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Major alterations in lignin composition lead to differential release of signals for activation of defense responses

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Short title: Lignin composition and release of elicitors

Summary

- Reducing lignin content in transgenic plants can induce ectopic expression of defense response genes, but the importance of altered lignin composition in such phenomena remains unclear.
- Two Arabidopsis lines with similar lignin contents but strikingly different lignin compositions exhibit different and non-overlapping patterns of transcriptional reprogramming, including alterations in defense gene expression. Plants with lignin composed primarily of guaiacyl units expressed genes responsive to oomycete and bacterial pathogen attack, whereas plants with lignin composed primarily of syringyl units expressed genes associated with cis-jasmone-mediate responses to insects and xenobiotics. These genes were differentially induced by water-soluble extracts from cell walls of plants of the two lines.
- Glycome profiling, fractionation and enzymatic digestion studies indicated that the different lignin compositions led to differential extractability of a range of heterogeneous oligosaccharide epitopes, with biological activity originating from different cell wall polymers.
- Lignin composition affects interactions with plant cell wall matrix polysaccharides to alter sequestration of latent defense signal molecules.

Introduction

Lignin is a complex biopolymer primarily present in plant secondary cell walls. Lignin biosynthesis begins with the deamination of the amino acid L-phenylalanine, and proceeds by successive hydroxylation and methoxylation of the aromatic ring, along with reduction of the side chain terminal acid to an alcohol group (Mottiar et al., 2016). These sequential reactions lead to the production of the three monolignols p-coumaryl, coniferyl and sinapyl alcohols (Boerjan et al., 2003) (Fig. S1). Following their biosynthesis, monolignols are transported to the apoplast for polymerization in the cell wall through combinatorial coupling of monolignol radicals generated

by the action of laccase and peroxidase enzymes (Mottiar et al., 2016). The final mechanical/physical features of the lignin polymer are defined by the relative abundance of each monolignol-derived unit and their bonding properties between themselves and with other cell wall components (Bonawitz and Chapple, 2010). Lignin is embedded in the cell walls of the plant vasculature along with pectins, hemicelluloses, cellulose and structural proteins (Burton et al., 2010). Pectins are acidic polysaccharides ranging in complexity from simple oligogalacturonans comprised of linear chains of free and methyl-esterified galacturonic acid, named homogalacturonan (HG), to the more complex rhamnogalacturonans I (RGI) and II (RGII), with 6 and 11 different glycosyl monomer units, respectively (Ridley et al., 2001).

Engineered plants with low lignin levels have low biomass recalcitrance, leading to enhanced sugar release for biofuel production and improved forage digestibility (Chen and Dixon, 2007), but strong reduction of lignin levels can lead to severe defects in plant growth (Bonawitz and Chapple, 2013) and altered plant immunity, potentially via multiple mechanisms (Zhao and Dixon, 2014). It is not clear whether reduction of lignin levels, changes in lignin composition, or both are responsible for these effects.

Lignin modification may affect pathogen response either through enhanced susceptibility (Miedes et al., 2014), or, paradoxically, enhanced resistance (Zhao and Dixon, 2014). The latter may be associated with activation of endogenous defense pathways in lignin-modified plants. For example, semi-dwarf alfalfa plants down-regulated in expression of HCT (hydroxycinnamoyl CoA: shikimate/quinate hydroxycinnamoyl transferase) constitutively express a suite of pathogenesis response (PR) proteins and exhibit enhanced tolerance to the fungal pathogen Colletotrichum trifolii as a result of increased accumulation of the signal molecule salicylic acid (SA) (Gallego-Giraldo et al., 2011a,b). This appears to be triggered by oligogalacturonide (OG) fragments released from the modified plant cell walls. Not all plant cell wall OGs are effective in plant defense, however, and the activity of OGs in in vitro assays depends on the plant species and the degree of polymerization (Côté and Hahn, 1994; Vorwerk et al., 2004). How lignin composition might affect elicitor release from cell walls is yet to be determined.

The enzyme ferulate/5-hydroxyconiferaldehyde 5-hydroxylase (F5H) is positioned at the entry point diverting precursors for formation of coniferyl alcohol leading to guaiacyl (G) lignin monomers towards the synthesis of sinapyl alcohol leading to syringyl (S units) in the lignin polymer. We here show that lines of Arabidopsis thaliana with similar lignin contents but

strikingly different lignin compositions resulting from over-expression or loss of function of F5H exhibit differential transcriptional reprogramming, with constitutive expression of different sets of defense response genes. These changes are associated with differential extractability of cell wall polysaccharide components, and the released molecules, which are heterogeneous with respect to composition and charge, include elicitors of the same defense response genes that are activated in the plants from which the elicitors originate. Our results indicate that lignin composition affects cell wall integrity in ways which impact the solubility of latent elicitor oligosaccharides.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana F5H-OE and fah-1 lines in ecotype Columbia-0 were obtained from Professor Clint Chapple, Purdue University, and were as previously described (Li et al., 2010). Seeds were exposed to 4˚C for 2 days and then sown in a seedling mix substrate (Metro-Mix 360, SUN GRO) with controlled fertilizer (20-10-20; 20 %N, 10 %P_2O_5 and 20 %K_2O) at 200 ppm per gallon at 21˚C under a 16h-light/8h-dark photoperiod. Light intensity was 110 µmol m^{-2} s^{-1}, supplied by both incandescent and fluorescent lights.

Microarray analysis

Transcriptomic analysis was performed on the main inflorescence stems of wild-type and lignin-modified lines growing under the same conditions. One and a half month old plants were harvested, stems from 5 different plants were pooled as one biological replicate, and three biological replicates were included for analysis. Stem material was frozen immediately in liquid nitrogen and stored at -80 ºC prior to RNA extraction. Total RNA was extracted with a Trizol kit (Invitrogen) and RNA was cleaned using the RNA cleanup kit (Qiagen). Purified RNA (1.5 µg) was quantified using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, http://www.nanodrop.com/) and evaluated for purity with an Agilent 2100 Bioanalyzer (http://www.home.agilent.com/). The Affymetrix GeneChip Arabidopsis Genome Array (Affymetrix, http://www.affymetrix.com/) was used for expression analysis. Probe labeling, hybridization, and scanning for microarray analysis were performed according to the

manufacturer's instructions and data analysis was performed as described previously (Gallego-Giraldo et al., 2011b).

Preparation of alcohol insoluble cell wall residues (AIRs)

Fresh plant material was frozen in liquid nitrogen and ground with a mortar and pestle. The AIR was prepared as described previously (Gallego-Giraldo et al., 2011b).

Preparation of water extracts from cell walls

AIR was suspended in water (1mg AIR/10 mL w/v) and incubated on a rotary shaker overnight at 100 rpm at room temperature. The suspension was then centrifuged at 4000 rpm at 24˚C for 15 min and the supernatant lyophilized to provide the water extract.

Determination of total sugar contents

Extraction of oligo- and poly-saccharides from AIRs was as described previously (Gallego-Giraldo et al., 2011b). The proportion of pectic material released by water extraction was determined as the proportion of total pectin. The uronic acid contents of elicitor fractions were estimated by the m-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973), using polygalacturonic acid PGA (Invitrogen) as standard. The sugar contents of neutral cell wall fractions were quantified by the orcinol method (McKay, 1964), and of acid fractions by the carbazole method (Dische and Borenfreund, 1951); in both cases glucose was used as standard.

Assay of elicitor activities in suspension cells

Arabidopsis cell suspension line T87 was obtained from the Arabidopsis ABRC stock center. Cells were grow in NT-1 medium in the dark at 24°C, shaken at 120 rpm (https://abrc.osu.edu/cell-culture), and sub-cultured every 8 days. Cells were harvested at 5 days for elicitor assays, and 4 mL batches of culture were distributed in each well of 6 well sterile plates. The elicitor volume ranged from 300-500 µL depending on the sugar concentration, with the final concentration adjusted to 0.1mg/mL. Six hundred µL of fresh NT-1 medium was added immediately and the plates were incubated for 12 h in the dark at 25°C and shaken at 100 rpm. Cells were harvested under vacuum on a nylon net, frozen in liquid nitrogen, ground with a pestle and mortar, and stored

at -80°C for subsequent RNA extraction. Each treatment/sample was run in triplicate and two independent mRNA extractions were done for each treatment.

Measurement of transcript levels by qRT-PCR

Total RNA was extracted with a Trizol kit (Invitrogen) and 3 µg of total RNA was subjected to DNase treatment with a DNA-free™ DNA removal kit (Thermofisher) followed by cDNA synthesis using a High-Capacity cDNA Reverse Transcription Kit (Thermofisher). Real time PCR was performed with a Quant-studio 6 Flex system (Apply Biosystems) using PowerUp™ SYBR® Green Master Mix (Thermofisher). qRT-PCR analysis was performed as described previously (Gallego-Giraldo et al., 2011b). Gene-specific primers are listed in Table S1.

