Morphological features of wheat grain and genotype affecting flour yield

Mark Andrew Edwards

Southern Cross University

Publication details
Copyright M Edwards 2010
Morphological Features of Wheat Grain and Genotype Affecting Flour Yield

Mark Andrew Edwards BSc (Hons)
Southern Cross Plant Science
and BRI Research

Thesis submitted for the fulfilment of the requirements for the Doctorate of Philosophy at Southern Cross University
Lismore, Australia
August 2010
### Table of Contents

**DECLARATION** ....................................................................................................................... V

**ABSTRACT** ................................................................................................................................ VI

**PUBLISHED CHAPTERS** ............................................................................................................. VII

**ACKNOWLEDGEMENTS** ........................................................................................................... VIII

**INTRODUCTION** ......................................................................................................................... 1

Research Aims ................................................................................................................................. 2

**CHAPTER 1. WHEAT: SIGNIFICANCE, PROCESSING AND QUALITY** ............ 3

1.1 Significance of Wheat ................................................................................................................. 3
  1.1.1 Historical Usage ...................................................................................................................... 3
  1.1.1.1 Domestication and Genetics ............................................................................................... 4
  1.1.2 Wheat Consumption and Trade ............................................................................................. 4
  1.1.3 Wheat Types .......................................................................................................................... 6

1.2 Wheat Milling ............................................................................................................................. 8
  1.2.1 First Break Rolls .................................................................................................................... 9
  1.2.2 Endosperm Fracture ............................................................................................................. 11
  1.2.2.1 Other Milling Stages ....................................................................................................... 12
  1.2.3 Conditioning of Wheat ......................................................................................................... 14
  1.2.3.1 Water Absorption Process by Wheat Caryopsis ................................................................ 15
  1.2.4 Commercial Milling Practice: Grist and Flours ................................................................... 16
  1.2.4.1 Flour Types ..................................................................................................................... 17
  1.2.4.2 Dough and Bread Structure ............................................................................................ 19

1.3 Grain Quality and Improvement ............................................................................................... 20
  1.3.1 Measurements Predicting Milling Quality ........................................................................... 20
  1.3.1.1 Weight ............................................................................................................................. 20
  1.3.1.2 Hardness .......................................................................................................................... 20
  1.3.1.3 Protein .............................................................................................................................. 22
  1.3.1.4 Alternative predictive Measurements for Flour Yield ..................................................... 22
  1.3.2 Classification of Wheat Varieties ....................................................................................... 23
  1.3.2.1 Regional Influence on Characterisation ........................................................................... 24
  1.3.2.2 Wheat Growers Choice of Varieties ............................................................................... 25
  1.3.3 Wheat Improvement ............................................................................................................. 25
  1.3.3.1 Grain Yield and Weight: Contributing Factors ................................................................. 25
  1.3.4 Wheat Breeding .................................................................................................................... 27
  1.3.4.1 Historical Background ...................................................................................................... 27
  1.3.4.2 Breeding for Grain Yield .................................................................................................. 28
  1.3.4.3 History of Kernel Weight Gain ....................................................................................... 30
  1.3.4.4 Modern Breeding Methods ............................................................................................... 31
  1.3.5 QTL Analyses ....................................................................................................................... 33
CHAPTER 4. INVESTIGATION OF THE EFFECT OF CONDITIONING ON THE FRACTURE OF HARD AND SOFT WHEAT GRAIN BY THE SINGLE-KERNEL CHARACTERIZATION SYSTEM: A COMPARISON WITH ROLLER MILLING .................................................. 106

4.1 Introduction ................................................................................................................. 106

4.2 Experimental .............................................................................................................. 108
  4.2.1 Samples ................................................................................................................ 108
  4.2.2 SKCS analysis ........................................................................................................ 108
  4.2.3 Experimental milling ............................................................................................ 109
  4.2.4 Particle size distribution analysis ......................................................................... 109
  4.2.5 Microscopy ........................................................................................................... 110

4.3 Results ....................................................................................................................... 110
  4.3.1 Conditioned versus Unconditioned Wheat ............................................................ 110
  4.3.2 Comparison of SKCS and first break roller milling ............................................. 110

4.4 Discussion .................................................................................................................. 115
  4.4.1 Crush-Response Profile (CRP) ............................................................................. 115
  4.4.2 CRPs of Conditioned versus Unconditioned Grain .............................................. 115
  4.4.3 SKCS modes of fracturing ................................................................................... 120
  4.4.4 First break roll modes of fracturing .................................................................... 121

4.5 Conclusions ............................................................................................................... 124

CHAPTER 5. EFFECT OF ENDOSPERM STARCH GRANULE SIZE DISTRIBUTION ON MILLING YIELD IN HARD WHEAT .................................................. 124

5.1 Introduction ............................................................................................................... 124

5.2 Materials and Methods ............................................................................................. 128
  5.2.1 Samples ................................................................................................................ 128
  5.2.2 Single Kernel Characterization System (SKCS) Analysis .................................... 128
  5.2.3 Imaging of grouped wheat samples using ESEM .............................................. 128
  5.2.4 Test Milling ........................................................................................................... 129
  5.2.5 Starch Extraction ................................................................................................ 129
  5.2.6 Laser diffraction analysis .................................................................................... 130
  5.2.7 Fluorescent staining of Spherosomes ................................................................ 130
Declaration

I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University's rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

Mark Edwards
Abstract

Although the wheat grain comprises 82 - 86% starchy endosperm, only approximately 76 - 78% is able to be separated using current milling technology. Suitability for primary processing depends on three main characteristics that are in turn influenced to varying degrees by the genetic origin of wheat and agro-climatic conditions during plant development: the endosperm to bran ratio, kernel hardness and ease of separation of bran and endosperm. Among these, hardness appears to be an essential factor in wheat milling behavior also important to the food processing industries. Accumulated evidence supports the prominent influence of the puroindoline (pin) proteins on grain hardness though additional factors influence milling quality.

The general aim of this project was to increase the understanding of grain characteristics in hard wheat varieties that influence flour yield. The first research question involved the validation of the rheological information derived from the Single Kernel Characterisation System (SKCS) as the most suitable indicator of milling quality for small sample quantities. The methodologies initially involved correlation of fragment/particle size distribution of conditioned and unconditioned hard wheat varieties produced by first break rolls and the SKCS. The use of the SKCS for studies in milling quality was validated (Chapter 4).

This was followed by a process relating qualitative observations gained from light microscopy, conventional and environmental electron microscopy with the crush response profiles produced by the SKCS. This study generated an hypothesis involving starch granule size distribution (SGSD) which was tested by correlating quantitative data produced by a laser diffraction technique with small scale milling and SKCS data. SGSD has generally been disregarded as having a significant influence on hardness or milling quality with much investigative effort instead directed towards the puroindolines. However the results presented indicate the contrary with significant correlations with hardness and flour yield across a range of hard varieties. This is partially a reflection of positive relationships between bonding area and cohesion within composite materials (Chapter 5).
Finally, standard DNA sequencing methods were used to screen puroindoline sequence variation in 120 varieties from the Australian Winter Cereal Collection harvested and milled from two different environments. Further SGSD analysis indicated significant correlations with flour yield though differing between pin genotypes. In addition quantitative analysis of starch granule-bound puroindoline using a micro-capillary electrophoresis method (Lab-on-chip) demonstrated a significant negative correlation with flour yield in the premium milling quality varieties with pin genotype, Pin-D1a, Pinb-D1b (Chapter 6). Several alternative influences on milling quality such as lipid content, cell wall characteristics, the regulation of the hardness locus (Ha) and function of the grain softness proteins require further investigation. Also as expected results suggested that seed weight itself has a significant influence on flour yield.

**Published Chapters**

**Chapter 4.**

**Chapter 5.**

**Chapter 6.**
Acknowledgements

Mark Edwards was the recipient of an Australian Post Graduate Award and scholarship from the Grain Foods CRC Ltd. The author wishes to thank Supervisors: Prof. Robert Henry (Centre of Plant Conservation and Genetics, Southern Cross University, Lismore, NSW) and Adjunct Prof. Brian Osborne (BRI Australia Pty Ltd, North Ryde, NSW) for their enduring support. The author wishes also to thank Dr. Mike Southan and Dr Daniel Skylas (BRI Australia Pty Ltd) for advice and carrying out the test milling, Dr Robert Sleigh (Food Science Australia) for assistance with laser diffraction systems and Katie McBean from the Microstructural Analysis Unit (University of Technology, Sydney) for assistance in the use of the ESEM and Maxine Dawes for assistance with the SEM (Southern Cross University (SCU)). The authors also thank Dr Linh Nguyen and Adam Benson from the Centre of Plant Conservation Genetics (SCU) for assistance with Puroindoline genotype analysis. Also, thanks to Dr Paola Tosi (Rothamsted Research, UK) for supplying standards of purified puroindoline A and B. In addition I would like to acknowledge journal reviewers and editors whose comments have assisted the writing of papers.
Abbreviations:

CRP        Crush-Response Profile
ESEM      Environmental Scanning Electron Microscopy
HI         Hardness Index
IA         Image Analysis
LDS        Laser Diffraction Sizing
QTL        Quantitative Trait Loci
SGSD       Starch Granule Size Distribution
SKCS       Single-Kernel Characterization System
WRI        Wheat Rheology Index
SGSD       Starch granule size distribution
WCCT       Winter Cereals Collection (Tamworth)
WRI        Wheat Rheology Index
SR         Shell response
EQR        Equilibrium response
TER        Time of the equilibrium response
TEC        Time of the endosperm collapse
MS         Milliseconds

Introduction

Apart from general subject background, the literature review has focused on three interrelated themes: 1) the wheat milling process, 2) milling quality determinants and enhancement, and 3) the morphology of the grain relative to fractionation. The wheat kernel contains typically 85% endosperm, so a perfectly efficient mill could in theory extract 85% pure white flour. However, in practice, as the maximum theoretical extraction rate is approached, bran contamination increases rapidly, so commercial mills tend to operate at extraction rates in the range 70-80% (Campbell et al., 2007a). Improving milling yields depends on increased understanding of grain characteristics. Also new milling objectives are aimed at dividing the plant material into its different components for use in chemical synthesis, biosynthesis or bioconversion to produce substances with higher added value. Knowledge of the morphology of cereal grains obtained from light, scanning and transmission electron microscopy has contributed
substantially to our understanding of structure–function relationships relevant to processing. Spectroscopic methods also are being used extensively for the analysis of cereal grains. In destructive biochemical analyses using bulk samples, the information about the spatial origin and distribution of the components of interest is lost. The ability to combine topographical and in situ chemical analysis of individual cereal grains using spectroscopic techniques, without recourse to fractionation, offers further opportunities to determine the distribution of structurally significant components (Mills et al., 2005).

**Research Aims**

The major aim of this study is to link spatial, compositional information with rheological properties as determined by the Single Kernel Characterization System (SKCS) and small scale milling methods, which will influence grain fracture and component separation during milling. In this way it will be possible to develop a more precise biophysical description of the grain particularly in relation to processing qualities. In the longer term, these results may help to identify the genetic origin of the quality traits desired by industry (Toole et al., 2005). Consequently the comparative study of a large number of wheat cultivars is required in order to be able to draw conclusions on possible correlations between grain component interactions and milling characteristics such as fracture propagation. Concerning structural properties, suitability for primary processing depends on three main characteristics that are in turn influenced to varying degrees by the genetic origin of wheat and agro-climatic conditions during plant development: the endosperm to bran ratio that should be as high as possible, kernel hardness and ease of separation of bran and endosperm. Among these, hardness is considered to be an essential factor in wheat milling behaviour (Haddad et al., 1999). Generally wheat breeders strive to develop wheat varieties of consistently uniform quality (Wrigley, 2002), the most pressing requirement of bakers. However the problem for millers is that, despite the best efforts of breeders and growers, wheat entering the mill is inherently variable (Pyler, 1958; Whitworth, 1999). Consequently millers are said to ‘mill wheat rather than varieties.’
Chapter 1. Wheat : Significance, Processing and Quality

1.1 Significance of Wheat

1.1.1 Historical Usage

Wheat, maize, and rice dominate world grain production. These grasses (family Poaceae, syn. Gramineae) are grown primarily for their grain (or caryopses). The importance of cereals appears easy to explain: relative to other grain crops, yields are both high and stable; compared to root and tuber crops, cereal grain is easier to grow, transport, and store (Evans, 1993; Gooding, 2009). Wheat is among the oldest and most extensively grown of all grain crops. Western agriculture is thought to have started around 10,000 BC somewhere along the Fertile Crescent in the Near East (Hillman & Davies, 1990; Araus et al., 2007). The cradle of wheat agricultural innovation was considered to be a small region of south-east Turkey and north-east Syria around the Middle Euphrates (average coordinates 37°00' N, 38°60' E) (Gopher et al., 2002; Salamini et al., 2002). However more recent archaeo-botanical studies support the model in which domestication occurred independently in several sites across the Levant. According to this view, the genes for non-brittleness were transferred to numerous wild emmer genotypes. Consequently, domesticated tetraploid wheat evolved as polymorphic populations rather than single genotypes (Feldman & Kislev, 2007; Brown et al., 2009). The nature of the crops first adopted, along with the selection pressure that was a result of cultural practices, are factors that are intimately related to water. Mediterranean savannahs and steppes of the Near East, have favoured plants with large annual seeds, able to survive for long dry periods and germinate when rains occur (Harlan, 1992). Most of the early domesticates appeared to be herbaceous annuals capable of selfing (Hancock, 2003), and the starchy cereals, complemented with high-protein legumes, were among the first plants cultivated. However, there is evidence that cultivation greatly preceded domestication (Moore et al., 2000; Tanno & Willcox, 2006).

The availability of wheat and other cereal grains are thought to be a major reason for the transition from the hunter-gatherer nomad to the settled agriculturalist. Solving the problems of cultivating cereal grains, developing crude methods for grinding, and processing the ground meal into useful foods led to the beginnings of civilization. In the Mediterranean region, centuries before recorded history, barley was
grown more extensively than wheat (Weaver, 1950; Takahashi, 1955). Later wheat played a dominant role in the Roman Empire. The word, *cereal* derives from Ceres, the Roman goddess associated with agriculture. Subsequent migrations from Europe caused wheat to be partly replaced by rye. Throughout the middle Ages, barley and rye were used as animal feed, whereas almost all wheat was used for human food. Likewise, wheat dominated domestic and international trade almost to the exclusion of all other cereals (Wrigley, 2009).

### 1.1.1.1 Domestication and Genetics

Domestication is thought to be present when archaeological plant remains show, most importantly among other traits, substantial increases in kernel weight (Salamini *et al.*, 2002; Willcox, 2004). Kernel weight is one of the three main agronomic components of grain yield in cereals and has direct implications for grain quality (Rharrabti *et al.*, 2003). Also commonly associated with the domestication process is an increase in local adaptation (Hancock, 2003). The free-threshings of tetraploid durum wheat and hexaploid bread wheat (*T. aestivum* L.) represent the final steps of wheat domestication (Salamini *et al.*, 2002; Araus *et al.*, 2007). Molecular marker-based studies of crop domestication make it possible to identify agronomically useful genes in wild relatives allowing introduction of these genes into the cultivated gene pool (Septiningsih *et al.*, 2003). These studies also identify genes involved in the domestication process or in subsequent selection events (Wright *et al.*, 2005). Assuming that the sample of dicoccoides accurately reflects the diversity of the wild progenitor of cultivated wheat 12,000 years ago, initial diversity was reduced by 69% in *aestivum* and 84% in *durum*. The loss of nucleotide diversity (total and silent) found during domestication is one of the largest reported so far for a crop species. Most crops have nucleotide diversities about 30% lower than that of their wild progenitor. Wright *et al.* (2005) estimated that 2-4% of maize genes were subject to selection during maize domestication.

### 1.1.2 Wheat Consumption and Trade

Written references to bread date back to about 2600 B.C.E. Possibly this is due to its desirable and unique, dough forming properties which are utilised for a wide range of products. Among these are pan bread, noodles, cakes, biscuits/cookies,
steamed bread, doughnuts, croissants, bagels, pizza, flat breads, and chapatti. Each of these products is ideally produced from wheat selected to provide flour with the required characteristics. Wheat is recognized as an important source of essential nutrients, providing energy, fibre, carbohydrate, protein, B vitamins, iron, calcium, phosphorus, zinc, potassium, and magnesium. The fractionation of the crushed grain during milling has critical implications for the distribution of many nutrients. The wholemeal product therefore provides better nutrition than does white flour. Lysine is the first limiting amino acid of wheat protein. Consequently cereal diets are traditionally supplemented with small quantities of legumes, nuts, fish, or milk. Cereal grains are now regarded as essential components of a healthy diet due to the suggested nutritional benefits of low-fat, high-fibre diets that is conferred by grains, including wheat-based foods (Painter et al., 2002; Wrigley, 2009).

Currently, bread in one form or another has become an important food for the Western world. Wheat bread in developing countries, has become preferred over rice, sorghum, and millet-based foods (Wrigley, 2009). Wheat provides between a fifth and a quarter of the energy and protein in the worldwide human diet. Two thirds of the crop is used directly in products for human consumption and much of the remaining third is used to feed livestock. The world production (millions of tonnes) of the main grain species in 2005 was as follows: corn, maize - 692; common (bread) and durum wheat - 626; rice and wild rice - 615 (as paddy); barley - 138; soybean - 210; sorghum - 57 (Source of production statistics: FAOSTAT data (2006), accessed via the website at www.fao.org.). Perhaps a better indicator would be production volumes (millions of tonnes) over a decade (1993-2002) for the top seven countries are: China (93.9), the European Union (91.7), India (68.8), the United States (53.3), the Russian Federation (46.9), Canada (20.6), and Australia (19.4) (Worden, 2004). Nearly half of the global wheat area is in developing countries. More than 80% of wheat produced is consumed within the source country, mainly as human food. The 80% of traded wheat that is produced in developed countries comes from the United States (28% market share, 1993-2002), Canada (16%), the European Union (EU) (15%), Australia (14%), and Argentina (8%) (Worden, 2004; Carson & Edwards, 2009; Wrigley, 2009).

The major factors used to distinguish wheats in trade are the hardness or softness of the grain, winter or spring habit, red or white bran colour, and protein
content (Cracknell & Williams, 2004). Within the resulting classes, wheat is further described according to bushel weight or test weight (a measure of bulk density), cleanliness (i.e., absence of contamination with foreign materials, including other cereal grains and weed seeds) and the level of screenings (i.e., small foreign seeds and broken or shrivelled wheat kernels), the degree of soundness (i.e., absence of sprouted grain), moisture content, and dough-quality attributes that determine suitability for end-product processing. These properties are influenced by a combination of genetic and environmental factors (genotype [G] and environment [E] and the interaction G x E). Some significant contributors to aspects of quality, such as protein content, are primarily the result of growth conditions: soil fertility, rainfall, and temperature during the growing season and at harvest. Generally, wheats can be closely matched to many different end uses according to their grain hardness and protein content (Wrigley, 2009).

1.1.3 Wheat Types

The genus name for wheat, *Triticum*, comes from the Latin, tero (I thresh). The current binomial name, *Triticum aestivum*, refers to hexaploid bread wheat (genomes A, B, and D), distinguishing it from tetraploid macaroni wheat (*Triticum durum*) (genomes A and B), which is used primarily for pasta production. Tetraploid forms of current domesticated wheats are derived from a wild tetraploid progenitor, identified as the wild emmer *Triticum turgidum* ssp. *dicoccoides* (referred to as *dicoccoides*). This species has an allotetraploid genome (AABB) resulting from spontaneous amphidiploidization between the diploid wild wheat *Triticum urartu* (AA genome, (Dvorak et al., 1998) and an unidentified diploid *Aegilops* species (BB genome), the closest current relative of which is *Ae. speltoides* (Khlestkina & Salina, 2001). Bread wheat (*Triticum aestivum*), the most widely cultivated wheat today, is a hexaploid form of free-threshing wheat (genome AABBDD). It is thought to have resulted from recent hybridization (no more than 8,000 years ago, according to Nesbitt and Samuel [1998]) between an allotetraploid wheat (AABB) and the diploid (DD) *Aegilops tauschii* var. *strangulata* (Dvorak et al., 1998; Nesbitt & Samuel, 1998; Haudry et al., 2007).

*T. aestivum* and *T. durum* have seven pairs of chromosomes (2n =14). Wheat is grown as a winter or spring crop. In severely cold regions, spring-wheat types are sown in spring, to develop and mature quickly for harvest before the onset of autumn.
snows. In more moderately cold regions, wheats of winter habit are sown before the arrival of winter snows, which overlay the seedlings, causing them to vernalize and permit prompt development as soon as the snow melts in the spring. In warmer climates, the distinction between spring and winter wheats is almost meaningless; the more important distinction is the maturity-late or early (Wrigley, 2009). Wheat varieties are often distinguished on the basis of seed coat colour, endosperm texture, dough strength, and sowing season. These are briefly described below (Gooding, 2009).

**Red and White Wheats:** Red varieties generally exhibit more dormancy than white varieties and so are favoured in climates conducive to preharvest sprouting. White wheats are more suited to areas that are dry during ripening and harvest and are favoured for the manufacture of certain types of flat bread and noodles.

**Hard and Soft Wheats:** The hardness of a variety or seed relates to the resistance encountered when it is milled. This important quality affecting milling performance is extensively discussed in following sections.

**Strong and Weak Wheats:** Leavened bread production is largely limited to the genomes coding for the proteins necessary to generate an elastic, strong dough suitable for the capture of gas bubbles during fermentation, thus allowing the dough to rise. The unique elastic properties of doughs made from wheat flours are largely the result of the type and amount of gluten present. The gluten contains proteins insoluble in water and alcohol, i.e., the prolamin storage proteins. These prolamins are divided into the monomeric gliadins and the polymeric glutenins (Shewry *et al.*, 1986). Varieties with a high gliadin-glutenin ratio tend to have viscous, extensible doughs that are often suitable for cookie (biscuit) making. Those having a low gliadin-glutenin ratio have more elasticity and strength, which are desired for breadmaking. Allelic variation in the high molecular weight (HMW) glutenins is closely linked with breadmaking quality and dough resistance. Varieties of bread wheat contain between three and five major HMW glutenin subunits. Two of these are coded by genes at *Glu-D1*, one or two by *Glu-B1*, and one or none by *Glu-A1* (Payne, 1987). More than 50% of the variation in baking potential and/or dough rheology within wheat collections of several countries has been explained on the basis of HMW glutenin subunit composition (see also topic below: Chapter 1, Flour Types).

**Winter and Spring Wheats:** Varieties differ in their requirement for a cold period to hasten, or permit, normal development toward reproductive development. This need
for vernalization (literally, "making ready for spring") is strongly affected by variation at the Vrn-1 loci located on each of the long arms of the group 5 chromosomes (i.e., Vrn-A1, Vrn-B1, and Vrn-D1) and their apparent regulation by minor vernalization genes (Loukoianov et al., 2005). A dominant Vrn-la allele on any of the three wheat genomes results in a spring habit, and the presence of recessive Vrn-Ib alleles on all three genomes results in a winter habit. However, Vrn-1 genes are closely linked to, and also interact with, other genes conferring cold tolerance (Reddy et al., 2006) and, therefore, survival over winter (Gooding, 2009). The rate of wheat development depends largely on variety, temperature, the need for a cold period (vernalization), and day length (photoperiod). As already described, maturation of winter wheat varieties is hastened following vernalization, i.e., exposure to low temperatures, typically 3-10°C, for six to eight weeks. Development can also be accelerated by exposure to long days; i.e., photoperiod-sensitive varieties are quantitative long day plants, although short days can sometimes substitute for vernalization. Because varieties vary in their response to temperature, vernalization, and photoperiod; in the extent to which these factors interact; and in relative sensitivity to them at different growth stages, varieties vary, apparently continuously, in their rates of maturation, thus contributing to the wide adaptation and distribution of wheat in world agriculture (Carson & Edwards, 2009; Gooding, 2009).

1.2 Wheat Milling
The aim of wheat milling is to isolate the endosperm without contamination by the outer parts of the grain and the germ. A specific characteristic of wheat milling results from the presence of a crease in the kernel. The crease presents the greatest difficulty for separation of starchy endosperm from other tissues. Investigation into the effect of crease shape on conventional milling performance showed that wheat grains with a closed crease tended to require a greater force to be applied for failure to occur than those with more open creases. However, the crack in the former case tended to be across the cheeks of the endosperm, causing more damage and, therefore, a high release of endosperm at first break. Conversely grains with a more open crease showed cracks occurring along the crease which was thought to be preferable as this would cause the bran to remain as larger pieces (Evers & Millar, 2002).
Campbell et al. (2007a) in an evaluation of fundamental parameters influencing milling performance made the following comments: Flour millers produce mainly for bakers, whose principal requirement is for a flour of consistent quality. Maintaining uniformity of flour quality was described by Scott (1951) as the miller's ‘golden rule’. The major tool employed by millers to deliver consistent flour quality is gristing, the correct blending of wheats (Scott, 1951; Pyler, 1973; Morris, 1992; Jones, 2001). The final flour is produced by blending together the flour produced at each milling and sifting stage, but those daughter flours have different histories and therefore different properties. These routes through the mill depend on the initial breakage characteristics of the grist (Campbell et al., 2007a). At the other end of the supply chain, breeders seek to develop wheat varieties of consistently uniform quality (Wrigley, 2002), thereby adding their contribution to the bakers' most pressing requirement. The problem for millers is that, despite the best efforts of breeders and growers, wheat entering the mill is inherently variable (Pyler, 1958; Whitworth, 1999). Kernels from the same wheat stalk vary as a result of their position on the spike during growth, and there will be variation even across a single field. More significantly, a given variety will be grown in different locations, under different agronomic practices and experiencing different weather conditions, and many varieties are grown each year (Campbell et al., 2007a).

1.2.1 First Break Rolls

Wheat millers first open the grain and then remove the endosperm from the bran stepwise, moving approximately from the inside towards the outside (refer section 2.2. Wheat Grain Structure, Figure 4). The milling process thus involves three operations: (i) the breaking and the dissociation of grains by roller mills such as break rolls or scratch rolls which produce a crushing as well as a shearing action; (ii) the size classification of the product by the plansifter to extract flour in the bread wheat milling process; (iii) the air classification of the product in a purifier to obtain pure semolina (coarse particles) in durum wheat milling (Haddad et al., 1999). During milling, starch damage involves molecular disruption of amylopectin and has the effect of increasing the capacity of starchy milled products to absorb water, a characteristic that is valued in flour destined for plain bread manufacture (see topic below: Chapter 2, Starch Damage). Soft wheat mills to give flours with relatively small irregular shaped
particles which do not flow easily and tend to block sieves in the mill, and are generally used for cake and biscuit production because of their fine particle size and low starch damage (Evers & Millar, 2002).

In modern flour milling, conditioned (pre-moistened) wheat kernels are broken open using first-break roller mills, comprising pairs of counter-rotating fluted rolls (Figure 1) having an asymmetric saw tooth profile and operating under a speed differential of up to 2.7 : 1 with a small gap between the rolls (Campbell et al., 2007a). The fluted rolls open up the wheat kernel such that the bran particles tend to remain large while the endosperm shatters into small particles, facilitating the separation of endosperm from bran by sifting. The endosperm material that is still too large to be considered as flour is then sent to the reduction system. The reduction system serves to reduce the size of these endosperm particles, again producing some flour at each stage. Reduction rolls are smooth and operate under pressure, which causes damage to starch granules and thereby affects the water absorption properties of the flour (see topic below: Chapter 2, Starch Damage). Managing the degree of starch damage in the composite flour is therefore one of tasks of the miller. First Break is therefore a critical control point in milling (Hsieh et al., 1980a).

**Figure 1.** Diagram of roll differential, fluting disposition and interaction. Adapted from Campbell et al. (2001)

First-break roller milling produces a wide range of particles from <200 μm to >2,000 μm. The two fluted break rolls have either, Sharp-to-Sharp (S - S) milling disposition, Sharp to - Dull, Dull - to - Sharp or Dull - to - Dull (D - D) dispositions (Figure 1). Campbell et al (2007a) developed models based on the breakage equation for roller milling to predict the output particle size distribution delivered by First Break roller milling of wheat based solely on Perten Single Kernel Characterisation System (SKCS) characteristics. Dull-to-Dull milling gives more of a crushing action, which encourages shattering of the brittle endosperm but leaves the bran layers
relatively intact, while the shearing action of Sharp-to-Sharp milling cuts through both
the bran and the endosperm material, slicing the kernel into smaller particles but not
shattering it to the same extent as D-D. These researchers observed that wheat
hardness and roll disposition appear to produce similar effects, such that a soft wheat
under D-D gives a pronounced U-shape (ie., predominantly large and small fragments
without intermediate sizes), while at the other extreme, a hard wheat under S-S gives a
distinct peak or an inverted U. This indicates that D-D milling is more sensitive to
wheat hardness than S-S. As millers tend to operate under D-D (because the U-shaped
distribution is readily separated into larger branny particle and smaller endosperm
particles), the greater sensitivity of D-D milling to kernel hardness and size is
commercially significant, as variations in the feedstock will have greater influence on
downstream operations. Campbell et al. (2007a) plotted the percentage of particles
smaller than a given aperture size used in a sieve analysis, versus average kernel hard-
ness (SKCS), for different roll dispositions and roll gaps. The consistent linear trends
imply that the effect of hardness on breakage is qualitatively similar for wheats of
varying hardness; that is, there are no sudden discontinuities that would indicate that
hard and soft wheats are different in their essential nature or exhibit fundamentally
different mechanisms of breakage (Campbell et al., 2007a; Anderssen & Haraszi,
2009).

In first-break roller milling of wheat, the factors affecting breakage of wheat
grains can thus be broadly classified into those arising from the physicochemical
properties of the wheat (size distribution, moisture content, hardness) and those related
to the design and operation of the milling equipment (Campbell et al., 2001a). The
hardness of the wheat is the most important factor affecting milling performance
(Pomeranz & Williams, 1990). Wheat hardness also affects the breakage patterns of
bran. During milling, shear forces are redirected through the endosperm of hard wheats
to the bran and thus cause the bran to break, while in soft wheat, the shear forces are
not redirected in this way and bran is less broken (Pomeranz & Williams, 1990). Some
researchers consider the thickness of the bran layers to be less influential in
determining the milling behaviour of wheat (Larkin et al., 1951).

1.2.2 Endosperm Fracture

A particle will break along the plane where its principal stress or maximum
shear stress exceeds its compressive, tensile, or shear strength depending on which
reaches the critical stress first. According to Glenn and co-workers (1990, 1991), the endosperm is approximately one order of magnitude stronger under compression than tension for the same wheat sample; therefore the kernel is most likely to be broken initially by tensile stress (Glenn & Saunders, 1990; Glenn et al., 1991). Deformation rate also affects the mechanical properties of materials in compression tests. Shpolyanskaya (1952) found that wheat endosperm loaded at higher rates was more brittle and produced courser fragments than grains loaded at lower rates. The deformation rate significantly affects $S_{\text{max}}$ (maximum stress) values for wheat endosperm. Considering other forces active during milling, results reported by Hsieh et al (1980a) indicate that the effect of rolls speed on kernel breakage is very small compared with roll gap and differential. In their study, the differential was set at 2.7 and the roll gap was 0.6 mm. Consort (a soft wheat from the 1999 UK harvest, 12.7% protein, dry basis (db); 79.4 kg/hL weight, conditioned overnight to 16 percent moisture content), was used for the observations. The combination of compressive stress applied in the horizontal direction and shear stress applied in the vertical direction means that the wheat kernel is most likely to break along the plane corresponding to the principal tensile stress, because of the weak tensile strength of endosperm compared with its compressor strength (Fang and Campbell 2002).

The particle size distribution resulting from first-break roller milling directly affects the subsequent system arrangement and machine settings, and thus determines the effectiveness of the milling process (Fang & Campbell, 2002b). Identifying the genetic influence on this critical factor is an underlying aim of our current studies. However from the perspective of commercial milling practice, mixtures of wheat cultivars are processed in flour mills rather than single cultivars of uniform physical characteristics, performed with very little adjustment of mill rolls to adapt to specific characteristics of grain to be milled. In addition apart from the initial break process involving intact wheat kernels, it could be considered that the stresses and moisture contents are almost identical for all wheat cultivars in subsequent milling phases (Mabille et al., 2001; Kweon et al., 2009).

1.2.2.1 Other Milling Stages
After first break stage, subsequent processes, typically four or more break passages, grading, purification, and eight or more reduction passages, mill and separate the endosperm and bran further (Figure 2)(Fang & Campbell, 2002a).
The production ratio between break and reduction flour may vary substantially according to the wheat hardness and according to agronomic conditions. The milling of soft wheat gives approximately the same percentage of break flour and reduction flour whereas with hard wheat break flour forms only about a quarter of the reduction flours yield. In fact, harder wheat tends to grind down to coarser particles referred to as semolina whereas soft varieties give flour particles directly (Haddad et al., 1999). The series of individual break and reduction operations in the milling process gives rise to as many as 150 different product streams in a modern flour mill (see flour extraction points in Figure 2).

**Figure 2.** Diagram of typical setup of processing stages in wheat milling with four break rolls (adapted from (Campbell et al., 2007a))

In general, there is a trend for gradual increase for starch damage, ash content, and total protein content in streams from later break and reduction stages (Sutton & Simmons, 2006). Flour streams are combined to yield the final product. When all the streams are combined, the result is called "straight" flour (see Chapters 3 and 4:
Methods). Frequently, the more refined streams are kept separate and sold at a premium as patent flours, while the remaining lesser streams yield so-called "clear" flours. The chemical composition of the flour depends upon the characteristics of the wheat and the extraction rate (Pyler, 1988; Bilheux et al., 1989).

1.2.3 Conditioning of Wheat

Conditioning (tempering) is a routine procedure that enhances the efficiency of flour extraction. In a majority of cases, conditioning is carried out by the addition of water to wheat in a worm-screw, followed by a time interval before milling. Its purpose is essentially to toughen the pericarp to enhance the separation of the endosperm. Fang and Campbell (2003) noted five purposes of conditioning as: (i) to toughen the bran, reducing formation of bran powder; (ii) to soften the endosperm, enhancing its millability and reducing the power consumed by the reduction rolls; (iii) to facilitate separation of bran from endosperm, reducing the power consumption of the break rolls and consequently reducing evaporative losses; (iv) to ensure easy and accurate sifting of stocks; and (v) to ensure the endosperm moisture content is sufficient to give a final flour moisture content of around 14-15%. This report also made an important observation that the amount of water added and the timescale over which it is allowed to penetrate into the kernel vary widely in practice, with no conditioning regime universally appropriate for all wheat types and milling systems. Typically soft wheats are conditioned to 15-15.5% moisture and hard wheats to 16-16.5% (Fang & Campbell, 2003). The moisture content that is optimal for milling represents a compromise between maintaining partial dryness for ease of sifting and sufficient moisture to soften the endosperm and toughen the bran that becomes more compliant and resilient with increasing moisture content. That is, it loses strength and stiffness but increases in elasticity and plasticity (Glenn et al., 1991).

Conditioning parameters of wheat grain also change the quality of the flour, yet most experimental milling systems use a standard conditioning without optimization. In a recent study by Kweon et al. (2009), the effect of conditioning parameters on the milling performance and flour functionality for soft red winter (SRW) wheat grain was tested. Flour yield was more reduced for all samples conditioned at 15% moisture than for samples conditioned to 12% moisture. Whereas flour quality of the 15% conditioned sample was better than the 12% conditioned samples due to less bran
contamination. Changing wheat moisture changed flour yield and quality much more than did changing the length of time for conditioning, the temperature when wheat is conditioned, or differences in the initial moisture of the wheat before conditioning. The last three effects could be used to improve flour yield in both the 12 and 15% conditioned wheat treatment with better quality at 15% moisture (Kweon et al., 2009). In view of the major influences conditioning has on milling performance, consideration of the way in which specific morphological features respond to water permeation is relevant. Conditioning additives have been devised in an attempt to improve these characteristics (see Chapter 7: Bran).

1.2.3.1 Water Absorption Process by Wheat Caryopsis

Hardness is known to be indicative of the rate and quantity of water uptake during the conditioning procedure, and although it is generally accepted that hard wheat endosperm diffuses water at a slower rate than soft wheat endosperm, the exact nature of the interaction is not well understood but appears to be affected by vitreousness and the agglomeration of starch and proteins within the endosperm (Pomeranz & Williams, 1990). Hinton (1955) reported that the testa was the rate limiting barrier to moisture uptake, but also reported a higher rate of moisture absorption in mealy than in vitreous wheat endosperm. It is well documented that the outer layers of the coat have wick-like properties and, due to the porous pericarp, are able to conduct water around the surface of the seed probably between the outer pericarp and the underlying testa layer. Water enters most rapidly through a pore, the micropyle, hydrating first the embryo then scutellum. The micropyle is situated close to the tip of the embryo, where the integuments do not meet and represents the remnants of the pollen tube in many species. Using a I$_2$/KI stain, Rathjen et al. (2009) demonstrated that water enters the endosperm around the edge of the scutellum and then moves progressively in a distal direction through the sub-aleurone endosperm (Rathjen et al., 2009). This path of moisture permeation appears to precede endosperm utilisation in early germination (see Chapter 2: Early Germination and Starch Granule Degradation). Stained tissue indicated that water had started to enter the endosperm by 7 h after the start of imbibition (Evers & Millar, 2002; Rathjen et al., 2009). There appears to be a preferential movement into the dorsal region and only later into the central and crease regions. Contrary to early research (Stenvert & Kingswood, 1976), a recent study reported that neither MRI (Magnetic Resonance Micro-Imaging),
or I$_2$/KI stain uptake experiments have provided evidence of direct permeation of water across the coat and into the underlying endosperm (Rathjen et al., 2009).

1.2.4 Commercial Milling Practice: Grists and Flours

When considering influences on the milling potential of wheat varieties, important factors contributing to practical flour production also should be taken into account. Given the variations in character among varieties within the major wheat classes, another vital operation of the miller is to blend wheats (gristing) of different varieties and from different sources to yield flours of the desired protein content and uniform baking performance. Furthermore, the blending of grain with diverse quality attributes can be used to achieve improved quality and market value compared to the original lots of grain. When considering the blending of wheat or flour consignments, knowledge of the varieties involved is of great potential value. Traditionally for wheat varieties from the same location, test weight is an indicator in predicting the flour yield. However, it is not true when widely varying varieties are used for blending (Hlynka & Bushuk, 1959). Although not ideal, PSI determination is also important to adjust the optimum hardness balances for the blend in terms of getting high flour yield. One of the reasons for the differences among the reported PSI values is the use of different mills and sieves, consequently in Australia, the scores from different laboratories are ranked when used for wheat classification purposes (Bekes & Wrigley, 1999).

Research is continuing towards providing a more comprehensive guide to predicting the outcome of blending (for example, the knowledge of expression and effect of glutenin subunits on dough properties). Success in the blending process depends first on knowing the quality characteristics of the components, and then on various practical factors, particularly difficulties associated with sampling and mixing. Prediction of the outcome of blending is a relatively simple task when formulating combined grain lots with respect to composition (e.g. protein content), because the relationships involved are linear. However, blending to achieve a specific target for other quality characteristics (e.g., dough properties) is difficult, because of the non-linearity of relationships involving such characteristics (Bekes & Wrigley, 1999). According to Sarkar (1988) the main considerations in wheat blending are the
availability of wheat (for example, using low grade wheat when supply is insufficient), price (maintaining desired final flour quality for minimum expenditure for wheat), and quality (Sarkar, 1988; Hayta & Cakmakli, 2001).

1.2.4.1 Flour Types

The main criteria in determining the bread-making quality of flour, regardless of the specific end product, are protein content, protein quality, dough strength properties or mixing properties, $\alpha$-amylase activity, and degree of starch damage. In terms of dough strength characteristics, weak flours produce dough that develops quickly, breaks down quickly, and has little tolerance to variations in mixing time. By comparison, overly strong dough requires long mixing times at high speed in order to develop properly, and it shows good tolerance to over-mixing and long fermentation processes. However, overly strong flour can cause difficulty in defining optimum processing conditions. Although this can be overcome by addition of proteolytic enzymes or reducing agents to shorten the mixing time. On the other hand, it is very difficult to correct weak mixing characteristics except by blending the weak flour with strong "corrector" flour to improve the overall strength (Carson & Edwards, 2009).

The evaluation of the quality of flour must be made in consideration of its end use. The technical information normally provided to the general user regarding wheat flours includes: flour type, for example, patent, high-gluten, all-purpose, bleached or unbleached, pastry, and others; whether or not the flour is made up of hard or soft wheat, or a blend; and a per cent protein content. The higher protein found in hard flour indicates a higher level of gluten, which results in a more elastic, better-textured bread. High-gluten flour is often blended by the baker with other low-gluten flours to give them more strength and elasticity as mentioned above. Straight flour is considered a good flour to use for bread making. It is 100 percent extraction flour, that is, based on 100 units of wheat, approximately 72 units of flour remains after extraction; the other 28 units is used for animal feed. Straight flour is used to make patent, clear, and low-grade flours. Patent flour is made from the centre portion of the endosperm. Short patent flour made from hard wheat is the most highly recommended commercially milled flour for bread baking and contains 70 to 80 percent straight
flour. All-purpose flour (general household use) is made from a blend of hard wheat flours or sometimes a blend of soft and hard wheat flours. For example, a blend of hard red winter wheat flour and soft winter wheat flour is suggested to perform consistently well when making Italian style bread (Pyler, 1988; Bilheux et al., 1989). Millers may blend wheat classes in a controlled fashion, mainly to minimize or contain grist cost, by adding high-quality wheat to lower-quality wheat. In reality, most millers would prefer to blend flours rather than wheat to create customized products because it affords them better control and more flexibility. However, the ability to blend flours is often limited by the storage capacity available for segregation of individual flour types (Carson & Edwards, 2009). The potential exists to further develop resilient wheat varieties with more specific qualities useful in blending and more rapid diagnostic tools to predict milling performance more precisely.

However to some extent, millers can manage the physical properties of the flour they produce through the grinding process and stream selection. When stream selection is used to produce low ash content (a measure of mineral content after incineration of ground wheat or flour), the resulting patent flour has greater dough strength properties, with longer farinograph dough development time and stability relative to straight-grade flour of higher ash content milled from the same wheat. An example of the difference that mill-stream selection can make to flour quality involved milled Canada Western red spring wheat. The protein content of the resulting flour was lower at lower extraction rates; however, the flour has greater strength, with longer development time and stability than higher-extraction-rate flour. The ash content decreases with decreasing extraction rate, while the flour colour grade improves. Also by altering the grinding conditions, a miller can reduce or increase the level of starch damage for hard wheat, thus affecting the flour's water-absorbing capacity. Mills add α-amylase to flour ground from sound wheat to achieve a desired level of enzyme activity for production of yeast-fermented products, necessitating careful control of the level of damaged starch that is to serve as substrate (Carson & Edwards, 2009). Consequently any improvement in flour yield through enhancement of specific traits must also take account of usage and potential extractions of protein and ash contents and susceptibility to starch damage.
1.2.4.2 Dough and Bread Structure

Bechtel et al. (1978) found that protein strands provide a matrix network in a mixed dough. Matrix formation requires adequate mixing and fermentation-produced gas vacuoles. In dough formation, both immediately after mixing and after fermentation, some structure formation involves "stringing" of small starch granules. Bechtel et al. (1978) suggested that the high numbers of small starch granules may make quite a significant contribution to dough structure though other researchers consider gluten strength is dominant. The large starch granules in the dough of white, wheat bread was thought to contribute little to structure formation. However in wheat bread, the well-leavened gluten system is reinforced during baking by the gelatinized starch (Bechtel et al., 1978). In the baked bread, most of the starch is gelatinized into fibrous strands that are in contact with thin protein strands. As shown by scanning electron microscopy (SEM), the contribution of small starch granules in the baked bread is relatively smaller than that of large granules, which swell and interact with protein. Therefore the structure of the baked wheat bread involves primarily interaction of denatured gluten, swollen starch (mainly large granules) and small starch granules that are strung together. Remaining small granules appear to retain their size and shape in "protected" areas inside the vacuoles especially in crust, possibly a result of water availability. The coherence and continuity of the protein matrix can be weakened by large amounts of bran particles (Pomeranz et al., 1984).

In summary and using examples, identification of components contributing to the processing quality of any grain class may be determined by the contribution of their roles (eg. response to conditioning, adhesion between bran and endosperm, vitreousness) to any stage of the applied processing methods during extraction, refinement or production/baking. Equally important, the component may contribute directly to the quantity of an essential end-product material (eg. patent flour quantity), or a specific product quality (eg. degree of starch damage or protein quality). The final end-products are the results of sometimes various combinations of wheat varieties and flours and their responses to the many process stages. Yet more detailed understanding of the significance of such components is useful to both breeders and millers.
1.3 Grain Quality and Improvement

1.3.1 Measurements Predicting Milling Quality

1.3.1.1 Weight

Generally considered as a guide to flour milling yield potential, test weight is a globally-used measurement of bulk density. It reflects the weight of kernels relative to their size and grain packing capacity (AACC, 2010). In Australia, the 74 kg/hl (hectolitre) target has been used to separate good and poor milling wheats for over 30 years (Cracknell & Williams, 2004). Wheat of different classes and of varieties within a class may exhibit different test weight values, but the greatest influence on test weight is from environment, not class. Factors such as weathering, kernel moisture, kernel protein content, and kernel physical shape and size all play a role in determining the test weight. All of these factors can be expected to affect wheat milling performance; hence, test weight is more often than not used by millers as a predictor of potential flour yield. However with proper mill setup, wheat classes of varying test weights but comparable physical conditions generally produce similar flour yields (Carson & Edwards, 2009).

Alternatively grain, or kernel weight, is reflective of grain size and the potential amount of flour within the kernel. Several techniques have been developed, all relying on measuring the weight of a known number of individual grains to provide an estimated weight of a single kernel. The common technique used in Australia has involved the counting of 1,000 grains, weighing the total mass and then calculating an average grain weight; referred to as the thousand-kernel weight. On the other hand, correlations between measurements of specific weight and flour yield are generally acknowledged to be poor if shriveled samples are excluded (Simmons & Meredith, 1979).

1.3.1.2 Hardness

Hardness in solids has been described as a property that is ultimately related to strength and ductility (Glenn et al., 1991). Wheat hardness has no universally accepted definition and has come to have several different meanings depending on the type of
test used to measure it. Fundamental studies on single kernel physical strength have been performed on intact kernels with simplified geometry (Arnold & Roberts, 1969; Glenn et al., 1991; Dobraszczyk, 1994; Delwiche, 2000; Morris et al., 2008a; Morris et al., 2008b). Considerable variation occurs in the mechanical strength of endosperm from different cultivars and from different kernels of the same cultivar. It is possible to have a genetically soft wheat which is physically hard; alternatively genetically hard wheat can be made soft by changing environmental and drying conditions (Hoseney, 1987). Consequently genetic classification alone may not necessarily describe the physical characteristics of the grain.

Indirect definitions of grain texture refer to the manner in which grain breaks down to a meal or flour and how that meal or flour behaves during processing. Measurement of grain hardness in Australia has focused on the Particle Size Index (PSI) (Symes, 1965). PSI is determined by grinding grain, usually 10-25 g, in a small-scale mill and calculating the percentage of the total weight that passes through a sieve of a pre-determined size then converting the resultant data into a relative hardness index (Worzella & Cutler, 1939). Hard wheats have smaller PSI scores than soft wheats due to their larger particle sizes not passing through the sieve. A single variety can have a range of PSI measurements but in relative terms a hard variety will always have PSI values that reflect harder measurements compared with those of a soft-grained variety. The considerable overlap of grain hardness between samples limits the discriminatory power of current tests for the hardness of bulk samples based on grinding, consequently research supports the Single Kernel Characterisation System (SKCS) 4100 analysis as providing a more pertinent measurement (Dobraszczyk et al., 2002). The endosperm texture or the relative hardness or softness of a grain can be defined as a measure of the resistance to deformation. This definition is at the basis of the measurement of hardness by the SKCS, which measures the force required to crush individual grains of a sample between two surfaces taking into account the weight, diameter, and moisture of the grain (see topic below: Chapter 3, Single Kernel Characterisation System). Numerous studies have dealt with efforts to standardize the measurement of wheat hardness, for example, visual inspection of crushed endosperm (Mattern, 1988) and, near-infrared reflectance spectroscopy (NIR) of ground meal (Williams & Sobering, 1986; Norris et al., 1989). Nevertheless the lack of a linear relationship between NIR hardness and any of the mechanical properties measured in
the study by Glenn et al. (1991) may be because hardness properties encompass more than one mechanical property (Turnbull & Rahman, 2002).

