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ORIGINAL PAPER

PCR-based isolation and identification of full-length low-molecular-weight glutenin subunit genes in bread wheat (*Triticum aestivum* L.)

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Abstract Low-molecular-weight glutenin subunits (LMW-GSs) are encoded by a multi-gene family and are essential for determining the quality of wheat flour products, such as bread and noodles. However, the exact role or contribution of individual LMW-GS genes to wheat quality remains unclear. This is, at least in part, due to the difficulty in characterizing complete sequences of all LMW-GS gene family members in bread wheat. To identify full-length LMW-GS genes, a polymerase chain reaction (PCR)-based method was established, consisting of newly designed conserved primers and the previously developed LMW-GS gene molecular marker system. Using the PCR-based method, 17 LMW-GS genes were identified and characterized in Xiaoyan 54, of which 12 contained full-length sequences. Sequence alignments showed that 13 LMW-GS genes were identical to those found in Xiaoyan 54 using the genomic DNA library screening, and the other four full-length LMW-GS genes were first isolated from Xiaoyan 54. In Chinese Spring, 16 unique LMW-GS genes were isolated, and 13 of them contained full-length coding sequences. Additionally, 16 and 17 LMW-GS genes in Dongnong 101 and Lvhan 328 (chosen from the micro-core collections of Chinese germplasm), respectively, were also identified. Sequence alignments revealed that at least 15 LMW-GS genes were common in the four wheat varieties, and allelic variants of each gene shared high sequence identities (>95%) but exhibited length polymorphism in repetitive regions. This study provides a PCR-based method for efficiently identifying LMW-GS genes in bread wheat, which will improve the characterization of complex members of the LMW-GS gene family and facilitate the understanding of their contributions to wheat quality.

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Introduction

Bread wheat (*Triticum aestivum* L., AABBDD, $2n = 6x = 42$) is widely used to prepare different types of food products, such as bread, cakes, and noodles; their quality is primarily determined by the unique viscoelastic properties of dough. Glutenin proteins are the major factors responsible for dough elasticity (Shewry et al. 1995) and are therefore major contributors to the end-product performance of dough (Payne 1987; Gianibelli et al. 2001; Shewry et al. 2003). Glutenins are polymeric proteins in wheat grains, linked together by intermolecular disulfide bonds. They form two groups of subunits, namely, high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs). Three to five members of HMW-GS genes are normally expressed in wheat varieties and the expression of certain subunits (e.g., 1Dx5 and 1Ax1) is correlated with superior bread-making

performance (Payne et al. 1981; Blechl and Anderson 1996; Barro et al. 1997). In contrast to HMW-GSs, substantially more LMW-GSs are expressed in the grains of bread wheat and are essential in determining dough properties, such as dough resistance and extensibility (Metakovsky et al. 1990; Andrews et al. 1994; Cornish et al. 2001; Tanaka et al. 2005; Dong et al. 2010). However, the exact role or contribution of individual LMW-GS genes to wheat quality is not well understood. This is, at least in part, because of the complexity of the LMW-GS gene family and the abundance of allelic variants in bread wheat (Branlard et al. 2003; D'Ovidio and Masci 2004).

The genes encoding LMW-GSs are located at the *Glu-A3*, *Glu-B3*, and *Glu-D3* loci on the short arms of homoeologous group 1 chromosomes (Singh and Shepherd 1988; D'Ovidio and Masci 2004; Dong et al. 2010). These LMW-GS genes compose a multi-gene family and their copy number has been estimated from 10–20 (Harberd et al. 1985; Zhang et al. 2011) to 30–40 (Cassidy et al. 1998; Huang and Cloutier 2008) in hexaploid wheat. Several hundred LMW-GS genes have been isolated from wheat varieties and deposited in GenBank. Their deduced LMW-GSs were classified into three types, based on the first amino acid of the N-terminal sequence of the mature subunits: LMW-i (isoleucine), LMW-m (methionine) and LMW-s (serine) (Tao and Kasarda 1989; Lew et al. 1992; Masci et al. 1995). On the other hand, LMW-GS genes were characterized and classified into 12 groups based on the deduced amino acid sequence identity in the N- and C-terminal conserved domains (Ikeda et al. 2002, 2006).

For isolating and characterizing members of the LMW-GS gene family in bread wheat varieties, cDNA or genomic libraries were initially used, but fewer than six full-length LMW-GS genes could be identified from each bread wheat variety (Bartels and Thompson 1983; Okita 1984; Okita et al. 1985; Pitts et al. 1988; Colot et al. 1989; Cassidy et al. 1998). Recently, 12 groups of LMW-GS genes (three at *Glu-A3*, two at *Glu-B3*, and seven at *Glu-D3*) were isolated from Norin 61 by cDNA library screening and genomic DNA polymerase chain reaction (PCR) (Ikeda et al. 2002). In Glenlea, 12 active LMW-GS genes and 7 inactive genes were identified by screening a bacterial artificial chromosome (BAC) library (Huang and Cloutier 2008). One active gene was assigned to chromosome *1A*, two to chromosome *1B*, and nine to chromosome *1D* (Huang and Cloutier 2008). 14 unique LMW-GS genes in a Chinese bread wheat variety, Xiaoyan 54, have been characterized by complementary approaches (genome wide characterization of gene members, expression profiling, and proteomics analyses; Dong et al. 2010). Four genes were located at the *Glu-A3* locus, three at *Glu-B3*, and seven at *Glu-D3* (Dong et al. 2010). Collectively, using cDNA or genomic libraries, more than ten LMW-GS genes were determined in each bread wheat variety (Norin 61, Glenlea, and Xiaoyan 54). These results indicated that the library screening was successful in characterizing members of the LMW-GS gene family with full-length sequences. However, constructing the cDNA or genomic library for a specific variety requires huge effort, which makes this method incapable of characterizing LMW-GS genes in many bread wheat varieties.

Based on the identified LMW-GS gene sequences, gene or locus-specific primers were designed and the well-known PCR based method was used for subsequent isolation and cloning (Masci et al. 1998; D'Ovidio et al. 1999; Zhang et al. 2004; An et al. 2006). Recently, LMW-GS genes at *Glu-A3*, *Glu-B3*, and *Glu-D3* loci in bread wheat were identified using gene-specific primers, and molecular markers were subsequently developed for specific haplotypes (Zhao et al. 2006, 2007; Wang et al. 2009, 2010). At the *Glu-A3* locus, three distinct LMW-GS genes were discovered in each bread wheat variety, and seven haplotype-specific markers were designed based on single nucleotide polymorphisms (SNPs) among allelic variants (Wang et al. 2010). Four active LMW-GS genes with 17 haplotypes were identified at the *Glu-B3* locus and ten allele-specific markers were designed (Wang et al. 2009). The *Glu-D3* locus had less variation in six active LMW-GS genes that were present in all bread wheat varieties studied (Zhao et al. 2006, 2007). Molecular markers were usually developed based on the polymorphic sequences among cloned LMW-GS genes, whereas the LMW-GS gene family in bread wheat was complex and the exact composition was not well understood (D'Ovidio and Masci 2004; Huang and Cloutier 2008; Dong et al. 2010). Thus, these molecular markers were useful in detecting known LMW-GS genes (Liu et al. 2010), but were limited and difficult for identifying and characterizing other gene variants. Moreover, little information about nucleotide sequences of specific LMW-GS genes was presented, which was a shortcoming common to all sequence-tagged-site (STS) markers (Long et al. 2005; Ikeda et al. 2006; Zhao et al. 2006, 2007; Wang et al. 2009, 2010; Dong et al. 2010).

