Australian Journal of Crop Science AJCS
AJCS 6(12):1642-1648 (2012) ISSN:1835-2707

Bicistronic expression and functional analysis of a novel LMW-m glutenin gene in wheat (*Triticum aestivum* L.)

Wen Zang[1], Jian Dong[1,2], Xiang Gao[1,2*], Qijiao Chen[1*], Zhoubo Hu[1], Xiaoyan Li[1,2], and Wanchun Zhao[1,2]

[1]College of Agronomy, Northwest A&F University, Yangling 712100, P.R. China
[2]Wheat Engineering Research Center of Shaanxi Province, Shaanxi 712100, P.R. China

[*]Corresponding authors: gx@nwsuaf.edu.cn; qjchen@nwsuaf.edu.cn

Abstract

Low-Molecular-Weight Glutenin Subunits (LMW-GS) play the major role in determining the viscoelastic properties of dough. DsbA, an efficient catalyst for disulfide bond formation, could increase the solubility of the co-expressed protein in *Escherichia coli*. To isolate large amounts of LMW-GS for *in vitro* functional analysis, the bicistronic expression method was employed in the present study. The *lmw-gs* and *dsbA* genes were obtained using gene-specific primers, and joined into *lmw/dsbA* through splicing by overlapping extension PCR (SOE-PCR). The combination molecule was ligated into pET32a (+) and co-expressed in *E.coli* BL21 (DE3) host cells. Meanwhile, pET32a-*lmw* was constructed as a negative control to assess the impact of DsbA on the solubility of LMW-GS. The expression products were confirmed by SDS-PAGE and Western-blot. The solution ability was evaluated by the concentration of LMW-GS in supernatants of pET32a-*lmw/dsbA* and pET32a-*lmw* under the same conditions. Small-scale dough testing was conducted using the standard Farinograph test (10-g flour). Results showed that three *lmw-gs* genes (from JF439428 to JF439430) were isolated from wheat Shaan253 (*Triticum aestivum* L.), and that one *dsbA* gene (JF755399) from *E.coli* strain DH5α, respectively. Due to owning more typical motifs of LMW-m type than the others, JF439428 was selected for co-expression with JF755399 and further functional analysis. Standard curve analysis revealed that DsbA could increase the solubility of LMW-GS 1.76-fold. Small scale testing suggested that JF439428 belongs to a neutral subunit on the dough quality. We also found the severe bias of each type among LMW-GS in common wheat.

Keywords: Bicistronic expression; DsbA; farinograph; LMW-GS; prokaryotic expression.
Abbreviations: DBT- dough breakdown time; DS- degree of softening; DvT- development time; FQN- farinograph quality number; HMW-GS- High-Molecular- Weight Glutenin Subunits; LMW-GS- Low-Molecular Weight Glutenin Subunits; MTI- mixing tolerance index; PDI- protein disulfide isomerase; SOE- splice overlap extension; ST- stability time.

Introduction

Low-Molecular-Weight Glutenin Subunits (LMW-GS) of wheat, which represent about one-third of the total seed storage protein and 60% of the total glutenin (Bietz and Wall, 1973), are essential in determining dough extensibility and gluten strength (Gale, 2005). The dough qualities of bread wheat (Gupta et al., 1994) and durum wheat (Pogna et al., 1990) are closely related to allelic variation in LMW-GS. LMW-GS were divided into three types: LMW-s, LMW-m, and LMW-i according to the first amino acid of mature subunits (Cloutier et al., 2001). LMW-GS have been divided into 12 groups based on the number and position of cysteine residues (Ikeda et al., 2002). Most of *lmw-gs* genes are located at the *Glu-A3*, *Glu-B3* and *Glu-D3* loci on the short arms of chromosomes1A, 1B and 1D, respectively (D'Ovidio and Masci, 2004). However, although the study on LMW-GS has been made a rapid progress, their contribution to quality and gluten strength has not been investigated to the same degree as the HMW-GS. Small-scale dough testing, such as 2 grams or 10 grams flour, has been of great value in the analysis of the properties of LMW-GS (Bekes et al., 1994; Sissons et al., 1998; Lee et al., 1999). Bekes et al. (1994) firstly employed this method to observe the acute change of mixograph and extension test parameters, including stability time, development time, peak time, and finally revealed the function of specific glutenin subunits. Then it was widely applied in the functional study of specific glutenin alleles (Uthayakumaran et al., 2000; Luo et al., 2001). Ferrante et al. (2006) investigated the mixing behavior of i-type LMW-GS using 2-g Mixograph parameters of durum and bread wheat doughs. Xu et al. (2006) studied the function of the LMW-GS subunit XYGluD3-LMWGS1 using 10-g Mixograph. Zheng et al. (2009) evaluated the effects of LMW-GS alleles at the *Glu-3* loci on dough-mixing properties with a 10-g Mixograph. Lee et al. (1999) conducted the functional testing of three bacterially synthesized LMW-GSs, LMWG-E2, LMWG-E4 and LMW-16/10, using 2-g Mixograph. Recombinant proteins from the *E. coli* are usually in the form of insoluble inclusion bodies which consist of insoluble proteins and RNA. To have biological activity, renaturation must take place leading to the correct formation of tertiary and possibly also quaternary structure, however, the yield of renatured protein will be reduced by the procedure. The solubility of expressed proteins in prokaryotic cells can be improved by the co-expression of homologous molecular chaperones (GroES-GroEL, DnaK-DnaJ-Grp, EClpB) and foldases (PPI's, DsbA, DsbC, PDI) in the same expression system. These enzymes could help the newly synthesized proteins reach a proper confirmation, prevent incorrect aggregation and reduce the formation of inclusion bodies (Lee

