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RESEARCH PAPER

# Analysis of the arabinoxylan arabinofuranohydrolase gene family in barley does not support their involvement in the remodelling of endosperm cell walls during development

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## Abstract

Arabinoxylan arabinofuranohydrolases (AXAHs) are family GH51 enzymes that have been implicated in the removal of arabinofuranosyl residues from the (1,4)- $\beta$ -xylan backbone of heteroxylans. Five genes encoding barley AXAHs range in size from 4.6 kb to 7.1 kb and each contains 16 introns. The barley *HvAXAH* genes map to chromosomes 2H, 4H, and 5H. A small cluster of three *HvAXAH* genes is located on chromosome 4H and there is evidence for gene duplication and the presence of pseudogenes in barley. The cDNAs corresponding to barley and wheat AXAH genes were cloned, and transcript levels of the genes were profiled across a range of tissues at different developmental stages. Two *HvAXAH* cDNAs that were successfully expressed in *Nicotiana benthamiana* leaves exhibited similar activities against 4-nitrophenyl  $\alpha$ -L-arabinofuranoside, but *HvAXAH2* activity was significantly higher against wheat flour arabinoxylan, compared with *HvAXAH1*. *HvAXAH2* also displayed activity against (1,5)- $\alpha$ -L-arabinopentase and debranched arabinan. Western blotting with an anti-*HvAXAH* antibody was used to define further the locations of the AXAH enzymes in developing barley grain, where high levels were detected in the outer layers of the grain but little or no protein was detected in the endosperm. The chromosomal locations of the genes do not correspond to any previously identified genomic regions shown to influence heteroxylan structure. The data are therefore consistent with a role for AXAH in depolymerizing arabinoxylans in maternal tissues during grain development, but do not provide compelling evidence for a role in remodelling arabinoxylans during endosperm or coleoptile development in barley as previously proposed.

**Key words:** AXAH, family GH51, glycosyl hydrolase, heteroxylan remodelling.

## Introduction

A key distinguishing feature of the grasses is that their cell walls differ significantly from those of other plants with respect to non-cellulosic polysaccharide constituents. Thus, walls of the grasses are relatively rich in heteroxylans compared with walls of other monocotyledons and the dicotyledons, where walls generally have higher levels of pectins and xyloglucans, and correspondingly lower levels of heteroxylans. Walls of the grasses also contain (1,3;1,4)- $\beta$ -glucans, although these polysaccharides are not present in all walls and therefore might not be crucial structural components of the walls (Fincher, 2009). Within walls of the grasses there is

a good deal of heterogeneity with respect to heteroxylan fine structure (Burton *et al.*, 2010). Here the family of genes encoding arabinoxylan arabinofuranohydrolases (AXAH) in barley is examined; they have been suggested to play a role in the generation of this heterogeneity in heteroxylan fine structure through the hydrolytic removal of arabinofuranosyl residues from the (1,4)- $\beta$ -xylan backbone of heteroxylans (Ferré *et al.*, 2000; Lee *et al.*, 2001).

Heteroxylans consist of a main chain of (1 $\rightarrow$ 4)-linked  $\beta$ -D-xylopyranosyl (Xylp) residues that carry various substituents. In the grasses, the (1,4)- $\beta$ -xylan main chain is commonly

substituted with  $\alpha$ -L-arabinofuranosyl (Araf) residues attached mainly at the O-3 position of the xylosyl residues, but also at the O-2 position and often at both O-2 and O-3 (Fincher and Stone, 2004). In certain tissues of the grasses and in many dicot heteroxylans, glucuronopyranosyl (GlcAp) residues or their 4-O-methyl ethers are common substituents of the (1,4)- $\beta$ -xylan backbone; acetyl groups are also found on the O-2 and O-3 positions of xylosyl residues in some heteroxylans. A proportion of the arabinofuranosyl residues of heteroxylans from the grasses are characteristically substituted at their O-5 positions with hydroxycinnamic acids, in particular ferulic acid, and in some cases heteroxylans carry short oligosaccharide substituents (Fincher and Stone, 2004). In endosperm of the cereals wheat and barley, the major substituents of the cell wall heteroxylans are Araf residues and, while reference is made to these polysaccharides as arabinoxylans, they are likely to contain minor amounts of other substituents. Cell walls in the peripheral layers of the grain also contain high levels of arabinoxylan, with significant variation in Araf substitution (Barron *et al.*, 2007).

The large number of potential substituents of the (1,4)- $\beta$ -xylan backbone allow a very large number of structures for heteroxylans, and the inherent heterogeneity afforded by different monosaccharide and hydroxycinnamic acid contents is compounded by different patterns of distribution of the substituents along the chain (Viator *et al.*, 1994; Izydorczyk and Biliaderis, 1995; Burton *et al.*, 2010). For example, there is evidence that heteroxylans of the grasses are synthesized in a form in which most xylosyl residues of the main chain are substituted with arabinofuranosyl residues, but that a varying number of the arabinosyl substituents are subsequently removed in a process that alters not only the fine structures of the polysaccharides, but also their physicochemical properties in the wall (Carpita and Gibeaut, 1993; Gibeaut *et al.*, 2005; Wilson *et al.*, 2006; Toole *et al.*, 2010). Heavily substituted arabinoxylans would be expected to be soluble in aqueous media (Andrewartha *et al.*, 1979), but the removal of a limited number of substituents in a local region of the polysaccharide would allow the discrete generation of 'junction zones' and hence gel formation. Further, the removal of a large proportion of the arabinosyl residues would allow the polysaccharide to interact via hydrogen bonding over long stretches of the backbone to form insoluble, tightly bound complexes between the arabinoxylan chains themselves and with other wall polysaccharides such as cellulose (Andrewartha *et al.*, 1979). The influence of arabinosyl substitution on water solubility is not straightforward, however, as more highly substituted arabinoxylans may have a higher degree of cross-linking through covalent di-ferulic acid and ester bridges, thereby causing them to be less soluble (Harholt *et al.*, 2010).

The enzymes involved in these modifications of heteroxylan fine structure, and hence functional properties, have not been characterized in detail. Ferré *et al.* (2000) and Lee *et al.* (2001) detected and purified AXAH activity from extracts of germinated barley grain. They demonstrated that the enzymes are members of the GH51 family of glycosyl hydrolases (Cantarel *et al.*, 2009; <http://www.cazy.org/>).

In kinetic comparisons using the two substrates wheat flour arabinoxylan and 4-nitrophenyl  $\alpha$ -L-arabinofuranoside (4NPA), Lee *et al.* (2001) showed that the family GH51 AXAH1 was about equally active on both substrates, but pointed out the difficulties in comparing kinetic parameters of enzymes against substrates with large differences in rates of diffusion. In contrast, bifunctional family GH3 glycoside hydrolases from germinated barley grain with both  $\alpha$ -L-arabinofuranosidase and  $\beta$ -D-xylopyranosidase activity hydrolysed 4NPA and oligo-arabinoxylides at much higher catalytic rates and efficiencies than the GH51 AXAHs, but hydrolysed wheat flour arabinoxylan relatively slowly (Lee *et al.*, 2003). It was therefore concluded that the AXAHs were most probably the more important group of enzymes for the removal of arabinofuranosyl residues from cell wall arabinoxylans during growth and development in barley (Lee *et al.*, 2001, 2003).

Here, advantage is taken of the increasing number of genome sequences available for the grasses, including rice, sorghum, *Brachypodium*, and barley, to characterize the AXAH gene families from grasses and to compare them with those from the dicots. AXAH gene structures and map positions in the respective genomes are defined, two of the genes have been successfully expressed in a heterologous system to compare their actions against various arabinofuranose-containing polysaccharides, the transcription patterns of individual members of the gene family have been examined, and the locations of the enzymes in developing barley grain have been defined.

## Materials and methods

### Cloning and sequence analysis

Total RNA was extracted from barley (*Hordeum vulgare*) 'Sloop' or 'Himalaya' tissues and used as a template for cDNA synthesis and SMART cDNA library generation (Clontech) as described in Burton *et al.* (2008). Primers were designed to amplify coding sequence for *HvAXAH1* and *HvAXAH2*, based on sequences in GenBank, and from *HvAXAH3*–*HvAXAH5*, based on sequences in online expressed sequence tag (EST) databases, and extended using the SMART RACE library. Two sets of degenerate primers were designed to amplify these and any other homologous sequences. Primer sequences are listed in Supplementary Table S1 available at *JXB* online. The barley *HvAXAH1*–*HvAXAH5* sequences were deposited in GenBank as accessions JQ303075–JQ303079, respectively. Isolated sequences were used to query online genome sequence databases to identify the orthologous genes from rice and sorghum ([www.phytozome.net](http://www.phytozome.net)), *Brachypodium distachyon* ([www.brachypodium.org](http://www.brachypodium.org)), and *Arabidopsis* ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Wheat sequences were isolated from similarly developed cDNA libraries. These sequences were assigned to the A, B, or D genomes where possible, as indicated on the phylogenetic tree, using the GrainGenes wEST BLAST facility with the 'monococcum' (A genome) and 'D genome non-repetitive (454)' databases (<http://wheat.pw.usda.gov/wEST/blast/>). Signal peptides and cellular targeting of putative protein sequences were predicted using SignalP and TargetP, respectively ([www.cbs.dtu.dk/services](http://www.cbs.dtu.dk/services)). Pairwise sequence identities and similarities were calculated using the BLOSUM50 similarity matrix in MatGAT (Campanella *et al.*, 2003). Phylogenetic trees were constructed using the Neighbor-Joining method with 1000 bootstrap replicates using MEGA4 ([www.megasoftware.net](http://www.megasoftware.net)).

