3 Characterisation of BP1 and LEA in relation to black point of barley grain

3.1 Introduction

Past research has indicated black point results from the enzymatic oxidation of phenolic compounds within the grain to form discoloured end products (see Chapter 1.2.3). There are possibly many biochemical pathways and proteins involved in this process. The research presented in Chapter 2 identified two proteins associated with black point formation. The proteins identified were a putative late embryogenesis abundant (LEA) protein and barley grain peroxidase 1 (BP1). LEA was more abundant in healthy grain indicating it might have a role in preventing black point, whereas BP1 was more abundant in black pointed grains indicating potential involvement in black point formation. While these proteins were associated with black point, it is still to be determined if they have a direct role in black point development and what that role is.

LEA proteins are a broad class of proteins proposed to be involved in protecting the cell against osmotic stress during events such as desiccation (Goyal et al., 2005). There have been at least six classes of LEA proteins identified in plants, which are based on unique sequence motifs (Wise, 2003). It is unclear what class the putative LEA protein identified in Chapter 2 falls into because the complete sequence was not available. LEA proteins have been previously identified in barley and found to confer dehydration tolerance by protecting the cell membrane (Babu et al., 2004). To date, no LEA proteins corresponding to the size of the one identified in this study have been reported in barley. Obtaining the complete sequence of this gene

will be paramount in beginning to understand its function in black point formation and barley grain development.

BP1 has been well characterised *in vitro*, its crystal structure has been determined and enzyme kinetics determined for a range of phenolic substrates (Rasmussen et al., 1995). On the other hand there is little information on its *in vivo* function. *BP1* gene expression has been shown to peak at 15 days post-anthesis, however, with transcript levels most abundant in the endosperm (Rasmussen et al., 1991).

The experiments that identified a putative LEA and BP1 in Chapter 2 analysed whole grain samples. Black point is exclusively located at the embryo end of the grain. If the putative LEA and BP1 were directly involved in black point it would therefore be expected that they would localise to the embryo region of the grain. LEA proteins have previously been shown to be located in the embryo tissue (Roberts et al., 1993). This would need to be confirmed for the LEA protein in this study due to its apparent uniqueness. To date no studies have used immunolocalisation to localise BP1 within barley grains. This knowledge would assist in understanding its *in vivo* role in grain development.

In an attempt to further understand the relevance of these two proteins in black point formation, the research presented in this chapter aimed to further characterise the putative LEA protein and BP1 in relation to black point. Furthermore both proteins were immunolocalised within the grain to see if they reside within the embryo region to further support a role for them in black point formation.

3.2 Materials and methods

3.2.1 Barley tissue samples

The barley grain samples used in this study were the same as used in Chapter 2.2.1. In addition, grain was also harvested from plants grown in the Waite Campus glasshouse (The University of Adelaide) at 20 to 24°C under natural light conditions and also sampled throughout development at the Zadoks' growth stages: medium milk (88), late milk (90), soft dough (92), hard dough (98) and maturity (100) (Zadoks et al., 1974). Collected samples were snap frozen in liquid nitrogen and stored at -80^{o}C until required.

3.2.2 RNA extraction and Northern blot analysis of *BP1* and *LEA*

Whole barley grains were ground in liquid nitrogen using a mortar and pestle. RNA was extracted from ground tissue using the TRIzol® reagent as per manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). Total RNA (10 μg) was separated on a 1.2% formaldehyde gel and blotted to N^{+} membrane as per the manufacturer's recommendations (GE Healthcare, Uppsala, Sweden). Probes for Northern analysis of *BP1* and *LEA* were PCR amplified from cDNA that was prepared from grain RNA using SuperScript® II reverse transcriptase as per the manufacturer's instructions (Invitrogen). PCR conditions were 2 min at 94^{o}C; then 32 cycles of 30 sec at 94^{o}C, 30 sec at 55^{o}C and 1 min at 72^{o}C; followed by 10 min at 72^{o}C. Primers used to amplify the BP1 probe were designed to the cDNA sequence (Accession number GI:167080) and the primer sequences were 5′-CACACACAAAGGAGAGAGGAGATGGCTCG-3′ and 5′-TGGACGAACTCGCGCACGATGG-3′. For the LEA probe, the primers were

designed to the barley UniGene sequence Hv.10490 and the primer sequences were 5′-TCGACTCAGCCTGCCACTGA-3′ and 5′-GTAGTCTCCAGTGGAGCCGAGCGT- 3′. These primers were designed to yield a PCR product of 195 bp and 823 bp respectively.

Probes were radioactively labelled with P^{32} (GE Healthcare) using the Ready-To-Go DNA Labelling Beads (GE Healthcare). Labelled probes were denatured for 2 min at 95°C and added to 20 mL of hybridisation buffer (0.5 M Na_2HPO_4 pH 7.2, 7% SDS and 1 mM EDTA). Membranes were hybridised overnight in bottles at 68°C and then washed consecutively with 2x, 1x and 0.5x SSC (0.15 M NaCl, 0.015 M Na citrate pH 7.0) containing 0.1% SDS for 15 min per wash at 68°C. Washed membranes were exposed to Hyperfilm MP (GE Healthcare) for 3 days at -80°C and then developed using a CP100 developer (AGFA-Geveart Group, Mortsel, Belgium).

3.2.3 Genome localisation of *BP1* and *LEA*

To determine the genetic map location of *BP1* and *LEA* within the Alexis x Sloop double haploid mapping population (Barr et al., 2003) Restriction Fragment Length Polymorphism (RFLP) analysis was performed using the following protocol. Genomic DNA (20 µg) from the cultivars Alexis and Sloop (kindly provided by Kerrie Willsmore, South Australian Research Development Institute, Adelaide) was separately digested with 20 units of the restriction enzymes *Eco*RI, *Dra*I, *Bam*HI, *Eco*RV, *Hind*III and *Xba*I (New England Biolabs, Ipswich, MA, USA) for 5 h at 37°C. Digested samples were separated in a 1% agarose gel in TAE buffer (40 mM Tris, 1 mM EDTA and 20 mM acetic acid) at 33 V for 15 h and then

blotted to N^+ nylon membrane (GE Healthcare) using 0.4 M NaOH as a transfer buffer, following the manufacturer's recommendations. Membranes were probed with the same radioactively labelled probe used for northern blot analysis (as per section 3.2.2). Membranes were exposed for 5 days at -80^{o}C and then developed using a CP100 developer.

If a RFLP was detected between gDNA from Alexis and gDNA from Sloop, digested with one of the restriction enzymes, that enzyme was then used to digest the DNA from 107 lines of the Alexis x Sloop double haploid mapping population. Digested DNA was separated and transferred to a nylon membrane and probed as described above.

The resulting polymorphic banding pattern was scored across the Alexis x Sloop double haploid mapping population as either Alexis or Sloop type. To map the genetic location of the polymorphism, the scores were entered into the program MapManager QTX (Manly et al., 2001) using the Kosambi mapping function. Linkage analysis was performed with existing markers on the Alexis x Sloop genetic map (kindly provided by Kerrie Willsmore) using the 'find best location' function to determine the map location.

3.2.4 Obtaining full length sequence of the LEA gene

The GeneRacer kit (Invitrogen) was used to obtain the unidentified 3′ sequence of the identified LEA partial coding UniGene sequence Hv.10490. Total RNA was extracted from mature grain as per section 3.2.2 and used to prepare cDNA. Amplification of the 3′ sequence was performed using a combination of the supplied primers and a primer designed to the 3′ region of the UniGene sequence

Hv.10490 (5'-CGCGCTCGGATTGACGGGAGACCAAACT-3'). The amplified fragment was cloned using the QIAGEN PCR Cloning Kit (Qiagen, Hilden, Germany) and sequenced by AgGenomics Pty Ltd (Melbourne, Australia) using BigDye™ chemistry (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the program Vector NTI Advance™ 10 (Invitrogen).

3.2.5 Western blot to detect biotinylated proteins

For western blots of 2-D gels, the electrophoresis method described in Chapter 2.2.7 was used except for the following changes. Protein samples (150 µg) were used to rehydrate 7 cm IPG dry strips pH 3-10 (GE Healthcare). Focussing was performed for a total of 8164 Vh consisting of the following steps: 6 hours of passive rehydration (0 V), 6 hours of active rehydration (0 V), 300 V for 2 hours, 30 min gradient to 1000 V, 80 min gradient to 5000 V then 5000 V for 3000 Vh. The second dimension was performed using NuPAGE® 4-12% Bis-Tris ZOOM gels with 4-morpholineethanesulfonic acid (MES) buffer and an XCell SureLock™ Mini cell gel tank (Invitrogen). Gels were electrophoresed at a constant 200 V for 40 min.

