Cell Walls of Developing Wheat Starchy Endosperm: Comparison of Composition and RNA-Seq Transcriptome[1][C][W][OA]

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The transcriptome of the developing starchy endosperm of hexaploid wheat (*Triticum aestivum*) was determined using RNA-Seq isolated at five stages during grain fill. This resource represents an excellent way to identify candidate genes responsible for the starchy endosperm cell wall, which is dominated by arabinoxylan (AX), accounting for 70% of the cell wall polysaccharides, with 20% (1,3;1,4)-β-D-glucan, 7% glucomannan, and 4% cellulose. A complete inventory of transcripts of 124 glycosyltransferase (GT) and 72 glycosylhydrolase (GH) genes associated with cell walls is presented. The most highly expressed GT transcript (excluding those known to be involved in starch synthesis) was a GT47 family transcript similar to Arabidopsis (*Arabidopsis thaliana*) IRX10 involved in xylan extension, and the second most abundant was a GT61. Profiles for GT43 IRX9 and IRX14 putative orthologs were consistent with roles in AX synthesis. Low abundances were found for transcripts from genes in the acyl-coA transferase BAHD family, for which a role in AX feruloylation has been postulated. The relative expression of these was much greater in whole grain compared with starchy endosperm, correlating with the levels of bound ferulate. Transcripts associated with callose (GSL), cellulose (CESA), pectin (GAUT), and glucomannan (CSLA) synthesis were also abundant in starchy endosperm, while the corresponding cell wall polysaccharides were confirmed as low abundance (glucomannan and callose) or undetectable (pectin) in these samples. Abundant transcripts from GH families associated with the hydrolysis of these polysaccharides were also present, suggesting that they may be rapidly turned over. Abundant transcripts in the GT31 family may be responsible for the addition of Gal residues to arabinogalactan peptide.

The wheat (*Triticum aestivum*) starchy endosperm is a tissue of huge importance to human nutrition. The starchy endosperm cell walls constitute 2% to 3% of white flour, have important effects on physical properties such as viscosity (Saulnier et al., 2007a; Faik, 2010), and provide a source of dietary fiber (Topping, 2007). Due to their importance, the composition of the cell walls of mature wheat starchy endosperm has been extensively studied and reported to comprise approximately 70% arabinoxylan (AX), 20% 1,3;1,4-β-D-glucan, 7% glucomannan, and 4% cellulose (Mares and Stone, 1973a; Bacic and Stone, 1980). The wheat starchy endosperm also represents an excellent system for studying grass cell walls, which differ fundamentally from those of dicots, being characterized by a greater abundance of xylan, which has more arabinosyl substitution, smaller amounts of xyloglucan and pectin and the presence of 1,3;1,4-β-D-glucan, and feruloylation of AX or glucuronoarabinoxylan (GAX; Carpita, 1996; Fincher, 2009; Scheller and Ulvskov, 2010). The grass-specific features of 1,3;1,4-β-D-glucan and feruloylated AX are present in the wheat starchy endosperm cell wall, in a large tissue composed of a single cell type with limited spatial variation, which can be dissected in a pure state for compositional and transcriptome analyses during grain fill, when the great majority of cell wall synthesis takes place. In particular, it provides a powerful system for studying grass xylan synthesis, given the predominance and relatively simple form of this polysaccharide.

However, the starchy endosperm cell wall composition does differ from those of most other wheat tissues in the very low cellulose content and also the apparent lack of pectin and xyloglucan, which, while minor components, are typically present in type II cell walls (Carpita, 1996). The AX component also differs from that in other tissues, including the outer grain tissues, in that it lacks glucuronyl substitution and acetylation and has much less feruloylation (Saulnier et al., 2007b). The structure of starchy endosperm AX,

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therefore, is comparatively simple, with the Xyl*p* backbone residues unsubstituted, monosubstituted at the 3-position, much more rarely at the 2-position, or disubstituted at the 3- and 2-positions by Ara*f*. About 25% of starchy endosperm AX is water extractable, with a molecular mass in the range of 200 to 300 kD, whereas the 75% that is water unextractable has a greater average molecular mass (Saulnier et al., 2007a, 2007b). Feruloylation occurs by ester linkage to the 5-position of a small fraction of Ara*f* that are 3-linked to monosubstituted Xyl*p*.

Wheat starchy endosperm cell wall composition is similar to that of barley (*Hordeum vulgare*) starchy endosperm, but with the proportions of AX and 1,3;1,4-β-D-glucan reversed; however, rice (*Oryza sativa*) starchy endosperm cell walls differ, having around 40% GAX and AX, 10% pectin, 30% cellulose, 15% glucomannan, and detectable amounts of xyloglucan (Fincher and Stone, 1986). Recently, the starchy endosperm cell wall of the model grass *Brachypodium distachyon* has been shown to differ from wheat and barley starchy endosperm cell walls, having higher (approximately 7%) cellulose content and detectable pectin; it also has much greater 1,3;1,4-β-D-glucan content than wheat (Guillon et al., 2011).

All of the above relates to mature starchy endosperm; however, there is a sequence of deposition of different polysaccharides in barley and wheat endosperm cell walls. Immunolabeling of polysaccharides in early barley endosperm development (0–8 d post anthesis [dpa]) showed initial callose and cellulose deposition, followed by 1,3;1,4-β-D-glucan, then glucomannan, then arabinogalactan peptides, and lastly AX (Wilson et al., 2006). The callose appears to be mostly transient, but some remains associated with plasmodesmata (Wilson et al., 2006); there appears to be more in the subaleurone than in the central endosperm in barley (Fulcher et al., 1977). In wheat starchy endosperm, callose is deposited during the cellularization stage but has apparently disappeared when cellularization is complete (Philippe et al., 2006), then 1,3;1,4-β-D-glucan is also deposited during cellularization and deposition continues after this, whereas AX deposition begins only toward the end of cellularization and then increases steeply (Philippe et al., 2006). The nature of the AX also changes during development, with disubstitution by Ara being more common early in development but monosubstitution at the 3-position becoming increasingly dominant (Saulnier et al., 2009; Toole et al., 2010). There have been no reports of pectin or xyloglucan in the cell walls of either mature or developing starchy endosperm of wheat (Shibuya and Nakane, 1984).

Relating cell wall composition to transcript abundance from transcriptome analysis has been widely used to derive lists of candidate genes responsible for synthetic or remodeling steps (Aspeborg et al., 2005; Brown et al., 2005; Mitchell et al., 2007b; Minic et al., 2009). Although the interpretation of transcript abundance must be treated with caution, as it is so far removed from the abundance of product synthesized by encoded enzymes (Fry, 2004; Dhugga, 2005), it has nevertheless proved a powerful first step in the identification of candidates for cell wall synthesis; for example, xylan glucuronyl transferase genes, the function of which has recently been demonstrated (Mortimer et al., 2010), were originally identified as candidates from coexpression with secondary wall-specific CESA transcripts (Brown et al., 2005). We have also exploited the relationship of transcript abundance to composition to identify candidate genes for the synthesis and feruloylation of AX, based on the greater transcript abundance in grasses compared with dicots (Mitchell et al., 2007b). Therefore, we would expect transcripts of AX synthetic genes to be abundant in wheat starchy endosperm but transcripts of BAHD candidate genes for feruloylation to be present, although at relatively low abundance compared with the whole grain. We have previously analyzed the transcriptome of whole wheat caryopsis during development using the Affymetrix wheat GeneChip microarray (Wan et al., 2008; Toole et al., 2010). However, this array only covers a portion of the wheat transcriptome, and the expression signal is highly dependent on the degree of cross-hybridization of the probe set with homeologs and with splice variants. Deep sequencing of cDNA using second-generation sequencing technology, therefore, offers great advantages of revealing both the absolute abundance and the exact sequences of transcripts.

Here, we report the results of sequencing of the transcriptome from pure developing starchy endosperm of hexaploid wheat (variety Cadenza). A recent study (Zeng et al., 2010) reported the relative abundance of six transcripts of cell wall GTs in wheat starchy endosperm at two developmental stages. Our RNA-Seq approach allows, to our knowledge, the first production of a complete inventory of absolute abundance of all cell wall-related transcripts from this single cell type through development during grain fill, the phase when the starchy endosperm undergoes rapid growth and the great majority of cell wall biosynthesis occurs. This comprehensive list of transcripts provides a definitive resource for the identification of candidate genes responsible for wheat starchy endosperm cell wall composition.

RESULTS

The time course of grain dry weight (Fig. 1A) reflects the deposition of storage materials, principally starch, in the endosperm, which requires a large expansion of starchy endosperm cells to accommodate it. The results presented here all relate to the region of endosperm manually dissected out at each of the five stages of development, as shown in Figure 1B.

