Short Communication

Changes in Cell Wall Carbohydrate Extractability Are Correlated with Reduced Recalcitrance of HCT Downregulated Alfalfa Biomass

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Abstract

*Downregulation of the expression of enzymes involved in lignin biosynthesis in alfalfa (*Medicago sativa L.*) has been shown to result in reduced cell wall recalcitrance and increased saccharification. Here we describe changes in the extractability of hemicellulosic and pectic polysaccharides in cell walls of alfalfa downregulated in the expression of hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT). Xylan and pectic polysaccharides are more readily solubilized from the cell walls of HCT downregulated transgenic lines than from empty vector control plants, suggesting alterations in lignin-polysaccharide interactions in the transgenic plants. Our studies show that reduced recalcitrance of HCT downregulated lines is also correlated with increased xylan extractability.*

Introduction

Secondary cell walls account for the bulk of plant biomass and are a major renewable resource for the biochemical production of biofuels.[1,2] However, the recalcitrance of these walls to deconstruction into fermentable sugars is an impediment to cost-effective production of liquid transportation fuels from lignocellulosic biomass.[3] Our current research is directed toward determining the specific cell wall components that contribute to recalcitrance. Lignin is one cell wall component that has already been shown to contribute to recalcitrance.[3] A recent study compared the effects on alfalfa recalcitrance when the amounts or structure of lignin were altered by downregulating the expression of cinnamate 4-hydroxylase (C4H), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), coumaroyl shikimate 3-hydroxylase (C3H), caffeoyl CoA 3-O-methyltransferase (CCoAOMT), ferulate 5-hydroxylase (F5H), or caffeic acid 3-O-methyltransferase (COMT).[4] C3H and HCT downregulated plants produced less lignin and yielded more fermentable carbohydrate than did control plants.[4] Further studies of these transgenic lines showed that they contain increased amounts of 4-hydroxyphenyl (H) lignin and produce lignin that is more easily extracted.[5] Moreover, modification of lignin in HCT downregulated plants results in the presence of more extractable cell wall pectic fragments that induce plant defense responses.[6]

In this study, we compared the effect of reduced/altered lignin on the extractability of cell wall polysaccharides and the composition of solubilized fractions obtained by treatment of the walls with aqueous buffers and alkali. We chose HCT downregulated transgenic lines because they are the least recalcitrant lines among various lignin-modified transgenic alfalfa lines available.[4,5] Downregulation of HCT activity leads to an approximate 50% reduction in acetyl bromide lignin levels, an approximate 75% decrease in lignin thioacidolysis yield, a very large increase in 4-hydroxyphenyl units within the lignin, reduced lignin chain length, and a small increase in the percent of total carbohydrate within the cell wall residue.[4,5,7,8] We focused on changes in the extractability of cell wall components, as this likely reflects altered polysaccharide-lignin interactions and may impact overall recalcitrance of the walls. Together, our results show that in HCT downregulated alfalfa, modification of lignin is accompanied by an increase in extractability of cell wall components, specifically xylan and pectic polysaccharides. We propose that such changes are due to alterations in polysaccharide-lignin interactions that reduce recalcitrance in these plants.

Materials and Methods

Plant biomass for transgenic lines HCT3a, HCT30a, and empty vector controls used in this study were obtained from the National Renewable Energy Laboratory (Golden, CO) and the Samuel Roberts Noble Foundation (Ardmore, OK).[4,5] The plants were originally generated at the Noble Foundation, and the HCT transcript levels and lignin properties of the independent lines HCT3a and 30a have been presented elsewhere.[7,8] On average, the lignin levels in the control and HCT downregulated lines were 210 and 100 mg/g cell wall residue, and the total cell wall carbohydrate contents were 650 and 700 mg/g cell wall residue, respectively.

