Cellular and Genetic Regulation of Coniferaldehyde Incorporation in Lignin of Herbaceous and Woody Plants by Quantitative Wiesner Staining

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Lignin accumulates in the cell walls of specialized cell types to enable plants to stand upright and conduct water and minerals, withstand abiotic stresses, and defend themselves against pathogens. These functions depend on specific lignin concentrations and subunit composition in different cell types and cell wall layers. However, the mechanisms controlling the accumulation of specific lignin subunits, such as coniferaldehyde, during the development of these different cell types are still poorly understood. We herein validated the Wiesner test (phloroglucinol/HCl) for the restrictive quantitative in situ analysis of coniferaldehyde incorporation in lignin. Using this optimized tool, we investigated the genetic control of coniferaldehyde incorporation in the different cell types of genetically-engineered herbaceous and woody plants with modified lignin content and/or composition. Our results demonstrate that the incorporation of coniferaldehyde in lignified cells is controlled by (a) autonomous biosynthetic routes for each cell type, combined with (b) distinct cell-to-cell cooperation between specific cell types, and (c) cell wall layer-specific accumulation capacity. This process tightly regulates coniferaldehyde residue accumulation in specific cell types to adapt their property and/or function to developmental and/or environmental changes.

Keywords: lignin, in situ quantification, coniferaldehyde, Wiesner test, phloroglucinol/HCl, cellular networks, image analysis

INTRODUCTION

Acquired by vascular plants 450 million years ago during the colonization of land (Edwards and Axe, 2000), lignin is deposited in the different cell wall layers of specific cell types to increase their structural rigidity, resistance to degradation, and/or impermeability (Vance et al., 1980; Naseer et al., 2012; Barros et al., 2015). Lignin is believed to result from the random coupling of phenoxy radicals, formed...
by phenoloxidases (such as peroxidases), of predominantly C₆C₃ monomers with different C₆ phenolic ring substitutions (hydroxyl, methoxyl, or none in 3 and 5 positions of the C₆ ring) and different C₃ aliphatic functions (acid, aldehyde, and alcohol; Boerjan et al., 2003). The specific changes in the C₆ and C₃ groups of lignin monomers are due to sequential and branching enzymatic steps grouped in a complex biosynthetic pathway (Figure S1). The concentration of lignin and its monomeric composition change between plant species, tissues, cell types, and different cell wall layers during development (Decou et al., 2019; Pesquet et al., 2019). In angiosperm xylem/wood, lignin concentration is high in primary cell wall layers, intermediate in secondary cell wall layers of vessels, and low in secondary cell wall layers of fibers (Terashima et al., 2012; Serk et al., 2015). Furthermore, the primary cell walls are enriched in C₆ phenolic unsubstituted residues (called p-hydroxyphenyl or H-units), vessel secondary cell walls in C₆ mono-methoxylated phenolic residues (called guaiacyl or G-units), and fiber secondary cell walls in C₆ di-methoxylated phenolic residues (called syringyl or S-units) (Vanholme et al., 2010). Yet, it is still undefined if the different C₆ aliphatic functions are as tightly spatially controlled as specific C₆ ring substitutions. Moreover, it remains unknown whether C₆C₃ residue(s) with both specific C₆ ring substitution and distinct C₃ aliphatic function are incorporated in different ways in various cell types and cell wall layers.

Herein, we investigated the developmental and genetic regulations controlling the accumulation of coniferaldehyde, a specific C₆C₃ residue with a G ring substitution and C₃ aldehyde function, at cellular and sub-cellular levels in herbaceous and woody plants. Changes in amount of coniferaldehyde residues were suggested to alter lignin biochemical, physical, and mechanical properties (Sibout et al., 2005; Holmgren et al., 2009; Fu et al., 2011; Fornalé et al., 2012; Bouvier D’Yvoire et al., 2013; Van Acker et al., 2017; Wang et al., 2018). We thus measured coniferaldehyde accumulation at the cellular levels by improving one of the oldest and most widely used histochemical methods for lignin detection, the Wiesner test (or phloroglucinol/HCl). Contrary to previous belief, we demonstrated its specificity to coniferaldehyde residues incorporated not only at the ends but also within lignin polymers, and also showed that synthetic C₆C₃ monomers reacted differently than C₆C₃ polymers to the Wiesner test. We hence established the quantitative capacity and set the high spatial resolution of this in situ method. This optimized method was used to unravel the genetic, cellular, and developmental regulation controlling the incorporation of coniferaldehyde into lignin. We thus identified for each cell type which genetic restriction(s) affected coniferaldehyde accumulation. Our findings demonstrate that coniferaldehyde incorporation into lignin during development depends on a combination of autonomous biosynthetic routes for each cell type, specific cell-to-cell cooperation between adjacent cells, and varying accumulation capacities of specific cell wall layers. Our study shows that coniferaldehyde accumulation is tightly controlled for each different lignified cell type to allow their specific cellular function(s).

**MATERIALS AND METHODS**

**Plant Material**

*Arabidopsis thaliana* plants were grown from seeds on 1:3 (v:v) vermiculite/soil and hybrid poplar in sterile magenta™ boxes on 0.5 × MS medium (Duchefa, M0222.0050) with 0.4% phytagel (Sigma, P8169) in controlled growth chambers under a 16/8 h and 22°C/18°C photoperiod with 60% humidity and 150 µmol m⁻² s⁻¹ illumination. *Arabidopsis* mutants in the Columbia-0 background included: 4cl-1 (SALK_142526; Van Acker et al., 2013), 4cl2-4 (SALK_110197; Li et al., 2015), ccr-3-3 (SALK_123-689; Mir Derikvand et al., 2008), cad4 (cad-c; SAIL_1265_A06; Lee et al., 2017), cad5 (cad-d; SAIL_776_B06; Lee et al., 2017), ecoomt1 (SALK_151507; Kai et al., 2008), fah1 (EMS mutant; Meyer et al., 1998), ont1 (SALK_135290; Tohge et al., 2007), and double mutants 4cl1-1x4cl2-4, ccr1-3xlah1 and cad4xsd5. All *Arabidopsis* plants were genotyped to select homozygous mutants using PCR with specific primer pairs (Table S1). Transgenic *Populus tremula × tremuloides* hybrid clones T89 were transformed and micro-propagated every 3 to 4 months as previously described by Nilsson et al. (1992) with 35S driven RNA interference constructs either targeting genes *CINNAMATE-4-HYDROXYLASE* (Potri.013G157900; Bjurhager et al., 2010) or *CINNAMOYL-COA REDUCTASE* (Potri.003G181400). Characterization of silencing efficiency, developmental and biomass changes in the transgenic poplar lines used has been previously reported in Escamez et al. (2017) and the best line for each construct was selected. Two centimeters portions of stem base (8 weeks after germination for *Arabidopsis* and 4 months after micropropagation for poplar) were stored in 70% ethanol for sectioning. 2-3 cm wide branches of mature spruce trees (*Picea abies*) were harvested from the university common garden.