1.3.1.3 Protein

Protein is a fundamental quality test of wheat since it forms the basis for payment to farmers and is related to its end-product processing potential. Protein level is an estimation based on the amount of nitrogen present in the grain. The level of protein can be accurately measured by chemical (Kjeldahl or Dumas methods) and NIR methods (Osborne, 2007). The level of protein is strongly influenced by environmental and management factors whereas the quality of protein is linked to genetic composition and environment interaction (Eagles et al., 2002). Protein quality measurements include determination of the actual amount of gluten, assessment of viscoelastic dough properties and molecular measurement of amino acids groups (Wesley et al., 2008). Modern techniques also allow the monitoring of the vitreousness of grain samples. Studies have demonstrated that classification of obviously vitreous or non-vitreous kernels by the NIR procedure agreed almost perfectly with inspector classifications. NIR classifications appear to be due, at least in part, to scattering effects and to starch and protein differences between vitreous and non-vitreous kernels (Dowell, 2000; Osborne, 2007).

1.3.1.4 Alternative predictive Measurements for Flour Yield

Apart from knowledge of the characteristics of individual cultivars, gained through experience or experimental evaluation and general estimation using weight measurements, there is no reliable means of predicting milling yield. Characteristics such as grain length and breadth, crease depth and embryo size have been considered. In some studies endosperm content has been determined and related to milling extraction rate and grain size (Simmons & Meredith, 1979). Previous research reports the feasibility of using image analysis (IA) to distinguish between wheat-grain samples according to quality attributes that relate to milling quality, particularly kernel vitreousity, grain color, or grain hardness class (Zayas et al., 1986; Draper & Keefe, 1989; Neuman, 1989; Bason et al., 1993; Sapirstein, 1995). For example, a promising, preliminary study by Evers and Withey (1989) produced
unexpected correlations with extraction rate using a length ratio defining the relative distance from the grain's widest point to grain tips (Evers & Withey, 1989). IA is one of the few techniques available to wheat breeders that leave whole-grain samples intact to plant after testing. The technique is based on the fact that experienced breeders can make predictions of milling quality by visual examination of whole-grain samples (see also Marshall et al., 1986). Berman et al. (1996) conducted a study of whole grains including eight genotypes grown at up to six sites (Berman et al., 1996). Results suggested that approximately 66% of the variation in flour yield for 38 grain samples could be explained by four factors computed from the images of 100 grains for each sample (mean of grain area, lengths of minor and major axes, and ellipsoidal volume), plus test weight. However Berman et al. (1996) concede that their predictive model is confined in its implications because of the limited extent of samples examined. As with many such studies, the approach needs to be extended and tested on a much wider basis, covering more seasons, sites, and genotypes, within a general quality type (such as Australian Prime Hard type). Nevertheless physical measurements by whole-grain image analysis in combination with other tests for example NIR might be expected to extend the predictive value (see topic: Chapter 1, QTL Analyses)(Berman et al., 1996).

### 1.3.2 Classification of Wheat Varieties

The formal classification of new wheat varieties is a subsequent and critical step in the development of agronomic research outcomes. In Australia, the classification process is a complex task involving an evaluation of the quality of a variety, within a defined geographic area, over several years of production. The broad categories of Australian wheat are described with reference to such things (with examples) as seed coat colour (white), grain hardness (hard or soft) end-use (noodle), species (durum), and overall rating (prime, premium, standard). The traditional classes of Australian wheat are Australian Prime Hard (APH), Australian Hard (AH), Australian Premium White (APW), Australian Standard White (ASW), Australian Standard White Noodle (ASWN), Australian Soft (ASFT), and Australian Durum (ADR) (source: http://www.wheatclassificationcouncil.com.au). Classification decisions for each candidate variety are made on the basis of quality data (including
grain quality, milling quality, dough rheology and performance in end-products) that is collected from breeding trials on a seasonal (over a minimum of three seasons) and regional (relating to a defined region of production - a Classification Zone) basis. As yet, no reference is made to genetic analyses. Other information (such as agronomic or pedigree information) may be submitted to support applications at the discretion of the Breeding Organisation. More specific measurements of physical grain required in the classification process include: Thousand Kernel Weight (g), Grain Hardness as measured by PSI, and/or SKCS and Starch Damage. Also important are measurements of end products, for examples: Loaf volume, crumb colour, appearance and final texture scores (source: http://www.wheatclassificationcouncil.com.au.; AWBI Wheat Classification Guidelines August 2008).

1.3.2.1 Regional Influence on Characterisation

Different classes are matched to specific regional conditions which also may enhance certain end-product qualities to supply a specific market. For example, Australian Prime Hard is grown only in the eastern Australian zones of Queensland, northern New South Wales, and southern New South Wales. This wheat has end-use functionality suitable for the production of high-volume European breads, Chinese style yellow alkaline noodles, fresh ramen noodles, dry noodles, and wonton skins. Australian Durum (ADR) is grown in northern New South Wales, Queensland and South Australia and is used for pasta products. Whereas Australian Standard White Noodle (ASWN) is grown mainly in Western Australia and was introduced for the Pacific Rim market being suitable for Japanese style udon white salted noodles and ramen noodles (Carson & Edwards, 2009). Endo et al (1989) recognized that Japanese consumers preferred wheats from Western Australia and suggested that this was due to the particular starch properties of lines derived from that source (Endo et al., 1989). Further investigations of this starch type (Crobbie, 1991; Miura et al., 1994), demonstrated that low amylose content, high starch paste viscosity, and higher swelling properties contributed to the desired characteristics. These characteristics are associated with the loss of the GBSS protein encoded by the GBSS locus on chromosome 4A (Zhao et al., 1998). This example demonstrates that very subtle changes in starch structure can translate into significant differences in starch functionality that, in turn, result in changes in the consumer appeal of high-value end
products (Stone & Morell, 2009). In conclusion, strategies to improve flour yields may differ between regions.

1.3.2.2 Wheat Growers Choice of Varieties

Agricultural research designs involving field trials require experiments over various locations and years to be truly informative to classification boards and ultimately growers. Grain quality (and potential flour yield) is one among many important characteristics to consider for the wheat grower including: maturity, winter hardiness, disease and insect resistance, lodging and shattering, acid tolerance, coleoptile length, and grazing potential. As perfect wheat varieties have not yet been developed, compromise and the assumption of risks are necessary to gain a wide advantage. It is recommended that several different varieties should be planted in order to reduce losses due to weather and pest problems. Varietal strengths such as yield potential, pest resistance, or strong straw, need to be matched against expected field problems (for example in the U.S., these include: soil-borne mosaic, Hessian fly infestation, or lodging). It is recommended that selection of varieties be prioritised on the basis of data over some years on tolerance and resistance, performance in several locations then processing quality. In addition, chosen varieties should have different pedigrees and different growing patterns (Paulsen, 1997).

1.3.3 Wheat Improvement

1.3.3.1 Grain Yield and Weight: Contributing Factors

Ignoring considerations of flour quality, and accepting that flour yield is partly a function of bran separation efficiency, flour yield is generally related to grain weight. Again, grain weight is commonly considered to be influenced by environment (however see section 1.3.4.3). Further gains in grain weight cannot be at the expense of overall grain yield unless marked quality improvements are also achieved to allow financial viability. The grain yield of wheat, in terms of mass per unit area can be described empirically in many ways. Two main approaches are used, one based on the concept of ‘sink’ and the other, ‘source’. These alternatives alter the design of agronomic inputs and breeding efforts. The sink concept understands yield in terms of yield components that is, grain numbers produced by mean grain weight. This may be further calculated in terms of: plants per square meter, seeds sown and viable proportions of all plant components. Therefore yield is formed throughout the growth
of the crop, with the environment and agronomic inputs at different growth stages influencing yield through their impact on specific yield components and susceptible to modification at particular times. Alternatively, the sink concept deals with the capacity of the grain population to contain yield. For example, developing ears (and then grain) compete for assimilate from other organs and because of hormonal and other factors, increasing the sink size increases the efficiency with which assimilate is partitioned to the ear and grain. Some take this approach a step further, supporting the idea that sink size affects resource capture and use-efficiency, for example, increasing grain numbers set may lead to increased photosynthetic rates. However the sink size may present a physical limitation on yield. For example, removing half of the spikelets on an ear does not double mean grain weight, even though it would seem that there should be twice as much assimilate available per grain. Grain yield is thought to be mostly a function of grain numbers (Gooding, 2009).

The second approach focuses on resource capture, utilization, and partitioning. That is, biomass is a function of the capture of a resource and the efficiency with which this resource leads to the production of biomass. Grain yield is then a function of biomass and the efficiency with which this biomass is partitioned to the grain, i.e., the ratio of grain yield to biomass yield, or harvest index (HI). The resource most commonly used to express yield in this way is light, or more precisely, photosynthetically active radiation (PAR). With regard to light, the ratio of biomass produced per unit of PAR intercepted is the radiation use efficiency (RUEPAR), so yield can be expressed:

\[
\text{Grain yield/m}^2 = \frac{\text{PAR interception/ m}^2 \times \text{RUEPAR} \times \text{HI}}{m^2}
\]

The production of biomass by a wheat crop during periods of leaf production is largely a linear function of light interception. The proportion of PAR that is intercepted depends on canopy size, canopy architecture, and canopy longevity. The yield component and resource capture methods of understanding yield are complementary because often grain numbers, and possibly also potential grain size, are in balance with the resources captured. However, harsh but transient climatic conditions at anthesis can reduce grain set and yield. It has also been suggested that the balance that the crop reaches between source and sink is conservative; i.e., the crop may always set fewer grains than could be potentially filled (Gooding, 2009). These considerations highlight
the overall contribution of the plant to grain quality and present alternative targets for development.

1.3.4 Wheat Breeding

Wrigley (2009) observes that the wheat breeder is responsible for the development of new varieties that suit the agronomic and quality needs of all participants in a so-called “grain chain”. The chain links include seed producers, farmers, subsequent stages of segregation, buying, storage, and transport, and those involved in milling and food manufacture. Consumer demands for specific flour qualities and their evaluations are communicated back to the breeder. The response to feedback may involve many years, due to the lag necessary for a breeder to change strategies (Wrigley, 2009). Wheat breeding has made great advances, but historical aims have remained similar that is, to choose parent lines that carry the desirable genetic attributes and to combine these in wheats adapted to the target region, thereby providing increased yield, improved resistance to pathogens, tolerance to abiotic stresses, and grain quality suited to market requirements (Bonjean & Angus, 2001) (see listing of identified genes that control many specific attributes: http://wheat.pw.usda.gov/ggpages/wgc/2003/Catalogue.pdf). There is also much more information on websites: Grainsenes, operated by the U.S. Department of Agriculture (http://wheat.pw.usda.gov), and the Wheat Genetics and Genomic Resources Centre, Kansas State University (www.k-state.edu/wgrc).

1.3.4.1 Historical Background

A history of wheat breeding was briefly reviewed by Wrigley (2009). Wheat cropping systems and selection pressures appeared to change significantly in Europe during the 1800s with more conscious selection, following careful observation and hybridization (Swaminathan, 2006). Input responsiveness, disease resistance, harvest index, and the relationship between these characters and crop height have been long appreciated by wheat farmers. For example: Roberts (1847) recommended that “on rich soils, where an abundance of straw is produced, short and stiff strawed wheat yields the best crop as the weak and long-strawed wheat is liable to be spoiled by being laid” (Roberts, 1847). Also Thomas Garnett wrote to the Manchester (U.K.) Guardian in 1852 about the advantages of short-strawed wheat, suggesting that it would bear high levels of manuring without lodging, and with much less liability to mildew, than
a long strawed wheat. Further comments note the proportion of grain to straw is
greater than in long strawed wheat. Also short-strawed wheat very rarely lodges and is
far better suited to the reaping machine (Garnett, 1883) (Wrigley, 2009).

Since the rediscovery of the Mendelian laws of genetics in 1900, an initial
phase from 1900 to 1930 involved selection from existing diversity and hybridization
using land races (Swaminathan, 2006). Particular targets were disease resistance
(hence yield stability) and also grain quality improvement. During this period,
William Farrer was also experimenting with cross-breeding in Australia, based on
wheats introduced from Africa, India, and the Middle East, as well as high-quality
germplasm such as Red Fife. He enlisted the assistance of the chemist Frederick
Guthrie to provide quality-testing facilities, thereby initiating the breeder-chemist
association. Interestingly, judgment for selection of promising varieties by breeders,
before the recent introduction of electronic devices was based on the "chewing test"
which is an indicator of grain hardness and gluten quality. The strongest flour is
obtained from those wheats which produce gluten having the greatest ability to
recover its shape. While the chewing test is certainly of value, supplementary baking
trials are recommended (Wrigley, 2009).

1.3.4.2 Breeding for Grain Yield

Factors that have been important in breeding for grain yield may also have
significant influence on grain filling potential and subsequent flour yield. Significant
limitations to grain yield have appeared to be those related to excessive height. Tall
varieties have poor yields partly because lodging risk prevents the economic use of
sufficient quantities of nitrogen to produce canopies large enough to intercept the
majority of available light during yield formation. With other agronomic variables
constant, grain yield is closely, and negatively, associated with straw dry weight
(Austin et al., 1980). Improvements in Harvest Index (see topic above, Grain Yield
and Weight: Contributing Factors) associated with reduced height can, therefore,
account for more than 80% of the improvement in yield potential of wheat varieties
within some twentieth century breeding programs (Gooding, 2009). Height is a
polygenic character, but a number of major reduced height (Rht) genes have been
identified. Historically these have been listed as Rht1 through Rht2. More recently
different alleles at the same locus have been identified. Rht genes have affected an in-
crease in HI, mostly through improved spikelet fertility while maintaining sufficient total biomass production, and consequently have had a hugely significant impact on worldwide wheat production. Genes that confer reduced sensitivity to gibberellic acid (GA), located on the long arms of 4B and 4D, have been particularly important. \textit{Rht-B1b} (formerly \textit{Rht1}) and \textit{Rht-D1b} (formerly \textit{Rht2}) can each reduce final crop height by about 15\% by reducing internode length. Both of these genes derived from the Japanese variety Norin 10, which itself was a descendant of the Daruma (semidwarf) and Turkey land races. The Norin 10 dwarfing genes are now present in more than 90\% of the world's semidwarf wheat production (Worland \textit{et al.}, 1998). Since the widespread adoption of semidwarf wheats, the rate of improvement in wheat yield has declined (Slafer \textit{et al.}, 2001).

Even accepting a value of 80\% for the contribution of Harvest Index increases to yield improvement, the implication is that 20\% is due to increased above ground biomass. Increased biomass production must necessarily derive either from increased light interception and/or increased radiation use efficiency (RUE, see above). Therefore there appears to be some possibilities for increased yield by extending the period of canopy closure. However without genetic advances to prolong grain filling, extra canopy longevity (or delayed senescence) is of little benefit. Taken together major advances in yield potential by further increasing Harvest Index, light interception, and RUE through manipulating canopy architecture and/or grain numbers will be difficult. Some commentators claim that real advances can only be possible by improving photosynthesis efficiency at a molecular level (Parry \textit{et al.}, 2007). Yield benefits may accrue through hybrid vigour, or heterosis, when two parents are crossed. However although wheat hybrids have been available in certain countries since the mid-1970s, until recently, hybrid wheat has never occupied more than 3\% of any national wheat area. This is a comparative failure that reflects a number of difficulties with this strategy. Alternatively breeding programmes can reduce yield loss by increasing resistance to disease (for example, by the translocation of a short chromosome arm from rye to homologous wheat chromosome 1B) (Schlegel & Korzun, 1997) and improving tolerance to abiotic stresses (Gooding, 2009). Although from a wider perspective, basic water availability is the most important limiting factor for wheat production (Zhao \textit{et al.}, 2009).
1.3.4.3 History of Kernel Weight Gain

Researchers have concluded that increases to present-day Kernel weight (KW) were already achieved centuries ago. KW is a key marker for early domestication (Cascon, 1934; Austin et al., 1989; Salamini et al., 2002; Willcox, 2004). KW is under complex polygenic control, and alleles having both positive and negative effects on the trait have been mapped for example, those involved in tillering see topic: Chapter 2, Tillering) and root systems (Cantrell & Joppa, 1991; Elias et al., 1996). Domesticated tetraploid wheats like durum wheat tend to have a comparatively low tillering capacity, making them more dependent on the early developed, deeper reaching seminal root system (MacKey, 2005). In such a context, a correlation exists between KW and seminal root system (Araus et al., 2007). Unconscious selection during a long phase of wild-plant cultivation can easily account for changes in traits with polygenic inheritance (Salamini et al., 2002) such as seed size (Willcox, 2004). Although strongly genetically determined, KW also depends on environmental constraints such as water availability or high temperatures during grain filling (Gooding et al., 2003; Rharrabti et al., 2003).

Araus et al (2007) in an attempt to reconstruct wheat cultivation c. 8.000BC, found that landraces from Morocco (overall, the best-yielding material in the rainfed trial) flowered earlier, were shorter and had a reduced total leaf area compared with the other origins. These are well-known attributes providing grain yield advantage, particularly the former, in drought-prone areas. KW is the most important grain yield component in durum wheat landraces originating from the north Mediterranean Basin (Moragues et al., 2005), an area characterized by moderate terminal water stress. Modern genotypes exhibited a clear yield superiority over the set of landraces as a consequence of breeding efforts resulting in reduced plant height, probably without significant decreases in total biomass, and improved partitioning (i.e. increased Harvest Index). The initial breeding material released by CIMMYT-ICARDA during the 1970s was clearly differentiated from the rest of the landraces as well as from the group of more recent cultivars. Overall, it displayed the highest KW and the shortest plant height, which contrasted with a relatively low number of kernels/spikes. In addition, modern cultivars showed larger minimum values in total leaf area and leaf
greenness, probably as a result of positive selection pressures pushing towards increased photosynthetic performance (Araus et al., 1997; Araus et al., 2007).

1.3.4.4 Modern Breeding Methods

Traditional wheat improvement relies on pedigree breeding, whereby initial hand-crossing produces heterozygotes, which are then ‘selfed’ for up to eight generations to increase homozygosity then selections are made. Selection, however, is often less efficient (though dependant on the heritability of the trait) in the early generations because heterozygosity complicates identification of suitable genotypes (Gooding, 2009). Selection is generally based on morphology, disease reaction, and yield components, although some biochemical selection (e.g., more recently by HMW-glutenin subunit composition as identified by electrophoretic systems) has been adopted in several commercial breeding programs. A more recent possibility is selection for DNA markers, linked or tagged, to single-gene traits (such as major disease resistance genes) or polygenic traits identified in chromosomal maps as Quantitative Trait Loci (QTLs). QTLs are stretches of DNA that are closely linked to the genes that underlie a quality trait (presented by a direct or indirect measurement thereof). Statistical analysis is required to demonstrate that different genes interact with one another and to determine whether they produce a significant effect on the phenotype. The last decade has seen increased use of techniques to produce doubled haploids, usually from the first generation of a cross (F1). This alleviates the complication of heterozygosity in the early generations of traditional systems. This procedure allows the production of homozygous lines from the F1 and thus has the potential to increase the rate of variety selection and release.

Therefore common breeding methodologies include: F2 Progeny, Back-crossing, doubled haploids (wheat x maize method) and single seed descent - routinely used, and Marker Assisted Selection. Wheat breeding objectives can encompass all of the following: Quality considerations - grade (protein content), receival characteristics (such as test weight, screenings, black point, low LMA), disease resistance; and Agronomical considerations – height (mainly semi-dwarf – advantage in ease of stubble handling), non shattering , ease of harvest, straw strength, presence of awns, amount of tillers, pre-harvest sprouting tolerance (using molecular markers), freedom from LMA , frost tolerance, water-logging tolerance. Environmental challenges
necessitate yet further demands on breeding programmes, for example, grain filling during increasing temperature and decreasing rainfall; also agronomic limitations such as nutrition (toxicities and deficiencies), coleoptile length (emerging sheath protecting first true leaf), time of sowing vs. maturity. A further priority of wheat breeders is to provide growers with a range of maturities in each quality grade/class for different sowing dates to hedge against possible frost or drought conditions (Wilson et al., 2007).

Considering wide crosses, intergeneric hybridization of wheat with related species has been increasingly used. In particular, different accessions of *Ae. tauschii* have been combined with *T. turgidum* to produce synthetic hexaploids. This increases the genetic base for hexaploid wheat breeding because, although more than 250 accessions of *Ae. tauschii* are known, it is thought that very few have been involved in the evolution of common wheat through donation of the D genome. This strategy leaves the tetraploid wheat genome intact while making improvements to resistance to disease (Mujeeb-Kazi et al., 2006) and to abiotic factors such as cold, drought, salinity, and waterlogging (Reynolds et al., 2005). There is also potential for intergenomic heterosis if novel alleles introgressed into the D genome interact positively with alleles at equivalent loci in the A and B genomes (Gill & Raupp, 1987). The genetic transformation of wheat has lagged behind that of other important crops. It was not until 1992 that the first reliable production of fertile transgenic wheat using particle bombardment was reported (Vasil et al., 1992). Subsequently Agrobacterium-mediated systems have also been developed (Hu et al., 2003). Release and use of genetically transformed wheat remains a politically sensitive issue in many parts of the world. In addition to herbicide tolerance, wheats with transformations for modified storage proteins, increased disease resistance, improved water-use efficiency, and drought tolerance are at various states of readiness for commercial exploitation should the political environment change (Gooding, 2009). Taken together, aspects of these various breeding methods can be readily applied to traits affecting milling performance once identified.
1.3.5 QTL Analyses

Increased flour yield is one of the most important traits in wheat breeding programs in Australia. However marker-assisted breeding for flour yield and flour quality traits is a major challenge because these traits are controlled by multiple-interactive and environmentally dependent quantitative trait loci (QTLs) that may have low heritability (Yin et al., 2003; Lehmensiek et al., 2006). Also an important consideration is the measurement of flour yield which has its own intrinsic variability within and between laboratories (Smith et al., 2001). Furthermore, reliably elucidating the genetic control of modestly heritable polygenic traits requires marker studies (observable variations in genes or sequences) of large populations in multiple environments (Griffiths et al., 2000; Nelson et al., 2006).

Lehmensiek et al. (2006) in a study of flour yield QTLs in three Australian doubled haploid wheat populations emphasised the importance of replicating sites in more than one environment in order to reliably identify QTLs associated with quality traits. For example, nine different QTL regions associated with flour yield were identified in the three populations used in their study. Only two of these coincided with regions associated with flour yield in other studies (Campbell et al., 2001b; Lehmensiek et al., 2006). These workers also noted that lack of consistency of QTLs over different crosses and trial site/year indicates that for flour yield, QTL expression is highly dependent upon the genetic background and its interactions with the environment. Consequently it was advised that breeding programs should base their marker selection strategies for this trait within pedigree groupings of regionally important core germplasm. Also special consideration must be given to which additional measurements need to be taken when focussing on one particular trait (Lehmensiek et al., 2006).

Selection of wheat germplasm for a range of quality traits has been a challenging exercise because of the cost of testing, the variation within testing data, and a poor understanding of the underlying genetics (Raman et al., 2009). Some studies conducted by the Queensland Department of Primary Industries and Fisheries and Australian universities have identified QTL for quality traits based on bi-parental
doubled haploid (DH) populations (Lehmensiek et al., 2006; Christopher et al., 2007). The majority of wheat germplasm grown in northern Australia has a significant genetic contribution from the ancestral varieties Cook or Hartog. These studies have identified significant QTL for milling yield on chromosomes: 1B, 2A, 2D, 4B, 4D, 3B, 5A, and 7D. Various QTLs identified as likely to influence flour milling yield have had negative relationships with protein content and water absorption and positive associations with both test weight and plant height. The Ha (Hardness) locus is known to have a strong influence on milling yield. Consistent with this result, past researchers have detected strong negative correlations between grain hardness and milling yield (Campbell et al., 1999; Martin et al., 2001; Nelson et al., 2006).

Using a pedigree mapping approach with Australian varieties, Christopher et al. (2007) identified 70 markers from 13 chromosomes for which at least one allele was associated with significant differences in flour milling yield (Christopher et al., 2007). In some cases, groups of markers may be associated with individual QTLs; alternatively they can be spread across a whole chromosome. Some of the regions identified by Christopher et al. (2007) were in common with those identified in biparental populations involving related genetic material but others were unique. For example, markers from nearly the entire length of chromosome 2B were found to be associated with significant differences in flour milling yield. This was thought to be associated with a large, interstitial translocation present in cv. Cook (and many of its derivatives) between chromosome 2B and T. timopheevii (also identified by Lehmensiek et al (2006)). The region identified on chromosome 4B associated with flour yield was also previously reported (see also Chapter 5, Introduction) (Schmidt et al., 2004; Lehmensiek et al., 2006). This region was the only one found to be associated with flour milling yield in more than one population in their study. In a second study, 4B QTL for flour milling yield were identified in all 3 year/sites by Lehmensiek et al (2006), with the high milling yield allele contributed by QT8766, the Hartog-derived parent. This part of chromosome 4B carries the Rht1 gene for plant height. Lehmensiek et al. (2006) found a QTL on 2AS and on 4DS which was identified in all 3 site/years with the high milling yield allele contributed by cv.Lang. Chromosome 4D also carries the Rht2 dwarfing gene. A QTL on 1B is associated with the Bx7 over-expression allele, derived from cv. Kukri.
Other chromosomes previously identified as associated with flour milling yield in the Kukri/Janz population, namely 2A, 2D, 3B, 4D and 7D also exhibited associations in this study (Christopher et al., 2007). In addition QTLs were also identified on 6AS (a gliadin gene is also located on 6AS), 2AS, and also on 3DL in 2 year/sites in concordance with Lehmensiek et al. (2006) (Lehmensiek et al., 2006). The region on chromosome 4A identified by Christopher et al. (2007) as associated with flour milling yield has not been identified in other studies. This region hosts major genes for resistance to pre-harvest sprouting (PHS) and has also been reported to be associated with late maturity alpha amylase (LMA). However it was suggested that the effect on milling yield of these genetic regions may be due to the effect of PHS or LMA rather than a QTL for milling yield. Additional regions found by Christopher et al. (2007) to be associated with milling yield but not reported in other studies of related material were on 5D and 5A. 5D hosts the Puroindoline genes associated with hardness, but the marker associated with flour milling yield in this study is genetically unlinked to these (Christopher et al., 2007).

Using a set of 131 recombinant inbred lines (RIL), the RIL and their two parental genotypes were evaluated for kernel length (KL), kernel width (KW), thousand-kernel weight (TKW), and test weight (TW) in four different environments. Eight QTL (40%) were detected in two or more environments. Two QTL clusters relating to KW, TKW, and TW were located on chromosomes 2A and 5D, and the co-located QTL on chromosome 6A involved a QTL for KW found in two environments and a QTL for TKW detected in four environments (Sun et al., 2009). Breseghello and Sorrells (2007) performed QTL analyses of kernel morphology (size and shape) and their relationship with yield and milling quality in two hexaploid wheat mapping populations, grown in New York and California. In the population W7984 x Opata 85, the strongest signal was detected on the chromosome 5B, for kernel length. In the population AC Reed x Grandin, the most important QTLs were detected on chromosome 2D, affecting the lateral dimensions of the kernel. This study agreed with previous reports that the genetic control of kernel length and width are largely independent. Additionally, it was shown that QTLs detected on different mapping populations, with identical evaluation methods, can be very distinct (Breseghello & Sorrells, 2007). Kunert et al. (2007) identified QTLs relevant to milling quality using an advanced backcross strategy, with two German winter wheat cultivars and
synthetics originating from hybridisations of wild emmer (T. turgidum spp. dicoccoides) and T. tauschii. This method enabled testing for exotic QTL effects on wheat genomes A and B in addition to genome D. In total, nine QTLs for hectolitre weight (HLW) were detected. In crosses, named B22 and Z86, the position of a QTL for HLW on chromosome 6B corresponded to a QTL detected by Elouafi and Nachit (2004). In population B22, the QTL at Xwmc596 also corresponds to QTw.crc–7A, a QTL located by Huang et al. (2006) on chromosome 7A (Huang et al., 2006; Kunert et al., 2007).

Milling performance is a complex quality with grain hardness being a highly significant component. Grain hardness is a genetic trait, although its expression is influenced by the prevailing environmental conditions during grain filling and during conditioning prior to flour milling (Pomeranz et al., 1985; Bechtel et al., 1996; Delwiche, 2000). The location of the major gene (Ha) controlling endosperm texture was determined using chromosome substitution lines. The Ha locus is located at the distal end of 5DS (Sourdille et al., 1996; Turner et al., 1999). Other, less significant, loci that modify endosperm texture have been located on homoeologous chromosomes 2, 5, 6, and 7 (Sourdille et al., 1996; Morris et al., 1999). Chalmers et al. (2001) have described in detail the establishment of the associated genetic maps (Chalmers et al., 2001).

In a study of the hardness locus in Australian wheat lines, Osborne et al. (2001) treated the main SKCS outputs: seed weight, hardness index, GompB (Gompertz function coefficient, (see Chapter 3: Single Kernel Characterisation System), and S\textsubscript{max} (maximum stress) as quantitative traits in a genetic analysis (Osborne et al., 2001b). Since notionally the QTL analysis works by examining the frequency of occurrence (association) of properties with matching markers; anomalies might arise if the subset were not representative, or genetically biased. The markers for the hardness locus used in this study were for the puroindoline-a gene (see topic below: Chapter2, Puroindolines) and the microsatellite marker wmc233. Osborne et al. (2001) reported that GompB output was the only one that showed an association with chromosome 4B. Also striking for this set of samples was that this same chromosome region also accounted for most of the variation in grain weight whereas S\textsubscript{max} provided a QTL that was independent of grain weight.
Another genetic region showing a significant association with hardness was located on chromosome 4D. The statistical associations between markers in the genetic map and trait values were based on regression analyses, and the likelihood ratio statistic (LRS) was estimated by interval analyses (Osborne et al., 2001b).

Osborne et al. (2001) also found an association of the hardness index with chromosome 1A to be of potential interest since it did not appear to be associated with any clear candidate genes such as the glutenin subunit protein loci located on this chromosome. These results implied that selection for large grain size might result in the selection of softer material with associated lower flour water absorption potential. It is evident that some major effects on grain hardness can be observed in Australian wheat lines that are not associated with the classical 5DS locus. Although there is wide acceptance for Pina-D1 and Pinb-D1 loci as the causal genes for grain hardness (Giroux & Morris, 1998), a survey of Australian wheats indicates (Turnbull et al., 2000) that other factors may limit their utility in predicting grain hardness; and at least one other factor is involved (Osborne et al., 2001b). Analysis of a population of Hobbit Sib x Avalon recombinant inbred lines by Turner et al. (2004) revealed year-on-year consistent, statistically significant, new QTL for protein content on chromosomes 2B, 6B and 7A, and for texture on chromosome 1B., with cv.Avalon contributing the allele for increasing hardness (Turner et al., 2004). Clearly, using the variation identified here, there are interesting possibilities for increasing the hardness of softer, higher yielding varieties for different end uses using these newly identified QTL. These results also indicated that the 5D locus is not the only genetic control of grain texture in UK wheats (Turner et al., 2004). The present study seeks to examine the significance of features additional to hardness and interactions affecting grain fractionation.
Chapter 2. Grain Morphology Relative to Milling

2.1 Grain Development

2.1.1 Growth Stages

Some general features of grain development from an agronomical perspective relevant to the present work were extracted from the Wheat Production Handbook (Kansas State University) by Paulsen et al. (1997). Considering winter wheat, growth and development are divided into stages: germination leads to seedlings, the first stage of plant growth, followed by tillering (formation of side shoots), overwintering (falling temperatures during autumn prompt the wheat plant to develop a high level of cold hardiness, also undergo a change from an upright to a prostrate growth form), jointing (stem nodes are first detected above the soil), boot (the swelling of the sheath of the ultimate leaf, the flag leaf, as the developing ear expands within it), heading (as the stem continues to elongate, the head is pushed out of the flag leaf sheath), and flowering. Maturation, or development of the grain, is divided into milk, soft dough, hard dough, and physiological maturity, the stage when kernel weight is at maximum. Ripening, the last stage, occurs as the grain loses moisture until it is ready to harvest (Paulsen, 1997).

Winter wheat is a cool-season crop and grows best under moderate temperatures, but it is able to resist both cold and hot weather. In the United States, this hardiness is essential for wheat to endure the freezing temperatures of winter, the late frosts of spring, the high summer temperatures, and the droughts that can occur anytime. Because of its winter growth habit, wheat is planted during autumn, becomes well established before winter, and “greens up” and starts growing quickly when conditions are favourable in spring. Winter wheat not only resists freezing temperatures during winter, it needs the cold to joint and flower so it can set grain in spring. Flowering also requires the lengthening days of spring, when the dangers of late frosts are usually past. The root system of winter wheat extends further than that of any other wheat class, enabling the plant to obtain moisture from deep in the soil profile in times of drought. These growth and development traits make wheat highly adaptable to harsh conditions (Paulsen, 1997).
2.1.1.1 Tillering

Some growth stages present potential for further optimization of grain yield or weight (see Chapter 7, Discussion) such as tillering, spikelet and floret formation and fertilisation. Nodes can give rise to side shoots, or tillers. Tillering allows the plant to expand and to produce more ears and therefore more grains per plant when interplant competition is low. Tillers thereby permit the wheat crop to partly compensate for adverse conditions. Tillering increases with increasing light and nitrogen availability during the vegetative phase and also depends greatly upon variety and plant density (Bunting & Drennan, 1966; Evans et al., 1975; Gooding et al., 2002). Tillering from a single plant in a fertile, low-competition environment can be substantial. The main stem gives rise to primary tillers, but primary tillers can also produce secondary tillers and, in some cases, even tertiary tillers. Excessive tillering, however, is undesirable. Maturity of the late-produced tillers tends to lag behind that of the main-stem tillers, resulting in an uneven crop, which is difficult to manage and harvest at optimum times. Not all tillers produced are fertile and reach maturity. Toward the flowering stage, the number of tillers per unit area is more a function of environmental conditions, although the genetic influence is still significant. Tiller death may represent a waste of resources (Berry et al., 2003). Much research has revealed numerous quantitative trait loci (QTL) associated with tiller production and death in wheat (Li & Gill, 2004). A few major genes conferring tiller inhibition (tin) have been identified (Richards, 1988), which can also be associated with other potentially useful traits such as increased mean grain weight and improved harvest index. Early tillering can also be useful in, for instance, competition against weeds and improved nutrient capture. Much of the nitrogen is retranslocated from senescing tillers, so infertile tillers may sometimes have a role in ensuring continuous nutrition to grain-bearing stems (Evans et al., 1975; Gooding, 2009).
2.1.1.2 Wheat Spike Components

Wheat grains are borne on a spike, or ear. In the case of wheat, the major axis, called the rachis, bears two rows of spikelets in alternating order (Figure 3). The mature spikelet will contain 3-6 fertile florets, each containing the reproductive organs necessary for kernel development. The mature wheat spike may contain 15-18 spikelets or more attached on alternating sides of the rachis (Kirby, 1974). The rachilla is the axis of a spikelet. It is also branched alternately, bearing a pair of empty (or nonflowering) glumes at the base and a series of up to six florets in the spikelet. Each floret consists of a pair of paleas (flowering glumes), the lower or outer lemma and the upper or inner palea, enclosing the ovary and later the caryopsis (Figure 3) At the base of the floret next to the lemma is a pair of lodicules that swell at the time of fertilization, pushing the floret open and allowing the anthers to emerge on elongated filaments. Floret size decreases from the base upward, and the largest grains are usually in the basal or second florets. Spikelet differentiation starts in the middle of the spike and proceeds toward the base and the tip. Within the floret, differentiation proceeds from the outside inward (Bonnet, 1966).

Figure 3. The relationship among parts of the wheat spike or ear. A, spikelets arranged alternately on the main axis or rachis. B, The structure of the mature spikelet. Its axis, or rachilla, bears (sterile) glumes at its base and florets arranged alternately along its length. In each floret, a grain is enclosed between two pales or fertile glumes [adapted from (Gooding, 2009)].
The number of spikelets formed on each ear depends on both duration and rate of initiation. Rapid initiation is often associated with shorter durations of spikelet production. Varieties can vary in both duration and rate, causing genetically determined variations in spikelet number and ear lengths. Spikelet number per ear usually increases with increased nitrogen availability, apparently resulting from an increase in rate of spikelet initiation (Whingwiri & Kemp, 1980). The duration from the terminal spikelet stage to ear emergence and then anthesis is dependent on both temperature and photoperiod. Despite the large numbers of florets initiated, only some develop green anthers, still fewer reach anthesis, and still fewer set grains (Evans et al., 1975). Floret survival may be limited by assimilate availability to the ear and thus also by the duration from the start of the reproductive phase to anthesis (Reynolds et al., 1999; Miralles et al., 2000). This determination has been supported by comparing different genotypes with different photoperiod sensitivities or by modifying photoperiods within controlled environments. The plant hormones abscisic acid (negative) and cytokinin (positive) have strong influences on floret development and fertility, possibly by influencing sucrose availability to the developing ear (Waters et al., 1984; Gooding, 2009).

### 2.1.1.3 Grain Variability within Plant

In early work done by Ali et al. (1969), researchers analysed kernels at various locations along the wheat spike. After dividing the spike into three equal sections they found the lowest third of the spike contained the highest protein content while decreasing towards the top (Ali et al., 1969; Bramble et al., 2002). Although these studies investigated the spike after being divided into thirds, other work has shown gradients at the single kernel level. Apical spikelets were also reported as having significantly lower kernel weight than other kernels in the spike (Kirby, 1974). In a study that compared hardness values for several varieties grown at locations around the world, Pomeranz et al. (1985) maintains that the protein content within a single variety is more telling of hardness, than protein content in general (Pomeranz et al., 1985). Recent research by Miller (2008) on variation in single kernel hardness within the wheat spike found that the top and bottom are not significantly different, with the centre portion having significantly softer kernels (Miller, 2008). A close examination of kernel weight and diameter shows that the top yields the smallest kernels followed by the bottom portion, and the middle producing significantly larger, heavier kernels.
This report also indicated that kernel weight follows a trend similar to that of diameter with central kernels being heavier on average (although larger diameter can result from larger endosperm cavity space). In addition the second floret has a significantly larger kernel weight than the first or third floret. Earlier, Evans et al. (1972) reported similar findings and also observed that the second floret kernels develop more rapidly than other kernels (Evans et al., 1972). Previous studies have reported protein content of the second floret (Ali et al., 1969; Bremner, 1972; Jie et al., 2005), to be greater than other floret positions.

Overall the developmental pattern of the third floret paralleled the first and second, indicating that time was the factor, rather than potential, limiting the size of that kernel. Nevertheless the results indicated that the largest source of variation for all attributes was found between spikes, accounting for up to 25% of the total variation. The variation within the spike accounted for little over 3%, meaning that spike to spike variation was a shift in mean hardness per spike, with each spike having a similar internal distribution. Miller (2008) comments that normally the smallest kernels are removed during the cleaning process which are shown to be the most variable kernels. However, research has shown that this fraction would include the portion of the spike containing the highest protein content. As 70% of kernels exist in the top and bottom portion of the spike, breeding programs that focus on developing this part of the spike, could bring improvements in grain yields and reduce variability.

Previous research has suggested that vascular effects are involved in the varied translocation of nutrients to the spike. It was suggested that breeding programs may target this, to ensure even levels of assimilate, which would likely promote uniformity within the spike. Perhaps ensuring that florets reach anthesis simultaneously may be an approach to reducing variability in wheat. Moreover, improvements to grain yield may be seen by targeting the third floret kernel. This kernel appears to have equal potential to become fully developed, given enough time for kernel filling (Miller, 2008). From a biological perspective, such variability in seed characteristics in a single plant may provide greater probability for eventual germination rates over time and transient conditions. Furthermore, studies have identified differing starch granule type expression according to grain position. For example, granule volume distribution in basal wheat grains exhibited a three peak curve, whereas that in distal grains exhibited
a two peak curve, indicating that the volume percentages of type C starch granules in basal grains were higher (Dai, 2009).

2.1.1.4 Fertilization

Considering fertilization, anthesis commences typically between three and eight days after ear emergence, depending on temperature and variety. Wheat is generally self-pollinated, but some cross-pollination is possible. All else being equal, self-fertilizing species are expected to have a lower diversity level than outcrossing species. Successful seed set is dependent on the production of viable pollen grains, transfer of pollen to the stigma, germination of the pollen grains and growth of pollen tubes down the style, union of the male gamete with a viable oocyte, and normal development of a zygote following fertilization. All these steps are temperature sensitive. When nutrition is also adequate, grain set in competent florets is usually more than 80% (Subedi et al., 2000). Sterility, particularly in distal spikelets, can be caused by both heat and cold stress, nutrient deficiency, particularly of boron; droughts and water-logging; extremes of humidity; and alkalinity. Most of these factors show strong interactions with variety (Subedi et al., 2001; Gooding, 2009). The relationship between weight and numbers of caryopses formed, consequent starch granule size distribution and milling quality are discussed in Chapter 7.

2.1.2 Endosperm Development

Physiologically, grain development proceeds in three phases. The first phase is one of grain enlargement as cells multiply and expand, with rapid accumulation of water into the grain (Pepler et al., 2006). Division of the endosperm nucleus occurs within a few hours of fertilization. The first cell walls appear about three days later. The rate of cell division slows until a maximum cell number (typically around 105) is attained 15-20 days after anthesis, at about the time when rapid water accumulation stops. Final grain weight under good growing conditions has often been correlated with the water mass per grain and/or the number of endosperm cells attained during the two to three weeks after anthesis (Brocklehurst, 1977; Borras et al., 2004). Researchers have observed that environmental treatments applied during the two to three weeks after anthesis have a much larger impact on final grain size than treatments applied
later. Often 40% or more of the photosynthate ultimately found in the grain derives from photosynthesis in the flag leaf. The flag leaf is the leaf in closest vascular proximity to the ear and is usually the most important leaf for light interception during grain filling (Gooding et al. 2003).

Table 1. Major events during endosperm development. Adapted from (Stone & Morell, 2009)

<table>
<thead>
<tr>
<th>Stage No.</th>
<th>Stage Name</th>
<th>Timing (DAF)a</th>
<th>Major Cellular Events</th>
<th>Major Events in Starch Granule Development</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Syncytial</td>
<td>0-5</td>
<td>Fertilization occurs. Coencytial nuclei proliferate. Vacuoles form in cytoplasm. Proplastids are found.</td>
<td>Spherical A granules are initiated.</td>
</tr>
<tr>
<td>2</td>
<td>Cellularization</td>
<td>6-8</td>
<td>Cell walls form. Amyloplasts differentiate and divide rapidly.</td>
<td>Equatorial plate on A granules develop.</td>
</tr>
<tr>
<td>3</td>
<td>Cell division</td>
<td>9-14</td>
<td>Cells divide rapidly.</td>
<td>B granules are initiated. Equatorial plate surrounding A granules is completed.</td>
</tr>
<tr>
<td>5</td>
<td>Maturation</td>
<td>22-35</td>
<td>Endosperm and aleurone cell divisions have ceased. Seed storage products are deposited.</td>
<td>A granules complete growth through lateral deposition of starch. Equatorial groove is less prominent. B granules continue to be initiated and expand radially. C granules are initiated and develop radially.</td>
</tr>
<tr>
<td>6</td>
<td>Desiccation</td>
<td>35 to maturity</td>
<td>Desiccation occurs.</td>
<td>Starch granules are compressed into the protein matrix. Amyloplast membrane integrity is lost.</td>
</tr>
</tbody>
</table>
Alternatively, the development of the endosperm in wheat and barley has been described as being divided into six phases: syncytial, cellularization, endosperm cell division, aleurone differentiation, maturation, and desiccation (Table 1). The timing of events occurring during endosperm development is highly temperature dependent (Bhullar & Jenner, 1986). The syncytial phase is concluded by the initiation of cell walls at about three to four days after fertilization. Cell division in this period becomes highly active, and proplastids begin to differentiate into amyloplasts. Characteristics of the amyloplast seen in the endosperm at this time are both evaginations of the granule membrane ("stromules") and internal invaginations of the membrane ("tubuli") (Buttrose, 1960; Briarty et al., 1979; Stone & Morell, 2009). As the endosperm cell division phase progresses, granules are observed to first form lobes on the surface of the granule. These lobes then develop bidirectionally from one side of the granule, extending to encircle the granule and thus forming an equatorial plate (Evers 1971).

2.1.2.1 Peripheral Cell and Starch Granule Differentiation

The next phase of development involves the differentiation of cells at the outermost periphery of the endosperm, giving rise to aleurone cells. Aleurone cells typically contain minimal amounts of starch, instead accumulating oils and pigments and forming thick cell walls (Evers & Millar, 2002). Toward the end of the aleurone differentiation phase, endosperm cell division ceases (Briarty et al., 1979). Two major events occur during this phase: first, the completion of the equatorial plate of the A granule population and, second, the initiation of a new population of small B granules. The initiation of B granules is thought to occur in two locations: first, in evaginations of the amyloplast outer membrane, in which clusters of very small B granules begin to appear from approximately 15 DAF, and, second, in the amyloplast stroma around the equatorial groove of the single large A granule within the amyloplast (Parker, 1985; Langeveld et al., 2000; Bechtel & Wilson, 2003).

Following the completion of the aleurone differentiation phase, cell expansion continues. The rate of increase in the diameter of the A granules through the plane at the equatorial groove decreases. At this time, deposition of starch occurs on the tangential faces of the starch granule, leading to an increase in granule thickness and volume. B granules continue to be initiated and to grow radially.
In cooler environments where grain develops over a longer period, a third class of granules can be observed, the "C" granules, which represent a third wave of granule initiation (Bechtel et al., 1990). A-type starch granules, the largest class of starch granules at maturity, typically attain a lenticular shape of 20-50 μm diameter by maturity. B-type (5-10 μm) granules are spherical and are initiated during the second and third weeks after anthesis (Stoddard, 2003) (see also Chapter 2. Starch Granule Formation). Storage protein appears about 10 days after anthesis in membrane-bound spherical bodies (0.5-1.5 μm) derived from the Golgi apparatus, closely associated with rough endoplasmic reticulum. Finally during desiccation as the endosperm dries out, it is thought that lipid membranes surrounding amyloplasts are ruptured, forcing proteins and lipids onto the surface of the starch granule (Stone & Morell, 2009).

2.1.2.2 Starch Synthesis

The primary source of substrates for starch synthesis in wheat is the sucrose delivered to the grain through the phloem as a result of either primary photosynthesis or remobilization of stored carbohydrates such as stem fructans (Stone & Morell, 2009). Sucrose, amino nitrogen, and mineral elements are transported through the sieve tubes of the phloem (living cells that transport soluble organic material made during photosynthesis (sap)) and unloaded through symplasmic pathways (via the inner side of the cell plasma membrane and between cells through plasmodesmata).

In developing wheat grains, the outermost cells of the filial tissues are differentiated transport cells containing secondary ingrowths of the plasma membrane, which greatly expand their plasma membrane surface to facilitate uptake and transport of sucrose from the apoplastic space (inside cell walls). These plasma membranes contain sucrose/H+ symporters (integral membrane proteins involved in movement of two or more different molecules or ions across a phospholipid membrane in the same direction) responsible for transporting sucrose from the apoplast to the transfer cells (Weschke et al., 2000). Once sucrose moves into the endosperm, concentration gradients drive sucrose delivery to the sites of utilization through a symplastic continuum of interconnecting endosperm cells. The point along this continuum at which sucrose cleavage occurs is not well defined. In higher plants, sucrose can be cleaved through either of two enzymatic reactions: via sucrose synthase or invertase. The products of action by invertase (glucose and fructose) and sucrose synthase (fructose and uridine
diphosphateglucose [UDPglucose, UDPG]) are interconverted into a pool of glucose-1-phosphate and glucose-6-phosphate. This pool is utilized in the synthesis of first, ADPglucose (adenosine diphosphateglucose) and then starch (Stone & Morell, 2009).

