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REVIEW

DNA Markers for Identifying *waxy* Mutations and Improving Noodle Quality in Wheat

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Abstract

Approximately 70% of wheat endosperm consists of starch, and variations in the quality and quantity of starch affect the processing characteristics of wheat flour. Amylose content in particular has a major effect on Asian noodle quality, and selection of wheat lines with slightly lower amylose levels is an important goal in Japanese wheat breeding programs. Accurately measuring amylose content by direct methods such as colorimetric assays was found to be problematic, suggesting there was a need for a more efficient and accurate method of screening for reduced amylose content. Therefore, we characterized mutations in the wheat *waxy* genes, which control amylose synthesis, and developed DNA markers for the identification of null *waxy* alleles. In this review, we describe the development of these markers and outline their utility for wheat breeding programs.

Discipline: Plant breeding
Additional key words: breeding, codominant marker, marker-assisted selection, starch

Introduction

The *waxy* gene encodes the waxy (Wx) protein, or granule-bound starch synthase I, which is a key enzyme for the synthesis of amylose in endosperm tissue. Mutations at the *waxy* locus that lead to a lack of enzyme activity affect amylose content and result in low-amylose or waxy (amylose-free) mutants. Although waxy mutations were reported in a number of plant species as early as the nineteenth century[4], only within the last fifteen years have such mutations been identified and studied in hexaploid wheat (*Triticum aestivum* L.).

Wheat has three *waxy* genes located on chromosomes 7A (*Wx-A1*), 4A (*Wx-B1*) and 7D (*Wx-D1*). Even in the absence of one or two waxy proteins, amylose production is maintained by the activity of the remaining protein(s), so that a completely waxy mutant was not easily identifiable in wheat germplasm. However, separation of the three Wx proteins allowed the identification of spontaneous mutations at each locus[14]. Null *waxy* alleles were found at the *Wx-A1* and *Wx-B1* loci of Japanese cultivars and at the *Wx-D1* locus of a Chinese cultivar[13,27]. These partial waxy mutants were combined to produce waxy wheat, which lacks all Wx proteins[15]. In addition to fully waxy wheat, six different partial waxy wheat lines can be produced by crossing, and the amylose content of these lines varies depending on the specific combination of *waxy* alleles (Table 1)[12]. Among the partial waxy types, the line carrying the *Wx-B1* null allele and two null combination lines show significantly lower amylose contents than wild type.

Amylose content has a significant role in determining noodle quality, and partial waxy wheat carrying the null *Wx-B1* allele is preferable for Japanese udon noodles. The suitability of Australian Standard White (ASW) wheat cultivars for producing udon noodles is due at least in part to the low amylose levels of these cultivars[16,23], and correspondingly, most ASW cultivars lack Wx-B1 protein[27]. Since low amylose content is an important trait in noodle breeding programs in Japan, breeders require an efficient method for the selection of partial waxy wheat types. However, in breeding programs handling large numbers of wheat lines, it is difficult to precisely and efficiently screen for partial waxy wheat using methods such as measurement of amylose content or electrophoretic separation of Wx proteins.

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Table 1. Classification of partial waxy wheats according to the presence (+) or absence (-) of each waxy protein and amylose contents (AM) of each type[12]

	Wx proteins			AM (%)
	Wx-A1	Wx-B1	Wx-D1	
Type 1	+	+	+	28.7
Type 2	-	+	+	28.5
Type 3	+	-	+	27.1
Type 4	+	+	-	28.0
Type 5	+	-	-	20.3
Type 6	-	+	-	25.8
Type 7	-	-	+	22.9
Type 8	-	-	-	0.9

AM: Amylose contents analyzed by an auto analyzer.

Recently, a large number of genes and quantitative trait loci (QTLs) related to important agronomic traits such as disease resistance, pest resistance and quality of wheat flour have been described. This information has allowed rapid progression in the development of DNA markers able to distinguish lines with desirable phenotypes based on plant genotypes. Due to the hexaploid nature of wheat, three copies of most genes are present, and recessive mutations are difficult to detect phenotypically. Therefore, selection using DNA markers able to exactly identify the required genotype is particularly important in wheat breeding.

In this review, we summarize the development of DNA markers for distinguishing *waxy* alleles, and outline the introduction of these markers into wheat breeding programs in Japan.

