Creation and Characterization of a Double Null *Puroindoline* Genotype in Spring Wheat

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ABSTRACT

Wheat (*Triticum aestivum* L.) grain hardness is controlled by the *Ha* locus, which is composed of two closely linked genes, *Puroindoline a* (*Pina*) and *Puroindoline b* (*Pinb*). Hard grain results from mutations in either of the *Pin* genes. Previous results have shown that the *Pina-D1b* (*Pina* null) allele has harder grain than other naturally occurring *Pin* alleles. Our goal was to create, identify, and characterize a double null *Pin* genotype by identifying a *Pinb* null mutation in a variety carrying the *Pina-D1l* null allele. Seeds of Fortuna, which has a premature stop codon in *Pina*, were treated with ethyl methanesulfonate. Two premature stop codon mutations were identified in *Pinb* using direct sequencing. The double null *Pin* haplotype was characterized after backcrossing to the parent variety Fortuna to create *Pina* null populations segregating for the presence of *Pinb*. The double null group was 6 units harder than the single null with no difference in other kernel characteristics. The milling characteristics differed between the two classes; the double null class had less break flour with a greater fraction of large and a smaller fraction of small flour particles compared with the single null class. Neither water absorption nor loaf volume was impacted by the change in grain hardness; however, Na_2CO_3 tests indicated greater starch damage in the double nulls. The double null *Pin* genotype may find a niche in hard wheat products for which flours with larger particle size are desired.

Wheat (*Triticum aestivum* L.) is the basic ingredient for nearly all bread and pastry products. It is a major contributor to human nutrition as a source of carbohydrates, protein, and dietary fiber (reviewed in Shewry and Hey 2015). Wheat is separated into two distinct classes depending on whether it has soft or hard endosperm texture. Flour extracted from hard wheats is used to produce bread and other leavened products, whereas soft wheat flour is used to produce cookies, cakes, and pastries (Pomeranz and Williams 1990; Morris and Rose 1996).

The hardness (*Ha*) locus, located on the short arm of chromosome 5D (Mattern et al. 1973; Law et al. 1978), controls much of the variation in wheat grain hardness (Campbell et al. 1999). This locus is made up of two closely linked genes, *Puroindoline a* (*Pina*) and *Puroindoline b* (*Pinb*). Each gene produces a small (approximately 13,000) cysteine-rich basic protein (Dubreil et al.1998). When combined, these two proteins are referred to collectively as friabilin. Friabilin is a marker protein for grain hardness and is found on the surface of endosperm starch granules. Friabilin is in greater abundance on the starch surface of soft wheat than on hard wheat (Greenwell and Schofield 1986; Giroux and Morris 1998; Hogg et al. 2004). Wild-type alleles of both *Pin* genes give rise to soft grain texture, whereas mutations in either of the *Pin* genes are found in all hard wheat genotypes (Giroux and Morris 1997, 1998). There is no difference in the starch granules or the storage protein between hard and soft wheats (Barlow et al. 1973; Simmonds 1974). Therefore, endosperm texture difference must be a result of degree to which friabilin binds to starch granules. Hogg et al. (2004) and Wanjugi et al. (2007) showed soft grain is produced when both PINs bind cooperatively to starch granules.

Cultivated soft wheat varieties have the same wild-type *Pina-D1a* and *Pinb-D1a* alleles (Chen et al. 2006), but both Gedye et al. (2004) and Massa et al. (2004) identified novel *Pina-D1/Pinb-D1* haplotypes in *Aegilops tauschii*, the donor of the D genome of bread wheat, which all conferred a soft grain texture. Four novel *Ae. tauschii Pina-D1/Pinb-D1* haplotypes were introgressed into a soft wheat background, and grain hardness was increased from 3.8 to 12.6 hardness units (Reynolds et al. 2010).

Naturally occurring *Pina* and *Pinb* mutations resulting in hard grain texture have been observed in germplasm surveys (Morris et al. 2001; Chen et al. 2013). One of two haplotypes, *Pina-D1a/Pinb-D1b* and *Pina-D1b/Pinb-D1a*, are present in the vast majority of U.S. hard wheats (Morris et al. 2001). The *Pinb-D1b* allele contains a glycine to serine substitution in PINB at the 46th amino acid (Giroux and Morris 1997, 1998), whereas *Pina-D1b* is a null mutation arising from a *Pina* gene deletion. Allelic variation within hard wheats results in grain hardness variation within the hard wheat market class, with the *Pina* null allele being harder than the other common *Pin* alleles. Ma et al. (2009) and Takata et al. (2010) using *Pin* near-isogenic lines and Martin et al. (2001) using a recombinant inbred line population showed that seeds containing the *Pina-D1b/Pinb-D1a* haplotype were 6–9 units harder than seeds containing the *Pina-D1a/Pinb-D1b* haplotype. Null mutations exist for *Pinb*, but *Pinb* null seeds are not as hard as the *Pina-D1b* null (Feiz et al. 2009b; Ma et al. 2009). Even though allelic variation at *Pin* loci exists within the hard wheat class, the total range in grain hardness is only about 10 units, with *Pina-D1b* ranking as the allele conferring the highest grain hardness (Takata et al. 2010). Feiz et al. (2009a, 2009b) identified mutations in both *Pin* loci that gave grain hardness outside the range of naturally occurring alleles within both the soft and hard classes after treating a soft wheat variety with ethyl methanesulfonate (EMS).

