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ORIGINAL PAPER

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Two quality-associated HMW glutenin subunits in a somatic hybrid line between *Triticum aestivum* and *Agropyron elongatum*

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Abstract High-molecular-weight glutenin subunits (HMW-GSs) from hybrid line II-12 between wheat (*Triticum aestivum* L.) and *Agropyron elongatum* (Host) Nivski were characterized with SDS-PAGE. Out of these HMW-GSs, two subunits, h1Bx and h1By, had mobilities similar to the subunits 1Bx13 and 1By16 from common wheat 4072, which was used as control. Polyclonal antibodies (pAbs) of h1Bx and h1By were prepared, and Western blotting showed that the pAbs had strong affinities for h1Bx and h1By, separately. The specificity of h1Bx-pAb was further checked; it preferentially recognized subunits h1Bx and 1Bx13. HMW-GS gene coding sequences were amplified by genomic polymerase chain reaction from hybrid II-12. Two of the five amplicons, marked *II2a* and *II31b*, were sequenced. Their coding sequences are clustered to *Glu-1Bx7* and *Glu-1By9* of common wheat. Three discrepant regions in deduced amino acid sequences of *II2a* and *31b* repeated one time more than *Glu-1Bx7* and *Glu-1By9*. N-terminal sequences of h1Bx and h1By were determined, which were identical to the published sequences of 1Bx13 and 1By16 and in agreement with that deduced from *II2a* and *II31b*, respectively. These results indicated that the two novel genes separated from the hybrid wheat derived from the allelic variation of *1Bx7* and *1By9* of the parent wheat. There is an additional cysteine residue positioned at 271st amino acid of the mature peptide of II2a, which may be related to the high quality of the flour.

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Introduction

The high-molecular-weight glutenin subunits (HMW-GSs) encoded by many allelic genes are one class of wheat prolamines. Two linked genes designated as x- and y-types are located on the long arms of chromosomes 1A, 1B, and 1D. The differences between them are the molecular weight (higher for x-type), number of cysteine residues (four in most x-type subunits, seven in most y-type), and repetitive motifs. Silencing of specific genes results in variation in the number of expressed subunits from three to five, while allelic variation in subunits encoded by the expressed genes leads to polymorphism of x-type and y-type subunits, which can be separated by SDS-PAGE. The association of HMW-GS with bread-making quality has enabled Payne et al. (1981) and Payne and Lawrence (1983) to assign "quality scores" for different alleles. The subunit pair 1Dx5 + 1Dy10 is positively associated with bread-making quality compared with subunit pair 1Dx2 + Dy12. The same holds true with the subunit pairs 1Bx17 + 1By18, 1Bx13 + 1By16, and 1Bx14 + 1By15 compared with 1Bx7 + 1By8, 1Bx7 + 1By9, and 1Bx6 + 1By8 or 1Bx7 and the subunits 1Ax1 or 1Ax2* compared with Null (Mills et al. 2000; Branlard et al. 2001).

Although the HMW-GSs compose around 8–10% of the total extractable flour protein (Halford et al. 1992), they are present as a network in dough and confer the visco-elastic property, which allows wheat to be processed into bread, pasta, and noodles, as well as a range of other food products. This makes their coding sequences as candidate genes to enhance grain-processing quality through crop genetic transformation (Shewry et al. 2001). Some of HMW-GS genes from wheat (Anderson and Greene 1989; Reddy and Apples 1993) and other related species (Wan et al. 2002; Liu et al. 2003; Feng et al. 2004) have been characterized, but only a few were located on the B genome.

The immunological method has been increasingly exploited in cereal research (Skerritt and Tatham 1996).

Polyclonal antibodies (pAbs) were used for detection of a synthetic HMW-GS in the induced cells by Western blotting (Kevin et al. 2001). A monoclonal antibody to a synthetic peptide of HMW-GS 1Dx5 was developed to investigate glutenin polymers (Mills et al. 2000).

Different allelic HMW-GS genes can be transferred via sexual crosses, and hence, different HMW-GS combinations were formed in wheat cultivars. However, only a few reports described the evolutionary origin and allelic variation of HMW-GS (Lawrence and Sheppherd 1981; Thompson et al. 1983; Anderson and Greene 1989; Shewry et al. 1995; Zhang et al. 1997). Some hybrid strains with different quality traits were generated by asymmetric somatic hybridization between wheat (*Triticum aestivum* L.) Jinan 177 and *Agropyron elongatum* (Host) Nivski (Xia et al. 2003) and inherited to F_6 stably (Chen et al. 2004; Wang et al. 2004). Many "new" HMW-GSs and combinations (absence in either parent) were identified in the hybrid lines (Zhao et al. 2003). These results provided a possibility to understand the allelic variation of HMW-GSs in wheat strains. In this report, we first characterize two quality-associated subunit genes, *II2a* and *II31b*, from hybrid line II-12. Furthermore, we discuss the possibility of allelic variation from *1Bx7 + 1By9* to *II2a* and *II31b*.

