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Molecular Characterization of a Novel HMW Glutenin Subunit Dx2.3*^{t} from *Aegilops tauschii*

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High molecular weight (HMW) glutenin subunits are important seed storage proteins in wheat and its related species. Novel HMW glutenin subunits in *Aegilops tauschii* accession of TA2484 were detected and characterized. SDS-PAGE analysis revealed the y-type subunit from TA2484 displayed similar electrophoretic mobility compared to that of 1Dy12 subunit. However, the electrophoretic mobility of x-type subunit was faster than that of 1Dx2 subunit. The primary structure of the two cloned subunits from TA2484 was similar to that of the x- and y-type subunits reported before. However, the 148 residues of the x-type subunit, which contained the sequence element GHCPTSLQQ, in the middle of the repetitive domain was quite different from other x-type subunits. Moreover, the 68 residues in this region were identical to those of the y-type subunits from the same accession. Consequently, 1Dx2.3*^{t} (x-type subunit of TA2484) contains an extra cystenin residue located at the repetitive domain, which is novel compared to the x-type subunits reported so far. Phylogenetic analysis indicated that two subunits from accession TA2484 were in the x- and y-type subunit cluster, but bootstrapping value of 100% gave high support for the spilt between two subunits (1Dx2.3*^{t} and 1Dy12.3*^{t}) and their alleles, respectively. A hypothesis on the genetic mechanism generating this novel sequence of 1Dx2.3*^{t} subunit is suggested.

Keywords: *Aegilops tauschii,* HMW glutenin subunit, 1Dx2.3*^{t}, phylogenetic analysis

Introduction

Wheat is one of the most important crop species in the world, with the highest annual yield worldwide. The seed storage proteins, especially the glutenins consist of high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits, are the main part of the endosperm (Wrigley 1996). Although the HMW glutenin subunits composed of 8–10% of the total extractable flour proteins, they play a crucial role in flour processing quality by network formation in dough by glutenin polymerization (Shewry et al. 1992). Due to this character, it allows wheat flour to be processed into bread, paste, noodles and other food products (Shewry et al. 1992; Shewry and Halford 2002; Ma et al. 2005).

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The structure, expression and function of HMW glutenin subunits have been intensively studied in common wheat and related species (Shewry et al. 2003). In hexaploid wheat, HMW glutenin subunits are encoded by the x and y genes localized on the *Glu-A1, Glu-B1* and *Glu-D1* loci (Payne 1987; Shewry et al. 2003). Three loci can encode up to six glutenin subunits in common wheat. However, usually three to five subunits were found in common wheat due to the silencing and allelic variation in these subunits. The x- and y-type subunits share a high similar primary structure, which contains a signal peptide, a N-terminal domain, a central repetitive domain and a C-terminal domain (Gupta and MacRitchie 1994; Shewry et al. 1995). The cystenin residues are important for the higher structure and functionality due to they can form disulphide bonds within and between subunits, which can shape the elastic properties of the gluten complex in dough (Shewry et al. 1995; Mackie et al. 1996). In x-type subunits, usually four conserved cystenin residues were detected (three in the N-terminal domain and one in the C-terminal domain), and mainly y-type subunits characterized seven conserved cystenin residues (five in the N-terminal domain, one in the repetitive domain and one in the C-terminal domain). In both x- and y-type subunits, short and repetitive peptide (tripeptide, hexapeptide and nanopeptide) were found, with tripeptide only detected in x-type subunits (Shewry et al. 1995; Sun et al. 2004).

Although different HMW glutenin subunits share the well-conserved primary structure, novel variations were found. Mostly, the changes in the number of cystenin residues, which can affect the bread-making quality, were commonly discussed. For example, an extra cystenin was detected in the N-terminal part of the repetitive domain of 1Dx5 subunit and this subunit has been frequently associated with improved processing quality in wheat varieties (Lafiandra et al. 1993). 1S^{s}x490771 contains an extra cystenin residue located at the C-terminal part of its repetitive domain, which is novel compared to x-type subunits reported before (Sun et al. 2006). Although the size of N- and C-terminal domains is highly conserved in either x- and y-type subunits, the repetitive domain size is usually variable. For instance, the repetitive domain of 1Dx2.2* from 1Ux subunit from *Aegilops umbellulata* is much larger than those of other reported x-type subunits (Liu et al. 2003). Analysis indicates that the extra section in 1Dx2.2* contains 111 amino acid residues (Liu et al. 2003). This large size of 1Dx2.2* repetitive domain has been suggested by the insertion or internally duplication (D'Ovidio et al. 1996; Wan et al. 2005).