### Transcript profiling

Quantitative real-time PCR (Q-PCR) was undertaken to determine transcript profiles of the *HvAXAH* genes across different tissues using conditions and normalization procedures described by Burton *et al.* (2008) and in the developing endosperm as described by Zhang *et al.* (2010).

### Antibody preparation and western blotting

A 14 amino acid peptide, ETIGPWEEERPGHYG, which was deduced from the barley *HvAXAH3* gene was synthesized and antiserum was generated in rabbits by GenScript (Piscataway, NJ, USA). The sequence is encoded within the seventh exon of the gene and most residues are conserved within the known *HvAXAH* genes, with the expectation that the antibody would be antigenic towards all members of the enzyme family. Protein was extracted from frozen tissue samples using extraction buffer [50 mM TRIS buffer, pH 7.5; 5% (v/v) glycerol; 5 mM EDTA with 4  $\mu\text{l ml}^{-1}$  protease inhibitor cocktail from Sigma] before centrifugation at 10 000 rpm for 5 min. After protein quantification using the Bio-Rad protein assay, 20  $\mu\text{g}$  of protein was subjected to electrophoresis on a NuPAGE Novex 4–12% BIS-TRIS gel (Invitrogen, Carlsbad, CA, USA) in MOPS buffer (50 mM MOPS, 50 mM TRIS, 0.1% SDS, 1 mM EDTA; pH 7.7) and transferred to a nitrocellulose membrane using the iBlot gel transfer system (Invitrogen) according to the manufacturer's instructions. Transferred proteins were stained with Ponceau S before blocking overnight at 4 °C in 10% (w/v) non-fat milk powder in TRIS-buffered saline with Tween-20 (TBS-T; 10 mM TRIS, pH 8; 150 mM NaCl; 0.1% Tween-20). The blot was incubated with anti-HvAXAH antibody (1:1000 dilution in TBS-T) at room temperature for 1 h. After washing with TBS-T three times (5 min each), the membrane was incubated with goat anti-rabbit IgG-conjugated horseradish peroxidase (HRP; 1:1000 dilution in TBS-T) at room temperature for 1 h. After washing the membranes three times with TBS-T, Amersham ECL detection reagent (GE Healthcare, Little Chalfont, Bucks, UK) was added according to the manufacturer's instructions before exposure to Amersham Hyperfilm™ chemiluminescence film.

### Gene mapping

*HvAXAH* genes were assigned to chromosomes using wheat–barley addition lines (Islam *et al.*, 1981) and gene-specific primers. Genes were also mapped in a doubled haploid Clipper×Sahara population (Karakousis *et al.*, 2003). Single nucleotide polymorphisms (SNPs) were identified by sequencing the genes from the parent lines, and flanking primers were used to amplify the SNP-containing region from lines in the mapping population known to have recombination events on the chromosome corresponding to the position of each *HvAXAH* locus. The products were sequenced and results analysed using MapManager QT version 0.30 (Manly *et al.*, 2001).

### Heterologous expression in *Nicotiana benthamiana*

Full-length AXAH coding sequences were amplified from the cDNA clones described above using PCR with primers (Supplementary Table S1 at JXB online) that incorporated a T7 epitope tag and *Xma*I and *Not*I restriction enzyme sites appropriate for cloning into a modified pORE02 expression vector (Coutu *et al.*, 2007). Plasmid pORE02 had first been modified to contain a single *Cauliflower mosaic virus* (CaMV) 35S promoter via digestion with *Sca*I. *Nicotiana benthamiana* infiltrations were conducted as described by Wood *et al.* (2009) using a bacterial density of 0.4 (OD<sub>600</sub>). Infiltrated leaves were harvested after 5 d, vacuum infiltrated in extraction buffer [50 mM MES, pH 5.5; 0.4% (v/v) protease inhibitor cocktail from Sigma], and centrifuged (900 g for 10 min) to provide an apoplastic extract. The remaining sample was ground in extraction buffer and centrifuged under the same conditions. Extracted protein was separated by electrophoresis in a 12% (w/v) TRIS-glycine gel for

western blotting with an anti-HvAXAH peptide antibody as described above.

### Assays for heterologously expressed enzyme activity

**Hydrolysis of 4-nitrophenyl  $\alpha$ -L-arabinofuranoside (4NPA):** The hydrolysis of 4NPA was used to measure  $\alpha$ -L-arabinofuranosidase activity. Assays were performed at 37 °C in 50 mM sodium acetate buffer, pH 4.9, containing 15  $\mu\text{g}$  of total protein and 0.04% (w/v) 4NPA. The reaction was stopped with the addition of 2 vols of sodium bicarbonate (0.5 M; pH ~12). The amount of released 4-nitrophenol (4NP) product was determined spectrophotometrically at 405 nm and through comparison with a standard curve of 4NP. The pH optimum was determined using 4NPA as a substrate.

**Substrate specificity and measurement of released arabinose:** Enzyme activity on wheat arabinoxylan was measured following incubation of 0.5% (w/v) wheat arabinoxylan (low viscosity arabinoxylan with an A:X ratio of 0.6; Megazyme, Ireland) in sodium acetate buffer, pH 4.9 with 45  $\mu\text{g}$  of total protein at 37 °C for 16 h. Enzyme activity was also measured on 10 mM (1,5)- $\alpha$ -L-arabinopentaose, 0.5% debranched arabinan from sugar beet (Megazyme), 0.5% larchwood arabinogalactan (Sigma), and 0.5% gum arabic (Sigma) under the same reaction conditions for 1 h. Released Araf was measured using a scaled-down variation to the K-LACGAR kit from Megazyme, whereby a 200  $\mu\text{l}$  sample was combined with 1 ml of water, 100  $\mu\text{l}$  of solution 2 (TRIS/EDTA buffer), and 50  $\mu\text{l}$  of solution 3 (NAD<sup>+</sup>), and incubated as recommended before 10  $\mu\text{l}$  of enzyme suspension (galactose dehydrogenase and mutarotase) was added. The arabinose concentration was determined spectrophotometrically at 340 nm after blank subtraction and comparison with a standard curve of arabinose.

**HPLC fingerprinting of AXAH-treated wheat arabinoxylan:** To produce oligosaccharide profiles, samples that had been treated with the protein extracts were heat inactivated before being further hydrolysed using *endo*-1,4- $\beta$ -xylanase M6 (rumen microorganism, Megazyme, 22.5 U) in 1 ml of 25 mM sodium acetate buffer, pH 6.5, for 16 h at 22 °C. The digests were centrifuged, and the supernatants were heated at 100 °C for 10 min and re-centrifuged.

Oligosaccharides were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP; Fu and O'Neill, 1995; Rozaklis *et al.*, 2002). To 15  $\mu\text{l}$  of oligosaccharide solution (supernatants described above), 5  $\mu\text{l}$  of 1 mM galactose (internal standard) and a mixture of 10  $\mu\text{l}$  of 0.5 M PMP in methanol and 8  $\mu\text{l}$  of 1 M ammonium hydroxide was added. The solutions were mixed, heated at 70 °C for 60 min, cooled, acidified with 10  $\mu\text{l}$  of acetic acid, and extracted twice with 1 ml of butyl ether to remove excess PMP. A further 5  $\mu\text{l}$  of acetic acid was added to the final solutions. Normal phase chromatography of PMP-oligosaccharides was carried out on a silica-diol column (Phenomenex Luna HILIC, 3  $\mu\text{m}$ , 150×2.1 mm) at 40 °C on an Agilent 1200 LC. The LC auto-sampler was used to dilute 2  $\mu\text{l}$  of sample with 60  $\mu\text{l}$  of acetonitrile immediately prior to injection. Eluents were (A) 95% acetonitrile, 0.2 M acetic acid, 5 mM ammonium hydroxide; and (B) 20% acetonitrile, 0.2 M acetic acid, 5 mM ammonium hydroxide. The gradient consisted of two steps: 1–24% B over 20 min, and 24–100% B over 5 min, followed by 3 min at 100% B, and column re-equilibration. PMP derivatives were detected by UV absorbance at 260 nm and were quantified as xylose equivalents.

Reversed phase chromatography was used to quantify the Araf and Xylp in the same samples (Phenomenex Kinetex C18, 2.6  $\mu\text{m}$ , 100×3 mm) at 30 °C on an Agilent 1200 LC. Eluents were (A) 10% acetonitrile, 40 mM ammonium acetate (pH ~6.8), and (B) 70% acetonitrile. The gradient was 8–16% B over 12 min, followed by 1.5 min at 100% B, and column re-equilibration, all at 0.6 ml min<sup>-1</sup>. PMP derivatives were detected by UV absorbance at 250 nm.



## Results

The barley *HvAXAH* gene family has at least five members

Five cDNAs of ~2 kb in length were amplified and genomic fragments carrying the corresponding barley genes were subsequently isolated using gene-specific PCR. The genes were between 4.6 kbp and 7.1 kbp in length and encoded putative proteins of between 656 and 677 amino acid residues (Supplementary Fig. S1 at JXB online). The encoded proteins had an estimated molecular mass of 72–75 kDa. The five genes each contained 16 introns ranging from 71 bp to 1167 bp (Fig. 1). Overall, the sizes of the introns were relatively small, with about half having lengths of close to 100 bp or less. The variation in gene length was largely attributable to differences in intron sizes. The positions of intron insertion and the lengths of the exons were relatively constant. The genes were numbered following on from the two sequences identified by Lee *et al.* (2001).

