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ORIGINAL PAPER

Creation and functional analysis of new *Puroindoline* alleles in *Triticum aestivum*

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Abstract The *Hardness* (*Ha*) locus controls grain texture and affects many end-use properties of wheat (*Triticum aestivum* L.). The *Ha* locus is functionally comprised of the *Puroindoline a* and *b* genes, *Pina* and *Pinb*, respectively. The lack of *Pin* allelic diversity is a major factor limiting *Ha* functional analyses and wheat quality improvement. In order to create new *Ha* alleles, a 630 member M_2 population was produced in the soft white spring cultivar Alpowa using ethylmethane sulfonate mutagenesis. The M_2 population was screened to identify new alleles of *Pina* and *Pinb*. Eighteen new *Pin* alleles, including eight missense alleles, were identified. F_2 populations for four of the new *Pin* alleles were developed after crossing each back to non-mutant Alpowa. Grain hardness was then measured on $F_{2:3}$ seeds and the impact of each allele on grain hardness was quantified. The tested mutations were responsible for between 28 and 94% of the grain hardness variation and seed weight and vigor of all mutation lines was restored among the F_2 populations. Selection of new *Pin* alleles following direct phenotyping or direct sequencing is a successful approach to identify new *Ha* alleles useful in improving wheat product quality and understanding *Ha* locus function.

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Introduction

Variation in wheat (*Triticum aestivum* L.) grain hardness is the single most important trait that determines wheat end-use properties (reviewed in Morris and Rose 1996). Grain hardness variation is controlled predominantly by the *Hardness* (*Ha*) locus, located on the extreme distal end of chromosome 5DS (Mattern et al. 1973; Law et al. 1978; Ram et al. 2002; Campbell et al. 1999). The *Ha* locus functionally consists of the *Puroindoline a* and *b* genes (*Pina* and *Pinb*, respectively) (Giroux and Morris 1998; Wanjugi et al. 2007a). Soft texture (*Ha*) is the result of both genes being in their wild-type allelic state (*Pina-D1a*, *Pinb-D1a*) while hard texture (*ha*) results from mutations in either *Pina* or *Pinb* (Giroux and Morris 1997, 1998). PINA and PINB are cysteine rich proteins which are unique among plant proteins in having a hydrophobic tryptophan-rich domain (Blochet et al. 1993) and together make up the grain hardness marker protein friabilin. Friabilin was originally described as a 15 kDa protein (Greenwell and Schofield 1986; Morris et al. 1994) present in higher levels on the surface of water washed starch prepared from soft wheats relative to the levels found on hard wheat starch. Similarly, Greenblatt et al. (1995) observed that water washed starch from soft wheats had more glyco- and phospholipids than hard wheat water washed starch. The correlation between friabilin and glyco- and phospholipid levels on the surface of starch granules led to the hypothesis that PINs are co-localized to the glyco- and phospholipid rich surface of the amyloplast membrane and

that this localization may be mediated by their tryptophan-rich domain (Giroux et al. 2000).

PINs were conclusively shown to alter grain texture in cereals via transgenic studies. We first demonstrated that PINs can modify grain texture and fungal resistance by transforming rice with both PINs (Krishnamurthy and Giroux 2001; Krishnamurthy et al. 2001). In addition, we demonstrated that *Pin* mutations are causative to hard wheat phenotypes via transgenic complementation studies in which soft phenotypes were achieved when hard wheat *Pin* alleles were complemented with the corresponding wild-type *Pin* allele (Beecher et al. 2002; Martin et al. 2006). Transgenic manipulations have also demonstrated that while both PINA and PINB levels limit grain softness in soft wheats, PINB is a greater limiting factor than PINA (Swan et al. 2006). Further, high levels of either PIN alone can lead to intermediate levels of grain hardness. However, full PIN starch association and a soft grain phenotype is achieved only with both PINs present (Hogg et al. 2004; Wanjugi et al. 2007a). Variation in PIN function affects not only grain hardness but nearly all product quality traits (Hogg et al. 2005; Wanjugi et al. 2007b; Martin et al. 2008) and associated milling effects are also seen in pilot scale milling studies (Martin et al. 2007).

While transgenic manipulation could be used to create nearly any desired level of grain hardness, non-transgenic manipulations of the puroindolines in hexaploid wheats are limited due to limited *Pin* allelic variation. The vast majority (>95%) of US hard wheat germplasm contains one of two *Ha* locus mutations (*Pina-D1b* or *Pinb-D1b*) (Morris et al. 2001). Despite this small level of diversity, it is known that hard wheats carrying the *Pinb-D1b* allele wheats are ~7 hardness units softer and superior in milling and bread baking properties to hard wheats carrying the *Pina-D1b* allele (Martin et al. 2001, 2007). Similarly, the addition of an A genome *Ha* locus to a soft wheat decreased grain hardness and modified milling yield and particle size (Campbell et al. 2007). From an end-product quality point of view the more *Pin* variation found or created the greater chance that some alleles will confer improved end-product quality. The *Pina* and *Pinb* missense alleles discovered in various germplasm surveys are shown in Table 1. While these alleles may vary in function somewhat, they each likely retain very little residual function as all were found in hard wheats. New functional *Pin* alleles could be introduced via direct hybridization between *Ae. tauschii* and hexaploid wheat (Gill and Raupp 1987; Cox 1998) or the production of synthetic hexaploids (Warburton et al. 2006) followed by crossing of the synthetic hexaploids to *T. aestivum*. While numerous Pin allelic variants have been reported (Gedye et al. 2004; Massa et al. 2004) (Table 1), they are not useful in terms of understanding PINA and PINB function for two reasons. First, a significant amount of the hardness variation is assignable to both *Ae. tauschii* and durum parents and secondly most of the haplotypes contain a large number of amino acid changes relative to *Pina-D1a* and *Pinb-D1a* (Table 1). In addition, all of the *Pin* alleles appear to condition a soft phenotype such that they appear limited as a source of grain hardness variation (Gedye et al. 2004).

