

Hormonal Regulation, Processing, and Secretion of Cysteine Proteinases in Barley Aleurone Layers

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Barley aleurone layers synthesize and secrete several proteases in response to gibberellic acid (GA₃). Two major cysteine proteinases designated EP-A (37,000 M_r) and EP-B (30,000 M_r) have been described [Koehler and Ho (1988). *Plant Physiol.* 87, 95–103]. We now report the cDNA cloning of EP-B and describe the post-translational processing and hormonal regulation of both cysteine proteinases. Three cDNAs for cysteine proteinases were cloned from GA₃-induced barley aleurone layers. Genomic DNA gel blot analysis indicated that these are members of a small gene family with no more than four to five different genes. The proteins encoded by two of these clones, pHVEP1 and 4, are 98% similar to each other and are isozymes of EP-B. The proteins contain large preprosequences followed by the amino acid sequence described as the mature N terminus of purified EP-B, and are antigenic to EP-B antiserum. The results of pulse-chase experiments indicated that the post-translational processing of large prosequences proceeds in a multistep fashion to produce the mature enzymes. Processing intermediates for EP-B are observed both in the aleurone layers and surrounding incubation medium, but only mature EP-A is secreted. The regulation of synthesis of EP-A, EP-B, and other aleurone cysteine proteinases was compared at the protein and mRNA levels. We conclude that barley aleurone cysteine proteinases are differentially regulated with respect to their temporal and hormonally induced expression.

INTRODUCTION

The hormonal regulation of hydrolases involved in the enzymatic degradation and mobilization of endosperm storage macromolecules in germinating cereal grains has been the focus of intense research (see Fincher, 1989, for a recent review). Because isolated barley aleurone layers synthesize and secrete many hydrolases in response to exogenously added gibberellic acid (GA₃) *in vitro*, they have been used as a model system for many of these investigations. The synthesis and/or secretion of α -amylase isozymes, nuclease, (1-3,1-4)- β -D-glucan endohydrolase, acid phosphatases, and proteases have been shown to be enhanced by GA₃. Abscisic acid (ABA) has been shown to antagonize most of the GA₃ responses (Ho, 1983). GA₃ enhances the accumulation of mRNA for α -amylase isozymes (Muthukrishnan et al., 1983; Chandler et al., 1984; Rogers et al., 1985), (1-3,1-4)- β -D-glucan endohydrolase (Stuart et al., 1986), and a putative cysteine proteinase designated aleurain (Rogers et al., 1985).

Barley aleurone layers synthesize a complex group of proteases. Jacobsen and Varner (1967) were the first to demonstrate that protease active against the wheat prolamine gliadin, at pH 4.8, is synthesized *de novo* in barley

(cv Himalaya) aleurone layers in response to GA₃. Approximately 80% of this protease activity is secreted, and 85% of the secreted protease activity is inhibited by bromate, an inhibitor of cysteine proteinases. The time course and the effect of GA₃ concentration on the accumulation of secreted protease are similar to that of α -amylase. Sundblom and Mikola (1972) detected at least four protease activities secreted from GA₃-treated barley aleurone layers that are qualitatively similar to those extracted from green malt. Three of these, described as sulfhydryl proteinases (endopeptidases) with pH optima of 3.9 and 5.0 to 6.5, accounted for the majority of the enzyme activity. The fourth was thought to be a metallo-proteinase. Hammerton and Ho (1986) also found that proteinase activity secreted from GA₃-treated Himalaya aleurone layers was inhibited by cysteine proteinase inhibitors and had a pH optimum of 4.0. Proteinase activity which is sensitive to cysteine proteinase inhibitors was identified as three bands on a native gel but as a single band of 37,000 M_r by 10% SDS-PAGE. This 37,000 M_r cysteine proteinase, designated EP-A, was subsequently purified (Koehler and Ho, 1988). The three isozymes were found to be closely related, with overlapping N-terminal amino acid sequences, indicating that they could be the product of a single gene. During the purification of EP-A, a second, more abundant proteinase with a M_r of 30,000 was observed. This cysteine proteinase,

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designated EP-B, has also been purified and its N-terminal amino acid sequence is 90% similar to that of EP-A (Koehler and Ho, 1990). Both EP-B and EP-A have pH optima in the range of 4.5 to 5.0.

Recently, cysteine proteinases with M_r of 29,000 and 30,000 and pH optima of 4.5 and 3.8, respectively, have also been purified from green malt of different barley cultivars (Pouille and Jones, 1988; Phillips and Wallace, 1989). Because the N-terminal amino acid sequence has not been determined for these green malt proteinases, it is not known to what extent they might be related to EP-B. All of these purified cysteine proteinases are capable of hydrolyzing hordein, the major seed storage prolamine, located primarily in the endosperm (Pouille and Jones, 1988; Phillips and Wallace, 1989; Koehler and Ho, 1990).

The hormonal regulation of expression of these purified cysteine proteinases has not yet been addressed. Two cDNA clones encoding putative cysteine proteinases, aleurain (Rogers et al., 1985) and pHV14 (Chandler et al., 1984; P. Chandler and L. Huiet, personal communication), have been identified whose corresponding mRNA in Himalaya aleurone layers are regulated by GA_3 . Another cDNA clone, pHV13, overlaps with the last 260 bp of pHV14 (a partial cDNA clone) to complete the coding region (P. Chandler and L. Huiet, personal communication). The proteins corresponding to aleurain and pHV14-13 have not yet been purified. Antibody raised against a fusion protein of aleurain expressed in *Escherichia coli* has been used to show that aleurain is not secreted (Holwerda et al., 1990).

In this report we present the characterization of two cDNA clones, pHVEP clones 1 and 4, which encode isozymes of EP-B. The complexity of this gene family was assessed by DNA gel blot analysis. Comparison of the deduced amino acid sequence of the clones with the purified protein has revealed that they are synthesized with large prosequences that are post-translationally cleaved to form the mature protein. This processing was analyzed by *in vivo* pulse-label and chase experiments, and multiple processing intermediates were observed. We describe the hormonal induction of the two groups of purified cysteine proteinases, EP-B and EP-A, at both the protein and mRNA levels. Using clone-specific probes, we were able to compare the hormonal regulation of different members of the EP-B family and aleurain at the mRNA level.

RESULTS

Isolation and Characterization of Proteinase cDNA Clones

A cDNA library was constructed from poly(A)⁺ RNA isolated from 24-hr GA_3 -treated aleurone layers. Preliminary time-course experiments indicated that *in vitro* translatable mRNA for both EP-B and EP-A isozymes was present in

relatively high abundance in this tissue. Because the N-terminal amino acid sequence of EP-B is similar to the sequence deduced for pHV14 in the region which most likely encodes the mature N terminus (only two out of 22 amino acids were different) (Koehler and Ho, 1990), it was anticipated that pHV14 could be used as a probe to isolate a cDNA clone encoding EP-B. Furthermore, pHV14 insert DNA was capable of hybrid-selecting mRNA that when translated *in vitro*, produced a protein immunoprecipitable by EP-B antiserum but not by EP-A antiserum (S.M. Koehler and T.-H.D. Ho, unpublished data). Therefore, the cDNA library was screened with pHV14 insert DNA. Out of eight positive clones, four had inserts of 1.4 kb to 1.5 kb, which also hybridized under relatively low stringency conditions to an oligonucleotide probe (20-mer) directed against the 5' region of pHV14 (gift of P. Chandler). Because pHV14 hybridizes to mRNA of approximately 1.4 kb to 1.5 kb (Chandler et al., 1984), these potentially full-length clones, designated pHVEP clones 1, 4, 3, and 7, were further characterized.

In vitro transcription and translation of these clones and immunoprecipitation of the resulting proteins are shown in Figure 1. The results revealed that pHVEP clones 1, 4, and 7 encode proteins that immunoprecipitate with EP-B antiserum and comigrate with protein immunoprecipitated by EP-B antiserum from *in vitro* translation of RNA from GA_3 -treated aleurone layers. These proteins migrate with a M_r of about 42,500 during electrophoresis in 10% SDS-PAGE, which is similar to the size predicted for the primary translation product of the protein encoded by pHV14-13. Protein immunoprecipitated by EP-A antiserum from products of *in vitro* translation of aleurone layer RNA has a higher M_r of about 47,000 (Figure 1, lane 15). These results indicated that these three clones encode proteins that are most likely related to EP-B. The *in vitro* transcription and translation product of pHVEP3 migrates as a major band of 19,000 M_r and a minor band of 22,000 M_r , which were slightly immunoprecipitable by EP-B and EP-A antisera (lanes 5 and 6).

Restriction endonuclease mapping and preliminary sequencing of the 5' and 3' ends of these clones indicated that pHVEP3 could be a full-length clone of pHV14 (despite the discrepancy in the predicted molecular weight of the encoded protein and the molecular weight of the protein resulting from the *in vitro* transcription and translation of pHVEP3). The other three clones had some obvious restriction site and sequence differences.

