Title
Reduction of a Triticum monococcum chromosome segment carrying the softness genes Pina and Pinb translocated to bread wheat

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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 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 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.

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*

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
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., 1995; Jolly et al., 1999; Turnbull et al., 2003). However, evidence 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 genotype 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 Phl gene (Sears, 1977) to induce homologous recombination between a large segment of the short arm of chromosome 5A and wheat chromosome 5A. The objective of this work was to produce lines with active Pina-A*1 and Pinb-A*1 within a short translocated T. monococcum chromosome segment that would be useful in soft wheat breeding programs.

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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequences†</th>
<th>Length (bp)‡</th>
<th>PCR conditions§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pina-A†</td>
<td>F 5’-GGTCAATCCAGCGACCTCA-3’</td>
<td>296</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>R 5’-GATTAAACACGCTATGCTA-3’</td>
<td>37 cycles (94°C 45 s, 65°C 50 s, 72°C 60 s)</td>
<td></td>
</tr>
<tr>
<td>Pinb-A†</td>
<td>F 5’-CTGGTACAGTAATTGTGGTC-3’</td>
<td>249</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>R 5’-AGTTGCTCTGGAATCCTC-3’</td>
<td>30 cycles (94°C 45 s, 65°C 50 s, 72°C 60 s)</td>
<td></td>
</tr>
<tr>
<td>Gsp-A†</td>
<td>F 5’-GCTCCACCCGGGATGGTTCT-3’</td>
<td>323</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>R 5’-TGCACTGTTTGTGTCC-3’</td>
<td>37 cycles (94°C 45 s, 65°C 50 s, 72°C 60 s)</td>
<td></td>
</tr>
<tr>
<td>Bggp (codominant)</td>
<td>F 5’-GCTGTTCACGAGAAGATA-3’</td>
<td>220</td>
<td>5 TD (1°C) (94°C 45 s, 56°C 45 s, 72°C 60 s)</td>
</tr>
<tr>
<td></td>
<td>R 5’-AACCCATCGTGTGTGAGCA-3’</td>
<td>34 cycles (94°C 40 s, 51°C 45 s, 72°C 60 s)</td>
<td></td>
</tr>
<tr>
<td>BF474606</td>
<td>F 5’-ACCATGGGCCAGCGCTGATT-3’</td>
<td>390</td>
<td>40 cycles (94°C 40 s, 65°C 40 s, 72°C 50 s)</td>
</tr>
<tr>
<td></td>
<td>R 5’-GCACCTTTGCGACTCCCT-3’</td>
<td>42 cycles (94°C 25 s, 65.5°C 25 s, 72°C 45 s)</td>
<td></td>
</tr>
<tr>
<td>BG606847</td>
<td>F 5’-TGTCTAAAGCTCGAACCCTA-3’</td>
<td>170</td>
<td>Null</td>
</tr>
<tr>
<td></td>
<td>R 5’-GAGGCTCAAGTTGTAGATGC-3’</td>
<td>42 cycles (94°C 25 s, 65.5°C 25 s, 72°C 45 s)</td>
<td></td>
</tr>
<tr>
<td>BQ168958</td>
<td>F 5’-AGCACAGATGGGGTCTCAT-3’</td>
<td>194</td>
<td>5 TD (0.5°C) (94°C 45 s, 65°C 45 s, 72°C 60 s)</td>
</tr>
<tr>
<td></td>
<td>R 5’-GGTGCTCAACATGACGGA-3’</td>
<td>36 cycles (94°C 45 s, 62.5°C 45 s, 72°C 60 s)</td>
<td></td>
</tr>
</tbody>
</table>

†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 = temperature decrease per cycle.
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\(^{m}\)1, Pinb-A\(^{m}\)1, and Gsp-A\(^{m}\)1). 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 F\(_1\) plants were grown and self-pollinated. The resulting F\(_2\) 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\(^{m}\)1 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 BC\(_1\) 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 MgCl\(_2\), 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\(^{m}\)1, Pinb-A\(^{m}\)1, Gsp-A\(^{m}\)1. These markers were validated in 75 F\(_2\) individuals derived from the cross CS (phph) × 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 et al., 2004). Finally, three STSs were designed from T. monococcum ESTs BQ168958 and BG606678 and common wheat EST BF474606 previously assigned to the most distal bins from homoeologous 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).
RESULTS AND DISCUSSION