Proteins were transferred to polyvinylidene fluoride (PVDF) membrane using an XCell II™ Blot Module following the manufacturer's instructions (Invitrogen). The direct blue 71 membrane staining method was used to visualise transferred proteins (Hong et al., 2000). Biotinylated proteins were detected using a 1:2000 dilution of streptavidin alkaline phosphatase (Sigma-Aldrich) and visualised via reaction with a nitroblue tetrazolium (NBT)/ Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine (BCIP) solution (Sigma-Aldrich) to yield a dark purple product.

3.2.6 Antibody design and production

The full-length protein sequence of BP1 and LEA were analysed for hydrophilic regions using the program ProtScale Tool (http://www.expasy.org/tools/protscale.html). The program was set to use the Hopp & Woods scale with a window size of 7 (Hopp and Woods, 1981). Hydrophobic regions were selected that spanned 15 to 20 amino acids for both BP1 and LEA as likely to be antigenic. The peptides were produced by Mimotopes (Victoria, Australia) and bound to the carrier protein diphtheria toxoid. Purified peptides (500 μg) were injected into two individual rabbits per peptide. Pre-immune serum was collected before injecting each rabbit with the antigen, with a test bleed collected after 55 days, and total serum collected a further 21 days later.

3.2.7 Testing antibody specificity

Western blots were prepared from healthy and black pointed whole grain samples and probed with LEA and BP1 antiserum respectively. The 2-D gel electrophoresis, transfer to membrane and total protein detection method was performed as described in section 3.2.5. Membranes were blocked for 2 h in 5% w/v skim milk powder then washed 3 x 5 min in PBS-T (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4). Membranes were then incubated for 1 h in a 1:5000 dilution of antiserum in PBS-T before they were washed for 3 x 5 min in PBS-T. Membranes were then incubated for 1 h in a 1:5000 dilution of mouse anti-rabbit biotin conjugate in PBS-T before they were washed 3 x 5 min in PBS-T. Membranes were then incubated for 1 h in a 1:5000 dilution of streptavidin alkaline phosphatase conjugate in PBS-T before 3 x 5 min washes in

PBST. Finally, bound protein was visualised via reaction with a NBT/BCIP solution (Sigma-Aldrich) to yield a dark purple product.

3.2.8 Preparation of grain tissue for immunolocalisation

Healthy and black pointed grains were prepared for immunolocalisation using the following method. Longitudinal cuts were made to remove 2 to 3 mm from either side of the grain to facilitate the penetration of the fixative solution into the grain. The following steps were carried out in 10 mL screw capped tubes and all volumes were approximately 5 mL. Cut grain samples were placed in fixative [0.25% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4)] overnight at room temperature. Samples were incubated in PBS buffer for 8 h, changing the solution three times during that period to remove residual aldehydes. Samples were then dehydrated through an ethanol series consisting of 3 x 20 min changes in 70%, 90%, 95% and then 100% ethanol. Samples were then subjected to a xylene:ethanol series consisting of 1 h in each of 25:75, 50:50 then 75:25 xylene:ethanol. This was followed by 3 x 1 h incubations in 100% xylene.

The xylene was replaced with molten paraffin and tubes incubated at 60^{o}C. The paraffin solution was changed twice per day for 3 days. Paraffin-infiltrated tissues were then cast into plastic moulds and allowed to solidify. Sections were cut at 8 µM thickness using a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and placed onto poly-L-lysine coated slides (Polysciences, Inc, Warrington, PA, USA). Slides were incubated on a 42^{o}C hotplate overnight to fix the sections to the slide.

3.2.9 Immunolocalisation of LEA and BP1

Tissue sections prepared as per section 3.2.8 were de-waxed in xylene for 2 x 10 min followed by 2 x 4 min washes in ethanol. Sections were rehydrated by incubating in 90% ethanol followed by 70% ethanol for 4 min each, then placed into PBS. To deactivate aldehydes, sections were incubated in 0.05 M glycine for 20 min. Sections were blocked in incubation buffer (1% bovine serum albumin in PBS) for 2 x 10 min followed by incubation in either the anti-LEA or anti-BP1 serum for 1 h (dilutions of 1:50 to 1:500 antiserum in incubation buffer were trialled). Sections were then washed for 3 x 10 min in incubation buffer. The primary antibody was detected by incubating the sections in a 1:200 dilution of red-fluorescent Alexa Fluor® 568 goat anti-rabbit IgG (Invitrogen). Sections were washed 3 x 10 min in incubation buffer and then mounted using 90% glycerol.

Sections were visualised first using light microscopy followed by fluorescence microscopy to visualise the bound secondary antibody using a LMD6000 microscope (Leica Microsystems) equipped with a N2.1 filter (excitation filter 515-560 nm band pass, barrier filter 590 nm long pass). All fluorescent images were captured with the inbuilt camera using an exposure time of 5 sec.

3.3 Results

3.3.1 Obtaining full length LEA sequence

Using 3' RACE-PCR and the partial coding sequence from UniGene Hv.10490 (933 bp), a full length cDNA clone of 2322 bp was obtained which encoded a protein 518 aa residues in length (Figure 3-1; assigned GenBank Accession EF535810) with a predicted size of 52 kDa and pI of 7. The newly obtained LEA

sequence matched to the two remaining MS/MS *de novo* peptide fragments identified in Chapter 2.3.4 (dashed line, Figure 3-1). This resulted in total sequence coverage by the MS/MS generated *de novo* peptides to be 10.8%.

3.3.2 Analysis of novel LEA protein sequence

To classify the newly identified LEA protein sequence, motifs were compared to those previously published for identified classes of LEA proteins (Wise and Tunnacliffe, 2004) (Table 3-1). The LEA sequence did not contain any of the conserved motifs previously identified for LEA groups 1, 2 and 3. The three LEA groups have been subsequently reclassified into super families which are based on over- and under-representation of individual amino acids and short motifs (Wise and Tunnacliffe, 2004). The amino acids and motifs associated with super families 5, 6, 7 and 9 were all consistent with the LEA protein identified in this study, for example the LEA sequence contained high representation of A, E, G, K, T, Q, AA, AE, AG, AQ, GG, GA, TA, and TG. However, an inconsistency was found in that super families 5 and 9 are characterised by having valine under-represented, whereas the putative barley LEA protein contained 23 valine residues.

3.3.3 LEA protein is biotinylated *in vivo*

Because LEA proteins of a similar size identified in pea and soybean (65 and 68 kDa respectively) are biotinylated *in vivo* (Duval et al., 1995; Hsing et al., 1998) and a potential atypical biotin binding site (GKF) (Job et al., 2001) was identified in the putative barley LEA, western blot analysis was performed on 2-D gels of healthy grain (Figure 3-1). The putative barley LEA protein isolated in this study bound streptavidin (Figure 3-2 B) indicating that this protein is also biotinylated *in*

vivo.

3.3.4 Transcript levels of BP1 and LEA

To determine if the increased abundance of LEA in healthy grain and BP1 in black pointed grain was controlled by gene transcription, northern blots were performed. Unexpectedly, gene expression levels for *LEA* were slightly higher in black pointed grain and could not account for the increased abundance of the LEA protein in healthy grain (Figure 3-3A). *BP1* gene expression was undetectable in both healthy and black pointed mature grain (Figure 3-3B).

During grain development *LEA* gene expression increased slightly from medium milk stage to late milk, then remained constant throughout the rest of grain development (Figure 3-4). *BP1* gene expression was detected at a constant level up until the soft dough stage and then was undetectable during grain development thereafter (Figure 3-4). This was consistent with no *BP1* expression detected in black pointed and healthy mature grain (Figure 3-3B).

3.3.5 Genome location of *BP1* and *LEA*

RFLP analysis revealed that *BP1* was polymorphic between Alexis and Sloop genomic DNA when digested with the restriction enzyme *Dra*I (Figure 3-5). This allowed the mapping of *BP1* within the Alexis x Sloop double haploid population. *BP1* was found to map to the long arm of barley chromosome 3H and was most closely linked to the existing markers P11/M62-183 and GBM1238 (Figure 3-6 A).

