Characterization and comparative analysis of HMW glutenin IAy alleles with differential expressions

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Abstract

Background: High-molecular-weight glutenin subunits (HMW-GSs) have been considered as most important seed storage proteins for wheat flour quality. IAy subunits are of great interest because they are always silent in common wheat. The presence of expressed IAy subunits in diploid and tetraploid wheat genotypes makes it possible to investigate molecular information of active IAy genes.

Results: We identified IAy subunits with different electrophoretic mobility from 141 accessions of diploid and tetraploid wheats, and obtained the complete ORFs and 5' flanking sequences of IAy genes including 6 active and 3 inactive ones. Furthermore, the 5' flanking sequences were characterized from 23 wild diploid species of Triticeae. All 6 active IAy possess a typical HMW-GS primary structure and some novel characteristics. The conserved cysteine residue within the repetitive domain of y-type subunits was replaced by phenylalanine residue in subunits of IAy (Tu-e1), IAy (Tu-e2), IAy (Ta-e2) and IAy (Td-e). Particularly, IAy (Ta-e3) has an unusual large molecular weight of 2202 bp and was one of the known largest y-type HMW-GSs. The translations of IAy (Tu-s), IAy (Ta-s) and IAy (Td-s) were disrupted by premature stop codons in their coding regions. The 5' flanking sequences of active and inactive IAy genes differ in a few base substitutions and insertions or deletions. The 85 bp deletions have been found in promoter regions of all IAy genes and the corresponding positions of 6 species from Aegilops and Hordeum.

Conclusion: The possession of larger molecular weight and fewer conserved cysteine residues are unique structural features of IAy genes; it would be interested to express them in bread wheat and further to examine their impact to processing quality of wheat. The IAy genes from T. urartu are closer to the genes from T. turgidum dicoccon and T. aestivum, than those from T. monococcum aegilopoides. The 85 bp deletion and some variations in the 5'flanking region, have not interrupted expression of IAy genes, whereas the defects in the coding regions could be responsible to the silence of the IAy genes. Some mutational events in more distant distal promoter regions are also possible causes for the inactivation of IAy genes.

Published: 6 February 2009

BMC Plant Biology 2009, 9:16 doi:10.1186/1471-2229-9-16

This article is available from: http://www.biomedcentral.com/1471-2229/9/16

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Background
In wheat and its relatives, seed storage proteins are mainly composed of glutenins and gliadins [1]. High-molecular-weight gluten subunits (HMW-GSs) are important storage proteins in endosperm of wheat and its related species [1]. HMW-GSs play a key role in determining wheat gluten and dough elasticity which promote the formation of the larger glutenin polymer [2,3]. The allelic variation in HMW-GS compositions has been reported to account for up to 70% of the variation in bread making quality among European wheats, even though they only account for about 10% of seed storage proteins [2,4]. Therefore, HMW-GS genes are important and useful in molecular modification to improve the wheat grain quality.

HMW-GSs are encoded by the Glu-1 loci on the long arms of chromosomes 1A, 1B and 1D, and each locus consists of 2 tightly linked genes encoding an x-type and a y-type subunit, respectively. Theoretically, hexaploid wheat could contain 6 different HMW-GSs, however, gene silence resulted in variation of HMW-GS number: from 3 to 5 subunits in hexaploid wheat [5,6]. Among all 6 HMW-GSs, 1Dx, 1Dy and 1Bx are always active, whereas 1Ax and 1By sometimes appear silent. In hexaploid wheat, the gene encoding 1Ay subunit is always silent. However, 1Ay subunits have been reported in some diploid and tetraploid wheats [7]. Although the expressed 1Ay subunits in 2 accessions of wheat have been reported [8,9], such subunits have never been confirmed by further molecular characterization. To date, more than 20 HMW-GS alleles have been isolated from wheat and its related species [10-30], and these information has greatly improved our understanding in structure, heredity and expression of HMW-GSs. However, our knowledge on 1Ay genes is still deficient. The expression of 1Ay subunits in some wild diploid and tetraploid wheats offers an opportunity to isolate and analyze nucleotide sequences of active HMW glutenin 1Ay genes [7,19,31,32].

Triticum urartu (AA, 2n = 14), Triticum monococcum aegilopoides (AA, 2n = 14) and Triticum turgidum dicoccon (AABB, 2n = 28) are important species possibly involved in the evolution process of hexaploid wheat. These species possess many excellent characteristics such as high content of seed protein and high resistance to stripe rust, scab and stress, which could be potentially employed to improve the agronomic traits of common wheat [33].

