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Technical Perspectives

## Ferulate and lignin cross-links increase in cell walls of wheat grain outer layers during late development



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## ABSTRACT

Important biological, nutritional and technological roles are attributed to cell wall polymers from cereal grains. The composition of cell walls in dry wheat grain has been well studied, however less is known about cell wall deposition and modification in the grain outer layers during grain development.

In this study, the composition of cell walls in the outer layers of the wheat grain (*Triticum aestivum* Recital cultivar) was investigated during grain development, with a focus on cell wall phenolics. We discovered that lignification of outer layers begins earlier than previously reported and long before the grain reaches its final size. Cell wall feruloylation increased in development. However, in the late stages, the amount of ferulate releasable by mild alkaline hydrolysis was reduced as well as the yield of lignin-derived thioacidolysis monomers. These reductions indicate that new ferulate-mediated cross-linkages of cell wall polymers appeared as well as new resistant interunit bonds in lignins. The formation of these additional linkages more specifically occurred in the outer pericarp.

Our results raised the possibility that stiffening of cell walls occur at late development stages in the outer pericarp and might contribute to the restriction of the grain radial growth.

## 1. Introduction

Cell walls of cereal grains contribute to grain quality since they impact the grain industrial and nutritional properties. Beneficial effects for human health are attributed to wall polymers of cereal grains the main components of dietary fibers which consumption reduces the risk of developing cardiovascular diseases, diabetes and certain cancers. Conversely, detrimental effects are associated with grain wall polysaccharides in animal feed especially for poultry. Among the industrial

processes affected by wall polymer properties, there are grain milling and tissue fractionation, bread, pasta and beer-making [1,2].

In the field, cell walls have also major biological functions in grain growth and seed protection. Grain cell walls are complex structures composed of various polysaccharides, proteins, lignins and lipid-based polymers. Their composition varies between species, tissues and developmental stages, which affects their extensibility and hydration degree. Cell walls of the wheat grain storage tissue, the endosperm, have been extensively studied due to their implication in flour quality [2,3].

**Abbreviations:** AIR, alcohol insoluble residues; CA, *p*-coumaric acid; DAF, celsius degrees days after flowering; DM, dry mass; FA, ferulic acid; FID, flame ionization detector; G, guaiacyl; H, *p*-hydroxyphenyl; GC-MS, gas chromatography-mass spectrometry; IF, inner fraction; S, syringyl; TMCA, trimethoxy-(*E*)-cinnamic acid; TMS, trimethylsilylated

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Less is known about the cell walls of the grain outer layers, which comprise different tissues: the pericarp, the seed coat or testa, and the nucellar epidermis. In wheat dry grain, these layers are reduced to a few compressed cell layers. They are mainly composed of polysaccharides (essentially cellulose and xylans), proteins, lignins and cutins in various proportions [4,5]. Xylans consist in a  $\beta(1-4)$  xylan backbone with several substituents among which arabinose residues that can be acylated by ferulic acid (FA) and, to a lower extent, by *p*-coumaric acid (CA). Grass lignins are essentially made of guaiacyl (G), and syringyl (S) units together with a low amount of *p*-hydroxyphenyl (H) units. Cutins are the main component of cuticles; they are composed of inter-esterified C16–C18 hydroxyfatty acids that also contain glycerol and FA and/or CA units [6]. FA is an important component of grass cell walls. It is involved in the formation of grass-specific polymer cross-linking through FA esters [7–10]. Ferulate bridges are thought to contribute to grass wall assembly and tissue cohesion [11]. Furthermore inducing FA cross-linking in strips of outer tissues from wheat grain by adding peroxidase was shown to increase the mechanical strength of these tissues [12].

Cell walls of the developing outer layers of the wheat grain have been scarcely studied although these layers constitute most of the grain volume at early developmental stage [13]. Both cellulose and mixed-linkage glucans have been evidenced in the cell walls of the outer layers at very early stages of development while xylan occurrence was assessed several days after flowering [14]. Lignins could be evidenced when grain weight was maximal, but not at earlier stages [4]. In this study, we monitored the composition of the grain outer layers of the wheat cultivar Recital during its development, with a particular focus on cell wall phenolics, namely lignins and cell wall-linked CA and FA units. The corresponding changes observed from early developmental stages to grain maturity are discussed with respect to the evolution of grain weight and dimensions.

## 2. Material and methods

### 2.1. Plant materials and growth conditions

Wheat plants (*Triticum aestivum* L. cv. Recital) were sown in containers filled with plain soil at INRA Clermont-Ferrand (France) in 2012 and grown under conditions of natural day length and temperature until flowering, then transferred to a tunnel where the conditions were 21 °C from 6 a.m. to 9.30 p.m. and 14 °C at night (average temperature 18.5 °C). To harvest grains at different developmental stages, individual ears were tagged when flowering of middle spikelets was observed and development was calculated on the basis of cumulative temperature in Celsius degrees days after flowering (°DAF).

### 2.2. Grain morphological measurements

For each stage, the two basal grains (noted G1 and G2) of the spikelets located in the middle of the ear of main tillers were collected. Ten grains per stage were sampled every day from flowering to 300°DAF and then every 2–3 days until maturity. Immediately after sampling, the grains volume was measured using a pycnometer and grain dimensions were measured under a microscope (length, width and thickness as shown in Fig.1E). Grains G1 were oven-dried (60 °C for 48 h) and weighed immediately afterwards. Grains G2 were dissected in order to collect separately the outer layers, the endosperm, and the embryo (from 250°DAF), all grain fractions were oven-dried and weighed. The dry mass of G2 grains was calculated as the sum of the masses of outer layers, endosperm and embryo. Observed data were statistically adjusted using the following functions: Gompertz for volume, width and thickness, 3-parameters logistic for dry mass, and a linear-segmented for grain length, and for the mass ratio. Thermal time when maximal value of a variable is attained was estimated from these adjustments.

### 2.3. Macroscopy and microscopy

Samples were collected from basal grains of spikelets in the middle of the ears, in the equatorial region of the grains (above the embryo) and all sections were cross-sections. In the case of dry grains, the embryo was removed and the grains were placed onto moist paper for 24 h at 4 °C to facilitate sectioning.

#### 2.3.1. Histochemical Staining

For staining with toluidine blue, samples were fixed overnight in 3% (w/v) paraformaldehyde and 0.5% (w/v) glutaraldehyde in 0.1 M Na-phosphate-buffered saline pH 7.2 and embedded with London Resin White acrylic as described in [15]. Semi-thin sections (1  $\mu$ m) obtained with an ultramicrotome (UC7, Leica) were stained with 1% (w/v) toluidine blue O as described in [16] before observation with a multi-zoom microscope (AZ100 M, Nikon). For lignin staining, grain samples were frozen and cut using a cryotome (HM 500 OM, Microm) into 50  $\mu$ m thick sections that were stained using the Maïle and the Wiesner (phloroglucinol-HCl) reagents as described in [17] and observed using a microscope. To detect cutin, samples embedded in paraffin, cut and dewaxed in HistoChoice Clearing Agent as described in [18]. Sections were stained using saturated and filtered Sudan Red solution in ethanol as described in [19].

