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A novel codominant marker for selection of the null *Wx-B1* allele in wheat breeding programs

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Abstract Waxy protein (granule-bound starch synthase I) 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 null *Wx-B1* alleles. 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. Using information from this analysis, a chromosome walk was initiated and the DNA sequence flanking the deletion breakpoints was obtained. The deletion included a 3,872 bp region downstream from the termination codon of *Wx-B1* gene. Based on similarity with *T. monococcum* sequences, it was estimated that approximately 60 kb upstream of the *Wx-B1* gene was also deleted. 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.

Keywords Wx-B1 · Wheat · Codominant marker · Breeding

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Introduction

Bread wheat (*Triticum aestivum* L.) has three granule-bound starch synthase I (*GBSSI*) genes (*Wx-A1*, *Wx-B1* and *Wx-D1*), also known as *waxy* genes. Waxy wheat is deficient in GBSSI, while partial waxy wheat lacks one or two waxy proteins. GBSSI is a key enzyme for amylose synthesis in the endosperm. Amylose content has a significant role in determining noodle quality, and partial waxy wheat is preferable for Asian noodles.

The suitability of Australian Standard White (ASW) wheat varieties for producing Japanese udon noodles is at least partly due to the low amylose

levels of these cultivars (Oda et al. 1980; Toyokawa et al. 1989). In support of this observation, most ASW wheat cultivars lack Wx-B1 protein (Yamamori et al. 1994). However, Zhao et al. (1998) reported that the high noodle-making quality of Australian wheat cultivars was not totally explained by the reduction in amylose content, which suggests the possibility that a gene linked to *Wx-B1* may also influence the quality of these cultivars.

The spontaneous mutation occurring in the *Wx-B1* gene of Kanto 107 (K107) has previously 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. A marker designed based on the null allele of K107 was used to analyze the mutated *Wx-B1* alleles occurring in wheat cultivars from many countries including Japan and Australia (Saito et al. 2004), and the results of this analysis suggested that the null mutations occurring in all these cultivars involved the deletion of the entire coding region. 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. It is also possible that a gene or genes affecting noodle making quality is deleted along with the *Wx-B1* gene. Determining the specific area that is deleted could lead to the identification of such a gene. Furthermore, determining the sequence flanking the deletion breakpoints is necessary for the development of a codominant marker. In breeding programs, continuous backcrossing is often employed for introducing a desired trait into an adapted cultivar. Clear identification of heterozygous plants is very important in backcross breeding programs, and can best be achieved using codominant markers.

Wheat genomic sequence information in the vicinity of *waxy* genes is not available. However, we felt that using DNA markers produced based on synteny with other plants would be an effective method for determining the deletion range and breakpoints. Using this strategy we earlier established that one of the deletion breakpoints was located approximately 3.3–6 kb downstream of the *Wx-B1* gene (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

Plant materials

Chinese Spring wheat 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 wheat cultivars carrying wild-type and null *Wx-B1* alleles used in this study included the cultivars Norin 61 (wild type), Kinuiroha (null), and Mochi-Otome (null) from Japan, and Bencubbin (wild type), Mintlor (wild type), Aroona (null), and Rosella (null) from Australia. In addition, breeding lines which are under selection for *Wx-B1* null genes in breeding programs in the USA were used to evaluate the newly developed codominant marker. The U.S. samples tested included the hard white wheat cultivars Anton (wild type) and RioBlanco (null), two fully waxy breeding lines NX03Y2489 and NWX03Y2459, and single seed selections from a heterogeneous breeding line, 03LNK6053-68 derived from the cross Redland (wild type)/99Y1436 (full waxy).

Sequence analysis

Multiple alignments of DNA sequences were performed using MultAlign (Corpet 1988). Database searches were conducted with the BLASTN program to identify wheat ESTs with high similarity (E-value $\leq e^{-100}$) to genomic sequences of BAC clones containing the *waxy* genes of *Hordeum vulgare* L. (AF474373) and *Triticum monococcum* L. (AF488415).

