Genotyping South African wheat germplasm for hardness alleles

by

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Dissertation presented for the degree of
Doctor of Philosophy (Food Science) in the
Faculty of AgriSciences at
Stellenbosch University

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April 2019

Declaration

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April 2019

Abstract

Wheat grain kernel hardness (GKH) is one of the most important quality properties of wheat (*Triticum aestivum*). The molecular basis of GKH is determined by the combination of *Puroindoline a* (*Pina-D1*) and *b* (*Pinb-D1*) alleles in a wheat cultivar. The current study investigated the *Pin* alleles present in commercial South African (SA) wheat cultivars. Wheat production regions in South Africa are diverse; and divided into the summer rainfall irrigation (SRI) and winter rainfall dryland (WRD) regions where spring wheat is planted, as well as the summer rainfall dryland (SRD) region where facultative and winter wheat are planted. Nine commercial wheat cultivars, differing in GKH, were planted at four locations per region, with three replications, over three production seasons (2012 – 2014). After each season, the wheat grain was harvested followed by determination of kernel characteristic, milling yield, flour and dough quality properties.

The *Pin* allele identities, of the 27 cultivars, were determined using polymerase chain reaction and allele sequencing. Four *Pin* allelic genotypes were identified. Wheat cultivars produced in the WRD region showed no diversity in *Pin* genotypes. GKH prediction models, based on the *Pin* allele identities of the samples, were thus developed for only the SRI and SRD production regions.

Following analysis of variance (ANOVA) and Pearson's correlations of the WRD region, where the cultivars had identical *Pin* genotypes, it was shown that genotype (G) primarily contributed to variation in GKH in the Swartland region. GKH correlated negatively with break flour yield (BFY), total flour yield (TFY) and α-amylase activity. Environment (E) primarily contributed to variation in GKH in the Rûens region, where GKH had negative correlations with BFY and TFY. In addition, negative GKH correlations were observed with kernel weight and diameter, and positive correlations with flour ash content, water absorption, dough strength, -stability, and -tenacity.

Wheat cultivars of the SRI and SRD regions were subjected to ANOVA, with cultivars nested within *Pin* genotypes. Wheat containing the *Pina-D1b*/*Pinb-D1a* genotype had increased GKH, flour water absorption (FWA), dough tenacity and alveograph P/L ratio; however, decreased kernel weight, diameter, BFY, TFY, dough extensibility, -strength, -stability, and tolerance to overmixing, compared to the *Pina-D1a*/*Pinb-D1b* genotype. The *Pinb-D1p* mutation had decreased kernel weight, diameter, dough extensibility, and swelling index; with increased BFY and TFY, FWA, dough development time, -strength and -tenacity compared to the *Pinb-D1b* and *Pinb-D1ab* mutations.

The molecular weight distribution of proteins within wheat cultivars of different *Pin* genotypes were determined with size exclusion high performance liquid chromatography. ANOVA with nested design and Pearson's correlations showed environmental influence, and G x E interaction, primarily contributed to the variation in all protein fractions. The *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes showed decreased sodium dodecyl sulphate (SDS) -soluble monomeric protein with increased kernel hardness. In contrast the *Pina-D1a*/*Pinb-D1b* genotype showed decreased SDS-soluble polymeric protein and increased SDS-insoluble monomeric protein.

This study contributed valuable knowledge on the *Pin* alleles present in SA wheat cultivars as well as the influence of *Pin* genotype combinations and *Pinb-D1* allele mutations on the GKH and processing quality. The influence of *Pin* genotype and GKH on the molecular weight distribution of proteins were also demonstrated. This will enable SA wheat breeders to select specific *Pin* allele combinations to more rapidly breed wheat for specific end-use purposes.

Uittreksel

Koring graankorrelhardheid (GKH) is een van die belangrikste eienskappe van koring (*Triticum aestivum*). Die molekulêre basis van GKH word bepaal deur die kombinasie van *Puroïndolien a* (*Pina-D1*) en *b* (*Pinb-D1*) allele teenwoordig in 'n koringkultivar. Die huidige studie het die *Pin*-alleel identiteit in kommersiële Suid-Afrikaanse (SA) koringkultivars ondersoek. Koringproduksie-streke in SA is uiteenlopend en word verdeel in die somerreënval besproeiing (SRI) en winterreënval droëland (WRD) streke waar lente koring geplant word, sowel as die somerreënval droëland (SRD) streek waar fakultatiewe- en winterkoring geplant word. Nege kommersiële koringkultivars, wat verskil in GKH, is op vier lokaliteite per streek geplant, met drie herhalings, oor drie produksieseisoene (2012 – 2014). Na elke seisoen is die koringkorrels ge-oes, gevolg deur die bepaling van korreleienskappe, maal opbrengste, meel- en deegkwaliteit eienskappe.

Die *Pin*-alleel identiteite, van die 27 kultivars, is bepaal deur gebruik te maak van polimerase kettingreaksie en alleel nukleotied volgordebepaling. Vier *Pin*-alleliese genotipes is geïdentifiseer. Koringkultivars wat in die WRD-streek geproduseer is, het geen verskil in *Pin*-genotipes gehad nie. GKH voorspellingsmodelle, gebaseer op die *Pin*-alleel identiteite van die monsters, is dus vir slegs die SRI en SRD produksie streke ontwikkel.

Na die analise van variansie (ANOVA) en Pearson se korrelasies van die WRD-streek, waar die kultivars identiese *Pin*-genotipes gehad het, is getoon dat genotipe (G) hoofsaaklik bygedra het tot die variasie in GKH in die Swartland-streek. GKH korreleer negatief met breek-meelopbrengs (BMO), totale meelopbrengs (TMO) en α-amilase aktiwiteit. Omgewing (E) het hoofsaaklik bygedra tot die variasie in GKH in die Rûens-streek, waar GKH negatiewe korrelasies met BMO en TMO gehad het. Daarbenewens is negatiewe GKH-korrelasies waargeneem met korrelgewig en -deursnee, en positiewe GKH-korrelasies met meel asinhoud, -waterabsorpsie (MWA), deegsterkte, -stabiliteit en -elastisiteit.

Koringkultivars van die SRI- en SRD-streke is onderwerp aan ANOVA, met kultivar gene binne *Pin*-genotipes. Koring wat die *Pina-D1b*/*Pinb-D1a* genotipe bevat, het in GKH, MWA, deegsterkte en alveograaf P/L-verhouding verhoog. Dit het egter verminderde korrelgewig, -deursnee, BMO, TMO, deegrekbaarheid, -sterkte, -stabiliteit en verdraagsaamheid teenoor oormeng getoon, in vergelyking met die *Pina-D1a*/*Pinb-D1b* genotipe. Die *Pinb-D1p* mutasie het verminderde korrelgewig, -deursnee, deegrekbaarheid en -swellingsindeks gehad; met verhoogde BMO en TMO, MWA, deegontwikkelingstyd, -sterkte en -elastisiteit in vergelyking met die *Pinb-D1b*- en *Pinb-D1ab*-mutasies.

Die molekulêre gewigsverdeling van proteïene binne koringkultivars van verskillende *Pin*-genotipes is bepaal met grootte-uitsluiting hoë-verrigting vloeistofchromatografie. ANOVA met ‘n geneste ontwerp en Pearson se korrelasies het omgewingsinvloed getoon, en G x E-interaksie het hoofsaaklik bygedra tot die variasie in alle proteïen fraksies. Die *Pina-D1b*/*Pinb-D1a* en *Pina-D1a*/*Pinb-D1b* genotipes het afname in natriumdodesielsulfaat (NDS) oplosbare monomeer

proteïene met verhoogde korrel hardheid getoon. In teenstelling hiermee, het die *Pina-D1a*/*Pinb-D1b* genotipe afgename in NDS-oplosbare polimeriese proteïene en toename in SDS-onoplosbare monomeer proteïene getoon.

Hierdie studie het bygedra tot waardevolle kennis oor die *Pin*-allele teenwoordig in SA koringkultivars, asook die invloed van *Pin*-genotipe-kombinasies en *Pinb-D1*-alleelmutasies op die GKH en verwerkingskwaliteit van koring. Die invloed van *Pin*-genotipe en GKH op die molekulêre gewigsverdeling van proteïene is ook gedemonstreer. Dit sal SA koringtelers in staat stel om spesifieke *Pin*-alleelkombinasies te selekteer om koring vir spesifieke eindgebruiksdoeleindes te kweek.

Acknowledgements

I would like to express my sincere gratitude to the following people and institutions for their professional contributions to my studies:

- My supervisor, Prof Marena Manley for her supervision, advice and guidance to make the completion of this study possible;
- My co-supervisor, Dr Glen Fox for his advice and reviewing of my work;
- Staff from Sensako (Pty) Ltd. (Bethlehem and Napier, South Africa) for their assistance in planting trails and conducting wheat quality analysis. To the staff at Bethlehem for their continued motivation, support and confidence in my abilities throughout this study;
- Dr Mardé Booyse (ARC Biometry Department, Stellenbosch, South Africa) for her advice, time and guidance with the statistical analysis of the data;
- Dr Barend Wentzel (ARC Small Grains Institute, Bethlehem, South Africa), for his contribution to my research, his assistance, guidance and reviewing of my work;
- Dr Renée Prins and staff at Cengen (Pty) Ltd. (Worcester, South Africa), for their assistance in identifying the *puroindoline* allele identity of the wheat samples, and guidance with the interpretation thereof;
- The sequencing unit of the Central Analytical Facility (Stellenbosch University, South Africa) for their assistance in identifying the *puroindoline* allele identity of the wheat samples;
- The Winter Cereal Trust for project funding and a study bursary to make this research possible;
- My father, mother, sister and friends for their continued interest, support and encouragement to complete this study;
- My daughters, Elizna and Roné, for all their patience and love;
- My wonderful husband, Stephan de Groot, who's assistance made the completion of this study possible. I am grateful for his interest in my research and continued motivation to persevere and complete this study.

Table of contents

This dissertation is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one or more research chapters (papers prepared for publication) and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter and culminating with a chapter for elaborating a general discussion and conclusion. The language, style and referencing format used are in accordance with the requirements of the *International Journal of Food Science and Technology*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

List of figures

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List of abbreviations used

ANOVA……Analysis of variance

A. ……Aegilops

AACC……..American Association for Cereal Chemists

BFY……Break flour yield

Bp……Base pair

C……Cultivar

cDNA……complementary deoxyribonucleic acid

CIMMYT……International Maize and Wheat Improvement Centre

CTAB……Cetyltrimethyl ammonium bromide

DNA……Deoxyribonucleic acid

E……Environment

ExAG……SDS-soluble albumin and globulin proteins

ExMP……SDS-soluble monomeric protein

ExPP……SDS-soluble polymeric protein

Fash……Flour ash content

Fmoist……Flour moisture content

FN……Falling number

Fprot……Flour protein content

FPT……Farinograph optimum development time

Fstab……Farinograph dough stability

Ftol……Farinograph tolerance to overmixing

FWA……Flour water absorption

FWG……Flour wet gluten content

G……Alveograph dough swelling index

G x E……Genotype by environment interaction

gDNA……Genomic DNA

GKH……Grain kernel hardness

Gprot……Grain protein content at 12% moisture basis

GSP……Grain softness protein expressed by alleles of *Gsp-1*

Gsp-1……*Grain softness protein* gene

Ha……Hardness locus

HMW-GS……High molecular weight glutenin subunits

IWGSC……International Wheat Genome Sequencing Consortium

kDa……Kilo Dalton

L……Alveograph dough extensibility

LMW-GS……Low molecular weight glutenin subunits

LSD……Least significant difference

MAS……Marker assisted selection

MLR……Multiple linear regression

MMT……Mixograph mixing time

MPH……Mixograph peak height

Mr……Molecular weight

MTH……Mixograph tail height

MTW……Mixograph tail width

NCBI……National Centre for Biotechnology Information

P……Alveograph dough tenacity/elasticity

P/L……Ratio of elasticity/extensibility

PC……Principal component

PCA……Principal component analysis

PCR……Polymerase chain reaction

PG……*Puroindoline* genotype

PIN……Puroindoline protein expressed by *Pin* genes

Pina……*Puroindoline a* gene, *Pina-D1*

PINA……Puroindoline-a protein expressed by alleles of *Pina-D1*

Pinb……*Puroindoline b* gene, *Pinb-D1*

PINB……Puroindoline-b protein expressed by alleles of *Pinb-D1*

QTL……Quantitative trait loci

RCBD……Randomised complete block design

RP-HPLC……Reverse-phase high-performance liquid chromatography

S……Alveograph dough strength

SD……Standard deviation

SDS……Sodium dodecyl sulphate

SDS-PAGE….Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SE-HPLC……Size-exclusion high-performance liquid chromatography

SKCS……Single kernel characterisation system

SKCS-HI……Single kernel characterisation system hardness index

SNP……Single nucleotide polymorphism

SRD……Summer rainfall dryland

SRI……Summer rainfall irrigation

T. ……*Triticum*

TFY……Total flour yield

TRD……Tryptophan rich domain

UAG……SDS-insoluble albumin and globulin proteins

UMP……SDS-insoluble monomeric protein

UPP……SDS-insoluble polymeric protein

WRD……Winter rainfall dryland

CHAPTER 1
General introduction

Wheat (*Triticum aestivum*) is cultivated worldwide as one of the most important staple foods, as it supplies high nutritional value to consumers, and it can be processed to produce a variety of food products (Wrigley, 2009). One of the most essential characteristics of wheat, for grading and trading purposes, is grain kernel hardness (GKH) which is a fundamental property that influences both milling and baking quality. Grain traders classify wheat into different classes, based on hard or soft endosperm texture, spring or winter growth habit, and red or white pericarp appearance (Orth & Shellenberger, 1988; Paulsen & Shroyer, 2004). Different wheat species, cultivars and hardness classes are used as primary ingredients for specific end products (Mahesh *et al.*, 2008) with hard bread wheat used primarily for leavened bread, and soft bread or white biscuit wheat used for cakes and biscuits. Some applications may have more specific criteria relating to wheat properties and the quality of flour or different end uses (O'Brien & DePauw, 2004).

It has been established that GKH is controlled by the hardness, *Ha*, locus located on chromosome 5DS (Mattern *et al.*, 1973; Baker & Dyck, 1975; Law *et al.*, 1978) which produces the 'non-stick' protein, i.e. 'friabilin' (Greenwell & Schofield, 1986; 1989). Friabilin influences the bond of protein and starch in the wheat endosperm and consist of puroindoline a (PINA), puroindoline b (PINB) and grain softness proteins; which are expressed by the *Ha* locus consisting of genes *Pina-D1*, *Pinb-D1* and *Gsp-1* (Blochet *et al.*, 1991; Gautier *et al.*, 1994; Rahman *et al.*, 1994). PINA and PINB proteins occur in different forms in the wheat endosperm depending on the *Pina-D1* and *Pinb-D1* alleles present in the wheat cultivar or breeding line. The respective wild-type alleles of *Pina-D1* and *Pinb-D1*, i.e. *Pina-D1a* and *Pinb-D1a*, cause soft grain kernels. If an alteration in the *Pina-D1* or *Pinb-D1* alleles exists, due to mutations or deletions in their deoxyribonucleic acid (DNA) sequence, hard wheat endosperm will be formed. A difference in the DNA sequence of alleles causes the expression of a PINA or PINB protein with a different tertiary structure and functional quality compared to the wild-type PIN proteins (Giroux & Morris, 1998; Lillemo & Morris, 2000; Morris *et al.*, 2001). In most geographic wheat production areas, bread wheat cultivars predominantly have the *Pina-D1a/Pinb-D1b* allelic genotype combination (Chen *et al.*, 2011; Chen, Li & Cui, 2013; Ma *et al.*, 2017).

Different combinations of *Pina-D1* and *Pinb-D1* alleles in wheat cultivars result in different levels of kernel hardness and thus different grain qualities (Giroux *et al.*, 2000; Martin *et al.*, 2001; Nagamine *et al.*, 2003; Cane *et al.*, 2004; Ikeda *et al.*, 2005; Eagles *et al.*, 2006). Research has been conducted globally to gain knowledge on the different *Pin* alleles present in *Triticum* and *Aegilops*, and their geographic distribution in wheat-producing countries. Countries where research regarding *Pin* allele identity has been conducted include China (Pan *et al.*, 2004; Chen *et al.*, 2005; 2006; 2007; 2012; Chen, Li & Li *et al.*, 2013; Xia *et al.*, 2005; Chang *et al.*, 2006; Wang, Li *et al.*, 2008; Wang,

Sun *et al.*, 2008), India (Kumar *et al.*, 2015), Japan (Tanaka *et al.*, 2008; Ikeda *et al.*, 2010), Korea (Park *et al.*, 2009), the International Maize and Wheat Improvement Centre in Mexico (Lillemo *et al.*, 2006; Chen, Li & Cui, 2013; Kumar *et al.*, 2015) and Southern Spain (Ayala *et al.*, 2016). To date, 25 *Pina-D1* and 35 *Pinb-D1* alleles have been identified by researchers globally. The discovery of new *Pin* alleles provides knowledge on wheat hardness, and quality characteristics associated with the different *Pin* alleles. One of the countries where no research on the *Pin* allelic diversity of adapted wheat cultivars has been conducted, is South Africa. Knowledge on the *Pin* alleles present in commercial wheat cultivars bred for South African environmental conditions, and which complies with the processing requirements of the South African wheat industry, would enable wheat breeders to more rapidly and efficiently produce cultivars with specific end-use purposes.

Kernel hardness has a definite effect on the milling performance of wheat (Wang, Li *et al.*, 2008), with harder grain kernels having better total flour yield (TFY), due to more efficient endosperm reduction (Oury *et al.*, 2017). Kernel hardness has been positively correlated with flour ash content and starch damage (Garland-Campbell *et al.*, 2001; Brites *et al.*, 2008; Ma *et al.*, 2009; Choy *et al.*, 2015). Increased starch damage is known to increase the flour water absorption (Morrison & Tester, 1994; Brites *et al.*, 2008; Li *et al.*, 2014; Liu *et al.*, 2014). Wheat grain protein content has been positively correlated with grain kernel hardness (Giroux *et al.*, 2000; Igrejas *et al.*, 2001; Martin *et al.*, 2001; Igrejas *et al.*, 2002). However, in wheat breeding programmes, wheat cultivars are selected for the end-use properties required, i.e. bread or biscuit making. Hard wheat for bread making typically has a protein content of 10 to 14%, while soft biscuit wheat typically contains 8 to 10% protein (Pauly *et al.*, 2013).

Grain kernel hardness is genetically determined by the *Pin* genotype of a cultivar or breeding line; however, the environment also influences GKH by producing vitreous or mealy grain kernels. Vitreousness is related to the packing of the starch granules and protein components in the grain endosperm, and these kernels are typically high in grain protein content (Oury *et al.*, 2015). High temperature (Bhullar & Jenner, 1985; Macleod & Duffus, 1988; Bechtel *et al.*, 1990; Blumenthal *et al.*, 1990; Tester & Karkalas, 2001; Park *et al.*, 2009) and water stress conditions (Brooks *et al.*, 1982; Kobata *et al.*, 1992; Altenbach *et al.*, 2003) alter the grain filling period of wheat kernels by decreasing the duration of grain fill and reducing starch accumulation in the endosperm. These environmental effects decrease kernel weight considerably, and results in vitreous kernels. A cultivar that is genetically hard may be affected by environmental conditions to vary in kernel hardness amongst growth environments, but never to the degree of becoming soft (Pomeranz & Williams, 1990).

Since the basis of wheat hardness results from puroindoline proteins, specific *Pin* alleles can be linked to the physical and rheological properties of wheat differing in hardness. The combination of *Pina-D1* and *Pinb-D1* alleles in a wheat cultivar or breeding line influences wheat GKH, and the wheat milling and processing properties associated with it. Various studies reported that wheat, with the *Pina-D1b/Pinb-D1a* genotype, displayed increased grain kernel hardness (Martin *et al.*, 2001;

Cane *et al.*, 2004; Eagles *et al.*, 2006) compared to wheat with the *Pina-D1a/Pinb-D1b* genotype. Chen *et al.* (2007) reported that wheat cultivars with the *Pina* null mutation (*Pina-D1b*) had poor milling quality and sub-standard processing quality compared to wheat cultivars with a *Pinb* null mutation. Wheat grain with both *Pina-D1* and *Pinb-D1* wild-type alleles, the genotype *Pina-D1a*/*Pinb-D1a* and soft grain endosperm, produce flour with reduced maximum dough resistance, dough development time and flour water absorption (FWA) in comparison to hard wheat grain with an allelic mutation in either *Pina-D1* or *Pinb-D1* genes (Cane *et al.*, 2004; Eagles *et al.*, 2006).

Wheat with increased hardness was shown to have increased TFY and loaf volume (Baker & Dyck, 1975), mixograph peak height (MPH), FWA, loaf volume (Baker & Dyck, 1975; Chen, Li & Li *et al.*, 2013), alveograph tenacity and P/L ratio; and a negative correlation with amylase activity and starch gelling (Chen, Li & Li *et al.*, 2013). Martin *et al.* (2001) reported mutations and deletions in *Pina-D1* and *Pinb-D1* alleles, to have a definite effect on properties involving particle size and milling quality, i.e. kernel hardness, TFY, break flour yield (BFY), milling score and flour ash content.

Variation in the molecular weight distribution of grain proteins is considered an essential factor affecting wheat GKH (Huebner & Gaines, 1992; Ohm & Chung, 1999; Giroux *et al.*, 2000; Ohm *et al.*, 2006). Puroindoline proteins have a mean molecular weight of 12.8 kDa (Blochet *et al.*, 1993) and form part of the 2S albumin proteins in the prolamin superfamily of proteins (Shewry *et al.*, 2002). Changes in the molecular weight distribution of protein fractions have been associated with increased grain kernel protein content. Although the protein quality characteristics are genetically determined, environmental factors strongly influence protein content (Cornish *et al.*, 2006; Vázquez *et al.*, 2012). Saint Pierre *et al.* (2008) found that both polymeric protein (PP) and monomeric protein (MP) contents increase with increased grain protein content, however, MP (gliadins) increase more rapidly than the PP (glutenins) as measured with size exclusion high performance liquid chromatography (SE-HPLC) (Triboï *et al.*, 2000). The albumin and globulin (AG) proteins respond the least to changes in the total grain protein content (Saint Pierre *et al.*, 2008). Kernel hardness primarily affects sodium dodecyl sulphate (SDS) extractable ω-gliadin (ExMP) as well as SDS-extractable albumin and globulin (ExAG) fractions (Ohm *et al.*, 2010). Grain kernel hardness has been positively correlated with SDS-extractable polymeric proteins (ExPP) (Katyal *et al.*, 2017), ExMP (Ohm *et al.*, 2010) and SDS-unextractable polymeric proteins (UPP) (Gupta *et al.*, 1993; Malik *et al.*, 2011; Katyal *et al.*, 2017); while negatively correlated with ExAG (Ohm *et al.*, 2010), ExMP (Katyal *et al.*, 2017), SDS-unextractable monomeric proteins (UMP) (Gupta *et al.*, 1993; Malik *et al.*, 2011; Katyal *et al.*, 2017) and SDS-unextractable albumin and globulin (UAG) (Ohm *et al.*, 2010).

The aim of this study was to genotype a number of South African wheat cultivars, selected based on differences in wheat grain hardness, for *puroindoline* alleles.

Specific objectives were to:

- determine the *Pin* allele identity of the selected cultivars;
- develop a model to predict grain kernel hardness based on the identified *Pina-D1* and *Pinb-D1* alleles present;

- investigate the effect of the *Pina-D1* null allele (*Pina-D1b*) expression compared to the *Pinb-D1b* mutation on grain-, milling- and flour quality properties in three wheat production areas;
- investigate the effect of different mutations at the *Pinb-D1* locus on grain-, milling- and flour quality properties;
- determine the effect of genotype (G), environment (E) and G x E interaction on the variation in grain kernel hardness in three wheat production areas;
- and evaluate the variation in grain kernel hardness of various cultivars with identical *Pin* allelic genotype;
- determine the correlation of grain kernel hardness with milling and flour properties; and
- investigate the influence of grain kernel hardness on the molecular weight distribution of protein within *Pin* genotypes.

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CHAPTER 2

Wheat (*Triticum aestivum*) grain hardness and the influence of puroindolines on milling and flour quality: a review

2.1. Introduction

Wheat is regarded as one of the world's most popular crops since it is easily adaptable agronomically, can effectively be stored as a food source, supplies high nutritional value, and can produce a variety of food products (Wrigley, 2009). Wheat provides more nutrition to humans than any other grain species (Paulsen & Shroyer, 2004). For these reasons, wheat is cultivated worldwide as one of the most important staple foods with the latest global production measured at 772 million tons for 2017 (FAOSTAT, 2018). Grain grading and trading worldwide are based on the quality of the wheat, that can be divided into three groups, namely botanical, physical and chemical characteristics. The botanical characteristics include the species, cultivar and growth type (spring or winter), while physical quality refers to kernel properties, such as hectolitre mass, hardness, weight, size and colour of the kernel. The chemical characteristics of importance include wheat moisture, protein and α-amylase content (Lusse, 2016).

Different wheat species and cultivars are used as primary ingredients for specific end products (Mahesh *et al.*, 2008). Bread wheat (*Triticum aestivum*) and durum wheat (*Triticum turgidum* ssp. durum) are characterised by different chemical and physical properties, which will affect the end product produced (Kent & Evers, 1994a; O'Brien & DePauw, 2004). Based on these different properties, wheat will differ in functional quality, nutritional contribution and, consequently, commercial value (Bietz, 1989). Bread wheat species comprise different classes with hard and soft endosperm texture, spring or winter growth habit, and red or white pericarp appearance (Orth & Shellenberger, 1988; Paulsen & Shroyer, 2004). Hard bread wheat is used primarily for leavened bread but is also suitably used for flat and steamed bread as well as noodles. Soft bread wheat, on the other hand, is used for cakes, pastries, biscuits and crackers. Some markets have more specific criteria relating to dough properties and the end-use quality of wheat (O'Brien & DePauw, 2004). Durum wheat grain is extremely hard, and is primarily used for the production of pasta and couscous (Paulsen & Shroyer, 2004).

Earlier reviews on wheat hardness addressed the discovery of puroindolines (Morris, 2002; Pauly *et al.*, 2013a), biochemical properties of puroindoline proteins (PIN proteins) (Bhave & Morris, 2008a), interaction of puroindolines and polar lipids (Pauly *et al.*, 2013a), implications of puroindolines for end product quality (Pauly *et al.*, 2013b), *puroindoline*-related genes, and expression of puroindoline in wheat and other cereals (Bhave & Morris, 2008b). The *puroindoline* (*Pin*) allele designations until 2007 are available in Morris and Bhave (2008).

This review aimed to examine the evolution of wheat, grain hardness and the discovery of friabilin and puroindolines. A complete list of discovered *puroindoline a* (*Pina-D1*) and *b* (*Pinb-D1*) alleles (until and including 2018) is provided (see Table 2.1) with specific reference to the allele designation and the genetic mutation differentiating it from the wild-type alleles. The influence of *Pina-D1* and *Pinb-D1* alleles and kernel hardness on wheat grain quality and processing is reviewed. The environmental influence on wheat grain kernel hardness (GKH) concludes the review.

2.2. Evolution of wheat

The origin of wheat is said to have occurred 2.5 to 6 million years ago in the Middle Eastern region (Wrigley, 2009), also referred to as the 'fertile crescent' (Encyclopedia Britannica, 2016). Today, this region comprises Israel, Jordan, Lebanon, western Syria, south-east Turkey, Iraq and the western borders of Iran (Zohary & Hopf, 2000). The tetraploid *T. turgidum* ssp. *durum* (AABB) was derived in the fertile crescent through the natural hybridisation of the diploids *T. urartu* (AA) and *Aegilops speltoides* (BB) (Orth & Shellenberger, 1988; O'Brien & DePauw, 2004; Chantret, 2005; Wrigley, 2009). During this polyploidisation process, the hardness (*Ha*) locus responsible for GKH was deleted from the A and B genomes of *T. turgidum* ssp. *durum* (Gautier *et al.*, 2000; Chantret, 2005). A subsequent polyploidisation event 7 000 to 9 500 years ago occurred between the tetraploid *T. turgidum* ssp. *dicoccum* (AABB) and the diploid *A. tauschii* (DD), which led to the origin of bread wheat (*T. aestivum*, AABBDD). During this second polyploidisation event, the *Ha* locus in bread wheat was restored from *A. tauschii* (Orth & Shellenberger, 1988; O'Brien & DePauw, 2004; Chantret, 2005; Wrigley, 2009; Morris *et al.*, 2011). The hybridisation event of *T. turgidum* ssp. *dicoccum* and diploid *A. tauschii* led to a definite influence on the processing properties of durum and bread wheat (Morris *et al.*, 2011), and narrow genetic diversity for hexaploid wheat. The wild relative of modern cultivated hexaploid wheat, namely *A. tauschii,* has remained mostly unchanged and can be viewed as an extension of the wheat gene pool (Pflüger *et al.*, 2001). This allows the introgression of new genes into the genepool of cultivated hexaploid wheat. These new genes could provide novel disease or pest resistance, but also valuable agronomic or quality traits (Eastwood *et al.*, 1991; Peña *et al.*, 1995; Pflüger *et al.*, 2001; Villareal *et al.*, 2001).

Today, two main species of wheat are grown around the world, namely common bread wheat (*T. aestivum*) and durum or pasta wheat (*T. turgidum* ssp. *durum*). Bread wheat occupies around 90% of the world's wheat cultivation area, and durum wheat accounts for around 8% of world wheat production (Paulsen & Shroyer, 2004).

2.3. Wheat grain hardness

Kernel hardness (or softness) is a milling characteristic of wheat, which relates to the way the wheat endosperm breaks down during milling (Kent & Evers, 1994b). The wheat kernel comprises the embryo and endosperm, enclosed by the nucellar epidermis, and a seed coat (bran) that envelops the kernel. The endosperm consists of cells that are filled with starch granules embedded in a

continuous protein matrix (Barlow *et al.*, 1973; Bradbury *et al.*, 1956). Starch is the major component of wheat endosperm as it makes up around 75% of the milled endosperm (Grundas & Wrigley, 2004; Delcour & Hoseney, 2010).

Wheat hardness can be defined as the measure of resistance to deformation of the wheat kernel (Turnbull & Rahman, 2002). It is a key criterion for wheat classification and global trading, as it determines the milling performance and ultimately the end-use quality of the wheat (Wang, Li *et al.*, 2008).

2.3.1. Influence of wheat hardness on grain-, milling- and processing quality

Wheat milling is performed to reduce the starchy endosperm of grain kernels to smaller particle sizes that can be used to produce various food products. Soft wheat is more friable than hard wheat, it requires less energy during milling, and produces flour with smaller particle size distribution and many intact starch granules compared to hard wheat. Hard wheat, on the other hand, requires more energy during milling, produces coarser flour than soft wheat, and has a higher amount of broken starch kernels and thus more starch damage (Devaux *et al.*, 1998).

During grain milling, the first point of fracture in the endosperm of soft wheat would occur through the cell contents due to the weaker binding of the starch-protein matrix, and this produces more intact starch granules than hard wheat kernels. In hard wheat, on the other hand, the first fracture would occur primarily at the endosperm cell walls due to the stronger binding of the starch-protein matrix. The subsequent reduction of endosperm particles to flour would occur through the cell contents and cause more broken starch granules than soft wheat kernels. This serves as evidence that the cell content is more tightly bound in hard wheat, resulting in a relative point of weakness through the starch granule (Grundas & Wrigley, 2004; Delcour & Hoseney, 2010).

Some researchers found that total flour yield is positively correlated with GKH (Martin *et al.*, 2001; Hogg *et al.*, 2005), while others showed the opposite (Kammeraad *et al.*, 2016; Martin *et al.*, 2017). Nonetheless, it is generally agreed that break flour yield is higher in soft grains (Brites *et al.*, 2008; Delcour & Hoseney, 2010). Although the total flour yield might not be affected by GKH, the average flour particle size is positively correlated with GKH, with hard kernels providing flour with larger flour particles compared to soft kernels. Grain with a harder endosperm texture results in a higher ash content, and more starch damage in the flour compared to wheat with soft endosperm (Brites *et al.*, 2008). The higher damaged starch in hard wheat increases flour water absorption, gas production during dough fermentation, and loaf volume; thus, it is an essential characteristic in bread-making (Pomeranz *et al.*, 1984; Martin *et al.*, 2001; Takata *et al.*, 2010).

Although grain protein content has been linked directly to GKH (Giroux *et al.*, 2000; Igrejas *et al.*, 2001; Martin *et al.*, 2001; Igrejas, Leroy *et al.*, 2002), soft and hard wheat cultivars are typically selected in wheat breeding based on their protein content and flour water absorption. Hard wheat cultivars generally yield flour with 10 to 14% protein, while soft wheat cultivars yield 8 to 10% protein (Pauly *et al.*, 2013b).

2.3.2. Environmental influence on grain hardness

Wheat is one of the most widely adapted agricultural crops, surviving temperature extremes from -35°C in the vegetative phase (Haji & Hunt, 1999) to 40°C during grain filling (Elahmadi, 1994). Wheat is cultivated over a wide range of latitudes and altitudes globally. The crop can survive drought stress while still producing a yield, and it can thrive in optimal conditions with record yields (Reynolds *et al.*, 2002). This adaptability reflects the tremendous genetic diversity that is contained in the wheat genome.

Bread wheat (*T. aestivum*) has the largest genome among commonly grown agricultural crops and is regarded in plant breeding as a highly complex crop due to its large genome size. The wheat genome (16 000 million deoxyribonucleic acid [DNA] nucleotide base pairs) is estimated to be five times the size of the human genome (3 000 million DNA nucleotide base pairs) (Colorado Wheat, 2013; Appels *et al.*, 2018). *Triticum aestivum* contains three sub-genomes (A, B and D) with seven chromosomes each. The mapping of the wheat genome has recently been completed by researchers of the International Wheat Genome Sequencing Consortium (IWGSC). The IWGSC was established in 2005 by a group of plant scientists, plant breeders and wheat growers. The goal was to lay a basis for research, to enable breeders to develop improved wheat varieties by making a high-quality genome sequence of *T. aestivum* publicly available (Appels *et al.*, 2018). With this sequence information available, wheat breeders will be able to more rapidly identify the fundamental genes and other regulatory elements that influence genetically complex traits such as yield, grain end-use quality and disease resistance in the breeding process. The breeding process will be improved, with greater speed and efficiency from a genetic viewpoint, while still successfully breeding for important traits. Which include stable and increased yield, and adapting the wheat line to specific biotic and abiotic stress factors (Appels *et al.*, 2018). Due to the great diversity within the wheat genome, the interaction between different wheat genotypes and environmental conditions is often highly significant.

The growth environment, i.e. temperature, daylight length, plant date, soil type, rain or irrigation of wheat has a considerable effect on its quality properties, such as GKH. The environmental effect on wheat grain can be moderate to extreme, depending on the growth conditions to which the wheat line or cultivar is exposed, and also the growth stage of the plant in which the exposure happens (Pomeranz *et al.*, 1985; Bushuk, 1998). Although the environment affects GKH, it does not affect the ranking of cultivars when compared at different locations (Hazen & Ward, 1997) as all cultivars are affected equally. A cultivar that is genetically hard may thus vary in GKH amongst growth environments but never to the degree of becoming soft (Pomeranz & Williams, 1990).

The vitreous or floury (mealy) appearance of grains are influenced by the bonding of all constituents in the endosperm of the kernel, and not by the genetic aspects of GKH. Wheat kernels can thus be vitreous but perform as soft kernels under mechanical resistance, and vice versa, as vitreousness is not a true reflexion of genetic hardness properties. Vitreousness depends greatly on environmental factors. Environmental conditions, especially the availability of water and nitrogen and

the temperature during maturation of wheat kernels, influence the appearance (vitreous or floury) of the grain endosperm (Pomeranz & Williams, 1990; Greffeuille *et al.*, 2006; 2007; Delenne *et al.*, 2008).

Amongst wheat sister lines with the same *Pin* allelic genotype, variation for milling and quality traits have been observed (Martin *et al.*, 2001). This variation indicates that other factors, such as the environment, also influence GKH, milling, and baking quality. Surma *et al.* (2012) confirmed that genotype has a considerable influence on GKH, while protein content, wet gluten content and Zeleny sedimentation are influenced more by the environment than by genotype. Grain hectolitre mass, starch content and alveograph strength were equally influenced by genotype as well as the environment (Surma *et al.*, 2012). Igrejas *et al.* (2001) studied 40 wheat cultivars over four different locations for puroindoline a (PINA) and puroindoline b (PINB) protein content and other quality traits. PIN protein contents and loaf volume did not differ significantly between environments, whereas tenacity and extensibility, Zeleny sedimentation, PINB protein content and GKH showed high heritability (Igrejas *et al.*, 2001).

Milling fractions were shown to be highly influenced by genetic factors (G) in the form of *Pinb-D1* alleles, moderately by the environment (E) in the form of location, year and nitrogen fertilisation, with little or no G x E interaction (Oury *et al.*, 2017). In contrast, Hazen and Ward (1997) and Morris *et al.* (1999) found that genotype, environment and G x E interaction affected GKH significantly. Severe G x E interaction would change a cultivar's rankings for a trait, while a mild interaction might increase the magnitude of difference but not the ranking order within a trait (Hazen *et al.*, 1997). The ranking of cultivars, however, remained stable, indicating that a breeding line's hardness relative to others should remain stable over environments, and that a reliable quality standard could be used as a benchmark for GKH. Wheat breeding for increased milling performance could thus be successful, regardless of environmental influence. Genotype by environment interactions could, however, assist wheat breeders to recognise differences amongst wheat lines. Besides grain yield, disease resistance and agronomic adaptability, breeding for better milling and baking quality are one of the main objectives in any wheat breeding programme (Symes, 1965).

2.3.3. Other physical and biochemical factors of wheat grain that influence kernel hardness

It has been established that GKH is genetically controlled. However, other factors, such as kernel vitreousness, size and moisture content, and the wheat kernel endosperm lipid, -protein, -starch and -pentosan content, might also influence GKH (Anjum & Walker, 1991; Turnbull & Rahman, 2002).

Kernel vitreousness and colour have been studied for their relationship to wheat grain hardness (Konopka, Kozirok *et al.*, 2005); however, increased grain protein content is generally associated with an increased vitreous appearance of grain endosperm (Oury *et al.*, 2015; 2017). Difference in starch granule size composition of wheat endosperm, as influenced by environmental conditions during grain filling, has been studied for its effect on GKH (Bechtel *et al.*, 1990; Igrejas, Faucher *et al.*, 2002; Li, Yan *et al.*, 2008; Park *et al.*, 2009). High temperatures influence the grain fill of wheat

kernels by decreasing the duration of grain fill. This influences the amounts of protein and starch accumulation in the kernel endosperm, which influence the GKH (Brooks *et al.*, 1982; Kobata *et al.*, 1992; Altenbach *et al.*, 2003).

The relationship between wheat GKH and kernel lipid content is not yet clear; however, many studies have been done on free glycolipids and the polar and non-polar starch surface lipids in wheat endosperm (Morrison *et al.*, 1989; Panozzo *et al.*, 1993; Kooijman *et al.*, 1997; Konopka, Rotkiewicz *et al.*, 2005). The reader is referred to Pareyt *et al.* (2011) for more information on wheat lipids and their function.

Pentosan content, which includes soluble and insoluble pentosan fractions, plays a vital role in water absorption of wheat flour and its relationship to rheological properties of dough. Pentosans make up 2–3% of wheat flour, and it can absorb 6–10 times its weight in water on a dry basis (Jelaca & Hlynka, 1971). In general, GKH is positively correlated with pentosan content (Hong *et al.*, 1989; Bettge & Morris, 2000). Flour with high water absorption is preferred for bread making, which implies selecting wheat with thicker cell walls that contains high amounts of arabinoxylan and which can absorb more water than wheat with thinner cell walls. Soft wheat, on the other hand, is commonly used for cookies and cakes where selection is made for lower flour water absorption, and thus wheat with thinner cell walls and lower arabinoxylan levels is selected (Simmonds, 1974; Delcour & Hoseney, 2010).

The relationship between the named physical and biochemical factors and GKH can be attributed to wheat breeder's selection for desired quality traits that are linked to GKH. Additionally, GKH can also be attributed to environmental influence on the accumulation of chemical compounds in the wheat kernel, thereby affecting GKH. A full discussion of these factors is beyond the scope of this review, and the reader is referred to reviews by Turnbull & Rahman (2002), Pasha *et al.* (2010) and Pauly *et al.* (2013b) for more information on these factors.

2.4. Discovery of friabilins

Studies on a fundamental understanding of wheat hardness and its inheritance started in the late 1800s, with the first known reference to wheat hardness by Cobb (1896), followed by Biffen (1908). The interaction between ears per plant, grain yield, and GKH was investigated by Aamondt *et al.* (1935) in an attempt to understand the basis of GKH. Early work was hampered by the absence of a reliable test for GKH, and several researchers tried to develop a reliable method for determining wheat hardness in their search for the basis of GKH (Worzella & Cutler, 1939; Worzella, 1942; Beard & Poehlman, 1954; Millington & Remilton, 1954; Symes, 1961).

In 1965, Symes reported the difference between hard and soft wheat as due to a single major gene, and several minor genes that explain differences in hardness amongst different wheat cultivars or breeding lines. A few years later, it was established that hardness is controlled by alleles at a single locus on the short arm of chromosome 5D (Mattern *et al.*, 1973; Baker & Dyck, 1975; Law *et al.*, 1978). In the study by Law *et al.* (1978), the researchers determined the dominant allele for

softness, *Ha*, was present in Chinese Spring wheat, and the allele for hardness, *ha*, was present in the other hard wheat cultivars studied. These findings were confirmed in a similar study by Morris and colleagues in 1999 using homozygous recombinant substitution lines (Morris *et al.*, 1999).

Despite findings regarding the *Ha* locus in wheat, researchers remained curious regarding the biochemical component(s) responsible for differences in GKH. Barlow *et al.* (1973) studied the isolated starch and protein components from hard and soft wheat and observed no difference in the amounts present between different wheat hardness classes. They concluded that the mechanism controlling GKH resulted from the nature of the starch–protein interface, and not the amounts of starch or protein present in the grain. They subsequently discovered a water-soluble protein, situated at the starch–protein interface, and surrounding starch granules. This protein formed an electrophoretically complex group, but could not be identified. Simmonds (1974) was also unsuccessful in identifying the compound responsible for hardness using 4 M urea and 0.01 M potassium pyrophosphate extractions.

A unique family of proteins were identified in the early 1980s, which interact with the surface of water-washed starch granules (Lowy *et al.*, 1981; Gough *et al.* 1985; cited in Greenwell & Schofield, 1986: p.379). Greenwell and Schofield (1986) investigated this family of starch granule surface proteins further using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and discovered a prominent band at 15 kDa molecular weight (Mr) for soft wheat. This was only a very faint band for hard wheat, and it was completely absent in the case of durum wheat. They concluded that the gene coding for this protein at 15 kDa must be located on the same chromosome as the major gene controlling endosperm texture, which is situated on the short arm of chromosome 5D. This explained the absence of the 15 kDa protein in durum wheat since durum does not contain the D-genome. The positive association of the 15 kDa protein and endosperm softness was demonstrated in Greenwell and Schofield's research, although the mechanism by which the protein causes this effect, was still unknown. Since the protein was associated with the starch granule surface, the assumption was made that it possessed some 'non-stick' property, which reduced the adhesion between starch granules and the protein matrix (Greenwell & Schofield, 1986).

This 'non-stick' protein was later referred to as 'friabilin' (Greenwell & Schofield, 1989) or 'grain softness protein' (GSP) (Jolly, 1991 and Jolly *et al.* 1990; cited in Jolly *et al.*, 1993: p.590). The mechanism causing variation in kernel texture and the protein responsible for this was, however, much more complex than initially assumed. It was unclear how many polypeptides were included within the 15 kDa protein band that was observed on the SDS-PAGE gels.

Several authors researched the composition of friabilin to understand the cause of GKH. Different extraction methods of friabilin protein were investigated during the early 1990s (Jolly *et al.*, 1993; Gautier *et al.*, 1994; Rahman *et al.*, 1994; Gaines *et al.*, 1996). As the studies progressed and friabilin was studied in much greater detail, it became clear that friabilin was composed of more than one protein. Different researchers reported that friabilin consists of one protein (Jolly *et al.* 1990; cited in Morris, 2002: p.637; Greenblatt *et al.*, 1992; Rahman *et al.*, 1994), two proteins (Morris *et*

al., 1994; Oda & Schofield, 1997; Rahman *et al.,* 1991; cited in Morris, 2002: p.637), four proteins (Oda, 1994; Sulaiman *et al.*, 1993; cited in Morris, 2002: p.637) and even a mixture of proteins (Greenwell & Brock, 1993; Greenwell, 1992; cited in Morris, 2002: p.636-637).

Amino acid sequence data of the proteins contained in friabilin was reported in a number of studies (Jolly, 1991; Rahman *et al.*, 1991 and Greenwell, 1992; cited in Morris, 2002: p.637; Oda *et al.*, 1992; Jolly *et al.*, 1993; Morris *et al.*, 1994; Giroux & Morris, 1997a; Oda & Schofield, 1997). This brought a new perspective to the friabilin phenomenon and supported the research of several studies suggesting that friabilin exists as a combination of at least two major polypeptides; however, their identities were still unknown and needed further investigation.

2.5. Puroindolines

The most effective extraction of friabilin was discovered by Blochet *et al.* (1991) who used the detergent Triton X-114 (TX-114) to extract flour lipid-binding proteins from *T. aestivum* variety Capitole. The detergent, TX-114, was used due to its unique ability to form phase separations when heated above 25°C and its high efficacy of solubilising polar lipids and proteins (Bordier, 1981). A protein product of less than 20 kDa was further separated on reverse-phase high-performance liquid chromatography (RP-HPLC) into six major proteins, of which three were purothionins, and the remaining three (peak 5, 6 and 7) were further analysed. The proteins of peaks 5, 6, and 7 were subjected to N-terminal amino acid sequencing, and all three proteins were previously uncharacterised (Blochet *et al.*, 1991).

Upon further investigation, peaks 5 and 7 (Blochet *et al.*, 1991) showed high homology with sequencing data of friabilin from previous studies (Oda *et al.*, 1992; Jolly *et al.*, 1993; Morris *et al.*, 1994; Oda & Schofield, 1997). Researchers realised that friabilin and these TX-114-soluble proteins were matching well, and that they were indeed the same entity (Blochet *et al.*, 1991; 1993; Gautier *et al.*, 1994).

2.5.1. Discovery of puroindolines

Blochet *et al.* (1993) isolated the more abundant of the friabilin proteins (peak 5) through TX-114 phase partitioning from *T. aestivum* variety Camp Rémy, and performed amino acid sequencing. This revealed a new, basic, and cysteine-rich protein of approximately 13 kDa molecular weight and an isoelectric point higher than 10. This protein contains ten cysteine residues that are organised in a cysteine skeleton, with a unique tryptophan-rich domain (TRD). Considering the presence of this TRD, the new protein was named ‘puroindoline’; ‘puro’ referring to wheat in Greek, ‘*puros’*, and ‘indoline’ referring to the indole ring of tryptophan (Gautier *et al.*, 1994).

Gautier *et al.* (1994) isolated two cDNA (complementary deoxyribonucleic acid) clones from *T. aestivum* var. Capitole. The two clones were sequenced in its whole, and the data was compared to earlier work. The major protein corresponded to the amino acid sequence data of ‘peak 5’ reported by Blochet *et al.* (1993) and was named the ‘*puroindoline a* (*Pina*) allele’ and expressed PINA protein.

The minor component corresponded to the amino acid sequence data of 'peak 7' (Blochet *et al.*, 1991), and was named '*puroindoline b* (*Pinb*) allele' and expressed PINB protein. *Pina-D1* and *Pinb-D1* displayed a 60% homology in their allele DNA sequence; however, the TRD in PINA contained five tryptophan (Trp) amino acids (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys), and PINB only three (Trp-Pro-Thr-Trp-Trp-Lys) (Blochet *et al.*, 1991; Gautier *et al.*, 1994).

It has been revealed in subsequent molecular studies that mutations in puroindoline (*Pin*) allele sequences were present in all hard endosperm wheat. These mutations could be in the form of a deletion, resulting in the absence of PINA or PINB proteins, a single nucleotide mutation causing a modified amino acid sequence in PINA or PINB protein, or the null expression of the PINA or PINB proteins. Hard-textured grains of American and European wheat have been surveyed, and mutations, deletions or null expressions were observed (Giroux & Morris, 1997a; 1998; Lillemo & Morris, 2000; Morris, Lillemo *et al.*, 2001). These results confirmed the theory of a direct effect of puroindoline proteins on GKH.

2.5.2. Interaction of puroindoline protein with the starch granule surface

The high tryptophan content and solubility of PIN proteins in TX-114 suggest that puroindolines are integral membrane proteins and that they could strongly bind to polar lipids. They could, therefore, form tight bonds to membranes. The fact that PINA contains five tryptophan amino acids compared to three in PINB indicated that PINA would form a much stronger bond to membranes. There seems to be an influence of bound polar lipids, such as glycol- and phospholipids, in the puroindoline–granule surface interaction. It has been shown that the TRDs form membrane-anchoring loops between α-helices in the starch membranes (Greenblatt *et al.*, 1995).

A mutation in the genes of *Pina-D1* or *Pinb-D1* causes an alteration in the DNA sequence of the PIN protein, which alters the secondary and tertiary structure of the protein. The structural change in the protein affects the hydrophobicity and strength with which PIN proteins can bind to polar lipids on the starch granule membrane, mainly when this DNA sequence alteration resides near the TRD (Giroux & Morris, 1997a; Ma *et al.*, 2009). This influences the strength of the bond between the starch granules and protein matrix in the wheat endosperm, and thereby the perceived hardness of the grain, affecting milling performance and end-use properties (Greenblatt *et al.*, 1995).

The different amounts of friabilin on the surface of water-washed starch of soft versus hard wheat cultivars were resolved by Jolly *et al.* (1993). The friabilin concentration difference was due to a 'partitioning phenomenon' related to the lipid-binding properties of PIN proteins. The water-washed starch-isolation procedure used to extract friabilin proteins also played a role, since the hydrophilic friabilin protein partitioned towards the gluten and starch fraction during the procedure, but not towards the water-soluble fraction. It was found that friabilin occurs at relative levels in both soft and hard wheat endosperm; however, it is the amount of friabilin that is directly associated with the starch granule surface that influences the perceived hardness of grain (Jolly *et al.*, 1993). The binding properties of friabilin to the starch granule surface are influenced by the *Pin* allele genotype of the

wheat cultivar, the DNA sequence of *Pina-D1* or *Pinb-D1*, and their respective amino acid structures of the expressed PIN proteins.

Although it is accepted that friabilin consists of two major polypeptides – PINA and PINB – and a third minor polypeptide, namely grain softness protein-1 (GSP-1), it has been shown that PINA and PINB proteins are the main contributors to endosperm texture (Beecher *et al.*, 2002; Martin *et al.*, 2006).

2.5.3. Grain softness protein

Initially, researchers used the term 'grain softness protein' (GSP) to refer to friabilin. However, there was a transition from these 15 kDa or friabilin protein studies to the discovery of *Pina-D1* and *Pinb-D1*, and the isolation of *Gsp* DNA-clones after which *Gsp* was used to refer to one component of the friabilin protein. The isolation of cDNA was accomplished by using an anti-Gsp serum that encodes a polypeptide derived from *Gsp-1*. This cDNA was used to characterise the *Gsp-1* family, and peptide sequencing revealed that GSP has a 40% amino acid similarity to PINA (Rahman *et al.*, 1994). The sequence data also corresponded with 'peak 6' reported by Blochet *et al.* (1991). The *Gsp-1* family includes *Gsp-1a*, *Gsp-1b* and *Gsp-1d*, with *Gsp-1* loci present on all three wheat genomes (Pauly *et al.*, 2013b). Initially, it was reported that GSP affects GKH (Jolly *et al.*, 1996), but it has since been suggested that this resulted from the tight linkage between *Gsp-1*, *Pina-D1* and *Pinb-D1* genes on the short arm of chromosome 5D, and that there is currently no evidence to suggest that GSP itself has any effect on GKH (Tranquilli *et al.*, 2002). More details regarding *Gsp-1* can be found in the reviews by Bhave and Morris (2008a; b).

2.5.4. Basic genetics of puroindoline (Pin) genes in wheat

The genes encoding the PIN proteins have been mapped to the distal part of chromosome 5DS. These genes are known as *puroindoline a* (*Pina-D1*) and *puroindoline b* (*Pinb-D1*) genes. Together with *Gsp-1* loci, they are part of the *Ha* locus on chromosome 5DS (Jolly *et al.*, 1993; Sourdille *et al.*, 1996; Ragupathy & Cloutier, 2008). The dominant allele for kernel softness is *Ha*, and *ha* is the recessive gene responsible for hard kernel texture (Law *et al.*, 1978).

