Identification and Characterization of Near-Isogenic Hard and Soft Hexaploid Wheats

Craig F. Morris, Garrison E. King, Robert E. Allan, and Marco C. Simeone

ABSTRACT

A complete understanding of the physical–chemical mechanism and underlying genetic control of wheat (*Triticum aestivum* L.) endosperm texture will contribute to defining optimal grain utilization while assisting the breeding and development of new cultivars. World trade in wheat grain primarily is based on the two main market classes, “soft” and “hard,” which are mostly determined by the expression of the puroindoline genes at the Hardness (*Ha*) locus. Here we identify and characterize new genetic stocks (near isogenic lines, NILs) in four different genetic backgrounds (20 NILs total, nine hard and 11 soft). Methods included identifying homogeneous or mixed texture lines by Single Kernel Characterization System and Near-Infrared Reflectance Spectroscopy. Puroindoline genes and *Ha* alleles were determined through nucleic acid sequence analysis. The four different genetic sources for NILs were (i) accessions of ‘Gamenya’ cultivar which were physical mixtures of hard and soft types, (ii) existing near-isogenic lines from the cultivars Heron and Falcon, (iii) advanced-generation backcross lines involving ‘Paha’ and ‘Early Blackhull,’ and (iv) ‘Nugaines’ and ‘Early Blackhull Derivative’. The NILs reported here provide new genetic materials for the study of wheat grain texture and the effect of puroindolines and the Hardness gene on end-use quality. Two of the four sets of NILs possess the Gly-46 to Ser-46 *Pib-D1b* hardness allele which has not been previously available in NILs. The results corroborate a model of wheat grain texture that identifies two major hardness classes, as opposed to one that accommodates intermediate texture classes such as “semi-hard” and “medium-soft.” A direct role of the puroindoline proteins in conferring soft grain phenotype is supported; conversely, no genetic basis for intermediate hardness was found. Rather, intermediate hardness resulted from mixtures of the soft and hard classes.

Kernel hardness (texture) is arguably the single most important determinant of wheat grain quality and utilization (Pomeranz and Williams, 1990; Morris, 1992; Morris and Rose, 1996; Campbell et al., 1999). The majority of phenotypic variation among cultivars and individual grain lots for kernel texture can be attributed to the *Ha* gene, located on the short arm of chromosome 5D (Mattern et al., 1973; Law et al., 1978; Symes, 1965; Baker, 1977). Defined genetic stocks that differ in either the presence or absence of the hardness gene, or differ in their allelic state (*Ha*, soft vs. *ha*, hard) are valuable in studying the biochemical and genetic bases of wheat grain hardness and provide material to compare the effect(s) of this gene on wheat and flour processing and end-use quality (Symes, 1969; Simmonds, 1974; Barlow et al., 1973; Rogers et al., 1993; Giroux and Morris, 1997, 1998; Bergman et al., 1998; Morris et al., 1999).

Regarding the underlying molecular basis of grain hardness, Greenwell and Schofield (1986) reported that the presence of the starch surface protein friabilin was associated with grain softness. The occurrence of friabilin was inherited additively (Bettge et al., 1995) and mediated by bound polar lipids (Greenblatt et al., 1995). Friabilin proved to be identical to the Triton X-114 soluble proteins, puroindolines a and b (Blochet et al., 1993; Jolly et al., 1993; Morris et al., 1994; Gautier et al., 1994). Recent studies revealed that as many as four specific mutations in the puroindoline genes confer wheat grain hardness (as opposed to soft which is the wild-type allele). The first mutation involves a complete absence of puroindoline a mRNA and protein (Giroux and Morris, 1998), whereas the second involves a single nucleotide change in puroindoline b such that the Gly-46 residue of the protein is altered to a Ser-46 (Giroux and Morris, 1997). The third and fourth mutations are also single-nucleotide changes such that Leu-60 is changed to Pro-60, and Trp-44 is changed to Arg-44, respectively (Lillermo and Morris, 2000). To date, a survey of several hundred hard wheats indicates that all *ha* (hard) alleles are attributable to specific changes in these puroindoline proteins.