Therefore, the creation of new *Pin* alleles in one background where each contains a single amino acid change via target-selected mutagenesis is the best approach for creation of new *Puroindoline* alleles in hexaploid wheat. The new alleles would have the potential to improve understanding of PIN function as well as improving wheat end

Table 1 Naturally occurring *Puroindoline* missense alleles in *T. aestivum* or *Ae. tauschii*

Allele[a]	Mutation[b]	Reference	Grain Hardness[c]
T. aestivum			
Pina-D1a	WT	Giroux and Morris (1997)	
Pina-D1f[d]	R86Q	Massa et al. (2004)	
Pina-D1 m	P63S	Chen et al. (2006)	
Pinb-D1a	WT	Giroux and Morris (1997)	
Pinb-D1b	G75S	Giroux and Morris (1997)	73 ± 14
Pinb-D1c	L89P	Lillemo and Morris (2000)	80 ± 14
Pinb-D1d	W73R	Lillemo and Morris (2000)	68 ± 14
Pinb-D1 l	K74E	Pan et al. (2004)	
Pinb-D1q	W73L	Chen et al. (2005)	
Pinb-D1t	G76R	Chen et al. (2006)	
Pinb-D1v	A8T, L9I	Chang et al. (2006)	
Pinb-D1w	S144I	Chang et al. (2006)	
Ae. tauschii			
Pina-D1i[e]	R86Q, R49S	Gedye et al. (2004)	
Pina-D1j[e]	R86Q, P136R	Gedye et al. (2004)	
Pinb-D1h[f]	14 Missense	Massa et al. (2004)	
Pinb-D1j	9 Missense	Massa et al. (2004)	

[a] The updated Allele designations from the Catalogue of Gene Symbols for wheat: McIntosh et al. (2007) were used for this table. Alleles *Pina-D1b*, *Pina-D1k*, *Pina-D1l*, *PinaD1n*, *Pina-D1P*, *Pina-D1q*, *Pinb-D1e*, *Pinb-D1f*, *Pinb-D1g*, *Pinb-D1p*, *Pinb-D1r*, *Pinb-D1s*, *Pinb-D1u*, *Pinb-D1aa*, and *Pinb-D1ab* result from gene deletion, base deletion (frame shift mutation), or nonsense mutations, and so are not included here. Allele *Pina-D1g* carries one silent mutation and encodes an identical protein to *Pina-D1a*

[b] Alleles are numbered relative to the starting methionine of the proteins

[c] Single Kernel Characterization System hardness index, average ± SD reported for the near isogenic lines developed from some of the *pinb* natural missense mutations in Alpowa background. The reference Alpowa parent had a SKCS hardness average of 24 ± 14 (Morris and King 2008)

[d] Alleles *Pina-D1c*, *d*, e, *f*, *h* and *o* encode an identical protein product

[e] Alleles *Pina-D1i* and *Pina-D1j* were identified in synthetic wheats

[f] Alleles *Pinb-D1h*, *I*, *k*, *m*, *n* and *o* encode an identical protein product

product quality. Direct phenotyping has been used in cases where the target genes confer a detectable phenotype (Zhu et al. 1998; Jander et al. 2003). Our approach described here was to test screening methods useful in identifying new ethylmethane sulfonate (EMS) induced *Pin* alleles. We utilized direct grain hardness phenotyping followed by direct sequencing to identify 18 new *Puroindoline* alleles. The results indicate that this approach will successfully create a large resource of new *Puroindoline* alleles to allow us to accomplish two goals. First to reveal the regions of PINA and PINB critical for conferring softness and second to improve wheat end product quality via incorporation of a broader range of PIN alleles into both soft and hard wheat varieties.

Materials and methods

Plant material

Creation and selection of EMS-induced population

A wheat EMS-induced M_1 population was created using a protocol similar to Slade et al. (2005) with some modifications. Approximately, 2,000 M_0 wheat seeds of the soft white spring cultivar Alpowa (PI566596) were soaked in 1% EMS for 18 h at room temperature followed by decanting of the EMS. Seeds were rinsed under cold running tap water for 5 h before planting individually in the greenhouse in 4 × 4 cm cells filled with a peat moss soil mix (Sunshine Mix #1, Sun Gro Horticulture Inc., Bellevue, WA). Greenhouse conditions consisted of target temperatures of 22°C and 14°C for day and night, respectively, with supplemental lighting providing 400 μE m^{-2} s^{-1} consisting of 1,000-W metal halide lamps on to provide a 16 h/day. Plants were watered as needed with a 100 ppm N–P–K solution (Peters General Purpose Plant Food, The Scotts Company, Marysville, OH). One thousand heads from surviving M_1 plants were planted as head rows in 0.6 m rows spaced 30 cm apart at the Arthur H. Post Field Research farm near Bozeman, MT in April 2006. Single heads from individual plants as well as a bulk harvest of all remaining heads were conducted on the 630 fertile rows by hand sickling followed by threshing using a single plant thresher (Bill's Welding, Pullman, WA). Kernel hardness and seed weight were determined using the Single Kernel Characterization System (SKCS) 4100 (Perten Instruments, Springfield, IL) (Martin et al. 1993) on 50 seeds from each bulk seed sample.

Creation of F_2 populations

The SKCS data from 630 M_2 families was sorted to give two subgroups consisting of lines enriched for those containing hard textured seeds. The first group consisted of 121 lines with a grain hardness average above 47 and the second group of 47 lines had grain hardness average less than 47 with a SD above 16. A seed to seed SD above 16 generally indicates a mixture of hardness genotypes (Giroux and Morris 1998). SKCS grain hardness analysis of at least ten individual seeds from each of ten individual heads from each of these M_2 families was used to enrich for homozygous *Pin* mutants for each family. A subsample consisting of candidate homozygote mutants from each family was planted in the greenhouse and used for direct sequencing of *Pina* and *Pinb*. Plants positive for *Pin* mutations were used as pollen source in crosses back to Alpowa non-mutant parent to produce an F_1. One hundred sixty F_1-derived F_2 seeds for each cross along with the 20 M_2:M_4 mutant and Alpowa parental seeds were planted at the Arthur H. Post Field Research farm near Bozeman, MT with within row plant spacing of 15 and 30 cm between rows in May of 2007. F_2:F_3 seeds were harvested from single F_2 plants and threshed using a single plant thresher (Bill's Welding, Pullman, WA). Twenty F_2 individuals per cross were analyzed by both SKCS and *Pin* genotyping and 1–10 individuals per each M_4 parental line as well as Alpowa non-mutant seeds were analyzed by SKCS. SKCS hardness average and SD was measured on a 50-kernel subsample from each individual F_2 and M_4 parent plant. A 5–10 kernel subsample of the same individuals were bulk planted in the greenhouse for DNA extraction followed by genotyping to identify the genotype of parental line.

