Engineering temporal accumulation of a low recalcitrance polysaccharide leads to increased C6 sugar content in plant cell walls

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Summary
Reduced cell wall recalcitrance and increased C6 monosaccharide content are desirable traits for future biofuel crops, as long as these biomass modifications do not significantly alter normal growth and development. Mixed-linkage glucan (MLG), a cell wall polysaccharide only present in grasses and related species among flowering plants, is comprised of glucose monomers linked by both β-1,3 and β-1,4 bonds. Previous data have shown that constitutive production of MLG in barley (Hordeum vulgare) severely compromises growth and development. Here, we used spatio-temporal strategies to engineer Arabidopsis thaliana plants to accumulate significant amounts of MLG in the cell wall by expressing the rice CslF6 MLG synthase using secondary cell wall and senescence-associated promoters. Results using secondary wall promoters were suboptimal. When the rice MLG synthase was expressed under the control of a senescence-associated promoter, we obtained up to four times more glucose in the matrix cell wall fraction and up to a 42% increase in saccharification compared to control lines. Importantly, these plants grew and developed normally. The induction of MLG deposition at senescence correlated with an increase of gluconic acid in cell wall extracts of transgenic plants in contrast to the other approaches presented in this study. MLG produced in Arabidopsis has an altered structure compared to the grass glucan, which likely affects its solubility, while its molecular size is unaffected. The induction of cell wall polysaccharide biosynthesis in senescing tissues offers a novel engineering alternative to enhance cell wall properties of lignocellulosic biofuel crops.

Introduction
Renewable transportation fuels derived from plant biomass (biofuels) have the potential to alleviate the world’s dependence on fossil fuel consumption and thus help curb the effects of climate change. In particular, the complex lignocellulosic polysaccharides present in plant cell walls have been targeted in recent years as an abundant source of sugars for microbial fermentation into fuels (Pauly and Keegstra, 2010; Vega-Sánchez and Ronald, 2010). However, overcoming the resistance of plant cell walls to deconstruction, commonly known as recalcitrance, presents a major challenge to the cost-effective and sustainable production of lignocellulosic biofuels (Himmel et al., 2007; Vega-Sanchez and Ronald, 2010).

A typical plant cell wall is composed of cellulose and matrix polysaccharides (i.e. hemicelluloses and pectin), as well as lignin, an aromatic polymer. This heterogeneous composition, as well as the arrangement of these polymers in the wall, is largely responsible for biomass recalcitrance (Himmel et al., 2007). More specifically, the arrangement of cell wall polysaccharides and lignin into hydrophobic cores prevents access of enzymes needed to break down biomass (Himmel et al., 2007). Lignin alone is a major determinant of recalcitrance, as plants genetically engineered to suppress its biosynthesis show dramatic increases in biomass saccharification, albeit generally with some growth and yield penalties, depending on the approach (Chen and Dixon, 2007; Eudes et al., 2014; Yang et al., 2013). In addition, pectins and major hemicellulose polymers (xylan, xyloglucan and arabinoxylan) consist of a mixture of pentose (C5) and hexose (C6) sugars (Scheller and Ulvskov, 2010). Biofuel fermenting microorganisms readily metabolize C6 sugars, such as glucose, as opposed to the slow or lack of fermentation of C5 monosaccharides (Young et al., 2010). Thus, reduced recalcitrance and higher C6 fermentable sugar content are desired properties for lignocellulosic biomass conversion into fuels.

With the advent of plant synthetic biology tools and tissue-specific transgene regulation, more sophisticated approaches of plant cell wall engineering are now available that allow researchers to tailor biomass composition for biofuel and other uses (Doblin et al., 2014; Eudes et al., 2014). The challenge lies in developing plants with optimized cell wall properties without impacting normal plant growth and development. Due to its predominant role in affecting recalcitrance, most of the cell wall...
engineering strategies have targeted lignin content and/or composition with various degrees of success (Eudes et al., 2014). Recently, the transcriptional machinery involved in secondary cell wall biosynthesis controlled by the NAC transcription factor NST1 in Arabidopsis thaliana was targeted to modify cell wall properties. Two approaches used fibre-specific regulation of the NST1 pathway resulting in saccharification yield improvements, one to enhance cell wall deposition by creating an artificial positive feedback loop (Yang et al., 2013), while the other used a chimeric repressor strategy (NST1-SRDX) to suppress secondary cell wall thickening (Hosse et al., 2009). The manipulation of the biosynthesis of specific cell wall polysaccharides to affect biomass properties in vegetative tissues has been approached in a less systematic way. For example, increased glucuronoxylan content in Arabidopsis stems was achieved by overexpression of C5IA genes (Goubet et al., 2009). Similarly, overexpression of the galactan synthase GALSL1, involved in pectin biosynthesis in Arabidopsis, resulted in a 50% increase in galactan content (Liwanag et al., 2012). Although the effect of the galactan and mannann modifications on biomass saccharification was not determined nor quantified in these plants, these results indicate that it is possible to target polysaccharide synthases to engineer the accumulation of certain cell wall components.