The coding regions of the two PIN proteins are 70% identical (Gautier *et al.*, 1994). *Pin* loci, namely *Pina-D1 and Pinb-D1*, have been detected on chromosome 5DS in all diploid *Triticum* (DD) and *Aegilops* (DD) as well as hexaploid wheat (AABBDD), while it was absent in tetraploid species such as *T. turgidum* (AABB) (Tranquilli *et al.*, 1999; Gautier *et al.*, 2000). The *Gsp-1* loci however, have been maintained in all three genomes, i.e. on chromosomes 5A, 5B and 5D (Dubcovsky & Dvorak, 1995; Dubcovsky *et al.*, 1996; Jolly *et al.*, 1996; Sourdille *et al.*, 1996; Giroux & Morris, 1997b; Tranquilli *et al.*, 2002).

2.6. *Puroindoline* alleles

2.6.1. Mutations in Pina-D1 and Pinb-D1 alleles

The first mutation in *Pin* alleles, *Pinb-D1b*, was discovered by Giroux and Morris (1997a). Numerous studies have been performed since then, and new *Pina-D1* and *Pinb-D1* alleles have been identified. *Pina-D1* and *Pinb-D1* allele designations and their effect on GKH, the molecular change on DNA and protein level according to which the alleles differ from the wild-type allele, and the researchers who had identified each allele are reflected in Table 2.1. *Puroindoline* alleles in germplasm from different countries and regions around the world have been studied extensively. Through these and other surveys on wheat cultivars, new *Pin* alleles and mutations have been discovered, whereby knowledge and understanding of *Pin* genes have improved. Valuable resources for the improvement of wheat in breeding programmes have also been unlocked through these studies.

Gene symbols and allele designations should be assigned in an orderly manner to facilitate the communication of genetic information amongst researchers. The Catalogue of Gene Symbols for Wheat (referred to as the 'Catalogue') has assumed this role under the leadership of Prof. R.A. McIntosh. The Catalogue is updated by annual supplements posted in the Annual Wheat Newsletter (Raupp, 2018) and on the Komugi website (Komugi, 2018). It is also published after the International Wheat Genetics Symposium (IWGS) held every five years (Morris & Bhave, 2008).

2.6.2. Discrepancies in allele designations

Reconciliation of designated *Pin* allele symbols and original sequence data published, were performed by Morris and Bhave (2008). No further official allele assignments or reconciliation have been performed since. Whilst researching *Pin* allele designations to date, some discrepancies have been observed. These discrepancies are described below, and reconciliation suggested.

There were two discrepancies in *Pina-D1* alleles, for *Pina-D1w* and *Pina-D1y*. Kumar *et al.* (2015) assigned a guanine to cytosine substitution at position 65 with an adenine to guanine substitution at position 86 to *Pina-D1w*, while Ali *et al.* (2015) assigned a guanine to adenine substitution at position 156 with a guanine to adenine substitution at position 257 to *Pina-D1w*. The assigned alleles were referenced against the National Centre for Biotechnology Information (NCBI) GenBank genetic sequence database. The NCBI GenBank accession number KJ446779.1 was assigned to *Pina-D1w* in a *T. turgidum* ssp. *durum* x *A. tauschii* cross (bio-material CIGM93.267) of the International Maize and Wheat Improvement Centre (CIMMYT) as reported by Ali *et al.* (2015). It is suggested that the guanine to cytosine substitution at position 65 with adenine to guanine substitution at position 86 of Kumar *et al.* (2015) be assigned the allele designation *Pina-D1z*.

Ali *et al.* (2015) assigned the allele *Pina-D1y* to a guanine to thymine substitution at position 242 and a guanine to adenine substitution at position 257, while Ma *et al.* (2017) assigned the synonymous mutation of cytosine to thymine at position 321 to *Pina-D1y*. No NCBI GenBank

accessions for *Pina-D1y* have been assigned. It is thus suggested that Ma *et al.* (2017) assign the synonymous mutation of cytosine to thymine at position 321 to *Pina-D1aa*.

The discrepancies encountered in *Pinb-D1* alleles were in *Pinb-D1x*, *Pinb-D1ac* and *Pinb-D1ad*. Both *Pinb-D1x* and *Pinb-D1ac* alleles had two single nucleotide polymorphisms of guanine to adenine substitution at position 257 and cytosine to thymine substitution at position 382, but different *Pinb-D1* alleles were designated to them (Wang, Sun *et al.*, 2008; Wang, Li *et al.* 2008). An NCBI GenBank accession was only assigned to *Pinb-D1x* (AM909618.1) in *T. aestivum* Kashibaipi (Wang, Sun *et al.*, 2008). It is suggested that the allele *Pinb-D1ac* was an assignment error, and that it should be assigned to *Pinb-D1x.* This would leave allele *Pinb-D1ac* currently unassigned.

Kumar *et al.* (2016) assigned *Pinb-D1ad* to a thymine to cytosine substitution at position 92, while Ayala *et al.* (2016) assigned *Pinb-D1ad* to a cytosine to thymine substitution at position 271. The NCBI GenBank accession of *Pinb-D1ad* was assigned to *T. aestivum* cultivar BGE018668 (accession number KT885199.1) by Ayala *et al.* (2016). It is suggested that the thymine to cytosine substitution at position 92 of Kumar *et al.* (2016) be assigned to *Pinb-D1aj*.

Table 2.1. *Pina-D1* and *Pinb-D1* alleles in *Triticum aestivum* and *Aegilops tauschii*

Allele designation	Kernel texture	Molecular change at DNA and/or protein level	References
Pina-D1			
Pina-D1a	Soft	Wild-type	Giroux and Morris (1997a); Chantret (2005)
Pina-D1b	Hard	Gene deletion, deletion of first 21 base pairs (null) Allele is defined as a 15 380 base pair deletion	Giroux and Morris (1998); Li, He *et al.* (2008)
Pina-D1c	Soft	One SNP. Arg58Gln	Morris, Simeone *et al.* (2001); Massa *et al.* (2004)
Pina-D1d	Soft	Two SNPs. Arg58Gln + one synonymous mutation	Morris, Simeone *et al.* (2001); Lillemo *et al.* (2002); Massa *et al.* (2004)
Pina-D1e	Soft	Two SNPs. Arg58Gln + one synonymous mutation	Massa *et al.* (2004)
Pina-D1f	Soft	Three SNPs. Arg58Gln + two synonymous mutations	Massa *et al.* (2004)
Pina-D1g	Soft	One SNP. One synonymous mutation	Massa *et al.* (2004)
Pina-D1h	Soft	Two SNPs. Arg58Gln + one synonymous mutation	Gedye *et al.* (2004)
Pina-D1i	Hard	Two SNPs. Arg58Glu + Arg21Ser	Gedye *et al.* (2004)
Pina-D1j	Hard	Three SNPs. Arg58Gln + Pro108Arg + one synonymous mutation	Gedye *et al.* (2004)
Pina-D1k	Very Hard	Multiple deletions in *Pina* and *Pinb.* 'Double null'	Tranquilli *et al.* (2002); Ikeda *et al.* (2005); Chang *et al.* (2006); Tanaka *et al.* (2008)
Pina-D1l	Hard	One C deletion, frame-shift Gln61Lys, then stop codon downstream (null)	Gazza *et al.* (2005); Chen *et al.* (2006)
Pina-D1m	Hard	One SNP. C-to-T substitution. Pro35Ser	Chen *et al.* (2006)
Pina-D1n	Hard	One SNP. G-to-A substitution. Trp43Stop	Chen *et al.* (2006)
Pina-D1o	Hard	Two SNPs. Arg58Gln + one synonymous mutation	Huo *et al.* (2006); Liu *et al.* (2016)
Pina-D1p	Hard	One SNP. Val13Glu in the leader peptide. Then one base deletion causing frame-shift at Cys110Ala, then stop codon downstream	Chang *et al.* (2006)
Pina-D1q	Hard	Two SNPs. Di-nucleotide inversion (CA to AC at position 417-418) causing Asn111Lys, Ile112Leu	Chang *et al.* (2006)

Table 2.1. Continued

Allele designation	Kernel texture	Molecular change at DNA and/or protein level	References
Pina-D1r	Hard	Complete locus deletion (null)	Chen *et al.* (2012)
Pina-D1s	Hard	Complete locus deletion (4 222bp deletion) (null)	Chen, Li and Cui (2013)
Pina-D1t	Hard	One SNP. Trp41Stop	Ramalingam *et al.* (2012)
Pina-D1u	Hard	Complete locus deletion (6 460bp deletion) (null)	Chen, Li and Cui (2013)
Pina-D1v	Hard	One SNP. C-to-T at position 41, occurring in pre-peptide part of signal peptide Ala15Val (null)	Kumar *et al.* (2015)
*Pina-D1w** (suggested *Pina-D1z*)	Hard	Two SNPs. G-to-C at position 65, occurring in pro-peptide part of signal peptide Ser7Thr. A-to-G at position 86 causing Asp1Gly in functional protein	Kumar *et al.* (2015)
*Pina-D1w**	Hard	Two SNPs. G-to-A at position 156, synonymous mutation. G-to-A at position 257, Arg58Gln	Ali *et al.* (2015)
Pina-D1x	Hard	Three SNPs. G-to-A at position 257, Arg58Gln C-to-T at position 330, synonymous mutation T-to-C at position 333, synonymous mutation (null)	Ali *et al.* (2015)
*Pina-D1y***	Hard	Two SNPs. G-to-T at position 242, Gly53Val G-to-A at position 257, Arg58Gln	Ali *et al.* (2015)
*Pina-D1y*** (suggested *Pina-D1aa*)	Soft	One SNP. C-to-T at position 321. Synonymous mutation	Ma *et al.* (2017)
Pinb-D1			
Pinb-D1a	Soft	Wild-type	Giroux and Morris (1997a); Gautier *et al.* (2000)
Pinb-D1b	Hard	One SNP. Gly46Ser	Giroux and Morris (1997a); Chantret (2005); Simeone *et al.* (2006)
Pinb-D1c	Hard	One SNP. Leu60Pro	Lillemo and Morris (2000)
Pinb-D1d	Hard	One SNP. Trp44Arg	Lillemo and Morris (2000); Corona *et al.* (2001)
Pinb-D1e	Hard	One SNP. Trp39 to stop codon (null)	Morris, Lillemo *et al.* (2001); Chen *et al.* (2007)

Table 2.1. Continued

Allele designation	Kernel texture	Molecular change at DNA and/or protein level	References
Pinb-D1f	Hard	One SNP. Trp44 to stop codon (null)	Morris, Lillemo *et al.* (2001); Pickering and Bhave (2007)
Pinb-D1g	Hard	One SNP. Cys56 to stop codon (null)	Morris, Lillemo *et al.* (2001)
Pinb-D1h	Soft	Twenty-nine SNPs. 14 amino acid substitutions	Turnbull *et al.* (2003); Massa *et al.* (2004); Ikeda *et al.* (2005)
Pinb-D1i	Soft	Thirty SNPs. 14 amino acid substitutions	Morris, Simeone *et al.* (2001); Massa *et al.* (2004); Chantret (2005); Chen *et al.* (2005); Simeone *et al.* (2006)
Pinb-D1j	Hard	Nineteen SNPs. 14 amino acid substitutions	Morris, Simeone *et al.* (2001); Massa *et al.* (2004)
Pinb-D1k	Hard	Thirty-one SNPs. 14 amino acid substitutions	Lillemo *et al.* (2002)
Pinb-D1l	Hard	One SNP. Lys45Glu	Pan *et al.* (2004)
Pinb-D1m	Soft	Twenty-eight SNPs. 14 amino acid substitutions	Gedye *et al.* (2004)
Pinb-D1n	Soft	Twenty-nine SNPs. 14 amino acid substitutions	Gedye *et al.* (2004)
Pinb-D1o	Soft	Twenty-eight SNPs. 14 amino acid substitutions	Gedye *et al.* (2004)
Pinb-D1p	Hard	One-base deletion, frame-shift at Lys42Asn, then stop codon at 60 (null)	Ikeda *et al.* (2005); Xia *et al.* (2005); Chang *et al.* (2006)
Pinb-D1q	Hard	One SNP. Trp44Leu	Tranquilli *et al.* (2002); Chen *et al.* (2005)
Pinb-D1r	Hard	G insertion at position 127, frame-shift at Glu14Gly, then stop codon at 48	Ram *et al.* (2005)
Pinb-D1s	Hard	G insertion + one SNP. Frame-shift at Glu14Gly, then stop codon at 48	Ram *et al.* (2005)
Pinb-D1t	Hard	One SNP. G-to-C substitution. Gly47Arg	Chen *et al.* (2006)
Pinb-D1u	Hard	One-base deletion (G at position 127), frame-shift at Glu14Ser, then stop codon at 18	Chen *et al.* (2007)
Pinb-D1v	Hard	Two SNPs, Ala8Thr and Leu9Ile in the leader peptide	Chang *et al.* (2006)

Table 2.1. Continued

Allele designation	Kernel texture	Molecular change at DNA and/or protein level	References
Pinb-D1w	Hard	One SNP. Ser115Ile	Chang *et al.* (2006)
Pinb-D1x	Hard	Two SNPs. G-to-A at position 257, causing Cys57Tyr C-to-T at position 382, causing Gln99stop	Wang, Sun *et al.* (2008)
Pinb-D1y	Original assignment of this allele was incorrect. Currently, this allele is unassigned		
Pinb-D1z	Original assignment of this allele was incorrect. Currently, this allele is unassigned		
Pinb-D1aa	Hard	One SNP, one synonymous mutation. Then one base deletion, frame-shift at Lys42Asn, then stop codon at 60	Li, He *et al.* (2008)
Pinb-D1ab	Hard	One SNP. C-to-T at position 382, causing Gln99stop	Tanaka *et al.* (2008)
Pinb-D1ac (suggested *Pinb-D1x*)	Hard	Two SNPs. G-to-A at position 257, causing Cys57Tyr C-to-T at position 382, causing Gln99stop	Wang, Li *et al.* (2008)
*Pinb-D1ad**** (suggested *Pinb-D1aj*)	Hard	One SNP, T-to-C at position 92, causing Val2Ala	Kumar *et al.* (2015)
*Pinb-D1ad****	Hard	One SNP, C-to-T at position 271, causing Gln62stop	Ayala *et al.* (2016)
Pinb-D1ae	Soft	One SNP, T-to-A at position 93. synonymous mutation	Kumar *et al.* (2015)
Pinb-D1af	Hard	One SNP, G-to-T at position 232, causing Glu49stop	Kumar *et al.* (2015)
Pinb-D1ag	Hard	One SNP, T-to-C at position 371, causing Leu95Pro	Kumar *et al.* (2015)
Pinb-D1ah	Hard	Null (Commonly found as 'double-null' *Pina-D1x*/*Pinb-D1ah*)	Ma *et al.* (2017)
Pinb-D1ai	Hard	Null	Ma *et al.* (2017)

Pina-D1 – *puroindoline a* gene, *Pinb-D1* – *puroindoline b* gene, SNP – single nucleotide polymorphism, bp – base pair, A – adenine, C – cytosine, G – guanine, T – thymine, Ala – alanine, Gly – glycine, Ile – isoleucine, Leu – leucine, Pro – proline, Val – valine, Trp – tryptophan, Tyr – tyrosine, Asp – aspartic acid, Glu – glutamic acid, Arg – arginine, Lys – lysine, Ser – serine, Thr – threonine, Cys – cysteine, Asn – asparagine, Gln – glutamine.
* Allele designation *Pina-D1w* was assigned by two authors (Ali *et al.*, 2015; Kumar *et al.*, 2015), but to different DNA sequences.
** Allele designation *Pina-D1y* was assigned by two authors (Ali *et al.*, 2015; Ma *et al.*, 2017), but to different DNA sequences.
*** Allele designation *Pinb-D1ad* was assigned by two authors (Kumar *et al.*, 2015; Ayala *et al.*, 2016), but to different DNA sequences.

2.6.3. Distribution of puroindoline alleles in wheat cultivars from wheat-producing countries

In most geographic areas of wheat production, bread wheat cultivars predominantly have the *Pina-D1a/Pinb-D1b* combination (Chen *et al.*, 2011; Chen, Li & Cui, 2013; Ma *et al.*, 2017). These countries include North America, Chili, Australia, Ukraine, China and Russia (Morris, Lillemo *et al.*, 2001; Chen *et al.*, 2011; Chen, Li & Cui, 2013; Kumar *et al.*, 2015).

The highest diversity in *Pin-D1* has been seen in Chinese landraces with allelic combinations, including alleles *Pina-D1a*, *Pina-D1b*, *Pina-D1l*, *Pina-D1n*, *Pina-D1r*, *Pina-D1s*, *Pina-D1u*, *Pinb-D1a*, *Pinb-D1b*, *Pinb-D1d*, *Pinb-D1e*, *Pinb-D1p*, *Pinb-D1q*, *Pinb-D1t*, *Pinb-D1u*, *Pinb-D1x*, *Pinb-D1aa*, *Pinb-D1ab*, *Pinb-D1ac* (Chen *et al.*, 2006; 2012; Chen, Li & Cui, 2013; Wang, Sun *et al.*, 2008; Wang, Li *et al.* 2008). Studies on Chinese wheat cultivars found that *Pina-D1a*, *Pinb-D1a*, *Pinb-D1b* and *Pinb-D1p* are the most frequent alleles found in cultivars of Chinese origin (Pan *et al.*, 2004; Chen *et al.*, 2005; 2006; 2007; Xia *et al.*, 2005; Chang *et al.*, 2006; Wang, Sun *et al.*, 2008), and the allelic combination *Pina-D1a/Pinb-D1p* was found more commonly in Chinese wheat cultivars than in cultivars from other countries (Chen *et al.*, 2006; Li, He *et al.*, 2008; Kumar *et al.*, 2015).

Pinb-D1ab was initially detected in the Japanese wheat line KU3062 (Tanaka *et al.*, 2008), but it was later also detected in Tuokexun 1 from the Xinjiang winter–spring wheat region of China (Wang, Sun *et al.*, 2008). To date, the novel allele *Pina-D1r* has only been found in Japanese wheat cultivars and landraces (Ikeda *et al.*, 2010).

Indian wheat cultivars also have a great diversity in *Pin* alleles with the appearance of *Pina-D1a*, *Pina-D1b*, *Pina-D1v*, *Pina-D1w*, *Pinb-D1a*, *Pinb-D1b*, *Pinb-D1e*, *Pinb-D1r*, *Pinb-D1ad*, *Pinb-D1ae*, *Pinb-D1af* and *Pinb-D1ag*. The most prevalent allele combination in Indian wheat cultivars is *Pina-D1b/Pinb-D1a* (Kumar *et al.*, 2015).

Korean and CIMMYT wheat cultivars had the least variation encountered thus far with only three *Pin-D1* allelic combinations, comprised of the alleles *Pina-D1a*, *Pina-D1b*, *Pinb-D1a* and *Pinb-D1b* (Park *et al.*, 2009; Chen, Li & Cui, 2013). The most frequent genotype in CIMMYT cultivars and advanced lines are PINA null, while the most prevalent allelic combination is *Pina-D1b/Pinb-D1a* (Lillemo *et al.*, 2006; Kumar *et al.*, 2015).

Andalusia (Southern Spain) predominantly has soft wheat endosperm with the most frequent *Pin* allele combination of *Pina-D1a*/*Pinb-D1a*, followed by *Pina-D1a*/*Pinb-D1d* and *Pina-D1a*/*Pinb-D1ad* (Ayala *et al.*, 2016).

Although the presence of *Pin* alleles has been widely studied, some countries or regions have not explored the *Pin* genetic diversity amongst their adapted wheat cultivars. Research on *Pin* alleles in South America, Europe, West Asia and Africa is proposed and will provide extremely valuable information to wheat breeders in these countries.

2.6.4. Influence of puroindoline alleles on grain hardness, flour properties and bread-baking quality

The significant effect of GKH on physical and rheology properties of wheat is generally accepted. Since the basis of GKH results from puroindoline proteins, specific *Pin* alleles can be linked to the effects on physical and rheological properties of wheat differing in hardness. Variation in *Pina-D1* and *Pinb-D1* alleles and the combination of alleles in a wheat cultivar or breeding line causes a difference in GKH. This difference has been observed in wheat milling and quality parameters, and different alleles could influence these parameters to different extents.

When *Pina-D1* and *Pinb-D1* are both in their wild-type (*Pina-D1a*/*Pinb-D1a*), the resulting texture of the grain is soft, but when the *Pina-D1* and *Pinb-D1* allele does not express a protein or when the allelic structure of the gene is mutated, then the grain texture is hard (Giroux & Morris, 1998; Lillemo & Morris, 2000; Morris, Lillemo *et al.*, 2001). Different combinations of *Pina-D1* and *Pinb-D1* alleles result in different hardness levels of wheat endosperm, and thus different influences on grain processing quality (Giroux *et al.*, 2000; Martin *et al.*, 2001; Nagamine *et al.*, 2003; Cane *et al.*, 2004; Ikeda *et al.*, 2005; Eagles *et al.*, 2006; Bhave & Morris, 2008a; b; Morris & Bhave, 2008).

Wheat containing the *Pina* null allele (*Pina-D1b*) has a significantly higher SKCS-HI value compared to wheat with null mutations in *Pinb* (*Pinb-D1b* or *Pinb-D1p*) (Giroux *et al.*, 2000; Chang *et al.*, 2006; Chen *et al.*, 2006; Geng *et al.*, 2013). Takata *et al.* (2010) compared near-isogenic wheat lines with the *Pina-D1b/Pinb-D1a* genotype to wheat lines with the double-null mutation *Pina-D1k* and found that wheat grain with the double-null genotype was harder than wheat grains with any other *Pin* allele combinations. This was confirmed by Chen, Li and Cui (2013) where the double-null genotype had the hardest grain kernels. Wheat grain containing the *Pina-D1a*/*Pinb-D1b* genotype has similar GKH as the *Pina-D1a*/*Pinb-D1e* and *Pina-D1v*/*Pinb-D1b* genotypes, but is harder than the *Pina-D1w*/*Pinb-D1b* genotype (Kumar *et al.*, 2015). The presence of *Pin-D1* genes in wheat is not only important for physical grain hardness, but also for wheat processing and flour quality properties (Ayala *et al.*, 2016).

Although the single major gene responsible for GKH has been identified, *Ha*, on chromosome 5DS (Mattern *et al.*, 1973; Baker & Dyck, 1975; Law *et al.*, 1978), there may still be minor differences in GKH within the same hardness genotypes. These differences can be attributed to environmental influence or the cooperation of several smaller genes and factors influencing GKH. Quantitative trait loci (QTL) are regions in the wheat genome at which genetic variation is associated with a quantitative trait, i.e. for wheat yield, kernel diameter, flour yield and various others. Quantitative trait loci are identified by a statistical association of genetic markers and measurable phenotypes. Likewise, some major genes affecting quality properties have been identified, but quality properties may display continuous variation within a class of wheat and appear to be controlled by multiple minor genes, i.e. QTLs (Smith *et al.*, 2011). Due to the number of genetic and environmental influences on the correlation of GKH with wheat quality properties, exceptional attention to experimental design is essential. Studies on GKH and its correlation with milling and baking

properties should be performed over enough years, locations and replicates to produce reliable results.

Some research studies where trials were not planted over enough years, locations and replications are discussed below. However, the results still indicated the effects of GKH on quality traits. Wheat with increased GKH was reported to have increased total flour yield, mixograph peak height, flour water absorption and loaf volume (Baker & Dyck, 1975). Chen, Li and Li (2013) reported GKH had a positive correlation with alveograph tenacity and P/L ratio, mixograph tolerance to over-mixing and water absorption, and a negative correlation with amylase activity and starch gelling. Giroux *et al.* (2000) found that GKH was positively correlated with grain protein content. Chen *et al.* (2007) reported that wheat cultivars with *Pina* null mutation (*Pina-D1b*) had poor milling quality and sub-standard processing quality compared to wheat cultivars with a *Pinb* null mutation.

Conversely, studies with good experimental design over years, locations and multiple field replicates (Martin *et al.*, 2001; Cane *et al.*, 2004; Eagles *et al.*, 2006) reported wheat with the *Pina-D1b/Pinb-D1a* genotype displayed increased GKH. This resulted in increased flour water absorption and ash content, with decreased break and total flour yield, dough development time, dough extensibility, loaf volume and a degrade in crumb grain score compared to wheat with the *Pina-D1a/Pinb-D1b* genotype. Wheat grain with the genotype *Pina-D1a*/*Pinb-D1a* and phenotype soft grain endosperm produced flour with reduced maximum dough resistance, dough development time and flour water absorption in comparison with allelic genotypes producing hard wheat grain (Cane *et al.*, 2004; Eagles *et al.*, 2006). Mutations and deletions in the *Pina-D1* and *Pinb-D1* alleles of wheat had the greatest effect on properties involving particle size and milling quality, i.e. grain hardness, total flour yield, break flour yield, milling score and flour ash content (Martin *et al.*, 2001).

It is evident that there are some differences in the findings of studies with good experimental design opposed to those that had too few years, locations and replicates. In the instance where there was too little variation, it is impossible to know whether the correlations are caused by the wheat genotype or by the environment.

Hogg *et al.* (2005) performed studies on a hard red spring cultivar, and genetically modified the expression of *Pina-D1a*, *Pinb-D1a* or both *Pina-D1a* and *Pinb-D1a* simultaneously. The amount of PIN protein expressed was the highest in lines with both *Pina-D1a* and *Pinb-D1a*, less in lines with *Pina-D1a*, and least in lines with only *Pinb-D1a*. Breeding lines with high PIN protein expression showed higher break flour yield, but lower total flour yield, lower protein and ash content in flour, decreased water absorption and loaf volume, but mixograph mixing time remained unchanged in comparison to other lines with less PIN protein (Hogg *et al.*, 2005). In general, wheat with the *Pina* null allele has an inferior processing quality compared to wheat with the *Pinb* null allele (*Pinb-D1b* or *Pinb-D1p*) (Martin *et al.*, 2001; Chen *et al.*, 2011).

Apart from wheat processing properties affected, PIN proteins have a high affinity for binding lipids, which is a beneficial property in the processing of cereals. During the foam stage in bread dough, puroindolines act at the air–water interface of gas cell walls to prevent the destabilisation of

foam by oil globules. This ensures a stable foam with a fine gas structure to form, a fine crumb structure, and high loaf volume in the final baked product (Dubreil *et al.*, 1998). This property of PIN proteins can even be implemented in beer processing to prevent the destabilisation of foams by neutral and polar lipids (Clark *et al.*, 1994). Although PINA and PINB possess similar foaming properties, PINA shows an enhanced foam formation in the presence of polar lipids (Wilde *et al.*, 1993). PIN proteins are also capable of encouraging favourable changes in dough tenacity and extensibility (Dubreil *et al.*, 1998).

2.7. Conclusion

Wheat kernel hardness is one of the most important grain characteristics that influence the complete wheat value chain and ultimately end-use properties and quality of food products delivered to the consumer. It is widely accepted that *puroindoline* genes on chromosome 5DS, are responsible for the genetic basis of GKH, although some minor genes may also be involved. It is essential that studies conducted on the correlation of GKH and *puroindoline* genes with wheat quality properties be well planned and executed. This is essential to keep the environmental influence to a minimum; otherwise those results could be deemed unreliable. Greater knowledge about the interaction of *puroindoline* alleles and their expressed proteins to portray a variety's GKH is necessary. The expressed hardness information in combination with correlation to wheat quality would enable the breeding of end-use-specific wheat cultivars, shortening the breeding time to release a suitable cultivar for different environments and applications.

Discrepancies with *puroindoline* allele designations have been observed in published studies, and a suggested solution for each has been proposed. The assignment of allele designations is critical to facilitate the communication and collaboration between researchers. A research gap in countries of South America, Europe, West Asia and Africa has been identified. Identification of *Pin* alleles in wheat cultivars adapted to these countries could further increase diversity and knowledge of GKH, and increase wheat breeding opportunities.

2.8. References

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CHAPTER 3

Determination of *puroindoline* allele identity and kernel hardness in commercial bread wheat (*Triticum aestivum*) from three production environments

Abstract

Puroindoline (*Pin*) *a* and *b* alleles, determining wheat grain hardness, were identified in 27 South African bread wheat cultivars. These cultivars were planted in each of three different wheat production environments. Spring wheat cultivars were planted in the summer rainfall irrigation region as well as in the winter rainfall dryland region, and facultative and winter wheat in the summer rainfall dryland region. A modified cetyltrimethyl ammonium bromide (CTAB) method was used for genomic deoxyribonucleic acid (DNA) extraction, and *Pin* alleles were amplified with specific polymerase chain reaction (PCR) markers. The PCR products were analysed on agarose gel and sequenced to determine specific *Pin* alleles. Only one mutant allele (*Pina-D1b*) in addition to the wild-type *Pina-D1a* allele for *Pina-D1* was observed. For *Pinb-D1*, the wild-type allele (*Pinb-D1a*) and three mutant alleles *(Pinb-D1b*, *Pinb-D1p* and *Pinb-D1ab*) were identified. Wheat grain kernel hardness (GKH) was determined for the 27 wheat samples over three planting seasons and four locations per production region. As found in various other studies, wheat with the allelic combination *Pina-D1a*/*Pinb-D1b* had lower GKH values, indicating that they were softer than wheat with the combination *Pina-D1b*/*Pinb-D1a*. Stepwise multiple linear regression models were developed for the summer rainfall irrigation and the summer rainfall dryland production regions each to predict wheat GKH, based on the *puroindoline* allele identity.

3.1. Introduction

Wheat, one of the most important cereal crops worldwide, serves a variety of purposes, but most importantly it provides more nutrition to humans than any other grain (Paulsen & Shroyer, 2004). Wheat is often used as the primary ingredient for a range of food products, depending on its physical, chemical and functional properties. Of these, GKH is one of the most important to be considered. Bread wheat (*Triticum aestivum*) can be divided into different hardness classes, each with its individual application. Hard bread wheat is utilised for leavened bread, while soft bread wheat is used to bake cakes and biscuits (Kent & Evers, 1994; O'Brien & DePauw, 2004). Extremely hard durum grain (*T. turgidum* ssp. *durum*) is used for pasta and couscous.

Wheat endosperm hardness is attributed to the hardness locus, *Ha*, that is situated on chromosome 5DS (Symes, 1965; Mattern *et al.*, 1973; Baker & Dyck, 1975; Law *et al.*, 1978). A study by Barlow *et al.* (1973) to investigate the biochemical mechanism controlling GKH, found that

the nature of adherence between endosperm starch granules and the protein matrix is ascribed to the presence of a water-soluble protein at the starch-protein interface, which controls perceived hardness. Subsequent research indicated the *Ha* locus to be responsible for the production of friabilin, a protein that is present on the surface of water-washed starch granules (Greenwell & Schofield, 1986). The *Ha* locus consists of three genes, namely *puroindoline a* (*Pina-D1*), *puroindoline b* (*Pinb-D1*) and *grain softness protein* (*Gsp-1*). According to various reports, the molecular basis of GKH results mainly from the two *puroindoline* genes (Giroux & Morris, 1997; Lillemo & Morris, 2000; Beecher *et al.*, 2002; Martin *et al.*, 2006; Bhave & Morris, 2008). There is currently no evidence of the effect of *Gsp-1* on GKH (Tranquilli *et al.*, 2002).

When *Pina-D1* and *Pinb-D1* are both in their wild-type, the resulting texture of the wheat kernel would be soft. When the *Pina-D1* and *Pinb-D1* allelic structure is altered so that it does not express a protein, or when the gene is mutated so that a different protein is expressed, the kernel texture would be hard (Giroux & Morris, 1998). To date, 25 *Pina-D1* and 35 *Pinb-D1* alleles have been identified by researchers globally. Different combinations of *Pina-D1* and *Pinb-D1* alleles in wheat cultivars result in different levels of GKH and thus different grain qualities (Giroux *et al.*, 2000; Martin *et al.*, 2001; Nagamine *et al.*, 2003; Cane *et al.*, 2004; Ikeda *et al.*, 2005; Eagles *et al.*, 2006).

Research has been conducted globally to gain knowledge on the different *Pin* alleles present in *Triticum* and *Aegilops* and their geographic distribution in wheat-producing countries. The highest diversity of *Pin* alleles has been found in Chinese wheat cultivars and landraces (Pan *et al.*, 2004; Chen *et al.*, 2005; 2006; 2007; 2012; 2013; Xia *et al.*, 2005; Chang *et al.*, 2006; Wang, Li *et al.*, 2008; Wang, Sun *et al.*, 2008). Indian wheat cultivars also have a great diversity of *Pin* alleles (Kumar *et al.*, 2015), with some novel and infrequent *Pin* alleles identified in Japanese wheat cultivars (Tanaka *et al.*, 2008; Ikeda *et al.*, 2010). The *Pin* allele diversity was not high in Korean wheat cultivars and in breeding lines from the International Maize and Wheat Improvement Centre (CIMMYT) (Lillemo *et al.*, 2006; Park *et al.*, 2009; Chen *et al.*, 2013; Kumar *et al.*, 2015). Wheat cultivars from Southern Spain were predominantly soft, and the *Pin* wild-type alleles were commonly identified in those wheat cultivars (Ayala *et al.*, 2016). The discovery of new *Pin* alleles brought with them genetic background knowledge of GKH. The knowledge of *Pin* alleles and their influence on GKH facilitate early generation selection in wheat breeding programmes, enabling breeding of wheat cultivars suitable for specific end products and novelty uses. Knowledge of specific *Pin* alleles thus enhances breeding and the commercial release of new and desirable wheat cultivars to be used by the food industry.

No information is available on the *Pin* allelic diversity in South African wheat germplasm. Such knowledge would enable breeders to select breeding parents more efficiently to develop a wheat cultivar with specific quality properties. The breeding process could be optimised by performing informed early generation selections at F5 stage based on *Pin* alleles present in breeding lines.

The objectives of the study on which this research chapter is based, were thus 1) to determine the *Pin* alleles present in South African wheat cultivars; and 2) to develop a model to predict wheat GKH from the identified *Pina-D1* and *Pinb-D1* alleles.

3.2. Materials and methods

3.2.1. Experimental population

Commercial wheat cultivars differing in GKH were selected for each of the three South African wheat production regions, i.e. summer rainfall irrigation (SRI), summer rainfall dryland (SRD) and winter rainfall dryland (WRD). A set of nine cultivars were selected for each production region and planted at four locations in each region. These cultivars consisted of the wheat quality standard for each region and eight commercial cultivars, ranging in hardness, agronomically adapted for production in each region (Table 3.1).

Table 3.1. Wheat cultivars selected based on differences in kernel hardness, planted in each of the three wheat production regions

Summer rainfall irrigation (SRI)	Summer rainfall dryland (SRD)	Winter rainfall dryland (WRD)
SST806*	Elands*	Kariega*
Duzi	PAN3161	PAN3434
Baviaans	PAN3144	Ratel
Buffels	Gariep	Baviaans
PAN3471	SST398	SST015
SST835	PAN3355	SST096
Olifants	SST347	SST056
PAN3478	SST356	SST087
SST875	PAN3379	SST88

*Wheat quality standard for each production region.

3.2.2. Field trials

Field trials, with the selected wheat cultivars, were planted for three consecutive seasons (2012–2014) in each of the three production regions. Trials were planted at four locations in each production region per year with three field replications per location (Fig. 3.1). The trials were all planted according to a randomised complete block design (RCBD) (Fig. 3.2). The RCBD is the standard design used when planting agricultural experiments. Three replicates of each experimental unit were grouped into a block, with randomisation of the cultivars within each block. This type of layout is used to control the variation within the experiment that might be caused by environmental factors, such as soil fertility, wind and water drainage (Clewer & Scarisbrick, 2001).

Figure 3.1. Map of South Africa with the three production regions and trial locations of each region indicated.

8	6	4	7	6	2	1	6	2
5	9	7	5	1	3	3	9	5
2	1	3	8	4	9	7	8	4
REP I			**REP II**			**REP III**		

Figure 3.2. A randomised complete block design layout with three replicate blocks. Numbers 1 to 9 represent the nine different cultivars.

The same agricultural practices were used as implemented by farmers for each specific area. The size of the trial plots and spaces between planted rows were thus different for each of the three production regions (Table 3.2). The plot sizes were as such to ensure adequate yield for the necessary laboratory analysis.

The locations for the SRI and WRD regions remained constant, although one trial at Winterton (SRI) was lost due to hail damage in 2014. Locations in the SRD region had to be reconsidered on a yearly basis due to a severe drought experienced in this production region during 2012 – 2014 (Table 3.2). The temperature and rainfall data are reflected in Appendix A (Tables A1 – A3).

The trials were harvested when the wheat plants reached physiological maturity, i.e. approximately in December for SRI and SRD, and in November for WRD. Following harvesting of the trials each year, the GKH of the wheat samples was determined.

Table 3.2. Field trial information for each of the three production regions, including plot information and locations

Production region	Plot information		Locations planted per season		
	Plot size (length x width)	Rows	2012	2013	2014
SRI	5.00 m x 1.02 m	Six rows 17 cm row width	Hartsvallei Lichtenburg Marblehall Winterton	Hartsvallei Lichtenburg Marblehall Winterton	Hartsvallei Lichtenburg Marblehall Winterton (Winterton trial lost due to hail damage)
SRD	8.00 m x 1.80 m	Four rows 45 cm row width	Bethlehem Bultfontein Clocolan Virginia (Virginia trial lost due to drought)	Bethlehem Bultfontein Ladybrand Reitz	Bethlehem Wesselsbron Ladybrand Reitz
WRD	5.00 m x 1.00 m	Five rows 25 cm row width	Moorreesburg Malmesbury Napier Riversdal	Moorreesburg Malmesbury Napier Riversdal	Moorreesburg Malmesbury Napier Riversdal

SRI – summer rainfall irrigation, SRD – summer rainfall dryland, WRD – winter rainfall dryland.

3.2.3. Wheat hardness determination

The single kernel characterisation system (SKCS) was used to determine the hardness of wheat kernels employing the physical force needed to crush a wheat kernel. The SKCS analysis was performed according to the American Association for Cereal Chemists (AACC) approved method 55-31.01 (AACC, 1999) using the SKCS model 4100 (Perten Instruments, Hägersten, Sweden). In a single analysis per sample, 300 kernels were used to determine the hardness index (HI), kernel moisture content, kernel weight and kernel diameter. Only HI values were used for further analysis, which could be related to different GKH classes (extra soft to extra hard) as shown in Table 3.3.

The SKCS-HI values of the cultivars for the three replicates at four locations in each of the three production regions were determined yearly. This resulted in SKCS kernel hardness data over 12 locations and three replicates each.

Table 3.3. The average hardness index values of different hardness classes wheat determined with the SKCS (AACC, 1999)

Hardness class	Hardness index (SKCS-HI) value
Extra hard	Above 90
Very hard	81–90
Hard	65–80
Medium hard	45–64
Medium soft	35–44
Soft	25–34
Very soft	10–24
Extra soft	Up to 10

3.2.4. Determination of puroindoline allele identity

The allele identity at the *Pina-D1* and *Pinb-D1* loci of the three cultivar sets were determined using deoxyribonucleic acid (DNA) extraction, polymerase chain reaction (PCR) and sequencing.

The 27 wheat cultivars were planted in pots with sand in the greenhouse at the research facility of Sensako (Pty) Ltd. (Bethlehem, Free State, South Africa). The greenhouse was kept at a constant temperature of about 20 °C, with automated daily watering of the pots. Once the seeds had germinated and reached the four-leaf phase (approximately fifteen days after planting), leaf samples were cut from three individual plants per cultivar and placed in a 96-well DNA extraction plate.

A modified cetyltrimethylammonium bromide (CTAB) DNA extraction method (Doyle & Doyle, 1990) was used to extract genomic DNA from 93 samples representing the 27 genotypes. Two additional DNA controls (Kariega and Avocet S) were included. The genomic DNA (gDNA) was quantified with a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to a working concentration of 25 ng/µL. The *Pina-D1* and *Pinb-D1* alleles were amplified with PCR markers as reported by Lillemo *et al*. (2006). The PCR tests were performed in GeneAmp® PCR system 9700 thermal cyclers (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and Veriti® 96-well thermal cyclers (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The reaction mix contained 0.2 mM of each deoxynucleotide (Kapa Biosystems, Wilmington, MA, USA), 1.5 mM $MgCl_2$ (Promega, Madison, Wisconsin, USA), 0.25 U GoTaq Flexi DNA polymerase (Promega, Madison, Wisconsin, USA) and 2 pmol of the forward and reverse primer each.

Aliquots of PCR products were analysed on a 1.5% agarose gel stained with ethidium bromide to determine the presence of an amplicon. The remaining PCR products of samples that produced an amplicon were sequenced. Sequencing was performed with the forward primer only (DNA Sequencer, Central Analytical Facility, Stellenbosch University, Stellenbosch, South Africa). The BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's protocol with slight modifications.

After cycle sequencing, the products were treated with sodium dodecyl sulphate (SDS) before they were transferred onto Sephadex columns (Merck, Darmstadt, Germany) using a Tecan EVO150 (Tecan Trading AG, Mannedorf, Switzerland) and centrifuged. Before DNA sequencing electrophoresis, the samples were denatured for 2 min at 95 °C in a water bath. Directly after heating, the samples were placed on ice for 5 min. Electrophoresis was performed on an ABI3730*xl* Genetic Analyzer using a 50 cm capillary array and POP7 polymer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Generated sequences were viewed with Sequence Scanner v1.0 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and aligned with the BioEdit sequence alignment editor (Hall, 1999) to a reference sequence for *Pina-D1a* (GenBank accession DQ363911) and *Pina-D1b* (GenBank accession AB262660) with numbering starting at the A of the ATG initiation codon. The *Pina-D1* and *Pinb-D1* alleles were compared to the reference sequences and literature, and alleles were identified for the 27 wheat cultivars.

3.2.5. Statistical analysis

A Shapiro–Wilk test for normality was performed before the standardised results could be assumed reliable (Shapiro & Wilk, 1965). The Pearson's product moment correlation matrix of the pairwise correlations was performed among the dependent variables to show their linear relationships. The Pearson's correlation was calculated using PROC CORR of SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA). For response variables X and Y, the correlation was denoted as r_{xy} and computed according to equation 3.1.

$$r_{xy} = \frac{\sum_{i-1}^{n}\{(x_i - \bar{x})(y_i - \bar{y})\}}{\sqrt{\sum_{i-1}^{n}(x_i - \bar{x})^2 \sum_{i-1}^{n}(y_i - \bar{y})^2}}$$

.... Equation 3.1

Where: r_{xy} = Pearson product moment correlation coefficient between two variables, x and y,
x_i = i^{th} factor (genotype, locality or year) for variable x,
$\bar{x}$ = mean of the factor, and
y_i and $\bar{y}$ similar for variable y.

If there is an exact linear relationship between two variables, the correlation is 1 or -1, depending on whether the variables are positively or negatively associated. If there is no linear relationship, the correlation tends toward zero.

Principal component analysis (PCA) with a Pearson's correlation matrix analysis was performed using XLSTAT software (Addinsoft, Version 2015, Paris, France). PCA is a useful multivariate analysis tool used on large datasets to remove noise and redundant data and thereby reducing the dimensionality of the dataset. PCA determines hidden patterns within a multivariate dataset and expresses these patterns as new variables called principal components (PCs). In PCA, the direction

in which the data varies is identified, i.e. eigenvectors or principal components. PCA assumes that the largest variation is the most important (i.e. principal) aspect of the data, and this will be PC1, i.e. the first principal direction. The largest variation in the remaining model is projected onto PC2, which is orthogonal to PC1, and subsequently PC3 and PC4 until all the variations in the data are attributed to PCs. This complete set of orthogonal eigenvectors, each with a corresponding eigenvalue, is a measure of the amount of variation retained within each PC (David & Jacobs, 2014; Kassambara, 2017). The interaction and contribution of each variable to the PCs were obtained and visualised on PC biplots as illustrated by Everitt and Dunn (1992).

Stepwise multiple linear regression (MLR) was used to determine which *Pin* alleles contributed most towards variation in SKCS-HI values, and to develop a prediction model accordingly. This analysis was performed according to the procedure described by Draper and Smith (1966). Stepwise MLR was performed using the PROC REG of SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA).

MLR calculates the relationship between a dependent variable Y (i.e. SKCS-HI) and one or more independent variables Xi (i.e. various *Pin* alleles). Stepwise MLR is an MLR equipped with a variable selection scheme. The variable that shows the highest correlation with Y is selected, and a regression coefficient is obtained for this selected variable Xi. The significance of this variable, Xi, to improve the coefficient of determination (R^2) was calculated using the F-test (Ott & Longnecker, 2001). If variable, Xi, contributes significantly ($P < 0.05$) to the determination of the dependent variable Y it is retained in the model. An additional variable may be selected according to the partial correlation coefficient of the variable partial correlation coefficient. Including new variables in the model may reduce the contribution of a previously included variable (Zhan *et al.*, 2013; Fritz & Berger, 2015). Therefore, the significance of all previous regression terms (independent variables) was tested after each new inclusion. Non-significant variables were subsequently eliminated from the model. This process was continued until the addition of new variables did not significantly ($P > 0.05$) improve the model. All variables that remained in the final model thus made a significant ($P < 0.05$) contribution in determining the dependent variable. In the case where independent variables were highly correlated, only one of them was entered into the model (Zhan *et al.*, 2013; Fritz & Berger, 2015).

Data analysis was performed on the data of each production region, and subsequently on the combined data set of all three regions.

3.3. Results

3.3.1. Grain hardness diversity among selected wheat cultivars and production regions

The average hardness value for each cultivar was determined across all data, i.e. trials over three years, all locations and replicates. The SKCS-HI is related to GKH since hard wheat requires a greater force to be crushed compared to soft wheat, and thus would have a higher HI value (Gaines

et al., 1996). The SKCS-HI values observed ranged from 46.8 ± 7.9 to 69.4 ± 8.8 (mean ± standard deviation [SD]) (Table 3.4), thus medium hard to hard category GKH (Table 3.3). The SKCS-HI value ranges per production region were 52.9 ± 10.8 to 69.4 ± 8.8 (WRD), 54.4 ± 6.6 to 64.3 ± 7.8 (SRD) and 46.8 ± 7.9 to 60.6 ± 9.4 (SRI) (Table 3.4). The WRD region produced the hardest set of grain kernels, followed by the SRD region, while the SRI region produced the softest set of grain kernels. Detailed SKCS-HI results are presented in Appendix A (Tables A4 – A6).

3.3.2. Identification of puroindoline alleles in selected wheat cultivars

The cultivars of the WRD region did not show diversity in *Pina-D1* and *Pinb-D1* alleles as *Pina-D1a* and *Pinb-D1b* were identified in all the cultivars. Cultivars of the SRI region showed more diversity, with the presence of *Pina-D1a*, *Pina-D1b*, *Pinb-D1a*, and *Pinb-D1b* alleles. The cultivars of the SRD region had the most diversity of all the regions, with two additional novel *Pinb-D1* alleles, *Pinb-D1p* and *Pinb-D1ab* (Table 3.4). These alleles have seldom been identified in wheat germplasm, and to date have been found in Chinese and Japanese wheat cultivars (Chen *et al.*, 2006; Li *et al.*, 2008; Tanaka *et al.*, 2008; Wang, Li *et al.*, 2008; Kumar *et al.*, 2015). Detailed PCR and sequence results are reflected in Appendix A (Table A7). Base pair and amino acid positions numbered from N-terminal end as reported by Gautier *et al.* (1994) are also included for clarity.

SST875 was not pure and segregated for both *Pina-D1* and *Pinb-D1* loci. Cultivars carrying the *Pina-D1a* allele (wild-type; soft) amplified a fragment of 524 bp as determined on an agarose gel. The absence of an amplicon with the *Pina-D1*-specific primers and the simultaneous amplification of *Pinb-D1* were taken as confirmation that the *Pina-D1* locus has the *Pina-D1b* allele (null mutation). The *Pina-D1a* allele was confirmed with sequencing.

The *Pinb-D1* specific primers produced a 597 bp fragment in all cultivars. Comparison of sequence data with a reference sequence (GenBank accession number AB262660) revealed the presence of four alleles. In addition to the *Pinb-D1a* wild-type allele (soft), the *Pinb-D1b* allele (223G > A, Gly75Ser) was detected at a high frequency (Table 3.4 and Fig. 3.3). Elands had a single nucleotide deletion (213delA) (Fig. 3.4), which causes a frame-shift at amino acid position 71, resulting in the introduction of a premature stop codon 18 amino acids downstream. The mutation has been reported as *Pinb-D1p* by Xia *et al.* (2005). PAN3355 had a substitution (382C > T) (Fig. 3.5), changing the glutamine at position 128 to a stop codon, therefore also truncating the protein. Tanaka *et al.* (2008) reported this allele as *Pinb-D1ab*.

Table 3.4. Average SKCS-HI values over three years and four locations per cultivar as well as *Pina-D1* and *Pinb-D1* allele identity of each cultivar for each production region

Cultivar	SKCS-HI (mean ± SD)	*Pina-D1* allele	*Pinb-D1* allele
Summer rainfall dryland region (SRD)			
PAN3144	54.4 ± 6.6	*Pina-D1a*	*Pinb-D1b*
PAN3161	56.0 ± 7.0	*Pina-D1a*	*Pinb-D1b*
Gariep	58.0 ± 6.8	*Pina-D1a*	*Pinb-D1b*
Elands*	58.4 ± 5.9	*Pina-D1a*	*Pinb-D1p*
PAN3355	58.5 ± 6.8	*Pina-D1a*	*Pinb-D1ab*
SST347	59.1 ± 6.6	*Pina-D1a*	*Pinb-D1b*
SST398	59.5 ± 7.2	*Pina-D1a*	*Pinb-D1b*
SST356	62.1 ± 6.9	*Pina-D1b*	*Pinb-D1a*
PAN3379	64.3 ± 7.8	*Pina-D1b*	*Pinb-D1a*
Summer rainfall irrigation region (SRI)			
Duzi	46.8 ± 7.9	*Pina-D1a*	*Pinb-D1b*
Buffels	52.0 ± 10.9	*Pina-D1a*	*Pinb-D1b*
Baviaans	52.6 ^{B} ± 10.7	*Pina-D1a*	*Pinb-D1b*
PAN3471	55.1 ± 8.6	*Pina-D1b*	*Pinb-D1a*
SST835	56.7 ± 7.6	*Pina-D1b*	*Pinb-D1a*
SST806*	57.2 ± 7.8	*Pina-D1b*	*Pinb-D1a*
SST875**	59.1 ± 8.5	*Pina-D1a*/*Pina-D1b*	*Pinb-D1a*/*Pinb-D1b*
PAN3478	59.3 ± 8.6	*Pina-D1b*	*Pinb-D1a*
Olifants	60.6 ± 9.4	*Pina-D1b*	*Pinb-D1a*
Winter rainfall dryland region (WRD)			
Ratel	52.9 ± 10.8	*Pina-D1a*	*Pinb-D1b*
SST015	55.5 ± 10.4	*Pina-D1a*	*Pinb-D1b*
Baviaans	56.7 ^{A} ± 10.2	*Pina-D1a*	*Pinb-D1b*
Kariega*	57.4 ± 10.4	*Pina-D1a*	*Pinb-D1b*
SST056	58.4 ± 8.5	*Pina-D1a*	*Pinb-D1b*
PAN3434	59.2 ± 9.5	*Pina-D1a*	*Pinb-D1b*
SST096	64.3 ± 7.3	*Pina-D1a*	*Pinb-D1b*
SST087	65.0 ± 7.5	*Pina-D1a*	*Pinb-D1b*
SST88	69.4 ± 8.8	*Pina-D1a*	*Pinb-D1b*

Means followed by the same letter did not differ significantly at $P < 0.05$, SD – standard deviation, *bread wheat quality standards, **SST875 were heterozygous for *Pin* alleles.