During the linear phase of grain growth, 80% of the photosynthate produced in the flag leaf is translocated to the ears (Thorne, 1982). When post-anthesis photosynthesis is curtailed by stress, however, the proportionate contribution of remobilized stem reserves to final grain yield may increase to more than 50% from only 5-15% (Blum et al., 1994). In good growing conditions, 70-75% of the nitrogen accumulated before anthesis is subsequently remobilized to the grain. This remobilized nitrogen typically accounts for 50-70% of grain nitrogen at harvest. The remaining 50-30% of grain N derives from post-anthesis uptake by the plant, for which partition rates to the grain can reach 90%. The second phase of endosperm development is a near-linear increase in grain dry matter, starting between 10 and 15 days after anthesis and continuing, depending on temperature, for 15-30 days. The mass of water per grain during this phase is relatively constant (Pepler et al., 2006); that is, water entry to the developing grain and water loss are approximately equivalent. Many protein bodies fuse, forming a continuous protein matrix that embeds the starch granules. The third phase describes processes subsequent to the attainment of maximum dry matter per grain. The time of maximum dry matter is often taken as physiological maturity, rather than harvest maturity (Gooding, 2009).

### 2.1.3 Early Germination and Starch Granule Degradation

Concerning germination, the embryo (germ) gives rise to the radicle (the seedling root), and the scutellum (the first leaf). The other important part of the seed is the endosperm, which contains food in the form of starch and protein for germination and emergence. The coleoptile (second leaf) penetrates the soil and results in emergence of the seedling, usually within 5 to 7 days after planting as observed in the USA (Paulsen, 1997) though can be much slower in southern regions of Australia. MacGregor and Matsuo (1982) observed starch degradation in endosperms of barley and wheat kernels during initial stages of germination. During initial stages of germination in kernels of barley and durum wheat, similar physical changes were noted. These events appear to follow the same path as water permeation (see topic
above: Chapter 1, Conditioning of Wheat). On imbibition of water, the grain can swell by more than 40% at the start of germination. Both $\alpha$- and $\beta$-amylases are present in developing wheat grain, but only $\beta$-amylase is at significant levels in the mature grain. Where significant levels of $\alpha$-amylase are present, they result from one or more of the four phenomena that contribute to low Hagberg falling number (measurement of high alpha-amylase activity in flour): retained pericarp $\alpha$-amylase, prematurity $\alpha$-amylase, prematurity sprouting, and postmaturity sprouting (Lunn et al., 2001). These all result from impacts of the environment during the development, maturation, and harvesting of the grain, resulting in an abnormal composition. The major role of $\beta$-amylase is in the mobilization of starch during germination. Extensive polymorphism has been reported. $\beta$-amylases in wheat and other cereals where they act as storage proteins in some respects; they accumulate during grain development and their amount responds to nitrogen availability (Giese & Hejgaard, 1984). In mature barley grains, the enzyme becomes associated with the periphery of starch granules, presumably as a result of desiccation (Hara-Nishimura et al., 1986; Lauriere et al., 1986). The location in wheat has not been determined but is presumably similar. Therefore the transition from free to bound $\beta$-amylase probably occurs during the desiccation phase when the protein becomes associated with the surface of the starch granule (Brijs et al., 2009).

Early research provided evidence that the scutellar epithelium is mainly responsible for the synthesis of $\alpha$-amylase during initial stages of germination. Starch degradation started at the endosperm-embryo junction, usually close to the ventral crease, and moved along the junction to the dorsal edge of the kernel. This degradation was preceded by extensive breakdown of cell wall material and the protein matrix of the endosperm. After 48 hr of germination, starch degradation had become more extensive still concentrated close to the crease edge, but it extended a little further into the endosperm. In this area, cell wall and proteinaceous material had disappeared, and many starch granules showed "pin-hole" damage as well as damage to the equatorial groove. After 72 hr the internal ring structure of the granules could be clearly seen. Close to the embryo, the starch granules were almost completely destroyed. Many small granules had roughened surfaces typical of $\alpha$-amylase attack. After 96 hr of germination, most of the detectable degradation appeared to be radiating from the embryo (MacGregor & Matsuo, 1982).
However, after 120 hr extensive starch degradation was apparent and few intact starch granules remained close to the embryo or aleurone layers. These results suggest that at this stage, α-amylase from the aleurone layer was playing a major role in granule degradation, but the degraded area did not extend very far into the endosperm. Large numbers of small starch granules were visible not far from the aleurone layer, again indicating small penetration of the aleurone α-amylase into the endosperm. Despite extensive hydrolysis of large granules, many small granules still remained, but this differs from the normal situation in barley, where very few small granules are found in areas of the endosperm containing highly degraded large starch granules. This suggests that small granules of wheat starch are less susceptible to α-amylase attack than are small granules of barley starch though this finding is not inconsistent with earlier reports (Dronzek et al 1972, Lineback and Ponpipom 1977, Palmer 1972). Several small granules also contained large corrosion holes as if the α-amylase had entered the granule and preferentially hydrolyzed the interior portion. Small starch granules from wheat, then, appeared to be hydrolyzed both by surface erosion and by interior hydrolysis via erosion channels from the granule surface. These different types of attack could be caused by different carbohydrate enzymes or as suggested by Meredith (1981) wheat might contain two different kinds of small starch granules (Meredith, 1981; MacGregor & Matsuo, 1982) (see topic, Chapter 2. Starch Granule Size Distribution and Development).

2.1.4 Grain Maturity

The grain begins growing immediately after flowering and reaches its maximum size (not weight) within about 2 weeks. The maximum weight occurs about 4 weeks after flowering (in Kansas, US). This period is determined largely by temperature and can extend up to 12 weeks in areas where the weather is cool. Grain development stages are determined by the hardness or consistency of the endosperm of the new kernel. Physiological maturity occurs when the kernel has accumulated its highest content of dry matter, has hardened, and changed colour. The kernel contains 30 to 35 percent water at physiological maturity. Most of the protein within the kernel comes from nitrogen previously accumulated in the leaves, and most of the starch is from sugars made by photosynthesis during the grain-filling period. The nitrogen
moves into the filling kernels to form protein during early grain development. If yields are low because the kernels do not fill properly, the grain is high in protein. Drought and high temperatures are usually responsible for this condition. If the grain fills normally and yields and test weights are high, grain protein is frequently lower because it is diluted by other materials. Under good growing conditions, grain protein can be increased with nitrogen fertilizer. Yields are high when favourable filling conditions, mild temperatures, and active leaves promote growth of large, plump kernels. High temperatures, especially when accompanied by winds and foliar diseases such as leaf rust produce shrivelled kernels, low test weights, and low yields. Ripening includes the changes that occur after the grain reaches physiological maturity. The most important change is the loss of moisture from 30 to 35 percent in mature grain to 12 to 13 percent in ‘combine-ripe’ grain. Grain must be harvested promptly after ripening to save the yield. Hail, lodging, and preharvest sprouting are ever-present threats to ripe grain (Paulsen, 1997).

2.2 Wheat Grain Structure

The wheat grain is composed of the husk (fused pericarp and testa), aleurone, endosperm, and embryo. However, because of the greater bulk of the endosperm, the hardness of the grain is largely determined by the properties of the endosperm. The embryo is soft and accounts for only a small proportion of the total grain. The starchy endosperm itself is a composite. The dried endosperm consists of cells filled by the protein/starch granule matrix and separated from the neighboring cells by cell walls. The resistance of the grain to deformation (hardness) would be determined by the weakest phase of this composite material. It is possible that many genes may alter the hardness of the grain; however, only those genes that show variation in the genetic analysis performed will be detected. So far the most well characterised source of variation is the Ha locus located on the chromosome 5DS (Piot et al., 2001a). The characterisation of the mechanical properties of materials is important in gaining a better understanding of their functional properties. Wheat caryopses are small and possess a complex geometry that makes such characterisation difficult. Consequently, a number of simple, rapid, bulk hardness methods such as near infrared analysis (NIR hardness) have become widely used in the industry (Pomeranz & Williams, 1990). Researchers have reported considerable variation in seed weight and diameter
within as well as between heads. In general the largest grains occur in the centre of the inflorescence and, in species with multiple tillers, the mean grain size decreases progressively from grain borne on the main culm (see topic below: Starch Granule Size Distribution and Development). Some of the best milling wheat types such as the hard classes grown in North America have characteristically small grains. More uniform wheat samples give more predictable milling performance. In addition large grains have a propensity to develop larger endosperm cavities (adjacent to the crease) and therefore do not always contain more endosperm than small grains (Evers & Millar, 2002). This observation infers that kernel weight should correlate more consistently with flour yield rather than kernel diameter (see topic above: Alternative Predictive Measurements of Flour Yield).

In wheat, endosperm cells have comparatively thin walls and can be subdivided into three populations: peripheral cells occurring as a single sub-aleurone layer and are approximately 60 μm in diameter, prismatic cells 128-200μm long x 40-60 μm wide, and central rounded or polygonal cells (72-144μm long x 69-120μm wide). Sub-aleurone and prismatic cells do not appear next to the modified aleurone cells in the crease region (Figure 4). To support the embryos early growth in germination, reserves stored in the endosperm are solubilised by hydrolytic enzymes (α-amylase), in which condition they can be assimilated into the new plant (Evers & Millar, 2002). Lignin distribution has been reported to affect the position of fracture within various cell wall layers. Together with the ferulate derivatives, the lignification pattern of bran layers could therefore affect the fractionation and mechanical behaviour of wheat grain. In addition, besides the influence of cell structure on its anisotropic character, the pericarp is characterised by higher cellulose content. Therefore it could be suggested that pericarp cellulose microfibrils take part in the higher rigidity of this tissue (Antoine et al., 2003).
Figure 4. Transverse section of (A) outer endosperm and (B) wheat grain (adapted from Fleckinger, 1935).

Mabille et al. (2003) proposes that three major factors of grain morphology affect milling performance: the endosperm/bran ratio, the endosperm vitreousness, and the separability of the starchy endosperm and the bran coat. This separability factor appears to be connected both to tissue adhesion and to tissue mechanical properties, which must be independently investigated (Mabille et al., 2003).
2.2.1 Bran Fractionation

The traditional aim of the wheat milling process is to obtain the best possible dissociation of the starchy endosperm from the other parts of the grain to yield the white flour. Pericarp, seed coats, nucellus and aleurone cells form the bran. The wheat germ is partly eliminated during grain preparation and the remainder is found as large flat particles in middlings and bran fractions. Studies have shown that the tissue composition of bran obtained by milling wheat grains is in the general range: starchy endosperm 15%, aleurone layer 49%, intermediate layer 30%, and outer pericarp 6% (Peyron et al., 2002b). However, the aleurone and intermediate layers which exhibit similar behaviour during the grinding process are difficult to separate by size classification. Future investigations to determine if specific conditions could be found for their dissociation and separation may involve the impact of moisture content on mechanical properties. In particular, the particle size reduction of the different tissues appears to be related to their intrinsic extensibility. To further this endeavour, Antoine et al. (2004) identified a dehydrotrimer of ferulic acid (DHT), and p-coumaric (p-CA) acid (also phytate contents) as efficient markers for the quantification of the outer pericarp and aleurone cells, respectively, in wheat grain fractions. Cereal cell walls are mainly composed of arabinoxylans and β-glucans, with smaller amounts of cellulose and lignin (Fincher & Stone, 1986; Schwarz et al., 1988; Mandalari et al., 2005). Arabinoxylans are known to be esterified by ferulic acid, which contributes to polymer cross-linking. The modulation of tissue mechanical properties by these polymer cross-links has been suggested in a number of studies (Tan et al., 1992; Wakabayashi et al., 2005). Also, Peyron et al (2002) showed that aleurone extensibility in durum wheat is affected by the degree of arabinoxylan cross-linking by ferulic acid dehydrodimers (Peyron et al., 2002a). Furthermore, the pericarp contains the highest level of dehydrodimers and is the least extensible caryopsis tissue (Antoine et al., 2003). These researchers concluded that components involved in controlling the rheological properties of bran tissues must be identified in order to improve dry fractionation processes (Antoine et al., 2004).
2.2.1.1 Aleurone Layer Features Determining Milling Performance

Related to both cell structure and cell wall organization, the extensibility of the aleurone layer is a critical component of milling behavior because it determines, in part, the dissociation between the bran coats and the starchy endosperm and, as a consequence, the purity of the flour or semolina. Investigations of the relationships between cell-wall structure of the different tissues and bran mechanical properties demonstrated that cell-wall cellulose or arabinoxylan contents are not determining factors of bran rheological behaviour. Rather, interactions between polymer and the degree of arabinoxlan cross-linking in the aleurone cell-wall expressed by the ferulic acid dehydrodimer/xylose ratio appear to be positively correlated with bran extensibility. Consequently such analysis has been proposed as a new indicator of wheat milling quality (Mabille et al., 2003).

2.2.1.2 Aleurone-Endosperm Interface

Earlier studies have not been able to identify any significant relationship between aleurone layer heterogeneity and the quantity of starchy endosperm eliminated in the bran fraction. Consequently research has focused on cell-wall components located at the aleurone-endosperm interface that are involved in tissue cohesion, and also to attempt to measure this adhesion (Mabille et al., 2003). This kind of investigation became possible using a Raman microspectrometer, which results from the coupling of a Raman spectrometer and a confocal microscope. The first studies performed on the aleurone cell-wall at the endosperm interface identified, in particular the high concentration in phenolic compounds that are known to play a part in cell-cell adhesion and consequently in tissue cohesion. Furthermore spectral images of aleurone cells displayed the distribution of phenolic acids in the cell-wall. In particular, a higher concentration of phenolics in anticlinal cell-walls and a lower concentration in the periclinal cell-wall, at the sub-aleurone interface were noted. This provided the first example of the high spatial resolution offered by this in situ analysis technique (Mabille et al., 2003).
The aleurone layer and nucellar epidermis may be the two cell layers that contribute most of the overall mechanical behaviour of the bran. The aleurone layer makes up over 50% of the overall bran thickness. It has a cell wall of intermediate thickness composed largely of heteroxylans and 1, 3:1, 4-β-glucan. Directly attached to the aleurone layer is the nucellar epidermis, which is a compressed cell layer with cell walls of the thickness and compositions similar to aleurone cell walls. In a study by Glenn and Johnston (1992) the tensile strength of the bran was five to tenfold greater than the tensile strength reported for the starchy endosperm (Glenn & Johnston, 1992b). The capability of bran to be deformed far beyond that of the starchy endosperm without breaking could be largely attributed to the structure of the aleurone layer and nucellar epidermis. The cell layers have moderately thick cell walls composed of microfibrils embedded in an amorphous matrix. The amorphous regions of cell walls are thought to be moisture absorbent and capable of swelling. This property renders the cell wall flexible and able to distort without breaking. The ability of the nucellar layer to deform without breaking may be further attributed to its cellular structure. The nucellar epidermis is a compressed cell layer consisting of lysed cells. Consequently, there is no resistance to deformation due to cell turgor. This could permit the cell wall structure to deform in a manner similar to a coiled or braided rope before breakage would occur (Glenn & Johnston, 1992b).

### 2.2.2 Cell wall composition & Arabinoxylans (AX)

The implication of the kernel wall in the cereal grain cohesion is not well documented. Bulk biochemical analysis has not shown any compositional differences between cell walls of hard and soft endosperm (Mares & Stone, 1973); the architecture of the cell walls and local compositional heterogeneity could also play a role. Plant cell walls give form and mechanical strength to the living plant. The mechanical properties of cell walls are controlled by their thickness and density and also by their complex architecture at the molecular level. In the physiological context of the wheat kernel, cell wall composition has to be appropriate to allow both resistance to the turgor pressure, and wall expansion during cell growth (Piot et al., 2001a).
Cell walls are thin in relation to cell contents. Walls around the peripheral cells are thickest, being up to 7 µm thick in the crease region and up to 4 µm elsewhere. The central cell walls are 2.6 µm thick, with a negative relationship between cell wall thickness and milling score (Larkin et al., 1952). The cell walls of wheat starchy endosperm comprise about 15% protein and 75% polysaccharide (Mares & Stone, 1973) the latter comprising about 70% arabinoxylans, 20% (1→3,1→4)-β-D-glucan, 7% β-glucomannan, and 2% cellulose (Bacic & Stone, 1980). Wood et al (1983) confirmed the presence of ferulic acid in subaleurone cell walls by its autofluorescence, and the mixed-linkage β-D-glucan was also detected by staining with fluorochromes Congo red and Calcofluor (Wood et al., 1983). Some cellulose was also detected. Guillon et al (2004) used antibodies against arabinoxylan (AX) and (1→3,1→4)-β-D-glucan to study mature wheat grain (Guillon et al., 2004). Two antibodies that recognize specific structural features of AX showing differences in their binding to the cell walls of aleurone, subaleurone, and central starchy endosperm cells, exhibited fine differences in cell wall structure, while the antibody to (1→3,1→4)-β-D-glucans bound most strongly to aleurone and subaleurone cell walls (Bechtel et al., 2009).

Arabinoxylans (AX) are hydrophilic non-starch polysaccharides found in wheat grain as minor constituents. In the primary cell wall, cross-linking between polysaccharides [such as AXs/cellulose and AX/AX through phenolic bridges] contributes to wall assembly, promoting tissue cohesion and regulating cell expansion and wall strength (Kamisaka et al., 1990; Fry et al., 2000). The walls that surround the cells in the starchy endosperm consist predominantly of AXs (60-70%), i.e. linear chains of β-1-4 xylene with branchings of arabinose (generally as monomers, but with a small percentage of oligomers) to which ferulic acid can be covalently linked via ester bonds. The esterified ferulic units can provide cross-linking between AX chains by formation of diferulic covalent bonds and, more rarely, formation of truxillic acid by photo dimerisation. The degree of branching, the spatial arrangement of arabinosyl substituents along xylan backbone, and the ferulic content are the main factors that determine the physico-chemical properties of the AX chains, such as their mechanical resistance or their elasticity. In another study, grain development was examined using Fourier transform infrared microspectroscopy.
A difference in the degree of AX substitution was found between peripheral and central parts of the grain at the cell differentiation stage; AX in central cells was less substituted (Philippe et al., 2006a). These properties are also likely to contribute in the mode of fracture propagation at endosperm cell walls and consequently to the easiness of endosperm fragmentation (Piot et al., 2001a). Nevertheless due to the thinness of cell walls in proportion to the mass of starch and protein, some researchers consider the quantity and effect of cell wall can almost be neglected (Wang & Jeronimidis, 2008).

2.2.2.1 AX and Hardness

The AXs in the endosperm of wheat are only composed of arabinose and xylose and for this reason are often referred to as pentosans. They are found as water-extractable (WE-AX) and water-unextractable (WU-AX) fractions. Total AX content in wheat flour is about 2.2% and approximately 1/4 of total AX are WE-AX. The arabinose to xylose ratio (A/X) is often used to characterize the structure of AX, and the average value of A/X ratio is 0.5 for WE-AX (Saulnier et al., 2007). Water extractable AX levels in wheat have been shown to vary much more than total AX levels. WE-AX content is primarily influenced by genotype, while WU-AX content is more greatly influenced by the environment (Finnie et al., 2006; Dornez et al., 2008). Polymers of xylose and arabinose (Pentosans) with a β-1,4 linked xylan providing the backbone and arabinose often occur as 1, 2 or 1, 3 linked substituents; however, there is variation in the nature and number of substituents and this may be the basis of the division into soluble and insoluble pentosan fractions. A study by Bettge and Morris (2000) found that the amount of pentosan appeared to modify the hardness within the soft category much more than within the hard and suggested that this could be one source of variation in grain hardness that was not controlled by the Ha locus. Alternatively in a recent study of 25 hard spring wheats grown in three environments, SKCS hardness was negatively correlated with WE-AX and proportion of total AX that was WE-AX (r = -0.46 and -0.51, respectively). When the location of AX in the grain is considered, hard wheat is clearly distinguishable from soft wheat (Barron et al., 2005). Cell walls in the peripheral layer in soft wheat have been characterized by a higher level of water-extractable AX than are those of hard wheat. The relation of this observation to conditioning response and milling performance is unclear and deserves further investigation. The differences between the walls of endosperm cells
of hard and soft wheats become apparent at about 15 dpa at the stage when the hard/soft characteristic is manifest (Bechtel et al., 1996). In later stages, changes in wall composition also occur, as shown in immunochemical studies (Philippe et al., 2006b; Stone & Morell, 2009).

2.2.2.2 AX and Water Diffusion

Li et al. (2009) concluded that the manipulation of arabinoxylan content of wheat grain was a reasonable breeding objective. Hong et al (1989) suggest that pentosan (AX) quality, rather than quantity, may be important in determining different levels of hardness (Hong et al., 1989; Turnbull & Rahman, 2002). Arabinoxylans can associate with large amounts of water through hydrogen bonding and can form oxidative gels. AX and cell walls are involved in water transport or diffusion during the different physiological stages of grain development, desiccation, and germination. It is therefore possible that structural variation is involved in modulating the hydration properties of the cells walls to regulate the water content of the grain (Saulnier et al., 2007). Consequently such function may have significant influence on the grains response to conditioning and subsequent milling performance. Nevertheless in a study of the breakage of endosperm cell walls in hard red winter wheat flours, Schulze and MacMasters (1962) observed that cell walls are broken transversely. Cell contents may be broken into two or more parts or may remain intact. In either case, none or part of the cell wall may remain with the particle. When the cell contents remain intact, the complete cell wall may also remain, but will then carry with it the adjacent cell walls of neighbouring cells, with the middle lamella. There is no evidence that there is any cleavage between cell walls, at least along the middle lamella. Walls between adjacent cells are cemented together, by a thin layer known as the "middle lamella". These researchers commented that what is known of plant cell-wall structure and properties supports their observations. Apparently the adhesiveness between cell walls and middle lamella is too strong to be broken during milling including various conditioning methods (Schulze & MacMasters, 1962).

Proteins are known to contribute in the cohesion of plant cell walls as, for instance, primary cell walls contain a typical protein named extensin possessing structural properties. Extensin forms a network by an intramolecular cross-linking
resulting from the formation of an isodityrosine bond between two tyrosine residues. In cereal cell walls, although few studies have been carried out on the possible role of protein networks, proteins covalently linked to arabinoxylan via ferulic units esterified to arabinose branches might be involved in maintenance of the structure. Lipids are also likely to play a role in the cell wall structure (Piot et al., 2001a). Taken together, cell-to-cell adhesion and intrinsic mechanical properties of the cell walls are also implicated in grain quality attributes contributing to variation in fractionation (Waldron et al., 1997; Peyron et al., 2002a).

2.2.3 Storage Proteins and Vitreousness

2.2.3.1 Protein Matrix Formation

The bread-making potential of wheat is largely derived from the quantity and quality of its protein content. Protein quantity is influenced mainly by environmental factors, while the quality of the protein is largely genetically determined. Wheat is unique among cereals in that its milled product, flour, alone when mixed with water is capable of forming a dough that will retain the gas evolved during fermentation and, on baking, will yield a light, well aerated loaf of bread. This unique characteristic of wheat is derived from its proteins which, on combining with water during the mixing process, result in gluten, the actual substance that imparts the property of gas retention to dough. Gluten is comprised of two insoluble proteins, glutenin (which is stable, and gives it its strength), and gliadin (which is soft and sticky, and gives it its elasticity). Previous studies provide evidence that gluten protein granules occur both within the lumen of the Endoplasmic Reticulum and the vacuole, with the latter arising from transport via the Golgi apparatus. Based on such observations, Galili and coworkers (Levanony et al., 1992; Galili et al., 1995; Galili et al., 1996; Galili & Herman, 1997) have proposed that two routes of protein body formation occur, with the populations of Endoplasmic Reticulum -derived and vacuole-derived bodies subsequently fusing. The mechanism of this fusion is not fully understood also it is not clear whether both routes operate throughout grain development or whether individual gluten proteins take similar or different routes. The formation of large numbers of protein bodies, coupled with starch granule enlargement, causes the cytoplasm to be isolated into small regions.
These regions condense with protein to form the continuous matrix during final stages of maturation associated with water loss (Bechtel et al., 2009).

2.2.3.2 Storage Protein Expression and Localization

It is well-established that gradients exist in the starchy endosperm with the outer sub-aleurone cells being richer in proteins with less starch than the central endosperm cells. The peripheral cells have the lowest starch content and, since all cells contain approximately the same mass of protein, the protein percentage is highest in these cells. Values as high as 54% protein have been found in sub-aleurone cells in a flour of 12.5% protein (Kent, 1966). The increasing starch content found toward the centre of the cheeks causes progressive dilution of other components as well as protein (Evers & Millar, 2002). In a study of protein content from different grain sizes, statistical analysis by Konopka et al. (2007) indicated that grain protein composition is affected by the cultivar and kernel size, as well as by interaction of these traits. The albumin/globulin and glutenin fractions showed a tendency to decrease with diminishing kernel size. The gliadin content was affected to a greater extent by the kernel size than by the wheat genotype. Interestingly, the highest content of gliadins was observed in the smallest and the largest kernel fractions (Konopka et al., 2007).

Protein accounts for approximately 10-13% of the grain and consists mainly of gliadins and glutenins. With near isogenic (similar genotype) Heron lines, the soft wheat cultivar was found to have a higher, protein content (13.7%) than the hard cultivar (11.8%) yet it could be clearly distinguished as a soft. Conversely, wheat lines with different storage protein patterns and dough properties can have very similar hardness scores (Turnbull & Rahman, 2002). Nitrogen fertilization has been demonstrated to affect mostly the synthesis of storage protein, especially glutenin and gliadin, whereas albumin and globulin were not or very little affected by fertilization (Triboi & Branlard, 1990; Wieser & Seilmeier, 1998). The gliadin-to-glutenin ratio increases with the level of nitrogen fertilization (Doekes & Wennekes, 1982; Triboi et al., 2000). According to Wieser and Seilmeier (1998), the increase of nitrogen quantity in soil involves an increase in minor proteins (omega-gliadin and high molecular weight glutenin (HMW-G)) and a reduction in hydrophobic proteins (gamma-gliadin and LMW-G) (Wieser & Seilmeier, 1998; Samson et al., 2005). Wheat crops sown at
their highest yielding times with legume based rotations can facilitate production of proteins in the appropriate ranges for premium paying grades.

Dexter et al (1989) found that the percentage of gliadin was greater in vitreous endosperm (Dexter et al., 1989). Moreover, the greater proportion of gliadin in vitreous kernels was associated with a harder texture (Gianibelli et al., 1991). Dexter et al. (1989) and Dexter and Edwards (2001) suggest that in vitreous endosperm, a high gliadin content will allow a better adhesion of the protein matrix on starch granules during kernel desiccation, leading to a compact endosperm structure (Dexter et al., 1989; Dexter & Edwards, 2001). Conversely, a lower gliadin content would provide a discontinuous protein matrix. The result is a more friable structure with air vacuoles that lower the density of endosperm (Matveef, 1963). In a recent study by Samson et al. (2005), biochemical analyses were performed on 270 kernels, mealy or vitreous, hand-picked from 148 different crops. The glia/glu ratio appeared to be a less accurate predictor of kernel vitreousness, indicating that, by itself, it cannot account for the change in kernel vitreousness. Studies have shown that endosperm vitreous texture rises above a threshold content of 9.7% protein within the endosperm (Samson et al., 2005). Few research articles have documented the interaction between gluten proteins (gliadin and glutenin) and the starch granule surface as it relates to flour functionality (Sandstedt, 1961; Hoseney et al., 1971; Larsson & Eliasson, 1997; Greffeuille et al., 2007). Nevertheless recent research by McCann et al. (2009) indicated that in gluten, glyco-lipids are likely to be associated with glutenins through both hydrophobic interactions and hydrogen bonds whilst phospholipids preferentially interact with gliadins and lipid binding proteins (McCann et al., 2009).

2.2.3.3 Vitrification

According to Hoseney (1986), the way endosperm dries and shrinks appears critical for cytoplasmic cohesiveness. Thus, vitreousness could be determined mainly by physical factors independent of the biochemical composition of the protein matrix (Hoseney, 1986; Greffeuille et al., 2007). Several studies on the effects of high temperature during grain filling and drying showed that flour from wheat harvested before maturity had quality traits superior to those of flour from wheat allowed to mature in the field (Finney, 1954; Finney & Fryer, 1957; Finney et al., 1962). Finney (1954) found that wheat that was harvested 10-14 days before maturity exhibited
optimum loaf potential as well as excellent crumb structure (Finney, 1954). This has implications for the effect on processing by the small starch granules expressed in the later stages of grain filling. The dough mixing requirement and mixing tolerance of flour from pre-ripe wheat were also generally superior to, but never worse than, those of dough from wheat harvested at field maturity. The maximum loaf volume potential was apparently related developmentally to the time that the storage protein bodies fused to form the protein matrix present in mature wheat endosperm (Bechtel et al. 1982b). The molecular and biochemical changes accompanying the structural changes during senescence require further investigation (Bechtel et al., 2009).

The agronomic conditions (water and nitrogen availability) and environmental conditions (temperature and light intensity) during grain filling and the rate of drying at maturity affect grain vitreousness (Parish & Halse, 1968; Bechtel et al., 2009). Based on studies on plant seeds, the vitrification hypothesis proposes that mixtures of accumulated non-reducing sugars and highly hydrophilic proteins enter a glassy state during dehydration and thereby immobilize membranes and macromolecules in the cytoplasm, protecting them from denaturation, coagulation, and disintegration (Sakurai et al., 2008). During drying, the cellular viscosity increases dramatically and in the dry state, the cytoplasm transforms into a glassy state. The storage stability of seeds is related to the packing density and molecular mobility of the intracellular glass, suggesting that the physico-chemical properties of intracellular glasses provide stability for long-term survival. Intracellular glasses exhibit slow molecular mobility and a high molecular packing, resembling glasses made of mixtures of sugars with proteins, which potentially interact with additional cytoplasmic components such as salts, organic acids and amino acids (Buitink & Leprince, 2008). The transition of developing seeds from the phase of reserve accumulation to desiccation is associated with distinct gene expression and metabolic switches. Interestingly, a significant proportion of the gene expression and metabolic signatures of seed desiccation resemble those characterizing seed germination, implying that the preparation of the seeds for germination begins already during seed desiccation (Angelovici et al., 2010).

2.2.3.4 Glutenin Distribution during Desiccation

Carceller and Aussenac (1999) reported a co-ordinated accumulation of different groups of storage proteins and polymer levels which was progressive until
seed maturity. The accumulation rate varied between different groups of proteins, indicating differential regulation of protein biosynthesis. Results indicated that regulation could be considered at three levels. First, the order of accumulation of protein was: albumins/globulins, monomers, and then polymeric proteins. Unlike monomers and polymeric proteins, the accumulation of albumins and globulins continued only until the early stages (during the cell division), confirming that these are metabolic or structural proteins. Second, the ratio of polymers/monomers was stable during the cell division and the cell enlargement stages (until 31 days after anthesis (DAA)) and then increased during the desiccation stage. However inconsistent results at this stage have been reported. In the opinion of Stone and Nicolas (1996), some of these differences may be related mainly to the method of analysis for polymer estimation, which varied between authors (Stone & Nicolas, 1996). For two wheat varieties (Soissons and Thesee) possessing Glu-D1 subunits 5+10 and 2+12, respectively, the period of most rapid accumulation of SDS-insoluble polymers coincided with the period of rapid water loss (after 32 DAA). Woodman and Engledow (1924) found that the first signs of gluten formation corresponded with the commencement of grain desiccation (Woodman & Engledow, 1924). Whatever the physiological stage of the grain during the cell enlargement phase (between 20 and 32 DAA), the massive loss of grain water results in the same phenomenon, that is, an increase of the polymeric content with a deposition of the insoluble fraction.

During the desiccation phase of the kernel that coincides with the breakdown of the protein bodies (Pernollet & Camilleri, 1983), the massive loss of water induces the in-solubilisation of a part of the polymeric material previously accumulated. Explanations for the late occurrence of glutenin polymer insolubility may include: the process of water loss from the grain may enhance disulfide bonding and the creation of SDS-insoluble polymers, and secondary interactions occurring during compression of protein bodies at the end of development. In this hypothesis, the loss of water from the kernel promotes the aggregation of the polymeric structures by facilitating in particular the interactions between the repetitive domains (inter-molecular (β sheets) of the glutenin subunits (Pezolet et al., 1992; Popineau et al., 1994). Taken together the results of this study provided evidence that the aggregation level obtained was highly influenced by the polymerisation level reached during grain development (Carceller &
Aussenac, 1999). Protein accumulation and quality and the extent of vitrification are primary quality traits affecting trading value, flour usage potential, and milling characteristics, for example, particle size (see topic below: Vitreousness).

### 2.2.4 Puroindolines

Greenwell and Schofield (1986) evaluated the surface components of water-washed starch granules and identified an unbroken molecular pattern between soft- and hard-textured wheat. A group of ~15 kDa proteins (friabilin) was found in greater quantities on water-washed starch from soft wheat than on equivalently treated starch from hard wheat and absent from durum wheat starch, the hardest wheat class (see topic introduced below: Theories of Wheat Endosperm hardness) (Greenwell & Schofield, 1986). Puroindolines are basic cysteine-rich polypeptides with a mean molecular weight of 12.8 kDa and contain a unique amphiphilic tryptophan-rich domain (Turnbull & Rahman, 2002). Large insert bacterial artificial chromosome (BAC) type clones have been isolated that contain all three Ha locus marker genes (puroindoline-a, puroindoline-b, and gsp-1) demonstrating that these genes are physically within 100 kb of each other in the genome. Analysis of the promoter regions from puroindoline-a, puroindoline-b, and gsp-1 genes has revealed a high level of sequence identity (72%) between the upstream regions; consistent with the hypothesis that puroindolines can act collectively to enhance grain softness.

The genes encoding puroindoline-a and -b share 55% nucleotide similarity. Both puroindoline-a and -b are believed to be synthesised as preproteins. They contain a cysteine skeleton that is also found in lipid transfer proteins (LTP) and α-amalyse inhibitors and the similarities between the primary and secondary structure of puroindolines and LTPs have been noted. The third and minor component of friabilin is GSP-1 which shares sequence homology with a 30 kDa arabinoxylanase isolated from wheat. The significance of this homology has yet to be determined (Turnbull & Rahman, 2002). In an exhaustive investigation of Gsp-1 genes in wheat, the interesting conclusion was made that despite the great sequence diversity, the functionally important amino acids involved in lipid binding, i.e., the 10 cysteines and two tryptophans, were retained in all putative proteins. The results suggest that these genes
may be functionally important, particularly in durums which lack puroindolines, and may have major roles in plant defence but only a minor influence on grain texture (Gollan et al., 2007). For comprehensive reviews of the molecular genetics of puroindolines see (Bhave & Morris, 2008a, b).

2.2.4.1 Location of puroindolines within the endosperm

Immunolocation studies using monoclonal antibodies raised against puroindoline-a and -b have shown that puroindoline-a is located primarily in the starchy endosperm. In an early study, Puroindoline-b in comparison, was indentified in the aleurone and possibly also in the starchy endosperm (Dubreil et al., 1998). In the aleurone, puroindoline-b was located in the small inclusion bodies and in the endosperm whereas puroindoline-a was located at the interface between the starch granule and the protein matrix. Interestingly, no immunolabelling of puroindoline-a occurred when starch granules were not surrounded by proteins. The location of the puroindoline proteins in these cells has more recently been confirmed by tissue printing of developing grain, using a highly specific monoclonal antibody for detection and an antibody to the aleurone-localised 8S globulin as a control. This study provided clear evidence that puroindolines are only synthesized and accumulated in the starchy endosperm cells of the wheat grain (Wiley et al., 2007). Accumulated evidence indicates puroindolines possess antifungal/antibacterial properties which may contribute to plant defence presumably of endosperm starch storage (Dubreil et al., 1998; Krishnamurthy et al., 2001; Charnet et al., 2003; Giroux et al., 2003; Jing et al., 2003; Faize et al., 2004; Capparelli et al., 2005; Llanos et al., 2006; Capparelli et al., 2007; Luo et al., 2008). For example, Puroindolines seem to aid in the protection of starch molecules from microbial digestion in the rumen, potentially increasing the amount of starch entering the small intestine (Swan et al., 2006a).

2.2.4.2 Puroindoline Protein Interactions

While the biological function of puroindolines is still a matter for further research, the mode of action of these proteins may be through their lipid binding properties. This may explain the association of a locus controlling the level of free polar (hydrophilic) lipids in the grain (Fpl-2) with the Ha locus (Panozzo et al., 1993)(see topic below: Lipids). Initial evidence suggesting a lipid-binding role for puroindolines was based on the homology between the primary structure of
puroindolines and wheat LTPs. With the exception of the tryptophan rich domain four of the five disulphide bridges of puroindolines are in identical positions to that found in LTPs. It has been proposed that puroindolines interact with lipids via the tryptophan-rich domain where the domain forms a membrane-anchoring loop between α-helices (Wilde et al., 1993; Marion et al., 1994; Kooijman et al., 1997). Previous studies have attempted to elucidate the mechanisms of puroindoline interactions with variable results. Polar glycolipids and phospholipids show patterns of distribution on starch granules similar to those of friabilin (Greenblatt et al., 1995). For example Dubreil et al. (1997) identified that PINA binds phospholipids and glycolipids tightly, whereas PINB only binds negatively charged phospholipids and forms lipoprotein complexes with glycolipids (Dubreil et al., 1997). Tryptophan residues are believed to facilitate interactions between proteins and membrane phospholipids (Dubreil et al., 1997; Le Guerneve et al., 1998). Furthermore, because complete or partial loss of functionality of either puroindoline a or b will result in hard kernel texture in common wheat, there must be some kind of interaction between them, either directly or indirectly. When either has a mutation, the interaction becomes weak and their binding activity to lipids will also be changed (Xia et al., 2005). Adhesion of PINB to the surface of starch granules is likely to be mediated by PINA, which seems to be tightly bound to membrane lipids (Gazza et al., 2005); therefore, the absence of PINA would result in a dramatic decrease in the association of PINB with the starch granule surface (Capparelli et al., 2003; Gazza et al., 2005).

As indicated in this study, PINA-null transgenic lines indeed had low levels of friabilin on the surface of their starch granules (Xia et al., 2008). The data therefore suggest that Pin-b mutants having single residue substitutions within their tryptophan-rich loop that are expressed in some hard-textured wheat varieties influence the degree of penetration of Pin-a and Pin-b into anionic phospholipid films. These findings highlight the key role of the tryptophan-rich loop in puroindoline-lipid interactions (Clifton et al., 2007a). The lipid binding capacity of puroindolines is important in many aspects of cereal processing. In bread, puroindolines are thought to prevent destabilisation of foams by oil globules. Puroindolines also affect dough extensibility and tenacity and, thereby, affect the texture of baked products (Dubreil et al., 1998).
A mutation in the tryptophan rich domain of the *puroindoline-b* gene has been highly correlated with grain texture. Each of the common three mutations in *puroindoline-b* are proposed to cause a 'loss-of-function', resulting in a hard wheat (Lillemo & Morris, 2000). Other results indicate that PINA or PINB can act alone leading to intermediate-textured grain or can function together to give a soft grain texture (Wanjigi *et al.*, 2007a). Other results indicate that increased PINB increases total PIN starch levels and decreases grain hardness more than increased PINA. This further demonstrates that PINB limits the binding of PINA to starch, and grain softness in soft wheats (Swan *et al.*, 2006b). Giroux and Morris suggested that either the loss of puroindoline-a or the alteration of the puroindoline-b sequence might alter the lipid binding capacity of the puroindoline complement and this could affect the way in which the membranes collapse during desiccation leading to alterations in the grain hardness (Giroux & Morris, 1998; Turnbull & Rahman, 2002). There have been several surveys of large collections of wheat varieties, germplasm, and other genetic resources resulting in the discovery of additional and less common puroindoline sequences (Bhave & Morris, 2008a; Morris & Bhave, 2008).

### 2.2.5 Lipids

Lipids occur in the various endosperm membranes, the aleurone layer and in starch granules of wheat. A previous study observed that the subaleurone region had the highest concentration of oil bodies, that is, free (non-membrane associated) lipids and the central region had the lowest (Hargin *et al.*, 1980). Lipids play a varied and often important role in many of the processes involved in milling, dough mixing, bread-making, and staling. The integral lipids of cereal starches are composed of lysophospholipids (LPL) and monoacyl lipids [free fatty acids (FFA)]. The relationship between endosperm texture and the amount and type of lipid is not clear but there have been several reports showing a correlation between these traits.

Non-polar (NL; hydrophobic) fractions have been found to dominate in the lipid composition and their proportion has been measured at 70.5–78.2%. The high content of NLs in kernels indicates maturity, as immature cereal grain is characterised by the prevalence of membrane structural lipids, mainly Phospholipid (PhLs). Flours of harder wheat varieties were found to contain more free non-polar lipids.
Most of the FNLs of grain and flour are represented by storage triacylglycerols (TAGs). Their sources are oil bodies, spherosomes. A majority of spherosomes are located in the aleuorone layer and germ and their concentration in starchy endosperm is considerably lower (Konopka et al., 2005).

**Figure 5.** The subdivision of wheat flour lipid classes. TAG = triacylglycerides/triacylglycerols, DAG = diacylglycerides/diacylglycerols, MAG = monoacylglycerides/monoacylglycerols, FFA = free fatty acids, SE = steryl esters, HCBN = hydrocarbons, LPC = lysophosphatidylcholines, LPE = lysophosphatidylethanolamines. (adapted from Chung et al., 2009)

Ohm and Chung (2002) reported that hardness scores obtained by NIR spectroscopy had significant and negative correlations with glycolipids (GL) \( r = -0.70, P < 0.05 \), monogalactosyldi-glycerol (MGDG) \( r = -0.70, P < 0.01 \), and digalactosyldiglyceride (DGDG) \( r = -0.57, P < 0.10 \) contents of the Free Lipids (FL) among 12 hard winter wheat cultivars (Ohm & Chung, 2002). This result suggests that free GL could influence the variation in kernel hardness of hard winter wheats by chemical interaction or by close genetic relationship to the hardness genes, as reported
by Morrison et al (1989) within hard winter wheats (Morrison et al., 1989). However, Konopka et al. (2005) found that simple linear correlation coefficients between endosperm hardness (as measured using an indentation method) and its lipid composition indicated that hardness was positively correlated with the content of free glycolipids ($r = 0.82$) and negatively with the content of surface lipids of starch, especially with their non-polar fraction ($r = -0.83$) (Konopka et al., 2005). FL in wheat obtained from six hard winter wheat varieties harvested at eight locations from 1995 to 1997 in Kansas were analysed to investigate the effects of variety, growing location, and year and their interactions (Hubbard et al., 2000). Variety had significant effects on variations in palmitic, stearic, and oleic acid contents, with interaction effects of year and location being insignificant (Chung et al., 2009).

2.2.5.1 Starch Lipids and Starch Surface Lipids

In barley and wheat the majority of the starch lipids are lysophospholipids (LPL), essentially all of the phosphorus is present in the LPL. The LPL and free fatty acids (FFA) have been postulated to form a complex with amylose, and thus the lipid concentration in starch is positively correlated with amylose content and accumulates with age (Davis et al., 2003). Results of previous research supports a quantitative and qualitative diversification of lipids occurring on the starch surface of wheat grains with different hardmesses (Greenblatt et al., 1995). Greenblatt et al. (1995) identified galactolipids and phospholipids in greater amounts on water-washed starch from soft wheat than from water-washed starch on hard wheat (Greenblatt et al., 1995). Moreover soft wheat grains were observed to contain higher amounts of polar lipids, more specifically lysophospholipids (phospholipid missing an acyl chain), associated with the starch granule surface (Greenblatt et al., 1995). Konopka et al. (2005) found a negative correlation between starch-surface lipids (polar and non-polar) and kernel hardness (Konopka et al., 2005). More recent examination of the variation in polar (hydrophilic) lipids located on the surface of wheat starch found the predominant starch surface polar lipids were digalactosyldiglyceride (DGDG), monogalactosyldiglycerol (MGDG) (neutral glycolipids constitute approx. 75% total flour lipid) and phosphatidylcholine (PC) polar lipid classes. The phospholipids, both negative and neutral, constitute approx. 25% total flour lipid. Flour from three soft textured wheat lines contained significantly greater concentrations of DGDG lipids, and hard wheat flour contained significantly greater concentrations of phosphatidylinositol lipids (PI;
phospholipid type, phosphoinositide structure) (Dubreil et al., 1997; Finnie et al., 2010b).

An associated study examined the relationship between starch bound polar lipid species and puroindoline haplotypes. Results indicated that the greatest quantities of polar lipids on the starch-surface occurred when both puroindoline proteins were present in their wild-type form (soft textured wheat). Starch surface polar lipid content dramatically decreased when one of the puroindoline proteins was null or pin-B was in the mutated form (Trp-44 to Arg). Among the hard-textured samples, more polar lipids were present on the starch-surface when pin-B was in its wild-type and the least amount of polar lipids were present when pin-B was in its mutated form (Trp-44 to Arg) suggesting that pin-B is more important than pin-A in binding lipids (Finnie et al., 2010a). The starch isolated from Hi-Line flour (Pina-D1a/Pinb-D1b, hard-textured wheat) contained greater amounts of phosphatidic acid (PA) and PI lipids than did the starch isolated from soft-textured wheat flour. Finnie et al. (2010) suggested that the activity of phospholipase D might be greater in the Hi-Line wheat sample than the other wheat samples as products of phospholipase D reactions are Phosphatidic Acid (PA) lipids (Finnie et al., 2010b).

Furthermore the typical feature of harder wheat varieties including durum was a substantially higher content of oleic acid (Fatty Acid 18:1) in lipids of the starch surface. A higher contribution of more oxidation-susceptible linoleic acid (Fatty Acid 18:2) on the surface of starch granules of softer varieties was suggested to initiate reactions with the sulphur-rich puroindolines. Konopka et al. (2005) also reported that apart from genetic factors, the fatty acid composition is also determined by grain maturity and climatic conditions. In barley and wheat starch, elevated growth temperatures increase the amount of starch lipids (Tester & Karkalas, 2001) and high temperature and low rainfalls enhance synthesis of less saturated acids. With maturity the concentrations of linolenic, palmitic and stearic acids decrease gradually in favour of linoleic acid (Konopka et al., 2005). Higher levels of lipids have been generally associated with smaller starch granules and results indicate that lipid may be preferentially associated with their biosynthesis (Raeker et al., 1998; Gaines et al., 2000). However Konopka et al. (2005) comments that these high levels mostly consisted of internal lipids (measured as total starch phosphorus). Phospholipids
influence the swelling behaviour of starch by inhibiting the movement of water into granules, resulting in reduced starch granule swelling, amylose leaching, and peak paste viscosity. It has been suggested that the lower pasting viscosities of B-type starch granules, compared with those of A-type starch granules, might be the result of the relatively higher phospholipid and lipid complexed amylose contents (Geera et al., 2006).

Starch lipids and starch surface lipids (SL and SSL) consist of 95-97% polar lipids, nearly 95% of which is made up of phospholipids. The lipid contents of starch differ depending on the size or type of starch granules. Soulaka and Morrison (1985) investigated the SL content of large A granules and small B granules and found that A granule starches have about 50 and 20% less FFA and lysophospholipids, respectively, than B granule starches (Soulaka & Morrison, 1985). Starches grouped by three sizes (fine, intermediate, and coarse) showed that the fine starch fraction contained the highest FA (1.51% of the starch fraction) followed by intermediate starch (1.20%) and coarse starch (0.97%), which made up 35.3, 13.1, and 51.6% of total starch weight, respectively (Whattam & Cornell, 1991). In conclusion, the compositions of SSL and SL are different based on the size of starch granules. The SSL and puroindolines a and b, are reported to play important roles in differentiating hard and soft wheats. Friabilins are composed of puroindolines a and b, which are known to complex with polar lipids (see topic above: Puroindolines) (Bhave & Morris, 2008b; Chung et al., 2009).

The genetic control of Free Lipids (FL) content and composition has especially been evidenced by variation in the glycolipids (GL) fraction of the FL. Free GL (the GL fraction in FL) content was found to be under the control of genes located on the short arm of chromosome 5D. At least two genes control Free polar lipid (Fpol) levels in wheat and have been mapped to the long (Fpl-1) and short (Fpl-2) arms of chromosome 5D. It has been suggested that Fpl-2 may be identical to the Ha locus and therefore may provide some explanation as to the control of Fpol level in wheat (Morrison et al., 1989). Turnbull and Rahman (2002) propose the possibility that Fpol levels may be influenced by the puroindolines (the puroindoline genes being tightly linked to the Ha locus). The linkage between Fpl-2 and Ha, however, has not been examined further (Turnbull & Rahman, 2002). However lipid analyses are challenging;
sometimes presenting inconsistent results. Extractions of starch surface lipids are prone to lipid contamination with fractions originating from the germ and aleurone layer therefore the study of dry starch is proposed. Taken together, the relationship between endosperm texture and the amount and type of lipid may partially be a result of expression levels of different starch granule types. Further study is required into the relationship between lipid, starch granule types, and hardness.

