

# Development of a codominant PCR-based marker for the wheat *Wx-B1* null allele

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## ABSTRACT

Waxy protein is a key enzyme in the synthesis of amylose in endosperm tissue. The amylose content of wheat flour plays a significant role in determining Japanese udon noodle quality. Most wheat cultivars suitable for producing udon noodles have a low amylose level due to a lack of Wx-B1 protein conditioned by the *Wx-B1* null allele. It was previously determined that the entire coding region of the wheat *Wx-B1* gene is deleted in the most common null allele. However, the extent and breakpoints of the deletion have not been established. In this study, the position of the 3' deletion breakpoint was refined by mapping with PCR-based markers. Based on information from this analysis, a chromosome walk was initiated and the DNA sequence flanking the deletion breakpoints was obtained. Using this sequence information, a codominant marker for the identification of the *Wx-B1* null allele was developed. This marker can unambiguously identify heterozygous plants, which will accelerate the selection of partial waxy mutants carrying the *Wx-B1* null allele.

## INTRODUCTION

Waxy protein, or granule-bound starch synthase I (GBSSI), is a key enzyme for amylose synthesis in the endosperm. Bread wheat (*Triticum aestivum* L.) has three *granule-bound starch synthase I* (GBSSI) genes (*Wx-A1*, *Wx-B1*, *Wx-D1*), also known as *Waxy* genes. Waxy wheat is deficient in waxy proteins, while partial waxy wheat lacks one or two waxy proteins. Amylose content has an influence on Japanese Udon noodle quality (Oda et al. 1980; Toyokawa et al. 1989). Among the partial waxy wheat types, cultivars lacking Wx-B1 protein are preferable for such noodles, partly due to their low amylose levels.

The spontaneous mutation occurring in the *Wx-B1* gene of Kanto 107 (K107) has been characterized (Vrinten et al. 1999), and it was established that the entire coding region of the *Wx-B1* gene was deleted in the null allele. Worldwide, most cultivars lacking the Wx-B1 protein carry the same mutation (Saito et al. 2004). However, neither the deletion breakpoints nor the extent of the *Wx-B1* deletion have been determined, therefore it is uncertain whether the range of the deletion in these cultivars is identical. Determining the sequence flanking the deletion breakpoints is also necessary for the development of a codominant marker. In breeding programs, clear

identification of heterozygous plants is important, and can best be achieved using codominant markers.

In a previous study, we demonstrated that one of the deletion breakpoints is located approximately 3.3 to 6 kb downstream of the *Wx-B1* gene using a comparative genomics analysis method (Saito et al. 2006). Here, we describe the genomic sequence flanking the deletion breakpoints in the null *Wx-B1* allele and outline the development of a codominant marker for distinguishing among *Wx-B1* alleles.

## MATERIALS AND METHODS

*Triticum aestivum* cv. Chinese Spring was used to determine the genomic sequence of the region downstream of the *Waxy* gene. Nullisomic-tetrasomic lines (Sears 1966) of 7A, 4A, and 7D chromosomes in a Chinese Spring background were used to assign markers to chromosomes. The Japanese and Australian cultivars carrying wild-type and null *Wx-B1* alleles used in this study included the cultivars Norin 61 (wild type), Kinuhiroha (null), and Mochi-Otome (null) from Japan, and Bencubbin (wild type), Mintlor (wild type), Aroona (null), and Rosella (null) from Australia. Database searches were conducted with the BLASTN program to identify wheat ESTs with high similarity (E-value < e-100) to genomic sequences from BAC clones containing the *Waxy* genes of *Hordeum vulgare* L. (accession number AF474373) and *Triticum monococcum* L. (AF488415). For the development of primers to obtain the region flanking the 3' deletion, the sequences of downstream regions of the *Wx-A1* and *-D1* genes were also determined using genomic sequence information from two BAC clones.

The genomic sequence flanking the deletion junction in the null *Wx-B1* allele was amplified by genome walking of Mochi-Otome using the BD GenomeWalker Universal Kit (Clontech), according to the manufacturer's instructions. The gene-specific primers 3'-R7 (5'-TTTTCTATCACAA GCTGTAAACGCTTGG-3') and 3'-R8 (5'-ACAGCCTTATTGTACCAAGACCCATGTGTG-3') were used for PCR-based DNA walking. PCR conditions for discrimination between wild-type and null *Wx-B1* alleles were identical to those used by Nakamura et al. (2002).