Even small differences in grain hardness within the hard wheat class cited above can lead to differences in end-use properties. Martin et al. (2001) showed seeds containing the *Pina-D1a/Pinb-D1b* haplotype gave more flour and break flour yield with lower ash and greater loaf volume compared with those containing the harder *Pina-D1b/Pinb-D1a* haplotype. Ma et al. (2009) found similar increased flour yield for these haplotypes. Kammeraad et al. (2016) compared milling behavior of *Pin* near-isogenic lines having grain hardness ranging from approximately 50 to 73 units and found flour yield was not related to grain hardness, whereas break flour yield was inversely proportional to grain hardness level. Flour particle size distribution was also altered, because softer genotypes had a greater proportion of smaller particles and a smaller proportion of larger particles.

Early reports identified the nonfunctional *Pina* null (*Pina-D1b* allele) mutation by the absence of a mutation in *Pinb*, a hard grain phenotype, failure to produce a *Pina* amplicon via polymerase chain

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reaction (PCR), and the absence of PINA from a Triton X-114 protein extraction (Giroux and Morris 1997). Morris and Bhave (2008) reported the *Pina-D1b* null has a 15,300 bp deletion. An additional *Pina* null mutation resulting from a stop codon in *Pina* has been described (Gazza et al. 2005) and designated *Pina-D1l* (Morris and Bhave 2008). Obtaining a *Pin* haplotype consisting of *Pina* and *Pinb* null mutations via recombination is unlikely because the two *Pin* genes are only separated by about 20 kb (Chantret et al. 2005). A deletion-derived double null *Pin* genotype was reported (Tranquilli et al. 2002; Ikeda et al. 2005) and designated by Morris and Bhave (2008) as *Pina-D1k*.

A haplotype in which both *Pin* genes are nonfunctional may have harder grain than the single *Pina* null. Takata et al. (2010) used near-isogenic lines to compare the *Pina-D1b/Pinb-D1a* haplotype with the *Pina-D1k* double null. The *Pina-D1k* double null was numerically, although not statistically, harder than *Pina-D1b/Pinb-D1a*.

Our goal was to create, identify, and characterize a double null *Pin* genotype by identifying a null mutation in *Pinb* in the variety Fortuna, which carries the *Pina-D1l* null mutation. The double null *Pin* haplotype was characterized after backcrossing to the parent variety Fortuna to create *Pina* null populations segregating for the presence of *Pinb*. The impact of these allelic variants was assessed on kernel, flour, milling, and bread baking traits.

MATERIALS AND METHODS

Approximately 5,000 M_0 seeds of the hard red spring wheat cultivar Fortuna (CI 13596) (Lebsock et al. 1967) were treated with 1% EMS using methods first described by Slade et al. (2005) and modified by Feiz et al. (2009a, 2009b), and surviving plants were advanced in the greenhouse. Fortuna is a single null genotype with the *Pin* haplotype *Pina-D1l /Pinb-D1a*. The M_1 population was advanced in the greenhouse, and 350 M_1-derived lines were planted in single rows in the field at the Post Agronomy Farm near Bozeman, Montana, in 2015. Leaf tissue for DNA preparations was collected and pooled at the two- to three-leaf stage from four plants per row. Lines were screened for mutations in *Pinb* via PCR and direct sequencing using the primers PB5 (Gautier et al. 1994) and Cat 3.4 (Swan et al. 2006). The PCR master mix for one sample contained 14.13 µL of H_2O, 5 µL of GoTaq 5X buffer (Promega, Madison, WI, U.S.A.), 2 µL of 25mM $MgCl_2$, 2 µL of 2 µM each of deoxynucleotide triphosphate, 0.52 µL of PB5, 0.52 µL of Cat 3.4, and 0.13 µL of GoTaq polymerase (Promega). The PCR program consisted of a four min initial denaturation at 96°C, followed by 35 cycles of 96°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 7 min. Amplicons were sequenced using the primer Cat 3.4, and resultant sequences were screened for mutations using the Seqman Pro 12 program (DNAStar, Madison, WI, U.S.A.).

