Pattern of deposition of cell wall polysaccharides and transcript abundance of related cell wall synthesis genes during differentiation in barley (*Hordeum vulgare*) endosperm


1 ARC Centre of Excellence in Plant Cell Walls, School of Botany, University of Melbourne, VIC, 3010, AUSTRALIA
2 ARC Centre of Excellence in Plant Cell Walls, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA 5064, Australia
3 School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA 5064, Australia
4 CSIRO Plant Industry, Canberra, ACT 2601, Australia.
Abstract
Immunolabelling, combined with chemical analyses and transcript profiling, have provided a comprehensive temporal and spatial picture of the deposition and modification of cell wall polysaccharides during barley grain development, from endosperm cellularization at 3 days after pollination (DAP) through differentiation to the mature grain at 38 DAP. (1→3)-β-D-Glucan appears transiently during cellularization but reappears in patches in the sub-aleurone cell walls around 20 DAP. (1→3, 1→4)-β-Glucan, the most abundant polysaccharide of the mature barley grain, accumulates throughout development. Arabino-(1-4)-β-D-xylan is deposited significantly earlier than previously reported. This was attributable to the initial deposition of the polysaccharide in a highly substituted form that was not recognized by antibodies commonly used to detect arabino-(1-4)-β-D-xylans in sections of plant material. The epitopes needed for antibody recognition were exposed by pre-treatment of sections with α-L-arabinofuranosidase; this procedure showed that arabino-(1-4)-β-D-xylans were deposited as early as 5 DAP and highlighted their changing structures during endosperm development. By 28 DAP labeling of hetero-(1→4)-β-D-mannan is observed in the walls of the starchy endosperm but not in the aleurone walls. Although absent in mature endosperm cell walls we now show that xyloglucan is present transiently from 3 until about 6 DAP and disappears by 8 DAP. Quantitative PCR of transcripts for glucan synthase-like (GSL), cellulose synthase (CesA) and cellulose synthase-like (CSL) genes were consistent with the patterns of polysaccharide deposition. Transcript profiling of some members from the glycosyl transferase CAZy families GT61, GT47 and GT43, previously implicated in arabino-(1-4)-β-D-xylan biosynthesis, confirms their presence during grain development.
Introduction

In cereal species the endosperm, the nutritive part of the grain, begins development as a syncytium, where the triploid endosperm nucleus undergoes numerous rounds of division without cytokinesis (Olsen, 2004). Thus, the syncytium consists of a highly vacuolated central cell with nuclei positioned around its perimeter. Approximately 3 days after pollination (3 DAP) and in the absence of mitosis and phragmoplasts, anticlinal cell walls grow out centripetally from the periphery of the central cell and form partitions between individual nuclei. Each nucleus then undergoes mitotic division, followed immediately by cytokinesis and the laying down of a cell plate and periclinal cell wall. This alternating cycle of ‘free-growing’ anticlinal cell walls followed by mitosis and the laying down of a periclinal cell wall continues in a centripetal fashion until the entire endosperm is compartmentalized into cells (Brown et al., 1994; Brown et al., 1997). This sequence of events makes grass (cereal) endosperm ideal for studying mechanisms of cell wall growth and development. Cereals are also the world’s major source of nutrition with much of their caloric content deposited as complex carbohydrates in developing and maturing endosperm cells. Given its unique biology and economic importance, it is not surprising that the cereal endosperm has attracted much attention from scientists with both pure and applied research interests.

The polysaccharide composition of the starchy endosperm cell walls in barley has been known for some time and chemical analyses have shown mature grain to contain approximately 70% (w/w) (1→3, 1→4)-β-D-glucan, 20% (w/w) arabinio-(1-4)-β-D-xylan, 3-4% (w/w) (1→4)-β-D-glucan (cellulose), 3-4% (w/w) hetero-(1→4)-β-D-mannan and some protein (Fincher, 1975). In contrast, walls of the aleurone consist of approximately 70% (w/w) arabinio-(1-4)-β-D-xylan, 25% (w/w) (1→3, 1→4)-β-D-glucan and minor amounts of (1→4)-β-D-glucan (cellulose) and hetero-(1→4)-β-D-mannan (Bacic and Stone, 1981a, 1981b). In a recent study we used immuno-light and -electron microscopy (EM) to describe the temporal and spatial deposition of key wall polysaccharides during the cellularization phase of barley grain development, 3-8 days after pollination (DAP) (Wilson et al., 2006). (1→3)-β-D-Glucan (callose) is the first polysaccharide to be laid down in the cellularizing grain, at ~3 DAP. However, its presence is only transient and by 6 DAP it can only be found in those cell wall regions surrounding plasmodesmata. (1→3, 1→4)-β-D-Glucan appears next between 4 and 5 DAP and continues to accumulate in the grain throughout
development. The hetero-(1→4)-β-D-mannan antibody detects hetero-mannans at 6 DAP while arabino-(1-4)-β-D-xylan was first detected at 8 DAP. During cellularization each polysaccharide is uniformly deposited in the endosperm cell walls except for arabino-(1-4)-β-D-xylan where labeling is restricted to those cell walls immediately adjacent to the grain crease (Wilson et al., 2006). Similar studies have been performed on developing rice (Brown et al., 1997) and wheat grains (Philippe et al., 2006).

Polysaccharides (in particular (1→3, 1→4)-β-D-glucan and arabino-(1→4)-β-D-xylan) of the cell walls in cereal grains are not digested by enzymes secreted into the small intestine of monogastric animals and are hence components of dietary fibre in human nutrition and therefore have positive effects on human health by reducing both the risk of cardiovascular disease by lowering plasma cholesterol levels (Topping, 2007) and on non-insulin-dependent diabetes by lowering postprandial blood glucose and insulin levels (Wood, 2007). Furthermore, recent pig feeding trials indicate that consumption of arabino-(1→4)-β-D-xylan attenuates the somewhat damaging effect of the western diet on gut health (Belobrajdic et al., 2011). Conversely, (1→3, 1→4)-β-D-glucan also reduces digestibility and hence available metabolizable energy in monogastric animals such as pigs and poultry and lowers extraction potential and reduces filterability during malting and brewing (Brennan and Cleary, 2005).

Considerable effort has been made towards identifying the genes and biochemical mechanisms that contribute to the biosynthesis of cell wall polysaccharides although progress in this area has been slow (Fincher, 2009; Doblin et al., 2010). Only relatively recently have specific genes and gene families been implicated in (1→3, 1→4)-β-D-glucan synthesis. After introducing the rice CslF genes into Arabidopsis, a species known not to ordinarily contain (1→3, 1→4)-β-D-glucan in its walls, (1→3,1→4)-β-D-glucan was detected in transgenic lines using immuno-electron microscopy (Burton et al., 2006). Similar gain-of-function experiments were performed by transforming Arabidopsis with the barley CslH gene. (1→3, 1→4)-β-D-Glucan was immunologically detected in the walls of transgenic plants and confirmed with biochemical analysis of wall extracts (Doblin et al., 2009). The genes encoding the xylan synthases and key side chain glycosyl transferases are largely unconfirmed biochemically but studies of mutant lines and transcript profiles of cereal species accumulating arabino-(1-4)-β-D-xylan implicate the GT43, GT47 and GT61 gene
families (Scheller and Ulvskov, 2010), Mitchell et al. 2007). Experimental evidence confirming the role of these genes, particularly the xylan synthases, is an area of intense interest given the importance of plant materials as feedstocks for biofuels and the potential human health benefits from diets inclusive of arabino-(1-4)-β-D-xylan.

