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Reduction of a *Triticum monococcum* Chromosome Segment Carrying the Softness Genes *Pina* and *Pinb* Translocated to Bread Wheat

Marcos Bonafede, Lingrang Kong, Gabriela Tranquilli, Herbert Ohm, and Jorge Dubcovsky*

ABSTRACT

Endosperm texture, i.e., the hardness or softness of the grain, is an important trait because it determines many end-use properties of wheat (*Triticum aestivum* L.). It is primarily controlled by the puroindoline genes (*Pina* and *Pinb*) at the *Hardness* (*Ha*) locus, mapped on the short arm of chromosome 5D. The introgression of functional *Pin* genes from diploid wheat *Triticum monococcum* L. chromosome 5A^m into hexaploid wheat resulted in softer grains, suggesting that this translocation might be useful for soft wheat breeders. However, the translocated segment includes a large portion of the 5A^m short arm and may carry detrimental genes for agronomic performance. In this study we have generated a backcross (BC) population of 210 individuals where 5A-5A^m homeologous recombination was induced by the *ph1b* mutation to recover individuals with a reduced translocated segment. A map of this region was constructed using specific sequence tagged site (STS) markers for the three *T. monococcum* *Ha*-related genes, the completely linked *BGGP* gene, three wheat ESTs (BG606847, BF474606, and BQ168958), and two microsatellite markers. Eight plants with recombination events between *XBggp* and the closest proximal locus BG606847 were identified. Of these, four have the desired *T. monococcum* allele at the *Ha* locus. These plants carry a 6.3-cM segment of *T. monococcum* chromatin proximal to the *Ha* locus. This germplasm, which will be publicly available, and the molecular markers developed in this study will be valuable tools for soft wheat breeding programs.

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Abbreviations: BC, backcross; CAPS, cleavage amplification polymorphic sequence; CS, Chinese Spring; EST, expressed sequence tag; *Ha*, hardness locus; PCR, polymerase chain reaction; SKCS, single-kernel characterization system; STS, sequence tagged site.

BREAD WHEAT is one of the major food crops in the world and is used to manufacture different products that require specific grain characteristics. Grain endosperm texture or grain hardness is an important trait that determines the end-use quality of wheat (Pomeranz and Williams, 1990; Morris, 2002). Soft wheat kernels require less energy to mill and produce flour particles with less starch damage after grinding or milling than hard wheats. Since broken and damaged starch granules absorb more water, hard wheats are suitable for bread and other yeast-leavened foods, whereas soft wheats are more suitable for cookies, cakes, and pastries (Tippless et al., 1994).

Grain texture is a simply inherited character, largely controlled by the *Ha* locus (Symes, 1965), which was mapped on the short arm of chromosome 5D (Mattern et al., 1973; Law et al., 1978). Though this main locus is referred to as hardness, softness is in fact the dominant trait.

A starch surface-associated protein (relative mass = 15 kDa) was found at high levels in soft wheat and at relatively low levels in hard

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Table 1. Sequence tagged site primer names, sequences, and expected sizes.

