Characterisation of a Novel High-Molecular-Weight Glutenin Subunit 1Dy12.3 from Hexaploid Wheat (*Triticum aestivum* L.)

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Abstract: A novel high-molecular-weight glutenin subunit encoded by the *Glu-1D* locus was identified in hexaploid wheat (*Triticum aestivum* L.) cultivar Noe and was designated as 1Dy12.3. This subunit differed in SDS-PAGE mobility from the well-known 1Dy10 and 1Dy12 subunits that are also encoded by this locus. An analysis of the gene sequences confirmed the uniqueness of 1Dy12.3 and revealed that it is most closely related to the 1Dy12 subunit. The size of the deduced protein was calculated to be 67 884 Da, which is different from the 1Dy10 and 1Dy12 subunits (67 475 Da and 68 713 Da, respectively). The 1Dy12.3 protein consists of 652 residues, with a highly conserved signal sequence and N- and C-terminal domains, although the central repetitive domain comprising motifs of hexapeptide (PGQGQQ) and nonapeptide (GYYPTSLQQ) repeats was less conserved. The 1Dy12.3 subunit demonstrates fewer QHPEQG hexapeptide motifs and exhibits an increased number of methionine residues in comparison to the other characterised high-molecular-weight glutenin subunits. The 1Dy12.3 subunit was cloned and expressed in *Escherichia coli* and was detected with a prolamin-specific antibody. The size of the detected immunocomplex corresponded to the native 1Dy12.3 protein isolated from grains. The existence and characterisation of this novel high-molecular-weight glutenin subunit increases the diversity of the glutenins encoded by the *Glu-1D* locus.

Keywords: 1Dy12.3; *Glu-1Dy* locus; high-molecular-weight glutenin subunit; *Triticum aestivum* L.

The edible seeds of wheat provide starch, proteins, and other substances for human nutrition, animal feed, and various products, with the annual global production of wheat reaching more than 685 million tons in 2009 (FAOSTAT final data for the year 2009, FAO Statistic Yearbook, http://faostat.fao.org/). The mature wheat grain contains an average of 10–12% proteins that are involved in structural, metabolic, storage, defensive, and other functions, thus wheat annually provides approximately 68.5–82.2 million tons of wheat proteins for human nutrition, animal feed, and other purposes. Storage proteins compose 80–90% of the total proteins, yet there has been a serious

debate regarding the amount of storage proteins since as early as in the mid-18th century when wheat gluten was described by Beccari (1745). The first systematic studies of wheat storage proteins were conducted at the turn of the 20th century and were later published by Osborn (1907, 1909, 1924). Osborn fractionated storage proteins according to their solubility in different solvents, and the basic principle of his classification is still used at today. One of the protein fractions consists of the so-called prolamins (Shewry & Tatham 1990), which significantly affect the technological quality of flour and dough, especially the elasticity and viscosity of dough during the bread-making process (Payne *et al*. 1979; Shewry *et al*. 1995, 2001). The term "prolamin" indicates that the proteins of this fraction contain an increased amount of proline and glutamine amino acids, and prolamins are classified into two major groups: gliadins and glutenins. The glutenins consist of two categories based on their molecular weight: low-molecular-weight glutenins (LMW-GSs) and high-molecular-weight glutenins (HMW-GSs). The molecular weights of the LMW-GSs range from 30 to 40 kilodaltons (kDa), whereas those of the HMW-GSs reach up to 65–90 kDa. Aggregated glutenins and gliadins are able to form large complexes (gluten) of approximately several million daltons.

The AABBDD genome of hexaploid wheat (*Triticum aestivum* L.) was created by the amphiploidisation of a tetraploid wheat species (*Triticum turgidum* L. ssp. *diccoccum* (Schrank) Thell., AABB) and diploid goat grass (*Aegilops tauschii* Coss., DD) (Kihara 1944; McFadden & Sears 1946a, b). The genes for the HMW-GSs of the *Glu-1* loci are located on the long arms of the homoeologous group 1 chromosomes (*Glu-1A*, *Glu-1B*, *Glu-1D*), whereas the LMW-GS-encoding gene families are located on the short arms of the same chromosomes. Each locus contains genes encoding one x-type HMW-GS, with a lower relative molecular weight, and one y-type HMW-GS, with a higher relative molecular weight (Payne *et al.* 1987; Shewry *et al.* 1992). Thus, hexaploid wheat can theoretically contain as many as six different HMW-GSs; however, due to the attenuation of some genes, the real number is five or less (Shewry & Halford 2002). All of the HMW-GS genes consist of a signal sequence that is a highly conserved domain within other cereal prolamins (Halford *et al*. 1987), N- and C-terminal domains composed of non-repetitive amino acid sequences having a high content of cysteine, and a central domain with oligopeptide repetitions containing mainly glutamine and proline residues. The cysteine residues located in the non-repetitive sequences play important roles in forming intermolecular disulfide bonds and significantly affect the bread-making quality of wheat flour. Differences in the glutenin size result from a variable number of repetitive nona- and hexapeptides within the central domain of the y-type subunit and nona-, hexa-, and tripeptides within the central domain of the x-type subunits.