Materials and methods

Plant materials

Agropyron elongatum (Host) Nevski [*Thinopyrum ponticum* (Podpera) Liu and Wang $2n = 10x = 70$], wheat Jinan 177 (*T. aestivum* L. $2n = 42$), Chinese Spring, and hybrid wheat II-12, I-4 which originated from intergeneric somatic hybridization between Jinan 177 and *A. elongatum* (Xia et al. 2003) and T177 lines derived from tissue cultures of Jinan 177 for F_3 and F_5 were stored in our laboratory. Shandong Academy of Agricultural Sciences, Jinan, China, kindly offered seeds of wheat cultivars 4072 and Yanyou 361. Jinan 177, T177, and hybrid lines were all planted in greenhouse separately in order to avoid cross-pollination from other wheat cultivars.

Determination of quality parameters of seed

The seed quality parameters of parent Jinan 177, hybrid II-12 F_3–F_5 were determined by Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences, Beijing, China.

SDS-PAGE separation of HMW-GS

HMW-GSs in the endosperm tissue were extracted from *A. elongatum*, Jinan 177 (1Dx2 + 1Dy12 and 1Bx7 + 1By9), T177, hybrid II-12 F_3–F_7, Chinese Spring (1Dx2 + 1Dy12 and 1Bx7 + 1By8), 4072 (1Ax1, 1Dx5 + 1Dy10 and 1Bx13 + 1By16), and Yanyou 361 (1Ax1, 1Dx5 + 1Dy10 and 1Bx17 + 1By18) with the same method as used by Zhao et al. (2003). SDS-PAGE was according to the procedure described by Feng et al. (2004).

Preparation of pAbs and Western blotting

After electrophoresis, parts of lanes on the gel with identical h1Bx and h1By segments were cut and stained with Coomassie BBG250. The stained lanes were laid back to the original position as markers for excising the h1Bx and h1By subunits on the other parts of the same unstained gel. The collected h1Bx and h1By subunits were used as antigens to immunize a rabbit. Until appropriate efficiency of antibody was obtained, the serum was collected and used for Western blotting with above HMW-GSs according to Kevin et al. (2001).

Cloning of the ORFs of HMW-GS

The seeds of hybrid wheat II-12 were grown for 5–7 days at 25°C. The extraction of genomic DNA adopted CTAB method according to Murray and Thompson (1980). In order to amplify the complete ORFs of HMW glutenin genes of hybrid II-12 via genomic polymerase chain reaction (PCR), a pair of degenerate primers specific for HMW-GS genes was designed. The sequences of the two primers are P1 (5′-ATGGCTAAGCGGC/TTA/GGTCCTCTTTG-3′) and P2 (5′-CTATCACTGGCTA/GGCCGA CAATGCG-3′) (Liu et al. 2003; Feng et al. 2004). A highly fidelity polymerase LA GC *Taq* (TaKaRa Biotechnology) with a buffer for a GC-rich template was used in PCR in order to reduce the risk of introducing errors into the sequence. The parameters for the reaction were one cycle at 95°C for 5 min, followed by 30 cycles of 94°C for 40 s, 68°C for 4 min, and a final extension step at 68°C for 10 min. The PCR products were recovered from agarose gels by a gel extraction kit and cloned into pUCm-T vector (Sangon, Shanghai, China) as described previously (Feng et al. 2004). By restriction-enzyme digestion analysis and terminal sequencing, the inserts of two novel clones, pUC *II2a* and pUC *II31b*, were deduced to represent the ORFs for the 1Bx- and 1By-type HMW glutenin subunits respectively. For complete sequencing of the inserts in pUC *II2a* and pUC *II31b*, a series of subclones were prepared using the nested deletion method (Sambrook et al. 1989). DNA was sequenced by a commercial company (TaKaRa). The full-length nucleotide sequences of the inserts were assembled from sequencing data from six to eight subclones. For sequence analysis, programs of the NCBI and EBI networks were used.

