THE ROLE OF PUROINDOLINE A AND B UPON GRAIN ENDOSPERM TEXTURE, END-USE QUALITY TRAITS AND PHYSICOCHEMICAL ASSOCIATION TO STARCH IN WHEAT

by

Humphrey Wainaina Wanjugi

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Plant Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

March 2007

©COPYRIGHT

by

Humphrey Wainaina Wanjugi

2007

All Rights Reserved

APPROVAL

of a dissertation submitted by

Humphrey Wainaina Wanjugi

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

Dr. Michael J. Giroux

Dr. John M. Martin

Approved for the Department of Plant Sciences and Plant Pathology

Dr. John E. Sherwood

Approved for the Division of Graduate Education

Dr Carl A. Fox

STATEMENT OF PERMISSION TO USE

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the library. I further agree that copying of this dissertation is allowed for scholarship purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this dissertation should be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part"

Humphrey Wainaina Wanjugi

March 2007

ACKNOWLEDGEMENTS

I am sincerely indebted to my major professors, Dr. Michael. J.Giroux and Dr. John.M. Martin for their enthusiastic supervision. Without their mentorship, inspiration and guidance, this dissertation would likely not have matured.

Special thanks to my committee members Dr. P. Bruckner, Dr. L. Talbert and Dr. N. Weeden for their endless support and input towards my doctoral program.

I would also like to thanks all the individuals at the Plant Sciences and Plant Pathology Department for providing a stimulating and fun environment to work that made my stay an unforgettable experience.

I am also thankful to all my friends who have inspired and given me emotional support. Particularly, I would like to express my sincere love and gratitude to Jim and Norma Halderson of American Falls, Idaho, for adopting me as their own. I am forever thankful.

Finally, thanks be to God (the author of knowledge and wisdom), parents Peter and Margaret Wanjugi, siblings and family for their love, prayers, and believing in my ability to succeed.

TABLES OF CONTENTS

1. THE ROLE OF PUROINDOLINE A AND B INDIVIDUALLY AND IN COMBINATION ON GRAIN HARDNESS AND STARCH ASSOCIATION.... 1

Introduction.....1
Materials and Methods.....4
Genetic Materials.....4
Herbicide Screening.....5
Field Trials.....6
Grain Hardness and Grain Protein Measurement 7
DNA Isolation and *Ha* Locus PCR Analysis.....7
TX-114 Protein Extraction and Analysis.....8
Friabilin Protein Extraction and Analysis.....8
Statistical Analysis.....10
Results.....10
Screening the *Ha* Locus.....10
Grain Analysis among *Pinb-D1e* and *Pinb-D1b* Genotypic Classes With added *Pina-D1a* or *Pina-D1b* 14
Grain Analysis Data among *Pina-D1b* and *Pinb-D1b* Genotypic Classes with Added *Pina-D1a* or *Pina-D1b* 15
Grain Analysis in Absence of Added *Pina* and *Pinb* Transgene.....19
TX-114 Extractable Puroindoline Levels 20
Starch Associated Puroindoline Levels 21
Discussion.....25
Conclusions.....28
Reference Cited.....30

2. THE INFLUENCE OF PUROINDOLINE A AND B INDIVIDUALLY AND IN COMBINATION UPON WHEAT MILLING AND BREAD BAKING TRAITS.....34

Introduction.....34
Material and Methods 37
Genetic Materials.....37
Milling and Baking Trait Analysis 40
Statistical Analysis.....40
Results.....41
Milling Trait Analysis.....43
Bread Baking Trait Quality Analysis.....47

TABLE OF CONTENTS – CONTINUED

Correlation Analysis between Milling and Baking Quality Trait....48
Discussion....53
Conclusions....56
Reference Cited....57

3. PUROINDOLINE CO-LOCALIZE TO THE STARCH GRANULE SURFACE AND INCREASE STARCH LIPID CONTENT....61
....
Introduction....61
Material and Methods....65
Genetic Materials....65
Field Planting and Seed Traits....68
Extraction of Total and Starch Bound Puroindolines....68
Immunofluorescent Localization....69
Extraction, Chromatography and Quantification of Lipid Fractions....70
Results....72
Grain Hardness and Protein Analysis....72
Total TX-114 and Starch Bound Protein Expression Levels....73
Immunofluorescent Localization of PINA and PINB....74
Polar Lipid Extractable from Starch or Polar Lipid Content....77
Discussion....81
Conclusions....85
Reference Cited....86

LIST OF TABLES

Table Page

1.1 Mean Grain Hardness, Kernel Weight and Protein Content Average for Parental Genotypes 12

1.2 Grain Hardness, Kernel Weight and Protein Content 13

1.3 Comparisons Among Genotype Mean Class for Grain Hardness Kernel Weight and Protein Content 18

1.4 Genotypic Mean Class Comparisons for Grain Hardness and Kernel Weight 20

1.5 Puroindoline Levels Quantified from TX-114 and Friabilin Extracts 22

2.1 Grain Hardness and Grain Protein Content 42

2.2 Flour Milling Traits Data 46

2.3 Bread Baking Quality Traits Data 49

2.4 Correlation Analysis for Milling and Baking Traits among *Pinb* nulls 51

2.5 Correlation Analysis for Milling and Baking Traits among *Pina* nulls 52

3.1 Grain Hardness and Grain Protein Content 72

3.2 Puroindoline Levels Quantified from TX-114 and Starch Extracts 74

3.3 Composition of Bound Polar Lipids from Whole Wheat Flour 78

3.4 Composition of Bound Polar Lipids from Water Washed Starch 79

LIST OF FIGURES

Figure Page

1.1 Cleaved Amplified Marker Test (CAPS)....12

1.2 Scatter Plot for Grain Hardness among *Pinb* nulls....16

1.3 Scatter Plot for Grain Hardness among *Pina* nulls....17

1.4 SDS-PAGE Gel of Total TX-114 Extractable Puroindolines....23

1.5 SDS_PAGE Gel of Starch-Associated Puroindolines....24

2.1 Scatter Plot for Grain Hardness versus Particle Size Distribution Among *Pinb* nulls....44

2.2 Scatter Plot for Grain Hardness versus Particle Size Distribution Among *Pina* nulls....45

3.1 Fluorescent Micrographs upon Addition of PINs to PINB null....75

3.2 Fluorescent Micrographs upon Addition of PINs to PINA null....76

ABSTRACT

Endosperm texture in wheat (*Triticum aestivum* L.) is controlled by the *Pina* and *Pinb* genes that together comprise the *Hardness* (*Ha*) locus, and is an important criterion affecting end-product quality. The *Ha* encodes friabilin, a 15 kD protein primarily composed of Puroindoline A (PINA) and Puroindoline B (PINB). Both PINA and PINB interact with starch leading to a soft endosperm, but the physicochemical mechanism controlling endosperm texture is not clearly understood. Here, I isolated the role of PINA and PINB individually and in combination upon grain hardness, milling and bread baking traits. I also determined the effect of overexpressing PINA or PINB on starch bound polar lipids and PIN cellular localization. F_3 recombinants homozygous for either the *Pina* or *Pinb* null *Ha* locus with or lacking a transgenically added *Pina* or *Pinb* were analyzed for grain hardness, PIN abundance, milling and baking traits, and levels of starch and flour polar lipids. Addition of *Pina* to the *Pinb* null *Ha* locus or *Pinb* to the *Pina* null *Ha* locus resulted in intermediate texture. Genotypes with added *Pina* to *Pina* null *Ha* locus and *Pinb* to *Pinb* null *Ha* locus were soft textured and had low flour yield, ash, small flour particles sizes, and increased PIN and polar lipid levels on starch. PINs localized to the starch granule surface in the presence or absence of the other protein but addition of *Pinb* to *Pina* null *Ha* locus resulted in more PINB associated to starch, increased affinity to bound polar lipids and greater negative effects on baking traits than addition of PINA. Results indicate that overexpression of PINs leads to reduced grain hardness, modified flour milling properties, negatively effects baking traits, and increased starch bound polar lipids. Further, PINA or PINB can act and localize to starch independently of the other protein leading to intermediate texture but soft texture requires both PINA and PINB but, PINB associates more with polar lipids on starch surface and negatively affect baking traits than PINA. My study shows that polar lipids mediate puroindolines association to starch and this mediation can influence endosperm texture, milling, and baking traits.

CHAPTER ONE

THE ROLE OF PUROINDOLINE A AND B INDIVIDUALLY AND IN COMBINATION UPON GRAIN HARDNESS AND STARCH ASSOCIATION

Introduction

Wheat (*Triticum aestivum* L.) is classified and traded as either hard or soft based on endosperm texture. Grain hardness in wheat is one of the most important characteristics affecting milling, baking, and end-use qualities. Hard textured wheat yields larger flour particles that have more starch damage and absorb more water than soft textured wheat flour (Symes 1965; Symes 1969). Soft textured wheats are used for cakes, cookies and pastries whereas hard wheats are generally used to make bread (Reviewed by Morris and Rose, 1996).

Wheat grain hardness is a simply inherited trait controlled by the *Hardness* locus (*Ha*) (Symes 1965; Baker 1977) and perhaps one or more minor genes (Anjum and Walker, 1991). The major gene (*Ha)* has been mapped to the short arm of chromosome 5D (5DS) (Mattern et al., 1973; Law et al., 1978). Soft wheats possess the dominant or wild type form (*Ha*) while hard wheats have the recessive or mutated form (*ha*) (Symes 1965; Baker 1977; Law et al., 1978). A hard vs. soft wheat marker protein termed friabilin was reported by Greenwell and Schofield (1986). Friabilin is a 15 kDa protein complex abundantly present on the surface of water washed starch from soft wheats and

scarce on hard wheat starch (Greenwell and Schofield, 1986). The physical interaction of friabilin with wheat starch is poorly understood, however, it has been suggested that friabilin regulates the degree of adhesion between starch granules and the protein matrix (Pomeranz and Williams 1990; Beecher et al., 2002; Hogg et al., 2004). N-terminal sequencing has shown that friabilin is composed primarily of two proteins, puroindoline a (PINA) and puroindoline b (PINB) (Blochet et al., 1993; Gautier et al., 1994; Rahman et al., 1994). The tightly linked genes puroindoline a (*Pina*) and puroindoline b (*Pinb*) code for the PINA and PINB proteins respectively, which together function as the *Ha* locus (Sourdille et al., 1996; Giroux and Morris, 1997, 1998). The puroindoline (PIN) active site may relate to their unique tryptophan-rich hydrophobic domain that is thought to be involved in the binding of lipids (Gautier et al., 1994; Marion et al., 1994). Molecular studies have revealed that variation in the coding sequence of *Pina* or *Pinb* is associated with the hard grain phenotype (Giroux and Morris, 1997, 1998). All soft wheats surveyed to date possess the *Pina-D1a* and *Pinb-D1a* alleles (Giroux and Morris, 1998; Lillemo and Morris, 2000; Morris et al., 2001). To date, numerous *Pin* gene mutations have been characterized among hard wheats, with a null mutation in *Pina* (*Pina-D1b*) or a point mutation in *Pinb* (*Pinb-D1b*) being the two most common *Ha* mutations (Giroux and Morris, 1997, 1998; Morris et al., 2001; Pan et al., 2004; reviewed in Morris, 2002)).

To validate the cause-effect relationship between puroindolines and grain hardness, Krishnamurthy and Giroux (2001) transformed rice, (*Oryza sativa* L.) which lacks *Pin* homologues, with the soft type *Pina-D1a* and *Pinb-D1a* coding sequences under control of the maize ubiquitin promoter. A decrease in rice kernel hardness was observed as well as reduced particle size and starch damage in whole seed meals after

milling. In addition, Beecher et al. (2002) demonstrated that expression of *Pina-D1a* in the hard wheat variety 'Hi-Line' complemented the native *Pinb-D1b* mutation present in Hi-Line and restored a soft grain phenotype. In a recent study, Martin et al. (2006) demonstrated that complementation of the *Pina-D1b* allele in hard wheats with the wild type *Pina-D1a* sequence also restored a soft phenotype. Further studies of the transgenic lines created in Hi-Line have shown that soft endosperm texture correlates with friabilin abundance and the presence of both PINA and PINB (Hogg et al., 2004). Hogg et al. (2004) however, did not demonstrate the individual role of PINA and PINB on grain hardness or starch binding because all genotypes studied contained a functional PINA. Further, the presence of *PINB-D1B* in Hi-Line with over-expressed PINA or PINB could have confounded the results of Hogg et al. (2004) preventing identifying the individual role of PINA or PINB on grain hardness and association to starch. In a study by Swan et al. (2006) a subset of the transgenic isolines created by Hogg et al. (2004) were crossed to the soft wheat Heron. They demonstrated that both PINs limit grain softness in soft wheat but PINB is more limiting to PINA starch association and grain softness in soft wheats than is PINA. This study however, was limited by the fact that genotypes with added PINA or PINB were in the soft wheat 'Heron' background and hence could not isolate the individual role of *Pina* or *Pinb* in grain hardness and association to starch.

To overcome the limitations inherent in the studies of Hogg et al. (2004) and Swan et al. (2006), a subset of the *Pin* over expressing transgenic lines of Hogg et al. (2004) were crossed with PINA or PINB null genotypes. This allowed us to determine the action of PINA and PINB on wheat grain hardness in the presence or absence of the other protein. To accomplish this, transgenic over expressing *Pina-D1a* or *Pinb-D1a*

lines created in Hi-Line were crossed to hard wheats that were either PINA or PINB null. The progeny segregated for the presence or absence of the transgene and the *Ha* locus. We identified random progeny from the four homozygous classes per cross which were then evaluated for grain hardness, kernel weight and protein content over two environments.

Materials and Methods

Genetic Materials

Two spring wheat cultivars, 'Canadian Red'(CR) (hard white) (Clark et al., 1926) and 'McNeal'(McN) (hard red) (Lanning et al.,1995) were crossed to a subset of the transgenic lines described in Hogg et al. (2004), which were created using the hard red spring wheat cultivar Hi-Line (Lanning et al., 1992). Four transgenic parents were used. Two were transgenic isolines created using the *Pina-D1a* coding sequence (HGA3, HGA1) while the other two were created using the *Pinb-D1a* coding sequence (HGB5, HGB12). Hi-Line has the soft type *Pina-D1a* and the mutant *Pinb-D1b* allele which contains a single point mutation in *Pinb* resulting in a glycine to serine substitution at the 46th residue of the peptide (Giroux and Morris 1997; Beecher et al., 2002). Canadian Red (CR) has the soft type *Pina-D1a* allele and a null *Pinb-D1e* allele (Morris et al., 2001). *Pinb-D1e* contains a single point mutation (TGG to a TGA) leading to a change in residue Trp-39 to a stop codon. McNeal (McN) carries a mutant *Pina* allele (*Pina-D1b*) which is an apparent deletion of the *Pina* coding sequence and the wild type *Pinb-D1a* allele (Giroux and Morris 1998).

The expression of the *Pina* and *Pinb* transgenes is under control of the *Glu1Dy10* seed specific high-molecular-weight glutenin promoter (Blechl and Anderson 1996). These transgenic plants also express the *Bar* gene (De Block et al., 1987) that confers resistance to bialaphos (Meiji Seika Kaisha Ltd, Japan) and glufosinate ammonium (AgrEvo, Wilmington, Del.). In each transgenic line used, the *Pin* transgene co-segregated with *Bar* (Hogg et al., 2004). Transgenic lines in this study were selected based on total PIN expression levels. HGB12 and HGA3 had slightly higher total PIN expression levels than HGB5 and HGA1, respectively (Hogg et al., 2004).

Four hundred single F_2 plants from each of the four Canadian Red (CR) crosses (CR/HGA3, CR/HGA1, CR/ HGB5, CR/HGB12) and the three McNeal (McN) crosses (McN/HGA3, McN/HGB5 and McN/HGB12) were planted in the greenhouse at Montana State University-Bozeman Plant Growth Center. These plants were segregating for the native *Ha* locus from Canadian Red, McNeal or Hi-Line, and for the added transgene (*Pina* or *Pinb*). Single heads (F_3 seeds) from F_2 plants were harvested and screened for herbicide resistance. Twelve F_2 derived F_3 seeds from each head in the crosses were tested in the greenhouse for the transgene as described below.

Herbicide Screening

To identify F_2 derived F_3 seed pools homozygous for the presence or absence of the transgene, eighteen F_2 derived F_3 seeds per line were planted in the greenhouse. Plants were sprayed with 0.1 % glufosinate ammonium (AgrEvo, Wilmington, Del.) at the two leaf stage. The plants were scored as being resistant or susceptible after 7 days. Resistant plants stayed green while susceptible plants were killed. The F_2 parent was classified as a

Pin transgene homozygous positive (*Pina* or *Pinb*) or a *Pin* transgene homozygous negative genotype if > 11 consecutive F_3 progeny were glufosinate-resistant or glufosinate-susceptible, respectively. F_3 progeny with mixed herbicide results, most often segregrating 3:1 resistant: susceptible, were considered to have a heterozygous-*Pin* F_2 parent. These lines were excluded from subsequent experiments.

Field Trials

Two seeds of each homozygous F_2 plant were grown at the Arthur H. Post Field Research Farm near Bozeman, MT in the summer of 2004 under rain fed conditions. The plants were then genotyped for the *Ha* locus. F_2 derived F_3 single plants homozygous for the *Ha* locus were harvested as individual plants. The number of plants homozygous for the segregating transgene and the native *Ha* locus within a cross varied from 14 to 42. In the summer of 2005, F_3 derived lines confirmed to be homozygous for the segregating transgene and native *Ha* locus plus parental controls were planted in a randomized block design with two blocks. The entries comprised of 441 F_3 derived F_4 lines and six parental controls. Each plot was a 3 m row seeded with 4 g with row spacing of 30 cm. The same trial was grown with and without irrigation in separate adjacent experiments at the Arthur H. Post Field Research laboratory near Bozeman, MT. Irrigated plots received 7.6 cm of water one week before and one week after anthesis. At maturity, plots were cut with a binder (Mitsubishi Agricultural Machinery Co., Ltd, Tokyo, Japan), threshed with a Vogel bundle thrasher (Bill's Welding, Pullman, WA), cleaned, and weighed. Grain hardness and grain protein were then determined on a sub-sample of seeds from each plot.

Grain Hardness and Grain Protein Measurement

The Single Kernel Characterization System (SKCS 4100, Perten Instruments, Springfield, IL.) was used to determine grain hardness. One hundred seeds from each plot in the 2005 replicated trial and 50 seeds from 2004 single plants were analyzed. Grain protein content was determined on whole grain samples using near-infrared transmission using an Infratec 1225 Grain Analyzer (Foss North America Incorporated, Eden Prairie, MN).

DNA Isolation and *Ha* Locus PCR Analysis

To identify the allelic state of the *Ha* locus, leaf tissues were taken from F_3 progeny lines arising from a single F_2 parent homozygous positive or negative for the transgene. Young leaf tissues were pooled from >10 individual F_3 plants from a single F_2 parent and genomic DNA extracted according to Riede and Anderson (1996). To determine the puroindoline genes resident at *Ha* in F_3 progeny lines, the polymorphisms inherent between the parental *Ha* locus genotypes were exploited (Giroux and Morris, 1997, 1998 and Morris et al., 2001). *Pinb* coding sequence was amplified by PCR from genomic DNA using the primers described by Massa et al. (2004) to generate a 469 bp product containing 447 bp of *Pinb* coding sequence. The temperature regime used consisted of a 4 min. initial denaturation step at 94 ^{o}C, followed by 40 cycles of 94 ^{o}C for 30 sec, 58^{o}C for 30 sec, 72 ^{o}C for 90 sec, and a final extension at 72 ^{o}C for 5 min. To distinguish *Pinb-D1e* from Canadian Red and *Pinb-D1b* from Hi-Line in CR/HG crosses, amplified *Pinb* PCR products were digested with *BstN1* at 60^{o}C for 1 hr and then separated on 2.5 % Metaphor Agarose 1 X TBE gels (Cambrex Bio-Science Inc.

Rockland, ME). *Bst*N1 cuts the *Pinb-D1b* PCR product once yielding 235 and 234 bp fragments while no *BstN1* sites are present in the *Pinb-D1e* PCR product. To differentiate *Pinb-D1a* from McNeal and *Pinb-D1b* from Hi-Line in McN/HG crosses, *Pinb* PCR products were digested with *BsrB1* at 37 ^{o}C for 1hr followed by separation using 2.5 % Metaphor Agarose gels. *Bsr*B1 cuts the *Pinb-D1a* PCR product once yielding 340 and 129 bp products while the *Pinb-D1b* PCR product is cut twice yielding 245, 129, and 95 bp products (Swan et al., 2006).

TX-114 Protein Extraction and Analysis

Fractionation of total puroindoline and separation via SDS-PAGE was done via Triton X-114 phase partitioning as previously described by Giroux et al. (2003). Whole meal UDY ground flour (UDY Co., Fort Collins, CO) from three random lines of each of CR/HGA3, CR/HGB12, McN/HGA3 and McN/HGB12 was used for TX-114 protein extraction. Puroindoline abundance was quantified visually using a scale of 1X to 8X. Heron (PI 290910) (soft white wheat) was used to construct the scale where 1X = 6 µl load (240 µl SDS-PAGE sample buffer/100 mg whole seed meal). Means for each group were computed from three randomly selected individual genotypes per group.

Friabilin Protein Extraction and Analysis

The method for friabilin extraction and analysis used combined the friabilin method of Bettge et al. (1995) with the addition of a $ZnSO_4$ starch purification step (Guraya et al., 2003). 100 mg of UDY milled whole wheat flour was steeped for 30 min in a 2 ml microfuge tube containing 0.5 ml of 0.1 M NaCl. The solution was vortexed and transferred to 2 ml pre-weighed microfuge tubes containing 1 ml of 80 % (w/v) $ZnSO_4$

and centrifuged at 13,000 g for 3 min. The $ZnSO_4$ solution and the supernatant consisting of gluten and bran were then decanted and the starch pellet re-suspended two more times in 80 % (w/v) 1ml $ZnSO_4$ by vortexing, followed by re-centrifugation, and decanting. The starch was then washed with 1 ml water by vortexing followed by a brief centrifugation, and the supernatant removed followed by two more identical water washes. To the tubes containing water washed starch 1 ml acetone was added and the tubes were vortexed, centrifuged briefly, and decanted. The pellet was allowed to completely dry before being weighed to allow equal loading of samples. To the dried starch, 200 µl of 50 % isopropanol, 0.5 M NaCl was added and the samples were vortexed. The samples were then incubated for 1 hr at room temperature. After incubation the samples were centrifuged for 3 min at 13,000 g and the supernatant was transferred to a new 2 ml tube. 520 µl of cold acetone was added to the supernatant, vortexed, and then incubated overnight at -20 ^{o}C. The samples were removed from -20 ^{o}C, centrifuged for 3 min at 13,000 g, and the supernatant aspirated off. The pellet was washed once with 500 µl of acetone and allowed to dry. After the pellet was completely dry, the correct amount of SDS sample buffer (240 µl buffer /100 mg starch) was added. The samples were heated for 10 min at 70 ^{o}C with occasional vortexing and then fractionated on 10-20 % Tris-HCL, 160 x 160 x 1.5 mm , 20 well polyacrylamide gels (Bio-Rad, Hercules, Calif.). Gels were then stained using Coomassie brilliant blue R-250 (Fischer Scientific, Hampton, and N.J). The amount of puroindoline associated with starch was quantified using a Heron (soft wheat) scale ranging from 1x to 5x where 1x = 10 µl. Means for each group were computed from three randomly selected individual genotypes per group.

Statistical Analysis

Four homozygous classes with varying numbers of random lines within each class were identified for each of the seven crosses. The two transgenes (*Pina* and *Pinb*), two null parents (Canadian Red and McNeal), and four homozygous classes within each cross gave 16 genotype classes with two independent events within each transgene except the HGA1 x McNeal cross was missing. Data obtained from F_3-derived F_4 lines were analyzed utilizing a mixed effects analysis of variance model for randomized block combined over environments using PROC MIXED in SAS (SAS Institute Inc, Cary, NC) 2006). Entries variation was partitioned into sources due to genotype classes, transgenic event within genotype classes and progeny lines within genotype class by event combination and their interactions with environment. All effects were considered fixed except blocks within environments, progeny lines within genotype class by event combination, and the interactions with environment were considered random. A similar model was applied to data from spaced F_3 plants. Specific comparisons among genotype class means were made using ESTIMATE statements in SAS.

Results

Screening for *Ha* locus

Seven homozygous classes were created by crossing transgenic isolines in Hi-Line (*Pina-D1a*/*Pinb-D1b*) background that over expressed *Pina-D1a* or *Pinb-D1a* coding sequences with Canadian Red (*Pina-D1a/Pinb-D1e*) and McNeal (*Pina-D1b/Pinb-D1a*) (Table 1.1). The homozygous classes segregated for the altered native *Pin* allele from Hi-Line (*Pinb-D1b*), Canadian Red (*Pinb-D1e*), or McNeal (*Pina-D1b*)

and the presence or absence of the transgene. The native *Pin* genotype of all lines was determined by cleaved amplified polymorphism marker (CAPS) analysis. Figure. 1.1 shows the CAPS marker test illustrating the segregation of *Pinb-D1e* from Canadian Red and *Pinb-D1b* from Hi-Line in Canadian Red crosses.