Amplification of downstream regions of wheat *waxy* genes

To obtain genomic sequence information from common wheat, the regions downstream of the *Wx-A1* and *Wx-D1* genes were amplified with the primers 1 (5′-ACGGAACACCTGGTTCTGATATTG-3′), 2 (5′-CTTCTAAGTAACCAGGCAAGGGGC-3′), 3 (5′-GATTTGATGGCTGCTAGCTGGAAC-3), 4 (5′-GGGACTTACTTGGTACTCCGCTC-3′), 5 (5′-TTGTACCTTTGTCCTAACAAATG-3′), and 3′-F2 (5′-TGCCATGGAGTAGTTGGTGCTA-3′) (Saito et al. 2006).

These primers were designed based on genomic sequences from *H. vulgare* and *T. monococcum*, and EST sequences from wheat. Each 50 µl reaction contained 100 ng of genomic DNA of Chinese Spring, 15 pmol each of the forward and reverse primers, 0.8 mM $MgSO_4$, 0.2 mM dNTP (each), 1× KOD-Plus-buffer, and 1 U KOD-Plus-polymerase (Toyobo, Osaka, Japan).

Cloning of PCR products and sequence analysis

PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Inserts were sequenced using a CEQ 8000 DNA analysis system (Beckman Coulter, Inc., Fullerton, CA, USA).

Molecular analysis using PCR-based markers

For markers used to distinguish chromosome 4A-derived fragments, PCR was performed as described by Nakamura et al. (2002) using annealing temperatures ranging from 59 to 65°C. Amplified products were separated by electrophoresis on 1% or 4% agarose gels.

Construction of GenomeWalker libraries and chromosome walking

The genomic sequence flanking the deletion junction in the null *Wx-B1* allele was amplified by genome walking using the GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA). Genomic DNA from Mochi-Otome was digested separately with four restriction enzymes, namely *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I, and libraries were constructed according to the manufacturer's instructions. The gene-specific primers 3′-R7 (5′-TTTTCTATCACAA GCTGTTAAACGTCTTGG-3′) and 3′-R8 (5′-ACAG CCTTATTGTACCAAGACCCATGTGTG-3′) were used for primary and secondary PCR, respectively. Each 50 µl reaction for primary PCR included 1 µl of DNA library, 15 pmol each of Adaptor Primer 1 (Clontech) and 3′-R7 primer, 0.8 mM $MgSO_4$, 0.2 mM dNTP (each), 1× KOD-Plus-buffer, and 1 U KOD-Plus-polymerase (Toyobo). For secondary PCR, 50-fold dilutions of primary PCR products were used as a template, and reactions included the specific primer 3′-R8 plus the non-specific primer Adapter Primer 2. The primary PCR cycle consisted of an initial 2 min denaturation at 94°C, followed by 7 cycles of 94°C for 15 s, 72°C for 3 min, and 32 cycles of 94°C for 15 s, 67°C for 3 min, with a final extension at 67°C for 4 min. The denaturation and extension conditions of secondary PCR were identical to those of primary PCR, except that the number of cycles was decreased from 7 to 5 for the first set of cycles and from 32 to 20 for the second set. The amplified products were cloned and sequenced as described above.

PCR conditions for the codominant PCR marker

PCR conditions for discriminating between wild-type and null *Wx-B1* alleles were identical to those used by Nakamura et al. (2002).

Results

Position of the 3′ deletion breakpoint

We previously determined the sequence of the 6,532 bp region immediately downstream of the *Wx-B1* gene in Chinese Spring (Saito et al. 2006) by employing primers based on sequences of BAC clones containing the *waxy* genes of *H. vulgare* (Hv, Accession number AF474373, 124,050 bp) and *T. monococcum* (Tm, AF488415, 168,746 bp). Using this sequence, we were able to determine that one of the deletion breakpoints was located approximately 3.3–6 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).

To further refine the position of the 3′ breakpoint, primers capable of amplifying downstream sequences of *Wx-A1* and *Wx-D1* genes (Fig. 1) were designed based on sequence information from *H. vulgare* and *T. monococcum* BAC clones, wheat EST sequences, and the Chinese Spring genomic sequence described above. Using these primers, three overlapping regions were amplified from Chinese Spring genomic DNA (Fig. 1). Sequences of several clones from each region were compared with those from adjacent regions, and two contigs, expected to contain

sequence downstream from either *Wx-A1* or *Wx-D1*, were constructed. Each contig was identified as containing sequence derived from chromosome 7A or 7D by nullisomic–tetrasomic analysis. Although the sequences for all three fragments shown in Fig. 1 were obtained for the D genome, only sequences for the two 3′ fragments were determined for the A genome.