The *waxy* mutations in wheat

The successful separation of the three waxy proteins, Wx-A1, Wx-B1 and Wx-D1, by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)[14] allowed the identification of waxy mutants lacking one or two waxy proteins[13,27]. The *waxy* mutations were first investigated at the molecular level using waxy wheat derived from crosses between Kanto 107, which lacks Wx-A1 and Wx-B1 proteins, and Bai Huo, which lacks Wx-D1 protein[25]. A 23-bp deletion and a 4-bp insertion was detected in the null *Wx-A1* allele (Fig. 1a), while a 588-bp deletion and a 12-bp insertion occurred in the null *Wx-D1* allele (Fig. 1c), and the entire coding region was deleted in the *Wx-B1* null allele (Fig. 1b).

PCR-based markers for each null allele of waxy wheat

Based on nucleotide sequences of the wild-type and null *waxy* alleles[11,25], primer sets capable of identifying the genotype at each *waxy* locus were designed[12] (Figs. 1 and 2). Since these primer sets can work under a single set of conditions, mutations at the *Wx-A1*, *Wx-B1* and *Wx-D1* loci can be identified in a single reaction using multiplex PCR. These primers can identify mutations occurring in *Wx-A1* and *Wx-B1* genes of wheat lines not only from Japan but also from the major wheat producing countries Australia, Canada and the USA. Therefore, the origins of the *waxy* mutations in lines from these countries appear to be identical to the mutations in Kanto 107, suggesting the markers will be useful in wheat breeding programs around the world.

Molecular comparison of *waxy* null alleles

The PCR markers described above were used to characterize *waxy* mutations in 168 wheat lines from 20 countries[19]. Although most cultivars lacking Wx-A1 protein had the same mutation found in Kanto 107, lines from Turkey produced a fragment 173 bp longer than the fragment from wild-type alleles. This newly identified mutation was caused by the insertion of a transposable element into exon 4 of the *Wx-A1* gene. The transposable element was 165 bp in size, with 12-bp terminal inverted repeats on each end. An 8-bp target site duplication consisting of *Wx-A1* sequence flanked each end of the insertion. It is noteworthy that this insertion was conserved only in lines from Turkey, while the more common deletion described above was found in lines from several areas of the world, suggesting that the insertion event occurred comparatively recently.

In all lines where the Wx-B1 protein was absent, the *Wx-B1* allele appeared to be identical to the mutation carried by Kanto 107, suggesting that the null *Wx-B1* mutation has a single origin. From the geographical distribution pattern of null *Wx-B1* alleles[27,28], we speculated that this mutation arose in western Asia and spread to other areas of Asia, with modern plant breeding programs facilitating the distribution of the allele to lines bred in Australia, Europe and North America.

Several spontaneous mutations caused by different mechanisms than those described above have been identified in *Wx-B1*[10,24] and *-D1*[10,21] alleles. Since these mutations were not found in multiple cultivars, they may have arisen relatively recently. Further molecular analysis of these mutations will provide additional insight into the origins and geographical distribution of mutations at the

