

ASP53, a thermostable protein from *Acacia erioloba* seeds that protects target proteins against thermal denaturation

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Abstract. ASP53, a 53 kDa heat soluble protein, was identified as the most abundant protein in the mature seeds of *Acacia erioloba* E.Mey. Immunocytochemistry showed that ASP53 was present in the vacuoles and cell walls of the axes and cotyledons of mature seeds and disappeared coincident with loss of desiccation tolerance. The sequence of the ASP53 transcript was determined and found to be homologous to the double cupin domain-containing vicilin class of seed storage proteins. Mature seeds survived heating to 60°C and this may be facilitated by the presence of ASP53. Circular dichroism spectroscopy demonstrated that the protein displayed defined secondary structure, which was maintained even at high temperature. ASP53 was found to inhibit all three stages of protein thermal denaturation. ASP53 decreased the rate of loss of alcohol dehydrogenase activity at 55°C, decreased the rate of temperature-dependent loss of secondary structure of haemoglobin and completely inhibited the temperature-dependent aggregation of egg white protein.

Additional keywords: circular dichroism spectroscopy, immunocytochemistry, protection of protein conformation, seed storage protein.

Introduction

Acacia erioloba, the Camel thorn tree, grows in the semi-arid regions of Southern Africa. The seed pods are shed in mid-winter and are an important source of nutrition for many species, especially the Gemsbok, *Oryx gazella*. Each pod contains ~20 seeds, which are indigestible and are excreted in the surrounding area. The seeds only germinate when the rains arrive in mid to late summer, before which they are exposed to ground temperatures regularly exceeding 70°C. Survival under these conditions might suggest that these seeds be classified as extremophiles and suggested the possibility of the presence of unusual late embryogenic abundant-like proteins in the mature seeds of this tree.

The survival of many plants is dependent to a large extent on the ability of the seeds they produce to withstand environmental conditions not conducive to vegetative growth (Roberts 1973). Seeds of most plant species undergo desiccation as part of a normal developmental program on the parent plant before shedding into the environment. They acquire tolerance to desiccation during the mid to final stages of reserve accumulation, before the onset of maturation drying (Sun and Leopold 1993; Vertucci and Farrant 1995). Acquisition of desiccation tolerance is coincident with the synthesis of non-reducing sugars and compatible solutes (Blackman *et al.* 1992; Horbowicz and Obendorf 1994; Scott 2000), antioxidants (Leprince *et al.* 1994), and stress-induced proteins (Bray 1997), all believed to play a protective role in tolerance of severe water

deficit (Vertucci and Farrant 1995). These molecules are rapidly degraded during germination to provide a source of amino acids for protein synthesis and metabolic energy (Leprince *et al.* 1993; Vertucci and Farrant 1995) in the developing seedlings, a process that has been shown to coincide with the loss of desiccation tolerance (Blackman *et al.* 1991).

Stress-induced proteins synthesised include the heat shock proteins and the late embryogenesis abundant (LEA) proteins. LEA proteins are a diverse group of poorly related proteins, which have been divided into at least six classes with different hydrophilicities and presumably different functions (for a recent review see Wise and Tunnacliffe 2004). LEA proteins can constitute up to 4% of total cellular protein (Roberts *et al.* 1993) and have been reported to be present not only in seeds but in vegetative tissue, bacteria and arthropods (Kikawada *et al.* 2006). Although the exact role of LEA proteins in plants has yet to be determined, these proteins are characterised by having a high hydrophilic amino acid content (Baker *et al.* 1988; Close *et al.* 1989; Dure *et al.* 1989), a feature that has been proposed to allow them to act as water replacement molecules thereby stabilising macromolecules against desiccation stress (Garay-Arroyo *et al.* 2000). An alternative hypothesis suggests that LEA proteins sequester ions accumulated during dehydration and interact with exposed hydrophobic surfaces of partially denatured proteins to prevent aggregation (Campbell and Close 1997; Close 1997). Most LEA proteins have been proposed to display no structure in hydrated solution, only gaining structure

on desiccation (Wise and Tunnacliffe 2004). However, a LEA14 protein from *Arabidopsis thaliana* (L) Heynh. has recently been shown with NMR spectroscopy to display a defined structure in solution (Singh *et al.* 2005). Strong circumstantial evidence exists that LEA proteins play a role in stress tolerance with specific roles suggested for some LEA and LEA-like proteins. The *Saccharomyces cerevisiae* LEA-like protein HSP12p has been proposed to act as a plasticiser in the cell wall of the yeast *S. cerevisiae* (Motshwene *et al.* 2004; Karreman *et al.* 2005) and the *Typha* pollen D7 protein was found to stabilise sugar glasses in an *in vitro* system (Wolkers *et al.* 2001).

The cupin family of proteins comprises a wide variety of proteins from both prokaryotes and eukaryotes and includes the seed storage proteins (for reviews see Dunwell 1998; Dunwell *et al.* 2000, 2004). The cupin domain, first identified in jack bean [*Canavalia ensiformis* (L) DC] canavalin (Ko *et al.* 1993), consists of six β -strands forming a β -barrel structure. Cupin domains have since been reported to be present in proteins from a wide variety of phyla within the bacterial and eukaryotic kingdoms and include not only seed storage proteins but also dioxygenases, isomerases, epimerases, decarboxylases, auxin-binding proteins, transcription factors as well as proteins that regulate seed germination and early seed development (Lapik and Kaufman 2003). It has been suggested that the widespread occurrence of this domain in a variety of proteins is on account of its thermal stability and resistance to proteolysis (Dunwell *et al.* 2004). Cupin domain-containing proteins would therefore be classed as genuine storage proteins, one feature of which is resistance to degradation during seed maturation (Shutov *et al.* 2003). Several cupin domain-containing proteins, for example anthocyanidin synthase (Wilmouth *et al.* 2002), quercetin dioxygenase (Fuzetti *et al.* 2002) and phosphomannose isomerase (Wilmouth *et al.* 2002) have biochemical activities associated with the cell wall. In addition, germin-like proteins have been shown to have enzymatic activities including oxalate oxidase and superoxide dismutase (Woo *et al.* 2000) as well as serine protease inhibitory activity (Segarra *et al.* 2003).

This paper reports the identification of a cupin domain-containing protein present in the cell walls and the vacuoles of the mature seeds of this tree. This protein was found to protect other proteins against thermal denaturation.

Materials and methods

Plant material

Newly shed *Acacia erioloba* E.Mey seed pods were collected from Auob in Southern Namibia and stored at -20°C until used. To test whether the presence of the protein within embryos correlated with seed desiccation tolerance, mature seeds of *A. erioloba* were allowed to germinate to different stages (denoted by radicle length) on water-saturated filter paper at 30°C in the dark. The variously germinated seedlings were subsequently desiccated at room temperature for 12 h with a flash-drying apparatus (Farrant *et al.* 1985), and thereafter rehydrated at 30°C in the light on a water-saturated filter paper to test for viability. Survival of seedlings was determined after 8 days, with seedlings exhibiting re-growth of the radicle and primary leaf formation scored as viable and, thus, tolerant of imposed drying. Total proteins were extracted from seedlings

before dehydration, after dehydration and after rehydration to determine protein expression patterns.

The ability of seeds to withstand elevated temperatures was tested by placing whole, mature seeds into incubators set at temperatures of 30, 40, 50, 55 and 60°C for 1 week. Seeds were germinated as outlined above to test for viability. Seed water content was determined gravimetrically by oven drying at 105°C for 24 h. Mature seeds had a water content of 7% ($\text{g H}_2\text{O g}^{-1}$ dry mass) and flash drying for 12 h reduced embryo water content to this value.

