The Genetics, Transcriptional Profiles and Catalytic Properties of the UDP-Arabinose Mutase Family from Barley

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ABSTRACT

Four members of the UDP-Ara mutase (UAM) gene family from barley have been isolated and characterized, and their map positions on chromosomes 2H, 3H and 4H have been defined. When the genes are expressed in *Escherichia coli* the corresponding HvUAM1, HvUAM2 and HvUAM3 proteins exhibit UAM activity and the kinetic properties of the enzymes have been determined, including \( K_m \), \( K_{cat} \) and catalytic efficiencies. However, the expressed HvUAM4 protein shows no mutase activity against UDP-Ara or against a broad range of other nucleotide-sugars and related molecules. The enzymic data indicate therefore that the HvUAM4 protein may not be a mutase. However, the *HvUAM4* gene is transcribed at high levels in all the barley tissues examined and its transcript abundance is correlated with transcript levels for other genes involved in cell wall biosynthesis. The UDP-L-Arap → UDP-L-Araf reaction, which is essential for the generation of the UDP-Araf substrate for arabinoxylan, arabinogalactan-protein and pectic polysaccharide biosynthesis, is thermodynamically unfavorable and has an equilibrium constant of 0.02. Nevertheless, the incorporation of Araf residues into nascent polysaccharides clearly occurs at biologically appropriate rates. The characterization of the *HvUAM* genes opens the way for the manipulation of both the amounts and fine structures of heteroxylans in cereals, grasses and other crop plants, with a view to enhancing their value in human health and nutrition, and in renewable biofuel production.
Grasses, including barley and other cereals, belong to the most ecologically dominant plant family (Poaceae) on the planet, and are estimated to cover more than 20% of the Earth’s land surface. One distinguishing feature of the Poaceae is that their cell walls differ significantly from those in other families of the angiosperms. In most dicotyledonous and in many monocotyledonous plants, the major constituents of primary cell walls are cellulose, pectic polysaccharides and xyloglucans, with smaller amounts of heteroxylans and heteromannans. In contrast, heteroxylans appear to be the predominant non-cellulosic polysaccharides of primary walls of the Poaceae, while levels of xyloglucans and pectin polysaccharides are generally much lower. In addition, fucosylated xyloglucans are generally absent from walls of the grasses, which often, but not always, contain (1,3;1,4)-β-glucans. Thus, (1,3;1,4)-β-glucans are widely distributed in primary walls of the Poaceae but much less so in other angiosperms, lower plants and fungi. Primary cell wall compositions in vegetative organs of the grasses are exemplified in 4 day-old barley coleoptiles, which consist of about 35% cellulose, 30% heteroxylan, 10% (1,3;1,4)-β-glucan, 10% xyloglucan, and 10% pectin on a weight basis. In a more specific and commercially important example, walls of the starchy endosperm of mature barley grain consist of about 70% (1,3;1,4)-β-glucan and 20% heteroxylan, with low levels of cellulose and heteromannans, while wheat and barley aleurone walls, together with the starchy endosperm cells of wheat, contain up to 85% heteroxylan and lower amounts of (1,3;1,4)-β-glucan. In both the aleurone and starchy endosperm of these important cereals, the heteroxylans are found predominantly as arabinoxylans with only low levels of glucuronyl and other substituents.

The arabinoxylans of cereal grains and other grasses consist of a backbone of (1,4)-linked β-d-xylopyranosyl residues (Xylp) that are substituted at the C(O)3, C(O)2 and sometimes at both positions, with α-L-arabinofuranosyl residues (L-Araf). The furanosyl form of L-
arabinosyl residues is found in most land plants rather than the thermodynamically more stable pyranosyl form (L-Arap)\(^{15}\). The L-Araf form is also found in arabinogalactan-proteins, which are abundant in cereal grains and in pectic Type II arabinogalactans, including those reported in maize coleoptiles and, at lower abundance, in rice endosperm walls\(^{16-19}\).

During the biosynthesis of arabinoxylans and the other Ara\(^f\)-containing polysaccharides, the activated donor molecule for arabinosyl transfer to the backbone polysaccharide is assumed to be uridine diphospho-L-arabinofuranose (UDP-L-Araf)\(^{20,21}\). Thus, UDP-L-Araf is an important building block precursor for arabinoxylan biosynthesis and is derived from a pool of nucleotide-sugars in plant cells\(^{22-25}\). The precursor nucleotide-sugar in this pool is considered to be UDP-D-glucopyranose (UDP-D-Glc), which is the activated sugar donor for the biosynthesis of cellulose, (1,3;1,4)-β-glucans, xyloglucans and other glycans that contain glucose. The UDP-D-Glc can be epimerized at C4 of the sugar residue to form UDP-D-Gal, which is used in galactan and other biosynthetic pathways. The UDP-D-Glc can also be oxidised by UDP-D-Glc dehydrogenase to form UDP-D-glucurionate (UDP-D-GlcA), which can be used as a sugar donor for the biosynthesis of glucuronorhabinoxylans\(^{22,23}\). The UDP-D-GlcA can also be epimerized to form UDP-D-GalA for pectic polysaccharide biosynthesis.

A key reaction in these nucleotide-sugar interconversions that commits carbon to the synthesis of pentose-containing polysaccharides is catalysed by UDP-D-GlcA decarboxylase, also known as UDP-D-xylose synthase (UXS)\(^{26}\). This enzyme forms UDP-D-Xyl through the essentially non-reversible oxidative decarboxylation of UDP-D-GlcA. The UDP-D-Xyl acts not only as the xylopyranosyl donor for arabinoxylan biosynthesis, but also as a substrate for UDP-xylose 4-epimerase (UXE) for the biosynthesis of UDP-L-Arap\(^{27}\). Although UDP-L-Arap can be detected in plant tissues, the furanosyl form UDP-L-Araf is much less abundant and the identity
of the enzyme or enzymes responsible for UDP-\(L\)-Araf formation from UDP-\(L\)-Arap remained unknown until the pioneering work of Konishi et al., who purified a UDP-\(L\)-arabinose mutase (UAM) from rice seedlings and showed that the enzyme reversibly catalyzes the conversion of UDP-\(L\)-Arap to UDP-\(L\)-Araf\(^{28}\). At the same time it was discovered that the sequence of the UAM enzyme matched orthologous genes and proteins that had been designated earlier as reversibly glycosylated polypeptides (RGP)s, on the basis that these proteins became glycosylated in the presence of UDP-\(d\)-Glc, UDP-\(d\)-Xyl, UDP-\(d\)-Gal and UDP-\(L\)-Ara\(^{29-31}\). It should be noted that the reaction catalyzed by UAM involves several steps, which presumably include cleavage of the arabinosyl residue from the UDP-Ara substrate, opening of the pyranosyl or furanosyl ring, reformation of the alternative ring form, and reconnection of the altered arabinosyl residue to the UDP molecule. However, the mechanism remains speculative and at this stage is not supported by any biochemical evidence.