et al., 2002). DsbA, a 21 kDa protein found in the periplasm of bacteria, is the first disulfide oxidoreductase found in the periplasm of bacteria and belongs to the strongest oxidant of the thioredoxin superfamily (Grauschopf et al., 1995). Mutation testing of *dsbA* in *E. coli* renders that DsbA could facilitate the formation of disulfide bond *in vivo* (Bardwell et al., 1991). Like thioredoxin and protein disulfide isomerase (PDI), the Dsb protein family possesses the thioredoxin-like CXYC active site and members can be distinguished by the different amino acids between the two cysteines (Selles et al., 2011). DsbA does show chaperone activity stronger than that of thioredoxin and similar to that of PDI and has beneficial effects on the accumulation of soluble heterologous proteins in the cytoplasm and periplasm of *E. coli* (Xu et al., 2008). In the present study, to isolate large amounts of LMW-GS for *in vitro* functional analysis, we discussed the possibility of the yields improvement of LMW-GS through increasing the solubility of proteins by co-expression of the target gene with the foldase DsbA. The *lmw-gs* and *dsbA* genes were obtained using gene-specific primers, and then joined into one unique complete gene *lmw/dsbA* through splicing by overlapping extension PCR (SOE-PCR). The combination molecule was inserted into pET32a (+) to get the recombinant bicistronic expression plasmid pET32a-*lmw/dsbA*, followed by co-expression in *E.coli* BL21 (DE3) host cells. Meanwhile, pET32a-*lmw* was constructed as a negative control to assess the impact of DsbA on solubility of LMW-GS. The expressed LMW-GS was isolated and conducted a quality test using 10-g Brabender Farinograph.

Results

Gene cloning and sequence analysis

The PCR bands with the fragment size about 0.5 Kb (Fig. 1, lane 1) and 1.0 Kb (Fig. 1, lane 2) were yielded with the primer pair *dsbA* F2/R2 (Table 1) using *E.coli* DH5α strain genomic DNA as template and with the primer pair *lmw-gs* F1/R1 (Table 1) using Shaan253 genomic DNA as template, respectively. Among them, the nucleotide sequence (accession no. JF755399), isolated by the primer pair *dsbA* F2/R2, was the same to the *dsbA* of *E.coli* strain DH1 (CP001637). On the basis of the deduced amino acid sequence, the identity was 100%. Although it seems like one band on agarose gel (Fig. 1), the amplification products of *lmw*F1/R1 actually were composed of three different alleles, which shared the same 903 bp long sequences (accession no. from JF439428 to JF439430). As shown in Fig. 2, three novel genes possessed a single unique ORF which coded the same number of 300 amino acids and without any internal stop coden. Sequence alignment revealed that the similarity of amino acid sequence among JF439428, JF439429 and JF439430 is 99.78%. Multiple sequence alignment showed that the similarity between the deduced amino acids of the three non-intron genes and the previously characterized LMW-m type glutenin subunits from the GenBank database were significantly higher than that with the subunits of LMW-i or LMW-s type from the same species (Fig. 2A). The same results were observed when using the first N-terminal amino acid of the mature protein (Masci et al., 1998; Ikeda et al., 2002) (full details see Fig. 2A). According to the review of D'Ovidio and Masci (2004), the characteristics of the present genes consisted of four typical structural regions of LMW-m type, successively including the typical 20 amino acid signal peptide (Sig), the N-terminal region (N-ter) of 13 amino acids starting with peptides of METSHIPGL, the repetitive domain (Rep) of 81 amino acids which contained repeats of a short peptide motif rich in glutamine and proline residues, and the C-terminal domain (C-ter) of 186 amino acids (Fig. 2A, 2B). The quantity and location of Cys residues were extremely conservative among these three subunits (Fig. 2B). A more thorough analysis of the present sequences showed that, amino acids substitution events were found. Among them, the first substitution (F↔L) occurred at position 53 in the start of the repetitive domain, the second (Q↔R) at the position 108 in the end of the repetitive domain, the last (F↔S) at the position 229 in the C-terminal II region (Fig. 2A). Compared to the others, JF439428 owned more typical motifs of LMW-GS, and therefore was picked out for further functional study. The 160 complete protein sequences, covered all three major kinds of storage proteins in the seeds of common wheat, were used to construct the phylogenetic tree. The corresponding genes included three novel LMW genes from this study and 157 sequences derived from GenBank database, including 105 LMW-m type, 37 LMW-s type, 10 LMW-i type, one alpha/beta gliadin, one gamma gliadin, one omega gliadin, one x-type HMW-GS, and one y-type HMW-GS. As shown in Fig. 3, the deduced amino acid sequences of the LMW-GS were classified into three types, namely LMW-m, LMW-s type, and LMW-i type. It is obvious that the LMW-m type subunit was clustered into four clear sub-branches, namely MI, MII, MIII, and MIV group. Among them, the former three groups were tightly linked each other. However, MIV group was separated by LMW-s type. On the basis of the above clustering results, the three novel genes of the present study (marked by dark dots in Fig. 3) therefore could be assigned to the LMW-m type. It's in good agreement with classification rules of first mature amino acid of N-terminal region (D'Ovidio and Masci, 2004). Similarly, LMW-s type subunits were clustered into two relatively independent groups: SI and SII. It's interesting that, LMW-i type was far away from two types of LMW-GS, and was clustered with the inclusion of other types of storage proteins in bread wheat. Another approximate 1.6 Kb combination molecule of *lmw/dsbA* was then obtained through splicing by overlapping extension PCR (SOE-PCR) using *lmw* F1/*dsbA* R2 as the concatenated primer and the positive clone of JF439428, together with *dsbA* of *E.coli* (JF755399) as the template (Fig. 1, lane 3; Table 1). Sequencing results revealed that the complete length of the combination molecule was 1539 bp, and the sequence contents were the same as JF755399 and JF439428, together with a piece of 15-bp enzyme recognition sequences between them (Table 1; Fig. 4).