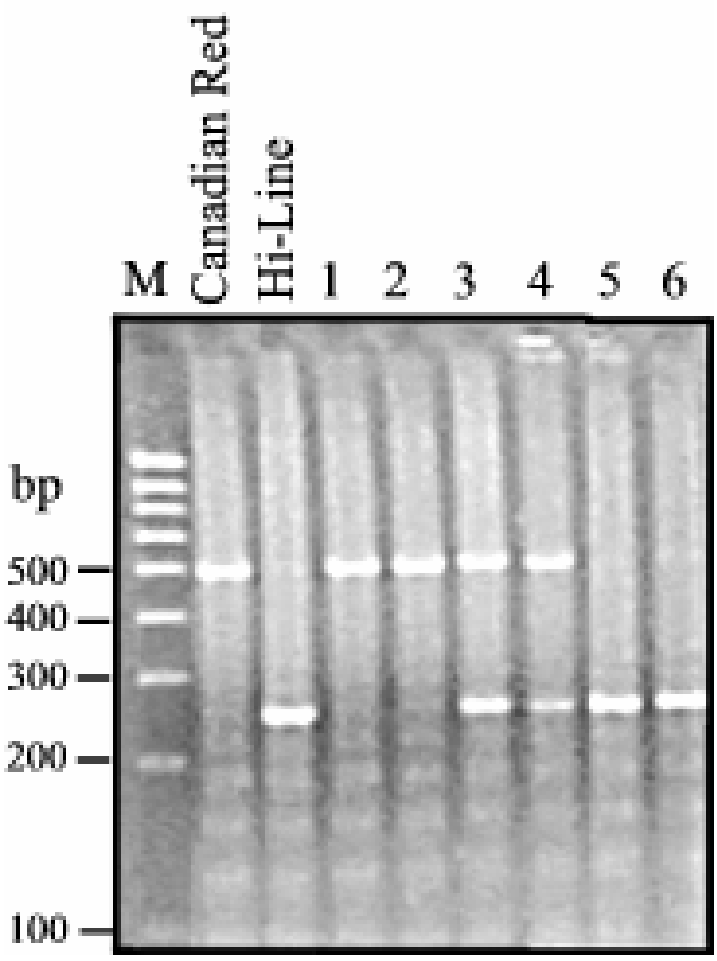


Figure 1.1. Cleaved amplified polymorphism sequence (CAPS) test used to distinguish between the *Pinb-D1e* (Canadian Red) and *Pinb-D1b* (Hi-Line) alleles at *Ha* locus in segregating populations among Canadian Red crosses. *Bst*NI does not digest the *Pinb-D1e* PCR product (469 bp) from Canadian Red. *Pinb-D1b* PCR product from Hi-Line is cut once yielding 234 and 235 bp products. Lanes 1 and 2 represent progeny lines possessing the *Pinb-D1e* allele while lanes 5 and 6 represent progeny lines carrying the *Pinb-D1b* allele. Lanes 3 and 4 are heterozygous for the *Pinb-D1e* and *Pinb-D1b* allele. 'M' denotes a 100 bp ladder marker (Promega, Madison, WI) that goes up to 900 bp.

Table 1.1. Mean grain hardness, kernel weight and grain protein content average for parental genotypes in 2005. Wild type Hi-Line (*Pina-D1a/Pinb-D1b*) is listed for comparative purposes only and was not used in any cross. Standard errors are presented in ( ).

Parental Line[†]	Transgene	*Ha Locus*[‡]	Grain Hardness[§]	Kernel Wt. (mg) [§]	Grain protein g/kg[¶]
Canadian Red	none	*Pina-D1a/Pinb-D1e*	71.6(2.12)	37.0(1.15)	136(0.81)
McNeal	none	*Pina-D1b/Pinb-D1a*	95.1(2.14)	30.8(2.35)	149(3.06)
Hi-Line	none	*Pina-D1a/Pinb-D1b*	78.9(1.63)	28.9(1.05)	152(3.37)
HGA3	Pina	*Pina-D1a/Pinb-D1b*	45.7(1.55)	28.6(0.88)	153(2.55)
HGA1-3[#]	Pina	*Pina-D1a/Pinb-D1b*	59.7(2.55)	28.6(1.67)	154(4.44)
HGB5	Pinb	*Pina-D1a/Pinb-D1b*	23.6(2.28)	28.5(1.14)	159(4.85)
HGB12	Pinb	*Pina-D1a/Pinb-D1b*	20.2(0.56)	29.3(1.43)	156(5.64)

[†] Hi-Line derived transgenic lines contain either *Pina* or *Pinb* transgene. Canadian Red, McNeal and Hi-Line are non-transgenic controls.
[‡] Puroindoline genes present at the *Ha* locus. The *Pina-D1a* and *Pinb-D1a* alleles produce functional PINA and PINB, respectively. The *Pina-D1b* and *Pinb-D1e* alleles are null alleles for PINA and PINB, respectively. The *Pinb-D1b* allele produces a non-functional PINB.
[§] Determined by single kernel characterization system (SKCS). Replicated four times.
[¶] Determined by infratec 1225 grain analyzer. Replicated four times.
[#] Parental line was heterozygous at the transgene locus.

Allele variation between *Pinb-D1a* present in McNeal and *Pinb-D1b* allele present in Hi-Line among McNeal crosses was determined as described by Swan et al. (2006). Based on herbicide screening and PCR analysis, four genotypic groups from each of the seven crosses were identified with 14 to 42 lines per class.

The McNeal parent had harder grain, lower seed weight, and higher grain protein than the Canadian Red parent (Table 1.1). Among the transgenic isoline parents in the Hi-Line background, the lines with added *Pina* had intermediate grain texture and the two lines with added *Pinb* had soft grain. The transgenic isoline parents did not vary for kernel weight or grain protein (Table 1.1).

Table 1.2. Genotype class means for grain hardness, kernel weight, and grain protein content for homozygous F_3 (2004) and F_3-derived F_4 progeny (2005) derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e)* or McNeal ((*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* (HGA) or *Pinb* (HGB). Progeny lines inheriting the Canadian Red locus are denoted as *Pina-D1a/Pinb-D1e*; those inheriting the McNeal locus are denoted as *Pina-D1b/Pinb-D1a* while those inheriting the Hi-Line locus are denoted as *Pina-D1a*/*Pinb-D1b.* Presence or absence of transgene is denoted as + or -, respectively. Standard errors of means are presented in ().

					2004†		2005‡		
Null Parent	Native Pin	Trans-genic Parent	Trans-gene	Number of Lines	Hardness	Kernel Wt. (mg)	Hardness	Kernel Wt. (mg)	Grain Protein (g/kg)
Canadian Red	*Pina-D1a/ Pinb-D1e*	HGA	+	29	40.4 (1.01)	38.7 (0.60)	43.5 (1.67)	35.1 (0.86)	146 (2.07)
			-	28	63.7 (1.03)	39.3 (0.52)	70.1 (1.75)	34.6 (0.82)	148 (1.72)
		HGB	+	26	8.4 (1.08)	39.8 (0.84)	15.9 (1.72)	34.5 (0.86)	150 (2.45)
			-	25	65.44 (1.09)	40.5 (0.58)	71.2 (1.79)	34.8 (0.85)	147 (2.29)
	Pina-D1a/ Pinb-D1b	HGA	+	21	38.7 (1.31)	38.3 (0.91)	42.5 (1.93)	34.8 (0.94)	144 (2.99)
			-	35	68.6 (0.95)	37.9 (0.60)	72.8 (1.62)	35.9 (0.85)	140 (2.33)
		HGB	+	26	8.3 (1.08)	38.3 (0.64)	17.3 (1.70)	33.6 (0.86)	150 (2.46)
			-	42	70.7 (0.84)	37.7 (0.53)	73.6 (1.6)	33.9 (0.83)	148 (2.14)
McNeal	*Pina-D1b/ Pinb-D1a*	HGA	+	28	24.3 (1.03)	35.0 (0.84)	28.2 (1.92)	30.8 (0.85)	157 (2.34)
			-	14	84.2 (1.41)	35.0 (0.61)	85.4 (1.65)	30.8 (0.97)	154.7 (3.27)
		HGB	+	26	39.5 (1.01)	37.5 (0.59)	45.5 (1.72)	30.9 (0.87)	158 (2.48)
			-	29	80.1 (0.99)	36.0 (0.59)	87.4 (1.65)	30.1 (0.84)	157 (2.31)

Table 1.2 Continued.

Null Parent	Native Pin	Trans-genic Parent	Trans-gene	Number of Lines	2004† Hardness	2004† Kernel Wt. (mg)	2005‡ Hardness	2005‡ Kernel Wt. (mg)	2005‡ Grain Protein (g/kg)
McNeal	*Pina-D1a/ Pinb-D1b*	HGA	+	29	39.4 (1.08)	37.2 (0.60)	40.5 (1.7)	31.1 (0.87)	156 (2.41)
			-	15	71.6 (1.41)	36.5 (0.84)	77.8 (1.94)	31.7 (0.96)	158 (3.08)
		HGB	+	30	13.0 (0.99)	37.1 (0.59)	23.7 (1.66)	31.0 (0.85)	156 (2.37)
			-	28	73.4 (0.95)	36.0 (0.60)	81.9 (1.66)	29.9 (0.85)	154 (2.34)

† Individual single F_2 derived F_3 plants determined to be homozygous positive or negative for the transgene and the *Ha* locus (Native *Pin*) inherited from Canadian Red, McNeal or Hi-Line.
‡ Means of replicated F_3 derived progeny lines grown under irrigated and rain-fed environments.

Grain Analysis among *Pinb-D1e or Pinb-D1b* Genotypic Classes with Added *Pina-D1a* or *Pinb-D1a*

To determine the role of PINA alone upon grain hardness in the absence of PINB, transgenic lines with added PINA in the Canadian Red (*Pinb-D1e*) *Ha* locus or Hi-Line (*Pinb-D1b*) *Ha* locus backgrounds were evaluated. In the presence of both native *Pinb* alleles (*Pinb-D1e or Pinb-D1b*) and an invariant *Pina-D1a*, addition of *Pina* gave intermediate grain texture (SKCS mean value of 43.5 and 42.5 respectively), while the addition of *Pinb* gave soft grain (SKCS mean value of 15.9 and 17.3 respectively) (Table 1.2 and Figure 1.2). Grain hardness, kernel weight, and grain protein did not differ among transgenic events with the same transgene in the presence of either *Pinb-D1e* or *Pinb-D1b* allele (Table 1.3). The grain texture was not different when comparing native *Pinb-D1e* versus *Pinb-D1b* with added *Pina* or with added *Pinb* (Table 1.4). The same relative

hardness differences upon addition of *Pina* or *Pinb* was observed in 2004 single plants as were observed in 2005 replicated trials.

Grain Analysis among *Pina-D1b* or *Pinb-D1b* Genotypic Classes with Added *Pina-D1a* or *Pinb-D1a*

The role of PINB alone upon grain hardness in the absence of PINA has not been previously demonstrated. Here, the addition of the *Pinb* transgene to *Pina-D1b/Pinb-D1a* progeny lines resulted in intermediate grain hardness (SKCS mean value of 45.5) (Table 1.2), while addition of *Pina* resulted in a soft phenotype (SKCS mean value 28.2) (Tables 1.2, 1.3 and Figure 1.3). *Pinb-D1b* with added *Pina* had a SKCS mean value of 40.5 while addition of *Pinb* gave a softer texture with a SKCS mean value of 23.7 (Table 1.2). The *Pina-D1b* lines with added *Pina* were softer (SKCS = 28.2) than *Pinb-D1b* with added *Pina* (SKCS = 40.5) (Table 1.2 and 1.4). We also observed that addition of *Pinb* to *Pina-D1b* had lesser effect on grain hardness (SKCS = 45.5) than the addition of *Pinb* to *Pinb-D1b* (SKCS = 23.7) (Table 1.2 and 1.4). In all the crosses, grain hardness among genotypic classes possessing the *Pinb-D1b* allele in the presence of added *Pinb* were consistent with those of Hogg et al. (2004) and Swan et al. (2006). When comparing events for the same transgene *Pina-D1b* and *Pinb-D1b* did not differ in grain hardness, kernel weight and grain protein (Table 1.3).

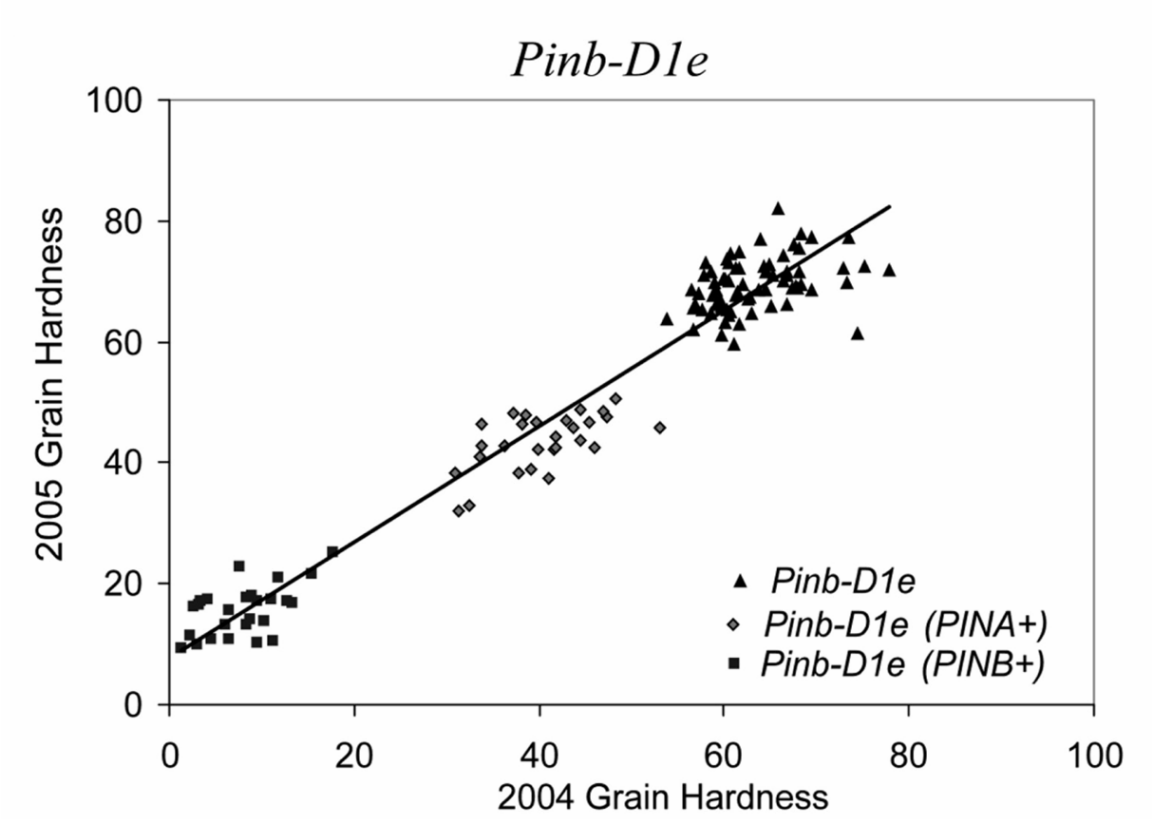


Figure 1.2. Scatter plot of F_3-derived F_4 progeny vs. F_3 single plant grain hardness for segregating progeny from crosses of Canadian Red (*Pina-D1a*/*Pinb-D1e*) x Hi-Line *Ha* locus homozygous lines with added *Pina* or *Pinb* and the Canadian Red *Ha* locus. *Pina-D1b* denotes genotypes possessing the *Pina-D1b/Pinb-D1a* alleles and lacking the transgene, *Pina-D1b* (PINA+) denotes genotypes possessing the *Pina-D1b/Pinb-D1a* alleles with added *Pina* transgene and *Pina-D1b* (PINB+) denote genotypes possessing the *Pina-D1b/Pinb-D1a* alleles with added *Pinb* transgene.

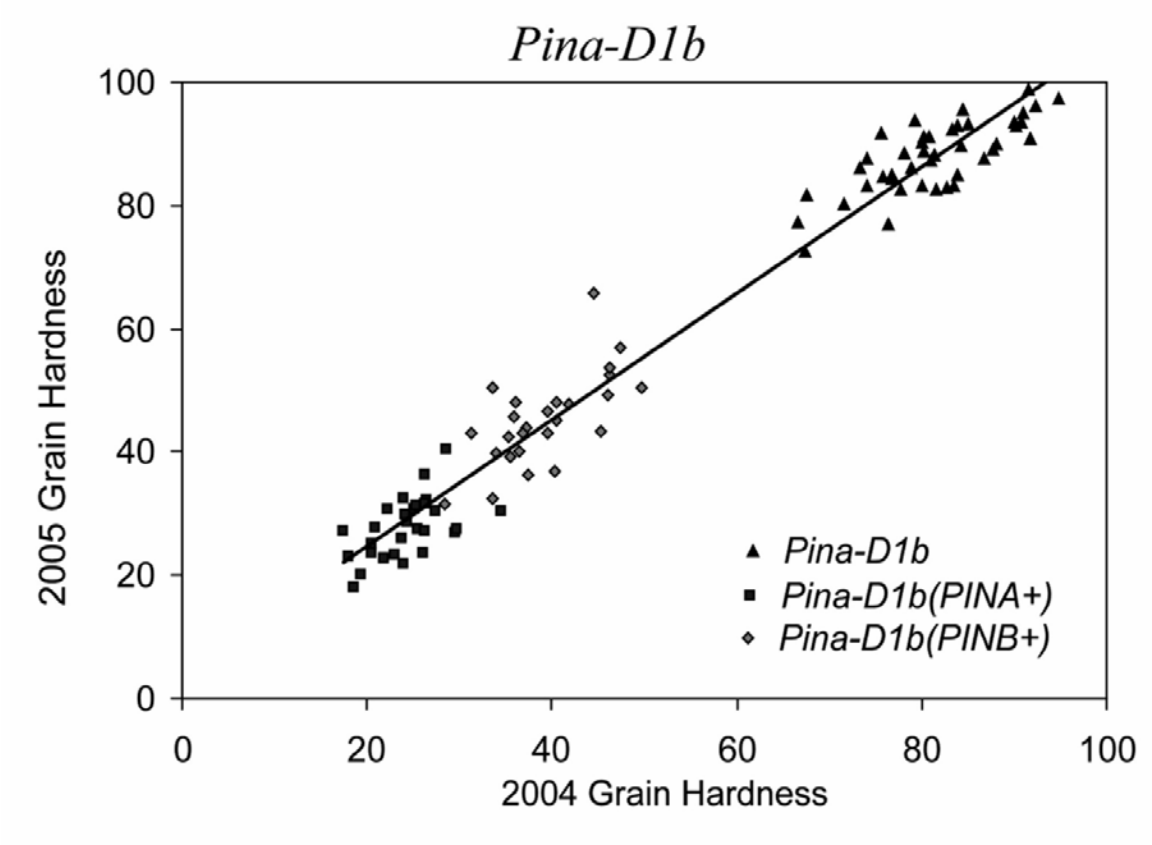


Figure 1.3. Scatter plot of F_3-derived F_4 progeny vs. F_3 single plant grain hardness for segregating progeny from crosses of: McNeal (*Pina-D1b*/*Pinb-D1a*) x Hi-Line *Ha* locus homozygous lines with added *Pina* or *Pinb* and the McNeal *Ha* locus. *Pina-D1b* denotes genotypes possessing the *Pina-D1b/Pinb-D1a* alleles and lacking a transgene, *Pina-D1b* (PINA+) denotes genotypes possessing the McNeal *Pina-D1b/Pinb-D1a* alleles with added *Pina* transgene and *Pina-D1b* (PINB+) denotes genotypes possessing the *Pina-D1b/Pinb-D1a* alleles with added *Pinb* transgene.

Table 1.3. *P*-values for comparisons among genotype class means for grain hardness, kernel weight, and grain protein content for homozygous F_3 (2004) and F_3-derived F_4 progeny (2005) derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e)* or McNeal ((*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* (HGA) or *Pinb* (HGB). Progeny lines inheriting the Canadian Red locus are denoted as *Pina-D1a/Pinb-D1e*; those inheriting the McNeal locus are denoted as *Pina-D1b/Pinb-D1a* while those inheriting the Hi-Line locus are denoted as *Pina-D1a*/*Pinb-D1b.*

		2004		2005		
Cross†	Comparison ‡	Hardness	Kernel Wt.	Hardness	Kernel Wt.	Protein
HGA or HGB X Canadian Red	*Pina-D1a/Pinb-D1e* without transgene					
	HGA1 vs. HGA3	0.599	0.142	0.827	0.818	0.571
	HGB5 vs. HGB12	0.926	0.060	0.257	0.927	0.132
	HGA vs. HGB	0.206	0.212	0.534	0.533	0.530
	Pina-D1a/Pinb-D1b without transgene					
	HGA1 vs. HGA3	0.201	0.422	0.042	0.901	0.827
	HGB5 vs. HGB12	<0.0001	0.0003	<0.0001	0.002	0.132
	HGA vs. HGB	0.096	0.792	0.596	0.203	0.011
	Pina-D1a/Pinb-D1e with transgene					
	HGA1 vs. HGA3	0.606	0.0002	0.567	0.821	0.207
	HGB5 vs. HGB12	0.029	0.819	0.029	0.625	0.018
	HGA vs. HGB	<0.0001	0.084	<0.0001	0.847	0.314
	Pina-D1a/Pinb-D1b with transgene					
	HGA1 vs. HGA3	0..4211	0.002	0.567	0.739	0.175
	HGB5 vs. HGB12	0.081	0.462	0.214	0.789	0.077
	HGA vs. HGB	<0.0001	0.996	<0.0001	0.004	0.089
HGA or HGB X McNeal	*Pina-D1b/Pinb-D1a* without transgene					
	HGB5 vs. HGB12	<0.0001	0.222	0.013	0.138	0.859
	HGA vs. HGB	0.017	0.801	0.229	0.796	0.480
	Pina-D1a/ Pinb-D1b without transgene					
	HGB5 vs. HGB12	0.932	0.042	0.475	0.560	0.058
	HGA vs. HGB	0.282	0.607	0.032	0.033	0.359
	Pina-D1b/Pinb-D1a with transgene					
	HGB5 vs. HGB12	0.068	<0.0001	0.049	0.118	0.282
	HGA vs. HGB	<0.0001	0.005	<0.0001	0.796	0.609
	Pina-D1a/Pinb-D1b with transgene					
	HGB5 vs. HGB12	0.288	0.173	0.653	0.851	0.187
	HGA vs. HGB	<0.0001	0.899	<0.0001	0.929	0.949

† HGA (transgenic Hi-Line with added *Pina*) and HGB (transgenic Hi-Line with added *Pinb*) were crossed to either Canadian Red (*Pina-D1a/Pinb-D1e*) or McNeal (*Pina-D1b/Pinb-D1a)*.
‡ Comparisons are made between two independent events transformed with *Pina* (HGA1, HGA3) and two with *Pinb* (HGB5, HGB12). HGA and HGB represent an average of both independent events within *Pina* and *Pinb*. For example 'HGB' averages were calculated from HGB5 and HGB12 data.

Grain Analysis in the Absence of Added *Pina* or *Pinb* Transgene

Comparisons between added *Pina* and added *Pinb* homozygous classes where the progeny are homozygous negative for the transgene represent average contribution from transgenic lines contributing *Pina* versus those contributing *Pinb*. The significant differences for these comparisons were confined to protein content in the presence of the *Pinb-D1b* native *Pin* allele inherited from the transgenic parent (Table 1.3) when crossed to the Canadian Red parent and both grain hardness and kernel weight in the presence of the *Pinb-D1b* allele inherited from the transgenic parent when crossed to the McNeal parent. However, these differences for grain hardness and kernel weight were not confirmed from the 2004 single plant trial (Table 1.3). Differences among events for the same transgene occurred only for HGB5 versus HGB12 for grain hardness and kernel weight in the presence of the *Pinb-D1b* allele from the transgenic parent and for grain hardness in the presence of the *Pina-D1b* allele from McNeal in crosses to McNeal. These same differences were confirmed in the 2004 single plant trial.