#### 2.3.2. Immunolabelling

Sections (150  $\mu$ m) were sampled, fixed by high pressure freezing, substituted and embedded in Lowicryl HM20 (Electron Microscopy Sciences) resin as described in [20]. Semi-thin sections (1  $\mu$ m) were cut with an ultramicrotome. Blocking, immunolabelling with KM1 monoclonal antibody [21] (ascites fluid 1:100 dilution in blocking buffer or supernatant, no dilution) incubation with the secondary antibody (with goat anti-mouse IgG Alexa Fluor 568 1:100 in blocking buffer), and observations were carried out as described in [22]. A control experiment was conducted omitting KM1 to check for autofluorescence and non-specific labelling.

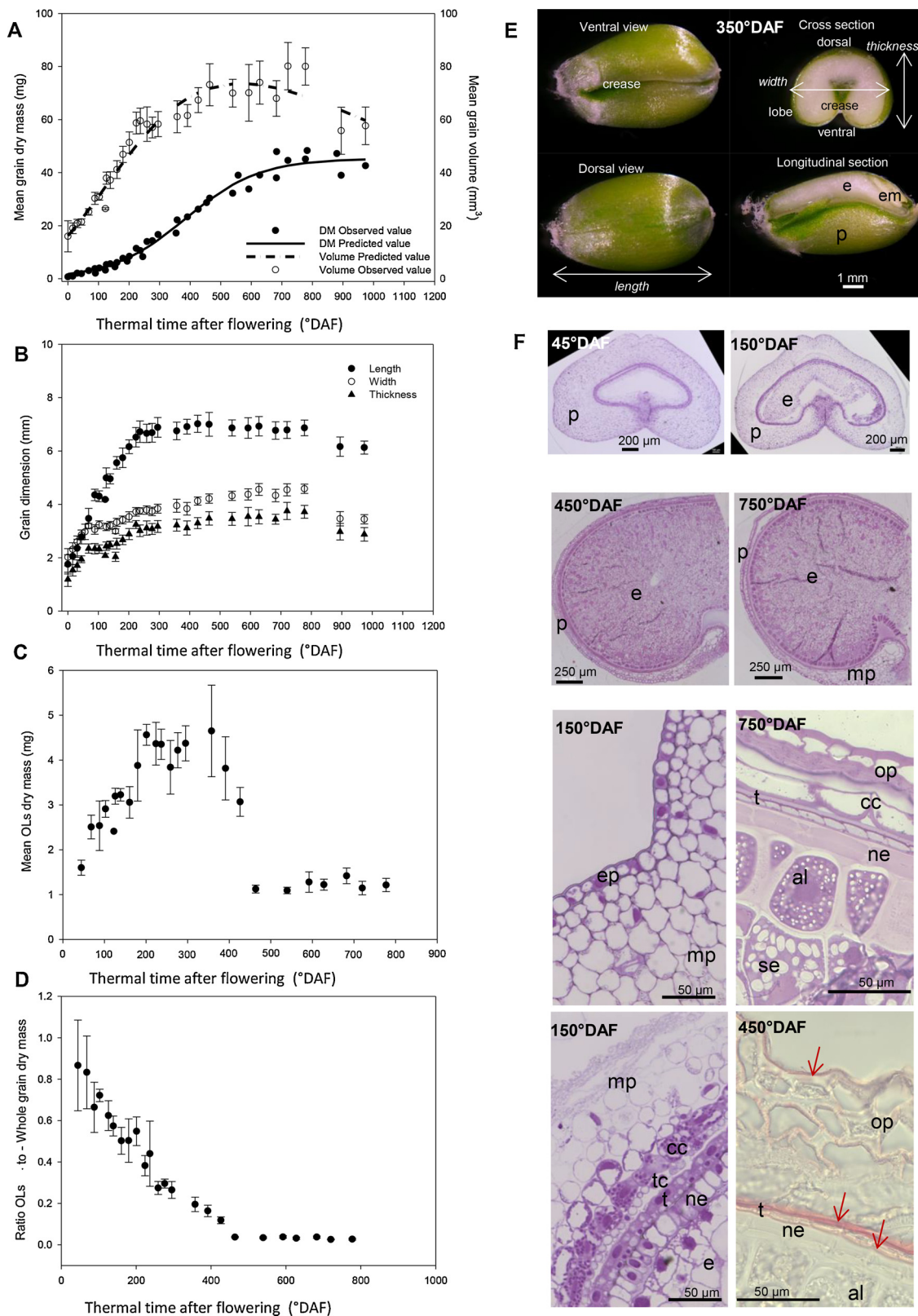
### 2.4. Biochemical analyses

#### 2.4.1. Tissue dissection and preparation of Alcohol Insoluble Residues (AIR)

Grains were harvested at several developmental stages, and in order to harvest enough material for tissue dissection, at least 200 grains were used per stage. Grains were manually dissected. Using tweezers, the colorless outermost tissues of the grains (outermost fraction), and the inner green tissues (inner fraction) were collected. The endosperm and embryo were discarded. The fractions were observed using a microscope under bright-field and UV. The outermost fraction was named outer pericarp fraction and the green fraction inner fraction. The aleurone layer was dissected from mature grains as described in [23]. Tissues were ground in liquid nitrogen and 50 mg of the resulting powder was mixed with 80% ethanol for 10 min in a boiling water bath to extract small sugars (glucose, sucrose, etc.). After centrifugation (6300g; 10 min) the alcohol insoluble residue was recovered and the procedure was repeated once. The residue was washed with 95% ethanol and again isolated by centrifugation (6300g; 10 min). The AIR sample was dried first in an oven at 40 °C for 24 h and then over P<sub>2</sub>O<sub>5</sub> in a vacuum oven at 40 °C for 48 h. All AIR analyses were performed at least in duplicate except for the protein content and some neutral sugar analyses of dissected mature grain because of a limiting quantity of material and priority given to lignin and phenolic acids. The AIR composition was analyzed as follows.

#### 2.4.2. Nitrogen content

The total amount of nitrogen was estimated according to the Kjeldahl method using a nitrogen protein conversion factor of 5.7 adapted for cereals [24].