Due to complex composition of the LMW-GS gene family, isolating nucleotide sequences of all members in bread wheat varieties remains a challenge. In our previous study, to dissect this complex gene family, a LMW-GS gene molecular marker system was developed based on conserved sequences and length polymorphisms among LMW-GS genes (Zhang et al. 2011). Using this system, more than 10 members of the LMW-GS gene family were successfully separated. Thus, the LMW-GS gene molecular marker system might be helpful in

Table 1 Primers designed for amplification of LMW-GS genes in bread wheat

Primer		Sequence (5′–3′)
LMWGS-Full	LMW-Full-Fa	ATGAAGACCTTCCTCRTCTTTG
	LMW-Full-R1a	CACACATGACGTTTGTGTGAC
	LMW-Full-R1b	TCACACATGACATTGTGTGAC
	LMW-Full-R1c	TCACACATGACGTTGTGTGAC
	LMW-Full-R1d	TCACACACGACGTTGTGTGAC
	LMW-Full-R1e	TCACACATGATGTTGTGTGAC
	LMW-Full-R1f	TCACACATGGTGTTGTGTGAC
	LMW-Full-R1g	TCACACACGACATTGTGTGAC
A3-1	A3-1-F	ATTGCGCAGATGGATACTAGCTG
	A3-1-R	GCTGCAAAAAGGTACCCTGTT
LMW-i-484	i-484-F	GTGTGGTTGTGCAAATTTCAC
	i-R1a	TTATCAGTAGRCACCAACTCCGA
	i-R1b	TTATCAGTAGGCACCAACTCCAG
LMW-i-type	i-F	GCCGTTGCGCAAATTTCAC
	i-R1a	TTATCAGTAGRCACCAACTCCGA
	i-R1b	TTATCAGTAGGCACCAACTCCAG
B3-2	B3-2-F	RCAACAAACATTATCGCACCA
	B3-2-R	CCAACTATATATTACTAGAGAYCTTTCCTTATT

The conserved primer set, LMWGS-Full, consisted of a combination of primers LMW-Full-F and LMW-Full-R. The primers LMW-Full-R1a, b, c, d, e, f, and g with unique nucleotides (underlined) were mixed to work as LMW-Full-R. The primers i-R1a and i-R1b were mixed as i-R1 and are specific to LMW-i-type genes

identifying nucleotide sequences of all LMW-GS genes in bread wheat. In addition, conserved primers were successfully used in amplifying full-length *Pm3* genes and HMW-GS genes (Srichumpa et al. 2005; Pang and Zhang 2008; Jiang et al. 2010). In the present study, consisting of conserved primers and the LMW-GS gene marker system, a PCR-based method was developed. By using this method, 16 or 17 LMW-GS gene sequences in individual bread wheat varieties were successfully identified and characterized.

Materials and methods

Plant materials

Wheat variety Xiaoyan 54 was used for validating the PCR-based method in identifying complete LMW-GS gene sequences in bread wheat. Three wheat varieties, Chinese Spring, Dongnong 101, and Lvhan 328, chosen from the micro-core collections of Chinese wheat germplasm, were used to check the efficiency of the method. Xiaoyan 54 was selected from variants of cv. Xiaoyan 6 (St2422/464/Xiaoyan 96), Dongnong 101 (Thacher/Lanshou) is a spring wheat variety bred and cultivated in northeast China, Lvhan 328 (Hanxuan 3/Jinnong 3) is a winter wheat variety bred in northwest China, and Chinese Spring is a well-known landrace, originating from southwest China. There is little genetic relationship among four wheat varieties.

Design of conserved primers for identifying complete LMW-GS gene sequences

With the exception of gene FJ755303, lacking a 3′-UTR, and gene FJ755307, with a coding region interrupted by a transposon insertion, the complete coding sequences and 3′-UTR of 12 LMW-GS genes identified from Xiaoyan 54 (Dong et al. 2010) were aligned using the Lasergene software (DNAStar; http://www.dnastar.com/). Conserved sequences assigned to signal peptide coding sequences and 3′-UTRs were used to design conserved primers for identifying complete sequences of LMW-GS genes. To optimize the conserved primers and ensure amplification of all known gene variants, these primer sequences were searched with NCBI BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Based on the hit sequences, the primers were modified and their specificity to LMW-GS genes was improved (Table 1). The primers were synthesized and purified using polyacrylamide gel electrophoresis (PAGE; Invitrogen Biotechnology Co., Ltd., Shanghai, China).

DNA isolation and PCR amplification

Genomic DNA was extracted from 10-day-old wheat seedlings grown in a glasshouse using the cetyl trimethyl

ammonium bromide (CTAB) procedure (Saghai-Maroof et al. 1984). PCR was performed in 20-μL reaction volumes containing 1.0 U *LA Taq* DNA polymerase (Takara Bio, Otsu, Japan), 100 ng of genomic DNA, 1× GC buffer I (Mg^{2+} plus), 6 pmol of each primer, and 8 nmol of each dNTP. PCR conditions were 95°C for 4 min, followed by 35 cycles of 30 s at 94°C, 40 s at 55–61°C, and 90 s at 72°C and a final extension of 10 min at 72°C using an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Analysis of PCR products using the LMW-GS gene molecular marker system

Using the conserved primers, LMW-GS gene sequences were amplified from genomic DNA of wheat varieties. PCR products were diluted 1:2,000 in DNA grade water. Diluted PCR products (1 μL) were analyzed with the LMW-GS gene molecular marker system according to Zhang et al. (2011).

Cloning and sequencing of LMW-GS genes

PCR products obtained using the conserved primers were separated on 1.2% agarose gels and the expected fragments were purified with the TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China). Purified DNA fragments were cloned into the pGEM-T vector (Promega). Recombinant clones were tested using the LMW-GS gene molecular marker system (Zhang et al. 2011). Positive clones were selected and sequenced by SinoGenoMax Co., Ltd. (Beijing, China). Each PCR and sequencing analysis was repeated three to five times to avoid technical errors. Sequence analysis was performed using the Lasergene software (DNAStar; http://www.dnastar.com/). Sixty-six LMW-GS genes were identified from four wheat varieties. Their coding sequences for mature proteins were used for constructing the phylogenetic tree, which was constructed by the neighbor-joining method using MEGA 4.1 (Tamura et al. 2007). Bootstrap tests were performed using 1,000 replications.

Accession numbers

The LMW-GS gene sequences identified from Xiaoyan 54, Chinese Spring, Dongnong 101 and Lvhan 328 were deposited in GenBank under accession numbers JF271917–JF271920 and JF339155–JF339203, viz. JF271917–JF271920 for the four LMW-GS genes newly identified from Xiaoyan 54, JF339155–FJ339170 for 16 genes from Chinese Spring, JF339171–JF339186 for 16 genes from Dongnong 101, and FJ339187–FJ339203 for 17 genes from Lvhan 328.

Results

Design of conserved primers for identifying full-length LMW-GS genes

Members of the LMW-GS gene family in Xiaoyan 54 have been extensively investigated in our previous studies (Dong et al. 2010; Zhang et al. 2011). To characterize full-length LMW-GS genes in bread wheat, nucleotide sequences of 12 LMW-GS genes containing untranslated regions identified from Xiaoyan 54 (Dong et al. 2010) were aligned using the Lasergene software. Sequence alignments showed that the sequences encoding signal peptides and those assigned to 3′-UTRs were highly conserved among LMW-GS genes (Fig. 1). Two sequence fragments, one containing the initiation codon (ATG), and the other being about 90 bp downstream of the stop codon (TAA), were used to design the conserved primers for amplifying full-length LMW-GS genes in bread wheat varieties. To optimize these primers, GenBank and Expressed Sequence Tag (EST) database were searched with the primer sequences using BLASTN. Based on hundreds of BLAST hits, the conserved primers were modified and supplemented (Table 1). For example, the primer LMW-Full-R1f was added to the conserved primers for amplifying LMW-i-type genes (*GluA3-3*) reported by Wang et al. (2010). The primer LMW-Full-R1g was designed based on the EST sequences corresponding to *GluB3-2* and *B3-2* (Wang et al. 2009; Dong et al. 2010). These modifications allowed a universal amplification of almost all the known LMW-GS genes and their allelic variants deposited in GenBank. As the primers were designed to amplify full-length LMW-GS genes in bread wheat, they were named as LMWGS-Full.

Validation of conserved primers LMWGS-Full in Xiaoyan 54

To evaluate the efficiency of the conserved primers in identifying LMW-GS genes in bread wheat, these primers were used to amplify the genes in Xiaoyan 54. Following electrophoresis with 1.2% agarose gels, two main bands were detected from the PCR products (Fig. 2). The experimental size of the DNA fragments ranged from 1,000 to 1,300 bp, in agreement with the expected size of the complete sequences of the LMW-GS gene family members (Fig. 1). To further separate DNA fragments with higher resolution, capillary electrophoresis (Applied Biosystems 3730 DNA Analyzer) was used. However, because the size of the DNA fragments was too large (>1,000 bp), the Analyzer was unable to distinguish the composition of PCR products (data not shown).

