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Synthesis, processing and export of cytoplasmic endo-β-1,4-xylanase from barley aleurone during germination

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Summary

We have identified the major endo-β-1,4-xylanase (XYN-1) in the aleurone of germinating barley grain, and show that it is expressed as a precursor of M_r 61 500 with both N- and C-terminal propeptides. XYN-1 is synthesized as an inactive enzyme in the cytoplasm, and only becomes active at a late stage of germination when the aleurone ceases to secrete hydrolases. A series of processing steps, mediated in part by aleurone cysteine endoproteases, yields a mature active enzyme of M_r 34 000. Processing and extracellular release of the mature enzyme coincide with the programmed cell death (PCD)-regulated disintegration of aleurone cells. We discuss the significance of delayed aleurone cell-wall degradation by endoxylanases in relation to the secretory capacity of the aleurone, and propose a novel role for aleurone PCD in facilitating the export of hydrolases.

Keywords: programmed cell death, xylanase, barley, aleurone, germination, secretion.

Introduction

In germinating cereal grains, the aleurone tissue secretes hydrolytic enzymes into the endosperm storage tissue that bring about the degradation of stored starch, protein and residual nucleic acids, providing a supply of nutrients for the growing embryo. Depolymerization of endosperm cell walls is essential for the mobilization of endosperm reserves, as it facilitates diffusion of hydrolases into the endosperm tissue, as well as itself providing a source of nutrients. The chemical composition of the aleurone and endosperm cell walls has been defined in detail, where arabinoxylans and (1,3–1,4)-β-glucans together account for 95% of wall polysaccharides with approximately 2% each of cellulose and glucomannans (Fincher, 1992). Their molecular organization has been likened to a cellulose-reinforced multicomponent gel, which provides the necessary strength required to maintain tissue integrity while allowing free diffusion of water, phytohormones and low molecular-weight metabolites (Fincher and Stone, 1986). However, it is unlikely that the cell-wall matrix would be freely permeable to secreted proteins of more than 20 kDa, based on the calculated pore diameter (4 nm) of cell-wall microcapillaries (Carpita, 1979). It is thus not surprising that (1,3–1,4)-β-glucanases capable of degrading the endosperm cell walls are among the first enzymes to be secreted by the aleurone of imbibed grain (Fincher, 1992). As *de novo* synthesized hydrolases secreted by the scutellum epithelium and aleurone move from the proximal to the distal end of the grain, the endosperm cell walls are progressively degraded, first with the loss of crystalline β-glucan, indicated by a loss of calcofluor-binding, and subsequently by their complete disintegration (Selvig *et al.*, 1986). In this context it is relevant to consider how the aleurone tissue retains its structural integrity, while facilitating the export of hydrolases required for endosperm nutrient mobilization.

The aleurone cell walls differ in both structure and composition from the walls of the starchy endosperm. First, the aleurone walls are significantly thicker, compris-

ing a thin inner wall and a thick outer wall; second, they show strong autofluorescence due to the abundance of ferulic acid esterified with arabinoxylan polymers in the wall (Bacic and Stone, 1981). The arabinoxylan content of the aleurone walls is, furthermore, significantly higher at 71% compared to 20% in the starchy endosperm (Fincher, 1992). Initially, secretion of hydrolases through the thick aleurone cell walls does not depend on extensive wall degradation, as α-amylases appear to move through plasmodesmata and channels which may be created by limited cell-wall hydrolysis (Gubler *et al.*, 1987). Depolymerization of the aleurone cell wall takes place after that of the β-glucan-rich endosperm cell walls (Schuurink *et al.*, 1997), consistent with the reported delay in the secretion/release of endoxylanases in comparison to β-glucanase, xylosidase and arabinofuranosidase (Banik *et al.*, 1997; Slade *et al.*, 1989). The outer wall of the aleurone is degraded first, while the inner wall is retained around actively secreting aleurone cells, suggesting that the composition of the two layers may differ (Fincher, 1992; Schuurink *et al.*, 1997). Hence the relatively resistant nature of the inner aleurone cell wall, in particular, and delayed release of hydrolases capable of degrading its component polymers may be critical for sustained enzyme secretion by aleurone tissue during germination. If endoxylanases were required for aleurone cell-wall degradation, the regulation of their synthesis and secretion should reflect this specific role.

Three endo-β-1,4-xylanase isoenzymes with an apparent M_r of 41 000 have been purified from germinating barley and shown to be endohydrolases on the basis of product analysis (Slade *et al.*, 1989). A cDNA and genomic clone encoding the endo-β-1,4-xylanase of M_r 41 000 have been characterized (Banik *et al.*, 1996, Banik *et al.*, 1997). The authors proposed that the enzyme was expressed with a 32-amino-acid presequence having characteristics of a signal peptide, based on a comparison of the deduced sequence of the encoded protein and the determined N-terminal sequence of the enzyme. However, the secretion of this M_r 41 000 enzyme and its contribution to aleurone cell-wall degradation has not been examined. Two previous reports describe secreted barley xylanases of apparent M_r 29 000 and 34 000, which were detected in the culture medium of gibberellic acid-induced aleurone layers after 28–32 h incubation (Benjavongkulchai and Spencer, 1986; Dashek and Chrispeels, 1977). It was suggested that the secreted xylanase remained bound to the aleurone cell wall during channel formation, and was only released following complete degradation of the outer wall. The enzymatic properties and structural similarity between these different barley xylanases have never been investigated. Furthermore, it is not possible from these studies to conclude if the viability and secretory role of the aleurone tissue during germination is due to its unique cell-wall composition and/or the regulated secretion and release of xylanases involved in cell-wall degradation.

In this study we have set out to identify the major endo-β-1,4-xylanase activity expressed in germinating barley grain, and to study in detail the timing of its synthesis and export in relationship to the physiological role of the aleurone tissue. Our investigations reveal that endo-β-1,4-xylanase is expressed later than the majority of hydrolases in the aleurone tissue during germination, and is synthesized as an inactive M_r 61 500 precursor in the cytoplasm. We demonstrate that both processing of the precursor, which involves removal of both N- and C-terminal peptides, and export of the mature, active M_r 34 000 enzyme from the aleurone cells, is regulated by aleurone programmed cell death (PCD).

Results

Characterization of a barley endo-β-1,4-xylanase purified from germinating barley

In order to identify and purify the major endo-β-1,4-xylanase activity in the germinating barley grain (*Hordeum vulgare* cv. Triumph), the activity of this enzyme was followed during germination at 15°C over a period of 16 days. The shoots and roots were removed in order to focus specifically on enzyme activity involved in endosperm metabolism. While endoxylanase activity was first detectable after 4 days of germination and started to increase significantly after 6 days, the activity level reached a peak only after 15 days (Figure 1a). The late accumulation of endoxylanase activity is in sharp contrast to α-amylase, whose synthesis is known to be initiated in the aleurone and scutellum epithelium shortly after imbibition (Figure 1a). Barley grain germinated for 14 days at 15°C was therefore used as the starting material for endoxylanase purification. A single endoxylanase activity was detected and purified to homogeneity by column chromatography (see Experimental procedures), and corresponded to a protein of apparent M_r of 34 000 on SDS–PAGE (Figure 1c, track 3). The purified protein had properties very similar to those of the M_r 34 000 barley xylanase purified by Benjavongkulchai and Spencer (1986) in terms of its pI of 4.6, optimum activity at pH 6.0, and sensitivity to inhibitors (not shown). The amino-terminal sequence of the M_r 34 000 protein was determined to be LDNAFPFGTCINTSVIQKPAFLDFFTNH. The sequences of several internal peptides, obtained by cleavage of the purified M_r 34 000 protein, were determined, and the C-terminal peptide sequence was found to be FLNLQKEWK. These peptide sequences were aligned with identity to the deduced sequence of the M_r 41 000 barley 1,4-β-D-xylan xylanohydrolase (EC 3.2.1.8) iso-