Table 1 Quality parameters of Jinan 177 (*JN 177*) and hybrid F_3–F_5 lines I-4 and II-12. Data are the mean of F_3–F_5 results

Species	SDS sedimentation (ml)	Content of protein (%)	Mixing time (min)	Mixing tolerance (min)
I-4	35.3	16.69	3	3–5
JN 177	32.5	14.48	3	3
II-12	49	20.64	12	16

N-terminal sequences of HMW-GSs

After SDS-PAGE separation, the HMW-GSs of hybrid II-12 were transferred to a PVDF membrane according to Bryan (1997). Staining of the PVDF membrane with 0.1% (W/V) Coomassie BBR250 and excision of the HMW-GS bands of h1Bx and h1By for protein sequencing were performed in an Applied Biosystems Precise Amino Acid Sequencer by Beijing University, Beijing, China.

Results

Quality parameters and HMW-GSs

The flour quality of mixing time and baking performance were tested. Among hybrid F_3–F_5 lines of II-12, I-4 and parent wheat, hybrid II-12 was the best. Parts of the quality data are shown in Table 1.

HMW-GSs in the endosperm tissue of Jinan 177, hybrid II-12, *A. elongatum*, and T177 were preferentially extracted and subjected to SDS-PAGE analysis. Two common wheat cultivars, Chinese spring and 4072, and T177 regenerated from embryo calli of Jinan 177 were used as controls. Jinan 177 showed protein pattern of 2 + 12 and 7 + 9 (Fig. 1). *A. elongatum* had more bands than wheat and hybrid, as being a tenfold subspecies. One of the bands had mobility similar to h1By of hybrid (Fig. 1a), while hybrid II-12 from F_3–F_5 appeared four fragments different from parent wheat Jinan 177, including h1Bx + h1By with mobility similar to subunits 1Bx13 + 1By16 from wheat 4072 (Figs. 1a, 3). T177 lines contained 2 + 12 and 7 + 9 in most lines. However, a segment substituted the subunit 9, having mobility similar to subunit 8 in a few lines (Fig. 1b).

Homologous detection of h1Bx + h1By with 1Bx13 + 1By16

After immunizing four times, two pAbs form h1Bx and h1By with high affinity were obtained. Western blotting with the pAbs displayed strong binding to the subunits h1Bx and h1By, but weak bindings to other x- and y-type subunits (Fig. 2). The pAbs' specificity was further checked by immunoblotting different HMW-GSs from cultivars Jinan 177, 4072, Yanyou 361, and somatic hybrid line II-12 between wheat and *A. elongatum* in Western blotting (Fig. 3). The subunit 1Bx 13 from 4072 showed strong signal similar to h1Bx. This indicated that the HMW-GS h1Bx from II-12 was highly homologous to HMW-GS 1Bx13.

Sequences of the ORF of *II2a* and *II31b* genes

Five DNA fragments were specifically amplified with primer pairs (Fig. 4). They represent different HMW glutenin genes. The fragments of 2.4 kb marked *II2a* and 2.2 kb marked *II31b* were selected to clone. Partial DNA sequencing indicated that *II2a* and *II31b* inserts represented the ORF of the x- and y-type of HMW-GS genes, respectively. Using subclones produced by nested deletion, the inserts in pUC *II2a* and in pUC *II31b* were all completely sequenced.

A comparison of the amino acid sequence derived from the *II2a* and *II31b* ORFs to those of published HMW-GSs showed that the primary structure of the II2a and II31b subunits were homologous with those of known x- and y-type subunits respectively. A dendrogram of the relative homologies among the two genes and 22 other published HMW-GS genes from wheat and related species was constructed based on the signal and N-terminal non-repetitive sequences by the Clustal W program of the EBI program net (Table 2; Fig. 5). It