About 3 million RNA-Seq 73-bp reads were obtained from each of the five time point samples (10, 14, 17, 21, and 28 dpa); transcript abundance is expressed as counts per million counts in the library. Reads were

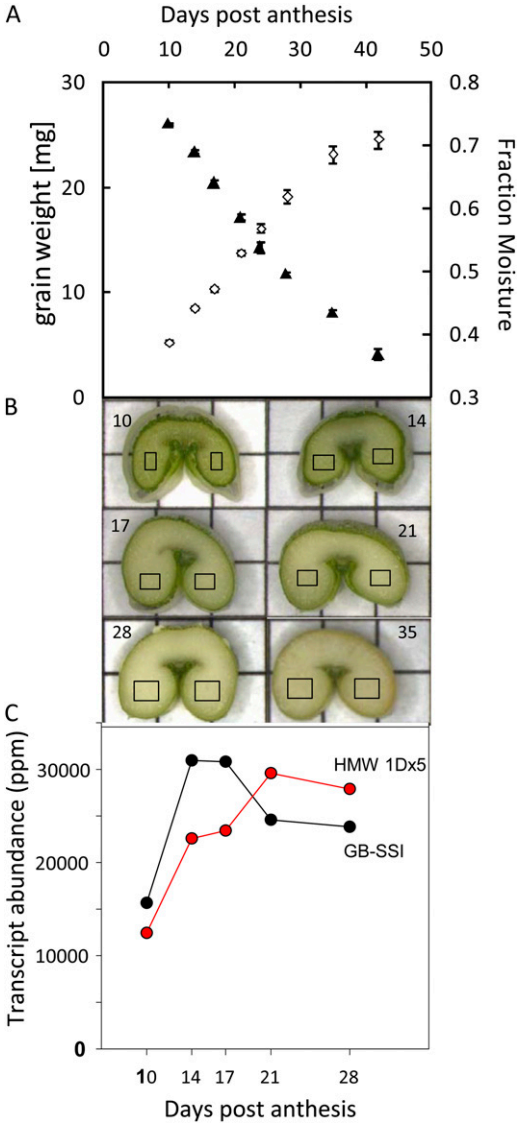

Figure 1. A, Dry weight (diamonds) and fraction moisture of fresh weight (triangles) of grain similar to those used for transcriptome and biochemical analyses (mean of four biological replicates ± SE). B, Grain sections at 10, 14, 17, 21, 28, and 35 dpa, with rectangles indicating sections manually dissected out for these analyses on a 2-mm grid. C, Expression profiles for highly abundant transcripts representative of starch synthesis (granule-bound starch synthase I [GB-SSI]) and storage protein deposition (high-molecular-weight glutenin [HMW 1Dx5]). [See online article for color version of this figure.]

mapped to rice gene sequences in the gene families of interest and assembled together with existing public domain wheat sequences to determine the numbers of genes and transcript abundances (see "Materials and Methods"). Here, we use "gene" to refer to an integration across the three homeologous forms from the three genomes of hexaploid wheat; these are 90% to 99% identical at the nucleotide level and are often identical at the amino acid level, so they can be assumed to perform identical functions. Two of the most abundant transcripts, granule-bound starch synthase I and high-molecular-weight 1Dx5 glutenin (Fig. 1C), indicate the dominance of storage product transcripts in this transcriptome and reflect a pattern of earlier increase for starch synthetic enzymes compared with storage protein (Shewry et al., 2009).

The validity of the methodology for estimating transcript abundance can be assessed by comparison with quantitative reverse transcription (qRT)-PCR determinations from biological replicate samples (Fig. 2). The three internal control genes used to normalize qRT-PCR expression determinations (Fig. 2A) have previously shown relatively constant expression in developing wheat whole grain relative to the total transcriptome (Wan et al., 2008); however, in pure starchy endosperm tissue, they show a decrease in sequence counts after 10 dpa. This is due to a large increase in storage protein transcripts between 10 and 14 dpa (data not shown), which results in a dilution of all other transcripts, expressed relative to the total transcriptome. To correct for this, qRT-PCR values were adjusted to reflect abundance relative to the total transcriptome, and these values were well correlated with sequence counts (Fig. 2B). This confirms that our methodology for counting transcripts, in the absence of a sequenced wheat genome, gives an accurate reflection of transcript abundance.

Figure 3 shows an overview of all the CAZy glycosyltransferase (GT) and glycosylhydrolase (GH) family genes expressed in wheat starchy endosperm (i.e. counts of wheat transcripts mapped to rice orthologs present in the CAZy database; Cantarel et al., 2009), excluding those where orthologs are known to have functions that are not cell wall related. A total of 124 GT genes and 72 GH genes are expressed in wheat starchy endosperm as defined using these criteria; 17 of the GT genes and three of the GH genes are not represented on the wheat Affymetrix array. It can be seen that the GT families with inverting activity (2, 31, 43, 47, 48, and 75) account for the majority of transcripts; while the GT families with retaining activity are less abundant and are dominated by families GT4 and GT8. Over half of the GH transcripts identified here are accounted for by the GH1, GH3, and GH9 families.

Clades in the GT43, GT47, and GT61 families contain candidates for the synthesis of AX (Mitchell et al., 2007b), and expression profiles for genes from GT43, GT47, and GT61 are shown together with their phylogenetic trees in Figure 4. This shows that *TaGT47_2*, closely related to IRX10 in Arabidopsis (*Arabidopsis thaliana*), is the most highly expressed gene of any in the four families and considerably more highly expressed than any other GT47. The second most highly expressed gene within these four families is *TaGT61_1*, which has a noticeably later peak of expression compared with *TaGT47_2*. *TaGT43_2*, which is similar to *IRX9* in Arabidopsis, is expressed at a similar level to *TaGT43_1*, a putative ortholog of *IRX14*. Only two other GT43 genes are expressed in starchy endosperm:

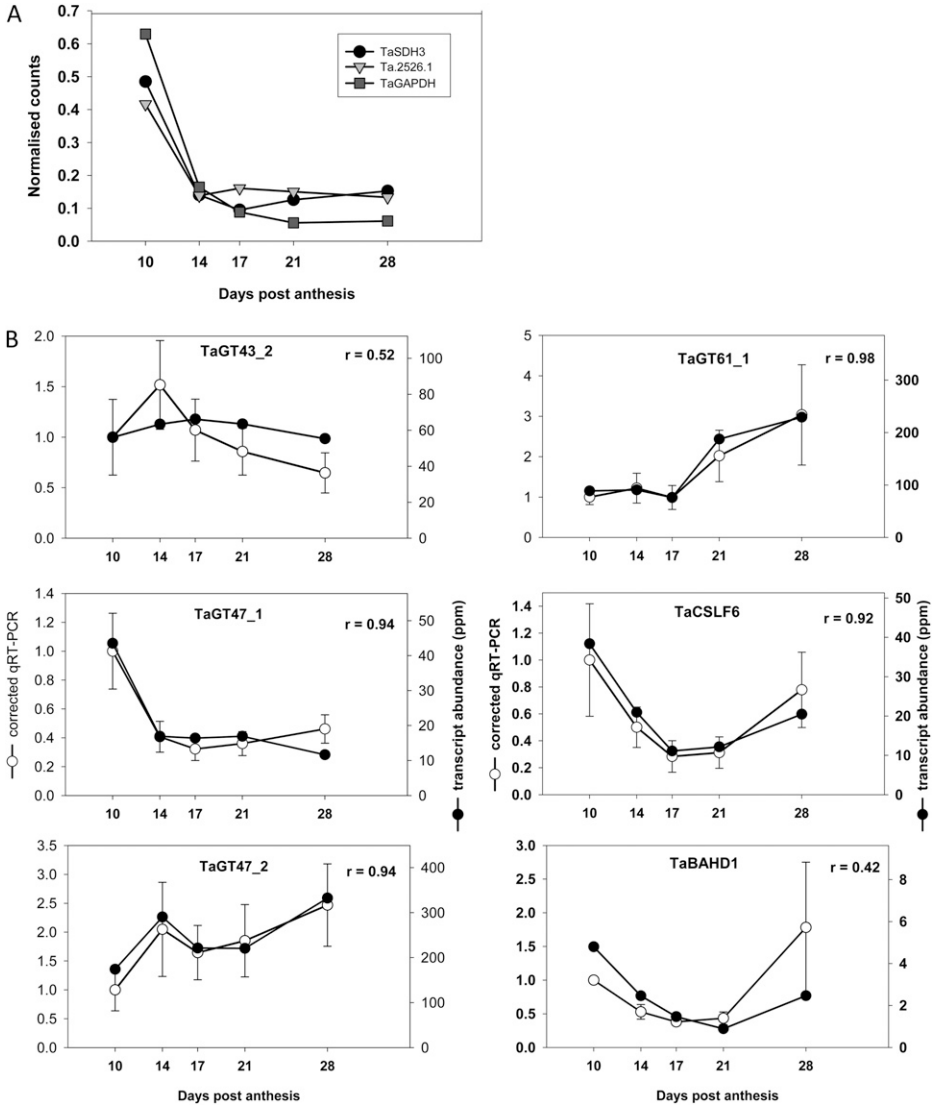

Figure 2. Comparison of transcript abundance estimated by sequence counts and qRT-PCR in developing starchy endosperm. A, Sequence counts for the three transcripts used as qRT-PCR internal controls, normalized to mean values for all time points. B, Sequence counts and qRT-PCR (mean of four biological replicates ± SE) for candidate genes for AX and 1,3;1,4-β-D-glucan synthesis. The correction applied to qRT-PCR for variation in internal controls was multiplication by geometric averages of values shown in A and expressed relative to 10 dpa.

TaGT43_3 and *TaGT43_4*, which show similar abundance and are in the same clade in the family. Knockout mutants in Arabidopsis, *irx9*, *irx10*, *irx10-L*, and *irx14*, all exhibit similar phenotypes of decreased xylan extension in secondary cell walls (Brown et al., 2007, 2009; Peña et al., 2007), so it is likely that *TaGT47_2*, *TaGT43_1*, and *TaGT43_2* are responsible for the synthesis of the AX backbone. The GT61 family was first implicated in AX synthesis by much greater expression in grasses than dicots and coexpression with other genes in the pathway (Mitchell et al., 2007b); recently, a collaboration between our laboratory and that of Prof. Paul Dupree (University of Cambridge) has provided evidence that genes in this family encode xylan arabinosyl transferases (Anders et al., 2012).