Table 1.4. *P*-values for comparisons among genotype class means for grain hardness, kernel weight, and grain protein content for homozygous F_3 (2004) and F_3-derived F_4 progeny (2005) derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e)* or McNeal ((*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* (HGA) or *Pinb* (HGB). Progeny lines inheriting the Canadian Red locus are denoted as *Pina-D1a/Pinb-D1e*; those inheriting the McNeal locus are denoted as *Pina-D1b/Pinb-D1a* while those inheriting the Hi-Line locus are denoted as *Pina-D1a*/*Pinb-D1b.*

		2004		2005		
Cross†	Comparison‡	Hardness	Kernel Wt (mg)	Hardness	Kernel Wt (mg)	Grain Protein
HGA or HGB X Canadian Red	*Pina-D1a/Pinb-D1e* vs. *Pina-D1a/Pinb-D1b*	*Without Pina Transgene*				
		0.0005	0.037	0.090	0.624	0.005
		Without Pinb Transgene				
		0.0002	0.0004	0.136	0.252	0.681
		With Pina Transgene				
		0.311	0.697	0.798	0.098	0.494
		With Pinb Transgene				
		0.967	0.109	0.436	0.218	0.848
HGA or HGB X McNeal	*Pina-D1b/Pinb-D1a* vs. *Pina-D1a/Pinb-D1b*	*Without Pina Transgene*				
		<0.0001	0.491	0.003	0.375	0.431
		Without Pinb Transgene				
		<0.0001	0.914	0.003	0.085	0.385
		With Pina Transgene				
		<0.0001	0.011	<0.0001	0.672	0.812
		With Pinb Transgene				
		<0.0001	0.618	<0.0001	0.941	0.423

† Hi-Line derived transgenic lines with added *Pina* (HGA) or *Pinb* (HGB) were crossed to either Canadian Red (*Pina-D1a*/*Pinb-D1e*) or McNeal (*Pina-D1b/PinbD1a).*
‡ Progeny segregated for either *Pinb-D1e* or *Pinb-D1b* among Canadian Red crosses or *Pina-D1b* or *Pinb-D1b* among McNeal crosses. Progeny lines also segregated for the presence and absence of the transgene.

TX-114 Extractable Puroindoline Levels.

To determine the total puroindoline levels present in *Pinb-D1e* and *Pina-D1b* genotypes with either added *Pina* or *Pinb*, TX-114 protein extracts from three randomly selected lines per genotypic class were fractionated using SDS-PAGE and visualized by direct staining. Amounts of PINA and PINB for the soft wheat Heron were used as

references. The Canadian Red parent had no PINB, but its PINA level was relatively equal to Heron.

The McNeal parent had no PINA and PINB was not detected with load concentrations used. Adding *Pina* increased PINA protein to more than 6 times that in Heron in both lines with the *Pinb-D1e* and the *Pina-D1b* alleles. Adding *Pina* to *Pinb-D1e* genotypes gave no PINB protein, while PINB in the *Pina-D1b* genotypes increased to amounts equal to the Heron control. Adding *Pinb* to either *Pinb-D1e* or *Pina-D1b* genotypes increased PINB protein about three times that in the Heron control (Figure 1.4 and Table 1.5). PINA protein did not increase over that in the respective parents with addition of *Pinb*. Total PIN protein levels, which were obtained as the sum of the two relative amounts, was higher with the addition of *Pina* than for addition of *Pinb* (Table 1.5).

Starch Associated Puroindoline Levels

Starch-associated PINA and PINB (friabilin) were extracted from the surface of starch granules from the same genotypes as TX-114 protein, fractionated using SDS-PAGE, and then visualized using Commasie blue staining. We assessed the relative amounts of starch PINA or PINB in the presence of native *Pinb-D1e* and *Pina-D1b* alleles with added *Pina* or *Pinb* respectively compared to the soft wheat Heron. PINA and PINB levels in Heron were assigned a value of 1X. Starch associated PIN protein (friabilin) results mirrored those for TX114 soluble protein (Figure 1.4 and 1.5). Adding *Pina* gave greater amounts of starch associated PINA and adding *Pinb* gave greater amounts of PINB in both *Pinb-D1e* and *Pina-D1b* backgrounds (Figure 1.5 and Table

1.5). As with TX114 protein, the only instances where both PINA and PINB proteins are present were when *Pinb* was added to *Pinb-D1e* genotypes and when *Pina* was added to *Pina-D1b* genotypes. Addition of *Pina* to *Pina-D1b* resulted in more PINA associated to starch than PINB. There was an increase in amount of PINB associated to starch upon addition of *Pinb* to *Pinb-D1e* with the amount of PINA being comparable to Heron. Therefore, these cases with both PINA and PINB present gave greatest amount of total friabilin.

Table 1.5. Puroindoline levels quantified from TX -114 and friabilin extracts for parent controls and lines derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e)* or McNeal (*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* or *Pinb*. Progeny were determined to be homozygous for the added transgene and the *Pina-D1b*/*Pinb-D1a* allele inherited from McNeal or *Pina-D1a/Pinb-D1e* allele from Canadian Red. Values are mean of two lines from each of the six crosses.

			Tx-114 Protein†			Friabilin‡		
Genotype	Pin Genotype	Transgene	*PINA*	*PINB*	Total PIN	*PINA*	*PINB*	Total Starch Associated PIN
Canadian Red	*Pina-D1a/Pinb-D1e*	None	1.3	0.0	1.3	0.0	0.0	0.0
McNeal	*Pina-D1b/Pinb-D1a*	None	0.0	ND	0.0	0.0	0.0	0.0
Heron	*Pina-D1a/Pina-D1a*	None	1.0	1.0	2.0	1.0	1.0	2.0
CR/PINA+	*Pina-D1a/Pinb-D1e*	*Pina*	6.4	0.0	6.4	1.5	0.0	1.5
CR/PINB+	*Pina-D1a/Pinb-D1e*	*Pinb*	1.3	3.2	4.4	1.0	3.0	4.0
McN/PINA+	*Pina-D1b/Pinb-D1a*	*Pinb*	6.3	1.1	7.4	3.0	0.6	3.6
McN/PINB+	*Pina-D1b/Pinb-D1a*	*Pina*	0.0	2.8	2.8	0.0	1.6	2.1

† Means of three randomly selected lines homozygous for the transgene and the *Ha* locus. Values are relative levels of TX-114 extractable PIN proteins compared to Heron (Soft) scale of 1 X to 8 X where 1 X = 6µl (240 µl SDS-PAGE sample buffer/100 mg ground seeds)

‡ Starch associated PINA and PINB (friabilin) levels from water washed starch. Values are relative levels of starch associated PIN proteins compared to Heron (Soft) scale of 1 X to 5 X where 1 X = 10 µl (240 µl SDS-PAGE sample buffer/100 mg ground seeds)

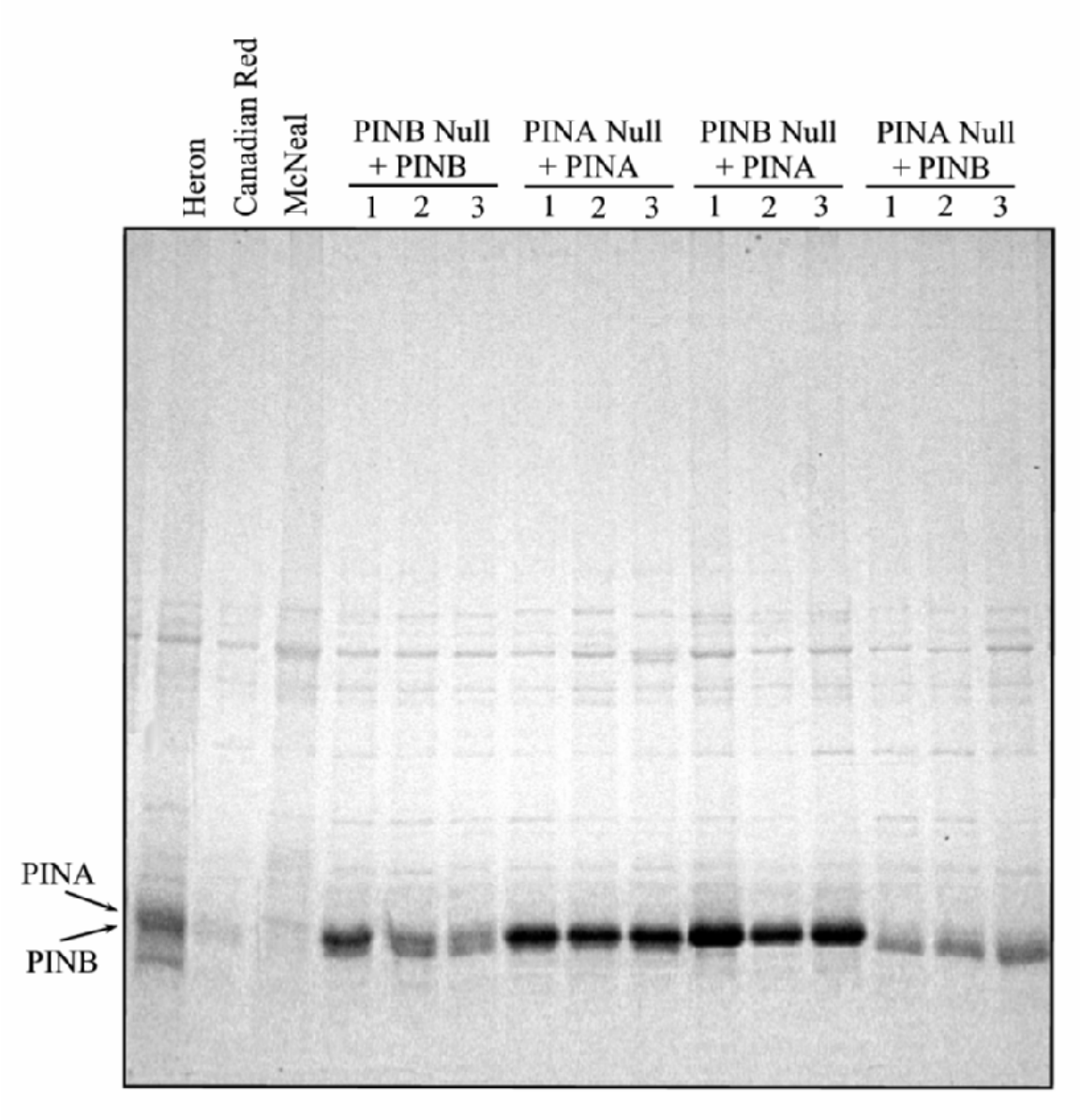


Figure 1.4. SDS-PAGE gel of total TX-114 extractable proteins levels from PIN null and transgenic over expressing lines. Heron *(Pina-D1a/Pinb-D1b)* is a soft wheat control while Canadian Red, PINB null *(Pina-D1a/Pinb-D1e)* and McNeal, PINA null *(Pina-D1b/Pinb-D1a)* are the hard wheat controls. TX-114 extractable puroindoline protein levels were performed on three randomly selected lines per group as shown. The position of PINA and PINB is marked.

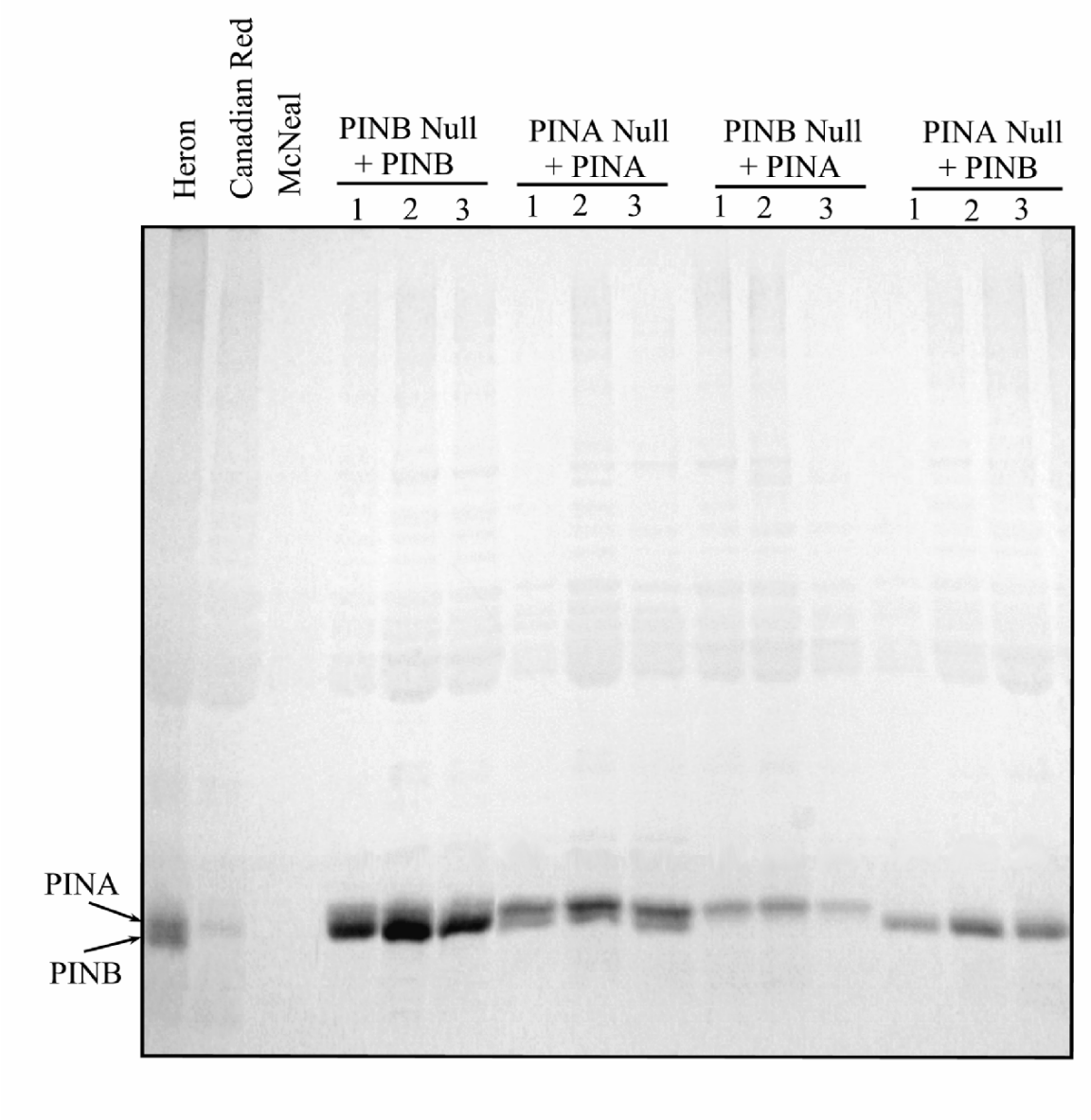


Figure 1.5. SDS-PAGE gel of starch associated puroindolines (PINA and PINB). Total puroindolines were extracted from water washed starch granules of control and soft and intermediate texture PIN over expressing lines. Heron *(Pina-D1a/Pinb-D1a)* is a soft wheat control while Canadian Red, PINB null *(Pina-D1a/Pinb-D1e)* and McNeal, PINA null *(Pina-D1b/Pinb-D1a)* are the hard wheat controls. Starch associated (PINA or PINB) proteins were extracted from three randomly selected lines per group as noted. The position of PINA and PINB is marked.

Discussion

Soft endosperm texture in wheat is associated with the presence of both PINA and PINB. All hard endosperm wheat cultivars contain an alteration in either the *Pina* or *Pinb* coding sequence that causes hard grain texture (Giroux and Morris 1997, 1998). The two most common *Pin* mutations are the *Pinb-D1b* and the *Pina-D1b* alleles (Morris et al., 2001). Demonstration that the *Pin-D1b* allele is causative to hard grain texture was presented by Beecher et al. (2002) who complemented the *Pinb-D1b* allele in the hard wheat Hi-Line with the wild type *Pinb-D1a* producing a soft wheat phenotype. Similarly, Martin et al. (2006) expressed *Pina-D1a* in the hard wheat cultivar Bobwhite that contains the *Pina-D1b* allele and observed soft grain texture. Both Beecher et al. (2002) and Martin et al. (2006) confirmed the assumptions made by Giroux and Morris (1997, 1998) and others in which *Pina* and *Pin's* involvement in grain hardness was believed to be more than simply genetic linkage.

In support of this theory, Hogg et al. (2004) characterized transgenic lines with added *Pina*, *Pinb,* or both in the hard wheat Hi-Line. They observed soft grain texture and increased puroindoline and friabilin abundance with addition of PINB, or both PINA and PINB to Hi-Line. Further, Swan et al. (2006) crossed Hi-Line transgenic lines expressing *Pina*, *Pinb,* or both *Pina* and *Pinb* to the soft wheat Heron and found that progeny lines with the soft *Ha* locus of Heron and increased PINB had softer endosperm texture than those with increased PINA. They concluded that PINB is the limiting factor in friabilin formation and reduction in grain hardness. The results of Hogg et al. (2004) and Swan et al. (2006) indicated that both PINA and PINB proteins are required for

friabilin formation and for soft grain phenotype. Our current results lend support to the theory that both PINA and PINB are required for a wild type soft grain endosperm phenotype in wheat. Expression of *Pinb* transgene in *Pinb-D1e* and *Pina* in *Pina-D1b* resulted in increased TX-114 soluble puroindoline levels, increased friabilin levels, and a soft phenotype (Table 1.2 and Figure 1.4 and 1.5). Although addition of *Pina* to *Pina-D1b* decreased grain hardness leading to a soft texture, the softest phenotype resulted from the addition of *Pinb* to *Pinb-D1e* (Table 1.2, Figure 1.3 and 1.4). These results concurred with Swan et al. (2006) that addition of *Pinb* leads to softer grain than the addition of *Pina,* and that *Pinb* is more limiting to grain softness than *Pina*. Further, addition of the *Pinb* transgene to *Pinb-D1b* also resulted in a soft phenotype (Table 1.2). Our study together with Beecher et al. (2002), Hogg et al. (2004), and Martin et al. (2006) demonstrates a that soft phenotype can be restored by complementing either the mutated *Pinb* (null), *Pinb* glycine-serine (*Pinb-D1b*), or *Pina* (null) with the corresponding functional *Pin* allele. It has been proposed that the absence of PINA in *Pina-D1b* and absence of PINB in *Pinb-D1e* hard wheats limits the interaction of PINB and PINA respectively with starch granules leading to hard endosperm phenotype (Giroux et al., 1997, 1998; Apparels et al., 2003 and Gazza et al., 2005). It has also been hypothesized that PINA and PINB interact to form friabilin and together affect grain texture (Hogg et al., 2004). Here, our main objective was to determine the individual role of each puroindoline protein in the absence of the other on grain hardness. Results obtained in this study show that addition of PINA in absence of PINB and addition of PINB in absence of PINA gave intermediate textured grain. A wild-type soft phenotype was obtained only in the presence of both PINA and PINB (Figure 1.2 and 1.3). High

amounts of either PIN individually led to an intermediate texture (Table 1.5, Figure 1.4 and 1.5). These results support the hypothesis that total puroindoline content does not dictate grain softness, rather the presence of both functional PINA and PINB.

Friabilin is abundant on water washed starch granules of soft wheat and little or none is found on water washed starch granules from hard wheat (Greenwell and Schofield, 1986). Here, we report that PINA and PINB weakly associate with starch granules in absence of the other protein leading to an intermediate textured endosperm (Table 1.3, Figure 1.2 and 1.3). However, friabilin was found in abundance only when both PINA and PINB were present. It is evident that the amount and association of friabilin components on the surface of starch granules affect endosperm texture. Over-expression of PINA in *Pinb* (null) and over-expression of PINB in *Pina* (null) resulted in starch granule associated puroindolines and intermediate texture with slightly more PINB associating to starch granule surface than PINA. Apparels et al. (2003) and Gazza et al. (2005) suggested that that in the absence of PINA, less PINB associates with starch granule surface. In this study, we observed that in the absence of PINA, over expressed PINB associates with starch granule surface more efficiently than PINA. Previously, Greenblatt et al. (1995) postulated that the association of puroindoline to starch granules involves either polar bound phospholipids or glycolipids. Regardless of the specific mechanism of interaction of starch granules with PINA or PINB leading to intermediate endosperm texture, our study shows that either PINA or PINB can bind independently with the starch granule surface to produce intermediate textured grain, or interact together to give wild type soft textured grain.

The effect of over-expressed puroindoline on a wide range of milling and baking traits was demonstrated by Hogg et al. (2005). In addition, the effect on dough properties and bread quality on puroindoline free flours reconstituted with puroindoline has been demonstrated (Dubreil et al., 1997). Martin et al. (2001) also observed an association of puroindoline sequence variation with several milling and baking traits in a recombinant inbred population segregating for *Pina-D1b* and *Pinb-D1b* alleles. However, to clearly understand the roles of puroindolines on milling and baking traits, a baking and milling study of the unique genotypes created in this study varying randomly in PINA and PINB abundance would further illustrate the individual role each puroindoline protein plays on baking and milling qualities. These unique populations would also be useful in studying the antifungal effects conferred by an individual PIN protein in the absence of the other in wheat.

Conclusions

In conclusion, this study has shown that PINA and PINB can act independently in the absence of the other protein leading to an intermediate endosperm texture or together to produce wild type soft textured grain. It is also evident that over-expressed PINA or PINB in absence of the other protein associates with starch granules leading to an intermediate phenotype, however, wild type soft phenotype leading to full friabilin abundance and function is observed when both functional PINA and PINB are present. The biochemical basis for the association of PINA or PINB to starch granules in the absence of the other leading to an intermediate grain texture is unknown. However, our

results support the hypothesis that *Pina* and *Pinb* are the causative factors in grain hardness and that wild-type *Pina* and *Pinb* genes are required for a wild type soft texture.

References Cited

Baker, R.J. 1977. Inheritance of kernel hardness in spring wheat. Crop Sci. 17:960-962.

Anjum, F.M., and C.E. Walker. 1991. Review on significance of starch and protein to wheat kernel hardness. J. Sci. Food Agric. 56:1-3.

Beecher, B., A. Bettge, E. Smidansky, and M. J. Giroux. 2002. Expression of wild-type *Pinb* sequence in transgenic wheat complements a hard phenotype. Theor. Appl. Genet.105:870-877.

Bettge, A.D., C.F. Morris, and G.A. Greenblatt. 1995. Assessing genotypic softness in single wheat kernels using starch granule-associated friabilin as a biochemical marker. Euphytica 86:65–72.

Blechl, A.E., and O.D. Anderson. 1996. Expression of a novel high-molecular weight glutenin subunit gene in transgenic wheat. Nature Biotechnology 14:875-879.

Blochet, J.E., C. Chevalier, E. Forest, E. Pebay-Peyroula, M.F. Gautier, P. Joudrier, M. Pezolet, and D. Marion. 1993. Complete amino-acid sequence of puroindoline, a new basic and cystine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS Lett. 329:336-340.

Capparelli, R., G. Borriello, M.J. Giroux, and M.G. Amoroso. 2003. Puroindoline A-gene expression is involved in association of puroindoline to starch. Theor. Appl. Genet. 107:1463-1468.

Clark, J.A., H.H. Love, and E.F. Gaines.1926. Registration of standard wheat varieties. J. Amer. Soc. Agron. 18(10):920-922.

De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V.Gossele, N.Rao Movva, C.Thompson, M.Van Montagu and J. Leemans. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enxyme. EMBO J.6:2513-2518.

Dubreil, L., J.P. Compoint, D. Marion. 1997. Interaction of puroindolines with wheat flour polar lipids determines their foaming properties. J. Agric. Food Chem. 45: 108–116.

Gautier, M.F., M.E. Aleman, A. Guirao, D. Marion, and P. Joudrier. 1994. *Triticum aestivum* puroindolines, two basic cystine-rich seed proteins: cDNA analysis and developmental gene expression. Plant Mol. Biol. 25:43-57.

Gazza, L., F. Nocente, P.K.W. Ng, and N.E. Pogna. 2005. Genetic and biochemical analysis of common wheat cultivar lacking puroindoline a. Theor. Appl. Genet. 110(3):470-478.

Giroux, M.J., and C.F. Morris. 1997. A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin. Theor. Appl. Genet. 95:857-864.

Giroux, M.J., and C.F. Morris. 1998. Wheat grain hardness results from highly conserved mutations in the friabilin components, *puroindoline a* and *b*. Proc. Natl. Acad. Sci. USA, 95:6262-6266.

Giroux, M.J., T. Sripo, S. Gerhardt, and J. Sherwood. 2003. Puroindolines: Their role in grain hardness and plant defense. Biotechnology and Genetic Engineering Reviews. 20:277-290. Editor: Stephen E. Harding. Intercept, Andover, Hampshire, UK.

Greenblatt, G.A., A.D. Bettge, and C.F. Morris. 1995. The relationship among endosperm texture, friabilin occurrence, and bound polar lipids on wheat starch. Cereal Chem. 72: 172–176.

Greenwell, P., and J.D. Schofield. 1986. A starch granule protein associated with endosperm softness in wheat. Cereal Chem. 63:379-380.

Guraya, H.S., C. James, E.T. Champagen. 2003. Physical Basis for separation of rice starch using various density gradient systems and its effect on starch recovery, purity, and pasting properties. Starch – Starke. 55: 450-456.