Here, we have used the barley (\textit{Hordeum vulgare}) genome reference sequence to define the number of \textit{UAM} genes in barley and to locate their positions on the barley genome\(^3^2\). Transcription profiles of the barley \textit{HvUAM} gene family members have been examined and can be related to the compositions of walls in various tissues and to the co-expression of other cell-wall related genes during barley growth and development. Four \textit{HvUAM} cDNAs have been cloned and expressed in \textit{Escherichia coli} not only for the confirmation of mutase activity, but also to define the substrate specificities of the enzymes, their enzymic properties and requirements for co-factors. Significant differences were observed in the activities, enzymic properties and specificities of the HvUAM enzymes.
EXPERIMENTAL PROCEDURES

Materials. All chemicals were reagent-grade and purchased from Sigma Aldrich and Carbosyn. UDP-Araf and UDP-Arap were obtained from the Complex Carbohydrate Research Centre (University of Georgia, Athens, GA, USA) and the Peptide Institute (Ibaraki-Shi, Osaka, Japan). Oligonucleotide primers were obtained from Geneworks (Thebarton, SA, Australia) and the site-directed mutagenesis kit was obtained from Stratagene (La Jolla, California, USA). The vectors and competent E. coli cells were obtained from Life Technologies (Carlsbad, California, USA). Barley (Hordeum vulgare) plants were grown in a glasshouse in the Australian Plant Phenomics Facility, at a maximum daytime temperature of 23°C and a minimum overnight temperature of 15°C. For transcript studies, spikelets were hand pollinated and tissues harvested for RNA isolation as described previously.\textsuperscript{32}

Amplification, Cloning and Heterologous Expression of \textit{HvUAM} cDNAs. Analysis of the barley genome sequence showed that the \textit{HvUAM} gene family contained four members. Total RNA extractions and cDNA synthesis were performed as described previously\textsuperscript{32}. The PCR primers (Table 1) were used to amplify fragments containing the full-length open reading frames from cDNA prepared from young barley shoots.

The cDNA fragments were inserted into the pCR8 vector by TA cloning (Invitrogen, Life Technologies) and the resulting constructs were transformed into One Shot Top10® chemically competent \textit{E. coli} cells (Invitrogen, Life Technologies). The pCR8-HvUAM plasmids were prepared using the Isolate II Plasmid Mini Kit (Bioline, London, UK) and their sequences and orientations verified at the AGRF (Adelaide, SA). Correct \textit{HvUAM} cDNAs were inserted into the pDEST17 vector from purified plasmids using the LR reaction catalyzed by LR Clonase II, resulting in HvUAM-pDEST17 constructs carrying the His6 tag coding sequence and ampicillin
The final constructs were transformed into BL21 cells (Invitrogen) by heat shock treatment and were grown in LB broth and 50 µg/ml ampicillin at 37 °C until the absorbance at 600 nm was 0.5-0.6, after which IPTG was added to a final concentration of 1 mM. The cells were grown for 16 h at 23°C before harvesting by centrifugation at 10,000 rpm. The cell pellet was re-suspended in X-tractor (Clontech, Mountain View, California, USA) at 4 °C for 1 h, and the soluble fraction was applied to a TALON metal affinity resin (Clontech) and eluted with 200 mM imidazole as described previously. The eluted material was desalted by buffer exchange using an Amicon centrifuge cartridge, and the protein concentration was measured using a colorimetric assay.

Site-directed Mutagenesis. Targeted mutations were introduced in HvUAM1 and HvUAM4 cDNAs using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA). Primer pairs were designed following the manufacturer’s guidelines (Table 2), and the mutagenesis reactions were carried out following the manufacturer’s instructions. Plasmids carrying putative mutations were sequenced to confirm the presence of changes at the AGRF (Adelaide, SA), verified HvUAM mutants were heterologously expressed in E. coli and the proteins were purified as described above.

Enzyme Activity Assay. The activities of the four expressed HvUAM enzymes were examined in different buffers and at various pH values, in the presence or absence of EDTA, and with or without bivalent cations such as Mn²⁺ or Mg²⁺ (5 mM, 15 mM and 50 mM). Substrate specificities of the HvUAMs were determined by incubating the enzymes with the following substrates: UDP-L-Araf, UDP-L-Arap, UDP-D-GalpNAc, UDP-D-GlcNAc, UDP-D-Galp, UDP-D-Glep, UDP-D-Xylp, UDP-D-GalpA, UDP-D-GlepA at a final concentration of 0.1 mM. All reaction products were monitored by chromatography using an Agilent 1260 HPLC equipped
with a UV detector at 262 nm. Separation was performed on a Kinetex 2.6u XB-C18 column (100Å, 100 x 3 mm; Phenomenex, Torrance, CA) at a flow rate of 0.6 ml min⁻¹ using isocratic elution with aqueous 50 mM triethylammonium acetate (TEAA).

The substrate specificities of HvUAM4 were further tested with GDP-L-Galp and α-L-Gal-1-phosphate, and reactions were monitored using an Agilent 1260 HPLC equipped with an Alltech ELSD800 detector (70°C, N₂ 3 bar) and UV detector at 262 nm. Separation was performed on a Hypercarb column (5 um, 100 x 4.6 mm) at a flow rate of 1 ml min⁻¹ using 50 mM ammonium acetate (Eluent A) and 90% acetonitrile (eluent B) at linear gradient of 0-80% B over 10 min.

The kinetic parameters of the HvUAM enzymes were determined from the rate of conversion of substrate (UDP-L-Araf or UDP-L-Arap) under optimum conditions. For conversion of UDP-L-Araf to UDP-L-Arap, the HvUAMs were incubated for 15 min at 37°C with different concentrations of UDP-L-Araf in 20 mM sodium phosphate buffer, pH 7 containing 5 mM MnCl₂, with continuous stirring. The reactions were terminated by the addition of 100% ethanol. Peak areas were converted to moles of product formed and data were fitted using non-linear regression to the Michaelis-Menten equation to calculate $K_m$, $V_{max}$ and $K_{cat}$. Similar conditions were used for the UDP-L-Arap to UDP-L-Araf reaction, except that the reaction mixture was buffered to pH 5.6.

Inhibition assays were performed in the presence of UDP-ᴅ-ᴅ-GalNAc, UDP-ᴅ-ᴅ-GlcNAc, UDP-ᴅ-ᴅ-Glc, UDP-ᴅ-Xyl, UDP, UMP, NAD⁺, NADPH, FAD, EDTA (0.1 mM final concentrations). To release any non-covalently bound co-factors, 1 μg expressed enzyme was extracted with 80% (v/v) ethanol and characterized as described previously⁶. 
Nuclear Magnetic Resonance. $^1$H-NMR spectra of isolated UDP-L-Araf and UDP-L-Arap were recorded in deuterated oxide at a frequency of 600 MHz, using an Agilent NMR spectrometer equipped with a cryoprobe.

