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Plant Science 169 (2005) 512–522
PLANT SCIENCE
www.elsevier.com/locate/plantsci

Differential expression of endo-β-1,4-xylanase isoenzymes X-I and X-II at various stages throughout barley development

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Received 19 April 2005
Available online 23 May 2005

Abstract

Barley endo-β-1,4-xylanases (EC 3.2.1.8) hydrolyse arabinoxylan, which is an abundant cell wall constituent in grass species. In addition to isoenzyme X-I, the major endo-β-1,4-xylanase released from the aleurone layer of germinating barley, another isoenzyme, named X-II, is expressed during germination. Using PCR on barley genomic DNA, we isolated the complete gene encoding sequence of this less-characterized isoenzyme. The gene, shown to be located on chromosome arm 5HL, has a coding sequence of 1665 bp interrupted by two introns. The corresponding protein sequence shows a high overall homology to X-I (88%) and reveals a modular structure consisting of a carbohydrate binding module and a glycosyl hydrolase family 10 domain but no signal peptide. Isoenzyme-specific sequence differences were exploited to develop X-I- and X-II-specific reverse transcriptase-PCR assays. These showed that X-I and X-II portray distinct expression profiles throughout plant development. Both isoenzymes are transcribed during germination but, in contrast to X-I, X-II transcripts accumulate in the developing shoot and root of the seedling embryo and not in the aleurone layer surrounding the endosperm. During subsequent stages of development, up to anthesis, X-II remains expressed in various organs. In developing grains, X-II is transcribed in the early stages of grain filling whereas X-I transcription is switched on during the later stages. The possible implications of these findings are discussed in regard to the distinct physiological role of the two isoenzymes.

Keywords: *Hordeum vulgare*; Endoxylanase; Gene; Isoenzyme-specific RT-PCR

1. Introduction

Endo-β-1,4-xylanases hydrolyse the 1,4-linked β-D-xylopyranosyl backbone of (hetero)xylan and, more specifically in cereal species, arabinoxylan in which the backbone may be singly or doubly substituted with 2-O- and 3-O-linked α-L-arabinofuranosyl residues. This polymer is abundantly present in the cell walls of the aleurone, endosperm, coleoptile, stem and leaves of barley [1]. The so far identified plant endo-β-1,4-xylanases are all classified into family 10 of glycosyl hydrolases (GH10), the general structure of which is described as an 8-fold β/α-barrel [2]. These enzymes hydrolyse the substrate with retention of their anomeric configuration by a double displacement mechanism in which one glutamic acid acts as catalytic acid/base and another one as catalytic nucleophile [3]. It has been shown that plants use these enzymes for the degradation of cell wall matrix components in processes such as the mobilisation of endosperm nutrients in germinating cereal grains [4–6], the lysis of the tapetum-containing anther wall at pollen maturation [7], the hydrolysis of the stigma surface for pollen tube penetration in maize [8] and secondary cell wall metabolism during vascular bundle development in *Arabidopsis thaliana* [9].

Especially the barley endo-β-1,4-xylanase, which is active during germination, has attracted attention because of its possible importance in commercial malting operations. Indeed, reduced arabinoxylan hydrolysis in these processes results in highly viscous malt extracts contributing to undesirable effects such as poor wort filterability and haze

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0168-9452/$ – see front matter
doi:10.1016/j.plantsci.2005.05.003

formation [10]. Dashek and Chrispeels [11] showed that this arabinoxylan hydrolysis could be associated with a 29 kDa protein released by GA_3-induced barley aleurone layers. This protein was purified and identified as an endo-β-1,4-xylanase with an apparent molecular weight of 34 kDa by Benjavongkulchai and Spencer [4]. When this enzyme was purified from extracts of germinated barley, it appeared as a 41 kDa protein [5]. Its N-terminal amino acid sequence was employed to isolate a clone from a GA_3-treated barley aleurone layer cDNA library encoding this 41 kDa endo-β-1,4-xylanase, designated isoenzyme X-I [12]. Further, screening of a 12 day germinated barley seedling cDNA library resulted in the identification of a clone encoding part of a related, yet distinct, endo-β-1,4-xylanase (isoenzyme X-II [12]). It was found that the genes for these two isoenzymes belong to a cluster of three endo-β-1,4-xylanase genes on the long arm of chromosome 5H [13]. Hormonal transcription control of the X-I gene in isolated aleurone layers was found to be comparable to barley (1,3-1,4)-β-glucanase EII, though important temporal differences were observed. Thus, endo-β-1,4-xylanase showed to be expressed and produced later in the germination process than (1,3-1,4)-β-glucanase [13]. Caspers et al. [6] revealed that the 34 kDa and 41 kDa endo-β-1,4-xylanases arise from an inactive, cytoplasmic 61.5 kDa precursor. Proteolytic processing, mediated in part by cysteine endoproteases, and extracellular release of active enzyme coincide with the programmed-cell-death-regulated disintegration of aleurone cells. The corresponding X-I gene sequence elucidated N- and C-terminal propeptide domains. Transient expression of truncated X-I in transfected aleurone protoplasts indicated the functional importance of these domains as chaperones of correct folding [6]. Although the module that precedes the catalytic GH10 domain, shows significant homology with microbial carbohydrate-binding modules, it lacks some putative sugar-binding sites. An alternative role for this domain in facilitating protein folding as well as in keeping the pro-enzyme in inactive state was suggested [3]. Hence, accumulation of the inactive endo-β-1,4-xylanase precursor within the aleurone cell ensures the structural integrity of the aleurone cell wall as long as hydrolytic enzymes are secreted into the non-living starchy endosperm for degradation of starch, protein and other reserve polymers. In the ultimate stages of endosperm mobilisation, the aleurone cells undergo programmed cell death leading to the release of cellular contents including cytosolic endo-β-1,4-xylanase precursor concomitantly with endo-peptidases. Consequently, endo-β-1,4-xylanase becomes processed and activated for final digestion of aleurone and endosperm cell wall (1,4)-β-xylans and the attendant recovery of constituent sugars for the developing seedling [3,6].