Grasses and related species in the order Poales uniquely accumulate large amounts of the hemicellulosic polysaccharide mixed-linkage glucan (MLG) in their cell walls. MLG, composed of both β1-3- and β1-4-linked glucose monomers, is an unbranched, amorphous polymer (Fincher, 2009). Unlike cellulose, which is made of only β1-4 linkages, the β1-3 bonds in MLG prevent the aggregation of the polysaccharide into hydrophobic, tightly wound microfibrils, which in turn renders MLG relatively soluble and viscous in aqueous environments (Fincher, 2009). This irregular conformation or low recalcitrance and the fact that it is composed entirely of glucose molecules (C6 sugars) make MLG a very attractive target for cell wall engineering. MLG is synthesized by members of the grass-specific Cellulose synthase-like F and H subfamilies of glycosyltransferase family 2 (Burton et al., 2006; Doblin et al., 2009). Previous results overexpressing either rice (Oryza sativa) CslF2 and CslF4 or barley (Hordeum vulgare) CslH1 in Arabidopsis resulted in very low levels of MLG (<0.1% w/w) (Burton et al., 2006; Doblin et al., 2009). More recently, others and we have shown that a single member of the family, CslF6, is required for MLG biosynthesis in vegetative tissues of rice and barley, as cslf6 knockout mutants are virtually devoid of this polysaccharide (Taketa et al., 2012; Vega-Sánchez et al., 2012, 2013). The overexpression of the CslF6 synthase with the constitutive 3SS promoter resulted in significant accumulation of MLG in vegetative tissues in barley. However, these plants displayed severe growth and developmental defects, as well as seedling death, which were attributed to what the authors termed ‘vascular suffocation’ (large amounts of MLG clogging water-conductive tissues) (Burton et al., 2011). To avoid the undesirable effects associated with constitutive accumulation of MLG observed in barley, we developed a strategy based on the spatio-temporal regulation of expression of the CslF6 synthase in Arabidopsis, a reference dicot plant that does not naturally accumulate MLG. The use of Arabidopsis as an experimental model for engineering of this trait allowed us to test the effects of fine-tuning MLG deposition with several tissue and developmentally regulated promoters in a background-free host. We show that Arabidopsis plants expressing the rice CslF6 gene under the control of a senescence-associated promoter accumulate large quantities of MLG, leading to four times more glucose in the noncellulosic polysaccharide fraction of the cell wall. This strategy resulted in a 42% increase in saccharification efficiency, without compromises in growth and development. The success of this approach opens a new path to engineer dedicated biofuel crops with increased amounts of other noncellulosic, C6-rich polysaccharides in plant cell walls such as galactans and mannans.

Results

MLG accumulation in heterologous hosts using the p3SS constitutive promoter correlates with poor growth and developmental defects

Barley plants overexpressing CslF6 with the constitutive cauliflower mosaic virus 3SS promoter (p3SS) show severe growth and developmental defects, likely due to ‘vascular suffocation’ (Burton et al., 2011). To confirm whether constitutive MLG accumulation in hosts outside the order Poales, which contains barley and other grasses, also leads to poor growth, we overexpressed the rice CslF6 gene in both Nicotiana benthamiana and Arabidopsis using transient and stable transformation methods, respectively. Previous studies had shown that N. benthamiana leaves accumulate significant amounts of MLG when transiently expressing either rice or barley CslF6 via Agrobacterium-mediated infiltration (Taketa et al., 2012; Vega-Sánchez et al., 2013). We found that N. benthamiana leaves displayed severe necrosis 5 days postinfiltration with Agrobacterium containing a p3SS:CslF6 construct (Figure 1a). When we generated stable p3SS:CslF6 Arabidopsis transformants, we observed that plants were only compromised in growth when accumulating a certain threshold of MLG content (above 1.5% w/w), indicating a dosage effect negatively correlating MLG levels with plant growth (Figure 1b, c). As shown in Figure 1b, lines with high amounts of MLG (>1.5% w/w) are small and have chlorotic leaves. Taken together, these results demonstrate that engineering high MLG content in both heterologous and native hosts using strong, constitutive promoters negatively impacts growth and development.

Spatio-temporal expression of CslF6 using secondary cell wall and senescence-associated promoters

Based on our results using the p3SS promoter, we reasoned that limiting the accumulation of MLG in cells that are no longer expanding, such as fibres, or during senescence could lead to more effective engineering of this trait. We first used constructs expressing CslF6 mediated by secondary cell wall promoters from the fibre-specific transcription factors NST1 (for NAC SECONDARY WALL THICKENING FACTOR 1) and NST2 (Mitsuda et al., 2005). In addition, to test the ‘vascular suffocation’ hypothesis from Burton et al. (2011), we used the secondary cell wall promoter from the Cellulose synthase 4 (CesA4, also known as IRX5 for IRREGULAR XYLEM 5) gene that is active in xylem and fibres tissues (Eudes et al., 2012; Taylor et al., 2003). As shown in Figure 2, lines expressing CslF6 with secondary cell wall promoters yielded either plants with low MLG content (pIRX5::CslF6) or plants with high MLG levels but with poor growth, reminiscent of the p3SS approach described above (pNST1::CslF6 and pNST2::CslF6). However, when MLG content was engineered by expressing CslF6 with the senescence-associated promoter from the cysteine protease gene SAG12 (Lohman et al., 1994; Noh and
Amasino, 1999), we obtained lines with high MLG polysaccharide content that grew and developed normally (Figures 3 and S1). Analysis of Arabidopsis pSAG12::CslF6 lines showed that accumulation of MLG was higher in senesced rosettes (>1.5% w/w; Figure 3c) than in senesced stems (<0.8% w/w; Figure S1b). As shown in Figure 3b, MLG was deposited in the cell walls of all tissues in senescing leaves of pSAG12 lines, including mesophyll and vascular bundles.