Protein purification

Seed coats were removed and the embryos were homogenised with a Kinematica CH-6010 Ultra-turrex homogeniser (Lucerne, Switzerland) in ice-cold extraction buffer, 10 mM Tris-HCl 50 mM NaCl 5 mM MgCl_2 1 mM phenylmethanesulfonyl fluoride (PMSF) pH 7.4, typically 10 mL g^{-1} . The protein was present in axes and cotyledons, and was extracted separately or pooled, depending on the experimental procedure to follow. The homogenate was centrifuged at $27\,000\text{ g}$ using a Beckman JA rotor (Beckham, Fullerton, CA, USA) for 10 min at 4°C after which the supernatant was incubated at 80°C for 30 min. Heat-coagulated protein was removed by centrifugation as above. Heat soluble protein was fractionated on a Whatman CM 52 cation-exchange column (Whatman, Kent, UK) previously equilibrated with 50 mM acetate (Na^+) 50 mM NaCl pH 5.0. Proteins were eluted with a gradient of 50 mM to 500 mM NaCl in this same buffer. Further purification was carried out by gel filtration with a Sephadex G-100 column (Sigma-Aldrich, Cambridge, MA, USA) using 20 mM HCl as the solvent. Final purification was performed by HPLC with a Jupiter C_{18} reverse-phase column (Phenomenex, Torrance, CA, USA) equilibrated in 0.1% heptafluorobutyric acid (HFBA). Proteins were eluted using a linear 0–70% gradient of acetonitrile in 0.1% HFBA. Protein used for secondary structure analysis was not heat-treated, and the gel filtration step was performed with 50 mM phosphate (K^+) buffer pH 7.0. Proteins were eluted with this same buffer, pooled, concentrated by Amicon (Millipore, Billerica, MA, USA) ultrafiltration and re-applied to this same column. No HPLC purification was applied to this protein preparation. Heat treatment and HPLC purification were not used in case these methods resulted in irreversible protein denaturation.

Peptide identification and sequencing

A gel piece of 1 mm in diameter containing ASP53 was excised from a Coomassie-stained gel and in-gel digested with trypsin (Westermeier and Naven 2002). Peptides were mixed with an equal volume of α -cyano-4-hydroxy-cinnamic acid and analysed by matrix assisted laser desorption/ionisation–time-of-flight (MALDI–TOF) mass spectrometry. The remainder of the digest was guanidinated with *O*-methylisourea hydrogen sulphate and sulfonated with the chemically activated fragmentation (CAF) reagent (Ettan, GE Healthcare, Amersham, Buckinghamshire, UK) before being analysed by MALDI–TOF mass spectrometry.

PCR and 5' RACE

Total RNA was extracted from mature seeds of *A. erioloba* using TRI-REAGENT (Molecular Research Center, Cincinnati, OH, USA). cDNA was synthesised from DNaseI-treated total RNA ($2\ \mu\text{g}$) with Moloney murine

leukaemia virus reverse transcriptase (M-MLV RT, Promega, Madison, WI, USA) and an anchored oligo dT primer (5'-GGGATCCT₁₈(CAG)I-3'). Complementary DNA together with the oligo dT primer and a degenerate primer, GSP 1, (5'-AA(CT)CA(AG)TA(CT)GA(CT)GCI(CTA)TI(CA)G-3') designed from the amino acid sequence of the 1577.7 Da peptide obtained by post source delay (PSD)-MALDI-TOF mass spectroscopy were used to amplify the ASP53 gene. The sample was denatured at 94°C for 4 min before being subjected to 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min in a GeneAmp PCR System 9700, Perkin Elmer (Applied Biosystems, Foster City, CA, USA). The purified PCR product was cloned into pGEM-T Easy vector and sequenced (Sanger *et al.* 1977).

5' RACE was performed to determine the sequence of the 5' end of the ASP53 gene. cDNA synthesis was carried out using an Expand Reverse Transcriptase kit (Roche, Basel, Switzerland) in the presence of SMART IV oligonucleotide (Creator SMART cDNA Library Construction Kit, BD Biosciences Clontech, Palo Alto, CA, USA) and an anchored oligo dT primer. To amplify the 5' end of the gene, a gene specific antisense primer, GSP 2, designed from the DNA sequence of the RT-PCR product was used in conjunction with the 5' PCR primer (complementary to the SMART IV oligonucleotide that was ligated to the 3' end of the cDNA). PCR was performed using the Expand High Fidelity^{PLUS} PCR System (Roche) in a GeneAmp PCR System 9700, Perkin Elmer (Applied Biosystems) by 30 cycles of sample denaturation at 94°C for 30 s, primer annealing at 61°C for 45 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The purified 5' RACE product was cloned into pGEM-T Easy vector and sequenced.

Circular dichroism and thermal denaturation

The circular dichroism spectra of samples in 100 mM phosphate (K⁺) pH 7.4 were determined at temperatures between 20 and 80°C. Spectra were obtained over the range 200–260 nm with a Jasco J-810 spectropolarimeter (Victoria, BC, Canada) with a 1-cm path length quartz cell at a protein concentration of 0.5 mg mL⁻¹ in 100 mM K₂HPO₄ pH 7.4. Subtraction of spectra was performed by subtracting the ellipticity ($y_{2i} - y_{1i}$) at each wavelength x_i (0.1 nm intervals between 200 and 260 nm) for each x, y data pair. Predictions of the relative secondary structure were made using CDNN software (<http://bioinformatik.biochemtech.uni-halle.de/cdnn>, accessed 2002).

Protein aggregation was monitored by measuring the light scattering of the solution in 100 mM K₂HPO₄ pH 7.4 at 350 nm with a Pye-Unicam SP1800 spectrophotometer (Pye-Unicam, Cambridge, UK) with a custom-made heating block interfaced to an IBM PC through an Oasis A/D converter. Absorbance values were recorded every 0.2°C between 20 and 90°C at a heating rate of 1°C min⁻¹. The maximum rate of thermal denaturation, the T_m , was determined from calculating the rate of change of light scattering as a function of the temperature.

The activity of yeast alcohol dehydrogenase (ADH, Sigma Chemical Co., St Louis, MO, USA) was determined from the spectrophotometric increase in NADH + H⁺ at room temperature at 340 nm with a Shimadzu UV-2201 spectrophotometer (Shimadzu, Kyoto, Japan). The assay mixture

contained 0.01 mM NAD⁺ 0.01 M ethanol 10 mM pyrophosphate pH 8.0.

Immunocytochemistry

Tissue processing for transmission electron microscopy (TEM)

Several (>20) 5-mm² cubes of cotyledons and axes from at least five different mature seeds were processed for TEM as previously described (Sherwin and Farrant 1996). Epoxy-resin (Spurr 1969) infiltrated specimens were sectioned at ~95 nm using a Reichert Ultracut-S microtome (Reichert, Vienna, Austria) and collected on nickel grids.

Antibody preparation and purification

The antibody used was prepared by injecting HPLC-purified protein in Freund's complete adjuvant into rabbits. Titres and specificity were determined by ELISA as described by Harlow and Lane (1988). Immunocytochemistry using this antibody was performed as described previously (Motshwene *et al.* 2004). The antibody detected the protein used to immunise the rabbits and failed to recognise any of the smaller proteins, possibly processing products, detected in Fig. 1.