In this study, we reported the identification of expressed 1Ay subunits from total 141 accessions of T. urartu, T. monococcum aegilopoides and T. turgidum dicoccon, and the characterization of the coding and promoter region sequences of 6 active and 3 inactive 1Ay genes. To further understand the control of this allele expression, we also characterized the 5' flanking sequences of y-type HMW-GS genes from 23 wild diploid species of Triticeae. The objectives of this study are: 1) to compare promoter and coding region structures of active and inactive 1Ay alleles, and further to understand the control of 1Ay gene expression; 2) to compare the primary structure of 1Ay subunits with other known HMW-GSs and analysis the evolution of Glu-A1-2 alleles; 3) to provide the basis of the genetic transformation of active 1Ay gene to verify their effect on wheat processing quality.

Results
SDS-PAGE profiles of HMW-GSs
The SDS-PAGE profiles of HMW-GSs showed that 1Ay subunits were differentially expressed in T. urartu, T. monococcum aegilopoides and T. turgidum dicoccon, whereas 1Ax subunits were expressed in all accessions of these 3 species (Figure 1). In T. urartu and T. turgidum dicoccon, 1Ay subunits displayed an electrophoretic mobility similar to that of 1Dy12 subunit. 1Ay subunits from T. monococcum aegilopoides migrated slower than those of T. urartu, showing a similar electrophoretic mobility with 1By8. Interestingly, 1Ay subunit in one accession [PI306526] of T. monococcum aegilopoides migrated slower than all y-type subunits and 1Bx7. To our knowledge, the y-type HMW-GS with such slower electrophoretic mobility has never been reported, indicating that this subunit might possess a molecular mass larger than other y-type subunits. We also found that for the expression frequency of 1Ay subunits, diploid wheats are higher than tetraploid wheats (Additional file 1).

Characterization of 1Ay coding sequences from diploid and tetraploid wheats
In genomic PCR, there is only one amplified fragment in each of T.urartu and T.monococcum aegilopoides, whereas 4 fragments were amplified in 2 T.turgidum dicoccon accessions. The amplified fragments in T. urartu and T. monococcum aegilopoides ranged from1800 to 2202 bp (Figure 2). It is close to the size of those typical y-type HMW-GS genes except for the fragment of 2202 bp. In T.turgidum dicoccon accessions, the molecular weight of fragments is between 1.8 and 2.5 kb (Figure 2). All amplified products were cloned. By terminal sequencing and enzyme digestions, the ORFs representing different 1Ay alleles were determined. The full length sequences of 1Ay ORFs were obtained by using the method of nested deletion. The 9 sequences were named as 1Ay (Tu-e1), 1Ay (Tu-e2) and 1Ay (Tu-s) to represent the ORFs of 1Ay subunits from T. urartu,1Ay (Ta-e1), 1Ay (Ta-e2) and 1Ay (Ta-s) to represent the ORFs of 1Ay subunits from T. monococcum aegilopoides; and 1Ay (Td-e) and 1Ay (Td-s) to represent the ORFs of 1Ay subunits from T.turgidum dicoccon (the letter e and s represent the expressed and silenced subunits, the numbers represent different alleles.). All sequences were deposited in NCBI database with Genbank accession numbers from:EU984503 to EU984511.

The primary structures of deduced 1Ay proteins
After translating the DNA into protein sequences, analysis of amino acid sequence indicated that the ORFs of 6 active
1Ay genes possess a typical primary structure shared by other published HMW-GSs, although these subunits differ greatly in sizes (Figure 3 and Table 1). Each of these deduced subunits consists of a signal peptide with 21 amino acids (aa), a conserved N-terminal region, a central repetitive domain and a C-terminal region. The N-terminal regions of these 6 subunits contain 104 aa and the C-terminal regions have 42 aa. Central repetitive domains of these subunits are composed of a similar repeat structure to other known y-type subunits. The subunit 1Ay (Ta-e3) is composed of 732 aa, larger than all other known y-type HMW-GSs. The difference between 1Ay (Ta-e3) and other y-type HMW-GSs were entirely due to variations of the number of repeat motifs. Compared to other 1Ay subunits, 13 extra hexapeptides and 5 extra nonapeptides have been inserted into the repetitive domain of 1Ay (Ta-e3), which resulted in 123 aa increases in its molecular mass.

