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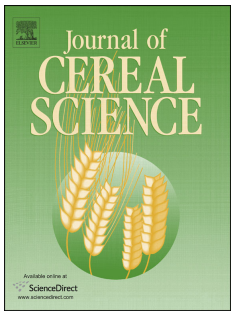

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A MALDI-TOF based analysis of high molecular weight glutenin subunits for wheat breeding

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Abstract

High molecular weight glutenin subunits play an important role in determining wheat dough quality as they confer visco-elastic properties to the dough required for mixing and baking performance. In this work, a collection of 103 genotypes of common wheat from 12 countries were used to analyse the composition of HMW-GS by SDS-PAGE and MALDI-TOF-MS. Results indicated that MALDI-TOF technology is suitable for analyzing most HMW-GS alleles. The allelic diversity at *Glu-B1* locus include subunits 6+8b*, 7, 7+8, 7+8a*, 7b*+8, 7^{OE}, 7^{OE}+8, 7^{OE}+8a*, 7^{OE}+8b*, 7+9, 13+16, 14+15, 17+18 and 20. The rapid identification of HMW-GS capability of MALDI-TOF-MS is discussed in relation to its value for screening lines in wheat breeding programs, especially in discriminating subunits 7^{OE}, 8a* and 8b* associated with superior quality. A new glutenin subunit 7b*+8 was found in Japanese germplasm Eshimashinriki.

Key words: *Triticum aestivum* L. - quality - SDS-PAGE - MALDI-TOF-MS - HMW-GS

Introduction

Gluten protein composition determines the rheological characteristics (strength and extensibility) of flour dough and is the main component responsible for differences in end-use suitability (Fu and Kovacs, 1999; Bekes et al., 2001; Butow et al., 2003a; Ma et al., 2005). Both major gluten protein groups, the monomeric gliadins and polymeric glutenins, are associated with quality differences among wheat cultivars (MacRitchie, 1987; Payne, 1987). The polymeric glutenin proteins, with molecular masses ranging from less than 300 kDa to greater than one million kDa are composed of two groups of subunits (Wrigley, 1996; Field et al., 1983; Payne, 1987; Shewry and Tatham, 1990; Stevenson and Preston, 1996). The low molecular weight glutenin subunits (LMW-GS) are similar in size and structure to the γ-gliadins (30-40 kDa). The high molecular weight glutenin subunits (HMW-GS) range in molecular mass from ~65-90 kDa (Shewry and Tatham, 1990; Zhang et al., 2007). The HMW-GS are encoded by tightly-linked "x" and "y" type genes at the *Glu-A1*, *Glu-B1* and *Glu-D1* loci on the long arms of chromosomes 1A, 1B and 1D, respectively (Payne et al., 1980). The LMW glutenin subunits are encoded by genes at the *Glu-A3*, *Glu-B3* and *Glu-D3* loci on the short arms of chromosomes 1A, 1B and 1D, respectively (Singh and Shepherd, 1988; Pogna et al., 1990).

Although HMW-GS are minor components in terms of quantity, they are key factors in the process of bread-making because they are major determinants of gluten elasticity by promoting the formation of larger glutenin polymers (Tatham et al., 1985; Shewry et al., 1992). The effect that different HMW-GS have on bread-making quality

has been widely studied (Fu and Kovacs, 1999; Bekes et al., 2001; Butow et al., 2003a; Ma et al., 2005). It has been shown that certain HMW-GS such as *GluB1 i* allele (17+18) and *GluD1 d* allele (5+10) have a positive influence, whereas others such as Null and *GluD1 a* allele (2+12) have a negative effect on dough characteristics and bread-making quality (Branlard and Dardevet, 1985; Payne et al., 1987; Lookhart et al., 1993; He et al., 2005; Liu et al., 2005). The HMW-GS alleles correlating with quality have been given different quality scores, and HMW-GS are extensively used as markers in wheat breeding programs for selecting preferable lines (Flæte and Uhlen, 2003).

The subunits 7+8 first described for bread wheat cultivar Chinese Spring are now known to be four alleles including 7+8, 7+8*, 7^{OE}+8, and 7^{OE}+8* (Gianibelli et al., 2001). It has been well-documented that the allele that contains over-expression of subunit 7^{OE}, designated *Glu-B1al*, has a large positive influence on bread-making quality (Marchylo et al., 1992; Lukow et al., 1992; D'Ovidio et al., 1997; Vawser and Cornish, 2004). The cultivars carrying subunit 7^{OE} formed dough with high strength as indicated by increased mixing times, maximum resistance to extension and decreased resistance breakdown (Bekes et al., 2001). Dough extensibility was also increased in cultivars containing subunit 7^{OE}, although this possibly results from the LMW-GS and gliadin present in respective cultivars (Gupta et al., 1994; Cornish et al., 2001). This association has recently been shown for a range of Australian and North American, Hungarian cultivars and breeding lines (Butow et al., 2002, 2003a, b, 2004; Radovanovic et al., 2002; Juhász et al., 2003a, b).