Marker	Primer sequences [†]	Length (bp) [‡]		PCR conditions [§]
		<i>Tm</i>	<i>Ta</i>	
Pina-A ^m 1 (dominant)	F 5' GGTCAATCCAAGGCGACCTCA 3' R 5' GATTAACACAGGCATACTGAA 3'	296	Absent	3 TD (1°C) (94°C 45 s, 65°C 50 s, 72°C 60 s) 37 cycles (94°C 45 s, 63°C 50 s, 72°C 60 s)
Pinb-A ^m 1 (dominant)	F 5' CTGGTACAATGAAGTTGGTGC 3' R 5' AGCTTGCCCTTGGATCATTCCT 3'	249	Absent	10 TD (1°C) (94°C 45 s, 60°C 50 s, 72°C 60 s) 30 cycles (94°C 45 s, 51°C 50 s, 72°C 60 s)
Gsp-A ^m 1 (dominant)	F 5' GCTCCACCGCGGATGGTTC 3' R 5' TGCACTGTTTTGGCTTTCAG 3'	323	Absent	3 TD (1°C) (94°C 45 s, 64°C 50 s, 72°C 60 s) 37 cycles (94°C 45 s, 62°C 50 s, 72°C 60 s)
Bggp (codominant)	F 5'-GCTGTCACGCAGGAAAGATA-3' R 5'-AACCCATCGTGTGTGTAGCA-3'	220	208	5 TD (1°C) (94°C 40 s, 56°C 45 s, 72°C 60 s) 34 cycles (94°C 40 s, 51°C 45 s, 72°C 60 s)
BF474606 (dominant)	F 5'-ACCATGGGCAACGCTCGATT-3' R 5'-GCACTTCTTGAGCTGCCTT-3'	390	390	40 cycles (94°C 40 s, 65°C 40 s, 72°C 50 s)
BG606847 (dominant)	F 5'-TGTTCAAAGATCTCGAACCTA-3' R 5'-CGAGCTCAAGTTTGTAGATATGC-3'	170	Null	42 cycles (94°C 25 s, 65.5°C 25 s, 72°C 45 s)
BQ168958 (dominant)	F 5'-AGCACAGAGTGGGTCTCCAT-3' R 5'-GGTGGCTCAACAATCAGGAA-3'	194	194	5 TD (0.5°C) (94°C 45 s, 65°C 45 s, 72°C 60 s) 36 cycles (94°C 45 s, 62.5°C 45 s, 72°C 60 s)

[†]F, forward primer; R, reverse primer.

[‡]*Tm*, *T. monococcum*; *Ta*, *T. aestivum*.

[§]All polymerase chain reaction conditions include a first cycle of 3 min at 95°C and a final extension cycle of 8 min at 72°C. TD(*n*), number of touchdown cycles, with *n* = temperature decrease per cycle.

wheat (Greenwell and Schofield, 1986). This protein, referred to as friabilin, provided a biochemical way to distinguish between hard and soft wheats. Subsequent work showed that friabilin is a composite of related lipid-binding proteins including three polypeptides called puroindoline a (PINA), puroindoline b (PINB), and the grain softness protein family GSP-1, which includes GSP-1a, GSP-1b, and GSP-1c (Jolly et al., 1993; Gautier et al., 1994; Morris et al., 1994; Rahman et al., 1994; Oda and Schofield, 1997; Turner et al., 1999).

The puroindoline proteins are encoded by the *Pina-D1* and *Pinb-D1* genes present on the short arm of chromosome 5D, whereas the GSP-1 proteins are encoded by the *Gsp-A1*, *Gsp-B1*, and *Gsp-D1* genes, located on the short arms of chromosomes 5A, 5B, and 5D, respectively. Since the *Pina-D1*, *Pinb-D1*, and *Gsp-D1* genes were mapped completely linked to the *Ha* locus on chromosome 5D, all three genes were initially considered as candidates for the differences in grain texture (Dubcovsky and Dvořák, 1995; Jolly et al., 1996; Sourdille et al., 1996; Giroux and Morris, 1997; Tranquilli et al., 1999; Turnbull et al., 2003). However, evidence from mutants and transgenic wheat suggest that the puroindolines and not the GSP-1 proteins are responsible for the differences in texture. Mutations in either the *Pina-D1* (null mutation) or *Pinb-D1* (single-point mutations) genes result in hard textures (Giroux and Morris, 1997, 1998; Lillemo and Morris, 2000; Morris et al., 2001; Chen et al., 2005, 2006; Ram et al., 2005; Xia et al., 2005), whereas deletions of the *GSP-1* genes result in no changes in texture (Tranquilli et al., 2002). In addition, expression of wild-type *Pinb* sequence in transgenic wheat complements the hard phenotype (Beecher et al., 2002).

Homeologous puroindoline genes on chromosomes 5A and 5B have not been observed in cultivated wheats. However these genes are present in the diploid donors of the A and B genomes, indicating that they have been deleted from

those chromosomes after polyploidization. Modern cultivated wheats have functional *Pina-D1* and *Pinb-D1* genes only on chromosome 5D (Tranquilli et al., 2002; Gautier et al., 2000). The presence of functional *Pin* homologs in wheat-related species opens the possibility to extend the range of grain soft textures. The introgression of functional copies of puroindoline genes from diploid species into hexaploid wheat resulted in softer grains, suggesting that these resources might be useful for soft wheat breeders (Tranquilli et al., 2002; See et al., 2004). However, these studies used either complete chromosome substitution lines (See et al., 2004) or large translocations, involving most of the short arm of chromosome 5A (Tranquilli et al., 2002), limiting their usefulness in commercial breeding programs.