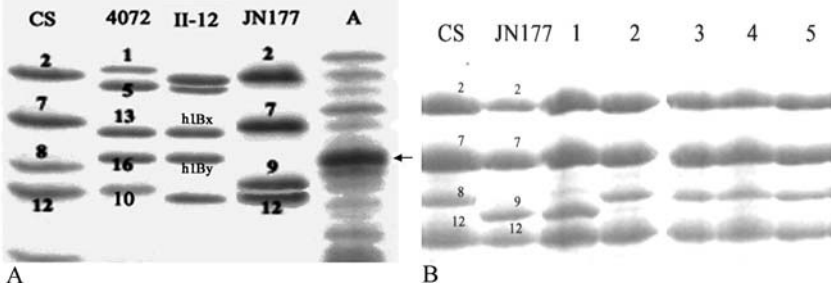

Fig. 1 SDS-PAGE analysis of high-molecular-weight glutenin subunits (HMW-GSs). The HMW subunits in the figure have been marked according to the nomenclature of Payne et al. (1981). **a** HMW-GS profile from different genotypes of wheat, somatic hybrid, and *Agropyron elongatum*. **b** HMW-GS profile from Chinese Spring, Jinan 177 and T177. *CS* Chinese Spring, *4072* wheat cultivars 4072, *II-12* hybrid wheat *II-12*, *JN177* Jinan 177, *AA. elongatum*, *lane 1* T177 F_3 line with 7 + 9 and 2 + 12, *lane 2* T177 F_3 line with 7 + 8 and 2 + 12, *lanes 3–5* three F_5 lines from 2

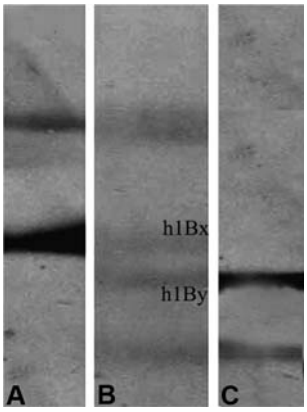

Fig. 2 Western blotting analysis of HMW-GS from hybrid wheat II-12, using pAb affinity to h1Bx (**a**) and h1By (**c**) from II2a. **b** The staining result of a PVDF membrane transferred with the SDS gel containing h1Bx and h1By

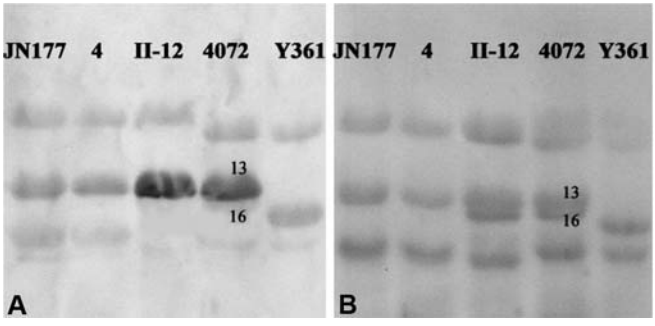

Fig. 3 Western blotting analysis of HMW-GS from various wheat varieties with different HMW-GS composition, using polyclonal antibody affinity to 1Bx from II2a. **a** Western blotting result. **b** The staining result of a PVDF membrane transferred with the SDS gel containing parent wheat Jinan 177, two hybrid lines between *T. aestivum* and *A. elongatum* 4 and II-12, as well as two common wheat cultivars 4072 and Yanyou 361

showed that *II2a* and *II31b* were clustered with *Glu-1Bx7* and *Glu-1By9* respectively. The ORF of the *II2a* gene, represented by the inset in pUC *II2a*, consists of 2,391 nucleotides. Its amino acid sequence contained 795 amino acids (Fig. 6). The ORF of the *II31b* gene, represented by the inset in pUC *II31b*, consists of 2,220 nucleotides. Its amino acid sequence contained 738 amino acids (Fig. 7). On the basis of the comparison, we can conclude that the primary structures of the two subunits include a signal peptide, an N-terminal region, a central repetitive domain, and a C-terminal region (Figs. 6, 7).

Compared with 1Bx7, II2a appeared a hexapeptide (PGQGQQ) addition and 22 amino acid residues replaced. In addition, there is a point mutation from A to G, which made the derived amino acid residue at the 271sh position of the mature peptide change from tyrosine (Y) to cysteine (C) (Fig. 6). Compared with 1By9, there were four hexapeptides (PAQGQQ, PGQGRQ, TRQGQQ, and LEQGQQ), a nonapeptide

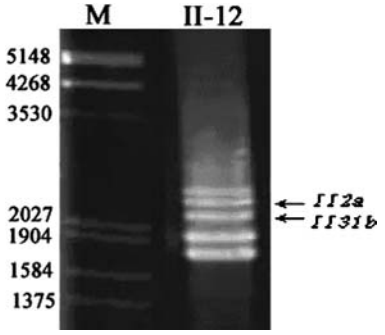

Fig. 4 PCR profile of II-12 DNA amplified with the primer pair. *M* Marker of *Eco*RI/*Hin*dIII-digested λ DNA, *II-12* PCR products from hybrid wheat II-12. The *II2a* and *II31b* segments are indicated by *arrows*

(GQYPASQQQ) addition, and 11 amino acid residues replaced in derived amino acid sequence of *II31b* (Fig. 7).