SDS-PAGE and HPLC methods have been used routinely in many breeding programs for selection of specific HMW and LMW subunits associated with superior quality (Dworschak et al., 1998). Identification HMW-GS using SDS-PAGE is based on their electrophoretic mobility and has been considered to be relatively straight-forward (Vawser and Cornish, 2004). However, some HMW-GS of near identical *M*r and electrophoretic mobility, such as 2 and 2^{*}, 14+15 and 20, can cause identification problems using these analytical procedures (Gianibelli et al., 2001). Although RP-HPLC (Marchylo et al., 1989) can resolve some of these ambiguities, subunits 7 and 7^{OE} cannot be differentiated on the basis of elution time using RP-HPLC (Marchylo et al., 1992).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has developed rapidly to become a powerful tool for characterizing wheat gluten proteins (Dworschak et al., 1998; Cozzolino et al., 2001; Cunsolo et al., 2002, 2003, 2004; Alberghina., 2005; Muccilli et al., 2005; Zhang et al., 2007; Chen et al., 2007). Compared with the common separation methods, the MALDI-TOF-MS technique appears to be much more accurate and sensitive, requiring only few minutes per sample to perform the measurement (Dworschak et al., 1998). Zhang et al. (2007) reported the characterization of HMW glutenin subunits in nine wheat cultivars by MALDI-TOF-MS. However, important subunits such as 7^{OE} and 8^{*} have not been addressed; and very little information about the molecular weight of subunits 1Bx20, 1By20, 1Dx3 and 1Dx4 was available. The feasibility of utilizing MALDI-TOF technology in wheat breeding programs has not been evaluated.

A large number of cultivars with all the possible HMW-GS combinations are required to establish the system of identifying HMW-GS by MALDI-TOF-MS in wheat breeding programs. The major objective of the present study is to evaluate the MALDI-TOF technology and establish analytical standards for identifying HMW-GS by MALDI-TOF for breeding programs with a particular focus on identifying the subunits 7^{OE} and 8^{*} associated with superior quality as well as the *M*r of subunits 1Bx20, 1By20, 1Dx3 and 1Dx4 by using 103 germplasm from 12 countries.

Materials and methods

Plant materials

In total, 103 genotypes of common wheat from 12 countries (Table 1) were used to analyse the composition of HMW-GS. These include 21 genotypes from China, 19 genotypes from Argentina, 15 genotypes from Australia, 14 genotypes from France, 10 genotypes from Japan, 7 genotypes from Canada, 8 genotypes from Mexico, 3 genotypes from America, 2 genotypes each from Italy and Netherlands, 1 genotype each from Finland and Germany. The composition of HMW-GS of most genotypes has been published using SDS-PAGE or RP-HPLC, including Liu et al (2005) for Chinese germplasms, Lerner et al (2008) for Argentinean germplasms, Branlard et al (2003) for German, French, Dutch, Italian, and Finnish germplasms, Rabinovich et al (2000) for germplasms from Mexico, Ng et al (1998) for germplasms from Canada, Bariana et al (1998) and Ma et al (2003) for Australian germplasms, and Nagamine et al (2000) for germplasms from Japan.

Protein extraction

Proteins were extracted from whole meal according to the sequential procedure of Singh et al. (1991). Whole meal (20 mg) was extracted with 1.0 ml of 55% propanol-1-ol (v/v) for 5 min continuous vortexing, followed by incubation (20 min at 65°C), vortexing (5 min), and centrifugation (5 min at 10, 000 × g). This step was repeated three times to remove gliadins completely. The HMW-GS present in the pellet was reduced with 55% propanol-1-ol, 0.08 M Tris-HCl solution containing 1% dithiothreitol (DTT). For SDS-PAGE analysis, the HMW glutenins were extracted as described previously (Marchylo et al., 1989). For MALDI-TOF analysis, 40% acetone was used to precipitate the HMWGS proportion followed by 80% acetone precipitation of the LMWGS proportion. The separation of HMWGS and LMWGS is essential since different mass ranges require different MALDI-TOF working parameters, ie, acceleration and grid voltages etc.

SDS-PAGE analysis of HMW-GS

HMW-GS were separated by SDS-PAGE using 5 μl of sample in a vertical gel (20× 20×0.1 cm) according to the protocol described by Singh et al. (1991). In order to achieve better resolution, the acrylamide/bisacrylamide concentration was constant and the gel concentration (T) and the cross linker (C) were modified as follow, T=14%, and C=1.3%. Electrophoresis was conducted at 16 mA/gel for 17 h and subunit bands visualised with 0.1% Coomassie Brilliant Blue R-250. HMW-GS was classified using the nomenclature of Payne and Lawrence (1983).

