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Molecular Characterization of Low Molecular Weight Glutenin Genes from Yunnan Hulled Wheat (*Triticum aestivum* subsp. *yunnanense* King)

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Abstract: Three new low molecular weight glutenin subunits (LMW-GS) genes, designated *LMWYN-1*, *LMWYN-2* and *LMWYN-3*, were isolated and characterized from Yunnan hulled wheat accession YN11 (*Triticum aestivum* subsp. *yunnanense* King). The length of complete nucleotide sequence of *LMWYN-1*, *LMWYN-2* and *LMWYN-3* were 1056, 960 and 897 bp, respectively. *LMWYN-1* and *LMWYN-2* encoded proteins with 330 and 299 amino acid residues, respectively, whereas *LMWYN-3* was a putative pseudogene due to the presence of three stop codons in the coding region. All deduced amino acid sequences of the three genes were the LMW-m type according to a methionine at position 1 of the putative mature protein. In repetitive domain of *LMWYN-1* two hydrophobic motifs PIIIL and PVIIL were observed. *LMWYN-1*, *LMWYN-2* and *LMWYN-3* were classified as groups 5, 2 and 8, respectively. Phylogenetic analysis showed that *LMWYN-1* and *LMWYN-3* were closely related to the genes on chromosome 1D and *LMWYN-2* was homologous with the genes on chromosome 1B.

Key words: *Triticum aestivum*, yunnan hulled wheat, LMW-GS, sequence analysis, phylogenetic analysis

INTRODUCTION

The processing quality is largely determined by seed storage proteins in bread wheat (Shewry *et al*., 1995). Wheat seed storage proteins are subdivided into two major fractions, glutenins and gliadins. Glutenins and gliadins are the primary determinations of dough elasticity and dough extensibility, respectively (Shewry *et al*., 2003). On the basis of their mobilities in SDS-PAGE under reducing conditions, glutenin subunits are classified into high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). The role of HMW-GS has been well characterized because of their importance, their relatively small number (3-5 subunits per hexaploid cultivar) and their ease of characterization by SDS-PAGE (Shewry *et al*., 1995). LMW-GS account for about 40% of the total seed storage proteins. LMW-GS also have significant effects on wheat quality via both additive and epistatic interactions, particularly with HMW-GS (Sissons *et al*., 1998; Luo *et al*., 2001; Tranquilli *et al*., 2002; Eagles *et al*., 2002). Despite their importance on the qualitative properties of wheat, the role of individual LMW-GS in the determination of wheat quality is still not clear, because large numbers of the LMW-GS with similar mobility and their overlapping mobility with the abundant gliadin proteins in SDS-PAGE makes the characterization difficult. Therefore it is very necessary to clone and identify LMW-GS genes for wheat quality improvement. LMW-GS genes are encoded by *Glu-A*3, *Glu-B*3 and *Glu-D*3 loci and located on the short arms of chromosome 1A, 1B and 1D, respectively (D'Ovidio and Masci, 2004). Structurally, LMW-GS are composed of a signal peptide, conserved N- and C-terminal domains with a central repetitive domain rich in glutamine residues (D'Ovidio and Masci, 2004). D'Ovidio *et al*. (1992) first cloned a LMW-GS gene from *T. durum* by Polymerase Chain Reaction (PCR). This direct cloning of genomic sequences provides an easy approach to obtain the LMW-GS genes from wheat and its related species. More recently, a number of partial and complete LMW-GS gene sequences from different wheat cultivars have been cloned and characterized (D'Ovidio and Masci, 2004).

Yunnan hulled wheat (*T. aestivum* subsp. *yunnanense* King), called Yunnan wheat, is a precious endemic wheat germplasm exclusively in Yunnan Province and one of the three exclusive wheat subspecies in China (Dong *et al*., 1981). Yunnan hulled wheat possesses

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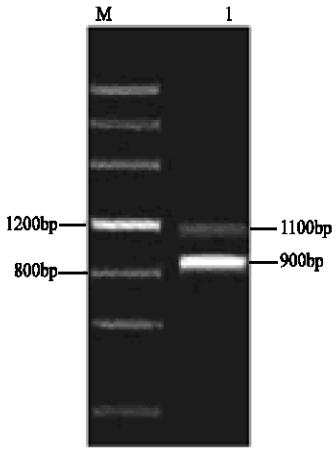

Fig. 1: The PCR amplification results of LMW-GS genes from Yunnan hulled wheat. Lane M: molecular marker; Lane 1: PCR amplification products

unique morphological peculiarity (i.e., glume colour, rachis fragility, long glume and glume tenacity) and many desirable characters such as good quality, high protein content (up to 18.24%), resistance to reharvest sprouting and yellow rust, tolerance to cold and infertile, etc (Chen, 1980). The genetic diversity of HMW-GS (Wei *et al*., 2001; Wang *et al*., 2005) and glaidins (Wei *et al*., 2001) in Yunnan hulled wheat had been analyzed, while the study of LMW-GS in Yunnan hulled wheat has not been reported. In this study, we isolate and characterize the LMW-GS genes in Yunnan hulled wheat, which may be helpful in obtaining more genetic information and effective utilization of Yunnan hulled wheat in wheat breeding.

