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Full Length Research Paper

Cloning and characterization of a y-type inactive HMW glutenin subunit gene from *Triticum durum* cultivar youmangbingmai

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The high molecular weight glutenin subunits (HMW-GS) are key factors of the breadmaking quality of common wheat flour. In the present study, one unexpressed *1By* gene from *Triticum durum* cultivar youmangbingmai was cloned and characterized. The results indicated that the silenced *1By* gene in youmangbingmai showed the highest homology to that of previously published *1By8*, suggesting that the unexpressed *1By* may have originated from *1By8*. However, compared to expressed *1By8*, the silenced *1By* in youmangbingmai had a deletion of 247bp at 5' segment. The deletion resulted in the intact of DNA sequences responsible for signal peptide and N- terminal domains. The deletion also resulted in frameshift mutation in C-terminal domains and central region. Therefore, *1By* gene in youmangbingmai had no the typical structure as did normal HMW-GS genes and thus this gene can be not translated into normal HWM-GS. This may be the molecular mechanism of silence for *1By* gene in youmangbingmai. This molecular mechanism is different from those previously reported. Previous studies indicated that the insertion of transposon elements or the presence of premature stop codon is the molecular mechanisms responsible for silence of HWM-GS genes. Moreover, this deletion of 247 nucleotides in YMBM produced a new reading frame. This reading frame may produce a new protein, which is different from HWM-GS.

Key words: *1By*, gene silence, glu-B1 locus, high molecular weight glutenin subunit.

INTRODUCTION

High-molecular-weight glutenin subunit (HWM-GS) is the most important seed storage protein, which determines bread making quality of wheat flour (Payne et al., 1981). They almost account for 10% of the total seed protein and 1% of the grain weight in common wheat, so they can be easily extracted and separated by SDS-PAGE (Xu et al., 2000). It was confirmed that the HMW-GS includes three loci, Glu-A1, Glu-B1 and Glu-D1, located on chromosomes 1A, 1B and 1D of hexaploid bread wheat, respectively. Each locus consists of two tightly liked genes which encode two types of subunits, the greater one termed x-type and the smaller one y-type (Harberd et al., 1986). By 2003, 22 Glu-A1, 56 Glu-B1 and 37 Glu-D1 alleles had been found in wheat and related species, which could be viewed as one of the most known gene in *Triticum* (Yang et al., 2006).

As seed storage proteins, the biological function of HMW glutenin subunits is to provide carbon, nitrogen and energy sources for seed germination and seedling growth. The mutation of silence of such genes is not lethal for the plant, so the selection pressure on these genes is much lower than on other functional genes in evolution (Liu et al., 2008). As a result, these genes can accumulate more mutation and record more accidences in evolution. The knowledge about these genes will give light on gene silencing mechanism.

Six HMW-GS genes are present in common wheat, but *1Ay* and sometimes *1Ax* and *1By*, are not expressed (Payne et al., 1983, Payne et al., 1987). Several reports have investigated the mechanisms underlying the silencing of glutenin in common wheat. The silencing of the

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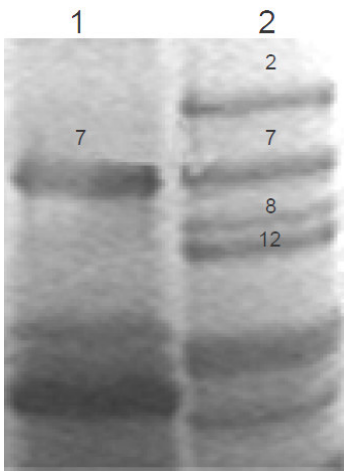

Figure 1. The electrophoretic characters of HMW-GS in YMBM, lane1 and Chinese spring (lane2).

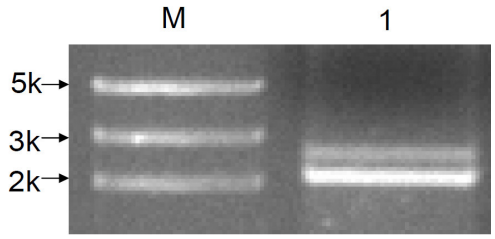

Figure 2. Agarose gel separation of PCR product of YMBM using primers P1 + P2 (lane 1) and DNA size Markers (lane M).