Temporal regulation of CslF6 expression prevents negative effects associated with elevated MLG accumulation in the cell wall

The poor growth of lines expressing CslF6 with the pNST1 and pNST2 promoters, but not pSAG12 or pIRX5 prompted us to analyse in more detail the differences associated with MLG deposition in these lines. The results with the pIRX5 promoter supported the previous hypothesis that accumulation of large amounts MLG in vascular tissues is detrimental to plant growth: we could only obtain 11 transformed lines of pIRX5::CslF6 compared to the other approaches (Table S1) and none of these lines were recovered with a significant amount of MLG (Figure 2 and Table S2), implying that pIRX5::CslF6 lines with MLG content above the 1.5% w/w threshold were not viable. In general, for all the strategies we tested, most of the hygromycin-resistant lines we recovered either did not present detectable MLG or accumulated low amounts of the polysaccharide (0.5% w/w or less; Tables S1 and S2). For this reason, we only characterized further the lines containing at least 1.5% w/w MLG.

Immunolabelling of tissue sections using an anti-MLG monoclonal antibody revealed that MLG accumulated in cell walls of vascular bundles and mesophyll of young, preflowering rosette leaves (Figure S2b), and in phloem, xylem and cortical parenchyma of mature stems (Figure S2d). Similar MLG deposition patterns were observed for pNST2 and pNST1 lines in young leaf and mature stem sections, with the added presence of detectable labelling in stem pith parenchyma, especially in pNST2::CslF6 lines (Figure S2e–l). In contrast, pSAG12::CslF6 lines did not accumulate MLG in young, preflowering rosette leaves, except for a few xylem cells in vascular bundles (Figure 4b). However, in senescing leaves postflowering stage, MLG was abundant in cell walls of spongy mesophyll, leaf vein parenchyma, xylem, phloem, epidermis, stomata and trichomes (Figure 4d–g). MLG was also detected in small amounts in stem sections of pSAG12::CslF6 lines, particularly in cortical and pith parenchymas, and less so in xylem (Figure 4i). To complement the immunolabelling studies, we performed gene expression analyses using publicly available data compiled from the Arabidopsis eFP browser at the University of Toronto (www.bar.utoronto.ca; Winter et al., 2007). We looked at the expression of the endogenous SAG12, IRX5 and NST2 genes throughout Arabidopsis development in vegetative tissues as a proxy for their respective promoter activities. Expression data for NST1 were not available on this developmental set and therefore were not included in the analysis. As shown in Figures S3–S5, there is almost no overlap in the expression pattern between SAG12 and both IRX5 and NST2 genes. The IRX5 and NST2 genes are expressed throughout development and various organs that contain secondary cell walls, albeit with a much stronger expression in first and second stem internodes (Figures S4 and S5). In contrast, SAG12 is almost exclusively expressed in senescing leaves, as expected, with a much lower expression in stem internodes (Figures S3). These results were confirmed by performing pairwise fold change expression comparisons between SAG12 and either IRX5 or NST2: both IRX5 and NST2 are clearly up-regulated compared to SAG12 in most Arabidopsis organs and developmental stages, with the notable exception of senescing leaves (Table S3). Taken together, the immunolabelling and gene expression analyses suggest that the temporal variations in promoter activity account for the differences observed between the pSAG12 and the secondary cell wall approaches for MLG engineering. Based on these results, a detailed characterization of the pSAG12::CslF6 lines was carried out to understand the effects of MLG accumulation on both biomass- and biofuel-related traits.

pSAG12::CslF6 lines accumulate four times more glucose in the cell wall matrix polysaccharide fraction compared to control lines

MLG is present in the noncellulosic, matrix polysaccharide fraction of grass plant cell walls (Carpita, 1996). We performed a cell wall...
monosaccharide analysis of the pSAG12 lines with alcohol insoluble residues (AIR) from senesced rosettes using high-performance anion exchange chromatography (HPAEC). As can be seen in Table 1, the amount of glucose (C6) derived from cell wall matrix polysaccharides of the transgenic lines was substantially increased, up to four times more than the control plants (Col-0), which resulted in a total glucose enrichment of at least 28% and up to 45% in the highest MLG producing line, pSAG12::CslF6-5 (Figure S6). The increase in glucose in the matrix cell wall fraction is consistent with the MLG content quantified for each line and shown in Figure 3c. As MLG biosynthesis uses the same substrate (UDP-Glc) as cellulose synthases, we checked whether cellulose content was affected in the pSAG12 lines. No significant differences in cellulose levels were seen in senesced rosette samples of transgenic lines compared to that of the Col-0 control (Figure S1c). Thus, MLG biosynthesis in Arabidopsis, mediated by the controlled expression of CslF6 in senescing tissues by the pSAG12 promoter, results in a significant increase in glucose content in the matrix polysaccharide fraction without competing with cellulose accumulation.

Figure 2. Phenotypes of Arabidopsis transgenic lines accumulating MLG mediated by secondary cell wall promoters. (a) Rosette size of lines expressing OsCsf6 with different secondary cell wall promoters; numbers below indicate the amount of MLG present in each line in percent of dry weight. Scale bar: 20 mm. (b) Whole plant size of lines expressing OsCsf6 with different secondary cell wall promoters; scale bar: 20 mm. (c) Rosette diameter of lines expressing OsCsf6 with different secondary cell wall promoters; n = 9 biological replicates; error bars: ±SEM. (d) Stem length of lines expressing OsCsf6 with different secondary cell wall promoters. n = 9 biological replicates; error bars: ±SEM. MLG content in rosettes (e) and stems (f) of lines expressing OsCsf6 with different secondary cell wall promoters; values for Col-0 represent background absorbance levels in the colorimetric assay as there is no MLG present in nontransgenic Arabidopsis. AIR: alcohol insoluble residue. *Significantly different from Col-0, t-test: P < 0.05.
While performing the monosaccharide analysis of rosette AIR samples, we noticed an unknown peak abundantly present only in the chromatograms of \textit{pSAG12::CsIF6} samples (Figure 5a). The peak eluted in the region corresponding to the NaOH gradient used to resolve the uronic acids (GalA and GlcA) in our HPAEC method (Figures 5a and S7). As the peak was present in AIR samples (alcohol insoluble), we tested whether hot water (100 °C) or acetone treatments would remove it. As shown in Figure S7, the peak is absent only in AIR samples washed with hot water but not acetone. Taking advantage of this solubility, hot water extracts from senesced \textit{pSAG12} and \textit{Col-0} rosettes were infused in a mass spectrometer and the spectra were analysed for compounds differing in abundance between the control and