Construction of the bicistronic expression vector and prokaryotic expression

Because of its special features, as mentioned above, JF439428 was selected for further investigation. The combination molecule *lmw/dsbA* (JF439428/JF755399) was diminished the signature peptides using the expression primer pair F3/R3 (Fig. 1, lane 3; Fig. 4A). The desired products and pET32a (+) were double digested with *Eco*R I and *Hin*d III, then ligated into the pET-32a (+) by T4 DNA ligase (Fig. 4A). The positive clone was validated by endonuclease digestion (Fig. 4A, 4B), colony PCR and sequencing and was designated pET32a-*lmw/dsbA* (Fig. 4A, 4C). Expression experiments were conducted using *E. coli* BL21 (DE3) as the host. The recombinant cells were harvested after 6 h induction with 0.6 mmol L^{-1} IPTG at 25°C and whole cell lysates were analyzed by 12% SDS-PAGE, which revealed two target bands, one was the recombinant protein, about 52 kDa fused with histidine-tagged thioredoxin (Trx) and the other was DsbA about 21 kDa, at the expected positions (Fig. 5A). Western blot analysis showed that a band with molecular weight of about 52 kDa was positively stained (Fig. 6). The control

Table 1. Primers used to clone overlapping DNA fragments of *lmw/dsbA*.

Name	Template	Sequence (5′→3′)		Function
lmw-gs	Shaan253	F1	ATGAAGACCTTCCTCGTCTTTGCCCCTCATG	Cloning
		R1	GCCAT***ATGTATATCTCCTTC***TCAGTAGGCACCAA	
dsbA	DH5α	F2	CTGA***GAAGGAGATATACAT***ATGGCGCAGTATG	Cloning
		R2	TTATTTTTTCTCGGACAGATATTTCACAGTA	
lmw/dabA	pMD19-T-*lmw*, *dabA*	F3	CCGGAATTCATGGAGACTAGCCACATCC *Eco*R I	Expression
		R3	CCCAAGCTTTTATTTTTTCTCGGACAG *Hin*d III	
lmw-gs	pMD19-T-*lmw*	R4	CCCAAGCTTTCAGTAGGCACCAACTC *Hin*d III	Expression

Overlapping regions at the concatenation sites were underlined and the RBS sequence is shown in bold italic. Enzyme reorganization sites marked by underline.

Table 2. The means of DvT, ST, DS, MTI, BDT, and FQN of the dough of base flour and which incorporated with LMW-GS encoded by JF439428.

Additive	DvT(min)	ST(min)	DS (BU)	MTI (BU)	BDT (min)	FQN (mm)
DTT, KIO_3	2.9 [b]	1.6	182[a]	158	4.00	40.0
DTT,KIO_3, JF439428	3.9 [a]	2.0	154[b]	136	4.65	46.5

Means with different small letters are significantly different at $P < 0.05$ level.

plasmid pET32a-*lmw* (without *dsbA*) was constructed and expressed using the similar procedure as above (Fig. 5A).

Protein purification and solubility testing

After purification, the expressed protein was detected as a 52 kDa target band at the expected position, proving that the fusion protein was purified successfully (Fig. 5B). The standard curve of bovine serum albumin (BSA) in serial dilution, ranging from 0 to 6 μg/mL, was constructed. The relationship between the densitometry readings of the BSA bands on SDS-PAGE gel and their corresponding concentration could be expressed by the equation (Fig. 7). The densitometry parameters of LMW-GS extracted from pET32a-*lmw/dsbA* and pET32a-*lmw* were 3465.85 and 2191.28, respectively. According to the standard curve, the deduced concentration of the soluble LMW-GS from pET32a-*lmw/dsbA* and pET32a-*lmw* were 4.04 and 2.29, respectively. Compared to the control, *in vitro* co-expressed DsbA improved the solubility of LMW-GS for 1.76-fold. It was therefore indicated that co-expressing DsbA could enhance the solubility of expressed LMW-GS in some degree.

Functional identification of LMW-GS

As shown in Fig. 8, the incorporation of JF439428 resulted in significant increase in Dough development time (DvT) while decreased Degree of softening (DS) significantly ($p < 0.05$). Stability time (ST), Breakdown time (BDT), and Farinograph quality number (FQN) values slightly increased, while Mixing tolerance index (MTI) decreased but not statistically significant (Fig. 8C). These farinographic parameters suggested that JF439428 could have positive effect on the end-use quality of wheat flour.