Analysis of sugar composition

Sulfuric acid hydrolysis was performed on 500 µL of the water soluble extracts from AIR using 500 µL of 72 % (w/w) H_2SO_4. Samples were shaking at RT for 1 h before add 9 mL of water and then autoclaving at 120°C for 1 h. Analysis of major monosaccharides was by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex (Sunnyvale, CA) ICS-2500 system. The method was modified from Thermo Scientific Application Note 1091, "Determination of Uronic Acids and Wood Sugars in Wood-Based Hydrolysates" (https://tools.thermofisher.com/content/sfs/brochures/AN-1091-IC-Uronic-Acids-Sugars-Wood-Based-Hydrolysates-AN71059-EN.pdf) and used a Dionex CarboPac PA20 guard column (3 x 30 mm) and analytical column (3 x 150 mm) held at 30 °C. The column was initially washed with 200 mM NaOH for 10 min and then equilibrated to 2 mM NaOH over 10 min. Sample injections were 10 µL. Eluent was held at 2 mM NaOH for 16 min, ramped to 100 mM NaOH over 2 min, and then held at 100 mM NaOH with 100 mM Na-Acetate for 37 min. Pulsed amperometric detection used a gold electrode with carbohydrate 4-potential waveform as recommended by the equipment manufacturer (Dionex). Sample peaks were integrated and quantified against sugar standards ranging from 5-2000 pmol per injection. The standards were fucose, arabinose, rhamnose, galactose, glucose, xylose, mannose, and galacturonic acid; all were well separated except arabinose and rhamnose, which co-eluted under these conditions.

Glycome profiling

AIRs were prepared from the various plant lines, and glycome profiling of these preparations performed as described previously (Pattathil et al., 2012). In brief, cell walls were extracted using increasingly harsh reagents (ammonium oxalate, sodium carbonate, 1 M KOH, 4M KOH, chlorite and 4 M KOH post-chlorite extraction (4MKOHPC)) and subsequent enzyme-linked immuno-absorbent assay (ELISA) screening of these extracts used a comprehensive suite of plant cell wall glycan-directed monoclonal antibodies (mAbs). mAbs were obtained from laboratory stocks (CCRC, JIM and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or from BioSupplies (Australia) (BG1, LAMP).

Extraction and ELISA screening of polysaccharides

Five mg of AIR was transferred to Qiagen tubes containing two ball bearings. The tubes were placed in the rack of a TissueLyser (Qiagen) that had been previously cooled to -80°C. Samples were ground twice at 50 Hz for 5 min. Cell wall components were extracted by addition of 1 mL of autoclaved distilled water in the TissueLyser for 5 min at the same speed and incubated overnight at 4^{o}C with slow rocking. Samples were then centrifuged at 14 000 g for 15 min, and supernatants collected. All samples were stored at -20°C until use. Total sugar content was estimated by the orcinol method (Bruckner, 1955), using galactose as standard. The ELISA screening of epitope occurrence in all extracts was performed by coating the water extracts onto microtitre plate wells (NUNC Maxisorp, Thermo Fisher Scientific #44-2404-21) for 5 h at room temperature using 100 µL of a 400-fold dilution of the 1 mL extract in 1x PBS (phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Plates were covered with aluminum foil to protect from light. Primary antibodies listed in Table S 3 were used at a 25-fold dilution in 5 % w/v milk powder/PBS, adding 100 µL per well and incubating for 2 h at room temperature. Secondary antibodies (anti-rat or anti-mouse horseradish peroxidase-conjugated IgG, Sigma-Aldrich #A9542 and #A9044 respectively) were added at 100 µL per well at 1000-fold dilution in 5 % w/v milk powder/PBS for 2 h at room temperature. All washes after blocking, primary and secondary incubation steps were done by a Zoom HT microplate washer machine (Titertek-Berthold)). Finally, after the last wash step cycle, microtitre plates were developed using 100 µL of substrate per well (0.1 M sodium acetate buffer, pH 6, 1 % tetramethyl benzidine, 0.006

% H_2O_2). The enzyme reaction was stopped after 4 min by addition of 40 µL of 2.5 M H_2SO_4 to each well, and the absorbance read at 450 nm by a microplate spectrophotometer. All samples were processed in parallel and assays performed in triplicate; the highest overall absorbance value from all antibodies was set to 100 and the others adjusted accordingly for data presentation.

Epitope detection chromatography (EDC)

Cell wall extracts were analyzed by anion exchange EDC (Cornuault et al., 2014) using three 5 mL HiTrap ANX FF columns (GE Healthcare, #17-5163-01) connected in line. For each anion-exchange chromatograph run, 150 µg of sugar (estimated by orcinol reagent), diluted in 2.5 mL of 20 mM sodium acetate buffer pH 4.5, was injected to allow comparison of EDC signals between genotypes. A multi-stepped salt elution gradient was used for the analyses at a flow rate of 5 mL/min starting with 20 mM sodium acetate buffer pH 4.5 for 22 mL. After this first elution of all the neutral material, a salt gradient in which 100 % is equivalent to 0.6 M NaCl in 50 mM acetate buffer pH 4.5, was designed as follows: 20 % for 18 mL, 30 % for 18 ml, and 40 % for 18 ml. The last step consisted in increasing from 40 % to 100 % over 16 mL. In total, 96 (15 mL) fractions were collected. Polysaccharides were eluted, collected and all the fractions in each gradient step were pooled and freeze-dried for further analysis. Each fraction was analyzed by ELISA following a similar protocol described above for the wide screening. Briefly, a 100 µL aliquot of each column fraction was coated into microtitre plates and incubated overnight at 4^{o}C. Primary and secondary antibodies were incubated for 1.5 h and 1 h respectively, at room temperature. Finally, the developing substrate was added and incubated for 7 min at room temperature and the reactions stopped as described above.

Enzymatic digestion of elicitor preparations

Plant cell wall degrading enzymes were purchased from Megazyme, Carbohydrate Active EnZymes (http://www.megazyme.com). Seven hundred µL of elicitor preparation from wild-type and lignin modified lines was digested with the following enzymes: endo-xyloglucanase from Paenibacillus sp. dissolved in sodium acetate buffer (100 mM) containing 1mg/mL BSA pH 5.0, incubation for 15 min at 50 °C with 3 µL (1U/µL) enzyme per sample; endo-1,5-α-arabinanase from Cellvibrio japonicus dissolved in potassium phosphate (100 mM) containing 1mg/mL BSA

pH 7.0, incubation for 15 min at 40 °C and 3 µl (1U/µL) enzyme per sample; endo-1,4-β-galactanase from Aspergillus niger dissolved in citrate phosphate buffer pH 4.0, incubation for 30 min at 50 °C and 3.5 µL enzyme per sample; endo-polygalacturonanase from Aspergillus aculeatus dissolved in citrate phosphate buffer pH 5.2, incubation for 15 min at 40 °C and 1.5 µl enzyme per sample; thermostable α-fucosidase from Thermotoga maritima dissolved in citrate phosphate buffer pH 5.0, incubation for 10 min at 90°C and 10 µL enzyme/sample. All reactions were stopped by heating the mixtures at 100 °C for 15 min. Preparations were cooled down on ice and spun down in the microcentrifuge at maximum speed for 5 min and applied to cell cultures as elicitors. Controls such as water and buffers without enzyme were run in parallel to ensure cell growth was not affected by different buffers.

Statistical analysis

One way ANOVA and all pairwise multiple comparison using Tukey test (SigmaPlot 12.3) was used to determine significant differences between groups of values (transgenic) with respect to their control (wild type). Asterisks above bars or lines indicate statistical significance (*, $p < 0.05$).

Accession numbers

All microarray data are deposited at NCBI under the following accession numbers:
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Results

Arabidopsis lines with altered lignin compositions

To address whether lignin composition is itself a determining factor underlying the transcriptional changes characteristic of lignin-modified plants, we made use of mutant or transgenic Arabidopsis lines with loss of function of F5H (fah-1 mutant; Meyer et al., 1996, 1998; Humphreys et al., 1999; Marita et al., 1999) and overexpression of F5H (F5H-OE) (Meyer et al., 1998). Loss function of F5H in the fah-1 mutant has been reported to result specifically in loss of S lignin (lignin therefore comprised primarily of G units), whereas overexpression of F5H has been

reported to result in lignin composed primarily of S units. To confirm the lignin phenotypes in the plants used in the present experiments, we first determined lignin content/composition by thioacidolysis. This revealed that the overall lignin content was similar in wild type, fah-1 and F5H OE lines, but that the lignin in the fah-1 line was almost exclusively composed of G units whereas that in the F5H-OE line was predominantly composed of S units, with low levels of G and p-hydroxyphenyl (H) units (Fig. S2). To check the cellular distribution of the lignin, we examined cross sections of inflorescence stems for lignin auto-fluorescence under UV light, and by Mäule staining which yields a red coloration with S lignin (Pradhan Mitra and Loque, 2014) (Fig. S3). The results confirmed loss of S lignin staining in the fah-1 line, and increased staining in the F5H-overexpressor (F5H-OE) line, without any significant alteration in the tissue-specific lignin distribution pattern.

Transcriptional changes in the fah-1 and F5H-OE lines

Affymetrix microarray analysis was performed to determine transcript levels in the stems of the fah-1 and F5H-OE lines compared to wild-type. Complete information is available in Dataset S1. The extent of transcriptional reprogramming in the F5H-OE line was greater than in the fah-1 line, as summarized in Venn diagrams (Fig. 1). Generally, most of the genes up-regulated in F5H-OE were repressed in fah-1 and vice versa, and few were up- or down-regulated in both lines (Fig. S4, Table 1).

We then specifically examined the differentially expressed genes associated with plant defense and cell wall biosynthesis/remodeling. Among the defense response genes, overexpression of F5H resulted in more than 10-fold increased transcript levels of three genes associated with response to cis-jasmone, comprising a cytochrome P450 (CYP81D11) that has been implicated in the production of volatile chemical signals that impact aphids (Bruce et al., 2008; Matthes et al., 2010) and two glutathione S-transferases (GSTs) associated with response to oxidative stress, herbicide detoxification, or inoculation with the downy mildew pathogen Peronospora parasitica (Wagner et al. 2002; Matthes et al., 2010) (Table 1). Another highly up-regulated gene in the F5H-OE line encoded the elicitor peptide 3 precursor (PROPE3), a defense response gene associated with jasmonic acid (JA) signaling and volatile release in response to herbivory (Klauser et al., 2015).