**Fig. 1.** Evolution of dry mass, dimensions and histology of wheat grain (cv Recital) during its development. (A) Mean grain dry mass and volume, observed data were adjusted by a logistic (3 parameters) function for dry mass and a Gompertz function for volume. (B) Mean grain dimensions. (C) Outer layers (OLs) dry mass per grain. (D) Ratio of OLs dry mass to grain dry mass. (E) Example of wheat grain at 350°DAF. (F) Grain tissues: cross sections were stained with toluidine blue or Sudan red for the last panel. P: pericarp, e: endosperm, em:embryo, ep:epicarp, mp:mesocarp, op: outer pericarp, cc: cross cells, tc: tube cells, t: seed coat, ne:nucellar epidermis, al:aleurone layer, se:starchy endosperm. Red arrows: cuticles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

#### 2.4.3. Carbohydrate analyses

The neutral sugar composition of cell wall polysaccharides was monitored after hydrolysis of AIR samples with 1 M H<sub>2</sub>SO<sub>4</sub> for 2 h at 100 °C. To fully release glucose from cellulose, a pre-hydrolysis step was carried out with 72% H<sub>2</sub>SO<sub>4</sub> for 30 min at 25 °C prior to the hydrolysis step. Individual neutral sugars were quantified after their derivatization into alditol acetates and gas chromatography analysis according to the method of [25]. Starch was analyzed according to AOAC procedures [starch, AOAC method 996.11] with modifications described in [26]. The starch content was subtracted from total glucose measured by gas liquid chromatography of alditol acetates.

#### 2.4.4. Cell wall linked FA and CA

Ester-linked FA and CA were analyzed by mild alkaline hydrolysis performed under oxygen-free atmosphere (Ar) at 35 °C in 2 M NaOH [23] for 2 h. An internal standard (2,3,5 trimethoxy-(*E*)-cinnamic acid (TMCA), T-4002, Sigma) was added before adjusting pH to 2. Phenolic acids were then extracted with diethylether and quantified by HPLC [23]. The FA content was calculated from the amount of *Z*- and *E*-isomers. In addition, several ferulate dimers (8-*O*-4', 8-5', 8-5' benzofuran, 5-5' forms) and one ferulate trimer (5-5'-8'-*O*-4'') were also measured.

#### 2.4.5. Thioacidolysis

Lignin structure of grain AIR was studied as previously described [27] using a modified thioacidolysis reagent (9/1 dioxane/ethanethiol mixture containing 0.1 M tetrafluoroboric acid dimethylether complex). Lignin-derived thioacidolysis H, G and S monomers were analyzed by GC-MS of their trimethylsilylated (TMS) derivatives as previously described [22].

#### 2.4.6. Cutin preparation and analysis

To study cutin composition and due to the low amount of cutin in the outer layers, outer layers were collected from at least 140 grains at different stages and analyzed. Since cutin is insoluble in organic solvents, outer layers were extensively delipidated by successive extractions in CHCl<sub>3</sub>:MeOH (2:1 v/v) overnight, CHCl<sub>3</sub>:MeOH (1:2 v/v) 8 h, MeOH 4 h, Propanol-1:water (70:30, 90 °C) 1 h, H<sub>2</sub>O 1 h, MeOH 1 h, CHCl<sub>3</sub>:MeOH (1:2 v/v) 1 h, CHCl<sub>3</sub>:MeOH (2:1 v/v) overnight, CHCl<sub>3</sub> 2 h. Cutin depolymerization was conducted by transesterification according to [28] and depolymerization products were identified and analyzed by GC-MS and GC-FID as previously described [29].

### 3. Results

#### 3.1. Evolution of the grain dimensions and tissues during grain development

The grain mass, dimensions and histology were studied in our growing conditions to evaluate the physiological stages corresponding to the harvested samples. The developing wheat grains increased in dry mass (DM) first slowly and then rapidly from 250°DAF until approximately 700°DAF -the grain filling phase- where the grain mass plateaued (Fig. 1A). The maximal DM (99%) was reached at 741 ± 32°DAF. Early after flowering, the grain volume and dimensions increased rapidly. The time necessary to reach the maximal volume was estimated at 550 ± 12°DAF. The grain length increased from 1.8 ± 0.4 mm to 6.5 ± 0.4 mm and this maximum length was reached at 215 ± 4°DAF (Fig. 1B). The grain width and thickness first increased rapidly up to 80°DAF, then the growth rate slowed down to reach the maximum value later in the development (width at 590 ± 10°DAF and thickness at 542 ± 13°DAF) (Fig. 1B). After 800°DAF the grain dimensions decreased slightly due to natural desiccation.

In regards to the grain tissues, the endosperm developed from 80°DAF and later differentiated into aleurone layer and starchy endosperm that fills with storage compounds (Fig. 1E, F). The grain outer layers, and especially the pericarp, made most of the grain volume and weight early in the development (Fig. 1C, D, F). The DM of these outer

layers increased early, from the first phases of development up to 323 ± 9°DAF (Fig. 1C, D). The pericarp is made of several tissues: the epicarp (epiderm of the pericarp), the mesocarp (central pericarp composed of parenchyma cells) and the endocarp or inner pericarp, which comprises the cross cells and, in the dorsal region of the grain, the tube cells (Fig. 1F). During this early development phase, the outer tissues are growing and some of them (the endocarp cells) are photosynthetically active as evidenced by the presence of chlorophyll (Fig. 1E), well differentiated chloroplasts and starch granules (Fig. S1). After 350°DAF, the outer layers DM drastically decreased, which induced a continuous decrease of the ratio of the outer layers DM to the whole grain DM between flowering and 434 ± 13°DAF (Fig. 1C, D, F). Mesocarp cells underwent cell lysis, cells of the seed coat and nucellar epidermis also degenerated but their cell walls were not degraded (Fig. 1F). The outer cell walls of the epicarp, seed coat, and nucellar epidermis, were found to be cutinized as revealed by staining with Sudan Red (Fig. 1F). At the end of the development, the outer layers of the grain comprise only a few compressed cell layers except in the crease region where mesocarp cells remained intact (Fig. 1F-750°DAF).