Aegilops tauschii (2n = 2x = 14, DD), generally accepted as the D-genome donor of the hexaploid wheat, has been used as a genetic resource of wheat improvement (Dvorak et al. 1998; Wan et al. 2002; Yan et al. 2003; Liu et al. 2010). Extensive allelic variations of HMW glutenin subunits have been detected and previous researches have indicated that HMW glutenin subunits from *Aegilops tauschii* have a significant influence on the processing quality of the synthetic hexaploid wheat (Belton 1999; Wieser et al. 2003; Yan et al. 2003, 2004; Zhang et al. 2006). In the present work, the electrophoretic and molecular analyses of HMW glutenin subunits from *Ae. tauschii* accession were studied. The complete open reading frames (ORFs) of the x and y genes were characterized. Comparative analysis of the deduced amino acid sequences of the cloned subunits with previously

reported subunits suggests the discovery of a novel x-type subunit from TA2484 that has not been reported in wheat and other *Triticeae* species.

Materials and Methods

Plant materials and SDS-PAGE experiments

Ae. tauschii accession TA2484 was used in this study. General conditions for extracting and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of HMW glutenin subunits were based on the protocol of Ren et al. (2008). For comparing the electrophoretic mobility of the HMW glutenin subunits, the HMW glutenin subunits from the bread wheat varieties Chinese Spring (having the HMW glutenin subunits 1Bx7+1By8, 1Dx2+1Dy12) and Chuanyu 12 (1Ax1, 1Bx7+1By8, 1Dx5+Dy10) were used as controls.

Cloning and sequencing Ae. tauschii *HMW glutenin gene ORFs*

Genomic DNA was extracted from TA2484 using the CTAB method as described by Cloutier et al. (2001). For amplifying the complete ORFs of the HMW glutenin genes in genomic PCR reactions, two degenerate primers, P1 (5'-ATGGTCAAGCGG/TTA/GGTCCTCTTTG-3') and P2 (5'-CTATCACTGGCTG/AGCCGACAATGCG-3'), were used (synthesized by Invitrogen, Shanghai, China). P1 contains the start codon of the genomic ORF and P2 contains the two tandem stop codons which present in HMW glutenin genes. The expected amplified amplicons were ~2 kb.

Genomic PCR reactions were conducted using the high fidelity DNA polymerase EX-Taq (TaKaRa). PCR was carried out on the Mastercycler thermal cycler (Eppendorf). A total volume of 50 µl reaction mix containing 100 ng of DNA, 25 µl of 2× Buffer II (including $MgCl_2$), 0.5 mM of each dNTPs, 0.5 µM of forward and reverse primers, and 2 units of EX-Taq polymerase. The PCR conditions were one cycle of 95ºC for 5 min, 32 cycles of 95ºC for 1 min, 68ºC for 3 min, followed by a final extension of 72ºC for 10 min.

The PCR products were fractionated using 1% agarose gels, and the fragments showing the anticipated size were cloned into the pGEM-T vector (Promega). Based on DNA sequencing, the inserts in the two plasmid clones p1Dx2.3*^{t} and p1Dy12.3*^{t} were found to contain the complete ORFs for the x- and y-type subunits of accession TA2484, respectively. Each product were sequenced at least three clones.

Deduced amino acid sequences comparison and phylogenetic analysis

The HMW glutenin gene ORFs from TA2484 were translated into amino acid sequences. Multiple alignment and sequence analysis of deduced amino acid sequences and those of previously published HMW glutenin subunits were conducted on BioEdit 7.0 software. For phylogenetic analysis using the amino acid sequence of the signal peptide plus the N-terminal domain, the MEGA 5 program was employed. During analysis, the complete deletion option was used to deal with gaps in the aligned sequences, and the PC distance was calculated for each pair of the aligned sequences. The bootstrap values were obtained on 1000 replications. The HMW glutenin subunits used in analysis were AF480485

(1Dx2t), X03346 (1Dx2), AB481100 (1Dx2.8), AB359016 (1Dx2.6), AJ893508 (1Dx2.2*), JX112756 (1Dx2.2), AF480486 (1Dx2.1t), AY517724 (1Dx2.1), AF497474 (Dtx2), X03041 (1Dy12), AY248704 (1Dy12.1), DQ307385 (1Dy12.2t), DQ681078 (1Dy12.1*t), EU266533 (1Dy12*), FJ226583 (1Dy12.2*), JQ293093 (1Dy12.1**t). The D-hordein protein from barley (GeneBank accession number Ay268139) was used as a control outgroup. The ORF sequences of 1Dx2.3*^{t}, 1Dy12.3*^{t} determined in this study were submitted to GeneBank with the accession numbers KC196062, KC196064, respectively.