The nucleotide sequence of pHVEP clones 1 and 4 is shown in Figure 2A. pHVEP7 was sequenced completely in one direction and partially in the opposite direction. Its sequence appears to be identical to pHVEP4 except for the following 12 additional bp at the 5' end: 5'-AGCA-AGCTAGCC-3'. Both pHVEP clones 1 and 4 contain 89-bp to 91-bp 5'-untranslated regions with 100% sequence identity, followed by translation initiation codons for the only long open reading frames. The deduced amino acid

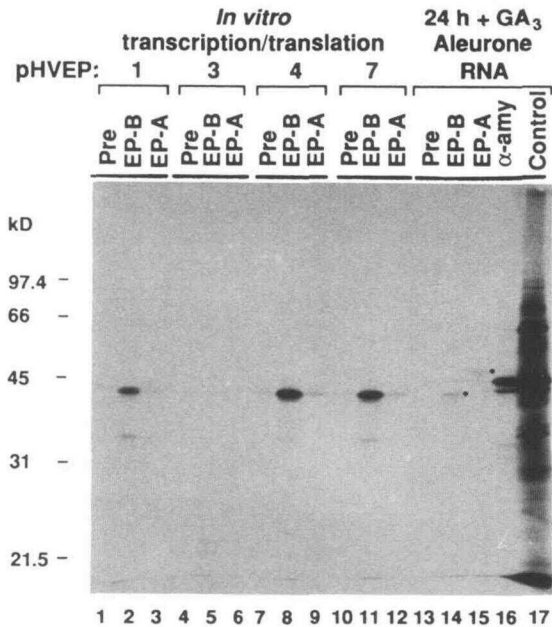


Figure 1. Immunoprecipitation of In Vitro Transcribed and Translated pHVEP Clones by Barley Aleurone Cysteine Proteinase Antisera.

In vitro transcripts of pHVEP clones 1 and 7 driven by the T3 promoter, and clones 3 and 4 driven by the T7 promoter were in vitro translated, and equal amounts of TCA-precipitable cpm were immunoprecipitated with preimmune serum (pre) or antisera against barley cysteine proteases EP-B or EP-A as indicated above each lane (lanes 1 to 12). An in vitro translation of RNA from aleurone layers incubated for 24 hr with GA₃ was immunoprecipitated with preimmune serum, EP-B antiserum, EP-A antiserum, and α -amylase antiserum as indicated (lanes 13 to 16). The positions of prepro-EP-B and prepro-EP-A are indicated by the dots. The same aleurone RNA in vitro translation reaction without immunoprecipitation is shown in lane 17. The migration of Bio-Rad low molecular weight protein standards is indicated on the left.

sequences of both clones are shown in Figure 2B. Both clones appear to encode functional cysteine proteinases that are 98% similar at the amino acid level. The overall similarity of the nucleotide sequence of these two clones is 94%. The first 396 bp of translated sequence for both clones encoding the prepropeptide of the proteases are 98% identical, and there is only one nonconservative substitution in the prepropeptide region. The following 66 bp are identical, and their deduced amino acid sequence is identical to that determined for the N terminus of purified EP-B (Koehler and Ho, 1990), lending further support to the conclusion that these clones encode isoenzymes of EP-B. In the mature protein coding regions of these clones, there are only five nonconservative nucleotide substitutions. Four of these, and a 6-bp insert in pHVEP4, result

in four amino acid differences and two additional amino acids, respectively, for the pHVEP4-encoded protein in the carboxy-terminal region.

The 3'-untranslated regions of these clones are more divergent, having 73% nucleotide identity with nine gaps introduced for optimal alignment. Both clones share approximately 90% overall sequence similarity to pHV14-13, but the 3'-untranslated region of pHV14-13 is more similar to the 3'-untranslated region of pHVEP1 (85%) compared with pHVEP4 (60%). To study the regulation of expression of each of these clones independently, oligonucleotide probes (18-mers) specific for pHVEP clones 1 and 4 (positions shown in Figure 2A) and pHV14 were synthesized. The pHV14-oligo (18-mer) was designed to hybridize to a region of pHV14 that shares 66% similarity to the 522-bp to 539-bp region of the other two clones. This region in pHV14 reads 5'-GACCGGAAGCTGGTGTC-3' (P. Chandler and L. Huiet, personal communication).

In contrast to most mammalian and plant mRNAs, no polyadenylation addition signals were present within 30 bp upstream of the poly(A) tail; however, AATAAA and AATAA sequences were identified 162 bp and 164 bp upstream of the poly(A) tail of pHVEP1 and pHVEP4, respectively. It is not certain whether AAUAAA-like sequences facilitate polyadenylation of plant mRNAs (Joshi, 1987; Hunt and MacDonald, 1989; Kursheed and Rogers, 1989).

The pHVEP4- and pHVEP1-encoded preproteins have predicted molecular weights of 40,466 and 40,328 and a total of 373 and 371 amino acids, respectively. The predicted molecular weights are approximately 2000 less than estimated by the molecular weight of the primary in vitro transcription/translation products of the clones (Figure 1). EP-B is a secreted protein and, therefore, probably contains a signal peptide that is cleaved cotranslationally in vivo. The first 28 amino acids deduced from these two clones have all the features of typical eukaryotic signal sequences described by Von Heijne (1983, 1985). These predicted signal peptides are followed by 104 amino acids of prosequence, and the mature proteins contain 241 and 239 amino acids for pHVEP4 and pHVEP1, respectively. Immediately preceding the mature N terminus is a potential Asn-linked glycosylation site in each, but the mature proteins lack such sites. The predicted molecular weights of the mature proteins encoded by pHVEP4 and pHVEP1 are 25,433 and 25,295, respectively, and the proteins are predicted to be acidic with slightly different charges. These properties are similar to purified EP-B, whose M_r is estimated to be 25,000 to 27,000 as determined by 10% to 20% linear gradient SDS-PAGE and which resolves into two bands with isoelectric points of 4.6 to 4.7 by native isoelectric focusing (Koehler and Ho, 1990).

Genomic DNA Gel Blot Analysis

Genomic DNA gel blot analysis, shown in Figure 3, was performed to determine the complexity of this gene family.

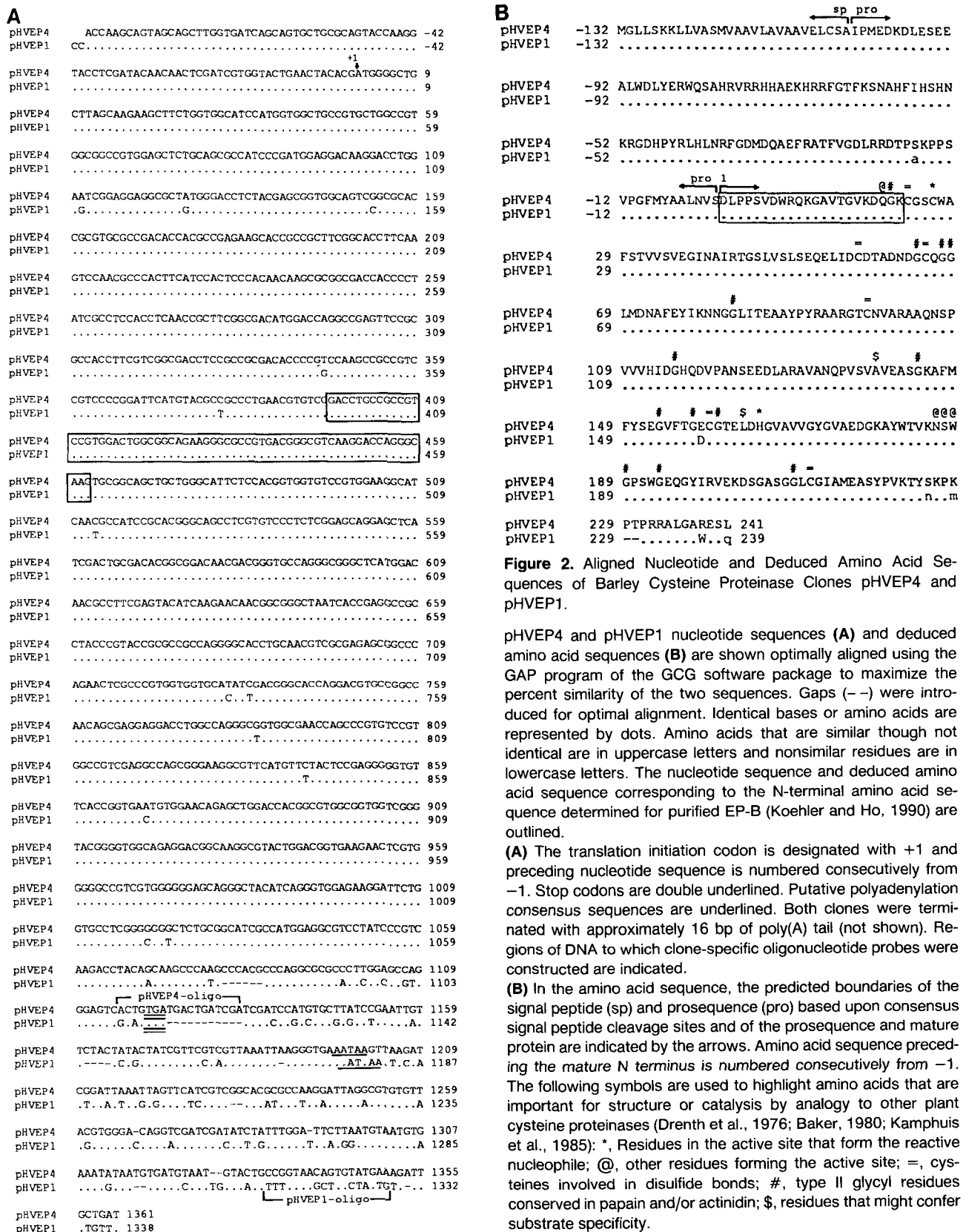


Figure 2. Aligned Nucleotide and Deduced Amino Acid Sequences of Barley Cysteine Proteinase Clones pHVEP4 and pHVEP1.

pHVEP4 and pHVEP1 nucleotide sequences (**A**) and deduced amino acid sequences (**B**) are shown optimally aligned using the GAP program of the GCG software package to maximize the percent similarity of the two sequences. Gaps (—) were introduced for optimal alignment. Identical bases or amino acids are represented by dots. Amino acids that are similar though not identical are in uppercase letters and nonsimilar residues are in lowercase letters. The nucleotide sequence and deduced amino acid sequence corresponding to the N-terminal amino acid sequence determined for purified EP-B (Koehler and Ho, 1990) are outlined.