An issue related to wheat grain hardness, utilization, and the breeding of improved cultivars is the existence of what is commonly referred to as “semi-soft” or “semi-hard” wheats. We have been keen to better understand and elucidate the genetic basis for such wheats as they do not apparently fit the current genetic model of hardness described above. One such semi-soft cultivar is Gamenya (Crobbie, 1989). A second example of possible semi-soft genotypes occurred among the set of ‘Falcon’ and ‘Heron’ hard and soft NILs (Symes, 1965; Giroux and Morris, 1998). However, the two lines that exhibited intermediate levels of grain hardness were found to be mixtures of hard and soft types (Giroux and Morris, 1998). To date, the set of Falcon and Heron NILs has been one of the primary genetic materials for studying the effects of *Ha* (Rogers et al., 1993). The recombinant inbred lines derived from ‘Clark’s Cream’ and ‘NY6432-18’ represent another (Campbell et al., 1999). Campbell et al. (1999) showed how the hardness gene was an often overwhelming contributor to variation in end-use quality among those Clark’s Cream X NY6432-18 lines.

During the course of our studies on Gamenya, we found that the semi-soft character of some earlier types of this cultivar appear to have resulted from mixtures of hard and soft types. Sibling lines have been isolated

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**Abbreviations:** NIL, near-isogenic line; NIR, near-infrared reflectance spectroscopy; NSGC, National Small Grains Collection; PCR, polymerase chain reaction; SKCS, Single Kernel Characterization System.
and characterized. Similarly, the heterogeneous Falcon and Herron lines have been reselected for homogeneity of hardness alleles, and characterized. Lastly, we have identified and researched two additional genetic backgrounds that provided the opportunity of recovering near-isogenic hard and soft lines. Hard and soft NILs from those two new genetic backgrounds have been isolated and characterized. These stocks will provide valuable resources for the study of wheat grain hardness and quality and have been released by the USDA-ARS, registered with the Crop Science Society of America (Morris and Allan, 2001), and deposited in the USDA-NSGC.

MATERIALS AND METHODS

Set 1. Gamenya Sibs

There were four germplasm accessions of the Australian cultivar Gamenya (PI 268329, PI 274503, PI 290909, and PI 349638) in the USDA National Small Grains Collection (NSGC) at Aberdeen, ID. Seed of each was obtained and increased through several cycles of seed amplification and grown in unreplicated field plots (approximately 1.2 by 20 m) at the Spillman Farm, Washington State Univ., Pullman, WA (annual crop) and the Dryland Research Unit, Washington State Univ., Lind, WA (summer fallow) in 1995 under common cultural practices. Grain was harvested and evaluated for grain hardness by near-infrared reflectance spectroscopy (NIR) (model 450, Technicon, Tarrytown, NY) (Method 39-70A; AACC, 1995) and a commercial prototype (P3) of the Single Kernel Characterization System (SKCS) (SKCS 4100, Perten Instruments, Springfield, IL) (Martin et al., 1993) (see Table 1).

New germplasm of those four Gamenya accessions was obtained from the NSGC and used to hand-sow directly 21 to 29 individual plants of each accession at the Western Wheat Quality Laboratory (WWQL) Barmore Farm, Pullman, WA, in 1996 under highly amended soil, irrigation, and manual culture techniques. Individual plants were harvested at maturity, threshed, and grain hardness was determined as described above on 45 to 200 individual kernels from each plant by the SKCS. For each accession, remnant grain of plants with SKCS hardness standard deviations from 12 to 19 was bulked according to soft or hard class (see Fig. 1). Grain from three plants with SKCS hardness standard deviations of 21 to 29 were not included (open bars, Fig. 1). These newly created hard and soft composites for each of the accessions PI 268329, PI 274503, and PI 290909 (PI 349638 was uniformly soft; see Table 1, Fig. 1, and Results) were increased near Brawley, CA, during the winter of 1997-1998 by Western Agricultural Research and Production, Inc., Pullman, WA, in a nonrandomized two-replicate design under common cultural practices. Grain from this increase was subjected to a final evaluation