In contrast, the *LEA* was not polymorphic between Alexis and Sloop for any of the six restriction enzymes tested. Because of this, the *LEA* could not be mapped

within the barley genome. The rice LEA protein XP_464846 that is most closely related to the LEA protein in this study is located on rice chromosome 2 at a genetic map position of 37 cM. Based on the established synteny between the rice and barley genomes (Cho et al., 2006), it is predicted that the barley LEA gene would reside on the short arm of barley chromosome 6H (Figure 3-6 B).

MASQQQSRKDAASKREEGQGGLGLEEIGKFRAEAQQHSADAIRAAQER
YNQNLQHGGGARGAVTVTQAPGATVVSYQEHKAIPEGAQQGRAHGHGT
NAPAGGTTASSRGTELQHGAKQEEGRGHGTGHKEHKGSAAVTRAADDK
ESAARGTNAPAGGTVASSRGAEKQHHTKQEEGRGHGAGHKEQKGSAAV
THATEDGGKEGHSARSAKDAAMHALGLTEDQTVGKGAGIKDAGAHGAH
VHGHDAGHREQKGSDSAARTLGSTGDYVSAKGAEAKDAGAHGAQVTAE
GTQEATATAAEYAKQAAAKAKEVTVSTGGTAAEYAKAAAEKAREAAL
AAGKTTAEYTQQAAVKGKDVTVSTGGTAAEYAKTAAEMAKDAALAAGK
TTAEYTQQAAVKTKDVTLSTGAQAAQKAKEVTAVTAQKVAEYTKEMAE
QGKATAAEVEEKAKEAAARAADKAEEPSLDTGSQAKGSAARAADKTR
DTAAQTMGRAKDATGETGDMTGSLTGQVKGHDRSHGAEGE

Figure 3-1 The entire coding region of the identified LEA protein was obtained using 3′ RACE. The derived amino acid sequence is shown with the peptides identified by MS/MS (from Figure 2-7) underlined. Those underlined with solid lines were identified in the UniGene sequence Hv. 10490 while those underlined with broken lines were identified in the additional sequence identified by 3′ RACE-PCR.

Table 3-1 LEA protein have been classified into at least 6 groups. Groups 1, 2, 3 historically have been distinguished by certain motif sequences. Recent reclassification using protein or oligonucleotide probability profile (POPP) analysis has grouped LEA sequence into 10 super families (SF) based on over (+) or under representation (-) of mono, di, and tri-peptides (Wise and Tunnacliffe, 2004). The number of these peptides present within the putative LEA protein is shown in brackets.

Group	Motifs	SF	Over or under represented amino acids from POPP analysis
1a	GGQTRREQLGEEGYSQMGRK	4	+E(46), +G(69), +EG(7), +GE(2), +GG(6), +KG(8), +QE(5), +RK(1), +GGE(0), +KGG(0)
1b		6	+E(46), +G(69), +DE(0), +EG(7), +ES(1), +GG(6), +GQ(2), +RE(3), +RK(1), +ARE(1), +DES(0), +REG(0)
2a	DEYGNP (Y domain)	1	+G(69), −L(12), +EK(3), +GG(6), +GT(9), +EKL(0), +IKE(0), +KEK(0), +KIK(0), +KKG(0), +KLP(0), +LPG(0)
2a	EEKK (K domain)	10	−F(1), +G(69), −I(4), +AG(9), +EK(3), +GG(6), +GQ(2), +KE(8), +SS(2), +EKL(0), +GAG(2), +IKE(0), +KEK(0), +KLP(0), +LPG(0), +SSS(0)
2b	S_n(S segment)	3	−F(1), −I(4), −L(12), −R(22), −W(0), +DK(3), +EK(3), +KK(0), +KL(0), +LP(0), +TH(1), +EKK(0), +KEK(0), +KLP(0), +LPG(0)
2		8	+EK(3), +SS(2), +EKI(0), +KEK(0), +KIK(0), +SSS(0)
2		9	−F(1), +G(69), −I(4), −L(12), −V(23), +AG(9), +EK(3), +GG(6), +GH(9), +GT(9), +TA(13), +TG(10), +GGT(4), +GTG(1), +TAG(0), +TGG(2)
3a	TAQAAKEKAGE	2	+A(117), −C(0), +E(46), −F(1), −I(4), +K(44), −L(12), −P(5), +AE(16), +AK(14), +EK(3), +ET(1), +GE(2), +GK(7), +KE(8), +AAE(6), +AKD(4), +EKA(2)
3b		5	+A(117), −I(4), +K(44), −L(12), −P(5), +Q(34), +T(50), −V(23), +AA(27), +AQ(8), +EK(3), +KE(8), +KT(5), +QA(6), +QQ(5), +QS(1), +QT(2), +TQ(4), +AAK(1), +AQA(1), +EKT(0), +QAA(4), +TQQ(2)
6		7	+A(117), −F(1), −L(12), +AA(27), +AE(16), +MQ(0), +QS(1), +VA(2), +AAA(3), +GVA(0), +QSA(0), +SAA(5)

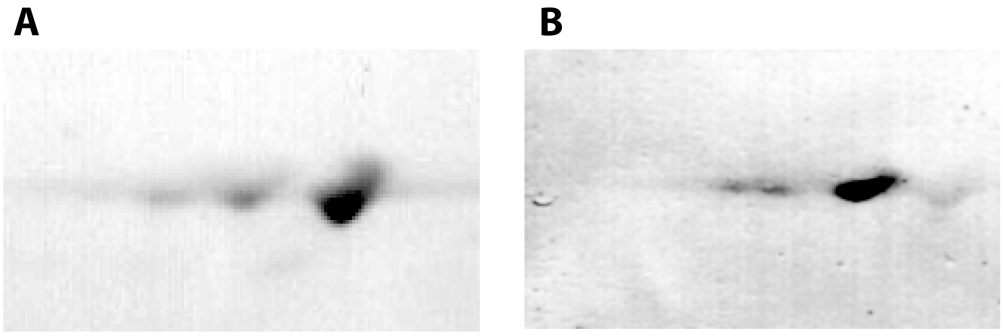

Figure 3-2 Western blot showing the LEA protein is biotinylated. To show that the LEA protein is biotinylated *in vivo,* a western blot was prepared from a 2-D gel of healthy grain protein and stained for total protein (A) and then re-probed with a streptavidin antibody (B). This picture is representative of two independent experiments (n=2).

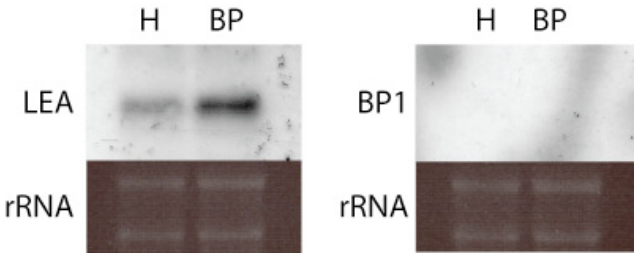

Figure 3-3 Northern blot analysis of LEA and BP1 from mature healthy (H) and black pointed (BP) grain. Ethidium bromide stained rRNA bands are shown as a loading control. This picture is representative of two independent experiments (n=2).

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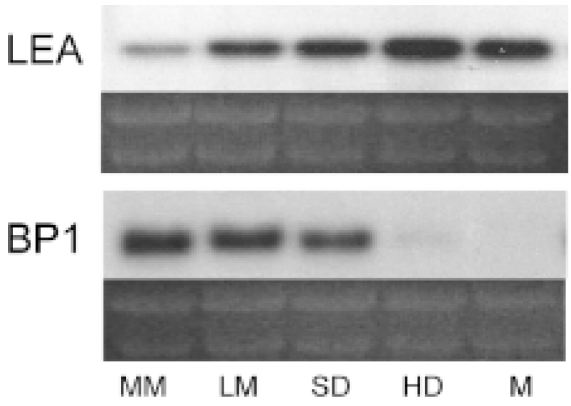

Figure 3-4 Gene expression of LEA and BP1 during healthy grain development. Total RNA was extracted from healthy grain at the following Zadoks' stages, medium milk (MM), late milk (LM), soft dough (SD), hard dough (HD) and maturity. Ethidum bromide stained rRNA bands are shown as a loading control. This picture is representative of two independent experiments (n=2).