2.2.6 Starch Granule Size Distribution (SGSD) and Development

The endosperm exhibits an ordered array of cells that radiate from the periphery to the centre of the flanks either side of the crease. The size of cells increases towards the centre and is compatible with the first formed cells (those at the centre) having had longer to accumulate storage products, particularly starch (see topic above: Wheat Grain Structure). In wheat, granule size declines systematically among cells from the centre to the periphery. A coincident decline in the numerical complement of lenticular (type A) granules has been suggested as compatible with a sequence of peripheral divisions in which each daughter cell received half the number of amyloplasts or proplastids (Evers & Millar, 2002). It is well documented that a higher percentage of small starch granules is typical of harder wheat (Evers & Lindley, 1977; Bechtel et al., 1993; Zayas et al., 1994; Stoddard, 1999b; Gaines et al., 2000; Li et al., 2008). Although no significant differences in the relative quantity of type A granules have been noted between the two wheat classes (Glenn et al., 1992; Bechtel et al., 1993). In a starch granule study of 12 soft wheat cultivars, Raeker et al. (1998) found highly significant differences among the cultivars for volume % of granules within the type A granule range (Raeker et al., 1998). It is proposed that granule type predominance directly affects milling quality and dough/baking quality.

Dependant on the type of granule size distribution analysis used, some studies support a trimodal distribution in wheat endosperm which Bechtel and Wilson (2003) class as large (type A), medium (type B), and small (type C) (Raeker et al., 1998; Bechtel & Wilson, 2003). Studies have identified differing granule type expression according to grain position. For example, a recent study displayed a granule volume distribution in basal wheat grains showing a three peak curve, whereas that in distal
grains exhibited a two peak curve, indicating that the volume percentages of type C starch granules in basal grains were higher (Dai, 2009). Similar results were also reported by Stoddard (1999), who concluded that grains from distal florets were always smaller and had lower B-granule and nitrogen contents than those from the two proximal florets on each spikelet (Stoddard, 1999b). A-type granules have been reported as 10 – 35 μm in diameter and account for more than 70% of the total starch weight but less than 10% of the granules by number. B and C-type granules account for over 90% of the granules by number, but less than 30% of the total starch by weight in wheat endosperm (Lindeboom et al., 2004). C-type granules have a diameter of less than 5.3 μm and represent 45.7% of the total number of endosperm starch granules and 3.4% of the total weight (Igrejas et al., 2002a). However the surface area of B-granules (including C granules) has been estimated at 0.7 m² per gram of starch and is about three times higher than that for A-granules producing a prominent contribution to endosperm cohesiveness and strength (Konopka et al., 2005). In summary the relationship between starch granule characteristics and fracture patterns requires further investigation.

2.2.6.1 Starch Granule Formation

Starch granules develop in a membrane bound organelle, the amyloplast. At maturity the plastid membrane is lost although some proteins remain associated with the granule surface. The mechanisms for starch granule formation are still being defined (Bechtel & Wilson, 2003). Bechtel and Wilson (2003) observed that the three granule classes are produced at specific times during wheat endosperm development (Bechtel & Wilson, 2003). Large A-type granules appear to be initiated in undifferentiated plastids in the coenocytic endosperm tissue at 2–4 d after anthesis. Following cellularization, the formation of additional B-type granules is initiated in stromules protruding from amyloplasts containing A-type granules in the sub-aleurone layer at 7–8 d after anthesis. Very small starch granules were observed in long stromules that branched and formed a network in the cytosol. Varied processes have also been observed in rice where small amyloplasts in the endosperm divide simultaneously at multiple sites, generating a beads-on-a-string structure. In addition, large amyloplasts divide by budding-type division, giving rise to small amyloplasts.
attached to their surfaces (Yun & Kawagoe, 2009). Stromules in wheat endosperm disappeared by 14 d after anthesis and amyloplasts containing single starch granules predominated, suggesting the growth of the B-type granules and the fragmentation of the stromules. Stromules reappeared in the sub-aleurone cells at 17 d after anthesis, and appear responsible for the initiation of a third round of starch granule formation. Eventually, three size classes of starch granules, each the result of a separate round of initiation, were present in the sub-aleurone layer of the seed: large A-type granules (>15 μm), medium B-type granules (5–15 μm) and small C-type granules (<5 μm). Similar development of starch granules and stromules was observed in cells of the central endosperm, although the two phases of stromule formation were delayed by several days compared with the sub-aleurone layer (Bechtel & Wilson, 2003; Natesan et al., 2005). It has been suggested that the long length of stromules and their projection into distant parts of the plant cell may allow plastids to sense the environment of the cell. Pyke and Howells (2002) observe that stromule frequency is inversely proportional to plastid frequency. They propose that stromules may allow plastids to reach out into the cell to sense the total number of plastids in the cell and thus control plastid division (Pyke & Howells, 2002; Kwok & Hanson, 2004). The formation of stromules provides a means of increasing the plastid surface area with only relatively small changes in volume. Stromule formation therefore provides the prospect of exchanging metabolites or signalling molecules over a much larger area (Natesan et al., 2005).

The cells in the centre of the "cheek" region of the endosperm are the oldest; they are cells formed during the cellularization phase. Such cells contain large A granules that go through the full developmental program (outlined in topic above, Grain Development). In contrast, at the periphery of the endosperm, the cells are youngest, and the A-granules found there are initiated at the end of the cellularization phase. Such cells contain A granules that have a smaller maximum diameter and are rounder than granules in the older cells, suggesting that such granules shift from a radial expansion process to a lateral expansion phase earlier than occurs for older granules. Between these two extremes are gradients of granule age and development across the endosperm. Researchers propose that the genes required for synthesis of the equatorial plate and groove may be expressed only during the early stages of
development and may no longer be expressed or active by the time B granules are initiated (Stone & Morell, 2009).

2.2.6.2 Environmental Influences on SGSD

The observation of significant environmental effects on granule size distribution is not surprising given that B granule synthesis is so dependent on the timing of events in grain development relative to the temperature regime and the duration of grain filling. For example, low temperatures during grain maturation extend the grain-filling period, allowing waves of B and eventually C granules to be initiated, leading to a high proportion of B and C granules. However, note that, in one study, C granules were shown to constitute just 3.4% of the total starch (Bechtel et al. 1990). In contrast, high temperatures accelerate grain development and cause premature maturation, limiting the opportunity for B granule initiation and growth and thus suppressing B granule numbers. Taken together, the extent and type of starch granule formation is partially determined by environmental influences such as ambient temperatures (Hurkman et al., 2003). Furthermore a recent study demonstrated that the proportion of A-type granules increased in response to water stress (WS) in all varieties, the extent of increase being greater at 15 DPA. The proportion of B-type granules decreased in most varieties in response to WS at 15 DPA. The starch from wheat exposed to WS at 15 DPA showed lower amylose content, lipids content and pasting temperature, and higher peak viscosity, final viscosity and setback. Changes in pasting and thermal properties of starch caused by WS were observed to be related to lipids, amylose content and distribution of granules (Singh et al., 2008). Consequently researchers suggest that granule size distribution in any given variety should have the potential to be a useful diagnostic describing the environmental conditions in operation during grain fill, which may, in turn, be useful for predicting other aspects of grain quality. Nevertheless previous research indicates that the control of the A-to-B granule ratio in hexaploid wheats is under complex multigenic control (Stoddard 2003)(Stone & Morell, 2009).

2.2.6.3 Genes in Amyloplast Division

Although the regulation of granule class differentiation is still unknown, genes playing a role in amyloplast division have been identified. For example, research has shown that modulation of the expression level of FtsZ protein results in altered starch
granule size and number in potato (de Pater et al., 2006). Related studies into chloroplast division have identified the tubulin-related GTPase FtsZ that assembles into a ring structure (Z-ring) at the mid-chloroplast division site, which is where invagination and constriction of the envelope membranes occur. Z-ring assembly is usually confined to the mid-chloroplast site by a well balanced counteraction of the stromal proteins MinD and MinE (Fujiwara et al., 2009). Mutants in these genes in bacteria divide asymmetrically and produce minicells (Addinall & Holland, 2002). Homologues of Min proteins have been detected in higher plants. In Arabidopsis, overexpressing either MinD or MinE gives drastic chloroplast division changes (Colletti et al., 2000; Kanamaru et al., 2000; Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002). Recent research demonstrates that the chloroplast division site placement involves a balance between the opposing activities of AtMinE1 and AtMinD1, which acts to prevent FtsZ ring formation anywhere outside of the mid-chloroplast (Fujiwara et al., 2008).

Furthermore Dynamin-like proteins, ARC 1, 5 and 6 also have a role in plastid division. Chloroplast division in plant cells is accomplished through the coordinated action of the tubulin-like FtsZ ring inside the organelle and the dynamin-like ARC5 ring outside the organelle. This coordination is facilitated by ARC6, an inner envelope protein required for both assembly of FtsZ and recruitment of ARC5. Recent research in this field showed that ARC6 specifies the mid-plastid positioning of the outer envelope proteins PDV1 and PDV2, which have parallel functions in dynamin recruitment. PDV2 positioning involves direct ARC6-PDV2 interaction, but PDV1 and ARC6 do not interact indicating that an additional factor functions downstream of ARC6 to position PDV1. This study indentified that PARC6 (paralog of ARC6), an ARC6-like protein unique to vascular plants, fulfills this role. However, whereas ARC6 promotes FtsZ assembly, PARC6 appears to inhibit FtsZ assembly, suggesting that ARC6 and PARC6 function as antagonistic regulators of FtsZ dynamics (Glynn et al., 2009). Arc1 mutants have an increased number of mesophyll chloroplasts relative to wild-type, with mutant chloroplasts having a smaller diameter than wild-type (Pyke & Leech, 1992). Double mutants of ARC 5 and ARC 1 have an intermediate phenotype, indicating that chloroplast division is not completely blocked in the ARC 5 background (Pyke & Leech, 1994).
In summary FtsZ1 and FtsZ2 are phylogenetically distinct homologues of the tubulin-like bacterial cell division protein FtsZ that play major roles in the initiation and progression of plastid division in plant cells. Both proteins are components of a mid-plastid ring, the Z-ring, which functions as a contractile ring on the stromal surface of the chloroplast IEM (inner envelope membrane). McAndrew et al. (2008) reported that the molar ratio between FtsZ1 and FtsZ2 remained constant at approx. 1:2, suggesting that this stoichiometry is regulated and functionally important. These researchers hypothesized that the FtsZ1-FtsZ2-ARC3-ARC6 complex represents an unpolymerized IEM-associated pool of FtsZ that contributes to the dynamic regulation of Z-ring assembly and remodelling at the plastid division site in vivo (McAndrew et al., 2008).

Other genes also have been identified which when overexpressed reduce starch granule size for example, Brittle-1 (adenosine diphosphateglucose (ADPG) transporter), Curcuma Starch Synthase, and oleosin (Kwok & Hanson, 2004). Peng et al. (2000) identified novel isozymes (SGP-140 and SGP-145) of starch branching enzyme (SBEIc) and suggested their contribution to the production of different granule size classes present only in cereal grains. The low presence of SGP-140 and SGP-145 on second phase production of small granules after DAF15 (transition of cellularisation to cell expansion or filling) was suggested to retard growth thereby producing the type B granules however this requires further confirmation. The study demonstrated no significant variation in concentration of the other major granule-bound polypeptides (60, 80, 92, 100, 108, and 115 kD) for both small- and large-size starch granules throughout endosperm development (Peng et al., 2000a). Alternatively, by increasing starch synthesis through overexpression of regulation-insensitive forms of ADPG PPase (thus overriding the allosteric regulation of the endogenous enzyme), researchers have increased the starch content in potato (Stark et al., 1992), rice (Sakulsingharoj et al., 2004), wheat (Smidansky et al., 2002), and maize (Wang et al., 2007; Stone & Morell, 2009).

In summary a range of genes have been identified that may have the potential to become markers or be genetically modified to produce starches with novel technological applications however the industrial techniques involved are challenging (for example, small granule extraction). Previous research has confirmed that the ratio
of A granules B granules has a genetic determinant (Stoddard, 1999a). In a recent study of the variations in characteristics of starches extracted from European soft wheats, starch damage varied from 13.2 to 19.9 CDU in function of the cultivars and contribution of the B-type starch granules (<10 μm) to the total volume ranged from 11.6% to 29.9%. These researchers concluded that starch properties were principally influenced by the wheat cultivar and slightly by the culture year (Massaux et al., 2008). The alternative applications of such manipulation either through selective breeding or molecular biology techniques include improved processing quality (milling) or production of alternative food products for consumers also with possible health benefits. Also patent applications have been submitted in recent years by others concerning methods for the manipulation of starch granules in cereals, potato and other plants for applications in the starch industry (Coates & Burrell, 2003; Singletary & Zhou, 2004; Waters & Henry, 2007). Wheat starches with different granular sizes not only have different degrees of enzymatic hydrolysis and thermal and pasting properties, but also different molecular characteristics such as varying amylose and protein content and branch chain length of amyllopectin relevant to other functional properties and digestibility (Liu et al., 2007).

2.3 Grain Mechanical Properties

Wheat hardness, a physical property, is important in the determination of milling throughput, equipment design, and energy requirements. In the general sense, hard wheat is easier to mill since it gives readier separation of bran from endosperm and the liberated flour is more mobile and easier to sift. Hardness is equally important to the baking and processing industries, which rely upon the differences in the textural properties of hard and soft wheats when forming various food products (Delwiche, 2000). From the perspective of material science, mechanical hardness is defined as the resistance of the material to deformation such as abrasion, indentation, or scratching (Glenn et al., 1991). Milling behaviour could also be influenced by endosperm vitreousness, a characteristic often confused with hardness. Vitreousness is an optical parameter which is mainly influenced by growing conditions such as temperature and light intensity, during grain filling. The rate of drying at maturity are also shown to be determinant factors affecting grain vitreousness rather than genetic factors (Parish & Halse, 1968). Vitreous endosperm has a glassy appearance that may, as suggested by
Dobraszczyk (2002), be due to a greater protein matrix density than those found in mealy grain. However studies have indicated that endosperm weakness is unrelated to the grain protein content, but to the overall porosity of the endosperm structure (Dexter et al., 1989; Sadowska et al., 1999; Dobraszczyk & Schofield, 2002; Greffeuille et al., 2006). Other researchers observe that hardness appears to be linked to genotype and insensitive to nitrogen supply. Therefore some researchers conclude that hardness and vitreousness are not related (Samson et al., 2005). Nevertheless evidence supports the combined effect of these qualities on milling performance (see topic below: Vitreousness).

2.3.1 Strength of Bran Layers

The mechanical properties of wheat seed coats also have important technological implications. Research by Glenn and Johnston (1992) reported that small differences in bran thickness are difficult to detect since bran thickness values vary depending on location and moisture content. Glenn and co-workers (1991, 1992) used stress-strain analysis to investigate the mechanical properties of machined endosperm samples and bran strips from 31 common hard and soft wheat samples (Glenn & Saunders, 1990; Glenn et al., 1991). They found that the compressive strength of wheat endosperm ranged from 11.6 to 61.3 MPa, while breakage strains were 2.71-7.11 %, depending on the hardness of the wheat. The tensile strength of the endosperm was 1.74-5.18 MPa, an order of magnitude smaller than the compressive strength. The tensile strength of bran was ~18 - 26 MPa and the strain to break was ~ 14-26%. This early study on the mechanical properties of wheat bran concluded that bran appears to be nearly isotropic with no consistent differences in the mechanical properties of bran from hard or soft wheat samples. Arnold and Roberts (1966) used a photoelastic model to study the stress distributions in wheat grains under compressive loading (Arnold & Roberts, 1966). They concluded that breakage of wheat grains is dominated by the fracture events occurring in the endosperm (Fang & Campbell, 2002b). Further studies are required to assess the effects of kernel morphology on the mechanical properties of wheat bran, with reference to flour yield and quality of different cultivars accounting for response of seed coats to conditioning (Glenn & Johnston, 1992b; Mabille et al., 2001; Anderssen & Haraszi, 2009). For example, differences in arabinose to xylose ratio (A/X) were found in bran layers of three hard and three soft wheats, being lower
in soft (0.76) than hard (0.86) wheats (Lee & Stenvert, 1973), with cultivars with highest A/X ratio having the most rapid water penetration during conditioning (Stenvert & Kingswood, 1976; Stone & Morell, 2009).

2.3.2 Effects of Moisture on Fracture of Endosperm

Early research by Hinton (1950) demonstrated that when the endosperm surface was moistened and allowed to dry for one to two minutes, the strains set-up caused the development of fine cracks in the endosperm which were visible as dark lines on a translucent ground. With a hard wheat, the pattern formed by these cracks reproduced to a considerable degree that of the cell walls. Other early research by Gosh and Milner (1959) showed that liquid water induced cracks in hard vitreous endosperm in advance of water movement (Grosh & Milner, 1959). In soft wheat, however, the cracks or lines of cleavage were not only much less noticeable but also they showed no similarity to the pattern of the cell walls and in many cases passed through the cells (Greer & Hinton, 1950). The microstructural features of vitreous endosperm are similar for different vitreous samples (Glenn & Saunders, 1990). In contrast, the micro-structural features of mealy endosperm can vary considerably. Not only can the size and distribution of the intracellular spaces vary among mealy samples, but there may also be vitreous regions interspersed among the mealy regions (Glenn & Saunders, 1990). Accordingly the water vapor diffusivity of mealy endosperm has also been reported to be higher and more variable than that of vitreous endosperm based on the limited number of cultivars tested. Diffusion studies that utilise vitreous caryopses from both hard and soft wheat cultivars or mealy caryopses from both wheats would be useful in determining what role, if any, hardness or softness has in diffusivity (Glenn & Johnston, 1994).

Water is a major component of wheat grains, whose content varies depending on environmental conditions. Importantly, moisture content affects wheat endosperm hardness. Of the mechanical properties tested by Glenn et al (1991), endosperm compressive strength ($S_{\text{max}}$) exhibits the most consistent relationship with moisture content (Glenn et al., 1991). Hard, wheat endosperm compressive strength was much more sensitive to moisture content than was a soft, wheat endosperm. This difference
in moisture response accounted for the differences in endosperm strength of the hard and soft (moisture contents below 22 %). Differences in the strength values with varying moisture contents may be due to moisture sensitivity of starch granule strength, storage protein strength and/or the strength of starch / protein adhesion. Historessis tests on cylinders of cv. Lerma Blanco endosperm showed that moisture levels of 17.5% and lower did not affect the amount of residual or elastic deformation from a 0.7 kg load. Initial residual deformation values were similar for both samples, which suggested that intracellular space was not a major contributing factor to residual deformation values (Glenn et al., 1991). Likewise Dobraszczyk (1994) concluded that irrespective of the degree of vitreousness, fracture toughness decreases as the moisture content increases (Dobraszczyk, 1994). Soft and hard wheats exhibit the same trend with moisture content; however, they do so at different response rates. Additionally, hard wheat samples generally have a more linear response between $S_{\text{max}}$ (a physical strength property) and moisture content than soft wheat samples (Delwiche, 2000). Such differing responses to water may be due to consistency of the endosperm composite bonding and strength of cell wall architecture (see Chapter 2: Early Germination and Starch Granule Degradation; Cell Wall Composition & Arabinoxylans).

### 2.3.3 Theories of Wheat Endosperm Hardness

The biochemical basis of wheat hardness has still not been fully elucidated. Accumulated research has proposed at least four theories to explain the physico-chemical bases of hardness. The first hypothesis proposed that grain hardness depended on the bond between starch granules and protein. The hypothesis suggests that a ‘cement’ provides the bond between starch granules and the protein matrix. However, these workers were unable to specifically identify such a protein at that time (Simmonds et al., 1976). There is clear evidence that particle-matrix adhesion affects the strength of materials. Spanoudakis and Young (1984) showed that fracture strength of epoxy resin reinforced with spherical glass particles was markedly increased when the glass particles were pre-coated with a coupling agent (Spanoudakis & Young, 1984). As expected fracture strength decreased when the glass particles were pre-coated with a release agent (Glenn & Johnston, 1992a). The
second hypothesis by Stenvert and Kingswood (1977) suggests that a non-continuous protein matrix around the starch granules is sufficient for there to be a significant reduction in mechanical strength (Stenvert & Kingswood, 1977). This theory unconfirmed by experiment gives priority to the physical interactions between starch and proteins and the effect of environmental factors rather than genetic factors. The third by Doekes (1985) advocates the cause of the differences in hardness resulting from the electrical charges of the proteins in the immature endosperm. If the net charge of proteins is high, they will stick together and the endosperm will be soft. In contrast, if the net charge is low, there will be no such repulsion and the endosperm will be hard. Although acceptable for proteins in solutions, endosperm moisture content falls rapidly from 40% to 12% during maturation (Doekes & Getreide, 1985).

The last hypothesis by Greenwell and Schofield (1986) demonstrated the presence of a protein with a low molecular weight (15 kDa) which remains attached to the surface of starch granules when they are purified (Greenwell & Schofield, 1986). Researchers have suggested that the protein has ‘anti-adhesive’ properties that would weaken the link between the starch and the proteins and give the endosperm its friable characteristic, whence its name ‘friabilin’ (see topic below: Puroindolines). Friabilin was later postulated to be a family of related proteins (Morris, 2002), producing a softening of wheat texture through a weaker bond between the endosperm matrix, the starch granule, and certain bound polar lipids (Greenblatt et al., 1995). The extent of this bond does not seem to become apparent until the action of cellular desiccation that immediately precedes grain maturity (Bechtel et al., 1996). The gene of this protein is located on the short arm of chromosome 5D. Durum wheats do not contain friabilin and harder wheats contain less than soft wheats (Haddad et al., 1999). This discovery has dominated research in grain rheology and is consistent with the genetic basis for hardness (Glenn & Johnston, 1992a).

### 2.3.4 Vitreousness

Researchers have suggested that wheat texture is brought on by a final mixing of cellular components during drying and an eventual binding of specific cellular components to the starch granule surface (Delwiche, 2000). Hoseney (1986) suggests that as the cytoplasm in the endosperm dries it shrinks and either still remains intact
(vitreous grains) or ruptures leaving air spaces (opaque kernels) (Hoseney, 1986). This interpretation may partly explain the results of Parish and Halse (1968) who found that both the temperature and light intensity during grain filling and the rate of drying at maturity can determine if the grain will appear vitreous or opaque (Parish & Halse, 1968; Turnbull & Rahman, 2002). Hardness is a mechanical property whereas vitreousness is an optical property related to the characteristics of cut surfaces examined by scanning electron microscopy and defined by two possible states of the endosperm (glassy or mealy appearance) (Rooney et al., 1983). As previously stated, vitreousness is strongly related to agro-climatic conditions whereas the hardness characteristic is controlled essentially by genetic factors. Nevertheless Dobraszczyk (1994) reported that vitreous kernels exhibited more than twice the fracture toughness as mealy kernels of the same variety. In a study of the rheological properties of wheat endosperm, Haddad et al. (1999) found that maximum failure stress and failure energy were affected very significantly by both the variety and the degree of vitreousness (Haddad et al., 1999). In addition, research by Haddad et al (1999) on average compression curves revealed substantially different behaviour according to cultivar.

Haddad et al. (1999) also observed that whatever their vitreousness, soft wheat display fragile failure whereas in hard wheat endosperm, plasticity is more important for vitreous endosperm. Furthermore the mechanical behaviour changes when the limit of elasticity was reached and the failure mode became ductile. Regarding ductile failure, the yield strength (point) of a material is defined in engineering and materials science as the stress at which a material begins to deform plastically. Prior to the yield point the material will deform elastically and will return to its original shape when the applied stress is removed. Haddad et al. (1999) concluded that soft wheats are characterised by a fragile mode of failure whereas hard wheats display either fragile or ductile failure according to their degree of vitreousness. Depending on the failure mode, the spread of cracks in the endosperm takes place at substantially different rates that result in the formation of particles of different size and shape. The results of their mechanical measurements were said to be in agreement with grain behavior during milling (Haddad et al., 1999). According to Dobraszczyk et al (2002) the relationship between endosperm density and fracture properties is non-linear, the failure stress and fracture toughness increasing rapidly as the density approached a limiting value corresponding to the density expected for pore-free endosperm.
This indicates that endosperm is a notch sensitive material, where the pores concentrate applied stresses and can act as sites of crack initiation. It is proposed for hard wheat, that differences in hardness are determined by the relative distribution of densities of single grains, which determine the distribution of mechanical properties. Hard wheat contains proportionally more dense grains, with very few grains of low density. In soft wheat, however, the discrimination of hardness by density is not clear, and it is suggested that the degree of adhesion may be more responsible for hardness (see topic above: Storage Proteins and Vitreousness) (Dobraszczyk et al., 2002).

2.3.5 Granule to Matrix Adhesion

However studies have indicated that at least some soft wheat varieties have the inherent ability to form endosperm with mechanical properties similar to hard and durum wheats, even in the presence of the 15 kDa polypeptide (Glenn & Saunders, 1990). Possibly endosperm hardness in certain wheat samples is determined by multiple factors (see Chapter 1 : QTL Analyses) and that the 15 kDa polypeptide plays only a role of minor importance (Glenn & Johnston, 1992a). The current consensus of research into the cause of mechanical hardness in wheat endosperm relates to the degree of starch-protein adhesion. However, the direct cause of starch-protein adhesion is still being elucidated. Glenn and Johnston (1992) reported that the range in hardness of individual caryopses from different wheat classes can overlap even though their bulk hardness scores do not. They concluded that further studies attempting to establish a direct relationship between a cell product and mechanical hardness would do well to correlate the quantity of the cell product with actual hardness data based on both single caryopses and bulk hardness tests (Glenn & Johnston, 1992a). Such direct analysis may be possible by performing SKCS measurements then extracting granule bound puroindolines from the resultant meal.

Visual differences between the cut surface of air dried soft and hard wheats can be detected by SEM from as early as 5 days after flowering also using near isogenic (genetically similar) lines. The SKCS 4100 measurements could classify hard and soft wheat as early as 15 days after flowering (Bechtel et al., 1996; Osborne & Anderssen, 2003; Turnbull et al., 2003). Therefore the air-drying process is particularly important in the manifestation of grain softness or hardness. This implies
that factors that cause the difference in grain hardness at maturity are already present in the immature grain possibly rendering some accepted theories of grain hardness doubtful. Using the same samples referred to above but with freeze-dried material, Turnbull et al. (2003) found that hard and soft grains could not be distinguished on the basis of their structure when fresh developing endosperm sections from 5 to 32 DAF were examined under a light microscope (Turnbull et al., 2003). Similarly, Bechtel and Wilson (1997) were unable to find significant differences between the ultrastructure of developing hard and soft wheat using TEM of material ranging in age from 14 DAF to maturity (Bechtel & Wilson, 1997). Bechtel and Wilson (1997) studied the ultrastructure of developing hard and soft red winter wheats after air- and freeze-drying and its relationship to endosperm texture. Wheat caryopses dried for 48 hr contained endosperm cells that were converted to mature-appearing tissue with starch granules embedded in the protein matrix and the cytoplasmic remnants relegated to the periphery of the cells. Their results further supported the notion that rather than accumulation of particular grain components, the process of senescence may cause changes in the starch granule surface such that surrounding components bind tightly in hard wheat varieties, whereas the binding is weaker in soft varieties (Bechtel & Wilson, 1997).

**2.3.6 Fracture Mechanics: Differences between Hard & Soft Wheat**

The endosperm of hard wheat has weak cohesion along the cell walls and strong adhesion internally, whereas that for soft wheat has strong cohesion along the cell walls and weak cohesion internally (Greer & Hinton, 1950). Greer et al (1951) found that hard wheat endosperm particles consisting of entire cells frequently had fragments of cell wall attached, suggesting that the contents played a greater role in maintaining cell integrity than the walls (Greer et al., 1951; Evers & Millar, 2002). Furthermore, soft endosperm has a greater and more variable porosity than does hard (Dobraszczyk et al., 2002). Wheat endosperm is a composite material comprising regular cells containing starch granules embedded in a protein matrix. Soft wheats tend to fracture along the (weaker) starch/protein interface. In hard wheats, where there is greater adhesion at the starch/protein interface, fracture occurs preferentially along cell boundaries and the effect of the (lower) porosity is negligible (Osborne & Anderssen, 2003). Whereas the rheological properties of the outer layers are generally determined
by tensile tests, the mechanical properties of the starchy endosperm are analysed by compression tests. Typical stress-strain curves plotted during the compression of endosperm samples display an elastic part related to the elastic behaviour followed by fracture with or without a plastic (deformation) stage. From these curves, the following parameters can be extracted:

1. Failure stress ($\sigma_{\text{max}}$, expressed in MPa (megapascals)), measured as the peak force of the stress-strain curve;
2. Failure strain ($\epsilon_{\text{max}}$, expressed in percent of initial length), which represents the maximum strain to induce failure;
3. Failure energy per unit volume (expressed in MJ/m$^3$ (Megajoules per cubic meter)), which is proportional to the area of the curve up to the peak force; and
4. Young’s modulus ($E$ expressed in $10^3$ N mm$^{-1}$) as the ratio of stress to corresponding strain below the proportional limit of elasticity (1 Newton/m$^2$ = 1 Pa).

Fracture mechanics measures the energy to separate surfaces. If the fracture area and fracture path can be accurately measured then fracture toughness can be related to interparticle adhesion. Particle-matrix adhesion has a significant effect upon fracture strength. Researchers have been able to predict the dependence of fracture strength of a composite on the strength of the particle matrix adhesion, enabling the actual strength of the bonding to be determined (Spanoudakis & Young, 1984; Dobraszczyk, 1994). More recently lattice-type discretization has been used for statistical analysis of mechanics of fracture in disordered media, and applied to the study of the fracture properties of concrete, ceramics and soils (Lilliu & van Mier, 2003; Topin et al., 2008). Vitreous kernels generally show higher strength in compression, higher density and larger protein content. However, no simple correlation can be established between vitreosity and grain hardness: vitreous and mealy kernels may be produced from ‘soft’, ‘hard’ and ‘durum’ wheats. Consequently endosperm can be very resistant either at low protein content with strong adherence between the protein and starch granules or with low adherence and high protein content. Features concentrating stresses such as bare contact zones between the granules and porosity make the structure of the wheat endosperm particularly variable, and the mechanical behavior is expected to depend in a complex manner on the filling volume and the nature of the matrix-particle interface. Starch damage occurs as a result of the penetration of the cracks into the starch granules. According to fracture
mechanics, this occurs if the particle (starch granule) is less tough than the matrix-particle interface. Otherwise, the crack will be deflected to the interface (see topic below: Starch Damage) (Topin et al., 2008). The complex geometric shapes of wheat grains and the presence of the deep crease further complicate the study of mechanical rheological properties.

Soft wheats tend to show only elastic deformation, with no plastic stage, and can be considered to be fragile with regard to failure, while durum wheats always show plastic deformation following elastic strain and experience ductile failure. Reviewing ductile failure, the yield strength (point) of a material is defined as the stress at which a material begins to deform plastically. Prior to the yield point the material will deform elastically and will return to its original shape when the applied stress is removed. Hard wheats also generally show plastic deformation, particularly if the grain is vitreous. Finally, the failure strain and failure energy depend on the grain hardness as well as on the vitreousness, whereas Young's modulus depends more on hardness (Bechtel et al., 2009). Lack of vitreousness (i.e., the mealy state) can be considered as a microstructural weakness related to endosperm porosity. The failure stress and fracture toughness increase rapidly as the density approaches a limiting value corresponding to the density expected for an endosperm without pores. This observation is consistent with models based on fracture mechanics theory, in which pores concentrate applied stress and can act as sites of crack initiation (Dobraszczyk et al., 2002). Both vitreousness and hardness impact the mechanical properties of the grain endosperm, with Young's modulus being principally determined by hardness, while failure energy varies widely with vitreousness. These observations led Haddad et al., (2001) to propose a classification of wheats on the basis of these rheological characteristics. This proposed that a Young's modulus of less than 0.5 GPa (pressure units: gigapascals ) corresponds to a soft wheat, whereas a value above 1.0 GPa corresponds to durum wheat. Furthermore, vitreous and mealy endosperms can be discriminated by a threshold value of 1.5 MJ/m3 (Haddad et al., 2001). However these measurements were performed at low speeds and may not determine the elastic and plastic properties of the endosperm at strain rates similar to those that occur in a roller mill (Bechtel et al., 2009).
2.3.6.1 Hard and Soft Wheat Fractionation During Milling

In milling, brittle fracture is the preferred mechanism since it gives rapid crack propagation with little permanent deformation of the surrounding material, producing sharp, angular fragments. Ductile rupture deforms the material irreversibly before failure, giving highly deformed, misshapen and irregular fragments. Using the energy balance fracture mechanics theory developed by A. A. Griffith in the 1920s, Kendall (1978) devised an analysis of the fracture of particles during the comminution process (Kendall, 1978). Large particles will fracture by crack initiation and brittle fracture, while small particles can break only as the result of plastic deformation and rupture. Vitreous endosperm has been shown to have higher fracture toughness than mealy endosperm in a single wheat cultivar. Fracture toughness was shown to decrease from the outside of grains, and increase again towards the central crease. Fracture mechanics theory suggests that the particle size produced during fracture of vitreous grains should be larger than for mealy grains (Dobraszczyk, 1994). Nevertheless any variability in the form of the grain or the properties of its envelopes introduces errors in the measurement of the rheological characteristics of the endosperm.

Farina is coarse ground wheat with particles mostly between 0.25 and 0.75 mm diameter, extracted from the mill purifiers and is generally free of bran coat and germ with less than 0.6% ash content (dry basis). In an analysis of the products of milling reduction stages of bread wheat farina, Greffeuille et al. (2007) concluded that hardness is one of the main characteristics influencing the yield of coarse farina (CF) since hard wheats produced noticeably more CF than soft ones (Greffeuille et al., 2007). However, when comparing hard wheats, it appears that other factors may have an impact on yield. At the end of three reduction steps, total flour yield reflects farina reduction ability. This study indicated that wheats can be classified according to their rate of farina reduction. Hardness or vitreousness may influence the mode of rupture at the cellular level and the coarse particle size production, whereas only hardness appeared to affect the small particle production and the protein/starch dissociation behaviour at the macromolecular level. Also Hardness could not solely explain the energy requirement to grind farina since cultivars with same degree of hardness display distinctly different $K'$ values. The energy needed to grind the CF was measured.
and the $K^l$ index (kJ/kg of flour), which corresponds to the energy necessary to produce 1 kg of flour (i.e. particles below 200 μm), calculated. These results suggested that endosperm porosity not only increases with softness but also with decreased vitreousness. Thus, vitreousness could be related to the uniformity and the compactness of the starchy endosperm as already suggested from microscopic observations with durum wheats and by density measurements (Dexter et al., 1989; Samson et al., 2005). In the endosperm, pores may constitute weakness zones where fracture can be more easily initiated. As a consequence, the greater porosity of farina from Soissons (medium hard variety) may explain its better capacity to produce fine particles compared with the other hard wheats (Greffeuille et al., 2007).

From a general milling perspective, it is desirable for hard wheat used for the production of bread and pasta products to contain a high percentage of vitreous kernels. High levels of vitreous kernels are important in the top grades of durum wheat for efficient production of high quality semolina (coarse particles). For hard common wheat, where the desired end product is flour, starchiness has little impact on milling performance when straight-grade types of flour are produced (Pomeranz et al., 1976; Dexter et al., 1988). However, starchiness reduces the yield of granular hard-wheat farina and hard-wheat (durum) semolina from the break roll passes of the mill, with more fine flour produced during the reduction passes, which could lower the potential for the production of low-ash top patent flours (Carson & Edwards, 2009). In milling, hard wheat produces less coarse bran than soft wheat and, as a consequence of the greater breakage of the aleurone, more aleurone cell contents are released into the flour at the reduction step from hard than from soft wheat (Greffeuille et al., 2005).

### 2.3.7 Starch Damage

#### 2.3.7.1 Processes Effected by Starch Damage

A significant effect of Hardness on bread-making qualities is attributed to higher starch damage during milling. In a study of fracture mechanics using numerical simulation, Vincent et al. (2009) found starch damage scales well with the relative toughness of the starch-protein interface (Vincent et al., 2009). This damage increases both water absorption and hydrolysis of starch into fermentable sugars that contribute to loaf volume. Damaged starch absorbs 2 to 4 times its weight in water as compared to 0.4 by native starch. Damaged starch granules are also subject to preferential attack.
by some specific enzymes (e.g., α-amylases). Some of these enzymes are incapable of attacking an intact native starch granule. The optimum starch damage value varies according to flour usage, and is greatly dependent upon the flour protein content, the α-amylase activity, and the kind of process used for bread making. Previous research suggests that regardless of mill type, 5 to 12% of starch granules are damaged on average during the milling process.

2.3.7.2 Starch Granule Type and Proneness to Damage

Reports vary as to the starch granule type which is more prone to damage. Dubois (1949) observed that the granule exhibits elastic properties that lead to different types of damage such as cracks and breaks. Two factors were noted which lead to damaged starch: (1) The surface factor corresponding to the scratching effect by the surface of grooved mill rolls; and (2) The internal factor appearing during the reduction phase when granules are broken or flattened (Dubois, 1949). In a study of the physicochemical properties of starches of wheats and flours, Kulp (1972) observed that greater damage occurred to the small granules than the regular ones during milling. Although this is contrary to the microscopic observations of Moss (1972), who found the large granules to be generally more damaged by milling than the small ones (Kulp, 1972; Moss, 1972). However Tester et al. (1994) also concluded that small starch granules are much more susceptible to damage during ball milling. Ball milling was said to be a suitable experimental equivalent to roller milling for this type of study. To account for the observed difference in susceptibility to damage, Kulp (1972) commented that smaller granules in wheat exhibited greater inherent imperfections than the larger ones (Kulp, 1972).

2.3.7.3 Causes of Starch Damage

Starch damage occurs as a result of the penetration of the cracks into the starch granules. According to fracture mechanics, this occurs if a particle (or starch granule) is less tough than the matrix-particle interface. Otherwise, the crack will be deflected to the interface (Topin et al., 2008). Taken together, these observations may suggest that either smaller granules with stronger bonding areas and fragile structure may indeed be more prone to damage or as expected larger granules would break more readily than smaller between roll gaps. However Hsieh et al. (1980) demonstrated that starch damage in First Break flour is increased significantly with increasing roll
differential whereas other parameters such as conditioning moisture, feed rate, roll speed had little effect; surprisingly a narrower roll gap slightly reduced starch damage at the first break stage (Hsieh et al., 1980b). Results indicate that the grinding stage leads to approximately 20% of the total starch damage incurred. The majority of the damage is produced by the front end reduction and sizing reduction stages. Increases in the level of starch damage during these stages were reported to be attained by: tightening the rolls, increasing the rate of feed, increasing the pressure exerted on smooth rolls, and decreasing roller speed. In summary the miller can affect the starch damage content of flours through wheat choice, grain preparation and the mill set-up and adjustments (Dubat, 2007).

The literature review has considered the following subjects relevant to the subject of wheat flour yield: the historical significance of wheat grain, current dry milling and flour production methods; quality evaluations, end-product usage; and the development of morphological components thought to influence milling quality and their mechanical responses to conditioning and fractionation.

Chapter 3. Methodologies

The contents of Chapters 4 – 6 are extracted from submitted journal articles. These include detailed coverage of methodology, therefore this section overviews research design and provides any additional explanation not appropriate for article format. The first research question in this project involved the validation of the rheological information derived from the Single Kernel Characterisation System (SKCS) as the most suitable indicator of milling quality for small sample quantities. The general aim of this project was to increase the understanding of grain characteristics in hard wheat varieties that influence flour yield. The methodologies initially involved correlation of fragment/particle size distribution of conditioned and unconditioned hard wheat varieties produced by first break rolls and the SKCS. The use of the SKCS for studies in milling quality was validated (Chapter 4).

3.1 Sampling

While the environment can strongly affect the hardness of a particular cultivar it does not seem to affect the relative ranking of cultivars (Hazen & Ward, 1997), with the environment affecting all cultivars in a similar manner (Turnbull & Rahman, 2002). These results have particular relevance to our study as a wide range of
Australian and international cultivars representing 188 wheat varieties were selected from the Australian Winter Wheat Collection and were grown at three different locations in Australia. In Chapter 6: Materials and Methods, sample selection is detailed as follows. To obtain sufficient quantities of grain for milling and other studies, the seeds were propagated twice. The first propagation took place at Tamworth, NSW in 2004. The seed from this propagation was of insufficient quantity so a second propagation was carried out. The second propagation was carried out at two locations (Biloela, QLD and Narrabri, NSW) in 2005 to produce sufficient quantities of grain for laboratory milling and starch granule size distribution. Not all of the varieties grew successfully at all three locations resulting in a reduced number of samples from the original set. 138 grain samples from the Narrabri propagation and 74 from the Biloela propagation were of sufficient quantity (> 0.35 kg) for milling. However, at this sample size there was only sufficient for a single milling of each sample.

One hundred and fifty one samples from the Australian Winter Cereals Collection Tamworth selection were also propagated in greenhouses at the Centre of Plant Conservation Genetics (Lismore NSW). Leaf tissue was dried or quick-frozen under liquid nitrogen for storage at -80 °C. DNA was extracted from frozen and dry leaf tissue using the DNeasy Plant Mini Kit according to supplied protocol (Qiagen).

Subsequent genotyping revealed the majority of samples to be the soft genotype (Pina-D1a, Pinb-D1a) though some producing high hardness index values. Only 9-12 of the samples grown in each environment were of the Pina-D1a, Pinb-D1b or Pina-D1b, Pinb-D1a genotype and only this very limited subset was available for studies into the relationships between flour yield, starch granule size distribution and starch-bound puroindoline -a content. Consequently, samples of a further 15 commercial Australian wheat varieties of the Pina-D1a, Pinb-D1b genotype grown at various locations around Australia were also used (see Appendices: Complete Data Sets & Sample Information).

3.2 The Single Kernel Characterisation System

Previously measurements of the mechanical properties of endosperm specimens isolated from single kernels have provided fundamental information useful to the understanding of milling operations, however the experimental
procedure is very laborious and time consuming (Osborne et al., 2001a). Consequently the SKCS 4100 was developed to provide an objective method for classification of wheat as hard, soft, or mixed according to the distribution of hardness values for a number of individual kernels (Figure 6)(Martin et al., 1993).

![Crushing Rotor](image_url)

**Figure 6.** The SKCS 4100 Crushing Mechanism.

The Standard Reference Materials Program of the U.S. National Institute of Standards and Technology, in cooperation with the USDA Federal Grain Inspection Service Program, has made available a set of ten wheat samples (Reference Material No. 8441) of specified hardness. The hardness of these samples has been characterized by the empirical scales used in NIR and in SKCS measurements for the purpose of allowing users the ability to standardize these instruments. The furnished values are based on normal ambient storage conditions (23 °C, 11-13% moisture content on dry basis). They comprise five soft (average Hardness Index = 25) and five hard (avg HI = 75) U.S. wheat samples. However Delwiche (2000) comments that these values do not provide an indication of the known sensitivity of hardness to moisture content or humidity levels (Delwiche, 2000). Turnbull and Rahman (2002) suggest that many of the difficulties associated with measuring hardness can be avoided if samples are equilibrated to similar moisture content before the hardness measurements are taken and only the results from apparatus that use similar mechanisms to measure hardness are compared.
The SKCS hardness value is calculated from measurements of the force required to crush each kernel, expressed as a crush force profile. Hard wheats have hardness scores greater than 50 while soft wheats have scores less than 50. The operating principles of the SKCS 4100 have been described previously (Sissons et al., 2000). The hardness index is based on the crescent load cell A/D counts measured over a period of 125 ms. These data can be recorded as a crush force time profile by manual insertion of one kernel at a time. A computer file collects the averages of the variables: weight (milligrams), peak force (maximum load cell force, A/D counts), area (area of the crush force profile, A/D count-second), and Gompertz function coefficients A and B (Gomp A and B). The crush force profile is best described by peak force, area, and Gomp B (Gaines et al., 1996; Osborne & Anderssen, 2003).

The Gompertz function provides a simple way of expressing the variation in the crush force plots by computing the differences between adjacent data points and creating a distribution plot of these differences. Gomp A and Gomp B are defined by a restricted form of the generalized Gompertz function \( Y = A^{B \cdot X} \), in which the data are normalized through dividing by the total number of A/D time periods in the crush force profile, thus making the Y range from zero to one for all profiles. X is the A/D change, \( dy/dt \) during a time period equal to the 1/ frequency of the A/D values and Y is the fractional estimate of the number of \( dy/dt \) occurring up to a corresponding value of X (i.e., an accumulation). Gomp A and Gomp B are both dimensionless fractions (Osborne et al., 2001a). Osborne et al. (2001) in comparing SKCS 4100 analysis with compressive strength measurements (maximum stress, \( S_{\text{max}} \); work to maximum stress, \( W \ [S_{\text{max}}] \), MJ m\(^{-3}\); modulus of elasticity, E, Mpa) as performed on machined endosperm specimens described by Delwiche (2000) concluded that the SKCS 4100 may provide a simpler, more practical method for estimating wheat endosperm compressive strength and that the data was traceable to fundamental physical measurements for example, the equation:

\[
S_{\text{max}} = 145 - 2.58 \times \text{weight} + 0.000252 \times \text{area under peak} - 98.6 \times \text{GompB}
\]

was derived to predict \( S_{\text{max}} \) from the SKCS 4100 average weight, area, and GompB (Delwiche, 2000; Osborne et al., 2001b).
3.2.1 SKCS 4100 Principles of Operation

The following principles of operation have been extracted from (Osborne & Anderssen, 2003). The design principle of the SKCS 4100 is based on sequential separation by means of an indented wheel (singulator) with the aid of a vacuum (Martin et al., 1992), of a sample of grain into individual seeds that are individually weighed and then crushed between a toothed rotor and a crescent at the rate of two seeds per second (Martin et al., 1992; Martin & Steele, 1996; Osborne & Anderssen, 2003). The sequence of measurements performed by the SKCS 4100 includes:

Weight (mg) measured as the electrical force required to return the boat, into which the individual seeds are dropped, to its original horizontal position. This force is proportional to the mass of the seed. The measurement is calibrated against mass determined using an analytical balance for single seeds with weights of 12-80 mg (U.S. method) or by means of the average mass of 1,000 seeds, (thousand kernel weight, Australian method). Diameter (mm, either lateral or longitudinal width, depending on the orientation of the seed as it is engaged between the crushing rotor and the crescent) is measured as the size of the gap formed between the crescent and the rotor at engagement. The position of the engagement and thereby the size of the gap is determined by the number of data scan intervals performed between engagement and exit. Moisture content (%) of each seed is regressed with the (natural) logarithm of electrical conductance (measured using a resistor connected through the rotor to a logarithmic ratio amplifier) and force terms. This is recorded more or less at the time of maximum crushing force because experimentation had established that this gave the most reliable estimate for moisture. In fact, the maximum crushing response is used in the calculation of the HI. The recorded average moisture content for a sample is calibrated against an appropriate oven-drying procedure. These parameters are substituted in the formulas derived by Martin et al (1993) to determine the hardness index (HI) (Martin et al., 1993; Osborne & Anderssen, 2003).