## RESULTS AND DISCUSSION

Previously, we determined the sequence of the 6532 bp region immediately downstream of the *Wx-B1* gene in Chinese Spring by employing primers based on sequences of BAC clones containing the *Waxy* genes of *H. vulgare* (AF474373) and *T. monococcum* (AF488415), and wheat EST sequences (Saito et al. 2006). From this data, we were able to ascertain that one of the deletion breakpoints was located approximately 3.3 to 6 kb downstream of the *Wx-B1* gene (Saito et al. 2006). The deletion encompassed a portion of an expressed gene that did not have homology to genes with known functions, but showed a high degree of similarity to cDNA clones of maize and rice (Saito et al. 2006).

In this study, we first developed four new PCR markers, 3'-3, 3'-4, 3'-5 and 3'-6, designed to include various portions of the unknown gene located downstream of *Wx-B1* gene (Fig. 1). For markers 3'-4, 3'-5 and 3'-6, the expected PCR products were amplified from all wheat cultivars. However, cultivars carrying the null *Wx-B1* allele did not produce an amplification product corresponding to fragment 3'-3 (Fig. 1). This indicated that a deletion breakpoint was located in the region between primers 3'-F3 and 3'-F4.

Next, sequences flanking the deletion junction were determined by chromosome walking using a genomic library from Mochi-Otome, using two gene-specific primers, 3'-R7 and 3'-R8, which were developed from sequences between 3'-F5 and -F6. A nested PCR produced a 709 bp fragment from the library. The region from nucleotide 394 to 709 in this fragment corresponded to the area found from 3873 to 4188 bp downstream of the translation termination codon of the *Wx-B1* gene in Chinese Spring (Fig. 2A). This indicated that an additional 3872 bp downstream of the termination codon of the *Wx-B1* gene was deleted in the null *Wx-B1* allele. A 159 bp sequence located immediately adjacent to the downstream breakpoint in the amplified product showed high similarity to the miniature inverted-repeat transposable element *Stowaway*. Additionally, the region from nucleotide 6 to 234 of the amplified fragment showed similarity to a *T. monococcum* sequence (accession no. AF488415, positions 14142-14376, E-value=3e-51, bit score=210). This sequence is located approximately 60 kb upstream of

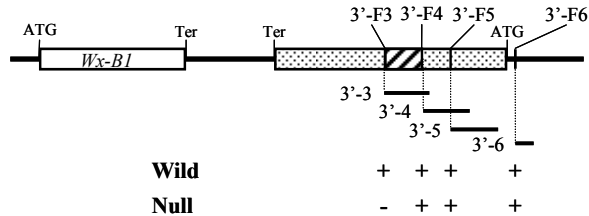


Figure 1. Molecular analysis of the 3' deletion breakpoint. Presence (+) and absence (-) of PCR products amplified from cultivars carrying wild-type and null *Wx-B1* genes are indicated. Bars represent positions of the markers. One of the deletion breakpoints is located between the 3'-F3 and 3'-F4 primers.

the *Waxy* gene in *T. monococcum*, and is therefore likely located approximately 60 kb upstream from the *Wx-B1* gene in *T. aestivum*. The intervening region appears to be deleted in the null *Wx-B1* allele. Thus, including the *Waxy* gene, the total size of the deletion is approximately 67 kb.

In the *T. monococcum* BAC clone, most of the region upstream of the *Waxy* gene is annotated as repetitive sequence, except for an 1828 bp region, which is annotated as coding for a hypothetical protein. However, it appears that this protein is not expressed, since BLAST searches did not identify cereal EST sequences with homology to this putative gene. Consequently, it appears that no expressed genes are found within the 60 kb region of the deletion upstream of the *Waxy* gene.

However, *T. monococcum* is more closely related to the A genome donor than the B genome donor of wheat. Gene order, orientation and number of genes often vary between corresponding regions of the A, B and D genomes of wheat (Chantret et al. 2005; Feuillet et al. 2001). Furthermore, sequence comparisons of orthologous genomic regions in the two closely related species *T. monococcum* and *T. turgidum* showed that the majority of the compared sequences were not conserved (Wicker et al. 2003). Genetic rearrangements such as insertions, deletions and duplications occurred in intergenic regions rich in repetitive elements, and different predicted genes were identified in each species. Therefore, it is quite possible that the size of the deleted area upstream from the *Wx-B1* start codon may differ from the 60 kb we estimated here, and furthermore, genes may be present in this area of