Discussion

LMW-GS accounts for about 30% of the total storage proteins and 60% of glutenins in the seeds of common wheat (Bietz and Wall, 1973). In wheat dough, glutenin polymers are composed mainly of LMW-GS. Therefore, this kind of storage protein often been regarded as the key element in quality decision (LMW-GS quality). Until now, the LMW-GS are still more difficult to purify than HMW-GS (Masci et al., 1998), and the natural sources rarely meet the requirements for quantity testing. Therefore, *in vitro* expression becomes the necessary method of choice to understand the quality effect of the

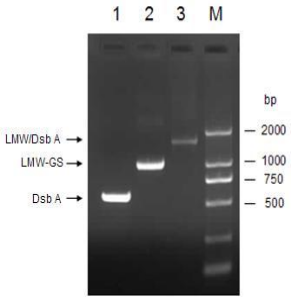

Fig 1. Amplification of *lmw/dsbA* by SOE-PCR. (M): molecular marker DL2000. (Lane 1): amplification product by using primer set *dabA* F2/R2. (Lane 2): amplification product by primer set *lmw-gs* F1/R1. (Lane 3): amplified by primer pair *lmw/dsbA*. Arrows indicate the location of desired PCR products of *lmw/dsbA*, *lmw-gs* and *dsbA* respectively.

members in this big family (Sissons et al., 1998; Lee et al., 1999; Xu et al., 2006). In spite of its high yield, expression of proteins in *E. coli* system often results in insoluble station known as inclusion bodies (Villaverde and Carrio, 2003). In the present study, we wish to test whether it is possible to improve the solubility of LMW-GS by using the co-expressed DsbA in *E.coli*. The SOE-PCR method was employed to get the unique fusion molecule of *lmw* and *dsbA*. The standard curve analysis results showed that, compared to the single gene expression system, co-expressed DsbA could increase the solubility of LMW-GS in some degree (Fig. 7). On one hand, former studies revealed that DsbA owned the strong ability for increasing the solubility of proteins. For example, 69 % of secreted leptin was induced as the soluble form without decreasing the amount of leptin produced by co-expression of DsbA (Jeong and Lee, 2000). The sorts of the co-expressed genes with DsbA were pretty plentiful, such as bovine pancreatic trypsin inhibitor (BPTI), alpha-Lactalbumin (Zapun and Creighton, 1994), and ragi bifunctional inhibitor (RBI) (Maskos et al., 2003). They also found that DsbA could markedly stimulated disulfide bond formation of denatured alkaline phosphatase and reduced bovine ribonucleaseA and exhibited chaperone activity by decreasing aggregation during refolding of denatured GAPDH and rhodanese containing no disulfide bonds (Akiyama et al., 1992). On the other hand, researchers found that many factors could affect the solubility of protein in *E.coli* system, and therefore these factors could be

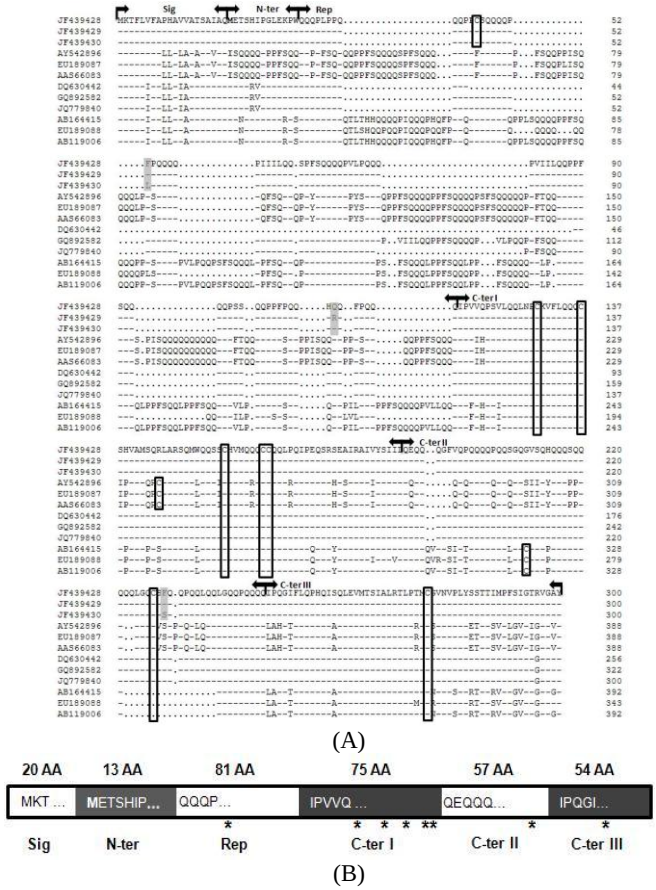

Fig 2. Multiple alignments and schematic diagram of the deduced amino acid sequences. Sig, signal peptide; N-ter, N-terminal region; Rep, repetitive domain; C-ter I, II and III represent the sub-region of the C-terminal domain of LMW-GS. (A): JF439428, JF439429 and JF439430 were deduced from the present study; others derived from the GenBank, including three LMW-m (DQ630442, GQ892582 and JQ779840), three LMW-s (AB164415, EU189088 and AB119006), and three LMW-i (AY542896, EU189087 and AAS66083) type of glutenin genes. The same and deletions of amino acids are indicated by dashes and dots, respectively. The cysteine residues are highlighted by a box. The shaded area stands for the substitution of amino acids among the three sequences in this study. (B): Schematic depiction of the structure of LMW-m type deduced from their coding sequences in the present study. The typical peptides together with the total number amino acid of each region were showed at each box. Cysteine residues and their relative locations were marked by asterisks.

employed to increase the solubility of co-expressed proteins, such as, cultivating temperatures (Blackwell and Horgan, 1991), compatible solutes, osmotic-resistant reagents and growth additives, etc (Chopra et al., 1994; Roberts, 2000). To this end, further researches are necessary to undertake so as to obtain the proper co-expression effect of *lmw* and *dabA*. 10-g farinograph testing in this study suggested that even though the LMW-GS encoded by JF439428 could increase DvT and decrease DS significantly compared with that of base flour, but there was no significant increase in ST, and resulted in only slight increase for the final index FQN. As a consequence, based on the present testing protocol, JF439428 belongs to a neutral subunit, which just has weak contribution to the quality improvement of wheat flour. It is also noteworthy that the present work confirmed its efficiency of clustering method in the classification of LMW-GS.