#### 3.2. Evolution of cell wall composition in developing outer layers

Wheat grains were harvested at different developmental stages. To gain information on the spatial variability of cell wall composition, the grain outer layers were hand-dissected in two fractions as described in material and methods. Macroscopic observations showed that the outermost fraction contained the epicarp and the mesocarp (Fig. S2). The second fraction contained the endocarp, seed coat, nucellus epidermis and aleurone layer, except for the mature grain sample that did not contain the aleurone layer (Fig. S2). The two fractions will be referred to as outer pericarp and inner fraction. Mature grain aleurone layer was collected separately. The composition of the mature grain inner fraction was calculated by adding the values of the aleurone layer to those of the dissected inner fraction, after correction for the relative weights of the tissues calculated by [5]. The composition of the samples was determined after alcohol treatment of the different fractions. Polysaccharides and proteins accounted for 77 to 89 percent of the AIR mass for all samples (Table 1), except for the mature inner fraction and aleurone layer. In the corresponding tissues, phenolic compounds, lipids, minerals and phytate inclusions contribute significantly to the tissue mass [30] and are not eliminated by treatment with 80% ethanol. The detailed composition of non-starch polysaccharides showed that in all samples they mainly contained glucose, arabinose and xylose (Table 1). Non-starch glucose most probably originated from cellulose and mixed-linkage glucans, while arabinose and xylose mainly originated from xylans [5,14]. During development, the content of non-starch polysaccharides increased especially in the outer pericarp. The relative amount of polymers of glucose and xylose was relatively stable. Xylose content was higher in cell walls of the inner fractions than in outer pericarp samples while arabinose to xylose ratio was higher in outer pericarp samples.

FA and CA units that are ester-linked to cell wall polymers can be released by mild alkaline hydrolysis. Both FA and CA were obtained from the outer pericarp and inner fractions (Table 2). FA was detected as a monomer at all investigated stages. Several dimers and one trimer of FA, resulting from the oxidative cross-linking of xylan chains, were also released by alkaline hydrolysis. 8-5' dimer was the more represented in all samples and its relative amount tended to slightly increase across development. CA was detected albeit to a much lower level. In inner fractions, the level of alkali-releasable FA strongly increased early in the development between 200 and 350°DAF to remain stable afterwards. Compared to inner fractions, considerably higher FA amount was released from outer pericarp fractions except for the mature sample. The level of both FA and CA obtained from the outer pericarp fractions reached their maximum value at 450°DAF and decreased sharply between 570°DAF and mature grain, while the level of

**Table 1**  
Protein, starch and cell wall neutral sugar composition of the outer layers in the developing wheat grain (Recital).

	200°D	250°D	350°D	450°D	570°D	Mature		
	IF*	IF*	IF*	IF*	IF*	IF	AL	IF + AL
Protein (N 5.7) <sup>1</sup>	24.0	17.6	14.1	16.6	16.3	1.9	19	15.4**
Starch <sup>1</sup>	32.7 (0.5)	38.0 (1.3)	41.4 (1.0)	40.7 (1.6)	41.6 (1.1)	1.5	2.1 (0.1)	1.9
Non starch neutral sugars <sup>1</sup>	32.7 (0.5)	21.7 (0.4)	23.3 (0.3)	21.9 (0.9)	21.4 (0.1)	64.0	32.0 (1.9)	42.2
Arabinose <sup>2</sup>	17.7 (0.8)	20.4 (0.7)	20.8 (0.2)	22.2 (0.0)	21.8 (0.1)	16.8	16.2 (0.3)	16.4
Xylose <sup>2</sup>	41.3 (1.3)	44.1 (0.0)	44.8 (1.3)	44.4 (0.7)	45.5 (0.4)	50.2	50.7 (0.3)	50.5
Mannose <sup>2</sup>	2.8 (0.0)	3.3 (0.1)	2.5 (0.1)	2.6 (0.3)	1.8 (0.0)	0.8	1.9 (0.2)	1.6
Galactose <sup>2</sup>	3.4 (0.1)	4.4 (0.1)	2.8 (0.6)	2.1 (0.1)	1.6 (0.3)	1.6	2.5 (0.8)	2.3
Non starch glucose <sup>2</sup>	34.7 (2.2)	27.7 (0.4)	29.1 (0.8)	28.8 (0.5)	29.3 (0.1)	30.7	28.7 (0.3)	29.3
<b>Outer pericarp</b>								
Protein (N 5.7) <sup>1</sup>	14.36	10.4	9.9	7.9	5.9	1.9		
Starch <sup>1</sup>	34.9 (1.2)	32.5 (1.0)	14.9 (0.1)	9.0 (0.5)	6.6 (0.4)	1.4 (0.3)		
Non starch neutral sugars <sup>1</sup>	38.6 (1.7)	44.0 (2.4)	59.9 (0.2)	66.4 (1.0)	69.8 (1.0)	81.1 (3.5)		
Arabinose <sup>2</sup>	26.6 (0.7)	25.6 (0.2)	29.2 (0.0)	30.1 (0.5)	30.5 (0.4)	33.3 (1.1)		
Xylose <sup>2</sup>	33.3 (1.7)	29.3 (1.0)	31.7 (0.0)	31.1 (0.4)	30.7 (0.2)	29.7 (0.2)		
Mannose <sup>2</sup>	2.2 (0.4)	2.2 (0.1)	1.4 (0.1)	1.1 (0.0)	1.0 (0.1)	1.0 (0.2)		
Galactose <sup>2</sup>	3.2 (0.2)	3.2 (0.1)	2.5 (0.1)	2.1 (0.1)	1.8 (0.1)	1.9 (0.2)		
Non starch glucose <sup>2</sup>	34.7 (2.2)	39.7 (1.0)	35.2 (0.1)	35.6 (0.8)	36.1 (0.6)	34.0 (1.7)		

Mean value (standard deviation). All analyses were carried out at least in duplicate except for protein analysis and analyses on inner fraction at mature stage. In these latter cases, the amount of material was limited and phenolic analyses were prioritized. N 5.7: nitrogen protein conversion factor of 5.7; IF : inner fraction ; AL : aleurone layer ; <sup>1</sup> expressed as weight percentage of AIR ; <sup>2</sup> expressed as weight percentage of non starch neutral sugars ; \*IF for developing grain contained the AL; \*\*for the mature sample “IF + AL”: average composition of the sum of inner fraction and aleurone layers; it was calculated assuming that inner fraction accounted for 32% and aleurone layer for 68% of the total weight [5]. Mean value (standard deviation).

diferulates remain stable and that of trimer increased (Table 2).

Lignin was studied using thioacidolysis [31]. This method provides lignin-derived H, G and S monomers specifically from H, G and S lignin units only involved in labile ether bonds. In agreement with previous studies performed on dry wheat grains [23,27,32], H, G and S thioacidolysis monomers could be evidenced from the thioacidolysis of AIR samples (Table 3). Whatever the developmental stage and even at early stages from 200°DAF, lignin-derived monomers could be evidenced both from the outer pericarp and from the inner fractions. For the inner fractions, the yield of thioacidolysis monomers increased with development. For the outer pericarp fractions, the yield increased from 200 to 450°DAF and then decreased in the dry mature grain. S/G ratio in outer pericarp fractions was fairly stable except for the mature grain where the value strongly decreased. For inner fractions, the S/G ratio increased between 200 and 570°DAF and then decreased in the mature

sample. The relative molar frequency of lignin-derived H monomers was found to vary in the 3–11% range, with the higher level observed for the mature outer fraction.