Results

Composition of HMW glutenin subunits in TA2484

Seed protein extracts were prepared from TA2484 and the common wheat Chinese Spring and Chuanyu 12. Based on SDS-PAGE, two subunits (Fig. 1A, indicated by arrows and arrowheads, respectively) were detected. By comparison with the electrophoretic patterns of the HMW glutenin subunits, the subunits showing faster mobility (marked by arrows) were likely to be y-type subunits, whereas those displaying slower mobility were probably of x-type subunits (marked by arrowheads) (Fig. 1A). Furthermore, the mobility of x-type subunits was faster than that of 1Dx2. The x-type HMW glutenin subunits of TA2484 was named as 1Dx2.3*^{t}. The mobility of y-type subunits was similar to that of 1Dy12. So we designated y-type HMW glutenin subunits of TA2484 as 1Dy12.3*^{t}.

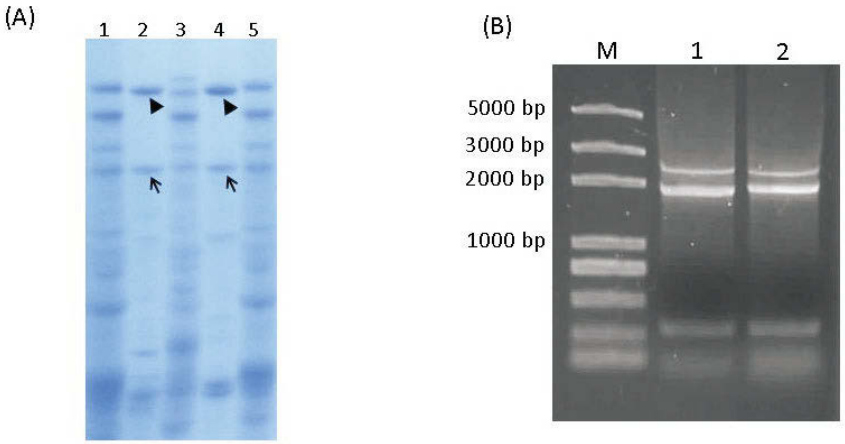

Figure 1. Identification of HMW glutenin subunits from TA2484. (A) SDS-PAGE analysis of HMW glutenin subunits in seed extract of the *Ae. tauschii* accession TA2484 (line 2). The Chinese Spring (1Dx2, 1Bx7, 1By8, 1Dy12, line 1) and Chuanyu 12 (1Ax1, 1Dx5, 1Bx7, 1By8, 1Dy10, line 3) were used as controls. Proteins with faster electrophoretic mobility were y-type subunits (indicated by arrows), while proteins with lower electrophoretic mobility were x-type subunits (indicated by arrowheads). (B) Two PCR fragments (representing complete ORFs sequences for the x- and y-type subunits) were amplified from genomic DNA samples of TA2484 (line 2). The DNA size markers were contained in line 1

Molecular characterization of HMW glutenin gene ORFs and derived amino acid sequences

The degenerate primers P1+P2 were used to amplify the complete ORFs of the two subunits in TA2484 (1Dx2.3*t and 1Dy12.3*t) (Fig. 1B). The two fragments were cloned and sequenced. They were characterized as typical HMW glutenin genes, including both the start and stop codons (two stop codons). No introns were detected, which is a general feature of HMW glutenin gene ORFs (Shewry et al. 2003). 1Dx2.3*t and 1Dy12.3*t consisted 818 and 597 amino acid residues, respectively.