Variation in the y-type HMW-GSs encoded by the D-genome of hexaploid wheat is low (Giles & Brown 2006), with only two HMW-GSs, 1Dy10 (627 amino acid residues) and 1Dy12 (639 amino acid residues), predominating in common wheat varieties (Xu *et al*. 2009). Another three HMW-GSs were identified in hexaploid wheat (genome AABBDD), with two in *T. aestivum* L., the 1Dy12* subunit in the Slovak obsolete cultivar Trebišovská (GenBank accession: EU266533, Mihálik & Gregová, unpublished) and the subunit 1Dy12.2* in the Chinese landrace Jiuquanjinbaoyin (GenBank: FJ226583, Guo *et al*. 2010), and the third subunit (1Dy10.1) in *T. petropavlovskyi* Udacz. et Migush (GenBank: AY695379, Jiang *et al*. 2006). However, the genes for the 1Dy subunits originating from diploid ancestors possess much higher variation in comparison to those isolated from hexaploid wheat varieties (Mackie *et al*. 1996; Dvorak *et al*. 1998; Gianibelli & Solomon 2003; Yan *et al*. 2004; Fang *et al*. 2009; Xu *et al*. 2010). Nevertheless, wheat breeders prefer to use more-related genomes as the donors for the improvement of bread wheat. Therefore, the aims of this study were as follows: (*i*) to characterise novel a HMW-GS, 1Dy12.3, identified in the wheat cultivar Noe (*Triticum aestivum* L. var. *lutescens*) (Mihálik *et al*. 2010); (*ii*) to compare the DNA and protein sequences of the novel HMW-GS 1Dy12.3 with the described 1Dy subunits originating from hexaploid wheat and *Aegilops tauschii*; and (*iii*) to express the gene encoding the 1Dy12.3 subunit in *E. coli*.

MATERIAL AND METHODS

Plant materials. The hexaploid wheat cultivars, Noe, Neepawa, and Chinese Spring, used in this study were obtained from the collection of wheat genetic resources maintained in the Gene Bank of

the Slovak Republic (Plant Production Research Center, Piešťany). Genotype TD122 of *Aegilops tauschii* was provided by Prof. Zeller (TU Munich, Germany).

Glutenin analyses. The seed storage proteins were extracted from mature kernels or from a part of the kernel without an embryo. The glutenins were extracted, separated by electrophoresis, and visualised according to the International Seed Testing Association standard procedure for SDS-PAGE (Wrigley 1992). Molecular weight standards, BenchMark^{TM} Protein Ladder (Invitrogen Corp., Carlsbad, California) and the HMW-GSs 1Dy12 (cv. Chinese Spring), 1Dy10 (cv. Neepawa) and 1Dy12.3^{t} (Genotype TD122 of *Aegilops tauschii*) were used as the molecular weight markers in the electrophoretic mobility evaluation of the novel 1Dy subunit expressed by the cultivar Noe.

DNA extraction and amplification. Total genomic DNA was isolated from 1–2 g of three-five week-old seedlings of *T. aestivum* L. cultivar Noe using the DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany). Primers for the polymerase chain reaction (PCR) were designed according to the sequences of the genes encoding HMW-GS 1Dy12 (*T. aestivum* L. cv. Chinese Spring, GenBank: X03041, Thompson *et al*. 1985) and 1Dy10 (*T. aestivum* L. cv. Cheyenne, GenBank: X12929, Anderson *et al*. 1989) and overlapping with the open reading frame (ORF). The primer sequences were as follows: (P1-reverse) 5'-ACCGAGCAGCCACAAAATAG-3', (P2-forward) 5'-GCATGGTGCTTGGGCTAGCAT-3', and (P3-forward) 5'-CACGCATCACGTTTATTGGA-3'. The DNA amplifications were performed with primer combinations P1 + P2 and P1 + P3 using a thermocycler Mastercycler®ep (Eppendorf, Hamburg, Germany). The expected PCR products were ranged between 2000 and 2100 base pairs (bp). The PCR conditions and composition of the amplification reaction in a final volume of 25 ml were as follows: 0.5 U Platinum® Taq DNA polymerase (Invitrogen Corp., Carlsbad, USA), 2.5 µl 10 × reaction buffer, 2.5 µl 50mM $MgCl_2$, 10 pmol each primer, 0.125 µl 10mM dNTP mix, and 25 ng template DNA. The PCR cycle consisted of 5 min of an initial denaturation at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. The final extension was 7 min at 72°C. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide. Lambda DNA cleaved with *Eco*RI and *Hind*III (Fermentas, St. Leon-Rot, Germany) was used as the molecular weight standards.

Cloning, sequencing, and phylogenetic analysis of HMW-GS genes. The PCR products were purified using the Agarose Gel DNA Extraction Kit (Roche Diagnostics GmbH, Manheim, Germany), ligated into the pGEM-Teasy vector (Promega, Madison, USA) and transformed into the competent cells of *E. coli* (strain TOP10'F). The isolated plasmid was sequenced by a commercial service (Ecoli s.r.o., Bratislava, Slovakia) using the primer walking method; both strands were sequenced. The ORF of the HMW-GS gene was translated into the amino acid sequence using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The alignments with the known sequences of HMW-GS were performed using the BioEdit 7.0.9 Sequence Alignment Editor program. The Clustal W program was used for the phylogenetic analysis (Thompson *et al*. 1994).

