



**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:

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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).

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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

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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.

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Publication and conference posters

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Conference posters

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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

Chapter 1 : Introduction

In the first part of the introduction, I will give a general overview of wheat, growing wheat in Australia, anatomy of the kernel, developmental stages and germination. Following this, I will discuss the milling process, products that are made from the different milling fractions, the conditioning process and how it can be potentially improved. The next part will cover proteins in wheat with respect to the different tissues, abscisic acid (ABA) and environmental stress proteins and current methods used in proteomic analysis. Finally, I will introduce the aim of my thesis and how I will address this.

1.1 Wheat

Wheat is a major cereal crop in Australia and on all other continents of the world (except for Antarctica). In 2009, Australia was ranked ninth in the world of all wheat producing countries (Figure 1.1) (<http://www.fao.org/es/ess/top/commodity.html?lang=en>). The grain from wheat is the main nutritional component, and is used in a wide variety of food products such as breads, cakes, pasta, breakfast cereals and animal feed (Aalami et al., 2007; Laubin et al., 2008; Shewry, 2009). There are two main types of wheat; bread wheat (soft wheat) and durum wheat (hard wheat). Bread wheat is mainly used in breads, cakes and biscuits. The latter is primarily used in making products such as pasta and noodles and some specialty breads. Bread wheat, *Triticum aestivum*, is a hexaploid and durum or pasta wheat, *Triticum turgidum*, is a tetraploid. These wheat species were developed over 8,000 years ago from ancestral diploid species (*Triticum* and *Aegilops*) that originated from the subfamily of grasses called Pooidae (Huang et al., 2002).

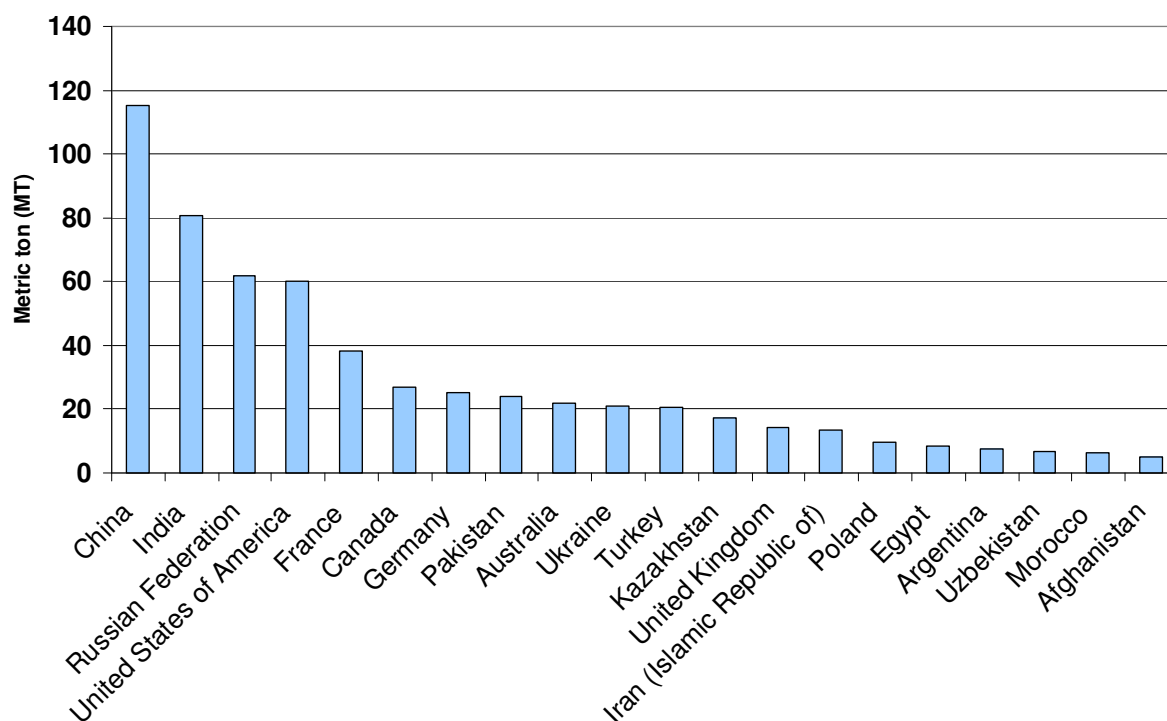


Figure 1.1. Production of wheat in metric tonne (MT) in 2009 from the top 20 major wheat producing countries. Data obtained from the Food and Agriculture Organization of the United Nations (<http://faostat.fao.org/site/339/default.aspx>).

1.2 Growing and harvesting wheat

Australian wheat is grown in an area stretching from regions of southeastern Queensland to Victoria and across to South Australia and additionally in the southern parts of Western Australia. These growing regions combined are commonly known as the Australian wheat belt (Figure 1.2). Terminals for transporting wheat are distributed along the coast in proximity to where it is grown (Figure 1.2).

Wheat sowing usually takes place prior to a high rainfall period and is harvested in a drier period. In the southern regions of Australia, wheat is sown in the austral autumn (March, April and May) – typically in April – so that it grows through the winter period and is harvested in spring (September, October and November). In the northern regions of Australia (Northern New South Wales and Queensland), wheat is sown in winter (June, July and August) – typically in July – and is harvested in late spring or early summer (January, February and March) (http://www.dpi.qld.gov.au/26_3808.htm).

When sowing wheat, the seeds are placed approximately 7 cm below the soil and 18 cm apart. Sprouting occurs around five to seven days after sowing and can take up to five to seven months to reach maturity. Once reaching maturity, the grain is ready for harvesting. During the growing period, a crop will require ~ 300 mm of rain for optimal growth (<http://www.rochedalss.eq.edu.au/wheat.htm>). In the event of drought, the crop will be depleted through loss of individual grain mass (endosperm volume) and therefore loss in value in terms of flour yield (Dupont and Altenbach, 2003; Toole et al., 2007).

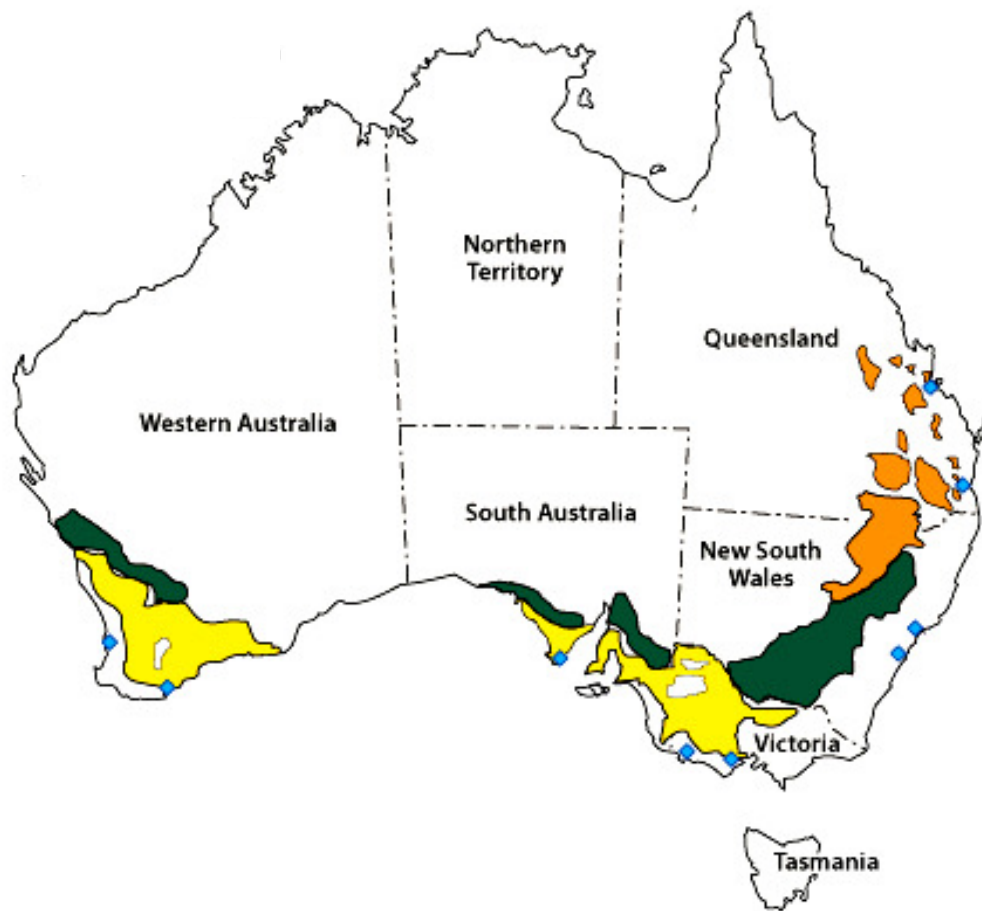


Figure 1.2. Map of Australian wheat belt showing premium white wheat areas (Yellow), premium white and hard wheat areas (Green), predominantly hard wheat areas (Orange) and wheat terminals (Blue). Modified from (http://www.abare.gov.au/interactive/09acr_sept/).

Wheat is generally harvested at grain maturation (or when the moisture level is at around 10%). It can also be harvested at a higher moisture level, up to around 20% if the grower wishes to avoid predicted rainfall. These grains can then be dried down with industrial scale driers. Blending wheat is also commonly used to control the moisture content. If the wheat on one end of a field is harvested in the morning and its moisture level is at 10% and the wheat on the other end of the field is harvested the following day with a moisture level at 13%, they can then be blended to equilibrate the moisture content. Rain can have detrimental effects on mature wheat crops, especially with repeated wetting and drying. Minimising exposure to moisture helps to prevent premature germination. If the grain absorbs moisture it can initiate the early processes of germination and as a result will affect starch/flour quality. This involves the secretion of hydrolases such as α -amylase from the aleurone cells, which degrades the starchy endosperm (Moss et al., 1972; Cejudo et al., 1995; Ritchie et al., 2000) and causes a loss in dough elasticity and downgrades the quality of the flour (Every et al., 2002). Once the crop is harvested, the straw and husk are separated (mostly by the harvesting machinery) and the grain is collected and stored on-site in Silo Bags, or shipped off to be stored in large silos (http://www.silobag.com.au/home/index.php?option=com_frontpage&Itemid=1).

1.3 Wheat based products

The average annual amount of wheat used for domestic flour production in Australia is approximately 10% of the total harvest (two million tons per annum). Of the remainder, approximately 15% is used for live stock feed, 50% is exported, 20% is held as stocks and 3% is used for seed in crop sowing

(http://www.grdc.com.au/director/research/Biosecurity.cfm?item_id=516586B9F31625C9704AFACBCA7454EB&pageNumber=1&filter1=&filter2=&filter3=&filter4=). However,

the amounts vary greatly from year to year depending on the quality of wheat (such as drought affected or rain damaged wheat).

Soft wheat generally contains less protein than durum and other hard wheat and is mainly used for making bread, pastry, cakes, and biscuits (Figure 1.3). This is due to baking properties of the lower dough elasticity of soft wheat, which results in smaller air pockets that form during baking and a crumbly texture (Belderok, 2000; Shewry, 2009).

Wheat that is of medium hardness and protein content is milled to produce domestic flour. This flour is used to make a wide variety of products such as breads, noodles and many other general baking products (Belderok, 2000; Aalami et al., 2007; Shewry, 2009).

Hard wheat such as the Durum variety is high in protein. Durum produces large endosperm particles (semolina) during milling, which is used in a wide variety of pasta products. Durum flour is also collected and is used to make specialty breads such as Turkish bread and pizza base. These have a different texture (chewy) and taste richer, compared to bread made from softer, lower protein wheat (more crumbly) (Figure 1.3).

Wheat bran is a by-product of flour milling, and is used in animal feed, biscuits, wholemeal breads and breakfast cereals. It is a good source of fiber for humans because the stomach acids do not break down the cellulose, hemicelluloses (arabinoxylans) and lignin tissue of the bran cell wall material (Beaugrand et al., 2004; Sorensen et al., 2010).

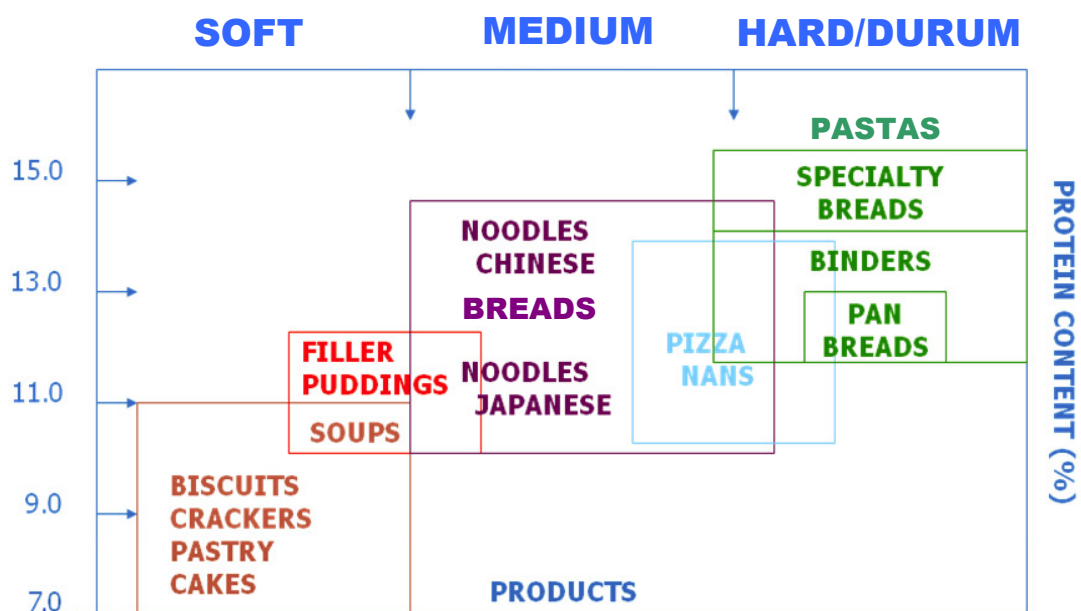


Figure 1.3. Relationship between wheat hardness, protein content and its use in making products. Figure modified from <http://www.regional.org.au/au/roc/1988/roc198815.htm>.

1.4 Anatomy of the wheat kernel

The wheat kernel consists of four major components; the embryo (germ), endosperm, bran and ventral groove (Figure 1.4). The embryo is the part of the kernel, which will develop into a new plant. It is comprised of scutellum, rudimentary stem and leaves and rudimentary primary root (Belderok, 2000). This tissue initiates many of the biochemical changes that occur in the grain at the early stage of germination (Ho et al., 2003), which occurs through signaling by phytohormones such as gibberellin (GA) and abscisic acid (ABA). GA is involved in activating the aleurone cells to produce the required protein machinery to begin the breakdown of starch and the transport of these nutrients to the developing embryo (Ho et al., 2003). ABA however, is antagonistic to GA and is involved in extending grain quiescence length and delaying germination (Belderok, 2000; Razem et al., 2006; Takasaki et al., 2008).

The endosperm (Figure 1.4 B) being the main mass component of the grain is mostly composed of starch, gluten and various proteins and enzymes (Skylas et al., 2000). It is the part of the grain that is extracted in milling in the form of flour and semolina (large endosperm particles).

Bran is the external protective barrier, which protects the nutrient-rich endosperm and germ. This tissue completely envelops the kernel and consists of several tissue layers of dead and compact cells. Within these dead tissues (Figure 1.5), there are various protective proteins that defend against biotic attack such as xylanase inhibitor proteins, α -amylase inhibitor proteins, non-specific lipid transfer proteins and chitinases (Jerkovic et al., 2010). The aleurone cell layer, forming part of the bran fraction and ventral groove is the only layer that is made up of live cells (Figures 1.5 and 1.6). This cell layer is functional and active at germination (Ho et al., 2003).

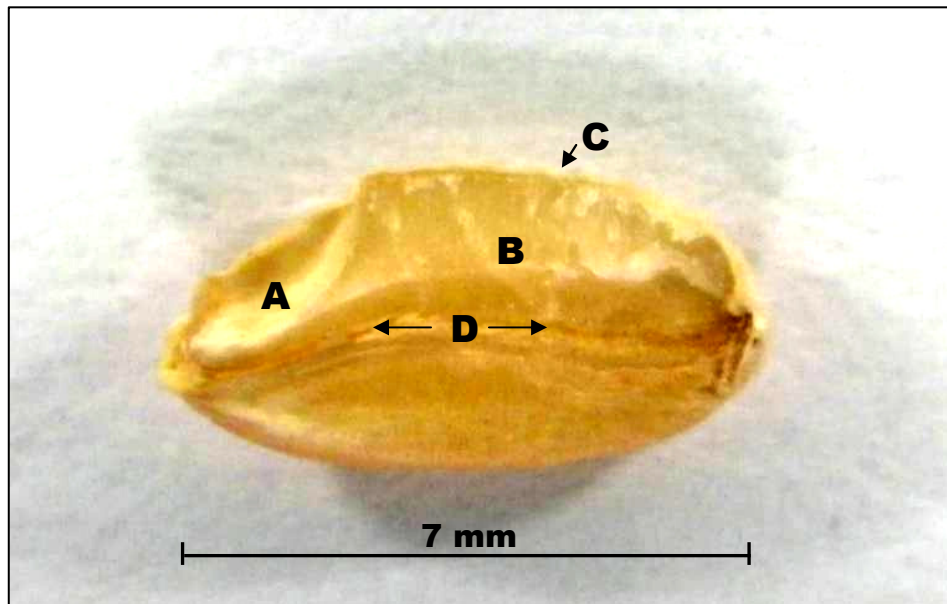


Figure 1.4. Longitudinal dissection of the wheat grain showing the four main components: (A) germ, (B) endosperm, (C) bran and (D) ventral groove.

The ventral groove (also referred to as the crease) runs longitudinally from the germ end to the opposite end of the grain (Figures 1.4 and 1.6). During grain development, the ventral groove may play an important role in delivering nutrients to the endosperm, however mechanisms for the movement of the nutrients are not understood (Frazier and Appalanaidu, 1965).

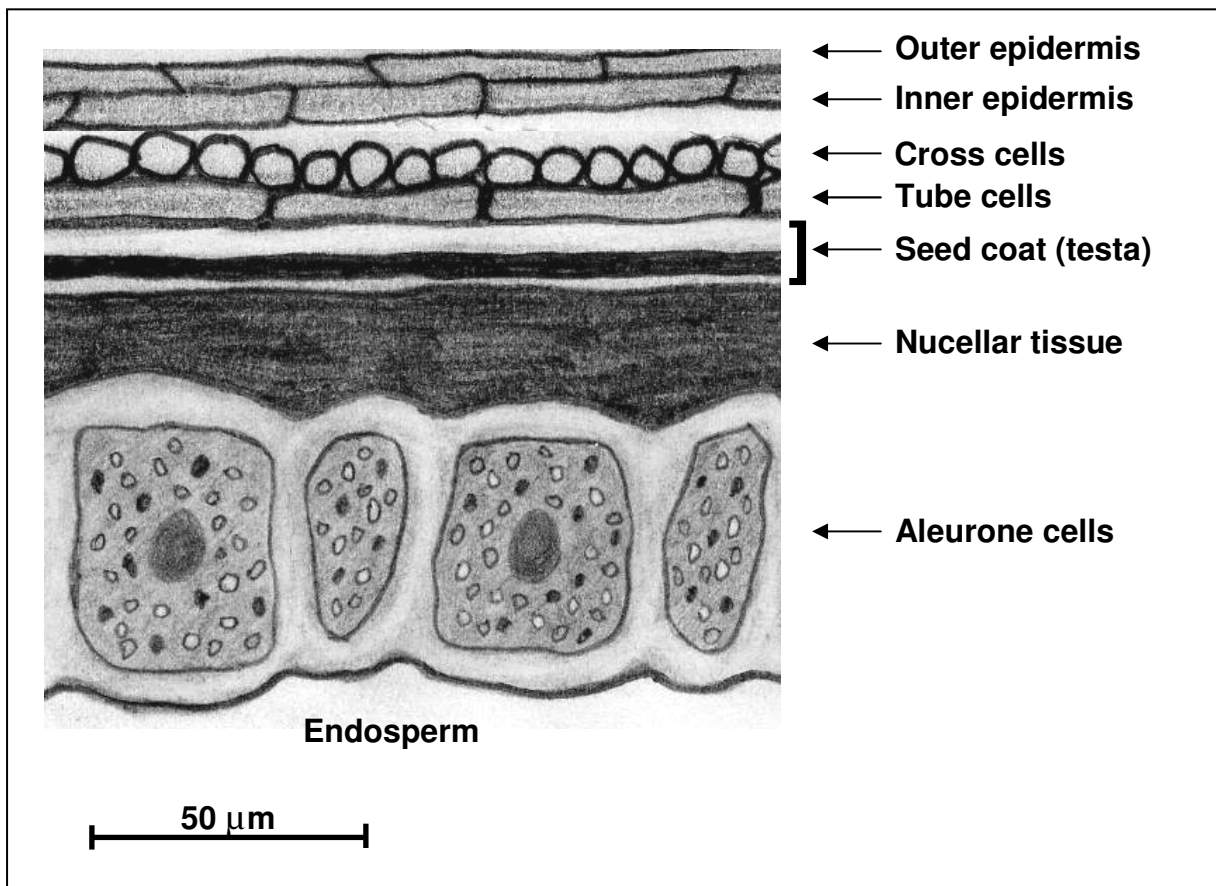


Figure 1.5. Schematic diagram of a cross section of wheat bran showing the different tissue layers.

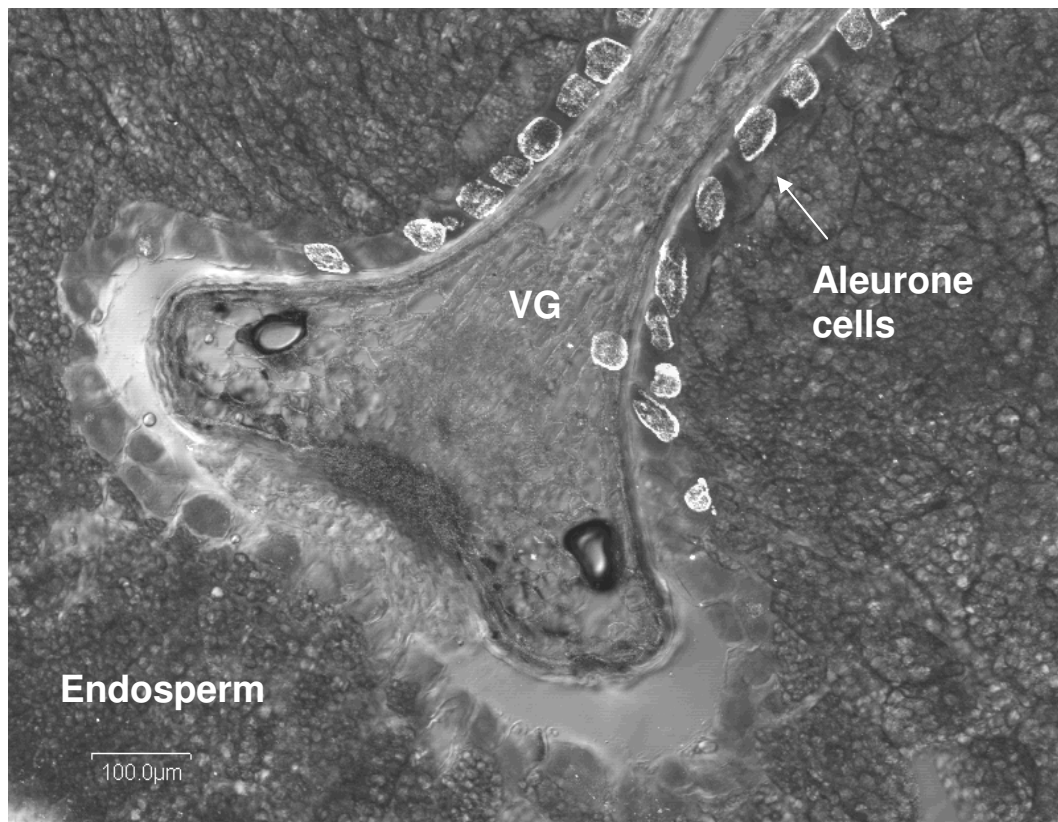


Figure 1.6. Micrograph of a cross-section of grain showing aleurone cells around the ventral groove (VG) and surrounding endosperm.

1.5 Grain developmental stages

Anthesis is the flowering stage of wheat where the ovary becomes fertilized and is the beginning of grain development (Percival, 1921). The embryo and endosperm develop separately in six distinct stages (Noda et al., 1994).

1.5.1 Embryo

The mitotic embryo enters a globular stage from 0 to 5 days post anthesis (DPA) that is characterized by cell division. From 5 to 10 DPA, the scutellum and embryo axis become differentiated and go through their growing phase. The scutellum develops from 10 to 15 DPA, followed by the formation of the embryonic axis from 15 to 20 DPA. From 20 to 30 DPA, the dry weight of the embryo increases and then approaches a desiccation stage from 30 to 50 DPA (Noda et al., 1994).

1.5.2 Endosperm

The endosperm is at the developmental and pre-milk stage from 0 to 10 DPA. At this time, the grain appears milky white, however, the contents of the grain is a clear liquid. Then from 10 to 20 DPA, the endosperm is at the milk stage. The outer surface of the grain appears green (caused by chlorophyll accumulation) and the grain content is a milky white liquid. From 20 to 30 DPA, the endosperm transforms into the soft dough stage, followed by a hard dough stage 30 to 35 DPA. At this stage the grain has begun its desiccation process and has reached its physical maturity. The grain is then in a ripe stage from 35 to 40 DPA and becomes completely mature at 50 DPA. The colour of the grain at maturity is

commonly a golden brown in Australian cultivars but this will vary depending on the variety (Noda et al., 1994).

1.6 Germination

Mature grain is quiescent and can stay that way for a long period of time, provided that they are kept dry. Given the right conditions for germination, the germ and aleurone cells become active and commence the process of developing into a plant (King, 1984). There are many phytohormones involved in the regulation of germination of which auxins (promote stem elongation), abscisic acid (fruit abscission and inhibitor of germination), gibberellins (promoter of germination is just one of the many functions of gibberellins), cytokinins (cell division) and ethylene (fruit ripening, shoot and root growth and differentiation and a stimulator in releasing dormancy) are important (Galston and Davies, 1969; Finkelstein et al., 2002; del Pozo et al., 2005; Kucera et al., 2005). The level of some of these hormones at grain maturity will vary depending on the cultivar and also the environmental conditions in which the plant and grain were grown (Wiedenhoeft et al., 1988). An over-production of a particular phytohormone due to an environmental stress, such as drought, may alter the timing of germination. For example, drought affected wheat will generally have higher levels of endogenous ABA (Morgan and King, 1984). Higher endogenous ABA levels can cause the germination process to halt or to slow down (Galston and Davies, 1969). Australian wheat is vulnerable to pre-harvest sprouting caused by rain. Studies of water uptake in wheat ears and grain show that 18% of variation in pre-harvest sprouting can be attributed to varietal differences (King, 1984). In Europe and North America, where they have a wetter climate, the wheat cultivars grown are more resistant to pre-harvest sprouting. This may be because of higher endogenous levels of

ABA, and/or the intrinsic physico-chemical properties of ear and grain water uptake (King, 1984; Morgan and King, 1984).

1.7 Milling

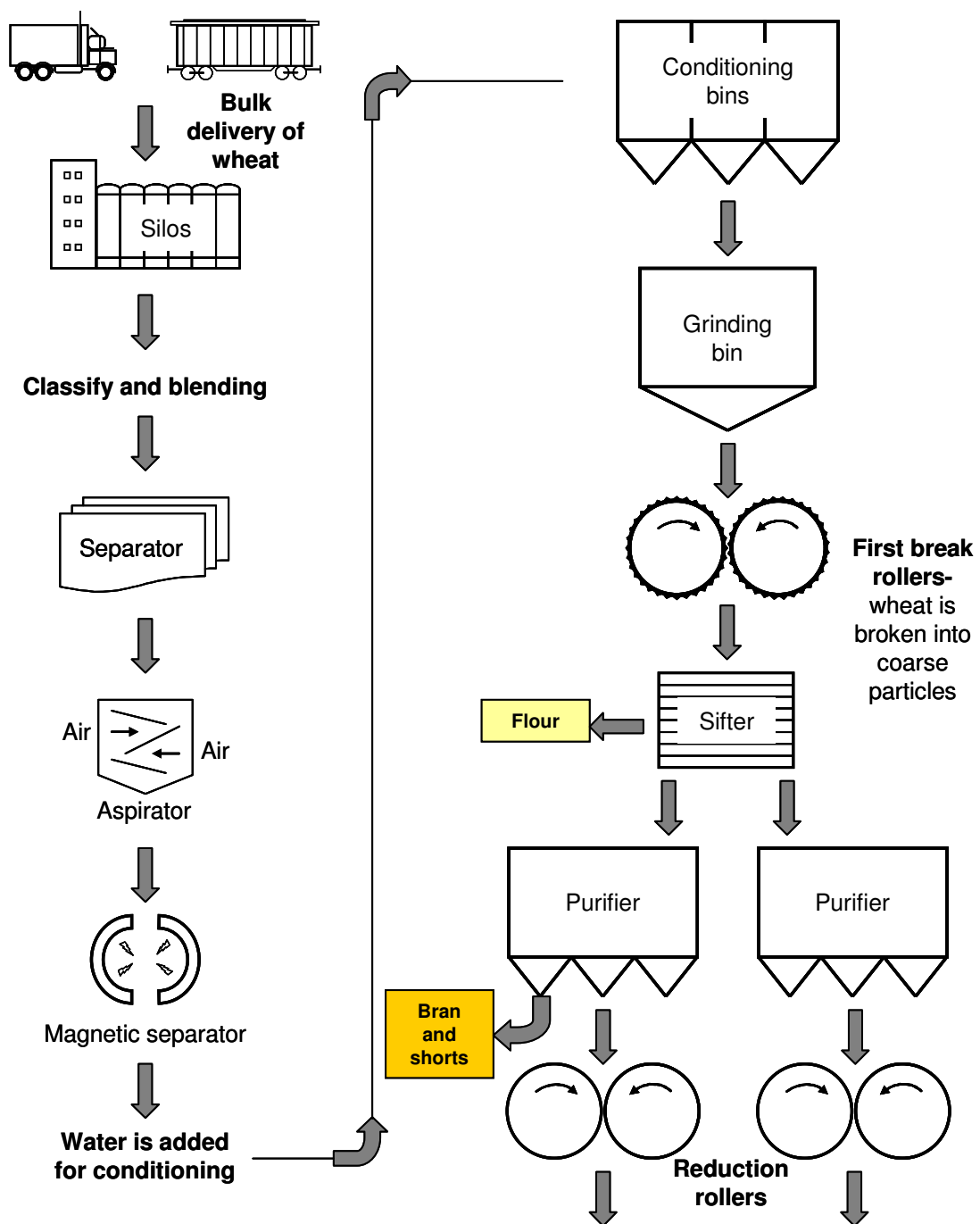
Improving the milling performance of wheat grain effectively requires separation of bran from endosperm with minimal bran contamination. Also, extracting and utilizing the potential nutritional benefits of the aleurone layer adds value to flour (Harris et al., 2005). In order to achieve this, it will require a better understanding of the biochemical processes that occur during the conditioning stage prior to milling.

In commercial flour milling, wheat grains are crushed and sheared through a series of narrow rollers and sieved through various sized sieves in order to separate the bran from the endosperm (Figure 1.7). This process is difficult because the aleurone layer (part of the bran fraction) is tightly bound to the endosperm at the aleurone/endosperm interface. Additionally, the shape of the grain, particularly the ventral groove, contributes to the difficulty in extracting endosperm. For example, if the grain were spherical, the bran could be removed by pearling. This is a method employed to remove the thin external layers of kernels by abrasion, commonly applied with rice (Mousia et al., 2004). Also, the ventral groove has a complex structure that is embedded within the endosperm (Figure 1.6), making it difficult to separate from the endosperm. Thus, the shearing and crushing forces required to separate the bran from the endosperm, causes the bran and ventral groove component of the grain to fracture into tiny fragments, contaminating the flour, and resulting in less pure product.

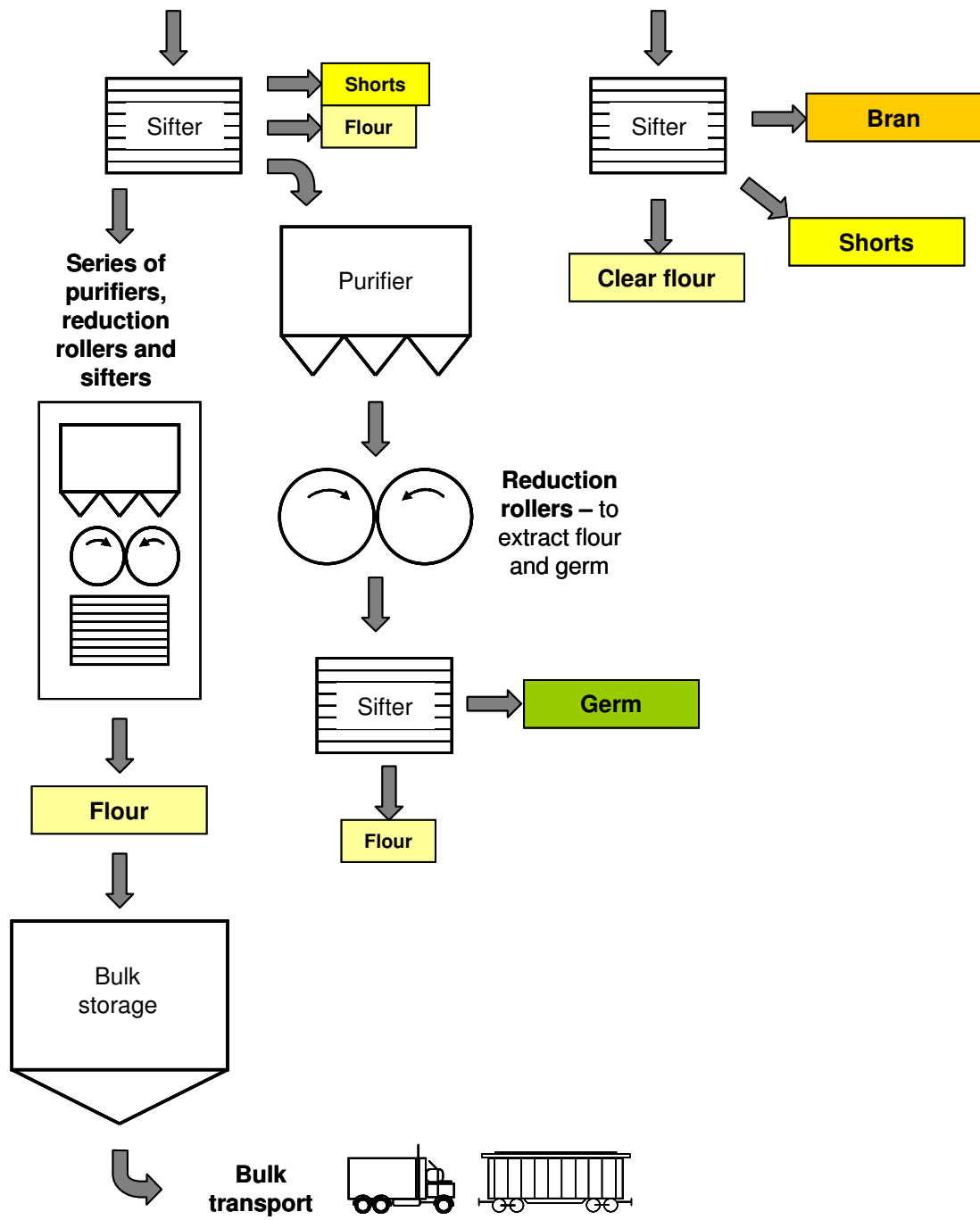
Figure 1.7. General outline of the milling process from raw material to product. The first stages are primarily classifying, blending and cleaning the wheat from debris and unwanted plant material. The next stage is conditioning (adding water and letting it rest for a period of hours). This is followed 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:

<http://www.wheatflourbook.org/Main.aspx?p=66>.

(see figure on following page)



(see legend on previous page)



1.8 Conditioning wheat

Conditioning (also referred to as tempering) wheat prior to milling is common practice in the milling industry. In this process, water is added to wheat to increase its moisture level from ~ 10% up to ~ 14.5% for soft wheat and ~ 16.5% for hard wheat. After the water has been added to adjust the moisture level, the wheat is rested/incubated for ~ 3 – 12 h for soft wheat and ~ 8- 24 h for hard wheat at room temperature (AACC, 2003). This process has been demonstrated to increase flour extraction and reduce bran contamination of the flour when compared with milling non-conditioned wheat (Kweon et al., 2009). It is suggested that the increase in moisture level toughens the bran by making it more elastic and softens the endosperm (Kweon et al., 2009). The early stages of germination are initiated as a result of raising the grain moisture level and will cause expression and secretion of α -amylase from the aleurone cells, which hydrolyse starch at the aleurone/endosperm interface, thus weakening the bond between the aleurone layer and endosperm. Therefore, conditioning for periods greater than the recommended resting time, may also begin to cause starch damage due to excessive starch hydrolysis (as occurs in water damaged wheat) and consequently the flour will lose its dough strength and baking properties (Every et al., 2002; Kweon et al., 2009).

1.9 Test milling

Test milling is used in industry to determine the quality and milling characteristics of wheat. It can be applied to assess physiological changes resulting from seasonal variation, water damage or to select for good milling and quality cultivars in selective breeding programs. Test mills range from industrial size down to experimental laboratory size mill such a Bühler mill (Bühler-Miag, Minneapolis, MN, USA) or an experimental laboratory

mill (Ross, Oklahoma City, OK). These smaller mills can process around half to several kilograms of wheat. However, when using the smaller type experimental laboratory mills, data obtain when measuring small improvements may not be detected due to the variable nature (such as size, shape and density) of wheat. Measuring these small changes in milling performance is critical in order to be able to assess quality, optimise conditioning treatments and to select for good wheat breeds. Although these test mills are valuable in estimating how wheat may perform in an industrial sized mill, they do require many replicates and large amounts of wheat in order to measure significant changes in milling properties.

1.10 Wheat defense proteins

The grain is rich in nutrients and thus is susceptible to insect and pathogen attack. Insects and plant pathogens such as fungi and bacteria use hydrolytic enzymes to break down plant material for food (Feng et al., 1996; Yamasaki et al., 2008). Proteomic analysis of wheat bran has shown that there are many defense proteins within the bran layers, such as xylanase inhibitors, thaumatin like proteins (TL), chitinases, α -amylase/subtilisin inhibitors and many classes of pathogenesis-related proteins, that protect it from biotic attack (Jerkovic et al., 2010). Together with the physically tough dead outer bran layers, these defense proteins make a formidable barrier to plant pathogens. These properties may also create difficulties when trying to use hydrolytic enzymes for wheat bioprocessing.

In past decades, adding enzymes during flour milling was performed for the purpose of improving flour yield and quality. The addition of the hydrolytic enzyme xylanase (during or after milling) has been shown to improve flour quality and baking properties, resulting in increased dough strength, bread volume and also to improve these properties in poor quality flour (Debyser et al., 1999; Haros et al., 2002); however, increases in flour yield have been unsuccessful. Novo Nordisk A/S, an enzyme manufacturing company, have developed a cocktail of plant cell wall degrading enzymes for addition to wheat in the conditioning stage prior to milling for the purpose of improving bran separation from the endosperm and increase flour yield. However, this also showed no improvement and the patent application was discontinued (Martinez et al., 1998).

As mentioned above, the bran proteomics studies (Jerkovic et al., 2010) revealed an array of inhibitory proteins within the dead outer bran layers that form a barrier, which may suggest that general enzyme treatment (especially by those derived from fungi) to hydrolyse bran cell walls may be inhibited (Payan et al., 2004). Research has shown that

xylanases derived from fungi are inhibited by xylanase inhibitor protein-I (XIP-I) but do not inhibit xylanases derived from bacteria (Flatman et al., 2002; Payan et al., 2004). Other work on the penetration of xylanase into bran tissue, showed that the intermediate tissue fractions (nucellar, testa, cross cells and tube cells) act as a barrier, thus preventing xylanase penetration into the adjacent aleurone tissue (Beaugrand et al., 2005). The removal of bran from endosperm and/or weakening the bran/endosperm bond, to improve flour extraction and quality in milling, will require enzymes that are not inhibited by these inhibitors and that are able to degrade specific bran tissue layers.

One tissue of interest, with regards to specific tissue hydrolysis using enzymes to separate bran from the endosperm, is the nucellar tissue. The nucellar tissue is located adjacent to the aleurone layer (Figure 1.9), and would be the ideal point to separate the rest of the bran layers from the endosperm in milling. This separation point will also introduce more protein and nutrients from the aleurone cells into the flour. However, the bran proteomic study revealed that the nucellar is highly saturated with inhibitory enzymes, especially the xylanase inhibitor proteins, which provides a challenge to researchers (Jerkovic et al., 2010). The concentration of these xylanase inhibitors within this tissue makes evolutionary sense, since the hemicellulose composition of this tissue is mostly xylose with a small amount of arabinose substitution, thus making it highly susceptible to enzyme hydrolysis by xylanase. In contrast, the arabinose and xylose composition in the outer dead tissue layers (from the seed coat and outwards) are almost at a ratio of 1:1 (Figure 1.8) (Parker et al., 2005), which makes it highly resistant to enzyme hydrolysis (Parker et al., 2005). Chitinases are another class of defense proteins located within the bran layers, especially the intermediate (nucellar, testa, cross cells and tube cells) and aleurone layers (Jerkovic et al., 2010). Finally, α -amylase/subtilisin inhibitors are located in the intermediate and aleurone tissues to protect the endosperm from biotic attack from fungi, bacteria and

insects that secrete amylases and proteases (Jerkovic et al., 2010). Collectively, these defense proteins, which are principally located within the intermediate and aleurone tissues, form a strong biochemical defense barrier against pathogens.

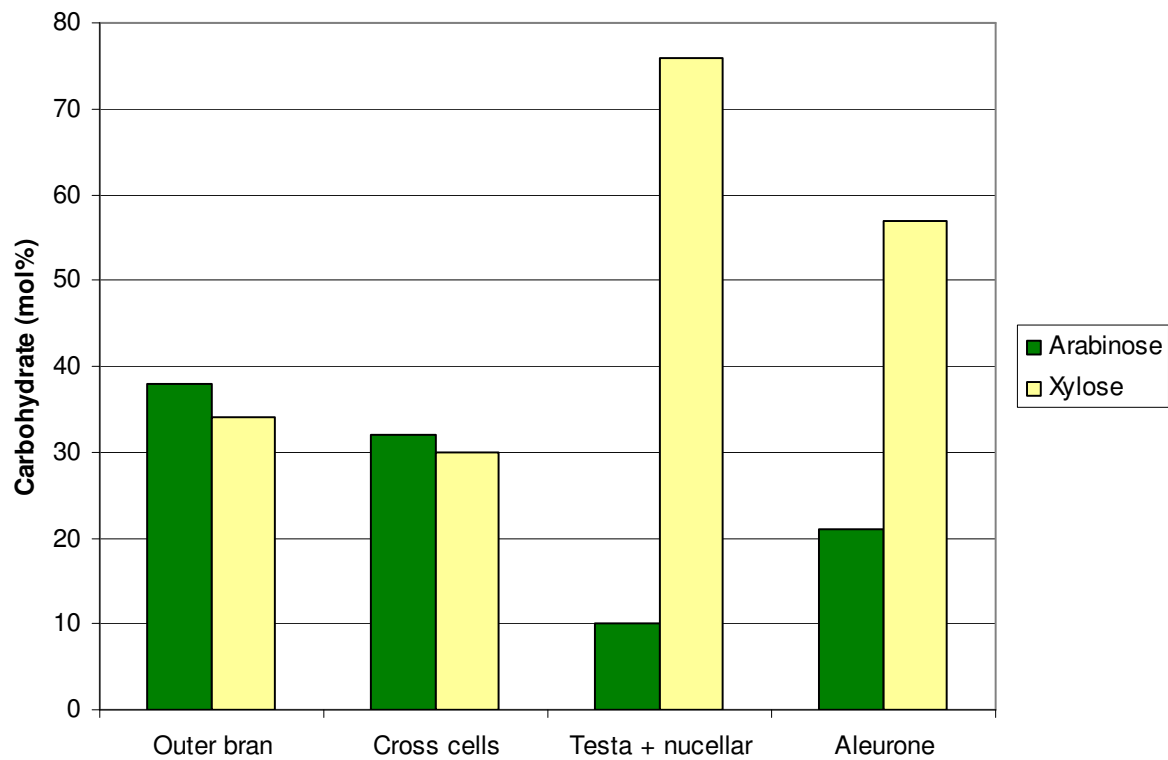


Figure 1.8. Carbohydrate composition of bran tissue layers showing relative levels of arabinose and xylose. The testa, nucellar and aleurone tissue comprise mostly of xylose. Modified from Parker et al. (2005).

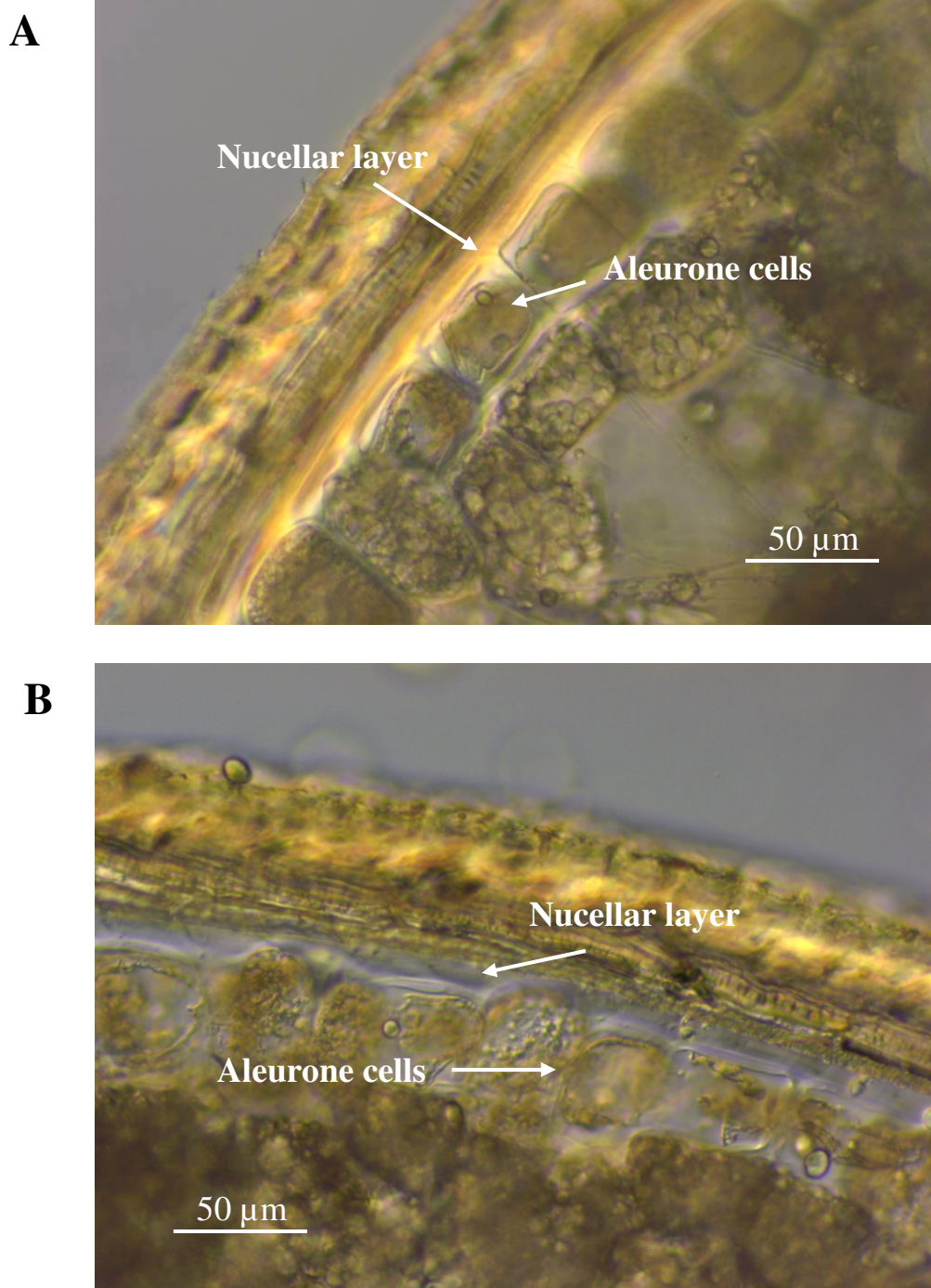


Figure 1.9. Photomicrograph of a cross-section of grain indicating the location of (A) nucellar tissue and aleurone cell walls and (B) nucellar tissue and aleurone cell walls that were hydrolysed by xylanase.

1.11 Environmental stress response proteins

The plant hormone abscisic acid (ABA) has many regulatory functions. Some of these functions are regulating protein expression, dormancy and biotic and abiotic stress response (Finkelstein et al., 2002; Zhang et al., 2006; Ohnishi et al., 2008). In particular, the level of ABA increases after an environmental stress on the plant, such as drought, freezing and salinity and signals many biochemical changes to allow it to adapt to these conditions (Kendall et al., 1993; Borovskii et al., 2002a; Borovskii et al., 2002b; Allagulova et al., 2003). As a result, there can be drastic changes to the grain phenotype. These changes are mainly due to the levels of protein expression, which may be vital for the plant to survive in adverse environments (Scarath, 1941; Bray, 1993). Proteins such as late embryogenesis abundant (LEA) proteins, LEA-2 (dehydrin) proteins, and anti-freeze proteins (AFP) are some of the common proteins found in plants that are involved in protection against environmental stresses. These proteins are up-regulated or expressed after exposure to such stressful conditions (Dure et al., 1989; Close, 1996; Black et al., 1999; Borovskii et al., 2002b; Allagulova et al., 2003; Herzer et al., 2003; Lopez et al., 2003; Kerepesi et al., 2004; Garnczarska et al., 2008). They all have strong hydrophilic and cryoprotective properties, which allow the plant to resist desiccation and freezing conditions respectively (Kendall et al., 1993; Close, 1996; Garnczarska et al., 2008).

Dehydrins are especially important in these environmental stress responses. The common features of dehydrins are their extreme hydrophilic properties, hydrophobic interactions (Ceccardi et al., 1994) and all contain a highly conserved region of 15 amino acids that forms an amphiphilic α -helix called the K-segment (Figure 1.10) (Allagulova et al., 2003; Rorat, 2006). They have the ability to bind to hydrophobic sites of proteins and lipid membranes and also prevent the growth of ice crystals, which reduces the freezing point and imparts cryoprotective properties (Kendall et al., 1993; Donoghue and Walker-

Simmons, 1999; Borovskii et al., 2002a; Borovskii et al., 2002b; Allagulova et al., 2003; Zhang et al., 2007).

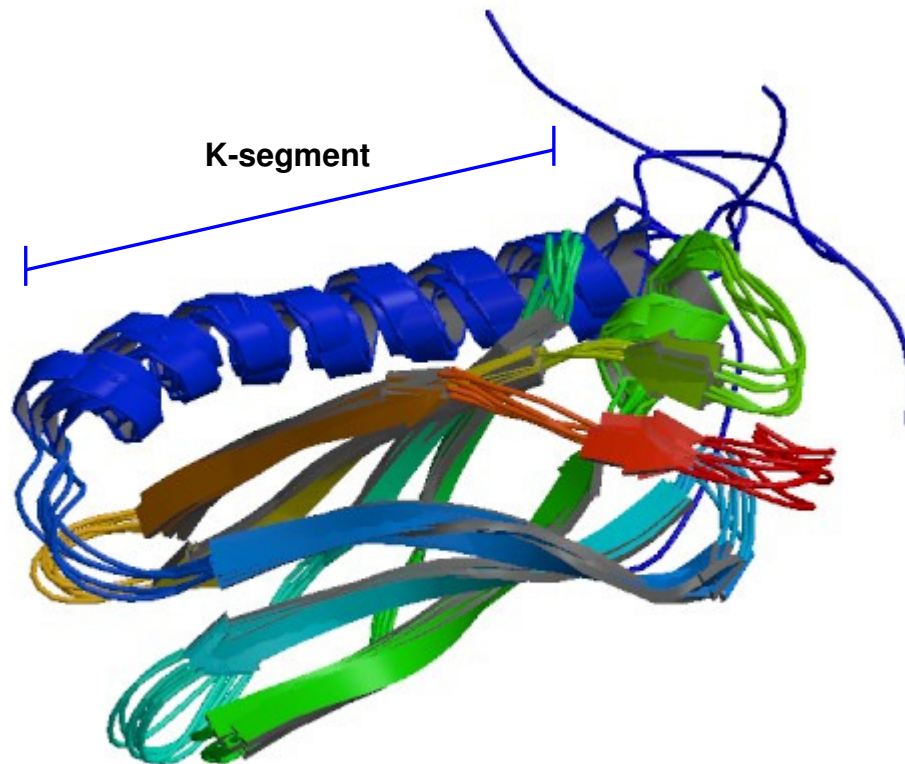


Figure 1.10. Solution structure of a putative late embryogenesis abundant (LEA) protein At2g46140.1. Image taken from RCSB Protein Data Bank. Deposited by Song J, Tyler RC, Lee MS, Markley JL, 2005/2/24 Center For Eukaryotic Structural Genomics (Cesg). (Web location: <http://www.rcsb.org/pdb/explore/images.do?structureId=1YYC>).

1.12 Proteomic methods in plant research

Proteomics has become widely used in plant research and has been applied in identifying the proteome of plant tissues and cells, proteins involved in disease response, metabolic processes and discovering protein biomarkers (Skylas et al., 2000; Islam et al., 2003; Wong et al., 2003; Wong et al., 2004; Lin et al., 2005; Mak et al., 2006b). Most of the early work in proteomics applied the traditional style two-dimension electrophoresis (2-DE) gels, which were of poor resolution and reproducibility and the protein identification was mostly obtained using N-terminal sequencing. More recently, there have been improvements in 2-DE gel technology (such as high resolution plastic backed gels) and protein identification techniques (such as high throughput mass spectrometry analysis). Also, there have been improvements in image analysis software and computer power, allowing for faster image analysis and relative protein quantitation. Other non-gel based proteomic techniques involve labeling, such as isobaric tags for relative and absolute quantitation (iTRAQ) and non-labeled (Shotgun) techniques. These approaches are generally more sensitive than gel based proteomics and are able to identify and measure low abundance proteins and their differential expression.

1.12.1 2-DE proteomics

2-DE essentially involves separating proteins according to two different separation techniques (O'Farrell, 1975). Firstly, proteins are separated according to their intrinsic isoelectric point (pI). The pI of any protein is the pH where its net charge is zero. Proteins are solubilised in a non-ionic detergent and are then applied to a polyacrylamide immobilized pH gradient (IPG) strip. The proteins on the IPG strip are then mobilized by applying a high voltage (up to 8000 V) across the strip. These IPG strips maintain a pH gradient so that proteins will migrate in the electric field until it reaches the point that

corresponds to its pI. This point on the IPG strip is where the net charge on the protein is zero.

The second dimension involves separating proteins according to their molecular weight. After first dimension separation, the IPG strip is equilibrated with SDS buffer in order to coat the proteins with a negative charge. It is then placed across the top of a polyacrylamide gel and a current is applied to mobilize proteins along the gel. Low molecular weight proteins will travel faster than high molecular weight proteins through the polyacrylamide gel, thus resolving proteins based on their relative molecular weight.

The protein in the gel can then be visualized by staining with a variety of stains, such as LavaPurple™, SYPRO® Ruby, or Coomassie G250. Lava Purple and Sypro Ruby are fluorescent stains that need to be imaged using a fluorescence scanner. These stains have better sensitivity than Coomassie stains and are more suitable in obtaining high quality images for gel analysis. Counter staining with Coomassie is usually performed to visualise protein spots for excision. The excised protein spots can then be identified by mass spectrometry (MS).

Staining using Differential In-Gel Electrophoresis (DIGE) allows for the comparison of three samples on a single gel, normally a control and two treatments (Ünlü et al., 1997). Each sample is labeled separately with different coloured fluorescent tags. Samples are pooled and separated using the 2-DE technique described above. The gel is scanned and protein spots associated with each sample are visualised according to the colour tag. The limitations of DIGE are its high cost and only two treatments can be analysed at a time. The current trend in proteomics is now moving towards high throughput methods involving label and label-free techniques that do not require 2-DE gels. Generally, these

techniques utilise column chromatography to separate peptides from trypsin digested protein.

1.12.2 Label and label-free proteomics

There are various high throughput proteomic techniques available for identifying proteins and measuring changes in protein levels without using 2-DE. Two of the more popular approaches are Shotgun proteomics with spectral counting and isobaric tag for relative and absolute quantitation (iTRAQ) proteomics (Ross et al., 2004; Aggarwal et al., 2006). The shotgun approach is label-free and combines sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with electrospray ionization-tandem mass spectrometry (ESI-MS/MS). The proteins from each sample are firstly separated by SDS-PAGE. The gel is stained with Coomassie and each lane is dissected into sections. The gel pieces are trypsin digested and the peptides are subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS). Peptide sequencing by MS/MS (Hunt et al., 1986) is used to identify proteins against a protein data base such as Mascot (Matrixscience, <http://www.matrixscience.com/>) or The Global Proteome Machine (The Gpm, <http://www.thegpm.org/>) and quantitation is achieved by spectral counting. For each sample run, the peak intensities are compared with a control and are usually performed in triplicate. Statistical methods are then applied to determine significant changes in protein levels (Choi and Nesvizhskii, 2007).

With the iTRAQ approach, samples are trypsin digested and peptides are labeled with an isobaric tag that has a unique mass reporter that is equalized with a balance group. The reactive group on the tag is amine specific and will bind to the N-terminus and lysine residues of a peptide (Figure 1.11). The peptides fragment into b and y ions during

collision induced dissociation (CID) and are used to determine the peptide sequence (Figure 1.12 A-B). The reporter ion and balance group are also liberated during MS/MS and the intensity of the reporter ion peak is used to calculate the relative abundance of the peptide between labeled samples. This reveals the relative abundance of peptides as a ratio between the control and treatments (Figure 1.12 C-D) (Ross et al., 2004; Sadowski et al., 2006). There is a standard 4plex or 8plex iTRAQ reagent kit (Applied Biosystems) that can be used to compare three or seven samples to a control respectively.