In contrast, loss of function of F5H resulted in more modest induction of a number of stress-response genes, including two genes, LURP1 and PCC1, associated with response to oomycetes (Knoth and Eulgem, 2008) and bacterial infection (Sauerbrunn **a**nd Schlaich, 2004), respectively (Table 1), but no induction of CYP81D11 or the two GSTs. Neither of the F5H-modified lines showed any induction of PR genes previously shown to be strongly activated in plants with reduced total lignin and elevated H-lignin as a result of down-regulation of HCT (Gallego-Giraldo et al., 2011b). Therefore, differentially altering lignin composition results in activation of discrete sets of defense response genes.

Modifying lignin composition may have significant effects on cell wall polysaccharide biosynthesis. Among the genes annotated as being involved in plant cell wall biosynthesis and remodeling, a much greater number was differentially expressed in the F5H-OE line (13 up-regulated, 2 down regulated) than in fah-1 (3 up-regulated, only one of which was up-regulated in F5H-OE) (Table 1). Most of these genes encoded either arabinogalactan proteins or enzymes involved in the remodeling of xyloglucan, cellulose and pectin.

Induction of defense responses in fah-1 and F5H-OE lines is triggered by water-soluble, cell wall-derived elicitors

To determine whether the differential patterns of defense gene expression in the fah-1 and F5H-OE lines result from the action of cell wall-derived elicitors, we prepared alcohol insoluble residues (AIRs) from stems of wild-type, F5H-OE and fah-1 lines, and extracted them in water at room temperature. Extracts were then added to suspension cells of Arabidopsis and the cells harvested after 12 h for RNA extraction and determination of defense gene transcripts by qPCR. We selected PCC1, LURP1 and CYP81D11 as target genes for analysis of elicitor-mediated induction of defense pathways. Because pectic oligomers and polymers have been shown to act as general elicitors of plant defense responses (Ridley et al., 2001), we included polygalacturonic acid (PGA) as a potential positive control elicitor, and also included sets of elicitations in which the water-soluble elicitors from the plant cell walls and the PGA had been pre-treated with PGase, to determine whether the elicitors were of pectic origin. Importantly, PGA did not significantly induce PCC1, LURP1 or CYP81D11 transcripts. Treatment of cells with extracts from cell walls of the fah-1 line resulted in induction of PCC1 and LURP1, but not CYP81D11, whereas extracts from cell walls of the F5H-OE plants induced CYP81D11 but not PCC1 or LURP1 transcripts

(Fig. 2a, b). Induction of CYP81D11 by extracts from F5H-OE plants was completely abolished by pre-treatment of the extracts with polygalacturonase (PGase), whereas induction of PCC1 by the water-soluble cell wall fraction from the fah-1 line was substantially, but incompletely, abolished by pre-treatment with this enzyme (Fig. 2a, b). Together, these data suggest that the differential induction of sets of defense response genes in the plants with different lignin compositions is associated with the generation and/or solubilization of different elicitor-active materials derived, at least in part, from pectin-like material. PROPE3 transcripts were also induced in cell cultures following application of the water-soluble fraction from cell walls of F5H-OE (Table S2). However, the glutathione S-transferases that were more highly induced than CYP81D1 in the F5H-OE plants were not induced by the wall-released elicitor(s) from this line (Table S2). Clearly, the wall released elicitors are not directly responsible for the totality of transcriptional changes observed in the lignin-modified plants.

Lignin modifications differentially affect cell wall integrity and extractability

To begin to address the nature of the cell wall changes resulting from alteration of lignin composition, we first determined the overall monomeric sugar composition of the AIRs from the wild type, fah-1 and F5H-OE lines by chromatography on a Dionex ICS-2500 system following acid hydrolysis. The composition of sugars in the total plant cell wall extracts was similar in all cases (Fig. 3a). However, the monosaccharide compositions of the water extracts from the different AIRs exhibited significant differences (Fig. 3b). In comparison to the wild-type, the extracts from the fah-1 mutant exhibited increased arabinose/rhammnose, glucose and galacturonic acid, whereas those from the F5H-OE line exhibited increased xylose and mannose.

To determine the changes in cell wall composition at the level of specific polymer types, we performed glycome profiling of solvent extracted AIRs from wild-type Arabidopsis and the two lignin-modified lines (Fig. 4). This technique uses ELISA with a large panel of epitope-specific monoclonal antibodies raised against various cell wall structural components (Pattathil et al., 2012). The glycome profiles for the water soluble cell wall extracts from the F5H-OE and fah-1 mutant were quite similar, showing, in comparison to the wild-type, reduced amounts of arabinogalactan and increased amounts of acetylated glucomannan and some xylan epitopes (Fig. 4a).

We next performed glycome profiling of sequentially extracted cell wall AIRs with a range of solvent treatments of increasing harshness (Fig. 4b), to assess broader differences in cell wall integrity. Analysis of the lower half of the profiles in Fig. 4b indicates that some rhamnoglacturonan/arabinogalactan epitopes were eluted with less harsh treatments from both lignin-modified lines compared to the wild-type. Overall, the glycome profiles indicate significant and differential changes in cell wall integrity leading to extractability of different cell wall polysaccharide fractions as a result of alterations in lignin composition.

Cell wall-derived elicitors are heterogeneous with respect to charge and composition

The components of the water-extracted AIRs were then separated by anion exchange (AE) chromatography coupled with ELISA-based epitope detection (Epitope Detection Chromatography (EDC), to determine the charge heterogeneity of the released polysaccharides. For separation, a two-step gradient was first tested, with the first step from 0-0.3 M and second step from 0.3 to 0.6 M NaCl. Prior to fractionation, the crude water extracts were screened by ELISA against a panel of 30 monoclonal antibodies (listed in Table S3) recognizing either HG, RGI, heteroxylan, xyloglucan, mannan and arabinogalactan protein epitopes). A set of 10 antibodies which generated good signals in the ELISA assays were selected for further analysis of the fractionated extracts, and the results indicated that some of these antibodies recognized epitopes that showed different elution profiles for the extracts from the fah-1 and F5H-OE lines (Fig. S5). These antibodies were then used to assay fractions obtained by a higher resolution AE-EDC method, in which the fractions were eluted from the anion exchange column by a step gradient of 20 %, 30 %, and 40 % followed by 40-100 % of 0.6 M sodium chloride (Fig. 5). There were both qualitative and quantitative differences between the relative amounts of some epitopes in the salt-eluted fractions from the different lines (Fig. 5). For example, extracts from the F5H-OE line exhibited reduced levels of RG-I-derived epitopes in the 20 % fraction (Fig. 5b), whereas the fah-1 extracts contained extra HG- and RG-I-reactive peaks in the 30 % fraction (Fig. 5a).

The fractions from the above separations were de-salted, and their ability to induce the selected defense genes tested in Arabidopsis suspension cells (Fig. 6). Induction of PCC1 was only observed for the 40 % fraction from the fah-1 line. Activity for induction of CYP81D11 by fractionated extracts from the F5H-OE line was found mainly in the 20 % and 40 % fractions.

However, no features of the epitope detection profiles corresponded to the elicitors inducing PCC1 or CYP81D11. Clearly, the epitopes involved in these specific elicitor activities could not be detected with the available antibodies. Conversely, when a new peak was detected by EDC (e.g. the new HG epitope peak in the 30 % extract of the fah-1 line (Fig. 5a), little or no associated elicitor activity was found (Figs 5 and 6). Overall, these data indicate that the released elicitors, at least from F5H-OE, are likely heterogeneous with respect to charge and epitope composition.

Elicitors originate from more than one class of cell wall polysaccharide

To determine whether the elicitor molecules released from cell walls of fah-1 and F5H-OE plants have common or distinct compositional/structural features essential for their function, we examined the effects of enzymatic digestion on the abilities of the cell wall extracts to induce defense genes (Table 2). After incubation and destruction of the added enzymatic activity by autoclaving, the extracts were assayed for ability to induce the defense genes induced by the untreated elicitors. Xyloglucanase specifically eliminated the ability of the extracts from F5H-OE plants to induce expression of CYP8D11, and fucosidase specifically eliminated the ability of the extracts from the fah-1 mutant to induce expression of PCC1 and LURP1. Interestingly, PGase treatment eliminated the ability of extracts from fah-1 cell walls to induce LURP1, but not PCC1, suggesting that different elicitors, or different epitopes on the same elicitor, are required for the induction of these defense genes.

Discussion

Lignin is an important component of both pre-existing and inducible defense responses in plants (Miedes et al., 2014). It is therefore paradoxical that reduction in lignin content (often associated with altered lignin composition) can sometimes enhance rather than reduce plant disease resistance (Gallego-Giraldo et al., 2011b; Zhao and Dixon, 2014). Various hypotheses have been put forward to explain this phenomenon, including antimicrobial activity of lignin pathway intermediates (Barber et al, 2000) and release of elicitor-active signal molecules from incorrectly assembled plant cell walls (Giberti et al., 2012).