The two selected *Pin* mutation-containing lines were crossed to the nonmutated Fortuna parent to create segregating populations. Both segregating F_2 populations were screened using methods described above to identify single null (*Pina-D1l/Pinb-D1a*) and double null (*Pina-D1l/Pinb-D1Q20* or *Pina-D1l/Pinb-D1W116*) homozygotes. Seed was harvested from single homozygous F_2 plants. Seeds from 10 single null (*Pina-D1l/Pinb-D1a*) and 10 double null (*Pina-D1l/Pinb-D1Q20Stop* or *Pinb-D1W116Stop*) homozygous F_2 plants from each population were planted as a single row in a winter nursery in Arizona for seed increase.

Seeds ($F_{2:4}$) from 10 single null and 10 double null lines from each of the two *Pinb* mutant cross populations plus the Fortuna parent were planted in a randomized block design with two replications at the Post Agronomy Farm near Bozeman, Montana, in 2016. Each plot consisted of two 3 m rows spaced 30 cm apart. Each plot was harvested with a plot combine at maturity. Grain samples were cleaned and weighed. Grain protein was measured using near-infrared reflectance (NIR) with the Infratec 1241 grain analyzer (Foss North America, Eden Prairie, MN, U.S.A.). Grain hardness, kernel weight, and kernel diameter were measured on 100 kernel samples with the single-kernel characterization system (SKCS) (Perten Instruments, Springfield, IL, U.S.A.). Five single and five double null lines from each population were selected for a milling study. Wheat grain was tempered to 14.5% moisture (fresh weight basis). Grain samples were milled with a Brabender Quadrumat Senior mill (C. W. Brabender, South Hackensack, NJ, U.S.A.) (AACC International Approved Method 26-21.02). Bran, shorts, break flour, and reduction flour were collected separately. Total flour yield was determined as the break plus reduction flour divided by total recovered product. Flour was size separated with 149, 75, and 53 µm U.S. standard sifting screens (Seedburo Equipment, Chicago, IL, U.S.A.) on a rotating sifter (Ro-Tap RX-29, Leval Lab, Quebec, Canada), and the sieve products were weighed. These data were used to determine flour particle size distribution. Flour protein was determined with an Infratec 1241 grain analyzer with a flour NIR attachment (Foss North America) and expressed at a 14.0% moisture basis proportional to the reference method, which utilizes a LECO FP-528 (LECO, Saint Joseph, MI, U.S.A.) nitrogen analyzer (AACCI Approved Method 46-30.01). Flour ash was predicted by a near-infrared method (AACCI Approved Method 08-21.01). The solvent retention capacity (SRC) test using sodium carbonate (Na_2CO_3) as the solvent was performed on all flour samples following AACCI Approved Method 56-11.01 with the modifications described by Bettge et al. (2002).

Mixograph dough properties were evaluated following AACCI Approved Method 54-40.02. Tolerance to mixing was scored on a 0–9 scale by visually comparing mixographs to standard reference mixograph charts, adjusted for protein content (Pomeranz 1987). Standard bake tests were conducted following AACCI Approved Method 10-10.03. Bake absorption was determined as the amount of water required to bring dough to proper consistency for bread baking. Bake mixing time was recorded as time to bring dough to optimum properties for baking. Loaf volume was determined by the volume of canola seeds displaced. Crumb grain was scored on a visual 1 (excellent) to 9 (unsatisfactory) scale by an experienced baker.

Each response variable was analyzed via analysis of variance using PROC MIXED in SAS version 9.3 software (SAS Institute Cary, NC, U.S.A.). The model included block, population, *Pin* genotype (single or double null) population × *Pin* genotype, and $F_{2:4}$ line within population × *Pin* genotype combination. The $F_{2:4}$ line within population × *Pin* genotype combination was considered random, and all other factors were considered fixed effects. *Pin* genotype means for each population and averages over populations were compared using a *t* statistic. Linear correlations among selected traits were computed by using the $F_{2:4}$ line means averaged over replications.

RESULTS

Two premature stop codon mutations were identified, *Q20** (CAA to TAA) and *W116** (TGG to TGA), following direct sequencing of *Pinb* from this EMS population. Each mutant was then crossed to the Fortuna parent to create segregating populations.

The double nulls (*Pina-D1l/Pinb-D1 Q20** and *Pina-D1l/Pinb-D1 W116**) were about 6 units harder than the single null (*Pina-D1l/Pinb-D1a*) genotype (Table I). The single and double null genotypes did not differ for kernel weight, kernel diameter, or grain protein. Grain yield did not differ between single and double null classes, but grain yield for the parent variety Fortuna was higher than that for the segregating populations. This is indicative of background mutations following EMS treatment.

