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ORIGINAL PAPER

Evolutionary origin of the segmental duplication encompassing the wheat *GLU-B1* locus encoding the overexpressed Bx7 ($Bx7^{OE}$) high molecular weight glutenin subunit

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Abstract Sequencing of a BAC clone encompassing the *Glu-B1* locus in Glenlea, revealed a 10.3 Kb segmental duplication including the *Bx7* gene and flanking an LTR retroelement. To better understand the evolution of this locus, two collections of wheat were surveyed. The first consisted of 96 diploid and tetraploid species accessions while the second consisted of 316 *Triticum aestivum* cultivars and landraces from 41 countries. The genotypes were first characterized by SDS-PAGE and a total of 40 of the 316 *T. aestivum* accessions were found to display the overexpressed Bx7 phenotype ($Bx7^{OE}$). Three lines from the 96 diploid/tetraploid collection also displayed the stronger intensity staining characteristic of the $Bx7^{OE}$ subunit. The relative amounts of the Bx7 subunit to total HMW-GS were quantified by RP-HPLC for all $Bx7^{OE}$ accessions and a number of checks. The entire collection was assessed for the presence of four DNA markers namely an 18 bp indel of the coding region of *Bx7* variant alleles, a 43 bp indel of the 5′-region and the left and right junctions of the LTR retrotransposon borders and the duplicated segment. All 43 accessions found to have the $Bx7^{OE}$ subunit by SDS-PAGE and RP-HPLC produced the four diagnostic PCR amplicons. None of the lines without the $Bx7^{OE}$ had the LTR retroelement/duplication genomic structure. However, the 18 and 43 bp indel were found in accessions other than $Bx7^{OE}$. These results indicate that the overexpression of the Bx7 HMW-GS is likely the result of a single event, i.e., a gene duplication at the *Glu-B1* locus mediated by the insertion of a retroelement. Also, the 18 and 43 bp indels pre-date the duplication event. Allelic variants *Bx7**, *Bx7* with and without 43 bp insert and $Bx7^{OE}$ were found in both tetraploid and hexaploid collections and shared the same genomic organization. Though the possibility of introgression from *T. aestivum* to *T. turgidum* cannot be ruled out, the three structural genomic changes of the B-genome taken together support the hypothesis of multiple polyploidization events involving different tetraploid progenitors.

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Introduction

Wheat flour has the unique ability to form dough that exhibits the rheological properties required for the production of leavened bread and for the wider diversity of foods that have been developed taking advantage of this attribute (Weegels et al. 1996). Storage proteins of the endosperm, namely the gluten complex, primarily determine bread-making ability (Shewry et al. 1992). Gluten content and composition are critical in providing the rheological properties of flour.

High molecular weight glutenin subunits (HMW-GS) are the most important fractions of the gluten because they

form large polymeric structures through disulphide bonds (Wrigley 1996). These polymeric structures are related to the molecular properties of dough and their composition alone may account for 47–60% variation in bread making quality of wheat (Payne 1987; Rakszegi et al. 2005). Both qualitative and quantitative effects of individual subunits on bread-making quality and dough functionality are important (Barro et al. 1997). The presence of the Dx5 and Dy10 subunit combination is associated with good bread-making quality (Payne 1987; Radovanovic et al. 2002). Similarly, the significant contribution of overexpressing Bx7 subunit towards the genetic variance for mixing characteristics important to dough strength has been reported (Butow et al. 2003; Radovanovic et al. 2002; Vawser and Cornish 2004). The dough strength, in turn, determines the suitability of the flour for bread making and other end uses (Bushuk 1998; Gale 2005; Lukow et al. 1992).

HMW-GSs are encoded at the *Glu-1* loci on the long arms of homologous chromosomes 1A, 1B and 1D (Payne 1987) and are designated *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively. Each *Glu-1* locus contains two tightly linked paralogous genes encoding two different types of HMW-GS, namely the x- and y-type subunits (Payne et al. 1981; Shewry et al. 1992). In bread wheat, most cultivars do not express the expected six HMW-GS but usually three to five subunits due to the silencing of some genes. The gene encoding the Ay subunit in hexaploid wheat is always silent. Each of these complex loci displays extensive allelic variation (Payne and Lawrence 1983).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used for the separation and identification of HMW-GS in different wheat lines where overproduction of the Bx7 subunit is visualized by a relatively higher intensity staining in a profile generated from the total protein fraction of the endosperm (Lukow et al. 1989). Allelic variation at the locus encoding HMW-GS subunit Bx7 and overexpression was detected quantitatively by reversed phase high performance liquid chromatography (RP-HPLC, Marchylo et al. 1992). In this study, the proportion of subunit Bx7 relative to the total amount of HMW glutenin subunits was significantly higher ($41.2 \pm 1.5\%$) in $Bx7^{OE}$ lines than it was in lines not overexpressing the Bx7 subunit ($27.1 \pm 0.9\%$). To our knowledge, this is the only HMW glutenin subunit to display the overexpression phenotype.

The presence of two functional copies of the gene encoding the HMW-GS Bx7 subunit and improved transcriptional and/or translational efficiency have been proposed to explain the overexpression of this subunit in certain accessions (Lukow et al. 2002). Southern analysis in TAA 36, a landrace from Israel, suggested that the gene encoding subunit Bx7 is present in two copies and their simultaneous expression lead to the overproduction of the subunit (Lukow et al. 1992). In the same study, however, the authors suggested that the mechanism responsible for the $Bx7^{OE}$ phenotype of cultivar Glenlea differed and was attributed to the increased efficiency of transcription caused by differences in the promoter sequence and/or translation. Southern analysis of ‘Red River 68’, another cultivar overexpressing the Bx7 subunit, revealed the presence of a stronger hybridization signal thereby supporting the gene duplication hypothesis (D’Ovidio et al. 1997). Recently, a BAC clone encompassing the G*lu-B1* locus of cultivar Glenlea was sequenced and a 10.3 Kb duplication including the gene encoding the Bx7 HMW-GS was identified (Cloutier et al. 2005). The structural organization of the locus revealed the presence of an LTR retrotransposon between the duplicated areas.