MS/MS Peptide Sequencing. The reversible-glycosylation activity of the HvUAM1 and HvUAM4 enzymes was assessed by LC-MS/MS after incubation of the enzymes with UDP-Glc.$^{30}$ Reaction mixtures were prepared in 20 mM sodium phosphate buffer (pH 7) with or without 5 mM MnCl$_2$ and contained 5 µg mutase and 10 mM UDP-Glc. After 30 min at 37°C under continuous stirring, proteins were separated on SDS-PAGE gels, excised and subjected to trypsin digestion as described by.$^{34}$ The tryptic peptides were dissolved in 2% (v/v) acetonitrile and 0.1% (v/v) formic acid, separated on a C18 column and analyzed using a 6550 Series quadrupole time-of-flight mass spectrometer (Agilent) fitted with a 1100 series nano pump (Agilent), using a gradient from 2% to 35% buffer B in 17 min and from 35% to 90% buffer B in 3 min. The MS spectra collected from the six most abundant ions were subjected to MS/MS from doubly and higher charge states.$^{34}$ Agilent D files were exported to Mascot generic files using the MassHunter (vB.06.00) software package from Agilent. Mascot generic files peak lists were searched against a concatenated list of protein sequences released for barley (ftp://ftp.ncbi.nlm.nih.gov/pub/Genomes/PlantGDB/barley_public_data/) and the HvUAM1 and HvUAM4 protein sequences using Mascot 2.3.

Phylogeny of HvUAM Genes. Sequences were aligned using MUSCLE within Geneious 8.1.3 (Biomatters Ltd, Auckland, New Zealand). The phylogenetic tree was produced from the Geneious tree builder module of the Geneious Pro 8.1.3 software package using the nearest neighbor joining method with 1000 replicates to obtain bootstrap values. Clade branches were labeled with % consensus support. The phylogram was constructed using the RAxML plugin.$^{35}$
The genetic map of the *HvUAM* gene family and the position of gene orthologues in the *Brachypodium* and rice genomes was drawn using Strudel software\(^{36}\).

**Quantitative PCR Analysis of Transcript Levels.** Total RNA preparations from developing endosperms, leaves, roots, coleoptiles, and other tissue types were extracted as described by Zhang et al.\(^{27}\) and converted to cDNA as described by Burton et al.\(^{32}\). Levels of barley *HvUAM* mRNA were determined by quantitative Q-PCR in a RG 2000 Rotor-Gene Real Time Thermal Cycler (Sydney, Australia) according to Zhang et al.\(^{27}\). Primer pairs for four members of the HvUAM gene family were designed and are listed in Table 3. Four genes, namely glyceraldehyde-3-phosphate dehydrogenase, heat shock protein 70, α-tubulin and cyclophilin, were used as controls to normalize the data and primer sequences were as reported previously\(^{26}\).

**RNASeq.** The publicly available barley transcriptomic data set was downloaded from the sequence read archive of the DNA Data bank of Japan (http://trace.ddbj.nig.ac.jp/DRASearch/). This dataset, associated with the barley MLOC sequences (ERP001600), represented eight barley tissues. The barley genome (Hordeum_vulgare.030312v2.18.dna_rm.toplevel.fa) was downloaded from the Gramene website (http://www.gramene.org/). This information was uploaded into the CLC Genomics workbench (CLC Bio) as a reference genome. The RNA-Seq data was mapped to the genome using the CLC genomics workbench RNA-Seq analysis tool and expression values were calculated in RPKM and converted to % maximum expression from all tissues. Expression data for every gene from replicate tissue samples were averaged.

**Accession Numbers.** The DNA sequences of the four UAM cDNAs have been deposited with the GenBank under accession numbers KT277671, KT227672, KT227673, and KT277674.
RESULTS

Barley HvUAM Gene Family and Chromosomal Locations. Four HvUAM sequences were identified in the barley genome database.\textsuperscript{37} PCR amplification from a cDNA preparation from barley shoots, using primer sets designed from the consensus sequences, generated four individual cDNAs. These cDNAs were 1113bp, 1083bp, 1068bp, and 1044bp in length and were designated as \textit{HvUAM1}, \textit{HvUAM2}, \textit{HvUAM3}, and \textit{HvUAM4}, respectively. The \textit{HvUAM1} gene is located on chromosome 4H at 45 centimorgan (cM) (Supplemental Figure S1), while the \textit{HvUAM2} and \textit{HvUAM4} genes map to the long arm of chromosome 2H at 55.2 cM and 114.4 cM, respectively. The \textit{HvUAM3} gene mapped to chromosome 3H at 63.9 cM. We have also defined the map positions of the \textit{UAM} orthologues in \textit{Brachypodium}\textsuperscript{38} and rice\textsuperscript{39}, using the Strudel program.\textsuperscript{36} The genes mapped to syntenic positions in the \textit{Brachypodium} and rice genomes, although no orthologous gene for \textit{HvUAM3} could be detected in the rice genome (Supplemental Figure S2).

Predicted Properties of Proteins Encoded by HvUAM Genes. The amino acid sequences of the proteins encoded by the four barley \textit{HvUAM} genes are aligned in Figure 1 and their sequence identities are summarized in Table 4. The mutases encoded by the \textit{HvUAM1}, \textit{HvUAM2} and \textit{HvUAM3} genes have relatively high sequence identities (83-86\%), while the \textit{HvUAM4} encoded protein is only about 45\% identical to the other proteins at the amino acid level (Table 4). An unrooted, radial phylogenetic tree of the \textit{UAM} gene families in barley, \textit{Brachypodium}, rice and sorghum show clear separation of four \textit{HvUAM} gene clades (Figure 2). Although there is no three-dimensional structure available for plant UAM proteins, we performed a secondary structure analysis using PSIPRED, MESTAT for transmembrane topology prediction and ENSATSVM for transmembrane helix prediction\textsuperscript{40,41} (Supplemental
Figure S3) in order to predict whether the enzymes were likely to be cytosolic or membrane-bound. These simulations suggested that all the barley mutases have a COOH-terminal transmembrane helix between amino acid residues 270 and 350. Putative transmembrane helices were also predicted in the region of amino acid residues 240-260 for HvUAM1, HvUAM2 and HvUAM3 (Supplemental Figure S3). In addition, the ENSATSV program predicted a putative pore-lining helix domain typical of membrane transporters in the HvUAM4 enzyme between amino acid residues 193 and 208. Nevertheless, we acknowledge that the predictive programs used can give different results and note that the UAM proteins isolated from rice were cytosolic\textsuperscript{28} and that the expressed proteins here were soluble. It therefore remains unclear whether or not the four barley UAM proteins are membrane-bound.

**Biochemical Characterization of Heterologously Expressed HvUAM Enzymes.** To confirm the enzymic action of the barley HvUAM proteins, cDNAs for the *HvUAM1, HvUAM2, HvUAM3*, and *HvUAM4* genes were cloned into the pDEST17 vector and expressed heterologously in *E. coli*. The expressed proteins were tagged with poly(His)\textsubscript{6} at their NH\textsubscript{2}-termini and purified by affinity chromatography on a cobalt column. The activities of the purified recombinant barley proteins (HvUAM1-4) were tested with a series of commercially available nucleotide-sugar substrates, including UDP-\textit{L}-Arap and UDP-\textit{L}-Araf.