**Chemicals**

Phenolic compounds included H-based compounds: C₆C₃-acid/p-hydroxybenzoic acid (Aldrich, H20059), C₆C₅-aldehyde/p-coumaldehyde (Toronto Research Chemicals, C755450), and C₆C₃-acid/p-coumaric acid (Sigma, C9008); G-based compounds: C₆C₁-aldehyde/vanilluin (Sigma-Aldrich, V1104), C₆C₁-acid/vanillic acid (Sigma-Aldrich, 94770), C₆C₅-aldehyde/coniferaldehyde (Aldrich, 382051), C₆C₅-alcohol/conifer alcohol (Aldrich, 223735), C₆C₅-aldehyde/furic acid (Aldrich, 12870-8), and C₆C₅-ester/ethyl ferulate (Aldrich, 320617); and S-based compounds: C₆C₁-aldehyde/syringaldehyde (Aldrich, S7602), C₆C₁-acid/syringic acid (Sigma, S6881), C₆C₅-aldehyde/sinapaldehyde (Sigma-Aldrich, 382159), C₆C₅-alcohol/sinapyl alcohol (Aldrich, 404586), and C₆C₅-acid/sinapic acid (Aldrich, D7927). LC grade solvents were: N,N-dimethylformamide (Sigma-Aldrich, 270547), acetonitrile (EMD Millipore, 100029), methanol (EMD Millipore, 106035), and formic acid (EMD Millipore, 533002), as well as lithium chloride (Aldrich, 203637), all purchased from Sigma-Aldrich. G C₆C₃-aldehyde without a β-unsaturation (dihydroconiferylaldehyde) was prepared using Pd-catalyzed hydrogenation of coniferylaldehyde in moderate yield. The
Wiesner reagent consisted of 1% phloroglucinol (Sigma, P3502) in 99.5% ethanol mixed with 12 M HCl (1:1, v:v).

**DHP Synthesis**

Dehydrogenation polymers (DHPs) were synthesized using the Zulauf method by incubating 6 mM of monomer with 5 µg ml⁻¹ horseradish peroxidase (Sigma-Aldrich, P8375) and 5 mM H₂O₂ (Sigma-Aldrich, 95299) in 10 mM sodium phosphate buffer (Sigma-Aldrich, S0876) at pH 6. The mixture was incubated at room temperature overnight under 24 rpm (rotation/min) using a Mini LabRoller H5500 (Labnet, USA). DHPs were purified by centrifugation at 13,300 g, washing the pellet twice in ultrapure water then resuspended in methanol.

**Spectrophotometric Analyses**

Liquid spectrophotometric analyses were performed using a Hitachi U2000 spectrophotometer (Shimadzu Co., Kyoto, Japan) for (i) monomers and DHPs before and after mixing with equal parts of Wiesner reagent, as well as (ii) for the collected LC fractions before and after adding equal parts of 6 M HCl. Solid spectrophotometric analyses were performed by drying 200 µl of DHPs onto Whatman parts of 6 M HCl. Solid spectrophotometric analyses and after mixing with equal parts of Wiesner reagent, as well as

**Liquid Chromatography Techniques**

For HPLC-DAD, reaction products of phenolic monomers or DHPs were synthesized in a Prominence LC system (Shimadzu Co., Kyoto, Japan) equipped with a Restek Raptor™ Biphenyl column (2.7 µm, 150 × 4.6 mm)/Restek Raptor C18 column-guard (2.7 µm, 5 × 4.6 mm) kept at 40°C, and separated with a mobile phase gradient of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at a flow rate of 0.6 ml min⁻¹: initial condition, 20% B; to 15 min, 30% B; to 2 min, 34% B; to 40 min, 44% B; to 50 min, 50% B, to 57.5 min, 99% B, to 58.5 min, back to initial conditions for equilibration. Elution was monitored using a SPD-M20A Diode Array Detector (DAD) at 280 nm with the flow cell kept at 40°C. Peak integration analyses were made using LabSolution v5.87 (Shimadzu Co., Kyoto, Japan). Fractionation of eluting compounds was made using a FRC-10A fraction collector set at 300 µl per peak.

UPLC-MS/MS analyses were carried out using an Acquity UPLC system coupled to a Xevo TQ mass spectrometer (MS) under the control of Masslynx software (Waters Co., Milford, MA, USA). Samples were separated on an Acquity UPLC BEH C18 column (1.7 µm, 150 × 2.1 mm)/Acquity UPLC BEH C18 VanGuard precolumn (1.7 µm, 5 × 2.1 mm) kept at 40°C (Waters Co., Milford, MA, USA). A mobile phase gradient at a flow rate of 300 ml min⁻¹ used (A) 0.1% formic acid in water/acetonitrile (99:1, v:v) and (B) 0.1% formic acid in acetonitrile/water (99:1, v:v); initial condition, 5% B; to 2 min, 10% B; to 30 min, 30% B; to 40 min, 50% B; to 43 min, 100% B; to 46 min, back to initial conditions for equilibration. Compound detection by MS was performed with an electrospray ionization source in negative ion mode with the following settings: capillary voltage, 2.4 kV; cone voltage, 22 V; desolvation temperature, 400°C; cone gas flow, 0 l h⁻¹; desolvation gas flow, 800 l h⁻¹. MS scans were first recorded between 50 and 1,000 m/z to detect peaks, which were integrated for quantification. The pseudo-molecular ions corresponding to the detected peak were selected for MS/MS fragmentation in daughter scan centroid mode using: collision gas flow, 0.15 ml min⁻¹; collision energy, 20 V; cone voltage, 25 V; and scan time, 0.5 s. MS/MS spectra were compared with pure standards of phenolic monomers and unknown peaks were identified based on their MS/MS fragmentation pattern and per se reaction properties of the compounds.