Immunogold labelling

Sections were sequentially incubated on drops of 1% (w/v) BSA in 0.1 M phosphate buffer pH 7.2 (PB) to prevent non-specific antibody binding, and on 0.02 M glycine to block unreactive aldehyde groups, before incubation with the primary

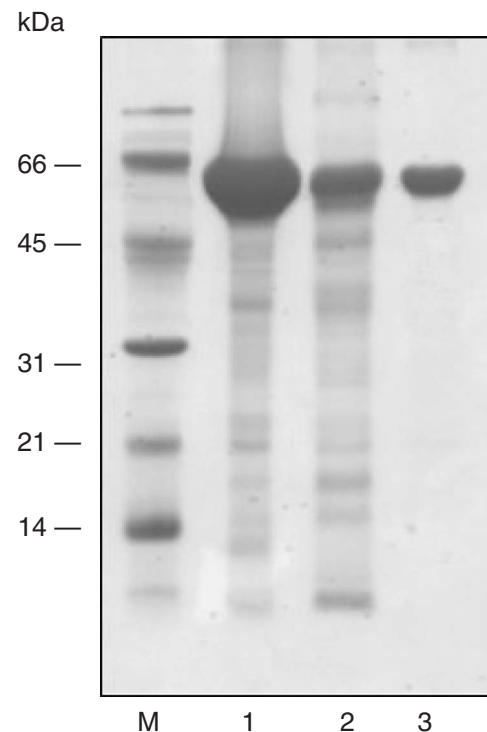


Fig. 1. SDS-PAGE of total proteins extracted from mature seeds of *Acacia erioloba* (lane 1), soluble proteins after incubation at 80°C for 30 min (lane 2) and HPLC-purified protein (lane 3). The molecular weights (in kDa) of markers (M) of known size are denoted on the left hand side of the figure.

antibody diluted 1:1000 in 1% (w/v) BSA/PB at room temperature for 16 h. After washing in 1% (w/v) BSA/PB, the grids were floated on a drop of goat-anti-rabbit immunoglobulin, to which was attached 10-nm diameter gold particles, diluted 1:50 in 1% (w/v) BSA/PB. Incubation was carried out for a minimum of 60 min at room temperature, after which the grids were rinsed with 1% (w/v) BSA/PB. Sections were fixed by floating the grids on 1% (v/v) glutaraldehyde for 10 min. After thoroughly rinsing the grids in water, samples were stained with 3% (w/v) uranyl acetate and 1% (w/v) lead citrate. Sections were examined for gold labelling with a Zeiss 200 SX (Zeiss, Göttingen, Germany) TEM. Control sections were probed with pre-immune serum in place of the antisera.

Results

A 53-kDa protein was found to be the most abundant in the total protein extracted from mature seeds of *A. erioloba*, constituting ~47% of the total soluble protein in whole seeds.

This protein together with a few other proteins remained soluble after incubation at 80°C for 30 min (Fig. 1), suggesting that they were hydrophilic in nature. The 53 kDa protein, named ASP53 (Acacia seed protein of molecular size 53 kDa), was purified to homogeneity (Fig. 1) and used to immunise rabbits for antibody production. Immunocytochemistry using this antibody showed label to occur only in the cell wall of the axis (Fig. 2A) and cell walls and protein storage vacuoles in cotyledons (Fig. 2B) of mature seeds. No gold particles indicative of the presence of ASP53 were detected when similar sections were probed with the pre-immune serum (Fig. 2C).

To identify ASP53, purified protein was subjected to Edman degradation. Although trace amounts of the sequence GSEQQRQDEPT were found, RT-PCR of mRNA extracted from *A. erioloba* seeds with a degenerate primer directed to this sequence together with an oligo-dT primer failed to produce a PCR product. We next digested ASP53 with trypsin to obtain a peptide mass fingerprint, which was used to determine whether

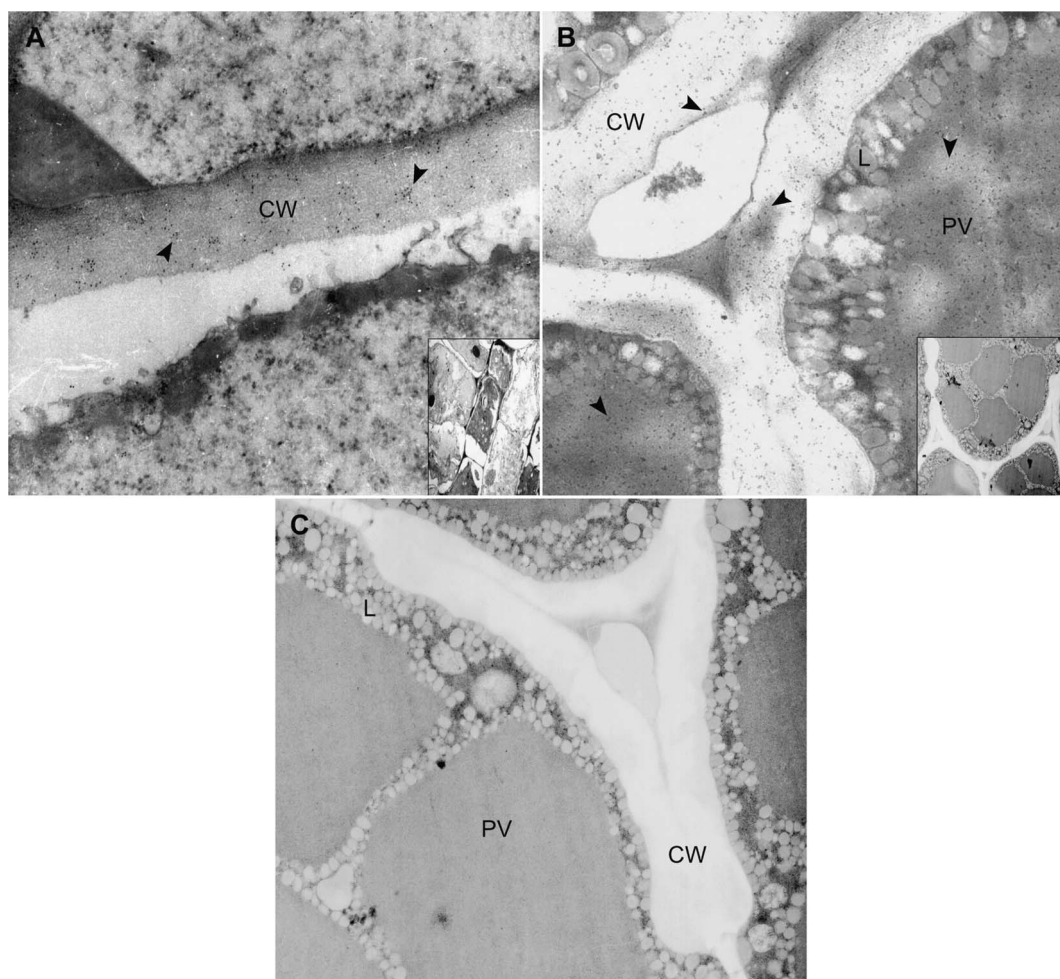


Fig. 2. Immunolocalisation of ASP53 in mature seeds of *Acacia erioloba* with the anti-ASP53 antibody. Sections of axes (A) and cotyledons (B) from mature seeds were examined. Arrows indicate gold label in the cell walls (CW) and protein vacuoles (PV). Sections of cotyledons from mature seeds were also probed with the pre-immune serum as a control (C). The insets in (A) and (B) are lower magnification views of axes and cotyledons from mature seeds included to show the area examined in the higher magnification views. Magnifications used (A) 45 000 \times , (inset) 1000 \times ; (B) 17 500 \times , (inset) 1588 \times ; (C) 9420 \times .