Some gene families have also been implicated in the synthesis of the other, less abundant, polysaccharides of the developing barley grain. For example, there is ample evidence associating the Glucan Synthase-Like (GSL) gene family with callose ((1→3)-β-D-glucan) biosynthesis (Brownfield et al., 2009). Heterologous expression experiments coupled with gene expression profiles have implicated the CslC gene family in the synthesis of the glucan backbone of xyloglucan (Cocuron et al., 2007) and cellulose (Dwivany et al, 2009) while members of the CslA gene family have been shown to have mannan or (gluco)mannan synthase activity (Dhugga et al., 2004).

In this study, we focus on the second phase, the differentiation phase, of barley endosperm development and apply antibodies to key wall polysaccharides from 10-28 DAP to describe their distribution, using both light and electron microscopy. We also have quantified the levels of (1→3, 1→4)-β-D-β-glucan and the monosaccharides arabinose, xylose and mannose from cellularization (3 DAP) through to the mature grain (28 DAP). In addition, RNA has been isolated from developing grains between 6 and 38 DAP and QPCR analysis performed in an attempt to determine whether transcript patterns of cell wall synthesis genes can be correlated with polysaccharide deposition and accumulation in the grain.
Results

Endosperm differentiation in barley from 10-28 DAP

During the differentiation phase, a number of changes to the endosperm were observed using light microscopy and toluidine blue staining of sectioned grain. The beginning of the differentiation phase in barley endosperm is marked by the appearance of 3 to 4 distinct layers of aleurone cells surrounding the starchy endosperm. At 10 DAP aleurone cells are easily distinguished from the rest of the endosperm by their small size, isodiametric shape, regular, “brick-like” arrangement and by their complete lack of starch granules (Fig. 1A). By 14 DAP a histologically distinct sub-aleurone layer separates the differentiating aleurone from the starchy endosperm. Sub-aleurone cells are larger than those of the aleurone but smaller than the starchy endosperm and contain small starch granules and protein bodies (Fig. 1B). Differentiation continues with the thickening of the endosperm cell walls, particularly those of the aleurone, and with the accumulation of starch granules and protein bodies. It is difficult to determine when differentiation ends and maturation of the grain begins but aleurone cell walls appear to reach their maximum thickness by ~22DAP (Fig. 1C). Beyond this stage, the barley grain continues to accumulate starch, progressively loses water and subsequently becomes difficult to section for either light or electron microscopy. Therefore, observations on grain development and polysaccharide distribution are only described up to 28 DAP.

(1→3)-β-D-glucan (callose) appearance in differentiating endosperm (10-28 DAP)

We (Wilson et al., 2006) and others (Brown et al., 1997; Philippe et al., 2006) have reported the transient nature of (1→3)-β-D-glucan during early endosperm development. Callose is the first polysaccharide to appear in the walls of the cellularizing endosperm at 3 DAP, persisting until around 6 DAP (Wilson et al., 2006). From 6 DAP onwards callose becomes restricted to those cell wall regions surrounding plasmodesmata throughout all endosperm cells. At 28 DAP, immunogold labeling with the (1→3)-β-D-glucan antibody reveals newly deposited callose in electron-dense, non-uniform patches at the edges of those sub-aleurone cell walls directly abutting the aleurone cells (Fig. 2A). Elsewhere in the 28 DAP endosperm callose is associated only with plasmodesmata (Fig. 2B).
Xyloglucan deposition during cellularization (3-8 DAP)

Since an antibody (LM 15) has only recently been generated (Marcus et al., 2008) that recognizes an epitope unique to xyloglucans and made commercially available, we were unable to confirm the presence of xyloglucan during cellularization in our previous paper (Wilson et al., 2006). The cellulose-degrading enzyme, cellulbiohydrolase II (CBHII or Cel6A) from the fungus *Trichoderma reesei*, coupled to gold was used to detect cellulose in the central cell wall at 3 DAP and the first-formed anticlinal walls at 4 DAP (see Fig. 5; (Wilson et al., 2006). However, CBHII also binds to the (1→4)-β-oligoglucoside structures in (1→3, 1→4)-β-D-glucan and xyloglucan (Henriksson et al., 1995; Amano et al., 1996). We assumed at 3 and 4 DAP, CBHII was binding to cellulose since (1→3, 1→4)-β-D-glucan is not deposited until after 4 DAP and xyloglucan is absent in the mature grain (Fincher, 1975; Bacic and Stone, 1981a, 1981b). This assumption was incorrect and we now show that xyloglucan is present transiently in the endosperm from 3 until about 6 DAP. It appears first in the central cell wall at 3 DAP just as the first anticlinal walls are forming (Fig. 3A). Labeling of the LM 15 antibody is present, albeit at low levels, at 4 and 5 DAP (Figs. 3B, C) but is barely detectable at 6 DAP (Fig. 3D). By 8 DAP no gold labeling can be found anywhere in the endosperm (images not shown). As yet, there is no specific probe for cellulose so we cannot comment when this polysaccharide is first deposited but chemical analysis shows cellulose to comprise ~3-4% (w/w) of the mature barley endosperm walls (Fincher, 1975).

(1→3, 1→4)-β-D-glucan deposition during endosperm differentiation (10-28 DAP)

Chemical analysis has shown (1→3, 1→4)-β-D-glucan to be the most abundant polysaccharide in the barley grain (Fincher, 1975). Unlike rice (Brown et al., 1997) but similar to wheat (Philippe et al., 2006), (1→3, 1→4)-β-D-glucan appears in the early walls of the cellularizing endosperm between 4 and 5 DAP and accumulates in the cell walls of the endosperm throughout development. At 10 DAP, immunolabeling for light and electron microscopy using the (1→3, 1→4)-β-D-glucan antibody shows (1→3, 1→4)-β-D-glucan to be distributed uniformly throughout the endosperm tissues (Fig 4A; 5A & B). However, at 12 DAP a few rows of starchy endosperm cells closest to the differentiating aleurone layers appear to have little or no (1→3, 1→4)-β-D-glucan in their walls (Fig. 4B). This specific lack of labeling in the peripheral endosperm cells has also been reported in wheat (Philippe et al., 2006). In wheat, this observed heterogeneity in labeling intensity within the starchy
endosperm persists throughout development whereas in barley, by 16 DAP the cell walls of the peripheral endosperm are labeled (Fig. 4C). Between 18 and 22 DAP, (1→3, 1→4)-β-D-glucan labeling is less intense in the thickened aleurone cell walls than the starchy endosperm (Fig. 4D & 5C, D). The cell walls of the sub-aleurone show similar levels of (1→3, 1→4)-β-D-glucan to the starchy endosperm. Indeed, the distribution of all polysaccharides in the sub-aleurone cell walls seems to resemble the starchy endosperm walls and not the aleurone walls. At 28 DAP there was substantial labeling in both the aleurone and starchy endosperm cell walls (Fig. 5E, F). This observation was expected since the level of (1→3, 1→4)-β-D-glucan in the developing endosperm shows greatest accumulation between 16 and 32-36 DAP (Fig. 8A). Similarly, (Coles, 1979) reported that the greatest accumulation of non-starchy polysaccharides in the barley grain occurs after 23 DAP in phytotron-grown plants.