The sequence similarities of the predicted protein sequences were between 71% and 91%, while sequence identities at the amino acid level ranged from 55% to 82% (Supplementary Table S2 at JXB online). Based on homology with protein domains in the Pfam database, the five predicted *HvAXAH* protein sequences all had a carbohydrate-binding domain (CBM\_4\_9; PF02018) and an  $\alpha$ -L-arabinofuranosidase domain ( $\alpha$ -L-AF\_C; PF06964) located towards the N- and C-terminus, respectively (Fig. 1). The CBM\_4\_9 family includes diverse carbohydrate-binding domains, including the CBM4 and CBM9 families of carbohydrate-binding modules. The CBM4 modules are ~150 residues in length and are found mostly in bacterial enzymes. Binding of these modules has been demonstrated with xylans, (1,3)- $\beta$ -glucans, (1,3;1,4)-

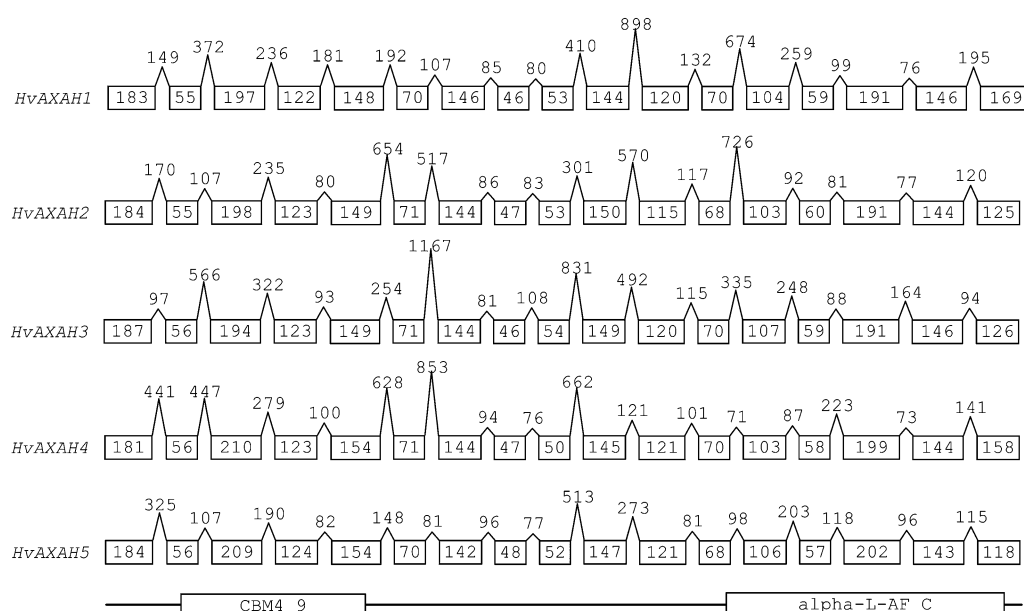
$\beta$ -glucans, (1,6)- $\beta$ -glucans, and amorphous cellulose (Boraston *et al.*, 2004; Cantarel *et al.*, 2009; <http://www.cazy.org>). Modules in the CBM9 family are ~170 residues in length and are found in xylanases (Cantarel *et al.*, 2009; <http://www.cazy.org>). The  $\alpha$ -L-AF\_C family represents the C-terminus of eukaryotic and bacterial  $\alpha$ -L-arabinofuranosidase enzymes (EC 3.2.1.55).

Two expressed pseudogenes with premature stop codons in the open reading frame were also identified in some barley varieties. Screening 16 barley varieties revealed the presence of one pseudogene in approximately half of the lines and the other pseudogene in a single line (data not shown). No additional *HvAXAH* genes could be amplified using degenerate primers designed to different regions of the coding sequence. Exhaustive analyses of the barley EST databases did not reveal any alternative splicing of introns (data not shown). Evidence does exist in EST databases for alternative splicing in rice *OsAXAH* genes Os11g03730, Os12g03470, and Os11g03780. None of these splice forms appears to change the protein sequence, as they are all located in the 5'-untranslated region or would lead to significantly truncated proteins (data not shown).

A 40 bp inverted repeat with a high level of homology to the *Stowaway* MITE (miniature inverted-repeat transposable element) consensus sequence was detected within intron 10 of the *HvAXAH3* gene.

The barley *HvAXAH* genes are located on three different chromosomes

Barley *AXAH* genes were assigned to chromosomes using PCR analysis of wheat–barley addition lines (Islam *et al.*, 1981). The *HvAXAH1* and *HvAXAH2* genes were assigned



**Fig. 1.** Schematic representation of the barley *HvAXAH* genes. Exons are displayed as blocks and contain their length in nucleotides starting from the initial ATG. Introns are displayed as lines between adjacent boxes with their length in nucleotides shown at the apex. The final exon length includes the TGA stop. No 5'- or 3'-untranslated region sequences have been included. The positions of the conserved carbohydrate-binding domain and  $\alpha$ -L-AF\_C domain in the putative protein sequence are indicated.

to chromosomes 5H and 4H, respectively. The *HvAXAH3* and *HvAXAH4* genes also mapped to chromosome 4H, and *HvAXAH5* to chromosome 2H.

Positions within chromosomes were assigned using SNP genotyping of a doubled haploid Clipper×Sahara population. The *HvAXAH1* gene mapped between markers *ksuA1* and *bcd276* on chromosome 5H. No polymorphisms existed between the parent lines across >6800 bp of *HvAXAH2*, which precluded the fine mapping of this gene. The other two genes on chromosome 4H, *HvAXAH3* and *HvAXAH4*, mapped together between markers *cd0358* and *awbma29*. The *HvAXAH5* gene mapped between *G9-406B* and *UXS3* on chromosome 2H.

Following the completion of this mapping work, a barley genome sequence scaffold became available (Mayer *et al.*, 2011). Examination of the positions of all the *HvAXAH* genes on the barley scaffold database confirmed the positions of *HvAXAH1* on chromosome 5H (bin 5), *HvAXAH2*, *HvAXAH3*, and *HvAXAH4* in a cluster on chromosome 4H (bin 6), and *HvAXAH5* on chromosome 2H (bin 6). The genes are located between the POPA markers AXAH1 2\_1260 and 1\_1260; AXAH2 and AXAH 3 1\_0492 and 1\_0577; AXAH4 1\_0411 and 1\_0480, and AXAH5 2\_1049 and 1\_1452 (Mayer *et al.*, 2011 Supplemental data sets 2–8).

#### *AXAH genes in other cereals and grasses*

Wheat *AXAH* gene orthologues were isolated from wheat cDNA. The sequence similarity and sequence identity between the predicted protein sequences of the wheat enzymes were similar to those observed for barley (Supplementary Table S2 at JXB online), while the sequence similarity between the barley and wheat homologues was between 95% and 99% at the amino acid level (Supplementary Table S2). The barley and wheat sequences were used to identify *AXAH* orthologues from the genomes of rice (nine genes), sorghum (six genes), and *Brachypodium* (five genes). The sequences of these genes, and an alignment of the predicted protein sequences and subsequent cluster analysis (Fig. 2), showed that barley, wheat, and *Brachypodium* contain five *AXAH* genes. An extra gene is present in sorghum that may be due to a duplication that has resulted in the *SbAXAH* genes annotated as Sb08g001540 and Sb05g001900. These genes are clustered closely together and share the highest level of sequence identity (87.6%) among the sorghum *AXAH* genes. Three of the rice *OsAXAH* genes are collinearly arranged within a 50 kb region on chromosome 11, and three syntenic genes are similarly arranged on chromosome 12. These genes are located within the first 3 Mb of the distal end of the short arm of both chromosomes that resulted from a segmental duplication event in the rice genome (Choisne *et al.*, 2005) and helps account for the larger gene family in rice.