Total flour yield did not differ, but the double null genotype had 2.2% less break flour ($P < 0.01$) than the single null genotype when averaged over the two populations (Table II). The double

null genotype tended to have less bran ($P = 0.03$) and more shorts ($P = 0.04$) when averaged over both populations. Flour ash was slightly higher for the double null than for the single null genotype ($P = 0.01$) averaged over populations. SRC using Na_2CO_3 is a proxy for starch damage, and on that basis the double null genotype suffered more starch damage than did the single null group ($P < 0.01$).

The two genotypes displayed different flour particle size distribution patterns (Table III). The double null genotype had a higher proportion in the >149 and >75 to <149 µm fractions and a lower proportion in the <53 µm fraction compared with the single null genotype when averaged over the two populations. No differences were detected for the >53 to <75 µm fraction. The differences between genotypes tended to be greater for the Fortuna/*Pina-D1l/Pinb-D1 W116** population, but the population × genotype interaction was not significant ($P < 0.12$) for any of the fractions.

The Fortuna/*Pina-D1l/Pinb-D1 W116** population did not show any difference between classes for any of the dough or bread characteristics (Table IV). But the Fortuna/*Pina-D1l/Pinb-D1 Q20** had a shorter mixograph mix time and bake mix time (data not shown) and less tolerance to mixing compared with the *Pina-D1l/Pinb-D1a* group. This gave significant population × genotype interactions for mixograph tolerance ($P < 0.05$), mixograph mixing time ($P < 0.01$), and bake mixing time ($P < 0.05$).

We examined the linear relation between grain hardness and milling-related traits. Grain hardness differences explained 74% of the variation in break flour yield (Fig. 1). In contrast, grain hardness was not related to flour yield ($r = 0.02$, $P = 0.93$). Grain hardness was also highly correlated with Na_2CO_3 SRC ($r = 0.86$, $P < 0.01$). Grain hardness correlated with the percent retained for the various particle size fractions (Table V). It was positively related to percent retained for >149 and >75 to <149 µm fractions, negatively related to the percent retained at <53 µm, but not related to percent retained for the >53 to <75 µm fraction.

DISCUSSION

Our goal was to create, identify, and characterize null alleles for both *Pina* and *Pinb* in a single genotype. We accomplished that by identifying two independent null mutations in *Pinb* in the variety Fortuna, which has a null mutation in *Pina* resulting from a premature stop codon (*Pina-D1l*). The two double null mutant genotypes were then crossed to the Fortuna parent to create segregating populations, which were evaluated for kernel, milling, and baking characteristics. A genotype with harder grain than currently available *Pin* genotypes could expand the phenotypic variation available for grain hardness and related milling traits. Sequencing of *Pinb-D1* from approximately 350 EMS Fortuna M_1 lines detected two nonsense mutations, *Pinb-D1Q20** and *Pinb-D1W116**. Feiz et al. (2009b) found a 1/11 kb mutation discovery rate in *Pinb-D1a* after EMS treatment in the soft white spring wheat Alpowa. They found 11% of their mutations were nonsense mutations. Our goal was to

TABLE I
Mean Values for Kernel Characteristics of Single Null (*Pina-D1l/Pinb-D1a*) and Double Null (*Pina-D1l/Pinb-D1Q20 and *Pina-D1l/Pinb-D1W116**) *Puroindoline* F_2-Derived Lines Obtained from Crossing Fortuna (*Pina-D1l/Pinb-D1a*) with Two Fortuna EMS-Derived Double Null Lines (*Pinb-D1Q20** and *Pinb-D1W116**)[a]**

Puroindoline Genotype	Number of Lines	Grain Yield (g/ha)	Grain Hardness	Kernel Weight (mg)	Kernel Diameter (mm)	Grain Protein (g/kg)
Fortuna *Pina-D1l/Pinb-D1a*		5,257 ± 63	63.2 ± 0.6	36.3 ± 0.8	2.74 ± 0.01	151 ± 0.0
Fortuna/*Pinb-D1 Q20**						
Pina-D1l/Pinb-D1a	5	3,935 ± 114	67.3 ± 1.1	36.0 ± 0.7	2.81 ± 0.02	156 ± 1.1
*Pina-D1l/Pinb-D1Q20**	5	3,693 ± 124	72.8 ± 0.8	35.7 ± 0.5	2.79 ± 0.01	159 ± 0.9
P value		0.13	<0.01	0.77	0.51	0.10
Fortuna/*Pinb-D1 W116**						
Pina-D1l/Pinb-D1a	5	4,079 ± 48	67.0 ± 1.3	36.3 ± 0.7	2.77 ± 0.02	154 ± 1.4
*Pina-D1l/Pinb-D1W116**	5	4,233 ± 234	73.9 ± 0.7	36.5 ± 0.4	2.81 ± 0.01	156 ± 0.8
P value		0.47	<0.01	0.82	0.11	0.44
Average						
Pina-D1l/Pinb-D1a	10	4,006 ± 63	67.2 ± 0.8	36.1 ± 0.5	2.79 ± 0.02	155 ± 0.9
Pina-D1l/Pinb-D1stop	10	3,918 ± 163	73.3 ± 0.6	36.1 ± 0.3	2.80 ± 0.01	157 ± 0.8
P value		0.55	<0.01	0.96	0.41	0.08