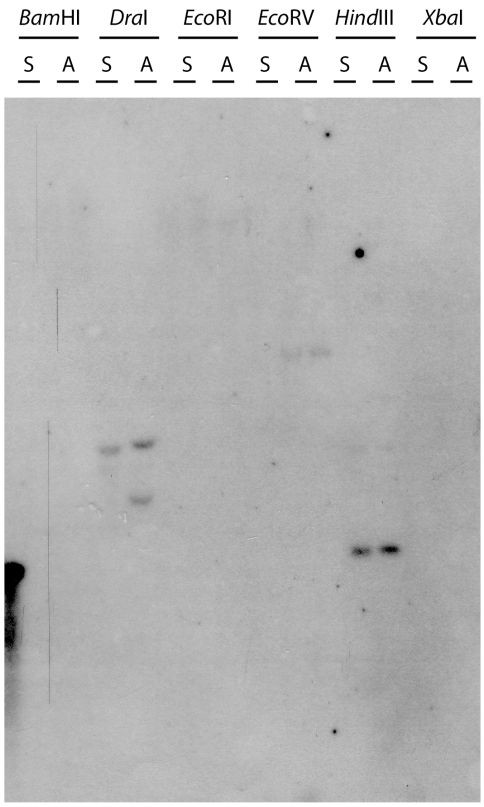

Figure 3-5 RFLP analysis for *BP1* between Alexis and Sloop. DNA from Alexis (A) and Sloop (S) was digested with six different restriction enzymes. The Southern blot above was hybridised with a probe for *BP1*. A polymorphism was identified with the enzyme *Dra*I. No polymorphisms were detected for LEA.

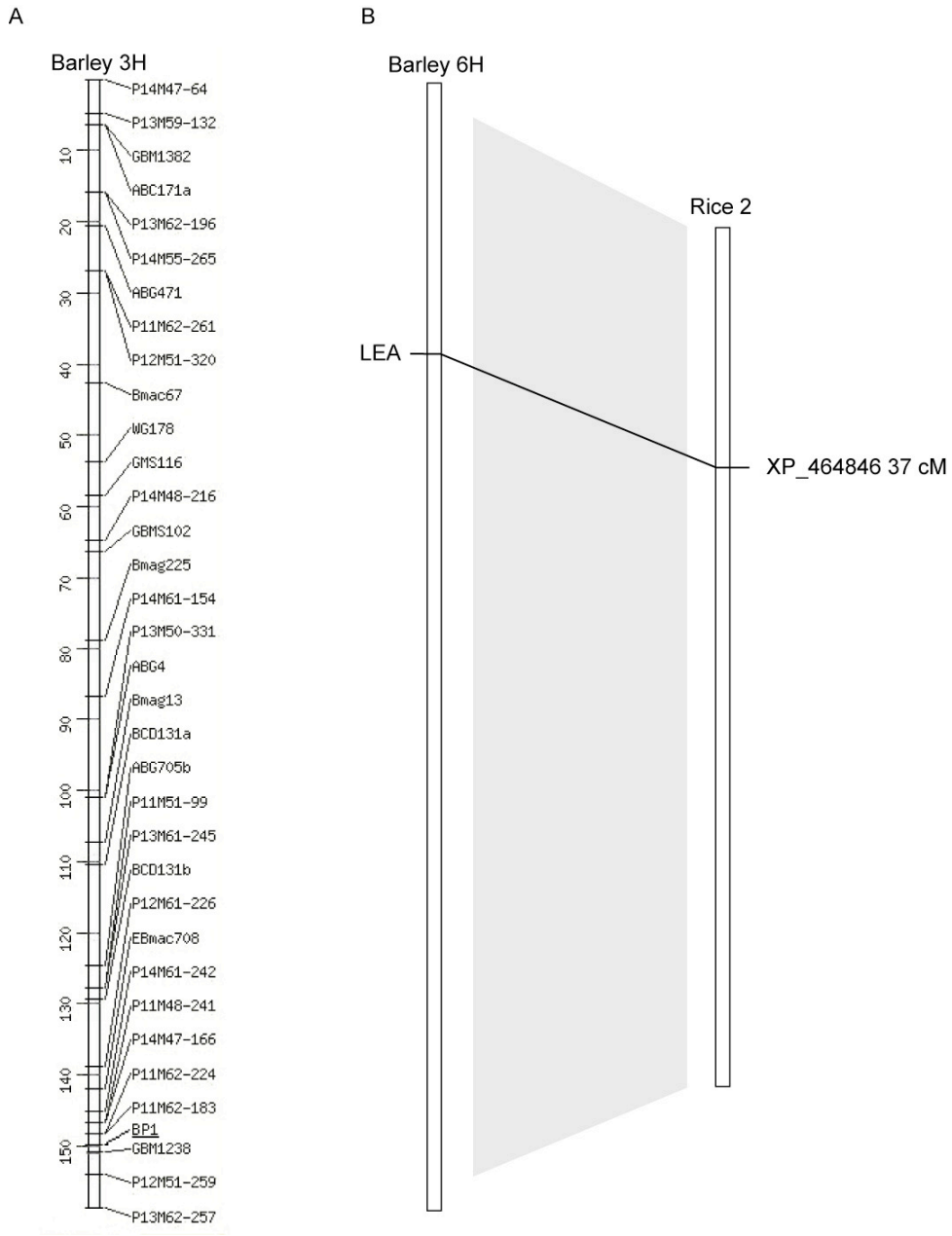

Figure 3-6 Genome location of BP1 and LEA. BP1 was mapped within the Alexis x Sloop double haploid mapping population. It was located to the distal end of the long arm of chromosome 3H (A, underlined). LEA was not polymorphic and thus could not be mapped *in vivo*, however based on the location of a similar LEA protein in rice (XP_464846) on rice chromosome 2 and the established synteny between rice chromosome 2 and barley chromosome 6H (Cho et al., 2006), the barley LEA gene was predicted to reside on the short arm of chromosome 6H (B).

3.3.6 Antibody design and specificity

Using the full-length protein sequence of LEA and BP1, hydrophobicity plots were generated to identify hydrophilic regions (Figure 3-7). From the graph, positive values indicate hydrophilic regions whereas negative values indicate hydrophobic regions. For BP1 and LEA the sequences AEPPVAPGLSFDFYRRTC and MASQQQSRKDAASKRC (shaded grey) were selected respectively as hydrophilic and thus likely to be antigenic. The LEA sequence was extremely hydrophilic as indicated by the hydrophobicity plot. BP1 is N-terminally processed within the cell (Rasmussen et al., 1991), and thus when determining antigenic regions, this was taken into account by not including that part of the sequence.

To determine the specificity of the generated rabbit antiserum, 2-D gel western blots were prepared from whole grain protein extracts (Figure 3-8) for black pointed and healthy grain samples. The anti-LEA serum detected three protein spots of the identical Mw and pI to the LEA isoforms. Several protein spots were also detected directly underneath the three LEA protein spots, as well as a horizontal smear across the membrane at the same size as the LEA protein. The anti-BP1 serum detected protein spots of Mw 34 and 32 kDA that were spread horizontally at five different pI positions. The size of the protein spots detected was identical with that of BP1. Two fainter spots of Mw 23 kDA were also detected that appeared unrelated to BP1.