The m/z of the different phloroglucinol-conjugates with phenolic aldehydes included: (i) H⁺-vanillyl-phloroglucinol (Figure S2F): 125(60), 135 (100); (ii) H⁺-syringyl-phloroglucinol (Figure S2G): 125(66), 165(100); (iii) coniferyl-(γ-)diphloroglucinol (Figure S2A): 285(100), 271(34), 161(70), 125(58); (iv) H⁺-coniferyl-(γ-)phloroglucinol (Figure S2B): 161(78), 125(38); and (v) diconiferyl-(γ-)diphloroglucinol (Figure S2D): 285(100), 446(58), 125(40).

For GPC-RID, DHPs were analyzed using a Prominence LC system (Shimadzu Co., Kyoto, Japan) on a PSS GRAM column (10 µm, 8 × 300 mm)/PSS GRAM precolumn (10 µm, 8 × 50 mm) kept at 50°C with a mobile phase made of 1% LiCl (Sigma-Aldrich, L9650) in dimethylformamide at a flow rate of 0.6 ml min⁻¹. Elution was monitored using a RID-20A Refractive Index Detector with flow cell at 50°C. Determination ofMp, Mn, Mw, PDI, and percentage contribution were conducted using LabSolution v5.87 with the GPC add-on and calibrated using ReadyCal-Kit Poly(styrene) low (PSS-pskitr4l) with Mp = 266–66,000 Da (PSS Polymer Standards Service GmbH, Germany).

**Histochemical Analysis**

Interfering pigments and extractives were removed by incubating cross-sections in 70% ethanol for several days before staining. Stems were embedded in 10% agarose (Sigma-Aldrich, A9539) and sectioned using a VT1000 vibratome (Leica, Sweden). Transverse cross-sections were stored in water at 4°C. Sections were mounted in water between a 1 mm thick microscopy glass-slide and a 150 µm thick glass coverslip and imaged. The cover slip was then removed and the section stained by adding 50 µl of Wiesner reagent before re-placing the cover slip. Live imaging was acquired using an Olympus BX60 brightfield microscope equipped with an Olympus UPLFN 40X objective (NA 0.75), an Olympus XC30 CCD color camera and yellow-corrected with a day light balanced filter (Olympus LBD, Japan). Irradiance, red/green/blue (RGB) adjustment, and gamma correction were kept constant for all image acquisitions.

**Image Analysis**

Real-time live imaging measurements included (i) the hue from HSV images to evaluate the color, as well as (ii) the optical density or absorbance from the 8-bit images. Acquired images were analyzed using ImageJ (Schindelin et al., 2012) by (i) compilation into single image stacks, (ii) registration using the
Arabidopsis (Goujon et al., 2003), as follows: CML, central 500 wall layers, according to previous studies performed IFs in impact across genotypes. The arrows were shaded according to the average of this relative absorbance of the cell type when adjacent to another cell type. average absorbance within one cell type to the average cooperativity in each mutant was expressed by the ratio of adjacent to themselves was then subtracted from

Supplementary Data Sheet 2 normalized cell wall width. All measurements are available in the

The Pearson correlation coefficient between pairs of cell types when adjacent to each other or themselves. In each cell type, the Wiesner stain absorbance was measured in cell walls directly adjacent to other cell types, as well as cell walls adjacent to the same cell type. The absorbance was averaged over 20 points of 4 pixels in five biological replicates. The Pearson correlation coefficient r between two cell types when adjacent to themselves was then subtracted from r when one cell type was adjacent to the other. The relative effect of this cooperativity in each mutant was expressed by the ratio of average absorbance within one cell type to the average absorbance of the cell type when adjacent to another cell type. The arrows were shaded according to the average of this relative impact across genotypes.

**Raman Confocal Microscopy** Raman spectra of interfascicular fiber, xylary fiber, and metaxytem vessel cell walls were acquired on 5 µm thick stem cross-sections, mounted in water between glass slide and coverslip, using a 100x objective (NA 0.9) using a confocal Raman microscope (Raman Touch-VIS-NIR, Nanophoton, Japan) with a 522 nm laser of 5 mW power. The linearly polarized laser light was focused on a 1 µm diameter spot of the secondary cell walls, avoiding cell corners and middle lamella. Spectra were measured using a CCD camera (Gatan Orius200D) behind a grating spectrometer (1,200 grooves mm⁻¹), from 80 to 4000 cm⁻¹ wavenumber bands with a spectral resolution of 1.6 cm⁻¹, and analyzed using the RAMAN Viewer software (Nanophoton, Japan) with baseline correction and smoothing.