Transposable elements play an important role in the evolution of the structure, function and regulation of expression of genes and genomes in eukaryotes (Bennetzen 2000; Grandbastien 1992; Kazazian 2004). They play a key role in evolution by driving structural changes such as duplication and deletion (Jiang et al. 2004; Morgante et al. 2005). Retrotransposons are the most abundant class of transposable elements in plant genomes such as maize and wheat (Kumar and Bennetzen 1999; SanMiguel and Bennetzen 1998; Vitte and Panaud 2005). They mediate structural chromosomal rearrangements by unequal homologous recombination and illegitimate recombination because of their abundant copy number and distribution across the genome (Bennetzen 2005). This study aimed at understanding the evolutionary origin of the *Glu-B1* locus in accessions overexpressing subunit Bx7. Specifically, whether the origin of the tandem segmental duplication driven by a retroelement leading to two copies of gene encoding Bx7 subunit occurred prior or post advent of hexaploid wheat. An understanding of the structural changes of the *Glu-B1* locus in diploid, tetraploid and hexaploid wheat could provide some insight into the origin(s) of hexaploid wheat.

Materials and methods

Plant materials

Germplasm was obtained from the Cereal Research Centre (CRC), the Plant Genetic Resources of Canada (PGRC), the United States Department of Agriculture-National Genetic Resources Program (USDA-NGRP), the International Wheat and Maize Improvement Centre (CIMMYT) and the European Cooperative Programme for Plant Genetic Resources (ECPGR) unit of the Research Institute of Crop Production (RICP). Two collections, one consisting of 96 diploid and tetraploid (*Aegilops* and *Turgidum*) accessions and the other of 316 *T. aestivum* cultivars and landraces from 41 countries were evaluated. The former collection

comprised 6 *T. monococcum* (A^mA^m), 2 *Aegilops speltoides* (BB), 11 *A. squarrosa* (syn. *Aegilops tauschii;* DD), 34 *T. turgidum* (AABB), 14 *T. dicoccoides*, 3 *T. durum*, 8 *T. dicoccum*, 12 *T. carthlicum*, 3 *T. turanicum* and 3 *T. polonicum* accessions.

SDS-PAGE analysis

To assess the overexpression of the Bx7 subunit, SDS-PAGE analysis was carried out on single kernels using a HMW glutenin extraction procedure and Coomassie blue staining as previously described (Radovanovic and Cloutier 2003).

RP-HPLC analysis

Five seeds were taken from each of the accessions and checks tested. Checks were selected for both tetraploid and hexaploid genotypes to have identical numbers of expressed subunits as the accessions tested. The embryo portion of the seeds was removed with a knife and the remaining endosperm and seed coat were crushed with a hammer and ground to a fine powder with a mortar and pestle. Extraction of insoluble glutenins and analysis of HMW-GS were done as described in Naeem and Sapirstein (2007) except that only 30 mg of sample was used for initial extraction. The data were acquired and analysed using Agilent ChemStation software (version 10.01). The elution profiles were used for the quantification of the Bx7 subunit relative to total HMW-GS.

PCR analyses

Plants were grown at CRC in a growth cabinet or in a greenhouse and genomic DNA was isolated from young leaf tissues using the Plant DNeasy 96 kit following the manufacturer's instructions (Qiagen, Maryland, USA). Genomic DNA was quantified by fluorometry and diluted to 100 ng/µl.

Primers designed to amplify an 18 bp indel characteristic of the *Bx7* coding region were from Butow et al. (2004) but the forward primer was modified to include an M13 tail (Schuelke 2000) for subsequent resolution on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primers for the dominant marker corresponding to the 43 bp indel of the promoter region were from Radovanovic and Cloutier (2003). Primer pairs flanking the LTR retrotransposon borders and the duplicated region were designed at the left and right junctions of the retroelement. The left junction primers were: TaBAC1215C06-F517, 5′-ACGTGTCCAAGCTTTGGTTC-3′ and TaBAC1215 C06-R964, 5′-GATTGGTGGGTGGATACAGG-3′. The right junction primers were: TaBAC1215C06-F24671, 5′-CCACTTCCAAGGTGGGACTA and TaBAC1215C06-R25515, 5′-TGCCAACACAAAAGAAGCTG-3′.

PCR reactions for the 18 bp indel were performed in 10 µl using 75 ng of genomic DNA as template and otherwise as previously described in Schuelke (2000). A touchdown program starting with 2 min at 94°C for 20 s, 64°C for 45 s, 72°C for 1 min decreasing the annealing temperature by 1°C every cycle to 54°C, followed by 26 cycles at 54°C annealing and a final 5 min extension at 72°C and cooling to 4°C. The amplification products were resolved on an ABI 3100 Genetic Analyzer (Applied Biosystems). PCR conditions and electrophoresis for the 43 bp indel marker were as previously described with the exception that 10 pmole of primers and 10 µl reaction volumes were used (Radovanovic and Cloutier 2003). PCR reactions for the retroelement right and left junction markers were performed in 25µl containing 200 ng of genomic DNA, 10 pmole of each primer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, and one unit of Taq DNA polymerase. The cycling conditions for amplifying the left junction were 94°C for 5 min followed by 34 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 1 min followed by final extension and cooling as above. Conditions were identical for the right junction with the exception that the annealing temperature was decreased to 59°C. The PCR products were resolved on 1.5% agarose gels in 0.5X TBE buffer, stained with ethidium bromide and visualized under UV.

Results

SDS-PAGE analysis of wheat accessions

HMW-GS profiles of the two wheat collections were first obtained by SDS-PAGE (Fig. 1). Accessions that displayed either a Bx7, Bx7* or $Bx7^{OE}$ were identified (Table 1). The presence of the $Bx7^{OE}$ was evaluated by assessing the staining intensity of the subunit. Accessions without Bx7, Bx7* or $Bx7^{OE}$ were classified as non-Bx7. Among the accessions surveyed, none of the diploids had a subunit Bx7 variant (Table 1). Among the 77 tetraploid accessions, 3 previously unreported *T. turgidum* (AABB) accessions (Branco, CN12222 and CN12225) were found to have the $Bx7^{OE}$ subunit. These lines were from Portugal and the Czech Republic. In the hexaploid wheat collection, 219 of the 316 accessions were found to express a Bx7 subunit variant among which 40 lines, all belonging to the subspecies *aestivum*, displayed the $Bx7^{OE}$ phenotype (Supplementary Table 1). The largest number of accessions (36) overexpressing the Bx7 subunit were found in the North and South American material. Three lines from Australia/New Zealand and one from Israel also displayed the overexpressing Bx7 phenotype. None of the 55 European and