The coding sequence of the *1Dy12.3* gene, without the signal sequence, was amplified using primers containing *Nde*I and *Eco*RI restriction sites, and the fragment was cloned into the pCRT7/NT-TOPO expression vector (Invitrogen Corp., Carlsbad, USA). The DNA sequences of the primers were as follows: forward primer, 5'-ggaattccatatgGCCTCTAGGCAACTACAGTGTGAGC-3', and reverse primer, 5'-ccggaattcTCACTGGCTAGCCGATAATGC-3'. The plasmid DNA was sequenced using the primer walking method.

Heterologous expression of the 1Dy12.3 gene in E. coli. The recombinant plasmid was transformed into the *E. coli* strain BL21(DE3)pLys, and the cells were grown overnight at 37°C on LB plates containing chloramphenicol and ampicillin. Single colonies were inoculated into 5 ml of LB medium, followed by the transfer of the inoculum to 100 ml of LB medium containing chloramphenicol. The cells were grown at 37°C until the desired level of cell growth (OD_{600} = 0.5) was reached. IPTG (up to final concentration of 0.5mM) and carbenicillin were then added, and the cells were grown overnight at 18°C and 30°C, respectively. The cell suspension was centrifuged at 15 000 × g for 1 min, and the supernatant was removed. The pellet was resuspended in 400 µl of buffer (100mM NaH_2PO_4, 10mM Tris-Cl, and 8M urea, pH 8.0), the cell lysate was centrifuged again (15 000 × g, at 4°C, 30 min), and the supernatant was used for the measurement of the protein concentration.

The bacterial cell lysate was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the Western blotting analysis was performed according to Wan *et al*. (2000), with the modification that the anti-gliadin (wheat) fractionated antiserum (Sigma, St. Louis, USA) was used at a dilution of 1:500. The ECL Rabbit IgG, HRP-Linked $F(ab')_2$ fragment (from donkey, diluted 1:4000, Amersham, Piscataway, USA) was used as the secondary antibody. The immunocomplex detection was performed according to Schaefer *et al*. (2005). The PageRuler™Plus pre-stained protein ladder (Fermentas, St. Leon-Rot, Germany) was used as the molecular weight marker.

RESULTS AND DISCUSSION

Glutenin extraction and HMW-GS analysis

The novel HMW-GS was discovered during the analysis of seed storage protein patterns, and an initial step in its discovery was the extensive screening of the wheat accessions maintained in genetic resource collections. The variation in the novel HMW-GS pattern was identified by SDS-PAGE using the known high-molecular-weight proteins, with the novel protein being identified by its different electrophoretic mobility from the 1Dy10 (cv. Neepawa) and 1Dy12 (cv. Chinese Spring) and 1Dy12.3^t (*Ae. tauschii*, genotype TD122, Yan *et al*. 2003) subunits (Figure 1). The novel subunit discovered in the cultivar Noe was termed 1Dy12.3 (Mihálik *et al*. 2010). Cultivar Noe is an obsolete

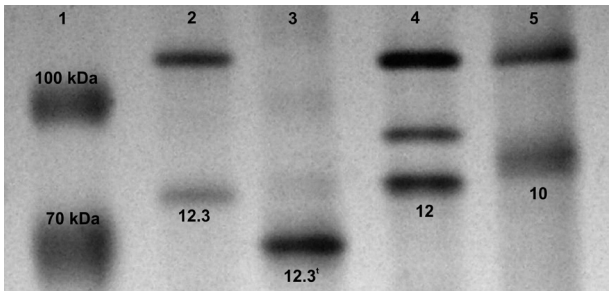


Figure 1. Electrophoretic mobility of HMW-GS 1Dy12.3, 1Dy12.3^t, 1Dy12 and 1Dy10 in SDS-PAGE: 1–5 – PageRuler™ PlusPrestained Protein ladder (Fermentas) size in kDa; 2 – HMW-GS fraction (cv. Noe); 3 – HMW-GS fraction (*Ae. tauschii*, genotype TD122); 4 – HMW-GS fraction (cv. Chinese Spring); 5 – HMW-GS fraction (cv. Neepawa)

French cultivar of hexaploid wheat with a genetic background of Russian winter wheat Odyssea and was released in the 19th century. According to our previous observations, the frequency of the discovery of a novel HMW-GS in hexaploid wheat (*Triticum aestivum* L.) is very low, especially within the modern cultivars. Nevertheless, the screening of the collections of the maintained landraces and obsolete cultivars could increase the probability of finding novel or very lowly expressed HMW-GSs, even using such a simple analytical approach as SDS-PAGE (Gregová *et al*. 2004) and landraces can contribute also breeding programes at present (Dotlačil *et al*. 2010). The nucleotide sequence of the gene *1Dy12.3* and the deduced amino acid sequence of the protein were deposited in the GenBank database (GenBank: EF472958, Gregová & Mihálik 2007, unpublished).