No activity was detectable for the HvUAM4 enzyme, but the other three HvUAMs possessed UDP-\textit{L}-Ara mutase activity, interconverting UDP-\textit{L}-Araf to UDP-\textit{L}-Arap and UDP-\textit{L}-Arap to UDP-\textit{L}-Araf, at pH optima of 7.0 and 5.6, respectively (data not shown). In preliminary experiments it was shown that only small amounts of UDP-\textit{L}-Araf were generated from UDP-\textit{L}-Arap. This indicated that the equilibrium of the reaction lies towards UDP-\textit{L}-Arap, as previously reported, and UDP-\textit{L}-Araf was therefore used as the preferred substrate for enzymic analyses.\textsuperscript{42}
The activities of the three enzymes were stable following repeated cycles of freezing and thawing.

To confirm the purity of the substrates and the conformational structures of both substrates and products, the compounds present in the reaction mixtures were separated by HPLC and collected. The structures of the nucleotide-sugars were characterized by LC-MS, $^1$H-NMR and HPLC (Supplemental Figure S4). The results clearly indicated that the interconverting substrates and products were indeed UDP-L-Arap and UDP-L-Araf. Minor impurities could be detected and were likely to be uridine monophosphate (UMP) and arabinosyl-1 phosphate released by Mn$^{2+}$-induced hydrolysis of the nucleotide-sugars$^{43}$. It is relevant to note that the rate of acid hydrolysis of glycosidic linkages adjacent to furanosyl residues is much higher than the rate of hydrolysis of linkages involving pyranosyl residues.$^{44}$ It was shown here that treatment with 50 mM Mn$^{2+}$ resulted in complete hydrolysis of UDP-L-Araf within a minute at room temperature and the presence of UMP in the reaction mixture was confirmed by HPLC (data not shown). This was consistent with the data reported by Nunez and Barker$^{43}$. Similarly, even when reactions were stopped by heating to 100°C in water, moderate hydrolysis of UDP-Araf was observed.

Activities of the three HvUAM enzymes were dependent on the presence of Mn$^{2+}$ ions. When the metal chelating agent 1 mM ethylenediaminetetraacetic acid (EDTA) was added to the reactions mixtures, there was a 90% reduction in the formation of UDP-L-Arap in the UDP-L-Araf→UDP-L-Arap reaction direction (Table 5) and complete inhibition of product formation in the UDP-L-Arap→UDP-L-Araf reaction direction. Thus, Mn$^{2+}$ ions are required for barley UAM activity and the low levels of activity in some reactions without added Mn$^{2+}$ is probably attributable to the presence of bound Mn$^{2+}$ on the purified enzyme or from low amounts of Mn$^{2+}$.
arising from the heterologous expression medium and subsequent purification steps. Other divalent cations, specifically Mg$^{2+}$, also increase the catalytic efficiency of the reaction (Table 6).

The addition of the potential co-enzymes nicotinamide adenine dinucleotide and flavin adenine dinucleotide in their oxidized forms showed no measurable stimulatory or inhibitory effects on the activity of HvUAM1 (Table 5). However, it has been shown for other nucleotide-sugar interconverting enzymes, including heterologously expressed barley epimerases and UXS$^{26,27}$, that the purified enzymes contain pre-bound NAD$^+$ or FAD. These pre-bound cofactors can be released by 80% (v/v) ethanol, but no NAD$^+$ or FAD was liberated from the HvUAM1 enzyme treated in this way.

As shown in Table 5, addition of UDP-GlcNAc, UDP-GalpNAc, or UDP-Xylp decreases HvUAM1 activity, while increases in activity were observed with UDP-Glc, NAD, NADP and UDP. At this stage we are unable to explain the mechanistic basis for these observations. Konishi et al. reported that a combination of different rice OsUAMs can enhance mutase activity by 1.3 to 2.1 fold$^{31}$, but we did not observe any changes in activity when the recombinant barley HvUAM mutases were combined in the reaction mixtures.

**Kinetic Properties of Expressed Barley HvUAM Enzymes.** Under optimal conditions at pH 7, the barley HvUAM1 had an apparent $K_m$ of 41.8 µM with a $V_{\text{max}}$ of 1.14 nmol min$^{-1}$ and a $K_{\text{cat}}$ of 4,009.6 min$^{-1}$, using UDP-L-Araf as the substrate (Table 6; for Lineweaver-Burke plot see Supplemental Figure S5). Values for HvUAM2 ($K_m = 16.6$ µM, $K_{\text{cat}} = 263.4$ min$^{-1}$) and HvUAM3 ($K_m = 4.4$ µM, $K_{\text{cat}} = 568.0$ min$^{-1}$) indicated that these enzymes had slower turnover rates when compared with HvUAM1.

When UDP-L-Arap was used as substrate, the reaction mixture was adjusted to pH 5.6 to achieve optimal activity. As mentioned above, use of UDP-L-Arap as substrate resulted in
almost an order of magnitude reduction in catalytic potency for all three active HvUAMs, compared with using UDP-L-Araf as a substrate. Thus, the HvUAM1 showed a 18-fold reduction in its $K_{\text{cat}}$ value when UDP-L-Arap ($K_{\text{cat}} 213.6 \text{ min}^{-1}$) was used as a substrate instead of UDP-L-Araf ($K_{\text{cat}} 4,009.6 \text{ min}^{-1}$) (Table 6).

Catalytic efficiencies ($K_{\text{cat}}/K_m$) were correspondingly lower when UDP-L-Arap was used as the substrate and showed that the least efficient enzyme among the three was HvUAM2. In addition, both Mg$^{2+}$ and Mn$^{2+}$ ions increased the UDP-L-Araf to UDP-L-Arap conversion efficiency by 3- to 33-fold, respectively (Table 6).

These kinetic data allowed us to calculate the equilibrium constant ($K_{\text{eq}}$) of the reaction. The value for $K_{\text{eq}}$ calculated from the Haldane equation [$K_{\text{eq}} = [P][S] = (K_{\text{cat}}/K_m)_A/(K_{\text{cat}}/K_m)_B$] for each of the three barley UAM enzymes was $K_{\text{eq}} = 0.02$. This was consistent with the equilibrium of the reaction lying strongly towards UDP-L-Arap.

**Amino Acid Residues Involved in Catalysis.** Several Arg residues are highly conserved in the HvUAM proteins and a mutagenic analysis of the UDP-galactose mutase (UGM) from *Klebsiella pneumonia* suggested that the guanidinium functional group of an arginine residue in the active site could interact with the pyrophosphoryl moiety of the nucleotide-sugar substrate$^{45}$. Three Arg mutants from HvUAM1 were therefore constructed, expressed and purified, and their activities compared (Table 7). A complete loss of activity was observed in the R158A and R165A mutants, but the R193A mutant remained active.

Next, the tri-aspartic acid DDD motif, which is conserved in all three barley HvUAMs and in their orthologues from other species, was examined. The DDD motif has been implicated in the coordination of Mn$^{2+}$ with the phosphate group of the nucleotide donor$^{46}$. The HvUAM1 D112A mutant, in which the motif was altered to DDA, showed no activity. The DDD motif
was changed to DDN in the inactive HvUAM4 enzyme, but restoring the DDD motif in HvUAM4 via a N100D mutation did not result in the restoration of UAM activity by this enzyme.