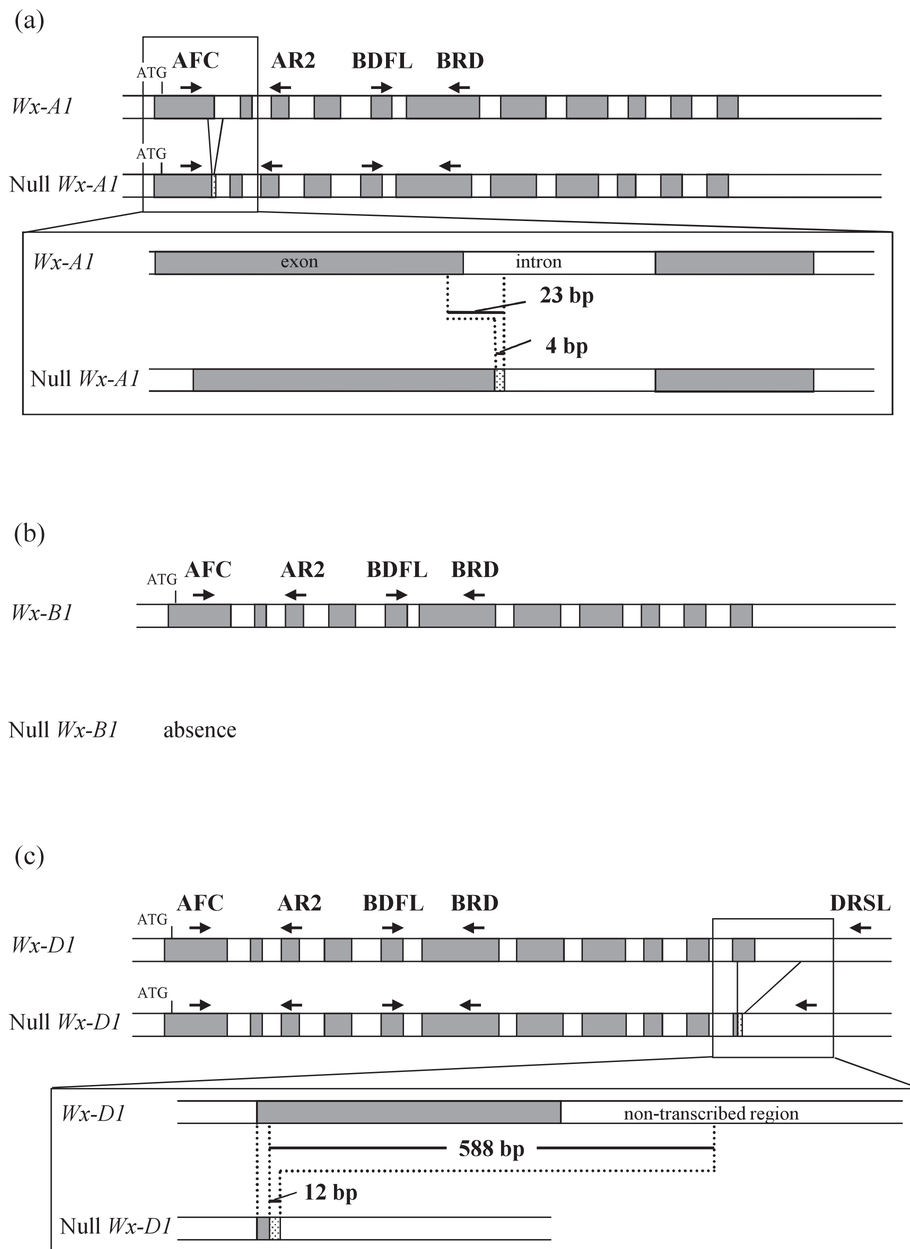


Fig. 1. Mutations in *Wx-A1* (a), *Wx-B1* (b) and *Wx-D1* (c) genes of waxy wheat
The stippled boxes represent filler DNA and gray boxes represent exons.
ATG indicates initiation codon.

waxy locus.

Development of a codominant marker for the *Wx-B1* null allele

In breeding programs, continuous backcrossing is often employed for introducing a desired trait into elite breeding lines. Clear identification of heterozygous plants is very important in this breeding method, and codominant markers are particularly useful since dominant markers cannot distinguish homozygous wild-type from heterozygous lines. The development of codominant markers for the *Wx-A1* and *Wx-D1* loci was relatively straightforward[12,21], as was the design of dominant markers for the mutation at the *Wx-B1* locus[9,12]. However, due to the large deletion in the null *Wx-B1* allele, the design of a codominant marker for this mutation was more challenging. While it has been known for some time that the entire coding region of the *Wx-B1* gene is missing in the most common null allele[25], the extent and breakpoints of the deletion were determined only recently[18,20]. This was accomplished using a comparative genomics analysis method