(**A**) The translation initiation codon is designated with +1 and preceding nucleotide sequence is numbered consecutively from -1. Stop codons are double underlined. Putative polyadenylation consensus sequences are underlined. Both clones were terminated with approximately 16 bp of poly(A) tail (not shown). Regions of DNA to which clone-specific oligonucleotide probes were constructed are indicated.

(**B**) In the amino acid sequence, the predicted boundaries of the signal peptide (sp) and prosequence (pro) based upon consensus signal peptide cleavage sites and of the prosequence and mature protein are indicated by the arrows. Amino acid sequence preceding the mature N terminus is numbered consecutively from -1. The following symbols are used to highlight amino acids that are important for structure or catalysis by analogy to other plant cysteine proteinases (Drenth et al., 1976; Baker, 1980; Kamphuis et al., 1985): *, Residues in the active site that form the reactive nucleophile; @, other residues forming the active site; =, cysteines involved in disulfide bonds; #, type II glycol residues conserved in papain and/or actinidin; \$, residues that might confer substrate specificity.

A blot containing Himalaya barley genomic DNA digested with restriction enzymes that do not cut within the inserts of pHVEP clones 1, 3, 4, or 7 was probed with pHVEP4 and pHVEP1. Copy number reconstructions for pHVEP clones 1, 4, and 3 were included, and, not surprisingly, both probes cross-hybridized to some degree with all three clones. At least four different EcoRI, BamHI, and HincII restriction fragments, greater than 2.8 kb in each case, hybridized to pHVEP1. Two of the BamHI fragments (7 kb and 3.2 kb) and HincII fragments (3.6 kb and 2.8 kb) hybridized exclusively to the pHVEP1 probe, whereas the other two BamHI fragments (8 kb and 5.6 kb) and HincII fragments (6.5 kb and 4.2 kb) and three of the EcoRI fragments also hybridized to the pHVEP4 probe. The largest HincII fragment could be a doublet, and there appears to be slight differences in the mobility of the second largest EcoRI fragment hybridizing to pHVEP1 and pHVEP4. All of the fragments appeared to hybridize with equal intensity as the single copy reconstructions. These results indicate that there are no more than four (and possibly five) different genes present in approximately a single copy each with homology to pHVEP1 and pHVEP4.

Post-Translational Processing of ProEP-B and ProEP-A

Pulse-chase experiments were employed to investigate the post-translational processing and secretion of pro-EP-B and pro-EP-A in GA₃-treated aleurone layers. ProEP-B processing is shown in Figure 4. After a 1-hr pulse, after 5 hr of incubation with GA₃, protein from aleurone layer extracts immunoprecipitable by EP-B antiserum had a M_r of 42,500 (lane 4) and comigrated with immunoprecipitable protein synthesized in vitro with RNA from 12-hr GA₃-treated aleurones and in vitro transcription/translation products of pHVEP1 and 4 (lanes 1, 2, and 3, respectively). After a 1-hr chase (lane 5), several additional immunoprecipitable species were observed with M_r of 41,000, 38,000 to 37,000, and 32,000, the latter of which comigrated with purified EP-B by 10% SDS-PAGE (lane 16). After 3 hr and 5 hr of chase (lanes 6 and 7, respectively), the percentage of immunoprecipitable counts associated with the lower molecular weight species increased. Similarly, after a 2-hr pulse after 10 hr of incubation with GA₃ (lane 8), the same processing intermediates were immunoprecipitated by EP-B antiserum. After a 2-hr chase (lane 9), most of the immunoprecipitate was in the 32,000 M_r species, and after a 12-hr chase (lane 10), the processing appeared to be complete with all of the immunoprecipitable counts in the 32,000 M_r species. No further processing products were detected after a 24-hr chase (lane 11).

A surprising observation was that these processing intermediates were observed in the incubation medium as well. In the incubation medium from layers that were pulsed for 1 hr and chased for 5 hr (Figure 4, lane 12), the only

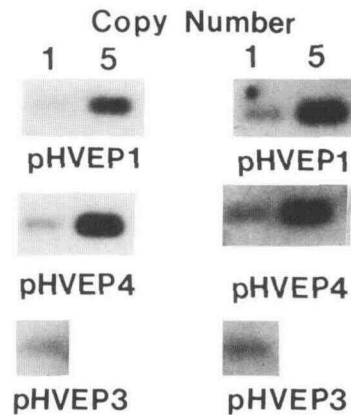
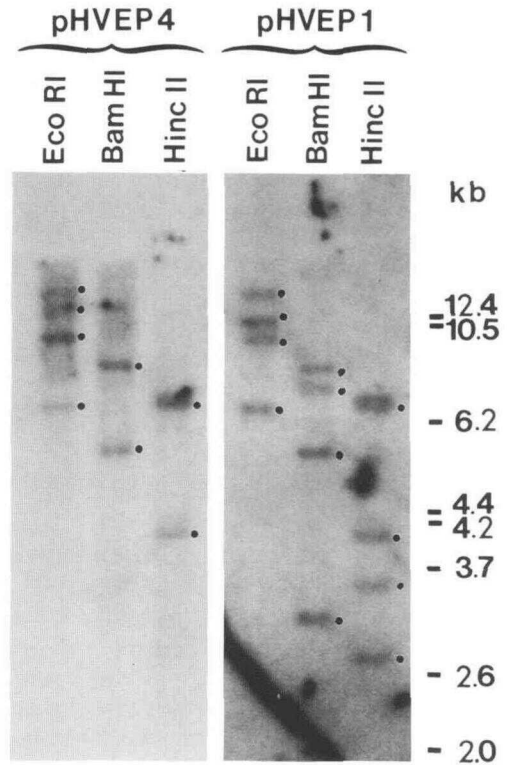


Figure 3. Genomic DNA Gel Blot Analysis.

Himalaya barley genomic DNA digested with EcoRI, BamHI, or HincII and copy number reconstructions of pHVEP clones 1, 4, and 3 (shown below) were electrophoresed on the same gel, DNA gel blotted, and probed with pHVEP4 as described in Methods. The same blot was stripped and reprobed with pHVEP1. A dot is positioned to the right of each hybridizing band. No bands were seen below 2.6 kb. The migration of λClnI restriction fragments is indicated to the right. The copy number reconstructions of pHVEP4 probed with pHVEP1 were slightly overloaded.

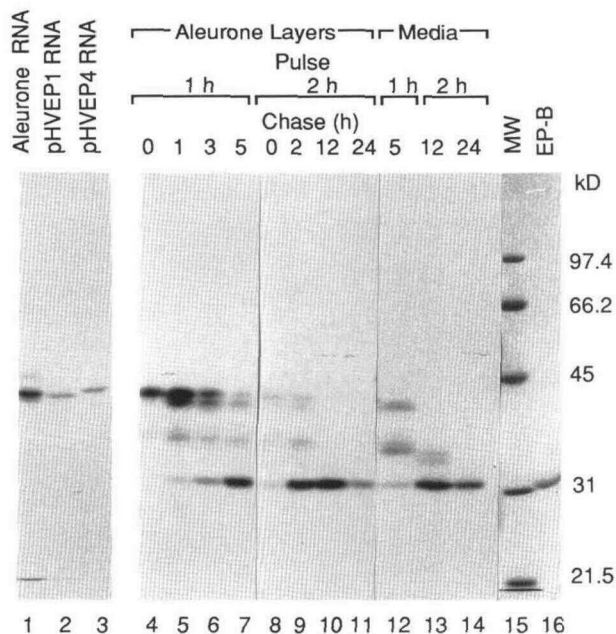


Figure 4. Post-Translational Processing of ProEP-B.