MALDI-TOF-MS

The dried mixtures of HMW-GS samples were dissolved in 60 μl acetonitrile (ACN)

/H_2O (v/v, 50:50) containing 0.05% v/v trifluoroacetic acid (TFA) for 1 hour. Sample preparation was carried out according to the dried droplet method (Kussmann et al., 1997), using sinapinic acid (SA) as matrix. The matrix solution was prepared by dissolving SA in ACN/H_2O (50:50 v/v) with 0.05% v/v TFA at a concentration of 10 mg/ml. The extracted HMWGS solution (total 60 µl) was mixed with SA solution at the ratio of 1:10 (v/v) and 2 µl of this protein-SA mixture was deposited on to a 96-sample MALDI probe tip, and dried at room temperature.

MALDI-TOF mass spectrometric experiments were carried out on a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with UV nitrogen laser (337 nm). The instrument was used with the following parameters: laser intensity 2 500, mass range 50-100 kDa, acceleration voltage 25 kV, grid voltage 92%, guide wire 0.3%, delay time 850 ns. The Bin size was set at 20 nsec and input bandwidth at 20 MHz. Spectra were obtained in positive linear ion mode and were averaged from 50 laser shots to improve the S/N level. All the samples were automatically accumulated in a random pattern over the sample spot to provide the final spectrum. Human transferrin (79 549 Da) was used as external standard for mass assignment.

Results and discussion

Composition of HMW-GS analysed by SDS-PAGE

SDS-PAGE, which separates proteins according to size, can differentiate between numerous HMW-GS subunit alleles on a single gel. Figure 1 represents the HMW-GS banding patterns associated with a selected 10 genotypes. Completed analysis of the

103 genotypes with SDS-PAGE revealed a total of sixteen HMW-GS alleles (Table 1). The variation includes subunits 1, 2* and Null encoded from the *Glu-A1* locus, subunits 6+8, 7, 7+8, 7+9, 13+16, 14+15, 17+18 and 20 encoded by the *Glu-B1* locus, and subunits 2+12, 2.2+12, 3+12, 4+12 and 5+10 encoded by the *Glu-D1* locus.

Characterisation of HMW-GS using MALDI-TOF-MS

The mass spectra of the HMW glutenin subunits for some cultivars are shown in Fig. 2. The mass spectra of HMW subunits show 4-5 distinct, well separated peaks in the spectrum of each sample. The compositions of HMW-GS identified by MALDI-TOF-MS are presented in Table 1. The spectra of most cultivars are consistent with SDS-PAGE results. The results confirmed the feasibility of using MALDI-TOF-MS to obtain rapid and complete profiles of HMW glutenin subunits. Automated and high-throughput sample analysis using this technique may prove particularly useful in wheat breeding programs where rapid isolation of lines containing subunits associated with superior quality is a major objective (Flæte and Uhlen, 2003).

The molecular weights of HMW-GS identified by MALDI-TOF are listed in Table 2. Most subunits can be well separated, allowing accurate molecular weight to be obtained. The apparent peaks with molecular ion signals at 82 300, 82 600, 83 600, 77 900+78 400, 82 100, 74 800, 73 300, 76 900, 86 400, 85 400, 87 900, 67 300 and 68 300 Da correspond to subunits 7, 7b*, 14, 17, 1Bx20, 8a*, 9, 16, 3, 4, 5, 10 and 12, respectively. The small difference in molecular weight of subunits 7^{OE} and 13 makes them difficult to be differentiated. Subunits 8, 15 and 1By20, 8b* and 18 have

identical molecular weights. However, subunits 16, 14, 17 and 1Bx20 show distinct molecular weights. Since subunits 13 and 16, 14 and 15, 17 and 18, 1Bx20 and 1By20 are coded by tightly linked genes, these pairs of proteins always appear together. The distinct molecular weights of subunits 16, 14, 17 and 1Bx20 can be used to differentiate allele 7^{OE}+8/8b* from subunits 13+16 and 17+18, allele 14+15 from 1Bx20+1By20. This indicates that subunits 7^{OE}, 8a* and 8b* can be discriminated from subunits 7, and 8 by using MALDI-TOF-MS. Although subunit 7^{OE} is reported to be slightly more mobile than subunit 7, differentiation between subunits 8 and 8* cannot be achieved using SDS-PAGE (Marchylo et al., 1992). The peak of subunit 1 overlaps with subunit 2 in the profile of MALDI-TOF-MS resulting from only a small difference in molecular weight. Therefore, differentiating the subunits Null and 1 at *Glu-A1* locus is still difficult by MALDI-TOF-MS. Closer examination of the results of Zhang et al. (2007) has revealed that only 4 obvious peaks were detected without the trace of subunits 1 and 2* presented in the profile of MALDI-TOF-MS. However, Jing 411 and Xiaoyan 6 had been confirmed to carry subunits 1 and 2*, respectively (Liu et al., 2005). In most cases, we find that it is necessary to carefully review the raw spectra data to achieve the discrimination of 1 (two tightly linked peaks) and null (one single peak). In addition, we cannot identify the subunit 2.2. This is consistent with Zhang et al (2007) for unknown reasons. However, in general, the advantages of the MALDI-TOF-MS method including rapidity, sensitivity especially in analyzing the allelic diversity of *Glu-B1* locus, far outweighed the disadvantages.

