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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 β(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 macroscope (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 µm) obtained with an ultramicrotome (UC7, Leica) were stained with 1% (w/v) toluidine blue O as described in [16] before observation with a multizoom macroscope (AZ100 M, Nikon). For lignin staining, grain samples were frozen and cut using a cryotome (HM 500 OM, Microm) into 50 µm thick sections that were stained using the Maüle and the Wiesner (phloroglucinol-HCl) reagents as described in [17] and observed using a macroscope. 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 µm) 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 µm) 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 macroscope 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 (6300*g*; 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 (6300*g*; 10 min). The AIR sample was dried first in an oven at 40 °C for 24 h and then over P_2O_5 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].

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