\textbf{pSAG12::CsIF6} lines accumulate significant amounts of gluconic acid in senesced rosettes

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transgenic lines. As shown in Figure S8, among the putative candidates, a compound corresponding to the mass of gluconic acid was identified (195.1 m/z [M-H\(^-\)]) that was more abundant in the pSAG12 samples. These results were subsequently validated and quantified by liquid chromatography–time-of-flight mass spectrometry (LC-TOF MS) analysis using a gluconic acid standard and water-extracted samples (Figure 5b and Table 2). Finally, HPAEC analysis showed that the chromatograms of hot water extracted samples from pSAG12 lines alone and spiked with the gluconic acid standard overlapped (Figure 5c). Taken together, these results show that in addition to MLG, pSAG12::CslF6::CslF6 lines accumulate significant amounts of gluconic acid in cell wall fractions. Based on LC/MS and HPAEC analyses, gluconic acid levels are 10 times higher in senesced rosettes of transgenic lines compared to those from Col-0 controls (Tables 1 and 2).

**pSAG12::CslF6 lines display increased enzymatic saccharification**

We have generated viable Arabidopsis transgenic lines with high MLG content and shown that C6 cell wall sugar levels are significantly increased. To test whether these changes affect saccharification, biomass from the lines with highest MLG content was tested for enzymatic sugar release following a mild pretreatment (hot water at 100 °C for 1 h). As seen in Figure 6, enzymatic saccharification was significantly increased in pSAG12::CslF6 lines, ranging from 23% up to 42% increase, depending on the line. These changes in sugar yield after enzymatic hydrolysis were due primarily to increased glucose, gluconic acid and glucuronic acid release in pSAG12::CslF6 lines (Figure 6b).

**MLG produced in Arabidopsis is qualitatively different than grass MLG**

In grasses, MLG content and fine structure have been extensively studied, primarily in grains, as species such as barley and oats (Avena sativa) contain large amounts of this polysaccharide in their endosperm cell walls (Collins et al., 2010; Fincher, 2009). MLG fine structure and solubility can be inferred from the analysis of the DP3/DP4 ratio of the major oligosaccharides released by enzymatic digestion of the polysaccharide with the MLG-specific endohydrodrolase lichenase (Collins et al., 2010). We analysed the DP3/DP4 ratios of MLG extracted from stems and rosettes of Arabidopsis lines expressing CslF6 with different promoters and compared them to DP3/DP4 ratios from rice. As shown in Figure S9 and Table 3, Arabidopsis MLG has a much lower DP3/DP4 ratio than rice MLG extracted from vegetative tissues (stems and leaves). Interestingly, MLG from Arabidopsis has a closer ratio to rice grain MLG (0.8 vs 1.3; Table 3). Moreover, sequential cell wall extraction of MLG showed that the majority of the polysaccharide (66–83%) is extracted with the pectin-rich fraction (CDTA) in Arabidopsis, while more than 85% of rice MLG is extracted with the hemicellulosic-rich fraction (4 N NaOH) (Figure 7 and Table S4). Despite these differences, size exclusion chromatography analysis of 4 N NaOH-extracted MLG revealed that, although rice seems to have a slightly higher proportion of high molecular weight MLG than Arabidopsis, the MLG molecular size did not vary significantly between the two species (Figure S10). Taken together, these results suggest that the physico-chemical properties, but not the size, of MLG polymers made in Arabidopsis vary significantly from the polysaccharide present in vegetative tissues of rice.

**Discussion**

High C6 sugar content and reduced cell wall recalcitrance are both traits of importance for dedicated biofuel crops. Theoretically, there are many ways to achieve these goals via cell wall engineering, but it remains challenging due to difficulties associated with tinkering with the plant cell wall environment, which is intimately linked to maintaining cellular homeostasis. Here, we have successfully engineered the accumulation of MLG, a low recalcitrance glucose polymer, in Arabidopsis without negatively impacting plant growth and development. We obtained up to four times more glucose in the matrix cell wall fraction and up to a 42% increase in saccharification without significantly affecting cellulose deposition. The strategy, using a senescence-associated promoter to drive the expression of the CslF6 synthase gene, constitutes a novel and powerful approach for engineering cell wall properties important for biofuel production. Although the pSAG12 promoter is mainly active in senescing leaf tissues, it is possible to use promoters with high expression in senescing stems, such as pSAG2 (Gribic, 2003), to drive the accumulation of MLG in tissues rich in secondary cell walls. Increasing MLG content in stems is thus an important future goal for the application of this engineering strategy as the majority of plant biomass is deposited in organs containing thick secondary cell walls. Stacking MLG engineering with other biofuel traits, such as reduced/modified lignin or xylan (Petersen et al., 2012; Yang et al., 2013), could further improve the sugar release yields of either trait alone. A previous study in Arabidopsis used the pSAG12 promoter to drive an RNA interference (RNAi) construct to suppress starch degradation at senescence by down-regulating the expression of the GLUCAN, WATER DIKINASE (GWDA) gene (Weise et al., 2012). Similarly, overexpression of the maize