All conserved cysteine residues presented in known HMW-GSs from wheat and its relative grasses were observed in the aa sequences of 1Ay (Ta-e1) and 1Ay (Ta-e3). For 1Ay (Ta-e1) and 1Ay (Ta-e3), the distributions of the 7 cysteine residues are conserved with 5 in N-terminal region, 1 at the end of central repetitive domain and 1 in C-terminal region. However, the conserved cysteine residues at the end of the central repetitive domain of 1Ay (Tu-e1), 1Ay (Tu-e2), 1Ay (Ta-e2) and 1Ay (Td-e) was replaced by phenylalanine residues (Figure 3, Table 1). The translation of the sequence of 1Ay (Tu-s) and 1Ay (Td-s) were disrupted by in-frame premature stop codons (Figure 3). In the coding sequences of 1Ay (Tu-s) and 1Ay (Ta-s), there is 1 stop codon located in the N-terminal and C-terminal region, respectively; and 4 stop codons were located in the repetitive domain of 1Ay (Td-s). If the premature stop codons were ignored, the resulted SDS-PAGE analysis of high-molecular-weight glutenin subunits (HMW-GSs) of diploid and tetraploid wheat species. a Diploid accessions of T. urartu (Tu1) PI428309, (Tu2) PI 428308, (Tu3) PI 428318, (Tu4) PI 428310; b, c: Diploid accessions of T. monococcum aegilopoides: (Ta1) PL 4277928, (Ta2) PI 427759, (Ta3) PI 428007, (Ta4) PI 427622, (Ta5–6) Citr 17665, (Ta7–8) PL 277123, (Ta9–10) PL 306526; d: Tetraploid wheat accessions of T. durum dicoccum: (Td1–2) PL 355475, (Td3–4) PL 355477; CS: Chinese spring. The SDS-PAGE profiles of HWM-GSs showed 1Ay subunits were differentially expressed in some accessions of T. urartu, T. monococcum aegilopoides and T. durum dicoccum while 1Ax subunits were expressed in all accessions (marked by tailed-arrows). The expressed 1Ay subunits were marked by solid and the hollow arrows indicated the area where the absent subunit band might have been.

Figure 1

PCR amplification of HMW-GS ORFs. Lane 1–3: PL 428309, PI 428318, PL 428308 (T. urartu); lane 4–7: PI 428007, PL 277123, PI 306526, PL 4277928 (T. monococcum aegilopoides) and lane 8 and 9: PI 355475, PI 355477 (T. durum dicoccum); M is 1 Kb DNA ladder.
peptides of 1Ay (Tu-s), 1Ay (Ta-s) and 1Ay (Td-s) would also have typical characteristics of HMW-GSs.

**Structural features of the 5’ flanking promoter regions of Glu-A1-2 alleles and those in 23 Triticeae species**

The 5’ flanking promoter regions of both active and inactive 1Ay from diploid and tetraploid wheat species were amplified using the primers P3 and P4. In previous study, regulatory elements (TATA box, complete HMW enhancer, partial HMW enhancer, E motif and N motif) have been identified in the study of promoter activity in wheat endosperm [34,35]. D’Ovidio (1996) previously reported the sequence locations of 5’ flanking promoter regions of 1Ay alleles in *T. urartu* to the positions -595 bp upstream of translational start codon. In this study, we extended the sequences to the positions -845 bp to cover all recognized elements mentioned above. It’s more scientific to carry out the promoter comparison using the sequences including all recognized elements. Although comparative analysis of promoter could not directly decide difference in function, it would useful in identification of regulatory elements variations which are relevant to gene function and evolution.

All characterized promoter regions of 1Ay were aligned to the homologous regions of 1Ay (Cheyenne) (from common wheat cv. Cheyenne), 1By9 and 1Dy10. The 5’ flanking promoter regions of both active and inactive 1Ay from *T. urartu*, *T. monococcum aegilopoides*, *T. turgidum dicoccon* and *T. aestivum* were compared. A few base substitutions and insertions or deletions were found even though the alignment showed high similarity (Figure 4). The N motif, E motif, complete enhancer and TATA box were well conserved in all compared alleles. An 85 bp deletion, in which the partial HMW enhancer was also included, was observed in the 5’ flanking promoter regions of all 1Ay genes from diploid, tetraploid and hexaploid wheats when compared to 1By9 and 1Dy10 (Figure 4). Our investigation in the region extended to -845 bp did not find any obvious basis for differential expression. The y-type HMW-GS promoter regions are conserved out to -1200 bp even though some of these genes diverged 4–5 million years ago and the non-coding sequences of wheat diverge fast. Some potential regulatory elements might be in the -845 to -1200 bp region.