The different lignin modified lines examined here exhibited widely different transcriptomic responses, with the most changes and highest fold-changes being observed in the F5H-OE line,

suggesting that modification to highly S-enriched lignin is perceived by the plant as more abnormal than the possession of all G-lignin. Interestingly, none of the differentially induced defense response genes was up-regulated in both F5H-OE and fah-1 lines. Likewise, only one of the 15 cell wall biosynthesis/remodeling genes induced in the F5H-OE line was also induced in fah-1. Deposition of lignin comprised primarily of G units leads to release of signals that activate genes associated with response to oomycete and bacterial pathogens, and deposition of lignin containing predominantly S units generates signals for induction of genes associate with responses to insect herbivory; a cytochrome P450 (CYP81D11) and two GSTs that are known to be induced by cis-jasmone (Matthes et al., 2010), and an elicitor peptide (PROPEP3) that can be released by wounding and is associated with JA-mediated responses to herbivory (Huffaker et al., 2013; Yamaguchi et al., 2011). Although the in vivo substrate of CYP81D11 is not known, constitutive expression of this gene in Arabidopsis thaliana leads to enhanced volatile release and subsequent attraction of parasitoids, mimicking the effects of cis-jasmone application (Bruce et al., 2008). PROPEP3 is a member of a group of endogenous peptide elicitors associated with the amplification of defense responses, hypothesized to be induced through recognition of microbe- or damage-associated molecular patterns (Klauser et al., 2015). This gene is not induced by cis-jasmone; rather, the maize ortholog of PROPEP3 is induced by insect oral secretions, and serves as a signal for subsequent activation of the JA-response pathway (Huffaker et al., 2013). Taken together, our results suggest that cell walls with lignin consisting of predominantly S units are modified in a way that mimics insect feeding via release of a specific elicitor or set of elicitors from cell wall polysaccharides, whereas these elicitors are not readily released from cell walls with lignin consisting of predominantly G units.

The elicitors released in response to modification of lignin composition are not responsible for the totality of the transcriptomic changes in the various low lignin-modified lines. It also cannot be ruled out that one or more metabolites that accumulate as a result of altered flux into the monolignol pathway might themselves act as signals for altered gene expression. Although we could show that cell wall-derived elicitors induce the expression of the elicitor peptide PROPEP3 and CYP81D11, induction of the GSTs, which represented the most highly induced genes relative to the wild-type control in the F5H-OE line, could not be mimicked by application of the corresponding wall-released elicitors to suspension cells. We also cannot rule out the possibility that cell cultures are inherently unresponsive to activation of these genes. However, alternative

assay systems (e.g. cut stem sections) present problems with elicitor application and uptake, as well as potentially confounding wound responses.

Modification of lignin composition through enrichment of G or S units impacts cell wall integrity. In the case of S unit enrichment as a result of F5H-OE overexpression, mannose and xylose levels are increased in the water-soluble fraction of the cell walls, in agreement with the enhanced extractability of xylan and acetylated glucomannann epitopes in glycome profiling. Xyloglucan, the major hemicellulose in the primary cell wall, was the cell wall component with extractability most affected by enrichment of S lignin. In the case of enrichment of G units following loss of function of F5H (fah-1), the cell walls release more arabinose/rhamnose, glucose and galacturonic acid following water extraction. This is consistent with the glycome profiling results, which reveal enhanced extractability of xylan and acetylated glucomannan epitopes as well as pectin. The pectin extracted from fah-1 AIR is enriched in HG, RGI and RGII. Similar increases in water-soluble pectin material have been observed previously with HCT-RNAi lines (Gallego-Giraldo et al., 2011b).

The biological activity of the elicitor fragments released by water extraction of F5H-OE cell walls was destroyed by the action of xyloglucanase but not pectinase, suggesting that the activity is associated with hemicellulosic xyloglucan epitopes, and not with the new peaks of homogalacturonan and RG1 epitopes released from cell walls of this line. The elicitor activity of the water extract from fah-1 cell walls was destroyed by the action of PGase (consistent with the increased extractability of HG, RGI and RGI) and fucosidase. Assuming that this activity resides in one class of molecule, its likely origin is RGII, with its homogalacturonan backbone and α-linked fucose in the A and B side-chains (Mohnen, 2008). Although fucose substitutions are also present in xyloglucan structures, xyloglucanase had no effect on the elicitor activity from fah-1 cell walls, in contrast to destruction of elicitor activity from F5H-OE cell walls. Additional complexity may be present in the case of the elicitors from fah-1 cell walls, because, although α-fucosidase destroyed induction of both AtCC1 and AtLUR, AtCC1 induction was not destroyed by incubation with PGase, indicative of the presence of more than one elicitor species.

Stimuli perceived in the cell wall, for example as a result of altered wall integrity, must be transduced to the nucleus to induce the responses which compensate for and/or alleviate either developmental growth processes or biotic/abiotic stresses (Nuhse, 2012). The lines studied here have altered cell wall composition throughout their lives, and this may result in continuous

signaling to activate the transcription of wall-modifying enzymes, perhaps as a mechanism to adjust the wall structure to compensate for the alterations. It is perhaps serendipitous that the responses to the types of cell wall modification we have studied mimic defense responses to distinct types of biotic stress.

In Fig. 7, we present a model to account for the differential expression of defense genes in plants with altered lignin compositions. Elicitors released in the F5H-OE line, from xyloglucan and AGPs, likely bind to yet to be characterized DAMP (damage associated molecular pattern) receptors, since both the CYP81D11 and PROPEP3 that are induced in this line have been identified as responsive to insect herbivory or damage (Klauser et al., 2015; Ortiz-Morea et al., 2016). However, the GSTs that are highly expressed in the F5H-OE plants are not directly induced by the cell wall-released elicitors, suggesting that additional signal amplification is required for their expression. Elicitors released from the fah-1 line may potentially work through the oligogalacturonide receptors of the type that have recently being characterized (Brutus et al., 2010; Benedetti et al., 2015), although the fact that PCC1 and LURP1 are not induced by simple PGA, and that PGase destroys the ability to induce LURP1 but not PCC1 suggests that the oligogalacturonides reelased from cell walls of the fah-1 mutant are not simple linear galacturonic acid oligomers/polymers. Further genetic analysis with signaling mutants will be necessary to determine which signaling pathways are involved in transducing changes in cell wall composition to generate the final pattern of defense responses.

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Author contributions

R.A.D., L.G.G and S.P.A. planned and designed the research. L.G.G, S.P.A, S.P, A.G.P, M.H, B.G.A, J.P.K and X.R performed experiments and analyzed data. L.G.G. and R.A.D. wrote the paper with input from all authors.

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Supporting information

Fig. S1. The lignin biosynthetic pathway.

Fig. S2. Lignin composition in Arabidopsis plants with different lignin compositions.

Fig. S3. Lignin deposition pattern in Arabidopsis plants with different lignin compositions.

Fig. S4. Heat map analysis comparing the most highly induced genes in the two Arabidopsis lines with different lignin compositions as compared to wild type.

Fig. S5. Epitope detection chromatography (EDC) of cell wall polysaccharides from water extracts from WT, F5H-OE and fah-1 plants.

Dataset S1. Microarray data from F5H-OE and fah-1 mutant plants.

Table S1. Gene-specific primers used for qRT-PCR.

Table S2. qPCR analysis of the ability of cell wall-released elicitors to induce the most highly induced genes in the lignin modified lines.

Table S3. Monoclonal antibodies used in this study and their specificity against cell wall oligosaccharide domains. All antibodies are rat hybridomas, except INRA-RU1 which is a mouse hybridoma mAb.

Legends to figures and tables

Fig. 1. Global gene expression data from stems of Arabidopsis plants with predominantly G lignin (fah-1) and predominantly S-lignin (F5H-OE). Venn diagram comparison from microarray analysis of wild type (WT), F5H-OE and fah-1 mutant.

(a) Numbers of genes differentially up-regulated with respect to wild type

(b) Numbers of genes differentially down-regulated with respect to wild type

Fig. 2. Induction of defense gene expression in Arabidopsis cell cultures in response to water-soluble elicitors from cell walls of wild type (WT) and Arabidopsis plants with predominantly G (fah-1) or S (F5H-OE) lignin.

(a) PCC1 and LURP1.

(b) CYP81D11.

Analysis of transcript levels in cell cultures was by qRT-PCR. Elicitors were prepared from the AIR of cell walls from wild-type plants (WT), F5H over-expressing (F5H-OE) and fah-1 lines. Elicitors were added directly to cell cultures (-), or pre-treated with PGase (+). Polygalacturonic acid (PGA) was also tested as elicitor for a positive control. The transcript analysis was performed with RNA from suspension cells harvested after 12 h of incubation at 25ºC in the dark. mRNA levels are expressed relative to AtPP2A and AtBT. Results are means ± SE of three biological

replicates. Asterisk indicate significant differences from WT ($P < 0.05$) by pairwise multiple comparison Tukey test.

Fig. 3. Sugar composition analysis of cell walls and water-soluble DAMP preparations.

(a) Total monosaccharide contents of non-extracted cell wall AIRs of wild-type (WT) and F5H-OE and fah-1 mutant Arabidopsis lines.

(b) Monosaccharide composition of water soluble extracts from AIR preparations of the above lines. Sugar composition was determined on 500 µL of water extract after sulfuric acid hydrolysis and anion exchange chromatography. Results are means ± SD of three technical replicates from two biological replicates. Ara/Rhamn indicates that arabinose and rhamnose were quantified as one peak). Asterisk indicate significant differences from WT ($P < 0.05$). by pairwise multiple comparison Tukey test

Fig. 4. Glycome profiling indicates that lignin modifications differentially affect cell wall extractability.

(a) Glycome profiles of cell wall polysaccharides from water-extracts

(b) Glycome profiles of cell wall polysaccharides released following sequential extractions o cell wall AIRs.