To examine the composition of the PCR products and to investigate whether primers LMWGS-Full efficiently

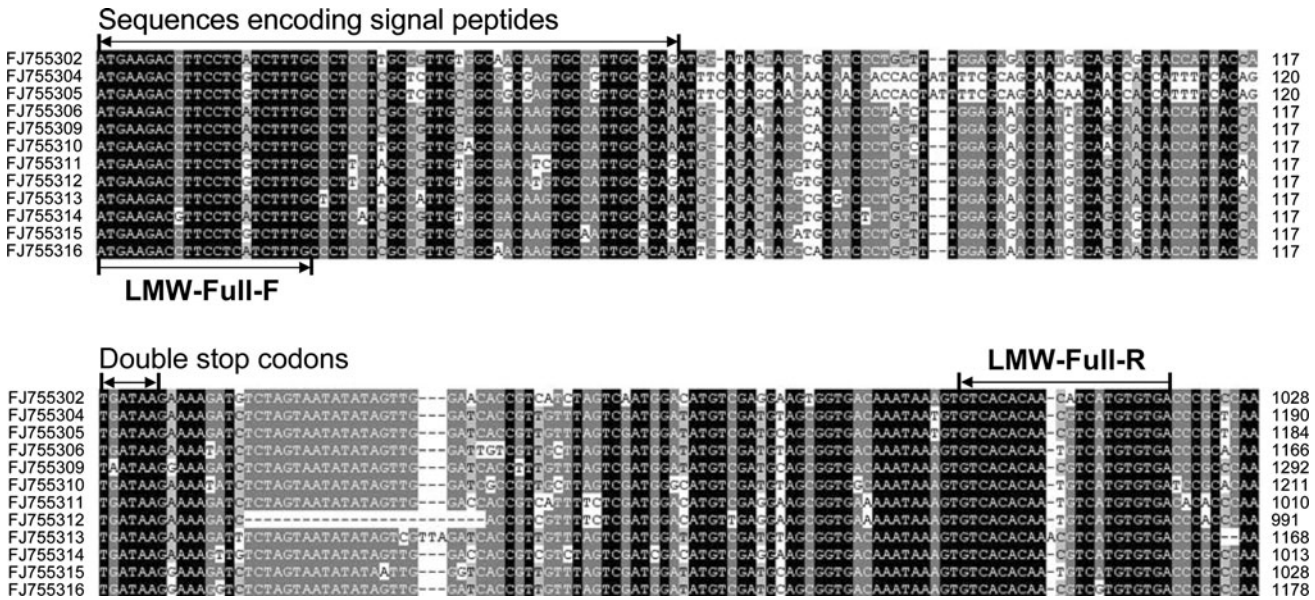

Fig. 1 Sequence alignment of LMW-GS genes in Xiaoyan 54 and conserved primer design for identifying full-length LMW-GS gene sequences. Multiple alignment was performed with full-length LMW-GS genes identified from Xiaoyan 54 (Dong et al. 2010) using Lasergene. Based on the conserved sequences, one pair of primers, LMWGS-Full, was designed. The forward and reverse primers, LMW-Full-F and LMW-Full-R, were matched with sequences encoding signal peptides and the 3′-UTR of the LMW-GS genes, respectively

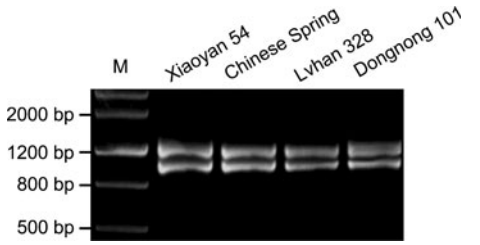

Fig. 2 Agarose gel electrophoresis of full-length LMW-GS genes amplified using conserved primers LMWGS-Full in four wheat varieties. *M*, DNA ladder marker (500, 800, 1,200, 2,000 bp; Tiangen Biotech Co., Ltd.)

amplified all members of the LMW-GS gene family, the LMW-GS gene molecular marker system was used, which can effectively separate complex members of the LMW-GS gene family (Zhang et al. 2011). As a positive control, LMW-GS genes in the genomic DNA of Xiaoyan 54 were also investigated with the marker system. Fifteen DNA fragments were detected using primer LMWGS1, except for DNA fragments 484 and 688, which can be identified by other primers in the molecular marker system (Fig. 3a; Table 2; Zhang et al. 2011). From the PCR products obtained using primers LMWGS-Full, except for DNA fragment 402, corresponding to gene *A3-1* (FJ55302), 14 fragments were detected and all of them were identical in size with those from genomic DNA (Fig. 3b; Table 2).

To obtain complete sequences of LMW-GS gene family members, each band of PCR products obtained using the primers LMWGS-Full in agarose gels was purified and cloned. To identify recombinant clones containing specific LMW-GS genes, the LMW-GS gene molecular marker system was used again. 46 transformed clones from each DNA band were detected. The clones containing specific LMW-GS genes with the proper DNA fragment were selected and sequenced. In Xiaoyan 54, 12 types of clones with a unique size fragment were detected. DNA fragments 566 and 568, corresponding to *A3-3* (FJ755305) and *A3-2* (FJ755304), respectively, were amplified but not cloned (Fig. 3b, c; Table 2; Zhang et al. 2011). Both genes were located at the *Glu-A3* locus and belonged to LMW-i-type genes (Dong et al. 2010). Corresponding to 12 types of clones, 12 full-length LMW-GS genes were obtained in Xiaoyan 54. Sequence alignments were performed with these full-length sequences and those previously identified from Xiaoyan 54 (Dong et al. 2010). Nine sequences were identical to the genes *A3-4*, *B3-1*, *D3-1*, *D3-2*, *D3-3*, *D3-4*, *D3-5*, *D3-6* and *D3-7* (Dong et al. 2010). The other three complete LMW-GS gene sequences in Xiaoyan 54 (i.e., genes corresponding to DNA fragments 370, 549 and 584; Table 2) were identified for the first time. These data suggested that LMWGS-Full primers and the LMW-GS gene marker system, composing a PCR-based method, could be used to identify and characterize LMW-GS genes in bread wheat varieties.

Identification of LMW-GS genes using the PCR-based method

To examine the efficiency of the PCR-based method in identifying LMW-GS genes, three wheat varieties, Chinese