So far, studies of barley endo-β-1,4-xylanases have been concentrating on the germination process. This has generated substantial knowledge about the X-I endo-β-1,4-xylanase in the aleurone of germinating barley grain. In contrast, little information is available on other barley endo-β-1,4-xylanases and their expression and occurrence throughout plant development. In addition to X-I, isoenzyme X-II also seems to be expressed during germination. However, the high level of sequence identity between the two sequences (about 91%) did not allow to obtain gene-specific probes for further isoenzyme-specific expression analysis via Northern blotting [12]. The present study reports on the identification and analysis of the complete gene-encoding sequence of X-II and the use of isoenzyme-specific primers in reverse-transcriptase-PCR (RT-PCR) to examine the expression of the X-I and X-II genes in different tissues during germination and later stages of development.

2. Materials and methods

2.1. Plant material and tissue preparation

Mature grains of barley (*Hordeum vulgare* L. cv. Hiro) were surface-sterilized for 1 min with 70% ethanol and for 10 min with 4% NaOCl, rinsed with water and germinated in sterile Petri dishes on wetted filter paper under etiolated conditions (continuous darkness at 16 °C). Germinating grains were harvested at 10 days after imbibition, and tissues were separated into endosperm (dead starchy endosperm surrounded by the aleurone), scutellum, roots and shoots by dissection. Following germination, the non-used seedlings were allowed to grow further in potted soil outside. During the subsequent growth stages (up to post-anthesis), tissues for RNA extraction were prepared by hand dissection and included leaf, root, culm (nodes and internodes), immature ear, rachis and spikelet material. Throughout grain growth, grains were collected at 3- to 4-day intervals (hence spanning different developmental stages: few days after fertilization, water ripe stage, milk stages, dough stages, kernel hard stage and harvest ripe stage). From the medium milk stage on embryos and endosperm parts were separated. Stage determinations were done according to the descriptions at http://www.psu.missouri.edu/wheatx/growth.html and http://www.wheatbp.net/cgi-bin/grain2.pl.

Wheat–barley disomic chromosome and ditelosomic addition lines (*Triticum aestivum* 'Chinese Spring'/*H. vulgare* 'Betzes') as well as *H. vulgare* cv. Betzes and *T. aestivum* cv. Chinese Spring were kindly provided by Dr. S. Reader (John Innes Centre, Norwich, UK) and were grown as described above. Genomic DNA was isolated from leaves of young plants using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany).

2.2. PCR and DNA sequencing

Primers XHv5 (CTCGAATTCCCGATCGACTC) and XHv3 (ATCACTTGACGTG-TTGCATG) were derived from the barley 1,4-β-D xylan xylanohydrolase sequence published by Caspers et al. [6] (GenBank accession AF287726). Primers XTa5 (TTTGTGCCTCGTTCGTTATC) and XTa3

(CAACCTTATTCCTTGACGTG) were designed from wheat 1,4-β-xylan endohydrolase AF156977 [3]. X-II-specific PCR was performed with XHvII5 (TCAGCCTTCACGGTTACTCC) in combination with XHvII3 (GTCCCTTCAATCACCATGG). Oligonucleotides were purchased from Proligo (Paris, France). PCR reactions contained 50 ng genomic DNA, 0.5 μM of each primer, 200 μM of each dNTP, 1.5 units HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany) in a total volume of 30 μl Qiagen PCR buffer with added Q-solution (Qiagen). PCR reactions were subjected to a preliminary denaturation step of 15 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 90 s at 56 °C or 58 °C and 2 min at 72 °C on a UNO II thermoblock (Biometra, Göttingen, Germany). For set XTa5/XTa3 an annealing temperature of 56 °C was used, while for sets XHv5/XHv3 and XHvII5/XhvII3 58 °C was used. The amplification products were electrophoretically analysed on 1% agarose gels. For reamplification of the XTa5/XTa3 products, 5 μl of the original amplicon product was used as template. Direct sequencing was performed on purified PCR products (Invisorb Spin PCRapid kit, Invitek, Berlin-Buch, Germany) using ABI PRISM Big Dye Terminator chemistry (Applied Biosystems, Foster City, USA) and analysis on a 377 DNA sequencer (Applied Biosystems). The sequences were deposited in the EMBL Nucleotide Sequence Database (accession numbers AJ849364–AJ849366).

2.3. Bioinformatic analysis

Molecular weight and theoretical isoelectrical points of deduced proteins were calculated with the 'ProtParam' tool at website http://www.expasy.org, where the protein sequences were also scanned for the occurrence of patterns and profiles stored in the 'Prosite' database. The sequences were compared to cereal sequences in the GenBank database using 'Blast' algorithm (available at http://www.ncbi.nlm.nih.gov) to confirm their identity and to uncover conserved protein domains ('pfam'). Predictions of N-terminal signal sequences and subcellular targeting of X-II deduced proteins were performed using 'Psort', 'SignalP' and 'TargetP' software at http://psort.nibb.ac.jp/form.htm and http://cbs.dtu.dk/services/SignalP, respectively. Pairwise sequence identity and similarity percentages were determined using 'Align-needle algorithm' at the EBI website (http://www.ebi.ac.uk). A putative phylogenetic tree was generated from sequence multiple alignments ('ClustalW') at the EBI website and visualised as unrooted tree via 'Treeview'. The 5'UTR region of the X-II sequence was analysed by PlantCare (http://intra.psb.ugent.be:8080/PlantCARE/) to identify putative regulatory elements.

2.4. RNA extraction and RT-PCR

Fresh tissues were grounded to a powder under liquid N_2 and stored for short times at −70 °C. Total RNA was extracted from these samples using an RNeasy Plant Mini kit (Qiagen, Hilden, Germany) and included an on-column digestion of DNA with the RNase-Free DNase set (Qiagen). Single-strand cDNA was synthesised via a Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) with oligo$(dT)_{20}$ primer according to the manufacturer's recommendations. Reverse transcription was performed at 50 °C for 60 min. Primers XHvIi15 (AAGCACAACTCAGGACGTGA) and XHvIIi15 (GGCAAACGCCCAGGACGTGC), designed to span the first intron site of the gene coding sequences, were used in combination with XHv3 and XHvII3, respectively, in subsequent PCR on 1 μl sample from the cDNA synthesis product. PCR conditions were as described above with an annealing temperature of 58 °C. The resulting amplification products were sequenced directly as described above. Two primers ActHv5 (GGTCGTACTACAGGTATTGTG) and ActHv3 (TGGATATCCACATCTGCTGG) were designed for a barley actin gene, assembled from four barley partial actin ESTs (GenBank AL504711, BJ462564, CB869672 and AV926925). This primer set targets a constitutively expressed actin gene and was used as a control RT-PCR experiment.