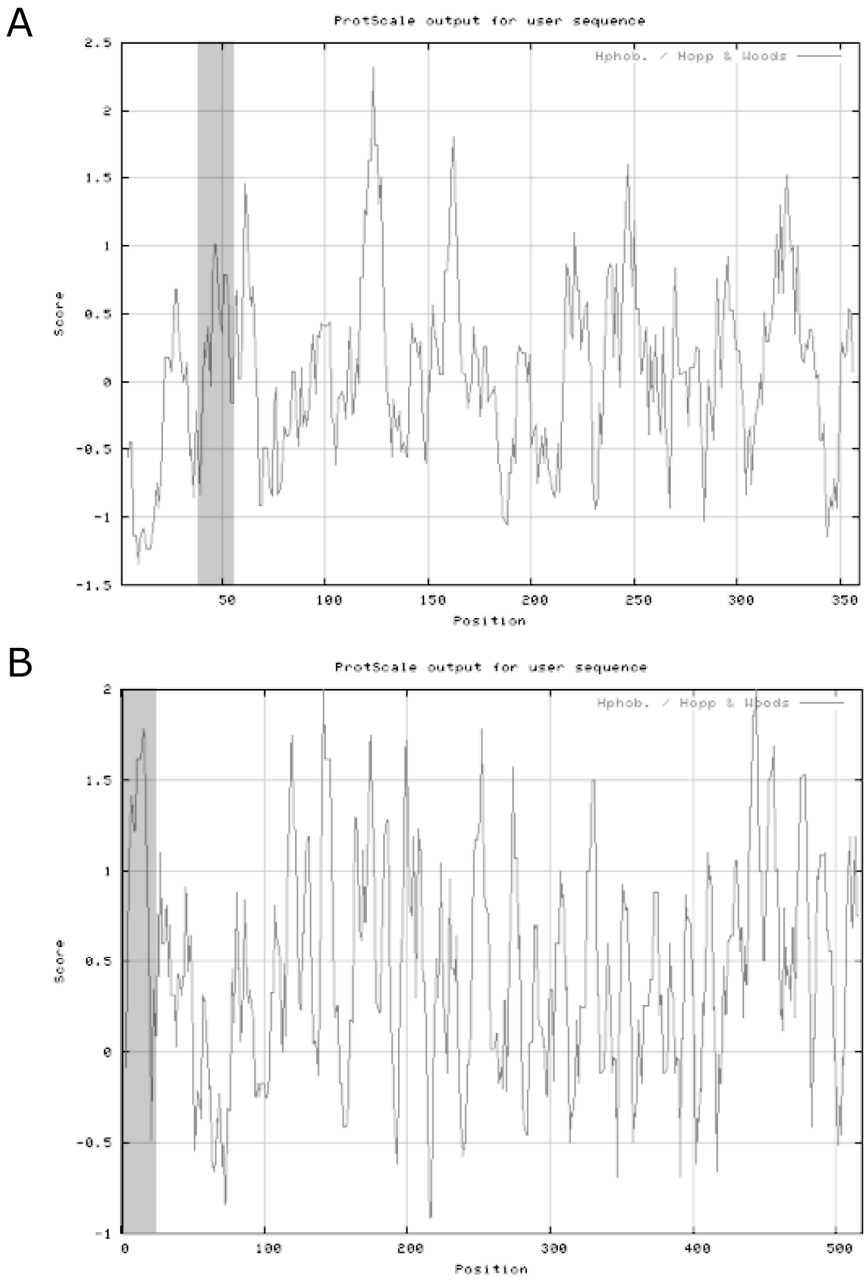

Figure 3-7 Hydrophobicity plots of BP1 (A) and LEA (B) protein sequences. Positive values indicate hydrophilic regions whereas negative values indicate hydrophobic regions. The shaded hydrophilic regions were selected for peptide synthesis and subsequently used as an antigen for injection into rabbits.

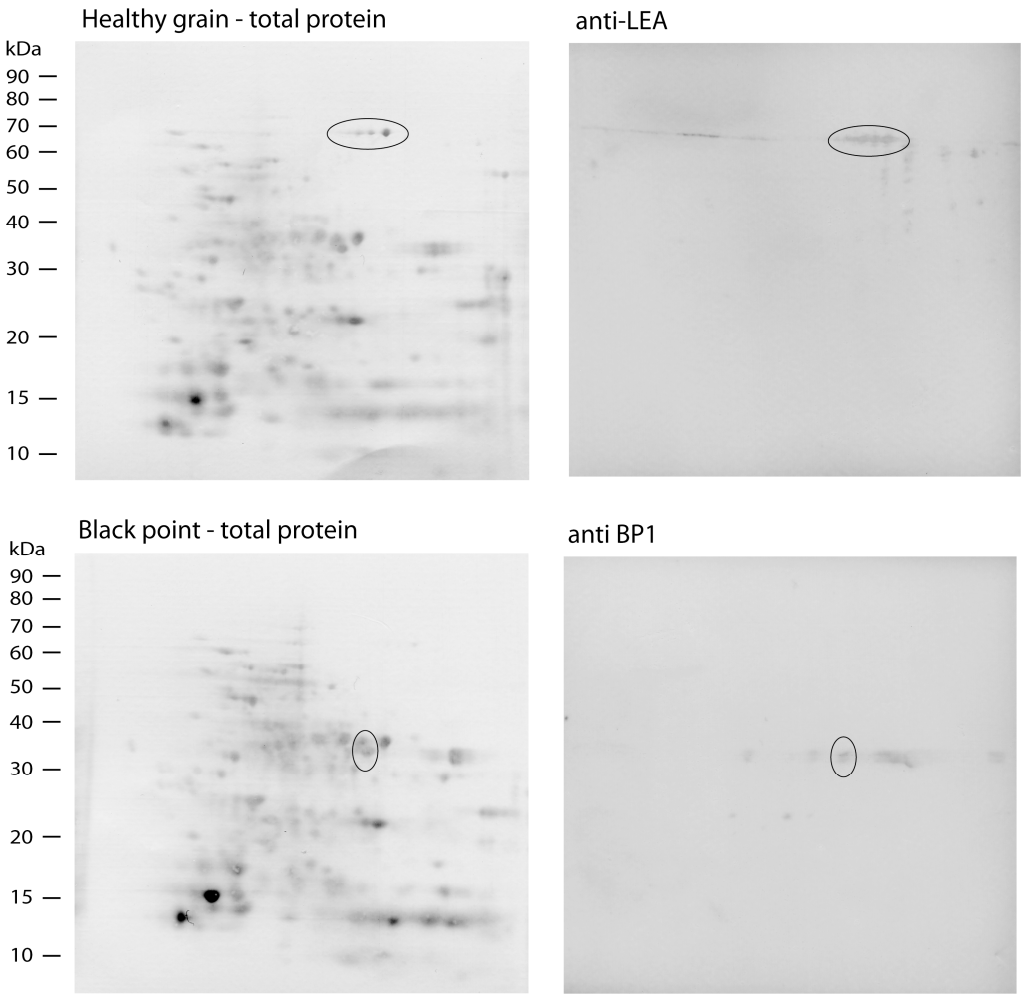

Figure 3-8 Testing specificity of the LEA and BP1 antiserum. 2-D western blots were prepared from grain protein and stained for total protein to check for protein seperation. Membranes were then de-stained and incubated with either LEA or BP1 antiserum from rabbit. Bound antibody was detected using the alkaline phosphatase colour reaction. Image is representative of two independent experiments (n=2).

3.3.7 Immunolocalisation of LEA and BP1 within barley grain

BP1 and LEA proteins were localised within longitude and transverse grain sections using the antibodies generated in section 3.2.6. The reaction between the antibody and the target protein was visualised using a fluorescent secondary antibody. In an initial experiment three different concentrations (1:50, 1:250, 1:500) of primary antibody were tested to find the optimum dilution. It was found that the 1:50 dilution gave the strongest signal without interfering background fluorescence, whereas the 1:500 dilution did not result in sufficient fluorescence (data not shown).

BP1 was detected throughout the grain (Figure 3-9) with slightly higher levels of BP1 being detected in the embryo compared with the endosperm tissue. Higher magnification (100x) revealed that there were higher levels of BP1 in the 3-cell layer thick aleurone tissue compared with the adjacent endosperm tissue (Figure 3-11). Comparison between black pointed and healthy grain showed similar distribution of BP1 throughout both grain samples.

LEA protein was most abundant in the embryo tissue (Figure 3-10). Comparison between the pre-immune control and the anti-LEA transverse sections showed no significant levels of LEA in endosperm tissue. There was a distinct lack of fluorescence detected in the scutellum tissue indicating that LEA is not present in that tissue. Higher magnification showed that LEA was present at even levels throughout the embryo (Figure 3-12). Similar to BP1, there were significantly higher levels of LEA in the aleurone layer compared with the endosperm tissue.

For all samples the pre-immune negative control showed insignificant fluorescence indicating that the reactivity of anti-serum was specific to LEA and BP1.