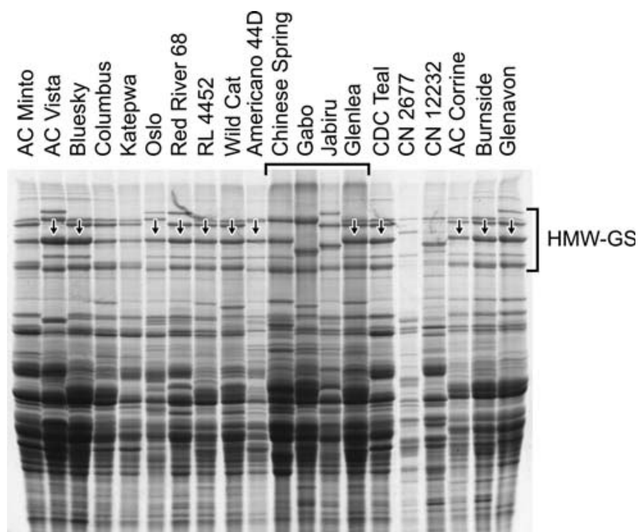

Fig. 1 SDS-PAGE of HMW-GS of a subset of *Triticum* accessions. Cultivars Chinese Spring, Gabo, Jabiru and Glenlea were used as checks on each gel for the profiling of the HMW-GS. Arrows indicate the $Bx7^{OE}$ subunit

12 African lines surveyed that had a Bx7 variant displayed the $Bx7^{OE}$ subunit. Relative overexpression of other HMW-GS was not observed in any of the accessions surveyed.

RP-HPLC analysis

The 3 *T. turgidum* accessions and the 40 *T. aestivum* accessions that displayed the $Bx7^{OE}$ subunit were analysed by RP-HPLC along with 1 tetraploid check and 12 hexaploid checks (Supplementary Table 2). To ensure that results on the proportion of Bx7 subunits to total HMW subunits were comparable between accessions and checks, these were chosen to have identical numbers of expressed subunits. All of the overexpressing accessions in our study had three and five expressed HMW-GS respectively for tetraploid and hexaploid accessions. The elution profiles were used for the quantification of the proportion of Bx7 subunit (% area) relative to the total amount of HMW-GS (Fig. 2). In tetraploid accessions, the proportion of the overexpressing Bx7 subunit relative to the total amount of HMW-GS averaged

Table 1 Survey of diploid, tetraploid and hexaploid wheat accessions for HMW-GS composition at the *Glu-B1* locus as assessed by SDS-PAGE. Hexaploid wheat accessions are categorized by their geographical origin

Species (Genome)	Number of accessions			
	Surveyed	HMW-GS at *Glu-B1x*		
		Non-Bx7	Bx7 or Bx7*	$Bx7^{OE}$
Diploid				
T. monococcum (A^mA^m)	6	6	0	0
Aegilops speltoides (SS)	2	2	0	0
Aegilops tauschii (DD)	11	11	0	0
Tetraploid (AABB)				
T. turgidum	31	15	13	3
T. turgidum subsp. *turgidum*	3	2	1	0
T. turgidum subsp. *dicoccoides*	14	13	1	0
T. turgidum subsp. *durum*	3	3	0	0
T. turgidum subsp. *dicoccum*	8	8	0	0
T. turgidum subsp. *carthlicum*	12	1	11	0
T. turgidum subsp. *turanicum*	3	2	1	0
T. turgidum subsp. *polonicum*	3	3	0	0
Hexaploid (AABBDD)				
T. aestivum subsp. *spelta*	4	2	2	0
T. aestivum subsp. *compactum*	4	2	2	0
T. aestivum subsp. *macha*	1	1	0	0
T. aestivum subsp. *spherococcum*	7	5	2	0
T. aestivum subsp. *aestivum*	300	87	173	40
Asia	84	17	66	1
Europe	78	23	55	0
North America	34	4	10	20
South America	51	14	21	16
Africa	29	17	12	0
Australia and New Zealand	24	12	9	3

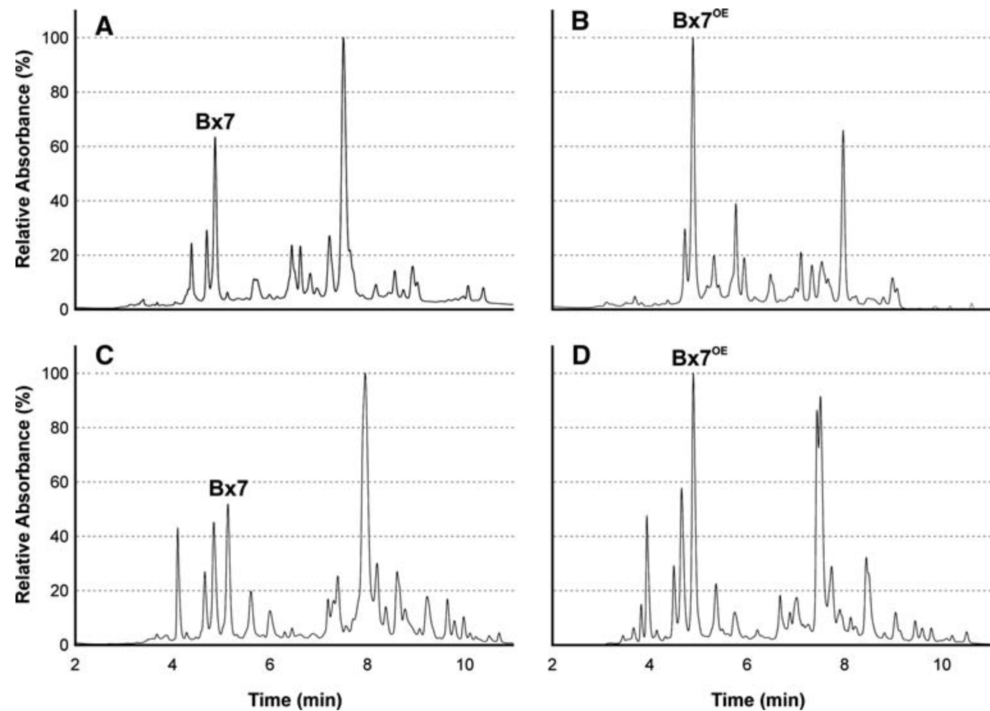

Fig. 2 RP-HPLC profiles of four wheat accessions. **a** *T. turgidum* check CN51263 has HMW-GS Bx7 that represented 57% of its total HMW-GS composition. **b** *T. turgidum* cv. Branco displayed HMW-GS Bx7^{OE} that represented 63.3 ± 4.7% of its total HMW-GS composition. **c** *T. aestivum* check cv. Klein Sin Rival has HMW-GS Bx7 that represented 30.4 ± 0.9% of its total HMW-GS composition. **d** *T. aestivum* cv. Glenlea displayed HMW-GS Bx7^{OE} that represented 38.40 ± 2.5% of its total HMW-GS composition

66.2 ± 4.6% in the three lines and was higher than the check (57.0%). Similarly, among the hexaploid accessions, the proportion of Bx7^{OE} subunit (41.7 ± 2.5%) was significantly higher ($P < 0.01$) in comparison to the check cultivars with non-overexpressed Bx7 subunit variants (29.6 ± 0.9%) (Table 2).