Sequences of N-terminal amino acid

The N-terminal sequences of the HMW-GS are compared (Table 3). All the sequences show high degrees of homology. In particular, the N-terminal amino acid sequences h1Bx and h1By were identical to 1Bx13 and 1By16 (Tatham et al. 1991), respectively. The deduced sequences from *II2a* and *II31b* showed a high homology with 1Bx13 and 1By16. This revealed that the h1Bx and h1By are encoded by *II2a* and *II31b* genes.

Discussion

Asymmetric somatic hybridization of common wheat with grass or cereal was successfully used for introgression of alien genome in our lab (Xia and Chen 1996; Xia et al. 2003; Zhou et al. 2001; Xiang et al. 2003; Xu et al. 2003). The hybrid strain II-12 and I-4 from the combination between *T. aestivum* and *A. elongatum* treated by UV (Xia et al. 2003) inherited from F_3 to F_7. II-12 had better quality for bread making than parent wheat and I-4 (Table 1). SDS-PAGE showed that the high quality of the flour was related to the composition of HMW-GSs in II-12, which was very different from I-4 (2 + 12, 7 + 9) and parent wheat (2 + 12, 7 + 9) (Figs. 1a, 3). Two of the HMW-GSs, h1Bx + h1By, in the hybrid were characterized. The comparison of N-terminal amino acid sequencing and Western blotting with specific pAbs revealed that they are homologous with 1Bx13 + 1By16 (Fig. 3). Two of the five DNA fragments, *II2a* and *II31b*, amplified by PCR from II-12 were confirmed to be encoding h1Bx + h1By by the analysis of sequencing (Figs. 5, 6, 7). Hence, we have cloned two novel HMW-GS genes on 1B chromosome from hybrid II-12.

In order to investigate the origin of HMW-GS genes in hybrids, we have cloned five HMW-GS y-type genes from parent grass *A. elongatum* (Feng et al. 2004;

Table 2 The information of high-molecular-weight glutenin subunits (*HMW-GSs*) in Fig. 5

HMW-GSs	The GenBank number of the coding sequence	The length of the coding sequence (bp)	Species
1Ax1	X61009	2,496	*Triticum aestivum*
1Dx2	X03346	2,520	*T. aestivum*
1Dx2t	AF480485	2,508	*Agropyron tauschii*
1Dx2.1t	AF480486	2,514	*Aegilops tauschii*
1Dx5	X12928	2,523	*T. aestivum*
1Ux	AF476961	2,982	*Ae. markgrafii*
1Cx	AF476959	2,391	*Ae. markgrafii*
R1x	AF216868	2,304	*Secale cereal*
1Bx7	X13927	2,373	*T. aestivum*
II2a	AY424400	2,391	*T. aestivum*
1Cy	AF476960	1,905	*Ae. markgrafii*
1Ay	X03042	1,812	*T. aestivum*
AgeloG5	AY319518	1,515	*A. elongatum*
AgeloG6	AY264065	1,494	*A. elongatum*
AgeloG7	AY263345	906	*A. elongatum*
R1y	AF216869	2,145	*S. cereal*
1By9	X61026	2,121	*T. aestivum*
II31b	AY263346	2,220	*T. aestivum*
1Dy12	X03041	1,986	*T. aestivum*
AgeloG2	AY263343	1,980	*A. elongatum*
1Dy12t	U39229	1,950	*A. tauschii*
1Dy10	X12929	1,950	*T. aestivum*
AgeloG3	AY263344	1,842	*A. elongatum*
1Uy	AF476962	1,971	*Ae. umbellulata*

Fig. 5). Clustering *II2a* and *II31b* sequences and many other HMW-GS genes from wheat cultivars and relatives in GenBank showed that *II31b* was more homologous with *Glu-1By9* than with all y-type genes of *A. elongatum* (Fig. 5), although the h1By subunit had a mobility similar with one segment in *A. elongatum* (Fig. 1a). The *II2a* was also clustered with *Glu-1Bx7* (Fig. 5). Thus, we suggest that the *II2a* and *II31b* genes were derived from the allelic variation of *Bx7*+*By9* genes in Jinan 177.