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**Table 1** Cell wall monosaccharide composition of senesced rosettes\(^1\) (µg/mg AIR)

<table>
<thead>
<tr>
<th></th>
<th>Fuc</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl/Man</th>
<th>GA</th>
<th>GalA</th>
<th>GlcA</th>
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<tbody>
<tr>
<td>Col-0</td>
<td>3.0 ± 0.3</td>
<td>6.9 ± 0.6</td>
<td>10.1 ± 0.7</td>
<td>14.5 ± 1.0</td>
<td>7.5 ± 0.1</td>
<td>14.7 ± 1.2</td>
<td>1.2 ± 0.1</td>
<td>35.5 ± 2.9</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>pSAG12::CslF6-5-22</td>
<td>2.2 ± 0.1*</td>
<td>6.3 ± 0.2</td>
<td>10.3 ± 0.3</td>
<td>14.4 ± 0.2</td>
<td>29.9 ± 0.3**</td>
<td>13.7 ± 0.2</td>
<td>12.2 ± 0.5**</td>
<td>42.4 ± 0.8</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>pSAG12::CslF6-11-22</td>
<td>2.3 ± 0.3</td>
<td>6.1 ± 1.1</td>
<td>10.3 ± 1.5</td>
<td>13.6 ± 1.3</td>
<td>20.3 ± 2.0**</td>
<td>14.0 ± 2.1</td>
<td>11.6 ± 0.9**</td>
<td>37.8 ± 7.4</td>
<td>1.4 ± 0.1*</td>
</tr>
<tr>
<td>pSAG12::CslF6-14-13</td>
<td>2.0 ± 0.3</td>
<td>5.7 ± 0.9</td>
<td>9.0 ± 1.2</td>
<td>11.8 ± 1.2</td>
<td>18.4 ± 0.9**</td>
<td>12.8 ± 2.0</td>
<td>10.0 ± 1.5**</td>
<td>33.0 ± 4.0</td>
<td>1.3 ± 0.2*</td>
</tr>
</tbody>
</table>

Fuc, fucose; Rha, rhamnose; Ara, arabinos; Gal, galactose; Glc, glucose; Xyl/Man, xylose/mannose; GA, gluconic acid; GalA, galacturonic acid; GlcA, glucuronic acid. Values are average of 3 biological replicates ± SEM. (*) and (**) statistically significant at \( P < 0.05 \) and \( P < 0.001 \), respectively. \(^1\)HPAEC analysis after TFA hydrolysis of AIR.
Corngrass (Cg1) microRNA was used to promote juvenile growth in switchgrass plants, increasing leaf starch content and saccharification yields (Chuck et al., 2011). Both approaches aimed at manipulating C6 sugar content in plants by targeting starch, another low recalcitrance glucose polymer. Our MLG strategy could then be combined with Cg1 or GWD-RNAi to further enhance the accumulation of easily degradable, C6 sugar polymers in biomass. Importantly, as senescence is universal to all plants, identifying endogenous promoters useful for cell wall engineering of relevant biofuel crops (i.e. poplar, switchgrass, Miscanthus) is readily feasible and should be the next step in translating this approach for bioenergy applications.

The fact that the strategies involving the use of the secondary cell wall promoters pIRX5, pNST1 and pNST2 were unsuccessful further highlights the difficulty in engineering high MLG content without impacting growth. Extensive studies, performed primarily with MLG extracted from cereal grains, have shown that this polysaccharide is soluble in water and that this characteristic depends on its fine structure determined by the ratio of the triose (DP3) and tetraose (DP4) oligosaccharides released after lichenase treatment (Collins et al., 2010; Fincher, 2009). MLG with DP3/DP4 ratios close to one are more soluble than glucans where either of the two diagnostic oligosaccharides is more abundant (Collins et al., 2010). MLG from our Arabidopsis transgenics expressing the rice CslF6 synthase gene has a DP3/DP4 ratio around 0.8 to 0.9, which would make it more soluble than rice MLG (with a ratio of 2 in vegetative tissues). This altered DP3/DP4 ratio was also observed for MLG made in N. benthamiana leaves transiently expressing the barley CslF6 homolog (Taketa et al., 2012). Thus, the highly soluble dicot MLG produced in Arabidopsis rosettes and stems during vegetative growth could explain

### Table 2 LC/MS data for gluconic acid in Col-0 and pSAG12::CslF6-5 senesced rosettes

<table>
<thead>
<tr>
<th>Sample</th>
<th>[M – H]⁻ (m/z)¹</th>
<th>RT (min)</th>
<th>Concentration (µM)²</th>
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</thead>
<tbody>
<tr>
<td>Col-0</td>
<td>195.05123</td>
<td>5.30</td>
<td>2.67 ± 0.1</td>
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<tr>
<td>pSAG12::CslF6-5-105</td>
<td>195.05084</td>
<td>5.33</td>
<td>20.2 ± 2.5*</td>
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<tr>
<td>Gluconic acid standard</td>
<td>195.05089</td>
<td>5.33</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a, not applicable.

*Significant at P < 0.01 (Student's t-test).

1Theoretical m/z is 195.05126.

2Retention time.

3±SD (n = 3).