#### *Transcript profiles of the barley HvAXAH genes*

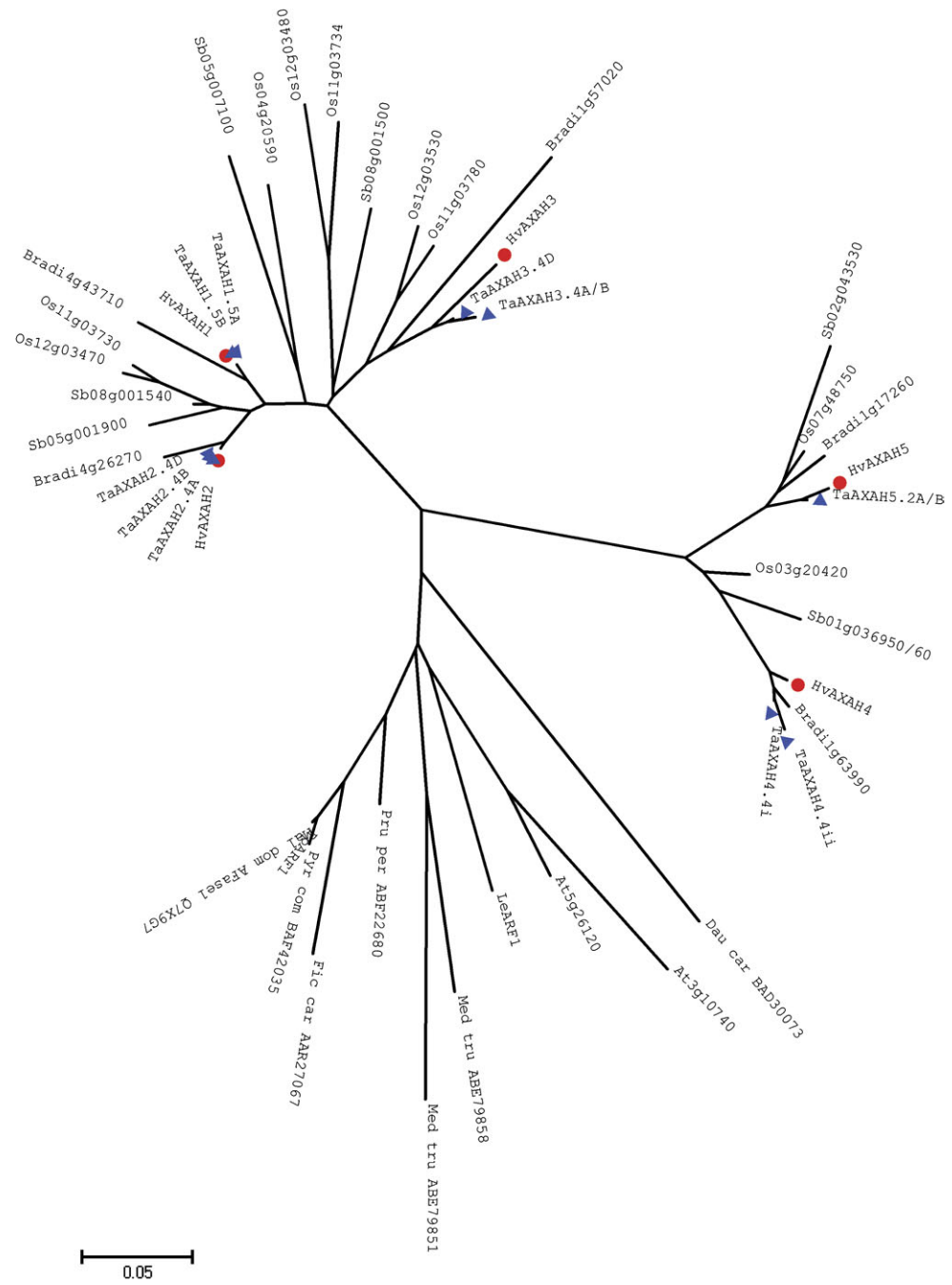
**Quantitative-PCR:** Transcript abundance of the five barley *HvAXAH* genes was determined in 16 tissue types (Fig. 3) using Q-PCR and gene-specific primers (Supplementary

Table S1 at JXB online). Transcript levels of *HvAXAH2* were significantly higher compared with the other genes, and the highest levels were detected in the leaf tip, leaf base, mature leaf, root base, peduncle, and whole flower at anthesis (Fig. 3A). The transcript levels of the other *HvAXAH* genes were more easily compared when plotted on re-scaled figures (Fig. 3B, C). The *HvAXAH1* mRNA was the second most abundant *AXAH* transcript across these tissue types and accumulated most in the anther before anthesis. The *HvAXAH3* gene was most highly transcribed in developing whole grains (Supplementary Fig. S2). Transcript levels of the *HvAXAH4* gene were the lowest in the tissue types examined here, but transcripts could be detected in the leaf base, peduncle, and anther before anthesis. The *HvAXAH5* gene was most highly transcribed in the leaf base and had low levels of transcript in several other tissue types (Fig. 3C).

Barley coleoptile and endosperm developmental series were used to determine *HvAXAH* transcript accumulation during development in these tissues (Fig. 4). In the developing endosperm, levels of transcripts for the five *HvAXAH* genes were very low throughout the sampling period of 6–38 DAP (days after pollination). Modest increases were observed in certain transcripts at 24 and 38 DAP, but levels were still low and quantitation errors relatively high (Fig. 3A). It is not believed that these increases are significant.

In contrast, relatively high levels of *HvAXAH2* mRNA were detected in developing coleoptiles 1 d after the initiation of germination and subsequently from 4 d to 7 d after germination (Fig. 4B). Low but significant levels of *HvAXAH1* and *HvAXAH3* were also detected at these later time points in the developing coleoptiles.

**Microarray analyses:** Sequences of the *HvAXAH* genes were used to query the PlexDB database ([www.plexdb.com](http://www.plexdb.com)) for homologous probe sets that are present on the Affymetrix 22K Barley GeneChip. Four of the genes were represented, with *HvAXAH1* represented by probe set Contig12712\_at, *HvAXAH2* by Contig3596\_at, *HvAXAH3* by Contig5239\_at, and *HvAXAH4* by Contig24807\_at. Transcript levels of the *HvAXAH* genes were compared across 21 tissues in a barley development series (Druka *et al.*, 2006; Supplementary Fig. S3 at JXB online). Six of the tissues were tested in two genotypes. The data indicated that *HvAXAH2* was more highly expressed than the other *HvAXAH* genes across most tissues, as observed here using Q-PCR (Fig. 3A). *HvAXAH1* transcripts increased in the embryo of germinating grains compared with the other tissues, but only low levels of *HvAXAH1* transcripts were detected in the embryo in the present study (Fig. 2B). *HvAXAH3* was highly expressed in the pistil before anthesis, in 5–16 DAP caryopsis, in 22 DAP caryopsis, and in the seedling of both genotypes (Supplementary Fig. S3). The Q-PCR data confirmed the relative abundance of *HvAXAH3* transcripts in whole developing grain, but not in the other tissues. Both the Q-PCR data and the microarray analyses indicated that *HvAXAH4* transcripts increased in the anthers before anthesis, compared with the other tissues (Fig. 3B, Supplementary Fig. S3).



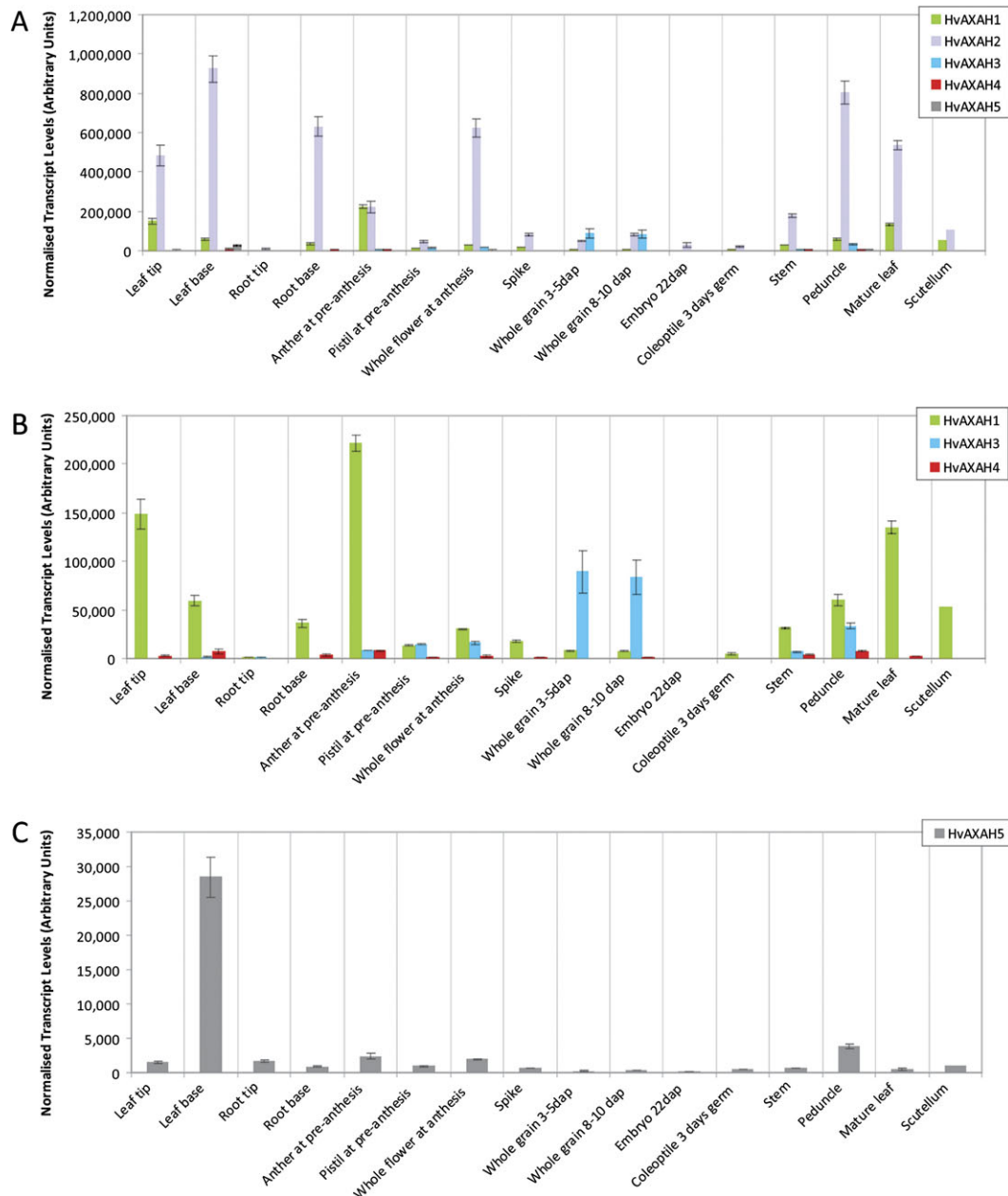
**Fig. 2.** Glycosyl hydrolase 51 arabinofuranosidases. Barley (Hv; circles) and wheat (Ta; triangles) GH51 proteins were aligned with sequences from rice (Os), *Brachypodium* (Bradi), sorghum (Sb), *Arabidopsis* (At), and selected dicotyledonous species. The tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates.