Four new markers, 3′-3, 3′-4, 3′-5 and 3′-6, were developed based on differences in genomic sequence between the regions downstream of the *Wx-A1*, *Wx-B1* and *Wx-D1* genes (Fig. 2a; Tables 1, 2). These

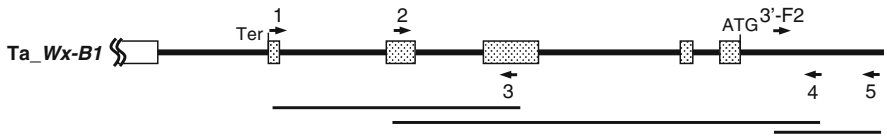

Fig. 1 Location of fragments used to determine genomic sequence downstream of the *waxy* gene. Primer positions are indicated by *arrows*. The *stippled boxes* represent exon regions of a gene found downstream of the *waxy* gene (*white box*). *Bars* represent DNA fragments amplified by PCR. *ATG* and *Ter* indicate initiation and termination codons, respectively

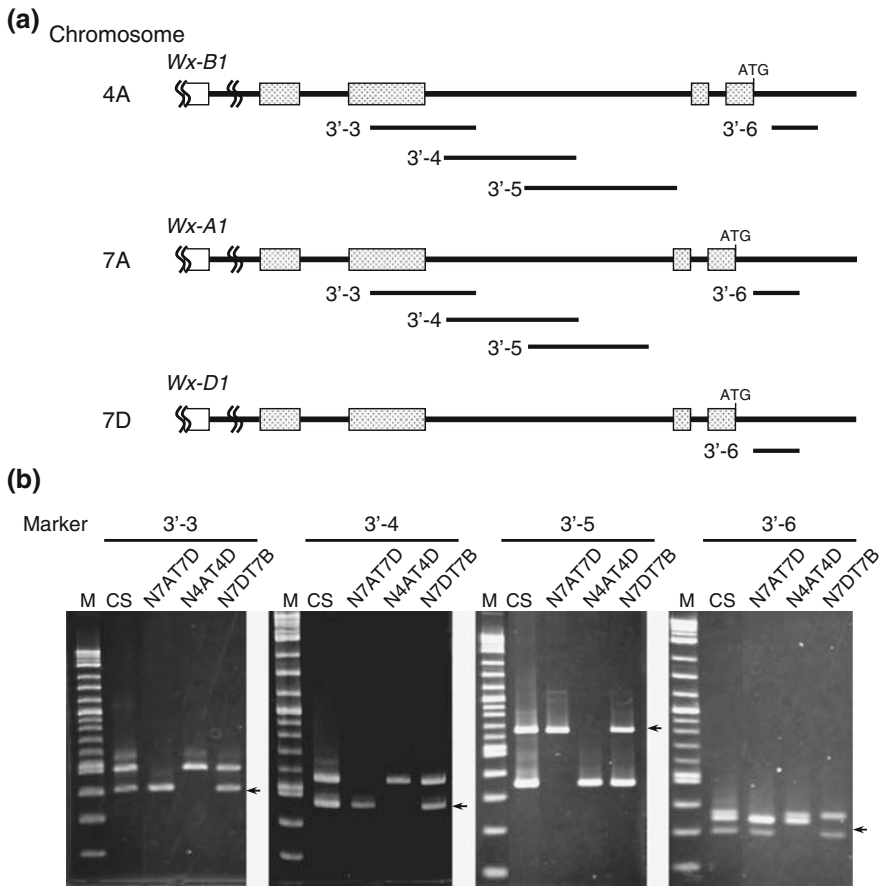

Fig. 2 Structure of the downstream regions of *waxy* genes and position of new markers specific for chromosome 4A. **a** Diagrammatic representation of the regions downstream of the three homoeologous *waxy* genes. *White boxes* represent *waxy* genes and *stippled boxes* represent exon regions of an expressed gene located downstream of *waxy*. *ATG* indicates initiation codon. *Bars* below the diagrams indicate the location of PCR-based markers. Marker names are indicated to the left of each fragment. **b** PCR markers for the detection of chromosome 4A-specific fragments. Fragments amplified from the chromosome carrying the *Wx-B1* gene (chromosome 4A) are indicated by *arrows*. M, 2-Log DNA Ladder (NEB). CS, Chinese Spring; N7AT7D, nullisomic 7A tetrasomic 7D; N4AT7D, nullisomic 4A tetrasomic 7D; N7DT7B, nullisomic 7D tetrasomic 7B

