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An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein

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In most higher plants a period of desiccation is the terminal event in embryogenesis. Excised barley embryos acquire desiccation tolerance at a precise developmental stage and cDNA clones have been isolated which are temporally linked with desiccation tolerance. One such clone (pG22-69) with a putative gene product of 34 kd displays high structural homology to mammalian genes encoding an NADPH dependent aldose reductase involved in the synthesis of sorbitol. This first aldose reductase gene of plants is expressed constitutively during embryo maturation and is modulated by the plant hormones abscisic acid (ABA) and gibberellic acid (GA). Immunohistochemistry showed that the protein is preferentially expressed in tissues formed at early stages in embryogenesis. Measurements of enzymatic activity indicate that pG22-69 encodes an active aldose reductase. The finding of this reductase activity and the cloning of the corresponding gene supports the existence of a metabolic pathway in plants playing a role in the synthesis of osmolytes like sorbitol. The significance of this work is that genes of related structure and functions are being used in diverse organisms to fulfil stress related biological requirements.

Key words: ABA/aldose reductase/barley embryo/desiccation tolerance/osmoprotection

Introduction

In the developmental programme of plants the maturation of the seeds involves desiccation and the embryo has to adapt to a changing osmotic environment without losing viability. This adaptation takes place during seed development when the embryo acquires tolerance to dehydration. After this stage the developing embryo can tolerate exposure to air with water potentials as low as 50% relative humidity (Gaff, 1980). The plant hormone abscisic acid (ABA) is thought to play a role in the development of desiccation tolerance (for a review see Kermode, 1990) but the biochemical basis of desiccation tolerance is still largely unknown.

A number of plant genes have been isolated which are abundantly expressed during late embryogenesis. These genes are characteristically ABA responsive and associated with seed maturity. Some of them appear to be expressed as a general response to osmotic stress (see Skriver and Mundy, 1990 for review). The function of their protein products is unknown, but based on their predicted amphiphilic secondary structure a physical role as osmoprotectants has been put forward (Dure *et al.*, 1989).

It has been recently demonstrated that the young barley embryo is able to withstand severe drying. Embryos isolated from barley grains 12 days after anthesis (12 DAP) do not germinate after a desiccation treatment to $<10\%$ water content: these embryos are desiccation intolerant. At a later, precise developmental stage barley embryos acquire desiccation tolerance: 100% of embryos isolated 18 days after anthesis (18 DAP) germinate despite a severe dehydration treatment. *In vitro* translation products were identified which first appeared during the developmental period leading to desiccation tolerance (12 DAP–18 DAP). ABA treatment of 12 DAP embryos leads to the induction of desiccation tolerance, and it further stimulates the appearance of a subset of proteins known to be present in the desiccation tolerant stage of embryos (Bartels *et al.*, 1988).

In order to understand the functions of those proteins which appear temporally correlated with the desiccation tolerant embryos, their corresponding genes were isolated. Embryo specific cDNA clones were selected whose mRNAs were present in 18 DAP and not in 12 DAP embryos and which were inducible by ABA. Here we report on the analysis of one such selected transcript, termed pG22-69. This cDNA clone encodes a protein which has significant homology to aldose reductases, a subgroup of the aldo-keto reductase superfamily (Bohren *et al.*, 1989). The aldo-keto reductases are cytosolic, monomeric oxidoreductases which catalyse the NADPH dependent reduction of carbonyl metabolites (Bohren *et al.*, 1989; Garcia-Perez *et al.*, 1989; Kawasaki *et al.*, 1989; Nishimura *et al.*, 1990). The expression of this first aldose reductase gene of plants correlates with enzymatic activity in barley embryos. We interpret these data as indicative of a role for plant aldose reductase in osmoregulation.

Results

cDNA isolation

A cDNA bank was constructed from RNA of 18 DAP desiccation tolerant embryos and screened with cDNA probes from RNAs of desiccation tolerant (18 DAP) and intolerant (12 DAP) embryos. As tolerance to protoplastic dehydration is specific for the embryo, selected cDNA clones were further hybridized with an RNA probe derived from 5 day old barley shoots. For further analysis cDNA clones were selected which hybridized preferentially with the 18 DAP embryo probe. One such selected clone, pG22-69, contained a *Pst*I-excisable insert of 1092 bp in pUC9.

RNA accumulation of pG22-69

The developmental and tissue specific distribution of pG22-69 related mRNAs was studied by Northern analysis: polyadenylated and total RNAs extracted from several tissues

and embryos of different developmental and physiological stages were probed with the ^{32}P-labelled insert of pG22-69. The hybridization data are presented in Figure 1. For all tissues tested pG22-69 hybridized to a transcript of 1400 bases. The hybridization data indicate that pG22-69 encodes a transcript which is developmentally regulated, embryo specific, ABA responsive and repressed by GA. No homologous mRNAs were detected in leaves or roots. The pG22-69 homologous transcripts started to accumulate during early embryogenesis, and reached a steady state level in embryos isolated between 16 and 18 DAP. A signal is obtained throughout embryogenesis even in dry mature embryos. The expression of pG22-69 homologous transcripts is modulated by ABA. When 12 DAP embryos were incubated on media containing ABA the transcript level was enhanced. Fluridone, which depresses the level of endogenous ABA (Moore and Smith, 1984) induced the germination of 12 DAP embryos and under these conditions the pG22-69 transcript was not detected. When fluridone and ABA were present at the same time (Figure 1B), a distinct hybridization was observed. As soon as the germination pathway was induced either precociously in isolated immature embryos or in mature embryos the pG22-69 homologous mRNAs disappeared.

Hybrid release translation

When barley embryo mRNA selected by hybridization to pG22-69 was translated *in vitro*, and the hybrid-selected product was separated in a two dimensional electrophoresis system it was resolved as one spot of 34 kd in the basic region of the gel (Figure 2A–C).