[a] Hardness, individual kernel weight, and kernel diameter mean were determined by the single-kernel characterization system. Grain protein was determined by NIR and is reported on a 12% moisture basis. EMS = ethyl methanesulfonate.

TABLE II
Mean Values for Flour Milling Characteristics of Single Null (*Pina-D1l/Pinb-D1a*) and Double Null (*Pina-D1l/Pinb-D1Q20 and *Pina-D1l/Pinb-D1W116**) *Puroindoline* F_2-Derived Lines Obtained from Crossing Fortuna (*Pina-D1l/Pinb-D1a*) with Two Fortuna EMS-Derived Double Null Lines (*Pinb-D1Q20** and *Pinb-D1W116**)[a]**

Puroindoline Genotype	Number of Lines	Flour Yield (%)	Break Flour (%)	Bran (%)	Shorts (%)	Flour Ash (%)	Flour Protein (g/kg)	Na_2CO_3 SRC (%)
Fortuna		74.0 ± 0.1	35.3 ± 0.1	22.0 ± 0.1	4.0 ± 0.1	0.40 ± 0.0	138 ± 0.0	81.3 ± 1.4
Fortuna/*Pinb-D1 Q20**								
Pina-D1l/Pinb-D1a	5	72.0 ± 0.5	33.4 ± 0.7	23.5 ± 0.4	4.6 ± 0.2	0.425 ± 0.004	142 ± 1.2	80.7 ± 0.6
*Pina-D1l/Pinb-D1 Q20**	5	71.9 ± 0.2	31.7 ± 0.2	23.2 ± 0.3	4.9 ± 0.1	0.44 0 ± 0.004	144 ± 1.1	86.1 ± 0.4
P value		0.92	0.03	0.52	0.18	0.03	0.16	<0.01
Fortuna/*Pinb-D1 W116**								
Pina-D1l/Pinb-D1a	5	72.5 ± 0.2	33.6 ± 0.5	23.2 ± 0.1	4.3 ± 0.2	0.411 ± 0.004	140 ± 1.6	80.5 ± 1.6
*Pina-D1l/Pinb-D1 W116**	5	73.0 ± 0.2	30.9 ± 0.3	22.4 ± 0.1	4.6 ± 0.1	0.422 ± 0.004	142 ± 1.3	86.0 ± 0.5
P value		0.30	<0.01	0.02	0.08	0.08	0.32	<0.01
Average								
Pina-D1l/Pinb-D1a	10	72.2 ± 0.3	33.5 ± 0.4	23.4 ± 0.2	4.4 ± 0.1	0.418 ± 0.003	141 ± 1.0	80.6 ± 0.8
Pina-D1l/Pinb-D1stop	10	72.4 ± 0.2	31.3 ± 0.2	22.8 ± 0.2	4.7 ± 0.1	0.431 ± 0.003	143 ± 0.9	86.1 ± 0.3
P value		0.50	<0.01	0.03	0.04	0.01	0.10	<0.01

[a] Flour protein and solvent retention capacity (SRC) reported on a 14% moisture basis. EMS = ethyl methanesulfonate.

identify nonsense mutations rather than all mutations in *Pinb*, so a mutation rate was not determined. We sequenced about 157.5 kb (approximately 350 M_1 lines × 0.45 kb); from this M_1 population, based on the results of Feiz et al. (2009b), we would have expected one or two nonsense mutations. Both Tranquilli et al. (2002) and Ikeda et al. (2005) reported double null *Pin* genotypes (designated *Pina-D1k*) that resulted from deletions of both *Pina* and *Pinb.* But the number of adjacent linked genes also deleted on 5DS is unknown. The double null genotypes we have created are unique compared with other reports of double null *Pin* genotypes in that closely linked genes have not been deleted.