**RESULTS** Specific Detection of Coniferaldehyde Residues in Lignin Using the Wiesner Test Although the Wiesner test has been widely used for 140 years, its target(s) and efficiency remain uncertain as it does not reflect total lignin amount (Black et al., 1953) or aldehyde residues in lignin (Adler and Ellmer, 1948; Kim et al., 2002). However, many lignin monomers have been shown to react positively to the Wiesner test such as G C₆C₃ aldehyde (coniferaldehyde), S C₆C₅ aldehyde (sinapaldehyde), H C₆C₅ aldehyde (p-coumaraldehyde), G C₆C₅ without function (eugenol), and G C₆C₅ alcohol (coniferyl alcohol) as well as various G and S C₆C₅ aldehydes (Adler and Ellmer, 1948; Black et al., 1953; Ishikawa and Ide, 1954; Bland, 1966; Geiger and Fuggerer, 1979; Pomar et al., 2002; Kim et al., 2002; Varbanova et al., 2011). To solve this conundrum and determine the exact target(s) and chemical reaction behind the Wiesner test, monomer analogues were used to monitor the production of chromatophore(s), their structure and stability as well as their absorbance. Monomers tested included C₆C₅ and C₆C₅ compounds with differences in (i) the substitution of their C₆ phenolic rings (H, G, or S), and/or (ii) in the terminal function (acid, aldehyde or alcohol) of their C₃ aliphatic chains. Liquid chromatography (LC) analysis before and after staining showed that compounds with C₃ aldehyde and alcohol but not acid could form condensation products with phloroglucinol (Figure S2A). However only C₆C₅ aldehydes produced the typical Wiesner purple chromatophore(s) at λₘₐₓ = 525 nm (hue = 343°) for H, λₘₐₓ = 550 nm (hue = 310°) for G, and λₘₐₓ = 561 nm (hue = 320°) for S (Figure 1A). The importance of the unsaturation in the C₃ chains was determined using dihydroconiferaldehyde, which, although condensation occurred (Figure S2A), did not allow the purple chromatophore to form (Figure 1A). Analysis using LC with tandem mass spectrometry (MS/MS) showed that the purple chromatophore(s) corresponded to resonance forms of H⁻-coniferyl-γ-phloroglucinol for G C₆C₅ aldehyde and H⁻-
FIGURE 1 | Reactivity of phenolic monomers to the Wiesner test. (A) Image acquired on the reactivity of 2.5 nmol of compound solubilized in methanol 1 min after adding either 6 M HCl (unstained), 0.5% phloroglucinol/6 M HCl (stained), 0.5% phloroglucinol/6 M HCl followed by 1 min neutralization with 6 M NaOH (neutralized) and followed by re-staining for 10 min in 6 M HCl (re-stained). (B) LC profiles of coniferaldehyde reaction to the Wiesner test in 50 mM HCl as well as the fractions collected in gray. (C) LC profiles showing the time-stability of coniferaldehyde reaction to the Wiesner test in 0.5% phloroglucinol/6 M HCl. The solutions were neutralized with 6 M NaOH before injection. All the chromatograms have the same absorbance scale. (D) Absorbance spectra of collected fractions before (dotted lines) and after addition of 6 M HCl (solid lines). Note that fraction 9 is the acid-dependent chromophore indicated by the purple background (hue = 319°). (E) Proposed chemical condensation reaction for the Wiesner test. Different tautomeric resonance forms of the carbocation intermediate are shown in Figure S3E.
sinapyl-γ-phloroglucinol for S C₆C₃ aldehyde (Figures 1B–E and Figure S3). These chromophores were however unstable over time (Figure 1C) and their color changed with acidity (Figure 1D and Figure S3). The color fading with time was due to the formation of stable, non-chromogenic, coniferyl-γ-diphenol (Figure 1E and Figure S3). Our results thus clearly confirmed that only C₆C₃ aldehyde monomers react positively to the Wiesner test.

Since compounds generally behave very differently in monomeric and polymeric form, synthetic lignin-like DHPs of known composition were produced in vitro using peroxidases and either H, G, or S C₆C₃ acid, aldehyde, or alcohol monomers. Unexpectedly, only H and G C₆C₃ aldehyde DHPs reacted positively to the Wiesner test (Figures 2A, B and Figure S4). Stained G C₆C₃ aldehyde DHPs produced the typical purple color of the Wiesner test in both liquid and solid states with an absorption maximum λₘₐₓ = 556–559 nm (hue = 305–330°) which faded with time (Figure 2B). Although H C₆C₃ aldehyde is not reported as a lignin residue (Vanholme et al., 2012a), its stained DHPs had λₘₐₓ = 544 nm (hue = 330°). The color fading was investigated by LC analyses for DHPs treated by the Wiesner test with or without phloroglucinol. After a few minutes, degradation products were readily detected for all DHPs even without phloroglucinol, indicating that acidolysis occurred independently of both DHP composition and phloroglucinol (Figure S2B). LC–MS/MS analyses of stained G C₆C₃ aldehyde DHPs with phloroglucinol revealed a gradual release of coniferyl-γ-diphenol during acidolysis (Figure 2C). Altogether, these results showed that the color fading of the Wiesner test over time was due to both the acidolytic break-down of lignin and the formation of stable non-chromogenic condensation products.

The fact that S C₆C₃ aldehyde DHPs were unstained contradicted previous published articles claiming that both H and S aldehyde end-residues positively reacted to the Wiesner test (Pomar et al., 2002). To determine the position(s) of the residues stained, DHPs made of G or S C₆C₃ acid or aldehyde were analyzed by gel permeation chromatography before and after staining. The molecular weight of G C₆C₃ acid and S C₆C₃ aldehyde DHPs were unaffected by the staining (Figure 2D and Table 1). In contrast, the molecular weight of G C₆C₃ aldehyde (coniferaldehyde) DHPs exhibited a homogeneous increase after staining (Figure 2D and Table 1), which suggested that multiple residues within and at the ends of the DHP reacted with phloroglucinol. In fact, the average polymer molecular weight shift from 1,595 to 2,431 Da (Table 1) indicated that about 67% of its residues formed condensation products, when considering 160 Da for coniferaldehyde and 125 Da for phloroglucinol. Contrary to previous reports (Pomar et al., 2002), our results demonstrate that the Wiesner test reacts only with coniferaldehyde residues incorporated both at the ends and within lignin polymers.