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Figure 5 Identification of gluconic acid in senesced rosette samples of pSAG12::CslF6 lines. (a) Monosaccharide analysis using high-performance anion exchange chromatography (HPAEC) of mature rosette samples following TFA hydrolysis of alcohol insoluble residue (AIR). The unknown peak elutes in the NaOH gradient region used to resolve uronic acids. 1: pSAG12::CslF6-14-21; 2: Col-0; 3: pNST2::CslF6-27-17; 4: pNST1::CslF6-1-15. GalA: galacturonic acid. (b) LC/MS analysis of hot water soluble extract from senesced rosettes of pSAG12::CslF6-5-105 AIR. Both the retention time and m/z correspond to gluconic acid. (c) HPAEC analysis of hot water soluble extracts from pSAG12::CslF6-5-105 (1) and of pSAG12::CslF6-5-105 sample spiked with gluconic acid chemical standard (2).
the poor growth of engineered lines, in agreement with the vascular suffocation hypothesis previously inferred for p35S::CslF6 barley plants (Burton et al., 2011). This is consistent with the pIRX5 promoter’s reported activity in water-conducting xylem vessels (Taylor et al., 2003) and with our results of the low recovery rate of hygromycin-resistant lines for pIRX5::CslF6. It is interesting to note that, in p35S::CslF6 Arabidopsis lines, a threshold of 1.5% w/w MLG determined whether plants grew normally or not. This is in agreement with the fact that we could not recover plants from the pIRX5 approach with more than 1% MLG, and thus all pIRX5::CslF6 lines grew normally. We infer that pIRX5::CslF6 plants with MLG content above the 1.5% threshold were not viable. The vascular suffocation hypothesis could also explain, in part, the poor growth of pNST1 and pNST2 lines. For example, promoter-GUS fusion assays have previously shown that the NST1 promoter is active in midrib of leaf veins and in phloem cells of leaf petiole vascular bundles, in addition to fibre cells in stems (Mitsuda et al., 2005), which is in agreement with our immunolabelling results. However, in pSAG12::CslF6 lines, MLG also accumulated in cell walls of vascular tissues (xylem and phloem) without compromising growth and development. We believe that the differences in timing of expression of the CslF6 transgene account for these contrasting results and for the success of the senescence strategy for MLG accumulation. Unlike the secondary cell wall promoters tested here, the pSAG12 promoter is tightly regulated at the onset of senescence, preventing the accumulation of MLG in younger tissues (i.e. prior to the transition to flower) and/or too early in the development of vascular and other tissues. MLG deposition at senescence would

![Graph](image-url)

**Figure 6** Increased enzymatic saccharification yield of pSAG12::CslF6 senesced rosettes. (a) Enzymatic saccharification following hot water mild pretreatment. (b) HPAEC analysis of monosaccharides present in hydrolysates from (a). n = 3 biological replicates; error bars: ±SEM; *Significantly different from Col-0, t-test: P < 0.05.

**Table 3** MLG DP3/DP4 ratios in wild-type rice and transgenic Arabidopsis lines

<table>
<thead>
<tr>
<th>Line/sample</th>
<th>DP3/DP4 ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNST1::CslF6-1-15 (rosette)</td>
<td>0.8</td>
</tr>
<tr>
<td>pNST2::CslF6-27-17 (rosette)</td>
<td>0.8</td>
</tr>
<tr>
<td>pSAG12::CslF6-5-22 (rosette)</td>
<td>0.8</td>
</tr>
<tr>
<td>pIRX5::CslF6-3-8 (stem)</td>
<td>0.9</td>
</tr>
<tr>
<td>pNST1::CslF6-1-1 (stem)</td>
<td>0.8</td>
</tr>
<tr>
<td>pNST2::CslF6-27-1 (stem)</td>
<td>0.8</td>
</tr>
<tr>
<td>pSAG12::CslF6-5-2 (stem)</td>
<td>0.9</td>
</tr>
<tr>
<td>Rice leaf</td>
<td>1.9</td>
</tr>
<tr>
<td>Rice stem</td>
<td>1.9</td>
</tr>
<tr>
<td>Rice grain</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*Calculated from peaks of MLG oligosaccharides resolved by HPAEC following lichenase digestion.

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bypass these negative effects on growth, as plants at that stage of development are transitioning to breaking down cellular components and remobilizing nutrients to feed source tissues (e.g. younger leaves, developing inflorescence and seed formation).

We have shown that MLG produced in Arabidopsis transgenics expressing the rice CslF6 synthase is structurally different from rice MLG from vegetative tissues. In addition to the altered DP3/DP4 ratio, MLG in Arabidopsis is more easily extracted with CDTA than with 4 M NaOH, which is opposite to the extraction profile of MLG from rice stems. This could be due to the fact that MLG is more soluble in Arabidopsis compared to rice (see discussion above). Alternatively, the differences in chemical fractionation of MLG could be explained by the nature of the cell wall environments differing in dicots from grasses. In Arabidopsis, MLG coexists with large amounts of pectin, xyloglucan and cellulose, while in rice it coexists with cellulose and arabinoxylan. In maize epidermal cell walls, MLG is tightly associated with cellulose and arabinoxylan. In maize epidermal cell walls, MLG is tightly associated with cellulose and arabinoxylan of a low degree of substitution (Carpita et al., 2001), which is in agreement with a requirement of a high concentration of base for extraction, as seen for rice MLG here. Thus, it is likely that the abundance of different polysaccharides in the dicot cell wall (i.e. pectins and xyloglucan) affects the molecular interactions of MLG with cellulose in Arabidopsis transgenics. Interestingly, the nonflowering plant Equisetum arvense has a cell wall containing abundant MLG coexistent with pectin and cellulose, which also differs from the grass cell walls (Sorensen et al., 2008). As E. arvense MLG is primarily composed of the tetraose oligomer (DP4), it is thus expected to be less soluble than both grass and Arabidopsis MLG (Sorensen et al., 2008). Our results confirm that the surrounding cell wall environment heavily influences the physico-chemical properties of MLG.