The wheat HI is an arbitrary measurement based on an interpretation of the incremental change histogram (Martin et al., 1991, 1993). Each of the measurements (weight, diameter, moisture, and HI) is indirect and must be calibrated against reference laboratory methods. After crushing the sample, which often contains hundreds of seeds, the HI can be calculated quickly using the U.S. hard-soft wheat HI previously developed by (Martin et al., 1991). However, when the need arises, the
crush-response profiles for the individual seeds can be recorded and averaged to obtain the much more informative (rheologically) crush-response profile. The choice of a numeric scale for the SKCS 4100 HI is based on the earlier use of an arbitrary scale for wheat hardness by NIR according to Approved Method 39-70A (AACC 2000) (Osborne & Anderssen, 2003). The force increments as they are recorded seed-by-seed, are binned into a single incremental change histogram and denoted by $H_f (\Delta f (k))$. It is this histogram that is then used to characterize the nature of the force response of the wheat sample to the crushing. In the SKCS 4100 software, this is achieved by fitting a two-parameter Gompertz model to the incremental change histogram data (Osborne & Anderssen, 2003). It is within such average crush-response profiles that the basic information about the rheology of the crushing performed by the SKCS 4100 is hidden. The two-parameter Gompertz model is then fitted to the cumulative distribution derived from this histogram data. The Gompertz model reduces the estimation of the unknown parameters to the fitting of a linear line to a log-log transformation of the data (Causton & Venus, 1981). This is a two-parameter model because the output contains two summary parameters, $GompA$ and $GompB$. However, the circumstantial evidence strongly suggests that the Gompertz model applied takes the form:

$$y(X) = A^B \times x \quad 0 < A, B < 1$$

where $A$ (Gomp A) defines the value of the cumulative distribution where it cuts the vertical axis ($y(0) = A$), and $B$ (GompB) characterizes the rate of growth of the cumulative distribution (Osborne & Anderssen, 2003).

Three sets of data are generated by the SKCS 4100:

1. On-line histograms of the four properties seed weight, diameter, HI, and moisture content from which are calculated the mean and standard deviation for each property.

2. X-Array data format comprising seed weight (mg), peak force (maximum load cell force, A/D count), conductance, area (area of the force-time crush profile, A/D count-second), $GompA$, $GompB$, length (length of crush period, number of data points in the crush-response profile), seed diameter (mm), seed moisture %, seed HI, seed conductivity ($XCON$), and crescent temperature (°F).

3. BIGFILE data format that expands the 12 X-Array variables to 40 variables using transform function such as exponentials, logarithms, inverse terms, and ratios (Osborne et al., 1997).
The computed histogram data for the four calculated properties provide measures of the uniformity of a sample. Those data can be used directly to predict the processing performance of that sample (Fang and Campbell 2001). Prediction of conventional wheat quality and end-use properties is achieved through the development of regression models. Another option is to utilize the underlying raw X-Array or BIGFILE data with the aid of principal components regression or partial least squares regression (Osborne et al., 1997).

3.2.2 SKCS 4100 Applications

Research by Osborne et al (2001) demonstrated the potential of SKCS 4100 analysis to provide a rapid means of assessing the fundamental wheat physical properties of individual samples which could open the way to novel means of characterizing wheat milling quality for grain segregation and mill intake (Osborne et al., 2001a; Dobraszczyk et al., 2002). The SKCS 4100 HI is not only a measurement in its own right with reliable precision (coefficient of variation 1%) but also one that can be correlated to other measurements. SKCS 4100 HI has correlated well with particle size index (PSI) (Osborne et al., 1997; Williams et al., 1998; Psotka, 1999) and NIR hardness (Chung et al., 1999).

In a study, using samples collected from five Kansas country elevators during the 1995 and 1996 seasons, a quality classification system for Hard Red Winter wheat was developed by using the SKCS 4100 data in combination with bulk NIR grain protein determination. Grain could be segregated independently of location and season into seven groups based on the dough factor. Zayas et al (1996) reported a 94% hard-soft recognition rate when SKCS 4100 data was combined with image analysis data in a pattern recognition algorithm (Zayas et al., 1996). The application of the SKCS 4100 for receival testing in Australia was reported by Osborne et al (Osborne et al., 1997). The instrument proved robust and rapid enough for use in receival testing, but adoption by the bulk handling industry depends on demonstrable use of the data in segregation and pricing of grain. Furthermore, it has been demonstrated that across a wide range of protein contents and falling numbers, within and across cultivars, while there was an observed effect on the crush-response profile, there was no effect caused by these properties on milling yield (Osborne et al., 1999; Osborne & Anderssen, 2003).
Osborne et al (1997) reported promising correlations between SKCS 4100 wheat data and flour yield and starch damage when the wheat was milled on a 650 kg/hr pilot mill (Osborne et al., 1997). Bettge and Morris (2000) reported similar results for break flour yield and starch damage (Bettge & Morris, 2000). Significant correlations between SKCS 4100 data and water absorption, dough elasticity, dough development time, and sedimentation volume were reported for Czech and Slozak wheats (Hubik, 2000). Thus, the ability of the SKCS 4100 to predict flour yield potential has been established across four different milling systems. Cox and Psotka (1996) reported the use of SKCS 4100 data to predict mixograph peak time (revolutions to peak dough development) using samples collected at intake in a commercial mill (Cox & Psotka, 1996). Osborne and Anderssen (2003) comment that remaining challenges include improved recovery of information from the crush-response curves, clear demonstration of the ability of the technology to meet the needs of grain traders and processors, traceability of calibrations, and transfer of calibrations between instruments. Furthermore they proposed the use of a rheological-based model as much of the detailed rheological information about the response of the wheat to crushing is lost in the analysis of SKCS data using the Gompertz model (Osborne & Anderssen, 2003).

### 3.2.2.1 Crush Response Profiles

Osborne and Anderssen observed that the crush-response profiles for a wide variety of hard and soft wheats indicate that they all have the same generic structure. Representative crush response profiles (CRPs) are obtained by averaging the data for a large number of individual whole grains. In addition the crush-response profile (CRP) may be considered as a (pseudo) stress-strain plot, even though the independent and dependent variables are recorded as time and force. Various observations concerning the phases of the CRPs were noted. The initial response as the kernel shell resists the crushing being performed between the rotor and the crescent suggests Kelvin-Voigt characteristics (Barnes et al., 1989). It is classified as a Kelvin-Voigt response to the crushing rather than Maxwell because it shows the type of emphatic elastic response associated with a spring being pressed in parallel, rather than in series with a dashpot. The initial resistance is followed by the sudden collapse of the shell (possibly supported by the internal cellular structure of the endosperm) producing a clear spring-back in the shape of the crush-response
profile. It shows that the shell has a stronger compressive yield than the uncompressed endosperm. The subsequent phase of the crush response of the endosperm is now Maxwell in character because it shows the type of stress relaxation indicative of a spring and dashpot in series (Barnes et al., 1989). Osborne et al. suggest that further rheological characterization may be possible using the Boltzmann model of linear viscoelasticity. Finally, the response of the endosperm reaches a maximum that identifies the onset of its collapse, with the rotor wheel pushing the pieces of the collapsed seed from between the wheel and the crescent (Osborne et al., 2001b). Consequently from a rheological perspective, the parameters that encapsulate successive rheological phases in the response to the crushing, are three shell characteristics:

1) SR, shell response; SS, shell springback; and TSC, time of the shell collapse. The shell of the grain is interpreted as the aleurone layer because it is vaulted, has a smaller cellular structure, and consequently has more cells per unit volume than the endosperm (Dobraszczyk et al., 2002). In addition, the aleurone cells are biochemically different from those in the endosperm. After the initial fracture of this cell layer, most of the energy is required to crush the bulk of the endosperm;

2) EQR, equilibrium response: TER, time of the equilibrium response;

3) ER, endosperm response: TEC, time of the endosperm collapse (Osborne & Anderssen, 2003). In a recent SKCS analysis of pearled and unpearled wheats, CRP data exhibited only a minor decrease in the Shell Strength of pearled wheat samples compared with the unpearled for both hard and soft wheat but the difference is greater for the soft. This implied that the Shell Strength response is not solely a property of the aleurone layer. After this phase, the responses to crushing of hard and soft pearled wheat became different and were consistent with the current understanding of hardness (see topic: Chapter 2, Theories of Wheat Endosperm Hardness). The overall strength of both the pearled hard and soft has been compromised as endosperm collapse occurs for a smaller strain than for the unpearled (Turnbull & Rahman, 2002; Osborne et al., 2005). Other aspects of the crush-response phases, such as the slopes of the responses, will be examined for biophysical significance in the current investigations. The underlying principle in the interpretation of CRPs involves the resistance of the grain to deformation being determined by the weakest phase of the composite structures. Work in progress may
provide insights into the relationships between rheological phases and botanical structure. It will focus on using the CRP information to link phenomenologically to grain processing and genetics. For example, the size of the shell springback (SS) can be interpreted as an indirect measure of the porosity within the grain type. Accordingly, structural endosperm parameters such as spatial disorder, which may introduce ductile behaviour and micro-cracks, and significant variations in the matrix distribution, could be used to classify the biophysical traits of a range of cultivars. This example represents the potential utility of the phases in the crush-response profiles of cereal cultivars for identifying quantitative and quite specific phenotypes with a direct physical interpretation which clearly differentiate between grain types and thereby represent more appropriate markers for QTL analysis than the more popular qualitative measures of GompA, GompB and HI. Specific quantitative phenotypes such as the appropriate rheological phases in a crush-response profile may be identified from a study of scatter-plots of the relevant parameters. The SKCS 4100 data have been used as quantitative traits to map the hardness locus of some Australian wheat lines as outlined above (see topic: Chapter 1, QTL Analyses) (Osborne et al., 2001b; Osborne & Anderssen, 2003).

3.3 Microscopy Methods: Differentiation of Soft and Hard Grains

Standard light microscopy allows visualisation of large lenticular shaped starch granules (A-type granules) and the smaller more spherical B-type starch granules in mature hard and soft grains. Using quantitative image analysis some red American winter cultivars have been classified as either hard or soft based on the diameter of the B-type granules (Bechtel et al., 1993). Differences in the amount of material adhering to the starch granules from hard and soft wheat have been reported (Bechtel et al., 1993). The cut surface of mature hard wheat examined by Scanning Electron Microscope (SEM) reveals a compact uniform endosperm structure with starch granules firmly embedded in the surrounding protein matrix (Stenvert & Kingswood, 1977). In contrast mature soft wheat has a much more disordered structure with the protein matrix in many cases being pulled away from the starch granules (Stenvert & Kingswood, 1977; Glenn & Saunders, 1990; Turnbull & Rahman, 2002).

While conventional scanning electron microscopy (SEM) provides valuable information on surface topography, its disadvantage lies in the need for complicated
sample preparation and observation of specimens under a high vacuum. Environmental scanning electron microscopy (ESEM) allows specimens to be studied at high resolution, close to their natural states, with minimal preparation. There is little information from ESEM studies of cereal grains, especially on the effects of processing (Dang & Copeland, 2004). In addition, heterogeneity in cereal endosperm has been measured on samples recovered from a fractionation process (Ciaccio & Dappolonia, 1982; Zheng et al., 2000), by direct visualisation using fluorescence microscopy (Fulcher et al., 1997), and by coupling imaging techniques and spectroscopic analysis (fluorescence, infrared, Raman, NMR; (Sen et al., 1994; Piot et al., 2001a; Wetzel et al., 2003; Barron et al., 2005). Our present work included a process of relating qualitative observations gained from light microscopy, conventional and environmental electron microscopy with the crush response profiles produced by the SKCS (see Appendices: Microscopy Images). This study generated an hypothesis involving starch granule size distribution (SGSD) which was tested by correlating quantitative data produced by a laser diffraction technique with small scale milling and SKCS data.

3.4 Test milling

The evaluation of fragment size distribution of soft and hard, conditioned and unconditioned wheat after the first break milling stage utilised a Vario Experimental Roll Stand (Miag, Braunscheig, Germany) (see Figure 7. below and Chapters 2: Experimental Milling). Milling data and seed samples samples over 120 wheat varieties from the Australian Winter Cereals Collection (Tamworth) were used for this study. Propagations took place at Tamworth, New South Wales (NSW) in 2004 and Biloela, Queensland and Narrabri, NSW in 2005. Both regions have similar climatic conditions of wet summers and low winter rainfall. Test milling processes were undertaken at the accredited Laboratory Mill at BRI Research (North Ryde, NSW, Australia). Cleaned, conditioned wheat was milled using a MLU 202 Laboratory Mill (Buhler Bros, Uzwil, Switzerland) at a feed rate of 100 gmin⁻¹. Bran and pollard fractions were further processed using two passes through a MLU 203 Laboratory Impact Finisher (Buhler Bros, Uzwil, Switzerland) and the finisher flours were passed through a 150 μm screen before incorporation into the straight-run flour. The extraction rate and the speck count by Branscan 2000 (Branscan Ltd, Evesham, UK)
were measured for each straight run flour (see Chapters 4 and 5: Materials and Methods, Test Milling).

Figure 7. Vario Experimental Roll Stand. (courtesy of BRI Research Pty Ltd, North Ryde, NSW)

3.5 Starch Granule Size Distribution (SGSD) Analysis

In regard to sampling, previous research has shown that grains of the centre portion were the most developed kernels in the spike and more reflective of genotype than the smaller more variable grains on the spike. Within the spikelet, the second kernels were found to be more developed and significantly softer than the first and third. However flour yield measurements are based on heterogeneous grain sizes with 70% of kernels coming from the top and bottom portion of the spike. Consequently confounding factors are to be expected in the attempt to relate the expression of complex morphological components with genotype then practically relate this data to the commercial milling scale. Numerous studies have been conducted on size distribution of starch granules in wheat grains, but the results are different. Some have reported a bimodal distribution (Morrison & Scott, 1986; Dai et al., 2009), and others showed a trimodal distribution (Bechtel et al., 1990; Raeker et al., 1998). The differences are probably attributable to the different grain positions in a spikelet, cultivars assessed as well as the different growing conditions. When a wheat cultivar with a higher content of distal grains is determined, the size distribution of starch granules may exhibit a bimodal distribution. Conversely, it may be a trimodal distribution. The environment is known to have a strong effect on the distribution of starch granules. Compared with the irrigated treatment, the volume percentage of C-type granules could be significantly increased under rainfed conditions. Hence, the soil water deficit may be associated with increase in percentage of small granules (Dai, 2009). The relationship between the size distribution of starch granules and wheat texture (hardness) has not yet been clearly established by the results of other authors (Pitts et al., 1989; Igrejas et al., 2002a). Differences observed in the range of A, B and C sizes, classes and hardness values for
the analysed cultivars may be the primary reason for the difference encountered. Discrepant results can be attributed to the different nature of the germplasm, and the analytical methodologies employed are not the same. In addition, different techniques for size determination (light microscopy, Coulter Counter, laser light scattering) and comparisons using different terms of expression (volume, number, or superficial area distribution) may also significantly affect results.

![Image of Mastersizer 2000 and basic diagram of operation](image)

**Figure 8.** Mastersizer 2000 and basic diagram of operation (Malvern Instruments Ltd Worcestershire UK).

The present SGSD analyses used the Mastersizer 2000 (Malvern Instruments Ltd Worcestershire UK). During the laser diffraction measurement, particles are passed through a focused laser beam. These particles scatter light at an angle that is inversely proportional to their size. The angular intensity of the scattered light is then measured by a series of photosensitive detectors. The number and positioning of these detectors
in the Mastersizer 2000 has been optimized to achieve maximum resolution across a broad range of sizes (Figure 8). In Chapter 5: Results and Discussion: SGSD methodology, further consideration is presented concerning possible causes of variability in reported results.

For example, Igrejas et al. (2002) detected a higher number of small starch granules in hard varieties than in soft ones, in a population derived from a synthetic amphihexaploid wheat (Igrejas et al., 2002a). Whereas Brites et al. (2008) found that soft varieties, with both puroindolines-a and -b [pin (a, a)], showed a significantly higher percentage of volume occupied by small (type C) and medium (type B) size granules, and a significantly lower percentage occupied by large (type A) size granules than was the case in hard [pin (a, b), pin (b, a) and pin (a, d)] wheat bread varieties (Brites et al., 2008). SGSD has generally been disregarded as having a significant influence on hardness or milling quality with much investigative effort instead directed towards puroindoline genotyping and the interactions of puroindoline proteins at the starch surface interface. However the results presented here indicate the contrary with significant correlations with hardness and flour yield across a range of wheat classes mainly hard varieties (see Chapters 5, 6).

3.6 Puroindoline Genotyping

Finally, standard DNA sequencing methods were used to screen puroindoline sequence variation in 120 varieties from the Australian Winter Cereal Collection harvested and milled from two different environments. Further SGSD analysis indicated significant correlations with flour yield though differing between pin genotypes (see Appendices: Puroindoline Genotype Study).

3.7 Puroindoline Protein Assay

In addition, quantitative analysis of starch granule-bound puroindoline using a micro-capillary electrophoresis method (BioAnalyzer Protein 80 chip (lab-on-chip) Figure 9, from Agilent, Santa Clara CA) demonstrated a significant negative correlation with flour yield in the premium milling quality varieties with pin genotype, Pina-D1a, Pinb-D1b (Chapter 6). The method used for starch granule isolation for surface bound puroindoline extraction was the same as used for SGSD analysis for consistency. However extraction yields may have been less than optimal as Greenblatt
et al. (1992) observed with the use of caesium chloride during starch isolation (Greenblatt et al., 1992). Also recent research by Finnie et al. (2010) has demonstrated that a batter method (before dough formation) of starch extraction improved extraction rates of both starch surface lipids and puroindoline proteins compared with a post-dough formation method (Finnie et al., 2009; Finnie et al., 2010b).

Figure 9. Agilent BioAnalyzer Protein 80 labchip using micro-capillary electrophoresis technology.

Individual proteins may also act as markers to select for specific traits, provided that reliable associations can be identified between the presence of the protein and the trait (Wrigley, 2009). The high molecular weight subunits (polypeptides) of glutenin are an example of successful protein markers. These proteins of the endosperm contribute to dough strength in the formation of the gluten complex (Shewry et al., 2006). Chip-based methodologies have been developed to identify and quantify glutenin subunits, using capillary electrophoresis (Uthayakumaran et al., 2006). In addition, antibody-based methods can detect the presence of such marker proteins more efficiently (Gale et al., 2001). Protein synthesis is a few steps away from the gene level, so growth environment can have an effect on the presence and amount of a potential marker protein. This G x E interaction may cause complications of interpretation that are not an issue for gene-based selection. Alternatively, knowledge of the effect of environmental factors may
also provide the breeder with some advantages, because ultimate selections must be based on phenotype, and phenotype must be understood for the target regions where the new variety is planned for commercial production (Wrigley, 2009).

3.8 Aims

The general aim of this project was to examine features of wheat grain structure that influence milling performance. Breeding better wheat varieties for discerning world markets requires a fundamental knowledge of the genetic expression of grain processing quality. However, the phenotypic expression of processing quality is complex and indirect. Starch granule size distribution and granule-protein matrix adhesion, vitreousness, and the response of the cell walls and bran to conditioning are some factors implicated in milling quality attributes (Waldron et al., 1997; Peyron et al., 2002a). Although the wheat grain comprises 85% starchy endosperm, only approximately 78% is able to be separated using current milling technology. Identification of significant features may lead to improving milling yields and facilitation of new milling objectives aimed at dividing the plant material into its different components to produce substances with higher added value. The specific aim of this study is to link spatial, compositional information with rheological properties as determined by the Single Kernel Characterisation System (SKCS 4100, Perten Instruments) and milling methods (shearing properties via first-break rolls)(BRI: Vario rolls and pilot mill). In this way it will be possible to develop a more precise biophysical description of the grain particularly in relation to processing qualities of which little research has been undertaken. In the longer term, these results will be used to identify the genetic origins of the quality traits desired by industry (Toole et al., 2005).

Chapter 4. Investigation of the effect of conditioning on the fracture of hard and soft wheat grain by the Single-Kernel Characterization System: A comparison with roller milling

4.1 Introduction

The Single-Kernel Characterization System (SKCS) 4100 was developed for the objective determination of admixtures of hard and soft wheat (Osborne & Anderssen, 2003). It measures the compressive force versus time (Crush-
Response Profile, CRP) as grains are crushed individually within a toothed crescent and rotor mechanism (Chapter 3. Figure 6). In routine use, a proprietary algorithm within the instrument software calculates, from the CRP, a Hardness Index on an arbitrary scale, and displays a histogram of the distribution of the Hardness Index values for 300 grains. This histogram provides a useful visualisation of the uniformity of the sample with respect to its hardness. Histograms are also displayed for grain weight, diameter and moisture content. Thus, the SKCS 4100 system provides rapid measurements of wheat physical properties that are important to the miller. In addition, these measurements or the raw data from which they are derived have been shown to be applicable to the direct prediction of processing quality (Osborne & Anderssen, 2003).

Recovery of grain rheological information requires the use of the raw crushing data. Thus, the crush-response profiles (CRPs) for the individual kernels can be recorded and representative and highly repeatable CRPs obtained by averaging the data for a suitable number of individual whole grains. The averaged CRPs can then be interpreted as a pseudo stress-strain characterisation of the crushing (Osborne & Anderssen, 2003). The characterisation can be expressed as a series of rheological phases corresponding to the forces resisting the crushing of the shell and the endosperm of the grain (Figure 1) (Osborne et al., 2005; Osborne et al., 2007). Justification for extrapolation of the rheological properties of individual grains to those of corresponding bulk samples was provided by experiments which confirmed that wheat grains mill independently, i.e., without interaction (Campbell & Webb, 2001).

Although there have been a number of papers describing the application of SKCS 4100 data to the prediction of wheat flour yield potential (Satumbaga et al., 1995; Osborne et al., 1997; Deyoe et al., 1998; Ohm et al., 1998; Baker et al., 1999; Psotka, 1999; Lyford et al., 2005; Morris et al., 2005), none of these has attempted to show that the SKCS measurement protocol involves crushing grain in a similar way to a flour milling operation. In particular, the SKCS measurement is carried out using unconditioned grain while milling is performed on grain that has been conditioned by the addition of water.

Muhamed and Campbell (2004) have, however, studied the breakage of wheat conditioned to a range of moisture contents in the SKCS but did not make a direct comparison with milling (Muhamed & Campbell, 2004). Psotka (1997) described an
empirical SKCS 4100 procedure for monitoring the progress of wheat conditioning prior to milling but did not report the detailed effect of conditioning on the SKCS data (Psotka, 1997). Thus, the aims of this study were to explore, for both hard and soft wheat, the relevance of the current SKCS testing protocol carried out using unconditioned whole grain to the first stage of roller milling, which includes conditioning. This involved (1) a study of the effect of conditioning on the SKCS CRPs and (2) a comparison of the fracture of both unconditioned and conditioned wheat grain as effected by the SKCS crushing mechanism and an experimental roll stand fitted with a pair of first break rolls set to the same gap as that between the crescent and rotor in the SKCS.

![Diagram of crush response profile](image)

**Figure 1.** The generic structure of the crush response profile, an average of 300 wheat kernels including stress-strain curve descriptors used in the text.

### 4.2 Experimental

#### 4.2.1 Samples

Samples of hard (cv Banks, Janz) and soft (cv Rosella, Tincurrin) commercially grown Australian wheat varieties were supplied by BRI Australia Ltd.
4.2.2 SKCS analysis

SKCS 4100 measurements were carried out on 300 grains of each sample according to AACC Method 55-31 (AACC, 2002). Following each analysis, the crushed material was recovered and the instrument was cleaned prior to the next analysis. The individual CRPs for each sample were exported from the SKCS 4100 directory in a text file format and Crush Curve Analysis Software (CCAS - BRI Australia Ltd, North Ryde, New South Wales, Australia) was used to calculate the average CRP for each set of curves. Then, the rheological parameters: strength (maximum stress, \( \sigma \)), ductility (strain at maximum stress, \( \varepsilon \)) and stiffness (Modulus of Elasticity, calculated as \( \delta \sigma / \delta \varepsilon \)) for both the shell and the endosperm phases were calculated. SKCS analyses were repeated 4 – 6 times for each variety and the crushed material from two tests was combined to reduce sieving error due to insufficient sample weight (see Appendices: SKCS Crush Response Profile Analyses).

4.2.2 Experimental milling

The moisture content of each wheat sample was measured using an Infratec 1229 NIR instrument (Foss, Hoganas, Sweden) then water was added to bring the samples to a conditioned moisture content of approximately 14.5 % for soft and 16.5 % for hard. Conditioning was carried out for 16 hours. A Vario Experimental Roll Stand (Miag, Braunscheig, Germany) fitted with a pair of first-break rolls (8 flutes per inch) were used in a sharp to sharp disposition. The rolls were operated at a differential of 2.5:1 with the speed of the first roll set at 240 rpm. A roll gap of 0.67 mm (0.0265 inches) was used to represent the minimum gap between the rotor and crescent in the SKCS crushing mechanism.

4.2.3 Particle size distribution analysis

The crushed samples were sieved using stainless steel wire mesh sieves (Endecotts Ltd., London, UK) mounted on a vibrator (Analysette 3 Pro, Fritsch, Germany) operating at 0.4 mm for 3 minutes per sample. The following sieve aperture sizes were employed: 4 mm, 2 mm, 1 mm, 710, 300, 125, 75, 38 μm. Particle size distributions were derived from masses of the various grades which were then graphed as a percentage of the whole sample. Analysis was replicated. Graph characteristics were also compared with CRPs to reveal possible associations.
4.2.4 Microscopy

Whole and crushed grain samples were examined using a model SZ40 dissecting microscope (Olympus, Japan) at approximately 40x magnification. Images were recorded using a digital camera (MicroPublisher, model B) and processed with Q Capture and Photoshop software.

4.3 Results

4.3.1 Conditioned versus Unconditioned Wheat

Moisture measurements taken before and after conditioning in this study indicated the change in average moisture content in the hard varieties was 4.4% (Banks) – 7.1% (Janz) (Table 1). According to commercial conditioning practice, less water was added to soft varieties subsequently reducing the change in moisture content: 1.7% (Tincurrin) – 4% (Rosella). Figures 3 and 4 show the CRPs and fragment size distributions for the four unconditioned wheat samples and Figures 5 and 6 show the corresponding results for the conditioned wheat samples. Table 2 records the rheological parameters calculated from the curves shown in Figures 3 and 5. Repeated SKCS analyses of the same sample batch generate CRPs of negligible variation. Figure 7 shows a direct comparison of the four pairs of conditioned and unconditioned samples.

4.3.2 Comparison of SKCS and first break roller milling

The fragmentation patterns revealed by low-resolution microscopy following fracture of the different wheat samples by the SKCS and Vario are shown in Figures 8-11. Figures 8 and 9 show a random selection of different crushed grain samples indicating the variation in fragmentation between hard and soft varieties when conditioned grain through Vario mill is compared with conditioned or unconditioned grain through the SKCS. The corresponding fragment size distributions are given in Table 3 and the comparisons for Banks are illustrated in Figure 12.
Table 1 Grain moisture content measured by SKCS (average of 4–6 samples, (U) unconditioned, (C) conditioned

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Moisture (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Janz-U</td>
<td>8.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Janz-C</td>
<td>16.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Banks-U</td>
<td>11.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Banks-C</td>
<td>16.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Rosella-U</td>
<td>10.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Rosella-C</td>
<td>14.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Tincurrin-U</td>
<td>12.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Tincurrin-C</td>
<td>14.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Figure 3. CRPs of unconditioned wheat ranging in decreasing hardness: cv. Janz and Banks (hard); cv. Rosella and Tincurrin (soft).
Figure 4. Average fragment size distribution of unconditioned wheat with decreasing hardness. cv. Janz and Banks (hard); cv. Rosella and Tincurrin (soft) after crushing with SKCS (n = 3).

Figure 5. CRPs of conditioned wheat ranging in decreasing hardness: cv. Janz and Banks (hard); cv. Rosella and Tincurrin (soft).
Figure 6. Average fragment size distribution of conditioned wheat ranging with decreasing hardness. cv. Janz and Banks (hard); cv. Rosella and Tincurrin (soft) after crushing with SKCS (n = 3).

Table 2. Rheological parameters calculated from the SKCS Crush-Response Profiles for unconditioned (U) and conditioned (C) hard and soft wheat. LSD = Least significant difference

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Shell Phases</th>
<th>Endosperm Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\sigma$ (N)</td>
<td>$\varepsilon$ (s)</td>
</tr>
<tr>
<td>Banks U</td>
<td>313</td>
<td>5.9</td>
</tr>
<tr>
<td>Banks C</td>
<td>258</td>
<td>9.0</td>
</tr>
<tr>
<td>Janz U</td>
<td>465</td>
<td>5.4</td>
</tr>
<tr>
<td>Janz C</td>
<td>448</td>
<td>9.8</td>
</tr>
<tr>
<td>Rosella U</td>
<td>458</td>
<td>7.0</td>
</tr>
<tr>
<td>Rosella C</td>
<td>454</td>
<td>8.9</td>
</tr>
<tr>
<td>Tincurrin U</td>
<td>391</td>
<td>6.9</td>
</tr>
<tr>
<td>Tincurrin C</td>
<td>382</td>
<td>8.9</td>
</tr>
<tr>
<td>LSD</td>
<td>31</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 3. Comparison of average fragment size distribution of conditioned wheat after crushing with SKCS and Vario first-break rolls with a gap of 0.67 mm (n = 3).

<table>
<thead>
<tr>
<th>Sieve size (μm)</th>
<th>Percentage of sample mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Janz (hard)</td>
</tr>
<tr>
<td></td>
<td>SKCS</td>
</tr>
<tr>
<td>4000</td>
<td>16.4</td>
</tr>
<tr>
<td>2000</td>
<td>56.2</td>
</tr>
<tr>
<td>1000</td>
<td>13.4</td>
</tr>
<tr>
<td>710</td>
<td>3.9</td>
</tr>
<tr>
<td>300</td>
<td>7.0</td>
</tr>
<tr>
<td>125</td>
<td>2.6</td>
</tr>
<tr>
<td>75</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Figure 7. Typical CRPs with consistent characteristics from 3 samples of unconditioned (thick line) and conditioned (thin line) wheat ranging in decreasing hardness: cv. Janz and Banks (hard); cv. Rosella and Tincurrin (soft). (A) Janz (B) Banks (C) Rosella (D) Tincurrin
4.4 Discussion

4.4.1 Crush-Response Profile (CRP)

In the SKCS, wheat grains are individually crushed between a toothed rotor and a crescent (Figure 1). The minimum gap between the rotor and the crescent is factory set at 0.67 mm by the use of spacers beneath the load cell (J. Kennedy, Perten Instruments, personal communication). The load cell mounting is then adjusted to a specified torque setting while using a special tool to minimize stress on the load cell. During crushing of the grain, the force exerted on a load cell, attached to the crescent, is measured in constant time steps of 0.25 ms. A plot of this force against time is referred to as a Crush-Response Profile (CRP), which can be interpreted as a pseudo stress/strain curve (Figure 2) (Osborne & Anderssen, 2003).

Typical CRPs for each variety indicate specific rheological characteristics differentiating between and within grain hardness classes (Figure 3) in which the endosperm strength and stiffness of unconditioned wheat appear to clearly differentiate between varieties of differing hardness (Table 2). The mode of crushing of the SKCS is demonstrated by the relative fragment and particle size distributions of the fragments (Figure 4) and is discussed below.

4.4.2 CRPs of Conditioned versus Unconditioned Grain

The standard SKCS method (AACC, 2002) involves crushing unconditioned wheat. On the other hand, milling practice involves conditioning (the addition of water to wheat before milling) to enhance the efficiency of separation of bran and endosperm. The moisture content that is optimal for milling represents a compromise between maintaining partial dryness for ease of sifting and sufficient moisture to soften the endosperm and toughen the bran, which becomes more compliant and resilient with increasing moisture content (Glenn et al., 1991). The response of different wheat varieties to moisture uptake is known to vary. Although it is generally accepted that hard wheat endosperm diffuses water at a slower rate than soft wheat endosperm, the exact nature of the interaction is not well understood (Pomeranz & Williams, 1990).

As a result of fracture mechanics studies carried out on isolated specimens of bran, Glenn and Johnston (1992) have shown that conditioning of wheat results in an increase in bran elasticity accompanied by a loss of its strength and stiffness (Glenn & Johnston, 1992b). Similarly, studies on isolated endosperm by Delwiche (2000) and
Glenn et al. (1991) have shown that its strength exhibits a consistent decrease with increased moisture content following conditioning (Glenn et al., 1991; Delwiche, 2000). The rheological data recovered from the SKCS CRPs (Table 2) shows a similar pattern, with shell stiffness and strength and endosperm strength all decreasing as a result of conditioning. These observations support the conclusions of Osborne et al. (2007) about the usefulness of SKCS measurements in the study of the mechanical properties of wheat grain (Osborne et al., 2007).

Of the mechanical properties tested by Glenn et al. (1991), endosperm compressive strength ($\sigma_{\text{max}}$) exhibited the most consistent relationship with moisture content. Hard wheat endosperm compressive strength was reported to be much more sensitive to moisture content than soft, wheat endosperm (Glenn et al., 1991). In addition, hard wheat samples generally have a more linear response between $\sigma_{\text{max}}$ and moisture content than soft wheat samples (Delwiche, 2000). This is confirmed in the present study in which conditioning had a more significant influence on the CRPs of the hard varieties, when conditioned to a higher moisture content than soft [Figure 7 (A-D)]. For example, Figure 7 (A, B) indicates that conditioning of hard varieties produced a marked reduction in the collapse of the ‘shell’ response with plateauing of the ‘equilibrium’ response. Figure 10(B) illustrates the enhanced plasticity of bran layers after conditioning of the hard wheat cv. Banks in comparison to the brittle fracture of unconditioned, vitreous endosperm producing smaller bran pieces [Figures 8(A) and 10(D)]. Glenn et al. (1991) suggested that differences in strength and stiffness values with varying moisture contents may be due either to moisture sensitivity of starch granule strength, storage protein strength and/or the strength of starch/protein adhesion though these relationships are still not well understood (Stenvert & Kingswood, 1977; Glenn et al., 1991).

The effect of conditioning on CRPs tended to remove many differentiating features between varieties conferring some of the characteristics of softer varieties to the harder varieties (compare Figures 3 and 5 and the rheological parameters for unconditioned and conditioned wheat given in Table 2). Accordingly, the resultant PSDs of the four varieties crushed by SKCS became more similar in profile to one another (Figure 6) presumably due to the toughened, more compliant bran layers as well as the effects of moisture and weakening of endosperm cohesion, particularly in hard varieties. This infers that biochemical properties influencing bran plasticity,
endosperm cohesion and cell wall structure are more sensitive to changes in moisture in hard varieties.

**Figure 8.** Fragment size variation in hard wheat grains (A) unconditioned and (B) conditioned (cv Banks) after fracturing by SKCS. (C) Fragmentation of conditioned wheat caused by first-break rolls. (D) unconditioned cv Janz crushed by SKCS. Imaging via dissecting microscope, scale bar represents 3mm.
Figure 9. Fragment size variation in (A) unconditioned and (B) conditioned soft wheat cv Tincurrin after fracturing by SKCS. Imaging via dissecting microscope, scale bar represents 3 mm.

Figure 10. (A, B) Hard, conditioned, wheat grain fragments (cv Banks) fractured using SKCS. (C, D) Hard, unconditioned, wheat grain (cv Banks) fragments fractured using SKCS. (E, F) Hard, conditioned, wheat grain fragments (cv Banks) fractured using Vario rolls. Imaging via dissecting microscope, scale bar represents 1mm.
Figure 11. (A, B) Soft, conditioned, wheat grain fragments [(A) Rosella, (B) Tuncurrin] fractured using SKCS. (C, D) Soft, unconditioned, wheat grain fragments fractured using SKCS [(C) Tuncurrin, (D) Rosella] (E, F) Soft, conditioned, wheat grain fragments [(E) Tuncurrin, (F) Rosella] fractured using Vario rolls. Imaging via dissecting microscope, scale bar represents 1mm.
The PSDs of conditioned wheat demonstrate the expected increase in deformability of the bran producing the fragments >4 mm in all varieties [Figures 6, 10 (B) and 11(A)]. In the SKCS crushing of unconditioned wheat samples, harder varieties fracture into more medium sized (1–2 mm), angular fragments shown in Figures 8 (A, D), 10 (C, D). By contrast, softer varieties tend to form larger fragments [Figure 9 (A)]. This is confirmed by the particle size distributions shown in Figure 4. After conditioning, on the other hand, similar particle size distributions occur for wheats of different hardness, as shown in Figure 6. This observation is in agreement with Muhamed and Campbell (2004). Also, the characteristic of soft wheat varieties to produce a higher first break flour release than hard varieties is indicated by particle sizes <710 μm in Table 3, by SKCS crushing forming particles <125 μm in Figure 4, and <710 μm in Figure 6 (Haddad et al., 1998; Haddad et al., 1999).

Microscopy studies suggest that fracture propagation in soft wheat varieties travels across cell walls and through the cell contents of porous endosperm [fragile failure, Figure 11(C, D)]. Whereas in hard varieties, the response to compression proceeds along cell walls (limiting plastic behaviour) [Figure 10 (E, F)]. Previous rheological studies by Osborne et al. (2007) have noted specific relationships between (1) varietal hardness and stiffness (modulus of elasticity) and (2) strength (maximum failure stress), vitreousness and variety; as indicated by a comparison of CRPs of (1) Banks (moderately hard) and (2) Rosella, a soft though vitreous variety (Figure 3) (Osborne et al., 2007). Grain with a more homogeneous structure such as vitreous durum wheat exhibits a plastic stage or plateauing of the endosperm response before failure (Haddad et al., 1998; Haddad et al., 1999). A similar plateauing can occur in very soft varieties such as cv Tincurrin having a consistent porous and mealy structure which may allow slight compression before final collapse [Figures 3, 5, 7 and 11 (B)].

4.4.3 SKCS modes of fracturing

The SKCS measures predominantly the compressive strength of whole wheat grain and produces significant rupturing of central endosperm cells [Figures 10 (A, C), 11 (C)]. These are known to be structurally weaker, possibly due to a larger and more spherical shape (Moss et al., 1980; Philippe et al., 2006a). Observations in this study also indicate that the central cells adjacent to the crease are particularly sensitive to conditioning.
SKCS crushing tends to produce diffuse fracture and rupture of cells producing mainly mealy fragments [Figures 10 (A, C) hard wheat, 11(A-D) soft wheat] except in the most vitreous regions (peripheral endosperm) of hard varieties [Figure 10 (D)]. The protein content of the sub-aleurone region of wheat endosperm is known to be higher than the central endosperm regions. Consequently, the extent of breakage of adhering bran layers without conditioning is dependant on the vitreousness of peripheral endosperm regions as shown by comparing the fracture of bran between unconditioned vitreous and mealy peripheral endosperm in [Figures 8 (D) and 9(A)]. Furthermore, SKCS analyses of pearled wheat indicates that the extent of grain hardness may be influenced by the composition of peripheral regions of the kernel (Osborne et al., 2005). Taken together, the resistance of endosperm to crushing would be the sum of both the weakness of the central cells and the structural strength of prismatic and sub-aleurone regions.

The crushing of conditioned wheat in the SKCS to mimic the commercial milling process produced a significantly greater proportion of larger sized fragments (> 2mm) and consequent reduction of fragments in the 1 – 2 mm range compared with the fragmentation of unconditioned samples. Soft, conditioned, wheat fractured using SKCS produced large fragments predominantly of mealy texture compressed into masses (with some small clumping) adhering to bran. Bran layers were widely split, possibly being comparatively thin and weak [Figures 9 (B) and in detail 11(A, B)]. However, a close association was indicated by the comparative PSDs of sizes less than 710 µm for conditioned wheat crushed by SKCS and first-break rolls (Table 3). In summary, the compression exerted by SKCS with less shearing action, tends to accentuate the deformability of conditioned bran thus reducing fragmentation [compare the large bran pieces in Figures 8 (B), 10(B) and 11(A) with the broken bran fragments from conditioned grain through first break rolls in Figures 8 (C) and unconditioned grain through SKCS in Figure 10 (C), and 11(D)].
4.4.4 First break roll modes of fracturing

A Vario experimental rollstand was used to represent the first break stage of milling. The particle size distribution resulting from first-break roller milling directly affects the subsequent flow and machine settings, and thus determines the effectiveness of the milling process (Hsieh et al., 1980a). The Vario mill is a single pass, fully variable test mill that uses commercial-scale rolls (250 mm diameter, 100 mm length) to represent commercial flour milling operations. The roll gap was set at 0.67 mm (0.026 inches) to match the minimum gap between the rotor and the crescent in the SKCS. Although a gap of this magnitude has been used for first break rolls in an experimental milling system by Gwirtz et al. (1996), it would be considered large for a commercial mill (Gwirtz et al., 1996). For example, this gap would represent the high extreme of the range used by Campbell and Webb (2001) to develop a model for commercial milling (Campbell & Webb, 2001).

In first-break roller milling of wheat, the factors affecting breakage of grains can be broadly classified into the physicochemical properties of the wheat (size distribution, moisture content, hardness) and those related to the design and operation of the milling equipment (Campbell et al., 2001a). With the occurrence of compressive stress in the horizontal direction and shear stress in the vertical direction, a kernel tends to break along a principal tensile stress plane because the tensile strength of the endosperm is much smaller than its compressive strength (Fang & Campbell, 2002b). Results reported by Hsieh et al. (1980) indicate that the effect of roll speed on kernel breakage is very small compared with roll gap and differential (Hsieh et al., 1980a). Therefore, first-break rolls exert more shearing forces through the differential rotation of fluted rolls and some compressive force from the roll gap. The compression force component possibly accounts for some of the mealy texture in larger fragments not seen in manually snapped or cut vitreous grains, however the extent of mealy or ruptured areas is much less evident compared to SKCS crushed samples [compare Figures 10 (A, C) with 10(E)].
Figure 12. Fragment size distribution of conditioned (c) and unconditioned (u) wheat (cv. Banks, hard) after crushing with SKCS (s) and first-break rolls with equal SKCS gap (0.67 mm) (v) (n=3).

Despite the different modes of crushing in the SKCS (compressive) and first-break rolls (shear and compressive), the fragment size distributions for conditioned wheat are similar to the first break roll throughs in ranges below 1 mm (Table 3). This would imply that, if the aim is to model milling performance, wheat should be conditioned prior to SKCS testing as carried out by Muhamed and Campbell (2004) (Muhamed & Campbell, 2004). However, Figure 12 shows that of the four modes of crushing tested, the particle size distribution produced by unconditioned grain crushed by the SKCS (the standard method) was, unexpectedly, the most comparable. This was the case for small and, more importantly, large fragment size ranges to conditioned grain crushed by the Vario mill. In Figure 12, intermediate range fragments between 300 μm and 1 mm for cv. Banks were significantly different (P < 0.05, n = 3). There is also similarity in the appearance of the fragments produced as seen by comparison of Figure 10 (C, D) and Figure 10 (E, F) (hard wheat) and Figure 11 (C, D) and Figure 11 (E, F) (soft wheat). Consequently these results
provide support, in terms of the relevance to flour milling, of the use of unconditioned grain and the configuration of the crushing mechanism in the standard SKCS test.

4.5 Conclusions

Conditioning of both hard and soft wheat results in reduction in the shell stiffness and strength and the endosperm strength, as measured using the SKCS 4100. This provides a rapid means to monitor the progress of conditioning.

The fragmentation patterns of both hard and soft wheat that result from crushing in the SKCS 4100 and in the first break stage of roller milling show that SKCS analysis of unconditioned wheat (the standard practice) is a suitable predictor of the milling performance of conditioned wheat within normal moisture ranges of delivered grain lots.

Chapter 5. Effect of Endosperm Starch Granule Size Distribution on Milling Yield in Hard Wheat

5.1 Introduction

Despite considerable gains in wheat flour milling yield through conventional breeding strategies and milling technologies, the theoretical maximum yield still has not been attained. Discovery of genes in wheat that control flour yield would provide a means for breeders to develop new wheats that fulfill their potential in relation to this trait. Such gene discovery is, however, made especially difficult by the number and complexity of linkages between the genetics and grain processing. For example, Lehmensiek et al. (2006) explored Quantitative Trait Loci (QTL) for flour yield in three Australian doubled haploid populations and found only one QTL in a similar location in more than one population (Lehmensiek et al., 2006). A more targeted approach is to improve the understanding of the role of grain microstructure in determining high flour yield. Hard wheat is generally considered easier to mill since it gives readier separation of bran from endosperm after conditioning, and the liberated flour is more mobile and easier to sift. Thus, the majority of research has focused on
elucidating the genetic mechanisms for variation in hardness, hence milling performance, within the hard phenotype. According to current understanding, for hard wheats, increased endosperm vitreousness is associated with higher flour yields (Dobraszczyk, 1994; Haddad et al., 1999). In terms of rheological properties of wheat endosperm, increased vitreousness may be described by increased stiffness and strength at a constant ductility (Mabille et al., 2003).

The SKCS 4100 has been used to measure endosperm rheological properties (Osborne & Anderssen, 2003; Osborne et al., 2005; Osborne et al., 2007). The SKCS provides for much higher throughput than the study of isolated endosperm specimens because it records relevant rheological information following the crushing of whole grains. Edwards et al. (2007) demonstrated, through microscopy and particle size analysis of the crushed material from the SKCS 4100 and a first break roll stand, that the SKCS data for unconditioned wheat averaged over 300 grains provided a useful indicator of milling performance of a wheat sample (Edwards et al., 2007). Osborne et al. (2007) showed that re-scaling SKCS data using correlations between Instron data on isolated endosperm and SKCS Endosperm Strength gave numerical values for Endosperm Strength in line with those in the literature (Osborne et al., 2007). On the basis of samples with contrasting Milling Quality Index it was then confirmed that better milling quality is characterised by increased Endosperm Strength and Stiffness and that these properties can be recovered from the Crush-Response Profile (CRP). This led to the combination of SKCS Endosperm Strength and Stiffness into a single value (Wheat Rheology Index) which resulted in a high rank correlation with commercial performance evaluation. The next step in the research was to establish relationships between endosperm rheological properties and its microstructure.

The *Pina-D1* and *Pinb-D1* alleles, tightly linked to the Ha locus on the short arm of Chromosome 5D, determine the hardness phenotype (Greenwell & Schofield, 1986; Turnbull & Rahman, 2002). However, this does not fully account for the observed genetic variation in hardness, especially within each hardness class, and it is thought that additional modifying genes account for the range of hardness within hard or soft classes (Turnbull et al., 2000; Martin et al., 2001; Osborne et al., 2001b). Several research groups have studied the role of the puroindolines in explaining within class variation in hardness. In hard wheats, the *Pina-D1b* allele was associated with
harder texture than the Pinb-D1b allele (Giroux & Morris, 1997). Martin et al. (2001) reported that the Pinb-D1b (softer texture) allele was associated with better flour yield in Hard Red Spring wheat (Martin et al., 2001).

Others have investigated the relationship between endosperm starch granule size and hardness. Bechtel et al. (1993) correctly identified wheat samples as hard or soft based on the size distribution of starch granules (Bechtel et al., 1993). Igrejas et al. (2002) reported that harder wheat had a higher content of small-sized starch granules but could not find a QTL for starch granule size on the 5D Chromosome; they concluded that “starch size distribution is influenced by genes which have yet to be analysed” (Igrejas et al., 2002b). This prompts a search for QTLs on chromosomes other than 5D. QTL studies have shown that other genes are also important in grain hardness regulation (Turnbull & Rahman, 2002). For example, results suggest genes on chromosomes 1A, 2A, 2D, 6D, 5B, and 6D also affect grain hardness while three others having interaction effects are located on chromosomes 5A, 6D and 7A (Sourdille et al., 1996; Perretant et al., 2000; Galande et al., 2001; Clarke & Rahman, 2005). Osborne et al. (2001) had earlier proposed a major new genetic factor for hardness and presented data that indicated it was associated with the microsatellite Xwmc 048 on Chromosome 4B (Osborne et al., 2001b). Interestingly, the same Xwmc 048 marker has also been reported to be associated with starch B granule content (Batey et al., 2001) and flour yield (Breseghello et al., 2005; Lehmensiek et al., 2006). Thus, it might be postulated that a genetic association exists between starch granule size distribution (SGSD) and flour yield.

Starch granules develop in a membrane bound organelle, the amyloplast. At maturity, the plastid membrane is lost although some proteins remain associated with the granule surface. The mechanisms for starch granule formation are still being defined (Bechtel & Wilson, 2003). The extent and type of starch granule formation is partially determined by ambient temperatures (Hurkman et al., 2003). Bechtel and Wilson (2003) observed that the three granule classes are produced at specific times during wheat endosperm development. A higher percentage of small starch granules is typical of hard in comparison with soft wheat (Evers & Lindley, 1977; Bechtel et al., 1993; Zayas et al., 1994; Stoddard, 1999b; Gaines et al., 2000), although no significant differences in the relative quantity of A-type granules have been noted between the
two wheat classes (Glenn et al., 1992; Bechtel et al., 1993). In a starch granule study of 12 soft wheat cultivars, Raeker et al. (1998) found highly significant differences among the cultivars for volume % of granules within the A-type granule range (Raeker et al., 1998). Granule type predominance is known to directly affect dough/baking quality (Park et al., 2004); however, little is known about how starch granule size distribution (SGSD) affects milling performance within the hard wheat class.