Alien chromosomes or chromosome segments do not recombine well with the wheat chromosomes because of the presence of the *Ph1* locus, which ensures correct homologous pairing (Riley and Chapman, 1958). Even closely related chromosome segments, such as those from the A^m genome of *T. monococcum* ($2n = 2x = 14$) and the A genome of polyploid wheat, recombine poorly (Dubcovsky et al., 1995; Luo et al., 1996, 2000). Consequently, translocated segments are transferred to progenies as large linkage blocks, limiting recombination in the translocated region. If genes with detrimental effects on agronomic characteristics were present in the large translocated segments, they would be difficult to separate from the *Pin* genes.

To overcome this limitation, we used a line carrying a deletion of the *Ph1* gene (Sears, 1977) to induce homeologous recombination between a large segment of the short arm of chromosome 5A^m and wheat chromosome 5A. The objective of this work was to produce lines with active *Pina-A^m1* and *Pinb-A^m1* within a short translocated *T. monococcum* chromosome segment that would be useful in soft wheat breeding programs.

MATERIALS AND METHODS

Plant Material

The Chinese Spring (CS) line carrying the *ph1b* mutation (Sears, 1977) was crossed with a CS 5A/5A^m recombinant substitution line number 25 carrying a 40-cM translocated segment from *T. monococcum* (Luo et al., 2000) including the *Ha* locus (*Pina-A^m1*, *Pinb-A^m1*, and *Gsp-A^m1*). This line was provided by Dr. J. Dvořák and Dr. M.-C. Luo (University of California, Davis). Since both parents carry the wild *Ha* allele on chromosome 5D (*Pina-D1a*, *Pinb-D1a*), all lines included in this study have the same *Pin* alleles on chromosome 5D. The F₁ plants were grown and self-pollinated. The resulting F₂ progenies were screened with molecular markers to select individuals homozygous for the *ph1b* mutation (sequence-characterized amplified region marker *Ph302.3* [Wang et al., 2002]), and heterozygous for the 5A/5A^m translocation (simple sequence repeat [microsatellite] locus *Xgwm154*). A dominant STS marker specific for *Pina-A^m1* was developed to confirm the presence of the *T. monococcum* *Ha* locus (see next section and Table 1). Selected plants were backcrossed to CS, and 210 grains were obtained. These plants were characterized with molecular markers previously mapped on chromosome 5A.

DNA Analysis

Genomic DNA was isolated following the protocol of Hoisington et al. (1994). Parental lines were screened with 10 microsatellite markers previously mapped on the short arm of chromosome 5A (Somers et al., 2004). Polymorphic microsatellite markers were used to characterize the 210 BC₁ plants using polymerase chain reaction (PCR) conditions described before (Röder et al., 1998). PCR amplifications were conducted in a PTC-100 Thermocycler (MJ Research, Inc., Waltham, MA) in a final reaction volume of 25 µL containing 1× standard PCR buffer, 3.0 mM MgCl₂, 0.3 µM of each primer, 200 µM of each dNTP, 1 U of Taq DNA polymerase, and 60 ng of template DNA.

In addition to the microsatellite markers we developed sequence tagged site (STS) markers for *Pina-A^m1*, *Pinb-A^m1*, *Gsp-A^m1*. These markers were validated in 75 F₂ individuals derived from the cross CS (*ph1b*) × CS 5A/5A^m. DNA analyses were correlated with values of hardness index determined by the single kernel characterization system (SKCS). An additional STS marker was designed for the *BGGP* gene, which is immediately adjacent to *Gsp* and distal to the *Pin* genes (Chantret