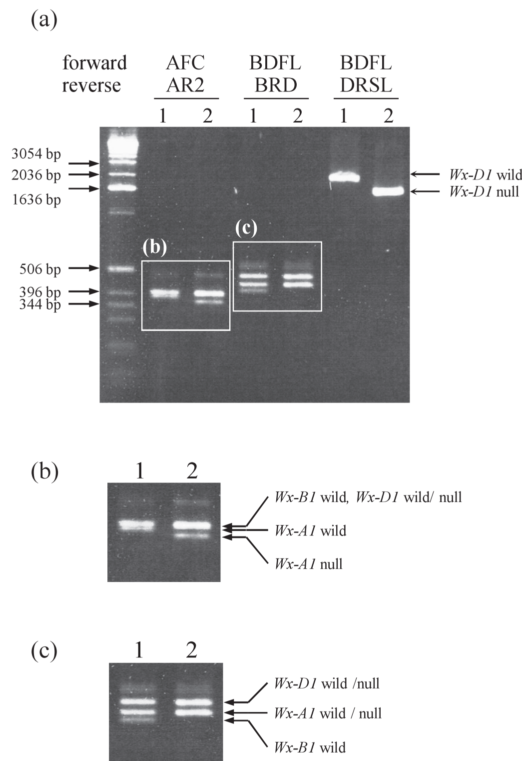


Fig. 2. PCR assays for the detection of null alleles of *waxy* genes in wild-type wheat (lane 1) and waxy wheat (lane 2)

(a) PCR assay using primers AFC and AR2 to distinguish the wild-type and null alleles of the *Wx-A1* gene, primers BDFL and BRD to distinguish the *Wx-B1* alleles and primers BDFL and DRSL to distinguish the *Wx-D1* alleles. (b) Enlarged view of products amplified with primers AFC and AR2. (c) Enlarged view of products amplified with primers BDFL and BRD. M: Marker; a 1 kb DNA ladder (Invitrogen) was used as a size marker.

based on the synteny between wheat and the related grass species; barley, einkorn wheat and rice, which allowed us to design PCR-based markers for the detection of deletion breakpoints[18,20]. The markers were used to refine the position of the 3' deletion breakpoint, and chromosome walking was used to determine the DNA sequences flanking the deletion breakpoints. As shown in Figure 3, the deletion included a 3872-bp region downstream from the termination codon of the *Wx-B1* gene[20]. Based on comparisons with *T. monococcum* sequences, it was estimated that the deletion also included approximately 60 kb of sequence upstream of the *Wx-B1* gene[20]. The sequence information obtained from these experiments was used to develop a codominant marker for the *Wx-B1* null allele (Fig. 4) which allows the clear identification of heterozygous plants.

The PCR conditions for this codominant marker are identical to those for the *Wx-A1* and *Wx-D1* markers. The sizes of the amplified fragments (778 bp for the wild-type allele and 668 bp for the null allele of the *Wx-B1* loci) allow the marker to be analyzed alongside markers for the *Wx-A1* and *Wx-D1* genes (Fig. 5)[20]. The practical utility of this marker has been tested in backcrossing programs, where we found it to serve as an efficient selection method.

Conclusions

In Japanese wheat breeding programs, the measurement of amylose content using colorimetric assays has been employed in screening to identify low amylose lines suitable for udon noodle production. Although this method is simple, inherent measurement errors renders

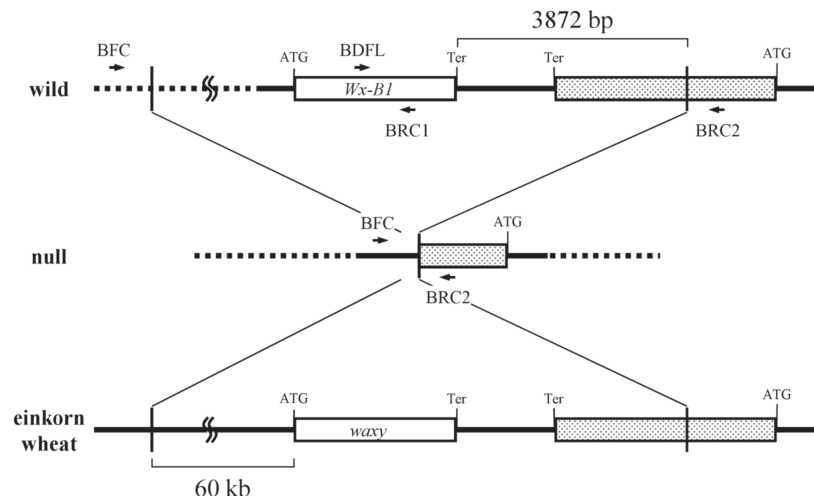


Fig. 3. Comparison of the *waxy* gene region in einkorn wheat with the wild-type and null *Wx-B1* gene regions in hexaploid wheat

Arrows indicate primer positions and orientations.

