



**Biochemical and physiological studies of
abscisic acid treated wheat (*Triticum aestivum*)
grain**

A thesis submitted in fulfilment of the requirements for the
degree of Doctor of Philosophy by:

Ante Jerkovic

Department of Chemistry and Biomolecular Sciences
Macquarie University, Sydney, NSW, Australia

June 2011

Table of Contents

Page Number

List of figures	xiii
List of tables	xxiii
Declaration	xxix
Acknowledgements	xxxi
Publication and conference posters	xxxiii
Summary	xxxv
Abbreviations	xxxvii
Chapter 1 : Introduction	39
<i>1.1 Wheat</i>	39
<i>1.2 Growing and harvesting wheat</i>	41
<i>1.3 Wheat based products</i>	43
<i>1.4 Anatomy of the wheat kernel</i>	46
<i>1.5 Grain developmental stages</i>	50
1.5.1 Embryo	50
1.5.2 Endosperm	50
<i>1.6 Germination</i>	51
<i>1.7 Milling</i>	52
<i>1.8 Conditioning wheat</i>	56
<i>1.9 Test milling</i>	56
<i>1.10 Wheat defense proteins</i>	58
<i>1.11 Environmental stress response proteins</i>	62
<i>1.12 Proteomic methods in plant research</i>	64
1.12.1 2-DE proteomics	64
1.12.2 Label and label-free proteomics	66
<i>1.13 Project aim and hypothesis</i>	70

Chapter 2 : Strategic distribution of protective proteins within bran layers of wheat

(*Triticum aestivum* L.) protects the nutrient-rich endosperm _____ 71

2.1 Introduction _____ 71

2.2 Materials and Methods _____ 73

2.2.1 Bran tissue microdissection _____ 73

2.2.2 Protein extraction of bran tissue fractions _____ 73

2.2.3 Water-soluble protein extraction from whole grain, isolated outer bran tissue and endosperm for enzyme activity assays and 1-D SDS-PAGE _____ 74

2.2.4 Protein estimation _____ 75

2.2.5 Two-dimensional electrophoresis (2-DE) _____ 75

2.2.6 Proteomic analysis _____ 76

2.2.7 Oxalate oxidase (OXO) and peroxidase (POX) activity assay _____ 79

2.2.8 OPD standard curve _____ 80

2.2.9 Polyphenol oxidase (PPO) assay _____ 80

2.2.10 Chitinase assay _____ 81

2.2.11 Generation of antibodies and affinity purification _____ 81

2.2.12 Sectioning and microscopy _____ 82

2.3 Results _____ 83

2.3.1 Light microscopy of bran tissue fractions _____ 83

2.3.2 Protein extraction from bran tissue fractions _____ 85

2.3.3 Protein identification from 2-DE gels _____ 85

2.3.4 Outer fraction _____ 87

2.3.5 Intermediate fraction _____ 87

2.3.6 Inner fraction (aleurone cells) _____ 88

2.3.7 Proteins identified in the supernatant from imbibed grain and isolated outer fraction _____ 92

2.3.8 Enzyme activity assays of the water-soluble proteins chitinase, OXO, POX and PPO _____	92
2.3.9 Immunofluorescence localization of oxalate oxidase (OXO), xylanase inhibitor protein-I (XIP-I) and pathogenesis-related protein 4 (PR-4) _____	94
2.4 Discussion _____	97
Chapter 3 : Proteomic analysis of water-and water + abscisic acid (ABA)-conditioned grain _____	103
3.1 Introduction _____	103
3.2 Materials and Methods _____	104
3.2.1 List of chemicals _____	104
3.2.1.1 Plant hormone _____	104
3.2.1.2 Protein extraction _____	104
3.2.1.3 Protein solubilisation _____	104
3.2.1.4 Protein quantification _____	104
3.2.2 Determining grain moisture content _____	105
3.2.3 Grain viability _____	105
3.2.4 ABA activity bioassay _____	105
3.2.5 Grain conditioning _____	106
3.2.6 Tissue collection _____	106
3.2.7 Protein extraction _____	108
3.2.8 Protein estimation by SDS PAGE _____	108
3.2.9 iTRAQ experiments for non-conditioned, water-and water + ABA-conditioned grains _____	109
3.2.9.1 Experiment 1 _____	109
3.2.9.2 Experiment 2 _____	109
3.3 Results _____	111