Genes in a clade within the BAHD acyl-CoA transferase family were shown to be vastly more highly expressed in grasses compared with dicots and coexpressed with some GT61 genes; from this, they were identified as candidates for AX feruloylation (Mitchell et al., 2007b). These predictions were tested by RNA interference constructs designed to repress the expression of several BAHD candidate genes in rice. Transgenic lines carrying these constructs exhibited a modest (19%) but significant decrease in cell wall feruloylation (Piston et al., 2010). Figure 5A shows the phylogenetic tree, and expression profiles for genes within this particular clade of the BAHD family are shown in Figure 5B; no genes outside this clade within the BAHD family were expressed in wheat starchy endosperm by our criteria. The known precursor steps for the AX synthetic pathway are catalyzed by UDP-Glc dehydrogenase and UDP-Glc decarboxylase to produce UDP-Xyl*p* and UDP-Xyl*p* epimerase to produce UDP-Ara*p*, which is then converted to UDP-Ara*f* by UDP-Ara*p* mutase encoded by UAM genes in the GT75 family (Konishi et al., 2010). The transcripts for these enzymes are shown in Figure 5, C and D; the normalized profiles for the total counts of transcripts for these families are all similar (Fig. 5E).

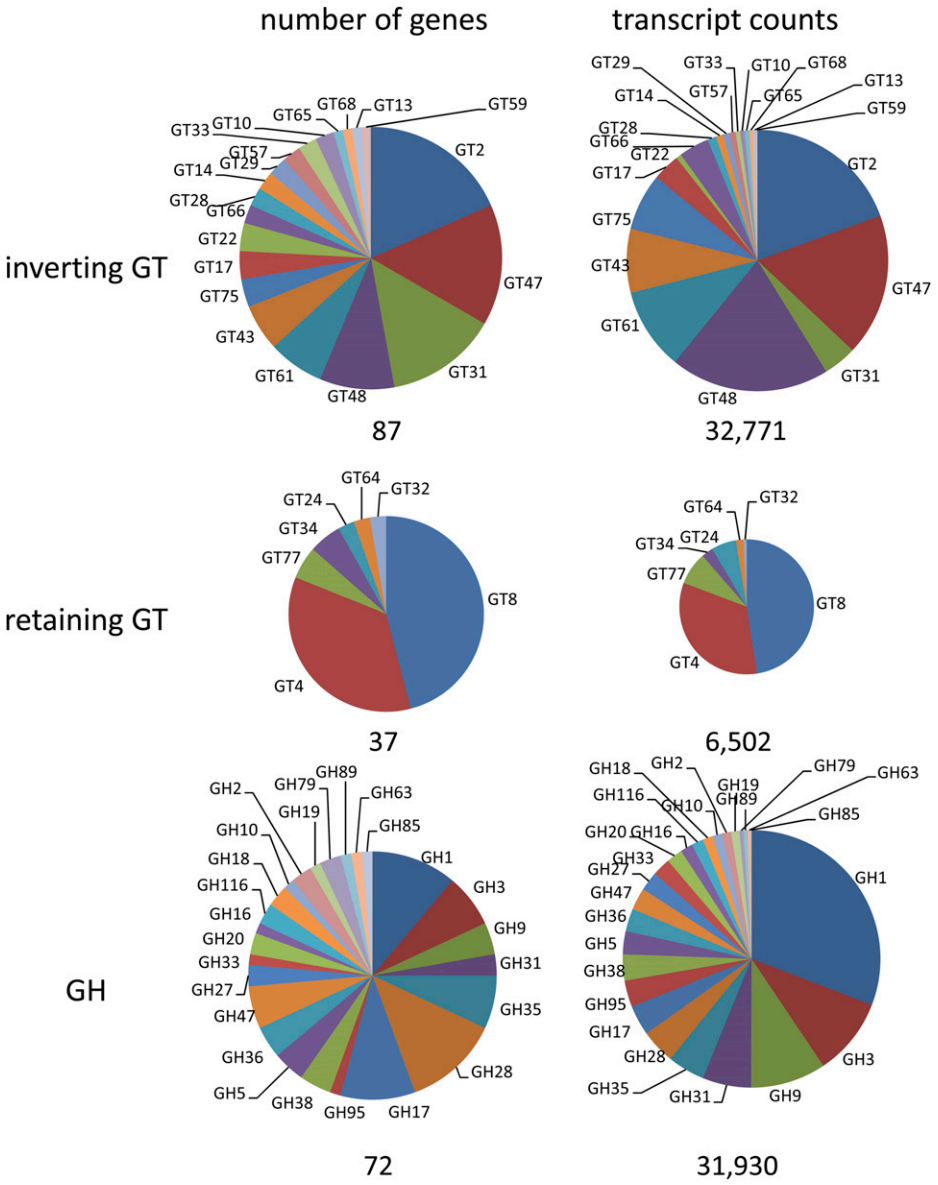

Figure 3. Overview of GT and GH genes expressed in wheat starchy endosperm. Totals for each pie chart are shown below that chart. [See online article for color version of this figure.]

The genes *TaGT43_2*, *TaGT47_2*, and *TaGT61_1* are candidates for AX synthesis, and *TaBAHD1* and *TaBAHD3* are candidates for AX feruloylation, in wheat starchy endosperm, and these have been cloned and full-length sequences deposited in the GenBank/EMBL/DDBJ databases (accession nos. AM701827.1, AM698096.1, FR846232.1, AM749044.1, and FR846233.1, respectively).

The expression profiles for these candidate genes putatively responsible for AX synthesis and feruloylation can be compared with compositional analyses from parallel samples taken from developing starchy endosperm. Figure 6 shows peak areas from high-performance anion-exchange chromatography (HPAEC) analyses of digestion products from cell walls using methodology developed by Saulnier et al. (2009). There is a continual decline in the amount of oligosaccharides derived from 1,3;1,4-β-D-glucan, which is consistent with the early expression peak for *TaCSLF6* (Fig. 2), which is required for 1,3;1,4-β-D-glucan synthesis in wheat starchy endosperm (Nemeth et al., 2010). The total amount of AX oligosaccharides (AXOS) does not change greatly on a dry weight basis until 25 dpa, showing that they are accumulating in line with increasing dry mass through development (Fig. 1A); thereafter, they decline in relative amount. There are some significant changes in two AXOS relative to other AXOS (Supplemental Table S1), indicating an increasing trend of AXOS monosubstituted with Ara and a decreasing trend of AXOS disubstituted, in agreement with previous analyses (Saulnier et al., 2009; Toole et al., 2010). The degree of feruloylation expressed relative to dry weight tends to increase until 28 dpa (Fig. 6), with some decline thereafter. The amount of ferulate per unit of AX can be estimated using the nonstarch monosaccharide

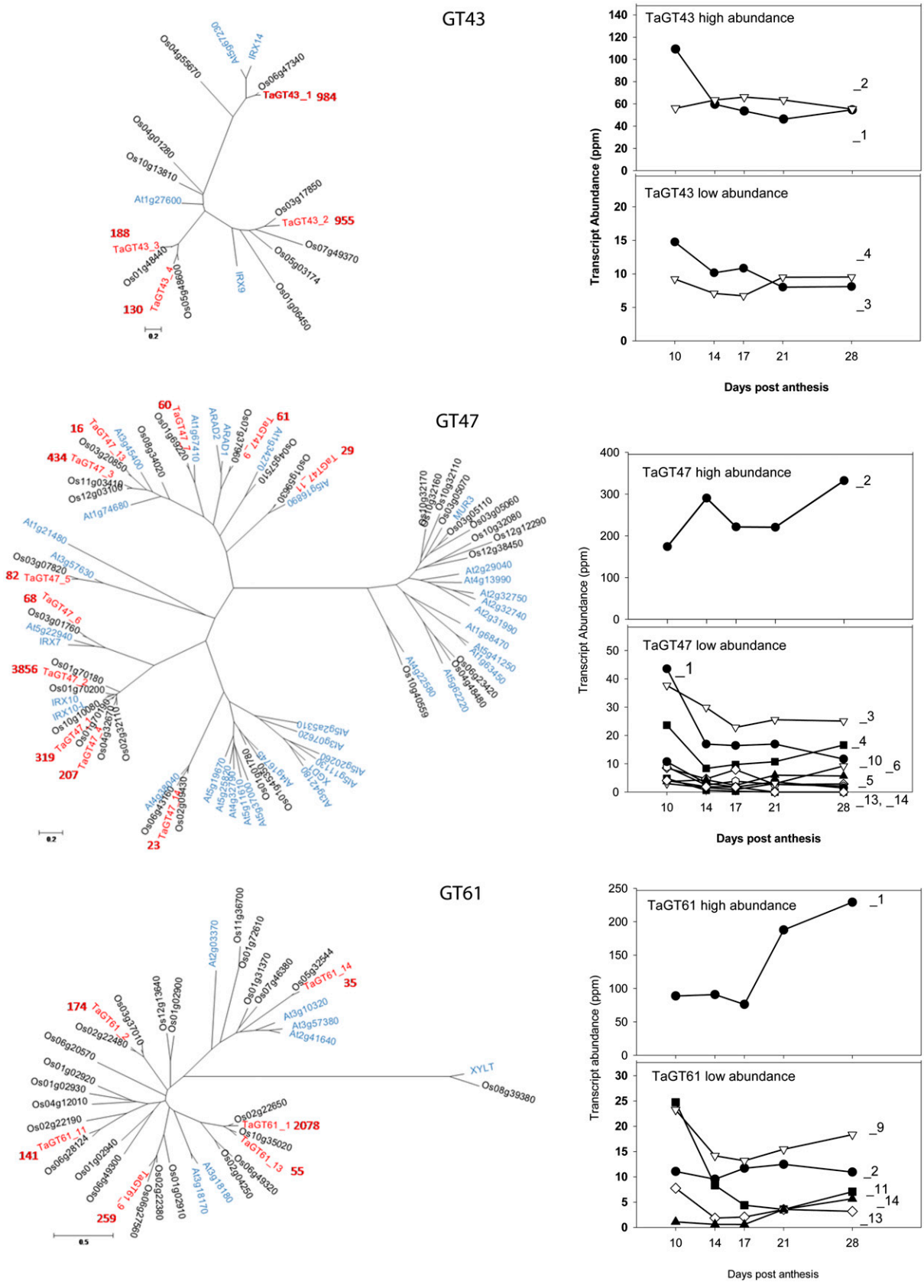

Figure 4. (*Legend appears on following page.*)