In order to further understand the control of HMW-GS 1Ay gene expression, we also characterized the corresponding 5’ flanking regions from 23 diploid species of Triticeae. The length of entire 5’ flanking regions in 23 Triticeae species varied from 845 to 915 bp (GenBank: EU4233–EU4245; EU4245–EU4257). Multiple sequence alignment showed the 5’ flanking of 23 Triticeae species regions were conserved but have more variations than those of Glu-A1-2 alleles (Additional file 2). A few substitutions were found in the elements of E motif, N motif, Partial enhancer and Enhancer. Interestingly, the 85 bp deletion was also found in the corresponding regions of y-type HMW-GS and D-hordein genes from six diploid species of *Aegilops umbellulata* (U), *Ae. uniaristata* (N), *Hordeum bogdani* (H), *H. brevisubulatum* (H), *H. bulbosum* (I) and *H. spontaneum* (H) (Figure 5).

**Evolutionary analyses of Glu-A1-2 alleles**

The phylogenetic analysis was conducted to investigate the evolutionary relationships among the alleles encoded by Glu-A1-2, Glu-B1-2 and Glu-D1-2 (Figure 6). The 5’ flanking sequences plus the sequences encoding the signal peptides and N-terminal domain were chosen to construct the phylogenetic tree under several principles for the sequence selections [36]. Firstly, we found that the regulatory elements that control the tissue specificity and expression level of different HMW-GS genes are well conserved in HMW-GS alleles from 23 diploid species. Secondly, the sequences encoding signal peptides and N-terminal domain are also relative conserved. Therefore,
Comparison of the primary structure of 1Ay subunits from different wheat species. Signal peptide was underlined; N-terminal and C-terminal regions were boxed, respectively. Conserved cysteine residues were indicated by solid arrows while the substitutions of cysteine residues with phenylalanine residue (F) were marked by hollow arrows. The in-frame stop codons were represented by asterisks and boxed.
Comparison of the 5' flanking sequences of 9 Glu-A1-2 alleles characterized in this study with those of Glu-A1-2, Glu-B1-2 and Glu-D1-2, represented by 1Ay (Cheyenne), 1By9 and 1Dy10. The regulatory elements E motif, N motif, partial HMW enhancer and complete HMW enhancer were boxed and labelled, respectively. TATA box was indicated by asterisks; and translational start codon was underlined. This comparison showed that the 85-bp fragment (marked by shadow) was deleted at the 5' flanking sequences of all alleles of Glu-A1-2. The 5' flanking sequences of Glu-A1-2 alleles from wild diploid, tetraploid and hexaploid wheat species shared high degree of homology.
high conservation with enough variations suggested these HMW-GS sequences are phylogenetically informative.

The resulted phylogenetic tree was divided into 2 clusters, comprising the Glu-A1-2 alleles at the top and the alleles of Glu-B1-2 and Glu-D1-2 at the bottom. In the cluster of Glu-A1-2 alleles, 1Ay genes from each species were clustered together, respectively. The 1Ay genes have been further divided into 3 clusters. 1Ay (Tu-e1), 1Ay (Tu-e2), 1Ay (Tu-s), 1Ay (Td-e), 1Ay (Td-s) and 1Ay (Cheyenne) were included one group showing close relationship; the genes in this group are from T. urartu, T. turgidum dicoccon and T. aestivum respectively. Three genes, 1Ay (Ta-e1), 1Ay (Ta-e2) and 1Ay (Ta-s) from T. monococcum aegilopoides, were clustered together while 1Ay (Ta-e3) was put outside of this cluster. In spite all 1Ay alleles from different wheats show a close relationship, we noted 1Ay genes from T. urartu, T. turgidum dicoccon and T. aestivum which were important species involved in wheat evolution, were tightly clustered together in one group; however the 1Ay genes from T. monococcum aegilopoides exhibited a more distant relationship to the genes of this group. And this group is supported by high bootstrap values, indicating that strong statistic support for the close relationship of the Glu-A1-2 alleles from T. urartu, T. turgidum dicoccon and T. aestivum.

Discussion
The HMW-GS 1Ay subunits are special because they are always silent in hexaploid wheat. Relative fewer researches have been conducted on this allele when compared to other loci of Glu-B1 and Glu-D1 [20,32,37]. These informations are not sufficient to understand the expression and heredity of 1Ay subunits. Our investigations on 1Ay alleles with differential expressions would be useful to enhance our knowledge on Glu-A1-2 alleles.