ATG and Ter indicate initiation and termination codons, respectively.

it unreliable for the accurate selection of partial waxy wheat lines. Conversely, the separation of waxy proteins using SDS-PAGE or 2D-PAGE can accurately identify partial waxy wheat lines. However, because procedures for purifying starch from seed and for protein separation are complicated, these methods are inconvenient for use in breeding programs that handle a large number of populations. Methods which are more suitable for high throughput screening of waxy and partial waxy wheat types include ELISA[5-8] and near-infrared reflectance spectroscopy[2,3]. While such methods can identify plants which are homozygous for null *waxy* alleles, they cannot distinguish heterozygous plants from those which are homozygous for wild-type alleles. In comparison, the use of DNA markers provides a more convenient and accurate screening method.

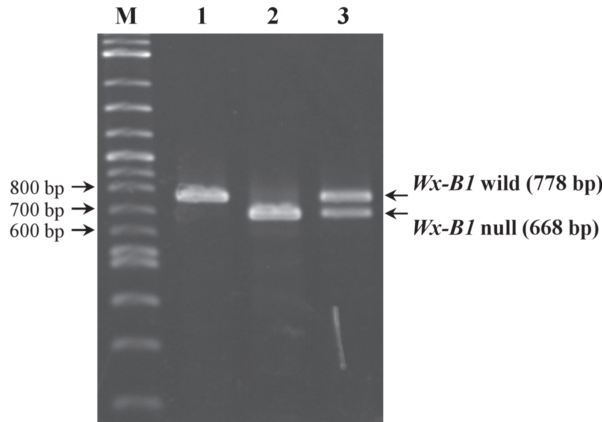
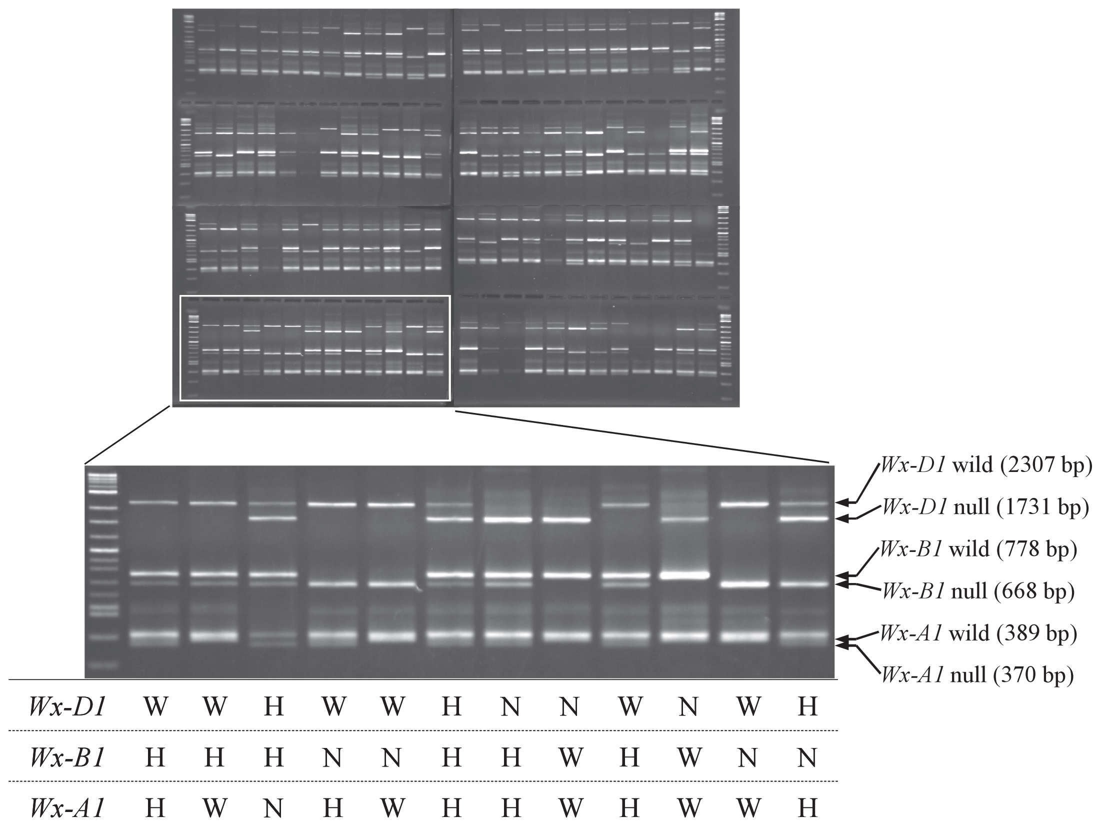


Fig. 4. PCR assays distinguishing wild-type and null alleles of the *Wx-B1* gene

Lane 1: Chinese Spring (wild-type), 2: Mochi-Otome (null), 3: individual heterozygous for *Wx-B1* gene, M: 2-Log DNA ladder (NEB).