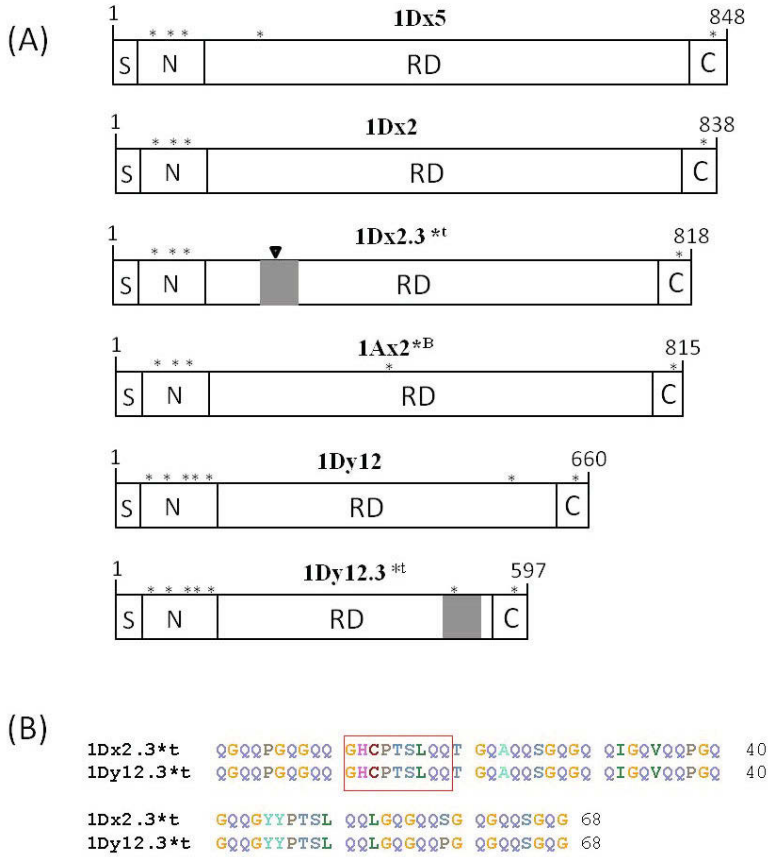

Figure 2. Analysis of the primary structure of two cloned *Ae. tauschii* subunits with reported subunits. (A) The primary structure of cloned subunits were identical to that of reported x- and y-type subunits from wheat, which contain a signal peptide (S), a N-terminal domain (N), a repetitive domain (RD) and a C-terminal domain (C). The conserved cysteine residues were indicated by asterisks. The identical region shared by 1Dx2.3*t and 1Dy12.3*t were represented by gray area. Consequently, the extra cysteine residue in the repetitive domain of 1Dx2.3*t was indicated by an arrowhead. (B) A comparison of the 68 residues of 1Dx2.3*t and 1Dy12.3*t. The extra cysteine residue was located in the nanopeptide GHCPTSLQQ (boxed region)

Table 1. The properties of the primary structure of 1Dx2.3*^{t} and 1Dy12.3*^{t} subunits in comparison with other HMW glutenin subunits

Subunit	Number of amino acid residues				Number of cysteine residues			
	N-terminal domain	Repetitive domain	C-terminal domain	Total	N-terminal domain	Repetitive domain	C-terminal domain	Total
1Dx5	89	687	42	818	3	1	1	5
1Dx2	88	687	42	817	3	0	1	4
1Dx2.3*^{t}	89	666	42	797	3	1	1	5
1Dy10	104	481	42	627	5	1	1	7
1Dy12	104	493	42	639	5	1	1	7
1Dy12.3*^{t}	104	430	42	576	5	1	1	7

Table 2. Repetitive peptide motifs in the repetitive domains of two subunits characterized in this study

Subunit	Number of tripeptide	Number of hexapeptide	Number of nanopeptide
1Dx2.3*^{t}	18	64	17
1Dy12.3*^{t}	0	43	17

Comparison of the amino acid sequences derived from the ORFs indicated that two subunits shared a primary structure identical to that of x- and y-type subunits, which contain a signal peptide, a N-terminal domain, a central repetitive domain and a C-terminal domain (Fig. 2A; Table 1; Fig. S1*; Fig. S2). The size of N- and C-terminal domains in the two subunits was identical to that of reported x- and y-type subunits (Table 1; Fig. S1). An extra cysteine residue was detected in the repetitive domain of 1Dx2.3*^{t}. This cysteine was located in the sequence element GHCPTSLQQ, which is not present in any of the x-type subunits characterized before (Fig. S1) (Shewry et al. 2003). Further examination revealed that the 148 residues (from residues 241 to 388) in the repetitive domain were quite different from that of other x-type subunits reported so far.