Table 1. Grain hardness of four accessions of Gamenya wheat cultivar grown at two Washington locations in 1995 as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).²

<table>
<thead>
<tr>
<th>Accession</th>
<th>Lind</th>
<th>Pullman</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIR</td>
<td>SKCS</td>
</tr>
<tr>
<td>PI 268329</td>
<td>42</td>
<td>55 ± 26</td>
</tr>
<tr>
<td>PI 274503</td>
<td>24</td>
<td>50 ± 28</td>
</tr>
<tr>
<td>PI 290909</td>
<td>23</td>
<td>39 ± 23</td>
</tr>
<tr>
<td>PI 349638</td>
<td>23</td>
<td>33 ± 19</td>
</tr>
</tbody>
</table>

² ANOVA on the combined accession—location data set produced an LSD (P = 0.05) of 23 for NIR and 7 for SKCS for comparison of accession means across locations.
Table 2. Grain hardness of hard and soft near-isogenic line (NIL) composites derived from three accessions of Gamenya wheat cultivar and grown near Brawley, CA, 1997-1998 as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).

<table>
<thead>
<tr>
<th>Accession</th>
<th>NIR</th>
<th>SKCS</th>
<th>N²</th>
<th>NIR</th>
<th>SKCS</th>
<th>N²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI268329</td>
<td>31</td>
<td>39±14</td>
<td>13</td>
<td>89</td>
<td>68±13</td>
<td>7</td>
</tr>
<tr>
<td>PI274503</td>
<td>36</td>
<td>34±14</td>
<td>8</td>
<td>93</td>
<td>70±16</td>
<td>12</td>
</tr>
<tr>
<td>PI290909</td>
<td>27</td>
<td>39±15</td>
<td>14</td>
<td>85</td>
<td>65±17</td>
<td>7</td>
</tr>
</tbody>
</table>

* Number of plants used to create the particular composite seed stock for that NIL.

of grain hardness using the above procedures (see Table 2) and represents the hard and soft near-isogenic sib lines (NILs) described herein. Germplasm of these lines was deposited with the USDA-NSGC (Morris and Allan, 2001).

Set 2. Australian Falcon–Heron NILs
Germlasm of 44 accessions of hard and soft NILs in the Falcon and Heron cultivar backgrounds was obtained from Dr. Michael Mackay, Australian Cereals Collection, Tamworth, NSW. Their AUS numbers are 10751, 10752, 90077, 90252-90261, 90264-90283, and 90286-90296. According to Dr. Mackay (1996, personal communication), accessions AUS10751, AUS10752, and AUS90077 were deposited by Dr. Albert Pugsley, whereas the remainder were deposited by Dr. K.J. Symes. Twenty-five lines had the pedigree Heron/7*Falcon and 19 had Falcon/7*Heron. All 44 lines were grown at Pullman, WA, in 1994 and at Lind, WA, in 1995 with the cooperation of Mr. Mike Davis. Grain of each line at each location was analyzed for grain hardness by the SKCS 4100 using 300 kernels as described above (see Fig. 2).

AUS90077 and AUS90254 were seeded at Barmore Farm in 1997 in unreplicated rows and individual spikes were harvested, threshed, and 10 to 12 kernels from each were subjected to SKCS 4100 grain hardness measurement. From the distributions presented in Fig. 3, remnant grain from 10 to 20 spikes from each accession–hardness class combination was bulked and increased near Brawley, CA, as described for Gamenya. Grain harvested from those plots was evaluated for grain hardness using the NIR and SKCS 4100 procedures (see Table 3).

To supplement the original set of 44 lines, the following additional accessions were obtained from Dr. Mackay (with supplemental identification): AUS90076 (Pugsley line), 90262 (Heron parent), 90263 (Falcon parent), 90284 (Symes Heron parent), and 90285 (Symes Falcon parent). Accessions, two pots of each, three plants per pot, were increased in a greenhouse during the winter of 1997-1998. Spikes were harvested, threshed, and the grain analyzed for SKCS hardness as above.