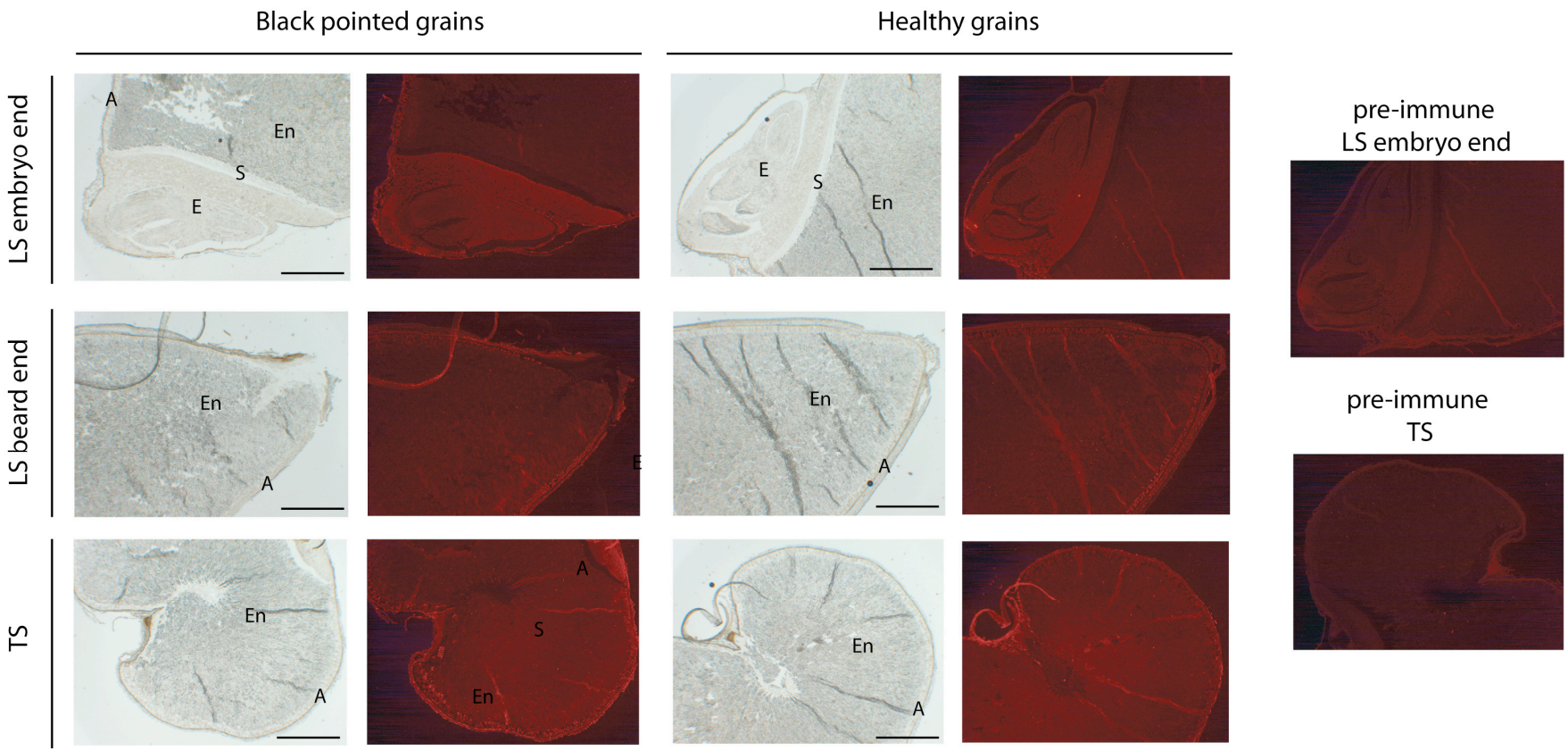

Figure 3-9 BP1 immunolocalisation within the barley grain. Longitudinal (LS) and transverse (TS) sections were prepared from healthy and black pointed grains. Tissues are labelled as embryo (E), scutellum (S), endosperm, (En) and aleurone (A). Sections were probed with either rabbit anti-BP1 serum or rabbit pre-immune serum. Light microscopy images were captured followed by fluorescent images to visualise the secondary antibody. Bar = 500 µm. Images representative of 3 replicates (n=3).

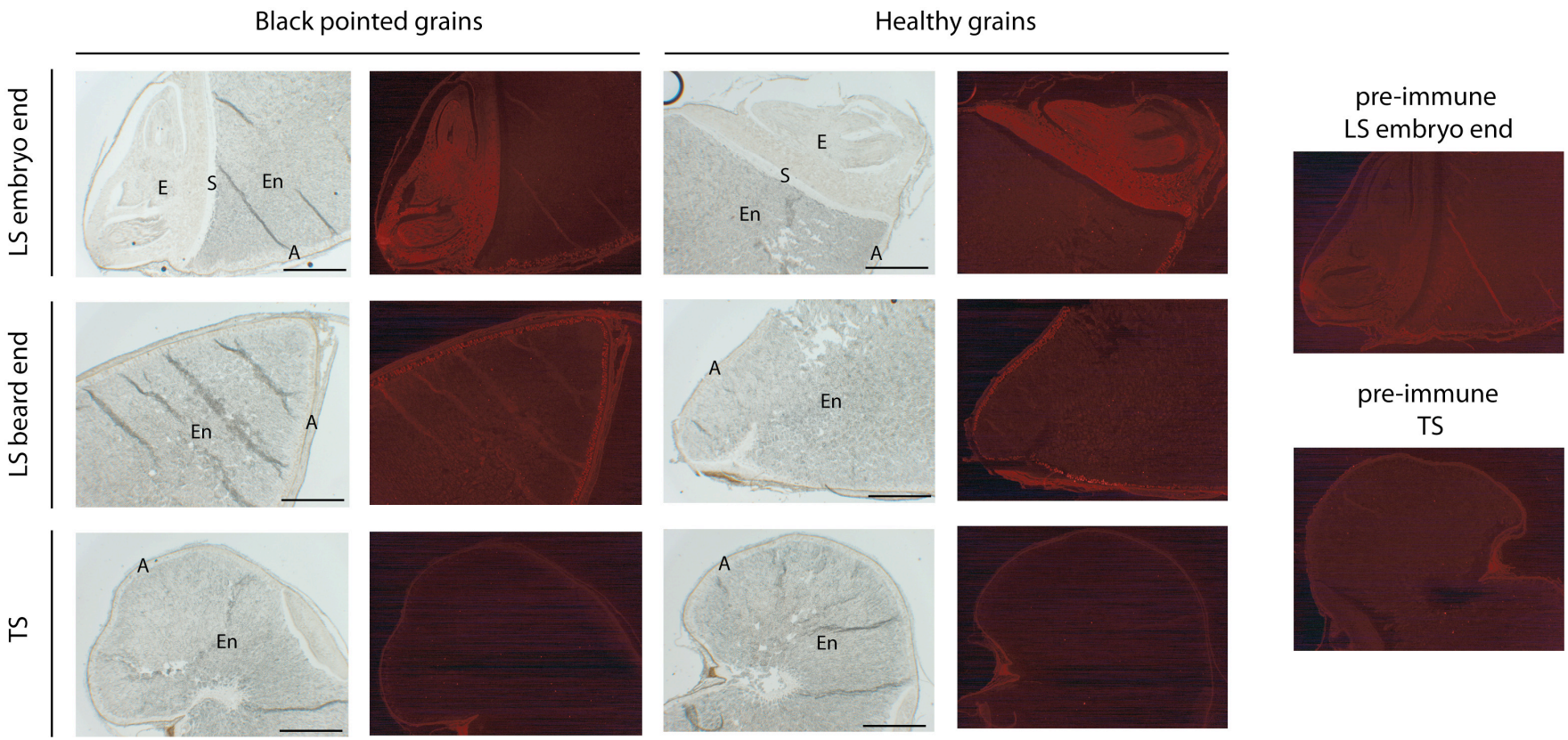

Figure 3-10 LEA immunolocalisation within the barley grain. Longitudinal (LS) and transverse (TS) sections were prepared from healthy and black pointed grains. Tissues are labelled as embryo (E), scutellum (S), endosperm, (En) and aleurone (A). Sections were probed with either rabbit anti-LEA serum or rabbit pre-immune serum. Light microscopy images were captured followed by fluorescent images to visualise the secondary antibody. Bar = 500 µm. Images representative of 3 replicates (n=3).