Glycome profiling was performed on alcohol insoluble cell wall fractions from wild-type plants (WT), and F5H over-expressing (F5H-OE) and fah-1 mutant lines. The cell wall AIR preparations from the lines shown were sequentially extracted with the solvents indicated, and glycome profiling by ELISA was performed with a suite of antibodies targeting the carbohydrate epitopes listed on the right. Heat map shows antibody reactivity, from weak (black) to strong (yellow).

Fig. 5. Anion exchange chromatography with epitope detection (EDC) of cell wall polysaccharides from water extracts of AIR residues from wild-type (WT) and F5H-OE and fah-1 mutant) Arabidopsis lines.

(a) Detection of HG epitopes by post-column ELISA with monoclonal antibodies (LM19 and LM20) specific for HG with low and high degree of esterification respectively.

(b) Detection of RGI epitopes by post-column ELISA with monoclonal antibodies (LM5, BR12-named LM6-M, and INRA-RU1) specific for galactan, arabinan and RGI backbone respectively.

(c) Detection of xylan epitopes by post-column ELISA with monoclonal antibodies (LM11 and LM28) specific for xylan and glucuronoxylan respectively.

(d) Determination of other epitopes by post-column ELISA with monoclonal antibodies (LM25, LM21, LM2) specific for galactosylated xyloglucan, heteromannan and AGP cell wall epitopes respectively.

The stepped salt elution gradient used for the analysis is shown as a dotted line above the chromatographic traces. Data are representative of two chromatographic runs. Further details of antibodies used are given in Table S3.

Fig. 6. Analysis of elicitor activity of the fractions in Fig. 5.

(a) PCC1 induction by fractions from cell walls of fah-1

(b) CYP81D11 induction by fractions from cell walls of F5H-OE

The elicitor activity of selected fractions was determined by measuring their ability to induce defense gene transcripts (PCC1 and CYP81D11) in cell suspension cultures. The qRT-PCR analyses were performed with RNA from suspension cells harvested 12 h post elicitation, and incubated in the dark at 25ºC. Transcript levels are expressed relative to AtPP2A. Results are means ± SE of three biological replicates.

Fig. 7. Model for the release of cell wall-derived elicitors from cell walls with different lignin compositions.

Model shows proposed events in F5H-OE and fah-1 mutant Arabidopsis lines. Lignin composition modifications lead to changes in cell wall integrity. These changes are perceived by the cell either directly through activation of plasma membrane-localized cell wall integrity receptors, or indirectly through mediation of released cell wall components (DAMPs); these initial signals induce the expression of cell wall biosynthesis and remodeling genes, including genes encoding enzymes that degrade specific cell wall components to release further DAMPs. The soluble DAMPs from differentially lignin-modified walls can induce discrete sets of defense response

genes. Abbreviations: WT, wild type; RGI, RGII rhamogalacturonan I and II; AGPs, arabinogalactan proteins. Genes: PCC1, pathogen and circadian controlled; LUPR1, oomycete pathogen mediated; PROPEP3, elicitor peptide 3 precursor; CYP81D11, Cytochrome P450 81D11. Other transcriptomic changes occurring in lignin-modified plants may result from secondary effects. Color coding links cell wall composition and origin of signals in different Arabidopsis lines.

Table 1. Up regulated defense and cell wall remodeling genes in A. thaliana F5H-overexpressor transgenic lines (F5H-OE) and fah-1 mutant plants, as compared to wild type. Transcriptome analysis was by DNA microarray. The list of genes is extracted from Dataset S1.

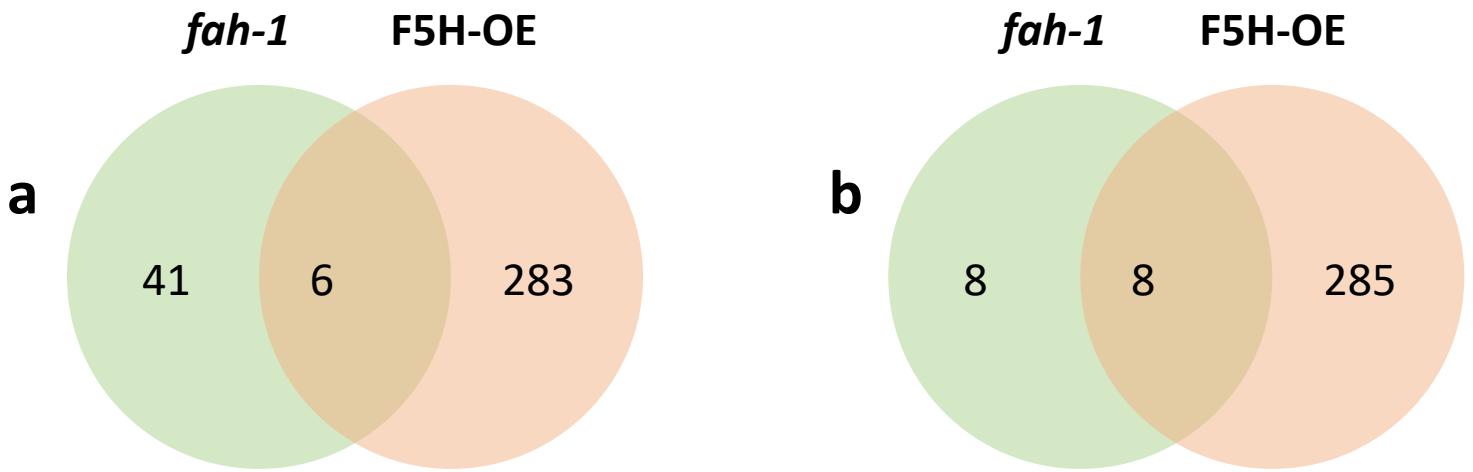
Table 2. Enzymatic digestion of elicitor-active water extracts from cell walls of four lignin-modified Arabidopsis lines. The data summarize defense gene transcript levels as determined by qRT-PCR in cell suspension cultures elicited with non-digested and digested water extracts from cell walls, expressed as ratios for enzyme-treated versus untreated extracts. Scale is from low (red) to high (blue) ratio, where 1.0 equals the transcript level in cultures treated with undigested extract. PCW, plant cell wall. Data are means and standard deviations from duplicate assays.

1. Defense response genes			
Line Gene ID	**F5H-OE** F5H-OE/WT	***fah-1*** *fah-1*/WT	***Gene Annotation***
At4g36220	7.23	0.11	Ferulate -5-hydroxylase (FAH-1)
At5g67080		3.52	MERKK subfamily, MAP kinase 19
At5g64905	5.58		Elicitor peptide 3 precursor (PROPEP3)
At2g14560	0.12	2.37	Related to response to the pathogenic oomycete *H. parasitica* mediated by RPR and RPP5 (LURP1)
At4g14400	0.23	1.37	Related to *Pseudomonas syringae* resistance (ACD6)
At2g29490	14.13	0.85	Glutathione transferase GSTU1. Response to plant stress, herbicide detoxification, and to *cis*-jasmone
At1g17180	24.05	0.72	Glutathione transferase GSTU1. Response to plant stress herbicide detoxification and to *cis*-jasmone
At1g31580	0.29	1.21	ECS1 cell wall associated, linked to a locus influencing resistance to *X. campestris*
At2g40750	0.26	1.58	WRKY54, regulation of defense response
At3g28930	0.48	1.24	AvrRpt2-induced gene after infection with *Pseudomonas syringae* pv. *maculicola*
At1g02450	0.87	2.63	Positive regulator of systemic acquired resistance (SAR)
At4g24350	0.35	1.61	Response to wounding, located in cell wall
At3g22231	0.24	2.07	Pathogen and circadian controlled 1(PCC1), response to *Pseudomonas* syringae pv. *tomato*
At1g06160	0.95	2.52	Ethylene response factor
At5g47220	0.40	1.82	Ethylene responsive element binding factor 2 (ATERF2)
At3g23240	0.69	1.66	Ethylene response factor 1 (ERF1)
At1g28370	0.78	1.82	Ethylene responsive element binding factor
At3g28740	13.81	1.01	CYP81D11, Cytochrome P450. Response to *cis*-jasmone. Over-expression induces synthesis of volatiles that affect chemical ecology and insect interactions
2. Cell Wall related genes			
At5g42830	5.40		HXXXD-type acyl-transferase family protein
At2g22470	4.71		Encodes arabinogalactan-protein (AGP2)
At4g30280	4.63	2.32	Xyloglucan endotransglucosylase/hydrolase (XET)
At4g24430	3.55		Rhamnogalacturonate lyase family protein (TAIR:At1g09890.1)
At1g13130	3.49		Cellulase (glycosyl hydrolase family 5) (TAIR: At3g26140.1)
At3g23730		1.71	Xyloglucan endotransglucosylase/hydrolase 16 (XTH16)
At5g64640	2.98		Plant invertase/pectin methylesterase inhibitor superfamily (TAIR:At5g09760.1)
At1g05310	2.57		Pectin lyase-like superfamily protein (TAIR: At2g36710.1)
At1g57590	2.55		Pectin acetylesterase family protein (TAIR: At5g26670.1)
At1g70500	2.54		Pectin lyase-like superfamily protein (TAIR: At1g23460.1)
At2g46760	2.01		D-arabinono-1,4-lactone oxidase family protein
At5g45280	1.84		Pectin acetylesterase family protein (TAIR: At4g19410.1)
At3g10720		1.50	Plant invertase/pectin methylesterase inhibitor superfamily
At5g56540	1.77		Arabinogalactan protein (AGP14). Mutants exhibit longer root hairs.