It is worth mention that all cultivars carrying subunit 17+18 present three peaks (77

900+78 400+75 000 Da) in MALDI-TOF-MS profile while there are two bands on SDS-PAGE. The additional peak is under investigation.

It is worth noting that human transferrin was used in this study to determine molecular weights for all glutenins. This serves well for our purpose in discriminating common subunits that are used and tracked in wheat breeding. It is worth noting, however, that the molecular weights determined by this method need to be treated with caution. We have assumed a molecular weight of 79549 for this protein for external calibration (Dworschak et al 1998). However, the human transferrin is a glycosylated protein and its molecular weight is uncertain. Recently, Wu et al (2008) reported mass values of 79492 and 79707 for a commercial and a laboratory prepared human transferrin. This suggests that some minor differences of MALDI-TOF measured glutenin Mrs may occur when different calibration chemicals are used"

Allelic variation of HMW-GS identified by MALDI-TOF-MS

A total of eighteen alleles of HMW-GS were found in the MALDI-TOF-MS profile and their frequencies are presented in Table 3. Only subunit 2^{*} can be reliably identified at *Glu-A1* locus. Thirteen *Glu-B1* alleles are presented, subunits 7+9, 7^{OE}+8 and 7+8 were found in high proportions (25.2, 17.5 and 16.5%, respectively), followed by subunit 17+18 (12.6%). The high proportion of subunit 7^{OE}+8 indicates that about one half of the subunit 7+8 separated by SDS-PAGE is ambiguous. Interestingly, there are contrasting effects on quality within these pairs and, hence, the score originally given to the pair 7+8 is sometimes misleading (Vawser and Cornish, 2004). Subunits 6+8b*, 14+15, 7^{OE} and 7^{OE}+8a* are found in seven, six, five and three

genotypes, respectively. Uncommon subunit(s) 7+8a* are present in the cultivars Jing 411 from China and Tasman from Australia, 13+16 in Argentinian cultivar ProINTA Isla Verde and Mexican cultivar Opata, 7 in Dutch cultivar Orca, 7b*+8 in Japanese cultivar Eshimashinriki, 7^{OE}+8b* in Argentinian cultivar Aca 601, and 20 in Chinese cultivar Wanmai 33. Four allelic variations are observed at the *Glu-D1* locus. The frequencies of subunits 5+10 and 2+12 are 53.4 and 36.9%, respectively, 4 + 12 is present in 4.9% of the genotypes, and 3 + 12 is observed only in American genotype Ernest. From the Japanese cultivar Eshimashinriki, a new HMW glutenin subunit 7b* was observed with the molecular weight of 82 600 Da, which was obviously different from subunits 7 (82 300) and 7^{OE} (82 900) in the profile of MALDI-TOF-MS.

Conclusion

The *Glu-B1* proteins *Glu-B1* proteins are highly variable and the variants are often related to different quality attributes and represent a group of biochemical factors that are not yet fully utilized for wheat quality improvement. Since these variants often have similar molecular weights, it is usually difficult to differentiate them by traditional SDS-PAGE methods. The current study indicated that MALDI-TOF-MS is a powerful technique for rapid identification of HMW-GS allele diversity at the *Glu-B1* locus. This has been demonstrated by its ability to discriminate protein subunits such as 7^{OE}, 8a* and 8b* associated with superior quality in wheat breeding programs, which are not possible to discriminate via SDS-PAGE method. Its high resolution also has led to the identification of a new HMW-GS 7b*+8 in Japanese germplasm Eshimashinriki. Another important HMW-GS allele is 5+10, which is

confers superior quality attributes for a wide range of wheat end-products. MALDI-TOF-MS technology can reliably differentiate 5+10 from other alleles. Overall, MALDI-TOF technology represents a powerful tool to fast and accurately analyse glutenin compositions for breeding purpose.

