An improved MALDI-TOF mass spectrometry procedure and a novel DNA marker for identifying over-expressed Bx7 glutenin protein subunit in wheat

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Wheat bread-making quality is mainly determined by glutenin proteins in the grain, which exist in a wide range of variable alleles with differential influence on processing attributes. A recently identified allele, Bx7 over-expression (Bx7^{oe}), has been showing highly significant positive effects on wheat dough strength over the normally expressed Bx7 allele. SDS-PAGE and normal RP-HPLC procedures failed to separate the two alleles. In the current study, an extensively optimised MALDI-TOF based procedure and a refined DNA based marker for efficiently differentiating Bx7^{oe} from normal Bx7 allele were established. Results indicated that the MALDI-TOF procedure is cost effective, high throughput, and proven reliable, while the refined PCR marker only amplifies Bx7^{oe} allele, a clear advantage over the previously developed codominant marker.

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Wheat storage protein, which consists of high molecular weight (HMW) glutenins, low molecular weight (LMW) glutenins and gliadins (Payne 1987), confer special visco-elasticity property and has made bread-making possible. Variation of the wheat gluten's visco-elasticity property has a major impact on bread and other end product qualities. Extensive researches have been conducted towards understanding the roles and functions of wheat storage proteins. It has long been understood that the encoding loci of both HMW and LMW glutenins are located on the group 1 chromosomes, with the HMW glutenins located on the long arms (designated as *Glu-A1, Glu-B1* and *Glu-D1*) and the LMW glutenins on the short arms (designated as *Glu-A3, Glu-B3* and *Glu-D3).* While each HMW glutenin consists of two genes, x- and y-type genes, encoding for x- and y-type protein subunits, each LMW glutenin locus consists of multiple genes, encoding multiple protein subunits. Although the HMW-GS are less abundant than the LMW-GS, they have a major impact on dough quality and are amenable to relatively straightforward analysis. They have been used extensively as markers in breeding programs (Cornish et al. 2001a, 2001b; Ma et al. 2003).

There is a wide variation among wheat varieties in the composition of gliadins and glutenin subunits (Nieto-Taladriz et al. 1994; Shewry et al. 1995). Usually, bread-making quality variations of wheat varieties can be largely accounted for by their glutenin compositions (Ma et al. 2005). Even though there are at least 15 alleles in the three HMW glutenin loci, a much small number of alleles have become predominant in commercial wheat varieties due to artificial selection of desirable alleles during the breeding process. For example, according to Ma et al (2003), in Australian varieties, there are two predominant alleles in each of the *Glu-A1*, *Glu-B1* and *Glu-D1* locus. At the *Glu-A1* locus, *Glu-A1a* (*Ax1* protein subunit) and *Glu-A1b* (Ax2 subunit) are the two predominant alleles, with allele rate 47.1% and 45.4%, respectively. *Glu-B1b* (Bx7 + By8 subunits) and *Glu-B1i* (Bx17 + By18 subunits) represent the two predominant alleles at the *Glu-B1* locus, with respective allele rate of 36.4% and 39.3%. For *Glu-D1* locus, *Glu-D1d* (Dx5 + Dy10 subunits) and *Glu-D1a* (Dx2 + Dy12 subunits) are the two main alleles with their allele rates being 30.5% and 67.8%, respectively.

Recently, a significant level of attention has been attracted towards a unique allele – Bx7 over-expression (Bx7^{oe}) subunit allele, which was found in both common wheat (D'Ovidio et al. 1997) and durum (Elfatih et al. 2013). It was observed that the over-expression of Bx7 results in an improved dough strength (D'Ovidio et al. 1997), which was confirmed later by studying a range of