In order to gain more information about tissue distribution of lignin, we conducted lignin staining and immunolabelling experiments. The most frequently employed lignin stainings are the Wiesner and the Maüle tests [33]. The Maüle test stains lignins containing S units in purple. It positively and slightly stained the endocarp (cross cells) at 450°DAF but not the other tissues. It reacted with the mesocarp at 530°DAF and at later stages (Fig. 2). The Wiesner test reveals *p*-hydroxycinnamaldehyde end-groups present in lignins mainly as coniferaldehyde end-groups by a reddish-purple coloration. The Wiesner test reacted in a consistent manner with the cell walls of the pericarp at 650°DAF (not at prior stages, data not shown) and in the dry grain (mature) sample (Fig. 2). A staining heterogeneity was observed - the

**Table 2**  
Amounts of FA (monomer, dimers and trimer) and of CA released by mild alkaline hydrolysis of the outer layers of developing wheat grain (Recital). Values are expressed as mg. g<sup>-1</sup> of AIR from which starch content was subtracted.

	200°D	250°D	350°D	450°D	570°D	Mature		
	IF*	IF*	IF*	IF*	IF*	IF	AL	IF + AL
FA**	4.37 (0.06)	3.70 (0.03)	6.25 (0.32)	6.55 (0.54)	5.81 (0.10)	4.68 (0.29)	6.94 (0.02)	6.22
FA dimers***	0.82 (0.04)	0.76 (0.02)	0.86 (0.05)	0.69 (0.04)	0.74 (0.01)	0.54 (0.06)	0.34 (0.00)	0.39
8-5'	0.40 (0.03)	0.35 (0.01)	0.42 (0.03)	0.37 (0.02)	0.40 (0.00)	0.32 (0.03)	0.18 (0.00)	0.22
8-O-4'	0.24 (0.01)	0.23 (0.01)	0.24 (0.02)	0.19 (0.01)	0.20 (0.01)	0.13 (0.02)	0.07 (0.00)	0.09
5-5'	0.18 (0.01)	0.18 (0.00)	0.20 (0.00)	0.13 (0.00)	0.14 (0.00)	0.09 (0.02)	0.09 (0.00)	0.08
FA trimer	0.13 (0.01)	0.11 (0.01)	0.10 (0.01)	0.10 (0.00)	0.08 (0.01)	0.06 (0.01)	0.03 (0.01)	0.04
CA	0.09 (0.00)	0.03 (0.00)	0.13 (0.01)	0.13 (0.00)	0.12 (0.01)	0.04 (0.00)	0.10 (0.00)	0.08
<b>Outer pericarp</b>								
FA**	13.80 (0.28)	14.22 (0.12)	18.27 (0.43)	18.96 (0.30)	18.05 (0.21)	2.72 (0.01)		
FA dimers***	1.95 (0.04)	1.96 (0.06)	2.17 (0.06)	1.71 (0.01)	2.14 (0.02)	2.13 (0.04)		
8-5'	0.90 (0.03)	0.85 (0.02)	0.99 (0.03)	0.85 (0.01)	1.18 (0.00)	1.21 (0.03)		
8-O-4'	0.60 (0.02)	0.60 (0.01)	0.63 (0.03)	0.45 (0.01)	0.50 (0.00)	0.51 (0.01)		
5-5'	0.45 (0.03)	0.51 (0.03)	0.55 (0.00)	0.42 (0.01)	0.46 (0.01)	0.41 (0.01)		
FA trimer	0.28 (0.00)	0.29 (0.00)	0.28 (0.05)	0.24 (0.01)	0.21 (0.01)	0.63 (0.01)		
CA	0.34 (0.00)	0.24 (0.02)	0.29 (0.00)	0.49 (0.02)	0.39 (0.02)	0.05 (0.00)		

Mean value (standard deviation). \*Inner fractions (IF) from developing grains contained the aleurone layer (AL). \*\*Ferulic acid monomer values were calculated by summing (E)-FA and (Z)-FA content; \*\*\*Ferulic acid dimers values were calculated by summing 8-5' (measured 8-5' and 8-5' benzo), 8-O-4' and 5-5' diferulate contents. For the mature sample “IF + AL”: average composition of the sum of inner fraction and aleurone layers; it was calculated assuming that inner fraction accounted for 32% and aleurone layer for 68% of the total weight [5].

**Table 3**

Lignification of the outer layers in the developing wheat grain (Recital) evaluated by thioacidolysis. Yields are expressed as nMole. g<sup>-1</sup> of AIR from which starch content was subtracted and lignin composition as relative molar % of H, G, S.

	200°D	250°D	350°D	450°D	570°D	Mature		
	IF*	IF*	IF*	IF*	IF*	IF	AL	IF + AL
Total yield (H + G + S)	1751 (164)	1405 (68)	2808 (12)	3688 (103)	7590 (712)	28,753 (1476)	546(64)	9569
% H	10.0 (0.1)	3.2 (0.1)	5.8 (0.6)	5.4 (0.2)	4.1 (0.1)	3.9 (0.2)	tr	3.7
% G	46.5 (0.2)	51.4 (0.5)	47.6 (1.2)	38.8 (1.4)	26.7 (1.1)	38.5 (1.1)	76.1 (2.4)	39.9
% S	43.6 (0.3)	48.6 (0.5)	46.7 (1.2)	55.9 (1.2)	69.2 (1.1)	57.6 (1.3)	23.9 (2.4)	56.3
S/G	0.94 (0.01)	0.94 (0.02)	0.98 (0.06)	1.44 (0.08)	2.60 (0.15)	1.50 (0.08)	0.32 (0.05)	1.41
<b>Outer pericarp</b>								
Total yield (H + G + S)	9977 (508)	13,156 (361)	16,098 (860)	26,001 (604)	20,879 (547)	15,977 (210)		
% H	5.8 (0.2)	2.8 (0.2)	3.9 (0.1)	6.2 (0.1)	4.8 (0.4)	11.4 (0.0)		
% G	44.9 (0.8)	42.0 (0.5)	42.0 (0.2)	40.6 (0.3)	38.9 (0.1)	45.7 (0.1)		
%S	49.4 (0.6)	55.3 (0.6)	54.2 (0.1)	53.4 (0.3)	56.4 (0.3)	42.9 (0.1)		
S/G	1.10 (0.03)	1.32 (0.04)	1.29 (0.01)	1.32 (0.02)	1.45 (0.01)	0.94 (0.00)		

Mean value (standard deviation). \*Inner fractions (IF) from developing grains contained the aleurone layer (AL). For the mature sample "IF + AL": average composition of the sum of inner fraction and aleurone layer; it was calculated assuming that inner fraction accounted for 32% and aleurone layer for 68% of the total weight [5].