3. Results

3.1. Gene isolation and characterisation

To isolate complete endo-β-1,4-xylanase sequences from barley two primer sets were tested. The first combination, XHv5/XHv3 (derived from barley endo-β-1,4-xylanase accession AF287726 [6]), resulted in the amplification of a clear 2094 bp PCR product on genomic DNA of cv. Hiro. Direct sequencing of this amplicon (hereafter named HiroX-I) revealed a perfect correspondence to AF287726. Primer set XTa5/XTa3 (designed from wheat endo-β-1,4-xylanase accession AF156977 [3]), however, amplified a weak product of approximately 2200 bp. After reamplification, sequencing resulted in the identification of a sequence (HiroX-II, Fig. 1) that corresponded well (99.4% DNA identity) to the partial cDNA sequence of barley endo-β-1,4-xylanase isoenzyme X-II (U59313 [12]). The first ATG and its flanking 6/4 nucleotides were found to correspond perfectly with the transcription start position of X-I (AF287726 [6]). Hence, HiroX-II was found to have a coding sequence of 1665 bp interrupted by two introns of 103 and 104 bp, respectively. Bioinformatic analysis of the deduced amino acid sequence revealed two protein family domains, a carbohydrate binding module (pfam02018) and the glycosyl hydrolase family 10 domain (GH10, pfam00331), respectively. Within this latter domain 'prosite' protein profile PS00591 for the glycosyl hydrolase family 10 active site, including the catalytic nucleophile, was recognized (Fig. 1). The glutamic acid acting as acid/base catalyst (A/B) could be assigned since this residue is totally conserved in plants [3]. One potential *N*-glycosylation site, NTTV, was predicted via 'Prosite' (located in the GH10

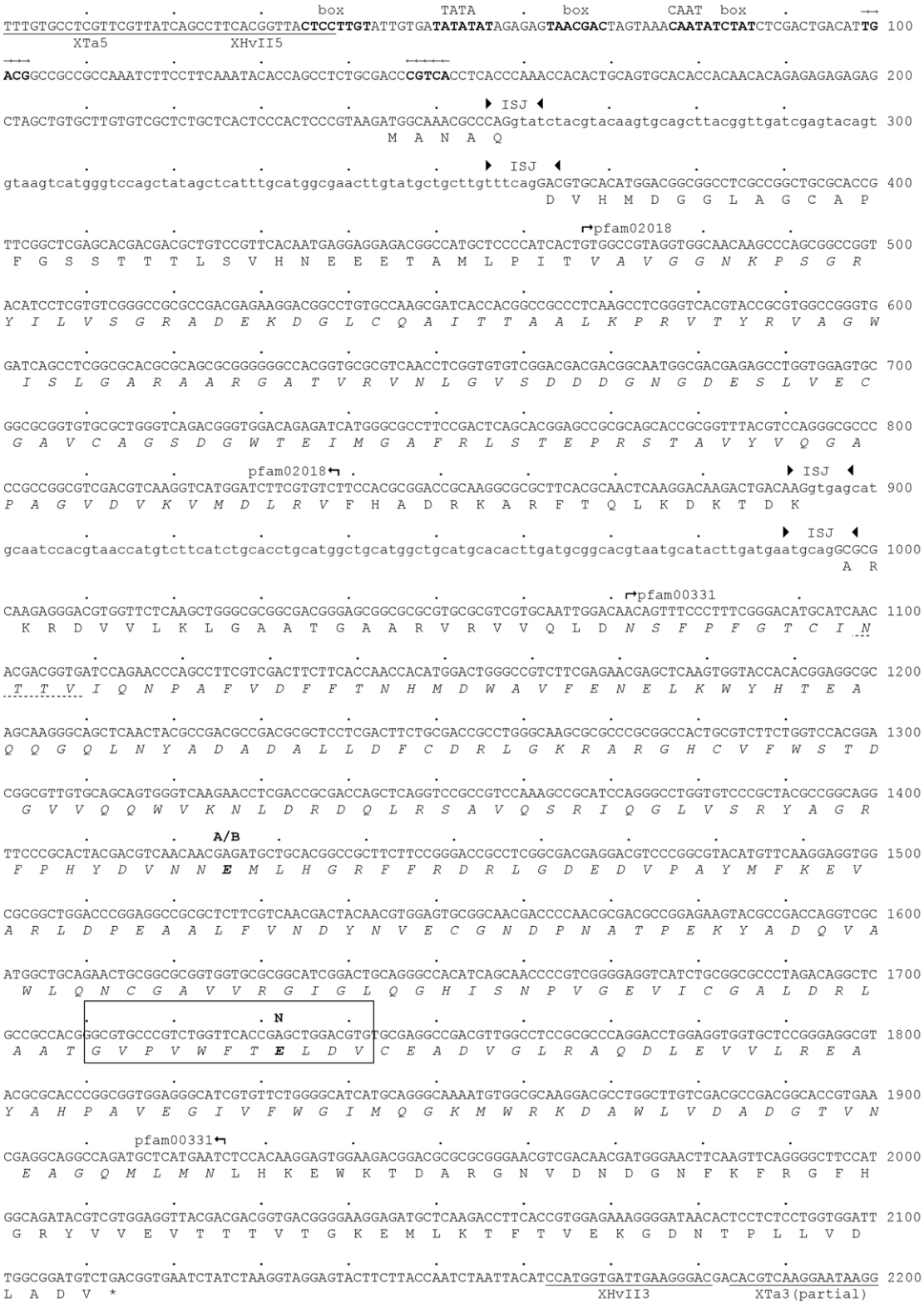

Fig. 1. Nucleotide and deduced amino acid sequence of the HiroX-II gene sequence amplified by primer set XTa5/XTa3. The intron sequences are shown in lower case letters and the presumed catalytic Glu residues comprising the acid/base catalyst (A/B) and the nucleophile (*N*) are in bold type. The stop codon is represented by an asterisk. The potential *N*-glycosylation site is indicated by dotted underlining. Protein families pfam02018 (a carbohydrate binding domain) and pfam00331 (glycosyl hydrolase family 10 domain) are indicated and 'prosite' protein profile PS00591 is boxed. The intron splice junction (ISJ) sequences that are perfectly conserved among X-I and X-II are indicated. Elements of the putative GA responsive complex (respectively the pyrimidine box, the TAACGAC box and the TATCTAT box) as well as the two TGACG motifs in opposite orientation and the putative TATA and CAAT boxes are shown in bold print. This sequence was submitted to EMBL Nucleotide Sequence Database: accession AJ849365.

