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Changes in cell wall polysaccharide composition, gene transcription and alternative splicing in germinating barley embryos

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ABSTRACT

Barley (*Hordeum vulgare* L.) seed germination initiates many important biological processes such as DNA, membrane and mitochondrial repairs. However, little is known on cell wall modifications in germinating embryos. We have investigated cell wall polysaccharide composition change, gene transcription and alternative splicing events in four barley varieties at 24 h and 48 h germination. Cell wall components in germinating barley embryos changed rapidly, with increases in cellulose and (1,3)(1,4)-β-D-glucan (20–100%) within 24 h, but decreases in heteroxylan and arabinan (3–50%). There were also significant changes in the levels of type I arabinogalactans and heteromannans. Alternative splicing played very important roles in cell wall modifications. At least 22 cell wall transcripts were detected to undergo either alternative 3′ splicing, alternative 5′ splicing or intron retention type of alternative splicing. These genes coded enzymes catalyzing synthesis and degradation of cellulose, heteroxylan, (1,3)(1,4)-β-D-glucan and other cell wall polymers. Furthermore, transcriptional regulation also played very important roles in cell wall modifications. Transcript levels of primary wall cellulase synthase, heteroxylan synthesizing and nucleotide sugar inter-conversion genes were very high in germinating embryos. At least 50 cell wall genes changed transcript levels significantly. Expression patterns of many cell wall genes coincided with changes in polysaccharide composition. Our data showed that cell wall polysaccharide metabolism was very active in germinating barley embryos, which was regulated at both transcriptional and post-transcriptional levels.

1. Introduction

The cell wall is unique to plants and is important for development, growth and survival in diverse environments. The plant cell wall is a complicated mixture of polysaccharides including cellulose, heteroxylan (HX), (1,3)(1,4)-β-D-glucan, pectin, lignin and other polymers. Cell wall composition varies between different parts of the barley grain. Cell walls of barley endosperm contain high levels of (1,3)(1,4)-β-D-glucans (75% v/v) and substantial amounts of HX (20% v/v), while cell walls in aleurone layers contain high levels of HX (71%,v/v) and moderate amounts of (1,3)(1,4)-β-D-glucans (26% v/v) (Fincher, 2010). Cellulose levels are extremely low in the cell walls of endosperm and aleurone layers (2%, v/v). Cell walls in barley grains also contain small amounts of other polysaccharides including glucomannans, arabinogalactans, arabinans and xyloglucans.

Biosynthesis and modification of plant cell wall polysaccharides require a suite of enzymes. The number of genes coding for these enzymes and regulatory proteins is more than 1200 (Burton et al., 2010). HX backbones are synthesized by members of the glycosyltransferase family 43 (GT43) and glycosyltransferase family 47 (GT47) (Scheller and Ulvskov, 2010). The

Abbreviations: AG, arabinogalactan; AIR, alcohol insoluble residues; AS, alternative splicing; A3S, alternative 3′ splicing; A5S, alternation 5′ splicing; IBSC, International Barley Sequencing Consortium; CesA, cellulose synthase A; CslF, cellulose synthase-like F; DEG, differentially expressed gene; EI, (1,3)(1,4)-β-D-glucan endohydrolase isoenzyme I; GT, glycosyl transferase; HvUGAE, UDP-glucouronic acid epimerase; HvUGE, UDP-Glc epimerase; HvUXE, UDP-Xyl epimerase; HvUXS, UDP-Xyl synthase; HX, heteroxylan; IR, intron retention; IUM, initially unmapped; QC, quality control; RPKM, reads per kilo base-pair per million mapped reads; TF, transcription factor; XET, xyloglucan endotransglucosylase/hydrolase.

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glucuronosyl substitution of the backbone is catalyzed by members of the glycosyltransferase family 8 (GT8) (Mortimer et al., 2010), while the α-(1,3)-linked arabinosyl substitution for the HX backbone is catalyzed by members of the glycosyltransferase family 61 (GT61) (Anders et al., 2012). Members of the cellulose synthase-like family F (*CslF*) and cellulose synthase-like family H (*CslH*) (both GT2) are responsible for the biosynthesis of (1,3)(1,4)-β-D-glucans (Burton et al., 2006; Doblin et al., 2009). Furthermore, a suite of hydrolytic enzymes may regulate polysaccharide levels in germinating barley grains. These include (1,4)-β-D-xylan endohydrolases (GH10), arabinoxylan arabinofuranohydrolase (AXAH, GH51), α-L-arabinofuranosidase (GH3) and β-D-xylosidase (GH3) for HX degradation (Fincher, 2010), and (1,3)(1,4)-β-D-glucan endohydrolase (GH17), (1,4)-β-D-glucan glucohydrolase (GH1) and β-D-glucan exohydrolase (GH3) for (1,3)(1,4)-β-D-glucan degradation (Fincher, 2010). Several glycosyl hydrolases in GH5, GH9 and GH16 may also be involved in modifications to (1,3)(1,4)-β-D-glucans, as implicated by association mapping with two-row spring and winter barley (Houston et al., 2014). Synthesis of cellulose in barley is catalyzed by cellulose synthases (CesA, GT2) with at least seven *CesA* genes identified in barley (Burton et al., 2004). Xyloglucan endotransglucosylase/hydrolase (XET) is another important cell wall enzyme family. It catalyzes the cross-linking between different polysaccharides including cellulose, (1,3)(1,4)-β-D-glucan and xyloglucans (Hrmova et al., 2009, 2007).