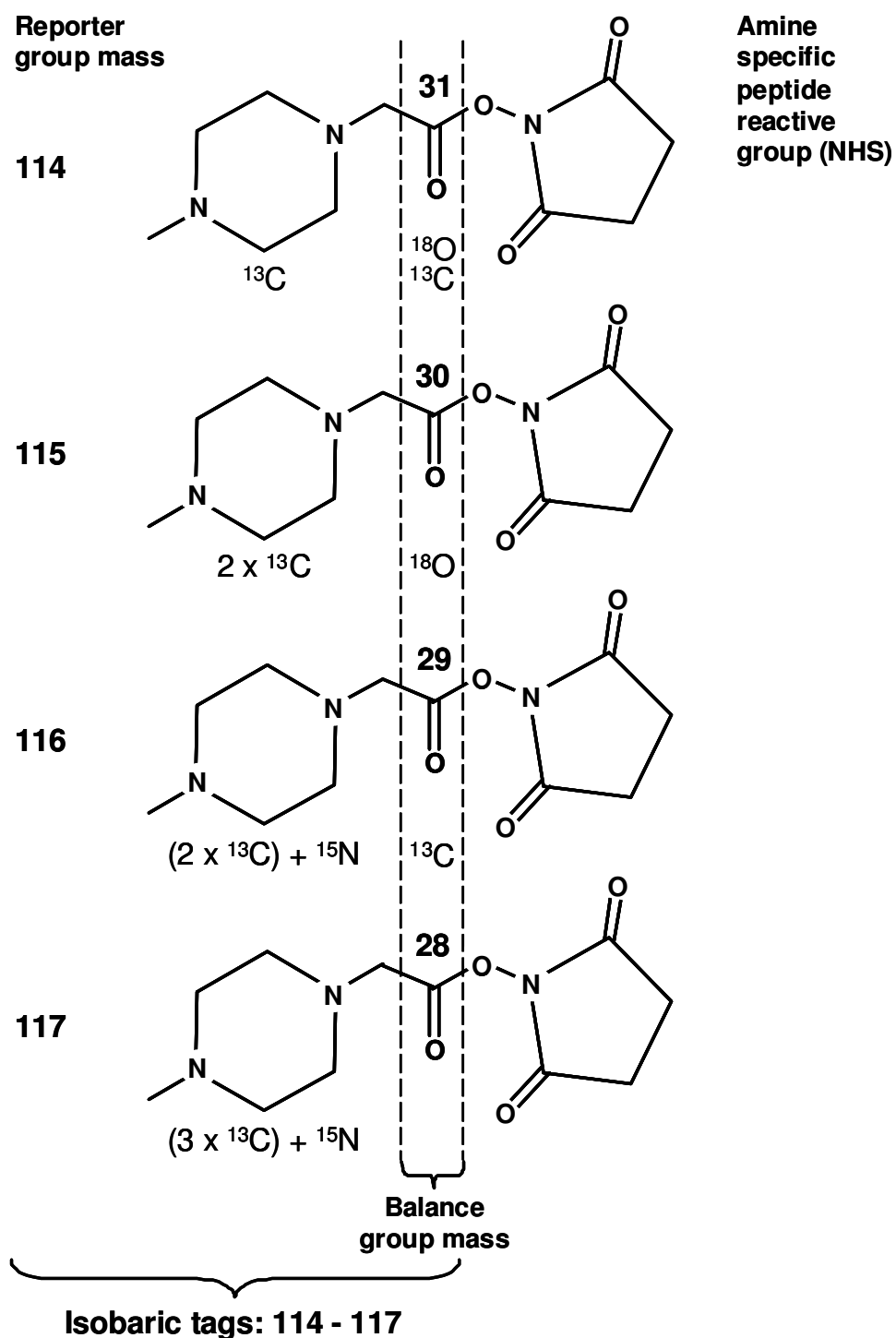


Figure 1.11. Chemical structures of isobaric tags. The balance group mass is lost at collision induced dissociation during MS/MS and intensities of each reporter ion is measured. Modified from Ross et al. (2004).

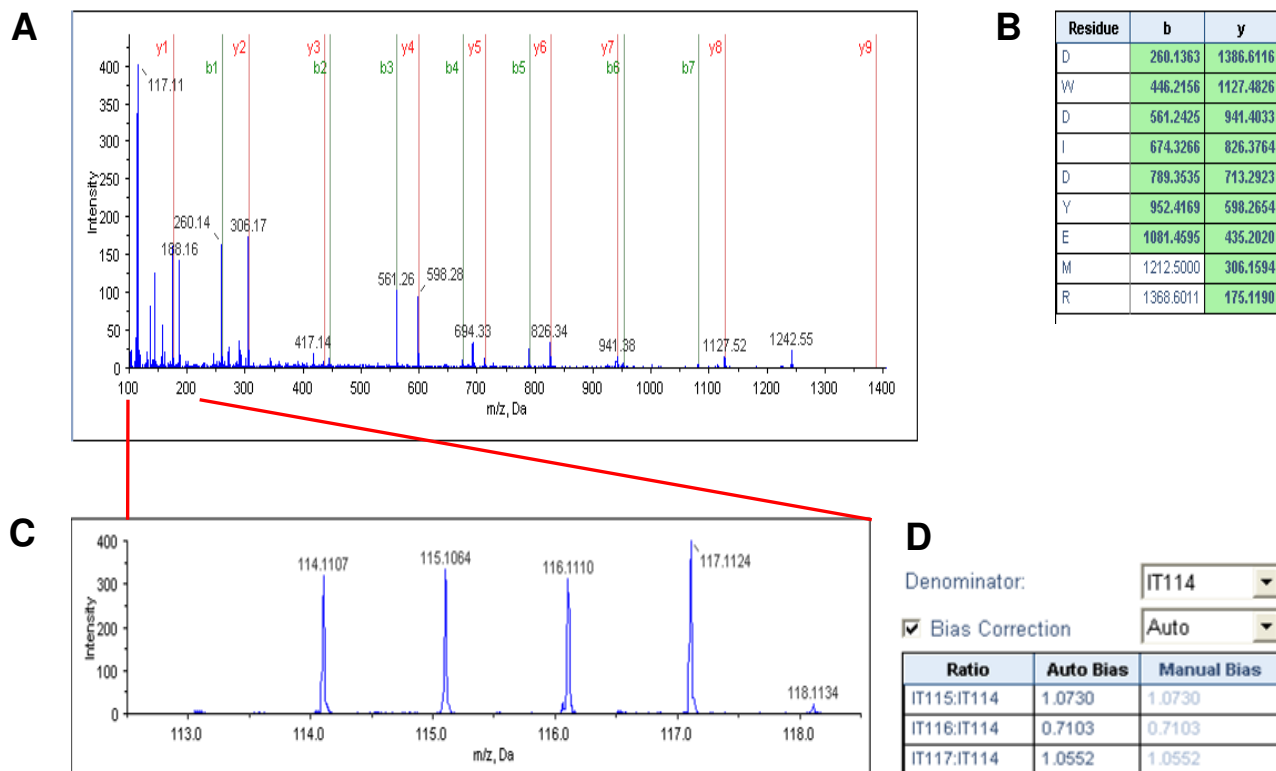


Figure 1.12. MS/MS spectra and iTRAQ reporter ions: (A) MS/MS spectra of a peptide showing the b and y ions, (B) theoretical amino acid sequence according to the b and y ions, (C) iTRAQ reporter ions and peptide quantitation information and (D) relative abundance of peptide according to the reporter ions.

1.13 Project aim and hypothesis

The aim of this project is to analyse the three main tissues (germ, bran and ventral groove) from ABA-treated wheat grain at the biochemical and physiological level to identify factors involved in milling performance. The approaches I have undertaken are: proteomics, to determine the biochemical mechanisms by which ABA improves milling performance; localize potentially important proteins discovered in the proteomics analysis using immunolocalisation microscopy; develop a laboratory based method for testing the effects of conditioning additives on milling performance of wheat; and measure the water potential (ψ) and mechanical properties of ABA and water-conditioned grain.

A suggested hypothesis is that ABA, when added in the conditioning water, prolongs quiescence/dormancy by either preventing water absorption into the germ or trapping water within the bran/aleurone tissues. Proteomic analysis of the proteins involved at this stage will potentially reveal signaling cascades in the early stages of germination that are responsible for the improved milling performance. The three main tissues in wheat grain likely to show changes in protein levels are the germ and the aleurone cells in the bran and the ventral groove. The ventral groove is likely to be responsible for aiding in the transport of water and nutrients to the germ.

Chapter 2 : Strategic distribution of protective proteins within bran layers of wheat (*Triticum aestivum* L.) protects the nutrient-rich endosperm

2.1 Introduction

Wheat grain (*Triticum aestivum* L.) is a major cereal crop and staple food in many parts of the world. The endosperm is the main nutritional component and is extracted in milling to produce base ingredients such as flour and semolina. Crop yield and quality may be compromised by both environmental and biological stresses. Wheat varieties are known to vary in their resistance to such stresses, probably due to individual differences in defense protein levels (Demeke and Morris, 2002; Bonnin et al., 2005; Yarullina et al., 2005). Cereal grain contains many defense proteins which have been categorized according to their mode of action and structural similarities. A major class of these is the pathogenesis-related (PR) proteins, which include PR-1, PR-2 (β -1,3-glucanases), PR-3 (chitinases), PR-4 (wheatwin1) and PR-5 (thaumatin-like proteins) (Selitrennikoff, 2001; Desmond et al., 2006). Other known defense proteins are xylanase inhibitor proteins (XIPs) and α -amylase inhibitor proteins (Mundy et al., 1984; Payan et al., 2003). All of these defense proteins have both general and specific roles that contribute to plant survival, although little is known of their location within the various grain tissues, particularly the multiple layers that comprise bran.

Proteomic analysis of wheat grain has previously been applied to identify proteins in the germ and endosperm (Skylas et al., 2000; Wong et al., 2004; Mak et al., 2006a) but analysis of bran and bran tissue fractions has not been reported. Collection of sufficiently pure bran tissue fractions has limited progress, mainly due to the strong bonds between the various bran tissue layers and endosperm in dry grain. Thus, a method to obtain bran layers free from contaminants, such as adjacent tissue and endosperm, is required to provide a sample suitable for proteomic analysis. Soaking whole grain in water causes the endosperm to soften, allowing it to be easily removed and washed from the bran; the bran becomes malleable enough to dissect. While this approach might not identify the proteome of dry grain fractions, it is the best available representation of the three distinct tissue fractions in grains, namely the outer layer (epidermis and hypodermis), intermediate layer (cross cells, tube cells, testa and nucellar tissue) and inner layer (aleurone cells) (Antoine et al., 2003; Antoine et al., 2004). Using this method, water-soluble proteins that diffuse from the grain can be collected and identified.

In this study we aimed (i) to dissect bran into the three separate tissue fractions described above and to identify the protein complement of each fraction using proteomics; (ii) to confirm the location of three major defense proteins identified (one from each micro-fraction) using immunolocalization and (iii) to identify water-soluble proteins and assay any defense-related proteins for enzymatic activity.

2.2 Materials and Methods

2.2.1 Bran tissue microdissection

Triticum aestivum L. (cultivar Babbler) grains (four lots of 0.5 g) were placed on separate weigh boats, wetted with Milli-Q water and left on the bench for 5 min. They were then stored at -20 °C for 30 min, followed by thawing and removal of the outer layers by using forceps with the aid of a dissecting microscope. Processing 0.5 g grains at a time was done to minimize migration and loss of water-soluble proteins. The collected tissue (195 mg total tissue from 2 g of grain) was placed into a 15-mL plastic tube and stored at -20 °C.

To obtain the intermediate and inner fractions, grains were soaked in Milli-Q water for 2 d at room temperature. This treatment caused softening of the endosperm, which allowed it to be scraped off and washed from the bran under a dissecting microscope. The bran was then separated into individual components by micro dissection (Figure 2.1). Total tissue collected from the intermediate layer was 42 mg and inner fraction 45 mg.

2.2.2 Protein extraction of bran tissue fractions

Proteins were extracted from the three bran tissue fractions according to Wang et al. (2003). Samples were kept on ice when in solution during protein extraction. The tissues were placed into 2-mL screw-cap plastic tubes and washed twice with 1 mL cold acetone, vortexed for 30 s and centrifuged at 18,000g for 3 min each time. The tissue was left to dry inside the plastic tubes at room temperature. Once dry, the tissue was ground down to a fine powder in a mortar and pestle with the aid of a small amount of acid-washed sand. The powder was returned to the 2-mL plastic tubes and washed three times with cold 10% TCA in acetone (1 mL). For each wash, the sample was vortexed for 30 s and then centrifuged at

14,000g for 3 min. The sample was then washed twice with cold 10% TCA in water (1 mL) and finally washed twice with cold 80% acetone (1 mL). Again, for each step the sample was vortexed for 30 s and centrifuged at 14,000g for 3 min. The tissue was left to dry at room temperature overnight. To the dry tissue we added 800 μ L of Tris-buffered phenol, pH 8.0, followed by 800 μ L of SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl at pH 8.0, and 5% 2-mercaptoethanol). The sample was then vortexed for 30 s and centrifuged at 14,000g for 3 min. The top phenol layer was removed and placed into a fresh 2-mL plastic tube. Five volumes of 0.1 M ammonium acetate in cold methanol was added to the phenol extracts and stored for 1 hr at -20 °C to allow for protein precipitation. The protein precipitate was centrifuged at 14,000g for 5 min and the supernatant was removed. The protein pellet was washed twice with cold methanol, 0.1 M ammonium acetate and then twice with cold 80% acetone. Each time, the pellet was vortexed for 30 s and centrifuged for 5 min at 14,000g. After the final wash, the pellet was left to air dry to evaporate any acetone. Protein pellets were solubilized in 500 μ L rehydration buffer (5 M urea, 2 M thiourea, 65 mM DTT, 2% [w/v] CHAPS, 2% [w/v] sulfobetaine 3-10, 1% [w/v] carrier ampholytes [Amersham Biosciences, GE], 40 mM Tris, 0.002% [w/v] bromophenol blue dye, milli-Q water) for isoelectric focusing (IEF).

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

Whole grain (10 g) and isolated outer bran tissue (100 mg) was placed into 50-mL and 1.5-mL plastic tubes in 15 mL and 1 mL Milli-Q water respectively. Endosperm was collected by passing whole grain (10 g) through a lab mill grain crusher (RM 001, custom made)

followed by sieving through a 180- μ m sieve (Endecotts). The collected endosperm (5 g) was placed into a 50-mL plastic tube in 15 mL Milli-Q water. The tubes were placed on a rotating wheel for approximately 3 h at 4 °C. Supernatant was collected into fresh 15-mL and 1.5-mL plastic tubes respectively, and centrifuged for 20 min at 5,500g. Supernatant was collected into 50-mL plastic tubes and freeze dried overnight. Protein extracts were resuspended in 500 μ L and 200 μ L Milli-Q water respectively and stored at -20 °C until needed for assaying and separation by 1-D SDS-PAGE.

2.2.4 Protein estimation

Protein concentration was estimated using the Bradford method (Bio-Rad) with BSA (Sigma-Aldrich) as the standard (Bradford, 1976).

2.2.5 Two-dimensional electrophoresis (2-DE)

Prior to IEF, the samples were reduced with 5 mM tributylphosphine (TBP) and alkylated in 10 mM acrylamide, followed by incubation for 1 h at room temperature. After incubation, the samples were centrifuged at 20,000g for 10 min at 4 °C. Sub-samples from each tissue (200 μ g protein from aleurone cells, 25 μ g protein from the intermediate layer and 13 μ g protein from the outer layer) were each separated by IEF in triplicate using immobilized pH gradient (IPG) strips; 17 cm IPG strips pH 4-7 (Bio-Rad) and 18 cm IPG strips pH 6-11 (GE Healthcare). In the acidic pH range, samples were loaded (300 μ L) onto the IPG strips using passive hydration and, for the alkaline pH range, the samples were cup-loaded (120 μ L). IEF was performed with a step-wise protocol to 128 kVh and 115 kVh respectively. After IEF, the strips were stored at -80 °C. The IPG strips were removed from the -80 °C freezer and then thawed at room temperature and equilibrated in (6 M urea,

2% SDS, 0.375 M Tris/HCl pH 8.8, 20% glycerol, 5 mM TBP, 2.5% acrylamide) for 15 min. This was repeated twice, and after each time, the equilibration solution was poured off and replaced with fresh solution. IPG strips were embedded on top of 8-18% gradient polyacrylamide gels (17 cm x 17 cm) using hot agarose (0.5% agarose, 0.001% bromophenol blue, 192 mM glycine, 0.1% SDS, 24.8 mM Tris base pH 8.3). The gels were electrophoresed in Protean II multi-cell tanks (Bio-Rad) using a power box (Bio-Rad Power Pac 3000), set at 5 mA per gel for 30 min, and then at 40 mA per gel for approximately 4.5 h, or until the dye front had run off the gel. After electrophoresis, the gels were removed from their casts and placed into fixing solution (30% methanol, 7.5% acetic acid) for at least 1 h to prepare for Lava Purple (FlouoroTechnics, Sydney, Australia) staining. The solution was poured off and replaced with 200 mM Na₂CO₃ for 1 h to alkalinate the gels. Sodium carbonate solution was replaced with water, adding approximately 10 x the gel volume. Lava Purple stain was added to the water to make a final dilution of 1 in 200. The gels were covered with foil and left to stain overnight on a rocker. The stain was poured off and destained twice with 1% acetic acid. The gels were immediately scanned using a Typhoon variable mode imager (Amersham Biosciences). The gel image was scanned in fluorescence mode, 610 BP Deep Purple emission filter, green (532) laser, and with 100 micron pixel resolution.

2.2.6 Proteomic analysis

The scanned images were uploaded into the image analysis software Progenesis Discovery version 2005 (Nonlinear Dynamics LTD) to overlay the triplicate gel images. The uploaded images were transferred to Progenesis PG240 version 2006 (Nonlinear Dynamics LTD) software to determine true protein spots and spot annotation for each of the triplicate gels. The gels were all counter stained with colloidal Coomassie Blue G-250 stain (17%

ammonium sulfate, 3% phosphoric acid, 0.1% Coomassie G-250, 34% methanol, Milli-Q water to make up 1 L total) (Neuhoff et al., 1988) and left on a rocker overnight. Background de-staining was done using 1% acetic acid. The Coomassie stained images of all the gels were scanned on an automated spot cutter (Bio-Rad, EXQuest) and only spots that could be clearly seen with Coomassie stain were manually selected for excision and identification.

Gel plugs were manually destained three times in 120 μL wash solution (50% [v/v] acetonitrile, 25 mM ammonium bicarbonate). For each wash, the gel plugs were placed onto an orbital shaker and incubated at 37 °C for 10 min, replacing wash solution each time. The gel plugs were vacuum dried using a Savant Speed Vac Plus SC210A.

To each dry gel plug were added 8 μL portions of 15 ng μL^{-1} sequencing grade trypsin (Promega) in 25 mM ammonium bicarbonate, pH 7.8, followed by incubation for 1 h at 4 °C to allow the trypsin to be absorbed. Excess trypsin was removed and the gel plugs were sealed and incubated over night at 37 °C. Peptides were extracted in 10 μL of extraction solution (0.1% TFA) with the aid of a water bath sonicator (Transsonic 700/H, Elma) for 20 min.

Zip Tips were activated with 10 μL of 70% acetonitrile, 0.1% TFA by pipetting 10 μL up and down three times. The tips were washed with 0.1% TFA in the same manner. The peptide extraction solution was taken up into the Zip Tip, drawing up and down 8 μL ten times to concentrate the peptides onto the column. The Zip Tip was further washed three times with 10 μL of 0.1% TFA. Four microlitres of extraction solution (4 mg.mL⁻¹ Matrix, α -cyano-4-hydroxycinnamic acid, 70% acetonitrile, 0.1% TFA) was drawn up into the Zip Tip. The extraction solution in the tip was drawn up and down at least five times forming a

drop at the end of the tip to elute the peptides from the column. Finally, 2 μ L of this solution was spotted onto a designated circle marked on the ABI plate. A standard (prep mix with matrix) was also spotted following each sample on the ABI plate to externally calibrate using near-point calibration with four peptide standards (bradykinin, angiotensin I, neurotensin and adrenocorticotrophic hormone [ACTH] fragment).

Samples were dried and analyzed using an Applied Biosystems 4700 MALDI MS/MS with TOF/TOF optics (Foster City, CA) in reflector mode for positive ion detection. A Nd:YAG laser with wavelength and repetition rate of 355 nm and 200 Hz, respectively, was used. All MS spectra resulted from accumulation of 4000 laser shots (20 sub-spectra were accumulated with 200 shots per sub-spectra). Laser intensity varied between 3000 and 4000. Data were collected over a mass range of 750 to 3500 Da. Mass spectral data were analyzed using Mascot (Matrixscience). Peak detection criteria for mass lists were MS: mass range 500-4000 Da, maximum 30 peaks per 200 Da, minimum signal to noise ration (S/N) 20, minimum area 200, maximum peak/spot 200, and for MS/MS: mass range 60 Da to precursor -15, maximum 20 peaks per 200 Da, minimum S/N 18, minimum area 300, maximum peak/spot 60. This converts the mass lists into Mascot and The Global Proteome Machine (The GPM) compatible text files. Mascot (Matrix Science, London, UK) and X-Tandem were used to search cereal entries in the NCBI non-redundant databases and a translated Wheat EST database (PlantGDB database *Triticum aestivum* EST assembly Feb 2006). Identification of proteins with high scores and low e-values (less than -1.0) with good peptide matches and coverage were tabulated with corresponding spot number and location on the 2-DE gel. Some spots that did not reveal significant protein identification were further analyzed using electro-spray ionization (ESI) MS/MS.

Digested peptides were separated by nano-LC using a CapLC system (Agilent 1100 Series, Agilent Technologies, Germany). Sample (39 μL) was injected onto a peptide trap (Michrome peptide Captrap) for preconcentration and desalted with 0.1% formic acid at 10 $\mu\text{L min}^{-1}$. The peptide trap was then switched into line with the analytical column containing C18 RP silica (SGE ProteCol C18, 300A, 3 μm , 150 $\mu\text{m} \times 10 \text{ cm}$). Peptides were eluted from the column using a linear solvent gradient, with steps, from $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (95:5 + 0.1% formic acid) to $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (20:80 + 0.1% formic acid) at 600 nl min^{-1} over 45 min. The LC eluent was subject to positive ion nanoflow electrospray analysis on an Applied Biosystems QSTAR XL mass spectrometer (ABI, CA, USA). The QSTAR was operated in an information-dependent acquisition (IDA) mode. In IDA mode a TOF/MS survey scan was acquired (m/z 400-2000, 1.0 s), with the four largest multiple-charged ions (counts >25) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 1 s (m/z 50-2000). The LC/MS/MS data was used to search cereal entries in the NCBI non-redundant protein database using Mascot (Matrix Science, London, UK). High scores in the database searches indicated a likely match, confirmed or qualified by inspection of the spectra and search results.

The GPM was used as a secondary search engine using the plantDB for *Triticum aestivum* to both confirm the Mascot identity and to calculate a false discovery rate (FDR) for all MS/MS spectra. FDR was calculated using the mixture model according to Choi and Nesvizhskii (2007). Search results and analysis from both Mascot and The GPM are provided as a supplementary table in Excel format.

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

To measure OXO activity, 50 μL of substrate solution (20 mM ammonium oxalate) was mixed with 50 μL of *o*-phenylenediamine dihydrochloride (OPD) (0.08 g) in 10 mL

substrate buffer (0.1 M citric acid / 0.2 M disodium phosphate buffer, pH 5.0). Substrate solution for measuring POX activity was 100 μ L of OPD (0.04 g) in 10 mL substrate buffer with 5 μ L 35% peroxide. A portion (50 μ L) of each protein extract was placed into wells of a 96-well flat-bottom microtitre plate (Greiner Bio-One) in triplicate, together with 50 μ L sodium succinate buffer. Finally, 100 μ L of the ammonium oxalate with OPD (OXO assay) and 100 μ L of the OPD with peroxide (POX assay) were added separately to each protein extract. Absorbance was read at 492 nm using a plate reader (Thermo Multiskan EX) at time intervals $t = 0, 30, 60$ and 120 min.

2.2.8 OPD standard curve

Excess horseradish peroxidase (Sigma) in sodium succinate buffer was added to the OPD solution (0.04 g of OPD in 10 mL substrate buffer [0.1 M citric acid / 0.2 M disodium phosphate buffer, pH 5.0] and 5 μ L of 35% peroxide). A 50% dilution series was prepared upon reaction completion. A portion (200 μ L) of each dilution was added in duplicate to wells of a flat-bottom 96-well microtitre plate. Absorbance at 492 nm on a 96-well plate reader was used to calculate the μ moles OPD in each well.

2.2.9 Polyphenol oxidase (PPO) assay

The protocol for the PPO activity assay was modified from Espin et al. (1998). Substrate solutions hydroquinone monomethyl ether (4HA) (25 mg) in 50 mL water and 3-methylbenzothiazolin-2-one hydrazone (MBTH) (20 mg) in 2 mL 70% EtOH were prepared separately. The two solutions were added together prior to the assay. 50 μ L of each sample, together with 20 μ L (1 M sodium acetate, pH 5) and 430 μ L Milli-Q water were placed into a 1-mL cuvette with a 1-cm light path. Finally, 500 μ L of substrate

solution was added to each sample in the cuvette (Sarstedt). Absorbance was read at 492 nm at time points 0, 30, 60 and 120 min. The reaction rate in nKat g⁻¹ protein was calculated using the 4HA extinction coefficient (Espín et al., 1998).

2.2.10 Chitinase assay

The chitinase assays for exochitinase (chitobioside substrate), exochitinase (β -N-acetylglucosaminidase), and endochitinase activity were performed in accordance with the manufacturer's instructions (Sigma, chitinase assay kit, colorimetric, product code CS0980). The assay was modified slightly such that the blank contained the sample in stop solution instead of the substrate in stop solution.

2.2.11 Generation of antibodies and affinity purification

Polyclonal antibodies were generated in New Zealand White Rabbits immunized with peptides to XIP-I (H-CNQNLGWEGSWDHKWTA-NH₂; H-AGGKTGQC SLIKYYA-OH), PR-4 (H-FTKIDTNGIGYQQGHC-NH₂; H-ATYHYRCDNN-OH) and oxalate oxidase (H-AKAGNTSTPNGSAVTC-NH₂; H-TDPDPLQCSKFAAGF-OH) covalently linked to diptheria toxoid using a thioether linkage. The polyclonal antibodies were affinity-purified by conjugating free peptides to an activated beaded agarose, SulfoLink® Coupling Gel (Pierce) according to the manufacturer's instructions.

2.2.12 Sectioning and microscopy

Wheat grains *Triticum aestivum* L. (cv. Babbler) were soaked in PBS for 0.5 to 2 h at room temperature before both ends of the grain were removed with a razor blade. Grains were then fixed in 2-4% paraformaldehyde in PBS for at least 2 d at 4 °C. Fixed grains were sectioned on a Leica VT 1000S Vibratome (speed 7, frequency 4) taking 60- to 100-µm sections directly into PBS. All processing was performed at room temperature. Grain sections were blocked with 10% fetal bovine serum (FBS) in PBS for 30 min followed by primary antibody diluted in 10% FBS for 1 h. Sections were washed five times in PBS (5 min per wash) before secondary antibody (anti-rabbit AlexaFluor 488, Molecular Probes) diluted in 10% FBS was applied for 1 h in darkness. Sections were again washed five times in PBS before mounting the sections with Gel/Mount (Biomedex) and sealing the coverslips with nail polish. Sections were stored in the dark at 4 °C overnight before visualizing on an Olympus IX 70 confocal microscope using Fluoview FV300 software.

2.3 Results

2.3.1 Light microscopy of bran tissue fractions

Microscopic examination of dissected tissue fractions showed that the cell types of each fraction were uniform and mostly free from cells of adjoining fractions. The distinctive cell patterns of the outer fraction (epidermis and hypodermis) (Figure 2.1 A) and the intermediate fraction cross cells (Figure 2.1 B) confirmed the purity of each fraction. Four tissues (cross cells, tube cells, testa and nucellar) that make up the intermediate fraction were also distinguished (Figure 2.1 C). Finally, the inner fraction (aleurone) cells were free from endosperm and were also largely intact (Figure 2.1 D).

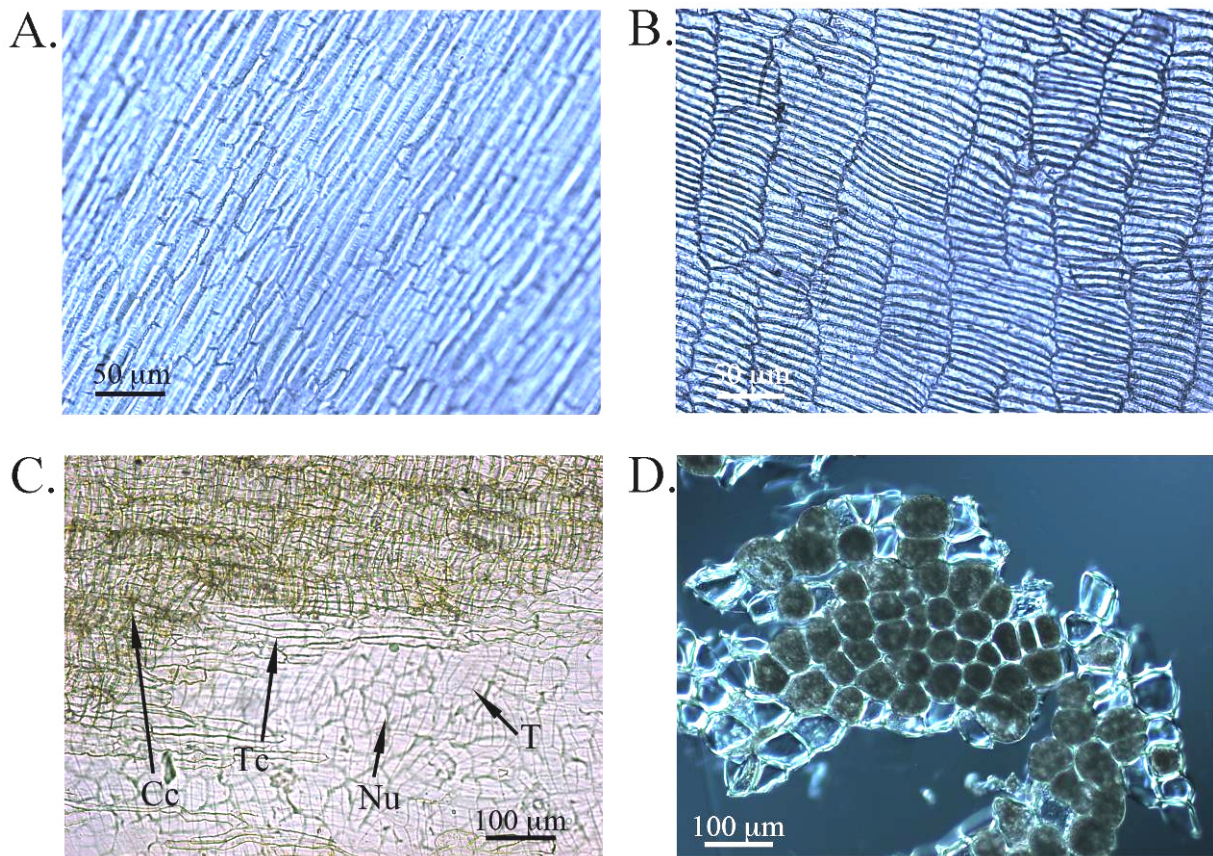


Figure 2.1. Micrographs of the isolated bran fractions. (A) Outer bran fraction (epidermis and hypodermis). (B) Intermediate bran fraction (cross cells, tube cells, testa, and nucellar tissue). (C) Detailed view of the individual layers in the intermediate fraction (Cc, cross-cells Tc, tube-cells T, testa Nu, nucellar tissue). (D) Aleurone cells.

2.3.2 Protein extraction from bran tissue fractions

The outer bran layers and intermediate fraction contained significantly less protein than the inner fraction (aleurone): 0.4 mg total protein per g was extracted from the outer layer (25% was water-soluble); 3.6 mg total protein per g was found in the intermediate fraction and 156 mg protein was extracted per g of inner layer.

2.3.3 Protein identification from 2-DE gels

The protein complement of the outer dead cell layers (outer layers and intermediate fraction) was much less diverse than that of the inner fraction (aleurone cells), as shown by the 2-DE gels (Figure 2.2 and Supplemental Figures S1-S6). Image analysis of triplicate 2-DE gels revealed 35 unique protein spots (30 identified using Mascot and 27 using The GPM) from the outer fraction (epidermis and hypodermis), 119 (106 identified using Mascot and 66 using The GPM) from the intermediate fraction (cross cells, tube cells, testa and nucellar tissue) and 672 (606 identified using Mascot and 310 using The GPM) from the inner fraction extract. Although some proteins were identified in more than one fraction, there was minimal overlap of the most intense and abundant protein spots between fractions. This observation, together with microscopic examination of the microdissected tissue, confirms that there was negligible cross contamination of proteins between the tissue types (Figure 2.1).

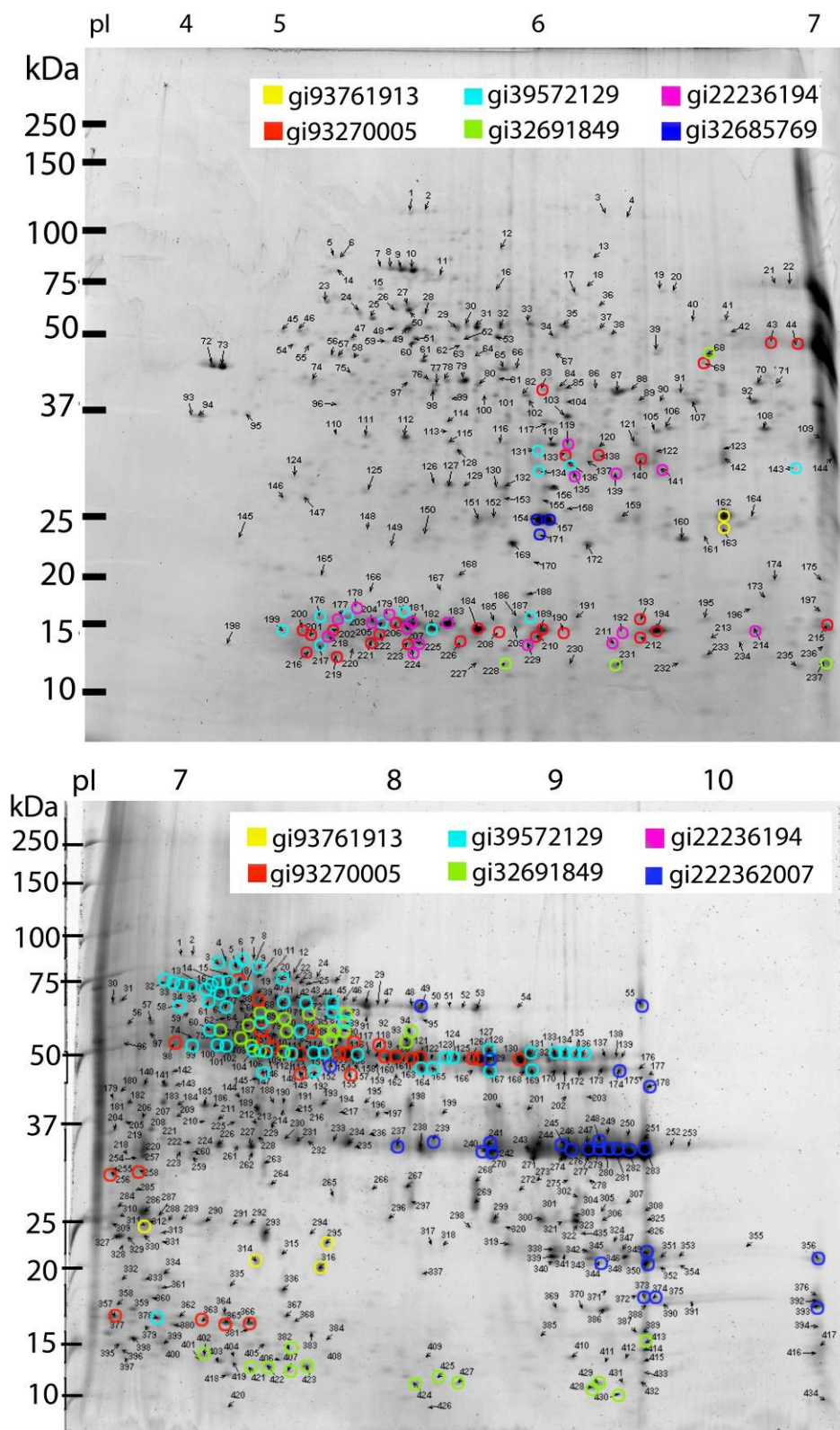


Figure 2.2. 2-DE gels of Inner bran layer (Aleurone). The highlighted spots show the different EST classes of 7S globulin with the EST GenBank gi number show in the legend.

The unhighlighted gels are shown in Supplementary figures S5 and S6 respectively.

A list of the major proteins identified in this study is shown in Figure 2.3. Complete protein identification tables (Mascot and The GPM results) and representative 2-DE images with numbered spots are provided in the Supplemental data (Supplemental Tables S1A-S1G; Supplemental Figures S1-S6). An individual false discovery rate (FDR) was only calculated for proteins identified using The GPM. This was because the proteins that were not identified in The GPM search, by both peptide mass and sequence from MS/MS, were only identified by peptide mass fingerprinting (PMF) in the Mascot search (FDRs could not be calculated in the same way from PMF identifications and are thus not comparable). The average FDR for the 453 proteins identified using The GPM was 1.4%.

2.3.4 Outer fraction

The proteins identified in this fraction were mostly oxidative-stress and defense-related proteins such as oxalate oxidase (OXO), lipid transfer protein and lipoxygenase (Supplemental Tables S1A and S1B).

2.3.5 Intermediate fraction

The intermediate fraction had a much more diverse set of defense proteins than the outer fraction. These proteins are oxidative-stress and defense-related proteins such as OXO, xylanase inhibitor-I protein (XIP-I), chitinase and endochitinase, α -amylase/subtilisin inhibitor (WASI), wheatwin1, thaumatin-like protein (TL) and benzothiadiazole (BTH)-clone of a wheat chemically-induced protein (cWCI-5) (Supplemental Tables S1C and S1D). OXO was the only protein identified in this fraction that was also identified in the outer fraction and supernatant.

2.3.6 Inner fraction (aleurone cells)

Mascot was used to search MS and MS/MS spectra against a cereal and wheat EST database, which identified 606 proteins. More than half (387 spots or 58%) were identified as globulin-like storage proteins from the cupin superfamily. Of the 387 spots, four were matched to a rice globulin-like protein, one each to a putative rice globulin and a barley embryo globulin, while 207 spots were matched to the wheat 7S globulin storage protein. The remaining 174 spots were matched to wheat ESTs, which were searched using NCBI BLAST. The BLAST search aligned 163 ESTs to the wheat 7S globulin storage protein and 11 to a rice cupin family expressed protein.

Additionally, the MS/MS spectra that were identified as cupin superfamily proteins were searched against the wheat EST database using The GPM. This search identified 212 of the 387 cupin superfamily spots identified in the Mascot search. The majority unidentified based on MS/MS data had been identified by Mascot based on PMF data alone. A BLAST search of these EST matches aligned 203 spots to a globulin 3 storage protein (homologous to the 7S globulin storage protein) and 9 spots to a cupin family protein. Within the 212 spots identified in the EST database, there were 7 distinct groups of ESTs. An unrooted phylogenetic tree was produced using the derived amino acid sequences from the ESTs of the 7 groups showing they are distinct but related to each other (Supplemental Figure S7). The 2-DE gels (pI 4-7 and 6-11) of the inner fraction show that the 7 groups are uniquely distributed as highlighted in Figure 2.2. Proteins from the cupin super family are thus distributed into 7 sequence-based groups with a total of 29 subgroups, according to significant differences in their molecular weight (MW) (Table 2.1).

The remaining 285 proteins identified in the Mascot search participate in a range of cell functions. They include carbohydrate metabolism (127 spots or 19%) and protein synthesis

(23 spots or 3.4%), stress and defense (31 spots or 4.5%), and other/miscellaneous functions (38 spots 5.7%) (Supplemental Tables S1E and S1F).

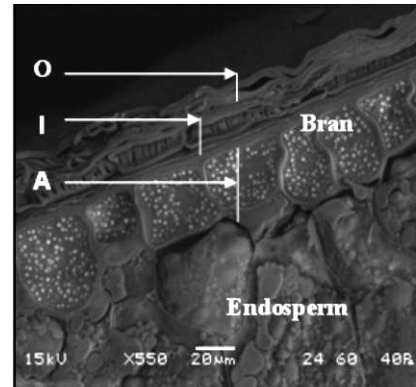
Table 2.1. Subgroups of globulin proteins separated on 2-DE gels.

GI number	Subgroup average MW (kDa)	Subgroup MW range (kDa)	Subgroup average pI	Subgroup pI range	Number of spots
gi 39572129	14.8	13 - 16	5.31	4.8 - 6.5	9
	30.3	29 - 32	5.78	5.7 - 5.9	3
	49.8	47 - 53	7.94	6.5 - 9.2	24
	59	58 - 60	7.08	6.9 - 7.35	3
	72.3	66 - 78	7.07	6.45 - 8.1	27
gi 93270005	14.2	12 - 15.5	5.71	4.85 - 7.05	23
	31	28 - 34	6.1	5.8 - 6.4	5
	49.5	45 - 53	7.49	5.75 - 8.7	20
	57.3	55 - 59	7.45	7.15 - 7.7	3
	73.5	73 - 74	7	6.95 - 7.05	2
gi 32691849	5.5	5.0 - 7.0	7.89	6.75 - 9.4	12
	10.8	10.0 - 11.0	6.91	5.2 - 9.65	4
	49.5	48 - 52	7.04	6.5 - 7.35	4
	57.4	55 - 60	7.39	6.9 - 8.0	7
	69.2	67 - 72	7.41	7.0 - 8.0	9
gi 222362007	18.7	16 - 21	9.85	9.3 - 10.75	7
	36.8	36 - 38	9.02	8 - 9.8	17
	47	44 - 50	8.78	7.5 - 9.6	4
	73.5	72 - 75	8.85	8.2 - 9.5	2
gi 222361947	5.15	5.1 - 5.2	5.18	5.1 - 5.25	2
	14.4	13 - 15	5.55	4.95 - 6.7	12
	30	29 - 33	6.03	5.85 - 6.25	4
gi 93761913	22.7	19 - 25	6.93	6.4 - 7.5	6
gi 32685769	24.5	23.5 - 25	5.78	5.75 - 5.8	3

A.

Protein	W	O	I	A
Polyphenol oxidase	•			
Peroxidase	•			
Oxalate oxidase	•	•	○	
Polycystin-1, Lipoygenase, Alpha-Toxin (PLAT)		•		
Type 1 non-specific lipid transfer protein precursor		•		
Benzothiadiazole-induced protein (clone WCI-5)			•	
Thaumatococcus-like protein			•	
26 kDa endochitinase 1 precursor			•	○
Pathogenesis related-4 protein (Wheatwin1)			•	○
Xylanase inhibitor protein I (XIP-I)			•	○
Xylanase inhibitor protein III (XIP-III)			•	○
Triticum aestivum xylanase inhibitor -I (TAXI-I)			•	○
Secretory protein			•	○
Class II chitinase			•	•
Alpha-amylase/subtilisin inhibitor (WASI)			•	•
7S Globulin storage protein				•
Enolase				•
Glyceraldehyde-3-phosphate dehydrogenase				•
Glucose and ribitol dehydrogenase				•
Formate dehydrogenase				•
Cytosolic NADP malic enzyme				•
Aldose reductase				•
HSP70				•

B.



W, Water-soluble pericarp
O, Pericarp layer
I, Intermediate layer
A, Aleurone layer
•, Major protein
○, Minor protein

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.

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

Water-soluble proteins in the supernatant from soaked grain and outer fraction were separated by 1-D SDS-PAGE. Only the five major protein bands from the whole grain supernatant were selected for identification (Supplemental Figure S8). The identified proteins were endochitinase, histone, OXO, peroxidase (POX) and polyphenol oxidase (PPO) (Supplemental Table S1G). The endochitinase and histone protein bands were not present in the outer fraction supernatant, suggesting that they have diffused from other parts of the grain such as the germ or inner bran layers.

2.3.8 Enzyme activity assays of the water-soluble proteins chitinase, OXO, POX and PPO

OXO, POX and PPO all showed enzymatic activity in the water-soluble protein extracts from whole grain and outer fraction but no activity was detected in the endosperm extract. The activities of OXO and POX were much higher (28- and 7-fold respectively) in the outer fraction supernatant compared with the whole grain supernatant, whereas PPO showed no significant difference in activity between these extracts.

The chitinase activity assays were done for three different chitinases (exochitinase with chitobioside substrate, exochitinase [β -N-acetylglucosaminidase] and endochitinase). Both endo- and exochitinase activity were detected in the whole grain water-soluble protein extracts and not in the outer fraction extracts (Table 2.2). The exochitinase (β -N-acetylglucosaminidase) showed some activity in the water-soluble protein extract from the endosperm but this was 6-fold less activity than in the whole grain water-soluble extract.

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.

Enzyme activity (nkat. g⁻¹ protein extract)	Whole grain	Pericarp	Flour
Oxalate oxidase	77 \pm 56	2219 \pm 184	0
Peroxidase	1998 \pm 140	14542 \pm 106	0
Polyphenol oxidase	188 \pm 7	230 \pm 100	0
Exochitinase (chitobioside)	6909 \pm 128	0	0
Exochitinase (β -N-acetylglucosaminidase)	4286 \pm 192	0	729 \pm 103
Endochitinase	6717 \pm 192	0	0

2.3.9 Immunofluorescence localization of oxalate oxidase (OXO), xylanase inhibitor protein-I (XIP-I) and pathogenesis-related protein 4 (PR-4)

Affinity-purified antibodies to OXO, XIP-I and PR-4 were used to confirm the localization of these proteins using cross sections of wheat grain from cv. Babbler (Figure 2.4). OXO had a mosaic distribution throughout the outer layers (Figure 2.4 B). XIP-I was found predominantly in the nucellar tissue (Figure 2.4 C) immediately adjacent to the inner layer. PR-4 was found predominantly within the inner layer but was also distributed throughout the intermediate bran layers, including the testa (Figure 2.4 D). Immunolocalization of these proteins in two other wheat varieties, Chara and Wedgetail, confirmed that the localization was consistent across genotypes, although the intensity of labeling and hence the relative levels of these proteins appears to vary considerably between varieties and developmental stages (Figure 2.5).

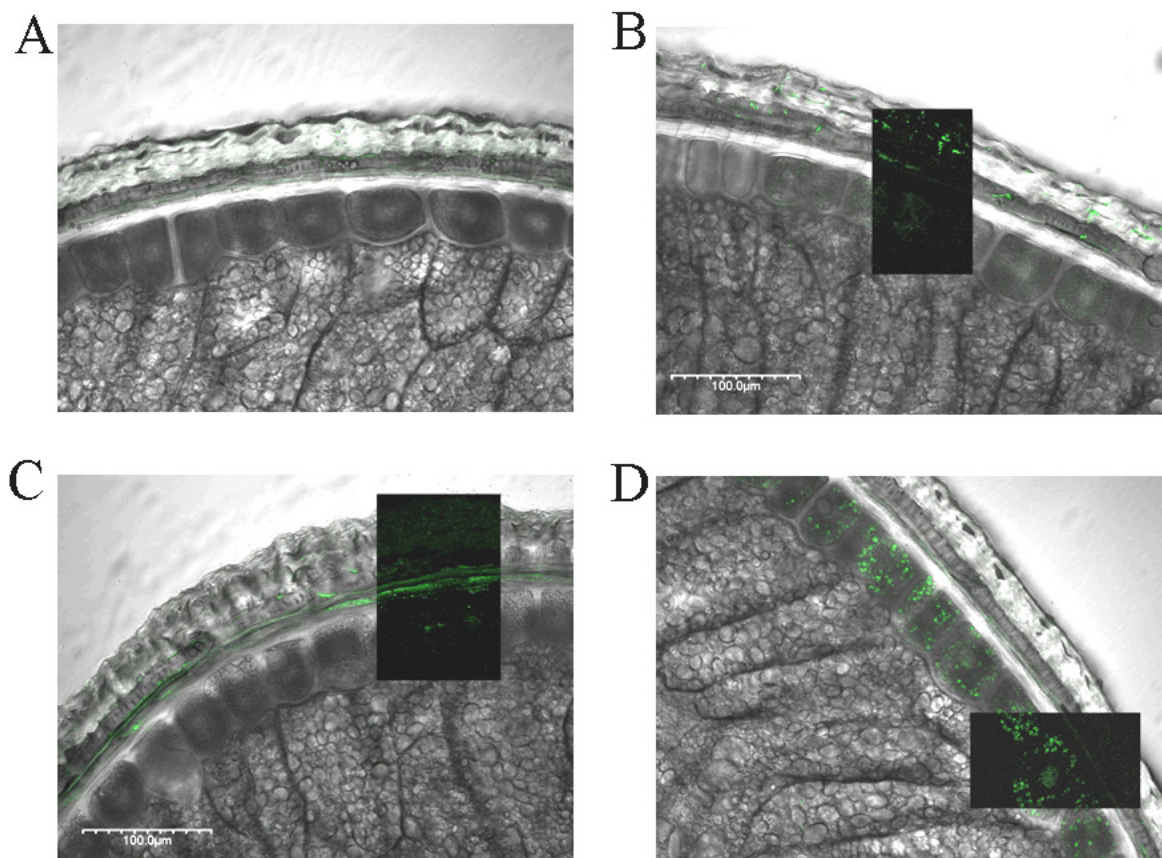


Figure 2.4. Fluorescence immunolocalization of defense proteins in bran cross-sections overlaid on DIC images of cross-sections (labelled). Dark inset overlays in images show fluorescence labeling without DIC overlay. (A) Control treated only with secondary antibody. (B) Oxalate oxidase antibody. (C)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

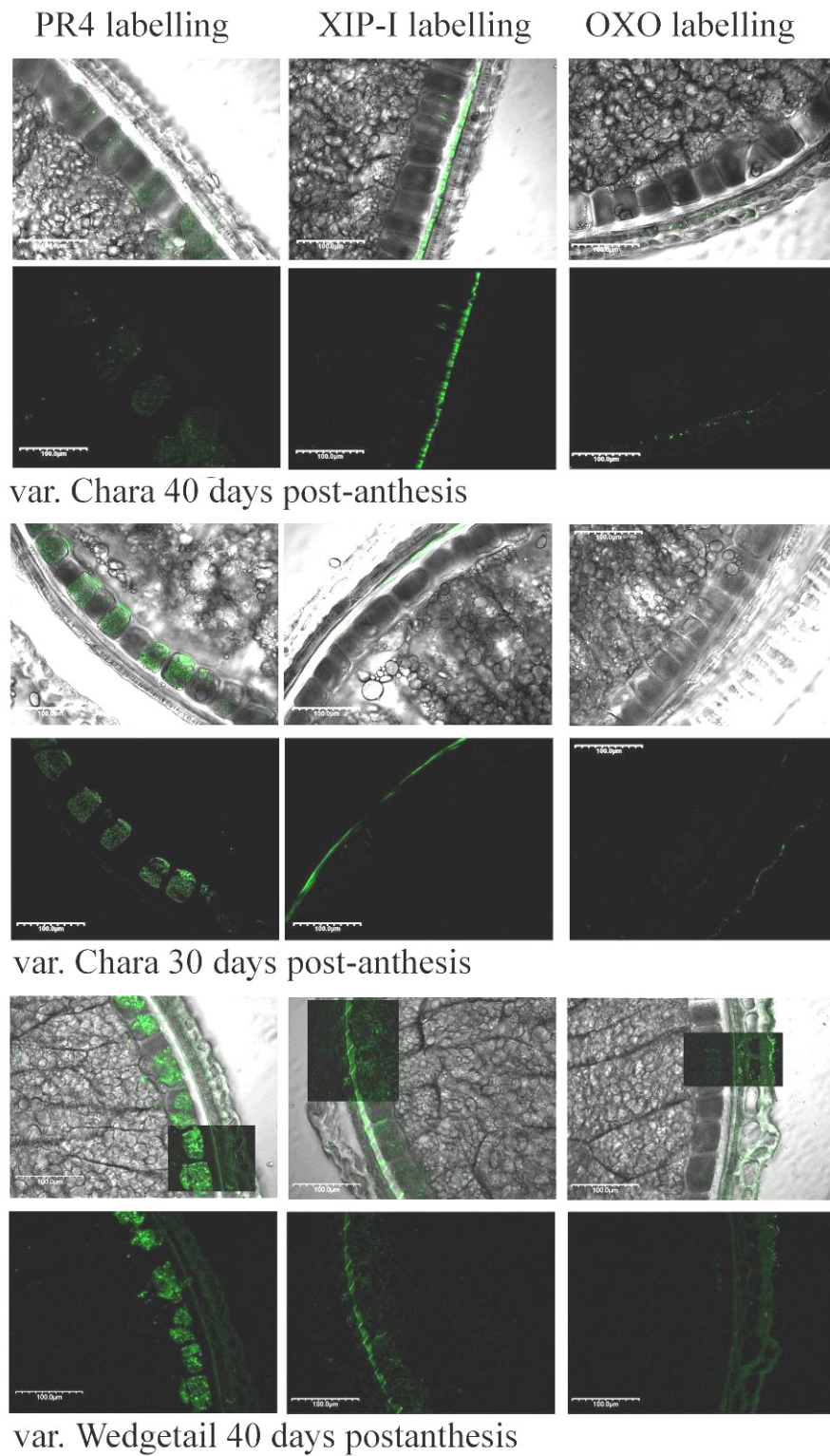


Figure 2.5. Immunofluorescence localisation of PR4, XIP-1 and OXO in wheat varieties Chara and Wedgetail. Images arrayed in alternating rows of the DIC-overlaid with immunofluorescence image, followed by immunofluorescence only image.

2.4 Discussion

Proteomic analysis of bran tissue fractions from wheat (*Triticum aestivum* L.) grain revealed the location and distribution of many common plant defense-related proteins, which appear to be specific to certain tissue layers within the bran. The proteins identified within the outer layers (epidermis and hypodermis) (Figure 2.3 A; Supplemental Tables S1A and S1B) provide resistance to fungal and bacterial colonization and so fulfil a general defensive role rather than targeting specific biotic stresses. For example, oxalate oxidase (OXO) can degrade a fungus-derived toxin (oxalic acid) and produce hydrogen peroxide as an antifungal agent (Lane, 2002). The location of OXO was also confirmed by confocal immunomicroscopy, showing its distribution throughout these outer layers (Figures 2.4 B and 2.5). Other proteins identified in the outer fraction, and the water-soluble extract from this fraction, have similar general defense roles. These outer layer proteins include peroxidase (POX), which in the presence of hydrogen peroxide may act to strengthen cell walls through lignin cross-linking, thus preventing fungal penetration (as in the oxidative burst response in live plant tissue) (Mellersh et al., 2002); polyphenol oxidase (PPO), which confers innate immunity against microorganisms in a number of plants (Niranjan Raj et al., 2006); polycystin-1 lipxygenase alpha-toxin (PLAT), which is expressed in acquired immune response against plant pathogens (Gorlach et al., 1996); and lipid transfer protein, which defends against fungi and bacteria (Blein et al., 2002; Breiteneder and Mills, 2005). Also, the activity of the enzymes OXO, POX and PPO was readily detectable in the supernatant from imbibed outer tissue layers and whole grain, suggesting that their protective role is enhanced by their mobility and stability in the aqueous phase (Table 2.2).

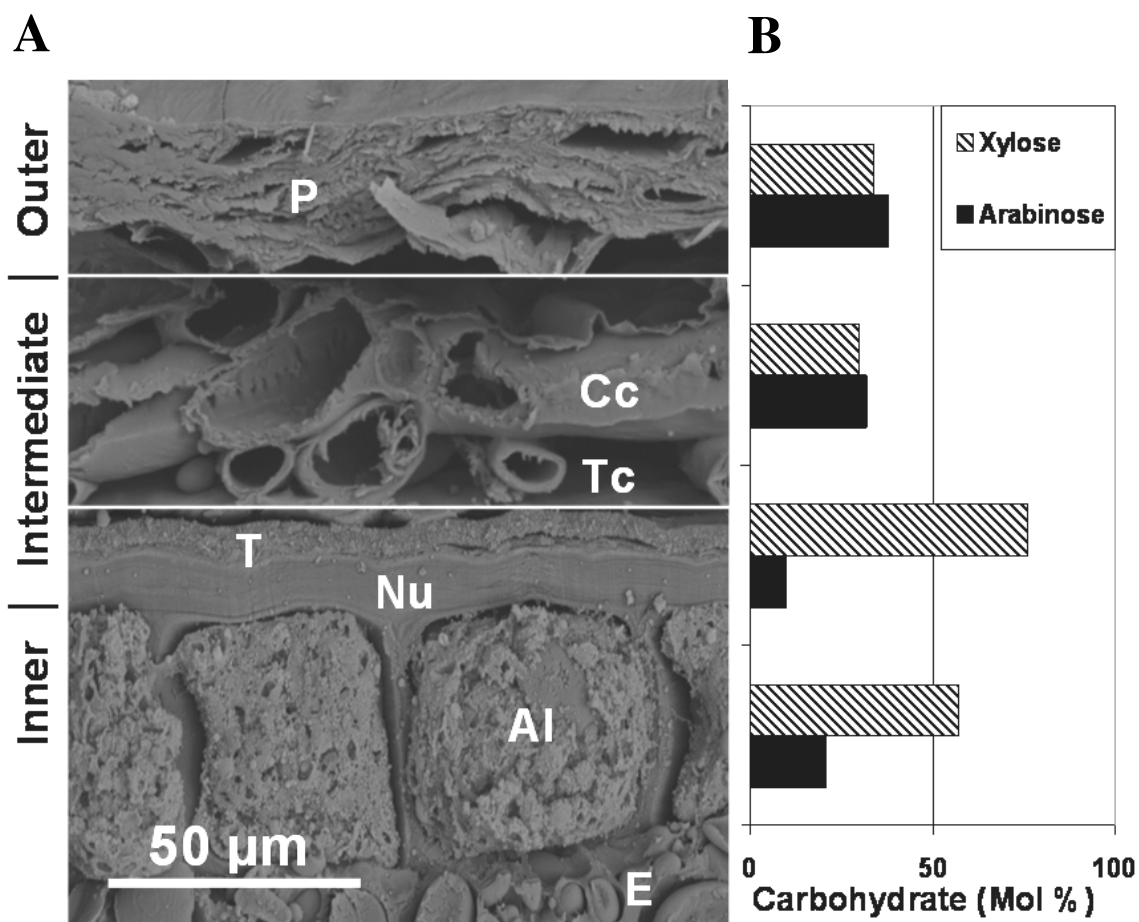


Figure 2.6. Scanning electron micrograph (SEM) of a cross-section of bran showing the bran tissue layers with corresponding xylose and arabinose content. (A) Outer (P, Pericarp), Intermediate (Cc, Cross-cells; Tc, Tube-cells; T, Testa (seed coat); Nu, Nucellar tissue), and Inner (Al, Aleurone cells;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

The cell layers of the intermediate fraction (cross cells, tube cells, testa and nucellar tissue) are the last line of defense against fungal hyphae, penetrating the metabolically active inner fraction (aleurone cells) and underlying endosperm. The protein complement of these intermediate fraction layers (Figure 2.3 A; Supplemental Tables S1C and S1D) revealed an array of defense-related enzymes and inhibitor proteins which, together, form a challenging environment for invading microorganisms.

The two xylanase inhibitor-type proteins, xylanase inhibitor protein-I (XIP-I) and *Triticum aestivum* xylanase inhibitor (TAXI), were identified in the intermediate fraction. XIP-I inhibits only fungal xylanases, whereas TAXI inhibits both fungal and bacterial xylanases (Gebruers et al., 2002). XIPs were first characterized as being the most abundant inhibitor-type proteins in wheat (McLauchlin et al., 1999; Goesaert et al., 2004). Their location within the grain has not been explicitly identified, however extraction and purification of this protein is generally from wheat flour (Gebruers et al., 2001). Other reported proteomic analyses of wheat grain did not identify these proteins in the endosperm (Skylas et al., 2000) or embryo (germ) (Mak et al., 2006a), suggesting they are likely to be exclusively located in the bran. Our proteomic and confocal immunomicroscopy results show that XIP-I is highly concentrated within the nucellar layer (Figures 2.4 C and 2.5). Based on this result, TAXI is also likely to be located within this layer and the inner layer (Figure 2.3 A). The carbohydrate composition of wheat bran (shown in Figure 2.6) clearly indicates that the nucellar layer has a higher ratio of xylose to arabinose than all the other bran tissues (Parker et al., 2005), which means that this tissue is highly susceptible to xylanase degradation. Thus the location of XIPs in these high-xylose tissues highlights their importance in protecting the inner layer and endosperm from biotic attack.