The structure variations and evolution of Glu-A1-2 alleles

Previous genetic researches suggested that there are two tightly linked HMW-GS genes for each genome of wheat and its wild relatives. However, we have only amplified one band representing y-type HMW-GS genes in genomic PCR of T. urartu and T. monococcum aegilopoides. Bai et al [32] reported their PCR amplification for HMW-GS ORFs could not obtain x-type genes either. Liu et al. have shown the similar results in the cloning of HMW-GS genes from decaploid Agropyron elongatum [30]. Only 15 of 20 genes can be isolated; the rest of 5 x-type ones can not be obtained, and they proposed that the failure in amplification was possibly due to the x-type genes were less conserved or polymeric than y-type ones. In addition, either deletion of sequences/genes or transposon insertions can also prevent the amplification of interest fragments. Therefore, sequence polymorphisms, deletion or transpon insertions may be the reason why we could not obtain the other fragment for x-type amplicon in diploid wheats.

The possession of larger molecular mass and fewer conserved cysteine residues are unique characteristics of 1Ay subunits tested in this study. 1Ay subunits differs from each other and those of known HMW subunits by substitutions, insertions or deletions involving single or more amino acid residues (Figure 3). The repetitive
domains of 1Ay subunits possess most variations, whereas the N- and C-terminal are relatively conserved only with some substitutions of single amino acid. With a larger molecular weight than other y-type subunits, 1Ay (Ta-e3) is one of the known largest y-type HMW-GS genes. The unusual large size of 1Ay (Ta-e3) is mainly due to the insertions of repeat units in central repetitive region. Belton [38] and Feeney et al. [39] proposed a model in which the gluten polymers interact via inter-chain hydrogen bonds between the subunit repetitive domains, and more stable interactions can be formed with longer subunits. The positive relationship between the size of HMW-GSs and their effect on dough strength has been reported [40]. The 1Ay (Ta-e3) is longer than other y-type subunits, so we predicted it may have a potential ability to strengthen the gluten polymer interactions.

The y-type HMW-GSs (i.e., Glu-B1-2 and Glu-D1-2 encoded subunits) in hexaploid wheat have a cysteine residue at the end of repetitive domain. We found that this cysteine residue is not always present in wild wheats. This cysteine residue is replaced by phenylalanine since whose codons, TTT or TTC can be easily converted from cysteine codons TGT or TGC. The number and distribution of cysteine residues in HMW subunit proteins are relevant to their ability to form high molecular polymers stabilized by inter-chain disulphide bonds [41]. In previous report, the substitutions of two cysteine residues in the N-terminal of subunit 1Bx20 resulted in its negative effect on dough strength [42]. This type of cysteine composition in 1Ay subunits has never been reported before our study, it is unknown what would be the effect of the cysteine substitution within repetitive domain to their high order structures. It would be important to express 1Ay subunits in bread wheat to verify their impact to flour quality. In addition, to expressing 1Ay subunit in bread wheat cultivar with 5 native expressed subunits to construct novel transgenic plants which allowed express all 6 x- and y-type HMW-GSs would be considerable interesting.

Because relatively fewer Glu-A1y alleles were identified and characterized, the evolution of these alleles has never been reported. Prior to our study, the molecular information on gene structure of Glu-A1-2 alleles was only available for 1Ay from T. urartu and T. timopheevi [19,32]. In this study, we are able to investigate the evolution of these alleles based on the identification of novel 1Ay alleles from more diploid and tetraploid wheats. The close relationship between 1Ay from T. urartu and those from T. turgidum dicoccon and T. aestivum is supported by
phylogenetic analysis and comparison of amino acid sequences; while the 1Ay genes from *T. monococcum aegilopoides* have a little more distant relationship to those of *T. turgidum dicoccon* and *T. aestivum* (Figure 3 and 6). For the close relationship of these alleles, it may be explained that *T. urartu* is generally accepted donor species of a genome of *T. turgidum dicoccon* and *T. aestivum* [43,44].

The evolution of new allelic subunit can be formed through the variations of number and distribution of cysteine residues [36]. For example, the good quality subunit 1Dx5 is a novel subunit with an extra cysteine residue in the repetitive domain [3]. In our finding, the conserved cysteine residue at the end of the repetitive domain were all replaced in 1Ay subunits from *T. urartu, T. turgidum dicoccon* and *T. aestivum* while this cysteine residue is still in 1Ay subunits of *T. monococcum aegilopoides*. We suppose that the substitution of this cysteine residue could be relevant to the evolution of Glu-A1-2 alleles. The difference in cysteine residue together with results of phylogenetic analysis and protein sequence comparison further supported that 1Ay genes from *T. urartu, T. turgidum dicoccon* and *T. aestivum* are closed, but differed from those of *T. monococcum aegilopoides*.

**Gene silencing in Glu-A1-2**

We focused on both promoter and coding regions to understand the silence of 1Ay alleles. The development of the primers specific for the 5' flanking promoters of 1Ay genes made it possible to extend these sequences to cover all recognized elements and compare them. Our investigations in the extended 5' flanking promoter sequences identified a few base substitutions and insertions or deletions among 1Ay alleles with differential expressions. Because these substitutions and insertions or deletions are not specific to active or inactive 1Ay genes, the correlation between these variations and expression of 1Ay genes has not been supported.