Table 1 Primer sequences of PCR-based markers near the *waxy* locus of wheat

Marker	Forward (5′–3′)		Reverse (5′–3′)	
3′-3	3′-F3	TCATCAAGAGCCATTCTTAGGG	3′-R3-A[a]	TGCTAATATTGTACTCCCTCCG
			3′-R3-B[b]	CTTCATTTTTCTGCAACCCTAGT
3′-4	3′-F4-A[a]	TTTCAACCAAGGTACTACCTCTGT	3′-R4	TCACAAGAACACCTTGAGACTGC
	3′-F4-B[b]	GAACTAAGGTACAATACTAGGGTTGC		
3′-5	3′-F5	ACGGAGCCGCTGGCATTG	3′-R5-A[a]	GAGGAGAGATGACGATAAACACC
			3′-R5-B[b]	GGAGGAGTTGGTGATACTGTGTATG
3′-6	3′-F6	CCTTCCTCTCTYTCGCTTGC	3′-R6	RTGGTGAAGCCATTCCTCCT

[a] Specific to the 7A genome

[b] Specific to the 4A genome

Table 2 Expected sizes of products amplified from each genome

Marker names	Forward primers	Reverse primers	Fragment sizes (bp)		
			7A (A genome)	4A (B genome)	7D (D genome)
3′-3	3′-F3	3′-R3-A, B	487	399	
3′-4	3′-F4-A, B	3′-R4	568	469	
3′-5	3′-F5	3′-R5-A, B	361	670	
3′-6	3′-F6	3′-R6	346	295	331

primers amplified products specific to chromosome 4A, the chromosome carrying the *Wx-B1* gene (Fig. 2b). Using the markers 3′-4, 3′-5 and 3′-6, PCR products from chromosome 4A were produced from all wheat cultivars (Fig. 3). With the marker 3′-3, amplification fragments were not produced from cultivars carrying the null *Wx-B1* allele (Fig. 3), indicating that a deletion breakpoint is located in the region between primers 3′-F3 and 3′-F4 (Fig. 4).

Position of the 5′ deletion breakpoint

Using a GenomeWalker library constructed from the cultivar Mochi-Otome as a template, sequences flanking the deletion junction were determined by chromosome walking. Chromosome 4A-specific primers 3′-R7 and 3′-R8, which were designed based on the sequence downstream of primer 3′-F4, were used for primary and secondary PCR, respectively. A 709 bp fragment amplified from a *Dra*I library contained genomic sequence flanking the deletion breakpoints (Fig. 5). The region from nucleotide 394–709 in this fragment corresponded to the area found from 3,873 to 4,188 bp downstream from the translation termination codon of the *Wx-B1* gene in wild-type wheat (Fig. 5, boxed letters). This indicated that an additional 3,872 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, a sequence similar to nucleotides 6–234 of the amplification fragment was found in a *T. monococcum* BAC clone (accession no. AF488415, positions 14142-14376, *E*-value = $3e^{-51}$, bit score = 210). This sequence is located approximately 60 kb upstream of 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 a 1,828 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