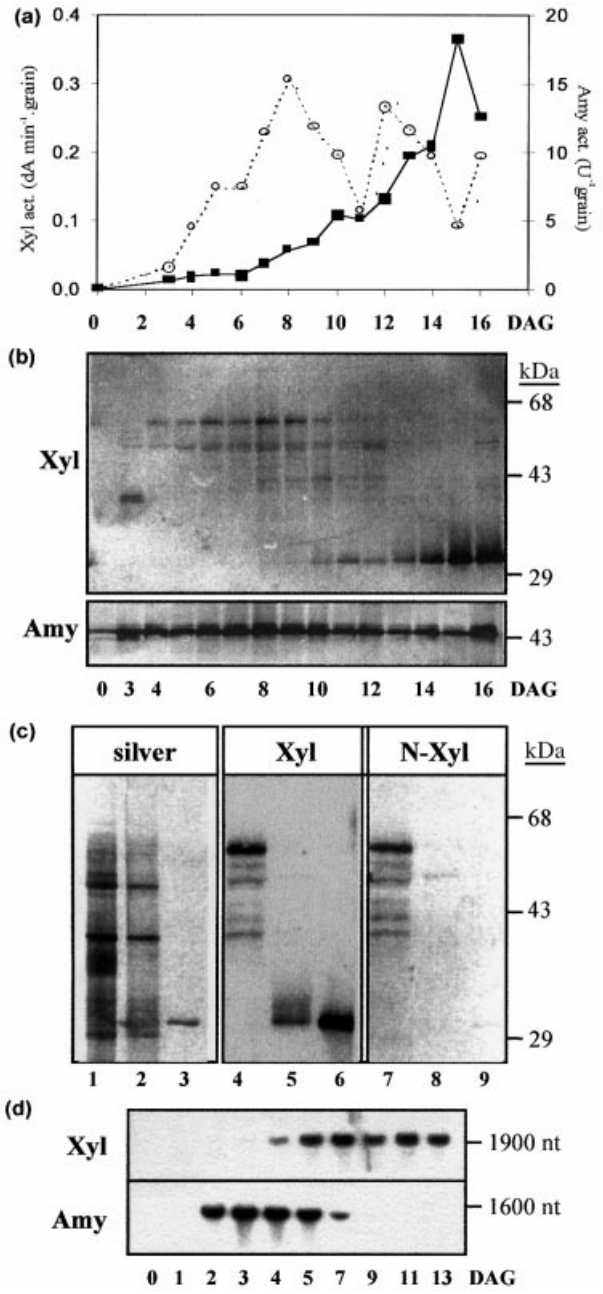

Figure 1. Expression of endo-β-1,4-xylanase and α-amylase in germinating barley grain.
(a) Endo-β-1,4-xylanase (■) and α-amylase (○) activity detected in extracts of grains of cv. Triumph (minus shoots and roots) from 0 to 16 days after germination (DAG) at 15°C.
(b) Western blot analysis of the grain extracts from (a) probed with anti-M_r 34 000 (Xyl) and anti-α-amylase (Amy).
(c) Extracts from (a) of grains germinated for 8 (tracks 1, 4, 7) and 14 days (tracks 2, 5, 8) analysed by SDS–PAGE/silver staining and Western blot analysis, probed with anti-M_r 34 000 (Xyl) or anti-N-peptide/M_r 41 000 (N-Xyl). Purified M_r 34 000 endo-β-1,4-xylanase analysed by silver-stained SDS–PAGE (track 3) and Western blot (track 6 and 9).
(d) Northern blot of RNA extracts of cv. Triumph grains from (a) probed with a partial *XYN*-1 cDNA, pFL0083 (Xyl) and α-amylase cDNA [790 bp *Sac*1 fragment of pM/C; Rogers (1985)] (Amy). Estimated mRNA size is indicated (nt = nucleotides).

enzyme X-1 (Slade *et al.*, 1989) encoded by the cDNA pMX1(1.6A) (Banik *et al.*, 1996).

Primary structure of barley endo-β-1,4-xylanase and its corresponding gene

A genomic clone (*Hv:XYN-1*) was isolated from a barley cv. Igri genomic library by hybridization to a partial cDNA clone encoding the M_r 34 000 endoxylanase (pFL400). The *Hv:XYN-1* gene was identified on a 5529 bp *Xba*I fragment whose nucleotide sequence (EMBL accession number AF287726) was scanned for ORFs using the GCG program. The first exon, initiated by an ATG codon, was predicted 126 nt 3′ downstream of the TATAA box, while two introns were predicted to interrupt the ORF (nucleotides +19/+101, +624/714) (Figure 2a). The predicted ORF sequence and introns in the *Hv:XYN-1* gene were confirmed by alignment with a full-length *XYN-1* cDNA sequence, obtained from overlapping 5′ RACE and the 3′ cDNA (pFL400) sequences (see Experimental procedures).

The *Hv:XYN-1* gene encodes an unexpectedly large polypeptide of M_r 61 500 with a predicted pI of 5.6. The determined N-terminal and internal peptide sequences of both the M_r 34 000 and the M_r 41 000 endoxylanase purified by Slade *et al.* (1989) show almost complete identity to the amino-acid sequence encoded by the *Hv:XYN-1* gene. The position of the peptide sequences (underlined residues shown in bold) of the M_r 34 000 protein with respect to the M_r 41 000 barley 1,4-β-D-xylan xylanohydrolase and the M_r 61 500 deduced translation product is shown in Figure 2(b). The M_r 41 000 enzyme, with the N-terminal sequence VYP...KLG (double underlined residues in Figure 2b), extends beyond the N-terminus of the M_r 34 000 protein. On the basis of these data it is proposed that the *Hv:XYN-1* gene encodes an M_r 61 500 polypeptide which subsequently undergoes a series of processing steps to yield the mature M_r 34 000 enzyme.

Synthesis and activity of barley endo-β-1,4-xylanase in germinating grain

To facilitate a detailed study of the synthesis of the M_r 34 000 endoxylanase, polyclonal antibodies were raised against the purified protein (Figure 1c, track 3) that recognize the enzyme in Western blots (Figure 1c, track 6). Additionally, polyclonal antibodies were raised against a 30-residue synthetic peptide (VYPVDHKARFRQLKD-KTDKARKRDVILKLG) homologous to the amino-terminus of the M_r 41 000 endoxylanase (Figure 2b; Slade *et al.*, 1989), to establish the relationship between the predicted precursor, intermediate and mature forms of barley endoxylanase.

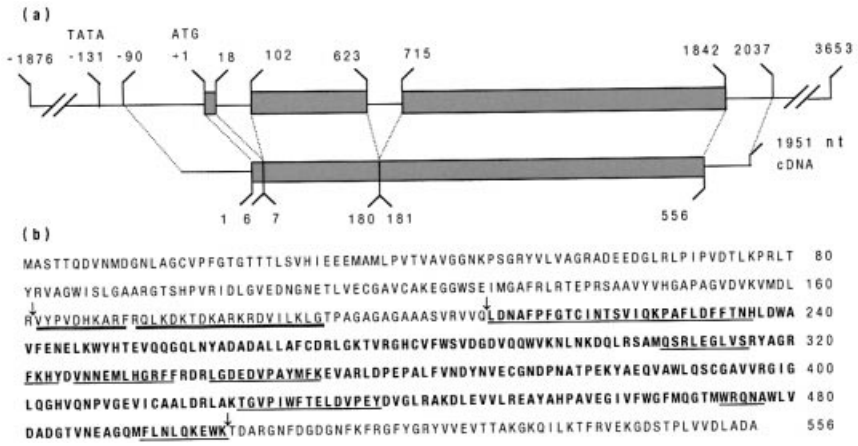

Figure 2. Structure of the endo-β-1,4-xylanase *Hv:XYN-1* gene and encoded protein.
(a) *Hv:XYN-1* gene structure, showing exons (shaded boxes) with nucleotide numbering, and *PXYN*-1 cDNA (given beneath) with amino-acid numbering.
(b) Deduced amino-acid sequence of XYN-1 endo-β-1,4-xylanase, showing processing sites (arrows) at the N-terminus of the M_r 41 000 (VYP.) and N- and C-terminus of the M_r 34 000 mature protein (residues in bold). Peptide sequences determined by authors (underlined) or by Slade *et al.* (1989) (double underlined) are indicated.

The activity profiles of endoxylanase and α-amylase in extracts of barley grain germinated at 15°C (Figure 1a) were compared with the relative levels of endoxylanase and α-amylase found by immunodetection, using antibodies raised against α-amylase (Juge *et al.*, 1993) and the M_r 34 000 endoxylanase (Figure 1b). The synthesis and accumulation of immunodetectable α-amylase mirrored the early detection and rise in α-amylase activity following the onset of germination.

In the first 4–7 days of germination, the polypeptides recognized by the M_r 34 000 antiserum had apparent M_r of approximately 60 000 and 50 000 (the presence of an approximately M_r 38 000 doublet in this 3-day sample was not reproducible and is considered an artefact). In 8-day-germinated grain, additional immunoreactive polypeptides of M_r approximately 40 000 appeared, and a polypeptide of M_r 34 000 was only detected after 10 days. The larger size and sequential appearance of the polypeptides in germinating grain, which share epitopes with the M_r 34 000 enzyme, suggest that they are the precursor and intermediate forms of the M_r 34 000 endoxylanase.

The level of detectable endoxylanase activity in grain remained low over the first 6 days, then steadily increased from 7 to 15 days germination. This suggests that barley endoxylanase may be synthesized as an inactive precursor which is activated during processing. From our sequence alignments it is likely that the M_r 41 000 endoxylanase is a processing intermediate of the mature M_r 34 000 endoxylanase. Since the M_r 41 000 endoxylanase was purified as an active enzyme from germinating grain (Slade *et al.*, 1989), we deduce that partial processing is sufficient to activate this enzyme. The rise in endoxylanase activity in germinated grain at 8 days coincides with the appearance of an immunodetectable approximately M_r 40 000 polypeptide.

Western blots of the grain extracts germinated for 8 and 14 days were then probed with either the M_r 41 000 endoxylanase N-terminal peptide antiserum or the M_r 34 000 antiserum. A major immunodetected polypeptide of approximately M_r 60 000 and minor amounts of polypeptides of M_r 40 000–60 000 in the 8-day extract were recognized by both antisera (Figure 1c, tracks 4 and 7). In the 14-day extract, a single polypeptide was detected with the M_r 34 000 antiserum (Figure 1c, track 5), that co-migrated with the purified and immunodetectable M_r 34 000 endoxylanase (track 6). The antiserum to the M_r 41 000 N-terminal peptide detected only trace amounts of a putative M_r 52 000 intermediate in the 14 day extract, but not the mature M_r 34 000 enzyme (Figure 1c, track 8). These data support the hypothesis that the major endoxylanase in barley grain is synthesized during germination as a precursor of M_r 61 500, and undergoes a series of processing steps to yield a mature enzyme of M_r 34 000. Furthermore, recognition of the larger precursor forms (M_r 40 000–60 000) by the antisera to both the M_r 41 000 N-terminal peptide and the M_r 34 000 confirms that the M_r 41 000 endoxylanase, purified by Slade *et al.* (1989), is a processing intermediate.

In order to provide additional evidence for the timing and regulation of endoxylanase synthesis during germination, Northern blot analysis was performed on total RNA extracted from the germinating grain samples. The blot was probed with a partial 3′ cDNA clone (pFL0083) encoding the C-terminal portion of the M_r 34 000 endoxylanase, identified in a barley aleurone cDNA library (see Experimental procedures), and with an α-amylase cDNA (Rogers, 1985) (Figure 1d). Transcripts of 1900 nucleotides that hybridized to the pFL0083 cDNA were detected after 4 days germination, coinciding with the appearance of M_r 55 000–60 000 polypeptides immunodetected by the M_r 34 000 antiserum (Figure 1b). Thus, compared with the AMY gene family, the *Hv:XYN-1* gene is transcriptionally upregulated relatively late in the germination programme.