*Co-expression analysis:* Although the microarray analyses did not always correspond to the Q-PCR data, especially where transcript levels were low, it must be noted that the preparation of tissues and the extraction of RNA samples are likely to have been different in the two sets of tissue samples. However, the microarray data did prove to be useful for the identification of genes for which mRNA levels were correlated with *HvAXAH* mRNA levels. Thus, ‘co-expression’ analyses using the same PlexDB database were undertaken for the four *HvAXAH* genes represented on the microarray. This revealed several cell wall-related genes with similar expression

patterns in the sampled tissue types, such as a putative  $\beta$ -fructofuranosidase being co-expressed with *HvAXAH4* in the anthers before anthesis. *HvAXAH2* also appeared to be co-ordinately transcribed with the three *HvCesA* genes that are putatively involved in secondary cell wall synthesis (Burton *et al.*, 2004; Supplementary Fig. S3 at *JXB* online).

*Location of HvAXAH protein*

High levels of *HvAXAH* mRNA were detected in whole developing grain in this study (Fig. 3B, Supplementary Fig. S2)



**Fig. 3.** Normalized transcript levels of *HvAXAH* genes in a range of barley tissues. Error bars indicate the SD for each transcript. (A) All five transcript levels. (B) Intermediate levels of expression of *HvAXAH1*, 3, and 4 in the absence of the more highly expressed *HvAXAH2* transcript. (C) Lower levels of *HvAXAH5* expression in the absence of the other transcripts.

and in previous studies (Druka *et al.*, 2006; Sreenivasulu *et al.*, 2006), but, when carefully dissected developing endosperm was examined, very low levels of *HvAXAH* mRNA were detected (Fig. 4A). This suggested that the *HvAXAH* transcripts in the whole developing grain samples probably originated from maternal tissues rather than from the endosperm itself.

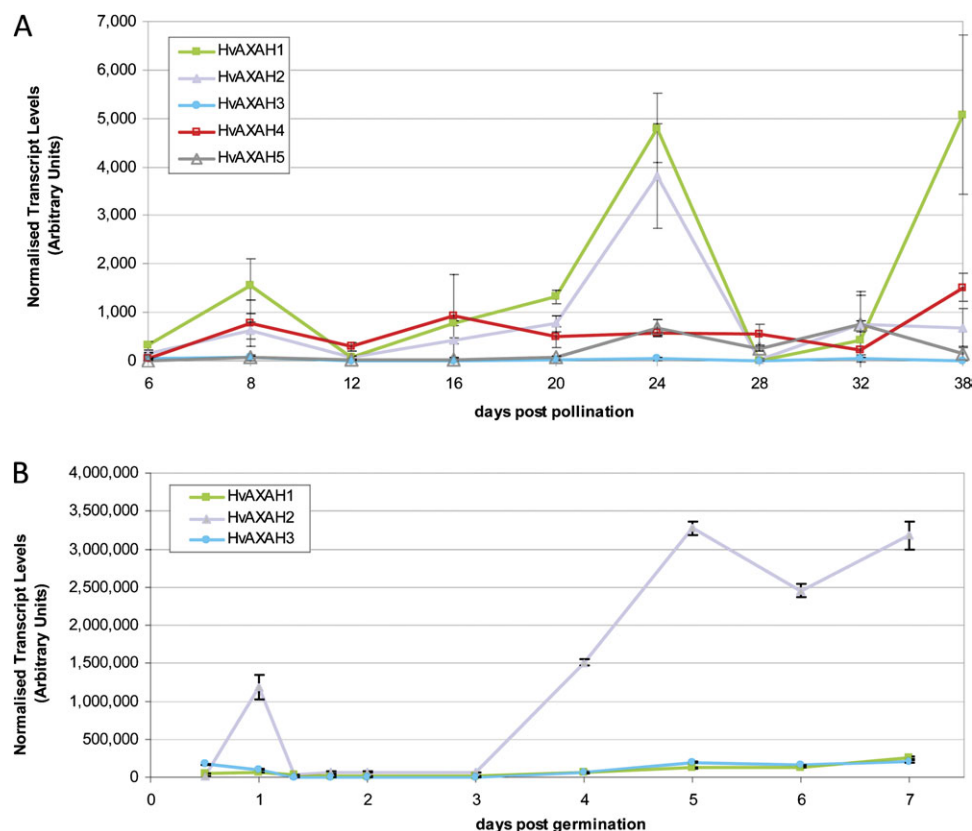
To investigate this possibility further at the protein level, total soluble protein was isolated from the starchy endosperm and from the pericarp and other outer layers of developing grain. Western blotting was used to detect the presence of the *HvAXAH* proteins, using an anti-*HvAXAH* peptide antibody that was expected to recognize all the known *HvAXAH* enzymes (Fig. 5). The antibody bound to

proteins extracted from the maternally derived outer grain layers at 4, 7, and 14 DAP and from the whole pistil at anthesis. Antibody binding to proteins from the endosperm extracts was low or undetectable (Fig. 5B), which was consistent with the low levels of *HvAXAH* mRNA detected in the developing endosperm (Fig. 4A).

#### *Heterologous expression of HvAXAH genes in Nicotiana benthamiana*

Previous attempts to express active *HvAXAH* enzymes heterologously in *Escherichia coli* and *Pichia* systems had been unsuccessful. Here it was attempted to express an active *HvAXAH* enzyme in the *N. benthamiana* transient





**Fig. 4.** Normalized transcript levels of *HvAXAH* genes in developing barley endosperm and coleoptile. Error bars indicate the SD for each transcript. (A) Transcript levels in isolated endosperm between 6 and 38 DAP. (B) Transcript levels in a coleoptile time series from 0.5–7 d post-germination.

leaf expression system (Schob *et al.*, 1997). The cDNAs of the five barley *HvAXAH* genes were fused with the CaMV 35S promoter and infiltrated into leaves of *N. benthamiana*. Transcript levels of the *HvAXAH* genes in the infiltrated *N. benthamiana* leaves were checked using Q-PCR, and confirmed that barley *HvAXAH* mRNA was present in each case.

The *HvAXAH* constructs contained sequences encoding AXAH signal peptides and it was therefore predicted that expressed enzyme would be secreted into the apoplast, where relatively few endogenous *N. benthamiana* leaf proteins would be located. Accordingly, apoplastic extracts of the leaf were used to measure enzyme protein levels and activities (Figs 6C, 7). Accumulation of the heterologously expressed proteins was determined by western blotting, using the anti-*HvAXAH* antibody (Fig. 6B). The *HvAXAH1* and *HvAXAH2* proteins accumulated to significant levels in the apoplastic fraction of the leaf. Small amounts of *HvAXAH3* and *HvAXAH5* proteins were also detected, but expression of the *HvAXAH4* protein could not be detected (Fig. 6B). These results were confirmed in several experiments and also in total soluble leaf protein extracts (data not shown).

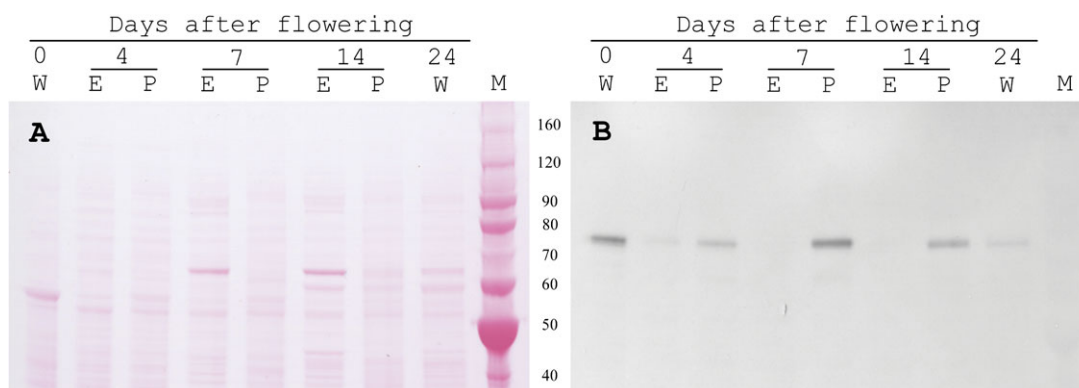
The apparent molecular size of the major protein band was ~80 kDa, which is larger than the predicted size of ~70 kDa observed by Lee *et al.* (2001) for the enzyme purified from barley coleoptile extracts. This discrepancy might be explained by different levels of glycosylation of the

expressed protein, compared with the enzyme extracted from barley, given that the enzymes have several potential *N*-glycosylation sites (Lee *et al.*, 2001). Variation in the molecular weight marker and electrophoretic conditions could also contribute to this difference. A second band ~5 kDa smaller than the main protein was often seen on the western blots (data not shown). The proteins expressed in this study contained a T7 epitope tag at the C-terminus, and western blotting using an anti-T7 antibody revealed that the C-terminus was missing from the second smaller product. Lee *et al.* (2001) also observed a truncated form of the enzyme that was of a similar size.