The intermediate fraction also contains a series of pathogenesis-related (PR) proteins, which are induced by the defense-signalling elicitor molecules, methyl jasmonate and ethylene, in response to biotic and abiotic stress (Mauch and Staehelin, 1989; Yu and Muehlbauer, 2001; Desmond et al., 2006). Of these PR proteins, the PR-3 chitinases were well represented in the intermediate fractions, as both precursors and mature proteins. PR-3 chitinases are fungal growth inhibitor proteins, which hydrolyze the chitin of the fungal cell walls and so disrupt hyphal entry to the live inner fraction and endosperm (Selitrennikoff, 2001; Singh et al., 2007). However, the PR-2 class II chitinase (β -1,3-glucanase) identified here and in the inner layer has a different function (Mauch and Staehelin, 1989). Rather than primarily inhibiting fungal growth, this enzyme has a signaling role, releasing elicitor molecules from invading fungal cell walls. These elicitor molecules induce the expression of both PR-2 and PR-3 proteins, which are concentrated in the vacuole. The vacuole bursts when fungal hyphae penetrate the cell, releasing a high concentration of these chitinases (Mauch and Staehelin, 1989; Singh et al., 2007). The PR proteins wheatwin1 (PR-4) and thaumatin-like protein (PR-5), are also both inducible as part of a chemically elicited defense response (Desmond et al., 2006). PR-4 has a well-described structure (Caporale et al., 1999) and is induced in response to fungal attack (Caruso et al., 1999). Immunolocalization of PR-4 indicated its presence primarily within the testa of the intermediate fraction and the inner fraction (Figures 2.4 D and 2.5). The PR-5 protein is strongly anti-fungal in common monocots (Juge, 2006), killing hyphae by one or more mechanisms involving cell wall assembly or water relations (Selitrennikoff, 2001). Another defense protein identified in the intermediate fraction is the wheat chemically-induced-5 (WCI-5) protein. It is induced by benzo (1, 2, 3) thiadiazole-7-carbothionic acid *S*-methyl ester (BTH) to elicit systemic acquired resistance (SAR) in plants (Schaffrath et al., 1997). The precise mode of action of this WCI-5 protein is unclear; however it does confer some resistance to powdery mildew but not to all fungal

pathogens (Gorlach et al., 1996; Yu and Muehlbauer, 2001). The spectrum of PR-and other defense-related proteins identified in the intermediate bran layer of wheat grain is certain to provide a hostile environment for invading hyphae. Identification of the inducible defense proteins in the dead cell layers suggests that they may be expressed in these cells during grain development, or are expressed in the aleurone cells and diffuse to the intermediate fraction upon hydration.

The inner fraction is associated with a much greater diversity of protein function than in the other bran layers (Supplemental Tables S1E and S1F). The majority of the proteins identified, corresponding to 150 of the 672 spots (22%), function in general metabolic activities such as protein synthesis, gene transcription and associated energy metabolism. However, the major protein class in terms of spot number (387 spots, 58%) was identified as the 7S globulin storage protein (globulin 3 protein) of the cupin superfamily. Although previously reported in wheat grain (Robert et al., 1985), this protein was not identified as a component in the endosperm (Skylas et al., 2000) or germ (Mak et al., 2006a) and has not been localized in wheat grain. It has been speculated that in addition to their storage protein function, 7S globulins may offer some protection against oxalic acid (Dunwell et al., 2000) produced by pathogenic fungi (Donaldson et al., 2001; Lane, 2002). It is also possible that some of our spots identified as 7S globulins may be the by-products of proteolytic processing to produce antimicrobial peptides, as has been found in other species (Marcus et al., 1999) and the large number of spots observed and the distinct MW groups found suggest that this may be a function in wheat as well. Another explanation for the large number of spots may be the accumulation of breakdown products of 7S globulin proteins during grain softening. Proteolytic degradation of proteins in our sample by endogenous proteases during grain softening could not be prevented because the cells were not lysed during tissue collection and therefore protease inhibitors could not be effectively

added. This grain softening inevitably triggers events associated with the very early stages of germination, which are known to involve the breakdown of specific storage proteins.

Two other biotic defense proteins that also appear as ‘major proteins’ in the inner fraction (Figure 2.3 A) are the class II chitinase, as described earlier, and a wheat α -amylase/subtilisin inhibitor (WASI). Alpha-amylase inhibitors were discovered in the 1970s (Deponte et al., 1976) and fall into several classes with a range of inhibitory activities against mammalian, invertebrate and, less commonly, endogenous α -amylases (Juge and Svensson, 2006). WASI, the wheat-specific form of this enzyme, has also been found in the endosperm in a previous study (Skylas et al., 2000). The family of α -amylase/subtilisin inhibitors has now been studied extensively and shown to inhibit a range of exogenous α -amylases but not all starch-degrading enzymes (Juge et al., 2004). Furthermore, α -amylase/subtilisin inhibitors disarm proteases from the subtilisin family, such as the subtilisin-like *Fusarium* proteinase (Pekkarinen and Jones, 2003).

A combination of bran layer microdissection, proteomics and immunomicroscopy has localized many proteins to specific wheat bran layers and placed them into a functional framework. The wheat bran proteome is predominantly a sophisticated defense structure which has evolved to fortify the bran layers and to protect the embryo and nutrient-rich endosperm.

Chapter 3 : Proteomic analysis of water-and water + abscisic acid (ABA)-conditioned grain

3.1 Introduction

Abscisic acid (ABA) is an endogenous phytohormone involved in various plant functions, such as grain quiescence/dormancy, biotic defense and in adverse environmental stress (Kucera et al., 2005; Rampino et al., 2006; Razem et al., 2006). Building on previous knowledge on how conditioning grain with water improves flour yield and quality, a recent patent by Atwell et al. (2007) claims that the addition of ABA to the conditioning water, further improves flour yield and quality. This suggests that ABA may change the biochemical properties of the live cells in the grain (such as the germ and aleurone cells) and impart some beneficial physical characteristic that improves milling quality. The mechanisms by which ABA improves flour yield and quality is not understood. Protein analysis after such treatments may reveal key proteins that are involved in the observed improvement.

The aim in this Chapter is to apply iTRAQ proteomics to determine the biochemical changes that occur after conditioning grain with water-and water + ABA, compared with non-conditioned grain as the control. The conditioned grain can be dissected into three major live tissue components, the bran (which will include the aleurone cells), germ and ventral groove. Proteomic analysis of these tissues was used to determine any biochemical changes that occur within and between these specific tissues. This is required for better understanding the initial biochemical changes that occur at germination and also, how ABA alters these changes.

3.2 *Materials and Methods*

Wheat grain *Triticum aestivum*, cv. Gregory (Australian hard wheat), harvested in 2008 was used for the primary analysis. This wheat was supplied by BRI Australia (now Grain Growers Limited) (1 Rivet Road, Riverside Corporate Park, North Ryde, NSW, Australia).

3.2.1 List of chemicals

3.2.1.1 *Plant hormone*

(+)-cis,trans-abscisic acid (ABA) (Sigma-Aldrich).

3.2.1.2 *Protein extraction*

Acetone (AJAX Finechem), glycerol (AJAX Finechem), 2-mercaptoethanol (Sigma-Aldrich), methanol (AJAX Finechem), SDS (Amresco), sucrose (AJAX Chemicals), trichloroacetic acid (TCA) (BDH Chemicals), Tris (Amresco), Tris-buffered phenol at pH 8.0 (Sigma-Aldrich).

3.2.1.3 *Protein solubilisation*

Triethylammonium bicarbonate (TEAB) (Sigma-Aldrich), urea (Bio-Rad), SDS (Amresco).

3.2.1.4 *Protein quantification*

Bovine serum albumin (BSA) (Sigma-Aldrich), Bradford reagent (Bio-Rad), G-250 Coomassie (Bio-Rad), methanol (Merck), 10-well 4-20% polyacrylamide gradient gels (Bio-Rad).

3.2.2 Determining grain moisture content

Grain moisture content was determined by weighing out approximately 10 g grain and placing them on a glass plate and left to dry in an oven at 60 °C until its mass stabilised. Grain was weighed at 24 h, 96 h and 168 h. The moisture level (%) was calculated using the equation, $100 - (\text{final mass}/\text{initial mass} \times 100)$.

3.2.3 Grain viability

Four layers of paper towel were placed in a zip lock plastic bag. Grains were evenly placed on top of the paper and 15 mL water was added. The zip lock bag was sealed and left on the bench at room temperature (~ 22 °C) for 48 h. Grains that were considered viable were those that exhibited the emergence of the coleoptile and roots.

3.2.4 ABA activity bioassay

ABA sensitivity was tested by germinating grains in water-and water + ABA. The germ-end of the grain was cut off (so as to not damage the germ) from 30 grains for each replicate and was placed on filter paper (exposed endosperm side down) in a petri dish. To each petri dish, 5 mL water or 5 mL water + 4 ppm ABA (as appropriate) was added. Each petri dish was sealed with Parafilm and left on the bench (~ 22 °C). Photographs and measurements of the coleoptile and root length were taken at each time point at 24 h, 48 h and 72 h to determine growth inhibition.

3.2.5 Grain conditioning

For each sample, 10 g of grain was weighed and placed into a 50-mL plastic tube. The grain was then conditioned with water-and water + 4 ppm ABA (ABA concentration in the conditioning liquid was adjusted to give a final concentration of 4 parts ABA to 1 million parts grain) according to standard milling practices. Sufficient water was added to each sample to increase the grain moisture content to ~ 16%. The required volume of conditioning water was determined by using the equation:

$$\text{Required conditioning water (mL)} = \left(\frac{[100 - \text{initial \% grain moisture}]}{[100 - \% \text{ final moisture}]} - 1 \right) \times \text{Mass of grain (g)}$$

Immediately following the addition of water, the tube was sealed and gently shaken for 1 min to evenly distribute the water. After shaking, the tube was left on the bench for ~ 16.5 h.

3.2.6 Tissue collection

Following the conditioning period, the germ, ventral groove and bran tissues were collected and stored at -20 °C.

The germ was manually dissected from the grain using a razor blade and with the aid of a dissecting microscope (Figure 3.1 A).

To remove the ventral groove, the grain was split in half longitudinally from the dorsal side and through the ventral groove using a razor blade. It was then dissected out using a razor blade with the aid of a dissecting microscope. Extra care was taken to remove as much ventral groove tissue as possible with minimal endosperm (Figure 3.1 B).

The remainder of grain (5 g) (Figure 3.1 C) was passed through a laboratory bench scale mill (RM 001). Bran was collected using a 1.18 mm sieve (Endecotts). The bran was further crushed in a mortar and pestle and passed through a fine sieve to remove as much endosperm as possible from bran.

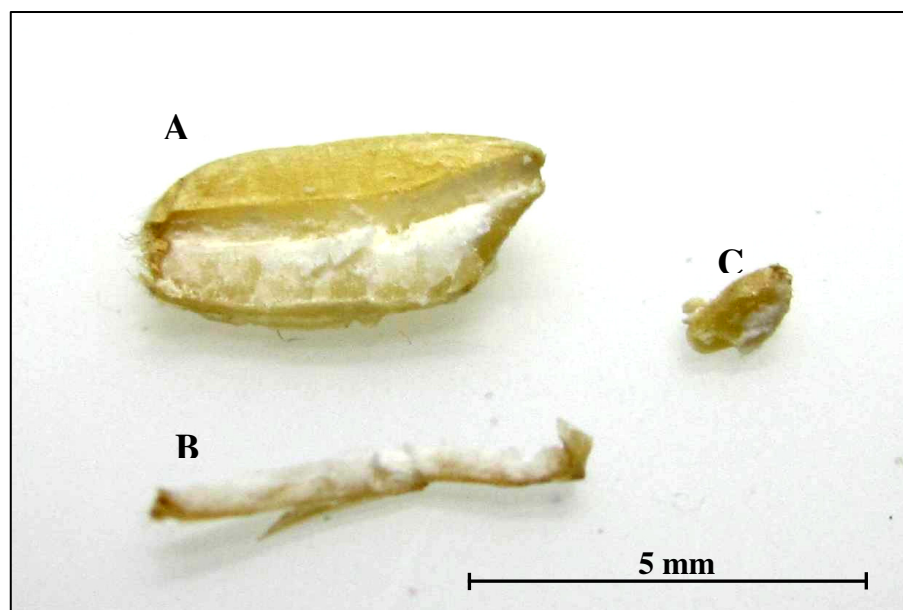


Figure 3.1. Micrograph of a longitudinal dissection of grain showing (A) bran and endosperm, (B) the removed ventral groove tissue and (C) germ.

3.2.7 Protein extraction

Proteins were extracted from each tissue sample (50 mg) by the method of Wang et al. (2003) described in Chapter 2 (Materials and Methods, 2.2.2). The only modification was 0.1 M ammonium acetate was excluded from the methanol when precipitating proteins from the phenol.

3.2.8 Protein estimation by SDS PAGE

Protein concentration was determined by SDS PAGE. Three pellets from each protein extract (precipitated from equal volumes of phenol from the phenol extraction) were placed in separate 1.5-mL plastic tubes. One pellet was solubilised into 6 M urea and the other in iTRAQ sample buffer (0.25 M TEAB, 0.1% SDS). The final pellet was kept in reserve. Protein estimations of the protein solubilised in 6 M urea were determined using Bradford reagent (Bradford, 1976). The protein concentration of the sample solubilised in 0.25 M TEAB, 0.1% SDS, was estimated by loading ~ 10 µg and ~ 20 µg (based on the concentration of protein solubilised in 6 M urea) of each sample into wells of the polyacrylamide gel. The gel was electrophoresed as per manufacturer's instructions. After electrophoresis, the gel was stained with Coomassie, and scanned using a flat bed scanner (Epson Perfection 2480 PHOTO). Densitometry, using ImageJ, version 1.34s (Rasband, 2008), was used for final correction of sample concentration (Appendix A, Figures A.7, A.8 and A.9).

3.2.9 iTRAQ experiments for non-conditioned, water-and water + ABA-conditioned grains

Protein pellets were solubilised in 0.25 M TEAB, pH 8.5, and 1% (w/v) SDS using a probe sonicator. The solubilised proteins from all tissue samples were processed, labeled and analysed according to the methods of Song et al. (2008) using ~ 75 µg of protein per sample.

3.2.9.1 Experiment 1

Isobaric tags 114 and 117 were allocated to protein extracts of the germ from non-conditioned grain (control). Isobaric tags 115 and 116 were allocated to protein extracts of the germ from water-conditioned and water + 4 ppm ABA-conditioned grain respectively (Figure 3.2).

3.2.9.2 Experiment 2

Isobaric tags 114, 115 and 116 were allocated to protein extracts of the bran from non-conditioned grain (control), water-conditioned and water + 4 ppm ABA-conditioned grain respectively. Isobaric tag 117 was allocated to protein extract of the ventral groove from non-conditioned grain (Figure 3.2).

3.2.9.3 Experiment 3

Isobaric tags 114, 115 and 116 were allocated to protein extracts of the ventral groove from non-conditioned grain (control), water-conditioned and water + 4 ppm ABA-conditioned grain respectively. Isobaric tag 117 was allocated to protein extract the of bran from non-conditioned grain (Figure 3.2).

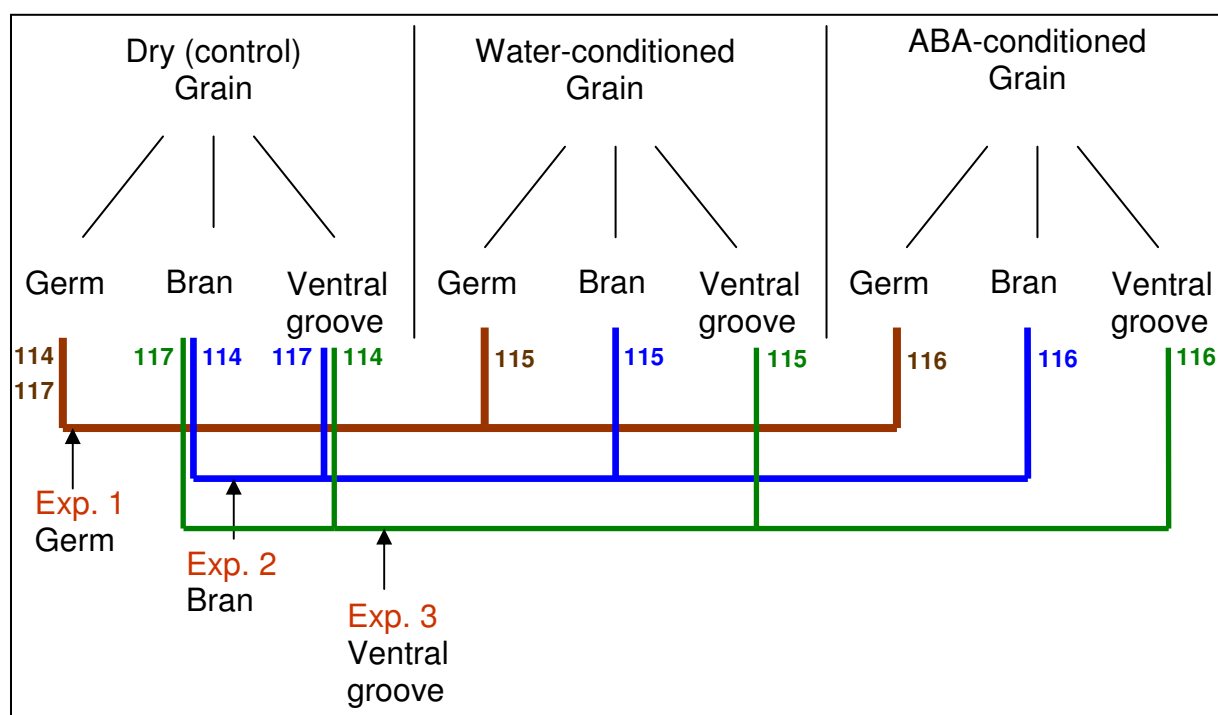


Figure 3.2. Experiment flow chart for each of the three grain tissues – germ (Experiment 1), bran (Experiment 2) and ventral groove (Experiment 3) – from the control (mature non-conditioned grain), water-conditioned and water + 4 ppm ABA-conditioned grain, showing the allocation of the iTRAQ labels 114,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

3.3 Results

3.3.1 Grain moisture level

Moisture rapidly decreased after 24 h at 60 °C from 10.006 g to 9.132 g. This was followed by a slower decrease to 9.05 g at 96 h. Finally, it slightly decreased to 9.042 g at 168 h. The calculated moisture content of the cultivar Gregory is, $100 - (9.042/10.006 \times 100) = 9.63 \%$.

3.3.2 Grain viability and ABA activity bioassay

3.3.2.1 Grain viability

Out of 100 grains tested three did not germinate, therefore the estimated grain viability for cultivar Gregory is 97 %.

3.3.2.2 Coleoptile and root growth of grain in water, 4 ppm and 40 ppm ABA

Grain in water germinated and grew rapidly from 0 – 72 h with the root growing much faster than the coleoptile. In contrast, the coleoptile and root of grain in 4 ppm ABA had ~ 1/3 and the coleoptile had ~ 1/2 of the overall growth rate respectively. The coleoptile and root of grain in 40 ppm ABA exhibited almost no growth in either the root or the coleoptile (Figures 3.3 A – I, Appendix A, Figures A.1 – 9 and Table 3.1).

The coleoptile growth of grain in water was 16.3-fold at 24 h, 5.4-fold at 48 h and 3.5-fold at 72 h higher when compared to grain in 4 ppm ABA. However, the root growth was less affected, 4.3-fold at 24 h, 2.6-fold at 48 h and 1.9-fold at 72 h higher when compared to grain in 4 ppm ABA.

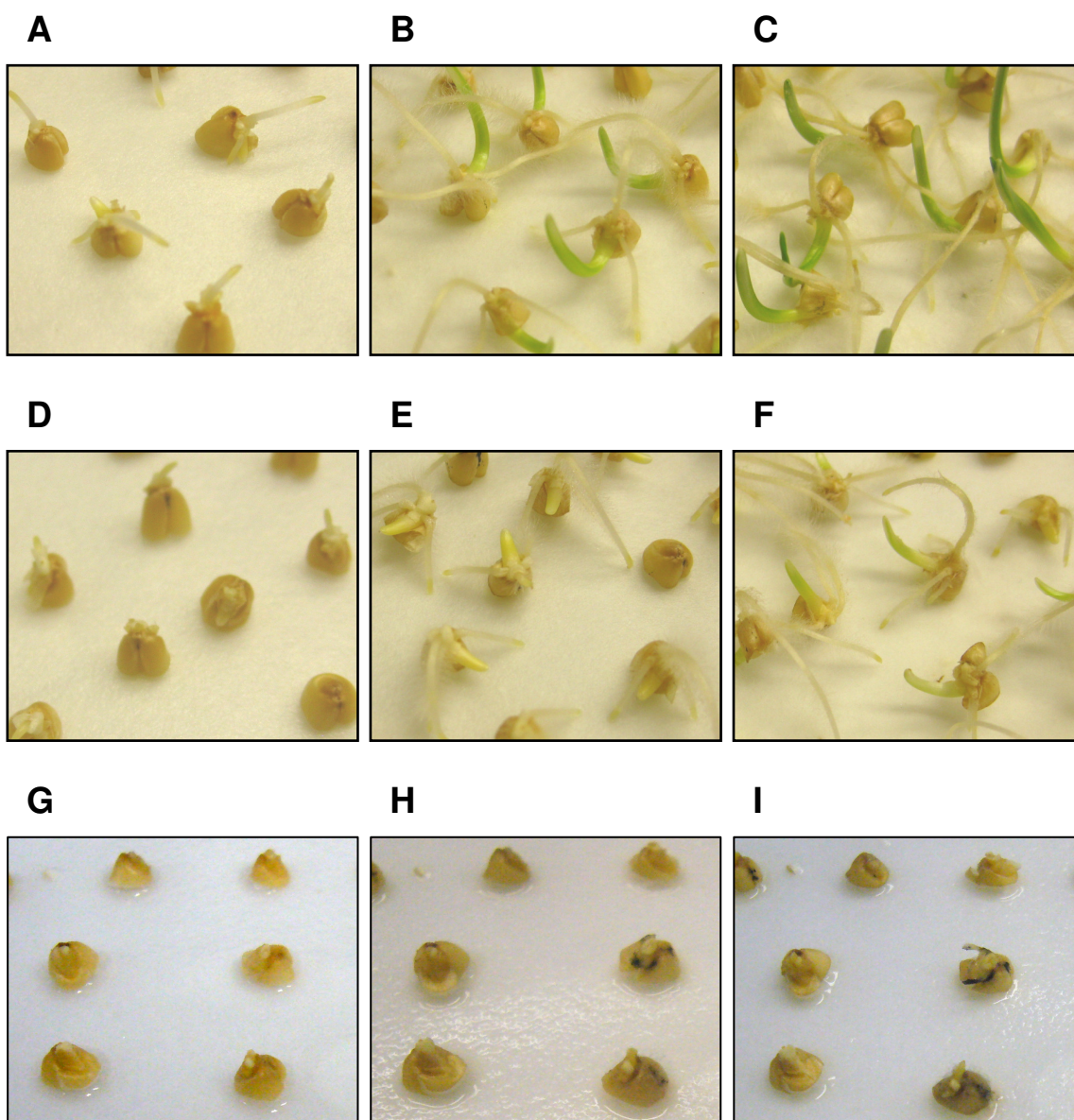


Figure 3.3. Bioassay of wheat to test ABA activity. Grain treated with water after (A) 24 h, (B) 48 h and (C) 72 h. Grain treated with 4 ppm ABA in water after (D) 24 h, (E) 48 h and (F) 72 h. Grain treated with 40 ppm ABA in water after (G) 24 h, (H) 48 h and (I) 72 h.

Table 3.1. Average coleoptile and root length and ratio of coleoptile and root length of water-and water + 4 ppm ABA treated grain.

Coleoptile length (mm)			
Time (h)	24	48	72
Water	0.54	6.08	12.93
4 ppm ABA	0.03	1.13	3.68
40 ppm ABA	0.02	0.075	0.34
Root length (mm)			
Time (h)	24	48	72
Water	3.38	17.85	23.33
4 ppm ABA	0.78	6.88	12.38
40 ppm ABA	0.17	0.23	0.33

3.3.3 Protein concentration estimation

Protein concentration of the germ protein extract was estimated to be $\sim 1 \mu\text{g}.\mu\text{L}^{-1}$ (Appendix A, Figure A.10) and bran and ventral groove were both $\sim 2 \mu\text{g}.\mu\text{L}^{-1}$ each (Appendix A, Figure A.11 – 12).

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

3.3.4.1 Germ

The number of changes in protein levels in the germ tissue from water + ABA-conditioned grain was significantly higher than the germ tissue from water-conditioned grain ($p = 0.0067$) (Figure 3.4).

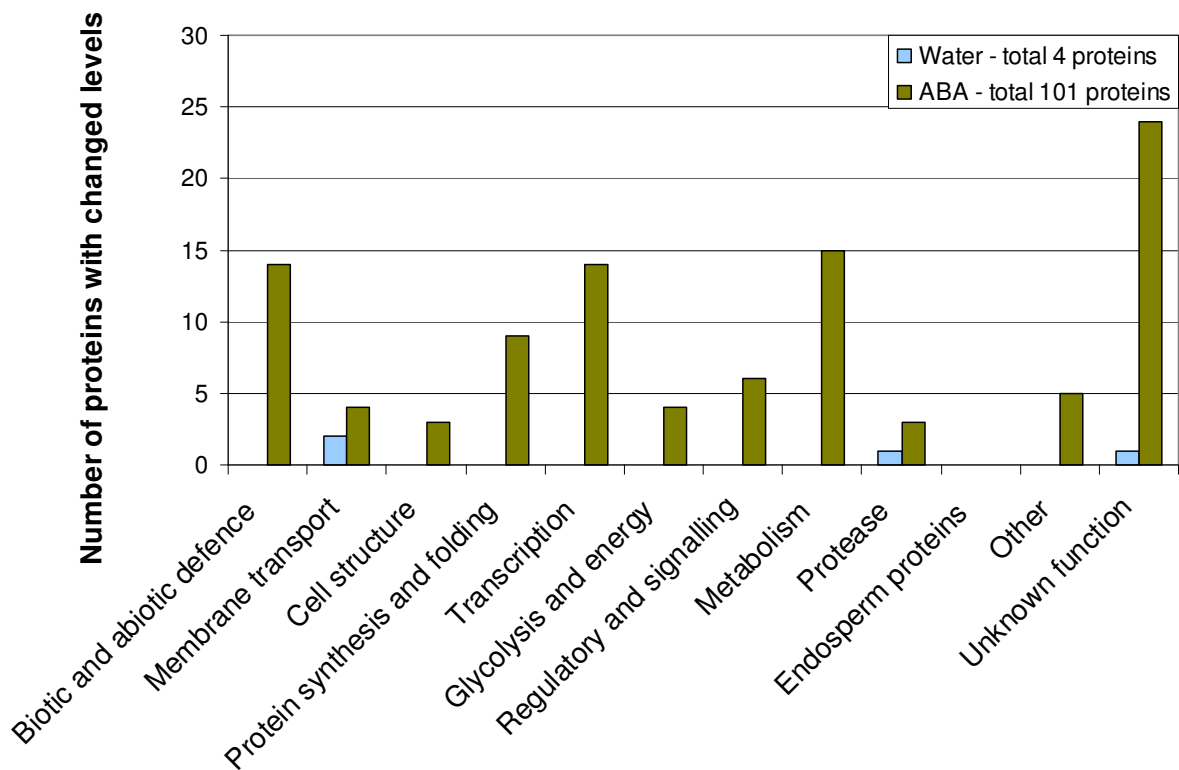


Figure 3.4. Protein level changes in the germ from water-and water + ABA-conditioned grain compared with germ from non-conditioned grain.

3.3.4.2 Bran

The number of changes in protein levels in the bran tissue from water + ABA-conditioned grain was significantly higher than the bran tissue from water-conditioned grain ($p = 0.041$) (Figure 3.5).

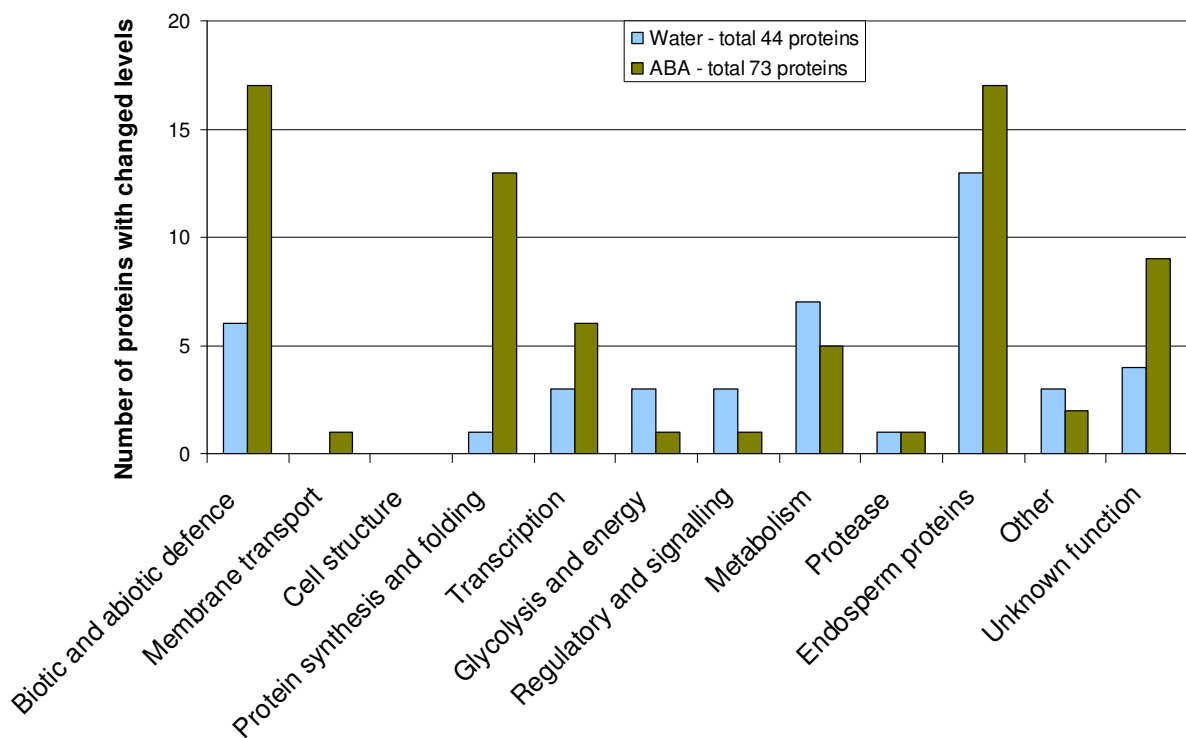


Figure 3.5. Protein level changes in the bran from water-and water + ABA-conditioned grain compared with germ from non-conditioned grain.

3.3.4.3 Ventral groove

The number of changes in protein levels in the ventral groove tissue from water-and water + ABA-conditioned grain were not significantly different from each other ($p = 0.056$) (Figure 3.6).

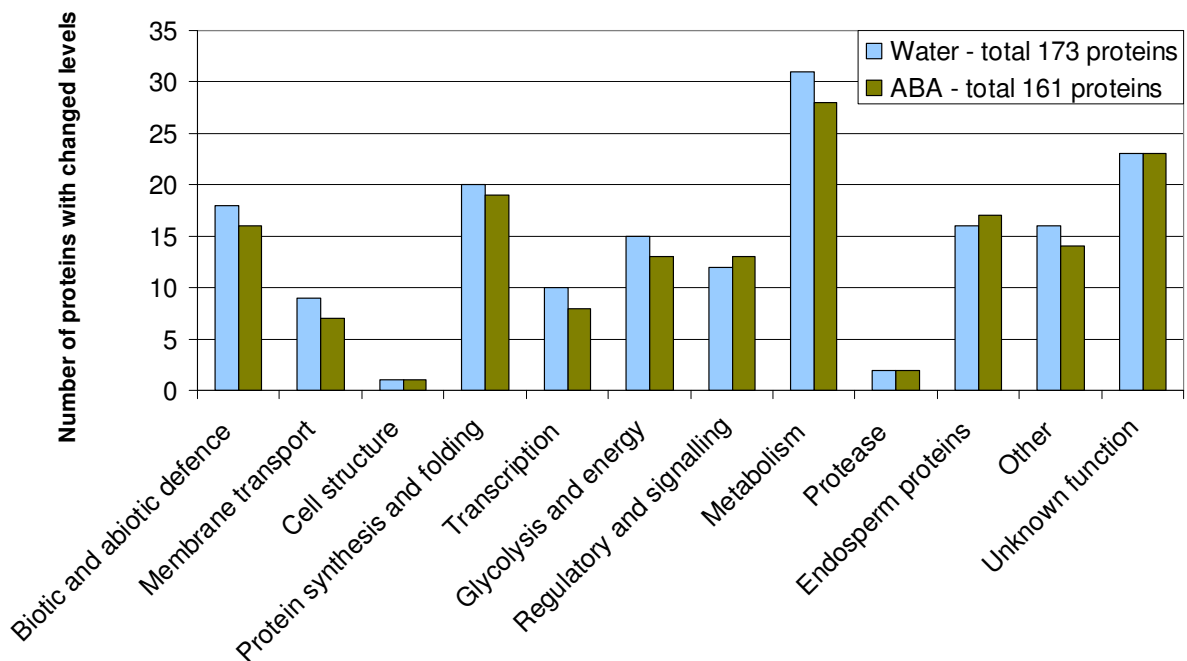


Figure 3.6. Protein level changes in the ventral groove from water-and water + ABA-conditioned grain compared with ventral groove from non-conditioned grain.

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

Changes in protein levels in the germ were minimal while the majority of changes in protein levels occurred in the bran and especially ventral groove tissues from water-conditioned grain.

The germ tissue, microdissected from water-conditioned grain, identified only four changes in protein levels compared to germ tissue from non-conditioned grain. Out of those, three proteins had increased and one had decreased (Figure 3.7 and Table 3.2).

The bran tissue collected from water-conditioned grain, identified 44 changes in protein levels compared to bran tissue from non-conditioned grain. The bran tissue from the water-conditioned grain had 12 proteins that increased and 32 that decreased (Figure 3.7 and Table 3.2).

The ventral groove tissue microdissected from water-conditioned grain identified 173 changes in protein levels compared to the ventral groove tissue from non-conditioned grain. The ventral groove tissue from water-conditioned grain had 92 proteins that increased and 81 that decreased (Figure 3.7 and Table 3.2).

The pattern of changes in protein levels in the germ, bran and ventral groove tissues from water-conditioned grain were significantly different between the tissue types – germ and bran ($p = 0.0046$), germ and ventral groove ($p = 0.0009$), and bran and ventral groove ($p =$

0.0067). All proteins that were identified with changes in levels were grouped according to their protein class (Table 3.2 and Appendix B, Tables B.1 – B.3).

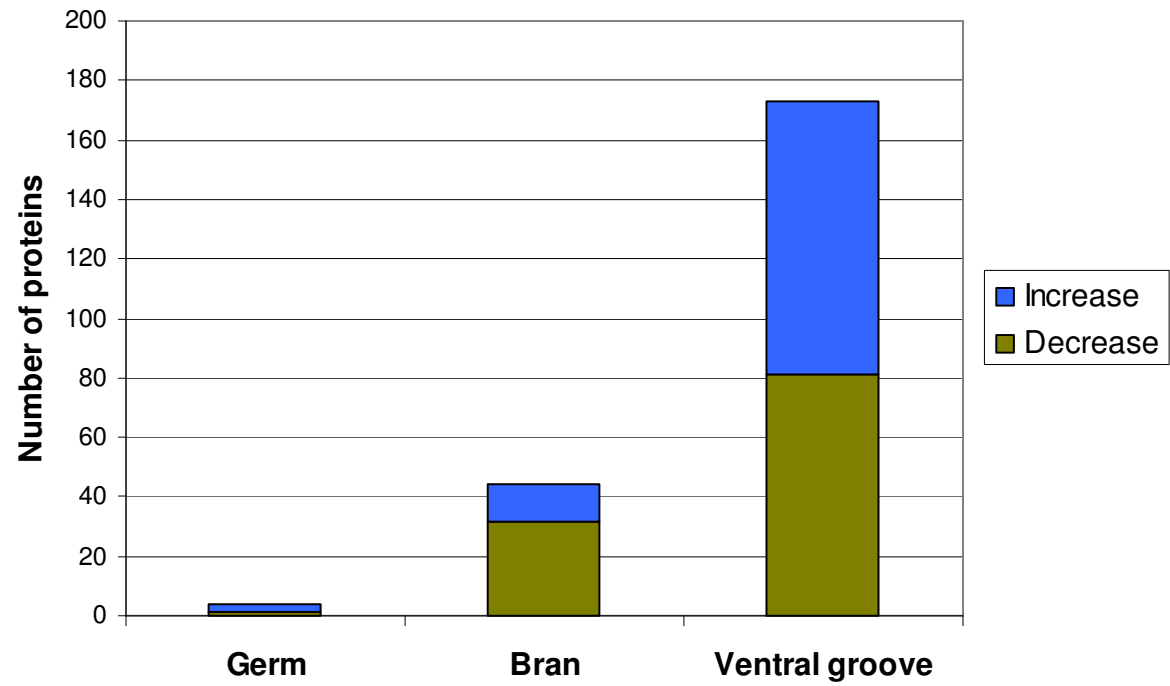


Figure 3.7. Changes in protein levels in the germ, bran and ventral groove tissues after water conditioning (total $n = 221$).

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.

Protein class	Germ Water		Bran Water		Ventral groove Water	
	Up	Down	Up	Down	Up	Down
Biotic and abiotic defense	0	0	0	6	12	6
Membrane transport	1	1	0	0	6	3
Cell structure	0	0	0	0	1	0
Protein synthesis and folding	0	0	1	0	12	8
Transcription	0	0	1	2	3	7
Glycolysis and energy	0	0	2	1	7	8
Regulatory and signalling	0	0	1	2	4	8
Metabolism	0	0	2	5	19	12
Protease	1	0	1	0	2	0
Endosperm-related proteins	0	0	2	11	11	5
Other	0	0	0	3	7	9
Unknown	1	0	2	2	8	15
Total number of proteins	3	1	12	32	92	81

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

The germ tissue, microdissected from water + ABA-conditioned grain, identified 101 proteins with level changes compared to germ tissue from non-conditioned grain. Out of those, 56 proteins had increased and 45 had decreased (Figure 3.8 and Table 3.3).

The bran tissue collected from water + ABA-conditioned grain, identified 73 proteins with level changes compared to bran tissue from non-conditioned grain. Of these, 20 proteins had increased and 53 had decreased (Figure 3.8 and Table 3.3).

The ventral groove tissue microdissected from water + ABA-conditioned grain, identified 161 proteins with level changes compared to the ventral groove tissue from non-conditioned grain. Out these, 82 proteins had increased and 79 proteins had decreased (Figure 3.8 and Table 3.3).

Proteomic analysis of the three grain tissues revealed that the ventral groove tissue was significantly more active in terms of total changes in protein levels compared with the germ tissue ($p = 0.0043$) and bran tissue ($p = 0.0028$). The bran and germ tissues were not significantly different from each other ($p = 0.305$). The identified proteins were grouped according to their protein class (Table 3.8 and Appendix B, Tables B.1 – B.3).

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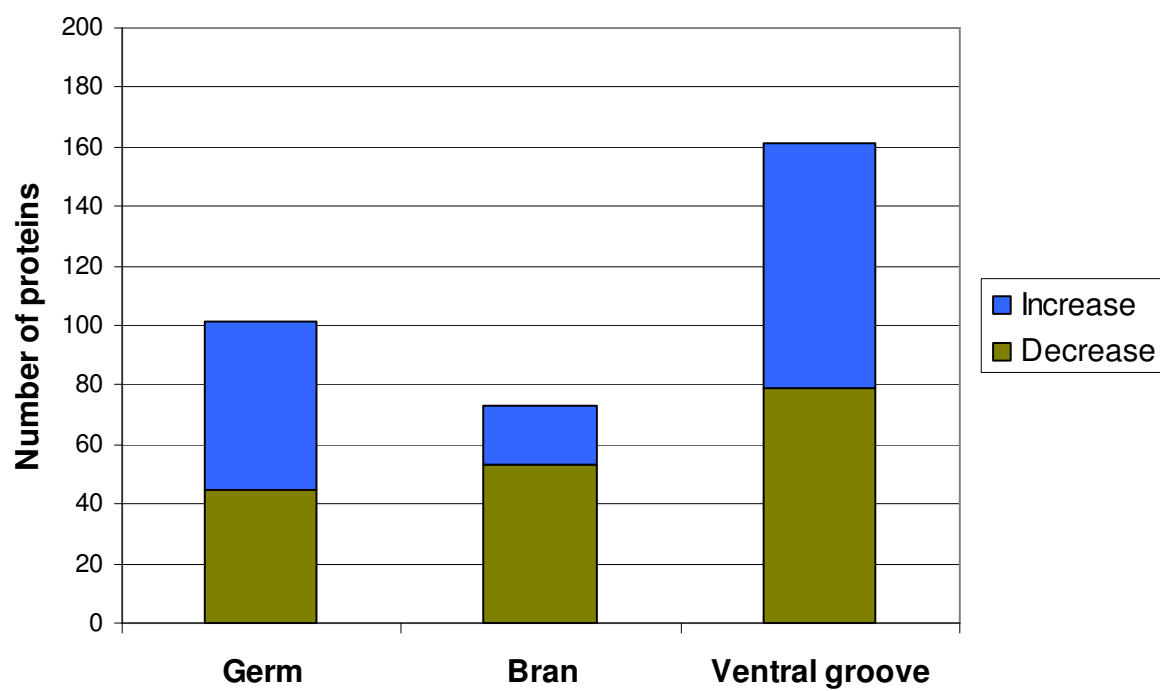


Figure 3.8. Changes in protein levels in the germ, bran and ventral groove tissues from water + ABA-conditioned grain compared to these tissue types from non-conditioned grain (total $n = 335$).

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.

Protein class	Germ ABA		Bran ABA		Ventral groove ABA	
	Up	Down	Up	Down	Up	Down
Bioitic and abiotic defense	9	4	4	13	10	6
Membrane transport	3	1	1	0	4	3
Cell structure	4	0	0	0	1	0
Protein synthesis and folding	4	5	4	9	12	7
Transcription	7	7	3	3	3	5
Glycolysis and energy	0	4	1	0	5	8
Regulatory and signalling	1	5	0	1	5	8
Metabolism	12	3	1	4	17	11
Protease	1	2	1	0	2	0
Endosperm-related proteins	0	0	1	16	10	7
Other	3	2	0	2	4	10
Unknown	12	12	4	5	9	14
Total number of proteins	56	45	20	53	82	79

3.3.7 Biotic and abiotic defense-related proteins

3.3.7.1 Germ, bran and ventral groove from water-conditioned grain

There were six changes in protein levels within this class of protein in the bran tissue from water-conditioned grain, all of which had decreased (Tables 3.2 and 3.4, and Appendix B, Table B.2). There were 18 changes in protein levels in the ventral groove tissue from water-conditioned grain. Of these, 12 had increased and six had decreased (Tables 3.2 and 3.4, and Appendix B, Table B.3).

The number of changes in protein levels within this class in the bran tissue and ventral groove tissue from water-conditioned grain were significantly different ($p = 0.0082$).

3.3.7.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

There were 14 changes in protein levels within this class in the germ tissue from water + ABA-conditioned grain. Of these, nine had increased and four had decreased (Tables 3.3 and 3.4, and Appendix B, Table B.1). There were 17 changes in protein levels in the bran tissue from water + ABA-conditioned grain. Of these, four had increased and 13 had decreased (Tables 3.3 and 3.4, and Appendix B, Table B.2). There were 16 changes in protein levels in the ventral groove tissue from water + ABA-conditioned grain, of which 10 had increased and six had decreased (Tables 3.3 and 3.4, and Appendix B, Table B.3).

The changes in protein levels within this class in the germ, bran and ventral groove tissues from water + ABA-conditioned grain were not significantly different from each other ($p > 0.05$).

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.

Biotic and abiotic defense proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
14-3-3 protein (ABA and stress induced).	-	-	*	-	-	-	-	-	-	-	-	-
Cold shock domain protein 2 (Putative glycine-rich protein).	-	-	*	-	-	-	*	-	-	-	-	-
Cold shock domain protein 3.	-	-	*	-	-	-	-	-	-	*	-	*
Late embryogenesis abundant protein (LEA) (Fragment).	-	-	*	-	-	-	-	-	-	-	-	-
Late embryonic abundant-like protein.	-	-	*	-	-	-	-	-	-	-	-	-
Peroxidase BP 1 precursor.	-	-	*	-	-	-	-	-	-	-	-	-
Putative bacterial-induced peroxidase (Class III peroxidase 86 precursor).	-	-	*	-	-	-	-	-	-	-	-	-
Putative disease resistance gene homolog 9N.	-	-	*	-	-	-	-	-	-	-	-	-
Putative late embryogenesis-abundant protein.	-	-	*	-	-	-	-	-	*	-	*	-
Betaine aldehyde dehydrogenase (synthesis of the osmoprotectant betaine)	-	-	-	*	-	-	-	-	-	-	-	-
Dehydrin DHN3 (B17).	-	-	-	*	-	-	-	-	-	-	-	-
OSJNBa0039K24.14 protein (Class III peroxidase 64 precursor).	-	-	-	*	-	-	-	-	-	-	-	-
Putative nodulin.	-	-	-	*	-	-	-	-	-	-	-	-
R40g3 protein (abiotic stress).	-	-	-	*	-	-	-	-	-	-	-	-
0.19 dimeric alpha-amylase inhibitor (Fragment) (Q5UHH6_WHEAT).	-	-	-	-	-	*	-	*	-	-	-	-
Alpha amylase inhibitor protein.	-	-	-	-	-	*	-	*	-	-	*	-
Pathogenesis-related protein 4 (Fragment).	-	-	-	-	-	*	-	-	-	-	-	-
Serpin (P93693_WHEAT).	-	-	-	-	-	*	-	*	*	-	*	-
Serpin (P93692_WHEAT).	-	-	-	-	-	*	-	*	*	-	*	-
Serpin (Q41593_WHEAT).	-	-	-	-	-	*	-	-	*	-	*	-
Em protein H2 (protective protein against desiccation, ABA induced).	-	-	-	-	-	-	*	-	-	-	-	-
Group 3 late embryogenesis abundant protein (Fragment).	-	-	-	-	-	-	*	-	-	-	-	-
Putative embryo-specific protein (LEA expressed).	-	-	-	-	-	-	*	-	-	-	-	-
0.19 dimeric alpha-amylase inhibitor (Fragment) (Q5UHH7_WHEAT).	-	-	-	-	-	-	-	*	-	-	-	-

(Continued next page)

Table 3.4. (Continued) 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.

Bioitic and abiotic defense proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Chitinase.	-	-	-	-	-	-	-	*	-	-	-	-
Endogenous alpha-amylase/subtilisin inhibitor (WASI).	-	-	-	-	-	-	-	*	-	*	-	*
Monomeric alpha-amylase inhibitor precursor.	-	-	-	-	-	-	-	*	-	-	-	-
Peroxidase 1.	-	-	-	-	-	-	-	*	-	-	-	-
PUP88 protein; member of trypsin/a-amylase inhibitors family from cereals precursor.	-	-	-	-	-	-	-	*	-	-	-	-
Thaumatococcus-like protein TLP7.	-	-	-	-	-	-	-	*	-	-	-	-
Xylanase inhibitor precursor.	-	-	-	-	-	-	-	*	-	-	-	-
Xylanase inhibitor protein 1 precursor (Class III chitinase homolog) (XIP-I protein).	-	-	-	-	-	-	-	*	-	-	-	-
Basic endochitinase C precursor (Rye seed chitinase-c) (RSC-c).	-	-	-	-	-	-	-	-	*	-	*	-
Cold regulated protein.	-	-	-	-	-	-	-	-	*	-	*	-
Putative activator of 90 kDa heat shock protein ATPase homolog 1.	-	-	-	-	-	-	-	-	*	-	-	-
Serpin (Q9ST58_WHEAT).	-	-	-	-	-	-	-	-	*	-	*	-
Subtilisin-chymotrypsin inhibitor.	-	-	-	-	-	-	-	-	*	-	-	-
Thaumatococcus-like protein.	-	-	-	-	-	-	-	-	*	-	*	-
Universal stress protein (USP) family protein.	-	-	-	-	-	-	-	-	*	-	-	-
UV-damaged DNA binding protein.	-	-	-	-	-	-	-	-	*	-	*	-
Basic endochitinase A precursor (Rye seed chitinase-a) (RSC-a).	-	-	-	-	-	-	-	-	-	*	-	*
Pathogenesis-related protein 4 precursor.	-	-	-	-	-	-	-	-	-	*	-	*
Putative stress related-like protein interactor.	-	-	-	-	-	-	-	-	-	*	-	*
Xylanase inhibitor TAXI-IV.	-	-	-	-	-	-	-	-	-	*	-	*

3.3.8 Membrane transport/transport proteins

3.3.8.1 Germ, bran and ventral groove from water-conditioned grain

There were two changes in protein levels within this class in the germ tissue from water-conditioned grain, one increase and one decrease (Tables 3.2 and 3.5, and Appendix B, Table B.1). There was no change in protein levels in the bran tissue from water-conditioned grain within this class (Tables 3.2 and 3.5, and Appendix B, Table B.2). There were nine changes in protein levels in the ventral groove tissue from water-conditioned grain, six had increased and three had decreased (Tables 3.2 and 3.5, and Appendix B, Table B.3).

There were significantly more changes in protein levels within this class in the ventral groove tissue compared to bran ($p = 0.018$) and germ ($p = 0.0007$) from water-conditioned grain. The number of changes in protein levels in the germ tissue and bran tissue from water-conditioned grain were not significantly different ($p = 0.155$).

3.3.8.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

There were four changes in protein levels within this class in the germ tissue from water + ABA-conditioned grain. Of these, three had increased and one had decreased (Tables 3.3 and 3.5, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain had an increase in one membrane transport/transport protein (Tables 3.3 and 3.5, and Appendix B, Table B.2). There were seven changes in protein levels in the ventral groove tissue from water + ABA treated grain. Of these, four had increased and three had decreased (Tables 3.3 and 3.5, and Appendix B, Table B.3).

The ventral groove tissue from the water + ABA-conditioned grain had significantly more changes in protein levels than in bran tissue ($p = 0.022$). The number of protein level

changes in the bran and ventral groove tissues from the water + ABA-conditioned grain were not significantly different from the germ tissue ($p = 0.166$ and $p = 0.322$ respectively).

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.

Membrane transport/transport proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Putative ATP-binding-cassette transporter protein.	*	-	*	-	-	-	-	-	-	-	-	-
Nonspecific lipid-transfer protein (LTP) (Fragment).	-	*	*	-	-	-	-	-	-	-	-	-
Putative vacuolar-type H ⁺ -translocating inorganic pyrophosphatase.	-	-	*	-	-	-	-	-	-	-	-	-
Delta tonoplast intrinsic protein TIP2;3 (aquaporin).	-	-	-	*	-	-	-	-	-	-	-	-
Synaptobrevin-like protein.	-	-	-	-	-	-	*	-	-	-	-	-
F1-ATPase (Fragment).	-	-	-	-	-	-	-	-	*	-	*	-
GTP-binding protein Rab6.	-	-	-	-	-	-	-	-	*	-	-	-
Predicted mitochondrial carrier protein.	-	-	-	-	-	-	-	-	*	-	*	-
Putative coatomer protein gamma 2-subunit.	-	-	-	-	-	-	-	-	*	-	-	-
Putative siderophore receptor.	-	-	-	-	-	-	-	-	*	-	*	-
Rab protein.	-	-	-	-	-	-	-	-	*	-	*	-
D-serine/D-alanine/glycine transporter.	-	-	-	-	-	-	-	-	-	*	-	*
Putative adapter-related protein complex 4 epsilon 1 subunit.	-	-	-	-	-	-	-	-	-	*	-	*
Ran.	-	-	-	-	-	-	-	-	-	*	-	*

3.3.9 Cell structure proteins

3.3.9.1 Germ, bran and ventral groove from water-conditioned grain

Only the ventral groove tissue from water-conditioned grain had an increased level in one cell structure protein (Tables 3.2 and 3.6, and Appendix B, Table B.3).

3.3.9.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had four changes in protein levels, all of which had increased (Tables 3.3 and 3.6, and Appendix B, Table B.1). There were no changes in protein levels in cell structure proteins in the bran from water + ABA-conditioned grain. The ventral groove from water + ABA-conditioned grain had an increase in one cell structure protein (Tables 3.3 and 3.6, and Appendix B, Table B.3).

There was significantly more changes in protein levels in germ tissue from the water + ABA-conditioned grain compared with bran ($p = 0.049$). The number of protein level changes in the germ and bran tissues were not significantly different from ventral groove tissue ($p = 0.26$ and $p = 0.341$ respectively).

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.

Cell structure proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Actin.	-	-	*	-	-	-	-	-	*	-	*	-
Drebrins and related actin binding proteins.	-	-	*	-	-	-	-	-	-	-	-	-
Profilin-1.	-	-	*	-	-	-	-	-	-	-	-	-

3.3.10 Protein synthesis and folding proteins

3.3.10.1 Germ, bran and ventral groove from water-conditioned grain

The bran tissue from water-conditioned grain had an increased level in one protein synthesis and folding protein (Tables 3.2 and 3.7, and Appendix B, Table B.2). The ventral groove tissue from water-conditioned grain had 20 changes in protein levels. Of these, 12 had increased and eight had decreased (Tables 3.2 and 3.7, and Appendix B, Table B.3).

The ventral groove tissue had significantly more changes in protein levels within this class than the bran tissue from water-conditioned grain ($p = 0.0067$).

3.3.10.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had nine changes in protein levels. Of these, four had increased and five had decreased (Tables 3.3 and 3.7, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain had 13 changes in protein levels. Of these, four had increased and nine had decreased (Tables 3.3 and 3.7, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had 19 changes in protein levels. Of these, 12 had increased and seven had decreased (Tables 3.3 and 3.7, and Appendix B, Table B.3).

The number of changes in protein levels in the ventral groove tissue from water + ABA-conditioned grain were significantly higher than the germ tissue ($p = 0.025$). There were no significant differences between protein level changes in the germ and ventral groove tissues from water + ABA-conditioned grain compared to bran tissue ($p = 0.364$ and $p = 0.177$ respectively).

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.

Protein synthesis and folding proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
2-cys peroxiredoxin BAS1, chloroplast precursor (Fragment).	-	-	*	-	-	-	-	-	-	-	-	-
40S ribosomal protein S21.	-	-	*	-	-	-	-	-	-	-	-	-
Chaperonin complex component.	-	-	*	-	-	-	-	-	-	-	-	-
Elongation factor 1 alpha (Q9M7E1_MAIZE).	-	-	*	-	-	-	-	-	-	-	-	*
40S ribosomal protein (Fragment).	-	-	-	*	-	-	*	-	-	-	-	-
Putative 60S ribosomal protein L31.	-	-	-	*	-	-	-	-	-	-	-	-
Putative CCT chaperonin gamma subunit.	-	-	-	*	-	-	-	-	-	-	-	-
Ribosomal protein L30.	-	-	-	*	-	-	-	-	-	-	-	-
Translation elongation factor EF-1 alpha/Tu.	-	-	-	*	-	-	-	-	-	-	-	-
Putative 60S ribosomal protein L27a.	-	-	-	-	*	-	*	-	-	-	-	-
60S ribosomal protein L7a.	-	-	-	-	-	-	*	-	-	-	-	-
Elongation factor 1-alpha (Q9M7E4_MAIZE).	-	-	-	-	-	-	*	-	-	-	-	-
Heat shock protein 90 (Fragment).	-	-	-	-	-	-	*	-	-	-	*	-
Putative 40S ribosomal protein S6.	-	-	-	-	-	-	*	-	-	-	-	-
Putative 60S ribosomal L28 protein (Q5TKP3_ORYSA).	-	-	-	-	-	-	*	-	-	-	-	-
Putative elongation factor 2.	-	-	-	-	-	-	*	-	-	-	-	-
Ribosomal protein L3.	-	-	-	-	-	-	*	-	-	-	-	-
Small heat shock protein HSP17.8.	-	-	-	-	-	-	*	-	-	-	-	-
Calnexin (Fragment).	-	-	-	-	-	-	-	*	-	-	-	-
HSP70 precursor.	-	-	-	-	-	-	-	*	-	-	-	-
Luminal-binding protein 3 precursor (BiP3).	-	-	-	-	-	-	-	*	-	-	-	-
EF-1 alpha (O49831_ORYSA).	-	-	-	-	-	-	-	-	*	-	*	-
Elongation factor 1 alpha (O50018_MAIZE).	-	-	-	-	-	-	-	-	*	-	*	-
Heat shock protein 82.	-	-	-	-	-	-	-	-	*	-	*	-
HSP70.	-	-	-	-	-	-	-	-	*	-	*	-

(Continued next page)

Table 3.7. (Continued) 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.

Protein synthesis and folding proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Putative 40S ribosomal protein S2.	-	-	-	-	-	-	-	-	*	-	*	-
Putative 60S ribosomal protein L4/L1.	-	-	-	-	-	-	-	-	*	-	*	-
Putative disulfide-isomerase.	-	-	-	-	-	-	-	-	*	-	-	-
Putative glycyl-tRNA synthetase.	-	-	-	-	-	-	-	-	*	-	*	-
Putative tRNA-glutamine synthetase.	-	-	-	-	-	-	-	-	*	-	-	-
Thioredoxin h.	-	-	-	-	-	-	-	-	*	-	*	-
Elongation factor 1 alpha (EF1A2_HORVU).	-	-	-	-	-	-	-	-	-	*	-	*
Elongation factor 1-alpha (Q6LAA4_HORVU).	-	-	-	-	-	-	-	-	-	*	-	*
Elongation factor 1-beta (EF-1-beta) (Elongation factor 1B-alpha 2) (eEF-1B alpha).	-	-	-	-	-	-	-	-	-	*	-	*
Heat shock protein HSP26.	-	-	-	-	-	-	-	-	-	*	-	*
Non-ribosomal peptide synthetase modules and related proteins.	-	-	-	-	-	-	-	-	-	*	-	*
Pre-mRNA processing factor.	-	-	-	-	-	-	-	-	-	*	-	-
Putative 60S ribosomal protein L28 (Q6K705_ORYSA).	-	-	-	-	-	-	-	-	-	*	-	-
Putative tRNA splicing protein.	-	-	-	-	-	-	-	-	-	*	-	*
40S ribosomal protein S24.	-	-	-	-	-	-	-	-	-	-	*	-
40S ribosomal protein S27 (Manganese efficiency-related protein 1).	-	-	-	-	-	-	-	-	-	-	*	-
Heat-shock protein DnaJ (Putative DnaJ-like protein).	-	-	-	-	-	-	-	-	-	-	*	-
Elongation factor 1 alpha (Q9M7E4_MAIZE).	-	-	-	-	-	-	-	-	-	-	-	*

3.3.11 Transcription-related proteins

3.3.11.1 Germ, bran and ventral groove from water-conditioned grain

There were three changes in protein levels within this class in the bran tissue from water-conditioned grain. Of these, one had increased and two had decreased (Tables 3.2 and 3.8, and Appendix B, Table B.2). There were 10 changes in protein levels in the ventral groove tissue from water-conditioned grain. Of these, three had increased and seven had decreased (Tables 3.2 and 3.8, and Appendix B, Table B.3).

The ventral groove tissue had significantly more changes in protein levels within this class than the bran tissue from water-conditioned grain ($p = 0.042$).

3.3.11.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had 14 changes in protein levels. Of these, half had increased and decreased (Tables 3.3 and 3.8, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain had six changes in protein levels. Also, half had increased and decreased (Tables 3.3 and 3.8, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had eight changes in protein levels. Of these, three had increased and five had decreased (Tables 3.3 and 3.8, and Appendix B, Table B.3).

The number of changes in protein levels within this class in the germ, bran and ventral groove tissues from the water + ABA-conditioned grain were not significantly different from each other (germ and bran, $p = 0.054$, germ and ventral groove, $p = 0.166$ and bran and ventral groove, $p = 0.576$).