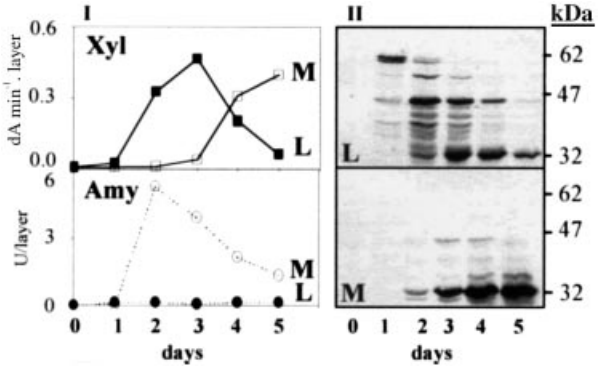

Figure 3. Expression of endo-β-1,4-xylanase and α-amylase in isolated aleurone layers.
Enzymatic activities (I) of endo-β-1,4-xylanase (Xyl) and α-amylase (Amy) and immunodetection (II) of endo-β-1,4-xylanase detected in extracts of GA_3-induced barley cv. Triumph aleurone layers (L) and incubation media (M) over a 5-day period.

Synthesis and activity of barley endo-β-1,4-xylanase in GA-induced aleurone layers

Isolated aleurone tissue is a valuable system to follow the temporal regulation of hydrolase synthesis as well as the fate of *de novo* synthesized proteins. Aleurone tissue isolated from *Hordeum vulgare* cv. Triumph was incubated at 25°C in the presence of gibberellic acid (GA_3). α-Amylase activity was secreted into the medium, reaching a maximum after 2 days, while endoxylanase activity was initially detected in extracts of aleurone tissue after 2 days incubation and rose to a maximum by 3 days (Figure 3, I). The appearance of endoxylanase activity in the medium was delayed by 2 days, suggesting that *de novo* synthesized endoxylanase is not immediately released. The proteins in the aleurone tissue and incubation medium were separated by SDS–PAGE, blotted and probed with M_r 34 000 antiserum (Figure 3, II). Immunodetectable polypeptides of M_r 60 000 were present in the aleurone after 1 days incubation, although endoxylanase activity was barely detectable. After 2 and 3 days incubation, immunodetectable polypeptides of M_r 40 000 and below were prominent within the tissue, concomitant with a sharp rise in endoxylanase activity. Release of endoxylanase activity into the medium was associated with the immunodetection of an M_r 34 000 polypeptide in the tissue and medium.

These data are consistent with the hypothesis that barley endoxylanase is expressed as an inactive M_r 61 500 precursor within the aleurone, which is activated during processing to the M_r 41 000 intermediate and is subsequently processed to the mature M_r 34 000 endoxylanase and simultaneously released. The synthesis of the barley endoxylanase as a precursor then raises the question of the function of the 213 N-terminal and 55 C-terminal amino-acid residues that are removed by post-translational processing.

Cellular localization of the Hv:XYN-1 *gene product*

As barley endoxylanase is synthesized in aleurone tissue, aleurone protoplasts provide a homologous model system for examining its synthesis and localization. Isolated protoplasts were co-transfected with plasmids pAMY6-4/XYN [1–556], comprising the *Hv:XYN-1* coding region (nt +1/+1845) translationally fused to the aleurone-specific AMY6-4 α-amylase promoter, and pAMY/[H(A12-M)ΔY13]GC-NOS encoding the cytoplasmic form of a heterologous thermostable (1,3–1,4)-β-glucanase (Jensen *et al.*, 1996). After 1 day of incubation, GA_3-induced transfected protoplasts over-expressed the M_r 61 500 endoxylanase precursor (Figure 4a, L), which co-migrated with endogenously expressed endoxylanase precursor detected in mock-transfected protoplasts (Figure 4a, track 5b). The immunodetection of lower M_r endoxylanase forms that accumulated in the protoplasts during the 5-day incubation indicates partial processing of the precursor. Transient expression of pAMY6-4/XYN [1–556] led to a progressive rise in endoxylanase activity within the protoplasts, which paralleled the appearance of processed intermediates. Endoxylanase activity first appeared in the medium at 3 days (Figure 4b), concomitant with the immunodetection of M_r 40 000 processing intermediates and the M_r 34 000 endoxylanase (Figure 4a, M).

The localization of co-expressed thermostable cytoplasmic (1,3–1,4)-β-glucanase was used to monitor the viability of the protoplasts during transient expression incubation. During the first 4 days, β-glucanase activity accumulated in the protoplasts (Figure 4c). β-Glucanase activity was detected in the medium from 3 days, and comprised 14% of the total β-glucanase activity by 4 days, indicating the onset of protoplast death and the release of intracellular proteins. As endoxylanase was released from protoplasts simultaneously with cytoplasmic β-glucanase, we conclude that its extracellular release was due to protoplast death rather than secretion. The percentage of endoxylanase activity found in the medium at 4 days (24% of total activity) was higher than that of β-glucanase. The predominance of the enzymatically active M_r 40 000 and 34 000 endoxylanases in the medium, in comparison to the protoplasts, indicates that processing and activation of the precursor coincides with extracellular release, leading to an increase in specific activity.

Parallel transfections were performed with plasmid pAMY/[αH(A12-M)ΔY13]GC-NOS encoding the thermostable (1,3–1,4)-β-glucanase fused to the α-amylase signal peptide. After 2 days incubation, the distribution of β-glucanase activity between the protoplasts and medium was 44 and 126 mU per 10^6 protoplasts, respect-

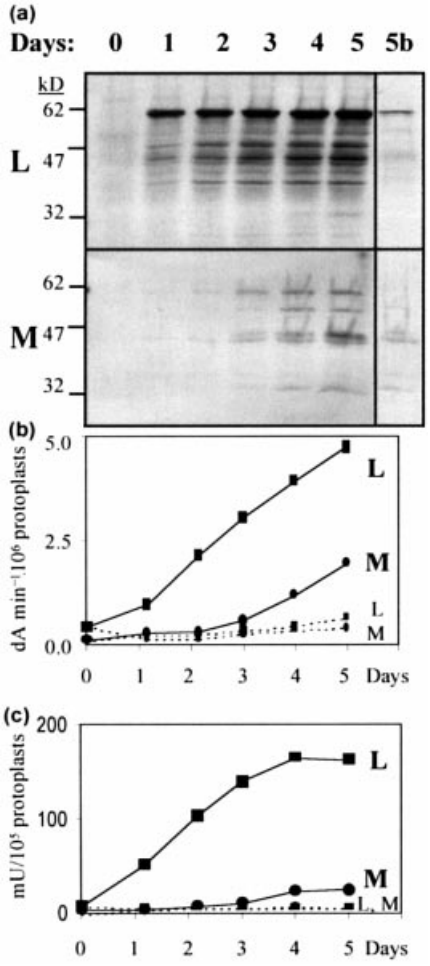

Figure 4. Transient expression of the barley endo-β-1,4-xylanase and heterologous thermostable β-glucanase in barley aleurone protoplasts.
Protoplasts were co-transfected with pAMY6-4/XYN [1–556] and pAMY/[H(A12-M)ΔY13]GC-NOS in a DNA ratio of 5 : 1.
(a) Western blot analysis of endo-β-1,4-xylanase in cell (L) and media (M) fractions of transfected protoplasts during 5 days incubation (5b: 5 days mock-transfected protoplasts), probed with anti-M_r 34 000.
(b) Endo-β-1,4-xylanase activity in cell (L) and media (M) fractions of transfected (solid lines) and mock-transfected (dashed lines) protoplasts from (a).
(c) Co-expressed (1,3–1,4)-β-glucanase activity in cell (L) and media (M) fractions of transfected (solid lines) and mock-transfected (dashed lines) protoplasts from (a).

ively. The secretion of 74% of the transiently expressed β-glucanase activity confirmed the secretory capacity of the protoplast preparation. In summary, these data support the conclusion that the *Hv:XYN-1* gene expresses an M_r 61 500 endoxylanase precursor, which is not targeted for extracellular secretion.

To reveal the intracellular localization of the *Hv:XYN-1* gene products, advantage was taken of green fluorescent protein (GFP) fusions expressed in aleurone protoplasts. As the location of an intracellular targeting signal within a polypeptide may be functionally important (Matsuoka and

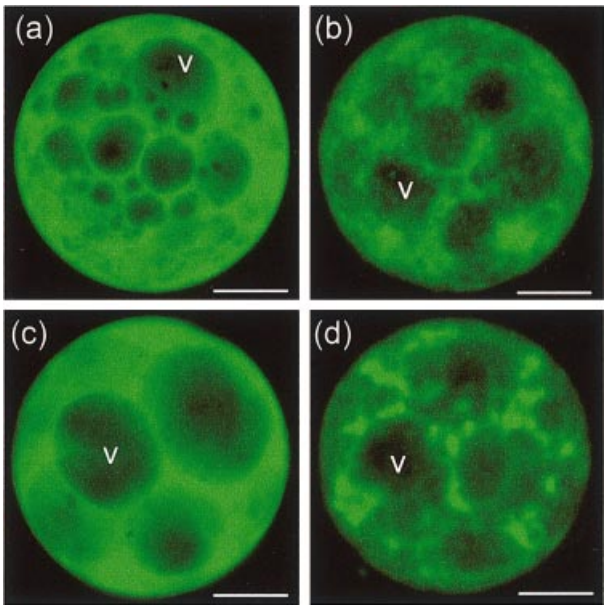

Figure 5. Transient expression of GFP fusion proteins in barley aleurone protoplasts.
Confocal images of aleurone protoplasts, incubated for 24 h with GA_3, following transfection with the chimeric GFP gene constructs (a) pXYN/[XYN1-556/L/GFP]; (b) pAMY6-4/[AMY/L/GFP]; (c) pXYN/[XYN1-213/GFP/XYN502-556]; (d) pAMY6-4/[AMY1-29/GFP/AMY419-423]. V, storage protein vacuoles; scale bar = 10 μm.