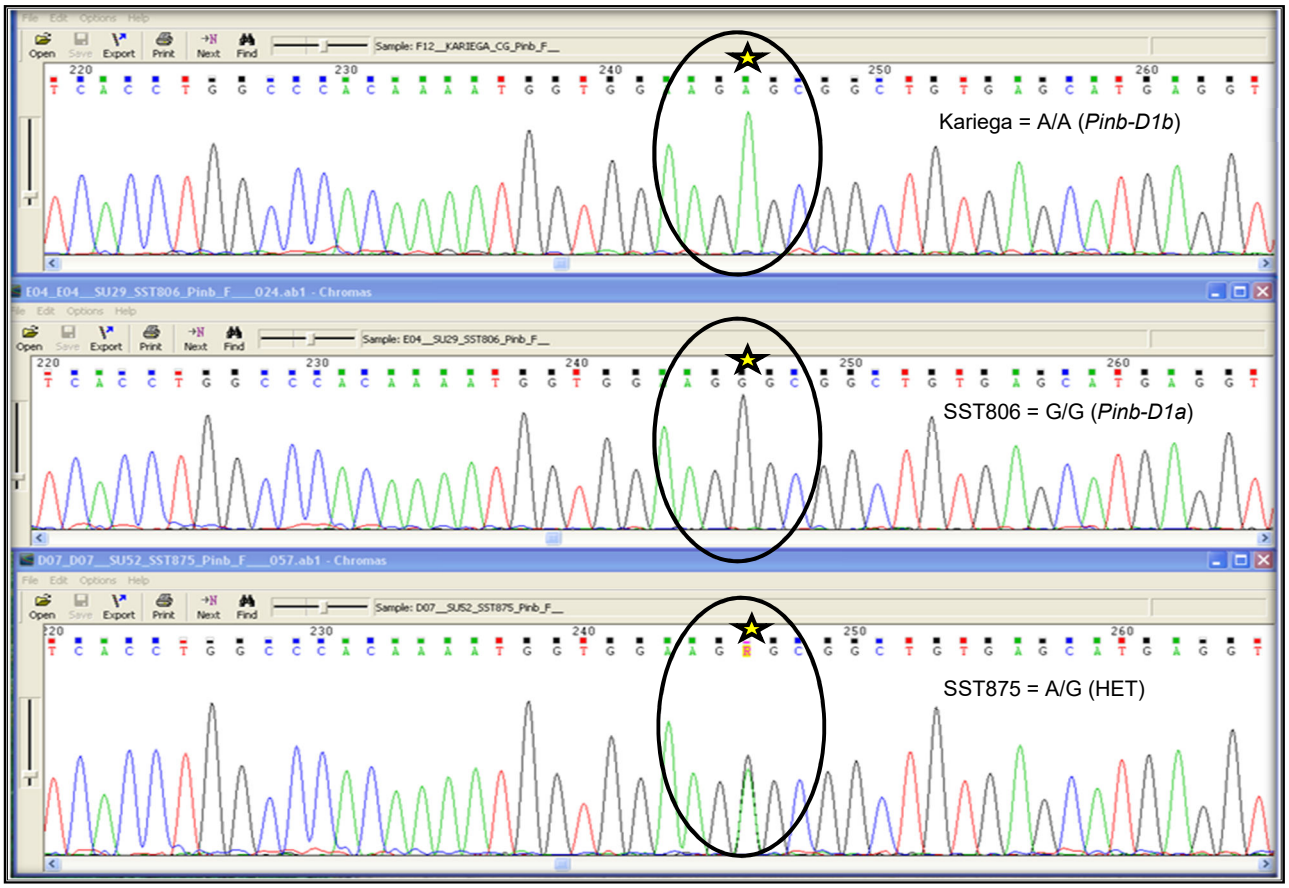


Figure 3.3. Electropherogram of *Pinb-D1a* (wild-type) and *Pinb-D1b* (mutation) alleles occurring in most cultivars. SST875 was heterozygous for these two alleles.

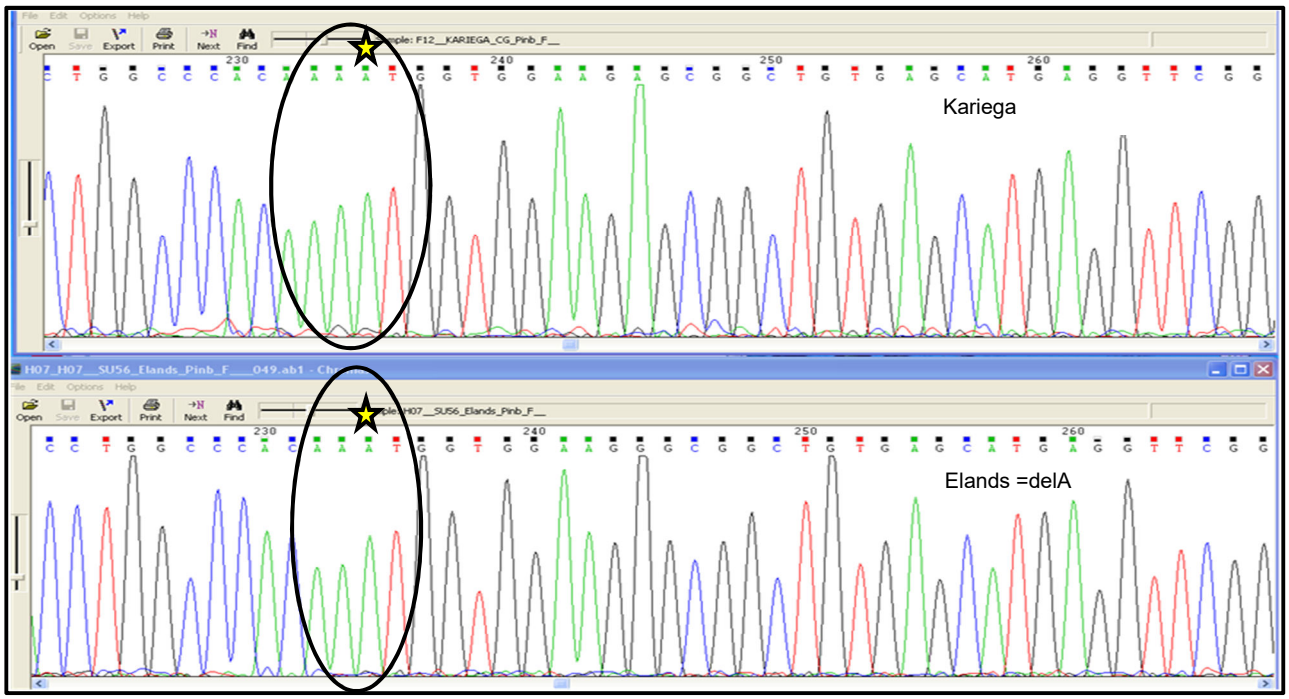


Figure 3.4. Electropherogram depicting the *Pinb-D1p* mutation in Elands compared to the *Pinb-D1b* mutation in Kariega.

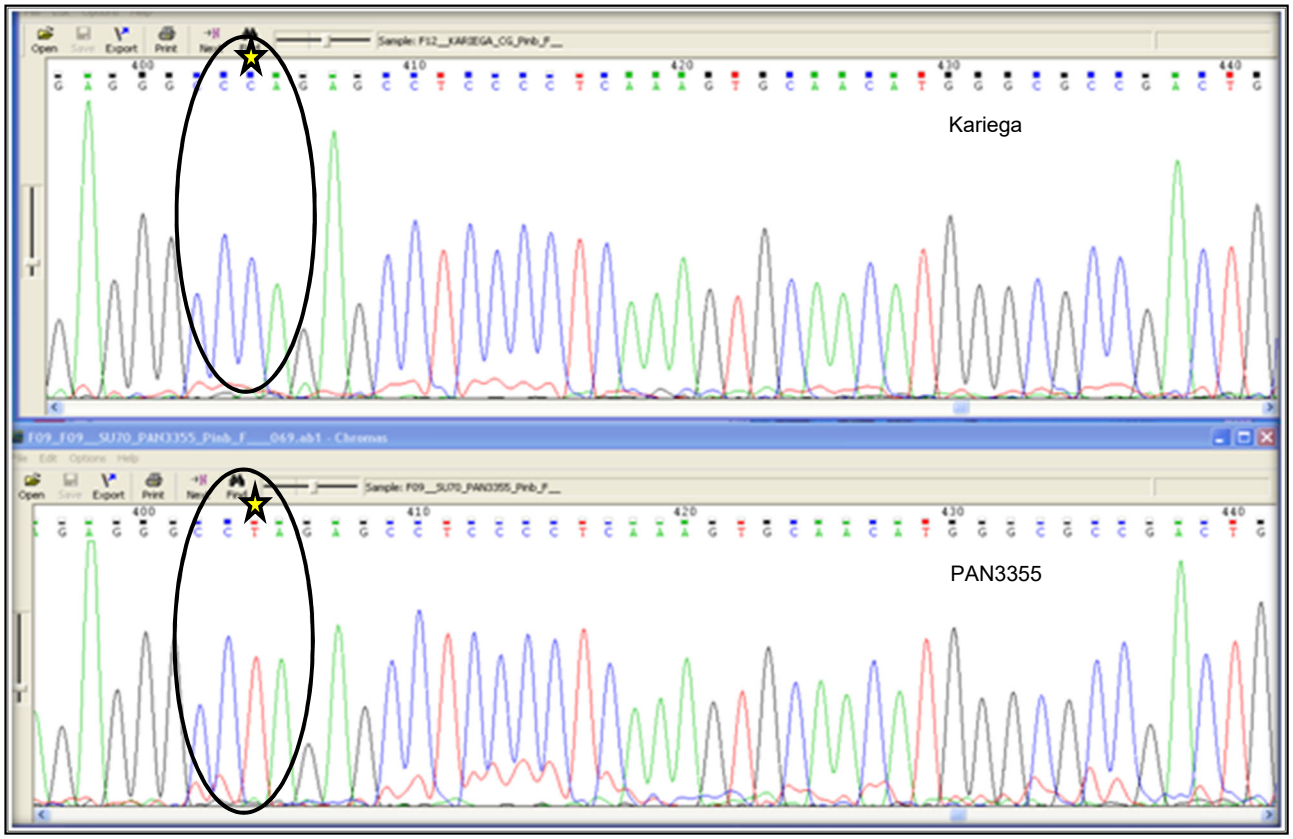


Figure 3.5. Electropherogram depicting the *Pinb-D1ab* mutation in PAN3355 compared to the *Pinb-D1b* mutation of Kariega.

3.3.3. Determination of grain hardness and puroindoline alleles

3.3.3.1. Summer rainfall dryland (SRD) region

Grain hardness and puroindoline allele frequency (SRD)

The occurrence of *Pina-D1a* was the highest (2.33 mean) of all the *Pina-D1* alleles. *Pinb-D1b* had the highest occurrence (1.67) for the *Pinb-D1* alleles, followed by *Pinb-D1a* (0.67). *Pinb-D1p* and *Pinb-D1ab* had the lowest occurrence of all *Pin* alleles with mean values of 0.33 each (Table 3.5).

The SKCS-HI values (mean 58.92 ± 2.93) ranged from 54.4 ± 6.6 to 64.3 ± 7.8; thus, the GKH of the kernels in the SRD region could be classified as medium-hard (Table 3.3).

Interaction of puroindoline alleles with grain hardness (SRD)

SKCS-HI had a significant ($P < 0.01$) negative correlation with *Pina-D1a* (-0.82) and significant ($P < 0.01$) positive correlations with *Pina-D1b* (0.82) and *Pinb-D1a* (0.82) (Table 3.6).

Principal component 1 (PC1) explained 60.12% of the variation in the data, followed by PC2 (20.29%), and PC3 (16.07%). A total variation of 96.48% was explained within the first three PCs (Table 3.7).

Table 3.5. Mean, standard deviation, range and standard error values for SKCS-HI and *Pin* alleles for the summer rainfall dryland (SRD) region

Variables	N	Mean ± SD	Range	SE
SKCS-HI	9	58.92 ± 2.93	54.45 – 64.26	0.98
Pina-D1a	9	2.33	0.00 – 3.00	
Pina-D1b	9	0.67	0.00 – 3.00	
Pinb-D1a	9	0.67	0.00 – 3.00	
Pinb-D1b	9	1.67	0.00 – 3.00	
Pinb-D1p	9	0.33	0.00 – 3.00	
Pinb-D1ab	9	0.33	0.00 – 3.00	

N – observations, Mean – mean values, SD – standard deviation, SE – standard error.

Table 3.6. Pearson's correlation matrix of SKCS-HI with *Pin* alleles for the summer rainfall dryland (SRD) region

Variables	*Pina-D1a*	*Pina-D1b*	*Pinb-D1a*	*Pinb-D1b*	*Pinb-D1p*	*Pinb-D1ab*
SKCS-HI	-0.82**	0.82**	0.82**	-0.61 ^{ns}	-0.06 ^{ns}	-0.06 ^{ns}

*P < 0.05, **P < 0.01, *** P < 0.001, ns – non-significant.

Table 3.7. Variation explained by each principal component and cumulative variation for the summer rainfall dryland (SRD) region

	PC 1	PC 2	PC 3	PC 4
Eigenvalue	4.21	1.42	1.13	0.25
Variation (%)	60.12	20.29	16.07	3.52
Cumulative %	60.12	80.41	96.48	100.00

Interpretation of PCA biplots was performed based on the following knowledge; the cosine of the angle between any two variable vectors is comparable to the variables' correlation with each other if the overall data fit is perfect. Thus, angles smaller than 90° between any two vectors indicate a positive correlation, i.e. they will similarly influence GKH; 90° angles indicate no correlation, and angles greater than 90° indicate a negative correlation (De la Vega & Chapman, 2006). A biplot uses points to represent scores of the observations on the principal components (PCs) (i.e. *Pin* allele identity presence in cultivars), and vectors are used to represent the coefficients of the variables on the PCs. The virtual location of points can be interpreted, i.e. points that lie close together indicate that observations have similar scores (i.e. cultivars have similar *Pin* allele identity). Vectors can be interpreted both in direction and in distance from the origin. Observations that point furthest in the same direction as a vector, have the highest amount of the variable represented by that specific vector; and similarly, observations of which the points lie furthest in the opposite direction, have least of that variable. Vectors that point in the same direction indicate that variables have similar response

profiles, and it can be interpreted that they have similar meanings in the context of the data set, i.e. GKH (Young & Valero, 1999).

The implementation of the PCA biplots was done to identify which *Pin* alleles correlate with SKCS-HI and could be used to predict GKH, should the *Pin* allele be present in a cultivar or breeding line. Knowledge of cultivar GKH (Table 3.4) was used to substantiate interpretation of the plots.

SKCS-HI, *Pina-D1a*, *Pina-D1b*, *Pinb-D1a* and *Pinb-D1b* contributed to the 60.12% variation in the data explained in PC1. *Pinb-D1b*, *Pinb-D1p* and *Pinb-D1ab* contributed to the 20.29% variation in the data explained in PC2. *Pinb-D1p* and *Pinb-D1ab* contributed to the 16.07% variation in the data explained in PC3 (Table 3.8).

The cosine angle (< 90°) between variables on the PCA biplots (Figs. 3.6a & b) indicates a positive correlation of SKCS-HI with *Pina-D1b* and *Pinb-D1a*. The vectors of these three variables point in the same direction and indicate that the variables have a similar response; thus, the presence of *Pina-D1b* and *Pinb-D1a* would cause an increase in SKCS-HI. Two cultivars, PAN3379 and SST356, were closely associated with SKCS-HI, *Pina-D1b* and *Pinb-D1a*. These two cultivars contain *Pina-D1b* and *Pinb-D1a* alleles and had the highest GKH in the SRD region (Table 3.4), verifying that *Pina-D1b* and *Pinb-D1a* cause an increased SKCS-HI.

The cosine angles (> 90°) indicated a negative correlation of SKCS-HI with *Pina-D1a* and *Pinb-D1b* (Figs. 3.6a & b). Observations of cultivars SST347, SST398, Gariep, PAN3161 and PAN3144 lie in the opposite direction of the vector representing SKCS-HI, indicating they have low values for SKCS-HI. These cultivars contain *Pina-D1a* and *Pinb-D1b* alleles and had the lowest GKH in the SRD region (Table 3.4), confirming the negative correlation of GKH with *Pina-D1a* and *Pinb-D1b*.

Table 3.8. Squared cosines of the variables for summer rainfall dryland (SRD) region

Variables	PC 1	PC 2	PC 3	PC 4
SKCS-HI	**0.80**	0.00	0.00	0.20
Pina-D1a	**0.97**	0.02	0.00	0.01
Pina-D1b	**0.97**	0.02	0.00	0.01
Pinb-D1a	**0.97**	0.02	0.00	0.01
Pinb-D1b	0.48	**0.51**	0.00	0.01
Pinb-D1p	0.01	0.43	**0.56**	0.00
Pinb-D1ab	0.01	0.43	**0.56**	0.00

Values in bold correspond to the PC for which the correlation per variable was the largest.

Prediction of grain hardness based on puroindoline allele identity (SRD)

Regression coefficients and the probability of the estimated variable(s), predicting SKCS-HI in the SRD region are reflected in Table 3.9. *Pina-D1a* contributed 68% of the total variation in SKCS-HI values. The negative parameter estimate for *Pina-D1a* confirms the negative correlation of *Pina-D1a* with SKCS-HI (Table 3.6). The prediction equation was calculated according to Leilah and Al-Khateeb (2005) and showed that the predicted (Ŷ) SKCS-HI in the SRD region could be calculated as shown in equation 3.2.

$$\hat{Y} = 63.17 - 1.82\,(Pina - D1a)$$

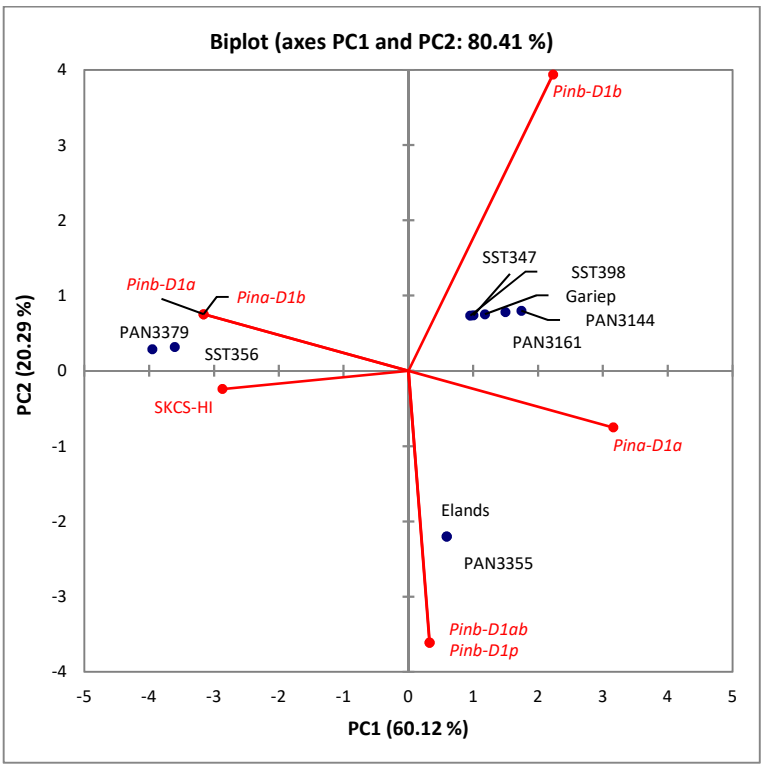
.... Equation 3.2

Table 3.9. Multiple linear regression model to predict SKCS-HI for the summer rainfall dryland (SRD) region

Variable entered	Parameter estimate	Partial R-square	Model R-square	F value	Pr > F	[a]Correlation R
Intercept	63.17			2529.91***	< 0.0001	
Pina-D1a	-1.82	0.68	0.68	14.72**	0.01	-0.82**

*P < 0.05, **P < 0.01, ***P < 0.001, ns – non-significant, a – Pearson's correlation values.

a)



b)

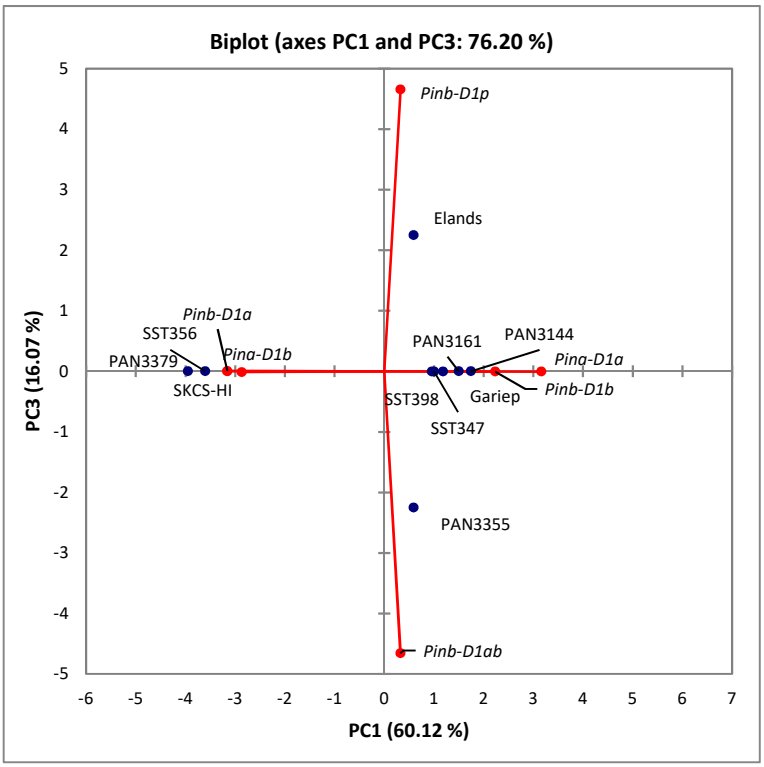


Figure 3.6. Grain hardness and *Pin* allele-centred biplots of a) PC1 vs. PC2, and b) PC1 vs. PC3 for grain hardness and *Pin* allele identity of the nine wheat cultivars across the summer rainfall dryland (SRD) region. Vectors and cultivars represent dependent variables (SKCS-HI and *Pin* alleles) by points.

3.3.3.2. Summer rainfall irrigation region

Grain hardness and puroindoline allele frequency (SRI)

For the SRI region, the occurrence of *Pina-D1b* was the highest (1.78 mean) for the *Pina-D1* alleles. *Pinb-D1a* had the highest (1.89) occurrence of all the alleles and the highest for the *Pinb-D1* alleles (Table 3.10). The SKCS-HI mean values (55.29) ranged from 46.56 to 60.43; thus, the kernels in the SRI region could be classified as medium-hard (Table 3.3).

Table 3.10. Means, standard deviation, range and standard error values for SKCS-HI and *Pin* alleles for the summer rainfall irrigation (SRI) region

Variables	N	Mean ± SD	Range	SE
SKCS-HI	9	55.29 ± 4.43	46.56 – 60.43	1.48
Pina-D1a	9	1.22	0.00 – 3.00	
Pina-D1b	9	1.78	0.00 – 3.00	
Pinb-D1a	9	1.89	0.00 – 3.00	
Pinb-D1b	9	1.11	0.00 – 3.00	

N – observations, Mean – mean values, SD – standard deviation, SE – standard error.

Interaction of puroindoline alleles with grain hardness (SRI)

SKCS-HI had significantly positive correlations with *Pinb-D1a* (0.81, $P < 0.01$) and *Pina-D1b* (0.73, $P < 0.05$) (Table 3.11). There were also significantly negative correlations of SKCS-HI with *Pina-D1a* (-0.73, $P < 0.05$) and *Pinb-D1b* (-0.81, $P < 0.01$).

Principal component 1 (PC1) explained 92.19% of the variation in the data, followed by PC2 (7.20%), with the total variation explained by the first two PCs being 99.38% (Table 3.12).

Table 3.11. Pearson's correlation matrix of SKCS-HI with *Pin* alleles for the summer rainfall irrigation (SRI) region

Variables	*Pina-D1a*	*Pina-D1b*	*Pinb-D1a*	*Pinb-D1b*
SKCS-HI	-0.73*	0.73*	0.81**	-0.81**

*P < 0.05, **P < 0.01, *** P < 0.001, ns – non-significant.

Table 3.12. Variation explained by each principal component and cumulative variation for the summer rainfall irrigation (SRI) region

	PC 1	PC 2	PC 3
Eigenvalue	4.61	0.36	0.03
Variation (%)	92.19	7.20	0.62
Cumulative %	92.19	99.38	100.00

SKCS-HI, *Pina-D1a*, *Pina-D1b*, *Pinb-D1a* and *Pinb-D1b* contributed to the 92.19% variation in the data explained in PC1. SKCS-HI contributed to the 7.20% variation in the data explained in PC2 (Table 3.13). The cosine angle (< 90°) between variables on the PCA variable plot (Fig. 3.7a) indicated a positive correlation of SKCS-HI with *Pina-D1b* and *Pinb-D1a*. The vectors of these three variables pointed in the same direction and indicated a similar response; thus, the presence of *Pina-D1b* and *Pinb-D1a* resulted in an increased SKCS-HI. Cultivars PAN3471, SST835, SST806, PAN3478 and Olifants, were closely associated with *Pina-D1b* and *Pinb-D1a* (Fig. 3.7b). These cultivars contained *Pina-D1b* and *Pinb-D1a* alleles and had the highest GKH in the SRI region (Table 3.4), verifying the positive correlation of SKCS-HI with *Pina-D1b* and *Pinb-D1a*.

Similarly, the cosine angles (> 90°) indicated a negative correlation of SKCS-HI with *Pina-D1a* and *Pinb-D1b* (Fig. 3.7a). Observations of cultivars Duzi, Buffels and Baviaans, laid in the opposite direction of the vector representing SKCS-HI, indicating low values for SKCS-HI in the cultivars (Fig. 3.7b). These cultivars contained *Pina-D1a* and *Pinb-D1b* alleles and had the lowest GKH in the SRI region (Table 3.4), verifying the observations of the negative correlation of GKH with *Pina-D1a* and *Pinb-D1b* (Table 3.13).

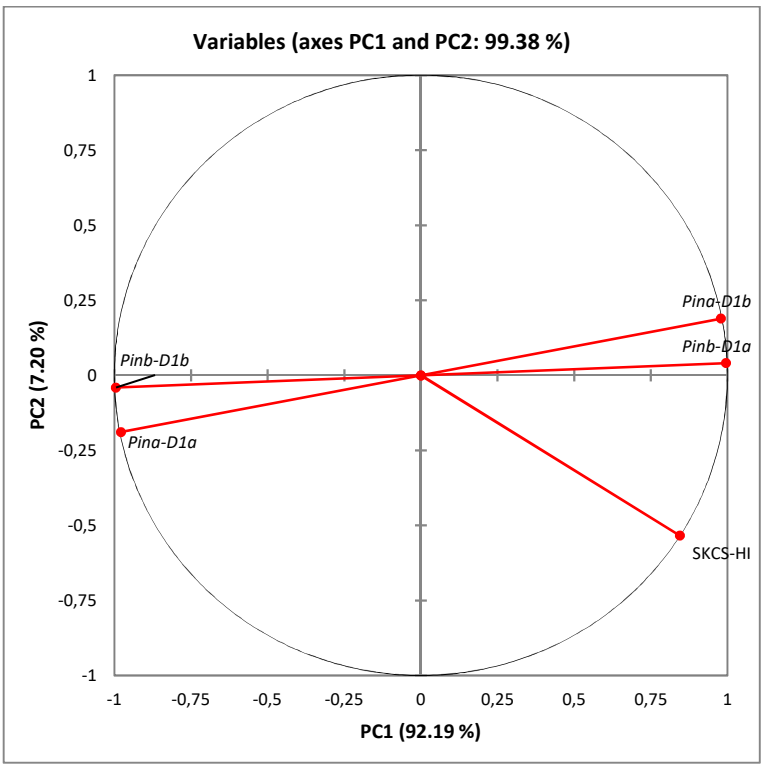
The heterozygous nature of cultivar SST875's *Pin* alleles settled the observation of SST875 in the middle of the PCA biplot (Fig. 3.7b), as it did not have high values for either of the variables (Table 3.13).

Table 3.13. Squared cosines of the variables for the summer rainfall irrigation (SRI) region

Variables	PC 1	PC 2	PC 3
SKCS-HI	**0.71**	0.29	0.00
Pina-D1a	**0.96**	0.04	0.01
Pina-D1b	**0.96**	0.04	0.01
Pinb-D1a	**0.99**	0.00	0.01
Pinb-D1b	**0.99**	0.00	0.01

Values in bold correspond to the PC for which the correlation per variable was the largest.

a)



b)

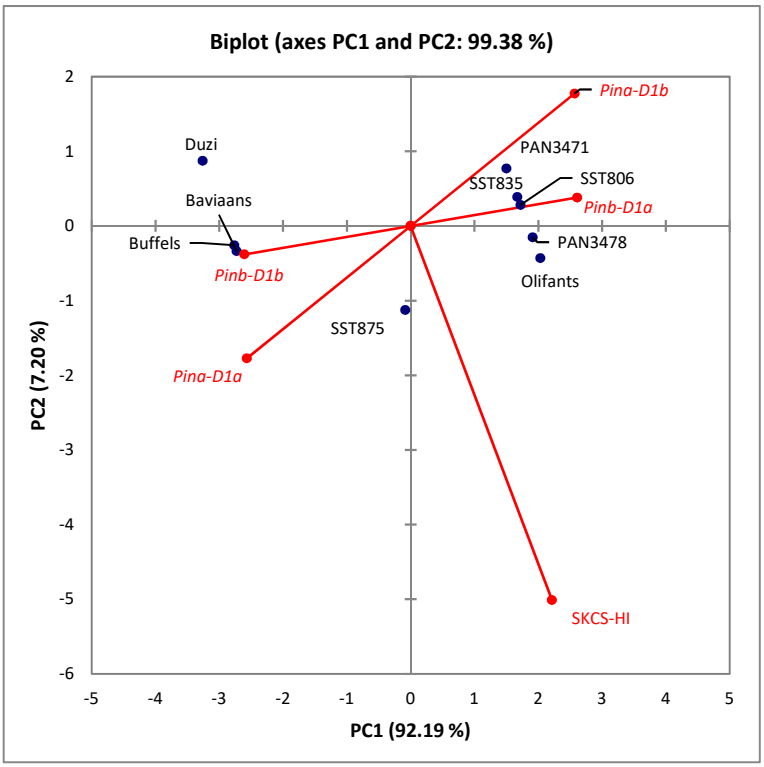


Figure 3.7. Grain hardness and *Pin* allele-centred a) variable plot of PC1 vs. PC2, and b) biplot of PC1 vs. PC2, for grain hardness and *Pin* allele identity of nine wheat cultivars across the summer rainfall irrigation (SRI) region. Vectors and cultivars represent dependent variables (SKCS-HI and *Pin* alleles) by points.

Prediction of grain hardness based on puroindoline allele identity (SRI)

Regression coefficients and the probability of the estimate variable(s) predicting SKCS-HI in the SRI region are presented in Table 3.14. *Pinb-D1a* contributed 66% of the total variation in SKCS-HI values. The positive parameter estimate for *Pinb-D1a* confirms the positive correlation of *Pinb-D1a* with SKCS-HI (Table 3.11). The prediction equation was calculated according to Leilah and Al-Khateeb, (2005) and showed that SKCS-HI for the SRI region (Ŷ) could be calculated as shown in equation 3.3.

$$\hat{Y} = 50.60 + 2.48\,(Pinb - D1a)$$

.... Equation 3.3

Table 3.14. Multiple linear regression model to predict SKCS-HI for the summer rainfall irrigation (SRI) region

Variable entered	Parameter estimate	Partial R-square	Model R-square	F value	Pr > F	[a]Correlation R
Intercept	50.60			1052.95***	< 0.0001	
Pinb-D1a	2.48	0.66	0.66	13.82**	0.01	0.81**

*P < 0.05, **P < 0.01, *** P < 0.001, ns – non-significant, a – Pearson's correlation values.

3.3.3.3. Winter rainfall dryland (WRD)

No variation in *Pina-D1* and *Pinb-D1* alleles in the WRD region was observed (Table 3.4), and thus no data analysis could be performed for this region.

3.3.3.4. SRI, SRD and WRD regions combined

Grain hardness and puroindoline allele frequency (combined regions)

The occurrence of *Pina-D1a* was the highest (2.19 mean) of all the *Pin* alleles, and it also had the highest occurrence for the *Pina-D1* alleles, followed by *Pina-D1b* (0.81). *Pinb-D1b* had the highest occurrence (1.93) of the *Pinb-D1* alleles, followed by *Pinb-D1a* (0.85). *Pinb-D1p* and *Pinb-D1ab* had the lowest occurrence of all the *Pin* alleles with mean values of 0.11 each (Table 3.15).

Table 3.15. Means, standard deviation, range and standard error values for SKCS-HI and *Pin* alleles for combined regions

Variable	N	Mean ± SD	Range	SE
SKCS-HI	27	58.02 ± 4.62	46.56 – 69.38	0.89
Pina-D1a	27	2.19	0.00 – 3.00	
Pina-D1b	27	0.81	0.00 – 3.00	
Pinb-D1a	27	0.85	0.00 – 3.00	
Pinb-D1b	27	1.93	0.00 – 3.00	
Pinb-D1p	27	0.11	0.00 – 3.00	
Pinb-D1ab	27	0.11	0.00 – 3.00	

N – observations, Mean – mean values, SD – standard deviation, SE – standard error.

Interaction of puroindoline alleles with grain hardness (combined regions)

SKCS-HI over the combined regions did not correlate significantly (P > 0.05) with any of the *Pin* alleles present (Table 3.16).

Principal component 1 (PC1) explained 54.07% of the variation in the data, followed by PC2 (17.18%), PC3 (14.84%) and PC4 (13.65%). A total variation of 99.74% was explained within the first 4 PCs (Table 3.17).

Table 3.16. Pearson's correlation matrix of SKCS-HI with *Pin* alleles for combined regions

Variables	*Pina-D1a*	*Pina-D1b*	*Pinb-D1a*	*Pinb-D1b*	*Pinb-D1p*	*Pinb-D1ab*
SKCS-HI	-0.16 ^{ns}	0.16 ^{ns}	0.16 ^{ns}	-0.17 ^{ns}	0.02 ^{ns}	0.02 ^{ns}

*P < 0.05, **P < 0.01, *** P < 0.001, ns – non-significant.

Table 3.17. Variation explained by each principal component and cumulative variation for the combined regions

	PC 1	PC 2	PC 3	PC 4	PC 5
Eigenvalue	3.79	1.20	1.04	0.96	0.02
Variation (%)	54.07	17.18	14.84	13.65	0.27
Cumulative (%)	54.07	71.25	86.09	99.74	100.00

Pina-D1a, *Pina-D1b*, *Pinb-D1a* and *Pinb-D1b* contributed to the 54.07% variation explained in PC1. *Pinb-D1b*, *Pinb-D1p* and *Pinb-D1ab* contributed to the 17.18% variation in the data explained in PC2. *Pinb-D1p* and *Pinb-D1ab* contributed to the 14.84% variation in the data explained in PC3, while SKCS-HI contributed to the 13.65% variation in the data explained in PC4 (Table 3.18).

The cosine angle (< 90°) between variables on the PCA biplot of PC1 and PC2 (Fig. 3.8a) indicated a positive correlation of SKCS-HI with *Pina-D1b*, *Pinb-D1a*, *Pinb-D1p* and *Pinb-D1ab*. The vectors of *Pina-D1b* and *Pinb-D1a*, and *Pinb-D1p* and *Pinb-D1ab* pointed in the same direction, which indicated that they had a similar response to increasing GKH. The cosine angle (< 90°) between variables on the PCA biplot of PC1 vs. PC3 (Fig. 3.8b) indicated a positive correlation of SKCS-HI with *Pina-D1b* and *Pinb-D1a*, and no correlation of SKCS-HI with *Pinb-D1p* and *Pinb-D1ab*. The vectors of SKCS-HI, *Pina-D1b* and *Pinb-D1a* pointed in the same direction, which indicated that they had a similar response to increasing GKH. Additionally, the cosine angles also indicated a negative correlation of SKCS-HI with *Pina-D1a* and *Pinb-D1b* (Figs. 3.8a & b). However, comparing to Pearson's correlation results (Table 3.16), these observed correlations in the biplot were non-significant (P > 0.05).

Table 3.18. Squared cosines of the variables for combined regions

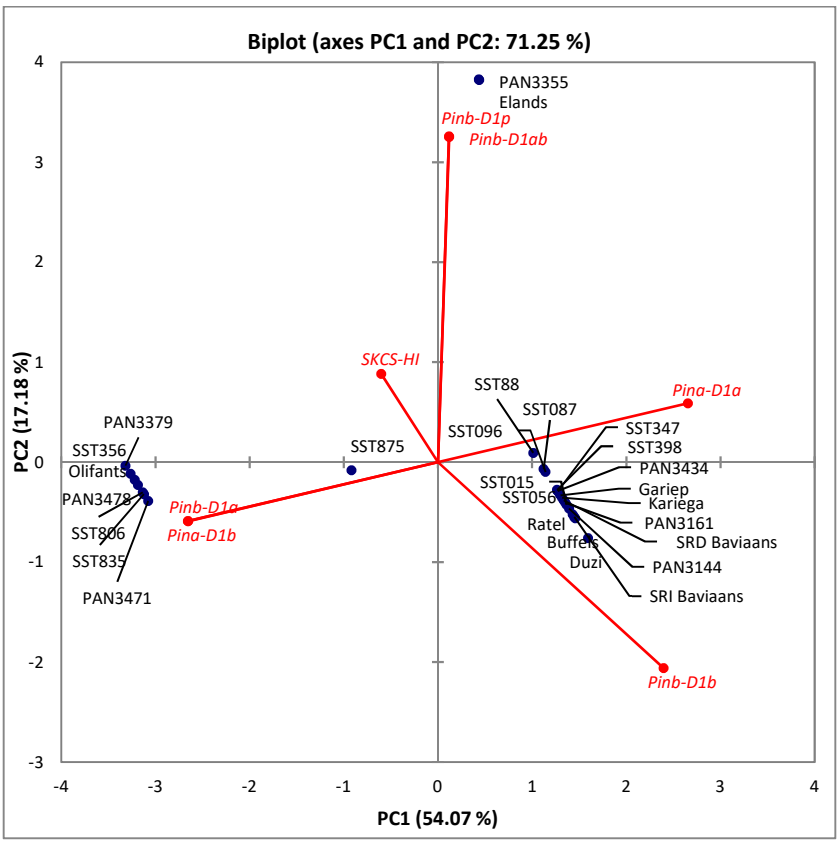
Variables	PC 1	PC 2	PC 3	PC 4	PC 5
SKCS-HI	0.05	0.03	0.00	**0.92**	0.00
Pina-D1a	**0.98**	0.02	0.00	0.00	0.00
Pina-D1b	**0.98**	0.02	0.00	0.00	0.00
Pinb-D1a	**0.98**	0.02	0.00	0.00	0.01
Pinb-D1b	**0.80**	0.19	0.00	0.01	0.00
Pinb-D1p	0.00	0.47	**0.52**	0.01	0.00
Pinb-D1ab	0.00	0.47	**0.52**	0.01	0.00

Values in bold correspond to the PC for which the correlation per variable was the largest.

Prediction of grain hardness based on puroindoline allele identity (combined)

There was no independent variable (i.e. *Pin* allele) that complied with the $P < 0.05$ significance level for entry into the stepwise MLR model; thus, no prediction of SKCS-HI could be made for the combined region.

a)



b)

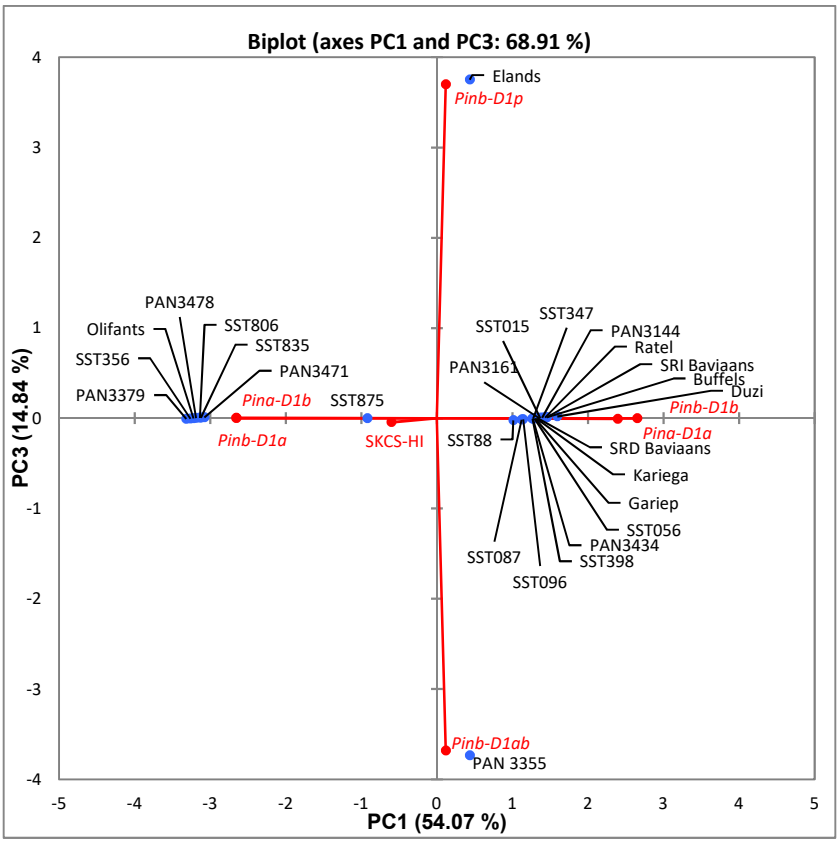


Figure 3.8. Grain hardness and *Pin* allele-centred biplot of a) PC1 vs. PC2, and b) PC1 vs. PC3 for grain hardness and *Pin* allele identity of 27 wheat cultivars across combined regions. Vectors represent dependent variables (SKCS-HI and *Pin* alleles), and points represent cultivars.

3.4. Discussion

Particular attention should be given to the method of determining phenotypic GKH. Most common measurements include either the single kernel characterisation system (SKCS) or near-infrared reflectance (NIR) spectroscopy. The SKCS method is based on the force needed to crush a wheat kernel, while the NIR spectroscopy method is based on the light scattering properties due to the difference in particle size of the flour after grinding. The NIR spectroscopy method has lower repeatability due to the required sample milling and potential error introduced in the sample preparation process (Boehm *et al.*, 2018). Thus, the SKCS method is more reliable to measure the true hardness properties of wheat grain kernels and is the method that was used in this study.

Perceived wheat hardness over different production regions

The WRD region produced the hardest set of grain kernels, followed by the SRD region, while the SRI region produced the softest set of grain kernels (Table 3.4). These regions all differed significantly in mean SKCS-HI values (Appendix A, Table A8). Grains kernels with the highest SKCS-HI were thus produced under dryland conditions. Growth requirements, i.e. spring vs. winter, were not a determining factor for GKH, since spring wheat (WRD) and winter wheat (SRD) both produced harder grains than SRI spring wheat. Grain kernel quality and size are typically somewhat affected by environmental conditions (Dupont & Altenbach, 2003). Saint Pierre *et al.* (2008) showed that an increase in water stress positively correlated with an increase in GKH.

The cultivar Baviaans is adapted to both the WRD and SRI regions and was planted in the trials for both these regions. As expected, the SKCS-HI was significantly higher (Table 3.4) for Baviaans grown in the WRD (56.7 ± 10.2) compared to that grown in the SRI (52.6 ± 10.7) region. Additionally, Baviaans' grain kernels produced in the WRD region had a higher kernel moisture content (12.66 ± 7.94% WRD vs. 12.03 ± 1.10% SRI), were heavier (42.38 ± 5.12 mg WRD vs. 40.50 ± 5.08 mg SRI) and had a bigger kernel diameter (2.66 ± 0.22 mm WRD vs. 2.59 ± 0.23 mm SRI) than grain kernels of Baviaans produced in the SRI region (Appendix A, Table A9). These differences illustrate the effect of the environmental impact on GKH when the same cultivar is planted in two production regions.

The diversity of puroindoline alleles in wheat germplasm

The spring wheat cultivars planted in the WRD region had *Pina-D1a* and *Pinb-D1b* present; while spring wheat cultivars planted in the SRI region had *Pina-D1a*, *Pina-D1b* and *Pinb-D1a, Pinb-D1b* alleles present. The facultative and winter wheat cultivars planted in the SRD region had two additional novel alleles, namely *Pinb-D1p* and *Pinb-D1ab*. *Pinb-D1p* is one of the most frequent alleles found in wheat cultivars of Chinese origin, and the allelic combination *Pina-D1a/Pinb-D1p* as identified in Elands is also commonly found in Chinese wheat cultivars (Chen *et al.*, 2006; Li *et al.*, 2008; Kumar *et al.*, 2015). PAN 3355 had the allele *Pinb-D1ab* present, which was originally detected

in a Japanese wheat line (Tanaka *et al.*, 2008) and later also in a Chinese wheat cultivar (Wang, Sun *et al.*, 2008). Elands and PAN 3355 are facultative winter wheat cultivars that require cold temperatures for optimal vernalisation and tillering; which implies, they could potentially have Japanese or Chinese ancestry.

The most abundant allelic combination in the South African cultivars was *Pina-D1a/Pinb-D1b*, which is also the most frequent combination in bread wheat produced globally (Chen *et al.*, 2013; Ma *et al.*, 2017). The other allelic combinations found were *Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*. One cultivar, SST 875, displayed a mixture of *Pina-D1a/Pinb-D1b* and *Pina-D1b*/*Pinb-D1a* genotypes. The grain kernels produced by this cultivar would thus differ in GKH. Other research studies indicated that it is not uncommon to find wheat cultivars that contain a mixture of *Pin* genotypes (Morris, King *et al.*, 2001; Morris, Lillemo *et al.*, 2001; Cane *et al.*, 2004). Morris, King *et al.* (2001) suggested that variation in GKH caused by a mixture of *Pin* genotypes could be desirable to produce Asian noodles. In breeding programmes, however, it is not desirable to use a wheat cultivar that is heterozygous, since the unintended use of a wrong single plant, as a parent during the crossing stage of wheat breeding, could have devastating effects (Cane *et al.*, 2004).

In South African breeding programmes, the quality selection is performed based on specified release criteria for baking quality acceptable to the South African milling- and baking industries. These criteria have been developed to ensure that newly released South African wheat cultivars have the quality suitable for bread making. It thus encourages the selection of medium to hard wheat (O'Brien & DePauw, 2004). Therefore, it is not surprising that the allelic combination of *Pina-D1a*/*Pinb-D1a*, expressing soft grain texture (Giroux & Morris, 1998) was not found in South African germplasm. It also explains the low variation of *Pin* alleles observed in general.

Interaction of puroindoline alleles with grain hardness

In the SRD region, the negative correlation of SKCS-HI with *Pina-D1a* was expected, since *Pina-D1a* is the wild-type *Pina-D1* allele. *Pin* alleles in their wild-type cause soft kernel texture (Giroux & Morris, 1998); hence, the negative correlation with GKH. The positive correlation of SKCS-HI with *Pina-D1b* could be expected since it is a null allele, and a mutation in the allelic structure or deletion of a *Pina-D1* and *Pinb-D1* gene causes harder grain kernels (Giroux & Morris, 1998). The positive correlation of *Pinb-D1a* with SKCS-HI values was unexpected, since *Pinb-D1a* is a wild-type allele that should cause soft grain endosperm (Giroux & Morris, 1998).

Studies have indicated that the amount of PINB protein associated with starch granules is highly reduced in the absence of the PINA protein (Corona *et al.*, 2001; Capparelli *et al.*, 2003; Turnbull *et al.*, 2003). This difference in the amount of PIN protein associated with the starch granule surface could be due to the different starch granule membrane affinities of PINA and PINB proteins. A null mutation in *Pina-D1* (*Pina-D1b*) results in almost no starch granule-associated PIN protein, while a null mutation in *Pinb-D1* (*Pinb-D1p*), or severely reduced function of the PINB protein as with *Pinb-*

D1b, still has a substantial amount of starch granule-associated PIN protein present. The expression of *Pina-D1* and the presence of PINA protein control the abundance of the total amount of PIN protein and the association thereof with the starch granule surface (Capparelli *et al.*, 2003). This implies that the influence of *Pina-D1* on perceived hardness is much greater than that of *Pinb-D1*. Comparing the presence of wild-type alleles, *Pina-D1a* would result in more PIN protein associated with the starch granule surface than *Pinb-D1a.* Thus, a softer grain kernel in the instance of the *Pina-D1* wild-type allele and in comparison, a harder grain kernel with the presence of the *Pinb-D1* wild-type allele. This explains the negative correlation of SKCS-HI with *Pina-D1a,* resulting in softer kernels; and the positive correlation of SKCS-HI with *Pinb-D1a*, which results in harder grain kernels.

It was evident that in the SRD region that the *Pina-D1b*/*Pinb-D1a* genotype caused harder grain kernels than the allelic combination of *Pina-D1a*/*Pinb-D1b*. This is in accordance with several other research studies that found the *Pina-D1b*/*Pinb-D1a* genotype to have harder grain kernels than the *Pina-D1a*/*Pinb-D1b* genotype (Giroux *et al.*, 2000; Chang *et al.*, 2006; Chen *et al.*, 2006; Geng *et al.*, 2013). The GKH of the *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab* genotypes are comparable to the GKH of the *Pina-D1a*/*Pinb-D1b* genotype.

Similar to the SRD region, the SKCS-HI had a negative correlation with *Pina-D1a* in the SRI region, since *Pina-D1a* is the wild-type *Pina-D1* allele. The positive correlation with *Pina-D1b* could be expected since it is a null allele that causes an increase in GKH (Giroux & Morris, 1998). The same phenomenon could explain the positive correlation of *Pinb-D1a* with SKCS-HI as in the SRD region due to the greater influence of *Pina-D1* and *Pinb-D1* on GKH. Despite both being wild-type alleles, *Pinb-D1a* unexpectedly resulted in higher SKCS-HI values compared to *Pina-D1a*. SKCS-HI showed a negative correlation with *Pinb-D1b*; however, *Pinb-D1b* express a PINB protein with severely reduced function and should produce harder grain kernels (Giroux & Morris, 1998). In this study, *Pinb-D1b* was present in the allelic combination with *Pina-D1a*. The definite effect of the presence of the *Pina-D1a* allele on the overall GKH can be related to earlier work concerning the PINA and PINB proteins (Corona *et al.*, 2001; Capparelli *et al.*, 2003; Turnbull *et al.*, 2003). The negative correlation of *Pinb-D1b* with SKCS-HI could be attributed to the association of *Pinb-D1b* with *Pina-D1a*. In the SRI region, it was evident that cultivars with the *Pina-D1b*/*Pinb-D1a* genotype had harder wheat kernels than cultivars with the *Pina-D1a*/*Pinb-D1b* genotype.

When *Pin* alleles and SKCS-HI over the combined production regions were analysed, there was no correlation ($P > 0.05$) of the *Pin* alleles with SKCS-HI. None of the correlations of *Pin* alleles with SKCS-HI complied with the $P < 0.05$ significance level for entry into the stepwise MLR model. Although there were no significant correlations ($P > 0.05$), the same trend was observed in the combined regions as in the SRD and SRI regions. SKCS-HI had a positive association with *Pina-D1b* and *Pinb-D1a* and a negative association with *Pina-D1a* and *Pinb-D1b*.

Although a diversity of *Pin* alleles was not identified in the WRD region of South Africa, there were significant differences in GKH between cultivars in this study. The significant difference in GKH

between cultivars with the same *Pin* genotype implies that there may be other genetic factors that influence wheat GKH in addition to *Pin* alleles. This is in accordance with the findings of other studies that also suggest the involvement of other genetic factors in wheat GKH (Surma *et al.*, 2012; Nirmal *et al.*, 2016).

3.5. Conclusion

The cultivars used in this study were commercial cultivars that have been adapted agronomically for production in each region. Cultivars adapted to the same environmental conditions and regions could be related to each other, due to use of the same parent/s in the breeding process. Thus, providing the possibility of related genes between cultivars that could influence the expression of wheat hardness and GKH results that could be influenced by genetic factors other than *Pin* alleles. All variables related to the environment were accounted for in the experimental design with data over several locations, replications and years being used.

Wheat breeding in South Africa is performed for agronomic adaptability of wheat lines, grain yield, disease resistance of the plant, and baking quality acceptable to the South African milling- and baking industries. The selected baking quality criteria used inherently forces the selection of other wheat grain traits, in combination with baking quality traits, such as GKH. The diversity of *Pin* alleles observed in South African wheat cultivars is such that it complies with local flour quality requirements and the related wheat GKH.

The WRD wheat-producing region of South Africa with spring wheat did not have any diversity in *Pin* alleles. The SRI region with spring wheat had more diversity, and the SRD region with facultative and winter wheat had the most diversity in *Pin* alleles.

The great diversity of wheat-planting regions in South Africa, and the unique environmental factors affecting each of them, make it challenging to predict SKCS-HI over the combined regions from the identified *Pin* alleles. Water and other environmental stress factors, such as those found under dryland conditions, caused harder grain kernels than in regions with fewer environmental stress factors, such as production under irrigation. Due to the considerable variation between the different regions, overall prediction models are not advisable, and models should rather be developed for each production region.

In both the SRD and SRI regions, the allelic combination of *Pina-D1a*/*Pinb-D1b* was more often identified in wheat with softer kernel texture than the allelic combination of *Pina-D1b*/*Pinb-D1a*. This could be due to the influence of *Pina-D1* expression on perceived hardness being much higher than that of *Pinb*. The *Pina-D1a*/*Pinb-D1a* genotype was not identified in the investigated cultivars. Based on the results obtained, the ranking of *Pin* genotypes to produce hard to soft wheat kernels would be *Pina-D1b*/*Pinb-D1a* > *Pina-D1a*/*Pinb-D1b* > *Pina-D1a*/*Pinb-D1a*. The *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab* genotypes produced wheat kernels with hardness comparable to that of the

Pina-D1a/*Pinb-D1b* genotype; however, a more substantial test population with these combinations is required to confirm this finding.

The current research is the first that provides insight into the *Pin* alleles present in South African wheat cultivars. The medium-hard to hard wheat classes to which all South African bread wheat cultivars belong, enable millers to provide flour suitable for the required end product, i.e. bread baking. By identifying the *Pin* alleles present in South African commercial wheat cultivars, the inherently selected *Pin* alleles that provide South Africa with acceptable flour processing quality and related GKH has been identified. Marker-assisted selection for wheat lines that contain a specific *Pin* genotype could be used to target GKH for soft bread wheat (*Pina-D1a*/*Pinb-D1a)* suitable for biscuits, and medium-hard (*Pina-D1a*/*Pinb-D1b*) or hard bread wheat (*Pina-D1b*/*Pinb-D1a*) suitable for leavened bread. This study was performed on a selected number of cultivars per production region. However, it would be beneficial to identify the *Pin* alleles present in all South African commercial wheat cultivars. This would enable wheat breeders to generate information regarding the diversity of *Pin* alleles in South African wheat cultivars that could be used in future wheat breeding.