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.

Transcription-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Diadenosine 5',5'''-P1,P4-tetraphosphate hydrolase.	-	-	*	-	-	-	-	-	-	-	-	-
FCA protein (Fragment).	-	-	*	-	-	-	-	-	-	-	-	-
Histone H2B.2.	-	-	*	-	-	-	-	-	-	-	-	-
MRNA splicing protein CDC5.	-	-	*	-	-	-	-	-	-	-	-	-
Putative high mobility group protein (Fragment).	-	-	*	-	-	-	-	-	-	-	-	-
Putative retroelement (Retrotransposon protein, putative, Ty3-gypsy sub-class).	-	-	*	-	-	-	-	-	-	-	-	-
Putative transcription elongation factor.	-	-	*	-	-	-	-	-	-	-	-	-
Histone H1.	-	-	-	*	-	-	-	-	-	-	-	-
Myb family transcription factor-like.	-	-	-	*	-	-	-	-	-	-	-	-
Putative DNA gyrase subunit.	-	-	-	*	-	-	-	-	-	-	-	-
Putative elongation factor 2.	-	-	-	*	-	-	-	-	-	-	-	-
Putative gag-pol (Retrotransposon protein, putative, Ty3-gypsy sub-class).	-	-	-	*	-	-	-	-	-	-	-	-
Putative histone H2A.	-	-	-	*	-	-	-	-	-	-	-	-
Retrotransposon protein, putative, Ty3-gypsy sub-class.	-	-	-	*	-	-	-	-	-	-	-	-
Putative histone H2A.	-	-	-	-	*	-	-	-	-	-	-	-
Putative fibrillarin.	-	-	-	-	-	*	-	-	-	-	-	-
Putative Glycine-rich protein 2.	-	-	-	-	-	*	-	-	-	-	-	-
Histone H3.	-	-	-	-	-	-	*	-	-	-	-	-
Nonsense-mediated mRNA decay 2 protein.	-	-	-	-	-	-	*	-	-	-	-	-
Protein H2A.	-	-	-	-	-	-	*	-	-	-	-	-
Putative iron inhibited ABC transporter 2.	-	-	-	-	-	-	-	*	-	-	-	-
Putative RNA helicase.	-	-	-	-	-	-	-	*	-	-	-	-
Superfamily II DNA/RNA helicases.	-	-	-	-	-	-	-	*	-	-	-	-
Putative copia-like retrotransposon Hopscotch polyprotein.	-	-	-	-	-	-	-	-	*	-	*	-
Putative gag-pol polyprotein.	-	-	-	-	-	-	-	-	*	-	*	-

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Table 3.8. (Continued) 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.

Transcription-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Ribosomal RNA apurinic site specific lyase, putative.	-	-	-	-	-	-	-	-	*	-	-	-
Eukaryotic translation initiation factor 3 subunit 10 (eIF-3 theta) (eIF3a).	-	-	-	-	-	-	-	-	-	*	-	*
Histone H3 (Fragment).	-	-	-	-	-	-	-	-	-	*	-	-
Nucleosome/chromatin assembly factor C.	-	-	-	-	-	-	-	-	-	*	-	*
Oxoprolinase.	-	-	-	-	-	-	-	-	-	*	-	-
Putative WD-40 repeat protein.	-	-	-	-	-	-	-	-	-	*	-	*
Transposon protein, putative, ping/pong/SNOOPY sub-class.	-	-	-	-	-	-	-	-	-	*	-	-
Transposon protein, putative, unclassified.	-	-	-	-	-	-	-	-	-	*	-	*
Retrotransposon protein, putative, Ty1-copia sub-class.	-	-	-	-	-	-	-	-	-	-	*	-
Structural maintenance of chromosome protein 3.	-	-	-	-	-	-	-	-	-	-	-	*

3.3.12 Glycolysis and energy proteins

3.3.12.1 Germ, bran and ventral groove from water-conditioned grain

There were three changes in protein levels within this class in the bran tissue from water-conditioned grain, two had increased and one had decreased (Tables 3.2 and 3.9, and Appendix B, Table B.2). There were 15 changes in protein levels in the ventral groove tissue from water-conditioned grain, seven had increased and eight had decreased (Tables 3.2 and 3.9, and Appendix B, Table B.3).

The ventral groove tissue had significantly more changes in protein levels within this class than the bran tissue ($p = 0.00137$).

3.3.12.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had four changes in protein levels, all of these had decreased (Tables 3.3 and 3.9, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain only had an increase in one glycolysis-and energy-related protein (Tables 3.3 and 3.9, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had 13 changes in protein levels. Of these, five had increased and eight had decreased (Tables 3.3 and 3.9, and Appendix B, Table B.3).

The ventral groove tissue had significantly more changes in protein levels than the germ and bran tissues from water + ABA-conditioned grain ($p = 0.015$ and $p = 0.0004$ respectively). The germ and bran tissues from water + ABA-conditioned grain had no significant difference in the number of protein level changes ($p = 0.171$).

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.

Glycolysis and energy proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Cytoplasmic aldolase.	-	-	-	*	-	-	-	-	-	-	-	-
Phosphoglucosylmutase; phosphomannomutase.	-	-	-	*	-	-	-	-	-	-	-	-
Putative alcohol dehydrogenase.	-	-	-	*	-	-	-	-	-	-	-	-
Pyruvate kinase, barrel domain.	-	-	-	*	-	*	-	-	-	-	-	-
Glucose-6-phosphate isomerase, cytosolic (GPI).	-	-	-	-	*	-	-	-	-	-	-	-
Putative NADH2 dehydrogenase (Ubiquinone) chain PSST.	-	-	-	-	*	-	-	-	-	-	-	-
Putative NADH dehydrogenase.	-	-	-	-	-	-	*	-	-	-	-	-
2,3-bisphosphoglycerate-independent phosphoglycerate mutase.	-	-	-	-	-	-	-	-	*	-	-	-
Cytosolic malate dehydrogenase (Fragment).	-	-	-	-	-	-	-	-	*	-	*	-
Enolase 2 (2-phosphoglycerate dehydratase 2) (2-phospho- D- glycerate hydro-lyase 2).	-	-	-	-	-	-	-	-	*	-	*	-
Glutaredoxin.	-	-	-	-	-	-	-	-	*	-	*	-
Glyoxysomal malate dehydrogenase (Fragment).	-	-	-	-	-	-	-	-	*	-	*	-
Putative malate dehydrogenase (Putative mitochondrial malate dehydrogenase).	-	-	-	-	-	-	-	-	*	-	*	-
Putative reductase.	-	-	-	-	-	-	-	-	*	-	-	-
Aldose reductase-related protein.	-	-	-	-	-	-	-	-	-	*	-	*
Cytosolic NADP malic enzyme.	-	-	-	-	-	-	-	-	-	*	-	*
Glucose-6-phosphate isomerase, cytosolic A.	-	-	-	-	-	-	-	-	-	*	-	-
Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3.	-	-	-	-	-	-	-	-	-	*	-	*
Glyceraldehyde-3-phosphate dehydrogenase. Xanthomonas oryzae pv. oryzae.	-	-	-	-	-	-	-	-	-	*	-	*
Putative fructose 1-,6-biphosphate aldolase (Fragment).	-	-	-	-	-	-	-	-	-	*	-	*
Putative glyceraldehyde-3-phosphate dehydrogenase (Phosphorylating).	-	-	-	-	-	-	-	-	-	*	-	*
Pyruvate orthophosphate dikinase (Fragment).	-	-	-	-	-	-	-	-	-	*	-	*
Putative fructose-bisphosphate aldolase.	-	-	-	-	-	-	-	-	-	-	-	*

3.3.13 Regulatory and signaling proteins

3.3.13.1 Germ, bran and ventral groove from water-conditioned grain

There were three changes in protein levels within this class in the bran tissue from water-conditioned grain, one had increased and two had decreased (Tables 3.2 and 3.10, and Appendix B, Table B.2). There were 12 proteins with changes in protein levels in the ventral groove tissue from water-conditioned grain, four had increased and eight had decreased (Tables 3.2 and 3.10, and Appendix B, Table B.3).

There were significantly more changes in protein levels within this class in the ventral groove tissue compared to bran tissue ($p = 0.0095$).

3.3.13.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had six changes in protein levels. Of these, one had increased and five had decreased (Tables 3.3 and 3.10, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain had a decrease in one regulatory and signaling protein (Tables 3.3 and 3.10, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had 13 changes in protein levels. Of these, five had increased and eight had decreased (Tables 3.3 and 3.10, and Appendix B, Table B.3).

The ventral groove and germ tissues from water + ABA-conditioned grain, both had significantly more changes in protein levels than the bran tissue ($p = 0.0003$ and $p = 0.049$ respectively). The germ and ventral groove tissues from water + ABA-conditioned grain had no significant difference in the number of changes in protein levels ($p = 0.067$).

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.

Regulatory and signaling	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Putative calcium-binding protein.	-	-	*	-	-	-	-	-	-	-	-	-
Hypothetical protein P0036D10.4 (Putative LRR receptor-like protein kinase).	-	-	-	*	-	-	-	-	-	-	-	-
NB-ARC domain, putative. (cell death)	-	-	-	*	-	-	-	-	-	-	-	-
Putative proteasome regulatory non-ATPase subunit.	-	-	-	*	-	-	-	-	*	-	*	-
Putative RNA-binding protein.	-	-	-	*	-	-	-	-	-	-	-	-
Two-component system regulatory protein.	-	-	-	*	-	-	-	-	-	-	-	-
Expressed protein.	-	-	-	-	*	-	-	-	-	-	-	-
Rab7 (Pearl millet).	-	-	-	-	-	*	-	-	-	-	-	-
Tritin.	-	-	-	-	-	*	-	*	*	-	*	-
NB-ARC domain, putative (apoptosis).	-	-	-	-	-	-	-	-	*	-	*	-
Protein synthesis inhibitor I (Ribosome-inactivating protein I) (rRNA N-glycosidase).	-	-	-	-	-	-	-	-	*	-	*	-
26S proteasome regulatory particle triple-A ATPase subunit1.	-	-	-	-	-	-	-	-	-	*	-	*
Casein kinase II alpha subunit (Hd6).	-	-	-	-	-	-	-	-	-	*	-	*
Lysosomal trafficking regulator LYST and related BEACH and WD40 repeat proteins.	-	-	-	-	-	-	-	-	-	*	-	*
Putative Glycine-rich protein 2.	-	-	-	-	-	-	-	-	-	*	-	*
Putative polyadenylate-binding protein.	-	-	-	-	-	-	-	-	-	*	-	*
Putative receptor-like protein kinase.	-	-	-	-	-	-	-	-	-	*	-	*
Putative wall-associated kinase 4.	-	-	-	-	-	-	-	-	-	*	-	*
Receptor-like protein kinase, putative.	-	-	-	-	-	-	-	-	-	*	-	*
ADP-ribosylation factor.	-	-	-	-	-	-	-	-	-	-	*	-

3.3.14 Metabolism-related proteins

3.3.14.1 Germ, bran and ventral groove from water-conditioned grain

There were seven changes in protein levels within this class in the bran tissue from water-conditioned grain. Of these, two had increased and five had decreased (Tables 3.2 and 3.11, and Appendix B, Table B.2). There were 31 changes in protein levels in the ventral groove tissue from water-conditioned grain. Of these, 19 had increased and 12 had decreased (Tables 3.2 and 3.11, and Appendix B, Table B.3).

The ventral groove tissue had significantly more changes in protein levels within this class than the bran tissue ($p = 0.0067$).

3.3.14.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had 15 changes in protein levels. Of these, 12 had increased and three had decreased (Tables 3.3 and 3.11, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain had five changes in protein levels. Of these, one had increased and four had decreased (Tables 3.3 and 3.11, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had 28 changes in protein levels. Of these, 17 had increased and 11 had decreased (Tables 3.3 and 3.11, and Appendix B, Table B.3).

The germ and ventral groove tissues from water + ABA-conditioned grain had significantly more changes in protein levels than the bran tissue ($p = 0.019$ and $p = 0.0025$ respectively). The ventral groove tissue had significantly more changes in protein levels than the germ tissue ($p = 0.026$).

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.

Metabolism-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Branching enzyme-3.	-	-	*	-	-	-	-	-	-	-	-	-
Dehydroascorbate reductase (Q84UH5_ORYSA).	-	-	*	-	-	-	-	-	-	-	-	-
Formate dehydrogenase (Fragment).	-	-	*	-	-	-	-	-	-	-	-	-
IDI1 protein.	-	-	*	-	-	-	-	-	-	-	-	-
Nucleoside diphosphate kinase I.	-	-	*	-	-	-	-	-	-	-	-	-
Phospholipid hydroperoxide glutathione peroxidase-like protein.	-	-	*	-	-	-	-	-	*	-	-	-
Putative amidase.	-	-	*	-	-	-	-	-	-	*	-	*
Putative aminoacylase.	-	-	*	-	-	-	-	-	-	-	-	-
Putative glycine dehydrogenase.	-	-	*	-	-	-	-	-	-	-	-	-
Putative nucleoside diphosphate kinase.	-	-	*	-	-	-	-	-	-	-	-	-
Putative phosphogluconate dehydrogenase.	-	-	*	-	-	-	-	-	-	-	-	-
Ubiquitin activating E1 enzyme-like protein.	-	-	*	-	-	-	-	-	-	-	-	-
Adenylosuccinate synthetase, chloroplast precursor (Fragment).	-	-	-	*	-	-	-	-	-	-	-	-
Chalcone isomerase.	-	-	-	*	-	-	-	-	-	-	-	-
Isopenicillin N epimerase-like.	-	-	-	*	-	-	-	-	-	-	-	-
Aldose reductase-related protein.	-	-	-	-	*	-	-	-	-	-	-	-
Putative ADP-glucose pyrophosphorylase.	-	-	-	-	*	-	-	-	-	-	-	-
1-Cys-peroxiredoxine.	-	-	-	-	-	*	-	-	-	-	-	-
Alanine aminotransferase 2.	-	-	-	-	-	*	-	-	*	-	-	-
Asparagine synthetase 2.	-	-	-	-	-	*	-	-	-	*	-	*
Glucose-1-phosphate adenylyltransferase large subunit, chloroplast precursor.	-	-	-	-	-	*	-	*	-	-	-	-
Small subunit ADP glucose pyrophosphorylase.	-	-	-	-	-	*	-	*	-	-	-	-
Putative 4-methyl-5(B-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme.	-	-	-	-	-	-	*	-	*	-	*	-
AmiB (Fragment).	-	-	-	-	-	-	-	*	-	-	-	-

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Table 3.11. (Continued) 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.

Metabolism-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Ferredoxin-dependent glutamate synthase, chloroplast precursor (Fd-GOGAT).	-	-	-	-	-	-	-	*	-	-	-	-
Acyl-CoA oxidase homolog.	-	-	-	-	-	-	-	-	*	-	*	-
Adenosine kinase-like protein (Fragment).	-	-	-	-	-	-	-	-	*	-	*	-
ADP,ATP carrier protein 2, mitochondrial precursor.	-	-	-	-	-	-	-	-	*	-	*	-
Alpha-glucosidase precursor (Maltase).	-	-	-	-	-	-	-	-	*	-	-	-
Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-I.	-	-	-	-	-	-	-	-	*	-	-	-
Dehydroascorbate reductase (Q84UH6_WHEAT).	-	-	-	-	-	-	-	-	*	-	*	-
GDSL-like Lipase/Acylhydrolase.	-	-	-	-	-	-	-	-	*	-	*	-
Glutathione S-transferase.	-	-	-	-	-	-	-	-	*	-	*	-
Glutathione transferase.	-	-	-	-	-	-	-	-	*	-	*	-
Hydrolase-like protein.	-	-	-	-	-	-	-	-	*	-	*	-
Putative beta-alanine synthases.	-	-	-	-	-	-	-	-	*	-	*	-
Putative endo-beta-1,4-glucanase.	-	-	-	-	-	-	-	-	*	-	*	-
Putative fructokinase I.	-	-	-	-	-	-	-	-	*	-	*	-
Pyrroline-5-carboxylate reductase.	-	-	-	-	-	-	-	-	*	-	*	-
RIB40 genomic DNA, SC009.	-	-	-	-	-	-	-	-	*	-	*	-
Sucrose-6F-phosphate phosphohydrolase SPP3.	-	-	-	-	-	-	-	-	*	-	*	-
2-alkenal reductase.	-	-	-	-	-	-	-	-	-	*	-	*
Catalase isozyme 1.	-	-	-	-	-	-	-	-	-	*	-	*
Methionine synthase (Fragment).	-	-	-	-	-	-	-	-	-	*	-	*
Methionine synthase 2 enzyme.	-	-	-	-	-	-	-	-	-	*	-	*
Monodehydroascorbate reductase (Fragment).	-	-	-	-	-	-	-	-	-	*	-	*
Putative cinnamate-4-hydroxylase.	-	-	-	-	-	-	-	-	-	*	-	-

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Table 3.11. (Continued) 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.

Metabolism-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Putative glucosyl transferase.	-	-	-	-	-	-	-	-	-	*	-	*
Pyridoxine biosynthesis protein.	-	-	-	-	-	-	-	-	-	*	-	*
Serine/threonine protein phosphatase 2A.	-	-	-	-	-	-	-	-	-	*	-	*
Thiamine pyrophosphate-requiring enzymes.	-	-	-	-	-	-	-	-	-	*	-	*
Putative isovaleryl-CoA dehydrogenase.	-	-	-	-	-	-	-	-	-	-	*	-
Putative S-adenosylhomocystein hydrolase 2.	-	-	-	-	-	-	-	-	-	-	*	-

3.3.15 Protease-related proteins

3.3.15.1 Germ, bran and ventral groove from water-conditioned grain

There was an increase in one protease protein in the germ tissue and bran tissue from water-conditioned grain (Tables 3.2 and 3.12, and Appendix B, Table B.1). There were two changes in protein levels that had increased in the ventral groove tissue from water-conditioned grain (Tables 3.2 and 3.12, and Appendix B, Table B.3).

The number of changes in protein levels within this class was not significantly different between any of the tissues ($p > 0.05$ for all tissues).

3.3.15.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

The germ tissue from water + ABA-conditioned grain had three protease proteins that increase in levels. Of these, one had increased and two had decreased (Tables 3.3 and 3.12, and Appendix B, Table B.1). There was one protease protein that increased in levels in the bran tissue from water + ABA-conditioned grain (Tables 3.3 and 3.12, and Appendix B,

Table B.2). There were two protease proteins that increased in levels in the ventral groove tissue from water + ABA-conditioned grain (Tables 3.3 and 3.12, and Appendix B, Table B.3).

The number of changes in protease protein levels was not significantly different between any of the tissues ($p > 0.05$ for all tissues).

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.

Protease-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
SKP1/ASK1-like protein.	*	-	-	-	-	-	-	-	-	-	-	-
Zinc protease.	-	-	*	-	-	-	-	-	-	-	-	-
Lon protease homolog 2, mitochondrial precursor.	-	-	-	*	-	-	-	-	-	-	-	-
Putative puromycin-sensitive aminopeptidase.	-	-	-	*	-	-	-	-	-	-	-	-
Oryzain beta chain precursor.	-	-	-	-	*	-	-	-	-	-	-	-
Serine carboxypeptidase II (Carboxypeptidase D) (CPDW- II) (CP-WII).	-	-	-	-	-	-	*	-	-	-	-	-
20S proteasome beta 4 subunit.	-	-	-	-	-	-	-	-	*	-	*	-
Aspartic proteinase.	-	-	-	-	-	-	-	-	*	-	*	-

3.3.16 Endosperm-related proteins

3.3.16.1 Germ, bran and ventral groove from water-conditioned grain

There were 13 changes in protein levels within this class in the bran tissue from water-conditioned grain, two had increased and 11 had decreased (Tables 3.2 and 3.13, and Appendix B, Table B.2). There were 16 changes in protein levels in the ventral groove tissue from water-conditioned grain, 11 had increased and five had decreased (Tables 3.2 and 3.13, and Appendix B, Table B.3).

The number of changes in protein levels within this class between the bran tissue and ventral groove tissues from water-conditioned grain were not significantly different ($p = 0.672$).

3.3.16.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

There were no changes in protein levels in the germ tissue from water + ABA-conditioned grain. The bran tissue from water + ABA-conditioned grain had 17 changes in protein levels. Of these, one had increased and 16 had decreased (Tables 3.3 and 3.13, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had 17 changes in protein levels. Of these, 10 had increased and seven had decreased (Tables 3.3 and 3.13, and Appendix B, Table B.3).

There was no significant difference in the number of changes in endosperm protein levels between the bran and ventral groove tissues from water + ABA-conditioned grain ($p = 0.841$).

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.

Endosperm-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
HMW glutenin subunit (Q6UKZ5_WHEAT).	-	-	-	-	*	-	-	-	-	-	-	-
Low-molecular-weight glutenin subunit group 3 type II (Fragment).	-	-	-	-	*	-	-	*	*	-	*	-
19 kDa globulin (Fragment).	-	-	-	-	-	*	-	*	-	-	-	-
Beta amylase (Fragment).	-	-	-	-	-	*	-	*	*	-	*	-
Endosperm-specific beta-amylase 1.	-	-	-	-	-	*	-	*	*	-	-	-
Glutelin.	-	-	-	-	-	*	-	-	-	-	-	-
LMW-s KS2.	-	-	-	-	-	*	-	*	-	-	-	-
Putative beta-amylase.	-	-	-	-	-	*	-	*	-	*	-	*
Putative beta-xylosidase (Fragment).	-	-	-	-	-	*	-	-	-	-	-	-
Starch branching enzyme 2.	-	-	-	-	-	*	-	*	-	-	-	-
Starch branching enzyme I precursor (Starch branching enzyme 1).	-	-	-	-	-	*	-	*	-	-	-	-
Starch synthase (GBSSI).	-	-	-	-	-	*	-	*	-	-	-	-
Sucrose synthase type 2.	-	-	-	-	-	*	-	*	-	-	-	-
Beta-amylase (1,4-alpha-D-glucan maltohydrolase) (Fragment).	-	-	-	-	-	-	*	-	-	*	-	*
Glutenin, high molecular weight subunit DX5 precursor.	-	-	-	-	-	-	-	*	-	-	-	-
HMW glutenin subunit Ax2*.	-	-	-	-	-	-	-	*	-	-	-	-
HMW glutenin subunit (Q52JL3_WHEAT).	-	-	-	-	-	-	-	*	-	-	-	-
HMW glutenin x-type subunit Bx7 precursor.	-	-	-	-	-	-	-	*	*	-	*	-
Low molecular weight glutenin.	-	-	-	-	-	-	-	*	-	-	-	-
Low-molecular-weight glutenin subunit group 5 type III.	-	-	-	-	-	-	-	*	-	-	-	-
Endo-1,3-beta-glucanase.	-	-	-	-	-	-	-	-	*	-	*	-
Grain softness protein-1 (GSP-1 Grain Softness Protein).	-	-	-	-	-	-	-	-	*	-	*	-
Low-molecular-weight glutenin subunit group 1 type I.	-	-	-	-	-	-	-	-	*	-	*	-
Low-molecular-weight glutenin subunit group 2 type I.	-	-	-	-	-	-	-	-	*	-	*	-
Low-molecular-weight glutenin subunit group 8 type IV.	-	-	-	-	-	-	-	-	*	-	*	-

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Table 3.13. (Continued) 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.

Endosperm-related proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Puroindoline b.	-	-	-	-	-	-	-	-	*	-	*	-
Starch synthase IIA.	-	-	-	-	-	-	-	-	*	-	*	-
Gamma gliadin.	-	-	-	-	-	-	-	-	-	*	-	*
High molecular weight glutenin subunit 1By15.	-	-	-	-	-	-	-	-	-	*	-	*
High-molecular-weight glutenin.	-	-	-	-	-	-	-	-	-	*	-	*
Gamma-gliadin/LMW-glutenin chimera Ch2 (Fragment).	-	-	-	-	-	-	-	-	-	-	-	*
High-molecular-weight glutenin subunit 1Dx2.1.	-	-	-	-	-	-	-	-	-	-	-	*

3.3.17 Other proteins

3.3.17.1 Germ, bran and ventral groove from water-conditioned grain

There were three changes in protein levels within this classification in the bran tissue from water-conditioned grain, all of which had decreased (Tables 3.2 and 3.14, and Appendix B, Table B.2). There were 16 changes in protein levels in the ventral groove tissue from water-conditioned grain, seven had increased and nine had decreased (Tables 3.2 and 3.14, and Appendix B, Table B.3).

The ventral groove tissue from water-conditioned grain had significantly more changes in protein levels within this classification than the bran tissue from water-conditioned grain ($p = 0.0008$).

3.3.17.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

There were five changes in protein levels in the germ tissue from water + ABA-conditioned grain. Of these, three had increased and two had decreased (Tables 3.3 and

3.14, and Appendix B, Table B.1). There were two decreases in protein levels within this class in the bran tissue from water + ABA-conditioned grain (Tables 3.3 and 3.14, and Appendix B, Table B.2). There were 14 changes in protein levels in the ventral groove tissue from water + ABA-conditioned grain. Of these, four had increased and 10 had decreased (Tables 3.3 and 3.14, and Appendix B, Table B.3).

The ventral groove tissue from water + ABA-conditioned grain had significantly more changes in protein levels compared to the germ and bran tissues ($p = 0.022$ and $p = 0.0009$ respectively). The germ and bran tissues from water + ABA-conditioned grain had no significant difference in the number of protein level changes ($p = 0.245$).

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.

Other function proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
AAA-type ATPase-like.	-	-	*	-	-	-	-	-	-	-	-	-
Embryo-specific protein.	-	-	*	-	-	-	-	-	-	-	-	-
Globulin-like protein.	-	-	*	-	-	-	-	-	-	-	-	-
Acetyltransferase 1-like.	-	-	-	*	-	-	-	-	-	-	-	-
Putative EBNA1-binding protein homolog; Ebp2p.	-	-	-	*	-	-	-	-	-	-	-	-
27K protein (Fragment).	-	-	-	-	-	*	-	*	-	-	-	-
Probable peroxiredoxin (Thioredoxin peroxidase) (Rehydrin homolog) (B15C).	-	-	-	-	-	*	-	-	-	-	-	-
Triticin (Fragment).	-	-	-	-	-	*	-	*	-	-	-	-
Amylogenin.	-	-	-	-	-	-	-	-	*	-	*	-
CM 17 protein.	-	-	-	-	-	-	-	-	*	-	*	-
Cu/Zn superoxide dismutase.	-	-	-	-	-	-	-	-	*	-	-	-
Manganese superoxide dismutase.	-	-	-	-	-	-	-	-	*	-	*	-
Putative abscisic acid-induced protein-rice.	-	-	-	-	-	-	-	-	*	-	*	-
Putative streptococcal hemagglutinin.	-	-	-	-	-	-	-	-	*	-	-	-
Putative vacuolar protein sorting-associated protein.	-	-	-	-	-	-	-	-	*	-	-	-
1-Cys-peroxiredoxine.	-	-	-	-	-	-	-	-	-	*	-	*
Adsorption protein-like.	-	-	-	-	-	-	-	-	-	*	-	*
Globulin-2 precursor.	-	-	-	-	-	-	-	-	-	*	-	-
LRR19 (apoptosis).	-	-	-	-	-	-	-	-	-	*	-	*
Probable peroxiredoxin (Thioredoxin peroxidase) (RAB24 protein).	-	-	-	-	-	-	-	-	-	*	-	*
Putative C2 domain-containing protein.	-	-	-	-	-	-	-	-	-	*	-	*
Putative cellular retinaldehyde-binding/triple function.	-	-	-	-	-	-	-	-	-	*	-	*
Putative globulin (With alternative splicing).	-	-	-	-	-	-	-	-	-	*	-	*
Storage protein.	-	-	-	-	-	-	-	-	-	*	-	*
Acyltransferase required for palmitoylation of Hedgehog.	-	-	-	-	-	-	-	-	-	-	-	*
Putative selenium binding protein.	-	-	-	-	-	-	-	-	-	-	-	*

3.3.18 Unknown function

3.3.18.1 Germ, bran and ventral groove from water-conditioned grain

There was an increase in one protein with unknown function in the germ tissue from water-conditioned grain (Tables 3.2 and 3.15, and Appendix B, Table B.1). There were four changes in protein levels in the bran tissue from water-conditioned grain, half had increased and half had decreased (Tables 3.2 and 3.15, and Appendix B, Table B.2). There were 23 changes in protein levels in the ventral groove tissue from water-conditioned grain, eight had increased and 15 had decreased (Table 3.2 and Appendix B, Table B.3).

The ventral groove tissue had significantly more changes in protein levels within this classification than the germ tissue and bran tissue from water-conditioned grain ($p = 0.0025$ and $p = 0.0067$ respectively). The number of changes in protein levels between the germ and bran tissues from water-conditioned grain were not significantly different ($p = 0.177$).

3.3.18.2 Germ, bran and ventral groove from water + 4 ppm ABA-conditioned grain

There were 24 changes in protein levels in the germ tissue from water + ABA-conditioned grain. Of these, half had increased and decreased (Tables 3.3 and 3.15, and Appendix B, Table B.1). The bran tissue from water + ABA-conditioned grain had nine changes in protein levels. Of these, four had increased and five had decreased (Tables 3.3 and 3.15, and Appendix B, Table B.2). The ventral groove tissue from water + ABA-conditioned grain had 23 changes in protein levels. Of these, nine had increased and 14 had decreased (Tables 3.3 and 3.15, and Appendix B, Table B.3).

Both the germ and ventral groove tissues from water + ABA-conditioned grain had significantly more changes in protein levels than the bran tissue ($p = 0.0049$ and $p =$

0.0078 respectively). There was no significant difference in the number of protein level changes between the germ and ventral groove tissues from water + ABA-conditioned grain ($p = 0.872$).

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.

Unknown function proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Hypothetical protein OJ1119_D01.23.	*	-	-	-	-	-	-	-	-	-	-	-
Hypothetical protein OJ1218_D07.4.	-	-	*	-	-	-	-	-	-	-	-	-
Hypothetical protein OJ1300_E01.11.	-	-	*	-	-	-	-	-	-	-	-	-
Hypothetical protein OSJNBb0024F06.8.	-	-	*	-	-	-	-	-	-	-	-	-
Hypothetical protein P0538C01.10.	-	-	*	-	-	-	-	-	-	-	-	-
Hypothetical protein P0696E01.34 (Hypothetical protein P0004A09.13).	-	-	*	-	-	-	-	-	-	-	-	-
Hypothetical protein (Q5GVK5_XANOR).	-	-	*	-	-	-	-	-	-	-	-	-
OSJNBa0043L24.17 protein. (Probable glutaredoxin)	-	-	*	-	-	-	-	-	-	-	-	-
OSJNBa0058K23.21 protein.	-	-	*	-	-	-	-	-	-	-	-	-
OSJNBa0088A01.24 protein.	-	-	*	-	-	-	-	-	-	-	-	-
OSJNBa0088A01.5 protein.	-	-	*	-	-	-	-	-	-	-	-	-
Putative F8K7.10 protein.	-	-	*	-	-	-	-	-	-	-	-	-
Putative negatively light-regulated protein.	-	-	*	-	-	-	-	-	-	-	-	-
Gb protein. Sorghum bicolor (Sorghum vulgare).	-	-	-	*	-	-	-	-	-	-	-	-
Hypothetical protein B1036C05.8.	-	-	-	*	-	-	-	-	-	-	-	-
Hypothetical protein OJ1655_B12.14.	-	-	-	*	-	-	-	-	-	-	-	-
Hypothetical protein OSJNBa0084C09.17.	-	-	-	*	-	-	-	-	-	-	-	-
Hypothetical protein OSJNBb0093E13.1.	-	-	-	*	-	-	-	-	-	-	-	-
Hypothetical protein P0486H12.21.	-	-	-	*	-	-	-	-	-	-	-	-
OJ000223_09.5 protein (OSJNBa0081L15.15 protein).	-	-	-	*	-	-	-	-	-	-	-	-
OSJNBa0074L08.10 protein.	-	-	-	*	-	-	-	-	-	-	-	-
OSJNBa0086O06.3 protein.	-	-	-	*	-	-	-	-	-	-	-	-
OSJNBb0022F23.11 protein.	-	-	-	*	-	-	-	-	-	-	-	-
OSJNBb0038F03.9 protein.	-	-	-	*	-	-	-	-	-	-	-	-

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Table 3.15. (Continued) 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.

Unknown function proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Putative r40c1 protein-rice.	-	-	-	*	-	-	-	-	-	-	-	-
Embryo-specific protein (probable lipoprotein).	-	-	-	-	*	-	-	-	-	-	-	-
Predicted protein (Q2UJ19_ASPOR).	-	-	-	-	*	-	*	-	-	-	-	-
Hypothetical protein (Q2QVK3_ORYSA).	-	-	-	-	-	*	-	-	-	-	-	-
OSJNBb0118P14.11 protein (OJ000315_02.8 protein).	-	-	-	-	-	*	-	-	-	-	*	-
Hypothetical protein OJ1679_B08.21.	-	-	-	-	-	-	*	-	-	-	-	-
OSJNBa0029H02.21 protein.	-	-	-	-	-	-	*	-	-	-	-	-
Predicted protein (Q2UUI9_ASPOR).	-	-	-	-	-	-	*	-	-	-	-	-
Hypothetical protein (Fragment).	-	-	-	-	-	-	-	*	-	-	-	-
Hypothetical protein OSJNBa0042H09.8.	-	-	-	-	-	-	-	*	-	-	-	-
Hypothetical protein OSJNBb0024N19.12.	-	-	-	-	-	-	-	*	-	-	-	-
Hypothetical protein precursor.	-	-	-	-	-	-	-	*	-	*	-	*
Predicted protein (Q2PIZ2_ASPOR).	-	-	-	-	-	-	-	*	-	-	-	-
Hypothetical protein B1435D02.9 (Hypothetical protein OSJNBa0042E12.26).	-	-	-	-	-	-	-	-	*	-	-	-
Hypothetical protein OSJNBa0082N11.24.	-	-	-	-	-	-	-	-	*	-	*	-
Hypothetical protein P0438E12.30 (Hypothetical protein P0410C01.9).	-	-	-	-	-	-	-	-	*	-	*	-
Hypothetical protein P0493G01.17.	-	-	-	-	-	-	-	-	*	-	-	-
Hypothetical protein (Q2R266_ORYSA).	-	-	-	-	-	-	-	-	*	-	*	-
OSJNBa0044M19.10 protein.	-	-	-	-	-	-	-	-	*	-	*	-
OSJNBb0069N01.1 protein (OSJNBa0013A04.20 protein).	-	-	-	-	-	-	-	-	*	-	*	-
Predicted protein (Q53K95_ORYSA).	-	-	-	-	-	-	-	-	*	-	*	-
Hypothetical protein OJ1014H12.11 (Transposon protein, putative, unclassified).	-	-	-	-	-	-	-	-	-	*	-	-
Hypothetical protein OSJNBa0011D16.50 (Hypothetical protein OSJNBa0069P02.4).	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein OSJNBa0037H03.17 (Hypothetical protein OJ1653_D06.2).	-	-	-	-	-	-	-	-	-	*	-	*

(Continued next page)

Table 3.15. (Continued) 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.

Unknown function proteins	Germ				Bran				Ventral groove			
	Water		ABA		Water		ABA		Water		ABA	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Hypothetical protein OSJNBa0090L05.5.	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein OSJNBa0091P11.25.	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein OSJNBa0093M23.12.	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein OSJNBb0058G04.14.	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein P0022B05.126.	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein P0229B10.35 (Hypothetical protein B1274F11.4).	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein tac7077.	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein (Q2QMF8_ORYSA).	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein. Xanthomonas oryzae pv. oryzae.	-	-	-	-	-	-	-	-	-	*	-	*
OSJNBa0014K14.15 protein.	-	-	-	-	-	-	-	-	-	*	-	*
Predicted protein (Q2UAM0_ASPOR).	-	-	-	-	-	-	-	-	-	*	-	*
Hypothetical protein OJ1743_B12.7.	-	-	-	-	-	-	-	-	-	-	*	-
Hypothetical protein (Q53JU2_ORYSA).	-	-	-	-	-	-	-	-	-	-	*	-

3.4 Discussion

iTRAQ analysis of the differential expression of proteins in the germ, bran and ventral groove tissue from water-and water + ABA-conditioned grain was compared to the same three tissues from non-conditioned grain. The tissues from grain conditioned with water + ABA had a distinctive protein complement when compared with the corresponding tissues from water-conditioned grain. This demonstrates the specific effect of ABA and confirms its role in both positive and negative regulation of gene expression in signaling pathways (Ohnishi et al., 2008). The dynamic protein level changes of the water-and water + ABA-conditioned grain are discussed separately. This is followed by a comparison between some specific differentially expressed biotic and abiotic defense proteins exhibited by the two treatments.

3.4.1 Biochemical changes in water-conditioned grain compared with non-conditioned grain

The ventral groove tissue from water-conditioned grain exhibited the majority of differentially expressed proteins. Bran tissue had significantly fewer differentially expressed proteins compared and the ventral groove and the germ the least number of differentially expressed proteins of all the three tissues.

The significant large number of differentially expressed proteins in the ventral groove suggests that it is likely that the biochemical processes that occur within this tissue are vital for the initial stages of germination. The bran tissue is also active during this early stage in germination and is possibly closely linked with the ventral groove in supplying energy and nutrients to the germ. The large number of protein level changes that occurred in the

ventral groove may also suggest that the majority of the water uptake by the grain is directed to this region. The potential movement of water towards the ventral groove may be initiated by late embryogenesis abundant (LEA) proteins. In this study, these proteins increased in the ventral groove from both water-and water + ABA-conditioned grain by ~ 6-and ~ 5-fold respectively (Appendix B, Table B.3). These proteins have protective functions against environmental stresses such as drought, salt and cold stress. Two of the most important biochemical properties of LEA proteins are extreme hydrophilicity and sugar binding ability. These properties help to protect the plant cells from desiccation and increase freezing tolerance (Black et al., 1999; Wisniewski et al., 1999; Borovskii et al., 2002b; Shakirova et al., 2005). The extreme hydrophilic characteristic of this protein may create a biochemical capillary action towards the ventral groove and the germ. Not only may the water be directed towards the ventral groove and also along the ventral groove to the germ, the process may also aid in carrying and delivering nutrients such as sugars and amino acids.

The ventral groove had the most changes in protein levels for ‘metabolic processes’, followed by ‘protein synthesis and folding’, ‘biotic defense’ and ‘glycolysis and energy’. Also, the ventral groove tissue had the highest level of expression of ‘membrane transport/transport’ proteins when compared with the germ and bran (Tables 3.2 and 3.3). This further supports its potential role in supplying nutrients to the germ, together with providing protection against pathogens and environmental stress during germination.

The iTRAQ analysis data showed evidence of protein migration. It revealed that glutenin protein levels had decreased in the bran and increased in the ventral groove (Table 3.13). Also, other proteins such as alanine aminotransferase 2 (Table 3.11), serpin (Table 3.4), tritin (Table 3.10) and β -amylase (Table 3.13) showed the same pattern of decreasing in the

bran and increasing in the ventral groove tissue. Since most of these proteins are already expressed during grain development (Flint et al., 1975), one would not expect to see increases in protein levels during the early stages of germination. These proteins might have migrated towards the ventral groove during conditioning or the bran and ventral groove tissues contained more or less of the endosperm when dissected. The latter is unlikely to be the case, since the results show that some of endosperm-related proteins from the bran had also increased and yet they decreased in the ventral groove of both the water-and water + ABA-conditioned grain.

3.4.2 Biochemical changes in water + ABA-conditioned grain compared with non-conditioned grain

Water + ABA-conditioned grain revealed a different pattern of differentially expressed proteins when compared to the water-conditioned grain. This was mostly observed in the germ and to a lesser extent in the bran tissue. The ventral groove tissue from water + ABA-conditioned grain, whilst similar to the corresponding tissue from the water-conditioned grain displayed fewer changes in protein levels. On the other hand, the germ was very sensitive to ABA, indicated by the large number of differentially expressed proteins when compared to the germ from water-conditioned grain (Tables 3.2 and 3.3).

Major changes in protein levels were observed within all protein classes in the germ from water + ABA-conditioned grain. This large shift in the protein complement towards defense may be responsible for inhibition of germination and growth rate. The cellular biochemical transformation in the germ may not be as necessary for the aleurone cells within the bran and ventral groove tissue, since these tissues are only required briefly during germination and very early growth.

The bran tissue was also sensitive to ABA in terms of increased activity in expression level changes in biotic and abiotic defense and protein synthesis and folding proteins (Tables 3.2 and 3.3). These protein classes were the only major changes in the bran tissue that were also observed in the germ. Since ABA is involved in a multitude of stress responses, it is not surprising to find that these types of proteins had increased or changed in expression levels. The only other protein class that was differentially expressed in the bran tissue from water + ABA-conditioned grain was protein synthesis and folding proteins. The other protein classes were much the same. Since the results did not show significant changes in transcription-related proteins, it suggests that biotic and abiotic defense and protein synthesis and folding proteins were a result of post transcriptional expression. This makes sense in evolutionary terms for both the germ and bran tissues, in that they need to be sufficiently prepared to contend with adverse conditions, whilst not compromising growth rate in good growing conditions. For example, in favorable conditions, fast growth rate will be advantageous in order to utilise or out-compete other plants for available nutrients and space in the surrounding environment. However, it is also beneficial for the grain to invest some energy in preparing itself for potential attacks by pathogens and/or environmental stress. Survival of the plant in adverse conditions will be compromised if it needs to transcribe all the mRNA required for the building of protective proteins. By transcribing some of the appropriate protective protein mRNA during grain development, it has created a middle ground in terms of being prepared but not over prepared.

An interesting similarity between the germ and bran tissues from water + ABA-conditioned grain was that there were increases in the level of LEA proteins (Table 3.4). It has been reported that Group 3 LEA proteins accumulate in the shoots arising from the germ after ABA treatment as part of the alleviation of dehydration stress (Ried and Walker-Simmons, 1993). However, this study has revealed for the first time that LEA

proteins begin to increase in expression levels in both the germ and bran tissues very early in germination and at relatively low grain moisture levels (~ 16.5% moisture). This suggests that the very early response to a water deficit (drought or salinity) would be to capture and retain as much water as possible in the germ and aleurone cells (which in milling is part of the bran tissue) to aid in intracellular stability and survival. Since it is not possible for the dead bran layers to synthesise proteins *de novo*, the major altered properties in the bran component is likely to be due to the aleurone layer. In terms of milling quality, these proteins may play an important role in retaining water in the bran tissues, qualities that are important in milling to prevent bran fracturing and contamination of the flour. An increase in the water absorptive property of bran makes it more elastic and therefore tougher, thus supporting the hypothesis that conditioning with water imparts this property (Kweon et al., 2009).

3.4.3 Changes in protein levels within the biotic and abiotic defense protein class exhibited by water-and water + ABA conditioning

This protein class exhibited differential expression of many biotic, abiotic and also both biotic/abiotic defense proteins (Table 3.4). Biotic and biotic/abiotic defense proteins were highly differentially expressed in the germ from water + ABA-conditioned grain when compared to bran and ventral groove tissues. Within the germ tissue, seven out of 14 were exclusively abiotic and two were dual function biotic/abiotic. These proteins were peroxidase, 14-3-3 protein (an ABA-and stress-induced protein), cold shock domain proteins 2 and 3, late embryogenesis abundant protein (LEA), nodulin and R40g3 protein. The only exclusively biotic defense proteins were a putative bacterial-induced peroxidase and a putative disease resistance gene homolog 9N protein. However, both the bran and ventral groove tissues had larger percentages of changes in proteins involved in biotic

defense. Within the bran tissue, 14 out of 19 were exclusively biotic, one was dual function biotic/abiotic and four were exclusively abiotic. Within the ventral groove tissue, 14 out of 19 in total were exclusively biotic, two were dual function biotic/abiotic and five were exclusively abiotic.

The distribution of these biotic and the abiotic defense proteins between the different grain tissues suggest that the germ is being preferentially prepared for abiotic defense because the growing embryo is more likely to struggle with abiotic stress compared with the rest of the grain. During germination, nutrients are released from the endosperm for the growing embryo, providing a food source for bacteria and fungi. Thus, the bran and ventral groove defense proteins are predominantly involved in biotic defense.

An endochitinase-a precursor and an endochitinase-c precursor has been identified in the ventral groove tissue (Table 3.4). These proteins were differentially expressed in both the water-and water + ABA-conditioned grain with no differences between the conditioning treatments. Previously, endochitinase was identified in the intermediate and aleurone tissue and the enzyme also exhibited activity in the whole grain water-soluble extract but not in the isolated pericarp or flour water soluble extract (Chapter 2; Table 2.2).

The endochitinase-a (class I chitinase homologous to rye seed) was ~ 2-fold lower in the ventral groove, whereas the endochitinase-c (class II chitinase, also a homolog to rye seed) was almost 2-fold higher (Appendix B, Table B.3). A previous study undertaken on rye seed chitinase reported that endochitinase-a has different activity on fungal hyphae when compared with endochitinase-c (Taira et al., 2002). Endochitinase-a was found to bind and degrade mature and nascent chitin. By contrast, endochitinase-c was found to only bind and degrade nascent chitin at the tips of the hyphae. The results from this study suggest

that when seed moisture level increases, endochitinase-c levels increase. Since endochitinase-c attacks the nascent chitin on the hyphal tips, it may be preventing fungi from penetrating and colonizing the ventral groove.

Chapter 4 : iTRAQ analysis of bran and ventral groove tissues from non-conditioned grain

4.1 Introduction

The ventral groove is potentially a vital tissue of the grain, which is believed to be involved in the mobilisation of nutrients and water to the embryo upon germination (Jenner et al., 1988; Rathjen et al., 2009). This tissue is also reported to deliver nutrients to the endosperm during grain development (Frazier and Appalanaidu, 1965). However, the function of this tissue at the biochemical level is not yet fully understood. Other functions of the ventral groove, may be involved in biotic and abiotic defense. Due to the concentration of water and nutrients in this tissue region, it is likely to be vulnerable to biotic attack and susceptible to drying in drought conditions.

The ventral groove has unique physiological features, such as the chalaza, nuclear projection and vascular bundles (Figure 4.1) that may contain unique proteins that are different to the bran tissue (Frazier and Appalanaidu, 1965). The aleurone cells in the bran tissue are continuous and envelop the whole endosperm. This includes the cells that form part of the ventral groove.

The results in Chapter 3 (Table 3.3) showed that the ventral groove had the most changes in protein levels in both the water-and water + ABA-conditioned grain between all the tissues analysed. iTRAQ analysis and comparison of this tissue to bran tissue from non-

conditioned grain, may reveal unique proteins and/or a higher abundance of particular proteins that may perform key biochemical functions within this tissue.

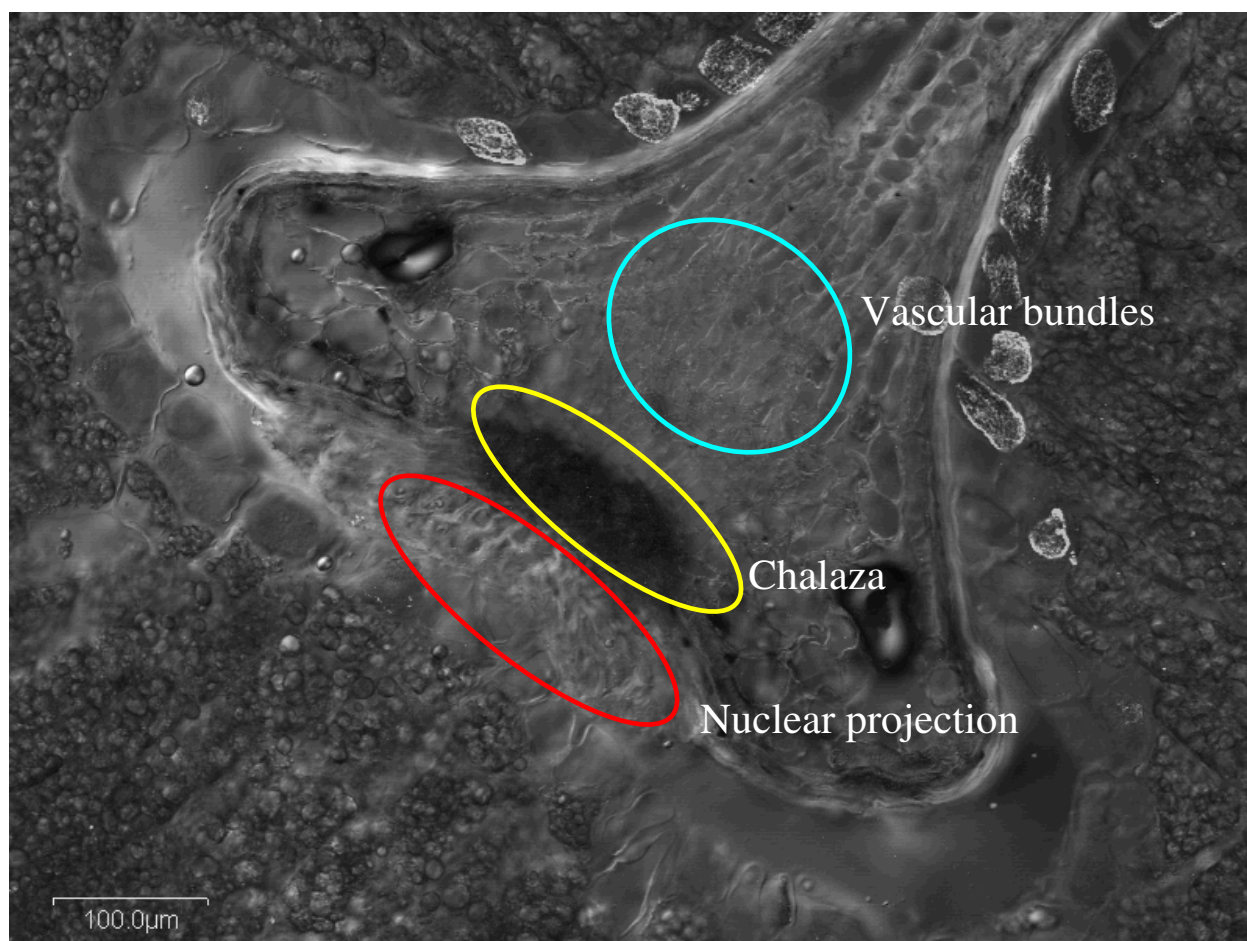


Figure 4.1. Photomicrograph of the ventral groove showing the vascular bundles, chalaza and nuclear projection.

4.2 Materials and Methods

The Materials and Methods for tissue collection, protein extraction and iTRAQ analysis are as per Chapter 2 (Materials and Methods, 2.2.2).

Experiment 4: data for the bran and ventral groove tissues from non-conditioned grain were obtained from experiments 2 and 3 in Chapter 3 (Materials and Methods, 3.2.9; Figure 3.1). A flow diagram of the iTRAQ label allocations are described below and also represented in Figure 4.2.

- From the iTRAQ experiment of the bran tissue proteins in Chapter 3 (Experiment 2), label 114 was used for bran and label 117 was used for the ventral groove protein extract from non-conditioned grain.
- From the iTRAQ experiment of the ventral groove proteins in Chapter 3 (Experiment 3), label 114 was used for ventral groove and label 117 was used for bran protein extract from non-conditioned grain.

The data from each of the above experiments were combined using Protein Pilot. Label 114 (bran proteins) was the denominator in experiment 2 and label 117 (bran proteins) was the denominator in experiment 3.

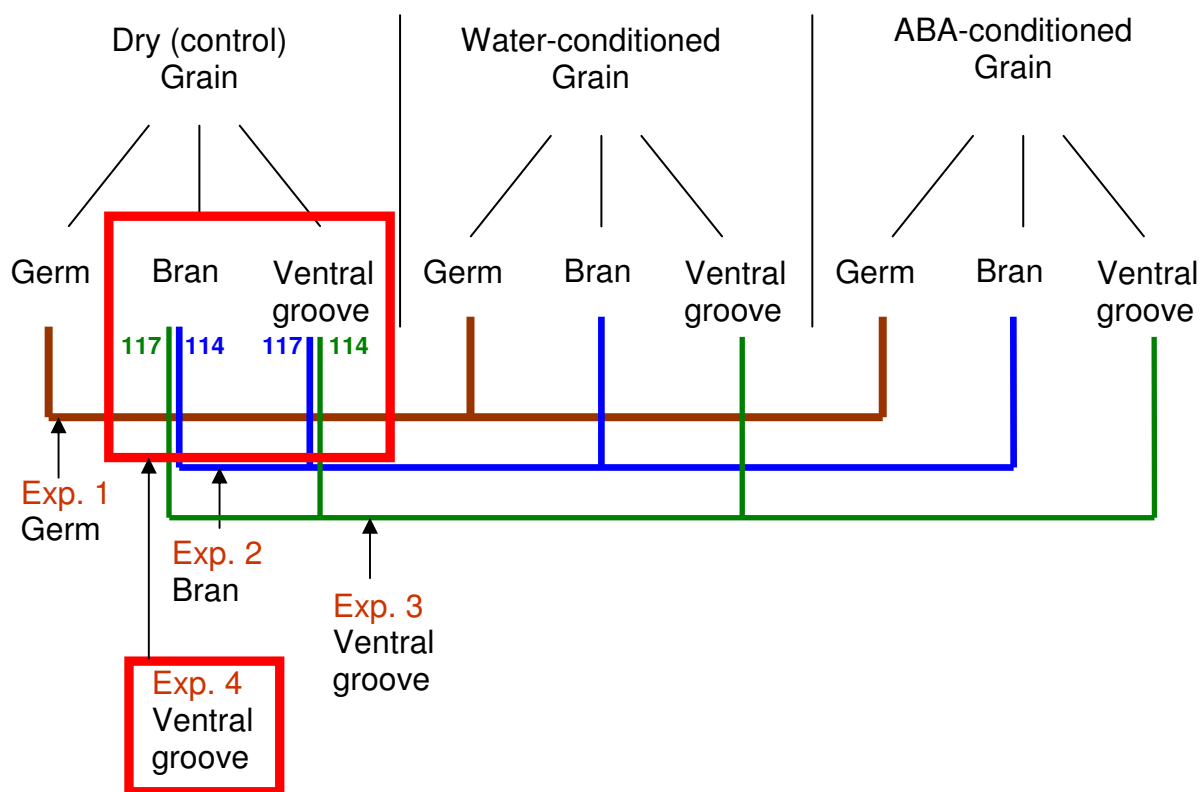


Figure 4.2. Flow chart of the iTRAQ label allocations for proteins extracted from bran and ventral groove from non-conditioned grain.

4.3 Results

Duplicate iTRAQ experiments revealed that 45 proteins out of the 318 (~ 14%) identified in both experiments of the ventral groove and the bran from the same non-conditioned grain were significantly different in abundance (Table 4.1). Of the 45 proteins that had different quantities between the two tissues, 19 were less abundant in the bran and 26 were more abundant compared with the ventral groove (Table 4.2). No proteins were uniquely identified in either of the two tissues.

4.3.1 Differences in protein levels between the ventral groove and bran from non-conditioned grain according to protein class

4.3.1.1 *Biotic and abiotic defense*

The ventral groove from non-conditioned grain had fewer biotic and abiotic defense proteins (six proteins) compared to bran (Tables 4.1 and 4.2).

4.3.1.2 *Membrane transport/transport*

There was ~ 2-fold higher level of secretory protein (one protein) in the ventral groove compared to bran (Tables 4.1 and 4.2).

4.3.1.3 *Cell structure*

There was ~ 1.5-fold less debris and actin binding related protein (one protein) in the ventral groove compared to bran (Tables 4.1 and 4.2).

4.3.1.4 Protein synthesis and folding

Most of the protein synthesis-and folding-related proteins were more abundant (seven proteins) in the ventral groove compared to bran; however, heat shock protein HSP 26 was 1.3-fold less abundant (Tables 4.1 and 4.2).

4.3.1.5 Transcription

There was no difference in transcription-related proteins between the two tissues.

4.3.1.6 Glycolysis and energy

All proteins associated with glycolysis and energy were higher (six proteins) in the ventral groove compared to bran (Tables 4.1 and 4.2).

4.3.1.7 Regulatory and signalling

There was no difference in proteins related to regulation and signaling between the two tissues.

4.3.1.8 Metabolism

Proteins involved in metabolism were only slightly more abundant (four proteins) in the ventral groove compared to bran with three less abundant proteins (Tables 4.1 and 4.2).

4.3.1.9 Proteases

There was no difference in protease proteins between the two tissues.

4.3.1.10 Endosperm-related proteins

All of the endosperm-related proteins identified were less abundant in the endosperm tissue interfacing the ventral groove (four proteins) compared to bran (Tables 4.1 and 4.2).

4.3.1.11 Other function

All other function proteins (eight proteins) had lower levels in the ventral groove compared to bran (Tables 4.1 and 4.2).

4.3.1.12 Unknown function

Finally, there were more unknown function proteins with lower levels (three proteins) in the ventral groove and one protein with higher levels when compared to bran (Tables 4.1 and 4.2).

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).

ID	% Cover	Accession_homologues	Biotic and abiotic defense	VG1:Bran1	VG2:Bran2
253	28.57	Q53YX8_WHEAT	Alpha amylase inhibitor protein.	-1.95	-1.75
19	75.89	Q8GV49_WHEAT	LEA1 protein.	-1.35	-1.22
55	58.94	Q8LK23_WHEAT	Peroxidase 1.	-2.01	-1.56
36	57.04	P93692_WHEAT	Serpin.	-1.26	-1.24
16	75.94	P93693_WHEAT	Serpin.	-1.48	-1.24
22	65.79	XIP1_WHEAT	Xylanase inhibitor protein 1 precursor (Class III chitinase homolog) (XIP-I protein).	-2.46	-3.09
Membrane transport/ transport					
72	54.91	Q9SWZ5_WHEAT	Secretory protein.	2.46	2.06
Cell structure					
686	19.80	Q2TVX9_ASPO	Drebrins and related actin binding proteins.	-1.31	-1.58
Protein synthesis and folding					
232	29.20	Q7X9K6_WHEAT	40S ribosomal protein (Fragment).	1.66	1.24
154	67.61	Q8L4F2_ORYSA	40S ribosomal protein S23.	1.54	1.55
262	47.68	Q5XUU9_WHEAT	Cytoplasmatic ribosomal protein S13.	1.38	1.26
80	56.30	Q9ZSR6_WHEAT	Heat shock protein HSP26.	-1.32	-1.33
30	54.28	Q40058_HORVU	HSP70 precursor.	1.87	1.27
3	72.43	Q7FYS2_WHEAT	Protein disulfide isomerase 1 precursor (EC 5.3.4.1).	1.69	1.35
774	25.34	Q8GSE9_ORYSA	Putative 60S ribosomal protein L27a.	1.71	1.33
125	45.21	Q6YY64_ORYSA	Putative 60S ribosomal protein L6 (RPL6C).	1.31	1.21
Glycolysis and energy					
807	77.57	Q94L27_HORVD	Alcohol dehydrogenase.	1.39	1.28
93	34.36	Q40676_ORYSA	Cytoplasmic aldolase.	1.50	1.51
213	81.28	Q9M4V4_WHEAT	Cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPDH (Fragment).	1.74	1.37
48	51.46	FDH_HORVU	Formate dehydrogenase, mitochondrial precursor (EC 1.2.1.2) (NAD- dependent formate dehydrogenase) (FDH).	1.31	1.35
27	64.04	Q8VWM9_WHEAT	Putative fructose 1-,6-biphosphate aldolase (Fragment).	1.61	1.36
524	12.72	Q7XKB5_ORYSA	Pyruvate kinase (EC 2.7.1.40).	2.28	1.49

(Continued next page)

Table 4.1. (continued) 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).