Grain hardness differences within the hard wheat class can manifest themselves in differential milling characteristics, including the yield and flour particle size distribution (Ma et al. 2009; Kammeraad et al. 2016). Our double null *Pin* genotype class was about 6 units harder than the single null genotype class, but other kernel-related traits did not differ between classes (Table I). In addition, the harder double null class had less break flour with a greater proportion of large flour particles (>149 and >75 to <149 µm fractions) but a smaller proportion of small flour particles, (<53 µm fraction) than the single null class (Table III). Despite the redistribution of flour particle sizes, total flour yield did not differ between classes. Tranquilli et al. (2002), using chromosome substitution lines in Chinese Spring, found the 5D substitution line with both *Pina* and *Pinb* deleted had harder grain than the 5D substitution line with only *Pina* deleted. Takata et al. (2010) created isogenic lines for the single null *Pina-D1b/Pinb-D1a* and double null (*Pina-D1k*) as well as for other *Pin* haplotypes. The double null *Pina-D1k* was not statistically different from the single null (*Pina-D1b/Pinb-D1a*) in grain hardness or flour yield, but they did find average flour particle size was larger for the *Pina-D1k* lines than for *Pina-D1b/Pinb-D1a* lines ($P < 0.05$). These comparisons may have been confounded by deletion of other closely linked genes.

Hard wheats have greater total flour yield but less break flour yield than do soft wheats across a wide range of grain hardness (Hogg et al. 2005; Martin et al. 2007). Our results showed a highly significant ($P < 0.01$) negative association between grain hardness and break flour yield (Fig. 1), but grain hardness was not related to total flour yield. Kammeraad et al. (2016) found similar results, in which grain hardness was negatively related to break flour yield but not related to total flour yield for 10 *Pin* alleles within the hard wheat class.

Starch granules are dislodged from the protein matrix during milling. For soft wheats, fractures tend to occur around rather than through starch granules, yielding a high proportion of intact starch granules. In hard wheats starch granules adhere more tightly to the protein matrix, requiring more energy to extract, and fractures tend to occur through starch granules, giving a higher proportion of fractured or damaged starch granules. SRC with Na_2CO_3 solvent has been used to estimate starch damage primarily in soft wheats. Studies have shown the Na_2CO_3 SRC test was highly correlated with starch damage across flours from soft and hard wheat (Duyvejonck et al. 2011) ($r = 0.91$, $P < 0.05$), within hard red spring wheat (Hammed et al. 2015) ($r = 0.67$, $P < 0.05$), and among hard

TABLE III
Mean Values for Flour Particle Size Distribution Fractions of Single Null (*Pina-D1l/Pinb-D1a*) and Double Null (*Pina-D1l/Pinb-D1Q20** and *Pina-D1l/Pinb-D1W116**) *Puroindoline* F_2-Derived Lines Obtained from Crossing Fortuna (*Pina-D1l/Pinb-D1a*) with Two Fortuna EMS-Derived Double Null Lines (*Pinb-D1Q20** and *Pinb-D1W116**)[a]**

Puroindoline Genotype	Number of Lines	>149 µm (%)	>75 to <149 µm (%)	>53 to <75 µm (%)	<53 µm (%)
Fortuna		4.2 ± 0.0	65.6 ± 0.7	17.7 ± 0.9	12.5 ± 0.1
Fortuna/*Pinb-D1Q20**					
Pina-D1l/Pinb-D1a	5	5.3 ± 0.5	66.2 ± 0.3	16.1 ± 1.6	12.4 ± 1.6
*Pina-D1l/Pinb-D1Q20**	5	6.4 ± 0.4	66.9 ± 0.3	17.3 ± 0.9	9.4 ± 0.9
P value		0.10	0.15	0.46	0.08
Fortuna/*Pinb-D1W116**					
Pina-D1l/Pinb-D1a	5	4.6 ± 0.5	66.4 ± 0.3	17.1 ± 1.3	11.9 ± 1.3
*Pina-D1l/Pinb-D1W116**	5	6.7 ± 0.3	68.1 ± 0.4	16.1 ± 0.7	9.1 ± 0.7
P value		<0.01	<0.01	0.53	0.11
Average					
Pina-D1l/Pinb-D1a	10	5.0 ± 0.4	66.3 ± 0.2	16.6 ± 1.0	12.1 ± 1.0
Pina-D1l/Pinb-D1stop	10	6.5 ± 0.2	67.5 ± 0.3	16.7 ± 0.6	9.2 ± 0.5
P value		<0.01	<0.01	0.93	0.02

[a] EMS = ethyl methanesulfonate.