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modern Australian and North American cultivars and lines (Butow et al. 2003; Radovanovic et al. 2002), and in old Hungarian varieties and landraces (Juhász et al. 2000). Butow et al. (2003) revealed that, compared to normal Bx7 subunit, Bx7 ^{oe} has an 18 bp insertion in the central repetitive region of its coding gene. This results in a six amino acids difference between the two proteins – the Bx7^{oe} subunit contains extra six amino acids in the central repetitive domain of the protein. Wheat breeders are highly interested in selecting the Bx7^{oe} allele in breeding programs, making the differentiation of Bx7^{oe} allele from normal Bx7 allele an important task for wheat breeding programs. Due to near identical molecular weight and same mobility, it is hard to differentiate the two alleles by SDS-PAGE electrophoresis. Normal RP-HPLC are also failed to directly differentiate Bx7^{oe} from normal Bx7 subunit based on their elution time. A co-dominant PCR based marker is available (Ma et al. 2003; Butow et al. 2003) for differentiating Bx7^{oe} allele from normal Bx7. However, its co-dominancy nature has prevented it from being converted to a high-throughput marker format suitable for screening a large number of samples in a short time, which is usually required in breeding programs. On the other hand, the marker was constructed based on an 18-bp difference between the coding genes of Bx7^{oe} and normal Bx7 subunits. Such subtle difference often results in inaccuracy in screening the breeding lines. Therefore, developing a better marker is essential for fast differentiation of Bx7^{oe} and normal Bx7 subunits in large breeding programs.

The first study of analysing HMW glutenin subunit by MALDI-TOF equipment was reported by Dworschak et al. (1998), which successfully characterised one Bx7 HMW glutenin subunit. Recently, Zhang et al. (2008) targeted 16 HMW glutenin protein subunits and were able to differentiate most subunits. However, the Bx7^{oe} allele wasn't targeted. Liu et al. (2009) demonstrated that MALDI-TOF procedure can identify Bx7^{oe} subunit based on the molecular mass difference. Nevertheless, the reported Bx7^{oe} spectrum did not reflect its over-expression nature, i.e. the height and area of the Bx7^{oe} peak were in the same scope comparing to others. This indicates that there is a scope for further optimisation of the experimental conditions for the MALDI-TOF procedure. In this study, a high throughput and cost effective MALDI-TOF procedure to differentiate Bx7^{oe} allele from normal Bx7 allele is established. Moreover, a dominant PCR marker was designed for efficiently differentiating Bx7^{oe} allele from the other alleles at the *Glu-B1* locus.

MATERIAL AND METHODS

Plant material

Fourteen common wheat cultivars including Chara (Bx7^{oe}), Kukri (Bx7^{oe}), CD87 (Bx7^{oe}), Katepwa (Bx7), Janz (Bx7), Frame (Bx7), Cheyenne (Bx7), Calingri (Bx13), Catalina (Bx17), EGABonnieR (Bx17), EGAJitarng (Bx17), Lumai 23 (Bx20), Halberd (Bx20), Wanmai 33 (Bx7) and another 40 lines from doubled haploid population CD87 × Katepwa (Butow et al. 2003) that contains 20 each of Bx7^{oe} and normal Bx7 lines were used in the study.

High molecular weight glutenin subunits (HMW-GS) extraction and sample preparation

HMW-GS was extracted from dry wheat seed or flour. The method used to extract HMW-GS was adopted from Zhang et al. (2008) with some modifications. One dry wheat seed was grinded into powder in a 2 ml tube and 600 μl of 70% ethanol was added followed by stirring for 30 min at room temperature. Then the tube was centrifuged at 13 000 g for 2 min and supernatant was discarded. After that, 600 μl of 55% iso-propanol was added into pellets and mixed well and put in 65° water bath for 30 min. The supernatant was discarded from the tube after centrifugation at 13 000 g for 2 min. This iso-propanol washing procedure was repeated once. Then 600 μl of the protein extraction buffer (50% iso-propanol, 80mM Tris-HCl, pH8.0, and 5% 2-mercaptoethanol) was added and incubated in 65° (water bath) for 1 h. After centrifugation at 13 000g for 10 min 480 μl of supernatant was transferred into a new tube. A volume of 320 μl of precold acetone (–20°) was added into the supernatant to a final concentration of 40 % (V/V). The samples was kept at –20° freezer for 1–2 h and then centrifuged at 13 000g for 2 min. After removing the supernatant 10 μl of 50% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA) was added to dissolve the precipitation.