Genomic Southern analysis

Barley genomic DNA digested with *Eco*RI or *Bgl*II gave a simple hybridization pattern when probed with pG22-69 suggesting the existence of between one and three copies of this gene per genome (Figure 3). The two *Eco*RI fragments (indicated by arrows) correspond in size to two *Eco*RI fragments of the genomic clone.

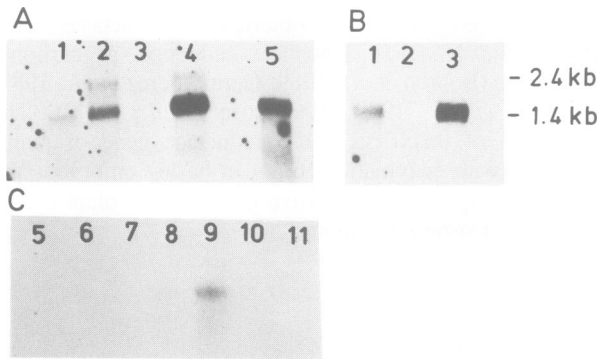

Fig. 1. Hybridization of the barley cDNA clone pG22-69 to Northern blots of RNAs isolated from different barley tissues. 2 μg of poly(A)$^+$ RNA (A and C) and 50 μg of total RNA (B) were separated from the following tissues: part **A**, lane 1, 12 DAP embryos; lane 2, 12 DAP embryos incubated with GA and ABA for 3 days; lane 3, 12 DAP embryos incubated with GA for 3 days; lane 4, 12 DAP embryos incubated with ABA for 3 days; lane 5, 18 DAP embryos. Part **B**, lane 1, 18 DAP embryos; lane 2, 12 DAP embryos treated with fluridone for 3 days; and lane 3, 12 DAP embryos incubated with fluridone and ABA for 3 days. Part **C**, lane 5, barley shoots; lane 6, barley leaves dehydrated (loss of >50% fresh weight); lane 7, roots; lane 8, endosperm; lane 9, embryos dissected from mature grains; lanes 10 and 11, embryos from mature grains incubated on GM medium for 48 h (10) and 24 h (11). To ensure equal loading of RNAs in all tracks the filters were hybridized with ^{32}P-labelled oligo(dT).

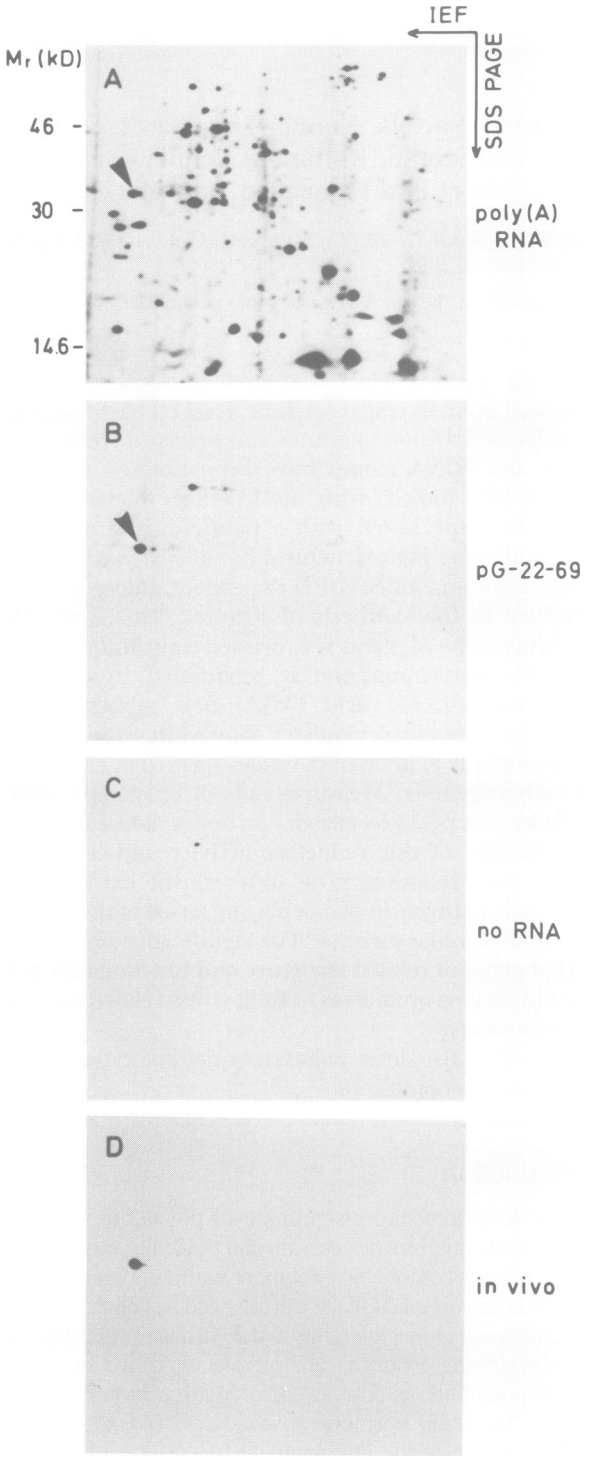

Fig. 2. Two dimensional separations of *in vitro* and *in vivo* synthesized proteins: the proteins were first separated by isoelectric focusing and then in a 12% SDS–polyacrylamide gel. A–C present results of *in vitro* translation experiments: **A** shows the proteins derived from the *in vitro* translation of poly(A)$^+$ RNA of 18 DAP embryos, **B** shows the hybrid-release translation product of pG22-69 and **C** presents the control with no RNA added to the *in vitro* translation assay. **D** is the result of a Western blot: 18 DAP embryo protein extracts were incubated with antibodies raised against the pG22-69 encoded protein.