DNA extraction, PCR amplification, and sequencing

Leaf tissue was collected at the 2–3 leaf stage from individual M_2:M_3 plants and DNA was extracted according to Riede and Anderson (1996). PCR reactions for direct sequencing contained 100 ng of genomic DNA, 15 pmol of each primer, 200 μM of each dNTP, 1× *Taq* DNA polymerase reaction buffer, 0.65 unit of *Taq* DNA polymerase (Promega, Madison, WI), and 2 mM of $MgCl_2$ in 20 μl. GenBank accession numbers are X69913 and X69912, respectively, for *Pina* and *Pinb* and are identical to the *Pina-D1a* and *Pinb-D1a* allele sequences present in Alpowa. The PCR primers were the *Pina* and *Pinb* 3′ primers of Gautier et al. (1994), which bind to the last 22 and 24 nucleotides of the 3′ end of the *Pina-D1* and *Pinb-D1* coding sequence respectively, combined with the *Pina* or *Pinb* forward primers designed by Massa et al. (2004) which bind 55 and 61 nucleotides upstream of the *Pina-D1* and *Pinb-D1* start codon's first nucleotide, respectively. The PCR product sizes of *Pina* and *Pinb* genes were 502 and 508 bp, respectively and covered the 447 bp uninterrupted reading frame of each gene (Simeone et al. 2006). While, the complete mRNAs are 631 and 598 bp, for *Pina* and

Pinb, respectively (Gautier et al. 1994) our ~500 bp PCR products allowed us to sequence all but the last 7–8 codons. The sequence for all four primers is as follows:

Pina-D1 forward: 5′-GGTGTGGCCTCATCTCATCT-3′
PA3: 5′-TCACCAGTAATAGCCAATAGTG-3′
Pinb-D1 forward: 5′-AATAAAGGGGAGCCTCAACC-3′
PB3: 5′-TCACCAGTAATAGCCACTAGGGAA-3′

Cycling parameters were 94°C for 3 min, followed by 40 cycles of 94°C 30 s, 55°C 30 s, 72°C 1 min, followed by a 5-min final extension at 72°C.

PCR products were separated on 1.5% agarose gels followed by gel purification (Qiagene, Valencia, CA) and were then direct sequenced using the forward amplification primer (SeqWright DNA Technology Services, Houston, TX). The GAP4 and Pregap4 programs from Staden Package v1.6.0, 2004, (http://staden.sourceforge.net/staden_home.html) were used to analyze the sequences. Sorting intolerant from tolerant (SIFT) (Ng and Henikoff 2003) was used to predict the tentative impact of mutations on protein function. The membrane affinity of individual amino acids were as reported by Thorgeirsson et al. (1996). Thorgeirsson et al. (1996) described the transfer free energies of synthetic peptides with different amino acids in the same position (relative to glycine) from bilayer to water ($\Delta\Delta G_{bilayer}$) and calculated an individual membrane affinity for each amino acid.

Genotyping of F_2 populations

The same primer pairs and PCR conditions were used to genotype F_2:F_3 lines for four of the crosses between *Puroindoline* mutants and their non-mutagenized parent Alpowa (Table 3). A minimum of four F_3 plants derived from each F_2 line were bulked for DNA extraction followed by genotyping to identify the genotype of each F_2 parent. Polymorphic co-dominant markers were developed based on the differential restriction digestion of *Pinb* mutant alleles *Pinb-D1C146T* and *Pinb-D1C200T* and *Pina* mutant allele *Pina-D1C187T* using restriction enzymes *Hin*dIII, *Tsp*451 and *Msp*1, respectively. Genotyping of the F_2:F_3 lines obtained from the cross between Alpowa and mutant *Pina-D1G212A* was performed using direct sequencing. The association between new alleles and grain hardness was analyzed via analysis of variance using PROC GLM in SAS Institute, Inc., Cary, NC (SAS 2004).

Results

Creation of new *puroindoline* alleles

To obtain and identify new *Ha* locus component gene alleles, we developed an EMS-mutagenized wheat population using the soft white spring variety Alpowa. Alpowa carries the functional alleles of *Puroindoline a* and *Puroindoline b* found in all hexaploid soft wheats, *Pina-D1a* and *Pinb-D1a*, respectively. SKCS analysis of the 630 M_2-derived M_3 bulk seed obtained from an average of four M_2 plants per M_2 family showed that the EMS treatment resulted in reduced average seed size (and vigor) and increased grain hardness relative to Alpowa (Fig. 1). The SKCS grain hardness mean was 41.1 and 24.4 for 630 M_2-derived M_3 bulk seed and ten Alpowa plants seed, respectively. The individual seed weight averages were 32.8 and 38.4 mg when averaged over the 630 M_2-derived M_3 bulk seed and ten Alpowa plants, respectively. From the whole population, a 168 member subpopulation was selected based upon grain hardness (121 lines with grain hardness average above 47 and variable SD) and grain hardness SD (47 lines with grain hardness average less than 47 and grain hardness SD greater than 16) for direct sequencing of *Pina* and *Pinb* coding sequences. The grain hardness of ten individual M_2-derived M_3 single heads from each selected family was

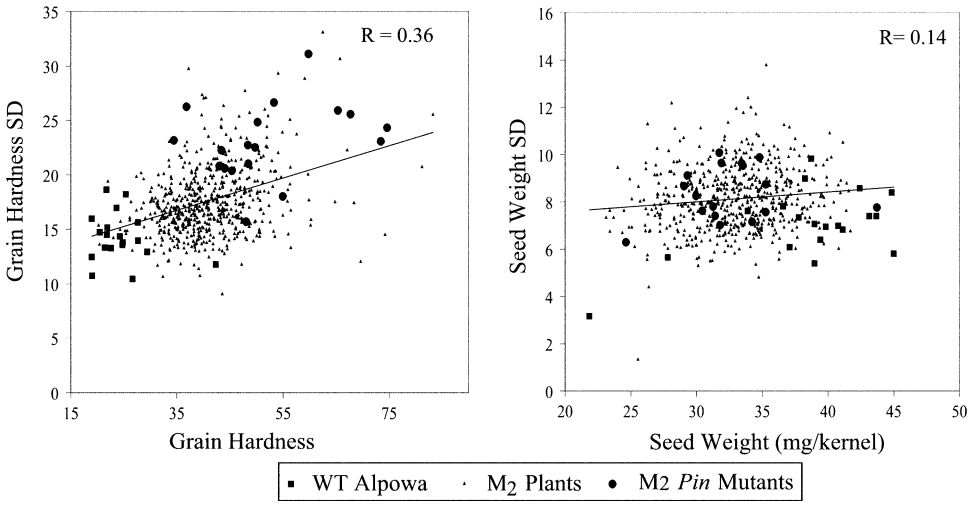

Fig. 1 Single Kernel Characterization System hardness (**a**) and weight (**b**); SD versus averages measured on 630 M_3 seed bulks obtained from EMS-induced M_2 families

measured to identify potential homozygote *Pina* or *Pinb* mutants. Candidate homozygote mutants were selected on the basis of increased grain hardness. *Pina* and *Pinb* were then sequenced from DNA extracted from plants grown from selected heads from each of the 168 lines. In total 18 new *Pin* alleles were identified and 16 of 18 were homozygous for the *Pin* mutation.