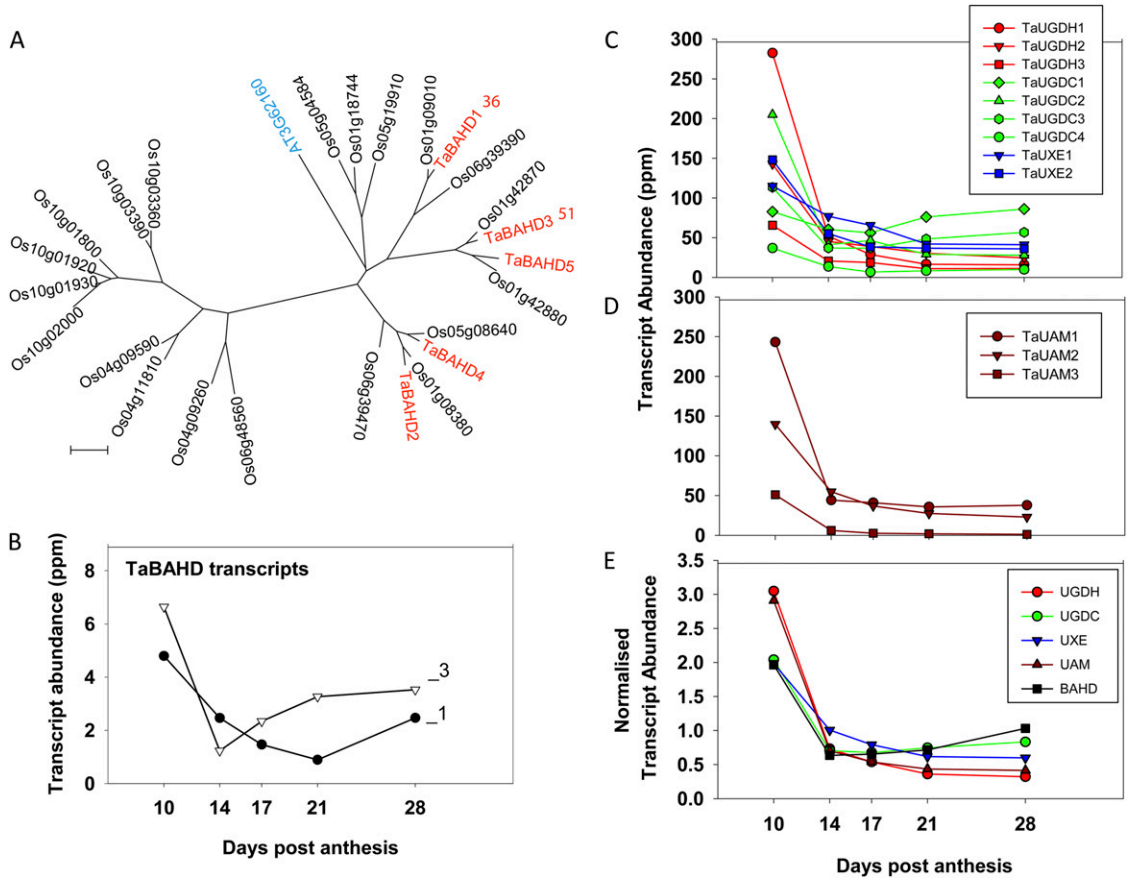

Figure 5. A, Phylogenetic tree of all rice and Arabidopsis loci, together with wheat genes expressed in grain, for the clade within the BAHD family identified as containing candidates for AX feruloylation (details are as for Fig. 4). B to D, Transcript abundance profiles for BAHD genes and genes believed to encode enzymes for precursor steps in the AX synthetic pathway: UGDH, UDP-Glc dehydrogenase; UGDC, UDP-Glc decarboxylase; UXE, UDP-Xyl*p* epimerase; UAM, UDP-Ara*p* mutase (numbers taken from closest rice genes; Konishi et al., 2010). E, Normalized profiles for total transcript abundance of genes in the different families.

analyses (Supplemental Table S2) as about 0.25% (w/w) at 28 dpa and 0.21% (w/w) at 42 dpa. This is at the lower end of the range found in wheat flour (Saulnier et al., 2007b), which may reflect low feruloylation of AX in the dissected lobe regions compared with the whole starchy endosperm. Given that grain dry weight also increases almost linearly until 28 dpa due principally to endosperm growth (Fig. 1), the rates of AX synthesis and feruloylation may peak around 25 dpa.

The total contents of nonstarch monosaccharide of the samples taken at 28 and 42 dpa (Supplemental Table S2) also show a decrease per unit dry weight after 28 dpa, suggesting that cell wall synthesis has ceased. The composition is consistent with AX constituting approximately 67%, 1,3;1,4-β-D-glucan plus cellulose approximately 24%, and glucomannan approximately 9%, similar to that typically reported for wheat flour: approximately 70% AX, approximately 20% 1,3;1,4-β-D-glucan, approximately 7% glucomannan, and approximately 2% cellulose (Fincher and Stone, 1986).

Comparison of transcript abundance in starchy endosperm and whole grain for candidate AX synthetic genes gives insight into their particular roles given the contrasting nature of the acetylated, highly feruloylated (G)AX present in grain outer tissues and the nonacetylated AX with a low degree of feruloylation in starchy endosperm. Relative transcript abundance from qRT-PCR is shown for a gene where expression

Figure 4. Phylogenetic trees of all rice and Arabidopsis loci, together with wheat genes expressed in starchy endosperm, for the AX synthetic candidate gene families. Rice loci are from Michigan State University gene models but omit the initial "LOC_" for clarity. Numbers in red are total numbers of counts in starchy endosperm for that transcript over the five time points; corresponding profiles of abundance are shown on the right. [See online article for color version of this figure.]

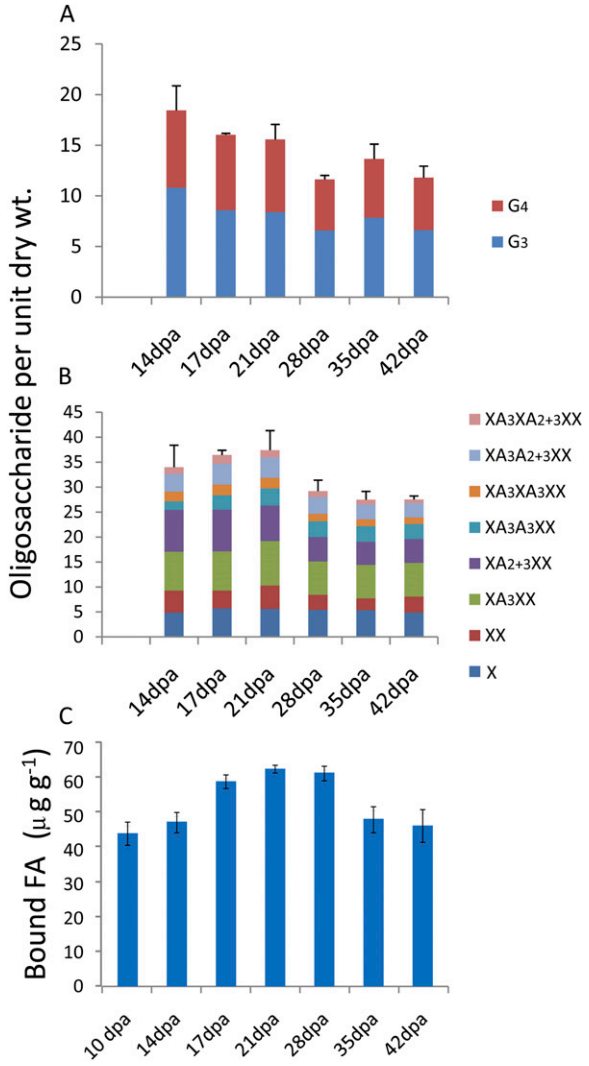

Figure 6. Cell wall composition in developing starchy endosperm determined by HPAEC analysis of oligosaccharides released by digestion with endoxylanase and lichenase (top and middle panels) and HPLC analysis of bound ferulic acid (FA; bottom panel). Amounts of oligosaccharides derived from the digestion of 1,3;1,4-β-D-glucan (top panel) and from AX (middle panel) are shown in arbitrary units per unit dry weight of sample; ferulic acid (bottom panel) is shown as $\mu g\ g^{-1}$ dry weight of sample. [See online article for color version of this figure.]

is known to be almost entirely restricted to starchy endosperm (starch-branching enzyme) or to aleurone (7S globulin), for TaCSLF6 and AX synthetic candidates in Figure 7A. TaGT47_2 and TaGT61_1 expression appears to be almost entirely confined to the starchy endosperm, given their similar whole grain-starchy endosperm ratios to that of starch-branching enzyme, whereas TaGT43_2, TaGT47_1, and TaGT61_2 must be expressed in outer tissues, given their relatively high abundance in whole grain. Five BAHD candidate genes also show much greater abundance in whole grain compared with starchy endosperm. This is consistent with a role in feruloylation of AX, since in wheat grain outer tissues (aleurone, testa, nucellar epidermis, and pericarp) it is about 5-fold greater per unit of Xyl*p* than for starchy endosperm (Barron et al., 2007; Saulnier et al., 2007a). Nearly all bound ferulic acid is believed to derived from ferulate ester linked to (G)AX in these tissues; therefore, it is a convenient measure of xylan feruloylation; bound ferulate was 30- to 100-fold higher per unit dry weight in whole grain compared with starchy endosperm, as determined in samples parallel to those used for expression measurements (Fig. 7B).