Sequence characterization of the *Glu-B1* locus

The three main types of Bx7 variants, namely Bx7*, Bx7 and Bx7^{OE} are differentiated by the presence of an 18 bp indel in the repetitive domain corresponding to an extra hexapeptide motif in the Bx7 and Bx7^{OE} subunits (Radovanovic and Cloutier 2003). The Bx7^{OE} subunit is however expressed at a higher level than the Bx7 subunit. A total of 27 of the 30 tetraploid accessions, including the 3 Bx7^{OE} accessions, displayed the 18 bp indel marker (Table 3). In the hexaploid collection, among the 219 accessions exhibiting a Bx7 variant, 57 Bx7 lines and all 40 lines with the Bx7^{OE} subunit were found to have the 18 bp indel marker. The allele encoding subunit *Bx7**, as characterized by the absence of the 18 bp indel, was found in 3 tetraploid and 120 hexaploid accessions. Hexaploid accession 01C0102607 was mixed (*Bx7/Bx7**) and CN9491 was not determined.

The presence of the 43 bp indel in the promoter region was found in four *T. turgidum* accessions including the three lines overexpressing the Bx7 subunit. Among the *T. aestivum* accessions, all 40 lines exhibiting the Bx7^{OE} subunits were found to have the 43 bp indel. In addition, 18 of the remaining 58 lines with the non-overexpressed Bx7 subunit also harbored this insert (Table 3; Supplementary Table 1).

The structural organization of the *Glu-B1* locus of Glenlea wheat is illustrated in Fig. 3. The tandem duplication of 10.3 Kb comprised two open reading frames (ORFs) including the *Bx7* gene, and flanked a complete and a partial LTR retroelement. The complete DNA sequence of TaBAC1215C06 has been deposited in GenBank (EU157184). Several primer pair combinations for the right

Table 2 Relative quantification of HMW-GS Bx7^{OE}, Bx7 and Bx7* by RP-HPLC

Species	Number of accessions	HMW-GS at *Glu-B1x*	Number of expressed HMW-GSs	Proportion of Bx7 subunit to total HMW-GS[a]
T. aestivum subsp *aestivum*	40	Bx7^{OE}	5	41.7 ± 2.5
T. aestivum subsp *aestivum (check)*	12	Bx7/Bx7*	5	29.6 ± 0.9
T. turgidum	3	Bx7 ^{OE}	3	66.2 ± 4.6
T turgidum (check)	1	Bx7	3	57.0

[a] Mean of duplicate injections ± standard deviation

Table 3 Presence of the 18 bp indel, 43 bp indel and right and left LTR junction DNA markers at the *Glu-B1* locus of tetraploid and hexaploid accessions having a Bx7 HMW-GS variant

Ploidy (*Glu-B1x*)	Number of accessions	18 bp Indel	43 bp Indel	Left and right LTR junction
Tetraploid (Bx7*)	3	0	0	0
Hexaploid (Bx7*)	120	0	0	0
Tetraploid (Bx7)	24	24	1	0
Hexaploid (Bx7)	57	57	18	0
Tetraploid ($Bx7^{OE}$)	3	3	3	3
Hexaploid ($Bx7^{OE}$)	40	40	40	40

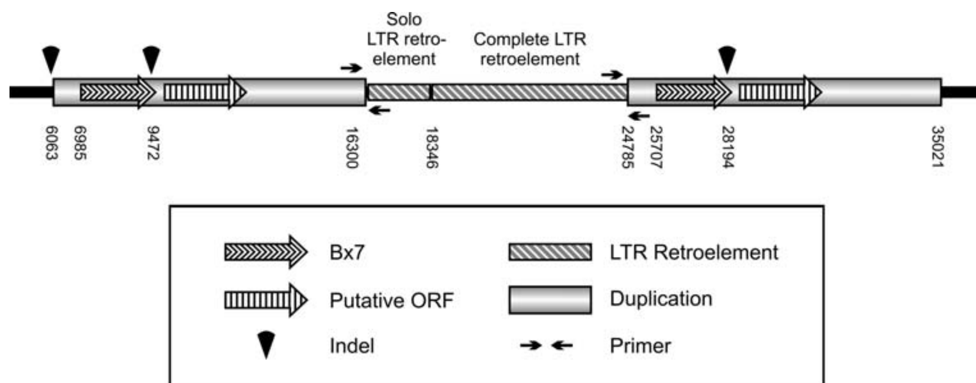

Fig. 3 Structural organization of the *Glu-B1* locus in cv. Glenlea showing a 10.3 Kb duplication encompassing the *Bx7* gene flanking a complete and a partial LTR retrotransposon. Nucleotide positions are indicated below. The first ORF of the duplication encodes the HMW-GS Bx7. The downstream predicted ORF encodes a putative protein kinase

and left junctions of the retroelement located at the *Glu-B1* locus were designed and tested (data not shown). Two primer pairs were selected for their specificity and robustness. Figure 4a and b illustrate the amplification of a 447 bp and an 884 bp fragment of the left and right junctions between the duplicated segments and the retroelement respectively. All 43 accessions (3 tetraploid and 40 hexaploid lines) identified to have the $Bx7^{OE}$ subunit by SDS-PAGE amplified both markers. Conversely, all other accessions that did not display $Bx7^{OE}$, including the 203 accessions expressing another Bx7 subunit variant, did not produce the PCR amplicons (Table 3). The *Glu-B1* locus specificity of the markers was confirmed by use of the Glenlea *Glu-B1* BAC clone TaBAC1215C06 as template.

The combined results of the three assessment methods for all $Bx7^{OE}$ accessions and checks are given in Table 4. References are listed for lines that had previously been reported to have $Bx7^{OE}$.