Cloning and sequence analysis of *1Dy12.3*

PCR primers flanking the ORF were designed considering the high homology between the 1Dy subunits, and the novel gene *1Dy12.3* was subsequently cloned, sequenced, and deposited in the GenBank database (accession number EF472958). The sequences of the DNA primers for the amplification of the hypothetical HMW-GS gene were designed according to the published nucleotide sequences of the 1Dy10 and 1Dy12 subunits. Due to the structural homology and similarity, only a minor difference in the lengths of the PCR products was expected. The primer pairs P1 + P2 and P1 + P3 amplified fragments of 2068 bp and 2101 bp, respectively (Figure 2). The conjunction of the nucleotide sequences of both of the amplified fragments after their cloning and sequencing confirmed the relevancy of the novel *1Dy12.3* gene.

The length of the ORF of the *1Dy12.3* gene was 1959 base pairs. The nucleotide sequences upstream and downstream of the stop codon were completely identical with the sequence of the *1Dy12* gene of cv. Chinese Spring (unpublished results). The deduced amino acid sequence of the signal peptide confirmed a high degree of conservation of this domain within all of the compared HMW-GSs (Figure 3). The N-terminal and C-terminal domains of all of the compared 1Dy subunits contained identical numbers of amino acids: 104 and 42, respectively (Table 1). The hot place for variation within the 1Dy HMW-GS is

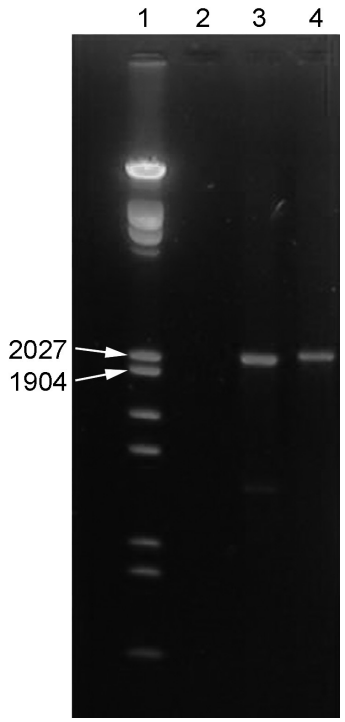


Figure 2. PCR analysis of Dy-subunit from cultivar Noe: 1 – lambda DNA/EcoRI, HindIII; 2 – negative controls; 3 – PCR fragment - Dy12.3 (P1 + P2 primers); 4 – PCR fragment (P1 + P3 primers)

the repetitive central domain that begins with two non-repeating peptides composed of 11 and 12 amino acid residues and consists of hexapeptide and nonapeptide repeats, with the consensus sequences PGQGQQ and GYYPTSLQQ, respectively. The subunit 1Dy12.3 contained 46 complete hexapeptide and 19 complete nonapeptide repetitions and the highest variation within the hexapeptide repetitions occurred at positions 1 and 4 and within the nonapeptide repetitions at positions 1, 6, 8, and 9 (Table 2). The total number of amino acid residues in the repetitive central domains of the compared 1Dy subunits was in the range of 457–506, and the novel subunit 1Dy12.3 differed from all of the compared subunits in the number of amino acid residues in the central repetitive domain of the entire protein (Table 1). However, the number of cysteine residues is conserved. The size of the deduced mature 1Dy12.3 protein was calculated to be 67 884 Daltons (Da), i.e., between the largest 1Dy12* (72 354 Da) and the smallest 1Dy13^t proteins (64 759 Da) (Table 1). The differences between 1Dy12.3 and the most frequented subunits, 1Dy10, 1Dy12 and 1Dy12.3^t, revealed by electrophoretic mobility (Figure 1) confirmed the different molecular weights of these subunits.

Comparison of the deduced amino acid sequences of the 1Dy12.3 subunit with other subunits

We detected a variation in the amino acid composition of the domains of the compared 1Dy subunits originating from *Triticum* L. and *Ae. tauschii*

Table 1. Complete protein and domain characteristics of 1Dy subunits

Subunit	Molecular mass (Da)	N-terminal domain	Repetitive central domain			C-terminal domain	Complete protein	SIM
		Res	Res	Hexa	Nona	Res	Res	
1Dy12.3	67 884	104	485	46	19	42	652	1.00
1Dy10	67 475	104	481	46	19	42	627	0.95
1Dy10.1	68 192	104	488	46	19	42	655	0.98
1Dy12	68 713	104	493	48	19	42	660	0.98
1Dy12*	72 354	104	506	48	20	42	673	0.96
1Dy12.2*	70 677	104	491	47	19	42	658	0.97
1Dy12^t	67 411	104	481	46	19	42	648	0.95
1Dy12.1^t	67 517	104	481	46	19	42	648	0.95
1Dy13^t	64 759	104	457	45	17	42	624	0.91

Res – number of amino acid residues; Hexa – number of hexapeptides; Nona – number of nonapeptides; SIM – sequence identity matrix

Figure 3. Comparison of deduced amino acid composition for selected 1Dy subunits of *T. aestivum* L. – 1Dy12.3, 1Dy10, 1Dy12, 1Dy12*, 1Dy12.2*, *T. petropavlovskyi* Udacz. et Migush – 1Dy10.1; *Ae. tauschii* – 1D^{t}y10, 1Dy12^{t}, 1Dy12.1^{t}