The complete list of all expressed genes from CAZy (Cantarel et al., 2009) putative cell wall GT families in developing starchy endosperm is presented in Supplemental Table S3. The two most abundant GT transcripts are *TaGT47_2* and *TaGT61_1*, consistent with the dominance of AX in mature starchy endosperm cell walls. However, the next most abundant are transcripts from the β-1,3-glucan synthase (GSL) and cellulose synthase (CESA) families (Fig. 8), despite the fact that callose and cellulose can together only account for less than 5% of cell walls in white flour, given that the great majority of nonstarch Glc is accounted for by 1,3;1,4-β-D-glucan. Both CESA and GSL transcripts are most abundant at 10 dpa, then decline rapidly, although they are still present at 28 dpa. Cellulose synthase-like (*CSL*) genes, which are similar to *CESA* and are also in the GT2 superfamily, contain known activities for the synthesis of mannan backbone (*CSLA*; Liepman et al., 2005), 1,3;1,4-β-D-glucan (*CSLF* and *CSLH*; Burton et al., 2006; Doblin et al., 2009; Nemeth et al., 2010), and the xyloglucan backbone (*CSLC*; Cocuron et al., 2007). Of these, the most abundant are *CSLA*, with *TaCSLA12* being abundant at 10 dpa but subsequently showing little expression and *TaCSLA7* having relatively consistent expression and being the most highly expressed CSL gene after 14 dpa (Fig. 8). It is surprising that these genes are so much more expressed than *TaCSLF6*, which is required for 1,3;1,4-β-D-glucan synthesis in wheat endosperm (Nemeth et al., 2010), given that mature starchy endosperm (white flour) contains about three times more 1,3;1,4-β-D-glucan than glucomannan. The other family shown to be capable of 1,3;1,4-β-D-glucan synthesis, *CSLH* (Doblin et al., 2009), is not expressed in wheat starchy endosperm. The next most abundant CSL transcript is *TaCSLD2*; there is also a low level of *TaCSLC1* expression, which is highest at 10 dpa and then declines.

The GT8 family has retaining activity and contains known activity for the synthesis of pectin galacturonic acid backbone (*GAUT1*; Sterling et al., 2006); other, similar Arabidopsis GT8 genes, designated as *GAUT* or *GAUT*-like (*GATL*), are implicated in the synthesis of the oligosaccharide present at the reducing end of xylan in dicots (*PARVUS* and *IRX8*; Brown et al., 2007; Peña et al., 2007). There is also evidence that other genes in the same clade as *IRX8*, *GAUT13*, and *GAUT14* may also have a role in xylan synthesis (Caffall et al., 2009). There is no precise ortholog for

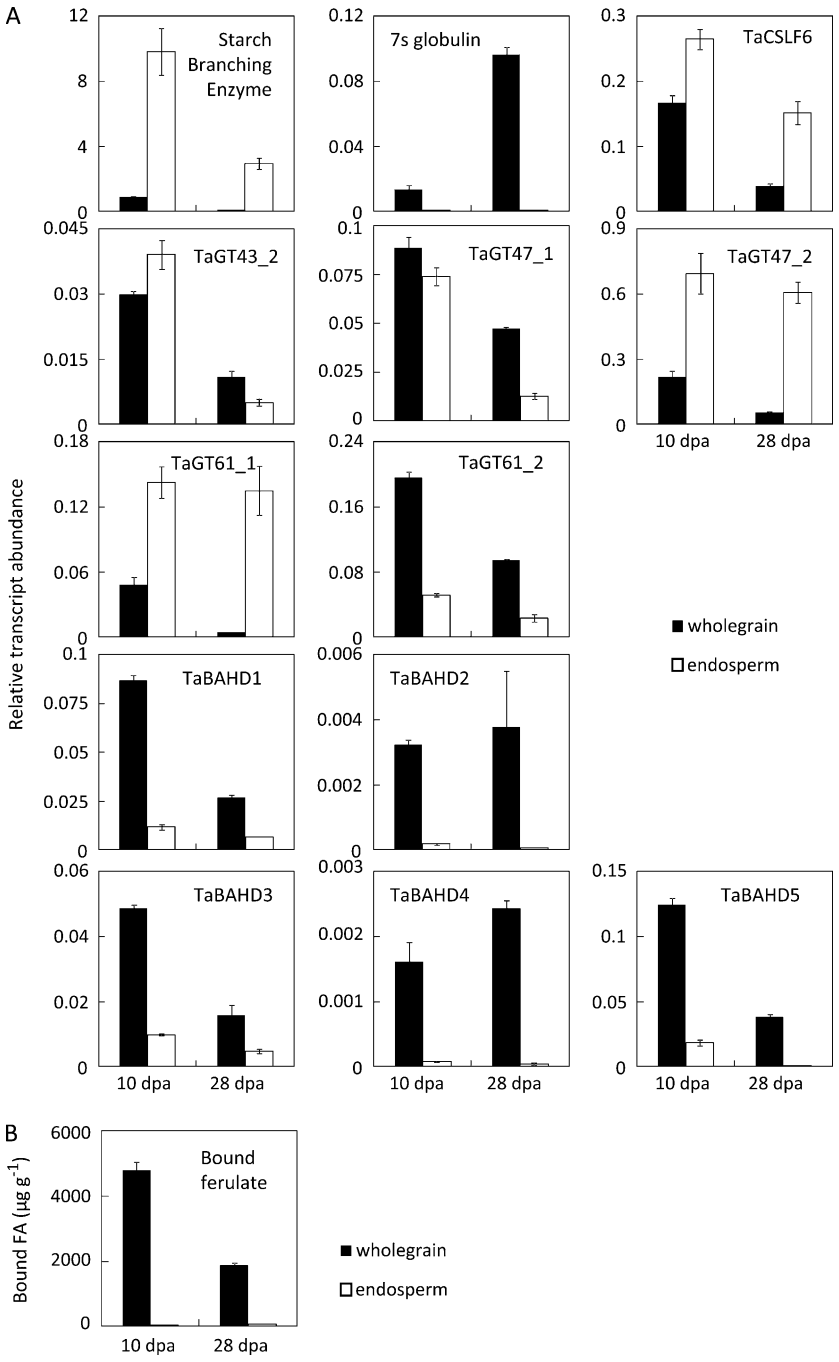

Figure 7. Comparison of starchy endosperm and whole grain fractions. A, Relative transcript abundance determined by qRT-PCR (mean of four biological replicates ± SE) for selected genes. The same correction factor used in Figure 2 was applied to starchy endosperm values at 28 dpa to account for variation in internal controls relative to 10 dpa. B, Bound ferulic acid (FA), shown as μg g^{-1} dry weight of sample.

IRX8 in grasses, but a putative ortholog of *GAUT13*, *TaGT8_4*, is expressed (Fig. 8). The *GUX* genes, also in GT8, which are responsible for glucuronyl substitution of xylan (Mortimer et al., 2010), are not expressed in starchy endosperm, which is consistent with the absence of glucuronic residues in starchy endosperm AX. The most highly expressed GT8, *TaGT8_1* (Fig. 8), is the apparent ortholog of *GAUT1* in Arabidopsis, and *TaGT47_9* is homologous to *ARAD1* and *ARAD2* (Fig. 4), which are responsible for pectin arabinan side chain synthesis in Arabidopsis (Harholt et al., 2006). However, no immunolabeling of cell walls in developing starchy endosperms with antibodies to pectin was detectable in our samples (including by JIM7, which labeled *Brachypodium* endosperm cell walls; Guillon et al., 2011), nor was pectin detectable by specific enzymic digestion of the same 10-, 17-, or 28-dpa samples (N. Anders, personal communication).