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CHAPTER 4

Effect of *puroindoline a* and *b* allelic variation on bread wheat (*Triticum aestivum*) grain characteristics and flour processing quality in two South African production regions

Abstract

Wheat cultivars, with known *puroindoline* (*Pin*) genotypes, were planted in two wheat production environments. Nine spring wheat cultivars were planted for three consecutive seasons at four locations in the summer rainfall irrigation (SRI) region. Similarly, nine facultative and winter wheat cultivars, were planted in the summer rainfall dryland (SRD) region, also at four locations for three consecutive seasons. Grain kernel characteristics, milling performance, flour components and various flour and dough quality properties were determined for the wheat samples. The objectives were to determine the influence of the two most common *Pin* genotypes, *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b*, on the measured quality properties. Additionally, the influence of three *Pinb-D1* allele mutations on the quality properties of wheat, produced in the SRD region, were determined. Wheat containing the *Pina-D1b*/*Pinb-D1a* genotype was found to have increased grain kernel hardness (GKH), flour water absorption, dough tenacity and alveograph P/L ratio, in comparison to wheat with the *Pina-D1a*/*Pinb-D1b* genotype. Wheat containing the *Pina-D1b*/*Pinb-D1a* genotype also resulted in decreased kernel weight, and -diameter, break flour yield (BFY), total flour yield (TFY), dough extensibility, -strength and -stability and tolerance to overmixing, in comparison to wheat with the *Pina-D1a*/*Pinb-D1b* genotype. Comparing three *Pinb-D1* mutations indicated that wheat with the *Pinb-D1p* mutation had decreased kernel weight and -diameter as well as decreased dough extensibility and swelling index in comparison to wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations. Wheat with the *Pinb-D1p* mutation resulted in increased BFY and TFY, flour water absorption, dough development time, dough strength and -tenacity in comparison to wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations. Knowledge on the influence of *Pin* genotype combinations and *Pin* allele mutations would provide valuable information for the wheat breeding and processing industries. When a wheat breeder implements marker assisted selection (MAS) on early generations (F5) of breeding lines, the desired grain-, milling- and flour quality properties can be selected based on the results obtained for the *Pin* genotypes and/or *Pin* mutations investigated in this study.

4.1. Introduction

Grain kernel hardness (GKH) is one of the essential characteristics of wheat grain, as it determines milling performance and flour processing quality. GKH is controlled by the hardness, *Ha*, gene located on chromosome 5DS (Mattern *et al.*, 1973; Baker & Dyck, 1975; Law *et al.*, 1978). The *Ha*

gene consists of three tightly linked genes coding for *puroindoline a* (*Pina-D1*), *puroindoline b* (*Pinb-D1*) and *grain softness* (*Gsp-1*) proteins (Blochet *et al.*, 1991; Gautier *et al.*, 1994; Rahman *et al.*, 1994). Combined, these three proteins form friabilin, that has been linked to GKH (Greenwell & Schofield, 1986; 1989). The binding properties of friabilin, to the starch-granule surface, are influenced by the *Pin* allele genotype of the wheat cultivar. The two PIN proteins are the primary contributors to GKH, with no current evidence on the involvement of grain softness protein (*Gsp-1*) (Beecher *et al.*, 2002; Tranquilli *et al.*, 2002; Martin *et al.*, 2006).

The combination of wild-type alleles of *Pina-D1* and *Pinb-D1*, which is *Pina-D1a* and *Pinb-D1a* respectively, both cause soft grain kernels. The alteration of the *Pina-D1* or *Pinb-D1* alleles, due to mutations or deletions in their deoxyribonucleic acid (DNA) sequence, causes the expression of a PINA or PINB protein with different functional quality compared to the wild-type proteins. The mutation in *Pina-D1* or *Pinb-D1* alleles influence the functional quality of the expressed PINA and PINB proteins, this causes increased GKH (Giroux & Morris, 1998; Lillemo & Morris, 2000; Morris *et al.*, 2001). Puroindolines are integral membrane proteins that strongly bind to polar lipids, and therefore, starch membranes. The tryptophan-rich domain (TRD) in the PIN protein form membrane-anchoring loops between α-helices in the starch-granule membrane (Greenblatt *et al.*, 1995). PINA protein contains five tryptophan amino acids compared to three in PINB protein, which implies that PINA will bind more strongly to starch-granule membranes.

The percentage of PINA protein expressed in the endosperm of wheat, is higher than the expression of PINB protein. Studies have indicated that the amount of PINB protein associated with starch granules are highly reduced in the absence of the PINA protein (Corona *et al.*, 2001; Capparelli *et al.*, 2003; Turnbull *et al.*, 2003). This difference in the amount of starch granule-associated PIN protein could be due to the different starch granule membrane affinities of PINA and PINB proteins. Wheat with a null mutation in *Pina-D1* results in almost no starch granule-associated PIN protein, while wheat with a null mutation in *Pinb-D1* still has a substantial amount of starch granule-associated PIN protein present. The expression of *Pina-D1* alleles, and the presence of PINA protein in wheat, control the abundance of the total amount of PIN protein and their association with the starch granule surface (Capparelli *et al.*, 2003). Puroindoline-lipid-binding affects GKH, but also has an essential function in flour and dough processing. Puroindoline protein assists by producing stable foams during dough formation (Dubreil *et al.*, 1998), and by stabilising the formation and expansion of gas cells in the dough (Igrejas *et al.*, 2001).

Various studies (Giroux *et al.*, 2000; Martin *et al.*, 2001; Ma *et al.*, 2009; Takata *et al.*, 2010) have found that wheat grain with the *Pina-D1b*/*Pinb-D1a* genotype has harder grain kernels and higher flour water absorption, but lower milling yield, than wheat grain with the *Pina-D1a*/*Pinb-D1b* genotype (Cane *et al.*, 2004; Eagles *et al.*, 2006; Edwards *et al.*, 2010; Chen *et al.*, 2013).

The mutations of *Pinb-D1* encountered in the current research comprised the *Pinb-D1b*, *Pinb-D1p* and *Pinb-D1ab* alleles. The *Pinb-D1b* allele expresses a PINB protein with severely reduced functionality compared to the *Pinb-D1* wild-type allele. Giroux and Morris (1997) discovered the first

mutation in either *Pina-D1* or *Pinb-D1*, namely the *Pinb-D1b* mutation. It has been suggested that the amino acid change of glycine to serine at amino acid 46 affects the interaction of the expressed PINB protein with the starch granule surface (Corona *et al*. 2001), possibly due to the influence on properties of the TRD which contains the lipid-binding loop. Xia *et al.* (2005) reported the *Pinb-D1p* allele as a single base deletion resulting in a lysine to aspartame change at amino acid 42. The *Pinb-D1p* mutation leaves only one tryptophan in the TRD and generates a premature stop codon downstream. A single nucleotide polymorphism (SNP) that results in a premature stop codon towards the carboxyl terminus is the mutation present in the *Pinb-D1ab* allele (Tanaka *et al.*, 2008).

With the *puroindoline* allelic genotype of nine South African spring wheat irrigation (SRI) cultivars and nine facultative winter wheat dryland (SRD) cultivars determined (Chapter 3), it would be valuable to compare the influence of these genotypes on grain-, milling- and flour quality properties. Knowledge on the wheat quality that could be expected with the presence of the *Pin* allele genotype of a breeding line, would enable wheat breeders to do selections for wanted and/or unwanted grain and flour quality properties. These selections could be done in early generations (F5) in the breeding process by using marker assisted selection (MAS).

The objectives of this study were to determine: 1) the effect of a null allele expression at the *Pina-D1* locus (*Pina-D1b*) compared to the *Pinb-D1b* mutation on grain-, milling- and flour quality properties in spring wheat planted in the summer rainfall irrigation (SRI) region, as well as, facultative and winter wheat cultivars planted in the summer rainfall dryland (SRD) region; and 2) the effect of different mutations at the *Pinb-D1* locus, i.e. *Pin-D1b*, *Pin-D1p* and *Pin-D1ab*, on grain-, milling- and flour quality properties.

4.2. Materials and Methods

4.2.1. Experimental population and field trials

Wheat samples used were planted in the summer rainfall irrigation (SRI) region and the summer rainfall dryland (SRD) region of South Africa for three consecutive seasons (2012 – 2014), as described in Chapter 3.

In the SRI region, the wheat quality standard (SST 806) and eight other commercial cultivars over a range of kernel hardness were included in the trials. The selected cultivars have been identified to have two *Pin* allelic genotypes (Chapter 3). Cultivars Duzi, Buffels and Baviaans, had the *Pin* genotype *Pina-D1a*/*Pinb-D1b*; while cultivars PAN3471, SST835, SST806, PAN 3478 and Olifants had the *Pin* genotype *Pina-D1b*/*Pinb-D1a*. The cultivar SST875 was heterozygous for the *Pin* alleles and was excluded for the purpose of the current study. The locations in the SRI region were represented by Hartsvallei, Lichtenburg, Marblehall, and Winterton (Fig. 4.1).

In the SRD region, the wheat quality standard (Elands) and eight other commercial cultivars over a range of GKH were included in the trails. The cultivars have been identified to have four *Pin* allelic genotypes (Chapter 3). Cultivars PAN3144, PAN3161, Gariep, SST347 and SST398 had the *Pin*

genotype *Pina-D1a*/*Pinb-D1b*; cultivars SST356 and PAN3379 had the *Pin* genotype *Pina-D1b*/*Pinb-D1a*; Elands had the *Pin* genotype *Pina-D1a*/*Pinb-D1p*, and PAN3355 had the *Pin* genotype *Pina-D1a*/*Pinb-D1ab*. The locations representing the SRD region were Bethlehem, Bultfontein, Clocolan, Ladybrand, Reitz, Virginia and Wesselsbron (Fig. 4.1).

Figure 4.1. Map of South Africa indicating the four trial locations in the summer rainfall irrigated production region, and seven locations in the summer rainfall dryland production region.

4.2.2. Grain analysis

4.2.2.1. Single kernel characterisation system

The Single Kernel Characterisation System (SKCS) was used to determine the physical hardness of wheat kernels, by determining the physical force needed to crush a wheat kernel. The SKCS analysis was performed according to the American Association for Cereal Chemists (AACC) approved method 55-31.01 (AACC, 1999a) using the SKCS model 4100 (Perten Instruments, Hägersten, Sweden). One analysis per sample was conducted, using 300 kernels per analysis to determine SKCS hardness index (SKCS-HI), kernel moisture content (SKCS-Moist), kernel weight (SKCS-Weight) and kernel diameter (SKCS-Dia).

4.2.2.2. Grain protein

The FOSS Infratec™ 1241 near-infrared transmittance (NIT) grain analyser (FOSS Analytics, Hillerød, Denmark), with FOSS wheat grain calibration no. 096126 was used to determine the grain protein content (Gprot) of the whole grain sample. The Infratec™ 1241 used near-infrared transmittance technology with a scanning monochromator, and silicon detector in the wavelength range of 570 to 1100 nm to capture 265 data points of each sample scanned to predict whole grain protein. This instrument is rapid, reliable and easy to use, it is accepted as the official system used

by many bulk grain handlers around the world. To ensure the reliability of results, the instrument was monitored daily with a reference sample and serviced yearly.

4.2.3. Milling characteristics

The first step in the milling process was to temper the wheat sample to the desired moisture level to facilitate better separation of the endosperm and bran during milling. The GKH and kernel moisture content as determined using the SKCS were used to calculate the desired amount of water to add to the wheat sample. Table 4.1 was used to determine the desired moisture content after tempering based on the SKCS-HI.

Table 4.1. Recommended final tempering moisture content for different kernel hardness classes

Kernel hardness (SKCS-HI)	Desired moisture (%)
0-15	15.0
16-30	15.3
31-45	15.5
46-60	15.7
61-75	15.9
76-90	16.1
91-100	16.3

SKCS-HI – single kernel characterisation system hardness index.

The amount of water required to temper 1.8 kg of wheat was determined using the AACC approved method 26-95.01 temper tables (AACC, 1999b). The original moisture content of the wheat sample and the desired final moisture content were used to determine the amount of water required to add to the sample. The wheat grain and water were combined in a container, and enough water dispersion was accomplished by rotating the containers for 25 min. The tempered grain was then stored in an airtight container for 24 h before experimental milling commenced.

Milling was performed using a Chopin CD1 laboratory mill (Chopin technologies, Paris, France), by following the AACC approved method 26-70.01 (AACC, 2015). The grain was passed through the break mill, resulting in 1st break flour, 1st break semolina and bran. Subsequently, the 1st break semolina was passed through the reduction mill, resulting in 2nd break semolina and 2nd break flour. The 2nd break semolina was passed through the reduction mill and the resulting 3rd break flour and shorts. After each break and reduction step, all fractions were weighed and recorded to the nearest 0.1 g. The break flour yield (BFY) and total flour yield (TFY) were determined according to equations 4.1 and 4.2.

$$\%\ \text{BFY} = \frac{(\text{Total first break flour (g)})}{(\text{Total tempered wheat weight before milling (g)})} \times 100$$

..... Equation 4.1

$$\% \text{ TFY} = \frac{(\text{Total flour [break 1, 2 and 3] (g)})}{(\text{Total tempered wheat weight before milling (g)})} \times 100$$

..... Equation 4.2

The three flour fractions were thoroughly mixed in a rotating drum for 15 min, after which the flour was used to perform various flour quality analyses.

4.2.4. Flour characteristics

4.2.4.1. Flour constituents

The FOSS Infratec™ 1241 NIT grain analyser (FOSS analytics, Hillerød, Denmark), with FOSS wheat flour calibration no. 133754, was used to determine the composition of a flour sample (AACC, 1999c). To ensure the reliability of results, the instrument was monitored daily with a flour reference sample. The instrument captured 265 data points in the wavelength range of 570 to 1100 nm with each sample scanned to predict flour protein (Fprot), moisture (Fmoist), ash (Fash) and wet gluten (FWG) contents.

4.2.4.2. Falling Number

The Falling Number (FN) test was performed according to the AACC approved method 56-81 (AACC, 1999d) to determine the α-amylase enzyme activity present in flour. The instrument used was the Perten Falling number 1400 instrument (Perten Instruments, Hägerstad, Sweden), and adjusted according to altitude values of 1580 m for the location of the laboratory. Distilled water was used to fill the water bath of the instrument and heated to boiling temperature. A volume of 25 mL distilled water at room temperature was added to each of two viscometer tubes, followed by adding 7.00 g of flour on a 14% moisture basis (mb). The tubes were sealed with a rubber stop and shook by hand, 30 times in an upright position. The viscometer-stirrer was used to scrape any slurry off the rubber stop and upper part of the tubes. The tubes with inserted viscometer-stirrer was placed in the water bath of the apparatus and analysis started immediately at 0 s. The time in seconds required for the metal stirrer to fall through the boiling flour-water suspension was recorded. This time, measured in seconds, measures starch quality and is a surrogate measure of α-amylase activity which influences the viscosity of the suspension when the starch granules in the suspension are gelatinised. A high falling number indicates sound starch granules with low α-amylase activity, while a low falling number indicates significant changes in starch quality due to high α-amylase activity. The falling number method indicates the α-amylase activity in wheat flour, by measuring the degradation of a gelatinised starch paste due to α-amylase hydrolysis of the starch (Perten, 1964).

4.2.4.3. Alveograph

The Alveograph (Chopin technologies, Paris, France) was used to measure the dough tenacity and extensibility of a sheet of dough with defined thickness according to the AACC approved method

54-30.02 (AACC, 1999e). The sheet of dough was expanded, using air pressure, into a bubble until it ruptured, while the internal pressure in the bubble was graphically recorded. The air generator flow and the air flowmeter were calibrated each day before using the alveograph, as per manufacturer instructions.

A 250 g flour sample was added to the flour mixer compartment. The amount of NaCl solution added to the flour was determined based on the moisture content of the flour, according to the AACC approved method 54-30.02 (AACC, 1999e). The mixer was started, and a 2.5% NaCl solution added to the flour, after 1 min the mixer was stopped, and sides of the bowl scraped down with a plastic spatula, then the mixing was resumed until 8 min. The extrusion process was started as described, and a total of five dough pieces were extruded and each placed on a plate in the resting compartment of the alveograph at 25°C ± 0.2°C.

After 28 min from the start of mixing, the stretching of dough pieces was performed, forming five test curves which were used to calculate the results. The alveogram curve provided information about the dough elasticity or tenacity (P), dough extensibility (L), the ratio of elasticity to extensibility (P/L), swelling index (G) and deformation energy (W). The swelling index (G) is calculated as the square root of air volume needed to inflate the bubble until it ruptures, while the deformation energy (W) is the energy required to inflate the dough bubble until it ruptures. The resistance strength (S) of the dough to deformation is more widely used in industry and is calculated by dividing the deformation energy (W) by 6.54.

4.2.4.4. Farinograph

The Brabender Farinograph (Brabender GmbH & Co. KG, Kulturstraβe, Duisburg) was used to determine the resistance of dough to mixing, flour water absorption and dough stability. The AACC approved method 54-21.02 with constant flour weight was followed (AACC, 2011), using the 300 g mixing bowl for the analysis. The water bath's temperature was maintained at 30 ± 0.2°C to ensure 30°C temperature at the entrance of the mixing bowl, and water circulating freely to maintain temperature throughout.

Sample flour was weighed to 300 g corrected to a 14% mb. The flour was added to the Farinograph's bowl, and the burette filled with water at 30°C. The instrument was turned on and when reaching zero minutes, a determined amount of water was added to the mixing bowl. The sides of the bowl were scraped off with a plastic scraper. The farinogram curve produced was required to have its maximum resistance centred on the 500 BU line. If this was not the case the amount of water was adjusted, and a second test performed until a curve was produced within 20 BU from the centre.

The farinogram was interpreted, providing flour water absorption (FWA), dough development or peak time (FPT), dough stability (FStab) and dough tolerance to over mixing (FTol). FWA relates to the required amount of water added to flour to produce a curve centred on the 500 BU line at optimum development. FPT is the point of optimum gluten development, and maximum consistency before

the dough weakens. FStab is the flour's tolerance to overmixing and weakening of dough consistency. FTol gives an indication of the stability of the dough and the rate of dough weakening due to overmixing.

4.2.4.5. Mixograph

The Mixograph with Mixsmart software (National MFG Co., Lincoln, Nebraska) was used to perform mixograph tests on the flour samples. This test provides the optimum dough development time, tolerance to overmixing and other dough characteristics.

The analysis was performed according to the AACC approved method 54-40.02 (AACC, 1999f). The 35 g mixing bowl was used for the analysis, with the spring attached in slot 12. Flour samples were weighed to 35 g on a 14% mb in the mixing bowls, with a hole shaped between the pins in the middle of the flour sample using a spatula. The bowl was placed in position on the mixograph, the correct amount of absorption water (determined according to equation 4.3) added to the hole in the centre of the flour, and the mixograph and mixsmart recording started simultaneously. The mixograph was allowed 6 min 30 s to finish.

$$Y = 0.35(1.5X + 45)$$

..... Equation 4.3

Where: X = percent flour protein content (14% mb), and
Y = absorption water in mL.

After interpretation of the mixogram, the optimum dough development or peak time (MMT), peak height (MPH) at peak time and tail height (MTH) and tail width (MTW) at 6 min were obtained. MMT is the time necessary to mix the dough to optimum gluten development, MPH indicates the strength of the dough at optimum development, MTH indicates the strength and stability of the dough, and MTW indicates the dough's tolerance to over-mixing.

Statistical analysis

The analysis of the SRI and SRD regions were performed separately, however using the same methods. The treatment design was a combined nested design with cultivar nested within two (SRI) or four (SRD) *Pin* allele genotypes (Montgomery, 2017). Normality of standardised residuals was confirmed by the Shapiro-Wilk test, to confirm that the data was reliable (Shapiro & Wilk, 1965). Levene's test was used to verify the homogeneity of genotype and locality and region variances (Levene, 1960). The sources of variation in the data were partitioned into years, localities, replications (per year and locality), *puroindoline* (*Pin*) genotype, cultivars (within *Pin* genotype), year and *Pin* genotype interaction, locality and *Pin* genotype interaction, year and cultivars (*Pin* genotype) interaction, locality and cultivars (*Pin* genotype) interaction, the interaction of *Pin* genotype, years and localities, and the interaction of cultivars (*Pin* genotype), years and localities. The data were

subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Fisher's least significant difference (LSD) was calculated at the 5% level to compare interaction means of quality parameters within *Pin* genotypes (Ott & Longnecker, 2001).

4.3. Results

4.3.1. Influence of two puroindoline allelic genotypes on grain-, milling- and flour properties in the summer rainfall irrigation (SRI) region

The wheat grain samples with *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes differed significantly ($P < 0.05$) in kernel hardness, -moisture content, -weight, -diameter, and grain protein content. Wheat with the *Pina-D1b*/*Pinb-D1a* genotype had higher SKCS-HI (*Pina-D1b*/*Pinb-D1a*, 58 ± 9 vs. *Pina-D1a*/*Pinb-D1b*, 50 ± 10); and lower SKCS-Moist (11.94 ± 1.06% vs. 12.04 ± 1.01%), SKCS-weight (36.42 ± 5.21 mg vs. 41.63 ± 5.48 mg), SKCS-Dia (2.42 ± 0.24 mm vs. 2.64 ± 0.23 mm) and Gprot (11.69 ± 2.61% vs. 12.06 ± 2.75%), compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype (Table 4.2).

The milling of wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes differed significantly ($P < 0.05$) in BFY and TFY. Wheat with the *Pina-D1b*/*Pinb-D1a* genotype yielded lower BFY (23.47 ± 5.19% vs. 27.64 ± 5.59%) and TFY (64.62 ± 3.76% vs. 68.44 ± 2.64%), compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype (Table 4.2). The flour of wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes differed significantly ($P < 0.05$) in flour protein, -ash, and wet gluten content. Flour from wheat with the *Pina-D1b*/*Pinb-D1a* genotype were lower in Fprot (10.95 ± 1.70% vs. 11.14 ± 1.48%), Fash (0.59 ± 0.05% vs. 0.58 ± 0.04) and FWG (30.93 ± 6.29 vs. 33.16 ± 5.43), compared to flour from wheat with the *Pina-D1a*/*Pinb-D1b* genotype (Table 4.2). There was no significant ($P > 0.05$) difference in falling number between the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes.

Flour from wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes differed significantly ($P < 0.05$) in alveograph dough tenacity, -extensibility, -P/L ratio, strength and swelling index. Wheat with the *Pina-D1b*/*Pinb-D1a* genotype were higher in P (83.22 ± 19.48 mm vs. 78.30 ± 20.56 mm) and P/L ratio (0.86 ± 0.36 vs. 0.73 ± 0.27); but lower in L (105.70 ± 30.05 mm vs. 113.07 ± 24.57 mm), S (37.82 ± 13.96 cm^2 vs. 39.10 ± 15.80 cm^2) and G (22.67 ± 3.18 cm^3 vs. 23.54 ± 2.55 cm^3) compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype (Table 4.2).

There was no significant ($P > 0.05$) difference in farinograph properties, i.e. FMT, FWA, FStab, FTol, between flour from wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes. Flour from wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes did not differ significantly ($P > 0.05$) in MPH and MMT (Table 4.2). However, flour from wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes differed significantly ($P < 0.05$) in MTH and MTW. Flour from the *Pina-D1b*/*Pinb-D1a* wheat genotype, produced dough with lower MTH (45.91 ± 4.74

mm vs. 46.95 ± 5.49 mm) and MTW (11.50 ± 3.54 mm vs. 14.61 ± 2.59 mm) compared to the *Pina-D1a*/*Pinb-D1b* genotype (Table 4.2).

The combined ANOVA (Tables 4.3 – 4.7) was used to determine the contribution of each variance component to the variation in grain characteristics, milling quality, flour components and dough properties. The cultivars were nested into two *puroindoline* genotypes (*Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b*) in the statistical analysis model. The objective was to determine the variable contribution of the *puroindoline* genotype (PG) as part of the total genotype effect, and the significance of PG to explain variation in the properties measured. The PG and cultivars within PG (C) represented the total genotype effect (G). C represented all other genetic components, apart from the PG of the cultivars. The year (Y), location (L), interaction of year and location (Y x L), and replicates of field trials per year and location (Reps) provided the total environmental effect (E). The combination of Y x PG, L x PG, Y x L x PG, Y x C, L x C, and Y x L x C represented the total G x E interaction.

The environmental effects (Y, L and Y x L) significantly ($P < 0.001$) and primarily, contributed to the variation in grain-, milling- and flour quality properties that were measured in the SRI region. However, the G effect and the G x E interaction, also contributed significantly to the variation; with certain quality properties being affected more by G or G x E than the other (Tables 4.3 – 4.7).

The G effect contributed significantly towards the variation in grain characteristics, i.e. SKCS-HI (12.26% of total variation attributed to PG, $P < 0.001$; 4.83% of total variation attributed to C, $P < 0.001$), SKCS-Moist (0.20% PG, $P < 0.01$; 0.76% C, $P < 0.001$), SKCS-Weight (18.52% PG, $P < 0.001$; 10.82% C, $P < 0.001$) and SKCS-Dia (16.80% PG, $P < 0.001$; 9.63% C, $P < 0.001$) (Table 4.3). The G effect contributed significantly towards the variation in milling- and flour components; i.e. BFY (12.57% PG, $P < 0.001$; 0.94% C, $P < 0.001$), TFY (23.15% PG, $P < 0.001$; 5.31% C, $P < 0.001$), FN (2.11% C, $P < 0.001$), Fprot (0.35% PG, $P < 0.01$; 2.88% C, $P < 0.001$), Fmoist (2.56% PG, $P < 0.001$; 6.40% C, $P < 0.001$), Fash (0.90% PG, $P < 0.01$; 13.74% C, $P < 0.001$) and FWG (3.19% PG, $P < 0.001$; 2.38% C, $P < 0.001$) (Table 4.4).

The variation in alveograph properties were significantly explained by the G effect; this included P (1.42% PG, $P < 0.001$; 17.60% C, $P < 0.001$), L (1.60% PG, $P < 0.001$; 18.37% C, $P < 0.001$), P/L ratio (3.45% PG, $P < 0.001$; 22.00% C, $P < 0.001$), S (0.18% PG, $P < 0.05$; 6.51% C, $P < 0.001$), and G (2.00% PG, $P < 0.001$; 17.68% C, $P < 0.001$) (Table 4.5).

The PG did not significantly ($P > 0.05$) explain variation in farinograph properties; however, variation in farinograph properties were attributed by C; i.e. FMT (5.70%, $P < 0.001$) FWA (14.33%, $P < 0.001$), FStab (5.44%, $P < 0.001$) and FTol (6.59%, $P < 0.001$) (Table 4.6). The G effect significantly contributed to the following mixograph properties; MPH (1.96% C, $P < 0.001$), MTH (0.99% PG, $P < 0.001$; 4.16% C, $P < 0.001$), MTW (6.34% PG, $P < 0.001$; 4.09% C, $P < 0.001$), and MMT (5.85% C, $P < 0.001$) (Table 4.7).

Table 4.2. *Puroindoline* allelic genotype class means, range and the least significant difference for grain-, milling- and flour quality properties in the summer rainfall irrigation (SRI) region

	Pina-D1b*/*Pinb-D1a				***Pina-D1a*/*Pinb-D1b***				
Quality property	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**LSD**
SKCS-HI	160	57.79 ^{A} ± 9.04	37.73 - 76.69	0.71	96	50.47 ^{B} ± 10.26	33.85 - 76.75	1.05	0.80
SKCS-Moist (%)	160	11.94 ^{B} ± 1.06	8.94 - 13.10	0.08	96	12.04 ^{A} ± 1.01	9.42 - 13.75	0.10	0.07
SKCS-Weight (mg)	160	36.42 ^{B} ± 5.21	23.84 - 51.19	0.41	96	41.63 ^{A} ± 5.48	27.13 - 51.56	0.56	0.44
SKCS-Dia (mm)	160	2.42 ^{B} ± 0.24	1.84 - 3.93	0.02	96	2.64 ^{A} ± 0.23	1.99 - 3.06	0.02	0.20
Gprot (%, 12% mb)	100	11.69 ^{B} ± 2.61	8.90 - 15.40	0.26	60	12.06 ^{A} ± 2.75	9.90 - 15.50	0.36	0.50
BFY (%)	160	23.47 ^{B} ± 5.19	16.36 - 41.33	0.41	96	27.64 ^{A} ± 5.59	17.13 - 41.22	0.57	0.30
TFY (%)	160	64.62 ^{B} ± 3.76	48.43 - 73.43	0.30	96	68.44 ^{A} ± 2.64	59.51 - 73.56	0.27	0.41
FN (s)	160	369 ^{A} ± 67	62 - 432	5	96	369 ^{A} ± 50	139 - 432	5	4.10
Fprot (%, 12% mb)	160	10.95 ^{B} ± 1.70	7.90 - 14.80	0.13	96	11.14 ^{A} ± 1.48	8.90 - 14.40	0.15	0.13
Fmoist (%)	160	15.67 ^{A} ± 0.51	14.50 - 17.50	0.04	96	15.51 ^{B} ± 0.41	14.60 - 17.10	0.04	0.06
Fash (%)	160	0.59 ^{A} ± 0.05	0.48 - 0.75	0.00	96	0.58 ^{B} ± 0.04	0.45 - 0.68	0.00	0.01
FWG (%)	160	30.93 ^{B} ± 6.29	17.40 - 43.00	0.50	96	33.16 ^{A} ± 5.43	17.70 - 43.40	0.55	0.52
P (mm)	160	83.22 ^{A} ± 19.48	44.00 - 126.00	1.54	96	78.30 ^{B} ± 20.56	43.00 - 126.00	2.10	1.52
L (mm)	160	105.70 ^{B} ± 30.05	47.00 - 199.00	2.38	96	113.07 ^{A} ± 24.57	63.00 - 199.00	2.51	3.35
P/L	160	0.86 ^{A} ± 0.36	0.27 - 1.45	0.03	96	0.73 ^{B} ± 0.27	0.22 - 1.86	0.03	0.40
S (cm^2)	160	37.82 ^{B} ± 13.96	17.43 - 84.78	1.10	96	39.10 ^{A} ± 15.80	20.33 - 84.25	1.61	1.07
G (cm^3)	160	22.67 ^{B} ± 3.18	15.30 - 31.10	0.25	96	23.54 ^{A} ± 2.55	17.70 - 31.40	0.26	0.36
FMT (min)	160	6.22 ^{A} ± 2.37	2.25 - 30.60	0.19	96	6.30 ^{A} ± 4.21	2.70 - 30.00	0.43	0.38
FWA (%)	160	59.44 ^{A} ± 2.89	53.80 - 65.00	0.23	96	59.56 ^{A} ± 2.62	53.80 - 65.25	0.27	0.23
FStab (min)	160	10.73 ^{A} ± 5.75	2.50 - 33.00	0.45	96	10.87 ^{A} ± 6.73	2.50 - 33.00	0.69	0.45
FTol (BU)	160	43.53 ^{A} ± 22.84	5.00 - 110.00	1.81	96	41.57 ^{A} ± 24.58	10.00 - 110.00	2.51	2.24
MPH (mm)	160	59.01 ^{A} ± 5.59	44.35 - 67.44	0.44	96	58.46 ^{A} ± 4.25	48.03 - 67.89	0.43	0.56
MTH (mm)	160	45.91 ^{B} ± 4.74	38.23 - 60.17	0.37	96	46.95 ^{A} ± 5.49	39.08 - 60.50	0.56	0.38
MTW (mm)	160	11.50 ^{B} ± 3.54	6.24 - 37.38	0.28	96	14.61 ^{A} ± 2.59	6.06 - 37.97	0.26	0.59
MMT (min)	160	2.94 ^{A} ± 0.66	1.64 - 5.98	0.05	96	2.93 ^{A} ± 0.78	1.66 - 5.18	0.08	0.06

Means followed by the same letter, did not differ significantly at $P < 0.05$, N – observations, Mean – mean values, SD – standard deviation, SE – standard error, LSD – least significant difference, SKCS-HI – single kernel characterisation system hardness index, SKCS-Moist – single kernel characterisation system kernel moisture, SKCS-Weight – single kernel characterisation system kernel weight, SKCS-Dia – single kernel characterisation system kernel diameter, FN – falling number, BFY – break flour yield, TFY – total flour yield, Fprot – flour protein, Fmoist – flour moisture, Fash – flour ash, FWG – flour wet gluten, Gprot – grain protein content at 12% moisture basis, P – alveograph elasticity, L – alveograph extensibility, P/L – alveograph ratio elasticity/extensibility, S – alveograph strength, G – alveograph swelling index, FMT – farinograph mixing time, FWA – farinograph flour water absorption, FStab – farinograph stability, FTol – farinograph tolerance, BU – Brabender units, MPH – mixograph peak height, MTH – mixograph tail height, MTW – mixograph tail width, MMT – Mixograph mixing time.

Table 4.3. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for wheat grain characteristics in the summer rainfall irrigation (SRI) region

Source	DF	SKCS-HI		SKCS-Moist		SKCS-weight		SKCS-Dia		Grain protein		
		Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	DF	Mean Squares	% of SS
Year	2	1488.38	11.37***	36.64	26.60***	250.76	5.69***	0.58	6.73***	1	58.44	5.20***
Lok	3	2042.26	23.39***	35.37	38.52***	691.16	23.54***	1.53	26.46***	3	87.20	23.29***
Y x L	5	1867.34	35.65***	12.03	21.84***	490.28	27.84***	0.84	24.16***	2	11.62	2.07**
Rep (Y x L)	21	15.69	1.26 ns	0.29	2.20***	11.40	2.72***	0.03	3.58***	13	11.04	12.77***
Total E			**71.67**		**89.16**		**59.79**		**60.93**			**43.33**
PG	1	3210.92	12.26***	0.56	0.20**	1630.68	18.52***	2.91	16.80***	1	5.14	0.46 ns
C	6	210.84	4.83***	0.35	0.76***	158.76	10.82***	0.28	9.62***	6	2.14	1.14 ns
Total G			**17.09**		**0.96**		**29.34**		**26.42**			**1.60**
Y x PG	2	33.60	0.26*	1.18	0.86***	0.33	0.01 ns	0.00	0.02 ns	1	3.69	0.33 ns
L x PG	3	48.69	0.56**	0.85	0.92***	8.08	0.28*	0.01	0.09 ns	3.	1.18	0.32 ns
Y x L x PG	5	59.35	1.13***	0.35	0.64***	24.45	1.39***	0.03	0.93**	2	0.07	0.01 ns
Total PG x E			**1.95**		**2.42**		**1.68**		**1.04**			**0.66**
Y x C	12	14.97	0.69 ns	0.20	0.85**	6.34	0.86 ns	0.01	0.80 ns	6	6.31	3.37*
L x C	18	23.79	1.63**	0.12	0.78 ns	5.17	1.06*	0.01	1.25 ns	18	10.19	16.33***
Y x L x C	30	12.83	1.47 ns	0.14	1.52*	7.11	2.42***	0.02	2.97***	12	14.43	15.41***
Total C x E			**3.79**		**3.15**		**4.34**		**5.02**			**35.11**
Total G x E			**5.74**		**5.57**		**6.02**		**6.06**			**35.77**
Error	147	9.82	5.51	0.08	4.31	2.91	4.86	0.01	6.59	91	2.38	19.30
R^2		0.95		0.96		0.95		0.93			0.81	
CV		5.69		2.37		4.45		3.51			13.04	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, SKCS-HI – single kernel characterisation system hardness index, SKCS-Moist – single kernel characterisation system kernel moisture, SKCS-Weight – single kernel characterisation system kernel weight, SKCS-Dia – single kernel characterisation system kernel diameter, Gprot – grain protein content at 12% moisture basis.

Table 4.4. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for wheat milling- and flour components in the summer rainfall irrigation (SRI) region

Source	DF	BFY		TFY		FN		Fprot		Fmoist		Fash		FWG	
		Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS
Year	2	2413.96	58.24***	63.44	3.34***	45835.00	9.59***	10.31	3.08***	6.01	20.45***	0.02	7.69***	213.82	4.56***
Lok	3	307.14	11.11***	214.50	16.96***	89950.63	28.24***	39.43	17.66***	2.67	13.64***	0.03	19.02***	707.02	22.59***
Y x L	5	161.10	9.72***	60.47	7.97***	70571.25	36.93***	81.56	60.88***	1.24	10.54***	0.03	25.65***	907.17	48.32***
Rep (Y x L)	21	2.07	0.52 ns	3.77	2.08 ns	192.18	0.42 ns	0.71	2.23***	0.14	5.03***	0.00	2.62 ns	11.49	2.57***
Total E			**79.59**		**30.35**		**75.18**		**83.85**		**49.66**		**54.98**		**78.04**
PG	1	1042.29	12.57***	878.51	23.15***	40.84	0.00 ns	2.35	0.35**	1.50	2.56***	0.00	0.90**	299.10	3.19***
C	6	12.96	0.94***	33.62	5.31***	3364.96	2.11***	3.21	2.88***	0.63	6.40***	0.01	13.74***	37.17	2.38***
Total G			**13.51**		**28.46**		**2.11**		**3.23**		**8.96**		**14.64**		**5.57**
Y x PG	2	37.20	0.90***	26.64	1.40***	3750.92	0.79***	3.45	1.03***	0.19	0.65*	0.00	1.20**	74.59	1.59***
L x PG	3	2.79	0.10 ns	24.72	1.95***	8671.60	2.72***	2.73	1.22***	0.38	1.94***	0.00	0.08 ns	37.35	1.19***
Y x L x PG	5	5.58	0.34**	33.94	4.47***	3231.07	1.69***	1.37	1.02***	0.21	1.78***	0.00	1.10 ns	8.58	0.46 ns
Total PG x E			**1.34**		**7.82**		**5.20**		**3.27**		**4.37**		**2.38**		**3.24**
Y x C	12	2.58	0.37*	19.87	6.28***	890.38	1.12***	0.45	0.81*	0.16	3.25***	0.00	2.26*	10.25	1.31**
L x C	18	4.88	1.06***	13.44	6.37***	2079.87	3.92***	0.45	1.20*	0.18	5.42***	0.00	2.31 ns	9.90	1.90***
Y x L x C	30	4.75	1.72***	13.45	10.63***	2701.93	8.48***	0.52	2.35**	0.33	16.60***	0.00	8.40***	11.12	3.55***
Total C x E			**3.15**		**23.28**		**13.52**		**4.36**		**25.27**		**12.97**		**6.76**
Total G x E			**4.49**		**31.10**		**18.72**		**7.63**		**29.64**		**15.35**		**10.00**
Error	147	1.36	2.41	2.60	10.07	258.19	3.97	0.24	5.29	0.05	11.75	0.00	15.05	4.09	6.40
R^2		0.97		0.90		0.96		0.95		0.88		0.85		0.94	
CV		4.65		2.44		4.35		4.46		1.39		3.90		6.36	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, BFY – break flour yield, TFY – total flour yield, Fprot – flour protein, Fmoist – flour moisture, Fash – flour ash, FWG – flour wet gluten.

Table 4.5. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for Alveograph properties in the summer rainfall irrigation (SRI) region

		P		L		P/L		S		G	
	DF	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS
Year	2	7772.56	15.25***	12295.62	12.05***	1.31	9.01***	4835.45	17.65***	131.95	11.59***
Lok	3	6416.63	18.88***	5440.72	8.00***	1.07	11.04***	2907.06	15.91***	62.49	8.23***
Y x L	5	4629.50	22.71***	10750.54	26.33***	0.57	9.74***	4979.88	45.43***	122.33	26.86***
Rep (Y x L)	21	127.11	2.62***	147.42	1.52 ns	0.03	2.08 ns	42.92	1.64***	1.77	1.63 ns
Total E			**59.46**		**47.90**		**31.87**		**80.63**		**48.31**
PG	1	1450.42	1.42***	3261.59	1.60***	1.00	3.45***	98.43	0.18*	45.59	2.00***
C	6	2989.86	17.60***	6250.09	18.37***	1.06	22.00***	594.76	6.51***	67.10	17.68***
Total G			**19.02**		**19.97**		**25.45**		**6.69**		**19.68**
Y x PG	2	805.40	1.58***	869.90	0.85**	0.43	2.94***	122.82	0.45***	11.88	1.04***
L x PG	3	547.55	1.61***	658.34	0.97**	0.10	1.05**	197.19	1.08***	6.21	0.82*
Y x L x PG	5	414.82	2.03***	1307.53	3.20***	0.37	6.46***	78.01	0.71***	17.42	3.83***
Total PG x E			**5.22**		**5.02**		**10.45**		**2.24**		**5.69**
Y x C	18	261.50	4.62***	544.86	4.80***	0.13	7.91***	54.44	1.79***	5.65	4.47***
L x C	12	77.29	0.91*	842.18	4.95***	0.07	2.77**	62.75	1.37***	8.20	4.32***
Y x L x C	30	190.65	5.61***	335.86	4.94**	0.09	8.83***	47.39	2.59***	3.36	4.43*
Total C x E			**11.14**		**14.69**		**19.51**		**5.75**		**13.22**
Total G x E			**16.36**		**19.71**		**29.96**		**7.99**		**18.91**
Error	147	35.72	5.15	172.54	12.43	0.03	12.71	17.46	4.68	2.03	13.09
R^2		0.95		0.88		0.87		0.95		0.87	
CV		7.34		12.11		19.48		10.91		6.19	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, P – alveograph elasticity, L – alveograph extensibility, P/L – alveograph ratio elasticity/extensibility, S – alveograph strength, G – alveograph swelling index.

Table 4.6. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for Farinograph properties in the summer rainfall irrigation (SRI) region

		FMT		FWA		FStab		FTol	
	DF	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS
Year	2	229.60	17.79***	2.76	0.28*	1065.04	22.27***	8350.75	11.88***
Lok	3	96.42	11.21***	152.39	23.05***	546.89	17.15***	9032.42	19.28***
Y x L	5	149.27	28.92***	148.39	37.40***	734.24	38.38***	11509.20	40.94***
Rep (Y x L)	21	2.47	2.01 ns	1.51	1.60*	8.06	1.77***	219.09	3.27***
Total E			**59.93**		**62.33**		**79.57**		**75.37**
PG	1	0.32	0.01 ns	0.97	0.05 ns	1.20	0.01 ns	230.10	0.16 ns
C	6	24.53	5.70***	47.37	14.33***	86.66	5.44***	1544.12	6.59***
Total G			**5.71**		**14.38**		**5.45**		**6.75**
Y x PG	2	19.01	1.47***	1.86	0.19 ns	29.23	0.61***	957.09	1.36***
L x PG	3	25.30	2.94***	15.58	2.36***	12.70	0.40**	57.40	0.12 ns
Y x L x PG	5	15.85	3.07***	7.64	1.93***	16.44	0.86***	323.82	1.15***
Total PG x E			**7.48**		**4.48**		**1.87**		**2.63**
Y x C	18	5.50	3.84***	3.85	3.49***	14.06	2.65***	71.78	0.92 ns
L x C	12	9.37	4.36***	2.76	1.67***	15.74	1.97***	229.79	1.96***
Y x L x C	30	5.45	6.34***	5.13	7.75***	12.14	3.81***	201.27	4.30***
Total C x E			**14.54**		**12.91**		**8.43**		**7.18**
Total G x E			**22.02**		**17.39**		**10.30**		**9.81**
Error	147	2.17	12.33	0.80	5.92	3.04	4.68	77.07	8.06
R^2		0.88		0.94		0.95		0.92	
CV		23.54		1.50		16.18		20.51	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, FMT – farinograph mixing time, FWA – farinograph flour water absorption, FStab – farinograph stability, FTol – farinograph tolerance.

Table 4.7. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for Mixograph properties in the summer rainfall irrigation (SRI) region

		MPH		MTH		MTW		MMT	
	DF	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS	Mean Squares	% of SS
Year	2	457.27	13.65***	600.79	18.50***	1992.02	43.31***	15.95	25.18***
Lok	3	484.22	21.68***	374.48	17.30***	280.98	9.16***	6.93	16.42***
Y x L	5	507.66	37.89***	559.53	43.08***	309.06	16.80***	7.30	28.81***
Rep (Y x L)	21	13.54	4.25***	8.56	2.77***	9.12	2.08***	0.28	4.68***
Total E			**77.47**		**81.65**		**71.35**		**75.09**
PG	1	18.34	0.27 ns	64.09	0.99***	583.52	6.34***	0.01	0.01 ns
C	6	21.90	1.96***	45.00	4.16***	62.77	4.09***	1.24	5.85***
Total G			**2.23**		**5.15**		**10.43**		**5.86**
Y x PG	2	9.99	0.30 ns	4.51	0.14 ns	13.41	0.29*	0.05	0.07 ns
L x PG	3	36.91	1.65***	7.74	0.36***	54.49	1.78***	0.81	1.91***
Y x L x PG	5	18.11	1.35***	14.73	1.13***	72.64	3.95***	0.71	2.79***
Total PG x E			**3.30**		**1.63**		**6.02**		**4.77**
Y x C	12	4.00	0.72 ns	6.35	1.17***	4.92	0.64 ns	0.19	1.83***
L x C	18	9.94	2.67**	9.45	2.62***	9.56	1.87***	0.15	2.07***
Y x L x C	30	6.77	3.03 ns	6.07	2.81***	11.08	3.61***	0.14	3.21***
Total C x E			**6.42**		**6.60**		**6.12**		**7.11**
Total G x E			**9.72**		**8.23**		**12.14**		**11.88**
Error	147	4.82	10.58	2.20	4.98	3.80	6.07	0.06	7.17
R^2		0.89		0.95		0.94		0.93	
CV		3.73		3.20		15.39		8.47	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, MPH – mixograph peak height, MTH – mixograph tail height, MTW – mixograph tail width, MMT – Mixograph mixing time.

4.3.2. Influence of four puroindoline allelic genotypes on grain-, milling- and flour properties in the summer rainfall dryland (SRD) region

Wheat samples in the four *Pin* genotype classes (*Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1b*, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*) differed significantly ($P < 0.05$) in GKH, -moisture content, -weight and -diameter. Wheat with the *Pina-D1b*/*Pinb-D1a* genotype (63 ± 8) had higher SKCS-HI than the other three *Pin* genotypes (*Pina-D1a*/*Pinb-D1b*, 57 ± 7, *Pina-D1a*/*Pinb-D1p*, 58 ± 6, and *Pina-D1a*/*Pinb-D1ab*, 58 ± 7); which did not differ significantly ($P > 0.05$) (Table 4.8). Wheat grain with the *Pina-D1b*/*Pinb-D1a* genotype (12.49 ± 0.83%) were higher in SKCS-Moist than wheat with the *Pina-D1a*/*Pinb-D1p* (12.37 ± 0.78%) and *Pina-D1a*/*Pinb-D1ab* (12.35 ± 0.68%) genotypes; while wheat with the *Pina-D1a*/*Pinb-D1b* genotype (12.46 ± 0.79%) did not differ significantly from any of the other *Pin* genotypes present (Table 4.8).

Wheat with the *Pina-D1a*/*Pinb-D1b* genotype (2.53 ± 0.23 mm) differed significantly ($P < 0.05$) in SKCS-Dia compared to the *Pina-D1b*/*Pinb-D1a* genotype (2.46 ± 0.22 mm), which differed significantly from the *Pina-D1a*/*Pinb-D1p* (2.41 ± 0.19 mm) genotype. Wheat with the *Pina-D1a*/*Pinb-D1ab* genotype (2.50 ± 0.21 mm) differed significantly ($P < 0.05$) from the *Pina-D1a*/*Pinb-D1p* genotype, but non-significantly ($P > 0.05$) from the *Pina-D1a*/*Pinb-D1b* and *Pina-D1b*/*Pinb-D1a* genotypes. Wheat grain with the *Pina-D1b*/*Pinb-D1a* (34.95 ± 7.54 mg) and *Pina-D1a*/*Pinb-D1p* (35.68 ± 4.72 mg) genotypes differed significantly ($P < 0.05$) in SKCS-Weight compared to wheat with the *Pina-D1a*/*Pinb-D1b* (37.16 ± 4.84 mg) and *Pina-D1a*/*Pinb-D1ab* (36.60 ± 4.41 mg) genotypes; the *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1ab* genotypes did not differ significantly ($P > 0.05$) in SKCS-Weight (Table 4.8).

Wheat samples of all four the *Pin* genotypes differed significantly ($P < 0.05$) for BFY and TFY. The milling of wheat with the *Pina-D1a*/*Pinb-D1p* genotype (BFY, 25.80 ± 7.96% and TFY, 65.09 ± 5.00%) yielded significantly ($P < 0.05$) higher BFY and TFY than the *Pina-D1a*/*Pinb-D1b* genotype (BFY, 25.08 ± 7.18% and TFY, 64.17 ± 5.75%), followed by the *Pina-D1a*/*Pinb-D1ab* genotype (BFY, 23.97 ± 7.12% and TFY, 62.00 ± 5.55%), and the *Pina-D1b*/*Pinb-D1a* genotype (BFY, 22.29 ± 5.10% and TFY, 60.95 ± 4.85%) with the lowest BFY and TFY (Table 4.8). The FN of flour from wheat within the four *Pin* genotype classes differed significantly ($P < 0.05$). Flour with the *Pina-D1b*/*Pinb-D1a* genotype (368 ± 46 s) were higher than flour with the *Pina-D1a*/*Pinb-D1b* (352 ± 58 s), *Pina-D1a*/*Pinb-D1p* (357 ± 39 s) and *Pina-D1a*/*Pinb-D1ab* (355 ± 52 s) genotypes.

There was no significant ($P > 0.05$) difference in Gprot between the *Pin* genotypes; however, after milling there was a significant difference ($P < 0.05$) in the Fprot from wheat in the different *Pin* genotypes. The Fprot of wheat with the *Pina-D1b*/*Pinb-D1a* genotype (12.38 ± 2.79%) were lower than the other three genotypes (*Pina-D1a*/*Pinb-D1b*, 12.67 ± 1.80%, *Pina-D1a*/*Pinb-D1p*, 12.87 ± 1.74%, and *Pina-D1a*/*Pinb-D1ab*, 12.65 ± 1.76%) (Table 4.8).

Dough from wheat in the different *Pin* genotype classes differed significantly ($P < 0.05$) in alveograph dough tenacity, -extensibility, -P/L ratio, strength and swelling index. Dough from wheat with the *Pina-D1b*/*Pinb-D1a* (P, 110.18 ± 8.85 mm and P/L, 1.38 ± 0.51) and *Pina-D1a*/*Pinb-D1p* (P,

113.81 ± 22.48 mm and P/L, 1.36 ± 0.59) genotypes produced dough with similar tenacity (P) and P/L ratio, while both had higher P and P/L values than the *Pina-D1a*/*Pinb-D1ab* (P, 105.00 ± 22.77 mm and P/L, 1.19 ± 0.62) genotype, followed by the *Pina-D1a*/*Pinb-D1b* (P, 98.80 ± 20.09 mm and P/L, 1.03 ± 0.41) genotype, which had the lowest P and P/L values. Dough from wheat with the *Pina-D1a*/*Pinb-D1b* (L, 104.81 ± 25.04 mm and G, 22.63 ± 2.73 cm^3) and *Pina-D1a*/*Pinb-D1ab* (L, 101.94 ± 28.96 and G, 22.24 ± 3.31 cm^3) genotypes produced dough with similar extensibility and swelling index, while they both had higher L and G values than the *Pina-D1a*/*Pinb-D1p* (L, 93.84 ± 26.91 and G, 21.36 ± 3.14 cm^3) genotype, and dough from wheat with the *Pina-D1b*/*Pinb-D1a* (L, 87.41 ± 23.88 and G, 20.62 ± 2.87 cm^3) genotype which had the lowest L and G values (Table 4.9). Dough from wheat with the *Pina-D1a*/*Pinb-D1p* genotype had the highest strength (S, 54.31 ± 12.94 cm^2) compared to the other three genotypes. Dough from wheat with the *Pina-D1a*/*Pinb-D1b* (S, 50.57 ± 13.17 cm^2) and *Pina-D1a*/*Pinb-D1ab* (S, 51.45 ± 45 cm^2) genotypes produced dough with similar strength, while the *Pina-D1b*/*Pinb-D1a* genotype produced the weakest dough (S, 47.52 ± 0.41 cm^2) (Table 4.9).

Flour from wheat in the different *Pin* genotype classes differed significantly ($P < 0.05$) in FWA. Flour from wheat with the *Pina-D1b*/*Pinb-D1a* genotype had the highest FWA (63.93 ± 1.23%), followed by the *Pina-D1a*/*Pinb-D1p* (63.24 ± 3.73%) genotype; and the *Pina-D1a*/*Pinb-D1b* (61.66 ± 3.80%) and *Pina-D1a*/*Pinb-D1ab* (61.44 ± 3.85%) wheat genotypes with the lowest FWA (Table 4.9).