Hogg, A.C., T. Stripo, B. Beecher, J.M. Martin, M.J. Giroux. 2004. Wheat Puroindolines interact to form friabilin and control wheat grain hardness. Theor. Appl. Genet. 108:1089-1097.

Hogg, A.C., B. Beecher, J.M. Martin, F.D. Meyer, S. Lanning, and M.J. Giroux. 2005. Hard wheat milling and bread baking traits affected by the seed-specific over expression of puroindolines. Crop Sci. 45:871-878.

Krishnamurthy, K., and M.J. Giroux. 2001. Expression of wheat *puroindoline* genes in transgenic rice enhances grain softness. Nature Biotechnology 19:162-166

Lanning, S.P., L.E. Talbert, F.H. McNeal, W.L. Alexander, C.F. McGuire, H. Bowman, G. Carlson, G. Jackson, J. Eckhoff, G. Kushnak, V. Stewart, and G. Stallknecht. 1992. Registration of Hi-Line wheat. Crop Sci. 32:283-284.

Lanning, S.P., L.E. Talbert, C.F. McGuire, H.F. Bowman, G.D. Carlson, J.L. Jackson, G.D. Eckhoff, R.N. Kushnak, R.N. Stougaard, and G.F. Stallknecht .1995. Registration of 'McNeal' wheat. Crop Sci. 34:1126-1127.

Law, C.N., C.F. Young, J.W.S. Brown, J.W. Snape, and, J.W.Worland. 1978. The study of grain protein control in wheat using whole chromosome substitution lines. P. 483-502. *In*

Seed Protein Improvement by Nuclear Techniques, International Atomic Agency, Vienna, Austria.

Lillemo, M., and C.F. Morris. 2000. A leucine to proline mutation in *puroindoline b* is frequently present in hard wheats from Northern Europe. Theor. Appl. Genet. 100:1100-1107.

Marion, D., M.F. Gautier, P. Joudrier, M. Ptak, M. Pezolet, E. Forest, D.C. Clark, and W. Broekaert. 1994. Structure and function of wheat lipid binding proteins. P. 175-180 *In* S. Martino al Cimino (ed.) Wheat Kernel Proteins- Molecular and Functional Aspects. 28-30 Sept. 1994. Universita Degli Studi Della Tuscia, Viterbo, Italy.

Martin, J.M., R.C. Frohberg, C.F. Morris, L.E. Talbert, and M.J. Giroux. 2001. Milling and bread baking traits associated with puroindoline sequence type in hard red spring wheat. Crop Sci. 41:228-234

Martin, J.M., F.D. Meyer, E.D. Smidansky, H. Wanjugi, A.E. Blechl, and M.J. Giroux. 2006. Complementation of the Pina (null) allele with the wild type Pina sequence restores a soft phenotype in wheat. Theor. Appl. Genet.113:1563-1570.

Massa A.N, C.F. Morris B.S. Gill. 2004. Sequence diversity of puroindoline-a (Pina-D1), puroindoline-b (Pinb-D1) and the grain softness protein (Gsp-1) genes in Aegilops tauschii Coss. Crop Sci 44:1808–1816.

Mattern, P. J., R. Morris, J.W. Schmidt, and V.A. Johnson. 1973. Location of genes for kernel properties in the wheat cultivar 'Cheyenne' using chromosome substitution lines. p. 703-707. *In* E.R. Sears and L.M.S. Sears (ed.) Proc 4th Int. Wheat Genetics Symp., Columbia, MO. 1-6 Aug 1973. Agric. Exp. Stn., Univ. of Missouri, Columbia, MO.

Morris, C.F., G.A. Greenblatt, A.D. Bettge, and H.I. Malkawi. 1994. Isolation and characterization of multiple forms of friabilin. J. Cereal Sci. 21:167-174

Morris, C.F., M. Lillemo, G.M. Simeon, M.J. Giroux, S. Babb, and K. Kidwell. 2001. Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci. 41:218-228

Morris, C.F. and S.P. Rose. 1997. Chapter 1: Quality requirements of cereal users, Wheat. Cereal Grain Quality R.J. Henry and P.S. Kettlewell, eds. Chapman Hall, London

Morris, C.F. 2002. Puroindolines: The molecular genetic basis of wheat grain hardness. Plant Mol. Biol. 48:633–647.

Pan, Z., W. Song, F. Meng, L. Xu, B.Liu, and J. Zhu, J. 2004. Characterization of genes encoding wheat grain hardness in Chinese cultivar GaoCheng 8901. Cereal Chem. 81:287-289.

Pomeranz, Y., and P.C. Williams. 1990. Wheat grain hardness: its genetic, structural and biochemical background, measurements and significance. In: Pomeranz Y (ed) Advances in cereal science and technology, vol.10. American Association of cereal chemistry, St Paul, Minn., pp 471-544.

Rahman, S., Jolly, J.C, Skerritt, J.H, Wallosheck, A. 1994. Cloning of a wheat 15-kDa grain softness protein (GSP). GSP is a mixture of puroindoline-like polypeptides. Eur.J. Biochem. 223: 917-925.

Riede, C.R., and J.A. Anderson. 1996. Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci. 36:905-909.

Sourdille, P., M.R. Perretant, P. Charmet, M.F. Leroy, P. Gautier, J.C. Joudrier, J.C. Nelson, M.E. Sorrells, and M. Bernard. 1996. Linkage between RFLP markers and genes affecting kernel hardness in wheat. Theor. Appl. Gen. 93:580-586.

Swan, C.G., F.D. Meyer, A.C. Hogg, J.M. Martin and M.J. Giroux. 2006. Puroindoline B Limits Binding of Puroindoline A to Starch and Grain Softness. Crop Science 46(4): 1656-1665.

Symes, K.J. 1965. The inheritance of grain hardness in wheat as measured by particle size index. Aust. J. Agric. Res. 16:113-123.

Symes, K.J. 1969. Influence of a gene causing hardness on the milling and baking quality of two wheats. Aust. J. Agric. Res, 20:971-979.

CHAPTER TWO

THE INFLUENCE OF PUROINDOLINE A AND B INDIVIDUALLY AND IN COMBINATION UPON WHEAT MILLING AND BREAD BAKING TRAITS

Introduction

Endosperm texture is an important criterion upon which wheat is classified and traded. Endosperm texture is simply inherited and controlled by a single locus termed *Hardness* (*Ha*) (Symes, 1965; Baker, 1977) located on chromosome 5DS (Mattern et al., 1973; Law et al., 1978). Greenwell and Schofield (1986) discovered a 15 kDa marker protein they termed friabilin that could distinguish between hard and soft wheat in that it was more abundant on water washed starch of soft wheats compared to hard wheat. At the time, it was thought that friabilin was a complex mixture of proteins (Morris et al., 1994; Oda and Schofield, 1997). The genetic control of grain hardness was further advanced when it was found that the puroindoline a (Sourdille et al., 1996) and b (Giroux and Morris, 1997) genes (*Pina* and *Pinb*, respectively) were linked to *Ha*. *Pina* and *Pinb* were cloned and shown to encode proteins 60 % similar in sequence with each containing a unique tryptophan rich domain (Gautier et al., 1994). Mutations in *Pina* and *Pinb* were then shown to be genetically linked to hard texture (Giroux and Morris, 1997, 1998) and comparison of the N-terminal sequence of friabilin with those of PINA and PINB (Gautier et al., 1994) indicated that friabilin consisted of PINA and PINB (Jolly et al.,

1993; Morris et al., 1994). Wheat bacterial artificial chromosome (BAC) clone sequencing has shown that *Pina* and *Pinb* are contained within a 25 kb region of 5DS in wheat (Chantret et al., 2004) rendering recombination between them extremely rare. Surveys of wheat genotypes has confirmed that soft wheat texture results from both *Pin* genes being in their wild type form, whereas hard wheats have a mutation in either *Pin* gene (Giroux and Morris, 1998; Lillemo and Morris, 2002; Morris et al., 2001). Nine *Pin* mutations among hard wheats have been reported (Giroux and Morris 1997, 1998; Morris et al. 2002; Gazza et al., 2005., Pan et al., 2005) with the most prevalent mutations being a null mutation in *Pina* (*Pina-D1b*) and a missense mutation in *Pinb* (*Pinb-D1b*) (Morris et al., 2001).

To demonstrate that *Pin* genes are the primary genetic elements controlling grain hardness, Krishnamurthy and Giroux, (2001) transformed the model cereal rice, which lacks genes homologous to *Pin*s (Caldwell et al., 2004), with "soft type" alleles *Pina-D1a* and *Pinb-D1a*. The result was a decrease in rice kernel hardness and particle size of whole seed meals after grinding. Direct evidence in wheat that mutations in *Pin*s are causative to hard texture was obtained by complementing the *Pina-D1b* (Martin et al., 2006a) and a *Pinb-D1b* mutations (Beecher et al., 2002) with the *Pina-D1a and Pinb-D1a* alleles, respectively. Successful complementation was observed in both cases in that increased friabilin levels and decreased grain hardness indicative of a soft phenotype were observed. Further understanding of how PINs modify grain texture has come from studies of PIN transgenic addition lines created in a hard wheat which have demonstrated that soft endosperm texture and friabilin formation coincide with the presence and abundance of both PINA and PINB (Hogg et al., 2004). Similarly, Swan et al. (2006)

used PINA and PINB overexpressing events to demonstrate that while both PINS limit grain softness in soft wheats, PINB is more limiting to grain softness than PINA (Swan et al., 2006). While much of the available evidence indicates that PINA and PINB interact to create grain softness, Wanjugi et al. (2007) reported that high levels of either PIN can act independently in the absence of the other protein leading to intermediate textured grain.

Grain endosperm texture affects a wide range of wheat milling and flour quality characteristics (Pomeranz and Williams 1990; Slaughter et al., 1992). In crosses between soft (*Ha*) and hard (*ha*) wheats, *Ha* lines were higher in softness equivalent, cookie diameter, and crumb grain score but lower in flour yield, damaged starch, and dough water absorption relative to *ha* genotypes (Campbell et al., 2001). Within hard wheats, the two most common *Pin* mutations (*Pina-D1b* and *Pinb-D1b*) confer different levels of grain hardness with those carrying the *Pinb-D1b* mutation being ~7 hardness units softer than those with the *Pina-D1b* allele (Martin et al., 2001). Further, Martin et al. (2001) observed higher flour yield, lower flour ash, improved loaf volume and better crumb grain score among progeny lines inheriting the *Pinb-D1b* allele. To examine the effects of transgenic additions of PINs to milling and baking quality, Hogg et al. (2005) characterized milling and bread baking traits in PIN overexpressing transgenic isolines created in a hard wheat background. They found that PIN overexpression was associated with decreased flour yield, mixograph water absorption, and loaf volume and crumb grain score and increased break flour yields. While the results of Hogg et al. (2005) demonstrated changes in milling and baking traits due to PIN overexpression, the study did not isolate the effect of individual PINs upon milling and bread baking traits and was

further complicated by the presence of soft type *Pina-D1a* and the variant *Pinb-D1b* allele in the "Hi-Line" derived transgenic isolines. The objective of this study was to isolate the role of PINA and PINB individually and in combination upon milling and bread baking traits by using transgenic lines that overexpressed either PINA or PINB alone in PINA or PINB null backgrounds.

Material and Methods

Genetic Materials

The genotypes used in this study were a subset of the recombinant lines described by Wanjugi et al. (2007). One transgenic isoline overexpressing PINA (HGA3) or PINB (HGB12) created in the variety 'Hi-Line' (HL) (Lanning et al., 1992) and described by Hogg et al. (2004) was crossed to either a PINB null, 'Canadian Red'(CR); hard white spring (Clark, 1926) or a PINA null, 'McNeal'(McN); hard red spring (Lanning et al., 1995) variety. The transgenic events were selected from the events described by Hogg et al. (2004, 2005) as having good plant vigor and relatively unaltered plant yield, seed size, and seed protein content. Canadian Red has the soft type *Pina-D1a* and a mutant *Pinb-D1e* allele (Morris et al., 2001). *Pinb-D1e* contains a point mutation (TGG –TGA) leading to a change in residue Trp-39 to a stop codon. McNeal possesses the soft type *Pinb-D1a* and a mutant *Pina* allele (*Pina-D1b*) which is an apparent deletion of the *Pina* coding sequence (Giroux and Morris, 1998). Hi-Line has the soft type *Pina-D1a* and the mutant *Pinb-D1b* allele which contains a single point mutation in *Pinb* resulting in a glycine to serine substitution at the 46th residue of PINB (Giroux et al., 2000). The Hi-Line derived transgenic lines (HGA3, HGB12) express the *Bar* gene (De Block et al.,

1987) that confers resistance to bialophos (Meiji Seika Kaisha Ltd., Tokyo, Japan) and glufosinate ammonium (AgrEvo, Wilmington, DE). The transgenic overexpression of the *Pina-D1a* (*Pina*) and *Pinb-D1a* (*Pinb*) coding sequences is under the control of the *Glu1Dy10* seed-specific high molecular weight glutenin promoter reported by Blechl and Anderson (1996). The crosses generated two populations in the HL/McN and HL/CR background, segregating for native *Ha* locus from HL, CR, or McN and the presence or absence of the transgene (*Pina* or *Pinb*). The F_2 generation for each cross was grown in the greenhouse. To identify F_2 derived F_3 seed pools homozygous for the presence or absence of the transgene, 18 F_2 derived F_3 seeds per line were planted in the greenhouse. Plants were sprayed with 0.1 % glufosinate ammonium at the two leaf stage. The plants were scored as being resistant or susceptible after 7 days. Resistant plants stayed green while susceptible plants were killed. The F_2 parent was classified as a *Pin* transgene homozygous positive (*Pina* or *Pinb*) or a *Pin* transgene homozygous negative genotype if > 11 consecutive F_3 progeny were glufosinate-resistant or glufosinate-susceptible, respectively. F_3 progeny with mixed herbicide results, most often segregating 3:1 resistant: susceptible, were considered to have a heterozygous-*Pin* F_2 parent. These lines were excluded from subsequent experiments.

To identify the allelic state of the *Ha* locus, leaf tissues were taken from F_3 plants derived from a single F_2 plant homozygous positive or negative for the transgene. Young leaf tissues were pooled from >10 individual F_3 plants from a single F_2 parent and genomic DNA was extracted according to Riede and Anderson (1996). The *Ha* locus genotype was determined using cleaved amplified polymorphic marker tests (CAPS) as described in Wanjugi et al. (2007). Identification of genotypes homozygous for the

presence or absence of the transgene and for the segregating native *Ha* locus resulted in four genotype classes for each cross. We used two of the four genotype classes in our experiment. The two classes were those inheriting the McN *Ha* locus in the crosses to McN or the CR *Ha* locus in crosses to CR in combination with the presence or absence of an added transgene. The classes inheriting the native *Ha* from HL were not included as the effect of PIN overexpression in the presence of the HL locus has been previously reported (Hogg et al. 2004, 2005). For this study, we used a randomly selected subset of F_3 derived lines homozygous for the CR or McN *Ha* locus and the presence of absence of the transgene. Genotypes were grown in 2005 in a randomized complete block design with two replications. Each plot was a 3 m row spaced 30 cm apart seeded with 4 g of seed. The same trial was grown in two separate adjacent experiments with and without added irrigation at the Montana State University-Bozeman Arthur H. Post Field Research Farm. Irrigated plots received 7.6 cm of water one week before and after anthesis. Rainfall from April to July 2005 was 18.7 cm. No nitrogen was supplemented, and average soil N was 177 kg ha^{-1} before planting. Plots were cut and bundled by hand and then threshed with a Vogel plot thresher (Bill's Welding, Pullman, WA). Grain hardness was determined using the Single Kernel Characterization System 4100 (SKCS, Perten Instruments, Springfield, IL) and grain protein was determined using near-infra red transmission using an Infratec 1225 Grain Analyzer (Foss North America Inc., Eden Prairie, MN) from a sub-sample of grain from each plot.

Milling and Baking Trait Analysis

Seed from the two replications from an environment was bulked for each genotype prior to milling. Four hundred g of grains per genotype per environment were tempered to 14.0 % moisture for soft and intermediate textured grains and 15.5 % for hards. Samples were milled on a Brabender Quadrumat Jr. Mill (Brabender GmbH, Duisburg, Germany). The resultant straight grade flour was analyzed for flour protein (Method 46-30) and flour ash (Method 08-01) (AACC, 2000). All flour characteristics are reported on a 14 % moisture basis. Mixograph analysis was performed using 10 g of flour following method 54-40A (AACC, 2000). Bread was baked according to method 10-10B (American Association of Cereal Chemists, 2000) and loaf volume determined using the canola seed displacement method. Crumb grain score was rated from 0 (unsatisfactory) to 5 (excellent). Following milling, 10 g of straight grade flour was size separated using 53, 74, 90, and 150 µm mesh International Standards Organization sieves by shaking for 10 min. using a RoTap shaker (Tyler Co., Mentor, OH).

Statistical Analysis

Data for milling and baking traits were analyzed using a mixed effect analysis of variance model for randomized block using PROC MIXED in SAS (SAS Institute Inc, Cary, NC). The model accounted for genotype classes and random lines within genotype classes. Comparisons among genotype class means were made using the ESTIMATE statement in SAS. The mean of the two genotype classes with the *Pina-D1b/Pinb-D1a Ha* locus from McN and without the added *Pin* transgene was used in comparisons with genotype classes with added *Pin* transgene. Correspondingly, the mean of the two

genotype classes with the *Pina-D1a*/*Pinb-D1e Ha* locus from CR and without the added *Pin* transgene was used in comparisons with genotypic classes with added *Pin* transgene. Correlations among traits were computed from entry means.

Results

A subset of the genotypes described by Wanjugi et al. (2007) segregating for both *Ha* and *Pin* transgene were used in this study. Grain hardness and protein content was measured on this subset (Table 2.1). The subset genotypes consisted of parental varieties and recombinant progeny lines from crosses of PIN overexpressing transgenic lines created in HL with either the CR (*Pina-D1a/Pinb-D1e* or *Pina* soft type, *Pinb* null) or McN *Ha* locus (*Pina-D1b/PinbD1a* or *Pina* null, *Pinb* soft type). Among the parental cultivars, McN had harder grain but lower grain protein than HL while CR had the lowest grain hardness and grain protein content (Table 2.1). The recombinant lines from crosses to CR consisted of genotypes homozygous for the CR *Ha* locus with added *Pina*, *Pinb* or lacking an added transgene with the negative control group consisting of negative lines from both Hi-Line transgenic x CR crosses. The recombinant lines from crosses to McN consisted of genotypes homozygous for the McN *Ha* locus, with added *Pina*, *Pinb*, or lacking an added transgene with the negative control group consisting of lines from both HL transgenic x McN crosses. Addition of *Pina* to McN *Ha locus* genotypes, and *Pinb* to CR *Ha* locus genotypes resulted in soft texture while the addition of *Pina* to CR *Ha* locus genotypes, and *Pinb* to McN *Ha* locus genotypes resulted in intermediate textured grain.

All genotypes lacking a *Pin* transgene were hard textured and didn't vary substantially in grain protein content within a cross group (HL x CR or HL x McN) relative to genotypes with an added *Pin* (Table 2.1).

Table 2.1. Genotype class means for grain hardness and grain protein content for a subset of F_3-derived progeny derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e Ha* locus) or McNeal (*Pina-D1b/Pinb-D1a Ha* locus) with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). Presence or absence of transgene is denoted as + or -, respectively. Standard errors are presented in ( ).

Hard wheat parent[†]	*Ha* locus[‡]	Transgenic parent[§]	Transgene	No. of lines	Grain Hardness[¶]	Grain protein (g/kg)[#]
Hi-Line(HL)	*Pina-D1a/Pinb-D1b*	None	None	2	73.1(1.71)	152(0.55)
Canadian Red (CR)	*Pina-D1a/Pinb-D1e*	None	None	2	71.6(2.12)	136(0.58)
McNeal(McN)	*Pina-D1b/Pinb-D1a*	None	None	2	95.1(2.14)	149(0.58)
Canadian Red (CR)	*Pina-D1a/Pinb-D1e*	HG-	-	13	69.3(0.88)	150(0.13)
		HGA3	+	13	42.2(1.06)	145(0.16)
		HGB12	+	12	16.9(1.71)	147(0.16)
McNeal(McN)	*Pina-D1b/Pinb-D1a*	HG-	-	15	86.5(1.46)	157(0.11)
		HGA3	+	17	29.7(1.48)	157(0.14)
		HGB12	+	12	42.9(1.82)	157(0.17)

[†] Hi-Line, Canadian Red and McNeal are hard wheat non-transgenic controls. Non-transgenic Hi-Line (*Pina-D1a/Pinb-D1b*) is listed for comparative purposes only and was not used in any cross

[‡] Puroindoline genes present at the *Ha* locus. The *Pina-D1a* and *Pinb-D1a* alleles produce functional PINA and PINB, respectively. The *Pina-D1b* and *Pinb-D1e* alleles are null alleles for PINA and PINB, respectively. The *Pinb-D1b* allele produces a non-functional PINB.

[§]Hi-Line derived transgenic lines contain either *Pina* or *Pinb* transgene. Recombinant progeny lines are homozygous for *Pina-D1a*/*Pinb-D1e Ha* locus from CR or *Pina-D1b*/*Pinb-D1a Ha* locus from McN with (+) or lacking (-) added transgene (*Pina* or *Pinb*).

[¶] Determined by Single Kernel Characterization System 4100 (SKCS) (Perten Instruments, Springfield, IL) from two replications in rain-fed and irrigated environments.

[#] Determined by Infratec 1225 Grain analyzer from two replications in rain-fed and irrigated environments.

Milling Traits Analysis**.**

The effect of *Pin* addition upon flour yield and particle size distribution was measured (Table 2.2). The results indicate significant differences in flour yield and particle size distribution upon addition of either *Pin* transgene (Table 2.2). Flour yield was significantly reduced for CR *Ha* locus genotypes with added *Pinb* and McN *Ha* locus genotypes with added *Pina*, when compared to their respective negative control group without an added transgene ($p<.0001$). However, flour yield was not significantly different when comparing genotypes carrying the CR *Ha* locus and a *Pina* transgene compared to CR *Ha* locus transgene negative controls and when comparing the effect of *Pina* vs. *Pinb* in McN *Ha* locus genotypes (Table 2.2). Seeds from all genotypes lacking a transgene were harder (Table 2.1) and yielded flours with larger particle sizes compared to genotypes with added *Pin* which had reduced grain hardness and relatively smaller particle sizes (Table 2.2). Genotypes with the CR *Ha* locus with added *Pinb* and the McN *Ha* locus with added *Pina* were soft (Table 2.1) and yielded flours with and increased amount of particles less than 53 µm ($p<.0001$) and a significantly smaller proportion of particles greater than 150 µm ($p<.0001$) relative to their hard textured genotypes lacking a transgene (Table 2.2, Figure. 2.1 and 2.2). Genotypes with the CR *Ha* locus with added *Pina* and the McN *Ha* locus with added *Pinb* were intermediate in texture and also produced relatively more medium sized particles mainly from 90 to 149 µm relative to transgene negative genotypes. Flour particle sizes differed significantly at 90 to 149 µm ($p<.0001$) particle size range when comparing addition of *Pina* versus *Pinb* to CR *Ha* locus and McN *Ha* locus genotypes.