Dependant on the type of granule size distribution analysis used, some studies support a trimodal distribution in wheat endosperm which Bechtel and Wilson (2003) class as large (A-type), medium (B-type), and small (C-type) (Raeker et al., 1998; Bechtel & Wilson, 2003). A-type granules have been reported as 10 – 35 μm in diameter and account for more than 70% of the total starch weight but less than 10% of the granules by number. Small granules (B- and C-type) account for over 90% of the granules by number, but less than 30% of the total starch by weight in wheat endosperm (Lindeboom et al., 2004). C-granules have a diameter of less than 5·3 μm and represent 45·7% of the total number of endosperm starch granules and 3·4% of the total seed weight (Igrejas et al., 2002b). However, the surface area of small granule types has been estimated at 0.7 m² per gram of starch and is about three times higher than that for A-granules producing a prominent contribution to endosperm cohesiveness and strength (Konopka et al., 2005). Also previous research has identified within the endosperm, predominantly in the subaleurone region, the presence of spherical lipid bodies with a 0.2-2.0 μm diameter (Hargin et al., 1980). These are similar to spherosomes, stored triglycerides bounded by a half-unit or monolayer membrane of proteins and diacylphospholipids which are abundant in aleurone and scutellar cells. However, most starch isolation methods should exclude contamination with such lipid bodies from the subsequent granule size distribution analysis. Nevertheless, Greenblatt et al. (1995) have pointed to quantitative and qualitative diversification of lipids occurring on the starch surface of wheat grains with different hardness (Greenblatt et al., 1995). Higher levels of lipids have been generally associated with smaller starch granules (Raeker et al., 1998; Gaines et al., 2000).

Many studies of wheat milling quality have focused on the structural differences between hard and soft wheat varieties. The aim of the present study was to test the hypothesis that a genetic association exists in hard wheat between starch
granule type and flour yield. This study is facilitated by the rheological analyses of the SKCS that is demonstrated to directly reflect variation in microstructural features as observed using Environmental Scanning Electron Microscopy (ESEM).

5.2 Materials and Methods

5.2.1 Samples

Seed samples representing 197 wheat varieties from the Australian Winter Cereals Collection (Tamworth) were used for this study. To obtain sufficient quantities of grain, the seeds were propagated twice. The first propagation took place at Tamworth, New South Wales in 2004 for an initial study using the SKCS and ESEM. The second propagation was carried out at two locations (Biloela, QLD and Narrabri, NSW) in 2005 to produce sufficient quantities of grain for laboratory milling and SGSD analysis. Not all of the varieties grew successfully at all three locations.

5.2.2 Single Kernel Characterization System (SKCS) Analysis

SKCS measurements were carried out on 300 grains from each sample from all three propagations according to AACC Method 55-31 (AACC, 2002). The individual CRPs for each sample were first exported from the SKCS 4100 directory in a text file format and then imported into Crush Curve Analysis Software (CCAS - BRI Australia Ltd, North Ryde, New South Wales, Australia). CCAS calculated the average CRP for each set of curves then, for each average CRP, calculated the rheological parameters strength (maximum stress, ζ) and stiffness (Modulus of Elasticity, calculated as δσ/δε) of the endosperm. The standard SKCS parameters (seed weight, diameter and Hardness Index (HI)), CRPs and the calculated rheological parameters (Edwards et al., 2007) were exported from CCAS in a text file format and further data processing was carried out using Microsoft Excel. The Wheat Rheology Index was calculated as (10 x endosperm stiffness + endosperm strength)/15 (Osborne et al., 2007).

5.2.3 Imaging of grouped wheat samples using ESEM

Whole wheat grains (three seeds of each selected variety) were manually scored with a scalpel and snapped transversely in two. The fractured grain was mounted on an aluminium stub using double-sided adhesive tape. Images were obtained without further specimen preparation using a Philips XL30 environmental scanning electron microscope (Philips, Denmark) with a gaseous secondary electron
(GSE) detector at a pressure of 1 Torr and an accelerating voltage of 15 kV. The working distance was 10 mm. Cell wall and granule diameter measurements were taken using ESEM image scale bars for a general comparison with the laser diffraction method.

5.2.4 Test Milling

Grain samples from the second (Biloela and Narrabri) propagations were available for milling. They ranged in size from 0.35 kg to 4.5 kg. Wheat was cleaned using a Dockage Tester (Carter-Day, Minneapolis, MN, USA) then conditioned at 16.5% moisture content for 16 h. The quantity of water to add to each sample was calculated from the NIR moisture content measured on the cleaned wheat by means of an Infratec 1229 NIR instrument (FOSS, Hoganas, Sweden) using conditioning tables (AACC Method 26-95). Cleaned, conditioned wheat was milled using a MLU 202 Laboratory Mill (Buhler Bros, Uzwil, Switzerland) at a feed rate of 100 gmin\(^{-1}\). Bran and pollard fractions were further processed using two passes through a MLU 203 Laboratory Impact Finisher (Buhler Bros, Uzwil, Switzerland) and the finisher flours were passed through a 150 μm screen before incorporation into the straight-run flour. The extraction rate and the speck count by Branscan 2000 (Branscan Ltd, Evesham, UK) were measured for each straight run flour then the commercial performance of each wheat was assessed using the Milling Quality Index: \((\text{speck count/flour yield %}) \times 100\) (Southan et al., 2001).

5.2.5 Starch Extraction

Triplicate subsamples of six grains were taken from each sample. Methods of starch extraction from flour and manually-ground kernels were adapted from (Stoddard, 1999a; Giroux et al., 2003; Hogg et al., 2004). Stoddard (1999b) demonstrated that proximal florets in the middle spikelets of the head have uniform grain sizes and their B-granule contents are both high and uniform. Accordingly, only the larger kernels were used in the present experiments to avoid within-head variation in grain filling duration and to maximise the chances of detection of genetic variation. Growing plants in the same environment ensured that grains experienced uniform environmental conditions excluding the variation in duration of grain filling affecting their variation in B-granule content (Stoddard, 1999b). The
grains were cracked into 2 ml Eppendorf tubes and soaked in 0.7 ml of 0.1M sodium chloride overnight. A plastic Eppendorf pestle attached to an electric drill was used to gently grind the samples in the soaking medium until the gluten formed a dough ball and the bran was broken into large flakes. The dough ball was pushed to the bottom of the tube and the aqueous starch suspension was transferred through a fine sieve (200 μm pore size) to the top of a 2 ml pre-weighted Eppendorf tube containing 1 ml of 80% cesium chloride. The solids were ground again in a further 0.5 ml of 0.1M sodium chloride, the slurry was decanted into the same tube as before, and the solids were ground and slurry decanted for a third time. The starch suspension and cesium chloride were then centrifuged at 13,000 G for 3 min. The cesium chloride was decanted and the starch was vortexed with 1 ml water until clumping dispersed (~40 s) and then re-centrifuged. The starch pellet was then washed by 3 min centrifugation at 13,000 G, through a sequence of 2% sodium dodecyl sulfate (SDS) then twice in water. The starch suspension itself was subsequently used for analysis.

5.2.6 Laser diffraction analysis

Granule size distribution was quantified in a Malvern Mastersizer 2000 laser-diffraction analyser (version 5.22, Malvern Instruments Ltd, Malvern, UK) using the flow-through, 100 ml reservoir. The starch suspension (1.7 ml) was vortexed and sonicated for 30 s at 6W then added to the reservoir until an obscuration value of between 12–17% was achieved (remaining sample was stored at 4°C). Settings were optimised for the refractive index of starch in water and an average of 3 consecutive measurements performed.

5.2.7 Fluorescent staining of Spherosomes

Aliquots (0.5ml) of extracted starch granule were stained for 30 - 60 s in 0.01 % aqueous Nile Blue A (Sigma-Aldrich), then washed gently in water for 2-3 min. The suspension was centrifuged at 13,000 G for 3 min and water removed then the pellet was re-suspended in water. A drop of the stained suspension was mounted under a cover glass and examined using a Nikon fluorescence microscope (model: Eclipse E600, Japan) equipped with an epi-fluorescence interference filter with maximum excitation at 450 - 490 nm. Control sections
were viewed without staining or were extracted with hexane for 2 -3 min before staining (Hargin et al., 1980).

5.3 Results and Discussion
5.3.1 Investigation of the microstructure of groups with contrasting Wheat Rheology Index

Samples of grain obtained from the first propagation at Tamworth, NSW in 2004 were of insufficient quantity for test milling. Instead, they were studied using the SKCS and ESEM. ESEM has only recently been used to study the microstructure of rice (Dang & Copeland, 2004) and wheat (Zakowsky & Donald, 2005). The technique is particularly useful when the natural moisture content of materials needs to be retained for example in the study of milling quality where moisture contents of between 9-16% play an important role. First, the 197 samples were ranked by means of the SKCS Wheat Rheology Index (Osborne et al., 2007) then three groups (four varieties per group) of contrasting WRI were examined by ESEM in an attempt to identify the structural differences associated with contrasting WRI (Figure 1). In accordance with previous rheological studies using the SKCS, specific relationships are apparent between groups of increasing hardness in relation to increasing endosperm stiffness (modulus of elasticity) and strength (maximum failure stress). These correspond with a similar trend in the initial ‘Shell Responses’ of the CRPs (Osborne & Anderssen, 2003; Osborne et al., 2005; Osborne et al., 2007).
Figure 1. Groups of three CRPs of hard wheat varieties with similar SKCS Wheat Quality Index ranking: Light gray : WRI = 40; Black : WRI = 68; Mid gray : WRI = 77).

Figure 2. ESEM images of outer endosperm prismatic cells (sub-aleurone cells left of frames). Varieties arranged from (A – D) according to CRP: (A) Sunco; arrow: continuous peripheral matrix surrounding interior of loosened granules (WRI = 77); (B) Dollarbird (WRI = 69.7); (C) Yitpi (WRI = 68.4); (D) Moldova (WRI = 40). Scale bars represent 100 μm.
The ESEM was used to explore the possible differences in endosperm structure between the samples with corresponding WRI. The low magnification images of inner and outer endosperm fractured surfaces are shown in **Figure 2.** **Figure 2 (A-D)** compares the fractured prismatic cells of the outer half of the endosperm of three rheological groups.

**Figures 2 (B, C)** show varieties in the harder group (WRI = 68) and display a wide granule size range with A-type granules being bound tightly with a greater presence of the smaller type spherical bodies. There is also prominent fracturing along cell walls typical of harder varieties. ESEM observations were also made using several high magnification images traversing the endosperm from the crease region to the aleurone layer of two large kernels from each variety (**Figure 3**). **Figure 3 (A-D)** exhibits ESEM images of fractured prismatic cells of hard wheat endosperm in the same regions as in **Figure 2** though at a higher magnification. **Figure 3 (C)** exhibits a structure of intermediate strength (WRI = 63.7) with most granules held in place though still allowing a diffuse fracture through cells rather than around cell walls compared with **Figures 2 (A-C) and 3 (A, B).** **Figures 2 (A) and 3 (A)** are both of the high flour yield variety, Sunco. This is a typical example of the hardest group (WRI = 77) with a higher number of small spherical bodies, possibly C-type granules, binding the large granules representing the major starch reserves.

The presence of a continuous amorphous layer around cell contents appears to create a zone of weakness between the boundary between the cell wall and cell contents. Hence cleavage tends to be intercellular. The contents of the cell act as a single entity and the whole cell is readily removed from the bran by the shear forces imparted during milling. These forces are also redirected towards the bran, causing fragmentation into relatively small pieces (Moss *et al.*, 1980). Similarly the more detailed view in **Figure 3 (B)** of variety, Saturno (WRI=77) indicates the peripheral segregation of bonded small granules of similar size forming a casing with the cell interior being more porous. More recent research on the structural changes in endosperms during drying indicates that this surrounding region may be partially composed of remnant cytoplasm (Bechtel & Wilson, 1997, 2003, 2005). **Figure 2 (D) and 3 (D)** are examples of the softer group (WRI = 40) indicating a prevalence of A-type granules surrounded by a semi-porous protein matrix embedded with small granules and fracturing across cell walls and around granules, causing a loss of many granules from the surface. A deficiency of smaller sized granules to fill gaps between
large granules may contribute to a weaker structural density. In summary, the two ‘harder’ groups exhibited an increasing predominance of the smallest range of spherical bodies possibly C-type granules in association with increasing endosperm strength and stiffness (WRI rating). Quantitative support for this relationship is presented in Section 5.3.3 (see also Appendices: Microscopy Images).

All hardness rankings appear to have an abundance of large A-type granules in central cell types of the endosperm (images not shown) though specific regional differences between varieties have not been quantified. Furthermore, research has shown that the large rounded shape of these cells tend to produce a weaker structure (Evers & Millar, 2002; Edwards et al., 2007). This may indicate that the shell response of the CRP may be influenced by the Type A granule content in central cells and may explain the correlation between the stiffness of the endosperm response and the volume ratio of large to small granule types (data not shown).

Figure 3. ESEM images of fractured prismatic cells of hard wheat endosperm indicating variation of granule size distribution between milling quality groups. WRI = 77: (A) Sunco, (B) Saturno: Arrow indicates a peripheral casing of bonded granules with a porous cellular interior; (C) WRI=63.7; Galaxy H45; (D) WRI=40: Vega. Scale bars represent 50 µm.
Similar studies of the variation in microstructure within the soft wheat class and other cereal grains may improve the understanding of their processing qualities. Other microstructural features such as cell wall structure and geometry may also contribute to grain hardness and flour yield. The influence of endosperm cell walls on processing quality is not well documented and further study in this area may be useful (Kamisaka et al., 1990; Fry et al., 2000). Recent research using microspectroscopy techniques has demonstrated regional variation in the physico-chemical properties of endosperm cell walls (Piot et al., 2001b; Philippe et al., 2006a).

5.3.2 SGSD methodology

Many methods of starch granule extraction have been reported. These include hand-washing, wet-milling, or enzymatic methods (EM), which are often tedious and time-consuming. Alternative rapid methods, such as a recently demonstrated sonication process have also proved effective (Park et al., 2006). These different methods can lead to some variation in results such as loss of the very small C-type granules. The method used in the present study only requires a few seeds producing results with more reproducibility than some methods requiring more material (Stoddard, 1999a, 2003). However, due to a strong tendency for starch granules to stick together, much care is needed in purification, vortexing and sonication to exclude aggregation which can distort size distributions (Figure 4). Care is also needed to ensure that large starch granules are not broken as a result of sonication.
Figure 4. Starch granule size distribution by laser diffraction showing the same hard wheat sample (NW25A) analysed twice. Light grey profile displays aggregations greater than 100 μm whereas black profile was subjected to additional pre-analysis sonication.

Figure 5 shows light microscopy images of starch granules suspended in water after the extraction process. In these examples most of the small granules are between 2 – 6 μm which approximates the distribution analysis results for the B-type granule mode. A proportion of the smallest granule range comprised irregular shaped particles possibly granule fragments, although this was not chemically confirmed. In a detailed microscopy study of wheat starch damage, Tester et al. (1994) reported the presence of similar particles possibly surface fragments of large granules whereas the study concluded that the small starch granules are more susceptible to damage during the normal milling process (Tester et al., 1994). In addition, a stain (0.01 % aqueous Nile Blue A) for the detection of lipid bodies in the starch isolate was applied to confirm their exclusion from the analysis (Hargin et al., 1980).
Several techniques can be used to determine particle size distributions: laser light scattering (LDS), microscopy, sieving, sedimentation analysis, permeability of a powder column, and an electrical-sensing zone technique. A wide range in particle size creates difficulties in accurately measuring starch populations. Particle size analysis by LDS is done because of its ease of operation and reproducibility. Digital image analysis (IA) coupled to light microscopy offers the ability to record physical parameters for each individual particle and to distinguish among individual granules, agglomerated granules, and non-starch particles (Wilson et al., 2006). However, Image Analysis (IA) is limited to a small sample size, and acquiring data and analysis can be time-consuming. In the study by Wilson et al. (2006), comparing the measurement of wheat SGSD using image analysis (IA) and laser diffraction technology, four laser diffraction sizing (LDS) instruments were used to measure granule distributions of four classes of wheat (Hard Red Winter, Hard Red Spring, Durum, and Spelt). In this study, the Malvern Mastersizer 2000 detected little variation from class to class compared with the other devices tested. On the other hand, the Microtrac instrument detected major shifts in both A- and B-type peaks with respect to diameter and volume. In the present study, the Mastersizer 2000 was able to make significant differentiation between hard wheat varieties with excellent repeatability.
**Figure 6** illustrates differences between (A) NW25A and (B) Gabo having 75% and 77.6% flour extraction and SKCS Hardness Indices of 60 and 76.5, respectively.

![Graph illustrating starch granule size distributions](image)

**Figure 6.** Triplicate starch granule isolation and LDS analyses of starch granule size distributions of six hard wheat kernels each from (A) NW25A and (B) Gabo.

LDS instruments base their algorithms on the Mie theory predicting the angular scattering intensity of a smooth, internally homogeneous sphere of known refractive index, illuminated by light of a given wavelength and polarization. Due to the unique oblate spheroid (lenticular) shape of large wheat starch granules, one pass of a granule through the instrument may diffract the laser on the flat surface of the granule, while a similar granule may diffract on the narrow edge or at some obtuse angle to these surfaces. This results in an underestimation of the diameter of an A- or B-type granule, and consequently an under-estimation of its volume when calculated as a sphere. LDS compared with IA resulted in a ~40% underestimation of the A-type granule diameter and a 50% under-estimation of the B-type granule diameter (Wilson *et al.*, 2006).

In this study, volume ranges from 35 to 10 μm were considered as A-type granule (*Figure 6*). Within the Type B and C classes, volumes were calculated between (6.6–2 μm) and (1.5–0.6 μm) respectively as measured by LDS (Malvern Mastersizer 2000). This tri-modal distribution is more apparent when viewed in terms of relative surface area (*Figure 7*). The large granules make the largest contribution to the starch volume whereas the small granules being numerous contribute most to the
bonding surface area. When converting volume data to particle numbers over a wide distribution of sizes, the graphical display of the cubic relationships involved, is beyond the resolution of the software. Therefore, the comparatively low number of large granules appear as zero on the graph.

**Figure 7.** Laser diffraction analysis (Malvern 2000) of starch granule size distribution of hard wheat (Janz) in terms of % volume, and secondary calculations of surface area and particle number.

The minimum intermodal diameters between A and B type granules ranged from 6.6 – 7.6 μm when considered in terms of volume or 7.6–10 μm in terms of specific surface area. Measurements taken from ESEM images of a similar range of hard wheat varieties indicate granule sizes ranged from 2-30 μm with predominant A-type sizes of ~ 24 μm and B-type sizes of 3–5 μm. In the present study, LDS measurements were under-estimated with maximum averages of 0.83 μm, 3 μm and 23 μm. Stoddard (1999a) also noted that LDS underestimated the diameter of B-type granule populations (Stoddard, 1999a). That study used the Malvern Mastersizer 2600C to analyse 1,000 accessions of wheat grown in Australia. It found the intermodal minimum cutoff of A- to B-type granule populations to be 6.0 μm in contrast to a commonly reported 10 μm (Morrison & Scott, 1986) or 15 μm (Bechtel et al., 1990). While these differences may represent environmental and varietal differences, as well as measurement techniques, this lends further credence to the
idea that the inconsistency being reported concerning starch size distribution is dependant on the method of analysis (Bechtel & Wilson, 2000). Therefore if results are to be considered beyond internal referencing in a comparison with research by others, this variability should be taken into account. Wilson et al. (2006) recently performed an exhaustive study demonstrating that LDS clearly underestimates the major diameter of oblate spheroid particles typical of wheat starch. However an adjustment was formulated and can be applied to the LDS data to produce size distributions (vol%) that approximate those of Image Analysis (IA). Nevertheless the adjustment was based on a limited data set and more work is needed to verify the approach (Wilson et al., 2006).

5.3.3 Correlations between flour yield, SKCS measurements and starch granule type

Sixteen wheat varieties grown at Biloela and Narrabri were selected to represent contrasting flour yields (Table 1). The origin and pedigree of wheat varieties used in SGSD analysis are shown in Table 2. Previous research has identified the cultivar, WW15 which contains the puroindoline allele (Pinb-Dlb) for good milling quality as an important parent of many Australian cultivars also evident in this set (Cane et al., 2004). The correlations between SKCS parameters, starch granule specific surface area (n=12) and milling quality are given in Table 3. In a larger sample set of 30 varieties having SKCS Hardness Index values greater than 55, the standard SKCS parameters, seed weight and diameter made a significant contribution to flour yield which has been noted in previous studies (data not shown). However other studies have demonstrated that small kernel sizes with closed creases can produce higher flour yields than those with large kernel size (Evers & Millar, 2002). On the other hand, the non significant correlation between SKCS HI and flour yield compared with a significant correlation between Rheology Index and flour yield confirms that information recovered from the rheological SKCS parameters is more relevant to milling quality. Interestingly, total starch granule surface area (predominantly a result of C-type granule content) positively correlated with increasing SKCS hardness index values.
Table 1. Sixteen wheat varieties grown at two locations selected to represent contrasting CRP characteristics and flour yield.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Origin</th>
<th>Pedigree</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amurskaja</td>
<td>Former Soviet Union</td>
<td></td>
</tr>
<tr>
<td>Arnhem</td>
<td>Australia</td>
<td>Pitic/2*Hartog</td>
</tr>
<tr>
<td>Diamondbird</td>
<td>Australia</td>
<td>Vicam//Ciano/7C/3/KAL/BB</td>
</tr>
<tr>
<td>EGA Hume</td>
<td>Australia</td>
<td>Batavia* 2/Pelsart</td>
</tr>
<tr>
<td>Ellison</td>
<td>Australia</td>
<td>Vicam / 3* Suneca// Sun231a</td>
</tr>
<tr>
<td>Gabo</td>
<td>Australia</td>
<td>Bobin/2/Bobin/Gaza</td>
</tr>
<tr>
<td>Galaxy H45</td>
<td>Australia</td>
<td>B1814//WW15/QT7605</td>
</tr>
<tr>
<td>Hartog</td>
<td>Australia</td>
<td>Vicam 71/2/Ciano S/Siete Cerros/3/Kalyansona/Bluebird</td>
</tr>
<tr>
<td>Janz</td>
<td>Australia</td>
<td>3Ag/4*Condor//Cook</td>
</tr>
<tr>
<td>Kite</td>
<td>Australia</td>
<td>Norin 10 Brevor (Seln.14) /2/4* Eureka 2/3/T-A/3<em>Falcon/4/T-A/4</em>Falcon/5/T-A/5*Falcon</td>
</tr>
<tr>
<td>Machete</td>
<td>Australia</td>
<td>Sonora 64/2/Tezanos Pintos Precos/ Yaqui 54/3/ * Gabo/ 4/ Madden</td>
</tr>
<tr>
<td>NW25A</td>
<td>Nepal (Chhuwa)</td>
<td></td>
</tr>
<tr>
<td>NW65A</td>
<td>Nepal (Kharikola)</td>
<td></td>
</tr>
<tr>
<td>Rhodesian</td>
<td>Zimbabwe</td>
<td></td>
</tr>
<tr>
<td>Sunco</td>
<td>Australia</td>
<td>Sun 9e - 27<em>4/3Ag14/2/WW15/3/3</em>Cook</td>
</tr>
<tr>
<td>Yitpi</td>
<td>Australia</td>
<td>(Chamlein* 8156)* (Mengavi* Siete Cerros)(Chamlein<em>8156)</em> Heron)<em>(Mengavi</em> Siete Cerros) * Frame/ Yitpi</td>
</tr>
</tbody>
</table>
Table 2. Origin and pedigree of wheat varieties used in SGSD analysis.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Flour yield (%)</th>
<th>Variety</th>
<th>Flour yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW65A</td>
<td>73.6</td>
<td>NW25A</td>
<td>72.6</td>
</tr>
<tr>
<td>Kite</td>
<td>74.6</td>
<td>Rhodesian</td>
<td>73.9</td>
</tr>
<tr>
<td>Machete</td>
<td>74.8</td>
<td>Diamondbird</td>
<td>75.4</td>
</tr>
<tr>
<td>Rhodesian</td>
<td>74.9</td>
<td>Yitpi</td>
<td>75.5</td>
</tr>
<tr>
<td>NW25A</td>
<td>75.0</td>
<td>Kite</td>
<td>75.5</td>
</tr>
<tr>
<td>H45</td>
<td>76.6</td>
<td>Hartog</td>
<td>75.6</td>
</tr>
<tr>
<td>Gabo</td>
<td>77.6</td>
<td>Arnhem</td>
<td>76.2</td>
</tr>
<tr>
<td>Janz</td>
<td>78.1</td>
<td>Amurskaja</td>
<td>76.7</td>
</tr>
<tr>
<td>Sunco</td>
<td>78.1</td>
<td>Sunco</td>
<td>77.6</td>
</tr>
<tr>
<td>Amurskaja</td>
<td>78.2</td>
<td>Janz</td>
<td>77.9</td>
</tr>
<tr>
<td>Arnhem</td>
<td>78.2</td>
<td>H45</td>
<td>78.2</td>
</tr>
<tr>
<td>Ega Hume</td>
<td>78.9</td>
<td>EGA Hume</td>
<td>78.7</td>
</tr>
<tr>
<td>Ellison</td>
<td>79.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This presumably accounts for the significant correlation between HI and C-type granule volume (Table 4). Table 4 shows, for the Narrabri site, the correlations between the SKCS parameters, milling quality and starch granule type volumes. All starch granule type volumes correlated significantly with flour yield, MQI, SKCS HI and the Rheology Index. This accounts for the observation of differences in starch granule types in samples of hard wheat of contrasting Rheology Index. Multiple regression analysis using the more significant correlations found the variables: seed weight, A/C granule volume ratio, and the Sauter Mean Diameter (μm) to predict 86% of straight run flour extraction % (ANOVA probability level: 0.0003).

ESEM observations in this study suggested that increasing SKCS Rheology Index ranking corresponded to an increasing predominance of smaller granules. Results of the present study (Table 4) indicated a positive correlation between the SKCS Rheology Index and C-type granule volumes (R²=0.34, P<0.05) and a negative correlation with the A/C volume ratio (R²=0.38, P<0.05). The correlations involving B/C-type volume ratios were not significant.
**Table 3.** Correlations indicated by $R^2$ values between milling data, SKCS measurements and granule surface area measured by laser diffraction for hard wheat with SKCS Hardness Index >55 (n=30).

<table>
<thead>
<tr>
<th>Measurement</th>
<th>surface area (n=12)</th>
<th>Flour ext. % (n=30)</th>
<th>SKCS HI (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MQI</td>
<td>(-) 0.31 *</td>
<td>(-) 0.24 **</td>
<td>(-) 0.23 **</td>
</tr>
<tr>
<td>SKCS Rheology Index</td>
<td>ns</td>
<td>0.34 ***</td>
<td>0.41 ***</td>
</tr>
<tr>
<td>SKCS HI</td>
<td>0.43 **</td>
<td>ns</td>
<td>N/A</td>
</tr>
<tr>
<td>Specific Surface Area</td>
<td>N/A</td>
<td>ns</td>
<td>0.43 **</td>
</tr>
<tr>
<td>SKCS Weight (mg)</td>
<td>ns</td>
<td>0.47 ***</td>
<td>0.20 **</td>
</tr>
<tr>
<td>SKCS Diameter (mm)</td>
<td>ns</td>
<td>0.43 **</td>
<td>0.38 ***</td>
</tr>
<tr>
<td>NIR Protein</td>
<td>ns</td>
<td>(-) 0.35 **</td>
<td>(-) 0.14 **</td>
</tr>
</tbody>
</table>

Note: (-) negative correlation. (***) $P$ value < 0.005; (**) $P$ value < 0.05; (*) $P$ value < 0.1.

**Table 4.** Correlations indicated by $R^2$ values of granule type volumes and granule volume ratios with milling data, SKCS measurements and granule surface area measured by laser diffraction for 12 hard wheat samples grown at one location. Correlations of B/C volume ratios were not significant.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>A</th>
<th>A/B</th>
<th>B</th>
<th>C</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIR Protein</td>
<td>(+) 0.36 **</td>
<td>ns</td>
<td>ns</td>
<td>(-) 0.31 **</td>
<td>(+) 0.38 **</td>
</tr>
<tr>
<td>MQI</td>
<td>(+) 0.34 **</td>
<td>(+) 0.31 **</td>
<td>(-) 0.32 **</td>
<td>(-) 0.41 **</td>
<td>(+) 0.46 **</td>
</tr>
<tr>
<td>Flour Extraction %</td>
<td>(-) 0.50 **</td>
<td>(-) 0.41 **</td>
<td>(+) 0.35 **</td>
<td>(+) 0.45 **</td>
<td>(-) 0.56 **</td>
</tr>
<tr>
<td>SKCS Rheology Index</td>
<td>(-) 0.27 *</td>
<td>(-) 0.29 *</td>
<td>ns</td>
<td>(+) 0.34 **</td>
<td>(-) 0.38 **</td>
</tr>
<tr>
<td>Specific Surface Area</td>
<td>(-) 0.61 ***</td>
<td>(-) 0.81 ***</td>
<td>(+) 0.88 ***</td>
<td>(+) 0.73 ***</td>
<td>(-) 0.71 ***</td>
</tr>
<tr>
<td>Surface Area Mean (µm)</td>
<td>*** 0.59</td>
<td>(+) 0.82 ***</td>
<td>(-) 0.88 ***</td>
<td>(-) 0.69 ***</td>
<td>(+) 0.69 ***</td>
</tr>
<tr>
<td>SKCS HI</td>
<td>(-) 0.28 *</td>
<td>(-) 0.39 **</td>
<td>(+) 0.37 **</td>
<td>(+) 0.52 ***</td>
<td>(-) 0.47 **</td>
</tr>
<tr>
<td>SKCS Weight (mg)</td>
<td>(-) 0.27 *</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>(-) 0.28 *</td>
</tr>
<tr>
<td>SKCS Diameter (mm)</td>
<td>(-) 0.37 **</td>
<td>(-) 0.29 *</td>
<td>ns</td>
<td>(+) 0.39 **</td>
<td>(-) 0.47 **</td>
</tr>
</tbody>
</table>

Note: (-) and (+) positive or negative correlation. (***) $P$ value < 0.005; (**) $P$ value < 0.05; (*) $P$ value < 0.1.
Significant correlations were observed between starch granule type in hard wheat varieties and flour yields (Table 4 & Figure 8). In particular, the results depicted in Figure 8 show a consistent sign and magnitude of the correlation between types A and C starch granules and flour yield across the two sites, suggesting that starch granule size is predominantly under genetic control. The trend for increased volume % of types B and C starch granules to be associated with increased flour yield confirms the current qualitative microscopic observations. A recent survey of flour yields and quality traits from wheat grown around Australia suggested a variation of 37% due to environment (Allen et al., 2006; Williams et al., 2006). Therefore, the A/C granule ratio would appear to account for most of the genetic component of the variation in the flour yield trait. The mechanism by which the relationship between large and small granules improves flour yield in hard wheat requires further research. Understanding of the nature and fracture properties of composite materials such as endosperm may be gained from studies of material science and civil engineering (Lilliu & van Mier, 2007). Endosperm may be considered as a three phase material like concrete: aggregate/ starch granules, an interfacial transition zone (ITZ) being the granule surface where puroindoline and lipid distribution affect bonding strength and the cement/ protein matrix (Lilliu & van Mier, 2003). The weaker of these phases as well as the structural encasement of kernel components comprising cell walls, bran layers and the kernel crease determine the fracture properties. Weakness may be due to ITZ continuity, structural stratification of granules or porosity, micro-cracking and moisture content of the matrix material.
Figure 8. Correlations between the percentage of sample volume contributed by starch granule size range (hard wheat) and straight run flour extraction percentage. Also indicated is a comparison of environmental effect between two sites (Biloela, Queensland, and Narrabri, NSW, Australia). (A) Type A granules (gray- Narrabri (R Squared= 0.50, p<0.05 ); Black- Biloela (R Squared= 0.34, p < 0.1) (B) Type C granules (grey- Narrabri (R Squared= 0.45, p<0.05 ); Black- Biloela (R Squared= 0.35, p < 0.1) (Data points are averages of three samples and flour extraction SD = 0.5%).
In concrete, the best gradation or particle size distribution is one that produces the maximum density (and thus minimizes the volume of cement paste) while preserving strength, and workability. This would involve a particle arrangement, where smaller particles are packed between the larger particles, which reduces the void space between particles. Well-graded aggregate has a gradation of particle sizes that evenly spans the size from the finest to the coarsest (Figure 10)(Zia et al., 1994; Muench, 2007). Such a SGSD appears to be a significant characteristic of varieties of higher milling quality. Figure 9 compares two typical SGSDs from varieties with a difference of 4.6% straight run flour yield. Interestingly for varieties with higher flour yield, the size distributions of both B-type and A-type indicate a shift to an increased granule diameter range by up to approximately 0.5 and 3 μm respectively. Poorly graded aggregate is characterized by small variations in size leaving relatively large voids in the concrete.

However, some minimum amount of void space is necessary and protects against extreme temperature fluctuations (Zia et al., 1994). Likewise, protein (considered as cement matrix) measurements using NIR indicated a strong negative correlation with flour yield and C-type (small) granule content (Tables 3 and 4). The general consensus is that smaller size aggregates should be used to produce higher strength concrete (Muench, 2007), apparently similar to harder endosperms. In an early study of wheat starch granules using image analysis, significant relationships were reported between hardness and the starch granule mean surface area in hard wheats and alternatively the coefficient of variation in granule area in soft wheats (Pitts et al., 1989). The ESEM study showed small granules well bound in the protein matrix whereas large smooth, lenticular granules having smaller surface to volume ratios present a weaker bonding interface. This is supported by SKCS measurements of shell strength (data not shown) producing a strong negative correlation with Type A granule content (R² = 0.63, P< 0.005). Accordingly these often appeared loose with clean surfaces. Softer wheat varieties are known to have fewer small granules to fill spaces causing porosity especially in an environment of low matrix availability. These conditions generally produce a high first break flour release.
Figure 9. Comparison of starch granule size distributions graphed with three different scales to clarify granule classes. Two hard wheat varieties with 4.6% difference in straight run flour yield indicated by heavy line: high yield and light line: low yield.
Figure 10. (A) SEM images of unconditioned, hard wheat grain (cv Banks) fractured using Vario rolls showing starch damage and planes of weakness along cell walls. (B) Three types of fracture in a composite structure either through (protein) matrix, bond failure at the interfacial transition zone or fracture through aggregate (granules). Micro cavities in the structure are also a source of fracturing. (C) Simplified diagrams of densities produced by differing granule size distributions.
5.4 Conclusions

ESEM observations on samples of hard wheat varieties confirmed the degrees of variation in endosperm structure ranked by SKCS crush response profile characteristics. The endosperm rheology associated with high flour yield was characterised by fracture along cell walls and the presence of a higher proportion of small spherical bodies, possibly C-type starch granules in prismatic cells. These observations have been confirmed by quantitative starch granule size distribution analyses which indicate that a higher milling quality results from a more evenly graded granule size distribution (Figure 10.C). In composite materials such a particle distribution produces a denser and stronger structure. In wheat endosperm with strongly bonded granules or associated low levels of puroindoline, fracture along cell walls and through starch granules may be enhanced. In addition, a significant part ($R^2 > 0.40$ (p < 0.05) at two sites) of the association appeared to be under genetic control despite known environmental effects on small granule number. This may provide a way of effecting genetic improvement in wheat flour yield through manipulating the genes regulating starch granule size distribution. Further confirmation is required on a wider sample set also accounting for varietal differences in granule bond strength. Studies into the regulation of starch granule classes and their influences on the processing qualities of other cereals may be challenging.
Chapter 6. Puroindoline Genotype, Starch Granule Size Distribution and Milling Quality

6.1 Introduction

Despite considerable gains in wheat flour milling yield through conventional breeding strategies and milling technologies, the theoretical maximum yield still has not been attained. Discovery of genes in wheat that control flour yield would provide a means for breeders to develop new wheat varieties that fulfill their potential in relation to this complex trait. Almost every aspect of the milling process is affected by grain hardness which is considered to be dominated by endosperm cohesion (Greenwell & Schofield, 1986; Pomeranz & Williams, 1990; Haddad et al., 1999; Piot et al., 2000; Dobraszczyk et al., 2002; Topin et al., 2008; Anderssen & Haraszi, 2009; Vincent et al., 2009). Among members of the Triticeae, most notably wheat, much of the variation in texture is controlled by a single locus comprised of the Puroindoline a, Puroindoline b and Grain Softness Protein-1 (Gsp-1) genes (Bhave & Morris, 2008a, b). The presence or absence of puroindoline proteins determines the three major texture classes of soft and hard common wheat and the very hard durum wheat (Bhave and Morris, 2008a, Bhave and Morris, 2008b). The soft phenotype results when the Puroindoline a and Puroindoline b genes are present and encode the wild-type puroindolines PINA and PINB, respectively, and various mutations in either or both gene(s) result in hard phenotypes (Bhave & Morris, 2008a, b; Nadolska-Orczyk et al., 2009).

A desirable property of hard wheats is the high degree of starch granule damage produced during milling. This is a result of the starch granule–protein matrix adhesion which is stronger. Also larger irregularly shaped particles mainly composed of whole endosperm cells are produced during initial fractionation (Pomeranz & Williams, 1990). Surveys of wheat genotypes across the world commonly show that soft wheats have the same pin alleles (pinA-D1a, pinB-D1a), while hard wheats have a mutation in either pina or pinb (Giroux & Morris, 1998; Lillemo & Morris, 2000; Morris et al., 2001). Studies over the past 15 years have identified numerous variants at the puroindoline loci within T. aestivum. The review by Morris and Bhave (2008) provide a suggested reconciliation of puroindoline allele designations for Triticum
aestivum, Aegilops tauschii, and synthetic hexaploids (Aegilotriticum) with the sequence data available at that time. The most prevalent pin mutations that have been identified include a null mutation in pina (Pina-D1b, Pinb-D1a) and a point mutation in pinb (Pina-D1a, Pinb-D1b) that results in a glycine-to-serine substitution at the 46th residue of the peptide (Giroux & Morris, 1997, 1998; Hogg et al., 2005). For example, a survey of SNP variations in the Pinb sequence of 493 European wheat varieties, of the three hardness alleles Pinb-D1b, Pinb-D1c, and Pinb-D1d detected, Pinb-D1b was the most predominant hardness allele in European hard wheats (Huang & Roder, 2005).

The protein products of these genes are clearly lipid-binding proteins. Both PINA and PINB form a tertiary structure very similar to that of non-specific lipid-transfer proteins (ns-LTPs) (Bhave & Morris, 2008b; Finnie et al., 2010a). Importantly the PIN proteins are associated with the surface of isolated starch as a protein fraction known as ‘friabilin’ (Greenwell et al., 1986). The genes involved (puroindoline-a, puroindoline-b, and gsp-1) are located near the Ha (Hardness) locus and are within 100 kb of each other on the short arm of chromosome 5D (Chantret et al., 2005). The gsp-1 gene whose function is not well understood is also located on A and B genomes in hexaploid wheat (Gollan et al., 2007). Nevertheless, results to date indicate that puroindoline content does not represent a linear explanation for variations in grain hardness. For example, one study found that the QTL at the Ha locus (5DS) explained only around 63% of the phenotypic variability in grain hardness (Igrejas et al., 2002b). Current research continues to provide further understanding regarding the spatial and temporal regulation of expression of Puroindoline genes (Haddad et al., 1999; Giroux et al., 2000; Morris, 2002; Capparelli et al., 2003; Hogg et al., 2004; Gedye et al., 2005; Day et al., 2006; Swan et al., 2006b). For comprehensive reviews of the biochemical properties and molecular genetics of puroindolines, refer to Bhave & Morris, (2008a, b).

Other researchers have investigated the relationship between endosperm starch granule size and hardness. Bechtel et al. (1993) correctly identified wheat samples as hard or soft based on the size distribution of starch granules. Igrejas et al. (2002a) reported that harder wheat had a higher content of small-sized starch granules but could not find a QTL for starch granule size on the 5D Chromosome; they concluded that “starch size distribution is influenced by genes which have yet to be analysed” (Igrejas et al., 2002a). It is well documented that a higher percentage of small starch
granules is typical of harder wheat (Evers & Lindley, 1977; Bechtel et al., 1993; Zayas et al., 1994; Stoddard, 1999b; Gaines et al., 2000), although no significant differences in the relative quantity of type A granules have been noted between soft and hard wheat classes (Glenn et al., 1992; Bechtel et al., 1993). In a starch granule study of 12 soft wheat cultivars, Raeker et al. (1998) found highly significant differences among the cultivars for volume % of granules within the type A granule range (Raeker et al., 1998). Granule type predominance is known to directly affect milling quality and dough/baking quality (Gaines et al., 2000; Chiotelli & Le Meste, 2002; Edwards et al., 2002; Goesaert et al., 2008; Wilson et al., 2008). Dependant on the type of starch granule size distribution analysis used, some studies support a trimodal distribution in wheat endosperm which Bechtel and Wilson (2003) class as large (type A), medium (type B), and small (type C) (Raeker et al., 1998; Bechtel & Wilson, 2003; Brites et al., 2008; Edwards et al., 2008; Dai, 2009). A-type granules have been reported as 10–35 mm in diameter and account for more than 70% of the total starch weight but less than 10% of the granules by number. B and C-type granules account for over 90% of the granules by number, but less than 30% of the total starch by weight in wheat endosperm (Lindeboom et al., 2004). C-granules have a diameter of less than 5.3 μm and represent 45.7% of the total number of endosperm starch granules and 3.4% of the total weight (Igrejas et al., 2002a). However the surface area of B-granules (including C granules) has been estimated at 0.7 m² per gram of starch and is about three times higher than that for A-granules producing a prominent contribution to endosperm cohesiveness and strength (Konopka et al., 2005).

Starch granule size distribution can also be affected by environmental influences. For example, B-granule development is initiated at 10–12 days after anthesis and continues to enlarge until maturity. Therefore researchers suggest that a high temperature event could cause a decrease in starch synthase activity and reduce the duration of grain filling, resulting in smaller size and number of B-granules in the endosperm (Bechtel et al., 1990; Park et al., 2009). Alternatively, a recent study demonstrated that accumulation rate of small starch granules are significantly increased by the deficit of soil water at 14–21 days after anthesis, when the B- and C-type starch granules are synthesized rapidly and development of A-type granules decreased significantly (Dai et al, 2009). Furthermore, a recent study supports the observation of a general increase in grain hardness in response to drought, and reports
for the first time that relative concentrations of PINs have been shown to be influenced by environment. Weightman et al. (2008) suggest that associated with the overall increase in protein content, the PINs may also increase in concentration under drought conditions, due to reduced starch accumulation during grain filling (Weightman et al., 2008). Various wheat varieties adapt to diverse growing regions and respond differently to the environment though remaining within the “hardness” class determined by puroindoline genotype. The aim of the present study is to examine the combined influence of starch granule characteristics and puroindoline genotype and expression on flour yield.

6.2 Materials and Methods

6.2.1 Samples

Seed samples representing 188 wheat varieties from the Australian Winter Cereals Collection Tamworth were available for this study. To obtain sufficient quantities of grain for milling and other studies, the seeds were propagated twice. The first propagation took place at Tamworth, NSW in 2004. The seed from this propagation was of insufficient quantity so a second propagation was carried out. The second propagation was carried out at two locations (Biloela, QLD and Narrabri, NSW) in 2005 to produce sufficient quantities of grain for laboratory milling and starch granule size distribution. Not all of the varieties grew successfully at all three locations resulting in a reduced number of samples from the original set. 137 grain samples from the Narrabri propagation and 75 from the Biloela propagation were of sufficient quantity (> 0.35 kg) for milling (note: not all the same varieties were successful at both sites). However, at this sample size there was only sufficient for a single milling of each sample.

Subsequent genotyping revealed that only 9-12 of the samples grown in each environment were of the Pina-D1a, Pinb-D1b or Pina-D1b, Pinb-D1a genotype and only this very limited subset was available for studies into the relationships between flour yield, starch granule size distribution and starch-bound puroindoline a content. Consequently, samples of a further 15 commercial Australian wheat varieties of the Pina-D1a, Pinb-D1b genotype grown at various locations around Australia were also used. 151 of the samples from the Australian Winter Cereals Collection Tamworth selection were also propagated in greenhouses at the Centre of Plant Conservation.
Genetics (Lismore NSW). Leaf tissue was dried or quick-frozen under liquid nitrogen for storage at -80 °C. DNA was extracted from frozen and dry leaf tissue using the DNeasy Plant Mini Kit according to supplied protocol (Qiagen).

6.2.2 Test milling

Grain samples from the second (Biloela and Narrabri) propagations ranged in size from 0.35 kg to 4.5 kg. Wheat Rheology Index was measured using the Single-Kernel Characterization System 4100 (Perten Instruments, Springfield, IL, USA) as described by Osborne et al. (2007) (Anderssen and Haraszi, 2009). Wheat was cleaned using a Dockage Tester (Carter-Day, Minneapolis, MN, USA) then conditioned at 16.5% moisture content for 16 h. The quantity of water to add to each sample was calculated from the moisture content measured on the cleaned wheat by means of an Infratec 1229 instrument (FOSS, Hoganas, Sweden) using temper tables (AACC Method 26-95). Cleaned, conditioned wheat was milled using a MLU 202 Laboratory Mill (Buhler Bros, Uzwil, Switzerland) at a feed rate of 100 g min⁻¹. Bran and pollard fractions were further processed using two passes through a MLU 203 Laboratory Impact Finisher (Buhler Bros, Uzwil, Switzerland) and the finisher flours were passed through a 150 μm screen before incorporation into the straight-run flour.

6.2.3 Starch granule size analysis

Australian Winter Cereals Collection Tamworth wheat varieties grown at Biloela and Narrabri were selected to represent contrasting flour yield. Triplicate subsamples of six grains were taken from each sample. Methods of starch extraction were adapted from Stoddard, (1999b); Giroux et al., (2000); and Hogg et al., (2004). The grains were cracked into 2 ml Eppendorf tubes and soaked in 0.7 ml of 0.1M sodium chloride overnight. A plastic Eppendorf pestle was used to gently grind the samples in the soaking medium until the gluten formed a dough ball and the bran was broken into large flakes. The dough ball was pushed to the bottom of the tube and the aqueous starch solution was transferred through a fine sieve (200 μm pore size) to the top of a 2 ml pre-weighed Eppendorf tube containing 1 ml of 80% cesium chloride. The solids were ground again in a further 0.5 ml of 0.1M sodium chloride, the slurry was decanted into the same tube as before, and the solids were ground and slurry decanted for a third time. The starch-water suspension and cesium chloride were then
centrifuged at 13,000 G for 3 min. The cesium chloride was decanted and the starch was vortexed with 1 ml water until the clumps dispersed (~40 s) and then re-centrifuged. The starch pellet was then washed by 3 min centrifugation at 13,000 G, through a sequence of 2% sodium dodecyl sulfate (SDS) then twice in water. Granule size distribution was quantified in a Mastersizer 2000 laser-diffraction analyser (Malvern Instruments, Malvern, UK) using the flow-through, 100 ml reservoir. Starch and ethanol (1.7 ml) was vortexed and sonicated for 30 sec at 6W before analysis and added to the reservoir until an obscuration value of between 12–17% was achieved. Settings were optimised for the refractive index of starch in ethanol and an average of three consecutive measurements performed.