**Glycosylation of Arg Residues.** Earlier reports suggested that the glycosylation of R158 in the rice enzyme OsUAM1 played a critical role in catalytic activity. Thus, we compared arginine glycosylation in the active HvUAM1 and inactive HvUAM4 enzymes. Based on MS/MS of tryptic digests of the enzymes after incubation with UDP-Glc, a glycosylated tryptic peptide containing R158 was observed in the HvUAM1 digest, indicating that the HvUAM1 is subject to reverse glycosylation. Conversely, no glycosylated tryptic peptides were detected in the HvUAM4 digests (Supplemental Figure S6).

**Transcription Profiles of HvUAM Genes.** Using gene-specific oligonucleotide primers (Table 3), Q-PCR was used to define mRNA abundance of individual HvUAM genes in various barley tissues (Figure 3). Transcripts for the HvUAM1 and HvUAM4 genes were detected in all of the tissues examined, but levels were highest in root tips and embryo 22 days after pollination (DAP) (Figure 3). Transcript levels of the HvUAM4 gene were highest of the four genes in most tissues examined. In contrast, the HvUAM2 transcripts were detected almost exclusively in roots, leaf base, anthers and developing endosperm, and in much lower abundance compared with HvUAM1 and HvUAM4 transcripts. The HvUAM3 transcripts were found at moderate levels in developing root tips and anthers, but at basal levels in most of the other tissues examined (Figure 3).

In the developing barley endosperm from 6-38 DAP, the HvUAM4 transcript abundance peaked at 8 DAP before dropping back to lower levels at DAP12 and thereafter remaining approximately constant (Figure 4). Transcripts of the HvUAM2 gene were the highest of the four
HvUAM genes in developing grain from 12-38 DAP. Thus, at 24 DAP, the HvUAM2 mRNA levels were 2-fold higher than HvUAM4 transcripts and 5-fold higher than HvUAM1 transcripts. In contrast, HvUAM1 transcripts remained at low but detectable levels for the duration of grain development and HvUAM3 transcripts could not be detected at any stage (Figure 4).

Co-expression studies by Q-PCR of the HvUAM genes in 16 tissue types against a subset of 200 cell wall-related genes showed that the HvUAM1 and HvUAM4 genes were co-transcribed with Pearson correlation coefficients greater than 0.95 (Figure 5). In addition, HvUAM3, expansin and cellulase transcripts correlated with a coefficient over 0.95 (Figure 5). No high level correlations between HvUAM2 transcript abundance and the 200 cell wall-related genes were detected.

To further examine correlations of transcript levels, publicly available RNA-seq data sets were used to investigate co-expression profiles against over 26,000 barley genes. The RNA-seq data from eight barley tissues revealed significant correlations between HvUAM2 and five other genes, two of which were genes that mediate the depolymerisation of pectic polysaccharides, namely polygalacturonase GH28 (MLOC_67885) and pectin esterase (MLOC_69251) (Figure 6). In addition, transcripts of two GDP-mannose pyrophosphorylase genes were correlated with the abundance of HvUAM2 transcripts, as was a family GT61 glycosyltransferase gene that could be a xylan (1,3)-α-arabinofuranosyltransferase.

The RNA-seq data confirmed the strong correlation between HvUAM1 and HvUAM4 transcripts and with mRNAs encoding membrane associated proteins (Figure 6). The latter included the MLOC_13546 and MLOC_54627 genes. Pfam analysis of MLOC_13546 suggested that this gene encodes a putative barley membrane permease that could be involved in amino acid transport. Orthologues of these two genes in Brachypodium and rice are also co-expressed.
with UAMs (Supplemental Figure S7). The other membrane-associated protein encoded by the MLOC_54627 gene is classified as a putative adaptor protein that might be involved in protein complexes during clathrin-coated pit and vesicle formation.

Additional co-expression partner genes were also found for HvUAM1, including two family GT34 glycosyltransferases (MLOC_25125 and MLOC_7517; Pfam family of galactosyltransferases) and MLOC_52073 (Pfam family ε-subunit protein involved in coatamer complexes). The GT34 gene product also comprises a characteristic DXD domain and belongs to the GT-A family.\footnote{47}
DISCUSSION

The four members of the barley HvUAM gene family have been cloned, sequenced and expressed in a heterologous system in the work described here. Similar small UAM gene families have been reported in other plant species, including Arabidopsis$^{48}$ and rice$^{28}$. In species of the Poaceae for which reference genome sequences are available, barley, sorghum (Sorghum bicolor) and Brachypodium also have four UAM genes members, while rice (Oryza sativa) has three genes and is missing the HvUAM3 orthologue (Figure 2). The HvUAM genes map to barley chromosomes 2H, 3H and 4H and their orthologues in other grass species are located in syntenic positions on their respective genomes (Supplemental Figure S2).

Within the barley HvUAM family, the HvUAM1, HvUAM2 and HvUAM3 proteins share more than 80% amino acid sequence identity, while the sequence identity of the HvUAM4 protein is much lower, at around 45% (Table 1). When cDNAs encoding the four barley enzymes are expressed in E. coli, the HvUAM1, HvUAM2 and HvUAM3 proteins actively interconvert UDP-L-Araf and UDP-L-Arap. However, the expressed HvUAM4 protein has no activity against UDP-L-Ara or a series of seven other nucleotide-sugar or related nucleotides that were tested. It has been reported that combining different rice OsUAMs can enhance mutase activity$^{31}$, but we did not observe any changes in activity when the recombinant barley HvUAM mutases were combined in the reaction mixtures (data not shown) and we could find no evidence for the formation of multi-enzyme complexes. Similarly, combinations of different Arabidopsis RGP proteins did not enhance enzymic activity$^{48}$.

A UDP-Galp mutase (UGM) from Mycobacterium tuberculosis$^{49}$ is a flavoenzyme that catalyzes the interconversion of UDP-d-Galp and UDP-d-Galf via a flavin-derived iminium ion. However, neither FAD or NAD$^+$ are required for HvUAM activity and no bound FAD or NAD$^+$
could be detected on the expressed barley HvUAM proteins, which is consistent with reports for rice and *Arabidopsis* UAMs\textsuperscript{28,48} but contrasts with the presence of bound NAD\textsuperscript{+} on barley UDP-\(\text{d-}\)glucuronate decarboxylases and UDP-\(\text{d-}\)Xylp epimerases\textsuperscript{26,27}.

The HvUAM1, HvUAM2 and HvUAM3 proteins have a conserved DDD divalent cation-binding motif, which is a distinctive structural feature in glycosyltransferases within the GT-A fold family\textsuperscript{50}. The DDD motif is absent in the HvUAM4 protein, which has a DDN sequence at the corresponding position (Figure 1). However, site-directed mutation of DDN to DDD in the HvUAM4 protein did not result in UAM activity in this protein. Indirect evidence suggests that the DDD motif might be involved in the co-ordination of Mn\textsuperscript{2+} with phosphoryl groups of the nucleotide-sugar substrate\textsuperscript{29,48,51}. This is supported by our data, because the D112A mutant of HvUAM1 has no activity and our Mn\textsuperscript{2+} depletion experiments show that Mn\textsuperscript{2+} is essential for activity.