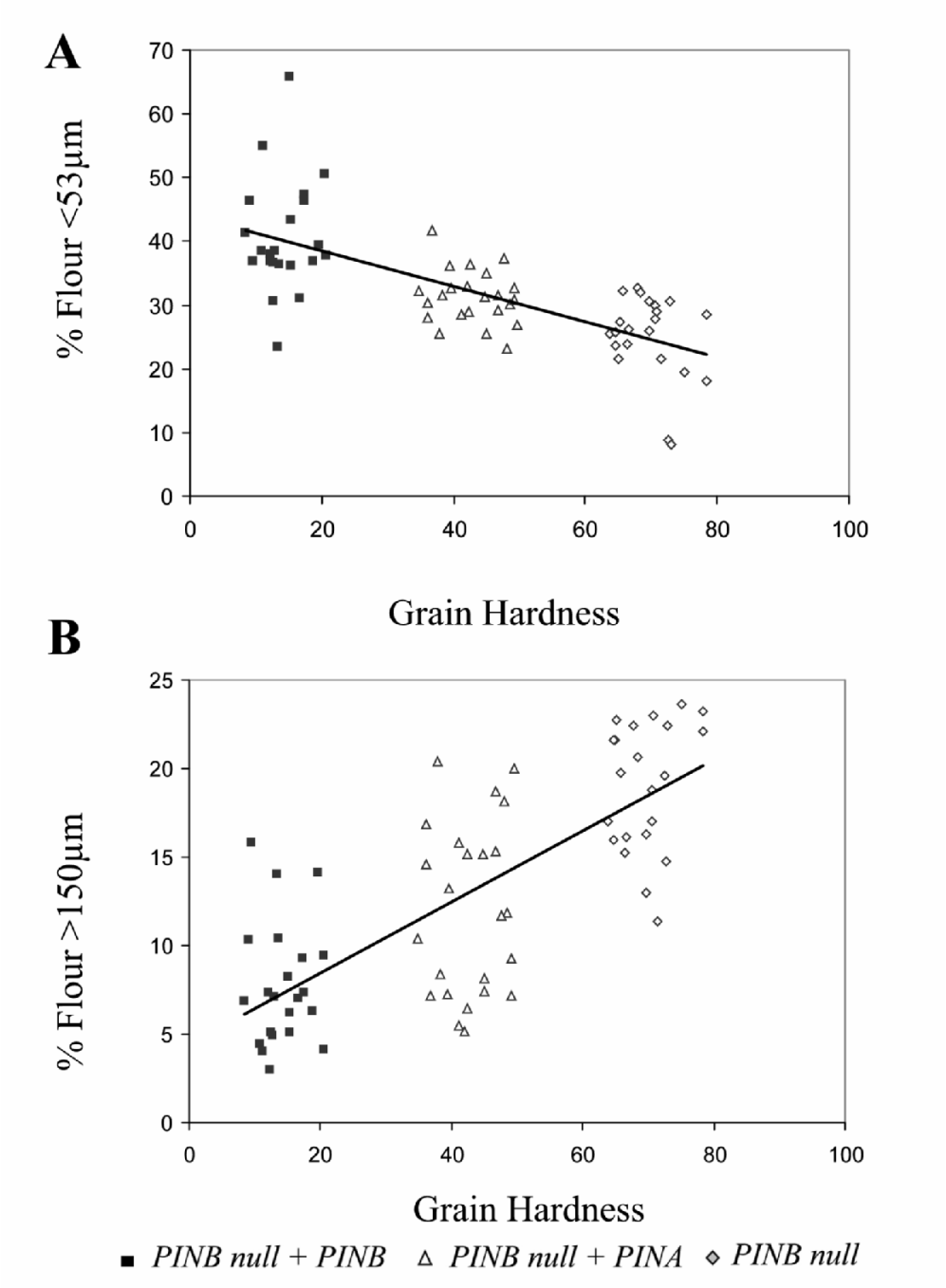


Figure 2.1. Effect of adding PINA and PINB to PINB null genotypes on grain hardness and flour particle size distribution. (A) Scatter plot showing relationship between % flour particles <53 µm and grain hardness. (B) Scatter plot relationship between % flour particles >150 µm and grain hardness. PINB null + PINA denotes genotypes with the *Pina-D1a/Pinb-D1e* locus with added *Pina* transgene, PINB null + PINB denotes genotypes with the *Pina-D1a/Pinb-D1e* locus with added *Pinb* transgene and PINB null denotes genotypes with the *Pina-D1a/Pinb-D1e* locus that lack the transgene. Data is based on means of two environments.

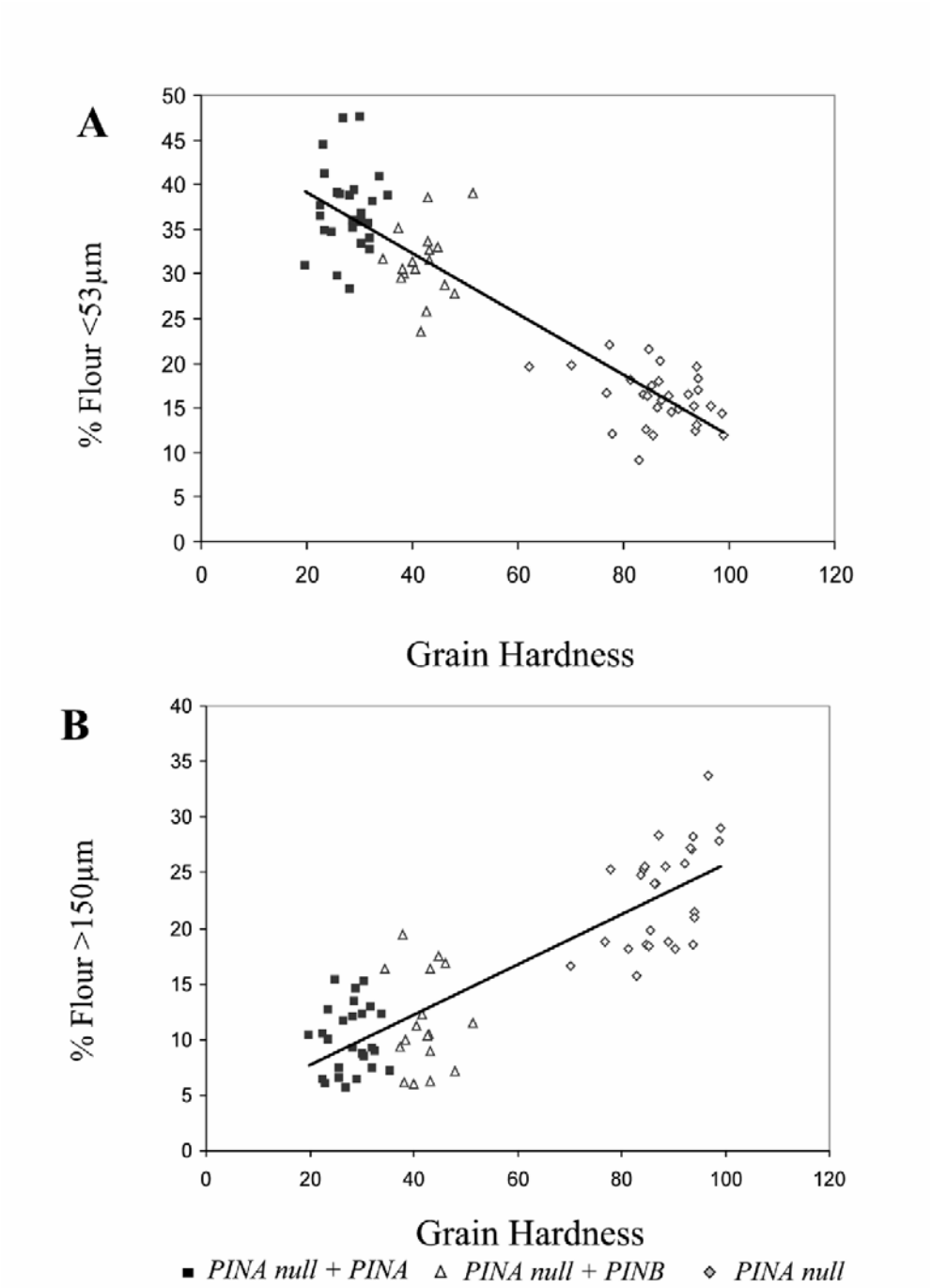


Figure 2.2. Effect of adding PINA and PINB to PINA null genotypes on grain hardness and flour particle size distribution. (A) Scatter plot showing relationship between % flour particles <53 µm and grain hardness. (B) Scatter plot relationship between % flour particles >150 µm and grain hardness. PINA null + PINA denotes genotypes with the *Pina-D1b/Pinb-D1a* locus with added *Pina* transgene, PINA null + PINB denotes genotypes with the *Pina-D1b/Pinb-D1a* locus with added *Pinb* transgene and PINA null denotes genotypes with the *Pina-D1b/Pinb-D1a* locus that lack the transgene. Data is based on means of two environments.

Table 2.2. Genotype class means for flour yield, particle size distribution and comparative statistics associated with flour traits of a subset of F_3-derived progeny derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e*) or McNeal (*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). Presence or absence of transgene is denoted as + or -, respectively. Standard errors are presented in ( ).

Hard wheat parentsa	***Ha***[b]	**Transgenic parent**[c]	**Transgene**	**No. of lines**	**Flour Yield**[¶]	**Size of flour particles (µm)**[d]				
						<53µm	**54 -73µm**	**74 -89µm**	**90 - 149µm**	**≥150µm**
						% of total recovery				
Hi-Line	*Pina-D1a/Pinb-D1b*	None	None	2	66.9(2.5)	16.6(3.8)	13.95(3.6)	21.7(2.2)	44.02.4)	3.6(3.0)
Canadian Red	*Pina-D1a/Pinb-D1e*	None	None	2	73.5(2.5)	17.9(3.8)	12.91(3.6)	16.4(2.2)	31.82.4)	20.9(3.0)
McNeal	*Pina-D1b/Pinb-D1a*	None	None	2	71.7(2.5)	14.8(3.8)	7.15(3.6)	15.8(2.2)	37.2(2.4)	24.9(3.0)
LSD(0.05)					7.1	10.6	10.2	6.3	6.7	8.6
CV%					5.4	18.2	40.4	20.8	11.5	31.0
Null parent			**F_3 lines homozygous for presence or absence of *Pin* Transgene and *Ha* locus**							
Canadian Red	*Pina-D1a/Pinb-D1e*	HG-	-	15	71.9(0.9)	22.8(1.2)	9.0(1.3)	17.1(1.0)	33.1(1.2)	17.9(1.1)
		HGA3	+	12	70.1(0.88)	32.72(1.2)	12.3(1.2)	13.5(0.9)	29.8(1.2)	11.8(1.1)
		HGB12	+	11	56.6(0.92)	40.54(1.2)	17.2(1.2)	15.3(1.0)	19.3(1.2)	7.7(1.10)
								***p* value**		
			HG-vs.HGA3		**0.15**	**<.0001**	**0.08**	**0.01**	**0.05**	**<.0001**
			HG-vs.HGB12		**<.0001**	**<.0001**	**0.00**	**0.02**	**<.0001**	**<.0001**
			HGA3 vs.HGB12		**<.0001**	**0.00**	**0.03**	**0.17**	**<.0001**	**<.0001**
McNeal	*Pina-D1b/Pinb-D1a*	HG-	-	16	70.7(0.8)	16.0(1.1)	10.2(1.2)	15.5(0.9)	36.8(1.1)	21.4(1.2)
		HGA3	+	16	64.5(0.8)	36.77(1.1)	13.7(1.2)	15.7(0.86)	23.0(1.0)	10.5(1.0)
		HGB12	+	12	62.9(1.1)	31.15(1.3)	13.8(1.4)	13.5(1.04)	29.8(1.1)	11.6(1.2)
								***p* value**		
			HG-vs.HGA3		**<.0001**	**<.0001**	**0.04**	**0.85**	**<.0001**	**<.0001**
			HG-vs.HGB12		**<.0001**	**<.0001**	**0.05**	**0.14**	**<.0001**	**<.0001**
			HGA3 vs.HGB12		**0.25**	**<.0001**	**0.95**	**0.12**	**0.00**	**0.48**

Table 2.2 Continued.

[†]Hi-Line, Canadian Red and McNeal are hard wheat non-transgenic controls. Non-transgenic Hi-Line (*Pina-D1a/Pinb-D1b*) is listed for comparative purposes only and was not used in any cross.
[‡]Puroindoline gene present at the *Ha* locus. The *Pina-D1a* and *Pinb-D1a* alleles produce functional PINA and PINB, respectively. The *Pina-D1b* and *Pinb-D1e* alleles are null alleles for PINA and PINB, respectively. The *Pinb-D1b* allele produces a non-functional PINB.
[§]Hi-Line derived transgenic lines contain either *Pina* or *Pinb* transgene. Recombinant progeny lines are homozygous for *Pina-D1a*/*Pinb-D1e Ha* locus from CR or *Pina-D1b*/*Pinb-D1a Ha* locus from McN with (+) or lacking (-) added transgene (*Pina* or *Pinb*).
[¶]Expressed as a percentage of total milling product recovered for each genotype class.
[#]Mean weight of the particles recovered for the given size ranges (μm) for each genotype class.

Bread Baking Quality Traits Analysis

To determine the role of PINs individually and together upon bread baking quality traits, milled straight grade flour from a subset of lines created by Wanjugi et al. (2007) described in the current experiment, together with hard parental controls, were baked and analyzed for their bread quality traits (Table 2.3). Hard wheat cultivars varied in all baking quality traits with McN and HL ranking higher than CR in all bread baking traits (Table 2.3). Hard wheat cultivars varied in all baking quality traits with McN and HL ranking higher than CR in all bread baking traits (Table 2.3). Addition of either *Pin* to CR *Ha* locus genotypes did not result in significant differences in mixing time, loaf volumes, and crumb grain score when compared to the CR *Ha* locus negative control group(Table 2.3). However, addition of either *Pin* to CR *Ha* locus genotypes resulted in significant reductions in mixograph water absorption ($p<0.0001$) relative to their transgene negative controls (Table 2.3). There were no significant differences between the addition of *Pina* vs. *Pinb* to CR *Ha* locus genotypes except for flour ash in which *Pina* addition to McN

Ha locus genotypes affected flour ash ($p<0.0001$) and mixograph water absorption ($p<0.0001$) but did not significantly impact flour protein, mixing time, loaf volume, and crumb grain score relative to transgene negative controls. Conversely, mixograph water absorption, mixing time and loaf volume among McN *Ha* locus genotypes with added PINB were significantly reduced ($p\leq0.0001$) relative to McN *Ha* locus negative controls but flour protein and crumb grain score were relatively unaltered (Table 2.3). There were no significant differences when comparing addition of *Pina* versus *Pinb* to McN *Ha* locus except for mixograph mixing time and loaf volume for which the effect of *Pinb* addition was more negative than that of *Pina*.

Correlation Analysis Between Milling and Bread Baking Quality Traits

A correlation analysis between milling and bread baking traits upon *Pin* addition was computed for both the CR *Ha* locus and the McN *Ha* locus populations (Tables 2.4 and 2.5). The percentage of flour particles in the <53 μm range correlated negatively to grain hardness for both the CR *Ha* locus (r =-0.68) and McN *Ha* locus (r =-0.75) populations (Tables 2.4 and 2.5; Fig. 2.1 and 2.2). In addition, flour particle size proportion >150 μm and grain hardness were strongly correlated in both populations (CR *Ha* r = 0.64; McN *Ha*, r = 0.76) (Tables 2.4 and 2.5, Figure. 2.1 and 2.2). Positive correlations were also observed for grain hardness vs. flour yield and flour ash in both populations and likewise for mixograph water absorption vs. flour yield, hardness, flour protein and flour ash (Tables 2.4 and 2.5). Loaf volume showed significant positive correlation with flour protein, flour ash, mixograph water absorption and mixing time upon addition of *Pin* transgene to either CR *Ha* or McN *Ha*.

Table 2.3. Genotype class means for bread quality traits obtained from straight grade flour of a subset of F_3-derived progeny derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e)* or McNeal (*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). Presence or absence of transgene is denoted as + or -, respectively. Standard errors are presented in ( ).

Hard wheat parents[a]	*Ha*[b]	**Transgenic parent**[c]	**Transgene**	**No. of lines**	**Flour protein (g/kg)**[d]	**Flour ash (%)**	**Mixograph water absorption (%)**	**Mixograph mixing time (min)**	**Loaf volume (cc)**	**Crumb grain score**[e]
Hi-Line	*Pina-D1a /P inb-D1b*	None	None	2	14.2(0.5)	0.375(0.033)	69.5(1.76)	6.1(00.93)	1345	3.0(0.34)
Canadian Red	*Pina-D1a /Pinb-D1e*	None	None	2	13.8(0.5)	0.385(0.033)	64.9(1.76)	2.50(0.93)	942	2.50(0.34)
McNeal	*Pina-D1b /Pinb-D1a*	None	None	2;	14.8(0.5)	0.520(0.033)	70.8(1.76)	7.00(0.93)	1282	4.00(0.34)
LSD(0.05)					1.3	0.093	5.0	2.65	149	0.95
CV%					4.6	11.6	3.7	27.2	6.1	13.4
Null parent	**F_3 Lines homozygous for presence or absence of *Pin* transgene and *Ha* locus**									
Canadian Red	*Pina-D1a /Pinb -D1e*	HG-	-	11	14.9(0.2)	0.401(0.012)	67.5(0.51)	3.59(0.36)	1180(23)	3.37(0.11)
		HGA3	+	13	14.4(0.2)	0.384(0.010)	65.7(0.48)	4.30(0.34)	1148(23)	3.65(0.11)
		HGB12	+	12	14.0(0.2)	0.345(0.012)	65.3(0.50)	3.82(0.36)	1116(27)	3.47(0.14)
						***P* value**				
			HG- vs.HGA3		**0.15**	**0.23**	**<.0001**	**0.15**	**0.33**	**0.09**
			HG- vs.HGB12		**0.00**	**<.0001**	**<.0001**	**0.65**	**0.08**	**0.61**
			HGA3 vs.HGB12		**0.08**	**0.01**	**0.58**	**0.33**	**0.37**	**0.32**
McNeal	*Pina-D1b /Pinb-D1a*	HG-	-	16	15.2(0.2)	0.505(0.010)	70.9(0.45)	6.19(0.32)	1325(14)	3.77(0.10)
		HGA3	+	16	15.0(0.2)	0.387(0.009)	66.5(0.43)	6.04(0.31)	1288(20)	3.65(0.10)
		HGB12	+	10	14.7(0.2)	0.40(00.011)	65.2(0.54)	4.28(0.38)	1155(25)	3.62(0.12)
						***P* value**				
			HG- vs.HGA3		**0.43**	**<.0001**	**<.0001**	**0.74**	**0.23**	**0.43**
			HG- vs.HGB12		**0.06**	**<.0001**	**<.0001**	**<.0001**	**<.0001**	**0.35**
			HGA3 vs.HGB12		**0.20**	**0.33**	**0.06**	**<.0001**	**<.0001**	**0.83**

Table 2.3 Continued.

† Hi-Line, Canadian Red and McNeal are hard wheat non-transgenic controls. Non-transgenic type Hi-Line (*Pina-D1a/Pinb-D1b*) is listed for comparative purposes only and was not used in any cross

‡ Puroindoline genes present at the *Ha* locus. The *Pina-D1a* and *Pinb-D1a* alleles produce functional PINA and PINB, respectively. The *Pina-D1b* and *Pinb-D1e* alleles are null alleles for PINA and PINB, respectively. The *Pinb-D1b* allele produces a non-functional PINB.

§Hi-Line derived transgenic lines contain either *Pina* or *Pinb* transgene. Recombinant progeny lines are homozygous for *Pina-D1a*/*Pinb-D1e Ha* locus from CR or *Pina-D1b*/*Pinb-D1a Ha* locus from McN with (+) or lacking (-) added transgene (*Pina* or *Pinb*).

¶Measured by near infrared reflectance (NIR) in (g/kg).

#Based on 0 (unsatisfactory) to 5 (excellent) scale

Table 2.4. Correlation among milling and bread quality traits of a subset of F_3-derived recombinant lines, homozygous for the Canadian Red (*Pina-D1a/Pinb-D1e Ha* locus) and the added *Pin* transgene (*Pina* or *Pinb*). The progeny lines were derived from crosses between Hi-Line (*Pina-D1a/Pinb-D1b Ha* locus) derived transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12) and Canadian Red (*Pina-D1a/Pinb-D1e Ha* locus). Correlations were computed from 38 entry means.

	% Flour <53µm	>150µm	Flour yield (%)	Grain hardness	Flour protein (g/kg)	Flour ash (%)	Mixograph water absorption(min)	Mixograph mixing time(min)	Crumb grain score
% Flour >150µm	-0.63***								
Flour yield	-0.64***	0.62***							
Grain hardness	-0.68***	0.65***	0.78***						
Flour protein	-0.29	0.11	0.28*	0.36***					
Flour ash	-0.44***	0.53***	0.49***	0.47***	0.37***				
Mixograph water absorption	-0.39***	0.34**	0.14	0.26*	0.64***	0.30**			
Mixograph mixing time	0.16	0.02	0.04	-0.06	-0.06	0.15	0.23*		
Crumb Grain score	0.09	0.04	0.03	-0.09	-0.06	0.18	0.03	0.35***	
Loaf volume	-0.03	0.32*	0.15	0.23	0.34***	0.32**	0.57***	0.63***	0.38***

*, **, and *** indicate statistical significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 2.5. Correlation among milling and bread quality traits of a subset of F_3-derived recombinant lines homozygous for the McNeal, (*Pina-D1b/Pinb-D1a Ha* locus) and the added *Pin* transgene (*Pina* or *Pinb*). The progeny lines were derived from crosses between Hi-Line (*Pina-D1a/Pinb-D1b Ha* locus) derived transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12) and McNeal (*Pina-D1b/Pinb-D1a Ha* locus). Correlations were computed from 42 entry means.

	% Flour <53µm	>150µm	Flour yield (%)	Grain hardness	Flour protein (g/kg)	Flour ash (%)	Mixograph water absorption(min)	Mixograph mixing time (min)	Crumb grain score
% Flour >150µm	-0.75***								
Flour yield	-0.49***	0.46***							
Grain hardness	-0.90***	0.76***	0.54***						
Flour protein	-0.05	-0.00	0.03	0.13					
Flour ash	-0.61***	0.66***	0.42***	0.72***	0.39***				
Mixograph water absorption	-0.61***	0.60***	0.49***	0.64***	0.59***	0.73***			
Mixograph mixing time	-0.09	0.18	0.08	0.13	0.35***	0.24*	0.48***		
Crumb grain score	-0.19	0.10	0.11	0.15	-0.21	-0.01	-0.02	0.09	
Loaf volume	-0.15	0.19	0.31***	0.26*	0.54***	0.34***	0.60***	0.49***	0.24*

*, **, and *** indicate statistical significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Discussion

Grain endosperm texture is a primary determinant of end-use quality traits in wheat (Reviewed by Morris and Rose 1996). Soft wheats have higher particle size index (PSI), increased break flour yield, reduced flour yield, and produce flours that are lower in ash and starch damage as compared to hard wheats (Symes, 1969; Rogers et al., 1993). To clearly understand the effect of puroindolines on milling and baking traits, we investigated the role of either PINA or PINB alone or together in recombinant line populations. This was accomplished by crossing transgenic isolines overexpressing PINA or PINB with a *Pinb* null (CR *Ha* locus) and a *Pina* null (McN *Ha* locus) hard wheat variety. Addition of *Pina* transgene to CR *Ha* locus genotypes and the *Pinb* transgene to McN *Ha* locus genotypes resulted in intermediate textured grain, while addition of *Pina* to McN *Ha* locus genotypes and *Pinb* to CR *Ha* locus genotypes conferred soft endosperm texture (Table 2.1), confirming the results of Wanjugi et al. (2007). Moreover, addition of either *Pin* transgene to McN *Ha* locus or CR *Ha* locus genotypes drastically affected milling behavior and most bread baking quality traits. Flour yield, flour ash and particle sizes decreased with reduction in grain hardness consistent with the results of Hogg et al. (2005). Flour yield, proportion of flour particles >150μm, hardness, and flour protein were all strongly positively interrelated (Tables 2.4 and 2.5). Flour ash was not significantly correlated with the proportion of flour <53 μm in particle sizes in either population but strongly correlated with % of flour >150 μm and grain hardness in both the McN and CR *Ha* locus populations (Tables 2.4 and 2.5) consistent with the expected effect of hardness upon milling. Soft wheats require less energy to mill and give more

efficient separation of bran during milling than hard wheats. As a result, soft wheats yield less flour with lower amounts of ash and protein. Milling behavior difference among soft and hard wheats is determined by the overall strength between starch granule and protein-matrix (Barlow et al., 1973). Soft wheats have less starch granule-protein matrix interaction and hence during milling, the plane of fracture occurs between starch granule and protein interface releasing a high proportion of intact starch granules. On the contrary, in hard wheats the starch-protein matrix interaction is stronger and hence larger and irregular shaped particles mainly composed of whole endosperm cells are produced during milling (Pomeranz et al., 1990). The analysis of milling behavior observed here (Table 2.2, Figure. 2.1 and 2.2) is typical of soft and hard wheats and confirms previous results of Campbell et al. (2001) and Hogg et al. (2005). Wanjugi et al. (2007) reported the association of PINA and PINB in the absence of the other protein on starch, with PINB demonstrating more independent binding to starch than PINA. Here, the presence of either PIN resulted in intermediate texture and a trend toward an increased proportion of medium sized flour particles (Figure. 2.1 and 2.2). Softer texture and higher proportion of smaller particle sizes observed on addition of PINB to CR *Ha* locus than addition of PINA to McN *Ha* locus would further indicate that adding PINB reduces PINs-starch matrix interaction more than adding PINA.