DNA sequence analysis and amino acid homologies

The nucleotide and deduced amino acid sequences of pG22-69 are presented in Figure 4. Only one predicted open reading frame matches the size of the protein selected by hybrid release translation (34 kd) (Figure 2). Since the cDNA does not contain the 5′ end of the transcript, the 5′ sequence was obtained by determining the sequence of the corresponding fragment from the genomic clone (λE3-22-69/1). (The coding region of the genomic clone is identical to that of the cDNA clone.) Based on sequence homology with related mammalian genes the translation start is in all probability the methionine at position 1. Without determining the N-terminal amino acid sequence of the protein the ATG codon at position 8 cannot be excluded as a possible translation start.

At the nucleotide level as well as at the amino acid level pG22-69 shows significant sequence homology with genes encoding aldose and aldehyde reductases (Figure 5): 41% and 39% of the amino acids are identical with rat lens and human placental aldose reductase and 35% with the human liver aldehyde reductase. These homologies include the tetrapeptide I-P-K-S which has been reported to be the active site for both aldehyde and aldose reductase (Carper *et al.*, 1987). In addition pG22-69 shares sequence homologies with two other reducing enzymes and a structural protein: prostaglandin F synthase from bovine lung (which reduces prostaglandin D_2 to prostaglandin F_2) (32% identical amino acids) (Watanabe *et al.*, 1988); 2,4 diketo-D-gluconate reductase from *Corynebacterium* (27% identical amino acids) (Anderson *et al.*, 1985) and rho-crystallin, a major structural eye lens protein from frog (25% identical amino acids) (Tomarev *et al.*, 1984) (Figure 5). In these comparisons only identical amino acids and not other conservative substitutions were considered.

Immunodetection of the barley aldose reductase gene and determinations of enzymatic activity

The protein encoded by pG22-69 was overexpressed in *Escherichia coli* as an N-terminal fusion protein. *E.coli* cells transformed with the glutathione-*S*-transferase−pG22-69 fusion construct expressed a 55 kd protein, a molecular size expected for the composite protein. Polyclonal antibodies were raised against the fusion protein. These antibodies were used to analyse the expression of the corresponding protein in barley (Figure 6). The antibodies detected a soluble protein of 34 kd molecular mass in developing embryos which was resolved as one spot in a two dimensional fractionation identical to the hybrid-release translation product (Figure 2D and B). In total protein extracts a minor protein of 36 kd was additionally observed; this protein is associated with the insoluble pellet fraction. The 34 kd protein accumulates during embryogenesis and is most abundant in mature embryos. A treatment of 12 DAP embryos with ABA for 3 days led to a significant induction of the detectable protein (Figure 6, lane 7). No signal was obtained for endosperm, barley shoots or roots (Figure 6, lanes 8−10). Leaves of the resurrection plant *Craterostigma plantagineum* (Bartels *et al.*, 1990) display desiccation tolerance like cereal embryos. The antibodies against the barley fusion protein pG22-69 detected a protein of 34 kd in desiccated *Craterostigma* leaves, but not in untreated leaves (Figure 6, lanes 11 and 12).

To link the presence of an aldose reductase homologous protein in barley embryos with aldose reductase activity an enzyme test was performed with fractions obtained after ammonium sulphate precipitations of S30 supernatants from the embryo. The results are summarized in Table I. The highest enzyme activity was found in the fraction saturated to 70% (w/v) with ammonium sulphate. The enzymatic activity in this fraction correlated with the largest amount of protein immunoprecipitated with the antibodies raised against the protein encoded by pG22-69 (Figure 7). The enzymatic activity was dependent on the substrate glyceraldehyde. Xylose and glucose were less efficient substrates. If antiserum against the pG22-69 fusion protein was added to the 70% ammonium sulphate fraction and the antigen complex was precipitated with protein A−Sepharose, the reducing activity in the supernatant was reduced by 45%. Statil (ICI, UK), an inhibitor of human aldose reductase (Poulsom, 1986), causes a 25% reduction of the activity.

Immunolocalization of the pG22-69 encoded protein

Immunocytological methods were used to determine the distribution of the pG22-69 encoded protein in barley embryos. Thin sections of developing embryos were incubated with anti-pG22-69 protein specific antibodies and visualized via indirect immunoperoxidase staining. Peroxidase-labelled secondary antibody showed a high concentration of the pG22-69 protein preferentially in the tissues which were formed earlier in embryogenesis (Figure 8B). The protein is not confined to a particular cell type. No appreciable staining was observed with the preimmune serum (Figure 8A).

Discussion

By differential hybridization a barley embryo cDNA clone was isolated which encodes a protein with significant homology to aldose and aldehyde reductases of mammals

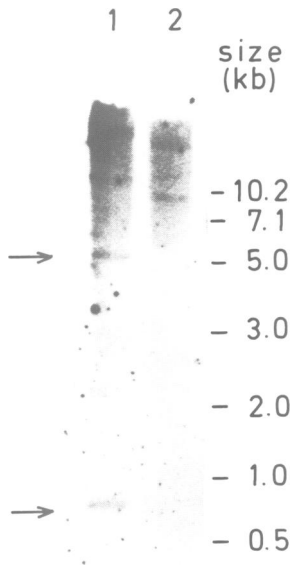

Fig. 3. Southern analysis of genomic DNA from barley (cv. Aura). DNA cut with *Eco*Ri (lane 1) and *Bgl*II (lane 2), was probed with the ^{32}P-labelled insert of pG22-69. Arrows point to the *Eco*RI fragments in size with fragments of a genomic clone.