Table 2. Enzymatic digestion of elicitor-active water extracts from cell walls of four lignin-modified Arabidopsis lines. The data summarize defense gene transcript levels as determined by qRT-PCR in cell suspension cultures elicited with non-digested and digested water extracts from cell walls, expressed as ratios for enzyme-treated versus untreated extracts. Scale is from low (red) to high (blue) ratio, where 1.0 equals the transcript level in cultures treated with undigested extract. PCW, plant cell wall. Data are means and standard deviations from two replicate assays.

		Defense genes	***AtCYP81D11***	***AtCC1***	***AtLUR1***
		Source of elicitor preparation	**F5H-OE**	***fah-1***	***fah-1***
PCW target	*PCW enzyme*	*Digestion mode or linkage*			
Hemicellulose	xyloglucanase	endo-β-1,4-glucanase.	0.09±0.01	0.99±0.06	0.86±0.02
RGI	arabinanase	arabinan endo-1,5-α-L-arabinosidase.	0.37±0.01	2.2±0.09	0.36±0.01
RGI	galactanase	arabinogalactan 4-β-D-galactanohydrolase.	1.5±0.06	1.3±0.01	0.27±0.01
HG	polygalacturonase	endo-polygalacturonanase M2	1.4±0.03	0.71±0.01	0.1±0.001
RGII	fucosidase	α-L-fucoside	1.2±0.02	0.05±0.001	0.08±0.01

Fig. 1. Global gene expression data from stems of Arabidopsis plants with predominantly G lignin *(fah-1*) and predominantly S-lignin (F5H-OE). Venn diagram comparison from microarray analysis of F5H-OE and *fah-1* mutant, as compared to wild type.
(**a**) Numbers of genes differentially up-regulated with respect to wild type
(**b**) Numbers of genes differentially down-regulated with respect to wild type



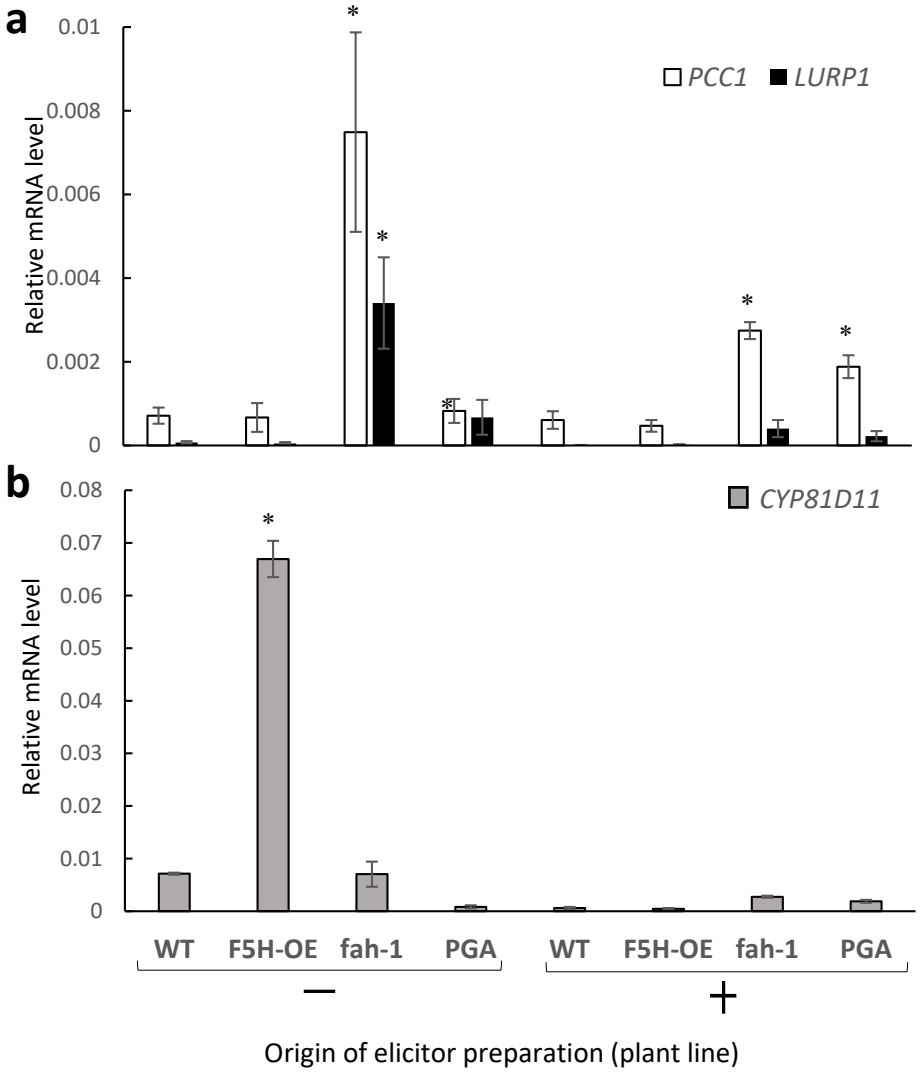


Fig. 2. Induction of defense gene expression in Arabidopsis cell cultures in response to water-soluble DAMPs from cell walls of wild type (WT) and lignin-modified Arabidopsis plants.
(a) *PCC1* and *LURP1*, **(b)** *CYP81D11*.
Analysis of transcript levels in cell cultures was by qRT-PCR. DAMPs were prepared from the AIR of cell walls from wild-type plants (WT), F5H over-expressing (F5H-OE) and *fah-1* lines. DAMPs were added directly to cell cultures (-), or pre-treated with PGase (+). Polygalacturonic acid (PGA) was also tested as elicitor for a positive control. The transcript analysis was performed with RNA from suspension cells harvested after 12 h of incubation at 25ºC in the dark. mRNA levels are expressed relative to *AtPP2A* and *AtBT*. Results are means ± SE of three biological replicates. Asterisk indicate significant differences from WT ($P < 0.05$) by pairwise multiple comparison Tukey test.

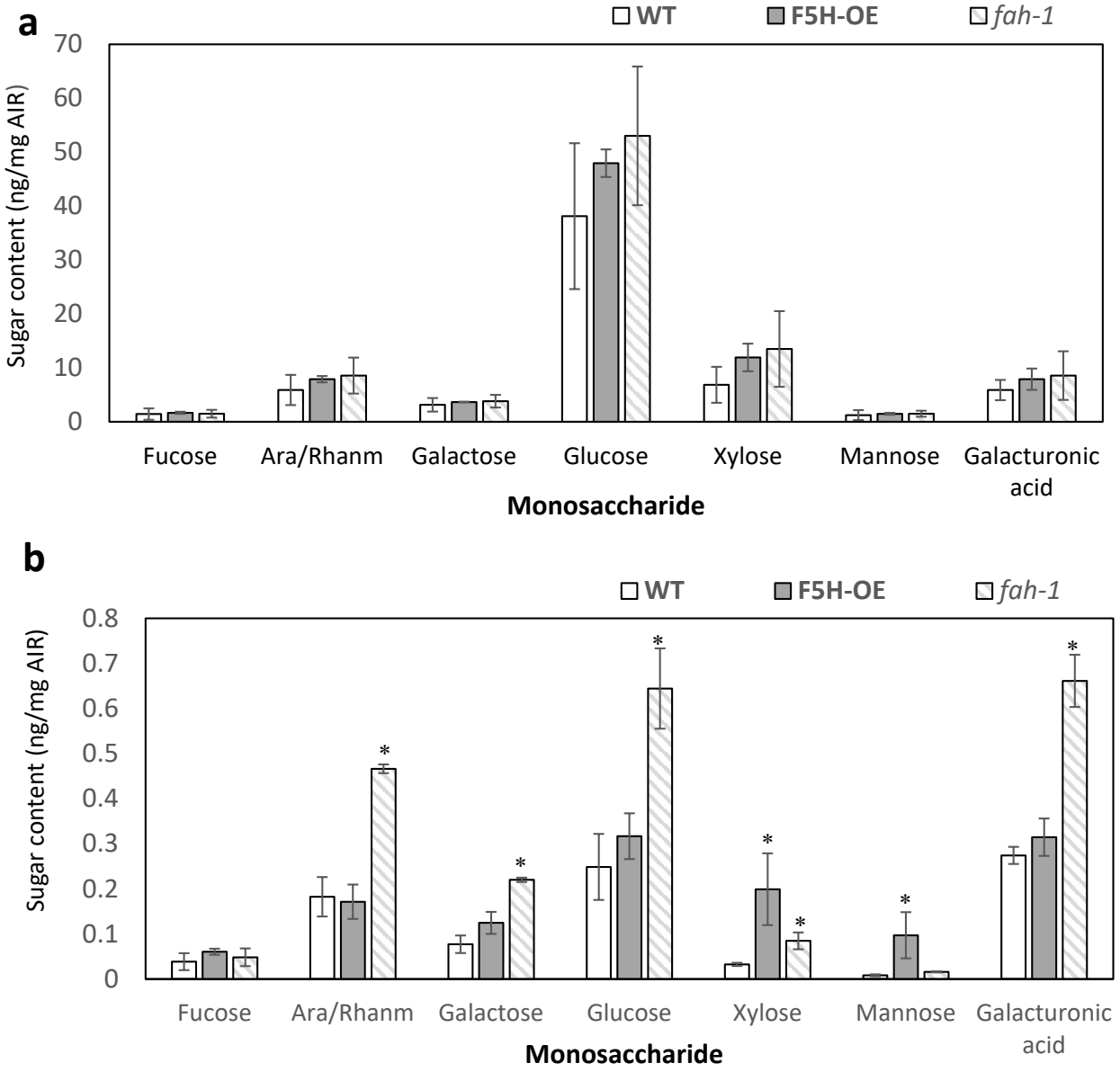


Fig. 3. Sugar composition analysis of cell walls and water-soluble DAMP preparations.
(a) Total monosaccharide contents of non-extracted cell wall AIRs of wild-type (WT) and lignin modified (HCT-RNAi, F5H-OE, *ccr-1* mutant and *fah-1* mutant) Arabidopsis lines.
(b) Monosaccharide composition of water soluble extracts from AIR preparations of the above lines. Sugar composition was determined on 500 µL of water extract after sulfuric acid hydrolysis and anion exchange chromatography. Results are means ± SD of three technical replicates from two biological replicates. Ara/Rhamn indicates that arabinose and rhamnose were quantified as one peak). Asterisk indicate significant differences from WT ($P < 0.05$). by pairwise multiple comparison Tukey test