Neuhaus, 1999), two types of fusion were tested. The GFP-coding sequence was either fused 3′ to the *Hv:XYN-1* coding sequence via a spacer sequence from an *Erwinia carotovora* CelN cellulase gene (Olsen *et al.*, 1996), such that the two functional domains were separated by a 19-residue Thr/Pro-rich linker; or alternatively the GFP coding sequence was substituted for the mature M_r 34 000 coding sequence within the *Hv:XYN-1* gene. Aleurone protoplasts transfected with the GFP fusions were cultured with GA_3, harvested after 24 h and GFP expression was analysed by confocal microscopy. The recorded images (Figure 5a,c) reveal that both endoxylanase–GFP fusion proteins were located in the cytoplasm. α-Amylase–GFP fusions were similarly expressed as an α-amylase–linker–GFP fusion or as a GFP substitution, flanked by the signal peptide and five C-terminal residues of α-amylase. The confocal images (Figure 5b,d) show that both α-amylase–GFP fusions were located in subcellular compartments, characteristic of ER. As the M_r 61 500 endoxylanase precursor polypeptide is shown to be a cytoplasmic protein, alternative functions must be sought for the N- and C-terminal propeptides.

Functional analysis of the barley M_r *61 500 endo-β-1,4-xylanase propeptides*

To investigate the functional importance of the propeptide domains, a series of *Hv:XYN-1* gene mutants (fused to the

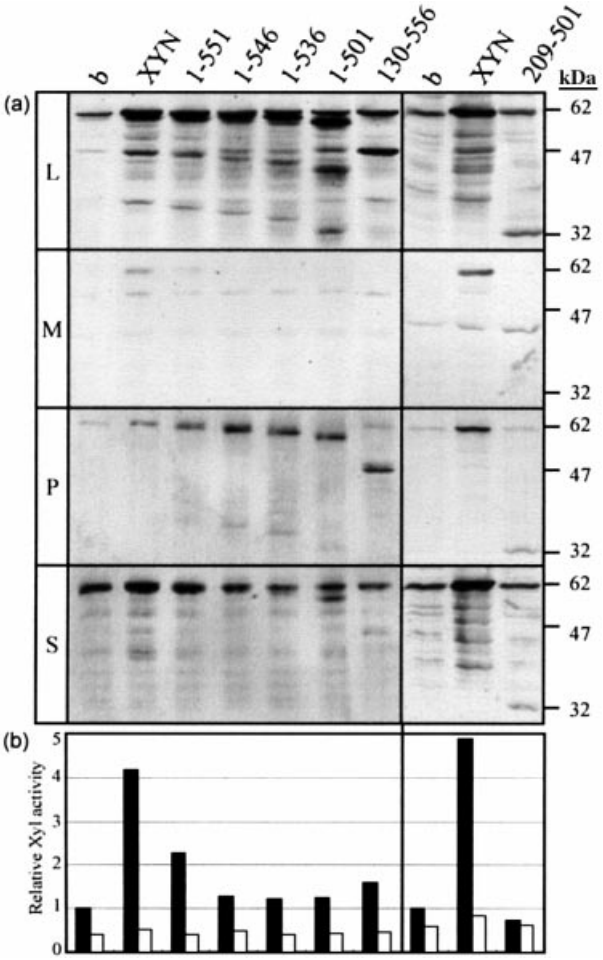

Figure 6. Transient expression of full-length and truncated barley endo-β-1,4-xylanase in barley aleurone protoplasts.
(a) Western blot analysis of endo-β-1,4-xylanase in cell (L) and media (M) fractions of protoplasts, mock-transfected (b) or transfected with pAMY6-4/XYN plasmids encoding full-length XYN (1–556, 61.5 kDa), and truncated 1–551 (60.9 kDa), 1–546 (60.4 kDa), 1–536 (59.4 kDa), 1–501 (55.4 kDa), 130–556 (48.0 kDa), 209–501(33.4 kDa) endo-β-1,4-xylanase, and incubated for 2 days in the presence of GA_3. Analysis of the cell fraction includes the cell pellet (P) and supernatant (S) of protoplasts after sonication and centrifugation at 1000 *g* for 5 min. Western probe: anti-M_r 34 000.
(b) Endo-β-1,4-xylanase activity in cell (■) and media (□) fractions of the transfected protoplasts from (a) relative to mock-transfected protoplasts, whose activity is set to 1.

AMY6-4 promoter) encoding endoxylanase, truncated at either the N- and/or C-terminus, were expressed in GA_3-induced aleurone protoplasts. The transfected protoplasts were incubated for 2 days, sufficient for the partial processing and activation of barley endoxylanase when Over-expressed in protoplasts (Figure 4b). Expression of the full-length M_r 61 500 endoxylanase, as well as each of the truncated polypeptides, was immunodetected and their migration on SDS–PAGE corresponded to their predicted M_r (Figure 6a, L,P). Over-expression of the full-length M_r 61 500 endoxylanase strongly enhanced endoxylanase activity compared to the mock-transfected control (Figures 4b, 6b). However, the step-wise truncation of the 55 amino-acid residue C-terminal propeptide strongly reduced endoxylanase activity (compare XYN with 1–551, 1–546, 1–536, 1–501 in Figure 6b). Similarly, a truncated endoxylanase lacking the first 129 amino acids of the N-terminal 213 residue propeptide (130–556) was inactive when expressed in protoplasts (Figure 6b). When the mature M_r 34 000 enzyme was expressed, where both N- and C-terminal propeptides were deleted (209–501), the immunodetectable protein was also inactive (Figure 6b). The loss of endoxylanase activity could not be attributed to a failure to express the truncated hydrolase (Figure 6a, L), or to its secretion into the medium (Figure 6a, M).

As endoxylanase activity was only found in protoplasts over-expressing the full-length M_r 61 500 endoxylanase, deletion of the propeptide domains may have impaired protein folding. To test this hypothesis, we examined the solubility of the full-length endoxylanase and the truncated forms expressed in barley protoplasts, on the grounds that insolubility is an indicator of misfolded proteins. The protoplasts expressing the different endoxylanase polypeptides were sonicated and centrifuged to yield a cell pellet and supernatant. The full-length endoxylanase expressed by the endogenous protoplast XYN-1 gene was immunodetected in all the supernatant fractions, consistent with its being a soluble enzyme (Figure 6a, S versus P). While the majority of over-expressed full-length endoxylanase was also found in the supernatant, a significant proportion of the truncated endoxylanases were immunodetected in the pellet fraction, indicating that the propeptide domains may be required for protein folding (Figure 6a, P versus S).

Processing, activation and release of barley endo-β-1,4-xylanase

The events that bring about the processing of the M_r 61 500 precursor and release of the mature M_r 34 000 endoxylanase in aleurone tissue (Figures 1b, 3) may depend on GA-induced PCD seen in this tissue during germination (Wang *et al.*, 1998). The morphological and biochemical events resulting in aleurone PCD have been convincingly demonstrated in isolated aleurone protoplasts in response to GA (Bethke *et al.*, 1999; Fath *et al.*, 1999). The association of these events with the processing and release of endogenously expressed endoxylanase was thus examined.

Aleurone protoplasts incubated for 2 days in the presence of GA_3, at a plating density of 2.5×10^4 protoplasts per well, contained numerous small protein storage vacuoles (PSVs; Figure 7a, 1) characteristic of protoplasts at an early developmental stage in the progression to PCD (Bethke *et al.*, 1998). Extracellular acidification, used as a