Phenotypic and genotypic characterization of new *Puroindoline* alleles

We found nine new alleles for both *Pina* and *Pinb*. Of the nine *Pina* alleles, four were missense and five were nonsense mutations. Of nine *Pinb* alleles, four were missense, three were nonsense mutations and two were silent (synonymous) (Table 2; Fig. 2). One of the *Pina* and one of the *Pinb* nonsense mutants were found in duplicate. Also, one of the *Pina* alleles (C187T)) had a mutation identical to a naturally occurring *T. aestivum Pina* allele (*Pina-D1m* in Table 1). Grain hardness analysis of the M_2-derived M_3 bulk seeds obtained from these mutants was used as an initial indicator of the mutations impact on the function of these genes (Table 2). The majority of M_2-derived M_3 bulk seeds obtained from the *Pina* or *Pinb* mutants showed a grain hardness average higher than the grain hardness mean of the whole population (41.2) and a grain hardness SD higher than 20. As expected most of the nonsense mutations led the list of mutants in grain hardness. The fact that two independent nonsense mutants with the same mutations in *Pina* showed a difference of 39 in grain hardness (Table 2, *Pina-D1G198A* found in M_2 lines 376 and 497) is a good indicator that M_2-derived M_3 bulk seeds are not

Table 2 Allelic series of mutations in *Pina-D1* and *PinbD1* genes found by phenotyping of EMS-mutagenized population

Mutation[a]	Effect[b]	M_2#	SIFT[c]	Wild type aa polarity/membrane affinity[d]	Mutant aa polarity/membrane affinity[d]	Grain Hardness[e]
Pina mutations						
G70A	V24I	155	0.4	NA[f]	NA[f]	36.8 ± 26
G77A	G26D	402	0.01	NA[f]	NA[f]	45.4 ± 20
C112T	Q38Stop	125				65.3 ± 26
C187T	P63S	363	0.01	Non-polar -0.76	Uncharged polar -0.25	50.1 ± 25
G198A	W66Stop	376				73.4 ± 23
G198A	W66Stop	497				34.4 ± 23
G212A	W71Stop	293				67.0 ± 26
C277T	Q93Stop	271				59.8 ± 31
G395A	C132Y	526	0	Uncharged polar	Uncharged polar 1.11	43.4 ± 22
Pinb mutations						
C58T	Q20Stop	498				43.0 ± 21
C146T	S49F	224	0	Uncharged polar -0.25	Non-polar 2.40	34.0 ± 17
C200T	T67I	421	0	Uncharged polar 0.05	Non-polar 2.36	48.7 ± 23
G254A	C85Y	592	0	Uncharged polar	Uncharged polar 1.11	44.0 ± 20
C336T	F112F (Silent)	599				48.0 ± 16
G348A	W116Stop	451				75.4 ± 24
G348A	W116Stop	529				53.2 ± 27
G377A	R126 K	11	0.18	Basic	Basic	55.8 ± 18
G384A	Q128Q (Silent)	303				50.5 ± 22

[a] Nucleotide changes are numbered relative to the starting methionine of each coding sequence. Notation gives original base, position within coding sequence, and altered base

[b] Protein amino acid changes are numbered relative to the starting methionine of the proteins with notation giving original base, position within peptide prior to processing of signal peptide, and altered base

[c] SIFT scores <0.05 are predicted to be deleterious

[d] Membrane affinity or ΔΔG bilayer to water (kcal/mol) of individuals were derived from Thorgeirsson et al. (1996). The membrane affinity of changed amino acids and cysteine were excluded from the experiment performed by Thorgeirsson et al. (1996). The values were ± 0.15 for all of the amino acids except for Q and N whose values were ±0.3

[e] The average and SD of kernel hardness was determined using the SKCS on the bulk of 50 M_3 seeds obtained from individual M_2 families in summer 2006

[f] The values are non-applicable for these mutants due to the occurrence of mutations in signal peptide

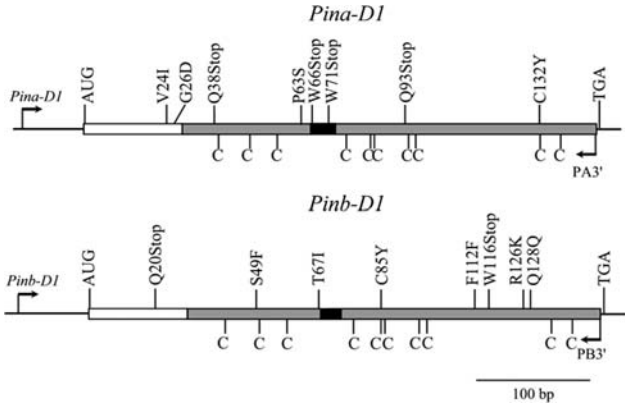

Fig. 2 The positions of the induced mutations within the coding regions (*boxed region*) of *Pina* and *Pinb*. The *white boxed* area denotes the signal peptide region while the *gray/black* boxed regions denotes mature peptide sequence with the *black boxed* region indicating the position of the tryptophan-rich domain. The position of cysteines within PINA and PINB are denoted by '*C*'s'. The position of the amplification primers is as shown with the forward primers residing within the promoter regions and the reverse primers within the 3′ end of the coding sequence

sufficient to test *Pin* gene function or to use for direct phenotypic screening. SIFT is a bioinformatics program which uses sequence homology to predict the impact of missense mutations on protein function and potential phenotype (Ng and Henikoff 2003). SIFT scores <0.05 are predicted to be deleterious. Of the eight *Pina* and *Pinb* missense mutations identified, only two were predicted to be tolerated by SIFT (*Pina-D1G70A*, *Pinb-D1G377A*) (Table 2). Membrane affinities of the mutated and wild type amino acids of PINA and PINB are shown in Table 2 as the individual amino acid affinities reported by Thorgeirsson et al. (1996). The PINA proline to serine change (P63S) in mutant *Pina-D1C187T* PINA resulted in a 0.51 kcal/mol increase in membrane affinity. This value was calculated as 2.15 for the serine to phenylalanine substitution in the *Pinb-C146T* mutant and 2.31 for the threonine 67 to isoleucine substitution in the *PinbD1C200T* mutant. One of the *Pina* mutations and two of the *Pinb* mutations were found in the signal peptides (Fig. 2).