ID	% Cover	Accession_homologues	Metabolism	VG1:Bran1	VG2:Bran2
87	55.19	ALA2_HORVU	Alanine aminotransferase 2 (EC 2.6.1.2) (GPT) (Glutamic--pyruvic transaminase 2) (Glutamic--alanine transaminase 2) (ALAAAT-2).	1.34	1.42
94	33.05	Q84LA5_HORVU	Asparagine synthetase 2 (EC 6.3.5.4).	-1.27	-1.23
34	57.00	AATC_ORYSA	Aspartate aminotransferase, cytoplasmic (EC 2.6.1.1) (Transaminase A).	1.22	1.22
44	49.19	CATA1_HORVU	Catalase isozyme 1 (EC 1.11.1.6).	1.69	1.76
68	75.94	Q84UH6_WHEAT	Dehydroascorbate reductase.	-1.67	-1.57
65	58.87	Q96185_WHEAT	Manganese superoxide dismutase (EC 1.15.1.1).	-1.23	-1.32
67	55.16	Q9ZR33_WHEAT	Reversibly glycosylated polypeptide.	1.68	1.20
Endosperm-related proteins					
413	21.80	Q6ERU3_ORYSA	Glutelin.	-1.72	-1.29
32	75.34	Q41553_WHEAT	HMW glutenin subunit Ax2*.	-1.57	-1.51
18	74.47	Q45R38_WHEAT	HMW glutenin x-type subunit Bx7 precursor.	-1.56	-1.35
294	50.34	Q8W3V4_WHEAT	Low-molecular-weight glutenin subunit group 8 type IV.	-2.13	-2.30
Other					
8	76.15	Q6W8Q2_WHEAT	1-Cys-peroxiredoxine.	-1.40	-1.25
112	41.38	Q7Y1Z2_WHEAT	27K protein (Fragment).	-1.53	-1.34
102	52.17	Q43769_HORVU	High molecular weight oleosin.	-1.47	-1.79
383	68.18	REHY_ORYSA	Probable peroxiredoxin (EC 1.11.1.15) (Thioredoxin peroxidase) (RAB24 protein).	-1.40	-1.26
96	75.69	REHY_HORVU	Probable peroxiredoxin (EC 1.11.1.15) (Thioredoxin peroxidase) (Rehydrin homolog) (B15C).	-1.46	-1.35
122	45.75	Q75KH3_ORYSA	Putative NAD-/NADP-dependent oxidoreductase.	-1.47	-1.28
217	49.78	Q7F8S5_ORYSA	Putative thioredoxin peroxidase.	-1.36	-1.25
12	69.52	Q08837_WHEAT	Triticin (Fragment).	-1.58	-1.39
Unknown function					
218	27.48	Q7X9M4_WHEAT	Hypothetical protein (Fragment).	-1.56	-1.32
250	46.48	Q8LHX6_ORYSA	Hypothetical protein P0022B05.126.	-1.27	-1.25
817	33.78	O24003_HORVU	Hypothetical protein precursor.	2.72	2.07
221	30.70	Q7XUW5_ORYSA	OSJNBa0027P08.9 protein.	-1.37	-1.27

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.

Protein class	Ventral groove	
	Higher	Lower
Bioitic and abiotic defense	0	6
Membrane transport/transport	1	0
Cell structure	0	1
Protein synthesis and folding	7	1
Transcription	0	0
Glycolysis and engergy	6	0
Regulatory and signalling	0	0
Metabolism	4	3
Protease	0	0
Endosperm-related proteins	0	4
Other	0	8
Unknown	1	3
Total number of proteins	19	26

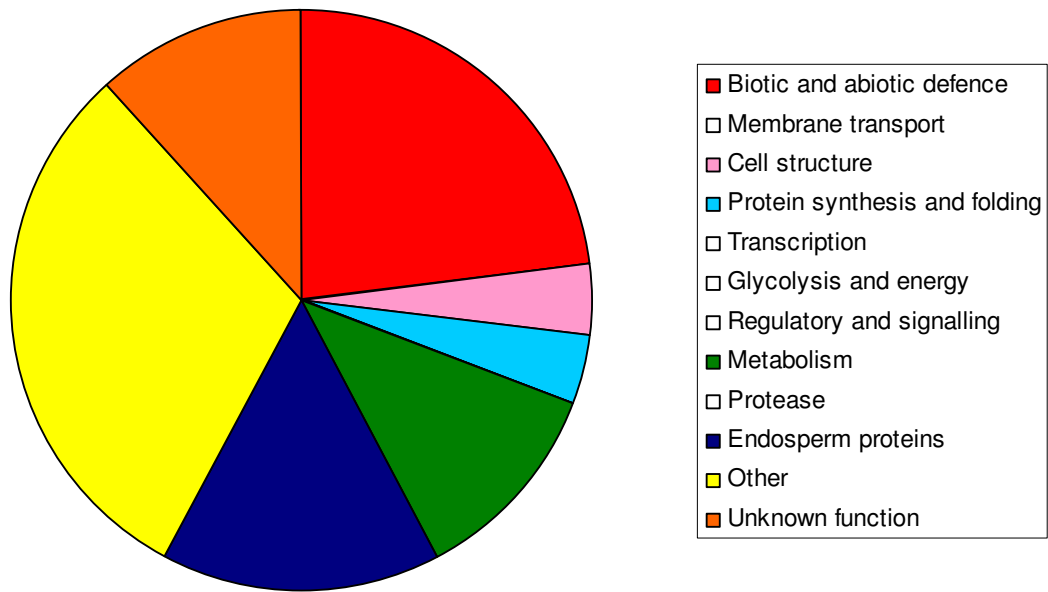
4.4 Discussion

The overall pattern of proteins in the ventral groove when compared to bran tissue from the same non-conditioned grain suggests that the ventral groove is adapted for protein synthesis and folding, energy production and metabolism. Surprisingly, there was a lower abundance of biotic and abiotic defense proteins in the ventral groove compared to the bran (Table 4.1).

The ventral groove tissue contained only a small number of proteins that varied significantly when compared with the proteins from bran tissue. This is not surprising since the aleurone cells in the bran tissue are contiguous around the ventral groove and thus constitute a major protein component of the ventral groove. The majority of these protein abundance variations can thus be attributed to proteins in parts of the ventral groove that do not include the aleurone cells; and/or the aleurone cells in this tissue are functionally different in combination with proteins in surrounding discrete ventral groove tissue.

There were three classes of proteins that were more abundant in the ventral groove compared to the same protein classes from bran tissue. They are proteins involved in membrane transport, protein synthesis and folding, glycolysis and energy and metabolism (Figure 4.3). The relative abundance of these proteins within the ventral groove suggests that the ventral groove may initiate metabolism of starch and storage proteins to supply the embryo during the early stages of growth. Metabolic activity in this tissue would require sufficient water, thus there must be mechanisms in place to transport and direct water to this region.

A



B

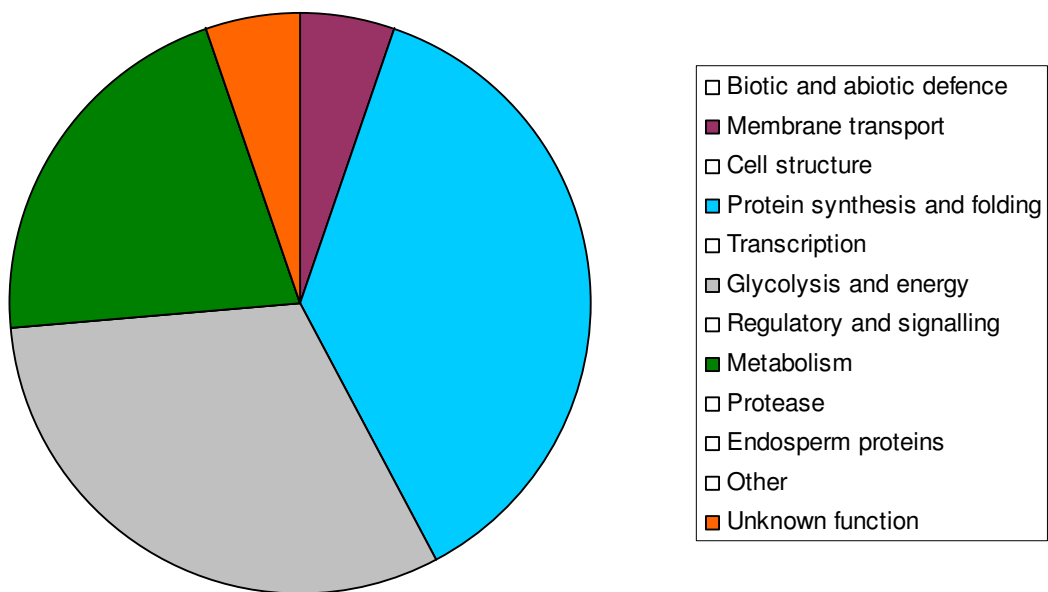


Figure 4.3. Major proteins of the (A) bran tissue ($n = 26$) and (B) ventral groove tissue ($n = 19$).

Another group of proteins that were less abundant in the ventral groove compared to bran were biotic and abiotic defense proteins (Table 4.1). Most of the types of defense proteins identified in the bran tissue were similarly identified in the ventral groove tissue. The relatively lower abundance of these defense proteins was unexpected since the ventral groove would be an ideal place for pathogens to grow due to its crevice like structure. Although, when water is added to the grain (such as in conditioning), the defense proteins increase in levels in the ventral groove, which is likely a result of post-transcriptional regulation of biotic and abiotic defense mRNA's stored during grain maturation (Rajjou et al., 2004). This suggests that the ventral groove is primarily adapted for germination, as illustrated by proteins involved in membrane transport, metabolism, energy and protein synthesis and folding; and secondly via post-translational regulation, the defense proteins are expressed after the start of germination when it becomes vulnerable to pathogen attack.

Serpins were also lower in abundance in the ventral groove compared with the bran. This was similar to the other defense proteins; however, upon hydration, serpin levels in the bran decreased whilst the levels in the ventral groove increased (Chapter 3; Table 3.4). It is unclear whether the serpins in the bran degrade upon hydration and are expressed in the ventral groove, or if they are mobilized towards the ventral groove from the bran.

Finally, it was observed that there were fewer glutenin proteins in the ventral groove tissue compared with the bran tissue. Similarly, both the serpins and glutenins tend to become more abundant in the ventral groove region only after conditioning with water (Chapter 3; Table 3.4) and in turn appear to decrease in the bran region. The movement of macronutrient elements, such as calcium, magnesium and phosphate, has been reported (Eastwood and Laidman, 1971), however, there have been no reports of the movement of proteins. This is interesting in that glutenins have already been expressed and deposited

during grain development (Flint et al., 1975) and thus it is not likely to be due to differential regulation. This suggests that serpins, glutenins and endosperm-related storage proteins may be mobilized towards the ventral groove during hydration/germination. However, due to the very high molecular weight of glutenins, it is more likely that these endosperm-related proteins surrounding ventral groove from the water-conditioned grain have become liberated as a result of partial starch hydrolysis as occurs in germination. Thus, the extraction of proteins from the ventral groove tissue from water-conditioned grain may have resulted in a higher representation of endosperm-related proteins when compared to similar proteins in ventral groove from non-conditioned grain.

Another function of the ventral groove may be to capture and channel water and nutrients to the germ. This could occur through physical contact with moisture from the soil or air. There is evidence that the grain does not require contact with the soil to gain moisture, instead it can germinate when the humidity is above 98.5% (Wuest et al., 1999). The ability of a grain to germinate without physical contact with water, suggests that there must be biochemical forces that are attracting and absorbing water from the humidity in the air. It is possible that the strong hydrophilic properties of the LEA proteins may be involved in this water catching process.

Interestingly, the LEA proteins that increased in levels in the ventral groove tissue of water-and water + ABA-conditioned grain (~ 6-fold and ~5 fold respectively) were less abundant in the ventral groove tissue compared to bran tissue from non-conditioned grain (Table 4.1; Appendix B, Table B.3). This suggests that the LEA proteins in the bran tissue may be attracting water to hydrate the aleurone cells first, and that the LEA proteins slowly begin to increase in levels in the ventral groove once the bran has been hydrated after a period of time. Hydrating the bran tissue first, allows the aleurone cells to hydrate and also

to begin to soften and hydrolyse sub-aleurone endosperm. This process may cause the aleurone-endosperm interface to become porous and when the ventral groove begins to express a much higher level of LEA proteins compared the bran, the water attractive forces in the ventral groove will override the water attractive forces of the bran, thus creating a biochemically induced capillary action towards the ventral groove. The evidence of this is seen in the apparent migration of proteins from the bran tissue to the ventral groove tissue after conditioning (Chapter 3; Table 3.13).

Other functions of the ventral groove may be to channel the signaling molecules such as ABA, GA and auxins. Upon germination the germ signals the aleurone cells to begin producing and secreting hydrolytic enzymes to break down the starchy endosperm (Ho et al., 2003). The mechanism of movement of the signaling molecules, nutrients and water across the ventral groove remains unknown.

Chapter 5 : Identification and immunolocalisation microscopy of late embryogenesis abundant (LEA) proteins

5.1 Introduction

Late embryogenesis abundant (LEA) proteins are involved in protecting cells in the grain during the desiccation process in grain maturation. They also have protective functions against various environmental stresses such as drought, salinity and freezing. The results in Chapters 3 and 4 (Tables 3.4 and 4.1) revealed the location and differential expression of these proteins to also suggest they may play an important role in water distribution within the grain upon hydration and during germination. A recent study on maturing pea seeds applying magnetic resonance imaging (MRI) and immunolocalisation microscopy has shown that water molecules and LEA proteins accumulate in vital areas of the seed during desiccation and suggest that these proteins promote the attraction of water to the vascular bundles (Garnczarska et al., 2008). Another study, applying magnetic resonance micro-imaging (MRMI) to wheat grain after imbibition, revealed that the water accumulates along the ventral groove, concentrating towards the embryo after 15-18 h (Rathjen et al., 2009). The evidence thus far supports a possible relationship between the strong Hydrophilic properties of LEA proteins directing water to parts of the grain where it is required – such as directing water to the embryo and ventral groove.

Immunolocalisation of the Group 2 LEA proteins (dehydrins) using confocal microscopy was applied to determine its distribution within germ cells and aleurone cells of the bran and ventral groove tissue. The distribution of these proteins was examined in non-

conditioned, water-conditioned and water + ABA-conditioned grain to complement the iTRAQ analysis results.

5.2 *Materials and Methods*

5.2.1 List of chemicals

5.2.1.1 *Microscopy*

Paraformaldehyde, potassium chloride, sodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), Tris (Sigma-Aldrich).

5.2.1.2 *Western blot and immunodetection*

Polyclonal primary antibody affinity purified rabbit IgG (Agrisera) raised against the dehydrin K-segment (15 amino acid sequence, EKKGIMDKIKEKLPG), secondary antibody goat anti-rabbit IRDye® 800 CW (LI-COR, Odyssey), secondary antibody goat anti-rabbit IgG (whole molecule) alkaline phosphatase (Sigma-Aldrich), methanol, glycine, Tris (Sigma-Aldrich), Tris-HCl (Sigma-Aldrich), Tween 20, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega), nitro blue tetrazolium (NBT) (Promega).

5.2.2 Determining grain moisture content and conditioning

Wheat grain (cv. Gregory) moisture level was determined and conditioned as described in Chapter 3 (Materials and Methods, 3.2.2 and 3.2.3).

5.2.3 Protein extraction

Grain (10 g) of each sample was ground in a coffee grinder to a fine powder in triplicate. The fine powder was then placed into 50-mL plastic tubes. The tubes were filled 20 mM Tris-HCl buffer, 5% (v/v) glycerol and 10 mM β -mercaptoethanol and held on ice for 1 h. After incubation, the tubes were centrifuged at 5000g for 30 min at 4 °C. Supernatant was

collected into fresh 50-mL plastic tubes and were then placed into a hot water bath at 80 °C for 15 min. The tubes were cooled on ice and then centrifuged at 5000g for 30 min at 4 °C. This method was modified from Garnczarska et al. (2008). Supernatant was precipitated in 100% cold acetone. Samples were centrifuged at 10,000g for 3 min and the pellets were washed three times with cold 80% acetone, 10% TCA. Each time, the samples were vortexed for 30 s and centrifuged at 10,000g for 3 min. The pellets were further washed with cold 10% TCA in water and finally in cold 80% acetone, in the same manner. The protein pellets were semi-dried at room temperature and were then solubilised in 6 M urea. Protein concentrations were estimated using Bradford reagent (Bradford, 1976).

5.2.4 Protein estimation

One μL of BSA standards ($5\ \mu\text{g}.\mu\text{L}^{-1}$, $2.5\ \mu\text{g}.\mu\text{L}^{-1}$, $1.25\ \mu\text{g}.\mu\text{L}^{-1}$ and $0.625\ \mu\text{g}.\mu\text{L}^{-1}$) in duplicate, followed by 1 μL of each sample, also in duplicate, were mixed with 200 μL of diluted Bradford reagent in Milli Q water (1 part Bradford reagent to 4 parts water) in a 96 well microtitre flat bottom plate. Absorbance was read at 595 nm.

5.2.5 One-dimensional SDS PAGE and semi dry blotting

5.2.5.1 *One-dimensional SDS PAGE*

Protein (10 μg) from each sample was heated at 70 °C in 10 μL NuPAGE loading buffer for 10 min and loaded onto 1D gel lanes (Invitrogen, NuPAGE 4-12%, bis-Tris gels, 1.5 mm, 10 wells). Electrophoresis was performed for 35 min at constant 200 V (Bio Rad, Power Pac 3000). After SDS PAGE, the gel was placed into semi dry blotting electrophoresis (Towbin) buffer (20% methanol, 192 mM glycine, 25 mM Tris, pH 8.3) for 20 min.

5.2.5.2 *Semi dry blotting*

Proteins were transferred to polyvinylidene fluoride (PVDF) Immobilon-P transfer membrane pore size 0.45 μm (Millipore), by semi dry blotting (Bio Rad, Trans-Blot SD). PVDF membrane, was cut to size of the 1D gel, soaked in methanol for 5 min, followed by equilibrating in Towbin buffer for 10 min. Three 5 mm thick filter pads were also equilibrated in Towbin buffer. The two filter pads were placed on the bottom electrode of the blotter. On top of the filter pads was placed the equilibrated PVDF membrane making sure to press out any bubbles between the filter pads and the membrane. The gel was placed on top of the PVDF membrane, also removing any bubbles. Finally, another filter pad was placed on top of the gel and the top electrode was attached. Transfer of proteins was at 20 V for 30 min (Bio Rad, Power Pac 3000). The PVDF membrane was removed and rinsed with Milli Q water and dried between filter paper. The membrane was placed between fresh filter papers and wrapped in aluminium foil and stored at 4 °C until used for immuno detection.

5.2.6 Immunodetection of dehydrin proteins

5.2.6.1 *Alkaline phosphatase*

The PVDF membrane was blocked using 5% skim milk in 1 x TBS.T buffer (20 mM Tris-HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.5) for 1 hr on a rocker at room temperature. After incubation, the membrane was washed three times with 1 x TBS.T for 5 min each time on a rocker. Polyclonal antibody affinity purified rabbit IgG (Agrisera) raised against the dehydrin K-segment was diluted 1:1000 in 1 x TBS.T (10 mL). The membrane was incubated with the primary antibody for 1.5 h on a rocker at room temperature and then washed three times with 1 x TBS.T for 5 min each time on a rocker. Secondary antibody goat anti-rabbit linked with alkaline phosphatase was diluted 1:2000 in

1 x TBS.T. The membrane was incubated with secondary antibody for 1 h. After incubation, the membrane was washed three times with 1 x TBS.T for 5 min each time on a rocker. The membrane was stained using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (20 µL) and nitro blue tetrazolium (NBT) (10 µL) in alkaline phosphate substrate solution (100 mM Tris, 100 mM sodium chloride, 5 mM magnesium chloride, pH 9.5) (10 mL). The substrate solution was poured over the PVDF and the membrane was kept in this solution until the first sign of colour appeared on the membrane. Once the colour appeared, the membrane was washed with water to stop the reaction then left to dry.

5.2.6.2 Infrared scan

The PVDF membrane was blocked using 1% casein in 1 x PBS.T (10 mM sodium phosphate, 150 mM sodium chloride, 0.05% Tween 20, pH 7.4) for 2 h on a rocker at room temperature. After blocking, the membrane was washed three times with 1 x TBS.T for 5 min each time on a rocker. Polyclonal antibody affinity purified rabbit IgG (Agrisera) against the dehydrin K-segment was diluted 1:1000 in 1 x TBS.T (10 mL). The membrane was incubated with the primary antibody for 1.5 h on a rocker at room temperature and then was washed three times with 1 x TBS.T for 5 min each time on a rocker. Secondary antibody goat anti-rabbit IRDye® 800 CW was diluted 1:2000 in 1 x TBS.T. The membrane was incubated with secondary antibody for 2 h. After incubation, the membrane was washed three times with 1 x TBS.T for 5 min each time on a rocker. The membrane was scanned at 800 nm using an infrared scanner (LI-COR, Odyssey).

5.2.7 Immunolocalisation of dehydrin protein on grain cross-sections

Grain (c.v Gregory) (10 g) was conditioned with water-and water + 4 ppm ABA to a moisture level of 16.5% for a 16 h period. After conditioning, the grains were stored at -20 °C.

Each end of the grain was dissected with a razor. The germ end of the grain was cut so that it cross-sectioned midway through the germ. The dissected grains were then stored in fixation solution (4% paraformaldehyde in 1 x PBS) overnight at 4 °C. Paraformaldehyde was removed and the grains were washed 3 times with 1 x PBS.

Cross-sections were taken at 60 µm thickness, feed speed 7 and frequency 4 using a vibratome (Leica VT 1000S). The cut sections were placed in cold 1 x PBS. Using a silicone pen, circles were drawn around each well of a 10 well slide to help contain buffer solutions. One section was placed into each well.

The sections were blocked with 10% FBS in 1 x PBS for 1 h at room RT. The blocking solution was removed using a fine tip pipette. Polyclonal antibody affinity purified rabbit IgG in 1 x PBS pH 7.4 (Agrisera) was diluted 1:1000. Approximately 30 µL was added to sections and incubated at RT for 1 h. The primary antibody was removed and washed five times with 1 x PBS, 5 min each time.

Secondary antibody with fluorescence label (anti-rabbit with Alexa Fluor® 488/546) was diluted 1:400 in 10% FBS in 1 x PBS. To each section, ~ 30 µL was added and incubated in a dark box to avoid light exposure, for 1 h at RT. The secondary antibody was removed and the sections were washed five times with 1 x PBS, 3 min each time. Sections were then

covered with ~ 30 μ L of 1.5 mM propidium iodide nucleic acid stain and incubated at RT for 5 min. The sections were washed five times with 1 x PBS, 3 min each time. Finally, the PBS was removed and onto each section was placed 5 μ L of gelmount. The cover-slip was placed onto the slide and sealed using generic nail polish to prevent the sections from drying out.

5.2.8 Fluorescence confocal microscopy

Micrographs were captured using an Olympus IX 70 confocal microscope. Absorbance and emission maximum wavelength for Alexa Fluor® 488/546 conjugated secondary antibody was 495 nm and 519 nm and for propidium iodide (nuclear stain) was at 535 nm and 617 nm.

5.3 Results

5.3.1 Immunolocalisation microscopy of dehydrin proteins in germ tissue

Immunolocalisation microscopy for dehydrin proteins revealed that they were all localised within the intracellular matrix of most but not all of the germ cells; however, they did not appear to be within any developing organs of the embryo such as the coleoptile, leaves or root (Figure 5.1 and 5.2). The pattern of dehydrin distribution within the cells were not uniform. Upon close observation, the dehydrin proteins appear to be concentrated on the inner cell wall surface and surrounding the nucleus. However, there were inconsistent patches of high dehydrin density on the inner cell wall surface. In some cells the dehydrins were distributed in a honeycomb or network like pattern throughout the intracellular matrix (Figure 5.2).

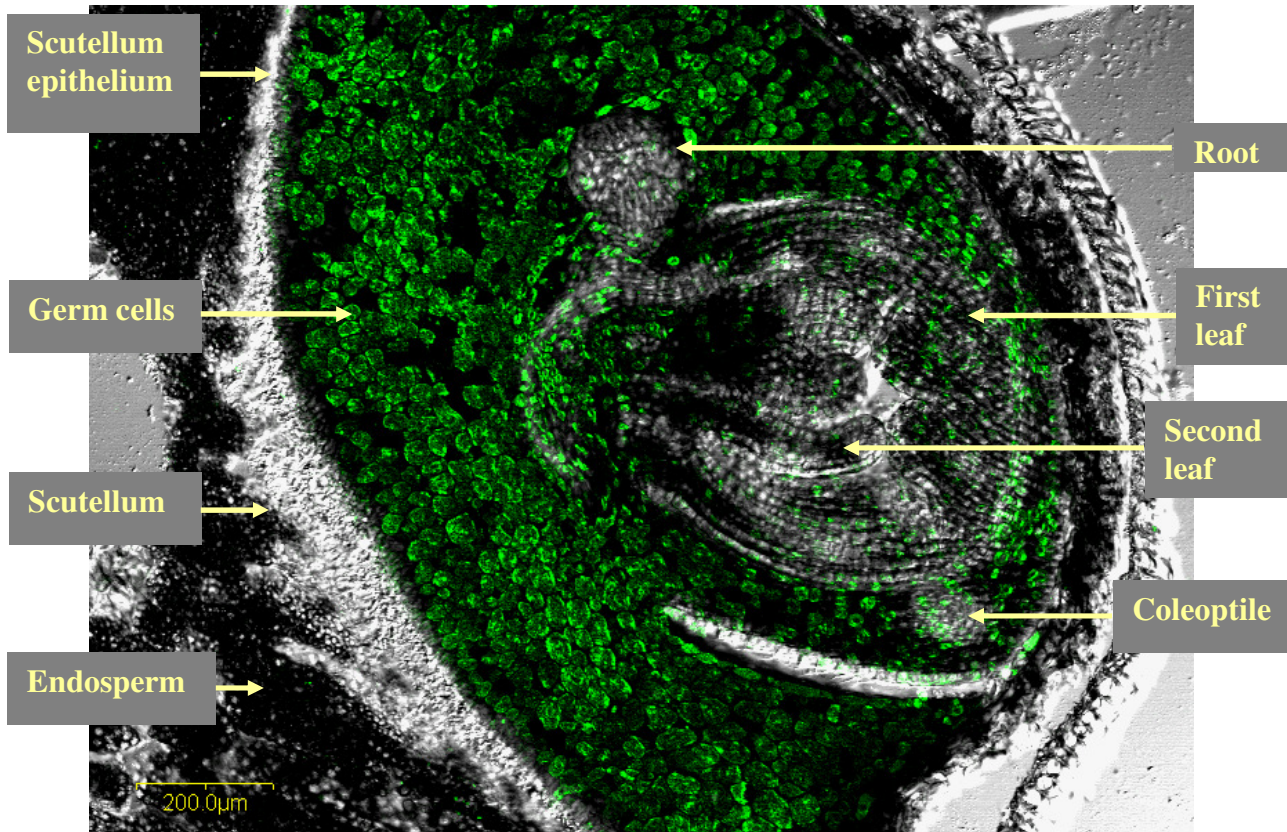


Figure 5.1. Photomicrograph of a cross-section through the germ highlighting the location (green) of dehydrin proteins within its tissue cells.

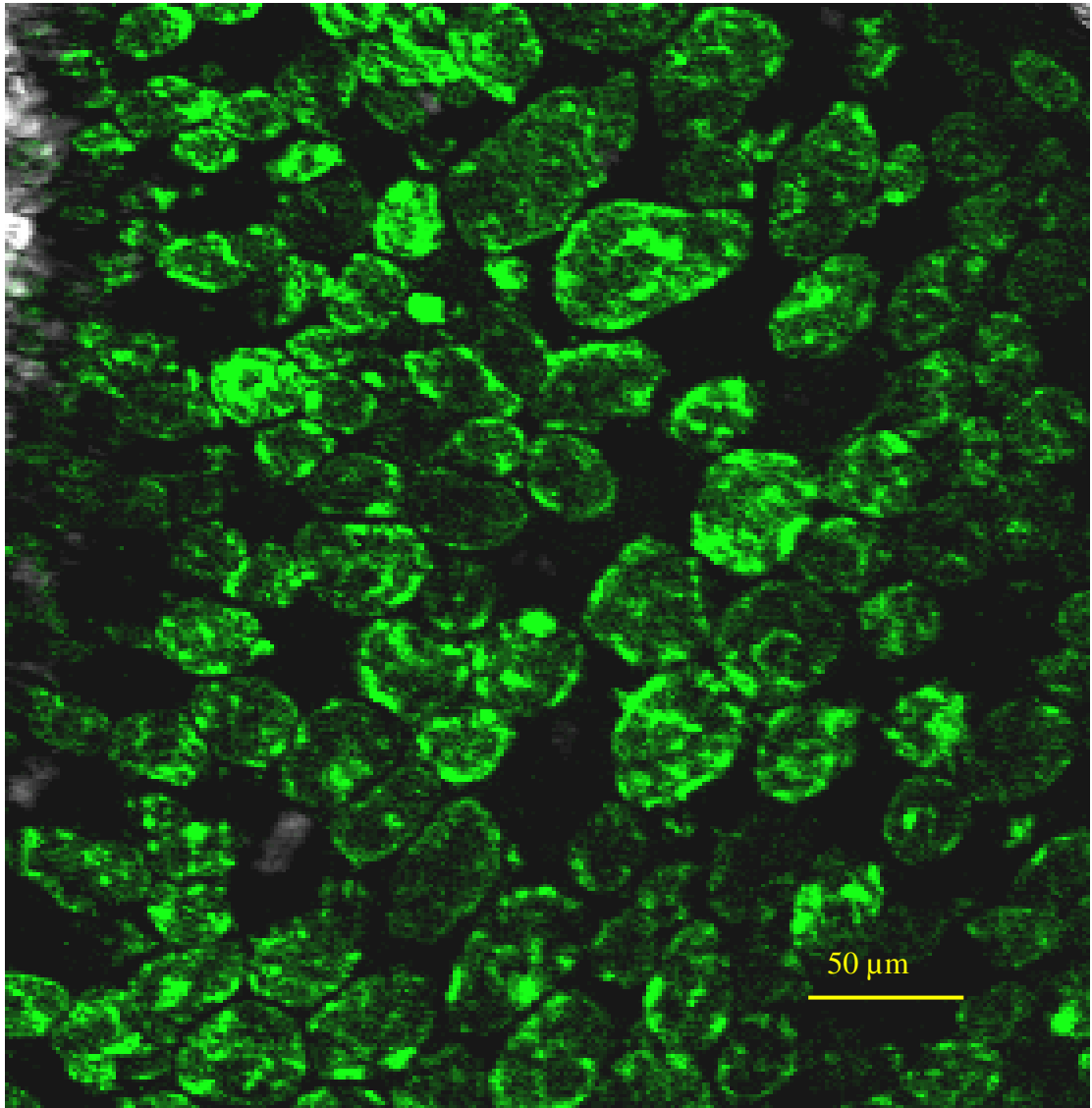


Figure 5.2. Enlarged photomicrograph of a cross-section through the germ highlighting the location (green) of dehydrin proteins within its tissue cells.

5.3.2 Immunolocalisation microscopy of dehydrin proteins in aleurone cells of the bran tissue

Immunolocalisation microscopy for dehydrin proteins revealed that they were all localised within the intracellular matrix of all of the aleurone cells in the bran and ventral groove tissues (Figure 5.3 and 5.4). The pattern of dehydrin distribution within the cells were consistent in all of the cells. Upon close observation, the dehydrin proteins were concentrated on the inner cell surface and surrounding the nucleus. As with the germ cells, dehydrins were consistently distributed in a honeycomb or network like pattern throughout the intracellular matrix in all cells. The location of the nucleus was confirmed using propidium iodide nuclear stain (Figure 5.4).

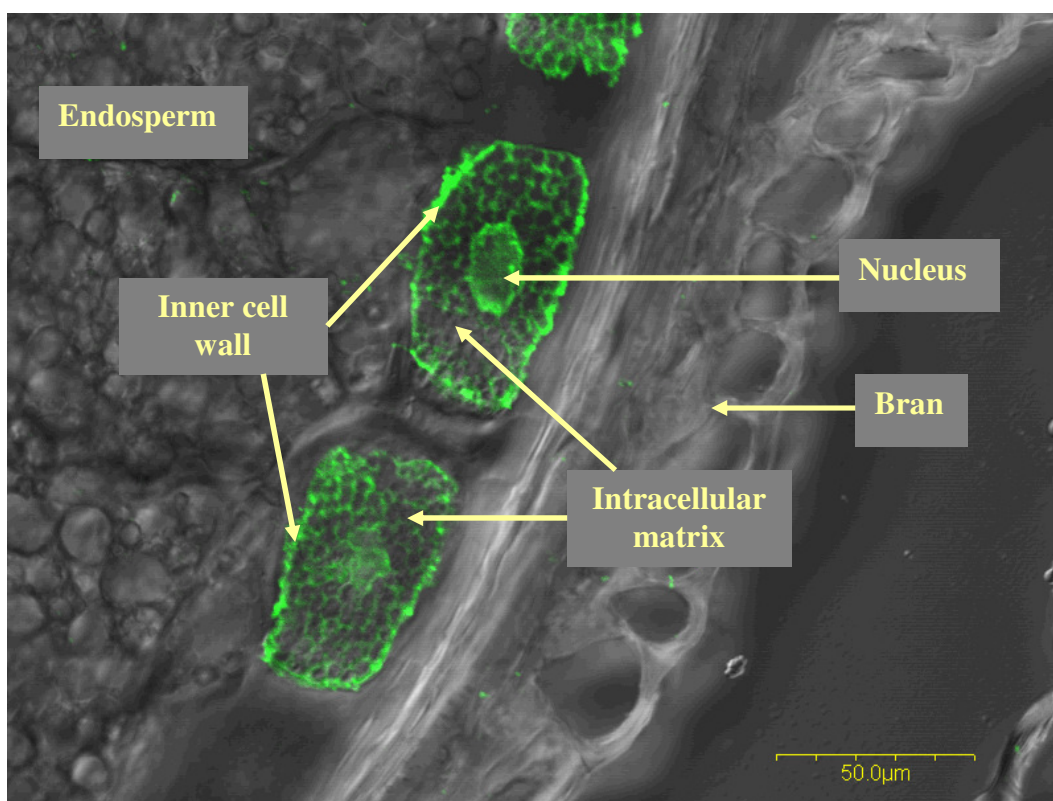


Figure 5.3. DIC photomicrograph of a cross-section of grain showing dehydrin proteins (in green) localised within aleurone cells of the bran tissue.

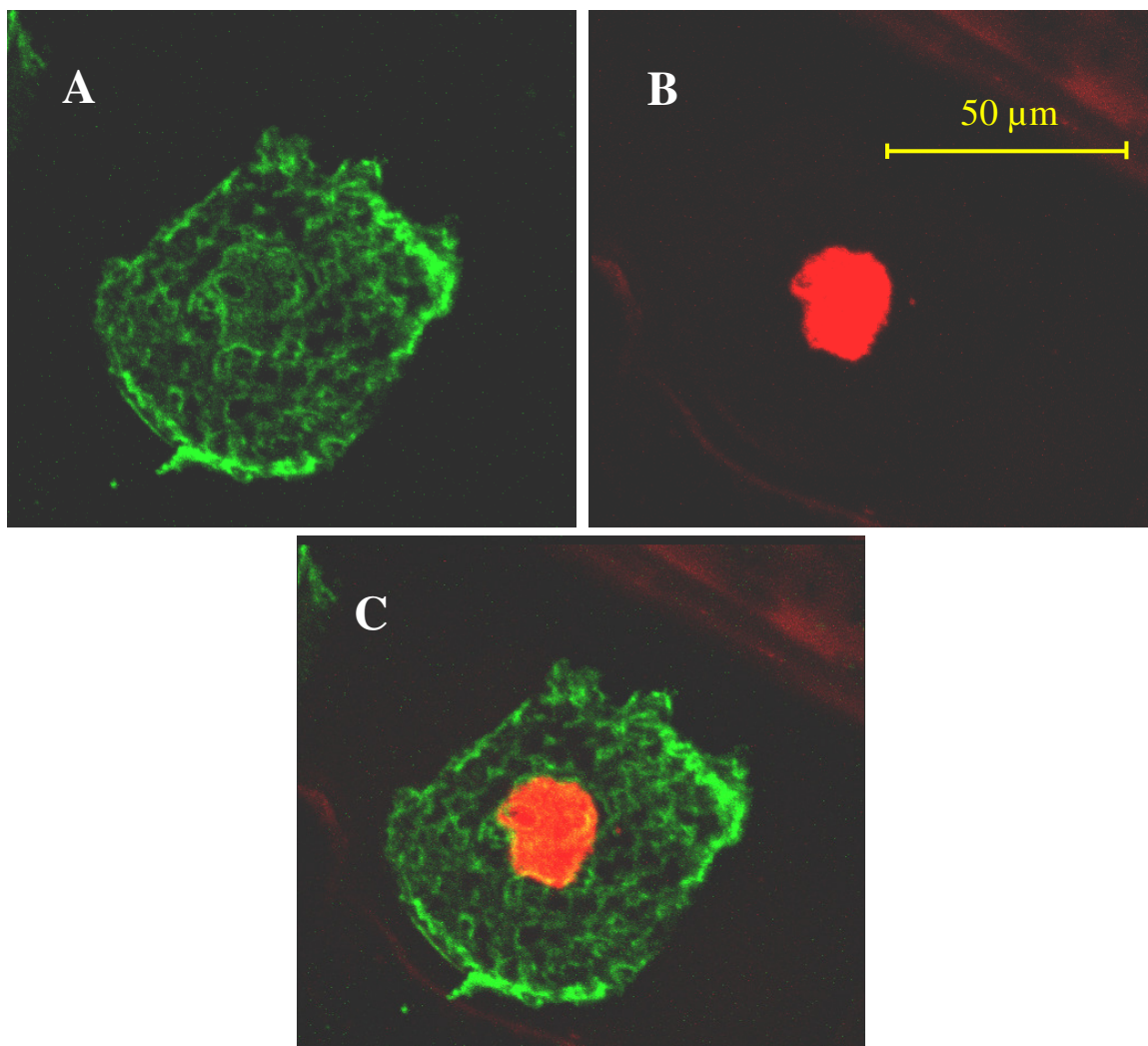


Figure 5.4. Photomicrographs of a cross-section of grain showing the localisation of (A) dehydrin proteins (coloured green) and the (B) nucleus (coloured red) within the aleurone cells of the bran tissue. (C) is an overlay of images (A) and (B).

5.3.3 Immunolocalisation microscopy of dehydrin proteins in the ventral groove tissue from non-conditioned, water-and water + ABA-conditioned grain

Immunolocalisation microscopy for dehydrin proteins revealed that they were all localised within the intracellular matrix of all of the aleurone cells in ventral groove tissues similar to that of the aleurone cells of the bran (Figure 5.5). In comparing the non-conditioned sections (Figure 5.5 A), with the conditioned sections (Figures 5.5 B and C), the intensity of the labeling of dehydrins always appeared to be much lower in the non-conditioned grain, suggesting an increase in dehydrin levels after conditioning. There were no dehydrins detected in areas of the vascular bundles, chalaza and nuclear projection in either the non-conditioned or conditioned grain.

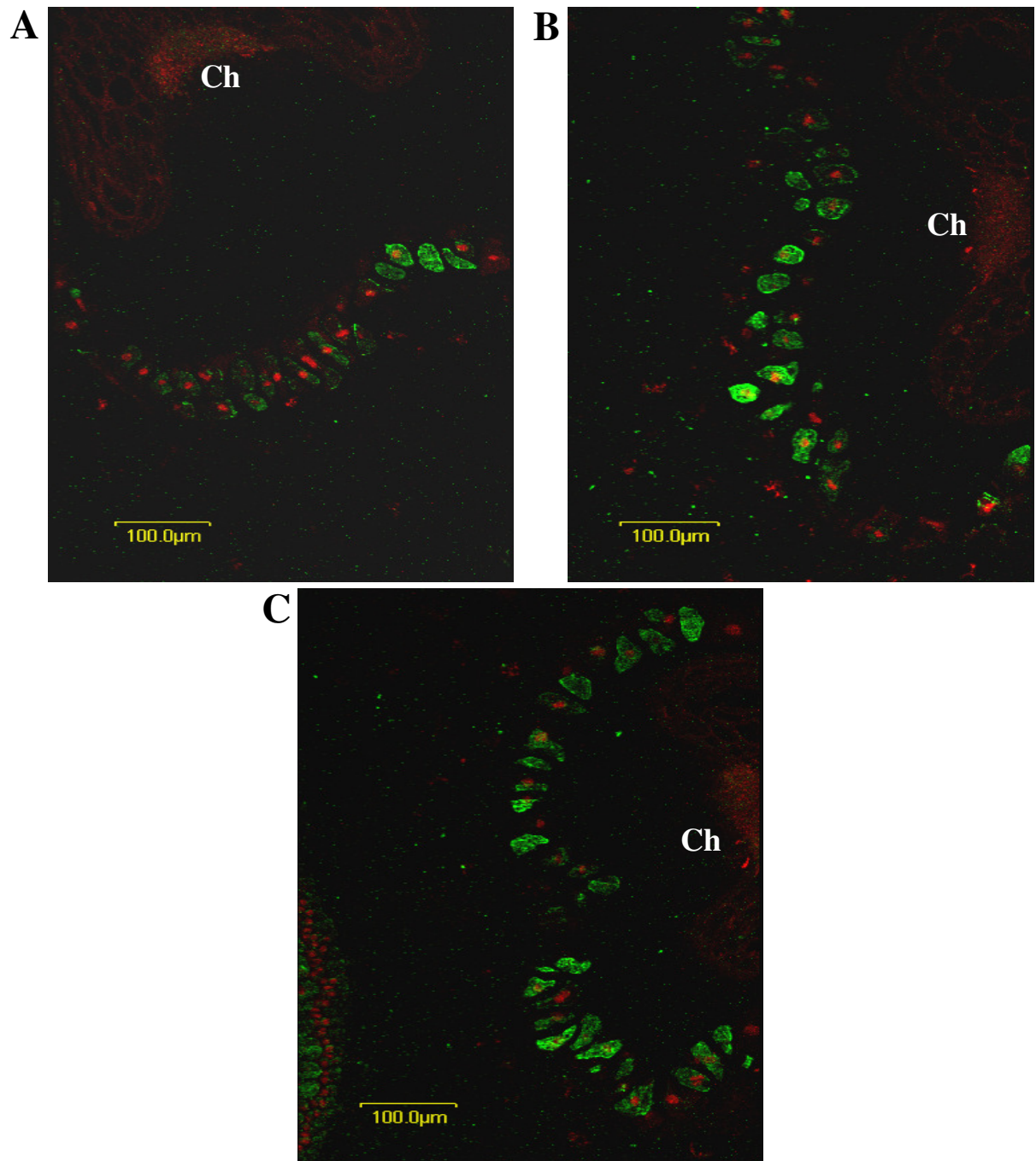


Figure 5.5. Photomicrograph of a cross-section of (A) non-conditioned, (B) water-conditioned and (C) water + ABA-conditioned grain showing the nucleus (red) and dehydrin proteins (green) localised within the aleurone cells of the ventral groove tissue. CH = Chalaza.

5.3.4 SDS PAGE and western blot of heat-stable proteins extracted from whole grain

SDS PAGE of the heat-stable protein extract from non-conditioned, water-and water + 4 ppm ABA-conditioned grain revealed multiple protein bands. The majority of protein bands were between the ranges ~ 100 kDa and ~ 10 kDa (Figure 5.6).

The western blot using a polyclonal primary antibody for the conserved K-segment of dehydrin proteins revealed fewer bands than in the 1-D gel (Figure 5.7). The molecular weights of the detected bands were, (1) ~ 75 kDa, (2) ~ 35 kDa, (3) ~ 13 kDa, (4) ~ 10 kDa and (5) ~ 7 kDa.

The protein bands (1), (2) and (4) were detected on the western blot, were barely visible on the Coomassie stained 1D gel. The protein bands (3) and (5) were visible with the Coomassie stain (Figures 5.6 and 5.7).

Qualitative analysis of the western blot of the three treatments suggests that the heat-stable protein extract from the water + ABA-conditioned grain appeared more abundant in dehydrins (Figure 5.7).

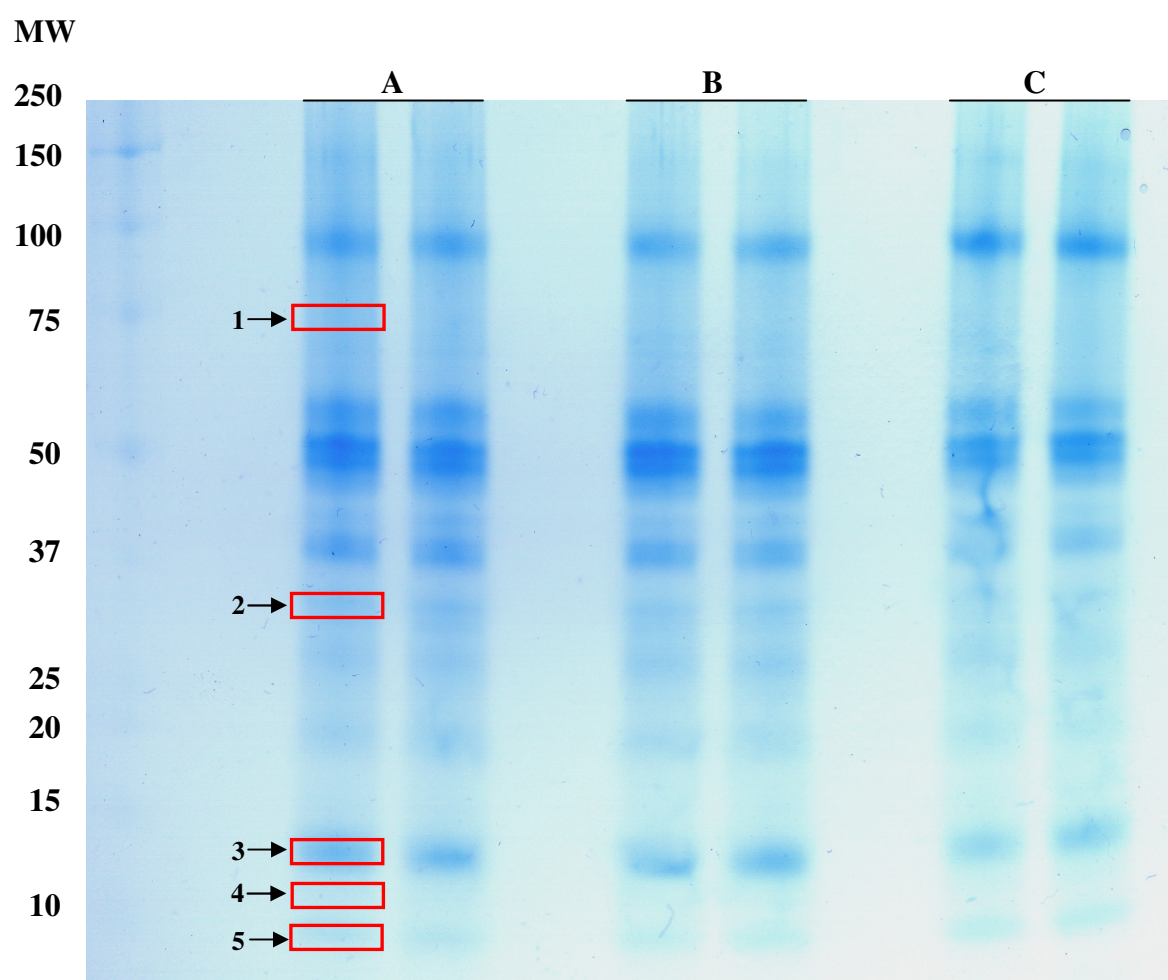


Figure 5.6. SDS PAGE of heat-stable proteins extracted from whole grain. (A) Non-conditioned, (B) water-conditioned and (C) water + ABA-conditioned grain. The protein bands boxed in red, numbered 1 – 5, correspond with the western blot (Figure 5.7).

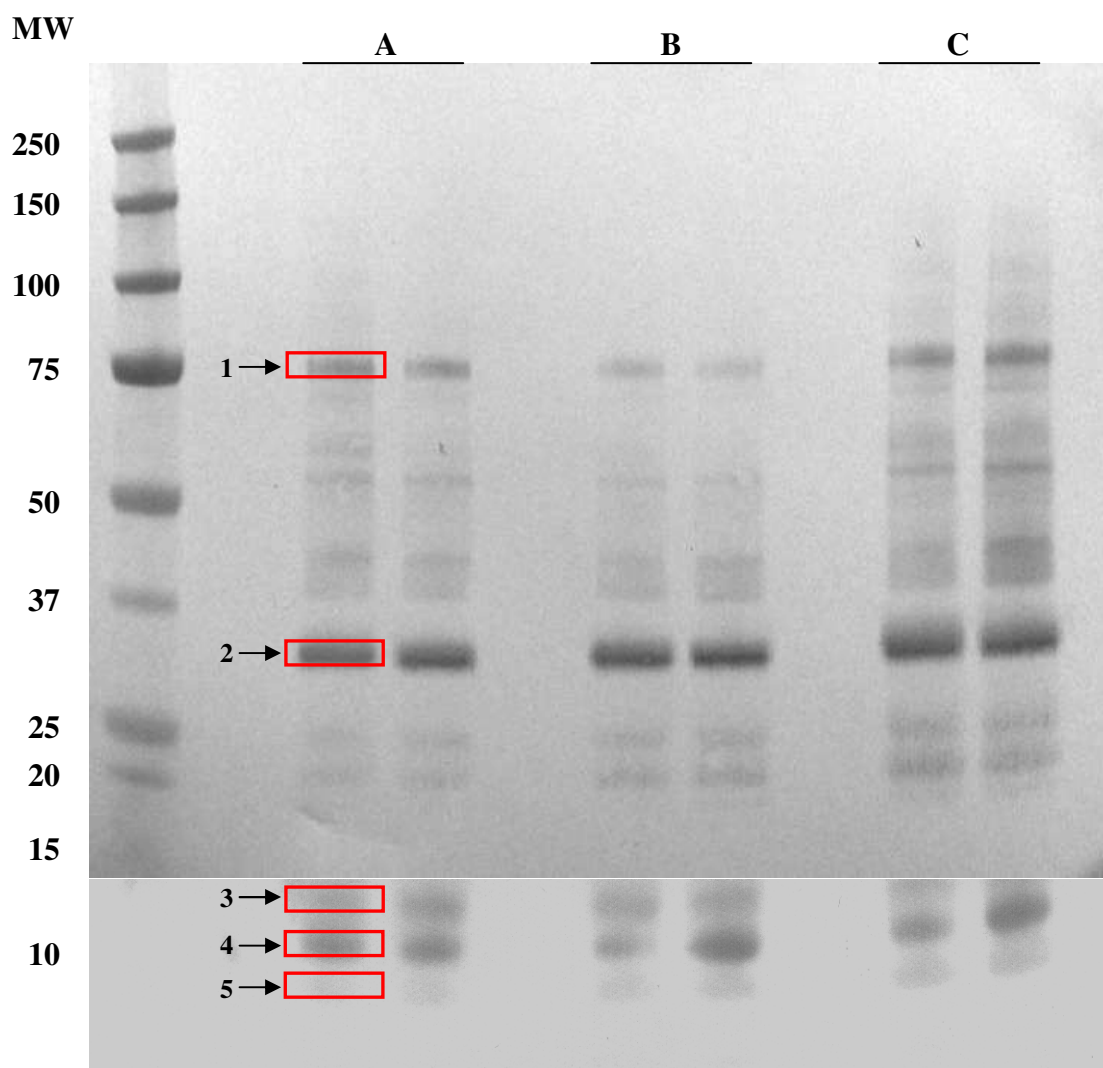


Figure 5.7. Western blot and dehydrin detection of heat-stable proteins extracted from (A) non-conditioned, (B) water-conditioned and (C) water + ABA-conditioned grain. The bands boxed in red, numbered 1 – 5, correspond with the 1D gel of the heat-stable proteins (Figure 5.2). The lower molecular weight range (bellow 15 kDa) was collaged to the upper 250 – 15 kDa molecular weight range.

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

5.3.5.1 *Germ*

The western blot detection of dehydrins in the germ protein extract from non-conditioned, water-and water + 4 ppm ABA-conditioned grain revealed six bands (~ 55, ~ 47, ~ 27, ~ 24, ~ 18 and ~ 17 kDa) (Figure 5.11 B). Of these bands, the ~ 24, ~ 27, ~ 18 and ~ 17 kDa bands were barely detected on the Coomassie stained SDS PAGE gel (Figure 5.8 A).

The dehydrins identified in the iTRAQ analysis of the germ matched to four dehydrins; 46.16 kDa dehydrin (Q9SPA6) at the ~ 47 kDa band; 28.19 kDa dehydrin-COR410 at the ~ 27 kDa band; 24.62 kDa dehydrin-4 at the ~ 24 kDa band; and 18.08 kDa dehydrin-7 at the ~ 18 kDa band. There were no proteins identified in the iTRAQ experiment that account for the ~ 55 and ~ 17 kDa bands on the western blot (Table 5.2).

Qualitative analysis of the western blot shows the only visibly more intense bands (~ 24, ~ 27, ~ 18 and 17 kDa) were the in the protein extract of the germ from water-conditioned grain. (Figure 5.8 B).

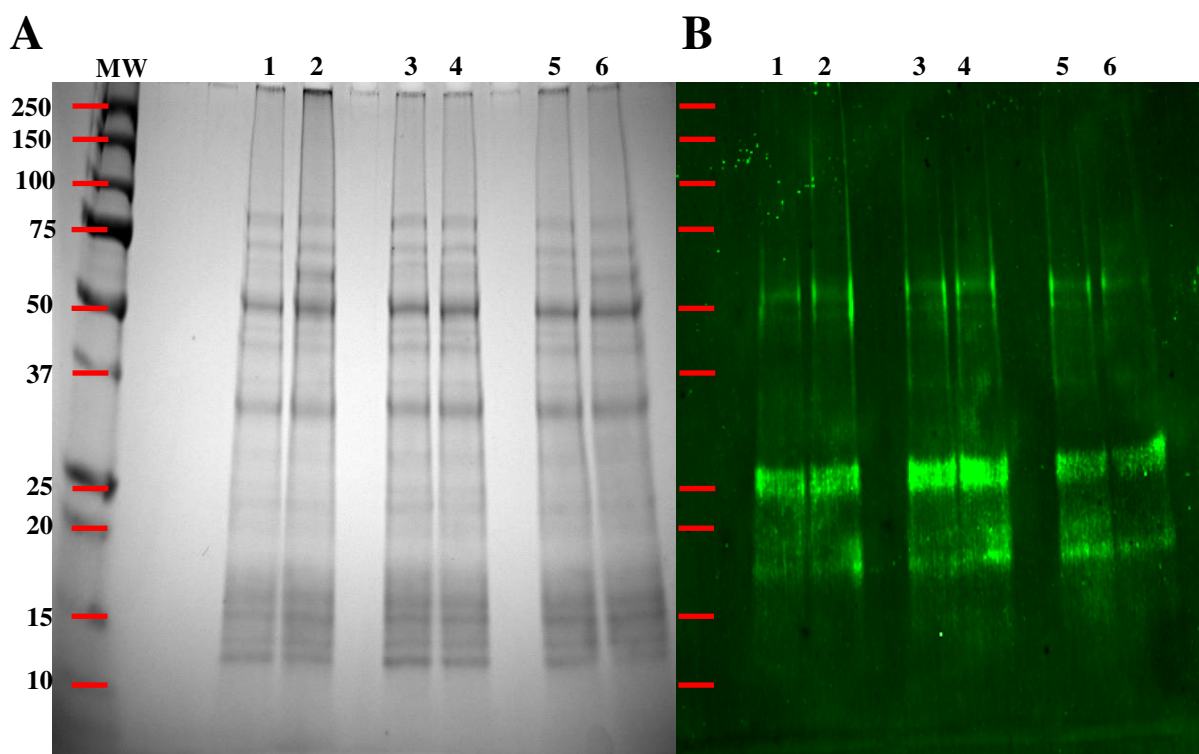


Figure 5.8. (A) 1D gel and (B) dehydrin detection western blot of proteins extracted from the germ from the iTRAQ experiment. Lanes 1 and 2 are germ protein extract from non-conditioned grain, lanes 3 and 4 are proteins extracted from water-conditioned grain and lanes 5 and 6 are proteins extracted from water + ABA-conditioned grain.

5.3.5.2 Bran

The western blot detection of dehydrins in the bran protein extract from non-conditioned, water-and water + 4 ppm ABA-conditioned grain revealed nine bands (~ 135, ~ 105, ~ 90, ~ 85, ~ 55, ~ 45, ~ 30 and ~ 28 kDa) (Figure 5.12 B). Of these bands, the ~ 135, ~ 30 and 28 kDa bands were barely detected on the Coomassie stains SDS PAGE gel (Figure 5.9 A).

The dehydrins identified in the iTRAQ analysis of the bran were only matched to 46.16 kDa dehydrin (QSPA6) (Table 5.2).

Qualitative analysis of the western blot shows that there were no visibly more intense bands in the bran protein extract from water-conditioned grain. (Figure 5.9 B).

5.3.5.3 Ventral groove

The western blot detection of dehydrins in the ventral groove protein extract from non-conditioned, water-and water + 4 ppm ABA-conditioned grain also revealed 9 bands (~ 135, ~ 105, ~ 90, ~ 85, ~ 55, ~ 45, ~ 30 and ~ 28 kDa) similar to that of the bran protein extract (Figure 5.12 B). Of these bands, the ~ 135, ~ 30 and 28 kDa bands were also barely detected on the Coomassie stains SDS PAGE gel (Figure 5.9 A).

The dehydrins identified in the iTRAQ analysis of the ventral groove were not matched to any of the dehydrins detected in the western blot (Table 5.2).

Qualitative analysis shows that the only visibly more intense bands (~ 105, ~ 90, ~ 85, ~ 55 and ~ 45 kDa) were the in the ventral groove protein extract from water + 4 ppm ABA-conditioned grain. (Figure 5.9 B).

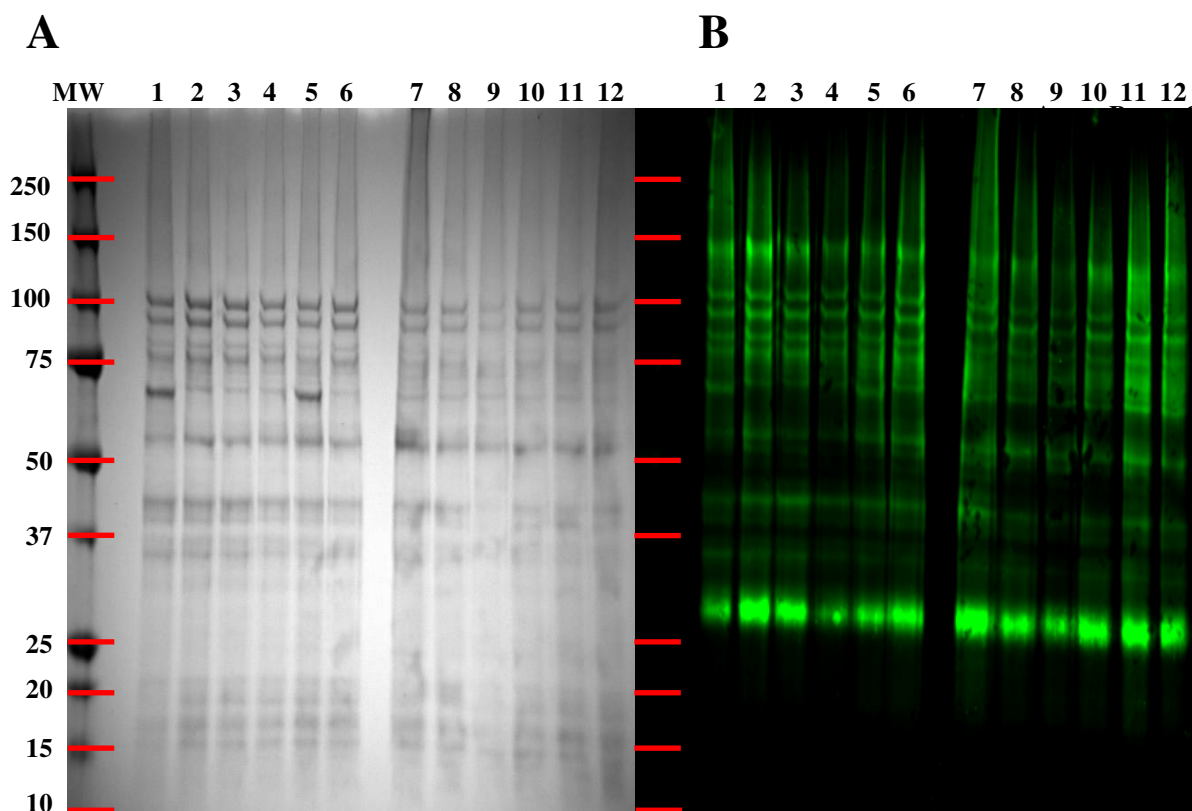


Figure 5.9. (A) 1D gel and (B) western blot probed with antibodies to dehydrin of proteins extracted from the bran and ventral groove tissues used in the iTRAQ experiments. Lanes 1 and 2 are bran protein extracts from non-conditioned grain, lanes 3 and 4 are protein extracts from water-conditioned grain 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.