The mixograph properties of flour from wheat in the different *Pin* genotype classes differed significantly ($P < 0.05$). The MMT of flour with the *Pina-D1a*/*Pinb-D1p* genotype were the highest (3.41 ± 0.58 min), followed by the *Pina-D1a*/*Pinb-D1b* (3.21 ± 0.63 min) and *Pina-D1a*/*Pinb-D1ab* (3.21 ± 0.80 min) genotypes, while flour with the *Pina-D1b*/*Pinb-D1a* genotype had the lowest MMT (2.89 ± 0.73 min) (Table 4.9). The MTH that indicates strength and stability of the dough, were the highest for flour with the *Pina-D1a*/*Pinb-D1p* genotype (50.88 ± 4.86 mm), followed by the *Pina-D1a*/*Pinb-D1b* (48.51 ± 3.69 mm), *Pina-D1a*/*Pinb-D1ab* (48.10 ± 4.35 mm) and *Pina-D1b*/*Pinb-D1a* (47.73 ± 8.00 mm) genotypes. The MTH of flour from wheat with the *Pina-D1a*/*Pinb-D1b* and *Pina-D1b*/*Pinb-D1a* genotypes differed significantly ($P < 0.05$), but neither differed significantly ($P < 0.05$) from *Pina-D1a*/*Pinb-D1ab* (Table 4.9). The MTW that indicates the dough's tolerance to overmixing, were the highest for flour with the *Pina-D1a*/*Pinb-D1p* (16.83 ± 7.70 mm) and *Pina-D1a*/*Pinb-D1ab* (17.04 ± 7.76 mm) genotypes, followed by the *Pina-D1a*/*Pinb-D1b* (14.72 ± 4.55 mm) genotype, with the *Pina-D1b*/*Pinb-D1a* genotype (13.02 ± 4.02 mm) lowest in MTW (Table 4.9).

Table 4.8. *Puroindoline* allelic genotype class means, range and the least significant difference for grain-, milling- and flour quality properties in the summer rainfall dryland (SRD) region

	Pina-D1b*/*Pinb-D1a				***Pina-D1a*/*Pinb-D1b***				***Pina-D1a*/*Pinb-D1p***				***Pina-D1a*/*Pinb-D1ab***				
Quality property	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**LSD**
SKCS-HI	66	63.17 ^{A} ± 7.51	42.91 - 76.48	0.92	165	57.42 ^{B} ± 7.11	41.89 - 75.84	0.55	33	58.43 ^{B} ± 6.03	48.91 - 70.09	1.05	33	58.45 ^{B} ± 6.90	42.66 - 72.30	1.20	1.25
SKCS-Moist (%)	66	12.49 ^{A} ± 0.83	11.01 - 15.26	0.10	165	12.46 ^{AB} ± 0.79	10.96 - 15.30	0.06	33	12.37 ^{B} ± 0.78	11.17 - 14.81	0.14	33	12.35 ^{B} ± 0.68	10.91 - 13.67	0.12	0.11
SKCS-Weight (mg)	66	34.95 ^{B} ± 4.54	25.85 - 47.07	0.56	165	37.16 ^{A} ± 4.84	24.72 - 47.90	0.38	33	35.68 ^{B} ± 3.72	27.56 - 42.20	0.65	33	36.60 ^{A} ± 4.41	28.48 - 45.06	0.77	0.76
SKCS-Dia (mm)	66	2.46 ^{B} ± 0.22	1.99 - 3.00	0.03	165	2.53 ^{A} ± 0.23	1.97 - 3.08	0.02	33	2.41 ^{C} ± 0.19	2.00 - 2.74	0.03	33	2.50 ^{AB} ± 0.21	2.12 - 2.85	0.04	0.04
Gprot (%, 12% mb)	48	14.03 ^{A} ± 1.40	11.35 - 17.72	0.20	120	13.95 ^{A} ± 1.61	10.38 - 17.93	0.15	24	14.30 ^{A} ± 1.24	12.52 - 16.50	0.25	24	14.05 ^{A} ± 1.64	10.58 - 17.10	0.33	0.30
BFY (%)	66	22.29 ^{D} ± 7.09	11.83 - 37.38	0.87	165	25.08 ^{B} ± 7.18	14.65 - 38.21	0.56	33	25.80 ^{A} ± 6.96	15.95 - 36.15	1.21	33	23.97 ^{C} ± 7.12	14.07 - 36.22	1.24	0.62
TFY (%)	66	60.95 ^{D} ± 5.85	47.63 - 71.06	0.72	165	64.17 ^{B} ± 5.75	49.08 - 78.25	0.45	33	65.09 ^{A} ± 6.00	52.80 - 75.23	1.04	33	62.00 ^{C} ± 5.55	50.81 - 71.03	0.97	0.83
FN (s)	66	368 ^{A} ± 46	285 - 432	5.68	165	352 ^{B} ± 58	122 - 450	4.49	33	357 ^{B} ± 39	250 - 423	6.73	33	355 ^{B} ± 52	270 - 432	9.10	9.71
Fprot (%, 12% mb)	66	12.38 ^{B} ± 1.79	7.90 - 15.90	0.22	165	12.67 ^{A} ± 1.80	8.60 - 16.40	0.14	33	12.87 ^{A} ± 1.74	8.70 - 15.20	0.30	33	12.65 ^{A} ± 1.76	9.40 - 15.70	0.31	0.26
Fmoist (%)	66	15.46 ^{A} ± 0.62	14.30 - 17.10	0.08	165	15.42 ^{A} ± 0.58	14.20 - 16.90	0.05	33	15.45 ^{A} ± 0.66	14.40 - 17.20	0.11	33	15.29 ^{B} ± 0.58	14.30 - 16.10	0.10	0.08
Fash (%)	66	0.63 ^{A} ± 0.07	0.53 - 0.80	0.01	165	0.62 ^{A} ± 0.07	0.41 - 0.83	0.01	33	0.64 ^{A} ± 0.09	0.33 - 0.84	0.02	33	0.63 ^{A} ± 0.08	0.53 - 0.85	0.01	0.02
FWG (%)	66	35.07 ^{C} ± 6.28	22.10 - 49.40	0.77	165	37.45 ^{B} ± 6.22	26.50 - 50.50	0.48	33	39.03 ^{A} ± 5.09	28.30 - 48.00	0.89	33	37.12 ^{B} ± 5.90	26.70 - 46.70	1.03	0.99

Means followed by the same letter, did not differ significantly at $P < 0.05$, N – observations, Mean – mean values, SD – standard deviation, SE – standard error, LSD – least significant difference, SKCS-HI – single kernel characterisation system hardness index, SKCS-Moist – single kernel characterisation system kernel moisture, SKCS-Weight – single kernel characterisation system kernel weight, SKCS-Dia – single kernel characterisation system kernel diameter, FN – falling number, BFY – break flour yield, TFY – total flour yield, Fprot – flour protein, Fmoist – flour moisture, Fash – flour ash, FWG – flour wet gluten, Gprot – grain protein content at 12% moisture basis.

Table 4.9. *Puroindoline* allelic genotype class means, range and the least significant difference for flour quality properties in the summer rainfall dryland (SRD) region

	Pina-D1b/*Pinb-D1a*				*Pina-D1a*/*Pinb-D1b*				*Pina-D1a*/*Pinb-D1p*				*Pina-D1a*/*Pinb-D1ab*				
Quality property	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**LSD**
P (mm)	66	110.18 ^{A} ± 15.85	70.00 - 150.00	1.95	164	98.80 ^{C} ± 20.09	60.00 - 152.00	1.57	31	113.81 ^{A} ± 22.48	64.00 - 154.00	4.04	32	105.00 ^{B} ± 22.77	61.00 - 144.00	4.03	4.27
L (mm)	66	87.41 ^{C} ± 23.88	39.00 - 173.00	2.94	164	104.81 ^{A} ± 25.04	49.00 - 184.00	1.96	31	93.84 ^{B} ± 26.91	50.00 - 155.00	4.83	32	101.94 ^{A} ± 28.96	48.00 - 175.00	5.12	6.02
P/L	66	1.38 ^{A} ± 0.51	0.52 - 2.87	0.06	164	1.03 ^{C} ± 0.41	0.39 - 2.59	0.03	31	1.36 ^{A} ± 0.59	0.46 - 2.80	0.11	32	1.19 ^{B} ± 0.62	0.35 - 3.00	0.11	0.12
S (cm^2)	66	47.52 ^{C} ± 13.41	24.15 - 82.00	1.65	164	50.57 ^{B} ± 13.17	25.99 - 85.62	1.03	31	54.31 ^{A} ± 11.94	30.73 - 80.58	2.14	32	51.45 ^{B} ± 12.92	31.80 - 79.51	2.28	2.13
G (cm^3)	66	20.62 ^{C} ± 2.87	13.90 - 29.30	0.35	164	22.63 ^{A} ± 2.73	15.60 - 30.20	0.21	31	21.36 ^{B} ± 3.14	15.70 - 27.70	0.56	32	22.24 ^{A} ± 3.31	15.40 - 29.40	0.59	0.66
FMT (min)	56	7.83 ^{A} ± 5.66	1.80 - 36.80	0.76	142	8.46 ^{A} ± 5.16	1.50 - 39.60	0.43	29	8.64 ^{A} ± 5.94	2.00 - 28.70	1.10	28	8.54 ^{A} ± 8.05	2.00 - 41.40	1.52	1.40
FWA (%)	56	63.93 ^{A} ± 3.23	56.75 - 69.20	0.43	142	61.66 ^{C} ± 3.80	53.10 - 70.80	0.32	29	63.24 ^{B} ± 3.73	55.80 - 68.60	0.69	28	61.44 ^{C} ± 3.85	55.80 - 68.50	0.73	0.47
FStab (min)	56	14.61 ^{A} ± 9.72	3.50 - 52.30	1.30	142	16.27 ^{A} ± 8.84	2.20 - 46.20	0.74	29	16.52 ^{A} ± 10.48	8.20 - 45.00	1.95	28	14.89 ^{A} ± 8.31	3.20 - 33.40	1.57	2.38
FTol (BU)	56	28.57 ^{A} ± 14.58	5.00 - 60.00	1.95	142	26.89 ^{A} ± 17.71	5.00 - 120.00	1.49	29	25.59 ^{A} ± 13.76	0.00 - 55.00	2.55	28	29.29 ^{A} ± 17.09	5.00 - 60.00	3.23	4.25
MPH (mm)	66	61.91 ^{A} ± 4.94	50.61 - 71.64	0.61	165	60.48 ^{B} ± 5.40	48.04 - 70.57	0.42	33	61.94 ^{A} ± 5.18	47.55 - 69.66	0.90	33	58.78 ^{C} ± 5.60	46.95 - 68.85	0.97	0.86
MTH (mm)	66	47.73 ^{C} ± 4.00	40.33 - 57.02	0.49	165	48.51 ^{B} ± 3.69	40.44 - 62.54	0.29	33	50.88 ^{A} ± 2.86	46.35 - 59.25	0.50	33	48.10 ^{BC} ± 4.35	41.42 - 58.66	0.76	0.72
MTW (mm)	66	13.02 ^{C} ± 4.02	6.79 - 27.61	0.50	165	14.72 ^{B} ± 4.55	7.49 - 32.40	0.35	33	16.83 ^{A} ± 5.70	7.99 - 31.12	0.99	33	17.04 ^{A} ± 7.76	8.23 - 35.35	1.35	1.12
MMT (min)	66	2.89 ^{C} ± 0.73	1.90 - 4.62	0.09	165	3.21 ^{B} ± 0.63	1.94 - 5.40	0.05	33	3.41 ^{A} ± 0.58	2.30 - 4.60	0.10	33	3.21 ^{B} ± 0.80	2.17 - 4.60	0.14	0.13

Means followed by the same letter, did not differ significantly at $P < 0.05$, N – observations, Mean – mean values, SD – standard deviation, SE – standard error, LSD – least significant difference, P – alveograph elasticity, L – alveograph extensibility, P/L – alveograph ratio elasticity/extensibility, S – alveograph strength, G – alveograph swelling index, FMT – farinograph mixing time, FWA – farinograph flour water absorption, FStab – farinograph stability, FTol – farinograph tolerance, BU – Brabender units, MPH – mixograph peak height, MTH – mixograph tail height, MTW – mixograph tail width, MMT – Mixograph mixing time.

Similar to the SRI region, the environment (Y, L and Y x L) primarily contributed to the variation in grain-, milling- and flour quality properties that were measured in the SRD region (Tables 4.10 – 4.14). While, the G x E interaction contributed with a greater effect in the SRD region compared to the SRI region. The PG effect contributed with a higher percentage to grain characteristics and milling quality in the SRI region compared to the SRD region; while the contribution of PG to the variation of alveograph-, farinograph- and mixograph properties were higher in the SRD region than in the SRI region (Tables 4.3 – 4.7 and 4.10 – 4.14).

The variation in some grain characteristics were significantly attributed to the G effect; i.e. SKCS-HI (9.75% PG, $P < 0.001$; 4.18% C, $P < 0.001$), SKCS-Weight (3.88% PG, $P < 0.001$; 2.00% C, $P < 0.001$) and SKCS-Dia (3.59% PG, $P < 0.001$; 5.45% C, $P < 0.001$) (Table 4.10).

The G effect contributed significantly towards the variation in the milling- and flour components; i.e. BFY (2.87% PG, $P < 0.001$; 0.58% C, $P < 0.001$), TFY (6.24% PG, $P < 0.001$; 1.20% C, $P < 0.001$), FN (1.35% PG, $P < 0.001$; 8.46% C, $P < 0.001$), Fprot (0.67% PG, $P < 0.01$; 3.29% C, $P < 0.001$), Fmoist (0.63% PG, $P < 0.001$; 0.42% C, $P < 0.05$) and FWG (3.69% PG, $P < 0.001$; 3.48% C, $P < 0.001$) (Table 4.11).

Alveograph properties that had a significant component explained by the PG included P (8.14% PG, $P < 0.001$; 6.94% C, $P < 0.001$), L (7.46% PG, $P < 0.001$; 2.94% C, $P < 0.001$), P/L ratio (9.72% PG, $P < 0.001$; 3.10% C, $P < 0.001$), S (2.73% PG, $P < 0.001$; 7.24% C, $P < 0.001$), and G (7.71% PG, $P < 0.001$; 3.01% C, $P < 0.001$) (Table 4.12).

The G effect significantly contributed to variation in only one farinograph property, namely FWA (6.08% PG, $P < 0.001$; 3.67% C, $P < 0.001$) (Table 4.13). The G effect significantly contributed to variation in all mixograph properties, i.e. MPH (3.24% PG, $P < 0.001$; 7.91% C, $P < 0.001$), MTH (5.26% PG, $P < 0.001$; 7.92% C, $P < 0.001$), MTW (6.47% PG, $P < 0.001$; 1.16% C, $P < 0.05$) and MMT (5.41% PG, $P < 0.001$; 1.01% C, $P < 0.05$) (Table 4.14).

Table 4.10. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for wheat grain characteristics in the summer rainfall dryland (SRD) region

Source	DF	SKCS-HI		SKCS-Moist		SKCS-weight		SKCS-Dia		Grain protein		
		Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	DF	Mean ± SD	% of SS
Year	2	2237.76	27.57***	15.28	16.84***	93.08	2.86***	0.24	3.27***	1	33.92	6.79***
Lok	5	771.98	23.78***	10.32	28.43***	571.77	43.99***	1.35	46.27***	4	43.40	34.75***
Y x L	3	91.76	1.70***	23.22	38.37***	312.39	14.42***	0.47	9.58***	2	35.65	14.27***
Rep (Y x L)	22	10.22	1.39 ns	0.16	1.88*	4.49	1.52 ns	0.01	1.07 ns	16	0.86	2.75*
Total E			**54.44**		**85.52**		**62.79**		**60.19**			**58.56**
PG	3	527.56	9.75***	0.22	0.36 ns	84.15	3.88***	0.18	3.59***	3	0.83	0.50 ns
C	5	135.84	4.18***	0.78	2.15***	25.98	2.00***	0.16	5.45***	5	8.14	8.15***
Total G			**13.93**		**2.51**		**5.88**		**9.04**			**8.65**
Y x PG	6	64.33	2.38***	0.23	0.76*	7.25	0.67 ns	0.01	0.46 ns	3	3.07	1.84***
L x PG	15	29.39	2.72***	0.10	0.82 ns	29.10	6.72***	0.07	6.97***	12	1.38	3.32***
Y x L x PG	9	51.00	2.83***	0.02	0.12 ns	14.11	1.95***	0.02	1.13 ns	6	1.87	2.24***
Total PG x E			**7.93**		**1.70**		**9.34**		**8.56**			**7.40**
Y x C	25	37.07	5.71***	0.09	1.28 ns	19.78	7.61***	0.04	6.41***	20	1.91	7.66***
L x C	10	44.63	2.75***	0.12	0.67 ns	13.97	2.15***	0.03	1.94**	5	2.81	2.81***
Y x L x C	15	50.35	4.65***	0.04	0.37 ns	10.14	2.34***	0.02	2.29**	10	2.20	4.41***
Total C x E			**13.11**		**2.32**		**12.10**		**10.64**			**14.88**
Total G x E			**21.04**		**4.02**		**21.44**		**19.20**			**22.28**
Error	176	9.77	10.60	0.08	7.96	3.65	9.89	0.01	11.59	128	0.41	10.49
R^2		0.89		0.92		0.90		0.88			0.90	
CV		5.31		2.30		5.24		3.93			4.56	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, SKCS-HI – single kernel characterisation system hardness index, SKCS-Moist – single kernel characterisation system kernel moisture, SKCS-Weight – single kernel characterisation system kernel weight, SKCS-Dia – single kernel characterisation system kernel diameter, Gprot – grain protein content at 12% moisture basis.

Table 4.11. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for wheat milling- and flour components in the summer rainfall dryland (SRD) region

Source	DF	BFY		TFY		FN		Fprot		Fmoist		Fash		FWG	
		Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS
Year	2	6035.63	78.71***	1687.82	32.38***	47845.67	11.52***	71.86	15.24***	34.63	65.38***	0.33	39.37***	547.78	9.72***
Lok	5	213.78	6.97***	467.94	22.45***	42460.93	25.57***	95.06	50.40***	2.00	9.44***	0.03	8.47***	988.11	43.86***
Y x L	3	111.51	2.18***	207.69	5.98***	15114.40	5.46***	20.22	6.43***	1.75	4.96***	0.06	10.87***	367.89	9.80***
Rep (Y x L)	22	1.48	0.21 ns	5.29	1.12 ns	637.98	1.69 ns	0.65	1.51 ns	0.05	1.07 ns	0.00	1.58 ns	10.60	2.07*
Total E			**88.07**		**61.93**		**44.24**		**73.58**		**80.85**		**60.29**		**65.45**
PG	3	146.58	2.87***	216.73	6.24***	3739.45	1.35***	2.11	0.67**	0.22	0.63***	0.00	0.70 ns	138.40	3.69***
C	5	17.79	0.58***	25.03	1.20***	14045.60	8.46***	6.21	3.29***	0.09	0.42*	0.01	1.51*	78.49	3.48***
Total G			**3.45**		**7.44**		**9.81**		**3.96**		**1.05**		**2.21**		**7.17**
Y x PG	6	14.73	0.58***	30.79	1.77***	1158.48	0.84 ns	2.29	1.46***	0.15	0.87***	0.00	0.64 ns	46.25	2.46***
L x PG	15	12.13	1.19***	32.33	4.65***	1685.12	3.04***	1.25	1.99***	0.19	2.71***	0.00	2.85*	29.02	3.86***
Y x L x PG	9	9.64	0.57***	20.59	1.78***	2486.77	2.70***	1.91	1.82***	0.21	1.77***	0.01	2.92**	15.74	1.26**
Total PG x E			**2.34**		**8.20**		**6.58**		**5.27**		**5.35**		**6.41**		**7.58**
Y x C	25	10.25	1.67***	24.70	5.92***	4328.01	13.03***	1.75	4.64***	0.12	2.93***	0.00	6.76***	21.79	4.84***
L x C	10	6.09	0.40**	29.06	2.79***	3010.17	3.63***	2.35	2.49***	0.09	0.83*	0.00	2.91***	23.29	2.07***
Y x L x C	15	13.42	1.31***	44.17	6.36***	5630.17	10.17***	1.46	2.32***	0.18	2.54***	0.00	3.00*	25.36	3.38***
Total C x E			**3.38**		**15.07**		**26.83**		**9.45**		**6.30**		**12.67**		**10.29**
Total G x E			**5.72**		**23.27**		**33.41**		**14.72**		**11.65**		**19.08**		**17.87**
Error	176	2.41	2.77	4.36	7.37	591.55	12.54	0.41	7.74	0.04	6.44	0.00	18.43	6.09	9.52
R^2		0.97		0.93		0.87		0.92		0.94		0.82		0.90	
CV		6.36		3.30		6.82		5.10		1.27		6.68		6.66	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, FN – falling number, BFY – break flour yield, TFY – total flour yield, Fprot – flour protein, Fmoist – flour moisture, Fash – flour ash, FWG – flour wet gluten.

Table 4.12. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for Alveograph properties in the summer rainfall dryland (SRD) region

		P		L		P/L		S		G	
	DF	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS
Year	2	1105.62	1.79***	17152.17	16.95***	3.81	10.25***	1952.67	7.74***	216.30	16.71***
Lok	5	5091.60	20.65***	6935.76	17.13***	2.19	14.76***	4420.71	43.81***	92.89	17.94***
Y x L	3	3240.50	7.89***	2093.48	3.10***	0.91	3.69***	1017.04	6.05***	28.41	3.29***
Rep (Y x L)	22	95.59	1.71 ns	251.39	2.73 ns	0.08	2.25 ns	36.98	1.61 ns	3.05	2.60 ns
Total E			**32.04**		**39.91**		**30.95**		**59.21**		**40.54**
PG	3	3343.37	8.14***	5034.77	7.46***	2.41	9.72***	458.59	2.73***	66.50	7.71***
C	5	1709.46	6.94***	1191.46	2.94***	0.46	3.10***	730.83	7.24***	15.60	3.01***
Total G			**15.08**		**10.40**		**12.82**		**9.97**		**10.72**
Y x PG	6	546.84	2.66***	410.89	1.22 ns	0.08	0.65 ns	137.73	1.64***	3.38	0.78 ns
L x PG	15	1048.21	12.76***	1449.92	10.74***	0.73	14.75***	104.36	3.10***	18.78	10.88***
Y x L x PG	9	874.67	6.39***	1082.10	4.81***	0.77	9.30***	122.67	2.19***	16.53	5.75***
Total PG x E			**21.81**		**16.77**		**24.70**		**6.93**		**17.41**
Y x C	10	498.24	4.04***	954.15	4.71***	0.22	2.90***	324.12	6.42***	11.03	4.26***
L x C	25	307.19	6.23***	572.07	7.06***	0.18	5.93***	59.32	2.94***	6.85	6.62***
Y x L x C	15	444.46	5.41***	334.18	2.48 ns	0.20	4.02***	173.05	5.14***	4.73	2.74*
Total C x E			**15.68**		**14.25**		**12.85**		**14.50**		**13.62**
Total G x E			**37.49**		**31.02**		**37.55**		**21.43**		**31.03**
Error	172	110.41	15.41	219.61	18.66	0.08	18.67	27.55	9.39	2.67	17.71
R^2		0.85		0.81		0.81		0.91		0.82	
CV		10.14		14.91		24.52		10.42		7.42	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, P – alveograph elasticity, L – alveograph extensibility, P/L – alveograph ratio elasticity/extensibility, S – alveograph strength, G – alveograph swelling index.

Table 4.13. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for Farinograph properties in the summer rainfall dryland (SRD) region

		FMT		FWA		FStab		FTol	
	DF	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS
Year	2	321.21	7.75***	382.62	20.95***	1875.43	17.59***	253.14	0.73^{ns}
Lok	5	998.28	60.24***	284.43	38.93***	1375.56	32.26***	2861.74	20.62***
Y x L	2	37.32	0.90*	42.71	2.34***	808.52	7.58***	4004.64	11.54***
Rep (Y x L)	20	1.81	0.44 ^{ns}	1.10	0.60 ^{ns}	12.06	1.13 ^{ns}	103.37	2.98 ^{ns}
Total E			**69.33**		**62.82**		**58.56**		**35.87**
PG	3	6.67	0.24 ^{ns}	74.00	6.08***	46.16	0.65 ^{ns}	93.91	0.41 ^{ns}
C	5	6.24	0.38 ^{ns}	26.78	3.67***	68.89	1.62 ^{ns}	499.34	3.60***
Total G			**0.62**		**9.75**		**2.27**		**4.01**
Y x PG	6	8.89	0.64 ^{ns}	14.25	2.34***	83.86	2.36*	770.10	6.66***
L x PG	15	15.18	2.75 ^{ns}	10.44	4.29***	26.52	1.87 ^{ns}	146.00	3.16 ^{ns}
Y x L x PG	6	5.52	0.40 ^{ns}	6.20	1.02***	40.71	1.15 ^{ns}	99.27	0.86 ^{ns}
Total PG x E			**3.79**		**7.65**		**5.38**		**10.68**
Y x C	10	21.64	2.61*	13.70	3.75***	69.36	3.25*	471.84	6.80***
L x C	24	14.65	4.24 ^{ns}	12.64	8.30***	52.80	5.94*	280.88	9.72***
Y x L x C	10	6.03	0.73 ^{ns}	10.54	2.89***	78.06	3.66**	861.50	12.42***
Total C x E			**7.58**		**14.94**		**12.85**		**28.94**
Total G x E			**11.37**		**22.59**		**18.23**		**39.62**
Error	146	10.60	18.67	1.21	4.85	30.58	20.94	97.47	20.51
R^2		0.81		0.95		0.79		0.79	
CV		38.98		1.77		35.05		36.06	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, FMT – farinograph mixing time, FWA – farinograph flour water absorption, FStab – farinograph stability, FTol – farinograph tolerance.

Table 4.14. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for Mixograph properties in the summer rainfall dryland (SRD) region

		MPH		MTH		MTW		MMT	
	DF	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS	Mean ± SD	% of SS
Year	2	906.49	21.33***	140.19	6.42***	605.91	15.30***	17.43	25.14***
Lok	5	398.30	23.43***	274.07	31.36***	428.24	27.03***	6.54	23.59***
Y x L	3	141.86	5.01***	33.32	2.29***	3.51	0.13 ns	1.69	3.66***
Rep (Y x L)	22	7.32	1.89 ns	3.00	1.51 ns	4.22	1.17 ns	0.08	1.33 ns
Total E			**51.66**		**41.58**		**43.63**		**53.72**
PG	3	91.71	3.24***	76.65	5.26***	170.83	6.47***	2.50	5.41***
C	5	134.52	7.91***	69.23	7.92***	18.38	1.16*	0.28	1.01*
Total G			**11.15**		**13.18**		**7.63**		**6.42**
Y x PG	6	26.71	1.89***	21.78	2.99***	54.49	4.13***	0.61	2.64***
L x PG	15	17.01	3.00***	19.86	6.82***	50.92	9.64***	0.33	3.52***
Y x L x PG	9	19.41	2.06***	11.82	2.43***	23.07	2.62**	0.28	1.81**
Total PG x E			**6.95**		**12.24**		**16.39**		**7.97**
Y x C	10	20.56	2.42***	21.79	4.99***	54.58	6.89***	0.96	6.95***
L x C	25	29.32	8.62***	13.00	7.44***	14.52	4.58*	0.33	5.98***
Y x L x C	15	53.84	9.50***	22.00	7.55***	18.27	3.46**	0.52	5.61***
Total C x E			**20.54**		**19.98**		**14.93**		**18.54**
Total G x E			**27.49**		**32.22**		**31.32**		**26.51**
Error	176	4.68	9.7	3.23	13.02	7.84	17.42	0.11	13.36
R^2		0.90		0.87		0.83		0.87	
CV		3.56		3.70		18.88		10.26	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares,% of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components in the cultivar, E – environment, Y – year, L – location, MPH – mixograph peak height, MTH – mixograph tail height, MTW – mixograph tail width, MMT – Mixograph mixing time.

4.4. Discussion

4.4.1. Influence of the puroindoline a null allele, Pina-D1b, in comparison to the Pinb-D1b mutation on wheat grain characteristics, milling quality and flour properties

The interpretation of results from the combined nested ANOVA, and Fisher's LSD test, provides clarity on the cause of significant differences between *Pin* genotype classes regarding quality properties. The environment substantially contributed to the variation of most grain and flour quality properties in the SRI region, with comparatively little contribution by G x E interaction (Tables 4.3 – 4.7). In the SRD region environment also substantially contributed to the variation in grain and flour quality properties, however, more variation was contributed by the G x E interaction (Tables 4.10 – 4.14) than observed in the SRI region.

Wheat samples containing the *Pina-D1b*/*Pinb-D1a* genotype had significantly harder grain kernels (SKCS-HI) compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype; with a mean difference of 8 SKCS-HI units in the SRI region and 6 SKCS-HI units in the SRD region (Tables 4.2 & 4.8). This observed difference in SKCS-HI were similar to those reported in earlier studies (Giroux *et al*., 2000; Martin *et al*., 2001; Ma *et al*., 2009; Takata *et al*., 2010). The variance contribution of *Pin* genotype (PG) was significant in the SRI and SRD regions, however, in the SRD region the PG x E interaction contributed more to the variation in SKCS-HI compared to the SRI region (Tables 4.3 & 4.10).

Wheat with the *Pina-D1b*/*Pinb-D1a* genotype had lower SKCS-Weight and SKCS-Dia compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype, in both the SRI and SRD regions (Tables 4.2 & 4.8). The contribution of PG to the variation in SKCS-Weight and SKCS-Dia was significant for both regions. There was, however, a higher contribution by PG x E interaction to the variation in SKCS-Weight and SKCS-Dia in the SRD region, compared to the SRI region (Tables 4.3 & 4.10). The contribution of PG to the variation in kernel weight and diameter corresponded to earlier work that found decreased kernel weight (Martin *et al.*, 2001) and -diameter (Martin *et al.*, 2001; Boehm *et al.*, 2018) in the *Pina-D1b*/*Pinb-D1a* genotype, compared to the *Pina-D1a*/*Pinb-D1b* genotype.

Milling quality of wheat, in terms of BFY and TFY, was lower in wheat with the *Pina-D1b*/*Pinb-D1a* genotype than wheat with the *Pina-D1a*/*Pinb-D1b* genotype, in both the SRI and SRD regions (Tables 4.2 & 4.8). Decreased BFY and TFY with the *Pina-D1b*/*Pinb-D1a* genotype were observed in earlier studies (Martin *et al.*, 2001; Cane *et al.*, 2004; Eagles *et al.*, 2006; Edwards *et al.*, 2010). The contribution of PG to the variation in BFY and TFY were considerably lower in the SRD region than the SRI region. This is attributed to the high G x E interaction in the SRD region. In both regions it was apparent that the variation in BFY is influenced by the E effect to a higher degree than the TFY. The PG contribution to the variation in TFY were higher than to the variation in BFY (Tables 4.4 & 4.11).

The protein content of flour obtained from wheat with the *Pina-D1b*/*Pinb-D1a* genotype was lower than flour with the *Pina-D1a*/*Pinb-D1b* genotype, in both the SRI and SRD regions (Tables 4.2

& 4.8). The contribution of PG to variation in flour protein content was minimal but significant in both regions, with a higher contribution of C than PG, while the environment primarily contributed to the variation in flour protein content (Tables 4.4 & 4.11). Contrasting results were observed in earlier research (Cane *et al.*, 2004). The discrepancy of findings between the current research and the findings of Cane *et al.* (2004) could be attributed to the large environmental effect on flour protein content.

The Fmoist and Fash were higher for wheat containing the *Pina-D1b*/*Pinb-D1a* genotype compared to the *Pina-D1a*/*Pinb-D1b* genotype in the SRI region (Table 4.8). The contribution of PG to variation in Fmoist and Fash was significant (Table 4.11). Martin and colleagues (2001) also observed an increased flour ash content for wheat containing the *Pina-D1b*/*Pinb-D1a* genotype compared to the *Pina-D1a*/*Pinb-D1b* genotype.

In both the SRI and SRD regions, flour from wheat with the *Pina-D1b*/*Pinb-D1a* genotype produced dough with higher alveograph P and -P/L ratio, but lower alveograph L, -S, and -G compared to flour from wheat with the *Pina-D1a*/*Pinb-D1b* genotype (Tables 4.2 & 4.9). The contributions of PG to the variation in alveograph properties were all significant, although the contribution of C to the variation in alveograph properties were higher. The variation S was primarily contributed by the E effect, and the G x E interaction, in both the SRI and SRD regions (Tables 4.5 & 4.12). The results regarding P, -P/L ratio, -L and -G were in accordance with the results reported in earlier work (Eagles *et al.*, 2006; Chen *et al.*, 2013). However, Chen *et al.* (2013) observed increased S in flour with the *Pina-D1b*/*Pinb-D1a* genotype, compared to the *Pina-D1a*/*Pinb-D1b* genotype. This could be due to the high environmental influence, and G x E interaction that contributed to the variation in S (Tables 4.5 & 4.12).

The higher FWA observed in the SRD region for flour with the *Pina-D1b*/*Pinb-D1a* genotype is in accordance with results of other researchers (Cane *et al.*, 2004; Eagles *et al.*, 2006; Chen *et al.*, 2013). The milling of hard wheat kernels resulted in more starch damage than the milling of soft wheat kernels. This implies that flour from wheat with the *Pina-D1b*/*Pinb-D1a* genotype should have increased starch damage compared to flour from wheat with the *Pina-D1a*/*Pinb-D1b* genotype. Increased starch damage in the wheat flour causes increased flour water absorption (Morrison & Tester, 1994; Brites *et al.*, 2008; Li *et al.*, 2014; Liu *et al.*, 2014). The contribution of PG to the variation in FWA in the SRD region was significant with 6.08% contribution, and higher than the contribution of C (3.67%) to the variation in FWA. In the SRI region, the contribution of PG to the variation in FWA was not significant, although C contributed (14.33%) significantly. The contribution of the total G x E interaction, to the variation in FWA, in the SRD region was higher than in the SRI region, which could explain the difference in results observed between the two regions (Tables 4.6 & 4.13).

The reduced MTH and MTW in flour from wheat with the *Pina-D1b*/*Pinb-D1a* genotype, from both the SRI and SRD regions, indicated a decrease in dough strength, -stability and tolerance to overmixing, compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype. This corresponds to the

decrease in alveograph S mentioned earlier. In previously reported work (Chen *et al.*, 2013; Katyal *et al.*, 2018) increased dough strength and -stability with an increase in GKH were observed, in contrast to the current findings. This discrepancy could be attributed to the substantial contribution of environment, and G x E interaction to the variation in MTH and MTW in the current study (Tables 4.7 & 4.14).

In the SRD region, the higher MPH and lower MMT in flour from wheat with the *Pina-D1b*/*Pinb-D1a* genotype refers to a decreased optimum dough development time, with increased dough strength at the optimum development time. A decrease in dough development time with an increase in GKH has been reported (Martin *et al.*, 2001; Eagles *et al.*, 2006; Katyal *et al.*, 2018). The contribution of PG to the variation in MPH and MMT was significant in the SRD region, but not significant in the SRI region. In the SRD region, the total genotype contribution and G x E interaction to the variation in MPH and MMT, were considerably higher than in the SRI region, which explains the difference in results between the two regions (Tables 4.7 & 4.14).

Eagles *et al.* (2006) suggested that the difference in flour quality observed between the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes might be due to the quantity of PIN protein expressed in the wheat endosperm rather than the functional quality of the expressed PIN protein as affected by the *Pina-D1* and *Pinb-D1* allele mutations. The quality of wheat proteins is determined genetically, while the environmental influence determines the quantity of protein. The environment and G x E interaction do not influence different protein components such as glutenin, gliadin, albumins and globulins equally (Cornish *et al.*, 2006; Vázquez *et al.*, 2012). The environmental influence on the content of different protein components could explain the differences in quality properties observed between the SRI and SRD regions. This could be attributed to the high E effect and G x E interaction contributing to the variation in the different dough quality properties.

4.4.2. Influence of puroindoline b allele mutations on wheat grain characteristics, milling quality and flour properties in the summer rainfall dryland region

All three *Pin* genotypes (*Pina-D1a*/*Pinb-D1b*, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*) contained the *Pina-D1a* wild-type allele, with different mutations at the *Pinb-D1* locus. Therefore, the *Pina-D1a*/*Pinb-D1b* genotype will be referred to as *Pinb-D1b*, the *Pina-D1a*/*Pinb-D1p* genotype will be referred to as *Pinb-D1p*, and the *Pina-D1a*/*Pinb-D1ab* genotype will be referred to as *Pinb-D1ab*.

Wheat with the *Pinb-D1p* mutation had lower SKCS-Weight and SKCS-Dia compared to wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations (Table 4.8). SKCS-Weight and SKCS-Dia have been shown to negatively correlate with GKH (Turnbull & Rahman, 2002) however no significant difference ($P > 0.05$) in SKCS-HI was observed between the three *Pinb-D1* mutations. Takata *et al.* (2010) reported *Pinb-D1p* to be significantly higher in SKCS-HI than *Pinb-D1b*, however, it was not observed in the current study.

Flour with the *Pinb-D1p* mutation had higher BFY, TFY and FN than flour with the *Pinb-D1b* and *Pinb-D1ab* mutations. The higher BFY and TFY of wheat with the *Pinb-D1p* mutation, in comparison

to wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations, could not be related to the variation in GKH, since the SKCS-HI results did not differ significantly. The alveograph P, -P/L ratio and -S, FWA and MPH of wheat with the *Pinb-D1p* mutation were higher; while the alveograph L and -G were lower, compared to wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations. Although the SKCS-HI of wheat with *Pinb-D1p* was not significantly higher ($P > 0.05$) than wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations, the flour and dough quality properties are in comparison with those of wheat with the *Pina-D1b*/*Pinb-D1a* mutation. The SKCS-HI of wheat with *Pina-D1b*/*Pinb-D1a* mutation were significantly higher than wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations (Table 4.8). Both the *Pina-D1b* and *Pinb-D1p* alleles are null alleles of *Pina-D1*, and *Pinb-D1* genes, respectively. The similar observed dough properties might be due to the lower PIN protein content expressed in the wheat endosperm.

The MTH values, referring to increased dough strength and stability, and MMT values of wheat with the *Pinb-D1p* mutation were higher compared to wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations. The MTW, referring to increased dough tolerance to overmixing, of wheat with *Pinb-D1p* and *Pinb-D1ab* mutations were similar. The latter two mutations, however, had MTW higher than *Pinb-D1b* mutation.

4.5. Conclusion

The knowledge obtained during this study can be implemented by wheat breeders, to select for *Pin* allele combinations with a specific end-use in mind. Similarly, the milling and baking industries can use the obtained results to select wheat cultivars with the desired *Pin* genotype that will provide flour with a required processing quality.

A breeding line with the *Pina-D1b*/*Pinb-D1a* genotype, would result in a wheat with increased SKCS-HI, but decreased SKCS-Weight and SKCS-Dia, BFY and TFY compared to a breeding line with the *Pina-D1a*/*Pinb-D1b* genotype. The flour of wheat with the *Pina-D1b*/*Pinb-D1a* genotype would have higher dough tenacity and FWA, but lower dough extensibility, -strength and tolerance to overmixing compared to flour from wheat with the *Pina-D1a*/*Pinb-D1b* genotype.

The differences in wheat and flour properties observed between the three *Pinb-D1* mutations, implies that the observed variation in flour and dough properties between wheat with the *Pinb-D1* mutations, are due to the functional quality of the expressed PIN protein caused by the mutation in the *Pinb-D1* allele. A breeding line that contains the *Pinb-D1p* mutation would result in wheat with lower SKCS-Weight and SKCS-Dia, but higher BFY and TFY compared to the *Pinb-D1b* and *Pinb-D1ab* mutations. Furthermore, the breeding line with the *Pinb-D1p* mutation would provide flour with increased dough tenacity, -strength, and FWA; but lower dough extensibility and swelling index compared to flour from wheat with the *Pinb-D1b* and *Pinb-D1ab* mutations.

With the knowledge on the expected grain-, milling- and flour quality properties of the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes; and, of the differences between the *Pinb-D1b*,

Pinb-D1p and *Pinb-D1ab* mutations, breeders can make a more informed selection regarding the end-use properties that they are breeding towards.

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CHAPTER 5

Influence of genotype and environment on bread wheat (*Triticum aestivum*) kernel harness and correlation with grain and flour quality traits in dryland spring wheat

Abstract

The winter rainfall spring wheat production region of South Africa can be divided into two main areas with different climate conditions, namely the Rûens region with moderate temperatures and possible summer rainfall, and the dry Swartland region with high temperatures. Nine bread wheat cultivars (including the wheat quality standard), were planted in two locations per area, over three planting seasons. Wheat grain kernel hardness (GKH), using the single kernel characterisation system (SKCS), as well as milling- and dough characteristics were determined. Significant differences between the SKCS hardness index (SKCS-HI) of wheat cultivars with identical *puroindoline* genotype confirms the influence of minor genes on the expression of GKH. ANOVA statistical analysis indicated that the variation in GKH was primarily due to genotype (G) in the Swartland region, and primarily due to environment (E) and G x E interaction in the Rûens region. Variation in GKH due to genotype had negative correlations with break flour yield, total flour yield and α-amylase activity. Wheat hardness variation due to environmental influence also had negative correlations with break flour yield and total flour yield. Additionally, environmental influence caused negative correlations with grain kernel weight and -diameter, and positive correlations with flour ash content, flour water absorption, dough strength, -stability, and -tenacity.

5.1. Introduction

Wheat grain quality is critical to various segments in the grain value chain; from the producer, wheat and flour processor, to the consumer. Grain quality refers to both the kernel characteristics and biochemical quality of the grain in a sample. However, wheat grain quality has different requirements depending on the intended end product for which it will be used. In the milling industry, the health and kernel characteristics of the grain are the most important, while properties like protein content, starch damage and α-amylase activity in the flour, is essential to the food production sector.

Different wheat species are used as primary ingredients for specific products (Mahesh *et al.*, 2008). Bread wheat (*Triticum aestivum*) and durum wheat (*T. turgidum* ssp. *durum*), are characterised by different chemical and physical properties (Kent & Evers, 1994a; O'Brien & DePauw, 2004). Based on these different properties, their wheat will differ in functional quality, nutritional contribution and consequently commercial value (Bietz, 1989). GKH is one of the most essential kernel characteristics that influence the processing of wheat. Hard bread wheat is primarily

used for leavened bread, while soft bread wheat is used for cakes and biscuits. The extremely hard grain of durum wheat is used for the production of pasta and couscous (Paulsen & Shroyer, 2004).

Wheat hardness is a grain kernel characteristic of wheat, which relates to the way wheat endosperm breaks down during milling (Kent & Evers, 1994b). GKH determines the milling performance, and ultimately the end-use quality of the wheat, and is therefore a key criterion for wheat classification (Wang *et al.*, 2008). Within the GKH range of very-soft to hard grain kernels, the harder grain kernels have better TFY, due to more efficient endosperm reduction. The efficiency of endosperm reduction comprises better separation of endosperm from the bran, less loss of wheat endosperm in the bran fraction, and a reduction in the total bran fraction percentage (Oury *et al.*, 2017).

Although the main contributor to GKH is genetically determined, the environment also has an influence on GKH, by producing vitreous or mealy grain kernels. Oury and colleagues (2017) found that the genetically influenced GKH, as determined by the single kernel characterisation system, was more important in affecting grain milling behaviour than grain vitreousness which is caused by environmental influence. Vitreousness is related to the packing of the starch granules and protein components in the grain endosperm, and is primarily influenced by grain protein content (Oury *et al.*, 2015).

High temperature alters the grain filling of wheat kernels by decreasing the duration of grain filling, and thus, the accumulation of protein and starch in the endosperm. Some wheat cultivars may adjust to the higher temperature by increased enzyme activity and metabolic processes; thus, filling the kernel to the same extent in a shorter time. However, other wheat cultivars may remain constant in the rate of grain filling during this shortened time; consequently, decreasing the wheat kernel's final weight. Studies have demonstrated that water stress conditions decrease kernel weight considerably, by shortening the grain filling period without increasing the filling rate (Brooks *et al.*, 1982; Kobata *et al.*, 1992; Altenbach *et al.*, 2003).

Typically, high temperatures during grain filling cause increased grain protein content, though certain cultivars may genetically produce a consistently higher grain protein content than other cultivars. Apart from the environmental influence of temperature, grain protein content may also be affected by drought, frost damage, and certain diseases (Delcour & Hoseney, 2010). Environmental factors strongly influence protein content; however, the protein quality is genetically determined (Cornish *et al.*, 2006; Vázquez *et al.*, 2012).

Wheat grain endosperm has a trimodal distribution of starch granules, which is formed and filled at different stages in the wheat kernel development. Large type A cells are first produced (initiated two days after flowering,), after which small type B starch cells are produced (initiated ten days after flowering), followed by smaller type C starch cells (initiated 21 days after flowering) (Bechtel *et al.*, 1990). Under normal environmental conditions, without temperature or water stress, harder wheat grain endosperm typically consists of a higher percentage of small starch granules (type B 2.8 – 9.9

μm and type C < 2.8 μm) and smaller starch granules (type A > 9.9 μm) compared to soft wheat endosperm (Igrejas *et al.*, 2002; Li *et al.*, 2008). The physicochemical properties, and end-use quality of the three types of starch granules differ slightly (Anjum & Walker, 1991; Raeker *et al.*, 1998; Kumar *et al.*, 2016). The surface area of small starch granules (type B and C), has been estimated at three times higher than that of large starch cells (type A), producing a higher contribution towards endosperm strength and cohesion of starch granules and the protein matrix (Konopka *et al.*, 2005).

The starch granules consist of amylose and amylopectin, which are two types of glucose polymers. Amylose is a mostly linear polymer of α-D-glucose linked by α-1,4 bonds, and has a low degree of α-1,6-glycosidic branches. Similar to amylose, amylopectin is composed of α-D-glucose linked by α-1,4 bonds, but contains a much higher degree of α-1,6-glycosidic branches. The amylose and amylopectin polymers form amorphous and crystalline regions in the starch granules, and are responsible for the birefringence and crystallinity of starch granules (Delcour & Hoseney, 2010). Kumar *et al.* (2016) reported larger granules (A type) had higher birefringence and crystallinity than smaller starch granules (type B and C). Soft wheat starch contains a higher percentage of amylose than hard wheat starch, and the amylose percentage decreases in the order of type A>B>C starch granules.

High temperature during grain filling causes a decrease in starch synthesis and reduces the duration of grain filling, resulting in less type B and C starch granules being produced in the wheat endosperm, and a comparatively higher amount of type A starch granules (Bhullar & Jenner, 1985; Macleod & Duffus, 1988; Bechtel *et al.*, 1990; Blumenthal *et al.*, 1990; Tester & Karkalas, 2001; Park *et al.*, 2009). In contrast to this, the deficit of soil water availability at 14 to 21 days after flowering, increases the accumulation of small starch granules (Dai, 2009). Environmental influences can thus influence the starch packing and composition of wheat grain endosperm, thereby influencing physical GKH.

GKH has been reported to be positively correlated with starch damage during milling (Garland-Campbell *et al.*, 2001; Ma *et al.*, 2009; Choy *et al.*, 2015), while increased starch damage is known to increase the flour water absorption (Morrison & Tester, 1994; Brites *et al.*, 2008; Li *et al.*, 2014; Liu *et al.*, 2014). Damaged starch leads to the crystalline region of the starch granules being broken, and consequently water can more easily enter into the starch granules (Liu *et al.*, 2014). Increased starch damage causes an increase in flour water absorption and dough development time, and a decrease in dough stability to overmixing (Liu *et al.*, 2014; Katyal *et al.*, 2018).

Several studies have been conducted on milling- and flour properties, affected by GKH. Some studies had good experimental design with many cultivars, years and locations (Martin *et al.*, 2001; Cane *et al.*, 2004; Eagles *et al.*, 2006); while others' field trials included too few seasons or locations to rely on the validity of the results (Baker & Dyck, 1975; Giroux *et al.*, 2000; Chen *et al.*, 2013). Seasonal effects can be greater than location effects (Bassett *et al.*, 1989), emphasising the importance of sufficient data capturing over planting seasons and locations.

Variation in GKH, milling- and quality traits have been observed amongst wheat cultivars with the same *puroindoline* genotype (Martin *et al.*, 2001), indicating that other genes also influence GKH. Although it is well accepted that *puroindolines* and the *Ha* locus, on chromosome 5DS, is the cause of wheat GKH, several major and minor genes have been identified on other chromosomes that have influenced GKH. These include the Glu-B3 locus on chromosome 1BS, 1AS, 1BL, 5AL, 5BL, 6BL and 7BS (Boehm *et al.*, 2018), 5BL (Sourdille *et al.*, 1996), 1A, 5A, 5B (Tsilo *et al.*, 2010), 1DL, 5AS, 5BL, 5DL, 7AL (Sun *et al.*, 2010), 1BL (Li *et al.*, 2009), 1BS, 4BS 5BS 2DS 4DS, 5DL (Wang *et al.*, 2012), 5A (Jernigan *et al.*, 2018).

The objectives of this research chapter were; 1) to evaluate the variation in GKH of various cultivars with an identical *Pin* allelic genotype, 2) to determine the genotype (G), environment (E) and G x E interaction of spring wheat produced in two winter rainfall dryland regions with different climates, and 3) to determine the correlation of GKH with milling- and flour properties.

5.2. Materials and methods

5.2.1. Experimental population and field trials

The wheat samples planted in the Western Cape winter rainfall dryland (WRD) region (as described in Chapter 3), were used for this research study. The wheat cultivars used were the wheat quality standard for the WRD region (Kariega) and eight commercial wheat cultivars, namely PAN3434, Ratel, Baviaans, SST015, SST096, SST056, SST087 and SST88. The selected cultivars have been identified to have the *puroindoline* (*Pin)* allelic genotype *Pina-D1a*/*Pinb-D1b* (Chapter 3).

The Western Cape wheat production region can be divided into two regions based on climate, i.e. Rûens and Swartland. The Swartland region is usually warmer with no summer rainfall, it receives most rain from the North West direction in winter, and it is very dry when the South East wind is blowing in summer. This region is very fertile and has been known as the breadbasket of Cape Town with wheat fields up to the foot of the mountains, interrupted by farms producing wine, fruit, and vegetables. The Swartland is divided from the Rûens region by the Hottentots-Holland mountain range. Rûens has a moderate and cooler climate compared to Swartland, with a possibility of some summer rain. This region receives rain both from the North West and South East directions, and the South East wind brings cloudy weather with high humidity. The Swartland region consists of Klipheuwel and Moorreesburg, while the Rûens region consists of Napier and Riversdal in this study (Fig. 5.1).

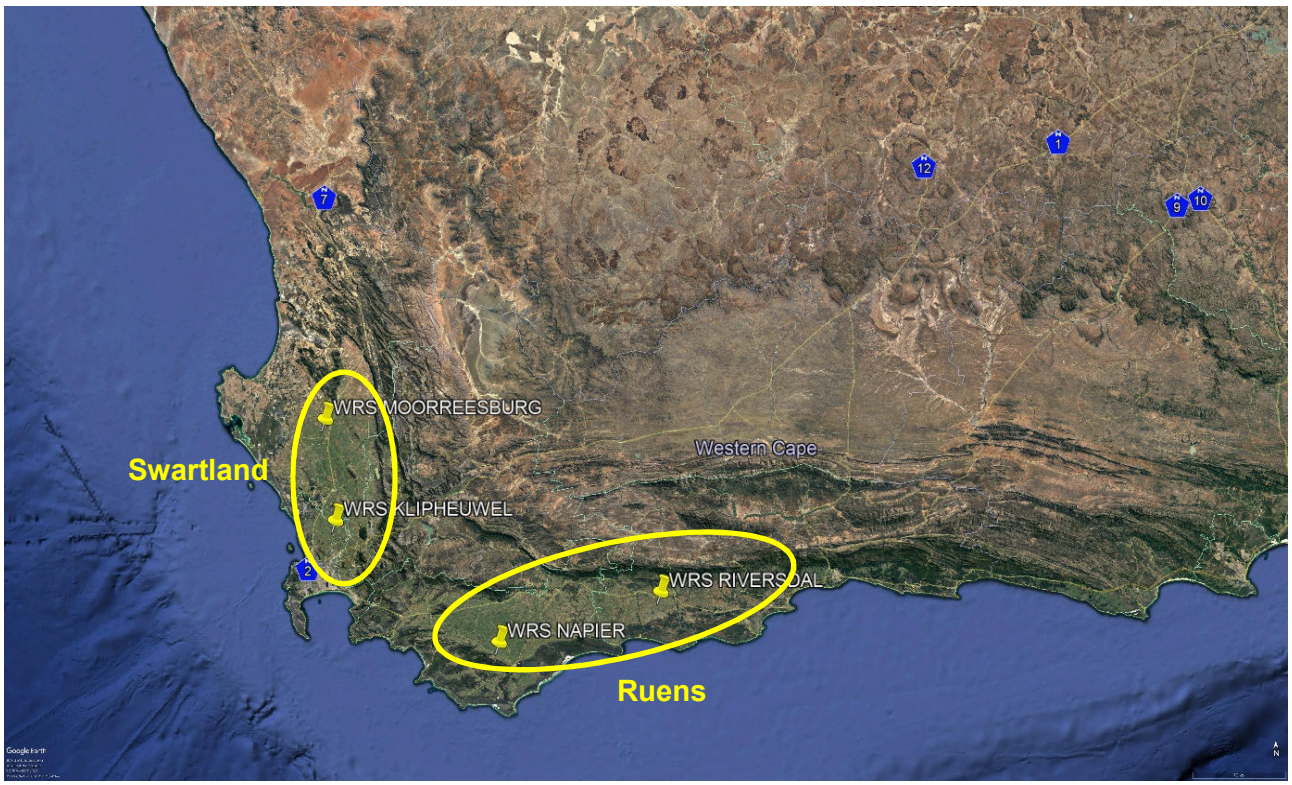

Figure 5.1. Map of South Africa indicating the Swartland and Rûens regions, and the trial locations situated in them.