Functional analysis of new *Puroindoline* alleles

In order to test the effects of the new *Pin* alleles upon grain hardness as well as to restore plant vigor and seed weight, the M_3 *Pin* mutant plants were crossed back to Alpowa. The *Pin* genotype of each F_1 plant was confirmed via sequencing before planting their F_2 seeds in the field. Twenty F_2 derived F_3 seed pools were evaluated for grain hardness and seed weight parameters for each of the tested *Pin* mutations. Three of the F_2 populations were created using missense alleles (*Pina*-D1C187T, *Pinb-D1C146T*, *Pinb-D1C200T*) along with a single control nonsense allele (*Pina-G212A*). A plot of grain hardness SD against hardness average showed that all new alleles segregated as expected for a single gene with the exception of the *Pinb-D1C146T* allele for which little variation in grain hardness was observed (Fig. 3). M_4 mutant and Alpowa parents were used as controls for seed characteristics (Table 3). The F_2 homozygote mutants derived from the *Pina* nonsense mutant *Pina-D1G212A* (a *Pina* nonsense mutant) showed a 39 unit increase in grain hardness versus their wild type *Pin* allele group (72.1 vs. 32.8 hardness units, $P < 0.0001$). Ninety-four percent of the variation in grain hardness in this population was attributable to the *Pina* mutation. Kernel weight of three of the F_2 populations was similar to Alpowa and almost 8 mg more than each parental mutant line. The F_2 *Pina-D1C187T* homozygote mutant group averaged 60 in grain hardness and an increase of 34 in grain hardness over its homozygous wild type control group (60.3 vs. 26.3 hardness units, $P < 0.0001$). Greater than 80% of the grain hardness variation in this population was attributable to the *Pina* nonsense mutation. The *Pinb-D1C200T* homozygous group proved as severe as the *Pina* nonsense mutation as its F_2 homozygote mutant group averaged 36.5 units harder than its wild type allele groups 35.1 hardness units (71.6 vs. 35.1 hardness units, $P < 0.0001$). The *Pinb-D1C200T* mutation controlled 90% of the variation in grain hardness in this population. Although the average kernel weight obtained for the *Pinb-D1C200T* F_2 homozygote mutant group of this cross was comparable to the Alpowa average (~1 mg less) the kernel weight average for the other two groups of this population were 4–5 mg less than Alpowa. A 19 mg decrease in the kernel weight of the M_4 parent relative to Alpowa shows that this mutant originally suffered from a severely low kernel weight. The F_2 population derived from *Pinb* missense mutant plant *Pinb-D1C146T* showed a very minor change in grain hardness (5 units) for the homozygote mutant allele group relative to its wild type allele sibling group (34.3 vs. wild type 29.3 hardness units, $P < 0.05$) and accounted for only 28% of the grain hardness variation. In summary for all crosses, the average seed weight measured for F_2 plants showed a near complete restoration of seed weight compared to the parental M_4 plants and Alpowa grown under the same field conditions. F_2 populations for the *Pina-D1G212A*, *Pina-D1C187T*, and the *Pinb-D1C146T* mutations showed approximately 1:2:1 segregation ratios (Table 3). The segregation ratio of F_2 population derived from the *Pinb-D1C200T* mutant deviated from expected Mendelian ratios with more homozygote mutant plants than expected.

Discussion

Induced mutagenesis in adapted elite lines followed by marker-assisted backcrossing is an efficient approach to

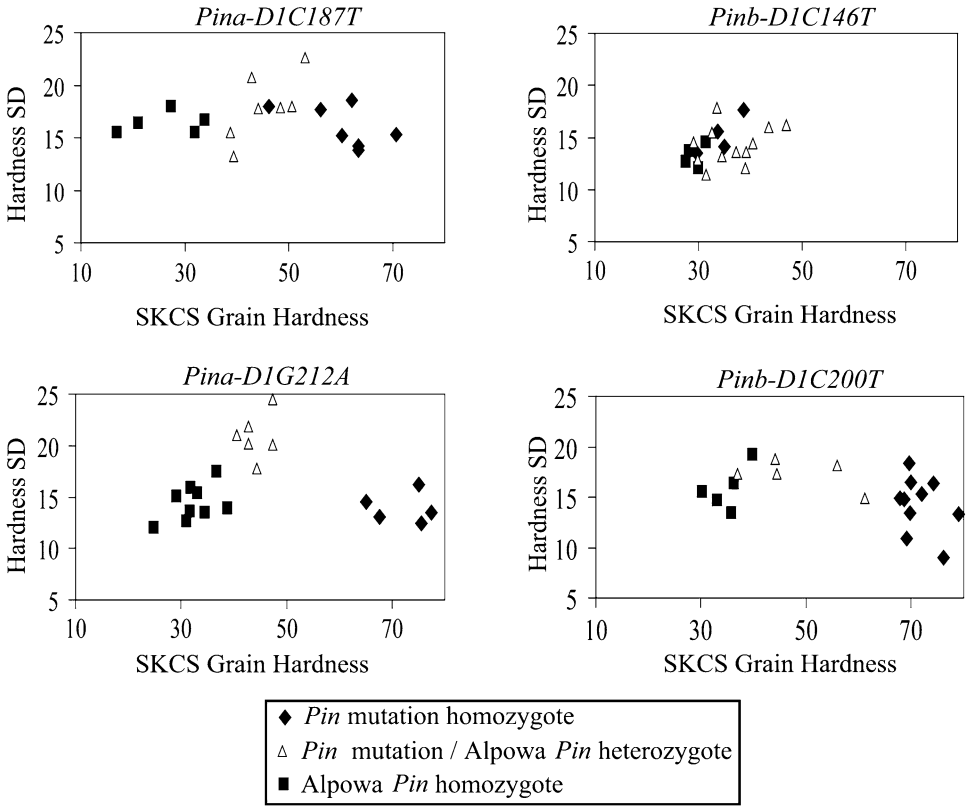

Fig. 3 Single Kernel Characterization System SD versus averages measured on 50-kernel subsamples from 20 individual F_2 plants from crosses of four select *Pin* mutants with WT Alpowa. *Pin* mutant parents are as noted above each graph with each F_2 line classified according to *Pin* allele genotype type as indicated

obtain favorable allelic variants for wheat, the most important staple crop world-wide (Bagge et al. 2007). *Puroindoline* allelic variation provides the genetic basis for most of the phenotypic variation in wheat grain hardness. In order to overcome the lack of *Pin* diversity (Table 1), our goal in this study was to create more allelic diversity for these proteins in hexaploid wheat and then to test the segregation pattern and function of these new alleles via segregating F_2 populations. These new alleles may be useful in improving wheat end use quality when they are incorporated into adapted genotypes. Specifically, hard wheats with reduced grain hardness may be improved in milling yield as suggested by the results of Martin et al. (2001, 2007). Soft wheats with reduced grain hardness may be improved in break flour yield (Hogg et al. 2005; Wanjugi et al. 2007b) or in starch extractability (Feiz et al. 2008). Soft wheats with increased grain hardness would likely have increased particle size but be more readily siftable (Campbell et al. 2007). Our first approach here was to advance an M_1 single head derived M_2 population and measure grain hardness of both bulk seeds and single plants from each M_2 family to identify new *Puroindoline* alleles (Fig. 1). Since we were time constrained we then measured grain hardness on M_3 seeds from M_2 plants, however the chimeric nature of M_1 plants makes them less than desirable for direct mutation screening. It would be more efficient to conduct direct screening after advancing all lines to homozygousity via single seed descent (SSD). Population sizes used for screening for new mutations via direct screening or targeting induced local lesions in genomes (TILLING) vary dramatically. The size of an original M_1- single seed derived M_2 library was reported to be 20,000 for barley (Caldwell et al. 2004) and 10,000 for hexaploid wheat (Slade et al. 2005).