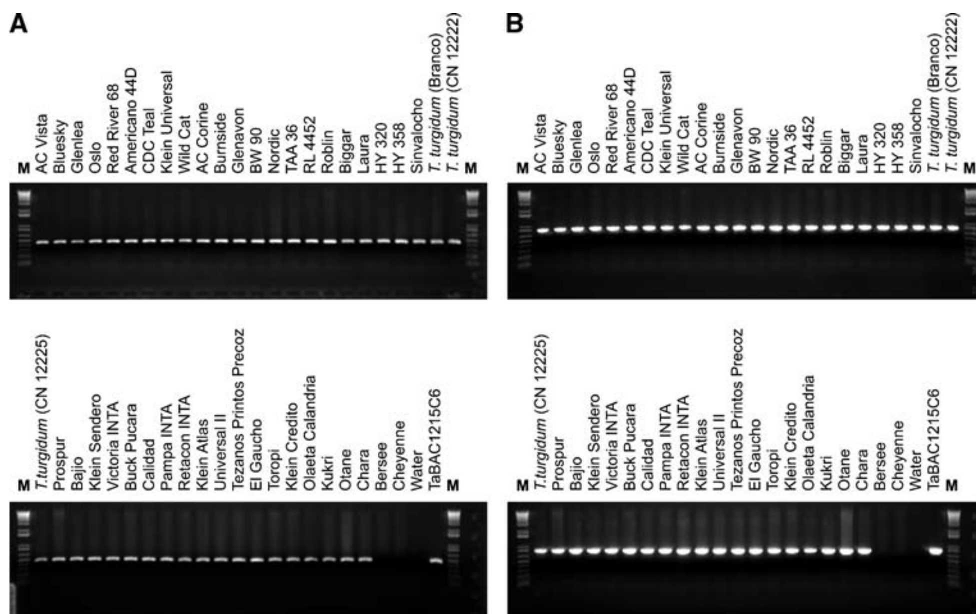

Fig. 4 PCR amplification of the *Triticum* accessions identified to have the $Bx7^{OE}$ HMW-GS by SDS-PAGE. Negative controls cv. Bersee (Bx7), cv. Cheyenne (Bx7*) and water (no DNA) and positive control BAC clone TaBAC1215C06 from Glenlea were included. **a** PCR amplification of the left junction of the retroelement and the duplicated region generated a 447 bp amplicon in all $Bx7^{OE}$ accessions. **b** PCR amplification of the right junction of the retroelement and the duplicated region generated an 844 bp amplicon. Marker (*M*) is 1 Kb Plus DNA ladder (Invitrogen, Mississauga, Canada)

Table 4 SDS-PAGE analysis, RP-HPLC quantification and PCR analyses for the 18 bp indel, the 43 bp indel and the *Glu-B1* retroelement left and right junction markers of tetraploid and hexaploid accessions with the $Bx7^{OE}$ phenotype and the check lines

Acc. No.	Name	Origin	HMW-GS $Bx7^{OE}$ (SDS-PAGE)	Relative proportion of Bx7 subunit to total HMW-GS (RP-HPLC)	18 bp Indel	43 bp Indel	Left and right LTR junction	Reference(s)
T. aestivum accessions with $Bx7^{OE}$ subunit								
–	AC Vista	Canada	+	42.49	+	+	+	
–	Blue sky	Canada	+	41.47	+	+	+	Butow et al. (2004), Marchylo et al. (1992), and Vawser and Cornish (2004)
CN 44438	Oslo	Canada	+	42.43	+	+	+	Marchylo et al. (1992), and Vawser and Cornish (2004)
CItr 14193	Red River 68	USA	+	40.67	+	+	+	Butow et al. (2004), D'Ovidio et al. (1997), and Vawser and Cornish (2004)
–	RL 4452	Canada	+	36.94	+	+	+	
–	Roblin	Canada	+	43.25	+	+	+	Butow et al. (2004), Marchylo et al. (1992), and Vawser and Cornish (2004)
CN 51820	Wild Cat	Canada	+	38.26	+	+	+	Butow et al. (2004), Marchylo et al. (1992), and Vawser and Cornish (2004)
PI 191937	Americano 44D	Uruguay	+	40.32	+	+	+	Butow et al. (2004)
–	CDC Teal	Canada	+	38.36	+	+	+	
–	AC Corinne	Canada	+	41.73	+	+	+	
–	Burnside	Canada	+	40.89	+	+	+	
–	Glenavon	Canada	+	41.06	+	+	+	
CN 43694	BW90	Canada	+	44.24	+	+	+	Marchylo et al. (1992)
–	Nordic	USA	+	44.24	+	+	+	Marchylo et al. (1992)
–	TAA 36	Israel	+	43.57	+	+	+	Lukow et al. (1989)
CItr 12606	Klein Universal	Argentina	+	39.76	+	+	+	Gianibelli et al. (2002)
CN 51812	Bigger	Canada	+	40.77	+	+	+	Butow et al. (2004), Marchylo et al. (1992), and Vawser and Cornish (2004)
CN 44167	Laura	Canada	+	42.93	+	+	+	Butow et al. (2004), Marchylo et al. (1992), and Vawser and Cornish (2004)
CN 42929	HY320	Canada	+	41.40	+	+	+	Marchylo et al. (1992)
CN 44146	HY358	Canada	+	39.22	+	+	+	Marchylo et al. (1992)
CN 11969	Sinvalocho	Uruguay	+	44.07	+	+	+	Butow et al. (2004), and Vawser and Cornish 2004
–	Glenlea	Canada	+	38.40	+	+	+	Butow et al. (2004), Lukow et al. (1989), and Vawser and Cornish (2004)
CWI 16281	Prospur	USA	+	37.16	+	+	+	Vawser and Cornish (2004)
BW 386	Bajio	Mexico	+	37.35	+	+	+	Vawser and Cornish (2004)