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174

175 **Table 1** Allelic variation at *Glu-A1*, *Glu-B1* and *Glu-D1* loci identified by
176 SDS-PAGE and MALDI-TOF-MS

Cultivar	Origin	*Glu-A1*	*Glu-B1*	*Glu-D1*
Ernest	America	Null/-	7+9/7+9	3+12/3+12
Splendor	America	1/1	7+8/7^{OE}+8	5+10/5+10
Verde	America	2*/2*	7+8/7^{OE}+8	5+10/5+10
Aca 303	Argentina	2*/2*	7+8/7^{OE}+8	5+10/5+10
Aca 601	Argentina	2*/2*	7+8/7^{OE}+8b*	5+10/5+10
Aca 801	Argentina	2*/2*	7+9/7+9	5+10/5+10
Buck Brasil	Argentina	1/1	7+8/7^{OE}+8	5+10/5+10
Buck Mejorpán	Argentina	2*/2*	7+8/7^{OE}+8	5+10/5+10
Buck Pingo	Argentina	1/1	17+18/17+18	5+10/5+10
Klein Capricornio	Argentina	2*/2*	7+8/7^{OE}+8	5+10/5+10
Klein Chaja	Argentina	2*/2*	17+18/17+18	5+10/5+10
Klein Flecha	Argentina	2*/2*	7+8/7^{OE}+8	5+10/5+10
Klein Jabal 1	Argentina	2*/2*	7+8/7^{OE}+8a*	5+10/5+10
Klein Martillo	Argentina	2*/2*	7+9/7+9	5+10/5+10
Klein Proteo	Argentina	1/1	7+9/7+9	5+10/5+10
Nidera Baguette 10	Argentina	Null/-	6+8/6+8b*	5+10/5+10
Nidera Baguette 20	Argentina	Null/-	7+8/7+8	5+10/5+10
ProINTA Amanecer	Argentina	1/1	17+18/17+18	5+10/5+10
ProINTA Colibr 1	Argentina	1/1	7+8/7^{OE}+8	5+10/5+10
ProINTA Isla Verde	Argentina	1/-	13+16/13+16	2+12/2+12
ProINTA Redomon	Argentina	1/1	7+8/7^{OE}+8a*	5+10/5+10
Thomas Nevado	Argentina	2*/2*	7+9/7+9	5+10/5+10
Angas	Australia	Null/-	7+8/7+8	2+12/2+12
Avocet	Australia	Null/-	7+8/7+8	2+12/2+12
Carnamah	Australia	2*/2*	7+9/7+9	2+12/2+12
Gabo	Australia	2*/2*	17+18/17+18	2+12/2+12
Grebe	Australia	Null/-	7+9/7+9	5+10/5+10
Halberd	Australia	1/1	14+15/14+15	5+10/5+10
Insignia	Australia	1/1	14+15/14+15	5+10/5+10
Millewa	Australia	Null/-	17+18/17+18	2+12/2+12
Pitic	Australia	1/-	7+8/7^{OE}+8	2+12/2+12
Spear	Australia	1/1	7+9/7+9	5+10/5+10
Stiletto	Australia	1/1	7+9/7+9	5+10/5+10
Tasman	Australia	2*/2*	7+8/7+8a*	5+10/5+10
Trident	Australia	1/1	7+9/7+9	5+10/5+10
Westonia	Australia	2*/2*	17+18/17+18	2+12/2+12
Wilgoyne	Australia	2*/2*	17+18/17+18	5+10/5+10
Ac. Vista	Canada	1/-	7+8/7^{OE} +8	2+12/2+12
Blu Sky	Canada	2*/2*	7+8/7^{OE}+8	5+10/5+10

177 Data preceding and following "/" are results by SDS-PAGE and MALDI-TOF-MS, respectively. Subunits not
178 being identified are indicated by "-".

179 **Table 1** (continued)