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   1  gaattcagaagaggagagaggtttgagattcagagaaggtgtgatcatggcgagcgccaa
   1                                                M  A  S  A  K  -

  61  ggcgacgatggggcaggggagcaagatcactttgttctcaagagcgggcatgccatGCC
   6   A  T  M  G  Q  G  E  Q  D  H  F  V  L  K  S  G  H  A  M  P  -

 121  GGCCGTCGGGTTGGGCACCTGGAGAGCTGGCTCCGATACTGCCCACTCCGTTCGGACGGC
  26   A  V  G  L  G  T  W  R  A  G  S  D  T  A  H  S  V  R  T  A  -

 181  CATCACCGAGGCTGGATACAGGCATGTGGACACAGCTGCTGAATACGGAGTAGAAAAGGA
  46   I  T  E  A  G  Y  R  H  V  D  T  A  A  E  Y  G  V  E  K  E  -

 241  GGTCGGCAAAGGGCTTAAGGCCGCAATGGAAGCGGGGATCGACAGGAAAGATTTGTTGT
  66   V  G  K  G  L  K  A  A  M  E  A  G  I  D  R  K  D  L  F  V  -

 301  CACGTCAAAAATATGGTGCACAAACTTGGCCCCTGAGAGGGTGCGGCCAGCATTAGAGAA
  86   T  S  K  I  W  C  T  N  L  A  P  E  R  V  R  P  A  L  E  N  -

 361  CACGCTCAAGGATCTACAGTTGGACTACATCGATCTTTACCACATCCATTGGCCGTTCCG
 106   T  L  K  D  L  Q  L  D  Y  I  D  L  Y  H  I  H  W  P  F  R  -

 421  ACTGAAAGATGGTGCACACATGCCTCCAGAAGCAGGGGAGGTGCTGGAATTCGACATGGA
 126   L  K  D  G  A  H  M  P  P  E  A  G  E  V  L  E  F  D  M  E  -

 481  GGGAGTGTGGAAGGAGATGGAGAACCTTGTGAAGGACGGGCTGGTTAAGGACATCGGCGT
 146   G  V  W  K  E  M  E  N  L  V  K  D  G  L  V  K  D  I  G  V  -

 541  CTGTAACTACACGGTGACCAAGCTCAACCGGCTGCTACGGTCTGCAAAGATTCCACCGGC
 166   C  N  Y  T  V  T  K  L  N  R  L  L  R  S  A  K  I  P  P  A  -

 601  CGTATGCCAGATGGAAATGCACCCTGGTTGGAAGAACGACAAGATTTTCGAGGCCTGCAA
 186   V  C  Q  M  E  M  H  P  G  W  K  N  D  K  I  F  E  A  C  K  -

 661  GAAGCACGGAATTCATGTTACGGCTTACTCCCCATTGGGTTCTTCAGAGAAGAACCTTGC
 206   K  H  G  I  H  V  T  A  Y  S  P  L  G  S  S  E  K  N  L  A  -

 721  CCATGACCCGGTGGTGGAAAAGGTGGCCAACAAACTGAACAAGACCCCGGGGCAGGTGCT
 226   H  D  P  V  V  E  K  V  A  N  K  L  N  K  T  P  G  Q  V  L  -

 781  CATCAAGTGGGCTCTGCAGAGGGGGACGAGTGTGATCCCCAAATCAAGCAAAGATGAGAG
 246   I  K  W  A  L  Q  R  G  T  S  V  I  P  K  S  S  K  D  E  R  -

 841  GATCAAGGAGAACATTCAGGTGTTCGGGTGGGAGATCCCCGAAGAGGACTTCAAGGTCCT
 266   I  K  E  N  I  Q  V  F  G  W  E  I  P  E  E  D  F  K  V  L  -

 901  GTGCAGCATAAAAGATGAGAAGCGTGTGCTGACCGGGGAGGAGCTGTTCGTGAACAAGAC
 286   C  S  I  K  D  E  K  R  V  L  T  G  E  E  L  F  V  N  K  T  -

 961  CCACGGGCCGTACAGGAGCGCAGCCGATGTCTGGGATCACGAGAACTGAGCTTAGCTGAG
 306   H  G  P  Y  R  S  A  A  D  V  W  D  H  E  N                -

1021  CTGAGCTCCCATGCATCATCGAGCTCAGCTCAAGACGCGCAGCTGATCCAAATAACAGAA

1081  GGGTGCTCTCTCCATAATCCATATGTATGTATGCTATGTAATAATAAGCACGGCTGTGTT

1141  CTGTACATGCAAATGCAATGAGTAAGAACGTACGTACGGCTCTTCTTCCTCCGTAAAAAA

1201  AAAAAAAAA
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Fig. 4. Nucleotide sequence (mRNA strand) and predicted amino acid sequence of pG22-69. The nucleotide sequence derived from the genomic clone is given in lower case letters and the sequence of the cDNA clone in upper case letters.

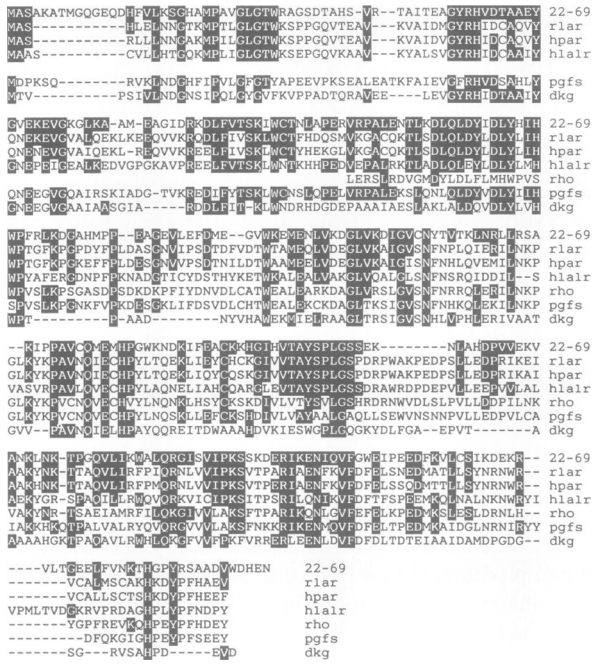

Fig. 5. Comparisons of the amino acid sequences of the barley clone pG22-69, the rat lens aldose reductase (rlar) (Nishimura *et al.*, 1989), the human placenta aldose reductase (hpar) (Bohren *et al.*, 1989), the human liver aldehyde reductase (hlalr) (Bohren *et al.*, 1989), the rho-crystallin of the frog lens (rho) (Tomarev *et al.*, 1984), the prostaglandin F synthase (pgfs) (Watanabe *et al.*, 1988) and the 2,5-diketo-D-gluconic acid reductase from *Corynebacterium* (dkg) (Anderson *et al.*, 1985). Amino acid identities between pG22-69 and any of the other sequences are indicated in black. Dashes were introduced to optimize sequence alignment.