Conserved Arg residues in UAMs have also been proposed to be involved in catalysis\textsuperscript{31,45} and this was confirmed for the barley HvUAM1 enzyme, where R158A, R165A, and D112A mutations resulted in the complete loss of activity. Based on the docking of UDP-Gal in the active site of a UGM from *K. pneumoniae*\textsuperscript{45}, it appears likely that the guanidinium groups of R158 and R165 in HvUAM1 are positioned in the active site, where they interact with the pyrophosphoryl moiety of UDP-Ara. There is evidence that the conserved Arg residues are also the sites of reversible glycosylation\textsuperscript{31,45}, a characteristic after which the UAM enzymes were originally named\textsuperscript{30}. Using MS/MS analyses of tryptic peptides generated from the HvUAM1 and HvUAM4 enzymes following incubation with UDP-Glc, it was shown that R158 of HvUAM1 is indeed glycosylated during the reaction, but no evidence for the glycosylation of any Arg or
other residues could be found for the HvUAM4 protein. In control experiments it was shown that the UAM enzymes expressed in *E. coli* were not glycosylated until UDP-Glc was added.

Kinetic analyses of the UDP-\(\text{-L-Araf}\) to UDP-\(\text{-L-Arap}\) conversion catalysed by the expressed barley UAM enzymes show that the recombinant barley HvUAM1 had a \(K_m\) 41.8 \(\mu\)M and \(V_{\text{max}}\) of 1.15 nmol min\(^{-1}\), which is comparable with the value reported for OsUAM1 (\(K_m = 55\) \(\mu\)M, \(V_{\text{max}} = 1.2\) nmol min\(^{-1}\)).\(^{28}\) When the pyranosyl UDP-\(\text{-L-Arap}\) form of the substrate was used in the reaction, the \(K_m\) values decreased by 30 times and the \(V_{\text{max}}\) for UDP-\(\text{-L-Arap}\) was almost 20 times slower than the rate measured with the UDP-\(\text{-L-Araf}\) substrate (Table 6).

The kinetic analyses of the expressed barley HvUAMs show that the equilibrium constant \((K_{\text{eq}})\) of the reaction direction that occurs in plant systems, namely UDP-\(\text{-L-Arap}\) \(\rightarrow\) UDP-\(\text{-L-Araf}\), is 0.02, which indicates that the equilibrium lies strongly towards UDP-\(\text{-L-Arap}\). This is consistent with observations that extremely low levels of UDP-\(\text{-L-Araf}\) are detected in plant extracts\(^{42,48,51}\).

The low \(K_{\text{eq}}\) value for the reaction from UDP-\(\text{-L-Arap}\) does not mean that the reaction cannot proceed *in planta*, because the removal of UDP-\(\text{-L-Araf}\) through the incorporation of \(\text{L-Araf}\) into polysaccharides such as arabinoxylans will pull the reaction forward from UDP-\(\text{-L-Arap}\) to generate more UDP-\(\text{-L-Araf}\). However, the precise mechanism of the reaction in a cellular context is not yet known and it is important to consider cellular aspects of the reaction, given that UDP-\(\text{-L-Araf}\) synthesis presumably occurs in the cytosol, while arabinoxylan synthesis is likely to occur in the Golgi. The kinetics of the reaction also raise the question as to why plants use the thermodynamically less stable and higher energy Araf residues in preference to the more stable Arap residues and therefore have evolved the UAM genes and enzymes? One possible explanation is that the difference in three-dimensional conformations of the Araf and Arap
residues facilitates the evolution of biosynthetic and hydrolytic enzymes that can more easily distinguish conformational differences between the Araf substituents and the Xylp backbone residues of arabinoxylans.

The importance of the UAM enzymes in the generation of UDP-L-Araf for arabinoxylan synthesis would suggest that there might be a relationship between HvUAM gene transcript levels and the appearance of arabinoxylans in the walls of developing cereal grains. In the present work the Q-PCR and RNAseq data show that there is a marked difference in transcript profiles between the four HvUAMs. The levels of HvUAM4 transcripts are much higher than those of the other three genes in most of the tissues examined (Figure 3). During barley grain development, HvUAM4 transcripts peak at 8 DAP, which corresponds to the time point at which endosperm cellularisation is complete and arabinoxylan deposition commences.\textsuperscript{52,53} After 12 DAP, the levels of HvUAM4 transcripts decrease, but are nevertheless found at quite high levels throughout the rest of grain development (Figure 4). The most highly transcribed HvUAM gene in developing grain is HvUAM2 and its transcript abundance mirrors the steady deposition of arabinoxylan in the starchy endosperm during barley grain development (Figure 4).\textsuperscript{53} This isoform is therefore likely to be important for the provision of UDP-L-Araf for the synthesis of arabinoxylan in the developing endosperm. Transcripts of HvUAM1 and HvUAM3 are low and undetectable, respectively, in developing grain (Figure 4), but HvUAM1 transcripts are relatively high in root tips and developing embryo, while HvUAM3 transcripts are most abundant in root tips and anthers post-anthesis. It can therefore be concluded that at least three of the four barley UAM proteins are involved in UDP-L-Araf synthesis in different tissues and at different stages of growth and development.
The transcript analyses also revealed some specific co-expression patterns in a wide range of barley tissues (Figure 5 and Figure 6). Some of the correlations clearly reflect overall changes in cell wall growth or extensibility, such as the correlation between HvUAM3 transcripts and the transcripts for cellulase and expansin genes (Figure 5). However, in other cases the correlations raise potentially important questions about gene function. For example, HvUAM1 transcript abundance is highly correlated with that of HvUAM4 and a GT34 gene (Figure 6) of the type that has been implicated in xylan biosynthesis in certain systems. This raises the possibility that the HvUAM4 protein, despite having no UAM activity, might nevertheless perform some other, as yet undefined, function in heteroxylan biosynthesis. In addition, the transcript profiles for HvUAM2 are correlated with mRNAs for enzymes that are required for pectic polysaccharide mobilization or modification, such as polygalacturonase and pectin methyl esterase (Figure 6).

CONCLUSION

In summary, we have shown that the HvUAM1, HvUAM2 and HvUAM3 enzymes are true UDP-Ara mutases that interconvert pyranosyl and furanosyl forms of arabinosyl residues in nucleotide-sugars during arabinoxylan biosynthesis. The high levels of specific HvUAM transcripts during critical stages of arabinoxylan deposition in developing grain are consistent with a role for the genes in controlling carbon flux into arabinoxylans. However, the data indicate that the enzyme designated HvUAM4 here and elsewhere is most probably not a UAM, because the expressed enzyme has no activity against a large number of potential substrates, it shows no evidence for reversible glycosylation and its sequence identity with the other three members of the gene family is much lower. Nevertheless, the HvUAM4 gene is unlikely to be a
pseudogene, given its high transcript levels in most tissues, and it may well catalyze a related mutase reaction, because it is co-transcribed with HvUAM1 and other genes involved in cell wall metabolism. The identification and characterization of the UAM genes of the Poaceae now provides opportunities to manipulate levels of UDP-L-Araf in both vegetative cells and in developing grain, and hence to increase the amount, fine structure and solubility of arabinoxylan as a source of dietary fibre in human health applications or to enhance the amount and structure of heteroxylans for the generation of renewable biofuels.
ASSOCIATED CONTENT

Supporting Information

Following data are included as Supporting Information and are available free of charge via [http://pubs.acs.org](http://pubs.acs.org):

Locations of HvUAM gene family on barley chromosome (Figure S1), map of the HvUAM gene family in the barley genome and the positions of their orthologues in the Brachypodium and rice genomes (Figure S2), secondary structure analysis using PSIPRED, MESTAT for the transmembrane topology prediction, and ENSATSVM for the transmembrane helix prediction (Figure S3), chemical characterization of enzyme products: UDP-L-Arap and UDP-L-Araf (Figure S4), Lineweaver-Burk plots (Figure S5), MS/MS peptide sequencing of HvUAM1 and HvUAM4 (Figure S6), cell wall related genes or orthologous genes (identified in Figure 6) that co-expresses with UAMs in other grasses genera (Figure S7).