Set 3. Paha–Early Blackhull NILs
Twenty-eight BC6 F3-derived lines of the pedigree ‘Paha’*/2//‘Early Blackhull’/5*Paha, developed primarily for club and lax head type NIL purposes, and the Paha parent were grown at Spillman Farm, Washington State Univ., Pullman, WA (two planting dates) and Central Ferry, WA, in 1994-1995 and 1995-1996 in unreplicated field plots. Early Blackhull was grown

Table 3. Grain hardness of hard and soft near-isogenic line (NIL) composites derived from two heterogeneous accessions of Falcon and Heron wheat cultivar NILs and grown near Brawley, CA, in 1997-1998 as determined by near-infrared reflectance spectroscopy (NIR) and the Single Kernel Characterization System (SKCS).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Soft NIL</th>
<th>Hard NIL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NIR</td>
<td>SKCS</td>
</tr>
<tr>
<td>AUS 90077</td>
<td>41</td>
<td>37±14</td>
</tr>
<tr>
<td>AUS 90254</td>
<td>38</td>
<td>35±14</td>
</tr>
</tbody>
</table>
in 1995-1996 only. Grain from all six environments was assessed for grain hardness by NIR and SKCS as described above (see Table 4). From these 28 lines, six were chosen on the basis of kernel texture, grain color, relative maturity and plant height, the specific parental plants crossed, and club and awnless spike morphology. Grain of the selected lines was hand harvested from plots grown at Spillman Farm in 1995-1996 and used as seed stock for the hard and soft NILs described herein (see Table 5). Germplasm of these lines was deposited with the USDA-NSGC (Morris and Allan, 2001).

**Set 4. Nugaines–Early Blackhull Derivative NILs**

Four lines (designated V60, V68, V75; and V112; Table 6) were originally chosen from a larger set of 12 BC$_2$F$_2$-derived lines based on NIR grain hardness data (Haro, 1990). The set of 12 lines included six with the pedigree, Early Blackhull Derivative/5*Nugaines and six with the pedigree, Early Blackhull/5*Nugaines. All four of the selected lines came from the first pedigree as the lines with the second pedigree were all soft. Those lines were originally developed to be near-isogenic for heading date (Haro, 1990; Haro and Allan, 1997). Haro (1990) grew the lines at the Spillman Farm and near Walla Walla, WA, in 1986-1987 and in 1987-1988 (with two planting dates at Spillman Farm in 1987-1988) for a total of five environments (see Table 6). Reselections of the lines were made in 1992 to verify their uniformity for heading date.

From two to five F$_2$-derived reselections from each of the original lines were grown again in 1993-1994 and 1995-1996 at the Spillman Farm, Pullman, WA, and characterized for hardness as described (see Table 6). Final line selection was based on kernel texture, relative heading date, and plant height (Morris and Allan, 2001).