5.2.2. Grain analysis

Single kernel characterisation system

Wheat kernel hardness was determined using the single kernel characterisation system method according to AACC approved method 55-31.01 (AACC, 1999a), as described in Chapter 3.2.3. One analysis per sample was conducted, using 300 kernels per analysis to determine grain hardness index (SKCS-HI), kernel moisture content (SKCS-Moist), kernel weight (SKCS-Weight) and kernel diameter (SKCS-Dia).

Grain protein

The grain protein (Gprot) content (12% moisture basis) was determined using wheat grain calibration no. 096126 with the FOSS Infratec™ 1241 grain analyser (FOSS analytics, Hillerød, Denmark), as described in Chapter 4.2.2.2.

5.2.3. Milling characteristics

The wheat samples were tempered to the desired moisture content based on their kernel hardness (SKCS-HI) and grain moisture content, as described in Chapter 4.2.3. The tempered grain was stored in airtight containers for 24 h before experimental milling commenced.

Milling was performed using a Chopin CD1 laboratory mill (Chopin technologies, Paris, France), according to the AACC approved method 26-70.01 (AACC, 2015), as described in Chapter 4.2.3.

The break flour yield (BFY) and total flour yield (TFY) were determined according to equations 4.1 and 4.2. The three flour fractions were thoroughly mixed and were subsequently used to perform various flour quality analyses.

5.2.4. Flour characteristics

Flour constituents

The chemical composition of the flour was determined using wheat flour calibration no. 133754 with the FOSS Infratec™ 1241 grain analyser (FOSS analytics, Hillerød, Denmark), as described in Chapter 5.2.4.1. The results obtained were flour protein- (12% moisture base) (Fprot), moisture- (Fmoist), ash- (Fash) and wet gluten (FWG) content.

Falling Number

The falling number (FN) test was performed according to the AACC approved method 56-81 (AACC, 1999b) to determine the α-amylase enzyme activity present in flour, as described in Chapter 4.2.4.2. The falling number method provides a surrogate measure of α-amylase activity in wheat flour, by measuring the degradation of a gelatinised starch paste due to α-amylase hydrolysis of the starch (Perten, 1964). Sound starch granules with low α-amylase activity will provide a high falling number, while a low falling number indicates high α-amylase activity that caused significant changes in starch quality.

Alveograph

The alveograph (Chopin technologies, Paris, France) was used to measure the dough tenacity (P), extensibility (L), resistance strength (S), and the swelling index (G) according to the AACC approved method 54-30.02 (AACC, 1999c), as described in Chapter 4.2.4.3.

Farinograph

The resistance of dough to mixing as measured using the Brabender Farinograph (Brabender GmbH & Co. KG, Kulturstraβe, Duisburg), dough development time (FMT), flour water absorption (FWA), dough stability (FStab), and tolerance to overmixing (FTol) were determined using the AACC approved method 54-21.02 with constant flour weight (AACC, 2011), as described in Chapter 4.2.4.4.

Mixograph

The was used to perform mixograph tests on the flour samples. The optimum dough development time, dough strength, stability and tolerance to overmixing were determined using the Mixograph with Mixsmart software (National MFG Co., Lincoln, Nebraska) according to the AACC approved method 54-40.02 (AACC, 1999d), as described in Chapter 4.2.4.5.

The optimum dough development or peak time (MPT), peak height (MPH) at peak time and tail height (MTH) and tail width (MTW) at 6 min were obtained from the mixogram. MPT is the time needed to mix the dough to optimum gluten development, MPH indicates the strength of the dough at optimum development, MTH indicates the strength and stability of the dough, and MTW indicates the dough's tolerance to over-mixing.

5.2.5. Statistical analysis

Analysis of variance (ANOVA) on the data of SKCS-HI in the Rûens and Swartland regions were performed individually using the PROC GLM in SAS software, Version 9.4 (SAS Institute Inc., Cary, North Carolina, USA). The Shapiro–Wilk test was performed to test the normality of residuals before it could be assumed that the data was reliable (Shapiro & Wilk, 1965). The sources of variation in the data were partitioned as replications (per year and locality), years, localities, genotypes (cultivars), year and genotype interaction, locality and genotype interaction and the interaction of genotype, years and localities.

This method is commonly used to analyse multi-environment data and is based on ANOVA, which requires homogenous variance-covariance of data since it is a fixed effects model. The homogeneity of variances was tested using Levene's test for homogeneity in the PROC GLM in SAS software, Version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) (Levene, 1960).

The Pearson's product moment correlation matrix of the pairwise correlations among the dependent variable (SKCS-HI) and 24 independent variables (quality data) was performed to calculate their linear relationships. The Pearson's correlation was calculated using PROC CORR of SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA).

The statistical model is given by equation 5.1.

$$Y_{ijkl} = \mu + Y_i + L_j + YL_{ij} + B(YL_{ijk}) + G_k + GY_{ik} + GL_{ik} + GYL_{ijk} + €_{ijkl}$$

..... Equation 5.1

Where: Yijkl = observed SKCS-HI value or quality parameter,

μ = general mean,

Yi = effect of the year,

Lj = effect of the locality,

YLij = interaction effect of the year and locality,

B(YLijk) = effect of block within year and locality,

Gk = effect of the genotype,

GYik = interaction effect of the genotype and year effect,

GLjk = interaction effect of the genotype and locality,

GYLijk = interaction effect of the genotype, year and locality,

€ijkl = error or residual effect, and

€ijkl ~ NID(0,σ2) (Shapiro & Wilk, 1965; Ott & Longnecker, 2001)

5.3. Results

5.3.1. Descriptive statistics

All quality properties differed significantly ($P < 0.05$) between the Swartland and Rûens regions, except for Fash content and MTH that differed non-significantly ($P > 0.05$). The mean SKCS-HI for the Swartland region (61.38 ± 7.80) was higher with a smaller standard deviation (SD) than the Rûens region (58.33 ± 12.66). The Gmoist was slightly higher in the Rûens region (12.90 ± 0.58%) compared to the Swartland region (12.26 ± 0.87%), while the SKCS-Weight and SKCS-Dia were also higher for the Rûens region (46.07 ± 4.55 mg and 2.79 ± 0.16 mm) than the Swartland region (40.95 ± 4.70 mg and 2.54 ± 0.18 mm) (Table 5.1).

The Gprot content was approximately the same for both regions (12.13 ± 1.15%, Rûens; 12.01 ± 1.33%, Swartland), however the flour yield differed (66.83 ± 3.28%, Rûens; 67.33 ± 2.53% Swartland), and the Swartland region had a higher Fprot percentage (11.29 ± 1.17%) compared to the Rûens region (10.59 ± 1.54%). The flour from the Swartland region had a higher FN (402.44 ± 27.64 s) compared to the Rûens region (354.19 ± 58.48 s) (Table 5.1), indicating there was less α-amylase activity in grain from the Swartland region.

Flour from the Swartland region had a higher alveograph L (96.58 ± 24.03 mm) compared to flour from the Rûens region (75.99 ± 25.76 mm), while the alveograph P had approximately the same values for both regions. Flour from the Swartland region had a higher alveograph S (43.62 ± 11.06 cm^2) compared to flour from the Rûens region (37.90 ± 9.06 cm^2) (Table 5.1).

Farinograph results indicated approximately the same values for FMT and FWA for both regions, however flour from the Swartland region had increased FStab (10.80 ±4.14 min) and better FTol (36.93 ± 14.33 min), compared to flour from the Rûens region (8.85 ± 3.04 min) and (45.65 ± 21.03 min) approximately (Table 5.1).

Flour from the Swartland region had a lower MTW at 6 min (12.78 ± 4.01 mm) compared to flour from the Rûens region (13.95 ± 3.91 mm) (Table 5.1).

Table 5.1. Means, minimum and maximum values, standard deviation and standard error values for all quality parameters for the Rûens and Swartland regions

	Rûens				Swartland				
Quality property	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**LSD**
SKCS-HI	162	58.33 ^{B} ± 12.66	37.90 – 83.80	0.99	162	61.38 ^{A} ± 7.80	33.30 – 78.10	0.61	0.94
SKCS-Moist (%)	162	12.90 ^{A} ± 0.58	12.00 – 14.00	0.05	162	12.26 ^{B} ± 0.87	10.30 – 13.60	0.07	0.26
SKCS-Weight (mg)	162	46.07 ^{A} ± 4.55	36.90 – 55.90	0.36	162	40.95 ^{B} ± 4.70	31.50 – 54.20	0.37	0.33
SKCS-Dia (mm)	162	2.79 ^{A} ± 0.16	2.46 – 3.16	0.01	162	2.54 ^{B} ± 0.18	2.16 – 3.01	0.01	0.02
Gprot (%, 12% mb)	108	12.13 ^{A} ± 1.15	9.90 – 14.60	0.11	108	12.01 ^{B} ± 1.33	9.80 – 14.40	0.13	1.98
FN (s)	162	354 ^{B} ± 58	212 – 432	4.59	162	402 ^{A} ± 28	330 – 435	2.17	2.46
BFY (%)	162	25.08 ^{B} ± 7.03	14.30 – 38.70	0.55	162	25.99 ^{A} ± 5.81	17.40 – 37.70	0.46	0.27
TFY (%)	162	66.83 ^{B} ± 3.28	57.90 – 74.40	0.26	162	67.33 ^{A} ± 2.53	53.60 – 71.80	0.20	0.32
FProt (%, 12% mb)	162	10.59 ^{B} ± 1.54	7.60 – 13.90	0.12	162	11.29 ^{A} ± 1.17	8.70 – 13.60	0.09	0.09
FMoist (%)	162	15.43 ^{A} ± 0.35	14.60 – 17.00	0.03	162	15.36 ^{B} ± 0.41	14.00 – 16.20	0.03	0.05
FAsh (%)	162	0.64 ^{A} ± 0.04	0.41 – 0.79	0.00	162	0.64 ^{A} ± 0.04	0.52 – 0.81	0.00	0.01
FWG (%)	162	32.47 ^{B} ± 4.92	22.80 – 43.40	0.39	162	34.82 ^{A} ± 4.78	22.80 – 43.30	0.38	0.35
P (mm)	161	107.12 ^{A} ±17.43	65.00 – 147.00	1.37	162	101.52 ^{B} ± 20.09	67.00 – 168.00	1.58	1.79
L (mm)	160	75.99 ^{B} ± 25.76	32.00 – 133.00	2.04	162	96.58 ^{A} ± 24.03	51.00 – 163.00	1.89	2.61
P/L	161	1.65 ^{A} ± 0.80	0.49 – 4.00	0.06	162	1.13 ^{B} ± 0.40	0.43 – 2.75	0.03	0.06
S (cm^2)	161	37.90 ^{B} ± 9.06	19.30 – 59.50	0.71	162	43.62 ^{A} ± 11.06	23.24 – 76.10	0.87	0.80
G (cm^3)	161	19.16 ^{B} ± 3.41	12.60 – 26.10	0.27	162	21.71 ^{A} ± 2.69	15.90 – 28.40	0.21	0.30
FMT (min)	161	5.38 ^{B} ± 1.71	2.00 – 9.50	0.13	162	5.68 ^{A} ± 1.64	2.00 – 9.50	0.13	0.14
FWA (%)	161	62.96 ^{A} ± 1.88	58.50 – 66.60	0.15	162	62.48 ^{B} ± 2.93	55.00 – 69.75	0.23	0.70
FStab (min)	161	8.85 ^{B} ± 3.04	3.30 – 17.00	0.24	162	10.80 ^{A} ± 4.14	5.00 – 30.00	0.33	0.42
FTol (BU)	161	45.65 ^{A} ± 21.03	5.00 – 130.00	1.66	162	36.93 ^{B} ± 14.33	5.00 – 70.00	1.13	2.03
MPH (mm)	162	61.07 ^{B} ± 4.68	50.30 – 70.00	0.37	162	62.22 ^{A} ± 5.51	45.20 – 71.20	0.43	0.35
MTH (mm)	162	47.44 ^{A} ± 3.29	41.90 59.40	0.26	159	47.46 ^{A} ± 3.71	39.30 – 60.30	0.29	0.51
MTW (mm)	162	13.95 ^{A} ± 3.91	7.70 – 25.50	0.31	162	12.78 ^{B} ± 4.01	7.70 – 25.30	0.32	0.36
MMT (min)	162	2.86 ^{A} ± 0.29	2.21 – 3.67	0.02	162	2.70 ^{B} ± 0.34	2.09 – 3.95	0.03	0.04

Means followed by the same letter, did not differ significantly at $P < 0.05$, N – observations, Mean – mean values, SD – standard deviation, SE – standard error, LSD – least significant difference, SKCS-HI – single kernel characterisation system hardness index, SKCS-Moist – single kernel characterisation system kernel moisture, SKCS-Weight – single kernel characterisation system kernel weight, SKCS-Dia – single kernel characterisation system kernel diameter, FN – falling number, BFY – break flour yield, TFY – total flour yield, Fprot – flour protein, Fmoist – flour moisture, Fash – flour ash, FWG – flour wet gluten, Gprot – grain protein content at 12% moisture basis, P – alveograph elasticity, L – alveograph extensibility, P/L – alveograph ratio elasticity/extensibility, S – alveograph strength, G – alveograph swelling index, FMT – farinograph mixing time, FWA – farinograph flour water absorption, FStab – farinograph stability, FTol – farinograph tolerance, BU – Brabender units, MPH – mixograph peak height, MTH – mixograph tail height, MTW – mixograph tail width, MMT – Mixograph mixing time.

5.3.2. The contribution of genotype, environment, and their interaction to the variation in grain kernel hardness

ANOVA (Table 5.2) was used to highlight the contribution of each variance component to the variation in SKCS-HI. The cultivar (C) represented the genotype effect (G). The year (Y), Location (L), interaction of year and location (Y x L) and replicates of field trials per year and location (Reps) provided the total environmental effect (E). The combination of Y x C, L x C and Y x L x C represented the G x E interaction.

The environment was the main contributor to variation in SKCS-HI of wheat produced in the Rûens region, representing a total of 68.21% of the variation in SKCS-HI. The significant effects of year (26.05% contribution to variation, P < 0.001), location (0.22%, P < 0.05) and Y x L (41.94%, P < 0.001) contributed to the total environmental effect. The G x E interaction was significant due to Y x C (10.86%, P < 0.001) and Y x L x C (2.05%, P < 0.01) and represented a total of 13.3% of the variation in SKCS-HI. Genotype contributed the least to the variation in SKCS-HI of wheat produced in the Rûens region, although the effect was significant with 12.93% contribution to variation(P < 0.001) (Table 5.2).

In the Swartland region, this scenario was reversed, with G contributing the most towards variation in SKCS-HI, explaining a total of 50.10% (P < 0.001) of the variation. The environmental effect contributed 16.76% to the variation in SKCS-HI, represented by Y (8.47%, P < 0.001), L (1.27%, P < 0.05) and Y x L (7.02%, P < 0.001). The G x E interaction was not significant (P > 0.05), although it did contribute 8.73% to the variation in SKCS-HI.

Table 5.2. Variance component contribution to variation in grain kernel hardness (SKCS-HI)

	G	E				GxE					
	Cultivar	**Year**	**Loc**	**YxL**	**Reps (YxL)**	**YxC**	**LxC**	**YxLxC**	**Error**	**R^2**	**CV**
Rûens											
DF	8	2	1	2	12	16	8	16	96		
Mean squares	416.67***	3358.62***	55.48*	5407.49***	10.48 ns	175.05***	12.50 ns	33.10**	13.64	0.95	6.33
Component contribution (% of SS)	12.93	26.05	0.22	41.94	0.49	10.86	0.39	2.05	5.08		
Swartland											
DF	8	2	1	2	12	16	8	16	96		
Mean squares	613.44***	414.78***	124.12*	343.72***	13.84 ns	36.63 ns	10.58 ns	11.58 ns	23.17	0.77	7.84
Component contribution (% of SS)	50.10	8.47	1.27	7.02	1.70	5.98	0.86	1.89	22.71		

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, C – cultivar, E – environment, Y – year, L – location.

The ranking of grain cultivars based on SKCS-HI remained relatively constant, with SST88 always being the hardest, and Ratel always being the softest cultivars in both regions (Table 5.3). The cultivars Baviaans, SST056, Kariega and SST015, occupied four ranks of SKCS-HI just harder than Ratel in both regions. However, their rank within these four slots changed order between the two regions.

The ranking of cultivars in the Swartland and Rûens regions, based on their BFY, showed the softest grain kernels (Ratel) had the highest BFY, and the hardest grain kernels (SST88) had the lowest BFY (Table 5.3). The ranking of cultivars based on their TFY was random for both regions and did not follow a trend according to ascending or descending GKH of the cultivars.

The ranking of cultivars in the Swartland region, based on their FN, indicated the softest grain kernels (Ratel) had the highest FN, and the hardest grain kernels (SST88) had the lowest FN. The ranking of cultivars based on their FN was random for the Rûens region, and did not portray a trend according to ascending or descending GKH of the cultivars (Table 5.3). It is noticeable that the standard deviation from mean values, for SKCS-HI and FN in the Rûens region, were higher than the standard deviation from mean values in the Swartland region (Table 5.3).

5.3.3. Correlation of grain and flour quality properties with grain kernel hardness

GKH (SKCS-HI), in the Rûens region, had significant positive, although weak correlations with Fash content (0.39, $P < 0.001$), FWG content (0.18, $P < 0.05$), Gprot content (0.25, $P < 0.01$), alveograph P/L ratio (0.17, $P < 0.05$), alveograph S (0.18, $P < 0.05$), MPH (0.21, $P < 0.01$), MTH (0.39, $P < 0.001$) and MTW (0.18, $P < 0.05$). SKCS-HI had significant positive and moderate correlations with alveograph P (0.48, $P < 0.001$) and FWA (0.50, $P < 0.001$) (Table 5.4). GKH, in the Rûens region, had significant negative, but weak correlations with FN (-0.16, $P < 0.05$) and FStab (-0.18, $P < 0.05$). GKH had significant negative and moderate correlations with SKCS-Weight (-0.63, $P < 0.001$), SKCS-Dia (-0.63, $P < 0.001$), and TFY (-0.44, $P < 0.001$); and additionally, a strong negative correlation with BFY (-0.77, $P < 0.001$) (Table 5.4).

GKH in the Swartland region had significant, although weak, negative correlations with SKCS-Moist (-0.28, $P < 0.001$), MTW (-0.19, $P < 0.05$), and TFY (-0.30, $P < 0.001$). The negative correlations of SKCS-HI with FN (-0.43, $P < 0.001$), and BFY (-0.39, $P < 0.001$) were moderate to weak (Table 5.4). The significant correlations of SKCS-HI and quality properties in the Rûens and Swartland regions are reflected in Fig. 5.2.

Table 5.3. Mean values and cultivar ranking of grain kernel hardness, break flour yield, total flour yield and falling number for Rûens and Swartland regions

Rûens									
		SKCS-HI		**BFY**		**TFY**		**FN**	
		LSD = 2.44		LSD = 0.93		LSD = 0.85		LSD = 7.80	
Cultivar	**N**	**Mean ± SD**	**Ranking**	**Mean ± SD**	**Ranking**	**Mean ± SD**	**Ranking**	**Mean ± SD**	**Ranking**
SST88	18	66.98 ± 11.25	A	23.28 ± 4.41	E	67.23 ± 1.43	BC	340 ± 35	C
SST087	18	62.74 ± 9.45	B	22.03 ± 5.95	F	65.93 ± 2.05	DE	349 ± 44	B
SST096	18	62.55 ± 9.52	B	24.23 ± 5.48	D	65.76 ± 2.67	E	365 ± 52	A
PAN3434	18	57.73 ± 12.51	C	25.67 ± 8.33	BC	66.61 ± 4.12	CD	352 ± 68	B
Baviaans	18	56.95 ± 11.52	C	26.29 ± 8.27	B	67.07 ± 5.11	BC	353 ± 73	B
SST056	18	56.72 ± 11.40	C	24.99 ± 6.11	CD	67.04 ± 1.86	BC	364 ± 52	A
Kariega	18	55.58 ± 14.23	CD	26.49 ± 8.18	B	67.69 ± 4.12	AB	351 ± 68	B
SST015	18	53.64 ± 13.53	DE	24.65 ± 6.84	D	66.08 ± 2.80	DE	364 ± 46	A
Ratel	18	52.11 ± 14.48	E	28.09 ± 8.12	A	68.09 ± 3.41	A	350 ± 80	B
Swartland									
		SKCS-HI		**BFY**		**TFY**		**FN**	
		LSD 3.18		LSD = 0.68		LSD 1.05		LSD = 7.07	
Cultivar	**N**	**Mean ± SD**	**Ranking**	**Mean ± SD**	**Ranking**	**Mean ± SD**	**Ranking**	**Mean ± SD**	**Ranking**
SST88	18	71.78 ± 4.85	A	23.73 ± 4.17	E	67.14 ± 1.66	C	370 ± 34	E
SST087	18	67.32 ± 4.48	B	21.58 ± 4.59	F	65.69 ± 3.58	D	373 ± 29	E
SST096	18	66.01 ± 3.83	B	25.06 ± 4.28	D	65.70 ± 2.84	D	411 ± 18.98	BC
PAN3434	18	60.63 ± 5.45	C	28.09 ± 5.97	C	68.59 ± 1.72	AB	409 ± 18.91	BC
SST056	18	60.14 ± 3.89	CD	25.27 ± 5.71	D	67.31 ± 1.62	C	401 ± 22.31	D
Kariega	18	59.16 ± 4.47	CD	28.79 ± 6.00	AB	69.28 ± 1.50	A	408 ± 16.15	C
SST015	18	57.31 ± 6.35	DE	23.85 ± 5.59	E	65.23 ± 2.52	D	415 ± 17.27	B
Baviaans	18	56.38 ± 9.41	EF	28.39 ± 6.18	BC	68.17 ± 2.09	BC	411 ± 18.80	BC
Ratel	18	53.69 ± 5.96	F	29.13 ± 5.24	A	68.49 ± 1.83	AB	425 ± 12.24	A

Means followed by the same letter, did not differ significantly at $P < 0.05$, Ranking based on highest to lowest of the quality property value, with A representing cultivar with the highest value and E or F the lowest value, N – observations, Mean – mean values, SD – standard deviation, LSD – least significant difference, SKCS-HI – single kernel characterisation system hardness; FN – falling number, BFY – break flour yield, TFY – total flour yield.

Table 5.4. Pearson's correlations of wheat grain hardness (SKCS-HI) with grain and flour quality properties

Quality property	Overall Rûens	Overall Swartland
SKCS-Moist	-0.10 ns	-0.28***
SKCS-Weight	-0.63***	-0.03 ns
SKCS-Dia	-0.63***	-0.05 ns
FN	-0.16*	-0.43***
BFY	-0.77***	-0.39***
TFY	-0.44***	-0.30***
FProt	0.03 ns	0.12 ns
FAsh	0.39***	0.10 ns
FWG	0.18*	0.15 ns
Gprot	0.25**	0.16 ns
P	0.48***	0.11 ns
L	-0.10 ns	0.09 ns
P/L	0.17*	-0.03 ns
S	0.18*	0.14 ns
G	-0.09 ns	0.10 ns
FMT	-0.06 ns	0.12 ns
FWA	0.50***	0.04 ns
FStab	-0.18*	-0.03 ns
FTol	0.12 ns	0.04 ns
MPH	0.21**	0.11 ns
MTH	0.39***	0.15 ns
MTW	0.18*	-0.19*
MMT	0.05 ns	0.10 ns

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, SKCS-HI – single kernel characterisation system hardness index, SKCS-Moist – single kernel characterisation system kernel moisture, SKCS-Weight – single kernel characterisation system kernel weight, SKCS-Dia – single kernel characterisation system kernel diameter, FN – falling number, BFY – break flour yield, TFY – total flour yield, Fprot – flour protein, Fmoist – flour moisture, Fash – flour ash, FWG – flour wet gluten, Gprot – grain protein content at 12% moisture basis, P – alveograph elasticity, L – alveograph extensibility, P/L – alveograph ratio elasticity/extensibility, S – alveograph strength, G – alveograph swelling index, FMT – farinograph mixing time, FWA – farinograph flour water absorption, FStab – farinograph stability, FTol – farinograph tolerance, BU – Brabender units, MPH – mixograph peak height, MTH – mixograph tail height, MTW – mixograph tail width, MMT – Mixograph mixing time.

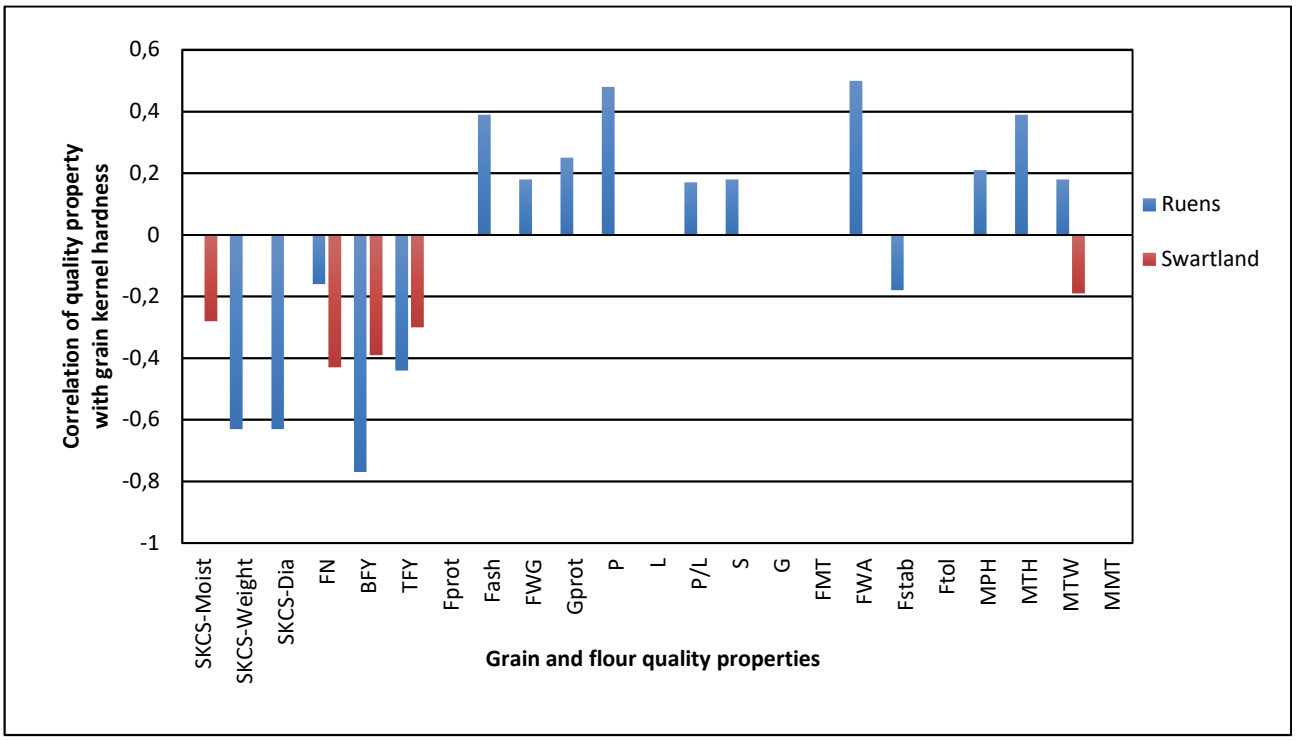

Figure 5.2. Chart indicating the significant correlation coefficients of grain kernel hardness with grain and flour quality properties in the Rûens and Swartland regions.

5.4. Discussion

5.4.1. Influence of genotype, environment, and their interaction on grain hardness

When changes in the ranking of a trait occur over different environments, it indicates severe G x E interaction, which changes the cultivar's phenotypic behaviour in each environment. Low G x E interaction may change the magnitude of difference amongst the cultivars or lines, but never the rank order (Hazen *et al.*, 1997). The ranking of four cultivars (Baviaans, SST056, Kariega and SST015) according to their SKCS-HI changed, when comparing the Swartland and Rûens regions. The change in the ranking of cultivars indicate that a strong G x E interaction was present in either of the regions. The ANOVA of SKCS-HI in the two regions (Table 5.2) indicate that the variation in GKH were mainly contributed by genotype (G) in the Swartland region, and environment (E) with G x E interaction in the Rûens region. The change in the ranking order of cultivars based on their SKCS-HI thus confirms the G x E interaction in the Rûens region. The environment and G x E interaction effects in the Rûens region, are also in accordance with the increased standard deviation from the mean SKCS-HI value observed in the Rûens region, which indicate a bigger variation in the phenotypic expression of the trait due to the environmental influence.

The *Pin* allelic genotype combination of all nine cultivars were identical, *Pina-D1a*/*Pinb-D1b* (Chapter 3), and cultivars were exposed to the same environmental influences. It is generally accepted that the *puroindoline* alleles lie at the basis of GKH. In the current research however, significant differences in GKH (Table 5.3) were observed between the cultivars in both the Rûens

(environmental influence) and Swartland (genotype influence) regions. The significant differences in GKH between cultivars, indicate that the difference in GKH was not due to the *Pin* genotype or the environmental influence; which implies that other genes and/or the interaction or minor genes, are also involved in the expression of GKH. Several minor genes have been identified on chromosomes other than 5DS, that influence GKH (Sourdille *et al.*, 1996; Li *et al.*, 2009; Sun *et al.*, 2010; Tsilo *et al.*, 2010; Wang *et al.*, 2012; Boehm *et al.*, 2018; Jernigan *et al.*, 2018). The extent to which each of these minor genes affect GKH were not reported to be high; however, their combined effect on GKH has not been determined yet. Pleiotropic gene effects, and linked genes, have been reported to be the primary cause for genetic correlation between traits (Mladenov *et al.*, 2012). Due to the massive size of the wheat genome, it would be impossible to control *Pin* alleles and all other genetic variables that could influence GKH. Research regarding the grain and flour properties that are correlated could however, be used to the wheat breeder's advantage during the wheat breeding process.

5.4.2. Correlation of grain kernel hardness with other wheat grain properties

The grain kernel characteristics i.e. grain hardness, -moisture, -weight and -diameter, differed significantly ($P < 0.05$) between the Swartland and Rûens regions. The Swartland region produced significantly ($P < 0.05$) harder wheat kernels (3 SKCS-HI units mean difference), but with lower moisture content, diameter and weight than the wheat kernels of the Rûens region (Table 5.1). Pearson's correlation results (Table 5.4) of the Swartland region indicated that SKCS-HI had a weak negative correlation with grain moisture content. In the Rûens region GKH had moderate to strong negative correlations with kernel weight and kernel diameter. Boehm *et al.* (2018) found SKCS-HI of wheat grain positively correlated ($P < 0.01$) with kernel weight and diameter. These positive correlations are in contrast with the negative correlations found in the current research; however, it could be explained due to the considerable environmental influence on GKH observed in the Rûens region (Table 5.2). High temperature and water stress are common to both the Swartland and Rûens regions (temperature and rainfall data are reflected in Appendix A, Tables A1 – A3); however, both temperature and water stress are usually higher in the Swartland region. High temperature and water stress are known to decrease kernel weight and diameter considerably, by shortening the grain filling period without increasing the filling rate (Brooks *et al.*, 1982; Kobata *et al.*, 1992; Altenbach *et al.*, 2003). The negative correlation of SKCS-HI with kernel weight and -diameter in the Rûens region, could thus be attributed to the environmental influence.

The grain protein content of wheat from the Swartland and Rûens regions differed significantly ($P < 0.05$), although there was not a big difference in the mean grain protein values. SKCS-HI had a weak positive correlation with grain protein content in the Rûens region. High temperatures during grain filling cause increased grain protein content; thus, the weak positive correlation of SKCS-HI with grain protein content can be attributed to the environmental influence observed in the Rûens region. This correlation was in agreement with results of Boehm *et al.* (2018) that found SKCS-HI of

wheat grain positively correlated ($P < 0.01$) with grain protein content. GKH from the Swartland region, thus genetically determined GKH, had no strong correlations with other grain kernel characteristics.

5.4.3. Correlation grain kernel hardness with wheat milling characteristics

SKCS-HI of wheat from the Swartland region, had weak to moderate negative correlations (Table 5.4) with break flour yield (BFY) and total flour yield (TFY). GKH in the Rûens region, showed a strong negative correlation with BFY and a moderate negative correlation with TFY. As GKH increased, BFY and TFY decreased, with the largest influence on the BFY. The effect of environment (Rûens region) on the negative correlation of GKH with BFY and TFY is more pronounced than the influence of genetically (Swartland region) determined hardness, although both regions showed the same tendency regardless of genotype or environmental influence (ANOVA Table 5.2). Oury *et al.* (2017) found that milling characteristics were profoundly influenced by genotype, and moderately by the environment, and no interaction of G x E. However, in the current research it was concluded that environment had a more substantial influence on milling characteristics than genotype.

In the current study, SKCS-HI was negatively correlated with BFY in both the Swartland and Rûens regions, implying that soft wheat grains had a better BFY compared to hard wheat grains. Several studies have concluded that soft wheat grain has increased BFY (Martin *et al.*, 2001; Hogg *et al.*, 2005; Carter *et al.*, 2012; Oury *et al.*, 2017), which is in accordance with the current research's findings.

The GKH in the current research, was negatively correlated with TFY in both regions, and did not increase with increased GKH. Several other studies (Garland-Campbell *et al.*, 2001; Ma *et al.*, 2009; Choy *et al.*, 2015; Oury *et al.*, 2017) found increased reduction of flour yield (RFY) and TFY with increased GKH in comparison to soft wheat grain, which is in contrast to the current research's findings. However, Oury *et al.* (2017) found that the contrasting effect of GKH on BFY and RFY had only a moderate effect on the TFY; therefore, there might not be a defined correlation of TFY with GKH classes. Oury *et al.* (2017) also observed that the softest and hardest grains on the SKCS-HI spectrum, produced the lowest TFY, while the highest TFY was obtained from medium hardness grain with SKCS-HI units of 30 to 50. The SKCS-HI units in the current research ranged from 54 to 72 for the Swartland region, and 52 to 67 for the Rûens region for the softest (Ratel) to hardest (SST88) cultivars (Table 5.3). The cultivars included in the current research were all bread wheat cultivars, within the medium-hard to hard wheat hardness classes (AACC, 1999a). Only the softest cultivars were close to the range of 30 to 50 SKCS-HI, that produces the best TFY according to Oury *et al.* (2017). The high hardness classes of wheat cultivars used in the current research, explain the negative correlation of SKCS-HI with TFY observed. If soft biscuit wheat had been included in the cultivar selection for the current research, the correlation of GKH with TFY might have been different.

5.4.4. Correlation grain kernel hardness with flour quality properties

Flour protein content from the Swartland region indicated a slightly higher positive correlation with GKH, than the correlation from the Rûens region, though both were very weak non-significant correlations ($P > 0.05$) (Table 5.4). The loss of protein content from grain to flour during milling was lower for the Swartland region (- 0.72%), in comparison to the Rûens region (- 1.54%). The lower protein loss in the Swartland region could be explained by the higher TFY obtained during milling of grain, indicating a more efficient separation of endosperm from bran during first break milling, and the higher protein endosperm cells in close proximity to the bran layer also being included in the extracted flour (Delcour & Hoseney, 2010).

SKCS-HI in the Swartland region, had a moderate negative correlation with falling number (FN), while the correlation of GKH with falling number in the Rûens region was very weak and negative (Table 5.4). The negative correlations of SKCS-HI with FN indicate increased α-amylase activity, and/or increased availability of starch granule content to hydrolysation, with an increase in GKH. The milling of hard wheat results in more starch damage than the milling of soft wheat (Brites *et al.*, 2008). Damaged starch molecules are more readily available to α-amylase enzymes to hydrolyse, leading to a decrease in the viscosity of the flour-water-suspension, and consequently a lower falling number. The decreased falling number leads to a negative correlation observed between starch damage and falling number (León *et al.*, 2006; Liu *et al.*, 2014). Due to the higher level of starch damage caused during the milling of hard wheat grain, flour from hard wheat grain would theoretically have decreased falling number in comparison to soft wheat grain. The decrease in falling number of flours from hard wheat grain is in accordance with our results of a negative correlation of GKH with falling number, thus a positive correlation of GKH with α-amylase activity.

The ranking of cultivars in the Swartland region, according to falling number value (Table 5.3) indicated the softest cultivar (Ratel) having the highest falling number, and the hardest cultivar (SST88) having the lowest falling number. The falling number in the Rûens region did not follow the same trend in the ranking of cultivars, since the ranking based on falling number values were random when compared to GKH ranking. Amylose content is positively correlated with the volume percentage of type A starch granules in the wheat endosperm (Li *et al.*, 2008), indicating that softer wheat endosperm, which has a higher amount of type A starch granules, has higher amylose content. A higher level of amylose content will provide a greater barrier against the hydrolysis effect of α-amylase, thus resulting in a higher falling number value. Consequently, flour from soft wheat should have a higher falling number, compared to flour from hard wheat grain. The ranking of cultivars in the Swartland region based on falling number were in accordance with this concept, with flour from soft wheat grain having a higher falling number compared to flour from hard wheat grain. The correlation of GKH and falling number, confirm that the variation of GKH in the Swartland region is due to genotype. The change in the ranking of cultivars in the Rûens region, with falling number

value in comparison to grain GKH, indicate that a strong G x E interaction was present, confirming the strong environmental influence on GKH observed in the Rûens region.

There was a very weak positive correlation of kernel SKCS-HI with flour ash content in the Swartland region, and a moderate to weak positive correlation (Table 5.4) in the Rûens region. The moderate positive correlation of SKCS-HI with flour ash content in the Rûens region, indicates that the environmental influence (Rûens region) on GKH affects flour ash content to a higher degree than the influence of genotype (Swartland region) on GKH.

There was a very weak positive correlation of SKCS-HI with dough tenacity (alveograph P value) in the Swartland region, but a moderate positive correlation in the Rûens region (Table 5.4). The positive correlation in the Rûens region indicates that the environmental influence (Rûens region) on GKH affects dough tenacity to a higher degree than the influence of genotype (Swartland region) on GKH. A strong positive correlation of GKH with alveograph tenacity and P/L ratio has been reported (Baker & Dyck, 1975; Chen *et al.*, 2013), and the results observed in the Rûens region is in accordance with it. Increased alveograph S with an increase in GKH at were also observed earlier (Chapter 4).

In the Swartland region, SKCS-HI did not correlate with flour water absorption; however, a moderate positive correlation was observed in the Rûens region (Table 5.4). The positive correlation in the Rûens region indicates that the environmental influence (Rûens region) on GKH affects flour water absorption to a higher degree than the influence of genotype (Swartland region) on GKH. A positive correlation of GKH with flour water absorption has been reported (Baker & Dyck, 1975; Martin *et al.*, 2001; Cane *et al.*, 2004; Eagles *et al.*, 2006; Chen *et al.*, 2013), and results from the Rûens region in the current research were in accordance with it.

In the Swartland region, SKCS-HI had a very weak negative correlation with MTW, while the Rûens region indicated SKCS-HI had a very weak positive correlation with the MTW (Table 5.4). Mixograph tail width indicates the dough's tolerance to overmixing, with a larger width corresponding to a higher tolerance. Results from the Swartland region, influenced by the genotypic influence on GKH, indicate a decreased tolerance to overmixing with an increase in GKH. However, results from the Rûens region, influenced by environmental influence on GKH, indicate an increase in tolerance to overmixing with an increase in GKH. A positive correlation of wheat GKH with mixograph tolerance to overmixing has been reported (Baker & Dyck, 1975; Chen *et al.*, 2013), and is in accordance with results from the Swartland region attributed, to the genotype influence on GKH. It has been reported that hard wheat grain has a decreased tolerance to overmixing compared to soft wheat grain (Hazen *et al.*, 1997), which is in accordance with results from the Rûens region in the current research, which was due to environmental influence. It is evident that the environment and genotype has a different influence on the dough's tolerance to overmixing; however, the correlation is weak with both the environmental and genotypic influence.

In the Rûens region, SKCS-HI had a moderate positive correlation with MTH (Table 5.4). The positive correlation of GKH with MTH indicated a positive correlation of GKH with dough strength and stability to overmixing. The positive correlation with MTH confirms the positive correlation with dough tenacity, reported in alveograph results of the Rûens region (Table 5.4), and the influence of environment on GKH with its correlation to dough strength, stability and tenacity.

Interactions between G x E can diminish the breeder's ability to recognise genotypic differences amongst breeding lines (Bassett *et al.*, 1989), emphasising the importance of careful experimental design. In the current research, it was clearly illustrated how the influence of G, E and G x E could provide conflicting results.

5.5. Conclusion

The genetic influence on GKH had the greatest effect on properties involving the milling quality of wheat grain, and the influence of the milling process on flour quality, i.e. the influence on properties which are influenced by the level of starch damage caused during milling. These properties comprised break flour yield, total flour yield, and α-amylase activity.

The environmental influence on GKH has also affected properties involving the milling quality of the wheat grain, i.e. break flour yield, total flour yield and flour ash content. However, the environmental influence on GKH has additionally affected kernel and dough properties comprising wheat kernel weight and diameter, flour water absorption, dough tenacity, strength, and tolerance to overmixing.

It is generally accepted that the *Pin* allele genotype of a cultivar forms the basis of GKH. In the current research, wheat cultivars with identical *Pin* allelic genotypes were used (*Pina-D1a*/*Pinb-D1b)*; however, there were significant differences ($P < 0.05$) in the SKCS-HI of the cultivars within the Swartland and Rûens regions respectively. Since the environmental conditions were constant for all the cultivars at a location, it is evident that GKH is more complex than only the *Pin* genotype combination of a cultivar.

This study was performed with careful experimental design, and with attention to the possible G x E interaction on GKH, therefore the results are considered of high quality and reliable in identifying that other genes are involved in the expression of GKH.

5.6. References

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Chapter 6

Influence of four *puroindoline* genotypes on SE-HPLC protein fractions in bread wheat (*Triticum aestivum*)

Abstract

Size exclusion high performance liquid chromatography (SE-HPLC) was used to determine the molecular weight distribution of proteins in wheat from two production regions. Nine spring wheat cultivars with the *puroindoline* (*Pin*) allelic genotypes, *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b*, were planted in the summer rainfall irrigation (SRI) region. A different set of nine facultative and winter wheat cultivars were planted in the summer rainfall dryland (SRD) region and they contained the *Pin* allelic genotypes *Pina-D1a*/*Pinb-D1b*, *Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*. The data analysis performed included analysis of variance (ANOVA) with cultivars nested within *Pin* genotype, to determine the variable contribution of environment (E), *Pin* genotype (PG), and other genetic components (C). Also, the interaction of PG x E and C x E was determined. Pearson's correlations were used to establish the linear relationship between grain kernel hardness (GKH) and SE-HPLC protein fractions.

The environmental influence was the primary contributor to variation in all the protein fractions of the SRI and SRD regions. Additionally, the SRD region showed a high contribution of G x E interaction to variation. The *Pin* genotypes, *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b*, both indicated a decrease in sodium dodecyl sulphate (SDS)-soluble proteins, and an increase in SDS-insoluble proteins, with increased GKH in both production regions. In the SRD region, all genotypes (*Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1b, Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*) showed positive correlations between SKCS-HI and albumin and globulin proteins (both SDS-soluble and -insoluble).

This study advanced on previous studies by investigating the interaction between single kernel characterisation system hardness index (SKCS-HI) and protein molecular weight distribution within different *Pin* genotypes. In wheat breeding programmes the knowledge of the *Pin* alleles present in a cultivar or breeding line would enable the breeder to predict the 'protein response' under high temperature and/or water stress conditions, within different *Pin* genotypes. Provision for acceptable wheat quality, even when influenced by the environment and G x E interaction, can be made by selecting for the desired *Pin* genotypes.

6.1. Introduction

In 1907, Osborne divided wheat grain proteins into four groups based on their solubility, i.e. albumins (soluble in water and dilute buffers), globulins (soluble in salt solutions), prolamins (soluble in 70 – 90% ethanol) and glutelins (soluble in dilute acid or alkali). These four groups were separated based

on their solubility, molecular weight and electrophoretic mobility into albumins, globulins, gliadins and glutenins (Chen & Bushuk 1970a). Chen and Bushuk (1970b) observed two additional albumin proteins in the disc electrophoresis pattern of *Triticum aestivum*, that were not present in *T. durum*. These two proteins could not be identified at the time, although Chen and Bushuk (1970b) assumed that they could be contributed by the D genome to *T. aestivum*.

The proteins in wheat are divided into structural or metabolic proteins (both non-gluten proteins), and storage proteins (gluten protein). The structural or metabolic proteins consist of albumin, globulin, and amphiphilic proteins (puroindoline) which have been reported to influence wheat grain hardness and dough quality properties (Dubreil *et al.*, 1998). The metabolic proteins occur in aleurone cells and germ, and also in the endosperm, where they contribute to endosperm cell structure and metabolism (Cauvain, 2003). These non-gluten proteins account for 15 to 20% of the wheat grain protein; while gluten proteins, composed of glutenins and gliadins, account for 80% of the total grain protein content (Shewry & Tatham, 1997). Monomeric proteins, i.e. gliadin, are responsible for the extensibility and viscosity properties of wheat flour dough; while polymeric proteins, i.e. glutenin, imparts elasticity and strength to the dough (Huebner & Bietz, 1985).

Gluten proteins contribute to the visco-elastic properties of wheat flour, and the wheat flour baking quality, with 70% of baking quality attributed to the quality and quantity of gluten proteins (Jones *et al.*, 2006). Gluten polymers vary in size from 500 kDa to 10 000 kDa, and are among the largest proteins observed in nature. Gluten polymers are heterogeneous mixtures of polymers, with disulphide-bonded (S-S) polypeptides; i.e. high molecular weight glutenin subunits (HMW-GS), low molecular weight glutenin subunits (LMW-GS), α/β/γ/ω-gliadin, albumin and globulin (Payne & Corfield, 1979). The S-S bonds must be reduced with SDS (SDS-soluble) that solubilise the small polymers; or sonification (SDS-insoluble) that solubilize the large polymers, for investigation of the protein fractions (Gupta *et al.*, 1993).

Variation in grain protein fractions are considered an important factor that affects wheat flour quality, while grain protein content has been positively correlated with grain hardness (Huebner & Gaines, 1992; Ohm & Chung, 1999; Giroux *et al.*, 2000; Ohm *et al.*, 2006). Wheat hardness is affected by variations in puroindoline proteins (PIN proteins) and lipids associated with the starch granule surface (Greenblatt *et al*. 1995; Giroux & Morris 1997). PIN proteins are cysteine-rich polypeptides with a mean molecular weight of 12.8 kDa (Blochet *et al.*, 1993). Two genes control PIN proteins, *Pina-D1* and *Pinb-D1* (Tranquilli *et al.*, 1999; Gautier *et al.*, 2000), located in the hardness (*Ha*) locus on chromosome 5DS (Mattern *et al.*, 1973; Baker & Dyck, 1975; Law *et al.*, 1978). The wild-type alleles, namely *Pina-D1a* and *Pinb-D1a*, cause soft wheat endosperm, while a mutation or deletion in nucleotides of the *Pina-D1* and *Pinb-D1* genes causes hard wheat endosperm (Giroux & Morris, 1997a). PIN proteins are integral membrane proteins that strongly bind to polar lipids, and form tight bonds with starch granule membranes, using the tryptophan-rich domain (TRD) present in PIN protein to form membrane-anchoring loops between α-helices in the starch granule membrane (Greenblatt *et al.*, 1995). The deoxyribonucleic acid (DNA) sequence of a *Pin* allele

determines the secondary and tertiary structure of the expressed PIN protein. Different *Pin* alleles express PIN proteins with different tertiary structures, and a difference in their interaction with polar lipids in the starch granule membrane, which affects the strength of the protein's bond to the membrane (Greenblatt *et al.*, 1995; Giroux & Morris, 1997b; Ma *et al.*, 2009). PIN proteins are part of the 2S albumin proteins in the prolamin superfamily of proteins. The 2S albumin proteins additionally comprise lipid-transfer proteins, purothionins and α-amylase inhibitors (Shewry *et al.*, 2002).

Starch granule-associated proteins can be divided into two groups based on their molecular weight, i.e. low molecular weight proteins of 5 to 30 kDa, which associate with the starch granule surface; and high molecular weight proteins of 60 to 149 kDa (Baldwin, 2001). These proteins, which associate with starch granules, generally consist of two distinct types. The first is storage proteins, i.e. glutenin and gliadin proteins, which remain attached to the starch granule surface after starch extraction. The second is starch granule-associated proteins, which are physiologically active proteins and are tightly-bound, surface or integral membrane components of the starch granule membrane (Skerritt *et al.*, 1990; Skerritt & Hill, 1992).

Although grain protein quality is genetically determined, protein quantity is influenced by the environment (Graybosch *et al.*, 1996; Huebner *et al.*, 1997; Zhu & Khan, 2001). The development of protein in the wheat grain kernel occur at three stages; cell division, cell enlargement, and dehydration and grain maturity. The accumulation of albumins and globulins (AG) occur only during the cell division stage, therefor a "reduction" in the percentage of AG during grain development will be apparent. Monomeric and polymeric proteins reportedly accumulated towards the end of the cell enlargement stage, and increased even more during the late stages of grain development. The formation of SDS-insoluble polymers occurred during the dehydration stage (Carceller & Aussenac, 1999).

Changes in the composition of protein fractions have been associated with the increase in grain kernel protein content, irrespective of the cause, i.e. water stress during grain fill, increased irrigation or fertilisation. Grain protein content increase considerably under water stress conditions (Guttieri *et al.*, 2000), such as dryland wheat production, due to the shortened grain fill period. A shortening of the grain fill period lowers the amount of starch accumulation in the wheat endosperm, and thus results in a relative increased grain protein content. Contrasting to this, irrigation practices may cause a decreased grain protein content due to the increased starch accumulation and grain yield, that cause a relative decrease of grain protein. Saint Pierre *et al.* (2008) found that the amount of both polymeric proteins (PP) and monomeric proteins (MP) increased with increased grain protein content, however, the percentage of MP (gliadins) increase more rapidly than the PP (glutenins) as evaluated by SE-HPLC (Triboï *et al.*, 2000). The proportion of MP to glutenins PP are affected by high temperature, with the relative content of PP decreasing at high-temperature environments (Blumenthal *et al.*, 1990; Panozzo & Eagles, 2000). The AG proteins respond the least to changes in the total grain protein content (Saint Pierre *et al.*, 2008).

Hard and soft wheat cultivars have different levels of PP and MP, even if produced under the same environmental conditions, indicating a genetic influence on protein fractions associated with GKH (Melnyk *et al.,* 2012). Specific fractions of gliadins had definite correlations with the particle size of milled wheat flour, which implies a correlation of those gliadin fractions with kernel hardness (Huebner & Gaines, 1992). Ohm *et al.* (2006; 2010) found that kernel hardness primarily affected SDS-soluble ω-gliadin (ExMP), and albumin and globulin (ExAG) fractions. Grain kernel hardness (GKH) has been reported to be positively correlated with SDS-soluble polymeric protein (ExPP) (Katyal *et al.*, 2017), ExMP (Ohm *et al.*, 2010) and SDS-insoluble polymeric protein (UPP) (Gupta *et al.*, 1993; Malik *et al.*, 2011; Katyal *et al.*, 2017); and negatively correlated with ExAG (Ohm *et al.*, 2010), ExMP (Katyal *et al.*, 2017), SDS-insoluble monomeric protein (UMP) (Gupta *et al.*, 1993; Malik *et al.*, 2011; Katyal *et al.*, 2017) and SDS-insoluble albumin and globulin (UAG) (Ohm *et al.*, 2010).

Polymeric proteins, as determined by SE-HPLC, have been reported to be positively correlated with kernel hardness, while ExMP was negatively correlated with kernel hardness in soft wheat (Huebner & Gaines, 1992). The variation of GKH in soft winter wheat affects protein fractions rich in gliadins (MP) (Ohm, Hareland *et al.*, 2009). Extra soft wheat cultivars showed higher MP content and lower PP content (Katyal *et al.*, 2017).

Although various researchers have studied the effect of kernel hardness on protein fractions, the effect of *Pin* genotypes on protein fractions has not yet been researched. The objective of this study was thus to determine the influence of GKH on protein fractions, as determined by SE-HPLC, within two *Pin* genotypes in the SRI region and four *Pin* genotypes in the SRD region.

6.2. Materials and methods

6.2.1. Experimental population and field trials

Wheat samples that were planted in the summer rainfall irrigation (SRI) and the summer rainfall dryland (SRD) regions of South Africa for two consecutive seasons (2013 – 2014) and at four locations per region, as described in Chapter 3, were used for this research study.