3.3.1 Grain moisture level _____	111
3.3.2 Grain viability and ABA activity bioassay _____	111
3.3.2.1 Grain viability _____	111
3.3.2.2 Coleoptile and root growth of grain in water, 4 ppm and 40 ppm ABA _	111
3.3.3 Protein concentration estimation_____	113
3.3.4 iTRAQ analysis of protein extracts from germ, bran and ventral groove tissues dissected from water-and water + ABA-conditioned grain compared to these tissue types from non-conditioned grain _____	114
3.3.4.1 Germ _____	114
3.3.4.2 Bran _____	115
3.3.4.3 Ventral groove _____	116
3.3.5 iTRAQ analysis of protein level changes in the germ, bran and ventral groove tissues from water-conditioned grain compared to non-conditioned grain _____	117
3.3.6 iTRAQ analysis of protein level changes in the germ, bran and ventral groove tissues from water + 4 ppm ABA-conditioned grain compared to non-conditioned grain _____	120
3.3.7 Biotic and abiotic defense-related proteins _____	123
3.3.7.1 Germ, bran and ventral groove from water-conditioned grain _____	123
3.3.7.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain _____	123
3.3.8 Membrane transport/transport proteins _____	126
3.3.8.1 Germ, bran and ventral groove from water-conditioned grain _____	126
3.3.8.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain _____	126
3.3.9 Cell structure proteins _____	128
3.3.9.1 Germ, bran and ventral groove from water-conditioned grain _____	128

3.3.9.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	128
3.3.10 Protein synthesis and folding proteins	129
3.3.10.1 <i>Germ, bran and ventral groove from water-conditioned grain</i>	129
3.3.10.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	129
3.3.11 Transcription-related proteins	132
3.3.11.1 <i>Germ, bran and ventral groove from water-conditioned grain</i>	132
3.3.11.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	132
3.3.12 Glycolysis and energy proteins	135
3.3.12.1 <i>Germ, bran and ventral groove from water-conditioned grain</i>	135
3.3.12.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	135
3.3.13 Regulatory and signaling proteins	137
3.3.13.1 <i>Germ, bran and ventral groove from water-conditioned grain</i>	137
3.3.13.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	137
3.3.14 Metabolism-related proteins	139
3.3.14.1 <i>Germ, bran and ventral groove from water-conditioned grain</i>	139
3.3.14.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	139
3.3.15 Protease-related proteins	142
3.3.15.1 <i>Germ, bran and ventral groove from water-conditioned grain</i>	142
3.3.15.2 <i>Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain</i>	142

3.3.16 Endosperm-related proteins	144
3.3.16.1 Germ, bran and ventral groove from water-conditioned grain	144
3.3.16.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain	144
3.3.17 Other proteins	146
3.3.17.1 Germ, bran and ventral groove from water-conditioned grain	146
3.3.17.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain	146
3.3.18 Unknown function	149
3.3.18.1 Germ, bran and ventral groove from water-conditioned grain	149
3.3.18.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain	149
3.4 Discussion	154
3.4.1 Biochemical changes in water-conditioned grain compared with non-conditioned grain	154
3.4.2 Biochemical changes in water + ABA-conditioned grain compared with non-conditioned grain	156
3.4.3 Changes in protein levels within the biotic and abiotic defense protein class exhibited by water-and water + ABA conditioning	158
Chapter 4 : iTRAQ analysis of bran and ventral groove tissues from non-conditioned grain	161
4.1 Introduction	161
4.2 Materials and Methods	163
4.3 Results	165
4.3.1 Differences in protein levels between the ventral groove and bran from non-conditioned grain according to protein class	165

4.3.1.1 Biotic and abiotic defense	165
4.3.1.2 Membrane transport/transport	165
4.3.1.3 Cell structure	165
4.3.1.4 Protein synthesis and folding	166
4.3.1.5 Transcription	166
4.3.1.6 Glycolysis and energy	166
4.3.1.7 Regulatory and signalling	166
4.3.1.8 Metabolism	166
4.3.1.9 Proteases	166
4.3.1.10 Endosperm-related proteins	166
4.3.1.11 Other function	167
4.3.1.12 Unknown function	167
4.4 Discussion	171
Chapter 5 : Identification and immunolocalisation microscopy of late embryogenesis	
abundant (LEA) proteins	177
5.1 Introduction	177
5.2 Materials and Methods	179
5.2.1 List of chemicals	179
5.2.1.1 Microscopy	179
5.2.1.2 Western blot and immunodetection	179
5.2.2 Determining grain moisture content and conditioning	179
5.2.3 Protein extraction	179
5.2.4 Protein estimation	180
5.2.5 One-dimensional SDS PAGE and semi dry blotting	180
5.2.5.1 One-dimensional SDS PAGE	180
5.2.5.2 Semi dry blotting	181