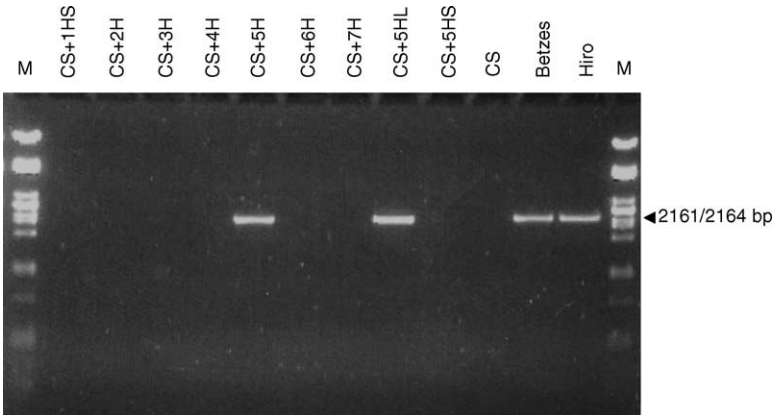

Fig. 2. Chromosome-arm assignment of the X-II gene was done via PCR with primer pair XHvII5/XHvII3 on genomic DNA of the seven wheat–barley chromosome addition lines and two ditelosomic addition lines (CS + 5HL and CS + 5HS). A 2161 bp product is amplified in CS + 5H, CS + 5HL and barley cv. Betzes, while a 2164 bp product is amplified in barley cv. Hiro. CS: wheat cv. Chinese spring. M: λ/*Pst*I size marker.

domain, Fig. 1). No signal peptide sequence could be deduced from the sequence, suggesting a cytosolic location of X-II. In the 5′ untranslated region (5′ UTR) a putative TATA- and CAAT-box were identified (Fig. 1). Further, three *cis*-acting regulatory elements, consisting of a pyrimidine box, a TAACGAC box and a TATCTAT box, showed high similarity (62.5%, 100% and 85.7%, respectively) to the elements of the putative GA response complex in the promoter of isoenzyme X-I [13]. In addition, the 5′ UTR contains the TGACG motif as inverted repeats. These palindromic sequences are also present in the promoter of barley lipoxygenase 1 (*Lox1*) gene that is expressed in embryos during grain development and germination and in leaves after methyl-jasmonate (MeJA) treatment [14]. This MeJA-responsive element probably functions as a binding site for bZIP transcription factors.

A more specific primer set (XHvII5/XHvII3) was developed to target HiroX-II (Fig. 1). This set as well as XHv5/XHv3 was used to check the chromosomal location of the X-II and X-I genes via PCR-based analysis of wheat–barley chromosomic and ditelosomic addition lines. Besides barley cv. Betzes and Hiro, both sets amplified only a PCR product in chromosome addition line CS + 5H and ditelosomic addition line CS + 5HL (illustrated for XHvII5/XHvII3 in Fig. 2), assigning the location of X-II and X-I to the long arm of barley chromosome 5H (in congruence with [13]). Direct sequencing of these amplification products revealed alleles of X-I and X-II in cv. Betzes: named BetzesX-I and BetzesX-II, respectively. The coding sequence of the latter showed a perfect match with U59313, proving that BetzesX-II encodes endo-β-1,4-xylanase isoenzyme X-II.

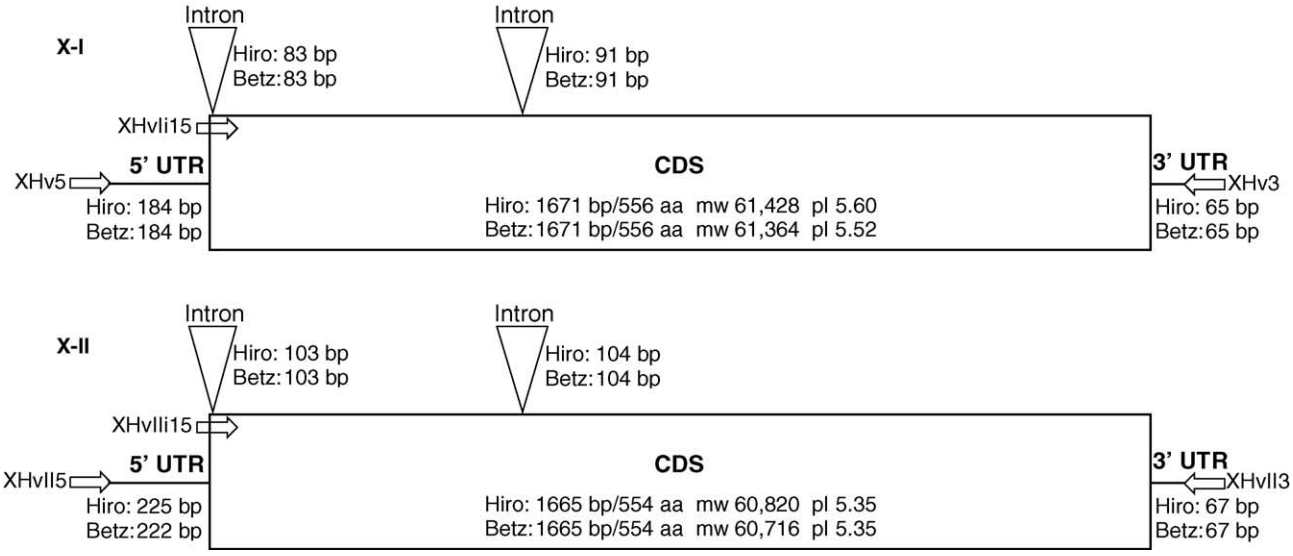

Fig. 3. Schematic representation of the X-I- and X-II-specific PCR products. Direct sequencing enabled to discern the different gene elements and to determine their exact size (UTR: untranslated region, CDS: coding sequence). The positions of the primers used for PCR analysis are indicated by block arrows. Betz: cv. Betzes. Nucleotide sequences BetzesX-I, HiroX-II and BetzesX-II are deposited under EMBL accession numbers AJ849364-AJ849366, while HiroX-I perfectly matches AF287726.

