhanced mannan synthase activity in tobacco leaves (31, 32). However, AtCSLD3 and AtCSLD5 have also been implicated in cellulose (33) and callose synthesis (34), respectively. GTs required for cell wall biosynthesis have at least one TM domain span, are typically expressed in very low abundance, and are difficult to purify in active form (35). Although in vitro activity assays were previously performed on *Pichia* microsomes containing CSLA proteins (8, 16, 18), these experiments only revealed that the enzymes incorporated exogenous GDP-Man and, to a lesser

extent, GDP-Glc into alcohol-insoluble compounds. Because the in vitro assays had limited yields, they relied on [14 C]-labeled nucleotide sugar donors. In contrast, we discovered that using only methanol as a carbon source, CSLA-expressing *Pichia* cells produce large amounts of plant HM (Figs. 1A and 2 and *SI Appendix*, Fig. S2D), which can be structurally characterized in detail using a variety of analytical tools. Because *Pichia* has a fast growth cycle and efficient homologous recombination, it also enables high-throughput studies of genetic permutations of these proteins that are not feasible *in planta* or in vitro.

In this study, we comprehensively analyzed the structures of HM polysaccharides produced by CSLA isoforms from different species. AkCSLA3 expression alone is sufficient for glucomannan production in *Pichia* cells (Fig. 1A), which can be enriched up to more than 70% purity in only two steps (Fig. 3C and *SI Appendix*, Fig. S3). The enriched HM from the AkCSLA3 yeast strain resembled the structure of konjac glucomannan, its native product (*SI Appendix*, Table S2). Consistent with hemi-cellulose synthesis in plants (2), AkCSLA3 enzymes were localized in small intracellular punctae in *Pichia*, and their HM products were deposited in the yeast cell wall (Fig. 1B and C and *SI Appendix*, Fig. S1 I and J). Indeed, another (gluco)mannan synthase (AtCSLA9) was previously shown to be Golgi-localized in *Pichia* and had an active site facing the Golgi lumen (18). Thus, *Pichia* likely has endogenous proteins capable of producing and supplying activated GDP-Man and GDP-Glc sugar donors to the CSLA active site. In contrast to AkCSLA3, the expression of two *Arabidopsis* CSLAs yielded relatively pure mannan in *Pichia*. Recombinant AtCSLA2 and AtCSLA7 proteins produced in insect cells were known to have (gluco)mannan and pure mannan synthase activities in vitro, respectively (13). Pointing to diverged roles, AtCSLA2 and AtCSLA9 form a phylogenetic clade distinct from that of the remaining seven CSLA isoforms in *Arabidopsis*, which includes AtCSLA7 (12). Consistent with its lower activity in vitro, AtCSLA7 (*SI Appendix*, Fig. S4D) showed reduced mannan production compared with AtCSLA2 in yeast cells (Figs. 2, 3, and 5). However, in contrast to the in vitro study, the AtCSLA2 protein alone did not incorporate significant amounts of 4-Glc in *Pichia*. These observations could be a result of distinct posttranslational modifications of the AtCSLA2 protein and/or the presence of other accessory proteins in *Drosophila* cells that are not present in yeast.

Heterologous expression in yeast enabled us to investigate the roles of MSR accessory proteins in HM biosynthesis, which were previously unclear (15). Although MSR1 proteins alone did not alter *Pichia* wall composition (*SI Appendix*, Figs. S4C and S5C), they modulated HM synthesis by CSLA enzymes. Potentially, the yeast wall was unchanged after AtMSR1 expression (*SI Appendix*, Fig. S4 C and E) because the encoded protein was inactive or unstable in the absence of a CSLA interaction partner. The coffee CcMSR1 was an indispensable cofactor for mannan production by CcMANS1 (Fig. 5). In contrast, AtMSR1 did not enhance the AtCSLA7 (*SI Appendix*, Fig. S4D) or the CcMANS1 (Fig. 5) mannan synthases, but was an optional enhancer of glucomannan synthases. The expression of AtMSR1 increased the content of glucomannan produced by AkCSLA3 in *Pichia* (Fig. 3A and *SI Appendix*, Fig. S4C), without altering its Glc:Man ratio. Furthermore, AtMSR1 enabled AtCSLA2 to produce glucomannan, its expected product in *Arabidopsis* (23–25), instead of mannan (Figs. 3 and 4). Because a single CSLA was sufficient for either glucomannan or mannan synthesis in yeast, MSR proteins are not required to initiate HM elongation. Indeed, *Arabidopsis msr1 msr2* double-mutant plants still contained mannan (15). Therefore, specific MSR cofactors are likely involved in the production of distinct types of HM by modulating CSLA activity. Plant MSR cofactors have sequence homology to mammalian PoFUT1 enzymes, which decorate proteins with O-glycan groups that are essential for cell development (28).

Mutagenesis of three AtMSR1 conserved amino acids, which are involved in nucleotide sugar binding by PoFUT1 enzymes (28, 29), significantly reduced 4-Glc as well as 4-Man incorporation by AtCSLA2 into AKI polymers (Fig. 5 and *SI Appendix, Fig. S5*). Furthermore, the reduced accumulation of HM in the AtCSLA2 + AtMSR1^{Δ28} strain (*SI Appendix, Fig. S5*) highlighted the importance of the AtMSR1 membrane-anchoring domain. Although AtMSR1 was an optional modulator of HM synthesis in *Pichia*, the mutated forms tested were detrimental even for the basal activity of the AtCSLA2 enzyme. Because the PoFUT1 sugar-binding motif was structurally conserved and important for the function of AtMSR1 in yeast, we hypothesize that plant MSR proteins may affect CSLA activity via protein glycosylation. Future studies should test this hypothesis by investigating whether CSLA proteins are glycosylated and/or whether they physically interact with the MSR cofactors.

In summary, we established *Pichia* as a convenient biofactory for plant HM production and gained mechanistic insights into mannan and glucomannan production and modulation. This study advances our knowledge of hemicellulose synthesis and also points to other poorly understood aspects that could be resolved using the powerful yeast platform. Furthermore, our approach can be extended to the study of other plant GTs, provided that the appropriate repertoire of substrates is present in yeast, and to investigate how

sugar units can be assembled into designer polymers with tailored properties for specific applications.

Methods

Plant genes were cloned and expressed in yeast cells, as recommended by the EasySelect *Pichia* Expression Kit (Invitrogen). After accumulating biomass using glycerol as a carbon source in culture tubes or in multiwell plates (Fig. 5 and *SI Appendix, Figs. S2, S4, and S5*), *Pichia* cells were induced to express recombinant proteins for 24 h, using methanol. For each genotype, at least three independent *Pichia* transformants showed a similar cell wall composition. Polysaccharides isolated from yeast were characterized via glycosidic linkage (36, 37) and monosaccharide analyses (23, 38), as previously described. After enzymatic digestion, solubilized carbohydrates were subjected to monosaccharide analysis, or the anthrone assay (39). *Pichia* cell walls were labeled with specific antibodies according to a protocol previously used for plant seeds (40, 41). Additional details on the protocols used to clone genes, transform and grow *Pichia*, quantify transgene expression, and extract and structurally characterize polysaccharides are presented in the *SI Appendix, Materials and Methods*.

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