The abundant GSL transcripts (Fig. 8) early in development may correspond to callose synthesis during cellularization, as shown in the barley endosperm (Wilson et al., 2006). However, immunolabeling of our wheat samples showed that callose was also present at 28 dpa (Fig. 9). Similarly, immunolabeling

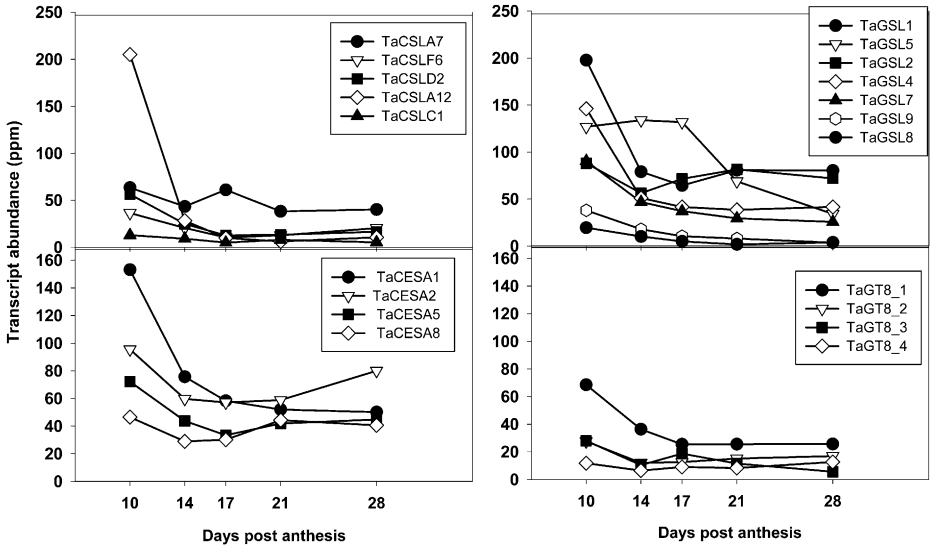

Figure 8. Transcript abundance of other GT genes. CESA represents cellulose synthase genes and CSL represents cellulose synthase-like genes (GT2). GSL represents β-(1,3)-glucan synthase genes (GT48), with numbers taken from the closest barley genes (Schober et al., 2009). The GT8 gene family encodes enzymes with retaining activity; the closest matching Arabidopsis genes for TaGT8_1, -2, -3, and -4 are GAUT1, GAUT7, GAUT1, and GAUT13, respectively.

showed the presence of mannan (all of which is considered to be present as glucomannan; Mares and Stone, 1973a) at all stages of development and the presence of traces of xyloglucan in early stages only (Fig. 9). These may reflect the high expression of CSLA and the low expression of CSLC genes, respectively (Fig. 8). Two GT34 transcripts encoding putative xyloglucan xylosyl transferases have similar profiles to *TaCSLC1*, peaking at 10 dpa (Supplemental Table S3), supporting the suggestion of early xyloglucan synthesis.

Another component of wheat flour is the arabinogalactan peptide (AGP; Mares and Stone, 1973b), the peptide component of which is encoded by the *Gsp-1* gene (Van den Bulck et al., 2005). Many other arabinogalactan peptides are associated with the cell wall, although it is still not clear whether this is the case with wheat starchy endosperm AGP. The Gal content of endosperm nonstarch polysaccharide is almost entirely derived from AGP and in our analysis accounts for approximately 10% of the nonstarch monosaccharides (Supplemental Table S2). The GT31 family encodes inverting GT enzymes including GALT1, which encodes an *N*-glycan β-1→3 galactosyltransferase in Arabidopsis (Strasser et al., 2007). The nature of AGP glycosylation has recently been characterized in detail in wheat cv Cadenza and shown to contain numerous Gal*p* β-1→6 and Gal*p* β-1→3 linkages requiring inverting activity (Tryfona et al., 2010). Therefore, it is possible that different but related GT31 genes could be responsible for the transfer of Gal to AGP. Figure 9 shows the phylogenetic tree for GT31 and expression profiles for the *Gsp-1* and GT31 genes (since *Gsp-1* encodes the actual product rather than a synthetic enzyme, it is not surprising that *Gsp-1* transcripts are orders of magnitude greater than for the GT31 genes).

Transcripts belonging to genes within CAZy (Cantarel et al., 2009) GH families are listed within Supplemental Table S4. Specific activities cannot be reliably ascribed to many GH plant genes, since extrapolation from sequence similarity may not be legitimate; for example, in the GH1 family (the most abundant GH family in starchy endosperm), very similar protein sequences have different hydrolytic activities (Opassiri et al., 2006). Members of this family could be involved in cellulose, callose, mannan, or xylan breakdown; GH5 and GH9 transcripts related to known cellulases are also highly expressed (Supplemental Table S4). Despite the lack of detectable pectin and the low abundance of xyloglucan, putative polygalacturonases in the GH28 family and a GH16 family member, *TaXTH4* (Liu et al., 2007), believed to encode a xyloglucan endotransglucosylase/hydrolase, are expressed, as are GH95 putative fucosidases. A relatively abundantly expressed GH17 (an ortholog of LOC_Os02g04670) is closely related to a glucan endo-1,3-β-glucosidase and may be involved in callose degradation. However, GH3 AX arabinofuranosidase transcripts, believed to be important in the degradation of AX in barley (Lee et al., 2003), show very few counts in wheat starchy endosperm and are classed as not expressed.

DISCUSSION

The relationship between transcript abundances and amounts of products synthesized by the encoded enzymes has many intermediate steps that may prevent simple correlations; transcripts may not be translated, encoded enzymes may not be activated or rapidly degraded, and substrates and cofactors may be limiting or absent. Nevertheless, abundant product does normally imply abundant transcripts encoding synthetic enzymes, and the general converse assumption that abundant transcripts must have a function seems usually to hold true, given the success in identifying candidates for cell wall synthesis from transcriptomics (Brown et al., 2005; Cocuron et al., 2007; Jensen et al., 2011). However, a counter example

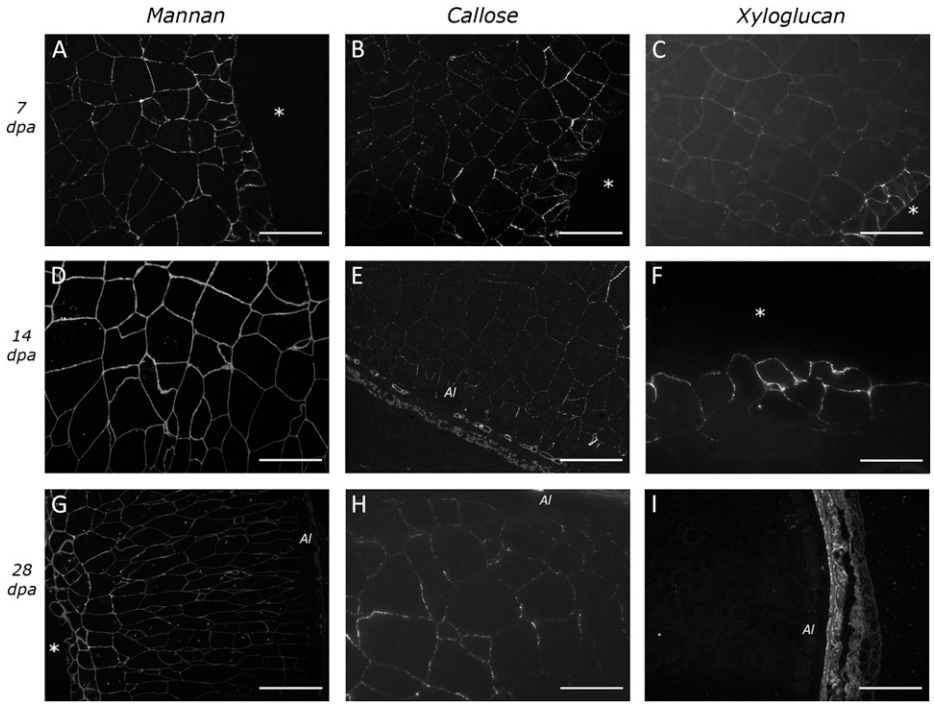

Figure 9. Immunofluorescence labeling of developing wheat grain with antibodies recognizing mannan (LM21), callose, and xyloglucan. A to C, Cells around the endosperm cavity. D, Inner endosperm in the lobes region. E, H, and I, Subaleurone region. F, Transfer cells. G, Endosperm of the dorsal region of the grain. Asterisks denote the endosperm cavity region. Al, Aleurone layer. Bars = 100 μm (A–E, H, and I), 50 μm (F), and 200 μm (G).

is the relatively low transcript abundance of the CSLA identified as encoding an enzyme responsible for the synthesis of abundant mannan (Dhugga et al., 2004).

AX

Due to its abundance and specific role in grasses and cereals, the study of grass xylan is an important topic for agriculture, for deriving biofuel from lignocellulose, and for its role in mechanical strength and pathogen resistance. Wheat starchy endosperm AX provides an excellent system for studying this because of its prevalence and relative simplicity in a large tissue consisting of a single cell type; furthermore, strong starchy endosperm-specific promoters have been successfully used to manipulate wheat starchy endosperm cell wall composition (Harholt et al., 2010; Nemeth et al., 2010). Until a few years ago, genes for the synthesis of xylans were unknown, since it has not been possible to synthesize xylan in heterologous systems (Fincher, 2009; Scheller and Ulvskov, 2010). However, studies of knockout mutants have shown that the GT47 IRX10 and GT43 IRX9 and IRX14 genes in Arabidopsis are necessary for secondary cell wall xylan backbone synthesis in Arabidopsis (Brown et al., 2007, 2009; Lee et al., 2007; Peña et al., 2007). An IRX10 ortholog was the most highly expressed GT in starchy endosperm (excluding those involved in starch synthesis), and orthologs for IRX9 and IRX14 were also highly expressed (Fig. 5), strongly suggesting that these genes synthesize the AX backbone. The second most abundant cell wall GT is *TaGT61_1*, recently shown to encode a xylan arabinosyl transferase (Anders et al., 2012).