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.

Accession_ homologues	Protein name	Molecular weight	Germ protein extract	Bran protein extract	Ventral groove protein extract	Heat- stable protein extract
Q9SPA6_HORVU	Dehydrin	46.16	++	++		
Q9SPA9_HORVU	Dehydrin	15.69				
Q40651_ORYSA	Dehydrin	16.67				
Q549A8_HORVU	Dehydrin 1	14.24				+
Q9ZTR6_HORVU	Dehydrin 4	24.62	++			+
Q9ZTR4_HORVU	Dehydrin 7	18.08	++			
Q9ZTR3_HORVU	Dehydrin 9	15.13			+ -	
Q9ZTR8_HORVU	Dehydrin 11	23.46			+ -	
CO410_WHEAT	Dehydrin COR410 (Cold-induced COR410 protein)	28.19	++			
DHN3_HORVU	Dehydrin DHN3 (B17)	16.17				
DHR15_WHEAT	Dehydrin Rab15	15.77				
Q8W192_WHEAT	Dehydrin WZY1-1	15.97				

5.4 Discussion

Dehydrins were mostly concentrated on the inner cell walls, intracellular matrix and also surrounding the nucleus. The dehydrins in the cytoplasm all appear to be distributed in a honeycomb or network like pattern (Figures 5.3 and 5.4). In terms of water distribution upon hydration, the inherent strong hydrophilic properties of dehydrins and the observed distribution within the aleurone and germ cells, are likely to have a strong influence on how water is distributed throughout the grain.

Dehydrins have previously been localised within the germ and aleurone cells of maize (Asghar et al., 1994). Their intracellular distribution in the aleurone cells of maize shows a similar distribution in wheat aleurone cells. Asghar et al. (1994) reported that in aleurone cells, the dehydrins are located in the cytoplasm and in the nucleus. However, in this study, dehydrins did not appear within the nucleus but instead were surrounding the nucleus (Figures 5.3 and 5.4).

The localisation of dehydrins within the germ cells and aleurone cells of the bran and ventral groove tissue, suggest that they may influence how water is distributed around the grain after contact with water. Early studies using autoradiography analysis on the movement of water during grain conditioning revealed that at the initial stage after water contact, it accumulated in the germ/bran interface and the bran tissue on the dorsal side of the grain. Only towards the end of the conditioning period did water accumulate in the ventral groove region of the grain (Stenvert and Kingswood, 1976).

More recent studies using magnetic resonance imaging of water distribution within grain at 20 % moisture content after 24 h revealed that its distribution was uniform (Chua et al., 2004). However, others have shown – applying magnetic resonance micro-imaging and also

iodine-potassium iodide in the imbibition water to track water movement within the grain through the staining of lipids and starch – that water initially accumulates in the embryo and scutellum via the micropyle (the pointy tip at the germ end of the grain) (Rathjen et al., 2009). Significant water distribution patterns appeared only after 18 h of imbibition. Rathjen et al. also reported that water was detected on either side of the seed coat, however, they found no evidence that it penetrated the seed coat.

As seen from these previous studies, it is difficult to obtain a clear picture of how water is distributed throughout the grain and also, what forces (biochemical or physical) are directing the water. The density and distribution of dehydrin and other LEA proteins (non dehydrin) in various locations throughout the grain may be responsible for influencing where water is directed. Also, the results in Chapter 3 (Discussion, 3.4.1), revealed that there was a ~ 5 to 6-fold increase in a putative LEA protein after the conditioning period (Appendix B, Table B.3).

The Coomassie stained 1-D gels of the protein extracts did not detect some of the dehydrins, even though they were detected in the western blot (Figures 5.8 and 5.9). The relatively low basic amino acid composition of dehydrins (high % basic amino acid decreases binding of Coomassie stain) is not a likely factor; however, the stain does require hydrophobic bonding for the electrostatic binding (Tal et al., 1985). Due to the strong hydrophilic nature of these proteins, it is likely that they may not be well represented when detecting via Coomassie staining methods.

The dehydrins detected in the western blots revealed that the germ and aleurone cells of the bran and ventral groove tissues contained different classes of dehydrins. Although dehydrins are similar in nature in their function, they must have subtle differences in

properties which make them suitable for the different tissue types and/or have other complex regulatory functions. It has been reported that in transgenic *Arabidopsis* plants that over expresses the wheat dehydrin-5 showed pleiotropic effects, which included changes in gene expression of biotic and abiotic, metabolic, regulation and osmotic stress (Brini et al., 2011). Over expression of dehydrin-5 also had an effect on jasmonate signaling. It caused extreme down-regulation of three negative regulator jasmonate-ZIM proteins, in turn decreasing the plants sensitivity to jasmonate (Brini et al., 2011). Others have also reported on the multiple-functions of various dehydrin subclasses (Rorat, 2006).

The LEA proteins identified in the iTRAQ experiments in Chapter 3 (Table 3.4), did not account for all of the protein bands detected in the western blot of the heat-stable protein extract from whole grain, germ, bran and ventral groove tissue from non-conditioned, water-and water + 4 ppm ABA-conditioned grain (Table 5.1). This suggests that this may be a limitation of iTRAQ in distinguishing between proteins that have such a large subclass that share many similar conserved regions.

Chapter 6 : Grain fractionation and physiological analysis of water-and water + ABA-conditioned wheat grain

6.1 Introduction

6.1.1 Grain fractionation

Grading the quality of wheat is important in industry to determine its market value, milling properties, end use and baking properties. Wheat varieties with known milling and baking qualities may also vary due to environmental and biotic factors such as drought, salinity, poor soil nutrients, water damage and microbial attack. Testing determines the value of wheat in terms of its protein content, protein quality, falling number, screening for shriveled grains and weed seeds, staining caused by black point and other fungal infections, hardness, moisture content and test weight (Simmonds, 1989). When the quality of wheat fails to meet criteria for milling and baking it will be classed as low grade. This is mainly due to poor or unsuitable baking properties. The nutritional content of low grade wheat is not affected and is usually sold off as animal feed.

The test weight of wheat is used as an indicator of milling performance. Further testing for milling performance is performed using milling simulators, such as large industrial scale test mills down to smaller scale mills. A smaller mill commonly used is a Bühler brand mill, which processes ~ 2 kg of sample, is commonly used to measure milling performance of wheat. The grain crushing motion is similar to that of an industrial mill, which simulates forces exerted on the grain as it would in an industrial mill. The fractions collected are analysed to determine milling performance by measuring flour extraction rate and bran

contamination. One such device to measure bran contamination in flour is called a Branscan (FOSS Pacific, North Ryde, Sydney, Australia) which counts bran specs in the flour. Together, these properties are used to calculate the milling quality index (MQI), which uses the equation “ $MQI = \text{Branscan spec number} \times 100 / \text{extraction rate}$ ” (Osborne et al., 2007). Thus, the lower the number denotes better milling quality. There is also a smaller version of the Bühler mill called a micromill (INRA-UTCA). It has only one set of break rollers and can process samples of ~ 100 g. It measures milling quality in terms of hardness and vitreousness, which have an effect on grinding resistance (Pujol et al., 2000). Another method used is the single-kernel characterization system (SKCS) (Osborne et al., 2007). The SKCS 4100 instrument, manufactured by Perten Instruments AB, Segeltorp, Sweden, measures the rheological properties of bran and endosperm to determine the milling quality. The measurement parameters used by Osborne et al. (2007), were strength, ductility, stiffness and toughness. They were used to generate a Rheology Index, which is a ranking of milling quality (Osborne et al., 2007). This method is much quicker and more efficient at generating milling quality data than the Bühler mill but it is unsuitable for testing conditioning treatments.

A fast and inexpensive method to determine wheat milling quality will be of great benefit in the laboratory. This is important if many samples and replicates are required to screen for good milling wheat in selective breeding programs or conditioning treatments to grain that can improve milling performance.

6.1.2 Psychrometry and mechanical property analysis

Psychrometry is essentially the science of measuring the thermodynamic properties between humidity and temperature. A psychrometer is used to measure humidity via a

thermocouple. Samples are sealed inside the psychrometer chamber and are allowed to equilibrate for several hours at a fixed temperature. Condensation of water on the thermocouple causes cooling, which alters the current (in millivolts) that passes through the thermocouple. There is a relationship between humidity and water potential (ψ). The relative humidity above sodium chloride solutions of known molality, hence ψ , is used to generate a standard curve to determine the ψ of unknown samples.

Psychrometry has been applied in plant research to determine the relationship between ψ and grain dormancy, quiescence and germination (Gummerson, 1986; Bradford, 1990; Christensen et al., 1996; Bradford, 2002). Most of this work has been concerned with germination rates and the ψ required for the initiation of germination. This can also be applied to determine water distribution in conditioned grain. Measuring the ψ of germ-end and bran/endosperm-end of conditioned grain will provide further information on where water is distributed and/or retained.

It is likely that any such changes in water distribution within the grain could also be measured or detected by measuring physical characteristics such as tissue softness or toughness. A specialised machine (Instron) that measures strain and physical resistance may be applied to measure the load required to penetrate the germ and bran-endosperm tissues of water-and water + ABA-conditioned grain and give a direct measure of tissue “toughness”.

Since water is critical in standard conditioning practices in milling, the addition of ABA may be involved in biochemical processes, which may alter cellular turgor pressure, water absorptive and/or retention properties of the grain.

This Chapter will examine a fast method in determining grain milling quality using minimal quantities of grain. Measuring how grain fractionates after passing through regulated crushing and shearing plates may reveal subtle changes that reflect milling quality. Since the contributing factors to how grain will fractionate are moisture and mechanical strength of the bran, these will also be measured to better understand what is causing improvement in milling quality.

6.2 Materials and Methods

6.2.1 Grain fractionation

The MicroMill (custom made grain crushing device, model no. RM-001, designed and built by Alan Donald Howard) was designed to uniformly crush grains so that they can be sieved and weighed to determine fractionation properties using minimal quantities (5 – 10 g) of grain. To achieve this at a very small scale, the MicroMill could not follow the traditional method of crushing grains through rollers. The main reason being is that the high speed required by the rollers to crush the grain generates a lot of dust and loss of fractionated material, which is also not ideal in a laboratory environment. An alternative method to roller milling is crushing and sheering the grain between two plates. This will minimise flour dust and loss of material.

The MicroMill (Figure 6.1) simply consists of one adjustable fixed plate and a moving plate. These plates are vertically orientated to allow for gravity feeding of the grains. Grain is fed through a hopper at the top of the opening above the two plates. It is then crushed and sheered between the two plates at a constant rate. As the grains pass between the plates, they are forced out of the bottom end of the plates through a fixed gap. This gap can be adjusted from 0.5 – 5 mm for various grain sizes and for milling optimization. The controlled gap distance which the grain finally passes through is to ensure that the grain is fractionated uniformly.

The grain fractions collected after milling are then passed through two different size sieves. The first sieve (~ 2.0 mm) is to separate the large bran particles. The second sieve (~ 0.5 mm) is to collect the next fraction that contains smaller fragmented bran particles and large endosperm particles (has similar texture to sand). The pass-through of this sieve is the flour fraction.

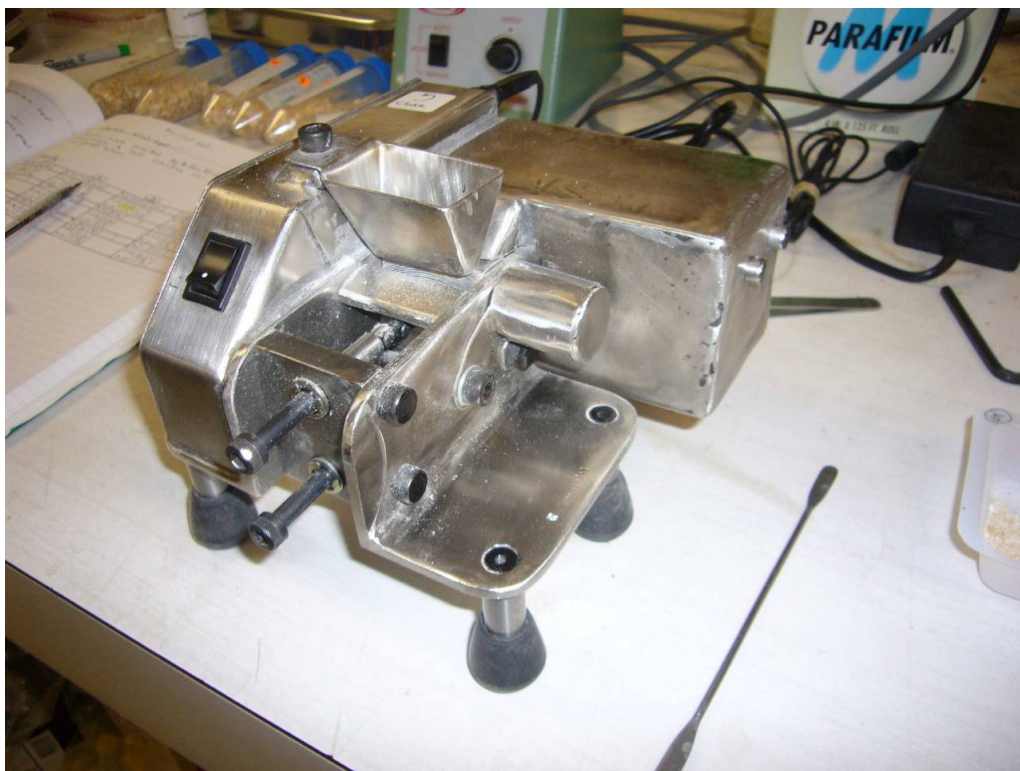


Figure 6.1. Photograph of the custom made laboratory bench scale mill (RM-001).

6.2.2 Wheat cultivars

2005 harvest: Drysdale, Kennedy.

2006 harvest: Chara Baldry M6948, H45 M6649, Strzelecki M6615, Ventura Baldry M6931, Wylah M6880.

2008 harvest: Gregory.

6.2.3 Estimating grain moisture content

Grain moisture content was determined as described in Chapter 3 (Materials and Methods, 3.2.2).

6.2.4 Conditioning

Grain (20 g) was weighed out and placed into a 50-mL plastic tube. Conditioning procedure was followed as described in Chapter 3 (Materials and Methods, 3.2.5). At the end of the conditioning period, each sample was divided into four equal (5 g) replicates and placed onto filter paper. The grain samples were left to dry for ~ 1.5 h prior to milling.

6.2.5 MicroMilling

The crushing plate gap of the MicroMill was adjusted to 1 mm and warmed up by passing 5 g of non-conditioned grain through one grain at a time with the aid of an automated grain feeder.

Each crushed 5 g sample (replicated four times) was sieved firstly with a 2 mm sieve and the pass-through was further sieved with a 0.5 mm sieve. The bran fraction collected in the 2 mm sieve, intermediated fraction collected in the 0.5 mm sieve and the flour fraction collected from the pass-through of the 0.5 mm sieve were weighed and recorded. The intermediate fraction, collected in the 0.5 mm sieve, was weighed and crushed again with the MicroMill with the gap now set at 0.5 mm. The intermediate fraction was again sieved using the 0.5 mm sieve and the resulting flour and the residual bran fraction collected in the 0.5 mm sieve were weighed and recorded.

The final mass recovered was determined by summing the mass of bran, flour and residual bran and flour from the intermediate fraction. The flour and residual bran percentages of the total mass recovered were used to calculate the fractionation quality score (FQS) applying the equation below. Scores were calculated for each of the replicates. Results were represented as side-by-side plots of the FQS and the ranked replicates. A paired t-test

was performed on the ranked data to determine the significance between the water-and water + 4 ppm ABA-conditioned grain.

6.2.6 Fractionation quality score (FQS)

An optimal method for determining changes in grain fractionation using the MicroMill was required. The method also had to be suitable for milling various cultivars. An ideal outcome would be to generate a milling quality score that represents important and favorable milling properties. Good milling properties include:

High % Flour yield

Low % Residual bran in the intermediate fraction

Applying the good milling properties to a ratio will generate a fractionation quality score (FQS):

$$\text{FQS} = \frac{\% \text{ Flour yield}}{\% \text{ Residual bran from the intermediate fraction}}$$

This ratio, which is based on the best theoretical outcome mention above, was used to determine the milling quality of non-conditioned, water-conditioned and water + ABA-conditioned grain. Thus, the ratio simply shows that high percentage values in the numerator and low percentage values in the denominator, result in a high number score indicating good milling wheat and a low number for poor milling wheat.

6.2.7 Setting up the psychrometer

6.2.7.1 Psychrometer connections and accessories

A twelve-channel thermocouple psychrometer (83 series, JRD Merrill Specialty Equipment, Logan, UT, USA) was connected to a current amplifier (Custom made by the Macquarie University electrical workshop), which was then connected to an oscilloscope (DS1M12 Stingray, USBInstruments). The oscilloscope data logger software (EasyLogger for DS1M12) was used to collect data measurements from the oscilloscope from a lap top computer.

6.2.7.2 Cleaning thermocouple sensor and sample chamber

The thermocouple sensor and sample chamber were rinsed with Milli Q water. After rinsing, they were sprayed with degreaser (CO Contact Cleaner, CRC Industries, Australia), rinsed again with Milli Q water and then left to dry in a 40 °C drying oven for 2 h.

6.2.7.3 Psychrometer settings

For each reading, the toggle switch was pushed to reset, wait for 3 s followed by pushing to start. Cooling current was 59 mA, cooling time was 30 s, and delay reading time was 3 s. To zero the instrument, only the first channel was zeroed during the cooling step. For all other channels, the off set value was subtracted from the sample measurement.

6.2.7.4 Sodium chloride standard curve

A standard curve was measured for each of the twelve channels using sodium chloride dilutions (0 M, 0.2 M, 0.5 M, 1.0 M, 1.5 M, and 1.9 M). Three μL of each dilution was pipetted onto a small circle of filter paper (cut using a standard size hole punch).

6.2.7.5 Water potential (ψ) measurements of samples

Wheat grain (cv. Gregory) moisture level was determined and conditioned as described in Chapter 3 (Materials and Methods, 3.2.2 and 3.2.5). However, grain needed to be conditioned at higher moisture contents (24, 30 and 34.5%) than the 16.5% moisture content done in milling due to the water potential (ψ) measuring limits of the psychrometer (Figure 6.4). The lowest measurable ψ with the psychrometer is ~ -6.5 MPa at 24% grain moisture content. Ψ was measured according to the methods outlined by Meinzer et al. (2003).

Each sample was placed into a separate thermocouple psychrometer chamber. The chamber cap was screwed on tightly. The chamber was then submerged into a hot water bath at 25 °C for 2.5 h to equilibrate.

After equilibration, the channel was selected (from 1-12, where channel 1 is zeroed during the 30 s cooling step) and the oscilloscope data logger was started. The toggle switch was pushed to reset, left for ~ 3 s and then followed by pushing the toggle switch to start. After 30 s, a beep is sounded, which marks a maximum mV time stamp. Data is recorded in the plateau region 3 s after the time stamp for ~ 2 to 3 s. This is repeated for each channel.

6.2.8 Instron measurements of the germ and bran-endosperm tissue of water-and water + ABA-conditioned grain

At the end of the conditioning period, water-and water + ABA-conditioned grain were sealed in 50-mL plastic tubes to prevent moisture loss.

Germ measurements were taken by cutting 1/3 of the grain at the germ end and placing the cut face down so as the germ end is pointing up. The Instron probe (1.5 mm diameter) was positioned just touching the end of the germ at the top. Once positioned, the Instron was set to penetrate 1.5 mm into the germ (Figure 6.2 A). Measurements were taken at 500 millisecond intervals, recording the distance in millimeters travelled and the flexure load in Newtons (N).

Bran/endosperm measurements were taken by placing the grain on its side on a concave mount. The probe was positioned centrally and perpendicular to the grain surface. Probe penetration and measurements were taken as described above (Figure 6.2 B).

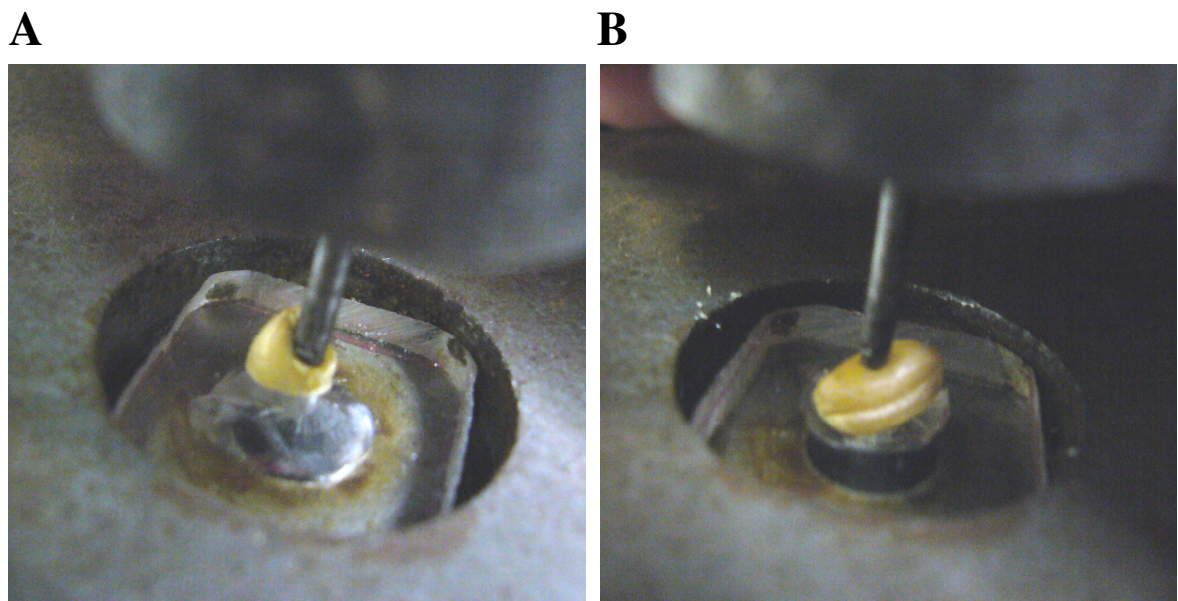


Figure 6.2. Photograph of the 1.5 mm diameter Instron probe after penetrating 1.5 mm into the (A) germ and (B) bran/endosperm.

6.2.9 Statistical analysis of grain fractionation, water potential and mechanical properties

Statistical analysis of the grain fractionation, water potential and mechanical property experiments considered variations in grain characteristics, such as grain size, shape, density, water uptake, viability and response to ABA. To account for these variations, the data set was ordered from lowest to highest forming ordered groups of ψ values. Statistical analysis applying a one-tailed distribution paired t-test between the ordered groups of values of water-and water + ABA was performed. The assumption was that the difference between each ordered replicate is significantly greater than 0.

6.3 Results

6.3.1 MicroMill optimisation

The optimal gap setting for the MicroMill was 1 mm. This setting allowed for all sizes and shapes of grain from the various cultivars that were used in the fractionation experiments. Gap settings that were less than 1 mm were problematic in that the crushed grain was unable to pass through smoothly, causing jams in the MicroMill. Gap settings that were larger than 1 mm were not effective in crushing the grain sufficiently.

6.3.2 MicroMilling various cultivars of non-conditioned, water-and water + 4 ppm ABA-conditioned grain

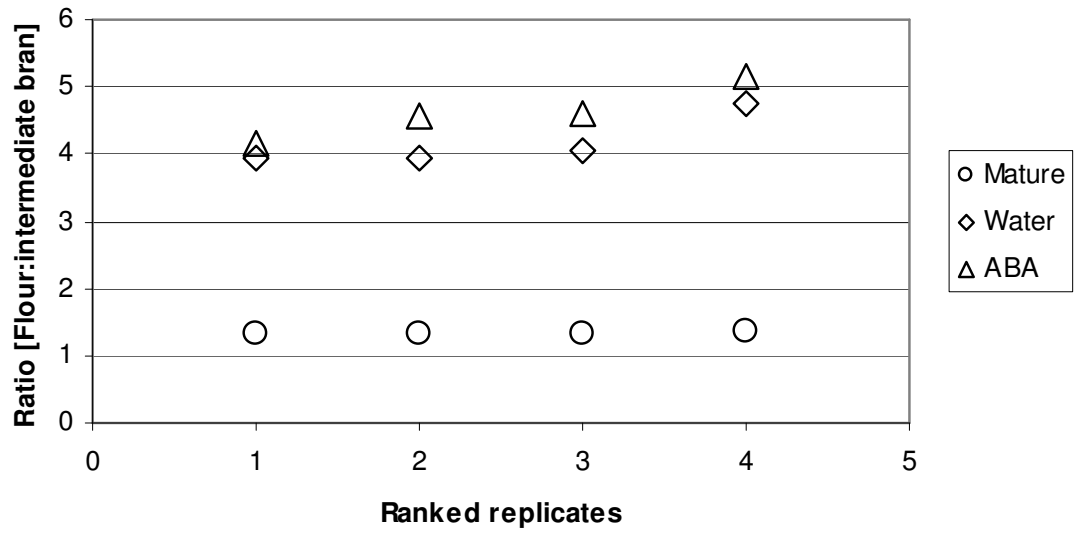
The estimated grain moisture content for the following cultivars were: 2005 harvest – Drysdale (8.7%), Kennedy (8.3%); 2006 harvest – Chara Baldry M6948 (8.2%), H45 M6649 (8.2%), Strzelecki M6615 (8.8%), Ventura Baldry M6931 (8.2%), Wylah M6880 (8.9%); and 2008 harvest – Gregory (9.6%).

By applying the ranked replicate analysis, results showed that the ABA-conditioned grain scored significantly higher than the water-conditioned grain (Figure 6.3 and Appendix B, Table B.4). Also, the non-conditioned grain scored significantly lower than either the water-and water + ABA-conditioned grain.

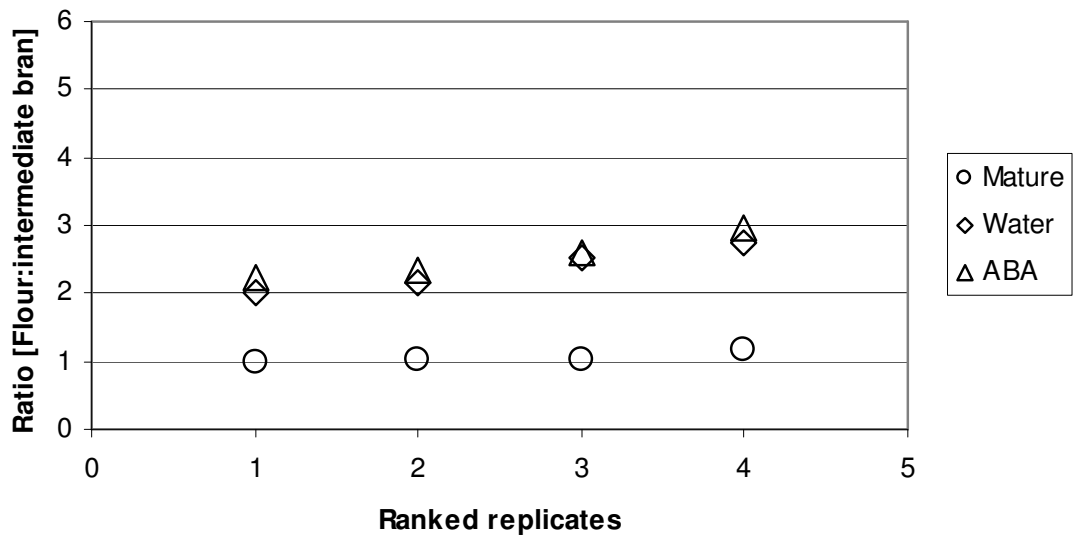
Of the eight cultivars tested, one showed no significant difference between the two treatments (Strzelecki, $p = 0.109$) and one cultivar showed a significant negative effect of

water + ABA conditioning compared with the water-conditioned (Wylah, $p = 0.023$) (Figure 6.3).

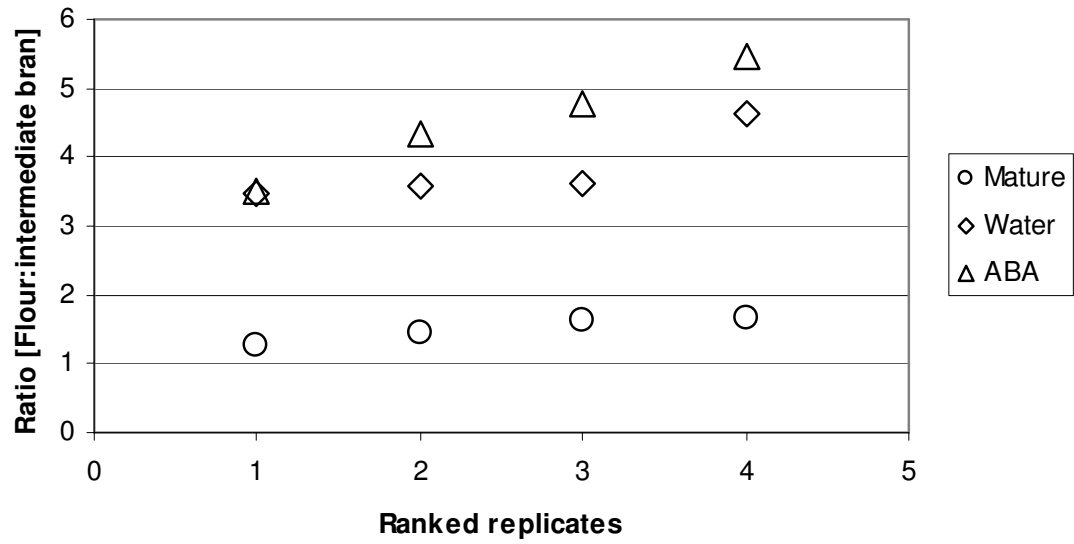
Chara Baldry, $p = 0.007$



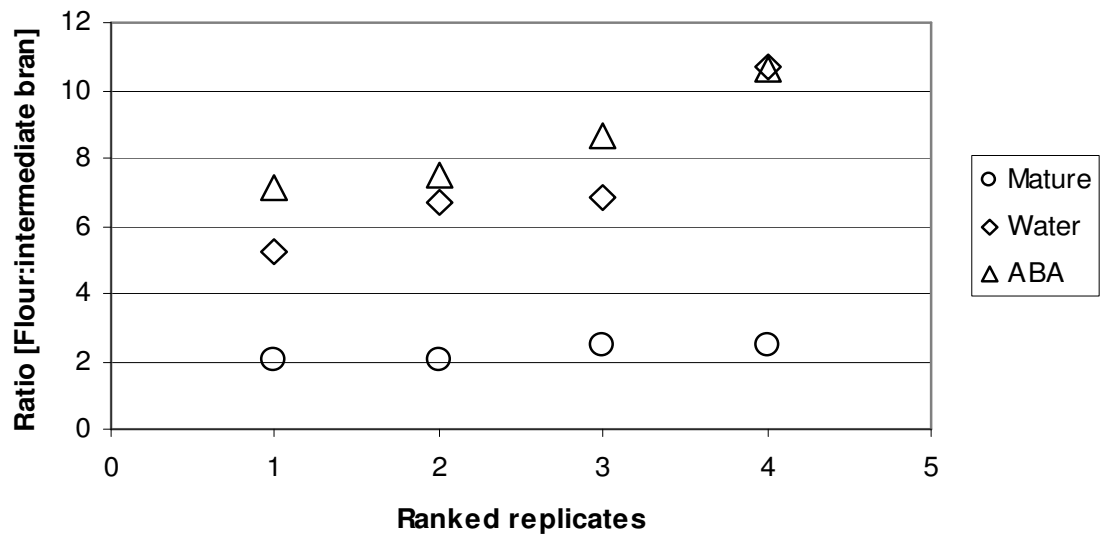
Drysdale, $p = 0.006$



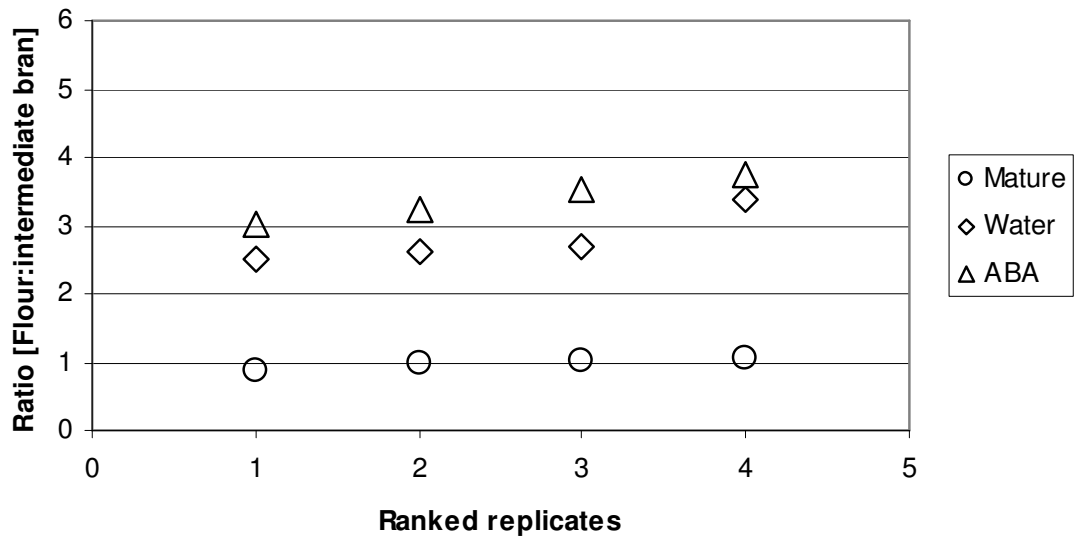
Gregory, $p = 0.031$



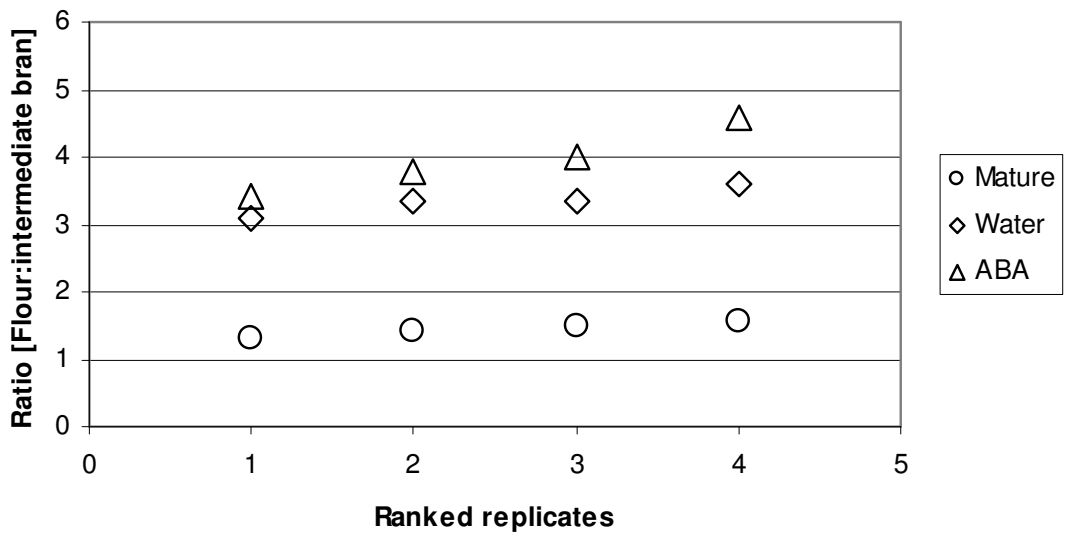
H45, $p = 0.049$



Kennedy, $p = 0.005$



Ventura Baldry, $p = 0.012$



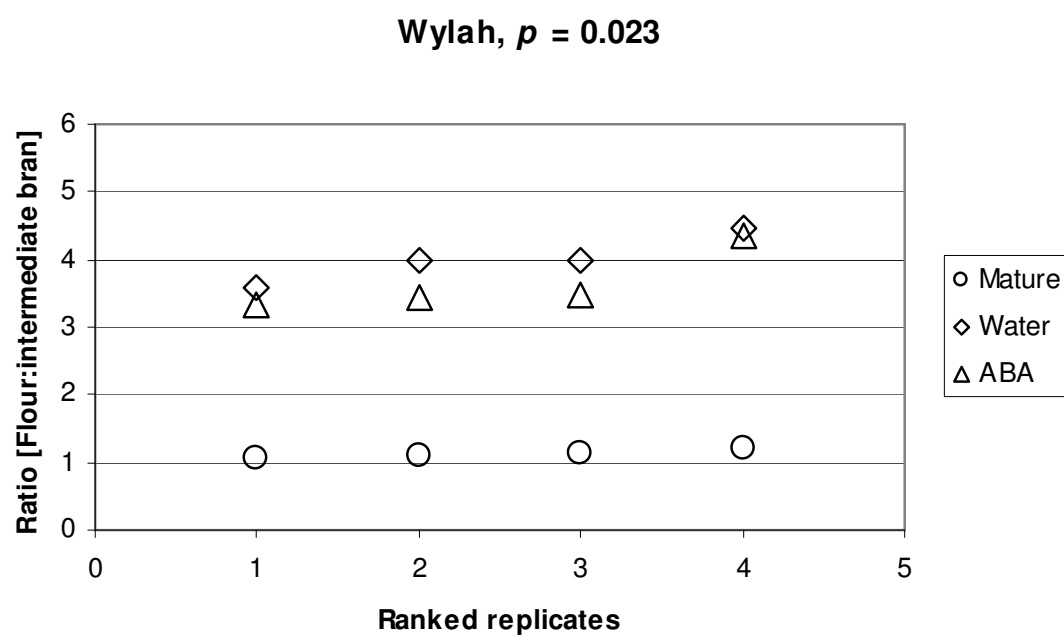
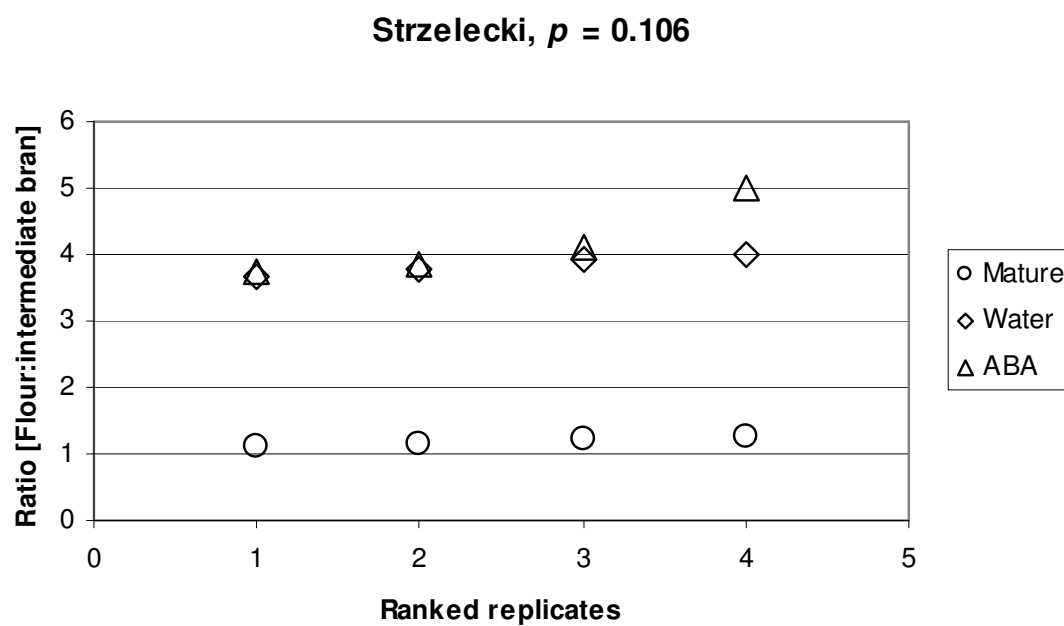


Figure 6.3. Ratios [% flour:% bran from the intermediate fraction] versus ranked replicates ($n = 4$) of various cultivars of the from non-conditioned (mature), water-and water + 4 ppm ABA-conditioned grain. A paired t-test was performed only on the ranked replicates between water-and water + 4ppm ABA-conditioned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

6.3.3 Water potential (ψ) of water-and water + 4 ppm ABA-conditioned grain

In general, there was no significant difference (applying standard deviation) between water-and water + 4 ppm ABA-conditioned grain at 24, 30 and 34.5% moisture content (Figure 6.4). Ψ generally followed the level of the conditioning moisture content of the grain. However, there was a distinct resistance to drying in the ABA treated grain as indicated by ψ measurements over a fixed period at fixed humidity levels (Figure 6.5). ABA treated grain showed, on average, a higher ψ than water-conditioned grain after drying.

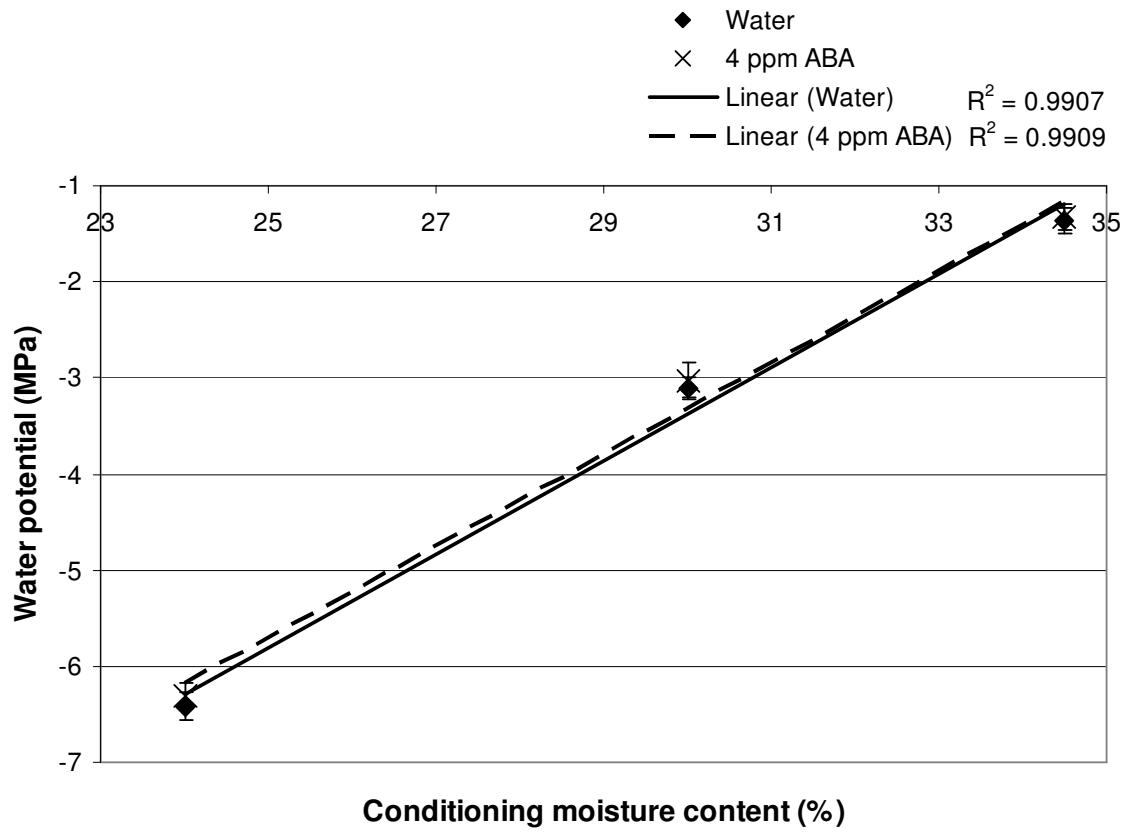


Figure 6.4. Ψ s of grain conditioned at 24, 30 and 34.5% moisture content. The lowest ψ measurable with the psychrometer is ~ -6.5 MPa at 24% grain moisture content. Error bars are \pm standard deviation ($n = 6$).

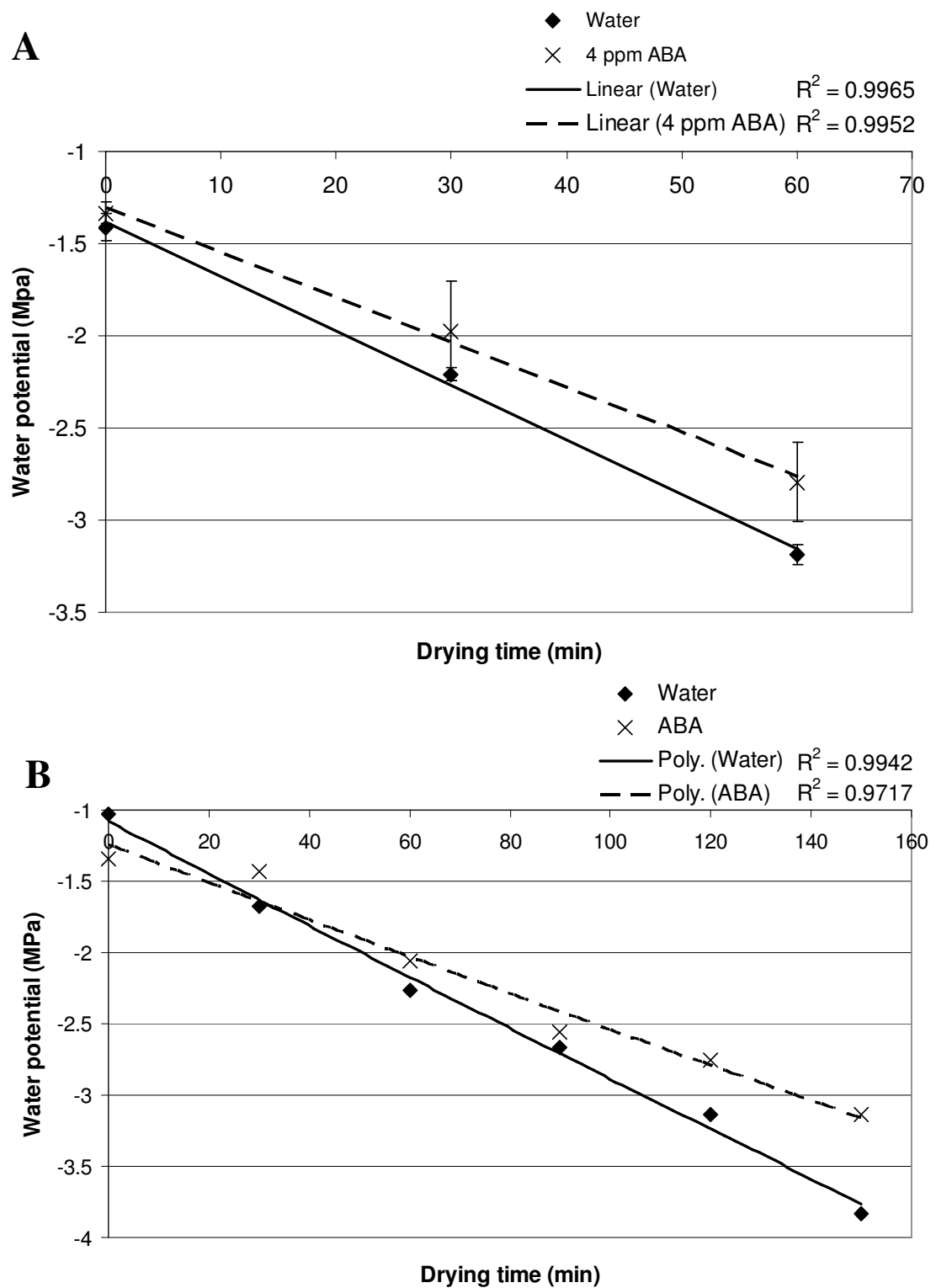
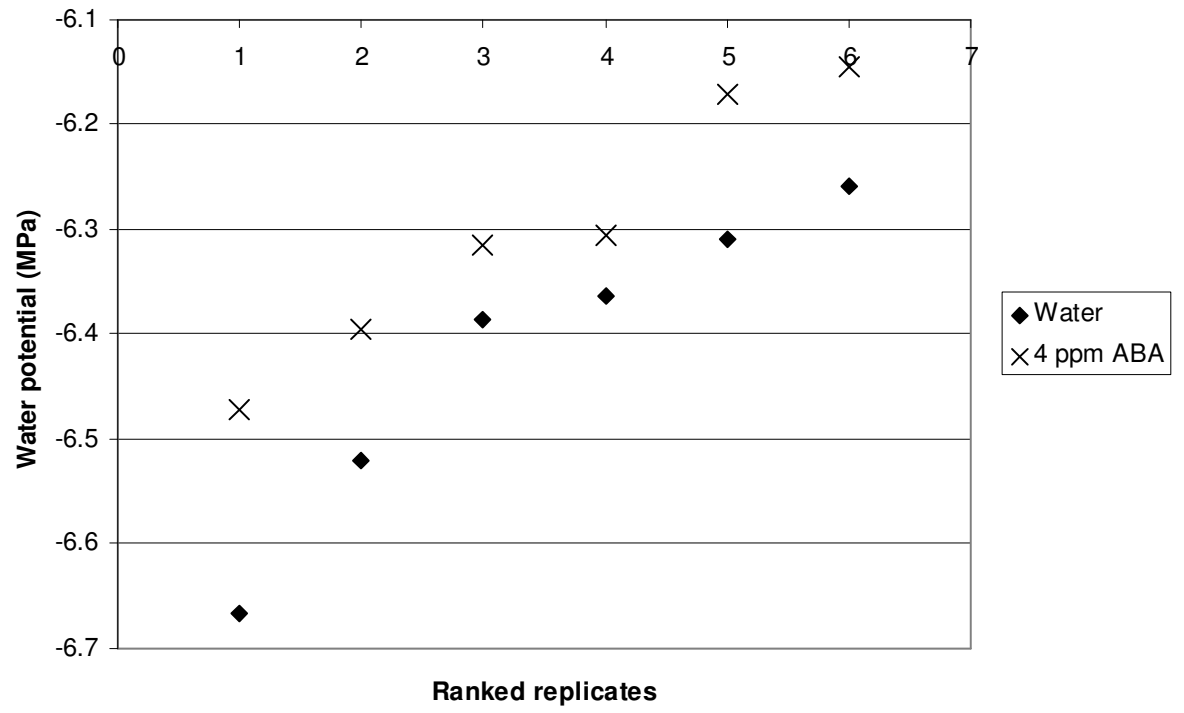
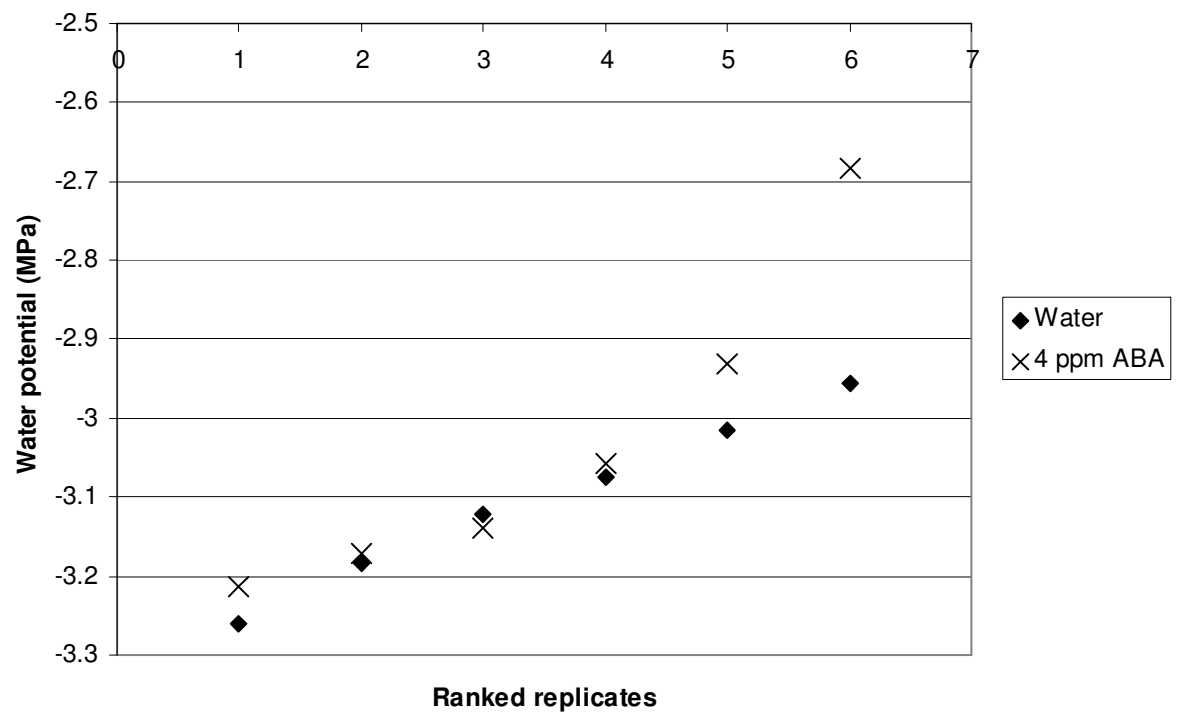


Figure 6.5. Drying time versus ψ of grain conditioned at 34.5 % moisture. (A) Drying at < 50% humidity, error bars are \pm half range and (B) drying at ~ 60% humidity.

At 24% moisture level, the ABA-conditioned grain had significantly higher ψ_s of the ranked replicates compared with the ranked replicates of water-conditioned grain ($p = 0.0011$) (Figure 6.6 A).

At 30% moisture level, the ABA-conditioned grain did not have significantly higher ψ_s of the ranked replicates compared with the ranked replicates of water-conditioned grain ($p = 0.085$) (Figure 6.6 B).

At 34.5% moisture level, the ABA-conditioned grain had significantly higher ψ_s of the ranked replicates compared with the ranked replicates of water-conditioned grain ($p = 0.046$) (Figure 6.6 C).

A**B**

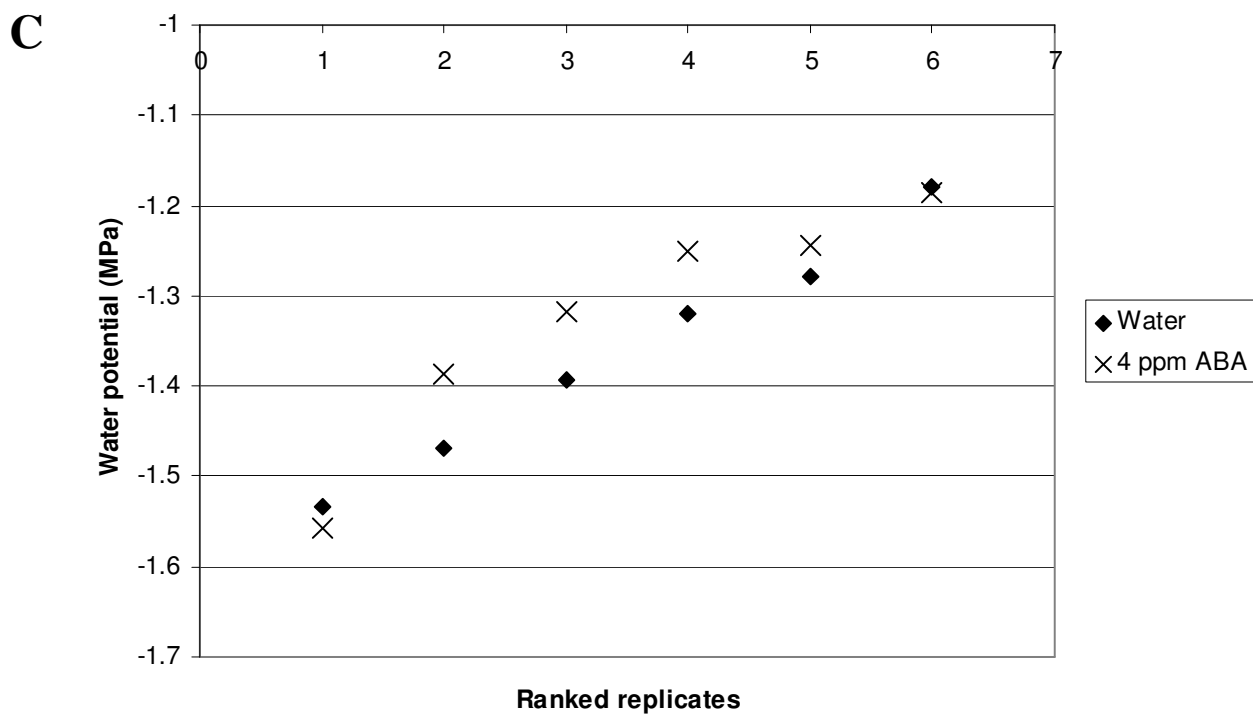


Figure 6.6. Ranked replicates ($n = 6$) of ψ_s of grain conditioned at (A) 24%, (B) 30% and (C) 34.5% moisture with water-and water + ABA for 16.5 h.

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

To further evaluate the effect of ABA, ψ was measured and compared between the germ-end and bran/endosperm-end of the grain after conditioning.

6.3.4.1 Grain conditioned at 30% moisture content

The germ-end from ABA-conditioned grain had significantly higher ψ s of the ranked replicates ($p = 0.019$) (Figure 6.7 A). There was no significant difference in ψ between the water-and water + ABA-conditioned grain with the bran-end ($p = 0.33$) (Figure 6.7 B).

6.3.4.2 Grain conditioned at 34.5% moisture content followed by 2 h drying

There was a significant difference between the water-and water + 4 ppm ABA-conditioned grain. The germ-end from 4 ppm ABA-conditioned grain had significantly higher ψ s between the ranked replicates of the germ from water-conditioned grain ($p = 0.041$) (Figure 6.8 A). Similarly, the bran-end from 4 ppm ABA-conditioned grain had significantly higher ψ s between the ranked replicates of the bran-end from water-conditioned grain and bran-end ($p = 0.0025$) (Figure 6.8 B).

Differences between not drying and drying grain prior to dissecting the germ-end and bran-end were only found in the bran-end. This suggests that the water absorbed during conditioning in the bran-end is similar between treatments. However, when the grain is dried for 2 h, the moisture is lost at a faster rate in the water-conditioned grain.