RNA from aleurone layers incubated for 12 hr with 1 μ M GA₃, and *in vitro* transcripts from pHVEP1 and pHVEP4 were translated *in vitro* and immunoprecipitated with EP-B antiserum (lanes 1, 2, and 3, respectively) as described in Methods. Immunoprecipitation by EP-B antiserum of 300,000 TCA-precipitable cpm of extracts from layers that were pulse-labeled for 1 hr after 5 hr of GA₃ treatment and chased for 0 hr, 1 hr, 3 hr, and 5 hr (lanes 4, 5, 6, and 7, respectively), or from layers pulse-labeled for 2 hr after 10 hr of GA₃ treatment and chased for 0 hr, 2 hr, 12 hr, and 24 hr (lanes 8, 9, 10, and 11, respectively). Immunoprecipitation by EP-B antiserum of 100,000 TCA-precipitable cpm of chase media from either 1-hr pulsed, 5-hr chased layers (lane 12) or 2-hr pulsed, and 12-hr and 24-hr chased layers (lanes 13 and 14). Bio-Rad low molecular weight protein standards (lane 15) and purified EP-B (3 μ g) (lane 16) stained with Coomassie blue before the gel was prepared for fluorography. The molecular weight of the protein standards is indicated on the right.

species not secreted was the primary 42,500 *M_r* species, and an additional product of 36,000 *M_r* was observed. In the medium of layers pulsed for 2 hr and chased for 12 hr (lane 14), processing intermediates of 36,000 *M_r*, and 35,000 *M_r*, were observed, and after a 24-hr chase, only the final 32,000 *M_r* product was found. Processing appears to occur more quickly in the layers because the percentage of lower molecular weight products including the final 32,000 *M_r* product in the pulse-chased layers always exceeds that of the corresponding incubation medium from those layers. None of these products was precipitated from layers or media by preimmune serum (not shown).

Compared with proEP-B, post-translational processing of proEP-A appears to be much less complex, and, at least

at these timepoints, proceeds at a much slower pace (data not shown). ProEP-A had a *M_r* of 45,000. A slightly smaller product of 44,000 *M_r* was observed after 5 hr of chase. No additional intermediates were observed, and the final product comigrating with purified EP-A did not appear until 24 hr of chase. Only the final form was observed in the media and similarly did not appear until 24 hr of chase.

GA₃-Mediated Temporal Expression of Aleurone Cysteine Proteinases

The time course of GA₃-mediated *in vivo* synthesis of proEP-A and proEP-B in aleurone layers is shown in Figures 5A and 5B, respectively. Equal amounts of TCA-precipitable counts were immunoprecipitated from the pulse-labeled layers to reflect changes in the amount of new proteases synthesized relative to total new protein synthesis. No protein was immunoprecipitated by preimmune serum from extracts of layers treated with GA₃ for 12 hr (Figure 5B, lane 12) or 24 hr (Figure 5A, lane 1). α -Amylase immunoprecipitated from 24-hr GA₃-treated aleurone layers was included as an internal control for a GA₃-induced protein (Figure 5A, lane 9). It always migrates in between protein immunoprecipitated by EP-B and EP-A antisera. Very low levels of proEP-B and proEP-A were detectable in uninduced aleurone layers at the beginning of the incubation period (time 0). GA₃-induced *in vivo* synthesis of proEP-B increased more rapidly than proEP-A and reached a maximum level approximately 6 hr to 12 hr earlier than proEP-A. The level of *in vivo* synthesis of proEP-B was relatively constant between 6 hr and 36 hr and then rapidly declined. ProEP-A reached its maximum at 12 hr to 24 hr, and then gradually declined. In the absence of GA₃, the synthesis of these proenzymes, relative to total new protein synthesis, did not increase over time.

The temporal accumulation of *in vitro* translatable mRNA for EP-A and EP-B in aleurone layers in response to GA₃ is shown in Figures 6A and 6B, respectively. The positions of preproEP-A and preproEP-B are indicated by arrows. The general trend is the same as for the *in vivo* synthesis results. GA₃-induced accumulation of *in vitro* translatable EP-B mRNA again increased more rapidly than EP-A mRNA, and it reached maximum levels at 12 hr, approximately 12 hr to 24 hr earlier than *in vitro* translatable EP-A mRNA. In both cases, however, the maximum level of *in vivo* synthesis relative to total new protein synthesis occurred 6 hr to 12 hr earlier than the maximum level of *in vitro* translatable mRNA. This could be due to additional translational regulation of these messages *in vivo*. α -Amylase mRNA was present in high amounts in GA₃-treated aleurone layers, and, therefore, α -amylase contaminated the immunoprecipitations and could be seen (as indicated by the dot) immunoprecipitated by preimmune serum from *in vitro* translations of RNA from aleurone layers treated with GA₃ for 24 hr (Figure 6A, lane 1).

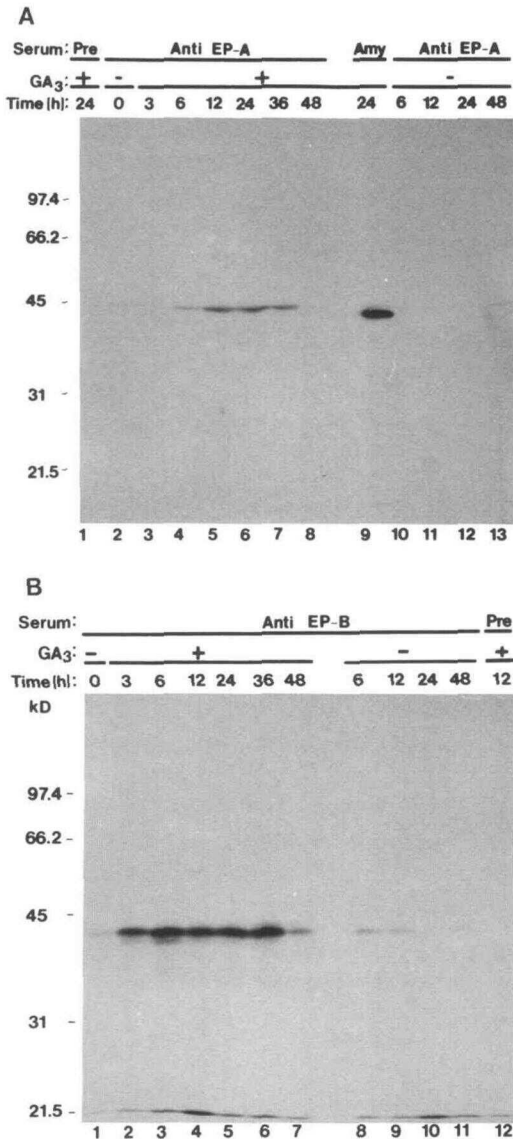


Figure 5. Time Course of GA₃-Mediated in Vivo Synthesis of ProEP-A and ProEP-B.

(A) Immunoprecipitation by EP-A antiserum of extracts from pulse-labeled aleurone layers incubated for the time indicated in the presence (lanes 3 to 8) or absence (lanes 2 and 10 to 13) of GA₃. Immunoprecipitation of extracts of pulse-labeled 24 hr GA₃-treated aleurone layers with preimmune serum (lane 1) or α -amylase antiserum (lane 9).

(B) Immunoprecipitation by EP-B antiserum of extracts from pulse-labeled aleurone layers incubated for the time indicated in the presence (lanes 2 to 7) or absence (lanes 1 and 8 to 11) of GA₃. Immunoprecipitation with preimmune serum of extracts of pulse-labeled 12 hr GA₃-treated aleurone layers (lane 12). For both **(A)** and **(B)**, layers were pulse-labeled during the last 30 min of the incubation period.

The time course of accumulation of aleurone protease mRNAs determined by RNA gel blot hybridization using clone-specific probes for pHVEP1, pHVEP4, pHV14, and aleurain is shown in Figure 7. The in vitro transcripts of pHVEP clones 1, 4, and 3 were included in the first three lanes to demonstrate the specificity of the probes under these hybridization conditions. Uninduced layers at the beginning of the incubation period (0 time) contained undetectable levels of pHVEP1 mRNA, very low levels of pHVEP4 and pHV14 mRNA, and very high levels of aleurain mRNA. In aleurone layers incubated in the presence of GA₃, mRNA for pHVEP clones 1 and 4 accumulated at a faster rate, reaching their maximum level by 12 hr, and then declined more rapidly. In contrast, pHV14 mRNA peaked at 24 hr and was still relatively abundant at 36 hr and 48 hr. Aleurone mRNA was present at very high levels in uninduced layers at 0 time. When layers were incubated in the presence of GA₃, the level declined slightly, then gradually increased approximately twofold by 24 hr before decreasing again. A similar profile of aleurain mRNA induction was reported by Rogers et al. (1985) and Nolan and Ho (1988). Of these protease clones, only aleurain mRNA was detectable in aleurone layers incubated in the absence of hormone. However, the high levels of aleurain mRNA present at 0 time were maintained only in the presence of GA₃. There appeared to be a decay of aleurain mRNA upon incubation in the absence of GA₃. The combined profiles of mRNA hybridizing to pHVEP1- and 4- and pHV14-specific probes agreed with the in vivo time course of protein immunoprecipitated by EP-B antiserum. Messenger RNAs for pHVEP clones 1 and 4 accumulated to their maximum levels more quickly than high pl α -amylase, which was included as a control. The expression of high pl α -amylase mRNA was similar to that previously reported (Rogers et al., 1985; Nolan and Ho, 1988).