Perhaps the best approach to obtain a high mutation frequency is to use the maximum amount of EMS that still permits plant germination. We started with an M_1 population greater than two times the expected size of the M_2 library and then used the M_1 single head-derived M_2 families (1,000) as individual members of the population planted as field head rows. The relatively low survivability we experienced indicates that there is a high risk of losing individuals in the early generations of selfing due to the segregation of deleterious mutations (Colbert et al. 2001). Since our M_1:M_2 families were segregating 1:2:1 for a new mutation, a sample over a large M_2 family should yield seeds that are equal parts wild type and mutant allele assuming similar viability and fertility between wild type and mutant alleles.

The increased grain hardness mean of 630 M_2-derived M_3 seeds over Alpowa (Fig. 1) was likely the result of reduced seed size and increased protein content. As expected, direct grain hardness phenotyping of the M_2-derived M_3 seeds increased *Pin* mutation frequency

Table 3 Kernel characteristics and *Puroindoline* allelic state of parental lines and F_2 families derived from four crosses between Alpowa non—mutant × new *Puroindoline* mutants

Pin allele group	*Pina-D1* allele	*Pinb-D1* alleles	Grain Hardness[b]	Kernel Wt[b] mg	*n*[c]	χ^2(*P* value)[d]	R^{2e}
F_2 progeny lines[a]							
Pina-D1G212A	*a/a*	*a/a*	32.8 ± 4.2	38.3 ± 4.2	9	4.8(0.09)	0.94
	a/G212A	*a/a*	44.2 ± 2.7	39.8 ± 3.0	6		
	G212A/G212A	*a/a*	72.1 ± 5.4**	38.6 ± 1.6	5		
Pina-D1C187T	*a/a*	*a/a*	26.3 ± 7.1	39.6 ± 5.1	5	1.2(0.54)	0.82
	a/C187T	*a/a*	44.7 ± 5.3	40.7 ± 2.4	8		
	C187T/C187T	*a/a*	60.3 ± 7.6**	42.6 ± 3.3	7		
Pinb-D1C200T	*a/a*	*a/a*	35.0 ± 3.6	34.8 ± 3.4	5	7.5(0.02)	0.9
	a/a	*a/C200T*	48.5 ± 9.8	34.1 ± 5.6	5		
	a/a	*C200T/C200T*	71.6 ± 3.7**	37.7 ± 2.1	10		
Pinb-D1C146T	*a/a*	*a/a*	29.3 ± 1.7	43.2 ± 3.4	4	0.8 (0.67)	0.28
	a/a	*a/C146T*	36.5 ± 5.6	44.5 ± 2.3	12		
	a/a	*C146T/C146T*	34.3 ± 3.7*	40.9 ± 1.9	4		
F_2 population parents							
Alpowa	*a/a*	*a/a*	29.6 ± 2.4	38.9 ± 1.3	10	–	–
Pina-D1G212A[f]	*G212A/G212A*	*a/a*	81.9 ± 6.4	30.6 ± 6.4	10	–	–
Pina-D1C187T[f]	*C187T/C187T*	*a/a*	58.1 ± 7.1	35.7 ± 4.4	9	–	–
Pinb-D1C200 T[f]	*a/a*	*C200T/C200T*	76.23	20.1	1	–	–
Pinb-D1C146T[f]	*a/a*	*C146T/C146T*	46.7 ± 7.1	35.1 ± 4.8	9	–	–

*, ** Denote significance at $P \leq 0.05$ and $P < 0.0001$, respectively, in comparisons of the homozygote wild type versus homozygote mutant within a cross

[a] Three F_2 groups, one parental group from each mutant family and one group of Alpowa non-mutant parent were used to determine kernel characteristics. *Pin* mutation alleles are named for the nucleotide change as listed in Table 2

[b] Mean of kernel characteristics averaged over number of plants per each genotype planted. Kernel characteristics were determined using the SKCS

[c] *n* is the number of plants analyzed per parental lines and the number of genotypes obtained and analyzed per each F_2 family comprised of 20 individuals

[d] χ^2 was obtained from the segregation pattern of F_2 progenies of the crosses between each mutant and Alpowa non-mutant parent

[e] R^2 value shows the proportion of the impact of new *Pin* alleles on grain hardness in comparison of homozygote wild type, homozygote mutant and heterozygote groups in each F_2 population

[f] M_4 parental individuals obtained from M_3 single parent used for sequencing

among selected lines. However, direct selection for *Pin* mutation containing M_2 lines likely did not detect most silent and missense mutations. EMS-induced mutagenesis is a random event; hence any deviations from randomness are due to bias from selection or mutation (Greene et al. 2003). While the overall predicted frequency of stop codon mutations is 4.7%, identification of 45% stop codon mutations from our pre-screened Alpowa-mutagenized population is a good indicator of the biases induced by selecting for hard phenotypes (Table 2). Another reason for an increase in stop codon mutations in our population could be the higher tryptophan content of both PINA and PINB relative to other proteins. Tryptophan and methionine are the only amino acids each coded by a single codon and therefore are not as tolerant as other amino acids to single nucleotide changes. A transition mutation in any of the two bases of the tryptophan codon results in a stop codon. If the frequency of nonsense mutations was used to predict the total mutation rate then the total mutation rate is at least ~1 mutation per 4 kb. However, the screening of a larger EMS-mutagenized population of Alpowa accomplished by direct sequencing over 1,500 kb of *Pina* and *Pinb* together resulted in a total mutation rate of 1 per 13 kb and a 17% rate of nonsense mutations (unpublished data). Our total mutation rate obtained by direct genotyping (unpublished data) is about 13 times higher than that reported for *A. thaliana* (Greene et al. 2003), 22 times higher than rice (Till et al. 2007), 77 times higher than barley (Caldwell et al. 2004) and ~2 times higher than previously observed in hexaploid wheat (Slade et al. 2005). The redundant nature of the hexaploid wheat genome is the most likely explanation for the higher mutation frequency observed in wheat relative to diploid plant species (Slade et al. 2005). All of the mutations were transitions (G to A or C to T) (Table 2),

in accordance with other EMS-mutagenesis studies, since EMS induces G-residue alkylation (Anderson 1995). Phenotyping of M_2-derived M_3 bulk seeds proved non-conclusive (Table 2) in terms of the functional analysis of *Pin* genes for three reasons. First, because M_2-derived M_3 bulk seeds are composed of homozygote mutant and wild type as well as heterozygote seeds. Second, because there is a chance of inequality between the mutant and wild type alleles in the M_2-derived M_3 bulk seeds which may result in a grain hardness bias toward wild type or mutant allele. Finally, mutation in other genes may affect grain texture independent of the *Puroindoline* locus.