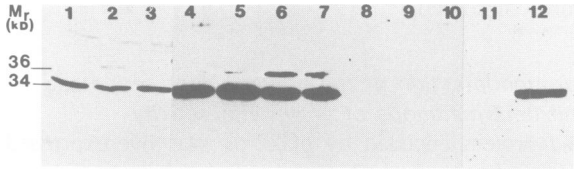

Fig. 6. Western blot of pG22-69 related proteins. Total protein extracts of the following tissues were prepared: lane 1, 10 DAP embryos; lane 2, 12 DAP embryos; lane 3,14 DAP embryos; lane 4, 18 DAP embryos; lane 5, 20 DAP embryos; lane 6, mature embryos; lane 7, 12 DAP embryos incubated in the presence of ABA for 3 days; lane 8, endosperm; lane 9, leaf tissue; lane 10, roots; lane 11, leaves of *C.plantagineum* (untreated); lane 12, desiccated leaves of *Craterostigma*. Always six embryos of each developmental stage were homogenized in 200 μl Laemmli sample buffer and 30 μl loaded per lane; for the preparation of the other samples see Materials and methods. The separated proteins were transferred onto a nitrocellulose filter, which was probed with the anti-pG22-69 antiserum.

(references listed in Figure 5 and Garcia-Perez *et al.*, 1989; Petrash and Favello, 1989; Schade *et al.*, 1990). The molecular structure, the biochemical characteristics of the protein (soluble protein of 34 kd), its enzymatic activity and substrate specificity, confirm the identification of this barley gene as aldose reductase. We show that barley embryo extracts are capable of reducing glyceraldehyde and, with lower efficiency, glucose and xylose. A relevant part of this enzymatic activity can be attributed to the characterized aldose reductase gene product, as evident from antibody inhibition of enzymatic activity (Table I) and activity data (Figure 7). As the enzyme has not been purified to homogeneity from barley, it cannot be excluded that other NADPH dependent enzymes contribute to the determined reducing activity. The expression of the aldose reductase transcript and the corresponding protein is under developmental and hormonal control. The steady state level of the transcript reaches its maximum in embryos of ~18 DAP and remains constant during further development. Incubation of embryos with ABA induces the synthesis of aldose reductase. This is supported by the observation that no transcript was detected when the embryos were treated with fluridone, an inhibitor of ABA. ABA has also been implicated in the control of a number of genes expressed during late embryogenesis which have been correlated with increased tolerance to osmotic stress (for a review see Skriver and Mundy, 1990). These genes and the barley aldose reductase gene share modulation by ABA. Our data indicate that the levels of aldose reductase mRNA and protein are determined by the interplay of ABA and GA. The two hormones are already known for their antagonistic roles in seed physiology, being germination supportive (GA) or inhibitory (ABA) (King, 1982).

In a number of mammalian organisms aldose reductase is induced under hyperglycaemic conditions and is associated

Table I. Aldose reductase activity in fractions obtained after ammonium sulphate fractionation of S30 supernatants from the embryo

Fraction	Substrate	Activity[a]
40% a.s. fraction	glyceraldehyde	20%
70% a.s. fraction	glyceraldehyde	100%
70% a.s. fraction	xylose	31%
70% a.s. fraction	glucose	26%
70% a.s. fraction	glyceraldehyde + Statil	75%
	incubation with	55%
	pG22-69 antiserum	
	→ glyceraldehyde	
	incubation with	100%
	preimmune serum	
	→ glyceraldehyde	

[a]Expressed in per cent of maximum activity as found in the 70% a.s. fraction incubated with glyceraldehyde as substrate.
a.s., ammonium sulphate.

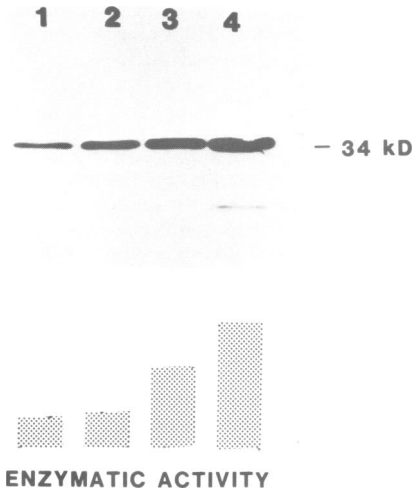

Fig. 7. Western blot analysis and enzymatic activity measurements of ammonium sulphate fractions of an S30 supernatant of barley embryos. The following samples were analysed via immunoblotting for the presence of pG22-69 related protein: lanes 1 and 2 respectively, 80 and 160 μg of protein of the 40% ammonium sulphate fraction; lanes 3 and 4 respectively, 80 and 160 μg of protein of the 70% ammonium sulphate fraction. The block diagram in the lower part of the figure reflects the relative aldose reductase activities measured in the fractions using glyceraldehyde as substrate.