Till now, over 30 HMW-GSs and many combinations were detected in a large number of wheat cultivars. Diversity came from sexual crosses between parents with different alleles. No novel allele was created in the strain from normal sexual procedure for wheat-quality breeding. The reason for which is the exact homologous genes crossover in meiosis of sexual hybridization. Allelic variation of wheat HMW-GS via tissue culturing was reported (Larkin et al. 1984; Zhang et al. 1996, 1997; Svec et al. 1999). However, there were no data of sequence verification on these allelic variations as described in this paper. In our experiment, somaclonal variation of HMW-GSs in parent wheat Jinan 177 (T177) was also checked (Fig. 1b); subunit 1By9 was substituted by a new segment having mobility similar to subunit 1By8. But the variation locus (only one) and

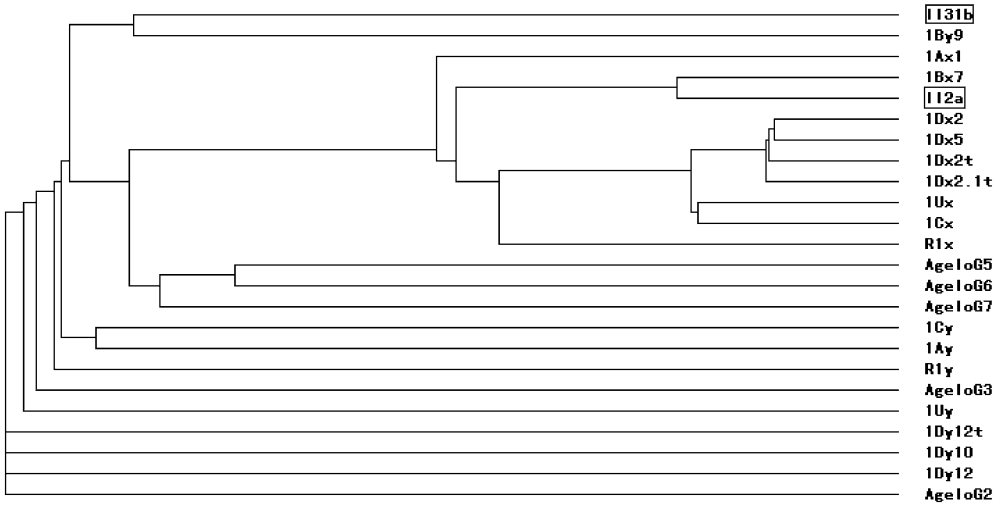

Fig. 5 Relative homology of the signal and N-terminal non-repetitive regions of two genes (*II2a* and *II31b*) and 22 HMW glutenin genes in common wheat and other related species. The dendrogram is derived from Clustal W program, European Bioinformatics Institute. The GenBank numbers of the genes and the species are listed in Table 2. *BoxesII2a* and *II31b*