In view of the low levels of expressed *HvAXAH3*, *HvAXAH4*, and *HvAXAH5* proteins, other promoters were tried. However, attempts to express the *HvAXAH3* gene driven by the maize ubiquitin promoter also failed to produce high levels of protein (data not shown). Initial experiments with constructs containing *HvAXAH1* with and without the T7 epitope tag revealed no difference in accumulation or activity against 4NPA when the tag was present.

#### Enzyme activities of heterologously expressed *HvAXAH* proteins

Enzyme activities of the soluble protein extract following heterologous expression of the barley *HvAXAH* genes in *N. benthamiana* leaves were assayed against the synthetic



**Fig. 5.** Soluble protein extraction of developing barley grain and detection of HvAXAH protein. (A) Total soluble protein extracted from developing grains at flowering (0 DAP) and 4, 7, 14, and 24 DAP. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S. Where possible, developing grains were dissected into endosperm (E) and outer seed layers (P) after removal of the embryo. Liquid endosperm was collected at 4 DAP and included some contaminating non-endosperm fluid. The whole pistil and grain with glumes removed was used at 0 and 24 d after flowering, respectively. The protein molecular weight ladder is shown in lane M with approximate sizes indicated (kDa). (B) Western blot of the same membrane using the anti-HvAXAH antibody.

substrate 4NPA and several arabinofuranose-containing polysaccharides. Activity against 4NPA was confirmed for the heterologously expressed HvAXAH1 and HvAXAH2 enzymes (Fig. 6C), both of which had activities that were similar to and significantly greater than those of the other HvAXAHs or the empty vector control. Some endogenous arabinofuranosidase activity was detected in the empty vector control sample and mostly in the apoplastic fraction; *N. benthamiana* is expected to contain both family GH51 AXAH genes and family GH3  $\alpha$ -L-arabinofuranosidase genes (Lee *et al.*, 2001, 2003; Chavez-Montes *et al.*, 2008). None of the expressed HvAXAH enzymes showed any xylosidase activity when measured using 4-nitrophenyl  $\beta$ -D-xylopyranoside (4NPX; data not shown).

Protein extracts assayed with wheat flour arabinoxylan showed that significantly higher amounts of Araf were released when this polysaccharide substrate was incubated with HvAXAH1 and HvAXAH2 extracts, compared with the other HvAXAH extracts and the empty vector control (Fig. 7). The activity of the expressed HvAXAH2 on the arabinoxylan was higher than that of the expressed HvAXAH1.

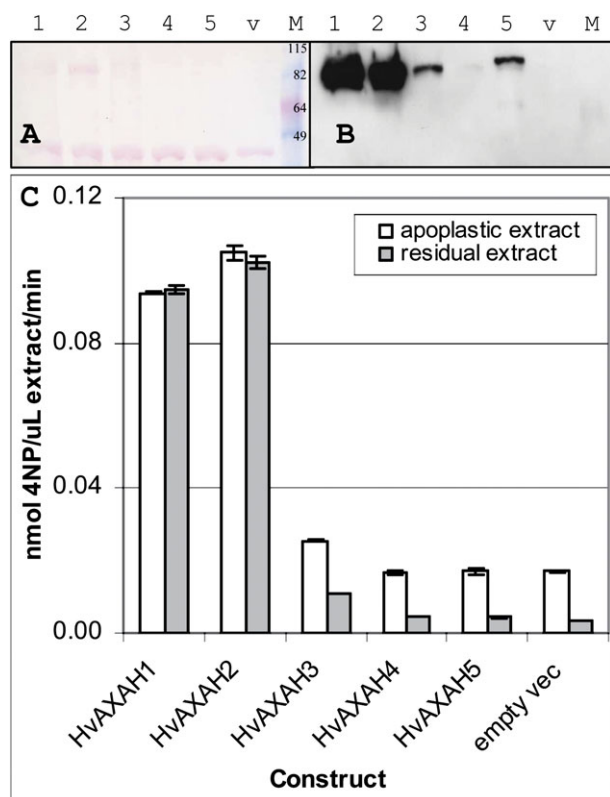
Protein extracts were also assayed with (1,5)- $\alpha$ -L-arabinopentaose, debranched arabinan from sugar beet, larchwood arabinogalactan, and gum arabic. A similar amount of Araf was released from the oligosaccharide as from wheat arabinoxylan, with the activity of HvAXAH2 being higher than that of the expressed HvAXAH1. Araf was released from debranched arabinan in the presence of the HvAXAH2 extracts, while a small amount also appears to have been released by the other four HvAXAH enzymes. A similarly small amount of Araf was released from larchwood arabinogalactan by HvAXAH2, 3, 4, and 5, but not HvAXAH1. No Araf was released by any of the protein extracts when incubated with gum arabic.

Oligosaccharide profiling using (1,4)- $\beta$ -xylan endohydrolase treatment and the quantitation of released products by

reversed phase chromatography was used to characterize changes in the fine structure of the wheat flour arabinoxylan following HvAXAH treatment (Fig. 8). Oligosaccharide peaks G, H, and I were identified as Xyl5Ara, Xyl4Ara2, and Xyl5Ara2, respectively, based on mass spectroscopy and linkage analysis. The release of Araf by the heterologously expressed HvAXAH1 and HvAXAH2 samples was confirmed and it could be calculated that  $\sim 3\%$  of the Araf in the initial wheat flour arabinoxylan was released following incubation with the expressed HvAXAH2 extract. The HvAXAH2 sample had increased amounts of xylose, xylobiose, and peak H (Xyl4Ara2), with a decrease in peak G (Xyl5Ara) and other oligosaccharides, all of which are consistent with the removal of Araf residues from the polysaccharide.

## Discussion

It has been proposed that AXAHs play a role in the remodelling of heteroxylan structure in the cell walls of the Poaceae, which include the commercially important cereals and grasses (Lee *et al.*, 2001; Farrokhi *et al.*, 2006; Toole *et al.*, 2009, 2010). The enzymes are classified with the family GH51 glycoside hydrolases (Cantarel *et al.*, 2009; <http://www.cazy.org>) and catalyse the hydrolytic removal of Araf residues from arabinoxylans. However, they also hydrolyse the synthetic glycoside 4NPA, (1,5)- $\alpha$ -L-arabinofuranohexaose, beet arabinan, and larchwood arabinogalactan (Ferré *et al.*, 2000; Lee *et al.*, 2001; Fulton and Cobbett, 2003; Chavez-Montes *et al.*, 2008). In view of the emerging evidence that arabinoxylans and other heteroxylans from the grasses are synthesized initially in a form in which most of the xylopyranosyl residues of the (1,4)- $\beta$ -xylan backbone are substituted with Araf residues and that these Araf residues are progressively removed from the backbone following deposition of the nascent arabinoxylans



**Fig. 6.** *HvAXAH* enzymes heterologously expressed in *N. benthamiana* and activity against 4NPA. Expression of each *HvAXAH* gene was driven by the 35S promoter. Total soluble protein was extracted from the apoplast of infiltrated leaves and from the residual sample. Western blots of protein fractions demonstrated variable accumulation of the *HvAXAH* enzymes in the transient expression system. Ponceau S was used to visualize proteins in the apoplastic extracts after transfer to a nitrocellulose membrane (A), before probing with the anti-*HvAXAH* antibody (B). Labels indicate *HvAXAH1*–5 (lanes 1–5), empty vector (v), and protein marker (M). Arabinofuranosidase activity was determined using the synthetic substrate 4NPA (C).

into the wall (Carpita and Gibeau, 1993; Gibeau et al., 2005; Wilson et al., 2006; Toole et al., 2010), the AXAH enzymes are prime candidates as the enzymes that remove the Araf residues from arabinoxylans. The attendant changes in fine structure are likely to carry functionally important consequences, insofar as removal of Araf residues will enhance the ability of arabinoxylan chains to align over extended regions and hence to bind more tightly through increased intermolecular hydrogen bonding. Increased binding between individual arabinoxylan chains or between arabinoxylans and other wall polysaccharides such as cellulose would be expected to reduce the flexibility and porosity of the wall, and therefore to decrease the wall's permeability to water and low molecular mass molecules, or the ability of potentially pathogenic microorganisms to penetrate the wall.

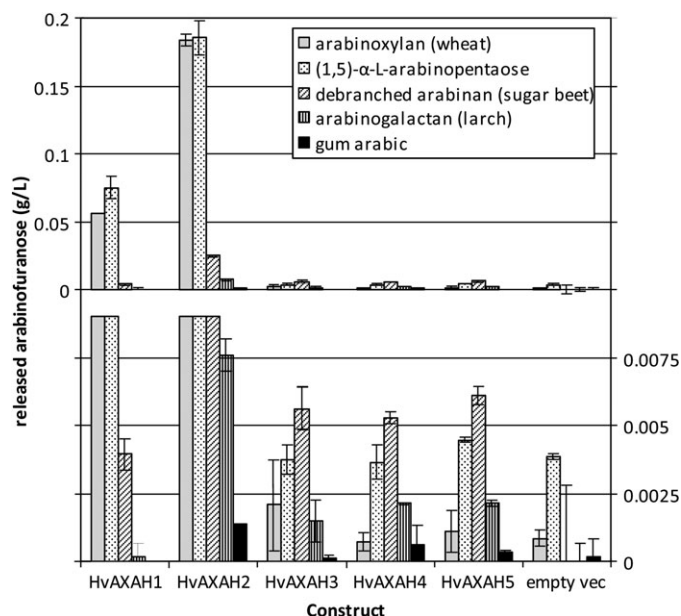
To examine these possibilities further, the *HvAXAH* gene family has now been characterized in detail. Five *HvAXAH*

genes have been isolated from barley based on sequence homology with a previously characterized *HvAXAH* and mapped to the barley genome. Single *HvAXAH* genes are located on chromosomes 2H and 5H, while a cluster of three tightly linked *HvAXAH* genes is located on chromosome 4H. However, the map positions of the genes do not correspond to any regions of the barley genome that have been shown to influence heteroxylan structure or levels. Thus, quantitative trait loci (QTL) related to phenotypic features such as total arabinoxylan, water-extractable arabinoxylan, the Ara:Xyl ratio, and the degree of arabinofuranosyl substitution of the polysaccharide have been mapped to wheat chromosomes 1A, 1B, 6A, 6B, and 7D (Saulnier et al., 2007; Quraishi et al., 2011); no *HvAXAH* genes are located in syntenic regions of the barley genome. In addition, there appear to be up to two *HvAXAH* pseudogenes in some barley varieties. Analyses of the *AXAH* gene families from other plants show that the families from the grasses are generally larger than those from dicotyledons. Thus, there are two *AXAH* genes in *Arabidopsis*, five in barley, wheat, and *Brachypodium*, six in sorghum, and nine in rice, which may reflect a higher incidence of gene duplication or the greater abundance of arabinose substitution in the heteroxylans of the Poaceae.