An unexpected result from engineering MLG deposition in Arabidopsis plants was the increased accumulation of gluconic acid in rosette leaves. The production of gluconic acid in bacteria and fungi has been well documented, and results from the oxidation of glucose by either glucose dehydrogenases or oxidases, respectively (Ramachandran et al., 2006). However, the biosynthesis and metabolism of gluconic acid in plants is poorly understood. In Arabidopsis, gluconic acid has been previously identified in metabolomic studies of rosettes (Fiehn et al., 2000), and it is increased in shoots acclimated to salt stress (Sanchez et al., 2008) and in transgenic plants expressing the maize C3 NAPD-malic enzyme undergoing dark-induced carbon starvation (Fahnenstich et al., 2007). In a recent study, transgenic poplar overexpressing the bacterial salicylic acid (SA) synthase gene ipr9 accumulated high amounts of gluconic acid, which correlated with elevated SA and SA-conjugated metabolite accumulation and the onset of oxidative stress on those plants (Xue et al., 2013). These results in Arabidopsis and poplar suggest that endogenous gluconic acid accumulation in plants is probably associated with oxidative stress and/or carbon re-allocation responses. However, unlike these previous reports, we have identified gluconic acid in both transgenic and Col-0 in cell wall extracts of metabolically inactive, senesced plant material. Interestingly, it has been recently shown that oxidative enzymes present in commercial cocktails commonly used for the hydrolysis of lignocellulose lead to the formation of both cellobionic and gluconic acids as products of the oxidation of cellobiose and glucose, respectively (Cannella et al., 2012). As a product of glucose oxidation, it is possible that increased gluconic acid in our Arabidopsis transgenic plants constitutes a by-product of MLG degradation resulting from the recycling and remobilization of nutrients that occur at senescence. Indeed, it is known that an array of glycosyl hydrolases and peroxidases, among other classes of proteins, are induced as part of the programmed cell death events associated with plant senescence (Lim et al., 2007). In this highly oxidative environment, a fraction of glucose residues in MLG synthesized and deposited during senescence could be partially oxidized and subsequently hydrolysed into gluconic acid. This is supported by the fact that only plants accumulating MLG with the pSAG12 approach contain increased gluconic acid levels in cell wall extracts from rosette leaves. In Col-0 rosette AIR samples, the small amount of gluconic acid likely comes from the oxidative degradation of glucose residues on xyloglucan or amorphous cellulose. Since gluconic acid constitutes a small but significant proportion of the monosaccharides present in AIR and after enzymatic saccharification, limiting its production may improve overall glucose yields in pSAG12::CslF6 lines. Based on our MS analysis of water-extracted pSAG12::CslF6 rosette samples, additional metabolites were also increased compared to Col-0 controls. A more detailed analysis, which is outside the scope of this study, is needed in order to further characterize these other metabolic changes associated with MLG engineering in Arabidopsis. Nevertheless, our results highlight the importance of phenotyping the effects of cell wall engineering strategies beyond the usual cell wall and growth characterizations.

In conclusion, we have established a novel approach for engineering cell wall polysaccharide accumulation in plants by inducing biosynthesis and deposition during senescence. This approach is particularly suited for tailoring biomass traits for biofuel applications in cases where plant growth and development are negatively affected by targeted cell wall modifications.

**Experimental procedures**

**Generation of pA6-pNST1-GW, pA6-pNST2-GW and pA6-pSAG12-GW binary vectors for transformation**

The IRX5 (At1g 44030), NST1 (At1g 46770), NST2 (At3g 61910) and SAG12 (At5g 45890) promoters corresponding to, respec-
seeds were germinated in soil and transgenic plants were

\[ \textit{pA6-pIRX5-GW, pA6-pNST1-GW, pA6-pSAG12-GW} \]

generate, respectively, the

generate, and

\[ \textit{pNST2} \]

transgenic lines in the T1 and T2 generations, seeds were

\[ \textit{A. tumefasciens} \]

strain GV3101. For selection of

\[ \textit{pBlunt-pNST2} \]

\[ \textit{pBlunt-pSAG12} \]

\[ \textit{pBlunt-pIRX5}; \textit{pBlunt-pNST1}; \textit{pBlunt-pNST2}; \textit{pBlunt-pSAG12} \]

were digested from the

\[ \textit{XhoI/AvrII} \]

\[ \textit{KpnI/AvrII} \]

\[ \textit{F-pSAG12-5/R-FYpSAG12AvrII-3} \]

\[ \textit{pBlunt-pSAG12} \]

\[ \textit{pBlunt-pIRX5} \]

\[ \textit{pBlunt-pNST1} \]

\[ \textit{pBlunt-pNST2} \]

\[ \textit{pA6-GW} \]

\[ \textit{pA6-pNST1-GW} \]

\[ \textit{pA6-pNST2-GW} \]

\[ \textit{pA6-pSAG12-GW} \]

binary vectors.

Generation of transgenic lines and plant care

All plant transformations were carried out using \textit{Arabidopsis thaliana} (L.) Heynh, Columbia-0 ecotype (Col-0), with the \textit{Agrobacterium-mediated} floral dip method as described in (Bechtold and Pelletier, 1998). Transformation vectors with Gateway cloning cassette (Life Technologies) and containing the secondary cell wall and senescence-associated promoters were made in the backbone of the binary plasmid pA6-GW, as described above. For the transformations involving the constitutive 3SS promoter, the pEarleyGate 100 destination vector was used (Earley et al., 2006). The pDONOR-CslF6 Gateway entry clone containing the coding sequence of the rice CslF6 gene has been described previously (Vega-Sanchez et al., 2012) and was used to create all expression constructs via LR recombination.