Dubreil et al. (1998) reported an increase in loaf volume and improved crumb grain score after reconstituting PINA null blend flours with PINA. Here, addition of *Pina* to CR and McN *Ha* locus genotypes did not result in significant differences in mixing time, loaf volume or crumb grain score. However, addition of either *Pin* to McN or CR *Ha* locus genotypes significantly ($p<.0001$) reduced mixograph water absorption

compared to the controls. Further, addition of *Pinb* to McN *Ha* locus genotypes significantly affected all bread baking traits, except for crumb grain score (p= 0.35) (Table 2.3). This trend was not seen upon addition of PINB to CR *Ha* locus genotypes except for flour protein, flour ash and mixograph water absorption ($p \leq 0.0001$).The significant reduction in loaf volume upon addition of the *Pinb* transgene to McN *Ha* locus genotypes observed here is consistent with the results of Hogg et al. (2005) and Martin et al. (2006b). The differences in mixograph properties when the *Pin* transgenes were added are not unexpected. These differences could be explained in part by diverse background of the CR and McN parents and by differences in flour yield caused by grain hardness differences. Ultimately, the differences in flour protein and mixograph properties are indicative of other segregating factors apart from overexpressed *Pin* transgene that affect bread baking traits e.g. glutenins, as indicated by a strong positive correlation between loaf volume, flour protein and mixograph properties (Tables 2.4 and 2.5). Puroindoline genes have been shown to significantly affect water hydration traits and milling yield with glutenin and puroindoline genes in combination accounting for more than 50% of the genotypic variance for these traits, except for milling yield (Eagles., 2005). Therefore, the changes in baking traits observed here can not be solely attributed to the overexpressed PIN transgenes, because we can not rule out the contribution of different glutenin alleles and possible puroindolines-glutenins interactions or other factors affecting bread baking quality traits like polar lipids (Chung and Ohm, 2002). However, it is clear that overexpressing *Pins* leads to reduced grain hardness and smaller proportion of particles sizes which could influence water hydration properties and therefore pronounced changes in baking traits.

The results obtained in this study indicate that PINB has a stronger negative effect than PINA on bread baking quality traits. This could be due to softer endosperm texture produced upon addition of PINB than PINA (Table 2.1; Hogg et al., 2004; Swan et al., 2006; Wanjugi et al., 2007) resulting in less desirable flour for bread baking together with a combination of other factors. Alternatively, the effects could be linked to the increased association of PINB to starch (Wanjugi et al., 2007) or specificity in binding residual polar bound lipids on starch than PINA (Wanjugi et al., 2007 unpublished results). Regardless of the mode of action of PINB in starch-lipid association and with other seed storage proteins that possibly affects baking traits, our results show that overexpressing PINs *in vivo* is detrimental to baking traits and supports the studies of Hogg et al. (2005)

Conclusions

These overall results have demonstrated the respective effects of overexpressing either PINA or PINB on milling and bread baking quality traits. Principally, addition of PINs led to increases in small flour particle sizes, and reduced flour yield and flour ash. In addition, increased PIN levels coincide with negative affects on bread baking quality traits especially upon addition of PINB. The effects on bread baking are in part likely the result of modified particle size, starch damage, and flour protein content.

Reference Cited

Baker, R.J. 1977. Inheritance of kernel hardness in spring wheat. Crop Sci. 17:960-962.

Barlow, K.K., M.S. Buttrose, D.H. Simmonds, and M. Vesk. 1973. The nature of starch-protein interface in wheat endosperm. Cereal Chem. 50, 443-454.

Beecher, B., A. Bettge, S E.Smidansky, and M.J. Giroux. 2002. Expression of wild-type pinB sequence in transgenic wheat complements a hard phenotype. Theor. Appl. Genet. 105:870-877.

Blechl, A. E, and O.D. Anderson. 1996. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nat. Biotech. 14:875-879.

Caldwell, K.S., P. Langridge, and W. Powell. 2004. Comparive sequence analysis of the region harboring the hardness locus in barley and its collinear region in rice. Plant Phys. 136:3177-3190.

Campbell, K.G., P.L. Finney, C.J. Bergman, D.G. Gualberto, J.A. Anderson, M.J. Giroux, D. Siritunga, J.Zhu, F. Gendre, C. Roue, A.Verel, and M.E. Sorrells. 2001. Quantitative trait loci associated with milling and baking quality in a soft hard wheat cross. Crop Sci. 41:1275-1285.

Cane, K., M. Spackman, and H.A Eagles. 2004. Puroindoline genes and their effects on grain quality in southern Australian wheat cultivars. Aust. J.Agric.Res. 55:89-95.

Chantret. N., A. Cenci, F. Sabot, O. Anderson, and J. Dubcovsky. 2004. Sequencing of the *Triticum monococcum Hardness* locus reveals good microcolinearity with rice. Mol. Gen. Genomics. 271:377-381.

Clark, J.A., H.H. Love, and E.F. Gaines. 1926. Registration of standard wheat varieties. J. Amer. Soc. Agron.18 (10):920-922.

De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N. Rao Movva, C.T hompson, M. Van Montagu, and J. Leemans. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J.6:2513-2518.

Dubreil, L., S. Meliande, H. Chiron, J-P.Compoint, L. Quillien, G. Branlard, and D. Marion 1998. Effect of puroindolines on the bread making properties of wheat flour. Cereal Chem. 75:222-229.

Eagles, A., K.Cane, R.F. Eastwood, G.J. Hollamby, H. Kuchel, P.J. Martin, and G.B. Cornish. 2006. Contributions of glutenin and puroindoline genes to grain quality traits in southern Australian wheat breeding programs. Aust. J. Agric. Res. 57:179-186.

Gautier, M.F., M.E. Aleman, A. Guirao, D. Marion, and P. Joudrier. 1994. Triticum aestivum puroindolines, two basic cystine-rich seed proteins: cDNA sequence analysis and developmental gene expression. Plant Mol. Biol. 25:43-57.

Gazza, L., F. Nocente, P.K.Ng, and N.E. Pogna. 2005. Genetic and biochemical analysis of common wheat cultivars lacking puroindoline a. Theor. Appl. Genet. 110:470-478.

Giroux, M.J., and C.F. Morris. 1997. A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin. Theor. Appl. Gen. 95:857-864.

Giroux, M.J., and C.F. Morris. 1998. Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. Proc. Natl. Acad. Sci. U. S. A. 95:6262-6266.

Giroux. M.J., L. Talbert., D.K. Habernicht., S. Lanning., A. Hemphill, and J. M. Martin. 2000. Association of puroindoline sequence type and grain hardness in hard red spring wheat. Crop Sci. 40:370-374.

Greenwell. P., and J.D. Schofield. 1986. A starch granule protein associated with endosperm softness in wheat. Cereal Chem. 63:379-380.

Hogg, A.C., B. Beecher, J.M. Martin, F. Meyer, L. Talbert, S. Lanning, and M.J. Giroux. 2005. Hard wheat milling and bread baking traits affected by the seed-specific overexpression of puroindolines. Crop Sci. 45:871-878.

Hogg, A.C., T. Sripo, B. Beecher, J.M. Martin, and M.J. Giroux. 2004. Wheat puroindolines interact to form friabilin and control wheat grain hardness. Theor. Appl. Genet. 108:1089-1097.

Krishnamurthy, K., and M.J. Giroux. 2001. Expression of wheat puroindoline genes in transgenic rice enhances grain softness. Nat. Biotechnol. 19:162-166.

Lanning, S.P., L.E. Talbert, C.F. McGuire, H.F. Bowman, G.D. Carlson, J.L. Jackson, G.D. Eckhoff, R.N. Kushnak, R.N. Stougaard, and G.F. Stallknecht. 1995. Registration of ‘McNeal’ wheat. Crop Sci. 34:1126-1127.

Lanning, S.P., L.E. Talbert, F.H. McNeal, W.L. Alexander, C.F. McGuire, H. Bowman, G. Carlson, G. Jackson, J. Eckhoff, G. Kushnak, V. Stewart, and G. Stallknecht. 1992. Registration of Hi-Line wheat. Crop Sci. 32:283-284.

Law, C.N., C.F. Young, J.W.S. Brown, J.W. Snape, and J.W. Worland. 1978. The study of grain protein control in wheat using whole chromosome substitution lines. P. 483-502. *In* Seed Protein Improvement by Nuclear Techniques, International Atomic Agency, Vienna, Austria.

Lillemo, M., and C.F. Morris. 2000. A leucine to proline mutation in *puroindoline b* is frequently present in hard wheats from Northern Europe. Theor. Appl. Genet.100:1100-1107.

Martin, J.M., F.D. Meyer, E.D. Smidansky, H. Wanjugi, A. E. Blechl, and M.J. Giroux. 2006. Complementation of the Pina (Null) allele with the wild type Pina sequence restores a soft phenotype in transgenic wheat. Theor. Appl. Genet.113:1563-1570.

Martin, J.M., F.D. Meyer, C.F. Morris, and M.J. Giroux. 2006. Pilot scale milling characteristics of transgenic isolines of a hard wheat over-expressing puroindolines. Submitted to Crop Sci.Sept. 2006.

Martin, J.M., R.C.Frohberg, C.F. Morris, L.E. Talbert, and M.J.Giroux. 2001. Milling and bread baking traits associated with puroindoline sequence type in hard red spring wheat. Crop Sci. 41:228-234.

Mattern, P.J., R. Morris, J.W. Schmidt, and V.A. Johnson. 1973. Location of genes for kernel properties in the wheat cultivar 'Cheyenne' using chromosome substitution lines. p. 703-707. *In* E.R. Sears and L.M.S. Sears (ed.) Proc 4th Int. Wheat Genetics Symp., Columbia, MO. 1-6 Aug 1973. Agric. Exp. Stn., Univ. of Missouri, Columbia, MO.

Morris, C.F., G.A. Greenblatt, A.D. Bettge, and H.I. Malkawi. 1994. Isolation and characterization of multiple forms of friabilin. Cereal Sci. 21:167-174.

Morris, C.F. 2002. Puroindolines: the molecular genetic basis of wheat grain hardness. Plant Mol. Biol. 48:633-647.

Morris, C.F., and S.P. Rose. 1996. Wheat. P. 3-34. *In* Cereal grain quality. Chapman and Hall, New York.

Morris, C.F., M. Lillemo, G.M. Simeon, M.J. Giroux, S. Babb, and K. Kidwell. 2001. Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci. 41:218-228.

Oda, S., and J.D. Schofield, J.D. 1997. Characterisation of friabilin polypeptides. Cereal Chem. 26:29-36.

Ohm, J.B., and O.K. Chung. 2002. Relationships of free lipids with quality factors in hard winter wheat flours. Cereal Chem. 79(2):274-278.

Pan, Z., W. Song, F. Meng, L. Xu, B. Liu, and J. Zhu, J. 2004. Characterization of genes encoding wheat grain hardness in Chinese cultivar GaoCheng 8901. Cereal Chem. 81:287-289.

Pomeranz, Y., and P.C. Williams. 1990. Wheat grain hardness: its genetic, structural and biochemical background, measurements and significance. In: Pomeranz Y (ed) Advances in cereal Science and technology, vol.10. American Association of cereal chemistry, St Paul, Minn., pp 471-544.

Riede, C.R., and J.A. Anderson. 1996. Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci. 36:905-909.

Rogers, D.E., R.C. Hoseney, G.L. Lookhart, S.P. Curran, W.D.A. Lin, and R.G. Sears. 1993. Milling and cookie baking quality of near-isogenic lines of wheat differing in kernel hardness. Cereal Chem. 70:183-187.

Slaughter, D.C., K.H.. Norris, and W.R. Hruschka.1992. Quality and classification of hard red wheat. Cereal Chem.69:428-432.

Sourdille, P., M.R. Perretant, P. Charmet, M.F. Leroy, P. Gautier, J.C. Joudrier, J.C. Nelson, M.E. Sorrells, and M. Bernard. 1996. Linkage between RFLP markers and genes affecting kernel hardness in wheat. Theor. Appl. Gen. 93:580-586.

Swan, C.G., F.D. Meyer, A.C. Hogg, J.M. Martin, and M.J. Giroux. 2006. Puroindoline a limits binding of puroindoline b to starch and grain softness. Crop Sci. 46:1656-1665.

Symes, K.J. 1965. Inheritance of grain hardness in wheat as measured by particle size index. Aust. J. Agric. Res. 16:113-123.

Symes, K.J. 1969. Influence of a gene causing hardness on the milling and baking quality of two wheats. Aust. J. Agric. Res, 20:971-979.

Wanjugi, H.W., A.C. Hogg, J.M. Martin, and M.J. Giroux. 2007. The role of puroindoline a and b individually and in combination on grain hardness and starch association. Crop Sci. 47(1):67-76.

CHAPTER THREE

PUROINDOLINES CO-LOCALIZE TO STARCH GRANULE SURFACE AND INCREASE STARCH POLAR LIPID CONTENT

Introduction

The '*Hardness*' (*Ha*) (Symes, 1965, 1969) locus on chromosome 5DS (Mattern 1973; Law et al., 1978) is the primary determinant of wheat grain end-use quality as it largely determines whether wheat is soft (*Ha*) or hard (*ha*) textured (Morris and Rose, 1996). The first evidence for which protein are involved in determining whether wheat was soft or hard came when a 15 kDa complex called friabilin was found to be associated with endosperm texture (Greenwell and Schofield, 1986). Water washed starch of soft wheats have high levels of friabilin while low levels are found in hards (Greenwell and Schofield, 1986). Comparison of friabilin N-terminal sequence with that of puroindoline a (PINA) (Jolly et al., 1993; Morris et al., 1994) and puroindoline b (PINB) (Blochet et al., 1993) indicated that friabilin was primarily a composite of PINA and PINB. PINA and PINB are 60 % homologous at the amino acid level and are characterized by a unique tryptophan-rich domain thought to be involved in binding polar lipids (Blochet et al., 1993; Marion et al., 1994). The PINA tryptophan-rich domain (Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys) includes five Trp and three basic residues, whereas the tryptophan-rich domain is partly truncated in PINB (Trp-Pro-Thr-Lys-Trp-Trp-Lys), consisting of only

three Trp and two basic residues (Blochet., 1993). *Pina* and *Pinb* have been cloned (Gautier et al., 1994) and reported to be tightly linked to *Ha* (Sourdille et al., 1996; Giroux and Morris, 1998). The hexaploid wheat *Pin* genes are located within a 25 kb segment of DNA (Chantret et al. 2004) rendering recombination between them negligible. Soft wheats possess the wild type *Pina* (*Pina-D1a*) and *Pinb* (*Pinb-D1a*) alleles, while mutation of either the *Pina* or *Pinb* coding sequence is associated with hard texture (Giroux and Morris, 1997, 1998; Lillemo and Morris 2000, Morris et al., 2001). To date, numerous *Pin* mutations have been reported among hard wheats (Giroux and Morris, 1998; Lillemo and Morris, 2002; Morris et al., 2001, Pan et al., 2004), with the most common mutations among North American hard wheat varieties being a glycine to serine mutation in *Pinb* (*Pinb-D1b*) and a null mutation for *Pina* (*Pina-D1b*) (Morris et al., 2001). Evidence to show the genetic role of *Pin*s in controlling endosperm texture has been demonstrated in rice (Krishnamurthy and Giroux, 2001) and wheat (Beecher et al., 2002; Hogg et al., 2004; Martin et al., 2006). In rice which lacks puroindoline homologue (Caldwell et al., 1994) transgenic expression of the "soft type" *Pina-D1a* and *Pinb-D1a* genes resulted in significantly softer endosperm texture (Krishnamurthy and Giroux, 2001). In wheat, expression of *Pinb-D1a* in the hard wheat cultivar 'Hi-Line' which contains the *Pinb-D1b* allele (Beecher et al., 2002; Hogg et al.,2004), and expression of *Pina-D1a* in a *Pina-D1b* hard wheat 'McNeal', restored full friabilin function and soft phenotype (Martin et al., 2006).

The mechanism of grain hardness or endosperm strength is most likely determined by the degree of adhesion between starch granules and the surrounding protein matrix (Barlow et al., 1973). Soft wheats have less starch granule-protein matrix

interaction and hence during milling, the plane of fracture occurs between the starch granule and protein interface releasing a high proportion of intact and less damaged starch granules (Barlow et al., 1973; Simmonds et al., 1974). On the contrary, in hard wheats, the starch-protein matrix interaction is much stronger and larger, and irregular shaped particles mainly composed of whole endosperm cells are produced during milling (Pomeranz et al., 1990). As a result, soft wheat flour takes up less water during dough formation and is therefore more suited for making cakes and pastries while hard wheat flour takes up more water and is preferred for baking of bread (reviewed by Morris and Rose, 1996).

The starch granule-protein matrix interaction is apparently regulated by friabilin (PINA and PINB) (Beecher et al., 2002). PINs have been demonstrated to localize on the surface of starch granules and on the aleurone layer (Capparelli et al., 2005; Dubreil et al., 1998) and perhaps form a “non-stick” polypeptide between starch granules and the surrounding protein matrix (Greenwell and Schofield., 1986) that is likely regulated by bound polar lipids. In support of this, Greenblatt et al. (1995) observed high levels of polar bound lipids (glycolipids and phospholipids) and friabilin, associated with water washed starch of soft but not hard wheat varieties. The observation led them to conclude that friabilin components associate with starch through residual bound polar lipids mediated interactions. Further results have shown that PINA and PINB interact on the surface of starch to form friabilin and soft texture (Hogg et al., 2004) but PINB is more limiting to friabilin formation, association to starch and soft endosperm texture than PINA (Swan et al., 2006). More recently, PINA and PINB have been reported to either associate with starch independently to confer intermediate texture or together leading to

full friabilin function and soft endosperm texture (Wanjugi et al., 2007). The association of PINs with the surface of starch granules has been speculated to be either direct, involving lipid bridges between starch or the protein, or indirect where lipids cause conformational change in the protein allowing binding to the starch matrix (Oda and Schofield, 1997). Regardless of the exact mechanism, the association of PINs to lipids is suspected to involve the tryptophan rich domain (Blochet et al., 1993), therefore a mutation that affects the structure and function of the tryptophan-rich region of either protein may influence grain texture.

Wheat lipids have been reported to influence a wide range of end-use quality traits (Graybosch et al., 1993; Dubreil et al., 1998; Ohm and Chung, 2002). Lipids are also essential in the developing and germinating wheat kernel (De La Roche and Andrews, 1973) and provide useful amounts of dietary essential fatty acids (Morrison, 1977). In wheat kernels, the distribution of various lipid classes within tissue fractions varies significantly (Hargin and Morrison, 1980) but can be extracted and separated by thin layer chromatography (TLC) (Morrison et al., 1980; Greenblatt et al., 1995) or HPLC (Ohm and Chung, 1999).The total lipid content of wheat flour consists of polar phospholipids (PL) and glycolipids (GL) and free non polar fractions lipids (FNLs) (Morrison et al., 1980). PL in wheat flour consists primarily of N- acyl phosphatidylethanolamine (NAPE), N-acyl lyso phosphatidlyethanolamine (NALPE), phosphatidylcholine (PC) and N-acyl phospatidylcholine (NAPC). The major GL of wheat seeds are monogalactosyldiglycerides (MGDG) and digalactosyldiglycerides (DGDG) (Morrison and Hargin 1980; Morrison et al., 1980; Ohm and Chung, 2002).

Although Greenblatt et al. (1995) reported that water washed starch prepared from several soft wheat varieties had more starch bound polar lipids than water washed starch of hard wheats, a definitive link between PIN expression and levels of starch associated polar lipids has not been demonstrated. However, some earlier data did indicate that *Ha* may be associated with the level of free polar lipids in wheat (Morrison et al., 1989). Morrison et al. (1989) demonstrated that a deletion of the short arm of chromosome 5D caused large decreases in the levels of free polar lipids and increased grain hardness. Therefore, they suggested that the gene for free polar lipid biosynthesis (*fpl-2*) may be allelic to *Ha*. These results however, did not demonstrate linkage between PIN expression levels and starch bound polar lipids or the individual lipid binding properties of PINA and PINB. In this study, we determined the effect of overexpressing PINA or PINB on flour and starch bound polar lipids as well as PIN immunolocalization. These were done by analyzing F_3 derived recombinant lines homozygous for either the *Pina-D1b/Pinb-D1a Ha* locus (PINA null) or the *Pina-D1a*/*Pinb-D1e Ha* locus (PINB null) with or lacking added *Pina-D1a* or *Pinb-D1a* transgene. The genotypes were tested for grain hardness, protein levels, PIN immunolocalization and polar lipids content of flour and starch.

Materials and Methods

Genetic Materials

The genotypes used in this study are a subset of the recombinant lines described by Wanjugi et al. (2007) and were developed by crossing one transgenic isoline overexpressing PINA (HGA3) or PINB (HGB12) created in the variety ‘HiLine’ (HL)

(Lanning et al., 1992) and described by Hogg et al. (2004) to either a PINB null, 'Canadian Red'(CR); hard white spring (Clark, 1926) or a PINA null, 'McNeal'(McN); hard red spring (Lanning et al., 1995) variety. The transgenic events were selected from the events described by Hogg et al. (2004, 2005) as having good plant vigor and relatively unaltered plant yield, seed size, and seed protein content. Canadian Red has the soft type *Pina-D1a* and a mutant *Pinb-D1e* allele (Morris et al., 2001). *Pinb-D1e* contains a point mutation (TGG –TGA) leading to a change in residue Trp-39 to a stop codon. McNeal possesses the soft type *Pinb-D1a* and a mutant *Pina* allele (*Pina-D1b*) which is an apparent deletion of the *Pina* coding sequence (Giroux and Morris, 1998). Hi-Line has the soft type *Pina-D1a* and the mutant *Pinb-D1b* allele which contains a single point mutation in *Pinb* resulting in a glycine to serine substitution at the 46th residue of PINB (Giroux et al., 2000). The Hi-Line derived transgenic lines (HGA3, HGB12) express the *Bar* gene (De Block et al., 1987) that confers resistance to bialophos (Meiji Seika Kaisha Ltd., Tokyo, Japan) and glufosinate ammonium (AgrEvo, Wilmington, DE). The transgenic overexpression of the *Pina-D1a* (*Pina*) and *Pinb-D1a* (*Pinb*) coding sequences is under the control of the *Glu1Dy10* seed-specific high molecular weight glutenin promoter reported by Blechl and Anderson (1996).

The crosses generated two populations in the HL/McN and HL/CR background, segregating for the native *Ha* locus from HL, CR, or McN and the presence or absence of the transgene (*Pina* or *Pinb*). The F_2 generation for each cross was grown in the greenhouse. To identify F_2 derived F_3 seed pools homozygous for the presence or absence of the transgene, 18 F_2 derived F_3 seeds per line were planted in the greenhouse. Plants were sprayed with 0.1 % glufosinate ammonium at the two leaf stage. The plants were

scored as being resistant or susceptible after 7 days. Resistant plants stayed green while susceptible plants were killed. The F_2 parent was classified as a *Pin* transgene homozygous positive (*Pina* or *Pinb*) or a *Pin* transgene homozygous negative genotype if > 11 consecutive F_3 progeny were glufosinate-resistant or glufosinate-susceptible, respectively. F_3 progeny with mixed herbicide results, most often segregating 3:1 resistant: susceptible, were considered to have a heterozygous-*Pin* F_2 parent. These lines were excluded from subsequent experiments.

To identify the allelic state of the *Ha* locus, leaf tissues were taken from F_3 progeny lines arising from a single F_2 parent homozygous positive or negative for the transgene. Young leaf tissues were pooled from >10 individual F_3 plants from a single F_2 parent and genomic DNA extracted according to Riede and Anderson (1996). The *Ha* locus genotype was determined using cleaved amplified polymorphic marker tests (CAPS) as described in Wanjugi et al. (2007). Identification of genotypes homozygous for the presence or absence of the transgene and for the segregating native *Ha* locus resulted in four genotype classes for each cross. We used two of the four genotype classes in our experiment. The two classes were those inheriting the McN *Ha* locus in the crosses to McN or the CR *Ha* locus in crosses to CR in combination with the presence or absence of an added transgene. The classes inheriting the native *Ha* from HL were not included as the effect of PIN overexpression in the presence of the HL locus has been previously reported (Hogg et al. 2004, 2005). For this study, we used a subset of three random lines from the two genotype classes of F_3 derived lines homozygous for the CR or McN *Ha* locus and the presence of absence of the transgene.

Field Planting and Seed Traits

Genotypes were grown in 2005 in a randomized block design with two replications at the Montana State University-Bozeman Arthur H. Post Field Research Farm as described in Wanjugi et al. (2007). Grain hardness was determined using the Single Kernel Characterization System 4100 (SKCS, Perten Instruments, Springfield, IL) and grain protein was determined using near-infra red transmission using an Infratec 1225 Grain Analyzer (Foss North America Incorporated, Eden Prairie,MN) from a sub sample of grain from each plot. The genotypes were also analyzed for PIN protein levels and polar lipid content. All analyses were replicated twice.

Extraction of Total and Starch Bound Puroindolines

Extraction and fractionation of total TX-114 soluble puroindoline proteins was done as described by Giroux et al. (2003). Quantification of total TX-114 soluble puroindoline proteins was carried out as described by Wanjugi et al. (2007). Water washed prime starch used for friabilin (starch bound PIN) extraction was prepared from straight grade flour as described by Wolf (1964) To obtain straight grade flour, whole grain samples were milled on a Brabender Quadrumat Jr. Mill (Brabender GmbH, Duisburg, Germany) as described by Campbell et al. (2007). Friabilin was extracted by adding 400 µl of 50 % isopropanol/0.5M NaCl to 150 mg of water washed starch, vortexed and samples were incubated for 1 h at room temperature. After incubation, the samples were centrifuged at 13,000 g for 3 min and supernatant transferred to a new tube. To the supernatant, 520 µl of acetone was added and the samples were incubated overnight at -20°C. After incubation at -20°C, the samples were centrifuged at 13,000 g

for 3 min. The supernatant was discarded and the pellet washed once with 500 µl acetone, dried, and suspended in SDS-PAGE sample buffer using 240 µl of sample buffer for each 100 mg starch used minus any reducing agents. Samples were heated at 70 ^{o}C for 15 min, and 10 µL (1X) was loaded into 160 by 160 by 1.5 mm 10 – 20 % Tris-HCl polyacrylamide gels (BioRad, Hercules, CA). Total friabilin was quantified using a Heron (soft wheat) scale ranging from 0.5X to 4X, where 1X equals 10 µl.