The deleted fragment of 85 bp contained partial HMW enhancer which is a partial copy of complete HMW enhancer. Halford et al. proposed that the 85 bp deletion was responsible for the silencing of 1Ay gene [45], whereas Colot et al. reported later that the corresponding fragment of 1Dy12 was not essential for gene regulation [46]. Prior to our study, it has been observed only in the 5' flanking regions of 1Ay alleles from *T. urartu, cv. Cheyenne* and *cv. Chinese spring* [13,37,45]. In this study, the 85 bp deletion was also found in the corresponding regions of all 1Ay genes from *T. monococcum aegilopoides* and *T. turgidum dicoccon* (Figure 4). Further examinations in the 5' flanking sequences of Glu-1-2 alleles from 23 wild diploid wheat species revealed that the 85 bp fragment deletion was also present in 6 species of *Aegilops* and *Hordeum*. Therefore, the 85 bp deletion is not specific for inactive 1Ay genes. Anderson et al. and Li et al. reported there was a 185 bp insertion in the 5' flanking regions of 1Bx7 and 1Bx14 when compared to 1Bx17 and 1Bx20 [36,47]. They concluded that this insertion has not disrupted the expression of 1Bx14 and 1Bx20. Because the 1Bx gene of *cv. Chinese spring* has apparently higher expression than the allelic of *cv. Cheyenne* 1Bx gene, the relationship of the 1Bx promoter cereal box duplication to protein synthesis levels was examined. Since both 1Bx genes contain the same duplication in the promoter, the relationship between different levels of hexaploid 1Bx genes and the duplication of regulatory elements is not supported [47]. These finding further supported the 85 bp deletion has not disrupted the control of 1Ay gene expression and is obviously not responsible for the silencing of 1Ay genes in diploid, tetraploid and hexaploid wheats.

Three silenced genes of 1Ay (Tu-s), 1Ay (Ta-s), 1Ay (Td-s) characterized in this study together with 1Ay (Cheyenne), showed that their translations were disrupted by the in-frame premature stop codons. It indicated that they were highly unlikely to be expressed as a full length protein. In fact, such information is consistent with our SDS-PAGE results. However, the silencing of 1Ay gene in *cv. Chinese spring* is accompanied by the insertion of an 8 kb transposon-like in its coding region [48]. The defects in the coding regions (premature stop codons and insertion of large transposon-like elements) would be possibly responsible for the silencing of the 1Ay genes in diploid, tetraploid and hexaploid wheats (Table 2). However, the mechanisms of gene expression and silencing are complicated and could involve the interactions of a number of factors, including specific nucleotide sequencing, chromosome rearrangement, and methylation, etc. Some mutational events in more distant distal promoter regions are possible causes for the inactivation of 1Ay genes; and more distal sequences are necessary to be examined. In addition, the experiments of 1Ay promoter function in wheat are required to further study the mechanism of the silencing of Glu-A1-2 alleles.

**Conclusion**

The possession of larger molecular mass and fewer conserved cysteine residues are unique characteristics of 1Ay subunits tested in this study. Particularly, 1Ay (Ta-e3) with an unusual large size, is one of known largest y-type HMW-GS gene and may contribute more to the gluten polymers than other known y-type subunits. It is also interested in observing that the conserved cysteine residue within the repetitive domain of the y-type genes of hexaploid wheat is not always present in wild wheats. The 1Ay genes from *T. urartu* have a closer relationship among, *T. turgidum dicoccon* and *T. aestivum* than those from *T. monococcum aegilopoides*. The 85 bp deletions are present not only in the promoter regions of Glu-A1-2 alleles with different expressions but also in the corresponding positions of 6 species of *Aegilops* and *Hordeum*. The 85 bp deletion and some variations in
the 5’flanking region, have not interrupted expression of 
1Ay genes, whereas the defects in the coding regions (pre-
mature stop codons and insertion of large transposon-like-
element) would be possibly responsible to the silencing of 
1Ay genes. Some mutational events in more distant distal 
promoter regions might also be the possible cause of the 
inactivation of 1Ay gene.

Methods

Plant materials

One hundred and forty-one accessions of T. urartu, T. mon-
occocum aegilopoides, T. monococcum dicoccon and T. aestivum 
were used in SDS-PAGE analysis. All accessions 
were kindly provided by USDA-ARS http://www.ars.
grin.gov. Fifty-three accessions with expressed 1Ay subunits 
were screened out from 141 accessions, and 6 accessions 
with expressed 1Ay subunit plus 3 ones without 1Ay subunit 
were chosen for cloning experiments (Table 3).