TABLE IV
Mean Values for Dough Mixing and Bread Characteristics of Single Null (*Pina-D1l/Pinb-D1a*) and Double Null (*Pina-D1l/Pinb-D1Q20** and *Pina-D1l/Pinb-D1W116**) *Puroindoline* F_2-Derived Lines Obtained from Crossing Fortuna (*Pina-D1l/Pinb-D1a*) with Two Fortuna EMS-Derived Double Null Lines (*Pinb-D1Q20** and *Pinb-D1W116**)[a]**

Puroindoline Genotype	Number of Lines	Mixing Tolerance	Mixograph Mixing Time (min)	Mixograph Water Absorption (%)	Loaf Volume (cm^3)	Crumb Grain Score
Fortuna		3.0 ± 1.0	2.5 ± 0.0	63.5 ± 0.8	1,160 ± 5	6.5 ± 0.5
Fortuna/*Pinb-D1Q20**						
Pina-D1l/Pinb-D1a	5	1.6 ± 0.3	2.19 ± 0.15	64.6 ± 0.3	1,132 ± 13	5.7 ± 0.3
*Pina-D1l/Pinb-D1Q20**	5	1.0 ± 0.0	1.85 ± 0.13	64.9 ± 0.5	1,100 ± 16	6.2 ± 0.3
P value		0.04	0.02	0.69	0.10	0.12
Fortuna/*Pinb-D1W116**						
Pina-D1l/Pinb-D1a	5	3.0 ± 0.3	2.41 ± 0.15	65.2 ± 0.4	1,133 ± 12	5.9 ± 0.2
*Pina-D1l/Pinb-D1W116**	5	3.2 ± 0.1	2.66 ± 0.13	65.9 ± 0.5	1,147 ± 11	6.0 ± 0.2
P value		0.47	0.08	0.32	0.44	0.76
Average						
Pina-D1l/Pinb-D1a	10	2.3 ± 0.4	2.3 ± 0.1	64.9 ± 0.4	1,132 ± 11	5.8 ± 0.2
Pina-D1l/Pinb-D1stop	10	2.1 ± 0.5	2.3 ± 0.2	65.4 ± 0.5	1,123 ± 17	6.1 ± 0.2
P value		0.32	0.64	0.33	0.50	0.19

[a] Mixograph mixing tolerance was measured on a 1–8 (weak to strong) scale. Crumb grain score was measured on a 1–9 scale with 9 being best. EMS = ethyl methanesulfonate.

Pin near-isogenic lines (Takata et al. 2010) ($r = 0.98$, $P < 0.05$). We used the Na_2CO_3 SRC test as a proxy for starch damage, and the harder double null class absorbed more solvent than the softer single null class (Table II), implying greater starch damage for the double null group. Takata et al. (2010) observed that the double null *Pina-D1k* near-isogenic lines suffered significantly more starch damage than the single null *Pina-D1b/Pinb-D1a* near-isogenic lines even though the two differed by only three units on the SKCS hardness scale.

Hard wheat flours absorb more water than do soft wheat flours over a range of grain hardness levels (Hogg et al. 2005; Martin et al. 2007), largely because damaged starch absorbs more water than intact starch. We did not detect a difference in mixograph water absorption between the single null and double null classes (Table IV). Martin et al. (2001) also did not find a difference for mixograph water absorption between *Pina-D1b/Pinb-D1a* and *Pina/D1a/Pinb-D1b* allelic classes in a recombinant inbred population, in which the two classes differed by only 6 hardness units. A 6 unit hardness difference and apparent starch damage difference were not sufficient to effect a detectable change in water absorption.

Loaf volume is the most important measure of bread quality. The impact of *Pin* allelic variation on loaf volume from previous studies has been inconsistent. Martin et al. (2001) found the softer *Pinb-D1b* had higher loaf volume than the harder *Pina-D1b* allele in two independent recombinant inbred populations of spring wheat, in which the two haplotypes differed by approximately 6 hardness units. In contrast, Kammeraad et al. (2016) found no difference in loaf volume when near-isogenic lines with *Pin* alleles conferring hard grain were compared with their soft wild-type counterparts. In the present study we found no difference between single and double null classes for loaf volume. Hard wheats generally suffer more starch damage during milling, and their flours absorb more water than soft wheats. The approximately 6 unit hardness difference in our populations did result in a difference in starch damage as measured by the SRC test using Na_2CO_3. But we did not detect any change in water absorption. That may not be surprising, because Martin et al. (2001) did not observe a difference in water absorption in their recombinant inbred population in which the *Pin* haplotypes differed by approximately 6 units.