PREPARATION OF ALCOHOL INSOLUBLE RESIDUES (AIR)

Air dried biomass was ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ) to a particle size that would pass through a #20 (0.841 mm) sieve, but be retained by a #80 (0.177 mm) sieve. A portion of this milled biomass was extracted overnight at 200 rpm

with aqueous 80% (v/v) ethanol. The insoluble biomass was collected by centrifugation, washed with acetone, and air dried.

SEQUENTIAL EXTRACTION OF AIR

Wiley-milled AIR (500 mg) was extracted for 16 h with 50 mM ammonium oxalate (20 mL, 200 rpm). The suspension was then centrifuged (10 min at 3,500 g). The soluble fraction was dialyzed (3,500 molecular weight cut-off) and stored at −20°C. The insoluble residues were then extracted with 1 M KOH containing 0.5% (w/v) $NaBH_4$ (16 h at room temperature), and then with 4 M KOH containing 0.5% (w/v) $NaBH_4$ for the same period of time. The 1 M and 4 M KOH soluble materials were neutralized with glacial acetic acid, dialyzed, and stored at −20°C. The residue remaining after 4 M KOH treatment was delignified for 4 h at 70°C with acetic acid/sodium chlorite.[9] The delignified residue was then extracted with 4 M KOH containing 0.5% (w/v) $NaBH_4$ and neutralized as described. The chlorite-acetic acid soluble and 4 M KOH soluble materials were dialyzed and stored at −20 °C. The volume of the dialyzed extracts was measured and 20% of the volume lyophilized. The bulk of the extracts were not lyophilized, as we have found previously that the dry extracts are not completely soluble subsequently in aqueous buffers.

QUANTIFICATION OF CARBOHYDRATES

Total uronic acid was determined colorimetrically using the *m*-hydroxydiphenyl method.[10] Total neutral sugars present in the soluble extracts were determined colorimetrically using orcinol and by gas chromatograph-flame ionization detector (GC-FID) analysis of the alditol acetate derivatives.[11,12]

Use of the orcinol assay for pentoses and hexoses and the *m*-hydroxydiphenyl assay for acidic sugars allowed us to quantify the total amounts of carbohydrate in each extract. Combining these data with the molar percentages of the glycoses determined by GC-FID allowed for a more accurate quantification of individual sugars than either method alone.

The above analyses were also performed on aliquots of the insoluble material from each extraction residue (data not shown). These results were inconsistent, probably due to a variety of factors relating to the insoluble material itself, such as particle size, acid lability, and degradation.

GLYCOME PROFILING

Glycome profiling was carried out using enzyme-linked immunosorbent assays (ELISAs) of the cell wall extracts and a large and diverse collection of plant glycan-directed monoclonal antibodies (mAbs), as described previously (*Supplementary Table S1;* Supplementary data are available online at www.liebertpub.com/ind).[13,14]

Results and Discussion

Biomass from empty vector control and HCT downregulated transgenic alfalfa plants were sequentially extracted with aqueous buffer and increasing concentrations of alkali before and after delignification to generate fractions enriched with various wall polysaccharides including pectin and hemicellulose. The yields—total

carbohydrate and glycosyl residue compositions—of each fraction were determined. The extracts were also analyzed by glycome profiling using a comprehensive suite of cell wall glycan-directed mAbs.

Oxalate, 1 M KOH, 4 M KOH, chlorite, and 4 M KOH post-chlorite extraction conditions used in this study establish a sequence of increasingly harsh extraction conditions that lead to the sequential removal/extraction of wall polysaccharides based on how tightly they are integrated into the cell wall matrix. Oxalate extraction has been shown to remove the most loosely bound pectic polysaccharides from the wall through chelation of Ca^{2+} ions; 1 M KOH has been shown to remove mostly xylan and pectin; and 4 M KOH is strong enough to remove xyloglucans in addition to xylan and pectins.[15,16] The acetic acid/chlorite treatment breaks down most of the lignin to release lignin-associated polysaccharides, and the last 4 M KOH treatment removes most of the residual polysaccharides that are otherwise integrated in the wall through association with lignin. The acetic acid/chlorite treatment step used in this study was previously shown to result in 90% delignification of black spruce wood after a 7 h treatment.[17] Our studies employed a 4 h acetic acid/chlorite treatment, and no quantitative measurement of delignification associated with this treatment was made as substantial lignin modification through chlorite treatment makes it nearly impossible to measure accurately the amount of residual lignin present. Due to the exposure of the extracted polysaccharides to strong alkaline extraction conditions, the possibility of some polysaccharide degradation cannot be ruled out, although we added a reducing agent to minimize this possibility. Furthermore, care was taken to maintain a consistent time of exposure for each extraction step and to neutralize the soluble alkali extracts immediately after removal from the extraction vessel to further minimize sample degradation.

