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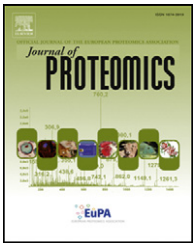

Identification of glycosyltransferases involved in cell wall synthesis of wheat endosperm

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ABSTRACT

Plant cell walls are complex structures critical for plant fitness and valuable for human nutrition as dietary fiber and for industrial uses such as biofuel production. The cell wall polysaccharides in wheat endosperm consist of two major polymers, arabinoxylans and beta-glucans, as well as other minor components. Most of these polysaccharides are synthesized in the Golgi apparatus but the mechanisms underlying their synthesis have yet to be fully elucidated and only a few of the enzymes involved have been characterized. To identify actors involved in the wheat endosperm cell wall formation, we used a subcellular fractionation strategy to isolate Golgi-enriched fractions from endosperm harvested during active cell wall deposition. The proteins extracted from these Golgi-enriched fractions were analyzed by LC–MS/MS. We report the identification of 1135 proteins among which 64 glycosyltransferases distributed in 17 families. Their potential function in cell wall synthesis is discussed. In addition, we identified 63 glycosylhydrolases, some of which may be involved in cell wall remodeling. Several glycosyltransferases were validated by showing that when expressed as fusion proteins with a fluorescent reporter, they indeed accumulate in the Golgi apparatus. Our results provide new candidates potentially involved in cell wall biogenesis in wheat endosperm.

1. Introduction

Plant cells are surrounded by cell walls composed of polysaccharides, proteins and, in some specialized cells, various non-carbohydrate substances (e.g., lignins) [1]. Cell wall composition varies between species and between cell types and developmental stages within a given plant species. Grasses are noteworthy for the unusual composition of their cell walls (type II walls), which contain less pectin and xyloglucan, but more heteroxylan than the walls of other higher plants. The chemical structure of the components and the way that they are assembled are key factors underlying cell wall properties. These properties are important not only for plant fitness but also for the quality of crop materials (e.g., degradation potential for biofuel production, dietary fiber and bread-making quality of wheat flour).

The cell wall composition of wheat grain endosperm is very specific, essentially composed of arabinoxylan (AX) (70%) and (1–3) (1–4) β-D-glucan or mixed-linked glucan (MLG) (20%) with minor amounts of cellulose (4%) and mannan (7%) [2,3]. The

Abbreviations: AX, arabinoxylan; MLG, (1–3) (1–4) β-D-glucan or mixed-linked glucan; GT, glycosyltransferase; GH, glycosylhydrolase.

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synthesis of cell wall polysaccharides is activated during wheat grain development. Drastic changes in cell wall composition and structure were observed in the developing endosperm [4–6].

The metabolism of cell wall polysaccharides requires the intervention of numerous enzymes. Enzymes acting on carbohydrates are listed and classified on the basis of their sequence similarities and activities in the Carbohydrate-Active enZYmes database (CAZy, http://www.cazy.org/) [7]. Plants are underrepresented in this database, essentially because there are less fully-sequenced genomes of plant species compared to other kingdoms such as bacteria. Cell wall polysaccharides are synthesized by enzymatic complexes in two major subcellular locations. While cellulose microfibrils and callose are synthesized at the plasma membrane surface [8–10], pectin and hemicellulose are synthesized in the Golgi apparatus to then be transported to the wall [11–15].

The Glycosyltransferase (GT) superfamily contains enzymes that synthesize polysaccharides such as starch and cell wall polysaccharides. GTs are also involved in the glycosylation of many other substrates. Several GTs involved in cell wall polysaccharide synthesis have been localized in the Golgi apparatus [16–18], some are membrane proteins containing transmembrane domain (TMD), others are devoid of TMD and associated to membrane GTs [19,20].

To gain access to the cellular machinery involved in the synthesis of the cell wall hemicelluloses in the wheat grain endosperm, a subcellular proteomic approach could be applied to focus on the direct actors of the polysaccharide biosynthesis, i.e., the enzymes [20]. However subcellular proteomics remains challenging for the endomembrane system. In the case of the Golgi apparatus and the ER, their similar density makes it very tedious to isolate them as pure fractions. Moreover, the large number of proteins trafficking from the ER to the Golgi apparatus inevitably leads to the identification of ER proteins, even in an ER-free Golgi preparation [21]. Efficiently monitoring the subcellular fractionation and assessing the possible contamination of the isolated fractions are additional difficulties [20,22]. Finally, many GTs are membrane proteins characterized by issues such as poor solubility, low abundance and recalcitrance to classical proteomic techniques.