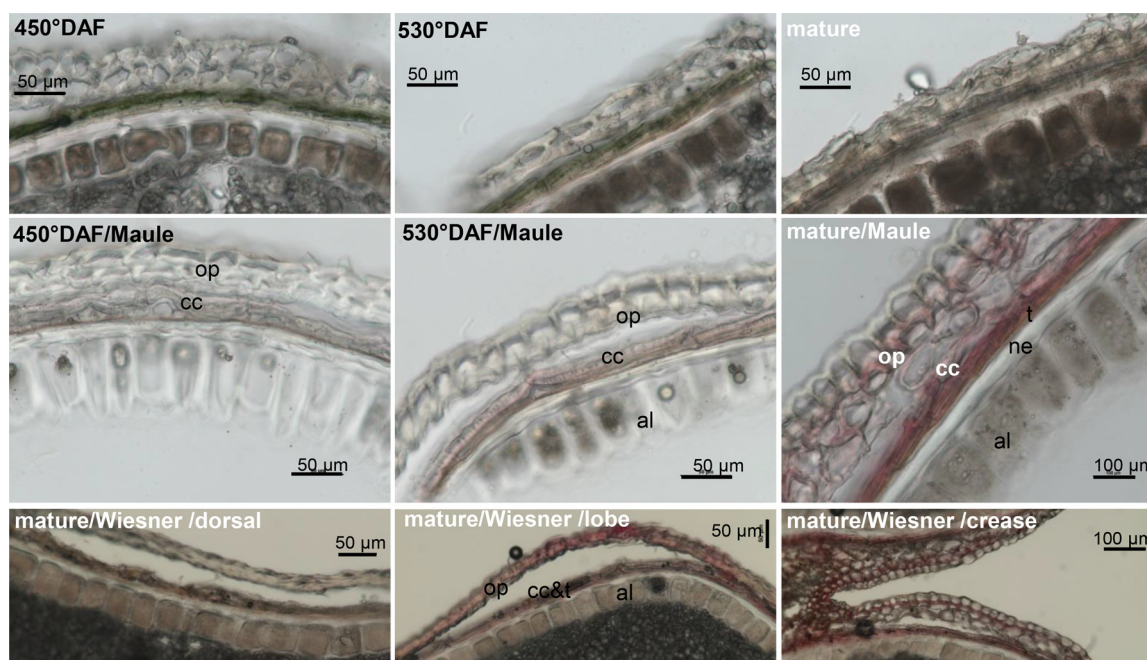
lobe and crease regions reacted more intensively to the dyes as compared to the dorsal region (Fig. 2). Immunolabelling experiments were carried out with an antibody targeting a  $\beta$ -5 dimer of coniferyl alcohol [21]. This antibody was previously used to detect lignins in grains of *Brachypodium distachyon*, a wild grass species related to wheat [22]. A positive labelling was obtained in the cell walls of the pericarp of wheat grains at all investigated stages (Fig. 3). The cell walls of the outer pericarp reacted positively as well as those of the cross cells and testa at 470°DAF. No signal was detected for cell walls of the nucellar epidermis and aleurone layer. At early stages such as 220°DAF, the labelling was more intense in the cell walls of the epicarp and in cell corners of subepidermal mesocarp cells.

Cutin composition was monitored in the developing and mature outer layers of wheat grains but without further dissection (Table 4). At all stages, the cutin composition was dominated by two monomers (i.e.) 18-hydroxyoctadecenoic acid and 9,10-epoxy-18-hydroxyoctadecanoic

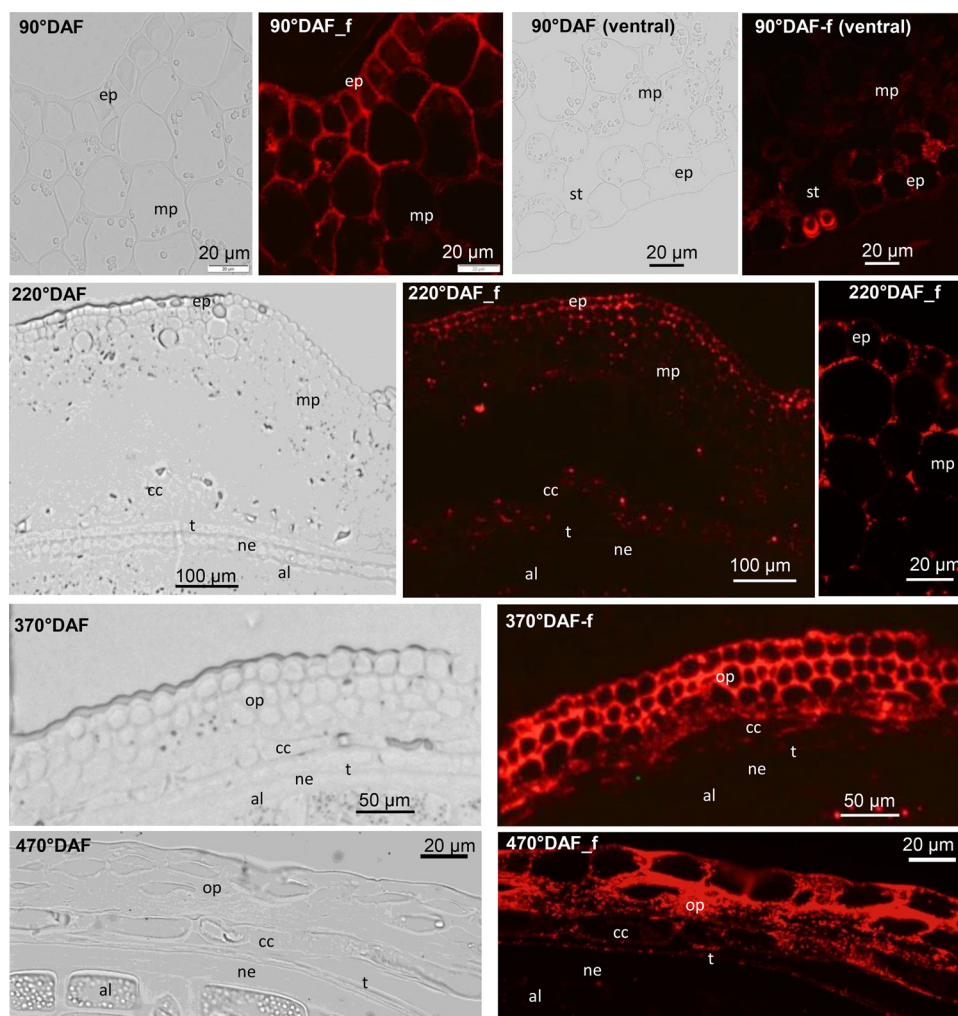
acid, in accordance with previous data obtained from different wheat cultivars [4,28]. Significant amount of FA was recovered from the transmethylation of the outer layers. A faint, but constant amount of CA was also identified throughout the development.