5.2.6 Immunodetection of dehydrin proteins _____	181
5.2.6.1 <i>Alkaline phosphatase</i> _____	181
5.2.6.2 <i>Infrared scan</i> _____	182
5.2.7 Immunolocalisation of dehydrin protein on grain cross-sections _____	183
5.2.8 Fluorescence confocal microscopy _____	184
5.3 <i>Results</i> _____	185
5.3.1 Immunolocalisation microscopy of dehydrin proteins in germ tissue _____	185
5.3.2 Immunolocalisation microscopy of dehydrin proteins in aleurone cells of the bran tissue _____	188
5.3.3 Immunolocalisation microscopy of dehydrin proteins in the ventral groove tissue from non-conditioned, water-and water + ABA-conditioned grain _____	190
5.3.4 SDS PAGE and western blot of heat-stable proteins extracted from whole grain _____	192
5.3.5 SDS PAGE and western blot detection of dehydrins in proteins extracted from germ, bran and ventral groove from non-conditioned, water-and water + ABA- conditioned grain _____	195
5.3.5.1 <i>Germ</i> _____	195
5.3.5.2 <i>Bran</i> _____	196
5.3.5.3 <i>Ventral groove</i> _____	197
5.4 <i>Discussion</i> _____	200
Chapter 6 : Grain fractionation and physiological analysis of water-and water + ABA-conditioned wheat grain _____	203
6.1 <i>Introduction</i> _____	203
6.1.1 Grain fractionation _____	203
6.1.2 Psychrometry and mechanical property analysis _____	204
6.2 <i>Materials and Methods</i> _____	207

6.2.1 Grain fractionation	207
6.2.2 Wheat cultivars	208
6.2.3 Estimating grain moisture content	208
6.2.4 Conditioning	209
6.2.5 MicroMilling	209
6.2.6 Fractionation quality score (FQS)	210
6.2.7 Setting up the psychrometer	211
6.2.7.1 Psychrometer connections and accessories	211
6.2.7.2 Cleaning thermocouple sensor and sample chamber	211
6.2.7.3 Psychrometer settings	211
6.2.7.4 Sodium chloride standard curve	211
6.2.7.5 Water potential (ψ) measurements of samples	212
6.2.8 Instron measurements of the germ and bran-endosperm tissue of water-and water + ABA-conditioned grain	212
6.2.9 Statistical analysis of grain fractionation, water potential and mechanical properties	214
6.3 Results	215
6.3.1 MicroMill optimisation	215
6.3.2 MicroMilling various cultivars of non-conditioned, water-and water + 4 ppm ABA-conditioned grain	215
6.3.3 Water potential (ψ) of water-and water + 4 ppm ABA-conditioned grain	221
6.3.4 Water potential (ψ) of the germ-end (1/3 of the grain) and bran-end (2/3 of the grain) of water-and water + 4 ppm ABA-conditioned grain	227
6.3.4.1 Grain conditioned at 30% moisture content	227
6.3.4.2 Grain conditioned at 34.5% moisture content followed by 2 h drying	227
6.3.5 Instron analysis of the germ of water-and water + ABA-conditioned grain	230

6.3.5.1 Toughness of the bran covering the germ	231
6.3.5.2 Toughness of the germ	232
6.3.6 Instron analysis of the bran-endosperm of water-and water + ABA-conditioned grain	233
6.3.6.1 Toughness of the bran	234
6.4 Discussion	236
Chapter 7 : Conclusion and future direction	241
References	245
Appendix A. Extension of Figures	263
Appendix B. Extension of Tables	279
Appendix C. Publication	299

DVDs (Discs 1 and 2) included on back cover:

DVD contents:

Disc 1

- Raw MS data from iTRAQ experiments of the germ tissue (Part 1)

Disc 2

- Raw MS data from iTRAQ experiments of the bran and ventral groove tissues (Parts 2 and 3)
- iTRAQ protein and peptide summaries for the germ, bran and ventral groove tissues
- Supplemental Figures and Tables for Chapter 2
- Mechanical properties analysis (Instron) Supplementary raw data for germ and bran/endosperm

Instructions to open raw MS iTRAQ data (for Parts 1, 2 and 3 only):

Disc 1 – AJerkovic Raw Data. Part 1.exe

Disc 2 – AJerkovic Raw Data. Part 2.rar

Disc 2 – AJerkovic Raw Data. Part 3.rar

To extract this data, copy the three above files from Disc 1 and Disc 2 to the computer's hard drive, double-click on the executable (exe) file and enter a directory for the data to be extracted to (~ 21 Gb uncompressed).

List of figures

Figure 1.1. Production of wheat in metric tonne (MT) in 2009 from the top 20 major wheat producing countries. Data obtained from the Food and Agriculture Organization of the United Nations (http://faostat.fao.org/site/339/default.aspx).	40
Figure 1.2. Map of Australian wheat belt showing premium white wheat areas (Yellow), premium white and hard wheat areas (Green), predominantly hard wheat areas (Orange) and wheat terminals (Blue). Modified from	42
Figure 1.3. Relationship between wheat hardness, protein content and its use in making products. Figure modified from http://www.regional.org.au/au/roc/1988/roc198815.htm . 45	45
Figure 1.4. Longitudinal dissection of the wheat grain showing the four main components: (A) germ, (B) endosperm, (C) bran and (D) ventral groove.	47
Figure 1.5. Schematic diagram of a cross section of wheat bran showing the different tissue layers.	48
Figure 1.6. Micrograph of a cross-section of grain showing aleurone cells around the ventral groove (VG) and surrounding endosperm.	49
Figure 1.7. General outline of the milling process from raw material to product. The first stages are primarily classifying, blending and cleaning the wheat from debris and unwanted plant material. The next stage is conditioning (adding water and letting it rest for a period of hours). This is followed by passing conditioned wheat through a series of break rollers (these are fluted rollers) which grinds it into coarse particles. These particles are passed through purifiers and sifters to separate into various particle sizes. The different particle sizes are further ground down by reduction rollers (smooth surface rolls) to extract flour from the middlings. Figure modified from web site:	53