**Hetero-(1→4)-β-D-mannan deposition during endosperm differentiation (10-28 DAP)**

Heteromannan is laid down after cellularization between 5 and 6 DAP and all endosperm cell walls are uniformly labeled with the hetero-(1→4)-β-D-mannan monoclonal antibody at this stage of development (Wilson et al., 2006). At 10 DAP heteromannans could not be detected in the aleurone cell walls using silver enhancement for light microscopy. However, intense labeling was present in the starchy endosperm, in particular, in those walls of the cells adjacent to the grain crease (Figs. 6A, B). Immunogold labeling of thin sections using TEM detects heteromannan in the aleurone cell walls at 10 DAP, albeit at low levels (Fig. 6C). Gold labeling in the starchy endosperm cell walls reaches its peak at this time, although this strong labeling is not indicative of the abundance of heteromannans in barley endosperm (Fig. 6D). It should be noted that differences in antibody avidity for their epitopes prevent us from making conclusions on polysaccharide (heteromannan) abundance based on antibody binding alone. Thus, the strong labeling in this case results from the antibody avidity, rather than from the presence of large amounts of the heteromannans since the levels determined by linkage analyses in starchy endosperm walls of mature barley grain is 3-4% (w/w) (Fincher, 1975). Sugar composition analysis of the endosperm (Fig. 8B) also shows that the amount of mannose in the endosperm is low and increases from 5 DAP and peaks at around 20 DAP. At 12 DAP heteromannan antibody labeling is absent in the walls of the aleurone and peripheral starchy endosperm while the central starchy endosperm remains well labeled (Fig. 6E). As was the case for
(1→3, 1→4)-β-D-glucan, this absence of labeling in the cell walls of the peripheral endosperm is observed until 16 DAP. At this time, the walls of the peripheral endosperm show a similar labeling intensity to other regions of the starchy endosperm (Fig. 6F). At 28 DAP the cell walls of the remnant maternal tissues, sub-aleurone and starchy endosperm, but not those of the aleurone, contain heteromannan (Fig. 6G, H & I).

Arabino-(1→4)-β-D-xylan deposition during endosperm differentiation 10-28 DAP

Arabino-(1→4)-β-D-xylan deposition, monitored with the LM 11 antibody, is the last polysaccharide to be laid down in the endosperm and first appears at 8 DAP but only in those endosperm cell walls nearest to the vascular strand in the crease region (Wilson et al., 2006). At 10 DAP arabino-(1→4)-β-D-xylan is still restricted to the crease region (Fig. 7A, B) but by 12 DAP, strong labeling can also be detected in the aleurone and central starchy endosperm cell walls (Fig. 7C). As was the case for (1→3, 1→4)-β-D-glucan and hetero-(1→4)-β-D-mannan, arabino-(1→4)-β-D-xylan appears to be absent from the cell walls of the peripheral starchy endosperm (Fig. 7C). At 16 DAP, all endosperm cell walls contain arabino-(1→4)-β-D-xylan including the sub-aleurone and peripheral starchy endosperm but the most intense labeling occurs in the aleurone (Fig. 7D). Silver enhancement for light microscopy shows very intense labeling in the thickened aleurone cell walls at 28 DAP and very light labeling in the sub-aleurone and starchy endosperm (Fig. 7E). Immunogold labeling for TEM shows the aleurone cell walls are intensely labeled while those of the sub-aleurone appear to have less arabino-(1→4)-β-D-xylan with a patchy distribution (Fig. 7F,G). The starchy endosperm cell walls adjacent to the crease continue to display intense labeling equal to that of the aleurone (Fig. 7H).

These immunolabelling results were inconsistent with the presence of arabinose and xylose in alcohol insoluble polymeric material, presumably as arabino-(1→4)-β-D-xylan, in the endosperm from 5 DAP (Fig. 8C, D). Thus, monosaccharide analysis of the developing endosperm shows a continual increase in arabinose and xylose from 5 to 38 DAP but the arabino-(1→4)-β-D-xylan is not detected at high levels with the antibodies until 12 DAP. A decrease in both arabinose and xylose is observed in the mature grain due to the removal of some of the aleurone layer during the pearling process. In an attempt to reconcile these data, we considered whether the xylan backbone may be so heavily substituted with either
arabinose or O-acetyl residues during early endosperm development that it is rendered inaccessible to the antibody. To test this hypothesis, we either pre-treated sections with alkali (to remove O-acetyl residues) or applied an α-L-arabinofuranosidase (to remove arabinose residues) to sections prior to LM11 labeling at 5, 8 & 12 DAP. Labeling was now observed in all cell walls of the endosperm that had been pretreated with the enzyme (Fig. 9A-F), confirming that heavily substituted arabinof(1→4)-β-D-xylan is present in walls of the early differentiating endosperm but was not detected in previous LM11 labeling experiments due to arabinose side chains. Pre-treating the sections with alkali did not change the pattern of LM 11 labeling.

5-O-(trans-feruloyl)-L-arabinose polyclonal antibody labeling of differentiating endosperm cell walls from 10-18 DAP

Barley endosperm arabino-(1→4)-β-D-xylan has been shown to vary in its degree of α-arabinofuranosyl substitution at the C(O)2 and/or C(O)3 position of the xylose and esterification on C(O)5 of the arabinofuranosyl residues by hydroxycinnamic acids, such as ferulic acid or p-coumaric acid (Fincher and Stone, 2004; York and O'Neil, 2008). To examine the temporal pattern of feruloylation throughout endosperm differentiation, we applied a polyclonal antibody specific for ferulic acid ester-linked at the C(O)5 position to α-L-arabinosyl residues of arabino-(1→4)-β-D-xylans (Philippe et al., 2007). At 8 DAP there appears to be very little, if any, ferulic acid substitution anywhere in the starchy endosperm which contrasts with the heavy labeling we observed with arabino-(1→4)-β-D-xylan labeling at the grain crease at this time point (Fig. 10A,B). Again at 12 DAP there are barely detectable levels of labeling at the grain crease whereas the aleurone cell walls, which by now are morphologically distinct from the endosperm, show considerable levels of labeling (Fig. 10C,D). A low level of feruloylated epitope was detected in the central starchy endosperm (Fig. 10E). This pattern of labeling continues to 18 DAP and beyond where the walls of the grain crease are unlabeled, the aleurone cell walls heavily labeled, while the starchy endosperm cell walls show barely detectable levels of the feruloylated epitope (Fig. 10F-G). The Golgi apparatus in aleurone cells is also well labeled with this antibody (data not shown) indicating the site of synthesis.

Transcript profiles of *Glucan Synthase-Like (GSL)* genes
There is now considerable biochemical and molecular evidence suggesting the GSL genes are responsible for the synthesis of (1→3)-β-D-glucan in either a developmentally-regulated or wound-activated manner in plants (Brownfield et al., 2009). Seven GSL genes have so far been identified in barley (Schober et al., 2009). Quantitative real-time PCR experiments using gene-specific primer pairs on cDNAs prepared from endosperm between 6 and 38 DAP show a number of patterns of steady-state transcript levels. HvGSL6 has the highest transcript level of all the GSLs early in endosperm development at 6 DAP being three-fold higher than any other GSL gene while HvGSL2 transcript dominates the GSL expression profile within the late differentiation stage, peaking at 24 DAP (Fig. 11A). The other GSL genes show lower and relatively even transcript levels throughout endosperm development. These data are similar to those presented by Schober et al. (2009), although in the earlier work GSL gene transcripts were only monitored up to 11 DAP.