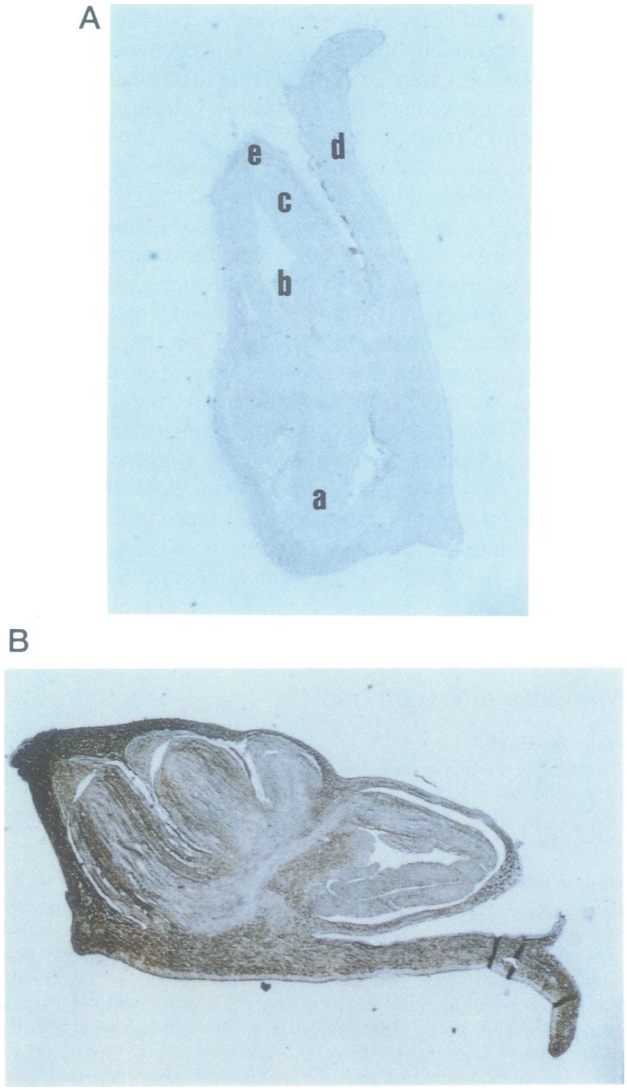

Fig. 8. Immunocytochemical localization of the pG22-69 encoded protein in developing barley embryos. Cross-sections of 18 DAP barley embryos were hybridized with preimmune serum (**A**) and anti-pG22-69, protein specific antibodies (**B**). The localization of the protein is indicated by the dark-brown peroxidase reaction products. Indicated are the different embryonic tissues: (a) embryonic roots, (b) shoot apex, (c) plumule with embryonic leaves, (d) scutellar tissue and (e) coleoptile.

with diabetic complications (Nishimura *et al.*, 1990; see also for further references). In renal medullary cells an increase in aldose reductase activity is a response to higher osmotic stress as mediated by NaCl (Garcia-Perez *et al.*, 1989; Moriyama *et al.*, 1989). In all these metabolic situations aldose reductase is considered a key enzyme in the polyol pathway leading to the accumulation of sorbitol (Jeffrey and Jörnvall, 1983). Sorbitol, like other polyols, is a common cell osmolyte which helps to balance the osmotic strength of the cytoplasm with that of the environment without affecting the function of important macromolecules. Pathways leading to the accumulation of osmolytes have been studied in bacteria and lower eukaryotes (Yanay *et al.*, 1982; Le Rudulier *et al.*, 1984). The fact that the antibodies directed against the barley protein also detected a protein of the same relative mol. wt (34 000) in the dried leaf tissue of the desiccation tolerant plant *C.plantagineum* (Figure 6) provides further evidence that the gene encoded by pG22-69 is related to osmoprotection.

Evidence for the occurrence of the sorbitol pathway in plants is given by the finding of this compound in germinating soybean axes (Kuo *et al.*, 1990), in fruits of members of the *Rosaceae* (Wallaart, 1980), in developing maize kernels (Shaw and Dickinson, 1984) and in barley seeds (Gohl *et al.*, 1978). It has been suggested that sorbitol in these tissues might represent a form of carbon storage. Alternatively it may play an osmoregulatory role as suggested for apples (Raese *et al.*, 1978) and of the salt tolerant marsh plant, *Plantago maritima* (Ahmad *et al.*, 1979). The functioning of the sorbitol pathway has recently been suggested for germinating soybean axes (Kuo *et al.*, 1990).

With the exception of the carboxy-terminal end, the amino acid sequence of the aldose reductase protein as deduced from the cDNA clone shows regions of perfect homology

with the mammalian structural genes encoding aldose reductases and aldehyde reductases. Sequence conservation among plants and animals suggests that functionally important regions must have been conserved during evolution together with similar metabolic pathways. As pointed out for mammalian aldose reductases (Nishimura *et al.*, 1989) the barley aldose reductase is also related to crystallin, the major structural protein of the frog eye lens (Tomarev *et al.*, 1984). A recent review revealed that functionally similar crystallins have evolved from stress responsive proteins like heat-shock proteins and stress inducible enzymes (de Jong *et al.*, 1989). Structural similarities exist between the barley aldose reductase and the bovine lung prostaglandin F synthase as well as the diketo-D-gluconate reductase from *Corynebacterium*. These proteins all belong to the NADPH dependent aldo-keto reductases with the conserved protein domains representing the active sites of these enzymes.

Our present findings support the existence of a metabolic pathway in plants directing the synthesis of osmoprotective molecules. The isolation of the structural gene is a first step towards the understanding of the metabolic context in which barley aldose reductase operates.

Materials and methods

Plant material

Culture of barley (*Hordeum vulgare* L.) plants and isolation of stage specific barley embryos are described by Bartels *et al.* (1988). If required ABA or GA (100 μm each) or fluridone (10^{-3} mg/ml) were added to the embryo culture medium (GM).