The bias caused by selection of harder phenotypes resulted in the identification of 16 homozygous Pin mutants among 18 total mutations which made it easier to cross the mutants back to non-mutant Alpowa. The backcross largely restored plant vigor and seed size as well as allowed us to measure the impact of each mutation on wheat grain texture (Table 3). Grain hardness of seed from individual F_2 plants divided each F_2 population into three distinct classes. The impact of the segregating mutant alleles on grain hardness indicated the efficiency of this approach in creating a broad range of grain hardness phenotypes. The comparison of grain hardness of the F_2 segregating populations with the predicted effects of mutations via SIFT (Table 2) indicates that SIFT was not capable of distinguishing severe mutants from very mild mutations. SIFT predictions of deleterious mutations relies solely on the sequence and the conservation pattern of amino acids (Ng and Henikoff 2003).

The tryptophan-rich domain of puroindolines is hypothesized to confer an affinity for lipids (Marion et al. 1994; Kooijman et al. 1997) and may result in PINA and PINB being localized to the glyco- and phospholipid rich surface of the amyloplast membrane (Giroux et al. 2000). A prediction of secondary structure and measurement of amino acids membrane affinity was used by Giroux and Morris (1997) to hypothesize that the *Pinb-D1b* allele had reduced function due to its glycine 75 to serine mutation. A close examination of our new induced alleles shows that a mutation in threonine 67 to isoleucine immediately adjacent to the tryptophan rich domain of PINB had a severe effect on grain hardness similar to a nonsense mutation (Tables 2, 3). All three missense mutations, P63S in PINA and T67I and S49F in PINB resulted in an increase in membrane affinity. However, the mutation in serine 49 to phenylalanine (*Pinb-D1C146T*) resulted in a significant change in membrane affinity comparable to the other PINB missense mutation (Table 2) but resulted in a very small grain hardness change (Table 3).

In conclusion, similar to SIFT the change in membrane affinity of substituted amino acids in *Puroindoline* mutants was not predictive in terms of the functional analysis (Table 2). Phenotype analysis of F_2 segregating groups after crossing the mutant back to the non-mutant parent is the most efficient way to understand the impact of mutation on the gene function. It decreases the effect of other mutations as well as restores the seed weight and vigor. More importantly after seed increase, this F_2 population will be used to analyze the effect of each *Pin* mutation upon a broader range of functions including milling, baking and other wheat end qualities. Further functional analysis of F_2 families carrying a wider range of missense mutations dispersed throughout PINA and PINB will help us better understand the importance of different amino acids and regions of the puroindoline proteins. The selected alleles with desirable end quality features could then be inserted into other elite germplasm adapted to different agricultural regions.

References

Anderson P (1995) Mutagenesis methods. Cell Biol 48:31–58

Bagge M, Xia X, Lübberstedt T (2007) Functional markers in wheat. Curr Opin Plant Biol 102:211–216

Beecher B, Bettge A, Smidansky E, Giroux MJ (2002) Expression of wild-type pinB sequence in transgenic wheat complements a hard phenotype. Theor Appl Genet 105:870–877

Blochet JE, Chevalier C, Forest E, Pebay-Peyroula E, Gautier MF, Joudrier P, Pezolet M, Marion D (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

Caldwell DG, McCallum N, Shaw P, Muehlbauer GJ, Marshall DF, Waugh RA (2004) A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L). Plant J 40:143–150

Campbell KG, Bergman CJ, Gualberto DG, Anderson JA, Giroux MJ, Hareland G, Fulcher G, Sorrels ME, Finney PL (1999) Quantitative trait loci associated with kernel traits in a soft hard wheat cross. Crop Sci 39:11841195

Campbell JB, Martin JM, Crutcher F, Meyer FD, Clark DR, Giroux MJ (2007) Effects on soft wheat (*Triticum aestivum* L.) quality of increased puroindoline dosage. Cereal Chem 84(1):80–87

Chang C, Zhang H, Xu J, Li W, Liu G, You M, Li B (2006) Identification of allelic variations of puroindoline genes controlling grain hardness in wheat using a modified denaturing PAGE. Euphytica 152:225–234

Chen F, He ZH, Xia XC, Lillemo M, Morris C (2005) A new puroindoline b mutation present in Chinese winter wheat cultivar Jingdong 11. J Cereal Sci 42:267–269

Chen F, He ZH, Xia XC, Xia LQ, Zhang XY, Lillemo M, Morris CF (2006) Molecular and biochemical characterization of Puroindoline a and b alleles in Chinese landraces and historical cultivars. Theor Appl Genet 112:400–409

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai l, Henikoff S (2001) High-throughput screening for induced point mutations. Plant Physiol 126:480–484

Cox TS (1998) Deepening the wheat gene pool. J Crop Prod 1:1–25

Feiz L, Martin JM, Giroux MJ (2008) The relationship between wheat (*Triticum aestivum* L.) grain hardness and wet-milling quality. Cereal Chem 85(1):44–50

Gautier MF, Aleman ME, Guirao A, Marion D, Joudrier P (1994) *Triticum aestivum* Puroindolines, two basic cystine-rich seed