In the SRI region, the wheat quality standard (SST 806) and eight other commercial cultivars, over a range of kernel hardness, were included in the trials. The selected cultivars have been identified to have two *Pin* allelic genotypes (Chapter 3), *Pina-D1a*/*Pinb-D1b* and *Pina-D1b*/*Pinb-D1a* (Table 6.1). The locations representing the SRI region were Marblehall, Lichtenburg, Hartsvallei and Winterton (Fig. 6.1).

In the SRD region, the wheat quality standard (Elands) and eight other commercial cultivars, over a range of kernel hardness, were included in the trails. The cultivars have been identified to have four *Pin* allelic genotypes (Chapter 3), *Pina-D1a*/*Pinb-D1b*, *Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*. The locations representing the SRD region were Bethlehem, Bultfontein, Ladybrand, Reitz and Wesselsbron (Fig. 6.1).

Figure 6.1. Map of South Africa with the two production regions and trial locations of each region, for the 2013 and 2014 seasons, indicated.

Table 6.1. Cultivars, from each production region, indicating the *puroindoline* genotype they represent

	Pina-D1a*/*Pinb-D1ab	***Pina-D1a*/*Pinb-D1b***	***Pina-D1a*/*Pinb-D1p***	***Pina-D1b*/*Pinb-D1a***
Summer rainfall irrigation region	-	Duzi Buffels Baviaans	-	PAN3471 SST835 SST806 PAN3478 Olifants
Summer rainfall dryland region	PAN3355	PAN3144 PAN3161 Gariep SST347 SST398	Elands	SST356 PAN3379

6.2.2. Grain analysis and -milling

6.2.2.1. Single kernel characterisation system

Wheat kernel hardness was determined using the single kernel characterisation system method according to AACC approved method 55-31.01 (AACC, 1999), as described in Chapter 3, section 3.2.3. One analysis per sample was conducted, using 300 kernels per analysis to determine the grain hardness index (SKCS-HI).

6.2.2.2. Grain protein

The grain protein (Gprot) content (12% moisture basis) was determined using wheat grain calibration no. 096126 with the FOSS Infratec™ 1241 grain analyser (FOSS analytics, Hillerød, Denmark), as described in Chapter 4, 4.2.2.2.

6.2.2.3. Grain milling

Approximately 5 g of grain per sample was dry-milled on a POLYMIX PX-MFC 90 D mill (Kinematica AG, Switzerland), equipped with a 0.8 mm sieve, to obtain whole wheat flour necessary for protein extraction and size exclusion high-performance liquid chromatography (SE-HPLC) analysis.

6.2.3. Protein extraction and size exclusion high-performance liquid chromatography

All protein extractions and SE-HPLC analysis were performed in duplicate, and the average values were used for data analysis. Protein extraction was performed according to the procedure of Gupta and Khan (1993), with some modifications. The extraction procedure consisted of two steps, to extract both sodium dodecyl sulphate (SDS) soluble and SDS-insoluble proteins.

Whole wheat flour (0.017 g) was weighed into Eppendorf tubes, in duplicate. Deionised water was used for the preparation of all solvents and eluants. The measured whole wheat flour samples were suspended in 1.5 mL of 0.5% (w/v) SDS-phosphate buffer (pH 6.9) and vortexed for 10 s. Subsequently, samples were stirred for 5 min at 1400 rpm and 21°C in a Thermomixer® comfort (Eppendorf AG, Hamburg, Germany) followed by centrifugation for 30 min at 10 000 rpm (HERMLE Z 233 M-2, HERMLE Labortechnik GmbH, Germany). The supernatant was filtered through a 0.45 µm HT Tuffryn Acrodisc® Syringe filter (Pall Corporation, Ann Arbor, Michigan, USA) into a glass vial. Filtration samples were heated for 2 min at 80°C immediately after filtration to suppress protease activity (Larroque *et al.*, 2000) and set aside for SE-HPLC analysis.

To extract the SDS-insoluble proteins, the pellet was resuspended in 1.5 mL of 0.5% (w/v) SDS-phosphate buffer (pH 6.9), vortexed for 10 s and sonicated using an ultrasonic disintegrator (Branson B12 Sonifier, Sigma, St. Louis, Missouri, USA). The ultrasonic disintegrator was fitted with a 3 mm exponential tip, and sonication performed for 30 s at amplitude 5. Samples were stirred, centrifuged and filtered into a glass vial as described previously. Filtration samples were heated, as described previously, to suppress protease activity (Larroque *et al.*, 2000).

Routine analyses of both the SDS-soluble and SDS-insoluble protein extractions were performed using a Thermo Finnigan™ Surveyor Plus HPLC system with a photodiode array (PDA) detector (Thermo Electron, San Jose, California, USA). The HPLC system was equipped with a ChromQuest™ 4.2 chromatography data system for integration events (Thermo Electron, San Jose, California, USA). A narrow bore column (NBC) (300 mm x 4.6 mm BioSep-SEC-S 4000 Phenomenex®, Torrance, California, USA) was used in this study (Ohm, Hareland *et al.*, 2009).

Separation was achieved within 15 min after injecting a 20 µL protein extract. The elution system consisted of (A) deionised water and trifluoroacetic acid (TFA, 99.9/0.1%, v/v, Sigma–Aldrich); and (B) acetonitrile (ACN) (ROMIL-SpS™ acetonitrile 200 far UV) + TFA solution (99.9/0.1%, v/v). Proteins were eluted by 50% ACN (B). The flow rate was set at 0.4 mL/min at ambient temperature, after which proteins were detected at 210 nm by the PDA detector.

Absorbance areas under the different peaks were calculated according to Gupta and Khan (1993), using the ChromQuest™ 4.2 chromatography data system. Fractions were measured at specific time intervals; namely F1 (4.64 – 5.45 min), F2 (5.46 – 7.15 min), F3 (7.16 – 7.74 min), F4 (7.75 – 8.60 min) and F5 (8.50 – where the trace cuts the baseline) (Ohm, Ross *et al.*, 2009). The HPLC profile represented different protein components; F1 represented high molecular weight glutenin polymers (HMW-GS), F2 represented low molecular weight glutenin polymers (LMW-GS), F3 represented ω-gliadin, F4 represented α/β/γ-gliadin, and F5 represented albumin and globulin (Larroque *et al.*, 2000). Protein fractions used for further analysis were calculated according to Table 6.2.

Table 6.2. Calculations of relative and absolute percentages of protein fractions

Protein fractions	Relative percentage	Absolute percentage
ExPP	$\frac{(F1\ ExP + F2\ ExP)}{[(F1\ ExP\ to\ F5\ ExP) + (F1\ UP\ to\ F5\ UP)]} \times 100$	$Gprot \times \frac{Relative\ \%\ ExPP}{100}$
ExMP	$\frac{(F3\ ExP + F4\ ExP)}{[(F1\ ExP\ to\ F5\ ExP) + (F1\ UP\ to\ F5\ UP)]} \times 100$	$Gprot \times \frac{Relative\ \%\ ExMP}{100}$
ExAG	$\frac{(F5\ ExP)}{[(F1\ ExP\ to\ F5\ ExP) + (F1\ UP\ to\ F5\ UP)]} \times 100$	$Gprot \times \frac{Relative\ \%\ ExAG}{100}$
UPP	$\frac{(F1\ UP + F2\ UP)}{[(F1\ ExP\ to\ F5\ ExP) + (F1\ UP\ to\ F5\ UP)]} \times 100$	$Gprot \times \frac{Relative\ \%\ UPP}{100}$
UMP	$\frac{(F3\ UP + F4\ UP)}{[(F1\ ExP\ to\ F5\ ExP) + (F1\ UP\ to\ F5\ UP)]} \times 100$	$Gprot \times \frac{Relative\ \%\ UMP}{100}$
UAG	$\frac{(F5\ UP)}{[(F1\ ExP\ to\ F5\ ExP) + (F1\ UP\ to\ F5\ UP)]} \times 100$	$Gprot \times \frac{Relative\ \%\ UAG}{100}$

ExP – SDS-soluble protein, UP – SDS-insoluble protein, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin, Gprot – grain protein content at 12% moisture basis.

6.2.4. Statistical analysis

The data analysis of the SRI and SRD regions were performed separately, although using the same statistical methods. A combined nested design was used (Montgomery, 2017), with cultivars nested within the *Pin* allele genotypes. The results were divided into two *Pin* genotypes (*Pina-D1a*/*Pinb-D1b* and *Pina-D1b*/*Pinb-D1a*) for the SRI region, and four *Pin* genotypes (*Pina-D1a*/*Pinb-D1b*, *Pina-D1a*/*Pinb-D1p*, *Pina-D1a*/*Pinb-D1ab* and *Pina-D1b*/*Pinb-D1a*) for the SRD region. The Shapiro–Wilk test confirmed the normality and standardised residuals of the data, for the data to be considered reliable (Shapiro & Wilk, 1965). The homogeneity of variances were verified by Levene's test (Levene, 1960). The sources of variation in the data were partitioned as years (Y), localities (L), replications (per Y and L, Reps), *puroindoline* genotype (PG), cultivars (within PG) (C), Y x PG interaction, L x PG interaction, Y x C interaction, L x C interaction, Y x L x PG interaction, and Y x L x C interaction. The data were subjected to analysis of variance (ANOVA) using the General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc,

Cary, USA). Interaction means of protein fractions within PG were compared using Fisher's least significant difference (LSD) calculated at the 5% significance level (Ott & Longnecker, 2001).

Pearson's product moment correlation matrix of the pairwise correlations among SKCS-HI and the protein fractions within each PG and region (SRI and SRD) were performed, to show their linear relationships. Correlations were calculated using PROC CORR of SAS statistical software version 9.4 (SAS Institute Inc., Cary, NC, USA). The amount of protein in a sample influences the relative and absolute protein fractions and complicates the interpretation of data.

6.3. Results

6.3.1. The influence of wheat grain hardness on SE-HPLC protein fractions within two puroindoline genotypes planted in the summer rainfall irrigation (SRI) region

The Gprot differed significantly ($P < 0.05$) between the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes. Wheat with the *Pina-D1b*/*Pinb-D1a* (12.05 ± 1.61%) genotype had higher Gprot compared to wheat with the *Pina-D1a*/*Pinb-D1b* (12.48 ± 1.60) genotype.

The relative ExAG, -UPP, -UMP and -UAG differed significantly ($P < 0.05$) between the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes. The *Pina-D1b*/*Pinb-D1a* genotype (ExAG, 12.27 ± 5.53%; UMP, 10.62 ± 2.50%; and UAG, 3.78 ± 1.65%) had higher relative ExAG, -UMP and -UAG contents compared to the *Pina-D1a*/*Pinb-D1b* genotype (ExAG, 11.37 ± 5.64%; UMP 9.87 ± 2.54%; and UAG, 3.45 ± 1.58%). Relative UPP contents were lower in the *Pina-D1b*/*Pinb-D1a* (21.63 ± 5.34%) genotype compared to the *Pina-D1a*/*Pinb-D1b* genotype (23.07 ± 4.88%).

The absolute ExPP, -ExMP, -ExAG, -UPP and -UAG differed significantly ($P < 0.05$) between the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes. The *Pina-D1b*/*Pinb-D1a* genotype (ExAG, 1.45 ± 0.71%; UAG, 0.45 ± 0.22%) had higher absolute ExAG and UAG contents compared to the *Pina-D1a*/*Pinb-D1b* genotype (ExAG, 1.35 ± 0.69%; UAG, 0.42 ± 0.21%). The *Pina-D1b*/*Pinb-D1a* genotype (ExPP, 2.75 ± 0.61%; ExMP, 3.45 ± 0.57%; UPP, 2.60 ± 0.78%) had lower absolute ExPP, ExMP and UPP contents compared to the *Pina-D1a*/*Pinb-D1b* genotype (ExPP, 2.90 ± 0.79%; ExMP, 3.64 ± 0.73%; UPP, 2.91 ± 0.69%).

Kernel hardness (SKCS-HI) differed significantly ($P < 0.05$) between the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes. The *Pina-D1b*/*Pinb-D1a* (55.44 ± 6.89%) genotype had higher kernel hardness compared to the *Pina-D1a*/*Pinb-D1b* (47.42 ± 8.49%) genotype (Table 6.3).

The variation in SKCS-HI was attributed primarily to the trial location (35.74%, $P < 0.001$) and the *Pin* genotype (PG) (21.21%, $P < 0.001$) of the cultivars. The interaction of year and location (Y x L) contributed 18.51% ($P < 0.001$), and C contributed 5.74% ($P < 0.001$) of the variation in kernel hardness (Table 6.4).

The environmental effect primarily contributed to the variation in Gprot (84.95%, $P < 0.001$), while PG (1.64%, $P < 0.001$), C (3.28%, $P < 0.001$) and G x E (5.09%) contributed the remaining variation in grain protein content (Table 6.4).

Table 6.3. Means, standard deviation, the range of values, and standard error values for all variables within two *puroindoline* allele genotypes of the summer rainfall irrigation (SRI) region

	Pina-D1b*/*Pinb-D1a				***Pina-D1a*/*Pinb-D1b***				
Variable	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**LSD**
Gprot	97	12.05 ^{B} ± 1.61	8.90 - 15.40	0.16	58	12.48 ^{A} ± 1.60	9.90 - 15.50	0.21	0.16
Relative ExPP	91	22.92 ^{A} ± 4.28	15.10 - 35.40	0.45	59	23.15 ^{A} ± 4.54	15.30 - 33.70	0.59	0.66
Relative ExMP	91	28.78 ^{A} ± 3.81	21.60 - 42.40	0.40	59	29.09 ^{A} ± 3.66	23.70 - 39.10	0.48	0.68
Relative ExAG	91	12.27 ^{A} ± 5.53	3.20 - 24.40	0.58	59	11.37 ^{B} ± 5.64	3.60 - 22.40	0.73	0.71
Relative UPP	91	21.63 ^{B} ± 5.34	7.10 - 32.30	0.56	59	23.07 ^{A} ± 4.88	8.80 - 33.70	0.63	1.21
Relative UMP	91	10.62 ^{A} ± 2.50	6.20 - 19.10	0.26	59	9.87 ^{B} ± 2.54	4.90 - 17.00	0.33	0.44
Relative UAG	91	3.78 ^{A} ± 1.65	1.10 - 8.00	0.17	59	3.45 ^{B} ± 1.58	1.00 - 6.80	0.21	0.19
Absolute ExPP	88	2.75 ^{B} ± 0.61	1.80 - 4.60	0.07	57	2.90 ^{A} ± 0.79	1.90 - 5.10	0.10	0.09
Absolute ExMP	88	3.45 ^{B} ± 0.57	2.40 - 4.90	0.06	57	3.64 ^{A} ± 0.73	2.60 - 5.60	0.10	0.10
Absolute ExAG	88	1.45 ^{A} ± 0.71	0.40 - 3.40	0.08	57	1.35 ^{B} ± 0.69	0.50 - 3.10	0.09	0.09
Absolute UPP	88	2.60 ^{B} ± 0.78	0.90 - 4.60	0.08	57	2.91 ^{A} ± 0.69	1.20 - 4.40	0.09	0.17
Absolute UMP	88	1.27 ^{A} ± 0.38	0.60 - 2.50	0.04	57	1.22 ^{A} ± 0.37	0.60 - 2.20	0.05	0.06
Absolute UAG	88	0.45 ^{A} ± 0.22	0.10 - 1.10	0.02	57	0.42 ^{B} ± 0.21	0.10 - 1.00	0.03	0.03
SKCS-HI	100	55.44 ^{A} ± 6.89	37.70 - 76.70	0.69	60	47.42 ^{B} ± 8.49	33.90 - 69.60	1.10	1.06

Means followed by the same letter, did not differ significantly at $P < 0.05$, N – observations, Mean – mean values, SD – standard deviation, SE – standard error, Gprot – grain protein content at 12% moisture basis, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin, SKCS-HI – single kernel characterisation number hardness index.

Table 6.4. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for grain kernel hardness and grain protein content in the summer rainfall irrigation (SRI) region

		SKCS-HI		Gprot	
	DF	Mean squares	% of SS	Mean squares	% of SS
Year	1	59.46	0.52*	11.03	2.75***
Lok	3	1354.10	35.74***	86.72	64.90***
Y x L	2	1052.17	18.51***	27.57	13.76***
Rep (Y x L)	13	19.91	2.28 ns	1.09	3.54***
Total E			**57.06**		**84.95**
PG	1	2411.01	21.21***	6.59	1.64***
C	6	108.67	5.74***	2.19	3.28***
Total G			**26.95**		**4.93**
Y x PG	1	17.80	0.16 ns	2.93	0.73***
L x PG	3	4.81	0.13 ns	1.33	1.00**
Y x L x PG	2	105.18	1.85***	0.22	0.11 ns
Total PG x E			**2.13**		**1.84**
Y x C	6	20.82	1.10 ns	0.32	0.49 ns
L x C	18	18.90	2.99*	0.33	1.50 ns
Y x L x C	12	11.52	1.22 ns	0.46	1.27*
Total C x E			**5.31**		**3.25**
Total G x E			**7.44**		**5.09**
Error	91	10.68	8.55	0.23	2.75
R^2		0.91		0.95	
CV		6.23		3.94	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components, C – cultivar, E – environment, Y – year, L – location, SKCS-HI – single kernel characterisation system hardness index, GProt – grain protein content at 12% moisture basis.

Variation in the ExPP was primarily contributed by the environmental effect (66.80% relative, 71.16% absolute). The contribution of PG to the variation in ExPP was non-significant, however C contributed significantly (1.88% relative, 3.77% absolute) to the variation in ExPP. There were significant contributions of Y x PG interaction (0.63% relative, 1.79% absolute), and Y x L x PG interaction (2.70% relative, 2.28% absolute) to the variation in ExPP. The C x E interaction also significantly contributed to the variation in ExPP (Y x C 6.13% relative, 4.46% absolute; Y x L x C 5.54% relative, 4.33% absolute) (Table 6.5).

The environmental effect was the main contributor to variation in ExMP with 54.13% contribution to variation in the relative values of ExMP, and 64.66% contribution to the variation of absolute values of ExMP. The PG significantly contributed 1.14% of the variation in absolute ExMP. The contribution of G x E interaction to the variation in relative and absolute ExMP was higher than the G x E contribution to the variation in ExPP (Table 6.5). The variation in ExAG was primarily attributed to the environmental effect (83.11% relative, 84.01% absolute), while the effect of genotype (both PG

and C) were non-significant. However, the C x E interaction (6.96% relative, 6.53% absolute) contributed significantly to the variation in ExAG (Table 6.5).

The variation in UPP could be attributed to the environmental influence (40.24% relative, 52.70% absolute), PG (2.50% absolute), and total G x E interaction (29.39% relative, 20.68% absolute) (Table 6.6). The variation in UMP was primarily contributed by environment (53.35% relative, 65.15% relative), PG x E interaction (6.91% relative, 7.06% absolute) and C x E interaction (21.97% relative, 15.37% absolute). The contribution of PG (1.42% relative) was small, but significant (Table 6.6). Environmental influence was also the primary contributor to variation in UAG (78.65% relative, 79.48% absolute), with much less contribution by the PG and total G x E interactions (13.51% relative, 13.04% absolute), compared to the ANOVA results of UPP and UMP (Table 6.6).

There was a moderate to strong positive correlation between kernel hardness and grain protein content in the *Pina-D1b*/*Pinb-D1a* (0.57, $P < 0.001$) genotype. Grain kernel hardness had a weak negative correlation with relative ExPP in the *Pina-D1a*/*Pinb-D1b* (-0.28, $P < 0.05$) genotype; and weak negative correlations with relative ExMP in the *Pina-D1b*/*Pinb-D1a* (-0.29, $P < 0.01$) and *Pina-D1a*/*Pinb-D1b* (-0.33, $P < 0.01$) genotypes (Table 6.7).

There were weak positive correlations between kernel hardness and absolute UPP in the *Pina-D1b*/*Pinb-D1a* (0.39, $P < 0.001$) and *Pina-D1a*/*Pinb-D1b* (0.32, $P < 0.05$) genotypes; and moderate to strong positive correlations with relative and absolute UMP in the *Pina-D1b*/*Pinb-D1a* (0.45, $P < 0.001$ and 0.60, $P < 0.001$ respectively) and *Pina-D1a*/*Pinb-D1b* (0.51, $P < 0.001$ and 0.56, $P < 0.001$ respectively) genotypes. A weak positive correlation was observed between kernel hardness and relative UAG, and a moderate positive correlation of kernel hardness with absolute UAG, in the *Pina-D1b*/*Pinb-D1a* (0.26, $P < 0.05$ and 0.40, $P < 0.001$ respectively) genotype (Table 6.7).

Table 6.5. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for relative and absolute values of SDS-soluble protein fractions in the summer rainfall irrigation (SRI) region

		ExPP				ExMP				ExAG			
		Relative		Absolute		Relative		Absolute		Relative		Absolute	
	DF	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS
Year	1	1646.96	57.95***	31.14	45.50***	838.62	40.09***	18.41	30.86***	3447.40	74.58***	45.44	64.09***
Lok	3	55.88	5.90***	4.43	19.43***	47.28	6.78***	12.05	20.20***	94.12	6.11***	2.65	11.22***
Y x L	2	19.52	1.37**	1.15	3.37***	37.02	3.54***	5.32	8.92***	30.01	1.30**	2.51	7.07***
Rep (Y x L)	13	3.44	1.58 ns	0.15	2.85*	5.99	3.72 ns	2.79	4.67**	4.02	1.13 ns	0.09	1.64 ns
Total E			**66.80**		**71.16**		**54.13**		**64.66**		**83.11**		**84.01**
PG	1	0.10	0.00 ns	0.31	0.45 ns	0.38	0.02 ns	0.68	1.14**	5.91	0.13 ns	0.03	0.04 ns
C	6	8.90	1.88*	0.43	3.77***	9.75	2.80*	3.24	5.44***	2.19	0.28 ns	0.08	0.72 ns
Total G			**1.88**		**4.21**		**2.81**		**6.58**		**0.41**		**0.76**
Y x PG	1	17.95	0.63*	1.22	1.79***	8.11	0.39 ns	0.92	1.54**	0.16	0.00 ns	0.00	0.01 ns
L x PG	3	9.38	0.99 ns	0.06	0.28 ns	18.52	2.66**	1.08	1.80**	15.89	1.03*	0.16	0.66 ns
Y x L x PG	2	38.34	2.70***	0.78	2.28***	19.47	1.86*	0.87	1.46**	10.61	0.46 ns	0.20	0.57 ns
Total PG x E			**4.32**		**4.34**		**4.9**		**4.81**		**1.49**		**1.24**
Y x C	6	29.03	6.13***	0.51	4.46***	24.81	7.11***	2.43	4.07***	3.55	0.46 ns	0.05	0.45 ns
L x C	18	6.66	4.22 ns	0.10	2.74 ns	10.70	9.20**	3.00	5.04*	9.95	3.88**	0.13	3.40*
Y x L x C	12	13.13	5.54***	0.27	4.33***	10.24	5.87**	2.75	4.62**	10.12	2.63*	0.17	2.68**
Total C x E			**15.89**		**11.53**		**22.19**		**13.72**		**6.96**		**6.53**
Total G x E			**20.21**		**15.87**		**27.09**		**18.53**		**8.46**		**7.76**
Error	91	3.90	11.11	0.08	8.76	4.12	15.96	6.11	10.24	4.58	8.02	0.07	7.46
R^2		0.89		0.91		0.84		0.90		0.92		0.93	
CV		8.58		9.94		7.02		7.99		17.96		18.61	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components, C – cultivar, E – environment, Y – year, L – location, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin.

Table 6.6. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for relative and absolute values of SDS-insoluble protein fractions in the summer rainfall irrigation (SRI) region

		UPP				UMP				UAG			
		Relative		Absolute		Relative		Absolute		Relative		Absolute	
	DF	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS
Year	1	480.11	11.93***	9.93	11.97***	301.21	31.60***	3.52	17.33***	238.93	60.53***	3.19	45.76***
Lok	3	322.29	24.02***	9.86	35.65***	48.55	15.28***	2.70	39.80***	11.99	9.11***	0.44	18.84***
Y x L	2	3.80	0.19 ns	0.66	1.60 ns	17.28	3.63***	0.47	4.59***	16.33	8.27***	0.49	13.93***
Rep (Y x L)	13	12.69	4.10 ns	0.22	3.48 ns	2.08	2.84 ns	0.05	3.44 ns	0.22	0.74 ns	0.01	0.95 ns
Total E			**40.24**		**52.70**		**53.35**		**65.15**		**78.65**		**79.48**
PG	1	50.29	1.25 ns	2.08	2.50**	13.56	1.42**	0.07	0.33 ns	1.49	0.38*	0.01	0.10 ns
C	6	16.14	2.41 ns	0.11	0.83 ns	2.27	1.43 ns	0.02	0.71 ns	0.67	1.01 ns	0.01	0.93 ns
Total G			**3.66**		**3.33**		**2.85**		**1.04**		**1.39**		**1.02**
Y x PG	1	17.53	0.44 ns	0.13	0.16 ns	5.18	0.54 ns	0.12	0.58 ns	0.13	0.03 ns	0.00	0.00 ns
L x PG	3	30.14	2.25 ns	0.69	2.48*	4.36	1.37 ns	0.16	2.42**	1.51	1.15**	0.04	1.54***
Y x L x PG	2	62.06	3.08*	0.98	2.36*	23.82	5.00***	0.41	4.06***	0.06	0.03 ns	0.00	0.14 ns
Total PG x E			**5.77**		**5.00**		**6.91**		**7.06**		**1.21**		**1.67**
Y x C	6	43.59	6.50**	0.51	3.66 ns	12.58	7.92***	0.17	5.03***	1.53	2.32***	0.03	2.64***
L x C	18	23.56	10.54*	0.34	7.26 ns	3.34	6.30*	0.04	3.88 ns	0.99	4.53***	0.01	3.81**
Y x L x C	12	22.10	6.59 ns	0.36	4.76 ns	6.16	7.75***	0.12	6.46***	1.79	5.44***	0.03	4.92***
Total C x E			**23.63**		**15.68**		**21.97**		**15.37**		**12.29**		**11.37**
Total G x E			**29.39**		**20.68**		**28.89**		**22.43**		**13.51**		**13.04**
Error	91	13.27	26.71	0.25	23.3	1.76	14.92	0.03	11.38	0.31	6.45	0.01	6.45
R^2		0.73		0.77		0.85		0.89		0.94		0.94	
CV		16.41		18.41		12.83		13.81		15.35		17.59	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components, C – cultivar, E – environment, Y – year, L – location, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin.

Table 6.7. Pearson's correlations between wheat grain hardness and grain protein content and protein fractions within two *puroindoline* allele genotypes of the summer rainfall irrigation (SRI) region

	Pina-D1b*/*Pinb-D1a		***Pina-D1a*/*Pinb-D1b***	
	Relative %	**Absolute %**	**Relative %**	**Absolute %**
Gprot	0.57***		0.23 ns	
ExPP	-0.16 ns	0.18 ns	-0.28*	-0.11 ns
ExMP	-0.29**	0.18 ns	-0.33**	-0.08 ns
ExAG	-0.09 ns	0.07 ns	-0.06 ns	0.05 ns
UPP	0.14 ns	0.39***	0.25 ns	0.32*
UMP	0.45***	0.60***	0.51***	0.56***
UAG	0.26*	0.40***	0.18 ns	0.26 ns

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, Gprot – grain protein content at 12% moisture basis, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin.

6.3.2. Influence of wheat grain hardness on SE-HPLC protein fractions in four puroindoline genotypes planted in the summer rainfall dryland (SRD) region

The Gprot differed significantly (P < 0.05) between the four *Pin* genotypes (*Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1b, Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*). Wheat with the *Pina-D1a*/*Pinb-D1p* genotype (14.30 ± 1.24%) had the highest Gprot, followed by the *Pina-D1b*/*Pinb-D1a* (14.03 ± 1.40%) and *Pina-D1a*/*Pinb-D1ab* (14.05 ± 1.64%), while wheat with the *Pina-D1a*/*Pinb-D1b* genotype (13.95 ± 1.61%) had the lowest Gprot. Wheat with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1ab* genotypes did not differ significantly (P > 0.05) from each other, neither from the *Pina-D1a*/*Pinb-D1p* or *Pina-D1a*/*Pinb-D1b* genotypes for Gprot (Table 6.8).

The relative and absolute percentages of ExPP, -ExMP and -ExAG differed significantly (P < 0.05) between the four *Pin* genotypes. Wheat with the *Pina-D1b*/*Pinb-D1a* (relative ExPP, 21.57 ± 3.42%; absolute ExPP, 3.01 ± 0.51%) and *Pina-D1a*/*Pinb-D1b* (relative ExPP, 22.48 ± 4.98%; absolute ExPP, 3.10 ± 0.86%) genotypes had lower relative and absolute ExPP contents, compared to the *Pina-D1a*/*Pinb-D1p* (relative ExPP, 24.09 ± 4.10%; absolute ExPP, 3.41 ± 0.71%) and *Pina-D1a*/*Pinb-D1ab* (relative ExPP, 23.88 ± 5.79%; absolute ExPP, 3.38 ± 1.01%) genotypes. Wheat with the *Pina-D1a*/*Pinb-D1p* genotype (relative ExMP, 31.65 ± 3.66%; absolute ExMP, 4.44 ± 0.59%) had higher relative and absolute ExMP compared to wheat with the *Pina-D1b*/*Pinb-D1a* (relative ExMP, 30.18 ± 4.48%; absolute ExMP, 4.25 ± 0.85%), *Pina-D1a*/*Pinb-D1b* (relative ExMP, 29.97 ± 2.92%; absolute ExMP, 4.13 ± 0.66%) and *Pina-D1a*/*Pinb-D1ab* (relative ExMP, 29.16 ± 3.54%; absolute ExMP, 4.12 ± 0.71%) genotypes.

Wheat with the *Pina-D1a*/*Pinb-D1p* genotype (relative ExAG, 10.87 ± 6.49%; absolute ExAG, 1.52 ± 0.93%) had the highest relative and absolute ExAG content, followed by the *Pina-D1b*/*Pinb-D1a* genotype (relative ExAG, 10.24 ± 5.64%; absolute ExAG, 1.43 ± 0.80%), while wheat with the *Pina-D1a*/*Pinb-D1b* (relative ExAG, 9.51 ± 5.03%; absolute ExAG, 1.27 ± 0.66%) and *Pina-*

D1a/*Pinb-D1ab* (relative ExAG, 8.97 ± 4.64%; absolute ExAG, 1.24 ± 0.60%) genotypes had the lowest relative and absolute ExAG content (Table 6.8). There were no significant differences ($P > 0.05$) between the *Pin* genotypes for relative and absolute UPP, -UMP and -UAG protein fractions.

The SKCS-HI differed significantly ($P < 0.05$) between the four *Pin* genotypes. Wheat with the *Pina-D1b*/*Pinb-D1a* genotype (65.45 ± 5.30) had the highest SKCS-HI, followed by wheat with the *Pina-D1a*/*Pinb-D1ab* genotype (61.05 ± 5.30); while wheat with the *Pina-D1a*/*Pinb-D1p* (59.43 ± 5.51) and *Pina-D1a*/*Pinb-D1b* (58.46 ± 6.01) genotypes had the lowest SKCS-HI (Table 6.8).

The variation in SKCS-HI was primarily due to the E effect (38.56% contribution to variation), G x E (23.84%), PG (19.75%) and C (8.19%). The variation in Gprot was primarily contributed to E (58.56%), C (8.15%), PG x E interaction (7.41%) and C x E interaction (14.88%), with no significant ($P > 0.05$) contribution by PG (Table 6.9).

The E effect primarily contributed (73.28% relative, 71.19% absolute) to the variation in ExPP; however, PG (1.78% relative, 1.61% absolute), C (1.27% relative, 1.24% absolute), PG x E interaction (8.94% relative, 9.12% absolute) and C x E interaction (5.31% relative, 8.24% absolute) also contributed significantly. The variation in ExMP was primarily contributed by G x E interaction, consisting of PG x E (13.10% relative, 9.55% absolute) and C x E interaction (25.50% relative, 26.18% absolute). The total genotype effect also significantly contributed to the variation in ExMP, with C (10.13% relative, 10.69% absolute) contributing to higher number than PG (3.46% relative, 2.49% absolute). The E effect contributed 21.81% and 34.70% to the variation in relative and absolute ExMP respectively.

The E effect primarily contributed to the variation in ExAG (91.58% relative, 89.92% absolute), with small but significant contributions by PG (0.76% relative and 1.59% absolute) and total G x E interaction (4.00% relative, 4.91% absolute) (Table 6.10).

The variation in UPP was primarily contributed by E (28.46% relative, 42.34% absolute), C (5.45% relative, 4.01% absolute), and PG x E interaction (12.60% relative, 9.97% absolute). The PG x E interaction explained a high amount of the variation in UMP (17.68% relative, 16.60% absolute); while the environment (20.36% relative, 22.47% absolute) and C (7.53% absolute) also contributed considerably to the variation in UMP. The environment (56.96% relative, 60.01% absolute) was the primary contributor to the variation in UAG, with PG x E interaction contributing 8.08% and 8.59% to the variation in relative and absolute UAG, respectively (Table 6.11).

Table 6.8. Means, standard deviation, the range of values, and standard error values for all variables within four *puroindoline* allele genotypes of the summer rainfall dryland (SRD) region

	Pina-D1b*/*Pinb-D1a				***Pina-D1a*/*Pinb-D1b***				***Pina-D1a*/*Pinb-D1p***				***Pina-D1a*/*Pinb-D1ab***				
Variable	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**N**	**Mean ± SD**	**Range**	**SE**	**LSD**
Gprot	48	14.03 ^{AB} ± 1.40	11.30 - 17.70	0.20	120	13.95 ^{B} ± 1.61	10.40 - 17.90	0.15	24	14.30 ^{A} ± 1.24	12.50 - 16.50	0.25	24	14.05 ^{AB} ± 1.64	10.60 - 17.10	0.33	0.30
Relative ExPP	42	21.57 ^{B} ± 3.42	13.80 - 27.90	0.53	105	22.48 ^{B} ± 4.98	12.80 - 35.00	0.49	21	24.09 ^{A} ± 4.10	18.90 - 33.60	0.90	24	23.88 ^{A} ± 5.79	14.40 - 34.40	1.18	0.93
Relative ExMP	42	30.18 ^{B} ± 4.48	22.20 - 39.00	0.69	105	29.97 ^{B} ± 2.92	22.30 - 38.00	0.28	21	31.65 ^{A} ± 3.66	27.40 - 43.80	0.80	24	29.16 ^{B} ± 3.54	23.60 - 39.80	0.72	1.14
Relative ExAG	42	10.24 ^{B} ± 5.64	3.10 - 19.60	0.87	105	9.51 ^{C} ± 5.03	2.90 - 17.50	0.49	21	10.87 ^{A} ± 6.49	3.20 - 22.60	1.42	24	8.97 ^{C} ± 4.64	3.60 - 15.00	0.95	0.60
Relative UPP	42	23.37 ^{A} ± 4.91	9.60 - 39.70	0.76	105	23.69 ^{A} ± 3.30	16.00 - 36.50	0.32	21	23.69 ^{A} ± 7.92	17.20 - 55.40	1.73	24	24.40 ^{A} ± 4.26	12.80 - 31.50	0.87	1.79
Relative UMP	42	11.56 ^{A} ± 2.19	7.30 - 17.60	0.34	105	11.11 ^{A} ± 2.29	5.80 - 17.30	0.22	21	11.37 ^{A} ± 5.96	6.90 - 32.40	1.30	24	10.58 ^{A} ± 2.53	5.70 - 16.80	0.52	1.28
Relative UAG	42	3.10 ^{A} ± 1.06	1.70 - 5.80	0.16	105	3.24 ^{A} ± 1.28	1.50 - 7.70	0.13	21	3.08 ^{A} ± 2.22	1.50 - 12.20	0.48	24	3.03 ^{A} ± 1.34	1.10 - 5.60	0.27	0.41
Absolute ExPP	42	3.01 ^{B} ± 0.51	1.60 - 4.00	0.08	105	3.10 ^{B} ± 0.86	1.80 - 5.70	0.08	21	3.41 ^{A} ± 0.71	2.50 - 4.80	0.15	24	3.38 ^{A} ± 1.01	2.10 - 5.50	0.21	0.15
Absolute ExMP	42	4.25 ^{B} ± 0.85	3.10 - 5.70	0.13	105	4.13 ^{B} ± 0.66	2.70 - 5.90	0.06	21	4.44 ^{A} ± 0.59	3.60 - 6.30	0.13	24	4.12 ^{B} ± 0.71	3.20 - 5.40	0.14	0.18
Absolute ExAG	42	1.43 ^{B} ± 0.80	0.40 - 2.80	0.12	105	1.27 ^{C} ± 0.66	0.40 - 2.50	0.06	21	1.52 ^{A} ± 0.93	0.40 - 3.30	0.20	24	1.24 ^{C} ± 0.60	0.50 - 2.10	0.12	0.09
Absolute UPP	42	3.27 ^{A} ± 0.79	1.40 - 6.30	0.12	105	3.27 ^{A} ± 0.66	2.00 - 5.20	0.06	21	3.36 ^{A} ± 1.22	2.30 - 8.00	0.27	24	3.45 ^{A} ± 0.76	1.70 - 4.50	0.16	0.28
Absolute UMP	42	1.62 ^{A} ± 0.34	1.00 - 2.60	0.05	105	1.53 ^{A} ± 0.36	0.90 - 3.00	0.04	21	1.61 ^{A} ± 0.85	1.00 - 4.70	0.18	24	1.50 ^{A} ± 0.41	0.80 - 2.40	0.08	0.19
Absolute UAG	42	0.43 ^{A} ± 0.15	0.20 - 0.70	0.02	105	0.44 ^{A} ± 0.17	0.20 - 0.90	0.02	21	0.44 ^{A} ± 0.33	0.20 - 1.80	0.07	24	0.43 ^{A} ± 0.20	0.10 - 0.80	0.04	0.06
SKCS-HI	48	65.45 ^{A} ± 5.30	53.90 - 76.50	0.76	120	58.46 ^{C} ± 6.01	47.70 - 75.80	0.55	24	59.43 ^{C} ± 5.51	51.40 - 70.10	1.12	24	61.05 ^{B} ± 5.30	52.90 - 72.30	1.08	1.20

Means followed by the same letter, did not differ significantly at $P < 0.05$, N – observations, Mean – mean values, SD – standard deviation, SE – standard error, Gprot – grain protein content at 12% moisture basis, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin, SKCS-HI – single kernel characterisation number hardness index.

Table 6.9. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for grain kernel hardness and grain protein content in the summer rainfall dryland (SRD) region

		SKCS-HI		Gprot	
	DF	Mean squares	% of SS	Mean squares	% of SS
Year	1	2734.94	31.56***	33.92	6.79***
Lok	4	44.24	2.04***	43.40	34.75***
Y x L	2	137.15	3.16***	35.65	14.27***
Rep (Y x L)	16	9.71	1.79 ns	0.86	2.75*
Total E			**38.56**		**58.56**
PG	3	570.55	19.75***	0.83	0.50 ns
C	5	142.02	8.19***	8.14	8.15***
Total G			**27.94**		**8.65**
Y x PG	3	19.07	0.66*	3.07	1.84***
L x PG	12	23.37	3.24***	1.38	3.32***
Y x L x PG	6	17.18	1.19*	1.87	2.24***
Total PG x E			**5.09**		**7.41**
Y x C	5	54.78	3.16***	2.81	2.81***
L x C	20	38.30	8.84***	1.91	7.66***
Y x L x C	10	58.55	6.76***	2.20	4.41***
Total C x E			**18.75**		**14.88**
Total G x E			**23.84**		**22.29**
Error	128	6.54	9.66	0.41	10.49
R^2		0.90		0.90	
CV		4.23		4.56	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components, C – cultivar, E – environment, Y – year, L – location, SKCS-HI – single kernel characterisation system hardness index, GProt – grain protein content at 12% moisture basis.

SKCS-HI had a weak negative correlation with Gprot in the *Pina-D1a*/*Pinb-D1b* genotype (-0.25< P < 0.01) and a moderate negative correlation (-0.44, P < 0.01) with Gprot in the *Pina-D1a*/*Pinb-D1p* genotype (Table 6.12). SKCS-HI had moderate to strong negative correlations with relative and absolute ExPP in the *Pina-D1a*/*Pinb-D1b* (-0.60, P < 0.001 and -0.53, P < 0.001 respectively) and *Pina-D1a*/*Pinb-D1ab* (-0.65, P < 0.001 and 0.50, P < 0.05 respectively) genotypes. There were weak negative correlations between SKCS-HI and relative and absolute ExMP in the *Pina-D1b*/*Pinb-D1a* (-0.37, P< 0.05 and -0.31, P < 0.05 respectively) and *Pina-D1a*/*Pinb-D1b* (-0.28, P < 0.01 and -0.26, P < 0.01 respectively) genotypes (Table 6.12). SKCS-HI had moderate positive correlations with relative and absolute ExAG in the *Pina-D1b*/*Pinb-D1a* (0.50, P < 0.001 and 0.51, P < 0.001 respectively) and *Pina-D1a*/*Pinb-D1ab* (0.55, P < 0.01 and 0.62, P < 0.001 respectively) genotypes, a moderate to strong positive correlation with relative and absolute ExAG in the *Pina-D1a*/*Pinb-D1b* (0.64, P < 0.001 and 0.65, P < 0.001 respectively) genotype, and a strong positive correlation with relative and absolute ExAG in the *Pina-D1a*/*Pinb-D1p* (0.80, P < 0.001 and 0.78, P < 0.001 respectively) genotype (Table 6.12).

Table 6.10. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for relative and absolute values of SDS-soluble protein fractions in the summer rainfall dryland (SRD) region

		ExPP				ExMP				ExAG			
		Relative		Absolute		Relative		Absolute		Relative		Absolute	
	DF	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS
Year	1	2835.14	65.97***	71.01	56.72***	261.73	11.20***	12.32	12.91***	4810.51	90.28***	85.74	87.05***
Lok	4	30.40	2.83***	3.62	11.56***	30.36	5.19***	3.91	16.38***	10.98	0.82***	0.37	1.50***
Y x L	2	66.49	3.09***	0.81	1.30***	36.77	3.15**	1.41	2.96***	0.07	0.00 ns	0.37	0.74***
Rep (Y x L)	16	3.72	1.39 ns	0.13	1.61 ns	3.32	2.27 ns	0.15	2.45 ns	1.57	0.47 ns	0.04	0.63 ns
Total E			**73.28**		**71.19**		**21.81**		**34.70**		**91.58**		**89.92**
PG	3	25.52	1.78***	0.67	1.61***	26.97	3.46**	0.79	2.49**	13.42	0.76***	0.52	1.59***
C	5	10.93	1.27*	0.31	1.24**	47.34	10.13***	2.04	10.69***	5.11	0.48**	0.02	0.12 ns
Total G			**3.05**		**2.85**		**13.59**		**13.18**		**1.24**		**1.70**
Y x PG	3	65.16	4.55***	2.08	4.99***	14.67	1.88*	0.47	1.47*	15.14	0.85***	0.51	1.54***
L x PG	12	12.43	3.47***	0.30	2.88***	17.42	8.94***	0.52	6.55***	2.02	0.46 ns	0.05	0.60 ns
Y x L x PG	6	9.88	0.92*	0.39	1.26**	13.28	2.27 ns	0.36	1.53*	3.30	0.25 ns	0.10	0.40*
Total PG x E			**8.94**		**9.12**		**13.10**		**9.55**		**1.56**		**2.55**
Y x C	5	5.30	0.62 ns	0.22	0.89*	51.45	11.01***	2.46	12.87***	2.73	0.26 ns	0.03	0.14 ns
L x C	20	7.70	3.58**	0.29	4.57***	14.38	12.30***	0.59	12.41***	5.48	2.06***	0.10	1.98***
Y x L x C	10	11.88	1.11*	0.87	2.78***	12.83	2.20 ns	0.21	0.89 ns	1.79	0.13 ns	0.06	0.25 ns
Total C x E			**5.31**		**8.24**		**25.50**		**26.18**		**2.45**		**2.36**
Total G x E			**14.25**		**17.37**		**38.60**		**35.72**		**4.00**		**4.91**
Error	128	3.62	9.42	0.10	8.59	5.43	26.00	0.14	16.4	1.51	3.18	0.03	3.46
R^2		0.91		0.91		0.74		0.84		0.97		0.97	
CV		8.40		9.82		7.74		8.93		12.6		13.10	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components, C – cultivar, E – environment, Y – year, L – location, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin.

Table 6.11. Analysis of variance, with cultivars nested in *puroindoline* genotypes, for relative and absolute values of SDS-insoluble protein fractions in the summer rainfall dryland (SRD) region

		UPP				UMP				UAG			
		Relative		Absolute		Relative		Absolute		Relative		Absolute	
	DF	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS	Mean squares	% of SS
Year	1	450.36	11.82***	15.23	13.19***	187.95	11.63***	2.30	6.25***	3.36	47.60***	193.60	54.07***
Lok	4	88.73	9.31***	5.89	20.40***	5.24	1.30 ns	0.43	4.70*	0.06	3.16*	1.76	1.97*
Y x L	2	26.83	1.41 ns	1.98	3.43**	17.90	2.22 ns	1.11	6.01***	0.08	2.41**	1.72	0.96 ns
Rep (Y x L)	16	14.12	5.93 ns	0.38	5.33 ns	5.27	5.22 ns	0.13	5.51 ns	0.02	3.79 ns	0.67	3.01 ns
Total E			**28.46**		**42.34**		**20.36**		**22.47**		**56.96**		**60.01**
PG	3	3.48	0.27 ns	0.10	0.26 ns	2.89	0.54 ns	0.10	0.84 ns	0.00	0.16 ns	0.36	0.30 ns
C	5	41.57	5.45*	0.93	4.01*	15.24	4.72 ns	0.55	7.53**	0.04	2.63 ns	1.11	1.55 ns
Total G			**5.73**		**4.26**		**5.25**		**8.36**		**2.79**		**1.85**
Y x PG	3	20.22	1.59 ns	0.28	0.74 ns	36.95	6.86**	0.59	4.81**	0.01	0.54 ns	1.28	1.07 ns
L x PG	12	32.71	10.30**	0.80	8.27**	14.31	10.63*	0.34	11.03*	0.04	6.72**	2.18	7.31***
Y x L x PG	6	6.75	0.71 ns	0.28	0.97 ns	0.79	0.2	0.07	0.76 ns	0.01	0.81 ns	0.19	0.21 ns
Total PG x E			**12.60**		**9.97**		**17.68**		**16.60**		**8.08**		**8.59**
Y x C	5	20.91	2.74 ns	0.36	1.56 ns	2.99	0.92 ns	0.08	1.15 ns	0.03	2.07 ns	2.02	2.83*
L x C	20	18.61	9.76 ns	0.35	6.13 ns	5.62	6.96 ns	0.10	5.65 ns	0.01	3.20 ns	0.84	4.70 ns
Y x L x C	10	14.30	1.50 ns	0.87	3.02*	6.75	1.67 ns	0.15	1.61 ns	0.01	0.63 ns	0.39	0.43 ns
Total C x E			**14.01**		**10.70**		**9.55**		**8.41**		**5.91**		**7.97**
Total G x E			**26.61**		**20.68**		**27.24**		**25.01**		**13.98**		**16.56**
Error	128	13.34	39.2	0.34	32.72	6.80	47.15	0.14	44.15	0.02	26.27	0.69	21.58
R^2		0.61		0.67		0.53		0.56		0.74		0.78	
CV		15.40		17.50		23.30		24.50		29.30		26.20	

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, DF – degrees of freedom, CV – coefficient of variation, R^2 – regression coefficient, SS – sum of squares, % of SS – variance component contribution, PG – *Pin* genotype, C – other genetic components, C – cultivar, E – environment, Y – year, L – location, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin.

Weak negative correlations were observed between SKCS-HI and relative and absolute UPP in the *Pina-D1a*/*Pinb-D1b* (-0.37, P < 0.001 and -0.31, P < 0.01 respectively) genotype. SKCS-HI had weak negative correlations with relative and absolute UMP in the *Pina-D1b*/*Pinb-D1a* (-0.38, P < 0.05 and -0.36, P < 0.05 respectively) genotype, weak to moderate positive correlations with relative UMP and absolute UMP in the *Pina-D1a*/*Pinb-D1b* (0.47, P < 0.001 and 0.40, P < 0.001 respectively) genotype, and strong positive correlations with relative and absolute UMP in the *Pina-D1a*/*Pinb-D1ab* (0.65, P < 0.001 and 0.65, P < 0.001 respectively) genotype (Table 6.12). There were moderate positive correlations between SKCS-HI and relative and absolute UAG in the *Pina-D1a*/*Pinb-D1p* (0.50, P < 0.05 and 0.45, P < 0.05 respectively) genotype, and moderate to strong positive correlations between kernel hardness and relative and absolute UAG in the *Pina-D1a*/*Pinb-D1b* (0.58, P < 0.001 and 0.60, P < 0.001 respectively) and *Pina-D1a*/*Pinb-D1ab* (0.63, P < 0.001 and 0.67, P < 0.001) genotypes (Table 6.12).

Table 6.12. Pearson's correlations between wheat grain hardness (SKCS-HI) and grain protein content and protein fractions within four *puroindoline* allele genotypes of the summer rainfall dryland (SRD) region

	Pina-D1b*/*Pinb-D1a		***Pina-D1a*/*Pinb-D1b***		***Pina-D1a*/*Pinb-D1p***		***Pina-D1a*/*Pinb-D1ab***	
	Relative %	**Absolute %**	**Relative %**	**Absolute %**	**Relative %**	**Absolute %**	**Relative %**	**Absolute %**
Gprot	-0.04 ^{ns}		-0.25**		-0.44*		0.12 ^{ns}	
ExPP	-0.04 ^{ns}	-0.10 ^{ns}	-0.60***	-0.53***	-0.24 ^{ns}	-0.33 ^{ns}	-0.65***	-0.50*
ExMP	-0.37*	-0.31*	-0.28**	-0.26**	0.27 ^{ns}	0.04 ^{ns}	-0.13 ^{ns}	-0.01 ^{ns}
ExAG	0.50***	0.51***	0.64***	0.65***	0.80***	0.78***	0.55**	0.62**
UPP	-0.08 ^{ns}	-0.12 ^{ns}	-0.37***	-0.31**	0.01 ^{ns}	-0.06 ^{ns}	-0.20 ^{ns}	-0.07 ^{ns}
UMP	-0.38*	-0.36*	0.47***	0.40***	0.18 ^{ns}	0.14 ^{ns}	0.65***	0.65***
UAG	0.18 ^{ns}	0.20 ^{ns}	0.58***	0.60***	0.50*	0.45*	0.63***	0.67***

* P < 0.05, ** P < 0.01, *** P < 0.001, ns – non-significant, Gprot – grain protein content at 12% moisture basis, ExPP – extractable polymeric protein, ExMP – extractable monomeric protein, ExAG – extractable albumin and globulin, UPP – unextractable polymeric protein, UMP – unextractable monomeric protein, UAG – unextractable albumin and globulin.

6.4. Discussion

This current study advanced on earlier work (Gupta *et al.*, 1993; Ohm *et al.*, 2010; Malik *et al.*, 2011; Katyal *et al.*, 2017) by investigating the influence of GKH on protein molecular weight distribution within individual *Pin* genotypes. Comparisons will be made to earlier work. It should, however, be kept in mind that the results in the current study were within *Pin* genotype classes, while the earlier work was correlated with GKH as a whole, i.e. unknown *Pin* genotypes.

The SKCS results (Tables 6.3 & 6.8) indicated that wheat with the *Pina-D1b*/*Pinb-D1a* genotype had the highest SKCS-HI, followed by wheat with the *Pina-D1a*/*Pinb-D1ab* genotype with the second-highest SKCS-HI, and the *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1p* genotypes that did not differ significantly (P > 0.05) in SKCS-HI. The SKCS-HI values were higher in the SRD region compared to the SRI region (Tables 6.3 & 6.8). The Gprot results (Tables 6.3 & 6.8) indicated the

Gprot in the SRI region was lower than in the SRD region. The lower grain protein content in the SRI region are attributed to the 'dilution' of grain protein due to increased starch accumulation and wheat kernel weight. This has been observed when wheat was produced under irrigation conditions (Guttieri *et al.*, 2000). The increase in starch accumulation and kernel weight, increased the relative ratio of endosperm to bran content. This led to a lower percentage of Gprot.

The variation in all the measured properties (Gprot, SKCS-HI and protein fractions) were highly contributed by E and G x E interaction in both regions. The SRI region normally has high day time temperatures and approximately 3 weeks prior to harvesting the irrigation was stopped. This caused water stress in the final wheat maturity stage. The SRD region has high day time temperature and water stress (Appendix A, Tables A1 – A3) throughout the wheat development stages.

In the SRI region, the positive correlation between SKCS-HI and Gprot in wheat with the *Pina-D1b*/*Pinb-D1a* genotype were in accordance with earlier work of Groos *et al.* (2004) and Katyal *et al.* (2017). The SDS-soluble fractions (ExPP, ExMP and ExAG) were not significantly correlated ($P > 0.05$) with SKCS-HI. The absolute UPP, UMP and UAG were positively correlated ($P > 0.001$) with SKCS-HI in the *Pina-D1b*/*Pinb-D1a* genotype, while the absolute UMP ($P < 0.001$) and UPP ($P < 0.05$) were positively correlated with SKCS-HI in the *Pina-D1a*/*Pinb-D1b* genotype. In both the *Pina-D1b*/*Pinb-D1a* and *Pina-D1b*/*Pinb-D1a* genotypes the correlation were the highest between SKCS-HI and UMP, followed by UPP with weak positive correlations (Table 6.7).