SDS-PAGE

HMW-GSs of T. urartu, T. monococcum aegilopoides and 
T. turgidum dicoccon were extracted from single half seed 
according to Mackie et al [49]. SDS-PAGE was conducted 
as described in Wan et al. [19]. HMW-GSs from hexaploid 
wheat cv. Chinese Spring (null, 1Bx7+1By9, 
1Dx2+1Dy12) were used as references.

Characterization of the complete ORFs of 1Ay from 
diploid and tetraploid wheats

CTAB method was carried out to extract genomic DNA 
from the leaves of two-week-old single plant [50]. For 
amplifying the complete coding sequence of 1Ay, a pair of 
primers, P1 (5’-ATGGCTAACGGGC/TTA/GGTCTCCCTTTGTTG-3’ 
and P2 (5’-CTATCACATGGCTG/ 
AGCGCGACCTACG-3’), were designed according to the 
nucleotide sequences conserved in the 5’ or 3’ ends of the 
ORFs of published HMW-GSs. The LA Taq polymerase 
(TaKaRa) with GC buffer for GC-rich template was used in 
the PCR amplification to avoid introducing errors into the 
sequence. The cycling parameters was 94 °C for 5 min, 
followed by 30 cycles of 94°C for 40 sec, 68°C for 5 min, 
and a final extension step at 68°C for 15 min[51]. PCR 
products were separated in 1% agarose gels and all DNA 
fragments were recovered and purified from agarose gels, 
and ligated into the pMD18-T vector (TaKaRa). Then the 
ligation mixtures were transformed into Escherichia coli 
DH5α competent cells. To obtain the full-length sequence, 
the strategy of primer walking and the nest dele-
tion method according to Sambrook et al. [52] were used. 
The sequencing was performed by Invitrogen Company 
(Shanghai, China). The final nucleotide sequences for 
each ORF of 1Ay were determined from the sequencing 
results of 3 independent clones.

Isolations of the 5’ flanking promoter region of 1Ay genes

Based on the alignment of the sequences of published 
HMW glutenin genes 1Ax1 (GenBank: X61009), 1Ax2* 
(GenBank: M22208), 1Bx7 (GenBank: X13927), 1Bx17 
(GenBank: JIC2099), 1Dx2 (GenBank: X03346), 1Dx5 
(GenBank: X12928), 1Ay (GenBank: X03042) 1By9 (Gen-
Bank: X61026), 1Dy10 (GenBank: X12929), 1Dy12 (Gen-
Bank: X03041) and 1Dy12.11 (GenBank: AY248704), a 
pair of primers (P3 and P4) specific for the promoter 
region of 1Ay was designed. The P3 primer (5’-AGCCGAAA-

| HWM-GS alleles | Species | Genome | Gene expression | 5'flanking regions | Coding Region | References |
|---------------|---------|--------|-----------------|-------------------|----------------|-----------|
| 1Ay (Tu-e)    | T. urartu | AA     | active          | 85 bp deletion    |                | This study |
| 1Ay (Tu-e2)   | T. urartu | AA     | active          | 85 bp deletion    |                | This study |
| 1Ay (Ta-s)    | T. urartu | AA     | inactive        | 85 bp deletion    | Stop codon     | This study |
| 1Ay (Ta-e1)   | T. monococcum aegilopoides | AA     | active          | 85 bp deletion    |                | This study |
| 1Ay (Ta-e2)   | T. monococcum aegilopoides | AA     | active          | 85 bp deletion    |                | This study |
| 1Ay (Ta-e3)   | T. monococcum aegilopoides | AA     | inactive        | 85 bp deletion    | Stop codon     | This study |
| 1Ay (Td-e)    | T. turgidum dicoccon | AABB   | active          | 85 bp deletion    |                | This study |
| 1Ay (Tds)     | T. turgidum dicoccon | AABB   | inactive        | 85 bp deletion    | Stop codon     | Forde et al. (1985) |
| 1Ay (Cheyenne) | T. aestivum | AABBD  | inactive        | 85 bp deletion    | transposon-like insertion | Harberd et al. (1987) |
| 1Ay (Chinese spring) | T. aestivum | AABBD  | inactive        | 85 bp deletion    | transposon-like insertion | Harberd et al. (1987) |