MATERIALS AND METHODS

Plant materials: Yunnan hulled wheat accession YN11 was obtained from the Institute of Crop Sciences of the Chinese Academy of Agricultural Sciences, Beijing, China. It was planted in greenhouse in March, 2007 and the leaves of the live plant were obtained for genomic DNA extraction in May, 2007.

DNA isolation and PCR amplification: Genomic DNA was extracted as described by Cloutier *et al.* (2001). In order to amplify the complete open reading frames (ORFs) of LMW-GS genes by genomic PCR, a pair of degenerate primers was designed according to published LMW-GS genes. The two primers were P1 (5'-ATGAAGACCTTCCTCG/ATCTTT-3') and P2 (5'-TTATCAGTAGG/ACACCAACTCC-3'). Primer P1 contained the start codon and primer P2 possessed the tandem stop codon of the LMW-GS ORF. Genomic PCR were carried out using the high-fidelity polymerase LA Taq with GC buffer (TaKaRa). The PCR reaction programmed at 94°C, t for 2 min to denature the DNA, followed by 35 cycles of 94°C, for 30 sec, 58°C, for 1 min and 72°C, for 1.5 min and a final extension step at 72°C, for 7 min. The PCR products were separated by 1.0% gel agarose.

Sequencing and analyzing of the LMW-GS genes: The PCR products were purified from the agarose gel. The fragment was ligated into the pMD18-T vector (TaKaRa) and the vector was used to transform competent cell of *Escherichia coli* DH5α strain, PCR amplification was carried out to identify the positive clones. The selected clones were sequenced on an automatic DNA sequencer (AuGCT biotechnology company, Beijing, China). The Open reading frames (ORFs) were translated into amino acid sequences using national center for biotechnology information (NCBI) ORF Finder program (http://www.ncbi.nlm.nih.gov). The sequence analyses were carried out using the program Clustal W version 1.83. The construction of phylogenetic trees was carried out with the software MEGA 3 (Kumar *et al*., 2004). The bootstrap values in the phylogenetic tree were estimated based on 1000 replications.

RESULTS

PCR amplification, cloning and sequencing: Because LMW-GS genes contain no introns, genomic DNA from Yunnan hulled wheat accession YN11 was used as a template for the amplification of the coding region of LMW-GS genes using the primers P1 and P2. The sizes of the two amplified fragments were approximately 900 and 1100 bp (Fig. 1). The PCR products were purified from the gel and ligated to the pMD18-T vector and then transformed into *E. coli* DH10B competent cells. In order to identify the clones with an insert, the white colonies were amplified using the primers P1 and P2. Three different positive clones were screened out and sequenced. Three LMW-GS genes, designated as *LMWYN-1*, *LMWYN-2* and *LMWYN-3*, were identified and submitted to the Genbank with the accession numbers EU286554, EU036695 and EU495301, respectively.

Sequence analysis and deduced amino acid sequences: The LMW-GS gene sequences *LMWYN-1* and *LMWYN-2* were 1056 and 960 bp without premature stop codons in coding region, respectively, while the LMW-GS gene sequence *LMWYN-3* was 897 bp with three premature stop codons in C-terminal domain. *LMWYN-1* could encode a putative 37.9 kDa mature peptide with 331 amino acid residues and *LMWYN-2* could encode a putative 34.2 kDa