The two independent *Pinb* mutations created through EMS mutagenesis were crossed to Fortuna to create two segregating populations. These two populations showed similar results across all traits except for the dough mixing traits, for which the results varied depending on the population (Table IV). In particular, the double null class had lower mixing tolerance and time than the single null class for the *Pina-D1l/Pinb-D1Q20** population, but no reduction was observed for the *Pina-D1l/Pinb-D1W116** population. It is noteworthy that both allelic classes for the *Pina-D1l/Pinb-D1Q20** population were well below the Fortuna parent for dough mixing tolerance and time (Table IV), whereas the two classes were more similar to the Fortuna parent for the *Pina-D1l/Pinb-D1W116** population. The difference in response for dough mixing traits occurred even though flour protein did not differ. One explanation is that the *Pina-D1l/Pinb-D1W116** parent had multiple mutations affecting dough characteristics.

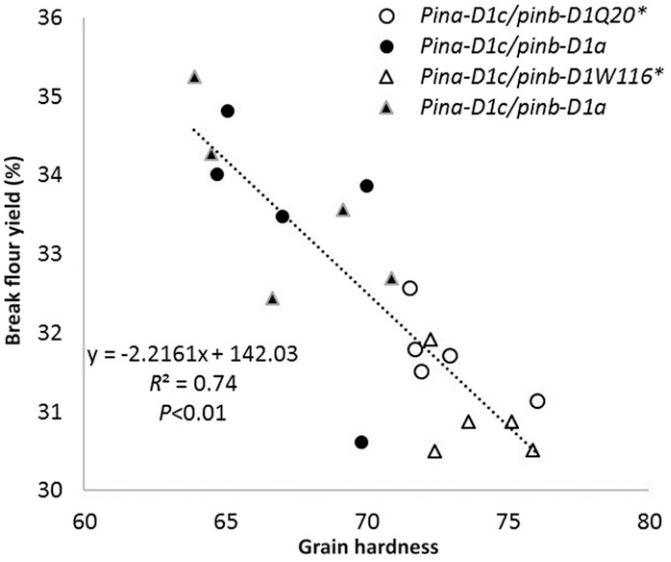

Fig. 1. Relationship between grain hardness and break flour yield for single null (*Pina-D1l/Pinb-D1b*) and double null (*Pina-D1l/Pinb-D1Q20** and *Pina-D1l/Pinb-D1W116**) *Puroindoline* F_2-derived lines obtained from crossing Fortuna (*Pina-D1l/Pinb-D1a*) with two Fortuna EMS-derived double null lines (*Pinb-D1Q20** and *Pinb-D1W116**).

CONCLUSIONS

We created and characterized two genotypes that have null mutations in both *Pina* and *Pinb* (double null) and then created populations segregating for the single *Pina* null (*Pina-D1l/Pinb-D1a*) and double null genotypes. The double null group was 6 units harder than the single null with no difference in other kernel characteristics. The milling characteristics differed between the two classes, in which the double null class had less break flour with a greater fraction of large flour particles and a smaller fraction of small flour particles compared with the single null class. Neither water absorption nor loaf volume was impacted by the change in grain hardness; however, Na_2CO_3 tests indicated greater starch damage in the double nulls. Results were consistent across the two populations, except mixing time and tolerance differed between classes for one population but not the other, probably because of an abundance of mutations in other genes in the double null parent. The double null *Pin* genotype may find a niche in hard wheat products for which flours with larger particle size, maximum starch damage, and higher water absorption are desired. The double null *Pin* genotype may have milling properties similar to durum wheat (*Triticum durum* subsp. *durum*) because both lack functional *Pin* genes, and it could have application for pasta manufacture. The harder double null *Pin* genotype may require more energy to mill, but that may be offset by less energy required during flour sifting than softer *Pin* haplotypes.

TABLE V
Correlations (*r*) Between Grain Hardness, Flour Particle Size Fractions, and Milling Yields[a]

Characteristic	Hardness	>149 µm (%)	>75 to <149 µm (%)	>53 to <75 µm (%)	<53 µm (%)	Break Flour (%)	Flour Yield (%)
>149 µm (%)	0.60**	…	…	…	…	…	…
>75 to <149 µm (%)	0.72**	0.25	…	…	…	…	…
>53 to <75 µm (%)	0.19	–0.41	0.22	…	…	…	…
<53 µm (%)	–0.69**	–0.18	–0.67**	–0.78**	…	…	…
Break flour (%)	–0.86**	–0.78**	–0.59**	–0.02	0.58**	…	…
Flour yield (%)	0.02	–0.17	0.38	–0.09	0.02	0.16	…
SRC Na_2CO_3 (%)	0.85**	0.62**	0.59**	0.19	–0.65**	–0.78**	0.06

[a] $n = 20$; ** indicates significantly different from 0.0 with $P < 0.01$. SRC = solvent retention capacity.

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