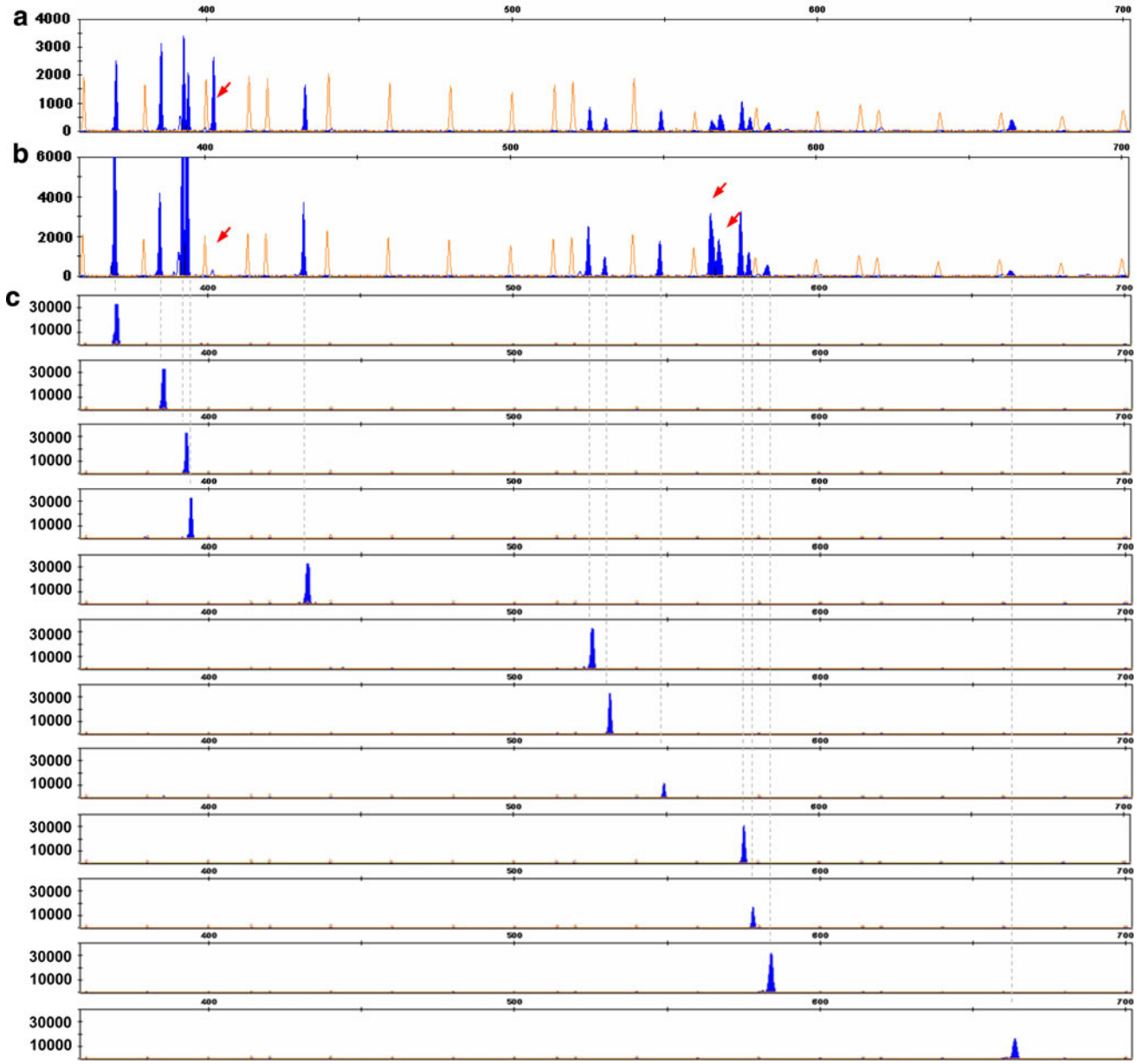

Fig. 3 Validation of conserved primers in identifying complete LMW-GS gene sequences in Xiaoyan 54. **a** Electropherogram showing capillary electrophoresis separation of the LMW-GS genes amplified from genomic DNA of Xiaoyan 54 with primer LMWGS1 of the LMW-GS gene marker system. The *horizontal axis* shows the size of the detected DNA fragments, while the *vertical axis* displays the concentration of DNA fragments. The *blue/solid peaks* represent DNA fragments in the PCR products. The *orange/hollow peaks* match the DNA fragments in the GeneScan 1200 LIZ size standard. The *numbers on the horizontal axis* are the size of the corresponding peak from the GeneScan 1200 LIZ size standard (*orange/hollow*). 15 LMW-GS genes from the genomic DNA were detected with LMWGS1 of LMW-GS gene marker system. **b** Electropherogram displaying LMW-GS genes amplified from Xiaoyan 54 using primers LMWGS-Full. LMW-GS genes in PCR products obtained using LMWGS-Full were amplified with LMWGS1. The PCR products were then separated by capillary electrophoresis. Fourteen LMW-GS genes shown in the electropherogram were amplified with LMWGS-Full from Xiaoyan 54. **c** Electropherogram of positive clones containing specific LMW-GS genes. After amplification with LMWGS-Full, LMW-GS genes were cloned. Twelve positive clones containing unique full-length LMW-GS genes were detected with the gene marker system

Spring, Dongnong 101, and Lvhan 328, were studied. In Chinese Spring, 15 DNA fragments were identified from the genomic DNA using primer LMWGS1 (Supplementary Fig. 1a). Thirteen of these fragments were detected from PCR products obtained using primers LMWGS-Full (Supplementary Fig. 1b). The other two DNA fragments 402 and 624 were absent (Supplementary Fig. 1b; Table 2). After cloning and clone selection, 12 clones containing DNA fragments of different size were identified and sequenced (Supplementary Fig. 1c). Only the LMW-GS

Table 2 LMW-GS genes identified from bread wheat varieties using the PCR-based method

Locus	Xiaoyan 54			Chinese Spring			Lvhan 328			Dongnong 101		
	DNA fragment[a]	Gene	Accession No.	DNA fragment[a]	Accession No.	Identity (%)[b]	DNA fragment[a]	Accession No.	Identity (%)[b]	DNA fragment[a]	Accession No.	Identity (%)[b]
Glu-A3	370[c]	–[d]	JF271917	391	JF339156	97.9	391	JF339187	97.9	370	JF339171	98.7
Glu-D3	385[c,e]	*D3-4*	FJ755311	385	JF339155	99.9	385	JF339203	99.9	385	JF339172	99.9
Glu-D3	393[c]	*D3-5*	FJ755312	393	JF339157	99.9	393	JF339188	100	393	JF339173	100
Glu-D3	394[c,e]	*D3-7*	FJ755314	394	JF339158	100	394	JF339189	99.9	394	JF339174	100
Glu-A3	402	*A3-1*	FJ755302[e]	402	JF339159	99.3	402	JF339190	99.3	408[e]	JF339175	99.4
Glu-D3	432[c,e]	*D3-2*	FJ755315	441	JF339160	99.7	432	JF339191	100	432	JF339176	100
Glu-A3	484	–	JF271918	502	JF339161	97.2	502	JF339192	97.2	502	JF339177	97.0
Glu-D3	525[c,e]	*D3-6*	FJ755313	525	JF339162	100	525	JF339193	100	528	JF339178	100
Glu-B3	530[c,e]	*B3-1*	FJ755306	530	JF339163	99.9	530	JF339194	99.9	530	JF339179	99.6
Glu-B3	–	–	–	–	–	–	544[e]	JF339195	–	–	–	–
Glu-B3	549[c]	–	JF271919	549	JF339164	100	549	JF339196	100	549	JF339180	100
Glu-A3	566	*A3-3*	FJ755305	–	–	–	–	–	–	–	–	–
Glu-A3	568[e]	*A3-2*	FJ755304	–	–	–	–	–	–	–	–	–
Glu-D3	575[c,e]	*D3-1*	FJ755316	575	JF339165	100	575	JF339197	100	575	JF339181	100
Glu-D3	578[c,e]	*D3-3*	FJ755310	578	JF339167	99.2	578	JF339199	100	578	JF339182	100
Glu-B3	–	–	–	578[c]	JF339166		578	JF339198	–	–	–	–
Glu-D3	584[c]	–	JF271920	589	JF339168	100	595	JF339200	99.9	589	JF339183	100
Glu-B3	–	–	–	593	N[f]	–	–	–	–	–	–	–
Glu-A3	664[c,e]	*A3-4*	FJ755303	620	JF339169	95.7	620	JF339201	95.7	646	JF339184	95.8
Glu-B3	688[e]	*B3-2*	FJ755309	624	JF339170	95.2	621	JF339202	95.2	688	JF339185	99.6
Glu-B3	–	*B3-3*	FJ755307	–	–	–	–	–	–	815	JF339186	–

[a] Experimental size of the DNA fragments amplified using primer LMWGS1 from the LMW-GS gene molecular marker system

[b] Sequence identity with the allelic variants in Xiaoyan 54

[c] Genes and their allelic variants identified from bread wheat varieties using conserved primers LMWGS-Full

[d] Not applicable

[e] Genes and their allelic variants possessing intact coding sequences

[f] Not detected

gene corresponding to DNA fragment 593 was not cloned (Supplementary Fig. 1b, c). This gene was located at the *Glu-B3* locus (Zhang et al. 2011), but has not been isolated. The similar situation occurred in Lvhan 328 and Dongnong 101, where the genes corresponding to DNA fragments 402/408 and 621/688 were not amplified and detected, and 12 positive clones containing specific LMW-GS genes were identified and sequenced (Table 2). Subsequent sequence analysis was performed with these cloned LMW-GS gene sequences. The clones corresponding to DNA fragment 578 in Chinese Spring and Lvhan 328 each contained two unique genes, one located at the *Glu-B3* locus and the other located at the *Glu-D3* locus, which was consistent with our previous results (Zhang et al. 2011). All the other clones each contained only one LMW-GS gene (Table 2). Thus, matched with 12 types of clones, 13 unique full-length LMW-GS genes were identified in Chinese Spring or Lvhan 328, and 12 complete LMW-GS gene sequences were isolated from Dongnong 101.