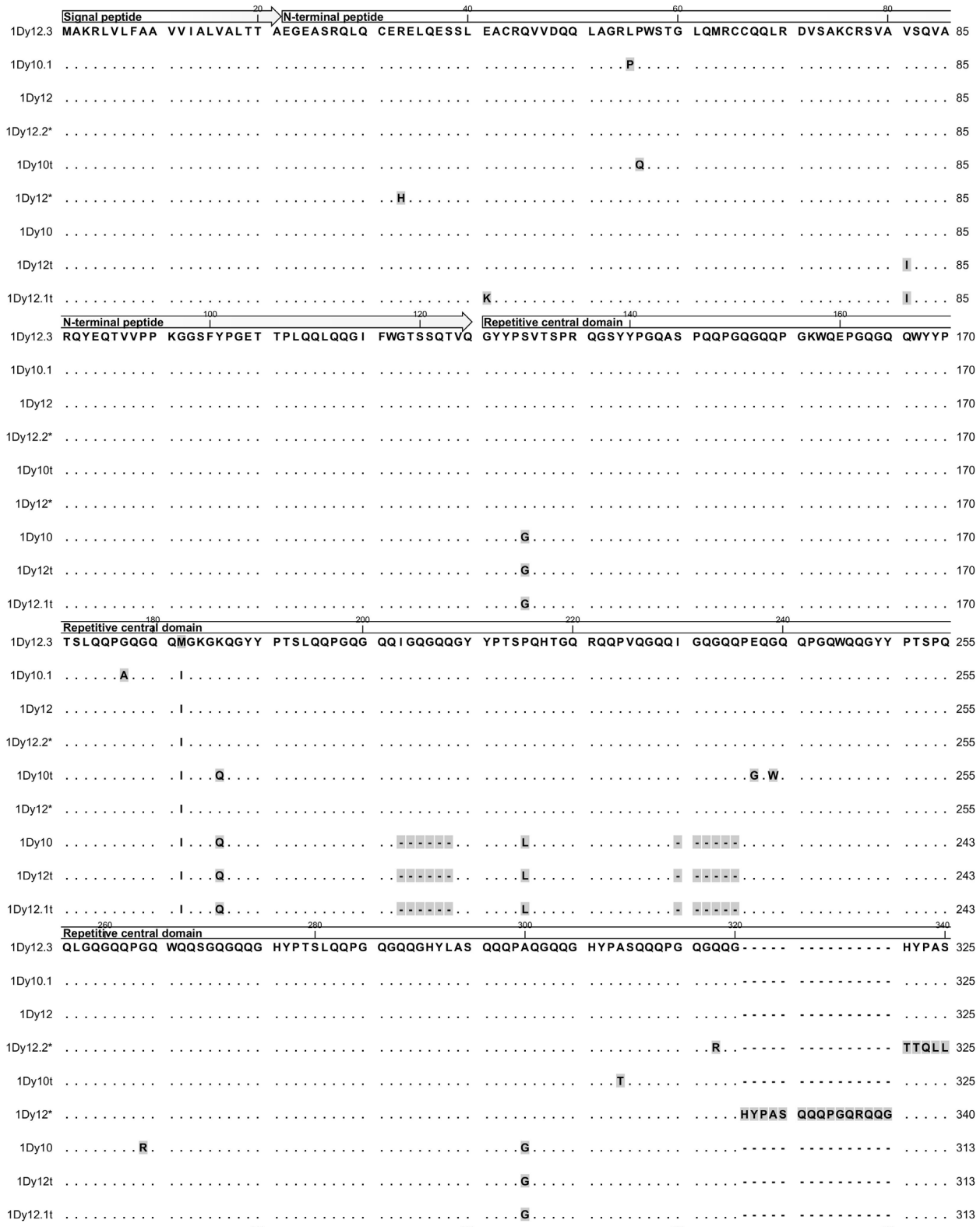
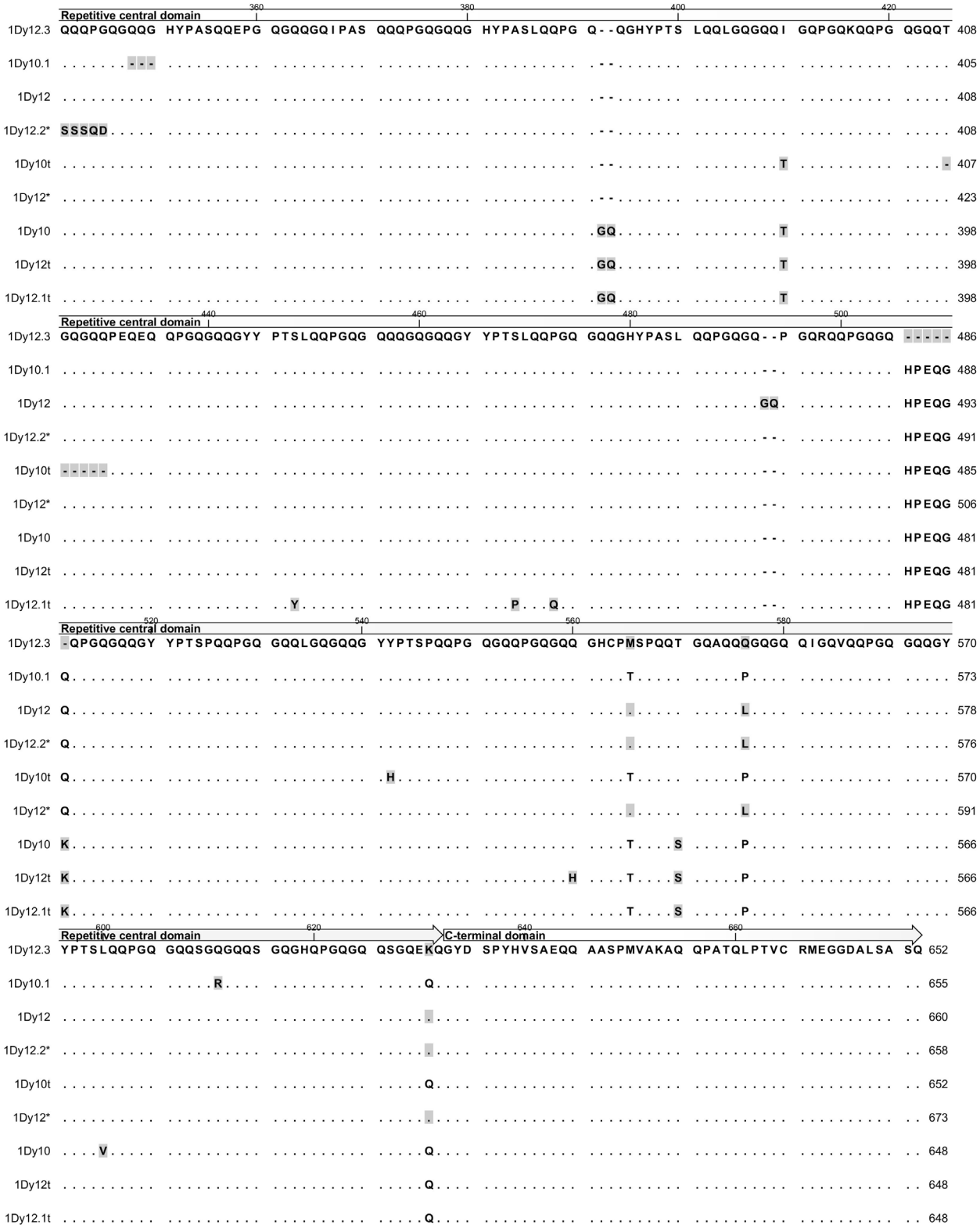