Construction and screening of a cDNA library

Four μg of poly(A)$^+$ RNA extracted from 18 DAP embryos were used as a template. Two libraries were constructed. For one library the cDNA was tailed with *d*-cytidine 5′-triphosphate and cloned into the pUC9 vector which was used to transform *E.coli* (strain TG-2) (Bartels *et al.*, 1990). For the other library *Eco*RI-linked cDNA was ligated to *Eco*RI-digested lambda NM1149 arms and packaged *in vitro*. Approximately 5×10^5 plaque forming units were plated on the selective strain POP13. This library was differentially screened with ^{32}P-labelled cDNAs synthesized from RNAs extracted from 12 DAP or 18 DAP embryos. Plaques hybridizing predominantly with the 18 DAP probes were purified and subcloned into pUC19. A fragment of one of these differentially hybridizing clones was used to screen the *E.coli* plasmid library to obtain longer cDNA clones.

Isolation of a genomic clone

Genomic clones were isolated from a barley genomic library made from DNA of *H.vulgare* L. var. NK 1558 using EMBL-3 as a cloning vector. The library was obtained from Clontech, CA, USA. The ^{32}P-labelled insert of pG22-69 was used for screening and plaque purification.

DNA sequencing and computer analysis

The nucleotide sequence of the cDNA clone was determined on both strands by subcloning of suitable restriction enzyme digest fragments into M13mp18 and 19 (Messing and Vieira, 1982) followed by dideoxynucleotide sequencing using the T7 polymerase kit (Pharmacia LKB, Freiburg, FRG). The program WISGEN (version 5.3) of the University of Wisconsin genetic group was used for nucleic acid and protein sequence analysis (Devereux *et al.*, 1984), and amino acid comparisons were done with the TFASTA program (Pearson and Lipman, 1988).

Other recombinant DNA techniques

Isolation of plasmid DNA, preparation of DNA fragments, ligation and transformation of *E.coli* cells were essentially carried out according to Maniatis *et al.* (1982). Isolation of barley DNA and Southern blot analysis are described by Thompson *et al.* (1983). RNA extraction, Northern analysis and hybrid release translations were performed according to Bartels *et al.* (1986, 1988).

Construction of expression vectors and analysis of expressed proteins

The insert of pG22-69 was digested with *Pvu*II resulting in a 847 bp fragment. This fragment was ligated into the *Sma*I site of the expression vector pGEX-1 (Smith and Johnson, 1988) to yield a translational fusion with the glutathione-*S*-transferase (GST). The expression of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 0.4 mM. Transformants were screened for expression of fusion proteins as described (Smith and Johnson, 1988).

Antibodies and immunoblotting

To purify the pGEX(1)-22-69−GST fusion protein from *E.coli* cells inclusion bodies were prepared (Schmidt *et al.*, 1986) and the proteins were separated on 12% preparative SDS−polyacrylamide gels. The proteins were visualized with KCl, excised and electroeluted (Nelles and Bamburg, 1976). Purified fusion protein (150 μg) was emulsified with an equal volume of complete Freund's adjuvant (Sigma) and was injected into a rabbit. Two more injections of antigen in incomplete Freund's adjuvant (Sigma) followed after 21 and a further 28 days. Serum was collected 10 days after each injection.

For immunodetection protein samples were prepared as follows: 100 mg of fresh tissue or 20 mg of dried tissue was ground and dissolved in 200 μl of Laemmli sample buffer (0.625 M Tris−HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue), and aliquots were loaded on to the gels. Electrophoretic transfer of proteins from SDS−polyacrylamide gels to nitrocellulose membranes was carried out as described (Towbin *et al.*, 1979). The membrane was probed with antiserum (1:1000 dilution) raised against the fusion protein followed by an incubation with anti-rabbit IgG conjugated horseradish peroxidase. The protein−antibody complex was detected by using the chemiluminescence (ECL) Western blotting detection system from Amersham (Braunschweig, FRG) according to the manufacturer's recommendations.

Assay of enzyme activity

Around 1 g of 18 DAP embryos was homogenized under liquid nitrogen with 10 ml extraction buffer (20 mM potassium phosphate buffer, pH 7.5, 5 mM mercaptoethanol, 0.5 mM EDTA). The thawed homogenate was centrifuged at 12 000 *g* for 20 min. The supernatant was saturated with ammonium sulphate to 40%. After centrifugation the ammonium sulphate concentration was raised to 70% saturation in the supernatant. The proteins precipitated by ammonium sulphate were dissolved in a small amount of extraction buffer, dialysed and aliquots were assayed for aldose reductase activity. All steps were done at 0−4°C.

Aldose reductase activity was photometrically determined by measuring the decrease in the concentration of NADPH at 340 nm for 5 min at room temperature according to Kawasaki *et al.* (1989) and Shiono *et al.* (1987). Assay mixtures (500 μl) contained 100 mM sodium phosphate buffer, pH 6.9, 0.15 mM NADPH and 10 mM DL-glyceraldehyde (Sigma) as substrate. The reaction was initiated by adding the enzyme. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation of 1 μmol NADPH per min under the conditions described here. The protein concentrations were determined using the Bio-Rad protein assay kit.

Immunohistochemistry

Tissue fixation, paraffin embedding, sectioning and the subsequent treatments of the sections are described by Schmelzer *et al.* (1989). The immunoperoxidase staining was done according to Jahnen and Hahlbrock (1988) with the following modifications: the sections (8 μm) were incubated for 1.5 h with the pG22-69 antiserum (dilution 1:100). After the washing procedure the sections were hybridized (1 h) with the peroxidase-labelled secondary antibody (Diagnostics Pasteur, Marne-La-Coquette, France).

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References

Ahmad,I., Larker,F. and Stewart,G.R. (1979) *New Phytol.*, **82**, 671−678.