As displayed by our phylogenetic analysis results (Fig. 3), all these three kinds of subunits (-m, -s, and -i type) were clearly separated and clustered into the proper stretch. And it's apparent that this method even could reflect the tiny differences among the same type into sub-group. It's very similar to the results of Long et al. (2005) who set the classification on the deduced amino acid sequences of N-terminal domain. Additionally, through the present analysis, we found the severe bias of each type among LMW-GS in common wheat. We collected all of the complete hexaploid-derived LMW-GS from GenBank database since 1993. Among the clustering individual subunits, LMW-m type has the percentage of 69.68 % in total, and is far more than the distribution of LMW-s (23.87 %) and LMW-i (6.45 %) types. Partially because of this feature, to date, lots of functional testing were generated among this type, for example, Sissons et al (1998), Xu et al (2006), and Chen et al (2011). And therefore, little information on the end-use quality as to the two other types was collected. On the other hand, when thinking about the diversification of LMW-GS, the feature of high redundancy of LMW-m type is remarkable. Of course, it's valuable to understand the basic reasons behind the phenomenon of bias in common wheat.

Materials and methods

Experimental materials

Shaan253 (*T. aestivum* L.), an important commercial wheat cultivar in the North China plain, was provided by the Yangling Branch of the China Wheat Improvement Center. Taq DNA polymerase, *E. coli* DH5α, *E.coli* BL21 (DE3), DNA gel recovery purification kit, plasmid extraction kit and Strengthen HRP-DAB Western Blotting Detection kit were purchased from Tiangen Biotech (Beijing). PrimerSTAR HS DNA Polymerase, dNTPs, pMD19-T, T4 DNA ligase, restriction enzymes *Eco*R I and *Hin*d III were obtained from TaKaRa (Dalian). Expression vector pET-32a (+) were purchased from MERCK (Germany).

Primer designing and gene splicing by overlap extension

Cloning and expression primers sets (listed in Table 1) for *lmw-gs*, *dsbA* and *lmw-gs/dsbA* genes were designed according to previously published sequences in GenBank (accession No: FJ876823 and Z54203) using the PCR Primers Design module of DNAMAN software package (Version 7.0.3, Lynnon Biosoft, Canada). Among them, the individual primer combination, template and other details were integrated in Table 1. The *lmw* and *dabA* genes were obtained using TaKaRa ExTaq kit (TaKaRa, Dalian) employing the same amplification systems and reaction programs which mentioned by Yu et al. (2009) and Wunderlich and Glockshuber (1993), respectively. The SOE-PCR reaction mixtures for *lmw*/*dabA* including: 5 μL 10 × PCR Buffer (Mg^{2+} free), 4 μL dNTP, 4 μL Mg^{2+}, 0.25 μL Taq DNA Polymerase, 1 μL of each primer *lmw*F1/*bsdA*R2, 1 μL of each PCR product of pMD19-T-*lmw* (the positive clone of JF439428) and *dsbA*, added ddH_2O to 50 μL. Corresponding programs as the following: 95ºC pre-denaturation for 4 min, followed by two steps: the first was 5 cycles of 30 s at 94°C, 30 s at 64°C, 2 min 30 s at 72°C, and 8 min at 72°C. The second was added 1 μL of each primer *lmw*F1/*bsdA*R2 into reaction system, 94°C denaturation for 3 min followed by 26 cycles of 30 s at 94°C, 30 s at 64°C, 2 min 30 s at 72°C, and 9 min at 72°C. The desired SOE-PCR products (*lmw*-linker-*dsbA*, namely *lmw/dsbA*) were recovered from

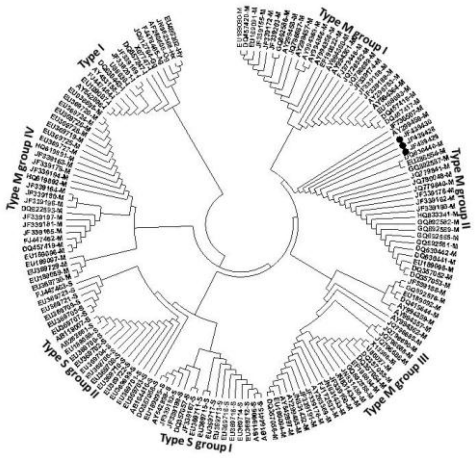

Fig 3. Phylogenetic relationship among JF439428, JF439429, JF439430 and other different types of storage proteins in common wheat. -M, LMW-m type; -S, LMW-s type; -I, LMW-I type; -Ag, alpha/beta gliadin; -Gg, gamma gliadin; -Og, omega gliadin; -Hx, HMW-GS x type; -Hy, HMW-GS y type. Three sequences in the present study were marked by black dots.