When comparing the DNA fragments obtained from genomic DNA with those obtained from PCR products of LMWGS-Full, fragments 402, 484, 566, 568, and 688 in Xiaoyan 54 (corresponding to *A3-1*, three LMW-i-type genes and *B3-2*) were not identified with primers LMWGS-Full. Three genes, absent in the experiments with Chinese Spring (corresponding to DNA fragments 402, 502 and 624), were homologous to *A3*-1, a LMW-i-type gene, and *B3-2*, which were also missing for Lvhan 328 and Dongnong 101 (Table 2; Zhang et al. 2011). Collectively, the LMW-GS genes, *A3-1*, LMW-i-type genes and *B3-2* were difficult to identify with conserved primers LMWGS-Full. To isolate these genes, gene-specific primers, covering nearly complete coding sequences for mature LMW-GS proteins, were designed based on the polymorphic sites

between the specific genes and the other members of the LMW-GS gene family (Table 1). Using these primers, those missing genes mentioned above were subsequently amplified, cloned, and sequenced, excluding the gene corresponding to DNA fragment 593 in Chinese Spring (Supplementary Fig. 2; Table 2). Thus, using LMWGS-Full and gene-specific primers, nucleotide sequences of all the genes detected by the LMW-GS gene marker system could be identified efficiently. Using the PCR-based method, 17 distinct gene members were identified in Xiaoyan 54 (four new genes, JF271917–JF271920), 16 in Chinese Spring (JF339155–JF339170), 16 in Dongnong 101 (JF339171–JF339186), and 17 in Lvhan 328 (JF339187–JF339203) (Table 2).

Characterization of LMW-GS genes in four bread wheat varieties

All the LMW-GS genes identified from the four varieties were subjected to sequence analysis using the Lasergene software and MEGA 4.1. Six pseudogenes, possessing internal stop codons in coding regions, were identified in Xiaoyan 54 (excluding *B3-3*; Table 2) and Dongnong 101, and seven in Chinese Spring and Lvhan 328. These data suggested that about one third of the LMW-GS gene family members were present in the bread wheat genome but not expressed in grains. Moreover, five pseudogenes corresponding to DNA fragments 370 and 391, 393, 484 and 502, 549, and 584, 589, and 595 were conserved among four wheat varieties (Table 2; Fig. 4). Regarding the expressed LMW-GS genes, 11 genes possessed intact open reading frames (ORFs) in Xiaoyan 54, which is consistent with those reported by Dong et al. (2010). 9, 10, and 10 LMW-GS genes with intact ORFs were identified in Chinese Spring, Dongnong 101, and Lvhan 328, respectively. The predicted proteins of the active genes had similar structures to previously characterized LMW-GSs (D'Ovidio and Masci 2004). Each consisted of four main structural regions: a signal peptide (20 amino acids), a short N-terminal region (13 amino acids), a repetitive domain, and a C-terminal domain. Genes corresponding to DNA fragments 568 and 664 in Xiaoyan 54, 620 in Chinese Spring and Lvhan 328, and 646 in Dongnong 101, encoded LMW-i-type subunits, lacking the N-terminal domain. LMW-s-type genes were also isolated, corresponding to DNA fragments 578 and 688 in Xiaoyan 54, 578 and 624 in Chinese Spring, 544, 578, and 621 in Lvhan 328, and 578 and 688 in Dongnong 101. The remaining active genes were all classified as LMW-m-type genes. LMW-m-type genes were clearly the most abundant in bread wheat, which was consistent with the results for Norin 61 and Glenlea (Ikeda et al. 2002; Huang and Cloutier 2008).

Sixty-six LMW-GS gene sequences identified from the above four varieties were aligned and clustered (Fig. 4). LMW-GS gene allelic variants corresponding to DNA fragments of the same size (i.e., 385, 393, 394, 530, 549, 575, and 578) shared extremely high sequence identities (>99%; Table 2). Some other allelic variants corresponding to DNA fragments of the similar size (i.e., 370 and 391; 402 and 408; 432 and 441; 484 and 502; 525 and 528; 584, 590, and 595; 621, 624, and 688; 620 and 646) also showed highly conserved structures (>95% identity) and were clustered into individual groups (Table 2; Fig. 4). Allelic variants of *D3-6* corresponding to DNA fragments 525 and 528, for example, shared identical N- and C-terminal sequences (METSRVPGLEKPW and IMPFSIGTGVGGY) and contained eight cysteine residues at conserved sites. Thus, genes clustered into the same group might be allelic variants, derived from a specific LMW-GS gene in bread wheat. Totally, 15 genes mentioned above, whose allelic variants were characterized with DNA fragments of the same or similar size, are universal in four varieties. In addition, several particular genes (e.g., the gene corresponding to DNA fragments 544 or 815 identified from only one variety; Table 2), should be investigated further to identify their allelic variants. Nonetheless, the sequence alignment above demonstrated that LMW-GS gene family members were highly conserved among wheat varieties, and the PCR-based method was successful in identifying and characterizing full-length LMW-GS genes in bread wheat.

Discussion

LMW-GS is one of the major determinants for the bread-making quality among bread wheat varieties. However, LMW-GSs display complex polymers and are encoded by a multi-gene family. To date, characterizing members of the LMW-GS gene family in bread wheat remains a challenge. In the present study, a PCR-based method was established to successfully elucidate the whole LMW-GS gene family in bread wheat.

Conservation and variation of LMW-GS genes in bread wheat

In previous studies, each LMW-GS gene identified in Xiaoyan 54 and Chinese Spring was located at a specific *Glu-3* locus (Dong et al. 2010; Zhang et al. 2011). Accordingly, locations of their allelic variants in Dongnong 101 and Lvhan 328 were determined (Fig. 4; Table 2). At *Glu-A3*, six LMW-GS genes were identified in Xiaoyan 54, and four in the other three varieties. Two genes corresponding to DNA fragments 370 and 402 (*A3-1*) in Xiaoyan 54 were conserved among four wheat varieties (Table 2; Fig. 4).

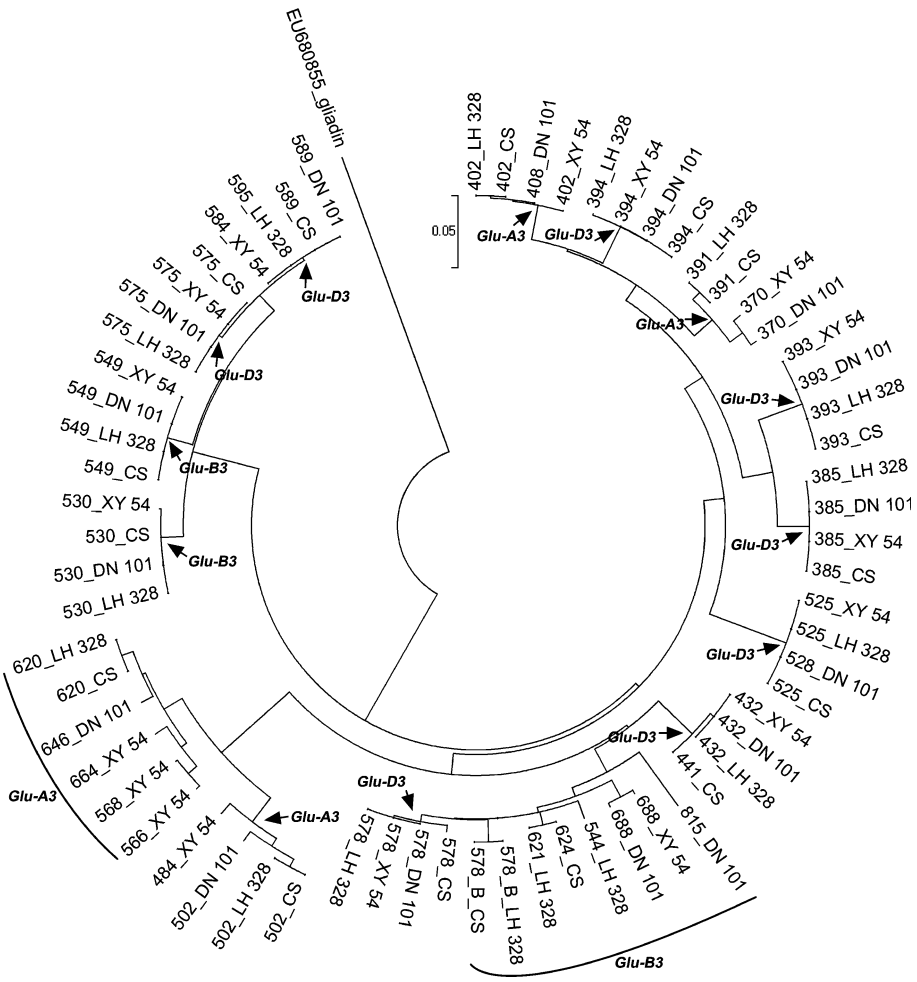

Fig. 4 Phylogenetic reconstruction of LMW-GS genes identified from Xiaoyan 54, Chinese Spring, Dongnong 101, and Lvhan 328. Phylogenetic tree of LMW-GS genes from Xiaoyan 54 (17 genes), Chinese Spring (16 genes), Lvhan 328 (17 genes), and Dongnong 101 (16 genes) was constructed using MEGA 4.1 (Tamura et al. 2007). Allelic variants of a LMW-GS gene in the four wheat varieties were generally clustered into an individual group. The *arrows* show branches containing four allelic variants of individual LMW-GS genes. In addition, some genes (corresponding to DNA fragments 566 and 568 located at *Glu-A3,* and 544 and 815 at *Glu-B3*) were only detected in single varieties. Moreover, based on the location of LMW-GS genes in Xiaoyan 54 and Chinese Spring (Dong et al. 2010; Zhang et al. 2011), allelic variants of each gene were assigned to a specific *Glu-3* locus