**Transcript profiles of Cellulose Synthase (CesA) genes**

The appearance and distribution of cellulose in the walls of developing barley grain could not be defined because currently there is no probe that specifically detects (1→4)-β-D-oligoglucosides within cellulose and not within (1→3, 1→4)-β-D-glucan and xyloglucan. Nevertheless, transcript abundance of cellulose synthase (CesA) genes involved in cellulose synthesis in primary cell walls was monitored throughout endosperm development, because the presence of small amounts of cellulose in the walls of mature endosperm (Fincher, 1975) indicates that these genes should be active. Burton et al. (2008) reported that HvCesA1, HvCesA2 and HvCesA6 are co-transcribed in tissues where primary wall synthesis is occurring. In the developing barley endosperm, relatively low levels of these transcripts were detected throughout starchy endosperm development (Fig. 11B). HvCesA4, HvCesA7 and HvCesA8, which tend to be co-expressed where secondary wall synthesis is occurring, showed extremely low levels of expression throughout development as expected (Burton et al. 2004; Fig. 11B).

**Transcript profiles of Cellulose Synthase-Like (Csl) genes**

The profiling was restricted to those gene families proposed to have roles in the assembly of the wall polysaccharides present in endosperm walls. Transcript profiles of members of the CslF family, that encode (1→3, 1→4)-β-D-glucan synthases (Burton et al., 2006; Doblin et al., 2009), are shown in Fig 11C. Two genes dominate the expression profile:transcripts
of CslF9 peak early in grain development at 8 DAP and decrease to negligible levels by 12 DAP whereas the transcript levels of CslF6 shows a concurrent increase to become the dominant transcript present throughout the majority of grain development (12-38 DAP). Significant levels of CslF6 transcript persist until late in grain development and despite a decline at 32 DAP (Fig 11C) mRNA levels of this gene are still far higher than any other CslF or indeed CslH gene, whose transcript levels are extremely low (< 200 normalized transcript units; data not shown). Thus, CslF6 is a likely candidate necessary for the synthesis of much of the (1→3;1→4)-β-glucan found in the mature grain (Burton et al., 2008).

The CslA genes from a broad range of species including Arabidopsis, guar (Cyamopsis tetragonoloba), poplar (Populus trichocarpa), loblolly pine (Pinus taeda), moss (Physcomitrella patens) and rice (Oryza sativa) have been shown to have either mannan or glucomannan synthase activity when heterologously expressed (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007). The expression levels of six known barley CslA genes were followed over the differentiation phase of endosperm development. Only one of the genes, CslA6, shows significant transcript abundance, albeit at a level well below the dominant transcripts of the other gene families described above. CslA6 expression levels began to rise around 5 DAP, peaking at 8 DAP and declining sharply to the low levels of the other CslA genes at around 12 DAP (Fig. 11D).

Low levels of HvCslC gene transcripts, proposed to encode the polysaccharide synthase for both the (1→4)-β-D-glucan backbone of xyloglucans (Cocuron et al. (2007)) and cellulose (Dwivany et al., (2007)), were also detected throughout endosperm development (data not shown; see Dwivany et al., (2007) for profiles from whole grain), but the functional significance of these gene transcripts is currently unknown.

**Transcript Profiles of Genes Implicated in Arabino-(1→4)-β-D-xylan Synthesis**

Although the genes involved in arabino-(1→4)-β-D-xylan biosynthesis have not been biochemically defined unequivocally, there are a number of studies that have implicated glycosyl transferases from the GT43, GT47 and GT61 CAZy families in the synthesis of this polysaccharide (Mitchell et al. 2007). The transcript abundance of selected genes from these families were therefore monitored and shown to be present throughout grain development, with relatively high levels of GT43 mRNA (Fig. 11F).
Discussion
This study extends our earlier work on endosperm cellularization in barley, insofar as it is focused on the temporal and spatial appearance of wall polysaccharides during the latter differentiation stage of endosperm development. Furthermore, observations made after application of newly available polysaccharide-specific probes have unveiled important new information about the deposition of polysaccharides within the developing barley grain. This has also led to a revision of some of our original observations on polysaccharide deposition during cellularization. We have examined the monosaccharide composition of the endosperm over the course of development, applied the recently generated xyloglucan antibody to early endosperm development and modified our approach to studying arabino-(1→4)-β-D-xylan using the LM11 antibody. Previous studies identifying genes involved in the synthesis of cell wall polysaccharides have shown that transcript accumulation correlates with the time of polysaccharide synthesis and deposition (Pear et al., 1996; Doblin et al., 2001; Dhugga et al., 2004; Brown et al., 2005; Persson et al., 2005; Cocuron et al., 2007; Mitchell et al., 2007). By coupling our immuno-EM observations with transcript profiles of candidate cell wall synthesis genes, we can now infer which sub-family members of these gene families is functional during key stages of endosperm development.

(1→3)-β-D-glucan (callose)
(1→3)-β-D-Glucan is the first polysaccharide to be deposited in the developing endosperm, appearing in the central cell wall at 3 DAP and within the newly formed anticlinal and periclinal cell walls that encapsulate the nuclei of the syncytium until cellularization is complete at 5-6 DAP (Wilson et al., 2006). Callose has a transient appearance in the cellularizing endosperm except in those regions surrounding plasmodesmata (Wilson et al., 2006). This pattern of callose deposition in the early endosperm is similar to what has been described for rice and wheat endosperm (Brown et al., 1997; Philippe et al., 2006).

Early studies on wheat and barley grains report callose in mature endosperm cell walls as evidenced by deposits of the aniline blue fluorochrome at the aleurone/starchy endosperm interface (Fulcher et al., 1977; Bacic and Stone, 1981a). Fulcher et al. (1977) described different labeling patterns in two barley varieties: one, cv Vanier, shows a continuous layer of the aniline blue fluorochrome while the other, cv Himalaya, has discrete patches of fluorescence along the thick walls directly adjacent to the aleurone. Similarly, Bacic &
Stone (1981a) observed a continuous layer of aniline blue fluorochrome staining at the sub-aleurone/aleurone interface in wheat (cv Insignia) while describing droplets of fluorescent material at the same location for barley (cv Himalaya). These aniline blue-fluorescent deposits match the non-continuous patches of (1→3)-β-D-glucan antibody labeling observed here in cv Sloop within the walls of sub-aleurone cells directly adjacent to the aleurone (Fig. 2A). Fulcher et al. (1977) suggests that these deposits may be the primary substrate for high levels of β-(1,3)-glucanase activity present at the onset of germination. Both Fulcher et al. (1977) and Bacic & Stone (1981a) recognized that variation in the pattern and size of callose deposits between barley varieties does not support a specific physiological function, although Fincher (1989) suggested that the variable patterns of callose distribution might have reflected a variable number of transient water stress events that caused plasmolysis and callose deposition during grain development. The deposits may either influence the movement of substances during germination or they could simply be a remnant of developing walls (Fulcher et al., 1977). However, another possible explanation is that callose is being synthesized as a wound response. The sub-aleurone cell would be expected to be under enormous mechanical pressure as they are pushed up against the thick aleurone cell during grain filling. It may also explain the variability in the pattern of callose deposition as it is likely the degree of pressure exerted and extent of “wounding” is non-uniform across grain varieties and individual grains.