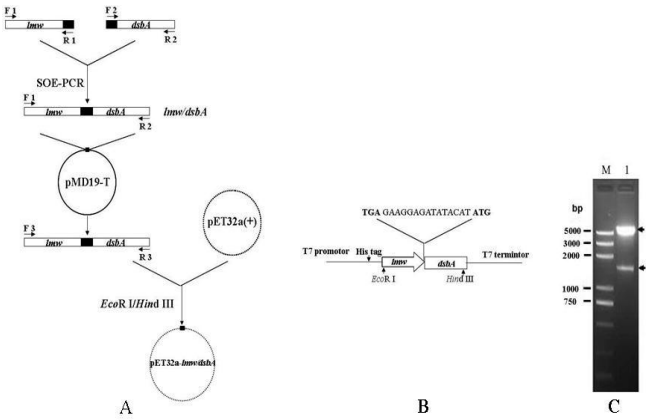

Fig 4. Construction of bicistronic expression vector containing *lmw-gs* (JF439428) and *dsbA* (JF755399). (A): schematic diagram of the construction of bicistronic expression vector by SOE-PCR. (B): a bicistronic transcription unit consisting of full-length of *lmw-gs* and *dsbA* is cloned between *Eco*R I and *Hin*d *III* on a pET-32a (+) vector for regulated expression by T7 promoter induction. A 15 nucleotide spacer locates between the *lmw-gs* stop codon and the *dsbA* start codon enabling co-expression from the bicistronic expression vector. (C): validation of pET32a-*lmw/dsbA* with *Eco*R I and *Hin*d III. (M): DNA marker. (Lane 1): pET32a-*lmw/dsbA* is digested with *Eco*R I and *Hin*d III. Arrows show the locations of digested fractions.

agarose gels, and then cloned into the vector pMD19-T. The recombinant vector was transformed into *E. coli* DH5a competent cells. Positive clones were identified by colony PCR, together with double digestion with *Eco*R I and *Hin*d III.

Sequence alignment and phylogenetic analysis

Multiple alignment of the deduced amino acid sequences of LMW-GS were carried out by DNAMAN 7.0.3 (Lynnon Biosoft, Canada). The complete length of LMW-GS (-m, -s and -i type), x- and y- type HMW-GS, together with three kinds of gliadins (α/β, γ, and ω gliadin), which used for phylogenetic analysis in the present study, were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). In addition, all of these candidate sequences shared the same origin of common wheat (*T. aestivum* L.). The phylogenetic tree was constructed with MEGA 5.0 software using maximum likelihood method (Tamura et al., 2011).

Construction of the bicistronic expression plasmid and prokaryotic expression

The positive vectors were sub-cloned using primers *lmw*F3/*dsb*AR3 to amplify the open reading frame of *lmw/dsbA*. The flowchart was described in Fig. 4A. The desired PCR product and pET-32a(+) were digested with *Eco*R I and *Hin*d III, ligated by T4 DNA ligase, then bicistronic expression plasmids were transformed into *E.coli* DH5α. The positive clones were validated by colony PCR, double digestion of restriction endonuclease and sequencing. Bicistronic expression plasmids were transformed into *E. coli* BL21 (DE3) and grown overnight in LB medium supplemented with ampicillin (100 mg mL^{-1}) at 25°C. The culture was diluted with fresh LB medium at 1:100 and the cells grown at 37°C until A_{600} = 0.6, then IPTG was added to obtain a final concentration of 0.6 mmol L^{-1}, followed by shaking at 25°C for an additional 6 h to allow for expression of the recombinant protein, then harvested by centrifugation at 2 000 × g for 10 min. The cells were resuspended in 100 µL 1 × SDS gel loading buffer, then boiled for 7 min. Lastly, 12% SDS-PAGE electrophoresis and a Western blot assay was executed to analyze protein expression. The open reading frame of the gene encoding the natural LMW-GS was amplified by PCR using expression primers sets *lmw*F3/*lmw*R4 (Table 1). Positive clones were purified, sequenced and then expressed in host cell *E. coli* BL21 (DE3).

Purification and solubility testing

Cells were collected by centrifuging at 2 000 × g for 10 min at 4°C. The cell pellet was suspended in breaking buffer (50 mmol L^{-1} Tris-HCl, 2 mmol L^{-1} EDTA, 100 mmol L^{-1} NaCl, 1 mg mL^{-1} lysozyme, pH = 8.5), placed on ice for 45 min, followed by ultrasound and then centrifuged at 12 000 × g for 10 min at 4°C. The precipitate was resuspended in 4 mol L^{-1} imidazole and collected at 17 000 × g for 10 min at 4°C. After that it was dissolved in 50 mmol L^{-1} PBS containing 8 mol L^{-1} Urea (pH = 7.4). The target protein was purified using a HisTrap HP column which was equilibrated with 10 mL balanced buffer (50 mmol L^{-1} PBS: 0.5 mol L^{-1} NaCl, 20 mmol L^{-1} imidazole, pH = 7.4). A 10 mL sample was then applied at 0.5 mL min^{-1}, followed by washing the column with 10 mL binding buffer and the wash fraction was collected. Lastly, the column was eluted with 5 mL elution buffer containing 0.5 mol L^{-1} NaCl, 4 mol L^{-1} urea, 500 mmol L^{-1} imidazole at 1 mL min^{-1} and the eluate collected in 2 mL fractions. After dialysis and lyophilization, the purified proteins were stored at -20°C for further use. The Bradford method was employed to quantify the soluble form of LMW-GS. The standard curve was developed using a series of Bovine Serum Albumin (BSA) standards ranging from the 100 µg/ml to 1,500 µg/ml (Bradford, 1976). The absorbance of band on the SDS-PAGE was recognized by Quantity One® v4.4.0 software (Bio-Rad, USA). The relationship between absorbance of band (x) and protein concentration (y) was

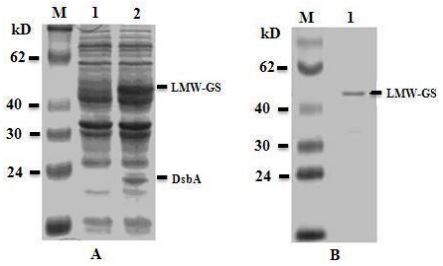

Fig 5. SDS-PAGE analysis of the expressed and purified proteins expressed in *E.coli*. (A): Comparative analysis of the solubility of LMW-GS in the supernatant between pET32a-*lmw* and pET32a-*lmw/dsbA* induced IPTG. (M) protein marker; (Lane 1) proteins of recombinant plasmid pET32a-*lmw*; (Lane 2) proteins of recombinant plasmid pET32a-*lmw/dsbA*. (B): Analysis of the purified LMW-GS using ÄKTA purifier 100. (Lane 1): purified from the expressed protein of pET32a-*lmw/dsbA*. (M): protein molecular weight marker.