Wheat breeding is time-consuming; the development and release of a new cultivar generally takes about ten years. However, with breeding goals changing rapidly based on producer and consumer demands, quicker progress is becoming a necessity. The use of continuous backcrossing combined with marker-assisted selection (MAS) can contribute to the acceleration of wheat breeding programs. The on-going progress in the molecular characterization of numerous pest resistance and quality traits is being accompanied by intensive development of



Wx-D1	W	W	H	W	W	H	N	N	W	N	W	H
Wx-B1	H	H	H	N	N	H	H	W	H	W	N	N
Wx-A1	H	W	N	H	W	H	H	W	H	W	W	H

Fig. 5. Genotype analysis for *waxy* mutations in individuals from a breeding line of wheat

A combination of three markers was used. Multiplex PCRs with markers for *Wx-B1* and *Wx-D1* and single PCRs for *Wx-A1* were conducted in 96-well formats. Amplified products from these reactions were mixed and separated on a 4% agarose gel. Alleles present for each *waxy* gene are indicated by W (wild-type allele), N (null allele) and H (heterozygous).

DNA markers linked to these phenotypes, resulting in a continual increase in the number of available markers. For example, the Coordinated Agricultural Projects for wheat (wheat CAP), which has the objective of incorporating modern selection technologies into wheat breeding programs, has close to sixty molecular markers listed on its website[26]. MAS in wheat has several advantages over traditional screening methods: 1) DNA for MAS can be extracted from any tissue, and tissue can be collected at almost all stages of plant growth. 2) Multiple markers can be combined in a single screening experiment. Breeders can determine genotypes at multiple loci using the same template DNA. 3) Desirable genotypes related to flour quality can be identified by MAS using DNA from a single seed, whereas other methods of screening for flour quality traits are difficult to use in early generations due to the difficulty of obtaining an appropriate quantity of flour for testing.

The PCR markers described here can distinguish individuals which are heterozygous, homozygous wild-type or homozygous null at all three *waxy* loci, and we have successfully used these markers for screening in continuous backcrossing programs. The cultivar "Yumeasahi", released in 2004, is the first wheat cultivar developed in Japan using these markers. These waxy markers are already being employed in breeding programs not only in Japan, but also in the USA and China, where they are used to assist in the development of both partial and fully waxy lines. Since partial waxy wheat flour is not only desirable for udon noodle production, but also has positive effects on the quality of bread and pasta products, including slower firming during bread storage and lower cooking losses for pasta[1,17,22], we expect that the use of the markers described here will continue to facilitate wheat breeding programs not only in Japan but throughout the world.