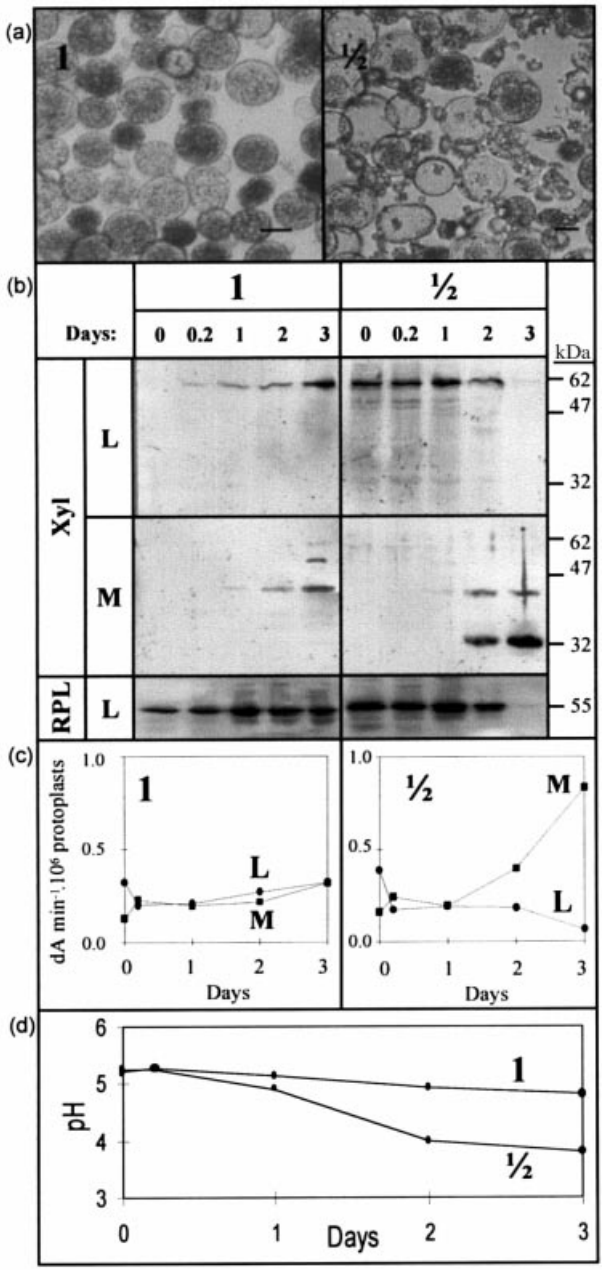

Figure 7. Release, processing and activity of endo-β-1,4-xylanase synthesized by GA_3-induced barley aleurone protoplasts.
(a) Light microscope image of protoplasts after 2 days culture at a plating density of $2.5 \cdot 10^4$ protoplasts per well (1) or $1.25 \cdot 10^4$ protoplasts per well ($\frac{1}{2}$) (bar = 20 μm).
(b) Western blot analysis of endo-β-1,4-xylanase in cell (L) and media (M) fractions of protoplasts cultured at 1 or 0.5 plating density over 3 days, using anti-M_r 34 000 X-1 (Xyl). Cell fractions were also probed with anti-RPL60 (RPL).
(c) Endo-β-1,4-xylanase activity detected in cell (L) and media (M) fractions of protoplasts from (b).
(d) pH of protoplasts incubation medium from (b).

monitor of GA-induced aleurone development, was also limited in protoplasts incubated for 3 days at this plating density (Figure 7d, 1). The precursor endoxylanase accumulated in these protoplasts over the 3 days, but processing to the M_r 34 000 enzyme was not detected and endoxylanase activity remained low (Figure 7b,c, 1). This expression pattern mirrors that seen in Figure 4, where GA-induced protoplasts accumulated the over-expressed endoxylanase over 3 days, but precursor processing remained minimal and the activity of the endogenously expressed gene (in mock-transformants) was barely detectable.

GA_3-induced aleurone protoplasts incubated at half plating density (1.25×10^4 protoplasts per well) were highly vacuolated after 2 days (Figure 7a) characteristic of protoplasts just prior to PCD (Bethke *et al.*, 1999). Furthermore, acidification of the medium was significantly greater in these protoplasts (Figure 7d), indicating a more rapid progression of the developmental events leading to PCD. Endogenously expressed endoxylanase was also immunodetected in these protoplasts (Figure 7b). However, after 2–3 days incubation, when the protoplasts were highly vacuolated, the endoxylanase precursor was both processed to the mature M_r 34 000 enzyme and released into the medium, suggesting that maturation of this enzyme coincides with the final stage of aleurone protoplast PCD. Endoxylanase activity rose sharply in parallel with maturation and release of the hydrolase (Figure 7b,c), further indicating that this enzyme is synthesized as an inactive precursor that is activated during processing.

Cell death, the final event in aleurone protoplast PCD, follows the loss of protoplast membrane integrity (Bethke *et al.*, 1999). The occurrence and timing of cell death in our two protoplast incubations was monitored by measuring retention of the resident protein, calreticulin (RPL), in the protoplasts. Calreticulin was lost from protoplasts within 24 h of their reaching the highly vacuolate stage after 2 days' incubation at half plating density (Figure 7b). Protoplasts delayed in the developmental progression of PCD by higher plating density (1), showed no evidence of calreticulin loss. Cell death is thus implicated as the mechanism for endoxylanase release from aleurone protoplasts, as this process coincided with the loss of protoplast membrane integrity.

Endo-β-1,4-xylanase maturation is mediated by cysteine endoproteases

A series of proteolytic cleavages brings about the progressive maturation of the endoxylanase synthesized in the aleurone of germinating grain (Figure 1b). We investigated the conditions required for processing of the M_r 61 500 precursor present in extracts of aleurone tissue of 3-day imbibed, embryo-less cv. Himalaya grain (Figure 8). When the aleurone extract was incubated at pH 5.4, the precursor was partially processed to an approximately M_r 50 000 intermediate, while at pH 4.2 a circa M_r 40 000 protein was

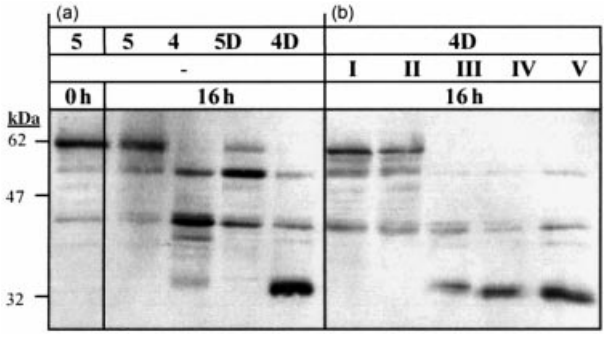

Figure 8. *In vitro* processing of the M_r 61 500 endo-β-1,4-xylanase precursor.
Western blot analysis of endo-β-1,4-xylanase in: (a) extracts of cv. Himalaya aleurone (L) incubated for 0 or 16 h at 25°C in APM buffer adjusted to pH 5.4 (5) or 4.2 (4), and with 4 mM DTT (4D, 5D), Western blot probe, anti-M_r 34 000; (b) extracts of cv. Himalaya aleurone incubated as in (a) in the presence of protease inhibitors: E-64 (I), leupeptin (II), PMSF (III), EDTA (IV) or pepstatin (V).

the major processing intermediate detected in Western blots (Figure 8a). The processing steps yielding the mature M_r 34 000 enzyme were strongly enhanced by the presence of a reducing agent (DTT). This implies that protease(s) capable of processing the M_r 61 500 precursor were present in the tissue, but in an inactive form and/or in a separate cell compartment.

Although several different types of endoproteases may contribute to the observed processing of endoxylanase, yielding various processed forms, maturation to the M_r 34 000 enzyme was found to be sensitive to inhibitors of cysteine proteases (E-64) and serine/cysteine proteases (leupeptin), and less sensitive to inhibitors of serine proteases (PMSF), metallo-proteases (EDTA) and aspartate proteases (pepstatin) (Figure 8b). Thus the major protease activity in germinating aleurone which is required for processing and activation of endoxylanase has the properties of cysteine endoproteases, because the observed protease activity was dependent on acidic pH, enhanced by reducing conditions, and sensitive to cysteine protease inhibitors.

Discussion

The major barley endo-β-1,4-xylanase in germinating barley grain has an apparent M_r *of 61 500 and is processed to a mature enzyme of* M_r *34 000*

Endo-β-1,4-xylanase activity was barely detectable in barley grains during the early stages of germination at 15°C, but its activity began to rise at 7 days and peaked at 15 days. This enzyme activity was purified to homogeneity and had an M_r of 34 000 and enzymatic properties similar those of an M_r 34 000 xylanase secreted by GA-induced isolated aleurone layers (Benjavongkulchai and Spencer, 1986).

Several lines of evidence suggest that this M_r 34 000 endoxylanase is derived from the M_r 41 000 endoxylanase purified by Slade *et al.* (1989), which in turn is derived from a larger precursor. All the determined peptide sequences of the purified M_r 34 000 enzyme can be identified within the deduced amino-acid sequence of the M_r 41 000 endoxylanase (Banik *et al.*, 1996). Sequence analysis of a full-length barley endoxylanase cDNA clone (1951 bp), *XYN-1* and the corresponding genomic clone *Hv:XYN-1* isolated in this study, revealed the primary structure of the precursor of the M_r 34 000. The gene encodes an M_r 61 500 polypeptide, whose amino-acid sequence includes those of the M_r 41 000, deduced from a homologous 1630 bp cDNA pMX1[1.6 A] (Banik *et al.*, 1996) and the M_r 34 000 endoxylanases. As pMX1[1.6 A] cDNA is a partial cDNA, the full-length ORF and 5′ intron were not recognized in the previously described endoxylanase genomic clone (Banik *et al.*, 1996, Banik *et al.*, 1997).

Northern analysis of germinating grain showed that Hn:XYN-1 is transcribed at 4 days germination and onwards. Antibodies were raised against both the purified M_r 34 000 endoxylanase, and a peptide corresponding to the N-terminus of the M_r 41 000 endoxylanase. The polypeptides immunodetected by anti-M_r 34 000 in 4 days germinated grain were between M_r 50 000 and 60 000, consistent in size with the *XYN-1* encoded polypeptide. Additional polypeptides of circa M_r 40 000 were immunodetected after 8 days germination, while the M_r 34 000 enzyme was not detected until 10 days germination. The sequential appearance of these immunologically related polypeptides during grain germination supports the hypothesis that barley endoxylanase is expressed as a precursor which undergoes post-translational processing. The antibody to the M_r 41 000 N-terminal peptide recognized similar polypeptides of M_r 40 000–60 000 in germinated grain, but did not detect the mature M_r 34 000 enzyme which lacks this peptide epitope. As the M_r 41 000 endoxylanase was purified from grain germinated for a shorter period, 5 days at 26°C (Slade *et al.*, 1989), we predict that it is a stable precursor of the M_r 34 000 enzyme. In isolated aleurone layers induced with GA_3, the first immunodetectable polypeptides had an M_r of circa 60 000, while a series of lower M_r forms appeared on further incubation. The M_r 34 000 enzyme was first detected in the layers after 3 days incubation, followed by its release into the medium, from where it has previously been purified (Benjavongkulchai and Spencer, 1986).