Figure 3 to be continued



(Figure 3). The amino acid composition of the N-terminal domain of the 1Dy12.3 subunit differed by 1 or 2 amino acids in comparison to the 1Dy10.1, $1D^t$y10, 1Dy12*, $1Dy12^t$, and $1Dy12.1^t$ subunits but was completely identical with the 1Dy10, 1Dy12, and 1Dy12.2* subunits. The C-terminal domain sequences were completely identical for all of the 1Dy subunits. The essential differences between the novel 1Dy12.3 subunit and other subunits were identified as single amino acid differences in the central repetitive domain, with particularly unique differences at positions 182 and 551 in the 1Dy12.3 subunit, with methionine and glutamine residues being specific only for the 1Dy12.3 subunit.

In addition, insertions/deletions of some amino acids in the central domain created differences between the compared 1Dy subunits. Deletions of amino acid pairs (glycine, glutamine) at positions 375–376 and 473–474 distinguished the 1Dy12.3 subunit from 1Dy10 and 1Dy12, respectively. Furthermore, longer insertions/deletions in the central domain region differentiated the 1Dy12.3 subunit from the other subunits of hexaploid wheat, e.g., deletions of the IGQGQQ motif at positions 203–208 and 230–235 in 1Dy10, unique insertions of the nonapeptide and hexapeptide at position 319–333 in 1Dy12*, and a unique deletion of the hexapeptide at position 477–482 in 1Dy12.3.

A remarkable characteristic of the HMW-GSs is the content of sulphur-containing amino acids that provide intermolecular disulfide bonds to form protein polymers. All of the compared subunits contain seven cysteine residues, whereas the 1Dy12.3 subunit contains the highest number of methionine residues (Table 3).

Phylogenetic comparison of 1Dy subunits

A phylogenetic analysis was performed by comparing the 1Dy sequences of six *Triticum* L. (1Dy10, 1Dy10.1, 1Dy12, 1Dy12*, 1Dy12.2*, and 1Dy12.3) and the $1D^t$y10 (GenBank: AY594360, Lu & Lu 2005), $1Dy12^t$ (GenBank: U39229, Mackie *et al*. 1996), and $1Dy12.1^t$ (GenBank: AY248704, Yan *et al*. 2004) *Aegilops tauschii* subunits. The compared subunits were separated into two main branches (Figure 5); the novel 1Dy12.3 subunit was clustered into the larger branch and was most closely related to 1Dy12. The 1Dy10 subunit, very frequented in hexaploid wheat, was clustered together with the subunits originating from *Aegilops tauschii*, i.e., from the D-genome donor $1Dy12^t$ and $1Dy12.1^t$, into the second branch of the phylogenetic tree.