**RESULTS AND DISCUSSION**

**Set 1. Gamenya Sibs**

Australian Standard White (ASW) wheat class from Western Australia was recognized in the late 1970s as having superior quality for Japanese white salted noodles (Nagao et al., 1977; Oda et al., 1980). At that time, Gamenya was the prominent cultivar of that region. Also, ASW at that time was a class that received both hard and soft-grain cultivars (Crosbie, 1989). As such, ASW was distinct from Australian soft (AS) and the hard classes, Prime Hard and Australian Hard. During the early 1980s, the WWQL received anecdotal information via U.S. wheat marketers that a desirable trait of noodle wheat cultivars was “medium” grain hardness. To learn more about the quality requirements of Asian noodle wheat cultivars, we obtained the four existing accessions of Gamenya from the USDA-NSGC for testing and evaluation. The preliminary results obtained on large field plots (after several generations of seed amplification) are presented in Table 1. Clearly, two features of the material became apparent. First, the accessions differed in grain hardness with PI349683 being the softest and PI268329 being the hardest, and second, grain hardness values and SKCS hardness standard deviations of all but PI349683 were indicative of physical mixtures of hard and soft kernels. The two traditional hardness classes of U.S. wheats are centered on about 25 for soft wheats and 75 for hard wheats (Norris et al., 1989). A major limitation with NIR grain hardness (as well as particle size index, PSI, Williams, 1979; Symes, 1965) has been that they are necessarily conducted on bulk, ground samples and therefore cannot provide information on mixtures beyond the general effect on the value obtained which may be intermediate between the traditional classes. With the development of the Single
Kernel Characterization System (SKCS) (Martin et al., 1993), this limitation was overcome. The SKCS crushes a population of individual kernels and produces a mean and standard deviation grain hardness value. Empirically, we at the WWQL have learned that an SKCS standard deviation on 300 kernels of approximately 13 to 17 is common and indicates an homogeneous sample—hard or soft, whereas a standard deviation of about 20 or greater is generally indicative that the grain lot is mixed. Again, the SKCS hardness value standard deviations of PI268329, PI274503, and PI290909 indicated that they were likely heterogeneous for grain hardness. Those results are consistent with our research on grain hardness, which indicates that medium hardness values of advanced generation, homozygous material are not consistent with the major effects and allelic states of the $Ha$ gene and therefore have no genetic basis as such (Giroux and Morris, 1997, 1998; Morris et al., 1999; Lillemo and Morris, 2000; cf. Bettge et al., 1995). Consequently, we investigated whether one or more of these Gamenya cultivar accessions was indeed a mixture of hard and soft grain types, and if so, to separate the genotypes.

To address this issue, individual plants were grown of each accession and analyzed for grain hardness. Grain hardness for each of the individual plants of each of the four Gamenya accessions are presented in Fig. 1. PI268329, PI274503, and PI290909 were harvested, threshed, and four soft lines on the basis of kernel texture, grain size, and medium-tending hardness and high standard deviations were suspect and were excluded from the bulks. They represent about 5% of the total 64 plants examined for these three accessions (Fig. 1 and Table 2). Analysis of the progeny of these bulks indicated that the hard component of these lines carried the $Pina-D1b$ hardness mutation ("puroindoline a null") (Giroux and Morris, 1998). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001). Apparently Gamenya was considered to be a semi-soft or medium hardness wheat cultivar on the basis of bulk analysis of grain hardness, traditionally PSI, or particle size index (Crosbie, 1989). From our results, it is clear that it was simply a mixture of soft and hard types. PI349638 appears to represent the "current" Gamenya cultivar since it is uniformly soft, consistent with current production of this cultivar in Western Australia (G. Crosbie, 1997, personal communication).

**Set 2. Australian Falcon–Heron NILs**

SKCS grain hardness values of the 44 Falcon and Heron soft–hard NILs from two separate locations and crop years are plotted in Fig. 2. Clearly, all but two lines clustered tightly in either the soft or hard grain hardness groups. Two lines, however, were intermediate between the two classes and differed significantly. ANOVA on the combined location hardness data produced an LSD ($P = 0.005$) of 5.2. The two intermediate lines, AU9077 and AU90254, were selected by Giroux and Morris (1998) for further study as to the molecular genetic basis of their intermediate phenotype. On the basis of individual kernels of these accessions, Giroux and Morris (1998) showed that they, too, were physical mixtures of hard and soft allelic types, in this case puroindoline a nulls ($Pina-D1b$) and soft, wild type ($Pina-D1a$), respectively.

To separate these two allelic types, a large number of individual spikes of the heterogeneous accessions, AU90077 and AU90254, were harvested, threshed, and a few kernels from each analyzed by SKCS 4100. A plot of the resulting data confirmed their mixed nature (Fig. 3). On the basis of these data, once again, hard and soft bulks were created for each accession and used as seed stock for increase. NIR and SKCS grain hardness analysis on the progeny of these bulks confirmed the separation of the two allelic types (Table 3). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

For completeness, additional Falcon and Heron lines not included in the original set, but available from the Australian collection were obtained, increased, and evaluated for SKCS grain hardness. Results were as follows: AU90076, 53 ± 14, 90262, 17 ± 11, 90263, 65 ± 11, 90284, 4 ± 13, and 90285, 49 ± 26. These results are consistent with expectations with one exception, that AU90285 appears to be heterogeneous for grain hardness, like the two lines AU90077 and 90254. The heterogeneous nature of the accession would not be consistent with its classification as “Symes’ Falcon parent.”