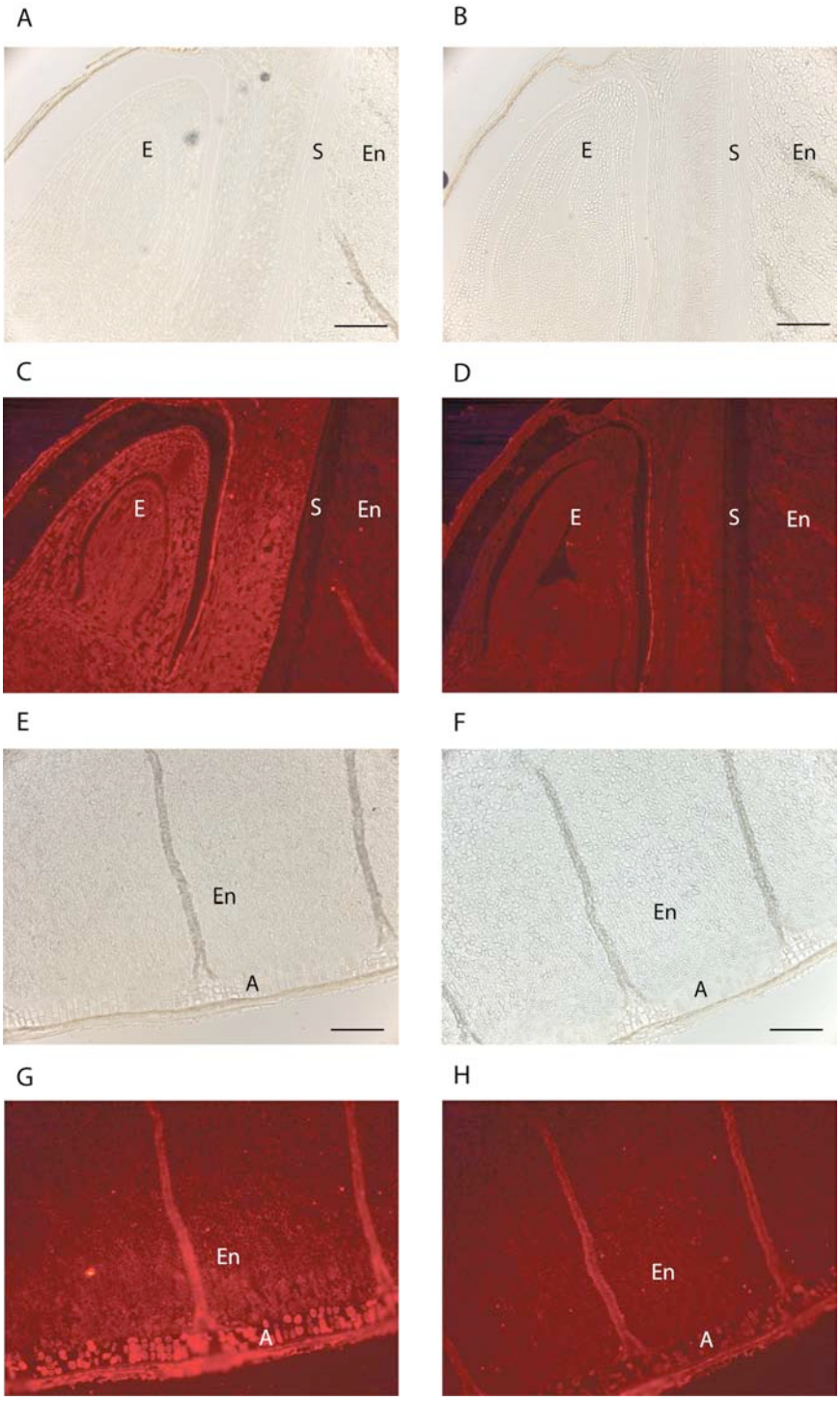

Figure 3-11 Close up of BP1 immunolocalisation in black pointed barley grain. Figures A, B, E & F showing light microscopy of sectioned grains. Tissues were labelled as embryo (E), scutellum (S), endosperm, (En) and aleurone (A). C & G are probed with anti-BP1 serum, while D & H were probed with pre-immune serum. Bar = 200 µm. Images representative of 3 replicates (n=3).

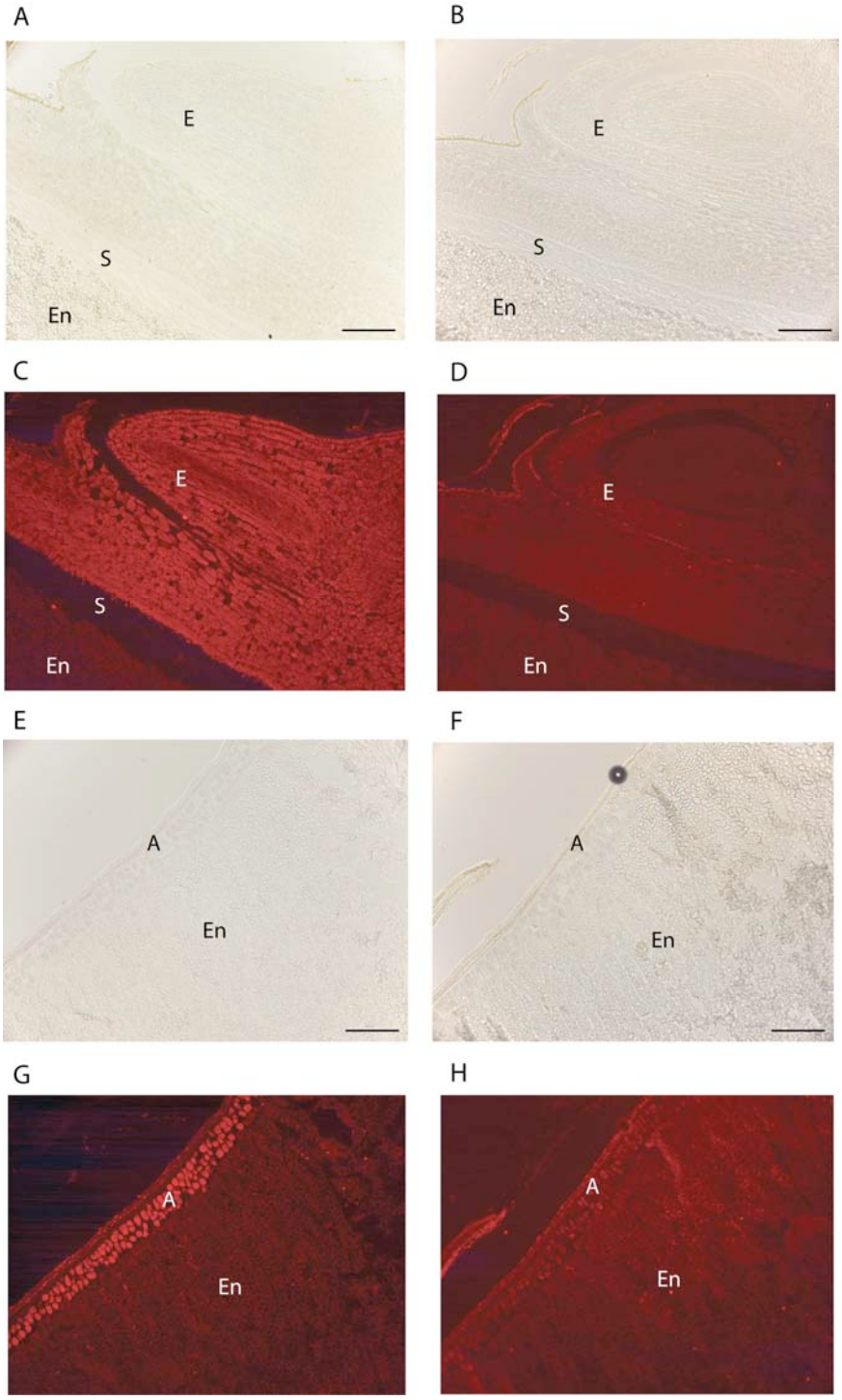

Figure 3-12 Close up of LEA immunolocalisation in healthy barley grain. Figures A, B, E & F showing light microscopy of sectioned grains. Tissues are labelled as embryo (E), scutellum (S), endosperm, (En) and aleurone (A). C & G were probed with anti-LEA serum, while D & H were probed with pre-immune serum. Bar = 200 µm. Images representative of 3 replicates (n=3).

3.4 Discussion

From the research presented in Chapter 2, it was determined that BP1 and a partial sequence of a putative LEA protein were associated with black point of barley grains. The experiments in this chapter were focussed on characterising and determining the role of these proteins in black point development.

To begin to characterise the novel LEA protein that was identified in Chapter 2 the full-length coding sequence of the gene was obtained using 3′RACE-PCR. This sequence was predicted to encode a protein of 49 kDa, which was smaller than the 75kDa protein observed on the 2-D electrophoresis gel. The size discrepancy may be due to the LEA protein being rich in alanine (23%). An over representation of alanine has been reported previously to contribute to proteins appearing larger than their predicted size when subjected to SDS-PAGE (Nolan et al., 2000)

LEA proteins are an extremely diverse class of proteins which are in part classified by their spatial and temporal association with the embryo and desiccation respectively (Roberts et al., 1993). Although prevalent in desiccated seeds, they decrease significantly once germination begins (Hsing et al., 1998). They are characterised by being rich in hydrophilic amino acids (such as lysine and glycine) ordered in repeated sequences (Shao et al., 2005), which results in hydrophilic side chains that are thought to interact with, and protect hydrophilic proteins during desiccation by causing aggregation (Goyal et al., 2005).