3.2. Homology among cereal endo-β-1,4-xylanase

Comparative analysis of the sequences of the four PCR products showed that the X-I and X-II genes have a similar structure (Fig. 3), with comparable intron positions bordered by completely conserved intron splice junction sequences (ISJ, Fig. 1). From the deduced protein sequences, X-I appeared to have a larger molecular weight and a slightly less acidic calculated p*I* than X-II. The carbohydrate binding modules of the two isoenzymes were found to be more divergent (on average 83.3% protein similarity) than the glycosyl hydrolase family 10 domains (93.9% similarity). The close relationship among the X-I and X-II paralogues is further evident from the sequence similarity percentages at the protein level (on average 88.0%, Table 1). When the entire sequences of the PCR products are compared, the similarity scores between paralogues show smaller values (on average 78.5%, Table 1). This can be attributed to significant sequence differences (including several indels) in the untranslated regions (UTR) and the introns.

The X-I and X-II protein sequences were aligned to deduced amino acid sequences from cereal endo-β-1,4-xylanase genes of wheat, maize and rice available in GenBank. An unrooted tree, derived from the ClustalW results showed that the cereal X-I and X-II proteins cluster closely together (Fig. 4). The nearest relatives represent two uncharacterised rice endo-β-1,4-xylanases mapping on chromosome 1, while the maize tapetum endo-β-1,4-xylanase [8] is most distally situated. Also the putative wheat endo-β-1,4-xylanase BAD06323, isolated form a developing seed cDNA library, differs clearly from X-I and X-II. Analysis of the barley EST database with the X-I and X-II DNA sequences revealed three related ESTs. One of these (AJ461435, 240 bp) corresponded perfectly with HiroX-II, while a large part of BI778071 showed an identity of 97% with BetzesX-I. The third EST, BI958652, had a 239 internal fragment showing 84–85% identity with X-I and X-II. It can be speculated that this latter EST corresponds to the third (so far unknown) gene of the endo-β-1,4-xylanase gene cluster on the long arm of chromosome 5H [13]. In addition, a tblastn search of the barley EST

Table 1
Pairwise X-I and X-II sequence similarity percentages

	HiroX-I	BetzesX-I	HiroX-II	BetzesX-II
HiroX-I	100	99.3	78.3	78.7
BetzesX-I	99.1	100	78.3	78.7
HiroX-II	87.7	87.9	100	99.1
BetzesX-II	88.1	88.3	99.6	100

Similarity percentages were calculated using the needle algorithm of the 'Align' programme. Results of the comparison of the protein sequences are presented below the diagonal, whereas comparisons of the complete PCR product sequences are presented above the diagonal.

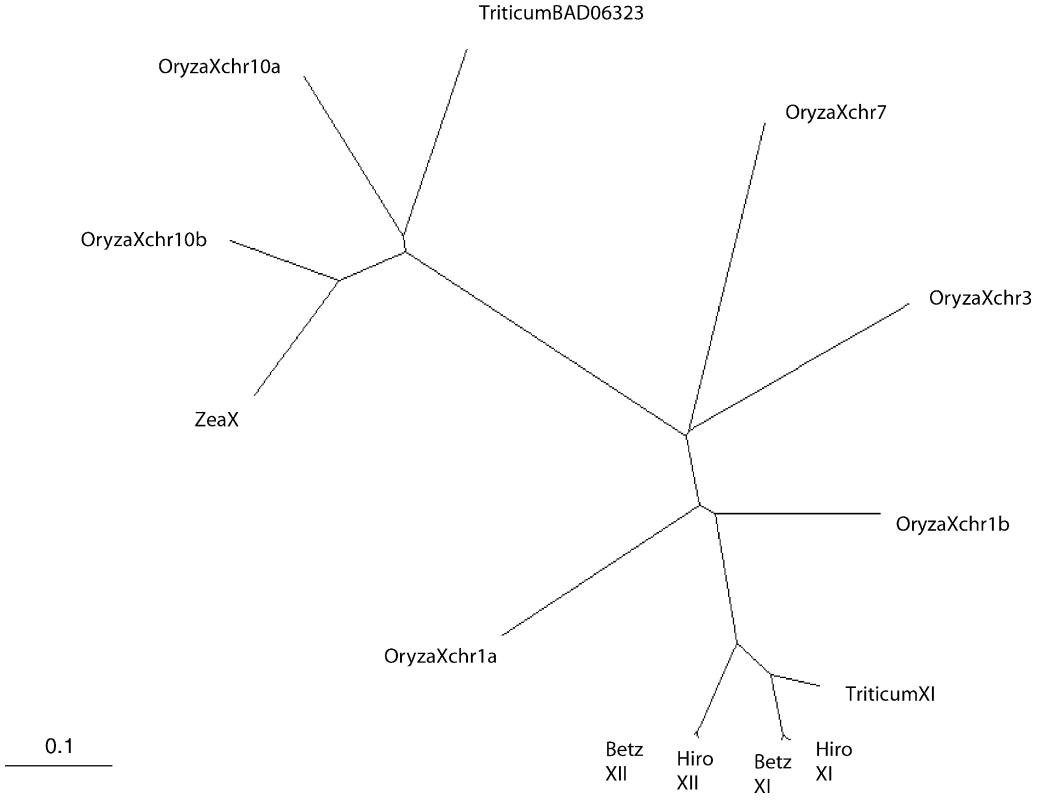

Fig. 4. Phylogenetic relationship of cereal endo-β-1,4-xylanase proteins, derived from available cDNA sequences. X-I and X-II of barley and wheat (TriticumX-I [3]) were aligned to sequences from maize (ZeaX [8]), wheat (GenBank accession BAD06323) and rice chromosomes 1, 3, 7 and 10 (OryzaXchr) via ClustalW and plotted using TreeView. The scale bar indicates a distance value of 0.1. OryzaXchr1a: BAB64624, OryzaXchr1b: BAB64626, OryzaXchr3: AAP03380, OryzaXchr7: BAC57375, OryzaXchr10a: AAM08565, OryzaXchr10b: AAM08566.

database resulted in the identification of five other endo-β-1,4-xylanase ESTs (BF259812, CA017926, BQ471249, CA018231 and CB882318), which are partially and loosely related to X-I and X-II (less than 55% identity at the amino acid level). As two of these ESTs (CA017926 and BQ471249) are almost identical, this indicates that the barley genome contains at least four other endo-β-1,4-xylanase genes in addition to the three genes clustering on 5H.