Effects of GA₃ and ABA on the Expression of Aleurone Cysteine Proteinases

Concentrations of 10⁻⁸ M and 10⁻⁷ M GA₃ have been shown to be sufficient for maximal induction of in vivo synthesis of low pl α -amylase and high pl α -amylase, whereas 10⁻⁴ to 10⁻⁵ M ABA counteracts the effects of 10⁻⁶ M GA₃ (Nolan and Ho, 1988). The effects of different concentrations of GA₃ and the addition of 2 × 10⁻⁵ M ABA on the in vivo synthesis of proEP-B and proEP-A are shown in Figure 8. Neither of the proteases was observed in the absence of hormone (lanes 1 and 11) or in the presence of ABA alone (lanes 6 and 16). The synthesis of EP-B was more sensitive to GA₃ at this timepoint than was EP-A (lanes 2 to 5 and 12 to 15, respectively). A concentration of 10⁻⁹ M was sufficient for maximizing the in vivo synthesis of proEP-B (lane 2), whereas proEP-A was barely detectable at this concentration (lane 12). GA₃ induction of proEP-A synthesis was more sensitive to ABA inhibition than was the synthesis of proEP-B. ABA was capable of

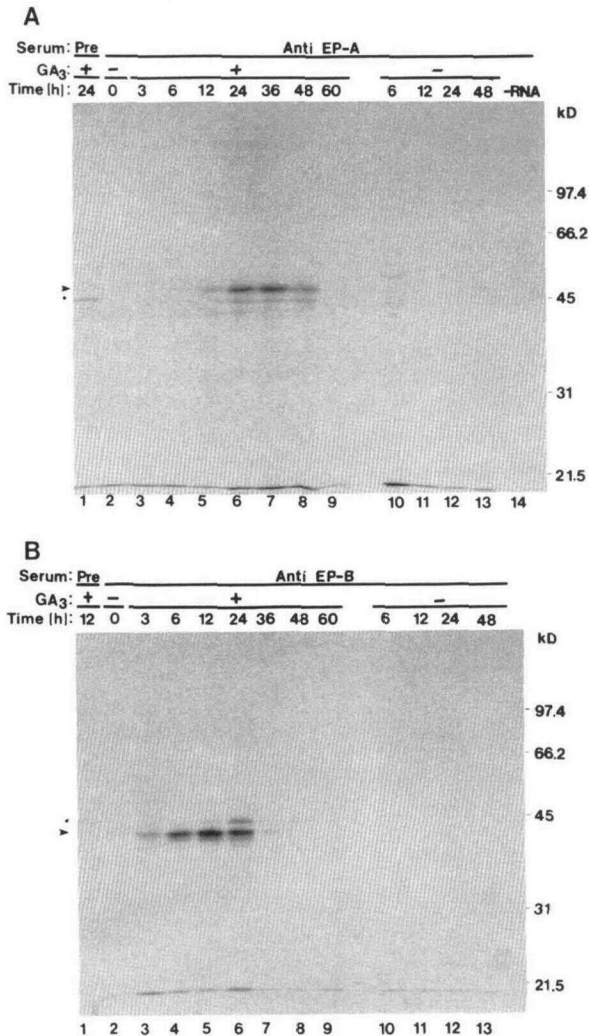


Figure 6. Time Course of GA₃-Mediated Accumulation of in Vitro-Translatable mRNA for EP-A and EP-B.

(A) Immunoprecipitation by EP-A antiserum.

(B) Immunoprecipitation by EP-B antiserum.

Immunoprecipitation was performed on equal amounts of TCA-precipitable cpm of in vitro translations of RNA from aleurone layers incubated for the time indicated in the presence (lanes 3 to 9) or absence (lanes 2 and 10 to 13) of GA₃. The positions of prepro-EP-A and prepro-EP-B are indicated by arrows. The dot indicates the position of contaminating α -amylase. Immunoprecipitation with preimmune serum of in vitro translations of RNA from either 12-hr GA₃-treated aleurone layers [(B), lane 1] or 24-hr GA₃-treated aleurone layers [(A), lane 1]. Immunoprecipitation by EP-A antiserum of equal TCA-precipitable cpm of an in vitro translation without added RNA is shown in (A), lane 14. The migration of Bio-Rad low molecular weight protein standards is indicated to the right.

eliminating the 10^{-7} M GA₃ induction of proEP-A synthesis (lane 17) and reducing the level of α -amylase induction (lanes 8 and 9), but it did not inhibit the synthesis of proEP-B at this concentration of GA₃ (lane 7). ABA at 2×10^{-5} M is capable of inhibiting proEP-B synthesis induced by lower concentrations of GA₃ (10^{-9} M) (S.M. Koehler and T.-H.D. Ho, unpublished observations).

The effects of GA₃ and ABA on the accumulation of in vitro translatable mRNA for EP-A (data not shown) are consistent with the in vivo synthesis of proEP-A. RNA gel blot analysis of the accumulation of aleurone protease mRNAs in response to GA₃ and ABA is shown in Figure 9. Consistent with the results of in vivo synthesis of proEP-B, mRNAs hybridizing to probes specific to pHVEP clones 1 and 4 and pHV14 were present at very low or undetectable levels in the absence of hormone, but at 10^{-9} M GA₃, the increase in mRNA levels was relatively low compared with in vivo synthesis of proEP-B. There was a dramatic increase in mRNA levels with 10^{-8} M GA₃. ABA (2×10^{-5} M) completely reversed the 10^{-7} M GA₃-induced accumulation of mRNA for pHV14 but only partially inhibited the GA₃-induced accumulation of mRNA for pHVEP clones 1 and 4. The hybridization patterns for pHVEP clones 1 and 4 and pHV14 combined paralleled the profile of in vitro translatable mRNA for EP-B (data not shown). In contrast to mRNA for EP-B and EP-A, aleurain mRNA was strongly induced at a 10-fold lower GA₃ concentration (10^{-9} M), and it was also present in low levels in the absence of hormone. Like pHVEP1 and pHVEP4, ABA only partially reversed the GA₃ response for aleurain mRNA. High pl α -amylase was less sensitive to lower concentrations of GA₃ than mRNA for pHVEP clones 1 and 4 and pHV14. The results for aleurain and high pl α -amylase mRNA were similar to those previously obtained (Nolan and Ho, 1988). The blot was probed with pHVA1 as an ABA-inducible control (Hong et al., 1988).

DISCUSSION

Several lines of evidence suggest that the pHVEP clones 1 and 4 represent isozymes of EP-B. (1) Both clones contain a sequence identical to the N-terminal amino acid sequence of the purified protein. (2) The in vitro transcription and translation products of the clones are immunoprecipitated by EP-B antiserum and comigrate with protein immunoprecipitated from in vitro translation of RNA isolated from GA₃-treated aleurone layers. (3) The molecular weight and acidic nature predicted for the mature proteins encoded by these clones are similar to those of purified EP-B.

The reaction mechanism and protein structure of plant cysteine proteinases, primarily papain and actinidin, have been well characterized by kinetic studies and x-ray diffraction in the presence and absence of substrate analogues (Drenth et al., 1976; Baker, 1980; Kamphuis et al.,

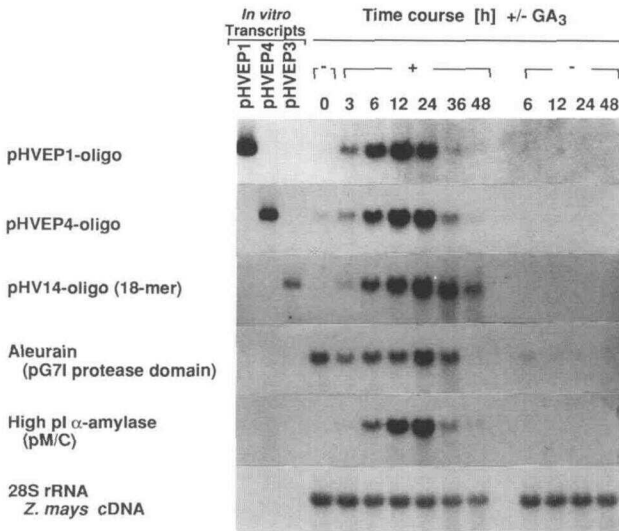


Figure 7. Time Course of GA₃-Mediated Accumulation of mRNA for Barley Aleurone Cysteine Proteinases.

RNA was electrophoresed, blotted, and hybridized to the probes indicated to the right of each panel as indicated in Methods. High pI α -amylase and rRNA probes were used as GA₃-induced and constitutively expressed controls, respectively. The number above each lane indicates the hours of incubation of aleurone layers in the presence (+) or absence (-) of 1 μ M GA₃. The first three lanes contain in vitro transcripts of pHVEP clones 1, 4, and 3 as indicated to demonstrate the specificity of the oligonucleotide probes under the hybridization conditions employed. The x-ray films were exposed to give approximately equivalent intensities between panels.

1985). By analogy to papain and actinidin, the mature proteins deduced from pHVEP4 and pHVEP1 contain conserved residues that constitute the reactive nucleophile, form and stabilize the active site, form disulfide bonds, and maintain proper main-chain conformation. The residues Ala-139 and Leu-163 align with Val residues 133 and 157 in papain. In papain, these residues are responsible for substrate specificity (Drenth et al., 1976).