Considerable molecular, biochemical and functional evidence has now accumulated supporting a role for GSL proteins in the synthesis of (1→3)-β-D-glucan in plants (for review see Brownfield et al., 2009). Two HvGSL genes show particularly interesting patterns during endosperm development. HvGSL6 has by far the highest transcript levels during cellularization, indicating that the encoded gene products are likely responsible for callose deposition in the early stages of endosperm development when the first cell walls are laid down (Wilson et al., 2006). In contrast, HvGSL2 transcript levels peak at 24 DAP within the late differentiation stage just as callose appears as deposits at the perimeter of sub-aleurone/aleurone interface. Hence, HvGSL2 may be involved in the synthesis of callose in this later stage of endosperm development.

Xyloglucan
We were not expecting to detect xyloglucan in the developing endosperm since previous chemical analyses were negative in both the starchy endosperm and aleurone cell walls of mature grain (Fincher, 1975; Bacic and Stone, 1981b) and the amount of xyloglucan in the primary cell walls of the commelinoid monocots is the lowest of any of the flowering plants. However, despite these assumptions, the \((1\rightarrow4)\-\beta\-oligoglucoside\) structures detected by the CBHII-AU probe at 3 and 4 DAP attributed to cellulose binding (Wilson et al., 2006), were at least partly belonging to the xyloglucan backbone. We can now draw this conclusion this because the recently generated xyloglucan-specific antibody, LM15 (Marcus et al., 2008), binds to the first formed endosperm cell walls at 3 DAP and persists till 6 DAP (Fig. 3). After this time, xyloglucan labeling diminishes in endosperm walls but persists in the surrounding maternal tissues, indicating that xyloglucan is either hydrolysed from the developing endosperm walls or undergoes modification such that the LM15 antibody does not recognize it (Dwivany et al., 2009). The former possibility seems the more likely given that xyloglucan-specific linkages are not detected in mature endosperm walls by either chemical analysis (Fincher, 1975; Bacic and Stone, 1981b) or by the more sensitive immuno-EM method used in this study (data not shown). Xyloglucan is therefore the second polysaccharide to be shown to undergo turnover in endosperm walls. The reason why xyloglucan is only temporarily deposited during endosperm cellularization is unknown but, like callose, it is a polysaccharide that is laid down early in phragmoplast formation and is subsequently removed (Samuels et al., 1995; Otegui and Staehelin, 2000a, 2000b; Otegui et al., 2001) , presumably because other polysaccharide/s supersede its role later in development. Whilst a definitive role for xyloglucan during early development cannot be deduced from this study, it is of interest that the low levels of xyloglucan often seen in the cell walls of the commelinoid monocots appears to be associated with young and/or expanding cells, although xyloglucan is present in the mature endosperm cell walls of rice (Kato et al., 1982; Kato and Nevins, 1984, 1991; Gibeaut et al., 2005) and has recently been reported to be present in mature tissues of specific cell types in some members of the Poales (Brennan and Harris, 2011).

Cocuron et al. (2007) proposed that the CSLC proteins function is to synthesize the xyloglucan backbone although (Dwivany et al., 2009) have also proposed a role for some CSLC family members in the synthesis of cellulose in certain specialized cell types. Quantitative RT-PCR analysis undertaken here showed that the abundance of barley CSLC
gene transcripts was extremely low (data not shown) consistent with the low levels of xyloglucan (Fincher, 1975; Bacic and Stone, 1981b).

**Cellulose**

The temporal or spatial deposition of cellulose in the developing endosperm is difficult to assess since the CBHII-Au probe used to detect cellulose in our previous study also binds the \((1\rightarrow4)-\beta\)-glucan backbone of xyloglucan. Over the years, a number of molecular probes have been used to detect cellulose including histochemical stains such as Congo Red, the fluorophore/optical brightener Calcofluor white (FB28) (Wood and Fulcher, 1978; Wood, 1980) and various carbohydrate-binding modules (CBM) (McCartney et al., 2006). However, to our knowledge, there is no probe for cellulose that does not also detect the \((1\rightarrow4)-\beta\)-oligoglucoside structures of \((1\rightarrow3, 1\rightarrow4)-\beta\)-D-glucan and/or xyloglucan, including CBM3a (S. Wilson, unpublished data) and the CBM from *Clostridium thermocellum* directed towards crystalline cellulose (Blake et al., 2006; Hervé et al., 2010).

Cellulose comprises 3-4% w/w of barley endosperm walls at maturity (Fincher, 1975) and must therefore be deposited sometime during endosperm development. It seems reasonable to assume that cellulose would also be deposited during the cellularization phase, concomitant with the other polysaccharides we have shown to be deposited during this time (Wilson et al., 2006). To our knowledge, there is no reported case of xyloglucan existing within a plant cell wall in the absence of cellulose in either eudicots or monocots. One of the postulated roles of xyloglucan in the primary wall is to non-covalently cross-link cellulose microfibrils so that the xyloglucan-cellulose network becomes the major load-bearing structure of the wall (Cavalier et al., 2008). Whether the low levels of xyloglucan permit or are necessary for such a role early in endosperm development is debatable; in any event it cannot fulfill this role in the endosperm beyond 8 DAP when LM15 labelling disappears in endosperm cells. Xyloglucan may serve a different purpose, possibly to coat nascent cellulose microfibrils as they are extruded into the early walls since endosperm walls are neither exposed to the load nor turgor that is experienced by the cell walls of vegetative tissues, thereby obviating the need for an extensive cellulose/xyloglucan network. Cellulose is considered a carbon-sink and is not turned over to any great degree after microfibril deposition hence it would be expected to persist in endosperm walls in the absence of xyloglucan during development.
QPCR analysis of barley CesA genes during endosperm development suggests that cellulose synthesis does occur during endosperm cellularization. The primary wall CesA genes, HvCesAs 1, 2 and 6 (Burton et al., 2004), were anticipated to show the highest steady-state transcript levels in developing endosperm and transcripts of these genes were indeed detected here. However, HvCesAs 4, 7 and 8 showed equally low levels of expression barely detectable on the transcript profile. This result was unexpected since these genes tend to be coordinately expressed in tissues where secondary wall synthesis is occurring (Burton et al. 2004).

(1→3, 1→4)-β-D-glucan
(1→3, 1→4)-β-D-glucan accumulates from about 5 DAP and is by far the most abundant polysaccharide of the mature barley grain (Fincher, 1975; Wilson et al., 2006). Its abundance is fairly uniform across the grain during early development then changes to being concentrated to the starchy endosperm and to a much lesser extent the thickened aleurone cell walls by the end of differentiation.