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Sample preparation for metabolite analysis

Leaf samples were collected from young rosette leaves prior to flowering (5-week-old plants) and from senescing (yellowing tips) rosette leaves from mature, 10-week-old, postflowering plants. Stem samples were collected from the bottom (first node) of 10-week-old, postflowering plants. All samples were fixed in 4% paraformaldehyde buffered in 50 mM PIPES, 5 mM EGTA, 5 mM MgSO\(_4\), pH 6.9 for 24 h at 4 °C. The base of the leaves (close to the petiole) or the stems were embedded in 7% agarose and sectioned to a thickness of 50 μm using a vibratome. Antibody labelling with the anti-MLG monoclonal antibody (Meikle et al., 1994) and fluorescence microscopy were performed as described previously (Vega-Sanchez et al., 2012).

Sample preparation for metabolite analysis

Based on its presence in the uronic acid portion of the chromatograms following TF hydrolysis, and cellulose content estimation were performed as previously reported in (Vega-Sanchez et al., 2012). Sequential cell wall extraction using CDTA and 4 N NaOH was performed as described in (Chiniquy et al., 2012). Sequential cell wall extraction using CDTA and 4 N NaOH was performed as described in (Chiniquy et al., 2012). Monosaccharides were analysed by HPAEC-PAD as in (Vega-Sanchez et al., 2012).

Enzymatic saccharification

De-starched AIR (5 mg) was used for enzymatic saccharification for 24 h following hot water pretreatment as described in (Chiniquy et al., 2012), with the following modification: AIR samples were suspended in water and incubated at 100 °C in a heat block for 60 min, prior to saccharification. After saccharification, sugars in the supernatant were quantified with a reducing sugar assay as described in (Chiniquy et al., 2012), and monosaccharides were analysed by HPAEC-PAD as in (Vega-Sanchez et al., 2012).

MLG oligosaccharide fingerprinting

Arabidopsis and rice AIR, as well as sequentially extracted samples, were digested with 1 U of lichenase from \textit{Bacillus subtilis} (Megazyme, Wicklow, Ireland) in 0.02 mM sodium phosphate buffer at 50 °C in a thermomixer at 800 RPM for 2 h. The supernatant containing the MLG oligomers was desalted by solid-phase extraction using Bond Elut graphitized carbon columns (Agilent Technologies, Santa Clara, CA, USA), and oligosaccharides were eluted in 50% v/v acetonitrile. Samples were dried in a speedvac overnight to remove solvent, and the pellet was re-suspended in deionized water. Resolution and quantification of the DP3 and DP4 MLG oligomers was performed by HPAEC using a CarboPac PA200 column with the method described in (Vega-Sanchez et al., 2012).

Immunofluorescence microscopy

Leaf samples were collected from young rosette leaves prior to flowering (5-week-old plants) and from senescing (yellowing tips) rosette leaves from mature, 10-week-old, postflowering plants. Stem samples were collected from the bottom (first node) of 10-week-old, postflowering plants. All samples were fixed in 4% paraformaldehyde buffered in 50 mM PIPES, 5 mM EGTA, 5 mM MgSO\(_4\), pH 6.9 for 24 h at 4 °C. The base of the leaves (close to the petiole) or the stems were embedded in 7% agarose and sectioned to a thickness of 50 μm using a vibratome. Antibody labelling with the anti-MLG monoclonal antibody (Meikle et al., 1994) and fluorescence microscopy were performed as described previously (Vega-Sanchez et al., 2012).
Low recalcitrance glucan engineering in plants

Metabolite analysis by LC-TOF MS

Samples were prepared as described above and reconstructed in 50% v/v methanol, including the gluconic acid standard purchased from Sigma-Aldrich. The separation and quantification of metabolites was carried out by HPLC–electrospray ionization TOF MS with the conditions described in (Juminaga et al., 2012).

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We thank Dr. Emilie Rennie for useful suggestions on sample purification for metabolite analysis. This work conducted by the Joint BioEnergy Institute was supported by the Office of Science, Office of Biological and Environmental Research, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. J. H. was supported by the Villum Foundation’s Young Investigator Program.

References


Supporting information
Additional Supporting information may be found in the online version of this article:

Figure S1 Additional phenotypes of pSAG12::CslF6 lines.
Figure S2 MLG detection in leaf and stem sections of lines expressing OsCslF6 with secondary cell wall promoters.
Figure S3 SAG12 gene expression profile throughout Arabidopsis development.
Figure S4 IRX5 gene expression profile throughout Arabidopsis development.
Figure S5 NST2 gene expression profile throughout Arabidopsis development.
Figure S6 Total cell wall glucose content in AIR as determined by sulfuric acid hydrolysis.
Figure S7 Gluconic acid in senesced pSAG12::CslF6 rosette AIR samples is extracted by hot water treatment.
Figure S8 MS analysis of hot water extracted sample from pSAG12::CslF6 senesced rosette AIR.
Figure S9 HPACE analysis of hot water extracted sample from pSAG12::CslF6 senesced rosette AIR.
Figure S10 Determination of the Mr distribution of MLG extracted form Arabidopsis lines and rice tillers.

Table S1 Hygromycin resistant lines recovered from T0 seed.
Table S2 MLG content of T0 lines.
Table S3 Hygromycin resistant lines recovered from T0 seed.
Table S4 MLG DP3/DP4 ratios from sequential extraction fractions.
Table S5 Primers used for cloning promoters.

Data S1 Materials and methods.

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Title:
Engineering temporal accumulation of a low recalcitrance polysaccharide leads to increased C6 sugar content in plant cell walls

Date:
2015-09-01

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