Both active and inactive 1Ay genes from T. urartu, T. monococcum aegilopoides, T. turgidum dicoccon and T. aestivum shared high homology promoter sequences including 85 bp deletions. Nevertheless, the defects (premature stop codons and transposon-like insertion) were found in the coding regions of all silenced 1Ay genes.
Table 3: Some accessions of diploid, tetraploid wheat species chosen for further cloning experiments based on the results of SDS-PAGE.

| Species                        | Accession No. | Genome | HMW-GS composition | 1Ay alleles | GenBank No. |
|--------------------------------|---------------|--------|---------------------|-------------|-------------|
|                                |               |        | Glu-A1 | Glu-B1 | Glu-D1 |               |
| T. urartu                      | PI 428309     | AA     | +  +   | -    |       | I1y (Tu-e) | EU984503 |
| T. urartu                      | PI 428318     | AA     | +  +   | -    |       | I1y (Tu-e2) | EU984504 |
| T. urartu                      | PI 428308     | AA     | +  -   | -    |       | I1y (Tu-s) | EU994505 |
| T. monococcum aegilopoides     | PI 428007     | AA     | +  +   | -    |       | I1y (Ta-e) | EU984506 |
| T. monococcum aegilopoides     | PI 277123     | AA     | +  +   | -    |       | I1y (Ta-e2) | EU984507 |
| T. monococcum aegilopoides     | PI 306526     | AA     | +  +   | -    |       | I1y (Ta-e3) | EU984508 |
| T. monococcum aegilopoides     | PI 427928     | AA     | +  -   | -    |       | I1y (Ta-s) | EU984509 |
| T. turgidum dicoccon           | PI 355477     | ABBB   | +  +  + |   +  |   +  | I1y (Td-e) | EU984510 |
| T. turgidum dicoccon           | PI 355475     | ABBB   | +  -  + |   +  |   +  | I1y (Td-s) | EU984511 |
| T. aestivum cv. Chinese spring  |              | AABBD  | -  -  7 | 8  2  | 12   |               |

Plus (+) and minus (-) signs indicate the presence or the absence of the corresponding HMW glutenin subunit, respectively.

GACAATGACATG-3’) was designed from the sequence which was strictly conserved in the 5’ flanking regions of all Glu-1 loci, whereas the P4 primer (5’-CATCTGGAGCCCCGTGCCTC-3’) was derived from the sequence coding for 6 amino acid residues (STGLQM) which existed only in y-type HMW-GSs. The amplification profile was 94°C for 5 min, followed by 35 cycles of 94°C for 40 sec, 60°C for 1 min, and 72°C for 1 min 30 sec, and a final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloned into pMD18-T, and sequenced. The final extension step at 72°C for 7 min. PCR products were purified, cloning and sequencing were the same 1Ay promoter characterization mentioned above.

Multiple alignments were carried out with Clustal W (V1.83) for comparisons of DNA or protein sequences [53]. The alignment was further improved by visual examination and manual adjustment. To investigate the phylogenetic relationship of 1Ay genes from different wheat species with previously characterized Glu-1-2 alleles (represented by 1By9 and 1Dy10), we selected the nucleotide sequences of the 5’flanking region plus the sequences encoding signal peptides and N-terminal domain (the corresponding region of 1Dx5 was used as outgroup) to create a multiple alignment by the Clustal W program. The software MEGA 4.02 was used to create phylogenetic trees by neighbour-joining (NJ) method [54].

Authors’ contributions
JQT contributed to design and carry out the experiments and wrote the paper; WYM did the cloning of HMW glutenin ORFs, and revised the manuscript; WF made contribution to SDS-PAGE analysis and promoter cloning of wild diploid species; WIR and YZH did the analysis of the data; ZYL contributed to improve research programme and review the manuscript. All authors have read and approved the final manuscript.

Additional material

Additional File 1
The summary of HMW-GS composition of 141 accessions from diploid and tetraploid wheats, identified by SDS-PAGE. Plus (+) and minus (-) signs indicate the presence or the absence of the corresponding HMW glutenin subunit, respectively. The expression frequency of 1Ay subunits is showed as percent, and the numbers in bracket represent the ratio of accessions with expressed 1Ay subunits to the total. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2229-9-16-S1.pdf]
Acknowledgements

The authors thank Drs John Lu and André Laroche of Agriculture and Agri-Food Canada for critical review of the manuscript. This work was supported by the National High Technology Research and Development Program of China (863 program 2006AA10Z179 and 2006AA10Z1F8), the National Natural Science Foundation of China (863 program 2006AA10Z179 and 2006AA10Z1F8), the FANEDD project (200357) from Ministry of Education, China. Dr. Y.-M. Wei was supported by the Program for New Century Excellent Talents in Universities of China (NCET-05-0814).

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