These data provide the first evidence that the major endo-β-1,4-xylanase in germinating barley grain is synthesized as an M_r 61 500 precursor polypeptide and that the mature enzyme of M_r 34 000 is the result of a series of

processing events. Based on the amino-acid sequences of the N- and C-terminus of the M_r 34 000 polypeptide, the mature protein sequence is flanked by N- and C-terminal propeptides of 213 and 55 residues, respectively. Although the *XYN-1* gene was expressed at 4 days germination, when endoxylanase precursors were first immunodetectable, endoxylanase activity was minimal. The appearance of endoxylanase intermediates of circa M_r 40 000 at 8 days germination coincided with a rise in activity, suggesting that processing is required for activation of the enzyme.

The M_r *61 500 endo-β-1,4-xylanase is a cytoplasmic pro-enzyme whose N- and C-terminal propeptide regions facilitate correct folding and activation of the precursor*

Transient expression of the *Hv:XYN-1* encoded endoxylanase in GA_3-induced aleurone protoplasts revealed that the M_r 61 500 precursor accumulates within the cell. This is in marked contrast to the majority of other hydrolases secreted by aleurone cells during germination (Fincher, 1992). Partial processing of the M_r 61 500 precursor was also observed in protoplasts, but the mature endoxylanase appeared in the protoplast medium only after prolonged incubation. The intracellular localization of the M_r 61 500 precursor, expressed as a GFP fusion protein in transfected protoplasts, was compared with that of α-amylase–GFP fusion proteins using confocal microscopy. While α-amylase–GFP fluorescence was typical of ER localization, endoxylanase–GFP was limited to the cytoplasm. The first 50 amino-acid residues of the M_r 61 500 precursor were submitted for signal peptide analysis at http://www.cbs.dtu.dk/services/SignalP. The absence of a signal peptide, characteristic of secreted proteins, supports our conclusion that the M_r 61 500 endoxylanase does not enter the secretory pathway.

The rise in endoxylanase activity in germinating grain, isolated aleurone layers or protoplasts was concomitant with the processing of the M_r 61 500 endoxylanase rather than its synthesis. This suggests that M_r 61 500 endoxylanase initially accumulates in the aleurone as an inactive zymogen. Partial processing is sufficient to activate the endoxylanase, as the M_r 41 000 processing intermediate was isolated as an active enzyme (Slade *et al.*, 1989). The function of the propeptide domains of the M_r 61 500 precursor was investigated by expressing N- or C-terminal truncated forms in aleurone protoplasts. After 2 days incubation, synthesis and activation of the wild-type enzyme was clearly detectable. Although the truncated polypeptides were also expressed and partially processed, they were largely inactive. A C-terminal truncation of just five amino acids was sufficient to halve endoxylanase activity. In contrast to the full-length endoxylanase, a significant proportion of the truncated forms expressed in protoplasts could be pelleted by low-speed centrifugation, indicative of insoluble misfolded proteins. These data suggest that the propeptide domains of the M_r 61 500 precursor may both facilitate protein folding and maintain the pro-enzyme in an inactive state. The vacuolar carboxypeptidase Y from *Saccharomyces cerevisiae* is one of the best-studied examples of a zymogen, where the pro-region facilitates *in vivo* folding of the native state and prevents enzymatic activity (Winther and Sørensen, 1991). The 91-amino-acid residue pro-region of CPY appears to lower the energy barriers for folding and unfolding in a similar manner to a molecular chaperone. Site-directed mutagenesis of putative processing sites may provide further insight into the function of the endoxylanase propeptide domains.

Maturation and release of barley endo-β-1,4-xylanase coincides with aleurone PCD

As our studies showed that barley M_r 61 500 endoxylanase does not enter the secretory pathway, alternative mechanisms leading to its release from the aleurone at a late stage of germination were considered. Recent studies have revealed that aleurone cells enter a developmental pathway in response to GA, culminating in PCD characterized by the metamorphosis of the PSVs and DNA degradation (Bethke *et al.*, 1999; Fath *et al.*, 1999; Wang *et al.*, 1996, Wang *et al.*, 1998). Acidification of the PSVs, leading to the activation of resident cysteine endoproteases and nucleases, α-amylase secretion and the onset of PSV fusion, are early events of this developmental pathway (Bethke *et al.*, 1998). Cell death, observed in highly vacuolated aleurone protoplasts, results from the abrupt loss of plasma membrane integrity and protoplast collapse (Bethke *et al.*, 1999).

We have followed the GA-induced events of extracellular acidification by aleurone protoplasts (Heimovaara-Dijkstra *et al.*, 1994), their vacuolization and ultimate death in relation to the fate of endogenously expressed barley endoxylanase. To monitor the final step of protoplast death, namely protoplast disintegration, we followed the loss of the ER-localized, soluble protein calreticulin. While the accumulation of the M_r 61 500 precursor was detected inside GA_3-induced protoplasts, the processing and release of the M_r 34 000 mature enzyme were observed only in highly vacuolated protoplasts in association with disintegration of their membranes.

The close timing of the maturation and release events suggests that loss of cell membrane integrity accompanying PCD releases aleurone vacuolar proteases capable of processing the M_r 61 500 precursor. The M_r 61 500 precursor, present in GA_3-induced aleurone layers, was processed following homogenization and incubation of the tissue, which supports this conclusion. This *in vitro* maturation of the M_r 61 500 precursor, which was

enhanced at pH 4.2 and by 4 mM DTT, was particularly sensitive to inhibitors of cysteine proteases. The cysteine endoproteases detected in isolated vacuoles from barley aleurone (Bethke *et al.*, 1996) are thus good candidates to process barley endoxylanase.

The role of barley endo-β-1,4-xylanase in germinating barley grain

We have demonstrated that the major barley endoxylanase synthesized in germinating barley grain is released from the aleurone at a stage of germination when α-amylase secretion is declining. This suggests that endoxylanase is not involved in the degradation of regions of the outer aleurone cell wall which are proposed to facilitate hydrolase secretion by this tissue (Gubler *et al.*, 1987). Furthermore, a delay in endoxylanase release from the aleurone until after the cessation of hydrolase secretion may be required to maintain the structural integrity of the inner aleurone cell wall, which remains intact until the cessation of secretion (Fincher, 1992). Differences in composition and molecular organization, which underlie the differential degradation of the inner and outer layers of the aleurone cell wall, have yet to be determined.

The hydrolysis of cell-wall polymers following the release of endoxylanase may provide the growing embryo with additional carbohydrate nutrients late in the germination process, which may be advantageous under poor germination conditions. This suggests a novel role for barley aleurone PCD in regulating the release of intracellular hydrolases involved in reserve carbohydrate mobilization. Barley limit dextrinase, which is also expressed in germinating aleurone, has recently been shown to have a presequence typical of a plastid transit peptide (Burton *et al.*, 1999). If limit dextrinase is indeed intracellularly targeted, only its release following aleurone PCD would allow this starch-debranching enzyme to contribute to the hydrolysis of limit dextrins *in vivo*.

Experimental procedures

Assay of endo-β-1,4-xylanase and α-amylase in germinating barley and GA-induced aleurone layers

For whole-grain analyses, *Hordeum vulgare* L. cv. Triumph grains were sterilized with $AgNO_3$ and germinated at 15°C, as described by Slade *et al.* (1989), in the dark. Samples (five grains minus roots and shoots) were homogenized in 2 ml ice-cold buffer A (50 mM Na-malate, 50 mM NaCl, 2 mM $CaCl_2$, 3 mM NaN_3 pH 5.2) using an ultra-Turrax and cell debris were removed by centrifugation (14 000 *g* for 10 min).

For GA-induced aleurone layer analyses, *H. vulgare* cv. Triumph grains were de-embryonated, halved, sterilized (1 min in 70% EtOH, 30 min in 50% H_2SO_4; each followed by four washes in H_2O) and imbibed in H_2O containing 50 μg ml^{-1} ampicillin, 5 μg ml^{-1} nystatin at 25°C for 3.5 days. The starchy endosperm was then removed and aleurone layers from five grains were cultured in 2 ml buffer B (20 mM Na-succinate, 20 mM $CaCl_2$, 50 μg ml^{-1} ampicillin, 5 μg ml^{-1} nystatin, 10 μM GA_3 pH 4.2) at 25°C. Samples of culture medium and aleurone layers were harvested and analysed separately. Aleurone layers were washed and homogenized in buffer B and the extracts clarified by centrifugation (14 000 *g* for 10 min).

Aliquots (50 μl) of the grain or aleurone extracts were mixed with 50 μl buffer A containing 0.2% BSA and incubated with 3 mg birchwood AZCL-xylan (Megazyme, Bray, Co. Wicklow, Ireland) at 40°C for 2–16 h. Reactions were stopped with 0.9 ml 1% alkaline Tris, clarified (200 *g* for 15 min) and measured spectrophotometrically [ΔA_{595} (1 cm cuvette) = 2.04 × ΔA_{620} (200 μl per titertek well)]. Endoxylanase activity is expressed as ΔA_{595} min^{-1} per grain or per aleurone layer in a 1 ml (stopped) reaction.

Grain and aleurone layer extracts were diluted in buffer A containing 0.02% BSA and assayed for α-amylase activity using the ceralpha:α-amylase assay kit (Megazyme) as described by Soor and Hinke (1990), where 1 mU is defined as the α-amylase activity required to release 1 μmole *p*-nitrophenol min^{-1} from *p*-nitrophenyl maltoheptaoside in the presence of excess α-glucosidase and glucoamylase, as described by the supplier.