proteins: cDNA sequence analysis and developmental gene expression. Plant Mol Biol 25:43–57
Gedye KR, Morris CF, Bettge AD (2004) Determination and evaluation of the sequence and textural effects of the Puroindoline a and Puroindoline b genes in a population of synthetic hexaploid wheat. Theor Appl Genet 109:1597–1603
Gill BS, Raupp WJ (1987) Direct genetic transfers from *Aegilops squarrosa* L to hexaploid wheat. Crop Sci 27:445–450
Giroux MJ, Morris CF (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 MJ, Morris CF (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 MJ, Talbert L, Habernicht DK, Lanning S, Hemphill A, Martin JM (2000) Association of puroindoline sequence type and grain hardness in hard red spring wheat. Crop Sci 40:370–374
Greenblatt GA, Bettge AD, Morris CF (1995) The relationship among endosperm texture, friabilin occurrence, and bound polar lipids on wheat starch. Cereal Chem 72:172–176
Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L, Henikoff S (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. Genetics 164:731–740
Greenwell P, Schofield JD (1986) A starch granule protein associated with endosperm softness in wheat. Cereal Chem 63:379–380
Hogg AC, Sripo T, Beecher B, Martin JM, Giroux MJ (2004) Wheat Puroindolines interact to form friabilin and control wheat grain hardness. Theor Appl Genet 108:1089–1097
Hogg AC, Beecher B, Martin JM, Meyer F, Talbert L, Lanning S, Giroux MJ (2005) Hard wheat milling and bread baking traits affected by the seed-specific overexpression of Puroindolines. Crop Sci 45(3):871–878
Jander G, Baerson SR, Hudak JA, Gonzalez KA, Gruys KJ, Last RL (2003) Ethylmethanesulfonate saturation mutagenesis in *Arabidopsis* to determine frequency of herbicide resistance. Plant Physiol 131:139–146
Kooijman M, Orsel R, Hessing M, Hamer RJ, Bekkers ACAPA (1997) Spectroscopic characterization of the lipid-binding properties of wheat puroindolines. J Cereal Sci 26:145–159
Krishnamurthy K, Giroux MJ (2001) Expression of wheat puroindoline genes in transgenic rice confers grain softness. Nature Biotech 19:162–166
Krishnamurthy K, Balconi C, Sherwood JE, Giroux MJ (2001) Increased tolerance to fungal diseases of rice plants transformed with puroindoline genes. Mol Plant Microbe Interact 14:1255–1260
Law CN, Young CF, Brown JWS, Snape JW, Worland JW (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, Vienna, Austria, pp 483–502
Lillemo M, Morris CF (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, Gautier MF, Joudrier P, Ptak M, Pezolet M, Forest E, Clark DC Broekaert W (1994) Structure and function of wheat lipid binding proteins. In: Martino al Cimino S (ed) Wheat kernel proteins—molecular and functional aspects, pp 175–180, Universita degli studi della tuscia Viterbo, Italy
Martin CR, Rousser R, Brabec DL (1993) Development of a single-kernel wheat characterization system. Trans ASAE 36:1399–1404
Martin JM, Frohberg RC, Morris CF, Talbert LE, Giroux MJ (2001) Milling and bread baking traits associated with puroindoline sequence type in hard red spring wheat. Crop Sci 41:228–234
Martin JM, Meyer FD, Smidansky ED, Wanjugi H, Blechl AE, Giroux MJ (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 JM, Meyer FD, Morris CF, Giroux MJ (2007) Pilot scale milling characteristics of transgenic isolines of a hard wheat overexpressing puroindolines. Crop Sci 47(2):497–504
Martin JM, Sherman JD, Lanning SP, Talbert LE, Giroux MJ (2008) Effect of variation in amylose content and puroindoline composition on bread quality in a hard spring wheat population. Cereal Chem 85(2):266–269
Massa AN, Morris CF, Gill BS (2004) Sequence diversity of puroindoline-a, puroindoline-b, and the grain softness protein genes in *Aegilops tauschii* cross. Crop Sci 44:1808–1816
Mattern PJ, Morris R, Schmidt JW, Johnson VA (1973) Location of genes for kernel properties in the wheat variety 'Cheyenne' using chromosome substitution lines In: Proceedings of the 4th international wheat genetics symposium. Columbia, MO, pp 703–707
McIntosh RA, Devos KM, Dubcovsky J, Rogers WJ, Morris CF, Appels R, Somers DJ, Anderson OA (2007) Catalogue of gene symbols for wheat: 2007 supplement. http://www.grain.jouy.inra.fr/ggpages/awn/53Textfile/WGC.html
Morris CF, King GE (2008) Registration of hard kernel puroindoline allele near-isogenic line hexaploid wheat genetic stocks. J Plant Reg 2:67–68
Morris CF, Rose SP (1996) Wheat. In: Henry RJ, Kettlewell PS (eds) Cereal grain quality. Chapman & Hall, London, pp 3–54
Morris CF, Greenblatt GA, Bettge AD, Malkawi HI (1994) Isolation and characterization of multiple forms of friabilin. J Cereal Sci 21:167–174
Morris CF, Lillemo M, Simeone MC, Giroux MJ, Babb SL, Kidwell KK (2001) Prevalence of Puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. Crop Sci 41:218–228
Ng PC, Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. Nuc Acid Res 31:3812–3814
Pan Z, Song W, Meng F, Xu L, Liu B, Zhu J (2004) Characterization of genes encoding wheat grain hardness from Chinese cultivar GaoCheng 8901. Cereal Chem 81:287–289
Ram S, Boyko E, Giroux MJ, Gill BS (2002) Null mutation in Puroindoline A is prevalent in Indian wheats: Puroindoline genes are located in the distal part of short arm of 5D chromosome. J Plant Biochem Biotech 11:1–5
Riede CR, Anderson JA (1996) Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Sci 36:905–909
SAS Institute, Inc. (2004) SAS/STAT 91 User's guide SAS Institute Inc., Cary
Simeone MC, Gedye KR, Mason-Gamer R, Gill BS, Morris CF (2006) Conserved regulatory elements identified from a comparative puroindoline gene sequence survey of *Triticum* and *Aegilops* diploid taxa. J Cereal Sci 44:21–33
Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nat Biotechnol 23:75–81
Swan CG, Meyer FD, Hogg AC, Martin JM, Giroux MJ (2006) Puroindoline B limits binding of Puroindoline A to starch and grain softness. Crop Sci 46(4):1656–1665
Till BJ, Cooper J, Tai TH, Colowit P, Greene EA, Henikoff S, Comai L (2007) Discovery of chemically induced mutations in rice by TILLING. BMC Plant Biol 11:7–19
Thorgeirsson TE, Russel CJ, King DS, Shin YK (1996) Direct determination of the membrane affinities of individual amino acids. Biochemistry 35:1803–1809
Wanjugi HW, Hogg AC, Martin JM, Giroux MJ (2007a) The role of puroindoline A and B individually and in combination on grain hardness and starch association. Crop Sci 47:67–76

Wanjugi HW, Martin JM, Giroux MJ (2007b) Influence of Puroindolines A and B individually and in combination on wheat milling and bread traits. Cereal Chem 54(6):540–547

Warburton ML, Crossa J, Franco J, Kazi M, Trethowan R, Rajaram S, Pfeiffer W, Zhang P, Dreisigacker S, van Ginkel M (2006) Bringing wild relatives back into the family: recovering genetic diversity in CIMMYT improved wheat germplasm. Euphytica 149:289–301

Zhu JK, Liu J, Xiong L (1998) Genetic analysis of salt tolerance in *Arabidopsis*: evidence for a critical role of potassium nutrition. Plant Cell 10:1181–1192