Figure 1. BLAST (NCBI, 2006) and ClustalX (Thompson et al., 1997) sequence alignment of nucleotide sequences from (A) TmPina5A (*T. monococcum* *Pina-A^m1*) and TaPina5D (*T. aestivum* *Pina-1*), (B) TmPinb5A (*T. monococcum* *Pinb-A^m1*) and TaPinb5D (*T. aestivum* *Pinb-1*), and (C) TmGsp5A (*T. monococcum* *Gsp-A^m1*) and TaGsp5A, 5B, 5D (*T. aestivum* *Gsp-1*). Primer sequences for sequence tagged site markers *Pina-A^m1*, *Pinb-A^m1*, and *Gsp-A^m1* are in underlined bold type. Shaded letters in DNA sequences correspond to the point mutations/insertion between *T. monococcum* and *T. aestivum*. Gel lanes: 1, DNA ladder; 2, donor of soft-textured grain (CS-5A^m); 3, Chinese Spring; 4–26, BC₁ segregating population; 27, DNA ladder. Arrows indicate the *T. monococcum*-specific bands.

et al., 2004). Finally, three STSs were designed from *T. monococcum* ESTs BQ168958 and BG606847 and common wheat EST BF474606 previously assigned to the most distal bins from homeologous group 5 physical maps (Linkiewicz et al., 2004). The relative order of the wheat ESTs within the bin was predicted from the order of the orthologous genes on rice chromosome 12 (Gramene, 2006). Database searches were done using BLAST (NCBI, 2006), and primers were designed with the Primer3 software (Rozen and Skaletsky, 2000).

tional in the *T. aestivum* background. The role of puroindolines in reducing grain hardness has been demonstrated before in transgenic plants. Krishnamurthy and Giroux (2001) showed that rice, a hard-textured cereal, has softer kernels when transformed with wheat puroindolines. Similar results were obtained by complementation of hard-textured wheats (Beecher et al., 2002; Hogg et al., 2005).

Reduction of the *T. monococcum* 5A^mS Chromosome Segment

Microsatellite Markers

To identify the recombinant BC₁ lines with the shortest *T. monococcum* segment, it was necessary to identify polymorphic markers covering the 5AS arm. We first screened 10 microsatellite markers previously mapped on this chromosome arm and found polymorphisms for 6 of them (*Xgwm154*, *Xbarc186*, *Xgwm205*, *Xgwm293*, *Xgwm304*, and *Xgwm415*). Two of these microsatellite markers (*Xgwm205* and *Xgwm293*) were mapped 18 cM apart and added to the map (Fig. 3A). The closest one to the *Ha* locus was *Xgwm205*, which was located 44 cM from the *XBggp* locus (Fig. 3A).

Since the segment identified by the microsatellite markers was still considerably long, we developed additional markers using the rice genome as a template to select markers in the targeted region.

STS Markers Development

We selected wheat ESTs mapped to the distal bins of the short arm of homeologous group 5 (Linkiewicz et al., 2004) and searched for the closest homologs in rice. Our first target was the *T. monococcum* EST BQ168958, corresponding to a rice gene located 740 kb proximal to *BGGP* on rice chromosome 12. The genome-specific primers used to develop a cleavage amplification polymorphic sequence (CAPS) marker for BQ168958 are indicated in Table 1. Digestion of the 194-bp PCR product with restriction enzyme *AluI* resulted in two fragments of 100 and 94 bp in *T. monococcum* and no digestion in CS (194 bp) (Fig. 2B). Locus BQ168958 was mapped on our BC₁ population 8.7 cM distal from microsatellite locus *Xgwm205*. The segment delimited by *XBggp* and BQ168958 was still relatively large (36 cM), so we developed additional markers (Fig. 3A).

The second CAPS marker was developed from *T. aestivum* EST BF474606, which corresponded to a rice gene located 160 kb proximal to *BGGP* on rice chromosome 12. Genome-specific primers for BF474606 (Table 1) amplified a PCR product of 390 bp that after digestion with restriction enzyme *MseI* produced differential fragments of 390 bp in CS and of 370 bp in *T. monococcum* (Fig. 2C). Locus BF474606 was mapped 26.5 cM distal to BQ168958 and 9.2 cM proximal to *XBggp*. Using this new marker we selected the 13 recombinant chromosomes with the shortest introgressed segments (Fig. 3).