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**Notes**

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**ABBREVIATIONS**

Ara, arabinose; Da, Dalton; DAP, days after pollination; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenosine dinucleotide; Glc, glucose; GlcA, glucuronic acid; HPLC, high performance liquid chromatography; MS, mass spectrometry; MW, molecular weight; NAD⁺, nicotinamide adenosine dinucleotide; NMR, nuclear magnetic resonance; Q-PCR, quantitative polymerase chain reaction; RNAsseq, ribonucleic acid sequencing; UAM, uridine arabinose mutase; UDP, uridine diphosphate; UDP-L-Araf, UDP-L-arabinofuranose; UDP-L-Arap, UDP-L-arabinopyranose; UDP-D-GlcA, UDP-D-glucuronate; UGM, UDP-D-galactose mutase; UMP, uridine monophosphate; UXE, UDP-D-xylose 4-epimerase; UXS, UDP-D-xylose synthase; Xyl, xylose.
REFERENCES


(22) Reiter, W.D., Vanzin, G.F. (2001) Molecular genetics of nucleotide sugar interconversion pathways in plants. *Plant Mol. Biol. 47*, 95-113


(38)  TIBI (2010) Genome sequencing and analysis of the model grass *Brachypodium distachyon*. *Nature* 463, 763-768


Table 1. The PCR primers sequence for amplify fragments containing the full-length open reading frames from cDNA prepared from young barley shoots.

<table>
<thead>
<tr>
<th>Name</th>
<th>Fwd Primer</th>
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<tr>
<td>HvUAM1</td>
<td>GCAGGGACGGTGACTGTGCC/CTACTTTGGCTGCTGCTTTGCTGC</td>
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<td>HvUAM2</td>
<td>ATGGCCGCGCCGCCGCCGCCGTCGATTCACTTTGGCTCTTTTGAGCAGGACGAGGTCGCTTTGCTGCTGG</td>
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<td>HvUAM3</td>
<td>ATGGCGCCGCTCTCAAGGAGCCGA/CTACTTCCCCTTTGGGCTGTGAGCTGAGGTCGCTTTGCTGCTGAGA</td>
</tr>
<tr>
<td>HvUAM4</td>
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</tr>
<tr>
<td>Primer Pair</td>
<td>Sequences</td>
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<td>---------------------</td>
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<tr>
<td>HvUAM1 D112A</td>
<td>ACCATCGACGACGCTGCTTCGTGGC/</td>
</tr>
<tr>
<td></td>
<td>GGCCACGAAGCGCCGTTCGATG</td>
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<tr>
<td>HvUAM1 R158A</td>
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<td>GAAGGGGTATCCAGCAACAAAGTCCGCCTTC</td>
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<td>CCTGCTGTTCCTCTCAACAGGTTTGACCATCTGTTG</td>
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<td>HvUAM4 N100D</td>
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</tr>
<tr>
<td></td>
<td>TCATCTCAATTTGACGATTTGCGCTCCCGAAGAAAG</td>
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Table 3  QPCR forward and reverse primer sequences, PCR product sizes and acquisition temperatures (Taq).

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<tr>
<th>Name</th>
<th>Fwd Primer</th>
<th>Rev Primer</th>
<th>Size (bp)</th>
<th>Taq</th>
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<tr>
<td>HvUAM1</td>
<td>TTGGGAAGTCAAGAGTTATGG</td>
<td>CAACAAACGGGTGCTTGATTTCTC</td>
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<td>76</td>
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<td>HvUAM2</td>
<td>GCAATCACCACCACAAACAG</td>
<td>CCCATTCCGACATTACAACCTG</td>
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<td>78</td>
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<tr>
<td>HvUAM3</td>
<td>CATGCTCAACTCCAAGGACTC</td>
<td>ACAGCCTGAACCTGGATAACCT</td>
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<td>82</td>
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<tr>
<td>HvUAM4</td>
<td>CGCCTAATAGACTGGACTGTGTC</td>
<td>TGAGCATACACAACCGAAGC</td>
<td>177</td>
<td>77</td>
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Table 4  Amino acid sequence identities of HvUAM proteins

<table>
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<tr>
<th></th>
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<th>HvUAM3</th>
<th>HvUAM4</th>
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<tr>
<td>HvUAM1</td>
<td>-</td>
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<td>83.8%</td>
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<tr>
<td>HvUAM2</td>
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<td>-</td>
<td>82.6%</td>
<td>45.6%</td>
</tr>
<tr>
<td>HvUAM3</td>
<td>83.8%</td>
<td>82.6%</td>
<td>-</td>
<td>45.9%</td>
</tr>
<tr>
<td>HvUAM4</td>
<td>45.9%</td>
<td>45.6%</td>
<td>45.9%</td>
<td>-</td>
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</table>
Table 5 Effects of various compounds (at final concentrations of 0.1 mM) on the activity of barley HvUAM1 preparations. The data are means of three replicates. The product conversion efficiencies (% of Control) were calculated for different substrates (UDP-Araf, or UDP-Arap) in the presence or absence of added Mn$^{2+}$. a No inhibitor added.