The comparison of the deduced amino acid sequence of the selected subunits encoded at the *Glu-1D^t* loci, i.e., originating from *Ae. tauschii*, were distributed into both of the main branches of the phylogenetic tree (Figure 4). The 1Dy and $1D^t$y subunits were not separated as discretely as

Table 2. Variation in repetitive central domains within 1Dy subunits

Subunit	Repetitive central domain														
	hexapeptids						nonapeptids								
	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9
	P	G	Q	G	Q	Q	G	Y	Y	P	T	S	L	Q	Q
1Dy12.3	30	42	44	36	43	45	18	9	17	18	11	19	9	19	17
1Dy10	33	41	44	36	44	44	18	9	17	18	12	19	9	19	18
1Dy10.1	30	40	43	36	44	44	18	9	17	18	12	19	9	19	17
$1D^t$y10	31	42	44	35	44	44	18	9	16	18	13	19	9	19	17
1Dy12	32	43	45	38	45	45	18	9	17	18	11	19	9	19	17
1Dy12*	32	43	46	37	45	46	19	9	18	19	11	20	9	20	19
1Dy12.2*	30	41	44	37	43	45	18	9	16	17	11	18	9	18	16
$1Dy12^t$	33	42	44	35	44	43	18	9	17	18	12	19	10	19	18
$1Dy12.1^t$	32	42	44	36	44	44	18	9	17	18	12	17	10	19	17
$1Dy13^t$	27	41	42	35	43	43	16	9	16	17	12	17	9	17	16

PGQGQQ, GYYPTSLQQ – hexapeptide and nonapeptide consensus sequence

Table 3. Comparison of essential amino acid content in selected HMW-GS 1Dy subunits of genus *Triticum* L. and *Ae. tauschii*

Essential amino acids	Subunits							
	1Dy12.3	1Dy10	1Dy12	1Dy10.1	1D^{t}y10	1Dy12^{t}	1Dy12.1^{t}	1Dy13^{t}
Phe	3	3	3	3	3	3	3	3
His	12	13	13	13	14	14	13	13
Ile	7	5	8	8	7	6	6	5
Lys	9	8	9	8	7	8	9	8
Leu	28	28	29	27	28	29	29	29
Met	6	4	5	4	4	4	4	4
Thr	25	26	25	26	27	26	26	27
Val	19	20	19	19	19	18	18	18
Trp	6	6	6	6	7	6	6	5

could be expected in the phylogenetic tree, but two facts must be considered: *Aegilops tauschii* is an ancestor of the D-genome of hexaploid wheat (Feldman *et al*. 1995), and notable *de novo* genetic variation in hexaploid wheat occurred subsequent to the hybridisation of tetraploid *Triticum turgidum* (AABB) and diploid *Aegilops tauschii* (DD) 8500 years ago (Liu *et al*. 2009).

Subunits 1Dy12.3 and 1Dy12 differed from subunit 1Dy10 at positions 264 (glycine/arginine), 393 (isoleucine/threonine), and 606 (lysine/glutamine). These substitutions result in a change of the physicochemical properties of the peptide chain and could lead to significant structural variation between the 1Dy12 and 1Dy10 subunits of hexaploid wheat. A consequence in the baking industry is that the presence of the 1Dx5+1Dy10 complex allele is associated with a good bread-making quality, whereas the presence of the allele pair 1Dx2+1Dy12 predicts a poor bread-making quality (Payne *et al*. 1987). A higher homology of the novel 1Dy12.3 subunit with 1Dy12 and a lower homology with 1Dy10 could, therefore, imply that the presence of the *1Dy12.3* gene and corresponding protein should negatively impact the bread-making quality of this wheat cultivar. This hypothesis, however, would have to be confirmed by the development of near-isogenic wheat lines and their comparison by experimental mixographic analyses. It is important to note that there are identical numbers of cysteine and tyrosine residues in the protein chain of the 1Dy12.3 and 1Dy10 subunits and that number of these amino acids significantly affects the bread-making quality of wheat flour, which is also influenced by the number and composition of repeats (Payne *et al*. 1987; Anderson *et al*. 1996).

Heterologous expression of *1Dy12.3* gene in *E. coli*

The novel 1Dy12.3 subunit was expressed in *E. coli* and was subsequently detected using an an-

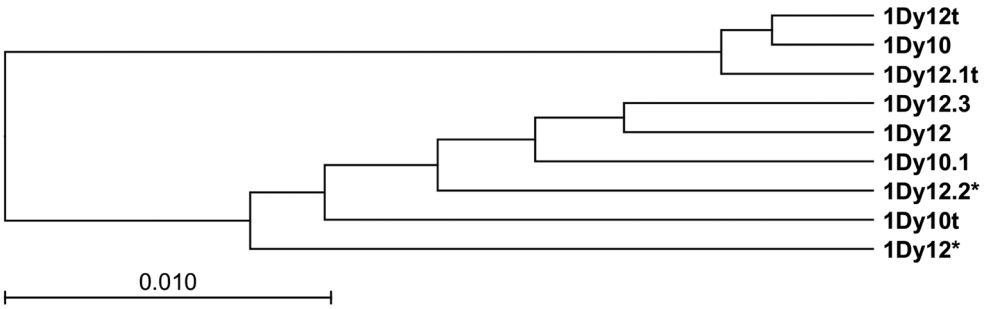


Figure 4. Phylogenetic relationships of HMW glutenin y-subunits in the genus *Triticum*; scale bar represents 0.01 nucleotide substitution per position