1Ay gene in wheat Cheyenne was associated with the presence of a premature stop codon within its coding region (Forde et al., 1985). Bustos et al. (2000) suggested the silence of the *1Ax* gene was due to the presence of a premature stop codon in the coding region. Premature stop codon also result the silence of 1Dx from *Aegilops cylindrica* (Wan et al., 2002). Harberd et al. (1987) found the insertion of a transposon-like element in the coding region of the *1Ay* gene in wheat Chinese Spring leads to this glutenin gene silence.

Silence of *1By* was observed in some wheats (Tahir, 2008). About 17% of Chinese wheats are silenced for *1By* gene (Wang et al., 2000). Yuan et al. (2009) cloned two silenced *1By* genes from *Triticum aesitvum* sp. *yunnanese* AS332 and *T. aesitvum* sp. tibetanum AS908. They found both of silenced *1By* genes in AS332 and AS908 were *1By9*. The silence of *1By9* was caused by premature stop codons via the deletion of base A in trinucleotides CAA, which lead to frameshift mutation and indirectly produced several premature stop codons (TAG) downstream of the coding sequence (Yuan et al., 2009).

T. durum (AABB, 2n = 4x = 28), is the probale ancestor of bread wheat (Zohary et al., 1969; Nesbitt, 2001). The character of silenced *1By* gene has not been studied in *T. durum*. To determine the molecular character of silenced Glu-*1By* in *T. durum*, the cloning and sequencing of the y-gene was attempted using PCR-based methods from youmangbingmai (YMBM), a durum cultivar planted in Qinghai-Tibet plateau of china. The silence mechanism of *1By* in YMBM was further discussed.

MATERIALS AND METHODS

Plant materials

Youmangbingmai (YMBM) is a *Triticum turgidum* cultivar durum cultivar, planted in Qinghai-Tibet plateau of China. It is deposited in Northwest Institution of Plateau Biology, China Academy of Science. Common wheat Chinese Spring with HMW-GS combination of 1Bx7, 1By8, 1Dx2 and 1Dy12 (N, 7+8, 2+12) was used as the control for SDS-PAGE analysis.

SDS-PAGE analysis

Extraction of HMW-GSs from wheat endosperm and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the extracted subunits were conducted as previously described by Tohver (2007).

DNA preparation and PCR amplification

Seeds were germinated in darkness at 20℃ for 2 weeks. Young leaves were harvested and crushed into powder with the aid of liquid nitrogen and the genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method. For amplifying the complete coding sequence of the Glu-*1By* gene by genomic PCR reaction, a pair of primers (P1: 5- GCCTAGCAACCTTCACAATC-3, P2: 5- GAAACCTGCTGCGGACAAG-3) for amplifying the coding region of HMW-GS genes were synthesized based on conserved nucleotide sequences at the 5 and 3 ends of the coding regions of published HMW-GS genes. PCR was carried out using a PTC-200 thermocycler (MJ Research, Massachusetts, USA). Each PCR reaction (50 µl) contained 200 ng of template DNA, 0.2 mM of each dNTPs, 1 µM of each of primer, 5 µl of 10× PCR buffer, 2.5 U of LATaq DNA polymerase with high fidelity (TaKaRa, Dalian, China) and ddH_2O to 50 µl volume. The PCR was programmed for 4 min at 94℃, followed by 30 cycles of 94℃ for 30 s, 60℃ for 30 s and 72℃ for 2 min 30 s. After the amplification, the final extension was kept for 10 min at 72℃. The PCR products were separated on the 1% agarose gel.

Cloning, sequencing and comparative analyses of the HMW-GS genes

The target PCR products were purified from agarose gel using Gel Extraction Kit (Biotech, china). The fragment was then ligated into T-easy plasmid vector (Premega). The ligated products were transformed into competent cells of Escherichia coli DH10B strain and the resulting plasmids were obtained. The full sequence of the coding region was obtained by sequencing a set of subclones, which were made by the nested deletion methods.