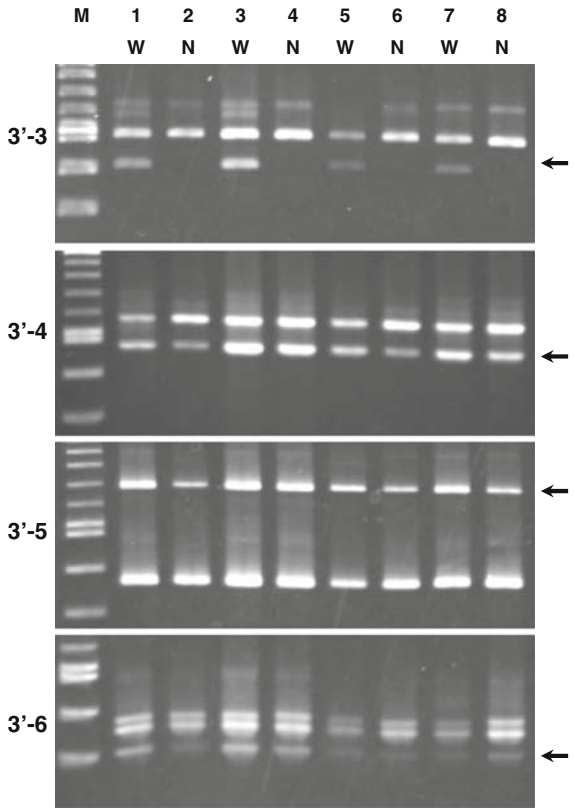

Fig. 3 PCR analysis of wheat lines using markers located downstream of the *Wx-B1* gene. Fragments amplified from the chromosome carrying the *Wx-B1* gene (chromosome 4A) are indicated by *arrows*. *Lane 1* Chinese Spring, *2* Kinuiroha, *3* Norin 61, *4* Mochi-Otome, *5* Bencubbin, *6* Aroona, *7* Mintlor, *8* Rosella. Chinese Spring, Norin 61, Bencubbin and Mintlor carry the wild-type *Wx-B1* allele (W), while Kinuiroha, Mochi-Otome, Aroona and Rosella carry the null *Wx-B1* allele (N). M, 2-Log DNA Ladder (NEB)

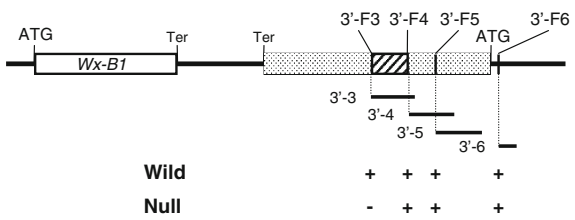

Fig. 4 Molecular analysis of the 3′ deletion breakpoint. Presence (+) and absence (−) of PCR products amplified from cultivars carrying wild-type and null *Wx-B1* genes are indicated. *Bars* represent positions of the markers. One of the deletion breakpoint is located between 3′-F3 and 3′-F4 primers

gene. Consequently, it appears that no expressed genes are found within the 60 kb region of the deletion upstream of the *waxy* gene.

Production of a PCR-based codominant marker

Primers for the identification of the null *Wx-B1* allele were designed based on sequences upstream and downstream of deletion breakpoints (Figs. 5, 6a, primers BFC and BRC2, 3′-R8 primer was renamed BRC2). Primers capable of amplifying the *Wx-B1* gene were employed for the detection of the wild-type *Wx-B1* allele (Fig. 6a, primers BDFL and BRC1). Primers BDFL and BRC1 (Table 3) anneal to the *Wx-B1* gene and amplify a 778 bp product, while primers BFC and BRC2 (Table 3) 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 only amplified 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. 6b, lanes 1, 3, 5 and 7) and 668 bp products were amplified from all cultivars carrying the null allele (Fig. 6b, lanes 2, 4, 6 and 8). When wheat genomic DNA of a heterozygous plant was used as a template, both products were detected (Fig. 6b, lanes 9–11). The marker also performed successfully in the U.S. derived breeding materials, where the 778 bp product was present in the wild-type cultivar Anton, and in approximately half of the tested seed of the heterogeneous breeding line 03LNK6053-68, whereas the 668 bp product was detected in the null cultivar RioBlanco, the two fully waxy breeding lines, and in half of the tested seed of 03LNK6053-68 (data not shown).

Discussion

A number of wheat varieties deficient in Wx-B1 protein have been identified in common wheat germplasm from throughout the world (Boggini et al. 2001; Urbano et al. 2002; Yamamori et al. 1994, 1998). At least two distinct mutations have been characterized; one is a deletion which includes the entire coding region of the *Wx-B1* gene, as is found in Kanto 107 (Vrinten et al. 1999) while the second characterized mutation does not involve the deletion