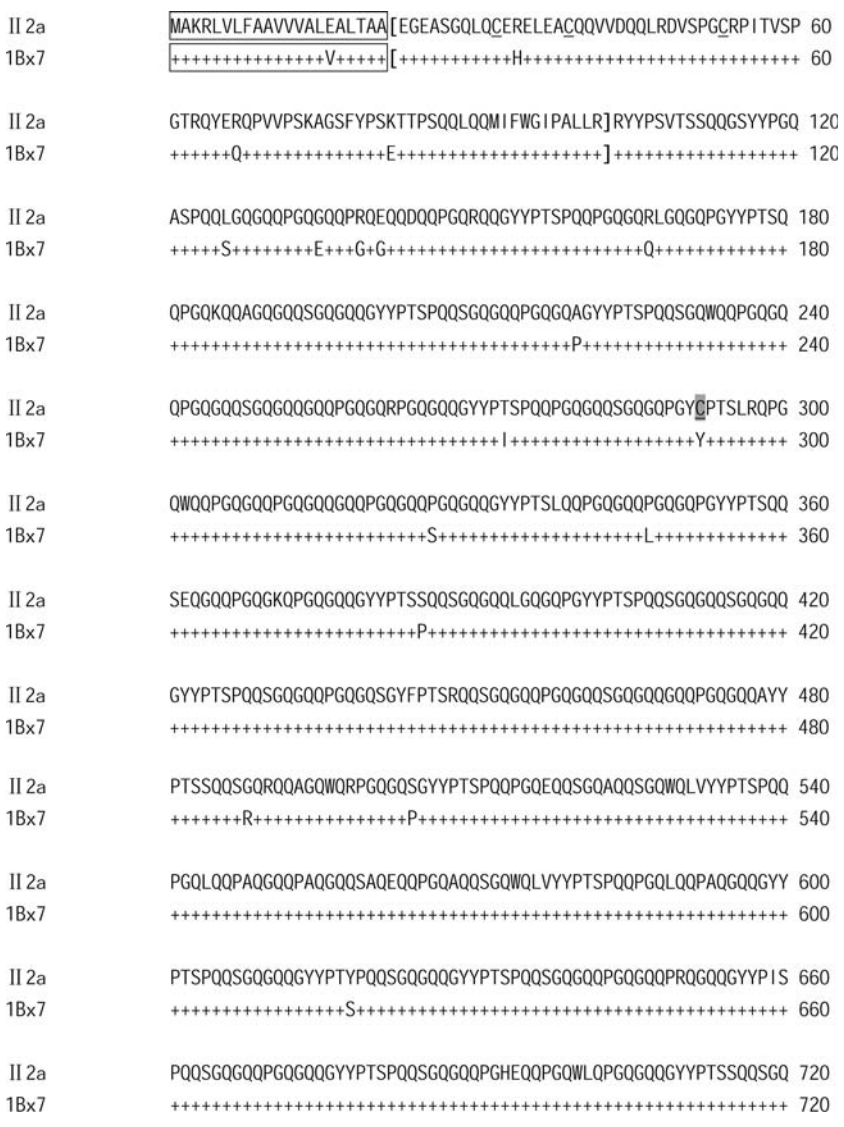

Fig. 6 Comparison of the deduced amino acid sequences of *Glu-1Bx7* from common wheat and *II2a* from the hybrid II-12. *Underlining* indicates the cysteine residues, *boxes* contain the signal peptides (21 amino acid residues), *Brackets*N-terminal region (81 amino acid residues) and C-terminal region (42 amino acid residues), + same amino acid residues in both 1Bx7 and II2a, – deleted peptide in 1Bx7

frequency (about 10%) were much lower than in the somatic hybrid strains (Zhao et al. 2003). Although these genes have not been sequenced completely, it is suggested that most of them originated from allelic variation of Null, 1Bx7 + 1By9, and 1Dx2 + 1Dy12 of Jinan 177, based on this experiment. Heritable allelic variation of HMW-GSs has been verified in the II-12 F_3–F_7 self-bred line (Z. Liu et al., personal communication). In the process of hybridization, many factors were involved in the genome variation and resulted in unequal crossover of homologous genes—for example, somaclonal variation in the parent and hybrid tissue culture, isolation and regeneration of cell wall of the protoplasts and fusion cells, UV treatment to the donor protoplast, the interaction and exclusion of parent genome, and the genetic unbalance, etc. We conclude that asymmetric somatic hybridization could not only introduce alien nuclear/cytoplasm genes (Wang et al. 2003) but also lead to allelic variation of HMW-GSs with high frequency. This technology can provide many novel strains for the quality improvement of wheat and for the study of allele-variation rule of HMW-GS genes.

We can deduce that this ''mutation'' likely derived from unequal crossover or slip mismatching during meiosis in homologous chromosomes and/or unequal crossing-over during mitosis in sister chromosomes. It was suggested that the evolutionary origin of HMW-GSs involved a same ancestor (Lawrence and Sheppherd 1981; Thompson et al. 1983; Anderson and Greene 1989; Shewry et al. 1995). Many kinds of HMW-GSs and combinations in the asymmetric somatic hybrid lines of wheat with *A. elongatum* (Zhao et al. 2003) likely reflect evolution process of HMW-GSs—a compressed ''episode'' of evolution from a single ancestral gene.

Biophysical studies have shown that the individual subunits have an extended rod-like shape, resulting from the central repetitive domain, which forms a loose spiral structure (Miles et al. 1991). The interactions (disulfide