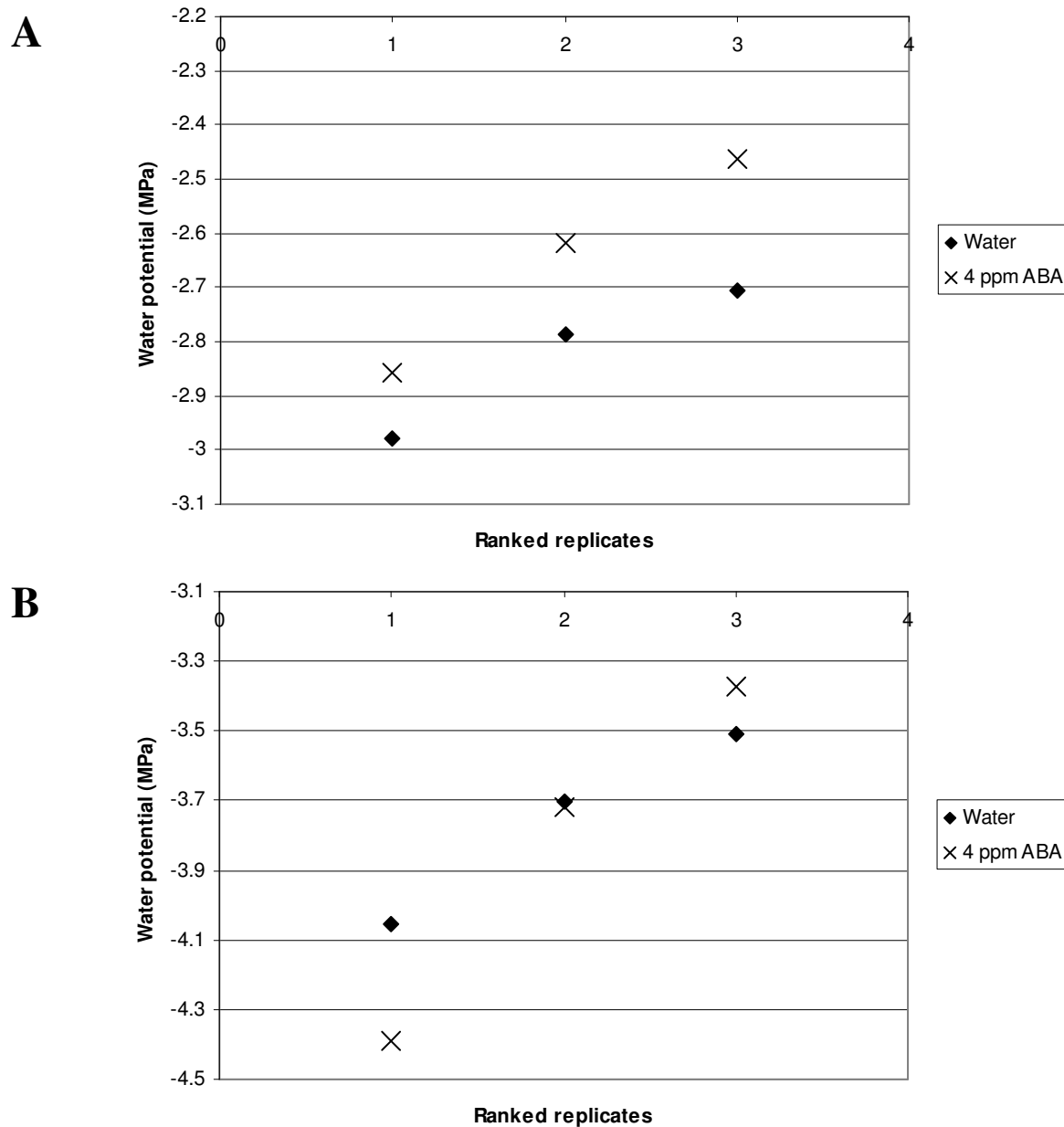


Figure 6.7. Ψ s of grain conditioned with water-and water + ABA at 30% moisture for 16.5 h. After conditioning, 1/3 of the germ-end of the grain was cut off and the ψ was measured for each the (A) germ-end and (B) bran-end.

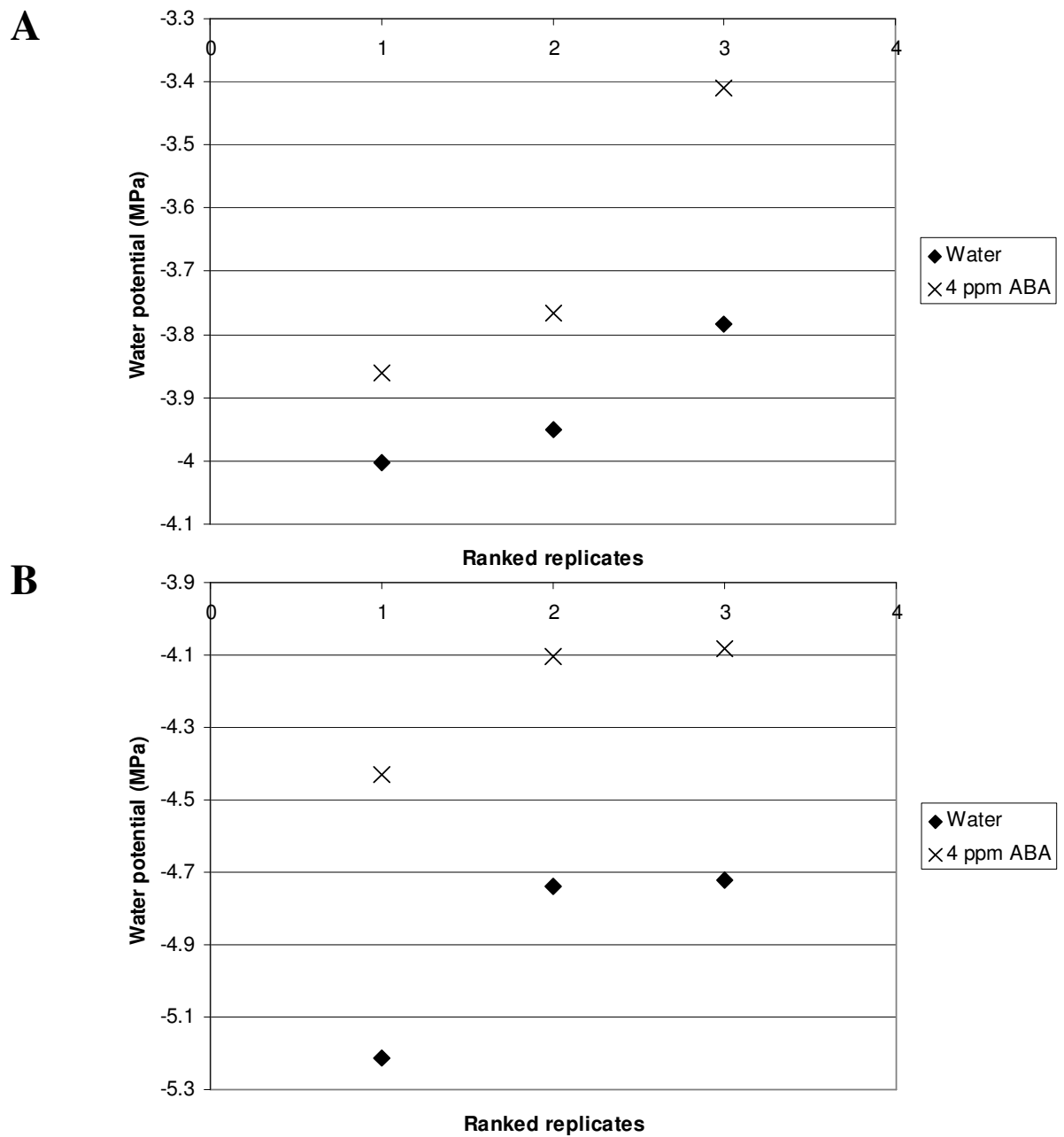


Figure 6.8. Ψ s of the (A) germ-end and (B) bran-end of grain conditioned at a moisture level of 34.5% with water-and water + ABA and left to dry at room temperature and 80% humidity for ~ 1.5 h.

6.3.5 Instron analysis of the germ of water-and water + ABA-conditioned grain

The gradient of the straight line equation (Appendix B, Table B.5) for all data points of flexure load (N) over a length of 1.5 mm penetration was taken for each replicate. The first tissue to penetrate was the bran covering the germ, followed by the germ and then finally the complete compaction of the germ. The ranked groups of replicates of the germ of water-and water + 4 ppm ABA-conditioned grain were not significantly different from each other ($p = 0.066$) (Figure 6.9).

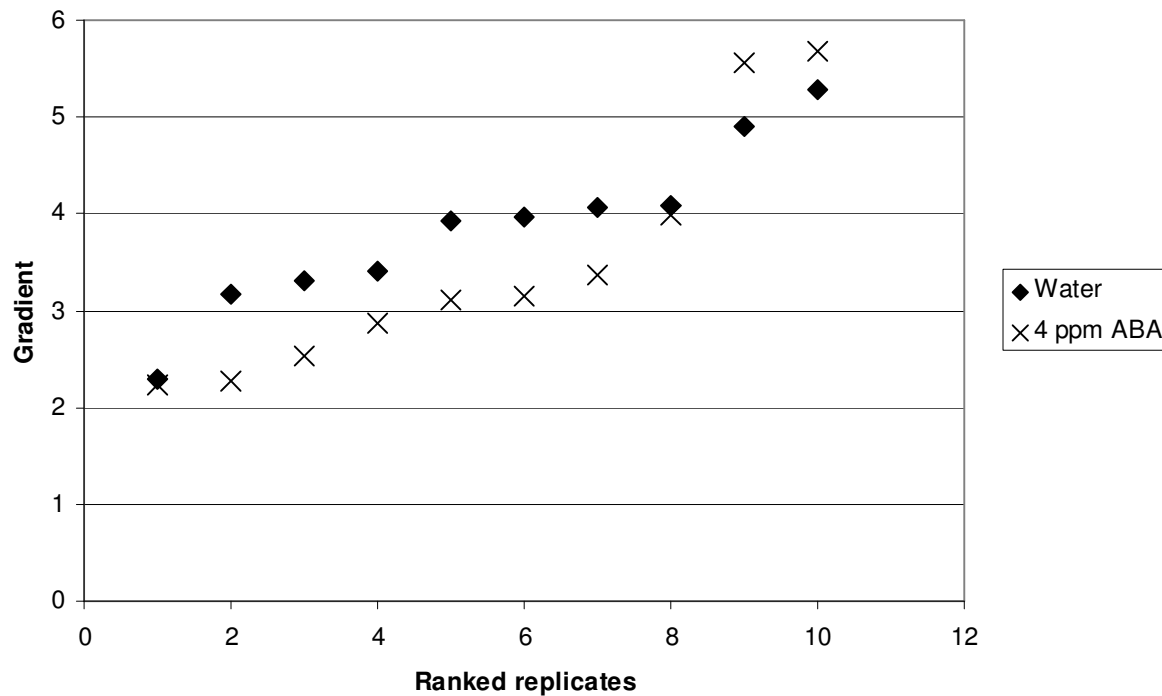


Figure 6.9. Germ tissue penetration of 1.5 mm into grain conditioned at 22.4% moisture. Gradient represents the toughness of the tissue (flexure load [N] over the length of penetration).

6.3.5.1 Toughness of the bran covering the germ

The gradient of the straight line equation (Appendix B, Table B.6) for data points of flexure load (N) over a length of 0.2 mm penetration was taken for each replicate. The ranked groups of replicates of the germ of water-and water + 4 ppm ABA-conditioned grain were significantly different from each other ($p = 0.0001$) (Figure 6.10).

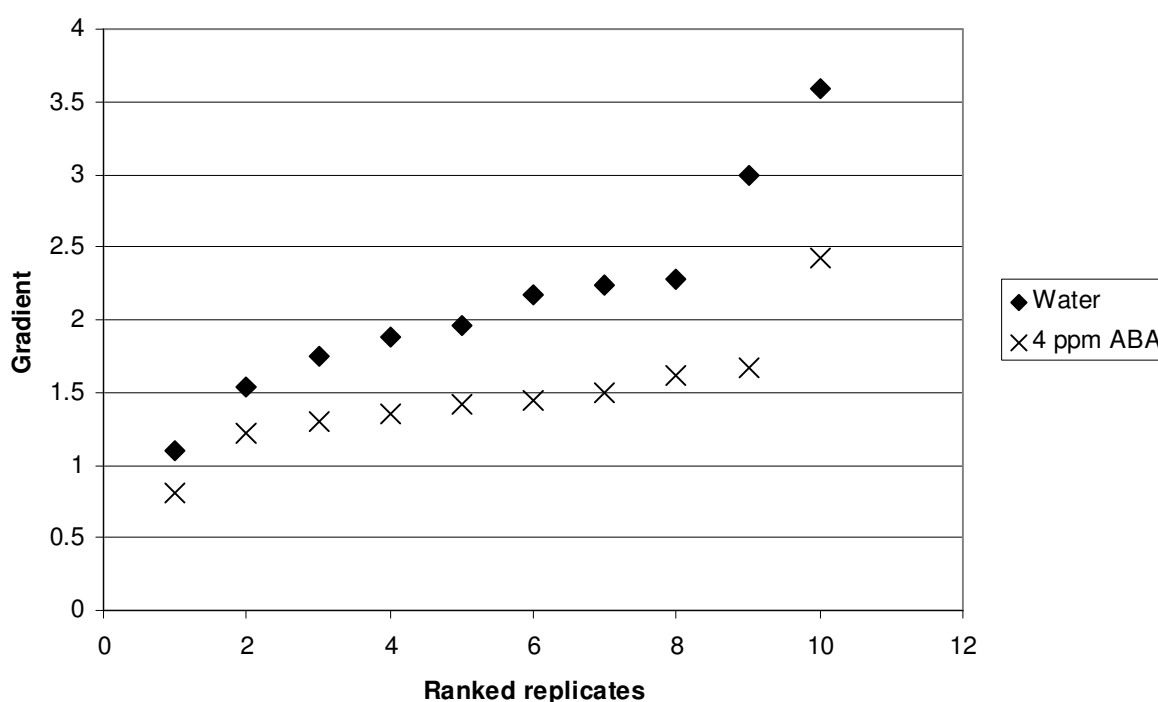


Figure 6.10. Germ tissue penetration of 0.2 mm into grain conditioned at 22.4% moisture. Gradient represents the toughness of the tissue (flexure load [N] over the length of penetration).

6.3.5.2 Toughness of the germ

The gradient of the straight line equation (Appendix B, Table B.7) for data points of flexure load (N) between the length of 0.8 to ~ 1.2 mm penetration was taken for each replicate. The ranked groups of replicates of the germ of water-and water + 4 ppm ABA-conditioned grain were significantly different from each other ($p = 0.000005$) (Figure 6.11).

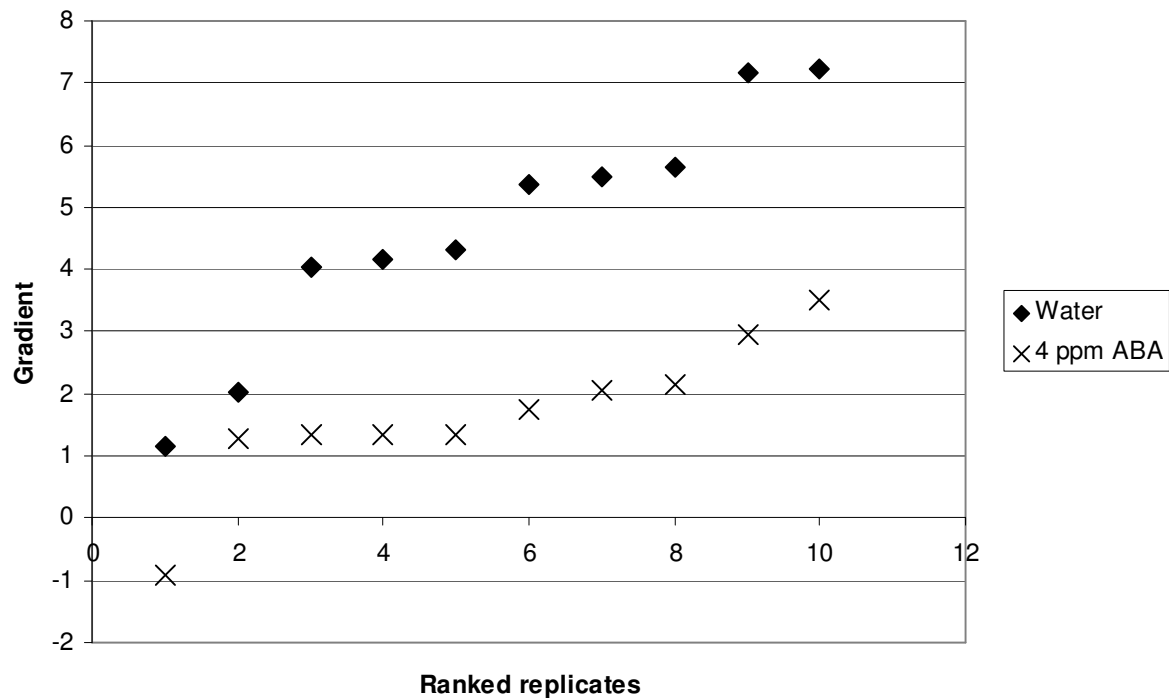


Figure 6.11. Germ tissue penetration from 0.8 to ~ 1.2 mm into grain conditioned at 22.4% moisture. Gradient represents the toughness of the tissue (flexure load [N] over the length of penetration).

6.3.6 Instron analysis of the bran-endosperm of water-and water + ABA-conditioned grain

The peak flexure load (N) (Appendix B, Table B.8) at the point of bran penetration for each replicate were ranked from lowest to highest. The ranked groups of replicates of the bran-endosperm of water-and water + 4 ppm ABA-conditioned grain were not significantly different from each other ($p = 0.109$) (Figure 6.12).

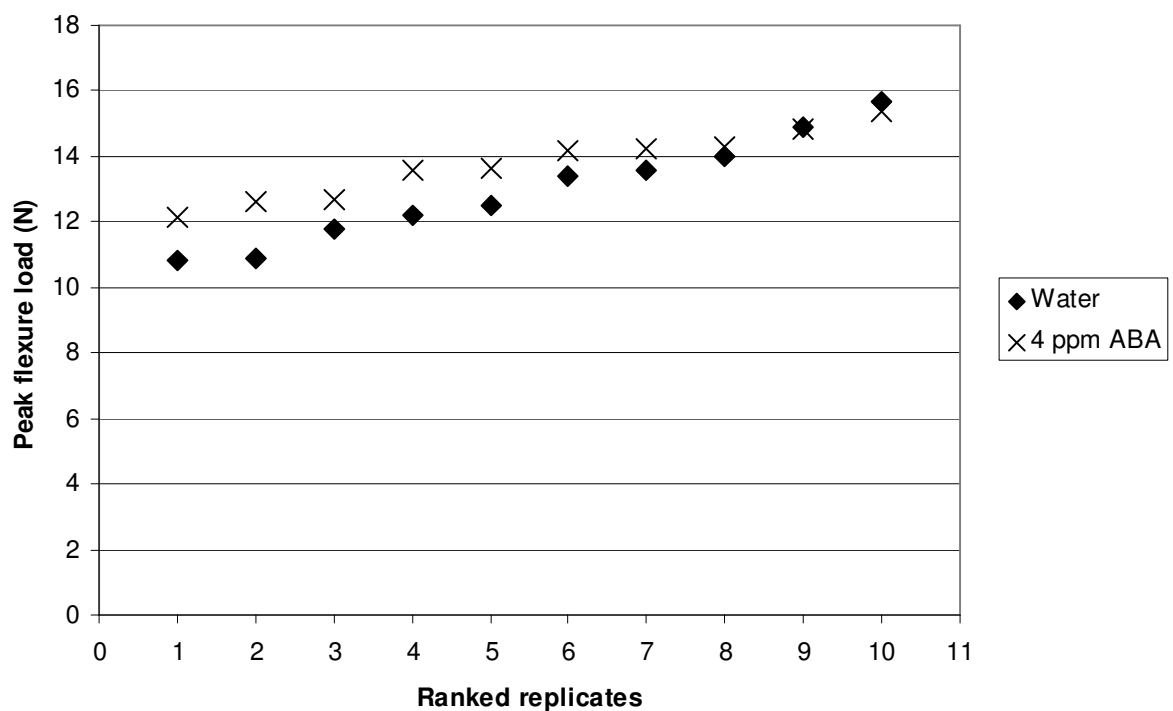


Figure 6.12. The peak flexure load (N) at the point of bran tissue penetration from 0 to ~1.5 mm into grain conditioned at 22.4% moisture.

6.3.6.1 Toughness of the bran

The gradient of the straight line equation (Appendix B, Table B.9) with the highest r-squared value for data points of flexure load (N) over the length of 0 to ~ 1.5 mm penetration was taken for each replicate. The ranked groups of replicates of the bran-endosperm of water-and water + 4 ppm ABA-conditioned grain were not significantly different from each other ($p = 0.063$) (Figure 6.13).

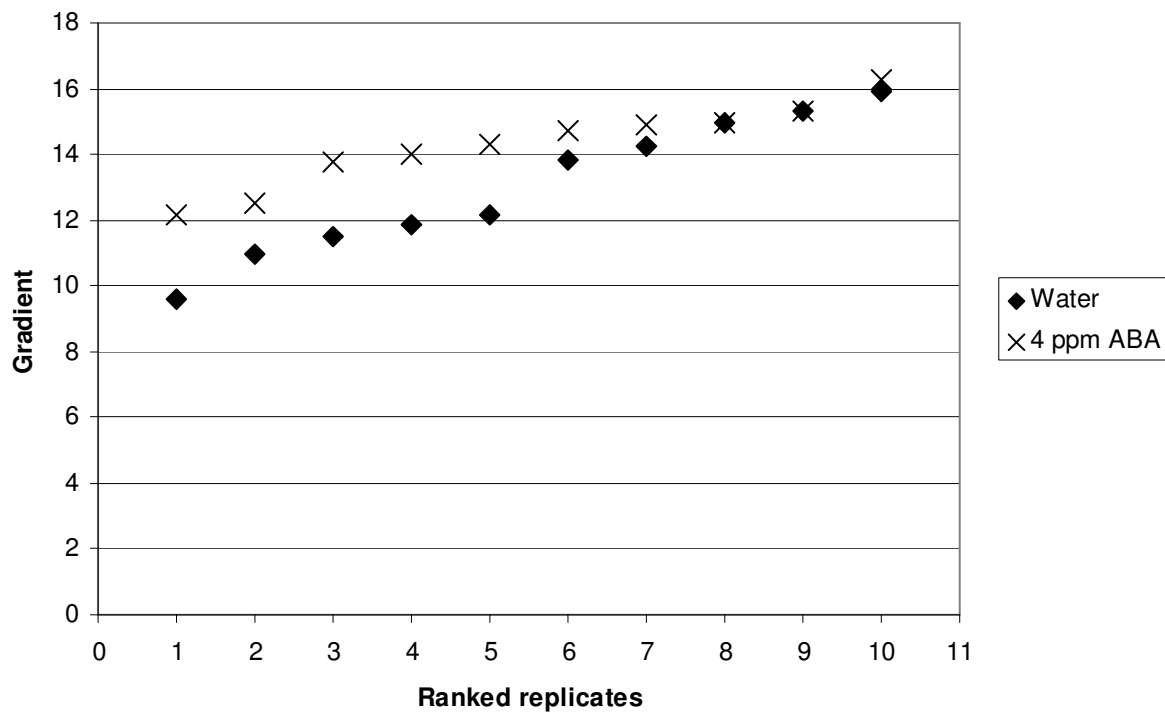


Figure 6.13. Bran-endosperm tissue penetration from 0 up to ~1.5 mm into grain conditioned at 22.4% moisture. Gradient was taken from the line equation with the maximum r-squared, which represents the flexure load (N) over the length the penetration.

The gradient of the straight line equation (Appendix B, Table B.10) for data points of flexure load (N) over the length of 0 to 1.0 mm penetration was taken for each replicate. The ranked groups of replicates of the bran-endosperm of water-and water + 4 ppm ABA-conditioned grain were significantly different from each other ($p = 0.0074$) (Figure 6.14).

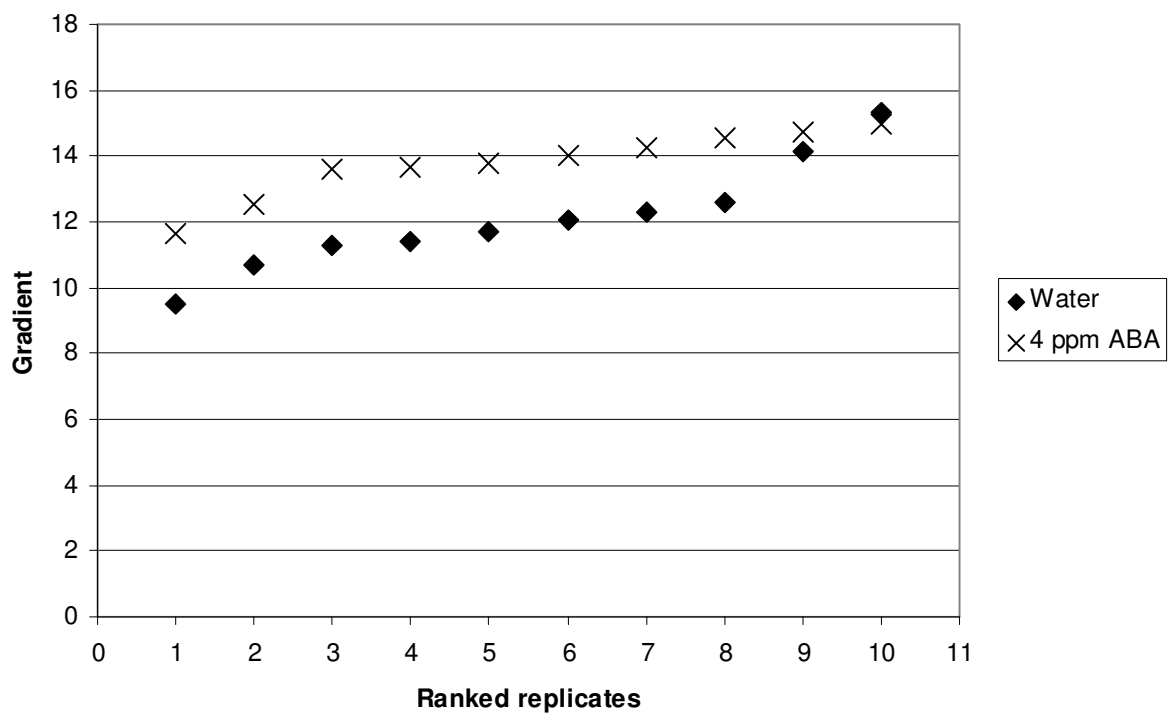


Figure 6.14. Bran-endosperm tissue penetration from 0 – 1 mm into grain conditioned at 22.4% moisture. Gradient from the line equation for all data points from 0 – 1 mm penetration, representing the flexure load (N) over the length the penetration.

6.4 Discussion

6.4.1 Grain fractionation of water-and water + ABA-conditioned grain

The design and purpose of the MicroMill was to gently crush and shear the grains through a narrow and adjustable gap to determine how well flour is liberated and how much the bran will fragment. In determining this, various fractions were separated and weighed to determine the milling quality. Theoretically, if a kernel was to fractionate perfectly into bran, germ and endosperm, you would generally obtain ~ 13.7% bran, ~ 3.1% germ and ~ 83.2% starchy endosperm (Barron et al., 2007). Grain which yields a higher flour fraction when passed through the MicroMill, may indicate that less mechanical exertion is required in an industrial mill and therefore minimise bran fragmentation. Thus, the mass of either starch granule or flour fraction will vary depending on how well the endosperm fractionates.

The mechanical motion by which the mill rollers crush grain has been studied over many years. Researchers have taken into account grain hardness or softness and starch granule size and distribution in the endosperm to adjust speed and distance between the break rollers to achieve the best fractionation (Fang and Campbell, 2002). It is known in the flour milling process that the more mechanical exertion placed on the grain the more flour you can extract, however there is likely to be more bran fragmentation and thus more bran contamination in the flour (Fang and Campbell, 2002; Peyron et al., 2002). Flour millers empirically balance this to achieve the maximum flour yield without compromising the flour quality in terms of bran contamination. The MicroMill, however, uses only one setting for all varieties of wheat being tested.

As mentioned in the Introduction to this Chapter, there are many methods which can be used to determine milling performance/quality. However none of these methods are able to analyse very small quantities of grain. The MicroMill has shown quantitatively that it can measure changes in fractionation properties between non-conditioned, water-conditioned and water + ABA-conditioned grain using small quantities of grain. Also, due to the small sample size, it is possible to analyse many samples in a short period of time.

It is likely that the fractionation effects quantified here are due to changes in water relations, such as its distribution and changes in tissue mechanical properties. These were investigated by measuring ψ s and mechanical strength changes in the germ-and bran-end of water-and water + ABA-conditioned grain.

6.4.2 Water potential (ψ) and mechanical properties of water-and water + ABA-conditioned grain

Ψ s of whole grain conditioned with water-and water + 4 ppm ABA initially showed that there was no significant difference between the treatments (Figure 6.4). Some explanations for this may be that because the grains were conditioned in sealed plastic tubes and were transferred directly into the psychrometer chambers, the ψ appeared similar in either treatment possibly due to either having similar matrix effects, or there is such large physical and biochemical variations between each grain that any treatment effects were not represented. Although, on average, the ABA-conditioned grain had a higher ψ comparatively, it was not statistically significant applying standard deviation. Thus, any biochemical changes that may have affected water distribution between water-and water + ABA-conditioned grain were possibly masked due to large variations in the samples.

To address the first issue, the grains were dried at low humidity over a fixed period after conditioning. This revealed a greater difference in ψ between the two conditioning treatments suggesting a resistance to drying (Figure 6.5). However, when applying standard deviation between the measurements, the differences were still not significant.

Biological variation is likely to have the greatest impact on reproducibility when performing any analysis on grain. The variations between each grain include size, shape, density, water uptake, viability and response to ABA to name a few. Since it is almost impossible to have the exact same properties, every grain must then be considered unique when performing physiological experiments. To account for this variation and consider all data as a real observation, each replicate from a particular experiment or treatment were ranked as a group and then compared to another ranked group. Applying a paired t-test revealed a significant difference between each ranked group. To avoid performing hundreds of replicates and eliminating outliers to show significance between treatments, the ranking of a smaller number of replicates allows inclusion of all measurements when trying to measure the physiological changes between treatments. This ranking normalizes biological variation and highlights the treatment effect. As a result, many of the experiments that appeared to be not significant were significant after the ranking comparison (Figures 6.6 to 6.8).

The semi-dried non-dissected grain conditioned with 4 ppm ABA revealed a significantly higher ψ than the water-conditioned grain (Figure 6.6). This suggested that the ABA treated grain potentially had a biochemical change that imparted water retaining properties. Rampino et al. (2006) have also observed that dehydrin expression after a water-related stress response in wheat grain imparted water retention properties. Also, there have been many reports on increased ABA and dehydrin levels after water-related stress on wheat

caused by salinity, drought and frost (Borovskii et al., 2002a; Borovskii et al., 2002b; Lopez et al., 2003; Rampino et al., 2006; Rorat, 2006). This suggests that conditioning grain with ABA may increase water retention and is likely to be the result of increased levels of dehydrins or other late embryogenesis abundant (LEA) proteins. If LEA proteins are responsible for the water retaining properties, then it is likely that the water movement will be influenced by these proteins.

After dissecting conditioned and non-dried grain into two sections (germ-end and the bran-end), it revealed a significantly higher ψ in the germ-end of ABA treated grain compared to the germ-end of water-conditioned grain. However, in the bran-end, there was no significant difference between either of the treatments (Figure 6.7). This suggests that in the bran-end, water may be exhibiting a matrix effect as was observed with the ψ levels of the non-dried whole grain.

A possible cause for the lower ψ observed in the germ-end from water-conditioned grain may be that the water has been taken up and contained in the germ cells as would occur in germination, thus exhibiting a lower ψ than the ABA treated grain. This is supported by the Instron data, which showed that the germ from water-conditioned grain was tougher (Figures 6.9 to 6.11), possibly due to high turgor pressure in the germ cells. However, in the ABA-conditioned grain, the germ may not readily take up the water and is possibly held back either in the ventral groove or surrounding bran tissue close to the germ. Supporting this, Morgan and King (1984) have reported the association between ABA levels and loss of turgor pressure in wheat leaves. Thus, is in line with the Instron data in this study, which showed that the germ from ABA-conditioned grain was softer than the germ from water-conditioned grain.

When repeating this experiment and allowing the grain to semi-dry before dissecting, both the germ-and bran-end had higher ψ_s in the ABA treated grain (Figure 6.8). This shows that semi-drying the grain does not effect the germ-end, such that water is absorbed and held in the germ from water-conditioned grain and is only liberated after cutting from ABA-conditioned grain. In the bran-end, water is possibly absorbed and retained in the aleurone cells and thus has a slower rate of evaporation after water + ABA conditioning. Supporting this, the Instron data also revealed that the bran tissue is significantly tougher in the ABA-conditioned grain (Figure 6.12 to 6.14).

Chapter 7 : Conclusion and future direction

In the pursuit of improving the milling performance (maximum flour extraction and minimal bran contamination in the flour) of wheat grain, there has been much interest in biochemical processing approaches by researchers. However, attempts to apply biological aids – such as various hydrolytic enzymes in conditioning to enhance the separation of bran from endosperm – has been unsuccessful. This has been primarily due to the lack of understanding in the protein composition of specific bran layers. Proteomic analysis of wheat bran in Chapter 2 has revealed an array of proteins related to biotic and abiotic defense that are likely to inhibit enzymes applied in grain bioprocessing. The localisation and activity of these inhibitory enzymes form a protective barrier against such hydrolytic enzyme treatments. One example is the xylanase inhibitor proteins concentrated in the nucellar layer, which will prevent the use of certain types of xylanases to degrade tissue. Detailed proteomic knowledge has opened the way to select for enzymes that are not inhibited by the inhibitor proteins in the bran layers. An alternative approach to applying hydrolytic enzymes in conditioning, is to apply plant hormones in conditioning that may alter the physiological characteristics of the grain to improve its milling performance.

One such hormone is abscisic acid (ABA), which has been shown by others to improve milling performance, however the mechanism by which it does this is not known. The following Chapters have attempted to address this question by investigating the biochemical and physiological properties that may be responsible for the improved milling performance. Since ABA is an important plant hormone involved in many other regulatory functions, such as biotic and abiotic stress, the analysis of grain treated with ABA has also reveal these other important biochemical changes.

In Chapter 3, iTRAQ proteomic analysis was applied to measure changes in protein levels in the germ, bran and ventral groove tissues from grain conditioned with water-and water + 4 ppm ABA after a 16 h conditioning period. The analysis revealed major differences in protein levels between germ, bran and ventral groove tissues from grain conditioned with water-and water + ABA when compared to the same tissues from non-conditioned grain. In water conditioned grain, the ventral groove tissue exhibited the most changes in protein levels, followed by the bran and lastly the germ tissue. However, the germ from water + ABA-conditioned grain showed a large increase in protein level changes. This highlighted the strong effect ABA has on germ and bran but to a lesser extent on the ventral groove. Of the identified proteins, two of them – late embryogenesis abundant (LEA) protein and tonoplast intrinsic protein (TIP) (aquaporin) – were considered important proteins, which may be factors in the improved in milling performance due their water-relation properties. The group-2 LEA (dehydrin) proteins were localised in Chapter 5 and highlighted their abundance and cellular distribution. The dynamic pattern of changes in LEA protein levels after conditioning with water-and water + ABA suggested they may have an influence on how water is distributed around the grain after hydration. These proteins were identified in all of the three tissues analysed. The large increase (~ 5-fold) in LEA proteins in the ventral groove after the conditioning period suggests that they have the potential to attract water to this region. Finally, the germ from ABA-conditioned grain showed a reduction in the level of tonoplast intrinsic proteins (TIPs). Thus, the decrease in TIP levels will result in less water movement in or out of the tonoplasts and prevent or slow down hydration of the germ. This was also supported by the mechanical properties of the germ from water + ABA-conditioned grain in Chapter 6, which showed it was softer, requiring less force to penetrate into the germ.

Chapter 4 revealed the specialised function of the ventral groove tissue. This tissue was primarily shown to be equipped with proteins involved in metabolic processes and membrane transport when compared to the bran tissue. In comparison, the bran tissue was shown to contain more defense proteins. However, Chapter 3 revealed that not only these metabolic proteins increase in levels after conditioning with water-and water + ABA, the biotic and abiotic defense protein also increase markedly.

In Chapter 6, a method for measuring grain fractionation and milling properties was developed. A very small custom made laboratory scale mill (MicroMill) was used to measure changes in grain fractionation between non-conditioned, water-and water + 4 ppm ABA-conditioned grain. This new method allowed for many replicates as it required only a small sample size for the analysis. Grain conditioned with water scored very high compared to non-conditioned grain, thus validating the scoring method. It is commonly known that non-conditioned grain has poor milling performance compared to conditioned grain, thus high scores indicated an improvement in milling performance. Examination of water + 4 ppm ABA-conditioned grain showed a further increase in the milling score. These scores were represented as ranked replicates to allow for statistical analysis (paired t-test on the grouped ranked replicates) to take into account the problem with measuring small changes in a sample that has dramatic inherent variability. This showed that the level of sensitivity of the laboratory mill was able to measure very small changes in milling performance.

This study has shown for the first time, the biochemical and physiological changes that occur in the germ, bran and ventral groove tissues from water-and water + ABA-conditioned grain. It also shows how these changes affect the physiological properties of

the grain that make it more favorable to milling; thus, presenting evidence that the efficiency of milling can be improved.

A number of further studies could be done to further characterise and confirm these findings. The hypothesis that dehydrin concentration within aleurone layers impacts on milling performance can be tested in a number of ways. Wheat varieties can readily be tested for dehydrin levels using antibodies to verify its correlation with milling performance. As ABA has an impact on dehydrin levels, environmental factors which affect ABA levels during grain development such as drought, could be tested on a single variety to see if such factors have an impact on milling performance of the grain. If these types of experiments prove that dehydrin levels in the aleurone layer are a contributing factor to milling performance, then selective breeding may be applied to produce high dehydrin levels within the aleurone of wheat. Alternatively, mutants could be selected for similar traits.

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Appendix A. Extension of Figures

A



B

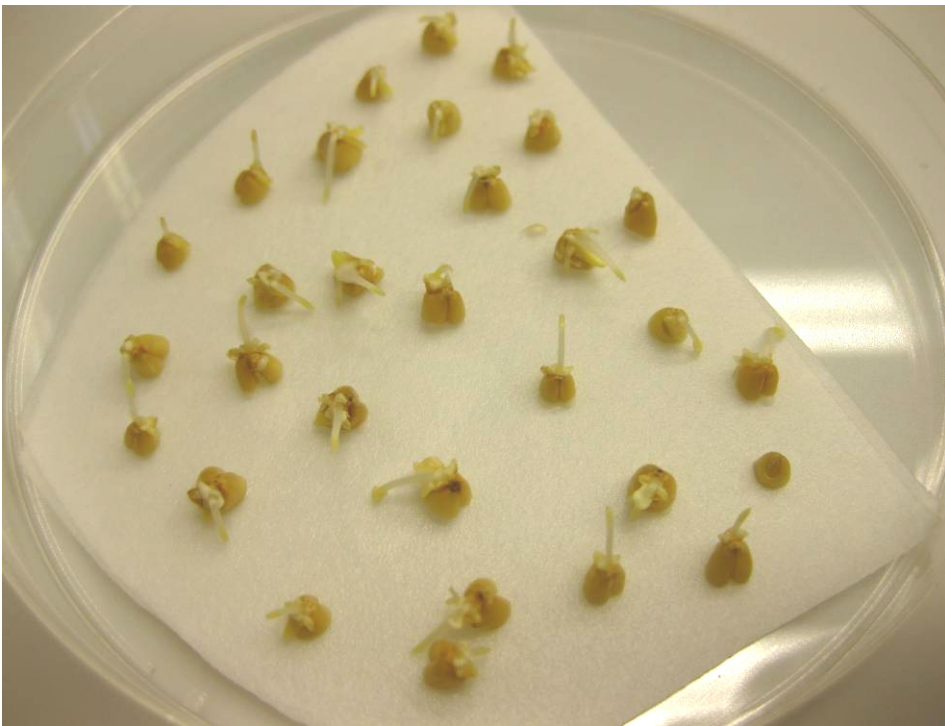


Figure A.1. After 24 h in Milli Q water. Replicates A and B.

A

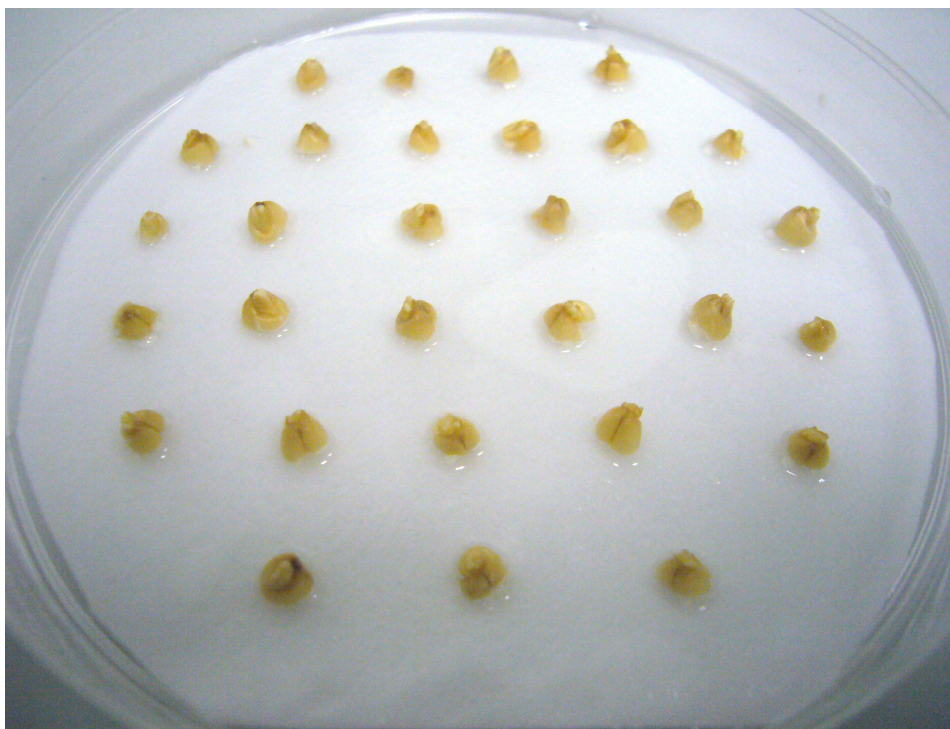


B



Figure A.2. After 24 h in 4 ppm ABA. Replicates A and B.

A



B



Figure A.3. After 24 h in 40 ppm ABA. Replicates A and B.

A



B



Figure A.4. After 48 h in Milli Q water. Replicates A and B.

A



B

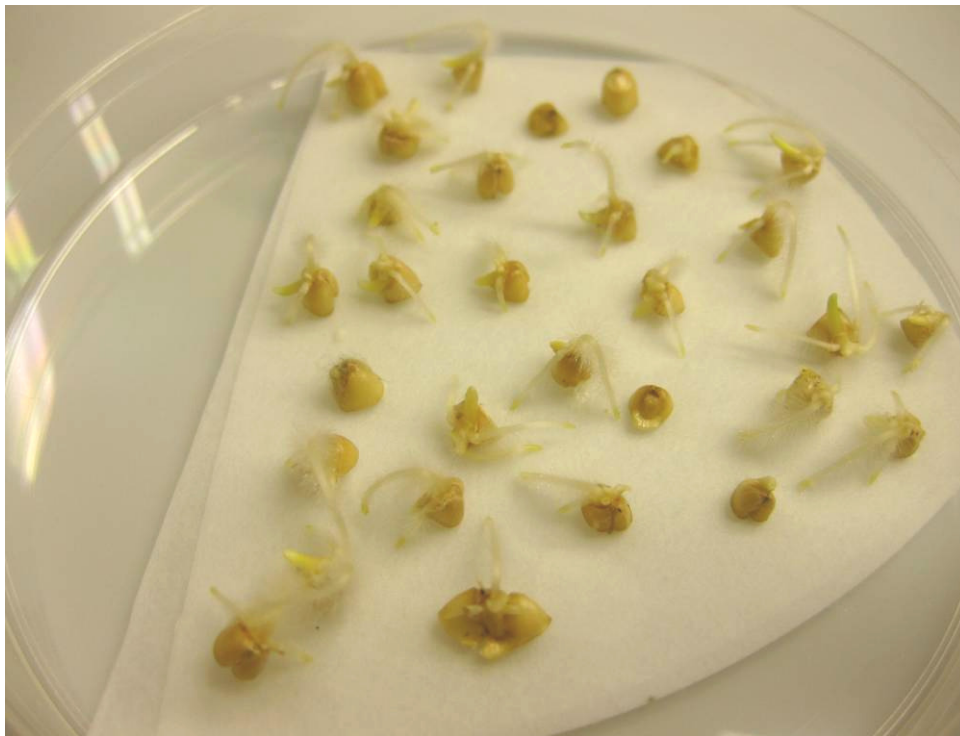
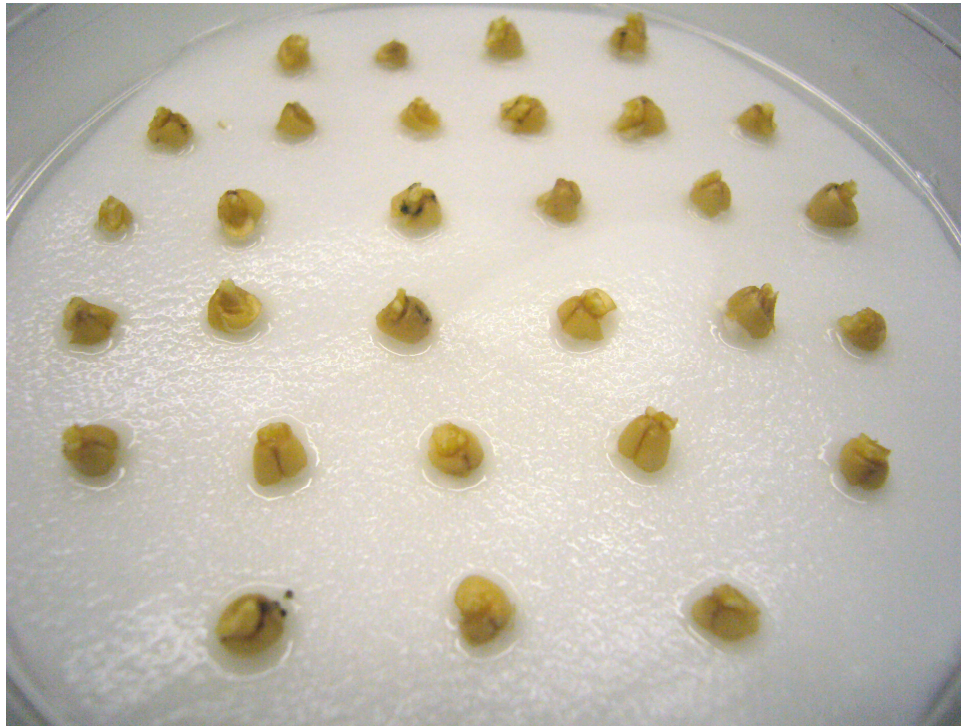


Figure A.5. After 48 h in 4 ppm ABA. Replicates A and B.

A



B



Figure A.6. After 48 h in 40 ppm ABA. Replicates A and B.

A**B**

Figure A.7. After 72 h in Milli Q water. Replicates A and B.

A



B

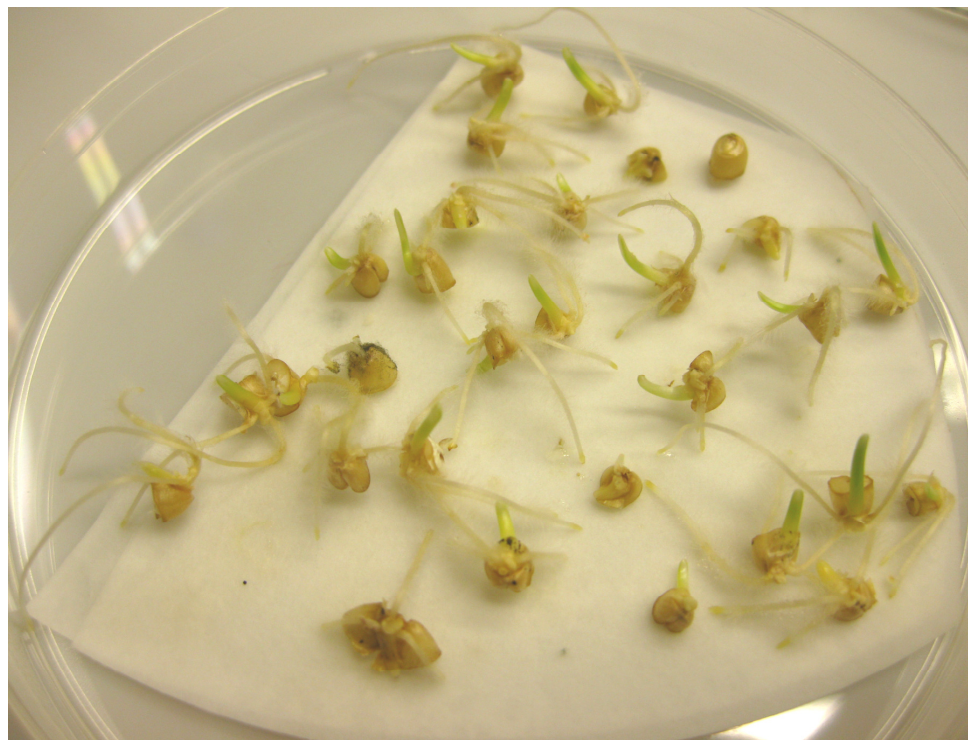
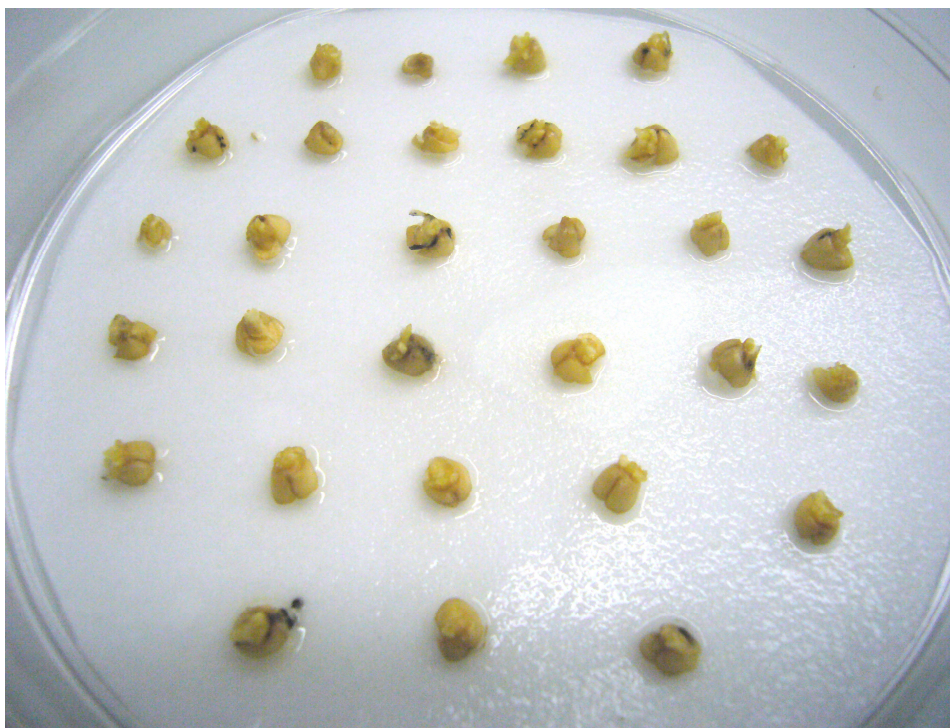


Figure A.8. After 72 h in 4 ppm ABA. Replicates A and B.

A



B

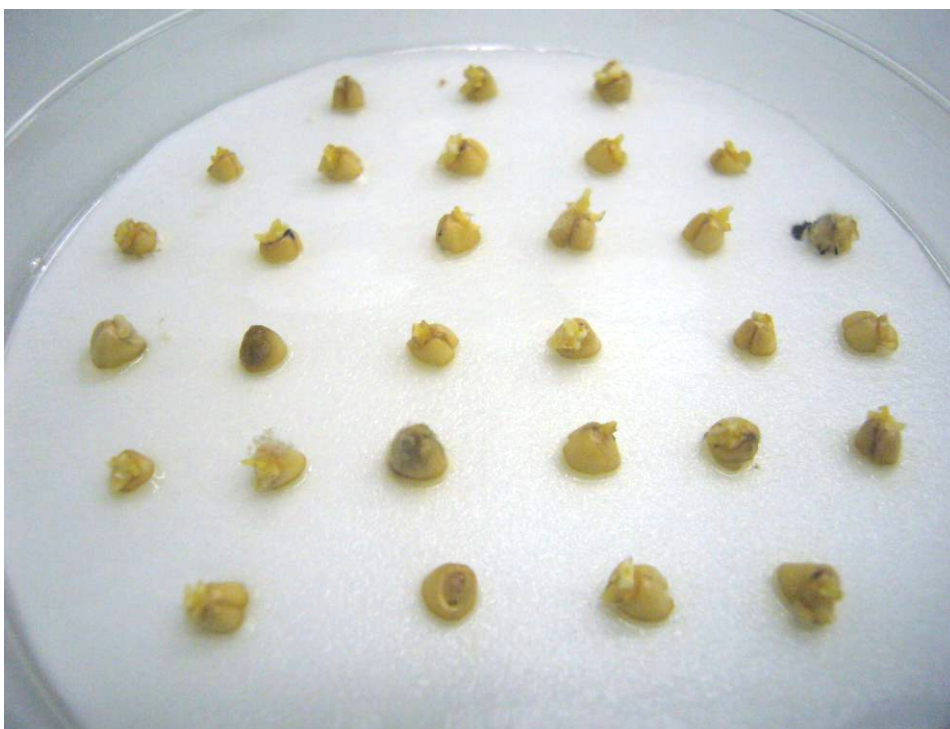


Figure A.9. After 72 h in 40 ppm ABA. Replicates A and B.

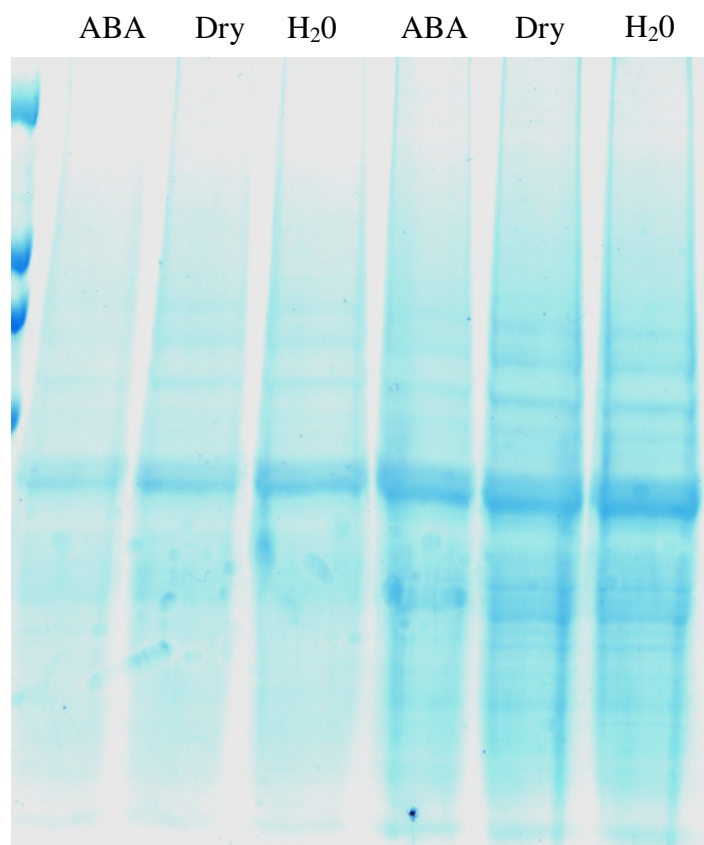


Figure A.10. SDS PAGE gel used to correct for protein quantification for iTRAQ analysis of germ. 20 µg and 10 µg of each sample Densitometry was used for final correction of sample concentration for iTRAQ analysis.

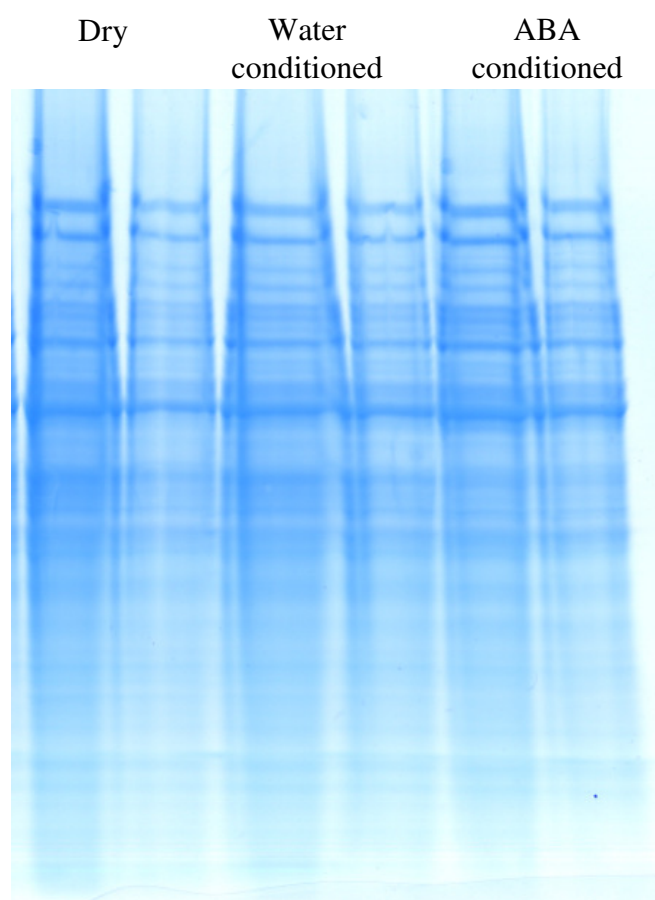


Figure A.11. SDS PAGE gel used to correct for protein quantification for iTRAQ analysis of bran. 10 μ g and 5 μ g of each sample Densitometry was used for final correction of sample concentration for iTRAQ analysis.

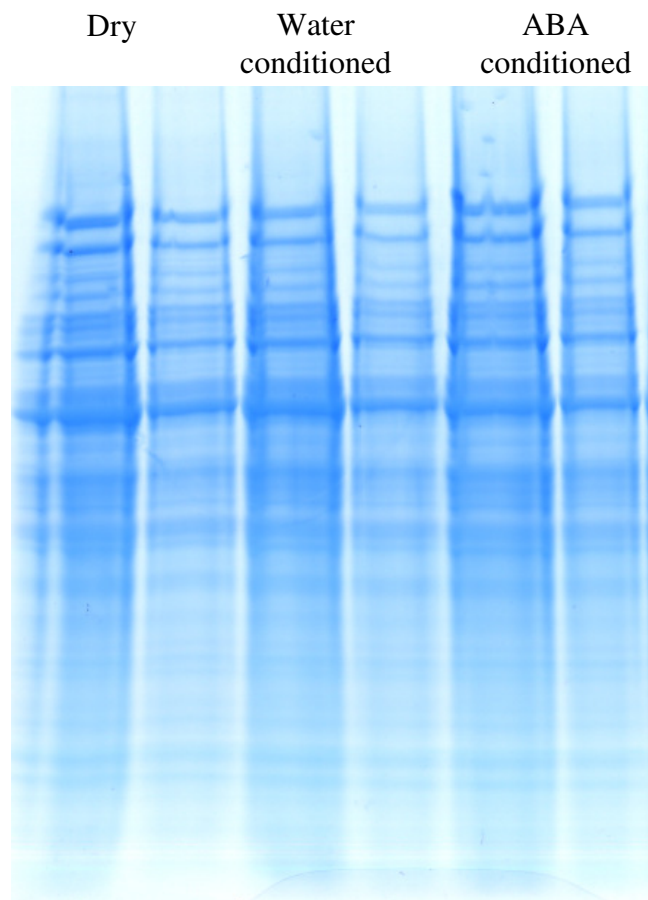


Figure A.12. SDS PAGE gel used to correct for protein quantification for iTRAQ analysis of ventral groove. 10 μ g and 5 μ g of each sample Densitometry was used for final correction of sample concentration for iTRAQ analysis.