The profile of cysteine proteinases in GA₃-induced Himalaya barley aleurone layers is obviously quite complex. Genomic DNA gel blot analysis indicates that not more than four (and possibly five) different genes exist that hybridize to pHVEP clones. These data agree with the cDNA clones that have been characterized. It is possible that pHVEP7 represents one of the two BamHI or HincII fragments that hybridize to pHVEP4. Alternatively, because it contains an additional 12 bp at the 5' end, pHVEP7 could represent a longer cDNA clone for pHVEP4 as opposed to a separate member of the gene family. The clones pHVEP1 and pHV14-13 represent two additional members of this gene family, the latter of which is more

divergent. Whether or not pHVEP3 is equivalent to pHV14-13 is unclear. The failure of in vitro transcripts of this clone to be translated in vitro into protein of the size predicted by pHV14-13 could be due to a frameshift resulting in a truncated protein. The in vitro transcript of pHVEP3 appears to be full length and hybridizes to the pHV14-specific oligonucleotide probe. The deduced amino acid sequence for pHV14-13 is 91% similar to the proteins predicted for pHVEP clones 1 and 4, and EP-B antiserum will recognize a Trp E/pHV14 fusion protein expressed in *E. coli* (B. Holwerda and J. Rogers, personal communication). EP-A and EP-B share 90% amino acid sequence similarity at their N termini (Koehler and Ho, 1990). cDNA cloning of EP-A would be beneficial to establish its degree of homology to members of the EP-B gene family.

Aleurain differs from these other proteases in many ways and probably has a different physiological role, possibly

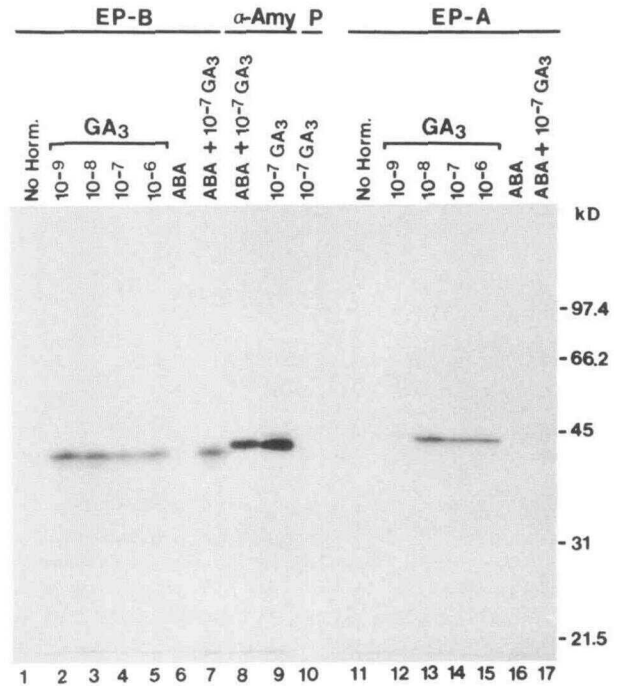


Figure 8. Effects of GA₃ and ABA on the in Vivo Synthesis of ProEP-B and ProEP-A.

Immunoprecipitation by EP-B antiserum (lanes 1 to 7), EP-A antiserum (lanes 11 to 17), preimmune serum (lane 10), or α -amylase antiserum (lanes 8 and 9) of equal amounts of TCA-precipitable cpm of extracts from 2-hr pulse-labeled aleurone layers incubated for 12 hr as indicated above each lane in the absence of added hormone, in the presence of GA₃ (at the molar concentration indicated) with or without the addition of 2×10^{-5} M ABA or in the presence of 2×10^{-5} M ABA alone. The migration of Bio-Rad low molecular weight protein standards is indicated to the right.

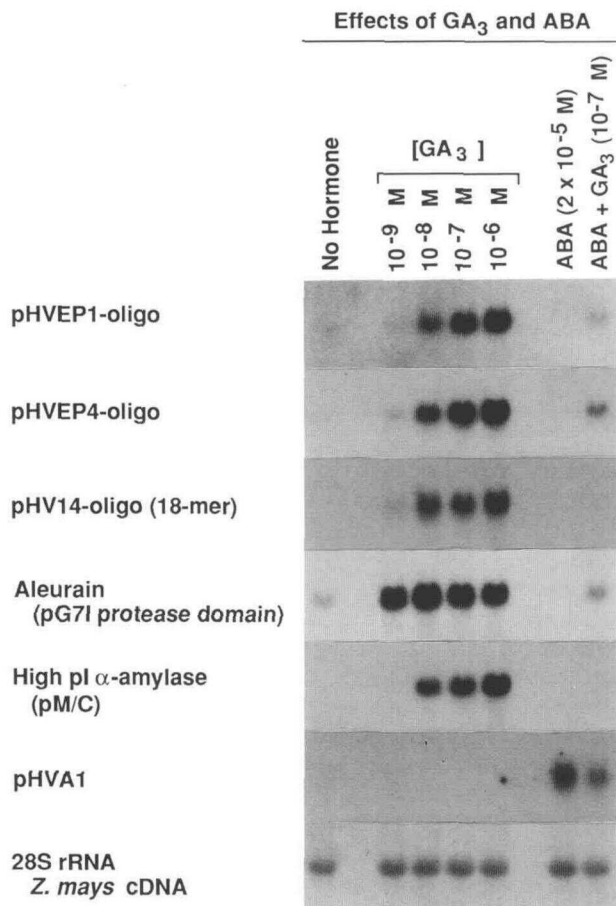


Figure 9. Effects of GA₃ and ABA on the Accumulation of mRNA for Barley Aleurone Cysteine Proteinases.

RNA from aleurone layers incubated for 12 hr in the presence or absence of hormones as indicated above each lane similar to Figure 8 was electrophoresed, blotted, and hybridized to the probes indicated to the right of each panel as indicated in Methods. High pI α -amylase, pHVA1, and rRNA probes were used as GA₃-induced, ABA-induced, and constitutively expressed controls, respectively. The blot was hybridized in the same bag with the time course RNA gel blot (Figure 7) whenever the same probes were used. The x-ray films were exposed to give approximately equivalent intensities between panels.

involving intracellular protein turnover or processing. Preproaleurain (Rogers et al., 1985) is only 57% similar to the preproteins encoded by pHVEP clones 1 and 4. Unlike EP-B and EP-A, the protein is not secreted from aleurone layers (Holwerda et al., 1990). The tissue-specific expression of aleurain appears to be different from these other proteases. In addition to its expression in aleurone layers, aleurain mRNA is expressed at very high levels in shoot

and root tissue (Rogers et al., 1985). EP-B and EP-A antisera failed to immunoprecipitate protein from 4-day germinated pulse-labeled roots, and mRNA for pHVEP4 (and cross-hybridizing messages) was undetectable on RNA gel blots of control and GA₃-treated peduncle internodes of 6-week-old barley shoots (S.M. Koehler and T.-H.D. Ho, unpublished observations). Aleurain mRNA, but not mRNA for pHVEP clones 1 and 4 and pHV14, was detectable at low levels in scutellum from 4-day imbibed seeds (S.M. Koehler and T.-H.D. Ho, unpublished observations).

Comparison of nucleotide sequences of cDNA clones encoding cysteine proteinases with the N-terminal amino acid sequence of the mature proteins in both plants and animals has revealed the common occurrence of an extensive pro-sequence that is apparently removed post-translationally. The only other report of multiple processing intermediates observed for plant cysteine proteinases is for SH-EP from germinating cotyledons of *Vigna mungo* (Mitsuhashi et al., 1986; Mitsuhashi and Minamikawa, 1989). A cDNA encoding this protease indicates that it also has a preprosequence (Akasofu et al., 1989). The mature protein has an M_r of 33,000 by 10% SDS-PAGE. The primary 45,000 M_r in vitro translation product is converted to 42,000 M_r when canine microsomal membranes are included in the translation reaction, indicating the cleavage of a signal peptide. In a cotyledonary extract incubated at neutral pH, a 43,000 M_r form was sequentially cleaved to the 33,000 M_r form via 39,000 M_r and 36,000 M_r intermediate polypeptides, as observed by protein gel blot analysis. Protease activity was associated with only the 33,000 M_r form. The results of these in vitro experiments have not yet been substantiated by in vivo pulse-labeling experiments. The mature proteins of pHVEP clones 1 and 4 are more similar to the mature protein of SH-EP (75%) than to aleurain (62%) (Rogers et al., 1985), papain (60%) (Cohen et al., 1986), or actinidin (70%) (Carne and Moore, 1978; Baker, 1980). Even the preprosequences of pHVEP clones 1 and 4 and SH-EP are 58% similar.

The processing of the prosequence has been studied most extensively for the mammalian lysosomal cysteine proteinases, cathepsins B, H, and L. The preproteins encoded by pHVEP clones 1 and 4 are more similar to rat preprocathepsin L (60%) (Ishidoh et al., 1987b) than they are to preprocathepsin H from rat (52%) (Ishidoh et al., 1987a) and B from mouse (45%) (Chan et al., 1986). In vivo processing of rat hepatocyte preprocathepsin L involves cotranslational cleavage of the signal peptide and core glycosylation with high-mannose-type phosphorylated oligosaccharides in the mature protein region to produce 39-kD procathepsin L. It is then transported from the Golgi to the lysosome, where the prosequence is cleaved, producing an active single chain form of 30 kD and then a stable, active two-chain form of 25 kD and 5 kD. No processing intermediates are observed between 39 kD and 30 kD (Nishimura et al., 1988). Processing of

preprocathepsins B and H seems to follow the same route (Nishimura and Kato, 1987).