Alternative splicing (AS) plays a key role in post-transcription regulations. It regulates many biological processes including hormone-mediated signal transduction and stress- or light-induced responses (Eckardt, 2013; Reddy et al., 2013; Staiger and Brown, 2013; Syed et al., 2012; Wu et al., 2014). It determines tissue-specific differentiation patterns and controls plant development and adaptation to environmental conditions. Several types of AS events have been detected in plants including alternative 3′ splicing (A3S), alternative 5′ splicing (A5S) and intron retention (IR).

Seed germination initiates a transition from metabolically inactive to active phases for many biological processes and eventually develops to a growing seedling. Cellular membrane and mitochondria repairs have been known to be important in the initiation of seed germination (Bewley, 1997). Transcription of cell wall genes is also very active in germinating barley seeds (An and Lin, 2011). However, little is known about cell wall polysaccharide biosynthetic activities and their regulations at transcriptional and post-transcriptional (e.g., AS) levels. In order to understand the cell wall biosynthetic activities during the seed germination, we have established the cell wall polysaccharide composition by monosaccharide linkage analysis at 24 and 48 h germination in four barley varieties. Monosaccharide linkage data showed that cell wall polysaccharide modification activities were very active during barley seed germination. We have also conducted RNA-sequencing experiments (RNA-seq) to investigate the involvement of AS events and differentially expressed genes (DEG) on cell wall biosynthesis. RNA-seq data showed that transcription of several cell wall genes was very active in germinating embryos and also coincided with changes in profiles of cellulose, HX, (1,3)(1,4)-β-glucan and arabinan. Thus, we concluded that cell wall modification was one of the most important biological processes during the seed germination. Transcription and post-transcription regulations play key roles in the cell wall modifications.

2. Materials and methods

2.1. Plant materials

The barley (*Hordeum vulgare* L.) varieties Bass, Baudin, Harrington and Stirling were used for this study. Seeds were sterilized with 1% (w/v) hypochrorite solution for 15 min, rinsed with 3–5 volumes of running water, transferred to a 14 cm Petri dish covered with two layers of filter paper and 10 mL water and incubated in the dark. Germinating embryos were separated from endosperms at 24 h and 48 h. Many small batches of barley seeds were used to induce germination and embryos were collected at 24 h and 48 h. The time needed for collecting embryos were not lasted for more than 20 min for each small batch. Different batch embryos were pooled and about 200–500 mg of embryo tissues were used for cell wall preparation and RNA purification. The embryos were stored at −80 °C before use.

2.2. Cell wall preparation and cell wall polysaccharide analyses

Three biological replications of embryos (collected at different days) were used for cell wall preparation. For preparation of alcohol insoluble residues (AIR), barley embryos were ground in liquid nitrogen to fine powder, extracted 5–6 times with 80% ethanol (v/v), and once each with acetone and methanol. AIR was de-starched by amylase according to Pettolino et al. (2012). Monosaccharide-linkage analysis was performed by methylation with methyl iodide in sodium hydroxide and DMSO (Ciucanu and Kerek, 1984) followed by hydrolysis, reduction and acetylation. Data were calculated as mol% of total AIR (Pettolino et al., 2012). Monosaccharide linkages (mol%) and relative polysaccharide proportions were deduced from the partially methylated alditol acetates that were separated and analyzed by GC–MS according to Pettolino et al. (2012).

2.3. Sequencing and bioinformatics analysis strategy

Total RNA was extracted from embryos with phenol-SDS reagents (http://onlinelibrary.wiley.com/doi/10.1002/0471142727.mb0403s09/pdf, accessed on 7 December 2015). RNA sequencing and bioinformatics analyses followed a flow chart in Supplemental Fig. S1.

2.3.1. Sequencing

We assessed the quality of the RNA preparation by the Agilent 2100 Bioanalyzer system with a RNA integrated number value greater than 8, after genomic DNA contamination was removed by DNase. We enriched messenger RNA by magnetic beads with Oligo (dT) and fragmented them. The first strand cDNA was synthesized with the mRNA fragments as templates using reverse transcriptase and random hexamer primers. The second-strand cDNA was synthesized with first strand cDNA as template after adding reaction buffer, dNTPs, RNase H, DNA polymerase and $MgCl_2$, purified with QIAquick PCR Purification Kit (Qiagen) and end-repaired with T4 DNA polymerase and Klenow DNA polymerase. After adding an "A" base to the 3′ end, cDNA was ligated to the sequencing adapters with a 'T' base overhang at the 3′ end. DNA fragments with length from 250 bps to 500 bp were selected from electrophoresis gels and purified with QIAquick PCR Purification Kit before PCR amplification (15 cycles). The quality of cDNA libraries were assessed by an Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR system. RNA sequencing was done on a HiSeq2000 (Illumina) using a paired-end sequencing protocol (Pease and Sooknanan, 2012).

2.3.2. Raw data quality control and alignment to reference genome

A quality control step was carried out on raw data by using FastQC software (version 0.11.2, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 7 December 2015). Briefly, the percentage of A/G should roughly equal that of T/C and the rate of N should be less than 1%, while the proportion of low