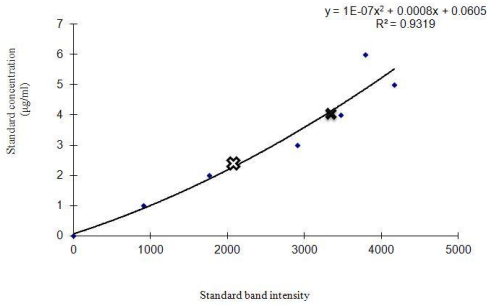

Fig 7. Standard curve for solubility testing of LMW-GS between pET32a-*lmw/dsbA* and the control pET32a-*lmw*. ⊠ indicated the protein expressed by pET32a-*lmw* (as a control); ✖ represented the co-expressed LMW-GS in pET32a-*lmw/dsbA*; ◆ meant the standard concentration of BSA.

determined using the Trendline module of Excel (Microsoft, Redmond, WA).

Farinograph testing of LMW-GS

The dough farinograph parameters, DvT, ST, DS, DBT, MTI and FQN were determined using a 10-g Brabender Farinograph®-AT according to AACC approved methods. Flour of the wheat cultivar Shaan556 was used as the base flour (control). Optimum water addition for mixing was based on flour protein content and flour water content. The steps were as follows: 10 g base flour were mixed with 125 mg purified LMW-GS, stirred at RT, then H_2O and 0.625 mL 50 μg mL^{-1} DTT added, mixed for 5 min, followed by adding 0.625 mL 200 μg mL^{-1} KIO_3 and mixed for another 5 min. Recording of the farinograph curve then took place for an additional 17 min. Control experiments were performed in the same way but without addition of the purified subunits. Statistical tests were carried out using SPSS software package (version 13.0, SPSS Inc., Chicago, ILL).

Conclusion

In order to find an effective way to obtain enough amount of soluble protein to be used in quality testing, we introduced the co-expression method of DsbA. Three *lmw-gs* genes were

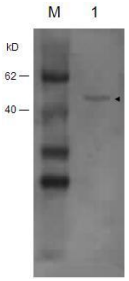

Fig 6. Western blot analysis of the expressed protein of pET32a-*lmw/dsbA*. (M): protein molecular weight marker. (Lane 1): induced recombinant plasmid pET32a-*lmw/dsbA*.

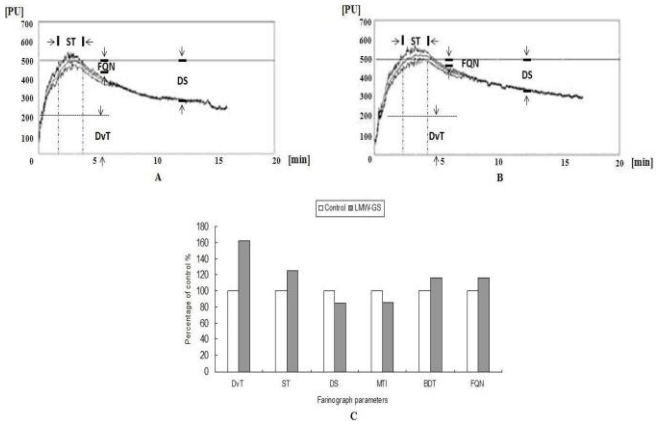

Fig 8. Quality effects of purified LMW-GS (JF439428). (A) the farinograph diagram of the basic flour Shaan556; (B) the farinograph diagram of the basic flour Shaan556 incorporated with JF439428; (C) histogram of the farinograph parameters between the control and flour incorporated LMW-GS. Dough development time (DvT), Stability time (ST), Degree of softening (DS), Mixing tolerance index (MTI), Breakdown time (BDT), Farinograph quality number (FQN).

obtained from the common wheat by using LMW-m type specific primers. Among them, JF439428 was selected to fusion with *dsbA* through SOE-PCR. Solubility testing revealed that co-expressed DsbA has a little effect on LMW-GS, and therefore further studies are needed to optimize this system. Quality testing showed that JF439428 belongs to a neutral subunit, although some positive parameters were changed drastically by this subunit. The results of the present study also suggested that clustering could be a useful solution to classification of LMW-GS. In addition, this research also shed light on the distribution feature of LMW-GS in common wheat. All the above information could benefit the understanding of the quality testing of individual glutenin subunit.

Acknowledgements

We would like to thank the anonymous reviewers, Prof. Scoles GJ, and the editor for their helpful suggestions. This work was supported by grants from National Science Foundation of China (30900896), A&F-Cyrus Tang Variety Breeding Foundation (A212020912), Fundamental Research Funds for

the Central Universities (QN2009007) and Special Capital for the Construction of a Modern Agriculture Technical System in Shaanxi province (NYCYTX-003).