Deletion analysis of recombinant cathepsin L constructs expressed in *E. coli* has shown that renatured procathepsin L can be autolytically cleaved in vitro by lowering the pH, and that enzymatic activity increases in parallel with this processing (Smith and Gottesman, 1989). Deletion of the prosequence resulted in a decreased recovery of enzymatic function following renaturation, suggesting that the propeptide might be necessary in protein folding and/or stability. Major excreted protein (MEP) (M_r , 39,000), a form of procathepsin L secreted from a transformed mouse fibroblast cell line, also autolyses at acidic pH to produce a more active 30,000 M_r form (Mason et al., 1987). Mason et al. (1987) suggest that the prosequence of the major excreted protein affects the stability of the enzyme at neutral pH.

EP-B and EP-A differ from the cathepsins B, H, and L in that the mature stable forms are single-chain forms. PreproEP-B and preproEP-A appear to be processed differently. Assuming preproEP-B involves cotranslational cleavage of a signal peptide, it is possible that the prosequence is glycosylated because the M_r of proEP-B is approximately equal to preproEP-B. The shift in M_r from 42,500 to 41,000 could be due to modification of the potential oligosaccharide or peptide cleavage, but whatever the cause, it occurs intracellularly because the 42,500 M_r species is not secreted. Processing of the 41,000 M_r species into the lower M_r species might occur in the cell wall or intercellular space, possibly mediated by a locally acidic environment and/or by other secreted proteases. These lower M_r species would then be free to diffuse into the incubation medium. The cause, consequence, and significance of the multiple proEP-B processing intermediates observed both in the aleurone layers and incubation media remain to be examined. The possibility exists that some of the processing intermediates could be due to differential processing sites in different proEP-B isozymes recognized by EP-B antiserum that are all eventually processed to a final form with the same M_r . In this case it would be useful to study protease processing using monoclonal antibodies specific to the propeptide or mature protein regions of pHVEP clones 1 and 4 and pHV14, or, alternatively, use a heterologous system. ProEP-A has a lower M_r than preproEP-A, which is consistent with the cleavage of a signal peptide. As for proEP-B, another small shift from 45,000 M_r to 44,000 M_r occurs in proEP-A, but then no other processing intermediates are observed until the final form is obtained and secreted. The functional importance of these different processing patterns remains to be elucidated.

Barley aleurone cysteine proteinases are differentially regulated with respect to their temporal and hormonally induced expression. GA₃ is necessary for the increase in de novo synthesis and accumulation of in vitro translatable mRNA of EP-B and EP-A. GA₃ induction of EP-B, as

measured by both the rate of in vivo protein synthesis and level of mRNA, precedes that of EP-A by 6 hr to 12 hr. Compared with EP-A, EP-B is induced at a 10-fold lower GA₃ concentration at the level of protein synthesis, and the induction is less sensitive to ABA inhibition. The effects of GA₃ concentration on the time course of protease accumulation were not assessed. Analysis of RNA gel blots with clone-specific probes demonstrated that mRNA for pHVEP clones 1 and 4 are coordinately regulated and begin to accumulate and reach maximal levels earlier than mRNA for pHV14. In contrast, aleurain mRNA is present at higher levels in the absence of hormone, but GA₃ is necessary to maintain high steady-state levels. Aleurain mRNA accumulation is enhanced at 10-fold lower GA₃ concentrations. In nuclear run-on transcription studies, Jacobsen and Beach (1985) demonstrated an increase in transcription of mRNA for pHV14 and α -amylase in nuclei isolated from GA₃-responsive aleurone cell protoplasts. Analysis of the promoter regions of these genes might provide insight into possible common mechanisms for GA₃ induction.

METHODS

Preparation and Incubation of Aleurone Layers

Barley (*Hordeum vulgare* L. cv Himalaya) seeds from the 1985 harvest were purchased from the Department of Agronomy and Soils, Washington State University, Pullman, WA. Double-cut half-seeds, with the embryo and distal ends removed, were imbibed for 4 days, and aleurone layers were peeled from the starchy endosperm and incubated in standard medium as described by Nolan et al. (1987). Antibiotic/antimycotic was used instead of chloramphenicol to maintain sterile conditions. Aleurone layers were preincubated for 4 hr at 25 layers per 5 mL or 12 layers per 2.5 mL in 25-mL flasks in medium without hormones before the beginning of all experimental treatments. At the end of the preincubation period, the medium was removed and replaced with half the volume of medium \pm 1 μ M GA₃ for time-course experiments or with GA₃ or ABA as indicated to examine the effects of these hormones. For incubation periods longer than 12 hr, the medium was replaced with fresh medium approximately every 12 hr.

In Vivo Labeling and Protein Extraction

For in vivo labeling, the medium was removed and replaced with 1.5 mL of fresh medium as before and a mixture of ³⁵S-methionine and ³⁵S-cysteine (*trans* ³⁵S-label from ICN, Irvine, CA) was added at 50 μ Ci of ³⁵S-methionine/mL during the last 30 min to 2 hr of the incubation period as indicated. Layers were then rinsed with 2 mL to 5 mL of ice-cold medium containing 1 mM methionine, blotted dry, and homogenized with an ice-cold mortar and pestle with acid-washed sea sand in 1 mL of PBS, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μ M leupeptin. The homogenates were microcentrifuged (12,000g) 15 min at

4°C, and the supernatant was stored at -20°C. TCA-precipitable counts were determined as follows. From 3 μ L to 5 μ L of each sample was applied to 2-cm² discs of filter paper (Schleicher & Schuell no. 596) in duplicate. These were washed twice (10 min each) with cold 10% TCA, 1 mM methionine (10 mL/filter), then once (10 min) with 95% ETOH (10 mL/filter), and finally acetone washed (5 mL/filter, 5 min).

RNA Isolation and In Vitro Translation

At the end of the incubation period, the layers were rinsed thoroughly with sterile water, frozen in liquid nitrogen, and then stored at -80°C before RNA extraction. Total RNA was isolated from aleurone layers using a guanidine HCl extraction procedure as described by Belanger et al. (1986) with the following modifications. From 25 to 100 layers were homogenized in 7.5 mL to 15 mL of 5 M guanidine-HCl grinding buffer and the final RNA pellet was precipitated with 0.1 volume of 3 M Na-acetate (pH 5.5) and 0.6 volume of isopropyl alcohol. For each treatment, 5 μ g of total RNA was in vitro translated in a nuclease-treated rabbit reticulocyte lysate system (Promega Biotec) in the presence of ³⁵S-methionine according to the manufacturer's instructions.

Immunoprecipitation

Polyclonal antibodies were raised against purified proteases EP-A (Koehler and Ho, 1988) and EP-B (Koehler and Ho, 1990). In most cases, the proteases were glutaraldehyde cross-linked to rabbit albumin (Bollum, 1975) and dialyzed against PBS before injection. The antigen was mixed with complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injections. Antigen was injected intradermally at 0.05 mL per site along the back of New Zealand White rabbits. For EP-A, three consecutive injections of 111 μ g, 66 μ g, and 66 μ g were made over 4-week intervals. For EP-B, four consecutive injections of 111 μ g, 67 μ g, 71 μ g, and 50 μ g were made over 1-month intervals. Blood was collected before the first injection and 5 weeks or 3 weeks after the last injection for EP-A and EP-B, respectively. The blood was placed on ice for 30 min or at 4°C overnight, then centrifuged 12,000g for 25 min. The serum was decanted and microcentrifuged for 5 min, and then stored in 1-mL aliquots at -80°C until ready for use.

For analysis of labeled in vivo or in vitro synthesized protease, protein extracts or in vitro translation reactions containing equal amounts of TCA-precipitable counts (100,000 cpm to 300,000 cpm) were brought up to 1 mL in PBS in 0.1% BSA-coated Eppendorf tubes on ice. Post-immune sera was added at 15 μ L/mL, and the samples were incubated at 4°C at low speed on a rotating wheel for 2.5 hr to 4.5 hr. Controls using the same amount of preimmune serum were always included, and antiserum against barley α -amylases (gift of Greg Heck, Washington University Biology Department) was sometimes used. Protein A-Sepharose CL-4B was equilibrated at 125 mg/mL PBS (0.2% Na₂S₂O₈), prewashed three times with 1 mL of wash solution (0.01 M Tris, 0.15 M NaCl, 5 mM cysteine, 5 mM methionine, 2 mM EDTA, 0.02% Na₂S₂O₈, 1.0% Nonidet P-40) followed by two 1-mL washes with PBS (0.02% Na₂S₂O₈), and then re-equilibrated at 125 mg/mL PBS (0.2% Na₂S₂O₈). Twenty microliters of prewashed protein A-Sepharose CL-4B was added to each Eppendorf tube to precipi-

tate the immune complex, and the immunoprecipitations were further incubated 2 hr to 4 hr. The protein A-Sepharose beads were pelleted by microcentrifuging 1 min. Nonspecifically bound protein was removed by washing three times with 1 mL of ice-cold wash solution, and then twice with 1 mL of wash solution without Nonidet P-40. The pellets were then boiled 5 min in 40 μ L of electrophoresis sample buffer and microcentrifuged 5 min. Analysis of immunoprecipitations by SDS-PAGE was performed using 10% acrylamide slab gels (Laemmli, 1970). Fluorography was performed with Kodak XAR-5 film following the APEX procedure of Jen and Thatch (1982).