6.2.4 Pin genotyping

Amplifications of the full-length *Pina-D1* and *Pinb-D1* genes from the genomic DNA by the polymerase chain reaction (PCR) were performed as described by Gautier et al. (1994), using the sense primer PINA2F (5’-AACACACTGA CAACATGAAGG-3’) and anti-sense primer PINAR (5’-TCACCAGTAAT AGCCAATAGTG-3’) for the *Pina-D1* gene and the sense primer PINBF (5’-ATGAAGACCTTATTCCTCCT-3’) and anti-sense primer PINBR (5’-TCACCAGTAATAGCCACTAGG-3’) for the *Pinb-D1* gene, the primer pairs being expected to yield products of 461 base pairs (bp) and 447 bp in length, respectively (Gautier et al., 1994). All PCRs were carried out in a palmcycler (Corbett), using total volumes of 50 µl, comprised of 45 µl of PCR Supermix (Invitrogen), 250 ng of genomic DNA and 25 pmol of each primer. The DNA samples were subjected to initial denaturation at 94 °C for 5 min, followed by 35 cycles of 45 s of denaturation at 94 °C, 45 s of annealing at 54 °C for both primer pairs, and 1 min of extension at 72 °C, then a final extension at 72 °C for 10 min. Five to 10 µl aliquots of the PCR products were separated on 1.7% agarose gels containing ethidium bromide and visualized under UV light (Pickering & Bhave, 2007). Sequencing analysis of a number of full-length *puroindoline* PCR products was used to test whether any novel mutations were present. Sequencing of PCR products employed a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, FosterCity, CA, USA) and analysed on an automated 3730 DNA analyser (Applied Biosystems) at the sequencing facility of the Centre of Plant Conservation Genetics, Lismore NSW. All sequences were
aligned and compared to the published wild-type sequences of Pina-D1 and Pinb-D1 (Gautier et al, 1994) using the sequence alignment editor, Sequencher™ version 4.5 - Build 1415 [Gene Codes Corp. Ann Arbor, MI – (http://www.genecodes.com)]. Sequencing of both strands of DNA for each PCR product was carried out to identify any areas of inconsistency, any sequencing results of poor quality were repeated, and any discrepancies in the sequence data resolved through analysis of the chromatograms.

6.2.5 Puroindoline Protein Assay

Puroindoline protein was isolated from the surface of starch granules by a modification of previously described methods (Bettge et al., 1995; Hogg et al., 2004). Eight of the larger grains of each variety were cracked into 2 ml Eppendorf tubes and soaked in 0.7 ml of 0.1M sodium chloride overnight. Starch was extracted using the same method as above (see 2.3 Starch granule size analysis). The starch pellet was allowed to dry completely before the tube was weighed to determine the amount of starch present. The starch pellets were then resuspended in 200 µl of 2-propanol and 200 µl of 0.5 M sodium chloride followed by incubation at room temperature for 30 min. The suspension was centrifuged for 3 min at 13,000 G, the supernatant was transferred to a 1.5 ml tube, and then 120 µl of acetone at -20°C was added to the solution. The tubes were then vortexed and incubated overnight (18 hr) at -20°C. Samples were then removed from -20°C, centrifuged for 3 min at 13,000 G, and the supernatant was removed. To the pellet 400 µl of -20°C acetone was added, followed by vortexing and overnight (18 hr) incubation at -20°C. The following day the samples were removed from the -20°C and centrifuged as described above. Next, the acetone was removed followed by an acetone wash to dry the pellet, care being taken not to dislodge the pellet. Protein pellets were suspended at a rate of 10 µl of 1% SDS for every 100 mg of extracted starch (necessary to normalize differences between varieties in kernel size and starch weights extracted). Granule bound protein comparisons were obtained on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the Protein 80 Lab Chip kits, which enables the separation of proteins in the 1.6-80 kDa range. Detection is based on laser-induced fluorescence of an intercalating dye, which interacts with the protein/SDS complex. The analytes are separated electrophoretically, detected by their fluorescence (670–700 nm) and these data are then translated into
individual electropherograms in which fluorescence units (FU) are presented against migration time in seconds (s). Each sample contained an internal standard comprising an upper marker of 95 kDa and a lower marker of 1.6 kDa to provide an internal calibration. Each chip included a ladder comprising reference proteins of 1.6, 3.5, 6.5, 15, 28, 46, 63, 95 kDa against which protein mobilities were compared for each analysis (see Supplementary Figure 5).

All reagents and samples were allowed to equilibrate to room temperature for 30 minutes before use. Sample preparation involved adding 4 µl of the analyte (diluted protein) to 2 µl of sample buffer (with 3.5% vol water for a non-reducing reaction) in 0.5 ml tubes. The sample buffer contains upper and lower marker standards identical to those in the ladder and is thus incorporated into each unknown sample for direct comparison against the ladder standard. Samples and ladder (6 µl in 0.5 ml tube) were then placed in a heating block at 100°C for 5 min then centrifuged for 15 s. A further 84 µl of deionised water was added to the samples and ladder then vortexed prior to loading onto the chip. The chip was initially loaded according to the supplied protocol with 12 µl Gel-dye mix in each of 4 wells (the first applied under pressure with a syringe, priming station assembly) and 12 µl of destaining solution in another well. The samples and ladder were then loaded into designated wells avoiding the insertion of bubbles or residues on well rims. The chip was then inserted into the Bioanalyzer and analysis started.

6.3 Results and Discussion

6.3.1 Puroindoline Genotypes of the Australian Winter Cereals Collection Tamworth Samples and Flour Yields

A subset of 151 Australian Winter Cereals Collection Tamworth wheat varieties was sequenced to identify puroindoline genotypes. The most prevalent was the *Pina-D1a, Pinb-D1a* (wild-type) genotype followed by the *Pina-D1a, Pinb-D1b* genotype found in good milling quality varieties (*Table 1*). The other most common genotype was the *Pina-D1b, Pinb-D1a* genotype which does not express puroindoline A protein, thus producing a very hard phenotype.
Table 1. Numbers of different varieties identified in the three common puroindoline genotypes grown at each site.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Site 1 (Qld)</th>
<th>Site 2 (NSW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pina-D1a, Pinb-D1a</em></td>
<td>51</td>
<td>105</td>
</tr>
<tr>
<td><em>Pina-D1a, Pinb-D1b</em></td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td><em>Pina-D1b, Pinb-D1a</em></td>
<td>12</td>
<td>15</td>
</tr>
</tbody>
</table>

Other less common mutations identified (data not shown) were the following: a rare stop codon (*Pinb-D1e*- amino position 39) was identified in two *sphaerococcum* subspecies including var: Rogue in Blu du Oasis and further point mutations in puroindoline B causing 12 amino acid changes were identified in wheat subspecies - *spelta*, a rust resistant Triticosecale (var. Yukuri, Russia) and a variety from Nepal (var. NW25A). Furthermore, a stop codon was identified at position 87 indicating a truncated puroindoline B protein in a spelt variety: Surhak Jubilenjnyj (Russia). Mutations in puroindoline A gene sequences included a novel deletion in subspecies: *vavilovii* (var. Gracum from Algeria). Single base changes were also evident in puroindoline A sequences in varieties: Conte Marzotto, Iraq 1, Daging Mang, Beladi 42, and Relin. The results of puroindoline genotype analysis of all those Australian Winter Cereals Collection Tamworth samples from two sites with associated milling data are summarized in Figure 1 and Figure 2 excluding the uncommon mutations which produced average or lower flour yield and low hardness measurements.
Figure 1. Comparison of three common puroindoline genotypes: Soft (Pina-D1a, Pinb-D1a), hard (Pina-D1a, Pinb-D1b), very hard (Pina-D1b, Pinb-D1a) in flour yield (extraction) percentages from 145 wheat varieties from the Australian Winter Cereal Collection (Tamworth) grown in NSW (Narrabri).

Figure 2. Comparison of three common puroindoline genotypes: Soft (Pina-D1a, Pinb-D1a), hard (Pina-D1a, Pinb-D1b), very hard (Pina-D1b, Pinb-D1a) in flour yield (extraction) percentages from 145 wheat varieties from the Australian Winter Cereal Collection (Tamworth) grown in Qld (Biloela).
In agreement with previous reports, the \textit{Pina-D1a, Pinb-D1b} genotype resulted in a higher average flour yield than either the \textit{Pina-D1b, Pinb-D1a} or the \textit{Pina-D1a, Pinb-D1a} (Cane \textit{et al.}, 2004; Eagles \textit{et al.}, 2006). However, the ranges of flour yields showed considerable overlap and so the gains in flour yield that might be achieved solely by selecting for the puroindoline genotype would not be expected to be substantial.

### 6.3.2 Starch granule size distribution and flour yield

The relationship between starch granule size distribution and flour yield has been studied in both soft (Gaines \textit{et al.}, 2000) and hard (Edwards \textit{et al.}, 2008) wheat varieties. Hardness was evaluated using laboratory mill and SKCS methods respectively. A microscopy study of twelve varieties indicated that higher proportions of small starch granules were associated with increased rheology index values. Subsequent starch granule size distribution analyses suggested decreased large to small granule volume ratios correlated with higher straight run flour yields (Edwards \textit{et al.}, 2008). However, the previous study did not consider the effect of puroindoline interactions. Therefore, this study has been extended to determine the relationships between starch granule size distribution and flour yield within the common hard puroindoline genotypes. Results of starch granule size distribution analysis of ten varieties of the \textit{Pina-D1b, Pinb-D1a} genotype grown in two sites (Table 2) indicated consistent and significant correlations between starch granule size distribution and flour yield (Figure 3 and Table 3); (see also Appendices: Starch Granule Size Distribution Data).

A study of starch granule size distribution and flour yield was undertaken on commercial hard wheat varieties grown around Australia of the \textit{Pina-D1a, Pinb-D1b} genotype (Table 4). Correlations between starch granule types and ratios thereof: A, C, A/B, A/C and flour yield were similar to the \textit{Pina-D1b, Pinb-D1a} varieties though of weaker significance (Table 5). Interestingly the \textit{Pina-D1b, Pinb-D1a} varieties exhibited a higher proportion of large to small granules compared to the \textit{Pina-D1a, Pinb-D1b} genotypes over a similar flour yield range.
Table 2. Flour yield and starch granule size distribution measurements (in terms of percentage of sample volume of granule type or ratio of percentages) of wheat varieties with *Pina*-D1b, *Pinb*-D1a null genotype grown at two sites.

<table>
<thead>
<tr>
<th>Variety (QLD)</th>
<th>FY%</th>
<th>A</th>
<th>B</th>
<th>A/B</th>
<th>C</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arnhem</td>
<td>76.2</td>
<td>65.0</td>
<td>16.0</td>
<td>4.1</td>
<td>4.9</td>
<td>13.3</td>
</tr>
<tr>
<td>Bowerbird</td>
<td>78.1</td>
<td>65.9</td>
<td>15.3</td>
<td>4.3</td>
<td>4.6</td>
<td>14.5</td>
</tr>
<tr>
<td>Dollarbird</td>
<td>75.4</td>
<td>65.4</td>
<td>15.8</td>
<td>4.1</td>
<td>4.6</td>
<td>14.4</td>
</tr>
<tr>
<td>Drysdale</td>
<td>75.1</td>
<td>62.7</td>
<td>17.4</td>
<td>3.6</td>
<td>5.1</td>
<td>12.4</td>
</tr>
<tr>
<td>Gabo</td>
<td>75.8</td>
<td>59.8</td>
<td>19.8</td>
<td>3.0</td>
<td>5.8</td>
<td>10.4</td>
</tr>
<tr>
<td>Hartog</td>
<td>75.6</td>
<td>65.2</td>
<td>15.8</td>
<td>4.1</td>
<td>4.9</td>
<td>13.4</td>
</tr>
<tr>
<td>Iraq 46</td>
<td>73.1</td>
<td>61.3</td>
<td>18.5</td>
<td>3.3</td>
<td>5.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Pakistan C273</td>
<td>75</td>
<td>61.2</td>
<td>19.3</td>
<td>3.2</td>
<td>5.7</td>
<td>10.8</td>
</tr>
<tr>
<td>Punjab 7</td>
<td>75.6</td>
<td>62.4</td>
<td>18.4</td>
<td>3.4</td>
<td>5.5</td>
<td>11.4</td>
</tr>
<tr>
<td>Rees</td>
<td>75.1</td>
<td>63.1</td>
<td>17.2</td>
<td>3.7</td>
<td>4.9</td>
<td>12.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variety (NSW)</th>
<th>FY%</th>
<th>A</th>
<th>B</th>
<th>A/B</th>
<th>C</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arnhem</td>
<td>78.2</td>
<td>69.1</td>
<td>13.8</td>
<td>5.0</td>
<td>4.3</td>
<td>16.1</td>
</tr>
<tr>
<td>Bowerbird</td>
<td>78</td>
<td>69.4</td>
<td>13.4</td>
<td>5.2</td>
<td>4.0</td>
<td>17.3</td>
</tr>
<tr>
<td>Dollarbird</td>
<td>77.7</td>
<td>67.9</td>
<td>14.2</td>
<td>4.8</td>
<td>4.6</td>
<td>14.9</td>
</tr>
<tr>
<td>Drysdale</td>
<td>77.8</td>
<td>67.0</td>
<td>15.5</td>
<td>4.3</td>
<td>4.7</td>
<td>14.2</td>
</tr>
<tr>
<td>Gabo</td>
<td>77.6</td>
<td>62.8</td>
<td>18.0</td>
<td>3.5</td>
<td>5.2</td>
<td>12.1</td>
</tr>
<tr>
<td>Hartog</td>
<td>79</td>
<td>69.6</td>
<td>13.9</td>
<td>5.0</td>
<td>4.3</td>
<td>16.3</td>
</tr>
<tr>
<td>Iraq 46</td>
<td>76.6</td>
<td>62.2</td>
<td>18.2</td>
<td>3.4</td>
<td>5.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Machette</td>
<td>74.8</td>
<td>64.4</td>
<td>16.8</td>
<td>3.8</td>
<td>4.9</td>
<td>13.1</td>
</tr>
<tr>
<td>Pakistan C273</td>
<td>76.4</td>
<td>61.0</td>
<td>19.3</td>
<td>3.2</td>
<td>6.0</td>
<td>10.1</td>
</tr>
<tr>
<td>Punjab 7</td>
<td>75.8</td>
<td>64.2</td>
<td>17.0</td>
<td>3.8</td>
<td>4.8</td>
<td>13.3</td>
</tr>
<tr>
<td>Rees</td>
<td>78</td>
<td>66.0</td>
<td>15.1</td>
<td>4.4</td>
<td>4.5</td>
<td>14.7</td>
</tr>
</tbody>
</table>

Table 3. R² correlation values of starch granule type percentage volume with flour extraction % wheat varieties with *Pina*-D1b, *Pinb*-D1a genotype from two sites (*p< 0.1, **p<0.05) (n=10).

<table>
<thead>
<tr>
<th>Granule type</th>
<th>Biloela</th>
<th>Narrabri</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.35*</td>
<td>0.46**</td>
</tr>
<tr>
<td>C</td>
<td>(-) 0.34*</td>
<td>(-) 0.32*</td>
</tr>
<tr>
<td>A/C</td>
<td>0.36*</td>
<td>0.40**</td>
</tr>
<tr>
<td>B</td>
<td>(-) 0.29*</td>
<td>(-) 0.43**</td>
</tr>
<tr>
<td>A/B</td>
<td>0.35*</td>
<td>0.47**</td>
</tr>
</tbody>
</table>
Figure 3. Correlation between starch granule size distribution and flour yield percentage from wheat varieties with *Pina-D1b, Pinb-D1a* genotype grown at two sites [Biloela, Qld (black) and Narrabri, NSW (grey)].
Table 4. Flour yield and starch granule size distribution measurements of commercial hard wheat varieties with puroindoline genotype: *Pina-D1a, Pinb-D1b* grown at various Australian sites (except Queensland).

<table>
<thead>
<tr>
<th>LOCATION</th>
<th>VARIETY</th>
<th>FY%</th>
<th>A</th>
<th>B</th>
<th>A/B</th>
<th>C</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA1</td>
<td>EGA Bonnie Rock</td>
<td>76.2</td>
<td>53.8</td>
<td>26.8</td>
<td>2.0</td>
<td>5.4</td>
<td>10.0</td>
</tr>
<tr>
<td>WA</td>
<td>Cascades</td>
<td>78.3</td>
<td>53.2</td>
<td>28.0</td>
<td>1.9</td>
<td>5.8</td>
<td>9.1</td>
</tr>
<tr>
<td>WA2</td>
<td>EGA Eagle Rock</td>
<td>76.0</td>
<td>54.7</td>
<td>25.3</td>
<td>2.2</td>
<td>5.8</td>
<td>9.4</td>
</tr>
<tr>
<td>NNSW</td>
<td>Ellison</td>
<td>78.9</td>
<td>66.6</td>
<td>15.7</td>
<td>4.3</td>
<td>5.1</td>
<td>13.0</td>
</tr>
<tr>
<td>SA</td>
<td>Frame</td>
<td>77.3</td>
<td>54.5</td>
<td>24.9</td>
<td>2.2</td>
<td>6.3</td>
<td>8.7</td>
</tr>
<tr>
<td>SA</td>
<td>Janz</td>
<td>76.7</td>
<td>56.8</td>
<td>23.6</td>
<td>2.4</td>
<td>6.2</td>
<td>9.2</td>
</tr>
<tr>
<td>WA2</td>
<td>Sapphire</td>
<td>74.3</td>
<td>52.8</td>
<td>26.8</td>
<td>2.0</td>
<td>6.7</td>
<td>7.9</td>
</tr>
<tr>
<td>NNSW</td>
<td>Ventura</td>
<td>78.4</td>
<td>67.9</td>
<td>13.9</td>
<td>4.9</td>
<td>4.9</td>
<td>13.9</td>
</tr>
<tr>
<td>SA</td>
<td>Yitpi</td>
<td>79.2</td>
<td>62.3</td>
<td>17.4</td>
<td>3.6</td>
<td>5.2</td>
<td>12.0</td>
</tr>
<tr>
<td>WA</td>
<td>EGA Bonnie Rock</td>
<td>77.9</td>
<td>55.8</td>
<td>24.4</td>
<td>2.3</td>
<td>6.6</td>
<td>8.5</td>
</tr>
<tr>
<td>VIC</td>
<td>Frame</td>
<td>77.4</td>
<td>54.5</td>
<td>23.9</td>
<td>2.3</td>
<td>6.9</td>
<td>7.9</td>
</tr>
<tr>
<td>VIC</td>
<td>Frame</td>
<td>77.2</td>
<td>54.7</td>
<td>23.9</td>
<td>2.3</td>
<td>7.1</td>
<td>7.7</td>
</tr>
<tr>
<td>VIC</td>
<td>Janz</td>
<td>76.7</td>
<td>54.1</td>
<td>24.9</td>
<td>2.2</td>
<td>7.4</td>
<td>7.3</td>
</tr>
<tr>
<td>VIC</td>
<td>Janz</td>
<td>77.1</td>
<td>55.3</td>
<td>24.1</td>
<td>2.3</td>
<td>6.9</td>
<td>8.0</td>
</tr>
<tr>
<td>WA1</td>
<td>Sapphire</td>
<td>77.9</td>
<td>59.7</td>
<td>19.3</td>
<td>3.1</td>
<td>5.8</td>
<td>10.3</td>
</tr>
<tr>
<td>WA</td>
<td>Wyalkatchem</td>
<td>76.8</td>
<td>58.6</td>
<td>21.4</td>
<td>2.7</td>
<td>6.9</td>
<td>8.5</td>
</tr>
<tr>
<td>VIC</td>
<td>Yitpi</td>
<td>77.6</td>
<td>60.5</td>
<td>19.6</td>
<td>3.1</td>
<td>6.6</td>
<td>9.2</td>
</tr>
<tr>
<td>VIC</td>
<td>Yitpi</td>
<td>77.4</td>
<td>60.2</td>
<td>19.1</td>
<td>3.1</td>
<td>6.5</td>
<td>9.3</td>
</tr>
<tr>
<td>WA2</td>
<td>Yitpi</td>
<td>75.4</td>
<td>58.4</td>
<td>20.5</td>
<td>2.9</td>
<td>6.5</td>
<td>9.0</td>
</tr>
<tr>
<td>WA1</td>
<td>Yitpi</td>
<td>75.4</td>
<td>57.2</td>
<td>22.5</td>
<td>2.5</td>
<td>6.8</td>
<td>8.5</td>
</tr>
</tbody>
</table>

Table 5. R² correlation values of starch granule type percentage volume with flour extraction % from hard wheat varieties with puroindoline genotype: *Pina-D1a, Pinb-D1b* grown at various Australian sites and New South Wales (Narrabri, NSW)(*p<0.05, **p<0.01).

<table>
<thead>
<tr>
<th>Granule type</th>
<th>NSW</th>
<th>Commercial</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.32*</td>
<td>0.31*</td>
</tr>
<tr>
<td>C</td>
<td>(-) 0.35*</td>
<td>(-) 0.24*</td>
</tr>
<tr>
<td>A/C</td>
<td>0.36*</td>
<td>0.34**</td>
</tr>
<tr>
<td>B</td>
<td>(-) 0.34*</td>
<td>(-) 0.27*</td>
</tr>
<tr>
<td>A/B</td>
<td>0.40*</td>
<td>0.31*</td>
</tr>
</tbody>
</table>

The study also included sets of the puroindoline genotype: *Pina-D1a, Pinb-D1b* varieties from the Australian Winter Cereals Collection Tamworth grown at sites in Queensland and NSW (Table 6). However, the results of starch granule size distribution analysis were inconsistent between sites probably because the Biloela samples were of pinched grain and far fewer were of sufficient quantity for milling due
to poor conditions. Nevertheless, the general correlations between starch granule size distribution and flour yield from the NSW site (Narrabri) were similar to those from the study of commercial varieties being mainly from Western Australia and Victoria (Figure 4). The overall finding from both genotypes is that the sample volume ratio of type A granules accounts for between 31% and 46% of the variation in flour yield (Tables 3 and 5). By comparison, in a study of 12 soft wheat cultivars, Gaines et al. (2000) found that milling yield was greatest from the wheats having larger, mean, starch granule diameters (Gaines et al., 2000).

Table 6. Flour yield and starch granule size distribution measurements of wheat varieties with puroindoline genotype: *Pina*-D1a, *Pinb*-D1b grown at two sites (QLD and NSW).

<table>
<thead>
<tr>
<th>Biloela- varieties</th>
<th>FY%</th>
<th>A</th>
<th>B</th>
<th>A/B</th>
<th>C</th>
<th>A/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMC 83</td>
<td>75.8</td>
<td>64.3</td>
<td>17.6</td>
<td>3.7</td>
<td>4.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Banks</td>
<td>75.1</td>
<td>73.4</td>
<td>11.5</td>
<td>6.4</td>
<td>3.3</td>
<td>22.5</td>
</tr>
<tr>
<td>Batavia</td>
<td>76.8</td>
<td>61.7</td>
<td>18.7</td>
<td>3.3</td>
<td>5.5</td>
<td>11.3</td>
</tr>
<tr>
<td>Canberra</td>
<td>76.5</td>
<td>68.7</td>
<td>13.5</td>
<td>5.1</td>
<td>4.0</td>
<td>17.1</td>
</tr>
<tr>
<td>Ellison</td>
<td>77.9</td>
<td>66.4</td>
<td>15.1</td>
<td>4.4</td>
<td>4.6</td>
<td>14.5</td>
</tr>
<tr>
<td>Galaxy H45</td>
<td>76.7</td>
<td>65.7</td>
<td>15.7</td>
<td>4.2</td>
<td>4.5</td>
<td>14.6</td>
</tr>
<tr>
<td>H45</td>
<td>78.2</td>
<td>63.3</td>
<td>17.3</td>
<td>3.7</td>
<td>5.2</td>
<td>12.2</td>
</tr>
<tr>
<td>Kite</td>
<td>75.5</td>
<td>59.2</td>
<td>19.2</td>
<td>3.1</td>
<td>5.6</td>
<td>10.5</td>
</tr>
<tr>
<td>Martinvasari 13T</td>
<td>76.8</td>
<td>60.8</td>
<td>18.2</td>
<td>3.3</td>
<td>5.1</td>
<td>11.9</td>
</tr>
<tr>
<td>Strezelecki</td>
<td>75.6</td>
<td>59.1</td>
<td>20.8</td>
<td>2.8</td>
<td>6.1</td>
<td>9.6</td>
</tr>
<tr>
<td>Tunis 24</td>
<td>74.8</td>
<td>68.3</td>
<td>14.9</td>
<td>4.6</td>
<td>4.3</td>
<td>15.8</td>
</tr>
<tr>
<td>Narrabri- varieties</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMC 83</td>
<td>75.6</td>
<td>67.8</td>
<td>15.4</td>
<td>4.4</td>
<td>4.1</td>
<td>16.7</td>
</tr>
<tr>
<td>Banks</td>
<td>78.4</td>
<td>71.9</td>
<td>12.4</td>
<td>5.8</td>
<td>3.5</td>
<td>20.6</td>
</tr>
<tr>
<td>Canberra</td>
<td>78.0</td>
<td>68.6</td>
<td>14.1</td>
<td>4.9</td>
<td>4.2</td>
<td>16.5</td>
</tr>
<tr>
<td>EGA Wedgetail</td>
<td>78.2</td>
<td>63.9</td>
<td>17.3</td>
<td>3.7</td>
<td>4.8</td>
<td>13.2</td>
</tr>
<tr>
<td>H45</td>
<td>76.6</td>
<td>62.7</td>
<td>18.3</td>
<td>3.4</td>
<td>5.3</td>
<td>11.8</td>
</tr>
<tr>
<td>Kite</td>
<td>74.6</td>
<td>62.0</td>
<td>18.7</td>
<td>3.3</td>
<td>5.5</td>
<td>11.2</td>
</tr>
<tr>
<td>Marombi</td>
<td>76.7</td>
<td>59.7</td>
<td>21.1</td>
<td>2.8</td>
<td>5.8</td>
<td>10.4</td>
</tr>
<tr>
<td>Martinvasari 13T</td>
<td>77.9</td>
<td>67.7</td>
<td>14.3</td>
<td>4.7</td>
<td>4.0</td>
<td>17.1</td>
</tr>
<tr>
<td>NW93A</td>
<td>75.6</td>
<td>66.5</td>
<td>16.4</td>
<td>4.0</td>
<td>4.4</td>
<td>15.0</td>
</tr>
<tr>
<td>Strzelecki</td>
<td>75.4</td>
<td>53.3</td>
<td>26.4</td>
<td>2.0</td>
<td>6.4</td>
<td>8.3</td>
</tr>
<tr>
<td>Tunis 24</td>
<td>77.7</td>
<td>71.8</td>
<td>13.2</td>
<td>5.4</td>
<td>3.6</td>
<td>20.1</td>
</tr>
<tr>
<td>Wylah</td>
<td>76.3</td>
<td>58.5</td>
<td>22.8</td>
<td>2.6</td>
<td>5.7</td>
<td>10.3</td>
</tr>
</tbody>
</table>
Figure 4. Correlation between the percentage of sample volume of Type A starch granules and flour yield percentage from hard wheat varieties with puroindoline genotype: \textit{Pina-D1a}, \textit{Pinb-D1b} from various Australian sites (grey) and Narrabri, NSW (black).

6.3.3 Significance of Granule Bound Puroindolines

The Agilent Bioanalyzer Protein 80 Chip was able to detect puroindoline proteins extracted from starch granules. Extraction yields were not as high as expected, possibly due to the use of caesium chloride during starch isolation as observed by Greenblatt 	extit{et al.}, (1992). Results were similar to those reported by others using alternative techniques (Capparelli 	extit{et al.}, 2003; Amoroso 	extit{et al.}, 2004). The size-based capillary electrophoresis device assigns a \(~17.5 – 18.2\) KDa size to Puroindoline A with migration times between \(24.4 – 24.8\) s and for puroindoline B: sizes \(15.6 – 16.3\) KDa with migration times between \(23.6 – 23.9\) s. Durum wheat \((4.6\ ng\ ml^{-1})\), hard \((\textit{Pina-D1a}, \textit{Pinb-D1b})\ \(83.9\ ng\ ml^{-1}\)) and soft \((934.2\ ng\ ml^{-1})\) wheat varieties have starch bound puroindoline within the expected ranges (see Supplementary Table 8).

Previous studies of the milling qualities of hard wheat often indicate that wheat varieties with the \textit{Pina-D1a}, \textit{Pinb-D1b} genotype produce the highest milling quality but express a wide range of puroindoline protein quantities (Capparelli 	extit{et al.}, 2003; Cane 	extit{et al.}, 2004; Eagles 	extit{et al.}, 2006). In the present study of this genotype results
suggest that varieties with less granule-bound puroindoline will be of a higher milling quality (flour yield and Wheat Rheology Index). Consistent trends were evident from both environments with starch-bound puroindoline A content accounting for 35-53% of the variation in flour yield (Table 7). Multiple regression showed that the combined effect of starch granule type and bound puroindoline content described by the equation

\[ \text{Flour yield} = 75.92 - 0.26 \text{ (puroindoline a)} + 0.19 \text{ (A/C granule ratio)} \]

accounted for 68% of the variation in flour yield in the \textit{Pina-D1a, Pinb-D1b} genotype.

Alternatively, higher flour yields may result from kernels with higher endosperm weight and a fuller expression of starch granule classes. Consequently lower apparent starch bound puroindoline A may indeed be an artifact of dilution in grains with higher starch weight. In a study of protein content from different grain sizes, statistical analysis by Konopka \textit{et al.} (2007) indicated that grain protein composition is also affected by kernel size and the cultivar, as well as by interaction of these traits (Konopka \textit{et al.}, 2007). Moreover, the greater proportion of gliadin (thought to bind to the starch surface) in vitreous kernels was associated with a harder texture (Gianibelli \textit{et al.}, 1991). Recent research by Miller (2008) on variation in single kernel hardness within the wheat spike found that the top and bottom are not significantly different, with the centre portion having significantly softer kernels (Miller, 2008).

\textbf{Table 7. Correlations (R^2 values) with starch granule bound puroindoline content of wheat samples (puroindoline genotype: Pina-D1a, Pinb-D1b) grown at Biloela and Narrabri.}

<table>
<thead>
<tr>
<th></th>
<th>Narrabri (n = 11)</th>
<th>Biloela (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flour Yield</td>
<td>(-) 0.53 **</td>
<td>(-) 0.35*</td>
</tr>
<tr>
<td>Type A</td>
<td>0.25 (ns)</td>
<td>0.33*</td>
</tr>
<tr>
<td>Type C</td>
<td>(-) 0.49**</td>
<td>(-) 0.31*</td>
</tr>
</tbody>
</table>

\((\text{ns) not significant, * p} \leq 0.1, \text{ ** p}\leq 0.05)\)

The results of previous research indicate that PINB is more specific and more limiting to starch binding than PINA which has been shown to have a lower amount of insertion into lipid monolayers though these mechanisms still require further clarification (Swan \textit{et al.}, 2006b; Clifton \textit{et al.}, 2007a; Clifton \textit{et al.}, 2007b; Wanjugi \textit{et al.}, 2007a; Evrard \textit{et al.}, 2008). Feiz \textit{et al.} (2009) concluded that several lines of
evidence indicate that Ha directly effects changes in starch polar lipid content via an unknown mechanism. Furthermore the most likely explanation proposed was that active PINs stabilize bound polar lipids on the surface of starch granule membranes preventing breakdown during seed desiccation and maturation. Consequently, lipid degradation may occur during kernel ripening and maturation in hard wheats supporting the observation of the hard phenotype at this stage (Feiz et al., 2009). Clearly, therefore, there is a variable interaction between the puroindolines and starch granule characteristics: size distribution and lipid content within hard wheat varieties that requires further investigation.

6.4 Conclusions

- On average, the *Pina-D1a, Pinb-D1b* genotype is associated with a higher flour yield than either the *Pina-D1b, Pinb-D1a* or the *Pina-D1a, Pinb-D1a*.

- Within each hard genotype (*Pina-D1a, Pinb-D1b* or *Pina-D1b, Pinb-D1a*), increased flour yield is provided by an increase in the proportion of large starch granules to small starch granules.

- For the *Pina-D1a, Pinb-D1b* genotype, increased flour yield is provided by a decrease in starch granule bound puroindoline A protein.
**Supplementary Figure 5.** Electrophoregram and SDS gel-like graphics output produced by Bioanalyzer 2100 software of supplied ladder for automated protein size calculation.

**Supplementary Table 8.** The size-based capillary electrophoresis device assigns a ~17.5 – 18.2 KDa size to Puroindoline A with migration times between 24.4 – 24.8 s and for puroindoline B: sizes 15.6 – 16.3 KDa with migration times between 23.6 – 23.9 s. Durum, hard (PinB mutant) and soft wheat varieties have the expected puroindoline contents of either none, intermediary or high respectively (* Total puroindoline a + puroindoline b).

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>MIGRATION TIME (S)</th>
<th>SIZE (KDa)</th>
<th>REL. CONC. (ng/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PinA Standard</td>
<td>24.41</td>
<td>17.5</td>
<td>288.4</td>
</tr>
<tr>
<td>PinB Standard</td>
<td>23.91</td>
<td>16.3</td>
<td>55.1</td>
</tr>
<tr>
<td>PinA Durum</td>
<td>24.41</td>
<td>17.4</td>
<td>4.6</td>
</tr>
<tr>
<td>PinA QAL2000</td>
<td>24.68</td>
<td>18.0</td>
<td>934.2*</td>
</tr>
<tr>
<td>PinA EGA Bonnie Rock</td>
<td>24.76</td>
<td>18.2</td>
<td>83.9</td>
</tr>
<tr>
<td>PinB EGA Bonnie Rock</td>
<td>23.63</td>
<td>15.6</td>
<td>36.5</td>
</tr>
</tbody>
</table>
Chapter 7. Summary Discussion

7.1.1 Properties relevant to flour yield

From our review of research to date relevant to flour yield and other wheat milling qualities, the following three groups of properties, many of which are interrelated appear to have significant influence: (1) factors relating to adhesion at the starch granule-matrix interface specifically types, affinities, and quantities of: 1 (a) puroindolines, 1 (b) lipids, and 1(c) starch granules; 2 (a) response to conditioning, 2 (b) separability of bran and endosperm, 2 (c) cell wall composition and architecture; 3 (a) vitreousness and porosity, 3 (b) endosperm response to senescence. The current research has involved microscopy studies of fractured endosperm, the measurement of grain rheology, the macro-structural response to conditioning, the relationship between SGSD and milling, and the relationship between starch surface bound puroindoline and flour yield.

7.1.2 Relevance of SKCS Measurements to Milling

Since milling is essentially a physical process involving fracture and separation of bran from endosperm, the use of physical measurements of wheat hardness is appropriate for varietal classification such as provided by the SKCS. The relation between wheat hardness and flour yield, depends on the initial and subsequent breakage characteristics of the grist (blended varieties)(Pomeranz & Williams, 1990; Ohm et al., 1998). The results of several studies exhibit strong correlations between SKCS measurements and wheat quality. For example, regression analysis was performed using the SKCS parameters and test weight against flour extraction rate for over 600 observations from multiple years from the hard red winter wheat production areas of the U.S. Their results suggested that the SKCS 4100 and test weight can be used to predict flour extraction rate in hard red winter wheat (Lyford et al., 2005). In another study evaluating 183 soft wheat cultivars and lines for milling quality characteristics (kernel hardness, kernel and flour protein, flour ash), and end-use properties, SKCS characteristics were significantly correlated with conventional wheat quality parameters such as kernel size, wheat protein content, and straight-grade flour yield. Of interest is the commonly reported inverse relationship between flour protein
contents and kernel weights or sizes (Park & Chang, 2007). This relationship is partially due to environmental conditions during grain filling and less starch content of under-developed grain.

Other results indicate that SKCS hardness is meaningful with respect to breakage during roller milling. Campbell et al. (2007) has shown that distributions of single kernel data, as measured by the SKCS, can be used directly to predict breakage during First Break roller milling (Campbell et al., 2007a). This was an unexpected finding, as the breakage mechanism in the SKCS, involving a single rotor with a relatively fine saw-tooth profile crushing kernels against a smooth stationary crescent with a large gap between, is very different from the breakage action during First Break roller milling (Martin & Steele, 1996). Our studies demonstrated that the fragmentation patterns of both hard and soft wheat which result from crushing in the SKCS 4100 and in the first break stage of roller milling confirmed that SKCS analysis of unconditioned wheat (the standard practice) is a suitable predictor of the milling performance of conditioned wheat. Our results also confirmed the use of SKCS crush response profiles as more informative in relation to milling performance than the automatically generated Hardness Index (HI) score. The crush response profiles also exhibited a high degree of sensitivity to the effects of conditioning on kernel structure. The conditioning of both hard and soft wheats produced a reduction in the shell stiffness and strength, and endosperm strength. Consequently this may provide a rapid means to finely calibrate the conditioning process for a proposed grist formulation and thereby optimize fractionation especially important at the first break stage. In addition, ESEM studies of mainly hard wheat varieties provided subjective support for a correlation between the degree of variation in endosperm structure and SKCS crush response profiles.

7.2 Gross Morphology relevant to Milling

Initial stages of this research involved image analysis utilising automated, low magnification and electron microscopy studies of varieties with a range of milling performance (see microscopy images in Appendix). Regarding kernel shape, the complex geometry of wheat grains and the presence of the deep crease have complicated the study of their rheological properties. Our experimentation also
confirmed that grains falling horizontally into mill rolls tend to break along the crease thereby producing large bran fragments (data not shown). The modern milling process has evolved many stages to minimize the difficulty that the crease presents.

Considering grain size, some of the best milling wheat types such as the hard classes grown in North America have characteristically small grains. Large grains have a propensity to develop larger endosperm cavities (adjacent to the crease) and therefore do not always contain more endosperm than small grains (Evers & Millar, 2002). More uniform wheat samples give more predictable milling performance. Accordingly coarse separation of grain sizes is able to provide further quality differentiation of grain lots. In the Chapter 1 topic, Alternative Predictive Measurements of Flour Yield, image and QTL analyses of grain dimensions have shown limited potential in the indication of underlying structure important to flour yield. In studying the influence of kernel size on wheat millability, Li and Posner (1987) found that larger wheat kernels produce higher break releases (Li & Posner, 1987). However in a similar study, 11 current commercial cultivars and breeding lines of wheat were grown at several sites in northern New South Wales. In samples of different cultivars grown at the one site, grain size was not correlated with milling yield. These contrasting results were taken to indicate that the contribution of differences in seed size to genotypic difference in milling yield were small relative to the effects of other factors. The effects on milling yield of other attributes of wheat grains such as the amount of germ tissue, thickness of bran, depth of crease, only appeared to make minor but cumulative contributions to differences in milling yield. No one factor in that study appeared to make a major contribution to genotypic difference in milling yield (Marshall et al., 1986).

Bechtel et al. (2009) comments that milling technology still relies more on technical know-how than on well established scientific facts. Wheat grains are very complex structures, heterogeneous in composition, morphology, and mechanical properties. To predict the milling behaviour of wheat grain, numerous factors must be taken into account (Bechtel et al., 2009). Small improvements in flour yield are possible by manipulation of any of these factors; although the alteration of certain features will be more economical and less complex than others. From the biological perspective and given milling techniques remain essentially the same, the first step is
to identify structural components that accommodate the current milling process. These features may either be altered through growth conditions and/or used as targets for conventional breeding or genetic techniques.

7.2.1 Blending Varieties for Specific Qualities

Given the variations in character among varieties within the major wheat classes, another vital operation of the miller is to blend wheats (gristing) of different varieties and from different sources to yield flours of the desired protein content and uniform baking performance. The blending of grain with diverse quality attributes can be used to achieve improved quality and market value compared to the original lots of grain. A single variety need not possess all desired quality traits to be useful for the final product. When considering the blending of either wheat or flour consignments, knowledge of the varieties involved is of great value. Research is continuing towards the aim of providing a more comprehensive guide to predicting the outcome of blending (for example, the knowledge of expression and effect of glutenin subunits on dough properties).

7.2.2 First Break Stage of Milling

The present research has given attention to the fractionation of grain in the first break stage of milling. The particle size distribution resulting from first-break roller milling directly affects the subsequent system arrangement and machine settings, and thus determines the effectiveness of the milling process (Fang & Campbell, 2002b). Apart from the initial break process involving intact wheat kernels, researchers conclude that the stresses and moisture contents are almost identical for all wheat cultivars in subsequent milling phases (Mabille et al., 2001; Kweon et al., 2009). In first-break roller milling of wheat, Campbell et al. (2001) broadly classifies the factors affecting breakage of wheat grains into those arising from the physicochemical properties of the wheat (size distribution, moisture content, hardness) and those related to the design and operation of the milling equipment (Campbell et al., 2001a). For example, millers tend to operate under D-D (because the U-shaped distribution is readily separated into larger branny particle and smaller endosperm particles), the greater sensitivity of D-D milling to kernel hardness and size is commercially significant, as variations in the feedstock will have greater influence on downstream
operations. The milling of soft wheat gives approximately the same percentage of break flour and reduction flour whereas with hard wheat, break flour forms only about a quarter of the reduction flours yield. Again, flour yield must be considered in the context of quality (protein and starch damage). Campbell et al. (2007) noted consistent linear trends in fragmentation response to varying hardness between hard and soft wheats (Campbell et al., 2007a). Observations detailed under morphological topics herein also highlight the continuous gradations of components both within the endosperm and between caryopses on the same plant.

7.2.3 Conditioning (Tempering)

The complex trait encompassing the response of grain to conditioning has significant influence on milling performance. Determinations on the extent of kernel moisture conditioning are based on a trade-off between reducing endosperm crumbliness and increasing seed coat deformability (Kweon et al., 2009). For example, a recent study demonstrated that flour yield was more reduced for all samples conditioned at 15% moisture than for samples conditioned to 12% moisture. Whereas flour quality of the 15% conditioned sample was better than the 12% conditioned samples due to less bran contamination. Millers generally accept that hard wheat endosperm diffuses water at a slower rate than soft wheat endosperm. Irrespective of the degree of vitreousness, fracture toughness decreases as the moisture content increases (Dobraszczyk, 1994). Fang and Campbell (2003) concluded that the amount of water added and the timescale over which it is allowed to penetrate into the kernel vary widely in practice, with no conditioning regime universally appropriate for all wheat types and milling systems (Fang & Campbell, 2003). Given that differences in moisture response have been shown to account for the differences in endosperm strength of the hard and soft grain (with moisture contents below 22 %), this trait is worthy of more efficient testing prior to conditioning and perhaps development if current milling processes are continued. The path and rate of water imbibition affected by structure and density is important to the germination process and differs according to class as demonstrated by our SKCS measurements of conditioned varieties.
7.2.4 Bran

In relation to conditioning and flour purity, the response of bran to the milling process is of critical importance. The capability of bran to be deformed far beyond that of the starchy endosperm without breaking could be largely attributed to the structure of the aleurone layer and nucellar epidermis. Furthermore interactions between polymer and the degree of arabinoxylan cross-linking in the aleurone cell-wall expressed by the ferulic acid dehydrodimer/xylose ratio appear to be positively correlated with bran extensibility. Consequently such analysis has been proposed as an indicator of wheat milling quality (Mabille et al., 2003). Previous research has also reported a higher deposition of \((1\rightarrow3,1\rightarrow4)-\beta-D\)-glucans in aleurone and sub-aleurone cell walls (Bechtel et al., 2009). Our examination of the SKCS crush response profiles (CRPs) from a range of varieties indicated differing shell responses partially attributable to bran characteristics. Varieties that possess thicker bran layers or those less prone to breakage after conditioning are possibly worthy of further investigation with the use of SKCS crush response profiles.

Wheat, on an average, contains about 85% of endosperm. Therefore the common extraction rate of 78% falls short of the potential yield. For the milling industry to produce high quality flours with very low bran contamination significant flour yield is sacrificed i.e. flour yields are reduced from 78% to 60% or even as low as 40%. This failure to extract all of the endosperm as flour, even with advanced milling methods, is caused by such firm adhesion of the peripheral zones of the endosperm to the aleurone and bran layers that complete separation is not practical under commercial milling conditions. Mabille et al. (2003) proposed that three major factors of grain morphology affect milling performance: the separability of the starchy endosperm and the bran coat, the endosperm/bran ratio, and endosperm vitreousness. This separability factor appears to be connected both to tissue adhesion and to tissue mechanical properties, which must be independently investigated (Mabille et al., 2003). Consequently the response of the mechanical properties of bran layers to the current conditioning process is of significant importance. Ideally a single step process to provide clean removal of bran through separation between the aleurone and sub-aleurone layers prior to crushing has not been achieved. Conditioning processes with enzyme or plant hormone additives have
been developed and patented over recent years (for example see International publications: WO 99/21656, WO 02/00910, WO 02/00731, WO/2007/106941). The conditioning solution used in the later invention (WO/2007/106941) included the plant hormone, abscisic acid and cell wall-degrading enzyme, cellulase. Apparently a slight increase in flour yield and reduction of conditioning times was achieved without adversely affecting baking quality. Further opportunities exist to develop processes targeting the separation between aleurone and sub-aleurone layers to accommodate this particular consumer demand.

7.2.5 Cell walls

Proceeding from outer to inner structures in gross morphology, the role of cell walls in milling performance is considered by some to be minor compared to that of the interface between starch granule and protein matrix. Nevertheless the strength of this architecture and the zones of weakness induced, may be sufficient to significantly influence endosperm cohesion and milling performance. So far, however, the significance of cell walls in endosperm cohesion is not well documented. The amorphous regions of cell walls are thought to be moisture absorbent and capable of swelling. Arabinoxylans (AX) within cell walls are involved in water transport or diffusion during different physiological stages: grain development, desiccation, and germination. It is therefore possible that structural variation is involved in modulating the hydration properties of the cells walls to regulate the water content of the grain (Saulnier et al., 2007). Consequently cell wall expansion from moisture diffusion may assist micro-fracture and weakening of the cellular framework after desiccation. Nevertheless there is no evidence that there is any cleavage between adjacent cell walls, along the middle lamella, rather fracture in hard and vitreous endosperm occurs down the cell wall inner surface. Apparently the adhesiveness between cell walls and middle lamella is too strong to be broken during milling including various conditioning methods (Schulze & MacMasters, 1962). Our microscopy observations (also supported by others), exhibit the endosperm of hard wheat as having weak cohesion along the cell walls and strong adhesion internally, whereas soft wheat has strong cohesion along the cell walls and weak cohesion internally (Greer & Hinton, 1950). Furthermore, soft endosperm tends to have a greater and more variable porosity producing a more fragile structure (Dobraszczyk et al., 2002).
7.3 Gradients in Endosperm Composition

7.3.1 Gradients in Cell Wall & Arabinoxylans (AX)

Walls around the peripheral cells are thickest, being up to 7 μm thick in the crease region and up to 4 μm elsewhere whereas the central cell walls are 2.6 μm thick. Our microscopy studies indicate a weaker structure in the central cells, generally larger and rounder in shape compared to the prismatic, peripheral cell types (see microscopy images in Appendix). Also studies using experimental first break rolls found that these cells types contribute to the initial fine particle release (whole starch granules) having less protein rich, vitreous particles especially in softer varieties. Previous research has found a difference in the degree of AX substitution between peripheral and central parts of the endosperm at the cell differentiation stage; AX in central cells was less substituted (Philippe et al., 2006a). Considering possible targets for genetics and breeding methods, water Extractable-AX content is primarily influenced by genotype (Dornez et al., 2008). Hong et al. (1989) also suggest that pentosan quality, rather than quantity, may be important in determining different levels of hardness (see Chapter 2: AX and hardness) (Hong et al., 1989; Turnbull & Rahman, 2002). Patent applications have been submitted by others for modulating the polysaccharide composition of the plant cell walls to improve the ability of cells to fracture thus improving milling characteristics of crop plants (US Patent Application 20080320613-Modulating Myo-Inositol Catabolism in Plants).

7.3.2 Lipid Gradients

As endosperm cell shape and size differ between peripheral to central cell types with corresponding changes in architectural strength, similarly other components are also observed to differ. For example, a previous study has observed that the subaleurone region had a higher concentration of oil bodies, that is, free (non-membrane associated) lipids compared to the central region (Hargin et al., 1980). Potential links between endosperm texture, puroindolines, and lipid profiles were examined by Greffeuille et al. (2007) using the two cultivars, Crousty (soft) and Caphorn (hard-mutant PINB), which showed extreme behaviour in their farina reduction ability: from FL1 [first reduction flour (<150 μm particle size)] to FL3 [third reduction flour (<150...
the proportion of non-polar lipids increased and those of phospholipids decreased two-fold. On the other hand, flours from Caphorn showed an increase in the amount of glycolipid between FL1 and FL3 whereas non-polar and phospholipid contents did not change significantly. It was also shown that the unequal lipid distribution in farina reduction products could be related to the different molecular associations between starch granules and the protein matrix (Greffeuille et al., 2007). This observation may be in concordance with the report of Konopka et al., (2005) where hardness was positively correlated with the content of free glycolipids \( r = 0.82 \) (Konopka et al., 2005).