Meanwhile, the 68 residues (from residues 229 to 297) were identical to those of 1Dy12.3*^{t} (Fig. 2B). Moreover, the nucleotide sequences encoding this region of the two subunits were also 99% identical (data not show). Compared to 1Dx2, 1Dx2.3*^{t} contains seven deletions in the repetitive domain and two insertions (one in the N-terminal domain and one in the repetitive domain). Comparison of 1Dy12.3*^{t} to 1Dy12 indicates that three deletions are present (Fig. S2). As their orthologs, the repetitive domains in the two subunits were composed of repetitive motifs (Table 2). The size of the repetitive domains in the two subunits were smaller to their orthologs, which was caused by less number of repetitive amino acid motifs forming the subunits (Table 2).

Phylogenetic analysis of two glutenin subunits

For constructing the phylogenetic tree, eight x-type (1Dx2 and its allelic variants) and seven y-type (1Dy12 and its allelic variants) HMW glutenin subunits, as well as the two subunits identified in this study were analyzed. The tree obtained by neighbor joining pro-

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

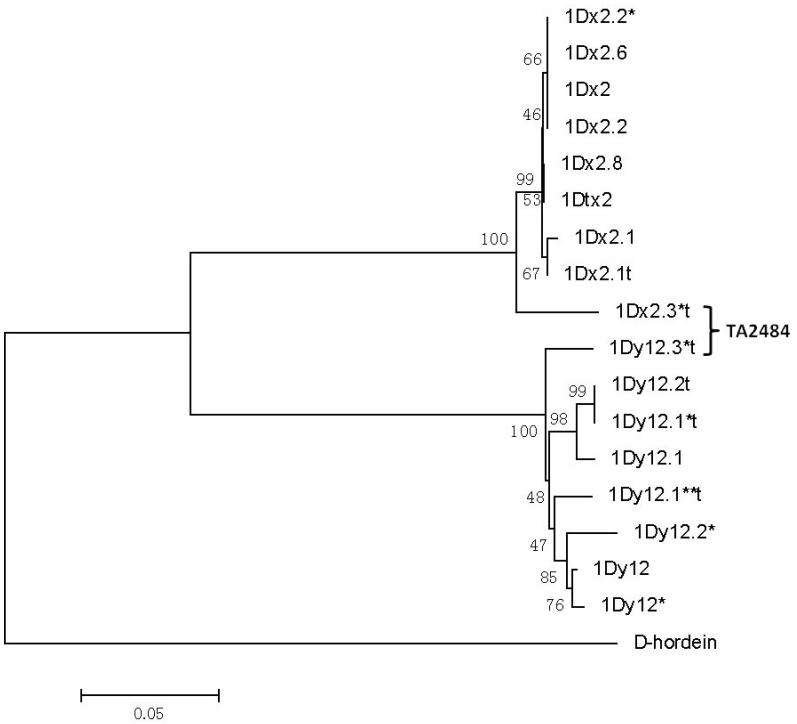

Figure 3. Phylogenetic analysis of two cloned subunits (1Dx2.3*t and 1Dy12.3*t) with reported 1Dx2 and 1Dy12 subunits and their allelic variants. The phylogenetic tree was built by neighbor joining method. The bootstrap values were obtained using 1000 replications

gram using P distance and pairwise deletion is presented (Fig. 3). As expected, these glutenin subunits divided in two clades, with x- and y-type subunits in each clade. Interestingly, although 1Dx2.3*t and 1Dy12.3*t were in two clades, they were not closely clustered into their orthologs. Within each clade, a bootstrapping value of 100% gave high support for the spilt between 1Dx2.3*t and the other alleles. The same result was found in 1Dy12.3*t (Fig. 3). For integrate view, 1Dx2.3*t and 1Dy12.3*t were most closet to each other, which has not been reported before.

Discussion

Owing to the important role in determining the flour processing properties of gluten complex and their high conservation in wheat and related species, continuous mining of novel HMW glutenin subunits from cultivars and germplasms not only improve wheat end use quality but also clarifying the evolution of these proteins. Although previously results had indicated that *Ae. tauschii* is a rich resource for detecting novel HMW glutenin subunits, characterization the primary structure at the molecular level remains not enough. In this

study, the expression and primary structure of the HMW glutenin subunits from *Ae. tauschii* were investigated using SDS-PAGE and molecular cloning approach.