In Situ Quantification of Incorporated Coniferaldehyde Residues in Lignin

Improvement of the Wiesner test for medium-throughput in situ quantification of coniferaldehyde content in lignin was then evaluated on Arabidopsis stem cross-sections. The resolution of the method was tested in the cell walls of different lignified cell types including protoxylem vessels (PX), metaxylem vessels (MX), xylary fibers (XF), interfascicular fibers (IF), and lignified pith parenchyma (LP) (Figure 3A). Live-imaging of the staining in different cross-section thicknesses, ranging from 12 to 150 µm (Figure 3A), showed that the staining plateaued after 2 min and faded within 24 h (Figure 3B). Once the staining faded away, cross-sections could not be re-stained by adding new reagent (Figure 3B). Distribution of hue and absorbance of different stained cells across section thicknesses indicated that 50 µm thick sections presented both the smallest variation as well as the most significant differences for both parameters between cell types (Figure S6A). In 50 µm thick Arabidopsis wild-type (WT) cross-sections, absorbance per square micrometer was highest for MX and XF, ~50% less in IF and LP, and ~75% less in PX (Figure 4B). The produced color had a hue between 310 and 320°, similarly to coniferaldehyde as monomers and DHPs (Figures 1A and 2B and Figure S5B). Using optimal conditions on a set of ten Arabidopsis loss-of-function (LOF) mutants, the quantitative capacity of the Wiesner test was compared to lignin concentration and composition measured using pyrolysis/GC–MS on the same mutants. Comparisons showed that the changes in Wiesner test absorbance directly corresponded only to changes in coniferaldehyde concentration (ranging from 1% to 25% of total lignin, Figure S6), but not to changes in total lignin amounts or its concentration in S, H, sinapaldehyde (S C₆C₃ aldehyde), or benzaldehydes (G/S C₆C₁ aldehydes) residues (Figure 4A and Table S2). In fact, the correlation between Wiesner test intensity and G lignin residues was weakened when including H and/or S residues (Table S2). These results showed that the Wiesner test absorbance increases linearly to allow the direct quantification of coniferaldehyde residues in lignin in situ across a wide range of concentrations.

To evaluate the in situ spatial resolution in stem cross-sections, the Wiesner test sensitivity was compared to confocal Raman microspectroscopy. Comparison between the two technologies was performed for measurements on the cell walls of three different lignified cell types (MX, XF, and IF) in two plant genotypes (WT and the LOF mutant 4cl1x4cl2, which is severely altered in lignin). The highest positive and significant correlation was observed between the Wiesner test absorbances and the 1,597 cm⁻¹ Raman band height, which corresponds to C₆ vibration of lignin residues (Gorzsás, 2017). However, no other significant correlations were observed between the Wiesner test absorbances and other Raman band heights, not even for the band shoulders 1,620 cm⁻¹ or at 1,140 cm⁻¹ suggested to reflect the vibrations of all phenolic aldehydes (Agarwal et al., 2011; Gorzsás, 2017) (Figure 4B). These results showed that the Wiesner test, in contrast to Raman microspectroscopy, specifically detects coniferaldehyde residues in situ with a high spatial resolution. Stained cross-sections using the Wiesner test could thus be converted into artificial color intensities to evaluate coniferaldehyde incorporation between cell types and across lignin.
FIGURE 2 | Reactivity of synthetic lignin-like dehydrogenation polymers (DHPs) with the Wiesner test. (A) Methanol solubilized H, G, or S C₆C₃ monomers and DHPs after staining for 1 min with 0.5% phloroglucinol/6 M HCl and subsequently adding a few droplets of 6 M NaOH without mixing, thus forming a pH gradient from top to bottom. (B) Absorbance spectra of G C₆C₃ aldehyde, alcohol, and acid DHPs solubilized in methanol (liquid) or dried onto 3MM Whatman paper (solid) and stained with the Wiesner test. Chromophores are indicated by the purple background in the corresponding hues for liquid (308°) and solid (326°) state. The small difference in hue suggests a minor influence of solvent effects on the sample reactivity to the Wiesner test. (C) LC-MS profiles of reaction products detected by staining with 6 M HCl/0.5% phloroglucinol following 0 and 4 h of 6 M HCl treatment. Both chromatograms are relative to total ion current. (D) GPC-RID profiles of G or S C₆C₃ acid and aldehyde DHPs solubilized in methanol either unstained (un) with 6 M HCl or stained (st) with 0.5% phloroglucinol/6 M HCl (compared to unstained methanol only in gray dotted lines) before neutralization with 6 M NaOH. M_p indicates the molecular weight at the peak maximum for each condition. All chromatograms have the same intensity scale.
This conversion revealed major differences in the incorporation levels of coniferaldehyde residues for similar cell types depending on both their adjacent cells and their position within the lignified tissue (Figure S5C). Altogether, these results establish the Wiesner test as the current most precise in situ method to discriminatingly detect coniferaldehyde residues incorporated in the lignin of specific cell types.

Incorporation of Coniferaldehyde Residues in Lignin of Herbaceous Plants Follows Cell Type Specific Biosynthetic Routes

The genetic control of coniferaldehyde accumulation in the lignified cell walls of the different cell types was investigated in Arabidopsis LOF mutants altered in lignin concentration and/or composition. The different LOF mutants...
affected specifically the lignin monomer biosynthetic pathway by modifying (i) the C₆ ring substitution using mutants in CCOAOMT1, FAH1, and OMT1; (ii) the C₃ terminal function (acid, aldehyde or alcohol) using mutants in 4CL1, 4CL2, CCR1, CAD4, and CAD5; as well as (iii) both with stacked mutants with LOF in both CCR1 and FAH1 (Figure S1).