Cultivar	Origin	*Glu-A1*	*Glu-B1*	*Glu-D1*
Glenlea	Canada	$2^{*}/2^{*}$	$7+8/7^{OE}+8$	5+10/5+10
Katpewa	Canada	$2^{*}/2^{*}$	7+9/7+9	5+10/5+10
Marquis	Canada	1/1	7+9/7+9	5+10/5+10
Neepawa	Canada	$2^{*}/2^{*}$	7+9/7+9	5+10/5+10
Pioneer	Canada	1/1	$7+8/7^{OE}+8a^{*}$	5+10/5+10
99G46	China	1/1	7+9/7+9	4+12/4+12
CA9641	China	Null/-	7+8/7+8	2+12/2+12
CA9722	China	Null/-	7+8/7+8	2+12/2+12
Chinese Spring	China	Null/-	7+8/7+8	2+12/2+12
Demai 3	China	1/-	$7+8/7^{OE}+8$	2+12/2+12
Fengmai 27	China	1/-	7+8/7+8	2+12/2+12
Guanfeng 2	China	Null/-	$7+8/7^{OE}+8$	5+10/5+10
Huaimai 16	China	Null/-	7+9/7+9	2+12/2+12
Jing 411	China	$2^{*}/2^{*}$	$7+8/7+8a^{*}$	2+12/2+12
Lumai 23	China	1/-	14+15/14+15	2+12/2+12
Neixiang 188	China	1/1	7+9/7+9	5+10/5+10
Shan 229	China	1/1	14+15/14+15	5+10/5+10
Wanmai 33	China	1/1	20/20	5+10/5+10
Yan 239	China	Null/-	7+8/7+8	2+12/2+12
Yangmai 158	China	Null/-	7+8/7+8	2+12/2+12
Yumai 54	China	1/1	7+9/7+9	4+12/4+12
Yumai 63	China	Null/-	14+15/14+15	4+12/4+12
Yumai 69	China	1/-	7+8/7+8	2+12/2+12
Zhongyou 9507	China	1/1	7+9/7+9	5+10/5+10
Zhongyou 9701	China	Null/-	7+8/7+8	5+10/5+10
Zhongyu 415	China	Null/-	14+15/14+15	4+12/4+12
Ruso	Finland	$2^{*}/2^{*}$	$6+8/6+8b^{*}$	5+10/5+10
Brimstone	France	Null/-	$6+8/6+8b^{*}$	2+12/2+12
Cappelle-Desprez	France	Null/-	$7/7^{OE}$	2+12/2+12
Chopin	France	1/1	7+9/7+9	5+10/5+10
Clément	France	Null/-	$6+8/6+8b^{*}$	2+12/2+12
Courtot	France	$2^{*}/2^{*}$	$7+8/7^{OE}+8$	2+12/2+12
Darius	France	Null/-	$7/7^{OE}$	2+12/2+12
Etoile De Choisy	France	Null/-	7+8/7+8	2+12/2+12
Festin	France	Null/-	$7/7^{OE}$	2+12/2+12
Magali Blondeau	France	Null/-	7+8/7+8	2+12/2+12
Magdalena	France	Null/-	7+9/7+9	5+10/5+10
Petrel	France	Null/-	$7/7^{OE}$	5+10/5+10
Renan	France	$2^{*}/2^{*}$	7+8/7+8	5+10/5+10
Soissons	France	$2^{*}/2^{*}$	7+8/7+8	5+10/5+10
Thesee	France	Null/-	$6+8/6+8b^{*}$	2+12/2+12

Table 1 (continued)

Cultivar	Origin	*Glu-A1*	*Glu-B1*	*Glu-D1*
Apollo	Germany	Null/-	6+8/6+8b*	2+12/2+12
Manital	Italy	2*/2*	17+18/17+18	2+12/2+12
Salmone	Italy	1/-	7+9/7+9	2+12/2+12
Aoba-komugi	Japan	Null/-	7+8/7OE+8	2+12/2+12
Eshimashinriki	Japan	Null/-	7+8/7b*+8	2.2+12/-
Haruyutaka	Japan	1/-	17+18/17+18	2+12/2+12
Kanto 107	Japan	Null/-	7+9/7+9	2.2+12/-
Kitanokaori	Japan	1/1	7+9/7+9	5+10/5+10
Nanbu-komugi	Japan	1/1	7+8/7OE+8	4+12/4+12
Norin 61	Japan	Null/-	7+8/7+8	2.2+12/-
Norin 67	Japan	Null/-	7+9/7+9	2+12/2+12
Shinchunaga	Japan	Null/-	7+8/7OE+8	2.2+12/-
Shirane-komugi	Japan	1/-	7+8/7+8	2+12/2+12
Amadina	Mexico	1/1	7+9/7+9	5+10/5+10
Attila	Mexico	2*/2*	7/7OE	5+10/5+10
Heilo	Mexico	2*/2*	17+18/17+18	5+10/5+10
Opata	Mexico	2*/2*	13+16/13+16	2+12/2+12
Pastor	Mexico	1/1	17+18/17+18	5+10/5+10
Pavon	Mexico	2*/2*	17+18/17+18	5+10/5+10
Rebeca	Mexico	1/1	17+18/17+18	5+10/5+10
Seri	Mexico	1/1	7+9/7+9	5+10/5+10
Orca	Netherlands	Null/-	7/7	2+12/2+12
Pepital	Netherlands	Null/-	6+8/6+8b*	5+10/5+10