Recently, a microsomal fraction derived from etiolated wheat seedlings with GAX synthetic capacity has been characterized and shown to contain *TaGT47_1* (*TaGT47-13* in the authors' nomenclature), *TaGT43_1* (*TaGT43-4*), and *TaUAM1* (*TaGT75-4*; Zeng et al., 2010). *TaGT47_1* is very similar to *TaGT47_2*, and both are likely orthologs of *IRX10* and *IRX10-L* (Fig. 3), but *TaGT47_1* tends to be more highly expressed in tissues other than starchy endosperm, including grain outer tissues (Fig. 7A). This analysis of the GAX-synthetic microsomal fraction is strongly supportive of the roles of *TaGT43_1* and *TaGT47_1* in grass xylan synthesis, but as it is not clear how many other proteins were present in the fraction, it is still not definitive evidence. Since glucuronyl and arabinosyl transferase activities were also demonstrated, it might be expected that GT8 GUX protein and GT61 proteins would be also present, but none were reported.

Although studies of wheat and Arabidopsis suggest that IRX9, IRX14, and IRX10 together form a xylan synthase complex, recent EST profiling from a *Plantago ovata* cell mucilage layer, which produces a large amount of xylan, showed that transcripts for *IRX10* orthologs were highly abundant, but those for *IRX9* and *IRX14* were 100-fold less abundant (Jensen et al., 2011). This work identified two DUF579 proteins, IRX15 and IRX15-L, which are involved in xylan chain extension in Arabidopsis; orthologs for these proteins are expressed in grasses, but their transcripts were not present in wheat starchy endosperm (data not shown). Therefore, there is evidence of considerable variation in the xylan synthetic machinery between species and tissues.

A specific oligosaccharide has been identified at the reducing end of xylan in dicots, and the *PARVUS*, *IRX8*

(both GT8 family), and *IRX7/FRA8* (GT47) genes have been implicated in the synthesis of this (Brown et al., 2007; Peña et al., 2007; York and O'Neill, 2008). No oligosaccharide has been reported at the reducing end of grass xylan, and there is no obvious ortholog for IRX8 in rice or *Brachypodium*. However, other GT8 genes in the same clade as *IRX8*, *GAUT13*, and *GAUT14* may also have a role in xylan synthesis (Caffall et al., 2009). A putative ortholog of *GAUT13*, TaGT8_4, is expressed (Fig. 8) and shows coexpression with *TaGT47_5* (r = 0.90), which is in the same clade as *IRX7/FRA8*. The presence of these transcripts may indicate an analogous requirement for genes from these GT8 and GT47 clades for normal xylan synthesis in grasses.

Five BAHD genes from the clade previously identified as candidates for AX feruloylation (Mitchell et al., 2007b) are expressed in wheat grain, although only two of these are classed as expressed in starchy endosperm by our criteria (Fig. 5A). The profiles of BAHD expression are more similar to those for the mutases that synthesize UDP-arabinofuranose (Fig. 5E) than those of GT43, GT47, or GT61 candidates (Fig. 4), so they may be responsible for the feruloylation of this precursor. All five BAHD genes show much greater expression in whole grain compared with starchy endosperm, correlating with the greater level of feruloylation (Fig. 7).

1,3;1,4-β-D-Glucan

It has been shown that CSLF (Burton et al., 2006) and the closely related CSLH (Doblin et al., 2009) genes are capable of conferring the capacity to make 1,3;1,4-β-D-glucan in Arabidopsis, which as a dicot does not normally synthesize 1,3;1,4-β-D-glucan. No CSLH genes and only one CSLF gene (*TaCSLF6*) are expressed in wheat starchy endosperm, and a RNA interference construct designed against this eliminated most 1,3;1,4-β-D-glucan synthesis during the period of transgene expression (Nemeth et al., 2010). Therefore, the relatively low level of expression of *TaCSLF6* (Figs. 2 and 4) is surprising given the abundance of 1,3;1,4-β-D-glucan. It has been suggested that 1,3;1,4-β-D-glucan is synthesized in the Golgi by a heterodimeric enzyme complex (Carpita, 2011), possibly a CSLF-CSLH heterodimer in some cases (Doblin et al., 2009). Since CSLH is absent from wheat starchy endosperm, TaCSLF6 may be one component of a 1,3;1,4-β-D-glucan synthase complex with another CSL gene product. One possibility in the case of wheat starchy endosperm is *TaCSLD2*, which showed some coexpression with *TaCSLF6* (Fig. 8).

Minor Cell Wall Polysaccharides

Abundant transcripts associated with the synthesis of cellulose, mannan, callose, and pectin are present in wheat starchy endosperm at 10 dpa, and there is also some expression of *TaCSLC1* and GT34 genes associated with xyloglucan synthesis (Fig. 8; Supplemental Table S3). All of these polysaccharides are probably synthesized during earlier development. Cellularization of cereal endosperm occurs at 3 to 6 dpa, with initial deposition of polysaccharides into the cell plates, which become the cell walls that separate the nuclei in the multinucleate syncytium. An immunolabeling study of developing barley endosperm showed that callose and cellulose were detected uniformly at 4 dpa, but callose labeling was subsequently

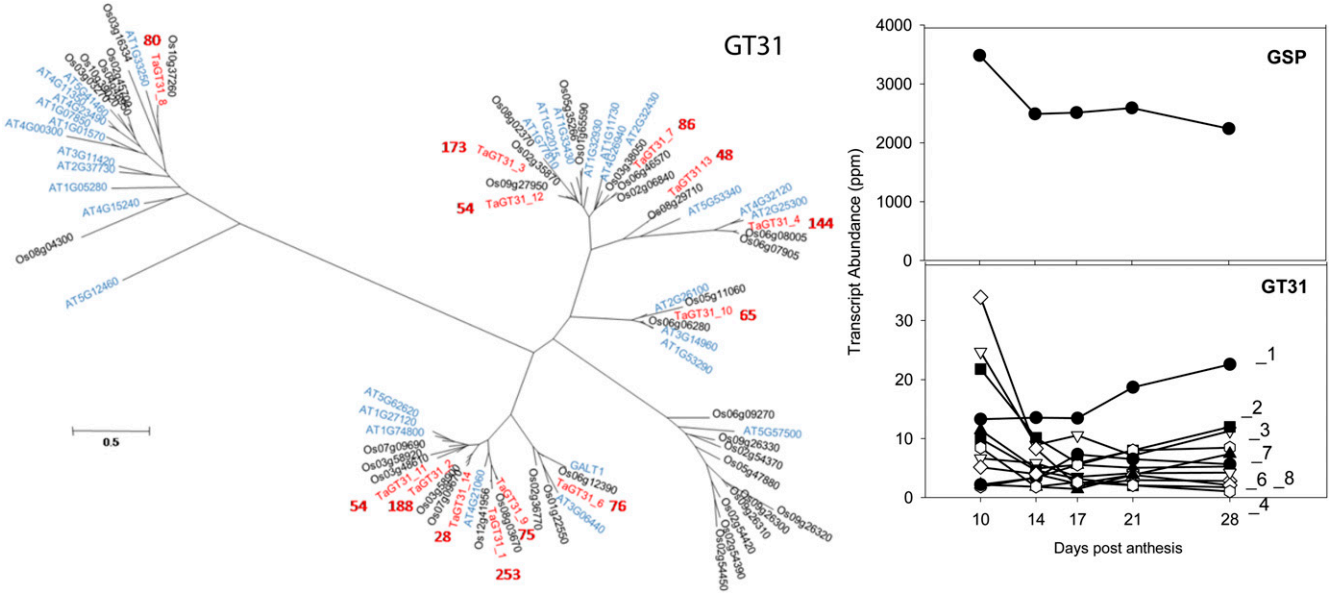

Figure 10. Phylogenetic tree for the GT31 family and transcript abundance profiles for the *Gsp-1* gene, which encodes AGP, and GT31 genes. Details are as for Figure 4. [See online article for color version of this figure.]

restricted to regions surrounding plasmodesmata (Wilson et al., 2006). The initiation of the synthesis of other components occurred in the order mannan, 1,3;1,4-β-D-glucan, AGP, and then AX. Callose and cellulose deposition to the cell plate seems to be common to most plants, as is the deposition of pectin to form the middle lamella (Fry, 2004). No xyloglucan has been reported in Triticeae endosperm and none was found in *B. distachyon* endosperm (Guillon et al., 2011), but we found evidence of trace amounts at 7 dpa (Fig. 9). There was also evidence of callose after 14 dpa in our samples (Fig. 9); however, no pectin was detectable in wheat endosperm at 10 dpa or later. The abundance of GSL transcripts may relate to a requirement for the rapid deposition of callose in response to wounding. The apparent discrepancy of the abundance of transcripts associated with the synthesis of cellulose, mannan, callose, and pectin with their apparent low abundance or absence could be due to the hydrolysis of these polysaccharides, which is consistent with abundant GH transcripts associated with their breakdown (Supplemental Table S2). This mixture of synthesis and disassembly could be required to allow the expansion of the starchy endosperm cells, particularly during the first part of grain fill.