ASP53 was homologous to known proteins; no significant match was found (data not shown). The digest was next derivatised with *O*-methylisourea–hydrogen sulphate and the CAF reagent in a 2-step reaction and the derivatised digest analysed by MALDI–TOF mass spectrometry. Two sulfonated peptides with molecular masses of 1357 Da and 1578 Da were selected for sequencing by PSD–MALDI–TOF mass spectrometry. The sequences of these peptides were determined to be HL(I)QF(Mo)L(I)QDYR and TFVTL(I)NQYDAL(I)R, respectively. A degenerate primer to the sequence NQYDAL(I)R of the latter peptide together with an oligo-dT primer were used to amplify the C-terminal part of the *ASP53* transcript. The complete transcript sequence was then determined with 5' RACE using an internal transcript-specific primer (Fig. 3). This sequence showed an open reading frame (ORF) of 1358 bp between positions 81 and 1434 encoding a protein of 453 amino acids with a molecular weight of 51 kDa. The ORF contained a start codon, a stop codon 1353 bp downstream as well as a stop codon 9 bp upstream of the putative start codon, indicating that the full-length transcript sequence had been obtained. The presence of the sequence of the second peptide identified by PSD–MALDI–TOF mass spectrometry confirmed that the correct product had indeed been obtained. Only one clone was sequenced in full although other clones yielded partial sequences, which were the same as that of the clone sequenced.

Comparison of the sequence obtained with that of known proteins in the Uniprot database using Fasta (<http://www.ebi.ac.uk/fasta>, accessed 23 January 2007) showed that ASP53 was related to the known storage proteins β -conglutin, β -conglycinin, canavalin and vicilin with amino acid homologies identities of 38, 38, 35 and 34% and amino acid similarities of 71, 70, 71 and 71%, respectively. With the Interproscan software (<http://www.ebi.ac.uk/interpro>, accessed 23 January 2007) to search for relatedness to all known domains and protein families, ASP53 was proposed to have two cupin domains between residues 49 and 182 and between residues 253 and 419, as well as a signal peptide between residues 1 and 26. Removal of this signal peptide would generate the sequence EQQGQDERT similar to the sequence determined earlier, namely GSEQQRQDEPT, suggesting that a limited amount of ASP53 stored in the *A. erioloba* seed was processed for export from the cells. In plants, bicupin-containing storage proteins have been proposed to protect plants against biotic and abiotic stress (Berna and Bernier 1999). Because these proteins have been shown to display high levels of thermal stability (Dunwell 1998), it has been further proposed that cupin-containing proteins may be involved *inter alia* in heat stress responses (Dunwell *et al.* 2001). Seeds of *A. erioloba* kept for 1 week in temperatures up to 60°C germinated with no loss in seed viability or in the rate of radicle protrusion. In contrast, the same treatment resulted in the complete loss of viability of *P. sativum* seeds used as a control (data not shown). We next investigated the effect of temperature on the secondary structure of ASP53. Circular dichroism (CD) spectroscopic measurements showed that considerable secondary structure was associated with ASP53, the conformation of which was largely unchanged up to 80°C (Fig. 4). Deconvolution of the spectrum suggested that the protein consisted mainly of anti-parallel β -pleated sheets (41%) with a further 33% of the protein in a random coil and

19% in a β -turn conformation. Only small parts of the protein were in an α -helical (7%) or in a parallel β -pleated sheet conformation (5%).

LEA proteins, with which ASP53 displays several common features including solubility at high temperature and being present in substantial quantities, have been proposed to protect biological systems against desiccation and thermal stress. Because the conformation of ASP53 was minimally effected by elevated temperature, we investigated whether ASP53 could protect other proteins against thermal stress. This was performed by determining the effect of the presence of ASP53 on changes in target protein activity and conformation brought about by increased temperature. Thermal denaturation of proteins has been reported to occur in three phases. In the first stage, loss of enzymatic activity is concomitant with undetectable conformational changes surrounding the active site. In the second phase, significant conformational changes occur, which eventually result in unfolding of the protein and aggregation (third phase), brought about by hydrophobic interactions. Progression between phases is brought about by increased temperature or by increased time at an elevated temperature (He *et al.* 1997).

To investigate whether ASP53 could prevent loss of enzymatic activity brought about by high temperature, yeast alcohol dehydrogenase (ADH) was incubated in the presence of varying concentrations of ASP53 at 55°C and the activity determined as a function of time. The presence of ASP53, even at very high concentrations ($> 10 \text{ mg mL}^{-1}$) was found to have no effect on the specific activity of ADH under the assay conditions used. Whereas incubation of ADH alone at 55°C resulted in the complete loss of enzymatic activity after 50 min, incubation in the presence of 3 mg mL^{-1} ASP53 resulted in $\sim 20\%$ of the enzymatic activity retained after this period (Fig. 5). This concentration of ASP53 reflected a ASP53 : ADH molar ratio of 37.5 : 1. The activity retained increased to $\sim 50\%$ when the ASP53 concentration was increased to 6 mg mL^{-1} (molar ratio = 75 : 1) and to $\sim 65\%$ in the presence of 12 mg mL^{-1} ASP53 (molar ratio = 150 : 1). We next determined whether the protection conferred by ASP53 was specific to this protein or whether other proteins had a similar effect. A protein of similar molecular weight, ovalbumin (45 kDa), was used in place of ASP53. Incubation of ADH in the presence of 12 mg mL^{-1} ovalbumin (molar ratio = 176 : 1) resulted in $\sim 20\%$ of the enzymatic activity retained after 50 min, showing that ASP53 was substantially more effective at protecting ADH against thermal denaturation. The secondary structure of ADH was determined with CD spectroscopy before and after 1 h incubation at 55°C, a procedure that resulted in the complete loss of enzymatic activity. No change in ADH secondary structure was found to occur; incubation at $\sim 85^\circ\text{C}$ was required to induce detectable conformational changes (data not shown).

Because ADH exhibited significant structural changes only at high temperature, a more labile target protein, haemoglobin, was chosen to investigate the effect of ASP53 on conformational changes brought about by increased temperature (Fig. 6). The protection afforded by other proteins was not investigated because the majority of commonly available proteins undergo conformational changes themselves as a function of increased temperature. Circular dichroism spectroscopy of haemoglobin


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1141   TAAACTGCAATGATC'TTTACATAGTCCC GG CAGGATATCCAACGGCCCTCAGTGCCTCAG
380   L N C N D L Y I V P A G Y P T A L S A S

1201   AGAACAACTTGGAGGTGGTCCAGTTCATCC'TTAATGCCCGCAACAACTCCAGAATGT
400   E N N N L E V V Q F I L N A R N N S R M

1261   TCCTCACAGGTGCTGGAGACAATGTGGTGAAGCATATACCCCGAGAGTTGCTTTCAAGGT
420   F L T G A G D N V V K H I P R E L L S R

1321   CCCCTGAACTAGTGAAGAAGCTAT'TGGGACAACAACAGCAGTCATTCTTTGTGAACCGTA
440   S P E L V K K L L G Q Q Q Q S F F V N R

1381   AGGATAAACCGAAGTCCCCAGAAAGGGAGATTGGGTTGCTTGGTCTTCATCCTAGTTTG
460   K D K P K S P R K G D W V A W S S S *

1441   AAGTCTATCATGTGAGTGATGAAGTGAGCGAGCGAGTGGTGTCCACTACTATGTAATAAAA
480                                     * *

1501   TAACGAATAAATAAATATATCAAAGTCTGTGGCCAATAAGGCAACTTACGTGACGGCT
500                                     *

1561   TATCCCCATGTGAGGAAGGAGTACTATGTATCTATCTATATCAATGTTATGAATAAATGA
520                                     *

1621   GGAGGTAGCTTCTGTCTTCCAAC TACCTCCTCTTTTCTCTCTAAAAAAAAAAAAAAAAA
540   *

1681   AAAGGATCCC
560

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Fig. 3. The full-length nucleotide and translated amino acid sequence of ASP53 together with 79 bp of 5' flanking sequence and the 3' untranslated region. The sequences of the gene specific anti-sense primer (GSP 2) and the 5' PCR primer used to amplify the 5' end of the *ASP53* gene are underlined. The sequences of the two peptides sequenced by PSD-matrix assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) mass spectroscopy are shown in bold type. The start and stop codons at positions 81 and 1434 are shown in bold type italics. No translation of the 5' and 3' untranslated regions is given except that stop codons are indicated (*).