Two gene families are known to be involved in (1→3, 1→4)-β-D-glucan synthesis, the CSLF (Burton et al., 2006) and CSLH (Doblin et al., 2009) gene families. Only one CSLH gene has been identified in the barley genome while there are seven CSLF genes. Previously published transcript profiles show that CSLH1 peaks at 4 DAP, one day before (1→3, 1→4)-β-D-glucan appears in endosperm cell walls (Doblin et al., 2009) and then declines to low levels by 8 DAP, implicating its involvement in early endosperm development. However, CSLH1 transcript levels are generally much lower than CSLF transcript levels in endosperm and in vegetative tissues. At their peak, CSLH1 transcript levels are considerably lower than both CSLF6 (<200 normalized transcripts levels; data not shown) which is steady in the early stages of endosperm development and CSLF9 which is highest at 8 DAP, declining to baseline levels by 12 DAP (Burton et al., 2008; Doblin et al., 2009). After this time, only CSLF6 has significant levels of expression throughout the developing endosperm, suggesting that this gene is responsible for the accumulation of (1→3, 1→4)-β-D-glucan during differentiation. Indeed, the barley blg ((1,3;1,4)-β-D-glucan less) mutant that has a mutation within the HvCSLF6 gene lacks (1→3, 1→4)-β-D-glucan in both endosperm and aleurone walls (Tonooka et al., 2009), confirming that this is the major gene contributing to (1→3, 1→4)-β-D-glucan content within the grain. Reduction of CSLF6 gene
expression in wheat using RNA interference constructs has shown that this gene is also a major contributor to wheat endosperm cell wall (1→3, 1→4)-β-D-glucan (Nemeth et al., 2010).

**Hetero-(1→4)-β-D-mannan**

Heteromannan first appears in the developing endosperm walls at 5 DAP and immunogold labeling reaches its maximum intensity in the starchy endosperm cell walls at around 10 DAP. Later in development heteromannan is present in the starchy endosperm but completely absent from aleurone cell walls. Experiments in which a number of CSLA genes from various plant species have been individually expressed heterologously in either plant or insect systems have shown that these genes encode (gluco)mannan synthases (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007; Doblin et al., 2010). However, this activity has not yet been demonstrated for any members of the cereal-specific clade of the CSLA family (van Erp and Walton, 2009). To date, six CSLA genes have been identified in barley (Schreiber et al., 2008). Of these, phylogenetic analyses show that two genes, HvCSLA2 and HvCSLA6, are located within the same clade as the known (gluco)mannan synthases are positioned and hence are predicted to encode enzymes with the same activities (MD unpublished data). The four other barley CSLA genes lie within the cereal-specific CSLA clade and, given their lower sequence similarity to the experimentally verified (gluco)mannan synthases, may encode enzymes with other polysaccharide synthase activities. Only one CSLA gene, CSLA6, shows significant levels of expression during endosperm development. The appearance and accumulation of heteromannan in the endosperm at 10 DAP coincides with an increase in CSLA6 expression. Given the transcript profiles of the HvCSLA genes as well as the phylogenetic evidence, it appears CSLA6 may be responsible for heteromannan deposition during endosperm development.

**Arabino-(1→4)-β-D-xylan**

In our first study we reported arabino-(1→4)-β-D-xylan, based upon LM 11 binding, as the last polysaccharide to be deposited in the endosperm at 8 DAP once cellularization was complete (Wilson et al., 2006). The distribution pattern of arabino-(1→4)-β-D-xylan deposition was different to the other polysaccharides in that it was restricted to the crease region until 12 DAP. All other polysaccharides were laid down uniformly across the
developing endosperm (Wilson et al., 2006). The observation that arabino-(1→4)-β-D-xylan appears later in development than the other polysaccharides is not supported by monosaccharide analyses which show xylose and arabinose to be present from 5 DAP, albeit in low amounts (Fig. 8C, D). In light of the chemical analyses, it seemed most likely that arabino-(1→4)-β-D-xylan was present earlier in endosperm development but in a highly substituted form that was not recognized by the LM11 antibody (McCartney et al., 2005). To test this hypothesis, we applied the enzyme α-L-arabinofuranosidase to thin sections in attempts to remove arabinosyl substituents from the polysaccharide and hence to expose the xylan backbone to the LM11 antibody (Fig. 9). Only those endosperm sections pre-treated with the enzyme had labeling along early endosperm cell walls confirming arabino-(1→4)-β-D-xylan does appear earlier in development but in a highly substituted form.

At 12 DAP, strong labeling was seen in the aleurone and central starchy endosperm but absent in the peripheral starchy endosperm cell walls. Similarly in wheat, arabino-(1→4)-β-D-xylan is reported to appear during early differentiation at 12 DAP and labeling is strongest in the differentiating aleurone layers and central starchy endosperm but scant in the cell walls of the peripheral endosperm (Philippe et al., 2007). The same pattern of labeling was seen using the (1→3, 1→4)-β-D-glucan antibody in wheat. In our study, none of the antibodies we applied to thin sections of endosperm between 12 and 16 DAP labeled the cell walls at the peripheral starchy endosperm. Given this puzzling result in both barley and wheat, we decided to treat endosperm sections at 12 and 14 DAP with the α-L-arabinofuranosidase enzyme prior to labeling with the LM11 antibody. As was the case for early cell walls at 5 DAP, those sections pre-treated with the enzyme had significant gold labeling in the cell walls of the peripheral endosperm while untreated sections did not (Fig. 9). This result suggests arabino-(1→4)-β-D-xylan in this region is in a highly substituted form that the LM11 antibody does not recognise. Consistent with this, members of the GT43 and GT47 gene families, which have been implicated in arabino-(1→4)-β-D-xylan synthesis in other plant systems ((Mitchell et al., 2007; York and O'Neill, 2008; Zeng et al., 2008) were transcribed throughout the developmental window used in the current study (Fig. 11E). Transcript levels of the GT43 gene were particularly high in comparison to levels for other genes but levels of mRNA encoding the GT61 gene examined here were low (Fig. 11E). This was somewhat surprising given that GT61 genes are proposed to encode arabinosyl transferases (Mitchell et al., 2007).
Conclusion
The availability of increasingly sophisticated antibodies that discriminate between the fine structural variations on polysaccharide backbones enables us to describe the structural variation during assembly or following deposition in a spatial and temporal manner by microscopy that is not possible by bulk chemical analysis techniques. Here we demonstrate the strength of these complementary approaches particularly for the heteroxylans, the most complex class of wall polysaccharides in the grass endosperm. These data are invaluable in informing the interpretation of transcriptomic data thereby identifying putative candidate synthase/glycosyl transferase genes involved in polysaccharide assembly and/or modification.
Materials and Methods

Plant Material
Barley grain (cv. Sloop) from 5 to 38 DAP was collected from glasshouse-grown plants grown in February and September as described in (Wilson et al., 2006). Samples of developing grain collected for quantitative real-time PCR were prepared as described in (Burton et al., 2008).

Immunolabeling for Light and Electron Microscopy
A complete description of the methods and the probes used is provided in Wilson et al. (2006). Grain samples from 10 to 28 DAP were prepared for microscopy and changes to the endosperm were monitored by taking transverse sections of the developing grains. The distribution and relative abundance of polysaccharides in the differentiating grain were detected on sections using monoclonal antibodies raised against specific polysaccharide epitopes including (1→3)-β-D-glucan (Meikle et al., 1991), xyloglucan LM15 (Marcus et al., 2008), (1→3,1→4)-β-D-glucan (Meikle et al., 1994), hetero-(1→4)-β-D-mannan (Pettolino et al., 2001) and arabino-(1-4)-β-D-xylan LM11 (McCartney et al., 2005). Sections for light microscopy (1 µm) were either stained in toluidine blue to highlight tissue architecture or processed for immunogold labeling. The unstained sections were dried onto glass microscope slides and incubated in diluted primary antibodies followed by either anti-mouse or -rat secondary antibodies conjugated to ultrasmall gold (average particle size 0.8 nm). These gold particles are made visible under the light microscope using a silver enhancement kit (Aurion, The Netherlands) that amplifies the labeling by coating the gold particles in silver to produce an increasingly intense black stain. Images of labeled samples were captured on a digital camera mounted to a DM2500 Leica compound light microscope (Vienna, Austria). Images were inverted in Adobe Photoshop so antibody labeling appears white against a black background. No control sections have been included as all were black images.