<table>
<thead>
<tr>
<th></th>
<th>UDP-Araf $\rightarrow$ UDP-Arap</th>
<th>UDP-Araf $\rightarrow$ 5mM Mn$^{2+}$ $\rightarrow$ UDP-Arap</th>
<th>UDP-Arap $\rightarrow$ UDP-Araf</th>
<th>UDP-Arap $\rightarrow$ 5mM Mn$^{2+}$ $\rightarrow$ UDP-Araf</th>
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</thead>
<tbody>
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<td>Control$^a$</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>UDP-D-GalpNAc</td>
<td>48.2 ± 3.5</td>
<td>84.6 ± 4.2</td>
<td>62 ± 1.7</td>
<td>85.2 ± 4.1</td>
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<tr>
<td>UDP-D-GlcNAc</td>
<td>54.3 ± 1.2</td>
<td>63.7 ± 3.1</td>
<td>116 ± 4.0</td>
<td>83.5 ± 2.8</td>
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<tr>
<td>UDP-D-Glcp</td>
<td>102.6 ± 4.4</td>
<td>141.8 ± 5.9</td>
<td>82 ± 5.8</td>
<td>103 ± 4.9</td>
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<tr>
<td>UDP-D-Xylopy</td>
<td>48.5 ± 5.8</td>
<td>60.2 ± 10.1</td>
<td>70 ± 4.1</td>
<td>104 ± 2.5</td>
</tr>
<tr>
<td>NAD</td>
<td>113 ± 4.9</td>
<td>140.3 ± 13.0</td>
<td>112 ± 4.3</td>
<td>110 ± 3.8</td>
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<tr>
<td>NADPH</td>
<td>94.7 ± 2.9</td>
<td>142.2 ± 11.2</td>
<td>48 ± 2.1</td>
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<tr>
<td>UDP</td>
<td>92.2 ± 4.0</td>
<td>145.9 ± 2.5</td>
<td>30 ± 1.4</td>
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<tr>
<td>UMP</td>
<td>100 ± 5.1</td>
<td>104.6 ± 7.7</td>
<td>74 ± 8.7</td>
<td>108 ± 3.0</td>
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<tr>
<td>EDTA</td>
<td>9.5 ± 1.4</td>
<td>102.2 ± 1.3</td>
<td>0</td>
<td>100.8 ± 5.9</td>
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<tr>
<td>FAD</td>
<td>89 ± 4.1</td>
<td>92.1 ± 4.1</td>
<td>76.9 ± 5.0</td>
<td>98.3 ± 4.2</td>
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</table>
Table 6 Kinetic constants of the HvUAMs. The data are the means of three replicates and show differences obtained with different substrates, pH and cations.

<table>
<thead>
<tr>
<th>UDP-Araf, pH 7.0, Mn^{2+}</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}} ) (nmol min(^{-1}))</th>
<th>( K_{\text{cat}} ) (min(^{-1}))</th>
<th>( K_{\text{cat}}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HvUAM1</td>
<td>41.8 ± 6.4</td>
<td>1.14 ± 0.12</td>
<td>4009.6</td>
<td>95.9</td>
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<tr>
<td>HvUAM2</td>
<td>16.6 ± 0.9</td>
<td>0.43 ± 0.01</td>
<td>263.4</td>
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<tr>
<td>HvUAM3</td>
<td>4.4 ± 1.2</td>
<td>0.13 ± 0.01</td>
<td>568.0</td>
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<th>( V_{\text{max}} ) (nmol min(^{-1}))</th>
<th>( K_{\text{cat}} ) (min(^{-1}))</th>
<th>( K_{\text{cat}}/K_m )</th>
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<tr>
<td>HvUAM1</td>
<td>1270.4 ± 56.1</td>
<td>0.06 ± 0.02</td>
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<tr>
<td>HvUAM2</td>
<td>223.2 ± 34.7</td>
<td>0.01 ± 0.001</td>
<td>7.6</td>
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<tr>
<td>HvUAM3</td>
<td>127.2 ± 17.5</td>
<td>0.01 ± 0.001</td>
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<td>0.28</td>
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</table>

<table>
<thead>
<tr>
<th>UDP-Araf, pH 7.0</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}} ) (nmol min(^{-1}))</th>
<th>( K_{\text{cat}} ) (min(^{-1}))</th>
<th>( K_{\text{cat}}/K_m )</th>
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<tbody>
<tr>
<td>HvUAM1</td>
<td>146.7 ± 54.1</td>
<td>0.09 ± 0.02</td>
<td>331.1</td>
<td>2.26</td>
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<tr>
<td>HvUAM2</td>
<td>390.1 ± 126.7</td>
<td>0.30 ± 0.05</td>
<td>187.0</td>
<td>0.48</td>
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<td>HvUAM3</td>
<td>182.5 ± 25.6</td>
<td>0.04 ± 0.01</td>
<td>166.8</td>
<td>0.91</td>
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<table>
<thead>
<tr>
<th>UDP-Araf, pH 7.0, Mg^{2+}</th>
<th>( K_m ) (µM)</th>
<th>( V_{\text{max}} ) (nmol min(^{-1}))</th>
<th>( K_{\text{cat}} ) (min(^{-1}))</th>
<th>( K_{\text{cat}}/K_m )</th>
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</thead>
<tbody>
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<td>HvUAM1</td>
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<td>0.16 ± 0.01</td>
<td>574.5</td>
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<td>HvUAM2</td>
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<td>1.49</td>
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<td>HvUAM3</td>
<td>62.4 ± 7.7</td>
<td>0.04 ± 0.01</td>
<td>186.6</td>
<td>2.99</td>
</tr>
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</table>
Table 7 Relative activity of mutants compared with HvUAM1

<table>
<thead>
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<th>Relative activity (%)</th>
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<tr>
<td>HvUAM1</td>
<td>100</td>
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<tr>
<td>HvUAM1 D112A</td>
<td>0</td>
</tr>
<tr>
<td>HvUAM1 R158A</td>
<td>0</td>
</tr>
<tr>
<td>HvUAM1 R165A</td>
<td>0</td>
</tr>
<tr>
<td>HvUAM1 R193A</td>
<td>99</td>
</tr>
<tr>
<td>HvUAM4 N100D</td>
<td>0</td>
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</tbody>
</table>
**Figure 1.** Protein sequence alignment of HvUAM family. The putative reversible glycosylation domain is highlighted in the red box.
Figure 2. Phylogenetic tree of the UAM protein family. Included on this tree are *Arabidopsis thaliana* (green), barley (blue), rice (purple), sorghum (tan) and *Brachypodium* (black). LOC_Os03g40270 corresponds to OsUAM3; LOC_Os03g40270 corresponds to OsUAM1; LOC_Os04g56520 corresponds to OsUAM2, AT5G16510 corresponds to AtRGP5; At5G15650 corresponds to AtRGP2; At5G02230 corresponds to AtRGP1; At5G08900 corresponds to AtRGP3. The scale bar provides a relative measure of branch length and the branches are labelled with percentage clade support.
Figure 3. Normalized transcript levels of *HvUAM* genes in a range of tissues presented as arbitrary units. Tissues examined included the first leaf tip, first leaf base (3 mm at leaf base), the root tip and the maturation zone of root (root m/zone), flower just before anthesis (anther pre-anthesis), flower at anthesis, spike, developing grain 3-5 DAP, 8-10 DAP, and embryo at 22 DAP, 3 days old coleoptiles. Error bars are standard deviations.
Figure 4. Normalized transcript levels of HvUAM genes in barley developing endosperm. DAP examined including 6, 8, 12, 16, 20, 24, 18, 32, 38 DAP. Error bars are standard deviations.
Figure 5. Normalized expression levels for *HvUAM* genes in 16 barley tissue types. These are as described earlier in Figure 3, in a set of ~200 cell wall genes. Correlation coefficients are all > 0.95.
Figure 6. Transcript profiles of a HvUAM1, b HvUAM2, c HvUAM3, and d HvUAM4 normalised as % maximum RPKM. These RNA-Seq data were mapped to the genome using the CLC genomics workbench RNA-Seq analysis tool and expression values were calculated in RPKM. Expression data for every gene from replicate tissue samples was averaged.
For Table of Contents

The genetics, transcriptional profiles and catalytic properties of the UDP-Arabinose mutase family from barley