The barley *HvAXAH* genes are highly fragmented, with 16 introns of lengths varying from 71 bp to 1167 bp inserted in the coding regions of the genes (Fig. 1). This characteristic raises the possibility that alternative splicing of the *HvAXAH* genes might occur, given the increasing recognition that alternative splicing of mRNA can be functionally important in the grasses, especially in response to abiotic stress (Simpson et al., 2010). However, in contrast to some rice *OsAXAH* genes, analysis of the EST databases revealed no evidence for alternative splicing in the barley *HvAXAH* genes (data not shown).

To check if the isolated genes did encode active AXAH enzymes, the five barley *HvAXAH* cDNAs were expressed in the *N. benthamiana* transient expression system. Western blot analyses indicated that *HvAXAH* protein could be readily detected when the *HvAXAH1* and *HvAXAH2* cDNAs were expressed in the heterologous system; apparently lower levels of *HvAXAH3* and *HvAXAH5* protein could also be detected, but little if any *HvAXAH4* protein was expressed (Fig. 6B). Attempts to express putative family GH51 AXAH enzymes from *Arabidopsis* and other plants, using heterologous systems such as *E. coli*, *Saccharomyces*, and *Pichia*, have generally been unsuccessful (Fulton and Cobbett, 2003).

The activities of the expressed *HvAXAH1* and *HvAXAH2* proteins were measured against 4NPA and other arabinofuranose-containing substrates. Activities against 4NPA were similar for both proteins; however, the *HvAXAH2* enzyme removed approximately three times more Araf from the arabinoxylan and (1,5)- $\alpha$ -L-arabinopentaose than *HvAXAH1* under the assay conditions used. *HvAXAH2* was also capable of hydrolysing debranched arabinan and arabinogalactan. This activity profile is similar to that reported previously for *HvAXAH1* (Lee et al., 2001).



**Fig. 7.** Activity of heterologously expressed *HvAXAH* enzymes on arabinofuranose-containing substrates. Arabinofuranosidase activity was measured via the release of Araf from wheat arabinoxylan, (1,5)- $\alpha$ -L-arabinopentaose, debranched arabinan from sugar beet, larchwood arabinogalactan, and gum arabic. Results are shown on two scales for clarity.

HvAXAH1 also hydrolysed debranched arabinan, which is consistent with the results of Lee *et al.* (2001) but not those of Ferré *et al.* (2000). The ability of these enzymes to hydrolyse (1,5)- $\alpha$ -linked arabinosyl raises the potential for participation in pectin depolymerization. Immunolocalization and gene expression studies have previously identified arabinan-containing pectins as potential *in vivo* substrates for a homologous GH51 hydrolase in *Arabidopsis* (Chavez-Montes *et al.*, 2008).

Analyses of the structures of the residual arabinoxylan substrates by oligosaccharide fingerprinting after incubation with the expressed enzymes (Fig. 8) showed that both could hydrolyse Araf from the polysaccharide and thereby provide more sites for endo-xylanase action. These results were consistent with those obtained by Lee *et al.* (2001) for the HvAXAH1 enzyme that they purified from germinated barley extracts and which could hydrolyse Araf substituents from wheat flour arabinoxylan. The purified barley enzyme rapidly hydrolysed Araf residues from the C(O)3 of backbone xylosyl residues, but could also hydrolyse Araf residues on doubly substituted xylosyl residues (Lee *et al.*, 2001).

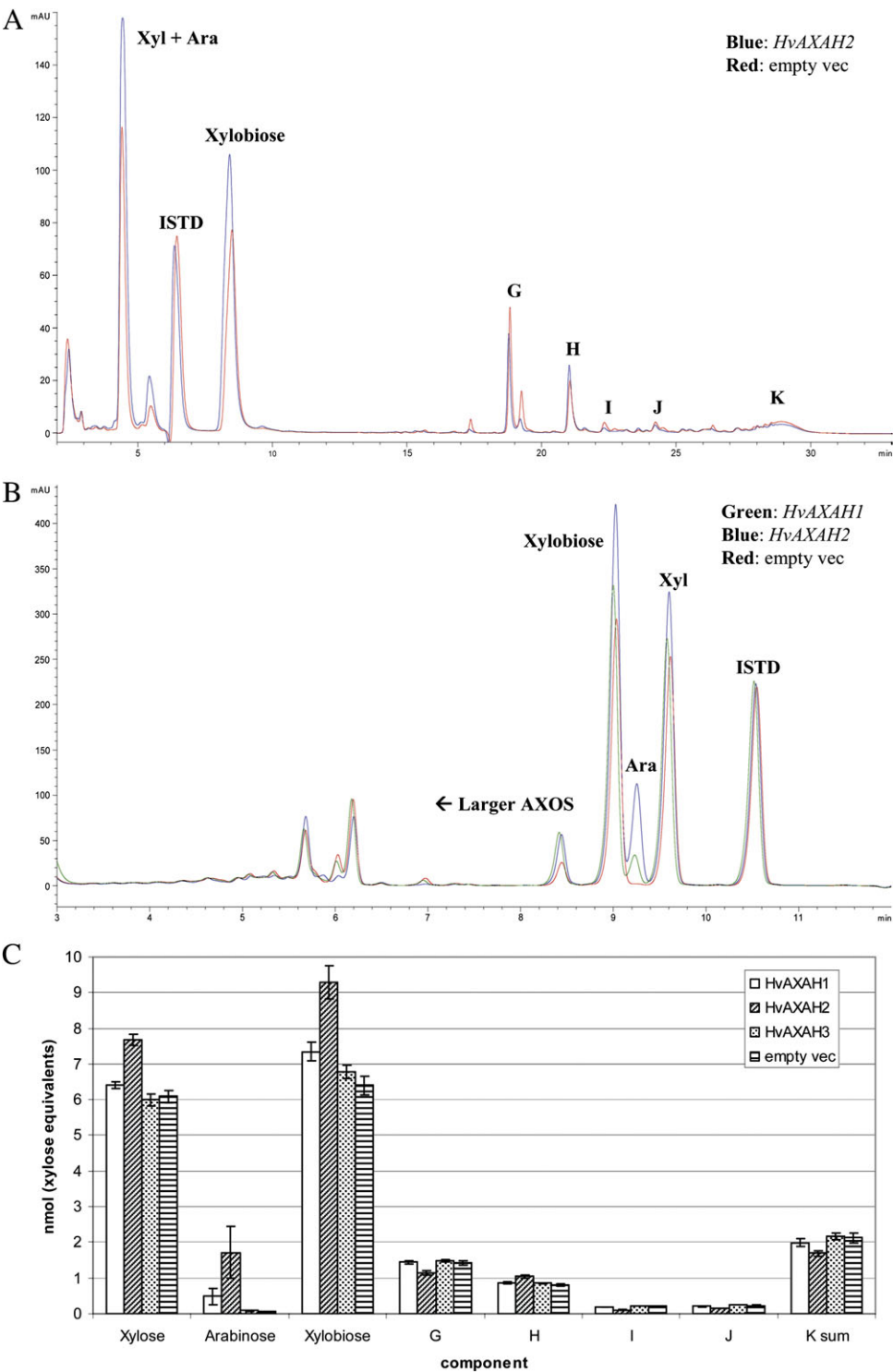
The expressed HvAXAH enzymes were not subjected to a comprehensive kinetic analysis here, mainly because meaningful kinetic parameters are difficult to obtain with polysaccharide substrates, where the products of the reaction are also substrates and the molarity of the substrate solution is very difficult to measure. A detailed kinetic analysis for purified barley HvAXAH1 has previously been undertaken by Lee *et al.* (2001). In addition, comparisons between kinetic parameters of an enzyme on small and large substrates that have vastly different diffusion rates must also be viewed with caution (Lee *et al.*, 2001). However, the comparisons of reaction conditions and rates of hydrolysis of the arabinoxylan substrate suggested that the proportion

of Araf released by the heterologously expressed HvAXAH enzymes here was lower than that expected from the kinetic analyses of Lee *et al.* (2001).