3.3. Expression analysis

We exploited DNA sequence differences between X-I and X-II to construct isoenzyme-specific primers XHvIi15 and XHvIIi15, for use in expression studies via RT-PCR. As it is important to ensure that PCR signals are due to the amplification of cDNA, and not to possible contaminating genomic DNA, these primers were designed to span the first intron site of the gene coding sequences (Fig. 3). The X-I-specificity of set XHvIi15/XHv3 and the X-II-specificity of set XHvIIi15/XHvII3 was confirmed by direct sequencing of the resulting amplicons. This also provided an extra control of the RT-PCR fidelity by recording the absence of the second intron sequence (Fig. 3).

X-I and X-II expression were examined in distinct organs throughout the various stages of barley plant development from germination up to anthesis. A control RT-PCR targeting an ubiquitously expressed barley actin gene was used to monitor variations in transcript levels among the samples. The rather weak signal in endosperm sections are in congruence with the low RNA yields from these tissues, while the transcript levels in the other samples were of comparable level (Fig. 5). From Fig. 5A it is clear that X-I expression is restricted to the aleurone (as the only living tissue of the endosperm) and the scutellum of germinating grains while X-II displays a much broader expression profile. X-II expression was especially predominant in developing shoot and roots of germinating grains and in roots of plants at the elongation stage. Further, a clear 1730 bp fragment was formed in scutellum at germination and in the culm at elongation; while weaker signals were found in roots of young plants, in leaves (in general), in the immature ears and in the rachis and spikelets at anthesis (Fig. 5A).

The same primer sets were also used to investigate X-I and X-II expression in the subsequent stages of grain development. To this end RNA was isolated from grains early after fertilization up to the harvest ripe stage. From the medium milk stage on, the rapidly developing embryo's (including the scutellum) were dissected out from the grains for separate RNA isolation. The complementary endosperm parts were also subjected to RNA isolations but these resulted only in very low yields. RT-PCR was performed on the different RNA samples and this showed that both isoenzymes are differentially expressed (Fig. 5B). X-II appeared to be transcribed with increasing intensity by the embryos in the successive milk stages. In the subsequent dough stages X-II expression vanished, whereas X-I transcripts started to accumulate in the embryos. Finally, in the harvest ripe stage, also X-I expression ceased (Fig. 5B).

4. Discussion

In this report we describe the PCR-based isolation of the complete coding sequence (Fig. 1) of a barley endo-β-1,4-xylanase that corresponds to a partial cDNA clone previously described to encode isoenzyme X-II [12]. The genomic organization of this gene resembles that of the well studied barley endo-β-1,4-xylanase isoenzyme X-I: both genes reside on 5HL (Fig. 2) and have a conserved gene structure (Fig. 3). Furthermore, the similarity between the coding sequences of X-I and X-II makes both isoenzymes group together when compared to other available cereal endo-β-1,4-xylanases (Fig. 4). Hence, the genes encoding X-I and X-II seem to have evolved from an ancestral endo-β-1,4-xylanase by a rather 'recent' duplication event. The high similarity suggests these isoenzymes are likely to have similar biochemical substrate specificities. However, evolutionary pressure has provided opportunities for functional evolution of endo-β-1,4-xylanase with respect to developmental regulation. It is clear that the expression of both genes has evolved in clearly distinct directions. Both genes are strongly expressed during germination, though showed distinct spatial expression patterns. Apart from a simultaneous expression in the scutellum, expression of X-I is predominant in the aleurone while that of X-II is restricted to the embryo (Fig. 5A). Throughout the different developmental stages from young to mature plant only X-II is expressed (Fig. 5A), whereas the two isoenzymes show a different temporal expression during grain formation (Fig. 5B).

The observed X-I transcription in the aleurone layers and scutellum of germinated grains is consistent with the previously described secretion of X-I from isolated GA_3-treated aleurone layers and with a role for the enzymes in the depolymerization of cell wall arabinoxylans of the aleurone-layer and the storage-endosperm cells in the germinated grain [4–6,11–13]. Furthermore, its expression coincides with the activity of other arabinoxylan-targeting enzymes like arabinoxylan arabinofuranohydrolases [15,16] and a β-D-xylosidase and an α-L-arabinofuranosidase in germinated barley grains [17]. Its expression and occurrence in developing grains has not been reported so far but, as we observed X-I expression in the embryos at the dough stages, it might be anticipated that X-I is synthesised by the scutellum to assist digestion of endosperm cells to free nutrients for the rapidly developing embryo. Indeed, at the later stages of grain filling, the scutellum is known to secrete hydrolytic enzymes, thus resulting in the formation of a crushed cell layer adjacent to it. So far only the presence of