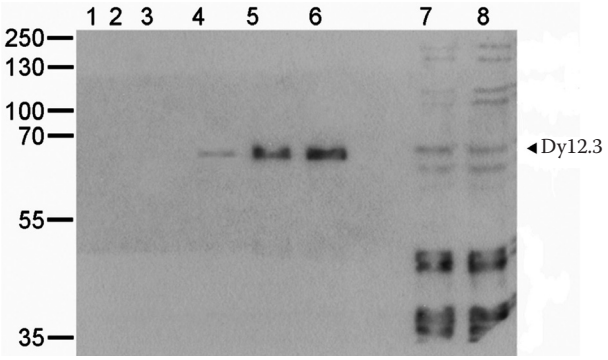


Figure 5. Heterologous expression of the gene encoding subunit 1Dy12.3 in *E. coli* by Western blotting analysis using primary antibody anti-gliadin (wheat), fractionated antiserum; 1 – ladder (size in kDa); 2 – cell lysate from strain BL21 (DE3)pLys(-IPTG,-Dy12.3-pCRT7/NT-TOPO), 30°C; 3 – cell lysate from strain BL21 (DE3)pLys(+IPTG,-Dy12.3-pCRT7/NT-TOPO), 30°C; 4 – cell lysate from strain BL21 (DE3)pLys (-IPTG,+Dy12.3-pCRT7/NT-TOPO), 30°C; 5 – cell lysate from strain BL21 (DE3)pLys(+IPTG,+Dy12.3-pCRT7/NT-TOPO), 18°C; 6 – cell lysate from strain BL21 (DE3)pLys(+IPTG,+Dy12.3-pCRT7/NT-TOPO), 30°C; 7 – prolamins fraction from cv. Noe (25 μg of proteins); 8 – prolamins fraction from cv. Noe (50 μg of proteins)

tibody primarily designed to identify wheat gliadins. The ORF without the signal sequence was cloned and expressed in a bacterial expression vector, producing a recombinant protein without any noticeable differences at both culture temperatures, i.e., 18°C and 30°C. Both the recombinant protein and native, mature protein were specifically detected by Western blotting using an antibody directed against wheat prolamins. A positive signal was detected only in the lysate of the cells harbouring the pCRT7/NT-TOPO plasmid with the inserted *1Dy12.3* gene (Figure 5). The antibody formed no immune-complexes with other bacterial proteins, though it did identify other proteins of the prolamin fraction isolated from the wheat grains (cv. Noe) used as the positive control. Other HMW-GS proteins of the cultivar Noe (1Ax1, 1Bx7, and 1Dx3), with higher molecular weights than 1Dy12.3, also produced strong signals with the polyclonal antibody. Thus, we proved that a commercial antibody is able to bind specifically both gliadins and glutenins.

The migration of the recombinant protein in SDS-PAGE corresponded fully to the predicted size (approx. 68 kDa), whereas the size of the mature, native protein was notably higher, at approximately 88 kDa. The same anomalies were also observed in the mobility of the 1Dy12 and 1Dy10 subunits: the mature proteins have a molecular weight of approximately 90 kDa (Figure 1), but the predicted sizes are approximately 68 kDa. This result may be because mature proteins have often been subjected to post-translational modifications, mainly glycosylation (Tilley 1997). Because the heterologous expression was performed in *E. coli*, it is likely that only a protein (68 kDa) having no post-translational modifications were present and detected. The lanes 7 and 8 on the Western blot containing the prolamin fraction of hexaploid wheat (cv. Noe) reveals a band with a size that is similar to the unmodified mature 1Dy12.3. This positive signal can be attributed to the immune-complex formed by the antibody and prolamin fraction of LMW-GS, as it is highly unlikely that the unmodified protein is present in protein bodies, unless, of course, such a protein is a storage protein transported into protein storage vacuoles without the participation of the Golgi apparatus (Levanony *et al*. 1992).

In summary, we demonstrated the presence of the novel HMW-GS 1Dy12.3 having a close similarity to the highly expressed HMW-GS 1Dy12 in the obsolete wheat cultivar Noe. The conservation of the D-genome of hexaploid wheat is much higher than that of the B-genome. The identification of a novel HMW-GS encoded by the *Glu-1D* locus increases the diversity at this locus. Our results also support the hypothesis that the study of landraces and obsolete cultivars of hexaploid wheat can be a source of new, potentially usable genes in wheat improvement, either by classical or modern (molecular breeding and gene transfer) breeding approaches (Juhasz *et al*. 2003). We are currently preparing transgenic wheat with this new subunit to monitor its effect on the bread-making quality of genotypes with well-defined HMW-GS compositions.

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