Figure 1.8. Carbohydrate composition of bran tissue layers showing relative levels of arabinose and xylose. The testa, nucellar and aleurone tissue comprise mostly of xylose. Modified from Parker et al. (2005).	60
Figure 1.9. Photomicrograph of a cross-section of grain indicating the location of (A) nucellar tissue and aleurone cell walls and (B) nucellar tissue and aleurone cell walls that were hydrolysed by xylanase.	61
Figure 1.10. Solution structure of a putative late embryogenesis abundant (LEA) protein At2g46140.1. Image taken from RCSB Protein Data Bank. Deposited by Song J, Tyler RC, Lee MS, Markley JL, 2005/2/24 Center For Eukaryotic Structural Genomics (Cesg). (Web location: http://www.rcsb.org/pdb/explore/images.do?structureId=1YYC).....	63
Figure 1.11. Chemical structures of isobaric tags. The balance group mass is lost at collision induced dissociation during MS/MS and intensities of each reporter ion is measured. Modified from Ross et al. (2004).	68
Figure 1.12. MS/MS spectra and iTRAQ reporter ions: (A) MS/MS spectra of a peptide showing the b and y ions, (B) theoretical amino acid sequence according to the b and y ions, (C) iTRAQ reporter ions and peptide quantitation information and (D) relative abundance of peptide according to the reporter ions.	69
Figure 2.1. Micrographs of the isolated bran fractions. (A) Outer bran fraction (epidermis and hypodermis). (B) Intermediate bran fraction (cross cells, tube cells, testa, and nucellar tissue). (C) Detailed view of the individual layers in the intermediate fraction (Cc, cross-cells Tc, tube-cells T, testa Nu, nucellar tissue). (D) Aleurone cells.....	84
Figure 2.2. 2-DE gels of Inner bran layer (Aleurone). The highlighted spots show the different EST classes of 7S globulin with the EST GenBank gi number show in the legend. The unhighlighted gels are shown in Supplementary figures S5 and S6 respectively.	86

Figure 2.3. Summary of the major proteins identified in bran tissue fractions, supernatant from imbibed grain, and outer tissue fraction. (A) Table of major bran tissue and water-soluble proteins identified. (B) Scanning electron micrograph (SEM) of a cross-section of bran showing the bran tissue fractions..... 91

Figure 2.4. Fluorescence immunolocalization of defense proteins in bran cross-sections overlaid on DIC images of cross-sections (labelled). Dark inset overlays in images show fluorescence labeling without DIC overlay. (A) Control treated only with secondary antibody. (B) Oxalate oxidase antibody. (C) XIP-1 antibody. (D) PR-4 antibody..... 95

Figure 2.5. Immunofluorescence localisation of PR4, XIP-1 and OXO in wheat varieties Chara and Wedgetail. Images arrayed in alternating rows of the DIC-overlaid with immunofluorescence image, followed by immunofluorescence only image. 96

Figure 2.6. Scanning electron micrograph (SEM) of a cross-section of bran showing the bran tissue layers with corresponding xylose and arabinose content. (A) Outer (P, Pericarp), Intermediate (Cc, Cross-cells; Tc, Tube-cells; T, Testa (seed coat); Nu, Nucellar tissue), and Inner (Al, Aleurone cells; E, Endosperm). (B) Mol % xylose and arabinose in corresponding bran tissue types (Parker et al., 2005). 98

Figure 3.1. Micrograph of a longitudinal dissection of grain showing (A) bran and endosperm, (B) the removed ventral groove tissue and (C) germ. 107

Figure 3.2. Experiment flow chart for each of the three grain tissues – germ (Experiment 1), bran (Experiment 2) and ventral groove (Experiment 3) – from the control (mature non-conditioned grain), water-conditioned and water + 4 ppm ABA-conditioned grain, showing the allocation of the iTRAQ labels 114, 115, 116 and 117 for each of the samples being examined..... 110