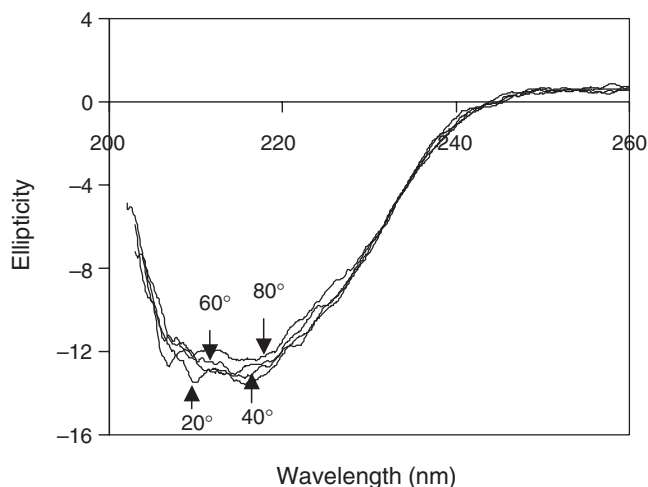


Fig. 4. Effect of temperature on the secondary structure of ASP53. Circular dichroism spectra of ASP53 were recorded in 100 mM K_2HPO_4 pH 7.4 at 20, 40, 60 and 80°C.

alone (Fig. 6A) demonstrated that little change in the secondary structure occurred up to 50°C. Increasing the temperature to 60°C and above resulted in more pronounced conformational changes being observed indicated by a general decrease in the ellipticity, with a correlation between the magnitude of the change and the temperature. To investigate whether the presence of ASP53 affected the conformation of haemoglobin or *vice versa*, haemoglobin was incubated together with ASP53 at a molar ratio of ASP53:haemoglobin of 3.4:1 and the circular dichroism spectrum of the solution (Hb + ASP53) determined, after which the spectrum of ASP53 was subtracted. The resultant spectrum of haemoglobin in the presence of ASP53 (Hb + ASP53) – (ASP53) (not shown) was found to be very similar, i.e. within experimental error of the 'noise' associated with any spectral determination, to that of haemoglobin alone, suggesting that the presence of ASP53 had no effect on the conformation of haemoglobin. The spectrum of the haemoglobin together with ASP53 was next determined at different temperatures up to 80°C and the contribution of haemoglobin assessed by subtracting the ASP53 spectrum at

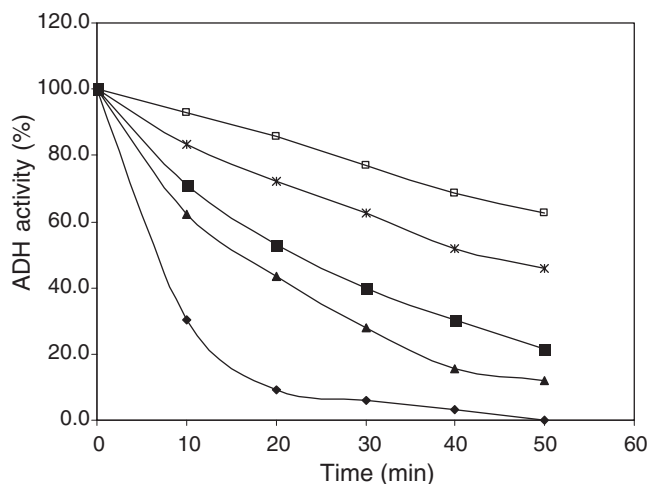


Fig. 5. Percentage alcohol dehydrogenase (ADH) enzymatic activity as a function of time of incubation at 55°C. ADH alone (◆—◆), in the presence of 3 mg mL⁻¹ ASP53 (■—■), 6 mg mL⁻¹ ASP53 (×—×), 12 mg mL⁻¹ ASP53 (□—□) or 12 mg mL⁻¹ ovalbumin (▲—▲).

each temperature. We found that incubation of haemoglobin together with ASP53 resulted in a general preservation of the secondary structure of haemoglobin at increased temperature (Fig. 6B), with a significant reduction in conformational changes observed as a function of temperature. Thus, the conformation of haemoglobin at 60°C was remarkably similar to that observed at 20°C with significant conformational changes only detected at 70°C and above (Fig. 6B). In an attempt to quantitate these changes, the effect of ASP53 on the α -helical content of haemoglobin, determined from the ellipticity at 225 nm, was determined as a function of temperature. This showed (Fig. 6C) that the presence of ASP53 protected the α -helical content of haemoglobin against thermal denaturation up to 50°C. Above this temperature the α -helical content decreased but at a far lower rate than that observed in the absence of ASP53.

To investigate whether ASP53 could prevent the aggregation of unfolded proteins, we used the temperature dependent aggregation of a diluted solution of total chicken egg white protein as a model system. Increasing the temperature of this solution resulted in no turbidity, determined from the 350 nm absorbance, up to 70°C, after which it increased markedly reaching a plateau at ~80°C (Fig. 7). Inclusion of ASP53 in the egg white protein solution at a molar ratio of 0.3 : 1 resulted in a marked decrease in the turbidity observed. A further increase in the concentration of ASP53 present resulted in a concentration-dependent decrease in the turbidity observed, with a molar ratio of 2.4 : 1 completely abolishing protein aggregation (Fig. 7). Concomitant with the decreased turbidity observed, the T_m of the denaturation transition increased from ~72°C in the absence of ASP53 to ~77°C in the presence of ASP53 at a molar ratio of ASP53 : egg white protein of 1.2 : 1 (data not shown).