The analyses of absorbance per area (µm²) of the different stained cell types revealed that changes in C₆ ring substitution...
only significantly altered IF, reducing coniferaldehyde concentration in ccooamt1 and increasing it in fah1 and omt1 compared to WT plants (Figures 5A–C). In contrast, the modification of C3 terminal functions significantly altered the different cell types specifically for each mutation (Figures 5A–C). Reduced coniferaldehyde incorporation in cell wall lignin was observed in the 4cl1, 4cl1x4cl2, ccr1, and ccr1xfsah1 mutants for IF, LP, and XF, only in ccr1 for MX but PX did not show any reduction (Figures 5A–C). In contrast, increased incorporation of coniferaldehyde in lignin was observed in both cad5 and cad4xcad5 mutants but not cad4 for IF, MX, and XF, whereas PX showed increases only in cad4xcad5 mutants (Figures 5A–C). Surprisingly, the loss of coniferaldehyde incorporation was partly reverted in ccr1xfsah1 for IF and LP but not in other cell types.
which remained like ccr1 (Figures 5A–C). A striking observation was the hue in the cad4xcad5 mutant both before (hue = 69°) and after (hue = 350°) staining compared to the other genotypes (Figures S7 and S8A). To evaluate whether the yellow background color influenced the Wiesner test, hues of cad4xcad5 mutants were analyzed using color deconvolution (Ruijrok et al., 2003) to identify the hue components before and after staining. The deconvolution of the hues merged in the stained cad4xcad5 sections was made by first setting a fixed primary channel with the characteristic purple hue of the Wiesner test, which resulted in a complementary hue of 66° similar to the cad4xcad5 unstained yellow background (Figure S8B). Inversely, setting the yellow background (hue = 64°) as a fixed primary channel resulted in a complementary hue of ~304° similar to the characteristic purple hue of the Wiesner test in WT plants (Figure S8C). Overall, our results revealed that the Wiesner test is not affected by sample background color and allows to unravel the impact of specific genetic mutations on the coniferaldehyde incorporation capacity of each specific cell type.

To further understand the genetic mechanisms controlling the incorporation of coniferaldehyde in the different lignified cell types, hierarchical clustering and principal component analysis (PCA) were performed to compare cell types and genotypes (Figures 5D, E). Surprisingly, these multivariate analyses revealed a differential influence of each mutation on the coniferaldehyde incorporation in the cell walls of each cell type (Figures 5D, E). Interestingly, LP, XF, and MX used similar biosynthetic routes to control the incorporation of coniferaldehyde residues in the lignin of their cell walls (Figures 5D, E). IF shared a similar regulation but exhibited the biggest amplitude of variation in response to LOF mutations (Figures 5D, E). All of these cell types reacted directly to the metabolic production and/or accumulation of coniferaldehyde. In contrast, PX presented a unique regulation of coniferaldehyde incorporation relatively unaffected by any mutation altering lignin monomer biosynthesis (Figures 5D, E). Altogether, our results demonstrated that different cell types possess unique biosynthetic routes to control the incorporation of coniferaldehyde residues into lignin.

**Incorporation of Coniferaldehyde Residues in Lignin of Woody Plants Changes During Tissue Development**

The cell type specific incorporation of coniferaldehyde in cell walls was then investigated in woody species (hybrid poplar) genetically altered in their lignin concentration and/or composition. Constitutive RNA interference (RNAi) lines using 35S promoters were used to alter (i) the CaC4 ring substitution by down-regulating the C4H gene; or (ii) the C3 terminal function by down-regulating the CCR gene (Figure S1).

The Wiesner test reaction in 50 µm thick poplar stem cross-sections was similar to that in Arabidopsis, exhibiting the characteristic Wiesner test hue between 320 and 350° (Figure S9). Absorbance per area (µm²) between lignified cell types were measured for vessels, fibers, and rays at different distances from the cambium, in three stages marking the gradual development of xylem/wood which includes the cell death of vessels according to Sundell et al. (2017) (Figure 6A). In WT cross-sections, absorbance per area (µm²) was highest for vessels, ~25% less in rays, and ~50% less in fibers (Figure 6B). All cell types presented increasing absorbance per area during xylem development, thereby revealing that the cell death of vessels did not prevent the continuous incorporation of coniferaldehyde residues in their cell walls (Figure 6B). The lower Wiesner test absorbances measured for the poplar plants were in accordance with the fact that coniferaldehyde residues represented only 0.2 ± 0.1% of lignin residues in the WT (Figure S6). RNAi of lignin biosynthesis genes altered the coniferaldehyde incorporation during the development of the different cell types. All cell types exhibited significantly reduced coniferaldehyde incorporation in CAH-RNAi plants during the early stages of xylem development, which were then restored to the WT levels in the third developmental stage (Figure 6B). CCR-RNAi plants did not show significant changes in coniferaldehyde incorporation compared to WT plants (Figure 6B). Hierarchical clustering analysis and PCA were then used to evaluate the mechanisms controlling coniferaldehyde incorporation during the development of each cell type (Figures 6C, D). All cell types presented similar developmental regulation to control the incorporation of coniferaldehyde residues in lignin of their cell walls. However, CAH-RNAi had a strong influence during the entire xylem formation compared to CCR-RNAi which only affected the earliest stages (Figure 6C). Altogether, our results demonstrated that coniferaldehyde incorporation is tightly and differently regulated during the development of each lignified cell type.