Pulse-Chase Analysis of Protease Processing

Aleurone layers were incubated 25 layers per 2.5 mL of standard incubation medium with 1 μ M GA₃. At 5 hr or 10 hr, layers were pulse-labeled for 1 hr or 2 hr, respectively. Medium was removed and replaced with 1.5 mL of fresh medium with 1 μ M GA₃ and 50 μ Ci/mL *trans*-label ³⁵S-Met/Cys. At the end of the labeling period, medium was removed, and the layers were rinsed with 5 mL of fresh medium (plus 1 μ M GA₃) containing 10 μ M cold methionine. The layers were then incubated in 1.5 mL of this same medium for the duration of the chase period. At the end of the chase period, the layers were extracted and the medium was microcentrifuged 15 min, and both were stored at -20°C for later analysis of in vivo labeled protease by immunoprecipitation.

cDNA Library Construction

Total RNA was isolated from aleurone layers incubated for 24 hr with 1 μ M GA₃ as described above. Poly(A)⁺ RNA was fractionated from 3 mg of total RNA by two rounds of selection over 0.4 mL of oligo(dT)-cellulose using a protocol described by Jacobson (1987). The yield was 26 μ g. Double-stranded cDNA synthesis was performed using a protocol patterned after that described by Watson and Jackson (1985) using 4.3 μ g of poly(A)⁺ RNA. Actinomycin D was omitted from the first-strand synthesis reaction. The double-stranded cDNA was ligated to EcoRI predigested and phosphatased Lambda ZAP II arms and packaged using Gigapack II Gold packaging extracts according to the manufacturer's instructions (Stratagene instruction manuals 236211 and 200214-200216). From 300 ng of ligated cDNA, a library of approximately 5 \times 10⁵ recombinant phage was obtained based on color selection by IPTG/X-gal in XL1-Blue host cells.

cDNA Library Screening

Insert DNA isolated from PstI-digested pHV14 was labeled by a random hexamer method modified from Feinberg and Vogelstein (1984). The final reaction volume was 15.5 mL containing about 0.5 μ g of insert DNA, twofold concentration of oligo-labeling buffer and ³²P-dCTP (Du Pont-New England Nuclear, 3000 Ci/mmol, 10 mCi/mL), and fivefold more Klenow fragment (Promega Biotec). The probe was used to screen one-fifth of the cDNA library in XL1-Blue host cells as described in the Stratagene instruction manual (no. 236211). Plaque lifts were prehybridized and hybridized in 6 \times SSC, 5 \times Denhardt's solution, 0.1% SDS, and 10 μ g/mL denatured, sonicated salmon sperm DNA at 65°C. Probe was

added to the hybridization solution at about 1×10^6 cpm/mL, and hybridization proceeded overnight. The filters were washed with $1 \times$ SSC, 0.1% SDS at room temperature, then at 65°C, and finally with $0.2 \times$ SSC, 0.1% SDS at 65°C. Eight positives with inserts ranging from 0.7 kb to 1.5 kb were obtained after three rounds of plaque purification. The *in vivo* excision protocol was used to generate pBluescript plasmids from these positive phages. An oligonucleotide probe (20-mer) directed against the 5' region of pHV14 (gift of Dr. Peter Chandler, CSIRO, Canberra, Australia) was 5'-end labeled using T4 polynucleotide kinase (Maniatis et al., 1982) and used to probe a plasmid DNA gel blot of the positives. Hybridization conditions were the same as for the pHV14 insert probe except that the hybridization temperature was 38°C, and the filter was washed in $1 \times$ SSC at room temperature and finally at 38°C for 20 min.

In Vitro Transcription/Translation and Immunoprecipitation of Positive Clones

Capped RNA transcripts were synthesized from the inserts of the positive clones in the pBluescript SK⁻ vectors according to the Stratagene pBluescript and mCap mRNA Capping Kit instruction manuals. Mini-prep plasmid DNA was linearized with Apal for transcription from the T3 promoter or with BamHI for transcription from the T7 promoter. A portion of the transcripts was *in vitro* translated in a nuclease-treated rabbit reticulocyte lysate system (Promega Biotec) in the presence of ³⁵S-methionine according to the manufacturer's instructions. Translation reactions from transcription reactions from both promoters were analyzed by 10% SDS-PAGE both before and after immunoprecipitation with antisera directed against EP-B and EP-A.

DNA Sequencing and Sequence Analysis

The dideoxynucleotide chain-termination method was used for double- and single-stranded sequencing of positive clones in pBluescript by Sequenase Version 2.0 polymerase using the sequencing kit as described in the manufacturer's instruction manual (United States Biochemical Corporation). A series of nested deletions was produced from the 5' and 3' ends of pHVEP clones 1, 4, and 7 using exonuclease III and S1 nuclease according to a protocol described by Henicoff (1987). The ligation reactions were allowed to proceed overnight at 16°C or 4°C before transformation of competent host cells using a 1:10 vol/vol ratio of ligation reaction to cells. DNA sequences were optimally aligned to find the maximum similarity between two sequences using the GAP program of the GCG software package (Devereaux et al., 1984) with a gap weight of 2.0 and length weight of 0.3 and end gaps weighted in the same way as other gaps. This program was also used to optimally align protease amino acid sequences to determine their similarity. In this case, a gap weight of 5.0 and length weight of 0.3 were used. The protease sequences were compared with other cysteine proteinase sequences obtained from the libraries of GenBank, EMBL, and NBRF.

Analysis of RNA Gel Blots and Genomic DNA Gel Blots

RNA gel blot analysis was performed as described in Nolan and Ho (1988). Briefly, 10 µg of total RNA for each treatment was

electrophoresed in formaldehyde-agarose gels as described in Maniatis et al. (1982), blotted onto GeneScreen membranes (New England Biolabs, Boston, MA), UV cross-linked, and baked for 1 hr at 80°C. Oligonucleotide probes (18-mers) (synthesized by Tom Keller, Washington University DNA Facility, St. Louis, MO) were purified over a Nensorb column according to the manufacturer's instructions (Du Pont-New England Nuclear Research Products, Boston, MA) and 5'-end labeled using T4 polynucleotide kinase (Maniatis et al., 1982). The RNA gel blots were prehybridized in a solution of $6 \times$ SSC, 50 mM sodium phosphate (pH 7.2), $5 \times$ Denhardt's solution, 1 mM EDTA, and sonicated, boiled salmon sperm DNA at 10 µg/mL for 4 hr to 16 hr at 38°C. The blots were hybridized together in 10 mL of prehybridization solution containing 10% dextran sulfate with approximately 10^6 cpm/0.2 pmol/mL labeled oligonucleotide probe overnight at 38°C. Blots were washed with $6 \times$ SSC at 4°C three times for 5 min and once for 30 min, and then with $6 \times$ SSC, 0.1% SDS at 25°C for 30 min, at T_d [2° (A + T) + 4° (G + C)] - 10°C for 10 min, and finally at T_d - 5°C for 10 min. The blots were exposed to XAR-5 film (Kodak) with an intensifying screen at -80°C for 25 hr to 48 hr. The same blots were stripped in $0.1 \times$ SSC, 0.1% SDS at 80°C before reprobing with the next oligonucleotide probe or at 100°C between hybridizations with cDNA clones. The blots were also probed with barley cDNA for high pI α-amylase (pM/C) and a subclone of the aleurain cDNA clone (G7i) containing only the mature protease domain obtained from Drs. J. Rogers and B. Holwerda, Washington University Medical School (Rogers, 1985; Rogers et al., 1985); an ABA induced cDNA clone, pHV A1, (Hong et al., 1988), and a *Zea mays* rRNA cDNA clone obtained from Dr. M. Sachs, Washington University, Biology Department. These clones were labeled by a random hexamer method described above. The blots were hybridized using the method of Church and Gilbert (1984) and exposed to XAR-5 film as above.

For genomic DNA gel blot analysis, 6 µg of Himalaya barley genomic DNA (gift of G. Heck, Washington University, Biology Department) was digested with restriction enzymes as indicated and electrophoresed on an 0.8% agarose gel, which was subsequently blotted onto GeneScreen (Maniatis et al., 1982) and UV cross-linked. Copy number reconstructions for pHVEP clones 1, 4, and 3 were included by loading the appropriately diluted EcoRI digests of these clones. The DNA gel blots were hybridized using the method of Church and Gilbert (1984) to pHVEP clones 1 and 4 labeled by the random hexamer method described above. The filters were exposed as above for approximately 10 days.

ACKNOWLEDGMENTS

We are especially grateful to Peter Chandler (CSIRO, Canberra, Australia) and Layne Huiet (University of California, Davis) for the use of their unpublished data, pHV14, and oligonucleotide probe. We thank the following individuals for the use of their clones: Bimei Hong (pHVA1), Marty Sachs (rRNA), John Rogers (pM/C, aleurain), and Barry Holwerda (aleurain probe containing only the mature protein domain). We thank Greg Heck for the genomic DNA and scutellum RNA, Csilla Fenczik for the use of her peduncle RNA gel blot, Tom Keller for synthesis of oligonucleotide probes, and Barry Holwerda for the protein gel blot of pHV14 fusion protein. We thank the following individuals for critical review of the manuscript: John Rogers, Barry Holwerda, Bimei Hong, Greg

Heck, Doug Russell, and Raphael Pont-Lezica. This research was supported by a National Science Foundation Grant DCB-8702299.

Received March 26, 1990; revised May 31, 1990.

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