Finally, because LEA-like proteins with properties akin to ASP53 have been shown to have accumulation and loss patterns correlated with onset and loss of desiccation tolerance (Baker *et al.* 1988; Close *et al.* 1989; Blackman *et al.* 1991, 1992; Russouw *et al.* 1995, 1997; Vertucci and Farrant 1995;

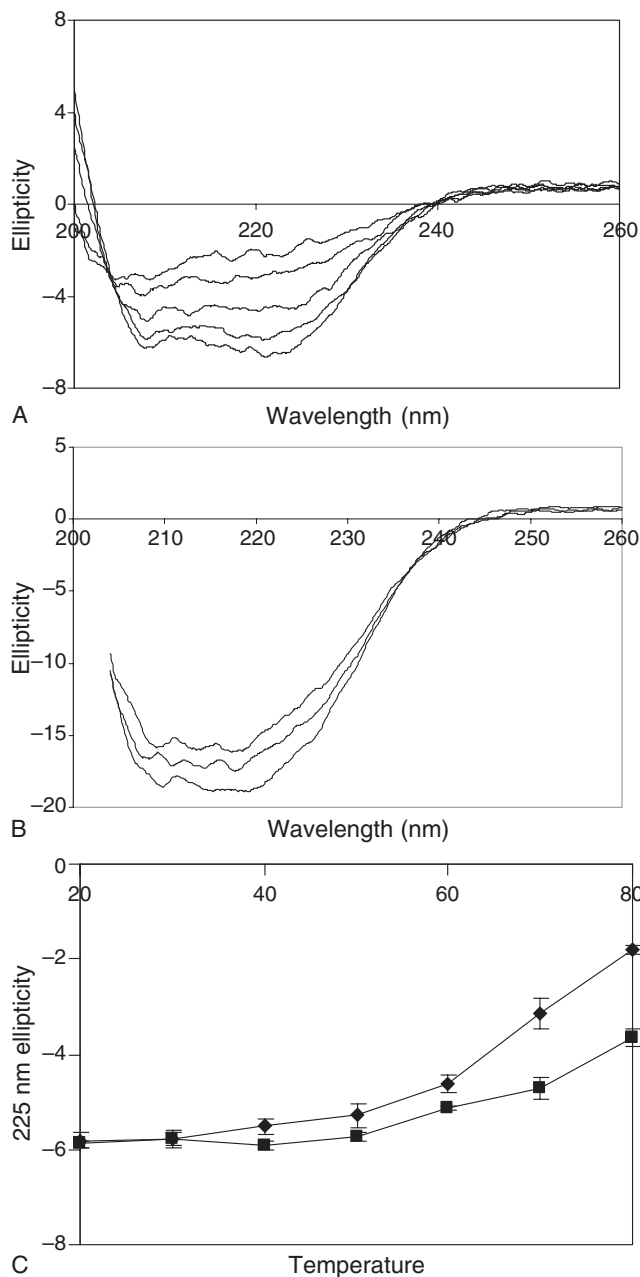


Fig. 6. The effect of ASP53 on temperature-induced conformational changes in haemoglobin determined by circular dichroism (CD) spectroscopy. (A) CD spectra of haemoglobin alone in 100 mM K₂HPO₄ pH 7.4 at 20, 50, 60, 70 and 80°C (decreasing ellipticity with increasing temperature). Spectra taken at 30 and 40°C (data not shown) were indistinguishable from that taken at 20°C. (B) CD spectra of haemoglobin together with 0.6 mg mL⁻¹ ASP53 in 100 mM K₂HPO₄ pH 7.4 at 20, 70 and 80°C (decreasing ellipticity with increasing temperature). Spectra taken at 30, 40, 50 and 60°C, (data not shown) were indistinguishable from that taken at 20°C. (C) α -Helical content of haemoglobin determined from the ellipticity at 225 nm as a function of temperature. The 225 nm ellipticity of haemoglobin alone (◆—◆) was determined from the spectra shown in (A); the 225 nm ellipticity of haemoglobin in the presence of 0.6 mg mL⁻¹ ASP53 (■—■) was determined by subtracting the spectra of ASP53 (Fig. 4) from the spectra shown in (B). The error bars reflect data from three separate experiments.

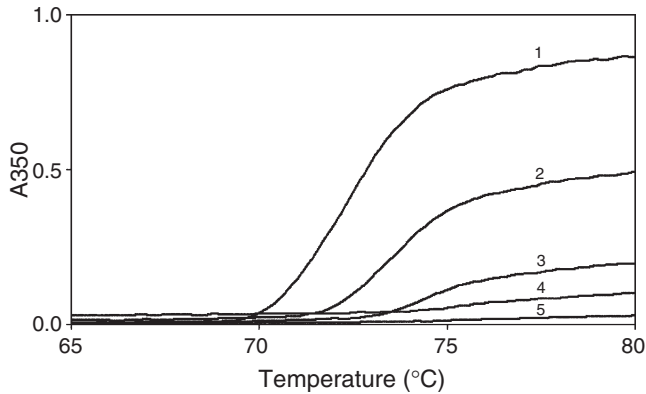


Fig. 7. The effect of ASP53 on temperature-induced aggregation of total chicken egg white proteins. The turbidity of the protein solution, determined from the 350 nm absorption, was assessed alone (1) or in the presence of increasing concentrations of ASP53 (2–5). Molar ratios of ASP53: egg white protein used were: (2) 0.3 : 1, (3) 0.6 : 1, (4) 1.2 : 1 and (5) 2.4 : 1. The egg white protein was assumed to have the molecular weight of ovalbumin, 45 kDa.

Bray 1997), we tested for the presence of this protein during loss of desiccation tolerance in germinating seedlings. The ASP53 content as determined by SDS-PAGE was measured on germination (Fig. 8) and after dehydration and subsequent rehydration of the germinated seedlings. Mature seeds were used as a control. ASP53 content, like that of most LEA proteins,

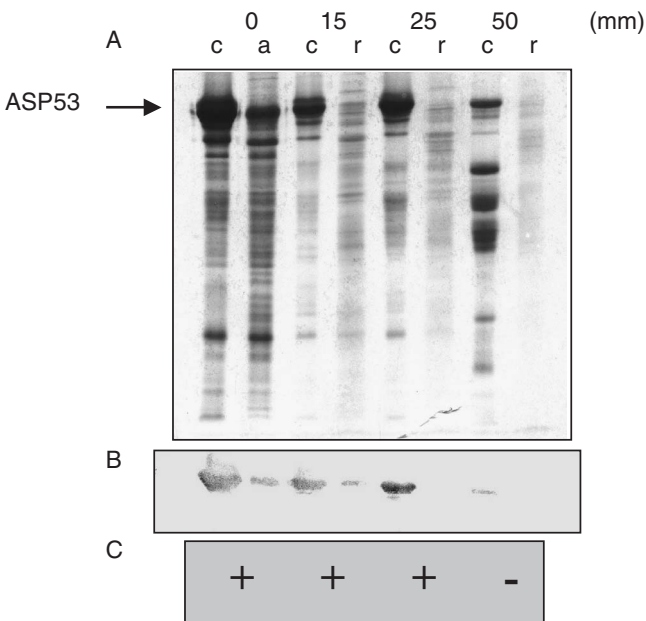


Fig. 8. ASP53 content of *Acacia erioloba* seeds during germination. (A) SDS-PAGE of total proteins extracted from the cotyledons (c) and the axis (a) of mature seeds (radicle length = 0) and from the cotyledons (c) and radicles (r) of germinated seedlings. The radicle length is shown above these lanes. Samples were lyophilised and equivalent masses of tissue were extracted and loaded onto the SDS gel. (B) Western blot of the ASP53-migrating portion of the gel using the anti-ASP53 antibody. (C) Desiccation tolerance (+) or desiccation sensitivity (-) of the seedlings used above.

decreased in both axes and cotyledons during germination and was undetectable in axes of seedlings with a radicle length of 50 mm (Fig. 8). Dehydration of seedlings with radicle lengths of 15 and 25 mm to 7% water content (dry mass basis) allowed normal re-growth on rehydration, whereas seedlings with a radicle length of 50 mm lost the ability to resume growth following such drying. This correlation between the loss of ASP53 content in the radicle and the inability of the germinated seedling to resume growth after desiccation and subsequent rehydration suggested a possible involvement of this protein in the acquisition of desiccation tolerance in *A. erioloba*.