**Incorporation of Coniferaldehyde Residues Into Lignin Depends on Cell Wall Layer Specific Accumulation Capacity**

The spatial regulation of coniferaldehyde incorporation in lignin was then investigated by comparing the absorbances of different cell wall layers spanning across two adjacent IF. This included the primary cell wall/middle lamella (CML) in the center and three mirrored concentric secondary cell wall layers—thin S1, thick S2, and thin S3—on each side. Line profiles of absorbances were measured in the different Arabidopsis LOF mutants to detect spatial differences in coniferaldehyde incorporation between cell wall layers (Figure 7A). WT plants exhibited a homogeneous incorporation of coniferaldehyde in all cell wall layers (Figures 7A, B). In contrast, mutants incorporated coniferaldehyde differently in specific cell wall layers (Figures 7A, B). For example, 4c11x4cl2 presented a reduction of ~60% in the S2 layer compared to its other layers reduced only ~30% (Figures 7A, B). In contrast, cad4xcad5 caused a ~25% increased incorporation in CML compared to its secondary cell wall layers which were only increased ~10% (Figures 7A, B). Comparing all the mutants moreover indicated that the different layers did not share the same level of regulation: CML, S1, and S2 were regulated similarly but the S3 layers was less sensitive to LOF mutations affecting coniferaldehyde formation (Figures 7A, B). Altogether our results demonstrated that the incorporation of
FIGURE 6 | Reaction of the Wiesner test on genetically modified lignin in poplar. (A) Unstained and 2 min stained transverse cross-sections of poplar WT, C4H-RNAi, and CCR-RNAi plants. Rays (R), vessels (V), and fibers (F) and xylem developmental stages according to Sundell et al. (2017) are indicated by dotted lines relatively to the cambium. Bars = 25 µm. (B) Changes in absorbance in response to the Wiesner test monitored in the different cell types, genotypes, and along xylem development. Circles indicate the average of each individual replicate and bars represent the average per genotype. The color of bars represents the respective average color of the stain. Quantification was done on 20 measured points of 5 pixels each per cell type in five individual plants, before binning the points according to their distance to the cambium (I, 0–50 µm; II, 50–100 µm; III, 100 µm–pith). Letters indicate significant differences according to a Tukey-HSD test (per panel; α = 0.05). (C) Hierarchical clustering of Wiesner test absorbance in the three lignified cell types at the three xylem developmental stages in the three genotypes of poplar using the range normalized Euclidean distance of their average difference from the WT. (D) Principal component analysis of the three lignified cell types at the three xylem developmental stages according to their absorbance in the three tested genotypes; 95% data ellipses are indicated.
Coniferaldehyde residues into lignin depended on the accumulation capacity of specific cell wall layers.

**Cell-to-Cell Relationships Contribute to Coniferaldehyde Incorporation in the Lignin of the Different Cell Types**

The differences observed in the incorporation of coniferaldehyde residues between cell types, cell wall layers, across tissues and during development after the cell death of vessels suggested the intervention of cell-to-cell cooperative processes (Figures 5–7). Since lignification depends on cell-to-cell cooperation between neighboring cell types (Pesquet et al., 2013; Serk et al., 2015), mathematical models were computed to define how specific mutation(s) altered the accumulation of coniferaldehyde residues between two neighboring cell types (Figure 8 and Figure S10A). Modelling allowed to evaluate the reciprocity in the cell-to-cell cooperation between different cell type pairs as well as the contribution of the different LOF mutations on the accumulation of coniferaldehyde residues between these cell type pairs (Figure S10B). The cellular relationships were first investigated in *Arabidopsis* to identify the genetic restriction(s) controlling the cell-to-cell cooperation between different cell types. Coniferaldehyde incorporation in MX was affected by a unidirectional negative influence from PX and a unidirectional positive influence of XF, neither relationships highlighted any specific genetic restrictions (Figure 8A and Figure S10B). In contrast, XF and IF exhibited a reciprocal but unbalanced cooperation, essentially affected by the *ccoaomt1, 4cl1, 4cl1 x 4cl2, and ccr1* mutations from XF to IF but restricted to only the *4cl1 x 4cl2* mutations from IF to XF (Figure 8A and Figure S10B). Lastly, LP also exhibited a unidirectional influence from IF mostly in *ccoaomt1 and ccr1* but also *fah1, omt1*, and *cad5* mutants (Figure 8A and Figure S10B). This suggested that the accumulation of coniferaldehyde residues in LP directly depended on the metabolic availability of coniferaldehyde, further explaining the partial restoration of LP observed in the *ccr1 x fah1* mutant (Figures 5A–C). The genetic regulation underlying cell-to-cell cooperation during the wood development was also investigated in poplar (Figure 8B and Figure S11A). Surprisingly, specific cell types showed an effect of neighboring cells only in certain development stages. Vessels benefited unidirectionally and positively from fibers during the early stage of xylem development (Figure 8B and Figure S11B). In contrast, fibers were influenced unidirectionally and positively from vessels only in the last stage of xylem maturation (Figure 8B and Figure S11B). Our results demonstrated the differential influence of distinct neighboring cells on the accumulation of coniferaldehyde residues into lignin depending on the accumulation capacity of specific cell wall layers.
coniferaldehyde residues during the development of other lignified cell types in both herbaceous and woody species.

**DISCUSSION**

In the present study, we demonstrate that the incorporation of coniferaldehyde residues in lignin is specifically and differently regulated between cell wall layers, cell types, across tissue and during development. These discoveries were possible because we uncovered the exact chemical reaction behind the Wiesner test 140 years after it was first described, and perfected it for *in situ* quantitative analyses with a high spatial resolution. In contrast to previous studies which concluded that both coniferaldehyde and sinapaldehyde residues at the end of polymers were stained (Pomar et al., 2002), we show that the Wiesner test reacts only with coniferaldehyde residues incorporated both at the ends and within lignin polymers. We also reveal the main reason behind this former mis-conclusion as we show that, alike the differences previously observed between amino-acid/protein, nucleotide/DNA, or glucose/cellulose, synthetic C6C3 monomers cannot be used as standards or models for lignin polymers (Figure 2A).