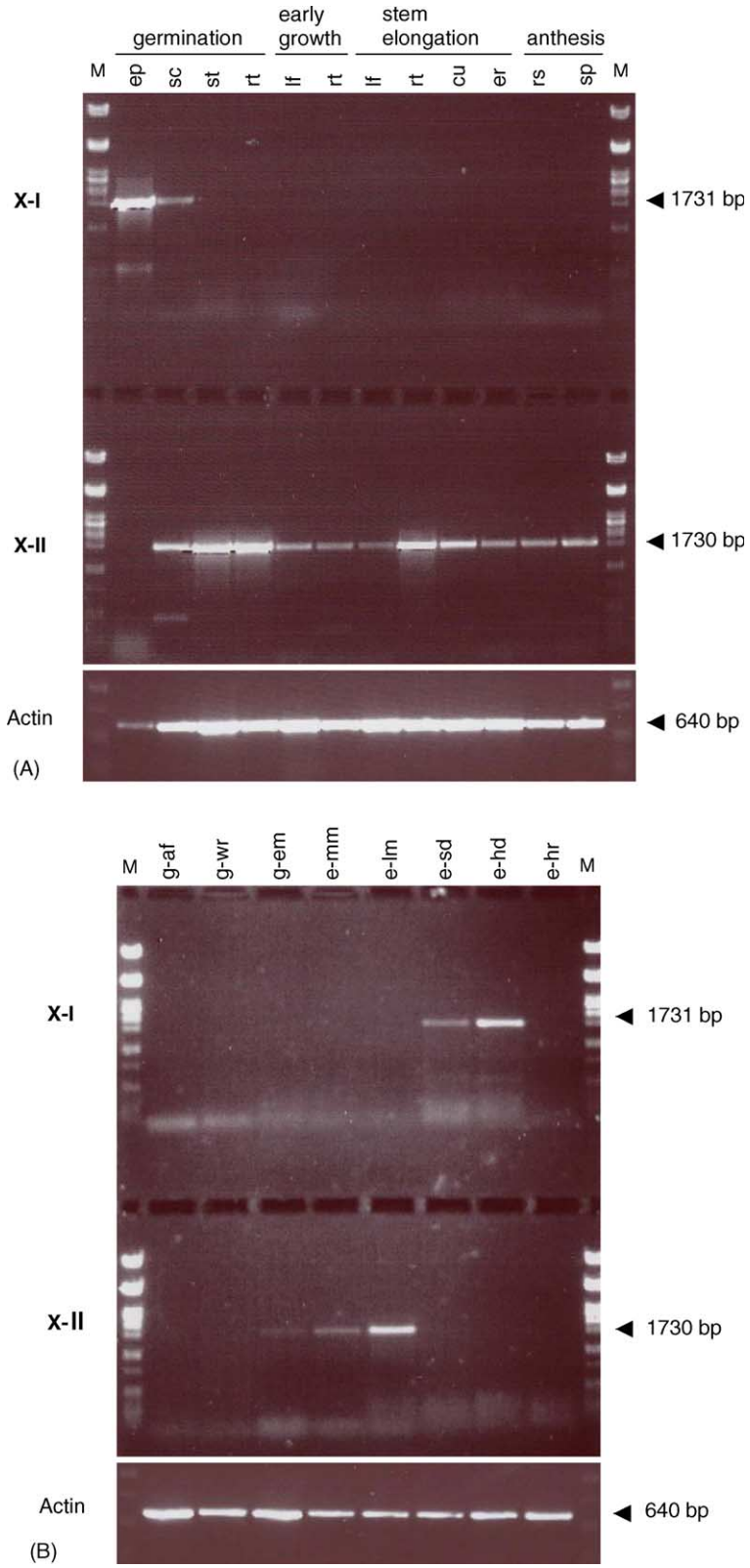

Fig. 5. RT-PCR analysis of X-I and X-II expression in different organs throughout barley development, from germination to anthesis (A) and in the subsequent stages of grain development (B). X-I-specific primer set XHvIi15/XHv3 and X-II-specific set XHvIIi15/XHvII3 were used in PCR on cDNA prepared from ep: endosperm; sc: scutellum; st: shoot; rt: roots; lf: leaf; cu: culm; er: ear; rs: rachis; sp: spikelets at indicated stages of development (A) and on cDNA prepared from g: grains and e: embryos at stages af: early days after anthesis; wr: water ripe stage; em: early milk stage; mm: medium milk stage; lm: late milk stage; sd: soft dough stage; hr: hard dough stage; hr harvest ripe (B). A control RT-PCR directed to a barley housekeeping gene (Actin) was used to monitor variations in transcript levels among the samples. M: size marker λ/P*st*I.

proteinases in this layer has been experimentally demonstrated [18], but in view of the analogy with the breakdown of endosperm during germination also xylanases are expected to be involved.

Explanation of X-II expression in the different organs throughout barley development is less obvious as so far barley xylanases have mainly been studied in relation to germination. In this context, it is remarkable that barley (1,3-1,4)-β-glucanases, involved in the degradation of the other major cell wall component (1,3-1,4)-β-glucan, also occur as two closely related but differentially regulated isoenzymes [19]. Transcription of one isoenzyme appears to be restricted to the germinating grain but the other isoenzyme is, comparable to X-II, concomitantly expressed in developing