#### 4. Discussion

In this work, the outer layers of the wheat grain (cultivar Recital) were examined during grain development with a particular focus on their cell walls. Previous studies showed that the major cell wall polymers of the wheat outer layers of mature grains were cellulose and heteroxylans, with a few percent of mixed-linkage glucans, lignins and cutin [5,14,23]. Immunolabelling experiments demonstrated that mixed-linkage glucans and cellulose were predominant in the outer layers early in the development. The use of xylan-targeted antibodies revealed that arabinoxylans were present from 90°DAF first in cell walls



**Fig. 2.** Lignification of the developing wheat grain studied by lignin staining. Cross sections of grains at different stages of development were imaged without (top) or with staining with Maule (middle) or Wiesner (Phloroglucinol – HCl) (bottom) reagents that revealed the presence of lignins by a red coloration. op: outer pericarp, cc: cross cells, t: seed coat, ne: nucellar epidermis, al: aleurone layer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



**Fig. 3.** Lignification of the developing wheat grain studied by immunolabelling with KM1. Cross sections of wheat grains were incubated with KM1, an antibody targeting a structure present in lignins. Brightfield (left) and fluorescence (right; *\_f*) images were acquired in the dorsal area of the grain except if stated otherwise. Sections for 90°DAF were incubated with KM1 (supernatant). Sections for 220°DAF, 370°DAF and 470°DAF were incubated with KM1 (ascite fluids). Positive signals were obtained for cell walls of the outer pericarp at all investigated stages. ep: epicarp, mp: mesocarp, op: outer pericarp, cc: cross cells, t: seed coat, ne: nucellar epidermis, al: aleurone layer, st: stomata.

of the nucellar epidermis and of the epicarp, and later in most cell walls of the grain [14,34]. Lignins were reported in wheat grain [4,23] but scarcely studied in developing grain (only in [4]).

To better monitor the spatio-temporal deposition of cell wall phenolics in the outer layers of developing wheat grain, we carried out lignin staining and immunolabelling experiments as well as chemical analyses of hand-dissected tissues. While all these methods consistently revealed lignins in the pericarp and at late stages of development, they provided different results at earlier stages. In this case and before 450°DAF, lignin-derived monomers could be evidenced and quantified from samples that were negatively stained by both the Maïle and Wiesner tests. These discrepancies are most likely assignable to the high sensitivity of GC–MS analyses of lignin-derived thioacidolysis

monomers, as compared to lignin histochemical tests and in agreement with a previous study on Brachypodium grains [22]. Immunolabelling with the antibody KM1 raised against the lignin-specific phenylcoumaran structure mostly gave positive results with the outer pericarp (epicarp and mesocarp) walls and even at early developmental stage (at 90°DAF). By contrast to the outer pericarp, the endocarp walls appeared labelled with KM1 only from 470°DAF while thioacidolysis yielded lignin monomers from inner fractions that contain the endocarp as soon as 200°DAF. These contrasting results suggest that immunolabelling may also suffer some detection limit. While the antibody KM1 is raised against a structural motif met in lignins (two G units linked via a phenylcoumaran bonding pattern), the presence of a free phenolic or alcoholic group is required for recognition by the KM1 antibody [21]

**Table 4**

Composition of cutin depolymerization products obtained by transesterification of whole outer layers from developing and mature grains of wheat (Recital).

Depolymerization products (%)	150°D	200°D	250°D	350°D	450°D	550°D	650°D	Mature
palmitic acid	1.40 (0.01)	1.15 (0.11)	0.15 (0.05)	0.4 (0.34)	0.61 (0.91)	0.35 (0.02)	0.69 (0.05)	2.02 (0.33)
CA	0.03 (0.001)	0.02 (0.003)	0.01 (0.001)	0.01 (0.001)	0.01 (0.001)	0.01 (0.002)	0.4 (0.001)	0.02 (0.003)
FA	6.20 (0.22)	5.03 (0.22)	1.01 (0.53)	3.27 (2.22)	1.6 (0.13)	2.98 (0.21)	2.44 (0.22)	2.87 (0.10)
16-hydroxyhexadecanoic acid	4.66 (0.07)	4.10 (0.56)	1.98 (0.28)	2.97 (0.97)	1.83 (0.05)	2.74 (0.04)	2.41 (0.18)	2.99 (0.16)
18-hydroxyoctadecenoic acid	41.61 (0.04)	39.62 (5.18)	38.1 (6.38)	51.45 (8.10)	38.15 (1.18)	51.87 (3.39)	47.24 (3.62)	44.7 (2.69)
8.16-/9.16-dihydroxyhexadecanoic acid	7.17 (0.02)	6.99 (0.91)	6.72 (1.13)	9.08 (2.14)	6.73 (0.21)	9.15 (0.07)	8.24 (0.64)	7.61 (0.46)
18-hydroxyoctadienoic acid	7.28 (0.19)	6.41 (1.59)	2.89 (0.51)	3.62 (0.80)	2.5 (0.09)	3.07 (0.11)	2.32 (0.20)	4.65 (0.23)
9.10-epoxy-18hydroxyoctadecanoic acid	27.89 (0.59)	33.1 (8.66)	48.15 (7.68)	27.7 (8.57)	47.23 (5.8)	27.28 (5.8)	35.72 (5.10)	33.08 (3.6)
9.18-dihydroxyoctadecanoic acid	1.33 (0.07)	1.42 (0.18)	0.65 (0.07)	1.03 (0.23)	0.96 (0.01)	1.8 (0.42)	0.75 (0.31)	1.24 (0.12)
9.10-epoxy-18hydroxyoctadecenoic acid	2.43 (0.14)	2.17 (0.09)	0.35 (0.09)	0.48 (0.12)	0.39 (0.02)	0.78 (0.07)	0.48 (0.03)	0.85 (0.04)

Values are means of triplicates (standard deviation) and are expressed in % of total depolymerization products.



and the precise epitope is unknown. This epitope might be hidden/masked by other wall components as described for other cell wall epitopes in wheat grain [15].