Sections for electron microscopy (80 nm) were collected on 200 mesh formvar coated gold grids and incubated in diluted primary antibodies followed by either anti-mouse or -rat secondary antibodies conjugated to 18 nm gold particles. Some grids were pre-incubated
for 30 min in a 1mg/ml solution of α-L-arabinofuranosidase (Megazyme, UK), washed and antibodies applied. Grids were post-stained in an aqueous solution of 2% (w/v) uranyl acetate, washed, dried and viewed on a Philips BioTwin Transmission Electron Microscope (Eindhoven, The Netherlands) and images captured on a Gatan multiscan digital camera (Wilson et al., 2006).

**RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR (QPCR)**

Total RNA was extracted from endosperm tissue homogenates using a phenol-guanidine isothiocyanate reagent (Trizol, Invitrogen) following the recommended protocol with the following exception. After the initial re-suspension of the tissue in Trizol, 200 µl of Plant RNA Isolation Aid (Ambion) was added. The sample was centrifuged at 13,200g for 10 minutes to pellet insoluble material and starch. The supernatant was removed to a clean tube and the standard extraction protocol continued. cDNA synthesis was carried out and used for quantitative real-time PCR (QPCR) as described in (Burton et al., 2008), using identical primers for control genes as described therein. QPCR primers for the various cellulose synthase-like genes examined are supplied in Table 1. Primers for the following genes are not included in the table because they have been published previously: HvGSL (Schober et al. 2009), HvCESAs (Burton et al. 2004), HvCSLFs (Burton et al. 2008) and HvCSLCs (Dwivany et al. 2009).

**Polysaccharide Quantitation**

Mature grain samples were pearled to remove external maternal tissue until 30% of the grain was removed. For quantification of levels of (1→3,1→4)-β-D-glucan and monosaccharides, endosperm material was harvested, freeze-dried and ground to a powder. (1→3, 1→4)-β-D-β-Glucan was measured using a small-scale version of the Megazyme mixed-linkage beta-glucan assay (McCleary and Codd, 1991). Initially 15 mg of sample was washed twice with ethanol (75% v/v, 1 ml) for 10 minutes at 75°C, centrifuged at 10,000 g for 10 minutes and the supernatant discarded. This was followed by a third wash at room temperature. Samples were dried at 37°C before analysis was undertaken according to the manufacturer’s instruction using appropriately decreased volumes. The quantitation of the monosaccharides xylose, arabinose and mannose was performed following acid hydrolysis by separating the monosaccharides on a LC using a Hypersil C18 5µm, 2.1x250 mm column as described in (Burton et al., 2011). Twenty five milligrams of
ground, freeze-dried endosperm was hydrolyzed with sulfuric acid (1M, 1 ml) for 3 hours at 100°C, cooled and centrifuged at 20,000 g for 5 minutes. The samples were diluted 20-fold before derivatization with PMP (1-phenyl-3-methyl-5-pyrazolone; (Burton et al., 2011). Talose was included with each sample as an internal standard.

Acknowledgments
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Fig. 1. Light micrographs of toluidine blue-stained sections through barley grains during endosperm differentiation.
A; At 10 DAP several cell layers of maternal tissue surround the “brick-like” arrangement of cells distinguishing the differentiating aleurone layers from the starch-rich central endosperm. Bar = 200 µm. B; At 14 DAP a sub-aleurone cell layer marked by small starch granules and protein bodies lies between the aleurone cell layers and starchy endosperm. Bar = 100 µm. C, By 22 DAP the aleurone cells appear to have fully differentiated with their cell walls attaining maximum thickness while large endosperm cells continue to accumulate starch. Bar = 100 µm. al, aleurone; se, starchy endosperm; sa, sub-aleurone.

Fig. 2. Transmission electron micrographs of endosperm cells at 28 DAP labeled with (1→3)-β-D-glucan antibody.
A; Deposits of callose were seen in electron-dense regions at the edges of sub-aleurone cells (arrows). B; In the starchy endosperm gold labeling was restricted to the callosic-rich plugs surrounding plasmodesmata (arrows). Bar = 0.5 µm. cw, cell wall.
**Fig. 3.** Transmission electron micrographs of developing endosperm cell walls labeled with xyloglucan antibody, LM 15, at 3-8 DAP.

A; At 3 DAP xyloglucan is present in the central cell wall but absent in the first forming anticlinal cell walls. B; At 4 DAP light labeling can be detected in the early endosperm cell walls. Inset shows enlarged image of cell wall with gold particles (arrows). C; At 5 DAP that xyloglucan labeling is at its most abundant in the endosperm. D; By 6 DAP gold labeling is barely detectable in the starchy endosperm but still present in the central cell wall. By 8 DAP xyloglucan labeling in the starchy endosperm is almost negligible (data not shown). Bar = 0.5 µm. acw, anticlinal cell wall; cw, cell wall; dcw, developing cell wall; ne, nucellar epidermis; V, vacuole.

**Fig. 4.** Light micrographs of silver-enhanced (1→3, 1→4)-β-D-glucan labeling of differentiating endosperm from 10-22 DAP.

A; At 10 DAP (1→3, 1→4)-β-D-glucan labeling was present in all cell walls of the differentiating endosperm. B; At 12 DAP the (1→3, 1→4)-β-D-glucan monoclonal antibody labeled the cell walls of the differentiating aleurone and central starchy endosperm. Labeling was absent from the cell walls of the peripheral endosperm. C; At 16 DAP (1→3, 1→4)-β-D-glucan was present in the cell walls of the peripheral endosperm. D; Increased labeling intensity implies (1→3, 1→4)-β-D-glucan is in greater abundance in the starchy endosperm cell walls than either the aleurone or maternal tissues. Bars = 100 µm. al, aleurone; cse, central starchy endosperm; mt, maternal tissues; pe, peripheral endosperm; se, starchy endosperm; sa, sub-aleurone.
Fig. 5. Transmission electron micrographs of (1→3, 1→4)-β-D-glucan labeling of differentiating aleurone and starchy endosperm cells 10-28 DAP.
A; At 10 DAP, there appears to be comparable levels of labeling in the cell walls of the aleurone and B, starchy endosperm. C; By 18 DAP there is less labeling in the thickened cell walls of the aleurone than the starchy endosperm (D). E & F; At 28 DAP, the density of gold labeling in the aleurone cell walls (E) is similar to that observed at 18 DAP. While labeling in cell walls of the starchy endosperm (F) appears less dense. Bars = 0.5 µm. pb, protein body.