Figure 3.3. Bioassay of wheat to test ABA activity. Grain treated with water after (A) 24 h, (B) 48 h and (C) 72 h. Grain treated with 4 ppm ABA in water after (D) 24 h, (E) 48 h and (F) 72 h. Grain treated with 40 ppm ABA in water after (G) 24 h, (H) 48 h and (I) 72 h.....	112
Figure 3.4. Protein level changes in the germ from water-and water + ABA-conditioned grain compared with germ from non-conditioned grain.	114
Figure 3.5. Protein level changes in the bran from water-and water + ABA-conditioned grain compared with germ from non-conditioned grain.	115
Figure 3.6. Protein level changes in the ventral groove from water-and water + ABA-conditioned grain compared with ventral groove from non-conditioned grain.	116
Figure 3.7. Changes in protein levels in the germ, bran and ventral groove tissues after water conditioning (total $n = 221$).	118
Figure 3.8. Changes in protein levels in the germ, bran and ventral groove tissues from water + ABA-conditioned grain compared to these tissue types from non-conditioned grain (total $n = 335$).....	121
Figure 4.1. Photomicrograph of the ventral groove showing the vascular bundles, chalaza and nuclear projection.	162
Figure 4.2. Flow chart of the iTRAQ label allocations for proteins extracted from bran and ventral groove from non-conditioned grain.	164
Figure 4.3. Major proteins of the (A) bran tissue ($n = 26$) and (B) ventral groove tissue ($n = 19$).	172

Figure 5.1. Photomicrograph of a cross-section through the germ highlighting the location (green) of dehydrin proteins within its tissue cells.	186
Figure 5.2. Enlarged photomicrograph of a cross-section through the germ highlighting the location (green) of dehydrin proteins within its tissue cells.	187
Figure 5.3. DIC photomicrograph of a cross-section of grain showing dehydrin proteins (in green) localised within aleurone cells of the bran tissue.	188
Figure 5.4. Photomicrographs of a cross-section of grain showing the localisation of (A) dehydrin proteins (coloured green) and the (B) nucleus (coloured red) within the aleurone cells of the bran tissue. (C) is an overlay of images (A) and (B).....	189
Figure 5.5. Photomicrograph of a cross-section of (A) non-conditioned, (B) water-conditioned and (C) water + ABA-conditioned grain showing the nucleus (red) and dehydrin proteins (green) localised within the aleurone cells of the ventral groove tissue. CH = Chalaza.....	191
Figure 5.6. SDS PAGE of heat-stable proteins extracted from whole grain. (A) Non-conditioned, (B) water-conditioned and (C) water + ABA-conditioned grain. The protein bands boxed in red, numbered 1 – 5, correspond with the western blot (Figure 5.7).....	193
Figure 5.7. Western blot and dehydrin detection of heat-stable proteins extracted from (A) non-conditioned, (B) water-conditioned and (C) water + ABA-conditioned grain. The bands boxed in red, numbered 1 – 5, correspond with the 1D gel of the heat-stable proteins (Figure 5.2). The lower molecular weight range (below 15 kDa) was collaged to the upper 250 – 15 kDa molecular weight range.	194
Figure 5.8. (A) 1D gel and (B) dehydrin detection western blot of proteins extracted from the germ from the iTRAQ experiment. Lanes 1 and 2 are germ protein extract from non-	

conditioned grain, lanes 3 and 4 are proteins extracted from water-conditioned grain and lanes 5 and 6 are proteins extracted from water + ABA-conditioned grain.196

Figure 5.9. (A) 1D gel and (B) western blot probed with antibodies to dehydrin of proteins extracted from the bran and ventral groove tissues used in the iTRAQ experiments. Lanes 1 and 2 are bran protein extracts from non-conditioned grain, lanes 3 and 4 are protein extracts from water-conditioned grain and lanes 5 and 6 are protein extracts from water + ABA-conditioned grain, lanes 7 and 8 are ventral groove protein extract from non-conditioned grain, lanes 9 and 10 are proteins extracted from water-conditioned grain and lanes 11 and 12 are proteins extracted from water + ABA-conditioned grain.198

Figure 6.1. Photograph of the custom made laboratory bench scale mill (RM-001).....208

Figure 6.2. Photograph of the 1.5 mm diameter Instron probe after penetrating 1.5 mm into the (A) germ and (B) bran/endosperm.213

Figure 6.3. Ratios [% flour:% bran from the intermediate fraction] versus ranked replicates ($n = 4$) of various cultivars of the from non-conditioned (mature), water-and water + 4 ppm ABA-conditioned grain. A paired t-test was performed only on the ranked replicates between water-and water + 4ppm ABA-conditioned grain.220

Figure 6.4. Ψ s of grain conditioned at 24, 30 and 34.5% moisture content. The lowest Ψ measurable with the psychrometer is ~ -6.5 MPa at 24% grain moisture content. Error bars are \pm standard deviation ($n = 6$).222

Figure 6.5. Drying time versus Ψ of grain conditioned at 34.5 % moisture. (A) Drying at $< 50\%$ humidity, error bars are \pm half range and (B) drying at $\sim 60\%$ humidity.....223

Figure 6.6. Ranked replicates ($n = 6$) of Ψ s of grain conditioned at (A) 24%, (B) 30% and (C) 34.5% moisture with water-and water + ABA for 16.5 h.....226