Discussion

We have identified a 53 kDa heat soluble protein, ASP53, as the most abundant protein present in the mature seeds of *A. erioloba*. ASP53 was found to be related to several globulin seed storage proteins, namely β -conglycinin, vicilin and canavalin, and to protect other proteins against thermal denaturation and also possibly act in a LEA-like manner to protect against desiccation damage. ASP53 was located largely in the cell wall of embryonic axis cells and cell walls and protein containing storage vacuoles of cotyledons from mature seeds. It was present in mature desiccation-tolerant seeds, but disappears from the axis tissue concomitant with loss of desiccation tolerance during seed germination. The dual subcellular location suggests a possible dual function for this protein – as a protectant against stress and as a storage reserve for metabolism on germination. Recently precedent has been set for such dual functions in glycolytic enzymes and the stress response protein Hsp12p in yeast. Thus, several reports have showed the presence of enzymes of the lower part of the glycolytic pathway in the cell wall of the yeast *S. cerevisiae* (Pardo *et al.* 2000; Delgado *et al.* 2001; Motshwene *et al.* 2003). Although some reports have suggested that these cell wall-located glycolytic enzymes might be ‘moonlighting’ proteins, i.e. having an alternative function in this alternate location (Jeffery 2003), we have showed that these enzymes are active, providing that the necessary substrates and cofactors are supplied in the medium (Motshwene *et al.* 2003). Moreover, the stress response protein Hsp12p is present in both the cytoplasm and the cell wall (Mtwisha *et al.* 1998; Motshwene *et al.* 2004). Thus Hsp12p is not only present in the total extract of *S. cerevisiae* cells after the cells have been disrupted by abrasion using a ball mill, but is also present in the alkali extract of whole cells, a process that selectively extracts proteins from the cell wall. This dual location has been confirmed with immunocytochemistry (Motshwene *et al.* 2004).

ASP53 was found to be soluble at high temperatures like the LEA proteins. Whereas LEA proteins have little secondary structure and are soluble at high temperature on account of their high hydrophilicities (Russouw *et al.* 1997), ASP53 displayed considerable secondary structure, which was maintained independent of the temperature. The demonstration that ASP53 can protect other proteins against thermal denaturation and aggregation is a novel activity for a protein, although the protection of proteins by compatible osmolytes is well known (for a review see Yancey 2005). We propose that this activity is mediated by ASP53 acting as a water replacement molecule by forming hydrogen bonds with

target macromolecules. The presence of the thermally stable cupin fold is presumably a requirement for ASP53 to maintain its hydrophilic exterior at high temperature. Although we have not investigated whether ASP53 has associated enzymatic or inhibitory activity, we have recently proposed that one function of the yeast *S. cerevisiae* hydrophilic stress response protein Hsp12 is to act as a plasticiser in the cell wall (Motshwene *et al.* 2004; Karreman *et al.* 2005, 2007). ASP53 may have a similar function, as it would inhibit hydrogen-bonded interactions between adjacent polysaccharide chains in the cell wall thereby leading to increased flexibility. Maintenance of the correct cell wall flexibility might be important in the desiccated state so as to retain cell wall integrity upon water ingress during germination. This process would be analogous to maintenance of membranes in the liquid crystalline phase during desiccation, a process brought about in yeast by trehalose (Mansure *et al.* 1994) and Hsp12 (Sales *et al.* 2000).

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References

- Baker JC, Steele C, Dure L (1988) Sequence and characterization of 6 LEA proteins and their genes from cotton. *Plant Molecular Biology* **11**, 277–291. doi: 10.1007/BF00027385
- Berna A, Bernier F (1999) Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H₂O₂-producing enzyme. *Plant Molecular Biology* **39**, 539–549. doi: 10.1023/A:1006123432157
- Blackman SA, Obendorf RL, Leopold AC (1992) Maturation proteins and sugars in desiccation tolerance of developing soybean seeds. *Plant Physiology* **100**, 225–230.
- Blackman SA, Wettlaufer SF, Obendorf RL, Leopold AC (1991) Maturation proteins associated with desiccation tolerance in soybean. *Plant Physiology* **96**, 868–874.
- Bray EA (1997) Plant responses to water deficit. *Trends in Plant Science* **2**, 48–54. doi: 10.1016/S1360-1385(97)82562-9
- Campbell SA, Close TJ (1997) Dehydrins: genes, proteins, and associations with phenotypic traits. *New Phytologist* **137**, 61–74. doi: 10.1046/j.1469-8137.1997.00831.x
- Close TJ (1997) Dehydrins: a commonality in the response of plants to dehydration and low temperature. *Physiologia Plantarum* **100**, 291–296. doi: 10.1111/j.1399-3054.1997.tb04785.x
- Close TJ, Kort AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Molecular Biology* **13**, 95–108. doi: 10.1007/BF00027338
- Delgado LM, O'Connor J, Azorin I, Renau-Piqueras J, Gil LM, Gozalbo D (2001) The glyceraldehydes-3-phosphate dehydrogenase polypeptides encoded by *Saccharomyces cerevisiae* TDH1, TDH2, and TDH3 genes are also cell wall proteins. *Microbiology* **147**, 411–417.
- Dunwell JM (1998) Cupins: a new superfamily of functionally diverse proteins that include germins and plant seeds storage proteins. *Biotechnology and Genetic Engineering Reviews* **15**, 1–32.
- Dunwell JM, Culham A, Carter CE, Sosa-Aguirre CR, Goodenough PW (2001) Evolution of functional diversity in the cupin superfamily. *Trends in Biochemical Sciences* **26**, 740–746. doi: 10.1016/S0968-0004(01)01981-8
- Dunwell JM, Khuri S, Gane PJ (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiology and Molecular Biology Reviews* **64**, 153–179. doi: 10.1128/MMBR.64.1.153-179.2000
- Dunwell JM, Purvis A, Khuri S (2004) Cupins: the most functionally diverse protein superfamily? *Phytochemistry* **65**, 7–17. doi: 10.1016/j.phytochem.2003.08.016
- Dure L, Crouch M, Harada J, Ho THD, Mundy J, Quatrano R, Sung ZR (1989) Common amino acid domains among LEA proteins of higher plants. *Plant Molecular Biology* **12**, 475–486. doi: 10.1007/BF00036962
- Farrant JM, Berjak P, Pammenter NW (1985) The effect of drying rate on viability retention of recalcitrant propagules of *Avicennia marina*. *Suid Afrikanse Tydskrift van Plantkunde* **51**, 432–438.
- Fuzetti F, Schroter KH, Steiner RA, van Noort PI, Pijning T, Rozeboom HJ, Kalk KH, Egmond MR, Dijkstra BW (2002) Crystal structure of the copper-containing quercetin 2,3-dioxygenase from *Aspergillus japonicus*. *Structure* **10**, 259–268.
- Garay-Arroyo A, Colmenero-Flores JM, Garcarrubio A, Covarrubias AA (2000) Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *Journal of Biological Chemistry* **275**, 5668–5674. doi: 10.1074/jbc.275.8.5668
- Harlow LD (1988) 'Antibodies: a laboratory manual.' (Cold Spring Harbour Laboratory Press: Cold Spring Harbour, NY)
- He B, Bai J, Zhou H (1997) Comparison of inactivation and unfolding of yeast alcohol dehydrogenase during thermal denaturation. *International Journal of Biochemistry & Cell Biology* **29**, 1021–1028. doi: 10.1016/S1357-2725(97)00021-6
- Horbowicz M, Obendorf RL (1994) Seed desiccation tolerance and storability: dependence on flatulence-producing oligosaccharides and cyclitols – review and survey. *Seed Science Research* **4**, 385–406.
- Jeffery CJ (2003) Moonlighting proteins. *Trends in Genetics* **19**, 415–417. doi: 10.1016/S0168-9525(03)00167-7
- Karreman RJ, Brandt WF, Lindsey GG (2005) The yeast *Saccharomyces cerevisiae* stress response protein HSP 12 decreases the gel strength of agarose used as a model system for the β-glucan layer of the cell wall. *Carbohydrate Polymers* **60**, 193–198. doi: 10.1016/j.carbpol.2004.12.004
- Karreman RJ, Dague E, Gaboriaud F, Quilès F, Duval JF, Lindsey GG (2007) The stress response protein Hsp12p increases the flexibility of the yeast *Saccharomyces cerevisiae* cell wall. *Biochimica et Biophysica Acta* **1774**, 131–137.
- Kikawada T, Nakahara Y, Kanamori Y, Iwata K, Watanabe M, McGee B, Tunnacliffe A, Okuda T (2006) Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid. *Biochemical and Biophysical Research Communications* **348**, 56–61. doi: 10.1016/j.bbrc.2006.07.003
- Ko T-P, Ng JD, McPherson A (1993) The three-dimensional structure of canavalin from jack bean (*Canavalia ensiformis*). *Plant Physiology* **101**, 729–744. doi: 10.1104/pp.101.3.729
- Lapik YR, Kaufman LS (2003) The *Arabidopsis* cupin domain protein AtPirin1 interacts with the G protein subunit GPA1 and regulates seed germination and early seedling development. *The Plant Cell* **15**, 1578–1590. doi: 10.1105/tpc.011890
- Leprince O, Atherthon NM, Deltour R, Hendry GA (1994) The involvement of respiration in free radical processes during loss of desiccation tolerance in germinating *Zea mays* L. (an electron paramagnetic resonance study). *Plant Physiology* **104**, 1333–1339.
- Leprince O, Hendry GAF, McKersie BD (1993) The mechanisms of desiccation tolerance in developing seeds. *Seed Science Research* **3**, 231–246.
- Mansure JJ, Panek AD, Crowe LM, Crowe JH (1994) Trehalose inhibits ethanol effects on intact yeast cells and liposomes. *Biochimica et Biophysica Acta* **1191**, 309–316.
- Motshwene P, Brandt W, Lindsey G (2003) Significant quantities of the glycolytic enzyme phosphoglycerate mutase are present in the cell wall of yeast *Saccharomyces cerevisiae*. *The Biochemical Journal* **369**, 357–362. doi: 10.1042/BJ20021352