Immunofluorescent Localization

Tissue preparation and fluorescent staining were carried out according to the methods of Dubreil et al. (1998) with some modifications. Three randomly selected mature wheat seeds from each genotype class and parental controls described above were fixed in a 4 % paraformaldehyde, 100mM phosphate buffer, pH 7, for two days. After rinsing twice in a 100mM phosphate buffer, pH 7, the seeds were tangentially cut into 2 mm^2 pieces and dehydrated with 70, 85, 100% (v/v) ethanol-water mixtures for two hours each. Samples were placed in 30, 40, 50, 60, 70, 80, 90 % (v/v) mixtures of medium grade London Resin white (Sigma Aldrich, Oakville, Canada)-ethanol for two hours each. Seeds were infiltrated three times in 100 % London Resin white for 12 hours each followed by polymerization at 60°C for 48 hours. 2 µm seed sections were cut with a Diatome MT485 diamond knife on a LKB Ultratome III type 8801A ultra microtome (Pharmacia-LKB, Sweden), collected in a glass well, and heated mildly to adhere the sample to a glass slide. Seed sections were rinsed in phosphate buffered saline (PBS) for 20 minutes and blocked with 2 % goat serum-PBS for one hour at room temperature. Three ten-minute PBS washes were performed on the sections, followed by a one hour

staining with either rabbit anti-PINA or anti-PINB antisera in 2 % goat serum-PBS. Sections were washed in PBS three times for ten minutes each. Anti-PINA and anti-PINB antisera (Dubreil et al., 1998) were diluted 1/400 in 2 % goat serum-PBS. Anti-PINA and anti-PINB antisera treated sections were incubated with a 1/400 dilution of goat anti-rabbit Alexa Fluor 488 conjugated antibody (Invitrogen, Burlington, Ontario) in 2 % goat serum-PBS. Sections were washed three times for 10 minutes each in PBS. A drop of ProLong® Gold Antifade (Invitrogen, Burlington, Ontario) was applied to each section to prevent photo bleaching before adding cover slips. Micrographs were obtained from an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with a Hamamatsu C5985-02 camera (Hamamatsu, Hamamatsu, Japan). Exposure times for phase contrast (0.1s) and fluorescent (1s) micrographs were kept constant for all seed samples. Fluorescent and phase contrast image overlays were performed with Metamorph® Imaging System software (Molecular Devices, Sunnyvale, California), using arithmetic "add" function.

Extraction, Chromatography and Quantification of Lipid Fractions

Whole wheat flour was prepared from seeds ground on a UDY mill fitted with a 0.5mm screen (UDY Co., Fort Collins, Co) while water washed prime starch was isolated from B Brabender Quadrumat Jr. Mill straight-grade flour as described by Wolf (1964). Extraction of bound polar lipids from whole wheat flour and prime starch and group separation of bound polar lipids and thin layer chromatography (TLC) of polar lipid fractions was performed as described by Morrison et al. (1980) and Greenblatt et al.

(1995). Free lipids were first extracted from flour and starch with hexane (sample to hexane ratio of 1:10) for 0.5 h and discarded. Bound lipids were then extracted sequentially from flour and starch with propan-2-ol and water (90:10) at 1:6 and 1:3 sample-to-solvent ratio respectively to ensure that extraction went to completion. Group separation of extractable polar lipids was accomplished as described by Greenblatt. (1995). Bound polar lipids extracts from each line in a genotype class possessing either the McN *Ha* locus or CR *Ha* with or without the added transgene were spotted in triplicate on glass plates (20 X 20 cm) coated with a 0.3 mm layer of Silica Gel (Whatman, Maidstone, UK). TLC plates were then developed with the appropriate solvent, visualized (Greenblatt et al., 1995) and quantified. Phospholipids (PL) and glycolipids (GL) were identified by comparing their R_F values (ratio of the distance traveled by a lipid species compared to the solvent) with commercially available glycolipid and phospholipid standards obtained from Sigma (Sigma Chemical Co, St. Louis, MO). Twenty five mg of each phospholipid standard was dissolved in 2.5 ml chloroform while 1 mg of each glycolipid standard was dissolved in 50 ml chloroform. A loading standard curve of lipid standards with known concentration on a TLC plate was used to quantify the amount of PL and GL from prime starch and flour samples. Data from each lipid species were analyzed via analysis of variance where the model included independent replication genotype class and random lines within genotype classes using PROC GLM in SAS (SAS Institute, Inc, 2004). Data were deleted from the analysis when 0 (non-detectable) was obtained for a lipid species. Least significant difference (LSD) was used to compare genotype class means.

Results

Grain Hardness and Grain Protein Analysis

Table 3.1: Mean grain hardness and grain protein content for a subset of F_3-derived progeny derived from crosses of either Canadian Red (*Pina-D1a/Pinb-D1e)* or McNeal (*Pina-D1b/Pinb-D1a)* with Hi-Line (*Pina-D1a/Pinb-D1b*) derived transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). Presence or absence of transgene is denoted as + or -, respectively. F_3 line values are means of three random lines from each genotype class with standard errors presented in ( ).

Parental Varieties[a]	***Ha***[b]	**Transgenic Parent**[c]	**Transgene**	**Grain Hardness**[d]	**Grain Protein (g/kg)**[e]
Heron	*Pina-D1a/Pinb-D1a*	None	None	30.4	133
Canadian Red	*Pina-D1a/Pinb-D1e*	None	None	71.6(2.12)	136(0.81)
McNeal	*Pina-D1b/Pinb-D1a*	None	None	95.1(2.14)	149(3.06)
HiLine	*Pina-D1a/Pinb-D1b*	None	None	78.9(1.63)	152(3.37)
Null parent	**F_3 lines homozygous for presence or absence of *Pin* transgene and *Ha* locus**				
Canadian Red	*Pina-D1a/Pinb-D1e*	HGA3+	+	43.5(1.37)	146(0.16)
		HGA3-	-	68.5(2.34)	148(0.23)
		HGB12+	+	15.9(1.72)	157(0.11)
		HGB12-	-	70.3(2.42)	152(0.23)
McNeal	*Pina-D1b/Pinb-D1a*	HGA3+	+	28.2(1.92)	157(0.14)
		HGA3-	-	88.4(2.16)	157(0.21)
		HGB12+	+	45.5(1.72)	157(0.17)
		HGB12-	-	84.5(2.10)	158(0.20)

[a]Hi-Line, Canadian Red, McNeal and Heron are hard wheat non-transgenic controls. Heron and non-transgenic Hi-Line and are listed for comparative purposes only and was not used in any cross.
[b]Puroindoline genes present at the *Ha* locus. The *Pina-D1a* and *Pinb-D1a* alleles produce functional PINA and PINB, respectively. The *Pina-D1b* and *Pinb-D1e* alleles are null alleles for PINA and PINB, respectively. The *Pinb-D1b* allele produces a non-functional PINB.
[c]Hi-Line derived transgenic lines contain either *Pina* or *Pinb* transgene. Recombinant progeny lines are homozygous for CR *Ha* locus (*Pina-D1a*/*Pinb-D1e*) or McN *Ha* locus (*Pina-D1b/Pinb-D1a*) with (+) or lacking (-) added transgene.
[d] Determined by single kernel characterization system (SKCS).
[e]Determined by Infratec 1225 grain analyzer.

Grain hardness and grain protein content were analyzed from a subset of three lines randomly selected from each of the two genotype classes, homozygous for CR or McN *Ha* locus and presence or absence of the added transgene. Genotypes lacking the added transgene did not substantially vary in grain hardness and grain protein in either the CR/HL or the HL/McN cross (Table 3.1). Addition of PINB to McN *Ha* locus genotypes and addition of PINA to CR *Ha* locus genotypes resulted in intermediate grain hardness with a mean value of 45.5 and 43.5 respectively (Table 3.1). Addition of PINB to CR *Ha* locus genotypes resulted in much softer texture (grain hardness mean value =15.9) than the addition of PINA to McN *Ha* locus genotypes (grain hardness mean value =28.2). Grain protein did not vary substantially with the addition of either PIN to either McN or CR *Ha* locus genotypes

TX-114 Extractable and Prime Starch- Associated Puroindoline Levels

Total TX-114 extractable and prime starch-associated proteins levels, were extracted as, fractionated using SDS-PAGE, and visualized by direct staining. The amounts of PINA and PINB present in Heron (soft wheat) were used as a reference to which other samples were compared. The amount of total TX-114 extractable PINA in CR was comparable to Heron though no PINB was present in CR (Table 3.2). McN had almost no detectable PINA present and no detectable PINB. Neither CR nor McN accumulated starch bound puroindoline (friabilin) (Table 3.2). The amount of TX-114 extractable puroindoline in PIN null genotypes with added PINA or PINB was comparable to that that observed by Wanjugi et al. (2007a). Addition of PINA to McN and CR *Ha* locus genotypes resulted in higher levels of total TX-114 extractable PIN than

addition of PINB to CR and McN *Ha* locus genotypes (Table 3.2). In contrast, genotypes with the CR *Ha* locus and added PINB had greater starch-bound PIN levels than the McN *Ha* genotypes with added PINA. Most of the added PINB in both McN CR *Ha* locus genotypes was associated with prime starch as opposed to PINA in which a smaller portion of TX-114 extractable protein was present on starch (Table 3.2).

Table 3.2: Puroindoline levels quantified from TX-114 and friabilin extracts for parental controls and F_3 lines derived from crosses of either Canadian Red (*Pina-D1b*/*Pinb-D1e*) or McNeal (*Pina-D1b*/*Pinb-D1a*) with Hi-Line (*Pina-D1a*/*Pinb-D1b*) transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). F_3 line values are means of three random lines from each genotype class.

Null Parent	*Ha*	Transgenic Parent	Transgene	TX-114 Protein [a]			Friabilin [b]		Total Starch-Associated PIN
				PINA	PINB	Total PIN	PINA	PINB	
Heron	*Pina-D1a/Pinb-D1a*	None	None	1.00	1.00	2.00	1.00	1.00	2.00
Canadian red (CR)	*Pina-D1a/Pinb-D1e*	None	None	1.10	0.00	1.10	ND	0.00	0.00
McNeal (McN)	*Pina-D1b/Pinb-D1a*	None	None	0.00	ND[c]	0.00	0.00	ND	0.00
Canadian red	*Pina-D1a/Pinb-D1e*	HGA3	+	4.10	0.00	4.10	1.58	0.00	1.58
		HGB12	+	1.00	2.80	3.80	0.71	2.54	3.25
McNeal	*Pina-D1b/Pinb-D1a*	HGA3	+	4.30	0.90	5.20	2.20	0.92	3.12
		HGB12	+	0.00	3.00	3.00	0.00	2.47	2.47

[a] Means of three randomly selected lines homozygous for the transgene and the *Ha* locus. Values are relative levels of TX-114 extractable PIN proteins compared to Heron (Soft) scale of 1 X to 8 X where 1 X = 6µl (240 µl SDS-PAGE sample buffer/100 mg ground seeds)
[b] Starch associated PINA and PINB (friabilin) levels from water washed starch. Values are relative levels of starch associated PIN proteins compared to Heron (Soft) scale of 1 X to 5 X where 1 X = 10 µl (240 µl SDS-PAGE sample buffer/100 mg starch).
[c] ND (non-detectable, <5% of Heron control value, with method used)

Immunoflourescent Localization of PINA and PINB

To accurately localize PINs in the wheat endosperm, the specificity and cross-reactivity of the anti-PINA and anti-PINB antisera were tested.

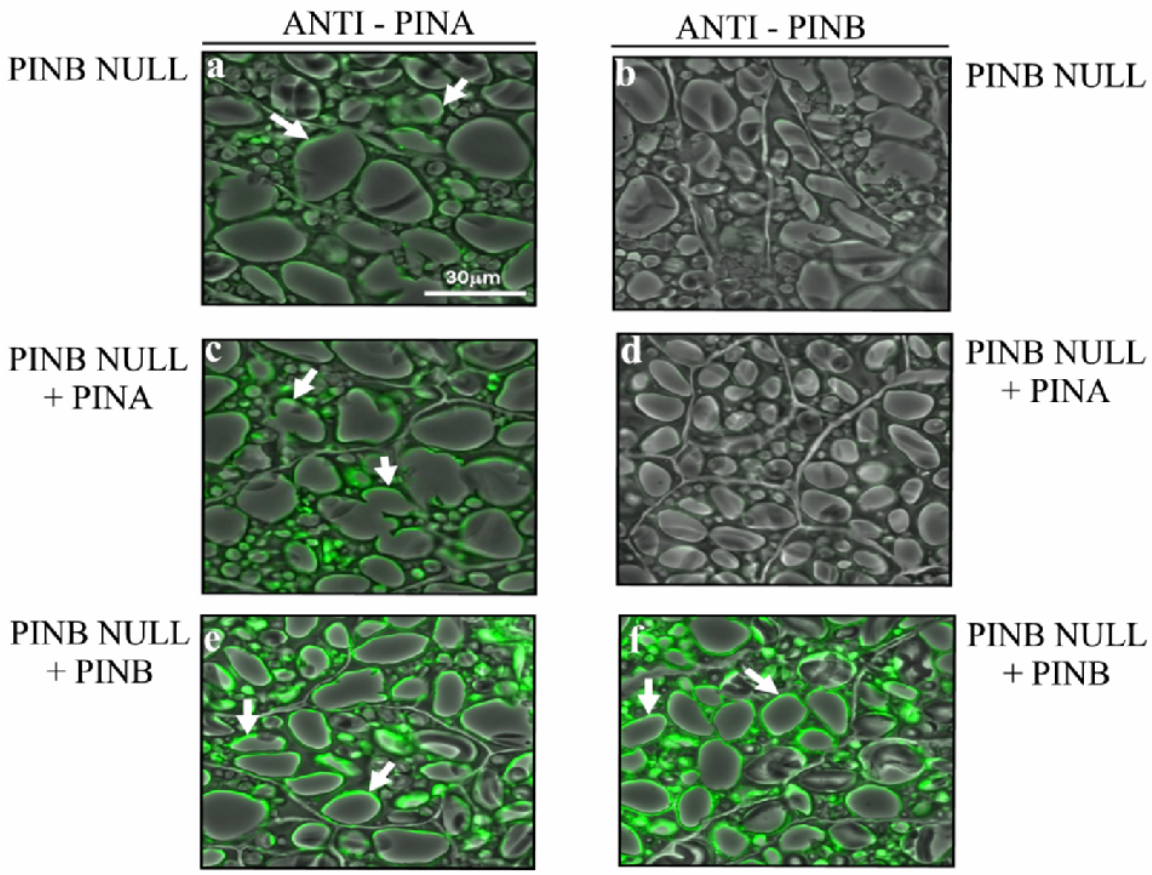


Figure 3.1. Immunolocalization of PINA and PINB using anti-PINA and anti-PINB antisera for random subset of F_3 lines derived lines from crosses of Canadian Red, PINB Null (*Pina-D1a*/*Pinb-D1e Ha* locus) with Hi-Line (*Pina-D1a*/*Pinb-D1b Ha* locus) transgenic isolines with added *Pina* (HGA) or *Pinb* (HGB12). Canadian Red is a hard wheat control. Progeny lines were determined to be homozygous for the added *Pin* transgene and the CR *Ha* locus. Phase contrast and fluorescent micrograph overlays of endosperm cells incubated with rabbit anti-PINA antiserum or anti-PINB antiserum and goat anti-rabbit$^{\text{Alexa 488}}$ antibodies. (A) Hard wheat control, Canadian Red, PINB null seed stained with anti-PINA antiserum and (B) stained with anti-PINB antiserum. (C) PINB null with added PINA seed stained with anti-PINA antiserum and (D) stained with anti-PINB antiserum. (E) PINB null with added PINA seed stained with anti-PINA antiserum and (F) stained with anti-PINB antiserum. Scale bar applies to all micrographs. Arrows indicate accumulation of anti-PINA or anti-PINB antiserum to the starch granule surface

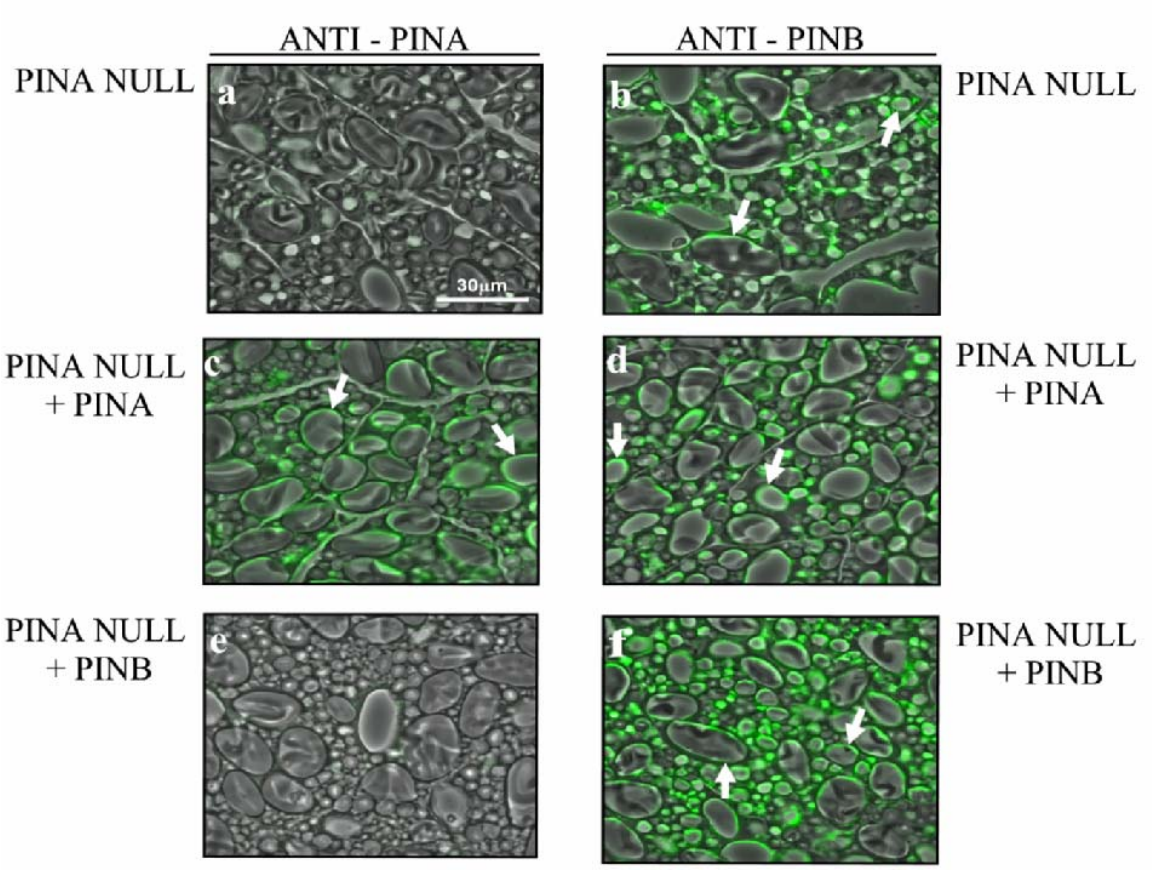


Figure 3.2. Immunolocalization of PINA and PINB using anti-PINA and anti-PINB antisera for random subset of F_3 lines derived lines from crosses of McNeal, PINA null (*Pina-D1b*/*Pinb-D1a Ha* locus) with Hi-Line (*Pina-D1a*/*Pinb-D1b Ha* locus) derived transgenic isolines with added *Pina* (HGA) or *Pinb* (HGB). McNeal is a hard wheat control. Progeny lines were determined to be homozygous for the added *Pin* transgene and the McN *Ha* locus. Phase contrast and fluorescent micrograph overlays of endosperm cells incubated with rabbit anti-PINA antiserum or anti-PINB antiserum and goat anti-rabbit$^{\text{Alexa 488}}$ antibodies. (A) Hard wheat control, McNeal, PINA null seed stained with anti-PINA antiserum, and (B) stained with anti-PINB antiserum. (C) PINA null with added PINA seed stained with anti-PINA antiserum and (D) stained with anti-PINB antiserum. (E) PINA null with added PINB seed stained with anti-PINA antiserum and (F) stained with anti-PINB antiserum. Scale bar applies to all micrographs. Arrows indicate accumulation of anti-PINA or anti-PINB antiserum to the starch granule surface.

CR *Ha* locus genotypes in the absence of a transgene (Figure 3.1b) or CR *Ha* locus genotypes with added PINA (Figure 3.1d) did not react with the anti-PINB antibody.

Similarly, McN *Ha* locus genotypes in the absence of a transgene (Figure 3.2a) or McN *Ha* locus genotypes with added PINB (Figure 3.2e) did not react with the anti-PINA antibody. These observations confirmed the absence of native PINA in McN *Ha* and PINB in CR *Ha* locus genotypes. We assessed the localization of PINA and PINB in the presence or absence of the other protein on starch granules using dry sections of mature seeds which were either expressing native *Ha* or transgenically overexpressed PINA or PINB. In all cases, PINs localized entirely on the surface of starch granules (arrow head) with little to no non-localized fluorescence. The results obtained with the anti-PINA antibody demonstrated that PINA localization is the same in the presence (Figure 3.1e and 3.2c) or absence of PINB (Figure 3.1a and 3.1c). Similarly, the use of the anti- PINB antibody demonstrated that PINB binds exclusively to the surface of starch in the presence (Figure 3.1f and 3.2d) or absence of PINA (Figure 3.2b and 3.2f).

Polar Lipids Extractable from Starch or Starch Polar Lipid Content

The effect of *Pin* addition upon polar lipids present in whole wheat flour and water washed prime starch was determined (Table 3.3). Glycolipids were abundant and mainly composed of DGDG which was 2-3 times higher than MGDG in whole wheat flour of all genotypes. Phospholipids were less (about 25 % of the total polar lipid content) and mostly composed of NAPE. Whole wheat flour of the hard wheats parental varieties, CR, McN and HL possessed lower amounts of glycolipids than the soft wheat Heron, but similar levels of phospholipids (Table 3.3).

Table 3.3. Composition of polar bound lipids from whole wheat flour (µg lipid /1g of flour) for parental varieties and F_3 lines derived from crosses of either Canadian Red (*Pina-D1b*/*Pinb-D1e*) or McNeal (*Pina-D1b*/*Pinb-D1a*) with Hi-Line (*Pina-D1a*/*Pinb-D1b*) transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). F_3 line values are means of three random lines from each genotype class with standard errors in ( ).

				(µg/1 g of whole wheat flour)						
Parental varieties	***Ha***	**Transgene**	**Transgenic Parent**	**Glycolipids[a]**			**Phospholipids[b]**			
				DGDG	**MGDG**	**Total GL**	**NAPE**	**DOPC**	**LPC**	**Total PL**
Heron	*Pina-D1a / Pinb-D1a*	None	None	90.10(5.77)	33.33(1.67)	123.43(17.12)	19.00(1.47)	4.12(1.39)	2.00(0.67)	23.13(4.82)
Canadian Red	*Pina-D1a/ Pinb-D1e*	None	None	65.23(2.88)	21.00(1.57)	86.23(9.42)	10.67(0.67)	2.50(0.29)	5.80(0.83)	18.97(2.84)
McNeal	*Pina-D1b/ Pinb-D1a*	None	None	48.33(4.40)	21.75(3.94)	70.08(9.61)	19.16(0.84)	1.96(0.84)	4.67(1.83)	25.79(3.56)
Hi-Line	*Pina-D1a/ Pinb-D1b*	None	None	45.00(5.00)	16.33(1.85)	61.33(7.27)	18.60(1.87)	3.83(0.44)	6.0(0.57)	28.43(2.76)
Null parent			**F_3 lines homozygous for presence or absence of *Pin* transgene and *Ha* locus**							
Canadian Red	*Pina-D1a/ Pinb-D1e*	+	HGA+	82.33.(5.14)	20.83(1.60)	103.16(9.67)	25.33(1.67)	3.08(0.39)	2.83(0.46)	31.24(2.61)
		-	HGA-	57.67(6.43)	14.33(1.17)	72.00(7.24)	26.33(2.84)	2.67(0.54)	3.50(0.57)	38.67(2.82)
		+	HGB+	110.00(8.16)	24.01(3.96)	134.01(13.68)	21.83(0.74)	3.00(0.22)	3.41(0.52)	28.24(2.15)
		-	HGB-	47.00(2.23)	12.33(1.31)	59.33(5.37)	24.00(0.96)	2.71(0.20)	3.10(0.16)	38.31(2.43)
McNeal	*Pina-D1b/ Pinb-D1a*	+	HGA+	100.00(5.42)	31.50(4.73)	131.5(11.72)	21.67(0.56)	4.67(0.80)	9.50(0.87)	35.84(2.43)
		-	HGA-	48.33(5.98)	18.83(2.07)	67.16(9.05)	28.33(3.57)	2.08(0.15)	3.58(0.52)	33.99(2.81)
		+	HGB+	81.67(7.03)	27.50(4.20)	109.17(9.05)	22.83(1.86)	6.75(0.53)	9.33(0.60)	38.75(1.74)
		-	HGB-	39.67(3.48)	14.17(1.07)	53.84(4.21)	24.17(2.38)	1.13(0.43)	3.33(0.36)	28.63(2.61)
			LSD (0.05) [c]	22.1	4.2	19.7	5.3	1.3	1.3	8.2

[a]Quantified values for whole wheat flour glycolipids, DGDG (digalactosyldiglyceride), MGDG (monogalactosyldiglyceride). Values are means of three randomly selected lines per genotype class homozygous for the transgene and *Ha*; replicated twice.
[b]Quantified values for whole wheat flour phospholipids. NAPE (N-acyl phosphatidylethalonolamine), PC, (Phospatidylcholine), NAPC (N-acyl Phospatidylcholine). Values are means of three randomly selected lines per genotype class homozygous for the transgene and *Ha*; replicated twice
[c]LSD values compares line means within a genotype class.