Figure 6.7. Ψ s of grain conditioned with water-and water + ABA at 30% moisture for 16.5 h. After conditioning, 1/3 of the germ-end of the grain was cut off and the ψ was measured for each the (A) germ-end and (B) bran-end.	228
Figure 6.8. Ψ s of the (A) germ-end and (B) bran-end of grain conditioned at a moisture level of 34.5% with water-and water + ABA and left to dry at room temperature and 80% humidity for ~ 1.5 h.	229
Figure 6.9. Germ tissue penetration of 1.5 mm into grain conditioned at 22.4% moisture. Gradient represents the toughness of the tissue (flexure load [N] over the length of penetration).	230
Figure 6.10. Germ tissue penetration of 0.2 mm into grain conditioned at 22.4% moisture. Gradient represents the toughness of the tissue (flexure load [N] over the length of penetration).	231
Figure 6.11. Germ tissue penetration from 0.8 to ~ 1.2 mm into grain conditioned at 22.4% moisture. Gradient represents the toughness of the tissue (flexure load [N] over the length of penetration).	232
Figure 6.12. The peak flexure load (N) at the point of bran tissue penetration from 0 to ~1.5 mm into grain conditioned at 22.4% moisture.	233
Figure 6.13. Bran-endosperm tissue penetration from 0 up to ~1.5 mm into grain conditioned at 22.4% moisture. Gradient was taken from the line equation with the maximum r-squared, which represents the flexure load (N) over the length the penetration.	234

Figure 6.14. Bran-endosperm tissue penetration from 0 – 1 mm into grain conditioned at 22.4% moisture. Gradient from the line equation for all data points from 0 – 1 mm penetration, representing the flexure load (N) over the length the penetration.	235
Figure A.1. After 24 h in Milli Q water. Replicates A and B.	264
Figure A.2. After 24 h in 4 ppm ABA. Replicates A and B.	265
Figure A.3. After 24 h in 40 ppm ABA. Replicates A and B.	266
Figure A.4. After 48 h in Milli Q water. Replicates A and B.	267
Figure A.5. After 48 h in 4 ppm ABA. Replicates A and B.	268
Figure A.6. After 48 h in 40 ppm ABA. Replicates A and B.	269
Figure A.7. After 72 h in Milli Q water. Replicates A and B.	270
Figure A.8. After 72 h in 4 ppm ABA. Replicates A and B.	271
Figure A.9. After 72 h in 40 ppm ABA. Replicates A and B.	272
Figure A.10. SDS PAGE gel used to correct for protein quantification for iTRAQ analysis of germ. 20 µg and 10 µg of each sample Densitometry was used for final correction of sample concentration for iTRAQ analysis.	273
Figure A.11. SDS PAGE gel used to correct for protein quantification for iTRAQ analysis of bran. 10 µg and 5 µg of each sample Densitometry was used for final correction of sample concentration for iTRAQ analysis.	274
Figure A.12. SDS PAGE gel used to correct for protein quantification for iTRAQ analysis of ventral groove. 10 µg and 5 µg of each sample Densitometry was used for final correction of sample concentration for iTRAQ analysis.	275

Figure A.13. Sodium chloride standard curves for channels 1 to 6.....	276
Figure A.14. Sodium chloride standard curves for channels 7 to 12.....	277

List of tables

Table 2.1. Subgroups of globulin proteins separated on 2-DE gels.....	90
Table 2.2. Enzyme activities of water-soluble protein extracts from whole grain, pericarp, and flour. Values are presented as means \pm SE (n = 3) for OXO, POX, and PPO and means \pm half-range (n = 2) for the chitinases.....	93
Table 3.1. Average coleoptile and root length and ratio of coleoptile and root length of water-and water + 4 ppm ABA treated grain.....	113
Table 3.2. Number of protein level changes in the germ, bran and ventral groove tissues from water-conditioned grain compared to non-conditioned grain. Proteins were grouped according to protein class indicating the number of proteins that increased (blue) or decreased (orange) in levels.....	119
Table 3.3. Number of protein level changes in the germ, bran and ventral groove tissues from water + ABA-conditioned grain compared to non-conditioned grain. Proteins were grouped according to protein class indicating the number of proteins that increased (blue) or decreased (orange) in levels.	122
Table 3.4. Biotic and abiotic defense proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	124
Table 3.5. Membrane transport/transport proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	127

Table 3.6. Cell structure proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.....	128
Table 3.7. Protein synthesis and folding proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	130
Table 3.8. Transcription-related proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.....	133
Table 3.9. Glycolysis-and energy-related proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	136
Table 3.10. Regulatory-and signaling-related proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	138
Table 3.11. Metabolism-related proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.....	140
Table 3.12. Protease-related proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.....	143