Fig. 6. Light and transmission electron micrographs of hetero-(1→4)-β-D-mannan labeling during endosperm differentiation from 10-28 DAP.
A; Silver-enhanced antibody labeling at 10 DAP revealing little or no hetero-(1→4)-β-D-mannan in the aleurone cell walls whereas the starchy endosperm is well labeled. Bar = 100 µm. B; At 10 DAP the thicker starchy endosperm cell walls of the crease region are heavily labeled. Bar = 200 µm. C; Antibody labeling at 10 DAP revealing low levels of hetero-(1→4)-β-D-mannan in the aleurone cell walls. Bar = 0.5 µm. D; The cell walls of the starchy endosperm are heavily labeled at 10 DAP. Bar = 0.5 µm. E; Silver enhanced antibody labeling at 12 DAP showing little labeling in the aleurone cell walls, no labeling in the walls of the peripheral starchy endosperm while hetero(1→4)-β-D-mannan is present in the walls of the central starchy endosperm. Bar = 100 µm. F; By 16 DAP the walls of the peripheral starchy endosperm are labeled. Bar = 100 µm. G; At 28 DAP, silver enhanced antibody labeling shows hetero(1→4)-β-D-mannan is present in the walls of the maternal tissues, sub-aleurone and starchy endosperm but absent in the thickened aleurone cell walls. Bar = 100 µm. H; Gold labeled sections confirm hetero(1→4)-β-D-mannan is absent in the aleurone cell walls while present in the starchy endosperm cell walls (I). Bars = 0.5 µm. al, aleurone; cse, central starchy endosperm; cr, crease region; mt, maternal tissues; pe, peripheral endosperm; sa, sub-aleurone; se, starchy endosperm; vb, vascular bundle.
**Fig. 7.** Light and transmission electron micrographs of arabino-(1-4)-β-D-xylan labeling using the LM 11 antibody during endosperm differentiation from 10-28 DAP.

A; Silver-enhanced antibody labeling at 10 DAP reveals no arabino-(1-4)-β-D-xylan in aleurone or starchy endosperm cell walls. Bar = 200 µm. B; Arabino-(1-4)-β-D-xylan is present in the starchy endosperm cells walls of the crease region at 10 DAP. Bar = 200 µm. Inset, An electron micrograph of a gold-labelled Golgi apparatus (arrow) located within a cell at the crease. C; At 12 DAP silver-enhanced antibody labeling reveals arabino-(1-4)-β-D-xylan is now present in the aleurone and central starchy endosperm cell walls but absent in the peripheral starchy endosperm. Bar = 100 µm. D; At 16 DAP the cell walls of the peripheral starchy endosperm are labeled with the arabino-(1-4)-β-D-xylan antibody. Bar = 100 µm. E; At 28 DAP the aleurone cell walls are intensely labeled with the arabino-(1-4)-β-D-xylan antibody while labeling in the starchy endosperm cell walls is barely detectable. Bar = 100 µm. F; At 28 DAP electron micrographs of antibody labeled sections show intense gold labeling in the aleurone cell wall. Bar = 0.5 µm. G; A heavily labeled aleurone cell wall abutting a sub-aleurone cell wall that shows patchy gold labeling with the arabino-(1-4)-β-D-xylan Ab. Bar = 0.5 µm. H; At 28 DAP the cell walls in the crease region continue to be strongly gold labeled. Bar = 0.5 µm. al, aleurone; cw, cell wall; cse, central starchy endosperm, cr, crease; pe, peripheral endosperm; sa, sub-aleurone; se, starchy endosperm.

**Fig 8.** The amounts of (1→3, 1→4)-β-D-glucan and the monosaccharides mannose, xylose and arabinose in the developing barley endosperm (cv Sloop).

The endosperms were collected from 5 to 38 days after pollination in February, 2008 (Feb) and September, 2008 (Sep) as described in the Materials and Methods. Mean of three replicates, error bars are standard deviation. A; (1→3, 1→4)-β-D-glucan, B; mannose (Sep only), C; xylose, D; arabinose expressed as µg per endosperm.
Fig. 9. Transmission electron micrographs of 5, 8 & 12 DAP endosperm cell walls labeled with the LM11 (left hand column) antibody for arabino-(1→4)-β-D-xylan and those pre-treated with α-L-arabinofuranosidase before labeling with LM11 (right hand column).

A: Immunogold labeled section of cellularizing endosperm at 5 DAP showing no gold particles in the cell walls. B; Treatment with α-L-arabinofuranosidase prior to LM11 labeling reveals arabino-(1→4)-β-D-xylan to be present in the early endosperm cell walls. C; No labeling using the LM11 antibody can be seen in the cell walls of the central starchy endosperm at 8 DAP. D; LM11 labeling after pre-treating 8 DAP sections with α-L-arabinofuranosidase reveals significant gold labeling along central starchy endosperm cell walls. E; Gold particles are absent when the peripheral starchy endosperm at 12 DAP has been labeled with the LM11 antibody. F; The presence of arabino-(1→4)-β-D-xylan is revealed in the peripheral starchy endosperm of 12 DAP sections when treated with α-L-arabinofuranosidase prior to LM11 labeling. Bars = 0.2 µm. cw, central starchy endosperm wall; pcw, peripheral starchy endosperm wall.

Fig. 10. Transmission electron micrographs revealing the labeling of the 5-O-(trans-feruloyl)-L-arabinose (5-O-Fer-Ara) epitope on arabino-(1→4)-β-D-xylan during barley endosperm differentiation from 10-18 DAP.

A; Immunogold labeled section at the grain crease showing barely detectable levels of binding at 10 DAP. B; The central starchy endosperm at 10 DAP also shows little labeling on cell walls although gold particles bind to starch granules non-specifically. C; Again at 12 DAP there is almost no detectable levels of the 5-O-Fer-Ara epitope at the grain crease. D; The aleurone cell walls which are beginning to differentiate (thicken) at 12 DAP are well labeled with the 5-O-Fer-Ara antibody. E; There is light labeling in the starchy endosperm at 12 DAP. F; No labeling was detected at the grain crease at 18 DAP. G; The aleurone cells are heavily and uniformly labeled throughout their thickened walls at 18 DAP. H; The starchy endosperm walls at 18 DAP are lightly and unevenly labeled with the 5-O-Fer-Ara antibody. Bar = 0.2 µm. al, aleurone; cr, crease; cw, cell wall; se, starchy endosperm.
Fig. 11. Normalized transcript levels of glycosyl transferase genes in developing barley endosperm from 6-38 DAP.
A; GSL gene family. B; CesA family genes. C; CSLF family genes. D; CSLA genes. E; representative CAZy GT 43, 47, and 61 gene family members.
### Primer sequences

**Table 1:** Gene-specific QPCR primers, with PCR product sizes in base pairs and optimal acquisition temperatures for the genes analysed.

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<th>Reverse Primer (5’ to 3’)</th>
<th>PCR product (bp)</th>
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References


Disrupting Two Arabidopsis thaliana Xylosyltransferase Genes Results in Plants Deficient in Xyloglucan, a Major Primary Cell Wall Component. Plant Cell 20: 1519-1537


Coles G (1979) Relationship of mixed link beta-glucan accumulation to accumulation of free sugars and other glucans in the developing barley endosperm. Carlsberg Research Communications 44


Fig. 8. The amount of polysaccharide in the developing endosperm from barley variety Sloop.

The endosperms were collected from 5 to 38 days after pollination (DAP) in February, 2008 (Feb) and September, 2008 (Sep). Mature grain was collected and pearled until 30% of the grain was removed. Mean of three replicates, error bars are standard deviation. A; β-glucan; B, mannose (Sep only); C, xylose; D; arabinose expressed as µg per endosperm.