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References

1. Baik, B. K. et al. (2003) Characteristics of noodles and bread prepared from double-null partial waxy wheat. *Cereal Chem.*, **80**, 627–633.
2. Delwiche, S. R. & Graybosch, R. A. (2002) Identification of waxy wheat by near-infrared reflectance spectroscopy. *J. Cereal Sci.*, **35**, 29–38.
3. Delwiche, S. R. et al. (2006) Single kernel near-infrared analysis of tetraploid (durum) wheat for classification of the waxy condition. *Cereal Chem.*, **83**, 287–292.
4. Eriksson, G. (1969) The waxy character. *Hereditas*, **63**, 180–204.
5. Gale, K. R., Blundell, M. J. & Hill, A. S. (2004) Development of a simple, antibody-based test for granule-bound starch synthase Wx-B1b (Null-4A) wheat varieties. *J. Cereal Sci.*, **40**, 85–92.
6. Gale, K. R. et al. (2001) Application of a high-throughput antibody-based assay for identification of the granule-bound starch synthase *Wx-B1b* allele in Australian wheat lines. *Aust. J. Agric. Res.*, **52**, 1417–1423.
7. Giersch, T. M. et al. (2007) Detection of mutations in the 7A allele of wheat (*Triticum aestivum*) granule-bound starch synthase (Wx-7A) with a monoclonal antibody produced by targeted peptide immunisation. *J. Cereal Sci.*, **45**, 162–171.
8. Graybosch, R. A., Schemmerhorn, K. J. & Skerritt, J. H. (1999) An enzyme-linked immunosorbent assay for the identification of wheats carrying null alleles at genetic loci encoding the granule-bound starch synthase. *J. Cereal Sci.*, **30**, 159–163.
9. McLauchlan, A. et al. (2001) Development of robust PCR-based DNA markers for each homoeo-allele of granule-bound starch synthase and their application in wheat breeding programs. *Aust. J. Agric. Res.*, **52**, 1409–1416.
10. Monari, A. M. et al. (2005) Molecular characterization of new waxy mutants identified in bread and durum wheat. *Theor. Appl. Genet.*, **110**, 1481–1489.
11. Murai, J., Taira, T. & Ohta, D. (1999) Isolation and characterization of the three *Waxy* genes encoding the granule-bound starch synthase in hexaploid wheat. *Gene*, **234**, 71–79.
12. Nakamura, T. et al. (2002) Rapid classification of partial waxy wheats using PCR-based markers. *Genome*, **45**, 1150–1156.
13. Nakamura, T. et al. (1993) Decrease of waxy (Wx) protein in 2 common wheat cultivars with low amylose content. *Plant Breed.*, **111**, 99–105.
14. Nakamura, T. et al. (1993) Identification of three Wx proteins in wheat (*Triticum aestivum* L.). *Biochem. Genet.*, **31**, 75–86.
15. Nakamura, T. et al. (1995) Production of waxy (amylose-free) wheats. *Mol. Gen. Genet.*, **248**, 253–259.
16. Oda, M. et al. (1980) A method of flour quality assessment for Japanese noodles. *Cereal Chem.*, **57**, 253–254.
17. Park, C. S. & Baik, B. K. (2007) Characteristics of french bread baked from wheat flours of reduced starch amylose content. *Cereal Chem.*, **84**, 437–442.
18. Saito, M. et al. (2006) Analysis of deletion breakpoints in a wheat null *Wx-B1* allele using molecular markers. *Ikushugaku Kenkyu (Breed. Res.)*, **8**, 9–16 [In Japanese with English summary].
19. Saito, M. et al. (2004) Molecular comparison of *waxy* null alleles in common wheat and identification of a unique null allele. *Theor. Appl. Genet.*, **108**, 1205–1211.
20. Saito, M. et al. (2009) A novel codominant marker for selection of the null *Wx-B1* allele in wheat breeding programs. *Mol. Breed.*, **23**, 209–217.
21. Shariflou, M. R., Hassani, M. E. & Sharp, P. J. (2001) A PCR-based DNA marker for detection of mutant and

normal alleles of the *Wx-D1* gene of wheat. *Plant Breed.*, **120**, 121–124.
22. Sharma, R. et al. (2002) The null-4A allele at the *waxy* locus in durum wheat affects pasta cooking quality. *J. Cereal Sci.*, **35**, 287–297.
23. Toyokawa, H. et al. (1989) Japanese noodle qualities. II. Starch components. *Cereal Chem.*, **66**, 387–391.
24. Urbano, M. et al. (2002) Waxy proteins in diploid, tetraploid and hexaploid wheats. *Plant Breed.*, **121**, 465–469.
25. Vrinten, P., Nakamura, T. & Yamamori, M. (1999) Molecular characterization of *waxy* mutations in wheat. *Mol. Gen. Genet.*, **261**, 463–471.
26. Wheat CAP: Wheat CAP. Available online at http://maswheat.ucdavis.edu/Index.htm.
27. Yamamori, M. et al. (1994) Waxy protein deficiency and chromosomal location of coding genes in common wheat. *Theor. Appl. Genet.*, **89**, 179–184.
28. Yamamori, M., Nakamura, T. & Kiribuchi-Otobe, C. (1998) Waxy protein alleles in common and emmer wheat germplasm. *Misc. Publ. Natl. Inst. Agrobiol. Resour.*, **12**, 57–104.