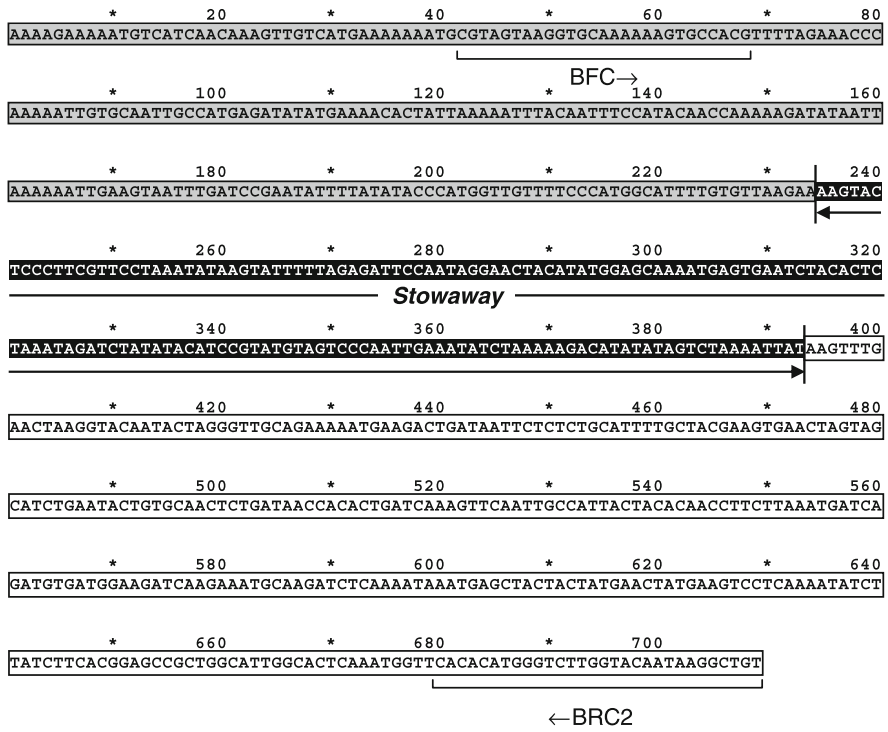

Fig. 5 Genomic sequence flanking the *Wx-B1* deletion. Nucleotide sequence was obtained from the cultivar Mochi-Otome. Sequence corresponding to a region 3′ of the *Wx-B1* gene is indicated by an *open box*. Sequence corresponding to the MITE "*Stowaway*" is shown on a *black background*. Sequence corresponding to an area 5′ of the *Wx-B1* gene is indicated by a *grey box*. Primers BFC and BRC2 were used for the detection of the null *Wx-B1* allele

Table 3 Primers used for the identification of the *Wx-B1* null allele

Primer names		Sequences (5′–3′)	Size (bp)
Wild type	BDFL	CTGGCCTGCTACCTCAAGAGCAACT	778
	BRC1	GGTTGCGGTTGGGGTCGATGAC	
Null	BFC	CGTAGTAAGGTGCAAAAAAGTGCCACG	668
	BRC2	ACAGCCTTATTGTACCAAGACCCATGTGTG	

of the entire gene (Urbano et al. 2002). An analysis of cultivars lacking the Wx-B1 protein collected from throughout the world indicated the entire *Wx-B1* gene appeared to be deleted in all 42 cultivars tested (Saito et al. 2004). This suggests that the deletion of the entire coding region is the major cause of Wx-B1 protein deficiency in common wheat germplasm. However, the extent and breakpoints of the deletion were not identified. In this study, we identified a nucleotide sequence flanked by the deletion breakpoints (Fig. 5). We also determined that an identical area is deleted in Japanese and Australian wheat varieties (Fig. 6). Furthermore, we found a miniature inverted-repeat transposable element, "*Stowaway*" adjacent to the deletion breakpoints (Fig. 5). It is possible that this element may have played a role in the deletion of the *Wx-B1* gene.

Wheat has a very large genome size (1.6×10^{10} bp), and relatively little genomic sequence information is available in public databases. Since genomic sequence information for the flanking regions of the *Wx-B1* gene was not available, a comparative genomics approach was used to obtain 6.4–7.0 kb of downstream sequence from wheat. PCR analysis revealed that a portion of a gene located downstream of the *Wx-B1* gene was deleted along with the *Wx-B1* gene. Although this gene did not show similarity to genes with known functions, it showed a high degree of similarity to cDNA clones of maize and rice (data not shown). Wheat EST clones derived from endosperm tissue also showed high similarity to this gene, suggesting the gene may be expressed and function in wheat endosperm tissue. It is therefore possible that the