The quantitative determination of lignins is not a trivial task when performed on grain samples. The gravimetric and sample-demanding Klason method is not adapted to samples available in low amount such as hand-dissected outer layers isolated from whole grains. It is not adapted either to samples containing aliphatic polymers which add to the Klason lignin content, or proteins, as proteins are prone to participate to condensation reactions with Klason lignin. The acetylbromide spectrometric method applied to poorly lignified samples can also suffer severe interferences between lignins and non lignin components, such as proteins or xylan-linked FA. Here, the lignin content was thus estimated using the specific thioacidolysis method [31] that provides H, G and S lignin-derived monomers from lignin H, G and S units, respectively. Whatever the developmental stages, H, G and S lignin-derived monomers could be evidenced, which further emphasizes the high sensitivity and specificity of thioacidolysis when combined with GC–MS analysis of lignin-derived TMS monomers. With the assumption that lignins of wheat grain are degraded with the same thioacidolysis yield as the lignins of wheat straw (i.e. 1 mg lignins approximately provides 1000 nmoles of H, G, S monomers [35], the lignin content of AIR from mature inner fraction (IF Table 3) could be roughly estimated at 2.9% by weight while that of AIR from the mature outer pericarp fraction was estimated to be about 1.6% by weight. These values are in agreement with the ones reported by [23] for hand-dissected tissues from dry wheat grains (outer pericarp fraction 1.13%, intermediate layers which corresponds to our inner fraction without aleurone layer 3.12%). Such a rough lignin estimation from thioacidolysis yields is however not straightforward as this yield relies not only on lignin content, but also on lignin structure. The strong decrease in thioacidolysis yield observed for the outer pericarp fractions between 450°DAF and the mature sample is unlikely assignable to a reduction of lignin content with cell wall maturation, but is more presumably linked to lignin structural changes. Interestingly enough, the maximum relative frequency of lignin-derived H monomers was found in the mature outer pericarp fraction (up to 11% molar percentage). As previously reported by microautoradiography, the incorporation of H units in native lignins increases the frequency of resistant interunit bonds (reviewed in [36]). The lower thioacidolysis yield observed for the mature outer pericarp richer in H lignin units can be related to the higher proportion of resistant interunit bonds. These results suggest that after 450°DAF and in the outer pericarp a change in lignin structure occurs which consists in a higher frequency of resistant interunit linkages.

Grass cell walls contain FA and CA linked to several polymers such as arabinoxylans, lignins and cutins. Here we studied the CA and FA units that are released by cutin depolymerization or by mild alkaline hydrolysis. In both cases, FA was more abundant than CA. FA and CA covalently bound to cutin have already been evidenced in cutin from different plant species [37,38]. In addition, an acyltransferase catalyzing the specific transacylation of hydroxyfatty acids and feruloyl-CoA or coumaroyl-CoA was characterized [39,40]. FA was recovered in cutin depolymerization products from outer layers of developing and mature grains. In percentage, FA decreased with the development. Although the experimental procedure is widely employed to study cutin composition, phenolics release upon cutin depolymerization could partly originate from other cell wall polymers. One challenge in future works would be to establish whether ferulate-mediated linkages could exist between cutins and xylans, and/or lignins.

The FA amount released by mild alkaline hydrolysis increased with the developmental stages up to 450°DAF probably reflecting an increased level of cell wall feruloylation. Globally more FA was released from the outer pericarp samples than from the inner fractions. In addition to FA, we could detect three ferulate dimers and one ferulate trimer which originate from the oxidation of ferulate units and which can act as cross-links between arabinoxylan chains. The amount of

ferulate dimers and trimer was not simply correlated with FA level. Interestingly, for the outer pericarp samples only, and concomitantly with the decrease in thioacidolysis yield, the yield of ester-linked FA drastically decreased between 450°DAF and the mature grain. This phenomenon was also reported by [4] (although they did not separate fractions of outer layers). The levels of FA in outer pericarp and inner fractions from mature grains measured by mild alkaline hydrolysis were consistent with those obtained by [5] on similar wheat grain tissues. Nevertheless an alkaline treatment conducted at high temperature (170 °C; 4 M NaOH) and designed to release ester-linked FA and FA ether-bound to lignins resulted in a 2-fold increase in released FA as compared to mild alkaline hydrolysis (35 °C; 2 M NaOH) both for outer pericarp and inner fractions [5]. In this study, even if we double the FA level obtained for mature outer pericarp fraction, to take into account FA engaged in ether bonds with lignin, we are far to reach the ester-linked FA level measured at 450°DAF. In addition to ester and ether linkages, other types of FA linkages between lignins and arabinoxylans have been described in cereal grains [41,42]. Furthermore, structures involving FA-bonds and currently not detected have been suggested from *in vitro* experiments which consisted in the enzymatic induction of oxidative cross-linking of arabinoxylan chains [43]. We can hypothesize that after 450°DAF special features of epicarp and mesocarp cell walls made FA units less easily releasable by alkaline hydrolysis possibly due to i) an increase of the oxidation of FA units ii) the formation of other types of bonds involving FA that are currently not detected.

By monitoring precisely the evolution of grain dimensions, we established that the grain length of Recital was set early in the development (around 220°DAF) and that the other dimensions were set later in the development (around 600°DAF for the width and 550°DAF for the thickness). Lignins were detected early in grain development by several experimental methods that establish that lignification of wheat grain outer layers begins earlier than previously reported and well before the grain reaches its final dimensions. Strong changes in the structure and properties of cell walls have been revealed at later stages in the epicarp and mesocarp starting between 450° and 570°DAF when both the grain maximal width and thickness are reached. These changes concomitantly reduced the recovery of lignin-derived thioacidolysis monomers and that of alkali-releasable FA and may be the respective signatures of increased frequency of resistant bonds in lignins and of ferulate-mediated polymer cross-linkage.

Structural modifications of grain cell walls are most likely prone to affect their mechanical properties and, thereby, the extensibility of grain tissues. Key factors governing the mechanical properties of cell walls are thickness, cellulose orientation, cross-linking of polymers, lignin deposition and cuticle occurrence [44–49]. In the present study, we provided the first evidence that lignin deposition occurs in grain cell walls even at early developmental stages, when conventional lignin stainings fail to reveal lignification. In addition, we have shown that at late developmental stage and concomitantly to the cessation of grain growth, major structural changes occurred in the phenolic components of grain outer layers, with an increased frequency of resistant interunit bonds in lignins and a dramatic reduction of alkali-releasable FA units. This reduction supports the hypothesis of additional FA-mediated cross-links between cell wall polymers. Such modifications in cell wall phenolics are likely to stiffen the cell walls and therefore the tissue itself. Considering the timing of the modifications, this study raised the possibility that, together with other factors (cell expansion and proliferation, restriction of endosperm growth) [50–52], phenolic-mediated cell wall stiffening in developing outer pericarp might participate to grain size by mechanically constraining grain growth. Additional works will be conducted to confirm this hypothesis, such as the investigation of the mechanical properties of the developing grain outer tissues in cultivars with different growth kinetics.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.plantsci.2018.08.022>.

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