We also reveal that coniferaldehyde incorporation is controlled by the cell wall itself: this mechanism appears to limit the incorporation of coniferaldehyde spilling and/or diffusing across tissue to other neighboring cell types, as observed in *ccr1* mutants between XF and neighboring IF (Figures 5, 7–9). This spatial regulation could depend on the type of coniferaldehyde-containing precursor used to lignify. Metabolomic analyses of lignifying tissues in *Arabidopsis* and poplar have in fact shown the presence of many phenolic compounds containing coniferaldehyde residues such as β-5-linked dilignols, β-O-4/β-5, and β-O-4/β-O-4-linked trilignols as well as glucosides (Vanholme et al., 2012b; Sundin et al., 2014; Van de Wouwer et al., 2016; Saleme M de et al., 2017). Our results therefore question whether the same coniferaldehyde-containing
precursor is used by the different cell types and cell wall layer for lignin biosynthesis. Our study clarifies the genetic and developmental mechanisms controlling the incorporation of coniferaldehyde residues in the lignin of different cell types and species (Figures 5–8). The distinction between cell-autonomous and cell-to-cell cooperative processes is technically difficult to resolve in biological tissues. To achieve this distinction, we evaluated how distinct genetic changes altered coniferaldehyde incorporation between neighboring cell types using mathematical modelling (Figure 8 and Figures S10 and 11). These models reveal that different cell-to-cell cooperation exists between different cell types and during development (Figure 8). Our study moreover defines the genetic restriction(s) controlling coniferaldehyde incorporation for different cooperation(s) between cell types: MX unidirectional cooperation with XF does not depend on any major genetic restriction whereas XF reciprocal cooperation with IF is greatly affected by ccoaomt1, 4cls and ccr1 mutations (Figure S10B). In most cases, the neighboring cells positively influence the accumulation of coniferaldehyde. However, for vessels, PX in Arabidopsis negatively influence coniferaldehyde accumulation in MX (Figure 8A) whereas, dead vessels in the most mature part of poplar xylem positively influence coniferaldehyde accumulation in fibers (Figure 8B). These results reveal that cooperating vessels, which are dead hollow interconnected structures conducting the sap over long distances, are able to enhance or deplete the presence of coniferaldehyde-containing lignin precursors to their neighboring cells. A simplified scheme of these complex regulations and cooperations for each specific cell type is presented in Figure 9. Altogether, we demonstrate that coniferaldehyde incorporation in each specific cell type depends on autonomous biosynthesis and cell-to-cell cooperation, each varying in amplitude, contribution and genetic restriction(s) depending on the cell type. Although we unraveled its genetic and cellular regulation, the molecular mechanisms (enzymes, metabolites) controlling the spatial accumulation of coniferaldehyde in specific cell wall layers still need to be clarified.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material. All R scripts and Fiji macros used are available at https://github.com/leonardblaschek.

AUTHOR CONTRIBUTIONS

EP conceived the study. LB, AC, CD, N, RD, SH, SuK, ShK, and EP performed the experiments. LB, AC, CD, ShK, and EP analyzed the data. EP wrote the article. All co-authors revised the manuscript.

FUNDING

This work was supported by Vetenskapsrådet (VR) research grants 2010-4620 and 2016-04727 (to EP), the Stiftelsen för...
Strategisk Forsknings ValueTree (to EP), and the Carl Trygger Foundation CTS 16:362/17:16/19:308 (to EP).

ACKNOWLEDGMENTS

We thank Veronica Bourquin, Magnus Hertzberg, and SweTree Technologies AB for producing and maintaining WT and transgenic poplar lines and Junko Takahashi-Schmidt and the Department of Ecology, Environment and Plant Sciences (DEEP) of Stockholm University.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00109/full#supplementary-material.
Moural, T. W., Lewis, K. M., Barnaba, C., Zhu, F., Palmer, N. A., Sarath, G., et al. (2017). Characterization of class III peroxidases from switchgrass. *Plant Physiol.* 175, 427–433. doi: 10.1090/pp/11014

Naseer, S., Lee, Y., Lapiere, C., Franke, R., Nawrath, C., and Geldner, N. (2012). Casparian strip diffusion barrier in *Arabidopsis* is made of a lignin polymer without suberin. *Proc. Natl. Acad. Sci. U. S. A.* 109, 1001–1006. doi: 10.1073/pnas.1205726109

Nilsson, O., Alden, T., Sitbon, F., Anthony Little, C. H., Chalupa, V., Sandberg, G., et al. (1992). Spatial pattern of cauliflower mosaic virus 35S promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. *Transgenic Res.* 1, 209–220. doi: 10.1007/BF02524751

Pesquet, E., Zhang, B., Gorzsás, A., Puhakainen, T., Serk, H., Escamez, S., et al. (2015). Cooperative regulation of Coniferaldehyde residue incorporation in lignin. *Plant Physiol.* 170, 1010–1019. doi: 10.1104/pp.15.00142

Van de Wouwer, D., Vanholme, R., Decou, R., Goeinmine, G., Audenaert, D., Nguyen, L., et al. (2016). Chemical genetics uncovers novel inhibitors of lignification, including p-iodobenzoic acid targeting CINNAMATE-4-HYDROXYLASE. *Plant Physiol.* 172, 198–220. doi: 10.1104/pp.15.01580

Vance, C. P., Kirk, T. K., and Sherwood, R. T. (1980). Lignification as a mechanism of disease resistance. *Annu. Rev. Phytopathol.* 18, 259–288. doi: 10.1146/annurev.py.18.090180.001355

Vanholme, R., Demeds, B., Morreel, K., Ralph, J., and Boerjan, W. (2010). Lignin biosynthesis and structure. *Plant Physiol.* 153, 895–905. doi: 10.1104/pp.110.153619

Vanholme, R., Morreel, K., Darrah, C., Oyarce, P., Grabber, J. H., Ralph, J., et al. (2012a). Metabolic engineering of novel lignin in biomass crops. *New Phytol.* 196, 978–1000. doi: 10.1111/j.1469-8137.2012.04337.x

Vanholme, R., Storme, V., Vanholme, R., Sibout, R., Christensen, J. H., and Goeinmine, G., et al. (2012b). A systems biology view of responses to lignin biosynthesis perturbations in *Arabidopsis*. *Plant Cell* 24, 3506–3529. doi: 10.1105/pp.112.102574

Varbanova, M., Porter, K., Lu, F., Ralph, J., Hammerschmidt, R., Jones, A. D., et al. (2011). Molecular and biochemical basis for stress-induced accumulation of free and bound p-coumarylaldehyde in cucumber. *Plant Physiol.* 157, 1056–1066. doi: 10.1104/pp.111.184358

Wang, J. P., Matthews, M. L., Williams, C. M., Shi, R., Yang, C., Tunlaya-Anukit, S., et al. (2018). Improving wood properties for wood utilization through multiomics integration in lignin biosynthesis. *Nat. Commun.* 9, 1579. doi: 10.1038/s41467-018-03863-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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