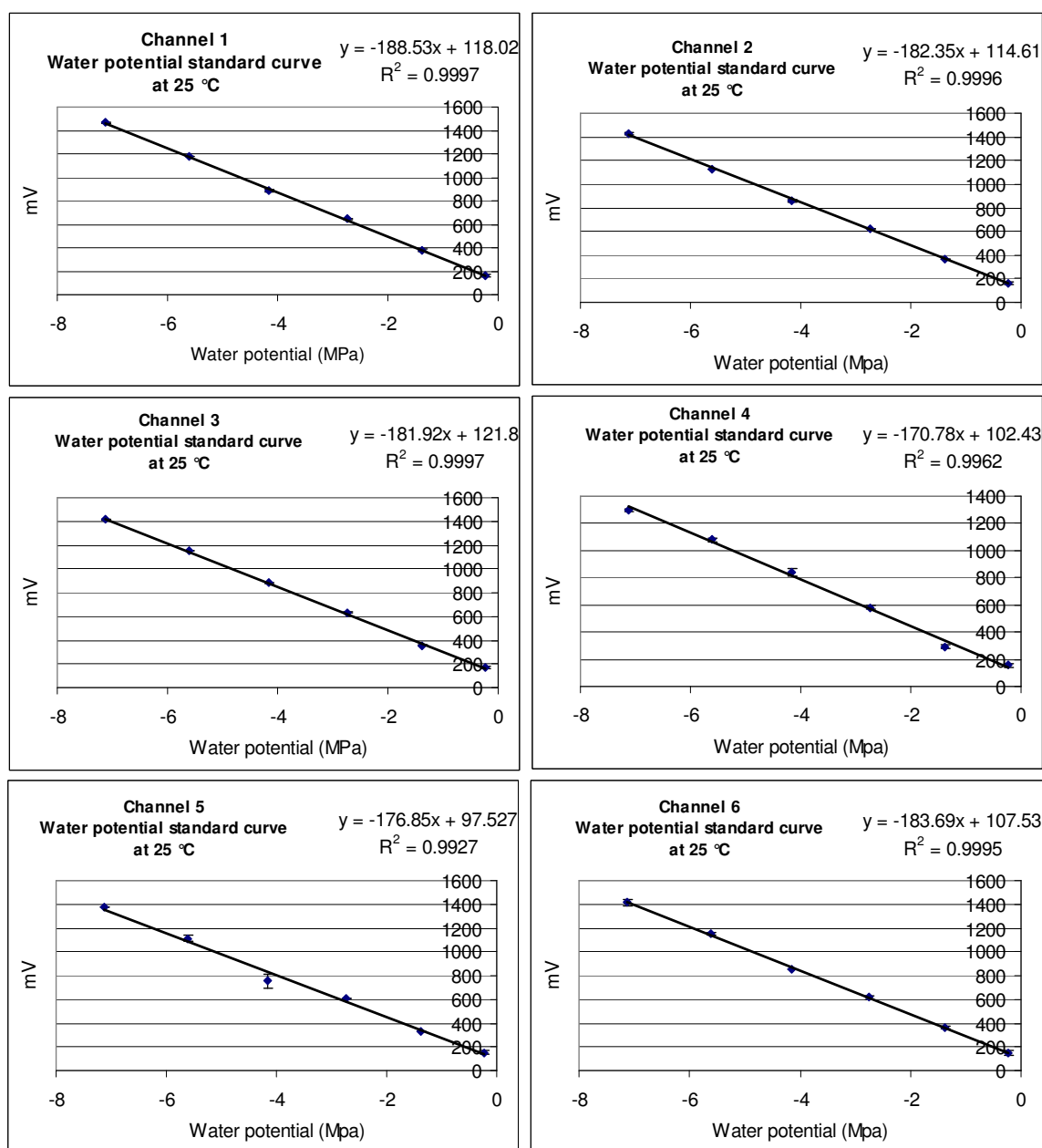


Figure A.13. Sodium chloride standard curves for channels 1 to 6.

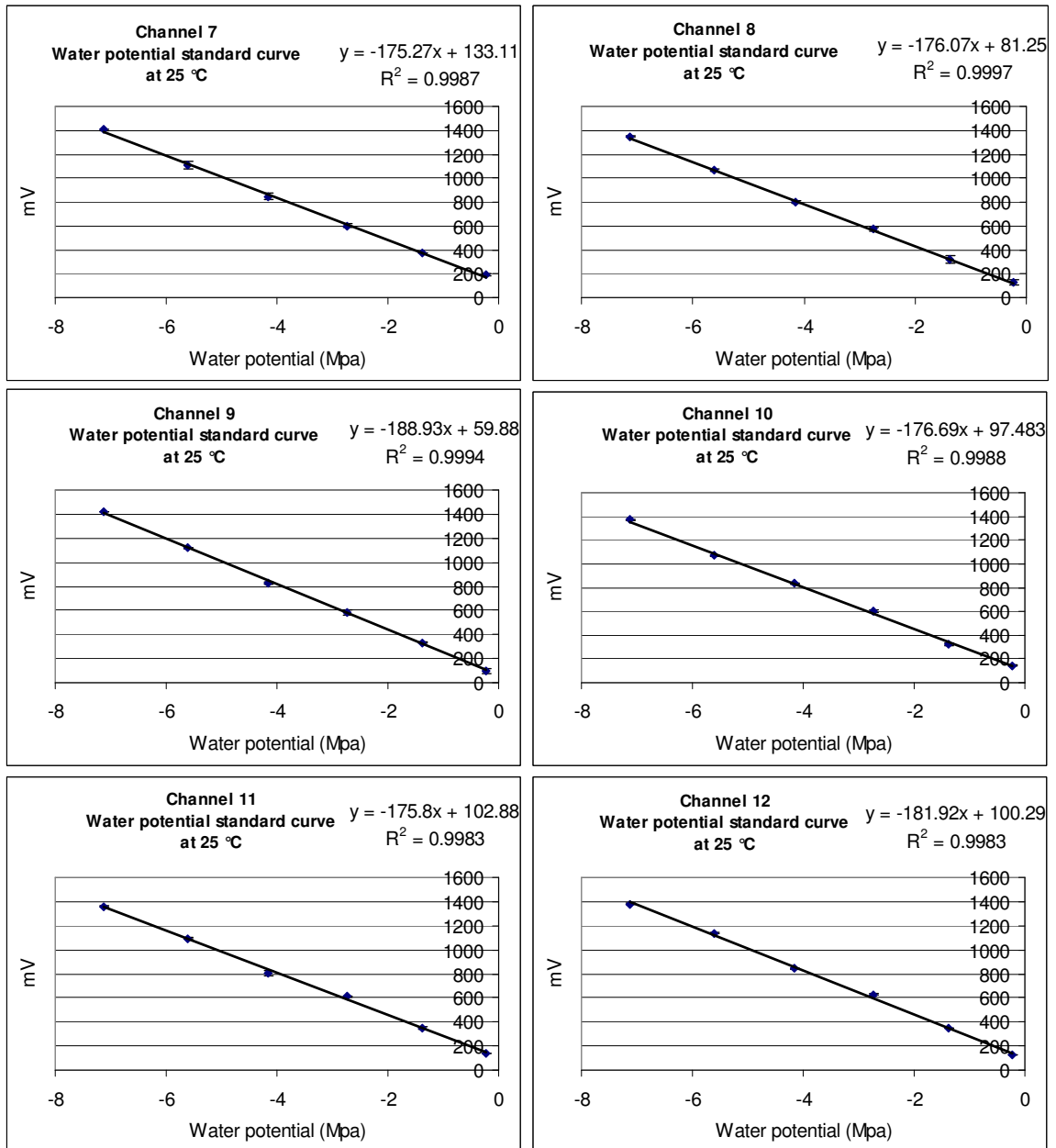


Figure A.14. Sodium chloride standard curves for channels 7 to 12.

Appendix B. Extension of Tables

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).

ID	% Cover	Accession_ homologues	Biotic and abiotic defense	Water	ABA
677	80.23	O24001_HORVU	14-3-3 protein (ABA and stress induced).	1.00	1.39
449	50.70	Q941C1_HORVU	Betaine aldehyde dehydrogenase (synthesis of the osmoprotectant betaine)	1.00	-1.31
235	72.20	Q75QN9_WHEAT	Cold shock domain protein 2 (Putative glycine-rich protein).	1.00	1.39
138	77.92	Q75QN8_WHEAT	Cold shock domain protein 3.	1.00	1.40
720	57.14	DHN3_HORVU	Dehydrin DHN3 (B17).	1.00	-1.34
2409	100.00	P83196_ORYSA	Late embryogenesis abundant protein (LEA) (Fragment).	1.00	1.36
2399	31.25	Q5MJ29_PENAM	Late embryonic abundant-like protein.	1.00	1.31
2536	40.24	Q7XSU2_ORYSA	OSJNBa0039K24.14 protein (Class III peroxidase 64 precursor) (EC 1.11.1.7).	1.00	-1.67
177	64.35	Q40069_HORVU	Peroxidase BP 1 precursor.	1.00	1.35
502	28.70	Q5Z7J2_ORYSA	Putative bacterial-induced peroxidase (Class III peroxidase 86 precursor) (EC 1.11.1.7).	1.00	1.90
2934	12.02	Q84YX3_ORYSA	Putative disease resistance gene homolog 9N.	1.00	1.72
627	78.15	Q9AWZ5_ORYSA	Putative late embryogenesis-abundant protein.	1.00	1.32
2185	8.83	Q7EYH8_ORYSA	Putative nodulin.	1.00	-1.48
1148	44.61	Q6Z4N4_ORYSA	R40g3 protein (abiotic stress).	1.00	-1.33
Membrane transport/transport					
1444	16.53	Q6QU77_WHEAT	Delta tonoplast intrinsic protein TIP2;3 (aquaporin).	1.00	-1.43
1202	72.34	Q9S877_WHEAT	Nonspecific lipid-transfer protein (LTP) (Fragment).	-1.51	1.41
2195	22.49	Q8H8V7_ORYSA	Putative ATP-binding-cassette transporter protein.	5.88	8.59
2127	7.88	Q6ER91_ORYSA	Putative vacuolar-type H ⁺ -translocating inorganic pyrophosphatase.	1.00	1.52
Cell structure					
23	84.35	Q9FUS4_SETIT	Actin.	1.00	1.67
1524	27.16	Q2TVX9_ASPOR	Drebrins and related actin binding proteins.	1.00	1.41
1515	35.88	PROF1_HORVU	Profilin-1.	1.00	1.32
Protein synthesis and folding					
257	58.10	BAS1_WHEAT	2-cys peroxiredoxin BAS1, chloroplast precursor (EC 1.11.1.15) (Thiol- specific antioxidant protein) (Fragment).	1.00	1.35
1803	91.14	Q7X9L6_WHEAT	40S ribosomal protein (Fragment).	1.00	-1.59
1204	39.02	RS21_ORYSA	40S ribosomal protein S21.	1.00	1.38
1772	35.75	Q2UN80_ASPOR	Chaperonin complex component.	1.00	1.39
762	74.27	Q9M7E1_MAIZE	Elongation factor 1 alpha.	1.00	1.50
2326	70.49	Q6Z3T3_ORYSA	Putative 60S ribosomal protein L31.	1.00	-1.34
130	57.53	Q5Z6U5_ORYSA	Putative CCT chaperonin gamma subunit.	1.00	-1.31
1159	49.11	Q5I7K9_WHEAT	Ribosomal protein L30.	1.00	-1.49
859	44.66	Q2U6V5_ASPOR	Translation elongation factor EF-1 alpha/Tu.	1.00	-1.77

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Table B.1. (continued) 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).

ID	% Cover	Accession_homologues	Transcription	Water	ABA
1169	28.90	O24005_HORVU	Diadenosine 5',5'''-P1,P4-tetraphosphate hydrolase (EC 3.6.1.17).	1.00	1.51
732	29.32	Q6XJS5_WHEAT	FCA protein (Fragment).	1.00	1.46
1138	45.83	O65795_WHEAT	Histone H1.	1.00	-1.58
1216	78.00	H2B2_MAIZE	Histone H2B.2.	1.00	1.92
2674	19.19	Q2UMQ4_ASPOR	MRNA splicing protein CDC5.	1.00	1.64
2951	32.84	Q6ER41_ORYSA	Myb family transcription factor-like.	1.00	-1.39
862	25.13	Q7XZF7_ORYSA	Putative DNA gyrase subunit.	1.00	-1.43
249	33.30	Q69V60_ORYSA	Putative elongation factor 2.	1.00	-1.39
1835	23.45	Q8S609_ORYSA	Putative gag-pol (Retrotransposon protein, putative, Ty3-gypsy sub- class).	1.00	-1.49
2057	17.86	Q4W1G2_WHEAT	Putative high mobility group protein (Fragment).	1.00	1.57
682	66.04	Q94E96_ORYSA	Putative histone H2A.	1.00	-1.33
1668	24.63	Q8LLY0_ORYSA	Putative retroelement (Retrotransposon protein, putative, Ty3-gypsy sub-class).	1.00	1.73
2527	39.86	Q8RUL1_ORYSA	Putative transcription elongation factor.	1.00	1.50
1739	16.65	Q53NJ8_ORYSA	Retrotransposon protein, putative, Ty3-gypsy sub-class.	1.00	-1.77
Glycolysis and energy					
126	62.57	Q40676_ORYSA	Cytoplasmic aldolase.	1.00	-1.31
1025	35.71	Q5H4R9_XANOR	Phosphoglucumutase; phosphomannomutase.	1.00	-1.34
2302	18.89	Q8H7N0_ORYSA	Putative alcohol dehydrogenase.	1.00	-1.56
1001	60.91	Q2QXR8_ORYSA	Pyruvate kinase, barrel domain.	1.00	-1.42
Regulatory and signalling					
2408	47.62	Q9FW38_ORYSA	Hypothetical protein P0036D10.4 (Putative LRR receptor-like protein kinase).	1.00	-1.30
2760	16.91	Q2R8L1_ORYSA	NB-ARC domain, putative. (cell death)	1.00	-1.48
765	19.42	Q8SAU4_ORYSA	Putative calcium-binding protein.	1.00	1.42
435	51.69	Q851Y6_ORYSA	Putative proteasome regulatory non-ATPase subunit.	1.00	-1.37
780	34.36	Q8H8C1_ORYSA	Putative RNA-binding protein.	1.00	-1.31
2929	26.12	Q5GVL1_XANOR	Two-component system regulatory protein.	1.00	-1.46
Metabolism					
205	39.71	PURA_WHEAT	Adenylosuccinate synthetase, chloroplast precursor (EC 6.3.4.4) (IMP-- aspartate ligase) (AdSS) (AMPSase) (Fragment).	1.00	-1.31
1254	25.33	Q6H6P8_ORYSA	Branching enzyme-3.	1.00	1.30
622	48.05	Q8S3X0_HORVD	Chalcone isomerase (EC 5.5.1.6).	1.00	-1.32
929	65.26	Q84UH5_ORYSA	Dehydroascorbate reductase.	1.00	1.48
1369	48.87	Q7X9L3_WHEAT	Formate dehydrogenase (Fragment).	1.00	1.42

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Table B.1. (continued) 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).

ID	% Cover	Accession_ homologues	Metabolism (continued)	Water	ABA
497	57.07	Q93XJ5_HORVU	IDI1 protein.	1.00	1.35
1111	26.30	Q5JNT6_ORYSA	Isopenicillin N epimerase-like.	1.00	-1.36
2803	32.21	Q6ZHK3_ORYSA	Nucleoside diphosphate kinase I.	1.00	1.50
658	47.93	Q852R3_HORVU	Phospholipid hydroperoxide glutathione peroxidase-like protein (EC 1.11.1.9).	1.00	1.31
1573	38.57	Q5VQ48_ORYSA	Putative amidase.	1.00	1.40
2578	19.52	Q6Z8P2_ORYSA	Putative aminoacylase.	1.00	1.44
1936	27.06	Q69X42_ORYSA	Putative glycine dehydrogenase.	1.00	1.31
1064	35.15	Q5TKF4_ORYSA	Putative nucleoside diphosphate kinase.	1.00	1.41
85	63.75	Q9LI00_ORYSA	Putative phosphogluconate dehydrogenase.	1.00	1.69
1876	17.39	Q2UMI3_ASPOR	Ubiquitin activating E1 enzyme-like protein.	1.00	1.70
Protease					
508	36.31	LONH2_MAIZE	Lon protease homolog 2, mitochondrial precursor (EC 3.4.21.-).	1.00	-1.53
1335	26.35	Q6K4E7_ORYSA	Putative puromycin-sensitive aminopeptidase.	1.00	-1.37
339	79.43	Q7XYF8_WHEAT	SKP1/ASK1-like protein.	1.62	1.00
2293	10.04	Q5GVJ7_XANOR	Zinc protease.	1.00	3.33
Other					
2762	27.20	Q69S61_ORYSA	AAA-type ATPase-like.	1.00	2.68
158	33.33	Q5ZDJ3_ORYSA	Acetyltransferase 1-like.	1.00	-1.31
297	39.92	Q9ZNS9_ORYSA	Embryo-specific protein.	1.00	1.38
433	38.72	Q8L8I0_ORYSA	Globulin-like protein.	1.00	1.34
725	30.93	Q84RU7_ORYSA	Putative EBNA1-binding protein homolog; Ebp2p.	1.00	-1.32
Unknown function					
1676	25.67	Q8W0T8_SORBI	Gb protein. Sorghum bicolor (Sorghum) (Sorghum vulgare).	1.00	-1.48
1617	19.39	Q5W6S3_ORYSA	Hypothetical protein B1036C05.8.	1.00	-1.41
1844	30.34	Q6ZKH7_ORYSA	Hypothetical protein OJ1119_D01.23.	1.67	1.00
2250	22.56	Q6ZHJ7_ORYSA	Hypothetical protein OJ1218_D07.4.	1.00	1.51
1627	43.28	Q6ZJM6_ORYSA	Hypothetical protein OJ1300_E01.11.	1.00	1.45
2235	54.22	Q6ERE0_ORYSA	Hypothetical protein OJ1655_B12.14.	1.00	-1.36
2437	20.24	Q94I55_ORYSA	Hypothetical protein OSJNBa0084C09.17.	1.00	-2.46
2813	20.83	Q5ZB09_ORYSA	Hypothetical protein OSJNBb0024F06.8.	1.00	1.45
2492	24.57	Q94GH5_ORYSA	Hypothetical protein OSJNBb0093E13.1.	1.00	-1.34
596	62.88	Q69XJ9_ORYSA	Hypothetical protein P0486H12.21.	1.00	-1.34
1468	53.43	Q658I0_ORYSA	Hypothetical protein P0538C01.10.	1.00	1.51
1652	40.46	Q5VNM9_ORYSA	Hypothetical protein P0696E01.34 (Hypothetical protein P0004A09.13).	1.00	1.82
3044	30.21	Q5GVK5_XANOR	Hypothetical protein.	1.00	1.69

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Table B.1. (continued) 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).

ID	% Cover	Accession_ homologues	Unknown function (continued)	Water	ABA
310	37.28	Q7X751_ORYSA	OJ000223_09.5 protein (OSJNBa0081L15.15 protein).	1.00	-1.31
2465	49.11	Q7FA28_ORYSA	OSJNBa0043L24.17 protein. (Probable glutaredoxin)	1.00	1.63
1597	20.93	Q7XTT4_ORYSA	OSJNBa0058K23.21 protein.	1.00	1.48
2948	21.46	Q7XUL8_ORYSA	OSJNBa0074L08.10 protein.	1.00	-1.41
1705	33.24	Q7XM06_ORYSA	OSJNBa0086O06.3 protein.	1.00	-1.45
1333	15.93	Q7XND9_ORYSA	OSJNBa0088A01.24 protein.	1.00	1.69
2025	16.18	Q7XUE0_ORYSA	OSJNBa0088A01.5 protein.	1.00	1.34
539	69.37	Q7XR19_ORYSA	OSJNBb0022F23.11 protein.	1.00	-1.33
2671	16.63	Q7XN02_ORYSA	OSJNBb0038F03.9 protein.	1.00	-1.40
1792	18.75	Q6AVR6_ORYSA	Putative F8K7.10 protein.	1.00	1.34
1016	34.13	Q9LIU9_ORYSA	Putative negatively light-regulated protein.	1.00	1.37
36	69.17	Q8H7M3_ORYSA	Putative r40c1 protein-rice.	1.00	-1.33

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).

ID	% Cover	Accession_ homologues	Biotic and abiotic defense	Water	ABA
169	45.97	Q5UHH7_WHEAT	0.19 dimeric alpha-amylase inhibitor (Fragment).	1.00	-1.51
468	57.26	Q5UHH6_WHEAT	0.19 dimeric alpha-amylase inhibitor (Fragment).	-1.51	-1.34
253	28.57	Q53YX8_WHEAT	Alpha amylase inhibitor protein.	-1.61	-1.48
935	11.14	Q42839_HORVU	Chitinase (EC 3.2.1.14).	1.00	-1.50
721	40.49	Q75QN9_WHEAT	Cold shock domain protein 2 (Putative glycine-rich protein).	1.00	1.31
175	64.52	EM3_WHEAT	Em protein H2 (protective protein against desiccation, ABA induced).	1.00	1.61
28	72.22	IAAS_WHEAT	Endogenous alpha-amylase/subtilisin inhibitor (WASI).	1.00	-1.24
62	62.78	Q03967_WHEAT	Group 3 late embryogenesis abundant protein (Fragment).	1.00	1.26
177	34.44	Q49956_WHEAT	Monomeric alpha-amylase inhibitor precursor.	1.00	-1.56
99	81.67	Q9SQG8_WHEAT	Pathogenesis-related protein 4 (Fragment).	-4.39	1.00
55	58.94	Q8LK23_WHEAT	Peroxidase 1.	1.00	-1.22
407	55.56	P93602_WHEAT	PUP88 protein; member of trypsin/a-amylase inhibitors family from cereals precursor.	1.00	-1.36
331	25.50	Q8S7U3_ORYSA	Putative embryo-specific protein (LEA expressed).	1.00	1.52
16	75.94	P93693_WHEAT	Serpin.	-1.28	-1.42
36	57.04	P93692_WHEAT	Serpin.	-1.22	-1.21
115	69.10	Q41593_WHEAT	Serpin.	-1.27	1.00
706	36.43	Q2QLS3_ORYSA	Thaumatin-like protein TLP7.	1.00	-1.35
113	38.89	Q6KE41_SECCE	Xylanase inhibitor precursor.	1.00	-1.24
22	65.79	XIP1_WHEAT	Xylanase inhibitor protein 1 precursor (Class III chitinase homolog) (XIP-I protein).	1.00	-1.26
Membrane transport and transport					
560	16.36	Q84SZ1_ORYSA	Synaptobrevin-like protein.	1.00	1.26
Protein synthesis and folding					
232	29.20	Q7X9K6_WHEAT	40S ribosomal protein (Fragment).	1.00	1.31
181	44.19	RL7A_ORYSA	60S ribosomal protein L7a.	1.00	1.22
401	10.51	Q41798_MAIZE	Calnexin (Fragment).	1.00	-1.43
311	49.44	Q9M7E4_MAIZE	Elongation factor 1 alpha.	1.00	1.21
49	43.77	Q2L3T8_WHEAT	Heat shock protein 90 (Fragment).	1.00	-1.31
30	54.28	Q40058_HORVU	HSP70 precursor.	1.00	-1.31
205	54.15	BIP3_MAIZE	Luminal-binding protein 3 precursor (BiP3).	1.00	-1.24

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Table B.2. (continued) 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).

ID	% Cover	Accession_ homologues	Protein synthesis and folding (continued)	Water	ABA
280	40.00	Q8LH97_ORYSA	Putative 40S ribosomal protein S6.	1.00	1.46
307	44.22	Q5TKP3_ORYSA	Putative 60S ribosomal L28 protein.	1.00	1.22
774	25.34	Q8GSE9_ORYSA	Putative 60S ribosomal protein L27a.	1.22	1.82
881	30.25	Q8W0C4_ORYSA	Putative elongation factor 2.	1.00	1.21
38	42.42	Q6V959_WHEAT	Ribosomal protein L3.	1.00	1.41
144	55.56	Q94KM0_WHEAT	Small heat shock protein HSP17.8.	1.00	1.24
Transcription					
187	50.74	Q53WY3_ORYSA	Histone H3.	1.00	1.51
627	10.53	Q2USJ1_ASPOR	Nonsense-mediated mRNA decay 2 protein.	1.00	1.26
161	55.86	Q43213_WHEAT	Protein H2A.	1.00	1.68
333	45.10	Q6K701_ORYSA	Putative fibrillarin.	-1.34	1.00
622	23.24	Q6YUR8_ORYSA	Putative Glycine-rich protein 2.	-1.21	1.00
659	41.10	Q6L500_ORYSA	Putative histone H2A.	1.21	1.00
663	20.70	Q650Z9_ORYSA	Putative iron inhibited ABC transporter 2.	1.00	-1.32
503	8.83	Q9FVY5_ORYSA	Putative RNA helicase.	1.00	-1.21
954	11.50	Q2UTR6_ASPOR	Superfamily II DNA/RNA helicases.	1.00	-1.41
Glycolysis and energy					
135	25.93	G6PI_MAIZE	Glucose-6-phosphate isomerase, cytosolic (EC 5.3.1.9) (GPI) (Phosphoglucose isomerase) (PGI) (Phosphohexose isomerase) (PHI).	1.32	1.00
419	21.03	Q8H2T7_ORYSA	Putative NADH dehydrogenase.	1.00	1.33
701	20.60	Q8W0E8_ORYSA	Putative NADH2 dehydrogenase (Ubiquinone) chain PSST.	1.44	1.00
129	36.43	Q2QXR8_ORYSA	Pyruvate kinase, barrel domain.	-1.26	1.00
Regulatory and signalling					
836	13.40	Q6ATR5_ORYSA	Expressed protein.	1.61	1.00
270	39.61	Q5MGQ9_PENAM	Rab7. Pennisetum americanum (Pearl millet).	-1.49	1.00
476	51.64	Q07810_WHEAT	Tritin.	-1.28	-1.38

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Table B.2. (continued) 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).

ID	% Cover	Accession_homologues	Metabolism	Water	ABA
8	76.15	Q6W8Q2_WHEAT	1-Cys-peroxiredoxine.	-1.26	1.00
87	55.19	ALA2_HORVU	Alanine aminotransferase 2 (EC 2.6.1.2) (GPT) (Glutamic--pyruvic transaminase 2) (Glutamic--alanine transaminase 2) (ALAAAT-2).	-1.23	1.00
194	55.80	Q43320_AVEFA	Aldose reductase-related protein (EC 1.1.1.21).	1.36	1.00
679	18.30	Q7X9M3_WHEAT	AmiB (Fragment).	1.00	-1.34
94	33.05	Q84LA5_HORVU	Asparagine synthetase 2 (EC 6.3.5.4).	-1.26	1.00
682	11.20	GLTB_MAIZE	Ferredoxin-dependent glutamate synthase, chloroplast precursor (EC 1.4.7.1) (Fd-GOGAT).	1.00	-1.38
51	53.26	GLGL2_WHEAT	Glucose-1-phosphate adenylyltransferase large subunit, chloroplast precursor (EC 2.7.7.27) (ADP-glucose synthase) (ADP-glucose pyrophosphorylase) (AGPase S) (Alpha-D-glucose-1-phosphate adenylyl transferase).	-1.35	-1.36
231	21.83	Q5Z790_ORYSA	Putative 4-methyl-5(B-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme.	1.00	1.32
749	16.44	Q6AVT2_ORYSA	Putative ADP-glucose pyrophosphorylase.	1.84	1.00
39	49.26	Q9M4Z1_WHEAT	Small subunit ADP glucose pyrophosphorylase (EC 2.7.7.27).	-1.25	-1.29
Protease					
731	19.53	ORYB_ORYSA	Oryzain beta chain precursor (EC 3.4.22.-).	1.25	1.00
76	37.59	CBP2_WHEAT	Serine carboxypeptidase II (EC 3.4.16.6) (Carboxypeptidase D) (CPDW- II) (CP-WII) (Contains: Serine carboxypeptidase II chain A; Serine carboxypeptidase II chain B).	1.00	1.25
Endosperm-related proteins					
45	61.41	Q7XYC3_WHEAT	19 kDa globulin (Fragment).	-1.53	-1.37
75	68.77	Q7X9M2_WHEAT	Beta amylase (Fragment).	-1.28	-1.35
86	68.92	AMYB_SECCE	Beta-amylase (EC 3.2.1.2) (1,4-alpha-D-glucan maltohydrolase) (Fragment).	1.00	1.28
6	61.50	Q84T19_HORVD	Endosperm-specific beta-amylase 1.	-1.26	-1.39
413	21.80	Q6ERU3_ORYSA	Glutelin.	-1.22	1.00
46	57.09	GLT5_WHEAT	Glutenin, high molecular weight subunit DX5 precursor.	1.00	-1.21
32	75.34	Q41553_WHEAT	HMW glutenin subunit Ax2*.	1.00	-1.31
11	65.20	Q52JL3_WHEAT	HMW glutenin subunit.	1.00	-1.31
534	59.50	Q6UKZ5_WHEAT	HMW glutenin subunit.	2.87	1.00

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Table B.2. (continued) 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).

ID	% Cover	Accession_ homologues	Endosperm-related proteins (continued)	Water	ABA
18	74.47	Q45R38_WHEAT	HMW glutenin x-type subunit Bx7 precursor.	1.00	-1.52
264	59.77	Q68AN2_WHEAT	LMW-s KS2.	-1.55	-1.76
298	51.26	Q6SPY8_WHEAT	Low molecular weight glutenin.	1.00	-1.40
670	65.72	Q8W3X4_WHEAT	Low-molecular-weight glutenin subunit group 3 type II (Fragment).	2.81	-1.90
336	61.78	Q8W3W1_WHEAT	Low-molecular-weight glutenin subunit group 5 type III.	1.00	-1.56
139	45.29	Q6Z5B2_ORYSA	Putative beta-amylase.	-1.22	-1.37
313	24.08	Q75RZ3_WHEAT	Putative beta-xylosidase (Fragment).	-1.31	1.00
88	24.79	Q9FUU7_WHEAT	Starch branching enzyme 2 (EC 2.4.1.81).	-1.30	-1.41
64	45.90	O04074_WHEAT	Starch branching enzyme I precursor (Starch branching enzyme 1) (EC 2.4.1.81).	-1.21	-1.25
84	44.13	Q9SXX3_WHEAT	Starch synthase (GBSSI) (EC 2.4.1.21).	-1.59	-1.89
9	54.23	Q43223_WHEAT	Sucrose synthase type 2.	-1.31	-1.29
ID	% Cover	Accession_ homologues	Other		
112	41.38	Q7Y1Z2_WHEAT	27K protein (Fragment).	-1.65	-1.40
96	75.69	REHY_HORVU	Probable peroxiredoxin (EC 1.11.1.15) (Thioredoxin peroxidase) (Rehydrin homolog) (B15C).	-1.28	1.00
12	69.52	Q08837_WHEAT	Triticin (Fragment).	-1.27	-1.21
ID	% Cover	Accession_ homologues	Unknown function		
180	37.82	Q9ZNS9_ORYSA	Embryo-specific protein (probable lipoprotein).	1.41	1.00
218	27.48	Q7X9M4_WHEAT	Hypothetical protein (Fragment).	1.00	-1.25
688	15.60	Q6Z2X0_ORYSA	Hypothetical protein OJ1679_B08.21.	1.00	1.35
647	6.14	Q8SB96_ORYSA	Hypothetical protein OSJNBa0042H09.8.	1.00	-1.24
961	36.14	Q6AVV3_ORYSA	Hypothetical protein OSJNBb0024N19.12.	1.00	-1.26
817	33.78	O24003_HORVU	Hypothetical protein precursor.	1.00	-1.23
804	12.90	Q2QVK3_ORYSA	Hypothetical protein.	-1.42	1.00
346	31.25	Q7XT72_ORYSA	OSJNBa0029H02.21 protein.	1.00	2.54
170	41.49	Q7X6I8_ORYSA	OSJNBb0118P14.11 protein (OJ000315_02.8 protein).	-1.21	1.00
960	12.79	Q2PIZ2_ASPOR	Predicted protein.	1.00	-1.22
916	11.81	Q2UUI9_ASPOR	Predicted protein.	1.00	1.21
883	17.54	Q2UJ19_ASPOR	Predicted protein.	1.27	1.25

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).

ID	% Cover	Accession_ homologues	Biotic and abiotic defense	Water	ABA
188	55.36	Q53YX8_WHEAT	Alpha amylase inhibitor protein.	1.00	1.22
110	65.11	CHIA_SECCE	Basic endochitinase A precursor (EC 3.2.1.14) (Rye seed chitinase-a) (RSC-a).	-3.21	-3.06
387	74.44	CHIC_SECCE	Basic endochitinase C precursor (EC 3.2.1.14) (Rye seed chitinase-c) (RSC-c).	1.92	1.51
197	58.18	Q8H0B8_WHEAT	Cold regulated protein.	1.85	1.74
268	41.56	Q75QN8_WHEAT	Cold shock domain protein 3.	-1.53	-1.54
55	85.00	IAAS_WHEAT	Endogenous alpha-amylase/subtilisin inhibitor (WASI).	-1.26	-1.24
1195	60.96	P93180_HORVU	Pathogenesis-related protein 4 precursor.	-1.90	-1.96
1063	18.54	Q6YUA7_ORYSA	Putative activator of 90 kDa heat shock protein ATPase homolog 1.	1.23	1.00
433	19.21	Q9AWZ5_ORYSA	Putative late embryogenesis-abundant protein.	6.17	5.45
1089	12.17	Q6ZBK6_ORYSA	Putative stress related-like protein interactor.	-2.87	-2.60
96	72.86	Q41593_WHEAT	Serpin.	1.49	1.52
75	74.37	Q9ST58_WHEAT	Serpin.	1.47	1.46
6	84.96	P93693_WHEAT	Serpin.	1.32	1.30
20	82.16	P93692_WHEAT	Serpin.	1.23	1.25
1548	20.00	Q5EII3_ORYSA	Subtilisin-chymotrypsin inhibitor.	1.26	1.00
1245	37.78	Q8S4P7_WHEAT	Thaumatin-like protein.	2.93	2.75
339	38.55	Q2TN84_WHEAT	Universal stress protein (USP) family protein.	2.09	1.00
1034	7.89	Q9FS08_ORYSA	UV-damaged DNA binding protein.	1.51	1.50
74	41.18	Q5TMB2_WHEAT	Xylanase inhibitor TAXI-IV.	-1.51	-1.54
Membrane transport/ transport					
1544	10.30	Q5GVI5_XANOR	D-serine/D-alanine/glycine transporter.	-3.03	-3.50
293	52.72	Q7X9K5_WHEAT	F1-ATPase (Fragment).	2.23	2.27
757	22.12	Q8H4Q9_ORYSA	GTP-binding protein Rab6.	1.75	1.00
744	23.81	Q2UDB1_ASPOB	Predicted mitochondrial carrier protein.	3.08	3.14
939	13.47	Q8RZX0_ORYSA	Putative adapter-related protein complex 4 epsilon 1 subunit.	-1.21	-1.28
179	18.56	Q6Z382_ORYSA	Putative coatomer protein gamma 2-subunit.	1.38	1.00
1305	15.83	Q8KNY6_9XANT	Putative siderophore receptor.	1.34	1.31
152	30.74	Q41579_WHEAT	Rab protein.	2.18	1.66
150	42.53	Q9XJ45_ORYSA	Ran.	-1.33	-1.27
Cell structure					
51	54.38	Q9FUS4_SETIT	Actin.	1.46	1.46

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Table B.3. (continued) 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).

ID	% Cover	Accession_homologues	Protein synthesis and folding	Water	ABA
620	30.66	Q9FUL7_MAIZE	40S ribosomal protein S24.	1.66	1.59
571	34.88	RS27_HORVU	40S ribosomal protein S27 (Manganese efficiency-related protein 1).	1.57	1.56
256	72.71	O49831_ORYSA	EF-1 alpha.	1.86	1.71
456	54.14	Q9M7E4_MAIZE	Elongation factor 1 alpha.	1.00	-2.52
286	74.50	EF1A2_HORVU	Elongation factor 1-alpha (EF-1-alpha).	-1.32	-1.30
591	68.90	O50018_MAIZE	Elongation factor 1-alpha.	2.05	2.01
4	71.81	Q6LAA4_HORVU	Elongation factor 1-alpha.	-1.28	-1.27
171	57.21	EF1B_WHEAT	Elongation factor 1-beta (EF-1-beta) (Elongation factor 1B-alpha 2) (eEF-1B alpha) (Elongation factor 1-beta') (EF-1-beta').	-1.53	-1.54
388	21.96	HSP82_MAIZE	Heat shock protein 82.	1.46	1.59
1232	37.84	Q2L3T8_WHEAT	Heat shock protein 90 (Fragment).	1.00	1.40
598	72.08	Q9ZSR5_WHEAT	Heat shock protein HSP26.	-4.39	-4.23
1210	8.31	Q9XFD9_ORYSA	Heat-shock protein DnaJ (Putative DnaJ-like protein).	1.00	1.22
2	71.76	Q9SAU8_WHEAT	HSP70.	1.38	1.40
1506	11.42	Q2U2A1_ASPOR	Non-ribosomal peptide synthetase modules and related proteins.	-1.39	-1.36
402	54.12	Q2QKC3_WHEAT	Pre-mRNA processing factor.	-1.41	1.00
389	33.33	Q6ZIW7_ORYSA	Putative 40S ribosomal protein S2.	1.68	1.64
642	30.61	Q6K705_ORYSA	Putative 60S ribosomal protein L28.	-1.47	1.00
1164	40.25	Q6ZLB8_ORYSA	Putative 60S ribosomal protein L4/L1.	2.25	2.44
203	37.70	Q75M08_ORYSA	Putative disulfide-isomerase.	1.39	1.00
181	30.09	Q6ZD35_ORYSA	Putative glycyl-tRNA synthetase.	1.44	1.43
752	23.80	Q6ERL4_ORYSA	Putative tRNA splicing protein.	-1.45	-1.35
281	14.70	Q5VRX8_ORYSA	Putative tRNA-glutamine synthetase.	1.21	1.00
439	31.20	Q9LXD4_WHEAT	Thioredoxin h.	2.19	2.57
Transcription and replication					
523	17.57	IF3A_MAIZE	Eukaryotic translation initiation factor 3 subunit 10 (eIF-3 theta) (Eukaryotic translation initiation factor 3 large subunit) (eIF3a).	-2.69	-2.60
368	43.31	Q42832_HORVU	Histone H3 (Fragment).	-1.68	1.00
563	21.63	Q94F77_MAIZE	Nucleosome/chromatin assembly factor C.	-1.33	-1.42
1132	3.84	Q2UTH5_ASPOR	Oxoprolinase.	-1.61	1.00
799	9.91	Q8RU52_ORYSA	Putative copia-like retrotransposon Hopscotch polypeptide (Retrotransposon protein, putative, Ty1-copia sub-class).	1.37	1.37
1354	7.66	Q8H6I8_MAIZE	Putative gag-pol polypeptide.	2.58	2.71

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Table B.3. (continued) 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).

ID	% Cover	Accession_ homologues	Transcription and replication (continued)	Water	ABA
624	11.30	Q69X61_ORYSA	Putative WD-40 repeat protein.	-1.64	-1.58
885	12.36	Q2R0A5_ORYSA	Retrotransposon protein, putative, Ty1-copia sub-class.	1.00	1.25
1434	12.83	Q2QXX2_ORYSA	Ribosomal RNA apurinic site specific lyase, putative.	1.95	1.00
1044	11.43	Q2UFN5_ASPO	Structural maintenance of chromosome protein 3.	1.00	-1.28
1037	17.32	Q2R4J2_ORYSA	Transposon protein, putative, ping/pong/SNOOPY sub-class.	-3.54	1.00
1432	29.96	Q2QQP1_ORYSA	Transposon protein, putative, unclassified.	-1.79	-1.70
Glycolysis/ citric acid cycle					
198	49.37	PMGI_MAIZE	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1) (Phosphoglyceromutase) (BPG-independent PGAM) (PGAM-I).	1.56	1.00
416	46.71	Q43320_AVEFA	Aldose reductase-related protein (EC 1.1.1.21).	-1.51	-1.59
366	36.56	Q6XEB8_WHEAT	Cytosolic malate dehydrogenase (EC 1.1.1.37) (Fragment).	1.51	1.50
97	53.16	Q6SZS7_ORYSA	Cytosolic NADP malic enzyme.	-2.55	-2.47
147	69.28	ENO2_MAIZE	Enolase 2 (EC 4.2.1.11) (2-phosphoglycerate dehydratase 2) (2-phospho- D-glycerate hydro-lyase 2).	1.79	1.73
961	29.98	G6PIA_ORYSA	Glucose-6-phosphate isomerase, cytosolic A (EC 5.3.1.9) (GPI-A) (Phosphoglucose isomerase A) (PGI-A) (Phosphohexose isomerase A) (PHI- A).	-1.37	1.00
320	65.49	Q7XY25_WHEAT	Glutaredoxin.	1.69	1.68
1235	58.46	G3PE_MAIZE	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3 (EC 1.2.1.12).	-6.68	-8.57
1066	33.43	Q5H3L4_XANOR	Glyceraldehyde-3-phosphate dehydrogenase. Xanthomonas oryzae pv. oryzae.	-10.71	-12.52
516	40.41	Q7XZE9_WHEAT	Glyoxysomal malate dehydrogenase (Fragment).	1.37	1.37
26	80.90	Q8VWM9_WHEAT	Putative fructose 1-,6-biphosphate aldolase (Fragment).	-1.40	-1.31
690	31.49	Q6YPF1_ORYSA	Putative fructose-bisphosphate aldolase.	1.00	-2.91
568	62.31	Q6K5G8_ORYSA	Putative glyceraldehyde-3-phosphate dehydrogenase (Phosphorylating).	-1.28	-1.21
435	55.59	Q94JA2_ORYSA	Putative malate dehydrogenase (Putative mitochondrial malate dehydrogenase).	1.51	1.46
230	32.93	Q8W317_ORYSA	Putative reductase.	1.36	1.00
135	50.51	Q7XYB5_WHEAT	Pyruvate orthophosphate dikinase (Fragment).	-1.42	-1.38

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Table B.3. (continued) 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).

ID	% Cover	Accession_ homologues	Regulation/ signalling	Water	ABA
113	45.54	Q6K8W1_ORYSA	26S proteasome regulatory particle triple-A ATPase subunit1 (26S protease regulatory subunit 7).	-1.60	-1.59
163	45.48	Q7F2H4_ORYSA	ADP-ribosylation factor.	1.00	1.42
1421	14.71	Q9AR27_ORYSA	Casein kinase II alpha subunit (Hd6).	-2.01	-1.87
968	11.75	Q2U427_ASPO	Lysosomal trafficking regulator LYST and related BEACH and WD40 repeat proteins.	-2.00	-1.94
1329	6.86	Q53P15_ORYSA	NB-ARC domain, putative (apoptosis).	7.46	7.94
381	34.29	RIP1_HORVU	Protein synthesis inhibitor 1 (EC 3.2.2.22) (Ribosome-inactivating protein I) (rRNA N-glycosidase).	1.82	1.78
392	23.65	Q6YUR8_ORYSA	Putative Glycine-rich protein 2.	-1.33	-1.32
356	46.06	Q6Z050_ORYSA	Putative polyadenylate-binding protein.	-1.82	-1.81
355	25.28	Q851Y6_ORYSA	Putative proteasome regulatory non-ATPase subunit.	1.59	1.60
1372	16.01	Q94DU4_ORYSA	Putative receptor-like protein kinase.	-3.47	-3.30
1501	8.71	Q653C0_ORYSA	Putative wall-associated kinase 4.	-3.60	-3.60
488	25.19	Q9FWV5_ORYSA	Receptor-like protein kinase, putative.	-3.54	-3.40
40	72.73	Q07810_WHEAT	Tritin.	1.44	1.54
Metabolism					
45	53.89	Q2KM86_HORVD	2-alkenal reductase (EC 1.3.1.74).	-1.23	-1.22
476	15.45	Q2R1G9_ORYSA	Acyl-CoA oxidase homolog.	1.44	1.39
374	30.27	Q84P58_ORYSA	Adenosine kinase-like protein (Fragment).	1.38	1.35
44	67.67	ADT2_WHEAT	ADP,ATP carrier protein 2, mitochondrial precursor (ADP/ATP translocase 2) (Adenine nucleotide translocator 2) (ANT 2).	1.51	1.55
199	38.38	ALA2_PANMI	Alanine aminotransferase 2 (EC 2.6.1.2) (GPT) (Glutamic--pyruvic transaminase 2) (Glutamic--alanine transaminase 2) (ALAAT-2).	1.98	1.00
264	23.60	AGLU_HORVU	Alpha-glucosidase precursor (EC 3.2.1.20) (Maltase).	1.74	1.00
449	14.44	Q9ATV8_HORVU	Arabinoxylan arabinofuranohydrolase isoenzyme AXAH-I.	2.57	1.00
154	34.08	Q84LA5_HORVU	Asparagine synthetase 2 (EC 6.3.5.4).	-1.43	-1.36
70	38.41	CATA1_HORVU	Catalase isozyme 1 (EC 1.11.1.6).	-1.78	-1.64
65	77.36	Q84UH6_WHEAT	Dehydroascorbate reductase.	1.42	1.37
1346	11.73	Q7XE38_ORYSA	GDSL-like Lipase/Acylhydrolase.	1.47	1.33
196	45.28	Q9SP56_WHEAT	Glutathione S-transferase.	1.35	1.39
81	55.86	Q8RW03_WHEAT	Glutathione transferase (EC 2.5.1.18).	1.53	1.49
1443	28.75	Q5N8H1_ORYSA	Hydrolase-like protein.	1.82	1.88
1065	45.82	Q8W529_MAIZE	Methionine synthase (Fragment).	-2.26	-2.26
233	61.62	Q4LB12_HORVU	Methionine synthase 2 enzyme (EC 2.1.1.14).	-1.57	-1.49

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Table B.3. (continued) 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).

ID	% Cover	Accession_ homologues	Metabolism (continued)	Water	ABA
1266	7.04	Q9SXX0_ORYSA	Monodehydroascorbate reductase (EC 1.6.5.4) (Fragment).	-3.19	-2.95
173	47.93	Q852R3_HORVU	Phospholipid hydroperoxide glutathione peroxidase-like protein (EC 1.11.1.9).	2.02	1.00
235	34.01	Q5Z790_ORYSA	Putative 4-methyl-5(B-hydroxyethyl)-thiazol monophosphate biosynthesis enzyme.	1.54	1.67
1076	20.68	Q5VQ48_ORYSA	Putative amidase.	-1.39	-1.41
1183	8.72	Q8H530_ORYSA	Putative beta-alanine synthases.	2.35	2.35
483	27.00	Q5W6F1_ORYSA	Putative cinnamate-4-hydroxylase.	-1.47	1.00
793	22.19	Q5NAT8_ORYSA	Putative endo-beta-1,4-glucanase.	1.33	1.29
522	43.03	Q944F4_ORYSA	Putative fructokinase I.	1.33	1.29
1001	16.94	Q5KQK5_ORYSA	Putative glucosyl transferase.	-3.98	-3.78
1112	12.96	Q75IM9_ORYSA	Putative isovaleryl-CoA dehydrogenase.	1.00	1.33
39	45.36	Q4LB19_HORVU	Putative S-adenosylhomocystein hydrolase 2 (EC 3.3.1.1).	1.00	1.36
267	37.26	Q3S861_WHEAT	Pyridoxine biosynthesis protein.	-1.23	-1.25
401	37.45	Q84UC6_HORVD	Pyrroline-5-carboxylate reductase.	1.42	1.46
876	6.24	Q2UUH3_ASPOR	RIB40 genomic DNA, SC009.	6.83	7.53
540	17.02	Q2UDJ0_ASPOR	Serine/threonine protein phosphatase 2A.	-2.37	-2.42
131	31.28	Q9ARG8_WHEAT	Sucrose-6F-phosphate phosphohydrolase SPP3 (EC 3.1.3.24).	1.73	1.76
1459	18.12	Q2U3G9_ASPOR	Thiamine pyrophosphate-requiring enzymes.	-1.91	-1.89
Protease					
140	68.54	Q5XUV7_WHEAT	20S proteasome beta 4 subunit.	1.60	1.47
23	56.10	Q401N7_WHEAT	Aspartic proteinase.	1.53	1.62
Endosperm-related proteins					
77	76.58	Q7X9M2_WHEAT	Beta amylase (Fragment).	1.67	1.62
246	54.95	AMYB_SECCE	Beta-amylase (EC 3.2.1.2) (1,4-alpha-D-glucan maltohydrolase) (Fragment).	-1.72	-1.66
771	17.03	Q5JKH9_ORYSA	Endo-1,3-beta-glucanase.	2.33	2.17
11	66.92	Q84T19_HORVD	Endosperm-specific beta-amylase 1.	1.22	1.00
1254	62.41	Q6EEX0_WHEAT	Gamma gliadin.	-1.84	-1.70
701	83.56	Q30DX7_WHEAT	Gamma-gliadin/LMW-glutenin chimera Ch2 (Fragment).	1.00	-1.28
1259	14.63	Q84QI5_WHEAT	Grain softness protein-1 (GSP-1 Grain Softness Protein).	3.17	3.33
242	47.16	Q4JHY1_WHEAT	High molecular weight glutenin subunit 1By15.	-2.30	-2.05
49	45.57	Q6R2V1_WHEAT	High-molecular-weight glutenin subunit 1Dx2.1.	1.00	-1.33
343	70.81	Q7M1M7_WHEAT	High-molecular-weight glutenin.	-1.66	-1.65
29	52.58	Q45R38_WHEAT	HMW glutenin x-type subunit Bx7 precursor.	2.11	1.80

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Table B.3. (continued) 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).

ID	% Cover	Accession_ homologues	Endosperm-related proteins (continued)	Water	ABA
259	58.63	Q8W3X6_WHEAT	Low-molecular-weight glutenin subunit group 1 type I.	4.42	2.88
43	57.43	Q8W3X5_WHEAT	Low-molecular-weight glutenin subunit group 2 type I.	1.99	1.63
1192	51.29	Q8W3X4_WHEAT	Low-molecular-weight glutenin subunit group 3 type II (Fragment).	6.98	2.14
224	55.03	Q8W3V4_WHEAT	Low-molecular-weight glutenin subunit group 8 type IV.	1.49	1.21
501	20.27	Q6J5P4_WHEAT	Puroindoline b.	3.18	3.34
612	33.61	Q6Z5B2_ORYSA	Putative beta-amylase.	-1.28	-1.28
249	35.79	Q9SPM9_WHEAT	Starch synthase IIA.	1.44	1.22
Other					
14	88.99	Q6W8Q2_WHEAT	1-Cys-peroxiredoxine.	-1.92	-1.89
1530	8.04	Q2ULQ8_ASPO	Acyltransferase required for palmitoylation of Hedgehog.	1.00	-1.89
1378	34.86	Q5Z9B0_ORYSA	Adsorption protein-like.	-3.13	-2.94
457	20.40	Q9ZR34_WHEAT	Amylogenin.	1.25	1.23
1298	7.69	Q41540_WHEAT	CM 17 protein.	1.56	1.61
308	62.69	Q96123_WHEAT	Cu/Zn superoxide dismutase.	1.35	1.00
292	23.56	Q7M1Z8_MAIZE	Globulin-2 precursor.	-1.24	1.00
1513	10.43	Q9ATQ8_WHEAT	LRR19 (apoptosis).	-22.98	-27.39
94	70.56	Q96185_WHEAT	Manganese superoxide dismutase (EC 1.15.1.1).	1.30	1.29
487	50.00	REHY_ORYSA	Probable peroxiredoxin (EC 1.11.1.15) (Thioredoxin peroxidase) (RAB24 protein).	-1.29	-1.25
755	15.87	Q8H813_ORYSA	Putative abscisic acid-induced protein-rice.	1.69	1.59
815	13.06	Q6ZBX9_ORYSA	Putative C2 domain-containing protein.	-2.18	-1.91
552	17.61	Q5TKJ2_ORYSA	Putative cellular retinaldehyde-binding/triple function.	-1.31	-1.30
531	20.46	Q75GX9_ORYSA	Putative globulin (With alternative splicing).	-1.45	-1.43
73	38.07	Q9AVA6_ORYSA	Putative selenium binding protein.	1.00	-1.30
878	24.76	Q6K675_ORYSA	Putative streptococcal hemagglutinin.	1.41	1.00
783	10.21	Q84SZ6_ORYSA	Putative vacuolar protein sorting-associated protein.	1.63	1.00
1	79.59	Q7DMU0_WHEAT	Storage protein.	-1.42	-1.43
Unknown function					
1339	34.40	Q5Z4C6_ORYSA	Hypothetical protein B1435D02.9 (Hypothetical protein OSJNBa0042E12.26).	1.39	1.00
745	70.00	Q8LMU0_ORYSA	Hypothetical protein OJ1014H12.11 (Transposon protein, putative, unclassified).	-1.45	1.00
1306	39.57	Q6ZH03_ORYSA	Hypothetical protein OJ1743_B12.7.	1.00	1.39
705	86.87	Q6K2A8_ORYSA	Hypothetical protein OSJNBa0011D16.50 (Hypothetical protein OSJNBa0069P02.4).	-2.10	-2.07

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Table B.3. (continued) 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).

ID	% Cover	Accession_ homologues	Unknown function (continued)	Water	ABA
1273	13.60	Q5W6B4_ORYSA	Hypothetical protein OSJNBa0037H03.17 (Hypothetical protein OJ1653_D06.2).	-1.73	-1.69
990	31.97	Q8S6W8_ORYSA	Hypothetical protein OSJNBa0082N11.24.	1.88	1.81
1386	12.92	Q7Y168_ORYSA	Hypothetical protein OSJNBa0090L05.5.	-2.56	-2.50
958	18.26	Q8S7V2_ORYSA	Hypothetical protein OSJNBa0091P11.25.	-2.43	-2.46
1309	6.89	Q7XZZ2_ORYSA	Hypothetical protein OSJNBa0093M23.12.	-2.09	-1.98
1105	23.20	Q6AVE5_ORYSA	Hypothetical protein OSJNBb0058G04.14.	-2.99	-3.10
323	33.10	Q8LHX6_ORYSA	Hypothetical protein P0022B05.126.	-1.28	-1.25
988	21.33	Q69JE1_ORYSA	Hypothetical protein P0229B10.35 (Hypothetical protein B1274F11.4).	-1.32	-1.32
1439	13.41	Q5Z6B4_ORYSA	Hypothetical protein P0438E12.30 (Hypothetical protein P0410C01.9).	1.66	1.55
1499	32.84	Q5ZDG4_ORYSA	Hypothetical protein P0493G01.17.	1.49	1.00
573	51.56	O24003_HORVU	Hypothetical protein precursor.	-1.96	-1.90
1351	27.19	Q8VY33_MAIZE	Hypothetical protein tac7077.	-1.32	-1.23
1111	20.44	Q2R266_ORYSA	Hypothetical protein.	2.00	2.25
831	18.26	Q53JU2_ORYSA	Hypothetical protein.	1.00	1.65
1060	6.85	Q2QMF8_ORYSA	Hypothetical protein.	-2.61	-2.66
1358	25.31	Q5GWR1_XANOR	Hypothetical protein. Xanthomonas oryzae pv. oryzae.	-5.61	-5.19
686	79.38	Q7X8B9_ORYSA	OSJNBa0014K14.15 protein.	-3.66	-3.22
1209	11.68	Q7XLP6_ORYSA	OSJNBa0044M19.10 protein.	2.33	2.65
1480	15.56	Q7X7I2_ORYSA	OSJNBb0069N01.1 protein (OSJNBa0013A04.20 protein).	1.73	1.72
143	51.39	Q7X6I8_ORYSA	OSJNBb0118P14.11 protein (OJ000315_02.8 protein).	1.00	1.33
817	12.04	Q53K95_ORYSA	Predicted protein.	1.43	1.53
1535	15.57	Q2UAM0_ASPO	Predicted protein.	-2.58	-2.86

Table B.4 Ranked FQS ratios of the various cultivars.

Cultivar	Replicate	Sample FQS (flour:intermediate bran)		
		Unconditioned	Water-conditioned	Water + ABA-conditioned
Chara Baldry	1	1.33	3.92	4.16
	2	1.33	3.93	4.57
	3	1.34	4.04	4.60
	4	1.35	4.73	5.14
Drysedale 05	1	0.97	2.01	2.24
	2	1.01	2.14	2.33
	3	1.04	2.51	2.60
	4	1.18	2.74	2.96
Gregory	1	1.28	3.48	3.50
	2	1.44	3.58	4.35
	3	1.61	3.62	4.78
	4	1.67	4.61	5.46
H45 M6649 2006	1	2.04	5.23	7.12
	2	2.05	6.73	7.48
	3	2.44	6.86	8.65
	4	2.47	10.69	10.64
Kenedy 05	1	0.89	2.50	3.03
	2	1.00	2.62	3.25
	3	1.03	2.68	3.52
	4	1.05	3.38	3.73
Ventura Baldry	1	1.32	3.08	3.42
	2	1.44	3.36	3.78
	3	1.48	3.36	4.01
	4	1.58	3.61	4.59
Strzelecki	1	1.09	3.66	3.76
	2	1.13	3.78	3.86
	3	1.21	3.91	4.11
	4	1.27	4.01	4.99
Whylah	1	1.07	3.60	3.35
	2	1.11	3.99	3.42
	3	1.13	4.00	3.47
	4	1.21	4.47	4.36

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).

Ranked replicates	Water-conditioned	4ppm ABA-conditioned
1	2.29	2.23
2	3.17	2.28
3	3.31	2.53
4	3.41	2.87
5	3.93	3.12
6	3.97	3.16
7	4.07	3.36
8	4.08	3.99
9	4.90	5.55
10	5.29	5.68

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).

Ranked replicates	Water-conditioned	4ppm ABA-conditioned
1	1.10	0.81
2	1.54	1.22
3	1.74	1.29
4	1.88	1.36
5	1.97	1.41
6	2.18	1.45
7	2.23	1.50
8	2.28	1.62
9	3.00	1.67
10	3.58	2.42

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).

Ranked replicates	Water-conditioned	4ppm ABA-conditioned
1	1.1673	-0.9163
2	2.0367	1.2907
3	4.0407	1.3453
4	4.1753	1.3474
5	4.3062	1.359
6	5.3638	1.7577
7	5.4869	2.0416
8	5.6519	2.1367
9	7.1729	2.9645
10	7.2387	3.5202

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).

Ranked replicates	Water-conditioned	4ppm ABA-conditioned
1	10.85	12.15
2	10.88	12.64
3	11.78	12.66
4	12.18	13.58
5	12.49	13.61
6	13.40	14.15
7	13.57	14.25
8	13.99	14.27
9	14.87	14.81
10	15.69	15.34

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).

Ranked replicates	Water-conditioned	4ppm ABA-conditioned
1	9.58	12.16
2	10.97	12.50
3	11.52	13.78
4	11.85	14.00
5	12.14	14.28
6	13.82	14.71
7	14.26	14.87
8	14.98	14.94
9	15.33	15.34
10	15.92	16.27

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).

Ranked replicates	Water-conditioned	4ppm ABA-conditioned
1	9.50	11.62
2	10.68	12.53
3	11.31	13.61
4	11.40	13.67
5	11.68	13.79
6	12.08	14.01
7	12.32	14.26
8	12.58	14.54
9	14.16	14.71
10	15.33	14.98

Appendix C. Publication