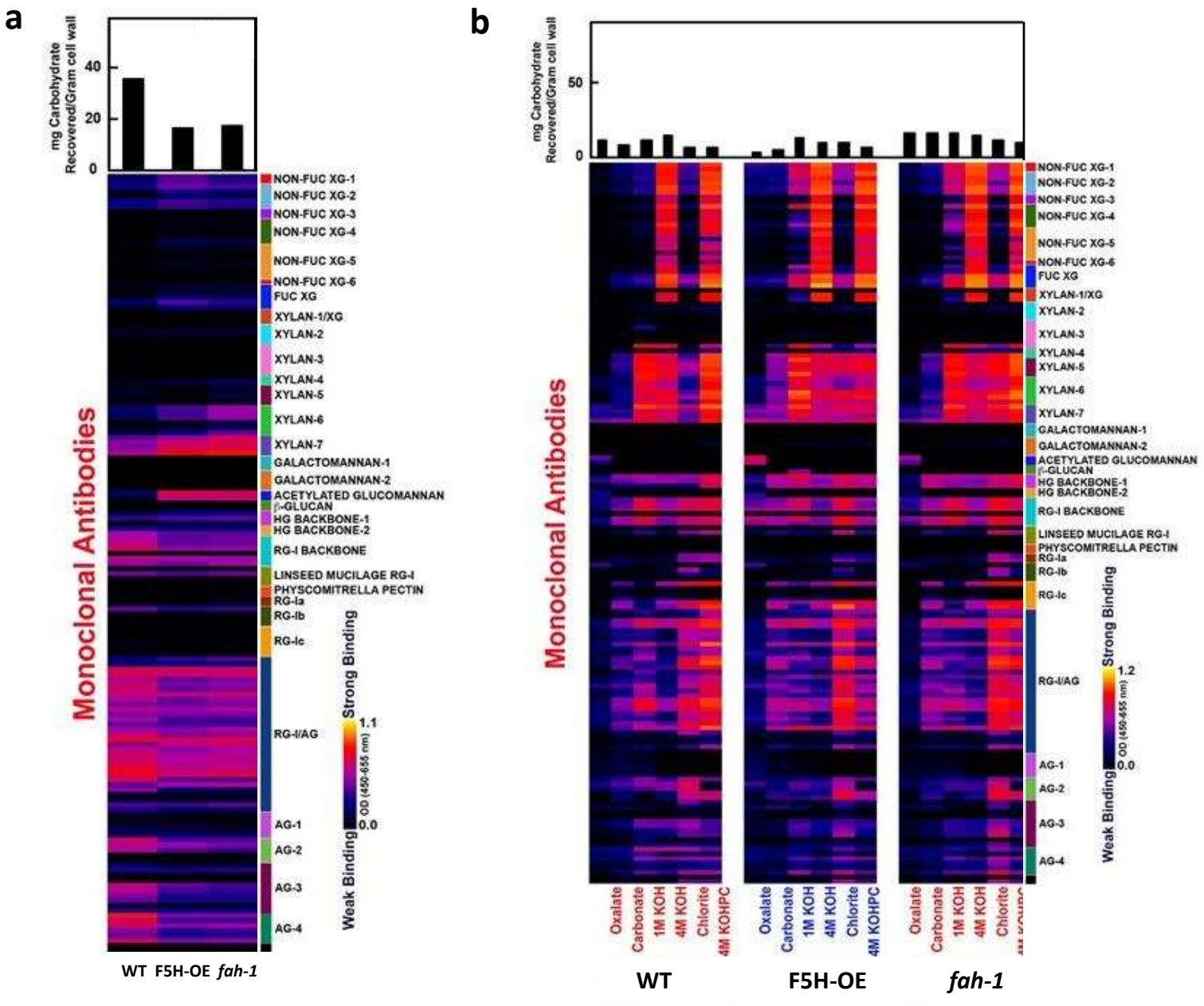


Fig. 4. Glycome profiling indicates that lignin modifications differentially affect cell wall extractability.

(**a**) Glycome profiles of cell wall polysaccharides from water-extracts

(b) Glycome profiles of cell wall polysaccharides released following sequential extractions of cell wall AIRs.

Glycome profiling was performed on alcohol insoluble cell wall fractions from wild-type plants (WT), HCT-RNAi, *ccr-1* mutant, F5H over-expressing (F5H-OE) and *fah-1* mutant lines. The cell wall AIR preparations from the lines shown were sequentially extracted with the solvents indicated, and glycome profiling by ELISA was performed with a suite of antibodies targeting the carbohydrate epitopes listed on the right. Heat map shows antibody reactivity, from weak (black) to strong (yellow).

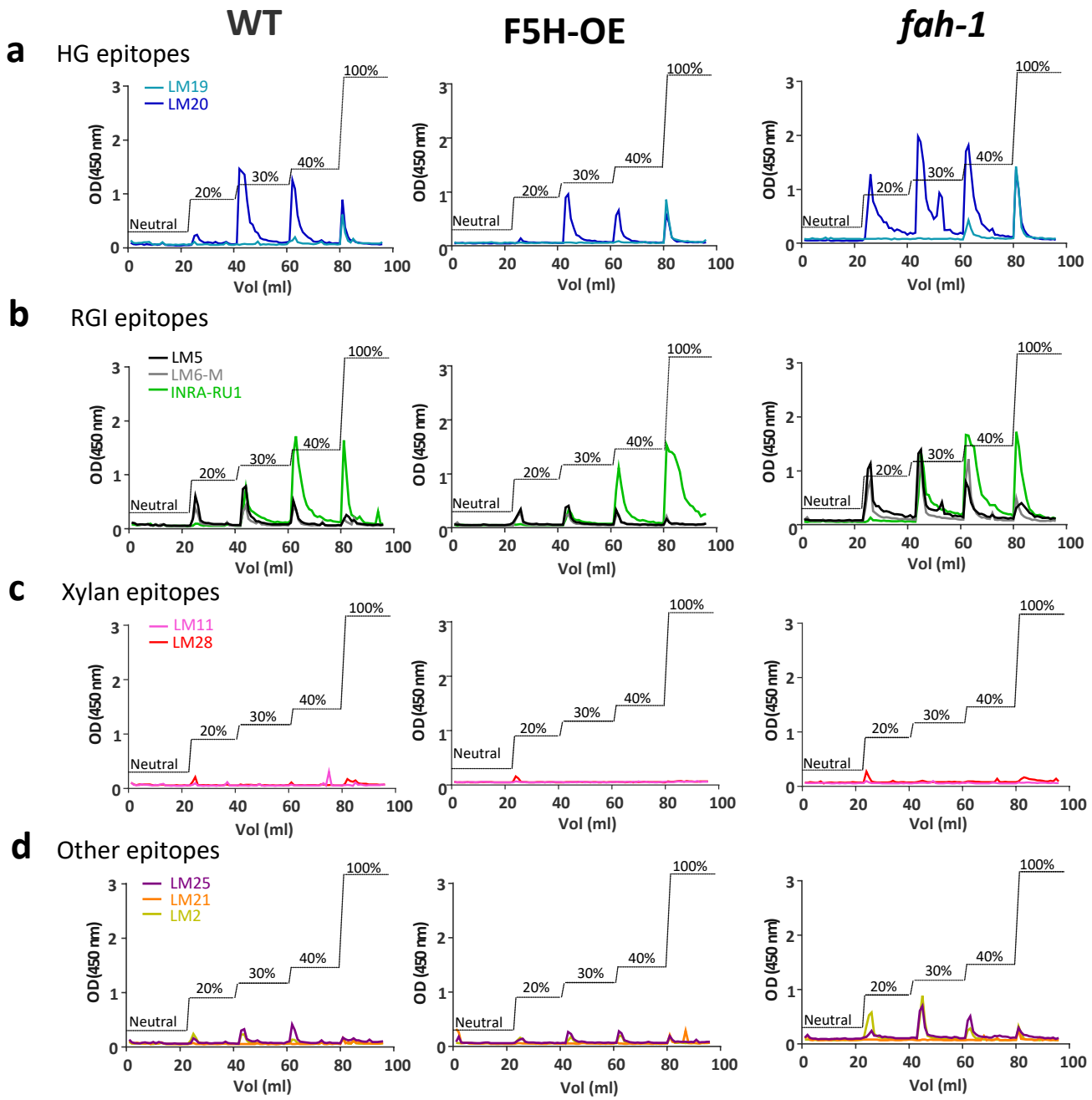


Fig. 5. Anion exchange chromatography with epitope detection (EDC) of cell wall polysaccharides from water extracts of AIR residues from wild-type (WT) and F5H-OE and *fah-1* mutant Arabidopsis lines. **(a)** Detection of HG epitopes by post-column ELISA with monoclonal antibodies (LM19 and LM20) specific for HG with low and high degree of esterification respectively; **(b)** Detection of RGI epitopes by post-column ELISA with monoclonal antibodies (LM5, BR12-named LM6-M, and INRA-RU1) specific for galactan, arabinan and RGI backbone respectively. **(c)** Detection of xylan epitopes by post-column ELISA with monoclonal antibodies (LM11 and LM28) specific for xylan and glucuronoxylan respectively. **(d)** Determination of other epitopes by post-column ELISA with monoclonal antibodies (LM25, LM21, LM2) specific for galactosylated xyloglucan, heteromannan and AGP cell wall epitopes respectively. The stepped salt elution gradient used for the analysis is shown as a dotted line above the chromatographic traces. Data are representative of two chromatographic runs. Further details of antibodies used are given in Supplemental Table 3.