Table 4 continued

Acc. No.	Name	Origin	HMW-GS $Bx7^{OE}$ (SDS-PAGE)	Relative proportion of Bx7 subunit to total HMW-GS (RP-HPLC)	18 bp Indel	43 bp Indel	Left and right LTR junction	Reference(s)
CWI 77253	Klein Sendero	Argentina	+	47.15	+	+	+	Butow et al. (2004)
BW 12005	Victoria INTA	Argentina	+	40.69	+	+	+	Butow et al. (2004)
BWI 1255	Buck Pucara	Argentina	+	41.95	+	+	+	
BW 464	Calidad	Argentina	+	44.33	+	+	+	Vawser and Cornish (2004)
BW 152416	Pampa INTA	Argentina	+	43.99	+	+	+	Butow et al. (2004)
CWI 33350	Retacon INTA	Argentina	+	41.06	+	+	+	Butow et al. (2004)
BW 4689	Klein Atlas	Argentina	+	47.24	+	+	+	
CWI 14048	Universal II	Argentina	+	41.36	+	+	+	Vawser and Cornish (2004)
BW 779	Tezanos Printos Precoz	Argentina	+	42.79	+	+	+	Butow et al. (2004), and Vawser and Cornish (2004)
CN 10020	El Gaucho	Argentina	+	45.06	+	+	+	
CN 44011	Toropi	Brazil	+	40.00	+	+	+	
CN 10856	Klein Credito	Uruguay	+	44.40	+	+	+	
CN 11243	Olaeta Calandria	Uruguay	+	40.41	+	+	+	
Aus 29472	Kukri	Australia	+	44.24	+	+	+	Butow et al. (2002, 2004), and Vawser and Cornish (2004)
Aus 30426	Otane	New Zealand	+	42.94	+	+	+	Butow et al. (2004), Sutton (1991), and Vawser and Cornish (2004)
Aus 30031	Chara	Australia	+	41.01	+	+	+	Butow et al. (2002, 2004), and Vawser and Cornish (2004)
T. turgidum accessions with $Bx7^{OE}$ subunit								
CN 2644	Branco	Portugal	+	63.29	+	+	+	
CN 12222	CN 12222	Czech	+	63.66	+	+	+	
CN 12225	CN 12225	Czech	+	71.60	+	+	+	
Checks (*T. aestivum* cultivars)								
PI 447404	Yang Mai No.1	China	–	29.60	–	–	–	
CItr 6731	Benefactor	UK	–	30.26	–	–	–	
01C0100613	Bankuti	Hungary	–	30.65	–	–	–	
CWI 14942	Klein Sin Rival	Argentina	–	30.40	–	–	–	
CN 10719	Kenya Farmer	Kenya	–	29.80	–	–	–	
01C0200129	Maja	Czech Republic	–	30.81	+	–	–	
CItr 8885	Cheyenne	USA	–	27.58	–	–	–	
CN 38927	Katepwa	Canada	–	29.00	–	–	–	
CN 11189	Neepawa	Canada	–	29.56	–	–	–	

Table 4 continued

Acc. No.	Name	Origin	HMW-GS Bx7^{OE} (SDS-PAGE)	Relative proportion of Bx7 subunit to total HMW-GS (RP-HPLC)	18 bp Indel	43 bp Indel	Left and right LTR junction	Reference(s)
PI 520297	Stoa	USA	–	28.93	–	–	–	
–	AC Minto	Canada	–	30.09	–	–	–	
–	Columbus	Canada	–	28.95	–	–	–	
Check (*T. turgidum* accession)								
CN 51263	CN 51263		–	57.04	–	–	–	

PCR analysis results of the three *Glu-B1* markers outlined in Table 3 clearly establish a relative timeline for these evolutionary events. Since none of the Bx7* lines have the 43 bp indel and none of the Bx7 lines have the left and right junction markers, and since the corollaries are also true, it can be inferred that the 18 bp insertion event pre-dates the 43 bp insertion event which, in turn, pre-dates the LTR retroelement mediated duplication at the *Glu-B1* locus (Fig. 5). Further, all these Bx7 variants were found in both tetraploid and hexaploid collections thereby supporting the multiple polyploidization event hypothesis of hexaploid wheat.

Discussion

Gene duplication generates the raw materials for evolutionary novelties such as new functions and expression patterns (Lynch and Conery 2000). Genome-wide dispersed duplicated regions originate from ancient polyploidization followed by chromosome fusions and translocations as observed in *Arabidopsis* (Vision et al. 2000; Wolfe 2001). In contrast, tandem segmental duplications result from unequal crossing over mediated by repetitive DNA such as retroelements (Zhang 2003; Dubcovsky and Dvorak 2007). Duplication of the ancestral *Glu-1* sequence leading to paralogous gene copies encoding x- and y-type subunits was dated at 7.2–10.0 million years ago (MYA) before the divergence of the wheat genomes 5.0–6.9 MYA (Allaby et al. 1999). In the absence of any direct evidence for the molecular mechanism involved in the duplication of the ancestral *Glu-1* gene, the predominance of repetitive elements (>80% of the genome, Smith and Flavell 1975) can indicate their possible role in duplication of this locus as previously suggested (Dubcovsky and Dvorak 2007). The presence of retroelements in the intergenic regions of genes encoding x- and y-type subunits of HMW glutenin (Anderson et al. 2003; Gu et al. 2006) suggests that the mechanism of inter-element ectopic recombination could have lead to these paralogous genes. Similarly, the presence of the LTR retroelements in the interval of the 10.3 Kb duplicated segments encompassing the Bx7 gene at the *Glu-B1* locus indicates their possible involvement in the origin of the duplication in the cultivar Glenlea (Fig. 3; Cloutier et al. 2005).

The duplication of the gene encoding the HMW-GS Bx7 has been proposed as the cause for the overexpression of this subunit in cultivar Glenlea (Cloutier et al. 2005). While most cultivars and accessions of hexaploid wheat express 3–5 subunits, lines with the Bx7^{OE} phenotype can also express up to five different subunits but likely have six functional genes encoding HMW-GS. A 43 bp indel located 572 bp upstream from the transcription initiation