The x-type subunit from the *Ae. tauschii* accession TA2484 is unusual because the 148 residues (from residues 241 to 388) in the repetitive domain were quite different from those of other x-type subunits reported so far. Meanwhile the 68 residues (from residues 229 to 297) were identical to the corresponding residues of the y-type subunit from the same accession. Theses variations may not be detected when the subunits from diverse accessions were compared in SDS-PAGE. However, the molecular investigations can uncover this using cloning the complete ORFs and comparison of deduced amino acid sequences.

What might be the mechanism underlying the occurrence and evolution of the novel x-type subunit in the *Ae. tauschii* accession TA2484? Previously, Anderson and Greene (1989) suggested the following modes may results in sequence alteration: (i) single base and single repeat; (ii) deletions or additions with a repeat; (iii) deletions or duplications of blocks of repeats, with possible unequal crossover and slip-mismatching. The unequal crossover was detected in *Aegilops searsii* accession IG49077, which results into the recombinant of *Glu-1* loci (Sun et al. 2006). Based on the result that the 68 residues in the repetitive domain of 1Dx2.3*^{t} were identical to those of the y-type subunits, we suggest that unequal crossing over between the two *Glu-1* loci on the sister chromatids of the homologous chromosomes during meiosis might have resulted into the formation of this novel hybrid ORF (Fig. 4). The occurrence of this hybrid subunit might be recently because the nucleotide sequences encoding the 68 amino acid residues in the x- and y-type subunits in TA2484 were 99% identical. Meanwhile, the mode we suggested also predicts that there would be a hybrid ORF encoded by a y-type subunit with its repetitive domain end identical to the corresponding region of x-type subunit.

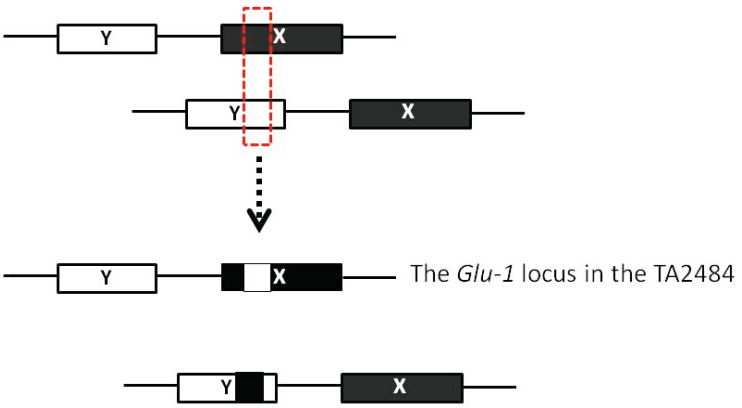

Figure 4. A hypothesis on the mechanism that might generate the ORF for the 1Dx2.3*^{t} in the *Ae. tauschii* accession TA2484. Unequal crossing over may occur between the *Glu-1* loci located on the sister chromatids of the homologous chromosomes. As a result, a double crossing over happened between x- and y-type subunits, which generated two hypothetical recombinant *Glu-1* loci (one of it was the *Glu-1* locus found in the *Ae. tauschii* accession TA2484)

Previously, the presence of extra cystenin residue in the repetitive domain was found in 1Dx5 and 1Ax2*^{B} subunits, which has been associated with the superior bread-making quality (Ciaffi et al. 1993; Lafiandra et al. 1993; Juhász et al. 2003; Feng et al. 2011). Compared to 1Dx2, a serine changed to cysteine residue at the front of the repetitive domain of 1Dx5. While, for 1Ax2*^{B}, the extra serine was occurred in the middle part of the repetitive domain compared to 1Ax2*. Both these serine residues from two subunits were generated by nucleotide exchange. Meanwhile, the extra cysteine residue of 1Dx2.3*^{t} was at the front of the repetitive domain and may generated by hybridization.

In this study, 1Dx2.3*^{t} contained an extra cysteine residue in the repetitive domain was characterized. Consequently, it will be important to find out if 1Dx2.3*^{t} could confer improved dough property to wheat flour when this subunit been expressed in bread wheat cultivars. For this purpose, the plant expression construct using the sequence of 1Dx2.3*^{t} subunit has been prepared, and the function of this subunit will be clarified using transgenic wheat lines.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Figure S1.* Multiple alignments of deduced amino acid sequences of 1Dx2 and 1Dx2.3*^{t}. The extra cystenin residue in 1Dx2.3*^{t} is marked by red star

Electronic Supplementary *Figure S2.* Multiple alignments of deduced amino acid sequences of 1Dy12 and 1Dy12.3*^{t}