Similarity comparison was conducted with program BLAST in the NCBI (www.ncbi.nlm.nih.gov). The multiple sequence alignment for

Figure 3. Comparison of the DNA sequences of *1By8* among *durum* (*1By8* (*durum*)), YMBM (*1By* (YMBM)) and common wheat (*1By8* (wheat)). Broken box shows the deletion of 247 base pairs in YMBM. Solid boxes point out the sites of difference among the three sequences. Arrow represents start code and line for stop code of the new reading frame of *1By* in YMBM.

DNA was carried out and Open reading frame was predicted by Vector NTI Advance 10.

RESULTS

SDS-PAGE analysis of HMW glutenin subunits

High molecular weight glutenin subunits from Youmang-bingmai (YMBM) were separated by SDS-PAGE (Figure 1). The HMW-GS composition of YMBM was determined using wheat lines Chinese Spring with HWM-GS composition of 1Bx7, *1By8*, 1Dx2 and 1Dy12 (N, 7+8, 2+12) as control. The results indicated that YMBM had the HMW-GS composition of 1Bx7 (N, 7). That means that the remaining three subunits *1Ax*, *1Ay* and *1By* were not expressed in YMBM. In this paper, we further analyzed the silence of *1By*.

Amplification and cloning of *1By* coding sequence

Genomic polymerase chain reaction (PCR) was carried out using degenerate primers P1, P2 specific for DNA fragments of glutenin that represented the complete coding region. The sizes of the amplified fragments were approximate 2.0 kb and 3.0 kb (Figure 2), which were close to the size of the complete coding region sequences of the HMW-GS genes published by previous investigators. The DNA fragments were cloned into the T-easy vector. After DNA sequencings using overlapping subclones, the nucleotide sequence of lower molecular weight segment was obtained and deposited in GenBank under the accession numbers (FJ959383.1). The bigger one was validated as 1Bx7 after sequencing (unpublished data).

Nucleotide sequence of the coding region of the silenced *1By* glutenin

BLASTn analysis indicated that the DNA sequence of silent *1By* gene in YMBM showed the highest homology to that of previously published *1By8* (AY245797) in *T. durum*. The DNA sequence of the silent *1By* in YMBM showed a homology of 88.40% with AY245797. These results suggested that the silent *1By* gene in YMBM was derived from *1By8*. Compared with the expressed *1By8* in common wheat (DQ537336) and *durum* (AY245797), the inactive *1By8* in YMBM has a deletion of 247 nucleotides from 17 base pairs downstream from the start sequence (Figure 3). The deletion in was verified by three overlapping sequences. Except the deletion, there was a substitution of A to G at 1390 base pair downstream from the start codon compared to the expressed gene in *durum*. Two more substitution, T to A at 1278 base pairs and G to A at 1325 base pairs after the start sequence, exist compared to common wheat (Figure 3).

DISCUSSION

Normal HWM-GS is composed of signal peptide, N-terminal domain, C-terminal domains and central region (Shewry and Halford, 2002). The first 63 bp DNA sequences are responsible for the signal peptide (the first 21 amino acid residues). The following 412 bp sequences are responsible for the N-terminal domain (104 amino acid residues) of y-type HWM-GS. However, the *1By* gene in YMBM has a deletion of 247 nucleotides from 17 base pairs downstream from the start sequence (Figure 3), which resulted in the intact of DNA sequences responsible for signal peptide and N-terminal domains. Meanwhile, this deletion caused the frameshift mutation in following C-terminal domains and central region. This made that *1By* gene in *T. durum* YMBM had abnormal structure and this gene can be not translated into normal HWM-GS. This may be the molecular mechanism of silence for *1By* gene in YMBM. This molecular mechanism is different from those previously reported. Previous studies suggested that the insertion of transposon elements or the presence of premature stop codon is the molecular mechanisms responsible for silence of other HWM-GS genes.

Moreover, this deletion of 247 nucleotides in YMBM caused the frameshift mutation and produced a new reading frame (Figure 3, arrow represents start code and line for stop code of the new reading frame). It has 1578 base pairs encode 526 amino acids about 57 KD (Figure 3). The protein is not the HMW-GS due to the absent of signal peptide and N-terminal and the changes of C-terminal domain and central region derived from frameshift mutation. The new reading frame may produce a new type of protein. Further works need to be made in order to prove that the presence or not of the new protein.

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