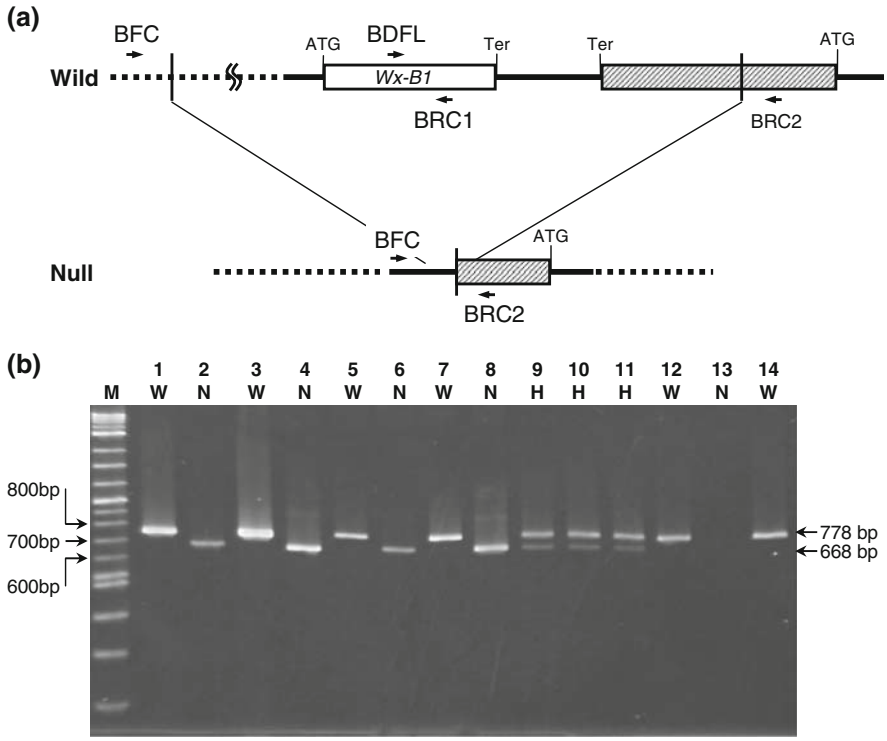

Fig. 6 Detection of the *Wx-B1* null allele by PCR-based markers. **a** Diagrammatic representation of the genomic area encompassing the wild-type and null *Wx-B1* genes. *Arrows* indicate primer position and orientation. **b** Identification of wild-type and null alleles of the *Wx-B1* gene using the primers BDFL, BRC1, BFC and BRC2. *Lane 1* Chinese Spring, *2* Kinuiroha, *3* Norin 61, *4* Mochi-Otome, *5* Bencubbin, *6* Aroona, *7* Mintlor, *8* Rosella, *9–11* heterozygotes for *Wx-B1* gene, *12* N7AT7D, *13* N4AT4D, *14* N7DT7B, *M* 2-Log DNA ladder. 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

deletion of a portion of this gene may affect endosperm and starch properties.

Based on the similarity between a sequence found upstream of the *Wx-B1* 3′ deletion breakpoint and a sequence found upstream of the *waxy* gene of *T. monococcum*, we estimated approximately 60 kb of DNA upstream of the *Wx-B1* gene was deleted in the null allele. Also, we hypothesized that no expressed genes are found within this region of the deletion, since expressed genes are not found in the corresponding region of the *T. monococcum* genome. 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. Similar observations were made for other genomic loci (Chantret et al. 2005). 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 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.

Determination of the genomic sequence flanking the deletion breakpoints in the null *Wx-B1* allele allowed us to produce a codominant marker for *Wx-B1* alleles. This marker can identify plants which are heterozygous at the *Wx-B1* locus, allowing efficiently selection in a continuous backcross program, and is also useful for selection in heterogeneous lines. When using dominant markers for *Wx-B1* alleles (Nakamura et al. 2002), it was only possible to tentatively identify heterozygotes based on the ratio of PCR products from wild-type *Wx-B1* alleles to those from *Wx-A1* or *Wx-D1* genes. However, using the codominant marker developed here, selection errors can be avoided, contributing to effective selection in wheat breeding programs. Furthermore, since the deletion used for the selection of partial waxy lines lacking Wx-B1 protein appears to be identical in Japanese, American, Australian, Chinese and Canadian wheat lines, the markers should be useful in a wide range of breeding programs.

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