At least six groups of LEA proteins have been identified based on expression profiles and sequence alignments (Wise, 2003). Of these the major ones are groups 1, 2 and 3. Group 1 LEA proteins contain a conserved 20 amino acid motif often

present as a single copy (Cuming, 1999). Group 2 LEA proteins contain up to three different sequence motifs which are known as the K domain (EEKK), the Y domain (DEYGNP) and the S segment (poly-serine stutter) (Close, 1996). Group 3 LEA proteins consist of up to 13 repetitions of a conserved 11 amino acid motif (Cuming, 1999). Since this initial classification of LEA proteins, several LEA proteins have been identified that do not contain the conserved motifs described above (Wise, 2003), including the LEA protein identified within this present study.

Grouping of LEA proteins based on sequence alignment is problematic due to low sequence similarity. To overcome this, new bioinformatics techniques such as POPP (protein or oligonucleotide probability profile) have been utilised (Wise, 2003). POPP is used to group LEA proteins based on over or under representation of mono-, di- or tri-peptides within the protein sequence. This analysis has resulted in the reclassification of LEA proteins into 10 super families (Wise, 2003) and resulted in LEA groups 4 and 5 amalgamating into LEA groups 2 and 3. Analysis of the LEA protein sequence from this study concluded that it may belong to either super family 5, 6, 7 or 9 but could not be placed exclusively into any single super family.

To date no full-length LEA protein sequence has been identified in barley that corresponds to the 75 kDa protein identified in this study. However, LEA proteins of 68 kDa and 65 kDa have been isolated from soybean and pea respectively (Dehaye et al., 1997; Hsing et al., 1998). These proteins were found to be biotinylated through an atypical covalent attachment of biotin at a lysine residue within a conserved motif of (V/M)GKF (Job et al., 2001). The proposed purpose of the biotinylation was to act as a biotin sink during desiccation, and then upon

germination when this protein is degraded the biotin is released to be used as a co-factor for enzymes involved with germination (Hsing et al., 1998).

Western blot analysis in this study revealed that the identified LEA protein was bound by streptavidin (Figure 3-2), indicating that it is biotinylated *in vivo*. This suggests that the LEA protein identified may have a similar role as a biotin sink as proposed in the pea and soybean studies (Dehaye et al., 1997; Hsing et al., 1998). Increased LEA protein in healthy grains is not a result of different levels of gene transcription (Figure 3-3) but likely to be due to the LEA protein being degraded in black pointed grains especially given transcript levels were higher in black pointed grain than healthy grain. With respect to black point development it suggests that grains with this condition have entered into the germination process where LEA is usually degraded (Hsing et al., 1998). Monitoring LEA protein levels during grain development and in response to environmental conditions conducive to black point development could be used to investigate this hypothesis.

As expected, based on the known function of LEA proteins, immunolocalisation found the LEA protein in this study to be highly abundant within the embryo tissue and present at insignificant levels in the endosperm. This expression pattern is consistent with that seen for LEA mRNA *in situ* hybridisation studies in soybean seeds (Hsing et al., 1998) and further supports a role for this protein in germination or at the very least embryo development. High levels of LEA were also detected in the aleurone layer. The aleurone layer along with the embryo are the only tissues of the barley grain that survive desiccation. Based on the proposed role of LEA in providing desiccation tolerance it is not surprising to see high levels in both these tissues.

Black point formation can be first observed on green grain prior to desiccation and has also been associated with increased rainfall and moisture during grain fill (Petr and Capouchova, 2001). One possibility, therefore, is that environmental conditions are prolonging the period of grain development in black pointed grain. This in turn may allow the grain to over-develop (Royo et al., 2000) and the embryo enters into a germination state resulting in the breakdown of LEA proteins. This notion of black pointed grains being over-developed is supported by evidence that black pointed wheat grains are larger than healthy grains, possibly as a result of prolonged endosperm development (Dimmock and Gooding, 2002).

The discolouration observed at the embryo end of the grain could therefore be a result of the over-developed embryo wounding the surrounding tissues. Previous observations of damaged germ aleurone tissue identified dark-brown discolouration which was proposed to be due to enzymatic oxidation of phenolic compounds by peroxidase enzymes (Cochrane, 1994a). In pearl millet grains it has been observed that during grain development the embryo crushes the surrounding transfer cells resulting in a dark pigmentation on the surface of the grain (Fussell and Dwarte, 1980). A similar phenomenon may result in black pointed grain if an enlarged embryo wounds the overlying germ aleurone cells, however, the microscopic images presented here did not shown any obvious morphological differences between healthy and black pointed grains. More detailed microscopic analysis of the embryo tissue may be required to further support this hypothesis.

In support of enzymatic oxidation induced by wounding, results indicated that barley grain peroxidase 1 (BP1) was more abundant in black pointed grains. BP1 belongs to the class III group of peroxidase enzymes (EC 1.11.1.7), which are

secreted into the extracellular space or the vacuole of plant cells (Welinder et al., 1992). BP1 contains both an N-terminal and C-terminal signal peptide that targets it to the secretory pathway and vacuole respectively. BP1 is a unique plant peroxidase in that it is inactive at pH > 5. Due to this, it has been proposed that BP1 is targeted to the vacuole rather than the cell wall as the vacuole provides an acidic environment. At its optimum pH, BP1 is able to oxidise the main phenolic compounds present in barley grain, ferulic and *p*-coumaric acid, into discoloured end products (Rasmussen et al., 1997).

In this study BP1 was identified as two separate spots on the 2D gel with a Mw difference of approximately 1 kDa. This has been observed before when BP1 was purified from barley grain as two distinct sized proteins (Rasmussen et al., 1991). The larger of the two proteins was found to be glycosylated.

BP1 gene expression has previously been reported to be high early in grain development and to cease approximately 15 days after flowering (Rasmussen et al., 1997). This was also confirmed in the present study. *BP1* transcripts were not detected in either healthy or black pointed mature grain. This is to be expected in healthy grain but cannot explain the increased levels of BP1 protein in black pointed grain.

BP1 transcript accumulation has been shown to be specifically present in the endosperm (Rasmussen et al., 1997) and enzyme activity staining has shown BP1 protein to be present specifically in the endosperm with no activity detected in the aleurone layer (Cochrane et al., 2000). Immunolocalisation performed in this study found BP1 to be present in consistently high levels in all grain tissues which is in

contrast to previous studies. This may be due to the fact that each study measured BP1 levels in a different way (that is, transcript levels, protein levels and functional enzyme activity) which are not necessarily directly correlated with each other. Furthermore, each study examined grains from different time points during grain development which makes comparison between studies difficult. The fact that the BP1 protein was detected in the embryo region of mature grain indicates it could play a role in oxidation of phenols leading to black point.

For both LEA and BP1, similar expression profiles and protein accumulation levels were observed between healthy and black pointed grain sections, despite differences in protein abundance being observed between 2-D gel electrophoresis experiments (Chapter 2). There is debate as to whether immunolocalisation is a quantifiable technique as the many manual experimental steps involved introduce variability (Taylor and Levenson, 2006; Walker, 2006). Variability can arise from differences in tissue fixation efficiency, variation due to uneven sectioning thickness, and variation in staining efficiency. Differences observed between samples from 2-D gels were representative of proteins contained within the 4 grams of grain used for the protein extraction, whereas, immunolocalisation compares differences between 10 μM sections of a single grain. A far greater sample average is therefore observed when comparing 2-D gel differences and differences in abundance will be greatly amplified.

Previous studies have identified several QTL influencing black point (see Chapter 1). If *BP1* and *LEA* play a role in black point tolerance or susceptibility they may be located within a previously identified QTL. *BP1* was found to map to the short arm of chromosome 3H in the identical position to that reported in an

earlier study using a different mapping population (Johansson et al., 1992). QTL for kernel discolouration have been located on chromosome 3H (Li et al., 2003) however they are not in the same position as where *BP1* maps. The *LEA* was predicted to map to chromosome 6H based on rice/barley synteny relationships. Several minor QTL have been located on chromosome 6H that influence kernel discolouration (Canci et al., 2004). Due to the *LEA* not being polymorphic it could not be accurately located on chromosome 6H thus it is not known if it truly resides within one of the aforementioned minor QTL. The possibility still exists that the identified black point QTL on 2H (Hadaway, 2002) may encode transcription factors or enzymes involved in post-translational modification that regulate BP1 and LEA accumulation within the grain but this hypothesis would require further research.

This present study has provided significant evidence to support the argument that black point is associated with physiological changes within the grain. Understanding precisely what environmental conditions induce black point formation will be of significant benefit in further understanding the molecular mechanism of the browning.