The other *Glu-A3* genes all belonged to LMW-i-type genes, disregarding the stop codons in the coding regions. Two LMW-i-type genes (DNA fragments 502 and 620/646) were identified from Chinese Spring, Dongnong 101, and Lvhan 328, which matched genes *GluA3-1* and *GluA3-3* reported by Wang et al. (2010), while four LMW-i-type genes (DNA fragments 484, 566, 568, 664) were identified from Xiaoyan 54, by far the largest number of LMW-i-type genes reported at the *Glu-A3* locus. Genes corresponding to DNA fragments 484 and 502 shared high identity, whereas genes corresponding to DNA fragments 620 and 646 and those corresponding to DNA fragments 566, 568 and 664 were clustered into different groups (Table 2; Fig. 4). These results suggested that LMW-i-type genes showed much variation among wheat varieties and allelic variants might be derived from different haplotypes in bread wheat (Fig. 4). At the *Glu-B3* locus, LMW-GS genes displayed extensive variation (Wang et al. 2009; Liu et al. 2010), except for two conserved genes corresponding to DNA fragments 530 and 549 (Fig. 4). LMW-GS genes corresponding to DNA fragments 621, 624, and 688 shared high sequence identities (>95%; Table 2), belonged to LMW-s-type genes, and might be allelic variants of a certain gene. The *Glu-B3* pseudogenes corresponding to DNA fragment 578 were detected only in Chinese Spring and Lvhan 328, and both shared 100% identity. Another pseudogene corresponding to DNA fragment 815 was identified only from Dongnong 101. Their allelic variants were not found in GenBank, suggesting that this gene was identified from the bread wheat genome for the first time. The LMW-GS gene corresponding to DNA fragment 544 was identified from Lvhan 328, whose allelic variants were also isolated from Glenlea and Aroona-B3g (Huang and Cloutier 2008; Wang et al. 2009). These data suggested that the three *Glu-B3* genes mentioned above might be present in some wheat varieties, but their genetic relationship with other LMW-GS genes should be investigated further. In addition, the gene corresponding to DNA fragment 593 in Chinese Spring should be isolated and whether it could be found universally in wheat varieties should be examined. At the *Glu-D3* locus, eight LMW-GS genes were identified from each variety, which were universal and conserved among four varieties. Except for two pseudogenes corresponding to DNA fragments 393 and 584 in Xiaoyan 54, the other six

LMW-GS genes were expressed and shared high nucleotide sequence identities with those reported in previous studies (Johal et al. 2004; Zhao et al. 2006, 2007; Ikeda et al. 2002; Dong et al. 2010). These data indicated that the *Glu-D3* locus possessed more genes, but displayed less allelic variation, than the other *Glu-3* loci (Fig. 4; Zhao et al. 2006, 2007; Liu et al. 2010). Collectively, 15 LMW-GS genes—four genes at *Glu-A3*, three genes at *Glu-B3*, eight genes at *Glu-D3*—were conserved and universal among bread wheat varieties, whereas LMW-i-type genes at *Glu-A3* and genes corresponding to DNA fragments 544, 578, and 815 at *Glu-B3* showed much variation in bread wheat (Fig. 4).

In addition, the *A3-1* genes were isolated from four wheat varieties, and their allelic variants (*GluA3-2*) were also identified by Wang et al. (2010). In the present study, although the allelic variants in Xiaoyan 54, Chinese Spring, and Lvhan 328 all corresponded to DNA fragment 402, only the gene in Xiaoyan 54 possessed an intact coding sequence and encoded the subunit with the N-terminal sequence MDTSCIPG (D'Ovidio et al. 1992; Ikeda et al. 2002; Dong et al. 2010; Wang et al. 2010). Sequence alignment showed that both genes in Chinese Spring and Lvhan 328 were identical and contained some specific SNPs compared with that in Xiaoyan 54 (Table 2). Thus, the *A3-1* genes in Chinese Spring and Lvhan 328 and that in Xiaoyan 54 might be derived from different haplotypes. Moreover, some SNPs also occurred among allelic varants of some other genes (e.g., genes corresponding to DNA fragments 502 and 578; Fig. 4). These data demonstrated that length polymorphisms were useful, but not sufficient, for characterizing LMW-GS genes in bread wheat, where nucleotide polymorphisms were extremely required. Thus, to elucidate the complex LMW-GS gene family, the PCR-based method was useful, which was successful in isolating full-length LMW-GS genes in bread wheat varieties.

Advantages of the developed PCR-based method in isolating full-length LMW-GS genes

As one of the most important elements that determine bread wheat quality, LMW-GS genes have been isolated and characterized with different approaches. Using cDNA or genomic DNA library screening, on the one hand, 12 groups of LMW-GS genes were identified from Norin 61 (Ikeda et al. 2002), 19 genes from Glenlea (Huang and Cloutier 2008), and 14 genes from Xiaoyan 54 (Dong et al. 2010). In the present study, using the PCR-based method, 18, 17 and 17 LMW-GS gene sequences were successfully isolated from Norin 61, Glenlea, and Xiaoyan 54, respectively, corresponding to all the genes detected with the LMW-GS gene marker system (data not shown; Zhang et al. 2011). Thus, the PCR-based method would work at least as well as, if not better than, the library screening in isolating LMW-GS genes. On the other hand, using gene-specific primers, three genes were isolated at the *Glu-A3* locus, four at the *Glu-B3* locus, and six at the *Glu-D3* locus (Zhao et al. 2006, 2007; Wang et al. 2009, 2010), which suggests that at least 13 genes could be isolated from a wheat variety. In the present study, using the newly developed PCR-based method, 16 or 17 LMW-GS genes were isolated from individual bread wheat varieties, which might compose the whole LMW-GS gene family. Thus, the PCR-based method was useful and efficient in identifying and characterizing LMW-GS genes in bread wheat. Moreover, this method has advantages over gene-specific PCR and the library screening in isolating LMW-GS genes.

First, conserved primers are efficient in amplifying most members of the LMW-GS gene family. Using conserved primers in the PCR-based method (Table 1), 16 or 17 LMW-GS genes were amplified from individual wheat varieties (Fig. 3; Supplementary Fig. 1; Table 2), which were far more than those isolated with gene-specific primers reported previously (D'Ovidio 1993; D'Ovidio and Porceddu 1996; Masci et al. 1998; D'Ovidio et al. 1999; Long et al. 2005; Zhao et al. 2006, 2007; Wang et al. 2009, 2010). Due to lack of information about composition of the LMW-GS gene family, gene(s)-specific primers were developed and used to identify LMW-GS genes in bread wheat and its relatives (Yan et al. 2003; Yue et al. 2005; An et al. 2006; Li et al. 2008; Jiang et al. 2008; Zhao et al. 2008; Huang et al. 2010; Wang et al. 2011). Although, more than 100 LMW-GS gene had been isolated using PCR amplification, only a few (<10) genes were isolated from individual wheat varieties. These studies contributed to characterizing specific LMW-GS genes or haplotypes, but were incapable of distinguishing the complex LMW-GS gene family in bread wheat. In the present study, members of the LMW-GS gene family identified from Xiaoyan 54 were used for primer design, which ensured that most gene members in bread wheat could be isolated. Using conserved primers LMWGS-Full, 12 or 13 full-length LMW-GS genes were amplified from genomic DNA in each PCR experiment (Figs. 3b, 4b), and the other genes were also successfully amplified using gene-specific primers (Supplementary Fig. 2).