Anderson,S., Berman Marks,C., Lazarus,R., Miller,J., Stafford,K., Seymour,J., Light,D., Rastetter,W. and Estell,P. (1985) *Science*, **230**, 144−149.

Bartels,D., Altosaar,I., Harberd,N.P., Barker,R.F. and Thompson,R.D. (1986) *Theor. Appl. Genet.*, **72**, 845−853.

Bartels,D., Singh,M. and Salamini,F. (1988) *Planta*, **175**, 485−492.

Bartels,D., Schneider,K., Terstappen,G., Piatkowski,D. and Salamini,F. (1990) *Planta*, **181**, 27−34.

Bohren,K.M., Bullock,B., Wermuth,B. and Gabbay,K.H. (1989) *J. Biol. Chem.*, **264**, 9547–9551.
Carper,D., Nishimura,C., Shinohara,T., Dietzchold,B., Wistow,G., Craft,C., Kadur,P. and Kinoshita,J. (1987) *FEBS Lett.*, **220**, 209–213.
de Jong,W.W., Hendiks,W., Mulders,J.W.M. and Blomendahl,H. (1989) *Trends Biochem. Sci.*, **14**, 365–368.
Devereux,J., Haeberly,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
Dure,L., III, Crouch,M., Harada,J., Ho,T.-H., Mundy,J., Quatrano,R., Thomas,R. and Sung,Z.R. (1989) *Plant Mol. Biol.*,**12**, 475–486.
Gaff,D.F. (1980) In Turner,W.C. and Kramer,P.J. (eds), *Adaptation of Plants to Water and High Temperature Stress.* Wiley Interscience, New York, pp. 207–209.
Garcia-Perez,A., Martin,B., Murphy,H.R., Uchida,S., Murer,H., Cowley,B.D., Jr, Handler,J.S. and Burg,M.B. (1989) *J. Biol. Chem.*, **264**, 16815–16821.
Gohl,B., Nilsson,M. and Thomke,S. (1978) *Cereal Chem.*, **55**, 341–347.
Jahnen,W. and Hahlbrock,K. (1988) *Planta*, **173**, 197–204.
Jeffrey,J. and Jörnvall,H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 901–905.
Kawasaki,N., Tanimoto,T. and Tanaka,A. (1989) *Biochim. Biophys. Acta*, **996**, 30–36.
Kermode,A. (1990) *Crit. Rev. Plant Sci.*, **9**, 155–195.
King,R.W. (1982) In Khan,A.A. (ed.), *The Physiology and Biochemistry of Seed Development, Dormancy and Germination.* Elsevier Biomedical Press, Amsterdam, pp. 157–181.
Kuo,T.M., Doehlert,D.C. and Crawford,C.G. (1990) *Plant Physiol.*, **93**, 1514–1520.
Le Rudulier,D., Strom,A.R., Dandekas,A.M., Smith,L.T. and Valentine, R.C. (1984) *Science*, **224**, 1064–1068.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laborator Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269–276.
Moore,R. and Smith,J.D. (1984) *Planta*, **162**, 342–344.
Moriyama,T., Garcia-Perez,A. and Burg,M.B. (1989) *J. Biol. Chem.*, **264**, 16810–16814.
Nelles,L.P. and Bamberg,J.R. (1976) *Anal. Biochem.*, **73**, 522–531.
Nishimura,C., Matsuura,Y., Kokai,Y., Akera,T., Carper,D., Morjana,N., Lyons,C. and Flynn,R.G. (1990) *J. Biol. Chem.*, **265**, 9788–9792.
Pearson,W.R. and Lipman,D.-J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2444–2448.
Petrash,J.M. and Favello,A.D. (1989) *Curr. Eye Res.*, **8**, 1021–1027.
Poulsom,R. (1986) *Biochem. Pharmacol.*, **35**, 2955–2959.
Raese,J.T., Williams,M.W. and Billingsley,H.D. (1978) *J. Am. Soc. Hortic. Sci.*, **103**, 796–801.
Skriver,K. and Mundy,J. (1990) *Plant Cell*, **2**, 503–512.
Schade,S.Z., Early,S.L., Williams,T.R., Kezdy,F.J., Heinrikson,P.L., Grimshaw,C.E. and Doughty,C.C. (1990) J. Biol. Chem., **265**, 3628–3635.
Schmelzer,E., Krüger-Lebus,S. and Hahlbrock,K. (1989) *Plant Cell*, **1**, 993–1001.
Schmidt,J., John,M., Wieneke,U., Krüssmann,H.-D. and Schell,J. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9581–9585.
Smith,D.B. and Johnson,K.S. (1988) *Gene*, **67**, 31–40.
Shaw,J.R. and Dickinson,D.B. (1984) *Plant Physiol.*, **75**, 207–211.
Shiono,T., Sato,S., Reddy,V.N., Kador,P.F. and Kinoshita,J.H. (1987) In Flynn,T.G. and Weiner,H. (eds), *Enzymology of Carbonyl Metabolism.* A.R.Liss, New York, pp. 317–324.
Thompson,R.D., Bartels,D., Harberd,N.P. and Flavell,R.B. (1983) *Theor. Appl. Genet.*, **67**, 87–96.
Tomarev,S.I., Zinovieva,R.D., Dolgilevich,S.M., Luchin,S.V., Krayer,A.S., Skryabin,K.G. and Gause,G.G., Jr (1984) *FEBS Lett.*, **171**, 297–302.
Towbin,H., Staehelin,T. and Gordon,T. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
Watanabe,K., Fujii,Y., Nakayama,L., Ohkubo,H., Kuramitsu,S., Kagamiyama,H., Nakanishi,S. and Hayaishi,O. (1988) *Proc. Natl. Acad. Sci., USA*, **85**, 11–15.
Yanay,P.H., Clark,M.E., Hand,S.C., Bowens,D.R. and Somero,G.N. (1982) *Science*, **217**, 1214–1222.

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