References

Akiyama Y, Kamitani S, Kusukawa N, Ito K (1992) *In vitro* catalysis of oxidative folding of disulfide-bonded proteins by the *Escherichia coli* dsbA (ppfA) gene product. J Biol Chem 267:22440-22445

Bardwell JC, McGovern K, Beckwith J (1991) Identification of a protein required for disulfide bond formation *in vivo*. Cell 67:581-589

Bekes F, Gras PW, Gupta RB (1994) Mixing properties as a measure of reversible reduction and oxidation of doughs. Cereal Chem 71:44-50

Bietz JA, Wall JS (1973) Isolation and characterization of gliadin-like subunits from glutenins. Cereal Chem 50:537-547

Blackwell JR, Horgan R (1991) A novel strategy for the production of a highly expressed recombinant protein in an active form. FEBS Lett 295:10-12

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Chen FG, Zhao F, Liu RK, Xia GM (2011) Functional properties of two low-molecular-weight glutenin subunits carrying additional cysteine residues from hybrid introgression line II-12 derived from *Triticum aestivum* and *Agropyron elongatum.* Food Chem 127:1773-1776

Chopra AK, Brasier AR, Das M, Xu XJ, Peterson JW (1994) Improved synthesis of *Salmonella typhimurium* enterotoxin using gene fusion expression systems. Gene 144:81-85

Cloutier S, Rampitsch C, Penner GA, Lukow OM (2001) Cloning and expression of a LMW-i glutenin gene. J Cereal Sci 33:143-154

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

Ferrante P, Gianibelli MC, Larroque O, Volpi C, D'Ovidio R, Lafiandra D, Masci S (2006) Effect of incorporation of an i-type low-molecular-weight glutenin subunit and a modified γ-gliadin in durum and in bread wheat doughs as measured by micro-mixographic analyses. J Cereal Sci 44:194-202

Gale KR (2005) Diagnostic DNA markers for quality traits in wheat. J Cereal Sci 41:181-192

Grauschopf U, Winther JR, Korber P, Zander T, Dallinger P, Bardwell JC (1995) Why is DsbA such an oxidizing disulfide catalyst? Cell 83:947-955

Gupta RB, Paul JG, Cornish GB, Palmer G, Bekes F, Rathjen A (1994) Allelic variation at glutenin subunit and gliadin loci, *Glu-1*, *Glu-3* and *Gli-1*, of common wheats. I. Its additive and interaction effects on dough properties. J Cereal Sci 19:9-17

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

Jeong KJ, Lee SY (2000) Secretory production of human leptin in *Escherichia coli*. Biotechnol Bioeng 67:398-407

Lee KH, Kim HS, Jeong HS, Lee YS (2002) Chaperonin GroESL mediates the protein folding of human liver mitochondrial aldehyde dehydrogenase in *Escherichia coli*. Biochem Biophys Res Commun 298:216-224

Lee YK, Bekes F, Gras P, Ciaffi M, Morell MK, Appels R (1999) The low-molecular-weight glutenin subunit proteins of primitive wheats. IV. Functional properties of products from individual genes. Theor Appl Genet 98:149-155

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

Luo C, Griffin WB, Branlard G, McNeil DL (2001) Comparison of low- and high-molecular- weight wheat glutenin allele effects on flour quality. Theor Appl Genet 102:1088-1098

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 the glutenin polymer. Plant Physiol 118:1147-1158

Maskos K, Huber-Wunderlich M, Glockshuber R (2003) DsbA and DsbC-catalyzed oxidative folding of proteins with complex disulfide bridge patterns *in vitro* and *in vivo*. J Mol Biol 325:495-513

Pogna NE, Autran JC, Mellini F, Lafiandra D, Feillet P (1990) Chromosome 1B-encoded gliadins and glutenin subunits in durum wheat: Genetics and relationship to gluten strength. J Cereal Sci 11:15-34

Roberts MF (2000) Osmoadption and osmoregulation in archaea. Front Biosci 5:796-812

Selles B, Jacquot JP, Rouhier N (2011) Comparative genomic study of protein disulfide isomerases from photosynthetic organisms. Genomics 97:37-50

Sissons MJ, Bekes F, Skerritt JH (1998) Isolation and functionality testing of low molecular glutein subunits. Cereal Chem 75:30-36

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731-2739

Uthayakumaran S, Stoddard FL, Gras PW, Bekes F (2000) Effects of incorporated glutenins on functional properties of wheat dough. Cereal Chem 77:737-743

Villaverde A, Carrio MM (2003) Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnol Lett 25:1385-1395

Wunderlich M, Glockshuber R (1993) Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. Protein Sci 2:717-726

Xu H, Wang RJ, Shen X, Zhao YL, Sun GL, Zhao HX, Guo AG (2006) Functional properties of a new low-molecular-weight glutenin subunit gene from a bread wheat cultivar. Theor Appl Genet 113:1295-1303

Xu Y, Yasin A, Tang R, Scharer JM, Moo-Young M, Chou CP (2008) Heterologous expression of lipase in *Escherichia coli* is limited by folding and disulfide bond formation. Appl Microbiol Biotechnol 81:79-87

Yu X, Gao X, Chen QJ, Wu D, Dong J, Zhao WC, Pang HX, Li ZQ (2009) 1B specific LMW-GS primers cloned a 1D located gene from wheat cultivar Xiaoyan 22. Sci Agric Sin 8:1419-1428

Zapun A, Creighton TE (1994) Effect of DsbA on the disulfide folding of bovine pancreatic trypsin inhibitor and alpha-lactalbumin. Biochem 33:5202-5211

Zheng SS, Byrne PF, Bai GH, Shan XY, Reid SD, Haley SD, Seabourn BW (2009) Association analysis reveals effects of wheat glutenin alleles and rye translocations on dough-mixing properties. J Cereal Sci 50:283-290