Second, the LMW-GS gene molecular marker system facilitates the characterization of the cloned LMW-GS genes. After amplification using LMWGS-Full, more than 10 full-length LMW-GS gene sequences were cloned and the recombinant clones needed to be selected and sequenced. Generally, SP6 and T7 primer system was used to detect the recombinant clones with agarose gel electrophoresis. However, agarose gels showed bad resolution (>30 bp) for more than 1 kb DNA fragments. Using agarose gel electrophoresis, only two main bands were detected from PCR products because the amplified

LMW-GS gene sequences displayed slight difference in size (Figs. 1, 2). Thus, SP6 and T7 primer system was incapable of characterizing clones containing LMW-GS genes. Using SP6 and T7 system, to cover all the LMW-GS genes amplified with LMWGS-Full, more than 100 recombinant clones needed to be sequenced, which was not efficient and greatly increased costs. Moreover, cloning LMW-GS genes might result in nucleotide deletions in repetitive regions (Masci et al. 1998; Ikeda et al. 2002), which made subsequent sequence assembly more complex. To overcome these problems, the LMW-GS gene molecular marker system was introduced. With this system, LMW-GS genes in individual clones were characterized with the unique size of their repetitive regions. Clones containing genes with nucleotide deletions were discarded and only clones with specific LMW-GS genes were selected and sequenced. Fewer than 50 positive clones sequenced were sufficient to identify all the LMW-GS genes amplified from one wheat variety, when three clones of each gene were sequenced. Because sequences with the same size of repetitive regions were generally derived from an identical LMW-GS gene (Zhang et al. 2011), these sequences were subsequently collected and assembled for identifying the specific gene. Comparing with SP6 and T7 system, the LMW-GS gene marker system really improved the efficiency of clone selection and reduced the difficulty of sequence analysis

Third, the PCR-based method is efficient in identifying full-length LMW-GS genes in bread wheat. In the present study, 17 LMW-GS genes were identified in Xiaoyan 54, which was consistent with the 17 DNA fragments detected by using the LMW-GS gene marker system (Table 2; Zhang et al. 2011). Sequence alignments revealed that, excluding the gene (*B3-3*) interrupted by a transposon insertion, 13 genes in Xiaoyan 54 were identical to those identified by screening a BAC library (Dong et al. 2010). The other four genes with full-length sequences (corresponding to DNA fragments 370, 484, 549, and 584) were first reported in Xiaoyan 54. In Chinese Spring, 16 LMW-GS genes were identified, which corresponded to the DNA fragments detected by the LMW-GS gene marker system (Zhang et al. 2011; Table 2). Database searching indicated that only ten of these genes were previously isolated from Chinese Spring (>99% identities; data not shown; Zhang et al. 2011). In addition, using the PCR method, 16 and 17 LMW-GS genes were isolated from Dongnong 101 and Lvhan 328, respectively. Sequence characterization and alignment showed that these genes belonged to the typical LMW-GS genes (D'Ovidio and Masci 2004), and shared high sequence identities (>95%) with those identified from Xiaoyan 54 and those previously reported (Table 2; Zhang et al. 2004; Zhao et al. 2006; Wang et al. 2009, 2010; Ikeda et al. 2002; Huang and Cloutier 2008; Dong et al. 2010). Thus, by using the PCR-based method, almost all LMW-GS genes in individual wheat varieties could be isolated and identified.

Fourth, the PCR-based method is simple and useful in high-throughput identifying LMW-GS genes in wheat varieties. Using PCR, cloning, clone selection with PCR and 3730 DNA analyzer, and sequencing, which are now very common at molecular biological libraries, more than 10 LMW-GS gene family members in bread wheat were successfully identified. Moreover, the PCR-based method was high-throughput and time-saving. Although the library screening is powerful in isolating LMW-GS genes (Ikeda et al. 2002; Huang and Cloutier 2008; Dong et al. 2010), construction and screening the library for one wheat variety would take more than half a year and need considerable labor and expense; whereas using the PCR based method, all the conventional molecular biological experiments could be finished by 1 person in 1 week, and more than 10 wheat varieties could be analyzed at once.

In summary, this study provided a PCR-based method, which was simple but efficient in isolating LMW-GS genes from bread wheat varieties. Using the PCR-based method, 16 or 17 LMW-GS genes were successfully identified and characterized in four individual varieties. At least 15 genes were universal in wheat varieties, and allelic variants of each gene shared high sequence identity. Composition of the LMW-GS gene family and conservation and variation of LMW-GS genes among wheat varieties were also discussed. This method may improve the characterization of complex members of the LMW-GS gene family and facilitate the efficient use of LMW-GS genes in further improvement of bread-making quality in bread wheat.

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References

An X, Zhang Q, Yan Y, Li Q, Zhang Y, Wang A, Pei Y, Tian J, Wang H, Hsam SLK, Zeller FJ (2006) Cloning and molecular characterization of three novel LMW-i glutenin subunit genes from cultivated einkorn (*Triticum monococcum* L.). Theor Appl Genet 113:383–395

Andrews JL, Hay RL, Skerritt JH, Sutton KH (1994) HPLC and immunoassay-based glutenin subunit analysis: screening for dough properties in wheats grown under different environmental conditions. J Cereal Sci 20:203–215

Barro F, Rooke L, Békés F, Gras P, Tatham AS, Fido R, Lazzeri PA, Shewry PR, Barcelo P (1997) Transformation of wheat with high molecular weight subunit genes results in improved functional properties. Nat Biotechnol 15:1295–1299

Bartels D, Thompson RD (1983) The characterization of cDNA clones coding for wheat storage proteins. Nucl Acids Res 11:2961–2977

Blechl AE, Anderson OD (1996) Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nat Biotechnol 14:875–879

Branlard G, Dardevet M, Amiour N, Igrejas G (2003) Allelic diversity of HMW and LMW glutenin subunits and omega-gliadins in French bread wheat (*Triticum aestivum* L.). Genet Resour Crop Evol 50:669–679

Cassidy BG, Dvorak J, Anderson OD (1998) The wheat low-molecular-weight glutenin genes: characterization of six new genes and progress in understanding gene family structure. Theor Appl Genet 96:743–750

Colot V, Bartels D, Thompson R, Flavell R (1989) Molecular characterization of an active wheat LMW glutenin gene and its relation to other wheat and barley prolamin genes. Mol Gen Genet 216:81–90

Cornish GB, Bekes F, Allen HM, Martin DJ (2001) Flour proteins linked to quality traits in an Australian doubled haploid wheat population. Aust J Agric Res 52:1339–1348

Dong LL, Zhang XF, Liu DC, Fan HJ, Sun JZ, Zhang ZJ, Qin HJ, Li B, Hao ST, Li ZS, Wang DW, Zhang AM, Ling HQ (2010) New insights into the organization, recombination, expression and functional mechanism of low molecular weight glutenin subunit genes in bread wheat. PLoS ONE 5(10):e13548. doi:10.1371/journal.pone.0013548

D'Ovidio R (1993) Single-seed PCR of LMW glutenin genes to distinguish between durum wheat cultivars with good and poor technological properties. Plant Mol Biol 22:1173–1176

D'Ovidio R, Marchitelli C, Ercoli Cardelli L, Porceddu E (1999) Sequence similarity between allelic *Glu-B3* genes related to quality properties of durum wheat. Theor Appl Genet 98:455–461

D'Ovidio R, Masci S (2004) The low-molecular-weight glutenin subunits of wheat gluten. J Cereal Sci 39:321–339

D'Ovidio R, Porceddu E (1996) PCR-based assay for detecting 1B-genes for low-molecular-weight glutenin subunits related to gluten quality properties in durum wheat. Plant Breed 115:413–415

D'Ovidio R, Tanzarella OA, Porceddu E (1992) Nucleotide sequence of a low-molecular-weight glutenin from *Triticum durum*. Plant Mol Biol 18:781–784

Gianibelli MC, Larroque OR, MacRitchie F, Wrigley CW (2001) Biochemical, genetic, and molecular characterization of wheat glutenin and its component subunits. Cereal Chem 78:635–646

Harberd NP, Bartels D, Thompson RD (1985) Analysis of the gliadin multigene loci in bread wheat using nullisomic–tetrasomic lines. Mol Gen Genet 198:234–242