Figure 3. (A) Genetic map for the 5A/5A^m recombinant arm, involving the *Ha* locus. Genetic distances are in cM; (B) Physical map of the collinear region in rice. The location of each marker in the rice physical map is indicated in parentheses (Gramene, 2006).

To identify lines with recombinant chromosomes carrying smaller *T. monococcum* chromosome segments, we developed an STS marker based on *T. monococcum* EST BG606847, which corresponds to a gene located in rice chromosome 12 only 90 kb proximal to *BGGP*. Genome-specific primers for BG606847, detailed in Table 1, amplified a PCR product of 170 bp in *T. monococcum*, and there was no product in CS (Fig. 2D). This locus was mapped on our BC₁ population 6.3 cM proximal to *XBggp* (Fig. 3A).

Map Construction and Comparison with Rice

In the absence of the *Ph1* gene, recombination between the *T. monococcum* chromosomes and *T. aestivum* chromosomes occur at a similar frequency as recombination

between *T. aestivum* chromosomes in the presence of the *Ph1* gene, and therefore, genetic distances in both situations are similar (Dubcovsky et al., 1995). A similar result was observed in this population. The genetic distance of 18 cM between microsatellite markers *Xgwm293* and *Xgwm205* observed in our population was almost identical to the 17 cM reported in the Synthetic $\times$ Opata-BARC 5A map (Song et al., 2005).

The *XBggp* and the three EST loci were colinear with the orthologous sequences on rice chromosome 12 (Fig. 3B). This was an expected result, based on the overall colinearity between the short arms of wheat homeologous group 5 and rice chromosome 12 reported before (Sorrells et al., 2003; Linkiewicz et al., 2004). The genetic distances between these four wheat markers showed a good correlation ($R = 0.994$) with the physical distances between these markers in rice. Therefore, in this case the rice genomic sequence was an excellent tool to predict the order and distance of the wheat markers.

Selected Recombinant Lines for Breeding Purposes

In the presence of the *Ph1* gene, the *T. monococcum* and *T. aestivum* chromosomes showed highly reduced recombination (Dubcovsky et al., 1995; Luo et al., 2000). Therefore, the *T. monococcum* chromosome segment is transmitted almost as a single linkage block, eliminating recombination within the translocated region. Consequently, the high-throughput codominant *XBggp* marker linked to the *Pin* genes should be sufficient for selecting the *T. monococcum Ha* allele. However, checking the final homozygous lines with the *T. monococcum Pin* markers is recommended as a confirmation of the transfer of the targeted allele.

Recombinant lines that have the shortest *T. monococcum* segment have a reduced probability of carrying undesirable linked genes and minimize the region of the chromosome that is locked out for additional recombination. Eight plants with recombination events between *XBggp* and the closest proximal locus *BG606847* were identified. Of these, four have the desired *T. monococcum* allele at the *Ha* locus, but one of them was discarded because of the presence of an additional proximal segment of *T. monococcum* detected by microsatellite marker *Xgwm293*. In each of the selected lines we confirmed with molecular markers the presence of the *T. monococcum Pina* and *Pinb* genes.

The *T. monococcum* segment proximal to *XBggp* in the original translocation line extended beyond *Xgwm293* (Fig. 3), indicating a genetic distance of more than 63 cM. The reduction of the *T. monococcum* segment proximal to *XBggp* to less than 6.3 cM represents a minimum 10-fold reduction of the genetic length of the alien segment. However, to estimate the total length of the *T. monococcum* segment it is necessary to add the segment between the *Ha* locus and the telomere. Although no markers distal to

Ha were included here, previous studies have shown that *Ha* is only 1.4 cM proximal to the *XNor* locus, the most distal marker on chromosome arm 5A^mS (Dubcovsky et al., 1996). By adding the *Ha* proximal and distal *T. monococcum* segments it is possible to estimate the total length of the 5A^m translocated segment at approximately 8 cM.

Two of these selected plants have been self-pollinated to recover homozygous recombinant lines where the *ph1b* deletion is eliminated. Seeds from the homozygous lines will be increased and deposited in the National Small Grain Collection. This publicly available germplasm, together with the markers developed in this study, will be useful to increase the range of soft textures available to soft wheat breeding programs.

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