- Motshwene P, Karreman R, Kgari G, Brandt W, Lindsey G (2004) The LEA-like protein Hsp12 is present in the cell wall and enhances the barotolerance of the yeast *Saccharomyces cerevisiae*. *The Biochemical Journal* **377**, 769–774.
- Mtwisha L, Brandt W, McCready S, Lindsey GG (1998) HSP 12 is a LEA-like protein in *Saccharomyces cerevisiae*. *Plant Molecular Biology* **37**, 513–521. doi: 10.1023/A:1005904219201
- Pardo M, Molina M, Gil C, Nombela C, Ward M, Bains S, Blackstock W (2000) A proteomic approach for the study of *Saccharomyces cerevisiae* cell wall biogenesis. *Electrophoresis* **21**, 3396–3410. doi: 10.1002/1522-2683(20001001)21:16<3396::AID-ELPS3396>3.0.CO;2-J
- Roberts EH (1973) Predicting the storage life of seeds. *Seed Science and Technology* **1**, 499–514.
- Roberts JK, DeSimone NA, Lingle WL, Dure L (1993) Cellular concentrations and uniformity of cell-type accumulation of two LEA proteins in cotton embryos. *The Plant Cell* **5**, 769–780. doi: 10.1105/tpc.5.7.769
- Russouw PS, Farrant J, Brandt W, Maeder D, Lindsey GG (1995) Isolation and characterization of a heat-soluble protein from pea (*Pisum sativum*) embryos. *Seed Science Research* **5**, 137–144.
- Russouw PS, Farrant JM, Brandt WF, Lindsey GG (1997) The most prevalent protein in a heat-treated extract of pea (*Pisum sativum*) embryos is a LEA group I protein; its conformation is not affected by exposure to high temperature. *Seed Science Research* **7**, 117–123.
- Sales K, Brandt WF, Rumbak E, Lindsey GG (2000) The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol induced stress. *Biochimica et Biophysica Acta* **1463**, 267–278.
- Sanger F, Niklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**, 5463–5469. doi: 10.1073/pnas.74.12.5463
- Scott P (2000) Resurrection plants and the secrets of eternal leaf. *Annals of Botany* **85**, 159–166. doi: 10.1006/anbo.1999.1006
- Segarra CI, Casalongué CA, Pinedo CL, Ronchi VP, Conde RD (2003) A germin-like protein of wheat leaf apoplast inhibits serine proteases. *Journal of Experimental Botany* **54**, 1335–1341. doi: 10.1093/jxb/erg139
- Sherwin HW, Farrant J (1996) Differences in rehydration of three desiccation-tolerant angiosperm species. *Annals of Botany* **78**, 703–710. doi: 10.1006/anbo.1996.0180
- Shutov AD, Baumlein H, Blattner FR, Muntz K (2003) Storage and mobilization as antagonistic functional constraints on seed storage globulin evolution. *Journal of Experimental Botany* **54**, 1645–1654. doi: 10.1093/jxb/erg165
- Singh S, Cornilescu CC, Tyler RC, Cornilescu G, Tonelli M, Lee MS, Markley JL (2005) Solution structure of a late embryogenesis abundant protein (LEA14) from *Arabidopsis thaliana*, a cellular stress-related protein. *Protein Science* **14**, 2601–2609. doi: 10.1110/ps.051579205
- Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31–43. doi: 10.1016/S0022-5320(69)90033-1
- Sun WQ, Leopold AC (1993) The glassy state and accelerated aging of soybeans. *Physiologia Plantarum* **89**, 767–774. doi: 10.1111/j.1399-3054.1993.tb05283.x
- Vertucci CW, Farrant JM (1995) Acquisition and loss of desiccation tolerance. In 'Seed development and germination'. (Eds J Kigel, G Galilli) pp. 237–271. (Marcel Dekker Inc.: New York)
- Westermeier R, Naven T (2002) 'Proteomics in practice: a laboratory manual of proteome analysis.' (Wiley: New Jersey)
- Wilmouth RC, Turnbull JJ, Welford RW, Clifton IJ, Prescott AG, Schofield CJ (2002) Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. *Structure* **10**, 93–103.
- Wise MJ, Tunnacliffe A (2004) POPP the question: what do LEA proteins do? *Trends in Plant Science* **9**, 13–17. doi: 10.1016/j.tplants.2003.10.012
- Wolkers WF, McCready S, Brandt W, Lindsey GG, Hoekstra FA (2001) Isolation and characterisation of a D-7 LEA protein from pollen that stabilises glasses in vitro. *Biochimica et Biophysica Acta* **1544**, 196–206.
- Woo EJ, Dunwell JM, Goodenough PW, Marvier AC, Pickersgill RW (2000) Germin is a manganese-containing homohexamer with oxalate oxidase and superoxide dismutase activities. *Nature Structural Biology* **7**, 1036–1040. doi: 10.1038/80954
- Yancey PH (2005) Organic osmolytes as compatible metabolic and counteracting cytoprotectants in high osmolality and other stresses. *Journal of Experimental Botany* **208**, 2819–2830.

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