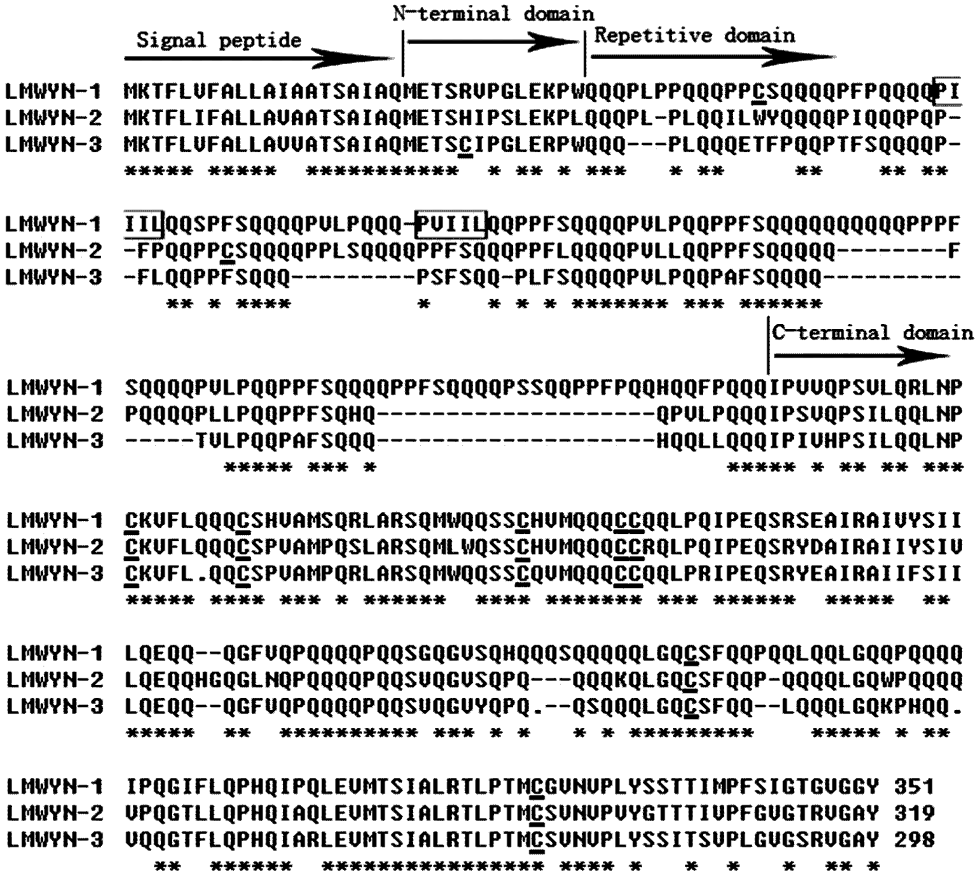

Fig. 2: Alignment of the three deduced amino acid sequences of LMW-GS from Yunnan hulled wheat. The dots indicate stop codon, the underlined letters indicates the position of cysteine, hydrophobic amino acids in the repetitive domain of *LWMYN-1* are boxed

Table 1: Comparison of the repetition of domain of three deduced of LMW-GS amino acid sequences from Yunnan hulled wheat

LMWYN-1	*LMWYN-2*	*LMWYN-3*
QQQ	QQQ	QQQ
PLPPQQQ	PLPLQQ	PLQQQ
PPCSQQQQ	ILWYQQQQ	ETFPQQ
PFPQQQQ	PIQQQ	PTFSQQQQ
PIIILQQ	PQPFPQQ	PFLQQ
SPFSQQQQ	PPCSQQQQ	PPFSQQQ
PVLPQQQ	PPLSQQQQ	PSFSQQ
PVIILQQ	PPFSQQQ	PLFSQQQQ
PPFSQQQQ	PPFLQQQQ	PVLPQQ
PVLPQQ	PVLLQQ	PAFSQQQHQQ
PPFSQQQQQQQQQQ	PPFSQQQQQ	TVLPQQ
PPPFSQQQQ	FPQQQQ	PAFSQQQHQQ
PVLPQQ	PLLPQQ	LLQQQ
PPFSQQQQ	PPFSQHQQ	
PPFSQQQQ	PVLPQQQ	
PSSQQ		
PPFPQQHQQ		
FPQQQ		

mature peptide with 299 amino acid residues. No complete ORF was identified in *LMWYN-3*, indicating that *LMWYN-3* was a putative pseudogene.

Sequence alignment of the deduced amino acid sequences of *LMWYN-1*, *LMWYN-2* and *LMWYN-3* was carried out using Clustal W (Fig. 2). All genes (including the pseudogene) encoded a predicted highly conserved signal peptide with 20 amino acid residues. In the highly conserved signal peptide domain, a valine (Val) residue was substituted by an isoleucine (Ile) residue at the corresponding position in *LMWYN-1* and *LMWYN-2*, respectively and a Val residue in LMWYN-3 was replaced with one alkaline (Ala) residue in *LMWYN-1* and *LMWYN-2*. Followed with a short N-terminal region with 13 amino acids, a central repetitive domain was rich in proline and glutamine residues with the most common repeat motif of PPFSQQQQ (Table 1). The size of the repetitive domain varied between the genes, mainly depending upon the number of repeats. *LMWYN-1*, *LMWYN-2* and *LMWYN-3* had 18, 15 and 13 repeat units, respectively. *LMWYN-1* was longer than *LMWYN-2* with three more successive repeat units from PPFSQQQQ to the last PPFPQQHQQ. Much more