Purification of barley endo-β-1,4-xylanase

Grain of cv. Triumph (approximately 500 g), germinated for 14 days (as above), were homogenized in 2 vol ice-cold buffer C (50 mM NaAc, 5 mM NaN_3, 10 mM EDTA, 3 mM β-mercaptoethanol, 3 mM PMSF pH 5.0) for 3 min in a Waring blender. After 30 min incubation at 0°C, the extract was clarified by centrifugation and precipitated with ammonium sulfate, taking the 20–40% cut. The precipitate was resuspended in buffer C, desalted (Sephadex G25 coarse, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK), concentrated by ultrafiltration (YM-10, Amicon, Millipore [UK], Walford, Herts, UK), and endoxylanase was then purified by two successive FPLC (Amersham Pharmacia Biotech) separations: MonoQ column (Pharmacia HR5/5), eluted with a 0–0.35 M NaCl gradient, pooling the 80–120 mM NaCl fractions containing the single activity peak, and a Merck Fractogel HW 50-superfine gel-filtration column (Pharmacia HR10/30) equilibrated and eluted in 20 mM Bis-Tris/HCl, 0.1 M NaCl pH 6.2. Fractions containing the single activity peak were pooled and concentrated on a MonoQ column (as above).

During purification, endoxylanase activity was assayed as described above. The amino-acid sequence of the N-terminus and tryptic- and cyanogen bromide-cleavage peptides of the purified enzyme were determined by Edman degradation (by Eurosequence B.V., Groningen, The Netherlands) and by Dr Ib Svendsen (Department of Chemistry, Carlsberg Laboratory, Denmark).

Preparation of barley endoxylanase antibodies and immunodetection

A synthetic peptide, having the sequence of the first 30 amino-acid residues of the M_r 41 000 endoxylanase (Slade *et al.*, 1989), was prepared by solid-phase synthesis using Fmoc-protected amino acids in an automated Milligan 9050 Continuous Flow Synthesizer (Millipore Co., Milford, MA, USA). The peptide was purified, essentially as described by Zegers *et al.* (1991), and the amino-acid composition determined as described by Janssen *et al.* (1986). To enhance the antigenicity of the peptide, it was conjugated to keyhole limpet hemocyanin, using *m*-maleimidobenzoyl-*N*-

hydroxysuccinimide as a coupling agent reactive for the terminal cysteine residue added to the peptide (Boersma *et al.*, 1992).

New Zealand white rabbits were subcutaneously immunized with either the peptide conjugate (250 μg) or the purified M_r 34 000 endoxylanase (100 μg) emulsified (1 : 1, v/v) with Freund's adjuvant followed by three booster immunizations. Immunodetection assays were performed on samples separated on 10% SDS–PAGE (Laemmli, 1970), transferred to nitrocellulose by semi-dry blotting. Blots were soaked in phosphate-buffered saline with 0.05% Tween 20 containing successively 1% BSA, antibody and alkaline phosphatase-conjugate or horse radish peroxidase-conjugate (Promega, Madison, WI, USA) and stained with nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Promega, USA) or Enhanced Chemi-Luminescence (Amersham Pharmacia Biotech, UK). Polyclonal antibodies raised against N-terminal peptide of M_r 41 000 barley endoxylanase (N-Xyl), barley endoxylanase (Xyl), barley α-amylase (Juge *et al.*, 1993) and tobacco calreticulin (anti-RPL60; Denecke *et al.*, 1995) were used at 1 : 500, 1 : 2000, 1 : 10 000 and 1 : 10 000 dilutions, respectively.

Isolation and analysis of RNA, cDNA and genomic clones

Total RNA, isolated according to Hensgens and Van Os-Ruygrok (1989), was separated on a denaturing glyoxal/DMSO 1.2% agarose gel (15 μg per lane), blotted to nylon membrane and hybridized to random-primed cDNA probes after standard protocols (Ausubel *et al.*, 1997). Partial barley endoxylanase cDNA clones (pFL0083, pFL400) were identified among ESTs in a cDNA library from 24 h GA_3-induced aleurone layers of cv. Himalaya (Leah *et al.*, 1991). The barley genomic clone (*Hv:XYN-1*) was screened from a Lambda FIX II, cv. Igri library (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. A 5′ region of the endoxylanase cDNA was obtained from total RNA of germinated cv. Himalaya grain by RT–PCR using the One-Step RT–PCR kit (Qiagen, Hilden, Germany) with forward 5′-CCACTCGCGTAAGAT-3′ and reverse 5′-ACGCGGTAAGTGAGA-3′ primers based on the *Hv:XYN-1* gene sequence. DNA sequences were determined by dideoxy chain termination using the Ampli Cycle Sequencing Kit (PE Biochemicals, Foster City, CA, USA) and sequencer (model 373A, Applied Biosystems, Foster City, CA, USA) and analysed by GCG (Devereaux *et al.*, 1984).

DNA constructs

The nucleotide sequences and protein translation products of all genes described in this article are deposited at EMBL with the following designated names and EMBL accession numbers. Barley endoxylanase DNA sequences: partial cDNA clones pFL400 (EMBL: AF287723) and pFL0083 (EMBL: AF287722); full-length cDNA pXYN-1 (EMBL: AF287731); and genomic clone *Hv:XYN-1* (EMBL: AF287726). Wild-type and mutant barley endoxylanase expression constructs fused to nt –737/–1 of the α-amylase promoter, AMY6-4 (Rogers, 1985;), [expressed amino-acid residues are shown in brackets]: pAMY6-4/XYN [1–556] (EMBL: AF287724), pAMY6-4/XYN [1–551] (EMBL: AF287725), pAMY6-4/XYN [1–546] (EMBL: AF287721), pAMY6-4/XYN [1–536], pAMY6-4/XYN [1–501] (EMBL: AF287728), pAMY6-4/XYN [130–556] (EMBL: AF370887), pAMY6-4/XYN [209–501] (EMBL: AF370888). Thermostable 1,3–1,4-β-glucanase [H(A12-M)ΔY13] expression construct, fused to the promoter and signal peptide [α] coding sequence of the high-pI α-amylase gene (Jensen *et al.*, 1996), is referred to as pAMY/[αH(A12-M)ΔY13]GC-NOS and without signal peptide as pAMY/[H(A12-M)ΔY13]GC-NOS. GFP-fusion protein expression constructs fused to the *Hv:XYN-1* (nt –1000/–1) or AMY6-4 (nt –737/–1) promoters: pXYN/[XYN1-556/L/GFP], pAMY6-4/[AMY/L/GFP], pXYN/[XYN1-213/GFP/XYN502-556] (EMBL: AF287730), pAMY6-4/[AMY1-29/GFP/AMY419-423] (EMBL: AF287729), where GFP is a fluorescence-enhanced variant encoded by the synthetic gene hGFP-C3 (Nielsen *et al.*, 1999) and L is the linker peptide from *Erwinia carotovora* CelN cellulase. The DNA constructs were assembled from PCR-amplified cDNA and genomic sequences according to standard molecular cloning protocols described by Ausubel *et al.* (1997), and their nucleotide sequences were verified by double-strand sequencing.

Preparation and analysis of transfected aleurone protoplasts

Hordeum vulgare L. cv. Himalaya grain (1988 harvest, Department of Agronomy, Washington State University, Pullman WA, USA) were de-embryonated, halved, sterilized (1 min 70% EtOH, 30 min 0.2% NaOCl, 10 min 10 mM HCl; each step followed by four washes in H_2O) and imbibed in H_2O containing 50 μg ml^{-1} ampicillin, 5 μg ml^{-1} nystatin at 25°C for 3.5 days prior to removal of the starchy endosperm. Harvested layers from 200 grains were rinsed in H_2O.

Protoplasts were prepared from isolated cv. Himalaya aleurone layers by cellulase treatment according to Jacobsen *et al.* (1985) and Skriver *et al.* (1991), modified by adjusting the isolation buffers to 800 mOsm with mannitol. Aliquots (1 ml) of protoplast ($2.5–10 \cdot 10^5$ ml^{-1}) were transfected at 0°C with 100–200 μg plasmid DNA or mock-transfected with 100–200 μg sheared salmon sperm DNA using PEG-mediated DNA uptake (Lee *et al.*, 1997). Transfected protoplasts were resuspended in 1 ml APM buffer (10 mM MES, 0.385% (w/v) Gamborg B5, 0.1 M glucose, 10 mM L-arginine, 20 mM $CaCl_2$, 0.67 M mannitol, 50 μg ml^{-1} ampicillin, 5 μg ml^{-1} nystatin, pH 5.4, 1080 mOsm) with 10 μM GA_3 and cultured in 96-well microtitre plates (100 μl per well), at 25°C in the dark. The culture medium was harvested from the wells after mixing and pelleting the protoplasts (10 *g*, 1 min). Protoplasts were washed, pelleted, and lysed (5 sec sonication) in APM buffer.

Aliquots (50 μl) of the incubation media and protoplast extracts were assayed for endoxylanase activity as described for germinating grain extracts. Expression of the thermostable H(A12-M)ΔY13 β-glucanase activity was assayed in aliquots (15 μl), mixed with 15 μl assay buffer (50 mM NaAc, 5 mM $CaCl_2$, 0.2% BSA pH 6.0) following 10 min incubation at 56°C to inactivate endogenous glucanase activity. The β-glucanase assay was performed in microtitre plates at 56°C with 30 μl Azo-barley glucan substrate (Megazyme) for 15–120 min and terminated with 180 μl stopping reagent, and 200 μl per well of the clarified reactions were measured spectrophotometrically (A_{620}). β-Glucanase activity is expressed as mU per 10^{-5} protoplasts (mU ml^{-1} = (ΔA_{620} h^{-1}) 42.19), where 1 U is defined as the release of 1 μmole reducing sugar equivalent min^{-1} by a standard malt extract determined according to Megazyme's instructions.

Transient expression of GFP fusion proteins in aleurone protoplasts was visualized by confocal laser scanning microscopy as described by Nielsen *et al.* (2000), where the specimens were excited with monochromatic light (λ 488 nm), and GFP fluorescence emission was detected at λ 500–530 nm, thereby avoiding aleurone autofluorescence between 555 and 605 nm.