In the present work, we have developed a subcellular strategy based on wheat endosperm endomembrane fractionation from developing grain harvested at a stage where cell wall polysaccharides are accumulating. We partitioned integral membrane and water soluble proteins associated with the endomembranes. Using mass spectrometry, we identified 1135 proteins, 64 of which were predicted to be GTs involved in glycosylation processes in the wheat endosperm. We also identified 63 GHs potentially involved in the remodeling or degradation of the endosperm carbohydrates.

2. Materials and methods

2.1. Plant materials

The plants *Triticum aestivum* cv. Recital were grown in pots in a greenhouse under conditions of natural day length (UMR Amélioration des Plantes et Biotechnologies Végétales, INRA-Rennes, France). To harvest grains at defined developmental stages, individual ears were tagged at flowering. Seed development was calculated on the basis of cumulated temperature in Celsius degrees days (°D) after flowering. Grains were harvested between 250 and 275°D and endosperms were isolated manually and maintained in buffer A (250 mM sucrose, 10 mM HEPES–NaOH, pH 7.4, 1 mM EDTA, 1 mM DTT and anti-protease cocktail) (Roche) on ice before further treatment.

For microscopy studies, the plants were grown in pots in a growth chamber at 23 °C day/15 °C night at 65% relative humidity under a 16 h/8 h day/dark photoperiod (UPR 2355/IFR 87, Gif Sur Yvette, France). Wheat grains were harvested at 250°D.

2.2. Electron transmission microscopy

2.2.1. Sample preparation

Pieces of tissue (1 mm^3) were sampled from halved wheat grains. For morphological assay, samples were loaded into copper hats filled with 1-hexadecene and high-pressure frozen using a HPF-EM PACT 2 freezer (Leica, http://www.leica-microsystems.com). The hats were immediately stored in liquid nitrogen until the freeze substitution procedure was initiated. Samples were transferred to a freeze substitution automate (AFS2/FSP, Leica), pre-cooled to −90 °C, then substituted in anhydrous acetone with 2% osmium tetroxyde at −90 °C for 125 h. The temperature was gradually raised to −30 °C for 60 h and stabilized for 12 h, then gradually raised to −4 °C (34 h) and stabilized for 3 h. Samples were rinsed in anhydrous acetone several times and slowly infiltrated and embedded in Epon's resin.

For immunolabeling, samples were prepared as described by Chevalier et al. [23]. High-pressure freezing was performed with the HPF-EM PACT I freezer. Samples were then transferred to an AFS. Samples were finally embedded in London Resin White (LRW, LRWhite R1280 HARD GRADE, London Resin Company Limited) and polymerization was performed in the AFS apparatus at −15 °C under UV light for 48 h.

2.2.2. Polysaccharide staining and immunolabeling

Periodic acid-thiosemicarbazide-silver proteinate staining (PATAg) [24] for polysaccharide detection. Ultra-thin sections (80 nm) were cut from Epon's embedded samples using an ultramicrotome (MICROM MT-7000) equipped with a diamond knife and directly floated in 1% periodic acid (MERCK) solution for 30 min. They were then rinsed 5 times in deionized water before incubation in thiosemicarbazide (0.2%) (MERCK) diluted in 20% acetic acid for 17 h. Sections were rinsed 5 times in decreasing acetic acid concentrations (20, 10, 5 and 2.5%), 4 times in deionized water, and stained with 1% aqueous silver proteinate (PROLABO) in the dark for 30 min. After rinsing 5 times in deionized water, sections were mounted on copper grids. Sections were examined with a JEOL 1230 transmission electron microscope with an accelerating voltage of 80 keV (IBISA/BioGenOuest Biopolymers, Interactions, Structural Biology platform (BIBS), UR 1268 BIA, INRA Angers-Nantes).

Immunolabeling. Ultra-thin sections (80 nm) were cut from LRW embedded samples and collected on nickel grids. Sections were incubated in a blocking solution of 3% (w/v) BSA in 20 mM PBS, pH 7.2, to block non-specific labeling for 30 min at room temperature. Sections were then incubated in a solution containing the mouse monoclonal antibody anti-AX1 [25] and the rabbit polyclonal antibody raised against the pea