183 **Table 2** Molecular weights of HMW-GS measured by MALDI-TOF

HMW-GS	Mr (Da) deduced from coding gene	Mr (Da) by MALDI-TOF*
1Ax2*	86 309	86 200
1Bx6	Unknown	86 500
1Bx7	82 524	82 300
1Bx7OE	83 134	82 900
1Bx7b*	Unknown	82 600
1Bx13	Unknown	83 000
1Bx14	84 012	83 600
1Bx17	78 607	77 900+78 400
1Bx20	Unknown	82 100
1Dx2	87 022	87 000
1Dx3	Unknown	86 400
1Dx4	Unknown	85 400
1Dx5	88 128	87 900
1By8	75 156	74 900
1By8a*	Unknown	74 800
1By8b*	Unknown	75 000
1By9	73 515	73 300
1By15	75 733	74 900
1By16	Unknown	76 900
1By18	Unknown	75 000
1By20	Unknown	74 900
1Dy10	67 473	67 300
1Dy12	68 652	68 300

184 *The Mr values reported are median values of the HMWGS in all genotype
185 backgrounds, being accurate to 100 daltons

Table 3 Allele frequencies of HMW-GS analysed by MALDI-TOF

Locus	HMW-GS	Number	Frequency %
Glu-A1	2*	30	29.1
	Others	73	70.9
Glu-B1	6+8b*	7	6.8
	7	1	1.0
	7^{OE}	5	4.9
	7+8	17	16.5
	7^{OE}+8	18	17.5
	7+8a*	2	1.9
	7^{OE} +8a*	3	2.9
	7^{OE} +8b*	1	1.0
	7b*+8	1	1.0
	7+9	26	25.2
	13+16	2	1.9
	14+15	6	5.8
	17+18	13	12.6
	20	1	1.0
Glu-D1	2+12	38	36.9
	3+12	1	1.0
	4+12	5	4.9
	5+10	55	53.4
	Others	4	3.9

206 **Figure legends**

207

208 **Fig. 1** One-step 1-demisional 14% SDS-PAGE of HMW-GS: Lane 1 and 2, Avocet;
209 3 and 4, Pastor; 5 and 6, Attila; 7 and 8, Rebeca; 9 and 10, Millewa; 11 and 12,
210 Marquis; 13 and 14, Ernest; 15 and 16, Spear; 17 and 18, Halberd; 19 and 20,
211 Amadina.

212

213 **Fig. 2** MALDI-TOF-MS profile of some HMW-GS. (a) Aca 601; (b) Chinese Spring;
214 (c) Eshimashinriki; (d) Jing 411; (e) Heilo; (f) Wanmai 33.