Table 3.4. Composition of polar bound lipids from water washed starch (µg lipid /1g of flour) for parental controls and F_3 lines derived lines from crosses of either Canadian Red (*Pina-D1b*/*Pinb-D1e*) or McNeal (*Pina-D1b*/*Pinb-D1a*) with Hi-Line (*Pina-D1a*/*Pinb-D1b*) transgenic isolines with added *Pina* (HGA3) or *Pinb* (HGB12). F_3 line F_3 line values are means of three random lines from each genotype class with standard errors in ( ).

Genotype	*Ha*	Transgene	Transgenic parent	(µg/1 g of prime starch)						
				Glycolipids [a]			Phospholipids[b]			
				DGDG	MGDG	Total GL	NAPE	DOPC	LPC	Total PL
Heron	*Pina-D1a/Pinb-D1a*	None	None	34.42(2.88)	3.33(0.89)	37.33(8.87)	7.50(1.44)	ND[a]	ND	7.50
Canadian red	*Pina-D1a/Pinb-D1e*	None	None	ND	ND	ND	ND	ND	ND	ND
McNeal	*Pina-D1b/Pinb-D1a*	None	None	ND	ND	ND	ND	ND	ND	ND
HiLine	*Pina-D1a/Pinb-D1b*	None	None	ND	ND	ND	ND	ND	ND	ND
Null parent			**F_3 lines homozygous for presence or absence of *Pin* transgene and *Ha* locus**							
Canadian Red	*Pina-D1a/Pinb-D1e*	+	HGA+	32.50(2.81	4.00(0.63)	37.00(4.51)	8.67(1.28)	ND	ND	8.67
		-	HGA-	ND	ND	ND	ND	ND	ND	ND
		+	HGB+	78.33(3.07)	13.41(1.49)	86.45(9.92)	12.50(1.18)	ND	ND	12.50
		-	HGB-	ND	ND	ND	ND	ND	ND	ND
McNeal	*Pina-D1b/Pinb-D1a*	+	HGA+	33.33(4.22)	4.67(0.80)	38.16(4.78)	12.08(1.00)	ND	ND	12.08
		-	HGA-	ND	ND	ND	ND	ND	ND	ND
		+	HGB+	45.00(5.47)	10.5(2.01)	54.72(5.89)	10.83(1.17)	ND	ND	10.83
		-	HGB-	ND	ND	ND	ND	ND	ND	ND
			LSD(0.05)[c]	12.3	2.8	12.4	3.5			3.5

[a]Quantified values for water washed prime starch glycolipids, DGDG (digalactosyldiglyceride), MGDG (monogalactosyldiglyceride). Values are means of three randomly selected lines per genotype class homozygous for the transgene (+/-) and *Ha*; replicated twice.

[b]Quantified values for water washed prime starch phospholipids. NAPE (N-acyl phosphatidylethalonolamine), PC, (Phospatidylcholine), NAPC (N-acyl Phospatidylcholine). Values are means of three randomly selected lines per genotype class homozygous for the transgene (+/-) and *Ha*; replicated twice.

[c]ND (non-detectable, <5% of Heron control value, with method used).

[d]LSD values compares line means within a genotype class.

The same trend was observed among hard textured progeny lines lacking the transgene. Whole wheat flour from transgenic soft genotypes had increased levels of bound polar lipids compared to intermediate or hard textured genotypes without the transgene (Table 3.3). In this respect, glycolipids were greatest for the soft genotypes (CR *Ha* locus with added PINB and McN *Ha* locus with added PINA) followed by intermediate textured genotypes (CR *Ha* locus with added PINA and McN *Ha* locus with added PINB), and least for hard genotypes (CR *Ha* and McN *Ha* without transgene). In contrast, the phospholipid content of flour of McN *Ha* and CR *Ha* locus genotypes with or lacking the transgene, remained relatively equivalent (Table 3.3).

To test if PIN expression affects the amount of starch polar lipids, we prepared water washed prime starch from straight-grade flour by standard methods (Wolf, 1964). This allowed us to determine extractable residual polar lipids on the surface of starch and eliminate contamination with fractions originating from the germ, bran, and aleurone layer. Starch polar lipids were much reduced in water washed prime starch compared to whole wheat flour with extractable glycolipids and phospholipids being essentially non detectable when starches from hard wheats were analyzed (Table 3.4). The presence of glycolipids (DGDG, MGDG) from starch of wild type soft (Heron) and upon the addition of either *Pin* to CR *Ha* or McN *Ha* locus genotypes, followed the same pattern to that of whole wheat flour (Table 3.3 and 3.4). However, glycolipids from water washed starch were more prevalent in CR *Ha* and McN *Ha* locus genotypes containing added PINB than in genotypes containing added PINA (Table 3.4). Phospholipids from water washed starch were mainly composed of NAPE, with transgenic soft genotypes having higher levels respectively (Table 3.4). In all cases, our results concurred with previous findings

that, DGDG>MGDG and GL>PL in whole wheat flour and water washed prime starch (Morrison et al., 1980; Morrison and Hargin, 1980). However, a decrease in grain hardness resulted in increased DGDG, NAPE, and total and starch PIN (friabilin) (Table 3.1, 3.2, 3.3, and 3.4).

Discussion

In this study, we investigated the physicochemical association of PINs with polar lipids on the surface of starch granules and their effect upon wheat grain hardness. It is believed that PINs bind membrane polar lipids on the surface of starch via their tryptophan-rich domain (Blochet et al., 1993). A mutation in either *Pina* or *Pinb* coding sequence disrupts or eliminates the ability of PINs to bind to starch, leading to a hard textured phenotype (Giroux and Morris, 1997). While the presence and interaction of both functional PINA and PINB on the surface of starch result in a soft texture (Hogg et al., 2004), PINA and PINB can function independently resulting in an intermediate textured grain (Table 3.1; Wanjugi et al., 2007) and there is no evidence reported to date indicating that PINA and PINB bind to one another. However, there is evidence that PINA and PINB bind cooperatively to starch such that the presence of one PIN increases starch association of the other PIN (Hogg et al. 2004). Moreover, in soft wheats, addition of PINB leads to a greater increase in grain softness than addition of PINA (Swan et al., 2006) and PINB binds in greater abundance to starch granules than PINA (Table 3.2; Wanjugi et al., 2007). Our current results together with others lend support to the hypothesis that soft endosperm texture is due to the presence and cooperative binding of both PINA and PINB on the surface of starch (Table 3.1 and 3.2). Soft genotypes (Table

3.1) had increased TX-114 total PIN (Table 3.2) and increased amounts of PIN on the surface of starch granules (Figure 3.1 and 3.2). Addition of PINB to CR *Ha* locus genotypes led to more starch associated PIN than addition of PINA to McN *Ha* locus genotypes (Table 3.2). Likewise, more PINB associated with starch upon addition of PINB to McN *Ha* locus than addition of PINA to CR *Ha* locus genotypes (Table 3.2). Increased association of PINB to starch was also confirmed by abundant immunofluorescence upon addition of PINB to McN *Ha* and to CR *Ha* locus genotypes (Figure 3.1e and 3.2f). These results confirm recent findings by Wanjugi et al. (2007) that PINB is more specific to starch binding than PINA and concurs with previous results by Swan et al. (2006) that PINB is more limiting to starch binding and grain softness than PINA. Further, immunofluorescent localization of PINA and PINB alone in the absence of the other protein on starch granule surface lend support to previous findings that PINA and PINB can bind to starch granules independently of each other leading to an intermediate endosperm texture. Capparelli et al. (2005) observed that both PINs localize on the aleurone layer and the starchy endosperm. Our results indicate that PINs localize most abundantly on the surface of starch when either or both are present.

The association of friabilin components (PINA and PINB) on the surface of starch granules is mediated by residual polar lipids whose occurrence on starch granules follows that of friabilin (Greenblatt et al., 1995). Whole wheat flour contained more bound polar lipids than water washed starch (Table 3.3 and 3.4). This is due to the occurrence of varying amounts of polar lipids in various kernel fractions such as bran, aleurone and the germ which are absent in starch (Hargin and Morrison, 1980). Higher contents of glycolipids especially digalactosydiglycerides (DGDG) were consistent in both flour and

starch, with addition of PINB to CR *Ha* locus resulting in the highest glycolipids content. The principle phospholipids in flour were negatively charged N-acyphospatidylethalonamines (NAPE) and neutral charged phosphatidycholines (LPC and PC) but only NAPEs were present in water washed starch of soft and non detectable in hard genotypes. Among polar lipids, GL are related to end product quality in hard wheat with increased GL content associated with improved milling, dough mixing, and bread making quality attributes (Ohm and Chung, 2002). In this study, we report that glycolipids (DGDG) and phospholipids (NAPE) residual membrane polar lipids predominate on the surface of starch, and their abundance corresponds with increased starch bound PINs (friabilin) and grain softness. These findings however contrast with Konopoka et al. (2005) who indicated that a higher content of DGDG corresponds to increased grain hardness. Because puroindolines are positively charged (Blochet et al., 1993), we suggest that the main electrostatic interaction of PINs with polar lipids on the surface of starch mostly involves DGDG, MGDG and negatively NAPEs, and that these lipid fractions can be used as a biochemical marker for grain hardness or could explain variation in baking and milling traits observed between hard and soft wheats. Dubreil et al. (1997) investigated differing lipid binding properties of PINA and PINB in puroindoline-lipid mixtures. They observed increased binding of PINA to both wheat PL and GL as opposed to PINB that formed loose lipoprotein complexes with GL. Here, our results show that PINB binds more to GL than PINA on starch; however, both PINs increase the abundance of GL in flour of soft relative to hard genotypes, with this effect, increasing greater in transgenic soft genotypes relative to wild type soft (Heron). Two extra tryptophan residues outside the tryptophan-rich domain in PINB likely enhance

polar lipid affinity of the protein and therefore increased lipid-PINB interaction on the surface of starch. Increased friabilin and bound polar lipids upon addition of PINB to CR *Ha* locus would also suggest that increasing PINB enhances PIN association to polar lipids and starch binding.

Overall, this study shows that PIN expression is strongly associated with GL and PL lipid levels in whole seed meal and starch. Morrison et al. (1989) presented a case for free polar lipid content and grain hardness both being controlled by *Ha*. The evidence was that recombinant lines varying in grain hardness also varied in polar lipid content and that both grain hardness and polar lipid content were linked to *Ha*. Several lines of evidence now indicate that *Ha* indeed directly effects changes in polar lipid content via an unknown mechanism. Certainly, there are several distinct possible explanations for the linkage between PIN expression and polar lipid content. The first is that PINs regulate the expression of polar lipids. The hypothesis seems quite unlikely as PINs are storage proteins with no known or predicted enzymatic activity. The second possibility is that the *Ha* locus contains gene(s) linked to *Pins* that regulate polar lipid content. This too seems unlikely as the *Ha* locus has been cloned from numerous *Triticeae* sp and only the *Pin*, *Gsp* (Grain softness protein), and several pseudogenes lie within close proximity (Chantret et al. 2005). Linked polar lipid biosynthetic genes also seem unlikely as our transgenic lines in which only *Pin* expression is altered contain large increases in polar lipids. The third and most likely possibility is that active PINs stabilize polar lipids on the surface of starch granule membranes preventing their breakdown during seed dry down and maturation. Alternatively, the low levels of GL and PL in hard and high levels in soft wheat flour and starch leaves the intriguing possibility that starch bound polar lipids are

formed during later stages of kernel maturation when PIN expression is highest in soft (Hogg et al., 2004) or lipid degradation occurs during kernel ripening and maturation in hard wheats. Nevertheless, these hypotheses together with that of genetic control of residual polar lipids biosynthesis in relation to *Ha* should be tested in further studies.

Conclusions

This study has shown that puroindoline expression is associated with increased bound polar lipids on starch. Glycolipids and phospholipids (DGDG and NAPE) contribute most to physicochemical interaction between PINA and PINB on the surface of starch that influence endosperm texture. It is also apparent that PINB binds polar lipids (glycolipids) on the surface of starch with higher affinity than PINA but, abundant polar lipid content is associated with the presence of both PINA and PINB and soft endosperm texture. A possible explanation for these results is that active PINs stabilize polar lipids on the surface of starch granule membranes preventing breakdown during kernel ripening and maturation. These results provide further evidence to support the hypothesis that the association of friabilin components (PINA and PINB) to starch is mediated by bound polar lipids that affect endosperm texture and that PINs do not directly bind to starch.

References Cited

Barlow, K.K., Buttrose, M.M., Simmonds, D.H., and Vesk, M. 1973. The nature of the starch-protein interface in wheat endosperm. Cereal Chem. 50, 443-454.

Beecher, B., A. Bettge, E. Smidansky, and M.J. Giroux. 2002. Expression of wild-type Pinb sequence in transgenic wheat complements a hard phenotype. Theor. Appl. Genet., 105:870-877.

Blechl, A.E., and O.D. Anderson. 1996. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nat Biotech 14:875-879.

Blochet, J.E., C. Chevalier, E. Forest, E. Pebay-Peyroula, M.F. Gautier, P. Joudrier, M. Pezolet, and D. Marion. 1993. Complete amino acid sequence of puroindoline, a new basic and cystine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS 329:336-340.

Caldwell. K.S., P. Langridge, and W. Powell. 2004. Comparative sequence analysis of the region harboring the hardness locus in barley and its collinear region in rice. Plant Phys. 136:3177-3190.

Campbell, J.B., J. M. Martin, F. Crutcher, F. D. Meyer, D. R. Clark, and M. J. 2007. Effects on soft wheat (*Triticum aestivum* L.) quality of increased *puroindoline* dosage. Cereal Chem. 84:

Capparelli, R., Amaroso. M.A, Palumbo.D, Iannacoone. M, Faleri.C, and M. Cresti. 2005. Two plant puroindolines colocalize in wheat seed and in vitro synergistically fight against pathogens. Plant Mol. Biol. 58:857-867.

Chantret, N.,J. Salse, F.Sabot, S. Rahman, A. Bellec, B Laubin,I. Dubois, C. Dossat, P. Sourdille, P. Joudrier, M.F. Gautier, L. Cattolico, M. Beckert, S. Aubourg, J. Weissenbach, M. Caboche, M. Bernard, P. Leroy, and B. Chalhoub. 2005. Molecular basis of evolutionary events that shaped the Hardness locus in diploid and polyploidy wheat species Triticum and Aegilops. Plant Cell. 17:1033-1045.

Clark, J.A., H.H. Love, E.F. Gaines. 1926. Registration of standard wheat varieties. J. Amer. Soc. Agron.18 (10):920-922.

De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gossele, N.Rao Movva, C. Thompson, M.Van Montagu, and J. Leemans. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J.6:2513-2518.

De La Roche, and C.J. Andrews. 1973. Changes in phospholipid composition of a winter wheat cultivar during germination at 2 C and 24 C. Plant Physiol. 51:468-473.

Dubreil, L., Meliande, S, Chiron, H, Compoint, J.-P, Quillen, L, Branlard, G, and Marion, D. 1998. Effect of puroindolines on the bread making properties of wheat flour. Cereal Chem.75, 222-229.

Dubreil, L., J. Compoint, and D. Marion. 1997. Interaction of puroindoline with wheat flour polar lipids determines foaming properties. J.Agric.Food Chem.45:108-116.

Giroux, M.J., and C.F. Morris. 1997. A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin. Theor. Appl. Genet., 95, 857-864.

Giroux, M.J., and C.F. Morris.1998. Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. Proc. Natl. Acad. Sci. USA., 95, 6262-6266.

Giroux, M.J., T. Sripo, S. Gerhardt, and J.E. Sherwood. 2003. Puroindolines: their role in grain hardness and plant defense. Biotechnol. Genet. Eng. Rev.20:227-290.

Graybosch, R., C.F. Peterson, K.J. Moore, J, M. Sterns, and D.L. Grant. 1993. Comparative effects of wheat flour protein, lipid, and pentosan composition in relation to baking and milling quality. Cereal Chem. 70:95-101.

Greenblatt, G.A., A.D. Bettge, and C.F. Morris. 1995. The relationship among endosperm texture, friabilin occurrence, and bound polar lipids on wheat starch. Cereal Chem. 72, 172 -176.

Greenwell, P., and J.D. Schofield. 1986. A starch granule protein associated with endosperm softness in wheat. Cereal Chem. 63, 379-380.

Hargin, K.D, and W.R. Morrison. 1980. The distribution of acyl lipids in the germ, aleurone, starch, and non-starch endosperm of four wheat varieties. J. Sci. Food. Agric.80: 877-900.

Hogg, A.C., T. Sripo, B. Beecher, J.M. Martin, and M.J. Giroux. 2004. Wheat puroindolines interact to form friabilin and control wheat grain hardness. Theor. Appl. Genet. 108:1089-1097.

Jolly, C.J., S. Rahman, A.A. Kortt, and T.J.V. Higgins. 1993. Characterization of the wheat Mr 15000 “grain-softness protein” and analysis of the relationship between its accumulation in the whole seed and grain softness. Theor. Appl. Genet. 86:589-597.

Konopka, I., D. Rotkiewicz, and M. Tanska. 2004. Wheat endosperm hardness. Part II. Relationships to content and composition of flour lipids. Eur.Food.Res.Technol.220:20-24.

Krishnamurthy, K., and M.J. Giroux. 2001. Expression of wheat puroindoline genes in transgenic rice confers grain softness. Nature Biotechnology 19:162-166.

Lanning, S.P., L.E. Talbert, C.F. McGuire, H.F. Bowman, G.D. Carlson, J.L. Jackson, G.D. Eckhoff, R.N. Kushnak, R.N. Stougaard, and G.F. Stallknecht .1995. Registration of 'McNeal' wheat. Crop Sci. 34:1126-1127.

Lanning, S.P., L.E. Talbert, F.H. McNeal, W.L. Alexander, C.F. McGuire, H. Bowman, G. Carlson, G. Jackson, J. Eckhoff, G. Kushnak, V. Stewart, and G. Stallknecht. 1992. Registration of Hi-Line wheat. Crop Sci. 32:283-284.

Law, C.N., C.F. Young, J.W.S. Brown, J.W. Snape, and J.W. Worland. 1978. The study of grain protein control in wheat using whole chromosome substitution lines. In: Seed Protein Improvement by Nuclear Techniques, International Atomic Energy Agency, and Vienna, Austria. pp. 483-502.

Lillemo, M., and C.F. Morris. 2000. A leucine to proline mutation in puroindoline b is frequently present in hard wheats from Northern Europe. Theor. Appl. Genet. 100:1100-1107.

Marion, D., M-F. Gautier, P. Joudrier, M.Ptak, M. Pezolet, E. Forest, D.C. Clark, and W. Broekaert. 1994). Structure and function of wheat lipid binding proteins. In: Wheat Kernel Proteins – Mol Functional Aspects. Universita degli studi della tuscia,Viterbo,pp 175-180.

Martin, J.M., F. D. Meyer, E.D. Smidansky, H. Wanjugi, A.E. Blechl, and M.J. Giroux (2006) Complementation of the Pina (null) allele with the wild type Pina sequence restores a soft phenotype in transgenic wheat. Theor Appl Genet 113:1563-1570.

Mattern, P.J., Morris, R, Schmidt, J.W, and V.A. Johnson. 1973. Location of genes for kernel properties in wheat variety 'Cheyenne' using chromosome substitution lines. P.703-707. In: Sears, E.R., and Sears, L.M.S. (eds) Proc. 4th Int. Wheat Genet. Symp., Columbia, MO. 6-11 Aug. 1973. Agric. Exp. Stn., Univ. of Missouri, Columbia, MO.

Morris, C. F., G.A. Greenblatt, A.D. Bettge, and H.I. Malkawi. 1994. Isolation and characterization of multiple forms of friabilin. J. Cereal Sci. 21, 167-174.

Morris, C.F., and S.P. Rose, 1996. Wheat. p. 3–54. In: Henry, R.J. and Kettlewell, P.S. (eds) Cereal Grain Quality. Chapman and Hall, London.

Morris, C.F., M. Lillemo, G.M. Simeon, M.J. Giroux, S. Babb, and K. Kidwell. 2001. Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci. 41:218-228.

Morrison, W. R., C.N. Law, L.J. Wylie, A.M. Coventry, and J. Seekings.1989. The effect of group 5 chromosome on the free polar lipids and bread making quality of wheat. J. Cereal Sci. 9:41-51.

Morrison, W.R. 1977. Cereal lipids. Proc.Nutr.Soc.1977, 36,143-148.

Morrison, W.R., S.L. Tan, and K.D. Hargin. 1980. Methods for the quantitative analysis of lipids in cereal grains and similar tissues.

Oda. S., and J.D. Schofield. 1997. Characterization of friabilin polypeptides. Cereal Sci. 26(1), 29-36.

Pomeranz, Y, and P.C. Williams. 1990. Heat grain hardness: its genetic, structural and biochemical background, measurements and significance. In: Pomeranz Y. (ed) Advances in Cereal Science and Technology, vol.10. American Association of Cereal Chemistry, St Paul, Minn., pp 471-544.

Ohm, J. B., and O.K. Chung. 1999. Estimation of free glycolipids in wheat flour by HPLC. Cereal Chem. 76:873-876.

Ohm, J.B., and O.K. Chung. 2002. Relationships of free lipids with quality factors in hard winter wheat flours. Cereal Chem. 79(2):274-278.

Pan, Z., W. Song, F. Meng, L. Xu, B. Liu, and J. Zhu. 2004. Characterization of genes encoding wheat grain hardness in Chinese cultivar GaoCheng 8901. Cereal Chem. 81:287-289.

Riede, C.R., and J.A. Anderson. 1996. Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci. 36:905-909.

SAS Institute Inc. 2004. SAS/STAT 9.1 User's guide. SAS Institute Inc. Cary, NC.

Simmonds, D.H. 1974. Chemical basis of hardness and vitreosity in wheat kernel. Baker's Digest 48(5) 16-29

Sourdille, P., M.R. Perretant, G. Charmet, P. Leroy, M.F. Gautier, P. Joudrier, J.C. Nelson, M.E. Sorrells, and M. Bernard.1996. Linkage between RFLP markers and genes affecting kernel hardness in wheat. Theor. Appl. Genet. 93, 580-586.

Swan, C.G., F.D. Meyer, A.C. Hogg, J.M. Martin, and M.J. Giroux. 2006. Puroindoline b limits binding of puroindoline a to starch and grain softness. Crop Sci. 46(4): 1656-1665.

Symes, K.J. 1965. Inheritance of grain hardness in wheat as measured by particle size index. Aust. J. Agric. Res. 16:113-123.

Symes, K.J. 1969. Influence of a gene causing hardness on the milling and baking quality of two wheats. Aust. J. Agric. Res, 20:971-979.

Tranquilli,G., D. Lijavetzky., G. Muzzi, and J. Dubcovsky. 1999. Genetic and physical characterization of grain texture-related loci in diploid wheat. Mol.Gen.Genet. 262:846-850.

Wanjugi, H.W., A.C. Hogg, J.M. Martin, and M.J. Giroux. 2007a. The role of puroindoline a and b individually and in combination on grain hardness and starch association. Crop Sci 47 (1):67-76.

Wolf, M.J. 1964. Wheat starch. Pages 6-9 in: Methods in carbohydrate chemistry, Vol 4.R.L.Whistler, ed. Academic press: New York.