AGP

The only known function within the inverting GT31 family is *N*-glycan β-1→3 galactosyltransferase encoded by GAL1 in Arabidopsis (Strasser et al., 2007). Similar linkages and Gal*p* β-1→6 linkages are abundant in wheat starchy endosperm AGP (Mares and Stone, 1973b; Tryfona et al., 2010), and while *TaGT31_6* appears to be the ortholog of *GAL1*, it seems likely that other members of this family, which are abundantly expressed (Fig. 10), are responsible for these.

CONCLUSION

The transcriptome of cell wall-related genes in developing wheat starchy endosperm presented here represents an excellent resource for the identification of candidate genes responsible for cell wall composition in this tissue. It also underpins the use of wheat starchy endosperm as a system for increased understanding of grass cell wall synthesis more generally, in particular the synthesis and feruloylation of AX, which has been characterized in great detail in wheat flour. The availability of strong wheat starchy endosperm-specific promoters provides the means of experimentally testing the role of these candidate genes.

MATERIALS AND METHODS

Sample Preparation

Bread wheat (*Triticum aestivum* 'Cadenza') plants were grown in a controlled environment as described (Sparks and Jones, 2009). Heads were tagged at anthesis, and pure starchy endosperm was dissected at 10, 14, 17, 21, and 28 dpa for transcript analysis. The outside two florets on the central four spikelets on each side of the ear, in total 16 caryopses, were dissected as shown in Figure 1 to yield pure central starchy endosperm of the developing grain. Each head was considered a biological replicate for the qRT analysis, and up to 10 replicates were pooled for the RNA sequencing and biochemical analysis. Further time points up to 42 dpa were dissected for biochemical characterization, but only small amounts of poor-quality RNA unsuitable for downstream applications were extracted from material older than 28 dpa. In addition, the moisture of the four caryopses immediately above and below the dissected florets was determined (Fig. 1). The strong correlation between age and moisture was used to standardize material from different batches. Dissected samples were immediately frozen in liquid nitrogen, lyophilized, and homogenized in a TissueLizer (Qiagen).

Transcript Analysis

Total RNA was extracted as described (Nemeth et al., 2010). Using the Illumina platform, unnormalized cDNA libraries for the five time points were end labeled, and single reads were sequenced by GATC Biotech. Sequences and quality values of all resulting RNA-Seq reads referred to in this paper are available at ArrayExpress (accession no. E-MTAB-861). For validation of sequence counts, the expression of selected genes was analyzed by qRT-PCR of three biological replicates as described (Pellny et al., 2008). Primer sequences used are listed in Supplemental Table S5.

Assignment of Reads and Assembly

In the absence of sequenced wheat genomes to act as a reference sequence, assignment of the 73-bp reads to transcript molecules was achieved utilizing a combination of rice sequences and the 1.5 million conventional Sanger-sequenced wheat (or progenitor) ESTs and high-quality cDNA sequences available in the public domain. A total of 1,742,122 sequences available from GenBank on October 1, 2010, were used. In the WhETS tool (Mitchell et al., 2007a), these were assigned to rice (*Oryza sativa*) genome loci and then assembled into contigs using CAP3 (Huang and Madan, 1999) software. A new version of WhETS that utilizes the sequenced *Brachypodium distachyon* genome (Vogel et al., 2010) was also utilized in parallel; results from mapping to rice and *Brachypodium* were very similar, but here we present the results for rice as the better established gene models. Reads were analyzed by BLAST against both the rice gene-coding sequences and their assigned wheat contigs; reads were then assigned to the best matching hit with an E-value cutoff of 1.e-10. No exact orthologs in rice or *Brachypodium* exist for the *Gsp-1*, *HMW 1Dx5*, or *GB-SSI* genes, and here reads were assigned directly to wheat sequences (GenBank accession no. AY255771.1 and The Institute for Genomic Research Plant Transcript Assembly contigs TA61310_4565 and TA57127_4565, respectively) by BLASTN with an E-value cutoff of 1.e-20. Reads were then assembled by CAP3 along with the preexisting wheat sequences into new contigs. Typically, three very similar wheat sequences were apparent matches to each rice locus, consistent with the three homeologous forms of the gene from the three genomes of hexaploid wheat; here, we do not distinguish these forms but present the results for each gene as an integration of counts over the three. However, to search for the possibility of multiple wheat genes (i.e. paralogs) matching to one rice gene, different wheat contigs that matched to the same region of a rice gene were manually inspected. Mostly, these could be interpreted as splice variants, but in three cases, there was good evidence of there being two expressed wheat genes corresponding to one rice gene, and these are presented as separate genes (in one of these cases, there were also two genes in *Brachypodium* corresponding to the rice gene). The total numbers of reads from each library were 2,708,600, 3,245,413, 3,412,567, 3,365,414, and 2,833,294 for 10, 14, 17, 21, and 28 dpa, respectively. Transcript abundance is given as the number of reads for a gene per million reads in the library.

Matching of the Michigan State University rice loci used here to those in the CAZy database (Cantarel et al., 2009), which uses the Rice Annotation Project gene models, was achieved using the Rice Annotation Project tool (http://rapdb.dna.affrc.go.jp/tools/converter) and checked with protein alignment to ensure that they were essentially identical.

For phylogenetic trees, the wheat contigs containing the reads were used to generate wheat protein sequences; in most cases, these appeared to be the full length, but in some cases, only a C-terminal fragment was possible. In some cases, the 5′ end was extended using the newly available wheat Chinese Spring genomic reads (http://www.cerealsdb.uk.net/). Final protein se-

quences used for phylogeny are available in Supplemental Table S6. Phylogenetic trees were derived from multiple alignments produced with the MUSCLE algorithm (Edgar, 2004), removal of gapped columns, followed by phylogeny analysis in the phyml package (Guindon et al., 2005) using the model of Whelan and Goldman (2001). An initial run optimized the γ and invariant proportion parameters; these were then held constant for 100 runs for bootstrap nonparametric analysis, and consensus trees are presented.

Nonstarch Monosaccharide Analysis

Analyses of the nonstarch monosaccharide content of dissected starchy endosperm samples were conducted following the protocol of Englyst et al. (1994).

Analysis of AX and 1,3;1,4-β-D-Glucan by HPAEC

The methodology for the analysis of starchy endosperm AX and 1,3;1,4-β-D-glucan by digestion with endoxylanase and lichenase followed by HPAEC of resultant AXOS was originally developed by Saulnier et al. (2009), and the procedure followed here was as described by Nemeth et al. (2010).

Ferulate Analysis

Bound phenolic acids were extracted and analyzed essentially according to Li et al. (2008). Endosperm samples (20 mg) were extracted with 1 mL of 80:20 ethanol:water. Suspensions were vortexed until all the flour was suspended, sonicated for 10 min, heated to 80°C for 15 min, and then centrifuged for 15 min at 5,000*g*, and the supernatant was discarded. Samples were then extracted two further times with sonication but no heating. A 20-μL internal standard (3,5-dichloro-4-hydroxybenzoic acid at 1.5 mg mL^{-1}) was added to the wet pellet, and samples were hydrolyzed by suspension in 0.8 mL of 2 M NaOH for 16 h in the dark. After centrifugation for 15 min at 5,000*g*, the supernatant was acidified to pH $<$ 2 by the addition of 220 μL of 12 M HCl. Bound phenolic acids were extracted into ethyl acetate (1$\times$ 600 μL, 1$\times$ 700 μL, and 1$\times$ 800 μL) by vortexing and centrifugation for 5 min. After evaporation, bound phenolic acids were dissolved in 100 μL of 2% acetic acid and centrifuged for 5 min. HPLC analysis was according to Li et al. (2008), except using a Shimadzu Prominence high-performance liquid chromatograph and column dimensions of 25 cm $\times$ 4.6 mm, 5 μm. About 10% to 20% of ferulic acid was found to be in the cis-form, but this is believed to be due to conversion in vitro (L. Saulnier, personal communication), so amounts of cis- and trans-forms were summed and total ferulic acid is reported here. All samples were extracted and analyzed in triplicate.

Microscopy

Transverse slices, approximately 1 mm thick, were cut from developing wheat grains at 7, 14, and 28 dpa and fixed in 4% paraformaldehyde in 50 mM potassium phosphate buffer, pH 7.4, for 3 h. They were then dehydrated in a graded ethanol series and infiltrated and polymerized in medium-grade LR White resin. Section preparation and immunofluorescence microscopy were carried out as described (Tosi et al., 2009). Primary antibodies LM15 (anti-xyloglucan; Marcus et al., 2008) and LM21 (anti-mannan; Marcus et al., 2010) were used at a 1:5 dilution; anti-callose antibody [(1$\rightarrow$3)β-glucan; catalog no. 400-2; Biosupplies] was used at a 1:25 dilution. Secondary antibodies (Alexa Fluor 488 anti-mouse and Alexa Fluor 633 anti-rat) were used at a 1:200 dilution. Sections were examined with a Zeiss Axiophot epifluorescence microscope. A Retiga Exi CCD digital camera (Qimaging) and MetaMorph software version 7.5.5 (Molecular Devices) were used to acquire the images.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AM701827.1, AM698096.1, AM749044.1, and FR846233.1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Details of HPAEC analysis of oligosaccharides produced by digestion of cell wall samples.

Supplemental Table S2. Nonstarch monosaccharide composition of starchy endosperm samples.

Supplemental Table S3. Transcript sequence counts from wheat starchy endosperm for GT families.

Supplemental Table S4. Transcript sequence counts from wheat starchy endosperm for all GH families.

Supplemental Table S5. Oligonucleotide sequences of primers used for qRT-PCR.

Supplemental Table S6. Wheat protein sequences used to produce phylogenetic trees.

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