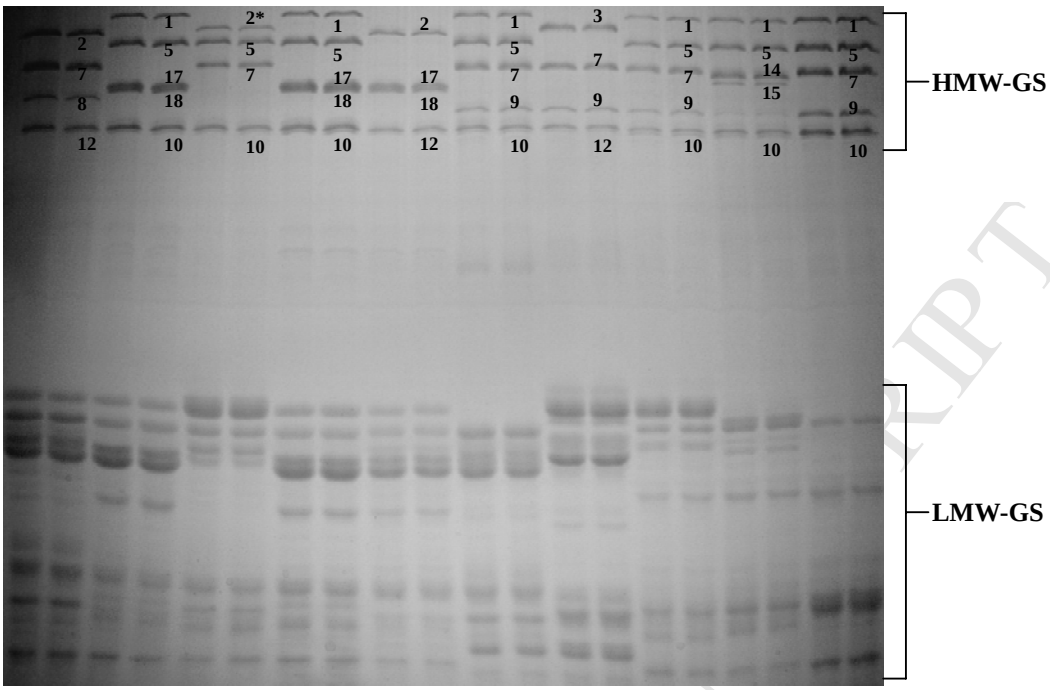

Fig. 1

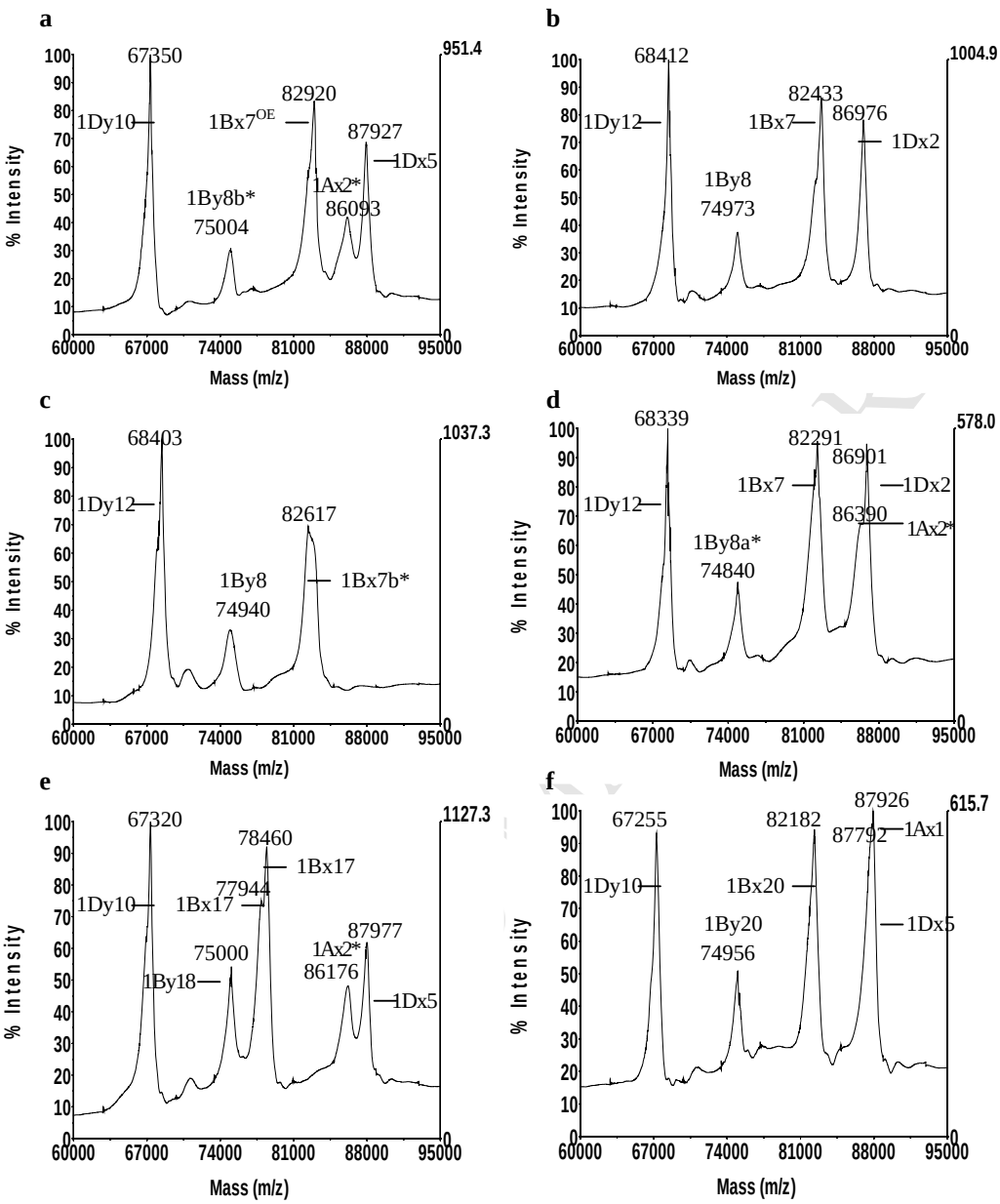
a
67350
951.4
1Dy10
1Bx7OE
82920
87927
1Dx5
1Ax2*
1By8b*
75004
86093
% Intensity
Mass (m/z)
b
68412
1004.9
1Dy12
82433
1Bx7
86976
1Dx2
1By8
74973
c
68403
1037.3
1Dy12
82617
1By8
74940
1Bx7b*
d
68339
578.0
82291
86901
1Bx7
1Dx2
1Dy12
86390
1Ax2*
1By8a*
74840
e
67320
1127.3
78460
1Bx17
1Dy10
1Bx17
77944
87977
1Ax2*
75000
1By18
86176
1Dx5
f
67255
87926
615.7
82182
87792
1Ax1
1Dy10
1Bx20
1By20
74956
1Dx5

Fig. 2