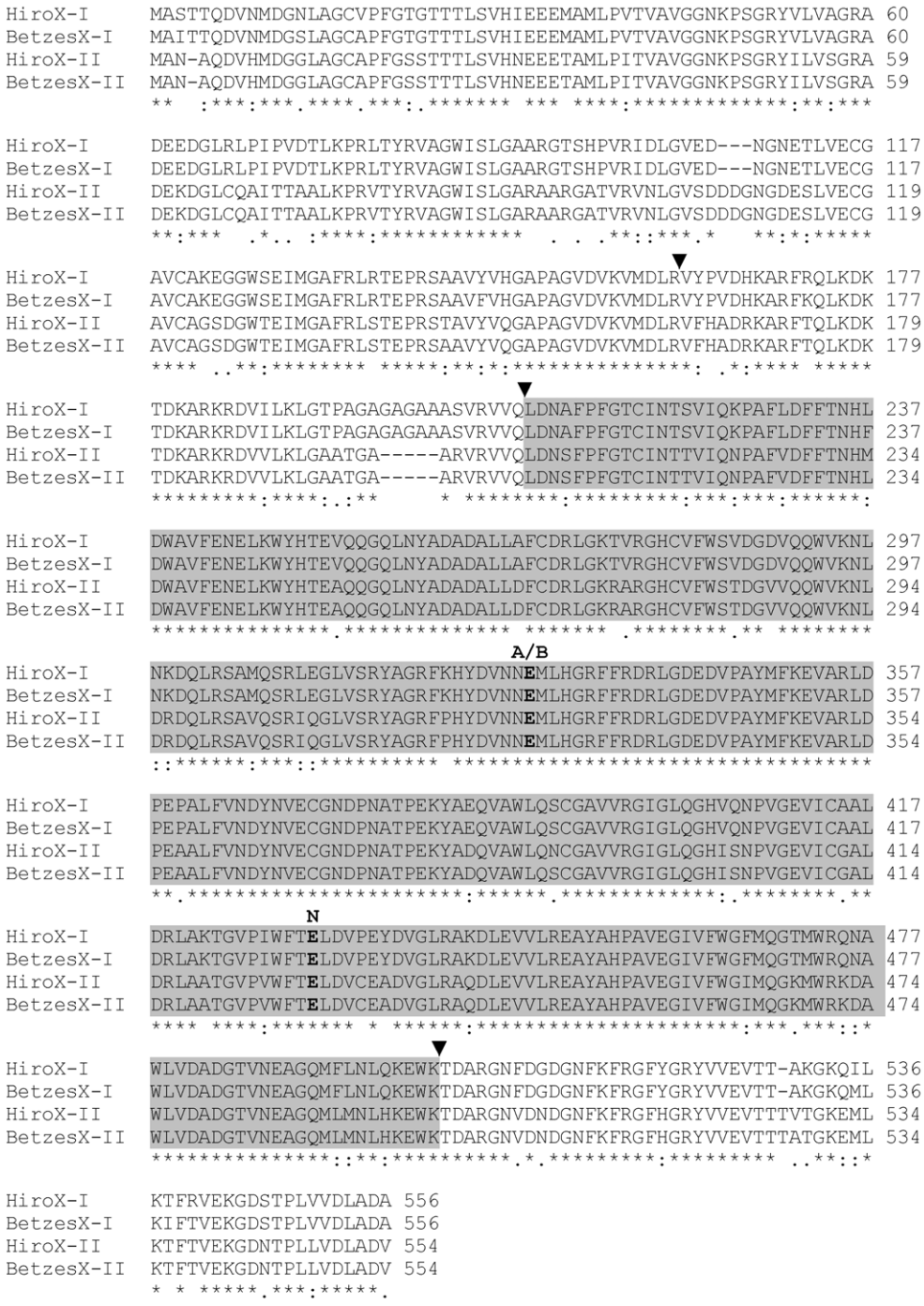

Fig. 6. Multiple alignment of the deduced amino acid sequences of the identified X-I and X-II barley endo-β-1,4-xylanase coding sequences (ClustalW, using default parameters). The processing sites determined for X-I [6] are indicated by arrows. The sequence corresponding to the 34 kDa mature active enzyme [6] is shaded. The presumed catalytic Glu residues comprising the acid/base catalyst (A/B) and the nucleophile (N) are in bold type. Consensus line: (*) identical residues; (:) conserved residues; (.) semi-conserved residues.

leaves and roots of young plants. It was hypothesized that the latter isoenzyme could participate in the differentiation of vascular tissues, a process by which unlignified primary cell walls disappear [19]. It is hence conceivable that X-II induction in diverse tissues throughout plant development is related to a specific expression in the vasculature of these organs. Moreover, the expression pattern of X-II parallels that of a β-xylosidase and an α-L-arabinofuranosidase in *A. thaliana*. Promoter-GUS expression analysis of the *AtBXL1*–encoded β-xylosidase, showed activity in the vascular region of organs like roots, leaves, stem, flowers, siliques and was proposed to be involved in secondary cell wall metabolism [20]. Analogous analysis of the *AtASD1* gene encoding α-L-arabinofuranosidase, detected expression in seedlings, roots, rosettes, flowers, stems, siliques and was suggested to coincide with cell proliferation and vascular development during vegetative growth and the morphogenesis, senescence and abscission of floral organs [21]. Possible implication of endo-β-1,4-xylanases in cell wall metabolism of vascular tissues is furthermore supported by the expression of AtXyn1 in tracheids and sieve elements of *A. thaliana* [9]. However, these considerations do not hold true in explaining X-II expression in embryos at milk stage as no differentiated vascular elements can be observed in young developing cereal embryos [22]. Remarkably, the barley α-L-arabinofuranosidase ARA-I and the barley β-D-xylosidase XYL, which both participate in further hydrolysis of oligosaccharides released from arabinoxylans by endo-β-1,4-xylanases are also transcribed in developing grains [17]. Although this supports the observed expression of X-II in young developing embryos, the role of these enzymes and X-II at this stage remains obscure.

Our analysis of the X-II sequence predicted absence of a signal peptide. In accordance with X-I, where a signal peptide is also lacking [6], this indicates that the X-II enzyme does not enter the protein secretory pathway. The X-II gene encodes an about 61 kDa primary translation product with a modular structure but, analogous to X-I, the carbohydrate-binding module lacks tryptophan and tyrosine at the putative sugar-binding sites [3]. In addition, the protein prosequence cleavage sites of X-I [6] can be matched with the corresponding protein sequences of X-II (Fig. 6). Taken together these data indicate that X-II is synthesised, processed and exported according to a mechanism that has been described for barley X-I and maize tapetum xylanase. More specifically, these endo-β-1,4-xylanases are synthesized as inactive precursors of about 60 kDa in the cytosol and processed to active enzymes of about 35 kDa by endoproteases during programmed-cell-death-dependent release [6,8]. The fact that programmed cell death occurs similarly during differentiation of tracheids and vessel elements [23] makes a role for X-II in maturation of xylem tracheary elements further plausible.

The presence of a possible GA response complex in the promoter region of X-II envisages a GA-induced transcription during germination, in analogy to X-I [13]. However, considering the absence of X-II expression in the aleurone layer, it might be that GA response of these elements is either impaired or restricted to certain tissues (e.g. scutellum) or other GA-regulated processes such as stem elongation. Its MeJA-responsive element, on the other hand, appears to be absent in the promoter of X-I and might be a pivotal factor in the differential regulation and hence expression of the two isoenzymes during development. After all, jasmonates (like MeJA) are not only known as signals in plant stress responses but they also play a role in plant development [24]. Their involvement in barley seedling development, for instance, can be deduced from the observed elevated levels of jasmonic acid in different seedling tissues [24,25]. Interestingly, in the light of the hypothesised commitment of X-II in vasculature differentiation, jasmonates seem to induce several proteins in vascular bundles of various organs at different developmental stages of plants [24,26,27].

Further research is necessary to understand the specific physiological role of the barley endo-β-1,4-xylanase isoenzymes. In this regard, in situ hybridisation and immunocytological experiments should allow to locate more precisely the endo-β-1,4-xylanase expression and occurrence within the barley plant.

Acknowledgements

We thank Gert Raedschelders for help with RNA isolations and Barbara Grymonprez for expert technical assistance. Dr. S. Van Campenhout is a postdoctoral fellow of the Flemish Fund for Scientific Research (FWO-Vlaanderen). This work was financed in part by the 'Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen' (IWT- GBOU project funding).

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