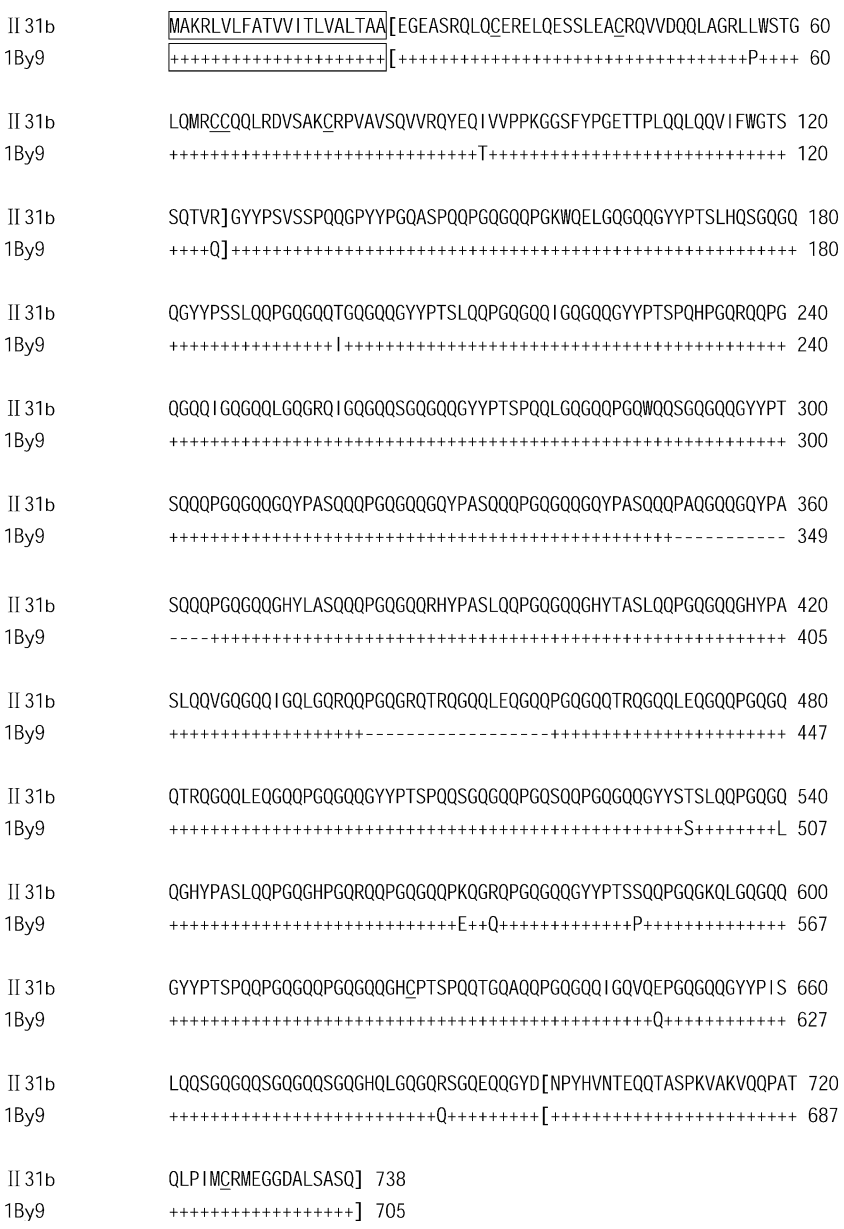

Fig. 7 Comparison of the deduced amino acid sequences of *Glu-1By9* from common wheat and *II31b* gene from the hybrid II-12. *Underlining* indicates the cysteine residues, *boxes* contain signal peptides, *Brackets* N-terminal region (104 amino acid residues) and C-terminal region (42 amino acid residues), + same amino acid residues in 1By9 and II31b; − deleted peptide in 1By9

Table 3 Comparison of derived N-terminal amino acid sequences of *II2a*, *II31b*, HMW-GSs h1Bx and h1By from hybrid wheat II-12, and 1Bx13 and 1By16 from bread wheat

Subunit	Residues	Reference
*h1Bx	EGEASGQLQCEREL	This paper
*1Bx13	EGEASGQLQCERELEACQQVVDQQLRDVSPGCRXIXVS	(Tatham et al. 1991)
**II2a	EGEASGQLQCERELEACQQVVDQQLRDVSPGCRPITVS	This paper
*h1By	EGEASRQLQCERELQ	This paper
*1By16	EGEASRQLQCERELQESSLEACRQVVDQQLAGRLPWSTGLQMRCCXXL	(Tatham et al. 1991)
**II31b	EGEASRQLQCERELQESSLEACRQVVDQQLAGRLLWSTGLQMRCCQQL	This paper

*, N-terminal residues of directly determined protein sequence; **, Deduced from nucleotide sequence.

cross-linking and hydrogen bonding) between subunits contribute to flour elastic property (Shewry et al. 1992). An extra cysteine residue (Cys^{97}) at the beginning of the repetitive domain of 1Dx5 subunit had a positive influence on dough properties (Lafiandra et al. 1993), whereas 1Bx20 has a detrimental effect on dough strength when it was compared with subunit 1Bx7. The major difference between them was the substitution of two cysteine residues in the N-terminal domain of subunit 1Bx20 by two tyrosine residues (Shewry et al. 2003). The impact of additional cysteine residue (Cys^{271}) in the repetitive domain of h1Bx subunit (Fig. 6) on dough quality is needed to study.

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Bryan JS (1997) Protein sequencing protocols: methods in molecular biology, vol 64. Humana, Totowa

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