A positive correlation between kernel hardness and UPP has been reported (Gupta *et al.*, 1993; Malik *et al.*, 2011; Katyal *et al.*, 2017). The amount of PP and MP increased with increased protein content, while the percentage of MP increased more rapidly (Triboï *et al.*, 2000; Saint Pierre *et al.*, 2008). High environmental temperature, as encountered in the SRI region, caused a quicker onset of the grain maturity stage. Earlier research showed that increased environmental temperature correlated with a decreased PP content (Blumenthal *et al.*, 1990; Panozzo & Eagles, 2000). In South Africa it is normal farming practice in the SRI region to stop irrigation approximately 3 weeks prior to harvesting; this forces the onset of the dehydration stage. The quicker onset of the dehydration stage increases the SDS-insoluble polymers and relatively decreases the SDS-soluble polymers (Carceller & Aussenac, 1999). Additionally, high environmental temperatures have been reported to be positively correlated with increased GKH (Kobata *et al.*, 1992; Altenbach *et al.*, 2003). The combination of high temperature and the final water stress, that cause the quicker onset of the dehydration and grain maturity stage, explain the positive interaction of SKCS-HI with unextractable proteins, and the stronger correlation with UMP in comparison to the weak correlations with UPP. The positive correlation of SKCS-HI with UAG in the *Pina-D1b*/*Pinb-D1a* genotype is also explained by the increased SKCS-HI and SDS-insoluble protein component due to high temperature and water stress.

In the SRD region, the total Gprot in all *Pin* genotypes were higher compared to the total Gprot in the SRI region (Tables 6.3 & 6.8). Unexpectedly, the *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1p* genotypes had weak negative correlations with Gprot, while the *Pina-D1b*/*Pinb-D1a* and *Pina-*

D1a/*Pinb-D1ab* genotypes showed no correlation of SKCS-HI and Gprot. Earlier work reported an increase in Gprot under high temperature and water stress conditions (Guttieri *et al.*, 2000; Dupont & Altenbach, 2003). The negative correlations between SKCS-HI and Gprot in wheat with the *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1p* genotypes can be attributed to the considerable contribution of G x E interaction to the variation in Gprot in the SRD region.

The *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1ab* genotypes showed moderate negative correlations between SKCS-HI and ExPP. The *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1p* genotypes also showed negative correlations between SKCS-HI and ExPP, although weak and not significant ($P > 0.05$). All the Pin genotypes (*Pina-D1b*/*Pinb-D1a, Pina-D1a*/*Pinb-D1b, Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*) showed a moderate to strong positive correlation between SKCS-HI and ExAG. The *Pina-D1a*/*Pinb-D1b* and *Pina-D1a*/*Pinb-D1ab* genotypes showed moderate to strong positive correlations between SKCS-HI and UMP, however, the *Pina-D1b*/*Pinb-D1a* genotype showed a weak negative correlation between SKCS-HI and UMP. The *Pin* genotypes (*Pina-D1a*/*Pinb-D1b, Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*) showed moderate to strong positive correlations between SKCS-HI and UAG.

The accumulation of albumins and globulins (AG) occur during the first stage of wheat development and then reaches a plateau. Under normal wheat development a ‘reduction’ in the percentage of AG during grain development will be apparent, as the MP and PP contents increase (Carceller & Aussenac, 1999). Under water stress conditions, the grain filling stage will be shortened, thus reducing the accumulation of SDS-soluble MP and PP (Guttieri *et al.*, 2000; Dupont & Altenbach, 2003). It has been confirmed that the SDS-insoluble fractions always accumulate in the dehydration stage of wheat maturation (Carceller & Aussenac, 1999). This implies that the SDS-insoluble fractions will increase and SDS-soluble will decrease with high environmental temperature. As discussed earlier both PP and MP have been reported to increase with an increase in grain protein content, and MP increase more rapidly, with the relative proportion of PP thus decreasing (Blumenthal *et al.*, 1990; Panozzo & Eagles, 2000; Triboï *et al.*, 2000). Saint Pierre *et al.* (2008) found that the albumin and globulin proteins, situated primarily in the bran layers of the wheat kernel, respond the least to the changes in total grain protein. Decreased grain kernel weight and diameter (Appendix A, Table A10) have been associated with increased GKH (Martin *et al.*, 2001; Boehm *et al.*, 2018; and Chapter 4 of this dissertation). This implies that AG protein fractions increase in relative percentage, as SKCS-Weight and SKCS-Dia decrease with the increase in SKCS-HI.

The interactions discussed above, between the high temperature and water stress in the SRD region, explains the positive correlations of SKCS-HI with ExAG and UAG. Also, the negative correlations between SKCS-HI and ExPP and ExMP; and the positive correlations between SKCS-HI and UPP and UMP. However, the correlation between SKCS-HI and UMP in the *Pina-D1b*/*Pinb-D1a* genotype was negative, and not positive as with the other *Pin* genotypes (*Pina-D1a*/*Pinb-D1b*, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*).

6.5. Conclusion

The current study was the first to investigate the influence of grain hardness on protein molecular weight distribution within *Pin* genotypes, and therefore different outcomes than those observed on mixed *Pin* genotypes in earlier work were anticipated. The current study's results indicated the response of the protein molecular weight distribution to variation in SKCS-HI, within different *Pin* genotypes. The results from the SRI region indicated the typical response of SKCS-HI and protein fractions to environmental influence, primarily in the form of high temperature. It was evident that wheat with the *Pina-D1b*/*Pinb-D1a* genotype, with higher SKCS-HI compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotype, increased in protein and particularly the SDS-insoluble fraction. If a wheat breeder encounters wheat breeding lines with the *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes, the information obtained in this study will inform the breeder on the wheat line's 'response' to environmental influence, and the typical wheat quality expected from it.

Environmental influence primarily attributed to the variation of all protein fractions measured in the different *Pin* genotypes. The SRD region additionally contained a higher degree of G x E interaction than the SRI region. The *Pin* genotypes that contain a *Pinb-D1* mutation responded similar to environmental influence and G x E interaction, however the *Pina-D1b*/*Pinb-D1a* responded differently with a negative correlation between SKCS-HI and UMP. This information would be valuable to implement in wheat breeding practices, by predicting a cultivar or breeding line's response to G x E interaction and increased SKCS-HI on protein fractions.

The *Pin* genotypes, *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*, were represented by only one cultivar each. To confirm the results obtained in the current study, it is recommended that the test population be increased with a higher representation of cultivars containing the *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab* genotypes.

6.6. References

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CHAPTER 7
General discussion and conclusions

Wheat hardness is one of the most important properties of wheat that impacts the processing of wheat grain, and end-use quality of the flour. The *puroindoline a* (*Pina-D1*) and *b* (*Pinb-D1*) alleles, located in the hardness locus on chromosome 5DS, form the molecular basis of grain kernel hardness (GKH).

In South Africa, selection during wheat breeding is based on the agronomic adaptability of wheat lines for each production region, optimum grain yield, adequate disease resistance of the plant, and finally baking quality that is acceptable to the South African milling- and bread baking industries. There are strict release criteria that a wheat line should adhere to before it can be commercially released and produced. The wheat quality release criteria are based on milling yield, and the suitability of the wheat line for bread baking, as determined by flour protein content, mixograph, alveograph, farinograph and falling number. All wheat breeding lines are compared to the wheat quality standard of each production region, and it can deviate within a set tolerance level from the wheat quality standard. The breeding line's quality must remain stable for three years with five locations each before it is approved for commercial release.

GKH is not one of the strict release criteria that a breeding line's quality is based on. However, GKH is an essential property that affects the processing quality of wheat and flour. The selection for bread baking quality in South Africa, inherently forces the selection of certain wheat grain traits, in combination with the required baking quality traits, such as GKH. The medium-hard to hard wheat classes to which all South African bread wheat cultivars belong, produce high grain protein content, high milling yields, and enable millers to provide flour suitable for the baking industry.

In this study, 27 South African wheat cultivars were selected to cover the available range of kernel hardness values, nine cultivars per wheat production region. The cultivars used were commercial cultivars that have been adapted agronomically for production in each region. Four *puroindoline* (*Pin*) allele genotypes were identified in the selected wheat cultivars, i.e. *Pina-D1b*/*Pinb-D1a, Pina-D1a*/*Pinb-D1b, Pina-D1a*/*Pinb-D1p and Pina-D1a*/*Pinb-D1ab*. The spring wheat cultivars from the winter rainfall dryland (WRD) and summer rainfall irrigation (SRI) regions showed the least diversity with only *Pina-D1b*/*Pinb-D1a* genotype in the WRD region, and *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes in the SRI region. Facultative and winter wheat from the summer rainfall dryland (SRD) region had the highest diversity with all four of the identified *Pin* genotypes represented. The diversity of *Pin* alleles observed in South African wheat cultivars is of such a nature that it complies with local flour quality requirements and the related GKH, namely medium-hard to hard wheat grain that is suitable for bread baking. By identifying the *Pin* alleles present in South African commercial wheat cultivars, the inherently selected *Pin* alleles that provide acceptable flour processing quality and related GKH has been determined. The two prediction models that were

developed for the SRI and SRD regions can be implemented to predict kernel hardness within the production regions, based on the *Pin* alleles present in a cultivar or breeding line.

The most common methods to determine GKH involve the single kernel characterisation system (SKCS) or near-infrared reflectance (NIR) spectroscopy. The SKCS method is based on the force needed to crush a wheat kernel, while the NIR method is based on the light scattering properties due to the difference in particle size of the wheat flour sample after grinding. The SKCS was reported to be the most reliable method, compared to NIR spectroscopy predictions, due to variation that might be introduced through the sample preparation and increased possible error with grinding and NIR methods (Boehm *et al.*, 2018). The SKCS was thus used to determine GKH in the current study.

Based on the SKCS hardness index (SKCS-HI) results, and published knowledge of the *Pina-D1* and *Pinb-D1* wild-type alleles (Giroux & Morris, 1997), the ranking of *Pin* genotypes from hardest to softest is *Pina-D1b*/*Pinb-D1a* > *Pina-D1a*/*Pinb-D1b* > *Pina-D1a*/*Pinb-D1a*. The GKH of *Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab* genotypes produced wheat kernels with hardness values comparable to that of the *Pina-D1a*/*Pinb-D1b* genotype.

The environmental influence, and genotype by environment (G x E) interaction, as determined by analysis of variance (ANOVA), contributed highly to the variation of GKH, other grain characteristics, milling performance, and flour quality properties of the wheat produced in the SRI and SRD regions. This influenced the correlations between GKH and the other grain characteristics, milling performance and flour quality properties. Wheat with the *Pina-D1b*/*Pinb-D1a* genotype produced wheat with GKH values from 6 to 8 SKCS-HI units higher compared to wheat with the *Pina-D1a*/*Pinb-D1b* genotypes. This was in accordance with earlier findings (Giroux *et al.*, 2000; Martin *et al.*, 2001; Ma *et al.*, 2009; Takata *et al.*, 2010). The *Pina-D1b*/*Pinb-D1a* genotype showed significantly ($P < 0.05$) decreased kernel weight, -diameter, break flour yield (BFY), total flour yield (TFY), dough extensibility, -strength, and tolerance to overmixing, but increased flour water absorption and dough tenacity, in comparison to the *Pina-D1a*/*Pinb-D1b* genotype.

Comparing the effect of three *Pinb-D1* mutations (*Pinb-D1b*, *Pinb-D1p* and *Pinb-D1ab*) on GKH, indicated no significant differences in SKCS-HI. In practice this information regarding the milling- and flour quality properties will still provide valuable information that can be implemented in wheat breeding programmes. Wheat with the *Pinb-D1p* mutation, had the highest BFY and TFY of all three *Pinb-D1* mutations genotypes, and also higher BFY and TFY compared to the *Pina-D1b/Pinb-D1a* genotypes. Wheat with the *Pina-D1b/Pinb-D1a* genotype had the lowest BFY and TFY of all the *Pin* genotypes identified in the current study. This is an undesirable property in wheat processing. If the wheat breeder thus encounters the *Pina-D1b/Pinb-D1a* genotype in a breeding line it would be advisable not to select it, due to its decreased performance in milling yield. Further significant differences, in wheat and flour properties, observed between the three *Pinb-D1* mutations included the *Pinb-D1p* mutation which showed increased dough tenacity, -strength, flour water absorption (FWA), mixograph peak height (MPH), mixograph tail height (MTH) and mixograph mixing time (MMT), but decreased dough extensibility and swelling index compared to wheat with the *Pinb-D1p*

and *Pinb-D1ab* mutations. This implies that the variation in flour and dough properties between wheat with the *Pinb-D1* mutations are due to the functional quality of the expressed PINB protein.

The cultivars from the WRD region did not have any diversity in *Pin* alleles as all nine cultivars had the *Pina-D1a*/*Pinb-D1b* genotype. This enabled the investigation of environmental influence on GKH and flour quality properties. The *Pina-D1a*/*Pinb-D1b* genotype primarily contributed the variation in GKH in the Swartland region. The genetic influence on GKH had the greatest effect on BFY, TFY, and α-amylase activity. The environment primarily contributed to the variation in GKH in the Ruens region. The environmental influence on GKH affected BFY, TFY, flour ash content, kernel weight, kernel diameter, FWA, dough tenacity, -strength, and -tolerance to overmixing. The additional properties that correlated with GKH in the Ruens region were attributed to protein characteristics, i.e. dough tenacity, -strength, and -tolerance to overmixing. A high environmental impact during grain development influences the ratio of protein components in the kernel endosperm, and decreases starch accumulation (Altenbach *et al.*, 2003).

Although it is generally accepted that the *Pin* genotype of a cultivar forms the genetic basis of GKH, the current study provided evidence that GKH remains a complex subject and is controlled by more than just the *Pin* genotype of a cultivar. Several major and minor genes have been identified (Martin *et al.*, 2001; Surma *et al.*, 2012; Nirmal *et al.*, 2016) on chromosomes other than 5DS. These genes have influenced GKH, although the extent to which they contribute to the variation in GKH has not yet been established. When studying the ANOVA results of this research, with cultivars nested in *Pin* genotype [Chapter 4 (Tables 4.3 & 4.10) and Chapter 6 (Tables 6.4 & 6.9)], it was evident that the *Pin* genotype of a cultivar contributes considerably more to the variation in GKH than the other genetic components present in the cultivars. It is suggested that the contribution of *Pin* genotype (PG) and other genetic components (C) justify further investigation, however, careful experimental design should be performed to minimise the contribution of environment (E), and genotype (G) x E interaction to the variation in GKH.

The current study was the first to investigate the influence of GKH on protein molecular weight distribution within the *Pin* genotype classes. Earlier studies were conducted on the correlation between GKH and protein molecular weight distribution, without knowledge on the *Pin* genotype class(es) of the test population (Gupta *et al.*, 1993; Ohm *et al.*, 2010; Malik *et al.*, 2011; Katyal *et al.*, 2017). The results obtained provide a valuable contribution to knowledge on the response of protein molecular weight distribution, within *Pin* genotype classes, to increased GKH. In the SRI region, where environmental influence primarily contributed to the variation in protein fractions, SKCS-HI in both *Pina-D1b*/*Pinb-D1a* and *Pina-D1a*/*Pinb-D1b* genotypes were positively correlated with the sodium dodecyl sulphate (SDS) insoluble protein fractions. In the SRD region, G x E interaction primarily contributed to the variation in protein fractions. All four *Pin* genotypes (*Pina-D1b*/*Pinb-D1a*, *Pina-D1a*/*Pinb-D1b, Pina-D1a*/*Pinb-D1p* and *Pina-D1a*/*Pinb-D1ab*) showed positive correlations between SKCS-HI and SDS-soluble and -insoluble albumins and globulins (ExAG and UAG). The knowledge of the *Pin* alleles present in a cultivar or breeding line would enable the wheat breeder to

predict the 'protein response' in a cultivar with known *Pin* genotype, to high temperature and/or water stress conditions. Provision for acceptable wheat quality, even when influenced by environmental and G x E interaction, can be made by the breeder when selecting for the desired *Pin* genotypes in breeding lines.

Obtained results demonstrated that the environment, and G x E interaction had a major influence on wheat grain-, milling- and flour quality properties. However, GKH is highly influenced by the *Pin* genotype of the wheat line or cultivar. When the wheat breeder encounters certain *Pin* genotypes within the breeding process, reliable predictions and selections for GKH and flour quality can be made. The knowledge obtained in this study, on the correlations of SKCS-HI in different *Pin* genotypes with milling- and flour quality properties, should be implemented in wheat breeding programmes. A further valued addition to this study would be to genotype all commercially released South African wheat cultivars for *Pin* alleles. Additional information regarding further diversity of *Pin* alleles in South African wheat cultivars would be useful in future wheat breeding practices. Currently released, and agronomically adapted cultivars, with known *Pin* genotype identity can be used as resources to select breeding parents or specific end-uses. This would enable the breeding of end-use-specific wheat cultivars, by selecting for specific *Pin* genotypes, while shortening the breeding process, and still breeding for suitable cultivars for different environments and applications.

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Appendix A

Table A1. Meteorological data for the summer rainfall dryland (SRD) region 2012 – 2014 with deviations from the long-term mean (2002 – 2011)

		2012 Bultfontein			2013 Bultfontein			2014 Wesselsbron		
		Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)
Pre-seeding	Dec – April	163 (-88.8)	14.3 (-0.80)	29.5 (+0.1)	163.6 (-89.0)	14.7 (-0.4)	29.8 (+0.5)	363.4 (+110.8)	14.4 (-0.7)	28.2 (-1.1)
seeding	May	0 (-11.6)	6.2 (0.0)	25.1 (+2.8)	0.0 (-11.6)	5.6 (-0.6)	23.1 (+0.8)	4.8 (-6.8)	5.8 (-0.4)	24.0 (+1.7)
early growth	Jun – Aug	3 (-20.3)	2.6 (-0.7)	20.6 (+0.2)	0.0 (-23.9)	3.0 (-0.3)	20.7 (+0.2)	7.8 (-16.1)	2.0 (-1.3)	19.5 (-0.9)
pre-anthesis	Sept	8 (-0.4)	6.8 (-2.2)	24.6 (-2.5)	0.0 (-8.6)	8.1 (-0.9)	26.0 (-1.1)	0.0 (-8.6)	9.1 (+0.1)	28.1 (+1.0)
post-anthesis & grain fill	Oct – Nov	48 (-34.7)	13.1 (-0.5)	30.4 (+0.7)	95.6 (+12.9)	12.6 (-0.9)	29.0 (-0.7)	129.6 (+46.9)	12.4 (-1.1)	27.9 (-1.8)
Total yearly rainfall		223.6 (-155.8)			259.2 (-120.2)			505.6 (+126.2)		
		2012 Clocolan			**2013 Ladybrand**			**2014 Ladybrand**		
		Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)
Pre-seeding	Jan – May	282.0 (-145.1)	8.8 (-0.9)	25.4 (+1.1)	156.0 (-265.7)	9.1 (-0.5)	24.8 (+0.5)	318.6 (-103.1)	9.4 (-0.2)	24.6 (+0.3)
seeding	Jun	0.0 (-20.1)	-1.4 (+0.3)	15.3 (-1.3)	0.0 (-20.6)	-3.3 (-1.6)	17.7 (+1.1)	0.0 (-20.6)	-3.6 (-1.9)	17.7 (+1.1)
early growth	Jul – Sept	47.0 (+13.6)	0.3 (-0.2)	19.4 (-0.9)	4.0 (-38.9)	0.7 (+0.2)	20.2 (-0.1)	12.5 (-30.4)	0.4 (-0.1)	20.7 (+0.4)
pre-anthesis	Oct	62.0 (+7.7)	9.2 (+0.6)	25.3 (-0.2)	62.6 (+5.9)	7.2 (-1.4)	25.2 (-0.3)	9.7 (-47.0)	8.1 (-0.5)	26.4 (+0.9)
post-anthesis & grain fill	Nov – Dec	226.0 (+32.7)	11.6 (-0.2)	26.5 (-0.8)	189.7 (+26.4)	11.2 (-0.6)	26.1 (-1.2)	311.3 (+148.0)	11.7 (-0.2)	25.9 (-1.3)
Total yearly rainfall		617.0 (-111.3)			412.3 (-292.9)			652.1 (-53.1)		
		2012 Bethlehem			**2013 Bethlehem**			**2014 Bethlehem**		
		Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)
Pre-seeding	Jan – May	281.0 (-83.7)	9.0 (-0.2)	24.8 (+1.4)	386.1 (+21.4)	9.2 (0.0)	23.9 (+0.4)	348.1 (-16.6)	9.7 (+0.5)	23.7 (+0.3)
seeding	Jun	50.6 (+31.3)	-1.5 (-0.2)	15.2 (-1.2)	0.0 (-19.3)	-1.3 (0.0)	17.8 (+1.4)	0.0 (-19.3)	-1.8 (-0.5)	17.9 (+1.5)
early growth	Jul – Sept	53.7 (+23.0)	0.6 (-0.2)	19.1 (-0.8)	8.9 (-21.8)	2.0 (+1.1)	19.9 (+0.1)	22.0 (-8.7)	1.3 (+0.5)	20.4 (+0.5)
pre-anthesis	Oct	66.7 (+1.1)	9.4 (+1.1)	24.1 (-0.4)	89.0 (+23.4)	7.6 (-0.7)	24.7 (+0.2)	28.2 (-37.4)	8.0 (-0.3)	25.2 (+0.7)
post-anthesis & grain fill	Nov – Dec	191.3 (-12.0)	11.9 (+0.7)	25.4 (-0.6)	269.4 (+66.1)	11.8 (+0.6)	24.6 (-1.4)	320.5 (+117.2)	12.1 (+0.9)	24.7 (-1.3)
Total yearly rainfall		643.3 (-40.4)			753.4 (+69.7)			718.8 (+35.1)		
		2012 Reitz			**2013 Reitz**			**2014 Reitz**		
		Rainfall (mm)			Rainfall (mm)			Rainfall (mm)		
Pre-seeding	Jan – May	123.5 (-241.4)			353.8 (-11.1)			260.2 (-104.7)		
seeding	Jun	31.0 (+13.9)			0.0 (-17.2)			0.0 (-17.2)		
early growth	Jul – Sept	97.7 (+61.1)			8.5 (-28.2)			41.9 (+5.3)		
pre-anthesis	Oct	131.5 (+78.5)			58.8 (+5.8)			15.3 (-37.7)		
post-anthesis & grain fill	Nov – Dec	176.3 (-12.5)			198.3 (+9.5)			279.9 (+91.1)		
Total yearly rainfall		560.0 (-100.50)			619.4 (-41.1)			597.3 (-63.2)		

Values in brackets indicate the deviation of the year's value from the long-term mean (2002 – 2011) (South African Weather Service). No temperature data was available for Reitz 2012 – 2014.

Table A2. Meteorological data for the summer rainfall irrigation (SRI) region 2012 – 2014 with deviations from the long-term mean (2002 – 2011)

		2012 Marblehall			2013 Marblehall			2014 Marblehall		
		Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)	Rainfall (mm)	Minimum temperature (°C)	Maximum Temperature(°C)
Pre-seeding	Nov – Apr	362.4 (-6.1)	15.9 (0.0)	31.3 (+1.1)	351.4 (-17.1)	14.8 (-1.1)	30.4 (+0.2)	485.4 (+116.9)	17.5 (+1.6)	31.3 (+1.2)
seeding	May	0.0 (-7.2)	9.1 (+1.4)	27.6 (+2.2)	11.2 (+4.0)	6.1 (-1.6)	25.1 (-0.3)	0.0 (-7.2)	8.2 (+0.5)	27.8 (+2.4)
early growth	Jun – Jul	0.0 (-3.8)	4.2 (+0.4)	24.1 (+1.2)	0.4 (-3.4)	3.5 (-0.4)	23.6 (+0.7)	1.0 (-2.8)	4.1 (+0.3)	25.1 (+2.3)
pre-anthesis	Aug	0.0 (-6.8)	5.7 (-0.3)	27.3 (+1.8)	1.2 (-5.6)	5.9 (-0.1)	26.1 (+0.6)	0.0 (-6.8)	6.6 (+0.6)	27.6 (+2.1)
post-anthesis & grain fill	Sept – Oct	138.4 (+80.5)	11.8 (-0.4)	26.7 (-3.3)	133.0 (+75.1)	13.8 (+1.6)	31.3 (+1.3)	31.8 (-26.1)	13.0 (+0.8)	32.0 (+2.0)
Total yearly rainfall		500.8 (+56.7)			497.2 (+53.1)			518.2 (+74.1)		
		2012 Lichtenburg			**2013 Lichtenburg**			**2014 Lichtenburg**		
		Rainfall (mm)	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**
Pre-seeding	Dec – May	349.80 (-103.9)	12.62 (-0.6)	27.48 (+1.0)	326.40 (-127.3)	12.47 (-0.8)	26.82 (+0.3)	482.40 (+28.7)	11.93 (-1.3)	25.27 (-1.2)
seeding	Jun	11.80 (-1.1)	2.30 (-0.3)	19.10 (+0.1)	0.00 (-12.9)	2.50 (-0.1)	20.80 (+1.8)	0.60 (-12.3)	0.70 (-1.9)	19.80 (+0.8)
early growth	Jul – Aug	0.20 (-7.2)	4.20 (+1.0)	21.45 (+1.0)	0.20 (-7.2)	3.55 (+0.4)	20.55 (+0.1)	0.00 (-7.4)	2.70 (-0.5)	20.30 (-0.2)
pre-anthesis	Sept	18.20 (+8.3)	5.80 (-3.3)	23.00 (-3.4)	0.60 (-9.3)	7.70 (-1.4)	26.50 (+0.1)	2.00 (-7.9)	9.20 (+0.1)	27.30 (+0.9)
post-anthesis & grain fill	Oct – Nov	126.00 (+22.6)	13.95 (+0.4)	29.45 (+1.2)	47.80 (-55.6)	12.20 (-1.3)	29.15 (+0.9)	79.00 (-24.4)	12.35 (-1.2)	27.65 (-0.6)
Total yearly rainfall		506.0 (-81.4)			375.0 (-212.4)			564.0 (-23.4)		
		2012 Hartsvallei			**2013 Hartsvallei**			**2014 Hartsvallei**		
		Rainfall (mm)	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**
Pre-seeding	Dec – May	194.2 (-126.4)	13.7 (-0.5)	30.7 (+0.9)	250.0 (-70.6)	14.8 (+0.6)	31.2 (+1.4)	169.4 (-151.2)	14.2 (-0.1)	31.1 (+1.4)
seeding	Jun	10.2 (-2.6)	2.6 (+0.4)	20.8 (+0.1)	0.8 (-12.0)	2.3 (+0.1)	22.6 (+1.9)	0.0 (-12.8)	1.2 (-1.0)	22.8 (+2.1)
early growth	Jul – Aug	0.6 (-10.4)	3.6 (+0.6)	23.3 (+0.6)	0.2 (-10.8)	3.7 (+0.8)	23.6 (+0.9)	21.6 (+10.6)	2.5 (-0.5)	22.5 (-0.2)
pre-anthesis	Sept	7.8 (-2.3)	7.8 (-0.7)	26.3 (-2.3)	0.0 (-10.1)	7.4 (-1.1)	28.7 (+0.1)	0.0 (-10.1)	7.2 (-1.3)	29.4 (+0.8)
post-anthesis & grain fill	Oct – Nov	8.2 (-51.9)	14.7 (+0.8)	33.1 (+1.5)	59.2 (-0.9)	13.4 (-0.5)	33.3 (+1.7)	102.8 (+42.7)	11.9 (-2.0)	30.3 (-1.3)
Total yearly rainfall		221.0 (-193.6)			310.2 (-10.4)			293.8 (-120.8)		
		2012 Winterton			**2013 Winterton**			**2014 Winterton**		
		Rainfall (mm)	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**
Pre-seeding	Dec – May	491.4 (+13.0)	14.4 (+1.3)	28.2 (+1.4)	409.4 (-69.0)	13.4 (+0.3)	27.0 (+0.2)	413.0 (-65.4)	14.3 (+1.1)	27.4 (+0.6)
seeding	Jun	10.6 (-0.1)	4.7 (+1.9)	21.7 (+1.9)	0.2 (-10.5)	3.4 (+0.6)	22.5 (+2.7)	0.4 (-10.3)	4.5 (+1.7)	22.9 (+3.1)
early growth	Jul – Aug	42.2 (+13.3)	6.0 (+2.0)	23.5 (+1.8)	16.0 (-12.9)	6.5 (+2.5)	22.7 (+1.0)	1.6 (-27.3)	4.9 (+0.9)	22.8 (+1.1)
pre-anthesis	Sept	166.0 (+146.8)	11.0 (+1.3)	23.7 (-2.4)	4.0 (-15.2)	9.1 (-0.6)	26.9 (+0.8)	24.0 (+4.8)	11.1 (+1.4)	30.0 (+3.9)
post-anthesis & grain fill	Oct – Nov	172.2 (+54.2)	14.2 (+0.6)	27.2 (-0.3)	86.6 (-31.4)	13.1 (-0.4)	27.7 (+0.2)	48.6 (-69.4)	13.3 (-0.2)	25.9 (-1.7)
Total yearly rainfall		882.4 (+227.3)			516.2 (-138.9)			487.60 (-167.5)		

Values in brackets indicate the deviation of the year's value from the long-term mean (2002 – 2011) (South African Weather Service).

Table A3. Meteorological data for the winter rainfall dryland (WRD) region 2012 – 2014 with deviations from the long-term mean (2002 – 2011)

		2012 Riversdal			**2013 Riversdal**			**2014 Riversdal**		
		Rainfall (mm)	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**
Pre-seeding	Dec – April	150.2 (+44.9)	15.8 (+0.3)	28.0 (+0.3)	102.6 (-2.7)	15.1 (-0.4)	28.2 (-0.4)	381.6 (+276.3)	15.6 (+0.1)	27.6 (+0.1)
seeding	May	17.4 (-27.0)	8.4 (-2.1)	22.1 (-2.1)	23.2 (-21.2)	8.6 (-1.9)	23.9 (-1.9)	20.0 (-24.4)	10.0 (-0.5)	21.9 (-0.5)
early growth	Jun – Aug	240.8 (+123.4)	6.5 (-0.8)	18.6 (-0.8)	153.2 (+35.8)	6.7 (-0.6)	19.7 (-0.6)	74.0 (-43.4)	6.4 (-0.9)	20.0 (-0.9)
pre-anthesis	Sept	15.4 (+2.6)	8.3 (0.0)	21.5 (0.0)	15.8 (+3.0)	6.6 (-1.7)	21.3 (-1.7)	54.6 (+41.8)	9.1 (+0.8)	22.2 (+0.8)
post-anthesis & grain fill	Oct – Nov	125.0 (-10.1)	11.7 (-0.4)	23.5 (-0.4)	256.0 (+120.9)	12.6 (+0.5)	24.5 (+0.5)	88.6 (-46.5)	12.7 (+0.5)	24.4 (+0.5)
Total yearly rainfall		548.8 (+133.8)			550.8 (+135.8)			618.8 (+203.8)		
		2012 Napier			**2013 Napier**			**2014 Napier**		
		Rainfall (mm)			**Rainfall (mm)**			**Rainfall (mm)**		
Pre-seeding	Dec – April	126.9 (-23.7)			137.2 (-13.4)			294.2 (+143.6)		
seeding	May	40.0 (-8.3)			40.0 (-8.3)			22.8 (-25.5)		
early growth	Jun – Aug	234.3 (+77.9)			318.7 (+162.3)			224.2 (+67.8)		
pre-anthesis	Sept	16.0 (-8.8)			69.5 (+44.7)			34.2 (+9.4)		
post-anthesis & grain fill	Oct – Nov	222.5 (+148.0)			235.7 (+161.2)			66.4 (-8.1)		
Total yearly rainfall		639.7 (+185.1)			801.1 (+346.5)			641.8 (+187.2)		
		2012 Klipheuwel			**2013 Klipheuwel**			**2014 Klipheuwel**		
		Rainfall (mm)	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**	**Rainfall (mm)**	**Minimum temperature (°C)**	**Maximum Temperature(°C)**
Pre-seeding	Dec – April	42.0 (-54.6)	16.2 (-0.3)	29.6 (-0.6)	103.8 (+7.2)	16.6 (+0.1)	30.1 (0.0)	126.0 (+29.4)	16.7 (+0.2)	30.1 (0.0)
seeding	May	89.5 (+12.5)	8.3 (-2.1)	20.6 (-0.8)	42.5 (-34.5)	9.7 (-0.7)	22.6 (+1.2)	75.2 (-1.8)	10.6 (+0.2)	21.3 (-0.1)
early growth	Jun – Aug	420.0 (+159.6)	6.6 (-0.6)	17.3 (-1.4)	387.6 (+127.2)	7.2 (+0.1)	18.0 (-0.7)	386.5 (+126.1)	7.5 (+0.3)	18.5 (-0.2)
pre-anthesis	Sept	33.0 (-17.6)	8.5 (-0.9)	19.8 (-1.7)	113.0 (+62.4)	7.5 (-1.9)	17.8 (-3.7)	13.5 (-37.1)	9.3 (-0.1)	22.0 (+0.5)
post-anthesis & grain fill	Oct – Nov	95.0 (+23.2)	13.0 (0.0)	25.4 (-0.4)	102.9 (+31.1)	13.5 (+0.5)	25.6 (-0.2)	38.7 (-33.1)	14.5 (+1.5)	27.6 (+1.8)
Total yearly rainfall		679.50 (+123.1)			749.8 (+193.4)			639.9 (+83.5)		
		2012 Moorreesburg			**2013 Moorreesburg**			**2014 Moorreesburg**		
		Rainfall (mm)			**Rainfall (mm)**			**Rainfall (mm)**		
Pre-seeding	Dec – April	43.0 (-26.0)			73.1 (+4.1)			67.6 (-1.4)		
seeding	May	21.9 (-29.7)			47.7 (-3.9)			32.3 (-19.3)		
early growth	Jun – Aug	218.7 (+16.7)			263.3 (+61.3)			219.0 (+17.0)		
pre-anthesis	Sept	44.5 (+15.5)			74.0 (+45.0)			8.5 (-20.5)		
post-anthesis & grain fill	Oct – Nov	15.4 (-36.0)			40.5 (-10.9)			23.5 (-27.9)		
Total yearly rainfall		343.5 (-59.5)			498.6 (+95.6)			350.9 (-52.1)		

Values in brackets indicate the deviation of the year's value from the long-term mean (2002 – 2011) (South African Weather Service). No temperature data was available for Napier and Moorreesburg 2012 – 2014.

Table A4. Single kernel characterisation system hardness index data for all cultivars and locations in winter rainfall dryland (WRD) region 2012 – 2014

		2012				2013				2014				Average per cultivar over 3 years
Cultivar	**Replication**	**Napier**	**Riversdal**	**Klipheuwel**	**Morreesburg**	**Napier**	**Riversdal**	**Klipheuwel**	**Morreesburg**	**Napier**	**Riversdal**	**Klipheuwel**	**Morreesburg**	
Kariega	1	76.4	60.2	61.0	64.0	43.1	42.0	58.5	55.1	44.2	63.8	57.3	62.6	57.4
	2	80.5	59.1	64.9	55.9	43.8	42.2	59.0	55.1	39.9	63.0	65.3	62.5	
	3	81.9	61.6	61.2	62.9	49.4	40.5	54.8	54.0	44.4	64.4	49.1	61.7	
PAN3434	1	77.1	55.6	63.6	62.6	45.6	42.0	56.9	57.0	54.4	69.4	64.0	63.0	59.2
	2	75.2	58.6	62.6	65.9	46.5	38.1	56.6	54.9	51.9	70.1	71.9	62.8	
	3	77.0	56.2	62.7	59.4	47.9	51.5	57.5	55.6	50.9	71.1	47.9	66.4	
Ratel	1	76.0	62.9	59.9	55.9	40.4	37.9	53.5	49.2	38.9	60.6	50.2	56.8	52.9
	2	77.2	53.3	58.2	57.6	41.2	38.3	53.6	49.2	39.1	60.4	55.0	57.1	
	3	78.6	53.5	57.7	56.1	44.7	39.8	54.1	52.3	38.6	56.6	33.3	56.8	
Baviaans	1	76.6	53.1	65.6	58.9	48.8	43.2	56.4	55.5	52.6	66.6	34.0	66.7	56.7
	2	76.6	53.0	64.3	58.6	48.9	41.5	59.7	58.1	52.8	68.2	42.1	67.4	
	3	72.6	57.9	60.8	60.8	48.8	42.7	59.1	56.9	53.6	67.6	51.7	38.2	
SST015	1	71.3	47.1	60.6	61.6	45.4	42.6	55.2	50.4	48.5	66.2	68.1	64.7	55.5
	2	77.5	47.3	60.0	55.2	43.2	44.2	53.9	53.2	44.9	65.8	40.1	61.5	
	3	82.4	45.8	61.4	60.7	42.4	44.2	52.5	53.8	42.2	64.5	57.5	61.1	
SST096	1	73.9	61.1	72.2	66.7	58.9	52.6	64.2	63.1	52.7	70.5	57.3	68.3	64.3
	2	74.1	68.5	66.6	68.0	61.7	54.0	63.2	61.5	53.1	74.2	64.1	69.5	
	3	81.0	57.1	70.3	72.8	59.2	55.1	65.9	64.5	48.9	69.3	64.6	65.3	
SST056	1	76.9	62.4	60.4	62.1	48.3	48.8	55.1	54.0	47.9	65.1	56.4	60.6	58.4
	2	74.1	51.0	64.7	61.7	47.1	49.7	57.1	58.1	51.0	68.6	61.1	66.9	
	3	72.5	42.9	64.2	64.2	47.4	50.0	55.0	57.0	47.3	70.0	59.1	64.9	
SST087	1	58.5	52.1	66.6	67.6	48.9	66.6	66.1	66.3	61.9	75.6	57.5	70.1	65.0
	2	72.5	52.1	66.7	70.5	61.0	61.6	67.5	63.4	59.7	74.5	62.1	68.9	
	3	75.1	46.0	66.9	75.6	61.8	63.9	64.8	76.7	60.3	77.3	64.5	70.0	
SST88	1	83.8	47.2	76.9	72.9	71.3	62.1	73.2	67.1	54.8	78.6	59.3	74.4	69.4
	2	82.9	65.8	78.1	73.9	67.5	61.3	71.3	71.5	55.5	81.7	63.0	72.3	
	3	67.9	63.6	77.1	76.1	68.2	61.1	72.3	67.8	50.9	81.4	72.2	72.7	

Table A5. Single kernel characterisation system hardness index data for all cultivars and locations in summer rainfall irrigation (SRI) region 2012 – 2014

		2012				2013				2014				Average per cultivar over 3 years
Cultivar	Replication	Hartsvallei	Lichtenburg	Marblehall	Winterton	Hartsvallei	Lichtenburg	Marblehall	Winterton	Hartsvallei	Lichtenburg	Marblehall	Winterton	
Baviaans	1	62.4	42.7	62.3	70.7	66.2	41.5	39.5	51.5	52.7	45.0	44.6	.	52.6
	2	57.0	36.2	63.5	65.1	63.7	41.1	38.1	48.7	48.1	44.0	49.6	.	
	3	64.4	38.8	63.6	76.8	65.3	.	39.2	51.9	47.5	53.0	47.8	.	
Buffels	1	55.7	41.1	64.7	65.4	67.5	49.7	35.2	53.3	50.3	36.8	47.5	.	52.0
	2	55.6	43.7	63.1	69.7	63.8	41.8	37.6	53.2	45.8	49.8	46.9	.	
	3	51.5	38.0	66.9	64.8	69.6	.	35.9	49.7	51.5	54.8	44.9	.	
Duzi	1	53.2	37.5	59.2	56.7	55.5	38.3	33.9	49.6	39.6	51.5	40.8	.	46.8
	2	53.3	38.0	57.0	58.4	54.5	40.7	35.3	47.8	40.4	44.1	41.2	.	
	3	54.1	36.8	54.4	57.9	54.5	.	37.1	44.6	41.1	41.6	48.3	.	
Olifants	1	70.0	46.7	73.7	75.7	71.0	54.2	46.6	62.6	65.4	50.2	57.2	.	60.6
	2	69.2	42.1	71.5	67.4	66.2	54.4	45.2	56.9	59.4	55.4	49.8	.	
	3	70.9	48.8	73.2	72.4	76.7	.	47.8	55.2	63.5	62.5	58.0	.	
PAN3471	1	60.5	38.0	68.5	66.8	61.2	37.7	39.2	52.3	56.5	58.5	50.6	.	55.1
	2	56.8	43.5	69.4	62.9	62.3	50.4	47.7	54.6	57.1	58.4	50.6	.	
	3	63.2	43.2	70.6	65.3	60.1	.	45.1	53.6	58.2	54.3	46.6	.	
PAN3478	1	61.9	49.3	76.5	69.8	69.0	52.9	50.2	61.8	59.3	42.4	50.5	.	59.3
	2	65.7	43.6	73.8	69.1	63.3	53.7	51.0	55.8	61.3	56.1	54.8	.	
	3	69.6	46.6	70.3	68.7	72.1	.	50.2	55.6	59.2	56.7	56.6	.	
SST806	1	62.7	44.2	68.5	72.4	66.4	52.9	51.7	55.4	53.4	51.5	54.2	.	57.2
	2	59.3	49.2	71.8	57.9	68.8	54.2	47.8	56.6	52.8	57.8	54.3	.	
	3	63.8	45.9	72.8	61.6	63.2	.	44.6	55.3	56.7	54.7	47.9	.	
SST835	1	57.8	46.8	72.6	62.5	63.9	51.8	45.2	59.4	53.6	54.0	47.4	.	56.7
	2	60.3	44.5	70.4	65.3	63.0	53.6	47.4	55.5	55.7	59.0	46.8	.	
	3	61.2	45.6	71.2	58.8	68.0	.	50.9	54.2	57.3	58.5	52.4	.	
SST875	1	66.0	46.2	75.1	73.1	68.8	56.4	46.2	59.1	57.8	50.3	47.5	.	59.1
	2	59.6	40.4	72.7	69.1	66.9	56.5	50.1	59.4	57.0	61.2	53.7	.	
	3	63.1	49.2	69.6	68.1	71.4	.	48.6	57.7	57.6	58.7	52.7	.	

Table A6. Single kernel characterisation system hardness index data for all cultivars and locations in summer rainfall dryland (SRD) region 2012 – 2014

		2012			2013				2014				Average per cultivar over 3 years
Cultivar	**Replication**	**Bethlehem**	**Bultfontein**	**Clocolan**	**Bethlehem**	**Bultfontein**	**Reitz**	**Ladybrand**	**Bethlehem**	**Wesselsbron**	**Reitz**	**Ladybrand**	
Elands	1	49.8	50.4	64.2	59.0	70.1	62.8	65.2	60.4	55.0	53.3	57.8	58.4
	2	48.9	54.6	66.6	58.2	68.2	62.4	62.8	59.5	53.6	53.3	53.4	
	3	51.6	52.9	63.0	65.1	69.4	57.8	61.3	58.9	54.9	51.4	52.4	
PAN 3161	1	44.5	50.2	68.9	59.9	58.1	72.1	56.3	59.3	51.0	49.2	52.5	56.0
	2	41.9	52.1	59.0	57.4	58.9	66.5	57.2	57.8	54.4	49.0	51.8	
	3	44.4	53.1	61.1	62.5	62.3	65.8	62.5	55.5	54.3	50.2	48.9	
PAN 3144	1	42.8	52.2	54.2	53.3	59.6	55.8	63.3	54.5	48.6	51.0	52.2	54.4
	2	45.3	58.8	53.0	52.5	55.1	62.6	63.6	55.3	47.7	48.6	51.8	
	3	45.6	54.5	75.5	50.7	63.5	58.8	63.2	53.5	50.5	48.8	50.6	
Gariep	1	46.7	58.0	65.7	62.4	65.6	61.9	62.3	52.5	55.7	51.9	49.5	58.0
	2	48.4	49.4	61.3	56.5	63.7	67.5	65.3	52.9	55.7	52.4	53.1	
	3	52.1	52.7	66.2	58.2	66.2	75.8	65.9	53.5	57.9	55.1	52.5	
SST 398	1	47.7	45.6	66.1	65.9	66.1	68.9	59.8	64.9	56.6	62.5	59.5	59.5
	2	42.8	50.5	70.8	66.8	67.1	68.6	58.9	61.3	59.6	60.6	53.0	
	3	46.0	49.5	64.8	61.7	65.0	57.3	60.9	61.0	58.0	59.1	55.0	
PAN 3355	1	51.1	50.4	54.5	63.7	67.7	72.1	58.9	63.3	52.9	55.6	59.8	58.5
	2	48.4	42.7	63.8	62.1	66.3	72.3	60.0	64.9	55.2	55.6	59.8	
	3	49.5	47.7	55.7	60.8	59.2	63.4	64.6	61.1	57.7	53.7	54.5	
SST 347	1	49.6	55.9	72.3	68.2	58.9	58.5	64.3	51.1	51.0	60.1	62.2	59.1
	2	43.0	56.8	66.5	67.3	64.6	67.1	63.4	51.5	53.1	61.1	58.58	
	3	51.8	55.6	66.2	63.2	64.5	52.1	67.4	53.7	54.6	59.2	58.36	
SST 356	1	54.2	54.6	64.7	61.3	68.5	69.9	74.7	62.7	53.9	57.2	65.09	62.1
	2	55.1	42.9	56.9	61.8	67.5	66.6	75.1	61.2	57.9	59.1	60.05	
	3	53.9	58.9	66.0	67.7	66.6	70.7	74.2	62.2	56.3	59.0	62.39	
PAN 3379	1	59.4	44.4	66.8	69.9	73.6	62.3	64.5	69.1	62.8	64.9	68.25	64.3
	2	53.3	46.5	75.9	67.9	76.5	67.7	60.6	70.0	62.3	67.2	65.01	
	3	59.6	44.8	69.8	66.6	73.5	66.8	61.0	69.5	60.1	67.8	62.33	

Table A7. Detailed sequence data for *Puroindoline a* and *b* allele determination of twenty-seven wheat cultivars

		PINA (GenBank reference sequence AB262660)			PINB (GenBank reference sequence DQ363911)								
Cultivar	Replicate	Gel fragment size	Coding sequence wild type	Pina allele	Gel fragment size	Base pair position	Amino Acid Position from N-terminal end	Base pair position from ATG transcription start	Amino Acid Position	Nucleotide change from wildtype Pinb-D1a	Amino acid produced	Pinb Allele	Homozygous/ Heterozygous status of Pinb allele
Kariega	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Kariega	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Kariega	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3434	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3434	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3434	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Ratel	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Ratel	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Ratel	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Baviaans	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Baviaans	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Baviaans	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST015	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST015	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST015	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST096	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST096	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST096	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST056	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST056	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST056	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST087	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST087	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST087	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST88	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST88	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST88	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A

Table A7. Detailed sequence data for *Puroindoline a* and *b* allele determination of twenty-seven wheat cultivars (continued)

		PINA (GenBank reference sequence AB262660)			PINB (GenBank reference sequence DQ363911)								
Cultivar	Replicate	Gel fragment size	Coding sequence wild type	Pina allele	Gel fragment size	Base pair position	Amino Acid Position from N-terminal end	Base pair position from ATG transcription start	Amino Acid Position	Nucleotide change from wildtype Pinb-D1a	Amino acid produced	Pinb Allele	Homozygous/ Heterozygous status of Pinb allele
SST806	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST806	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST806	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
Duzi	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Duzi	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Duzi	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Baviaans	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Baviaans	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Baviaans	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Buffels	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Buffels	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Buffels	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3471	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3471	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3471	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST835	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST835	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST835	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
Olifants	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
Olifants	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
Olifants	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3478	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3478	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3478	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G

Table A7. Detailed sequence data for *Puroindoline a* and *b* allele determination of twenty-seven wheat cultivars (continued)

		PINA (GenBank reference sequence AB262660)			PINB (GenBank reference sequence DQ363911)								
Cultivar	Replicate	Gel fragment size	Coding sequence wild type	Pina allele	Gel fragment size	Base pair position	Amino Acid Position from N-terminal end	Base pair position from ATG transcription start	Amino Acid Position	Nucleotide change from wildtype Pinb-D1a	Amino acid produced	Pinb Allele	Homozygous/ Heterozygous status of Pinb allele
SST875	1	524	confirmed	Pina-D1a	597	MIXED	MIXED	MIXED	MIXED	MIXED	MIXED	HET	A/G
SST875	2	524	confirmed	Pina-D1a	597	MIXED	MIXED	MIXED	MIXED	MIXED	MIXED	Pinb-D1b	A/A
SST875	3	null		Pina-D1b	597	MIXED	MIXED	MIXED	MIXED	MIXED	MIXED	Pinb-D1a	G/G
Elands	1	524	confirmed	Pina-D1a	597	126	42	213	71	delA	AAA (Lysine)>AAdelA frameshift resulting in stop codon at position 60 from N-terminal end	Pinb-D1p	delA/delA
Elands	2	524	confirmed	Pina-D1a	597	126	42	213	71	delA		Pinb-D1p	delA/delA
Elands	3	524	confirmed	Pina-D1a	597	126	42	213	71	delA		Pinb-D1p	delA/delA
PAN3161	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3161	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3161	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3144	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3144	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3144	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Gariep	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Gariep	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
Gariep	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST398	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST398	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST398	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
PAN3355	1	524	confirmed	Pina-D1a	597	295	99	382	128	C>T	CAG (Glutamine)>TAG stopcodon	Pinb-D1ab	C/C
PAN3355	2	524	confirmed	Pina-D1a	597	295	99	382	128	C>T		Pinb-D1ab	C/C
PAN3355	3	524	confirmed	Pina-D1a	597	295	99	382	128	C>T		Pinb-D1ab	C/C
SST347	1	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST347	2	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A
SST347	3	524	confirmed	Pina-D1a	597	136	46	223	75	G>A	Serine	Pinb-D1b	A/A

Table A7. Detailed sequence data for *Puroindoline a* and *b* allele determination of twenty-seven wheat cultivars (continued)

		PINA (GenBank reference sequence AB262660)			PINB (GenBank reference sequence DQ363911)								
Cultivar	Replicate	Gel fragment size	Coding sequence wild type	Pina allele	Gel fragment size	Base pair position	Amino Acid Position from N-terminal end	Base pair position from ATG transcript on start	Amino Acid Position	Nucleotide change from wildtype Pinb-D1a	Amino acid produced	Pinb Allele	Homozygous/ Heterozygous status of Pinb allele
SST356	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST356	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
SST356	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3379	1	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3379	2	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G
PAN3379	3	null		Pina-D1b	597	136	46	223	75	Wild-type	Glycine	Pinb-D1a	G/G

Table A8. Means, standard deviation and least significant difference of weighted analysis on SKSC-HI over three production regions

	Observations	Sum of weights	SKCS-HI Mean ± SD Least significant difference = 0.55
Summer rainfall irrigation	288	31.99	55.43 ^{C} ± 3.43
Summer rainfall dryland	297	30.65	58.83 ^{B} ± 2.38
Winter rainfall dryland	324	18.87	59.46 ^{A} ± 2.70

SKCS – single kernel characterisation system; SD – standard deviation.

Table A9. Means and standard deviation for kernel characteristics of Baviaans in the summer rainfall irrigation (SRI) and winter rainfall dryland (WRD) regions 2012 - 2014

		SKCS-HI	SKCS-Moisture (%)	SKCS-Weight (mg)	SKCS-Diameter (mm)
	Observations	**Mean ± Standard deviation**	**Mean ± Standard deviation**	**Mean ± Standard deviation**	**Mean ± Standard deviation**
Summer rainfall irrigation	32	52.58 [B] ± 10.7	12.03 ± 1.10	40.50 ± 5.08	2.59 ± 0.23
Winter rainfall dryland	36	56.66 [A] ± 10.2	12.66 ± 7.94	42.38 ± 5.12	2.66 ± 0.22

SKCS – single kernel characterisation system; Least significant difference for SKCS-HI = 2.28; Means followed by the same letter, did not differ significantly at $P < 0.05$.

Table A10. Means, standard deviation and range of values for kernel characteristics in the SRI, SRD and WRD regions (2012 – 2014)

		SKCS-HI		SKCS-Moisture (%)		SKCS-Weight (mg)		SKCS-Diameter (mm)	
	Observations	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
Summer rainfall irrigation region	288	55.49 ± 10.09	33.90 - 76.80	11.98 ± 1.04	8.90 - 14.10	57.93 ± 5.86	23.80 - 51.60	2.48 ± 0.26	1.84 - 3.10
Summer rainfall dryland region	297	58.92 ± 7.39	41.89 - 76.48	12.44 ± 0.78	10.91 - 15.30	36.44 ± 4.68	24.72 - 47.90	2.50 ± 0.22	1.97 - 3.08
Winter rainfall dryland	324	59.86 ± 10.59	33.29 - 83.8	12.58 ± 0.81	10.26 - 14.00	43.51 ± 5.27	31.53 - 55.94	2.67 ± 0.21	2.16 - 3.16

SKCS – single kernel characterisation system; SD – standard deviation.