7.3.3 SGSD Gradients

In wheat, starch granule size is known to decline systematically among cells from the centre to the periphery. A coincident decline in the numerical complement of lenticular (type A) granules has been suggested as compatible with a sequence of peripheral divisions in which each daughter cell received half the number of amyloplasts or proplastids (Evers & Millar, 2002). Between these two extremes are gradients of granule age and development across the endosperm. This gradation has implications for studies focused on the time course of gene expression, the variable hardness of inner and outer zones within the endosperm, also the developmental differences and variable hardness between kernels on the same plant and between varieties as demonstrated in the present research.

7.3.3.1 Implications of Different SGSD between Grain Positions

Further to the consideration of the internal gradation of components within the endosperm, individual grains of wheat cultivars have shown significant variation in small starch granule content, grain mass and nutrient concentration depending on their position within the spike (Morrison & Scott, 1986; Park et al., 2004). In summary, hardness and milling quality are affected by protein and starch packaging characteristics which in turn affect vitreousness and porosity. All these factors can vary according to position on the plant and presumably are functions of nutrient availability, transport reticulation and seed development rate. Related results suggest that the lower nutrient concentrations found in distal grains could be related to the transport of nutrients from the rachis to distal positions. The ratio of phloem to xylem
concentration of nutrients and the mobility of the different nutrients within the phloem may contribute to the observed differences in nutrient concentration in individual grains (Calderini & Ortiz-Monasterio, 2003). An altered expression of transport mechanisms may also be worthy of further investigation.

7.3.4 Protein Gradients

It is well-established that protein gradients exist in the starchy endosperm with the outer sub-aleurone cells being richer in proteins with less starch than the central endosperm cells. The peripheral cells have the lowest starch content and, since all cells are thought to contain approximately the same mass of protein, the protein percentage is highest in these cells. Values as high as 54% protein have been found in sub-aleurone cells in a flour of 12.5% protein (Kent, 1966). The increasing starch content found toward the centre of the cheeks causes progressive dilution of other components as well as protein (Evers & Millar, 2002). This may partially account for the report by Dobraszczyk (1994) who observed that a decrease in cutting force occurred from the outside of the grain toward the center (Dobraszczyk, 1994). These transitions are also evidenced during the milling process where there is a trend for the gradual increase of starch damage, ash content, and total protein content in streams from later break and reduction stages associated with peripheral cells (Sutton & Simmons, 2006). As mentioned above, lipid characteristics also vary between early and later milling stages.

7.4 Vitreousness

The results of a study by Greffeuille et al. (2007) indicated that both vitreousness and hardness impact on the dissociation behaviour of the endosperm. Hardness mainly influences the proportion of small particles i.e. the ability of the endosperm to release starch granules, whereas the size of the coarse particles produced during farina reduction is mainly influenced by vitreousness, which itself could be related to the porosity level (Greffeuille et al., 2007). Nevertheless Phillips and Nierberger (1976) concluded that degree of vitreousness has no effect on total milling yield (Phillips & Niernberger, 1976; Dexter & Edwards, 1997). Typically, it is desirable for hard wheat used for the production of bread and pasta products to contain a high percentage of vitreous kernels. High levels of vitreous kernels are important in the top grades of durum wheat for efficient production of top-quality semolina (coarse
particles). For hard common wheat, where the desired end product is flour, starchiness has little impact on milling performance when straight-grade types of flour are produced (Pomeranz et al., 1976; Dexter et al., 1988). However, starchiness reduces the yield of granular hard-wheat farina and hard-wheat (durum) semolina from the break roll passes of the mill, with more fine flour produced during the reduction passes, which could lower the potential for the production of low-ash top patent flours (Carson & Edwards, 2009). In milling, hard wheat produces less coarse bran than soft wheat and, as a consequence of the greater breakage of the aleurone, more aleurone cell contents are released into the flour at the reduction step from hard than from soft wheat (Greffeuille et al., 2005). These observations highlight contributing factors to flour yield optimisation and that more consistent vitreousness and efficient bran separation must be targeted as well increased yield weight.

Dobraszczyk (1994) has shown that the starch-protein matrix is largely dependent on the degree of vitreousness in the kernel, with vitreous kernels exhibiting more than twice the fracture toughness as mealy kernels of the same variety (Dobraszczyk, 1994). Haddad et al. (1999) found that maximum failure stress and failure energy were affected very significantly by both the variety and the degree of vitreousness (Haddad et al., 1999). Interestingly a recent study using computational modelling to simulate endosperm structure, found that the protein volume fraction and the starch-protein adherence thought to be major parameters controlling hardness, play nearly the same role as far as the tensile strength is concerned (Topin et al., 2008). However wheat lines with different storage protein patterns and dough properties can have very similar hardness scores (Turnbull & Rahman, 2002). Again, this is dependant on how the hardness measurements are performed. The traits of seed vitreousness and grain hardness are highly correlated yet not all hard wheat necessarily contains vitreous seeds. Seed vitreousness and hardness therefore should be considered as separate traits that are most likely linked as seen by overlapping QTLs on chromosome 5DS. In addition evidence implies the influence of other chromosomes (see topic: Chapter 1, QTL Analysis). Nevertheless more research regarding texture needs to be focused on mechanisms of storage protein deposition and vitrification.
7.4.1 Senescence

The most important change in maturation of wheat grain is the loss of moisture from 30 to 35 percent in mature grain to 12 to 13 percent in ‘combine-ripe’ grain. The formation of large numbers of protein bodies, coupled with starch granule enlargement, causes the cytoplasm to be isolated into small regions. These regions condense with protein to form the continuous matrix during final stages of maturation associated with water loss (Bechtel et al., 2009). The air-drying process is suggested to be particularly important in the manifestation of grain softness or hardness. Bechtel & Wilson, (1997) demonstrated that factors that cause the difference in grain hardness at maturity are already present in the immature grain possibly rendering some accepted theories of grain hardness doubtful. In our studies, SKCS - CRPs of kernels collected at 14 days DPA showed similarities to those measured at maturity (see Appendix CD: SKCS Crush Response Profile analyses). Consequently results suggest that rather than accumulation of particular grain components, the process of senescence may cause changes in the starch granule surface such that surrounding components bind tightly in hard wheat varieties, whereas the binding is weaker in soft varieties (Bechtel & Wilson, 1997). Therefore the extent of starch/ protein matrix bond does not seem to become apparent until the action of cellular desiccation that immediately precedes grain maturity (Bechtel et al., 1996; Delwiche, 2000). Finally during desiccation as the endosperm dries out, it is thought that lipid membranes surrounding amyloplasts are ruptured, forcing proteins and lipids onto the surface of the starch granule (See details on the process of vitrification under the topic: Chapter 2, Vitrification)(Stone & Morell, 2009).

7.4.2 Agronomic Conditions & Protein Content

Most of the protein within the kernel comes from nitrogen previously accumulated in the leaves, and most of the starch is from sugars made by photosynthesis during the grain-filling period. Nitrogen moves into the filling kernels to form protein during early grain development. If yields are low because the kernels do not fill properly, the grain is high in protein. Drought and high temperatures are usually responsible for this condition. If the grain fills normally and yields and test weights are high, grain protein is frequently lower because it is diluted by other materials (starch). The agronomic conditions (water and nitrogen availability) and environmental conditions (temperature and light intensity) during grain filling and the
rate of drying at maturity also affect grain vitreousness (Parish & Halse, 1968; Bechtel et al., 2009). The molecular and biochemical changes accompanying the structural changes during senescence have not yet been investigated (Bechtel et al., 2009). Obviously the timing of agronomic inputs (nitrogen) and time of harvest can affect the degree of vitreousness apart from environmental and varietal influences. Taken together, vitreousness and protein characteristics are very important to the milling quality of hard and durum wheats. However these qualities are under strong environmental influence, though may be manipulated by agronomic management. Identification of genetic regulation of specific aspects of water, nitrogen and nutrient accumulation and the process of vitrification itself are worthy of further research, especially in regions prone to abiotic stress and soil degradation.

7.5 SGSD & Rheology

It is well documented that a higher percentage of small starch granules is a characteristic of harder wheat (Evers & Lindley, 1977; Bechtel et al., 1993; Zayas et al., 1994; Stoddard, 1999b; Gaines et al., 2000; Li et al., 2008). Although no significant differences in the relative quantity of type A granules have been noted between the two wheat classes (Glenn et al., 1992; Bechtel et al., 1993). The surface area of B-granules has been estimated at 0.7 m² per gram of starch and is about three times higher than that for A-granules producing a prominent contribution to endosperm cohesiveness and strength (Konopka et al., 2005). Accordingly, Ahmed and Jones (1990) cited several studies on filled synthetic polymers where an increase in modulus with decreasing particle size was evident, suggesting that increasing surface area provided more efficient interfacial bonding (Ahmed & Jones, 1990; Edwards et al., 2002). Likewise in another study, the small-granule starches presented a higher ratio of surface area per unit weight of starch, and thus hydrated and swelled more efficiently than the large-granule starches. Previous research provides evidence that granule type predominance directly affects dough/ baking quality. The present research proposes that granule type ratio affects milling quality. High endosperm responses in SKCS crush response profiles were characterised by fracture along cell walls and the presence of a higher proportion of C-type starch granules in prismatic (peripheral) cells. Conversely the same regions contained less type A granules in size and number compared to central cells (see Appendix CD: Microscopy Images).
7.5.1 Starch Damage

The potential or level of starch damage (fractured starch granules) in flour is a critical and defining quality trait of hard wheat classes but is not important to high flour yield from soft wheat classes. Hard wheats with higher protein content and consequent prevalence of vitreous endosperm facilitate greater control over starch damage subsequent to the first break milling stage. The majority of starch damage is produced by the front end reduction and sizing reduction stages. The miller can affect the starch damage content of flours through wheat choice, grain preparation and the mill set-up and adjustments (Dubat, 2007). Also it is proposed that specific starch granule characteristics of size distribution, structural strength, and surface bonding properties contribute to this desirable quality. Manipulation of starch characteristics may have possible benefits in the control of this quality important to starch metabolism and water absorption in dough and baking processes. Previous research has produced variable results as to the type of starch granules which are more prone to damage. Either smaller granules with strong bonding areas and fragile structure may be more prone to damage as often reported; alternatively larger granules would break more readily through shearing forces or in the small roll gaps of reduction stages.

7.5.2 Hardness & Puroindolines

As puroindolines are closely associated with starch granules, and given their effects on grain texture and the influence of starch on the quality of wheat-based end products, the aim of the present study was to contribute to the knowledge of these and other factors influencing flour yield variability. It is not proposed that SGSD directly affects the regulation of puroindoline deposition. The three major classes of endosperm texture: soft, hard common, and durum wheat represent and define one of the leading determinants of the milling and end-use quality of wheat (Morris et al., 2008a). Given the high heritability and, therefore, the strong cultivar effect on hardness it is possible to predict the relative cultivar performance for kernel hardness from a single location or a composite sample from multiple locations (Hazen & Ward, 1997). Consequently much research in grain rheology has been dominated by studies involving the puroindoline genotyping of varieties and the elucidation of the puroindoline protein mechanism of action. The value of high flour yield to the end-user is dependant on protein quality and content, the levels of bran contamination and starch damage.
Delwiche (2000) comments that wheat hardness is also important in the determination of milling throughput, equipment design, and energy requirements. The quality of ‘Hardness’ becomes a determining factor in the designation of class to a particular variety. Nevertheless, it is entirely possible to have a genetically soft wheat which is physically hard; alternatively genetically hard wheat can be made soft by changing environmental and drying conditions (Hoseney, 1987). Classification is presently based on physical measurements rather than genetic analyses.

7.5.2.1 Hardness – Factors Other Than Puroindolines

Although the \textit{Pina-D1} and \textit{Pinb-D1 loci} have gained wide acceptance as the causal genes for grain hardness (Giroux & Morris, 1998), a survey of Australian wheats indicates that other factors may limit their utility in predicting grain hardness. It is evident that some major effects on grain hardness can be observed in Australian wheat lines that are not associated with the classical 5DS locus (Turnbull \textit{et al.}, 2000; Osborne \textit{et al.}, 2001b). In addition, Eagles \textit{et al.} (2006) concluded that although significant effects of puroindoline genes on milling yield were found in their study, the reduction of the genotypic variance due to these genes was only modest, indicating that other genes are important determinants of milling yield in southern Australian breeding programs (Eagles \textit{et al.}, 2006). As with all searches for genes of commercial interest, it is possible that many genes affect processing quality; however, only those genes that show variation in the genetic analysis performed will be detected. For example, alternative research can be challenging in the identification of significant regulatory elements, and mRNA processing which can all effect quality phenotypes. So far the most well characterized source of variation is the \textit{Ha locus} located on the chromosome 5DS (Piot \textit{et al.}, 2001a).

Variation within hardness (puroindoline) class is poorly understood and kernel hardness itself has no absolute definition. For example, in a study of thirteen Italian cultivars, seven soft and six hard according to puroindoline genotype, though genetically unrelated, Gazza \textit{et al.} (2008) concluded that variation in grain hardness within each texture class was largely determined by growing conditions (soil, climate, diseases, water and nutrient levels), and thus a matter of agronomic practices (Gazza \textit{et al.}, 2008). In a study of US soft wheat cultivars, Morris \textit{et al.} (2005) found significant differences in kernel texture. These researchers suggested the existence of different
minor gene(s) among these cultivars for kernel texture or macromolecular composition that could potentially be exploited in wheat improvement. Morris et al. (2005) also suggested that varieties identified as sources of exceptional kernel softness may also be useful for crossing and research purposes (Morris et al., 2005). In our surveys of over one hundred varieties at two sites, the Queensland site produced high SKCS Hardness Index values of 101 - 96 for cv. Dollarbird, Rees, Iraq 46, Machette and lowest values 7 - 4 for cv. Tota 63, Lerma Rojo, and Yaktana. Alternatively the NSW site produced SKCS Hardness Index values of 93 - 85 for cv. Marombi, Strzelecki, India 37 and lowest values 6.5 - 4 for cv. Yaktana, Sion, Lerma Rojo, and Tota 63. The SKCS is known to operate more consistently for harder varieties (considered as having values greater than 50).

7.6 Present Research in SGSD & Puroindoline Genotype

Our last phase of experiments selected varieties grouped according to puroindoline genotype. When considering the pin genotype which expresses the lowest quantity of granule bound puroindoline (Pina-D1b, Pinb-D1a) wheat varieties with higher ratios of large to small granules produced higher straight run flour yields. Considering fractionation of endosperm, comparative SGSDs that present a reduction in total bonding area may induce a more fragile structure if a direct mechanical relationship is concluded. The correlation of flour yield to an increased large to small granule ratio was weak with varieties of the genotype: Pina-D1a, Pinb-D1b. Previous studies of the milling qualities of hard wheat often indicate that wheat varieties with the Pina-D1a, Pinb-D1b genotype produce the highest milling quality. Although possessing a single point mutation, varieties with this genotype are known to deposit a wide range of puroindoline protein quantities at the starch surface. In the present study of this genotype and using micro-electrophoresis technology to quantify the starch bound proteins, results suggest that varieties with less granule-bound puroindoline will be of a higher milling quality (flour yield and Wheat Rheology Index). However as suggested above, this phenomenon may result from the dilution of proteins with higher starch contents. Consistent trends were evident from two environments with starch-bound puroindoline content accounting for 31-35% of the variation in flour yield. This suggests that only a minor component of granule/protein matrix debonding is necessary to improve flour extraction from this premium genotype (normally found within the high protein, Australian Hard classification). This should be compared to
the very hard varieties (puroindoline genotype: Pina-D1b, Pinb-D1a) having strong granule bonding and subsequently higher levels of starch damage but generally producing slightly lower flour yields. Multiple regression showed that the combined effect of starch granule type and bound puroindoline content described by the equation

\[
\text{Flour yield} = 75.92 - 0.26 \text{ (puroindoline a)} + 0.19 \text{ (A/C granule ratio)}
\]

accounted for 68% of the variation in flour yield in the Pina-D1a, Pinb-D1b genotype. The results of previous research indicate that PINB is more specific and more limiting to starch binding than PINA which has been shown to have a lower amount of insertion into lipid monolayers. Although these mechanisms still require further clarification (Swan et al., 2006; Clifton et al. 2007a,b; Wanjigi et al., 2007; Evrard et al., 2008).

### 7.6.1 Lipid Interactions affecting Hardness

Regarding lipid interactions, Feiz et al., (2009) concluded that several lines of evidence indicate that the Ha locus directly effects changes in starch polar lipid content via an unknown mechanism. Furthermore the most likely explanation proposed was that active PINs stabilize bound polar lipids on the surface of starch granule membranes preventing breakdown during seed desiccation and maturation. Consequently, lipid degradation may occur during kernel ripening and maturation in hard wheats supporting the observation of the hard phenotype at this stage (Feiz et al., 2009). Clearly, there is a variable interaction between the puroindolines and starch granules. Starch surface characteristics and lipid content within hard wheat varieties require further study as there have been several reports showing a correlation between these traits.

A full profile of the lipid species found on the starch granule surface and the relationship of these molecules to endosperm hardness have not been reported (Finnie et al., 2010a). Previous workers have suggested that Free polar lipid levels may be influenced by the puroindolines (the puroindoline genes being tightly linked to the Ha locus). The linkage between Fpl-2 and Ha loci, however, has not been examined further (Turnbull & Rahman, 2002). In conclusion, the compositions of starch surface lipids and internal starch lipids are different based on starch granule class. The relationship between lipid distribution, starch granule types, and weakness zones in the endosperm is worthy of further investigation (Greffeuille et al., 2007).
7.6.2 Puroindoline Affinity for Different Starch Granule Types

Puroindoline was negatively correlated with total starch granule surface area. Interestingly, these results may infer that puroindoline has a higher affinity for the surface of the type A granules, as these contribute least to bonding surface area in the endosperm composite structure. Such affinity has yet to be supported by immuno-fluorescent localization studies. As the starch granule types possess different physical and compositional characteristics and result from differing developmental processes, presumably surface interactions are also expected to vary. From a biological perspective, puroindolines have been allocated a defence function due to demonstrated ability to bind to lipid membranes. Accumulated evidence indicates puroindolines possess antifungal/antibacterial properties which may contribute to plant defense presumably of endosperm starch storage predominantly accumulated in the large type A starch granules (Dubreil et al., 1998; Krishnamurthy et al., 2001; Charnet et al., 2003; Giroux et al., 2003; Jing et al., 2003; Faize et al., 2004; Capparelli et al., 2005; Llanos et al., 2006; Capparelli et al., 2007; Luo et al., 2008).

In conclusion the functional significance of differential expression of endosperm starch granules remains unclear. Possibly the production of numerous small granules in the later stages of grain filling through interior-sensing stromules (see Chapter 2 topics: Endosperm Development and Starch Granule Formation) maximises storage capacity within physical constraints as well as providing packaging support for the main storage compartments, that is, the large type A granules. SGSD is also regulated according to environmental limiting factors such as temperature and water allowing seed viability within normal ranges of stressors. During germination, different rates of metabolism by enzymes are indicated between large and small granule classes; the sequence initially utilising cell walls and protein then the interior of the large granules via channels. Compared with small granules, surface degradation of large starch granules appears to be inhibited.
7.7 Defining Flour Yield

Flour yield is the product of complex inter-relationships between variations in the milling process and wheat quality traits. These qualities are themselves inter-related to various degrees dependant on genetic and environmental factors. The flour yield of a specific wheat variety as expressed as a weight ratio of straight run flour extracted to milled grain, does not indicate which aspects of milling and flour quality are important. For example, growers are interested in market values on the basis of wheat protein; moisture, screening levels and physical measurements. Alternatively a high priority for a miller would be potential flour yield also accounting for the specifications of customer requirements. Moreover food manufacturers require specific flour functionality. The main criteria in determining the bread-making quality of flour, regardless of the specific end product, are protein content and quality, dough strength properties or mixing properties, $\alpha$-amylase activity, and degree of starch damage. Short patent flour made from hard wheat is the most highly recommended commercially milled flour for bread baking and contains 70 to 80 percent straight flour. Patent flour is made from the centre portion of the endosperm.

To some extent, millers can manage the physical properties of the flour they produce through the grinding process and stream selection. Consequently any improvement in flour yield through enhancement of wheat varieties must also take account of potential extractions of protein and ash contents and susceptibility to starch damage. Therefore, flour yield must also be considered in relation to the various stages of processing and popular end–usage. Of course, the traditional acceptance of whole grain flour greatly simplifies processing. Currently, nutritionists advocate the consumption of whole grain as the healthier alternative. A recently patented process describes a bleached whole wheat flour that is obtained having the colour and taste of white flour. This is achieved by bleaching whole wheat kernels with a peroxide solution to lighten the colour of the bran layers prior to conventional flour milling (US Patent 7101580 - Method of bleaching cereal grains - Sept. 5, 2006). Optimising flour yield in such instances relates more to grain weight improvement rather than extraction efficiency.
7.7.1 Environment, Flour Yield and Weight

Test weight is used by millers as a crude predictor of potential flour yield especially for wheat varieties from the same location. However, it is not true when widely varying varieties are used for blending (Hlynka & Bushuk, 1959). In the present study, a subset of 30 hard varieties with SKCS Hardness Index values greater than 55, including data from standard SKCS parameters, seed weight and diameter measurements made significant correlations with flour yield (Edwards et al., 2008). Ignoring considerations of flour quality, and accepting that flour yield is partly a function of separation efficiency, flour yield is generally related to caryopsis weight. However further gains in grain weight cannot be at the expense of overall grain yield unless marked quality improvements are also achieved to allow financial viability.

A rapid method of measuring test weight remains widely used for the determination of price in cereals. There are instances when this parameter gives no indication about quality or flour yield (Kleijer et al., 2007). Carson and Edwards (2009) comment that with proper mill setup, wheat classes of varying test weights but with comparable physical condition generally produce similar flour yields. The greatest influence on test weight is considered to be from the environment, not class (Carson & Edwards, 2009). This has implications for the association of breeding strategies and agronomic management to specific regions. Also significant contributors to aspects of quality, such as protein content, are the result of growth conditions - soil fertility, rainfall, and temperature during the growing season, at harvest, and beyond. Generally, wheats can be closely matched to many different end uses according to their grain hardness and protein content (Wrigley, 2009).

7.7.2 Environmental Effects on SGSD

Water is the most important limiting factor for wheat production. Soil water conditions greatly affect mineral contents in the grains of winter wheat, particularly with regard to the major minerals (P, K, Ca and Mg). Water deficit during grain filling can also result in a decrease in lipid contents in wheat grains. Although, a mild water deficit during grain filling can be beneficial to grain filling, starch compositions and
subsequent bread-making quality. Zhao et al. (2009) concluded that good management of wheat field water at post-anthesis stage was helpful to improving grain quality relevant to processing and human nutrition (Zhao et al., 2009). Another recent study demonstrated that the accumulation rate of small starch granules is significantly increased by the deficit of soil water (rain-fed conditions without irrigation) at 14–21 days after anthesis, when the B- and C-type starch granules are synthesized rapidly and development of A-type granules decreases significantly (Dai et al., 2009). The suggested reason was that the starch accumulation rate and the activities of the related enzymes are significantly increased by the deficit of soil water at 14-21 days after anthesis (Stoddard, 1999b), when the B- and C-type starch granules are synthesized rapidly. A switching of starch synthesis rate and diversion of substrate is suggested from a slower, large volume output to a more rapid though significantly smaller volume output via the stromule mechanism as a result of water deficit. At the same time, the protein content in grains also increased under water deficit conditions (Dai, 2009). As mentioned above, this is also an effect of a lower volume of starch (predominantly the large type A fraction) presenting as a higher proportion of protein content per kernel. Importantly, subtle changes in granule packing within endosperm cells with specific protein characteristics and moisture contents may produce more vitreous and less porous composite structures, possibly the increased pervasion of small starch granules facilitating the vitrification process.

The observation of significant environmental effects on granule size distribution is not surprising given that B granule synthesis is so dependent on the timing of events in grain development relative to the temperature regime and the duration of grain filling. For example, low temperatures during grain maturation extend the grain-filling period, allowing waves of B and eventually C granules to be initiated, leading to a high proportion of B and C granules. However, note that, in one study, C granules were shown to constitute just 3.4% of the total starch (Bechtel et al 1990). In contrast, high temperatures accelerate grain development and cause early maturation, limiting the opportunity for B granule initiation and growth and thus suppressing B granule numbers. Taken together, the extent and type of starch granule formation is partially determined by environmental influences such as ambient temperatures.
Consequently researchers suggest that granule size distribution in any given variety should have the potential to be a useful diagnostic describing the environmental conditions in operation during grain fill, which may, in turn, be useful for predicting other aspects of grain quality. Nevertheless previous research indicates that the control of the A-to-B granule ratio in hexaploid wheats is under complex multigenic control (Stoddard, 2003; Stone and Morell, 2009). Such variation in SGSD between kernels of the same plant also has the potential to confound analyses therefore sample selection confined to position or at least size is advised (see Chapter 3, Starch Granule Size Distribution Analysis).

7.7.3 Regional Qualities

As observed, there are important environmental influences interacting with wheat lines from various regions that produce specific end-use requirements. For example, harder-grained wheats are deliberately cultivated in areas known to produce high protein contents, because the combination of high protein and hard kernels results in the flour most suited to products such as pan breads and Cantonese noodles. High protein content is also required for durum-wheat semolina. It is for this reason that certain wheat types are dominant in each wheat-growing region of the world. The combination of quality characteristics genetically built into varieties and those influenced by environment is carefully balanced to produce wheats with particular end uses in mind. Much research effort has been directed at determining which types from which regions are best suited to individual products. The classification of wheat into specific grades is essential for pricing and trading purposes because it allows customers to specify their requirements according to the particular products they manufacture (Cracknell and Williams 2004). Millers require this information as mixtures of wheat cultivars rather than single cultivars of uniform physical characteristics are processed in flour mills, with very little adjustment of mill rolls to adapt to specific characteristics of grain to be milled. In Australia, the classification process is a complex task involving an evaluation of the quality of a variety, within a defined geographic area, over several years of production. As stated different classes are matched to specific regional conditions which also may enhance certain end-product qualities to supply a specific market. Accordingly strategies to improve flour yields may differ between regions. As perfect wheat varieties have not yet been developed, compromise between traits and the assumption of risks are also necessary.
to insure profitability. Agronomists recommend that several different varieties should be planted in order to hedge against weather and pest problems. In addition, chosen varieties should have different pedigrees and different growing patterns (Paulsen, 1997). In conclusion, breeding strategies to improve flour yield may differ between regions.

7.8 Breeding Methods and Trait Interactions

7.8.1 Breeding for Increased Weight

Substantial increases in kernel weight are primary markers in archaeological plant remains for the domestication of wheat. The development of this trait continues today as evident by the current research to improve flour yield partially through optimisation of the endosperm to bran ratio. Considering the differences in granule size distribution between grain positions within the spikelet, researchers conclude that there are important implications for future wheat breeding strategies. Dai (2009) reported that the distal florets are detrimental to average grain mass and grain protein content, therefore a readjustment of yield components against grains per spikelet may be of value. A promising breeding strategy would be to improve grain yield by increasing average grain weight rather than grain number within a spikelet. This would resolve the inconsistency between high yield and quality (Dai, 2009). Such improvements in grain weight are likely to improve flour yield. The different qualities in basal and distal grains can also affect food processing and qualities. As for millers, the most practical alternative available to ensure sample uniformity may be through seed size segregation at the time of grain cleaning.

In the past, wheat yield gains have been associated with increases in the number of grains rather than in grain size (Loss & Siddique, 1994; Slafer & Savin, 1994; Calderini & Slafer, 1999). Calderini et al. (2003) comments that if plant breeders continue to strive for increased wheat yields by selecting for grain set in distal positions of the spike, the inherent lesser grain weight potential at these distal positions will likely limit advances in grain yield (Calderini & Ortiz-Monasterio, 2003). Increases in grain number have been due mainly to improved grain set in more distal positions of the spikelet (Slafer & Savin, 1994). Moreover, the added grains will have progressively lower mineral concentrations compared with proximal grains of central
spikelets. Miralles and Slafer (1995) showed there was a greater proportion of grains in distal positions in semi-dwarf wheat than in standard-height isogenic lines (Miralles & Slafer, 1995). The weight of these distal grains was clearly lower than that of grains from florets more proximal to the rachis (Calderini & Ortiz-Monasterio, 2003).

### 7.8.2 Genes affecting Yield and Weight

In a study by Rattey et al. (2009), Seri/Babax lines with both high grain yield and grain weight were associated with a combination of several traits: earlier flowering, reduced tillering, a greater proportion of tillers that produce grain-bearing spikes at maturity, high water-soluble carbohydrate stem reserves at anthesis, a higher proportion of competent florets at anthesis to maximise grains per spikelet leading to a high harvest index, and possibly a greater capacity to extract soil water. Therefore grain yield and weight were associated with a combination of several traits including earlier flowering and reduced tillering (implicating advantageous allele combinations of Vrn and tin genes respectively)(Richards, 1988; Rattey et al., 2009). These traits were suggested as a suitable ideotype for breeding high-yielding wheat cultivars with high grain weight adapted to environments with hotter, drier conditions during the post-anthesis period (Rattey et al., 2009).

Improvements in Harvest Index (HI) associated with reduced height can account for more than 80% of the improvement in yield potential of wheat varieties within some twentieth century breeding programs. Rht genes have affected an increase in HI, mostly through improved spikelet fertility while maintaining sufficient total biomass production, and consequently have had a hugely significant impact on worldwide wheat production. QTLs identified for flour yield have also been related to the location of Rht genes. A few major genes conferring tiller inhibition (tin) have been identified, which can also be associated with other potentially useful traits such as increased mean grain weight and improved harvest index (Richards, 1988). Genetic advances to increase grain-filling rate or prolong grain filling including the protection of the flag leaf from premature senescence is particularly important to increase yields associated with increased mean grain weight (Bancal et al., 2007). The rate of wheat development depends largely on variety, temperature, the need for a cold period (vernalization), and day length (photoperiod) (Gooding, 2009). The present
development of diagnostic markers to test for desirable combinations of the various important vernalization, photoperiod and height–reducing alleles should accelerate progress for wheat breeders (Eagles et al., 2009).

There are great advantages to the breeder by identifying specific traits in wheat lines on the basis of gene presence or absence. Appropriate selection at an early stage of the process avoids the need for ongoing propagation of undesirable lines. Simply inherited traits are selected early. However attributes such as yield and grain quality that involve many genes (quantitative traits) are more difficult to select for, so they have traditionally been part of the mid- to late-generation selection schedule, determined largely on the basis of actual phenotype (measurable aspects of yield and quality performance), rather than according to the presence or absence of relevant genes. Grain yield depends on the number and mass of grains per unit of growth area. The components of this measure include plant density (plants per square meter), tillers (thus heads per plant), spikelets and grains per head, and the mass distribution of the grains. These factors are partly determined by genotype (variety, designed by the breeder) but largely by agronomic practice, which aims to optimize these components. Maximizing all these components is not an option, as they are interactive; for example, the number of tillers per plant is reduced if plant density is too high, and excessive numbers of tillers may cause fewer grains per head.

Final grain weight under good growing conditions has often been correlated with the water mass per grain and/or the number of endosperm cells attained during the two to three weeks after anthesis (Brocklehurst, 1977; Borras et al., 2004). Researchers have observed that environmental treatments applied during this period have a much larger impact on final grain size than treatments applied later. During the linear phase of grain growth, 80% of the photosynthate produced in the flag leaf is translocated to the ears (Thorne, 1982). The understanding of major pathways in grain development can assist gene expression studies to identify significant or limiting factors. Potential exists in farming practice to promote features important to milling performance given that this complex trait is under significant environmental influence.
In summary, prominent features important to breeding and agronomic practice contributing to combined gains in grain weight, number, quality and consequent flour yield are water uptake regulation, plant height, flowering efficiency, tiller inhibition, flag leaf health and time to maturity. At a cellular level, the regulation of photosynthesis efficiency, endosperm cell numbers attained during the two to three weeks after anthesis, sucrose/H+ symporters, aquaporins, and other transport channels are all possible targets of investigation. In association with increased kernel weight through prolonged grain filling, a current priority of wheat breeders is to provide growers with a range of maturity rates in each quality grade/class for different sowing dates to hedge against possible frost or drought conditions.

7.9 Implications of Present Results

The present research indicated that a significant part ($R^2 > 0.40, p< 0.05$) at two sites) of the association between starch granule size distribution (SGSD) and flour yield appeared to be under genetic control despite known environmental effects on small granule number. Nevertheless this may provide a way of effecting genetic improvement in wheat flour yield through manipulating the genes regulating SGSD. Further confirmation is required on a wider sample set also accounting for varietal differences in granule bond strength. In composite materials a more evenly graded particle distribution is known to produce a higher density and stronger structure (Chapter 5, Figure 10). In wheat endosperm with strongly bonded granules often filled with vitreous protein matrix or associated low levels of puroindoline, initial fracture along cell walls and subsequent fracture through starch granules in reduction milling stages may be enhanced. Although the effect of SGSD on the endosperm composite structure may directly influence extraction efficiency, SGSD in any given variety could have the potential to be a useful diagnostic describing the response to environmental conditions during grain fill and rate of maturation.

Accordingly the results and methods described in Chapter 5 indicate that across a range of hardness phenotypes, the analysis of larger kernels of high flour yield varieties exhibited a smaller ratio of large to small granule types which can be a marked response by these varieties to a period of water deficit or a longer grain fill duration that allows for small granule development. Whereas from larger sample sets
within specific puroindoline genotypes (especially *Pina-D1b, Pinb-D1a*), high flour yield varieties were characterized by higher volumes of large granule types and less deposition of starch bound puroindoline protein. This may be a response of these varieties to higher temperatures or maturation rate which also relates to grain position on the plant. In the wider context, kernel weight was significantly correlated with flour yield in these studies. The large type A granules are known to contribute to 60-75% of total starch mass and total starch forms approx. 80-85 % of flour dry mass (Roman-Gutierrez et al., 2002). Therefore higher comparative volumes of the type A granules may be an expected characteristic of high flour yield varieties.

Analyses of starch granule size distribution and flour yield were performed using two different experimental designs and different sample sets, resulting in inconsistent relationships between milling quality and the ratio of A-type to C-type starch granules. In Chapter 5 the relationships between morphological and rheological phenotypes and flour yield were considered in a select set of heterogeneous puroindoline genotypes whereas in Chapter 6 the interrelationships between puroindoline content, genotype, SGSD and flour yield were examined using samples derived from a single genotype. Consequently, variation in ratios of A-type to C-type starch granules relative to milling quality appeared between sample sets. This variation is likely due to the rheological effects of differences in the deposition of starch granule bound puroindoline, particularly in the *Pina-D1a, Pinb-D1b genotypes*.

### 7.9.1 Manipulation of SGSD

Previous research has identified a range of genes (see Chapter 2, Genes in Amyloplast Division) that may have the potential to become markers or be genetically modified to produce starches with novel technological applications. However the industrial techniques involved are challenging for example, extraction of small granule types. Manipulation of SGSD for specific industrial applications is potentially demanding given the complex synergies involved in the formation of amyloplasts and starch structure. The alternative application of such manipulation either through selective breeding or molecular biology techniques resides in improved processing quality (milling) or production of alternative food products including possible health benefits for consumers. A recent study by Zhang et al. (2010) confirms previous research on the formation of wheat starch granules. Starch granule types A-, B-, C-
were initiated at 4, 8, 20 DAF, respectively, which was consistent with the patterns of starch synthase activities and relative gene expression levels. For example, activities of soluble and granule-bound starch synthases (SSS and GBSS) peaked on 20 and 24 DAF. Genes encoding isoforms of starch synthases were expressed at different grain filling periods. In addition, SS I was generally expressed over the grain filling stage; while SS II and SS III were expressed over the early and mid grain filling stage, and the GBSS I was expressed during the mid to late grain filling stage (Zhang et al., 2010). Researchers propose that the genes required for synthesis of the equatorial plate and groove producing enlargement of type A granules may be expressed only during the early stages of development and may no longer be expressed or active by the time B granules are initiated (Stone & Morell, 2009). Related genes may soon be identified through current mRNA expression studies during development assuming appropriate selection of different time points.

Very little is known about the processes determining starch granule size (see Chapter 2: Genes in Amyloplast Division). A few studies have indicated that Starch Branching Enzyme I (SBEI) may be involved. Studies using antisense SBEI constructs have reported increased granule size in transgenic potato, and an increased proportion of large A-type granules in transgenic wheat. It has also been suggested that SBEIc may play a role in the determination of starch granule size and morphology in wheat and barley, based on the observations that: i) it is only present in starches from plants with bimodal granule size distributions, and ii) it is preferentially associated with A type granules. This promising research requires further confirmation. If SBEI or SBEIc are key factors in the bimodal starch granule size distribution in wheat and barley, then down-regulation or a mutation in either protein should alter the proportion of A- and B-type granules. Consequently, researchers concluded that a transgenic antisense SBEI and/or SBEIc approach or use of traditional plant breeding techniques and/or mutagenesis could be taken to investigate this possibility. Given the diploid and hexaploid genomes of barley and wheat respectively, barley is suggested to be the plant of choice for initial investigations (Peng et al., 2000b; Davis et al., 2003). Further genes of interest identified by others related to SGSD include: Limit dextrinase inhibitor (a starch debranching inhibitor) (de Pater et al., 2006), heteromultimeric isoamylases (Bustos et al., 2004), and ATP binding membrane transporter (Ycf 16) (Gemstar and Burrell et al. (2006) Patent WO/2006/059130).
There is also potential to identify variation in novel attributes such as starch granule shape, amylose to amylopectin ratio, crystallinity and gelling properties in the lower ploidy species for use in the hexaploid species. These modifications could significantly alter the technological properties of starch for use in both food and non-food industries (Martin & Smith, 1995). Starch granule size distribution is affected by both genotype and environment (Blumenthal et al., 1994; Blumenthal et al., 1995; Stoddard, 2000, 2003). In a survey of starch particle-size distribution in wheat and related species, Stoddard (1999) found the B granule content of hexaploid wheat starch to be commonly around 30% by volume (Hughes & Briarty, 1976; Brocklehurst & Evers, 1977; Evers & Lindley, 1977) and surveys have shown values as low as 13% (Soulaka & Morrison, 1985) and as high as 47% (Stoddard, 1999a). Tetraploid wheats had a similar range of B granule content and A. tauschii lines often have lower values, down to 15%. In addition results indicated some synthetics had very high values above 50%. Six synthetic hexaploid samples had over 44% B granules (less than 10 μm in diameter). Stoddard (1999) concluded that there are prospects for increasing the variation in granule size of hexaploids, by use of synthetics or tetraploid germplasm with higher B granule contents (Stoddard, 1999a). This would be potentially valuable for bread-making, where higher starch granule surface area is associated with greater water uptake, and for Japanese white salted noodles, where higher swelling power and lower gelatinisation temperature is desirable (Konik-Rose et al., 2009). The relationship between starch granule characteristics and milling quality requires further investigation.

7.9.2 Puroindoline Manipulation

The results of various experiments increasing puroindoline gene dosage in grain thereby producing softer phenotypes support the current understanding of the influence of puroindoline on grain texture. For example, genotypes with added Pin genes on the A genome produced seeds that were softer by 7.4 hardness units. These softer double Ha genotypes were lower in flour yields, but produced flour with lower ash content, reduced starch damage, and smaller mean particle size. Soft wheats with increased Ha dosage may be useful in improving soft wheat quality through its effects on particle size and starch damage reduction (Campbell et al., 2007b). Turnbull and
Rahman (2002) comment that other genes carried on the short arm of chromosome 5D in wheat are possibly also important in the regulation of grain hardness (Turnbull & Rahman, 2002). Therefore PIN over-expression can cause a reduction in grain hardness and reduced flour yield, flour ash, and flour particle size. Increased PIN expression can also result in reduced loaf volume and flour water absorption (Wanjugi et al., 2007b). Conversely a reduction of puroindoline expression is likely to facilitate water absorption, the result of increased starch damage. Ideally studies attempting to establish a direct relationship between a cell product and mechanical hardness should correlate the quantity of the cell product with actual hardness data based on both single caryopses and bulk hardness tests. Such analyses have become more efficient and less subjective with micro-electrophoresis (Lab-on-Chip) technology to profile and quantify proteins as used in this project.

7.9.3 Puroindoline Genotyping Studies

Part of the present research endeavoured to identify novel puroindoline genotypes producing useful milling qualities. In over 150 wheat varieties from the Australian Winter Cereal Collection (Tamworth), the most prevalent genotype was the \textit{Pina-D1a, Pinb-D1a} (wild-type soft) followed by the \textit{Pina-D1a, Pinb-D1b} genotype, a widely published characteristic of good milling quality varieties. The other most common genotype was the \textit{Pina-D1b, Pinb-D1a} genotype which does not express puroindoline A protein, thus producing a very hard phenotype. Many studies have reported the processing characteristics of these three dominant genotypes. The \textit{Pina-D1b, Pinb-D1a} genotype is often found to possess significantly higher values in grain hardness, protein content and starch damage than other genotypes (Martin et al., 2001; Cane et al., 2004; Ma et al., 2009). Water absorption has been associated with starch damage during milling, which tends to increase with increasing grain hardness (Williams, 1967). The effect of \textit{Pina-D1a, Pinb-D1b} was to significantly reduce water absorption, and increase milling yield compared with \textit{Pina-D1b, Pinb-D1a} (Martin et al., 2001; Cane et al., 2004). Previous large scale surveys of milling performance including the results presented generally demonstrate that the \textit{Pina-D1a, Pinb-D1b} genotype is associated with a higher flour yield than either genotypes: \textit{Pina-D1b, Pinb-D1a} or \textit{Pina-D1a, Pinb-D1a}. Taken together, conventional breeding or genetics techniques to reduce the expression of starch bound puroindoline thereby improving
milling performance of superior varieties is worthy of further study. To date an increased expression of puroindoline has been more achievable with consistent results presenting softer endosperm phenotypes and release of undamaged starch (Hogg et al., 2004; Campbell et al., 2007b; Xia et al., 2008).

7.10 Conclusion

Our milling quality measurements on similar sample sets from diverse regions indicate significant environmental effects on the complex trait of flour yield. The understanding of puroindoline genotype provides some explanation for general differences between varieties regarding hardness and some milling characteristics mostly irrespective of environmental influences. To date other influences on milling quality appear to be multigenic and/or strongly affected by environment and therefore present difficult targets for development, though breeding programmes confined to specific growing regions have potential. In associated research on flour yield QTLs in three Australian doubled haploid wheat populations, Lehmensiek et al. (2006) concluded that lack of consistency of QTLs over different crosses and trial site/year indicates that for flour yield, QTL expression is highly dependent upon the genetic background and its interactions with the environment. Consequently breeding programs should base their marker selection strategies for this trait within pedigree groupings of regionally important core germplasm (Lehmensiek et al., 2006). Single or simple gene combinations with significant effects on flour yield have either attracted little attention or remain to be identified.

In summary, the progressive stages of our research has:

- Demonstrated, through microscopy and particle size analysis of the crushed material from the SKCS 4100 and a first break roll stand, that the SKCS data averaged over 300 grains provides a useful indicator of milling performance of a wheat sample.

- Utilized Environmental Scanning Electron Microscope (ESEM) to demonstrate that microstructural differences between wheats of contrasting Rheology Index and flour yield is partially due to starch granule size.

- Confirmed a consistent, quantitative method for starch granule size analysis (SGSD) using laser diffraction. The results, using contrasting germplasm grown at
two sites, support ESEM observations indicating that high flour yield is associated with a higher proportion of small (Type C) starch granules relative to large (Type A) granules. These results were based on a small number of heterogeneous puroindoline genotypes and further confirmation is required on wider sample sets. In addition, a significant part ($r^2 > 0.44$ across the 2 sites) of the association appeared to be under genetic control. This finding resulted in a patent position.

- Conducted puroindoline genotyping of 140 samples from The Australian Winter Cereal Collection (Tamworth) and analyzed in relation to a range of milling quality measurements. The $Pina-D1a$, $Pinb-D1b$ (Pin B) genotype resulted in a higher average flour yield than either the $Pina-D1b$, $Pinb-D1a$ (Pin A Null) or the $Pina-D1a$, $Pinb-D1a$ (soft). However, the ranges of flour yields showed considerable overlap and so the gains in flour yield that might be achieved solely by selecting for puroindoline genotype without additional phenotypic measurements (such as SKCS weight and diameter) would not be expected to be substantial.

- Developed a new method for the analysis of starch-bound puroindoline proteins using the Agilent Bioanalyzer 2100 (“Protein 80 Chip”). Results showed that for the $Pina-D1a$, $Pinb-D1b$ (Pin B) genotype, starch granule size accounted for no more than 24% of the variation in flour yield between varieties, with 31-35% accounted for by starch bound puroindoline protein content. The combined effect of starch granule A/C ratio and starch bound puroindoline a accounted for 68% of the variation in flour yield in the $Pina-D1a$, $Pinb-D1b$ set. For the $Pina-D1b$, $Pinb-D1a$ (Pin A Null) genotype, which does not express starch bound puroindoline protein, starch granule size accounted for up to 47% of the variation in flour yield between varieties. Further confirmation is required on wider sample sets.

In addition, our results are expected to guide current genome sequence and transcriptome analyses aimed to further understanding of the genetic regulation of milling quality and starch granule size distribution. In conclusion, our research has identified through data analysis and literature review, a number of promising targets for further investigation in the improvement of wheat flour yield. Flour yield is a complex trait which may be improved through different approaches dependant on the flour qualities desired by end-users.
Appendices

See disc attached inside back cover

Disc Contents & Brief Descriptions

Microscopy Images

Microscopy & SKCS Milling Quality Grouping
- ESEM - SKCS high & low quality groups
- ESEM - SKCS medium quality group-Janz related
- ESEM combined group comparisons & index
- ESEM -high mag- SKCS group comparisons
- ESEM- low & high mag- subset -soft & hard wheat
- ESEM -low mag- SKCS group comparisons
- SKCS groupings - compare SKCS data & microscopy

Microscopy conditioned-uncond- fracture
- 40X mag- hard & soft fracture comparison
- 40X mag- vars-Tincurrin & Banks
- Microscopy-40X-ESEM-SEM-phase1
- SEM Fracture types vars- Banks & Tincurrin
- SKCS Data & CRPs - Conditioned & Uncon

Microscopy- ESEM -snapped caryopsis cross sections
- ESEM -snapped cross sections- Banks & Tincurrin
- ESEM -snapped cross sections- janz & roSELLa
- Initial granule & misc measurements

SKCS Crush Response Profile Analyses

- CRPs Mature & 14 DAF
- Crush Response profile analyses (WCCT)
- Crush Response profile data (2 sites)
- Grain crushing diag-1
- SKCS CRP groups & particle size

Starch Granule Size Distribution Data

- Commercial wheat SGSD data summary Table
- SGSD- 2 sites
- SGSD- PinA null & mixed Pin genotypes summary

Durum SGSD
- Durum - SGSD Analysis
- Durum SKCS & SGSD & Milling Data
Complete Data Sets & Sample Information

- Flour vs weight & kernel diameter
- Flour yield- SKCS crush response profile diagnostic
- Milling & protein & bran data (Narrabri)
- Milling & SKCS data-2 sites- SGSD data subsets
- Sample subset pedigrees
- Winter Cereal Collection background info.

Puroindoline Genotype Study

- Data Tables- pin genotypes & flour yield & milling quality index
- Genotype survey tables & plate layouts
- Pin genotyping preliminary report
- Puroindoline Genotype groups
- 3 Pin genotypes- flour averages

Starch Bound Puroindoline Quantification

- Examples Protein80 chip outputs (2 sites)
Combined References


American Association of Cereal Chemists, St. Paul, MN.


Bekes F & Wrigley CW. (1999). Prediction of the dough properties of blended flours based on variety mix. CSIRO Plant Industry & Quality Wheat CRC Ltd, North Ryde, NSW


Berry PM, Spink JH, Foulkes MJ & Wade A. (2003). Quantifying the contributions and losses of dry matter from non-surviving shoots in four cultivars of winter wheat. Field Crops Research 80, 111-121.


Stoddard FL. (1999b). Variation in grain mass, grain nitrogen, and starch B-granule content within wheat heads. *Cereal Chemistry* 76, 139-144.


Wanjugi HW, Hogg AC, Martin JM & Giroux MJ. (2007a). The role of puroindoline A and B individually and in combination on grain hardness and starch association. Crop Science 47, 67-76.