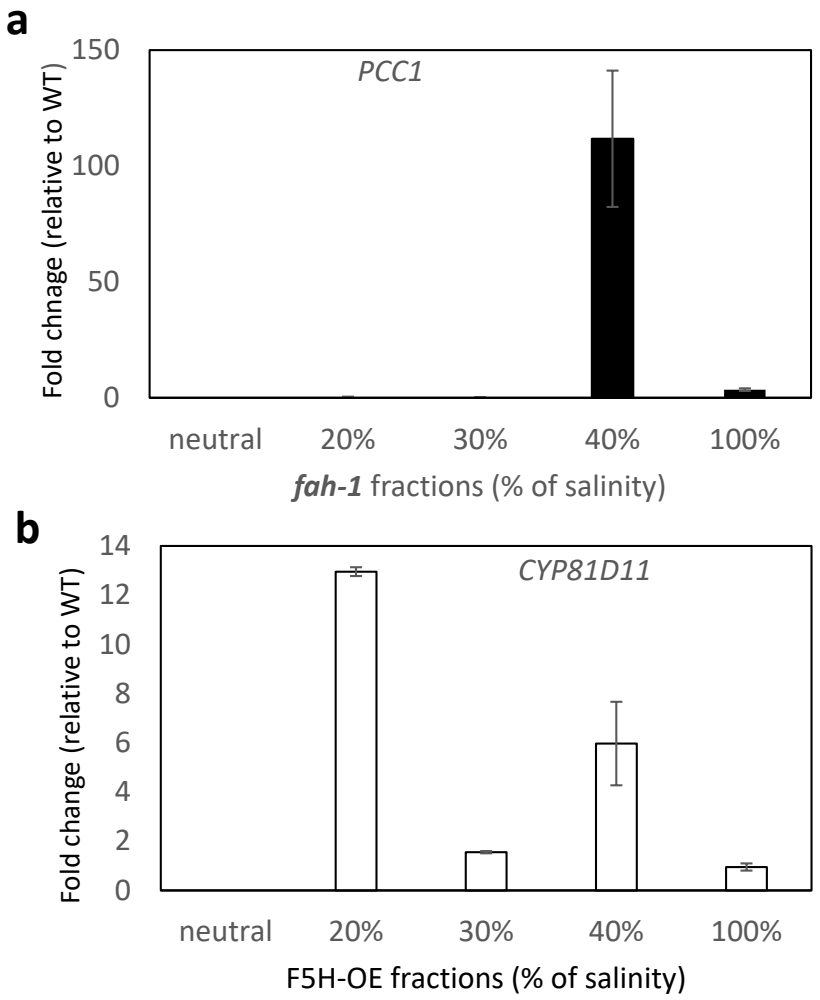


Fig. 6. Analysis of elicitor activity of the fractions in Figure 6
(a) *PCC1* induction by fractions from cell walls of *fah-1*
(b) *CYP81D11* induction by fractions from cell walls of F5H-OE
The elicitor activity of selected fractions was determined by measuring their ability to induce defense gene transcripts (*PCC1* and *CYP81D11*) in cell suspension cultures. The qRT-PCR analyses were performed with RNA from suspension cells harvested 12 h post elicitation, and incubated in the dark at 25ºC. Transcript levels are expressed relative to *AtPP2A.* Results are means ± SE of three biological replicates.

Fig. 7.

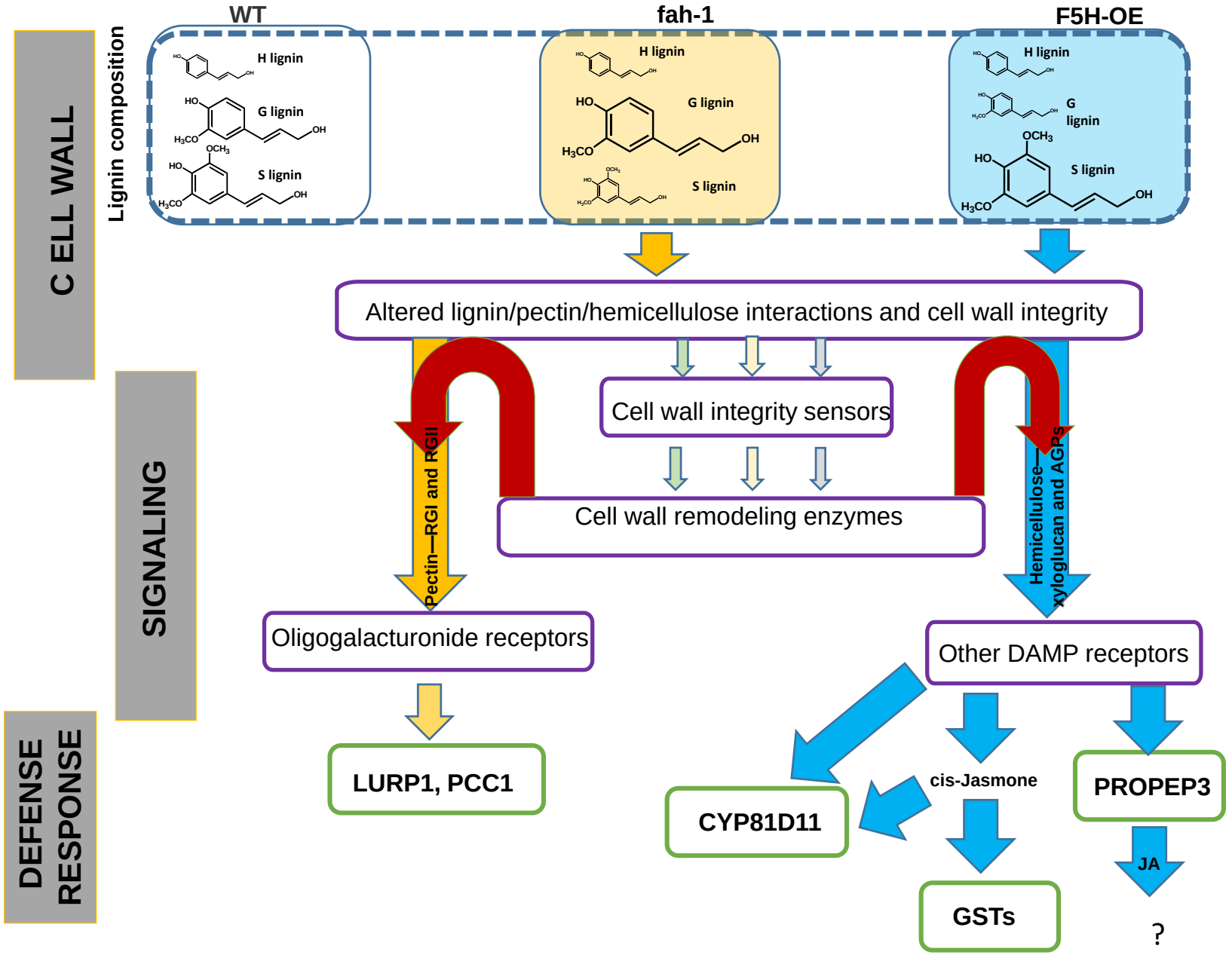

C ELL WALL
Lignin composition
WT
H lignin
G lignin
S lignin
fah-1
H lignin
G lignin
S lignin
F5H-OE
H lignin
G lignin
S lignin
Altered lignin/pectin/hemicellulose interactions and cell wall integrity
Cell wall integrity sensors
Cell wall remodeling enzymes
SIGNALING
Pectin—RGI and RGII
Hemicellulose—xyloglucan and AGPs
Oligogalacturonide receptors
Other DAMP receptors
DEFENSE RESPONSE
LURP1, PCC1
CYP81D11
cis-Jasmone
PROPEP3
JA
GSTs
?

Fig. 7. Model for the release of cell wall-derived DAMPs in lignin-modified cells.
Model shows proposed events in F5H-OE and *fah-1* mutant Arabidopsis lines. Lignin composition modifications lead to changes in cell wall integrity. These changes are perceived by the cell either directly through activation of plasma membrane-localized cell wall integrity receptors, or indirectly through mediation of released cell wall components (DAMPs); these initial signals induce the expression of cell wall biosynthesis and remodeling genes, including genes encoding enzymes that may degrade specific cell wall components to release further DAMPs. The soluble DAMPs from differentially lignin-modified walls can induce discrete sets of defense response genes. Abbreviations: WT, wild type; RGI, RGII rhamogalacturonan I and II; AGPs, arabinogalactan proteins. Genes: PCC1, pathogen and circadian controlled; LUPR1, oomycete pathogen mediated; PROPEP3, elicitor peptide 3 precursor; *CYP81D11,* Cytochrome P450 81D11; JA, jasmonic acid. Color coding links cell wall composition and origin of signals in different Arabidopsis lines.