The HMW-GS sample was diluted into 10 times in 50% ACN and 0.5% TFA. One μl of the diluted sample was added into 9 μl SA solution and mixed by shortly votexing and 0.5 μl of each sample/matrix was spotted onto a MALDI-TOF Voyager DE Pro 100 sample size plate and air-dried thoroughly. The spotting was repeated 2 times for each sample. Matrix for HMW-GS analysis was Sinapinic acid (SA), which dissolved in 50% ACN and 0.05%TFA (10 mg ml^{-1}).

Instrument settings for MALDI TOF

Applied Biosystems Voyager DE Pro MALDI-TOF mass spectrometer was used and the instrument was set in linear mode. Mass spectra were collected automatically with the followings settings: 25 kv acceleration voltage; 0.3% guide wire voltage and 92% plate voltage; 1000 ns delay time; collection range from 60000–95 000 Da with a 10 000 Da low mass gate. Laser intensity was set to a maximum 2500. Ten spectra of 50 laser shots were collected in a random pattern of centre biased over the

sample spot for each sample, all which were automatically accumulated to provide the final spectrum.

DNA extraction and PCR primer design

Genomic DNA of single dry seeds was extracted following the procedure of An et al. (2006). PCR primers were designed based on the previously reported sequences of Bx7 (Anderson and Greene 1989) and a BAC clone of Bx7^{oe} (Ragupathy et al. 2008). Comparing the two sequences, there was a 12 bp insertion in Bx7^{oe} in the upstream region. The primers were designed based on the insertion, including forward 5′TGGTCTATTGGG TGGTCTAT3′ and reverse 5′AGGCGGTCGCTTAGT CAGAT3′.

PCR amplifications were carried out in a reaction volume of 20 µl containing 5 U Taq polymerase, 1 × PCR buffer (Axgen, containing 1.5 mM $MgCl_2$), 5 pmoles of each primer, 200 µM of each dNTP and 50 ng of template DNA. Amplification conditions for the PCR reaction were an initial cycle at 94°C for 4 min, cycled 32 times at 94°C for 30 s, 61°C for 30 s and 72°C for 20 s, and a final extension at 72°C for 1 min. PCR products were analyzed by agarose gel electrophoresis with 1.5% gel in Tris-acetic acid-EDTA buffer.

RESULTS

Differentiating over-expressed and normal Bx7 subunits by MALDI-TOF

By analysing set of wheat cultivars containing Bx7^{oe} or Bx7 and a doubled haploid population (CD87 × Katepwa) that is segregating at the *GluB1* locus for Bx7^{oe} versus normal Bx7 subunits. The results demonstrate that the new HMW glutenin analysis procedure has the potential to differentiate the Bx7^{oe} and normal Bx7 subunits accurately. The differences are at two aspects (Fig. 1): firstly, the molecular weight of Bx7^{oe} is about 600 Dalton greater than that of normal Bx7 and secondly, the Bx7^{oe} subunit peak is proportionally (to the other peaks) higher than that of normal Bx7 subunit. The method has been successfully optimised to improve the accuracy and resolution in differentiating all the other HMWGS alleles as well. For instance, the Ax1 subunit was rather difficult to score when it is coupled with Dx5 subunit. Under the optimised condition, accurately scoring of Ax1 is no longer an issue.