**Set 3. Paha–Early Blackhull NILs**

We serendipitously encountered both soft and hard lines in advanced-generation backcross programs aimed at producing NILs for other traits. The first of these programs involved club (c) versus lax (l) spike type using the cultivars Paha (club and soft) and Early Blackhull (lax and hard). Results of six environment-years of hardness data are presented in Table 4 and highlight the allelic difference for hardness among these lines. All the lines had low SKCS grain hardness standard deviations and appeared uniformly hard or soft (data not shown). From the set of 28 lines, we chose two hard and four soft lines on the basis of kernel texture, grain color, relative maturity and plant height (Table 5), and the specific parental plants crossed (data not shown). All are winter habit with awnless, red-chaff, club-type spikes.

PCR analysis and puroindoline gene sequencing indicated that the hard lines carry the Gly-46 to Ser-46 *Pnb-D1b* hardness mutation from Early Blackhull (Table 7). This hardness allele most certainly traces it origin to...
introductions of ‘Turkey Red’ (Bayles and Clark, 1954). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

### Set 4. Nugaines–Early Blackhull Derivative NILs

The second set of hard-soft NILs was encountered in a set of “heading-date” NILs derived from the soft white winter cultivar Nugaines and Early Blackhull Derivative, a hard red winter heading date variant of Early Blackhull (Haro, 1990). The original observation of variation in kernel texture was made on the BC2F2-derived lines described by Haro (1990) and Haro and Allan (1997). The 2 to 5 F2-derived reselections within each of the original lines were all uniformly soft or hard, consistent with the hardness class of the original line (data not shown). Consequently, one each of the reselected lines was chosen to be advanced as parental stock for the hard–soft NILs (Table 6). Final line selection was based on kernel texture, relative heading date, and plant height (Morris and Allan, 2001).

PCR analysis and puroindoline gene sequencing confirmed that these hard NILs also carry the Gly-46 to Ser-46 Pinb-D1b allele like Early Blackhull and Early Blackhull Derivative (Table 7). Germplasm of these lines has been released by the USDA-ARS and deposited with the USDA-NSGC (Morris and Allan, 2001).

### CONCLUSION

The results presented here support the model that the two major alleles (Ha and ha) at the Hardness locus confer the major variation in grain hardness between hardness classes. Further, prior situations involving “medium hardness” Gamena cultivar and Falcon-Heron hard-soft NILs proved to be simply physical mixtures of the two types of alleles. Consequently, the genetic basis, if any, to support “medium hard” or “medium soft” wheats remains to be substantiated in other materials.

An elaboration of the hardness gene model which involves the expression of puroindoline proteins was supported without exception by the present study. Giroux and Morris (1997, 1998) and Lillemo and Morris (2000) described specific mutations in the puroindoline proteins a and b that confer hard kernel texture. The first two and most prevalent of these hardness mutations, Pina-D1b and Pinb-D1b, were both encountered in the present study. Gamena carries the puroindoline a null mutation (Pina-D1b), whereas the hard lines derived from Early Blackhull and Early Blackhull Derivative carry the Pinb-D1b allele. This latter allele putatively originated with Turkey Red introductions to Kansas in the latter half of the 19th century. A survey of historically important hard red winter wheat cultivars of North America indicates that greater than 95% carry this hardness mutation (Morris et al., 2001).

These NILs described herein should prove useful in studies aimed at understanding the role of the hardness gene on end-use quality. In addition to adding three new genetic backgrounds to the existing set of NILs derived from Falcon and Heron, these NILs provide a second hardness mutation, winter and spring-habit lines, and lines with a high degree of genetic similarity to the Pacific Northwest soft white club and common cultivars Paha and Nugaines (respectively).

### REFERENCES