Table 2: Identity of the deduced amino acid sequences of *LMWYN-1*, *LMWYN-2* and *LMWYN-3* with those known LMW-GS

LMW-GS	GenBank accession	Identity (%)	Cultivar	References
LMWYN-1	DQ357052	99.14	*T. aestivum* cv. Tasman	Zhao *et al.* (2006)
	AY831780	98.86	*T. aestivum* cv. Glenlea	Ozdemir and Cloutier (2005)
	AY831781	98.86	*T. aestivum* cv. Glenlea	Ozdemir and Cloutier (2005)
	DQ357053	98.86	*T. aestivum* cv. Tasman	Zhao *et al.* (2006)
	AY831777	98.00	*T. aestivum* cv. Glenlea	Ozdemir and Cloutier (2005)
LMWYN-2	AB062852	91.25	*T. aestivum* cv. Norin 61	Ikeda *et al.* (2002)
	EF188290	90.00	*T. turgidum* subsp. *dicoccoides*	Jiang *et al.* (2006) (unpublished)
	EF188292	90.00	*T. zhukovskyi* PI 355707	Jiang *et al.* (2006) (unpublished)
	Y14104	89.43	*T. turgidum* subsp. *durum* cv. Langdon	D'Ovidio *et al.* (1997)
	EF371505	86.33	*T. turgidum* subsp. *polonicum*	Song *et al.* (2006) (unpublished)
LMWYN-3	AY841015	99.66	*Ae. tauschii*	Huang *et al.* (2004) (unpublished)
	AY829370	99.66	*Secale sylvestre*	Shang *et al.* (2005)
	AY585351	99.33	*Ae. tauschii* AUS18913	Johal *et al.* (2004)
	AY841016	98.99	*Ae. triuncialis*	Lin *et al.* (2004) (unpublished)
	EF437430	95.97	*Ae. tauschii*	Zhao *et al.* (2007) (unpublished)

variation could be observed between *LMWYN-3* and the other two genes in this region (Table 1, Fig. 2). Two hydrophobic repeats (PIIIL and PVIIL) were observed in *LMWYN-1*. The same hydrophobic structure was reported by Ikeda *et al.* (2002). The repetitive domain was followed by the C-terminal domain rich in glutamine interspersed proline residues, which comprised more than half of the protein and was less variable in size than the repetitive domain. Based on LMW-GS conserved N- and C-terminal sequences, LMW-GS sequences were classified into 12 groups (Ikeda *et al.*, 2002). In study research, *LMWYN-1*, *LMWYN-2* and *LMWYN-3* could be classified as groups 5, 2 and 8, respectively.

All the deduced amino acid sequences (including the pseudogene) possessed 8 conserved cysteine residues. Ikeda *et al.* (2002) found the positions of the first and the seventh cysteine residues in LMW-GS were variable. In *LMWYN-1*, the first cysteine residue is present at position 26 (central repetitive domain). In *LMWYN-2*, the first cysteine residue is present at position 45 (central repetitive domain). In *LMWYN-3*, the first cysteine residue is present at position 5 in the N-terminal domain. The positions of the other seven cysteine residues are much conserved and all are present in the C-terminal domain. Based on the position of the cysteine residues, the genes *LMWYN-1*, *LMWYN-2* and *LMWYN-3* could be classified as types III, I and IV, respectively, according to the nomenclature of Ikeda *et al.* (2002).

Homology analysis: BLASTN (NCBI) was conducted to investigate the homologous relationships of among the three genes from Yunnan hulled wheat and those known LMW-GS genes present in the databases. Five best matches obtained for each of the three genes are listed in Table 2. *LMWYN-1* and *LMWYN-3* had >95% identity to the known LMW-GS from wheat and its relatives. *LMWYN-2* showed identity between 86.33 and 91.25% to the known LMW-GS from wheat. *LMWYN-1* had up to 99.14% identity to a LMW-GS gene from *T. aestivum* cv Tasman (Zhao *et al.*, 2006). *LMWYN-3* also had 99.66% identity to two LMW-GS genes in *Ae. tauschii* (Huang *et al.*, 2006, unpublished) and *Secale sylvestre* (Shang *et al.*, 2005).