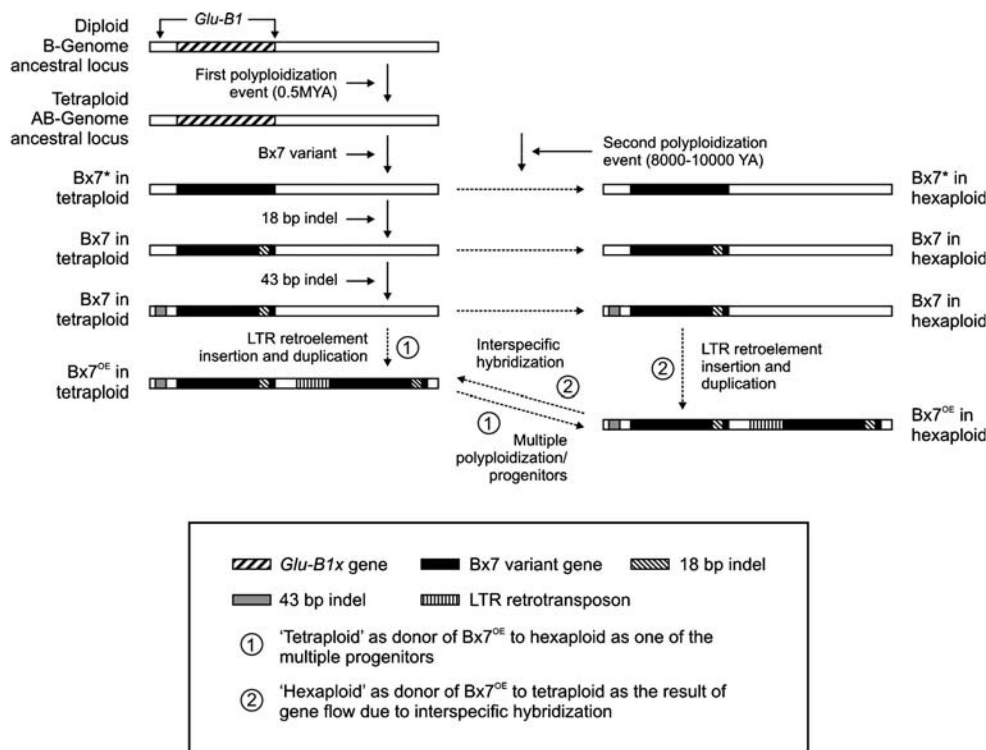

Fig. 5 Diagram of the chronological events that occurred at the *Glu-B1* locus of wheat lines with a *Bx7* allele variant illustrating the possibilities of several independent polyploidization events involving different tetraploid ancestors. Elements not drawn to scale to better illustrate the insertion and deletion events

site had previously been described and used to develop a marker associated with the Bx7^{OE} subunit (Radovanovic and Cloutier 2003; Butow et al. 2004). This marker was used in two independent studies to characterize a Canadian and an Australian segregating population and to establish the significant genetic contribution of the *Bx7^{OE}* allele to dough strength characteristics (Butow et al. 2003; Radovanovic et al. 2002). While this 43 bp indel was always present in Bx7^{OE} lines, the reverse was not always true i.e., this indel was found in some non-Bx7^{OE} lines (Butow et al. 2004). Our extensive study corroborated these findings. Butow et al. (2004) also used RP-HPLC and the presence of an 18 bp indel (one hexapeptide motif) in the coding region of the allele to characterize lines from eight different *Glu-B1* allelic groups but found that neither the 43 bp nor the 18 bp indel was perfectly linked with the overexpression phenotype. These short tandem insertions were also found in the accessions of *T. turgidum* indicating that these indels occurred prior to the polyploidization event that led to the formation of hexaploid wheat 8,000–10,000 years ago. Indeed, the duplication of the gene encoding the Bx7 subunit hypothesized by Lukow et al. (1992) and D'Ovidio et al. (1997) based on Southern hybridization and recently demonstrated by BAC sequencing (Cloutier et al. 2005) was confirmed in the present study. Without exception, all 43 accessions overexpressing HMW-GS Bx7 shared the same locus structure with the LTR retroelement flanked by the duplication encompassing the *Bx7* gene, and, all 369 accessions that were non-Bx7^{OE} lacked this genomic structure. The 43 bp insert was found in four *T. turgidum* accessions including the three Bx7^{OE} lines indicating that this short tandem insertion occurred prior to the retrotransposon mediated duplication. Similarly, the 18 bp indel in the coding region also pre-dates the duplication corroborating the finding of Butow et al. (2004) who reported these two indels in accessions of *T. turgidum*.

DNA markers are being employed as tools to improve the efficiency of selection in the pre-breeding and breeding activities in wheat (Eagles et al. 2001; Gale 2005). Marker assisted selection for Bx7^{OE} subunit will be useful since it contributes to dough strength (Butow et al. 2003; Lukow et al. 1992; Marchylo et al. 1992; Radovanovic et al. 2002). DNA markers were developed based on both the coding region of the gene (Butow et al. 2003, 2004; Ma et al. 2003) and the promoter region of the gene encoding HMW-GS Bx7 (Butow et al. 2004; Juhasz et al. 2003; Radovanovic and Cloutier 2003). However, they were employed in the selection strategies along with SDS-PAGE and/or RP-HPLC profiling because of their imperfect association with the Bx7^{OE} phenotype. The dominant left and right junction markers developed in this study are perfectly linked to the Bx7^{OE} phenotype and can be applied in the selection schemes of wheat breeding programs.

Pedigree analysis indicated that an Argentinean landrace, namely Klein Universal II released in 1922, was the source of worldwide dissemination of the *Glu-B1al* allele (Bx7^{OE} + By8) through the CIMMYT germplasm used in modern wheat breeding programs (Butow et al. 2004).

Further, it was suggested that Americano 44D, a Uruguayan landrace of unknown origin could be the donor of the allele found in Klein Universal II. However, the origin of the *Glu-B1al* allele found in the Israeli landrace (TAA36) and the Hungarian landrace Bankuti 1201 could not be explained by the Argentinean ancestor. Historical information indicated that Eastern European landraces introduced by immigrants in the nineteenth century could be the possible source of the *Glu-B1al* allele found in Americano 44D, TAA36 and Bankuti 1201 (Butow et al. 2004). The presence of the gene duplication in the tetraploid accessions of European origin as reported in the present study reinforces the possible European origin of the Bx7^{OE} allele found in these landraces.

The survey also found previously unreported lines with the HMW-GS Bx7^{OE} in the hexaploid cultivars namely, Klein Credito and Olaeta Calandria from Uruguay and Toropi from Brazil. The high frequency of the Bx7^{OE} subunit reported in the Argentinean wheat cultivars was also confirmed (Gianibelli et al. 2002; Vawser and Cornish 2004). However, the Hungarian landrace Bankuti 1201 previously reported to have HMW-GS Bx7^{OE} (Juhasz et al. 2003), did not exhibit the genome organization corresponding to the duplication nor did it show this subunit on SDS-PAGE. The presence of different biotypes in this accession was reported earlier and is presumed to be the reason for the discrepancies between the various reports to date (Butow et al. 2004; Juhasz et al. 2003).

Evolutionary mechanisms in the form of retroelement mediated segmental chromosomal duplication can have significant phenotypic impacts on agriculturally important loci such as the *Glu-B1* locus. Gene duplication by helitron-like transposons in maize and gene fragment acquisition by pack-MULES in rice were reported (Jiang et al. 2004; Morgante et al. 2005). Unlike the *cut and paste* mode of transposition of these mobile elements, the *copy and paste* mode of propagation of retroelements distribute their homologous sequences across the genome, which in turn, increases the probability of ectopic recombination leading to deletions and duplications. Deletions as an evolutionary force are implicated in drastic reductions in genome sizes (Bennetzen 2002; Ma et al. 2004; Vitte and Panaud 2005). However, the phenotypic impacts of duplications have not been frequently discovered in plant genomes of agricultural importance. The observed segmental duplication resulting in two functional copies of the gene encoding HMW-GS Bx7 signifies that the evolutionary dynamics of the genome driven by retroelements may have played a role in shaping the structural organization of not only the biologically important loci discovered earlier but also agriculturally important loci (Gaut et al. 2007).