Downregulation of HCT resulted in a dramatic increase in the total amount of extractable carbohydrate released from Wiley-milled alfalfa biomass from both HCT downregulated lines compared with the empty vector control biomass (*Figure 1A*). Between 43% and 48% of the HCT downregulated biomass was solubilized by the combined oxalate, alkali, and chlorite extractions, whereas only 28% of the control biomass was solubilized. Most of the more readily extractable glycans were released from the HCT downregulated biomass with 1 M KOH treatment. In contrast, there was about a 3- to 4-fold reduction in carbohydrate released by post-chlorite 4 M KOH from the HCT downregulated biomass compared with control biomass. These results suggest that HCT downregulation leads to a loosening of the most tightly bound carbohydrates in the wall allowing them to be extracted under the less harsh conditions of 1 M KOH. Xylose accounts for between 70–80% of the glycoses in the 1 M KOH extract, suggesting that xylan is the major polysaccharide present in this extract (*Table 1*). An increased uronic acid content was also observed in the 1 M KOH extract, possibly arising from the glucuronic acid substituents typically present on dicot xylans. In this study, individual uronic acids were not quantified. Together these results suggest that altering lignin by downregulating HCT results in a considerable increase in the ease with which biomass polysaccharides are solubilized.
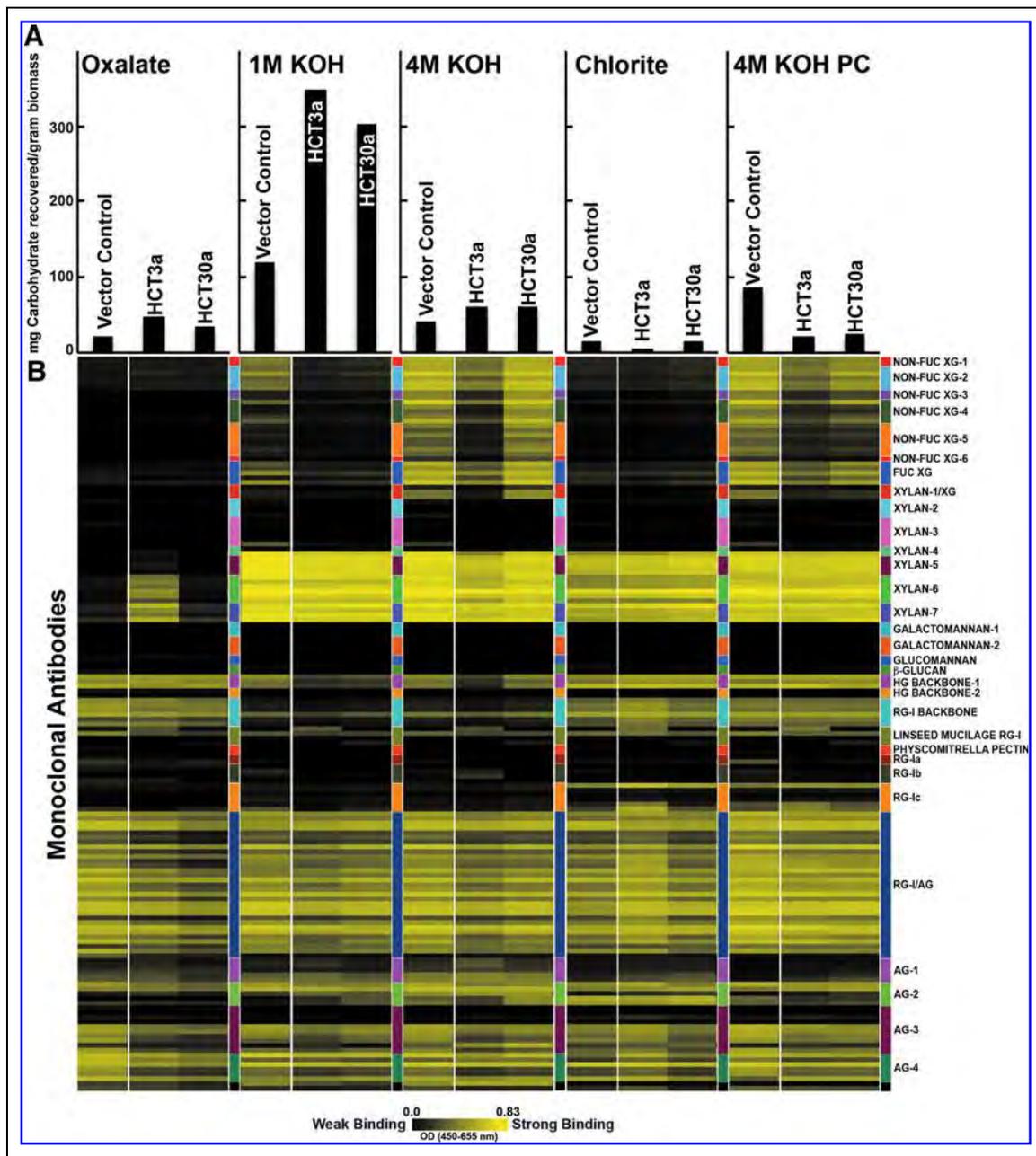
Fig. 1. Glycome profiles of HCT downregulated and empty vector control alfalfa biomass. Alfalfa biomass was sequentially extracted with oxalate, 1 M KOH, 4 M KOH, acetic acid/chlorite, and 4 M KOH post-chlorite (PC). **(A)** Total carbohydrate recovered in each extraction; **(B)** Each extract was probed against an array of plant glycan-directed monoclonal antibodies. Antibody binding (average value of three independent replicates) is depicted as colored heat maps, with black signifying no binding and yellow representing the strongest binding. The panel on the right of the figure indicates the principal cell wall epitopes recognized by each group of mAbs used in this study (*Table S1*).