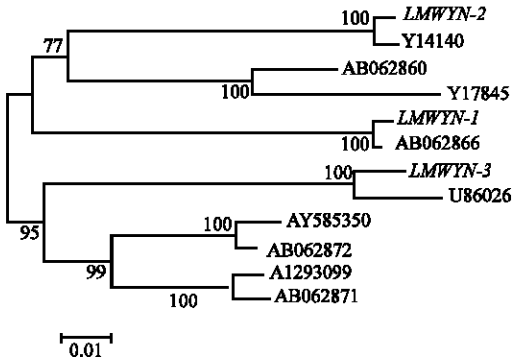

Fig. 3: Phylogenetic analysis for LWM-GS nucleotide sequences from Yunnan hulled wheat and the representatives of known LMW-GS

Phylogenic analysis: In order to investigate evolutionary relationships between LMW-GS genes from Yunnan hulled wheat and the representatives of known LMW-GS genes, a phylogenetic tree was constructed based on the nucleotide sequences of the coding regions (Fig. 3). In the evolutionary analysis, the nine representatives of known LMW-GS genes located in chromosome 1A (AB062871 and AJ293099), chromosome 1B (AB062860, Y14104 and Y17845) and chromosome 1D (DQ357050, AB062872, AB062866 and U86026) were retrieved from the NCBI. Y14104 and AJ293099 were derived from *T. durum* (AABBB = 28), AY585350 was derived from *Ae. tauschii* (DD = 14)and the other five genes isolated from *T. aestivum* (AABBDD = 42). The twelve

genes were clearly clustered into four groups (Fig. 3). *LMWYN-1* and *LMWYN-3* were closely related to AB062866 and U86026, respectively, which were located in chromosome 1D, while *LMWYN-2* was more homologous with Y14104 in chromosome 1B.

DISCUSSION

It is known that both HMW-GS and LMW-GS are responsible for the dough elasticity, but scientists have paid much attention to HMW-GS rather than LMW-GS in past 30 years. Therefore, in order to understand the mechanism of the high quality in a precious wheat subspecies named Yunnan hulled wheat existed only in China (Chen, 1980; Dong *et al*., 1981) and to find out the new LMW-GS with good properties which might be useful for wheat genetic improvement, the aim of this study work was to isolate and characterize the LMW-GS genes in this high quality wheat subspecies.

LMW-GS are classically subdivided into B, C and D groups according to their electrophoretic mobility in SDS-PAGE and their isoelectric points (Jackson *et al*., 1983). The molecular weights of LMW-GS in the B and C groups are about 30-40 and 40-50 kDa, respectively (Jackson *et al*., 1983). In this research, *LMWYN-1* and *LMWYN-2* encode predicted LMW-GS with molecular weights of 37.9 and 34.2 kDa and are thus classified as C groups LMW-GS. On the basis of the first amino acid residue of N-terminal sequences, LMW-GS have been classified into three classes: LMW-m, LMW-s and LMW-i types possessing methionine, serine and isoleucine, respectively (Cloutier *et al*., 2001). All the proteins of *LMWYN-1*, *LMWYN-2* and *LMWYN-3* are LMW-m type.

Disulphide bonds play a critical role in determining the structure and properties of wheat gluten proteins (Shewry *et al*., 1995). It has been proposed that the six cysteine residues present in LMW-GS are involved in intramolecular disulphide bond formation, while the remaining two cysteine residues are likely to participate in intermolecular disulfide bond formation (D'Ovidio *et al*., 1999). LMW-GS were classified into 6 types based on the position of functional cysteine residues (Ikeda *et al*., 2002). *LMWYN-1*, *LMWYN-2* and *LMWYN-3* are classified into types III, I and IV, respectively. So we inferred *LMWYN-1* and *LMWYN-2* could have similar effects on bread-making quality with LMW-GS of types III and I, respectively. Phylogenetic analysis showed that *LMWYN-1* and *LMWYN-3* were closely related to AB062866 and U86026, respectively, which were located in chromosome 1D, whereas *LMWYN-2* was more homologous with Y14104 in chromosome 1B.

The composition of HMW-GS in Yunnan hulled wheat had been analyzed (Wei *et al*., 2001; Wang *et al*., 2005), but no good quality subunits were observed. Consequently the high quality in Yunnan hulled wheat may be related to LMW-GS, gliadin. In this study, three LMW-GS genes in Yunnan hulled wheat had been characterized. However, these three isolated LMW-GS genes did not have special structures which possibly related to good processing quality. Therefore, the mechanism of the high quality in Yunnan hulled wheat is still worth being investigated further.

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