In vitro *endoxylanase processing*

Extracts containing M_r 61 500 endoxylanase precursor were prepared as follows: aleurone layers were isolated from 200 imbibed cv. Himalaya grain as described above, rinsed and homogenized by ultra-Turrax in 20 ml H_2O at 0°C, and the extract was clarified by centrifugation (14 000 *g* for 10 min).

The aleurone extract (9 μl) was incubated with 9 μl APM buffer pH 5.4 or 4.2 (adjusted with 3 μl 200 mM Na succinate buffer pH 4.1), with or without addition of 4 mM DTT and adjusted to a final volume of 30 μl with H_2O. Aleurone extracts incubated with the following inhibitors were similarly tested: 10 μg ml^{-1} E-64 (Roche Diagnostics, Basel, Switzerland), 10 μg ml^{-1} leupeptin (Sigma, St Louis, MO, USA), 200 μg ml^{-1} PMSF (Sigma), 2 mM EDTA, 10 μg ml^{-1} pepstatin (Sigma).

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References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1997). *Current Protocols in Molecular Biology*. New York: Wiley Interscience.

Bacic, A. and Stone, B.A. (1981) Isolation and ultrastructure of aleurone cell walls from wheat and barley. *Aust. J. Plant Physiol.* **8**, 453–474.

Banik, M., Garrett, T.P.J. and Fincher, G.B. (1996) Molecular cloning of cDNAs encoding (1→4)-β-xylan endohydrolases from the aleurone layer of germinated barley (*Hordeum vulgare*). *Plant Mol. Biol.* **31**, 1163–1172.

Banik, M., Li, C.-D., Langridge, P. and Fincher, G.B. (1997) Structure, hormonal regulation, and chromosomal location of genes encoding barley (1→4)-β-xylan endohydrolases. *Mol. Gen. Genet.* **253**, 599–608.

Benjavongkulchai, E. and Spencer, M.S. (1986) Purification and characterization of barley-aleurone xylanase. *Planta*, **169**, 415–419.

Bethke, P.C., Hillmer, S. and Jones, R.L. (1996) Isolation of intact protein storage vacuoles from barley aleurone. *Plant Physiol.* **110**, 521–529.

Bethke, P.C., Scanson, S.J., Hillmer, S. and Jones, R.L. (1998) From storage compartment to lytic organelle: the metamorphosis of the aleurone protein storage vacuole. *Ann. Bot.* **82**, 399–412.

Bethke, P.C., Lonsdale, J.E., Fath, A. and Jones, R.L. (1999) Hormonally regulated programmed cell death in barley aleurone cells. *Plant Cell*, **11**, 1033–1045.

Boersma, W.J., Bogaerts, W.J., Bianchi, A.T. and Claassen, E. (1992) Adjuvant properties of stable water-in-oil emulsions: evaluation of the experience with Specol. *Res. Immunol.* **143**, 503–512.

Burton, R.A., Zhang, X.-Q., Hrmova, M. and Fincher, G.B. (1999) A single limit dextrinase gene is expressed both in the developing endosperm and in germinating grains of barley. *Plant Physiol.* **119**, 859–871.

Carpita, N., Sabularse, D., Montezinos, D. and Delmer, D. (1979) Determination of the pore size of cell walls of living plant cells. *Science*, **205**, 1144–1147.

Dashek, W.V. and Chrispeels, M.J. (1977) Gibberellic-acid-induced synthesis and release of cell-wall-degrading endoxylanase by isolated aleurone layers of barley. *Planta*, **134**, 251–256.

Denecke, J., Carlsson, L.E., Vidal, S., Hoglund, A.S., Ek, B., van Zeijl, M.J., Sinjorgo, K.M.C. and Palva, E.T. (1995) The tobacco homolog of mammalian calreticulin is present in protein complexes *in vivo*. *Plant Cell*, **7**, 391–406.

Devereaux, J., Haerberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387–395.

Fath, A., Bethke, P.C. and Jones, R.L. (1999) Barley aleurone cell death is not apoptotic: characterization of nuclease activities and DNA degradation. *Plant J.* **20**, 305–315.

Fincher, G.B. (1992) Cell wall metabolism in barley. In *Barley: Genetics, Biochemistry, Molecular Biology and Biotechnology* (Shewry, P.R., ed.). Wallingford UK: CAB International, pp. 413–437.

Fincher, G.B. and Stone, B.A. (1986) Cell walls and their components in cereal grain. *Adv. Cereal Sci. Technology*, **8**, 207–295.

Gubler, F., Ashford, A.E. and Jacobsen, J.V. (1987) The release of α-amylase through gibberellin-treated barley aleurone cell walls. *Planta*, **172**, 155–161.

Heimovaara-Dijkstra, S., Heistek, J.C. and Wang, M. (1994) Counteractive effects of ABA and GA_3 on extracellular pH and malate in barley aleurone. *Plant Physiol.* **106**, 359–365.

Hensgens, L.A.H. and van Os-Ruygrok, P.E. (1989) Isolation of RNA from different rice tissues. *Rice Gen. Newslett.* **6**, 163–168.

Jacobsen, J.V., Zwar, J.A. and Chandler, P. (1985) Gibberellic-acid-responsive protoplasts from mature aleurone of Himalaya barley. *Planta*, **163**, 430–438.

Janssen, P.S.L., van Nispen, J.W., Melchert, P.A.T.A., van den Boogaardt, B., Hamelink, R.L.A.E. and Janssen, B.C. (1986) HPLC analysis of phenylthiocarbamyl PTC amino acids. *Chromatographia*, **22**, 345–350.

Jensen, L.G., Olsen, O., Kops, O., Wolf, N., Thomsen, K.K. and von Wettstein, D. (1996) Transgenic barley expressing a protein-engineered, thermostable (1,3–1,4)-β-glucanase during germination. *Proc. Natl Acad. Sci. USA*, **93**, 3487–3491.

Juge, N., Søgaard, M., Chaix, J.-C., Martin-Eauclaire, M.-F., Svensson, B., Marchis-Mouren, G. and Guo, X.-J. (1993) Comparison of barley malt α-amylase isoenzymes 1 and 2: construction of cDNA hybrids by *in vivo* recombination and their expression in yeast. *Gene*, **130**, 159–166.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991) Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**, 1564–1573.

Lee, B., Murdoch, K., Topping, J., Kreis, M. and Jones, M.G.K. (1997) Transient gene expression in aleurone protoplasts

isolated from developing caryopsis of barley and wheat. *Plant Mol. Biol.* **13**, 21–29.

Matsuoka, K. and Neuhaus, J.-M. (1999) Cis-elements of protein transport to the plant vacuoles. *J. Exp. Bot.* **50**, 165–174.

Nielsen, K., Olsen, O. and Oliver, R. (1999) A transient expression system to assay putative antifungal genes on powdery mildew infected barley leaves. *Physiol. Mol. Plant Patho.* **54**, 1–12.

Nielsen, K.A., Nicholson, R.L., Carver, T.L.W., Kunoh, H. and Oliver, R.P. (2000) First touch: an immediate response to surface recognition in conidia of *Blumeria graminis*. *Physiol. Mol. Plant Pathol.* **56**, 63–70.

Olsen, O., Thompsen, K.K., Weber, J., Duus, J.Ø., Svendsen, I., Wegener, C. and von Wettstein, D. (1996) Transplanting two unique β-glucanase catalytic activities into one multienzyme, which forms glucose. *Bio/Technol.* **14**, 71–76.

Rogers, J.C. (1985) Two barley α-amylase gene families are regulated differently in aleurone cells. *J. Biol. Chem.* **260**, 3731–3738.

Schuurink, R.C., Bakhuisen, R., Libbenga, K.R., Boulanger, F. and Sinjorgo, K.M.C. (1997) Dormant barley aleurone shows heterogeneity and a specific cytodifferentiation. *J. Cereal Sci.* **25**, 27–36.

Selvig, A., Aarnes, H. and Lie, S. (1986) Cell wall degradation in endosperm of barley during germination. *J. Inst. Brew.* **92**, 185–187.

Skriver, K., Olsen, F.L., Rogers, J.C. and Mundy, J. (1991) *Cis*-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proc. Natl Acad. Sci. USA*, **88**, 7266–7270.

Slade, A.M., Høj, P.B., Morrice, N.A. and Fincher, G.B. (1989) Purification and characterization of three (1→4)-β-D-xylan endohydrolases from germinated barley. *Eur. J. Biochem.* **185**, 533–539.

Soor, S.K. and Hinke, M.T. (1990) Microplate reader-based kinetic determination of α-amylase activity: application to quantitation of secretion from rat parotic acini. *Anal. Biochem.* **188**, 187–191.

Wang, M., Oppedijk, B.J., Lu, X., Van Duijn, B. and Schilperoort, R.A. (1996) Apoptosis in barley aleurone during germination and its inhibition by abscisic acid. *Plant Mol. Biol.* **32**, 1125–1134.

Wang, M., Oppedijk, B.J., Caspers, M.P.M., Lamers, G.E.M., Boot, M.J., Geerlings, D.N.G., Bakhuizen, B., Meijer, A.M. and Van Duijn, B. (1998) Spatial and temporal regulation of DNA fragmentation in the aleurone of germinating barley. *J. Exp. Bot.* **49**, 1293–1301.

Winther, J.R. and Sørensen, P. (1991) Propeptide of carboxypeptidase Y provides a chaperone-like function as well as inhibition of the enzymatic activity. *Proc. Natl Acad. Sci. USA*, **88**, 9330–9334.

Zegers, N.D., Claassen, E., Neelen, C. *et al.* (1991) Epitope prediction and confirmation for the human androgen receptor: generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochem. Biophys. Acta*, **1073**, 23–32.

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