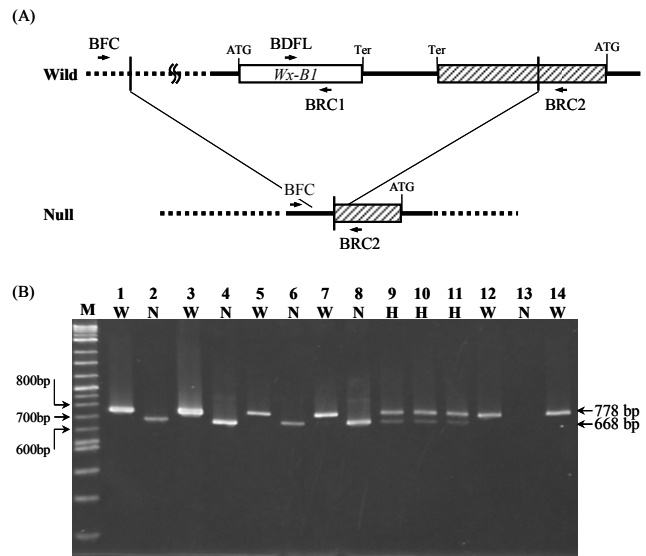


Figure 2. Detection of the *Wx-B1* null allele by PCR-based markers. (A) Diagrammatic representation of the genomic area encompassing the wild type and null *Wx-B1* genes. Arrows indicate primer position and orientation. (B) Identification of wild-type and null alleles of the *Wx-B1* gene using the primers BDFL, BRC1, BFC and BRC2. Lane 1: Chinese Spring, 2: Kinuhiroha, 3: Norin 61, 4: Mochi-Otome, 5: Bencubbin, 6: Aroona, 7: Mintlor, 8: Rosella, 9-11: heterozygous for *Wx-B1* gene, 12: N7AT7D, 13: N4AT4D, 14: N7DT7B, M: 2-Log DNA ladder (NEB). Lines homozygous for the wild-type allele are indicated by a W above the lane, those homozygous for the null allele are indicated by an N, while heterozygous lines are indicated by an H.

wild-type wheat that are not found in the corresponding region of *T. monococcum*. The sequence of the deleted region upstream from the *Wx-B1* gene can be determined precisely by sequencing of BAC clones from a wheat cultivar with a wild-type *Wx-B1* locus. This will also establish whether additional genes are present in the area of the deletion, and whether there are alterations in colinearity between wheat and the related plants used in this study.

Finally, primers for the identification of the null *Wx-B1* allele were designed based on sequences upstream and downstream of deletion breakpoints (Fig. 2A, primers BFC: 5'-CGTAGTAAGGTGCAAAAAAGTGCCACG-3' and BRC2: 5'-ACAGCCTTATTGTACCAAGACCCATGTGTG-3'). Primers capable of amplifying the *Wx-B1* gene were employed for the detection of the wild-type *Wx-B1* allele (Fig. 2A, primers BDFL: 5'-CTGGCCTGCTACCTCAAGAGCAACT-3' and BRC1: 5'-GGTTGCGGTTGGGGTCGATGAC-3'). Primers BDFL and BRC1 anneal to the *Wx-B1* gene and amplify a 778 bp product, while primers BFC and BRC2 anneal to genomic sequences of both wild-type and null *Wx-B1* alleles. However, no amplification product is obtained from the wild-type allele using the latter primer set because the distance between the two primers is too large. Consequently, a 668 bp fragment is amplified only from cultivars carrying the null *Wx-B1* allele. The PCR products of these two primer sets represent a codominant marker for the identification of *Wx-B1* alleles. Using this codominant marker, 778 bp products were amplified from all wheat cultivars with the wild-type *Wx-B1* allele (Fig. 2B, lanes 1, 3, 5 and 7) and 668 bp products were amplified from all cultivars carrying the null allele (Fig. 2B, lanes 2, 4, 6 and 8). These primer sets could also identify plants which are heterozygous at the *Wx-B1* locus (Fig. 2B, lanes 9-11). Therefore, this co-dominant marker allows efficiently selection in continuous backcross programs aimed at breeding new *Wx-B1* null cultivars. This marker has been validated in breeding programs in Japan and the USA, and since the most *Wx-B1* null cultivars appear to carry the same mutation, the marker should be useful in wheat breeding programs throughout the world.

## ACKNOWLEDGEMENTS

We thank the NIAS (National Institute of Agrobiological Sciences) Genebank for providing wheat seeds.

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