Glycome profiling using mAbs that recognize diverse cell wall polysaccharide epitopes was used to determine the major classes of polysaccharides solubilized by oxalate, alkali, and chlorite (*Fig, 1*).[13,14] Epitopes characteristic of pectins and arabinogalactans were present in all of the extracts. By contrast, epitopes characteristic of xylans were particularly enriched in the alkali- and chlorite-

extractable fractions. Epitopes characteristic of xyloglucan were present predominantly in the 4 M KOH extracts before and after delignification.

Downregulation of HCT caused three notable changes in the glycome profiles of the extracts prepared from the three transgenic plant lines (*Fig, 1*). First, some xylan epitopes (recognized by the xylan-6

Table 1. Total Carbohydrate and Monosaccharide Compositions of the Oxalate-, Alkali-, and Acetic Acid/Chlorite-Soluble Extracts Obtained from HCT Downregulated and Empty Vector Control Alfalfa Biomass.*

PLANT	EXTRACT	CARBOHYDRATE (MG/G EXTRACTED AIR)								
		RHA	FUC	ARA	XYL	MAN	GAL	GLC	URONIC	TOTAL
Control		2	< 1	5	2	2	4	2	5	22
HCT3A	Oxalate	5	1	7	13	2	8	2	7	47
HCT30A		3	< 1	4	10	1	6	1	9	33
Control		6	< 1	4	82	1	2	2	23	120
HCT3A	1 M KOH	5	1	5	300	3	3	3	37	360
HCT30A		7	1	7	240	3	4	4	29	300
Control		1	< 1	7	23	3	4	4	3	42
HCT3A	4 M KOH	1	< 1	10	25	7	8	8	6	62
HCT30A		1	1	2	32	5	11	11	5	60
Control		1	< 1	4	3	3	1	1	3	14
HCT3A	Chlorite	< 1	< 1	2	< 1	1	< 1	< 1	2	4
HCT30A		1	< 1	5	1	4	1	1	3	15
Control		1	< 1	3	55	3	4	7	12	86
HCT3A	4 M KOH PC	< 1	< 1	3	6	2	2	5	2	22
HCT30A		< 1	< 1	2	12	1	1	4	3	24

*Data presented here are the averages of three independent replicates.

RHA: rhamnose; FUC: fucose; ARA: arabinose; XYL: xylose ; MAN: mannose; GAL:glactose GLC: glucose.

and -7 groups of mAbs) were present in the oxalate fraction of the HCT3a line, and this was accompanied by a small increase in total extractable sugars recovered in this fraction compared to control and HCT30a lines. Secondly, there was a decrease in the relative xyloglucan epitope content in the 1 M KOH extract of the HCT downregulated lines in comparison with the control. Third, the relative abundance of pectic backbone epitopes in the 1 M KOH, and possibly in the 4 M KOH-soluble fractions of HCT down regulated lines, was lower than in the controls (as indicated by the reduced binding intensities of HG backbone-1, HG backbone-2, and RG-I backbone groups of mAbs). These observations suggest that changes in lignin brought about by downregulation of HCT causes changes in overall wall structure that differentially affect the extractability of at least some glycan epitopes. These changes implicate lignin as having a central role in overall wall structure, perhaps through direct covalent connections to diverse wall polysaccharides or through strong noncovalent interactions with these polymers. There is evidence in the literature supporting covalent or very strong noncovalent interactions between lignin and xylan and lignin and arabinogalactan-containing polymers.[9, 18] To our knowledge, there is no experimental evidence supporting a direct interaction between lignin and xyloglucan, though an indirect interaction via connections with other wall components is possible. More in-depth studies are required to

delineate the exact nature of the interactions/connections of lignin with wall polysaccharides and the cell wall structural modifications in the HCT downregulated lines that cause altered polysaccharide extractability as revealed by the glycome profiling analyses.

Although the total amount of extractable carbohydrate changed as a result of downregulating the HCT gene, the glycosyl compositions and epitope compositions of the extracts were not dramatically changed (*Table 1*, *Fig. 1*). Nearly all of the carbohydrate that could be extracted from the genetically modified cell walls was released prior to chlorite treatment. In contrast, a significant amount of carbohydrate was released from the control after the chlorite treatment. All alkali–extracted fractions were enriched in xylan epitopes. These observations suggest that altering lignin structure by down-regulating HCT increases the extractability of most, if not all, wall polysaccharides and are consistent with the notion that lignin interferes with solubilization of wall polysaccharides. This may be due to strong (covalent or noncovalent) interactions of lignin with the polysaccharides, leading to a recalcitrant cell wall architecture. Indeed, a number of studies have demonstrated that altering lignin content and/or composition leads to reduced recalcitrance, that is, increased sugar release.[3,4,19] In the case of the HCT downregulated lines studied here, this effect is quite dramatic, with a near doubling of enzymatic hydrolysis efficiency after acid pre-treatment.[4] Our

observations also suggest that a detailed understanding of glycan-lignin interactions in the plant cell wall would be a fertile area of investigation to gain a better understanding of the structural features of the wall that control recalcitrance.

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Author Disclosure Statement

No competing financial interests exist.

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