Molecular markers for **T. monococcum** Ha Locus

To facilitate the introgression of the *T. monococcum* Ha locus (softer grain textures) into commercial soft wheat varieties, we developed genome-specific PCR markers for this locus. We first compared the sequences of the *Pina* and *Pinb* genes from *T. monococcum* with those from *T. aestivum* D genome (Fig. 1A and B). For the tightly linked *XGsp-1* locus the *T. monococcum* sequence was compared with those from the three genomes of hexaploid *T. aestivum* (Fig. 1C). Since the primers were designed in regions carrying mutations specific for the *T. monococcum* sequence, the resulting markers are dominant (Fig. 1). Fragments of 296 bp, 249 bp, and 323 bp were amplified by *Pina-A"I*, *Pinb-A"I*, and *Gsp- A"I* primer combinations, respectively, only when the *T. monococcum* chromosome segment was present (Fig. 1).

**BGGP Marker**

The dominant Ha-related markers described above cannot differentiate between homozygous and heterozygous carriers of the *T. monococcum* chromosome segment, limiting their use in marker-assisted selection. Consequently, a high-throughput codominant marker was developed from the closely linked gene for β-1,3-galactosyl-O-glycosyl-glycoprotein (BGGP). This gene is distal and very close to *Gsp-1* both in *T. monococcum* (1.8 kb, AY491681) and *T. aestivum* (2.1 kb, CR626929) (Chantret et al., 2004, 2005).

A 12-bp region (ATTTTGTTTTCTT) found in the third intron was present in *T. monococcum* (both DV92 and G1777) and absent in *T. aestivum* (GenBank CR626929.1). The primers flanking this deletion are specific for the A genome as confirmed by the absence of PCR amplification product in the DNA of the N5AT5D nullitetrasomic line (Fig. 2A). The specificity of this marker to trace the *T. monococcum* chromosome segment was also verified by analyzing a set of 34 U.S. wheat varieties from different market classes currently used as parents of 17 mapping populations (Wheat CAP, 2006). None of these varieties showed the *T. monococcum* 220-bp diagnostic band.

In summary, this marker is codominant, does not require digestion by restriction enzymes, and can be easily visualized in agarose or vertical polyacrylamide gels, facilitating its use in high-throughput marker-assisted selection strategies.

**Validation of the Ha Markers**

No recombination events were found between the *XBggp* and the puroindoline genes in our BC lines. Seventy five F$_2$ lines derived from the cross CS(5A$^m$) × CS(*ph1b *ph1b*) were genotyped and their grain texture was measured using a SKCS to confirm the linkage between these markers and the softer texture of the grain. All lines have a CS 5D Ha allele and therefore functional *Pina-D1* and *Pinb-D1* alleles. Consequently, segregation is observed only for the presence of the 5A$^m$ puroindoline genes.

The average SKCS values from the 20 lines homozygous for the presence of both the *T. monococcum* and *T. aestivum* puroindoline genes (38.6 ± 6.7) showed a 41% reduction in hardness relative to the 17 lines carrying only the *T. aestivum* puroindolines on chromosome 5D (65.6 ± 12.9). The 38 heterozygous lines showed intermediate values (46.5 ± 9.3), which are smaller than the midpoint between the two homozygous classes (52.1). The degree of dominance was $d = -0.41$ ($d = [H - ((AA + BB)/2)]/[(AA - BB)/2]$), indicating a predominantly additive effect with a slight dominance of the *T. monococcum* allele.

The observed reduction of hardness values associated with the presence of *T. monococcum* Ha locus confirmed previous observations (Tranquilli et al., 2002; See et al., 2004). These results can be explained by the addition of *T. monococcum* puroindoline proteins, which remain func-
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<sup>m</sup>S Chromosome Segment**

**Microsatellite Markers**

To identify the recombinant BC<sub>1</sub> 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 *Alu*I 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 BC<sub>1</sub> 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 *Mse*I 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).

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*. Genomespecific 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 BC<sub>1</sub> 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 × 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 homoeologous 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* *Pin* 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*′*S (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*′* 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.

**Acknowledgments**

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