In relation to the question of whether or not the barley HvAXAH enzymes do indeed play a role in the removal of Araf residues from arabinoxylan chains, one would expect to observe spatial and temporal correlations between expression patterns of the *HvAXAH* genes and changes in arabinoxylan fine structure, as measured by Ara:Xyl ratios. While it must be emphasized that transcript abundance does not necessarily reflect the final level of an active gene product and that the turnover rates of different mRNAs will vary greatly, the transcription patterns of the five *HvAXAH* genes were nevertheless examined in the expectation that their transcripts would probably be relatively more abundant where Araf residues were being removed from nascent arabinoxylans. Gibeaut *et al.* (2005) monitored changes in Ara:Xyl ratios and hence arabinoxylan fine structure in developing barley coleoptiles, and Obel *et al.* (2002) observed in 7-day-old wheat seedlings an Ara:Xyl ratio gradient that ranged from high values at the base of the leaf, which is close to the meristem, to low values at the top of the leaf (Obel *et al.*, 2002). In the developing endosperm of wheat and barley grain, arabinoxylan deposition in the walls has been monitored (Philippe *et al.*, 2006b; Wilson *et al.*, 2006), and arabinoxylan fine structure has been shown to change as the grain matures (Philippe *et al.*, 2006a; Saulnier *et al.*, 2009; Toole *et al.*, 2010). The coleoptile and developing endosperm systems were therefore used to investigate any correlations between *HvAXAH* mRNA abundance and the removal of Araf residues from arabinoxylans of the cell wall.

Barley coleoptiles grown under the same conditions used by Gibeaut *et al.* (2005) increased in length for 5 d after





**Fig. 8.** HPLC AXOS analysis of *HvAXAH*-treated wheat AX. Peaks G, H, and I have been identified as Xyl5Ara, Xyl4Ara2, and Xyl5Ara2 respectively, based on mass spectroscopy and linkage analysis. (A) Example chromatogram of normal phase HPLC with samples *HvAXAH2* (blue) and empty vector (red). (B) Example chromatogram of reversed phase HPLC for monosaccharide quantification with samples *HvAXAH1* (green), *HvAXAH2* (blue), and empty vector (red). (C) Quantification of xylose, arabinose, and AXOS components (as xylose equivalents). Error bars indicate the SD of the mean. Analysis of samples treated with the residual protein extractions followed the same trends as the apoplastic fraction displayed here.

germination of the grain, and thereafter stopped elongating. Transcripts of the *HvAXAH2* gene increased transiently at 1 d after germination to be 80-fold more abundant than at 0.5 d. They then decreased to low levels (2- to 5-fold the

abundance at 0.5 d after germination). At 4 d after germination, transcript levels were 100-fold higher and remained >150-fold higher from 5 d to 7 d (Fig. 4B). Levels of mRNA for the other *HvAXAH* genes were low. The

appearance of high levels of *HvAXAH2* transcript after 4 d therefore coincides more closely with the cessation of coleoptile growth at about day 5, after which the coleoptile begins to shrivel and twist before ultimately senescing, rather than any correlation with the continuous decrease in xylose substitution observed across the entire 0 to 9 d time-course (Gibeaut *et al.*, 2005). It should be noted that the normalized *HvAXAH2* transcript levels observed after day 4 are actually very high and are comparable with or higher than those of the *HvCsIF* (Burton *et al.*, 2008) and *HvCesA* (Burton *et al.*, 2004) genes.

During the 7 d period of coleoptile growth examined in this work (Fig. 4B), arabinoxylan levels in the wall remain essentially constant at 25–30% (mol/mol), while the degree of substitution of xylosyl residues steadily declines from ~3:1 to ~0.9:1 over the same period (Gibeaut *et al.*, 2005). Thus, the pattern of *HvAXAH2* transcript accumulation in the developing coleoptiles did not match the changes in xylosyl substitution in the arabinoxylan. Given that the barley *HvAXAH* enzymes are also capable of hydrolysing (1,5)- $\alpha$ -L-arabinan, another function of the enzyme might be in the turnover or remodelling of this arabinan component of pectic polysaccharides. Gibeaut *et al.* (2005) showed that (1,5)- $\alpha$ -L-arabinan levels in walls from developing barley coleoptiles decreased sharply in the first 2 d after germination, while the pectic polysaccharides decreased steadily from ~25% (mol/mol) of the wall to ~2% (mol/mol) after 7 d.

In the case of developing barley endosperm, analyses of the transcript abundance of individual *HvAXAH* genes revealed that levels remained very low in comparison with those observed in the coleoptile and other tissues (Fig. 3, 4A). In early experiments in which extracts from whole developing grain were examined, very high levels of *HvAXAH3* transcripts were detected during early developmental stages (Supplementary Fig. S2 at JXB online). However, it was subsequently deduced that the very high levels of *HvAXAH3* mRNA in the whole grain samples were located predominantly in the maternally derived cell layers around the developing endosperm (Fig. 5), where arabinoxylans are found in the nucellar epidermis and cross cells (Philippe *et al.*, 2006b; Wilson *et al.*, 2006). The maternally derived tissue undergoes considerable cell expansion during cellularization (Radchuk *et al.*, 2011), so a role for AXAH in the loosening and expansion of these cell walls cannot be excluded. In addition, the cellularization period coincides with highly regulated programmed cell death of specific cell layers within the maternal tissues (Radchuk *et al.*, 2011), and a role for AXAHs in the depolymerization of these cell walls is also possible.

These observations are consistent with microarray data derived from isolated maternal tissues and from the developing caryopsis (Druka *et al.*, 2006; Sreenivasulu *et al.*, 2006), and emphasize the importance of dissecting out the starchy endosperm for transcript profiling of developing endosperm; data from whole grain extracts will almost certainly be complicated by the presence of the maternal tissues.

During starchy endosperm development in barley, arabinoxylans are first detected at ~8 DAP after pollination (Wilson *et al.*, 2006) and at a similar time in developing

wheat endosperm (Phillipe *et al.*, 2006a). Wilson *et al.* (2006) commented on the fact that the antibody they used to detect arabinoxylans did not recognize highly substituted arabinoxylan chains and suggested that a highly substituted arabinoxylan might have been present earlier than 8 DAP. They have subsequently shown that this is indeed the case and that arabinoxylans are present in walls of the developing endosperm at 6 DAP and possibly earlier (S.M. Wilson and A. Bacic, unpublished data). Against this background information, one might expect to observe significant levels of *HvAXAH* gene transcription during the early cellularization stage at 4–5 DAP and subsequently throughout endosperm development, if the encoded enzymes were involved in the removal of AraF residues from the backbone of the nascent arabinoxylan. However, this is not the case. Although one can again argue that transcript levels might not accurately reflect levels of active enzyme, the very low levels of *HvAXAH* mRNA in developing endosperm (Fig. 4A), which are nearly three orders of magnitude lower than those measured in the developing coleoptile (Fig. 4B), and the absence of any significant *HvAXAH* protein accumulation in isolated endosperm fractions (Fig. 5B), does not provide compelling evidence for a role for AXAH enzymes in the remodelling of the arabinoxylans in walls of the developing endosperm.

Other enzymes that have  $\alpha$ -L-arabinofuranosidase activity have been purified from germinated barley grain, including the family GH3 bifunctional  $\alpha$ -L-arabinofuranosidase/ $\beta$ -D-xylosidase designated ARA-1 by Lee *et al.* (2003). However, this enzyme released arabinofuranosyl residues from wheat arabinoxylan very slowly, compared with the purified barley *HvAXAH* (Lee *et al.*, 2003), and its role in the re-modelling of arabinoxylans remains to be demonstrated. Another possibility is that the changes in Ara:Xyl ratios in arabinoxylans can be attributed to progressive decreases in levels of arabinosylation of the nascent polysaccharide, which in turn might be controlled by synthase complexes containing xylan synthases, arabinosyl transferases, and other enzymes. Arabinoxylan fine structure might be regulated by relative activities of component enzymes of the complex or by the supply of nucleotide sugar substrates. For example, complementation of an *Arabidopsis* mutant, *mur4*, that is deficient in a functional UDP-D-xylose 4-epimerase by multiple copies of the functional gene led to increased incorporation of arabinosyl residues into the cell wall (Burget *et al.*, 2003).

In summary, there is strong evidence to suggest that arabinoxylans newly deposited into the cell walls of the Poaceae have higher levels of arabinofuranosyl substitution than those isolated from older walls, but the mechanisms by which these changes in fine structure are achieved remain unclear. However, there are continuing incentives to examine these mechanisms, not only from the point of view of the fundamental biology of cell wall synthesis, modification, and degradation, but also because the fine structures of wall arabinoxylans from the Poaceae are crucially important in the performance of cereal grains in such diverse applications as the physical and functional properties of wheat flour (Izydorczyk and Biliaderis, 1995; Courtin and Delcour,

2002; Saulnier *et al.*, 2007), the role of non-starchy wall polysaccharides as key components of soluble and total dietary fibre content of foods that are increasingly recognized for their beneficial effects on human health (Gebruers *et al.*, 2008; Collins *et al.*, 2010), and the conversion of arabinoxylans in plant biomass to fermentable sugars during biofuel production.

## Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Multiple alignment of putative wheat and barley AXAH proteins.

**Figure S2.** Transcript levels in developing caryopsis from 2 to 11 DAP.

**Figure S3.** Transcript levels of four barley *HvAXAH* genes extracted from the array experiment of Druka *et al.* (2006) in the PLEXdb.

**Table S1.** PCR primers used.

**Table S2.** Sequence identity and similarity of the predicted AXAH proteins of barley and wheat.

## Acknowledgements

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