Table 3.13. Endosperm-related proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	145
Table 3.14. Other function proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	148
Table 3.15. Unknown function proteins of the germ, bran and ventral groove from water-and water + ABA-conditioned grain compared with non-conditioned grain, showing the number of proteins up (blue) and down (orange) regulated.	151
Table 4.1. Protein level differences in the ventral groove compared with bran from the same non-conditioned grain. Positive values (blue) represent ~ fold more protein and negative values (orange) represent ~ fold less protein in the ventral groove (VG) ($p < 0.05$, Error Factor < 2.00 and Unused > 1.50).	168
Table 4.2. Summary of the number of proteins with higher (blue) or lower (orange) levels in the ventral groove from non-conditioned grain compared with bran from non-conditioned grain according to protein class.	170
Table 5.1. Molecular weights and locations of dehydrins identified in the iTRAQ experiment of germ, bran and ventral groove tissues compared to their corresponding western blots and western blot of the heat-stable protein extract. (+) Present in western blot, (+ +) present in both iTRAQ identification and western blot and (+ -) present in iTRAQ identification and not in the western blot.....	199
Table B.1. Protein level changes in germ from water-and water + 4 ppm ABA-conditioned grain grouped on protein function. Blue = ~ fold increase and orange = ~ fold decrease ($p < 0.05$, Error Factor < 2.00 and Unused > 1.50).	280

Table B.2. Protein level changes in bran from water-and water + 4 ppm ABA-conditioned grain grouped on protein function. Blue = ~ fold increase and orange = ~ fold decrease (p<0.05, Error Factor <2.00 and Unused >1.50).....	284
Table B.3 Protein level changes in the ventral groove manually dissected from water-and water + 4 ppm ABA-conditioned grain grouped on protein function. Blue = ~ fold increase and orange = ~ fold decrease (p<0.05, Error Factor <2.00 and Unused >1.50).	288
Table B.4 Ranked FQS ratios of the various cultivars.	295
Table B.5 Gradient of the straight line equation for the data points of flexure load (N) and 0 – 1.5 mm penetration into germ tissue of water-and water + 4 ppm ABA-conditioned grain (Instron Supplementary Figures, Disc 2).	296
Table B.6 Gradient of the straight line equation for the data points of flexure load (N) and 0 – 1.5 mm penetration into germ tissue of water-and water + 4 ppm ABA-conditioned grain (Instron Supplementary Figures, Disc 2).	296
Table B.7 Gradient of the straight line equation for the data points of flexure load (N) and 0.8 – 1.2 mm penetration into germ tissue of water-and water + 4 ppm ABA-conditioned grain (Instron Supplementary Figures, Disc 2).	297
Table B.8 Peak flexure load (N) of 0 – 1.2 mm penetration into bran/endosperm tissue of water-and water + 4 ppm ABA-conditioned grain (Instron Supplementary Figures, Disc 2).	297
Table B.9 Gradient of the straight line equation (with maximum r-squared) for the data points of flexure load (N) between 0 – 1.2 mm penetration into bran/endosperm tissue of water-and water + 4 ppm ABA-conditioned grain (Instron Supplementary Figures, Disc 2).	298

Table B.10 Gradient of the straight line equation for the data points of flexure load (N) and 0 – 1 mm penetration into bran/endosperm tissue of water-and water + 4 ppm ABA-conditioned grain (Instron Supplementary Figures, Disc 2).....	298
--	-----

Declaration

The work presented in this thesis was carried out between January 2007 and June 2011 on a full-time basis. This work represents original research, which has not been submitted for any other degree or to any other University or institution. All work was carried out by the author unless otherwise acknowledged.

Candidates Signature

Ante Jerkovic

Acknowledgements

I would like to thank Associate Professor Robert Willows for his guidance, help and support during my PhD. Thank you for this great opportunity.

Thank you to Ron Bradner, Alison Kriegel, Artur Sawiki, Andrew Scafaro and Phyllis Farmer with whom I share the office. A special thank you to Ron for your wealth of suggestions and for being so helpful in keeping me organised and on track.

I thank my parents for all their great support over the years and I am grateful to have such lovely parents. Not to forget the other really important people in my life, I thank my brother, sister and all my friends for their encouragement and support also.

I would also like to thank the following people who have helped me during this project: Debra Birch and Nicole Vella from microscopy, for their help and suggestions in getting wonderful images for my publication and thesis. Thiri Winn, Alamgir Khan, Brett Cooke, Dana Pascovici, Vidya Nelaturi and Xiaomin Song from APAF who have been so friendly and helpful in the proteomics work. My co-supervisor Associate Professor Brian Atwell for his guidance and suggestions throughout this project.

Finally, I thank the GrainFoods CRC for the scholarship and financial support for this project. I have thoroughly enjoyed the experience being part of the GrainFoods CRC.

Chapter 2: This Chapter was published in Plant Physiology in March 2010 and a copy of this publication is attached in Appendix C. The bran tissue collection and 2-DE gels and protein identification was completed by me as part of my Masters thesis. Antibody purification and immunomicroscopy was performed by Alison Kriegel and John Bradner.