Application of the newly designed PCR marker

Using the Bx7^{oe} primers, a clear DNA band (270 bp) was amplified in the cultivars containing the Bx7^{oe} gene. No

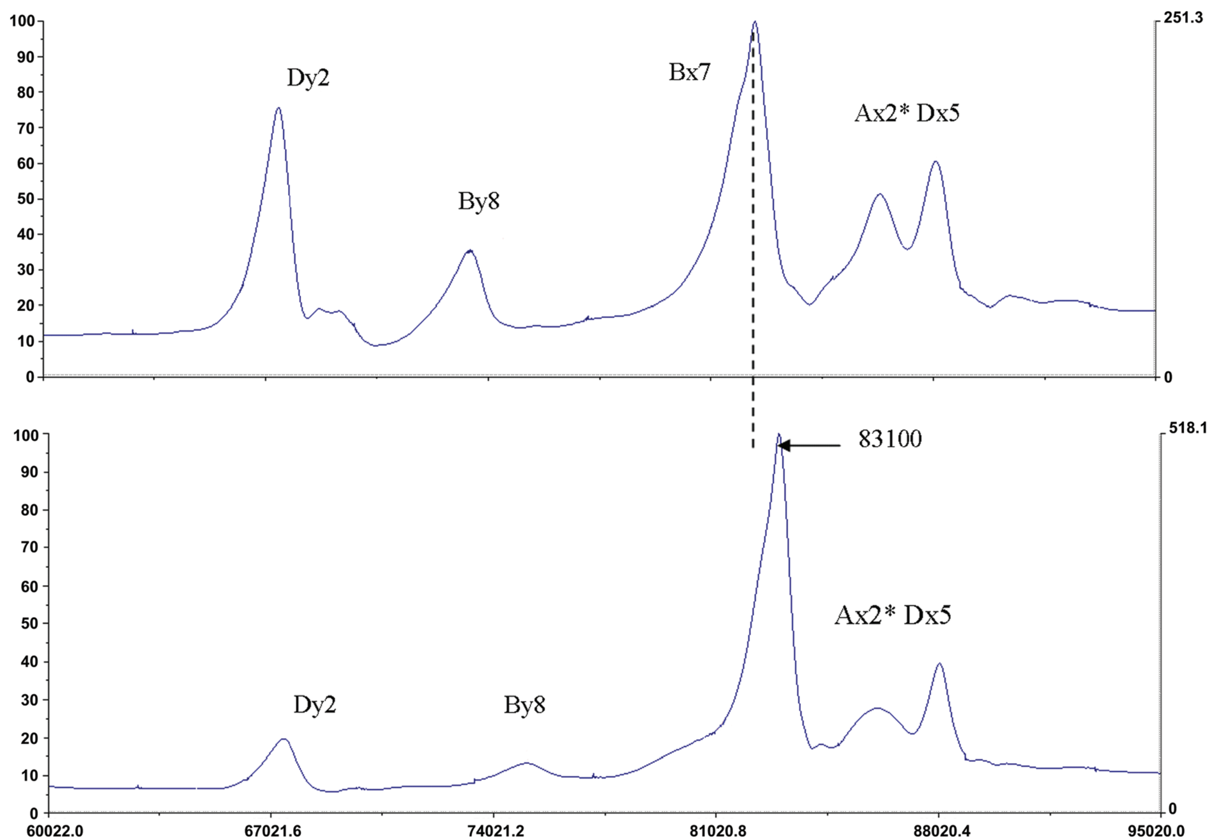

Fig. 1. MALDI-TOF spectra of Bx7^{oe} and normal Bx7 alleles. The top figure is a profile of normal Bx7 allele while the bottom figure is the profile for Bx7^{oe} allele.