Comparative sequence analyses of the coding regions of the *Glu-1* alleles and its immediate upstream regions were carried out extensively (Halford et al. 1987; Anderson and Greene 1989; Mackie et al. 1996; Allaby et al. 1999; Shewry et al. 2003). However, the genome organization of the orthologous *Glu-1* loci with its distal flanking regions covering a few 100 Kb emerged only after the construction of large insert BAC libraries. The observed intergenic distances between genes encoding x- and y-type subunits of HMW glutenin are 140 Kb, 168 Kb and 51 Kb respectively in the A, B and D genomes (Gu et al. 2004; Kong et al. 2004; Anderson et al. 2003). Though there is conservation of gene order and orientation at the orthologous *Glu-1* loci, microcolinearity at the intergenic region is disrupted mainly due to the insertion of retrotransposons as observed in the maize genome (SanMiguel et al. 1996). The segmental duplication encompassing the gene coding for the Bx7 subunit described herein, originated as a consequence of the mechanism of unequal homologous crossing over driven by the LTR retroelement inserted into the locus (Cloutier et al. 2005). The models of the retroelement mediated origin of this tandem segmental duplication at the *Glu-B1* locus are presented in supplementary Figure 1(A) and 1(B). It is possible to estimate the time of insertion of LTR retroelements by comparing observed nucleotide substitutions between right and left LTRs because point mutations accumulate over time (Gaut et al. 1996; SanMiguel et al. 1998). Cloutier et al. (2005) identified no base substitutions between the LTRs of the retroelement at the *Glu-B1* locus. However, nucleotide substitution rate of the duplicated region yielded an estimated time of the duplication of 15,000 years ago $\pm$ 11,000 years, which overlaps with the polyploidization event of hexaploid wheat estimated to be 8,000–10,000 years ago (Huang et al. 2002). Stress can activate transposons and lead to their retrotransposition into new sites (Grandbastien 1992). Polyploidization per se could have caused the transposition of the LTR retroelement at the *Glu-B1* locus followed by inter-element recombination leading to the segmental duplication. In this case, the *Glu-B1al* allele would not be found in diploid or tetraploid progenitors but would be restricted to hexaploid wheat accessions. Our results clearly showed that this was not the case because three tetraploid accessions displayed the *Bx7^{OE}* allele and were structurally identical at the genomic level to the Bx7 ^{OE} hexaploid lines i.e., they had the duplicated region flanking the LTR retroelement, the 18 bp and the 43 bp indels. Moreover, they showed high staining intensity on SDS-PAGE and had a higher proportion of Bx7 HMW-GS when compared to the check. Butow et al. (2004) had hypothesized the presence of the Bx7 ^{OE} phenotype in *T. turgidum* var. Portugal 170 but could not confirm the overexpression phenotype.

Many polyploid species were hypothesized to have formed recurrently from several crosses involving different gene pools of their progenitor species (Soltis and Soltis 1999).

Polyploid wheat exists in both tetraploid and hexaploid forms which could have originated independently from hybridizations between distinct diploid or tetraploid ancestors (Feldman 2001). Comparative analysis of orthologous regions for shared genome organization between the D-genome of hexaploid wheat and *Ae. tauschii*, its diploid D-genome donor, indicated the existence of more than one shared allele (Caldwell et al. 2004; Dvorak et al. 1998; Giles and Brown 2006; Talbert et al. 1998). These shared alleles suggested that the hexaploid wheats were formed by recurrent hybridizations involving more than one genotype of *Ae. tauschii*. Gu et al. (2006) compared nucleotide substitution rates for the A and B genomes of tetraploid and hexaploid wheats at the *Glu-1* loci and found that they differed significantly despite co-evolving in the same nuclei in their respective species thereby supporting the hypothesis of more than one tetraploid ancestor with distinct A genome lineage in the origin of hexaploid wheat. The present study provides additional evidence for the multiple tetraploid ancestor hypothesis for hexaploid wheat, however based on evidence from the B-genome. The existence of two different shared genome organizations at the *Glu-B1* locus of *T. turgidum* and *T. aestivum* indicates that at least one *T. turgidum* line with the *Bx7* duplication and LTR retrotransposon and one without could have served as progenitors in the formation of hexaploid wheat. Findings supporting the same hypothesis were reported at the orthologous *Lr10* loci where two conserved deletion point haplotypes were described in the A genome at the three ploidy levels i.e. *T. monococcum*, *T turgidum* and *T. aestivum* (Isidore et al. 2005). The three *T. turgidum* lines described to have a $Bx7^{OE}$ phenotype and the retroelement could have acquired it through inter-specific hybridization with hexaploid lines in either natural habitats or in classical plant breeding efforts aimed at introgression of desirable traits. These scenarios are however unlikely because two of the lines are landraces and the third one, cultivar Branco, was described as a released landrace not improved by breeders. Aside from the $Bx7^{OE}$ versus *Bx7* allele presence in both tetraploid and hexaploid wheat collections, the multiple ploidization hypothesis involving different tetraploid ancestors is further supported by the findings of *Bx7** and *Bx7* alleles with and without the 43 bp indel in both collections as well.

Conclusion

In this study, the genomic organization of the *Glu-B1* locus and the expression level of the Bx7 subunit were simultaneously assessed in a number of diploid, tetraploid and hexaploid accessions. The perfect correlation between the presence of the gene duplication and the overexpression of the Bx7 HMW-GS reinforce the causal link between genotype and phenotype. The duplication described herein occurred as a consequence of the transposition of an LTR retroelement.

The structural organization associated with the segmental duplication encompassing the *Glu-B1* locus in three tetraploid accessions indicated that the retroelement mediated recombination event occurred prior to the polyploidization event resulting in hexaploid wheat speciation. Our data also supports the proposal of multiple polyploidization events in the origin of the hexaploid wheat genome, primarily based on the presence of two independent genome organizations at the *Glu-B1* locus between tetraploid and hexaploid accessions. However, the possibility of gene flow as the result of interspecific hybridization between *T. aestivum* and *T. turgidum* in the natural habitats was not excluded. The result also serves as evidence for the role of retroelements on the evolution of agriculturally important loci. Finally, the DNA markers identified in the present study can be used as perfectly linked markers for the *Glu-B1al* allele encoding the $Bx7^{OE}$ subunit in wheat breeding programs.

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