Brian Atwell, Thomas Roberts and Robert Willows contributed to writing and editing. All other work in this Chapter and in the publication, which includes collating and interpreting all the information, drafting the manuscript, image analysis and enzyme assay experiments was part of my PhD.

Chapter 7: The MicroMill design and construction – to fulfill the required task for grain fractionation in Chapter 7 – was done by Alan Donald Howard.

Publication and conference posters

Publication

Jerkovic A, Kriegel AM, Bradner JR, Atwell BJ, Roberts TH, Willows RD (2010)

Strategic distribution of protective proteins within bran layers of wheat protects the nutrient-rich endosperm. *Plant Physiology* 152: 1459-1470

Conference posters

Jerkovic A and Willows RD (2007) **Proteomics of wheat bran.** COMBIO, Sydney Convention and Exhibition Centre, Darling Harbour, Sydney, Australia, 22-26 September 2007.

Jerkovic A and Willows RD (2010) **iTRAQ analysis of abscisic acid treated wheat seeds.** Gordon Research Conference (Salt and Water Stress in Plants), Les Diablerets Conference Center, Les Diablerets, Switzerland, 13-18 June 2010.

Summary

Absciscic acid (ABA) is a well known plant hormone that is involved in many biotic and abiotic stress responses. Application of ABA in the milling of wheat has been shown to improve flour yield and quality. This suggests that there may be biochemical processes which impart a physiological change that is favorable to improving milling performance. In this study, I have attempted to better understand the biochemical and physiological changes of water + ABA-conditioned grain. The strategy involved four stages: (1) apply proteomic analysis to measure and compare the differential expression of proteins in the germ, bran (aleurone layer) and ventral groove of non-conditioned grain (control) and grain conditioned with water-and water + ABA, (2) localize proteins of interest that were identified in the proteomic analysis using immunolabeling and confocal microscopy, (3) test for flour yield and quality by analysing variations in grain fractionation using a laboratory scale mill (MicroMill) to generate fractionation quality scores (FQS's) (4) measure physiological changes in water relations by determining grain water potential (ψ) and mechanical properties of the germ and bran/endosperm of following treatments.

The proteomic analysis of the water-conditioned grain showed that there were many changes in protein levels in the bran and ventral groove tissues, however, there were almost no changes in protein levels in the germ tissue. In the tissues of the ABA-conditioned grain, there were many differentially expressed proteins in the germ, bran and ventral groove tissues, especially those involved in biotic and abiotic stress response. Of these proteins, two are involved in water relations; late embryogenesis abundant proteins (LEA's) and tonoplast intrinsic protein's (TIP's). The ventral groove showed little variation in protein levels between water-and water + ABA-conditioned grain; however, both exhibited a ~ 5-fold increase in LEA's compared to non-conditioned grain. LEA protein abundance also increased in the germ and aleurone cells after ABA treatment but

only by ~ 1.5-fold. Confocal microscopy of immunolabeled grain cross-sections, revealed that group 2 LEA (dehydrin) proteins are distributed throughout the intracellular matrix in a ‘honeycomb-like’ arrangement and also surrounding the nucleus and inner cell walls within the germ cells, aleurone cells in the bran layer and in the aleurone cells surrounding the ventral groove. TIP levels decreased by ~ 2-fold exclusively in the germ, which is likely to reduce water movement in and out of the tonoplast. An increase in FQS’s of ABA-conditioned grain compared with water-conditioned grain may indicate improved fractionation, leading to improved flour quality and yield. Psychrometric measurements of the germ-end and bran/endosperm-end of ABA-conditioned grain showed a slightly elevated ψ when compared to water-only treatment after drying at room temperature for 1.5 h. This suggested that the grain somehow retains more moisture if treated with ABA. Finally, mechanical property analysis of the germ and bran tissue showed that the germ was softer and bran was tougher after conditioning with ABA.

Collectively, these results suggest that ABA may induce changes in biochemical processes of the germ and aleurone cells of the bran and ventral groove tissues, such as increased levels of LEAs and a reduction in TIPs in order to prevent moisture loss in response to an environmental stress such as drought. Consequently it is suggested that this altered the water distribution within the grain, thus transforming its physiological properties, making it better adapted to surviving a desiccating environment. From an applied science perspective, this physiological change allows the grain to be more amenable to milling, thus improving flour yield and quality.

Abbreviations

ABA	Absciscic acid
CID	Collision induced dissociation
1-DE	One-dimensional electrophoresis
2-DE	Two-dimensional electrophoresis
DIC	Differential interference contrast
DPA	Days post anthesis
EST	Expressed sequence tag
FBS	Fetal bovine serum
FQS	Fractionation quality score
GA	Gibberellic acid
TheGPM	The Global Proteome Machine
iTRAQ	Isobaric tags for relative and absolute quantification
LC	Liquid chromatography
LEA	Late embryogenesis abundant
MALDI	Matrix assisted laser desorption ionisation
FQS	Fractionation quality score
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
TOF	Time of flight