Huang XQ, Cloutier S (2008) Molecular characterization and genomic organization of low molecular weight glutenin subunit genes at the *Glu-3* loci in hexaploid wheat (*Triticum aestivum* L.). Theor Appl Genet 116:953–966

Huang Z, Long H, Jiang QT, Wei YM, Yan ZH, Zheng YL (2010) Molecular characterization of novel low-molecular-weight glutenin genes in *Aegilops longissima*. J Appl Genet 51:9–18

Ikeda TM, Araki E, Fujita Y, Yano H (2006) Characterization of low-molecular-weight glutenin subunit genes and their protein products in common wheats. Theor Appl Genet 112:327–334

Ikeda TM, Nagamine T, Fukuoka H, Yano H (2002) Identification of new low-molecular-weight glutenin subunit genes in wheat. Theor Appl Genet 104:680–687

Jiang C, Pei Y, Zhang Y, Li X, Yao D, Yan Y, Ma W, Hsam SL, Zeller FJ (2008) Molecular cloning and characterization of four novel LMW glutenin subunit genes from *Aegilops longissima*, *Triticum dicoccoides* and *T. zhukovskyi*. Hereditas 145:92–98

Jiang QT, Wei YM, Lu ZX, Pu ZE, Lan XJ, Zheng YL (2010) Structural variation and evolutionary relationship of novel HMW glutenin subunits from *Elymus glaucus*. Hereditas 147:136–141

Johal J, Gianibelli MC, Rahman S, Morell MK, Gale KR (2004) Characterization of low-molecular-weight glutenin genes in *Aegilops tauschii*. Theor Appl Genet 109:1028–1040

Lew EJL, Kuzmicky DD, Kasarda DD (1992) Characterization of low-molecular-weight glutenin subunits by reversed-phase high performance liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and N-terminal amino acid sequencing. Cereal Chem 69:508–515

Li X, Ma W, Gao L, Zhang Y, Wang A, Ji K, Wang K, Appels R, Yan Y (2008) A novel chimeric low-molecular-weight glutenin subunit gene from the wild relatives of wheat *Aegilops kotschyi* and *Ae. juvenalis*: evolution at the *Glu-3* loci. Genetics 180:93–101

Liu L, Ikeda TM, Branlard G, Peña RJ, RogersWJ, Lerner SE, Kolman MA, Xia XC, Wang LH, Ma WJ, Appels R, Yoshida H, Wang AL, Yan YM, He ZH (2010) Comparison of low molecular weight glutenin subunits identified by SDS-PAGE, 2-DE, MALDI-TOF-MS and PCR in common wheat. BMC Plant Biol 10:124

Long H, Wei YM, Yan ZH, Baum B, Nevo E, Zheng YL (2005) Classification of wheat low-molecular-weight glutenin subunit genes and its chromosome assignment by developing LMW-GS group-specific primers. Theor Appl Genet 111:1251–1259

Masci S, D'Ovidio R, Lafiandra D, Kasarda DD (1998) Characterization of a low-molecular-weight glutenin subunit gene from bread wheat and the corresponding protein that represents a major subunit of glutenin polymer. Plant Physiol 118:1147–1158

Masci S, Lew EJL, Lafiandra D, Porceddu E, Kasarda DD (1995) Characterization of low-molecular-weight glutenin subunits in durum wheat by RP-HPLC and N-terminal sequencing. Cereal Chem 72:100–104

Metakovsky EV, Wrigley CW, Békés F, Gupta RB (1990) Gluten polypeptides as useful genetic markers of dough quality in Australian wheats. Aust J Agric Res 41:289–306

Okita TW (1984) Identification and DNA sequence analysis of a γ-gliadin cDNA plasmid from winter wheat. Plant Mol Biol 3:325–332

Okita TW, Cheesbrough V, Reeves CD (1985) Evolution and heterogeneity of the alpha-/beta-type and gamma-type gliadin DNA sequences. J Biol Chem 260:8203–8213

Pang BS, Zhang XY (2008) Isolation and molecular characterization of high molecular weight glutenin subunit genes *1Bx13* and *1By16* from hexaploid wheat. J Integr Plant Biol 50:329–337

Payne EI, Corfield KG, Holt LM, Blackman JA (1981) Correlations between the inheritance of certain high molecular weight subunits of glutenin and bread-making quality in progenies of six crosses of bread wheat. J Sci Food Agric 32:51–60

Payne PI (1987) Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality. Ann Rev Plant Physiol 38:141–153

Pitts EG, Rafalski JA, Hedgcoth C (1988) Nucleotide sequence and encoded amino acid sequence of a genomic gene region for a low molecular weight glutenin. Nucl Acids Res 16:11376

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Shewry PR, Halford NG, Tatham AS, Popineau Y, Lafiandra D, Belton PS (2003) The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. Adv Food Nutr Res 45:219–302

Shewry PR, Tatham AS, Barro F, Barcelo P, Lazzeri P (1995) Biotechnology of breadmaking: unraveling and manipulating the multiprotein gluten complex. Nat Biotechnol 13:1185–1190

Singh NK, Shepherd KW (1988) Linkage mapping of genes controlling endosperm storage proteins in wheat. 1. Genes on the short arms of group 1 chromosomes. Theor Appl Genet 75:628–641

Srichumpa P, Brunner S, Keller B, Yahiaoui N (2005) Allelic series of four powdery mildew resistance genes at the *Pm3* locus in hexaploid bread wheat. Plant Physiol 139:885–895

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tanaka H, Shimizu R, Tsujimoto H (2005) Genetical analysis of contribution of low-molecular-weight glutenin subunits to dough strength in common wheat (*Triticum aestivum* L.). Euphytica 141:157–162

Tao HP, Kasarda DD (1989) Two-dimensional gel mapping and N-terminal sequencing of LMW-glutenin subunits. J Exp Biol 40:1015–1020

Wang K, Gao L, Wang S, Zhang Y, Li X, Zhang M, Xie Z, Yan Y, Belgard M, Ma W (2011) Phylogenetic relationship of a new class of LMW-GS genes in the M genome of *Aegilops comosa*. Theor Appl Genet 122:1411–1425

Wang LH, Li GY, Peňa RJ, Xia XC, He ZH (2010) Development of STS markers and establishment of multiplex PCR for *Glu-A3* alleles in common wheat (*Triticum aestivum* L.). J Cereal Sci 51:305–312

Wang LH, Zhao XL, He ZH, Ma W, Appels R, Peña RJ, Xia XC (2009) Characterization of low-molecular-weight glutenin subunit *Glu-B3* genes and development of STS markers in common wheat (*Triticum aestivum* L.). Theor Appl Genet 118:525–539

Yan Y, Hsam SL, Yu JZ, Jiang Y, Ohtsuka I, Zeller FJ (2003) HMW and LMW glutenin alleles among putative tetraploid and hexaploid European spelt wheat (*Triticum spelta* L.) progenitors. Theor Appl Genet 107:1321–1330

Yue YW, Long H, Liu Q, Wei YM, Yan ZH, Zheng YL (2005) Isolation of low-molecular-weight glutenin subunit genes from wild emmer wheat (*Triticum dicoccoides*). J Appl Genet 46:349–355

Zhang W, Gianibelli MC, Rampling LR, Gale KR (2004) Characterization and marker development for low molecular weight glutenin genes from *Glu-A3* alleles of bread wheat (*Triticum aestivum* L.). Theor Appl Genet 108:1409–1419

Zhang XF, Liu DC, Yang WL, Liu KF, Sun JZ, Guo XL, Li YW, Wang DW, Ling HQ, Zhang AM (2011) Development of a new marker system for identifying the complex members of the low-molecular-weight glutenin subunit gene family in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 122:1503–1516

Zhao X, Yang Y, He Z, Lei Z, Ma W, Sun Q, Xia X (2008) Characterization of novel LMW-GS genes at *Glu-D3* locus on chromosome 1D in *Aegilops tauschii*. Hereditas. 145:238–250

Zhao XL, Xia XC, He ZH, Gale KR, Lei ZS, Appels R, Ma W (2006) Characterization of three low-molecular-weight Glu-D3 subunit genes in common wheat. Theor Appl Genet 113:1247–1259

Zhao XL, Xia XC, He ZH, Lei ZS, Appels R, Yang Y, Sun QX, Ma W (2007) Novel DNA variations to characterize low molecular weight glutenin *Glu-D3* genes and develop STS markers in common wheat. Theor Appl Genet 114:451–460