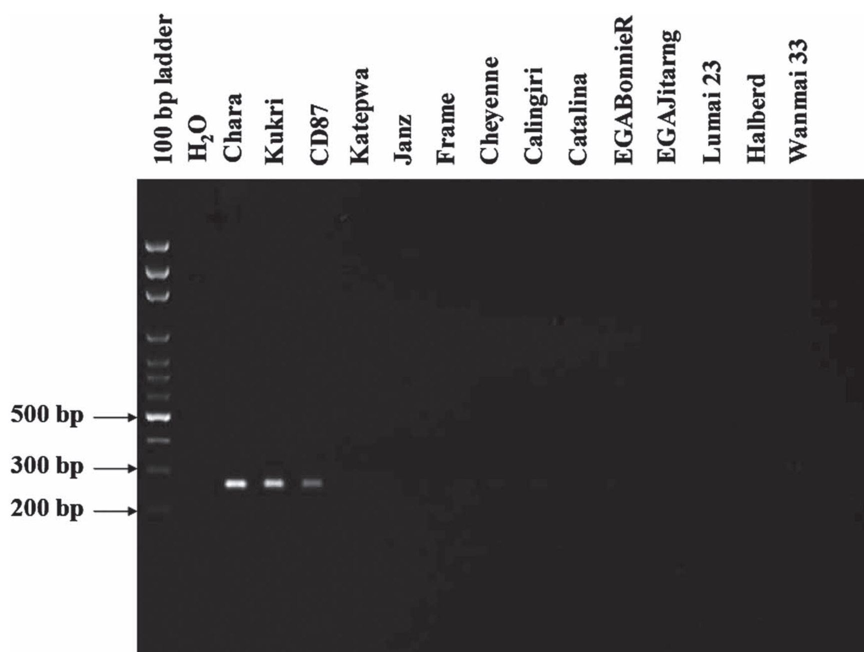

Fig. 2. PCR assay for screening cultivars containing $Bx7^{oe}$ allele. Cultivars with $Bx7^{oe}$ allele: Chara, Kukri and CD87 were all amplified with a fragment of 270 bp. The other cultivars: Katepwa (Bx7), Janz (Bx7), Frame (Bx7), Cheyenne (Bx7), Calingri (Bx13), Catalina (Bx17), EGABonnieR (Bx17), EGAJitarng (Bx17), Lumai 23 (Bx20), Halberd (Bx20), Wanmai 33 (Bx7) all have no PCR amplification.

PCR amplification was detected for the non-$Bx7^{oe}$ wheat cultivars (Fig. 2). The PCR results exactly matched the previous genotype results. Therefore, the refined marker is ideal for differentiating $Bx7^{oe}$ allele from normal Bx7. This dominant PCR marker made it possible to convert the marker into a high throughput format for fast screening the presence of this subunit in wheat lines. Moreover, the dominant nature of the marker makes the screening result highly reliable.

DISCUSSION

Liu et al. (2009) demonstrated that MALDI-TOF procedure could identify $Bx7^{oe}$ subunit. However, the $Bx7^{oe}$ spectrum reported in their study did not reflect its overexpression nature, ie, the height and area of the peak were same as that of the normal Bx7 subunit. This indicates that there is a scope for further optimisation of experimental conditions of the MALDI-TOF procedure. In fact, MALDI-TOF machine is a highly sophisticated, and often requires extensive optimisation of a wide range of parameters to give the maximum optimal performance. Comparing to Liu et al. (2009), which used machine settings of mass range 50–100 kDa, grid voltage 93%, guide wire 0.2%, and delay time 850 ns, in this study, we have changed the parameters to mass range 60–95 kDa, grid voltage 92%, guide wire 0.3%, and delay time 1000 ns. After such changes, the molecular mass associated with the $Bx7^{oe}$ and Bx7 spectrum became 83 100 Da and 82 500 Da instead of 82 900 Da and 82 300 Da, respectively. The results well corresponded to the molecular mass deduced from $Bx7^{oe}$ sequences (83 100 Da) and Bx7 (82 500 Da). Therefore, the changes made the results more accurate and more reliable. Moreover, the spectrum peak stands out from the rest of the subunits, making the other subunits visually insignificant. This simple change in the machine setting has made the scoring of $Bx7^{oe}$ subunit easier and more accurate.

As to the PCR marker, we redesigned the primer based on polymorphism outside the coding region, making the PCR marker dominant. We have thoroughly examined the coding region of the gene but failed to optimise the primers. The newly available DNA sequence information in the Genbank has made the refinement possible. When screened by the new primers, only the $Bx7^{oe}$ subunit can be amplified; all the other subunits appeared as negative in PCR. This dominant nature has made the screen of $Bx7^{oe}$ allele more accurate and can be readily converted into high-throughput marker format.

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